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The Comparative Ultrastructure and Possible Function of Eyespots: *Euglena granulata* and *Chlamydomonas eugametos** ** ***

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Summary. The structure of *Euglena granulata* and *Chlamydomonas eugametos* has been studied using polarization and electron microscopy, cinematography, and chemical extraction procedures, with the main focus on the structure of the eyespot.

The 50—60 granules which form the extrachloroplastic eyespot of *E. granulata* are large bodies, up to 1200 mμ in diameter. They are found in the cytoplasm near the base of the reservoir and are associated with the parabasal body which contains a large crystal. The eyespot granules are contained within membranes having a unit membrane structure; 2 or 3 are usually present in a single eyespot packet; microtubules are also contained within the packet. The eyespot granules have the structure of a positive spherite and clearly exhibit birefringence; this structure is modified by fixation.

The granules of the chloroplastic eyespot of *C. eugametos* are about 75 mμ in diameter and are contained within the chloroplast in an ordered array. Occasionally, the eyespot contains elongate or helical bodies mixed with the granules. Extraction with organic solvents caused the removal of materials which formed the eyespot granules as well as that of the osmophilic globules in the chloroplasts.

Several hypotheses which concern the function of the eyespots in these and other species are discussed in the light of our results. The possible origin and demise of the eyespot granules are also discussed.

Introduction

The structure and function of the eyespot present in many microorganisms have aroused interest and speculation among microscopists since before the turn of the century. A number of early investigators noted the similarity in the color of the eyespot to that of the true eyes of certain lower animals and assumed parallels in function.

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STRASBURGER (1878) observed, however, that some eyespot-less flagellates were sensitive to light and suggested that reactivity to light was a property of the entire protoplasm. In experiments with *Euglena*, ENGELMANN (1882) noted that light had no effect on the organism until it impinged on the region of the eyespot.

MAST (1927) postulated that the eyespot was the photoreceptor involved in directing flagellar movement, and hence cell movement, and suggested increasingly complex eyespot structure in the progression from unicellular to colonial organisms.

HARTSHORNE (1953) concluded from studies with *Chlamydomonas* that perception of light was not restricted to the eyespot, although the latter undoubtedly had a role in controlling phototactic responses. More recently, HALLDAL and WOLKEN have come to alternative conclusions as to the role of the eyespot. HALLDAL (1958, 1964) suggests that the eyespot has an auxiliary role in phototaxis, whereas WOLKEN (see WOLKEN, 1967 for a summary of his views) postulates that the eyespot is the principal photoreceptor for light perception; LEEDALE (1967) has summarized the arguments for both views.

BATRA and TOLLIN (1964) isolated eyespot granules of *Euglena gracilis* and found a close correlation between the absorption spectrum of the isolated granules and the action spectrum for positive phototaxis. They also identified some of the carotenoid pigments of the eyespot granules as lutein, cryptoxanthin, and β-carotene.

Ultrastructural investigations of eyespots of algae have been carried out by MANTON and CLARK (1956), SAGER and PALADE (1957), LANG (1963), LEMBI and LANG (1965), LEEDALE et al. (1965), WALNE and ARNOTT (1966) and ARNOTT and BROWN (1967). These studies have not substantiated the eyespot structure proposed by earlier workers such as MAST (1927).

The purposes of the present investigation were, (a) to compare the structure of chloroplastic and extrachloroplastic eyespots, in the hope that a better understanding of the structure may provide insight into the role of the eyespot as a primary or auxiliary photoreceptor; (b) to compare the eyespot granules with the dense osmophilic globules observed in chloroplasts, in the hope of elucidating the origin of eyespot granules; and (c) to compare eyespot granules to the paraflagellar body in *Euglena*, in order to ascertain whether the two are identical in structure and composition.

Materials and Methods

Cells of *Euglena granulata* (KLEBS) Lemm. were obtained from a water bloom in a pond on the University of Texas campus. Identification and single-cell isolates of the organism were made by Professor HAROLD C. BOLD. The resulting clonal cultures were grown in enriched soil-water medium.

Axenic cultures of *Chlamydomonas eugametos* MOEWUS were maintained on agar slants of BOLD's Basal Medium (BBM) (WALNE, 1966) solidified with 1.8% agar, and were transferred to liquid BBM to induce motility about 1 hour prior to fixation for electron microscopy.

The condition and configuration of the eyespot in living cells were studied by use of polarization and phase contrast microscopy and cinematographic procedures utilizing a Kodak 16 mm camera. The Zeiss polarization microscope was equipped with a rotary mica compensator which proved to be essential in the determination of the optical character of the eyespot granules.

Cells of both organisms were subjected to a variety of fixation procedures for electron microscopy, and the following were selected as most satisfactory. *Euglena* was fixed in 3% acrolein plus 3% glutaraldehyde in 0.2 M cacodylate buffer, followed by post-fixation in 2% osmium tetroxide using the same buffer (HESS, 1966). *Chlamydomonas* was fixed for 1 hour in 5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, followed by post-fixation in either 2% osmium tetroxide using the same buffer, or in unbuffered 2% aqueous potassium permanganate. Materials were dehydrated and then embedded in a mixture of Araldite and Epon (MOLLENHAUER, 1964), and standard procedures were followed in their preparation for observation with RCA EMU 3-F and Siemens Elmiskop I electron microscopes.

In addition to the observations on untreated cells, we also studied the ultrastructure of cells from which the lipo-carotenoid components of eyespot granules had been extracted with a variety of solvents, namely, acetone, benzene, carbon-disulfide, chloroform, ethyl alcohol, ethyl ether, methanol, methanol/acetone. To prevent loss of structure, cells were fixed in cacodylate-buffered glutaraldehyde and rinsed in the same buffer prior to extraction. Following extraction for 15 minutes the cells were rinsed again with buffer, post-fixed in 2% osmium tetroxide, and after this they were processed like the controls.

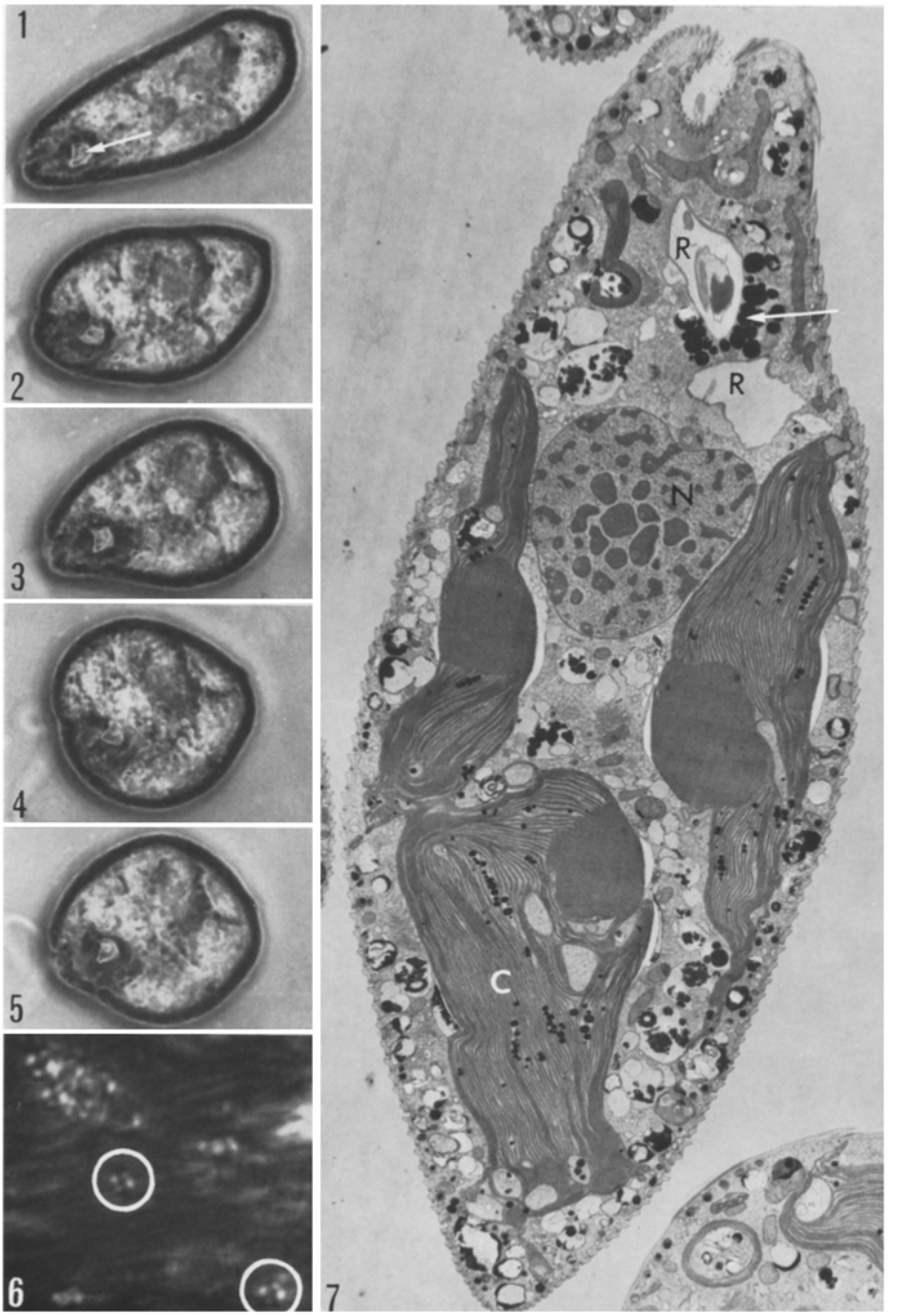
Absorption spectra of the extracted pigments were obtained with a Beckman DB Recording Spectrophotometer. On the basis of spectral data and in conjunction with the degree of fluorescence exhibited under ultraviolet inspection, certain extracted materials were selected for intensive investigation by electron microscopy.

Observations

1. Euglena granulata

a) Eyespot Structure in Untreated Cells. A typical cell of *E. granulata* is spindle-shaped with a rather blunt anterior end and ranges in size from approximately 75—100 μ in length and 25—30 μ in width (GOJDICS, 1953) (Figs. 1—5, 7). Light microscopy shows that the eyespot is a conspicuous extra-chloroplastic organelle. It is located in the cytoplasm at one side of the base of the reservoir in a subanterior to median position within the cell and is always found in close association with the parabasal body (the "flagellar swelling" of earlier investigators). Cinematographic and time-lapse studies show that the curved eyespot can move freely in the cytoplasm at the base of the reservoir as the organism changes shape during metaboly (Figs. 1—5). Its movement seems to be correlated with that of the parabasal body.

The eyespot in living cells usually measures 7×8 μ and consists of a cup-shaped group of orange-red granules. These stain blue-black after



Figs. 1—7 (for Legends see p. 329)

application of iodine (IKI) solution, this being a typical carotenoid reaction. When living cells that have been flattened or squashed are viewed under crossed nicols each granule exhibits a birefringent pattern, forming a cross similar to that produced by starch grains. This indicates that each granule has the ordered internal structure of a spherite (Fig. 6). Using a rotary mica compensator it was possible to determine that the optical character is positive. At the same time it was determined that the paramylon grains also exhibit the characteristics of a positive spherite. When fixed and embedded in plastic and sectioned for light microscopy the eyespot granules no longer exhibit this pattern of birefringence.

As seen with the electron microscope the eyespot consists of a series of dense osmophilic bodies. Some of these bodies are associated with membranes and with microtubules. From 3 to 5 granules may be contained within a common membrane, forming a packet (Figs. 9, 10, 12, 13). The membrane bounding the eyespot packet, the packet membrane, is single and has the morphology and dimensions of a "unit membrane", its tripartite construction being evident in numerous sections (Figs. 10, 11). Some eyespot granules are not contained in packets and are free of membranes; the eyespot as a whole has no bounding membrane.

In contrast to other eyespots which we have studied (e.g., Figs. 20 and 21), the arrangement of granules in *Euglena* is neither linear nor uniseriate; in longitudinal section, 15—20 granules are grouped together, either in packets or singly, to form a curved or irregular body (Figs. 7,8). Tangential sections show that the eyespot may consist of as many as 50—60 granules (Fig. 9). Because of the close packing and somewhat irregular arrangement of the granules, the best estimates of their size are

Figs. 1—5. A series of negative enlargements (positive reversals) of original 16 mm movie film showing a *Euglena granulata* cell undergoing changes of shape during metaboly. Arrow indicates eyespot. ×1200.

C = Chloroplast
CB = Connecting body
CG = Chloroplast osmophilic globule
CV = Contractile vacuole
E = Eyespot or eyespot granule
EP = Eyespot packet
ER = Endoplasmic reticulum
F = Flagellum
FC = Flagellar crystal
G = Golgi apparatus
M = Mitochondrion
N = Nucleus
P = Pyrenoid
PM = Packet membrane
R = Reservoir
RM = Reservoir microtubules
T = Tubules within the eyespot packet
S = Starch
W = Cell wall

Fig. 6. Eyespot granules (encircled) of *Euglena granulata* under crossed nicols showing spherite birefringence. ×5400

Fig. 7. Electronmicrograph of typical cell of *Euglena granulata*. Arrow denotes eyespot granules and the adjacent reservoir in which the parabasal body can be seen. ×5250

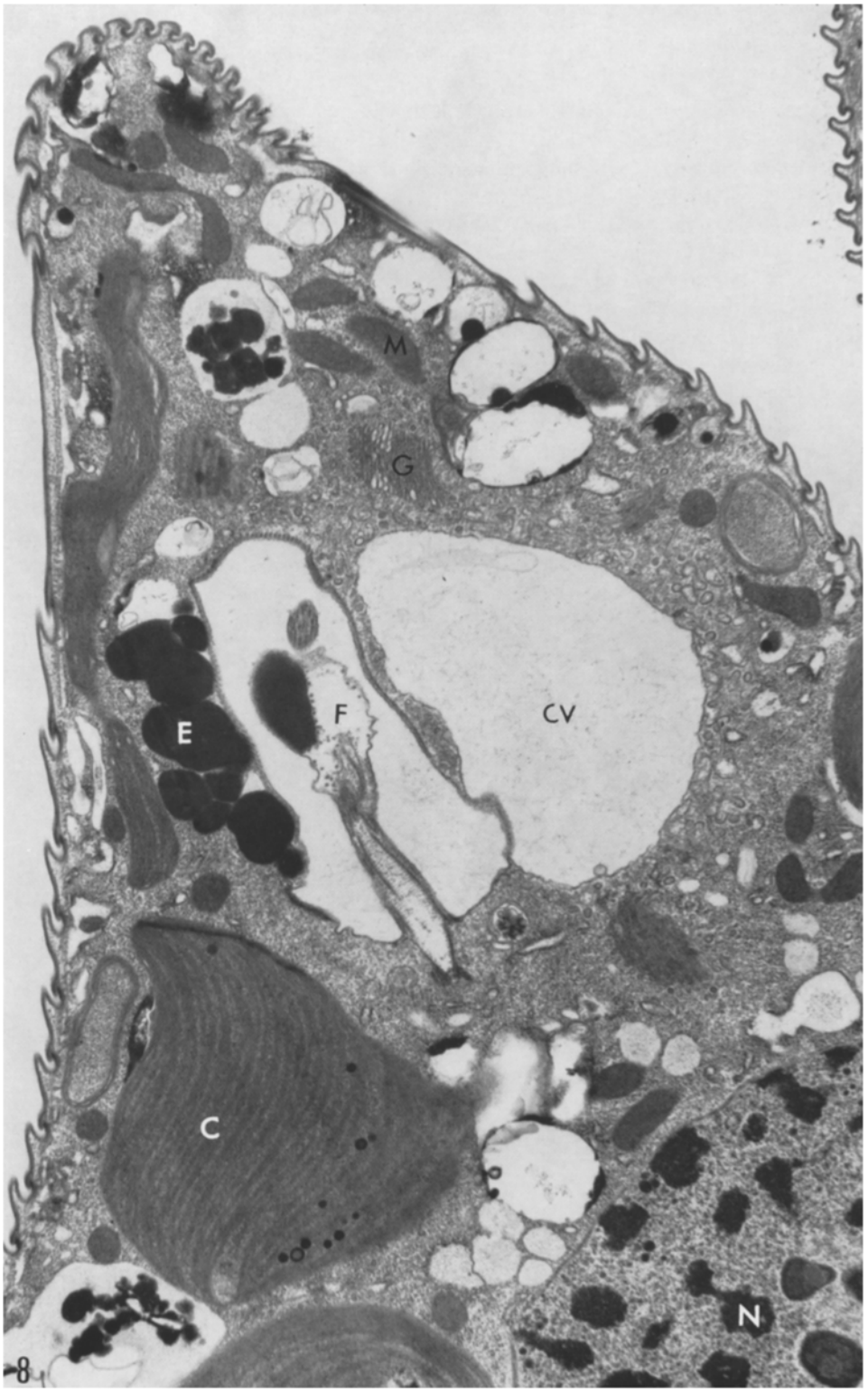


Fig. 8 (for Legend see p. 331)

based on measurements made from tangential sections. Such measurements show variation in diameter from 240 to 1200 mμ (Figs. 9, 10, 12, 13). Although some differences in granule size may be attributed to the planes or levels of sectioning, we believe that real size differences do exist; some of the sizes may represent ontogenetic or degenerative stages.

Mature granules show a fine reticulate pattern; in certain areas this may approximate a crystal lattice (Fig. 11). However, since fixation changes the pattern of birefringence, in fixed granules we can only expect to see a part of the structure present in the living state. Even the ordered areas in the granules must therefore be subject to question.

The eyespot granules appear to develop from the fusion of small, often polygonal or hexagonal particles found within the packet (Figs. 9, 10, 12). The origin of the packet membrane and of the small granules which fuse to form the eyespot granules is not certain. However, in many cases the endoplasmic reticulum appears to be closely associated with the developing eyespot packets. The eyespot granules which result from this fusion may appear both spherical-ovoid and polygonal (Figs. 9, 12).

A number of curved microtubules may be seen within the packet membrane (Fig. 12). These tubules are about 260 Å diameter but do not appear to have the same substructure consisting of 13 subunits that has been reported in higher plants (LEDBETTER and PORTER, 1964) or as seen in other microtubules of *Euglena granulata* (WALNE and ARNOTT, 1966). An ordered array of microtubules, in the latter sense, is associated with the reservoir; these measure ca. 240 Å in diameter and are spaced with regularity just inside the plasmalemma (Figs. 9, 12). A single contractile vacuole can be observed in close proximity to the reservoir; a specialized portion of the reservoir wall allows for the exit of materials from the contractile vacuole (Fig. 8). Coated vesicles, apparently originating from the Golgi apparatus, can be seen in all stages of fusion with the membrane of the contractile vacuole (Fig. 8).

The parabasal body lies near the base of the exserted flagellum and is closely associated with the eyespot (Figs. 7, 8, 16). It contains the flagellar axis which runs through the swelling and two other major elements, a large crystal and a connecting body which seems to interconnect the flagellar axis with the crystal; all these structures are surrounded by the plasmalemma which forms the continuous surface of the flagellum.

A large crystal fills the greater portion of the parabasal body. It has a lattice with a repeat pattern of ca. 75 Å (Fig. 16). Because of these

Fig. 8. Anterior end of *Euglena granulata* cell showing non-linear arrangement of packets of granules to form eyespot (*E*), the reservoir containing flagella and the parabasal and connecting bodies. Note specialized portion of reservoir wall adjoining the contractile vacuole and coated vesicles in all stages of fusion with the membrane of the contractile vacuole. ×13,300

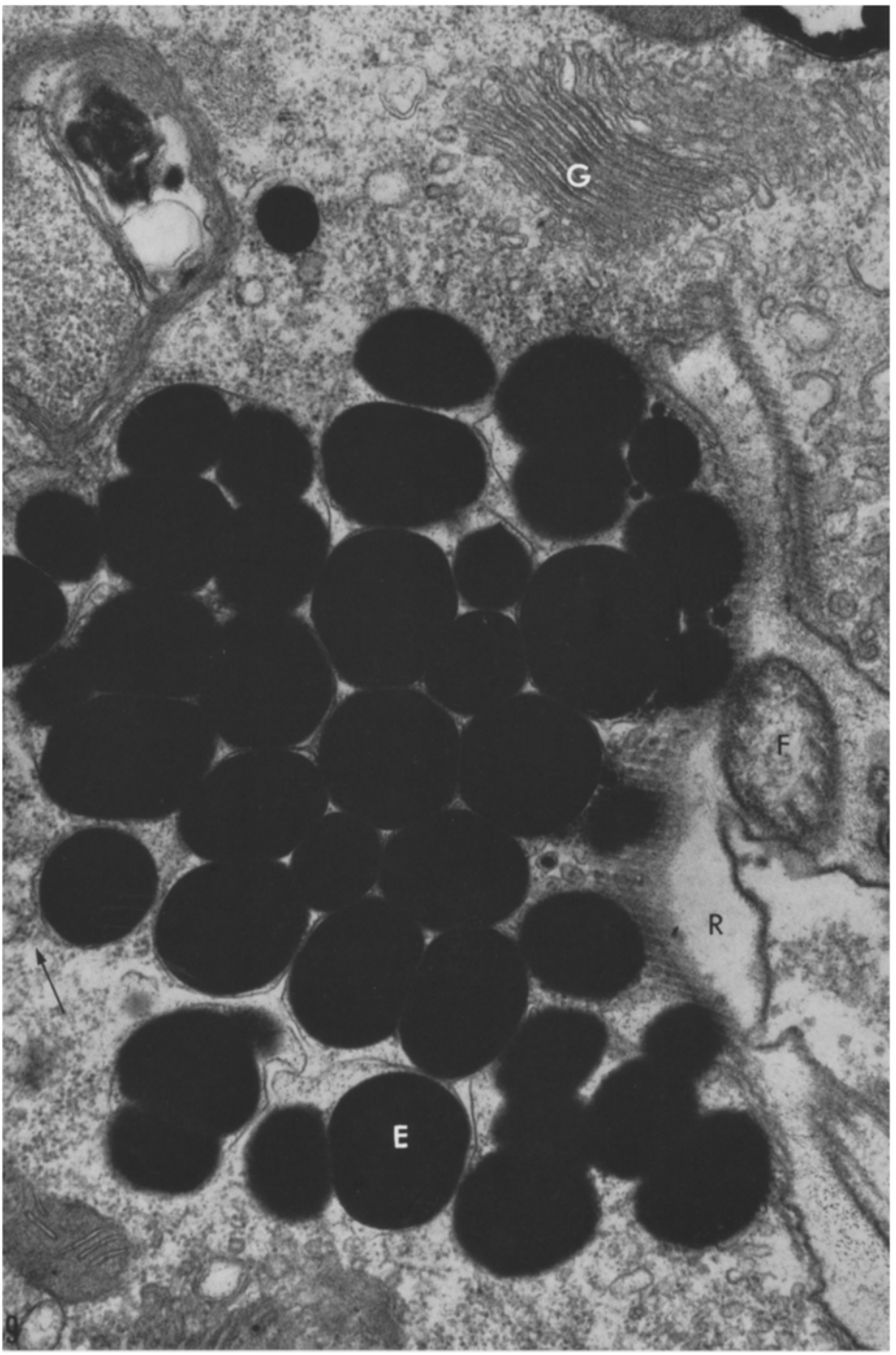


Fig. 9 (for Legend see p. 333)

large lattice parameters the crystal may be partly protein. The connecting body is made up of a series of membrane-like components (Figs. 8, 16). Similar structures in this region have been reported in other euglenoids (LEEDALE et al., 1965; ARNOTT and WALNE, 1966; MIGNOT, 1966).

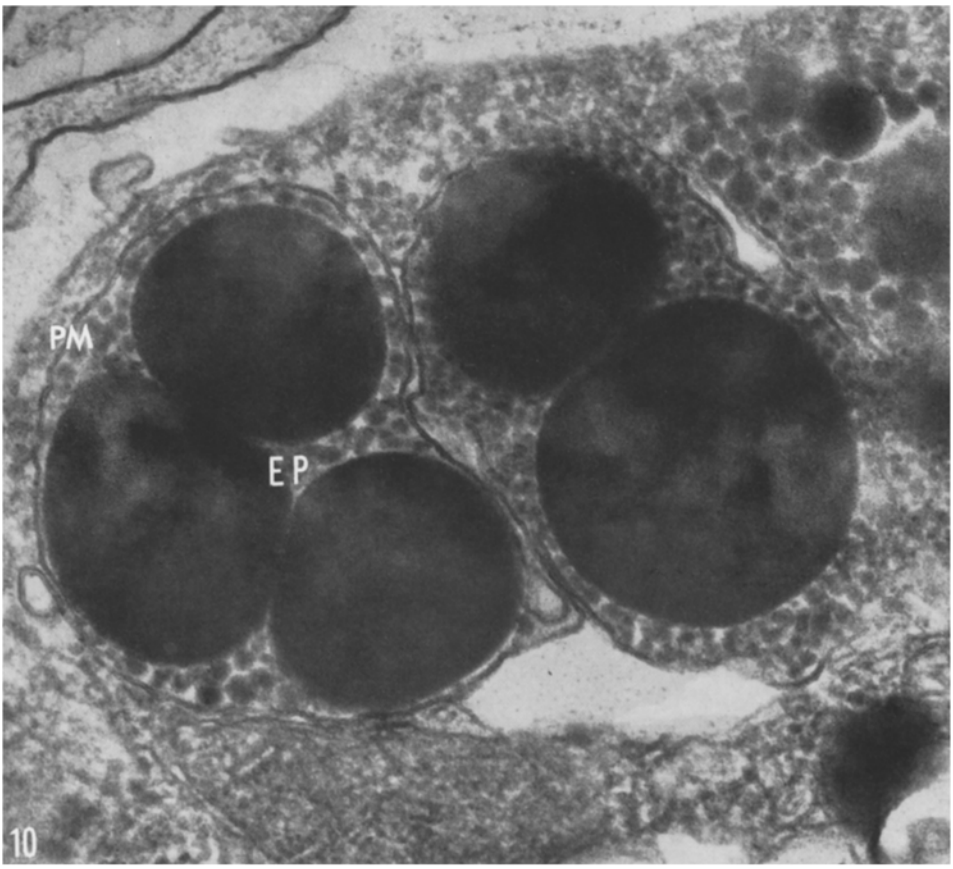
Because of the extra-chloroplastic nature of the *Euglena* eyespot, we are not primarily concerned with the chlorplasts in this organism, as we will be in *Chlamydomonas* which has a chloroplastic eyespot. However, the dense osmophilic granules commonly seen in rows between the chloroplast lamellae should be mentioned (Figs. 14, 15). In density and substructure, but not in size, these granules are very similar to the granules composing the eyespot and may possibly also contain carotenoids. In longitudinal section, they are aligned as are the eyespot granules in *Chlamydomonas* and differ only in that they occupy an internal rather than a peripheral position in the chloroplast. In tangential section, their arrangement, plate-like appearance, and occasional polygonal configurations approximate those of the eyespot in *Chlamydomonas* and other organisms with chloroplastic eyespots (compare Figs. 14 and 21).

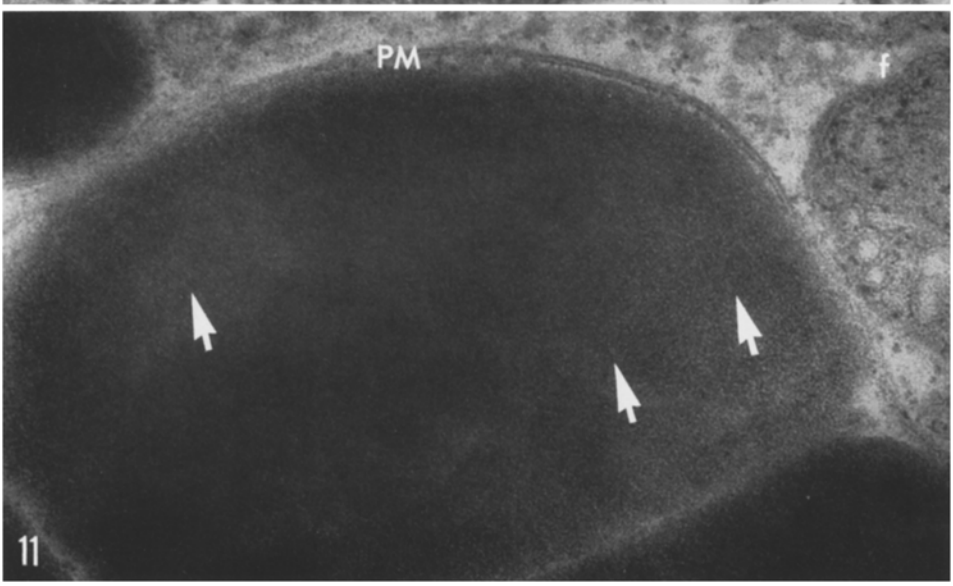
b) Structure of Cells after Extraction. After stabilization with glutaraldehyde the cells were extracted with various organic solvents, and the absorption spectra of the extracts as well as their fluorescence under ultraviolet light (360 mμ) were determined. All of the *Euglena* extracts had very similar absorption spectra (Fig. 17). Very strong, bright-red fluorescence was shown by the acetone and chloroform extracts. Benzene extracts had a pink fluorescence; ethyl-ether extracts showed very little fluorescence and carbon-disulfide extracts none at all.

From the absorption spectra and fluorescence data it can be seen that the solvent extracts contained primarily chlorophyll and carotenoid pigments, as might be expected with whole-cell extracts of green organisms. Ideally and for complete accuracy, data should be obtained from isolated eyespot granules, and such studies are in progress. In this investigation, however, our intent was to compare the ultrastructure of the intact eyespot granules before and after extraction in order to compare them with the parabasal body and with the osmophilic globules in the chloroplast.

Because of the very different fluorescence properties of chloroform and carbon disulfide extracts, which effect extraction of different pigments, *Euglena* cells which had been extracted by these two solvents were selected for electron-microscopic investigation. Despite the widely different fluorescence in chloroform and carbon-disulfide extracts the effects of the two solvents on the ultrastructure of the cells were quite similar.

Fig. 9. Tangential section of eyespot of *Euglena granulata* showing 50—60 polygonal-hexagonal granules, many arranged in packets of 3—5 granules. Microtubules are seen in association with the reservoir. ×34,000





Figs. 10 and 11 (for Legends see p. 335)

The effect of chloroform extraction on the ultrastructure of *E. granulata* cells are shown in Figs. 18 and 19. The most obvious and severe ones are the disappearance of the chloroplast lamellae, although changes may also be noted in the parabasal body and in some eyespot granules. Much of the granular matrix and many of the delimiting membranes have also disappeared although some membranes can still be seen, i.e., the plasmalemma and the chloroplast envelope. The eyespot granules may be either absent and replaced by a clear zone or the granules may have become confluent and only partly extracted (Fig. 19).

The parabasal body and eyespot granules which had certain similarities in structure and density prior to extraction exhibited considerable differences afterwards. The most obvious difference is in density, and in contrast to the eyespot material, which appears amorphous or absent, the parabasal body retains a semblance of crystallinity or ordered internal structure. It, however, stains less densely and apparently some extraction has taken place.

2. *Chlamydomonas eugametos*

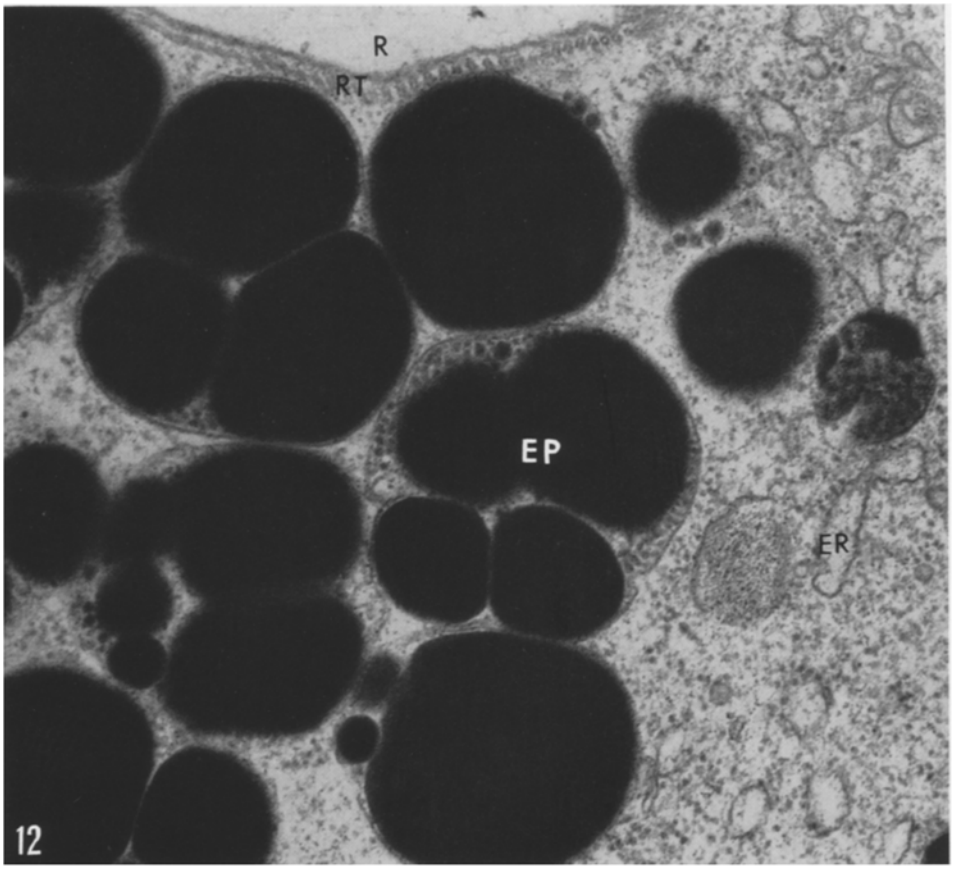
a) Eyespot Structure in Untreated Cells. The vegetative cell of *C. eugametos* is biflagellate, ellipsoidal-subcylindrical, and varies in size from 9—12 μ in length and 4—6 μ in width. With light microscopy, the sublinear, anterior-median stigma can be seen in the periphery of the large, cup-shaped, parietal chloroplast. The longitudinal axis of the eyespot lies in a plane with the long axis of the cell and the anteriorly extended flagella but, unlike *Euglena*, not in close association with the flagellar bases or contractile vacuoles.

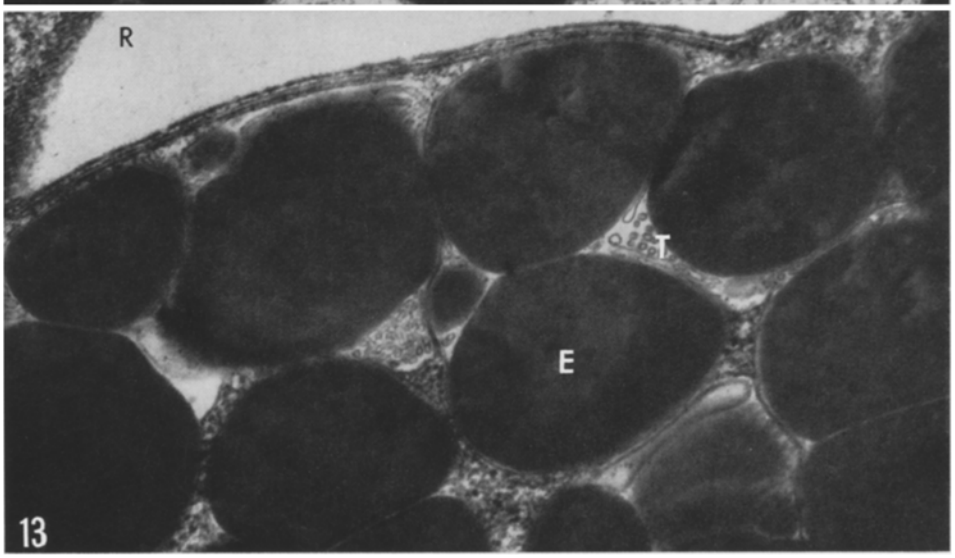
Although quite small (2—3 μ long), the orange-red eyespot is clearly observable in the optical microscope. Because of its small size, however, no staining or polarization studies were successful, and the structure appears to be a single body at this level of investigation.

Electron microscopy reveals the eyespot, in longitudinal section, to consist of a single layer of closely appressed, electron-dense granules of variable number, ca. 15 to 45. It is slightly convex, situated immediately below the plasma membrane and the chloroplast envelope, and is subtended by a chloroplast lamella (Fig. 20). In tangential section, the eyespot appears as a single paracrystalline plate consisting of polygonal granules of variable density which is probably due to the plane of section of the individual granules (Fig. 21). A similar number and configuration of

Fig. 10. Packets of eyespot granules (*EP*) in *Euglena granulata* showing clearly the tripartite arrangement of the packet membrane (*PM*). Numerous small granules within the membranes may be fusing to form larger granules. ×99,000

Fig. 11. High magnification micrograph of one eyespot granule of *Euglena granulata* showing reticulate substructure (arrows) approximating a crystal lattice. Also shown is the tripartite nature of the packet membrane. (*PM*). ×128,000





Figs. 12 and 13 (for Legends see p. 337)

granules has been reported for the eyespot of other unicellular algae, including other species of *Chlamydomonas* (ARNOTT and BROWN, 1967). Based on the number of granules seen in various planes of section our data suggest that about 100 granules are present in the eyespot of this species with possible variation from about 70 to 120.

Individual granules vary in size from 75 to 100 mμ. As in *Euglena*, some of the size differences may be attributed to the plane of section, but others are undoubtedly real and may represent stages in development or degeneration. Although granules are spherical or appressed laterally in longitudinal section, they are clearly polygonal (usually hexagonal) in tangential view; many granules exhibit hexagonal close- packing. The granules as seen in high-resolution consist of a fibrous reticulum in an irregular or unordered configuration (Fig. 21, inset).

The eyespot components of some cells of *Chlamydomonas* are very different from those normally observed. In place of the usual spherical granules, the eyespot consists of mixtures of granules, plates, and irregular curving bodies (Figs. 22—24). The proportion of granules to the alternate types of structures varies from cells having mostly granules (Fig. 24) to those having only a few granules (Fig. 22). Sometimes long rod-shaped bodies are seen mixed with the granules (Fig. 23). The general nature of these structures is very similar to those reported in chromoplasts where it is commonly stated that these represent crystalline deposits of carotenoids.

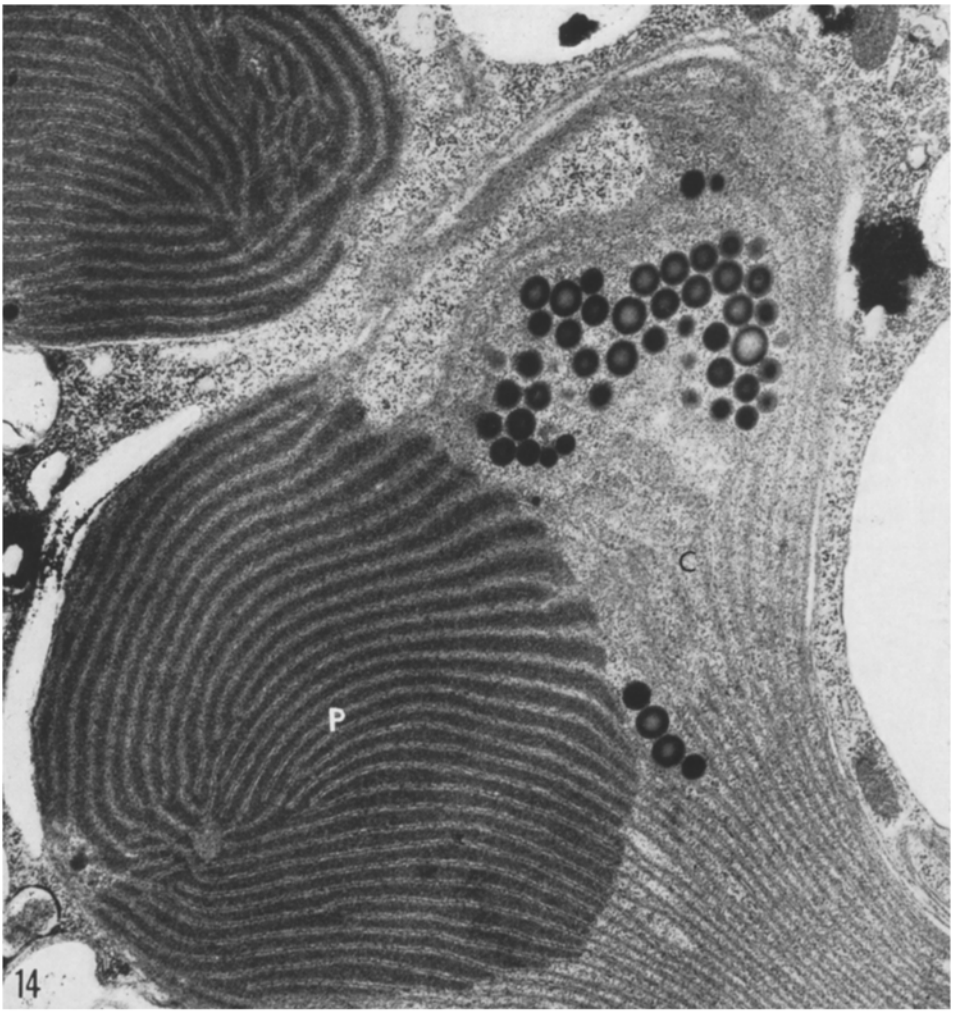
In *Chlamydomonas*, unlike *Euglena*, neither individual nor groups of eyespot granules are membrane bound. However, small fibrils like those in the reticulum extend between granules and possibly serve to interconnect those adjacent to one another (Fig. 21). The eyespot is associated with the subtending chloroplast lamellae and the overlying chloroplast envelope and plasmalemma.

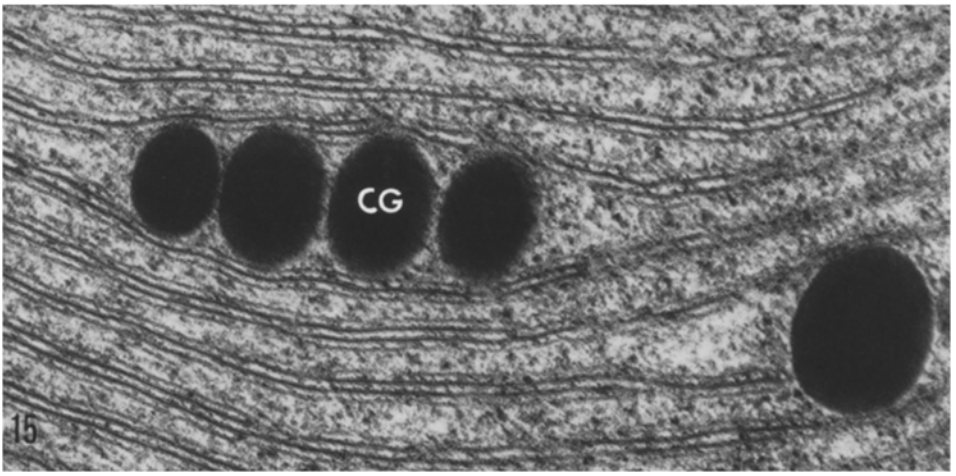
Modifications of the plasma membrane occur in the eyespot region. Not only is the membrane slightly thicker (100—120 Å) than its usual unit-membrane dimension of 70—80 Å, but also it protrudes in a slightly convex fashion within the confines of the cell wall, as it covers the convex eyespot area (Fig. 20).

Some modification in the chloroplast envelope is also apparent where it covers the eyespot granules. Like the plasmalemma, the chloroplast membranes also conform to the eyespot convexity and protrude slightly in this region. As described by earlier workers, the space between the eyespot and the periphery of the cell is clear. In many other areas of the

Fig. 12. Several packets of polygonal-hexagonal eyespot granules in *Euglena granulata*. Small granules in some cases appear to be fusing. Microtubules (*RM*) are seen in association with the reservoir (*R*). ×46,200

Fig. 13. Eyespot packets of *Euglena granulata* within which are seen "microtubules" (*T*) of ca. 260 Å diameter. ×41,600





Figs. 14 and 15 (for Legends see p. 339)

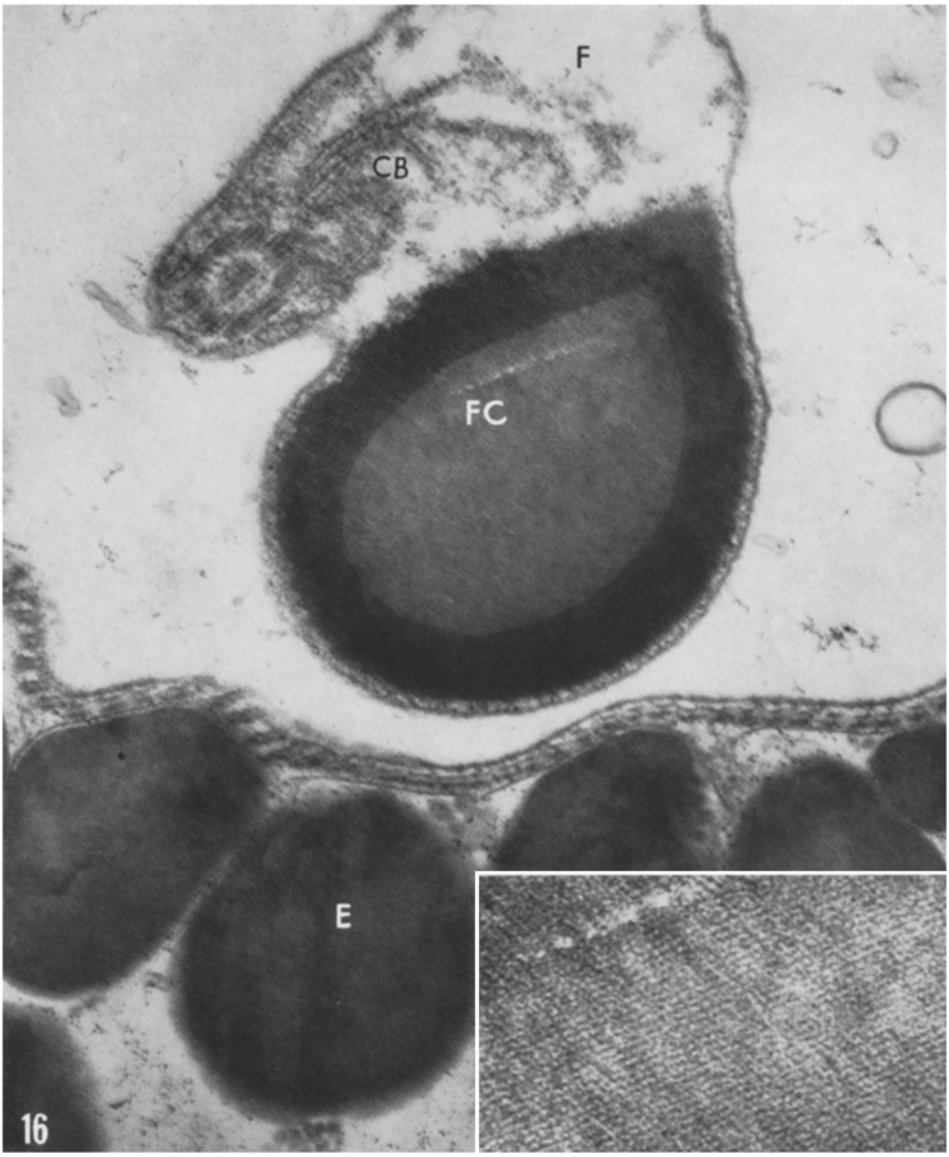


Fig. 16. Reservoir region of *Euglena granulata* showing eyespot granules (*E*), flagellar crystal and connection body. ×66,000. Inset shows crystal lattice substructure of flagellar crystal with 75 Å repeat pattern. ×160,000. The central portion of the crystal was printed with a mask in order to demonstrate more clearly the structure

Fig. 14. Portion of *Euglena granulata* chloroplast showing pyrenoid (*P*), linear arrangement of 4 osmophilic globules, and a tangential view of many other chloroplast osmophilic globules. ×35,100

Fig. 15. Interlamellar osmophilic globules in chloroplast of *Euglena granulata*. ×81,000

cell, the chloroplast envelope and plasmalemma are often closely appressed, but in the eyespot region, no contiguity of the two has been observed (Fig. 20). Although ribosomes and an occasional strand of ER may be seen immediately below the plasmalemma in other regions of the cell, no organelles occur in the area between the granules and cell periphery. ARNOTT and BROWN (1967) have found a similar situation in *Tetracystis excentrica*.

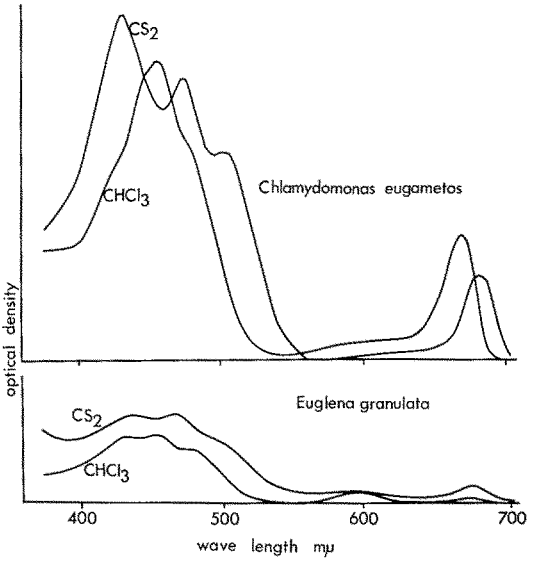


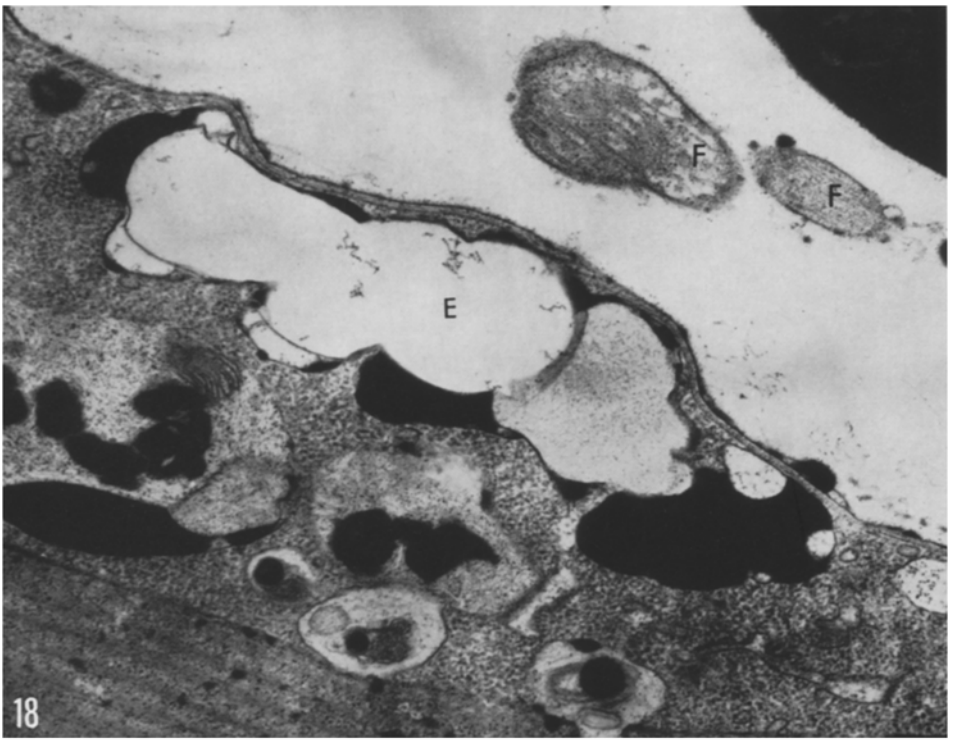
Fig. 17. Absorption spectra for chloroform and carbon disulfide extracts of *Euglena granulata* and *Chlamydomonas eugametos*

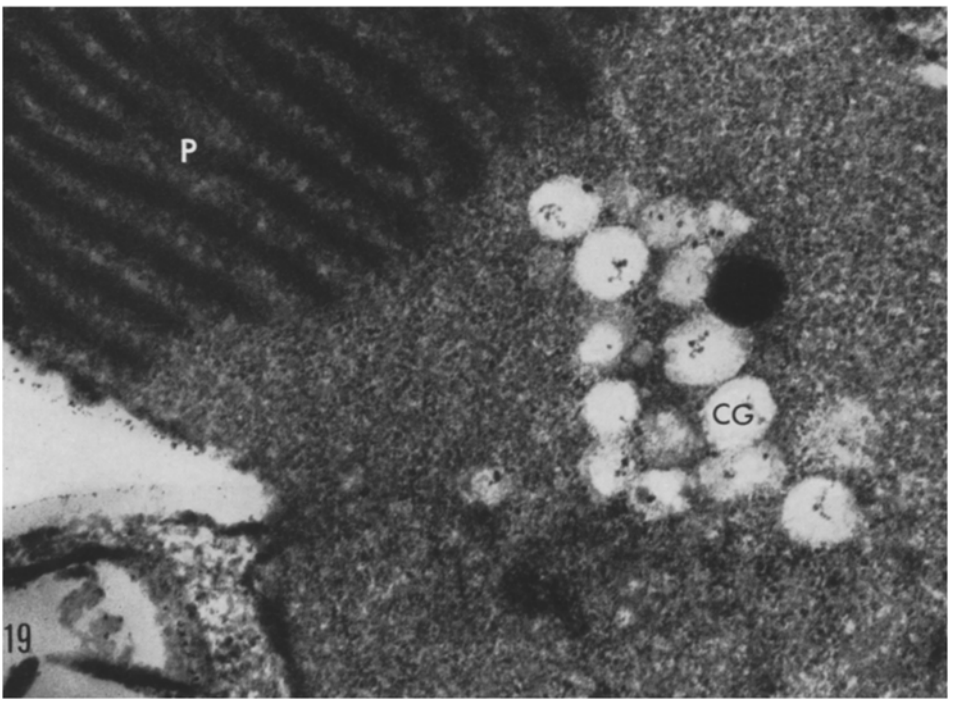
In addition to the eyespot granules, other similar osmophilic globules occur singly or in small linear groups in internal positions throughout the chloroplast (Fig. 20).

b) Structure of Cells after Extraction. For reasons stated previously for *Euglena*, cells of *Chlamydomonas* extracted by chloroform and carbon-disulfide were selected for further study. As shown in Fig. 17 the absorption spectra of extracts of the alga are similar, though not identical. Extracts irradiated with ultraviolet light of 360 mμ fluoresced the same as did those of *Euglena*.

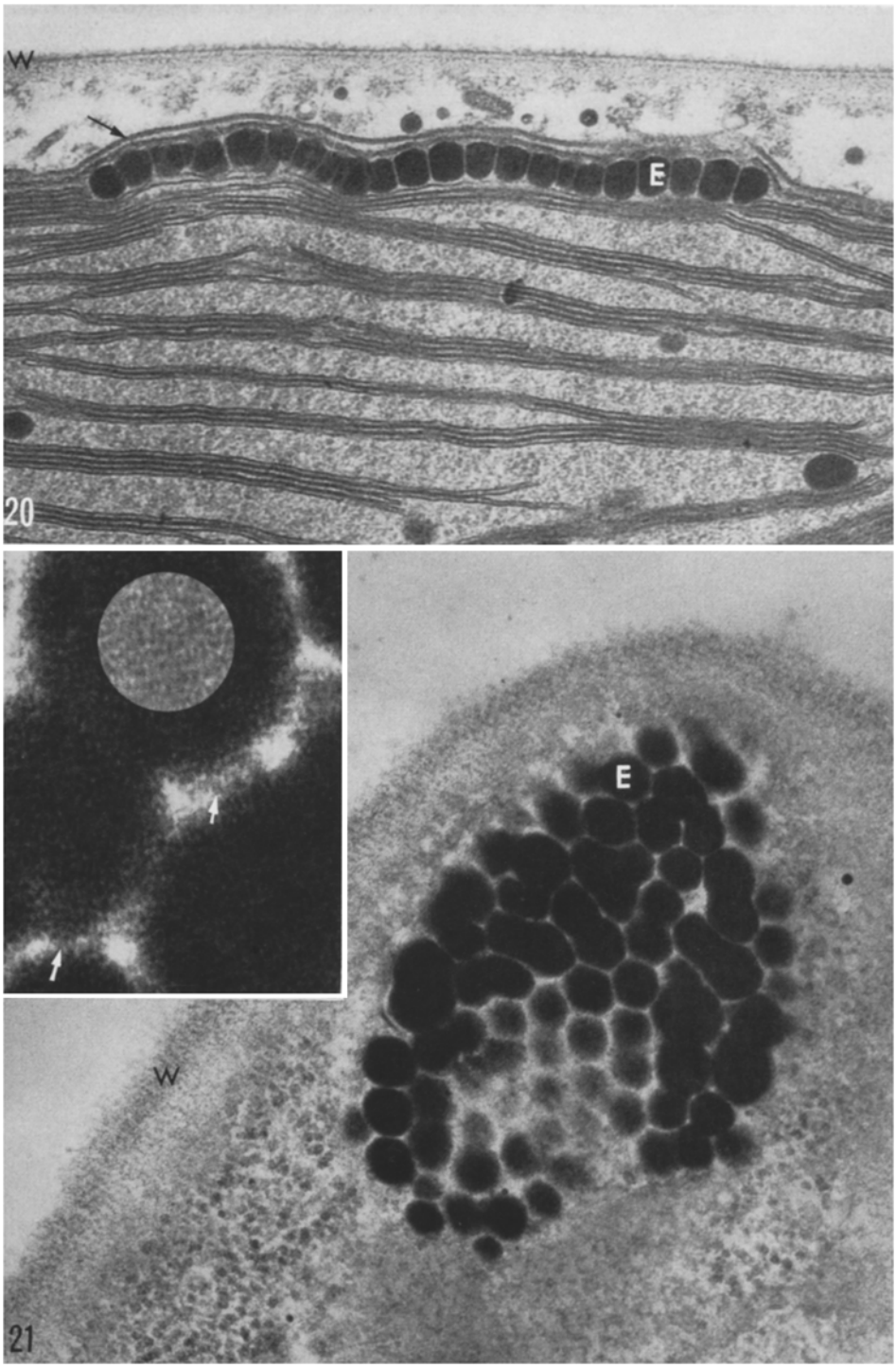
Fig. 18. Portion of *Euglena granulata* cell extracted with chloroform. Note deletion of chloroplast lamellae (lower left) and most of matrix material and delimiting membranes from eyespot granules (*E*). Eyespot residuum appears amorphous. ×34,000

Fig. 19. Chloroform-extracted *Euglena granulata* chloroplast showing pyrenoid region (*P*), deletion of lamellae and matrices of most osmophilic globules (*CG*). ×80,000





Figs. 18 and 19 (for Legends see p. 340)



Figs. 20 and 21 (for Legends see p. 343)

Preliminary results of extraction with chloroform on eyespot ultrastructure are seen in Figs. 25 and 26. The central portions of some granules have been removed and appear relatively clear. In others, some matrix material remains which may have a vesicular appearance. The granules sometimes appear chamberlike with individual delimiting "membranes" (Fig. 26). In density, structure, and residual components after extraction, osmophilic globules in the chloroplast are similar to eyespot granules (Fig. 27).

Discussion

1. Comparative Ultrastructure

This investigation has afforded a comparison of the chloroplastic and extra-chloroplastic eyespots in *Chlamydomonas* and *Euglena*, respectively. Results are summarized in the Table.

One of the major differences between the eyespots of the two organisms is that of position. The cytoplasmic nature of the *Euglena* stigma is rather unique, for the eyespots of most other algae studied are within the chloroplast envelope (see tabular summary by ARNOTT and BROWN, 1967).

In its position at the base of the reservoir, the *Euglena* eyespot is in close association with the flagellar bases, and WOLKEN (1967) suggests that this relationship is necessary for phototactic movements of the alga. Other interesting, possibly significant, relationships between flagella and stigmata exist in *Fucus*, a brown alga, in *Chromulina*, a marine chrysomonad, and in *Tetracystis*, a green alga.

The recurrent flagellum of the *Fucus* biflagellate spermatozoid is basally flattened and is closely appressed to the plasmalemma in the region of the eyespot (MANTON and CLARK, 1956). The association in *Chromulina* is more complex. A single flagellum extends from the cell, but a second, modified, short flagellum with a typical basal body remains internal and is oriented perpendicular to the base of the exserted flagellum. The short flagellum lies in an invagination of the cell membrane and adheres closely to the plastid surface in the eyespot region (FAURÉ-FREMIET and ROUILLER, 1957). In *Tetracystis* (ARNOTT and BROWN, 1967), as in *Chlamydomonas eugametos*, the two flagella and the eyespot are regularly located in the same plane, the plane of flagellar beating; it

Fig. 20. Eyespot (*E*) of *Chlamydomonas eugametos* composed of a monolayer of ca. 23 granules lying below the plasma membrane and the chloroplast envelope and subtended by a chloroplast lamella. Note modification of plasma membrane in this region and no contiguity of the latter with the chloroplast envelope. ×74,100

Fig. 21. Tangential section of eyespot of *Chlamydomonas eugametos* showing paracrystalline plate of ca. 100 granules. In some cases interconnecting fibrils (arrow) are seen. ×72,000. Inset shows substructure of a granule as a fibrous reticulum in an irregular or unordered configuration. ×280,000. Circular area in inset produced by printing in order to demonstrate more clearly the structure

Table
Characteristics of eyespots in Chlamydomonas eugametos and Euglena granulata

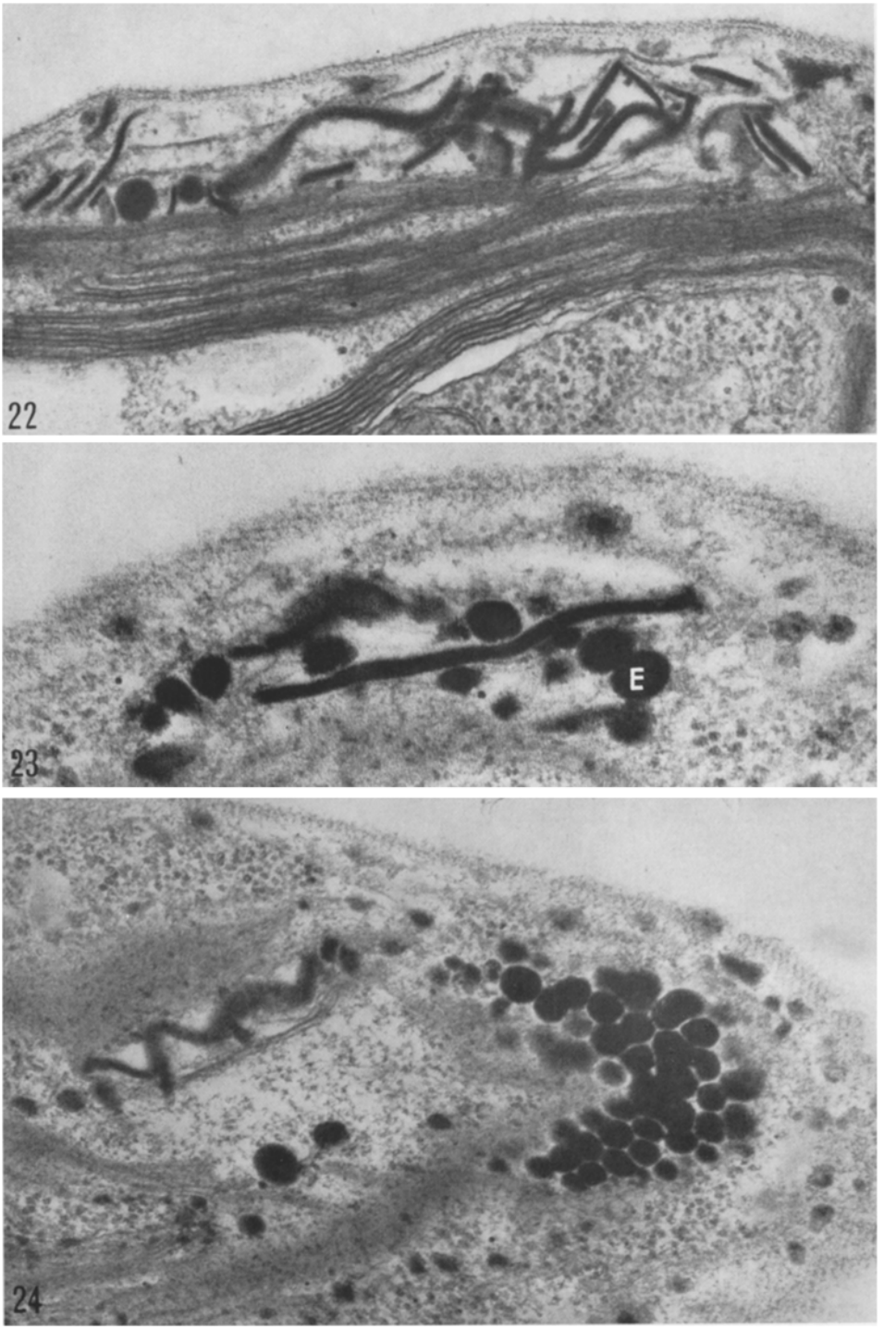
	Chlamydomonas eugametos	*Euglena granulata*
Position	Chloroplastic, anterior-median	Extra-chloroplastic, sub-anterior median
Flagellar association	None apparent	Close association with flagellar bases
Size of stigma region	$2 \times 3\ \mu$	$7 \times 8\ \mu$
Size of stigma granules	75—100 mμ	240—1,200 mμ
Shape of granules	Polygonal, largely hexagonal	Spherical, oval, polygonal
Number of granules	70—120	50—60
Arrangement of granules	Uniseriate in longitudinal section; plate-like in tangential section	Associated into packets near base of reservoir; plate-like in tangential section
Membrane and tubule association	Granules subtended by thylakoid or chloroplast lamella; overlain by chloroplast envelope and modified plasmalemma; interconnecting fibrils	Granule packets surrounded by membrane of unit type. Tubules regularly associated with packets
Substructure of granules	Unknown in living state; unordered fibrous reticulum in E.M.	Positive spherite in living state; unordered fibrous reticulum in E.M.
Polarization	None	Positive spherite
Extraction results	Few granules extracted completely; most with marginal residual component	Some granules extracted completely; some membranes destroyed: many granules with marginal residual component; some coalescence of matrices to form amorphous mass

has been suggested that this may indicate a possible functional relationship (ARNOTT and BROWN, 1967). In contrast to the situation in *Fucus* and *Chromulina*, however, no such obvious positional eyespot-flagellar relationship exists in either *Chlamydomonas* or *Tetracystis*.

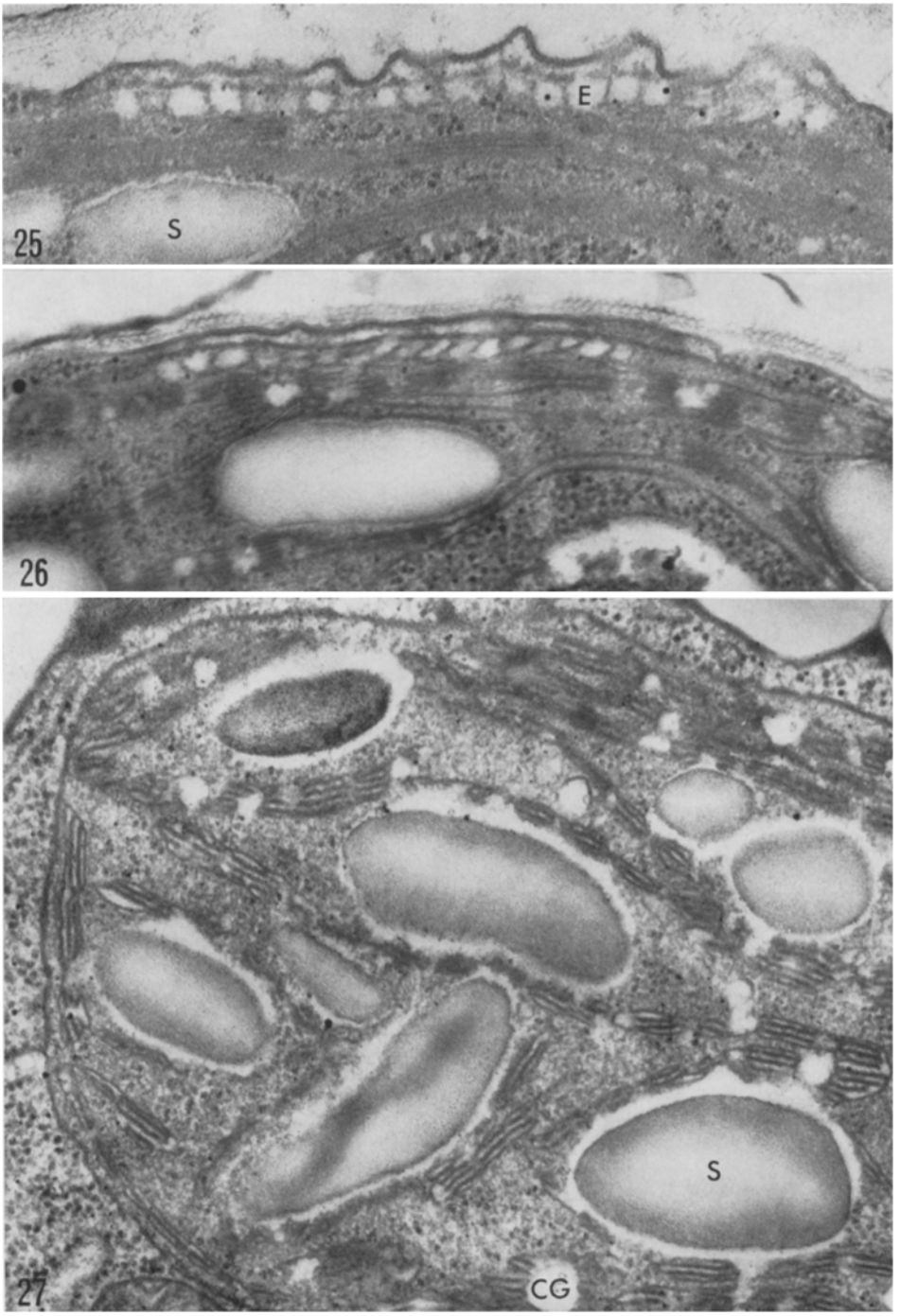
Fig. 22. Eyespot region of *Chlamydomonas eugametos* with a mixture of irregular curving bodies, plates, and a few granules. ×77,000

Fig. 23. Long rod-shaped bodies intermixed with eyespot granules of *Chlamydomonas eugametos*. ×165,000

Fig. 24. Tangential section of eyespot region of *Chlamydomonas eugametos* showing numerous eyespot granules and a few rod-shaped and curving bodies. ×56,000



Figs. 22—24 (for Legends see p. 344)



Figs. 25—27 (for Legends see p. 347)

The present paper represents the first report that granules forming the eyespot in *Euglena* are membrane bound, at least in certain stages of their development. The single unit membrane surrounding several eyespot granules may also contain many curving microtubules about which we have little information. This is also the first demonstration that the granules are positive spherites, which indicates that the granules, in the living state, possess a radial organization at the molecular level. One could speculate that the elongate carotenoid molecules may be arranged with regard to this radial organization. In the living state light passing through these granules would be modified; this would be true also for polarized light. Because of their small size similar studies were not possible in *Chlamydomonas*. It is worth noting that the granules fixed by our methods no longer exhibit this birefringence; such a loss indicates the molecular architecture of the granules has been changed by fixation. The small areas of apparent order noted above (Fig. 11) may represent the remnants of a more completely ordered system present in the living state.

Our studies show that the eyespot structure is severely modified or completely removed by extraction with organic solvents. These extractions show that a considerable degree of similarity exists between the eyespot granules and the osmophilic globules of the chloroplast, suggesting that they may have common properties. The "membranes" apparent around some extracted eyespot in *Chlamydomonas* are reminiscent of those demonstrated by JACKS et al. (1967) in their study of lipid droplets from peanut cotyledons. In our material we feel they probably represent an interface between the granule and the surrounding chloroplast matrix.

2. Eyespot Function

Although most investigators are in general agreement concerning the structure of the eyespot, there is no such agreement on the function. A variety of suggestions have been made but at the present two hypotheses, the photoreceptor and the shading hypothesis, are given serious consideration (LEEDALE, 1967).

Fig. 25. Eyespot region of chloroform-extracted cell of *Chlamydomonas eugametos*. Central portion of most granules and most lamellar structure has been removed. ×72,000

Fig. 26. Eyespot region of a chloroform-extracted cell of *Chlamydomonas eugametos*; extraction of granule matrices gives a vesicular appearance to the eyespot components, which appear chamber-like by a simulation of delimiting membranes. ×64,000

Fig. 27. Chloroplast of chloroform-extracted *Chlamydomonas eugametos* cell showing chamber-like osmophilic globules, after removal of matrix material. Compare with Fig. 19, 26. ×68,000

a) Photoreceptor Hypothesis. Studies of *Euglena gracilis* have shown that the action spectrum of phototaxis is very similar to the absorption spectrum of eyespot granules (BATRA and TOLLIN, 1964; COBB, 1963; WOLKEN, 1967; WOLKEN and SHIN, 1958). This led some investigators to the conclusion that the eyespot is the primary photoreceptor in the phototaxis response. On the other hand, experiments with certain Volvocalean algae (HALLDAL, 1958, 1964) have failed to show any correlation between the two spectra. Additionally, it is difficult to understand how any directional vector could be interposed on an eyespot acting by itself. The spherite nature of the eyespot granules in *Euglena* indicates that they have a radial molecular organization; such bodies would presumably have the same reaction to light from any direction.

b) Shading Hypothesis. If this hypothesis is correct (and many of the available data seem to indicate that it may be), then what region or structure is shaded by the eyespot? In *Euglena* the parabasal body is a logical possibility, and its proximity to the connecting body and flagellar axis would facilitate information transfer. The parabasal body can be shaded effectively from one side by the eyespot; as the organism changes its orientation (e.g., with respect to a stationary light source), the eyespot could shade, or not shade, the parabasal body. If the parabasal body is sensitive to light (e.g., the crystal might generate an electrical impulse via piezo-electrical response), this could represent a mechanism utilizing a photoreceptor (the parabasal body) and a shading device (the eyespot) to provide the organism with clues as to its orientation with respect to light.

No situation directly comparable to *Euglena* exists in *Chlamydomonas.* When earlier investigators (MAST, 1927) speculated about the lens-like structure of the eyespot, they suggested the existence of a photosensitive substance between the pigmented region and the cell periphery. The ultrastructure of the organelle does not show any such structure or region, other than the modification of chloroplast envelope and plasmalemma in the eyespot region and the clear area resulting from their protrusions.

A configuration similar to *Chlamydomonas* has been found in other organisms by LANG (1963) and ARNOTT and BROWN (1967), and LANG suggests an analogy between the lens postulated earlier and the clear region seen with electron microscopy. ARNOTT and BROWN propose the modified plasma membrane of *Tetracystis* as a zone which could serve as a photoreceptor and as a possible pathway for conduction of information to the flagella; they noted a clear relationship between the plane of flagellar beat and the position of the eyespot in the cell. Unlike *Euglena,* the eyespot in *Chlamydomonas* is confined within the chloroplast envelope and no body similar to the parabasal body of *Euglena* is present. How-

ever, a unidirectional shading device similar to that proposed for *Tetracystis* might operate here. In such a hypothesis, the eyespot would be the shading device and the thickened plasmalemma would be the photoreceptor.

The controversy about the function of the eyespot, narrowed generally to the photoreceptor and the shading hypotheses, remains unresolved, although investigators seem to agree that the eyespot is an integral part of the phototactic mechanism. In the species studied here it seems likely that the eyespot and flagella function together in the phototactic response, and the plasmalemma apparently plays an important though yet undetermined role in coordination of these two organelles.

3. Communication between the Eyespot and Flagella

In *Euglena*, microtubules are clearly associated with the base of the reservoir where the flagella are inserted and where the eyespot is located. If the eyespot is the primary photoreceptor, then microtubules could provide a method of communication between the flagella and the eyespot; the close association with the plasmalemma as it covers the surface of the reservoir and extends to form the surface of the flagella might also be a pathway of communication. On the other hand, if the eyespot is merely a shading device, communication would only entail the interruption or modification of the incoming light prior to the time it impinges upon the parabasal body. In this case the crystal and the connecting body in concert with the eyespot would provide the major elements of the light-sensing mechanism. Because the eyespot is involved in such a system, one would expect the action spectrum of phototaxis and the absorption spectrum of the eyespot to be very similar as has been pointed out already. If the photoreceptor function is accepted for the eyespot, then the function of the paraflagellar body must be explained in some other manner.

In *Chlamydomonas* the component granules of the eyespot are aligned at the chloroplast surface seemingly far removed from the flagellar bases, and a direct association between the two organelles is not evident. Their possible interaction in phototaxis should not be discounted, however. Recently, RINGO (1967) has demonstrated that microtubules in ordered arrays extend from the basal bodies of the flagella along the periphery of the cells where they would pass near the eyespot. In the region of the eyespot, the plasmalemma is modified; it is continuous with the membrane which forms the outer boundary of the flagellum. Information from the eyespot, or from shading caused by the eyespot, might be conducted along the plasma membrane to the flagella, as has been suggested in *Tetracystis* (ARNOTT and BROWN, 1967); microtubules might also be suggested as an alternate mechanism for conduction.

4. The Origin of the Eyespot Granules

The origin of the eyespot remains enigmatic; however, several possibilities may be considered. Some investigators (LEMBI and LANG, 1965) have suggested that granules may originate within the chloroplast from the osmophilic globules, which subsequently migrate to a position in the eyespot. Such a suggestion is reasonable, for as shown with extraction experiments, both kinds of granules are similar in structure and composition, and in some electron micrographs of *Chlamydomonas* a few granules can be seen occasionally near the plastid periphery but not within the eyespot region. Similar observations have recently been reported in *Tetracystis* (ARNOTT and BROWN, 1967).

Osmophilic globules similar in arrangement to eyespot granules are seen frequently in chloroplasts of mosses (SITTE, 1963) and other plants, as well as in *Euglena* chloroplasts (Fig. 14). In studies of zoosporogenesis in *Tetracystis*, apparently no osmophilic globules are present in the plastid prior to eyespot formation, even though they are found in chloroplasts of vegetative cells (ARNOTT and BROWN, 1967). In that organism the eyespot presumably develops *de novo*, since no precursors have been found.

Some micrographs of *Euglena* seem to contain evidence that the eyespot granules develop by fusion of smaller granules within the packet membrane (Fig. 10); we also noted an association of developing eyespot packets with the endoplasmic reticulum (Fig. 9, 12). These observations however, give only the first hints as to how the eyespot develops, to say nothing of the problem of how it replicates during cell division.

In colchicine-treated cells of *Chlamydomonas* (WALNE, 1967), as the cells enlarge, lose their flagella and become non-motile, the granules dissociate until eventually an eyespot is no longer discernible. When treated cells are removed to a colchicine-free medium, one of the first manifestations of the recovery phase and imminent motility is the reassociation of granules into a recognized eyespot.

5. The Fate of the Eyespot

The fate of the eyespot granules is as enigmatic as their origin. In *Euglena* the eyespot granules often lack a surrounding membrane (Fig. 12). Perhaps the packet membranes degenerate and the components contained within are released into the cytoplasm where they are attacked and dissolved by enzymes. Or, conversely, the carotenoid portions may degenerate, leaving a membrane-bounded vesicle which is no longer recognizable as an eyespot component. As we have observed eyespot granules which were not membrane bound, the former possibility seems more likely. Bodies of similar size and staining reaction to the eyespot granules are often seen in other parts of the cells (Fig. 7).

In older cells of *Chlamydomonas*, linear, helical, and irregular structures are sometimes seen in or near the eyespot region; these are morphologically similar to the carotenoid deposits seen in the plastids (chromoplastids) of higher plants such as the tomato and carrot (Rosso, 1967; Straus, 1961; Trabucchi, 1964). These bodies are intermixed with eyespot granules, and the proportion of the two varies in individual cells. These bodies may represent a change in the oxidation state of the contained carotenoids; for example, α to β carotene or a carotene to lycopene. Such a change might result in a change from a lipid-soluble to a crystalline state, and with the subsequent loss of the lipid fraction, only the crystalline carotenoid would remain. Whether this represents the senescence of the eyespot or some functional state is not clear, but the former seems more likely.

6. Parallel Light-Sensing Systems; Polarized Light

In *Euglena*, as Wolken (1967) pointed out, the involvement of the flagellum in the light-sensing apparatus strikes an interesting parallel to the light-sensing apparatus of other organisms, e.g., the outer segment of the retinal cells of vertebrate eyes which are modified cilia containing carotenoid pigments (De Robertis, 1956; Lasansky and De Robertis, 1960; Mann, 1928; Wilmer, 1955). At the present time it seems clear that much work must attend any thoughtful decision as to the functional nature of the eyespot (in *Euglena* and *Chlamydomonas*). The possible modes of action given here or elsewhere in the literature are still only unproven hypotheses. Much experimental work must be done before we will have a real understanding of the eyespot's function.

It is well known that the polarization of light in the sky is important in the orientation of certain organisms; bees maintain their body positions by allowing brightness patterns to remain constant in each ommatidium. Here each of the elements in the bee's eye functions in the perception of polarized light. The plate-like arrangement of granules in the eyespot in a similar way might also function through appropriate analysis of polarized light. Wolken (1967) found that the action spectrum of phototaxis in *Euglena gracilis* was markedly shifted toward the longer wavelengths with the use of polarized light. Further experiments with polarized light would seem to be in order, especially since it is now known that the granules are spherites capable of modifying polarized light in a characteristic manner.

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