PROTEIN SYNTHESIS IN MICRASTERIAS THOMASIANA

by

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Abstract

The desmid Micrasterias thomasiana Arch. is a single cell consisting of two identical hemicells each containing a large chloroplast. The cell cycle may be divided into an interdivisional phase of five days duration and a developmental phase, of eight hours duration. During the developmental phase the hemicells separate each producing a new hemicell, thus doubling the cell volume.

Relative protein concentration, determined by the Millon reaction, and total chlorophyll concentration were measured in individual cells cytophotometrically. Results show that chlorophyll and protein concentrations remain constant throughout the interdivisional phase until about twenty-four hours before cell division at which time there is a doubling indicating the time of chloroplast replication. During the later stages of development, as the chloroplast spreads out into the new hemicell, the chlorophyll concentration decreases and the protein concentration returns to the interdivisional level.

The amount of H^3leucine incorporated into protein per hour, determined by autoradiography, is nearly constant throughout both the interdivisional and developmental phases, but is significantly higher in the former. This indicates that the enlargement of the new hemicell, while accompanied by some protein synthesis, does not involve a burst of protein synthesis nor a doubling of the chloroplast.

The results of autoradiography show equal distribution of label in the chloroplasts of both the mature and new hemicell after incubation in isotope during the interdivisional phase followed by incubation in unlabeled medium until a new hemicell develops. This indicates that

chlorplast growth may occur by intercalation of amino acids or protein into the pre-existing chloroplast structure.

Although the pyrenoids contain a high protein concentration as indicated by the Millon reaction, the results of autoradiography show that little or no protein synthesis occurs at these sites.

In order to investigate the question of cytoplasmic versus nuclear control over the early development of the new hemicell, cells displaying the same abnormal development as physically enucleated cells were produced by Actinomycin D treatment. The results of autoradiography reveal that these cells synthesize protein in the absence of demonstrable nuclear RNA synthesis, indicating that stable m-RNA in the cytoplasm may be involved in the development of the major lobes. An attempt to identify the axial fibrillar framework which is postulated as the unit of cytoplasmic inheritance was made. The results of the present study show no evidence for either synthesis of new protein or accumulation of newly synthesized protein forming such a framework. Therefore, the weight of the evidence presented here is in favor of nuclear control over early development.

Introduction

Micrasterias thomasiana Arch. is a unicellular desmid approximately 200 microns in diameter (Fig. 1). The cell is discoform and is composed of two half cells, (designated elsewhere as semicells (51), here as hemicells). Each hemicell contains one large chloroplast. The nucleus, which is approximately 20 microns in diameter, lies in the isthmus between the two hemicells. Each hemicell consists of a polar lobe which is centrally located between two or, occasionally, more "wings." The number of "wings" determines the degree of "radiation" of the cell (61). Each wing consists of one major lobe which in the course of development is subdivided several times. The tips of the lobes are characterized by elaborate species specific dentition. Micrasterias cells occur in either the haploid or diploid condition. The common form of Micrasterias thomasiana and the one worked with here is the haploid biradiate form.

Desmids reproduce sexually by conjugation, but in Micrasterias this is not readily observed in the laboratory. Therefore, only asexual reproduction by cell division is considered in the present study. Desmids have an interesting form of development. The first noticeable event is the elongation of the isthmus that joins the two hemicells. The nucleus then divides, and a septum forms between the two nuclei across the isthmus, separating the two hemicells. Each hemicell then forms an exact replica of itself which starts as a small round bulge, (Fig. 2, stage I), which enlarges, (Fig. 2, stage II). Next the polar and side lobes become discernible, (Fig. 2, stage III), referred to by others (51) as the "puckered" bulge stage. Thereafter, the side lobes

develop and progressively subdivide (Fig. 2, stages IV and V). The stages have been numbered for reference in the text. It can be seen that the chloroplast starts to enter the newly developing hemicell by the beginning of stage III and progressively fills it. The development of the new hemicell, during which the cell volume doubles, takes about eight hours. The time between divisions, when no change in volume occurs, is about five or six days. In contrast to most other cells and unicellular organisms which grow continuously, the doubling in volume of Micrasterias occupies only about one-fifteenth of the cell cycle. Replication involves both the increase in size of the new hemicell and the doubling of the chloroplast. Previous work has not shown when protein is made nor when chloroplast replication occurs. These questions were investigated in the present study by autoradiography and by cytophotometry.

Two different interpretations of hemicell morphogenesis have been offered. Kiermayer (32, 33, 36) believes that turgor pressure and differential thickening and extensibility at the cell periphery are the determining factors. This is based on the observation that, when developing Micrasterias cells are placed in high osmotic strength sucrose, the the region of the cell membrane shows thickened areas where lobe formation will take place and thinner areas where the lobe incisions will be. If proteins involved with wall synthesis are either synthesized in greater amounts <u>in situ</u>, or accumulated in these thicker regions, this should be observed by autoradiography following incorporation of labeled amino acids into protein. On the other hand, Waris and Kallio (26, 27, 30, 57, 61) argue that the development of the major lobes of the new hemicell involves

the outgrowth of axial cytoplasmic fibrils from the parent hemicell (the "cytoplasmic framework"). The distribution of newly synthesized protein in the new hemicell during early development should show the growth of such fibrils if they exist.

The following questions concerning the normal growth of Micrasterias have also beeninvestigated: 1. How does chloroplast replication take place? 2. Are pyrenoids involved in protein synthesis? 3. Does transport of protein to or from the nucleus take place?

In addition to these questions the second part of this study investigates the possibility of cytoplasmic inheritance of the symmetry of the new hemicell which has been proposed for Micrasterias (26, 27, 57). While cytoplasmic inheritance of plastid or mitrochondrial characteristics is well known in plants, cytoplasmic involvement in the transmission of form is not. In Acetabularia, although cytoplasmic inheritance may appear to be involved, the evidence clearly indicates nuclear determination of shape through the influence of stable m-RNAs (10, 21). The evidence for cytoplasmic control of early development in Micrasterias has been obtained from observations made on "Janus" cells, (one hemicell differing from the other) (26, 27, 57). The forms of "Janus" cell observed in this connection were those with one or both wings missing in one hemicell, (the uniradiate and aradiate defect respectively). These cells contain one nucleus. When the cell divides the defective hemicell produces its like, with the missing wing on the same side as that of the parent hemicell defect, and the normal hemicell produces its like, suggesting that the nucleus is not involved in the transmission of this defect. Additional evidence favoring cytoplasmic inheritance of the major lobes

of the new hemicell has been derived from experiments using physically enucleated cells (57). When cells in mitosis are centrifuged the nucleus is displaced into one hemicell. After the septum forms, one hemicell has two nuclei, the other none. The hemicell without a nucleus produces a daughter hemicell with three lobes in most species and usually five lobes in M. thomasiana (57, 61). The enucleated Micrasterias cell survives for only about twenty-four hours (58) in contrast to enucleated Acetabularia and Spirogyra cells which live for several months (21, 22) suggesting a different basis for development in enucleate Micrasterias cells. Waris and Kallio (61) have postulated that three cytoplasmically inherited fibrillar axes control the development of the major lobes of the new hemicell and that the branching of these axes under nuclear control causes the subdivision of the major lobes. There is good evidence for nuclear control over the subdivision of the major lobes and the appearance of species specific dentition (28, 29, 51, 60) but there is no direct evidence for cytoplasmic axes.

The question of truly cytoplasmic control or nuclear control, mediated by stable m-RNA, of the development of the major lobes has been investigated here using cells treated during cytokinesis with Actinomycin D. These cells develop like physically enucleated cells and both survive only about twenty-four hours. The lobes formed are simple, vacuolate and elongate as in Figs. 17 and 18. The Actinomycin D treated cells have been compared to physically enucleated cells with respect to the absence of nuclear RNA production during their development. Whether protein synthesis occurs during this abnormal development has been investigated in order to ascertain whether stable m-RNA might be involved in the early development of M. thomasiana as in Acetabularia (10).

Materials and Methods

Micrasterias thomasiana Arch. haploid, biradiate cells #LB543 were obtained from the culture collection of algae at Indiana University. The cells were cultured in sterile Waris medium (59), under cool white fluorescent lights averaging 150 foot candles light intensity. The light regime was 12 hours light, (6 PM to 6 AM), 12 hours dark, (6 AM to 6 PM). Since the cells only divide in the dark, the dark period was given during the day.

For convenience in interpreting the experimental results, the cell cycle has been divided into the following categories: The interdivisional phase - that portion of the cell cycle extending from the completion of development of the daughter hemicells until the onset of the following mitosis. The premitotic stage - that part of the interdivisional phase which is characterized by intense greening of the chloroplast, an event which occurs several hours before mitosis. The developmental phase - which includes mitosis, cytokinesis and development of the new hemicell, the latter is divided into five stages as shown previously in Fig. 2. At 11°C, the cultivation temperature employed, the interdivisional phase lasts five or six days and the development of the new hemicell occurs in about eight hours.

Since the cultures were not axenic, a routine washing procedure for all cells was used in order to prevent incorporation of labeled precursor into micro-organisms associated with the cells during incubation in isotope. The cells were washed nine times individually prior to incubation in penicillin "G" 250 µg/ml and streptomycin sulphate 250 µg/ml in Waris medium. The cells were then washed three additional times in

Waris medium alone. The efficacy of this washing procedure was tested by plating equal numbers of washed and unwashed cells on nutrient agar and on Waris medium agar supplemented with 1 percent glucose at both 37°C and 25°C and examining the plates after 48 hours. Plates containing unwashed cells were grossly contaminated. Plates containing washed cells had four or fewer bacterial colonies. Autoradiograms showed the absence of labeling at the cell borders which might be expected to occur under conditions of significant bacterial contamination.

Unless otherwise stated, the following procedure was used for investigating protein synthesis in the developing and interdivisional cells. The cells were incubated for one hour in tritiated leucine, 10 µc/ml in Waris medium at 11°C and then fixed in 10% formalin. All experiments on interdivisional cells were carried out at approximately the same time of the day during the dark period. In one set of experiments, the determination of the length of the interdivisional phase was based on observations of the length of the interdivisional phase for sister cells.

In some experiments a chase of cold amino acid was used. These cells were washed in Waris medium three times immediately following the one hour incubation in isotope and then immediately incubated in cold leucine 100 times the concentration of the isotope, (0.26 mg/ml in Waris medium). The second incubation period was two hours at 11°C.

In addition to studying H^3 leucine incorporation, C^{14} valine was used as a protein precursor. In this experiment cells were incubated in L valine C^{14}, 5 µc/ml for two hours at 11°C.

All cells were fixed in 10% neutral formalin for one or more hours after incubation in isotope or cold amino acid. In all experiments

in which tritium incorporation was analyzed, the cells were dehydrated after fixation and individually embedded in maraglass. The sections were cut with a glass knife at a microtome setting of 0.5 microns on a Porter-Blum MT-1 or MT-2 microtome. Since section thickness variation is of critical importance in obtaining quantitative autoradiographic data in thin sections, the uniformity of section thickness was tested in the following way. Four randomly chosen sections from the same cell and four randomly chosen sections from different cells were measured with the filar micrometer on folded areas and found to vary from 0.55 microns to 0.65 microns. The variation between section thickness from the same cell was the same as between sections from different cells and, since more than two sections of each cell were used for grain counts, it was concluded that no consistent error resulted from the method used.

In the experiments utilizing C^{14} valine, unlike those utilizing tritiated leucine, whole cells were placed on slides after fixation and processed in the same manner as the sections. This method could not be used with a tritium precursor because the path length of the beta emission is short and the cell walls are thick.

Slides were processed with Kodak AR 10 stripping film. Most autoradiograms were exposed for two weeks in the dark at 4°C then developed in Kodak D19 developer and cleared in Kodak acid fixer. A few autoradiograms were exposed for seven to eight weeks. All autoradiograms were unstained.

The quantitative data were obtained by counting silver grains within a $25\mu^2$ area over sections and 30 areas per section were counted. Four central sections for each cell were analyzed (in three cases fewer than four central sections were used). The use of four sections per cell for grain counts minimizes the differences in grain counts which

might arise from differences in section thickness, as discussed above. Background counts for each slide were determined by counting grains in twenty $25\mu^2$ areas close to the section and averaging these counts. In all cases the data are presented with background counts subtracted.

In order to demonstrate that the measured incorporation was into trichloroacetic acid (TCA) insoluble material, the following two types of experiments were done. First, whole cells, which had been incubated in H^3leucine 10 µc/ml, for one hour at 11°C, were treated after fixation with 5% TCA at 4°C for ten minutes. Grain counts over sections of these cells were compared with grain counts over sections of similar cells not treated with TCA. In the second type of experiment, some of the sections of one cell which had been incubated in H^3leucine, 10 µc/ml, for one hour at 11°C were treated with 5% TCA at 85°C for ten minutes, and the grain counts over these sections compared with grain counts over untreated sections of the same cell. These data are presented in Table I. In both cases there is no loss of grains following TCA treatment, indicating that the H^3leucine that remains after processing for autoradiography is TCA insoluble and is, therefore, assumed to be present in protein.

The anucleate type of development in M. thomasiana was obtained by incubating cells during cytokinesis in a solution of Actinomycin D, 100 µg/ml at 25°C for one hour. Some cells were incubated in H^3leucine, 10 µc/ml, simultaneously with Actinomycin D treatment. Others were incubated in H^3leucine, 10 µc/ml, at 25°C for one hour in the first, third, and twenty-fourth hour after the one hour Actinomycin D treatment. A control cell was incubated in H^3leucine, 10 µc/ml, for one hour at 25°C at the same stage of development and was not treated with Actinomycin D. In one experiment a chase of cold leucine, 0.26 mg/ml, for two hours at

25°C was used.

In some cells displaying the anucleate type of development, the extent and localization of RNA synthesis was determined by means of the following procedure. Normal cells in cytokinesis through Stage I of development were incubated in H^3 adenosine, 50 uc/ml, for one hour at 25°C. These were compared with cells treated with Actinomycin D, 100 ug/ml, for one hour at 25°C during cytokinesis. Some of the latter cells were incubated in H^3 adenosine, 50 uc/ml, for one hour at 25°C during treatment and some, during the first or third hour after treatment. After fixing, embedding and sectioning these cells, as described previously, some sections were left untreated while the remaining sections were treated in one of the following ways: 1.incubation in RNAase, 1 mg/ml in distilled water at pH 6.8 at 37°C for twenty-four hours; 2. incubation in DNAase, 0.2 mg/ml in 0.003M $MgSO_4$ at pH 6.5 at 37°C for twenty-four hours; 3. incubated in distilled water at pH 6.5 at 37°C for twenty-four hours. Since it is difficult to stain the DNA of the nucleus in Micrasterias sections the effectiveness of the DNAase digestion was determined using formalin fixed, maraglas embedded frog liver sections. All sections were then filmed and processed as described.

The relative concentration of total chlorophyll in live cells was determined by measuring a complete absorption spectrum and extinctions at 650 mu through a plug with a five micron diameter using a Leitz microspectrophotometer. From four to sixteen measurements per cell through different regions of the chloroplast were taken to eliminate variation due to differences in cell thickness.

Protein determinations were done on whole fixed cells selected from the developmental and interdivisional phases of the life cycle.

The cells were fixed in 10% neutral formalin, dehydrated and the chlorophyll extracted with acetone until no green color was discernible. The cells were then rehydrated and the Millon reaction done according to the procedure of Mirsky and Pollister as modified by Rasch and Swift (43, 47). The intensity of the staining reaction was determined by measuring the extinction at 500 mμ with a Leitz microspectrophotometer through a plug of five microns in diameter over the cytoplasm and the nucleus and 2.5 microns in diameter over the pyrenoids. A mechanical slit width of 10 was used giving a spectral slit width of 4 mμ. An attempt was made to match the refractive index of the cells using an oil of 1.516 refractive index. Extracted unstained cells were measured at 500 mμ to determine non-specific light loss. From four to eight measurements were taken per cell to obtain an average through different areas of different thicknesses of the cell.

The sources of the chemicals used were as follows:
DL leucine 4,5 tritiated, sp. act. 500 mc/mM, Nuclear Chicago
L valine C^{14}, uniformly labeled, sp. act. 11.71 mc/mM, Volk
Penicellin "G", Nutritional Biochemicals Corporation
Streptomycin Sulphate, Nutritional Biochemicals Corporation
Dactinomycin (Actinomycin D), donated by Merck, Sharpe and Dohme
Adenosine, tritiated, G.L., sp. act. 8.76 C/mM, New England Nuclear Corp.
Ribonuclease and Deoxyribonuclease I, Worthington Biochemicals Corporation
L leucine, Nutritional Biochemicals Corporation

All autoradiograms were photographed under a Leitz 54 power oil immersion lens in order to include a sufficiently large area of the cell to observe grain distribution. All grain counts were made under a Zeiss 90 power oil immersion lens.

Results

When a live interdivisional cell is observed in face view the chloroplast extends to the edges of the cell leaving only a clear border of cell wall. When the cell is viewed from the side the chloroplast appears to fill the entire cell. In sectioned material an indication of a thin layer of cytoplasm can be seen in side view in the autoradiograms, Fig. 3, a side view section toward the periphery of the cell shows a thin region of extrachloroplastic cytoplasm at the edge of the section. Fig. 4 is a side view section through the central region of the cell showing the nucleus and the chloroplast. No large central vacuole is observed in these cells.

The chlorophyll absorption spectrum was measured through a region of the live cell chloroplast. There is a peak from 420 to 440 mμ and another sharp peak at 660 mμ with almost no absorption from 520 to 590 mμ. Since extinctions at 660 mμ through dividing cells were too great to be accurate, the extinction at 650 mμ, slightly off the peak, was used for determining chlorophyll concentration instead. The data in Table II are expressed as extinctions through the chloroplast area of whole cells and therefore express relative chlorophyll concentrations. Since the volume of the cell does not change during the interdivisional phase, these data also represent the relative chlorophyll content per cell. The data in Table II show that the chlorophyll concentration does not change significantly between the beginning of the interdivisional phase and the premitotic stage. The variation which is evident is probably primarily due to non-specific light loss since living cells were used and no attempt to match the refractive index could be made. The chlorophyll concentration

doubles at the premitotic stage and this concentration is maintained until the chloroplast spreads out into the new hemicell in stage III. As the developing hemicell gets larger the chloroplast spreads out in it and the extinction at 650 mμ (and, therefore, the concentration) decreases.

The changes in protein concentration in whole cells throughout the cell cycle as determined cytophotometrically following the Millon reaction are reported in Table III. As in Table II, the data are expressed as extinctions per cell and therefore relative protein concentration. Measurements were taken through the nucleus, pyrenoids, chloroplast and cytoplasm exclusive of the chloroplast (extrachloroplastic cytoplasm). The extrachloroplastic cytoplasm lies above and below the chloroplast and within the new hemicell before the chloroplast enters. During the interdivisional phase there is no volume change, therefore the measurements reflect relative protein content as well as relative concentration. In spite of the fact that the limit of accuracy of this method is about 20% (47), the data indicate that the protein concentration stays the same from the beginning of the interdivisional phase until the premitotic stage, when it approximately doubles. The concentration then returns to the interdivisional value by stages IV and V of development. These changes in protein concentration are similar to the changes seen in chlorophyll concentration as reported in Fig. 5. Each point in this figure represents the mean extinction for each individual cell. Table III also shows the high concentration of protein in the chloroplast of the new hemicell in stage III when the chlorophyll concentration is also high. The protein concentration in the extrachloroplastic cytoplasm in stages I and II, (comparable to the clear areas seen

in the new hemicell Fig. 2, stages I and III), is about the same as that of the mature hemicell during the interdivisional phase. These data are also given in Table III.

The pyrenoids and the nucleus stain deeply with the Millon reagent as seen in Fig. 6. Table IV shows the relative protein concentration for the cytoplasm, nucleus and pyrenoids expressed as extinctions per micron thickness of each cell component. The approximate thickness of the nucleus and pyrenoids and both the central and peripheral regions of the cytoplasm were measured with the firm adjustment of the microscope. Measurements of five cells were averaged. Extinction of nuclei and pyrenoids were corrected for chloroplast or cytoplasmic absorption using the following formulae: $E_{nucleus} = E_{whole\ cell} - 0.28\ E_{cytoplasm}$ and $E_{pyrenoids} = E_{whole\ cell} - 0.5\ E_{cytoplasm}$. The nuclei have about three or four times, and the pyrenoids about five times, the protein concentration of the cytoplasm.

The distribution of H^3leucine incorporation shows several unexpected features in view of the results of protein concentration presented above. There are areas of the chloroplast which are not labeled which correspond in size and position to the pyrenoids observed in whole cells, (compare Figs. 6 and 7). These areas also stain intensely with fast green at low p^H. The pyrenoids do not accumulate label after a two hour chase in cold leucine nor after twenty-four hours in isotope, despite their high protein content (Fig. 8). Table V reveals that the label over the chloroplast is about twice that of the extrachloroplastic cytoplasm while the protein concentration of these two cell components is the same (Table III). The label in the chloroplast of the new hemicell and the

chloroplast of the old hemicell is equal in all cells observed. These same relationships hold for cells chased for two hours in cold leucine. This is shown in Figs. 9 and 10. In Table V, as in all subsequent tables on which grain counts are given, the quantitative data are presented as mean counts for one cell $\pm$ the standard error. The standard errors are for 30 counts of $25u^2$ areas of four sections each with background counts subtracted. The data in Table V indicate that significant differences may exist between cells with the same treatment. Biological variability of this kind is also indicated by differences in the length of the interdivisional phase of cells observed within one culture.

The nucleus in both the developing and interdivisional cells is more heavily labeled than the cytoplasm (Fig. 7). This is comparable to the high nuclear protein concentration indicated by the Millon reaction (Fig. 6).

Table VI shows the specific activities of three cell components expressed as silver grains per unit of dye bound in accordance with the following formula:

$$\text{sp. act.} = \frac{\text{number of grains per micron}^2}{\text{Millon } E_{500} \text{ per micron of cell component thickness}}$$

The specific activity of the chloroplast protein appears to be about twice that of the cytoplasmic protein exclusive of the chloroplast. The specific activity of the nuclear protein is about the same as that of the chloroplast protein.

The incorporation of H^3 leucine into the chloroplast protein per hour has been investigated throughout the cell cycle and the results of this set of experiments are shown in Tables VII and VIII. For the interdivisional cells both the interdivisional day when the cell was incubated

in isotope and fixed and the total length of the interdivisional phase, based on the length of the interdivisional phase of a sister cell, are indicated. For the developing cells the stage of development indicates the stage the cell had reached at fixation. The transition between one stage and another at 11°C is a little over one hour. The combined data for Tables VII and VIII are shown graphically in Fig. 11. The highest level of incorporation occurs on the first three interdivisional days. The incorporation then decreases until cytokinesis and stage I of development, after which there is a gradual rise. In general, the amount of incorporation of H^3leucine into the protein of the chloroplast per hour during cytokinesis and development is lower than during the interdivisional phase. This difference is significant at the 1% level. An analysis of variance shows no significant difference in incorporation between cells incubated on different days of the interdivisional phase or in different stages of development. The data indicate clearly that the amount of incorporation is not greater during mitosis and cytokinesis when the protein concentration is double.

Since no premitotic cell was included in Table VII and VIII and since this is the stage when chlorophyll and protein concentrations are doubled, a separate set of experiments was performed to analyze incorporation at this stage. The criterion for selecting the premitotic cell was its darker green color.

An analysis of variance on this set of experiments, Table IX, shows no significant difference in the level of incorporation between cells incubated in isotope on each day of the interdivisional phase and cell labeled in the premitotic stage of the interdivisional phase. The data for the set of experiments presented in Table IX show a lower level of

incorporation than the cells presented in Table VII due to experimental variability, but each set of experiments is self consistent.

To gain insight into the stability of the newly synthesized protein and to determine whether there was any obvious migration of protein between cellular compartments, cells which had been incubated in H^3leucine for one hour were chased immediately after incubation for two hours in cold leucine 100 times the concentration of the isotope, (Tables X and XI). The chase was limited to two hours because developing cells if incubated longer in leucine of this concentration develop more slowly than normal and show abnormal morphology. Interdivisional cells are probably also adversely affected. In Table X, the increase in area attained by the developing cells during enlargement of the new hemicell is indicated. Since enlargement occurred during a chase, a dilution of the isotope might be expected. However an increase in the total number of grains over the cytoplasm is apparent. The number of grains over the nucleus appears to be somewhat less than in unchased cells but the difference is not statistically significant. Table XI compares cells in the interdivisional phase which were labeled for one hour in H^3leucine with cells chased for two hours in cold leucine after incubation in isotope. In most cases there is an increase in labeling over both the cytoplasm and the nucleus. In spite of the variability from cell to cell, the overall means of grain counts over the cytoplasm and nuclei show approximately the same percent increase.

Cells were also incubated for one hour in H^3leucine during a given interdivisional day and then transferred to medium without isotope for several days until division and development of the new hemicell occurred

at which time they were fixed. Table XII shows that despite the increase in volume due to the enlargement of the new hemicell the number of grains per unit area is the same as in cells fixed immediately after incubation in isotope (Table VII). The data from Tables VII and XII are directly comparable because these experiments were performed at the same time. Table XII also shows that the number of grains per unit area is the same in the chloroplasts of both the old and new hemicell. Fig. 12 shows a cell which remained in unlabeled medium for seven days before the new hemicell developed. This section of the cell includes the nucleus and shows that it is heavily labeled compared to the cytoplasm. Fig. 13 shows a cell which remained in unlabeled medium for three days after incubation in isotope. The proportion of label in the chloroplast compared with that in extrachloroplastic cytoplasm is about the same as that of cells labeled during development (Fig. 9). In both Fig.12 and Fig.13 the equal distribution of label over the chloroplasts of both the old and new hemicells can be seen.

The distribution of the label in the extrachloroplastic cytoplasm in the new hemicell does not indicate the presence of central axes nor peripheral distribution of newly synthesized protein even with long term exposure of autoradiograms nor when cells are chased in cold leucine. Fig. 14 is an autoradiogram of a cell in stage II of development where central axes in the new hemicell might be observed. The presence of vacuoles in the developing hemicell,(Fig.2,stage II), probably accounts for the lack of homogeneity in labeling over the new hemicell. Fig.15 is an autoradiogram of a cell incubated in H^3leucine during mitosis and chased two hours in cold leucine until stage II of development. The uniformity of label in the new hemicell indicates that protein synthesized prior

to the development of the new hemicell is not assembled into axial patterns nor accumulated at the periphery of the new hemicell. Figs. 9 and 10, which show cells in stage III of development, also show uniform distribution of grains over the extrachloroplastic cytoplasm both with and without a chase in cold leucine. In Fig. 16 a cell incubated in C^{14} valine in stage V of development is shown. Despite the fact that in whole cell autoradiographs density differences in regions of the chloroplast are evident, one can observe that there is a uniform distribution of grains over regions outside of the chloroplast as well as over the chloroplast.

Part Two

Cells incubated in Actinomycin D(100 µg/ml) for one hour at cytokinesis show the typical anucleate morphology when allowed to continue development (Figs. 17 and 18). Cells were inclubated in H^3adenosine at the same time, and subsequent to, Actinomycin D treatment to determine whether RNA synthesis was shut down during treatment and whether or not the cells recovered the ability to synthesize RNA thereafter. Fig. 19 is an autoradiogram of a normal cell labeled for one hour with H^3adenosine. The cytoplasmic label is neither RNAase nor DNAase removable, as shown in Figs. 20 and 21. When a cell incubated in H^3adenosine simultaneously with Actinomycin D treatment, there is no nuclear incorporation (Fig. 22). An adjacent section stained with Azure B, Fig. 23, indicates that the nuclei are present in the section that was filmed. Only the cytoplasmic label is present which is not RNAase digestible, indicating that no demonstrable RNA synthesis occurs at the time the cells are in Actinomycin D. Cells incubated in H^3adenosine three thours after

Actinomycin D treatment also show no nuclear label (Fig. 24). This indicates that the cells do not recover the ability to synthesize RNA within three hours after Actinomycin D treatment.

Cells were also labeled with H^3leucine for one hour both simultaneously with Actinomycin D treatment and at different intervals following treatment to determine whether they synthesize protein at these times. Table XIII indicates that H^3leucine incorporation occurs at 26% of the normal level during Actinomycin D treatment. By three hours after treatment incorporation is normal. The data also indicate that the label persists after a chase in cold leucine was given following simultaneous incubation in H^3leucine and Actinomycin D. Fig. 25 is of a cell incubated one hour in H^3leucine during the first hour after Actinomycin D treatment. Label appears in both the old and new hemicells. Note the absence of any pattern suggesting axial structures. Fig. 26 shows a cell incubated in H^3leucine during the third hour after Actinomycin D treatment which shows typical anucleate morphology. In this cell the chloroplast and the extra chloroplastic cytoplasm are both evident in the new hemicell. As in untreated cells, the extra chloroplastic cytoplasm has less label than the chloroplast. At sometime between three and twenty-four hours after Actinomycin D treatment the cells lose their ability to synthesize protein. This is indicated by the absence of incorporation in cells incubated in H^3leucine for one hour 24 hours after treatment which is consistent with the short lifetime of the anucleate cell.

Discussion

The cell volume of Micrasterias thomasiana doubles in the course of development of the new hemicell. This doubling occurs abruptly; occupying only one-fifteenth of the cell cycle. Two alternative courses of events could accompany this doubling. Rapid synthesis of protein could be involved or the desmid could resemble higher plant cells, (e.g. root tip cells), in which rapid enlargement results substantially from water uptake and vacuolar enlargement. In elongating root tip cells the amount of protein does not appear to increase although the rate of incorporation of labeled amino acids may (25) suggesting balanced synthesis and breakdown. The results presented here demonstrate that the chloroplast has doubled before cytokinesis. The results also show that the incorporation of labeled leucine into the chloroplast protein is less at the time of cell enlargement than during the rest of the cell cycle. Incorporation of labeled leucine into the extrachloroplastic cytoplasm during development cannot be compared to that during the interdivisional phase, since this cytoplasm takes up such a small part of the interdivisional cell. The specific activity of the extrachloroplastic cytoplasm is only one-half of that of the chloroplast and nucleus. These results tend to support Kiermayer's evidence that the enlargement of the new hemicell is largely by turgor pressure (32, 36) rather than by intense protein synthesis. Enlargement of the new hemicell is not entirely a passive process as indicated by some incorporation of labeled leucine at this time. However, it appears to involve mainly the redistribution of previously replicated material between the old and new hemicells. During the development of the new hemicell the chloroplast entering the new hemicell can be seen to slide over the old hemicell chloroplast, (Fig. 2, stage III),

reflecting the fact that the chloroplast divides in a plane parallel to the face of the cell.

Among the possibilities to be considered for the control of the shape of the new hemicell are: 1. uneven deposition of material at the periphery of the new hemicell and 2. the outgrowth of cytoplasmic axes. The results of autoradiography presented here show no localization of newly synthesized protein or accumulation of protein in any region of the new hemicell. Thus this evidence does not support either theory. However, it is possible that deposition or accumulation of substances other than protein (e.g. wall material) at the cell periphery may occur. There is no precedent for nonprotein elements within plant cells corresponding to the cytoplasmic axes. The assumption is made that these axes are protein. Therefore the data showing no protein axes tends to support the first theory.

Algal chloroplasts arise by division of pre-existing chloroplasts and their division may be independent of cell division (6, 12, 19, 38). The time in the cell cycle at which chloroplast replication occurs differs in different algae. In Euglena deses, when the cell divides the chloroplasts are apportioned between daughter cells. They replicate and divide during the interdivisional phase of the cell cycle (14). In Spirogyra the largest increase in chloroplast length occurs during the night following cell division (55). In Ulva thallus cells on the other hand, increase in the chloroplast material, as indicated by increased oxygen evolution, (40) occurs just before cell division which is concurrent with chloroplast division (41). The results presented here show that in M. thomasiana, the synthesis of the chloroplast components occurs about twenty-four hours before cell division. The chlorophyll and protein content

approximately double concurrently at this time while remaining constant throughout the rest of the cell cycle. The finding of concomitant increases in chlorophyll and in protein content agrees with the findings of others that chlorophyll and chloroplast membrane synthesis occur together (23, 24, 45, 50). Since algal chloroplasts have ribosomes (18) and synthesis protein (13) an increase in the rate or amount of incorporation of labeled amino acids into chloroplast protein might be expected at the time of chloroplast replication. In Euglena, during chloroplast greening, an increase in the amount of chloroplast protein occurs (52) and incorporation of labeled amino acids into chloroplast and other cellular protein increases (3). The data presented here show a relatively constant rate of incorporation of labeled leucine into chloroplast protein throughout the cell cycle in M. thomasiana. There are several possible explanations for the failure to observe a rise in incorporation at the time in the cell cycle prior to or during the observed doubling of the chloroplast protein: 1. The labeled amino acid given twenty-four hours before cell division might be diluted by a larger endogenous amino acid pool existing at this time compared to the rest of the cell cycle. 2. The protein synthesized at the time of chloroplast replication might be stable, as indicated by increase in protein content, while the protein synthesized at other times in the cell cycle might be unstable, as indicated by amino acid incorporation without increase in protein content. The evidence from the chase experiments shows gain of label in all phases of the cell cycle. This suggests retention of a large pool of unchased labeled amino acid. Therefore, the question of differential protein stability cannot be answered.

The equal distribution of label in the chloroplasts in the old and

new hemicells has been demonstrated. This is particularly evident in cells which were incubated in isotope early in the interdivisional phase and allowed to develop the new hemicell several days later in unlabeled medium. This strongly suggests that the chloroplasts in this organism grow by random intussusception of protein into chloroplast membranes. This type of membrane growth has also been found in mitochondria (42) during their genesis. Random intussusception of lipid into the membranes of chloroplasts in greening Chlamydomonas cells has been found (16). As a result of the evidence presented here it is likely that the same method of growth occurs during normal chloroplast replication as during greening.

Pyrenoids occur in the chromatophores of all green algae and in all major algal divisions except Cyanophycophyta (4). Their function has been postulated to involve starch synthesis or they may serve as areas of stored protein in other algae (37). They are differentiated regions of the chloroplast stroma which in some genera including Micrasterias, have lamellae continuous with the chloroplast lamellae (9). They have been reported to contain chlorophyll (39). The results presented here show that the pyrenoids in whole cells of M. thomasiana stain deeply with the Millon reagent which is specific for the tyrosine and tryptophane residue of proteins. In contrast to their high protein content, the results of autoradiography show that little or no protein synthesis occurs in the pyrenoids. There is evidence that the accumulation of protein must occur at a very slow rate. As a result of the present investigation it can be stated that protein synthesis is not a function of the pyrenoids. While pyrenoids have been reported both to divide (17) and to arise

de novo (62) a distinction between these two possibilities cannot be based on their lack of protein synthesis.

The results presented here show that the nucleus is heavily labeled during one hour of incubation in H^3 leucine in all stages of the cell cycle. It is possible that this represents nuclear protein synthesis or the accumulation of protein synthesized in the cytoplasm or both. Whether cell nuclei can synthesize protein has been investigated by others. Thymus cell nuclei in vitro do carry out protein synthesis (1); while ribosomes from tobacco cell nuclei cannot support protein synthesis (11). There is evidence for cytoplasmic synthesis of protein fractions which are subsequently transferred to the nucleus, as has been found for histones in HeLa cells (48) and for a nuclear protein fraction in Amoeba (5). If newly synthesized protein were transferred from the cytoplasm to the nucleus, one would expect a decrease in cytoplasmic grains with a concomitant increase in nuclear grains during a chase. During the interdivisional phase both the nucleus and the chloroplast gain label to the same extent after a chase implying retention of a pool of labeled amino acids. In the developmental phase, where the extrachloroplastic cytoplasm as well as the chloroplast and the nucleus can be seen, there is relatively little gain in label in the chloroplast and extrachloroplastic cytoplasm and no gain in nuclear label. The ratios of grains in the three compartments do not change significantly from those in unchased cells. Therefore there is no evidence for transfer of protein. However, the labeling time of one hour is too long to exclude perinuclear protein synthesis with rapid transfer of protein into the nucleus. Support for this idea comes from evidence for lack of nuclear ribosomes (46) and also from the failure to observe ribosomes in electron microscopic studies of the

M.rotata nucleus (8).

The question of the occurrence of cytoplasmic inheritance in the early development of the new hemicell of M. thomasiana may be considered in the light of the autoradiographic evidence from both the normal and Actinomycin D treated cells. Two ideas will be considered: first, whether there is any evidence for the existence of the proposed "cytoplasmic framework" and second, whether there is any evidence for the existence of stable nuclear RNA at the time of development of the major lobes.

The cytoplasmic framework postulated by Waris and Kallio,(26,57,61) is an unusual kind of cytoplasmic inheritance. It is not comparable to cytoplasmic inheritance involving mitochondria and chloroplasts or cortical pattern in Paramecium (54) or inheritance of flagellae in Trypanosomes (63). It is possible that a massive array of microtubules is involved in the postulated cytoplasmic axes. In some cells microtubules play a role in determining shape (56). If the outgrowth of fibrillar axes occurs during the development of the new hemicell - normal or anucleate - then it ought to be possible to detect labeled amino acid incorporation into these structures in cells labeled during early development. If protein units which are synthesized before division, are assembled during the early development of the new hemicell to form these axes, then it should be possible to detect such an event in cells labeled before division and allowed to form the early stages of the new hemicell in a chase. The results presented here show that no such event occurs. This makes it unlikely that the synthesis of microtubules is involved in the formation of these axes. Microtubules

have been found in M. rotata but their distribution within the cell in no way suggests the postulated framework (34). No other fine structure or arrangement of organelles occurs in M. rotata corresponding to fibrillar axes or suggesting their formation (35).

These results show that M. thomasiana cells treated at cytokinesis with Actinomycin D not only develop like the physically enucleated cells but also lack demonstrable nuclear RNA synthesis, as expected (15), both during Actinomycin D treatment and for at least three hours following treatment. The Actinomycin D treated cells carry out protein synthesis at reduced levels initially but protein synthesis returns to normal by the third hour after treatment at which time there is still no nuclear RNA synthesis. This suggests that if there is any secondary effect of Actinomycin D on protein synthesis, as found with Ilyanassa embryos at 200 ug/ml (7), the cells have recovered from it within three hours of treatment. Development in the absence of DNA directed RNA synthesis indicates that gene transcription directing the observed developmental event has occurred at some time prior to the event and implies that the m-RNA produced at that time is stable. A schedule for the relationship between the time of gene transciption and the time of appearance of certain proteins or morphological events utilizing Actinomycin D has been studied for slime molds (49), Ilyanassa embryos (7) and Micraterias thomasiana (51). In Micrasterias the morphological events recorded are the splitting of the side lobes and the appearance of dentition. In Selman's experiments (51), the same concentration of Actinomycin D used at a somewhat later time than in the present work, produced cells slowing reduction in side lobe number and simplification of lobes and with greater longevity than the physically enucleated cells. These experiments indicate

a time lag of between 30 and 155 minutes between transcription and the observation of a given morphological change. In the work presented here the cells were treated with Actinomycin D at cytokinesis and a lag of at least three hours was observed from the time of transcription and the appearance of the major lobes. The effect of treatment at an earlier time to find out when transcription for information for three lobes takes place could not be studied because cells treated at mitosis or before die. The results presented here strongly support Selman's interpretation that the nucleus controls the splitting of the side lobes since they show that protein synthesis occurred during the absence of RNA synthesis indicating stable m-RNA. The results presented here also suggest that the nucleus affects the early development of M. thomasiana by the production of relatively stable m-RNA sometime prior to cytokinesis. There is a great deal of accumulated evidence for the presence of stable m-RNA which directs the protein synthesis necessary for developmental events. A stiking instance of m-RNA stability is in Acetabularia (2, 10). Stable material m-RNA also direct protein synthesis and early development in amphibia and sea urchins (44) although some gene transcription takes place even in very early development in the sea urchin (20, 44). In view of the widespread occurrence of stable m-RNA it is not unexpected that there is some evidence for it in Micrasterias.

The possibility exists that early development in Micrasterias might be directed by extranuclear DNA. Since the incorporation of H^3adenosine which occurs in the cytoplasm of both the Actinomycin D treated and the untreated cells is not RNAase digestible no conclusion can be drawn about the effect of Actinomycin D on the extranuclear DNA. However it seems unlikely that mitochondrial or chloroplast DNA could direct morphogenesis.

The possibility that proteins synthesized in the chloroplast as opposed to extrachloroplastic cytoplasm may be involved in morphogenesis could be investigated using protein synthesis inhibitors specific for 70S or 80S ribosomes.

There is some additional evidence from experiments done by Kallio (31) indicating that the formation of the three major lobes in Micrasterias sol may be directed by the nucleus at some time before cell division, (although this is not his interpretation). Ultraviolet irradiation of whole interdivisional cells, using the dose which causes anucleate type development when given at mitosis, shows no effect until development occurs. At this time some cells with missing wings are produced (31). The time in the interdivisional phase when the irradiation was given was not reported. If one knew on which day of the interdivisional phase the ultraviolet irradiation produced its effect, it might be possible to determine the time of the nuclear effect on the formation of the major lobes.

The evidence presented here suggests the presence of stable nuclear RNA which is sufficient to account for the early development of M. thomasiana without the necessity of invoking a form of cytoplasmic inheritance. While the evidence for stable nuclear RNA does not in itself rule out the possibility of the presence of a "cytoplasmic framework," there is no evidence for the growth of such a protein framework in developing cells. Although the mode of transmission of the uniradiate and aradiate defects in the "Janus" cell remains difficult to explain, the weight of the evidence presented here favors the idea of nuclear control over the development of the new hemicell in Micrasterias thomasiana.

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Table I

H^3 leucine incorporation in M. thomasiana cells, developmental phase, following TCA treatment of whole cells and sections.

	Untreated		Treated	
	Number of Cells	Mean Grains/25u^2 ± Standard Error	Number of Cells	Mean Grains/25u^2 ± Standard Error
Whole Cells				
Cytoplasm	4	10.4 ± 0.4	3	9.9 ± 0.6
Nucleus	1	35.0	1	32.5
Sections				
	Number of Sections		Number of Sections	
Cytoplasm	2	7.5 ± 0.7	2	8.5 ± 0.9
Nucleus	2	19.5	2	21.0

Whole cells were treated with 5% TCA at 4°C for 10 minutes.
Sections were treated with 5% TCA at 85°C for 10 minutes.

Table II

Relative chlorophyll concentration of living M. thomasiana cells throughout the cell cycle.

Interdivisional Day	Number of Cells	Mean Extinction at 650 mμ
1	4	.555
2	3	.430
3	2	.342
4	3	.463
5	2	.527
premitotic	2	.900
Developmental Stage		
cytokinesis	1	1.004
III	1	.903
V	2	.745

Each cell was measured through a plug of 5μ diameter.
From 4 - 16 areas per cell were measured.

Table III

Relative proten concentration(Millon) of the cytoplasm of whole *M. thomasiana* cells throughout the cell cycle.

Interdivisional Day	Number of Cells	Mean Extinction at 500 mμ Mature Hemicell
1	4	.158
2	2	.193
3	2	.160
4	2	.173
5	2	.184
premitotic	1	.450

Developmental Stage		Mature Hemicell	New Hemicell	
			Chloroplast	Extrachloroplastic cytoplasm
cytokinesis	2	.409		
I	1	.350		.189
III	2	.250	.433	.131
IV	2	.141	.188	
V	2	.186	.203	

Each cell was measured through a plug of 5μ diameter.
From 4-8 areas per cell were measured.

Table IV

Relative protein concentration(Millon) of four cellular components measured in whole cells throughout the cell cycle.

Cell Component	Number of Cells	Number of Measurements	Mean Extinction at 500 mμ ± Standard Error	Approximate Thickness of Cell Component in μ	Mean Extinction at 500 mμ per μ Thickness of Cell Components
Chloroplast	22	132	.230 ± .022	18.5	.013
Nucleus	17	34	.609 ± .047		
			(.546)*	13.6	.040
Pyrenoid	14	28	.512 ± .042		
			(.397)*	8.0	.050
Extrachloroplastic cytoplasm	2	7	.160	13.5	.012

*Corrected extinctions as described in the text.
Standard errors are calculated from means of individual cells.

Table V

H^3 leucine incorporation into the protein of the chloroplast and extra-chloroplastic cytoplasm of the new hemicell of *M. thomasiana* cells, stage III of development.

Chloroplast		Extra-chloroplastic Cytoplasm	
Mean Grains/25μ^2 ± Standard Error	Mean of Two Cells	Mean Grains/25μ^2 ± Standard Error	Mean of Two Cells
13.5 ± 0.4		5.5 ± 0.4	
9.1 ± 0.5	11.3	3.9 ± 0.3	5.2
Cell chased in cold leucine, from stage II - III in chase			
8.5 ± 0.3		4.9 ± 0.3	

Table VI

Specific activity of protein of three cell components of *M. thomasiana* cells.

Cell Component	Number of Cells	Mean Grains per 25 μ^2	Mean Grains per μ^2	Extinction (Millon) per Micron Thickness	Specific Activity
Extra-Chloroplastic Cytoplasm	2	5.2	0.21	.012	17.5
Chloroplast	23	11.8 ± 0.4	0.47	.013	36.1
Nucleus	18	34.6 ± 1.2	1.4	.040	35.0

$$\text{Specific Activity} = \frac{\text{Mean Grains per micron}^2}{\text{Extinction at 500 m}\mu\text{ per micron thickness of cell component}}$$

Table VII

H^3leucine incorporated into protein of the chloroplast of *M. thomasiana* cells during the interdivisional phase of the cell cycle.

Interdivisional Day Labeled	Total Number of Interdivisional Days of the Sister Cell	Mean Grains/25μ^2 ± Standard Error
1	6	14.3 ± 0.4
1	5	16.4 ± 0.4
2	6	15.8 ± 0.7
3	6	13.5 ± 0.3
3	6	15.8 ± 0.3
4	6	13.7 ± 0.3
4	5	11.2 ± 0.2
5	6	11.5 ± 0.3
6	6	11.8 ± 0.4
overall mean		13.7 ± 0.2

Table VIII

H^3leucine incorporation into protein of the chloroplast of *M. thomasiana* cells during the development phase of the cell cycle

Developmental Stage	Mean Grains/25μ^2 ± Standard Error Individual Cells	Mean Grains/25μ^2 ± Standard Error Each Stage
cytokinesis	6.9 ± 0.3	
I	7.8 ± 0.3	
I	6.7 ± 0.5	7.3
II	11.8 ± 0.8	
II	10.9 ± 0.4	
II	8.3 ± 0.3	10.3 ± 0.8
III	8.9 ± 0.4	
III	12.6 ± 0.3	10.7
IV	9.5 ± 0.4	
IV	10.2 ± 0.5	
IV	10.9 ± 0.5	10.2 ± 0.3
V	11.5 ± 0.4	
V	10.7 ± 0.4	
V	12.1 ± 0.3	11.4 ± 0.3
overall mean		9.9 ± 0.5

Table IX

H^3 leucine incorporation into protein of the chloroplast of M. thomasiana cells during the interdivisional phase of the cell cycle, including the premitotic stage.

Day Labeled	Mean Grains/25μ^2 ± Standard Error
1	5.9 ± 0.2
1	7.6 ± 0.2
2	2.6 ± 0.1
2	4.1 ± 0.2
3	5.1 ± 0.2
3	3.7 ± 0.2
5	3.5 ± 0.2
premitotic	6.2 ± 0.2

Table I

Comparison of H^3 leucine incorporation in cells fixed immediately after one hour isotope incubation with cells chased for two hours in cold leucine.

Fixed Immediately		Chased		
Developmental Stage at Fixation	Mean Grains/25μ^2 ± Standard Error	Transition of Stages in Chase	Increase in Area as Percent of Whole Cell Area	Mean Grains/25μ^2 ± Standard Error
		Cytoplasm		
II	9.4 ± 0.4	II - III	3	11.4 ± 0.4
II	10.9 ± 0.7	I - II	1	12.2 ± 0.4
IV	9.9 ± 0.4	III - IV	8	9.4 ± 0.4
mean	10.1	mean		11.0
		Nucleus		
	31.6 ± 1.9(10)*			28.8(2)*

*Number of nuclei counted.

Table XI

Comparison of H^3 leucine incorporation of cells fixed immediately after one hour isotope incubation with cells chased for two hours in cold leucine.

Interdivisional Day Labeled	Fixed Immediately: Mean Grains/25μ^2 ± Standard Error Individual Cells	Chased: Mean Grains/25μ^2 ± Standard Error	Percent Increase in Average Number of Grains During Chase
	Chloroplast		
1	5.9 ± 0.2	10.0 ± 0.3	
1	7.6 ± 0.2	10.7 ± 0.3	
2	2.6 ± 0.1	5.0 ± 0.2	
2	4.1 ± 0.2		
3	5.1 ± 0.2	4.0 ± 0.2	
3	3.7 ± 0.2	3.0 ± 0.2	
5	3.5 ± 0.2	8.2 ± 0.2	
overall mean	4.6 ± 0.1	6.8 ± 0.1	48
	Nucleus		
1	15.7	30.0	
2	13.8	20.5	
3	15.3	18.5	
5	16.5	24.1	
overall mean	15.2	23.3	53

Table XII

Cells incubated one hour in H^3 leucine during the interdivisional phase of the cell cycle and transferred to unlabeled medium until division and development of the new hemicell.

Number of Days in Unlabeled Medium Until Development	Developmental Stage at Fixation	Mean Grains/25μ^2 ± Standard Error	
		New Hemicell	Mature Hemicell
7	5	11.0 ± 0.3	11.9 ± 0.3
3	4	12.5 ± 0.4	13.8 ± 0.4

Table XIII

H^3leucine incorporation into cytoplasmic protein of M. thomasiana cells treated with Actinomycin D for one hour during cytokinesis.

Time of Incubation in Isotope Relative to Actinomycin D Treatment	Stage of Isotope Incubation	Stage at Fixation	Mean Grains/$25\mu^2$ ± Standard Error	Level of Incorporation Percent of control
Control Untreated Cell	cytokinesis	I	9.4 ± 0.4	
Simultaneous	cytokinesis	cytokinesis	3.1 ± 0.2	26
	cytokinesis	I	1.9 ± 0.3	
One Hour After Actinomycin D	cytokinesis-I	II	5.7 ± 0.4	60
Three Hours After Actinomysin D	II	III	9.3 ± 0.4	
Cells chased with cold leucine for two hours after simultaneous incubation in H^3leucine and Actinomycin D.				
	cytokinesis	I	3.4 ± 0.3	37

Figure 1

Typical Micrasterias thomasiana cell x 150.

Wings (W) Polar lobe (P)

Figure 1

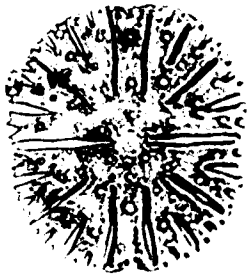


Figure 2

The stages of development of *Micrasterias thomasiana*.

Stage I x 100.

Stage II Vacuoles are seen in the cytoplasm of the new hemicell. x 100.

Stage III The arrow indicates the region where the chloroplast entering the new hemicell has pulled away from the chloroplast in the old hemicell. x 100.

Stage IV x 100.

Stage V x 100.

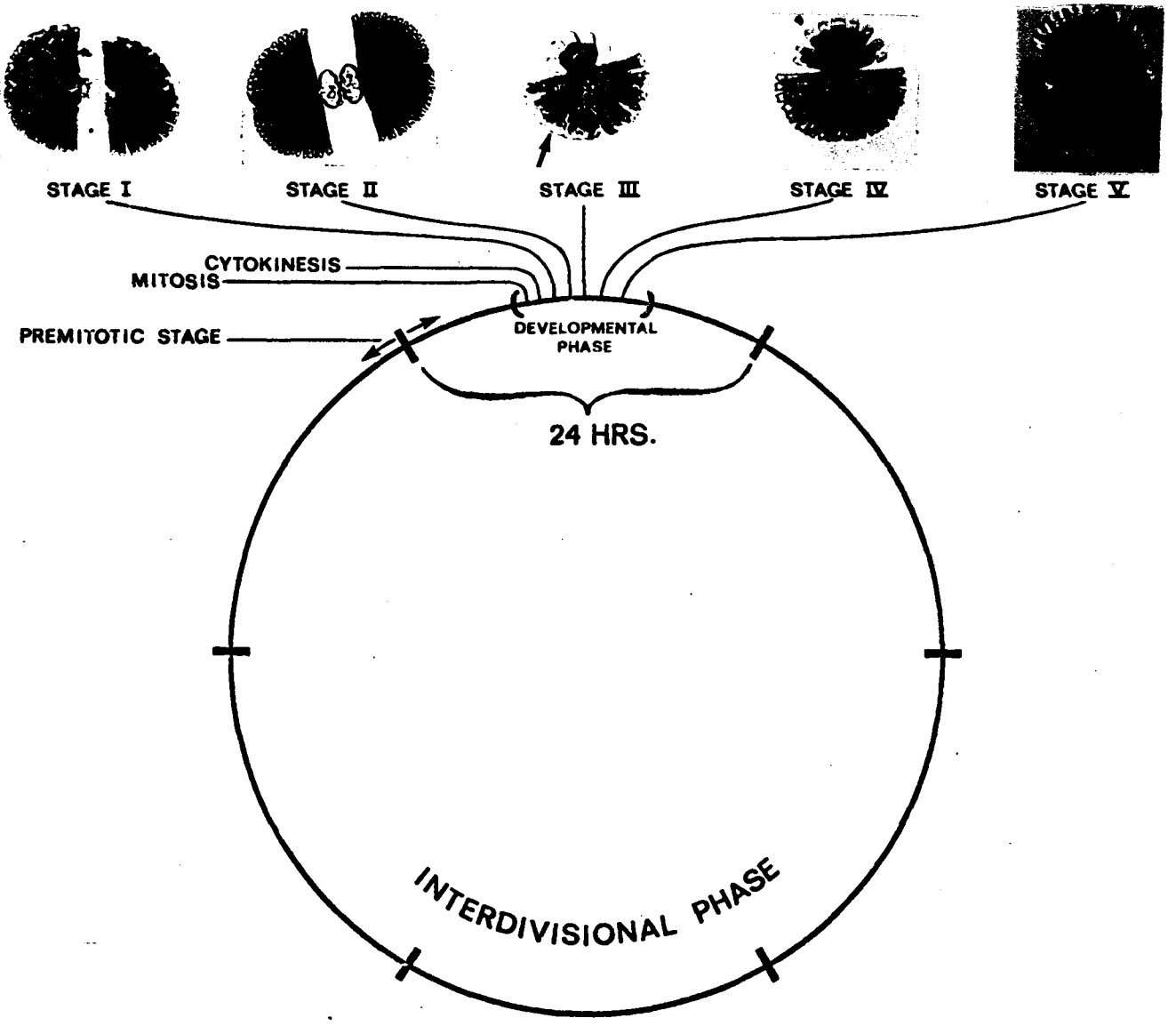


Figure 2. MICRASTERIAS THOMASIANA CELL CYCLE

Figure 3

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3leucine for one hour during the interdivisional phase; side view section through the lobe tops. There are fewer grains over the cytoplasm (C) peripheral to the chloroplast (CH). Cell border (CB). x 325.

Enlargement aprox. x750.

Figure 3

Figure 4

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3 leucine for one hour during the interdivisional phase, central side view section. The nucleus (N) is more heavily labeled than the cytoplasm. x 325.

Enlargement Aprox. x 750.

Figure 4

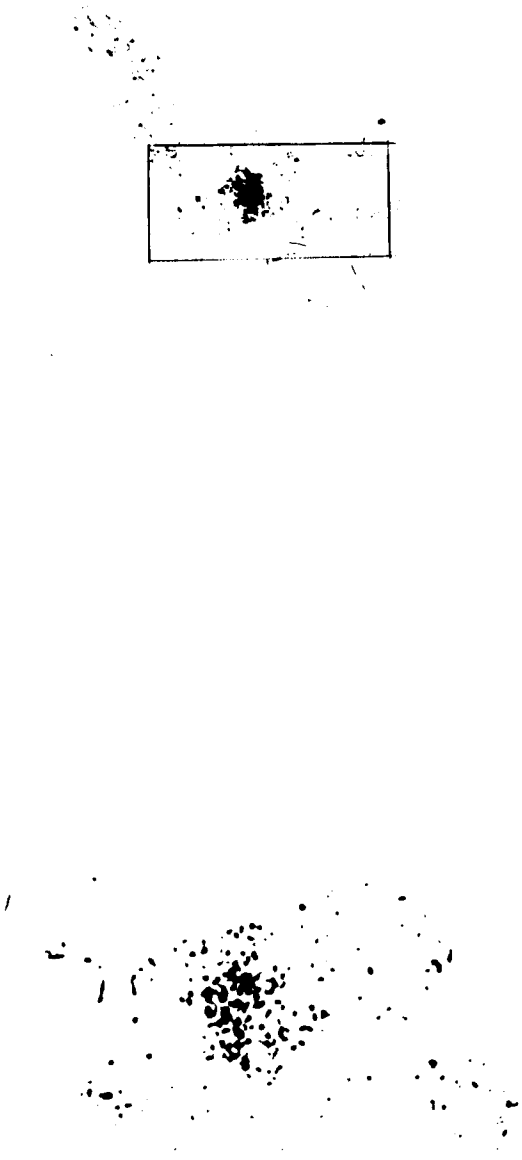


Figure 5

Relative chlorophyll and protein concentrations throughout the cell cycle.

Average number of interdivisional days is five.

The developmental stage lasts about one-third of a day.

P = premitotic stage of the interdivisional phase. The fact that this is not precisely timed is indicated by the arrow on either side.

C = cytokinesis

Roman numerals denote the stages of development.

The solid line indicates chlorophyll concentration (E_{650}).

The broken line indicates protein concentration (Millon, E_{500}).

RELATIVE CHLOROPHYLL AND PROTEIN CONCENTRATIONS
IN MICRASTERIAS THOMASIANA (MATURE HEMICELL)

FIG. 5

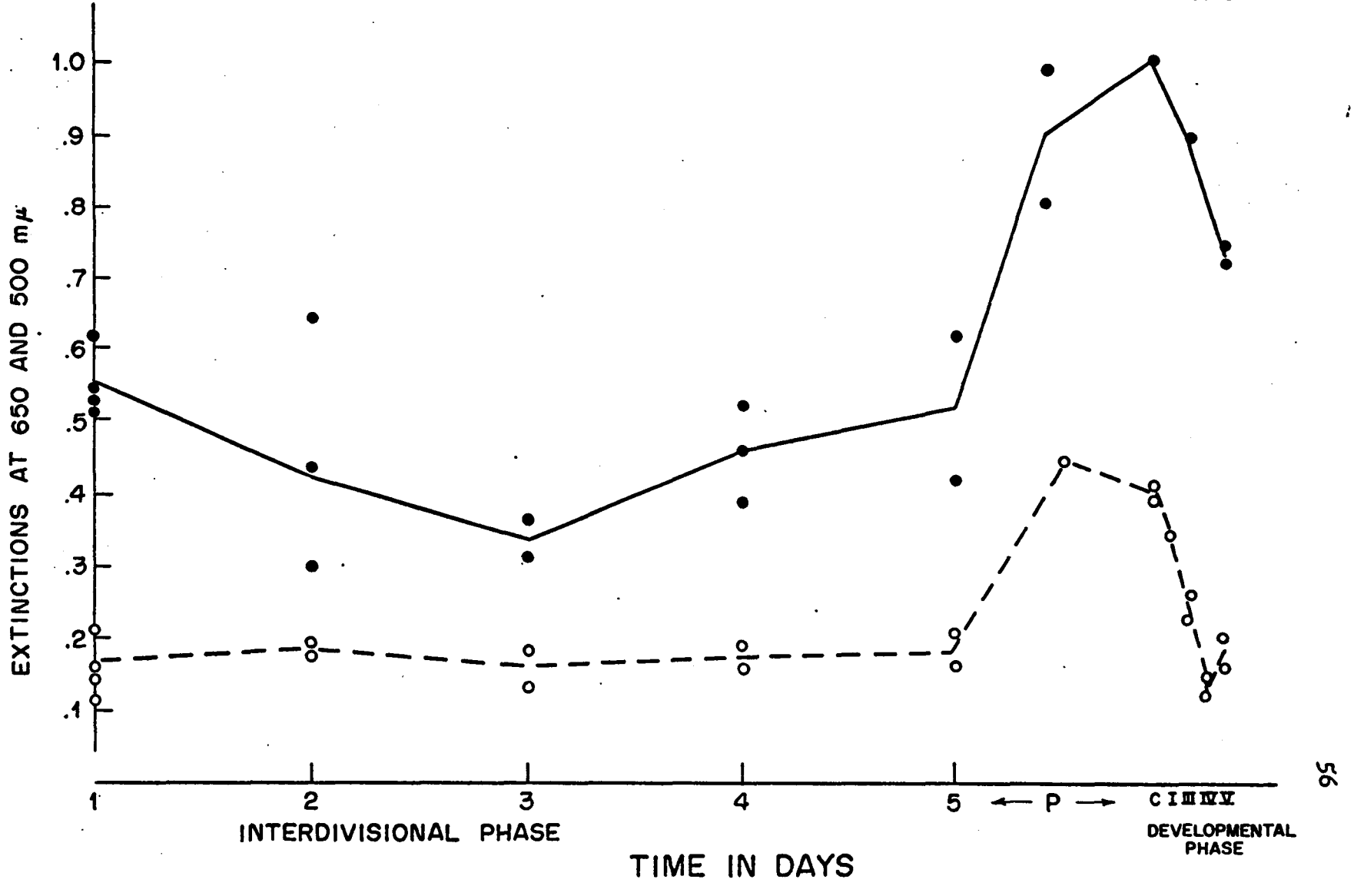


Figure 6

Micrasterias thomasiana, whole cell stained with the Millon reagent. Nucleus (N), pyrenoids (P). x 230.

Figure 6

Figure 7

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3 leucine for one hour. The nucleus (N) is more heavily labeled than the cytoplasm. The pyrenoids (P) are unlabeled, see Figure 6. x 375.

Enlargement Aprox. x 750

Figure 7

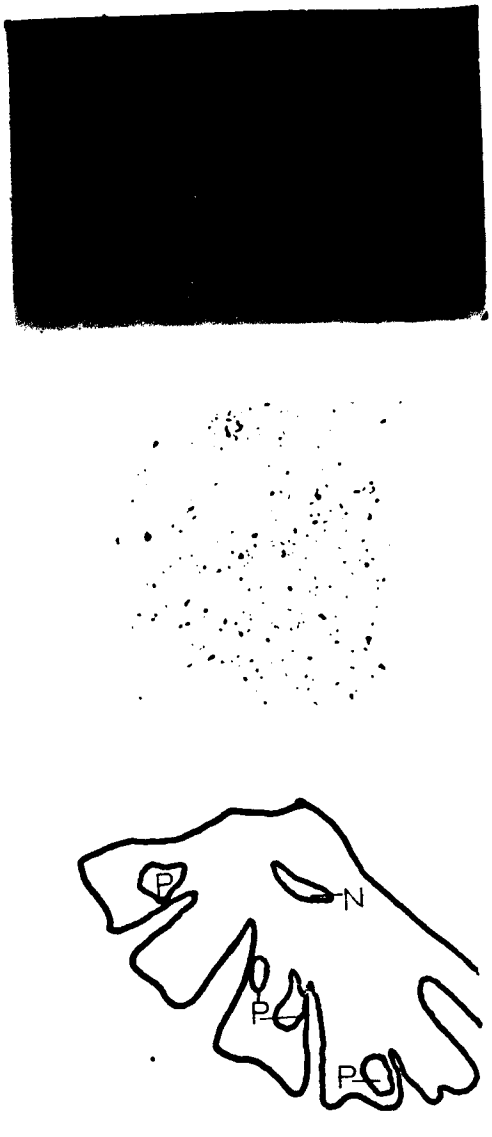


Figure 8

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3 leucine for twenty-four hours. The pyrenoids (P) are unlabeled. x 375.

Enlargement Aprox. x 900

Figure 8

Figure 9

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3leucine for one hour; fixed in stage III of development. Mature hemicell (MHC), new hemicell (NHC), extrachloroplastic cytoplasm (C), chloroplast (CH). x 375.

Enlargement Aprox. x 900.

Figure 9

Figure 10

Autoradiogram of an unstained 0.6 micron section of Micrasterias thomasiana cell incubated in H^3 leucine for one hour then chased for two hours in cold leucine; fixed in stage III of development. Mature hemicell (MHC), new hemicell (NHC), Chloroplast (CH), extrachloroplastic cytoplasm (C). x 375.

Enlargement Aprox. x 900.

Figure 10

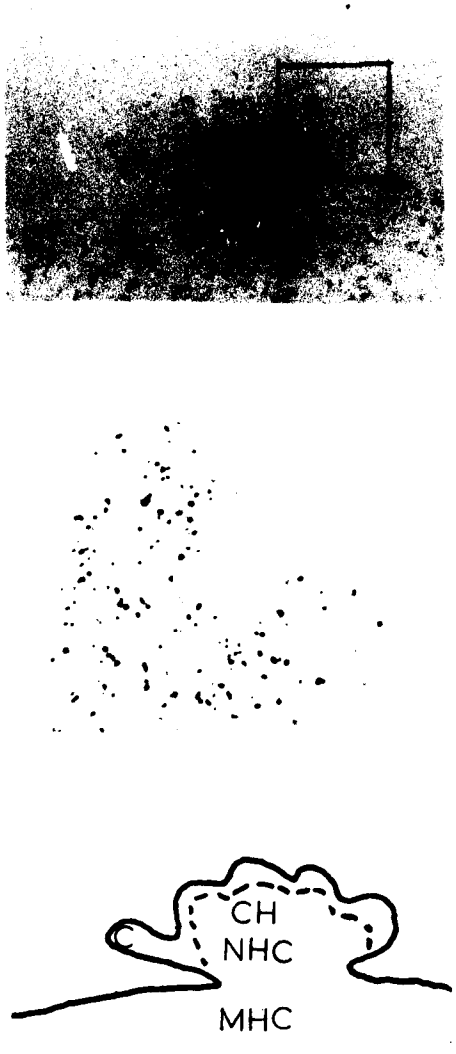


Figure 11

Incorporation of H^3leucine into chloroplast protein throughout the cell cycle.

The number of interdivisional days is six, based on the length of the interdivisional phase of sister cells.

C = cytokenesis

Roman numerals denote stages of development.

INCORPORATION OF H^3 LEUCINE INTO PROTEIN OF THE CHLOROPLAST PER HOUR.

FIG. 11

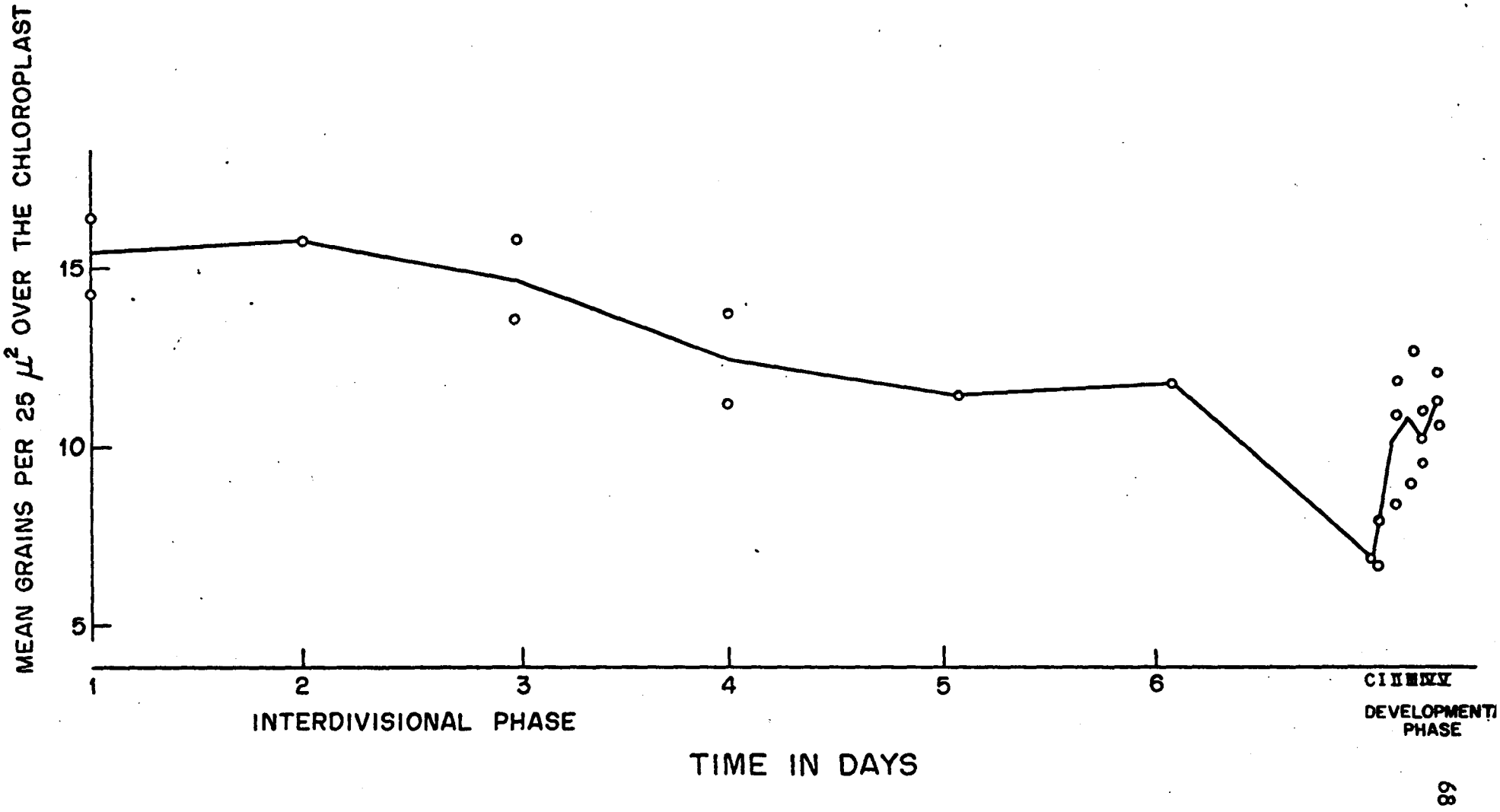


Figure 12

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3 leucine for one hour during the interdivisional phase then placed in unlabeled medium for seven days until the development of the new hemicell; fixed in stage V of development. The nucleus (N) is more heavily labeled than the chloroplast. The grains are equally distributed over the chloroplast (CH) in the mature (MHC) and new (NHC) hemicells. x 375.

Enlargement Aprox. x 900.

Figure 12

Figure 13

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3 leucine for one hour during the inter-divisional phase then placed in unlabeled medium for three days until development of the new hemicell; fixed in stage III of development. Mature hemicell (MHC), new hemicell (NHC), chloroplast (CH), extra-chloroplastic cytoplasm (C). x 375.

Enlargement Aprox. x 1000

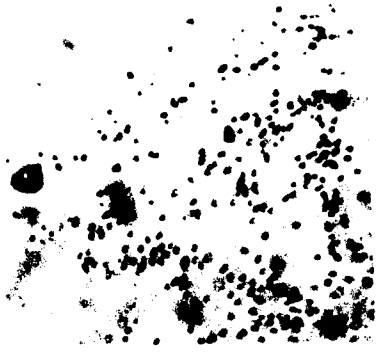
Figure 13

Figure 14

Autoradiogram of an unstained 0.6 micron section of Micrasterias thomasiana incubated in H^3leucine for one hour; fixed in stage II of development. There is no indication of incorporation of H^3leucine into axial structures. Compare this to Figure 2, stage II. Mature hemicell (MHC), new hemicell (NHC). x 375.

Enlargement Aprox. x 900.

Figure 14



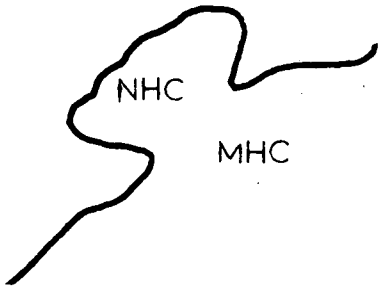


Figure 15

Autoradiogram of an 0.6 micron tangential section of a Micrasterias thomasiana cell incubated in H^3 leucine for one hour during mitosis then chased for two hours in cold leucine; fixed in stage I of development. Mature hemicell (MHC), new hemicell (NHC), nucleus (N), chloroplast (CH), extrachloroplastic cytoplasm (C), artifact (A). x 375.

Enlargement Aprox. x 900.

Figure 15

Figure 16

Autoradiogram of a whole Micrasterias thomasiana cell incubated in C^{14}valine for one hour; fixed in stage V of development. The extra-chloroplastic cytoplasm (C) is indicated by the arrow. x 200.

Figure 16

Figures 17 and 18

Typical range of the results of treating *Micrasterias thomasiana* cells with Actinomycin D (100 µg/ml) for one hour during cytokinesis. These cells were then placed in medium without Actinomycin D for twenty-four hours and have reached their full development. The lobes are simple, elongated and vacuolated. Fig. 17 x 85, Figure 18 x 100.

Figure 17

Figure 18

Figure 19

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3adenosine for one hour; fixed in stage I of development, untreated section. x 375.

Figure 20

Autoradiogram of an adjacent unstained 0.6 micron section treated with RNAase. The nucleus is unabeled. x 540.

Figure 21

Autoradiogram of an adjacent unstained 0.6 micron section treated with DNAase. The nuclear label is heavy. x 375.
Nucleus (N), mature hemicell (MHC), new hemicell (NHC).

Figure 19

Figure 20

Figure 21

Figure 22

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3 adenosine and Actinomycin D (100 µg/ml) simultaneously for one hour at cytokinesis. The nucleus is unlabeled. x 375.

Figure 23

Adjacent section stained with Azure B (0.3 mg/ml at pH 4.0) for two hours at 37°C. Note the presence of the nucleus (N). x 375.

Figure 22

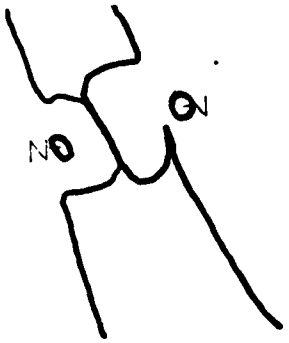


Figure 23

Figure 24

Autoradiogram of an unstained 0.6 micron section through the nucleus of a Micrasterias thomasiana cell incubated in H^3 adenosine for one hour during the third hour following treatment with Actinomycin D 100 ug/ml for one hour at cytokinesis. The lobes in the developing hemicell are typically elongated. There is no label over the nucleus (N). Mature hemicell (MHC), new hemicell (NHC). x 375.

Enlargement Aprox. x 900.

Figure 24

Figure 25

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3leucine for one hour during the first hour following treatment with Actinomycin D (100 µg/ml) for one hour at cytokinesis. There is no incorporation of H^3leucine into axial patterns in the new hemicell (NHC). The nucleus (N), seen in the new hemicell of one cell, is more heavily labeled than the cytoplasm. Mature hemicell (MHC). x 375.

Enlargement Aprox. x 900.

Figure 25

Figure 26

Autoradiogram of an unstained 0.6 micron section of a Micrasterias thomasiana cell incubated in H^3 leucine for one hour during the third hour following treatment with Actinomycin D (100 µg/ml) for one hour at cytokinesis. The lobes in the developing hemicell are typically elongated. Mature hemicell (MHC), new hemicell (NHC), chloroplast (CH). x 375. Extra chloroplastic cytoplasm (C).

Enlargement Aprox. x 900.

Figure 26