ULTRASTRUCTURAL STUDIES ON THE BLUE-GREEN ALGAL SYMBIONT IN *CYANOPHORA PARADOXA* KORSCHIKOFF

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ABSTRACT

Studies conducted on the ultrastructure of *Cyanophora paradoxa* Korschikoff (a cryptomonad) have shown that its intracellular symbiont is closely related to unicellular blue-green algae. Due to its peculiar habitat, the intracellular symbiont lacks the characteristic cyanophycean double-layered cell wall, but is surrounded by a thin protoplasmic membrane. The protoplasm itself is differentiated into a lamellated chromatoplasm containing photosynthetic pigments, polyphosphate granules, and possible oil droplets, and a non-lamellar centroplasm with a large centrally located electron-opaque body surrounded by a fibril-containing halo. This halo-central body complex may be nuclear in nature. Binary fission of the organism is described. Since this cyanelle has not yet been classified, we name it *Cyanocyta korschikoffiana* nov. gen. nov. sp.; and because of its structural peculiarities, we find it necessary to create a new family for it, Cyanocytaceae, in the order Chroococcales.

INTRODUCTION

With the term "Syncyanosen," Pascher (1914) described the general phenomenon of a blue-green alga living symbiotically with a colorless protist. In his paper, he reported cases in which the cyanophyte was attached on the external surface of the host organism. In 1924, Korschikoff published a description of a flagellate containing in its protoplasm colored bodies which bore a striking resemblance to unicellular blue-green algae. In spite of this similarity, he himself expressed doubt about their cyanophycean nature and supposed that he had found an organism *sui generis*. With an accumulation of reports on symbiotic blue-green algae, some occurring within the host's protoplasm, others on its external surface, Pascher (1929) distinguished between "Endocyanosen" and "Ectocyanosen."

In the present paper, we wish to deal with the internal type of relationship and in particular with that organism found within the flagellate *Cyanophora paradoxa* Korschikoff (Fig. 1). Although this intracellular symbiont is one of the most thoroughly studied of the cyanelles ("Cyanellen"—Pascher, 1929), its nature still remains a paradox. Investigators since Korschikoff point out the great similarity of the organism to blue-green algae. However, Geitler (1959) enumerates several arguments against this conventional theory. His main points are the following: (1) the negative response of the inner body to nuclear stains even when using a special technique developed by Lietz (1951); (2) the possibility of separating the inner body from the isolated cyanelles without causing any apparent damage; (3) the absence of an ergastic membrane (wall?) around the organism which thus permits swelling without rupturing, contrary to observed blue-green algal responses; and (4) the prolonged existence

of the division-furrow, which characteristically is a transient feature of normal cyanophycean division. In an attempt to clairfy the issue we have undertaken a complementary light- and electron-microscope study.

Since, to our knowledge, the cyanelles of the species of the *Cyanophora* Korschikoff genus were never named, we propose the following generic and specific name to designate the specimens described below: *Cyanocyta korschikoffiana* nov. gen. nov. sp.

The host organism will be described in detail elsewhere.

MATERIALS AND METHODS

Culture material was obtained through the courtesy of Dr. Luigi Provasoli (Haskins Laboratories, New York) from the original isolate of Pringsheim (1958). Both of these investigators accepted the cultures as *Cyanophora paradoxa* Korschikoff, although the flagellate usually contains more than two cyanelles, which is the characteristic number originally reported by Korschikoff. A second species, *C. tetracyanea*, reported by Korschikoff in 1941, contains, as the name suggests, four cyanelles, whereas the flagellates in the material we investigated occasionally possessed as many as nine. Therefore, the identity of the flagellate component of the symbiosis is questionable.

Feulgen staining was performed according to the method of Conn (1953). An ortholux phase contrast microscope with 70 and 90× oil immersion objectives and 10 and 25× oculars was employed.

For electron microscope investigations, ultrathin sections were prepared. The material was centrifuged at 1000 RPM for 10 minutes until a pellet was formed. This was cut into small pieces, fixed in phosphate-buffered (pH 7.2) 1 per cent osmium tetroxide for 1 hour, dehydrated through a series of alcohol concentrations, put into propylene oxide for 20 minutes, and then transferred into a 1:1 propylene oxide-Epon mix, which was placed in an oven at 57°C. for 2 hours. To assure better infiltration, the material was then put into pure Epon and allowed to stand overnight at room temperature before it was placed in capsules and finally embedded in Epon, which was allowed to harden for 2 days at 57°C. The Epon was mixed in the following proportions;

Epon 812	100 ml.
DDSA[1]	180 ml.
HHPA	10 ml.
BDMA	3 ml.

Sections were made with a glass knife on a Porter-Blum microtome and those showing gray interference patterns were placed on 300-mesh bare grids. Sections were stained with PbOH using the method of Feldman (1962) and were observed under a Philips EM 100B.

[1] DDSA: dodecenylsuccinic anhydride.
HHPA: hexahydrophthalic anhydride.
BDMA: benzyldimethyl amine.

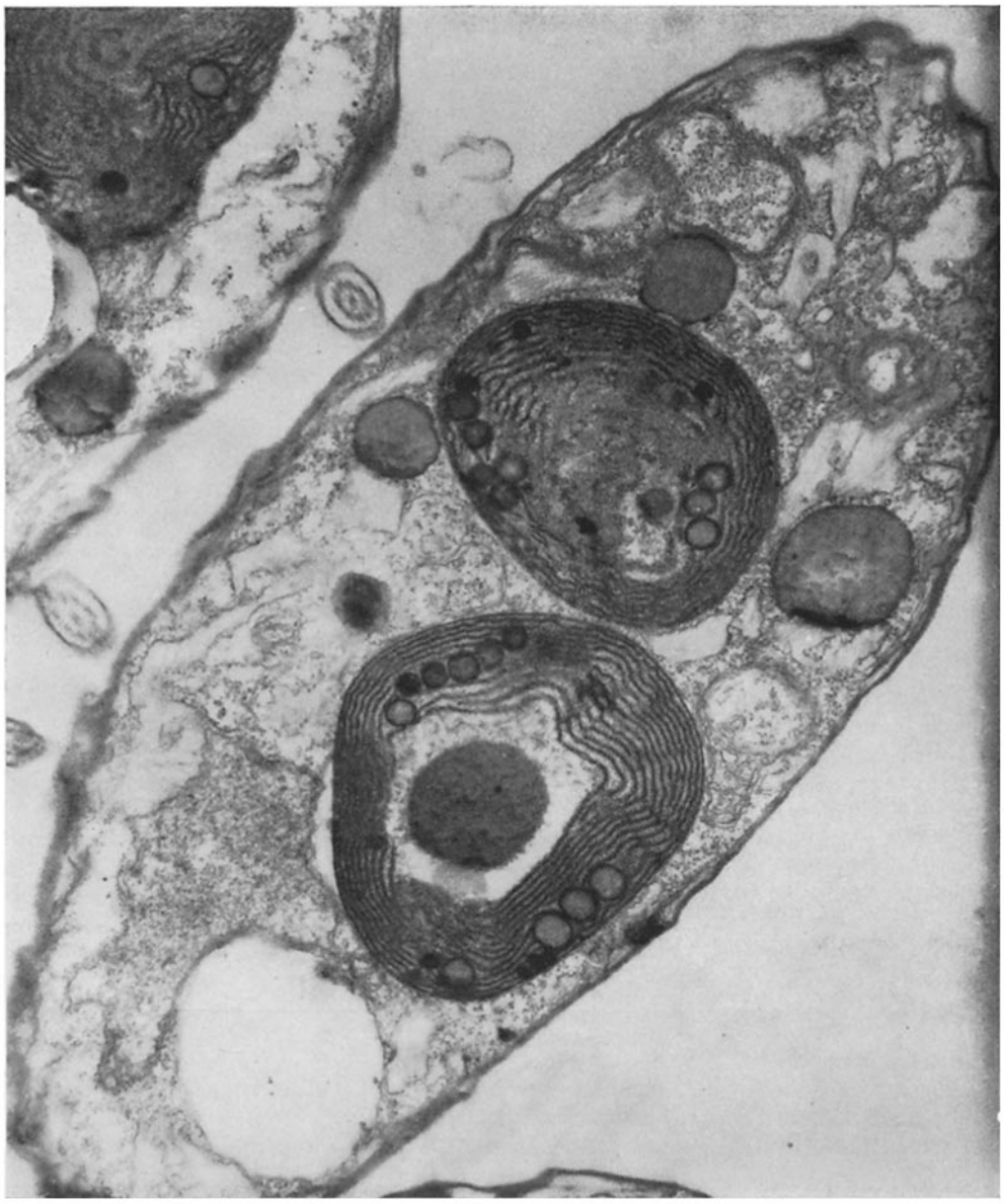
RESULTS

Cyanocyta korschikoffiana, a more or less spherical body with diameter of 1.5 to 3.7 micra, is randomly distributed throughout the protoplasm of the host flagellate. The number of these bodies present in any single host organism varies from one to several, usually 4 to 6. Their bluish-green color is restricted to a peripheral zone, whereas in their center, a usually spherical, but sometimes irregularly shaped, seemingly colorless area is visible. Between the chromatoplasm and the more refractile central area, a less refractile thin halo is discernible. After Feulgen staining (repeated in five different experiments), the halo area turned magenta, whereas the chromatoplasm could be observed as an unstained surrounding capsule. Only the nuclei of the host flagellates showed any positive response to the Feulgen treatment. A species of *Oocystis* admixed to the culture served as an internal control for the staining. In this case also, only the nuclei of the green alga gave a positive reaction. These results would indicate that chromatin material is contained within the halo-central body complex of the intracellular symbiont. To what extent, if any, the actual central body responds to the Feulgen stain cannot be determined because the stained halo impedes investigation of the core.

The division of *C. korschikoffiana* is apparently independent of the division of its host, and the cyanelles are not necessarily equally distributed to the resulting daughter cells of the host. The furrow frequently observed on their surface is a centripetally progressing division furrow which invariably results in the production of two daughter cells, although it may temporarily lag in the process. Ultimately, the central body is cleaved by the invaginating furrows.

Ultrastructurally, *C. korschikoffiana* shows a thin limiting membrane directly apposed to a protoplasmic membrane of the host (Figs. 1 to 3). The algal limiting membrane is visibly thicker than the lamellar membranes (Fig. 3) and in some cases appears to be double. Pores were not observed in this membrane, nor could a direct connection with the cytoplasmic lamellae be detected.

The chromatoplasm, readily discernible in the light microscope by its blue-green color, is equally distinctive in the electron microscope. Composed



Figures 1 to 19 are all electron micrographs.

FIGURE 1 Section showing the flagellate, *Cyanophora paradoxa*, with two blue-green algae lying in the cytoplasm. There seems to be no particular pattern of distribution, though when several algae are present they often are in close proximity to the nucleus of the host. × 20,000.

of more or less concentric lamellae and interlamellar spaces, the area presents an abrupt contrast to the non-lamellated centroplasmic area (Fig. 4). Because the lamellae are not perfectly concentric, but seem rather to form an interrupted labyrinthine pattern, the ground substance of the central body halo is in contact with that of the interlamellar spaces and presumably miscible with it (Figs. 4, 6, 7).

The lamellae themselves are double-membraned

structures, 175 to 200 A thick, with a total period from one lamella to the next of about 400 A. Their distribution is reminiscent of the orderly membrane arrangement exemplified by *Oscillatoria chalybea* Mertens (Hall and Claus, 1962) (Fig. 5). Along the lamellar membrane there is a more or less uniform distribution of small electron-opaque particles, probably the chlorophyll content of the cell (Fig. 3). In some areas along the membrane, the intralamellar spaces widen to form small vesicles (Fig. 9) of the type previously observed occurring regularly in *O. chalybea*.

The space between lamellae is about twice the width of the lamella and is filled with a finely granulated cytoplasmic material. In addition to this, two types of larger granules are present in the interlamellar spaces. The first, very electron opaque, varies in size from 1700 to 3000 A, exhibits no internal structure nor limiting membrane, and shows a great resemblance to the polyphosphate bodies of the blue-green algae (Fig. 6, *P*). The second type is larger, less electron opaque, and seems to possess a surface layer more dense than the interior (Fig. 6, *L*). The whole appearance of these larger granules suggests a lipoidal composition, a feature foreign to cyanophytes in general, and they respond positively to Sudan Red staining. That these granules are present early in the development of the plant seems evident from the fact that they cause the lamellae to deviate from their normal position and wind around them (Fig. 6). What seem to be interlamellar continuities appear in some sections (Fig. 3, arrows), but since they are thinner than the lamellae they may be sectioning artefacts rather than true bridges.

The centroplasm can be divided into two portions, a rather electron-opaque central body surrounded by a less dense halo which possesses fibrillar strands extending from the fringe of the lamellae up to and, perhaps, into the central body (Fig. 7). In their structure, these fibrillar elements of the halo are rather similar to the fine fibrillar elements of the nucleoplasm described in blue-green algae by Ris and Singh (1961) and Hopwood and Glauert (1960), and they probably represent the chromatin material of the organism.

As was previously noted, the central body has been separated from the isolated organism without producing any apparent damage (Geitler, 1959). On the basis of this observation, earlier authors were wont to conclude that the central body is some kind of reserve material, possibly leucosin. The fact that the central body can be easily separated may have its foundation rather in its compact structure and seemingly tenuous connection with the surrounding cytoplasm. It is also possible that the innermost lamella of the chromatoplasm could, during certain stages of development, function as a retaining membrane around the whole centroplasmic area (Figs. 9 and 18) and thus serve as a primitive nuclear membrane.

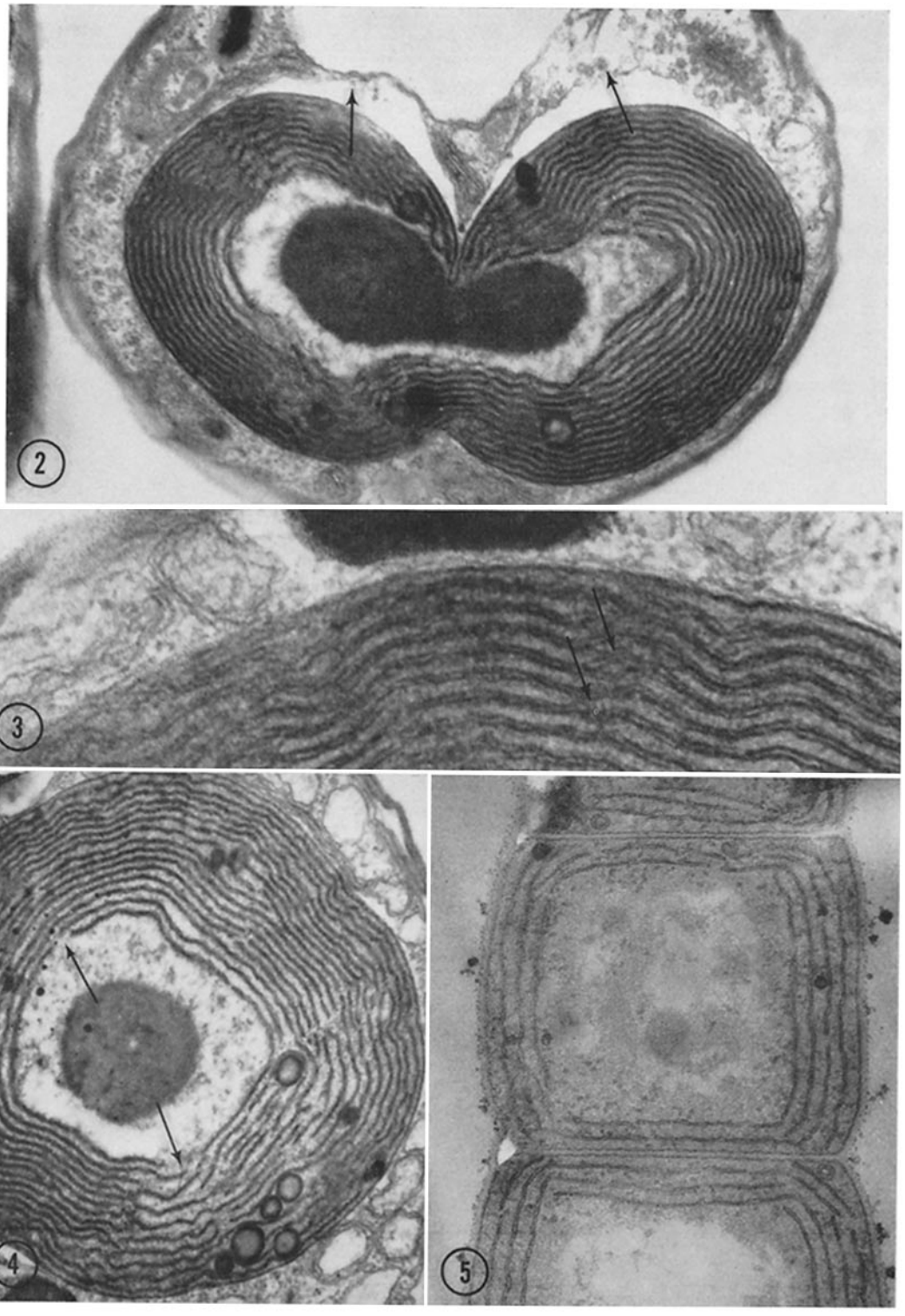
Several features, however, deny the possibility that the central body is merely reserve material. Differences in electron opacity occur throughout it due to its complex ultrastructure (Fig. 7). It

FIGURE 2 A dividing alga which has pulled away from the cytoplasm of the flagellate, showing the protoplasmic membrane (arrows) of the host and the relative thinness of the algal external membrane. × 34,000.

FIGURE 3 A greatly enlarged section showing the chromatoplasmic membranes and the outer membrane of the commensal and the plasmic membrane of the host. Granules are visible both between and along the chromatoplasmic lamellae. Arrows indicate interlamellar bridges. × 100,000.

FIGURE 4 Cross-section through *Cyanocyta korschikoffiana*. The differentiation into chromatoplasm and centroplasm is clearly evident. The lamellae are not perfectly concentric, but rather labyrinthine. Interruptions (arrows) occur which allow the centroplasm to come into contact with the interlamellar chromatoplasm. × 24,000.

FIGURE 5 Section through the blue-green alga, *Oscillatoria chalybea* Mertens, demonstrating several structural similarities between this filamentous cyanophyte and the unicellular symbiont. Note orderly, peripheral arrangement of lamellae, large electron-opaque body, and fine filaments in central region. × 6500 (courtesy of *Protoplasma*).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seems to be composed basically of intertwined fibrils whose meshes are filled with small, very dense, spherical granules. The fibrils of the surrounding halo come into very close contact with the central body and also with the chromatoplasm, thereby providing a rather intimate continuity among all the elements of the organism. No structure similar to the discrete nuclear membrane existing in higher plants is present.

In several preparations, extremely dense granulations are visible in the central body (Figs. 8, 12, 17, and 18) which seem to fuse and ultimately build up a network. Their precise function is unknown, but they show some similarities to the paranucleolar bodies described by Claude (1961, 1962) in mouse renal carcinoma cells and found by Seshachar in *Blepharisma* (private communication).

The division of *C. korschikoffiana* follows the general pattern of cell division in the blue-green algae which we have described previously on the submicroscopic level. The invaginating limiting membrane first pushes the lamellae inwards (Fig. 10) and subsequently ruptures them (Fig. 11). It next enters the halo (Figs. 12 and 13) and finally dissects the central body (Figs. 14 and 15), fusing with that portion of the membrane invaginating from the opposite side (Fig. 16). During this process, the whole organism becomes elliptical and later assumes the shape of a figure eight (Fig. 17), prior to separating into discrete daughter cells and resuming the original spherical form (Fig. 18). After fusion of the invaginated portions of the limiting membrane, the two daughter cells ultimately pull apart, the central bodies round up, and the severed lamellae reconstitute their concentric arrangement (Fig. 19).

Because the invaginated portions of the limiting membrane do not always form symmetrically, a temporary asymmetry may result (Fig. 12). In cross-section, this would manifest itself as a deep invagination of the membrane on one side, with little or no centripetal movement of the membrane on the other side. This asymmetrical stage may be retained for a prolonged period. Perhaps for this reason Geitler thought it to be a permanent stage of the organism. Ultimately, however, both sides of the membrane will invaginate completely and fuse in the middle.

The chromatoplasmic granules seem to take no active part in any of the divisional processes.

DISCUSSION

Both light and electron microscope investigations point to the strong similarity between *C. korschikoffiana* and free-living, colored blue-green algae. The four points of Geitler, enumerated earlier, that would separate the present species from the Cyanophyta deserve further discussion.

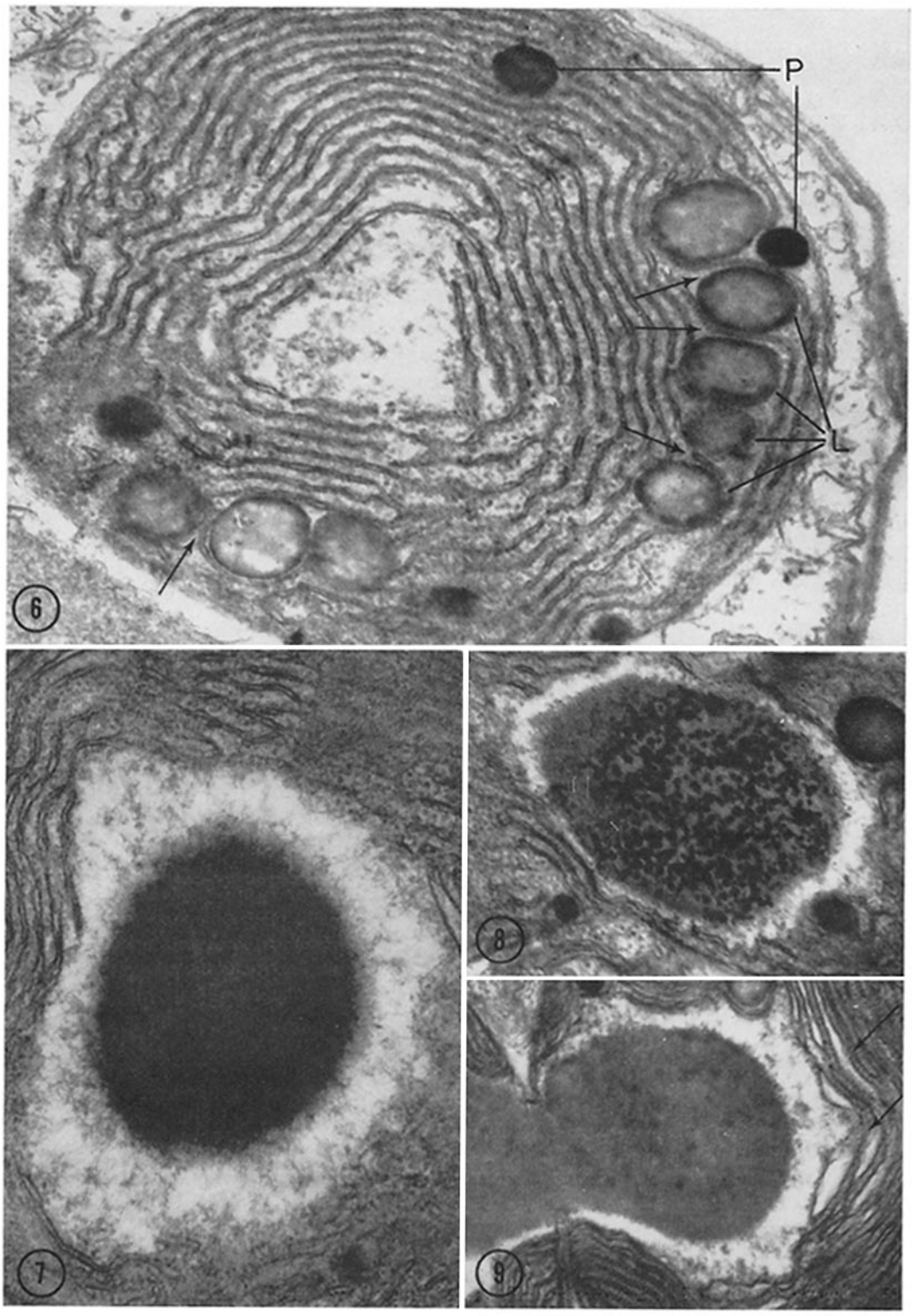
The presence of a plasma membrane in place of a double-membraned cell wall can be better understood if we take into consideration the intracellular habitat of the organism. Such a symbiosis, especially in the light of adaptations made by the host organism (to be discussed in a later paper), must be the result of a long evolutionary process, during which it is not at all unlikely that a double-layered cell wall was replaced by a possibly more permeable membrane to facilitate adequate and mutual interchange of nutrients between the

FIGURE 6 Double-membraned nature of the lamellae is clearly exemplified. Where granules occur, the membranes deviate from their normal path to curve around them (arrows). The larger granules are probably lipoid in nature (*L*), whereas the smaller ones (*P*) may be polyphosphate bodies. × 62,000.

FIGURE 7 Central body and surrounding halo which is in contact with the adjacent chromatoplasm. Small interlamellar granules are shown. The substance of the halo appears to come into intimate contact with the electron-opaque central area and the interlamellar space. × 60,000.

FIGURE 8 The central body with very electron-opaque granulations. In some sections the granulations are only sparsely present, and in others rather numerous. This may reflect physiological differences in the cells. × 51,000.

FIGURE 9 In several places, the lamellar membranes pull apart to form intralamellar vesicles (arrows). × 43,000.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symbionts. Therefore, differences in wall structure do not seem to be a basic differentiating criterion in this particular instance.

The prolonged presence of the division furrow has been discussed above and, as was pointed out, is not a differentiating characteristic, but a matter of degree rather than kind.

The halo is certainly Feulgen positive, but the exact nature of the central body is not yet known and must be the object of a more detailed investigation.

The only remaining difference between *C. korschikoffiana* and the members of the Cyanophyta can be found in the removability of the central body of the former. And this is a real difference. One could, however, speculate that *C. korschikoffiana* actually represents a rather advanced stage among the blue-green algae, where the nuclear equivalent begins to assume the more compact nature of a true nucleus with the DNA confined to the region of the halo and the central body anticipating a nucleolus. Admittedly, no nuclear membrane as such actually exists, but in several sections the innermost lamella of the chromatoplasm is closely associated with the centroplasmic halo, suggesting a possible primordial stage in the development of a nuclear membrane.

It seems to us, then, that *C. korschikoffiana* could be classified as a member of the Cyanophyta, belonging to the order Chroococcales. The peculiarities of the central body and cell membrane, however, would tend to exclude it from any of the existing families. For this reason, the establishment of a new family for this genus and species is deemed necessary. We propose the family name, Cyanocytaceae nov. fam.

Descriptions of New Taxa

Cyanocytaceae nov. fam.

Unicellular, spherical, chlorophyll containing, intracellularly living blue-green algae with plasmic cell membranes (no real cell wall exists) and compact central body located in the centroplasm. Propagation by binary fission. Type genus: *Cyanocyta* nov. gen.

Cyanophyta, unicellularia, sphaerica, chlorophyllum continentia, intracellularie symbiotica, membrana plasmatica praedita, muri cellularii expertia; corpore et denso et medio in cytoplasmate posito. Per fissionem binariam propagant. Typus familiae: Cyanocyta nov. gen.

Cyanocyta korschikoffiana nov. gen., nov. sp.

Unicellular, spherical, intracellularly living blue-green alga with diameter from 1.5 to 3.7 μ. Cell wall absent, but thin plasma membrane present. Protoplasm clearly differentiated into bluish-green chromatoplasm and colorless centroplasm, the latter with large, compact, central body, surrounded with Feulgen-reactive halo.

Host: *Cyanophora paradoxa* Korschikoff.

Type species: *Cyanocyta korschikoffiana* nov. sp.

Illustrations: 1 to 20; slides: 1 to 2.

Cyanophyta, unicellularia, sphaerica, intracellularie symbiotica, extremis per medium 1.5 ad 3.7 μ; muri cellularii expertia, praedita membrana plasmatica. Et chromatoplasma coeruleum et centroplasma acoloratum inter se manifeste discrepant; quod illud magno densoque medio corpore, circumdata Feulgeniano positive infecto regione.

Hospes: *Cyanophora paradoxa* Korschikoff.

Typus speciei: *Cyanocyta korschikoffiana* nov. sp.

Figurae I–XX nostrae, Preparationes I–II.

The type of the new species will be lodged with the Rijksmuseet in Stockholm, Sweden.

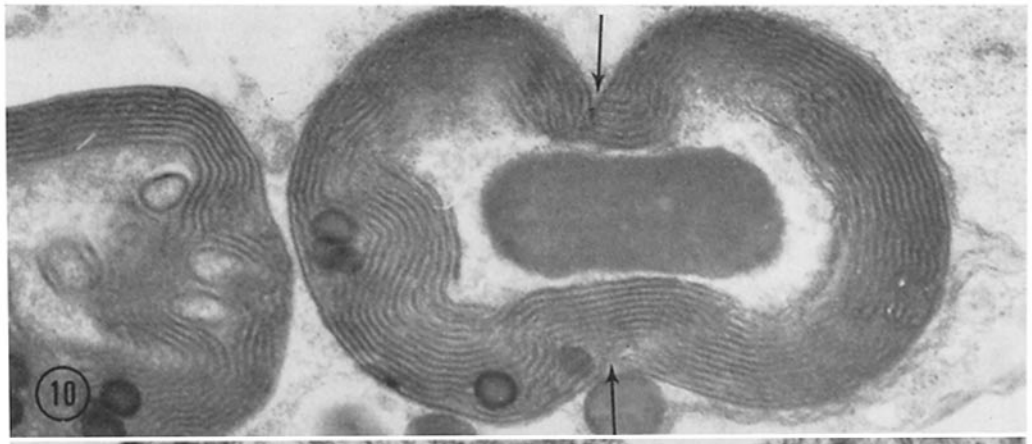
Dr. Hall is a Visiting Investigator, United States Public Health Training Program, (Grant 2G-70).

Received for publication, February 3, 1963.

FIGURE 10 Early stage in cytokinesis. At the top of the figure, the invagination (upper arrow) of the outer membrane has almost completely passed through the lamellae, whereas in the lower part of the figure the invagination (lower arrow) is just beginning. All of the lamellae shown in the lower region are still intact. × 28,000.

FIGURE 11 The invaginations of the outer membrane shown at the top and bottom of the figure have passed through the chromatoplasm and are beginning to enter the centroplasmic halo (arrows). × 36,000.

FIGURE 12 The dense central body is bending before the invaginating outer membrane. In this section, the membrane is moving centripetally only on one side. The membrane will eventually invaginate also on the other side. × 27,000.



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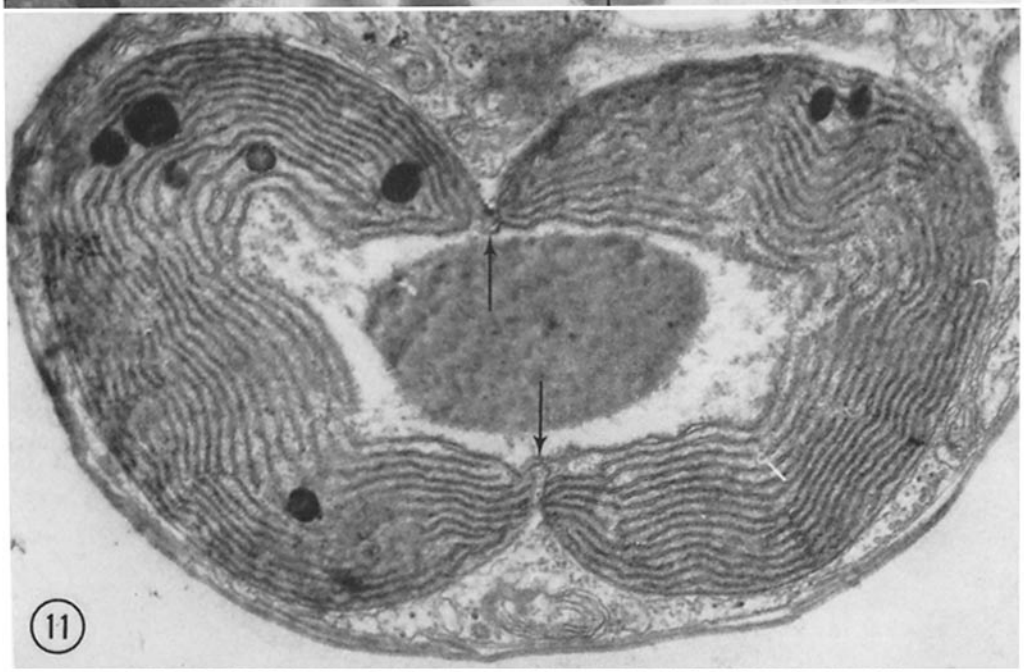

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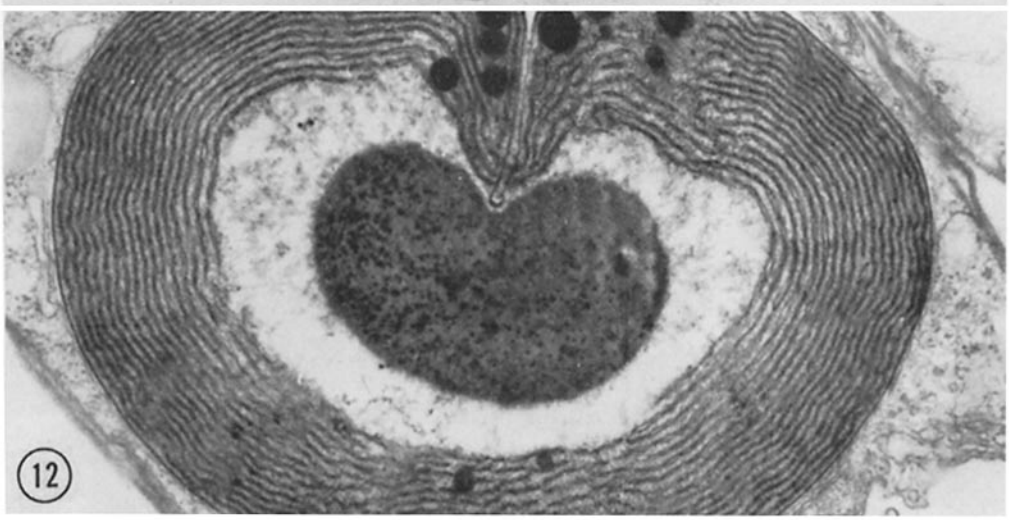

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FIGURE 13 Division phase about the same as in Fig. 12, but in this case the membrane is invaginating on both sides. × 34,000.

FIGURE 14 The division process on one side is complete, whereas that on the other side is nearly complete. Much of the centroplasm has already been pinched off. × 25,000.

FIGURE 15 Division stage very near that of Fig. 14. The section passes through the periphery of the central body. × 31,000.

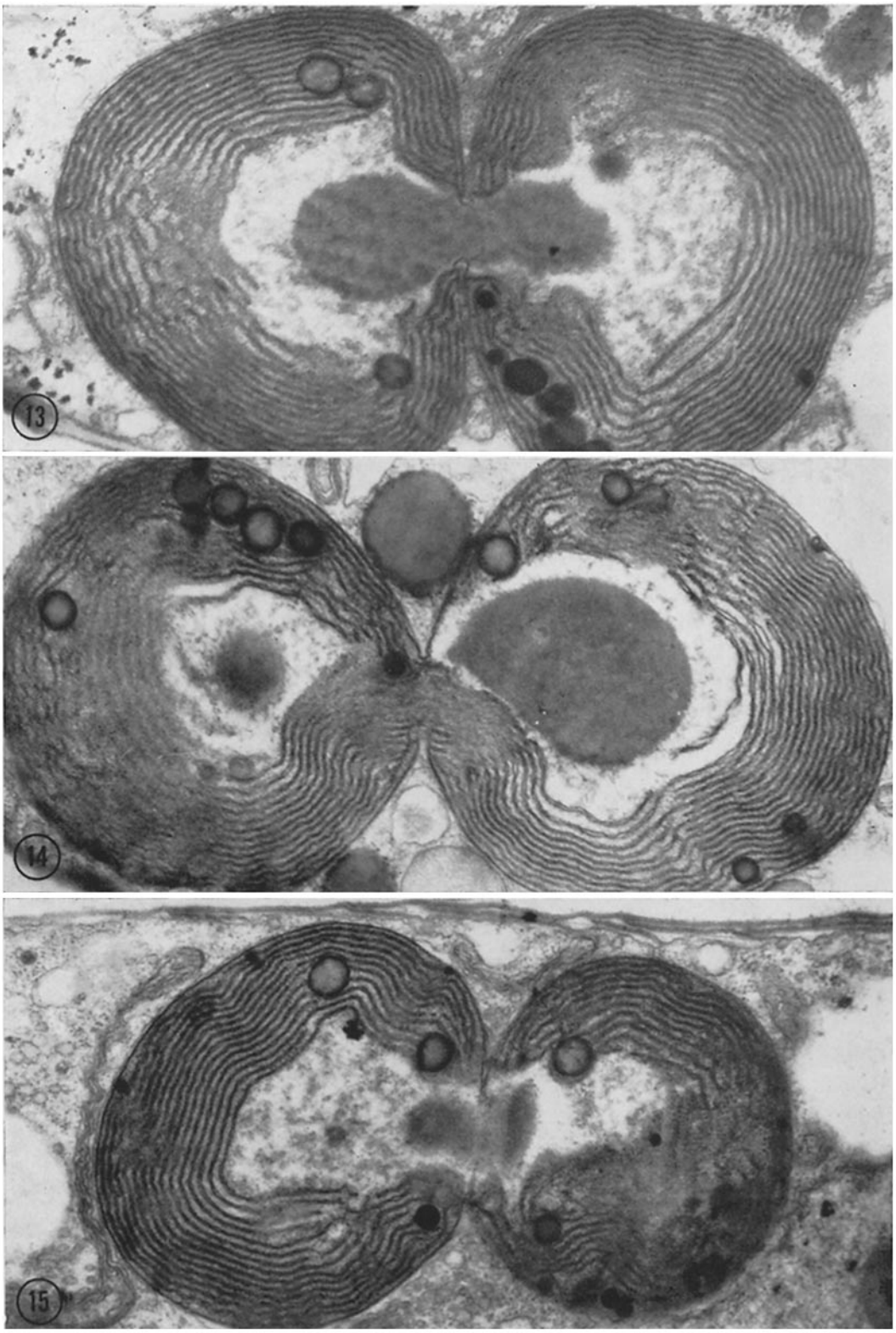
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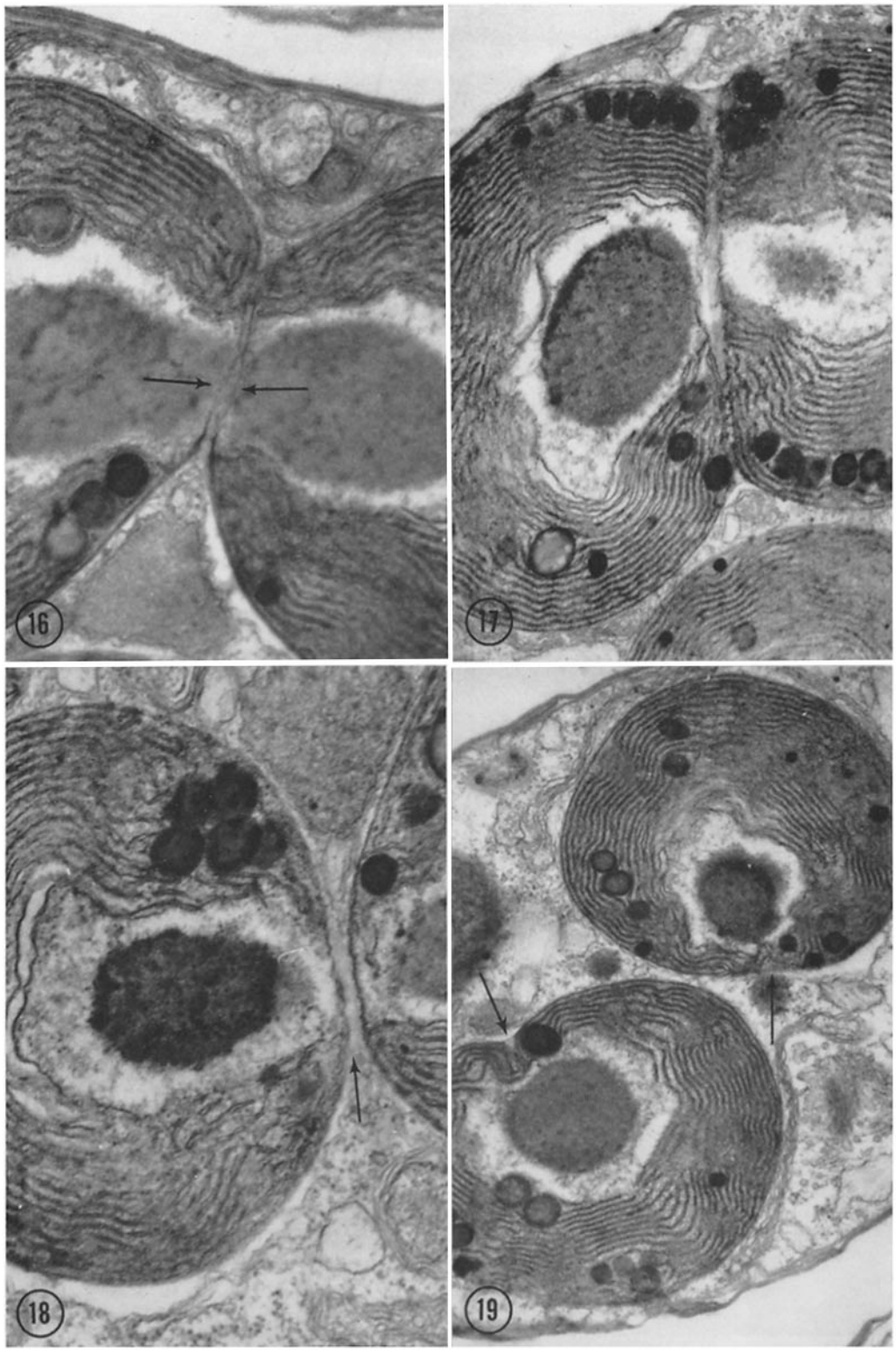
FIGURE 16 The invaginating membrane has completely passed through the central body and fused with its counterpart from the opposite side (arrows). The cell is now divided. × 39,000.

FIGURE 17 A later stage in the algal division after fusion of the membrane invaginations. × 33,000.

FIGURE 18 The divided cells are beginning to move apart and the severed membranes of the chromatoplasm start to move around the surface of the cleavage plane (arrow). × 46,000.

FIGURE 19 The divided cells have moved apart and several of the severed chromatoplasmic membranes are now reconstituted (arrows). × 33,000.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