THE FINE STRUCTURE OF GREEN BACTERIA

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ABSTRACT

The fine structure of several strains of green bacteria belonging to the genus *Chlorobium* has been studied in thin sections with the electron microscope. In addition to having general cytological features typical of Gram-negative bacteria, the cells of these organisms always contain membranous mesosomal elements, connected with the cytoplasmic membrane, and an elaborate system of isolated cortical vesicles, some 300 to 400 A wide and 1000 to 1500 A long. The latter structures, chlorobium vesicles, have been isolated in a partly purified state by differential centrifugation of cell-free extracts. They are associated with a centrifugal fraction that has a very high specific chlorophyll content. In all probability, therefore, the chlorobium vesicles are the site of the photosynthetic apparatus of green bacteria.

INTRODUCTION

The photosynthetic green bacteria constitute a small and homogeneous biological group, readily distinguishable from the other major photosynthetic groups among procaryotic organisms, namely purple bacteria and blue-green algae. Each of these three groups has a characteristic photosynthetic pigment system (Table I). As shown by van Niel (1), the metabolism of green bacteria closely resembles that of the purple sulfur bacteria. Both are strictly anaerobic photolithotrophs, which use H_2S as the principal electron donor for photosynthesis.

Most green bacteria obtained in pure culture belong to a single genus, *Chlorobium*, defined as comprising small, permanently immotile rods, which sometimes occur in short chains. Following Larsen (2), there are two principal species: *C. thiosulfatophilum*, which can use thiosulfate as an electron donor, and *C. limicola*, which cannot. Subsequent work (3) has shown that strains of *C. thiosulfatophilum* may differ with respect to the nature of their principal chlorophyll, some containing chlorobium chlorophyll-650 and others chlorobium chlorophyll-660. Pfennig has found that strains of *C. limicola* are likewise heterogeneous in this respect. A motile green bacterium, *Chloropseudomonas ethylicum*, has recently been isolated (4). Apart from possessing typical bacterial polar flagella, it is morphologically indistinguishable from the members of the genus *Chlorobium*.

Pringsheim (5) has demonstrated that some organisms which had been originally observed in nature and described as green bacteria are in fact blue-green algae; upon cultivation, they proved to contain chlorophyll *a* and phycocyanin, and to evolve oxygen in the light. It accordingly follows that immotile green bacteria cannot be distinguished, on gross morphological criteria alone, from small, unicellular blue-green algae.

As this summary shows, the green bacteria are an isolated group of photosynthetic organisms, which share certain metabolic features with the

purple sulfur bacteria but differ from all purple bacteria with respect to the chemical nature of their principal photosynthetic pigments. The green bacteria should thus provide very valuable material for the comparative study of the structure of the photosynthetic apparatus at the lowest level of cellular differentiation. Surprisingly, there have been only two brief reports concerning the cellular organization of green bacteria. In the first survey of the structure of photosynthetic bacteria as revealed by the electron microscopy of thin sections, Vatter and Wolfe (6) examined a strain of *C. limicola*. They were unable to detect vesicular elements, which are such conspicuous cytoplasmic constituents of most purple bacteria, but observed that the cytoplasm contained numerous, electron-opaque granules 150 to 250 A in diameter, which they interpreted as the "chromatophores" of *Chlorobium*. To judge from their illustration, these granules could be more reasonably interpreted as inclusions of metaphosphate, which are always abundant in the cells of green bacteria grown with an excess of inorganic phosphate in the medium.

Bergeron and Fuller (7) examined thin sections of a strain of *C. thiosulfatophilum* and were unable to detect either vesicular or lamellar elements in the cytoplasm. Apart from metaphosphate inclusions, the only cytoplasmic structures which they observed were numerous particles about 150 A in diameter (*i.e.* of ribosomal dimensions). They also examined the pigment-bearing fractions in extracts of *C. thiosulfatophilum* prepared by mechanical abrasion of the cells. After low-speed centrifugation to remove coarse debris, the clear supernatant fraction was sedimented in the analytical ultracentrifuge; most of the chlorophyll was associated with a component that had a sedimentation coefficient of 50 S. Bergeron and Fuller tentatively concluded that the photosynthetic structural unit of *C. thiosulfatophilum* is a particle with a maximal dimension of 150 A, probably spherical, and with a molecular weight of about 1.5 million. In electron micrographs of thin sections of cells, such units could probably not be distinguished from ribosomes. Hence the analytical data on extracts seemed in good accord with the apparently simple cytoplasmic organization revealed in thin sections.

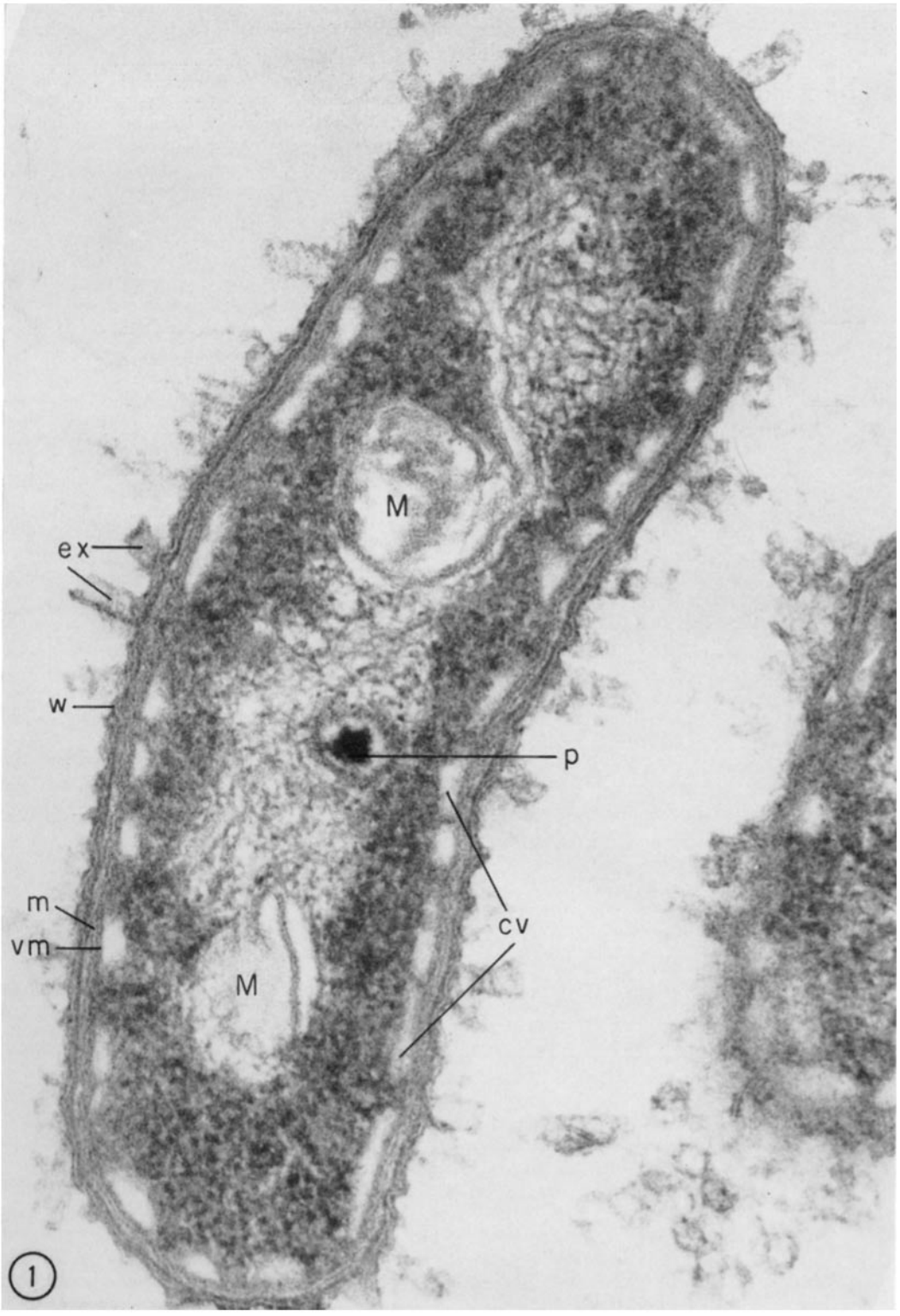
Despite the differences of intepretation, both

TABLE I

Principal Photosynthetic Pigments of Procaryotic Organisms

Pigments	Organisms		
	Blue green algae	Purple bacteria	Green bacteria
Chlorophylls	Chlorophyll *a*	Bacteriochlorophyll	Chlorobium chlorophyll-650 or Chlorobium chlorophyll-660; traces of Bacteriochlorophyll
Carotenoids	β-carotene, other group-specific bicyclic carotenoids	Aliphatic carotenoids, often bearing methoxyl groups (*e.g.*, spirilloxanthin)	Monocyclic carotenoids
Phycobilins	Present	Absent	Absent

FIGURE 1 Longitudinal section of *Chlorobium thiosulfatophilum* T embedded in Vestopal, showing the complex cell wall (*w*) with its rod-shaped extensions (*ex*), the cell membrane (*m*), and the large, electron-transparent chlorobium vesicles (*cv*) surrounded by an electron-opaque membrane (*vm*), adjacent to but distinct from the cell membrane. Two large mesosomal elements (*M*) are visible, as well as a granule of polymetaphosphate (*p*). Main fixation, 2 hours. × 140,000.


M
ex
w
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m
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M
cv
1

these reports suggest that green bacteria have a photosynthetic apparatus different from and considerably simpler in structure than that of either purple bacteria or blue-green algae. In an attempt to throw additional light on this question, we have undertaken a comparative cytological study of several strains of green bacteria, the results of which are described below. A preliminary report of some of our findings has appeared elsewhere (8).

MATERIALS AND METHODS

As biological material, we have used a collection of *Chlorobium* strains isolated from various sources by Pfennig[1], and in addition a subculture of the Russian strain of *Chloropseudomonas ethylicum*.[2] Four strains judged representative of the range of biotypes that occur in the genus *Chlorobium* were studied in considerable detail, and all the illustrative material included in this paper is derived from them. Two of these strains, Tassajara (T) and Carmel River (CR), can use thiosulfate, and thus conform to Larsen's definition of *C. thiosulfatophilum*. Two strains, Reyershausen (R) and Moss Landing (ML) cannot do so, thus conforming to Larsen's definition of *C. limicola*. Strain ML contains chlorobium chlorophyll-650, and the other three strains, chlorobium chlorophyll-660.

The *Chlorobium* cultures were grown in screw-cap bottles in a synthetic medium originally developed for the cultivation of *Chromatium okenii* by Pfennig (9). Chloride salts were used instead of sulfates. The culture medium was supplemented with 1 per cent NaCl in the case of *C. limicola* ML, a strain isolated from brackish water. *C. ethylicum* was grown in the culture medium of Shaposhnikov and coworkers (4). Most cultures were incubated at room temperature (20 to 23°C); a few cultures were grown at 15°C. The light intensity was usually 40 ft-c or less, a 40-watt tungsten lamp serving as the light source. For experimental studies the cells were harvested from actively growing cultures.

Fixation, staining, and dehydration of cells for electron microscopic examination were performed as described by Ryter and Kellenberger (10), except that the period of main fixation was normally reduced to 2 hours. Vestopal, Epon, and prepolymerized methacrylate were used as embedding materials. Sections were cut with a diamond knife in a Porter-Blum microtome. Vestopal-embedded sections were mounted on uncoated, 300- or 400-mesh grids; Epon- and methacrylate-embedded sections were mounted on Formvar-coated 200-mesh grids. All sections were poststained with lead hydroxide by the procedure of Millonig (11).

Negatively stained preparations were made by the method of Huxley and Zubay (12). The material to be stained was suspended in 0.5 per cent ammonium acetate containing 10^{-3}M $MgSO_4$. A drop of this suspension was placed on a 400-mesh grid covered with a carbon film. Most of the liquid was sucked off by capillarity, after which a solution of 2 per cent potassium phosphotungstate (pH 7) containing 10^{-3}M $MgSO_4$ was added. All electron microscopic observations were made with a Siemens Elmiskop I, operating at 80 kv.

The chlorobium chlorophylls were measured spectrophotometrically on methanolic extracts of cells or cell fractions, using the absorption coefficients reported by Stanier and Smith (3). Determinations of protein were made by the Folin-Lowry method (13). The techniques for breakage of cells and isolation of pigmented fractions were similar to those used with *Rhodospirillum rubrum* by Cohen-Bazire and Kunisawa (14).

RESULTS

General Features of the Cell

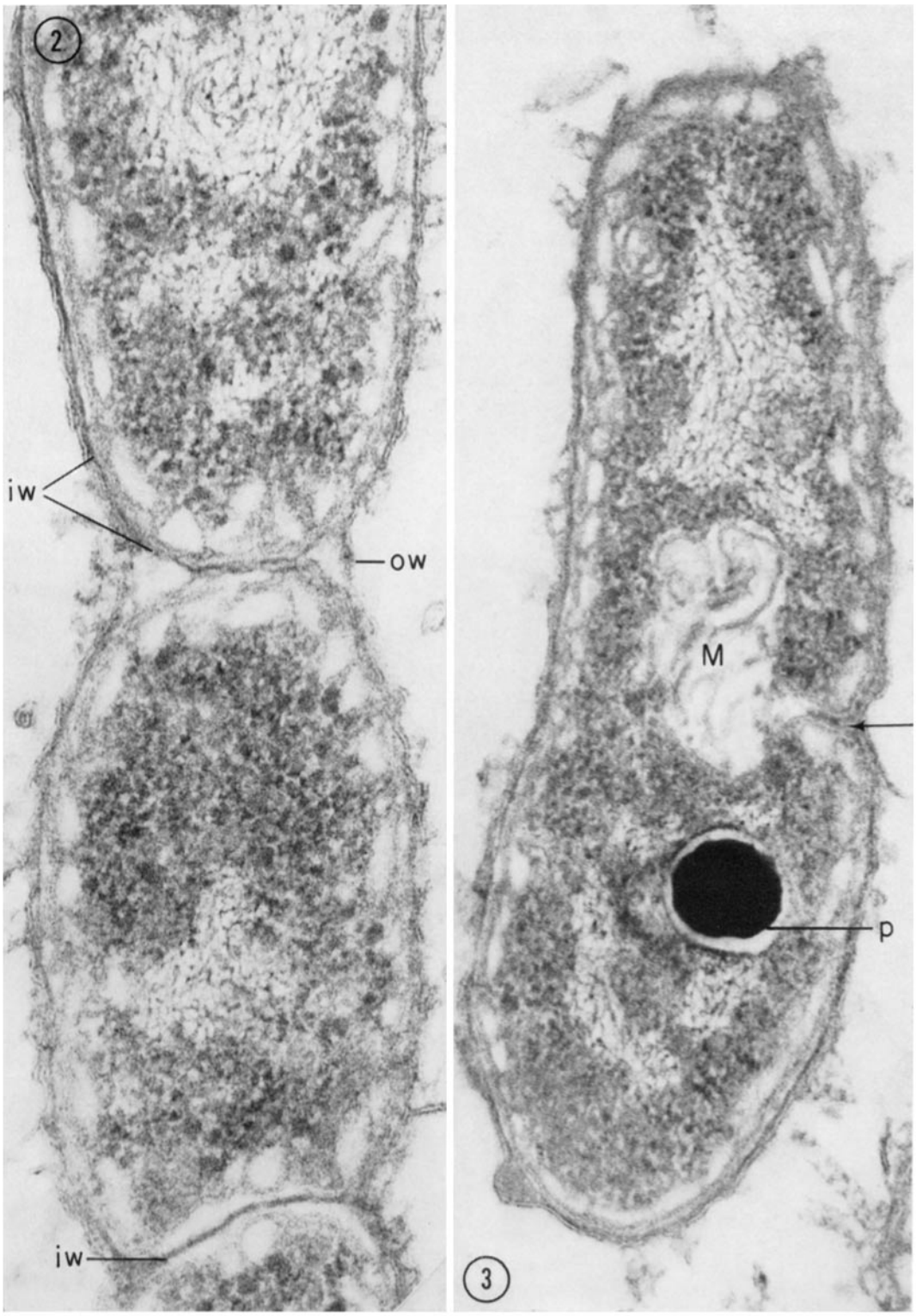
Typical sections of Vestopal-embedded cells of the four *Chlorobium* strains studied in detail are shown in Figs. 1, 12, 13, and 16. The cell wall

[1] Most of these strains were isolated at the Hopkins Marine Station, Pacific Grove, California, in the laboratory of Professor C. B. van Niel.

[2] We are indebted to Dr. E. Leadbetter for the provision of a subculture of this strain.

FIGURE 2 Section through a chain of three cells of *Chlorobium thiosulfatophilum* T, embedded in Vestopal. The two lower cells are separated only by the newly synthesized common inner layer of the cell wall (*iw*). Between the two upper cells, the inner layer of the cell wall is double, but the cells are still held together by the outer layer of the wall (*ow*). Main fixation, 2 hours. × 120,000.

FIGURE 3 Section of *Chlorobium thiosulfatophilum* T embedded in Vestopal. A large mesosomal structure (*M*) is connected with the cell membrane (arrow) at the site of formation of a transverse wall. Note also polymetaphosphate granule (*p*). Main fixation, 2 hours. × 100,000.


2
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3

has the multilayered structure generally characteristic of Gram-negative bacteria: there is an electron-opaque inner layer about 40 A thick, covered by a wider and more irregular outer layer. In *C. thiosulfatophilum* T (Fig. 1) and *C. limicola* R (Fig. 13), the outer layer of the cell wall is ornamented with rod-shaped extensions, projecting at right angles from the surface. These extensions are 300 A in diameter and have a helicoidal structure, most clearly evident in phosphotungstate-stained preparations of broken cells (Figs. 18 and 19). Cells of *C. limicola* ML are surrounded, at some distance from the wall, by a loose halo of short fibrils, probably interpretable as vestiges of the slime layer (Fig. 15).

The cytoplasmic membrane, a unit membrane 80 A thick, is infolded at several points to form membranous internal structures of variable size, similar to the mesosomes which have been described in a variety of non-photosynthetic bacteria (*e.g.* references 15–17). All strains of green bacteria examined contain these structures, which seem to be frequently associated with transverse wall formation (Figs. 3, 4, and 8).

An interesting feature of transverse wall formation in *C. thiosulfatophilum* strain T is shown in Figs. 2 and 4. Separation between two daughter cells is achieved by growth across the plane of division of only the thin inner layer of the wall. As a result, each of the two cells is completely surrounded by the inner wall layer, but both are initially maintained as a single structural unit by the common enclosing outer layer of the wall.

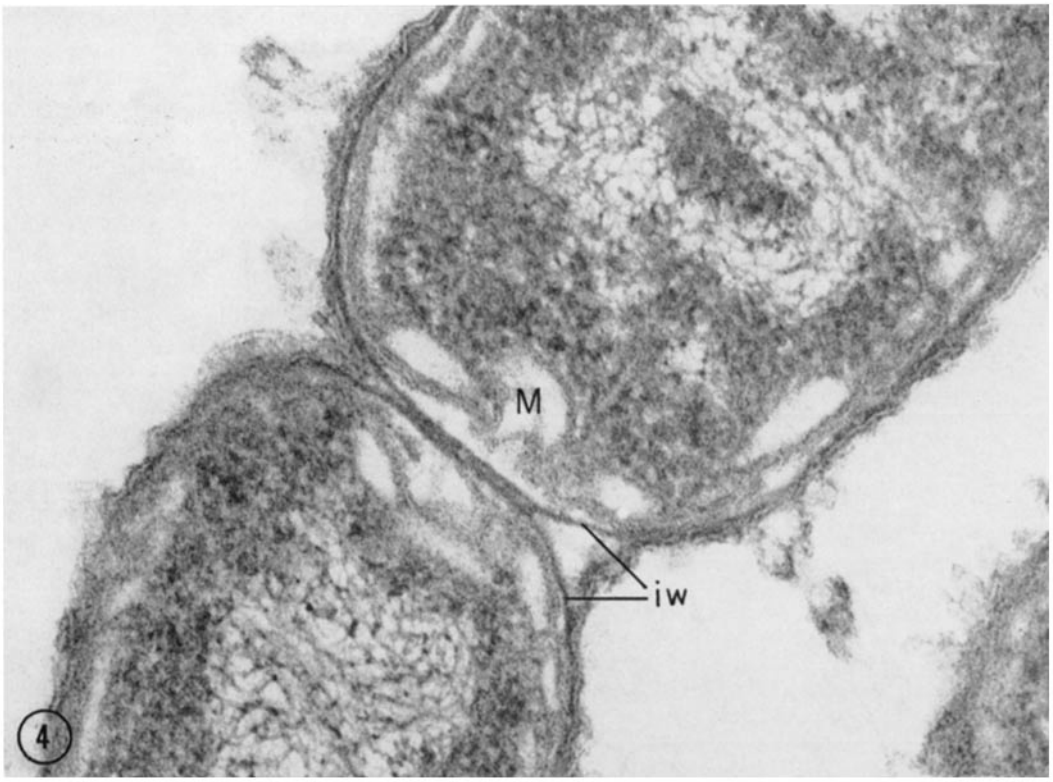
The most unusual cytological elements in green bacteria are the structures which we have earlier (8) termed chlorobium vesicles. These structures immediately underlie the cytoplasmic membrane, and are more or less evenly distributed through the whole cortical region of the cell. Each vesicle is oblong, some 300 to 400 A wide and 1000 to 1500 A long, and surrounded by a very thin (50 A) electron-opaque membrane. The enclosing membrane, most clearly shown in Fig. 15, separates the vesicles from the cytoplasmic membrane on the outer side, and from the ribosomal region on the inner side. The disposition of the vesicles in the cortical region is best seen in tangential sections (Figs. 5 to 7): they seem to comprise a fairly closely packed layer. In sections of cells which have been embedded in Vestopal (Fig. 1) or methacrylate (Figs. 7 and 14), the vesicles appear transparent and could be interpreted as empty. A different internal appearance is revealed in Epon-embedded cells (*e.g.* Figs. 9 and 10): here, the contents of the vesicles are very electron-opaque and show indications of a regular internal fine structure in sections cut at a favorable angle (Fig. 10). Each vesicle appears to be filled with fibrils 12 to 20 A wide, arranged more or less parallel to its long axis.

Localization of the Photosynthetic Pigment System in Green Bacteria

In the case of purple bacteria, there is good evidence that the photosynthetic pigment system is strictly associated with membranes that arise from the cytoplasmic membrane and are organized as either vesicles or lamellae (18, 19). When purple bacteria are grown photosynthetically at low light intensities (50 ft-c or less), the cytoplasm becomes so densely filled with internal membranous structures that ribosomes and even nucleoplasm are difficult to observe in thin sections (8, 19). Under such conditions of cultivation, the specific chlorophyll content of a number of different species of purple bacteria ranges between 50 and 70 μg per milligram of cell protein. As shown in Table II, green bacteria grown at low light intensities have a considerably higher

FIGURE 4 Section of *Chlorobium thiosulfatophilum* T embedded in Vestopal, showing formation of a transverse wall in association with a mesosomal element (*M*). As in Fig. 2, only the inner layer (*iw*) of the cell wall separates the two daughter cells. At left, the outer layers of the wall have already begun to intrude into the septum. Main fixation, 2 hours. × 150,000.

FIGURES 5 and 6 Tangential sections of *Chlorobium thiosulfatophilum* T embedded in Vestopal. Such sections show the arrangement of the chlorobium vesicles (*cv*) in the cortical layer of the cytoplasm. The section shown in Fig. 6 cuts across the surface of the cell, and hence includes transverse sections of several cell wall extensions (*ex*), which can be seen to be hollow. Main fixation, 2 hours. Fig. 5, × 120,000; Fig. 6, × 100,000.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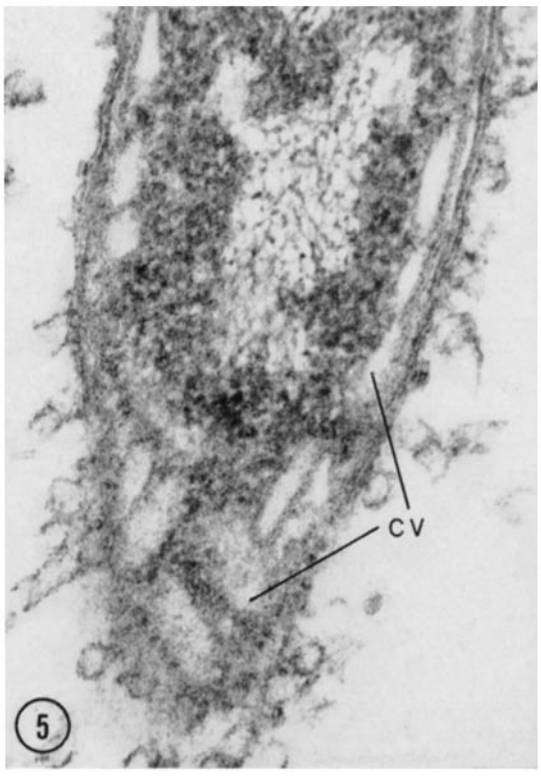
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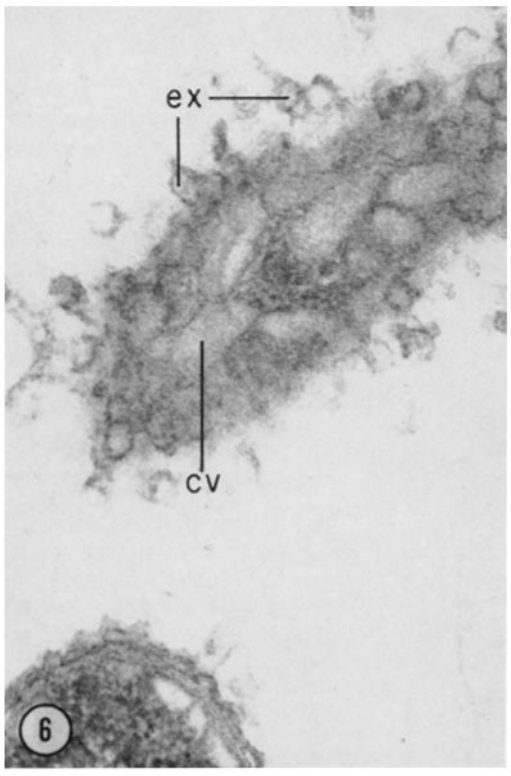
5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specific chlorophyll content, in the range of 100 to 200 μg per milligram of cell protein. It is evident, therefore, that in the cells of green bacteria the photosynthetic apparatus must occupy a substantial fraction of the total volume of the cell. With which of the detectable elements of structure is this apparatus associated? The mesosomes appear to have the same origin and fine structure as the internal vesicular or lamellar elements of purple bacteria, and hence by analogy might be considered a possible site for the photosynthetic apparatus of the green bacteria. Against this interpretation is the fact that mesosomes are never abundant and at best represent a very minor fraction of the cell volume. Hence it seems unlikely that they could accommodate more than a small fraction of the photosynthetic pigment system. In terms of their abundance, the chlorobium vesicles are clearly more favorable candidates. They make up an almost continuous layer underlying the cell membrane, approximately 300 to 400 A thick as judged from thin sections. Rough calculation shows that the chlorobium vesicles account for at least 25 per cent of the volume of the protoplast; the true value may be considerably greater, since the apparent width as measured in thin sections is probably an underestimate.

TABLE II

Chlorophyll content of green bacteria grown at low light intensity

Strain	*Chlorophyll content of cells, in micrograms per milligram cellular protein*
Chlorobium limicola R (660)	142
Chlorobium limicola ML (650)	120
Chlorobium limicola 17 CR (650)	118
Chlorobium thiosulfatophilum B (660)	115
Chlorobium thiosulfatophilum T (660)	190
Chlorobium thiosulfatophilum 6 CR (660)	100

The numbers in parentheses indicate the type of chlorobium chlorophyll present in each strain.

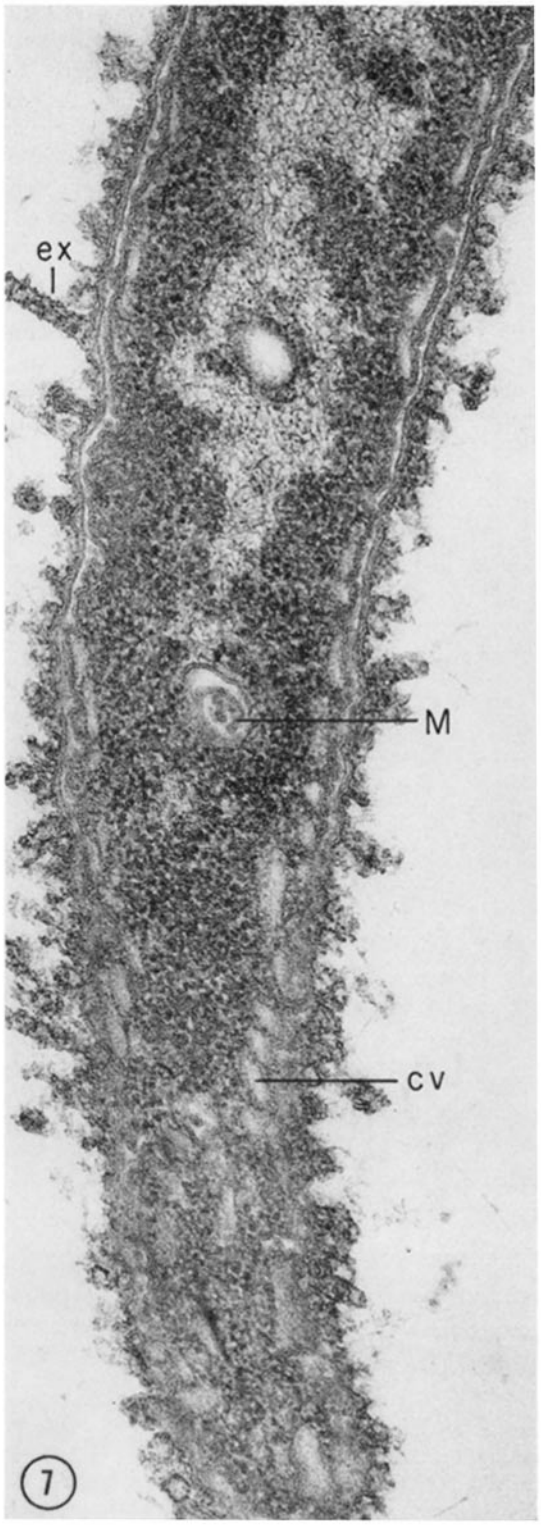
Direct evidence which points to the association of much of the cellular chlorophyll with the chlorobium vesicles has been obtained by the fractionation of cell-free extracts. Cells of *C. thiosulfatophilum* T were broken in the French pressure cell. The resulting extract was first centrifuged at low speed to remove residual unbroken cells. The supernatant layer was then centrifuged for 2 hours at 100,000 *g*., and the pellet, which contained nearly all the chlorophyll, was resuspended in Tris buffer (0.01 M, pH 7.5) containing 10^{-3} M $MgSO_4$. This material was placed on a linear sucrose gradient (0.5 to 2.0 M) and centrifuged for 2 hours at 25,000 RPM. The main pigmented band, containing 87 per cent of the chlorophyll recovered from the gradient, was isolated, washed free of sucrose, and examined by negative staining (Fig. 17). Although this material is clearly heterogeneous, the main structures present consist of oblong bodies, 1000 to 1500 A long and 500 to 750 A wide, which can be reasonably equated with the chlorobium vesicles seen in thin sections. The specific chlorophyll content of this fraction was 240 μg per milligram of protein, representing an enrichment of approximately twofold, relative to the chlorophyll content (107 μg per milligram protein) of the original unbroken cells.

DISCUSSION

At the level of resolution provided by the light microscope, the cells of green bacteria present no distinctive features, and cannot be distinguished on structural grounds from other small, rod-shaped bacteria or from the smaller unicellular blue-green algae. Examination by the techniques of electron microscopy has revealed, however, that green bac-

FIGURE 7 Longitudinal section of *C. thiosulfatophilum* T embedded in methacrylate. All the structural features found in Vestopal-embedded cells are also evident here: rod-shaped wall extensions (*ex*), chlorobium vesicles (*cv*), mesosomal element (*M*). At the lower end of the cell, the section traverses the cortical region of the cytoplasm, revealing the arrangement of the chlorobium vesicles. Main fixation, 2 hours. × 80,000.

FIGURE 8 Longitudinal section of a chain of cells of *Chlorobium thiosulfatophilum* T, embedded in methacrylate. A mesosome (*M*), associated with the formation of a transverse wall, can be seen in the upper cell. As in Fig. 7, the sites of polymetaphosphate deposits (*p*) are visible, but the contents have disappeared. Main fixation, 2 hours. × 80,000.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cv
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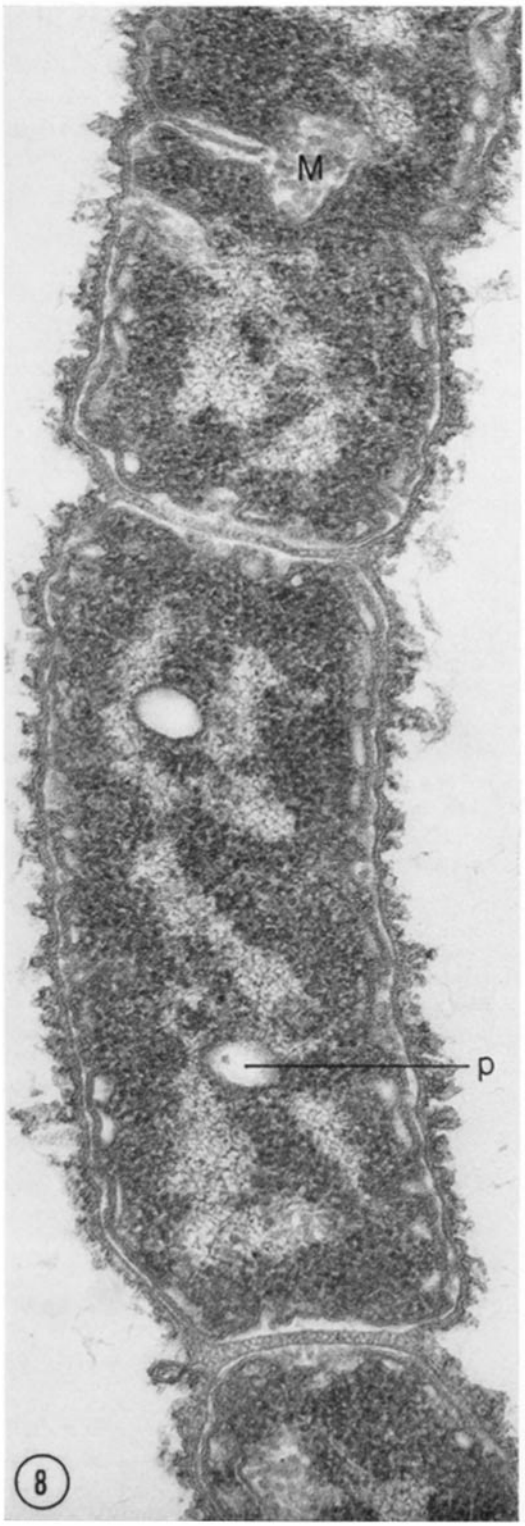
M
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8

teria possess a remarkably complex and distinctive internal fine structure, unlike that of any other major group of procaryotic organisms so far studied with the electron microscope. In particular, it should be noted that, on fine structure alone, the green bacteria are readily distinguishable from both purple bacteria and blue-green algae. Although we have documented the major structural features of green bacteria with reference to only four *Chlorobium* strains, more cursory examination of several other strains, including *Chloropseudomonas ethylicum*, has established that the characteristic fine structure is common to all members so far grown in pure culture.

The unique feature of the green bacterial cell is the presence of a cortical layer composed of large, oblong, membrane-bounded chlorobium vesicles which is located between the cytoplasmic membrane and the ribosomal layer. Observations on fractions of cell-free extracts indicate that the photosynthetic pigment system is associated largely, if not exclusively, with these structures.

We cannot readily explain the failure of previous workers (6, 7) to detect such a conspicuous and distinctive element as the chlorobium vesicles in thin sections of green bacteria. It is possibly attributable to the rather poor internal contrast obtained in cells which have not been poststained with lead hydroxide. A major discrepancy between our findings and those of Bergeron and Fuller (7) concerns the physical state of the photosynthetic pigment system in cell-free extracts. After breakage of cells with the French press, we found a major part of the pigment system associated with a readily sedimentable fraction, largely composed of chlorobium vesicles. Bergeron and Fuller (7), who used more drastic mechanical methods to break the cells, reported that the pigment system was associated with particles of ribosomal dimensions, several orders of magnitude smaller than chlorobium vesicles. The most plausible explanation for this discrepancy is that the methods of cell breakage used by Bergeron and Fuller comminuted the chlorobium vesicles, with the resultant formation of much smaller, pigment-bearing fragments. Only further work can show whether these small particles represent the ultimate functional units of the photosynthetic apparatus, as proposed by Bergeron and Fuller, or are simply products of a random fragmentation of the vesicles.

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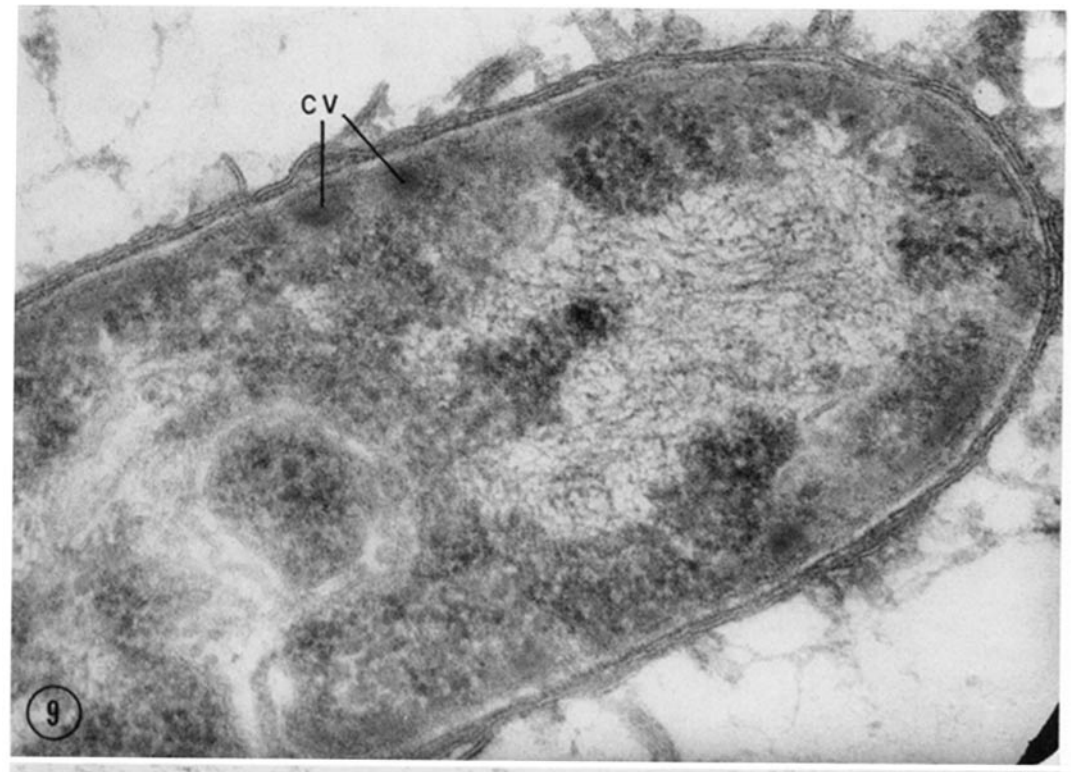
FIGURE 9 Section of *Chlorobium thiosulfatophilum* T embedded in Epon. Although other cell structures have much the same appearance as in sections embedded in Vestopal or methacrylate, the chlorobium vesicles (*cv*) are very electron-opaque. Main fixation, 2 hours. × 120,000.

FIGURE 10 A highly magnified section of part of a cell of *Chlorobium thiosulfatophilum* T embedded in Epon, showing internal fine structure of the chlorobium vesicles (*cv*). Each vesicle appears to be filled with fibrils 12 to 20 A wide, arrayed more or less parallel to the long axis of the vesicle. Main fixation, 2 hours. × 400,000.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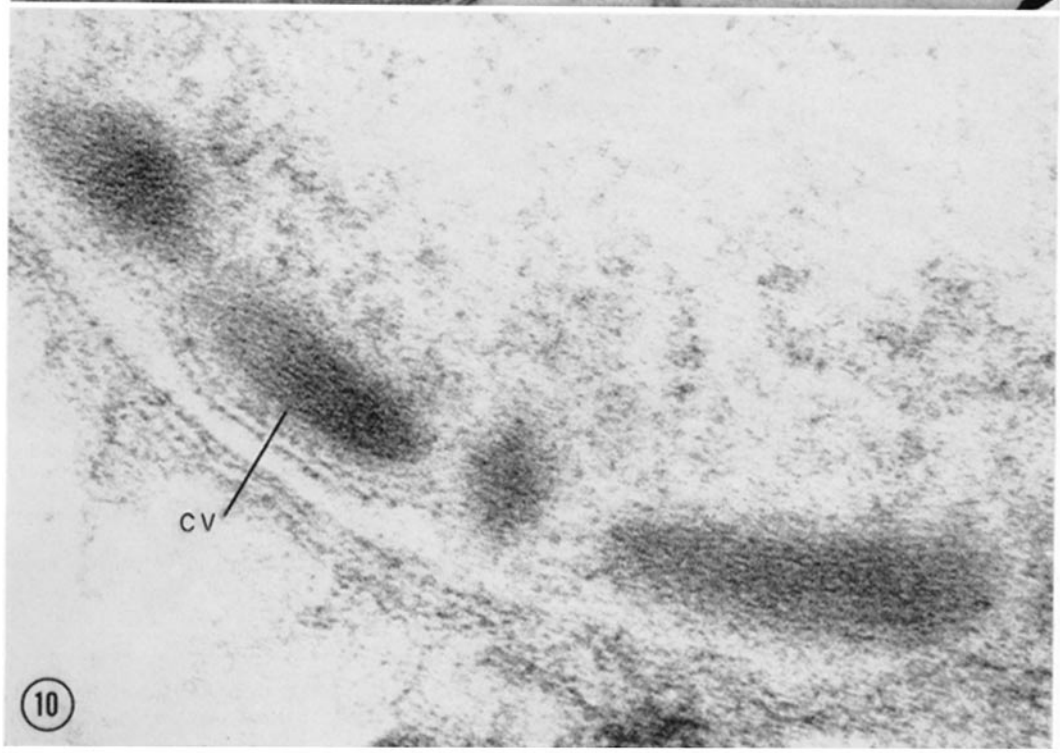
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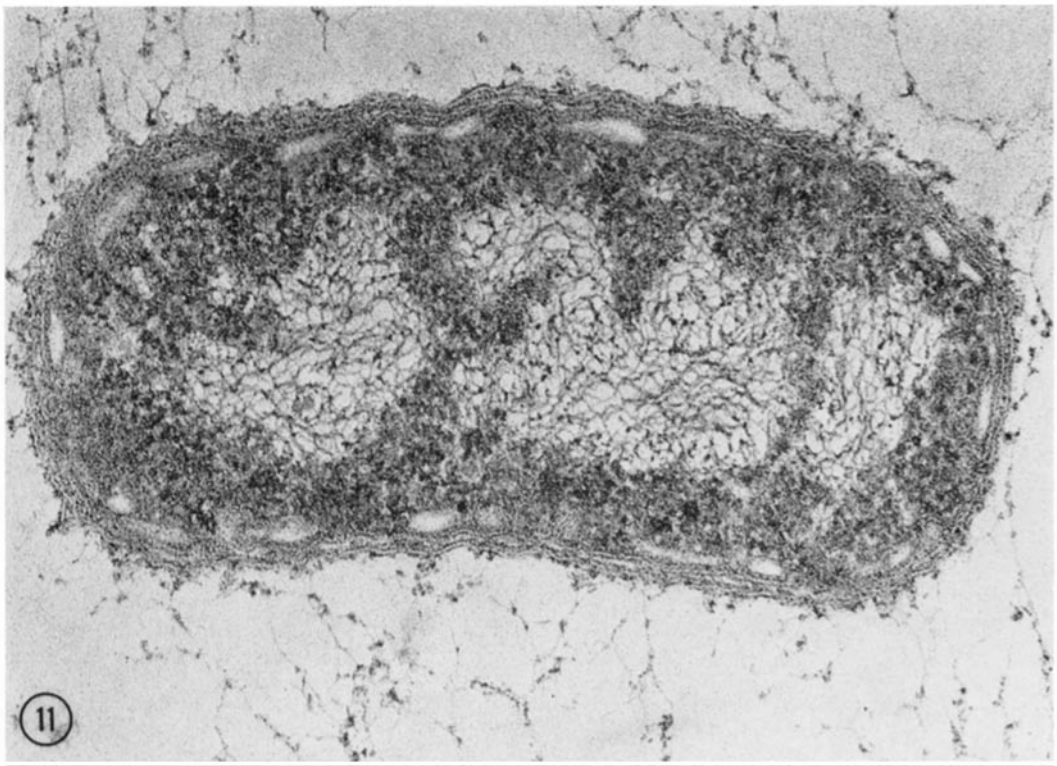
FIGURES 11 and 12 Sections of *Chlorobium thiosulfatophilum* 6 CR, embedded in Vestopal. In this strain, the cell wall does not bear the rod-shaped extensions characteristic of strain T. The internal structure of the cell is, however, very similar. Chlorobium vesicles (*cv*); mesosomal element (*M*). Main fixation, 2 hours. × 120,000.



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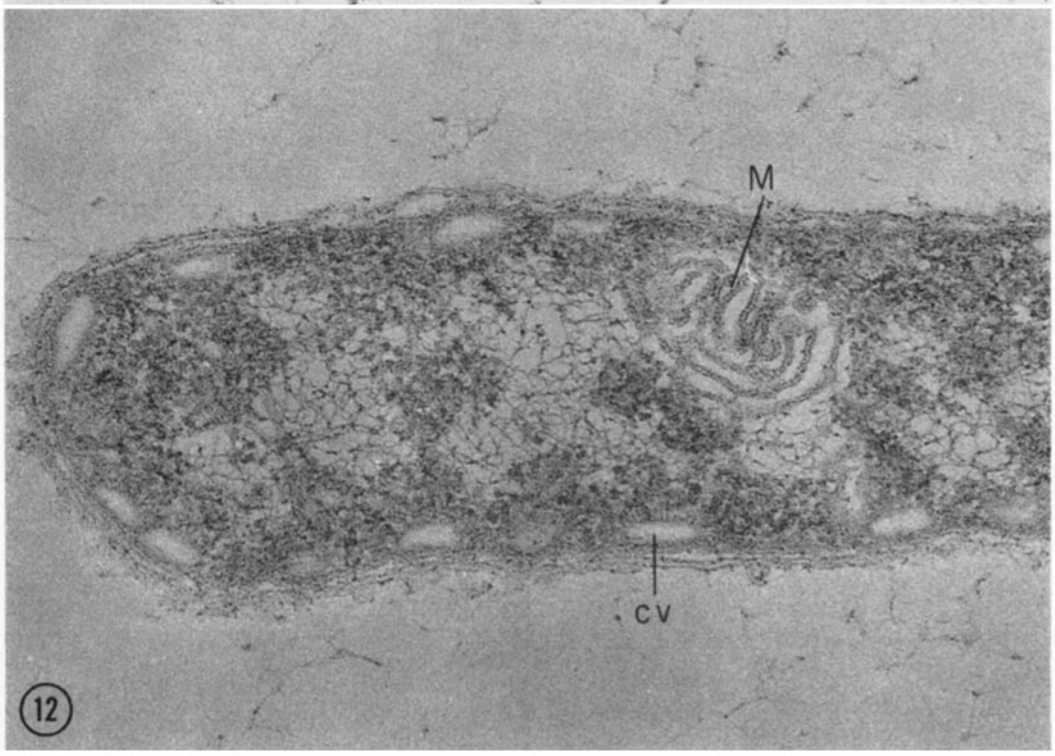

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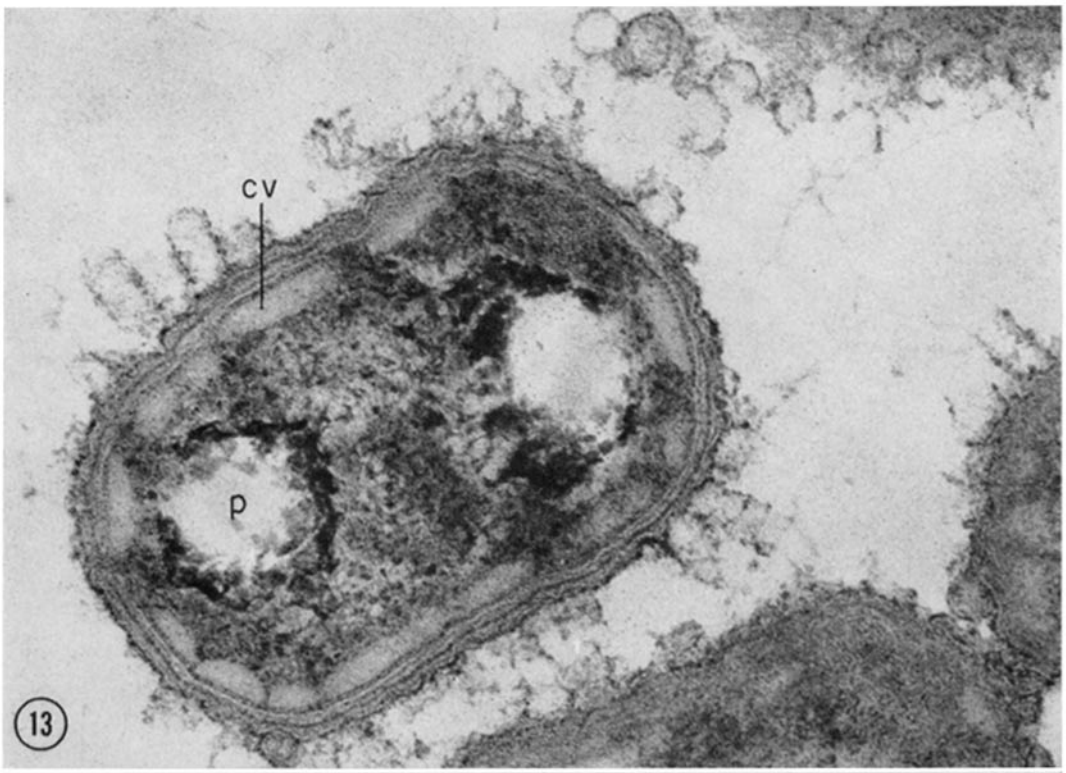
FIGURES 13 and 14 Sections of *Chlorobium limicola* R, embedded in Vestopal (Fig. 13) and methacrylate (Fig. 14). The cell wall carries large, hollow, rod-shaped extensions, which are particularly evident in Fig. 13; they are cut in cross-section at the upper right of this figure. The fixation is rather poor, but the characteristic chlorobium vesicles (*cv*) and a small mesosomal element (*M*) are evident. Large polymetaphosphate desposits (*p*) are present. Main fixation, 2 hours. Fig. 13, × 135,000; Fig. 14, × 100,000.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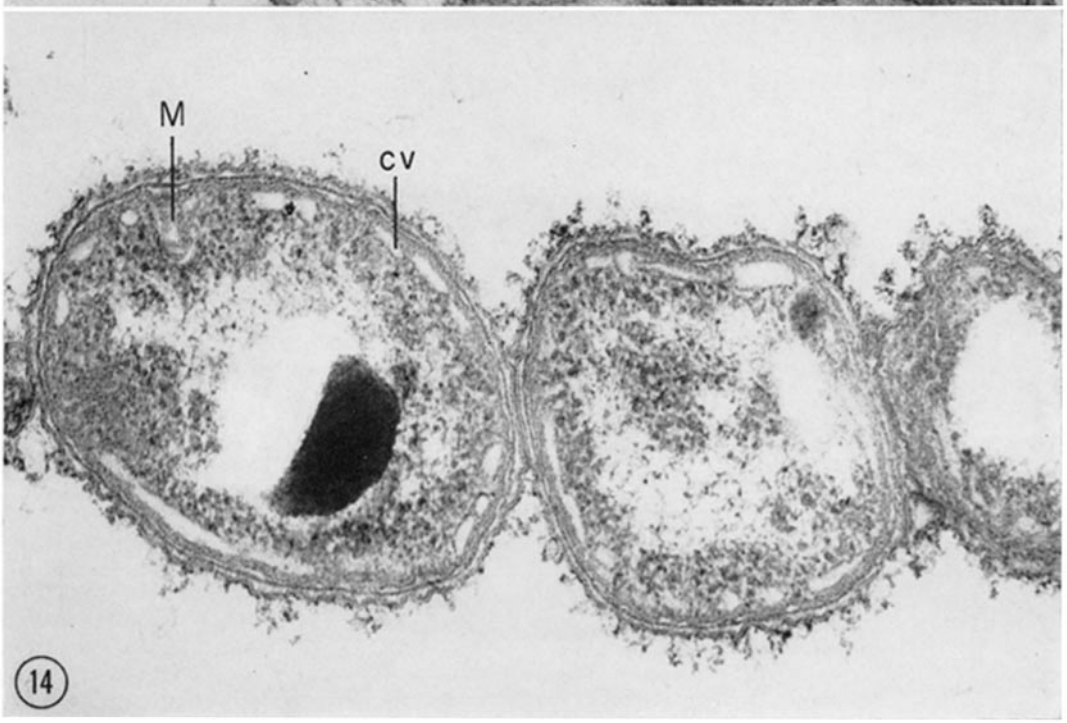
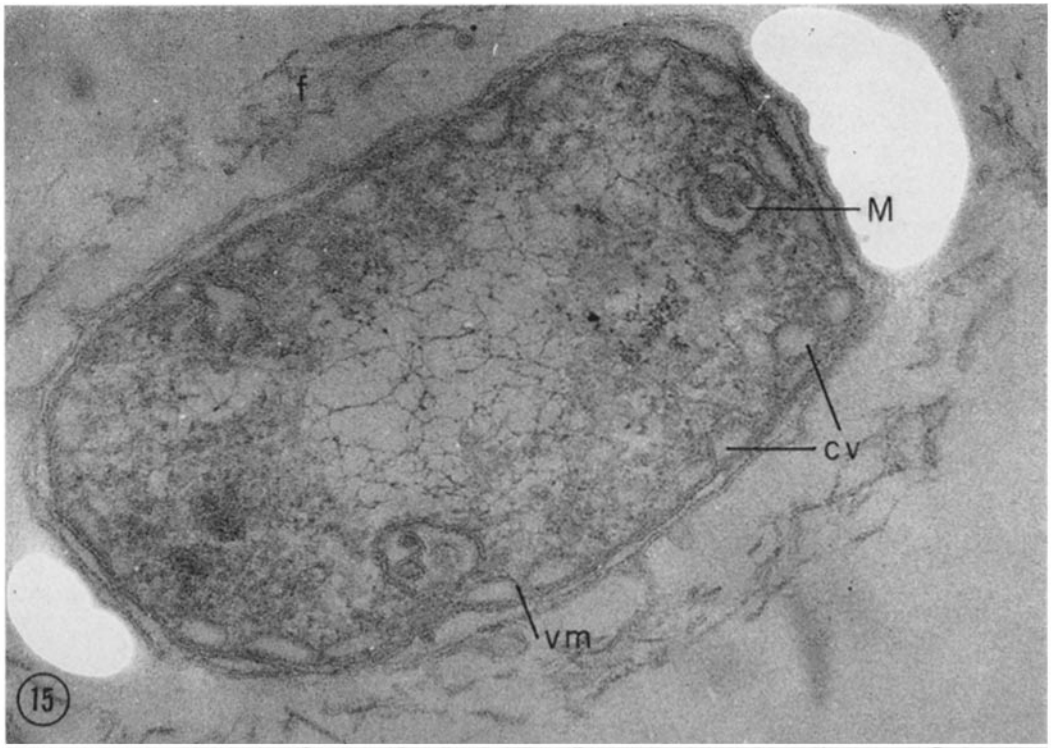

M
cv
14

FIGURE 15 Section of *Chlorobium limicola* ML, embedded in Vestopal. Note the halo of fibrils (*f*) at some distance from the cell. Two small mesosomal elements (*M*) are present. The membrane (*vm*) which surrounds each chlorobium vesicle (*cv*) is readily distinguishable. Main fixation, 18 hours at 4°C. × 120,000.

FIGURE 16 Section of *Chlorobium limicola* ML, embedded in Vestopal. Preparation identical with that of Fig. 15, except that main fixation was conducted at room temperature. The general preservation of structure is superior to that in Fig. 15, but the membranes surrounding the chlorobium vesicles are less distinct. × 90,000.


f
M
cv
vm
15

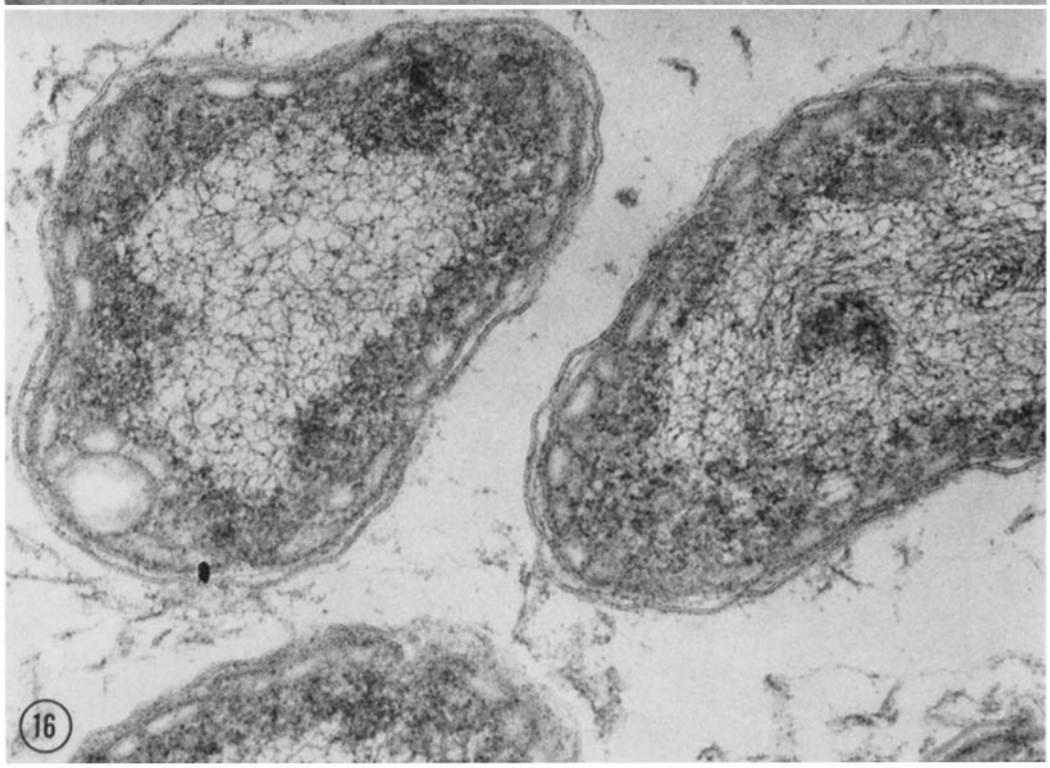
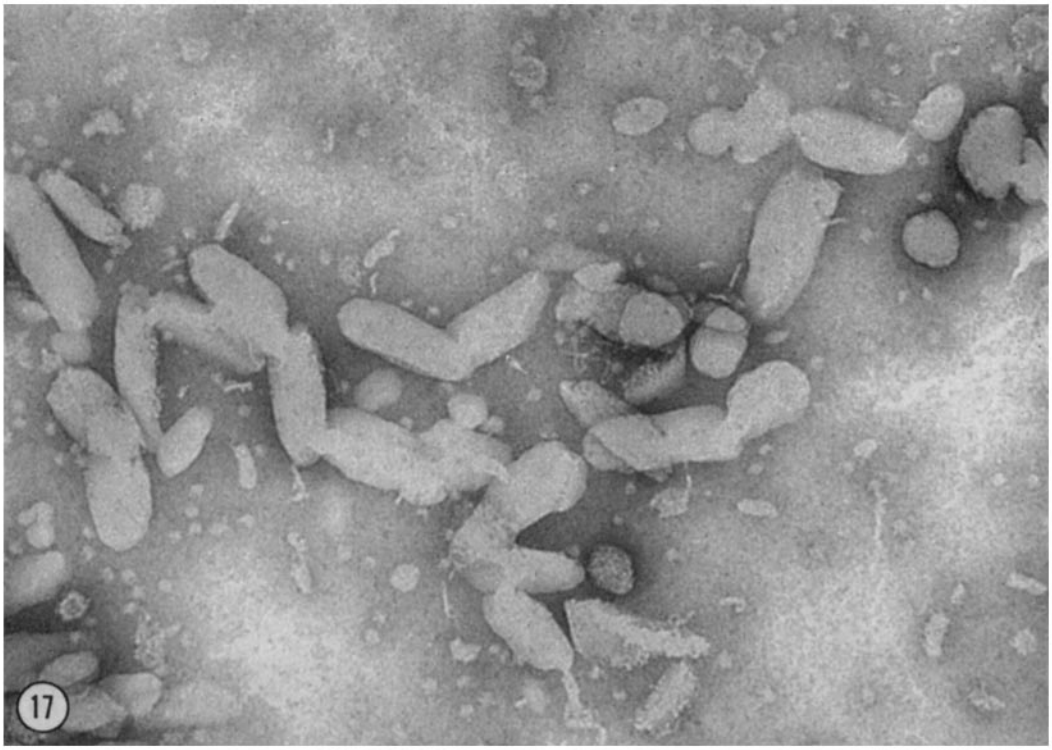
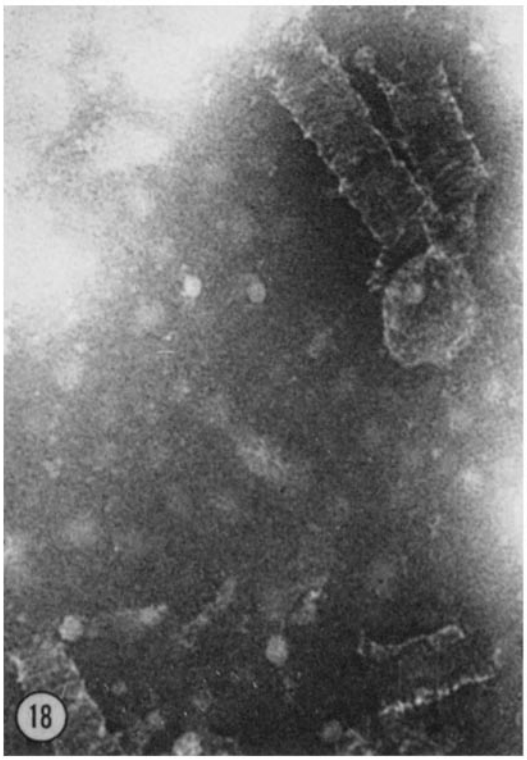
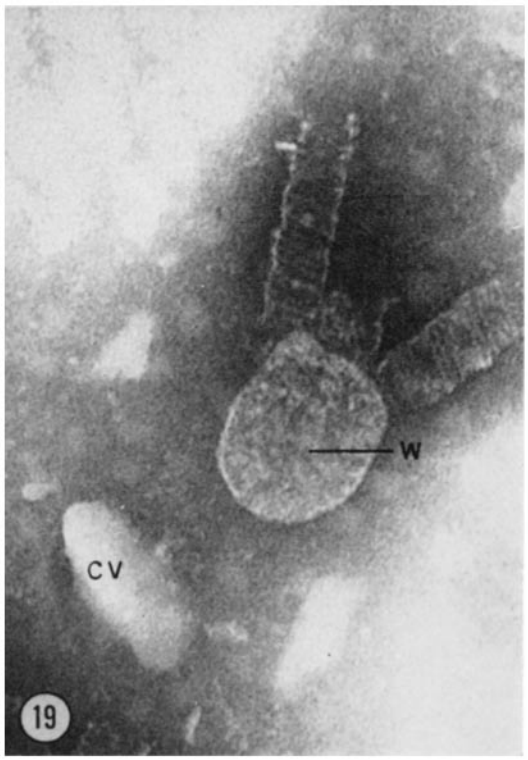

16

FIGURE 17 The main pigmented fraction from an extract of *Chlorobium thiosulfatophilum* T, isolated from a sucrose gradient (see text) and negatively stained with potassium phosphotungstate. The most conspicuous elements are oblong structures which resemble in size and form the chlorobium vesicles seen in thin sections. Some of these structures have obviously been damaged during isolation, and appear to be disintegrating with the liberation of smaller fragments. The preparation contains other particulate elements, including some ribosomes. × 120,000.

FIGURES 18 and 19 Sample of the heavy fraction with a low pigment content isolated from a sucrose gradient of an extract of *Chlorobium thiosulfatophilum* T and negatively stained with potassium phosphotungstate. This fraction consists predominantly of fragments of the cell wall (*w*), some of which still have attached to them the rod-shaped extensions characteristic of strain T. These extensions appear to be hollow tubes, the walls of which are formed from three closely apposed filaments, wound in helical fashion. In Fig. 19, a chlorobium vesicle (*cv*) is also present. × 200,000.


17


18


W
CV
19