JOURNAL OF BACTERIOLOGY, Dec. 1973, p. 1405-1411
Vol. 116, No. 3

Electron Microscopy of the Carboxysomes (Polyhedral Bodies) of *Thiobacillus neapolitanus*

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Received for publication 30 August 1973

The carboxysomes of *Thiobacillus neapolitanus* are shown, by electron microscopy, to consist of a paracrystalline array of 10-nm particles surrounded by a "membrane." The 10-nm particles have a center hole or depression and have been previously identified as ribulose diphosphate carboxylase. The membrane is a monolayer approximately 3.5-nm thick.

Many, if not all, of the blue-green algae, and several chemolithotrophic bacteria, contain polyhedral inclusion bodies (3, 13, 18, 20). In thin section, these inclusions have polygonal profiles (four to six sides); range in size from 50 to 500 nm in diameter, and are commonly associated with the nuclear material (3, 5, 18, 20). It is surmised, because of the similarity in structure and staining properties, that the bodies in all these organisms are similar, if not identical, chemically and functionally.

We recently isolated the polyhedral bodies of *Thiobacillus neapolitanus* and discovered that they possess a surrounding "membrane" and contain the enzyme D-ribulose-1,5-diphosphate carboxy-lyase (EC 4.1.1.39; trivial name ribulose diphosphate carboxylase; the name carboxysomes has been proposed (17a).

This report describes the ultrastructural properties of the carboxysomes of *T. neapolitanus*.

MATERIALS AND METHODS

The organism, obtained from E. Johnson, Department of Microbiology, Tulane University, New Orleans, La., was cultured, harvested, and stored, and the carboxysomes, the carboxysome membrane, and the ribulose diphosphate carboxylase were isolated, as previously described (J. M. Shively et al., Science, in press). All isolation, dialysis, dilution, fixation, and dehydration procedures were carried out at 0 to 4 C. Dialysis was accomplished with several changes of deionized water. All negative stains were achieved with 1% potassium phosphotungstate (pH 7.0) on carbon-coated, Formvar-covered grids.

Isolated carboxysomes and carboxysome membranes in 60 and 55% (wt/wt) sucrose, respectively, were treated for 2 h with a final concentration of 3% (vol/vol) glutaraldehyde, and the resulting fixed specimens, as well as unfixed ribulose diphosphate carboxylase in 25% sucrose, were dialyzed for 24 h. Negative stains were prepared from each dialyzed preparation.

In addition, samples of the glutaraldehyde-fixed, dialyzed carboxysomes and carboxysome membranes were placed in Microfuge tubes (Beckman Instruments, Inc., Fullerton, Calif.) and centrifuged for 5 min in a Beckman Microfuge operated at maximal speed; 0.5-mm pellets were obtained. The supernatant fluid was removed, and the pellets were treated with 2% osmium tetroxide for 2 h. The resulting doubly fixed specimens were dehydrated with a series of increasing ethyl alcohol concentrations (50 to 100%) and embedded in Epon 812 essentially as described by Luft (9).

Thin sections were cut on an LKB ultratome equipped with a diamond knife, collected on unsupported 500-mesh grids, and stained with uranyl acetate and lead citrate.

Also, samples of carboxysomes in 60% sucrose were either dialyzed without fixation, or diluted with an equal volume of deionized water, fixed for 2 h with 3% glutaraldehyde, and then dialyzed. Negative stains were made from both preparations.

All specimens were examined in a Siemens Elmiskop 1A equipped with a short-focal-length objective at an acceleration voltage of either 60 or 80 kV.

RESULTS

As observed in thin section, each cell of *T. neapolitanus* contains between 1 and 15 carboxysomes, with four to six being most common (Fig. 1). No cells, sectioned longitudinally, have been found to be devoid of carboxysomes. The number per cell appears to be directly related to cell size, i.e., large cells have the most and small cells have the least (Fig. 1 and 2).

The bodies vary in size from 90 to 150 nm in diameter, have polygonal profiles, exhibit a

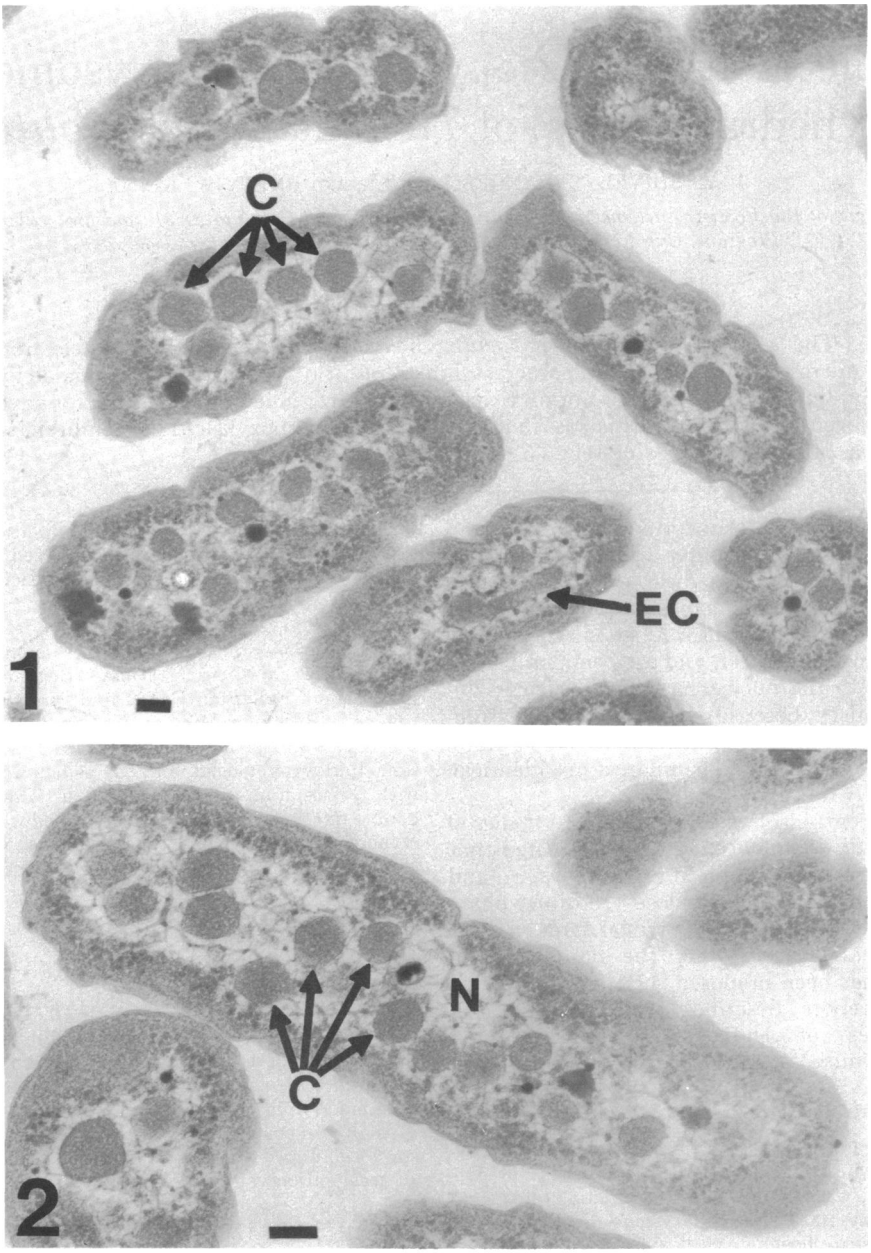
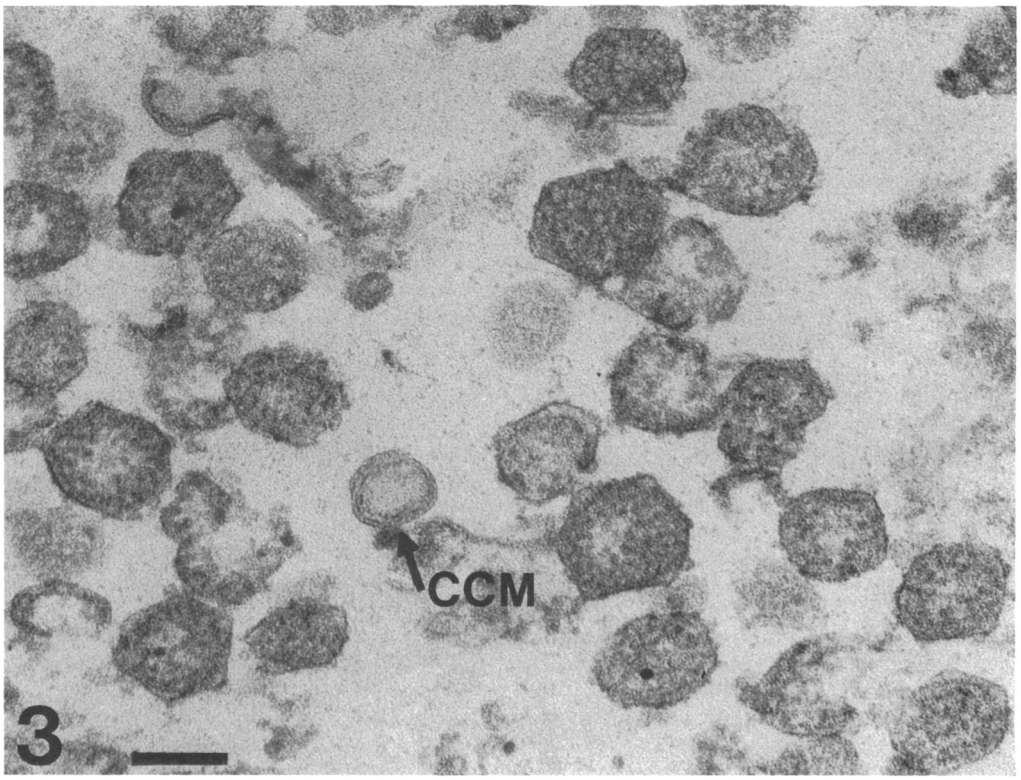


FIG. 1. *Thin section of T. neapolitanus showing general cell structure and carboxysomes. Abbreviations: N, nuclear area; C, carboxysomes; and EC, elongated carboxysome. In all micrographs, the marker bar represents 100 nm.*

FIG. 2. *Thin section showing an elongated cell just prior to division.*

granular substructure of medium electron density, appear to have a surrounding barrier, and are associated with nucleoplasm (Fig. 1; reference 18). The carboxysomes appear reasonably uniform in size and shape, but some variation, e.g., elongation, has been observed (Fig. 1; reference 18).

The isolated bodies in thin section (Fig. 3 and 4) have the same appearance as in the intact cell, i.e., granular substructure, medium electron density, 90 to 150-nm diameter, and polygonal profiles. The granular material is surrounded by a monolayer "membrane" approximately 3.5-nm thick; a beaded substructure is



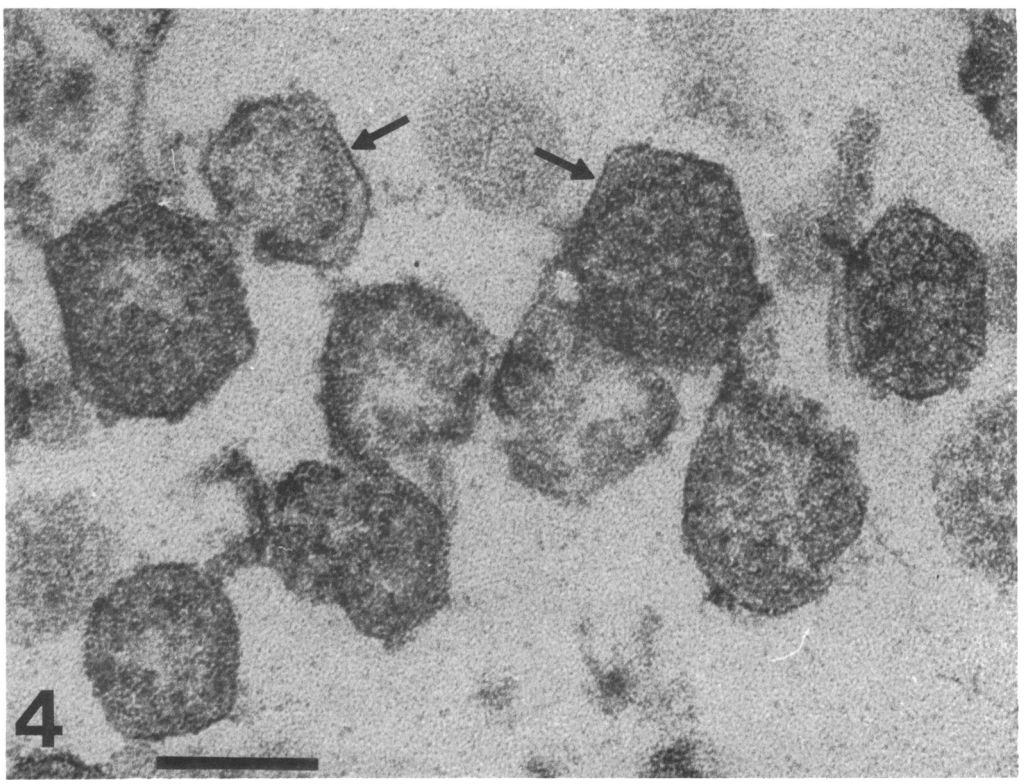


FIG. 3. *Thin section of isolated carboxysomes. Abbreviations: CCM, contaminating cell membrane.*

FIG. 4. *Enlargement of the right half of Fig. 3. Arrows: beaded substructure of the carboxysome monolayer "membrane."*

evident in some cases (Fig. 4). Contaminating cell membrane, probably outer membrane, is present in Fig. 3; contrast the trilaminar appearance of this membrane to the appearance of the carboxysome membrane.

As observed by negative staining, the isolated carboxysomes consist of particles surrounded by a membrane (Fig. 5). The inner component particles have been identified as ribulose diphosphate carboxylase. It appears that the isolation procedure disturbs the intrainclusion organization, i.e., the enzyme exists in a paracrystalline array in well-preserved, unswollen bodies (Fig. 6). Shape and size variability is also demonstrated in Fig. 6 and 7.

If the carboxysomes in 60% sucrose are diluted or dialyzed without prior glutaraldehyde fixation, they swell and rupture, releasing the ribulose diphosphate carboxylase (Fig. 8; 17a). The empty membrane sacks still retain their polyhedral properties (Fig. 9).

The ribulose diphosphate carboxylase (10-nm inner component particles) and membranes are readily isolated by density gradient centrifugation in sucrose (17a). The enzyme is approximately 10 nm in diameter and shows one very characteristic profile—a "doughnut" with a densely staining center hole or depression (Fig. 10).

The isolated membranes, as seen in thin section, retain their polyhedral profile and appear as a single layer approximately 3.5-nm thick; a beaded appearance is occasionally evident (Fig. 11). Note the trilaminar appearance of the contaminating membrane. It appears that the membranes have retained some internal material. This may be ribulose diphosphate carboxylase or other carboxysome components.

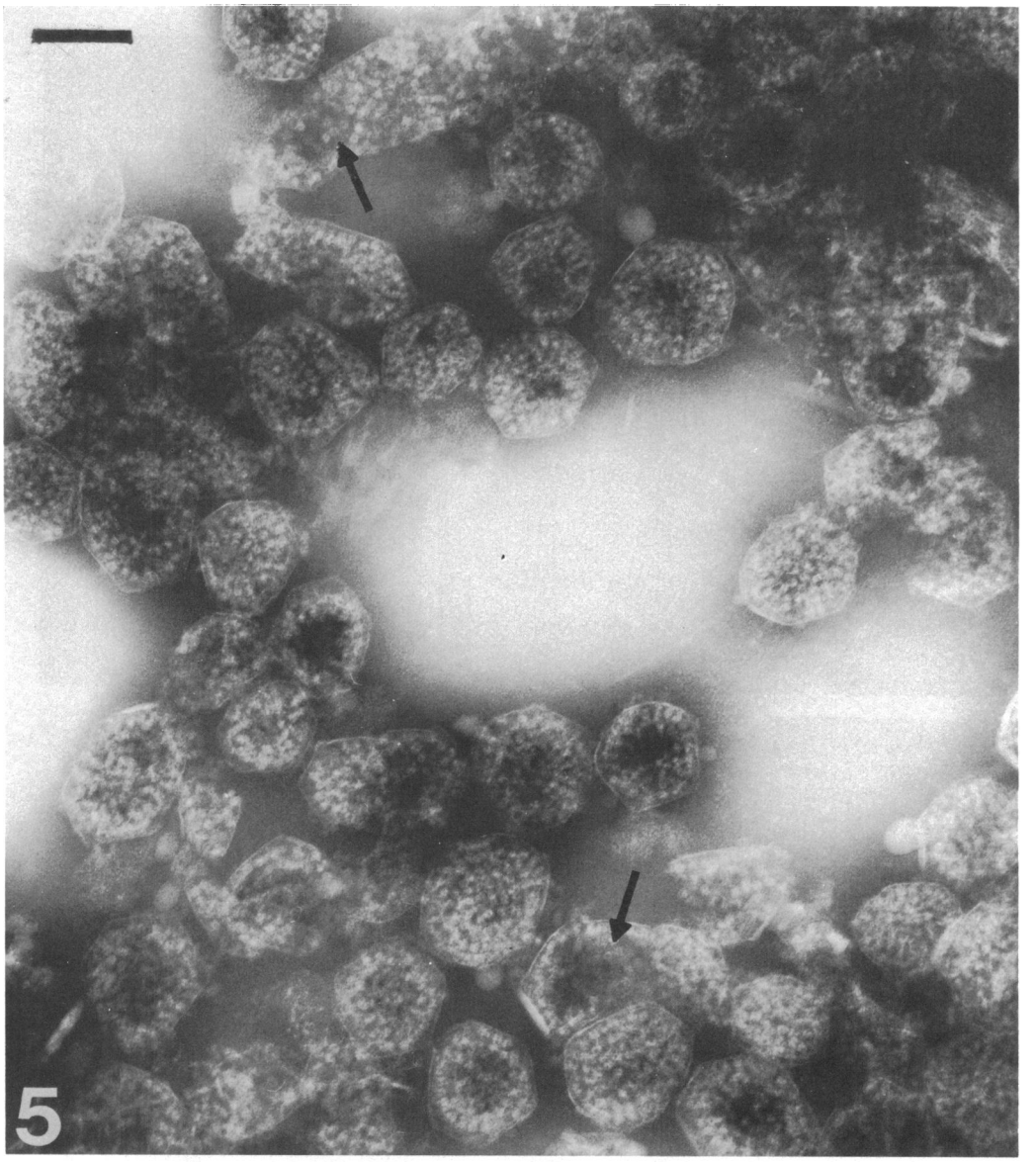
DISCUSSION

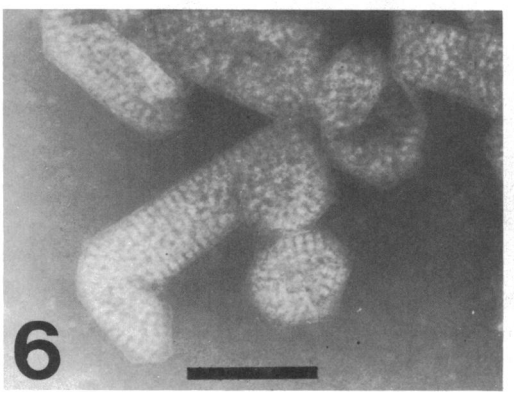
The electron microscopy image, i.e., size (approximately 10 nm in diameter) and shape (doughnut with a central hole or depression), of the ribulose diphosphate carboxylase of *T. neapolitanus* is very similat to this enzyme in spinach chloroplasts or *Chromatium* (1, 16). The fact that the ribulose diphosphate carboxylase of *T. neapolitanus* has an estimated sedimentation coefficient of 17*S* supports this observation (10). Both the higher plant and *Chromatium* enzymes have *S* values and masses in excess of 18 and 500,000 daltons, respectively (1, 8).

The membrane of the carboxysome, a single layer about 3.5-nm thick in thin section, appears to be similar to the membrane surrounding the sulfur granules of *Chromatium* and the gas vacuoles of blue-green algae (2, 14, 17). Both of these membranes are monolayers 2.0- to 2.5-nm thick and are composed entirely of protein (6, 7, 14, 17); this implies that the carboxysome membrane is devoid of lipid. This contention is supported by the following observations. The carboxysome membrane retains its polyhedral shape after breaking, i.e., it does not vesiculate, and its density, being about the same as the outer, trilaminar, lipopolysaccharide membrane of gram-negative bacteria, is much higher than that of cytoplasmic membrane (15; 17a). The stabilizing effect of sucrose suggests that the carboxysome membrane is osmotically active.

The reasonably uniform size and shape and constant number of polyhedral bodies per cell, coupled with the observation that the numbers increase as the cells enlarge during each division cycle, suggest that the carboxysomes function in carbon dioxide fixation and are not just enzyme-storage bodies. It is indeed interesting that the heterocysts of blue-green algae lack polyhedral bodies and fix little, if any, carbon dioxide (19). Furthermore, MacElroy et al. (10) reported that phosphoribulokinase and ribulose diphosphate carboxylase purify as a single component; this implies that the carboxysomes will have at least one other enzyme of the Calvin cycle. However, after cell disruption in the French pressure cell, we find 15 to 40% of the ribulose diphosphate carboxylase in the soluble form (17a). We have attributed this to carboxysome breakage, but one could argue that this represents the active enzyme fraction. Our assay conditions do not insure stability of the carboxysome. Also, one must consider that closely related organisms, e.g., *Thiobacillus denitrificans*, which have ribulose diphosphate carboxylase, do not have polyhedral bodies (11, 18).

Micrographs of negative-stain preparations of the polyhedral bodies of *Anacystis nidulans* and of the nitrifying bacteria show particles in a paracrystalline array (3; personal communication from S. W. Watson, Woods Hole Oceanographic Institution, Woods Hole, Mass.). This, and other observed similarities, namely, size, shape, granularity, electron density, and cellular location, support the contention that the polyhedral bodies form the thiobacilli, the nitrifying bacteria, and the blue-green algae are homologous. If indeed, this is the case, it will provide additional evidence of an evolutionary link between these groups of microorganisms. Furthermore, it will lend some support to Mereschkowsky's (12) theory that the chloroplasts of eukaryotic plant cells evolved from endosymbiotic blue-green algae. The chromatophores of





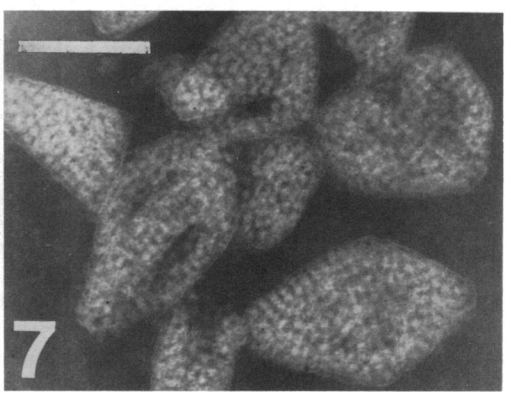


FIG. 5. *Negative stain (potassium phosphotungstate) of isolated carboxysomes. Arrows: characteristic "doughnut" particles of ribulose diphosphate carboxylase.*

FIG. 6. *Negative stain of isolated carboxysomes showing size and shape variability and paracrystalline arrangement of ribulose diphosphate carboxylase.*

FIG. 7. *Negative stain of isolated carboxysomes, demonstrating size and shape variability.*

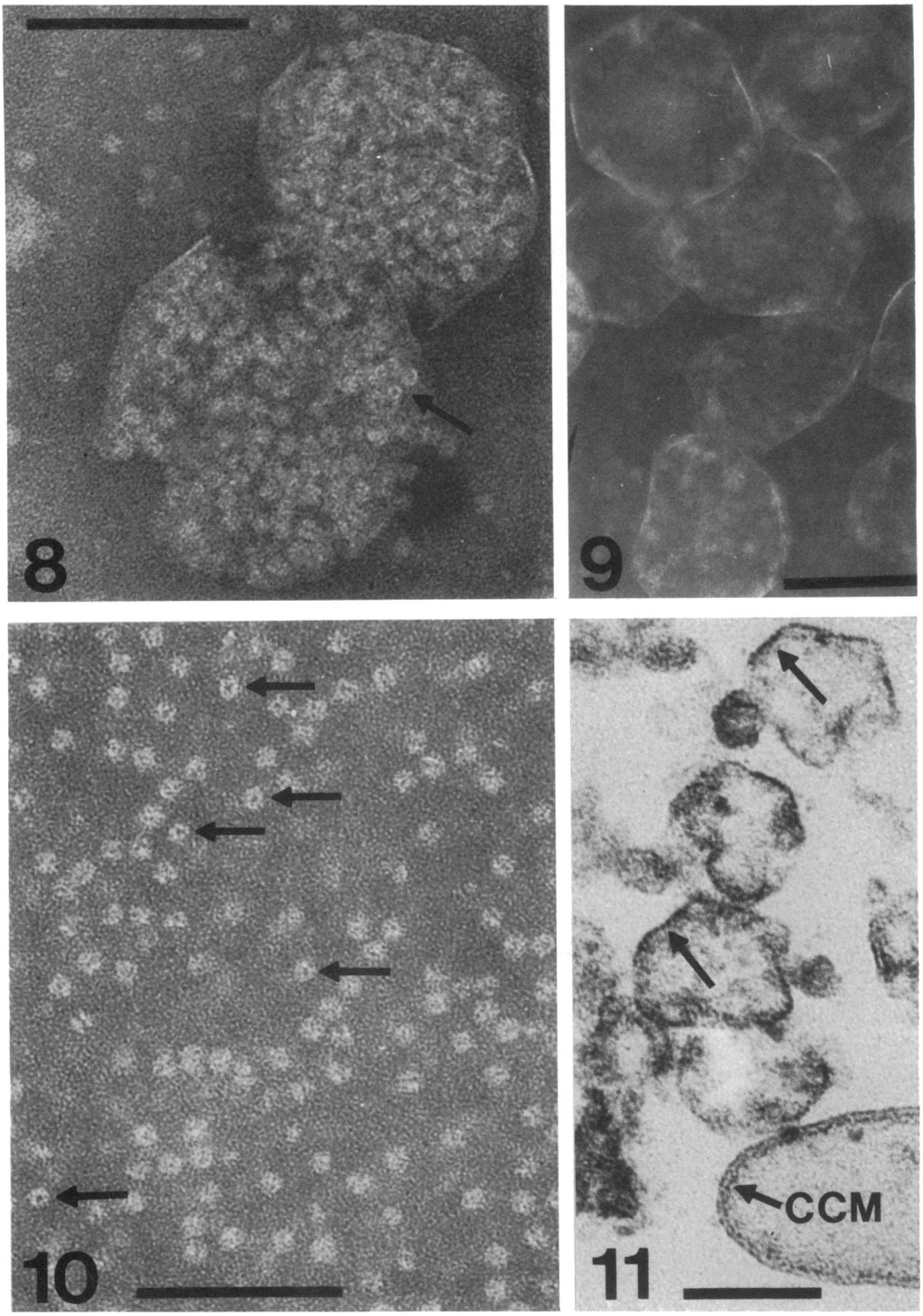


FIG. 8. *Negative stain showing two carboxysomes in the process of rupturing. Arrow: characteristic "doughnut" particles of ribulose diphosphate carboxylase.*

FIG. 9. *Negative stain of empty carboxysomes.*

FIG. 10. *Negative stain of isolated ribulose diphosphate carboxylase. Arrows: characteristic "doughnut" profile of the enzyme.*

FIG. 11. *Thin section of isolated carboxysome "membranes." Arrows: beaded substructure.*

the lower red algae, *Bangiales*, have pyrenoids similar to the polyhedral bodies. Also, the protein core of the chloroplast pyrenoid of the green alga, *Eremosphaera viridis*, consists of a paracrystalline array of ribulose diphosphate carboxylase (approximately 90%) and other Calvin cycle enzymes (4).

Obviously, considerable research is yet to be accomplished to attain a full understanding of the structure and function of the carboxysomes.

ACKNOWLEDGMENTS

The micrographs used in Fig. 1 and 2 were prepared while one of the authors (J. M. S.) was a Public Health Service Special Postdoctoral Fellow (1-F03-GM-39661-01, from the National Institute of General Medical Sciences) with J. W. Greenawalt, Johns Hopkins University Medical School, Baltimore, Md. This research was supported by a participation award to one of the authors (J. M. S.) by Oak Ridge Associated Universities. Oak Ridge National Laboratory is operated for the U.S. Atomic Energy Commission by the Union Carbide Corporation.

LITERATURE CITED

1. Asazawa, T., H. Kondo, T. Shimazne, M. Nishimura, and T. Sugiyama. 1972. Further studies on ribulose-1,5-diphosphate carboxylase from *Chromatium* strain D. Biochemistry **11**:1298-1303.
2. Bowen, C. C., and T. E. Jensen. 1965. Blue-green algae: fine structure of the gas vacuoles. Science **147**:1460-1462.
3. Gantt, E., and S. F. Conti. 1969. Ultrastructure of blue-green algae. J. Bacteriol. **97**:1486-1493.
4. Holdsworth, R. H. 1971. The isolation and partial characterization of the pyrenoid protein of *Eremosphaera viridis*. J. Cell Biol. **51**:499-513.
5. Jensen, T. E., and C. C. Bowen. 1961. Organization of the centroplasm in *Nostoc pruniforme*. Iowa Acad. Sci. **68**:86-89.
6. Jones, D. D., and M. Jost. 1970. Isolation and chemical characterization of gas-vacuole membranes from *Microcystis aeruginosa* Kuetz. emend. Elenkin. Arch. Mikrobiol. **70**:43-64.
7. Jones, D. D., and M. Jost. 1971. Characterization of the protein from gas-vacuole membranes of the blue-green alga, *Microcystis aeruginosa*. Planta **100**:277-287.
8. Kawashima, N., and S. G. Wildman. 1970. Fraction I protein. Annu. Rev. Plant Physiol. **21**:325-358.
9. Luft, J. L. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. **9**:409-414.
10. MacElroy, R. D., E. J. Johnson, and M. K. Johnson. 1968. Characterization of ribulose diphosphate carboxylase and phosphoribulokinase from *Thiobacillus thioparus* and *Thiobacillus neapolitanus*. Arch. Biochem. Biophys. **127**:310-316.
11. McFadden, B. A., and A. R. Denend. 1972. Ribulose diphosphate carboxylase from autotrophic microorganisms. J. Bacteriol. **110**:633-642.
12. Mereschkowsky, C. 1905. Über Natur und Ursprung der Chromatophoren in Pflanzenreiche. Biol. Centralbl. **25**:593-604.
13. Murray, R. G. E., and S. W. Watson. 1965. Structure of *Nitrocystis oceanus* and comparison with *Nitrosomonas* and *Nitrobacter*. J. Bacteriol. **89**:1594-1609.
14. Nicolson, G. L., and G. L. Schmidt. 1971. Structure of the *Chromatium* sulfur particle and it's protein membrane. J. Bacteriol. **105**:1142-1148.
15. Osborn, M. J., J. E. Gardner, E. Parisi, and J. Carson. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*: Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962-3972.
16. Park, R. B. 1966. Chloroplast structure, p. 283-311. *In* L. Vernon and G. Seely (ed.), The chlorophyls. Academic Press Inc., New York.
17. Schmidt, G. L., G. L. Nicolson, and M. D. Kamen. 1971. Composition of the sulfur particle of *Chromatium vinosum* strain D. J. Bacteriol. **105**:1137-1141.
17a. Shively, J. M., F. L. Ball, D. H. Brown, and R. E. Saunders. 1973. Functional organelles in procaryotes: polyhedral inclusions (carboxysomes) of *Thiobacillus neapolitanus*. Science **182**:584-586.
18. Shively, J. M., G. L. Decker, and J. W. Greenawalt. 1970. Comparative ultrastructure of the thiobacilli. J. Bacteriol. **101**:618-627.
19. Wolk, C. P. 1968. Movement of carbon from vegetative cells to heterocysts in *Anabaena cylindrica*. J. Bacteriol. **96**:2138-2143.
20. Wolk, C. P. 1973. Physiology and cytological chemistry of blue-green algae. Bacteriol. Rev. **37**:32-101.