GAMETIC DIFFERENTIATION IN *CHLAMYDOMONAS REINHARDTII*

I. Production of Gametes and Their Fine Structure

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ABSTRACT

Gametogenesis in *Chlamydomonas reinhardtii* has been studied in mating-type *plus* cells utilizing several different culture conditions, all of which are shown to depend on the depletion of nitrogen from the medium, and the fine structure of gametes prepared under these conditions has been compared by using thin sections of fixed material. We document alterations in ribosome levels, in chromatin morphology, in starch levels, in the organization of chloroplast membranes, and in the appearance of nuclear envelope and endoplasmic reticulum membranes during gametogenesis. We also note the acquisition of two new organelles: a mating structure (Friedman, L., A. L. Colwin, and L. H. Colwin. 1968. *J. Cell Sci.* **3**:115–128; Goodenough, U. W., and R. L. Weiss. 1975. *J. Cell Biol.* **67**:623–637), and Golgi-derived vesicles containing a homogeneous material. We chart the time course of these morphological changes during synchronous gametogenesis. We note that many of these changes may represent adjustments to nitrogen starvation rather than direct features of gametic differentiation, and we also document that cells can differentiate so that they survive conditions of nitrogen starvation for many weeks after they become gametes. We conclude that metabolic alterations, the acquisition of mating ability, and the preparation for long-term survival are all elicited in this organism by nitrogen withdrawal, and we discuss how the various structural alterations observed in this study may relate to these three interrelated avenues of cellular differentiation.

When the unicellular flagellate *Chlamydomonas reinhardtii* is induced to differentiate from a vegetative cell into a gamete, a number of changes occur in its biochemistry, morphology, and behavior that have been described by earlier investigators. The differentiation period itself, which can be as short as 10–12 h in synchronized cultures (16), requires both RNA and protein synthesis (14) and is marked by an extensive breakdown and resynthesis of nucleic acids (20) and an alteration in the activity of several enzymes (15). Synchronous differentiation normally concludes with a mitotic division, and the gametic cells that emerge from this mitosis are able to mate with 100% efficiency (3). Mating is induced by mixing gametes of mating-type plus (mt^+) with those of mt^-. The cells first agglutinate by the tips of their flagella; there follow a shedding of cell walls (4) and a cell fusion between pairs of mt^+ and mt^- gametes to form quadriflagellated zygotes (18).

We initiated a fine-structural study of gametogenesis in *C. reinhardtii* as a prelude to analyzing

the process at a biochemical and genetic level. In particular, we sought to define the morphological features of competent gametes so that we might detect any aberrations present in nonmating mutants. This seemingly simple project became complex when we discovered that the morphology of competent gametes was dependent on the culture conditions used to produce them. We therefore developed highly standardized procedures for obtaining gametes both in liquid and on solid agar medium and compared the fine structure of gametes prepared by these procedures.

This paper presents the results of these studies. We first summarize our methods for obtaining gametes and demonstrate that all known modes of gametogenesis in *C. reinhardtii* in fact depend on the same underlying stimulus, namely, the withdrawal of nitrogen from the growth medium. We then present a fine-structural analysis of gametes prepared by these various conditions and a time-course study of the changes that cells undergo during synchronous gametogenesis. We conclude that many of these changes are probably degradative events stimulated by nitrogen starvation and/or events geared to insure the long-term survival of gametes in the face of a nitrogen-free environment, and we define those few phenotypes that are directly related to the acquisition of mating ability. Two of these gamete-specific traits are explored more extensively in the accompanying papers in this series (1, 9).

MATERIALS AND METHODS

Strains and Culture Conditions

Wild-type *C. reinhardtii*, strain 137c, mt^+, was used throughout this investigation. The final structural phenotype of mt^- gametes was also examined and found to be comparable to mt^+ cells, but detailed studies of mt^- cells were not made.

Two kinds of liquid growth media were used: high-salt minimal medium (HSM) (21) and Tris-acetate-phosphate medium (TAP) (10). These media are referred to as N-free HSM and N-free TAP when prepared without nitrogen. Solid media were prepared by adding 1.5% agar (Difco Laboratories, Detroit, Mich.) to TAP (TAP-agar) or N-free TAP (N-free TAP-agar).

Cells were grown vegetatively in both liquid and solid media, and gametic differentiation was induced in both kinds of media. The most reproducible protocols for each mode of growth were used in the present study and are described below.

VEGETATIVE LIQUID CULTURES: Vegetative cells were grown synchronously in HSM on a 12 h light-12 h dark cycle (16). Cultures were maintained at 21° ± 1°C and illuminated with daylight fluorescent lamps giving an intensity of 9,000 lx on the shelves where the flasks stood. The cultures were stirred constantly and aerated with a mixture of air and 5% CO_2.

VEGETATIVE PLATE CULTURES: Vegetative cells, cultured as above, were harvested at 6 h into that light period when the cultures had reached a density of $2–3 \times 10^6$ cells/ml. They were suspended into fresh HSM at a known cell concentration, and 0.1-ml aliquots were applied to the surface of TAP-agar contained in petri plates. The cultures were maintained at constant temperature and in continuous light at 3,600 lx. Several such subcultures were prepared from a given vegetative culture so that it would be possible to sample cells at a number of different growth intervals. Cell number was determined by thoroughly washing the lawn of cells off the agar surface into a known volume of liquid and counting an aliquot using a hemacytometer.

GAMETIC LIQUID CULTURES: Vegetative cells were again harvested at 6 h into that light period when the cultures had reached a density of $2–3 \times 10^6$ cells/ml and were resuspended into N-free HSM. Two distinct growth regimes were then imposed. In the first the cells were maintained in continuous light throughout the duration of gametogenesis; the resultant gametes are referred to as *liquid-grown light gametes*. Alternatively, the cells were maintained on the light-dark cycle so that the light was extinguished 6 h after suspension into N-free medium; the resultant gametes are referred to as *liquid-grown light-dark gametes*.

GAMETIC PLATE CULTURES: Synchronous vegetative cells were harvested as described above for the preparation of vegetative plate cultures. Gametogenesis then proceeded in two distinct media. In the first case, cells were inoculated from the vegetative liquid culture to TAP-agar medium; as such cells grow, they use up the nitrogen in the medium and become gametic (see Results). These are referred to as *TAP plate gametes*. In the second case, cells were inoculated to N-free TAP-agar medium plates, whereupon they differentiate much more rapidly into gametes (see Results section). These are referred to as *N-free plate gametes*. In both cases, a number of plate subcultures were prepared from a given liquid vegetative culture so that cell number and mating efficiency could be followed for several days.

Determination of Mating Efficiency

The extent of gametic differentiation was determined by mating efficiency tests (3). Tester gametes of mt^- were prepared from either plate or liquid cultures and their numbers were determined with a hemacytometer. These gametes were mixed with an equal number of the mt^+ gametes being tested; in the case of plate gametes, mt^+ cells were taken from plates and suspended into liquid HSM for 1 h before mixing with mt^- cells. Zygote formation was allowed to proceed for 1.5 h. Counts were then made of the number of cells (both zygotes and any unmated cells) in the mixture, and the following equation

was used to determine mating efficiency:

$$\% \text{ mating} = 2\left(1 - \frac{\text{cell number after mating}}{\text{cell number before mating}}\right) \times 100.$$

All types of gametes used in this study were tested to determine whether they were capable of giving rise to normal zygotes. Zygotes were obtained and allowed to germinate according to the procedure of Ebersold and Levine (5). Germination was found to be consistently greater than 85%.

Determination of Ammonium in Solid Media

The method described by Burris and Wilson (2) was used, with minor modifications, to determine the ammonium content of TAP medium. Samples of liquid TAP media were diluted 10-fold; aliquots of the diluted medium, ranging between 0.1 ml and 1.5 ml, were brought to 1.5 ml with double-distilled water; 1 ml of Nessler's Reagent (Fisher Scientific Co.,) was slowly added to each tube, followed by 1 ml of 2 N NaOH; color was allowed to develop for 20 min at room temperature; and the absorbance at 490 nm was determined using a Zeiss spectrophotometer. Standard curves using NH_4Cl solutions demonstrated that the reaction was linear, and extremely accurate, in the range between 1 and 5 μg of NH_3, after which the reaction product began to precipitate. A 10-fold dilution of TAP medium was determined to yield appropriate-sized aliquots containing ammonium in this concentration range.

To determine the ammonium content of TAP-agar medium, plates were prepared each containing 32 ml of TAP-agar. Medium that had been used for cultures was first completely cleared of cells by using a razor blade and a cotton swab. Fresh, uninoculated plates served as controls. The agar was taken out of each plate being tested and was placed in a stoppered graduated cylinder, melted in an autoclave, and brought to 32 ml with hot distilled water. 1-ml samples were diluted 10-fold with hot distilled water, and the diluted samples were stirred vigorously with a magnetic stirrer while being allowed to come to room temperature. The resulting solutions were visibly clear; their agar and any contaminating cell debris were subsequently removed by centrifugation at 12,000 *g* in a refrigerated centrifuge. Aliquots of the supernates were analyzed for ammonium as described above.

Photosynthetic CO_2 Fixation

Synchronous liquid-grown light gametes were tested for ability to fix CO_2 by following the methods of Togaski as described by Goodenough et al. (7): 2 ml of cells containing 25 μg chlorophyll were placed in a small flask and agitated with a constant stream of air. The flask was placed in a waterbath maintained at 25°C and illuminated at 4,000 lx for 5 min before $NaH^{14}CO_2$ was added. Samples of 0.1 ml were taken at 1-min intervals for 5 min and plated on stainless steel planchets containing 0.1 ml of a mixture of HCl and glacial acetic acid (4:1). Samples were dried and counted in a gas flow counter. Correction for light-independent CO_2 fixation was obtained from control experiments performed in the dark.

Electron Microscopy

Gametic cell structure was found to be poorly preserved by many standard fixation procedures. The following protocol was therefore developed. Cells were harvested by centrifugation and suspended in cold 4 mM potassium phosphate buffer, pH 7.0, containing 1 mM $CaCl_2$ (in which medium they are capable of mating normally). An equal volume of cold 4% glutaraldehyde in the same buffer was added to yield a final glutaraldehyde concentration of 2%. The fixation vial was left on ice for 5 min. The cells were then centrifuged and resuspended in cold 2% glutaraldehyde mixed with 0.5% OsO_4 in the same phosphate buffer. A 1-h fixation was followed by rapid dehydration in ethanol; cells that are well preserved lose little chlorophyll into the 100% ethanol, a criterion followed in the development of this fixation protocol. After embedding in Epon-Araldite (13), thin sections were cut, stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-11C or an RCA EMU-3A electron microscope.

Analysis of Ribosomes in Electron Micrographs

To determine the numbers of chloroplast and cytoplasmic ribosomes present in electron micrographs, cut-and-weigh estimates were made (8): for each sample, portions of 10 different thin-sectioned cells were photographed at an original magnification of 20,000, micrographs were printed at × 98,000, areas of chloroplast stroma and cytoplasmic ground substance were cut from the micrographs, the total number of ribosomes in the cuttings was counted, and the cuttings were weighed to yield a value of ribosomes per unit weight.

RESULTS

The Acquisition of Mating Ability

Gametes were prepared for this study by the various procedures outlined in Materials and Methods. Figs. 1-4 summarize the growth and mating properties observed with each protocol; in later sections we describe the fine structure of gametes prepared by each procedure.

DIFFERENTIATION IN LIQUID MEDIUM: Fig. 1 illustrates several features of the gametogenesis that occur when vegetative cells in the 6th h of a synchronous cycle (16) are suspended into

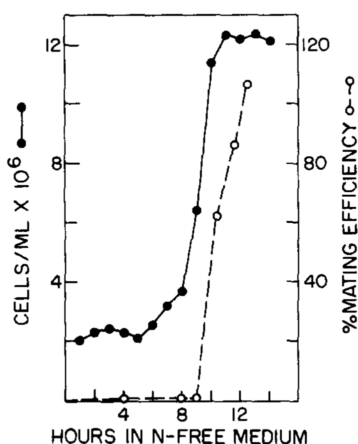


FIGURE 1 Growth curve (●——●) and percent mating efficiency (O——O) during synchronous gametogenesis in liquid culture in continuous light.

N-free liquid medium. A synchronous cell division is seen to occur after 6–8 h in N-free medium, and mating ability develops in parallel with the increase in cell number until it reaches 100%.

Our experiments have indicated an additional feature of gametogenesis in liquid medium: we find that cells do not require continuous light (14) in order to differentiate. Gametes that arise from a synchronous cell division and mate with 100% efficiency are routinely obtained if the light is extinguished 6 h after suspension into N-free medium (i.e. the usual hour for the lights to go off in the synchronous cycle). Indeed, light appears to be required for gametogenesis only if photosynthesis is the only available means of obtaining fixed carbon, for if 0.2% sodium acetate is provided to cells shortly before and during the time they are placed in N-free minimal medium, differentiation proceeds normally even when the culture is maintained in total darkness.

DIFFERENTIATION ON SOLID MEDIUM: The typical method for obtaining gametes from agar-grown cultures is to take plates on which cells have been growing for several weeks and flood them with water or N-free medium for several hours or overnight; the cells that swim into the liquid are found to be gametic (6, 18, 19). This method is not only difficult to standardize, but also leaves open the question as to whether differentiation actually occurred on the agar medium or whether it occurs in the flooding liquid.

We therefore modified the procedure in the following way. Cells grown on TAP-agar were suspended into liquid medium during the hour required for the acquisition of motility (cells growing on solid medium lack flagella, so that their ability to undergo agglutination and fusion cannot be assessed). The medium, however, contained nitrogen to prevent any (further) gametic differentiation from occurring in the liquid. Using this assay, we found that vegetative cells indeed differentiate into gametes when maintained on TAP-agar plates so that they are able to mate with 100% efficiency even after 2.5 h in liquid HSM. Fig. 2 gives a growth curve for such cells and plots the acquisition of mating ability. Mating ability is seen to develop as the cells approach stationary phase, a feature of plate gametogenesis that is underscored by the experiments summarized in Fig. 3. Here plates were inoculated with relatively low, medium, and high cell densities; the low-density culture is seen to reach stationary phase later than the high-density culture and to exhibit a parallel lag in gametic differentiation. That this lag is not more pronounced is due to the fact that all the cultures reach stationary phase at approximately the same time, the density of plating being inversely related to the increment in cell number at each sampling time.

Such growth experiments suggest two interpretations: (*a*) some essential component(s) of the medium, including nitrogen, become limiting at the agar surface so that the cells no longer divide and instead differentiate into gametes; or (*b*) the cells in the lawn somehow respond to a particular

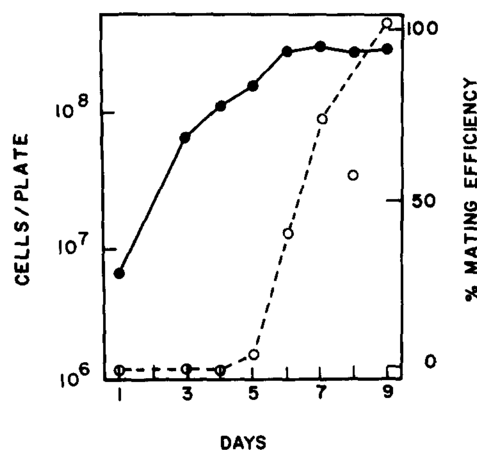


FIGURE 2 Growth curve (●——●) and percent mating ability (O——O) during plate gametogenesis after the application of 6.3 × 10^6 vegetative cells to TAP-agar plates as described in Materials and Methods.

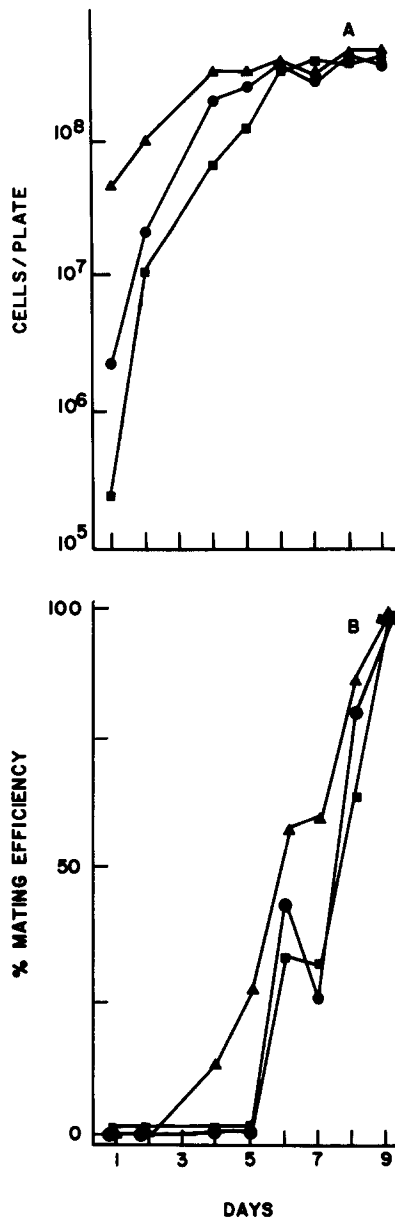


FIGURE 3 (A) Growth curves during plate gametogenesis of cultures plated at different initial cell densities. Initial cell numbers were 2.4 × 10^5 (■), 2.4 × 10^6 (●), and 4.8 × 10^7 (▲). (B) Percent mating efficiency of the above cultures.

level of "crowding" by a cessation of growth and an onset of gametic differentiation, one that is perhaps independent of nitrogen starvation and thus distinct from the differentiation that occurs in N-free liquid culture. To distinguish between these possibilities, vegetative cells were inoculated at low densities to either TAP-agar plates or N-free TAP-agar plates. As is evident in Fig. 4, the cells on the N-free plates stop dividing after 24 h even though they have not approached the usual "crowding" level. Since mating activity is always detected after 24 h on N-free plates, "crowding" does not appear to be a relevant factor in gametogenesis.

To establish that cells indeed use up the nitrogen present in TAP-agar medium and that this depletion stimulates plate gametogenesis, the following experiments were performed. Plates were prepared such that each contained identical amounts of TAP-agar medium, and several of these were inoculated with identical aliquots from the same vegetative culture. After 2 days and after 6 days of growth, several plates were scraped clean of cells and the medium was tested for its content of ammonium (the sole form of inorganic nitrogen utilized by strain 137c of *C. reinhardtii*). Ammonium levels were found to have dropped by 50% after 2 days and were less than 2% of control levels after 6 days (cells on these 6-day plates mated with 80% efficiency). When such TAP-agar plates on which gametes had differentiated were scraped clean and reinoculated with vegetative cells, moreover, gametic activity was found to develop after 24 h, in contrast to 5 days on fresh control plates.

Despite the fact that a lawn of *C. reinhardtii* cells exhausts the ammonium present in a TAP-

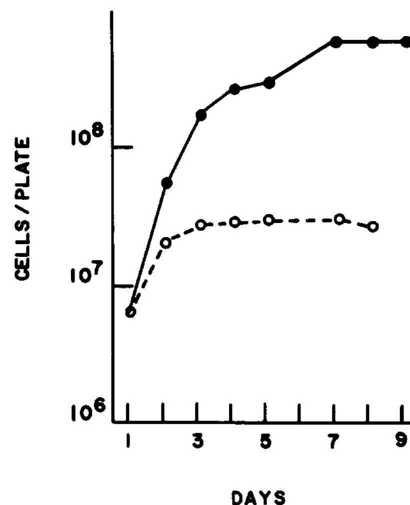


FIGURE 4 Growth curve on TAP (●——●) and N-free TAP (O——O) plates after the application of 6.2 × 10^6 vegetative cells to each.

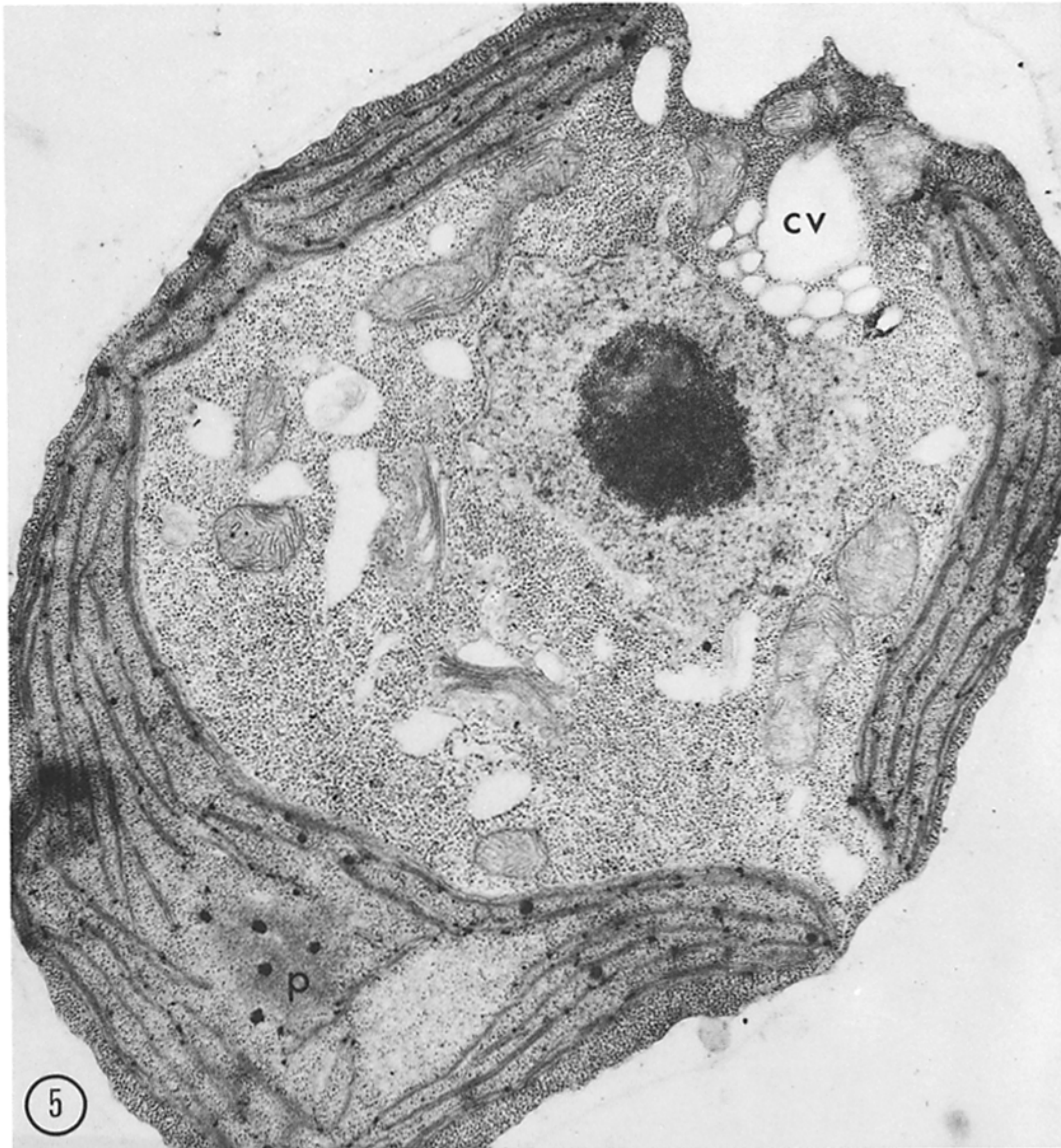
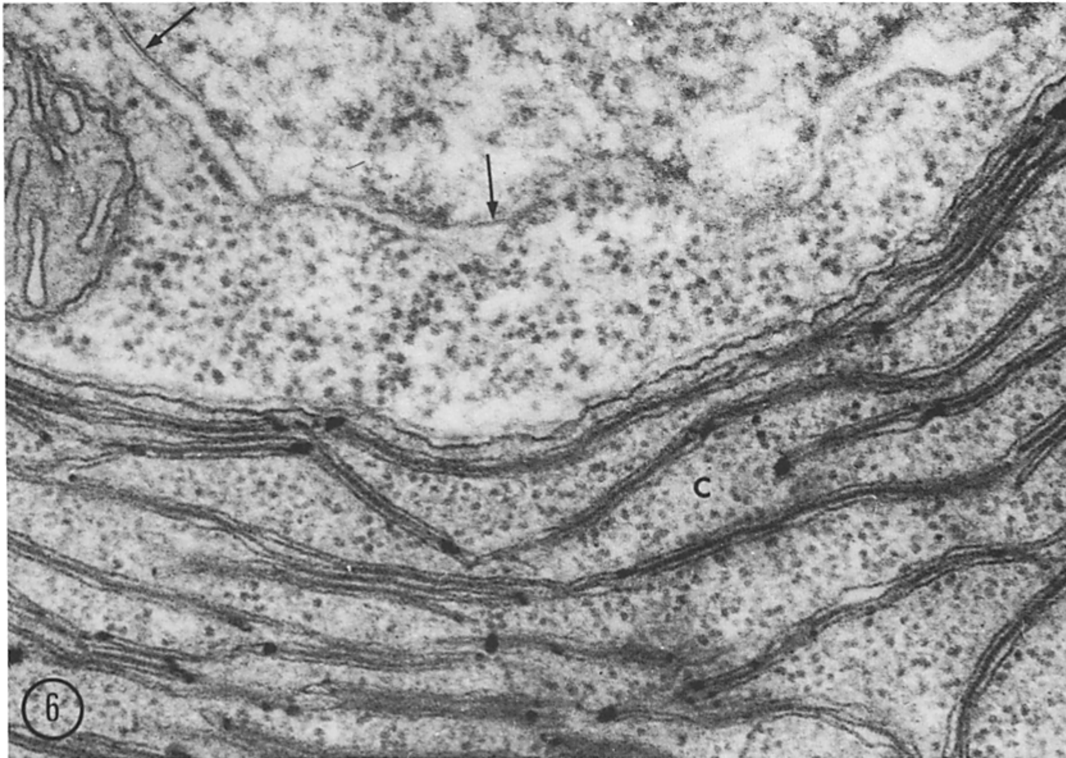


FIGURE 5 Vegetative cell at 6 h into the light period of the synchronous cell cycle at which cell density had reached 2–3 × 10^6 cells/ml. A cup-shaped chloroplast lies beneath the cell membrane with a pyrenoid (*p*) at the base. Other visible organelles include a nucleus, mitochondria, Golgi regions, and contractile vacuole (*cv*). A cell wall surrounds the cell. × 20,000.

agar plate within 1 wk, these cells continue to be fully viable and capable of mating with 100% efficiency for at least 3 wk. In the remaining sections of this paper, reference will be made to "young" plate gametes that have just completed differentiation and to "old" plate gametes that have remained on nitrogen-depleted plates for several weeks.

Fine Structure of Reference Vegetative Cells

Before comparing the fine structure of gametes grown under the various culture regimes described above, we investigated whether any differences existed between the vegetative cells from which the gametes derived. No important differences were



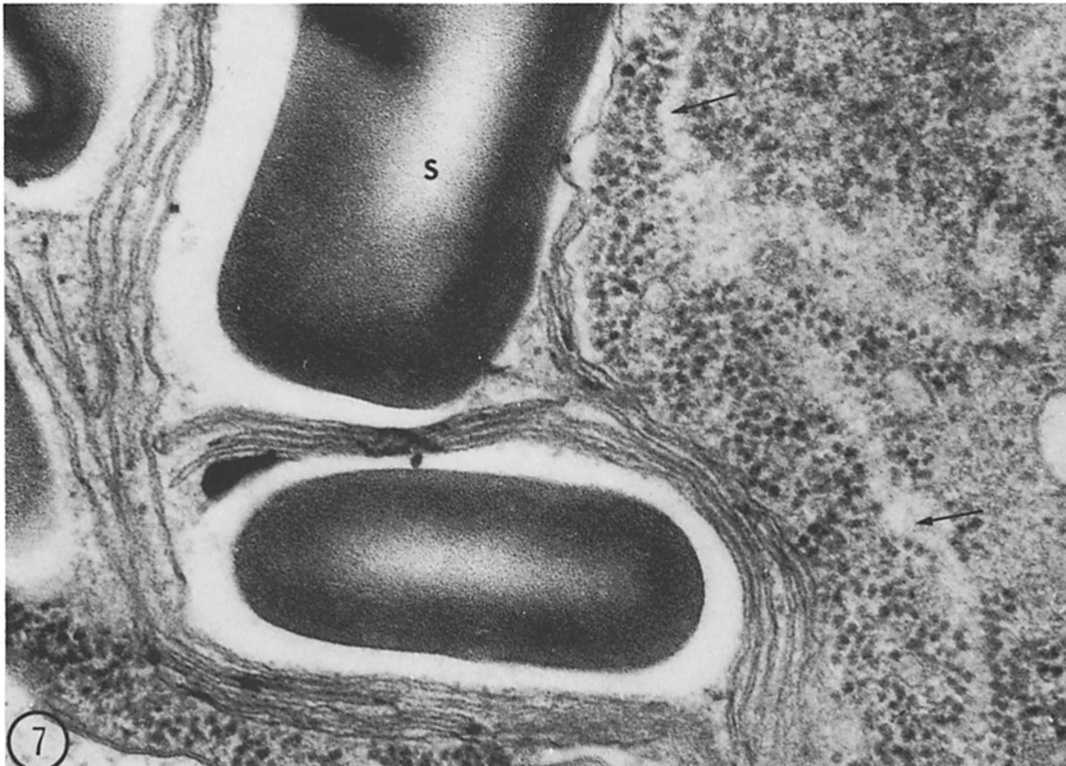


FIGURE 6 Portion of a vegetative cell as in Fig. 5. The nucleus is delimited by an envelope (arrows). The chloroplast (*c*) contains anastomosing stacks of thylakoids and a dense population of ribosomes in the stroma. × 76,000.

FIGURE 7 Portion of a liquid-grown light gamete from a synchronous culture. The membranes of the nuclear envelope and endoplasmic reticulum have not been preserved, but the cisternae can be detected (arrows). The chloroplast contains large starch grains (*s*) and unstacked thylakoids. The stroma is virtually free of ribosomes. × 76,000.

found (11). Therefore, the vegetative cells depicted in Figs. 5 and 6 are typical of all the vegetative cells that were subjected to nitrogen starvation in our experiments, with the minor exception that vegetative cells growing on TAP medium contain more starch in their chloroplasts than cells growing in HSM. The features of Figs. 5 and 6 that should be noted include the euchromatic nucleus with a prominent nucleolus and a nuclear envelope, the presence of numerous free ribosomes in the cytoplasm and chloroplast, and the cup-shaped chloroplast containing thylakoids that are arranged in small, anastomosing stacks.

Fine Structure of Liquid-Grown Light Gametes

Fully differentiated liquid-grown light gametes are shown in Figs. 7 and 8. The most dramatic differences between the gametes and their refer-

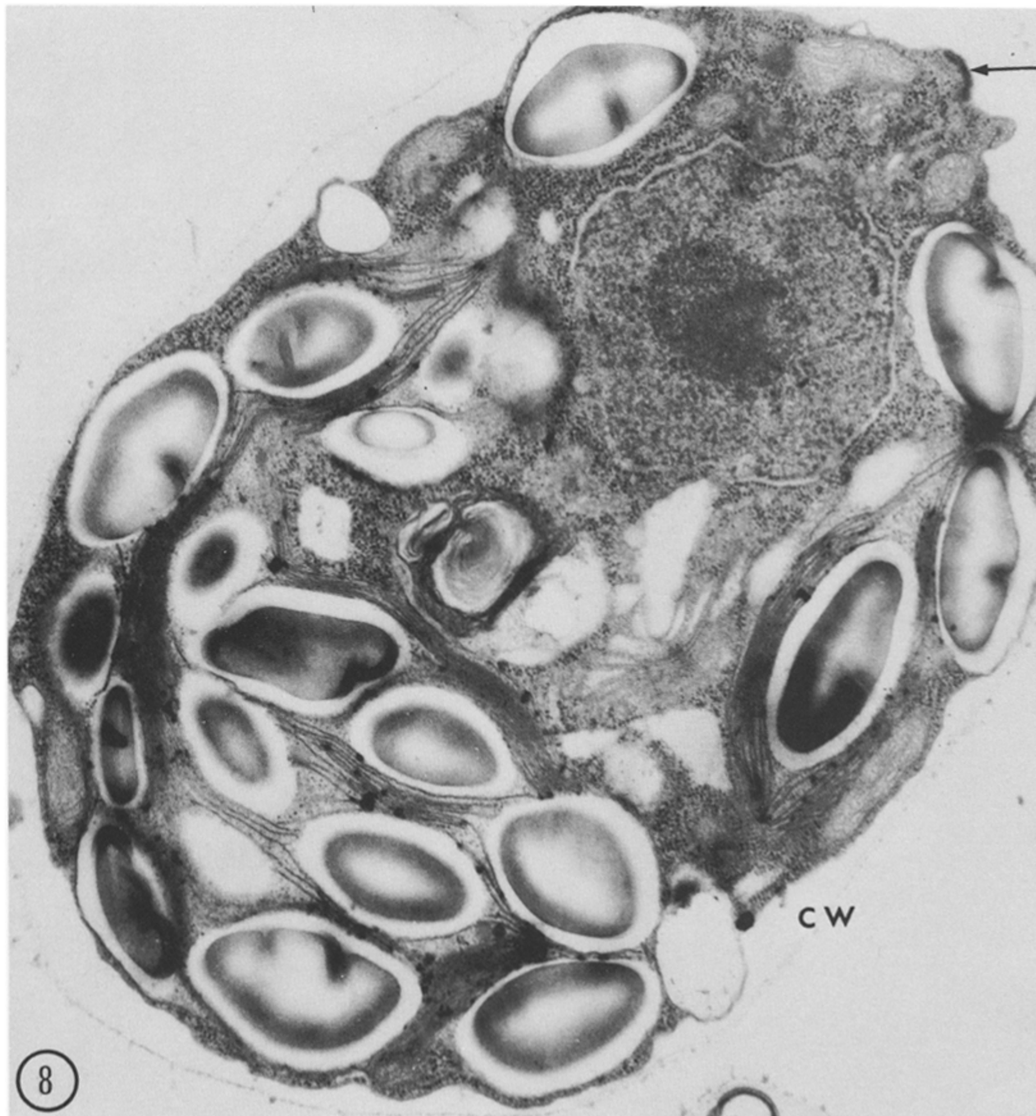


FIGURE 8 Liquid-grown light gamete. A cell wall (*cw*) surrounds the cell. The cup-shaped chloroplast is filled with starch, some of which is stained and some unstained (the significance of this variability in starch staining is unknown). A mating structure lies at the anterior end of the cell (arrow). × 26,000.

ence vegetative cells (Figs. 5 and 6) are found in the chloroplast. The gamete chloroplast is seen to contain an abundance of starch (Fig. 8). Moreover, the chloroplast stroma is less electron dense and contains greatly reduced numbers of ribosomes (compare Figs. 6 and 7), a reduction quantitated in Table I. The pattern of chloroplast membrane organization is greatly disrupted, the thylakoids being either single or compressed into large stacks (Figs. 7 and 8). Finally, the pyrenoid in the gametic chloroplast is typically smaller and more irregular in shape than in the vegetative cell chloroplast (not shown).

Because these alterations in chloroplast fine structure suggested an impairment of function, we examined the photosynthetic capacity of liquid-grown gametes compared to vegetative control cells. Table II shows that gametes indeed fix CO_2 at poor rates, both on a chlorophyll and on a cell basis (gametes commonly possess about half the chlorophyll of their vegetative counterparts, possibly in part because of their smaller size). A preliminary investigation of the ability of the two cell types to carry out light-induced photosynthetic electron-transport reactions in vitro showed that gametes are capable of rates that are at least half the vegetative rates. Therefore, the 10-fold reduction in photosynthetic capacity indicated by the CO_2-fixation assays cannot be explained simply by alterations in membrane-associated photosynthetic electron-transport reactions, despite the obvious disruption of chloroplast membrane organization seen in electron micrographs of gametes. Preliminary investigations also demonstrated no change in the ribulose-1,5-diphosphate carboxylase activity of gametes, but other enzymes of the Calvin-Benson cycle were not examined.

Differences between liquid-grown light gametes and vegetative cells are also apparent outside the chloroplast. The nuclear envelope and endoplasmic reticulum (ER) membranes are rarely present in micrographs of gametes, and outer mitochondrial membranes are often absent as well; clear areas exist where these membranes should be found (Fig. 7), indicating that some form of membrane was probably present in the living cell but was not preserved during the fixation procedure. In addition, the gametic chromatin often appears condensed (heterochromatic) compared to the vegetative (compare Figs. 5 and 8, Figs. 6 and 7). Finally, a reduction in numbers of free cytoplasmic ribosomes is generally noted in gametes (Table I), although it is not apparent in the micrographs selected for this publication, and gametic ribosomes are found packed along the surface of the nuclear envelope and ER to a much greater extent than is observed in vegetative cells.

In addition to these structural modifications,

TABLE I

Variation in Ribosomes during Gametogenesis

	Ribosomes/gram paper		% Reduction*	
Cell type	Cyto-plasm	Chloro-plast	Cyto-plasm	Chloro-plast
Vegetative liquid	520	369	—	—
	485	342	—	—
Vegetative plate	454	389	—	—
Liquid-grown light gamete	322	169	—	—
	346	133	34	58
Liquid-grown light-dark gamete	442	213	12	40
TAP plate gamete	322	332	—	—
	332	330	25	19
	365	284	—	—
N-free plate gamete	370	211	19	46

* The percent reduction was calculated after averaging the data from replicate determinations; it equals the vegetative value minus the gamete value divided by the vegetative value.

TABLE II

Photosynthetic CO_2 Fixation in Vegetative Cells and Synchronous Light Gametes

μmoles CO_2 fixed/h · mg chlorophyll		μmol CO_2 fixed/h · 10^6 cells	
Vegetative	Gametes	Vegetative	Gametes
108.0	28.0	0.460	0.035
78.5	7.4	0.204	0.016
75.0	8.8	0.165	0.011
80.0	21.8	0.192	0.031

Data were obtained from four separate cultures. Vegetative cultures were grown under the same conditions as liquid-grown light gametes but suspended in fresh HSM instead of N-free HSM at 6 h after the beginning of the light period in the synchronous cycle. Procedures are described in Materials and Methods.

two new organelles are present in thin sections of gametes. The first is a group of cytoplasmic vesicles that can be called *gametic vesicles*. As illustrated in Fig. 9, they contain a homogeneous material that is moderately electron opaque. They are membrane limited (Fig. 9), in contrast to the lipid droplets that are also encountered in both vegetative cells and gametes. They also differ from microsomes (Fig 9, *mi*) in that the microsomal contents are more flocculent and microsomes are found in both vegetative cells and gametes. We have no evidence that the gametic vesicles discharge their contents at any time during differentiation or the mating reaction, and indeed, the fact that we find identical vesicles in zygotes fixed 18 h after mating argues against any exclusive role for the vesicles in the mating process. Otherwise, their function is unknown.

The second new organelle visible in gametes is the structure which Friedmann et al. (6) have designated the "choanoid body;" we prefer to use the term *mating structure* for reasons that are given elsewhere (9). A mating structure is evident at the anterior end of the gamete shown in Fig. 8 as an electron-dense band of material beneath the cell membrane. A more detailed description of this organelle is reserved for the accompanying paper (9).

The liquid-grown gametes described above were maintained in continuous light throughout gametogenesis. If the light is extinguished after 6 h so that the cells are maintained on their accustomed light-dark cycle, the resultant gametes appear less disrupted than their continuous-light counterparts (Fig. 10). Starch is less abundant; the chloroplast stroma is more electron dense and contains more ribosomes (Table I); the chloroplast thylakoids tend to exist less often as single units and more often in large stacks; the pyrenoid is larger and more regular in shape; and the cytoplasmic ground substance contains many more ribosomes (Table I). Thus, the extreme alterations in the fine structure that occur during the continuous light regime are not required for normal gametogenesis

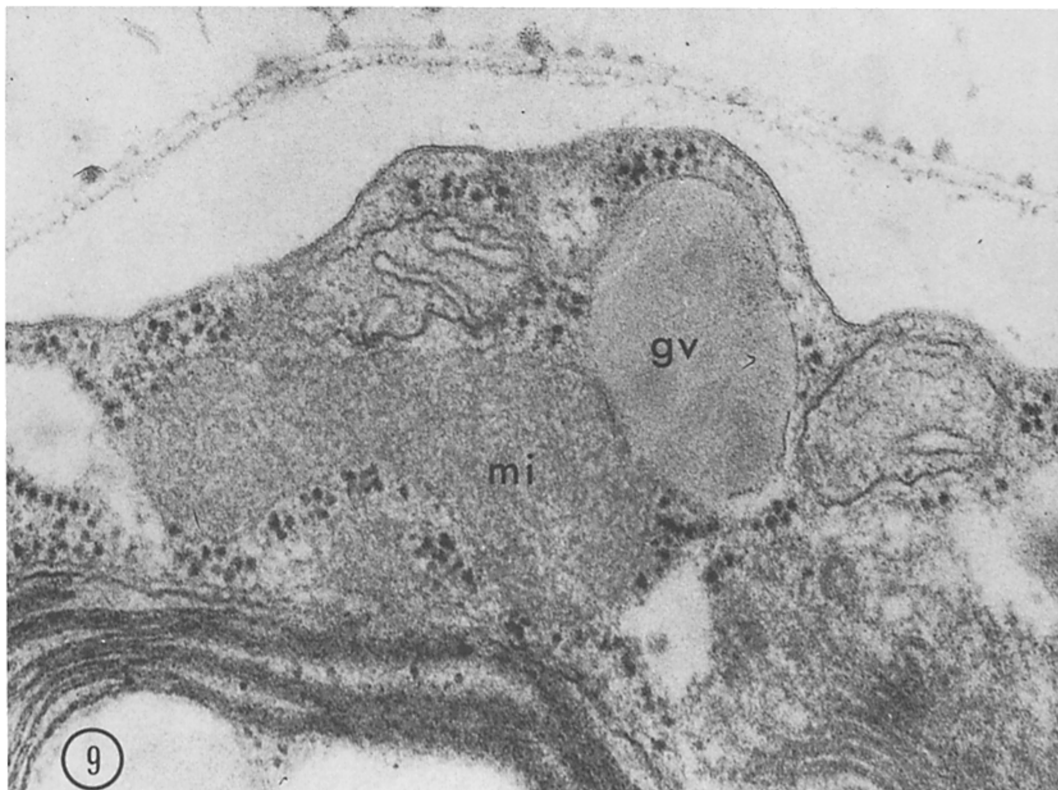


FIGURE 9. Portion of a liquid-grown light gamete showing a gametic vesicle (*gv*) and an adjacent microsome (*mi*). × 87,000.

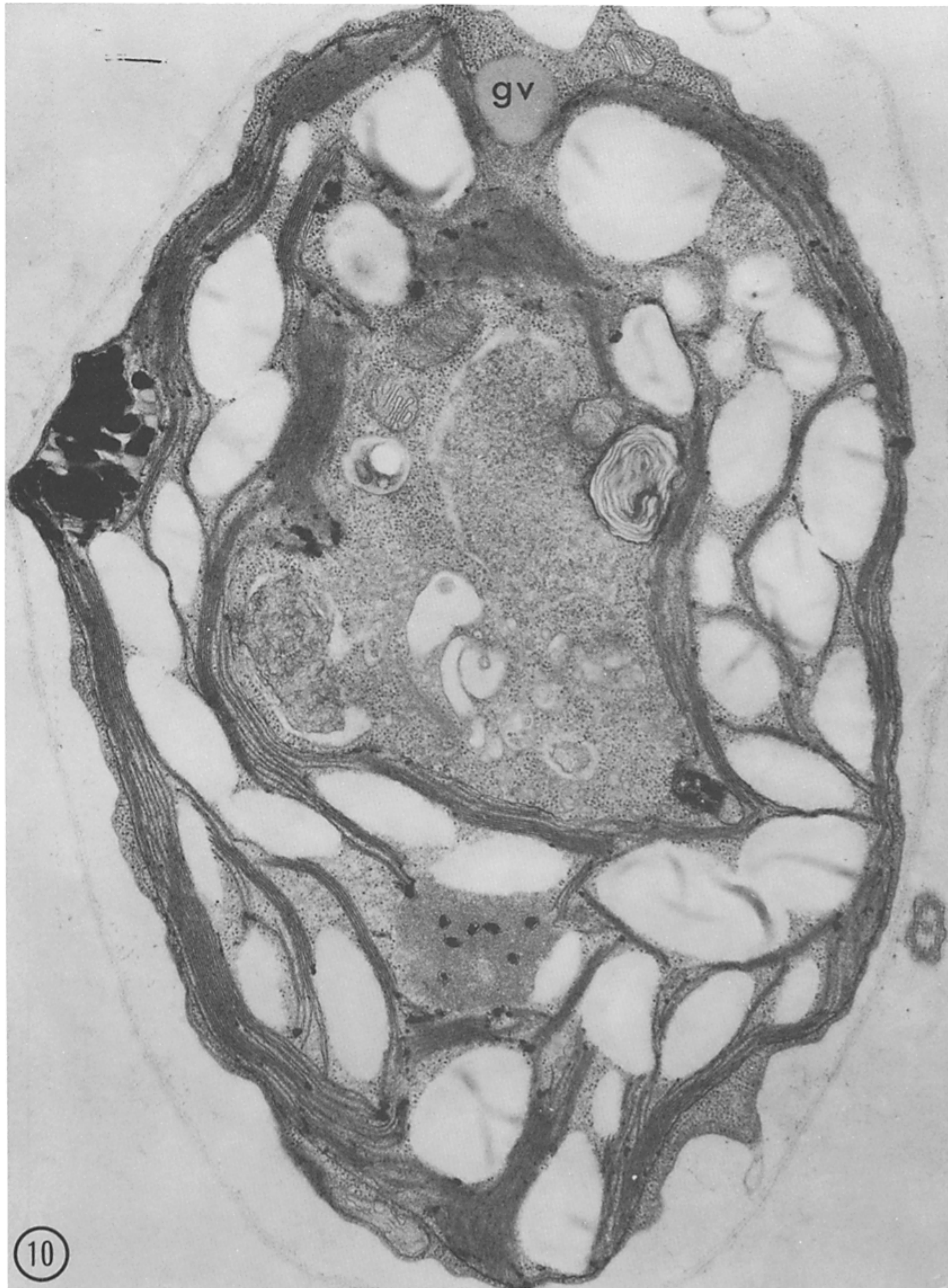


FIGURE 10 Liquid-grown light-dark gamete, showing a cup-shaped chloroplast, a central nucleus, and a gametic vesicle (*gv*). × 28,000.

since liquid-grown light-dark gametes mate with 100% efficiency.

Liquid-grown light-dark gametes are indistinguishable from their light-grown counterparts in the possession of gametic vesicles (Fig. 10, *gv*), mating structures, and cytoplasmic membranes that fix poorly. The nucleoplasm is far less apt to appear heterochromatic, however, and the association of ribosomes with the nuclear envelope and ER is less pronounced.

Fine Structure of Plate-Grown Gametes

Plate gametes fixed at the time they have reached 100% mating efficiencies (Fig. 2) are far less homogeneous in size and in cellular fine structure than are gametes prepared in liquid culture. For example, some cells are found to contain an abundance of starch, such as the plate gamete depicted in Fig. 11, while others contain very little starch. Such variation is evidently unrelated to gametic potential since mating efficiencies of 95–100% are routinely obtained from plate-grown gametes.

When TAP plate gametes are compared with vegetative cells maintained on plates for 2 days, differences are less dramatic than when liquid-grown gametes are compared with their reference vegetative cells. Chloroplast membrane stacks tend to be slightly larger, and a decrease is noted in levels of both chloroplast and cytoplasmic ribosomes (Table I).

When TAP plate gametes are instead compared with liquid-grown gametes, both similarities and differences can be found. In common is the possession of mating structures, gametic vesicles, and nuclear envelopes and ER that are poorly preserved by fixation. A difference in degree is that the plate gamete nucleus is more apt to be heterochromatic. The plate gamete chloroplasts fail to show the striking increase in single thylakoids found in the liquid-grown gametes. Finally, large vacuoles, filled with a fibrous material, are found in the cytoplasm of plate gametes (Fig. 11) but not in any other kinds of *C. reinhardtii* cells that we have studied.

TAP plate gametes were also compared in this study with N-free plate gametes, the latter having experienced a sudden withdrawal of nitrogen rather than a more gradual depletion. Cells cultured on N-free plates for 48 h appear quite similar to TAP plate gametes, except that larger areas of the cytoplasm are given over to the fiber-filled vacuoles, gametic vesicles are larger, and the chloroplast membrane stacks are smaller in size. Chloroplast ribosomes appear to be far more extensively depleted in N-free plate gametes than in TAP plate gametes whereas the depletion of cytoplasmic ribosomes is comparable (Table I).

Finally, plate gametes that had just completed gametic differentiation (Fig. 2) were compared with cells that had been maintained on TAP-agar plate for 3 wk. These "old" plate gametes, which also mate with 100% efficiency, are generally comparable in structure to "young" plate gametes with a notable exception: they posess highly heterochromatic nuclei (Fig. 12), the chromatin appearing far more dense than in any preparations of "young" gametes from liquid or agar cultures.

Time Course Studies

Because plate gametogenesis produces gametes with variable fine structure, a structural analysis of the time course of plate gametogenesis did not seem worthwhile. In contrast, gametes from synchronous liquid cultures are strikingly uniform. We therefore fixed samples of differentiating synchronous cells at 1- or 2-h intervals in several separate experiments in order to chart a time course for the various structural changes that occur during synchronous gametogenesis. Changes noted during the first 6 hours after nitrogen withdrawal apply to both light and light-dark cultures; changes occurring in the next 6 h were observed separately for each of the two culture regimes.

STARCH: By 1 h after transfer to N-free medium, starch has begun to accumulate as small grains around the pyrenoid. By 2 h, starch is evident in the chloroplast stroma, and it continues to increase steadily throughout the time that the cells remain in the light so that, by 5 hours, starch levels have nearly reached those of a mature gamete (Fig. 13). Accompanying this increase in starch is a reduction in the size and integrity of the pyrenoid (Fig. 13).

Light-dark gametes show a decreased starch content after 1 h in the dark, and starch is steadily depleted during the 6 h of darkness, presumably because it is being utilized as a source of fixed carbon in the absence of photosynthesis (17).

CHLOROPLAST MEMBRANES: The organization of chloroplast membranes is observed to change gradually; it becomes unambiguously gamete-like by the 7th h.

NUCLEAR AND ER MEMBRANES: The nuclear and ER membranes begin to appear frag-

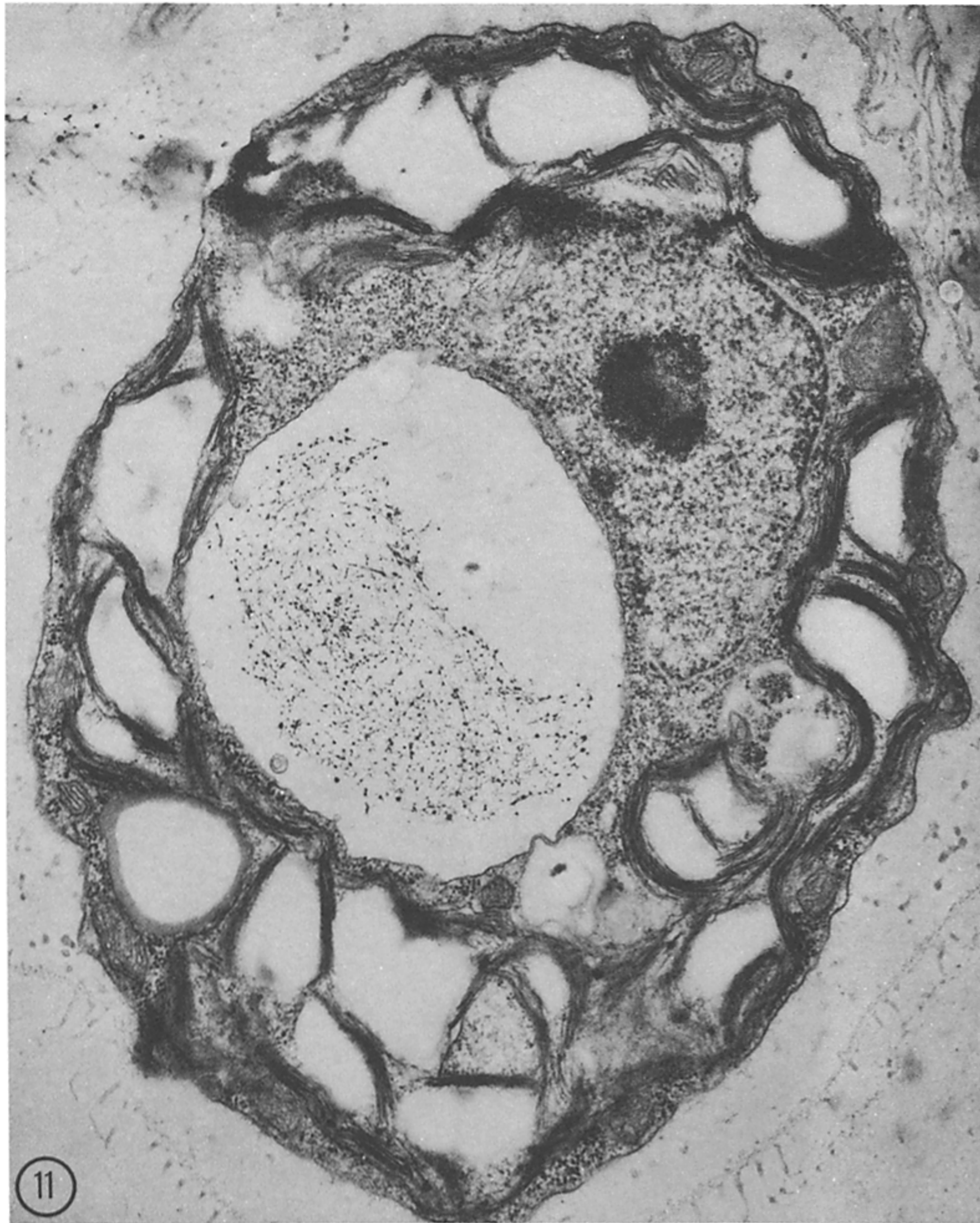


FIGURE 11 TAP plate gamete fixed after 5 days of growth on TAP-agar medium. A fiber-filled vacuole is found in the middle of the cell. × 24,000.

mentary by 1 h after transfer and are usually undetectable after 4 h. An increase in ribosomes associated with the nuclear membrane or "membrane spaces" is noticeable after 1 h and becomes increasingly apparent as differentiation proceeds.

GAMETIC VESICLES: Small gametic vesicles are first detected at 4–5 h after transfer to N-free medium (Fig. 13) and they increase in size throughout differentiation. Their appearance is preceded by a dramatic development in the size and apparent number of Golgi regions, and the vesicles are first found associated with Golgi membranes.

MATING STRUCTURES: The small size of the

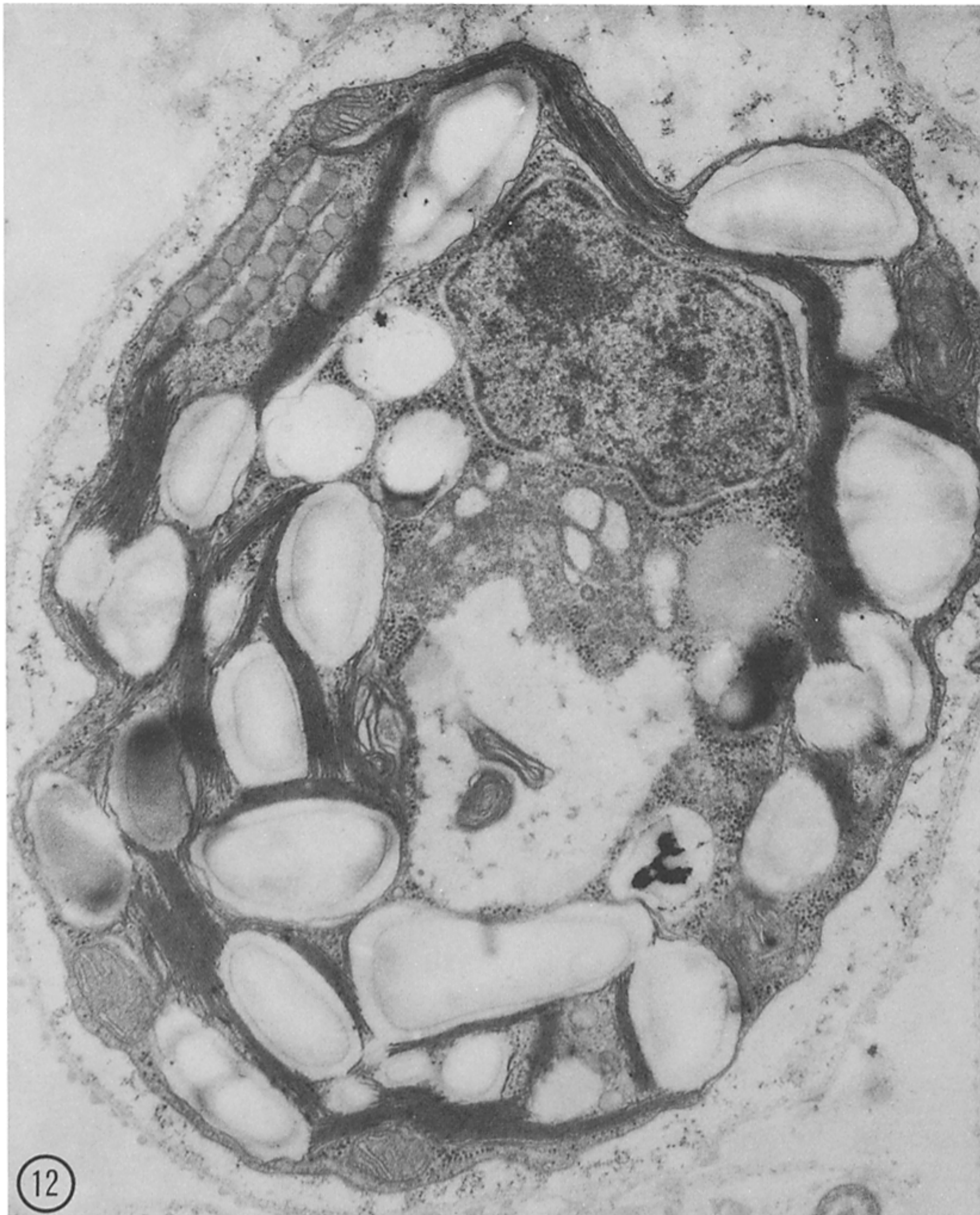


FIGURE 12 Plate gamete fixed after being maintained for 3 wk on a plate containing what was originally TAP-agar medium. × 38,000.

mating structures renders them relatively difficult to find even in sections of fully differentiated cells; therefore, a failure to find them in early stages of differentiation does not provide compelling evidence that they are absent. Nonetheless, an extensive search failed to reveal any mating structures in cells fixed before the gametic cell division. That the mating structure is probably assembled during the gametic mitosis is indicated by Fig. 14, where a mating body is found associated with the cleavage

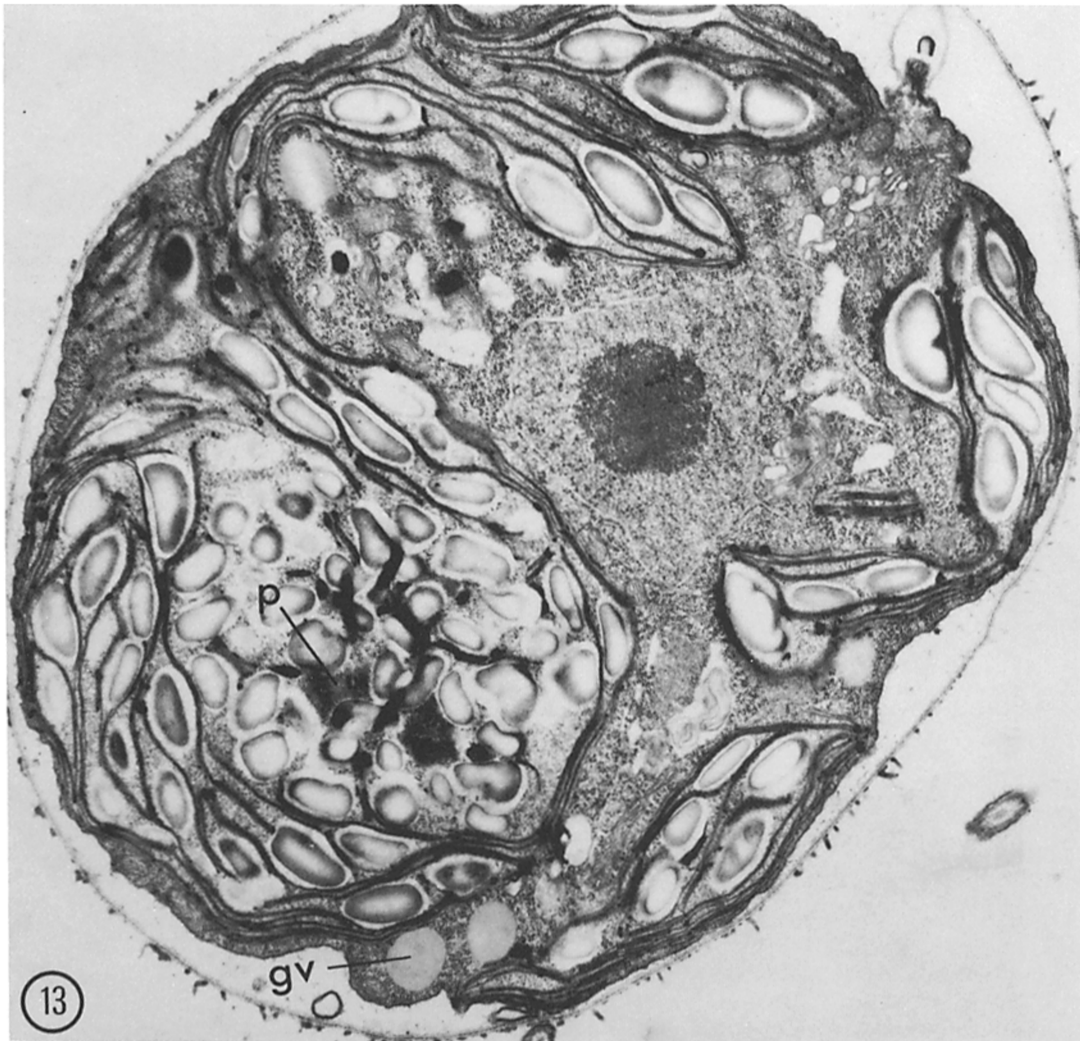


FIGURE 13 Liquid-grown light cell fixed 5 h after being deprived of nitrogen. *p*, Pyrenoid; *gv*, gametic vesicle. × 21,000.

furrow membrane (13) separating two daughter gametes.

DISCUSSION

Nitrogen starvation represents an extreme alteration in an organism's environment, one that presumably places cells under considerable metabolic stress, and one that might well be expected to have a number of effects on cellular integrity. Therefore, as Schmeisser et al. (19) have also pointed out, caution must be used in ascribing all the differences found between vegetative and gametic cells of *C. reinhardtii* to the development or maintenence of the gametic state; certain differences may be unrelated to gametogenesis.

In this context, we have found the following concepts useful. We first propose that nitrogen starvation triggers in *C. reinhardtii* a *degradative program* designed to utilize existing sources of nitrogen to best advantage. It is clear from the present study that such a program in no way brings about an indiscriminate autophagocytosis, since certain structures (inner mitochondrial membranes, microtubules, basal bodies, and cell membranes) are left morphologically intact while other structures (ribosomes, pyrenoids, thylakoids, ER,

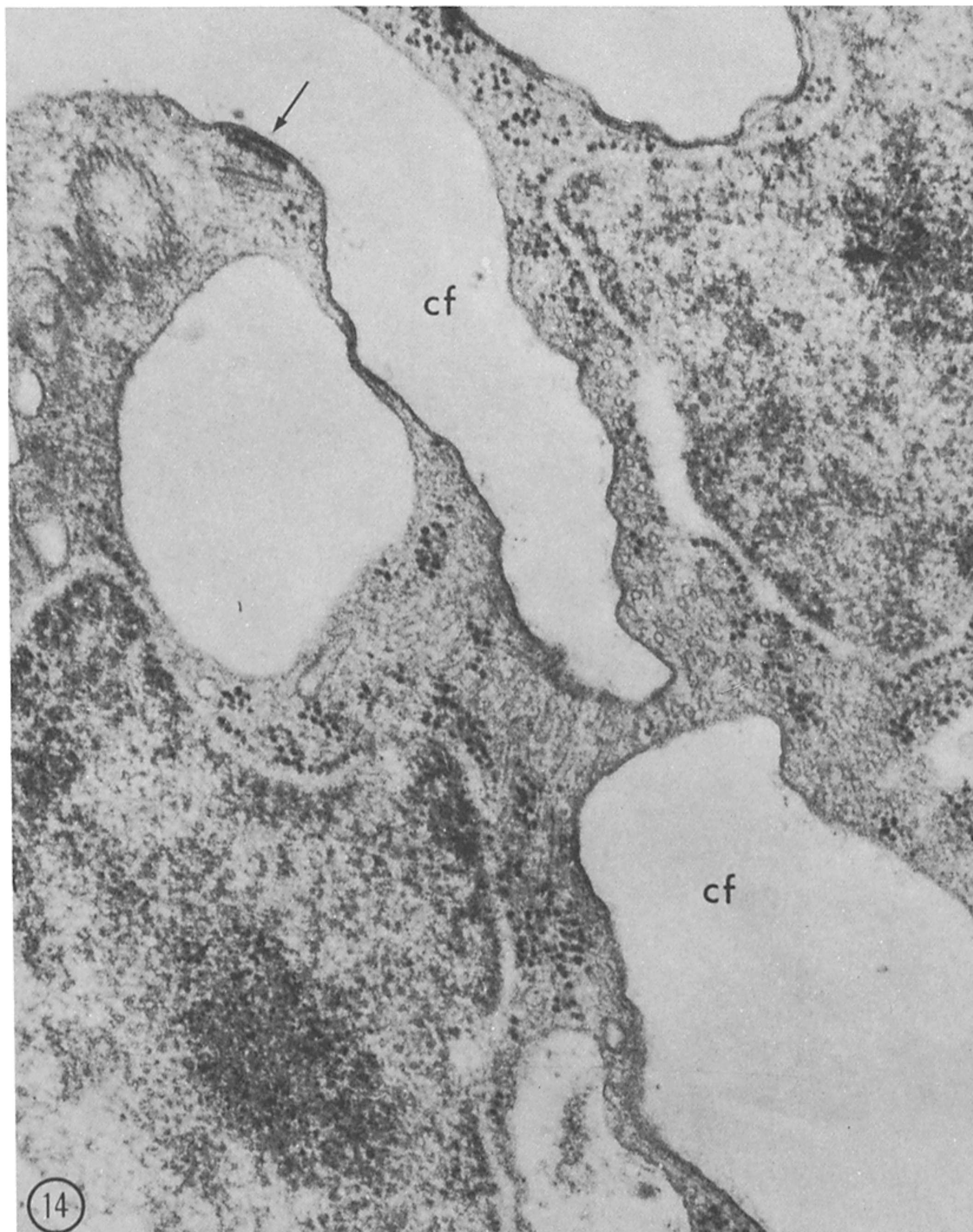


FIGURE 14 Liquid-grown light cells fixed 15 h after being deprived of nitrogen. Two daughter cells have just completed mitosis and remain connected by a strand of cytoplasm. Cleavage microtubules are evident along the cleavage furrow (*cf*). A mating structure (arrow) has formed in association with the cleavage furrow membrane. A basal-body associated microtubule (12) lies just beneath the mating structure. × 68,000.

and outer mitochondrial membranes) are being modified or broken down. It is therefore likely that the degradative program is carefully regulated, but it would also be expected to produce variant phenotypes depending on the rapidity with which nitrogen is removed, the availability of other nutrients and light, and so on.

We secondly propose that a *gametogenic program* is activated at some point between the withdrawal of nitrogen and the acquisition of mating ability which results in the production of new, gamete-specific phenotypes designed to bring about mating and zygote formation. These phenotypes would be expected to be less variant and less dependent on culture conditions. The gametogenic program might prove to be independently triggered by nitrogen starvation; alternatively, it might be triggered by some critical level of degradation or some degradative event.

Finally, our observations indicate the existence of a long-term gametic state in *C. reinhardtii*: cells that have completed gametogenesis but fail to find mating partners for zygote formation are capable of living for at least 3 wk as gametes on solid medium in the absence of nitrogen. Thus, the gametic stage of the life cycle appears geared not only to effect zygote formation and sexual reproduction, but also to provide an alternative to zygote formation so that survival is possible in a nitrogen-depleted environment in which sexual partners are lacking; such an environment might well be encountered by a mitotic clone of *C. reinhardtii* in its natural soil habitat. The development and maintenance of such a long-term gametic state presumably entail yet another form of cell specialization that might be termed a *gametic survival program*. This program could also be visualized either as being stimulated directly by nitrogen starvation or as being triggered by some manifestation of the degradative program.

We stress that in using such terms as "program" we are not implying any detailed mechanisms, and we realize that certain degradative events may, for example, constitute gametogenic events as well. The point is that if gametogenesis in *C. reinhardtii* is to be regarded as a model for studying eukaryotic differentiation, a conceptual separation of degradation from short-term and long-term differentiation is important to both experimental design and interpretation.

We have been unable to find any stimulus other than nitrogen starvation that will induce gametogenesis, and we have found no way to subject cells to an N-free environment and not induce gametic differentiation and, presumably, long-term gametic survival at the same time. Because we have found no way to separate these "programs" experimentally, we have had to rely on more inferential approaches to sort out what takes place between the time nitrogen is withdrawn, the time cells can mate, and the time cells become capable of surviving long-term nitrogen starvation.

One approach is to reject as essential to either degradation or gametic differentiation any morphological feature that is restricted to gametes prepared by only one protocol. Thus, the fiber-filled vacuoles of plate gametes, the cell wall-less state of unmated gametes reported by Friedmann et al. (6), and the four basal-bodied state of gametes reported by Friedmann et al. (6) are characteristic of only one kind of gamete and, therefore, presumably reflect peculiarities of particular growth regimes.

Similarly, one can accept as essential to gametic differentiation any features that are found in all gametes and whose role in the mating process is clear. Three such phenotypes can be cited: (*a*) the mating structure described by Friedmann et al. (6, 9); (*b*) the flagellar agglutinability of gametes described by Wiese (23) and in an accompanying paper (1); and (*c*) the lysis of cell walls when gametes of opposite mating types are mixed, a reaction reportedly catalyzed by an enzyme called endolysin (4; see also reference 9). A fourth phenotype, the possession of gametic vesicles, is shown in the present study to be a new feature acquired during all types of gametogenesis, but until the contents of the vesicles are known, it is not possible to state whether they relate to gametogenesis, degradation, and/or long-term gametic survival.

The remaining morphological changes described in this paper require more discussion.

STARCH: Starch accumulation has been shown to be a common feature of nitrogen starvation in various algae (22). The considerable variation in starch content observed in gametes prepared by different methods argues against a direct relationship between starch accumulation and mating ability. On the other hand, starch accumulation may well be a manifestation of the gametic survival program in that 3-wk old plate gametes and gametes maintained in liquid culture for 36–48 h contain considerably less starch than their newly

differentiated gametic counterparts (unpublished observations). If this proposal proves to be correct, then the long-term gametic survival program must be operative shortly after nitrogen is withdrawn, since starch accumulation is shown in the present study to commence almost immediately thereafter.

MEMBRANE BREAKDOWN AND REORGANIZATION: The protein-rich intracellular membranes seem logical targets for a degradative program geared to mobilizing nitrogen, and the thylakoids of the chloroplast are particularly likely candidates for degradation since the cells greatly reduce their overall photosynthetic capacity during the course of gametogenesis. We propose, therefore, that the various changes observed in chloroplast membrane organization reflect a degradative process and are not in themselves necessarily significant to gametic differentiation. The wide variability seen in thylakoid organization from one kind of gamete preparation to the next supports this concept.

The modifications that occur in other cellular membranes (mitochondrial, ER, and nuclear) are more subtle: the resulting membranes are still present and in their usual positions but are only rarely preserved by fixation, regardless of the fixative used (see also reference 6), perhaps because of a dearth of proteins available for cross-linking. Again it seems reasonable to propose that such modifications relate to a degradative program, at least in part. Whether the modified membranes have altered functional properties has not been determined.

NUCLEAR HETEROCHROMATIZATION: Sparse heterochromatin is found in liquid-grown light-dark gametes that mate with 100% efficiency, indicating that gametic differentiation per se can proceed without a detectable change in the morphology of nuclear chromatin. In contrast, "old" gametes that have been on agar plates for 3 wk exhibit highly heterochromatic nuclei; since these gametes have been in stationary phase for several weeks, it cannot be argued that they are preparing for, or emerging from mitosis. This heterochromatization may well reflect a reduction in gene activity, dictated by the gametic survival program, which allows gametes to remain semidormant in the prolonged stationary phase that follows nitrogen starvation. Such a proposal, which we are currently testing, implies that the gametic survival program may be quite as sophisticated and interesting as the program leading to the ability to mate.

RIBOSOMES: Table I indicates the existence of considerable variation in the extent to which ribosomes are reduced in thin sections of various types of gametes. Sampling error does not appear to be a major factor contributing to this variation since the numbers obtained in repeated experiments fall within the same range. A more important question is whether differences in cell volume are a factor: a given number of ribosomes spread out over a large cytoplasmic volume, for example, would exhibit a lower density in thin section than this number in a small volume. Therefore, while we conclude from this study that at least some loss of cytoplasmic and chloroplast ribosomes accompanies all forms of gametogenesis, we hesitate to emphasize quantitative differences based on the cut-and-weigh assay except in the case of liquid-grown light and light-dark gametes. Here the two types of cells appear generally indistinguishable in size and morphology, yet the light-dark cells have many more ribosomes. This "less drastic" phenotype of the light-dark gametes parallels the more moderate appearance of these cells in a number of other respects. Thus, we would propose that whereas gametogenesis is always accompanied by a loss of ribosomes, the extent of this loss may depend on the mode of gametogenesis that occurs.

Ribosome breakdown during gametogenesis would clearly mobilize nitrogen for utilization in other biosynthetic processes (20), and would thus appear to be a likely candidate for the postulated degradative program. Ribosome breakdown has been found, however, to be accompanied by a synthesis of new ribosomes throughout the differentiation period (11). This finding, to be reported in detail elsewhere,[1] leaves open the possibility that there may be some features of ribosome turnover that are important to gametic differentiation and/or to the long-term survival of gametes.

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