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A COMPARATIVE CYTOLOGICAL STUDY OF FOUR SPECIES OF CHLAMYDOMONAS[1]

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Relatively few investigators have engaged in cytological studies of *Chlamydomonas* and karyological investigations in particular have been neglected. Due to the extremely small size of the chromosomes, and because conventional techniques for staining and identifying individual chromosomes have not always yielded good results, those reports which have been made of chromosome counts in various species are confusing and contradictory.

Dangeard (1899) described in detail the morphology of several volvocacean species, giving particular attention to the mitotic process. He reported the following (haploid) chromosome numbers for species of *Chlamydomonas:*

Chlamydomonas monadina	30.
Chlamydomonas variabilis	10.
Chlamydomonas dilli	10.

Among other observations, one which appears to be important in connection with the present work was that the formation of chromosomes in these species is preceded by a stage at which the number of chromatinic granules is larger than that of the chromosomes as seen at their fully formed, condensed condition.

In a lengthy study of the comparative morphology of several protozoa, algae and fungi, Bêlaî (1926) gave passing attention to *Chlamydomonas.* In one of his figures of an unidentified species, 8 anaphase chromosomes are depicted passing toward each pole.

Kater (1929), in a study of the cytology of *Chlamydomonas nasuta,* reported the chromosome number to be 8. Like Dangeard, he found that the appearance of a large number of chromatinic granules during early prophase is characteristic of mitosis, and that condensation of these leads to the development of fully formed, late prophase chromosomes. He reported the appearance of centrioles and spindle fibers. According to his account, the nuclear membrane does not disappear until late anaphase, a

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phenomenon which is not in general agreement with the observations of other investigators. Kater also found that at the second division both cells divide at right angles to the original plane of cleavage and at right angles to each other.

In reviewing the work of Franz Moewus on the nature of sexuality in *Chlamydomonas,* Hartmann (1934) reported the chromosome numbers of *C. paupera* and *C. eugametos,* which Moewus succeeded in crossing, as 10 in each species; according to this account, the hybrid of this cross also exhibited this number, and behavior of a sex chromosome was described. Moewus (1936), himself, reviewed this same work and included data concerning the inheritance of certain morphological, physiological and zygotic characters, reporting specific cases of genetic linkage. The figures accompanying this paper include a zygote in the first meiotic division exhibiting 10 bivalents. In a later publication (1940), Moewus presented evidence for the existence of 10 linkage groups in *C. eugametos* obtained by crossing various mutants.

Akins (1941) made a cytological study of *Carteria crucifera* which, along with other members of this genus, is morphologically similar to *Chamydomonas,* the most obvious difference being that the former is quadriflagellate and the latter biflagellate. One of the goals of Akins' study was to determine whether any correlation could be found between the double number of flagella in *Carteria* and the chromosome number as compared with that of *Chlamydomonas* species. Inasmuch as Akins found the chromosome number in this species to be $n = 9$, and since previous reports of chromosome counts in *Chlamydomonas* ranged from 8 to 30, no relation between chromosome number and the number of flagella was obvious. However, the chromosome number itself is of interest, as are many other details which are reported. Nuclear division was described by Akins as occurring after a revolution of 90° by the protoplast, just before which the flagella had been either lost or withdrawn. The single pyrenoid usually divided before the nucleus. Prophases of division were characterized by the appearance of numerous chromatinic granules. The nuclear membrane and nucleolus disappeared at this stage, and definitely organized chromosomes then arranged themselves on the equatorial plate. At telophase, nucleoli reappeared and nuclear membranes were formed about each group of chromosomes. Although a number of different stains were employed in Akins' study, centrioles were not observed. In second division, the two nuclei did or did not divide simultaneously, according to this investigator.

Cave and Pocock (1951a), in studying nuclear behavior in the colonial Volvocales, have reviewed accounts in which chromosome numbers are given for members of this group previous to their own investigations. They succeeded in counting the chromosomes of several species, the cells of which

bear some resemblance to those of *Chlamydomonas*. Chromosome numbers in this group are consistently low, ranging from 5 in several species of *Volvox* to 17 in *Gonium pectorale*. No essential variation in the mitotic cycle was observed among these species, and it may be summarized as follows. At the beginning of prophase, small chromatinic bodies appeared throughout the nucleus. The nucleolus was well stained, but became fainter as prophase progressed. The attenuated chromosomes, of which the chromatinic bodies are a part, shortened and thickened, reaching maximum contraction at metaphase, where they became aligned on the equator. Centrioles were not observed. The aceto-carmine technique, which Cave and Pocock used exclusively, failed to reveal the presence of spindle fibers. The nuclear membrane disappeared before metaphase and reappeared with the formation of daughter nuclei. Telophase chromosomes stained heavily, but rapidly lost their stainability as the nuclei proceeded toward interphase. After the nucleus had divided, nucleoli reappeared in the daughter nuclei, and cytokinesis followed. Cave and Pocock concluded that mitosis in these species is similar to that in higher plants.

Schaechter and DeLamater (1954, 1955) studied mitosis in 4 species of *Chlamydomonas,* reporting chromosome numbers as follows:

C. moewusii Gerloff	36 ± 2.
C. eugametos Moewus	36 ± 4.
C. reinhardti Dangeard[3]	18 ± 2.
C. dysosmos Moewus	16 ± 1.

They found the pattern of mitosis to be similar in all 4 species and essentially like that in most plants. Details of the process are described briefly; nothing is said of the fate of the nuclear membrane, nucleolus or spindle fibers, nor is the presence of centrioles reported.

These investigators also studied meiosis in *C. moewusii* (1954, 1956). They described configurations which are suggestive of diplotene loops, the number of which appeared to be on the order of 8 to 10. In view of the difficulty of reconciling this number with their previous haploid chromosome count of 36 ± 2, these authors suggest that meiosis in this organism cannot be explained according to a classic pattern, or if it does occur as such, that the difficulty of resolving such small chromosomes precludes a correct interpretation.

In a study of polyploidy in *C. eugametos* and *C. reinhardti,* Wetherell and Krauss (1956) reported the chromosome number to be 16 in *C. reinhardti.*

Materials and methods. Stock cultures of three of the four organisms used in this study, *C. eugametos* Moewus, *C. reinhardti* Dangeard and *C.*

[3] Trainor (1957) states that the correct specific epithet is *"reinhardti,"* not *"reinhardi"* as usually given.

moewusii Gerloff, were maintained in bacteria-free cultures on slants of Knop's agar, compounded as follows:

10% $Ca(NO_3)_2$	10 ml.
5% KNO_3	5 ml.
5% $MgSO_4 \cdot 7H_2O$	5 ml.
5% KH_2PO_4	5 ml.
Distilled water	1575 ml.
Agar-agar	24 gm.

C. chlamydogama Bold, which has been shown to be deficient for vitamin B_{12} (Hutner *et al* in Lwoff 1951, Bell 1953), was grown successfully on Knop's agar supplemented with soil extract. In some cultures of *C. chlamydogama* vitamin B_{12} was supplied as "Cobione" (Merck and Company) at the rate of 15 micrograms/150 ml. of Knop's agar.

Cultures were grown at temperatures of 19° to 22° C. Except where otherwise specified, illumination was at an intensity of 800 foot-candles produced by a battery of "daylight" fluorescent bulbs which burned continuously. Stock cultures were maintained, however, at 100 foot-candles for 12 hours daily.

In preparing cultures of organisms for staining, the following method was employed. Cultures were grown for three days on appropriate media, at the end of which a quantity of material was transferred and spread over the same medium in fresh culture dishes. The maximum number of division figures was obtained in all species at 8–16 hours' illumination after such transfer.

Glass slides were cleaned in a solution of acid alcohol, wiped dry, and one end of each slide was smeared with egg albumin. After drying, a few drops of one of the fixatives described below were placed upon the end of the slide, into which cells were introduced, having been removed from the culture plate by a platinum loop or edge of a cover glass. When the cells were just at the point of drying, the slide was immersed in a Coplin jar of the same fixative into which the cells had first been introduced.

Because the small size of the chromosomes of these species necessitates extreme care in preparation and observations, four different techniques were employed in the preparation of material of nuclear and cell division. These were the iron-alum haematoxylin, Feulgen, Azure A (DeLamater 1951) and aceto-carmine techniques. Two modifications of the last named method were developed for use in the present work. The first, which is a modification of the technique developed by Cave and Pocock (1956) as applied to the colonial Volvocales, was used whenever cellular details were to be studied. The fixative, which Cave and Pocock modified from Johansen (1940), is prepared as follows:

Iodine	0.5 gm.
Potassium iodide	1 gm.
Acetic acid, glacial	4 ml.
Formalin	24 ml.
Distilled water	400 ml.

Slides were allowed to remain in the fixative for at least 15 minutes to about 5 days; after longer periods in the fixative the cells did not respond satisfactorily to further treatment. Upon removal, excess fluid was drained from the slide, two drops of aceto-carmine prepared according to the method described by Cave and Pocock (1951b) were placed upon the area occupied by fixed cells, a cover glass was gently lowered, and the slide heated over a low flame. It was found that the method of heating followed by Cave and Pocock (1951b), namely, allowing the fluid to reach almost the boiling point, did not result in stained chromosomes in any of the species of *Chlamydomonas* which were utilized. Staining was inadequate unless the fluid beneath the cover glass was actually boiled. In utilizing this method, great care must be taken to prevent overstaining; the exact and delicate point to be reached between under-staining and over-staining was learned in the present study only after much trial and error.

The second aceto-carmine method which was developed for use in the present work is an adaptation of the technique developed by Ray (1956) in staining *Tetrahymena* cells. While this method brought out few cellular details, it was superior in the staining of chromosomes to the other aceto-carmine method described above. Thus the two methods supplemented each other admirably in the present work.

Since considerable modification of Ray's method was required, it is felt that a detailed account of it also should be given. In fixing cells, a drop of distilled water was transferred to a glass slide, into which were deposited a quantity of cells which had been scraped from the surface of a plate by means of a clean cover glass. These were distributed as evenly as possible within the drop of water. Next, a small drop of Nissenbaum's Fluid (Nissenbaum 1953) was placed upon the suspension of cells and thoroughly mixed with a dissecting needle. As soon as evaporation almost to the point of dryness had occurred, the slide was immersed in a Coplin jar of Carnoy's 1:3 acetic acid-absolute ethyl alcohol. Primary fixation in Carnoy's solution did not prove to be quite as satisfactory as did the use of Nissenbaum's Fluid.

Glass slides containing cells fixed by this method were stored in Carnoy's solution for 1–24 hours, after which time they were transferred to 70% ethyl alcohol, where they could be left indefinitely. Upon carrying the slides to completion, they were transferred to 50%, 30% and 15% ethyl alcohols,

and then into water. Hydrolysis then was accomplished by placing the slides in 1-normal hydrochloric acid in the following series: 2 minutes at room temperature, 6 minutes at 60° C., and 2 minutes a second time at room temperature. Following this treatment, the slides were washed in at least 5 changes of distilled water within a period of 5 minutes.

Slides then were transferred to 45% acetic acid, where they were allowed to remain for 5–7 minutes. Following this treatment, they were removed to a Coplin jar containing aceto-carmine stain, where they were stored for

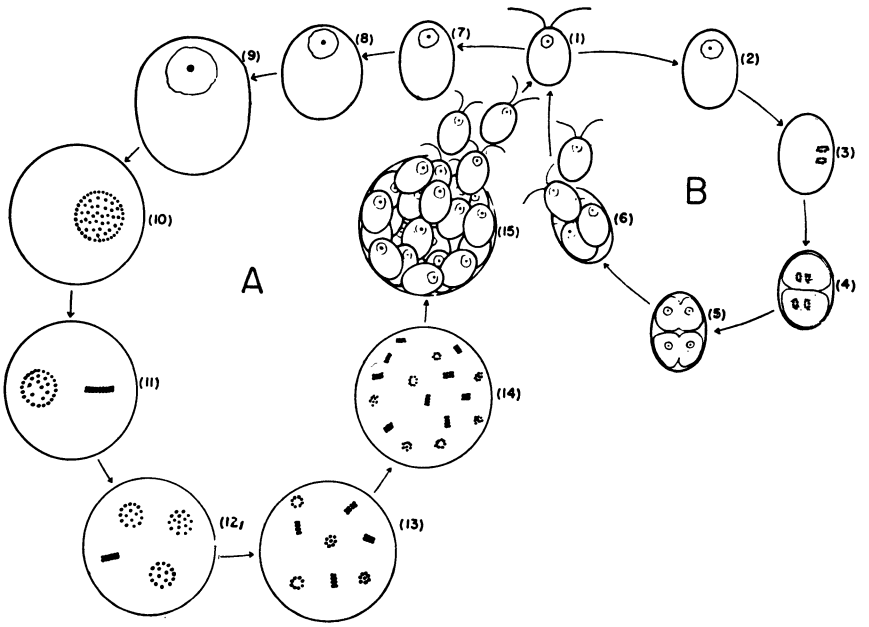


FIG. 1. Diagram to illustrate the effects of light intensities of 800 as compared with 100 foot-candles on *Chlamydomonas eugametos* and *C. moewusii*. The cycle at A (1, 7–15) occurs at 800 foot-candles and that at B (1–6) at 100 foot-candles or less. The cycle at B reaches stage (3) approximately 4 hours after the cultures are inoculated. Note relatively small size of cell at inception of division (3) as compared with a similar stage (10) in cycle A. In the cycle at B, 8 chromosomes are visible at each mitosis. In cycle A, nuclear and cell division are delayed through a period of 8–12 hours during which cell enlargement and endomitosis (presumably) occur. Note the progressive decrease in chromosome number at successive mitoses between (10) and (14) and formation of 32 cells (15) as compared with 4 or 8 at 100 foot-candles (6). See text for amplification.

at least 4 hours to obtain adequate staining; 4–8 hours in the stain proved to be ideal. If slides were allowed to remain in the stain for a longer time than this, the cells tended to become overstained. However, good results could be obtained from slides which had been in the stain for several days if, before being heated, they were removed to 45% acetic acid for a period which had to be determined experimentally, a period proportional to the

time the slide had been in the stain. This varied in the present work from about 1 to 8 hours.

Following the 4–8 hour period of staining, or after the slides had been placed in 45% acetic acid for an appropriate length of time as described above, they were removed individually for heating. Excess stain was wiped from the slide, an additional drop of aceto-carmine was placed on the area occupied by the cells, and a cover glass gently lowered. Heating did not need to be as intense as in the modified Cave and Pocock method described above, but gentle boiling for an instant usually was necessry.

In employing both of these methods, it was found that best results were obtained from a study of cells which had been freshly stained, and all drawings and photographs which were made in connection with the present study were made from such material. Preparations could be rendered permanent with a certain degree of success, however, by following the method of Manton (1950).

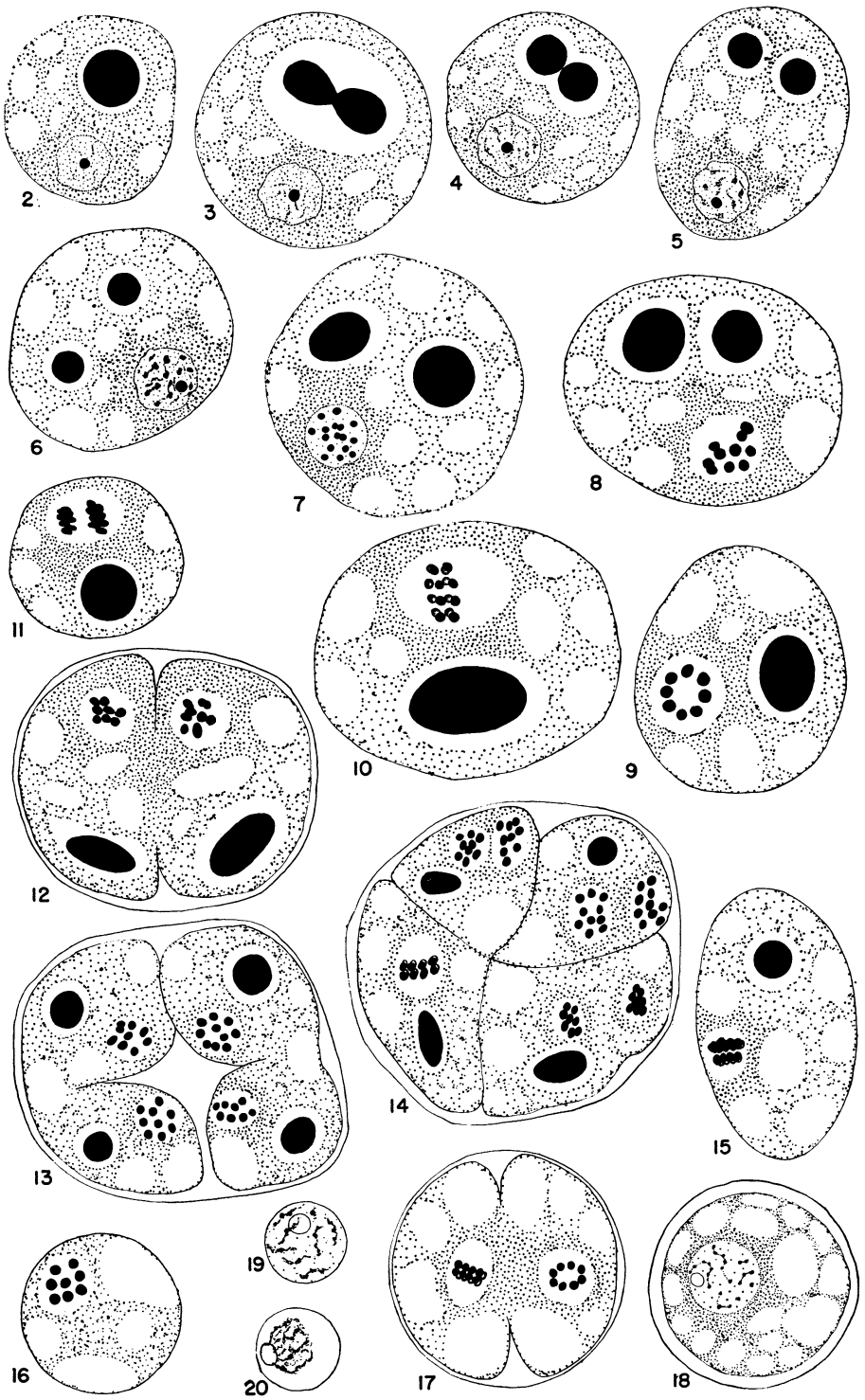
Fixation of cells preparatory to staining by the Feulgen and Azure A techniques was accomplished by means of Carnoy's solution, while Schaudinn's Protozoological Fluid, compounded as follows, was employed in connection with the iron-alum haematoxylin method:

Saturated aqueous $HgCl_2$	200 ml.
Ethyl alcohol, 95%	100 ml.
Acetic acid, glacial	9 ml.

For the study of meiosis, zygospores of the 4 species were obtained by mixing opposite mating types of each species under optimal conditions for the sexual reaction in each case which have been defined by Trainor (1957). Conditions under which these zygospores would germinate were found to vary considerably. Those of *C. chlamydogama* and *C. reinhardti* responded favorably to the method of Starr (1949); those of *C. moewusii* germinated readily when they were produced within the limits of initial illumination which have been stated by Lewin (1949), and when they were kept in the dark according to his methods. Although zygospores of *C. eugametos* were treated in a variety of ways, a very low percentage of germination was obtained. The Starr method resulted in only about 10% germination, but it proved to be the best that was tried.

In the staining of zygotes, only the iron-alum haematoxylin and Azure A methods, of the four listed above, were employed. Neither of the variations of the aceto-carmine technique, used so successfully with vegetative cells, could be satisfactorily adapted to the staining of zygotes.

Comparative cytology of vegetative cells. Cytokinesis is accomplished in all 4 species by furrowing (figs. 12, 17), the first division always occurring at a 90° angle to the longitudinal axis of the cell. Observations on living



cells of the 4 species reveals that the protoplast turns 90° within the cell wall just prior to the first nuclear division, a phenomenon also described by Kater (1929) for *C. nasuta* and by Akins (1941) for *Carteria crucifera*. The second division generally occurs at right angles to the first (fig. 13). It seems evident that cytokinesis begins most readily in areas where the cytoplasm is least dense, and since this often is true in the central portion of the cell, the process may begin there and at the periphery of the cell simultaneously.

There is no exact synchrony between division of the nucleus and division of the pyrenoid. Within a given population, some cells may be seen whose pyrenoids are dividing but whose nuclei are not (figs. 3, 35) or the reverse may be true (fig. 11). In general, it was observed that in cells which are grown at a light intensity of 100 foot-candles, division of the pyrenoid precedes nuclear division (figs. 2–8), while in cells which are subjected to a light intensity of 800 foot-candles nuclear division precedes division of the pyrenoid (figs. 9–11). In the formation of daughter cells, nuclear and pyrenoidal divisions eventually become adjusted so that before each cytokinesis, nuclei and pyrenoids always lie near each other in pairs (fig. 13).

Observation of the behavior of the stigma during division of living cells revealed that one daughter cell retains the original stigma, and the other division products develop theirs *de novo*.

Cytokinesis usually is initiated between the two contractile vacuoles, one of the latter being transmitted to each new daughter cell. Although the process was not observed, it is assumed that each cell develops an additional contractile vacuole, as two are apparent very shortly after division. The details regarding behavior of the pyrenoid, stigma and contractile vacuoles during cell division are essentially similar in the 4 species studied.

Preliminary observations indicated that *C. reinhardti* was most favorable for the study of mitosis, chiefly because greater numbers of division

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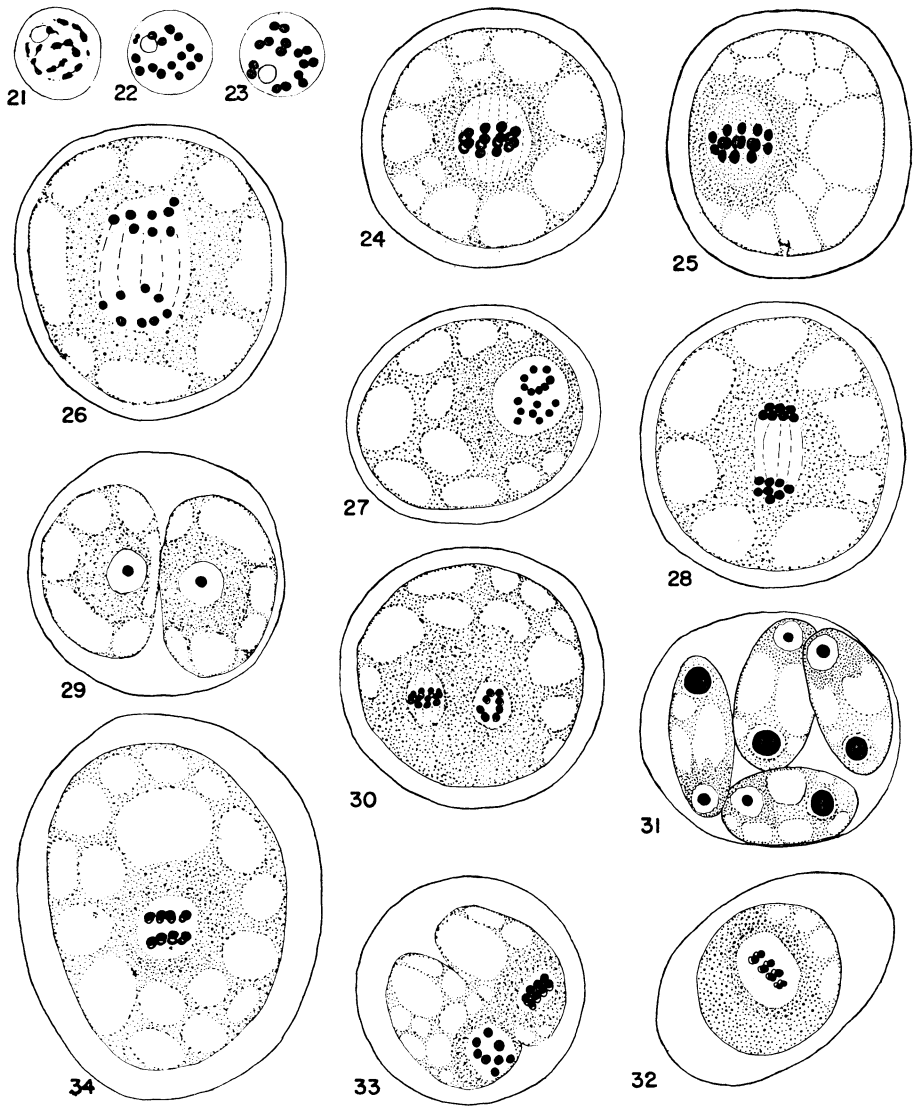
FIGS. 2–14. *Chlamydomonas reinhardti*. FIGS. 15, 18–20. *Chlamydomonas chlamydogama*. FIG. 16. *Chlamydomonas eugametos*. FIG. 17. *Chlamydomonas moewusii*. FIGS. 2–17. Mitosis. FIGS. 2–8. Nuclei in successive stages of prophase showing condensation of chromatin into chromosomes, division of pyrenoid. FIG. 9. Nucleus in metaphase, 8 chromosomes in ring formation (polar aspect); pyrenoid undivided. FIGS. 10, 11. Anaphasic separation of two rings of 8 chromosomes. FIG. 12. Late prophase of second division. FIG. 13. Late prophase of third division. FIG. 14. Late prophase of fourth division exhibited by 6 nuclei, one nucleus still in early anaphase of the previous division. FIG. 15. Anaphasic separation of two rings of 8 chromosomes. FIG. 16. Late prophase, one chromosome yet unoriented in ring. FIG. 17. Metaphase of second division, 8 chromosomes in ring formation in each nucleus. FIGS. 18–20. Meiosis; nuclei in successive stages of prophase showing condensation of chromatin. FIG. 20. Chromatin condensed into synizetic knot (compare Figs. 55, 56). FIGS. 2–17. drawn with aid of camera lucida from aceto-carmine preparations; FIGS. 18–20. drawn from iron-alum haematoxylin preparations. All figures × 3035.

figures could be obtained in cells of this species than in others. This may be an indication that the process of mitosis requires more time in cells of *C. reinhardti* than in cells of *C. chlamydogama, C. eugametos* and *C. moewusii. C. reinhardti* proved to be a fortunate choice, not only for this reason, but also because there is no complicating phenomenon evoked at high light intensity such as appeared later in connection with cells of *C. eugametos* and *C. moewusii,* a feature which is to be described in detail later in this report.

The mitotic process is illustrated for *C. reinhardti* in figures 2–14 and figures 36–39. The interphase nucleus (fig. 2) is approximately 3 μ in diameter and exhibits one nucleolus. Azure A-positive granules are scattered about the nucleus or organized into short thread-like groups (fig. 2). During early prophase (figs. 3–5), these become organized into indefinite groups, which eventually condense in later prophase (fig. 6) into scattered bodies which are loosely connected by threads. Still later (fig. 7), a number of bodies, approximately twice the haploid chromosome number, are seen within the nucleus. Meanwhile, the nucleolus disappears (fig. 7) as does the nuclear membrane (fig. 8) and the condensing chromatinic bodies lie within a clear area, about which there is an accumulation of dense cytoplasm. At late prophase (fig. 8), it is common to observe 8–12 large bodies, but upon final condensation at metaphase, a ring of 8 chromosomes[4] is seen in polar aspect (figs. 9, 37, 38). It seems probable that a condensation process occurs between the prophase stage represented by figure 7 and metaphase as illustrated by figure 9, and that the variable number of "chromosomes" as seen at late prophase (fig. 8) is due to a lag in condensation on the part of some of these bodies. Figure 36 shows a cell of *C. reinhardti* with two nuclei in very late prophase of the second division, that to the left exhibiting a ring of 7 chromosomes with an eighth yet in the center, while the other nucleus, which is not in exact focus in this illustration, exhibits about 10 bodies. Figure 39 shows a cell whose nucleus is in anaphase of division, 4 chromosomes being seen in each separating group in this lateral view. There are, of course, 4 other chromosomes in each group which are not visible in this focus (fig. 10).

In cells which are grown at a light intensity of 800 foot-candles, cytokinesis usually lags somewhat behind mitosis; in figure 11, there is no evidence that cytokinesis has begun, even though the nucleus is in anaphase of division, and in figure 12, in which the two nuclei are in late prophase, cytoplasmic division following the first mitosis is just beginning. This same

[4] Although the writer is reasonably certain of the chromosome numbers, as stated in this paper, their extremely minute size makes it impossible to be absolutely sure. Therefore, when any number is stated, it should be considered the approximation which can be made under the conditions of observation. In any case, the error would be in the range of 1 or 2, at most.



FIGS. 21–31. *Chlamydomonas chlamydogama.* FIG. 32. *Chlamydomonas moewusii.* FIG. 33. *Chlamydomonas reinhardti.* FIG. 34. *Chlamydomonas eugametos.* FIGS. 21–34. Meiosis (continued). FIG. 21. Chromatin condensing into connected pairs of bodies. FIG. 22. Late prophase, paired chromatinic bodies no longer visibly connected. FIG. 23. Diakinesis, 8 bodies visible. FIG. 24. First metaphase, lateral aspect; chromosomes arranged in ring of 8 on spindle. Compare with figure 59. FIGS. 25, 26. Anaphasic separation of 2 rings of 8 chromosomes. FIG. 27. Anaphase, 2 rings of 8 chromosomes slightly flattened. Compare with figure 61. FIG. 28. Telophase. FIG. 29. End of first division, cytokinesis completed. FIGS. 30, 31. Second division. FIG. 32. Metaphase of first division showing ring of 8 double chromosomes. FIG. 33. Metaphase of second division, nucleus at upper right in lateral aspect showing ring of 8 chromosomes; nucleus at lower left in polar aspect, ring of 8 chromosomes slightly crushed and distorted. FIG. 34. Anaphase of first division, lateral aspect, showing 2 separating rings of 8 chromosomes. Compare with figure 62. Camera lucida drawings made from iron-alum haematoxylin preparations. All figures $\times 3035$.

sort of lag is seen in figures 13 and 14. Eventually, a new cell wall is formed about each daughter cell, the old wall breaks open, and each new cell escapes. In *C. reinhardti,* this may occur at the 2-, 4- or 8-celled stages, and rarely at the 16-celled stage. Characteristically, 4 daughter cells form in *C. chlamydogama,* while as many as 32 may be produced in *C. eugametos* and *C. moewusii.*

Mitosis, as observed in dividing cells of *C. chlamydogama,* proved to be essentially similar to that described and figured for *C. reinhardti,* and the same chromosome number was observed (figs. 15, 40, 41). The chromosomes were quite similar in size and shape to those of *C. reinhardti.*

While no essential differences were observed in the mitotic process in cells of *C. eugametos* and *C. moewusii* as compared with that of *C. reinhardti,* the basic haploid number being 8 (figures 16, 17, 42, 44, 45, 51), the writer was at first somewhat perplexed to discover certain cells, in both species, in which there appeared metaphase plates with approximately 16 chromosomes and still others with even more (figs. 43, 46–50). That these were true metaphases and not prophases in which maximum condensation had not occurred was shown by the fact that the plates were flat, not rounded, as would have been the case in prophase. This was seen to be true by focusing and also by observing cells which, when prepared and stained according to the modified aceto-carmine technique of Ray, could be made to change position upon the slide by gentle tapping. In the lateral aspect, such metaphase or anaphase plates appeared as broad lines similar to those illustrated in figure 46. In polar aspect, the same nuclei exhibited 16, 32 or possibly more chromosomes. These chromosomes were not arranged in a hollow ring, but as a solid plate (fig. 50).

It already had been observed, before studying mitosis, that cells of all 4 species, when grown at 800 foot-candles, behaved differently from those grown at 100 foot-candles. If, for example, a culture of *C. moewusii* was grown upon a Knop agar slant at 100 foot-candles until all cells were uniform in size, and if such cells then were spread upon Knop agar plates, some of them maintained at 100 foot-candles and others at 800 foot-candles, distinct differences became observable. At 100 foot-candles, cells could be seen to undergo a period of growth of about 4 hours' duration, after which widespread division was in evidence. However, cells did not appear to reach a size exceeding twice their original volume before dividing. At 800 foot-candles, no division was evident at all before 10 hours' growth under the new conditions, and most cells did not divide before 15 hours. Some cells failed either to grow or divide. Figures 52–54 show cells of *C. moewusii* which enlarged in the manner indicated; the extent of growth of the large cells thus may readily be seen.

With some minor variations, this pattern was observed in all 4 species.

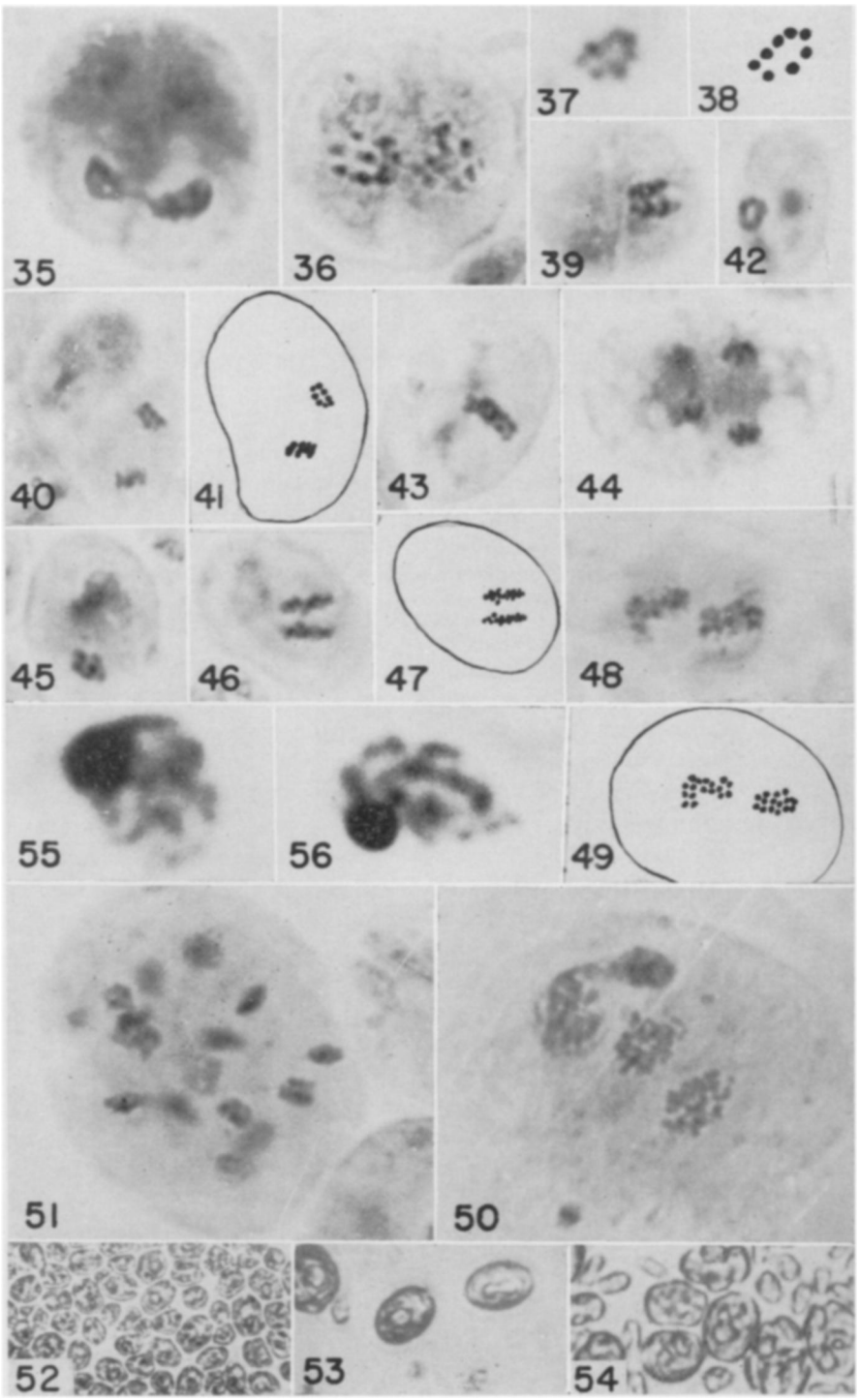
Furthermore, a direct correlation was established between light intensity and the size which cells attained befare dividing, by growing cells of all species at 100, 200, 400, 600 and 800 foot-candles. In general, it was observed that the higher the intensity, the longer cells remained undivided and the larger they became. Cells of *C. eugametos, C. chlamydogama* and *C. reinhardti* showed no more marked response to 800 foot-candles than to 600, whereas cells of *C. moewusii* did, becoming larger before division and taking longer to initiate the division process than did cells of other species as long as they were maintained in the light.

Cells of all species, when placed in total darkness following intense light treatment, immediately began to divide, indicating that light intensities which produce unusually large cells provide an artificial block to mitosis and cytokinesis; the continued synthesis of materials within the cell causes it to grow to an unusual size when in such light, division seemingly being inhibited by a disturbance of balance between the rate of synthesis and the production of division-initiating substances within the cell.

In view of the fact that those nuclei which exhibited about 16 or more chromosomes in *C. eugametos* and *C. moewusii* usually were observed in comparatively large cells, and because they were seen at the earliest divisions of these cells as they progressed toward the production of 8, 16 and 32 daughter cells, the following hypothesis suggested itself: a light intensity of 800 foot-candles, while inhibiting cell division for several hours, induces the cells of these two species to undergo endomitosis, so that polyploidy is exhibited at the first division. This hypothesis suggested that somatic reduction would occur with the formation of daughter cells.

The possibility could not be ruled out, however, that genetically stable polyploid strains had arisen in these species. Because the original strains of each organism maintained as stock cultures were clonal, having been produced from single-cell isolates made at the beginning of the present study, the possibility of the existence of haploid and polyploid strains within the stocks seemed highly unlikely, unless such strains had arisen independently within stock cultures after the original isolations. Furthermore, such an event would have had to occur 4 times independently, since the phenomenon of chromosomal multiplication was observed in both mating types of *C. eugametos* and *C. moewusii.*

Nevertheless, in order to rule out such a possibility altogether, new isolations were made in the following manner. Cells of each mating type, taken from cultures which had been maintained for many generations at low light intensity, and which had never been subjected to high light intensities, were spread on Knop agar plates. Two plates, one containing one mating type of *C. eugametos* and the other one mating type of *C. moewusii,* were grown at 800 foot-candles. Two plates containing the opposite mating



type in each case were grown at 100 foot-candles. After 18 hours' growth, 8 isolations were made from each dish, single cells being picked up and transferred to small tubes with sterile medium. In the case of those cells which had been grown in the brighter light, an attempt was made to isolate as large cells as possible, since it had been indicated that it was within such large cells that the higher chromosome numbers could be found.

Within 10 days, growth was evident in most of the tubes. One tube was selected from each of the 4 types of cells which were isolated, cells were concentrated by centrifugation, and spread upon Knop agar plates. These then were exposed to a light intensity of 800 foot-candles. Within 12 hours, a similar situation to that shown in figure 53 was evident in each of the 4 plates. This was considered presumptive evidence against the hypothesis that stable polyploid strains were involved from the beginning. Fixation and staining of these cells revealed in all four cases the presence of nuclei some of which exhibited the haploid number (8) and others of which were characterized by larger numbers at metaphase and early anaphases. On the basis of this experiment, therefore, the phenomenon of chromosomal multi-

FIGS. 35–39. *Chlamydomonas reinhardti.* FIGS. 40, 41, 55, 56. *Chlamydomonas chlamydogama.* FIGS. 42–45. *Chlamydomonas eugametos.* FIGS. 46–54. *Chlamydomonas moewusii.* FIG. 35. Division of pyrenoid. × 2650. FIG. 36. Two nuclei in prophase, that at left showing ca. 7 chromosomes in a ring; the 8th is yet unoriented. Right-hand nucleus not in exact focus; 10–12 fodies in late prophase. × 2650. FIG. 37. Nucleus in metaphase (polar aspect) showing ring of 8 chromosomes. × 5500. FIG. 38. Interpretive drawing of figure 37. × 5500. FIG. 39. Second anaphase (only 1 nucleus visible) in lateral view. Four chromatids visible in each separating ring of 8. × 2650. FIG. 40. Lateral aspect of two nuclei in late anaphase showing two separating rings of 8 chromosomes; note lateral position of division figure effected by earlier migration of nucleus. × 2650. FIG. 41. Interpretive drawing of figure 40. × 2650. FIG. 42. Polar aspect of metaphase showing ring of ca. 8 chromosomes. × 2650. FIG. 43. Lateral aspect of polyploid nucleus in early anaphase; compare width of spindle with that of division figure in figure 42. × 2650. FIG. 44. Two haploid nuclei at late anaphase, portion of spindle visible in nucleus at the right. × 2650. FIG. 45. Cell with 2 nuclei (only 1 in focus), ca. 8 chromosomes visible in late prophase. × 2650. Fig. 46. Cell showing polyploid nucleus at anaphase. × 2650. FIG. 47. Interpretive drawing of figure 46. × 2650. FIG. 48. Two polyploid nuclei in metaphase, plates of chromosomes slightly tilted and distorted. × 2650. FIG. 49. Interpretive drawing of figures 48. × 2650. FIG. 50. Cell with 4 polyploid nuclei at metaphase, the lower 2 of which are seen as flat plates containing many chromosomes; upper 2 not in focus. × 2650. FIG. 51. One focus of cell whose 16 nuclei are dividing to form 32. Note small ("reduced") number of chromosomes exhibited by division figures. × 2650. FIG. 52. Actively growing vegetative cells immediately following transfer to Knop agar and prior to exposure to a light intensity of 800 foot-candles. × 530. FIG. 53. Living cells from the same plate as those shown in figure 52 after growth at 800 foot-candles of light intensity for 12 hours. × 530. FIG. 54. Living cells from the same plate as those shown in figures 52 and 53 after growth at 800 foot-candles of light intensity for 15 hours. Cells in all stages of division to produce daughter cells. × 530. FIG. 55. Nucleus of germinating zygote approaching synizesis with chromatinic threads oriented; note large nucleolus. × 11,000. FIG. 56. Post-synizetic nucleus of germinating zygote, chromatinic threads loosening. × 11,000. FIGS. 35, 36, 39–51. Aceto-carmine preparations. FIG. 37. Azure A preparation. FIGS. 55, 56. Iron-alum haematoxylin preparations.

plication was shown to be a light effect, and not a genetically controlled phenomenon.

At first metaphase in such enlarged cells of *C. reinhardti* and *C. chlamydogama,* there proved to be no disturbance of chromosome number, that is, 8 chromosomes could be seen as a hollow ring in polar view at any given metaphase. However, in cells of *C. eugametos* and *C. moewusii,* the first metaphase of such large cells always exhibited more than 8 chromosomes. In such cells, approximately 16, 32 or 64 were observed. Subsequent division of the original large cell to form 2, 4, 8, 16 or 32 daughter cells invariably was accompanied by a reduction in chromosome numbers (fig. 51). This reduction occurred regardless of whether daughter cell formation occurred at a light intensity of 800 foot-candles, one of 100 foot-candles, or in total darkness.

Although a direct correlation was not always observed between cell size and the degree of polyploidy, it was generally true that large cells produced at high light intensities were polyploid, and that small cells were not. However, some cells which were relatively quite small were seen to exhibit more than 8 chromosomes at first division (figs. 46, 47).

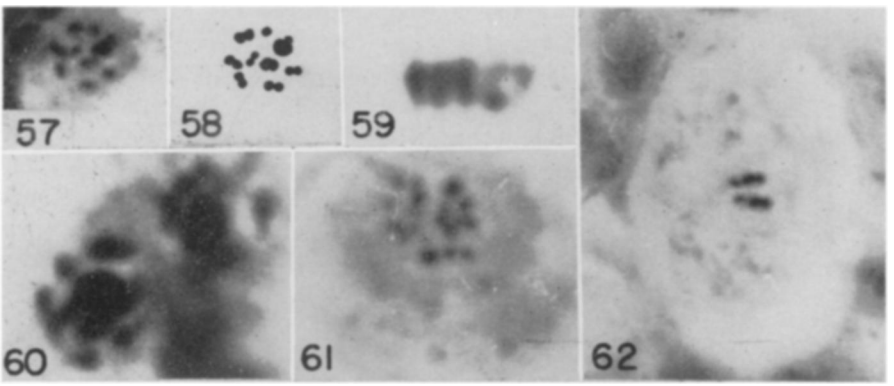
The phenomena of endomitosis and somatic reduction as they apparently occur in vegetative cells of *C. eugametos* and *C. moewusii* are illustrated diagramatically by figure 1. Upon a given plate of Knop agar, when maintained at 800 foot-candles of continuous illumination, these phenomena are accomplished repeatedly by the enlargement of small cells initially spread upon the surface of the medium, production by these of haploid daughter cells, their enlargement again accompanied by endomitoses, and so on, until crowding of cells upon the plate obviates a further manifestation of these phenomena. Observation of such a plate upon which a solid growth of cells is seen, usually evident by 72 hours if the initial inoculum was fairly heavy, reveals the fact that all cells are small in size. If these, in turn, are spread upon another plate, the increase in cell size and polyploidy again become evident.

Meiosis. Details of the meiotic process as studied in germinating zygotes of each species proved to be essentially similar; because preparations of *C. chlamydogama* exhibited the greatest number of critical division stages and were in general more revealing than those of the other species, the process will be described in detail as it occurs in that organism.

During the time between formation of the mature zygote and initiation of prophase of the first meiotic division, a thick wall develops, from which the zygotic protoplast appears to be shrunken in fixed and stained preparations (fig. 18). This shrinkage is no doubt due to the slow penetration of the fixative through this wall, as living zygospores do not exhibit this characteristic. The zygospore wall also proves to be impermeable to stains.

With the initiation of meiosis, however, a change in permeability occurs, and the nuclear stains used in this study enter the cell readily. It is not until after the formation of 4 daughter cells, however, that fast green counterstain is able to penetrate the wall readily.

At early prophase, a number of chromatinic bodies may be seen within the nucleus, these being connected by strands (fig. 18). This material becomes more and more condensed (fig. 19), and eventually a synizesis-like pattern is evident (figs. 20, 55, 56). Further condensation results in a number of bodies many of which are connected (fig. 21). At a slightly later stage, these bodies, which now number approximately 16, no longer give evidence of being connected (fig. 22). However, a stage next occurs



FIGS. 57–61. *Chlamydomonas chlamydogama.* FIG. 62. *Chlamydomonas eugametos.* FIG. 57. Diakinesis in germinating zygote. ×5500. FIG. 58. Interpretive drawing of figure 57. FIG. 59. Metaphase of first meiotic division of zygote, lateral aspect. Four pairs of chromosomes separating with 4 others behind (not visible). Separating pair at right slightly crushed apart from the others. ×11,000. FIG. 60. Late prophase of second meiotic division in germinating zygote. Note ca. 8 chromosomes and nucleolus in nucleus at left. ×11,000. FIG. 61. Anaphase of first meiotic division, separating rings of 8 chromosomes slightly crushed. See figure 27 for interpretive drawing. FIG. 62. Anaphase of first meiotic division showing 2 separating rings of ca. 8 chromosomes in lateral aspect. See figure 34 for interpretive drawing. All figures from iron-alum haematoxylin preparations.

in which bodies are somewhat larger and clearly paired, this stage being diakinesis (figs. 23, 57, 58). After this, a metaphase ring of 8 double chromosomes become oriented upon a spindle whose fibers are clearly evident with the technique employed (figs. 24, 59). Anaphasic separation reveals the movement of 8 chromosomes toward each pole (figs. 25–28, 61), the telophase stage also revealing approximately this same number (fig. 28). Two daughter cells form within the original zygote wall (fig. 29), the second meiotic division occurs (figs. 30, 60) and the 4 daughter cells within the original wall (fig. 31) constitute the final meiotic products (in *C. chlamydogama*).

The patterns of meiosis as observed in *C. reinhardti, C. eugametos* and *C. moewusii* appear to be identical to that described for *C. chlamydogama.* Figures 32–34 and 62 show representative stages of meiosis as it was observed in these species.

Spindle fibers were clearly observed in several mitotic figures of all species when cells were stained by the modified Cave and Pocock aceto-carmine method and in meiotic figures of cells prepared by the iron-alum haematoxylin technique. No centrioles were observed in cells of any species.

Discussion. The fact that the haploid chromosome number of all 4 species of *Chlamydomonas* studied proved to be n = 8 suggests the possibility that all species of the genus exhibit a relatively low number. It has been pointed out that both Bêlaî (1926), working with an unidentified species of *Chlamydomonas,* and Kater (1929), who studied *C. nasuta,* reported a chromosome number of 8 in each species. Dangeard (1899) reported the number of *C. variabilis* and *C. dilli* to be n = 10. While the writer disagrees with Moewus' (1936) count of 10 chromosomes in *C. eugametos,* it is evident that both it and *C. paupera,* which he states are interfertile, exhibit a low number.

It is felt that Schaechter and DeLamater's (1955) counts of 36 ± 2 for *C. moewusii,* 36 ± 4 for *C. eugametos,* 18 ± 2 for *C. reinhardti* and 16 ± 1 for *C. dysosmos* are open to question. In the first place, these authors apparently were not aware of the light effect described above which results in temporary polyploidy in cells of *C. moewusii* and *C. eugametos.* While they do not state the light intensity which they employed, their figures of preparations made from cells of these two species give every indication that they made their counts from such polyploid cells. A second factor which may have given rise to error in their work lies in the fact that nuclei which are in earlier prophase exhibit a number of chromatinic bodies which exceeds that of the chromosomes at metaphase and anaphases, a phenomenon which was also observed by Dangeard (1899) and Kater (1929). The technique of Schaechter and DeLamater consisted of squashing cells apparently before they examined them microscopically; in such preparations, it would be difficult to determine with certainty the actual stage of division of any given nucleus. Furthermore, squashing may tend to separate chromatids at late prophase or metaphase, and it has been pointed out by Steere, Anderson and Bryan (1954), in their work on the chromosomes of mosses, that in cells which have been strongly flattened, first anaphase or second metaphase plates may flow together and appear as a single figure, thus presenting a misleading picture. While the squash technique is a standard procedure in cytological work, counts made in this fashion in cells of *Chlamydomonas* are somewhat suspect for the reasons given.

From a critical examination of Schaechter and DeLamater's figures, it

seems evident that a combination of these factors may be responsible for their interpretations of chromosome number in *C. moewusii* and *C. eugametos*. Their photographs and interpretive drawings of the chromosomes of *C. reinhardti* are somewhat more difficult to reconcile with counts which were made in connection with the present study, but there seems to have been a certain degree of crushing apart of chromatids. It is quite possible that their two photographs (18, 20) of *C. reinhardti* chromosomes, presenting 18 and 19 bodies respectively, represent late prophase figures in which full condensation had not occurred in the case of some of the bodies, while others were sufficiently advanced that the squashing apart of chromatids could occur. The writer has observed many cells of *C. reinhardti* at late prophase in which 10–12 bodies could be seen; the right-hand nucleus in figure 36 is an example of this.

In their observations on the meiotic chromosomes of *C. moewusii,* Schaechter and DeLamater (1954) reported "the number of loops and of diakinesis-like configurations," in the first meiotic division, to be "in the order of 8 to 10." However, they later (1956) suggest "the possibility that the mechanism of meiosis in this organism is of some aberrant form" since the counts of 8 to 10 did not correspond to their report of 36 ± 2 in vegetative cells of this species which they had made previous to studying meiosis.

Krauss and Wetherell (1956) have reported the chromosome number of *C. reinhardti* to be 16, and they have published photographs described as "metaphase," showing approximately this number of bodies. However, in view of the spherical form of the figure, which required photographs at three focal levels to include all the chromatinic bodies, the nucleus photographed seems to be in prophase, and it is probable that they counted bodies which had not yet condensed into chromosomes.

The only other case of which the writer is aware in which a species of *Chlamydomonas* has been reported as exhibiting more than a haploid chromosome number of 10 is Dangeard's (1899) count of 30 in *C. monadina.* It is possible that this species is subject to the same light effect as *C. eugametos* and *C. moewusii,* reported in this paper, and that this effect escaped Dangeard. One of his figures shows a second division in a vegetative cell in which one of the nuclei is in early anaphase, as seen in lateral aspect. The other nucleus apparently is in metaphase and exhibits a polar aspect of a flat plate of chromosomes. These figures are strikingly similar in appearance to comparable stages in *C. eugametos* and *C. moewusii* when cells of these two species are still polyploid.

In the transformation from small to large cells under the influence of high light intensities, the species of *Chlamydomonas* studied show a remarkable parallel to the behavior of *Chlorella ellipsoidea,* where "dark

cell'' and ''light cell'' forms have been described (Tamiya, Iwamura, Shibata, Hase and Nihei 1953). It seems likely that the mechanisms responsible for this parallel behavior are the same. An extension of the PNA and DNA determinations of Iwamura (1955) on *Chlorella ellipsoidea* to *Chlamydomonas eugametos* and *C. moewusii,* especially, should go far toward revealing the degree of similarity involved.

That light may be an important factor in stimulating or inhibiting degree of polyploidy is clear from the results of v. Witsch and Flügel (1951). Dividing nuclei of callus produced by mesophyll cells of short day plants of *Kolanchoë Blossfeldiana* exhibited chromosome numbers up to 32n (= 544!) while comparable nuclei of long-day plants showed much lesser degrees of polyploidy.

It is of interest that in haploid cells of *C. eugametos* and *C. moewusii* the 8 chromosomes are characteristically arranged in a ring at metaphase (fig. 42) while polyploid nuclei exhibit flat plates of chromosomes (fig. 50). Fankhauser and Humphrey (1950) have shown that this same pattern of chromosomal behavior appears in polyploid nuclei of the axolotl, that is, metaphase chromosomes of normal diploid cells appear in a ring, while those of polyploid cells become crowded into the central portion of the equatorial plate.

Although it is possible to describe the details of chromosomal behavior during somatic reduction in those organisms where the chromosomes are of sufficient size to allow it, this has not been possible for *C. eugametos* and *C. moewusii* in the present study due to the extremely minute size of the chromosomes. It is to be hoped that techniques will be developed which will render a closer study possible, but at the present time these details cannot be described. The meiotic process seems clearly to follow a classical pattern.

SUMMARY

1. Four species of *Chlamydomonas, C. eugametos* Moewus, *C. chlamydogama* Bold, *C. reinhardti* Dangeard and *C. moewusii* Gerloff, were studied cytologically and compared. Particular study was devoted to mitosis and meiosis. These processes proved to be essentially similar in all 4 species and to follow the classical patterns observed among plants. No dissimilarities were noted in mitosis as it occurs in these organisms and the pattern which has been described for other species of *Chlamydomonas* by earlier investigators.

2. The haploid chromosome number was found to be 8 for all 4 species; this constitutes a much lower count than those reported by recent workers for *C. eugametos, C. reinhardti* and *C. moewusii.*

3. It was found that when vegetative cells of *C. eugametos* and *C. moewusii* are grown at a light intensity of 800 foot-candles, temporary

polyploidy is exhibited by certain cells. Somatic reduction, the exact mechanism of which was not determined, eventually results independently of light.

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