ULTRASTRUCTURE OF DNA-CONTAINING AREAS IN THE CHLOROPLAST OF *CHLAMYDOMONAS*

HANS RIS, Ph.D., and WALTER PLAUT, Ph.D.

From the Department of Zoology, University of Wisconsin, Madison

ABSTRACT

The chloroplast of *Chlamydomonas moewusii* was examined by electron microscopic and cytochemical methods for the possible presence of DNA. Both the Feulgen reaction and acridine orange indicated the presence within the chloroplast of one or more irregularly shaped DNA-containing bodies generally in the vicinity of the pyrenoid. Electron micrographs revealed 25 A microfibrils in these areas which correspond to DNA macromolecules with respect to their location, morphology, and sensitivity to deoxyribonuclease digestion. The possibility that this material is the genetic system of the chloroplast and the hypothesis that the chloroplast represents an evolved endosymbiont are discussed.

Except in certain viruses, primary genetic systems appear to be associated with deoxyribonucleic acid (DNA). Electron microscope studies of the DNA-containing cell components have so far revealed two different types of ultrastructure. In bacteria (Kellenberger, 1960), *Streptomyces* (Hopwood and Glauert, 1960 *a*), and blue-gree algae (Hopwood and Glauert, 1960 *b*. Ris and Singh, 1961), one finds areas of low density and variable shape which contain fibrils about 25 to 30 A thick. By spreading protoplasts on the surface of water, these fibrils can be obtained intact for electron microscopy; under these conditions the fibrils are found to be very long, with few free ends visible. They represent DNA macromolecules; the linkage group of a bacterial cell may consist of one or several such macromolecules (Kleinschmidt and Lang, 1960). In most cells of animals and plants the DNA is associated with histone-type protein and more complex proteins to form fibrils 40 and 100 A thick (Ris, 1961). These fibrils, probably as multistranded bundles, are organized into chromosomes which show complicated patterns of replication and structural changes in the mitotic cycle. In those cells the primary genetic system is in the chromosomes, but transmission of hereditary characteristics through cytoplasmic units has been described for a number of cases. One of the best known of these "plasmids" (Lederberg, 1952) is the chloroplast of plant cells (*cf.* Rhoades, 1955). It seemed to us of interest to investigate the possibility that plastids contain structurally organized DNA and thus provide a basis for the supposition that the genetic system of the plastid is basically similar to the known nuclear systems.

The presence of DNA in plastids has been the subject of considerable debate. Cytochemical tests and chemical analyses on isolated chloroplasts (*cf.* Granick, 1955) and incorporation studies with tritiated thymidine (Stocking and Gifford, 1959) have been claimed by some investigators to show the presence of DNA. However, the data are not conclusive at the present time. At least one cytochemical investigation (Littau, 1958) has failed to demonstrate DNA; the chemical analysis of isolated plastids is complicated by the fact that nuclear contamination is

This paper was dedicated to Dr. F. Schrader on the occasion of his 70th birthday. We are saddened by his death in March of this year and dedicate this paper to his memory.

difficult to avoid in the isolation of plastids (Jagendorf and Wildman, 1954); lastly, the incorporation of tritiated thymidine is not a sufficient test for the presence of DNA unless the demonstration of incorporation is at least accompanied by a suitable enzymatic control experiment.

In the course of a search for plastids that give a positive Feulgen reaction we found that the chloroplast of *Chlamydomonas* contains several small bodies which are Feulgen-positive; this alga was therefore chosen for a study of the ultrastructure of the plastid.

MATERIAL AND METHODS

Chlamydomonas moewusii Gerloff (Indiana University Algae Collection #96-) were grown in liquid medium (modified Myer medium) or on agar slants. For light microscopy cells were fixed in alcohol-acetic acid for 10 minutes and squashed between slide and coverslip. After freezing in liquid air the coverslips were removed and some slides were subjected to hydrolysis for 10 minutes in N HCl at 60°C and stained with Schiff's reagent for 45 minutes. Control slides were stained without hydrolysis. Other slides, with cells fixed in the same way, were washed in 0.1 M acetate buffer, pH 4.5, stained for 15 to 30 minutes in acridine orange (Harleco) (0.02 mg/ml in 0.02 M acetate buffer, pH 4.5), and then washed with several changes of 0.1 M acetate buffer at the same pH. Fluorescence of acridine orange-stained cells was observed with a 2 mm Zeiss apochromat, NA 1.4, Xenon arc illumination, a BG-12 exciter filter, and a Bausch and Lomb ocular barrier filter, and photographed with High Speed Ektachrome film (Daylight). Some of the preparations stained with acridine orange were treated, before staining, with ribonuclease (Worthington, 0.3 mg/ml in distilled water, pH adjusted to 7.0, 1 hour at 50°C), others with deoxyribonuclease (Worthington, 1X crystallized, 0.3 mg/ml in ¼ strength McIlvaine buffer at pH 7.0, with 4×10^{-3} M $MgSO_4$, 5 hours at 40°C), and still others with both enzymes in sequence.

For electron microscopy, cells growing as a film on liquid medium were fixed in Kellenberger's fixative for 1 hour, post-treated with uranyl acetate or versene (Ryter *et al.*, 1958), and embedded in Epon 812 according to the method of Luft (1958). Similar cells were fixed in 10 per cent formalin in veronal-acetate buffer (final pH 7.8) for 10 minutes, washed in distilled water, and digested with deoxyribonuclease (1 mg of enzyme per ml) at 40° for 5 hours. A control group was incubated in buffer only. The cells were then postfixed in Kellenberger's buffered OsO_4, treated with uranyl acetate, and embedded in Epon 812. One-micron-thick sections were dried on glass slides, hydrolyzed in N HCl at 60°C for 20 minutes, and stained in Schiff's reagent for 45 minutes.

Electron micrographs were taken with a Siemens Elmiskop II b electron microscope at original magnifications of 7500 × and 15,000 × on Gevaert Scientia plates 19D50 and developed in D-19.

RESULTS

1. *Demonstration of DNA in the Chloroplast of Chlamydomonas*

In Feulgen-stained cells the chloroplast contains one or more small bodies of irregular shape which give a reaction of about the same intensity as the nucleus (Fig. 1). In shape and size these bodies vary from spheres 0.5 micron in diameter to oblongs 0.5 by 1.5 micra; they are located mostly around the pyrenoid. Without prior hydrolysis these structures, like the nucleus, do not stain and the only positive reaction is found in the cell walls of some of the cells. After digestion with deoxyribonuclease, the Feulgen reaction of formalin-fixed cells is very faint or absent in both nucleus and chloroplast.

A more sensitive demonstration of DNA is the yellowish-green fluorescence obtained after staining with acridine orange (Rustad, 1958). *Chlamydomonas* cells fixed in alcohol-acetic acid (3:1) and stained with acridine orange show a strong orange-red fluorescence which covers the yellowish-green fluorescence of the nucleus (the latter can be seen with suitable filtration). After ribonuclease treatment, the red fluorescence is absent, and one sees now a moderately strong yellowish-green fluorescence in the nucleus and also in the bodies within the chloroplast which were found to be stained with the Feulgen reaction (Fig. 2). In addition, there is a faint dull green fluorescence in the rest of the cytoplasm. In cells digested with deoxyribonuclease after ribonuclease treatment, the yellowish-green fluorescence of the nucleus and the chloroplast bodies cannot be seen and only the faint green background fluorescence remains (Fig. 3).

We conclude from these observations that in *Chlamydomonas moewusii* small DNA-containing bodies are closely associated with the chloroplast. (DNA in this context is operationally defined as an acid-insoluble substance which is stained by the Feulgen reaction after hydrolysis, gives the characteristic yellowish-green fluorescence after acri-

dine-orange staining, and is sensitive to deoxyribonuclease but not to ribonuclease digestion.) The fact that two other species of *Chlamydomonas*, *rheinhardi* and *eugametos*, were found upon examination of Feulgen-stained preparations to have similar bodies associated with the chloroplasts of every cell suggests that these elements are not restricted to the single species chosen for detailed examination. The specific location of the DNA-containing bodies was studied in the *C. moewusii* cells fixed for electron microscopy, sectioned at one micron thickness and stained with the Feulgen reaction. These preparations showed that the stained bodies are always located within the chloroplast, usually around the pyrenoid, and never outside the chloroplast.

This observation also suggests that the DNA-containing structures of the *Chlamydomonas* chloroplast are not directly attributable to bacterial contaminants which might per chance have been present in our cultures. A subsequent test for bacterial contamination in the cultures from which our experimental cells were taken gave negative results.[1] A Feulgen analysis of *Chlamydomonas* cells derived from a culture known to be infected showed numerous bacteria with typical chromatin bodies outside but never inside the algae. Moreover, the shape of the bacterial chromatin bodies is more regular and clearly different from the DNA-containing structures of the *Chlamydomonas* chloroplast.

If we can thus rule out chance contamination by bacteria as an explanation for the presence of extranuclear DNA in *Chlamydomonas*, we must consider the possibility that a less autonomous but still distinct biological system is associated with

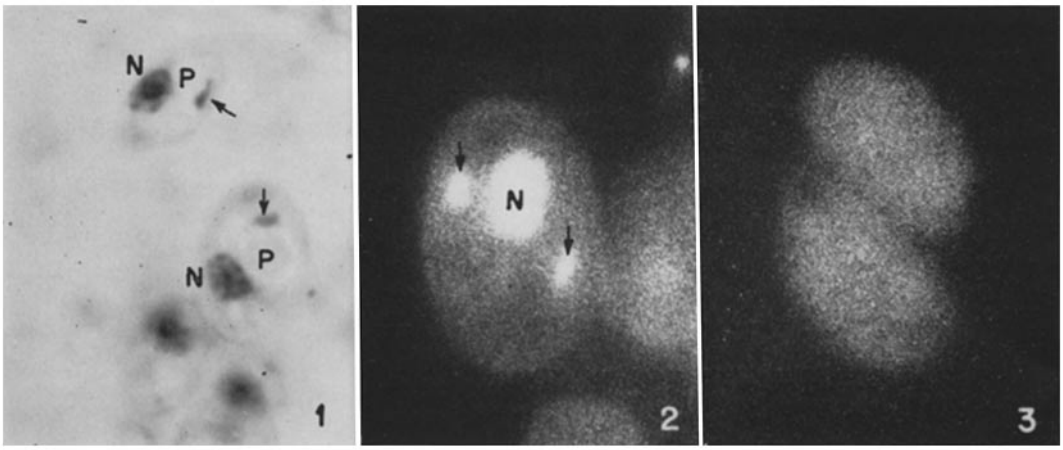


FIGURE 1

Two cells of *Chlamydomonas moewusii* fixed in ethanol-acetic acid 3:1 and stained with the Feulgen reaction. *N* = nucleus, *P* = pyrenoid. The arrows point to the DNA-containing structure in the chloroplast. × 2500.

FIGURE 2

Cell fixed in ethanol-acetic acid 3:1, treated with ribonuclease, stained with acridine orange. Copy of fluorescence micrograph taken on High Speed Ektachrome film. The nucleus (*N*) and the bodies in the chloroplast (arrows) show bright yellowish-green fluorescence. × 3000.

FIGURE 3

Two cells of *Chlamydomonas moewusii* fixed in ethanol-acetic acid 3:1, digested with ribonuclease followed by deoxyribonuclease, stained and photographed as in Fig. 2. The fluorescence in nucleus and chloroplast structures has disappeared. × 3000.

[1] We wish to thank Mr. L. McBride for testing our culture for bacterial contamination.

the chloroplast. As shown below, analysis with the electron microscope suggests a degree of structural integration of the DNA-containing material with the rest of the chloroplast which makes this unlikely.

2. *The Ultrastructure of the DNA-Containing Bodies*

The finely fibrillar nucleoplasm of microorganisms is sensitive to fixation and is only preserved under certain conditions (Ryter *et al.*, 1958). Since we were interested in comparing the DNA-containing regions of the chloroplast with the nucleoplasm of microorganisms, we used fixative and post-treatments developed for bacteria (Ryter *et al.*, 1958).

The identification of the Feulgen-positive regions in the sections proved difficult at first because their small size makes the study of adjacent thin and thick sections almost impossible. However, the general size, shape, and location on the periphery of the pyrenoid led to the recognition of special areas of characteristic appearance located between chloroplast lamellae. Once seen, they are easily identified in every cell. (Figs. 4 to 6). The membranes of chloroplast lamellae are thicker and denser than the outer chloroplast membrane and thus can be distinguished from it (*cm*, Figs. 5 and 6). The Feulgen-positive areas are bounded by chloroplast lamellar membranes and are, therefore, part of the chloroplast and not cytoplasmic pockets extending into the chloroplast. These regions contain granules which resemble the ribosomes in the cytoplasm (*R*, Figs. 5 and 6). Similar granules were found between photosynthetic membranes throughout the chloroplast. In the DNA-containing regions they are associated with areas of low density which contain fibrils about 25 to 30 A thick (arrows, Figs. 5 and 6). There is a striking similarity between these regions and the nucleoplasm in blue-green algae (Hopwood and Glauert, 1960 *b*; Ris and Singh, 1961) and bacteria. In bacteria these fibrils were shown to be DNA (Kleinschmidt and Lang, 1960; Lee, 1960). To test the hypothesis that the 25 A fibrils in the chloroplasts are also DNA, algae fixed in formalin were treated with deoxyribonuclease (controls were treated with buffer without enzyme) and then postfixed with Kellenberger's method.

While the over-all preservation of structure is not so good as in the cells fixed directly in buffered OsO_4, the DNA-containing regions are easily identified in the controls by the ribosome-like granules and the light areas with characteristic 25 A fibrils. After deoxyribonuclease no such fibrils can be found anywhere in the chloroplast, but the ribosome-like granules remain. This suggests that, as in bacteria, the fine fibrils, seen in sections through the Feulgen-positive regions, correspond to the DNA macromolecules which are removed with deoxyribonuclease.

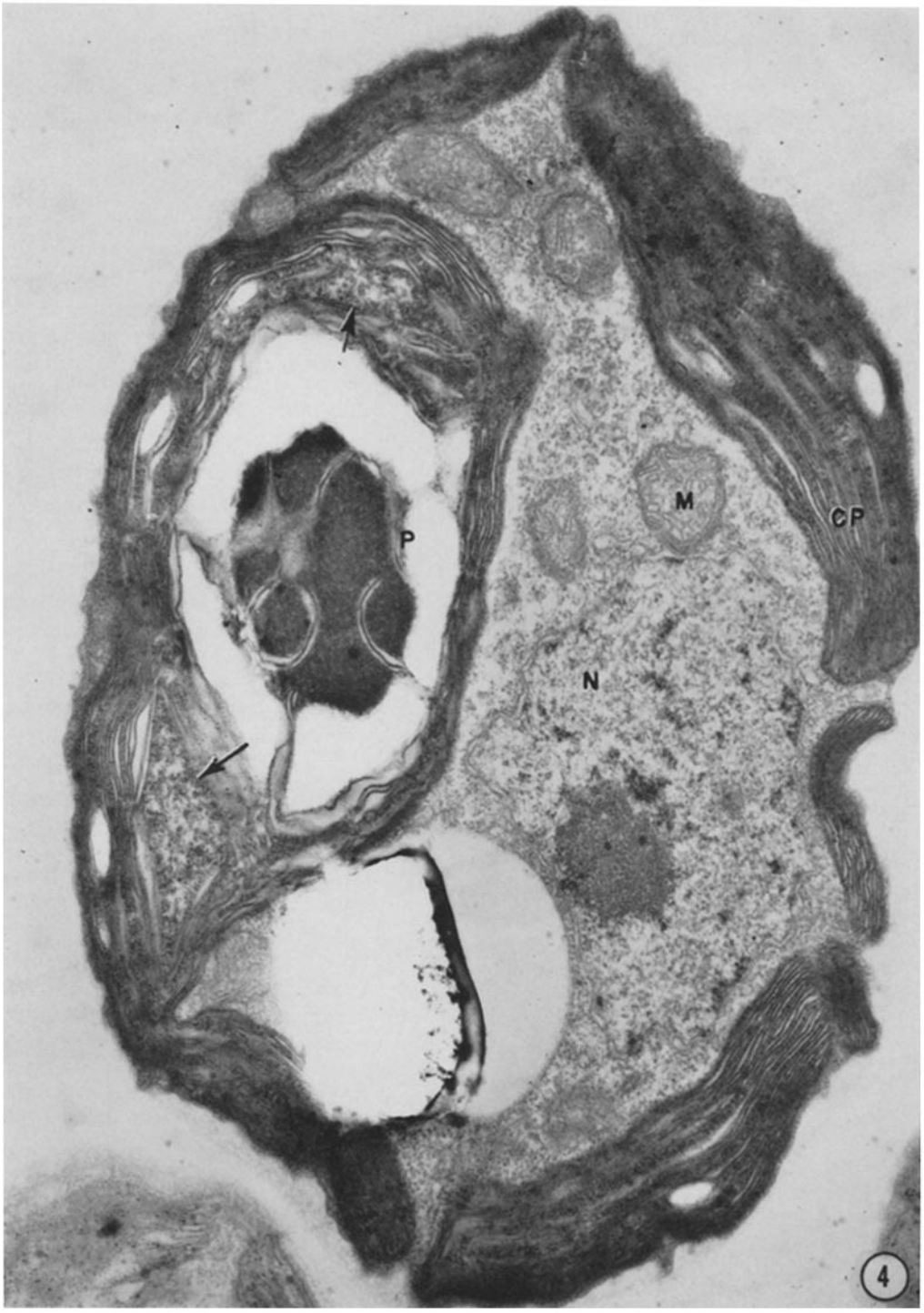
It is of interest to compare this result with the effect of deoxyribonuclease on the nucleus. Thick sections of the same block were stained with the Feulgen reaction and showed staining of nuclei in the control, but not in the enzyme-treated material. Electron micrographs of control and treated nuclei, stained with uranyl acetate, show the same general structure, namely random sections through the characteristic chromosome fibrils. In the enzyme-treated material, however, these fibrils are considerably less dense than in the controls. The nucleolus on the other hand shows no change in density after deoxyribonuclease treatment. If the DNA is combined here with protein as in other chromosomes that have been analyzed chemically, these results could mean that after formalin fixation DNA can be removed from the chromosome fibrils without completely destroying their structure.

DISCUSSION

The involvement of plastids in non-chromosomal, cytoplasmic inheritance has now been well established (*cf.* Rhoades, 1955). In several plants, experiments have shown that plastids are self-dependent (Lederberg, 1952), *i.e.*, they are derived

FIGURE 4

Electron micrograph of section of *Chlamydomonas moewusii*, fixed with Kellenberger's procedure. *CP* = chloroplast; *M* = mitochondrion; *N* = nucleus; *P* = pyrenoid. The arrows point to the DNA-containing structures in the chloroplast. × 21,000.


P
M
CP
N
4

from existing plastids or from undifferentiated proplastids and cannot be regenerated by the cell after they have been experimentally eliminated by antibiotics or by abnormal temperatures under conditions which inhibit reproduction of plastids but not of the cell (Pringsheim and Pringsheim, 1952; De Deken-Grenson and Messin, 1958; Provasoli *et al.*, 1951). This partial autonomy of plastids has suggested that they possess some kind of genetic system. In view of the finding that genetic systems in viruses and in nuclear elements of cells are associated with RNA or DNA, many authors have looked for nucleic acids in plastids. The most interesting observations that suggest the presence of DNA in chloroplast are Iwamura's (1960) studies on *Chlorella*.

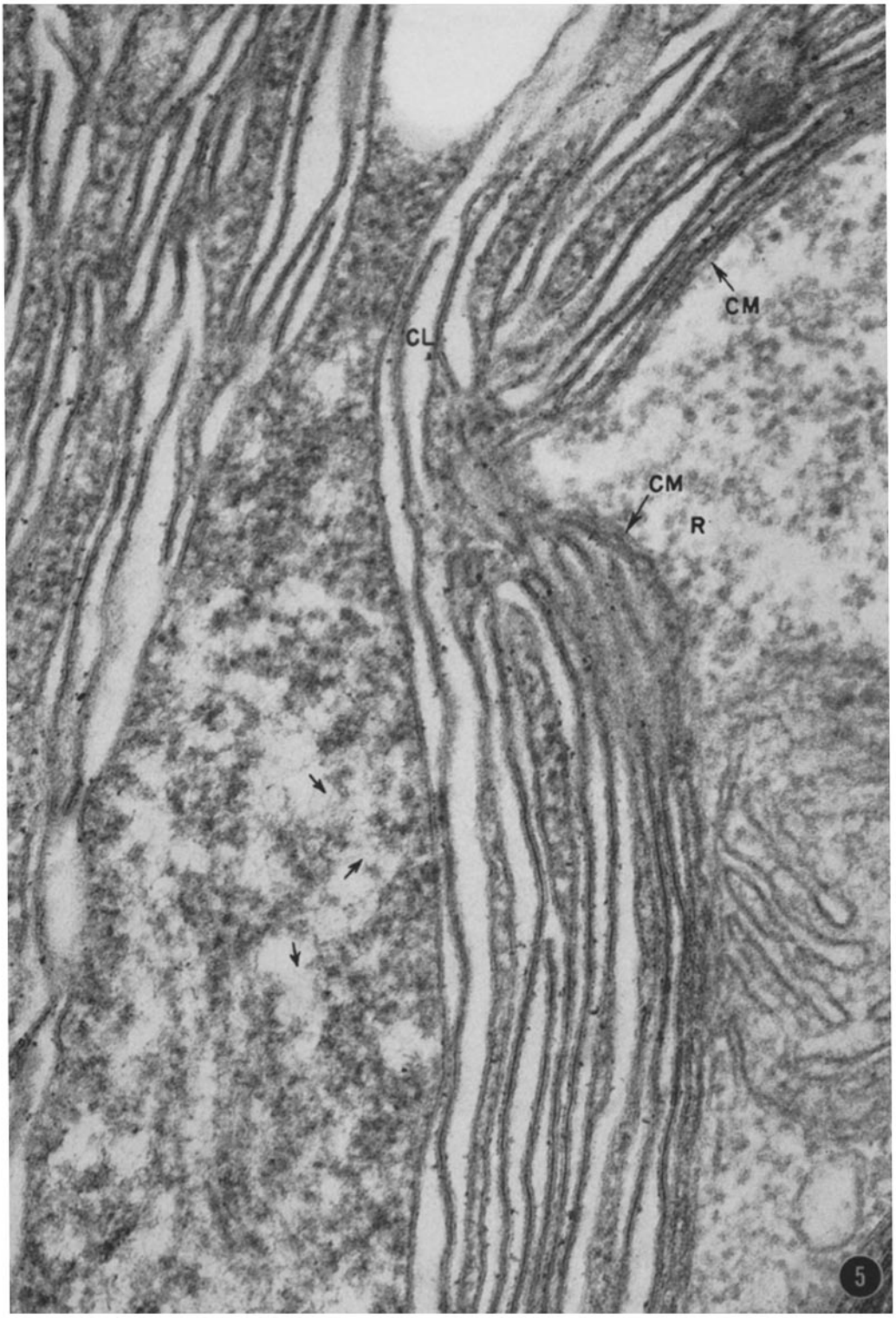
Chlamydomonas apparently has not been previously investigated for chloroplast DNA. Our cytochemical studies indicate that certain localized areas in *Chlamydomonas* chloroplasts contain DNA in concentrations high enough to give an unmistakably positive Feulgen reaction. This alga was, therefore, especially favorable for investigating the ultrastructure of the DNA-containing material. Sager and Palade (1957) have published an electron microscope study of *Chlamydomonas reinhardi*. They do not mention any special regions in the chloroplast which might correspond to the DNA-containing structures. Since the 25 A fibrils characteristic of these regions show up clearly only with the method of Ryter *et al.* (1958) it is not surprising that they were not visible in the micrographs of Sager and Palade.

The similarity in organization of the DNA-containing regions in the chloroplast and the nucleoplasm of bacteria and blue-green algae (Monera) which we have demonstrated here is of considerable theoretical interest. It is most attractive to assume that the DNA and the corresponding fibrils represent the genetic system of the chloroplast which their genetic properties and autonomy in reproduction imply. This genetic system resembles the nuclear equivalent of Monera (Dougherty's protocaryon, 1957). The "plasmagene" as represented by plastids thus resembles the "chromogene" of the Monera. Is the chloroplast of *Chlamydomonas* unique or are structually similar systems present in plastids of higher plants? In a preliminary study we have found these low density areas containing 25 A fibrils in chloroplasts of a number of plants where the concentration of DNA may not be high enough to give a positive Feulgen reaction (*Elodea canadensis*; *Zea mays*, both normal green chloroplast and albino mutant; *Helianthus tuberosus* and *Anthoceros* sp.). The electron microscope in combination with DNase digestion is a most sensitive tool for the demonstration of such DNA-nucleoplasm and can reveal its presence where direct cytochemical methods fail.

With the demonstration of "nucleoplasm" in chloroplasts, the similarity in ultrastructural organization of a chloroplast and a blue-green algal cell becomes indeed striking. Both are enveloped in a double membrane. Both contain the photosynthetic apparatus in membrane systems of similar organization (*cf.* Mühlethaler, 1960; Ris and Singh, 1961; Lefort, 1960). Both contain particles which look like ribosomes in the electron microscope. Whether they are in fact ribosomes remains to be established by isolation and biochemical analysis. Both contain DNA in the form of a nucleoplasm; *i.e.*, areas of low density which contain fibrils about 25 A thick. We suggest that this similarity in organization is not fortuitous but shows some historical relationship and lends support to the old hypothesis of Famintzin (1907) and Mereschkowski (1905) that chloroplasts originate from endosymbiotic blue-green algae. Endosymbiotic blue-green algae occur today in several types of protozoa. They act as chromatophores and endow these cells with photosynthetic ability (Geitler, 1959 *a*, *b*). This hypothesis also explains why the photosynthetic apparatus is associated

FIGURE 5

Region of the chloroplast of *Chlamydomonas moewusii* which gives a Feulgen-positive reaction. The widened space between the chloroplast membranes contains dense particles, which resemble the ribosomes in the cytoplasm (*R*), and 25 A thick fibrils (arrows) which presumably represent the DNA. *CM* = external chloroplast membrane, *CL* = chloroplast lamellae. × 110,000.


CM
CL
CM
R
5

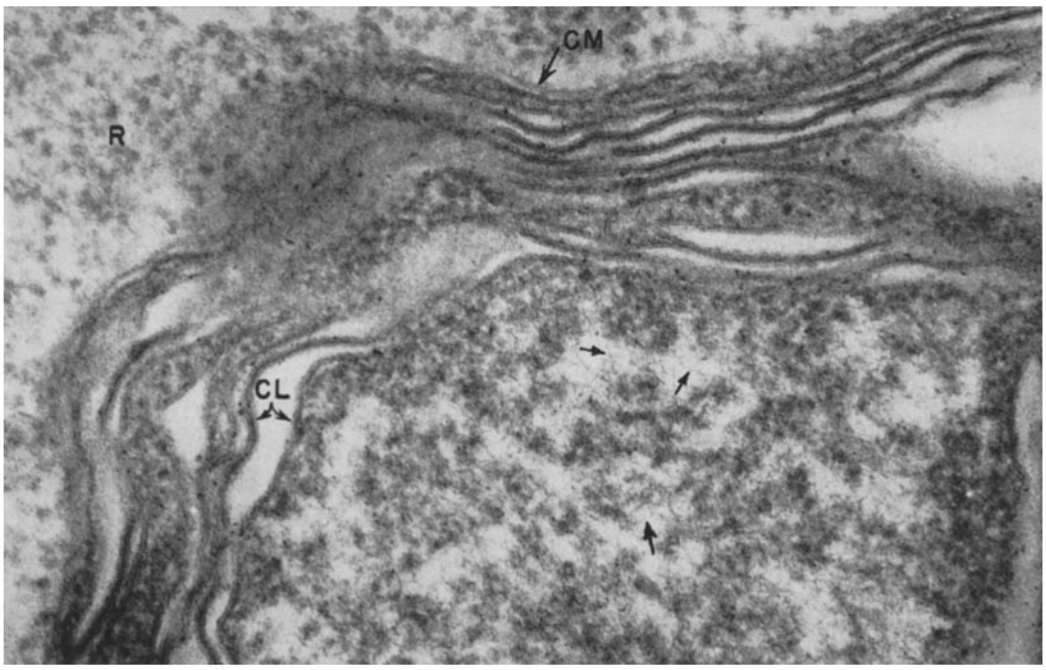


FIGURE 6

Part of the chloroplast of *Chlamydomonas moewusii* with the Feulgen-positive area. *R* = ribosomes in the cytoplasm. *CL* = chloroplast lamellae. The arrows point to the 25 A fibrils which are taken to represent the DNA. *CM* = external chloroplast membrane. × 95,000.

with membrane systems which traverse freely the cytoplasm in blue-green algae but which in higher plants are incorporated into complex cell organelles having a high degree of genetic individuality and containing just about every classified organelle found in free-living blue-green algae. The evolution of the complex cell, with its array of more or less autonomous organelles, from the simpler organization found in Monera is a question that has been neglected. With the demonstration of ultrastructural similarity of a cell organelle and free living organisms, endosymbiosis must again be considered seriously as a possible evolutionary step in the origin of complex cell systems.

Received for publication, September 10, 1961.

BIBLIOGRAPHY

DE DEKEN-GRENSON, M., and MESSIN, S., *Biochim. et Biophysica Acta*, 1958, **27**, 145.

DOUGHERTY, E. C., *J. Protozoology*, 1957, **4**, Suppl., 14.

FAMINTZIN, A., *Biol. Centralblatt*, 1907, **27**, 353.

GEITLER, L., *Oesterreich. Bot. Z.*, 1959 *a*, **106**, 464.

GEITLER, L., *Encyclopedia Plant Physiol.*, 1959 *b*, **11**, 530.

GRANICK, S., *Encyclopedia Plant Physiol.*, 1955, **1**, 507.

HOPWOOD, D. A., and GLAUERT, A. M., *J. Biophysic. and Biochem. Cytol.*, 1960 *a*, **8**, 267.

HOPWOOD, D. A., and GLAUERT, A. M., *J. Biophysic. and Biochem. Cytol.*, 1960 *b*, **8**, 813.

IWAMURA, T., *Biochim. et Biophysica Acta*, 1960, **42**, 161.

JAGENDORF, A. T., and WILDMAN, S. G., *Plant Physiol.*, 1954, **29**, 270.

KELLENBERGER, E., *in* HAYES and CLOWES, Bacterial Genetics, Cambridge University Press, 1960, 39.

KLEINSCHMIDT, A. and LANG, D., *Proc. European Reg. Conf. Electron Micr.*, Delft, 1960, **2**, 690.

LEDERBERG, J., *Physiol. Rev.*, 1952, **32**, 403.

LEE, S., *Exp. Cell Research*, 1960, **21**, 252.

LEFORT, M., *Compt. rend. Acad. sc.*, 1960, **251**, 3046.

LITTAU, V. C., *Am. J. Bot.*, 1958, **45**, 45.

LUFT, J. H., *J. Biophysic. and Biochem. Cytol.*, 1958, **9**, 409.

MERESCHKOWSKI, C., *Biol. Centralblatt*, 1905, **25**, 593.

MÜHLETHALER, K., *Z. Wissensch. Mikr.*, 1960, **64**, 444.

PRINGSHEIM, E. C., and PRINGSHEIM, O., *New Phytol.*, 1952, **51**, 65.

PROVASOLI, L., HUTNER, S. H., and PINTNER, I. J., *Cold Spring Harbor Symp. Quant. Biol.*, 1951, **16**, 113.

RHOADES, M. M., *Encyclopedia Plant Physiol.*, 1955, **1**, 19.

RIS, H., *Canad. J. Genetics and Cytol.*, 1961, **3**, 95.

RIS, H., and SINGH, R. N., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 63.

RUSTAD, R. C., *Exp. Cell Research*, 1958, **15**, 444.

RYTER, A., KELLENBERGER, E., BIRCH-ANDERSON, A., and MAALØE, O., *Z. Naturforsch.*, 1958, **13b**, 597.

SAGER, R., and PALADE, G. E., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 463.

STOCKING, C. R., and GIFFORD, E. M., *Biochem. Biophys. Res. Communic.*, 1959, **1**, 159.