COLCHICINE ALTERATIONS IN EUDORINA

COLCHICINE INDUCED ALTERATIONS IN COLONY DEVELOPMENT AND DIFFERENTIATION IN EUDORINA

by

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ABSTRÄCT

A detailed study of colony development and differentiation in <u>Budorina elegans</u> Ehrenberg was carried out at both the light and electron microscope levels. In untreated colonies the sequence of events in plakeal development and inversion were studied. Significant alterations in these processes were observed when colonies were treated with a 0.2% colchicine solution. These alterations are discussed as well as the role of microtubules, golgi and colonial envelope formation in colony development. Colchicine treatment also induced a high level of polyploidy in colonies, and following recovery

in colchicine-free media, haploid and induced stable diploid colonies were found.

Differentiation between somatic and generative cells in <u>Eudorina californica</u> (Shaw) Goldstein was found to occur following the fourth plakeal division.

This is the first clear demonstration of the stage in plakeal development at which differentiation is initiated in <u>Eudorina californica</u>.

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RESUME

Une étude détaillée du développement et de la différenciation des colonies dans <u>Eudorina elegans</u>

Ehrenberg a été faite au niveau du microscope composé et du microscope électronique. On a étudié le déroulement des événements dans le développement et l'inversion pla-kéels dans les colonies non traitées. On a observé des changements significatifs dans ces processus quand on a traité les colonies avec une solution de colchicine à 0.2%. Il y a discussion de ces changements aussi bien que des rôles des microtubules, des gölgi, et de la formation d'enveloppes coloniales dans le développement des colonies.

Le traitement au colchicine a aussi créé un niveau élevé de polyploïdie dans les colonies et, après avoir remis la colonie dans un milieu sans colchicine, on a trouvé de l'haploïde et des diploïdes stables.

On a trouvé que la différenciation entre les cellules somatiques et génératives dans <u>Eudorina californica</u>
(Shaw) Goldstein s'établit après la quatrième division
plakéelle. C'est la première démonstration nette de l'étape
dans le développement plakéel à laquelle la différenciation
s'établit dans <u>Eudorina californica</u>.

COLCHICIME INDUCED ALTERATIONS

IN EUDORINA

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"A women of worth who can find? For her price is far above rubies. The heart of her husband trusteth her, and he shall have no lack of gain." (Proverbs)

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Colchicine Induced Alterations in Colony Development and Differentiation in Eudorina

Statement of Originality

To the writer's knowledge, the research described within this thesis contains the following original contributions:

- 1. The first detailed account of the ultrastructure of nuclear division in <u>Eudorina</u>.
- 2. The first detailed account of the ultrastructure of cytokinesis in <u>Budorina</u>, which revealed the following:
 - a. The presence of cytoplasmic bridges between plakeal cells.
 - b. The presence of the "phycoplast" microtubules and their role in colony development.
- 3. The first detailed account of the ultrastructure of inversion in <u>Eudorina</u>. This study revealed the possible involvement in inversion of cytoplasmic bridges and colonial envelope material.

- 4. The first observation in <u>Eudorina</u> of microtubules in the chloroplasts of dividing and young vegetative cells.
- 5. The first study of the effects of colchicine on the Volvocaceae at the light and electron microscope levels. These effects included:
 - a. Formation of projections or "stellations" at the cell surface.
 - b. Production of extra-cytoplasmic particles.
 - c. The disorientation and inhibition of cytokinesis.
 - d. The inhibition of inversion.
 - e. Production of flattened gonium-like colonies.
- 6. The first detailed light microscope observations of the nuclear cycle of <u>Eudorina</u>, during plakeal development.
- 7. The first study to demonstrate the effects of colchicine on the nuclear cycle of the Volvocaceae.

- B. The first study to clearly demonstrate polyploid production with colchicine in the Volvocaceae, and to isolate colchicine-induced stable polyploids.
- 9. An investigation into the ability of treated cells of Eudorina, to recover from colchicine treatment.
- 10. The discovery of when differentiation of somatic and generative cells occurs in E. californica.

INTRODUCTION AND LITERATURE REVIEW

Since the time of the Greeks, colchicine has been used for various medicinal purposes and is mentioned in such terms by Dioscorides. However, modern studies on colchicine were not commenced until the early 1930's. It was in the laboratories of Professor A. P. Dustin in Brussels, that the effects of colchicine were looked at for the first time in detail (Dustin, 1934). It was found that colchicine had an antimitotic effect in both animal and plant cells. In fact, in 1938 Levan had already completed a detailed study on the effects of colchicine on mitosis in root tips of Allium. This, and much subsequent work established that colchicine was destroying the spindle apparatus and was resulting in polyploidy of the cells (Eigsti and Dustin, 1955).

Though most of the original effort was concentrated on the anti-mitotic effect, it was noticed that colchicine had

other effects too. Levan (1942) was perhaps the first to notice that these effects were not related to the antimitotic effects, i.e.polyploidy. Working with Allium he discovered that the so-called colchicine tumours produced on the roots were not due to polyploidy. Soon the literature on these aspects of colchicine effects became quite profuse, particularly on higher and lower plants and protozoa (Eigsti and Dustin, 1955).

I. Effects of Colchicine on the Algae at the Light Microscope Level

Algae as a rule appear much less sensitive to colchicine treatment than higher plants (Sarma, 1961). In fact, a few species, such as Euglena (Leedale, 1958) and Pithophora (Patel, 1971) appear to be totally unaffected by concentrations of colchicine, many times greater than that which will kill higher plant cells. It is also interesting to note that the number of algae which have been induced to form polyploids are few, although in a number of algae, particularly green algae, the effects of colchicine treatment have been studied. The most intensive study of colchicine treatment on a green algae has been carried out with the unicellular

showed that colchicine treatment produced abnormalities in growth and division, and also resulted in the formation of both polyploids and aneuploids. However, Cornman (1942), . failed to produce polyploids, and it was left to Wetherall and Krauss (1956) to definitely induce polyploidy in Chlamydomonas. Buffaloe (1957) noted that after prolonged treatment the normal vegetative cells became quite distorted, indicating that the colchicine was not just affecting the nucleus.

In fact, colchicine treatment has produced changes in nearly every part of the algal cell. In Spirogyra, colchicine treatment has produced changes in the morphology and distribution of the plastids (Yamaha and Ueda, 1940; Kartoshova, 1945), as well as a disruption of cross wall formation (Mairold, 1943). Treatment of Hydrodictyon has resulted in swelling which occurs at the extreme ends of segments, as well as a general thickening of the cell wall (Gorter, 1943, 1945; Sarma, 1962). Further, a high degree of polyploidy has been observed in this genua, (Sarma, 1958, 1962). Polyploidy has also been produced in species of Sphaeroplea (Sarma, 1957), Microspora (Sarma, 1958),

Drapanaldia (Godward, 1966). Cladophora (Patel, 1971), and Oedogonium (Tachermack, 1942). Both Oedogonium (Gorter, 1943, 1945). and Cladophora (Gorter, 1943; 1945; Mairold, 1943) also underwent a considerable thickening of their cell walls, though these thickenings were limited in Cladophora to the cross-walls. Henningsen (1963) treated several species of Oedogonium with colchicine to produce c-metaphase chromosomes in order to facilitate karyotyping of the species. Other green algae investigated have included Pediastrum (Jakob, 1951), Scenedesmus, (Jakob, 1951) and Chara (Delay, 1958) all of which showed a disruption in the normal pattern of growth and development.

Other algae, for unknown reasons have appeared to be much less affected by colchicine. Levan and Levring (1942), for instance, found that both Ectocarpus and Spermatochnus were only very slightly affected by colchicine treatment.

II. Effects of Colchicine on the Algae at the Electron Microscope Level

More recently, investigators have turned to the electron microscope for its higher resolution and have demonstrated a number of effects at the ultrastructural level, not all of

which are apparently directly related to its effect on the nucleus.

A. Non-Mitotic Effects

i. Effects on Golgi Apparatus and Cell Wall Deposition

In <u>Chlamydomonas</u>, Walne (1967) observed a marked increase in cell wall material in colchicine-treated cells. This cell wall material was laid down in layers with abscissed portions of the cytoplasm between the cell wall layers. These portions of cytoplasm included pieces of the chloroplast, mitochondria, sections of endoplasmic reticulum, and other cell organelles. Further, Walne also observed a considerable increase in the activity of the golgi apparatus with the release of numerous golgi-associated vesicles. Wanka (1968) working with <u>Chlorella</u>, noted that cell wall formation was disrupted and disorientated. He also observed that the golgi apparatus showed an increase in activity following colchicine treatment.

The effects of colchicine on spermatogenesis in

Nitella (Turner, 1970), resulted in the disruption of cell

wall formation. Coombs et al., (1968), showed that colchicine

affected the silica shell formation in Mavicula and

postulated that colchicine apparently affected the golgi

apparatus, which is known for its secretory function in many organisms (Morré et al., 1967).

It is also interesting to note that in both higher plants and mammalian cells, colchicine has been noted in certain cases to have similar non-mitotic effects on the cell fine structure. Pickett-Heaps (1967), on treating xylem cells with colchicine, noted that the pattern of cell wall disposition was considerably disrupted. Robbins and Gonatas (1964), on examining colchicine-treated HeLa cells, noted a build up within cells of golgi bodies, lysosomes and various osmiophilic granules.

ii. Other Non-Mitotic Effects of Colchicine

In Chlamydomonas, Walne (1967), noted a considerable increase in cell size, as well as in the size of the nucleus, which also underwent lobing. Further, the amount of nuclear membrane and endoplasmic reticulum increased considerably, the nuclear membrane becoming convoluted. After about 18 hrs. of treatment with colchicine the cells lost their motility and eventually their flagella. However, on removing the treated cells from the colchicine and allowing them to

morphologically normal cells. Wanka (1968), found a similar increase in the amount of endoplasmic reticulum in colchicine-treated Chlorella cells. Finally, Turner (1970) found that when sperm of Nitella, were treated with colchicine, branched flagella were produced.

B'. Mitotic Effects

It has already been mentioned, that under the light microscope, colchicine was observed to break down the spindle apparatus. However, the structure of some cell components, such as the spindle apparatus, were not resolvable at the ultrastructural level until the advent of gluteraldehyde as a fixative (Sabatini et al., 1963). After fixation in gluteraldehyde, the spindle apparatus is seen to be made up of a number of microtubules of varying length, and with a diameter of 200-250 Å. Borisy and Taylor (1967 a,b) established, by using ³H=colchicine, that colchicine bound to the microtubule sub-unit, now known as tubulin (Feit et al., 1971; Witman et al., 1972). The microtuble sub-unit has been found to be a protein molecule of about 120,000 M.W. (Shelanski and Taylor, 1967), that contains a number of specific sites to

which GTP binds (Adelman et al., 1968). Colchicine treatment thus results in the disappearance of the microtubules that make up the spindle apparatus, as has been observed in many drganisms including the alga Nitella (Turner, 1970). Further, microtubules are also found widely distributed outside the nucleus in the rest of the cell, where they are referred to as cytoplasmic microtubles, or in flagella as flagellar fibres. Slautterback (1963) has reported on their distribution in animal cells whilst Ledbetter and Porter (1963) and Newcomb (1969) have reported on their distribution in plant cells in general.

C. Effects on Cytoplasmic Microtubules

Kiermayer (1968, 1970), working with Micrasterias, showed that colchicine affected cytoplasmic microtubules that were possibly involved in the migration of the nuclei and chloroplasts in dividing cells. Both Werz (1969) and Woodcock (1971) treated Acetabularia undergoing reproduction, and colchicine was found to affect the cytoplasmic microtubules that were responsible for the anchoring of the dividing nuclei in the reproductive crown. Brown and Bouck (1973) found

that colchicine affected cytoplasmic microtubules that appeared to control the cell shape of Ochromonas.

D. Cytoplasmic Microtubules

Recently, particular attention has been paid to the role of the cytoplasmic microtubules in the cell. review\paper on plant microtubules, Newcomb (1969) discussed our present knowledge of microtubules in plant cells. Firstly, they compose the mitotic and meiotic spindle fibres, and are involved in chromosome separation. Other microtubules have been found to play an important role in the formation of the phragmoplast of dividing cells. They are present in the cytoplasm next to the growing primary wall and also next to the developing layers of the secondary wall, where they may control the deposition of the cellulosic microfibrils. Very often they occur in the cytoplasm, arranged beneath the cell surface or at random throughout the cell. In Fucus sperm they are postulated to be involved in the alignment of eyespot granules (Bouck, 1970). Recently, Pickett-Heaps (1972) has noted that many species of green algae have their cleavage furrows directed by a group of cytoplasmic

microtubules, which he has termed "the phycoplast". Microtubules are also, of course, very important components of centrioles and basal bodies and constitute the 9 + 2 axonemal elements of the flagella. Special microtubule-like structures have been found in the chloroplasts of several green algal species (Hoffman, 1967; Brown et al., 1968; Braten and Lévlie, 1968; Pickett-Heaps, 1968; Sabnis, 1969; McBride, 1970; Pickett-Heaps and Fowke, 1970; Deason and Darden, 1971; Jacobs and Ahmadjian, 1971; Smith and Butler, 1971; Moestrup and Hoffman, 1973; Pickett-Heaps, 1973b).

In animal cells and protozoa, the microtubes that are distributed in the cytoplasm under the cell surface are believed to play a prominent role in controlling cell shape, particularly in developing organisms (Tilney, 1968). However, as most plant cells possess a cell wall, the cytoskeletal function of these microtubules may be less important.

There is no doubt from studies on the effects of colchicine on the algae and other organisms, that microtubules do play an important role in the development of plant cells.

The Volvocaceae and Eudorina

The colonial green flagellate. Eudorina is one of several genera belonging to the family Volvocaceae in the green algae (Chlorophyta). The family Volvocaceae which includes most genera of the colonial green flagellates is characterised by a unique and simple form of multicellular organisation made up of chlamydomonad-like cells. Colony development results in the formation of plakea, which undergo inversion to form daughter colonies, a process which is unique to the Volvocaceae. Though plakeal development and inversion at the kight microscope level are similar in all species of the Volvocaceae, it is clear these developmental processes result in colonies of increasing complexity, and degree of cellular differentiation, culminating in the most complex genus of the family, Volvox. Colony development has been studied with the light microscope in the following genera: Gonium (Stein, 1958a), Pandorina (Coleman, 1959), Volvulina (Stein, 1958b; Carefoot, 1966), Platydorina (Harris and Starr, 1969), Eudorina (Gerisch, 1959; Goldstein, 1964, 1967), and Volvox (Starr, 1968, 1969, 1971b; Kochert, . 1968; McCracken and Starr, 1970; Vande Berg and Starr, 1971),

and at the ultrastructural level in <u>Volvox</u> (Bisalputra and Stein, 1966; Deason et al., 1969; Pickett-Heaps, 1970a; Deason and Darden, 1971). However, some of the processes of colony development, such as inversion, are still poorly understood, particularly at the ultrastructural level. To date, only the ultrastructure of vegetative colonies and cells has been studied in <u>Eudorina</u> (Lang, 1963; Hobbs, 1971, 1972).

<u>Eudorina</u> is of particular interest for two reasons:

1. The developmental process in <u>Eudorina</u> is simpler than in <u>Volvox</u>, and this simplicity may facilitate an understanding of the general process of colony development as it occurs throughout the Volvocaceae.

Further, a comparison of the ultrastructural details of colony development in <u>Eudorina</u> with <u>Volvox</u>, may add additional support to the proposed evolutionary scheme of the Volvocine line (Klein and Cronquist, 1967).

2. In some species of <u>Eudorina</u>, all the cells are potentially generative and capable of daughter colony

spp., colony development and differentiation results in somatic and generative cells in a pattern specific to the species. The mechanism of the differentiation process is little understood, and its elucidation could be very significant particularly as the Volvocaceae, and Eudorina in particular, exhibit one of the simplest forms of cell differentiation that occurs in nature. Further, a better understanding of such a simple form of differentiation, as that which occurs in Eudorina, may eventually give some insight into differentiation as it occurs in more advanced plants and animals.

It has already been mentioned how recent studies have shown the importance of microtubules in developmental processes in the algae (Kiermayer, 1968; Newcomb, 1969; Bouck, 1970; Woodcock, 1971).

It was, therefore, decided to look at the effects of colchicine on the colonial green flagellate, <u>Eudorina</u>

elegans, investigating the developmental effects under the light and electron microscopes. It was felt that the treatment of <u>Eudorina</u> might reveal the role, particularly of

cytoplasmic microtubules, in the cellular developmental processes, with emphasis on nuclear division, cytokinesis and inversion. Further, the colchicine treatment would also enable one to look with greater detail at the nuclear cytology of a group of organisms whose nuclear cytology has not been intensively studied (Cave and Pocock, 1951b).

In examining the developmental process in <u>Eudorina</u>, it was decided to adopt a broad approach, i.e., to examine all the various events of colony development. This approach was decided upon because it was felt <u>essential</u> to obtain an overall view of development, rather than study a few isolated events. Further, this type of approach would also help in the understanding of the relationship of the various events that make up colony development in <u>Eudorina</u>.

Therefore, the broad overall objectives of this study were to more clearly elucidate the inherent mechanisms controlling colony development and the patterns of colony differentiation in <u>Eudorina</u>, as well as, to shed light on more general questions concerning cell differentiation and multicellular development.

MATERIALS AND METHODS

I. <u>Isolation</u>, <u>Purification and Maintenance</u> of <u>Eudorina</u>

A heterothallic strain of <u>Budorina elegans</u>, 56-1 (f), 56-2 (m), was isolated from a dried mud sample collected by M.E. Goldstein from a pond just south of Greencastle, Indiana, on Rt. 41 (6.7.59). The technique for clonal isolation involved the acquisition of colonies with a micropipette, washing, and inoculation of single colonies into soil-water medium (Starr, 1964) according to the method of Goldstein (1964). Similarly, the technique for preparing axenic cultures in which colonies were washed by centrifugation, plated out on soil-extract agar (Starr, 1964), and bacteria-free colonies inoculated into Volvocacean medium (Starr, 1964) was like that of Goldstein (1963).

The maintenance of stock cultures and some of the experimental work was carried out under controlled environmental conditions. The temperature in the culture chambers was maintained at 20 [±] 1°C. The light source consisted of banks of cool-white standard fluorescent tubes (40W.CW) with illumination of 250-350 ft.c.intensity. The lights were automatically controlled by a clock which allowed 16 hrs. of light and 8 hrs. of dark daily.

The strains were maintained either in half pint bottles (250 ml) of soil-water medium for light microscopy or in 250 ml. Erlenmeyer flasks of Volvocacean (Starr, 1964) or Volvox medium (Starr, 1971a) for electron microscopy.

Stock cultures were also maintained on slants of soil-extract agar. The formulas and methods of preparation of these and other media used in this study are given in Appendix II. When grown in soil-water medium, the strains were transferred every three weeks, while those grown in either liquid Volvocacean or Volvox medium were transferred every 10 days. Those strains grown on Volvocacean or Volvox agar were transferred every 2-3 months. The newly transferred strains were placed in bright light (250-350 ft.c.

intensity) for 7-10 days and then moved to a place where the light intensity was only 20-60 ft. c.

II. Colchicine Treatment

Dividing material of both strains of Eudorina was treated with an 0.2% aqueous solution of colchicine (General Biochem. Lots 82934, 407671). To obtain dividing material, colonies from 10-12 day old stock cultures were transferred to watch glasses containing fresh, soil-water supernatant, Volvocacean or Volvox medium. A 5% solution of NaHCO3 Was added to the bottom of the petri dish, in which a watch glass was supported on a glass triangle. The watch glass setup was then placed under illumination of 250-350 ft.c. about 5 hrs. before the 8 hr. dark period. Then 3 or 4 hrs. after the onset of the light period, good dividing material was obtained. For light microscope preparations, the colchicine solution was prepared with sterile soilwater supernatant whilst for electron microscope preparations, the colchicine solution was prepared with sterile Volvocacean or <u>Volvox</u> medium.

Dividing material was then pipetted from the watch glasses into 12 ml. centrifuge tubes and centrifuged for 2 mins. at 3000 r.p.m. The supernatant was poured off and the colonies resuspended in an excess of 6 ml. of an 0.2% colchicine solution. The suspension was then placed in a watch glass setup for the period of treatment. At the end of the treatment, the colonies were washed through a number of successive changes of distilled water by centrifugation.

III. Staining Techniques

For the cytological study, untreated dividing colonies or those treated with the 0.2% colchicine were fixed (except for aceto-orcein staining) in a 3:1 solution of absolute alcohol and glacial acetic acid saturated with ferric acetate (Cave and Pocock, 1951a) and were then stained with several different stains.

- A. Aceto-carmine (Cave and Pocock, 1951a)
- B. Aceto-orcein (after Darlington and LaCour, 1960)
- C. Schiff reagent (Graumann, 1952/53).

For the recipes of the various stains, see Appendix III.

A. Aceto-carmine

For aceto-carmine staining, the method according to Goldstein (1964) was used, but instead of ringing the coverslip with wax, clear nail polish was found to be more satisfactory.

B. Aceto-orcein

The treated and untreated colonies were individually picked up using a capillary pipette under the dissecting microscope, and then placed on a clean microscope slide.

The water was allowed to evaporate until the material was almost dry, and then 1-2 drops of the aceto-orcein were placed on the material. Using a rusty iron needle, the material and stain were mixed together on the slide. A coverslip was then placed carefully over the material and the sides blotted wery lightly between sheets of bibulous paper to remove excess stain. The coverslip was then sealed with colourless nail polish. When the polish dried, the slide was ready for observation.

It was found that this method was particularly useful when correlations between the effects of colchicine on the

cellular organisation and on the nucleus were studied.

Colonies which showed certain morphological effects could be transferred individually to a slide and stained to observe effects on the nucleus.

C. Schiff Reagent

The Schiff reaction used to stain the treated material was adapted by Bowers (1968) from a method devised by Kapoor and Beaudry (1966) and further modified by the author. The details were as follows:

- 1. Fixed colonies were concentrated in a pellet by low speed centritugation and washed through a number of successive changes of distilled water.
- 2. The colonies were hydrolysed in n-HCl for 30 mins., at 60°C, the hydrolysis time being determined by running a hydrolysis series between 5 and 60 mins. The time chosen was that which gave the highest percentage of differentially stained nuclei.
- 3. The colonies were washed immediately through 3 changes of distilled water.

- 4. Then colonies were stained in leucobasic fuchsin (Schiff's reagent) for 2-4 hrs. Treatment with SO₂ water was generally unnecessary.
- 5. A small amount of the stained material was then placed on a clean slide and squashed in aceto-orcein by tapping on the coverslip followed by moderate digital pressure. For observation and photography of the chromosomal preparations, the coverslip was sealed to the slide with colourless nail polish.

IV. Preparation of Material for the Electron Microscope

Because of the nature of the problem. two approaches were used in the preparation of the material for the electron microscope. 1. Fixation and embedding of a large number of colonies using the standard methods described below.

2. Fixation and embedding of individual colonies utilizing capillary pipettes and a dissecting microscope.

For the first method, colonies were spun down for 5 mins. at 3000 r.p.m. in a clinical centrifuge. The material was then washed in the Volvocacean or Volvox medium

depending on which was used for growth. Considerable difficulty was initially found in fixing <u>Eudorina</u>, and a number of different concentrations of gluteraldehyde were used for varying periods of time at different temperatures. The main problem appeared to be a lack of penetration of the fixative. To solve this problem, it was decided to use the penetrating agent dimethyl sulfoxide (Rammler and Zeffarain, 1967) as part of the fixation process. The use of the dimethyl sulfoxide considerably enhanced the degree of penetration of the fixative and therefore the quality of fixation. Subsequently, all material was fixed for 30 mins. in 2.5% gluteraldehyde and 2% dimethyl sulfoxide at pH 7.2 using an 0.05M phosphate buffer.

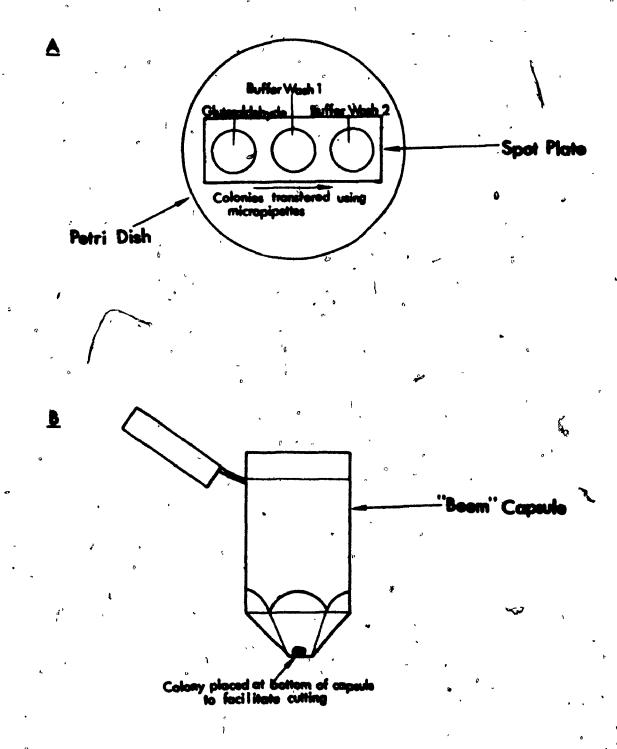
After this first fixation step the colonies were spun down and washed three times (10 mins. for each washing) in 0.1M phosphate buffer at a pH of 7.2. This was followed by a post-fixation with 1% osmium tetroxide in 0.1M phosphate buffer at a pH of 7.2, for 30 mins. The fixed colonies were then centrifuged and washed through two changes of the buffer, each of 10 mins. duration. The material was then passed through a series of alcohol concentrations (50%,

70%, 95%, 100%, 100%, 100%) to dehydrate the cells, each step of dehydration lasting 5 mins.

suspended in two series of propylene oxide and plastic resin (Spurr, 1969) for another 30 mins. Then the plastic mixture was spun off and replaced with pure Spurr plastic for an hour. Using a pipette, a small amount of material was then transferred to a plastic "Beem" capsule (Diagram 1 B) and further plastic resin added. Subsequently, the capsule was topped off with plastic and polymerized at 70°C for 8 hrs. Upon cooling, the capsules were ready for cutting.

The segond technique was developed because it allowed for a correlation of the effects of colchicine at the light and electron microscope levels. Colonies which had been subjected to colchicine treatment were observed at different stages of development under the dissecting microscope. Then, at increasing time intervals, individual colonies were picked up with a capillary pipette and placed in spot plates (Diagram 1 A) for fixing and embedding. In all cases, the times in the fixatives, alcohols, and plastics, were the same as described previously. The colonies were transferred

DIAGRAM 1 COLONIES FOR THE ELECTRON MICROSCOPE



through the various fixations and washings using capillary pipettes. However, when the colonies were transferred into the plastic, pipettes were used in which the inner surface of the shaft had been coated with liquid silicon (Siliclad) to reduce surface tension. Finally, the single colonies were placed in the capsules and oriented, as shown in Diagram 1B. They were then covered with plastic and polymerized.

Ultrathin sections were cut using glass knives, on a Porter-Blum MT-2 Microtome. The sections were placed on 200 mesh copper grids covered with a 2% formvar film and shadowed with carbon.

Staining (see Appendix IV for recipes)

The sections were stained in lead citrate (Venable and Coggeshal, 1965), for 15 mins., followed by uranyl acetate for another 15 mins. The sections were then examined under a Philips EM 200.

V. Photography

Photomicrographs at the light microscope level were taken with a Kodak Bantam camera on a Zeiss microscope, using Kodak Plus X and High Contrast Copy film, developed in Kodak Microdol X and D-19 respectively at 68°C. Agfa Brovira paper was used for printing.

Electron microscope images were photographed with Kodak fine Grain Positive film, Ilford Ilfobrom paper was used for printing.

VI. Colony and Cell Structure of Eudorina elegans

To appreciate the effects of colchicine on colony development and differentiation, it is necessary to understand the basic structure of the colony and the vegetative cells. The strain of <u>Budorina elegans</u> used in this study normally contains 16 or 32 biflagellate cells, arranged in tiers at the periphery of the colonial envelope (Fig. 1).

As seen under the light microscope, each cell of <u>E. elegans</u> is chlamydomonad-like in structure, possessing a pair of apically inserted flagella, a cup-shaped chloroplast with an

<u>Eudorina elegans</u>

Fig. 1. A mature vegetative colony. x 400.

Ultrastructure of Vegetative Cell of Eudorina

- Pig. 2. A longitudinal section of a mature vegetative cell of <u>Budorina</u>. Note the cup-shaped chloroplast (c) which possesses a number of projections or lobes (pr). Within the chloroplast two of several pyrenoids (p) are shown, surrounded by starch plates (sp). Also apparent is the peripheral arrangement of the mitochondria (m) and the central location of the nucleus (n). The cell itself is embedded in the colonial envelope (ce) which is bounded by a colonial boundary (cbn). x 9,900.
- Fig. 2A. Detail of the colonial envelope which shows its fibrous nature (arrows). Note also the colonial boundary (cbn). x 85,100.



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eyespot, and several pyrenoids. The nucleus is centrally located and there are two contractile vacuoles beneath the points of insertion of the flagella.

Eudorina reveals the most conspicuous cell component to be the chloroplast, which contains several pyrenoids surrounded by starch plates (Fig. 2). The pyrenoid (Fig. 4) possesses a number of tubular extensions which arise from the thylakoids and are embedded in an opaque matrix. The pyrenoid structure is essentially the same as that described in <u>B. illinoisensis</u> by Hobbs (1971). Also present within the chloroplast is an eyespot (Fig. 3). The mitochondria are peripheral in arrangement (Lang, 1963), while the nucleus has a central location (Fig. 2). The nucleus has a single nucleolus which often contains one or more light cores (Fig. 17). The golgi apparatus (Fig. 42) is partially surrounded by characteristic extensions of the nuclear envelope, or amplexi, that have been described previously by Lang (1963).

Each cell contains two apically disposed contractile vacuoles with a number of attached vacuoles (Fig. 51).

Ultrastructure of Vegetative Cells

- Fig. 3. An eyespot (ey) present within the chloroplast. Note the chloroplast thylakoids (ct):
- Fig. 4. A pyrenoid from a mature cell surrounded by starch plates (sp). Note the chloroplast thylakoids (ct) running into the pyrenoid as tubular extensions (te). x 20,825.
- Fig. 5. One flagellum within its flagellar canal (fc) and bounded by the colonial envelope (ce).

 Note the transitional region (cyn), where the central microtubules terminate, and the basal body (bb) forms. x 55,600.

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The flagella are contained within flagellar canals that are bounded by the colonial envelope (Fig. 5), and have been described by Lang (1963) and Hobbs (1971).

observed in many plant and animal cells, is the cytoplasmic microtubule. In <u>Budorina</u> there are two types of cytoplasmic microtubules: flagellar root microtubules (Fig. 6); and microtubules distributed throughout the cytoplasm, but not directly associated with any organelle (Fig. 6). The cytoplasmic microtubules have a diameter of 190-230 % and are primarily distributed in the peripheral region of the cell where they are generally associated with the cell surface (Figs. 7, 7A). Similar observations on cytoplasmic microtubules were made by Hobbs (1971) in E. illinoisensis.

The cells of the colony are embedded in an envelope which appears to be of a fibrous nature (Fig. 2A). However, the exact chemical nature of the envelope is unknown. The envelope is bound by two membrane-like structures (Fig.2A), as observed by Lang (1963).

Ultrastructure of Vegetative Cells

Cytoplasmic Microtubules

- Fig. 6. An oblique section of the apical portion of a cell. Note the flagellar bases (flb) and basal bodies (bb). Also present is a flagellar root microtubule (frm) and scattered cytoplasmic microtubule (cm). x 40,425.
- Fig. 7. A series of cytoplasmic microtubules (cm)
 which run just beneath the cell surface.
 x 44,100.
- Fig. 7A. A higher magnification view of the cytoplasmic microtubules (cm) of Fig. 13. x 112,700.



RESULTS

I. Light and Electron Microscopy of Colony Development

In <u>E</u>. <u>elegans</u>, each cell of the colony possesses the potential to divide and form a daughter colony, though there is a slight delay in the onset of division in the anterior tier of cells (Fig. 8). Cell division usually involves 4 or 5 successive bi-partitions in which all the planes of division are parallel to the longitudinal axis of the mother cell. Division results in a curved plate of cells or plakea containing 16 or 32 cells (Fig. 8).

The nuclear division cycle has been recorded for the first time and consists of typical prophase (Figs. 10, 11), metaphase (Figs. 12, 13, 14), anaphase (Fig. 15) and telophase (Fig. 16) stages. Nuclear divisions in the formation of a single plakea are consistently synchronous from the 2 cell

- Fig. 12. A lateral view of a nucleus indergoing metaphase with the chromosomes a igned along the
 equatorial plate. Note that the fibrous
 nature of the spindle apparatus (sd) can be
 clearly seen. Aceto-orcein stain. x 2,000.
- Fig. 13. Both a lateral and polar view of metaphase chromosomes. Aceto-orcein stain. x 2,000.
- Fig. 14. A 2-celled plakes showing metaphase chromosomes in lateral view. Note the cell membrane (cmb) dividing the cells. Aceto-orcein stain.
- Fig. 15. Late anaphase, showing chromosomes, near or at the pole. Aceto-orcein stain. x 2,600.
- Fig. 16. A 2-celled plakea in late telophase with the chromosomes starting to uncoil. Aceto-orcein stain. x 2,600.

Light Microscopy of Asexual Reproduction

- Note the lack of synchrony among the plakea,
 the anterior tier of cells having undergone
 fewer divisions. x 240.
- from their maternal colonial envelope. Note each daughter colony now has its own distinct colonial envelope. x 240.

Normal Nuclear Division 🗅

- Fig. 10. An early stage in prophase of a 4-celled plakea. Note the apparent lack of membranes dividing the cells due to the squash technique. Aceto-carmine stain. x 2,600.
- Fig. 11. A late stage in prophase of a 4-celled plakea with greater condensation of the chromatin.

 Cf. with Fig. 10. Schiff reaction. x 2,600.

(over)



stage onward (Figs. 10, 11; 13-16). However, nuclear divisions and plakeal development in different cells within a colony are by no means synchronous (Goldstein, 1964). When division is complete, the parental eyespot is found in one of the newly formed peripheral plakeal cells. observed by Goldstein (1964). The two parental flagella are attached to peripheral cells on opposite sides of the plakea, as reported by Gerisch (1959), or to a single peripheral cell, as observed by Doraiswami (1940). distribution of the parental flagella is dependent on the initial plane of cleavage. Upon completion of cell division, plakeal inversion takes place, (see Diagram 2, p. 50) which results in daughter colonies composed of a compact sphere of bi-flagellate cells (Fig. 9). At the onset of inversion, each cell of the plakea produces a new pair of flagella, and a colonial envelope is produced around the cells. Depending on the number of cell divisions in plakeal development, the whole process of daughter colony formation takes from 2-4 hrs., with each nuclear division taking from 20-30 mins. Following daughter colony formation, the maternal colonial envelope breaks down and the daughter colonies are released.

daughter colonies will enlarge over a 3 day period, and then divide to form the next generation.

Under the electron microscope, a number of additional. structures and events have been observed in plakeal development, beginning with nuclear division. At the start of division, the interphase nucleus (Fig. 17) moves close to the cell surface (Fig. 18). This movement does result in some reorganization of the cell components, but not of the cytoplasmic microtubules which remain peripherally orientated (Fig. 18).

Upon entering prophase, the chromosomes begin to condense and the nucleolus disappears (Figs. 10, 11). The chromosomes then become fully condensed and align themselves along the equatorial plate at the start of metaphase (Figs. 12-14). The nuclear membrane is present around the metaphase chromosomes (Fig. 19), and remains intact throughout division. The chromosomes are associated with the microtubules of the nuclear spindle (Fig. 19A), which radiate from the nuclear poles (Fig. 19B). However, special points of attachment of microtubules to the chromosomes (i.e., kinetochores) were not observed. At the

Ultrastructure of Asexual Reproduction

- rig. 17. A typical interphase nucleus with single nucleolus (nl), which contains two light cores (lc). The nucleus is surrounded by a typical double nuclear membrane (nm) containing numerous pores (np). x 20,825.
- Fig. 18. A nucleus (n) with nucleolus (n1) just prior to entering prophase. Note that the nucleus has moved to a position next to the cell surface. Further, the cytoplasmic microtubules (cm) are still peripherally orientated. x 49,000.



Ultrastructure of Asexual Reproduction

- Fig. 19. A 2-celled plakea, undergoing division. The nucleus (nl) is of a polar view of metaphase, looking down on the chromosomes (ch) aligned on the equatorial plate. It can be seen that the nuclear membrane (nm) remains intact during nuclear division. The nucleus (n2) is of the metaphase chromosomes in lateral view. Note the incomplete furrow (f) with the cytoplasmic bridges (cb) connecting the cells. x 14,350.
- Fig. 19A. A more detailed view of part of nucleus nl (Fig. 19). Note the presence of part of a chromosome (ch) and numerous spindle microtubules (sm).

 x 78,400.
- Fig. 19B. A more detailed view of the polar region of nucleus n2, (Fig. 19). The spindle microtubules can be seen radiating from the pole, where there is an opening (arrows) in the nuclear membrane (nm). x 37,050.
- Fig. 20. A lateral view of a nuclear pole at metaphase.

 The spindle microtubules (sm) appear to radiate from a special region (mc). x 60,125.



envelope (Fig. 19B), into which the spindle microtubules pass. The area, from which the microtubules radiate, is visible at the extreme poles of the nucleus, and appears more densely stained (Fig. 20) than the nucleoplasm. This area may possibly be similar to the Microtubule Organizing Centre (MTOC), described by Pickett-Heaps (1969). Of note is the total lack of association of basal bodies or centrioles with the spindle microtubules. In fact, when basal bodies or centrioles are observed in plakeal development, they are always at the periphery of the cells (Fig. 21), and are never found in close association with the nuclear poles.

At anaphase, the daughter chromatids separate and proceed to the poles (Fig. 15), where they uncoil during telophase (Fig. 16). Following telophase, the daughter nucleoli form (Fig. 22). Subsequently, an elaboration of the persistent nuclear membrane results in the formation of the daughter nuclear envelopes. Often associated with newly formed daughter nuclei are parallel bands of internuclear ER (Fig. 23). Following the completion of nuclear division, the daughter nuclei remain close together, near the periphery of the cell (Fig. 24) until the onset of cell cleavage.

Ultrastructure of Asexual Reproduction

- Fig. 21. A centriole (cn) is present at the surface of a plakeal cell. Note the nucleus, which is not visible in this micrograph, is located some distance from the centriole. Also shown is a cytoplasmic bridge (cb) and cleavage furrow (f) between the two cells. x 42,466.
- Fig. 22. A nucleus in late telophase showing the two daughter nucleoli (nll and nl2) which have just formed. x 51,450.
- Fig. 23. Two recently formed daughter nuclei (nl and n2)

 are shown together with bands of endoplasmic

 reticulum (er) between the daughter nuclei.

 x 46,550.
- Fig. 24. Two daughter nuclei (nl and n2) prior to cytokinesis. Note that the daughter nuclei remain
 closely associated. x 24,225.



Cell cleavage is discontinuous and results in a furrow interkupted by cytoplasmic bridges which join adjacent daughter cells (Fig. 25). These bridges often contain ER, but are never seen to contain mitochondria or portions of the chloroplast as observed in Volvox (Bisalputra and Stein, 1966). Closely associated with the cleavage furrow are portions of ER (Fig. 25A) as well as cytoplasmic microtubules of the phycoplast (Fig. 26), which may direct the orientation of the plane of cleavage.

Not reported before in <u>Budorina</u> is the presence of distinct microtubule-like structures in the chloroplast. These are only evident in actively dividing plakeal cells (Fig. 27) and cells of newly formed daughter colonies. The microtubule-like structures are 250-300 Å in diameter. Chloroplast microtubules usually occur in groups (Fig. 28), which may contain up to 15 individual microtubules. In cross-section, the microtubules are made up of sub-units (Fig. 28); while in longitudinal section, they possess a distinct banding (Fig. 29). These bands occur at regular intervals of about 250-270 Å, as reported by Hoffman (1967) in Oedogonium.

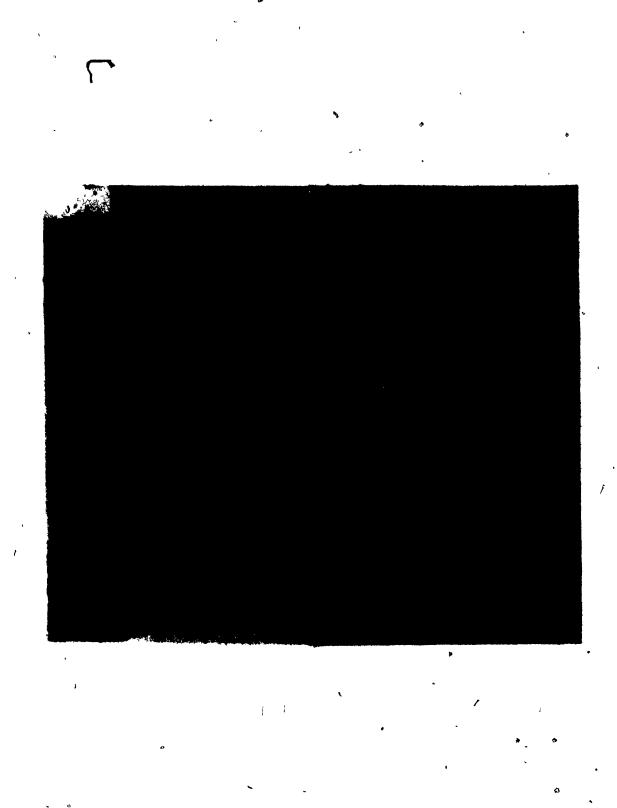
Ultrastructure of Asexual Reproduction

Cytokinesis

- rig. 25. The cell has just completed cytokinesis, the incomplete cleavage furrow (f) results in the production of cytoplasmic bridges (cb) between the plakeal cells. At this early stage, the furrow is narrow and the bridges are broad.

 x 29,600.
- Fig. 25A. A portion of the cleavage furrow (f), from Fig. 25. Note the band of endoplasmic reticulum (er) lying close to the furrow. x 75,850.
- Fig. 26. A cleavage furrow showing the phycoplast microtubules (fm) that run parallel to the furrow.

 Note also the endoplasmic reticulum (er) running through the cytoplasmic bridge (cb). x 49,000.

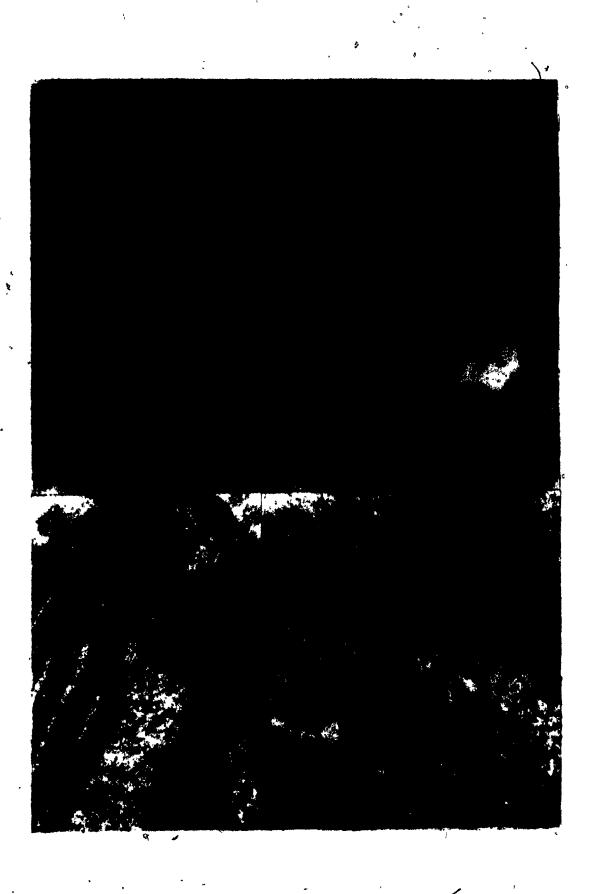


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Ultrastructure of Asexual Reproduction

Chloroplast Microtubules

- Fig. 27. Chloroplasts of plakeal cells which contain a number of chloroplast microtubules (chm) in cross-section. Note the cytoplasmic bridge (cb) connecting two of the cells. x 34,200.
- Fig. 28. A section of the chloroplast from a young daughter colony with a typical group of chloroplast microtubules (chm), seen in cross-section. Note the microtubules appear to be made up of a number of sub-units (sb). x 115,150.
- Fig. 29. A lateral view of a group of chloroplast microtubules (chm). Note the distinct bands or gyres
 of the microtubules. x 109,600.



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Chloroplast division occurs during cytokinesis, in a manner similar to that reported by Goodenough (1970) in Chlamydomonas. The replication of the pyrenoids, however, was not observed, although plakeal cells are often seen to contain more than a single pyrenoid (Fig. 30).

One organelle that shows considerable activity during the development of the plakea is the golgi apparatus. This activity involves the production of a large number of vesicles. These vesicles are small (35-70 mm in diameter) with electron opaque contents (Fig. 30A).

As soon as nuclear division and cytokinesis are complete, the plakeal cells commence inversion (Diagram 2), which leads to the formation of the daughter colony.

Inversion, as seen under the light microscope, has already been described by Gerisch (1959) and Goldstein (1964).

Just prior to inversion plakeal cells are closely appressed such companies and classification with

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Ultrastructure of Asexual Reproduction

- Fig. 30. This plakes has just completed its 3rd division and is in the 8-celled stage. The cleavage furrows (f) are narrow, while the cytoplasmic bridges (cb) are broad. Note the two pyrenoids (p) within one plakes cell. x 12,000.
- Fig. 30A. A golgi apparatus (g) from a typical plakeal cell (Fig. 30). Note the numerous opaque vasicles (vm) associated with the golgi. x 27,000.
- Fig. 31. A plakea just prior to inversion. A separation of the plakeal cells has taken place. Note the elongation of the cytoplasmic bridges (cb) and the broadening of the furrows (f). x 11,500.
- rig. 32. An 8-celled plakes just prior to inversion showing considerable cell separation. Note the
 stretched cytoplasmic bridges (cb). x 9,900.



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material (Fig. 31). This separation of the plakeal cells results in an elongation and narrowing of the cytoplasmic bridges (Fig. 32).

The cytoplasmic bridges, however, do not occur at random. An examination of many sections, both before, and especially during inversion (Fig. 33), indicates that the cytoplasmic bridges are most numerous in the region of the posterior facets of the plakeal cells (see Diagram 2).

Peripheral plakeal dells start inversion movement which results in a reorientation of the convex and concave surfaces of the plakea (see Diagram 2). A 16-celled plakea, which has undergone partial inversion, is illustrated (Fig. 33) and an equivalent stage in development is shown in Diagram 2C. At this stage, the anterior facets of the plakeal cells now form the convex surface of the partially inverted plakea (see Diagram 2C).

At the completion of inversion, the cells form a compact sphere of usually 16 or 32 cells, that constitutes the young daughter colony. The cells then completely separate and the cytoplasmic bridges disappear. This is

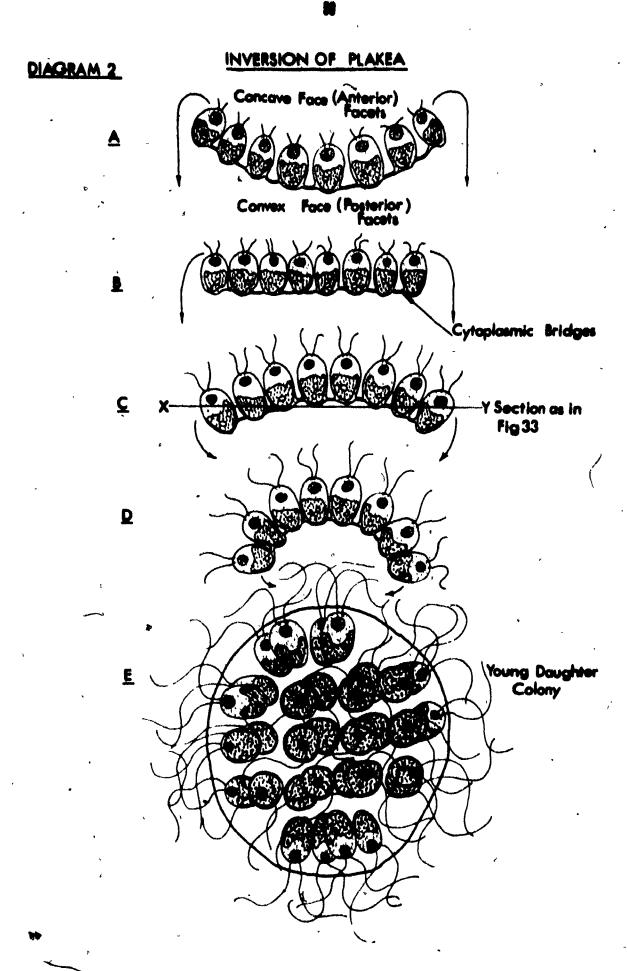
Ultrastructure of Asexual Reproduction

Inversion

- Fig. 33. This section is of a partially inverted 16-celled plakes. Note the cytoplasmic bridges (cb) are numerous between the central plakes cells (cc). However, there are few connections between the peripheral cells (pc). x 19,800.
- released from the maternal envelope. Note that it now has its own colonial envelope (ce).

 x 4,400.





partly due to a considerable expansion of the colonial envelope, and also to the stress created between cells during inversion. The young daughter colony is soon released as a result of the breakdown of the maternal envelope (Fig. 34).

II. Colchicine Alterations in Colony Development

A. Short-Term Effects (0-5 hrs.) on Cells Initiating Division

so far as it can be determined, short-term treatments (0-5 hrs.) with a 0.2% solution of colchicine have no apparent visible effect at the light microscope level on non-dividing cells of <u>Eudorina</u>. Only cells which have initiated cell division or are actively dividing are visibly altered by colchicine treatment. (See Diagram 3, p. 72).

The first structural (non-nuclear) effects of colchicine on cells which have initiated their first division,
are visible in live cells after 90 mins, of treatment (Fig. 36).

Compared with the non-dividing cell (Fig. 35), the treated
cell no longer has a smooth surface, but possesses distinctive
separate short (a) and long (b) projections (Fig. 36).

Under the electron microscope only the long projections

- Fig. 39. Colchicine treatment: 2 hrs. A stellate cell.

 Note that the flagella (fl) still appear to be normal. x 860.
- Fig. 40. Colchicine treatment: 2 1/2 hrs. The stellation (st) has become considerably expanded and can be seen to contain portions of cytoplasm and the chloroplast. x 1,700.
- Fig. 41. Colchicine treatment: 2 1/2 hrs. A
 stellation (st) under the electron microscope.

 Note that it contains mitochondria (m),
 vacuoles (v), part of the chloroplast (c),
 and profiles of the endoplasmic reticulum
 (er). x 27,075.

Short-Term Effects of Colchicine on Cells Initiating Division

- Fig. 35. A mature vegetative cell not treated with colchicine. x 2.240.
- Fig. 36. Colchicine treatment: 90 mins. The cell initiating division shows 2 types of projections.

 Small stout projections (a) and longer, thin projections (b). x 2,000.
- Fig. 37. Colchicine treatment: 90 mins. A long, thin

 "b" (as in Fig. 36) projection as seen under the
 electron microscope, with an expanded basal
 portion containing cytoplasm (cy), and a long,
 thin membrane-bound extension (lb). x 35,075.
- Fig. 38. Colchicine treatment: 2 hrs. The short "a" (as in Fig. 36) projections or stellations (st) have considerably expanded and elongated. Note the size of the dividing cell (dc) compared with the unaffected vegetative cell (vc) to the left.

 x 1.500.



are visible. The short projections are difficult to locate, due to the fact that the cell surface becomes somewhat distorted during fixation. The long (b) projections (Fig. 37) consist of an expanded basal portion containing cytoplasm and a continuous, long, thin, cytoplasmic extension bound by a membrane. The contents of the treated cells appear normal except for the total absence of cytoplasmic microtubules, which are no longer present after 30 mins. treatment, in both dividing and non-dividing cells. Further, the spindle microtubules are also not present after 30 mins. of treatment. The short (a) projections in cells initiating division expand and elongate, and are more readily visible (Figs. 38, 39) following 2 hrs. of colchicine treatment. These expanded projections give the cells a stellate appearance (Fig. 38) as compared to unaffected vegetative cells (Fig. 38).

After 2 1/2 hrs. of treatment, the continued elongation and expansion of the projections or stellations dramatically alters the appearance of the dividing cells (Fig. 40). Under the light microscope, cytoplasm and portions

of the chloroplast are evident in the stellations and with the electron microscope, mitochondria, profiles of the endoplasmic reticulum, ribosomes, and even vacuoles are evident (Fig. 41).

In addition to the above short-term effects observed with the light and electron microscope, there are a number of other effects visible only with the electron microscope. One of these effects is on the golgi apparatus. After treatment for 1 hr., the golgi appear quite normal, as in untreated dividing cells (Fig. 42). However, after 2 hrs. of colchicine treatment, there is an increase in the number of golgi-associated vesicles (Fig. 43). These vesicles are small, opaque (35-70 mu in diameter) and similar to those in untreated cells (Fig. 42). The vesicles become more numerous with longer exposure to colchicine.

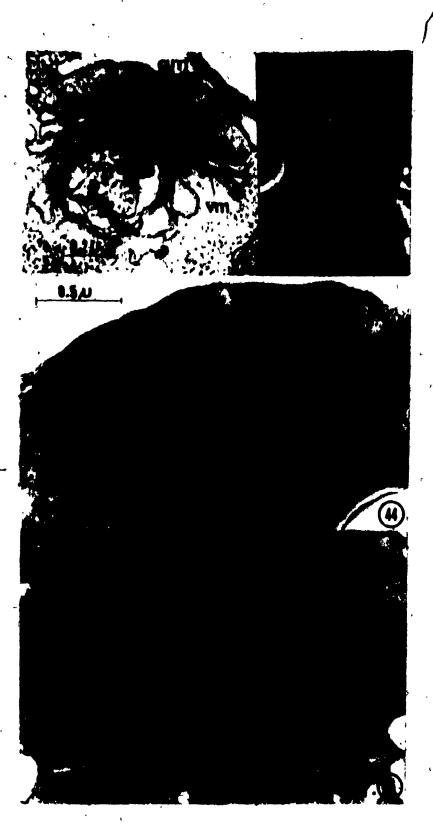
Following 2 hrs. of treatment, effects on the nucleus are also observed. One such effect is the presence within the nuclei of both a nucleolus and condensed chromatin (Fig. 44). Eurther, the presence of 2 nucleoli and areas of condensed chromatin within a single nucleus also occurs (Fig. 45).

Short-Term Effects of Colchicine

- Fig. 42.. A golgi apparatus (g) partially surrounded by characteristic extensions of the nuclear envelope known as amplexi (am). Note the vesicles (vm) associated with golgi lamellae. x 56,350.
- (g) are producing small opaque (vm) vesicles.
- rig. 44. Colchicine treatment: 2 hrs. A nucleus that can be seen to contain both a nucleolus (nl) and condensed chromatin (ch). x 46,473.
- Fig. 45. Colchicine treatment: 2 hrs. A nucleus with

 2 nucleoli (nll and nl2) and condensed chromatin

 (ch). x 41,825.



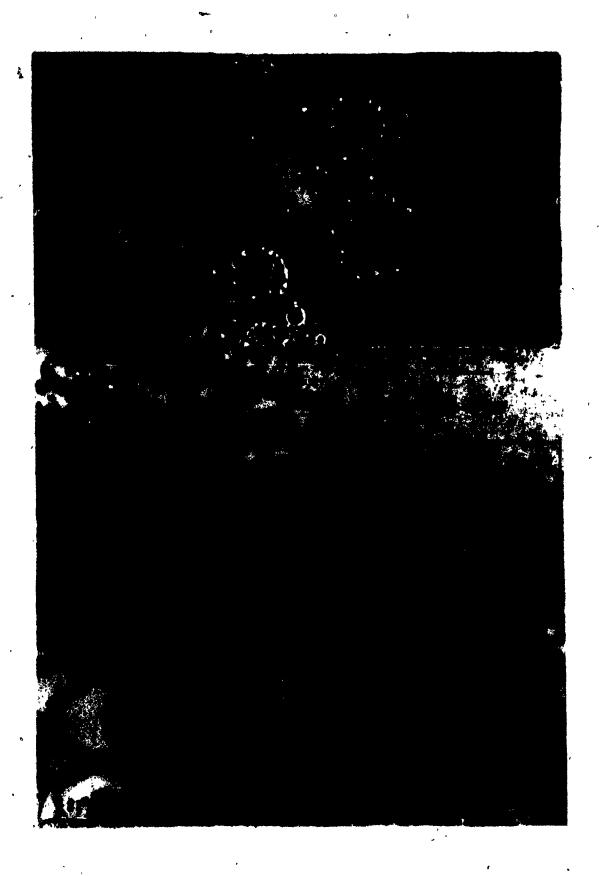
B. Long-Term Effects (5-70 hrs.) on Cells 'Initiating Division

After about 5 hrs. exposure to colchicine, the stellations begin to disappear and numerous cytoplasmic particles start to form a ring around the treated cell (Fig. 47). Under the electron microscope, it is difficult to observe the formation of these extra-cytoplasmic particles. However, light microscope observations indicate that the particles appear to result from the abacission of the elongated projections, or stellations (Fig. 46). Under the electron microscope (Fig. 48), the particles consist of membrane-bound portions of the cytoplasm, parts of the chloroplast thylakoids, starch grains, and membrane profiles. These extra-cytoplasmic particles are a consistent feature of cells treated for 5 hrs. or more (Figs. 47, 48), and are persistent for up to 50 hrs. of colchicine treatment.

External to the particles, the colonial envelope is observed to be altered in structure. The envelope appears to have a distinct lamellate appearance in certain areas (Fig. 48A) which is never observed in untreated cells (Fig. 2A).

Long-Term Effects of Colchicine

- Fig. 46. Colchicine treatment: 5 hrs. A stellation (st) in the process of "breaking off", which results in the production of extra-cytoplasmic particles (ep). x 2,500.
- Fig. 47. Colchicine treatment: 5 hrs. A typical treated cell with numerous extra-cytoplasmic particles (ep) around the outside of the now smooth cell surface. x 1,060.
- Fig. 48. Colchicine treatment: 12 hrs. A cell which has produced extra-cytoplasmic particles. This particular cell has a number of small particles and a few larger ones (gp). The cell itself appears to have a normal chloroplast (c) and the usual type of mitochondria (m). Unusual, however, are the large numbers of opaque vesicles (vm) and vacuoles (v) present within the cell. x 17,325.
- Fig. 48A. A more detailed view of the colonial envelope of Fig. 48. Note the numerous "lamellar" structures (lm). x 37,950.



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The long-term treatments (5-70 hrs.) also result in changes in a number of the organelles within the cell. These changes, however, are not exclusively limited to cells initiating division but are also observed in cells at various stages of plakeal development. There is a further increase in the number of small golgi-associated vesicles (Figs. 49, 50), whose presence throughout the cell is especially evident after 12 hrs. of treatment (Fig. 48). The number of non-contractile vacuoles also increase throughout the cells (Fig. 48). In addition, there are numerous membranous structures closely associated with contractile vacuoles (Fig. 52).

After 24 hrs., certain cells have nuclei which are quite irregular in appearance, with the nucleoplasm being more fibrillar than granular (Fig. 53). Also, the two membranes of the nuclear envelope appear to separate in certain regions (Fig. 54). In addition, a number of membrane-bound inclusions are evident in the nucleus after 24 hrs. of treatment (Fig. 55), and these may well represent a lobing of the nucleus or nuclei.

Long-Term Effects of Colchicine

- Fig. 49. Colchicine treatment: 24 hrs. Note the numerous opaque vesicles (vm) associated with the golgi. x 30,000.
- Fig. 50. Colchicine treatment: 24 hrs. Golgi (g)
 surrounded by numerous opaque vesicles (vm).
 x 46,550.
- Fig. 51. A typical contractile vacuole (cv), surrounded by numerous attached vacuoles (v). x 46,250.
- Fig. 52. Colchicine treatment: 24 hrs. The contractile vacuole (cv) with a number of associated membranous structures. x 61,250.



Long-Term Effects of Colchicine

- Fig. 53. Colchicine treatment: 24 hrs. The nucleoplasm (npl) appears fibrillar and the double nuclear membrane (nm) can be seen to have separated.

 The nucleolus (nl) appears normal. x 20,625.
- Fig. 54. Colchicine treatment: 24 hrs. The nucleoplasm (npl) appears to have a fibrillar structure and the double nuclear membrane (nm) has separated (arrow). x 19,600.
- Fig. 55. Colchicine treatment: 24 hrs. The nucleus contains a number of membrane-bound structures (nx), which may be indicative of a lobing of the nucleus. x 18,025.



within the cell, however, some of the organelles appear to be little affected by prolonged colchicine treatment. One of these organelles is the chloroplast, which appears to have no unusual internal structural alterations (Fig. 48). The thylakoids, pyrenoids, and chloroplast microtubules are unaltered and persist even after 24 hrs. of treatment (Fig. 56). The mitochondria also show little structural change (Fig. 48). Unaltered both structurally and functionally appear to be the flagella of vegetative colonies, even after 50 hrs. of colchicine treatment. In cells that are visibly affected by colchicine, the flagella may persist during the stellate stage (Fig. 39). However, with the production of extra-cytoplasmic particles, the flagella disappear.

Eventually, after 70 hrs. of treatment, all cells that initiate division die (Fig. 57). The dead cells are characterised by a thick, wall-like structure, that surrounds a cytoplasm apparently devoid of organelles. Non-dividing cells are observed to die after the same treatment time, and they also have an appearance similar to the treated dividing cells (Fig. 58). However, some non-dividing cells may still

Long-ferm Effects of Colchicine

Fig. 56. Colchicine treatment: 12 hrs. Numerous

extra-cytoplasmic particles (ep) are present.

The chloroplast microtubules (chm) appear

structurally unaltered, but cytoplasmic micro
tubules are not present. Note that the rest

of the chloroplast including the pyrenoid (p)

also appears unaltered. x 70,000.





Long-Term Effects of Colchicine

- Fig. 57. Colchicine treatment: 70 hrs. The cells are dead, with a loss of cytoplasm and a thick wall-like structure (w). x 340.
- Fig. 58. Colchicine treatment: 70 hrs. Vegetative colonies that have died. Note the thick wall-like structure around the cells.



appear to have a fairly normal cell structure, even after 70 hrs. of treatment.

C. Short-Term Effects (0-5 hrs.) on Plakeal Stages

plakeal stages of <u>Eudorina</u> are the disorientation and inhibition of cytokinesis. Based on observations of cells undergoing first and second cleavage, it appears that disorientation of the cleavage plane precedes inhibition of cytokinesis.

The effects on cytokinesis are first observed soon after the onset of colchicine treatment (see Sec. IVA, p. 73). However, they only become evident in unstained cells after 2-3 hrs. of treatment. The disorientation of cytokinesis results in unequal division of the plakeal cells (Pig. 59). The inhibition of cytokinesis results in "semi-divided" (Pig. 60) and highly irregular plakeal cells (Fig. 61). This is especially evident after 5 hrs. of treatment.

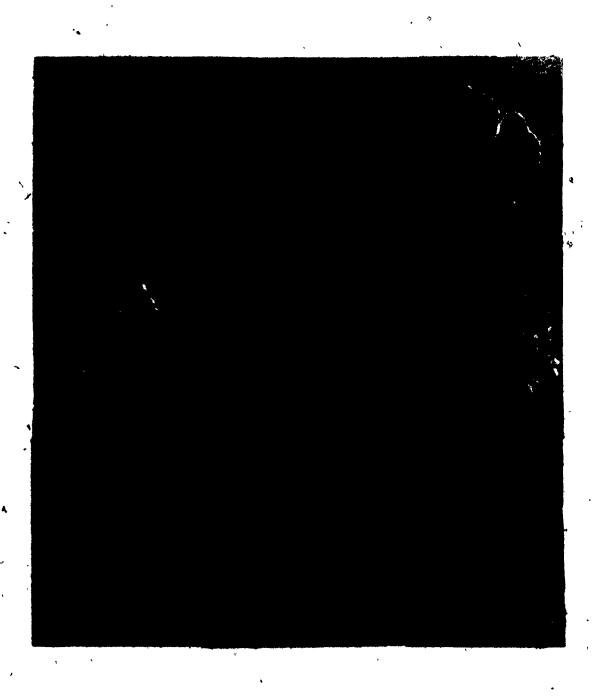
After that time, the treatment with colchicine results in the production of plakes that possess a highly unusual

- Fig. 64. Colchicine treatment: 5 hrs. A 6-celled plakes. x 1,050.
- Fig. 65. Colchicine treatment: 2 hrs. An unequally divided plakea. Note the small stellations (st). x 960.
- plakea. Note the small stellations (st).
 - Fig. 67. Colchicine treatment: 5 hrs. A 4-celled plakea. Note the extra-cytoplasmic particles (arrows). x 1,050.

Short-Term Effects of Colchicine on Plakeal Stages

- rig. 59. Colchicine treatment: 5 hrs. A 2-celled plakea that is the result of unequal division.
- Fig. 60. Colchicine treatment: 5 hrs. A plakeal cell with incomplete division due to an inhibition of cytokinesis. Note the small extracytoplasmic particles around cell. x 1,200.
- Fig. 61. Colchicine treatment: 5 hrs. A 2-celled plakea whose second division has been inhibited.
- Fig. 62. Colchicine treatment: 5 hrs. A 3-celled plakea. x 960.
- Fig. 63. Colchicine treatment: 5 Mrs. A 3-celled plakea surrounded by extra-cytoplasmic particles (arrows). x 1,050.

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number of cells such as 3 and 6 (Figs. 62-64). These types of plakea are never observed in untreated cells because of the apparent synchronous nature of plakeal divisions.

However, in these cases, one or more of the plakeal cells has failed to divide, which indicates that the colchicine either has a differential effect on the plakeal cells, or disrupts the synchrony of plakeal divisions.

after 2 hrs. of treatment (Fig. 65), although they are always much smaller than stellations of cells initiating division (Figs. 38-40). After 5 hrs. of treatment, these stellations also give rise to extra-cytoplasmic particles (Figs. 59, 60, 63, 64). Many normal appearing 2-, 4-, and 8-celled plakea are also observed to produce small stellations (Fig. 66) and subsequently, extra-cytoplasmic farticles (Fig. 67).

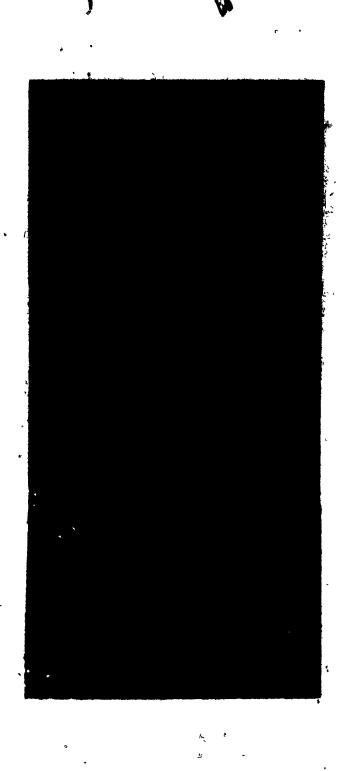
Often, treated plakea exhibit an abnormal separation of cells which is never observed in untreated plakea (Figs. 68-70). The separation of plakeal cells becomes very evident following 5 hrs. of treatment (Fig. 69). This phenomenon occurs in all stages of plakeal development (Figs. 68, 69) and

Short-Term Effects of Colchicine on Plakeal Stages

- Pig. 68. Colchicine treatment: 5 hrs. Separation of cells occurs in all stages of plakeal development.

 Note that the vegetative colony (vcl) is unaffected. x 350.
- Pig. 69. Colchicine treatment: 5 hrs. The separation is very apparent together with an increase in, envelope material between the cells (arrow).
- Pig. 70. Colchicine treatment: 5 hrs. Note that plakeal cells have become slightly disorientated.

 x 850.



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results in the inhibition of inversion. Further, there appears to be an increase in colonial envelope material between the plakeal cells (Fig. 69). The appearance of these plakea is quite similar to the flattened colonies of Gonium and they remain so for at least one generation when placed in colchicine-free medium. Subsequently, these gonium-like colonies become free-swimming and give rise to typical colonies of Eudorina (see Sec. V, p. 83).

Cytoplasmic particles are sometimes associated with separated plakeal cells (Fig. 67), while in similar treated plakea, the extra-cytoplasmic particles are absent (Fig. 70). Examination of many treated plakea reveals that only plakeal cells that form stellations (Fig. 66) produce cytoplasmic particles.

Onergfurther aspect of plakeal development affected by colchicine treatment, was that of flagella development. As it has been described previously in Section I, (p. 37), the flagella are produced in plakeal cells just prior to inversion. Examination of numerous late-plakeal stages reveals an absence of flagella development during colchicine treatment:

At the electron microscope level, plakeal cells, after 2 hrs. of treatment, are commonly found to contain 2 nuclei (Fig. 71), a situation that occurs much less frequently in untreated cells. This binucleate condition appears to result from an inhibition of cytokinesis. A very peculiar situation was observed where apparently 4 nuclei were found within one plakeal cell that had been treated with colchicine for 2 1/2 hrs. (Fig. 72).

D. Long-Term Effects (5-70 hrs.) on Plakeal Stages

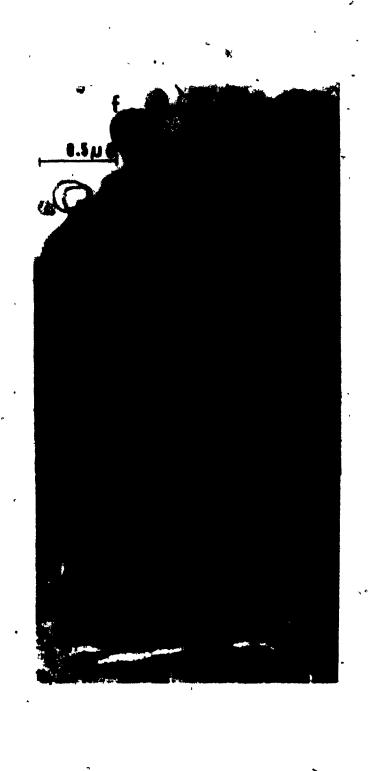
After 7 hrs. of treatment, the separated plakeal cells become disorientated (Fig. 73). With 48 hrs. exposure to colchicine, the cells of the disorientated plakea extrude their cytoplasmic contents and degenerate (Fig. 74). Eventually, after 70 hrs. of exposure, these colonies are killed by colchicine.

Diagram 3 is a pictorial representation of the major effects of colchicine on cells of <u>Eudorina</u> in various stages of development. This diagram puts into perspective all the different effects of colchicine mentioned above and will be discussed later.

Short-Term Effects of Colchicine on Plakeal Stages

- Fig. 71. Colchicine treatment: 2 hrs. A plakeal cell of a colony with 2 nuclei (nl and n2).

 Note the short flagellum (f). x 41,825.
- Fig. 72. Colchicine treatment: 2 1/2 hrs. A plakeal cell with 4 nuclei (nl, n2, n3, and n4), or possibly 1 lobed nucleus. x 27,390.



Long-Term Effects of Colchicine on Plakeal Stages

- Fig. 73. Colchicine treatment: 7 hrs. Plakea are considerably disorientated with the cells arranged at random. Note the blurred swimming vegetative colony unaffected by colchicine preatment. x 240.
- rig. 74. Colchicine treatment: 48 hrs. Some plakeal cells have extruded their cytoplasmic contents.



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DIAGRAM 3 COLCHICINE INDUCED ALTERATERATIONS IN THE MORPHOLOGY OF DIVIDING CELLS

LEGEND
CELLS UNDERGOING NUCLEAR DIVISION
CEUS THAT ARE UNDERGOING
- OR HAVE COMPLETED CYTOKINESIS

LENGTH	8	COLCHICINE	TREATMENT	- 4	IN HOURS
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III. Colchicine Alterations in the Muclear Cycle

A. Short-Term Effects (0-5 hrs.) of Colchicine

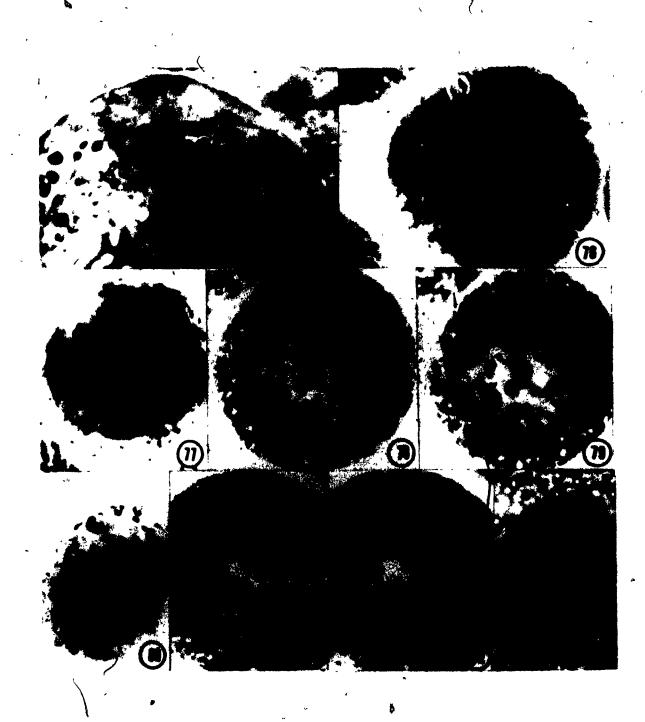
The first noticeable alterations in the nuckear cycle are evident after 15-20 mins. of treatment and result in typical c-metaphase configurations of chromosomes (Fig. 75). Unlike the normal metaphase configuration (Fig. 13), the chromosomes are widely spaced as a result of the breakdown of the spindle apparatus. In addition, chromosome doubling occurs (Fig. 75). Subsequently, the sister chromatids separate at random. Then the nucleus enters interphase which may last up to 15 mins., even though cytokinesis appears to be inhibited. Following the prolonged interphase, the nuclei re-enter prophase with a diploid complement of chromosomes, at 40 mins. of treatment (Fig. 76). At 45 mins. of treatment (Fig. 77), a diploid complement of c-metaphase chromosomes is apparent and at 50 mins. of treatment, the chromosomes double once again (Figs. 78, 79). Figures show chromosomes at two optical levels in the same nucleus to illustrate the diploid number of chromosomes.

- in Fig. 78, but at different focal or optical level to illustrate diploid complement of chromosomes. Aceto-carmine stain. x 2,400.
- Fig. 80. Colchicine treatment: 75 mins. A cell with a tetraploid complement of 56 chromosomes. Acetocarmine stain. x 2,000.
- Fig. 81. Colchicine treatment: 80 mins. A cell with a tetraploid complement of chromosomes that are starting to double. Aceto-carmine stain. x 2,400.
- Fig. 82. Colchicine treatment: 80 mins. The same cell as in Fig. 81, but at a different optical level.

 Aceto-carmine stain. x 2,400.
- Pig. 83. Colchicine treatment: 95 mins. Cell with large chromosome complement (octoploid?). Aceto-carming stain. x 2,400.

Short-Term Effects of Colchicine on Nuclear Division

- Fig. 75. Colchicine treatment: 20 mins. A typical c-metaphase chromosome configuration with widely separated chromosomes revealing 14 distinct chromosomes (unmarked arrows, point to 3 chromosomes at a slightly lower optical level). Also note the doubling of the chromosomes (x). Schiff reaction. x 4,160.
- Fig. 76. Colchicine treatment: 40 mins. A cell entering prophase again, but with a diploid number of chromosomes. Aceto-carmine stain.
- Fig. 77. Colchicine treatment: 45 mins. A cell in c-metaphase with a diploid complement of 28 chromosomes. Aceto-carmine stain. x 2,220.
- Fig. 78. Colchicine treatment: 50 mins. The chromosomes are seen to be doubling again. Aceto-carmine, stain. x 2,400.



apparatus has been rendered nonfunctional, chromosome replication continues to take place and the phases in the nuclear division cycle are limited to interphase, c-prophase, and c-metaphase, with normal anaphase and telophase stages being absent. Further treatment (75 mins.) results in tetraploid nuclei (Fig. 80), which undergo chromosome doubling at 80 mins. of treatment (Figs. 81, 82). Subsequently, chromosome doubling occurs again at 95 mins. (Fig. 83).

Additional stages in chromosome doubling are more difficult to follow, due to the large number of chromosomes to be surveyed. However, it is clear that further doubling does occur in the sequence described (see Table 1).

The normal nuclear cycle is compared to the colchicineaffected cycle in Table 1. It should be noted that the
lengths of exposure to colchicine and the resulting cytological changes refer only to mother-cell nuclei. Daughter
nuclei, arising from nuclear division prior to colchicine
treatment, appear to respond similarly, but give rise to
polyploid complements containing fewer chromosomes, a fact
which will be discussed later.

TABLE 1

Comparison of Muclear Division Cycle in Untreated and Colchicine-Treated Cells 1

UNTREATED CELLS			TREATED CELLS .		
Time in minutes	Division	Muclear Division Cycle	Time in	Division	Nuclear Division Cycle
0		Interphase	0		Interphase
5	lst	Prophase	5	lst	Prophase
10	1.	Metaphase	10		Metaphase
/ 15		Anaphase Telophase	15	•	c-Metaphase, chromosomes appear double
20		Cytokinesis 2-celled plakea	20	•	Separation of chromosomes at random
25	٥	Interphase	25		Interphase
30	2nd	Prophase a	30		Interphase
3 5		Metaphase	35	,	Interphase
40		Anaphase Telophase	40	2nd	Prophase-diploid number of chromosomes

¹Division cycle in treated material refers only to chromosomes within a mother cell nucleus.

TABLE 1 (Continued)

UNTREATED CELLS			TREATED CELLS		
Time in	Divisiòn	Nuclear Division	Time in	•	Nuclear Division
minutes	DIAISTON	Cycle	minutes	Division	Cycle
45		Cytokinesis 4-celled plakea	45 ,		c-Metaphase chromosomes appear double
50		Interphase	50		Separation of chromosomes at random
55	3rđ	Prophase	55		Interphase
60 *		Metaphase'	60		Interphase
65		Anaphase Telophase	65		Interphase
70		Cytokinesis 8-celled plakea	70	3rd	Prophase - tetr ploid number of chromosomes
75 、	,	Interphase	175		c-Metaphase, chromosomes appear double
80	4th	Prophase .	80,	•	Separation of chromosomes at random
85	•	Metaphase	85	•	Interphase

In daughter nuclei of plakes, it is difficult to ascertain the exact degree of polyploidy produced because the squash technique results in an aggregation of the separate chromosome complements.

B. Long-Term Effects (5-24 hrs.) of Colchicine

pistinct from the production of polyploidy and other short-term effects of colchicine, further nuclear cytological effects were observed at the light and the electron microscope levels after more prolonged colchicine treatment. After 12 hrs., densely stained nuclei appear in treated cells (Fig. 84), and certain single cells appear to contain a number of densely stained nuclei (Fig. 85). Often, these nuclei appear to be interconnected in a similar way to that observed in Chlamydomonas (Walne, 1966). These connections between nuclei appear to be present in many cells that have been treated for 12 hrs., or more, and perhaps indicate a lobing of the nucleus. As an example, what appears to be 5 "nuclei" seen in Figure 86, could be the equivalent of 4, 3, 2, or only 1 highly lobed nucleus.

- Fig. 88. Colchicine treatment: 12 hrs. A 3-celled plakes, with heavily stained nuclei. Note that one nucleus is much larger than the other two.

 Aceto-carmine stain. x 2,500.
- Fig. 89. Colchicine treatment: 12 hrs. A typical multi-lobed nucleus, the arrows pointing to the lobes. Aceto-carmine stain. x 3,375.
- Fig. 90. Colchicine treatment: 12 hrs. Photograph of a typical heavily stained nucleus, to bring out the nuclear structures. Note that the nucleus apparently consists of numerous condensed chromosomes. Aceto-carmine stain. x 4,000.

Long-Term Effects of Colchicine on Nuclear Cytology

- Fig. 84. Colchicine treatment: 12 hrs. A single plakeal cell containing a nucleus with heavily stained condensed chromatin material. Aceto-carmine stain. x 3,375.
- Fig. 85. Colchicine treatment: 12 hrs. A single plakeal cell containing two nuclei with heavily stained condensed chromatin. Aceto-carmine stain.

 x 2,000.
- Fig. 86. Colchicine treatment: 12 hrs. A single plakeal cell with either 5 nuclei or 1 highly-lobed nucleus. Note the arrow points to a connection between 2 "nuclei". Aceto-carmine stain.
- Fig. 87. Colchicine treatment: 12 hrs. A 2-celled plakes, each cell containing a heavily stained nucleus.

 Aceto-carmine stain. x 2,500.

(over)



Two-celled plakes, with separated cells following colchicine treatment, also possess densely stained nuclei (Fig. 87) as do 3-celled plakes (Fig. 88). In the latter case, the size difference between the nuclei appears to relate to the difference in cell size (Fig. 88). The lobed nature of the nucleus is clearly seen (Fig. 89), as well as the large relative volume of the nucleus, as compared to the nucleus in a normal cell.

The nature of the heavily stained nuclei was fortuitously resolved when it was found (cf., nuclei Figs. 89, 90), through careful dodging of the photographs, that the dense material consisted of a large number of apparently condensed chromosomes. The large numbers of chromosomes present seemed to indicate a high degree of polyploidy and were most obvious in mother-cells and in 2-, 3-, and 4-celled plakes.

IV. Recovery Cycle

The ability of dividing cells of <u>Eudorina</u> to recover from colchicine treatment varied greatly, even when the cells were-subjected to the same period of treatment. In the study undertaken on the recovery cycle, this variation produced complex results. To facilitate the understanding of the main events of recovery, a Diagram (4) has been drawn to illustrate these major events in chronological order.

Cells treated for 20 mins. to 4 hrs. were allowed to recover in colchicine-free medium for up to 10 days. It should be noted that colonies were only treated up to 4 hrs. since longer treatment resulted in very distorted colonies, whose recovery was difficult to assess. However, colonies treated for periods of up to 8 hrs. did show some degree of recovery.

Certain plakeal cells undergo a number of unequal divisions soon after removal from colchicine. These distorted plakea are called plakeal "fragments", and always degenerate and die after about 5 days of recovery (Figs."

- Fig. 96. Colchicine treatment: 4 hrs. Recovery time:

 76 hrs. A flattened uninverted 32-celled

 plakea, showing less separation of the plakeal

 cells. x 220.
- Fig. 97. Colchicine treatment: 4 hrs. Recovery time:
 96 hrs. An uninverted plakea commencing to
 divide. Note that some of the cells appear to be
 dividing normally (arrow). x 270.
- Fig. 98. Colchicine treatment: 4 hrs. Recovery time:

 96 hrs. An uninverted plakea commencing to

 divide. Note that some cells appear to be

 forming plakeal fragments (arrow). x 270.

Recovery Cycle .

- Fig. 91. Colchicine treatment: 60 mins. Recovery time: 6 hrs. Distortion of colonial envelope and formation of plakeal fragments (arrow): x 220.
- Fig. 92. Colchicine treatment: 90 mins. Recovery time:

 76 hrs. A plakeal fragment following release
 from the maternal envelope. x 300.
- Fig. 93. Colchicine treatment: 20 mins. Recovery time:

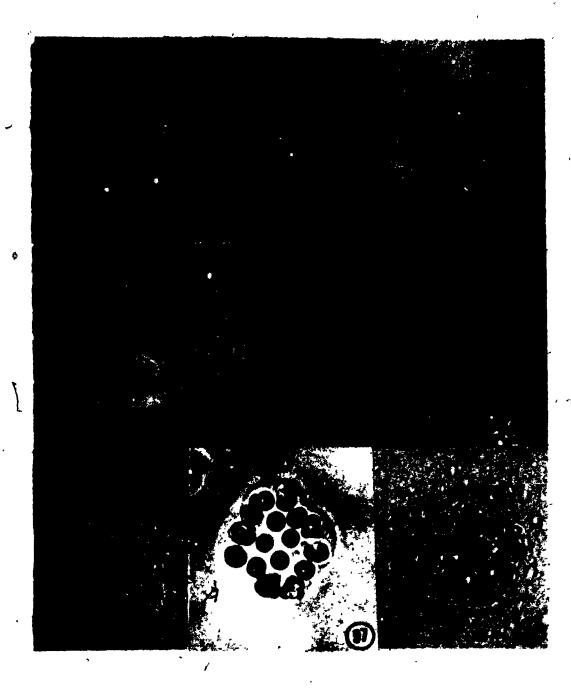
 110 hrs. Plakeal fragments degenerating and

 dying. x 220.
- Pig. 94. Colchicine treatment: 20 mins. Recovery time:

 26 hrs. Typical separated plakeal cells and
 plakeal fragments present. However, some plakea
 do invert (arrow). x 220.
- Pig. 95. Colchicine treatment: 2 hrs. Recovery time:

 26 hrs. Note the considerable separation of
 plakeal cells. x 250.

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91-93). The degenerating fragments bear a striking similarity to the nonviable polyploid progeny that resulted from interspecific hybridization in <u>Eudorina</u> (Goldstein, 1964).

At the other extreme, a number of plakea, particularly 16- and 32-celled stages, treated for only 20 mins. appear to be little affected. These plakea often invert normally to form typical daughter colonies (Fig. 94).

Most of the plakes are observed to undergo a separation of their cells and to produce flattened gonium—like plakeal colonies (Figs. 95, 96). The degree of cell separation can vary considerably (cf., Figs. 95, 96).

Generally, however, plakes that have undergone a greater number of divisions before colchicine treatment show less separation of plakeal cells (Fig. 96). These separated plakes never invert and, when released from the maternal envelope, often develop flagella and motility. Eventually, these flattened colonies divide again, some normally, to produce typical daughter colonies (Fig. 97). Others appear to undergo unequal division to produce fragments (Fig. 98).

These fragments eventually degenerate and die.

Occasionally, the flattened plakes give rise to very peculiar colonies. For example, colonies which appear to be made up of cells closely appressed to each other, much like colonies of Pandorina (Fig. 99); or colonies which remain within their maternal envelope and divide yet again to form colonies containing two generations of cells (Fig. 100). Both of the above cases were at first thought to be possible mutants. However, when their progeny were released, they formed perfectly normal colonies.

One aspect of the recovery cycle studied with considerable interest, was the production of permanent polyploids. As has already been illustrated, colchicine produces quite a high degree of polyploidy.

To find out if permanent polyploids were produced, individual colonies, which had been treated for 20 mins. up to 4 hrs. and formed flattened plakes, were isolated following 7 days of recovery. Within 3 weeks, clonal populations were produced and then dividing colonies were fixed and stained to ascertain their chromosome numbers. Examination of a number of these clonal isolates revealed a few polyploids with a maximum of 28 chromosomes (Fig. 101). The

- pig. 103. ColchicIne treatment: 40 mins. A polar view of metaphase chromosomes. Note a haploid number, i.e., 14 chromosomes. Aceto-carmine stain. x 2,800.
- Pig. 104. Colchicine treatment: 2 hrs. A polar view of metaphase chromosomes. Note a haploid number, i.e., 14 chromosomes. Aceto-carmine stain. x 3,600.

Recovery Cycle

- Pig. 99. Colchicine treatment: 4 hrs. Recovery time:

 10 days. A group of pandorina-like daughter

 colonies just being released. x 220.
- Fig. 100. Colchicine treatment: 4 hrs. Recovery time:

 10 days. Two generations of daughter colonies

 all within the original maternal colonial

 envelope. x 220.

Colchicine Polyploids

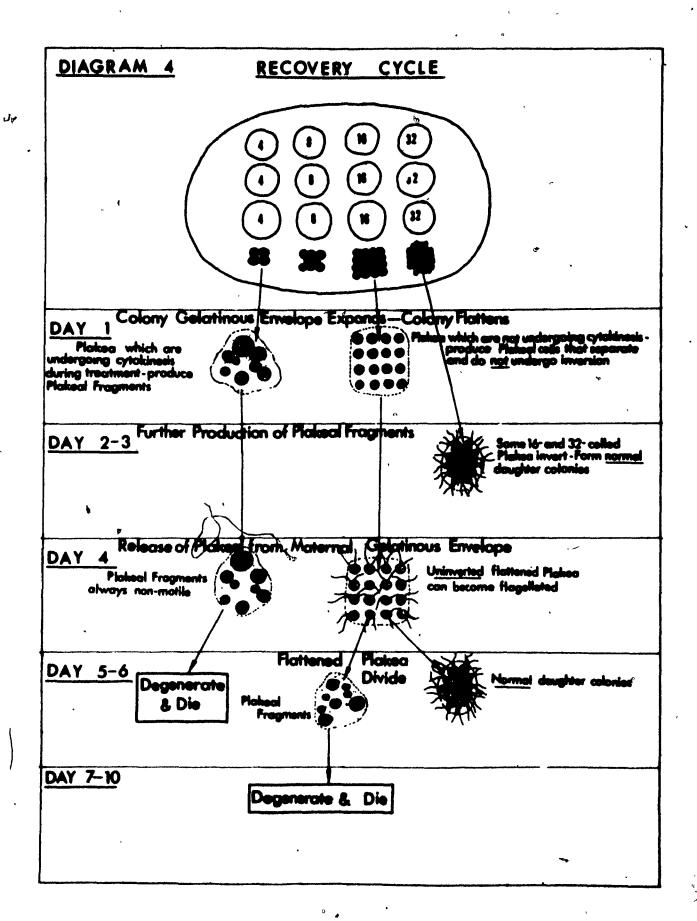
- Pig. 101. Colchicine treatment: 20 mins. A polar view of metaphase chromosomes. Mote a diploid number, i.e., 28 chromosomes. Aceto-carmine stain. x 2,800.
- Pig. 102. Untreated colony. A polar view of metaphase chromosomes. Note that there are 14 chromosomes.

 Aceto-carmine stain. x 3,600.



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normal untreated cell contains a complement of 14 chromosomes (Fig. 102). These stable polyploids arose only from dividing colonies treated for 20 mins. Colonies which had been subjected to longer periods of colchicine treatment were found to contain a normal haploid complement of chromosomes (Figs. 103, 106).



DISCUSSION

I. Structural Alterations Induced by Colchicine

In the one previous detailed study of colchicine's effects on the cell form and ultrastructure of an alga-(Chlamydomonas, Walne, 1966, 1967), the first effects of colchicine treatment, visible under the light microscope occurred after about 6 hrs. of treatment. However, Walne (1967) did report effects on the golgi apparatus after 2-3 hrs. and transitory effects on the mitochondria after 4-5 hrs. contrast, the first structural effects visible under the light microscope in Eudorina occurred after only 90 mins. of treatment, even though the same concentrations of colchicine were used. Walne (1966, 1967), in her studies on Chlamydomonas did not differentiate between non-dividing and dividing cells. This may well account for the apparent discrepancy between the treatment times, so that this would not appear to be significant.

A. Stellations

The most spectacular morphological response to colchicine is the production of stellations, which to my knowledge, have never been produced in any other organism treated with colchicine. However, in response to prolonged colchicine treatment, grasshopper neuroblasts were observed to produce small blebs at the cell surface (Gaulden and Carlson, 1951).

The stellations are primarily produced in cells that are initiating division. The most dramatic event during the initial stages of division, is the pre-prophase migration of the nucleus from the center to the periphery of the cell, which must result in a considerable reorganization of the cell components. During pre-prophase, peripheral cytoplasmic microtubules are most evident and may be responsible for maintaining the smooth shape during the reorganization at this stage. Therefore, the absence of these microtubules could possibly result in the formation of stellations. Further the actual form of the stellations could be a reflection of the fact that the chloroplast's

exterior surface does not appear smooth, but rather consists of a number of projections (Fig. 2). These projections are visible in the light microscope as striations of the chloroplast (Goldstein, 1964, Fig. 4, p. 31) and have also been observed in Pandorina (Coleman, 1959). The movement of the nuclei during subsequent pre-prophase stages is minimal, since the nuclei are already at the cell surface (Fig. 19). Consequently, only very small stellations are produced in cells entering their second, third, fourth, and fifth divisions.

The question then arises as to why Chlamydomonas, which appears to have pre-prophase nuclear migration and a similar arrangement of cytoplasmic microtubules (Johnson and Porter, 1968), does not produce stellations. A possible explanation may be that the presence of a cell wall in Chlamydomonas results in the restriction of the stellations.

In conclusion, the production of the stellations would appear to indicate that the peripheral cytoplasmic microtubules have an important cytoskeletal function during the pre-prophase cellular reorganization. A cytoskeletal function has also been ascribed for cytoplasmic microtubules in Ochromonas (Brown and Bouck, 1973).

B. Production of Extra-Cytoplasmic Particles

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Following about 5 hrs. of colchicine treatment, the stellations are observed to rupture. This results in cells surrounded by numerous extra-cytoplasmic particles.

Chlamydomonas (Walne, 1966, 1967) was also observed to be surrounded by similar type particles, following colchicine treatment. In Chlamydomonas, Walne (1967) postulated that a continuous synthesis of cellular components resulted in an abscission of portions of the cytoplasm, which formed the cytoplasmic particles.

In <u>Eudorina</u>, the abscission of the stellations may be due to the excessive stretching of the cell membrane of the elongate stellations. The evidence then seems to indicate that the production of the cytoplasmic particles may be an indirect response to the colchicine treatment. A somewhat similar production of extracytoplasmic particles has been observed in cells that were both injured (Clegg, 1964) and dying (Mookerjee and Gangluly, 1965). The production of extra-cytoplasmic particles in <u>Eudorina</u> and <u>Chlamydomonas</u> may also be the

result of an injury or indirect response, rather than a specific response to the colchicine treatment.

C. Colonial Envelope

A major structural change caused by the colchicine treatment was the abnormal expansion of the colonial envelope around the plakeal cells. Similarly, in her study on Chlamydomonas. Walne (1967) reported a considerable increase in cell wall material. The question arises as to how the colchicine treatment is affecting the production of envelope material.

Examination of the cell components during the expansion of the envelope reveals that the only organelle that shows any increase in activity is the golgi apparatus.

Walne (1967) reports a similar increase in golgi activity in Chlamydomonas after colchicine treatment.

In <u>Budorina</u>, this activity primarily consists of the production of small (35-70 mm in diameter) opaque vesicles, similar to those found in the untreated cells of plakes and young daughter colonies but in much greater numbers.

The evidence then suggests that the colchicine treatment results in an increase of golgi-associated vesicles, and that they may possibly contain the envelope material. A similar origin for the envelope material has also been postulated in <u>Volvox</u> by Bisalputra and Steifi (1966) and Deason and Darden (1971).

An important point that could either support or refute this hypothesis concerns the nature of the envelope material. If the envelope is of a carbohydrate nature, as suggested by Lang (1963) in <u>Eudorina</u> and by Deason and Darden (1971) in <u>Volvox</u>; then, it is quite possible that golding yesicles may contain envelope material since golding are associated with synthesis of carbohydrates (Whaley et al., 1966; Robards, 1970). However, some workers claim that the envelope in <u>Volvox</u> (e.g. Kochert, 1968) is proteinaceous in nature. Kochert (1968) was able to break down the envelope by using a number of proteolytic enzymes. In contrast to carbohydrates, proteins in the algae do not appear to be associated with the golgi.

The exact reason for the increase in the colonial envelope is unclear. However, the evidence seems to indicate

a correlation between the increase in colonial envelope material and golgi activity.

D. Contractile Vacuoles

prolonged periods of colchicine treatment. The same was noted by Walne (1967) in <u>Chlamydomonas</u>. A possible explanation for the large number of membranous and vesicular particles that are associated with the vacuoles, is that the contractile vacuoles may be "pumping out" the colchicine from the cell. A similar situation has been reported in <u>Paramecium</u> (Pickett-Heaps, personal communication).

E. Nuclear Morphology

After the production of polyploid nuclei, longer colchicine treatment (12 hrs. or more), produced a number of very heavily stained nuclei. The staining intensity was thought to be due to a large number of condensed chromosomes, which were not expected at this time in the nuclear cycle. Upon completion of chromosome division, in colchicine-treated cells, the chromosomes appeared to uncoil and enter interphase.

The chromosomes were not expected to condense again until the treated cells were about to enter their next series of divisions, i.e., approximately 3 days after the initial treatment. Yet chromosomes appeared to be condensing after; only 12 hrs. of colchicine treatment.

These apparent highly polyploid nuclei also exhibited a degree of lobing and irregularity of form, not found in normal untreated nuclei. The lobed nuclei were seen under the light and the electron microscope and appeared very similar to the nuclei described by Walne (1966, 1967) in Chlamydomonas. The electron microscope examination of these lobed and heavily stained nuclei in Eudorina revealed a fibrous nucleoplasm rather than their normal granular appearance, but the nucleolus appeared normal. This fibrous nature of the nucleoplasm may account for the condensed nature of the stained nuclei (Figs. 84-90). If this is the case, it would tend to discount the possibility of the lobed nuclei containing condensed chromosomes. Dustin (1947) has reported that following prolonged periods of treatment of mouse intestinal tissue with several mitotic poisons, there is a degeneration of chromosomes. This degeneration eventually leads to the presence of clumps of basiophilic

material which stain with Schiff's reagent. It is possible, therefore, that the heavily stained nuclei actually contain degenerating nuclear material and the ultrastructural evidence would tend to support this hypothesis.

Further, the nuclear membrane also undergoes considerable change as the two membranes expand and come apart (as seen in <u>Chlamydomonas</u>, Walne, 1967). A similar response was produced in colchicine-treated ascites tumors (Guerritore et al., 1967). The nuclear membrane reaction appears to be similar to events in cells undergoing degeneration (Mollenhauer et al., 1960). It seems probable, therefore, that the more prolonged treatments of colchicine elicit a nonspecific response in the nucleus, rather than a specific one, such as that due to its effects on the spindle microtubules.

F. Flagellar Microtubules

Another interesting aspect of colchicine treatment in <u>Eudorina</u> was the ability of the flagella to function normally when treated up to 50 hrs. In contrast, Walne (1966, 1967) found that flagella in <u>Chlamydomonas</u> were affected after only 18 hrs. of colchicine treatment. In

Chlamydomonas, when cells enter division they normally lose their flagella. Walne (1966, 1967) was working with cells that appeared to be entering division during colchicine treatment, which may explain why shorter treatments resulted in a loss of the flagella.

In <u>Budorina</u>, one interesting facet of colchicine's effect on the flagellar apparatus was its ability to inhibit the formation of flagella. Similar colchicine effects have been observed by Rosenbaum et al. (1969) on regenerating flagella of <u>Chlamydomonas</u>. Thus, the indications from the present study and others (Tilney and Gibbons, 1968) are that flagella are less readily affected by colchicine except when regeneration of elongation is occurring.

G. Chloroplast Microtubules

This is the first observation of chloroplast microtubules in <u>Eudorina</u> and adds <u>Eudorina</u> to the growing list of
members of the green algae in which they have been observed
(see Table IV. p. 120).

Chloroplast microtubules, from the observations of their distribution in Eudorina cells, seem to be almost exclusively associated with the chloroplasts of differentiating and rapidly growing cells (<u>i.e.</u>, the plakea and young daughter colonies). This distribution is similar to that in other green algae, as noted by McBride (1970) and suggests that the chloroplast microtubules may be involved in chloroplast division and expansion.

The colchicine treatment has no apparent affect on the chloroplast microtubules, even after 24 hrs. These results indicate that chloroplast microtubules are distinct from cytoplasmic microtubules and confirm the observations of Pickett-Heaps and Fowke (1970).

In view of their structural and functional differences with respect to cytoplasmic microtubules, the alternative name of "plastotubules" for these structures is suggested. Further their possible taxonomic significance in the algae will be dealt with later in the discussion (see p. 118).

II. Recovery Cycle

One of the most interesting questions to be asked in relation to the effects of colchicine on <u>Budorina</u> is the degree of reversibility of the effects. Walne (1966) placed colchicine-treated cells of <u>Chlamydomonas</u> in colchicine-free medium and then allowed them to recover. She reported that cells underwent a number of successive unequal divisions that resulted in some recovered vegetative cells which ultrastructurally appeared normal.

In the present study, numerous treated plakea were allowed to recover, and cells were found to be affected both reversibly and irreversibly. The irreversibly affected cells eventually formed plakeal fragments; while the reversibly affected cells eventually formed normal colonies.

The normal colonies arose both from inverted plakea and from plakeal colonies which failed to undergo inversion (see Diagram 4). The plakeal fragments occurred at two different stages of the recovery cycle (see Diagram 4). The first occurrence was immediately following the colchicine treatment. These plakeal fragments appear to arise from

plakeal cells that undergo a disorientation or inhibition of cytokinesis during and following colchicine treatment (see Diagram 4). The second occurrence was at the start of division of the flattened uninverted plakes. In this case, the results suggest that the fragmentation occurs as a result of chromosomal imbalance due to the unequal distribution of chromosomes at division. The fragmentation may represent an attempt by the cells to re-establish their normal chromosome complement. .Therefore, the ability of the flattened plakes to form normal daughter colonies would appear to be dependent on the degree of ploidy of the cells. Further, the cytological evidence indicates that cells with greater than a diploid number did not recover. It was not clearly established, however, if the polyploid cells were able to successfully reduce their chromosome numbers or not. would appear though, that this was not the case as unequal divisions always appeared to result in the production of plakeal fragments which died.

It appears then that the treated cells do not recover if cytokinesis is affected. However, if inversion is affected, the cells may recover depending on their level of ploidy.

III. Nuclear Division

A. Normal

from the present results, it appears that nuclear division in <u>Eudorina</u> bears a considerable similarity to the, same process in both <u>Chlamydomonas</u> (Johnson and Porter, 1968) and <u>Volvox</u> (Deason and Darden, 1971). This is in many ways expected, as the cell structure of all three organisms are clearly related. The similarities are listed in Table II (see Features 1-5), together with a number of features (6-8) not observed in <u>Eudorina</u>, and some features (9-11) only observed in <u>Eudorina</u>. The features (1-8, Table II) previously observed in <u>Volvox</u> and/or <u>Chlamydomonas</u>, have already been discussed in previous studies (Johnson and Porter, 1968; Deason and Darden, 1971). Therefore, emphasis will be placed only on those features which appear exclusive to Eudorina (9-11, Table II).

One feature is the formation of the daughter nucleoli prior to the formation of the daughter nuclei. This appears to be different to the situations in both <u>Chlamydomonas</u>

(Johnson and Porter, 1968) and <u>Volvox</u> (Deason and Darden,

1971) where the nucleoli form following the formation of the daughter nuclei.

Another feature concerns the presence of darkly stained areas at the nuclear poles from which the microtubules appear to radiate. It is tentatively suggested in the results that these areas might correspond to the Microtubule Organizing Centers (MTOC), described and defined by Pickett-Heaps (1969). If this area is the MTOC, it would tend to support the hypothesis that basal bodies and centrioles are not involved, in the organization of the spindle apparatus.

A third feature of Eudorina is the presence of ER, between the newly formed daughter nuclei, in distinct bands., A possible explanation for the internuclear ER is that it is merely the result of an excessive elaboration of the nuclear envelope during daughter nuclei formation. (This feature will be further discussed in the section on cytokinesis (see sec. IVA, p.108).

TABLE II

Some Features of Muclear Division of Chlamydomonas

Volvox, and Eudorina

	Feature	Chlamydomonas	Volvox	Eudorina
1.	Nuclear envelope remains intact throughout except for two polar fenestra	* *	+	+
2.	Kinetochores not associated with chromosomes	+ · · · · · · · · · · · · · · · · · · ·	+	+
3.	Basal bodies/centrioles present at periphery of cell, not associated with nuclear poles or spindle microtubules	* *	+	,
4.	Daughter nuclear envelopes formed through an elaboration of the persistent nuclear envelope		+	+
5.	ER present between newly formed daughter nuclei	+	c +	+
6.	Metaphase band of microtubules present	+	+	- "
7.	Extra-nuclear sheath of micro- tubules radiating from the base bodies	al -	+	-

TABLE II

(Continued)

Feature

Chlamydomonas Volvox Eudorina

- 8. Internuclear microtubules between newly formed daughter nuclei
- Formation of daughter nucleoli prior to the formation of the daughter nuclei
- 10. Microtubule Organizing Centers (MTOC) may be present
- 11. Presence of internuclear ER (Peature 5) in <u>distinct</u> bands

B. <u>Colchicine Treatment</u>

The effects of colchicine on nuclear division in <u>Budorina</u> result in considerable alteration to the nuclear division cycle (see Table I). These alterations appear to be the result of the continued replication of the chromosomes following the breakdown of the spindle microtubules. This continued replication of the chromosomes is only at a slightly reduced rate from normal (see Table I), and is still rapid enough to result in a very high degree of ploidy after relatively short exposures to colchicine. This situation has not been reported before in the algae. Further, the degree of polyploidy exhibited within each cell appears to be directly related to their stage of plakeal development prior to colchicine treatment.

Eudorina is the first algal species in which c-mitosis, including c-metaphase, has been induced by short (15-20 mins.) treatments of colchicine at concentrations of only 0.2%. The only other genus in which c-metaphase has been observed is Oedogonium (Henningsen, 1963) but then only with prolonged treatments and at higher concentrations of colchicine. The spatial separation of chromosomes at c-metaphase is of

particular value in <u>Eudorina</u>, where flattened squash preparations of chromosomes are not possible, due to the nature of the colonial envelope. In view of these results, it is now possible to consider a detailed cytotaxonomic investigation of <u>Eudorina</u>, to define more clearly speciation in the genus (Goldstein, 1964).

Another significant effect of colchicine on nuclear division in <u>Eudorina</u> is the production of permanent polyploids, as a result of short-term (20 mins.) treatment with 0.2% colchicine. This is of extreme interest because permanent polyploidy is questionable in <u>Chlamydomonas</u> (Buffaloe, 1957). In the given algal species in which polyploidy has been observed (Godward, 1966), it occurs only after more prolonged treatments, using higher concentrations of colchicine.

cells with a high degree of polyploidy (see Table I), but these polyploid cells have never been recovered. This evidence suggests that with a greater than diploid number of chromosomes, the chromosome complement is unbalanced and the cell is unstable, which results in poor viability and death.

IV. Cytokinesis

A. Normal

In all three species, Chlamydomonas, Eudorina and Volvox, a prominent feature of normal cytokinesis is the presence of the phycoplast system of microtubules. These microtubules appear to direct the orientation of the cleavage furrow between the closely associated daughter nuclei. Pickett-Heaps and Marchant (1972) consider the presence or absence of a phycoplast a highly significant feature of the green algae, and have devised a phyletic scheme based upon its presence or absence. In both Volvox (Deason and Darden, 1971) and Eudorina, the phycoplast appears to contain fewer microtubules than in Chlamydomonas (Johnson and Porter, 1968).

A most significant observation in <u>Eudorina</u> is the formation of a discontinuous furrow with cytoplasmic bridges between adjacent cells as in <u>Volvox</u> (Deason and Darden, 1971), but distinct from the continuous furrows of <u>Chlamydomonas</u> (Johnson and Porter, 1968). Unlike <u>Gonium</u> (Stein, 1965) and <u>Volvox</u> (Darden, 1966), the cytoplasmic

bridges are not evident in colonies under the light microscope. This is the first report of their existence in the genus. In view of the significance of these bridges for inversion (to be discussed below), and the fact that inversion is a characteristic process of the Volvocaceae, this evidence would tend to suggest their existence in all members of this family. In fact, there has already been a brief mention of their presence in Pandorina (Lefort, 1963).

associated with the cleavage furrow. It is possible that this ER plays a role in cleavage furrow formation. Further, orientation of the internuclear bands of ER along the presumptive plane of cleavage, suggests a possible connection between the two sets of bands. However, further studies will be necessary to establish the above hypothesis.

B. Colchicine Treatment

Colchicine treatment of cells that are undergoing cytokinesis results in two striking effects: (1) the discrimination, and (2) the inhibition of cytokinesis.

It has been suggested by Pickett-Heaps (1972) that phycoplast microtubules direct the orientation of the cleavage furrow. In the present study, short-term colchicine treatment of dividing cells results in the breakdown of the phycoplast. The disorientation of the cleavage furrow, which results in unequal divisions could be explained in terms of the breakdown of the phycoplast, thus supporting the hypothesis of Pickett-Heaps (1972).

The subsequent complete inhibition of cytokinesis would suggest further that the phycoplast could possibly be involved in the actual formation of the furrow. This may turn out to be an interesting hypothesis as it suggests an additional role for the phycoplast. It may be, therefore, that the phycoplast plays a dual role and is essential for both the direction and the formation of the cleavage furrow in Eudorina.

Another possible explanation for the complete inhibition of cytokinesis is that the colchicine is directly inhibiting membrane elaboration for the formation of the furrow, but at present, evidence in the literature does not support such a hypothesis.

V. Inversion

A. Normal

The examination of inversion in Eudorina, under the electron microscope, reveals a number of features not observed under the light microscope. The only other colonial green flagellate in which inversion has been studied at the ultrastructural level, is Volvox (Pickett-Heaps, 1970a). There appears to be a number of similarities in the inversion processes of the two species, though they differ in colony complexity. Most obvious, is the presence and arrangement of cytoplasmic bridges in the region of the posterior facets of the plakeal cells (see Diagram 2). The bridges appear to hold the cells together, while acting as "hinges", as reported by Pickett-Heaps, (1970a). Also present in both genera are peripherally orientated microtubules. In Volvox these microtubules may be involved in the cell elongation that occurs just prior to inversion (Pickett-Heaps, 1970a). Found exclusively in Volvox are striations on the plasmalemma around the cytoplasmic bridges (Pickett-Heaps, 1970a).

With regard to the actual process of inversion,

Pickett-Heaps (1970a) postulated that some coordinated

contractile movement of the cell membrane around the cyto
plasmic bridges, together with other factors, such as cell

elongation, may be responsible for inversion.

In Eudorina, neither cell elongation nor striations on the cytoplasmic bridge membranes are observed in inverting plakea. Rather, the most prominent activity during inversion is the expansion of the colonial envelope around the plakea. To understand how this expansion may result in inversion, let us examine the uninverted plakea (see Diagram 2A), and note that the cells are mainly connected at their posterior facets. Upon expansion of the envelope, considerable pressure is likely to be created within the plakeal cup. In response to this pressure, the cells tend to separate; but they can only move apart at their anterior facets, due to the position of the bridges. This, then results in movement that begins with the peripheral cells and results in the formation of a sphere of cells (i.e., daughter colonies). This formation of a sphere is a natural tendency of objects that are pushed apart while attached at only one end.

is because a sphere allows for the greatest separation of the cells at their unattached ends. Therefore, it may be that inversion is simply the result of pressure created within the plakeal cup by the expansion of the colonial envelope between the plakeal cells.

pushed apart and move passively in inversion, in contrast to their active role in plakeal inversion in Volvox, postulated by Pickett-Heaps (1970a). It is felt that this difference may be reflected in the much simpler nature of inversion in <u>Eudorina</u> as compared to <u>Volvox</u>. However, the author feels that inversion in <u>Volvox</u> may also involve a similar expansion of the colonial envelope. This hypothesis also lends additional functional significance to the arrangement of the cytoplasmic bridges. It will be interesting to see if bridges are arranged in <u>Gonium</u> in a manner similar to that observed in <u>Eudorina</u> and Volvox, as Gonium does not undergo complete inversion.

B. Colchicine Treatment

Colchicine treatment completely inhibits the process of inversion. The most obvious explanation would be to consider the involvement of microtubules in the normal inversion process of <u>Eudorina</u>.

Peripheral microtubules are present in plakeal cells and could possibly be involved, as has been suggested for Volvox (Pickett-Heaps, 1970a). However, in <u>Eudorina</u> cell elongation normally does not take place during inversion.

Inversion appears to be inhibited by the breakage of the cytoplasmic bridges. This breakage may be due to an abnormal production of colonial envelope material as compared to plake undergoing normal inversion (cf. Figs. 15, 71). It may be, therefore, that this excessive expansion of the envelope puts an increased strain on the bridges, causing them to break before inversion takes place. Evidence to support this hypothesis was obtained from studying recovering plakes.

Examination of these plakes indicated that plakes!

cells that were still tightly appressed, following colchicine

treatment, would often invert normally when allowed to recover. However, plakeal cells that had become separated and flattened were never observed to invert.

colchicine treatment, then, appears to add further evidence to the importance of the bridges in the inversion process in colonial green flagellates. In this light, it would be interesting to examine certain species of Volvox, such as V. aureus, which have permanent cytoplasmic connections, to see if inversion has been inhibited, following colchicine treatment.

In conclusion, the ability of plakes to invert would appear to depend upon the production of colonial envelope material and the arrangement and presence of cytoplasmic bridges. In contrast, a breakdown of the bridges due to excessive production of colonial envelope material, inhibits inversion, as observed in colchicine-treated plakes.

'VI. Taxonomic and Phylogenetic Implications

A. Events of Cell Division

In recent years, a number of ultrastructural characteristics have become important taxonomic and phylogenetic

Criteria within the green algae (Round, 1971). Pickett-Heaps (1969, 1972) and Pickett-Heaps and Marchant (1972) have placed particular emphasis on the ultrastructural details of cell division. They noted three main characteristics which they considered important in distinguishing phyletic groups in the green algae: (1) the presence of a phycoplast during cytokinesis; (2) a closed spindle; and (3) the persistence of the interzonal spindle. Further, Pickett-Heaps (1969, 1972) has stressed the involvement of the MTOC rather than the centrioles or basal bodies in spindle organization. He also postulated that basal bodies (centrioles) are primarily involved with the flagella apparatus.

Pickett-Heaps and Marchant (1972) used one of the above criteria, the presence of a phycoplast, to divide the green algae into two main phyletic groups. Recently, this same characteristic together with some others, has been used in a taxonomic and phylogenetic evaluation of the Ulotrichales (Stewart et al., 1973). The phycoplast-containing algae were further distinguished (Pickett-Heaps and Marchant, 1972) by the collapse of their interzonal spindle and the closed nature of their spindle.

Based on the above criteria, <u>Eudorina</u> would appear to be a typical member of the phycoplast-containing algae. Further, the author believes that other characteristics of taxonomic significance have been observed in this and other studies to further distinguish the Volvocales from the other phycoplast-containing algae. Examining Table III, it can be seen that <u>Eudorina</u>, <u>Chlamydomonas</u>, and <u>Volvox</u> can be distinguished from the other genera by two unique characteristics, the absence of basal bodies or centrioles from the nuclear pole, and the presence of internuclear ER.

The absence of the basal bodies from the nuclear poles and their presence at the cell surface is a particularly significant taxonomic characteristic. Of all the members of the phycoplast-containing algae, only the Volvocales are permanently flagellated. Therefore, it may be that the position of the basal bodies or centrioles within a cell is related to the permanency of flagellation in the organism's life history. However, further studies on cell division in other Volvocales will be necessary to support the validity of the above characteristics as taxonomic criteria for this group of organisms.

TABLE III

Comparison of Phycoplast-Containing Green Algae

Organism		Reference	Centrioles/ Basal bodies associated with nuclear poles	Inter- nuclear ER
λ.	VOLVOCALES			
1.	Eudorina	Gottlieb	•	+
2.	Volvox	Deason and Darden (1971)	*	+ .
3.	Chlamydomonas	Johnson and Porter (1968)	- 9	+
В.	CHLOROCOCCALES	134	•	
4.	Chlorella	Atkinson <u>et al.</u> (1971)	+	7
5.	Kirchneriella	Pickett-Heaps (1970b)	+	•
6.	Hydrodictyon	Marchant and Pickett Heaps (1970)	+ -	; -
c.	ULOTRICHALES			
7.	Ulothrix	Floyd et al. (1972)	+	- *(
8.	Stigeoclonium	Floyd et al. (1972)	+	-
9.	Microspora	Pickett-Heaps (1973a)	+	-
10.	Schizomeris	Stewart et al. (1973)	+	. ,
D.	OEDOGONIALES		1	
u.	<u>Oedogonium</u>	Pickett-Heaps and Fowke (1969)	≖ ♦ . ° ,	·
12.	Bulbochaete	Pickett-Heaps (1973b)	_+ /	_

^{*} No centrioles were observed in normal dividing cells.

[?] No observations made of post-telophase stage of division.

B. Chloroplast Microtubules

In recent years, chloroplast microtubules have been found within a number of members of the green algae (Table IV). Further, the features of chloroplast ultrastructure have been used for some time to delineate different groups of algae (Gibbs, 1970). Gibbs distinguished the Chlorophyceae (sensu, Christensen, 1966) by the following features of their chloroplast. The chloroplasts are limited solely by a double membrane, the bands are made up of a variable number (2-6) of thylakoids or grana or both, and the starch grains are stored inside the chloroplast. In the author's view, the presence of chloroplast microtubules is an additional characteristic that can help define the limits of the Chlorophyceae.

In examining the distribution of chloroplast microtubules (Table IV), their presence is revealed within the Chlorophyceae (sensu, Christensen, 1966) and Chlorophyta and Charophyta (sensu, Round, 1971).

At the moment, chloroplast microtubules have not been observed in the two other classes of the green algae,

the Prasinophyceae and Euglenophyceae. This absence, if proved conclusively, would tend to confirm their placement into separate classes (sensu, Christensen, 1966) or phyla (sensu, Round, 1971). However, there has been insufficient information to make definite conclusions. The presence of chloroplast microtubules however does suggest that perhaps the Chlorophyta (sensu, Round, 1971) should include the Charophyta reduced to a class.

TABLE IV

Distribution of Chloroplast Microtubules within the Green Algae

Phylum.	Class	Order .	Species	Reference		
Chlorophyta	Chlorophyceae	Volvoçales	1. Volvox	Pickett-Heaps, 1968 Deason & Darden, 1971		
r		٠	2. Eudorina	Gottlieb		
			3. Chlamydomonas	Brown et al., 1968		
\sim	•	Chlorococcales	1. Trebouxlá	Jacobs & Amadjan, 1971		
-		Ulotrichales	1. Fritschiella	McBride, 1970		
		0	2. Stigeoclonium	Pickett-Heaps, unpublished		
		Ulvales	1. <u>Ulva</u>	Braten & Løvlie, 19 6 8		
Ď	•	Oedogoniales	1. Oedogonium	Hoffman, 1967		
	g wee		2. Bulbochaete	Smith & Butler, 1971 ° Pîckett-Heaps, 1973b		
	•	Zygnematales	1. Closterium	Pickett-Heaps & Powke, 197		
		∛Bryopsidales	1. Caulerpa	Sabnis, 1969		
•	,		2. Dichotomosiphon*	Moestrup & Hoffman, 1973		
		Chärales	1. Chara	Pickett-Heaps, 1968		
	Prasinophyceae		•			
~ *	Euglenophyceae		_	·		

^{*} Structure reported to be unlike other types of chloroplast microtubules.

SUMMARY AND CONCLUSIONS

- 1. Colchicine treatment was found to affect colonies that were undergoing division. Affected dividing colonies were found to exhibit the following structural, and nuclear alterations, depending on the stage of development the dividing cell had reached prior to colchicine treatment.
 - A. The production of stellations that were most prominent in cells that were initiating division.

 It was hypothesized that the stellations were the result of the breakdown of peripheral microtubules during the pre-prophase migration of the nucleus, and that the microtubules were performing a cytoskeletal function at this crucial stage of development.
 - B. The production of extra-cytoplasmic particles that occurred around the treated cells after more prolonged colchicine treatment. The particles appeared to be the result of a rupture of the stellations.

- C. Blakes were produced that did not invert and which resembled the flattened colonies of Gonium.

 These gonium-like colonies appeared to be the result of an abnormal increase in colonial envelope material around the cells. A possible explanation for this increase was suggested by the considerable increase in the production of small opaque golgiassociated vesicles.
- D. An alteration was induced in the normal nuclear cycle due to the breakdown of the spindle microtubules. These microtubules were observed to disappear after short (25-30 mins.) periods of colchicine treatment. This resulted in a considerable increase in the number of chromosomes in the treated plakeal cells. Furthermore, a number of permanent diploid colonies were obtained following recovery in colchicine-free medium.
- E. Colchicine treatment also resulted in alterations in the nuclear morphology. These included changes from a granular to a fibrous nucleoplasm and the condensation of nuclear material. Also observed was

an expansion and lobing of the nuclei and a separation of the two nuclear membranes. It was hypothesized that these nuclear effects could possibly be due to the general poisonous nature of the colchicine.

- F. The disorientation and complete inhibition of cytokinesis was induced in treated plakeal cells, and appeared to be the result of the breakdown of the phycoplast band of microtubules. This suggested that these microtubules could be responsible for both the direction and formation of the cleavage furrow.
- G. Colchicine treatment resulted in a complete inhibition of inversion that appeared to be due to an abnormal production of colonial envelope material. It was suggested that this abnormal production resulted in the breakage of the cytoplasmic bridges between the cells. These results then, seemed to confirm the importance of the bridges in the inversion process.
- 2. On allowing treated cells to recover in colchicine-free medium, plakeal cells were observed to be affected, both reversibly and irreversibly. Plakea, in which cytokinesis

had been disrupted formed "plakeal fragments" and never recovered. However, plakea in which only inversion had been affected, did sometimes recover, but this recovery appeared to be dependent on the degree of polyploidy of these flattened plakea.

- 3. A detailed ultrastructural study was made of cell division and colony development in <u>Eudorina elegans</u>, which resulted in the following observations and conclusions.
 - A. Nuclear division was similar to that in Chlamydomonas reinhardi and Volvox aureus with the exception of a few features. These features included the following: 1. the possible presence of a Microtubule Organizing Centre; and 2. the formation of daughter-nucleoli prior to the formation of daughter nuclei.
 - B. A typical "phycoplast" band of microtubules was found to be involved in cytokinesis. However, cytokinesis was incomplete which resulted in a number of cytoplasmic bridges between the plakeal cells.

These bridges had never been observed previously in <u>Mudorina</u> and it was suggested that they may be a characteristic of all members of the Volvocaceae.

- C. The inversion process appeared to involve the production of colonial envelope material and the arrangement of the cytoplasmic bridges. A new hypothesis concerning inversion was postulated' based on these two features.
- D. A number of taxonomic criteria were identified at the ultrastructural level with which it was suggested that the Volvocales could be distinguished from other members of the phycoplast-containing algae (Pickett-Heaps and Marchant, 1972). These included the presence of internuclear ER between newly formed daughter nuclei, and the lack of association of basal bodies/centrioles with the nuclear poles.
- B. Microtubules were observed for the first time in the chloroplasts of dividing and young cells.

 An examination of the literature revealed these

Chlorophyceae (sensu Christensen, 1966). It was, therefore, proposed that their presence might indeed be a useful taxonomic feature of the class. Purther, in light of these microtubules' structural dissimilarity to regular microtubules, and their resistance to colchicine treatment, it was suggested that they be given the name "plastotubules".

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APPENDIX I

COLONY DIFFERENTIATIÓN IN EUDORINA CALIFORNICA

entiation in E. (Pleodorina) californica, Gerisch (1959) and Goldstein (1967) postulated the stage in plakeal development when differentiation of somatic and generative cells was initiated. These postulates, however, have not been confirmed experimentally, since no suitable methods were available to inhibit plakeal development and to study differentiation of uninverted plakeal cells. The effective inhibition of plakeal development with colchicine has enabled the present study to reveal the stage of development at which the onset of differentiation occurs in E. californica.

The strain (198 hd) of E. californica used in this study was obtained from the Indiana University culture

collection. To inhibit plakeal development, dividing colonies were treated with a 0.2% colchicine solution in sterile soil-water supernatant for 40 mins. Following colchicine treatment, colonies were washed in fresh soil-water medium, and then 4, 8, 16, and 32 cell plakea were excised from their maternal colonial envelopes. Individual plakea were then placed in depression spot plates containing fresh soil-water medium, for observations over a 6 day period.

Normal vegetative colonies have a number of momatic and generative cells arranged in distinct rows or tiers.

The small somatic cells always makeup the anterior tiers of the colony, while the larger generative cells makeup the posterior tiers of the colony (Fig. ,105). Further, the number of tiers of each cell type varies with the number of cells in the colony (Figs. 105-107).

Under normal growth conditions in culture, 4-, 8- and 16-celled colonies are never observed. On entering division, the generative cells divide (Fig. 108) and form plakes which eventually invert to produce daughter colonies (Fig. 109). The generative cells divide while the somatic cells are

- rig. 109. 64-celled colony with plakes undergoing inversion to form daughter colonies. Note that the anterior tiers of cells are not dividing. x 200.
- Fig. 110. A young vegetative colony. Note that cell differentiation is not yet apparent. x 352.

Differentiation in Eudorina californica

Vegetative Colony

- Fig. 105. Vegetative colony of <u>Eudorina californica</u>

 containing 32 cells. Note the reduction in size

 of the two anterior tiers of cells. x 320.
- Fig. 106. Vegetative colony of <u>Eudorina californica</u>

 containing 64 cells. Note the reduction in size

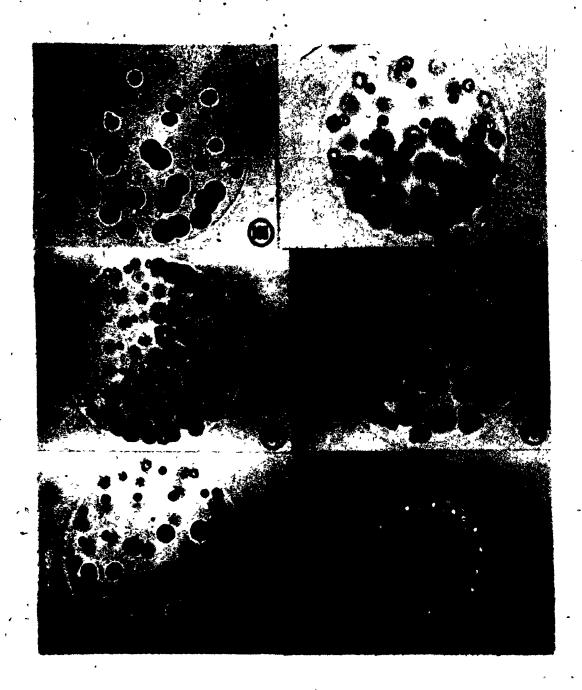
 of the three anterior tiers of cells. x 275.
- Fig. 107. Vegetative colony of <u>Eudorina californica</u>

 containing 128 cells. Note the reduction in size

 of the four anterior tiers of cells. x 200.

Asexual Reproduction

Fig. 108. 64-celled colony undergoing division. Note that the anterior three tiers of cells do not undergo division. x 200.



observed to undergo senescence and death. In newly formed daughter colonies (Fig. 109), and those recently released from the maternal envelope (Fig. 110), all the cells are equal in size. Only as the young colony enlarges does differentiation of the cells gradually become apparent, eventually resulting in a fully differentiated colony (Fig. 105).

Shortly after treatment, the plakea appeared normal and showed no signs of the colchicine treatment (Figs. 111, 114, 116, 119). The plakea were then examined every day until they divided which was usually after 6 days of recovery.

Following 48 hrs. recovery in colchicine-free medium, cells of all plakeal stages separated, and underwent some enlargement, concomitant with an expansion of the colonial envelope (Figs. 112, 117, 120). After 6 days recovery, all the cells of the 4-, and 8-celled plakea proved to be generative (Figs. 113, 115). In <u>E. californica</u>, the peripheral cells of 16- and 32-celled plakea enlarged and divided while the central plakeal cells remained somatic (Figs. 118, 121, 122).

16-celled Plakea

- Fig. 116. 5 hrs. recovery. Plakeal cells still closely appressed to one another. x 560.
- Fig. 117. 48 hrs. recovery. Plakeal cells separated.

 Expansion of colonial envelope around cells.

 x 560.
- rig. 118. 6 days recovery. Peripheral plakeal cells undergoing division. Note the four central cells (arrows) do not divide and are strictly somatic. x 220.

32-celled Plakea

- Fig. 119. 5 hrs. recovery. Plakeal cells still closely appressed to one another. x 560.
- Fig. 120. 48 hrs. recovery. Plakeal cells separated; expansion of colonial envelope. x 560.
- Fig. 121. 5 days recovery. Difference in size between central and peripheral cells is apparent. x 560.
- Fig. 122. 6 days recovery. Note central cells do not divide, but remain somatic. x 200.

Patterns of Differentiation in Plakes of E. californica Following Colchicine Treatment

4-celled Plakea

- Fig. 111. 5 hrs. recovery. Plakeal cells still closely appressed to one another. x 560,
- Fig. 112. 48 hrs. recovery. Plakeal cells separated;
 expansion of colonial envelope around cells.
 x 560.
- Fig. 113. 6 days recovery. Plakeal cells considerably enlarged about to enter division. All cells generative. x 560.

8-celled Plakea

- Fig. 114. 5 hrs. recovery. Plakeal cells still closely appressed to one another. x 560.
- Fig. 115. 6 days recovery. Plakeal cells considerably enlarged and about to enter division. All cells generative. x 560.

(over)



In Diagram 3, the patterns of successive cleavage planes, up to the 32-celled stage, are clearly illustrated. The origin of the 4 central plakeal cells, which in recovered 16-celled plakea become somatic can easily be traced, as can the origin of the 12 central plakeal cells in recovered 32-celled plakea. Thus, in treated plakeal stages in <u>E. californica</u> the onset of differentiation is first evident in 16-celled plakea which arise at the 4th successive bipartition of the mother cell.

These results provide direct confirmation of the postulates of Gerisch (1959) and Goldstein (1967).

Goldstein (1967) further postulated that the somatic nature of the central plakeal cells may arise through an unequal distribution of a peripherally oriented cytoplasmic component of the mother cell. This author suggested that mitochondria may be the organelle involved, since the mitochondria are peripherally oriented in vegetative cells (Fig. 2). Therefore, based on this hypothesis, one would expect differentiation to occur at the 16-celled plakeal stage when peripheral and central cells are first cut out by cleavage.

PATTERNS OF DIFFERENTION IN EUDORINA CALIFORNICA

DIAGRAM 5

Recovered colchine

Pattern of successive cleavage planes in the formation of the planest stage

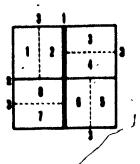
A.4 CELL STAGE



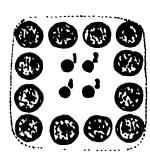
1			LEGEND
	1	2	☐ Generative cell
2	4	3	Sometic cell

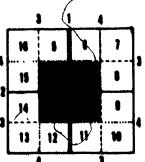
B.8 CELL STAGE



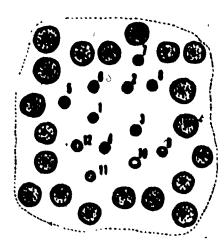


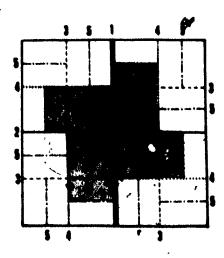
'<u>C</u>.16 CEL_IL STAGE





D.32 CELL STAGE





A possible explanation of how the mitochondria are involved is to consider the fact that mitochondria normally supply the energy for DNA synthesis. If unequal distribution of mitochondria occurs in E. californica, there may be very few mitochondria in the central plakeal cells. Then following inversion the now-anterior tiers of cells of the colony may subsequently be unable to produce sufficient energy to carry out DNA synthesis.

This, in turn, would render the cells somatic in nature.

californica also seem to imply some type of cytoplasmic control. Gerisch (1959) after a number of expesiments hypothesized that there was an early determination, possibly in the undivided cells, of a somatic and generative cytoplasmic area. In Volvox, Starr, (1969, 1971b) has postulated that differentiation occurs following an unequal division of cells in the developing plakea. The larger cells are believed to give rise to gonidia while the smaller cells give rise to somatic cells. Translating this evidence into the hypothesis just postulated to

explain differentiation in R. californica, it may be that the smaller plakeal cells of Volvox, receive less mitochondria during development and subsequently in maturing colonies, may produce insufficient energy for DNA synthesis.

APPENDIX II

RECIPES OF GROWTH MEDIA

Pringsheim's Soil-Water Medium Containing CaCO3

A pinch of CaCO₃ is placed in a test tube (20 ml.) or half-pint milk bottle (250 ml.), and sufficient soil (obtained from a field near Burlington, Vermont) is added to make a layer 1/4 to 1/2 an inch deep in the vessel. Pyrex distilled water is then added to the vessels until they are about 2/3 full. The test tubes are covered with metal caps and the bottles are covered with small petri dishes and then both are steamed on two successive days. The test tubes for one hour each day and the milk bottles for one and a half hours each day.

Soil-Extract Agar

(Starr, 1964)

For each 1000 ml. of medium required:

modified Bristol's solution												
soil water supernant	•	•	•	•	•	•	•	•	٠	•	¥0.0	ml,
Ama w											15 0	~

Modified Bristol's Solution

Six stock solutions, 400 ml. in volume, are employed. Each contains one of the following salts in the amounts listed:

$NaNO_3$	 • • **•		• • • •	10.0 g.
cacl ₂				1.0 g.
$\frac{\text{MgSO}_4.7\text{H}_{2}\text{O}}{2}.$				3.0·g.
к ₂ нро ₄	 	• • • •		3.0 q.
кн ро	 	• • • •		7.0 g.
NaCl	 			1.0 g.°

10 ml. of each stock solution are added to 940.0 ml. of Pyrex-distilled water. To this is added a drop of 1.0% Pecl3 solution.

Volvox Medium (Starr, 1971a)

For each 1000 ml.of medium required place the following amounts of stock solutions in 943 ml. of Pyrex distilled water:

	Number of ml.	Stock Solutions
	10	$Ca(NO_3)_2.4H_20$ 1.19g/100 ml.
	10	MgSO .7H 0 0.4g/100 ml.
	. 10	Na ₂ glycerol phosphate. 5H ₂ O 0.5g/100 ml.
	10	KCl 0.5g/100 ml.
	10	Glycylglycine 5.0g/100 ml.
	1	B ₁₂
•	1.	Biotin
	<i>-</i> 5	PIV metal solution as given below.

PIV Metal Solution

Add the following amounts of chelating agent and salts to 500 ml. of Pyrex-distilled water.

ૄૡ૽ૼૢઌૣ૿ૺૺૺ	h FeCl ₃ ,6H ₂ 0 0.097 g.
•	Mncl ₂ .4H ₂₀
•	zncl ₂ 0.005 g.
,	сосі, 6H ₂ 0 0.002 g.
	Na_Mo0
	Ná ₂ EDTA 0.75 g.
just	final solution to pH 7.0 using 1N NaOH. "

Volvocacean Agar (Starr, 1964)

For each 1000 ml-of medium required:

Waris solution						pH).		•, •	800.0 ml.
Euglena medium	• 12 • 2	• •	• (• •	• •		•,•		200.0 ml.
• • •	•					e,		•	1
Agar (Difco) .						٠. •	`• · •.		15.0 g.

Waris Solution

To 1000 ml.of	Pyrex-distilled	water add	l ml.of	the following
•	~ ,		•	
solutions: .	,	-	/ .	•

10% KNO₃; 2% MgSO₄.7H₂0; 2% (NH₄)₂.HPO₄; 5% CaSO₄; Iron Sequestrine solution.

Iron Sequestrine solution is composed of the following:

 Sequestrine AA
 ...
 2.61%

 FeSO .7H 0
 ...
 2.49 g.

 1N KOH
 ...
 ...
 ...

 Pyrex distilled water
 ...
 ...
 ...

Euglena Medium

To 1000 ml of Pyrex distilled water add:

Sodium acetate .	•	•	•	. •	•	• .	• ``	•	•	•	1.0 g.
Beef extract		•	• '	•	•	•	•	•	•	•	,1.0 g.
Tryptone	•	•	•	•	•	•	•	•	•	•	2.0 g.
Yeast extract	•	•	•	•	•	•	•	•	•	•	2.0 g.
Caloium chloride	•		_	_		ተ _			0	4.1	0.01-a.

APPENDIX III

Recipes of the Chromosomal Stains

1. Aceto-carmine (Cave and Pocock, 1951a)

2 g. of carmine (National Aniline, Cert. No. NCa-13) are refluxed in 400 ml. 45% glacial acetic acid for 4 hours, allowed to cool and filtered and bottled.

2. Aceto-orcein (after Darlington and LaCour, 1960)

2 g. of orcein (Gurr, Cert. No. 375) are refluxed in 100 ml. of 70% glacial acetic acid for half an hour. The solution is then cooled, filtered and bottled. The stain was improved by adding a few bits of iron needle to the refluxate.

Schiff Reagent (Graumann, 1952/53)

0.5 g. basic fuchsin (Basic Fuchsin, Fisher Scient., Cert. No. 790325) is shaken in 15 ml. of normal HCl until dissolved. Then 0.8 g. of $K_2S_2O_5$ is dissolved in 85 ml. of distilled water and added to the basic solution. After 24 hrs., the solution is shaken for about 5 mins. with 0.3 g. of activated charcoal, filtered using No. 50 paper, and stored in the dark at 4° C.

APPENDIX IV.

Recipes for Electron Microscope Stains

1. Lead Citrate (Venable and Coggeshall, 1965)

0.06 g of Lead Citrate is added to 20 ml. Pyrex-distilled water in a 30 ml screw capped cultured tube.

To this solution is added 0.4 ml. of 5N CO₂-free NaOH and a piece of "Parafilm" is placed over the top of the tube, the top being screwed on tightly. The mixture is then shaken vigorously until all the lead citrate is dissolved.

The mixture is then filtered through No. 50 filter paper and the stain used immediately.

2. Uranyl Acetate

A saturated solution of 3% Uranyl Acetate in 70% ethyl alcohol is prepared and stored in a tightly stoppered bottle wrapped in aluminum foil. Immediately before use, a few ml. are filtered through No. 50 filter paper.