

EFFECTS OF CHLORAMPHENICOL ON CHLOROPLAST AND MITOCHONDRIAL ULTRASTRUCTURE IN *OCHROMONAS DANICA*

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ABSTRACT

The effect of chloramphenicol (CAP) on cell division and organelle ultrastructure was studied during light-induced chloroplast development in the Chrysoophyte alga, *Ochromonas danica*. Since the growth rate of the CAP-treated cells is the same as that of the control cells for the first 12 hr in the light, CAP is presumed to be acting during that interval solely by inhibiting protein synthesis on chloroplast and mitochondrial ribosomes. CAP markedly inhibits chloroplast growth and differentiation. During the first 12 hr in the light, chlorophyll synthesis is inhibited by 93%, the formation of new thylakoid membranes is reduced by 91%, and the synthesis of chloroplast ribosomes is inhibited by 81%. Other chloroplast-associated abnormalities which occur during the first 12 hr and become more pronounced with extended CAP treatment are the presence of prolamellar bodies and of abnormal stacks of thylakoids, the proliferation of the perinuclear reticulum, and the accumulation of dense granular material between the chloroplast envelope and the chloroplast endoplasmic reticulum. CAP also causes a progressive loss of the mitochondrial cristae, which is paralleled by a decline in the growth rate of the cells, but it has no effect on the synthesis of mitochondrial ribosomes. We postulate that one or more chloroplast ribosomal proteins are synthesized on chloroplast ribosomes, whereas mitochondrial ribosomal proteins are synthesized on cytoplasmic ribosomes.

INTRODUCTION

Both chloroplasts and mitochondria are known to contain DNA and RNA and to have all the components required for the synthesis of nucleic acids and proteins (34, 37). Although these organelles are thus potentially autonomous, current evidence favors the view that both organelles synthesize only some of their proteins while the rest are synthesized on cytoplasmic ribosomes. In the case of mitochondria, the large majority of their proteins, including cytochrome *c*, malic dehydrogenase, and ATPase, are synthesized on cytoplasmic ribosomes, whereas only a small number of mitochondrial proteins, all apparently

insoluble inner membrane proteins, are believed to be synthesized on mitochondrial ribosomes (3). If one assumes that only mitochondrial-coded messenger RNAs are translated on mitochondrial ribosomes, the limited number of proteins believed to be synthesized within the mitochondrion is in good agreement with the limited information content of animal mitochondrial DNA (35). Chloroplast DNA is a larger molecule (30) and recent analyses of its kinetic complexity (40, 41, 44) indicate that it has 10–20 times the information content of 5 μ mitochondrial DNA. A correspondingly greater number of chloroplast

proteins, including ribulose diphosphate carboxylase (10, 24, 36), ATPase (33), and cytochromes 553 and 563 (2, 36), are believed to be synthesized within the chloroplast, but this is not established with certainty (26).

A tool widely used in studies of the site of synthesis of organelle proteins is the antibiotic, chloramphenicol (CAP), which selectively inhibits protein synthesis on organelle ribosomes. We have looked at the effects of this antibiotic on the ultrastructure of the chloroplast and mitochondria of *Ochromonas danica* during light-induced chloroplast development. This communication reports the effects of CAP on the synthesis of chloroplast and mitochondrial ribosomes and on the number and organization of internal organelle membranes.

MATERIALS AND METHODS

Physiological Studies

Stocks of *Ochromonas danica* Pringsheim were obtained from the Culture Collection of Algae at Indiana University (Culture No. 1298). Cells were grown at 29°C in 250-ml Erlenmeyer flasks containing 160 ml of complete *Ochromonas* medium (1) either in light-tight incubators or under a bank of fluorescent and incandescent lamps adjusted to give a light intensity of 450 ft-c. All operations on dark-grown cultures were performed using safelights covered with both a blue (Rohm and Haas No. 2424) and a green (Rohm and Haas No. 2092) sheet of Plexiglas.

For the greening experiment, dark-grown cultures which were in the logarithmic phase of growth and contained between 0.6 and 1.0×10^6 cells per ml were placed in the light on a rotary shaker set at low speed and samples were taken for cell number and pigment determinations at appropriate intervals. D-threo chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) was added in powder form at a concentration of 300 µg/ml to replicate dark-grown cultures 30 min before illumination. No contamination was observed.

Cell counts were made with either a hemacytometer or a Model B Coulter Counter. For the latter instrument, the cells were diluted in a millipore-filtered solution of 0.3% NaCl and 0.5% glutaraldehyde and counted using a 100 µ aperture.

Chlorophyll *a* and carotenoid pigments were extracted in 80% acetone and measured at 663 nm and 470 nm, respectively, with a Beckman DB-G Spectrophotometer Beckman Instruments, Inc., Fullerton, Calif. Pigment concentrations were calculated as described previously (16).

Ultrastructural Studies

Flasks of control and CAP-treated cells were prepared for electron microscopy after 0, 3, 6, 9, 12, 24, 48, and 96 hr in the light. Cells of each type were collected by centrifugation (5 min at approximately 700 *g*) and samples were fixed by two different methods. The first method was a standard fixation of 30 min in 2.5% glutaraldehyde in 0.1 M Na phosphate buffer, pH 7.2, at room temperature, followed by two rinses in the same buffer and 1 hr of postfixation in 1% osmium tetroxide in 0.1 M Na phosphate buffer, pH 7.2, at room temperature. The second fixation was a simultaneous glutaraldehyde-osmium tetroxide fixation which Falk (11) has shown gives excellent preservation of chloroplast ribosomes. Cells were fixed for 30 min at room temperature in a solution containing 2% glutaraldehyde and 2% osmium tetroxide in 0.05 M Na phosphate buffer, pH 7.2. This was mixed just before use from two double-strength stock solutions. After two rinses in 0.1 M Na phosphate buffer, pH 7.2, the cells were postfixed in 1% osmium tetroxide in 0.1 M Na phosphate buffer, pH 7.2, for 1 hr at room temperature.

Cells fixed by both methods were dehydrated rapidly in an ethanol series and embedded in a low-viscosity epoxy resin (39). Sections were cut on a Porter-Blum MT-2 Ultramicrotome, stained for 15 min with lead citrate (43), and examined in a Philips EM 200 electron microscope.

A variety of counts and measurements was made on the electron micrographs. Counts of thylakoids per chloroplast section and thylakoids per band were made on every chloroplast which was sectioned approximately perpendicular to the plane of the thylakoids. A line was drawn at the midpoint of each section at right angles to its long axis and the number of intersected thylakoids was counted.

Since chloroplast ribosomes were not well preserved in cells fixed by standard methods, all counts of chloroplast and mitochondrial ribosomes were made on cells fixed by the method of Falk. Electron micrographs were printed at a total magnification of 45,600 and a square window $1/16 \mu^2$ in area was placed at random over the organelle and counts were made. Each count given in Tables II and IV represents the average of approximately 100 counts from a minimum of 15 different chloroplasts or 50 different mitochondria.

To measure the growth of the chloroplast during greening in control and CAP-treated cells, the per cent volume of the cells occupied by the chloroplast at each time interval was determined from the fractional area occupied by the chloroplast in random sections of 120–130 different cells. Chloroplast and cell areas were determined from micrographs printed at a final magnification of 16,800 by the tracing and weighing method described previously (17). The

per cent chloroplast volume determined for each time interval was converted to absolute chloroplast volume by multiplying by the mean cell volume of the population at that time. Mean cell volume for cells in complete growth medium was determined using a Model Z Coulter Counter, calibrated with paper mulberry pollen. The absolute chloroplast volumes so obtained were used in calculating changes in the total number of chloroplast ribosomes and the total amount of thylakoid membrane.

To determine the ratio of the length of the inner mitochondrial membrane to that of the outer mitochondrial membrane, 25–35 mitochondrial profiles were measured at each of a variety of times for the control greening and CAP-treated cells. Measurements were made with a map reader on micrographs printed at a magnification of 45,600.

RESULTS

Effects of CAP on Pigment Synthesis and Cell Division

Figs. 1 and 2 show the effects of CAP on pigment synthesis and cell division in light-induced cells of *Ochromonas danica*. CAP was added at a concentration of 300 $\mu\text{g/ml}$ to log-phase dark-

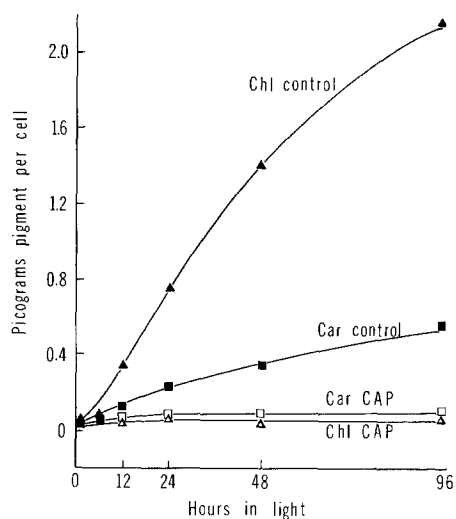


FIGURE 1 Effect of chloramphenicol (CAP) on chlorophyll (*Chl*) and total carotenoid (*Car*) synthesis in greening cultures of *Ochromonas danica*. CAP at a concentration of 300 $\mu\text{g/ml}$ was added to dark-grown cultures 30 min before illumination. Each point is an average of replicate flasks. ▲—▲, chlorophyll content of control cells; △—△, chlorophyll content of CAP-treated cells; ■—■, carotenoid content of control cells; □—□, carotenoid content of CAP-treated cells.

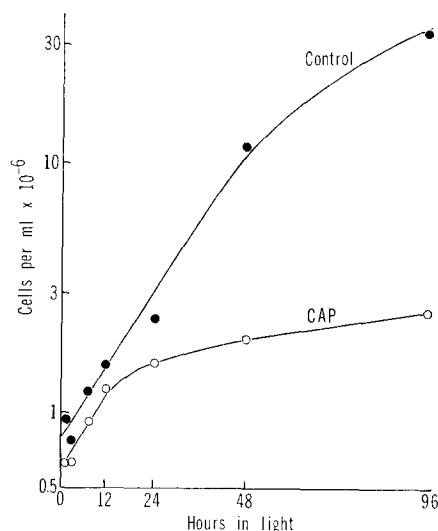


FIGURE 2 Effect of chloramphenicol (CAP) on the division rate of greening cells of *Ochromonas*. Data from the same experiment as Fig. 1. Each point is an average of two or four replicate flasks. ●—●, control cells; ○—○, CAP-treated cells.

grown cultures 30 min before the cultures were exposed to light. It can be seen in Fig. 1 that both chlorophyll and carotenoid synthesis are strongly inhibited by CAP, but chlorophyll synthesis is more sensitive to the drug. Fig. 2 shows that the growth rate of the CAP-treated cells is normal during the first 12 hr in the light, but then decreases markedly. As will be shown later (Table III), this decline in growth rate occurs at the same time as the mitochondria display a marked reduction in the number of cristae. Thus, the decrease in the growth rate is probably due to the secondary inhibition of mitochondrial adenosine triphosphate (ATP) production caused by a direct inhibition by CAP of proteins essential for the formation of functional cristae.

Since the growth rate of the cells declined markedly after 12 hr of exposure to CAP, it was important to determine whether extended treatment with CAP was killing any of the cells. Since *Ochromonas* is a flagellated organism, cell motility is a convenient measure of whether or not cells which have stopped dividing are in fact alive. In all CAP-treated and control cultures, 80–90% of the cells were motile. The remaining cells had the same color and refractility as the swimming cells but were stuck to the cover glass.

Effects of CAP on Chloroplast Development

CHLOROPLAST GROWTH AND DIVISION: During normal chloroplast development in *Ochromonas*, the single plastid grows 10-fold in volume. Table I shows that CAP markedly inhibits the light-induced growth of the chloroplast. During the first 12 hr in the light, the chloroplast of the control cells grows 3.5-fold in volume, whereas the plastid of the CAP-treated cells increases only 1.6-fold in volume. In cells exposed to CAP for longer times, the inhibition of chloroplast growth is proportionally greater.

Chloroplast division appears to be less sensitive to CAP than is cell division or chloroplast growth. In cells treated with CAP for 24 hr or longer, one sees an increasing number of cell sections containing four to six small chloroplast profiles (Fig. 3). It is unlikely that these profiles are all sections through a single, many-lobed chloroplast since multilobate tangential sections are not observed; thus it seems that the small chloroplast has divided once or twice without a corresponding cell division. Also, in cells treated with CAP for 9 hr or more, one sometimes observes that at least in the plane of the section, the chloroplast has divided without a corresponding division of the layer of endoplasmic reticulum which surrounds the chloroplast (Fig. 4). Such images are never seen in control cells. Apparently, in normal chloroplast division, the chloroplast envelope and the chloroplast endoplasmic reticulum (CER) pinch in simultaneously. Treatment with CAP somehow uncouples this synchrony.

CHLOROPLAST THYLAKOIDS: Before the effect of

CAP on the synthesis and organization of the internal membranes of the chloroplast is described, it is necessary to describe the normal development of chloroplast thylakoids during greening. The proplastid of dark-grown cells of *Ochromonas* typically contains a single fenestrated thylakoid which in some places may already be developed into a band of two appressed thylakoids. When dark-grown cells of *Ochromonas* are placed in the light, there is a rapid synthesis of new thylakoid membrane. Fig. 5 summarizes the steps in this process. During the first 24 hr in the light, three processes occur simultaneously. Single thylakoids develop into bands of two appressed thylakoids and then into bands of three appressed thylakoids (Fig. 7). At the same time, the average number of bands per chloroplast section increases threefold. Since the chloroplast grows in both width and length during this interval, the thylakoids also increase in surface area. During the second 24 hr in the light, two processes occur: the number of three-thylakoid bands per chloroplast section continues to increase and simultaneously the thylakoids continue to grow in surface area. By 48 hr in the light, the chloroplast has the appearance of a mature chloroplast containing an average of eight three-thylakoid bands per section. However, during the subsequent 48 hr in the light, the chloroplast continues to elongate as well as to accumulate chlorophyll (Fig. 1), and this is accompanied by a continued growth in surface area of the existing thylakoids.

CAP has a dramatic effect on the number, size, and organization of chloroplast thylakoids. Fig.

TABLE I
Effect of CAP on Chloroplast Growth

Hours in light	Control			CAP		
	Cell volume*	Chloroplast volume		Cell volume*	Chloroplast volume	
	μ^3	% of cell	μ^3	μ^3	% of cell	μ^3
0	450	2.4	11	450	2.4	11
6	545	5.5	30	565	2.6	15
12	560	6.8	38	600	2.8	17
24	560	9.9	55	625	3.1	19
48	560	15.6	87	625	3.7	23‡
96	560	19.4	109	625	5.0	31‡

* Figures are an average of values from three experiments.

‡ At these times most cells appear to contain more than one chloroplast (see text). These figures thus refer to the total volume of all the chloroplasts in the cell.

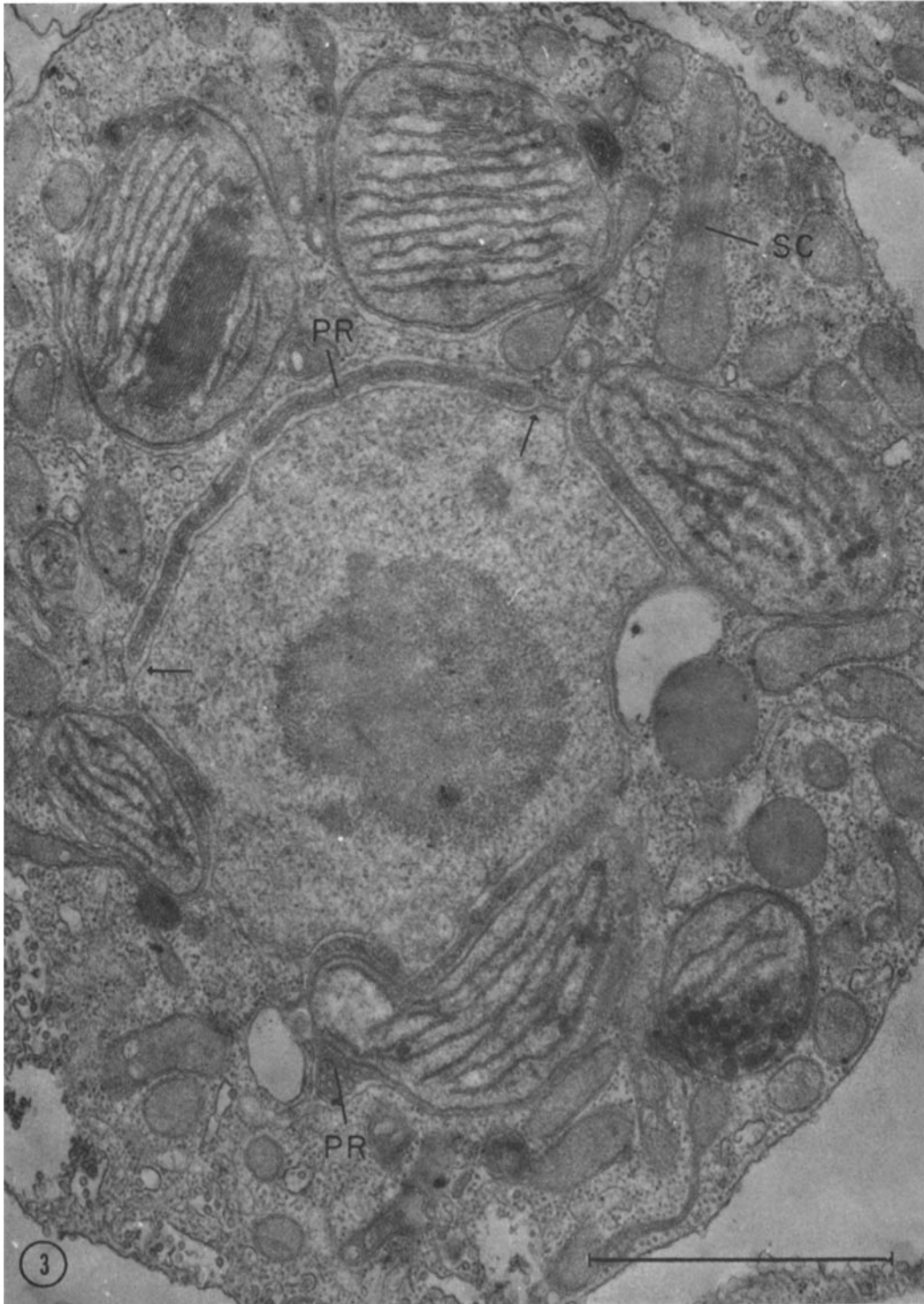


FIGURE 3 96-hr light cell which illustrates many of the effects of CAP treatment. The six chloroplast profiles may be lobes of a single plastid, but are more likely separate structures arising from chloroplast division in the absence of cell division. The chloroplast thylakoids are reduced in size and number and are abnormally organized either as single thylakoids or into large stacks of thylakoids. The perinuclear reticulum (*PR*) has proliferated to a great extent, apparently displacing the point of bifurcation (arrows) of the chloroplast ER and nuclear envelope. Mitochondrial cristae are almost completely absent. A striated core (*SC*), however, is still visible despite the increased density of the mitochondrial matrix. Standard fixation. $\times 26,400$.

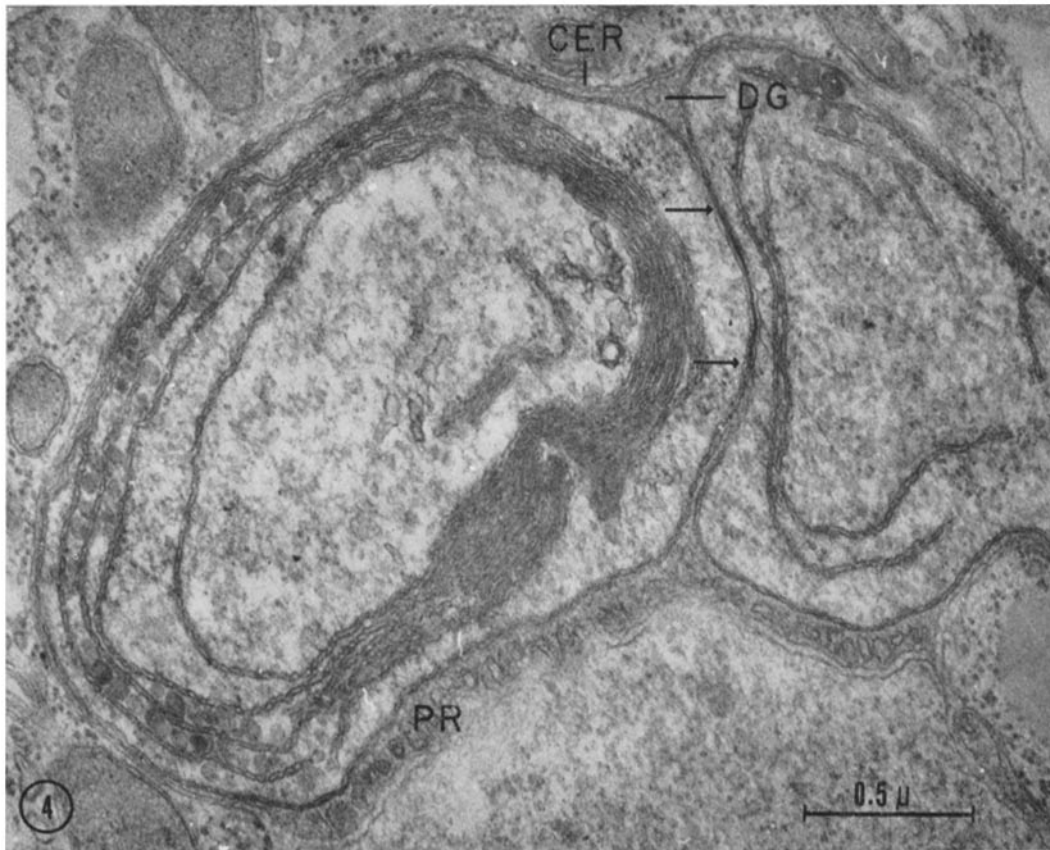


FIGURE 4 48-hr light CAP-treated cell. At the plane of section the chloroplast is divided, yet remains within a single sac of chloroplast ER (*CER*). Between the arrows the outer membranes of the adjacent chloroplast envelopes are appressed. Observe the abnormal arrangement of the chloroplast thylakoids in this CAP-treated cell and the mitochondrial profiles which have no cristae. *DG*, dense granules. *PR*, perinuclear reticulum. Standard fixation. $\times 45,600$.

6 shows that in the CAP-treated cells the total number of thylakoids per chloroplast section is markedly reduced. Since the chloroplasts of the CAP-treated cells increase relatively little in size, there is also a marked reduction in thylakoid size in the drug-treated cells. The total amount of thylakoid membrane synthesized in control and CAP-treated cells was calculated from chloroplast volume, chloroplast thickness, and the number of thylakoids per section. During the first 12 hr in the light, when CAP has no secondary effects on cell division, the amount of thylakoid membrane per chloroplast in the control cells increases 17-fold, whereas in the CAP-treated cells the amount of thylakoid membrane per chloroplast increases only 2-fold. If one considers the fact that both control and CAP-treated cells divide once during

this interval, then it can be seen that CAP during the first 12 hr in the light inhibits the synthesis of new thylakoid membrane by 91%. Chlorophyll synthesis is inhibited 93% during the same interval.

In addition to greatly reducing the number and size of chloroplast thylakoids, CAP also disrupts their normal organization in bands of three. The main abnormality of thylakoid organization which appears during the first 12 hr of CAP treatment is the appearance of bands containing more than three thylakoids. Four-thylakoid bands are present as early as 3 hr of light, and bands of five or six thylakoids first appear at 12 hr of light (Fig. 16). A second abnormality which appears early is that the bands of thylakoids terminate abruptly at the rim of the chloroplast instead of looping around

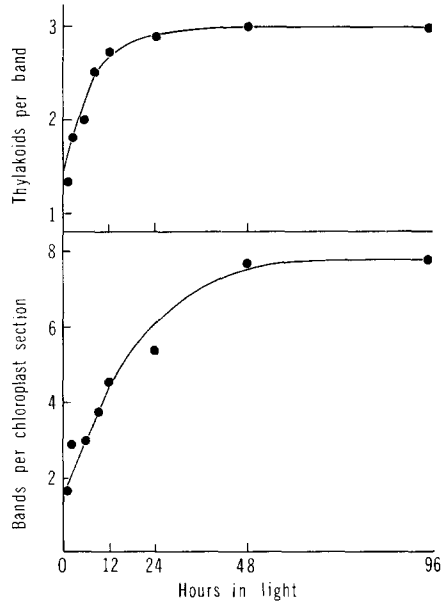


FIGURE 5 Number and organization of the chloroplast thylakoids during normal greening in *Ochromonas danica*. Counts were made at the midline of each chloroplast section which had been cut approximately perpendicular to the plane of the thylakoids, i.e., at right angles to the plane of the platelike chloroplast.

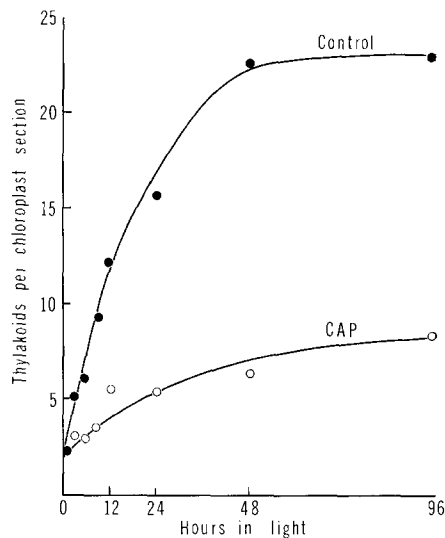


FIGURE 6 Effect of chloramphenicol (CAP) on the number of thylakoids per chloroplast section. ●—●, control cells; ○—○, CAP-treated cells.

the chloroplast rim to form girdle bands. With the loss of the girdle bands, the chloroplast DNA no longer has its characteristic peripheral location, but appears to be scattered throughout the chloro-

plast. Since Hooper et al. (21) observed that CAP impairs the fusion of thylakoids in *Chlamydomonas*, it was expected that CAP might affect the degree of appression of thylakoids within a band. However, at 12 hr of light, the width of the inter-thylakoid space measured approximately 30 Å in both control and CAP-treated cells.

In cells which have been treated with CAP for 24 hr or more the organization of chloroplast thylakoids is strikingly abnormal. In these cells almost all thylakoids either are arranged singly, often being separated from each other by rows of lipid globules (cf. Figs. 3 and 4 with Fig. 7), or they are piled into abnormal stacks of five to 30 thylakoids (Fig. 3). Some of these large stacks consist of a whorl of concentric thylakoids; others have a striking resemblance to the grana of higher plant chloroplasts (Fig. 8).

PROLAMELLAR BODIES: Prolamellar bodies are first observed in chloroplasts of cells which have been exposed to CAP for 6 hr (Fig. 9) and they become progressively larger and more numerous with extended treatment (Figs. 10 and 11). In some chloroplasts, the prolamellar bodies consist of an irregular network of tubules (Figs. 9 and 10) and resemble the transformed prolamellar bodies of higher plant plastids. More commonly, the prolamellar bodies are made up of a mass of dense material in which profiles of vesicles and tubules can be discerned (Fig. 11). Numerous lipid globules are also often found in close association with the prolamellar bodies. On rare occasions similar, but smaller, structures have been observed in untreated dark-grown and early greening cells of *Ochromonas*.

CHLOROPLAST RIBOSOMES: During normal chloroplast development in *Ochromonas*, there is a dramatic increase in the number of chloroplast ribosomes. Figs. 12, 14, and 7 and Table II show that the concentration of chloroplast ribosomes doubles during the first 6 hr in the light and continues to increase during the next 18 hr in the light. At the same time the chloroplast is growing rapidly in volume (Table I), so that there is an even larger increase in the total number of ribosomes per chloroplast. During the first 6 hr in the light, the total number of ribosomes per chloroplast increases 6-fold; during the following 90 hr, there is a further 5-fold increase. CAP markedly inhibits this light-induced synthesis of chloroplast ribosomes. In the CAP-treated cells, the number of chloroplast ribosomes per area stays approximately constant (cf. Fig. 13 with

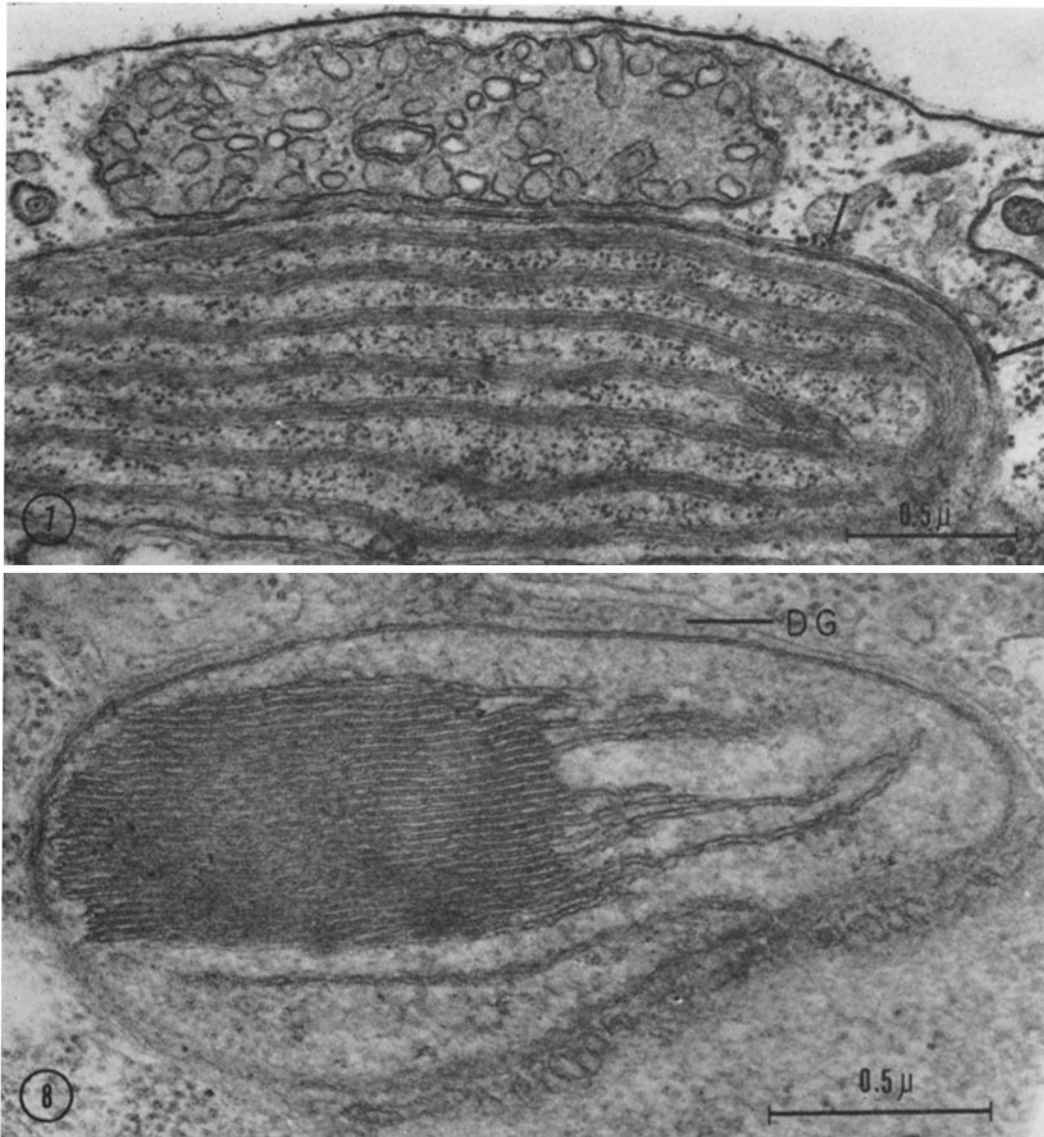
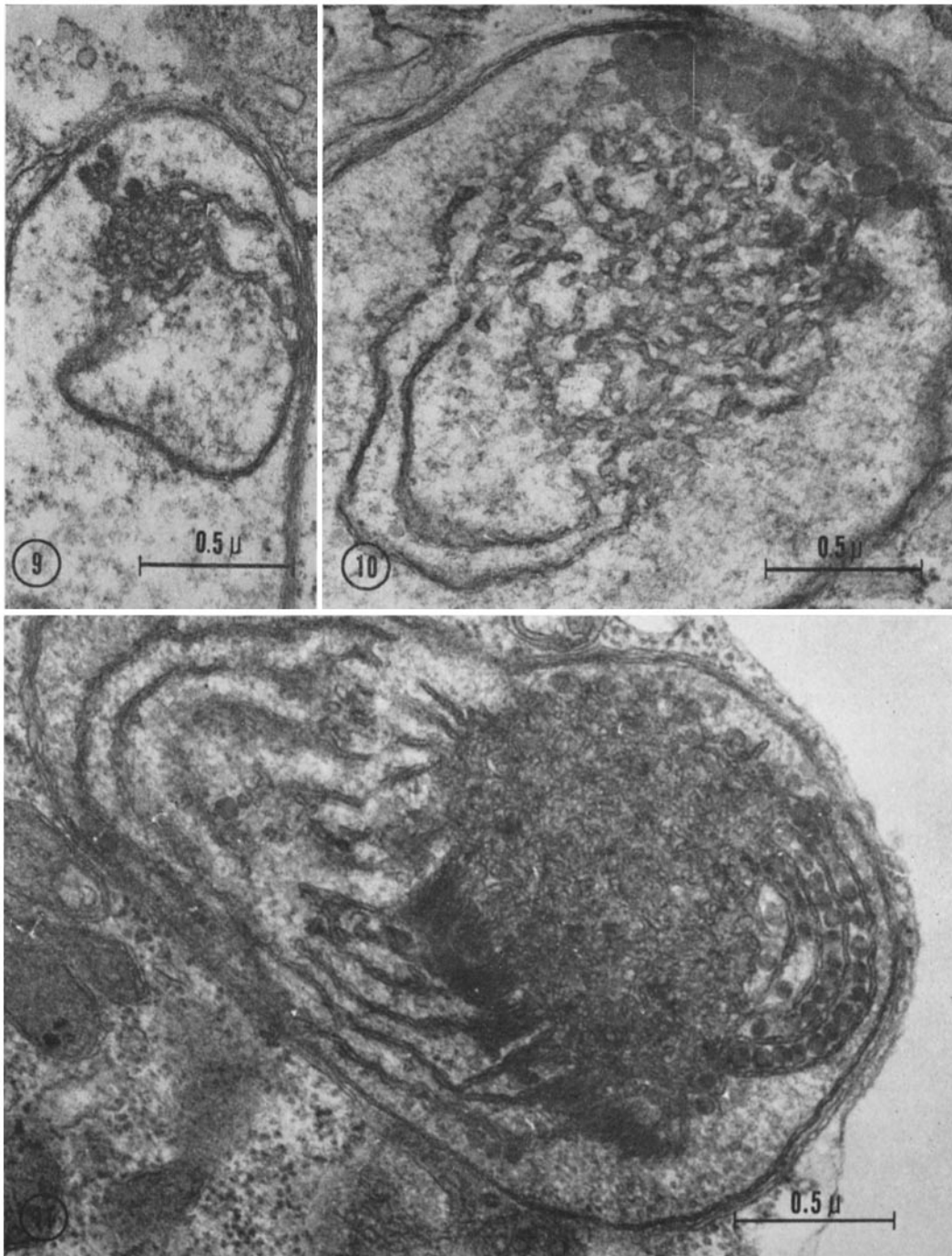


FIGURE 7 24-hr light control cell. This micrograph illustrates the normal organization of chloroplast thylakoids in bands of three. At this stage of development, the concentration of chloroplast ribosomes has reached its maximum, although the total number of chloroplast ribosomes will continue to increase as the chloroplast grows. Compare the mitochondrion in this control cell with that in a 24-hr light CAP-treated cell (Fig. 20 c). CAP has a marked effect on the number of tubular cristae, but little effect on the concentration of mitochondrial ribosomes. This micrograph also illustrates that ribosomes are present on the outer surface of the chloroplast ER (arrows). Falk's fixation. $\times 45,600$.

FIGURE 8 Chloroplast of a 48-hr light CAP-treated cell containing a large granum-like stack of thylakoids. Dense granular material (DG) fills the space between the chloroplast envelope and chloroplast ER. Standard fixation. $\times 58,800$.



FIGURES 9-11 Chloroplasts of CAP-treated cells containing prolamellar bodies. The prolamellar bodies in Figs. 9 and 10 are open networks of tubules whereas that in Fig. 11 consists of tubular structures embedded in a dense matrix. Concentric single thylakoids radiate from the prolamellar bodies. Fig. 9, 6-hr CAP; Figs. 10 and 11, 96-hr CAP. Figs. 9 and 10, Falk's fixation; Fig. 11, standard fixation. $\times 45,600$.

TABLE II
Effect of CAP on Chloroplast Ribosomes

Hours in light	Ribosomes per $\mu^2/16$		Ribosomes per chloroplast (Ratio light/dark)	
	Control (Average \pm SEM)	CAP (Average \pm SEM)	Control	CAP
0	8.8 \pm 0.4	8.1 \pm 0.4	1.0	1.0
3	14.2 \pm 0.5	8.6 \pm 0.6	2.9	1.3
6	18.4 \pm 0.4	7.8 \pm 0.6	5.8	1.3
12	18.9 \pm 0.6	9.3 \pm 0.3	7.5	1.8
24	23.0 \pm 0.5	9.6 \pm 0.3	13.3	2.1
48	22.3 \pm 0.7	7.4 \pm 0.3	20.5	1.9
96	23.8 \pm 0.6	7.1 \pm 0.3	27.0	2.6

Fig. 12), and there is a slow increase in the total number of chloroplast ribosomes per cell, paralleling the increase in chloroplast volume (Table II). If one calculates the number of new chloroplast ribosomes synthesized in the control and CAP-treated cultures during the first 12 hr in the light, remembering that the cells in each culture divide once in this interval, it can be seen that CAP inhibits the synthesis of chloroplast ribosomes by 81%.

PERINUCLEAR RETICULUM: In *Ochromonas danica* the single chloroplast lies close to the nucleus and is surrounded by a sac of ER. At the borders of the region where the chloroplast lies against the nucleus, this sac of chloroplast ER is continuous with the outer membrane of the nuclear envelope (15). In control cells, the narrow space lying between the chloroplast envelope and the nuclear envelope frequently contains a single layer of tubules and vesicles (Fig. 15). Tangential sections (Fig. 17) reveal that the tubules form a branched network interspersed with vesicles, so we shall call this structure the perinuclear reticulum. In control cells, the perinuclear reticulum is almost exclusively localized in the narrow space separating the nucleus from the chloroplast.

In cells treated with CAP for 9 hr or more, the perinuclear reticulum becomes progressively hypertrophied. In many sections, the space between the nucleus and the chloroplast is swollen and filled with several layers of tubules (Fig. 16). In other sections the perinuclear reticulum has proliferated into the space between the chloroplast envelope and the chloroplast ER (Fig. 3). In the CAP-treated cells the junction of the chloroplast ER and the outer membrane of the nuclear envelope is usually not at the border of the chloroplast,

but a considerable distance away. The perinuclear reticulum fills this space also (Fig. 3). In fact, the proliferation of the perinuclear reticulum may be the cause of this displacement of the chloroplast ER-nuclear envelope junction. The significance of the CAP-induced proliferation of the perinuclear reticulum is not understood; nor, in fact, is any function known for this structure.

In cells which have been treated with CAP for 6 hr or more, masses of dense granular material are also observed in the space between the chloroplast ER and the chloroplast envelope (Figs. 4, 8, and 19) and in the space between the nuclear envelope and the extended chloroplast ER (Fig. 18). Dense material has never been found in this location in control cells.

Effect of CAP on Mitochondrial Ultrastructure

CAP causes profound changes in mitochondrial ultrastructure. The most striking change is the progressive loss of the tubular cristae in the mitochondria of the CAP-treated cells. The representative mitochondria in Figs. 20 a-20 e illustrate these changes. Cells which have been in CAP for 9 hr show no reduction in the number of their mitochondrial cristae (cf. Fig. 20 b with Fig. 20 a). After 24 hr in the drug, the number of mitochondrial cristae is greatly reduced (Fig. 20 c), and after 96 hr, almost no cristae remain (Figs. 20 e and 3). These changes are expressed quantitatively in Table III which gives the ratio of inner mitochondrial membrane (the inner limiting membrane of the mitochondrion plus the membrane of the cristae) to outer mitochondrial membrane in control and CAP-treated cells.

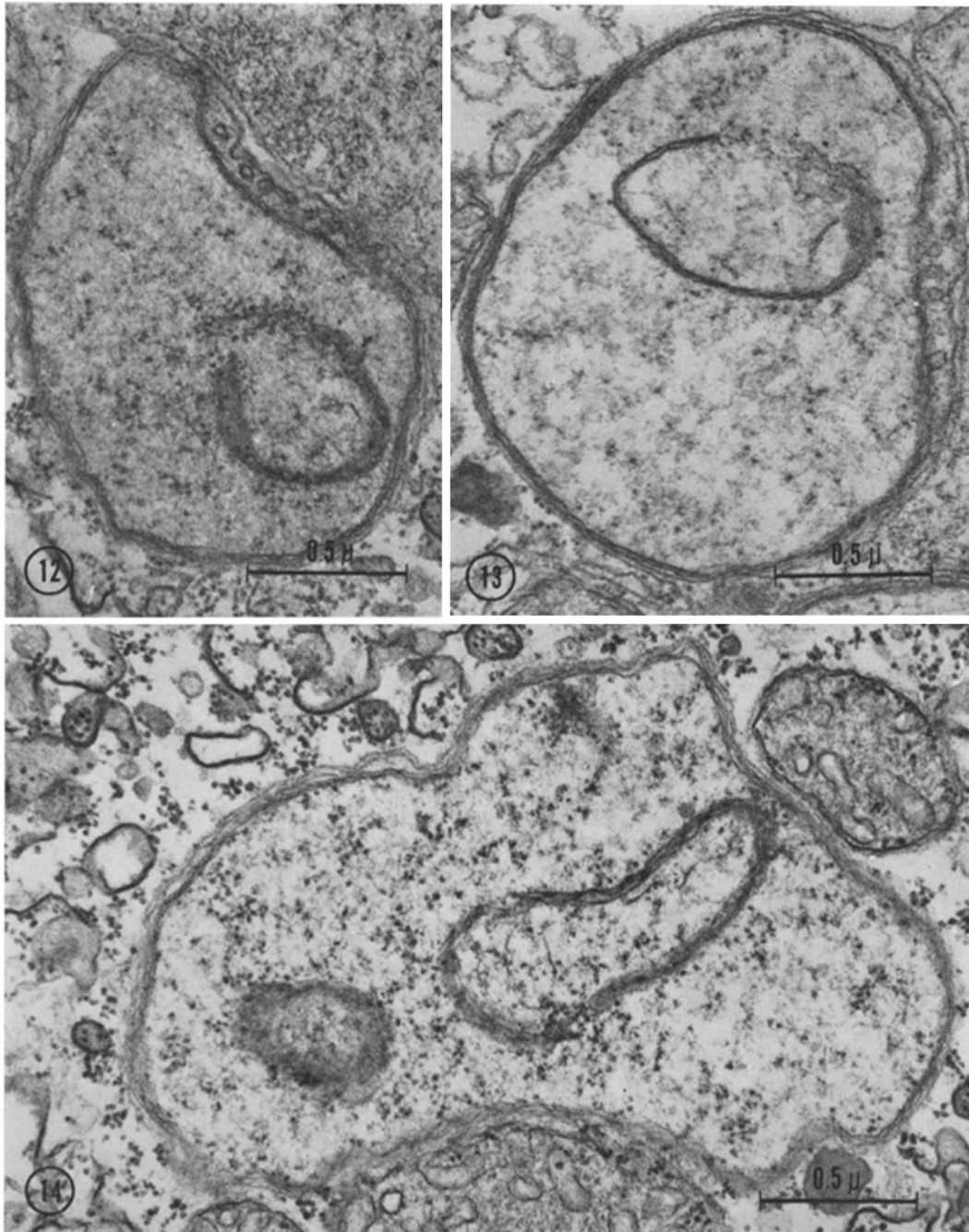


FIGURE 12 Proplastid of a dark-grown cell fixed by the method of Falk to demonstrate chloroplast ribosomes. $\times 45,600$.

FIGURES 13-14 6-hr light cells. The concentration of ribosomes in the chloroplast of the control cell (Fig. 14) is considerably greater than that in the chloroplast of the CAP-treated cell (Fig. 13). Since the chloroplast of the control cell is also larger than the chloroplast of the CAP-treated cell, the control cell at this stage of development contains approximately four times as many chloroplast ribosomes as the CAP-treated cell. Fig. 13, CAP-treated cell; Fig. 14, control cell. Falk's fixation. $\times 45,600$.

With extended CAP treatment, the matrix of the mitochondria becomes denser (Figs. 3 and 20). Smith et al. (38) have also observed a CAP-induced increase in the matrix density of mitochondria from human bone marrow. Furthermore, in cells treated with CAP for 24 hr or longer, the mitochondria become smaller and more numerous (Figs. 3 and 20). It appears that mitochondria, like the chloroplast, continue to divide in the absence of cell division.

Table IV shows that the number of mitochondrial ribosomes per area stays essentially constant in both the control and CAP-treated cells during the first 12 hr in the light and that in both types of cells the concentration of mitochondrial ribosomes declines slightly when the rate of cell division decreases. Although we did not determine mitochondrial volume in this study, counts of the number of mitochondrial profiles per area and measurements of mitochondrial diameters showed that there is no apparent change in mitochondrial size or number in either the control or CAP-treated cells during the first 12 hr in the light. Since both the control and CAP-treated cells divide once during this interval, the total number of mitochondrial ribosomes in both the control and CAP-treated cultures must approximately double. Thus, we conclude that CAP has no effect on the synthesis of mitochondrial ribosomes.

DISCUSSION

Specificity of CAP Action

It is now established by studies on both lower and higher plants (8, 9, 28) that CAP at concentrations similar to that used in this experiment specifically inhibits protein synthesis on organelle ribosomes, but has no effect on protein synthesis on cytoplasmic ribosomes. In view of these studies, we believe that CAP exerts its primary effect on cells of *Ochromonas danica* by specifically inhibiting protein synthesis on chloroplast and mitochondrial ribosomes.

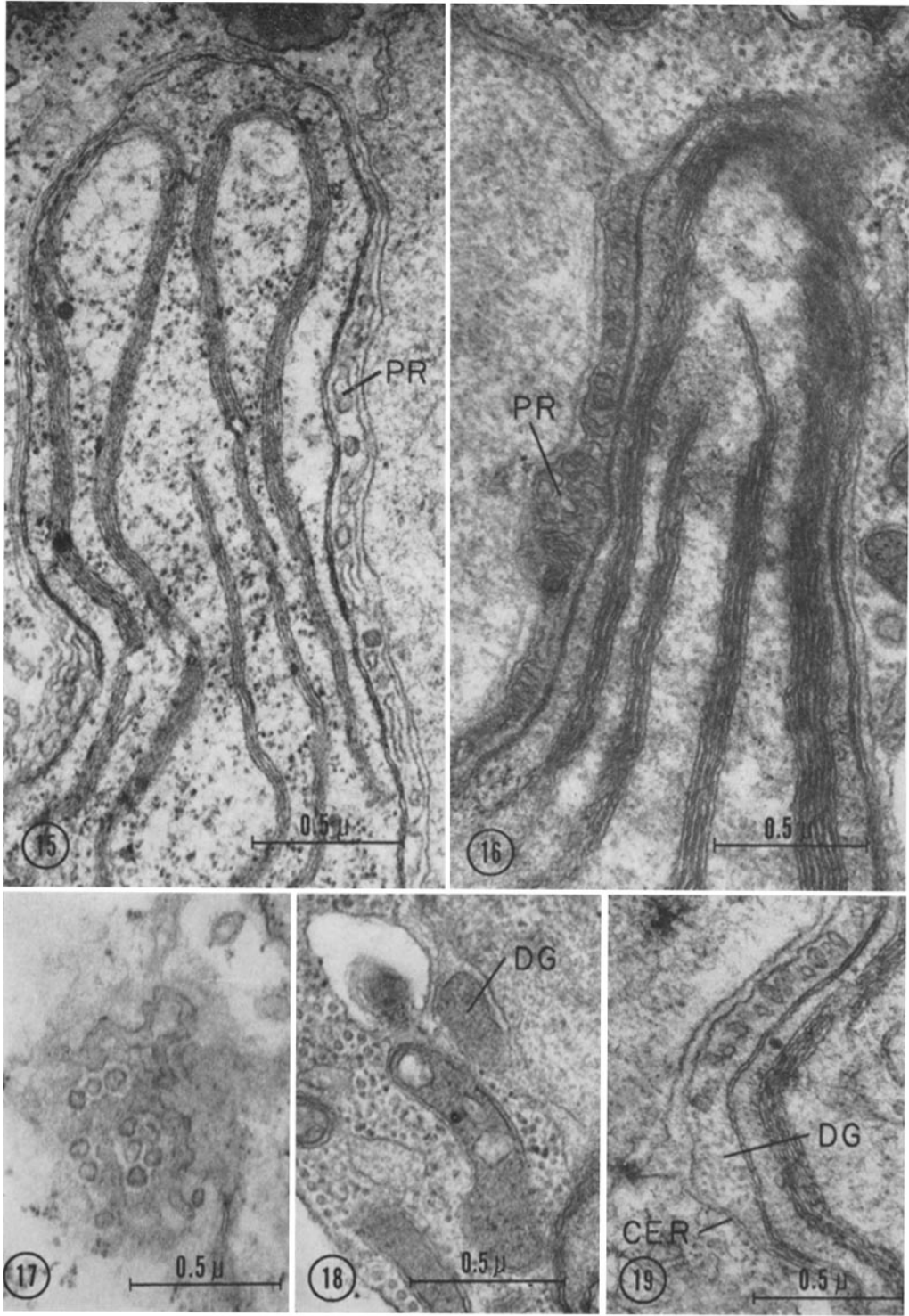
Several authors (4, 12, 14, 20) have reported that CAP at high concentrations may also directly inhibit mitochondrial respiration. Fortunately, the fact that CAP had no effect on the growth rate of *Ochromonas* during the first 12 hr of treatment argues strongly against the possibility that CAP at the concentration employed directly inhibits respiration in *Ochromonas*. In the HeLa cell system studied by Firkin and Linnane (12),

cell division was immediately inhibited by those concentrations of CAP which inhibited respiration, and one would expect to see a similar immediate inhibition of growth in *Ochromonas* if CAP were affecting respiration directly. Instead, a decline in growth rate was observed only after 12 hr of treatment with CAP. This decrease in growth rate paralleled the observed decrease in the number of mitochondrial cristae and can easily be explained as resulting from decreased ATP production by the cristae-deficient mitochondria. In the discussion which follows, only those ultrastructural changes which occurred in the CAP-treated cells before mitochondrial cristae or growth rate were affected are considered to result from the inhibition of protein synthesis on organelle ribosomes.

Chloroplast Thylakoids and Prolamellar Bodies

In *Ochromonas*, CAP has an immediate and marked effect on the amount of thylakoid membrane synthesized during greening. CAP has been shown to have a similar inhibitory effect on the amount of thylakoid membrane synthesized during greening in beans (31), *Euglena gracilis* (5), and in the *y-1* mutant of *Chlamydomonas reinhardtii* (21, 22). We interpret the large decrease in the amount of thylakoid membrane formed in greening CAP-treated cells of *Ochromonas* as indicating that some protein or proteins essential for the synthesis of thylakoid membranes is synthesized on chloroplast ribosomes. The identity of this protein(s) is unknown. It could be a protein of the membrane itself, or it could be an enzyme involved in the synthesis of a membrane lipid or of chlorophyll. Likewise, we do not know whether CAP inhibits chlorophyll synthesis in *Ochromonas* by interfering with the synthesis on chloroplast ribosomes of a chlorophyll-binding membrane protein or of an enzyme involved in chlorophyll synthesis. Clearly, biochemical studies are needed to answer these questions.

The formation of prolamellar bodies in the CAP-treated cells is probably a secondary consequence of the inhibition by CAP of thylakoid membrane synthesis. It seems likely that these bodies are accumulations of material (protein and/or lipid) which has continued to be synthesized in the CAP-treated cells but has been prevented from being incorporated into thylakoid membranes.



Chloroplast Ribosomes

The marked inhibition by CAP of the synthesis of chloroplast ribosomes suggests that one or more chloroplast ribosomal proteins are synthesized within the chloroplast of *Ochromonas*. Our observations on *Ochromonas* are in agreement with those of Ingle (23) who showed that CAP inhibits the synthesis of chloroplast ribosomal RNA in radish seedlings. Recently, Ellis and Hartley (10) have shown that another inhibitor of protein synthesis on chloroplast ribosomes, lincomycin, also prevents the synthesis of chloroplast ribosomes in greening pea apices. However, Goodenough (18) has shown that in light-grown wild-type cells of *Chlamydomonas reinhardtii* there is no reduction in the concentration and, thus, the presumed total number of chloroplast ribosomes during three generations' growth in CAP. A possible explanation of the discrepancy between our results and those of Goodenough is that light-grown cells of *Chlamydomonas* may have a very large pool of chloroplast ribosomal proteins, as has been demonstrated to be present in mixotrophic *ac-20* cells of *Chlamydomonas* (19), whereas dark-grown cells of *Ochromonas* have a relatively small pool of chloroplast ribosomal proteins. However, it seems more likely that a genuine species difference is involved since *Ochromonas* belongs to an algal group phylogenetically distant from *Chlamydomonas*.

Mitochondrial Cristae and Ribosomes

Growth of *Ochromonas* in CAP causes a progressive and ultimately almost total loss of the mitochondrial cristae. Growth in CAP has also been

shown to cause a reduction in the number of mitochondrial cristae in two species of yeast (6, 25), in *Tetrahymena pyriformis* (42), in HeLa cells (29), and in regenerating rat liver (13), although, in most of these cells, the loss of cristae is not as dramatic as in *Ochromonas*. Although, in our system, the growth rate declines as soon as mitochondrial cristae begin to be lost, we believe that CAP is directly affecting the synthesis of mitochondrial cristae by inhibiting the synthesis on mitochondrial ribosomes of a protein or proteins essential for formation of the cristae membrane. The results of biochemical studies on other species (3) suggest that this could be a protein of the cristae membrane itself.

Mitochondrial ribosomes, unlike those of the chloroplast, continue to be synthesized at a normal rate during the first 12 hr of treatment with CAP. In both the CAP-treated and control cultures, the total number of mitochondrial ribosomes in the culture approximately doubles as the cell number doubles. Unless these ribosomes are assembled from preexisting proteins, it appears that the synthesis of mitochondrial ribosomal proteins is unaffected by CAP. This indicates that in *Ochromonas*, as in yeast (7) and *Neurospora* (27, 32), mitochondrial ribosomal proteins are synthesized on cytoplasmic ribosomes.

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FIGURE 15 9-hr light control cell showing the normal appearance of the perinuclear reticulum (PR), a network of tubules and vesicles occupying the narrow space between the chloroplast and nuclear envelope. Falk's fixation. $\times 45,600$.

FIGURE 16 24-hr light CAP-treated cell demonstrating the hypertrophy of the perinuclear reticulum (PR). The proliferation of the perinuclear reticulum has distended the perinuclear space. Note the abnormal bands of four and six thylakoids in the chloroplast. Standard fixation. $\times 45,600$.

FIGURE 17 3-hr light control cell. Tangential section through the perinuclear reticulum demonstrating that it has the form of a branched network of tubules with vesicles filling the interstices. Falk's fixation. $\times 45,600$.

FIGURE 18 24-hr light CAP-treated cell. Dense granular material (DG) has accumulated in the space between the nuclear envelope and the extended chloroplast ER. Standard fixation. $\times 45,600$.

FIGURE 19 12-hr light CAP-treated cell. Granular material (DG) is present in the space between the chloroplast envelope and the chloroplast ER (CER). Falk's fixation. $\times 45,600$.

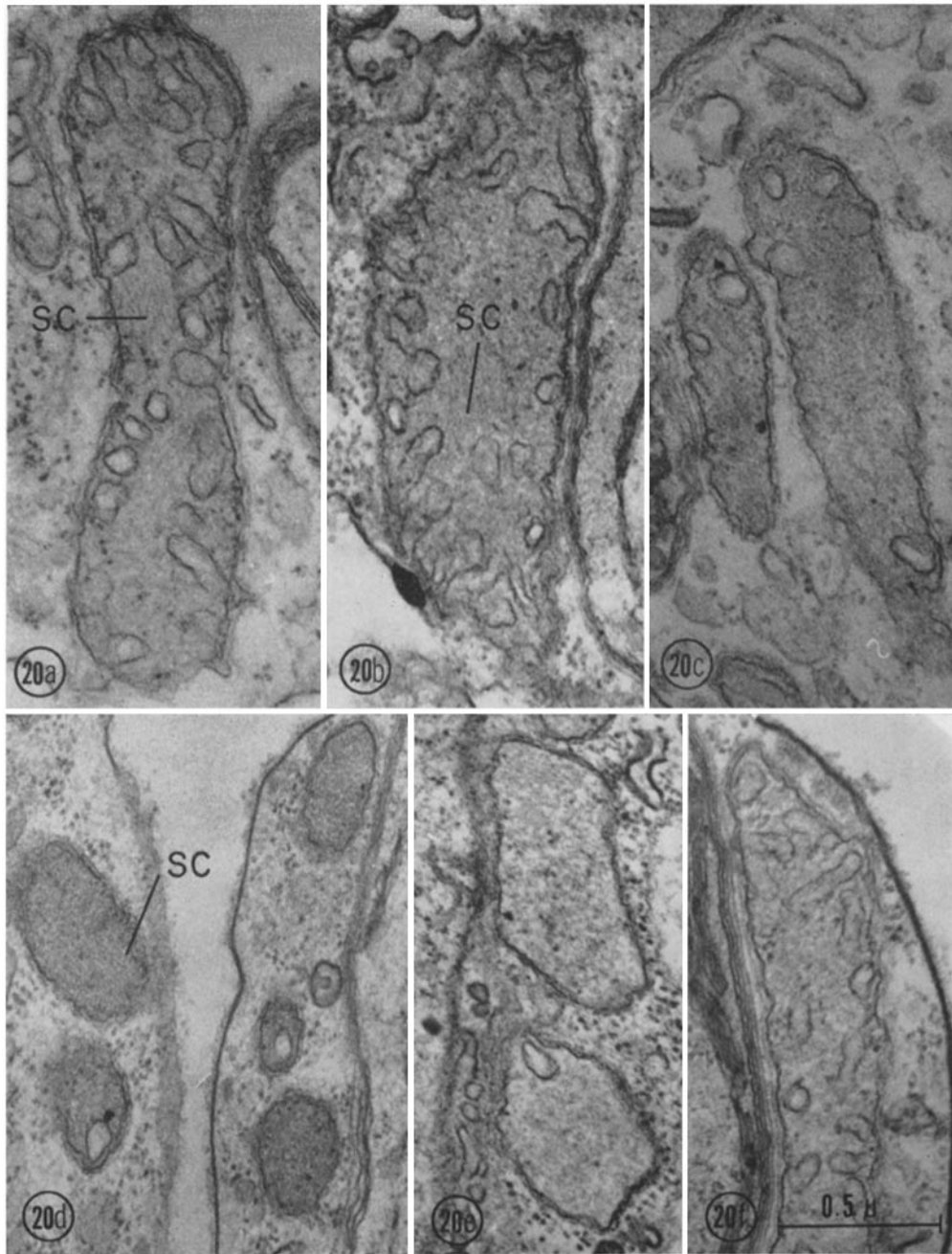


FIGURE 20 These micrographs illustrate the effects of CAP on mitochondrial ultrastructure. Depicted are representative mitochondria from (a) a dark-grown control cell, (b) a 9-hr light CAP-treated cell, (c) a 24-hr light CAP-treated cell, (d) a 48-hr light CAP-treated cell, (e) a 96-hr light CAP-treated cell, and (f) a 96-hr light control cell. The decrease in the number of tubular cristae is first observed after 12 hr of treatment and becomes progressively more pronounced. Mitochondrial ribosomes, however, persist even when the cristae are completely lost. With CAP treatment, the mitochondrial profiles also become smaller and more numerous. SC, striated core. Falk's fixation. $\times 45,600$.

TABLE III
Ratio of Inner to Outer Mitochondrial Membranes:
Changes during Exposure to CAP

Hours in light	Ratio inner/outer membrane	
	Control	CAP
0	2.4	2.5
12		2.5
24		1.6
48		1.2
96	2.5	1.1

TABLE IV
Effect of CAP on Mitochondrial Ribosomes

Hours in light	Ribosomes per $\mu^2/16$	
	Control (Average \pm SEM)	CAP (Average \pm SEM)
0	6.7 \pm 0.3	6.6 \pm 0.3
6	6.7 \pm 0.5	6.5 \pm 0.4
12	6.6 \pm 0.3	5.4 \pm 0.2
24	7.6 \pm 0.3	4.8 \pm 0.2
48	5.4 \pm 0.3	3.6 \pm 0.2
96	5.5 \pm 0.3	4.3 \pm 0.2

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