GROWTH AND MORPHOGENESIS IN *OEDOGONIUM CARDIACUM*

A STUDY OF GROWTH AND MORPHOGENESIS

IN

OEDOGONIUM CARDIACUM

By

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SCOPE AND CONTENTS:

The investigations concerned the effect of some environmental factors on growth and zoospore production in Oedogonium cardiacum. Media and culture techniques were developed which favoured optimum growth, and zoosporulation within a definite time period.

The normal sequence of cell divisions was established for young filaments. This was a problem of fundamental interest in algal ontogeny.

The normal sequence of cell divisions in filaments was disturbed by treatment with ionizing radiation. A different ontogenetic sequence was induced by ionizing radiation and this sequence was often associated with those individuals which were classified as non-survivors.

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INTRODUCTION

This work was undertaken in an attempt to discover whether the constituent cells of a simple, colonial plant exercise any influence over, and are, similarly, partially controlled by, their neighbouring cells in the plant. That is, do the cells in the colony behave in a strictly individualisti way or do they grow and develop in a manner, presumably, advantageous to the organism as a whole? In other words, do the cells of the organism function as a type of symplast?

It has been shown that the cells of higher organisms, both plant and animal, do exert a controlling influence by direct or indirect chemical and/or physical means over other cells close by or at some considerable distance in the same organism. (Discussions of this are to be found in Wetmore, 1958; Steward and Shantz, 1959: Wardlaw, 1955, 1965, concerning plants and in Brachet, 1950, 1960, concerning animals.) These many, complex interacting controls have evolved in such a way as to allow the organism to develop from a single cell, the spore or zygote, into a fully functional adult capable of reproducing its own kind. When any of these controls are lacking, abnormal individuals usually result. Hence, these morphogenetic and functional control mechanisms are of utmost importance in the

development and existence of the organism.

However, in many so-called lower plants this interaction of one cell with its neighbours has not received much consideration. This is especially true of the simpler genera of the green algae despite the fact that in the embryogeny of higher plants early development has been claimed to be a reversion to a filamentous algal type of organization (Bower, 1922). The term "lower" usually indicates a lack of structural complexity and a low position on the evolutionary scale of development for evolution seems to deal with elaboration of organization (Wardlaw, 1965). It is anticipated that developmental studies of these simple plants may ultimately lead to some understanding of the mechanisms that control growth and differentiation. Here one is dealing with simple organisms unencumbered with multitudinous morphogenetic sequences which tend to obscure one another and, thereby, hinder investigations.

The term colonial as it is applied to these simple plants implies a collection of individuals (cells) with little regard for the integrity of the plant as a whole. Therefore, a major goal of this study was to ascertain whether the cells form a type of symplast rather than behaving as individuals. If the developmental sequence of the plant can be changed directly or indirectly by external factors, insight may be gained into some of the processes involved in the normal sequence of events. For as Wardlaw (1965, p. 331)

has stated on the experimental investigation of organization: "It is held that the reaction system in an embryonic region ·· ·· normally gives rise to a pattern as a whole. Accordingly, if we wish to test this idea, we must interfere with the normal working of the reaction system in some characteristic way, e.g., by introducing new components, by changing the balance of existing components, by modifying environmental factors, or by disturbing the system mechanically; and we must try to relate the induced changes in the system to the manifestation of organization." The production of a different developmental sequence emphasizes the actual existence of the original organization.

The use of Oedogonium cardiacum Wittrock for developmental studies is based upon several properties that this alga possesses, some of which are unique. The cylindrical cells of this colonial organism are linearly arranged to form a single filamentous structure. This formation implies a greater measure of organization within the plant than is found in those algae with amorphous colonies. The unique caps of the vegetative cells, many aspects of cell division, the apical and basal cells and even the asexual zoospores exhibit a marked apical-basal polarity. This must be a manifestation of organization among the cells of a filament. [The source of Oedogonium cardiacum Wittrock, female strain, was the culture collection of algae at Indiana University (Starr, 1964).]

The radiobiological studies by Horsley and his co-workers (1962, 1963) on Oedogonium cardiacum indicate that its sensitivity to ionizing radiation is not unlike that of many higher organisms. This condition allows for radiobiological damage to the cells of a filament to be interpreted, perhaps, in the light of processes already partially known for higher organisms. This is contrary to the condition in some simple organisms such as certain members of fungi, bacteria and protozoa which have high resistance to ionizing radiation. The bases for the resistance appear to be rather obscure. Some algae have several nuclei per cell and damage to one nucleus may not be manifested morphologically or physiologically, for the control of development is most likely to be under the direction of all the separate nuclei. Damage to one of many nuclei is probably of little consequence to the cell and this increases the complexity of such a system.

An assumed autotroph such as Oedogonium cardiacum should be able to survive more exacting environmental conditions than are commonly required for other cells and tissues often used for investigations into developmental phenomena. Frequently, plant or animal cells isolated from whole organisms are used for such inquiries and must be cultured away from their complex, natural habitat. In order to evaluate the results in such instances much effort must be extended to provide an environment closely approximating

the natural one from where the cells have been obtained. (Steward et al., 1964, and other investigators have explored the intricate conditions necessary for the culture of isolated plant cells.) Species of Oedogonium may be grown in relatively simple culture media within a reasonably wide temperature range and in cultures where microorganisms co-exist usually without detrimental effect to the algae. Several of the culturing techniques for members of this genus including light intensity, temperature, growth media are considered to be already well known.

One of the most important reasons for using Oedogonium cardiacum in developmental studies is that the vegetative progeny of a single cell can be followed visually throughout subsequent cell divisions for some period of time, which is unlike the conditions that exist in most other cell culture systems. This alga has two inherent features which facilitate the observation of cell progeny. The apical caps which are characteristic of the cells are a direct result of cell division and give a precise indication of how many times a cell has divided as one cap results from each cell division. Also, the filaments of Oedogonium cardiacum can be grown on glass microscope slides because the asexual zoospores which subsequently develop into filaments are able to attach onto solid objects by means of a pectic secretion. If the microscope slide is marked or divided into many small sections each of which is coded in some manner as in the New

England Finder slides *(see Plate 1), a filament which has become attached in a certain region of the slide may be referred to repeatedly since once attached to an object the filament rarely moves away.

Oedogonium cardiacum belongs to the one family of the order Oedogoniales, the Oedogoniaceae, and is a species of the true green algae, the Chlorophyceae (Tiffany, 1924). Only three genera comprise the Oedogoniaceae - Oedogonium (found in quiet waters), Bulbochaete (similar to Oedogonium but has branched filaments) and Oedocladium (a rather rare, southern, terrestrial alga).

All the vegetative cells of a filament of Oedogonium cardiacum are similar, if not identical, except for the most basal and apical ones. The cells are cylindrically elongate, approximately 60 to 100 microns long by 20 microns in diameter and abut one another squarely producing a straight, unbranched filament. The basal cell is pyriform with a branched protoplasmic extension, the holdfast, emanating from the narrow, bottom end of the cell. The apical cell is similar to any other vegetative cell but has a rounded or subacuminate distal end (Plate 1).

Except for the holdfast section of the basal cell the lateral cell walls of Oedogonium and Bulbochaete are said to consist of three concentric layers - an inner

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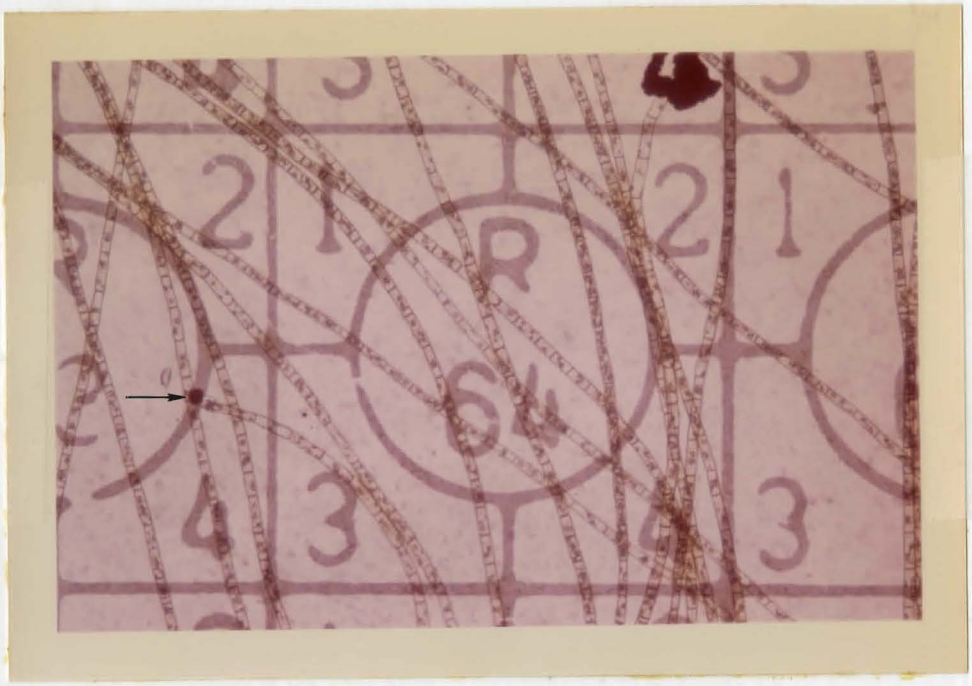
*Available from Graticules Ltd., London E.C.1, England

PLATE 1

1. Section of a New England Finder Slide showing the coding system used to locate filaments. Long filaments of Oedogonium cardiacum are growing on the surface of the slide. The arrow points to the position of attachment of one of these filaments to the slide (X 60)

2. Germlings and filaments of Oedogonium cardiacum - the single-celled germlings are growing attached to a fibre in the culture solution. One germling has just divided to produce a 2-celled filament. The filaments traversing the photomicrograph are older filaments of this alga. (X 200)


2
1
R
2
1
64
3
3

cellulose wall bounded exteriorly by a layer of pectose with an outer layer of chitin (Tiffany, 1924). Just within the lateral cell wall and bounding on all sides lies the single, large, parietal chloroplast. It is a reticulate sheath with most of the chloroplast threads running parallel to the long axis of the cell (Smith, 1950). The writer has found that the actual gross chloroplast morphology seems to depend upon the age of the cells and the medium in which they are grown. Young, rapidly growing cells show the recticulate nature of the chloroplast clearly but in older cells or those grown under some culture conditions where growth is slow the structure of the chloroplast is obscured by the large number of starch grains present in or near the chloroplast. The interphase nucleus usually lies just under the chloroplast sheath midway between the ends of the cell. Although colourless, the nucleus may sometimes be seen in rapidly growing, young cells where the chloroplast reticulum is often thin and sparse and does not obstruct one's vision. The central region of the cell is occupied by a large, colourless vacuole.

Two aspects of development must be considered before the main problem of the thesis can be attacked. These are growth and asexual reproduction. The culturing of algae has been reviewed by several investigators including Bold (1942), Pringsheim (1946), Burlew (1953), Krauss (1958), Lewin (1962) and Starr (1964). According

to most reports the various species of Oedogonium can be grown in aqueous soil extract solutions. Although these solutions appear satisfactory for stock cultures, which are necessary as a continuous source of the algae, they are not so desireable for experimental purposes. This type of culture medium is undefined as to contents, especially the micro and organic nutrients, and varies from one lot to another depending upon the soil used and the treatment given to the soil-water mixtures. A defined medium has the advantage of always being uniform and, thereby, assists in effecting reproducible experimental results.

Since Oedogonium cardiacum is supposedly an autotrophic organism, it should be able to exist in a simple, inorganic salts solution supplying the necessary ions required for metabolism. The other requirements, carbon, oxygen and hydrogen are present in abundance in the aquatic environment, natural or artificial. But which inorganic salts are required? Various defined media have been employed in culturing species of Oedogonium (Buhnemann, 1955; Hoffman, 1961; Kim, 1961; Horsley and Fucikovsky, 1962; Machlis, 1962). Since a variety of media have been employed by these investigators it would indicate that these media have proved somewhat less than satisfactory in the culture of the algae in vitro. Therefore, some effort should be made to determine what constitutes a suitable defined medium for the in vitro culture of Oedogonium cardiacum.

The Oedogoniales increase in quantity not only by vegetative growth but also by sexual and asexual reproduction. Sexual reproduction has been explored by several investigators including Spessard (1930), Hoffman (1961, 1965) and Rawitscher-Kunkel and Machlis (1962), but this form of reproduction was not studied in the present work. Asexual reproduction has also received some attention (Gussewa, 1930; Mainx, 1931; Bühnemann, 1955). This form of vegetative propagation is of prime importance to the investigation since the resulting zoospores become germlings which develop directly into young filaments suitable for developmental studies. There is some controversy as to the stimulus which initiates asexual reproduction. A change in environmental conditions, e.g., in light intensity, temperature or culture medium, is reported to effect zoosporulation. However, these results are qualitative not quantitative. Attempts are to be made to control asexual reproduction in order that zoospores may be had when needed.

Thus, there are three lines of pursuit to this investigation:

(1) The control of asexual reproduction;

(2) The culture of Oedogonium cardiacum;

(3) The determination of a developmental sequence in this alga and attempts at revealing some of the controlling factors involved.

TECHNIQUE

The basic procedure underlying many of the experiments was to obtain young filaments of Oedogonium cardiacum, culture them, and observe their development into mature filaments. To this end the following techniques were employed.

Stock cultures of Oedogonium cardiacum were grown in a soil extract medium, modified after Pringsheim (1946), in 500 ml. borosilicate Erlenmeyer flasks. The soil extract was prepared by adding 10 to 20 percent pulverized clay loam, by volume, to 400 ml. of distilled water in the flasks. The flasks were then autoclaved for one hour at 16 to 18 psi. pressure. (The pressure must be released very slowly in order to prevent the contents of the flasks from boiling over.) The need for double autoclaving, that is, autoclaving the same solution on two separate occasions in order to kill any algal, bacterial or fungal spores that might have germinated following the first sterilizing period, was shown to be unnecessary. Singly autoclaved solutions remained sterile for more than a year.

New stock cultures could be started about one week after the sterilization of the flasks. During the week most of the suspended soil particles settled out of the soil-water

mixtures and the clear solution above the level of the soil could then be inoculated with filaments of Oedogonium cardiacum. These new cultures were kept under reasonably bright illumination of 200 foot-candles (as measured with a Gossen Lunasix exposure meter) or 0.8×10^4 ergs/cm.^{2}-sec. (as recorded on a Yellow Springs Instrument, model 65, radiometer) incident upon the table top supporting the culture flasks. The light was provided by a bank of lamps consisting of a mixture of "cool-white" and "Gro-Lux/WS" 40 watt fluorescent lamps. ["Gro-Lux/WS" is one manufacturer's brand of wide spectrum fluorescent lamp which has been shown to be more suitable for most plant growth than standard fluorescent lamps since its energy spectrum matches the action spectra of photosynthesis and chlorophyll synthesis more closely (Sylvania Electric (Canada) Ltd. engineering bulletins # 0-278, 0-283, 0-285). Their superiority in algal culture has not been demonstrated.] The diurnal photoperiod was 16 hours of light followed by 8 hours of darkness.

The cultures were maintained in this state at 18° to 21°C. for at least one month after which the intensity of illumination was reduced considerably, to about 35 foot-candles, in order to preserve the cultures in a healthy state (Pringsheim, 1946; Starr, 1964). Continuous high light intensity often led to oogonia formation in the female strain with the subsequent deterioration of the filaments

after the oogonia were not fertilized because the sexes were kept separate. Also, high light intensity facilitated continuous, rapid growth and the culture solutions were quickly exhausted of nutrients. This led to the disintegration of the cells' cytoplasm and the filaments died.

For culturing the alga 500 ml. flasks were found to be satisfactory. In larger flasks the alga tended to grow near the wall of the flask and its growth there shaded the inner part of the culture resulting in little growth at the centre. Increased illumination provided sufficient light at the centre but at the expense of causing disintegration of the filaments near the wall of the flask. Thus, there was little increase in the amount of alga for an increase in the volume of the culture flasks beyond 500 ml. Bubbling the cultures with air in order to increase the amount of alga produced by causing a turnover of filaments from the centre to the outer regions of the culture usually resulted in the twisting of many of the filaments into long, inseparable cords. Flasks much smaller than 500 ml. simply allowed for less growth.

During the actual experimental work the alga was grown in two defined and one undefined culture media all reported as being adequate for the culture of Oedogonium cardiacum. The first defined medium was a modified version of Molisch's solution (Horsley and Fucikovsky, 1962) and consisted of the following inorganic salts dissolved in a

litre of distilled water:

$(NH_4)_2HPO_4$	0.8 grams
K_2HPO_4	0.4 grams
$MgSO_4.7H_2O$	0.4 grams
$Ca(NO_3)_2.4H_2O$	0.4 grams

A white precipitate (calcium phosphate and magnesium phosphate) usually formed upon standing. This was unfortunate since the medium no longer remained an accurately defined one.

The second solution was one modified after Machlis (1962). In order to make Machlis' medium "E" the following compounds were dissolved in a litre of distilled water:

KH_2PO_4	0.20 grams
K_2HPO_4	0.09 grams
$CaCl_2$	0.06 grams
$MgSO_4.7H_2O$	0.25 grams
KNO_3	2.02 grams

Cyanocobalamin (vitamin B_{12}) was added to this solution at the rate of 1 ppb. One ml. of a chelated trace element solution was also added. The trace element solution (after Hutner et al., as reported in Stein, 1958) was compounded from the following chemicals:

H_3BO_3	1.00 grams	$MnCl_2.4H_2O$	0.50 grams
$CuSO_4.5H_2O$	0.15 grams	$FeSO_4.7H_2O$	0.50 grams
EDTA (disodium)	5.00 grams	$CoCl_2.6H_2O$	0.15 grams
$ZnSO_4.7H_2O$	2.20 grams	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.10 grams

These salts were added to 100 ml. of distilled water but they did not always dissolve readily even when heated as suggested by Stein. However, the writer found that if the pH value of the mixture was slowly increased from its original low value of 2 by the addition of sodium hydroxide pellets with constant stirring, the entire contents went into solution as the pH changed from 2.9 to 3.0. The pH of the solution was increased further to approximately 4.5. (An original pH of about 6.5, as recommended by Stein, drifted to more than 7.0 on standing overnight with the concomitant precipitation of the iron.) Fungi could be prevented from contaminating the trace element solution by autoclaving the solution for 10 minutes at 16 to 18 psi. pressure. The colour of a freshly made trace element solution was green but it was found to change to magenta upon standing. This colour change did not seem to affect its value.

This culture solution, hereafter called medium J, appeared to be quite stable and could be autoclaved for sterilization.

The only other commonly used culture medium was a soil extract solution prepared by filtering any particulate matter existing in the supernatant of aqueous soil extract mixtures as used for stock cultures (cf. p. 13). An efficient method found for filtering soil extract supernatant was to use vacuum filtration with a Buchner funnel employing

glass fibre filter paper*. The flow rate was satisfactory and the retention was good. This solution could also be sterilized in the autoclave and kept for extended periods of time before use without showing any obvious deterioration. The composition of this medium was quite undefined especially with regard to the micronutrients and organic compounds.

Since this alga reproduces asexually by means of motile zoospores and since these zoospores germinate directly into young filaments upon attaching to the substratum, zoospores constituted a source of young filaments that were suitable for use in the developmental studies. Zoospores could be obtained from filaments of mature cultures that were at least 6 to 8 weeks old. Filaments from a stock culture were rinsed several times with distilled water to remove loose debris and excess bacteria and then were transferred to a 250 ml. flask containing 150 ml. of fresh, sterile culture medium. The flask was then placed in darkness at a constant temperature of 21°C. for at least 1 1/2 days. During this period air containing an excess of carbon dioxide (3% CO_2) was bubbled through the culture medium by way of a cotton plugged, sterilized dropping pipette inserted through a foamed plastic stopper in the top of the flask. This procedure usually provided a sufficient stimulus to effect zoosporulation. (Plate 2 shows several

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*Manufactured by the Reeve Angel Co., Clifton, New Jersey

such flasks, some aerated and some not, in the incubator.)

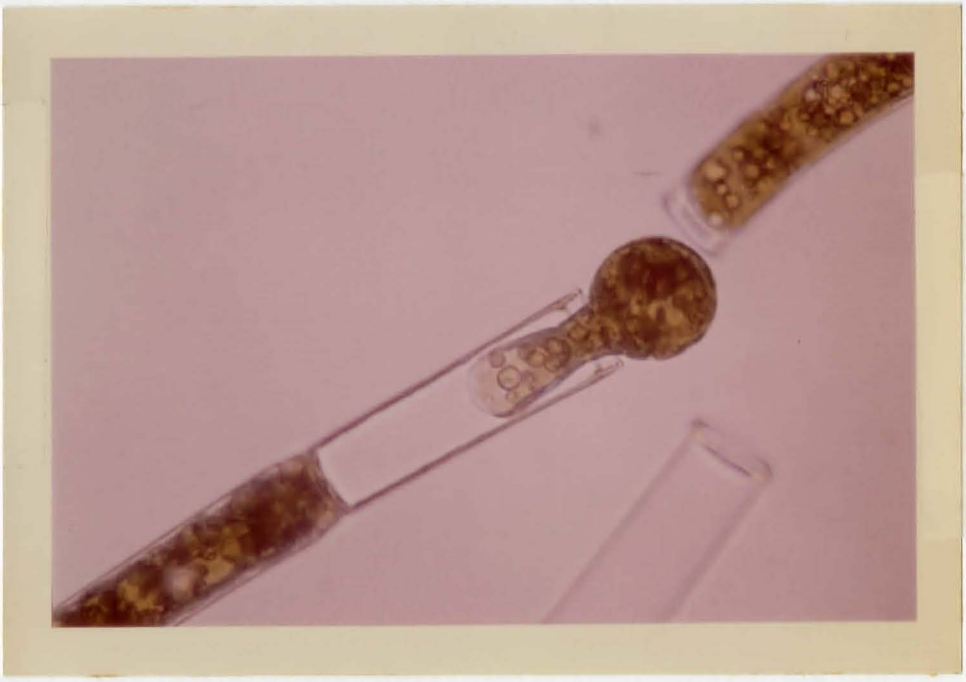
The zoospores were harvested and cultured on standard 3 x 1 inch, glass microscope slides or on gridded New England Finder slides (after Horsley and Fucikovsky, 1962). The culture medium containing the sporulating filaments was placed in a flat-bottomed, glass container which was often darkened on the sides and top to keep out extraneous light. The bottom of the vessel was covered with a single layer of slides and the size of the collecting vessel was determined by the number of slides of young filaments desired. The vessel was illuminated from beneath by fluorescent lamps providing an intensity of 200 foot-candles at the bottom surface of the vessel. Since the motile zoospores were positively phototrophic, they were attracted to the light source and swam to the bottom of the container where they came to rest on the slides. The collection of zoospores on these slides was carried out at 21°C.

Here the zoospores lost their flagella, developed holdfasts and anchored onto the slides by means of a secretion. Once these zoospores had attached to the slides they could be rinsed with a strong stream of distilled water from a washing bottle to remove loose debris, such as empty zoospore mother cells and poorly anchored zoospores, without dislodging them from their firm attachment. The anchored zoospores, now called germlings, could then be transferred

PLATE 2

1. Incubator containing 14 250 ml. culture flasks. Seven cultures are being bubbled with CO_2 enriched air from an external cylinder by way of a glass manifold on the top shelf of the incubator. The "circline" fluorescent lamps and plastic diffuser are used for supplying the illumination for collecting zoospores on glass slides and their subsequent growth.

2. Late stage in the release of a zoospore. (X 550)



from one vessel to another en masse and subjected to various experimental studies.

Microscopic examination of these developing germlings was facilitated by placing the slide containing the alga in a shallow, rectangular glass receptacle, devised by the writer, that was partially filled with the culture medium being used in the experiment. The rectangular container allowed for the use of the mechanical stage of the microscope for easier positioning of the slide in the microscope field of view. The container simply consisted of a small, rectangular sheet of glass, 3 1/2" x 1 5/8" x 1/16", with a raised border made from 3/8" diameter glass rod cemented to the glass sheet with epoxy adhesive.

Direct observations of the living plant were supplemented with cytological investigations especially those pertaining to the cells' nuclei for assisting in the determination of various stages of the cell cycle. To this end the Feulgen technique of chromosome staining was adapted to this alga. Since acid hydrolysis is part of this procedure, the algal filaments would normally be lost from the slides during the staining process because the pectic secretion of the holdfast that anchors the filaments to the slides would be hydrolyzed. To prevent this the filaments were retained on the slides by a film of collodion which was quite permeable to the solvents employed in the staining process and which could be removed prior to the final mounting of the coverslip.

Because of its length the staining procedure is described in Appendix 1.

The black and white photomicrographs were taken on 35 mm Kodak High Contrast Copy film rating the ASA exposure index of the film at 12 to 16. The film was developed at 20°C. in Agfa Rodinal film developer, diluted 1:100 with tap water, for 10 minutes with 5 seconds of agitation at each 30 second interval. This produced negatives of high resolution and enhanced contrast.

Most of the colour photomicrographs were taken on Kodachrome II daylight colour reversal film. Since this film was colour balanced for 5500 to 6000° Kelvin, it was exposed using B_{18} decamired filtration over a light source rated at 2800°K. This rendered the colours approximately true to their natural appearance in daylight. The use of daylight colour film was preferred to tungsten balanced colour film because the heavy blue filtration required by the daylight film reduced the heat absorbed by the slides and organisms being photographed although the filtration necessitated somewhat longer exposures.

The irradiation of the germlings was performed at the University with high energy, ionizing radiation from a caesium ^{137} source. The general method was to rinse the slides containing the alga with distilled water and place them in a glass Petri dish or similar glass vessel, without a lid, containing distilled water or some other culture medium.

The slides were covered to a depth of at least 5 mm. with the liquid medium in order to provide a sufficient build-up of radiation to occur before it reached the alga on the slides.

The dish was then placed under the high energy source at a predetermined distance and irradiated for a given length of time to provide a calculated dose of ionizing radiation. The doses given were calculated from tables supplied by Dr. Schneider and the radiation fields used were such that in no case was there more than a 10% difference in the dose rate between the centre of the radiation field and the perimeter - the tolerance was usually dept within a 5% limit. The dose rate most commonly employed was 100 rads/min which necessitated a source to subject distance of approximately 33 cm. After irradiation the slides were removed from the Petri dish and rinsed well with distilled water and then returned to the main culture vessel.

When irradiating large numbers of slides at one time, as was sometimes required, they were irradiated in a rectangular, glass staining vessel where the slides were held horizontally on their edges. This allowed for a smaller radiation field and a concomitant higher dose rate than would normally have been available if all the slides were spread out flat in a large Petri dish or other container. The difference between a dose delivered to the centre of the slide and the edges was less than 10%.

EXPERIMENTS AND RESULTS

ON THE PRODUCTION OF ZOOSPORES

Two of the problems encountered throughout the experiments were the lack of sufficient numbers of zoospores with which to work and the inability of the investigator to determine when the zoospores would be produced. The objective was not only to have a large number of zoospores at one time, often in the order to 10^4 to 10^5, but also that they be of a nearly equal age. This was essential for the radiation studies since the sensitivity of a cell to ionizing radiation depends to a large extent upon the stage in the cell cycle that the nucleus is in during the radiation period (Davies and Evans, 1966, - a review; Das and Alfert, 1962; Dewey and Humphrey, 1965; Horsley and Fucikovsky, 1963; Horsley et al., 1967; Terasima and Tolmach, 1961).

In a later section it is shown that zoospores which were released from the parent filaments and attached onto the glass slides at approximately the same time, plus or minus 15 minutes of each other, divided synchronously for the first cell division. That is, the cells progressed through the first cell cycle at about the same rate. (The cell cycle is after Howard and Pelc, 1953, and is described in a later section.) This meant that the large numbers of zoospores often required must be released from the parent

filaments and collected on the glass slides within a period of about 30 minutes. To this end several experiments were executed in an attempt to discover some of the factors governing zoospore production.

Filaments of Oedogonium cardiacum produce zoospores at the rate of one zoospore per cell. The entire cytoplasmic content of a cell is incorporated into a zoospore. This process is a metamorphosis from a non-motile to a flagellated, motile stage. Zoospore production appears to occur at the expense of stored energy since the transformation took place in the dark as well as in the light. The actual change from the vegetative to the reproductive stage has not been fully reported in the literature.

The writer recognized the onset of zoospore production microscopically by the constrictions or bands of dark green colour which developed in the chloroplast; that is, the chloroplast "condensed" in seemingly random areas of the cell resulting in dark green areas interspersed within the normal, light-green chloroplast reticulum. In early stages of zoospore development only a few of these condensations existed per cell but, eventually, more were formed. (Plate 3 shows a sequence of developmental stages in the production and release of zoospores.)

The spore mother cell wall would split at the apical end of the cell at the region of the last formed apical cap and the zoospore would emerge quickly - in one to two minutes.

PLATE 3

Various stages in the release of a zoospore.

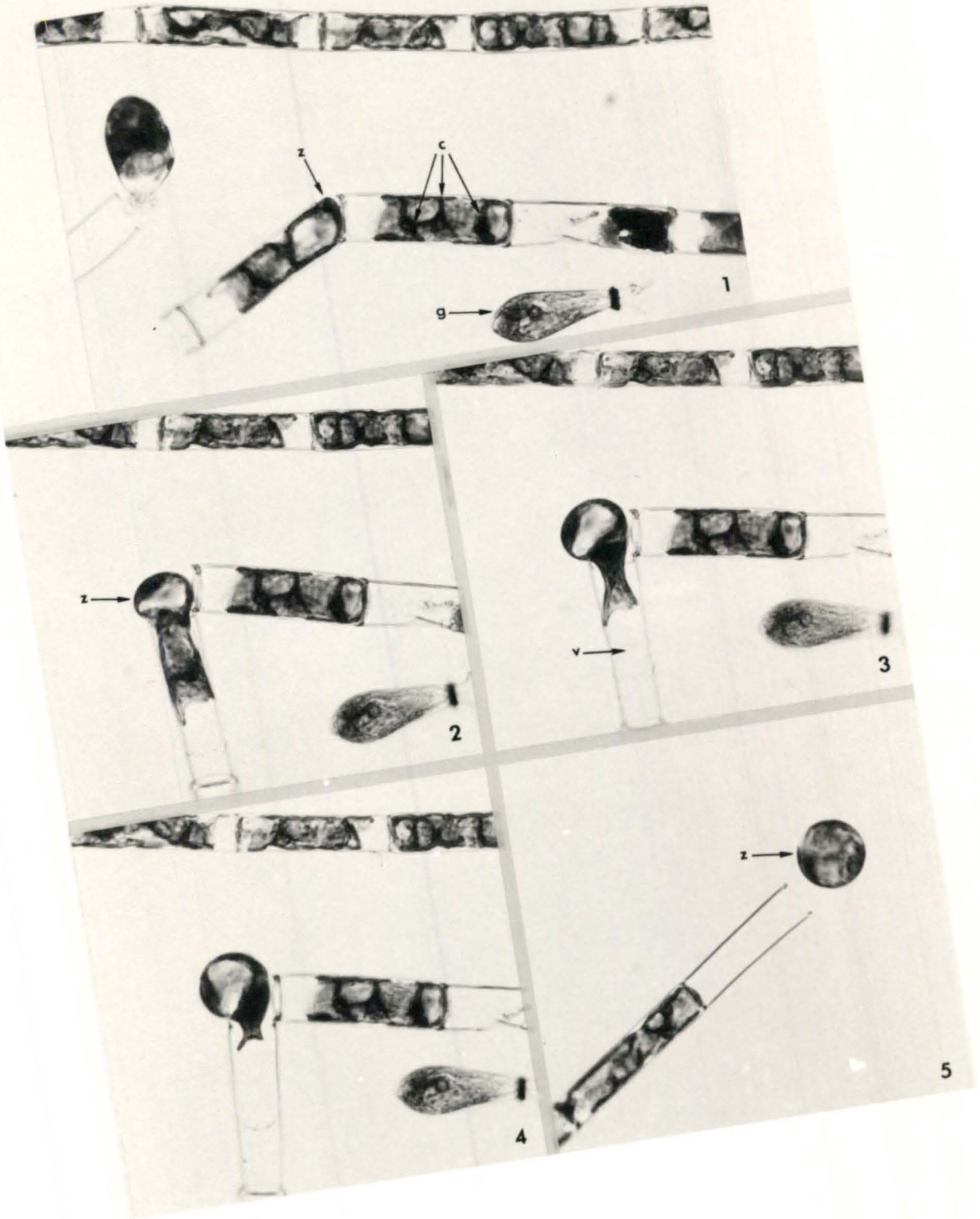
1. Early - mother cell wall has just split at the apical end of the cell and the zoospore (z) is just beginning to emerge; c indicates regions of chloroplast "condensations"; g is a germling.

2. Mid - mother cell wall is split nearly around entire circumference and the top of the cell has been pushed aside by the emerging zoospore.

3. Somewhat later - zoospore emerging; v points to the large colourless region of a forming zoospore that is last to exist from the zoospore mother cell.

4. Later

5. Zoospore has emerged and is oscillating within a enclosing membrane (not visible). (all X 350)


z
c
g
1
z
2
v
3
4
z
5

The splitting of the cell wall and the emergence of the zoospore was probably due to imbibition of water as a result of a colloidal change in the cytoplasm (Cook, 1959). Evidence for this appears in Plates 2 and 3 which show a large, clear area of cytoplasm the size of which varied from one zoospore to another and this cytoplasm was last to emerge from the cell during the release of the zoospore. This colourless portion, at times, approximated the size of the original cell and, most likely, was the result of cytoplasmic swelling.

The spore mother cell wall would split at one side only and was not separated completely around the circumference of the cell in the early stages. The emerging zoospore pushed back the upper part of the old cell wall and usually remained just outside the apical end of the mother cell imprisoned within a transparent sheath. The zoospore escaped within one to two minutes as the sheath disappeared (dissolved?). The actual morphology of the formed zoospore is discussed in various texts (e.g., Smith, 1950).

When a zoospore stopped swarming, usually well within an hour, it came to rest with its flagellated anterior end against the substratum. The flagella were withdrawn (or shed?) and a clear, raised area of the zoospore membrane that occupied the centre area in the whorl of flagella swelled outward rapidly, often equal in length of the diameter of the original zoospore, and usually branched considerably.

This branched structure was the holdfast and it anchored the zoospore to the substratum by means of a secretion. The newly attached zoospore, now called a germling, displayed metabolic polarity with the chloroplast at the distal end and clear, colourless cytoplasm at the basal end of the cell.

That zoospore production was not a random event among cells of a culture was demonstrated by the fact that during zoosporogenesis there was not only an interfilament gradation in the number of chloroplast constrictions per cell, which was anticipated, but also an intrafilament gradation. That is, within a filament there was a gradation in zoosporogenesis from one region of the filament which showed no visible signs of zoospore production to another where the cells were ready to release their zoospores. This developmental gradation occurred from one end of the filament to the other or spread out in either direction from various regions of the filament. This implied that there might have been a stimulus which travelled up or down the filament and which was responsible for the initiation of zoospore production. There is no previous report of this characteristic.

In the laboratory when a mature culture, i.e., one that was at least 6 to 8 weeks old, was transferred from its stock culture container to a fresh soil extract medium, it eventually underwent zoosporulation to a greater or lesser degree. Greater zoospore production was sometimes effected by placing the culture in darkness of 72 hours

and then returning it to the light. However, this procedure was a "hit and miss" affair. Even at best this method (used by other investigators and previously by this one) rarely realized the total zoospore production capacity of the culture as a large percentage of the filaments often failed to sporulate.

The best method found in this investigation to elicit zoospore production was the following. A stock culture of Oedogonium cardiacum was rinsed well with distilled water to remove loose debris and bacteria; this meant 5 to 6 changes of distilled water in a beaker containing the alga. The alga was then placed in a 250 ml. flask containing 150 ml. of sterile culture medium containing soil extract solution. (Larger flasks, 500 ml., containing proportionately more medium and alga were sometimes employed if very large numbers of zoospores were needed.) The actual composition of the medium is discussed below but it had to contain some soil extract for abundant sporulation. The culture was then placed in darkness in an incubator at 21°C. and slowly bubbled (about 14 ml. air/min.) with air containing an excess of carbon dioxide (cf. p. 16).

After 36 1/2 hours, plus or minus 1 hour, some of the newly formed zoospores were released into the culture medium. This time was supported by the results of 8 experiments. Times of 36, 37, 37, 37, 36, 37, 36 and 36 hours were recorded for the time interval that occurred

between placing the culture in fresh medium and aerating it and the first signs of major zoospore release. These times were approximate and, therefore, the deviation from the mean was doubled to take this into consideration. In no case was there any failure of the cultures to sporulate as was commonly experienced using the original methods mentioned previously. That zoospores were being released could be detected macroscopically since the filaments broke apart where the zoospores were escaping; the long filaments disappeared and the remaining short filament fragments settled to the bottom of the flask. This was the first stage of zoospore release and it continued until the whole culture had so fragmented.

The second stage of zoospore production occurred when these filament fragments released their zoospores. Groups of fragments tended to sporulate together and this resulted in several large discharges of zoospores. These zoospores could be seen with the unaided eye as green clouds emerging from the fragmented filaments at the bottom of the flask. The time interval between the initial fragmentation of the culture and the final release of all the zoospores seemed to vary somewhat extending from one to several hours. Actual figures for this time interval were not kept for a sufficient number of cases to make any results meaningful.

The stimulative effect of carbon dioxide on zoospore production was noted by Gussewa (1930) and there is some controversy as to its action (Carr, 1961). Mainx (1931) doubted that CO_2 produced any stimulative effect on zoosporulation. Aerating the cultures with air containing 3% CO_2 did affect the pH of the culture media. The pH values of the various solutions were lowered about 1/2 a pH unit in the media that were bubbled over the dark incubation period. (Table 2 has the actual values from one experiment.) However, the amount of pH change seemed rather inconsequential when the pH values of the various media were considered in relation to their ability to effect zoospore production (Table 1). Although the initial pH values were not taken for all the solutions, those for soil extract and Machlis E media were taken and these remained the same in the unaerated cultures from the beginning to the termination of the experiment. Therefore, those values in the unaerated column were assumed to represent the initial pH values of the solutions.

One is probably justified in concluding that some direct effect of CO_2 was involved here rather than the indirect one of change of pH in the culture medium. Whatever the action, direct or indirect, bubbling the cultures with air containing 3% CO_2 certainly stimulated zoospore production in many more filaments than was normally the case without it and in certain media it led to total sporulation of the

TABLE 1

An Experiment on Sporulation

Medium Employed	Relative Zoospore Production after				
	36 hrs.	37 hrs.	39 hrs.	59 hrs.	61 hrs.
1. SE		X	XX	XX	XXXX
2. Medium J			X	XX	XXXX
3. SE:Medium J, 1:1		X	XXX	XXX	XXXX
4. SE:aqu TE, 1:1		X	XXX	XXXX	XXXX
5. SE:aqu B_{12}, 1:1	X	XXX	XXX	XXXX	XXXX
6. Machlis E			X	XX	XXXX
7. SE:Machlis E, 1:1		X	XXX	XXXX	XXXX
					85 hrs.
1a SE				XX	XX
2a Medium J				X	X
3a SE:Medium J, 1:1				XXX	XXX
4a SE:aqu TE, 1:1				XXX	XXX
5a SE:aqu B_{12}, 1:1				XXX	XXXX
6a Machlis E				X	X
7a SE:Machlis E, 1:1				XXX	XXX

1.-7. - indicates media aerated slowly with air containing 3% carbon dioxide

1a-7a - denotes unaerated cultutes

SE - soil extract solution prepared as in technique section

Medium J - Machlis E + trace elements (1 ml./litre) and vitamin B_{12} (100 ppb)

aqu TE - trace elements as described in technique section, diluted with distilled water (1 ml. trace elements/ litre water)

aqu B_{12} - vitamin B_{12} diluted to a final concentration of 100 ppb B_{12} with distilled water

X - the X's represent relative zoospore production in the medium indicated; one X signals the initiation of filament fragmentation and zoospore release while four X's means total sporulation of the culture; 2 and 3 X's signify intermediate degrees of sporulation

TABLE 2

pH of Culture Media after Dark Incubation with Alga

Medium Used	pH (aerated 3% CO_2)	pH (unaerated)
1. SE	6.3	6.6
2. Medium J	5.9	6.3
3. SE:Medium J, 1:1	6.1	6.6
4. SE:aqueous TE, 1:1	6.2	6.8
5. SE:aqueous B_{12}, 1:1	6.3	6.9
6. Machlis E	5.9	6.4
7. SE:Machlis E, 1:1	6.0	6.7

TABLE 3

An Experiment on Sporulation

Medium Employed	36 hrs.	38 hrs.	60 hrs.
1. DW			
2. DW +B_{12}			
3. DW:SE, 9:1	XXX	XXXX	XXXX
4. DW:SE, 9:1, +B_{12}	XXX	XXXX	XXXX
5. DW:SE, 9:1, +B_{12}	X	XX	XXX
6. DW:SE, 9:1, +B_{12}		X	XXX

1.-4. - cultures aerated with air containing 3% CO_2

5. - unaerated

6. - aerated with atmospheric air, unsupplemented with extra CO_2

DW - distilled water

B_{12}- vitamin B_{12} used at the rate of 100 ppb of the total solution volume

culture (Table 1).

The results of one experiment (Table 1) demonstrated that slow bubbling of cultures with air containing an excess of CO_2 caused zoosporulation in culture media that normally did not support abundant zoospore production, that is, those cultures that lacked soil extract. After 61 hours of aeration with 3% CO_2 even these cultures gave rise to total sporulation. Similar cultures that were not bubbled did not produce significant sporulation even after 85 hours although there was some fragmentation of the filaments. In another experiment (Table 3) bubbling with air alone did not stimulate sporulation but, rather, inhibited it slightly.

The effect of various media on zoospore production was somewhat more obscure than the CO_2 results. Certainly, soil extract was necessary for rapid, copious zoosporulation (Tables 1 and 3). Whether it was the addition of vitamin B_{12}, which at first was suspected, or the addition of the other solutions used, or the dilution of the soil extract by these solutions that promoted zoospore production was not certain. Vitamin B_{12}, alone, used in conjunction with distilled water as the culture medium did not induce sporulation. This result could have been due to the low osmotic concentration of this medium compared to the other media employed. An experiment to determine the effect, if any, of the concentration of vitamin B_{12} on zoosporulation, using B_{12} at the rate of 1 ppb and 100 ppb in diluted

soil extract medium (diluted 1:1 with distilled water), showed that those filaments which were exposed to the lower concentration of the vitamin were slower in reaching the second stage of zoosporulation than the others. However, the time of the initial fragmentation of the cultures was approximately the same.

In the experiment reported in Table 3 dilute soil extract gave rise to much sporulation with or without the addition of vitamin B_{12}. This indicated that the dilution of the soil extract was more important than what was added to it in increasing zoospore output. Perhaps the only concrete results from these experiments were that slow bubbling of the cultures with air containing 3% CO_2 hastened zoospore production and that soil extract had to be present in the culture medium for rapid zoosporulation.

Other investigators have claimed that mature cultures, several months old, are necessary for adequate zoosporulation. That this notion is without foundation was recently demonstrated with a young culture, 14 days old. The culture had been growing in defined medium J and was transferred to a soil extract - medium J solution (1:1) and bubbled with air containing 3%CO_2. Almost total sporulation occurred within 42 hours and the germlings which resulted were the most uniform in appearance that this investigator had ever seen. All were of about equal size, shape and colour and all that were recorded grew into long filaments. This indicated that

young cultures might be more useful as a source of viable germlings than was formerly considered.

One reason for the need of a large number of zoospores at a defined time was stated at the beginning of this section. There was another reason why a rapid production and release of zoospores was often desireable. If zoospores were not produced and collected within 2 to 3 days the culture often became degraded. The degree of deterioration usually depended upon the length of time that the culture remained in the dark. Although the cultures were not axenic, the alga lived in harmony with the bacteria and fungi present in the culture in the light. After a few days in total darkness the algal cells sometimes died with a concomitant increase in the number of other microorganisms. Whether or not these orgnaisms were the cause or the result of the death of the alga was not ascertained but there was often a tremendous increase in their number. Hence, with a shorter dark incubation period it was less likely that the filaments would die without producing zoospores.

From Tables 1 and 3 it was concluded that the best medium in which to elicit zoosporulation was a dilute solution of soil extract. However, the medium most often employed in subsequent experiments was a soil extract - medium J solution mixed in a 1:1 ratio. The reason for this was that the germlings were cultured in medium J after they were collected on glass slides since this solution was found

to be the best defined medium for the vegetative growth of the filaments. The use of medium J in the sporulation as well as in the culture medium circumvented any period of adaptation that might have been required if two entirely different media had been used during these two periods.

EXPERIMENTAL CULTURES

As stated in the introduction Oedogonium cardiacum was expected to grow in a simple, aqueous, inorganic salts solution provided that other culture conditions, such as temperature and light intensity, were within accepted limits. Dr. Horsley (1962 and personal communication) claimed success with his modified version of Molisch's solution (cf. p. 14). Although the major cations required for plant growth were available in such a solution e.g., those of nitrogen, phosphorus, potassium, magnesium and sulphur, there was a marked absence of those elements reported to be necessary for many aspects of metabolism in trace quantities, i.e., those elements which, generally, occur in the third and fourth groups of the periodic table (Wiessner, 1962; Myers, 1962).

The argument in support of using such a simple, salts solution was that the chemicals employed, even though they were of reagent grade, probably did contain very small amounts of some of the trace elements as impurities. Only a few of these micronutrients, e.g., iron and manganese, had been shown to be essential for all algal growth but the need for others, e.g., molybdenum, vanadium, cobalt, zinc, copper, sodium and boron had been demonstrated for several specific aglae. The requirement for only small quantities

of these elements in the culture solution was demonstrated by the fact that copper, although necessary for growth as a constituent of certain oxidizing enzymes, was quite toxic to many algae in anything but minute amounts (Wiessner, 1962). The fundamental requirement of any medium used to culture the alga for developmental studies was that of supporting growth. The alga had to be able to grow consistently in the medium, not sporadically, as to time and place.

In filaments of Oedogonium cardiacum any cell is capable of cell division; that is vegetative growth is not relegated to any one cell or group of cells as occurs in some other algae. Therefore, logarithmic rather than linear growth is anticipated under ideal culturing conditions. The reason for this is that one cell division in a filament results in the formation of two daughter cells; both of these now divide to produce four cells; the four cells divide to give eight cells and so on. That is, the number of cells per filament theoretically doubles at each cell division cycle. Naturally, it is expected that such a growth rate cannot be maintained over a long period of time in the same culture medium since nutrients that are consumed by the growing alga are not replaced. Eventually, the amount of nutrients remaining in the culture medium must become the limiting factor for further growth and when one necessary ion is totally consumed, growth ceases. Thus, with Oedogonium cardiacum there is a criterion with which to judge growth.

If the culture medium employed does not support logarithmic growth, it may be considered to be inadequate. However, logarithmic growth alone does not necessarily signify the best medium for all aspects of development of the alga for other factors such as the ability to reproduce sexually and asexually may not be supported by such a medium. So far as vegetative growth is concerned, rapid, logarithmic development indicates maximum utilization of the alga's growth potential.

The modified Molisch's solution (hereafter referred to as MM) did not consistently support logarithmic growth under the writer's culturing conditions. Also, MM did not support the growth of stock cultures for such cultures usually turned white or pale green within two weeks - the colour change was due to the loss of chlorophyll from the cells and this signaled their death. Although this appeared to be of little direct significance since this medium was used only for developmental studies that usually lasted for no longer than one week, the lack of continued growth and the rapid deterioration of the filaments in such a solution over a longer period emphasized an inadequacy of MM as a culture solution for this alga.

Another aspect of this lack of consistency involved the development of germlings once they had been collected on glass slides. In several experiments using MM as the culture medium a large percentage of the germlings resporulated. That is, the single-celled germlings formed zoospores within

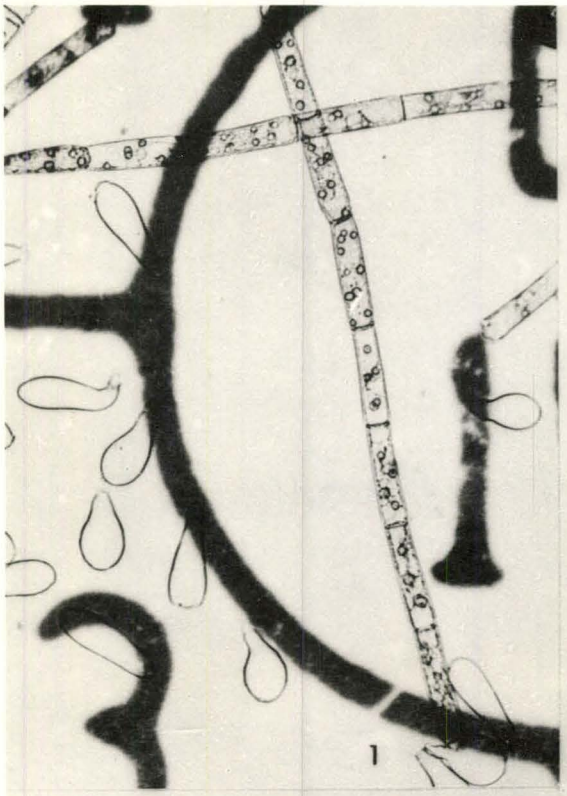
themselves and the zoospores were released into the environment and, presumably, attached to the substratum elsewhere. (Plate 4 shows evidence of this resporulation.) In one experiment 50 percent of the recorded germlings (positioned on the gridded slides) resporulated; in another 33 percent resporulated. The loss of germlings made the tracing of cell progeny difficult. Hence, MM was not desirable as a culture medium from this point of view. Since a change of culture medium from that used by Horsley in his radiobiological studies of Oedogonium cardiacum might have elicited different growth rates, patterns of development and, even, radiosensitivities, several attempts were made by this investigator to elucidate the source of limitation in MM for supporting growth in order to be able to use MM even in a modified form.

However, considerable experimentation led to the adoption of medium J (cf. p. 14) as an adequate culture medium for this alga. The growth of the alga in medium J (with and without the addition of soil extract - Machlis recommended the addition of soil extract) was compared to that in MM and the results are recorded in Figure 1 and Plate 5. The growth rate was logarithmic for a longer period of time in medium J than in MM and after 9 days of growth the filaments in MM were so short in length and pale in colour as to be invisible in the photomacrographs.

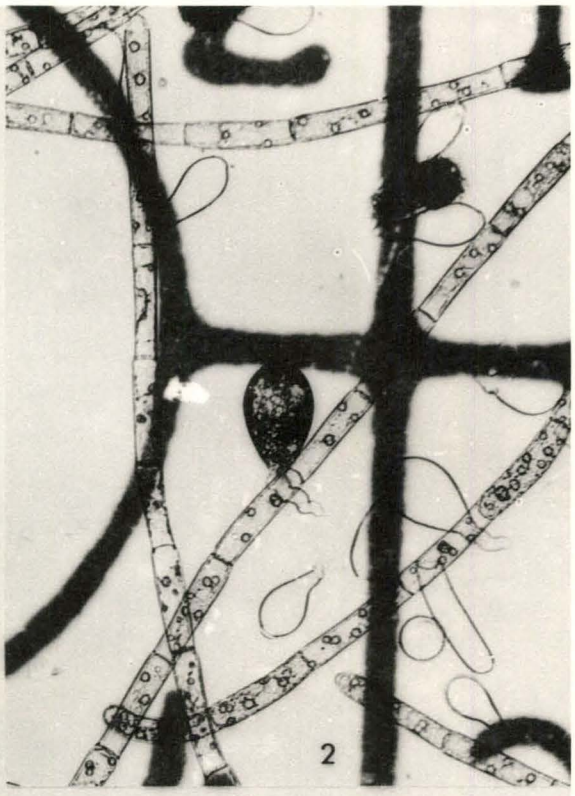
PLATE 4

Evidence of resporulation

Empty single cells anchored to the glass slides are all that remain after these germlings resporulated. a points to an apical cap of a germling that was pushed ajar to allow the newly formed zoospore to escape. b shows a zoospore that has just emerged from the empty germling. (X 200)



1



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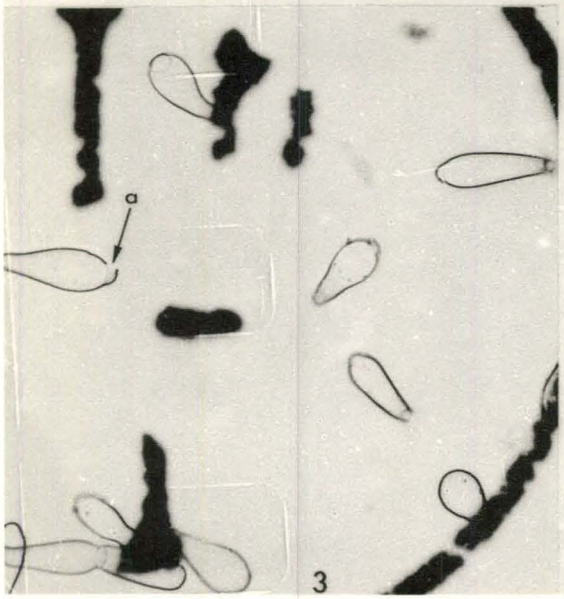

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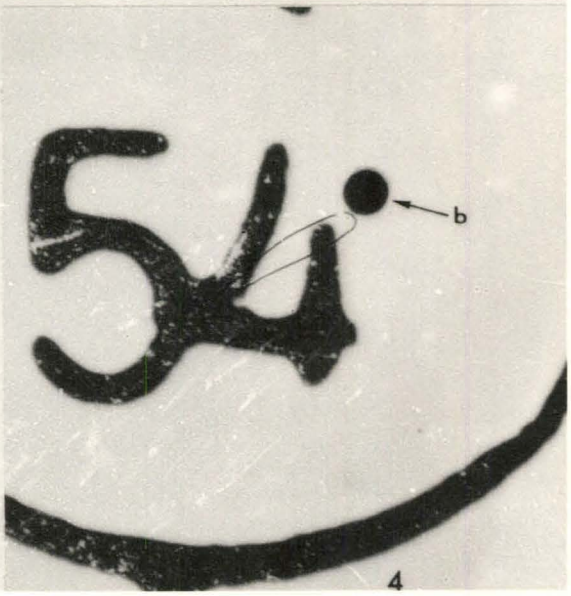


4

FIGURE 1

Growth in Medium J vs. MM

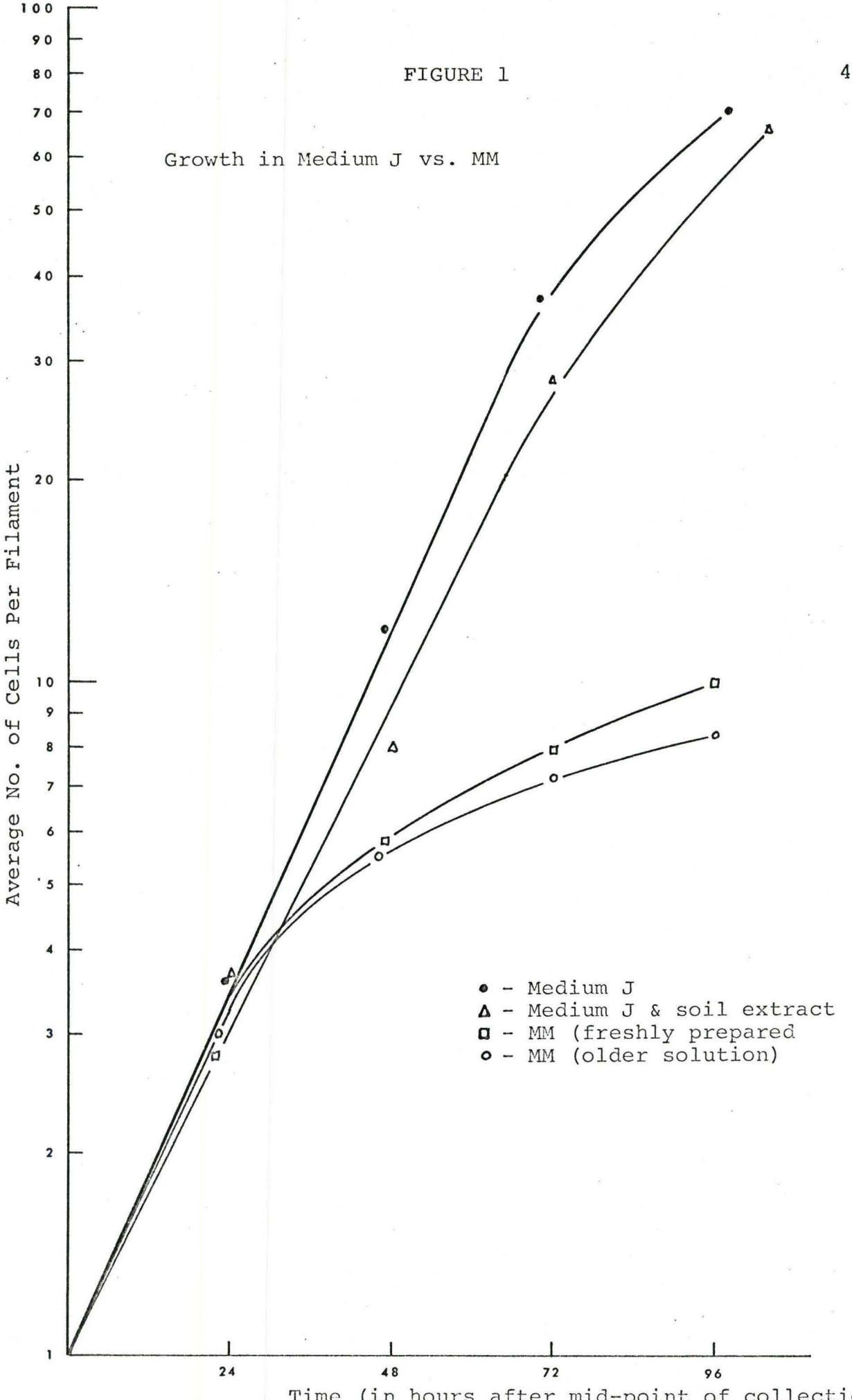
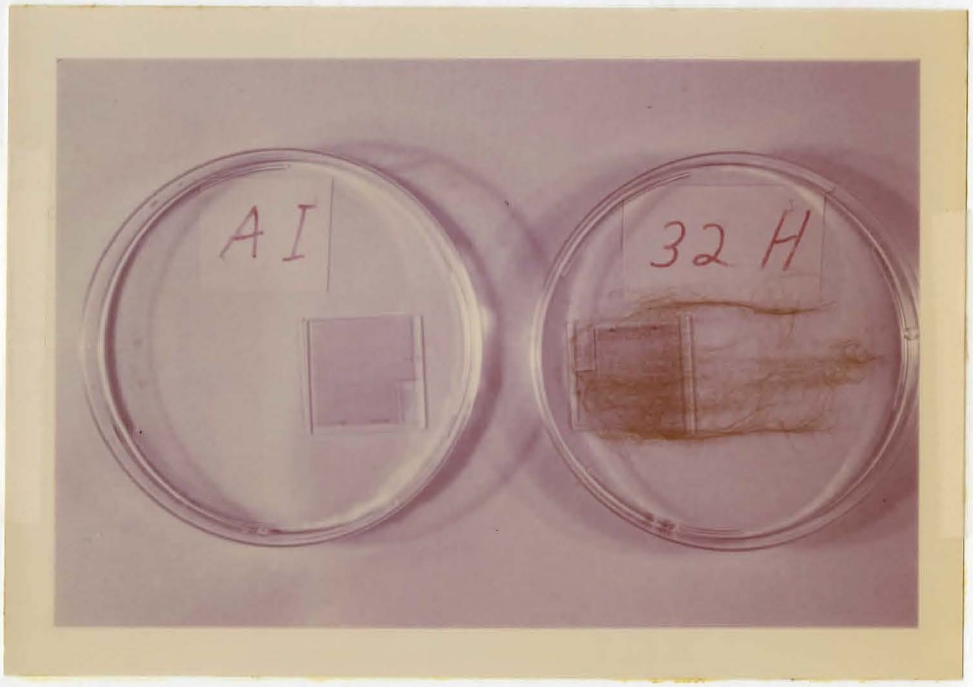


PLATE 5

1. Growth in medium J with soil extract added (27T) and in medium J (32H) after 9 days. Filaments are growing attached to rectangular sections of the gridded slides.

2. Growth in MM (A1) and medium J (32H) after 9 days.


27T
32 H


A I
32 H

The growth in medium J was strongly influenced by the presence of the trace elements and to a much lesser extent by the vitamin B_{12} content. Without the chelated trace elements and vitamin B_{12} there was little growth of the alga in the basic salts solution of medium J (i.e., Machlis E). The addition of vitamin B_{12} stimulated growth slightly but it was the addition of the chelated trace elements that produced the rapid growth reported above. The supplementation of MM with micronutrients did not produce such a large increase in growth (see Figure 2). The growth that occurred after 10 days in some of these solutions is shown in Plate 6.

The results of a comparison of the growth of the alga in medium J and soil extract are shown in Table 4 - soil extract was assumed to simulate the natural culture medium. For the recorded interval the growth rates were similar in each culture solution but many filaments were lost from the gridded slides in the soil extract. Soon after the recorded interval ended growth ceased in the soil extract solution but resumed upon the addition of nitrogen (Plate 7). This indicated that the nitrogen supply was a limiting factor for growth of the alga in soil extract supernatant solution.

There was also a marked difference in the cell size between those filaments grown in soil extract and those cultured in medium J (Plate 8). The cell diameter was about

FIGURE 2

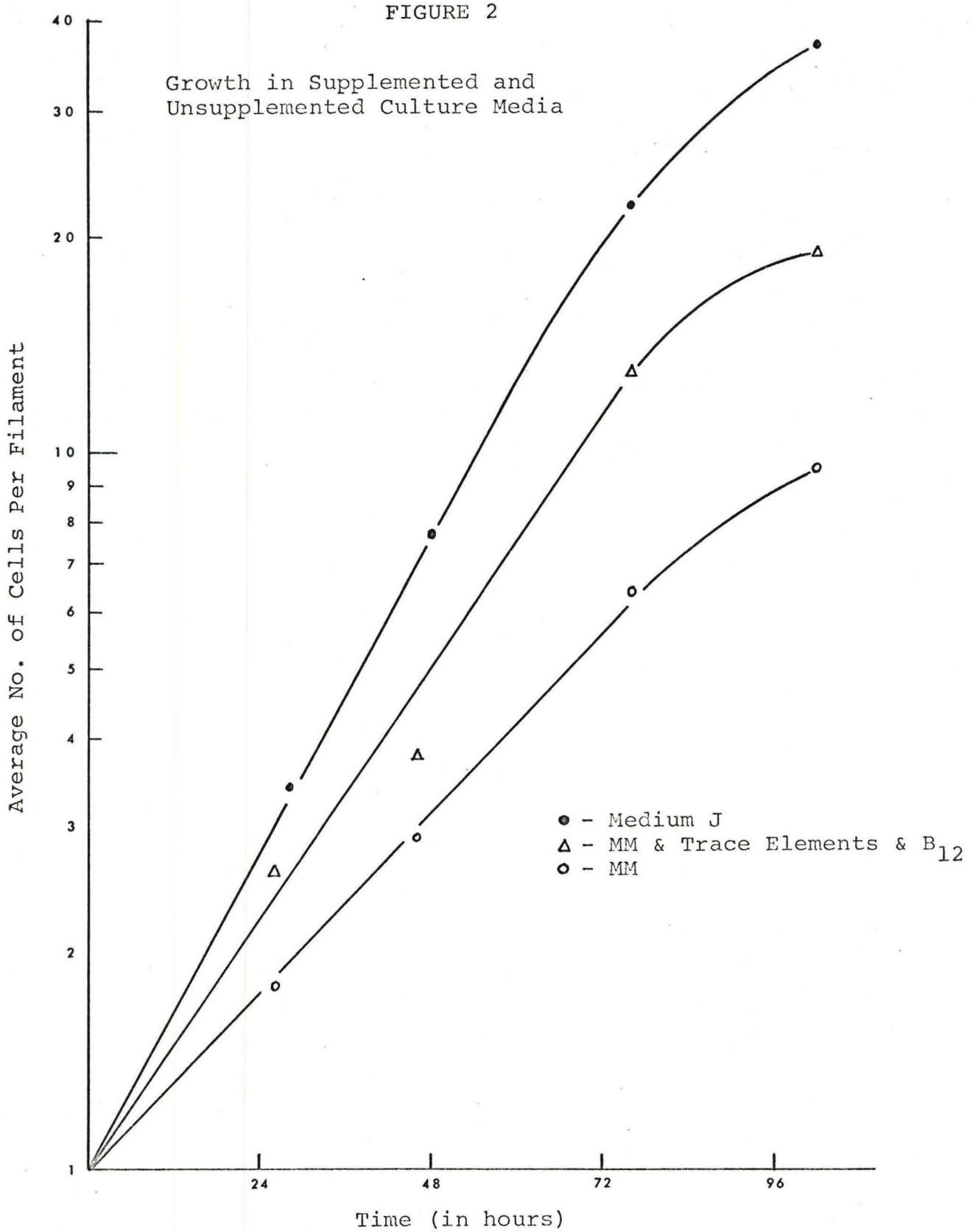

Growth in Supplemented and
Unsupplemented Culture Media
Average No. of Cells Per Filament
40
30
20
10
9
8
7
6
5
4
3
2
1
24
48
72
96
Time (in hours)
● - Medium J
Δ - MM & Trace Elements & B_{12}
○ - MM

PLATE 6

Comparison of growth in 4 media after 10 days of growth

1. medium J
2. MM
3. MM + trace elements + vitamin B_{12}
4. medium J (only 0.5 grams KNO_3/litre solution)

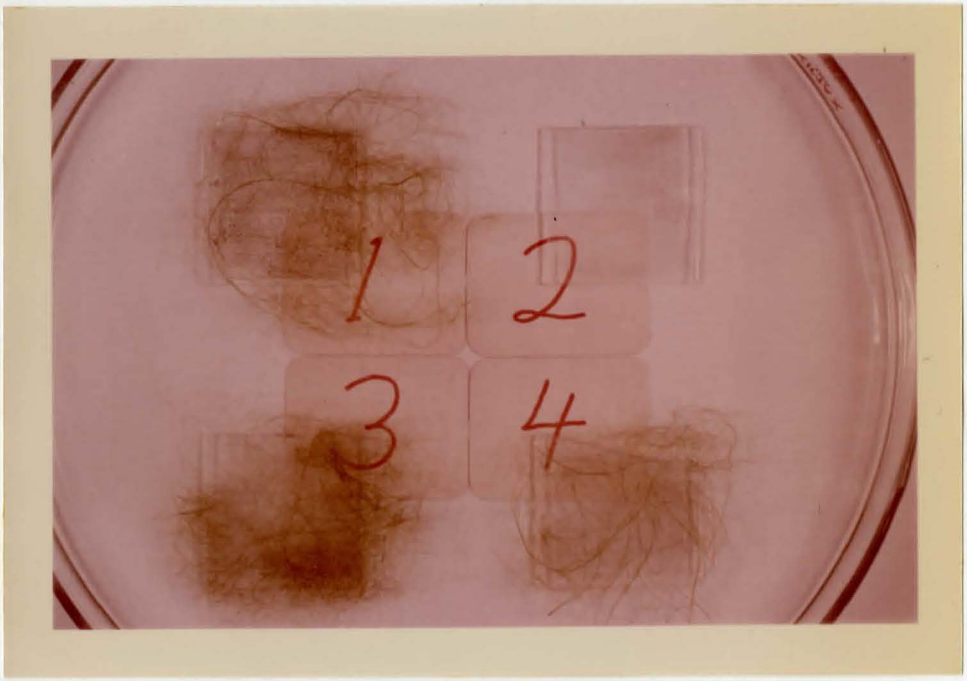

1
2
3
4

TABLE 4

Soil Extract vs. Medium J

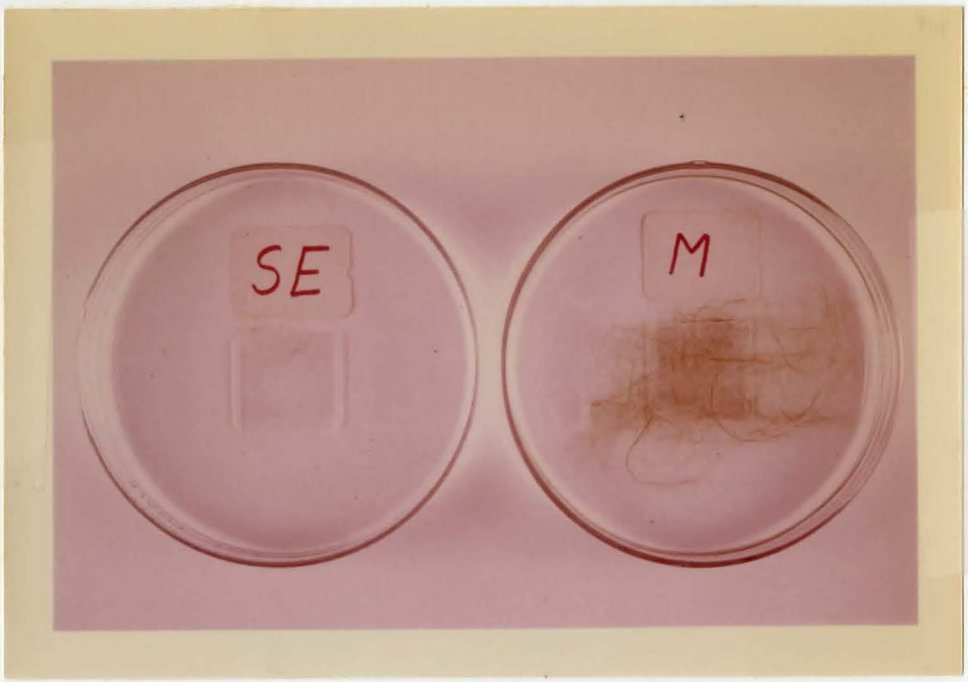
Culture Medium	Average No. cells/filament at time indicated after collection of germlings			Percent filaments lost
	29 hrs.	50 hrs.	72 hrs.	
Medium J	4.22	12.20	28.18	0
Soil Extract	4.00	12.46	25.42	52

PLATE 7

Comparison of growth in medium J (M) and soil extract (SE) before and after the addition of KNO_3 (2 grams/litre) to the soil extract solution.

1. After 13 days of growth the filaments in soil extract are so short as to be difficult to see in macrophotograph; added KNO_3 to soil extract culture at this time.

2. Growth of the two cultures 14 days later - filaments in medium J have grown longer but are now turning brown as the nutrients in the culture solution are exhausted; additional growth has occurred in the supplemented soil extract solution.


SE
M


M
SE

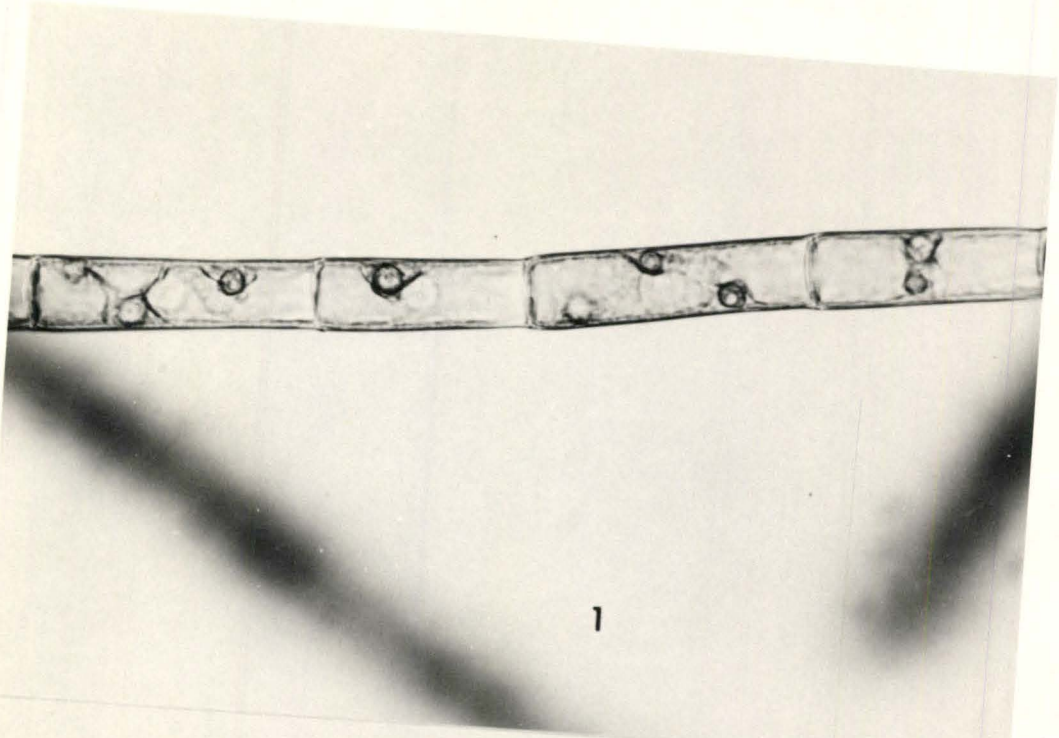
the same in both cultures but those cells which were grown in soil extract were about 48 percent longer than those cultured in medium J. From the foregoing data, it was concluded that medium J fulfilled the basic requirements of a culture solution for this alga and that the limitations of MM were obviated.

PLATE 8

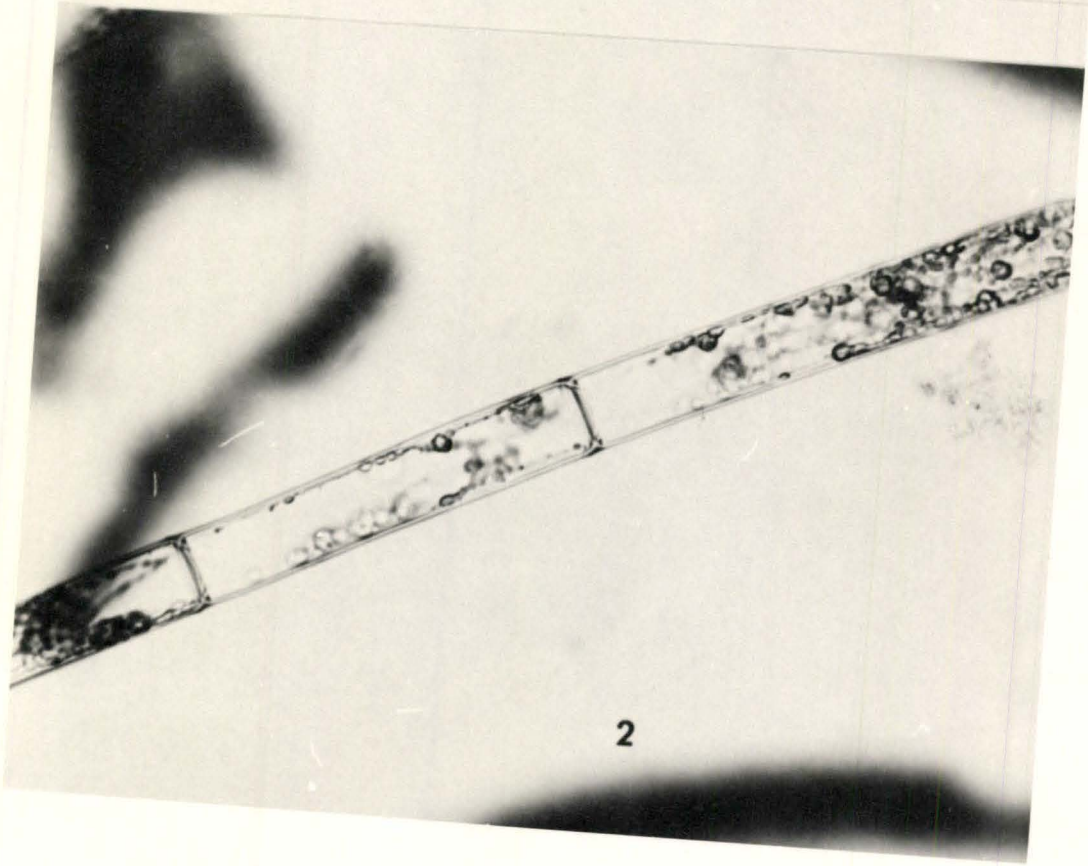
Cell size and structure as they occurred in two different media after 5 days of growth.

1. These cells were growing in medium J.

2. These cells were cultured in soil extract - note the disintegration of the chloroplast. (both X 550)



1



2

THE VEGETATIVE SEQUENCE IN *OEDOGONIUM CARDIACUM*

The following is a resume intended to locate the Oedogoniales on the evolutionary scale of developmental complexity. There appear to have been three main lines of evolutionary organization that the green algae have followed and each is assumed to have begun with a simple, flagellated, motile unicell (Blackman, 1900; West, 1904, Smith, 1950). The first line of development, represented by such genera as *Chlamydomonas*, *Eudorina* and *Volvox*, is termed the volvocine tendency. Herein, flagellated, motile cells have become aggregated, ultimately, into symmetrical structures displaying definite organization. The cells in these colonies retain their flagella and, thus, their motility throughout their entire life even when part of a complex colony. It is unlikely that this development led to larger plants because of the need for motility of all the cells in a colony.

Another evolutionary pathway, the tetrasporine, results in the loss of motility except during reproduction as in higher plants. Primitive stages of this succession consist of colonies, each comprised of several non-motile cells embedded in a gelatinous matrix. While in the matrix these cells do not have flagella but may revert to the

flagellated state seemingly at random. A further development restricts this reversion to motility to defined periods - during reproduction. The cells may still divide vegetatively and, if the daughter cells separate after division, two non-motile cells result. If the daughter cells remain united and division continues in one plane, a filamentous structure is obtained as in Oedogonium. Branched filaments, as they occur in Bulbochaete, a related genus, are an advance over the simple filaments.

The third or chlorococcine route is very restrictive. Except during reproduction, there is not only a loss of motility, but there is also a loss of the ability to divide vegetatively. The siphonaceous algae are included in this group (Smith, 1950). The nuclei of these algal cells can still divide but the cells cannot even though the cells may elongate considerably.

Thus, Oedogonium appears to be a relatively simple genus that is slightly advanced over a cluster of cells, with little obvious symplastic relationship among the cells of a filament. In 1908 C. van Wisselingh noted in Oedogonium sp. that if the second cell division occurred in the apical cell of a 2-celled filament, then the basal cell in the filament produced the third cell division. But, if the second cell division took place in the basal cell of the filament, then the apical cell divided for the third cell division. This implied that there was some order to the

occurrence of cell divisions in a filament of the alga; i.e., cell division did not appear to be a random process. To test this hypothesis several experiments were carried out but before describing them in detail some additional information pertaining to the alga must be presented for a fuller appreciation of the procedures and results.

Although cell division has been described previously (Hirn, 1900; Kraskovits, 1905; van Wisselingh, 1908; Ohashi, 1930; Smith, 1950; Ueda, 1960) a brief summary is included as it is pertinent to the interpretation of some of the observations. The onset of cell division is indicated by the formation of a narrow ring of hemicellulose near the apical end of the cell (Steineke, 1929). This band gradually increases in size and eventually forms most of the cell wall of the new, distal, daughter cell. This new wall material appears to either invaginate slightly or partially split about the outside rim of the ring and becomes somewhat hollowed. At the region of this split a rent appears in the outer, old cell wall that separates within a duration of one second all around the circumference of the cell. This frees the top part of the old cell wall from the bottom part. The ring of hemicellulose now stretches longitudinally to about the length of a normal cell. (See Plate 9 for certain aspects of normal cell division.)

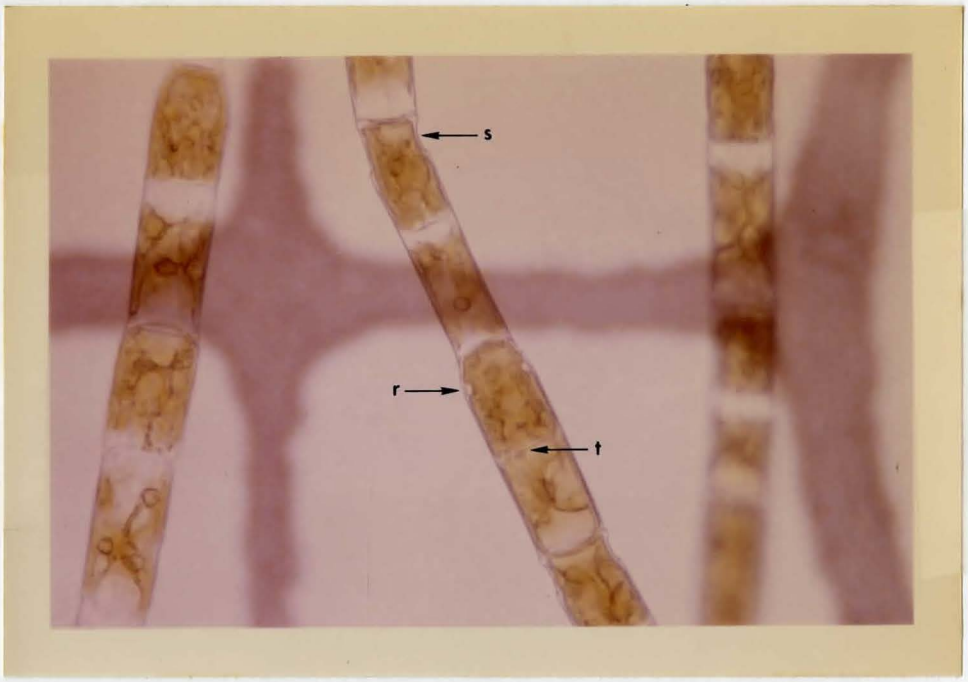
Previous to the stretching of the ring the nucleus of the cell has divided mitotically and the cytoplasm has

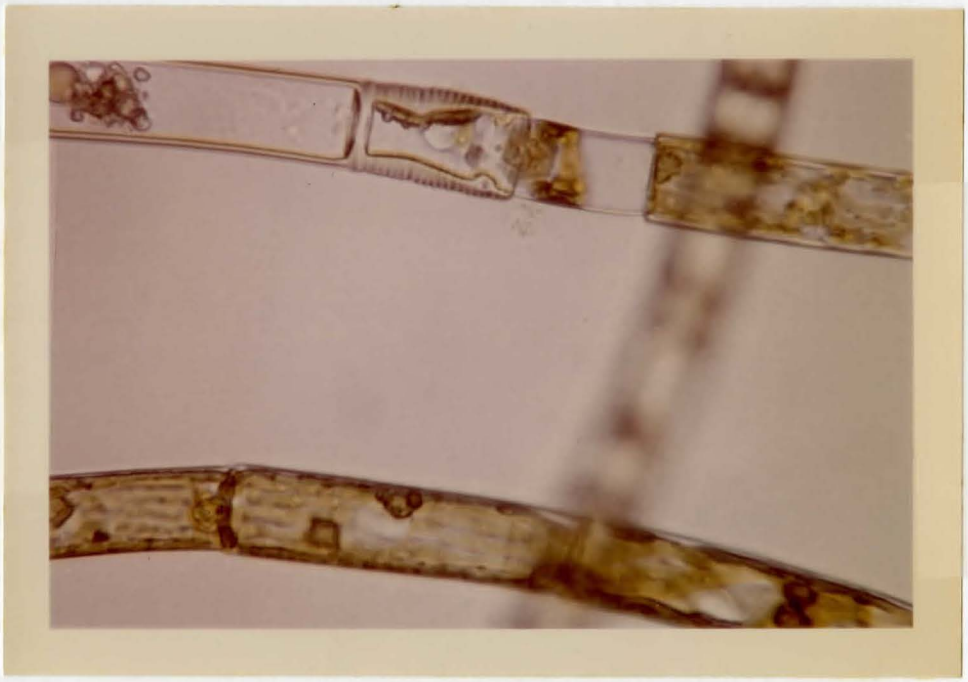
been cleft in half transversely. As the ring elongates so does the cytoplasm of both daughter cells. At full extension of the ring the lower daughter cell is bounded by the original mother cell wall. The apical daughter cell is bounded by the new cell wall material of the extended hemicellulose ring except for a small region of the old wall material that remains at the very top of this distal cell. This apical, old part of the original cell wall remains distinct from the new lateral wall material below indefinitely and can be distinguished microscopically. It is termed the apical cap.

When the upper cell with its apical cap divides again, its distal daughter cell results with two caps of old cell wall material, one cap from the previous division and one from the present division. There seems to be a limit to apical cap formation because the writer has observed that no more than one-half of the lateral wall of a cell is ever covered with apical caps (Plate 9). This indicates that some cells attain a certain stage of differentiation imposed upon them by these apical caps such that further cell division is improbable. The apical cap serves as an indicator of cell division and the number of caps denotes the exact number of times that the cell has divided. A cell containing no caps occurs in the basal daughter cell of each division and the number of times such cells have divided cannot be ascertained. These features of cell division enable the investigator to

PLATE 9

1. Shows typical, vegetative cells in various stages of cell division. r points to hemicellulose ring; s indicates stretching ring; t points to the transverse cleft in the divided cytoplasm (X 500)
2. The middle cell in the upper filament is approximately half covered with apical caps (X 600)


s
r
t

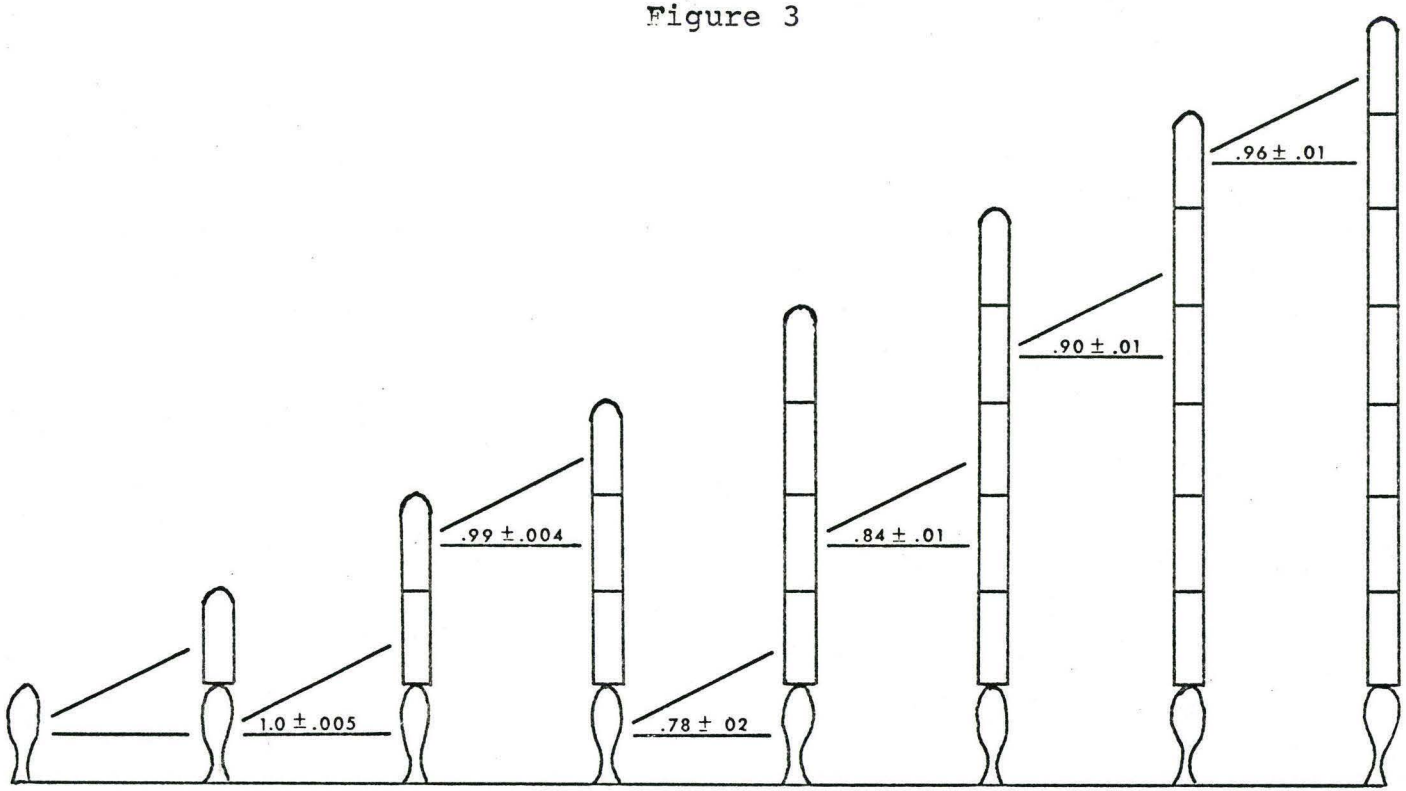


more easily observe and record the production of cell progeny in a given filament than is the case in other filamentous organisms.

The results of nine experiments designed to determine whether or not there was a pattern to the order in which cell divisions occurred in young filaments are summarized in Figure 3 where the numbers represent the probability of that cell dividing in the filament in lieu of any other cell in that filament. The actual observations and calculations appear in Appendix II where it is demonstrated that cell division was definitely not a random process in filaments of this alga.

From the sequence of cell divisions indicated in Figure 3, cell divisions in a filament took place in recurring cycles. A germling divided once to produce a 2-celled filament; then, the basal cell divided again and this was closely followed by the division of the original second cell which resulted in a 4-celled filament. Cell division started again at the base of the filament and proceeded upwards through the four cells until an 8-celled filament had been obtained. Once this stage of development had been reached the writer found difficulty in pursuing longer filaments. (Various stages in this developmental sequence of cell divisions are shown in Plate 10.)

Figure 3



The numbers represent the probability of such cell divisions occurring

From empirical observations cell division appeared to commence again near the basal end of a filament and proceeded distally usually resulting in the formation of a 16-celled filament, although, apparently, not with the regularity of cell division sequence stated to occur for the shorter filaments. Few observations were recorded beyond the 16-celled stage.

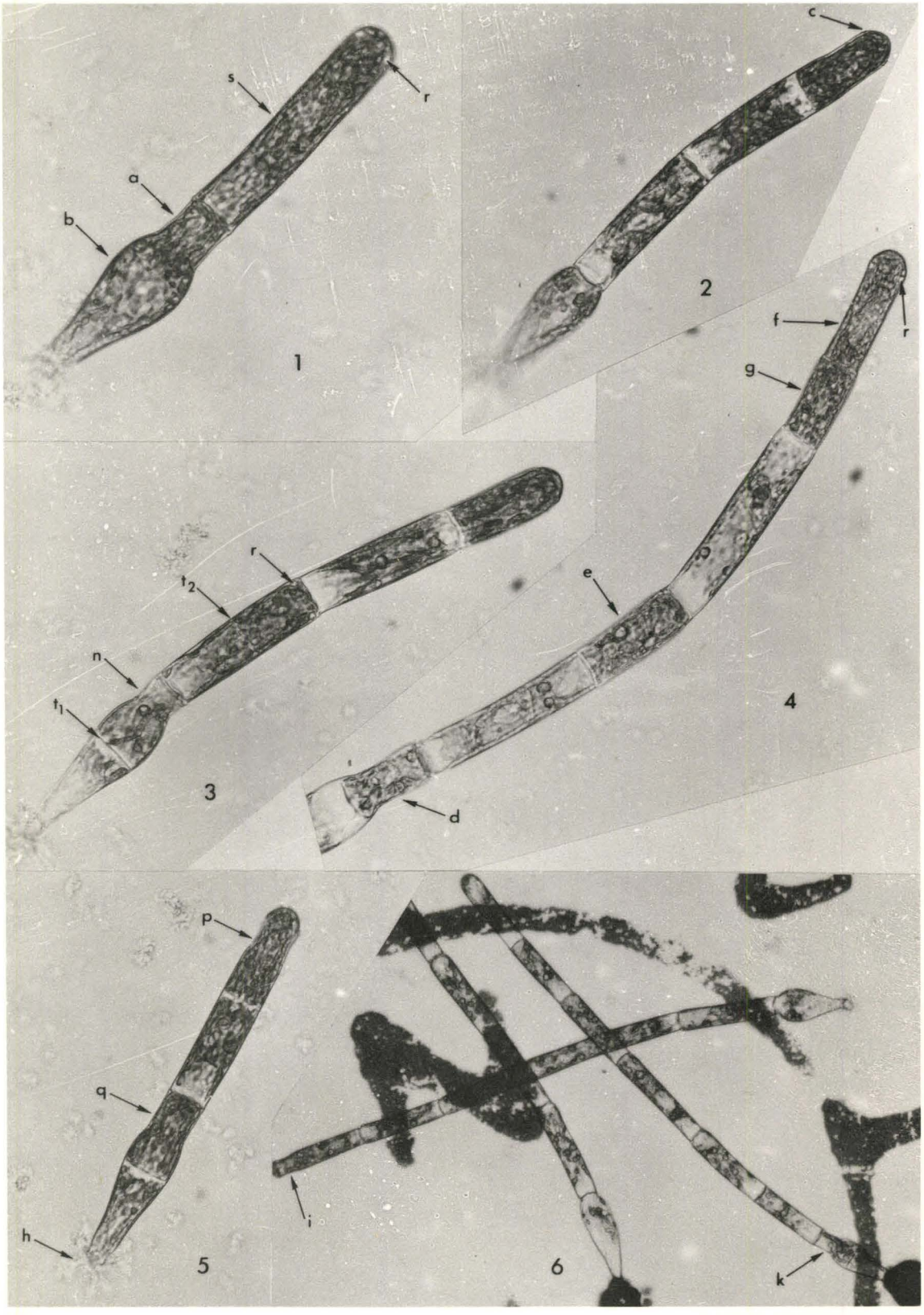
The lowest probability of a certain cell dividing as the main division in a filament as shown in Figure 3 occurred in the 4-celled filaments. Here the probability of the basal cell dividing was only .78 ± .02. The main reason for this low value was a delay in the basal cell division which lasted until other cells in the filament had divided.

This delay in the basal cell division had been noted in the progression from the 8- to 16-celled stage where sometimes the basal cell never did divide. This resulted in the formation of 15- rather than 16-celled filaments. It was not unlikely that some basal cells attained a stage of differentiation where further cell division was improbable; for in long filaments basal cells were seen, on occassion, to contain only small chloroplasts which denoted scant photosynthetic activity and, thus, little capacity for growth. Such a state could well account for the remark by Smith (1950, p. 201) that the basal cell divides only once during the growth of a filament.

PLATE 10

Cell division sequences

1. 2+1,1 b - basal cell, s - second cell, a - the +1 (new cell), r - ring in second cell indicating cell division is soon to occur in this cell;
2. 3+1,3 c points to very slight, double protuberance - the two apical caps;
3. 4+1,1 n points to stretching ring of the distal daughter cell, t_1 and t_2 indicate transverse septa, r points to the ring in the second cell which signifies that cell division is soon to occur;
4. 4+3,1,2,3 this is a 4-celled filament in which 3 cells are dividing and the distal daughter cells are labelled d, e, g. f shows the original fourth cell which contains a ring r.
5. 2+2,1,2 a 2-celled filament in which the two cells are dividing, h - holdfast; p and q - stretching rings.
6. Two 8-celled filaments - in one the 8^{th} cell (i) is being formed in the usual manner, by division of the 7^{th} cell; in the other the 8^{th} cell (k) arises from the basal cell. (2,3,4,5 x 500) (1 X 600) (6 X 200)


s
r
a
b
1
c
2
f
r
g
e
4
r
t_2
n
t_1
3
d
p
q
h
5
i
6
k

The problem then arising was what might influence this developmental pattern of cell divisions. To this end radiobiological studies were performed in an attempt to discover whether or not this morphogenetic sequence could be changed. As stated in the introduction, it was anticipated that if such changes could be elicited in the filaments some insight might be gained into the controlling factors involved. However, before the effects of ionizing radiation on the sequence of cell divisions could be investigated, certain other features pertinent to filament growth had to be revealed.

One such item concerned the sensitivity of these algal cells to ionizing radiation. This depended upon the stage that the cell nucleus was in during the period of irradiation. The radiosensitivity of Oedogonium cardiacum had been shown by Horsely, Fucikovsky and Banerjee (1967) to have been greatest after chromosome duplication and least before this period.

The cell cycle consists of four basic stages starting with G_1 period where there is but a single set of chromosomes in the haploid alga. This is followed by the S phase which corresponds to the period of DNA synthesis. After this comes the G_2 stage where each original chromosome now consists of two individual chromatids. (See Taylor et al., 1957, and Monesi et al., 1967, for relevant discussions of this subject.)

The cell cycle is completed by the period of visible, physical mitosis or M stage.

Usually, just after the M phase the cell, itself, divides and two daughter cells result each with its own nucleus containing one member from each pair of the original chromatids. The cell cycle now repeats itself in each of the daughter cells and so on as a long filament is produced. According to Banerjee and Horsley (1968) the time intervals for the various phases of the first cell cycle of a germling of Oedogonium cardiacum cultured in MM are as follows: G_1 - 3 1/3 hrs., S - 6 ± 1 hrs., G_2 - 6 1/2 ± 1 hrs. and M - 2 hrs. This gives a total first cell duration of 18 ± 2 hours.

Hence, it had to be shown that the germlings of the investigator's cultures passed through the cell cycle within a relatively uniform time period; for, if the cells progressed at various rates, they might have been in different cell stages when irradiated and, thus, have had different radiosensitivities. The results of such cases would have been difficult to interpret from any point of view as various degrees of cell damage would have resulted.

Therefore, three growth experiments were performed using medium J as the culture solution and these were aimed at correlating the cell cycle duration with temperature and any influence this might have had on cell division synchrony. For these experiments 3 X 1 in. glass microscope slides were used to support the algal growth. Several slides containing newly attached germlings were obtained during as brief an interval as possible and the filaments were allowed to grow for predetermined periods of time after which the slides supporting the developing filaments were immersed in a killing-fixing solution (see Appendix I). This stopped growth instantly and preserved the alga so that the average number of cells per filament for each time interval could be determined.

The results of the three experiments were recorded in Figures 4 and 5. At 16°C. cell division was delayed by several hours from that recorded at 21°C. When the alga was cultured at 26°C. cell division was first noted as soon as nine hours after the mid-point of the germling collection (this collection lasted for 60 minutes, whereas, in the previous two cases 30 minutes was sufficient for an abundant collection of germlings).

From the high temperature experiment the slope of the curved part of the graph was shallow indicating that some of the germlings were stimulated to divide quickly by the warm temperature while others were not. From the curve

obtained for growth at 21°C. the germlings appear to have divided together in a reasonably uniform manner. After the 2-celled stage in a filament had been attained there was a stationary period of about two to four hours, presumably, during which the nuclei went through the G_1, S and G_2 stages again, before visible cell division resumed. Nevertheless, synchrony of cell division appeared lost after 30 hours of growth at 21°C. as the curve in Figure 5 no longer remained stepwise beyond this point.

For the high and low temperature curves the results were obtained for each time interval from the first 200 filaments encountered under the microscope upon traversing across each slide several times. The curve obtained for growth at 21°C. resulted from different counts per time interval. The first 12 points in Figure 5 were obtained by observing the first 1000 filaments on each of the corresponding slides and the remaining points from the first 500 filaments.

The reasonably high degree of cell division synchrony exhibited by the germlings at 21°C. constituted one reason for growing subsequent experimental cultures at this temperature. (The slope of the rising section of a synchrony curve implies the degree of synchrony in the cell divisions - the steeper the slope, the greater the division synchrony - and the slope of the curve obtained at 21°C. was the steepest of the three.) However, too much weight should not be laid upon this aspect without further experimentation for it might

FIGURE 4

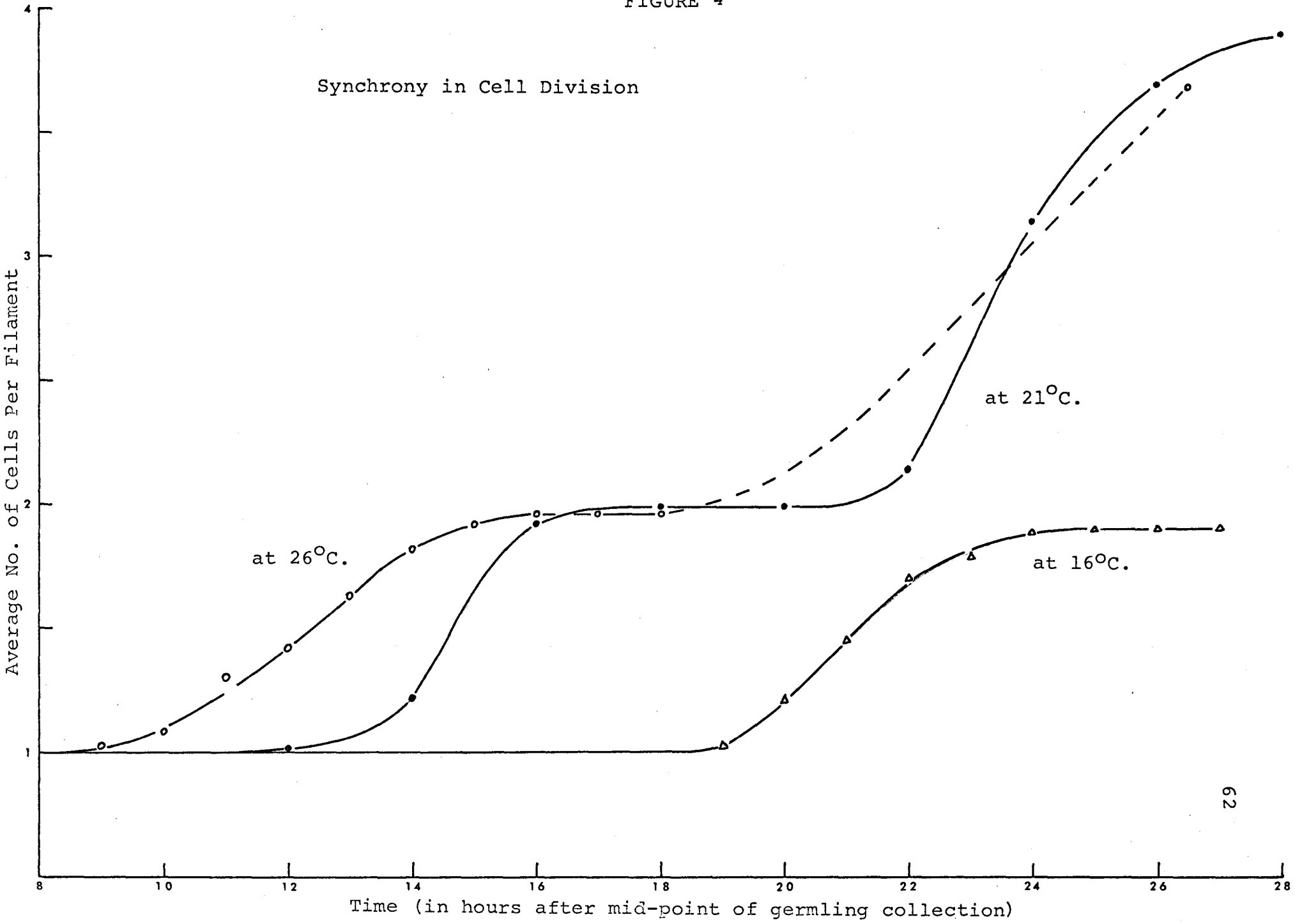
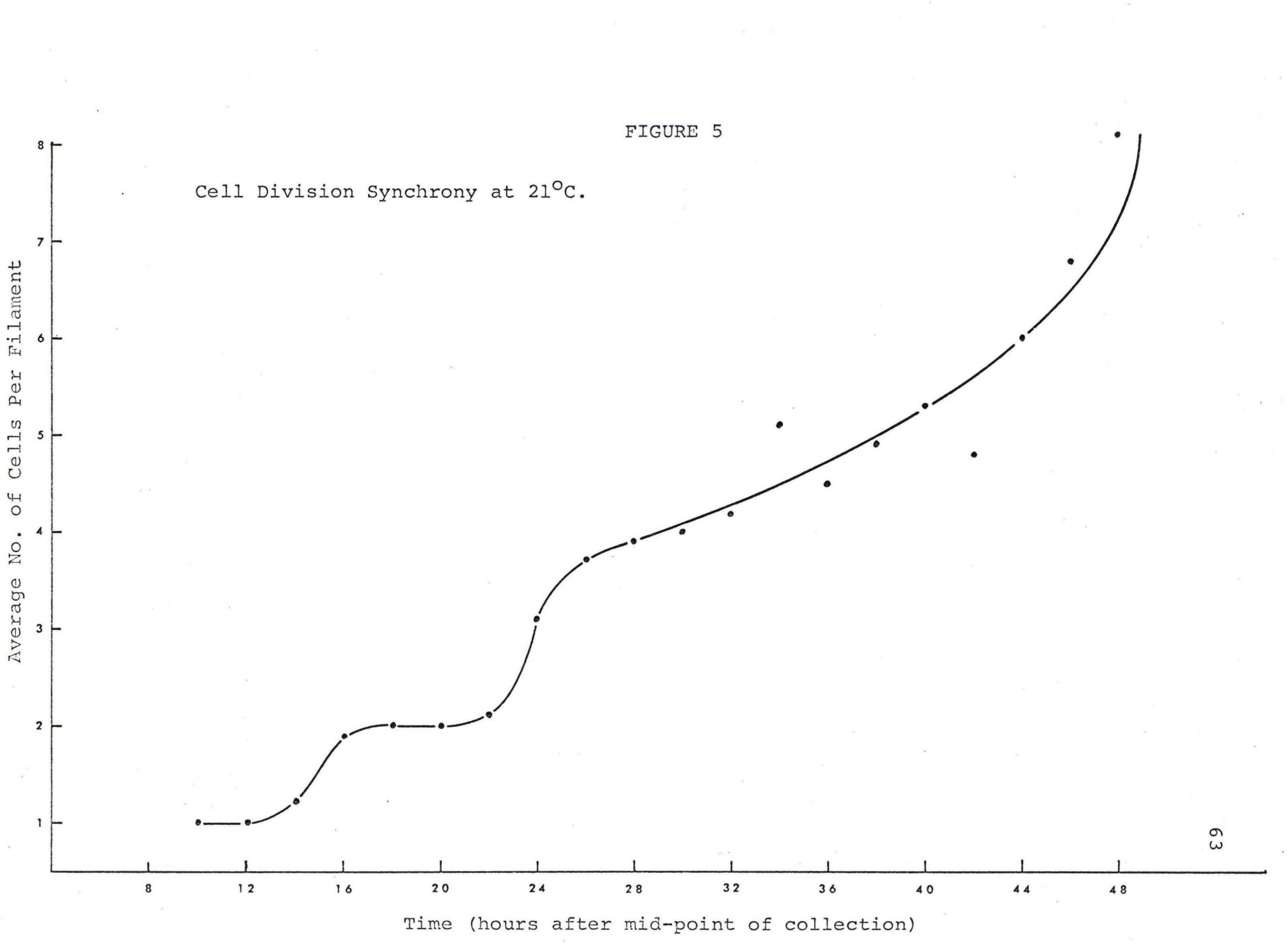

Synchrony in Cell Division
Average No. of Cells Per Filament
1
2
3
4
at 26°C.
at 21°C.
at 16°C.
8
10
12
14
16
18
20
22
24
26
28
Time (in hours after mid-point of germling collection)

FIGURE 5


Cell Division Synchrony at 21°C.
Average No. of Cells Per Filament
1
2
3
4
5
6
7
8
8
12
16
20
24
28
32
36
40
44
48
Time (hours after mid-point of collection)

be that the observed slope differences were due to experimental variation. Another reason for culturing the alga at 21°C. was that at this temperature the cell cycle of the alga cultured in medium J was 14 to 16 hours in duration which approximated that obtained by Horsley and his co-workers for their cultures of Oedogonium cardiacum. Thus, the writer might be able to use their cell cycle sub-stage times and interpret his results in the light of some of their experiments.

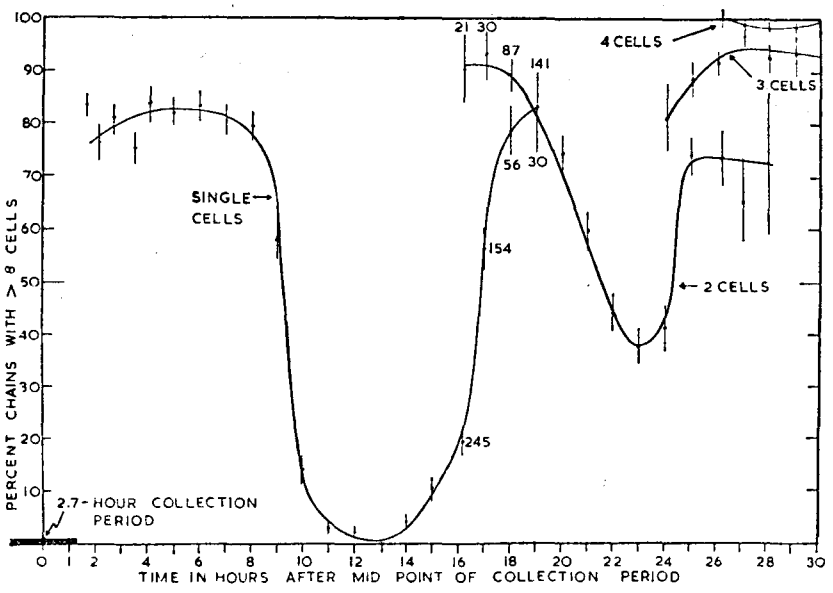
However, the aforementioned evidence alone did not provide proof of the actual duration of the various sub-stages of the cell cycle, i.e., the G_1, S, G_2 and M periods, in the writer's system. The M period could be determined microscopically since here the nucleus changed in gross morphology but the other stages could not be so identified. Nevertheless, Horsley and Fucikovsky (1963) showed that the radiosensitivity of Oedogonium cardiacum varied throughout the cell cycle according to the curve in Figure 6. The sensitivity to ionizing radiation remained constant throughout the G_1 and S phases and then increased sharply during G_2. If the writer's alga had a similar range of radiosensitivity in relation to the time of application of the radiation, the change from the S to the G_2 stage might be demonstrated.

The procedure for this experiment was to collect germlings on 20 glass microscope slides within as short a period of time as possible - for this experiment an abundant

collection of germlings was obtained within a 15 minute interval. The slides were then rinsed with distilled water to remove debris and loosely attached germlings and then were placed in a rectangular glass culture vessel of sufficient size to accommodate all the 20 slides horizontally in the bottom of the vessel. The culture solution used was medium J. At hourly intervals commencing one hour after the mid-point of the germling collection a slide of alga was removed from the glass container and was irradiated with a dose of 1000 rads and then returned to the main culture vessel. Here the alga was allowed to grow for 6 days at 21°C. after which survival was determined.

Survival after radiation was ascertained by noting the number of cells in each filament. Those filaments which had 12 or more cells were considered to be survivors while those with fewer cells were said to be non-survivors. The use of this criterion was based upon work by Horsley and Fucikovsky (1963) and their interpretation of histograms showing the final length attained by filaments following radiation. The writer noticed in his cultures that if a filament attained the 12-celled stage within 6 days, it usually continued to grow much longer; but, if the filament did not reach this length within the 6 days alloted for growth, the constituent cells were usually observed to be in various stages of cytoplasmic disintegration and incapable of further growth. The filaments used for determination

FIGURE 6



Radiosensitivity of single cells and short chains versus time after 1960 rads ^{60}Co radiation. Curves partly overlap at end of cell-cycles. For example, samples from 16 to 19 hours contain both single cells and 2-cell chains. Figures besides a point indicate the number of cells scored if less than 200.

(from Horsley and Fucikovsky, 1963)

of survival were the first 300 filaments encountered upon traversing the slides microscopically and the results were reported in Table 5 and were expressed graphically in Figure 7.

Inasmuch as only 16 of the original 20 slides were used for this part of the experiment, the remaining 4 slides were employed as indicators of the nuclear stage of the germlings during the radiation. That is, at the 10, 12, 14 and 16 hour periods after the mid-point of the germling collection, one of these slides was removed from the culture vessel and placed in the acetic acid-alcohol fixative. These slides of alga were then stained to elucidate their nuclear structure. The results concerning the nuclear stage were recorded in Table 6 and were obtained from the first 200 filaments encountered on each slide.

By 12 hours only 1% of the cells had passed into the M phase. At 14 hrs. 18% had entered M. By 16 hours all but 10% had entered M and 65% had reached the 2-celled stage. That is, most germlings divided between the 14 and 16 hour stages. (Two-celled implies that there were two, distinctly separate nuclei, one in the basal cell and one in the stretching, if not fully extended, second cell.) The radiosensitivity curve plotted in Figure 7 closely approximates that obtained by Horsley and Fucikovsky (1963) with the radiosensitivity beginning to increase rapidly after 8 hours and decreasing after 12 to 13 hours. This 8 hour

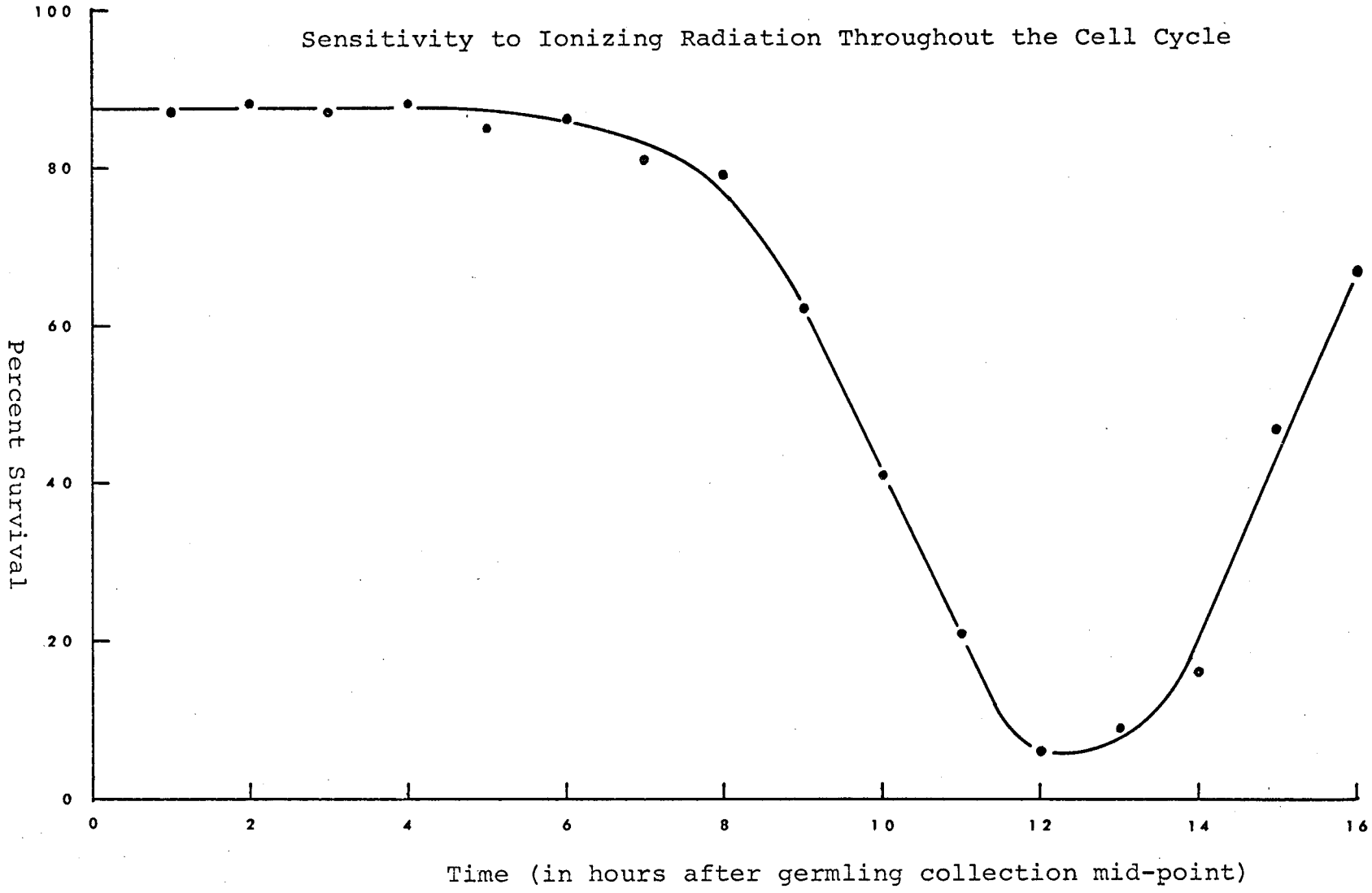
TABLE 5

Cell Cycle Survival

Time	Survivors	Non-Survivors	%Survival	1-celled	Broken	%100
1 hr.	187	28	87 ± 2	1	-	
2	264	36	88 ± 2	8	1	87
3	87	13	87 ± 3	-	-	
4	263	37	88 ± 2	2	-	87
5	254	46	85 ± 2	1	-	
6	257	43	86 ± 2	1	-	92
7	242	58	81 ± 2	-	1	
8	237	63	79 ± 2	4		74
9	187	113	62 ± 3	1	-	63
10	123	177	41 ± 3	1	-	38
11	62	238	21 ± 2	1	-	19
12	18	282	6 ± 1	2	-	7
13	28	272	9 ± 2	4	-	11
14	49	251	16 ± 2	-	1	14
15	141	159	47 ± 3	-	-	45
16	202	98	67 ± 3	1	1	64

%Survival is based on 12 cells or more/filament criterion.
1-celled - indicates ungerminated germlings
%100 - this is the survival value based on the first 100 filaments observed and since these values closely approximate the value obtained after counting 300 filaments it is assumed that counting more than 300 filaments is unnecessary for an accurate determination of survival.
Time - indicates the hours after the mid-point of the collection of the germlings.
Dosage - 1000 rads (100 rads/min.) at each time interval.

FIGURE 7


Sensitivity to Ionizing Radiation Throughout the Cell Cycle
Percent Survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Time (in hours after germling collection mid-point)

turning point corresponded to the end of the S phase as determined by Banerjee and Horsley (1968) for a cell cycle duration of 16 hours.

From the data presented only the M period was actually determined. However, since the cell cycle time was similar for the author's work and that of Banerjee and Horsley (1968) and a similar variation in radiosensitivity had been found throughout the cell cycle by this investigator and Horsley and Fucikovsky (1963) and that a similar M period was obtained by Banerjee and Horsley (1968) and the author, it was assumed that the periods of the cell cycle were as reported by Banerjee and Horsley (1968).

Before the influence of ionizing radiation on the pattern of cell division in this alga could be studied survival of the germlings after various doses of ionizing radiation had to be ascertained for the two genetically different periods of the cell cycle, G_1 and G_2. In order to interpret developmental phenomena arising after radiation during these periods the alga might have to be irradiated not with equal doses but with doses that effect similar survival values. In Figure 7 survival was shown to vary throughout the cell cycle where a dose of 1000 rads nearly destroyed the whole population when absorbed during G_2 while only 13% of the germlings were seriously affected when the radiation was given in G_1. A similar situation regarding developmental patterns might occur following radiation treatment.

TABLE 6

Nuclear Stages

Time	Inter	Pro	Meta	Ana	Telo	Two
10 hrs.	200		-	-	-	-
12 hrs.	198	2	-	+	-	-
14 hrs.	163	10	12	3	7	15
16 hrs.	20	6	9	8	28	129

Time - indicates time after the mid-point of germling collection.
Inter, Pro, etc. - means interphase, prophase etc.
Two - implies two separate nuclei in a stretching if not fully formed two-celled filament.

Horsley et al. (1963, 1967) plotted the radiosensitivity of Oedogonium cardiacum with respect to survival for various times throughout the cell cycle under their culture conditions. Inasmuch as variations in environment during and after radiation had been shown to influence survival (Alper, 1965) and the conditions employed by the author and Horsley and his co-workers differed somewhat, the writer thought it advisable to investigate survival in his culturing system. The results of two such experiments performed during the G_1 period are summarized in Table 7. In both cases the collection period for obtaining germlings was approximately one-half hour. The zoospores were collected on gridded slides and were allowed to remain in a quiescent state for about one-half hour before being subjected to gamma radiation. Seven gridded slides were employed in the first experiment with two slides serving as controls; i.e., they were not irradiated. The others received 1000, 2000, 4000 (2 slides) or 8000 rads. The positions of 100 germlings on each gridded slide were recorded after the radiation treatment since there was too little time for doing so beforehand.

From the results which appear in Table 7 resporulation was prevalent in the germlings on all the gridded slides except for those that received 8000 rads. The resporulation was probably the result of having aerated the culture with air containing 3% CO_2. The culture was so aerated because at that time the natural concentration of

TABLE 7

Survival after G_1 Radiation

Dose (rads)	No. filaments lost	No. germlings resporulated	Percent Survival
	Experiment 1		
control a	7	5	100
b	6	3	100
1000	9	11	93 ± 3
2000	9	10	74 ± 5
4000 a	8	8	21 ± 4
b	16	5	18 ± 4
8000	13	-	0
	Experiment 2		
control	0	0	99
1000	0	0	85 ± 4
2000	1	0	80 ± 4
4000	0	1	20 ± 4
6000	0	0	2 ± 1
8000	2	0	0

Note: 100 germlings were recorded initially for each dose.

carbon dioxide in the culture medium was considered to be a factor that might limit growth. The experiment was repeated and these results are also found in Table 7. Similar doses of ionizing radiation were employed but the culture was not subsequently aerated with CO_2 enriched air. In this case resporulation occurred in only 1 of 600 recorded germlings and this fact tended to confirm the aforementioned suspicion that CO_2 was able to stimulate zoospore production even in very young filaments, e.g., in germlings. A survival curve for the alga irradiated in G_1 is plotted in Figure 8. (The data is from the second experiment in Table 7.)

Similar experiments were carried out for determining survival after irradiation during the G_2 period which resulted in the characteristic 2-stage curve found by Horsley et al. (1967) and the results of one experiment are summarized in Figure 9. The other G_2 experiment confirmed the results reported above. In both cases the radiation treatment commenced approximately 12 hours after the mid-point of the germling collections.

Now that these various preliminary studies were completed, the writer was able to investigate the effects of ionizing radiation on the sequence of cell divisions in the algal filaments.

FIGURE 8

Survival Following Radiation during G_1

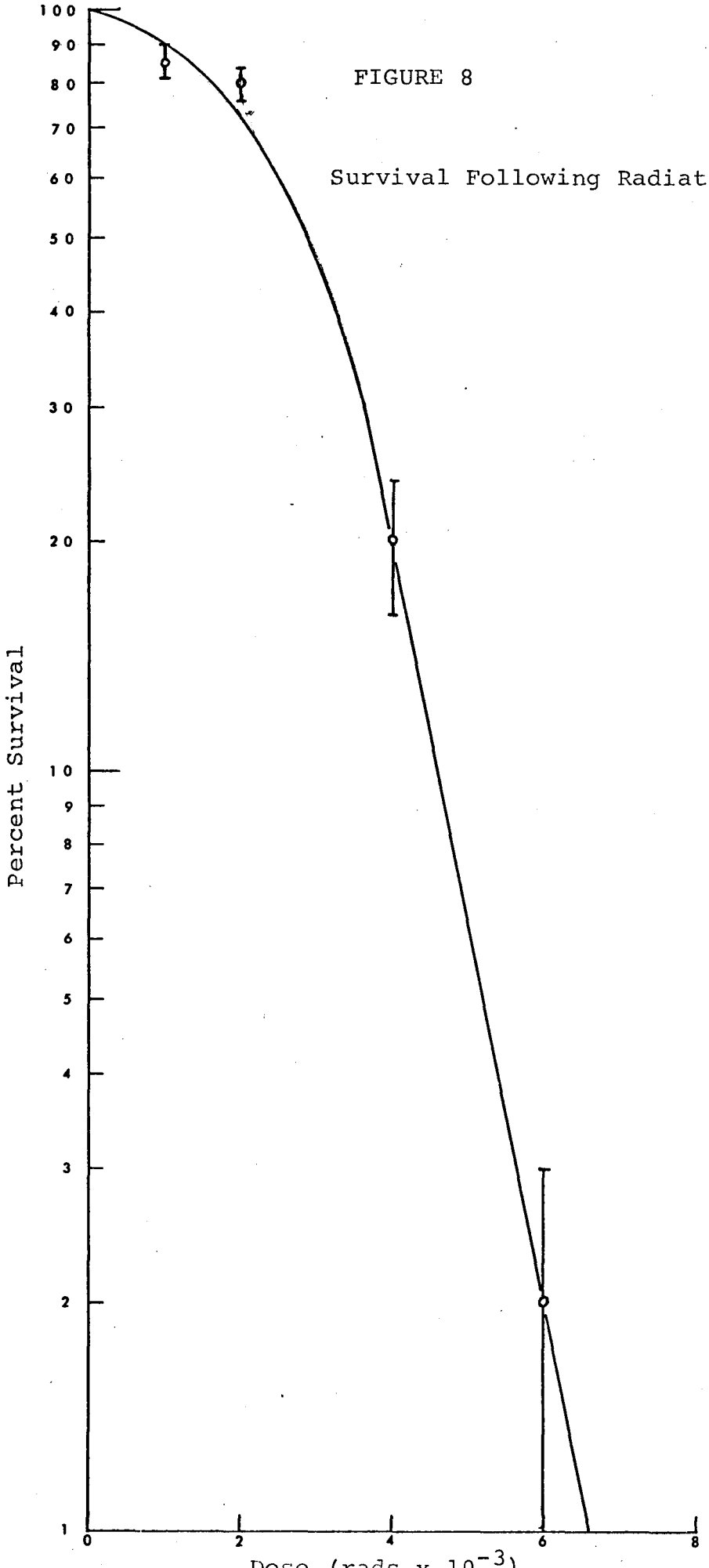
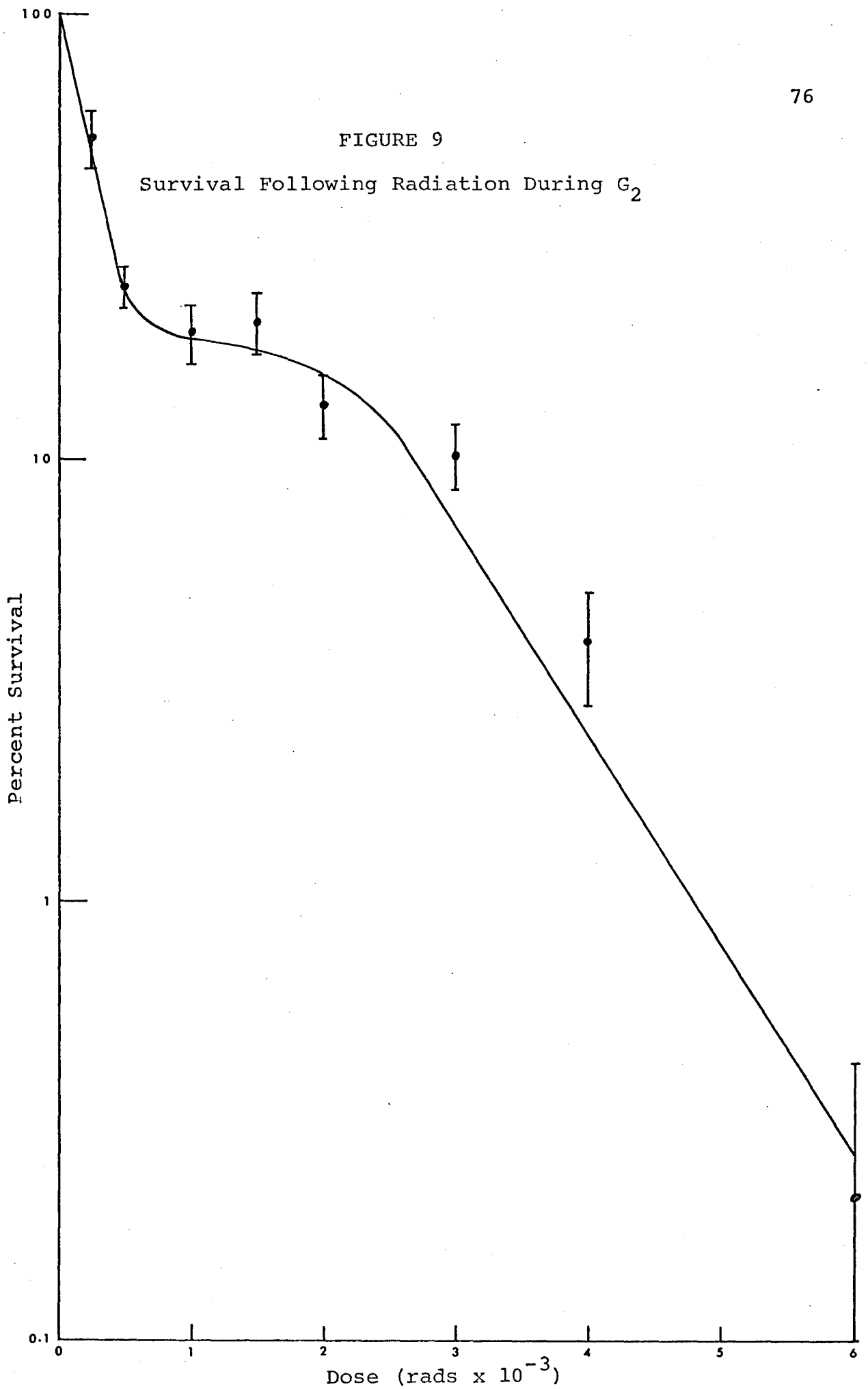


FIGURE 9

Survival Following Radiation During G_2



IONIZING RADIATION AND THE DEVELOPMENTAL SEQUENCE OF CELL DIVISIONS

The sequence in which cell divisions ordinarily occur in a developing filament has been presented. This section reveals some of the developmental anomolies that were expressed by filaments which had been subjected to gamma radiation in the germling stage. Since the actual cytoplasmic and nuclear effects of ionizing radiation on organization as it is manifested by growth and development are really little understood, one must be satisfied with morphological observations for these are the ultimate expressions of more subtle interactions within the disturbed cells. The G_2 period of the cell cycle was the first stage investigated for only here had morphological cellular variations been noticed in profusion following radiation.

The procedure was to collect zoospores on gridded slides, determine the position of some of the germlings on each of these slides, subject the alga to various, predetermined amounts of ionizing radiation within the time limits of the G_2 period and, finally, observe the actual development of the individual filaments.*

---

*This observation, involved for each dose, following the progress of 100 filaments every two or three hours for a period of about 48 hours. Because of the limitation of time, it was possible to examine simultaneously the development for only three or four different samples.

In the first experiment germlings were obtained within a relatively short collection period of 30 minutes. This short period helped to insure synchronous passage of the similarly aged germlings through the first cell cycle. The location of 100 germlings was determined on each of the four gridded slides. At 11 3/4 hours into the first cell cycle the radiation treatment was started and three exposures were given - equivalent to 250, 1000 and 4000 rads.

The amount of radiation given had been arrived at from interpretation of Figure 9. A dose of 250 rads was shown to have reduced survival to about 52 percent. One thousand rads lowered the survival to, approximately, 20 percent while 4000 rads was sufficient to diminsh the surviving population to an insignificant fraction (about 4%). These three doses of radiation covered the range of survival for this alga when irradiated during the G_2 period. One slide of alga was retained as a control and received no radiation treatment.

The second G_2 experiment was executed in exactly the same manner except that the collection period employed for obtaining germlings was only 20 minutes in duration. The third G_2 experiment was carried out in a slightly different manner. Here, 12 gridded slides were used for collecting zoospores and these provided additional material so that a more complete dose range might be covered. A

one hour collection period was necessary in this case. The radiation doses used in this experiment are specified in Table 8 where the survival data for all three experiments are presented. These data did not conflict with the results obtained earlier for G_2 survival (cf. p. 75) although there was some variation from one experiment to another. In the third G_2 experiment 200 germlings were located per slide at the beginning of the experiment. However, these were not followed individually throughout their early development and were only observed after the six day period alloted for growth.

1. Filament Length, Developmental Sequence and Radiation Dose

The first area of investigation concerned the relationships, if any, among filament length (and thus, survival), the developmental sequence of cell divisions and the radiation dose. From Table 9 the number of short filaments (this term was used to designate those filaments that contain more than 2 cells but fewer than 12 cells) was seen to have increased with increasing dose concomitant with a decrease in the number of survivors. That is, increasing the dose did not reduce the population to only 1- or 2-celled filaments but, rather, there was an increase in the frequency of occurrence of the so-called short filaments (see also Figures 10 and 11). The histograms in Figure 10 showed the actual frequency of filament lengths recorded as precentages of the total number of filaments observed for each dose given.

TABLE 8

Survival Data For G_2 Developmental Sequence Experiments

Dose (rads)	Percent Survival	Percent Resporulation	Number of Filaments Lost or Broken
	Experiment 1		
control	95 ± 2 %	4	10
250	67 ± 5 %	2	3
1000	20 ± 4 %	6	3
4000	1.2 ± 1.2%	6	8
	Experiment 2		
control	100%	0	0
250	50 ± 5 %	0	3
1000	14 ± 4 %	0	20
4000	1.1 ± 1.1%	0	4
	Experiment 3		
control	100%	1	1
100	91 ± 2 %	3	4
250	63 ± 4 %	8	3
500	36 ± 4 %	4	4
1000	27 ± 3 %	4	4
1500	24 ± 3 %	6	2
2000	14 ± 3 %	8	2
3000	11 ± 2 %	5	1
4000	7.8 ± 1.9 %	3	0
5000	6.5 ± 1.8 %	6	1
6000	0.5 ± 0.05%	6	2
8000	0.0%	5	0

Percent Resporulation - percentage of original population that underwent resporulation - rounded off to nearest whole number.

TABLE 9

Filament Length, Developmental Sequence & Radiation Dose

Dose (rads)	Number of Cells per Filament		
	2-celled	3-11 (non-survivors)	12 or +
Experiment 1			
control	1	2 (50%)	71 (100%)
250	5	8 (75%)	50 (98%)
1000	39	21 (19%)	13 (100%)
4000	29	48 (4%)	1 (100%)
Experiment 2			
control	--	---	99 (100%)
250	4	29 (83%)	41 (100%)
1000	25	39 (13%)	7 (86%)
4000	8	84 (10%)	1 (100%)

Note - bracketed values represent the percentage of the observed number of filaments (unbracketed numbers) that followed the normal cell division sequence.
2-celled - number of germlings capable of only one cell division.
3-11 - indicates the number of filaments that are classified as non-survivors.
12 or + - number of survivors based on the 12 cells or more per filament criterion.

Figure 10

Frequency of Filament Length

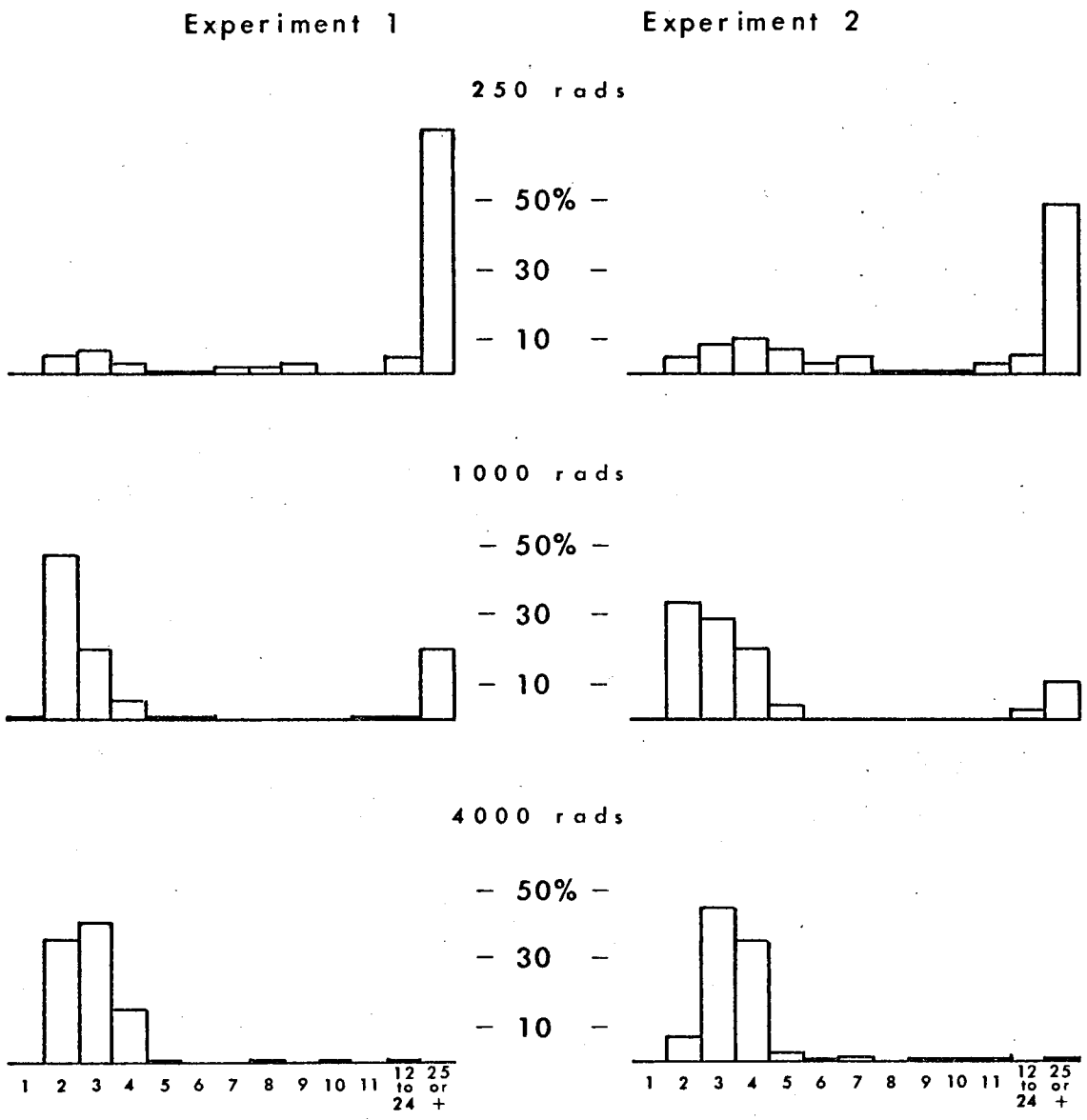
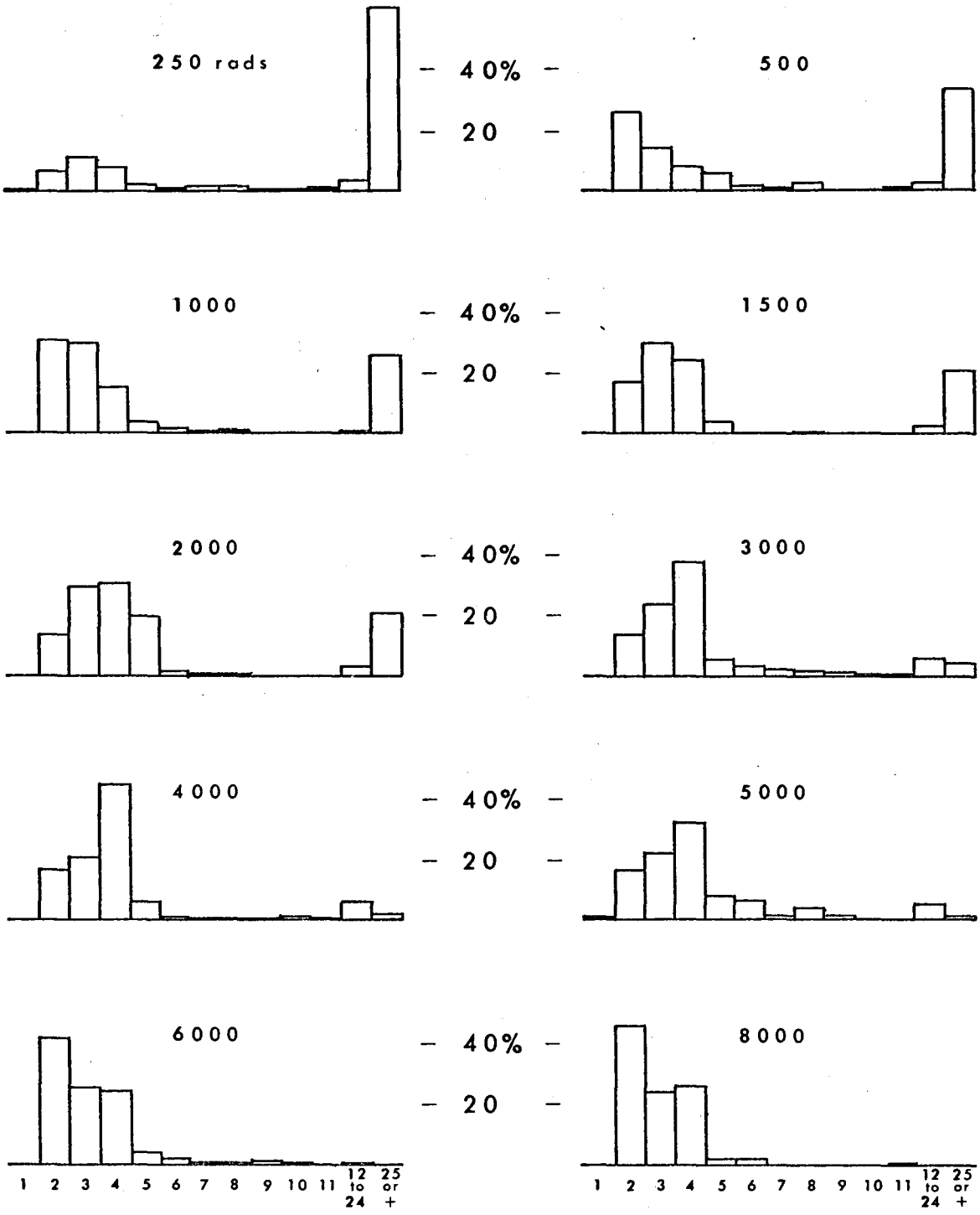


Figure 10 (continued)

Experiment 3



Following 250 rads of ionizing radiation there were some short filaments present in the population. However, after 1000 rads the number of short and 2-celled filaments outnumbered all others while a few filaments still grew to more than 25 cells in length within the 6 day observation period. Nearly the whole population fell into the 2- to 4-celled filament categories after 4000 rads of high energy radiation.

The values given in brackets in Table 9 and summarized in Table 10 for the doses common to the three experiments are concerned with the developmental pattern of cell divisions in the filament and represent the percentage of filaments observed (unbracketed numbers) that followed the normal cell division sequence. These values disclose that non-survivors were disposed to follow the normal vegetative cell division sequence after having received a low dose of 250 rads. After higher amounts of radiation, 1000 and 4000 rads, the majority of non-survivors followed an abnormal development pattern of cell divisions. Survivors generally took the normal pathway to the 4-celled stage following any of the given doses.

The change in cell division sequence mentioned above was simply a change from intercalary to apical development. In apical growth only the most distal cell in a filament remained capable of cell division. One might conclude from this that a normal cell division

TABLE 10

Developmental Sequence of Cell Divisions

Dose (rads)	$P_{(T)}$	$P_{(S)}$	$P_{(NS)}$
0	.98 ± .01		
250	.94 ± .06	.99 ± .01	.81 ± .06
1000	.35 ± .05	.95 ± .05	.15 ± .05
4000	.09 ± .02	1.0	.08 ± .02

$P_{(T)}$ - probability that the normal cell division sequence is followed to the 4-celled stage

$P_{(S)}$ - the same but considering only the surviving population

$P_{(NS)}$ - the same but considering the non-surviving population only

Sample Calculation

For 1000 rads (see Table 9);

$$\text{Let } a = \frac{\#\text{ survivors following NDS}}{\#\text{ survivors (for 1000 rads)}}$$

$$= \frac{(100\%\text{ of } 13) + (86\%\text{ of } 7)}{13 + 7} = \frac{19}{20}$$

$$\text{Let } b = \frac{\#\text{ non-survivors following NDS}}{\#\text{ non-survivors (for 1000 rads)}}$$

$$= \frac{(19\%\text{ of } 21) + (13\%\text{ of } 39)}{21 + 39} = \frac{9}{60}$$

The population that underwent more than one cell division = 60 + 20 = 80

$$P_{(T)} = \frac{\#\text{ survivors} + \#\text{ non-survivors}}{\text{total population}} = \frac{9 + 19}{80} = .35$$

$$\text{Standard Error} = \pm\sqrt{\frac{.35\ (1 - .35)}{80}} = \pm.05$$

i.e., $P_{(T)} = .35 \pm .05$

$P_{(S)} = a = \frac{19}{20} = .95 \pm .05$

$P_{(NS)} = b = \frac{9}{60} = .15 \pm .05$

NDS = normal developmental sequence of cell divisions

sequence was essential for survival. However, not all survivors divided normally and not all non-survivors followed the abnormal division sequence. This implied that the factors that governed survival and cell division sequence were not directly related to each other; although, they both appeared to be affected similarly by ionizing radiation.

2. Developmental Sequence, Cell Anomalies and Radiation Dose

The relationships among the developmental sequence of cell divisions, the production of cell "abnormalities" and radiation dose are presented in Table 11 and summarized in Table 12. Those filaments which were observed to have developed in accordance with the normal cell division sequence all had cells of about normal external morphology. [The values given in brackets in Table 11 refer to the percentage of the number of observed filaments (unbracketed numbers) that contained all normal cells.] There was an increase with dose of those filaments which developed in an abnormal fashion that contained abnormal cells. That is, the abnormal (apical) development was not directly related to the production of abnormal cells such that each abnormally developing filament contained an abnormal cell(s) but, rather, both phenomena were dependent upon the amount of ionizing radiation that had been given.

What was meant by an "abnormal" cell? The most common abnormal cell type (in relation to a "normal" unirradiated vegetative cell) that was encountered after

TABLE 11

Developmental Sequence, Cell Anomalies & Radiation Dose

Dose (rads)	Cell Division Sequence	
	Normal	Abnormal
	Experiment 1	
250	55 (100%)	3 (100%)
1000	17 (100%)	17 (100%)
4000	3 (100%)	46 (0%)
	Experiment 2	
250	65 (100%)	5 (100%)
1000	11 (100%)	35 (3%)
4000	9 (100%)	76 (4%)

Note - the bracketed values represent the percentage of the recorded number of filaments (unbracketed values) containing all normal cells.

- normal and abnormal refer to the order in which cell divisions occur in a filament.

- the numbers are the total number of filaments seen to follow the different developmental sequences.

TABLE 12

Ionizing Radiation and Abnormal Cell Size

Dose (rads)	$P_{(AC)}$	$P_{(NDS,AC)}$	$P_{(ADS,AC)}$
0	0		
250	.00	.00	.00
1000	.42 ± .06	.00	.65 ± .07
4000	.89 ± .03	.00	.98 ± .01

$P_{(AC)}$ - the probability of observing one or more abnormally sized cells in a filament following treatment of the germlings with ionizing radiation.

$P_{(NDS,AC)}$ - same as above except that it considers only those filaments that followed the normal developmental sequence.

$P_{(ADS,AC)}$ - same as $P_{(AC)}$ except that it considers only those filaments that follow the abnormal cell division sequence.

radiation during the G_2 period was the large or giant cell. These cells were often considerably larger than normal cells although their size was not constant and varied greatly. (Plates 11, 12, 13 and 14 show photomicrographs of various abnormal cells including these large and giant cells.) A distinguishing characteristic of these large, abnormal cells in addition to their increased size was their shape. Often it might be defined as being laterally swollen as well as being elongate. That is, the cells were generally somewhat elliptical in longisection rather than rectilinear. Included in the tabulation of large cells were the few ovoid cells which may or may not have been larger than normal cells when observed but which were noticed on several occasions to swell into giant cells at later dates.

3. Anomolous Second Cells

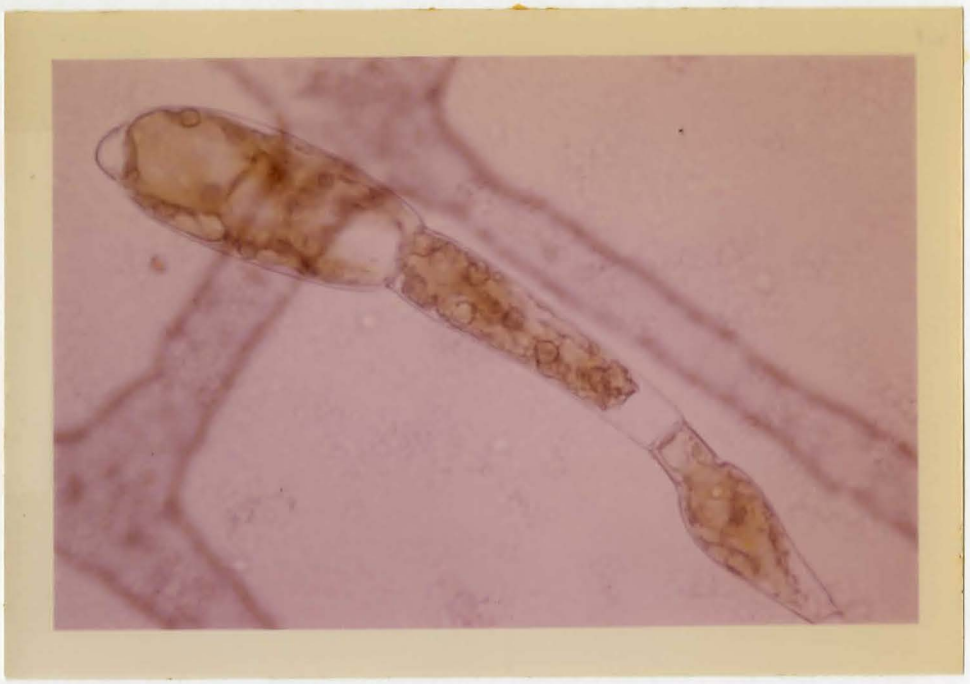
Another organizational feature was the observed development of these large cells, ultimately, from abnormal second cells in the filaments (see Plates 13 and 14 for evidence of such sequences). Sometimes there was more than one cell between the abnormal second cell and the large cell(s). These abnormal second cells fell into one of three morphological categories based upon the length of the cell. A short second cell was exactly as stated - the cell was less than one-half the length of a normal cell and, generally, had a stout cell wall displaying considerable thickening of the internal cell wall layer. The long second

PLATE 11

Typical G_2 radiation damage

1. 4-celled filament - second cell is short and lacks transverse cell wall, third cell elongate and fourth cell is a giant cell; there is a transverse wall between the short second cell and the third cell.

2. 3-celled filament with basal cell attempting to divide, cells about normal size but cytoplasm is quite dense when compared to "normal" cells in corner of photo (X 400)



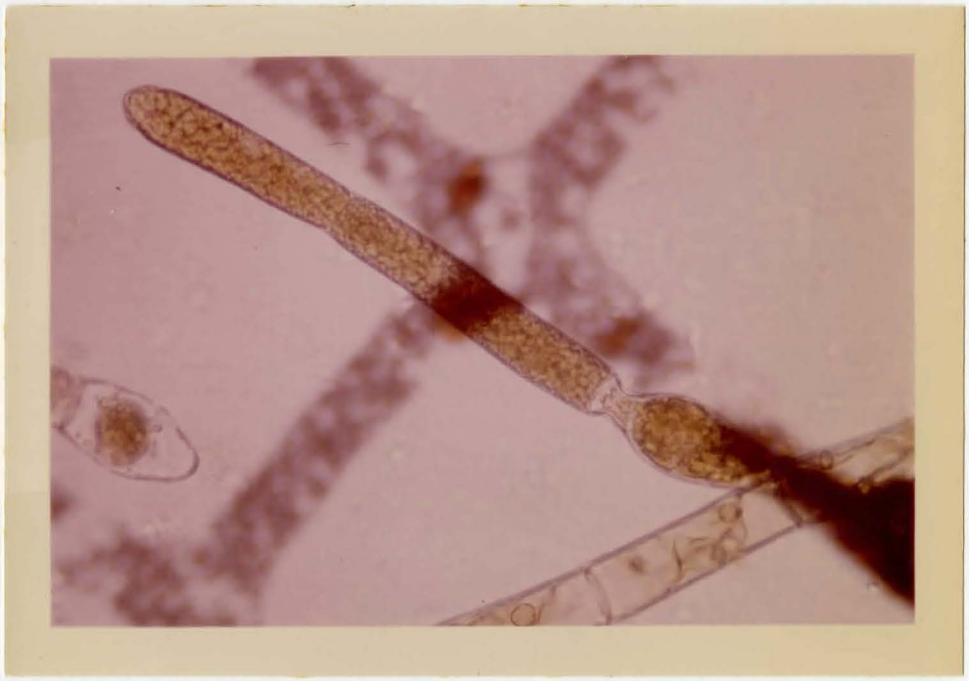


PLATE 12

G_2 radiation damage

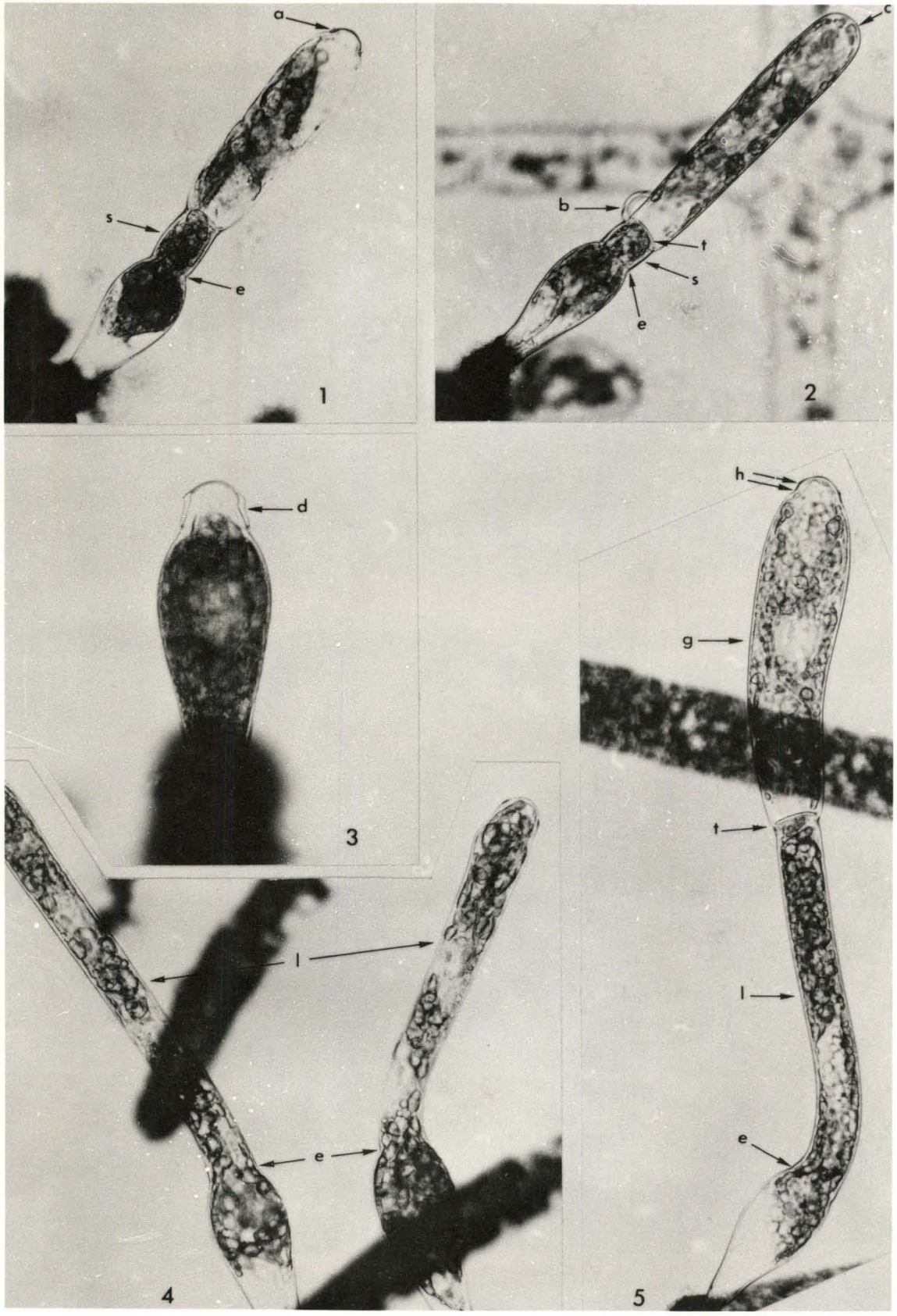
1. 3-celled filament, 2nd cell short (s), e points to lack of transverse cell wall in all photos, a - apical cap,

2. 3-celled filament, t - transverse cell wall, b - apical cap at top-side of second cell, c - smooth apex of third cell (i.e., no apical caps),

3. "short" 2-celled filament, d - thick cell wall of the short 2nd cell,

4. two "long" 2-celled filaments, l - long second cell,

5. 3-celled filament with a long 2nd cell (l), g - giant 3rd cell, h points to two apical caps as would be expected if cell division followed the abnormal, apical development. (1. 2. 4. 5. X 430) (3. X 700) approx.


a
s
e
1
c
b
t
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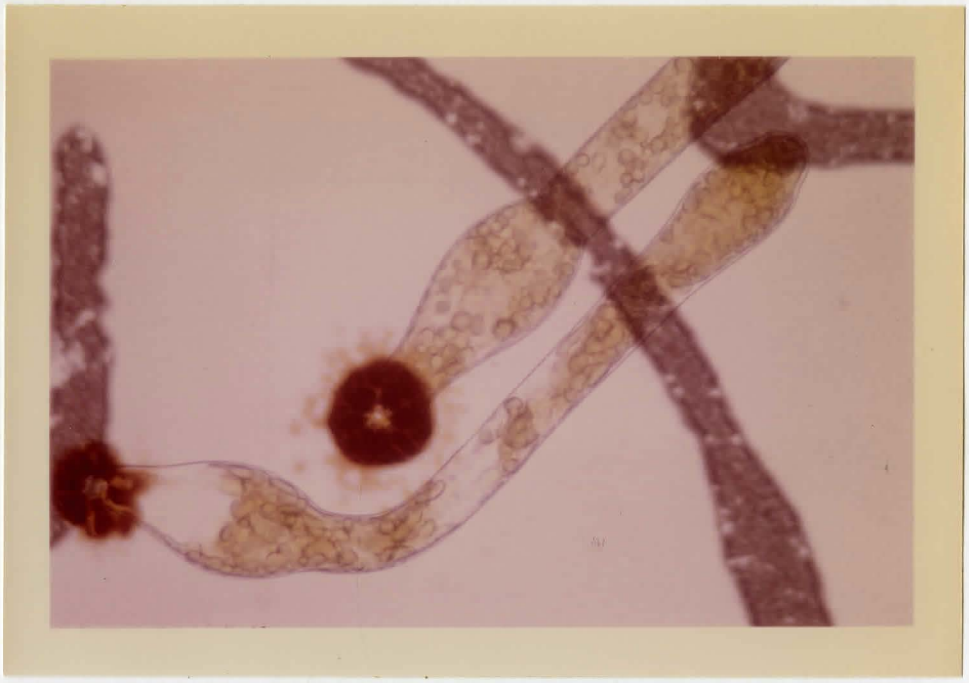
2
d
3
h
g
t
l
e
4
l
e
5

PLATE 13

1. Shows typical 2-celled filament containing a long 2nd cell. (Note absence of transverse cell wall between 1st and 2nd cells.) (X 550)

2. The long second cell went on to divide and produce this 4-celled filament with large 3rd and 4th cells. (X 220)



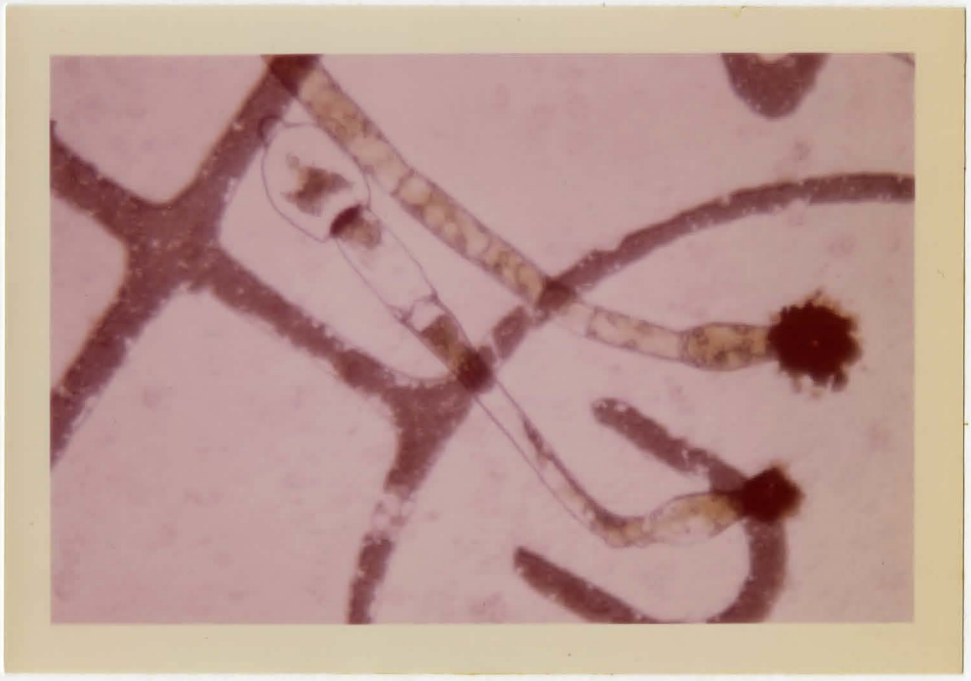


PLATE 14

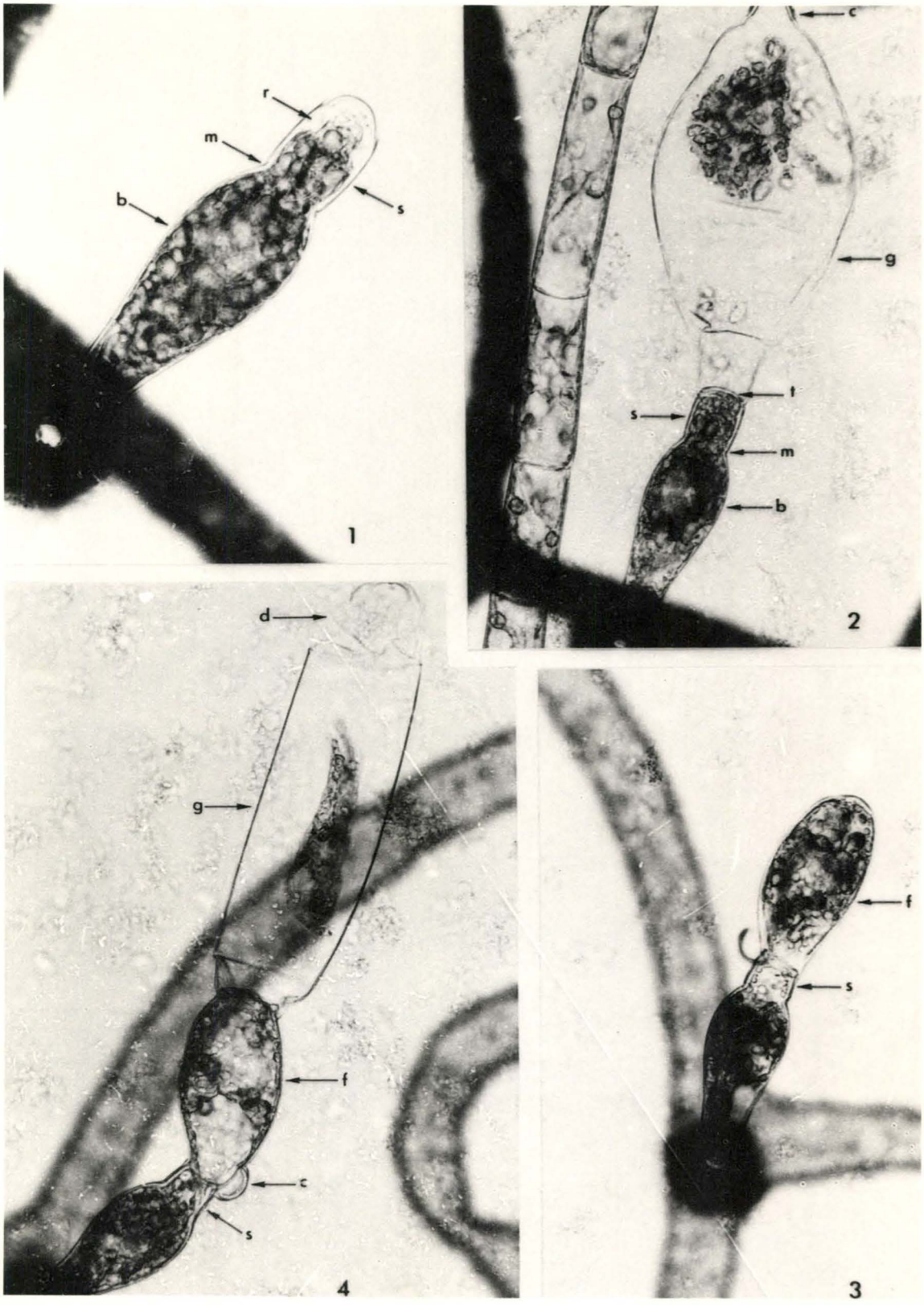
Cell division sequences from short 2nd cells

1. 2-celled filament with short 2nd cell (s), b - basal cell, m - no transverse cell wall, r - ring indicating cell division is to occur,

2. this 3-celled filament developed from that in 1. by division of the short 2nd cell, t - transverse wall, g - giant 3rd cell, c - apical caps,

3. 3-celled filament which developed from a filament with a short 2nd cell (s), apical cap is at side of short 2nd cell, f - ovoid 3rd cell.

4. same filament after another cell division, f divided to produce the giant 4th cell (g), c - apical cap at side of 2nd cell, d - large hemicellulose ring disintegrating, indicating giant cell attempted to divide.
(1. X 900), (2. 3. 4. X 600)


r
m
b
s
1
c
g
t
s
m
b
2
d
g
f
c
s
4
f
s
3

cell was just the opposite in being slightly longer to several times the length of a normal cell. These long second cells did not show the thick internal wall layer that was so evident in the short second cells. The long cells were typically narrower than normal, vegetative cells but this feature varied considerably. A medium second cell attained normal length but might be narrower than normal. Nevertheless, the most distinguishing feature of all three types of abnormal second cells other than their physical dimensions was the absence of the transverse cell wall that normally delineated the boundary between these cells and the basal cells in filaments.

The relationship between the type of abnormal second cell that produced large cells following abnormal development and radiation dose was recorded in Table 13. The percentage of abnormally developing filaments that contained large/giant cells which had been produced by short second cells increased with dose or remained about constant after a certain value had been obtained as in experiment No. 3. There was a decrease in the percentage of these filaments that contained long second cells with increasing dose. (There was also a decided increase in the total number of filaments that contained large/giant cell(s) to a maximum at 4000 rads after which they declined in number.*)

---

*Horsley, Banerjee and Banerjee (1967) found that the maximum number of giant cells occurred after 8000 rads when the germlings were irradiated in the G_2 period.

TABLE 13

Types of Second Cells Producing Large Cells

Dose	Types of Second Cells: Short	Medium	Long	Normal	Total Filaments Observed
	Experiment 1 (100 germlings positioned)				
250	3	--	--	--	3
1000	5 (42%)	--	5 (42%)	2	12
4000	41 (89%)	3	2 (4%)	--	46
	Experiment 2 (100 germlings positioned)				
250	--	--	--	--	--
1000	9 (26%)	4	21 (62%)	--	34
4000	30 (41%)	6	37 (51%)	--	73
	Experiment 3 (200 germlings positioned)				
250	3	--	4	2	9
500	3	--	5	1	9
1000	17 (34%)	4 (8%)	28 (56%)	1	50
1500	38 (44%)	4 (5%)	47 (53%)	--	89
2000	58 (59%)	1 (1%)	38 (39%)	1	98
3000	46 (44%)	9 (9%)	49 (47%)	--	104
4000	95 (81%)	3 (3%)	18 (15%)	1	117
5000	36 (44%)	8 (10%)	35 (43%)	2	81
6000	34 (46%)	20 (27%)	20 (27%)	1	74
8000	39 (57%)	16 (23%)	13 (19%)	1	69

Note - numbers in columns represent the actual observed number of filaments containing short, medium, long or about normal second cells.

- bracketed figures represent the percentage of each type of abnormal second cell of the total number of filaments producing large or giant cells for each dose.

It was also noted that the percentage of medium second cells increased at the higher doses. These facts suggested that long, medium and short, abnormal second cells were closely related phenomena which were expressed differently after various doses of ionizing radiation; that is, short second cells were simply stunted long second cells.

4. Apical Cap Transfer

In the production of large cells from short second cells two systems were encountered. In the first method normal cell division of the short second cell resulted in its thick apical cap remaining at the distal end of the larger, third cell. By the second mode the apical cap of the second cell did not become part of the third cell but, rather, it was pushed aside by the emerging third cell and often remained attached at the top side of the short second cell. This appeared to indicate that the apical cap was really not an integral part of the developing new cell wall. (Plate 15 contains photomicrographs of these conditions.) As stated previously long second cells did not have thick cell walls or caps and they seemed to follow the normal cell division routine of apical cap transfer to the distal daughter cell. In both cases of short and long second cells these cells were only associated with the abnormal, apical cell division pattern.

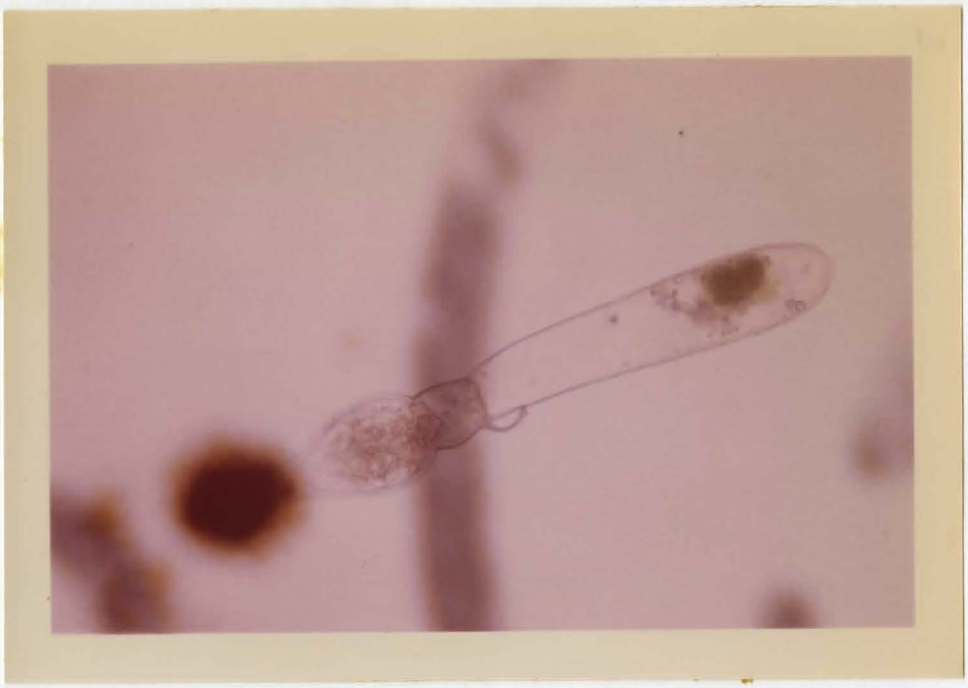
PLATE 15

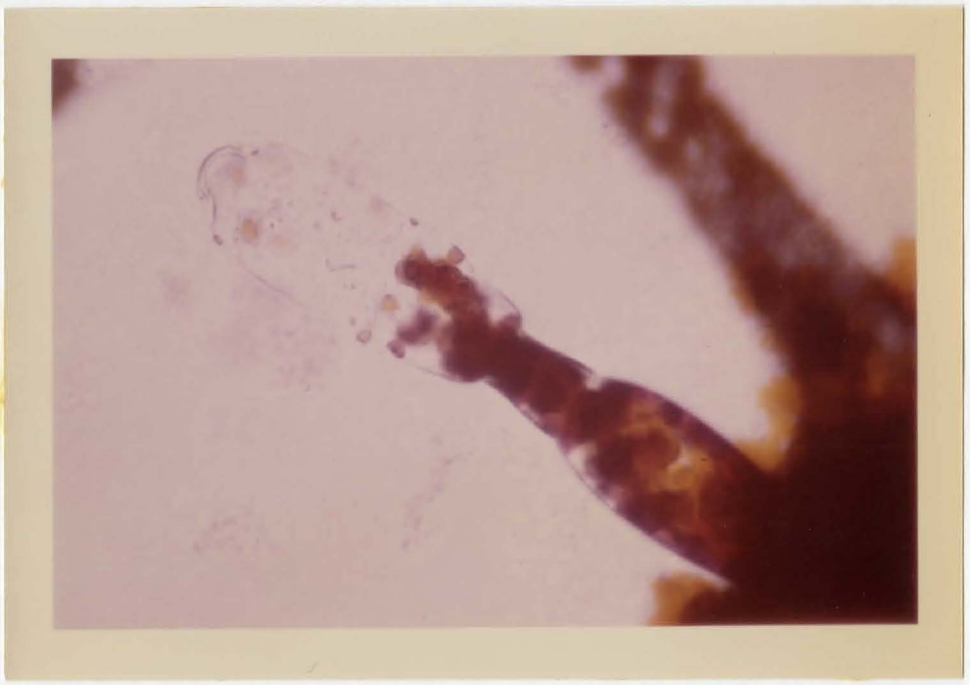
Apical cap transfer in radiation damaged filaments

1. Apical cap of short second cell has remained at the side of the cell resulting in a 3rd cell with a smooth apex.

2. Apical cap of short second cell has been transferred to apex of 3rd cell producing 2 caps on the 3rd cell.

Note: both filaments are dead, i.e., they could not grow beyond the 3-celled stage.
(1. X 400) (2. X 900) approx.





5. 2-celled Filaments

So far little attention has been paid to 2-celled filaments which formed a distinct part of the population following exposure to ionizing radiation. Two-celled filaments developed from germlings capable of only one cell division. As in the short filaments previously discussed, the second cell in these filaments took on various abnormal morphological characteristics. Some of the second cells exhibited normal external morphology showing only a dense chloroplast and an increased number of starch grains that were typical of cells of this alga when the cells grew slowly. There were also short and long second cells which were similar to those previously described in other filaments and these also lacked the transverse cell walls between themselves and the basal cells beneath. There was another group of second cells designated "others" in Table 14 into which fell small ovoid cells and protrusions which did not fit in with the other established categories of abnormal cells. These cells constituted only a small portion of the population.

Although the importance of cell size was not fully appreciated during the first G_2 experiment certain trends were evident among 2-celled filaments with regard to frequency of occurrence and cell size in this and the second G_2 experiment which were substantiated by the third trial. The actual observations were recorded in Table 14 and are summarized here. After 250 rads of ionizing radiation there

TABLE 14

Occurrence of Two-celled Filaments

Dose	Types of Second Cells				
	Normal	Short	Long	Others	Total
	Experiment 1 (100 positioned germlings)				
250	4 (80%)	--	1 (20%)		5
1000	30 (77%)	3	6 (15%)		39
4000	7 (24%)	19 (66%)	2 (7%)	1	29
	Experiment 2 (100 positioned germlings)				
250	4 (100%)	--	-	--	4
1000	11 (44%)	--	11 (44%)	3	25
4000	1 (12%)	7 (88%)	-	--	8
	Experiment 3 (200 positioned germlings)				
250	8 (67%)	1	3 (25%)	--	12
500	14 (30%)	--	32 (70%)	--	46
1000	25 (46%)	1	25 (46%)	3	54
1500	16 (50%)	1 (3%)	14 (44%)	1	32
2000	9 (38%)	2 (8%)	10 (42%)	3	24
3000	10 (48%)	6 (29%)	3 (14%)	2	21
4000	2 (7%)	26 (87%)	1	1	30
5000	-	25 (89%)	-	3	28
6000	1	70 (92%)	1	4	76
8000	5 (6%)	68 (79%)	3 (4%)	10	86

Note - figures in columns represent the actual observed number of the various groups of two-celled filaments

- bracketed figures are the percentage values of the same for the dose given

were just a few filaments that were capable of only one cell division and the majority of these had second cells of normal morphology. One thousand rads effected the greatest number of these very short filaments (except at very high doses in experiment #3) and there were an equal number of filaments that contained normal and long second cells in the last two experiments. Four thousand rads elicited a smaller quantity of these filaments and in all three cases the number of filaments that contained short second cells far exceeded the total of those which belonged to the other classifications. After 4000 to 5000 rads (in experiment #3) the number of 2-celled filaments again increased.

Two-celled filaments containing long second cells were most numerous and constituted the highest percentage of these abnormal filaments following 500 rads of ionizing radiation. There was a gradual decline in their number to zero at 5000 rads, a point at which those 2-celled filaments that contained short second cells were near a maximum. This lent support to the previously mentioned deduction that these two cell types, long and short, were basically one phenomenon which was expressed by different morphological characteristics following various amounts of radiation.

In experiment #3 the trends were more obvious because of the larger number of radiation doses given and the results were more mathematically satisfying because of the

larger number of germlings positioned on each slide at the beginning of the experiment. However, the percentage values determined for the various categories corresponded adequately in all three experiments. The results are summarized in Table 15 for the doses common to the three experiments.

6. Division Delay

Accompanying the decrease in the number of survivors with increasing exposure to high energy radiation was an increase in the delay of the first cell division of the germlings. This aspect of development in Oedogonium cardiacum was being investigated by other workers.

Another aspect of development brought forward in the first G_2 experiment concerned a time delay (concerned mainly with non-survivors) in the occurrence of the second cell division of filaments that were radiated in the G_2 period of the germling stage. (In the following discussion division refers to the second cell division in the filaments unless otherwise stated.) In the control filaments second cell divisions took place within the expected time interval, i.e., on or by the second day. After receiving 250 rads 54% of the filaments that followed the normal developmental cell division sequence divided on the second day while 44% followed on the third day. But, in the filaments that developed abnormally only 25% divided on the second day while 75% took until the third day to divide. This delayed

TABLE 15

2-celled Filaments

Dose (rads)	$P_{(O)}$	$P_{(S)}$	$P_{(L)}$	$P_{(N)}$
0	.003 ± .002			
250	.05 ± .01	.05 ± .05	.19 ± .09	.76 ± .09
1000	.30 ± .02	.03 ± .02	.36 ± .04	.56 ± .05
4000	.17 ± .02	.78 ± .05	.04 ± .02	.15 ± .04

$P_{(O)}$ - probability of occurrence of 2-celled filaments.

$P_{(S)}$ - probability that any of these 2-celled filaments contain short second cells.

$P_{(L)}$ - probability that any of the filaments in $P_{(O)}$ contain long second cells.

$P_{(N)}$ - same as $P_{(L)}$ only is concerned with the appearance of second cells of about normal morphology.

division condition was more extreme following higher doses of radiation. After 1000 rads 100% of those filaments which were to develop normally had divided by the second day but only 26% of those classified as abnormal had so divided. By the fourth day there were still some filaments, 9%, which had not yet divided; but, these did divide before the sixth day. Following 4000 rads 5 of the 7 normal filaments had divided before the third day, whereas, of the abnormal filaments, only 6% had divided by the fourth day. This implied that even if a filament was to be a non-survivor but divided by the normal sequence (2 + 1, 1 followed by 3 + 1, 3) it did so as quickly as in the control filaments. However, those filaments that followed the abnormal (apical) division often showed a greatly delayed second cell division cycle.

The results of the second G_2 experiment confirmed the existence of this second division delay discussed above. No such results concerning this aspect of development were available from the third G_2 experiment as the filaments were not followed on a daily basis.

7. G_1 radiation

The irradiation of germlings during the G_1 period did not produce the cellular anomalies elicted by radiation in G_2. There were few, if any, large cells at any of the doses employed (see Table 16) and abnormal second cells were quite rare and, if present, they did not take on the extreme

morphological features characteristic of those produced after G_2 radiation. Even the abnormal (apical) development was not frequently apparent until a dose of 4000 rads had been absorbed by the culture where 16% of the recorded population followed this sequence (see Table 16). As in the results obtained after G_2 radiation, abnormal filament development was almost entirely restricted to non-survivors and it increased in frequency with an increase in radiation dose. But, even after 6000 rads where survival was very scant, those filaments that followed the abnormal pathway constituted only 51% of the population (excepting 2-celled filaments) observed at this dose. During G_2 studies when survival had been reduced to a negligible quantity the abnormal pathway was followed by 90 to 96% of the filaments observed.

The frequency of the various filament lengths was similar to that obtained after G_2 radiation with most non-survivors falling into the 2- to 5-celled categories (Figure 11). One of the more obvious features of G_1 radiation was the ability of several of the filaments to grow to 12 or more cells in length, that is, those classified as survivors, and then die. Death in most of the filaments observed was denoted by the disintegration of the chloroplast reticulum.

Because of the paucity of developmental abnormalities noted after G_1 radiation, only one formal G_1 experiment was performed specifically concerned with the sequence of cell

divisions during filament growth and the results are those recorded in Table 16. As in the G_2 experiments, gridded slides were employed to support the algal filaments and 100 germlings were located on each slide directly after the G_1 radiation treatment. Six days were alloted for growth for the survival aspect of this experiment. The survival data were also included in Table 16 and they corresponded closely with that obtained in earlier sections concerned with the G_1 period of the cell cycle (cf. p. 75).

In summary, one might draw the following conclusions concerning the effects of ionizing radiation on germlings which were radiated in the G_2 and G_1 stages of the cell cycle. With increasing radiation dose delivered during G_2 there was, in addition to the decrease in survival,

1. an increase in the number of filaments which followed the atypical (apical) developmental sequence of cell divisions,
2. an increase in the occurrence of cellular anomalies, expecially, the production of abnormal second cells and large/giant cells associated with the apical division sequence,
3. a change from long to short second cells in filaments displaying the apical division sequence,

TABLE 16

Filament Length, Developmental Sequence and Radiation Dose

	Number of Cells per Filament			
Dose (rads)	2-celled	3 - 11	12 or +	25 or +
control	-	2 (100%)	86 (100%)	85 (100%)
1000	1	4 (100%)	53 (94%)	48 (96%)
3000	9	29 (90%)	35 (100%)	30 (100%)
4000	17	40 (80%)	18 (95%)	12 (100%)
6000	31	46 (46%)	3 (100%)	2 (100%)

Note - bracketed values represent the percentage to the observed number of filaments (unbracketed figures) that followed the normal cell division sequence.
2-celled - germlings capable of only one cell division
3 - 11 - 3 to 11 cells/filament, i.e., non-survivors
12 or + - filaments containing 12 or more cells
25 or + - filaments containing 25 or more cells

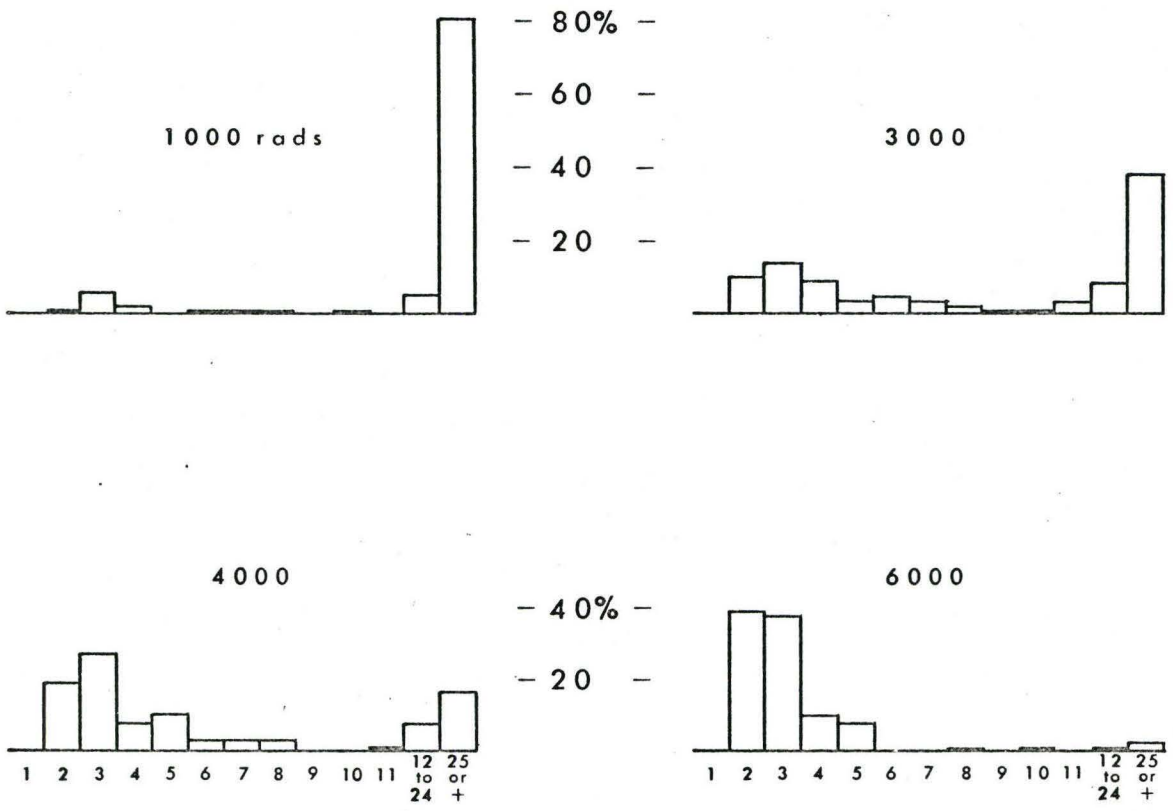
Survival

Dose (rads)	Percent Survival
control	98 ± 1 %
1000	86 ± 4 %
3000	47 ± 5 %
4000	25 ± 4 %
6000	3.8 ± 1.9%

Figure 11

Frequency of Filament Length

G_1 Period



4. an increase followed by a decrease in the number of 2-celled filaments and a change from long to short second cells,
5. an increase in the delay of the second cell division, especially, in those filaments that followed the atypical apical sequence.

Following G_1 radiation there was an increase in the number of filaments which followed the apical development but to a lesser extent than that seen in G_2 for equivalent doses. The other developmental anomalies mentioned above for the G_2 period occurred at very low frequencies.

DISCUSSION

The Production of Zoospores

There are several references in the literature pertaining to the production of zoospores in the Oedogoniales including those by Gussewa (1930), Mainx (1931), Bühnemann (1955), Cook (1959), Howard and Horsley (1960), Kim (1961) and Hoffman (1961). In most cases the quantities of zoospores released have not been clearly stated and the time interval required for their production seems to have been variable. Kim (1961) claimed a "tremendous" discharge of zoospores in most cases with 36 hours from Oedogonium cardiacum (male). He employed a diurnal photoperiod of 15 hours of light (125 foot-candles) followed by 9 hours of darkness at 25° ± 1°C. in a defined, inorganic culture medium which contained chelated trace elements. Kim made no comment as to when during the diurnal light-dark period the alga had been placed under these conditions for the purpose of eliciting zoospores or as to whether this would have had any effect upon zoosporulation.

In this regard Bühnemann (1955), as reported in Bünning (1956), discovered that the release and germination of zoospores of Oedogonium cardiacum were subject to regulation to a large extent by light-dark cycles and that

in some cases the rhythmic production of zoospores continued for 64 days under conditions of constant darkness. That is, there appeared to be a diurnal rhythm to the production of zoospores. The fact that the author's cultures were usually inoculated, for the purpose of obtaining zoospores, at about the same time of day, early to mid-evening, may have had some influence upon the consistency of the time intervals which were observed to have been necessary for zoospore production (36 1/2 ± 1 hour).

Cook (1959) studying the physiology of Bulbochaete hiloensis, of the same family as Oedogonium, reported that low light intensity and increased temperature (27°C) promoted zoospore differentiation and release. Also, more zoospores were formed when 10 to 12 rather than 16 hours of light were given per day. Gussewa (1930), as reported in Carr (1961), showed that zoospore production in Oedogonium was triggered when the alga's respiration had elevated the extracellular concentration of carbon dioxide to a level equivalent to a few percent of the gas phase. The production of zoospores was shown not to have been due to a change in the pH of the medium; but, a decline in the pH within the cells was not excluded as a possible cause of sporulation. High culture temperatures as employed by Cook would probably favour retention of the carbon dioxide from respiration within the cell as the solubility of carbon dioxide decreases with increasing temperature (Carr, 1961).

[Carbon dioxide lowers the pH of distilled water from 5.7, when the atmosphere is at equilibrium with the water, to 3.9, where the partial pressure of carbon dioxide equals 1, (see Carr, 1961)]. Gussewa believed that it was the dissolved gas in the medium rather than the hydrated forms (carbonic and bicarbonic acids) that had been responsible for the production of zoospores. [Felfoldy (1962) demonstrated that unicellular green algae can utilize both free CO_2 dissolved in water and hydrocarbonate ions in their photosynthetic process.]

Certainly, in the writer's work, bubbling of the cultures with CO_2-enriched air assisted zoospore production, especially in those media which normally did not support much zoosporulation. The use of CO_2-enriched air and/or the rate of aeration may have had some effect upon the fate of zoospores after they had been released from the parent filaments. Gussewa pointed out that although 50 mg./litre carbon dioxide (3% CO_2 in the gas phase) effected sporulation the resulting zoospores were somewhat abnormal in being rather amoeboid. This might explain why, on occasion, in some of the writer's cultures, zoospores were released in abundance following bubbling of the culture with CO_2-enriched air but many were reluctant to attach to the glass slides employed for collecting the zoospores within the usual short time period of about 30 minutes. On other occasions, slower rates of aeration might have provided sufficient CO_2 for stimulation

of zoosporulation but not enough to have so injured the zoospores. (The rate of aeration of the cultures tended to vary somewhat from one experiment to another although the time required for zoosporulation remained constant.)

Mainx (1931) disagreed with Gussewa that high CO_2 tension constituted the stimulus for zoosporogenesis since he had found that zoospores could be formed in alkaline solutions, where the hydrated forms of CO_2 predominate over the dissolved gas, and in CO_2 free media. Nevertheless, he listed various other factors that assisted zoospore production in Oedogonium but noted that all factors did not work equally well with all species. These factors included darkening and warming the culture, stirring of still water, transfer to a fresh medium, dilution of the culture medium and transfer of the alga to water. That is, the algae were subjected to changes in the environment. In the writer's process for eliciting zoosporulation the environmental conditions were changed from alternating light-dark periods to constant darkness, from one culture medium to another, somewhat different solution, and from a quiescent state to one of constant motion caused by the aeration with CO_2-enriched air.

The fact that zoospores were produced in dilute soil extract culture solutions (cf. p. 33) substantiated Mainx' statement concerning the dilution of the culture medium and the ability of such a solution to support zoospore

production. Mainx also found that the physiological condition of the vegetative cells influenced their ability to sporulate. Rapidly growing cells were generally unsuitable, whereas, cells of old, long filaments from dense cultures were much more capable of producing zoospores. This work influenced, no doubt, subsequent investigators to employ older cultures as a source of zoospores. However, the writer noted (cf. p. 33) that the most uniform appearing zoospores in relation to external morphology and chloroplast colour and content had been obtained from a young culture.

Howard and Horsley (1960) stimulated zoospore production in Oedogonium cardiacum by placing a mass of vegetative filaments into a darkened bottle containing distilled water. The alga was then maintained at 25°C. for 48 to 72 hours. However, they noted that sufficient numbers of zoospores were not always subsequently released within a short enough period of time for their experimental purposes. This is in accordance with the present investigator's results concerning zoosporulation in distilled water. No zoospores were elicited in distilled water after 60 hours (cf. p. 31) but there was considerable sporulation in another unrecorded experiment where the alloted time was longer - 4 days.

In later experiments by Horsley and Fucikovsky (1962), the distilled water incubation medium was replaced with one which contained, in part, soil extract. Soil extract

was also found to have been a necessary component of the culture medium for consistently rapid and abundant zoosporulation. The role of soil extract in the support of zoospore production is unknown but it is not unreasonable to assume that it is due to some dissolved organic compound(s) in the soil extract.

In summary, the actual initiating factor(s) for zoospore production still remain rather obscure but the importance of adding additional CO_2 by bubbling has demonstrated a more controlled production of zoospores.

Effectiveness of Culture Media

Different culture media have been used to grow various members of the Oedogoniales. The most universal media employed have been modifications of the soil extract solutions after Pringsheim (1946). These have been used "especially for isolation purposes and for growing algae in order to secure 'normal' growth forms" (Starr, 1964, p. 1038). As has been stated earlier, soil-water mixtures are assumed to approximate the natural environment of most fresh-water algae. For those algae that require an alkaline medium the pH of the soil extract solutions can be raised by the addition of $CaCO_3$ (Starr, 1964).

Pringsheim (1946) steamed rather than autoclaved the soil-water mixtures and this was also the technique recommended by Starr (1964). Nevertheless, Machlis (1962) in studying the growth of Oedogonium cardiacum and Oedogonium

geniculatum autoclaved his soil-water mixtures. He noted that only by autoclaving was the growth factor in soil, which he subsequently demonstrated to be necessary for the growth of these algae, released into the liquid phase of the mixture. That this growth factor was an organic compound was demonstrated by drying and combusting the soil extract after which it lost all its ability to support the growth of these algae. This substantiated the earlier observations of Cook (1959) that ashed soil was of no value in making soil extract solutions for the culture of Bulbochaete. This indicates that some members, at least, of the Oedogoniales are partially heterotrophic or are auxotrophs, i.e., they need organic substances as growth factors but not as sources of energy.

The potency of this soil extract factor was shown by Machlis in 1962 who noted that the impressive growth which could be attained by Oedogonium in purely inorganic culture media supplemented with vitamin B_{12} was simply due to a carry over of this soil extract growth factor with the algal inoculum if the algae had been exposed previously to soil extract. In a similar fashion a dependency upon such a carry over effect for growth in inorganic media was also shown for vitamin B_{12}. However, recently Machlis (via personal communication, Dr. Horsley) has been able to grow axenic cultures of Oedogonium cardiacum without the addition of soil extract. Also Horsley et al. have recently grown

large, 5 gallon stock cultures of non-axenic Oedogonium cardiacum using a Machlis medium bubbled with 3% CO_2.

Droop (1962) listed references which supported the notion that vitamins as they occur in the natural habitat are mostly of bacterial origin. This could explain why the writer did not observe a strong dependency upon B_{12} by his cultures of Oedogonium cardiacum. The writer's cultures contained bacteria while those of Machlis' were axenic and, therefore, had no microorganisms that might have been capable of producing the vitamin for the algae to utilize. Nevertheless, the author observed that there appeared to have been a slight improvement in growth in a strictly inorganic medium when it was supplemented with B_{12} even when bacteria had not been excluded from the culture.

Evidence that bacteria played a useful role in the writer's stock cultures, which were maintained in the diphasic soil-water extract medium, was provided by the following: When two equal volumes of soil extract solution, one containing the soil fraction (diphasic) and the other with the particulate matter removed (monophasic), were inoculated with filaments of Oedogonium cardiacum, both cultures grew well at first; but, within a short time (19 days) the culture without the soil turned brown and died. The continued growth in the diphasic medium could have been due to the constant production of growth factors by bacteria but there were also bacteria in the monophasic culture.

It was possible that the continuous growth of the alga in the diphasic medium was due to the release of available nitrogen from the soil fraction, perhaps by bacterial activity. (On page 43 it was demonstrated that growth which had ceased early in the monophasic soil extract solution resumed when nitrogen (KNO_3) was added and this implied that the soil extract supernatant solution contained only a limited supply of nitrogen.)

The necessity of employing a defined culture medium for experimental purposes was indicated in an earlier section. Different defined media have been employed to culture various members of the Oedogoniales. Some of these solutions are not truly defined media because they contain various quantities of soil extract, although they are defined as to the inorganic salts used and the organic supplements added. Bühnemann (1955) in his early work used a slightly modified solution after Uspenski that contained 100 ml. of soil extract per litre of solution. Later he grew Oedogonium cardiacum in a much altered version of the same solution from which he deleted the soil extract. Like the writer's cultures, those of Bühnemann were not bacteria- free and this fact might explain the growth of both investigators' cultures in media that lacked soil extract if bacteria were responsible for the production of vitamins and growth factors. Hoffman (1961) maintained his axenic cultures of Oedogonium in a modified version of Machlis' Medium A which contained

4% soil extract. The defined medium employed by Horsley and his co-workers has already been presented (cf. p. 14). Kim (1961) used a 1:1 mixture of Bristol-Bold and Kock's media (neither contained soil extract) with the addition of chelated trace elements for algae as diverse as Anabaena (Cyanophyta), Oedogonium (Chlorophyta) and Vaucheria (Xanthophyta) in axenic cultures.

The basic elements necessary for most algal growth have been stated (cf. p. 36) but the fact that they have been included in the culture solution does not always mean that they are readily available to the algae. This is especially true of those trace elements which easily form highly insoluble oxides and hydroxides (Krauss, 1958). To this end various chelating agents have been employed to keep the supply of micronutrients available for the algae. The most commonly used sequestering compound has been EDTA and its various salts. It reacts with most metallic ions to form nonionic metal chelates which are soluble even in alkaline solutions (Machlis and Torrey, 1956). Spencer (1957) deduced from his work that EDTA was stable and not metabolized. However Krauss and Specht (1958), as reported in Wiessner (1962) showed that EDTA chelates were absorbed by certain algae and then broken down to free the necessary ions.

The writer's work confirmed the value of using chelated trace elements in defined, inorganic culture media. There

is little to be gained by discussing the various roles that the major and minor elements play in algal metabolism for some are as yet unknown, e.g., that of boron, and others have been well documented (see Krauss, 1958; and Wiessner, 1962 for reviews).

Another aspect of culturing is the influence that media have on the morphology of the organism, e.g., the observed difference in cell length when filaments of Oedogonium cardiacum were cultured in different media. The cells were shown to have been considerably longer when grown in soil extract than when cultured in medium J. Rettlack and von Maltzahn (1968) state a cell length of about 120 μ, and width of 30 μ for vegetative cells of Oedogonium cardiacum grown in Chu 10 medium. Tiffany (1924) referred to the earlier studies of Livingston who concluded that differences in the form of the alga Stigeoclonium grown in Knop's solution were due to variations in the osmotic concentration of the culture solution rather than to the chemical nature of the salts themselves. High osmotic concentration in the medium produced, among other features, a change in cell shape. From Livingston's and the writer's observations concerning cell size and shape, one must exercise caution when describing a "normal" or "typical" cell of an alga for this appears to vary depending upon the culture medium employed.

The Sequence of Cell Divisions

The evidence presented earlier demonstrated a definite

sequence to the occurrence of cell divisions in filaments of Oedogonium cardiacum (female) during the period of early development. It might have been considered that this early developmental sequence was just a chance happening. Following the first cell division of a germling both daughter nuclei were assumed to be of equal age in relation to their positions in the cell cycle. In the work of Ohashi (1930) and Ueda (1960) Oedogonium were shown to have nuclei typical of higher organisms and, thus, they would be expected to have had equal potential for self-replication and division. This assumed that the processes of differentiation (Bonner, 1965) had not set in to hinder nuclear and/or cell division in one or both of the daughter cells. Such an assumption appeared correct since each of these cells did, eventually, divide.

Under a state of equal mitotic potential for both daughter cells there was a probability of 0.5 that either the original basal cell or the second cell in a 2-celled filament would have divided first. This was not the actual situation (see page 55b). The observed occurrence of the second cell dividing before the basal cell in a "normal", unirradiated filament was practically zero. The very high probability of the basal cell dividing first in 2-celled filaments indicated that the cell divisions did not occur randomly and this inferred that there might be some controlling factor which governed when the cells in a young filament divided.

If there were some controlling factor it began to lose its influence over the cells in a filament when the 4-celled filaments divided and the amount of loss appeared to vary somewhat from one experiment to another. In the majority of cases (78%) cell division in 4-celled filaments began again with the basal cell and proceeded in turn up the filament to the fourth cell. At other times the basal cell was often slow to divide and its division was preceded by that of other cells in the 4-celled filaments.

Nevertheless, similar conditions existed in 4-celled as in the 2-celled filaments discussed above regarding nuclear age. The nuclei in the basal and second cell of a filament were of equal mitotic age following division while those of the third and fourth cells were somewhat younger than the basal pair but were of equal age themselves in relation to their passage through the cell cycle. From this it was expected that the two most proximal cells would divide before the distal pair and this was, generally, the observed situation. [It was accepted from the work of Ohashi (1930) and Horsley and Fucikovsky (1963) that nuclear division in Oedogonium is followed immediately by cell division.] However, considering the equal mitotic age of the nuclei in the basal and second cells in 4-celled filaments, the expected probability of one cell dividing before the other is 0.5. A similar probability would be anticipated for the occurrence of cell divisions in the third and fourth cells if chance

alone governed the occurrence of these cell divisions. But these were not the observed cases and, therefore, chance alone did not explain the sequence of cell divisions in 4-celled filaments.

There are at least two explanations for the observed sequence of cell divisions in short filaments. One is that the basal cell somehow influences cell division in cells further up the filament. In Bulbochaete, a related, branched genus, cell division occurs mainly in basal cells and, thus, basal cells hold a very important position in filament growth. Therefore, the influence of the basal cell over cell division in filaments of Oedogonium cardiacum cannot be overlooked. The second explanation relies on a delay of cell division produced by the necessity of lateral wall completion in distal daughter cells. A distal daughter cell, following cell division, must complete its lateral wall before it can divide. The lateral wall of a proximal daughter is received intact from the parent cell. This delay caused by cell wall completion would account for the second cell in 2-celled filaments dividing after the basal cell. Similar conditions regarding order of division would exist in 4-celled filaments. Whether or not either explanation is accurate awaits further investigation.

For cells irradiated in G_2 evidence was presented to show that the normal, intercalary growth pattern could be replaced by apical growth. This change in growth pattern

was infrequent at very low doses (250 rads) even though about 40% of the germlings did not survive. At higher dose levels (1000 and 4000 rads) growth in the short filaments containing atypical cells occurred always by apical growth. However, short, non-surviving filaments which contained normal appearing cells grew either by intercalary or apical growth. For germlings irradiated in the G_1 period there were relatively few short filaments containing atypical cells observed even at dose levels as high as 6000 rads. Some of the short, normal appearing, non-surviving filaments grew by apical development (see Table 16). An explanation for these observed phenomena cannot be presented.

The formation of transverse cell walls between the radiation damaged, atypical second cells (which lacked transverse walls between themselves and the basal cells) and the third cells in short filaments suggested that during the divisions of these second cells they regained the ability to form transverse cell walls. Only on rare occasions was a completely coenocytic condition found in 3-celled radiation-damaged filaments regardless of the morphological state of the third cell. Generally, if there were more than two cells in these filaments there was almost always a transverse wall between the second and third and between the third and fourth cells. (Most such radiation damaged filaments contained only three or four cells.) Thus, the mechanism responsible for the production of

transverse cell walls did not appear to have been essential for cell division since cell division could occur without such wall formation and the capacity for transverse wall formation was regained on subsequent divisions.

In summary, a definite developmental sequence has been shown for the order in which cell divisions occurred in young filaments of Oedogonium cardiacum (female) and this sequence could be altered dramatically by ionizing radiation. Two suggestions have been presented concerning possible causes of this ordered sequence. The basal control suggestion supports a symplastic relationship among cells in filaments whereas the delay caused by lateral cell wall formation does not support such a concept.

Evidence for a symplastic relationship can be seen in the pattern of zoosporogenesis in a filament. As discussed earlier (cf. p. 23) the formation of dark green constrictions which always appeared prior to zoospore release were prominent in cells adjacent to one in which a zoospore had formed. These dark constrictions became less evident in cells further away from the cell in which the zoospore was formed. This gradual change in the pattern of constrictions suggested the passage of a stimulus from the cell which had formed a zoospore. In other words, the formation of a few zoospores, perhaps at random throughout the filaments and even in the same filaments, promoted the formation of zoospores in the total mass of filaments.

THE FUTURE

Whether or not a symplastic relationship exists among cells of this colonial organism remains unresolved. Quantitative evidence regarding the pattern of constrictions in cells of filaments is necessary to substantiate the conception that developing zoospores exert influence over the formation of zoospores in neighbouring cells. To show whether or not the basal cell in young filaments does exert a controlling influence on cell division sequence, experiments may be performed wherein only the basal cell in a short filament is inactivated, e.g., by mechanical manipulation or by radiation. Any significant increase or decrease in the time taken for division of the distal cells would indicate that the basal cell exerts a controlling influence in an undamaged filament. Irradiation of short filaments, 2 to 4 cells long, rather than germlings, could provide evidence of whether or not survival is dependent upon the number of cells in the filament. That is, does each cell survive independently from its neighbour?

SUMMARY

In the thesis the following points were elucidated:

1. A method for rapid zoosporulation was established.
2. An adequate, defined culture medium for growth was confirmed for this alga.
3. A definite ontogenetic sequence of cell divisions was found in young filaments of this species.
4. Irradiation of the germlings with ionizing radiation altered the sequence of cell divisions. Differences in the behaviour between germlings irradiated in the G_1 and G_2 stages of the cell cycle were observed.
5. Concomitant with the change in cell division sequence various anomalies, e.g., cell size, lack of wall formation, apical cap transfer, second division delay, were recorded. The frequencies of these anomalies were dose and cell cycle stage dependent.

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APPENDIX 1

Fixing:

The most commonly used killing-fixing solution was a solution of absolute ethanol and glacial acetic acid mixed in a ratio of 3:1 (v/v).

Staining:

(The duration of time in each solution was 3 to 5 minutes unless otherwise stated.) After being fixed for at least 24 hours, the alga on the slides was transferred through the following solutions:

1. 80% ethanol
2. 95% ethanol
3. 100% ethanol containing 10 ml. each of collodion and ether in 100 ml. solution
4. 80% ethanol containing 10 ml. chloroform in 100 ml. solution
5. 70% ethanol
6. 50% ethanol
7. 30% ethanol
8. 15% ethanol
9. distilled water - 2 changes
10. 1N hydrochloric acid at 60°C. for 8 minutes

11. leuco-basic fuchsin (Coleman, 1938)
12. SO_2 water (Gatenby and Beams, 1950, par. 1386) 2 or 3 changes of 10 min. each
13. slowly running tap water for 5 min.
14. dehydration through a graded ethanol series to absolute ethanol
15. 100% ethanol containing 10 ml. ether in 100 ml.
16. 100% ethanol
17. 100% ethanol:xylol (2:1)
18. 100% ethanol-xylol (1:2)
19. 100% ethanol:xylol (1:4)
20. xylol - 15 minutes
21. xylol - 30 minutes
22. slides mounted with a solution of xylol and "Permount" (2:1)

APPENDIX II

DATA FOR THE DEVELOPMENTAL SEQUENCE OF CELL DIVISIONS

There were only two conditions which could have existed in the developing filaments of Oedogonium cardiacum in relation to the order in which cell divisions occurred - either the cell divisions occurred on a random or non-random basis. In order to test whether the observed frequencies of cell divisions demonstrated random or non-random conditions Chi^2 tests were performed on the data with the assumption that cell divisions occurred in a random manner. For example, in a 3-celled filament there were three cells capable of cell division. Did the observed frequency of 3 + 1, 3 cell divisions indicate a random or non-random basis for cell division in a 3-celled filament.

Filament Stage	Number of Observations	Division Considered	Observed Frequency	Chi^2	Probability
2-celled	182	2 + 1, 1	1.00 ± .005	178	<.001
3-celled	765	3 + 1, 3	.99 ± .004	1465	<.001
4-celled	639	4 + 1, 1	.78 ± .02	997	<.001
5-celled	604	5 + 1, 1	.84 ± .02	1580	<.001
6-celled	542	6 + 1, 5	.90 ± .01	2126	<.001
7-celled	337	7 + 1, 7	.96 ± .01	1835	<.001

Since in all cases the probability that the stated division would occur on a random basis was less than 0.001, the original assumption must have been incorrect and one might conclude that cell division did not occur on a random basis.