CHLOROPLAST DEVELOPMENT IN *OCHROMONAS DANICA*

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ABSTRACT

When dark-grown cells of *Ochromonas danica* are placed in the light, the amount of chlorophyll *a* per cell increases 82-fold; the content of carotenoid pigment, 24-fold. Concomitantly with this increase in chlorophyll and carotenoid pigment, the small proplastid of dark-grown cells develops into a large lamellate chloroplast. During the first 12 hours in the light, vesicles appear within the loose clusters of dense chloroplast granules, enlarge, align themselves into rows (plates in three dimensions), and fuse into discs. Double discs may form from the more or less simultaneous fusion of two adjacent plates of vesicles or by the addition of vesicles to an already formed single disc. Three-disc bands arise by the addition of a disc to an already formed two-disc band through the approach and fusion of more vesicles. After 24 hours in the light, most of the chloroplast bands contain three discs, but the chloroplasts are still small. After 48 hours in the light, almost all the cells contain full-sized chloroplasts with a full complement of three-disc bands. However, at this time the amount of chlorophyll *a* and carotenoid pigment is only one-half of maximum. During the next 3 days in the light, as the number of chlorophyll and carotenoid molecules per chloroplast approximately doubles, there is a compression of the discs in each band (from 180 to 130 A) and a precise alignment of their membranes. Changes also occur in the nucleus when dark-grown cells are placed in the light. There is an increase in the number of small nucleolar bodies, many of which lie directly against the nuclear envelope, and in a few cells a dense mass of granules is seen between the two membranes of the nuclear envelope.

Many studies of chloroplast ultrastructure have shown that chlorophyll is necessary for the normal development of the chloroplast lamellae. However, the exact relationship of the chlorophyll and carotenoid pigments to the lamellar discs of the chloroplast remains highly speculative. It was hoped that an electron microscope study of chloroplast development in a unicellular organism in which the amount of chlorophyll and carotenoid pigment present per cell could be accurately determined might show more precisely the effect of pigment content on chloroplast ultrastructure. The Chrysophyte alga *Ochromonas danica* was selected for this study, for it is one of very few algae which is yellowish white when grown in the dark and rapidly greens when placed in the light. In addition, it has the advantages of having only a single chlorophyll, chlorophyll *a*, and of containing only one chloroplast, so that determinations of pigment present per cell are actually pigment per chloroplast.

MATERIALS AND METHODS

Culture Methods

Stocks of *Ochromonas danica* were kindly provided by Dr. S. H. Hutner. Cells were grown at 26°C in 250 ml. Erlenmeyer flasks containing 160 ml of Aaronson and Baker's medium (1) adjusted to pH 5.0. The cultures were not aerated, but were shaken by hand for a few seconds each day. Light-grown cells were grown under fluorescent lights at an

intensity of 400 to 450 ft-c. Dark-grown cells were grown in large light-tight boxes. The boxes were opened for shaking flasks or transferring cultures in a dark room, the only illumination being provided by a Bunsen burner. Cell density was determined by counting approximately 400 cells in a Levy hemacytometer. This method gave a maximum variation from the mean of 2.5 per cent.

suspensions. However, not all the small fragments are discarded in the supernatant, and these contribute pigment but are not counted as cells. Hence, there is a slight overestimation of the amounts of chlorophyll and carotenoid pigment per cell.

The extracts were clarified by filtration and their optical densities measured at 663 mμ and 470 mμ with a Beckman DU spectrophotometer or a Zeiss

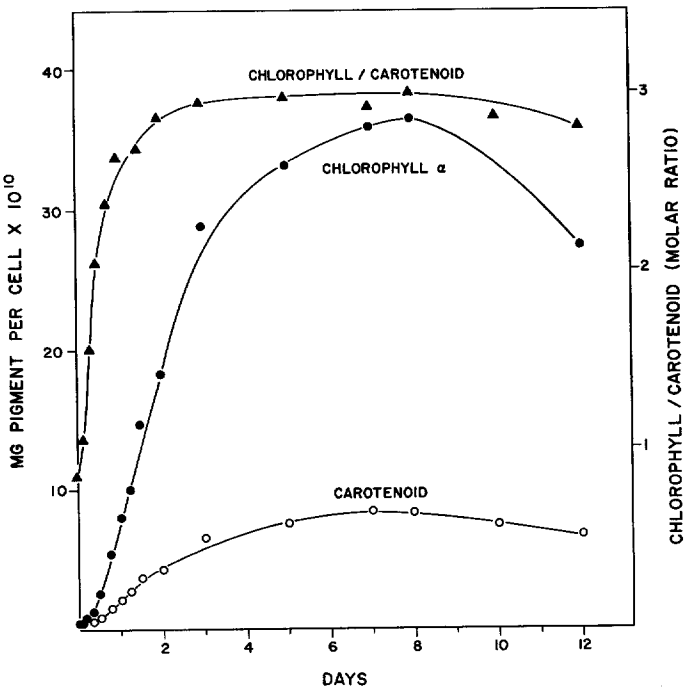


FIGURE 1

Chlorophyll *a* (●) and carotenoid pigment (○) content of dark-grown cells of *Ochromonas danica* after exposure to light. Cultures which had been grown in the dark for 7 days from a dark-grown inoculum were placed in the light at zero time. At this time the cultures contained 8 × 10^6 cells/ml and at the termination of the experiment (day 12), 57 × 10^6 cells/ml. The chlorophyll/carotenoid ratio (molar) is also shown (▲). An average molecular weight of 615 for the carotenoid pigments was used.

Pigment Analysis

When cultures contained sufficient pigment, a 1 or 2 ml sample was pipetted directly into 8 ml of acetone and then 1 ml of distilled water was added, when necessary, to give a final acetone concentration of 80 per cent (*v/v*). Dark-grown cultures and cultures in the early stages of greening were concentrated 10 to 40 times by centrifuging at 700 *g* and resuspending the cells in distilled water. 2 ml of the concentrated suspension were pipetted into 8 ml of acetone. This method is less accurate than that for the green cells because some cells are broken during centrifugation. This error was partly compensated for by making cell counts on the concentrated

PMQ II spectrophotometer in 1 cm cuvettes. Since chlorophyll *a* is the only pigment in *Ochromonas danica* which absorbs at 663 mμ, its concentration could be determined directly, using MacKinney's (25) value for $E_1^{1\%}_{cm}$ of 820.4. Spectra of the acetone extracts of light- and dark-grown cells are very similar in the carotenoid region and both have a small peak at 470 mμ. Since chlorophyll *a* absorption is still low here ($E_1^{1\%}_{cm}$ = 17.7 (25)), this wavelength was selected as a measure of the total carotenoid content. The carotenoids of *Ochromonas danica* have been determined (2) to consist of 17 per cent β-carotene, 73.8 per cent fucoxanthin, and 9.2 per cent unidentified pigment. Fucoxanthin and β-carotene were assumed to constitute 100 per cent of the

carotenoids, and Richards' (27) values for the extinction coefficients of these two carotenoids in 90 per cent acetone were used to calculate an approximate value for $E_{1\ cm}^{1\%}$ of 2300 for the carotenoid absorption at 470 mμ.

Electron Microscopy

Cultures grown in the dark for 7 days were placed under fluorescent lights (450 ft-c.), and flasks were removed for the electron microscope studies after 0, ½, 1, 2, 4, 8, 12, 18, 24, 36, and 48 hours and 3, 5, and 8 days in the light. A part of the cells in each flask was used for pigment analysis; the rest of the cells were collected by centrifugation and fixed for 8 hours in cold 1 per cent OsO_4, buffered to pH 7.3. The composition of the fixative and the method of methacrylate embedding have been described previously (13). All sections were stained with 1 per cent potassium permanganate according to the method of Lawn (23) before being observed with an RCA EMU-2D electron microscope.

RESULTS

A. Pigment Synthesis

With the culture methods used, *Ochromonas danica* grows almost as well in the dark as in the light. The doubling time for dark-grown cells is 15 hours; that for light-grown cells is 14 hours. Dark-grown cultures, however, are yellowish white, whereas light-grown cultures are dark brownish green. Measurements of pigment content reveal that dark-grown cells contain only 1 to 2 per cent as much chlorophyll *a* as light-grown cells and 4 to 7 per cent as much carotenoid pigment. Attempts to obtain chlorophyll-free cells by many generations of growth in the dark with repeated transfers did not succeed. Daily analyses of dark-grown cultures inoculated with dark-grown cells showed that the amount of chlorophyll *a* per cell stayed approximately constant (about 3.5×10^{-11} mg) while the cells were still dividing, but doubled during the first 9 days following the cessation of cell division. It appears, therefore, that chlorophyll synthesis continues in the dark in this species, but at a very low rate. The presence of protochlorophyll could not be detected in spectra of acetone extracts of dark-grown cells.

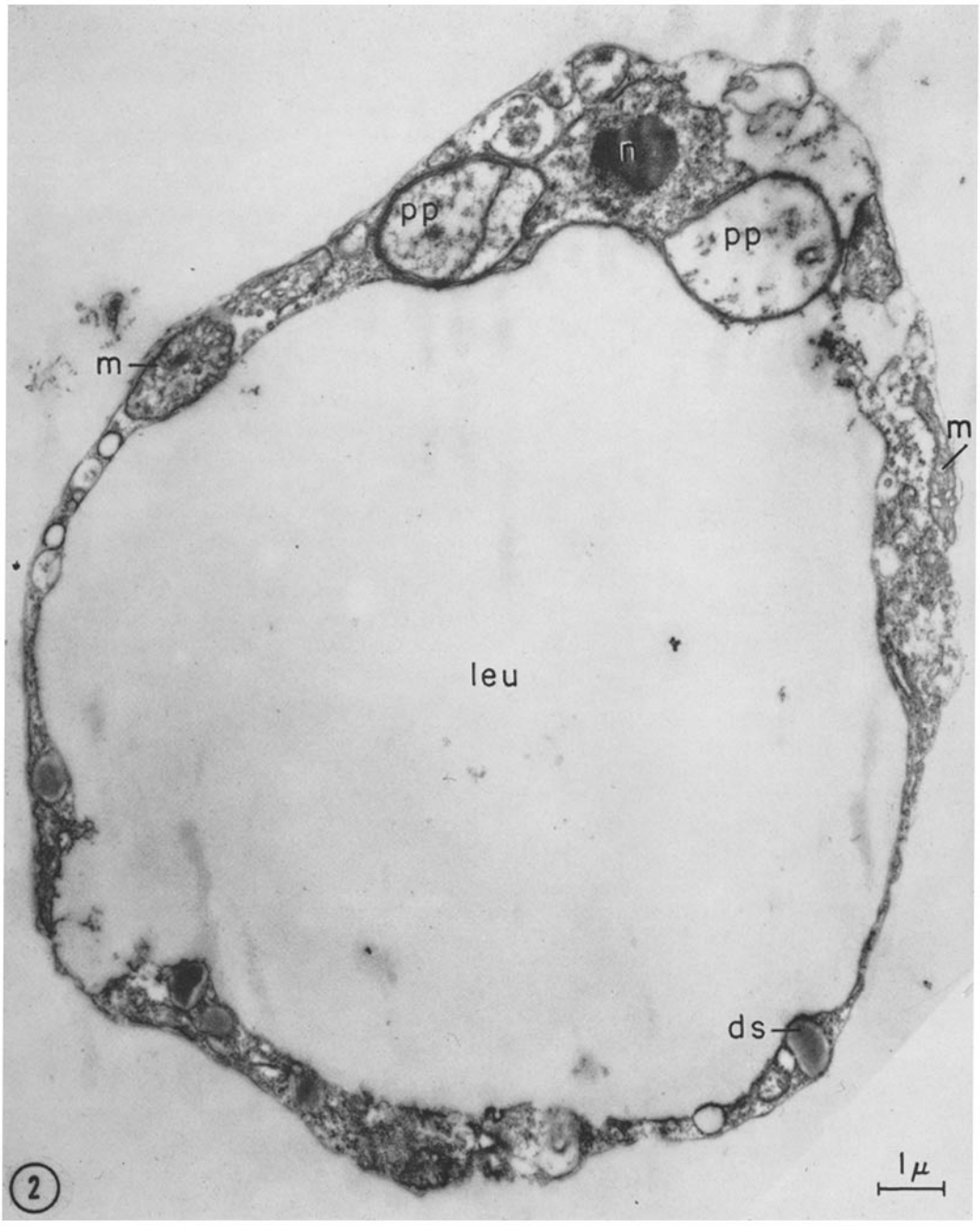
For the electron microscope studies, it was necessary to find conditions under which the cells would show a large and rapid increase in chlorophyll. Preliminary experiments revealed that younger dark-grown cultures display a greater and more rapid increase in chlorophyll per cell when they are placed in the light than do older cultures, despite the fact that more cell divisions occur in the younger cultures. Yet the cultures could not be too dilute, since they had to contain enough cells to collect for electron microscopy and pigment analysis. Consequently 7-day dark-grown cultures which contained 8×10^6 cells per ml were used.

Fig. 1 shows that when these dark-grown cells are placed in the light, chlorophyll per cell increases at a high rate after a lag period of about 2 hours. There was no increase in cell number until 12 hours in the light, so there apparently is a short lag period in chlorophyll synthesis. The amount of chlorophyll per cell increases rapidly for 3 days, then more slowly, reaching a maximum on day 8. An 82-fold increase in the amount of chlorophyll *a* per cell was observed (from 4.4×10^{-11} mg to 3.6×10^{-9} mg). Carotenoid pigments are also synthesized when dark-grown cells are placed in the light, but at a slower rate (Fig. 1). A 24-fold increase in the amount of carotenoid pigment per cell was observed (from 3.5×10^{-11} mg to 8.4×10^{-10} mg). The upper curve in Fig. 1 shows the corresponding changes in the chlorophyll/carotenoid ratio (molar). When dark-grown cells are placed in the light, the chlorophyll/carotenoid ratio increases very rapidly during the first 24 hours (from 0.86 to 2.6) and by 48 hours has risen to almost maximum (2.9).

B. Chloroplast Development

Fig. 2 is a longitudinal section through a representative cell from the 7-day dark-grown cultures used as the starting cultures in these experiments. The presence of a large posterior vacuole and an anterior nucleus gives the cell its characteristic signet ring appearance. The two proplastids which are present on either side of the nucleus are actually the two arms of a single U-shaped proplastid which partly encircles the nucleus. The vacuole is limited by a single membrane (Fig. 9, *vm*) and in the living cell contains a large granule of leucosin, the storage carbohydrate of the Chrysophyceae. Mitochondria and small vacuoles containing a dense homogeneous substance are present in the peripheral cytoplasm.

When these dark-grown cells are placed in the light, the large leucosin granule becomes progressively reduced in volume and the small proplastid rapidly grows into a large deeply pigmented chloroplast. At the same time, the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cells lose their rounded shape and take on the terete shape characteristic of light-grown cells. Surveys with the light microscope showed that these changes are changes of the population as a whole. At any one time after exposure to light, most of the cells show essentially the same degree of development with only small percentages displaying notable advancement or retardation. Under the light microscope, almost all the cells of the 7-day dark-grown cultures were indistinguishable from light-grown cells after only 2 days in the light.

Fig. 3 illustrates the changes which occur in cell fine structure when dark-grown cells are placed in the light. This cell is from a culture which was grown 15 days in the dark, followed by 10 days in the light. The leucosin vacuole is greatly reduced in size, and a large lamellate chloroplast now fills most of the cell. The difference in magnification between this figure and Fig. 2 should be noted. This cell measures approximately 12 μ in length (exclusive of the tail, which is not cut in this plane of section); the dark-grown cell in Fig. 2 is 17 μ long.

The ultrastructure of the chloroplast of light-grown cells of *Ochromonas danica* has been previously described (14, 16), and only the salient features will be noted here. It can be seen in Figs. 3, 19, and 20 that the chloroplast lamellae are arranged in groups of four, of which the inner two are wider (65 to 75 A) than the outer two (40 to 50 A). Each such group represents a stack or band of three closely appressed discs (14). The chloroplast of this species is also characterized by being enclosed within a double-membraned outer envelope which is an outfolding of the outer membrane of the nuclear envelope (16). This outer envelope (*oe*) is best illustrated here in Figs. 6 to 9, 12, 17, and 19. The narrow space which separates the chloroplast from the nucleus usually contains a number of tubules 250 to 350 A in diameter (Figs. 3, 4, 12, and 19), but in places they are absent (Figs. 8 and 9).

Early Events in Chloroplast Development

Fig. 4 is a section through a typical proplastid of a dark-grown cell. It contains a single disc 200 A in width (*sd*), a few small vesicles (*v*), and a large number of small dense granules which tend to be grouped in loose clusters (*cg*). These dense granules usually measure 90 to 120 A in diameter, occasionally 150 A. Sections of the proplastids of dark-grown cells commonly show one or two centers of tightly packed granules. Such a dense cluster of granules is illustrated in Fig. 5 (*dc*), which is a section of a cell which has been exposed to light for 1 hour. The small dense granules are abundant in chloroplasts at all stages of develop-

Abbreviations

bb, basal body of a flagellum
c, chloroplast
ce, chloroplast envelope
cg, chloroplast granules
cm, cell membrane
dc, dense center of chloroplast granules
dd, double disc
ds, dense substance
e, eyespot
f, flagellum
if, intraenvelope fibrils
ig, intraenvelope granules
leu, dissolved-out leucosin granule
m, mitochondrion
mg, mitochondrial granules
N, nucleus
n, nucleolus
nb, small nucleolar body
ne, nuclear envelope
oe, outer envelope of the chloroplast
pp, proplastid
r, ribosomes
sc, striated core of a mitochondrion
sd, single disc
t, tubule lying between nucleus and chloroplast
v, vesicle
vm, vacuole membrane

FIGURE 2

Dark-grown cell of *Ochromonas danica* containing a large leucosin vacuole (*leu*) and a small shield-shaped proplastid (*pp*) which partly encircles the nucleus. Mitochondria (*m*) and vacuoles containing a homogeneous dense substance (*ds*) are present in the peripheral cytoplasm. This cell is from a culture which had grown in the dark for 7 days. Mean chlorophyll per cell was 1.2 per cent of maximum (see text); mean carotenoid per cell was 4.2 per cent of maximum. × 10,000.

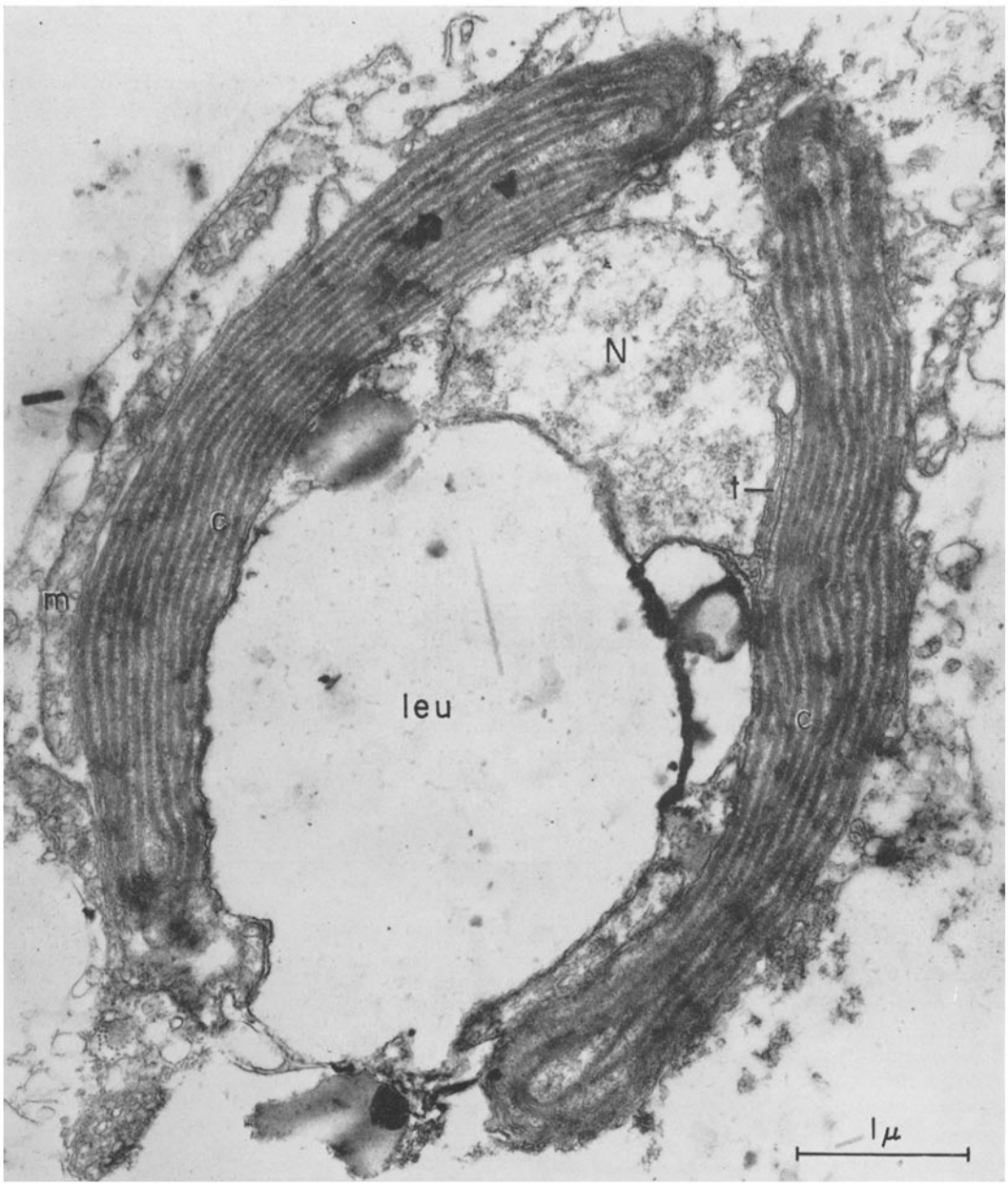


FIGURE 3

Light-grown cell of *Ochromonas danica* containing a much reduced leucosin vacuole (*leu*) and a large lamellate chloroplast (*c*). Tubules (*t*) are present in the narrow space which separates the nucleus (*N*) from the chloroplast. This cell is from a culture which had grown in the light for 10 days following 15 days' growth in the dark. × 24,000.

ment, but the dense centers of granules are characteristic of the early stages of chloroplast development and disappear after the cells have been in the light for 18 hours.

There is variation within a population of dark-grown cells in the degree of development of lamellar structures in the proplastids. In some sections only dense granules and a few small

vesicles are present; in other sections rows of vesicles are observed; in others a few mostly fused single or double discs may be present. It is unfortunate that these dark-grown cells could not be completely depleted of chlorophyll, so that one could determine whether lamellar discs are present in this species in the absence of chlorophyll.

Figs. 5, 8, and 9, which are sections of cells which have been exposed to light for 1 or 2 hours, illustrate the early stages in the formation of discs. In Fig. 5, several single discs appear to be forming through the fusion of vesicles. In Fig. 8, two adjacent rows of vesicles (in three dimensions, two plates) are fusing to form a double disc (*dd*). In Fig. 9, vesicles have lined up into a number of rows and appear to be in the process of fusing into discs, at some places into single discs, at others directly into double discs. In some sections, single discs are seen which have one or two small vesicles adjacent to them; hence it appears that double discs may arise either by the more or less simultaneous fusion of two adjacent rows of vesicles or by the addition of a disc to an already formed disc through the approach and fusion of more vesicles.

Rows of vesicles which appear to be more or less fused into single or double discs are such a common feature of the early stages of plastid development (up to 12 hours of light) that it seems reasonably certain that the discs originate from the fusion of vesicles, as they do in the proplastids of numerous higher plants. The origin of the vesicles, however, is problematical. It is well known that in the developing proplastids of higher plants, some, if not all, of the vesicles originate as infoldings of the inner proplastid membrane. Invaginations of the inner membrane of the chloroplast envelope have also been observed in several species of algae (3, 6, 9). However, it has not been possible in this study to demonstrate that any of the vesicles originate from the inner proplastid membrane. Instead one commonly sees, in the proplastids of cells which have been in the light for a few hours, loose clusters of dense granules and small vesicles of different sizes. Several such clusters are shown at relatively high magnification in Figs. 6 and 7. Some of the vesicles are almost as small (160 A) as the largest dense granules (Fig. 6, arrows); others may be as large as 1000 A (Fig. 5). It is possible, therefore, that the vesicles may arise *de novo* within the clusters of dense granules, enlarge, and then migrate out to fuse into discs (Fig. 7).

Nuclear Events

Fig. 10 is a section through the nucleus and adjacent proplastid of a dark-grown cell which has been exposed to light for 1 hour. It can be seen that the nucleus contains a large central nucleolus and a number of small bodies of nucleolar granules (*nb*), which may have detached from the the nucleolus. Frequently, these small nucleolar bodies lie against the inner membrane of the nuclear envelope. The presence of a large number of small nucleolar bodies is a characteristic feature of the nuclei of cells which have been in the light for ½ to 36 hours. This is the period of rapid chloroplast growth. Small nucleolar bodies are much less numerous in dark-grown cells and in cells which have been in the light for 2 or more days.

Another phenomenon was observed in the nuclei of greening cells which was never observed in dark- or light-grown cells. In a very few micrographs (seven out of more than a thousand), a dense mass of granular material was present between the two membranes of the nuclear envelope (Figs. 9, 11, and 14, *ig*). In six of the seven micrographs, the dense clumps of granules were present in the region of the nuclear envelope which lies adjacent to the chloroplast envelope (Figs. 9 and 11); in one case, a dense cluster of granules was present within the part of the nuclear envelope adjacent to the cytoplasm (Fig. 14). Of these seven cells, two had been exposed to light for 1 hour, two for 2 hours, two for 8 hours, and one for 5 days. On the basis of this limited sample, it appears that dense masses of granules are most frequently observed within the nuclear envelope in cells which have been exposed to light for only a few hours.

These dense masses of intraenvelope material appear to be nucleolar material which has crossed the inner nuclear membrane. They contain dense granules 120 to 160 A in diameter (Fig. 14) as well as small vesicles (Fig. 11, arrows). The nucleolus and nucleolar bodies also contain dense granules 120 to 160 A in diameter (Fig. 8) as well as small vesicles (Fig. 14, *v*). Nucleoli are also occasionally observed in which the usual granules are partly replaced by hexagonally packed vesicles 300 to 350 A in diameter (Fig. 15). The presence of a hexagonal array of vesicles within

the nucleolus did not show any correlation with the amount of light a cell had received.

In addition to the dense masses of granules already described, a regular array of widely spaced structures was occasionally observed within the nuclear envelope in the widened area where the outer nuclear membrane outfolds to form the outer envelope of the chloroplast (16). In some sections, these structures appear solid (Fig. 12, arrow), but in others they have light centers (Fig. 13). Fig. 19 suggests that these structures are short hollow fibrils (*if*) 160 to 200 A in diameter. These fibrils were observed in dark-grown cells as well as in cells exposed to light for short or long periods.

Later Events in Chloroplast Development

It has been seen that during the first 12 hours in the light, a number of single and double discs form within the proplastid of each cell through the fusion of vesicles. At the same time, the proplastid grows larger. During this interval, the amount of chlorophyll *a* per cell increases from 1.2 per cent to 7 per cent of the maximum amount attained by the 8-day-light cells. Bands of three discs are occasionally observed in cells which have had as little as 2 hours of light, but they are not common until the cells have been in the light for 12 hours. At this time most sections through the proplastid show three to six bands of discs; two-disc bands predominate, but single discs and three-disc bands are also common. Rows of vesicles not yet fused into discs are also still present in some sections. After 18 hours in the light (15 per cent maximum chlorophyll), three-disc bands begin to predominate, and by 24 hours of light (22 per cent maximum chlorophyll), most of the bands consist of three discs (Fig. 16). After 36 hours of light (40 per cent maximum chlorophyll), the chloroplasts are much larger, and by 48 hours (50 per cent maximum chlorophyll), the chloroplasts are full sized and contain a normal complement of three-disc bands (Fig. 19).

FIGURE 4

Proplastid of a dark-grown cell of *Ochromonas danica* showing a single disc (*sd*), scattered small vesicles (*v*), and loose clusters of dense granules (*cg*). This cell is from a culture which had grown in the dark for 7 days. Mean chlorophyll per cell was 1.2 per cent of maximum; mean carotenoid per cell was 4.2 per cent of maximum. × 34,000.

FIGURE 5

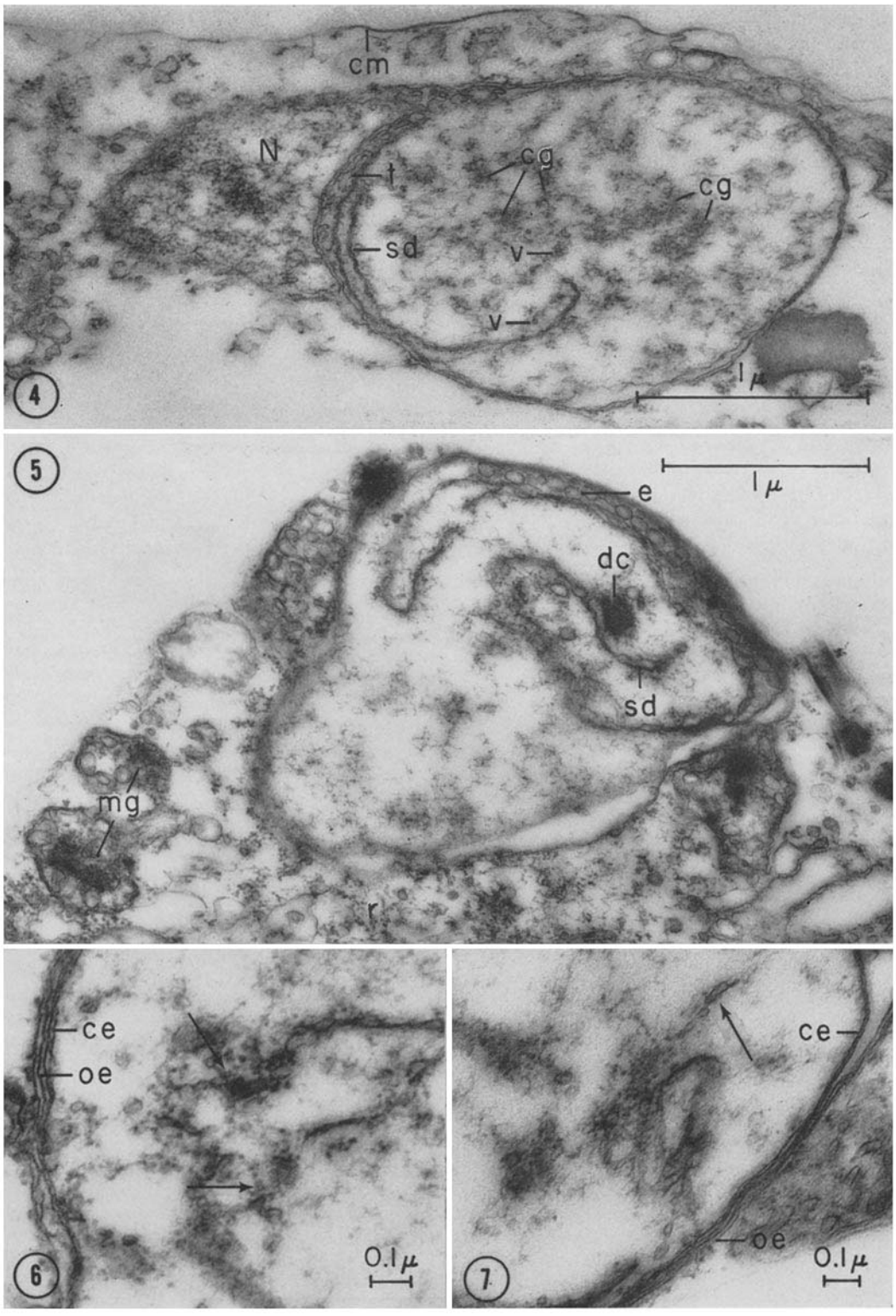
Developing proplastid of *Ochromonas danica*. A dense center (*dc*) of tightly packed granules is present and several single discs (*sd*) appear to be forming through the fusion of vesicles. Dense clusters of granules 120 to 160 A in diameter are frequently observed in the mitochondria at this stage (*mg*). This cell is from a 7-day dark-grown culture which had been exposed to light for 1 hour. Mean chlorophyll per cell was 1.4 per cent of maximum; mean carotenoid per cell was 4.6 per cent of maximum. × 32,000.

FIGURE 6

Loose cluster of dense granules and vesicles in a developing proplastid of *Ochromonas danica*. The arrows point to small vesicles which are almost as small (about 160 A) as the dense granules. In this figure and the next one, the double-membraned chloroplast envelope (*ce*) and the double-membraned outer envelope (*oe*) are both well resolved. This cell is from a 7-day dark-grown culture which had been exposed to light for 2 hours. Mean chlorophyll per cell was 1.3 per cent of maximum; mean carotenoid per cell was 4.6 per cent of maximum. × 62,000.

FIGURE 7

Clusters of dense granules and vesicles in a developing proplastid of *Ochromonas danica*. At the arrow, vesicles have begun to fuse into discs. This cell is from a 7-day dark-grown culture which had been exposed to light for 4 hours. Mean chlorophyll per cell was 2.3 per cent of maximum; mean carotenoid per cell was 6.4 per cent of maximum. × 52,000.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Transition stages between two-disc bands and three-disc bands are not seen so commonly as one would expect. However, in a number of sections one observes a two-disc band which along part of its length has a small adjacent third disc (Fig. 17, arrow) or one or two small vesicles. Whether the third disc which is added to the two-disc band arises solely from the fusion of vesicles or whether it starts from one or two vesicles and then expands to cover the entire band is not known. The same uncertainty exists in the case of the addition of a second disc to an already formed single disc.

The same question must be asked for the bands of discs in general. It has been seen that two- and three-disc bands form while the developing chloroplasts are still small and thus they must grow greatly in diameter as the chloroplasts grow to maximum size. This growth could be accomplished by a uniform intercalary growth of the membranes of the discs, or possibly the membranes grow only at the borders of the expanding discs or possibly discs grow by the addition of new vesicles peripherally. Fig. 18 illustrates a phenomenon which is commonly observed in the chloroplasts of cells which have been in the light for 2 to 3 days. The bands of three discs do not extend the whole length of the chloroplast but are interrupted in one or several areas. In these areas, the three discs of a band are frayed apart; also the dense granules tend to be more concentrated in these areas. These areas give the impression of being growing zones; the single discs appear to be forming within the areas of dense granules and then coming together into bands of three appressed discs. Only occasionally are vesicles present in these areas. The presence of these interrupted areas along the bands of discs in growing chloroplasts suggests that discs are extending at their peripheries, but it by no means rules out the possibility of intercalary growth as well.

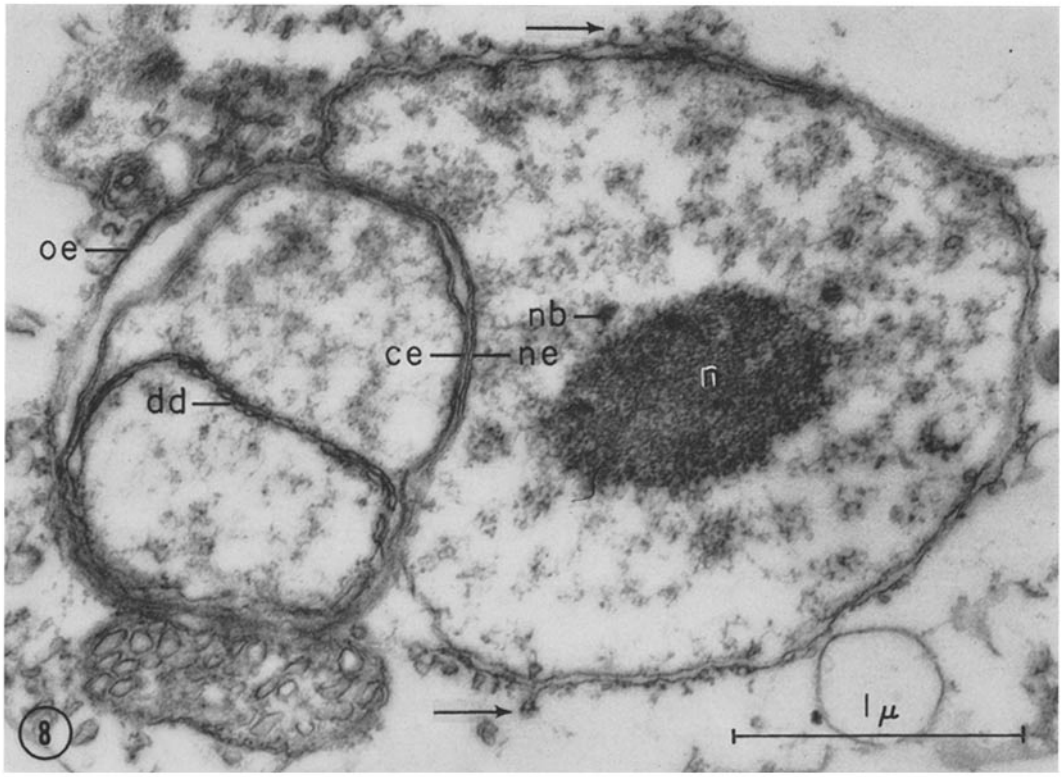
By the time the cells have been in the light for 2 days, the chloroplasts are essentially full grown and contain as many lamellae (*i.e.*, bands of three discs) as 5- or 8-day-light cells. Yet the amount of chlorophyll *a* and carotenoid pigment per cell is only half that in an 8-day cell. The only observable change in chloroplast ultrastructure which occurs as the number of chlorophyll and carotenoid molecules in the chloroplast doubles is a compression of the discs and an exact aligning of their membranes. This can be seen by comparing the 2-day-light cell in Fig. 19 with the 5-day-light cell in Fig. 20. In the chloroplast of the 2-day-light cell, the discs of each band are very irregular and the bands are moderately wide (about 520 to 550 A). In the 5-day-light cells, the three-disc bands are only 370 to 400 A wide (so that individual discs are only approximately 130 A wide instead of 180 A), and the membranes of the discs are very regularly aligned. It may be significant that this tightening of the discs and aligning of their membranes occurs only after the chlorophyll/carotenoid ratio reaches normal levels (compare with Fig. 1).

FIGURE 8

Nucleus and developing proplastid of *Ochromonas danica*. Two adjacent rows of vesicles appear to be fusing to form a double disc (*dd*). The arrows indicate blebs of the outer membrane of the nuclear envelope (*ne*). *nb*, small nucleolar body (see Figs. 9 and 10). This cell is from a 7-day dark-grown culture which had been exposed to light for 2 hours. Mean chlorophyll per cell was 1.3 per cent of maximum; mean carotenoid per cell was 4.6 per cent of maximum. × 38,000.

FIGURE 9

Nucleus and developing proplastid of *Ochromonas danica*. In the proplastid single and double discs appear to be forming through the fusion of vesicles. In the nucleus a small body of nucleolar granules (*nb*) appears to be detached from the central nucleolus (*n*), and a dense mass of granules (*ig*) is present between the two membranes of the nuclear envelope. At the arrows the outer membrane of the nuclear envelope outfolds to form a double-membraned outer envelope which encloses the chloroplast. The leucosin vacuole (*leu*) is limited by a single membrane (*vm*). This cell is from the 2-hour-light culture described above. × 33,000.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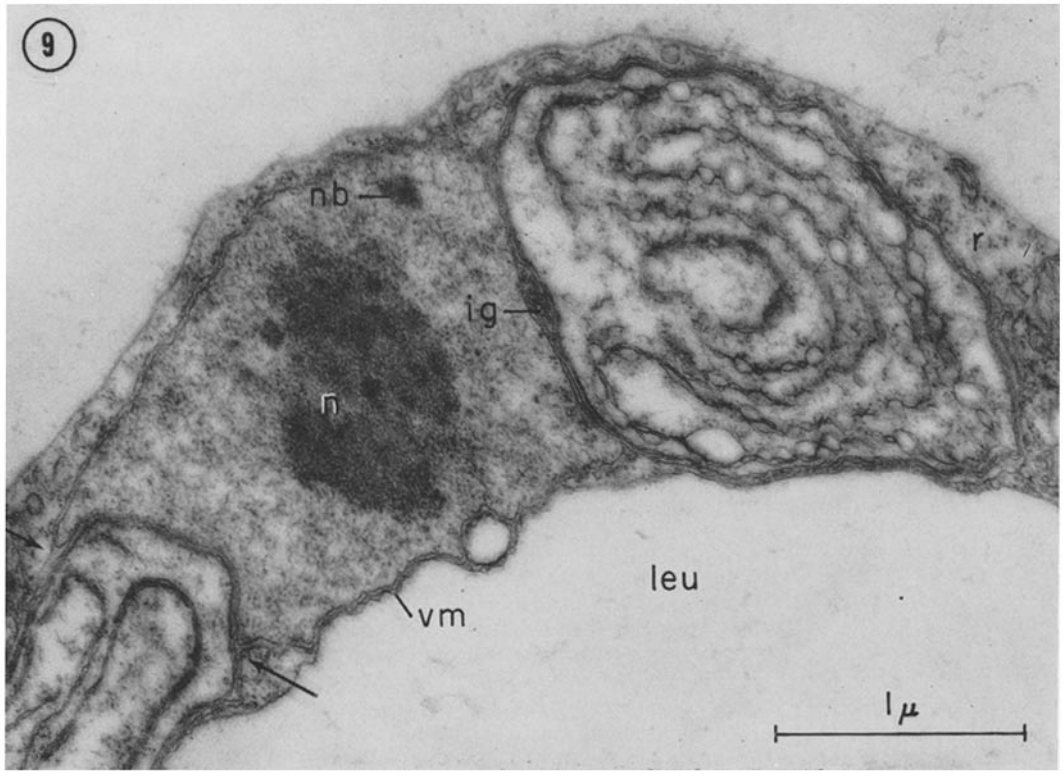
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DISCUSSION

Chlorophyll—Lamellar Disc Relationships

The role chlorophyll plays in the development of the lamellar structures of chloroplasts is not known. Many investigators believe that chlorophyll is an essential constituent of the chloroplast lamellae (*i.e.*, the membranes of the chloroplast discs) and that in the absence of chlorophyll synthesis no lamellae are formed. Hodge (18, 19) early reported that lamellar discs are not present in etiolated seedlings of *Zea mays*, and other investigators noted the complete absence of lamellar structures in the plastids of chlorophyll-deficient mutants of both higher plants (24, 28) and algae (29).

However, as more plants were studied with the electron microscope, it became evident that the presence of chlorophyll is not an absolute requirement for the formation of lamellar discs. Single discs were observed in proplastids of etiolated seedlings of a number of plants (11, 21, 30, 32, 35, 36). In addition, von Wettstein (36) showed that the chloroplasts of the *xantha*-10 mutant of barley, which is unable to synthesize chlorophyll, contain large, concentrically arranged discs. These discs, however, are never aggregated together. This observation, coupled with the observation that light is necessary for the stacking of discs into grana in normally developing chloroplasts, led von Wettstein (37–39) to suggest that chlorophyll is necessary for the aggregation of discs, perhaps in the form of a monomolecular film between discs.

The observations presented here on *Ochromonas*

FIGURE 10

Section through the anterior end of a cell of *Ochromonas danica*. A number of small bodies of nucleolar granules (*nb*), which may have detached from the main body of the nucleolus (*n*), are present in the nucleus. A flagellum (*f*) and its basal body (*bb*) have also been sectioned. This cell is from a 7-day dark-grown culture which had been exposed to light for 1 hour. Mean chlorophyll per cell was 1.4 per cent of maximum; mean carotenoid per cell was 4.6 per cent of maximum. × 33,000.

FIGURE 11

An enlargement of the dense mass of intraenvelope granules (*ig*) seen in Fig. 9. The outer membrane of the nuclear envelope (*ne*) bulges out over the granules and lies close to the chloroplast envelope (*ce*). A few vesicles can also be discerned in the dense mass of granules (arrows). × 61,000.

FIGURE 12

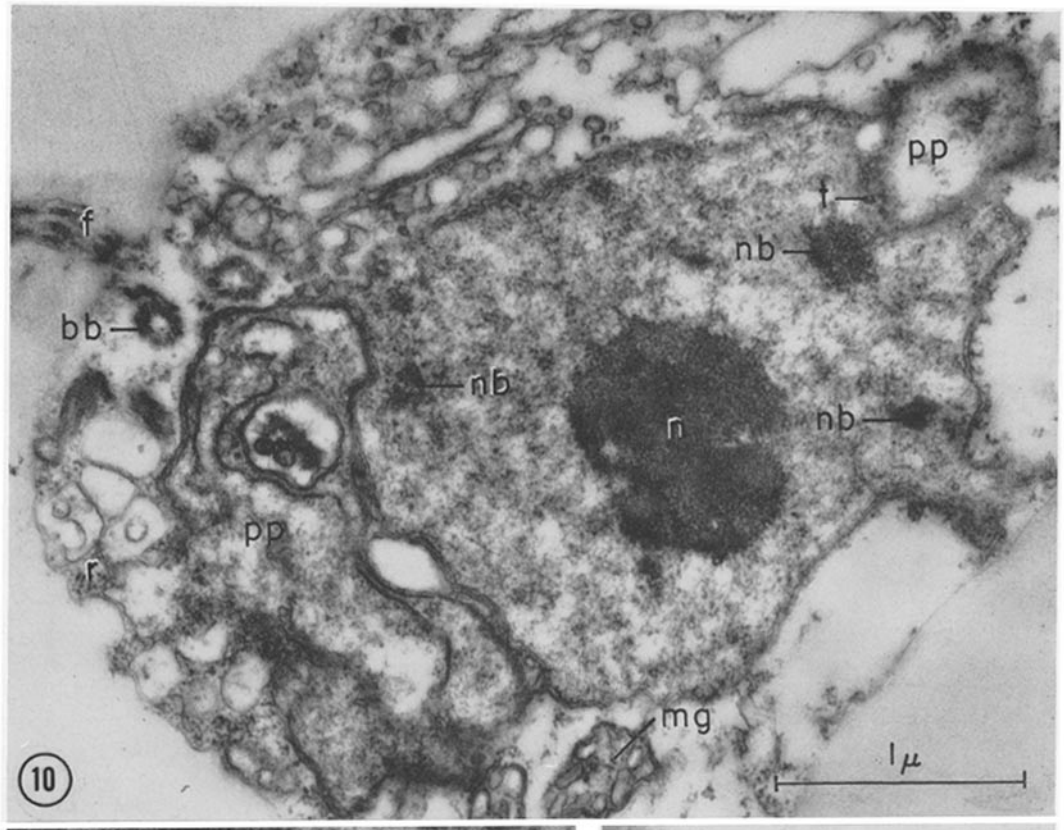
Section through the widened area of the nuclear envelope (*ne*) where the outer membrane outfolds to form the outer envelope (*oe*) of the chloroplast. Widely spaced dense structures 160 to 200 A in diameter are present (arrow). A tubule (*t*) can be seen in the narrow space separating the nuclear envelope from the chloroplast envelope (*ce*). This figure is an enlargement of part of Fig. 3. × 77,000.

FIGURE 13

Section through the widened area of the nuclear envelope of *Ochromonas danica*. The regularly arranged dense structures appear in this section to have less dense centers. Fig. 19 (*if*) indicates that they are short hollow fibrils. This cell is from the same 24-hour-light culture as the cell in Fig. 16. × 73,000.

FIGURE 14

Section through part of the nucleus and adjacent proplastid (*pp*) of *Ochromonas danica* A mass of granules (*ig*) is present with the nuclear envelope where it borders on the cytoplasm. Both vesicles (*v*) and granules can be seen in one of the small nucleolar bodies (*nb*). This cell is from the same 1-hour-light culture as the cell in Fig. 10. × 45,000.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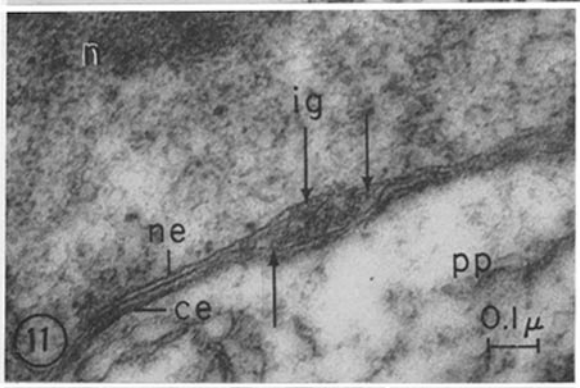
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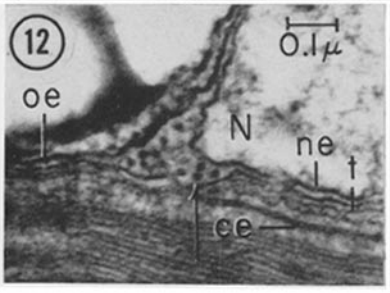
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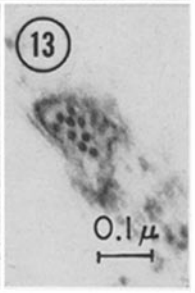
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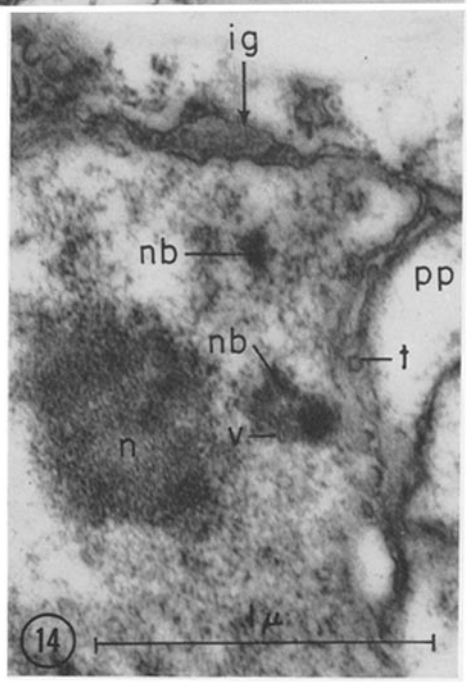
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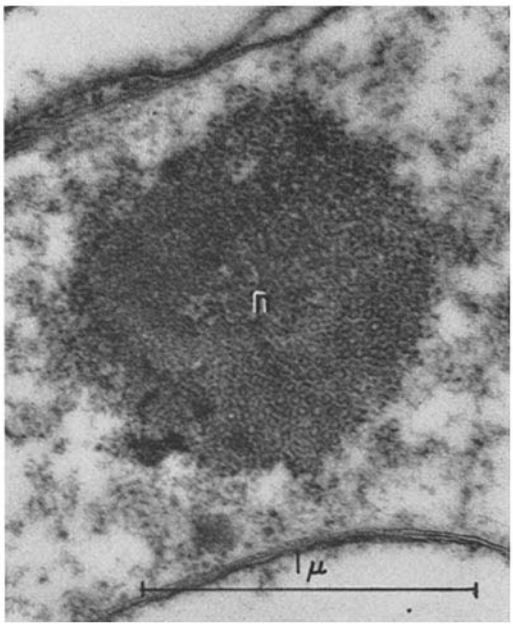


FIGURE 15

Nucleolus of *Ochromonas danica*. Below and to the right of the *n*, hexagonally packed vesicles 300 to 350 A in diameter are present. Above the *n*, dense granules 120 to 160 A in diameter are present. This cell is from a 7-day dark-grown culture which had been exposed to light for 2 days. Mean chlorophyll per cell was 50 per cent of maximum; mean carotenoid per cell was 52 per cent of maximum. × 44,000.

danica suggest that chlorophyll (and possibly also the carotenoid pigments) plays another role in the development of the lamellar discs. During the first 2 days in the light there is a concurrent increase in the amount of chlorophyll per chloroplast and the number and size of the lamellar discs, in agreement with the observations on *Chlamydomonas* (29) and *Euglena* (9, 40). Since the formation of the discs and their aggregation into bands of three take place simultaneously, it is not possible to say whether chlorophyll is playing a role in membrane formation or in disc aggregation or both. However, after dark-grown cells of *Ochromonas danica* have been in the light for 2 days, the synthesis of three-disc bands is complete, whereas chlorophyll *a* and the carotenoid pigments have reached only one-half their normal level. The further increase in pigment is accompanied by a compression of the chloroplast discs and by an alignment of their membranes. These observations suggest that the synthesis of chlorophyll plays a role not only in the formation or aggregation of the discs, but also in determining their final structure. Since in potassium permanganate–fixed material (14) it can be seen that when the discs are most compressed their limiting membranes are actually appressed together internally, it is possible that during the later stages of chloroplast development chlorophyll molecules are arranged in the membranes of each

FIGURE 16

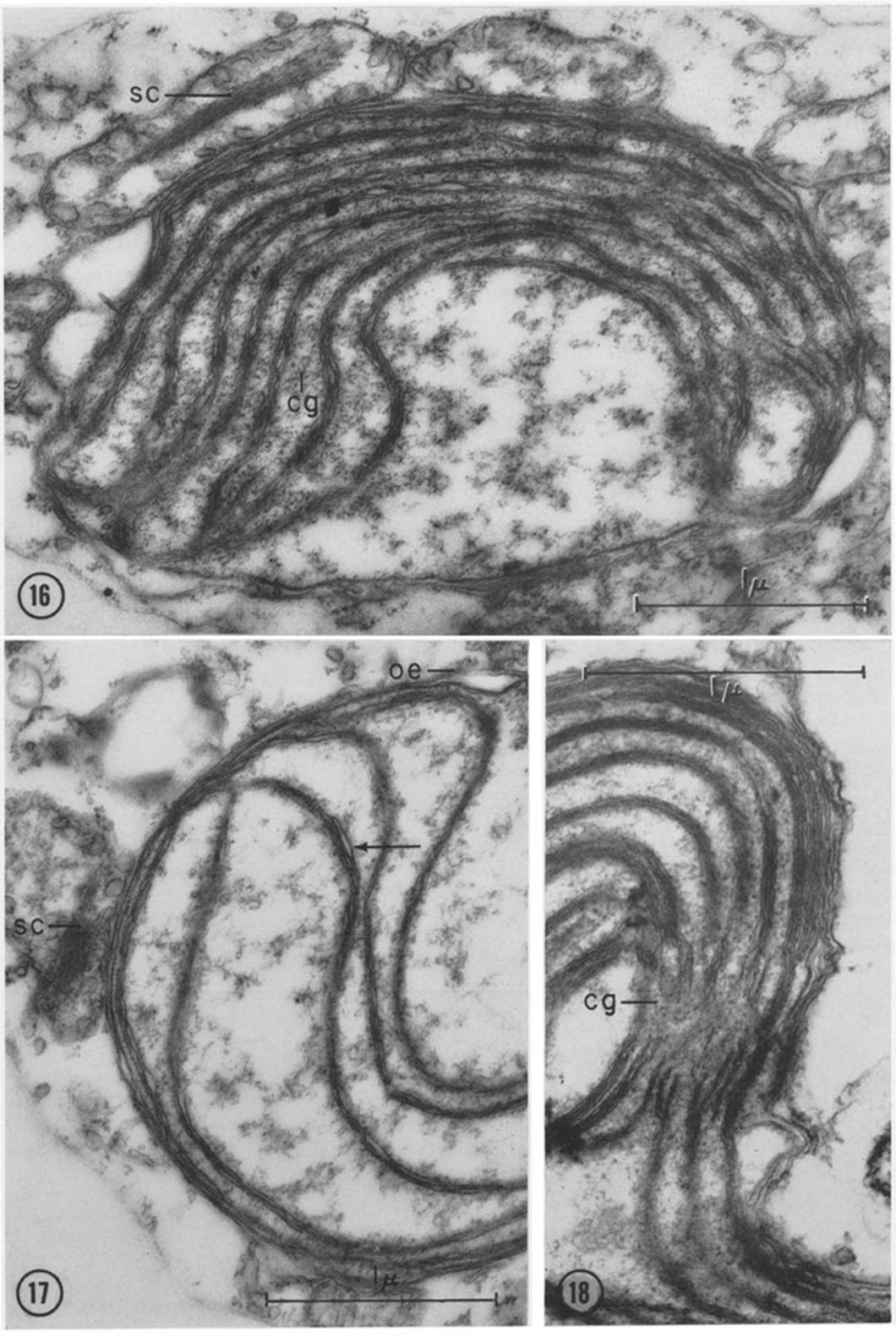
Developing chloroplast of *Ochromonas danica*. This cell is from a 7-day dark-grown culture which had been exposed to light for 24 hours. Mean chlorophyll per cell was 22 per cent of maximum; mean carotenoid per cell was 24 per cent of maximum. At this stage, most of the bands already consist of three discs. Note that the chloroplast granules (*cg*) are abundant in the region of the chloroplast where the three-disc bands have formed or are forming, and are sparse elsewhere in the chloroplast. The upper left mitochondrion has a dense striated core (*sc*). × 36,000.

FIGURE 17

Developing chloroplast of *Ochromonas danica*. At the arrow a small disc lies appressed to a two-disc band. This probably represents a stage in the formation of a three-disc band from a previously formed two-disc band. The sectioned mitochondrion has a dense striated core (*sc*). This cell is from the 24-hour-light culture described above (Fig. 16). × 36,000.

FIGURE 18

Section through part of a developing chloroplast of *Ochromonas danica* showing an area of dense granules (*cg*) where the bands of discs are interrupted. Individual discs may grow at their peripheries in such an area and subsequently come together to form three-disc bands. This cell is from an 8-day dark-grown culture which had been exposed to light for 40 hours. Mean chlorophyll per cell was 44 per cent of maximum; mean carotenoid per cell was 43 per cent of maximum. × 44,000.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16
1μ
oe
sc
17
1μ
1μ
cg
18

disc in such a way as to bring them together internally.

The association observed here between an increase in chlorophyll and the narrowing of chloroplast discs is supported qualitatively by several other studies. Gerola (10, 11) observed that a flattening of granal discs occurs during the later stages of chloroplast development in *Pisum sativum*. Crawley (5) noted that during chloroplast development in *Lupinus albus* the spacing between the granal lamellae is reduced from 170 to 125 A. Conversely, Signol (31) observed that in the chlorotic areas of corn leaves treated with dihydrostreptomycin the granal discs are notably dilated. Swollen granal discs have also been observed in the chloroplasts of iron-deficient *Tradescantia* leaves (22) and TMV-infected tobacco leaves (12). Similarly, the lamellar discs present in algal pyrenoids, areas shown by absorption or fluorescence microscopy to contain little or no chlorophyll, are usually swollen and irregular (15).

Nuclear Events Associated with Chloroplast Development

When dark-grown cultures of *Ochromonas danica* are placed in light, the cells are suddenly stimulated to synthesize the chlorophyll and carotenoid pigments and presumably also the proteins and lipids necessary to form a large complexly structured chloroplast from a small undifferentiated proplastid. The fact that at the time of rapid chloroplast formation numerous small bodies of nucleolar granules are found between the nucleolus and the nuclear envelope, many lying directly against the inner nuclear membrane, suggests that the nucleolus is transferring some of its material to the cytoplasm or directly to the chloroplast. The occasional presence within the nuclear envelope of clumps of small granules and vesicles similar to those of the nucleolus and nucleolar bodies suggests that nucleolar granules may have been transferred as such across the inner nuclear membrane or at least that granules have been reconstituted from smaller subunits which crossed the membrane. It is tempting to speculate that the rapid formation of a chloroplast which occurs when dark-grown cells are put in the light requires additional RNA of one or more kinds which is transferred from the nucleolus to the chloroplast or cytoplasm. Such a hypothesis is in line with the accumulating biochemical evidence for the transfer of RNA from the nucleolus to the cytoplasm (7, 8, 26, 33).

Chloroplast Granules

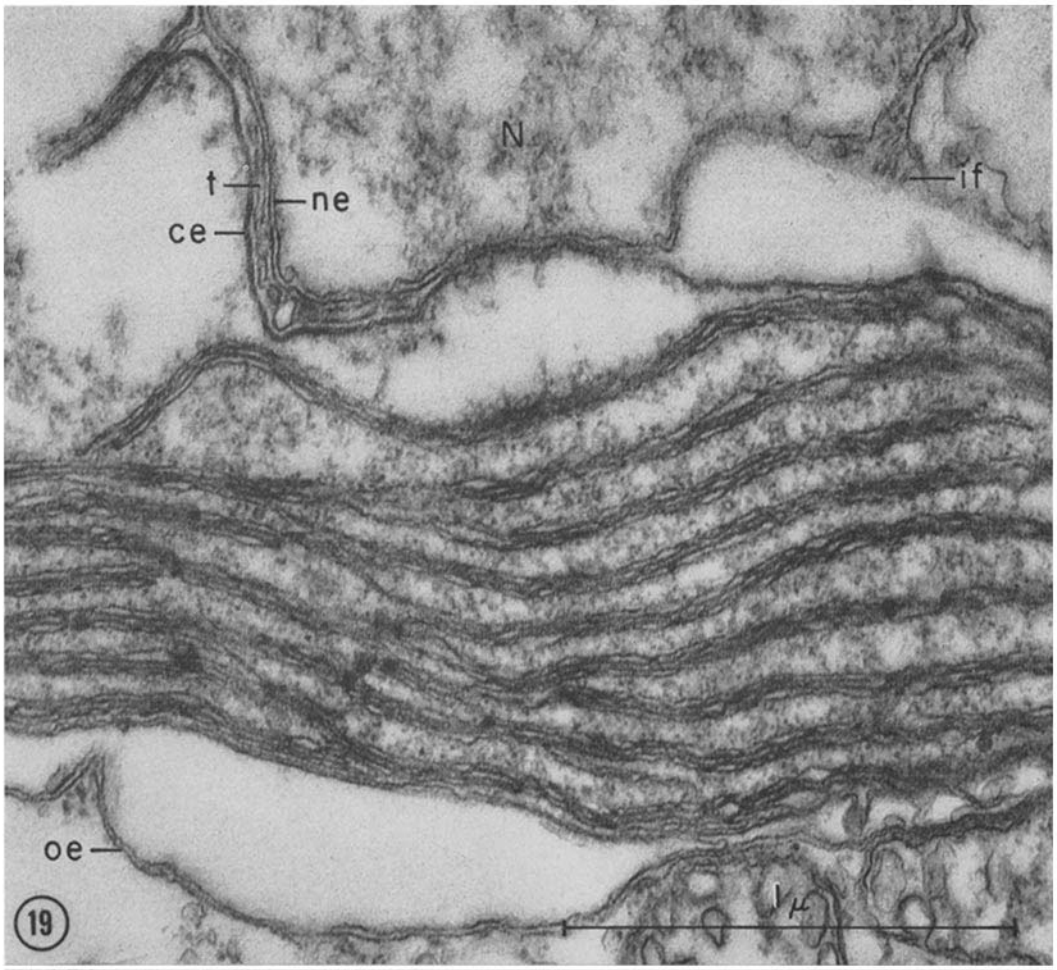
It is now generally agreed that chloroplasts contain RNA (17), and it seems likely that they can synthesize their own proteins (34). Some of the results of this study suggest that the small dense granules, 90 to 120 A in diameter, which are such a conspicuous feature of the chloroplast of *Ochromonas danica* may contain RNA and be the chloroplast equivalent of ribosomes. The cytological picture indicates that they are most abundant in the regions of the chloroplast where membranes are being formed and thus might function in their synthesis. The first small vesicles which appear in the developing proplastids are usually associated with the loose clusters of granules. In Fig. 16, it can be seen that the chloroplast granules are abundant in the region of the chloroplast where lamellar discs have formed or are forming, but are sparse elsewhere. Similarly, the granules are concentrated in the interrupted areas along the bands of discs where the discs appear to be still

FIGURE 19

Section through part of a chloroplast and the adjoining nucleus (*N*) of *Ochromonas danica*. This cell is from a 7-day dark-grown culture which had been exposed to light for 2 days. Mean chlorophyll per cell was 50 per cent of maximum; mean carotenoid per cell was 52 per cent of maximum. In this cell, the discs of each band are very irregular and the bands are moderately wide. Short hollow fibrils (*if*) are present within the nuclear envelope. × 60,000.

FIGURE 20

Section through part of a chloroplast of *Ochromonas danica*. This cell is from a 7-day dark-grown culture which had been exposed to light for 5 days. Mean chlorophyll per cell was 91 per cent of maximum; mean carotenoid per cell was 90 per cent of maximum. In this cell, the discs of each band are narrower (130 instead of 180 A) and their membranes are much more regularly aligned than those shown in Fig. 19. × 60,000.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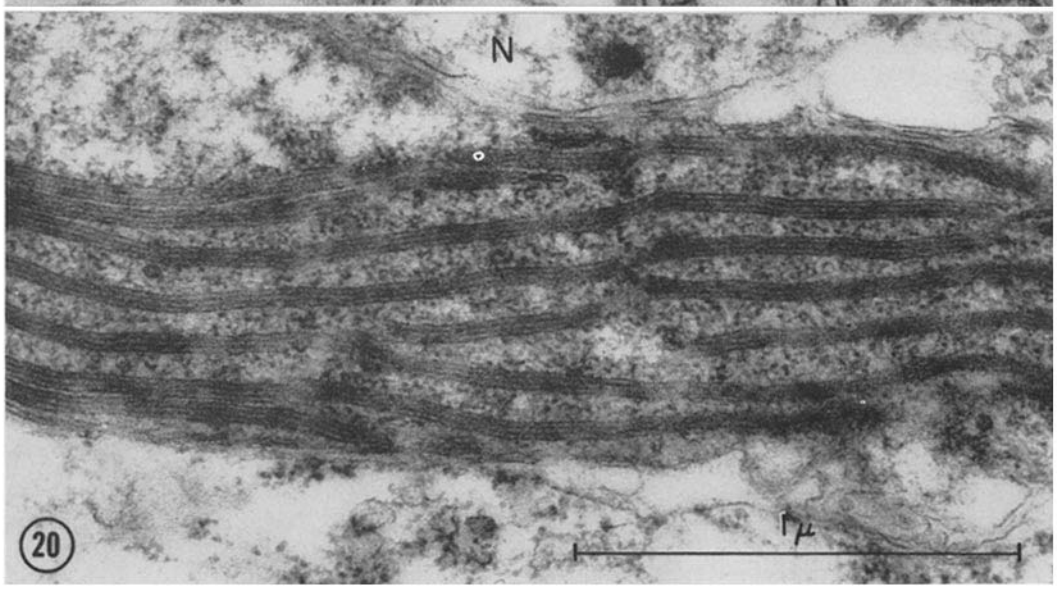
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growing (Fig. 18). It was also observed that the chloroplast granules, like the ribosomes of the cytoplasm, are absent in cells fixed in potassium permanganate, a fixative known to remove RNA (4). Histochemical tests have not yet been made, but Jacobson and Swift (20) have recently reported that ribonuclease partially digests the small chloroplast granules of *Zea mays*.

This work was performed while the author was a National Science Foundation Predoctoral Fellow.

The author wishes to express her gratitude to Dr. George B. Chapman and Dr. William R. Sistrom for their instruction and guidance during the course of this study and to Dr. Ralph A. Lewin for the hospitality of his laboratory during the summer of 1960.

Received for publication, May 29, 1962.

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