OBSERVATIONS ON THE FINE STRUCTURE OF SPHEROPLASTS OF *RHODOSPIRILLUM RUBRUM*

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ABSTRACT

Spheroplasts of the photosynthetic bacterium *Rhodospirillum rubrum* were prepared from cultures grown in either the presence or absence of light. Cells were converted into spheroplasts by using lysozyme and Versene and fixed in a sucrose-veronal-acetate buffer mixture containing osmium tetroxide. Some preparations were shadow-cast and examined whole; others were embedded in Epon 812 and sectioned. The action of lysozyme and Versene appears to result in removal of the cell wall in strips. The relationship of the chromatophores to the cytoplasmic membrane is readily visualized in sections of broken spheroplasts, and in areas the chromatophores are seen to be continuous with the membrane. In all preparations examined, no definite connections between individual chromatophores were observed. In some cells large spherical granules were evident which either possessed or lacked a clearly visible limiting membrane. On serial sectioning, all granules appeared bounded by a single membrane 40 A wide. The granule membrane was well defined only if the section came from the center of the granule. Sections at other levels showed either a diffuse membrane or no membrane at all. The reasons for this are discussed.

INTRODUCTION

Previous studies on the fine structure of the photosynthetic bacterium *Rhodospirillum rubrum* have shown the cytoplasm of this species to contain large numbers of membranous structures. These structures consist essentially of chromatophores, granules containing organic reserve materials, and compressed lamellated bodies of various configurations.

The discovery that the photosynthetic pigments of *R. rubrum* were associated with the chromatophores (1–3) raised the question of the origin of these structures. The immunological work of Newton (7) suggested that the chromatophore membrane was similar in composition to the cytoplasmic membrane, and the observations of Flexer, Sistrom, and Chapman (5) indicated that chromatophores arose at the periphery of the cell. Our own preliminary observations (9), as well as those of Cohen-Bazire and Kunisawa (8) and Giesbrecht and Drews (14), have led to the conclusion that chromatophores are formed by invagination of the cytoplasmic membrane.

Chromatophores were first isolated from alumina ground preparations of *R. rubrum* (2) and were described as distinct vesicular entities which numbered several thousand per bacterial cell. This concept was questioned, however, by Karunairatnam, Spizizen, and Gest (12), and Tuttle and Gest (6). These workers observed that the pigment system of lysed protoplasts of *R. rubrum* sedimented at low centrifugal force, suggesting that the photochemical apparatus of the cells was associated with the cytoplasmic membrane or membranous extensions in the cytoplasm.

The granules, which are most prevalent in

older cultures grown under certain conditions and with specific substrates, have been isolated and have been shown to contain poly-β-hydroxybutyrate (4). By light microscopy, the granules are most clearly seen on staining with Sudan black B (1, 3). Cytological observations as to whether these granules in both *R. rubrum* and other bacteria are bounded by a membrane have proved inconclusive.

The present paper deals with the origin of chromatophores in *R. rubrum* and the question of whether the photosynthetic apparatus consists of a membranous continuum or a system of separate, discrete bodies. In addition, observations are present on the effect of lysozyme and Versene on the cell wall of *R. rubrum*, and on the conditions necessary to resolve the limiting membrane which surrounds the cellular granules of poly-β-hydroxybutyrate.

MATERIALS AND METHODS

Spheroplasts were prepared from cells grown under the following conditions: (*a*) anaerobically in the presence of low light intensity—conditions where chromatophores are abundant; (*b*) aerobically in the dark with maximum aeration—conditions where chromatophores are not detectable; (*c*) subaerobically in the dark with minimum aeration—conditions where a photosynthetic pigment system is acquired by the cells and the development of chromatophores initiated.

1. *Culture*

The culture of *Rhodospirillum rubrum* was supplied by Dr. R. S. Wolfe. The medium used was identical to that used previously (10), with the exception that in the subaerobic growth experiments 0.2 per cent sodium succinate was substituted for ethanol, and sodium sulfide and sodium bicarbonate were omitted. For anaerobic growth in the presence of light, the apparatus and culture volumes employed were also identical to those used previously.

For aerobic growth, screw-capped bottles with a volume of 65 ml were filled with 10 ml of medium and shaken at 84 OPM[1] at 30°C for 5 days in the dark. These cultures were subcultured serially at 5-day intervals until no bacteriochlorophyll was observable by spectrophotometric examination of cell suspensions in 32 per cent bovine serum albumen (Barer, 11).

For subaerobic growth, 65-ml screw-capped bottles containing 50 ml of medium were inoculated with the serially cultured, aerobic-grown cells and shaken at 84 OPM[1] at 30°C in the dark. The time at which bacteriochlorophyll synthesis began was determined by hourly spectrophotometric examinations of aliquots of the cultures.

2. *Preparation of Spheroplasts*

Cells were converted into spheroplasts by using the lysozyme-Versene technique of Karunairatnam, Spizizen, and Gest (12). The stability of the spheroplasts was improved prior to fixation by suspending them in 40 per cent sucrose w/v and storing at +4°C for 3 days. About 70 per cent of the cells were converted to spheroplasts.

3. *Fixation and Embedding*

After storage at +4°C, spheroplast preparations were centrifuged at 2,500 RPM for 30 minutes at +2°C. The cells were resuspended in 30 per cent sucrose w/v in veronal-acetate buffer pH 6.1 (13) containing 1 per cent osmium tetroxide. Fixation was carried out at room temperature for 2 hours in the dark. Centrifugation was repeated and the cells were resuspended in 0.5 per cent uranyl acetate in veronal-acetate buffer and allowed to stand for 2 hours at room temperature, after which they were again centrifuged. The deposits were then placed between layers of agar, dehydrated, embedded, and sectioned as described previously (10).

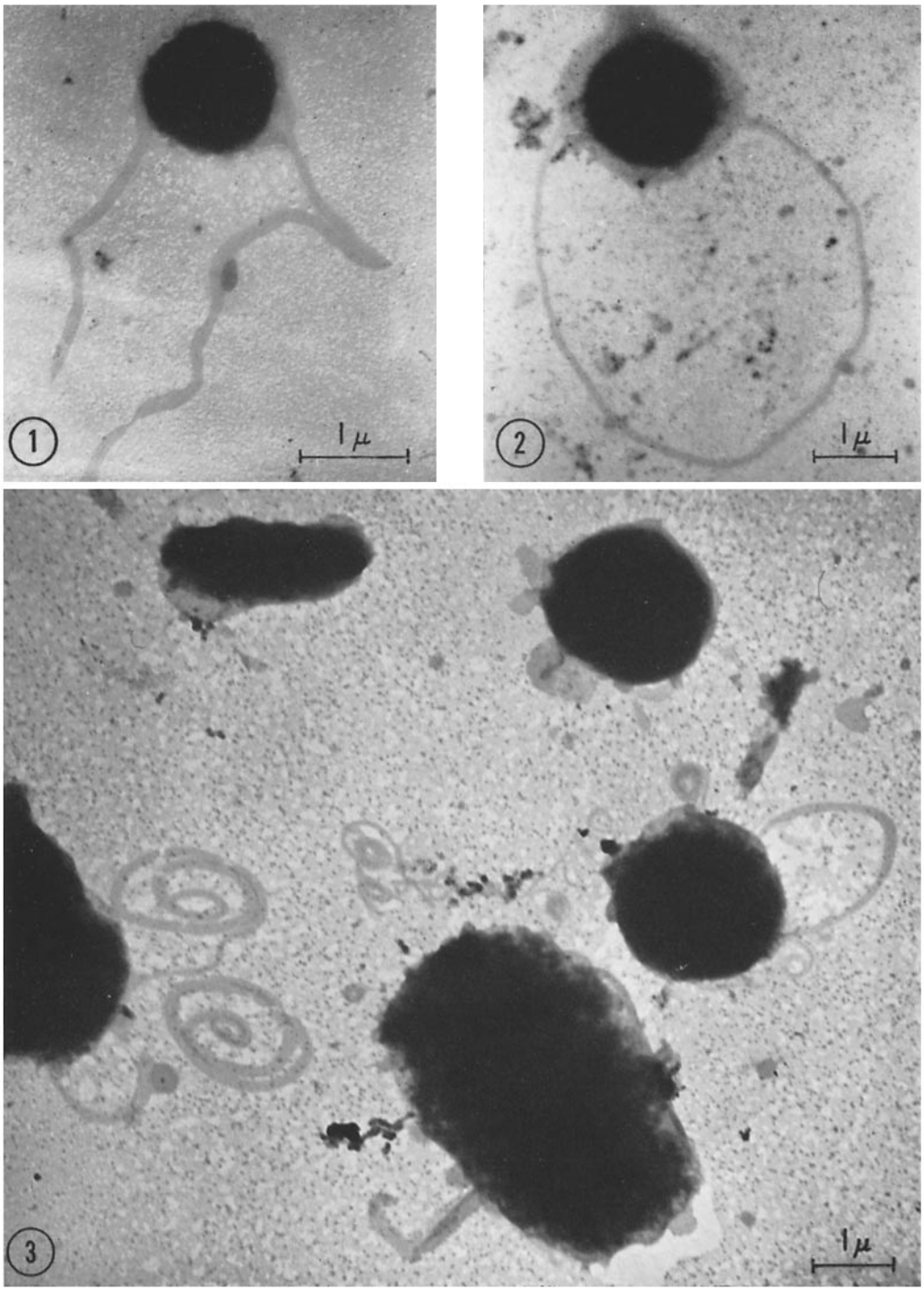
Specimens were examined with an RCA EMU-2C and photographed with Kodak fine-grain positive film.

[1] Oscillations per minute.

FIGURE 1 Electron micrograph of an osmium tetroxide-fixed, unsectioned spheroplast from cells grown anaerobically in the light. Shadow-cast with palladium. Note strips of presumably cell wall. Strips are approximately 100 mμ wide. × 15,500.

FIGURE 2 A fixed, unsectioned spheroplast from cells grown anaerobically in the light. Shadow-cast with palladium. Note greatly extended cell wall devoid of obvious free ends. × 12,000.

FIGURE 3 Fixed, unsectioned spheroplasts from cells grown anaerobically in the light. Shadow-cast with palladium. Numerous coils of cell wall material surround or extend from the cells. × 11,500.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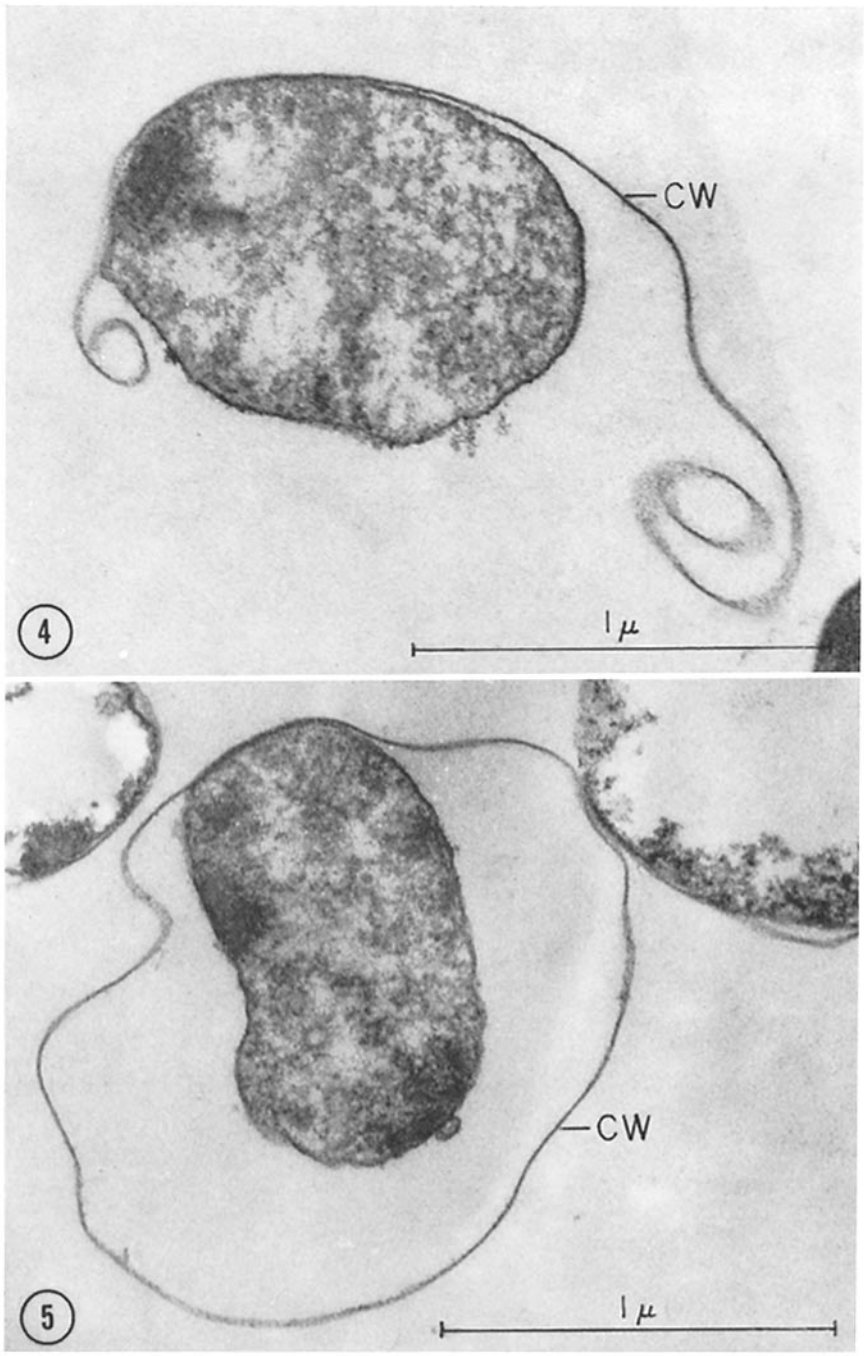


FIGURE 4 Section of a spheroplast prepared from cells grown aerobically in the dark. In this plane of section the cell wall (*CW*) appears almost completely retracted from the spheroplast. Note coiling at the free ends of the wall. × 55,000.

FIGURE 5 Section of a spheroplast prepared from cells grown aerobically in the dark. Cell wall (*CW*) is greatly expanded but appears continuous. × 52,000.

OBSERVATIONS

1. *Effect of the Lysozyme-Versene Treatment on the Cell Wall of R. rubrum*

Figs. 1 to 3 are electron micrographs of fixed, unsectioned spheroplasts of light-grown cells which were shadow-cast with palladium at an angle of 15°. It is evident from the coils of material surrounding the cells that the cell walls have been partially or completely removed in strips. The strips in Figs. 1 to 3 are about 100 mμ wide and in Fig. 1 measure about 10 μ in length. Often a strip of cell wall appeared greatly extended without any free ends being visible (Fig. 2). If such a cell were sectioned in the appropriate plane an appearance similar to that of the cell in Fig. 5 would be obtained.

Thin sections of spheroplasts of dark-grown aerobic cells showing various configurations of cell wall structure are presented in Figs. 4 to 6. Since unsectioned cells show similar configurations, it is probable that what is seen represents a section through relatively narrow strips of cell wall. Fig. 4 shows a cell in which the cell wall is almost completely retracted from the spheroplast. The structure shown at *I* in Fig. 6 is thought to be an isolated piece of cell wall.

2. *Sections of Cells Grown Anaerobically in the Light*

Fig. 7 represents a longitudinal section of a light-grown cell and illustrates the difficulties encountered with intact cells in determining the relationship of the chromatophores to the cytoplasmic membrane. The seven large spherical bodies in this cell appear to be empty and probably represent the storage sites of polymerized β-hydroxybutyrate which was extracted during processing. The interesting feature of these bodies is that some of them appear to be bounded by a membrane, as shown by the sharply defined black line, while in others the boundary appears diffuse or lacking entirely. As will be demonstrated later, these bodies are probably all membrane-bounded, but the resolution of the membrane depends on the level of sectioning.

The relationship of the chromatophores to the cytoplasmic membrane is much more readily visualized in sections of broken spheroplasts. Fig. 8 presents such a section, and in at least three places (double arrows) the chromatophores appear to be continuous with the cytoplasmic membrane. A unit membrane about 85 A wide can be detected surrounding the chromatophores.

3. *Sections of Cells Grown Subaerobically in the Dark*

Figs. 9 and 10 show sectioned spheroplasts prepared from a culture grown subaerobically for 4 hours, at which time the absorption spectrum of the cells indicated a small content of bacteriochlorophyll. Only a few chromatophores were present in these cells as compared with those grown in the presence of light (Figs. 7 and 8), and they were situated mainly in close relationship to the cytoplasmic membrane.

4. *Serial Sections of Spheroplasts of Cells Grown Anaerobically in the Light and Containing Cytoplasmic Granules*

Figs. 11 to 13 represent three serial sections of a single spheroplast, whereas Fig. 14 *a* to *e* represents five serial sections. The cells contained numerous chromatophores and several empty granules; in certain areas the cytoplasmic membrane was well defined, and in Fig. 14 *b*, *d*, and *e* the chromatophores were seen to be continuous with this membrane. Perusal of the serial sections indicates that the membrane surrounding the spherical granules changes in appearance, depending upon the level of cut, from a single membrane to a widely spaced double membrane to a diffuse membrane. The double and diffuse appearances always appear above and below the center (indicated by a dense single membrane) of the spherical granules. This is well shown in the series in Figs. 11 to 13, Granules *1* to *3*, and in the series in Fig. 14, Granules *1* to *4*, and *5*.

Granules ranged from 2,200 A to 4,400 A in diameter when measured at the single membrane level. A comparison between the cytoplasmic unit membrane of *R. rubrum* and the single membrane of the granule is shown in Fig. 15. The granule membrane is about 40 A wide, which is approximately half the width of the cytoplasmic membrane.

DISCUSSION

Lysozyme and Versene have been used for many years to convert bacteria cells into protoplasts, and their effects on certain gram-positive bacteria, particularly *Bacillus megaterium*, have been well documented. In *B. megaterium*, lysozyme normally

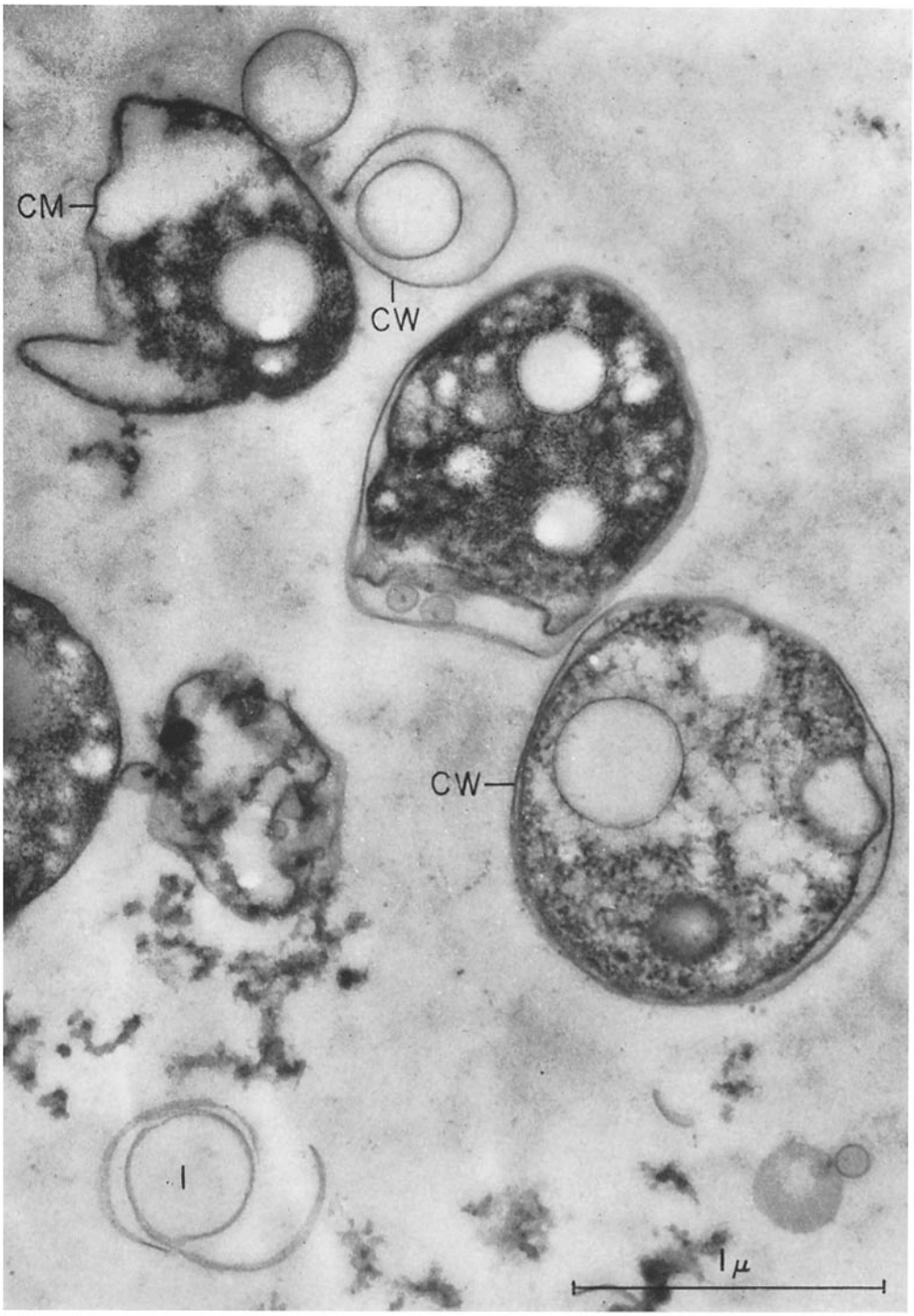


FIGURE 6 Section of spheroplasts of cells grown aerobically in the dark. Cell wall (*CW*) and cytoplasmic membrane (*CM*) are indicated. Note coiling of cell wall. Structure *I* is interpreted as being an isolated piece of cell wall. × 45,000.

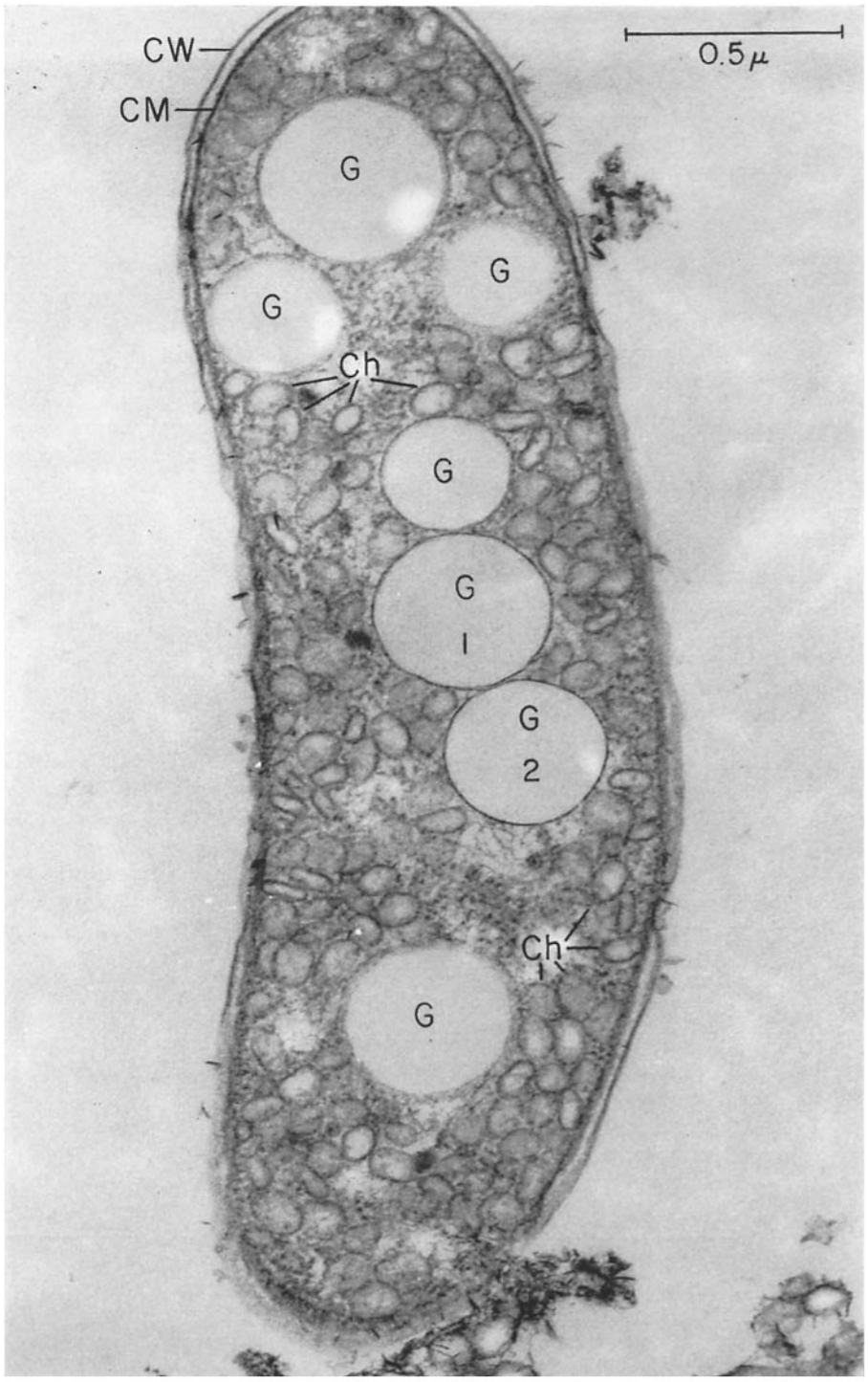


FIGURE 7 Longitudinal section of an intact cell grown anaerobically in the light. Cell wall (*CW*), cytoplasmic membrane (*CM*), chromatophores (*Ch*), and seven empty granules (*G*) are shown. Granules *1* (400 mμ diameter) and *2* (330 mμ diameter) possess a distinct limiting membrane. The periphery of the remaining granules is diffuse. × 61,000.

completely solubilizes the cell wall (Weibull, 15), and no residual wall structure can be detected under the electron microscope (Salton and Williams, 16). The action of lysozyme and Versene on a few gram-negative species has also been observed. For example, Lee (17) prepared spheroplasts from *Escherichia coli* and found them surrounded by the remains of the cell wall in different stages of disintegration, somewhat resembling the spheroplast in Fig. 5 of this study. Observations on unsectioned spheroplasts were not made, so that it was not possible to visualize the actual three-dimensional appearance of the detached or partially detached cell wall. This also applies to the work of Hofschneider (18) who also used *E. coli* and obtained similar results. From our study, it was not possible to say whether the cell wall was ever removed completely from the spheroplast. However, it does appear that, with *R. rubrum* at least, the cell wall may be removed under the action of lysozyme and Versene by a stripping process. Mitchell and Moyle (19) found that autolysis of *Staphylococcus aureus* at pH 5.8 resulted in the selective degradation of a band or ribbon of cell wall material from the cells, leaving two hemispherical shells remaining. Mechanical disintegration of the bacteria, on the other hand, produced typical punctured bags.

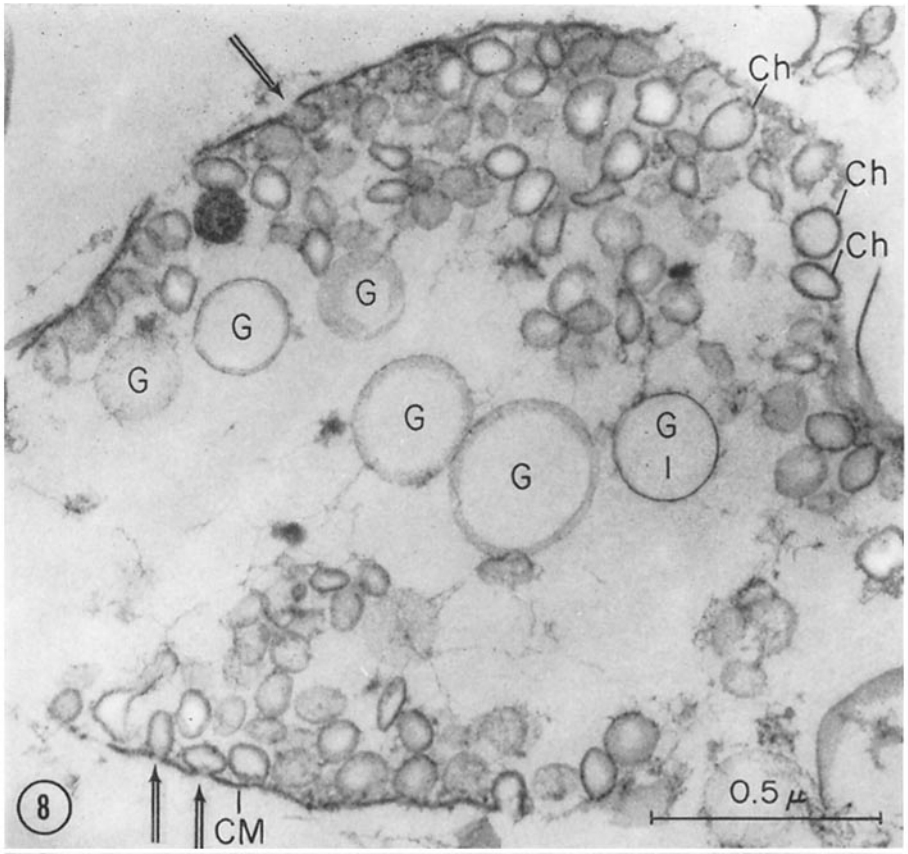
In the present work, preparations of cell walls from cultures of *R. rubrum* grown anaerobically in the presence of light and subjected to mechanical disintegration with glass beads yielded on purification mainly typical bag-like structures with no evidence of ribbons. In some instances, structures of the dimensions of chromatophores were seen trapped within the cell wall envelope.

Conversion of cells to spheroplasts allowed visualization of direct connections between the chromatophores and the cytoplasmic membrane of the cell. From our observations, the evidence would imply that chromatophores are formed initially at the cytoplasmic membrane of the cell. No definite connections between the chromatophores themselves were observed, although occasionally suggestive evidence was obtained (Fig. 14 *d*, dotted area). The fact that two sections of 500 A each would completely traverse a chromatophore might make it difficult to visualize the continuity of the presumably small connections that might exist, and extremely thin sections may be necessary, if contrast permits to unequivocally demonstrate such structures. It is difficult even to trace a single chromatophore through any two serial sections. Although the spheroplasts were fixed in buffered sucrose-osmium tetroxide and were not subjected to osmotic changes until after fixation, the trauma necessary to lyse those cells observed to have broken membranes may have been sufficient to break the chromatophore connections also. Because of the uniformity in size of the apparently unconnected chromatophores seen in sections of broken spheroplasts (a uniformity not observed in preparations of other membranous structures subjected to breakage), it would appear that the chromatophore interconnections, if they exist, must behave as weak links in the membranous continuum of the chromatophore system. The aspect of continuity between chromatophores is important in the light of the findings of Karunairatnam *et al.* (12), Tuttle and Gest (6), and Cohen-Bazire (8), who suggest that because virtually all the pigmented material in "lysates" of *R. rubrum* spheroplasts is readily sedimented at low centrifugal force the chromatophores are, therefore, associated with the membrane (cytoplasmic membrane) of the cell, Lascelles (20) also refers to this point and suggests that "these observations might, however, be attributed to trapping of the particles in the ruptured membranes."

Although the large cytoplasmic granules have been shown to be storage sites for reserve organic materials, evidence for the existence of a limiting

FIGURE 8 Section of a broken spheroplast from cells grown anaerobically in the light. The unit membrane of the chromatophores (*Ch*) is well defined. In three places the chromatophores appear continuous (double arrows) with the cytoplasmic membrane (*CM*). Six granules (*G*) are shown; only Granule *1* has a distinct membrane. × 61,000.

FIGURE 9 Section of a spheroplast from cells grown for 4 hours subaerobically in the dark. Some of the chromatophores (*Ch*) are situated at the cytoplasmic membrane (*CM*) of the cell. × 67,000.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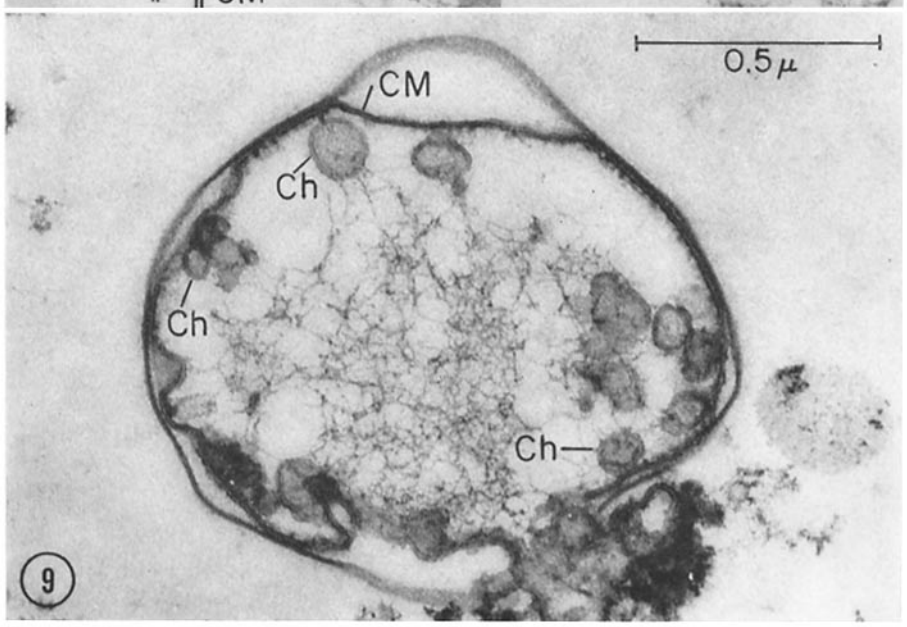
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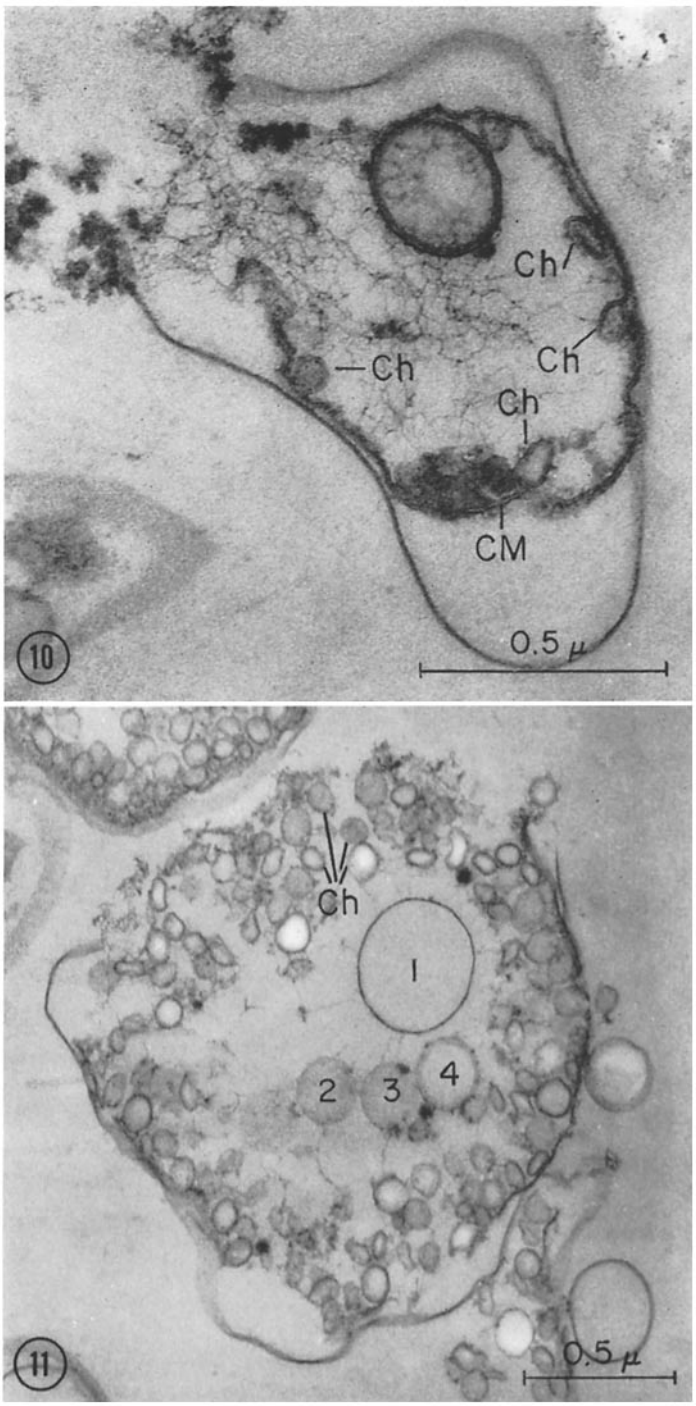


FIGURE 10 Section of a spheroplast from cells grown for 4 hours subaerobically in the dark. Chromatophores (*Ch*) are seen situated at the cytoplasmic membrane (*CM*) of the cell. × 67,000.

FIGURE 11 Section 1 of a spheroplast from light-grown cells containing chromatophores (*Ch*) and four granules. Granule *1* has a distinct membrane. × 41,000.

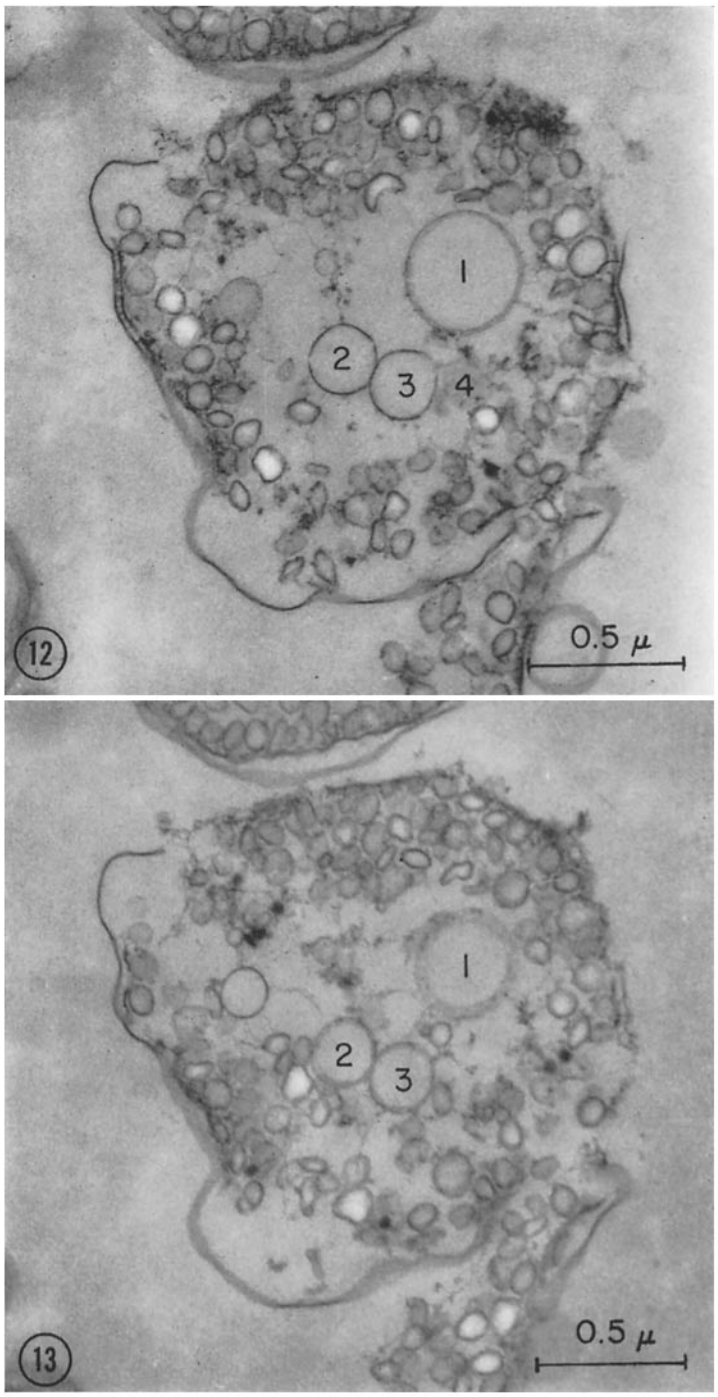


FIGURE 12 Section 2 of cell shown in Fig. 11. Note the membrane of Granule *1* is less distinct. Granules *2* and *3* now have distinct membranes; Granule *4* has almost disappeared. × 41,000.

FIGURE 13 Section 3 of cell shown in Fig. 11. The remaining Granules *1*, *2*, and *3* show diffuse boundaries. × 41,000.

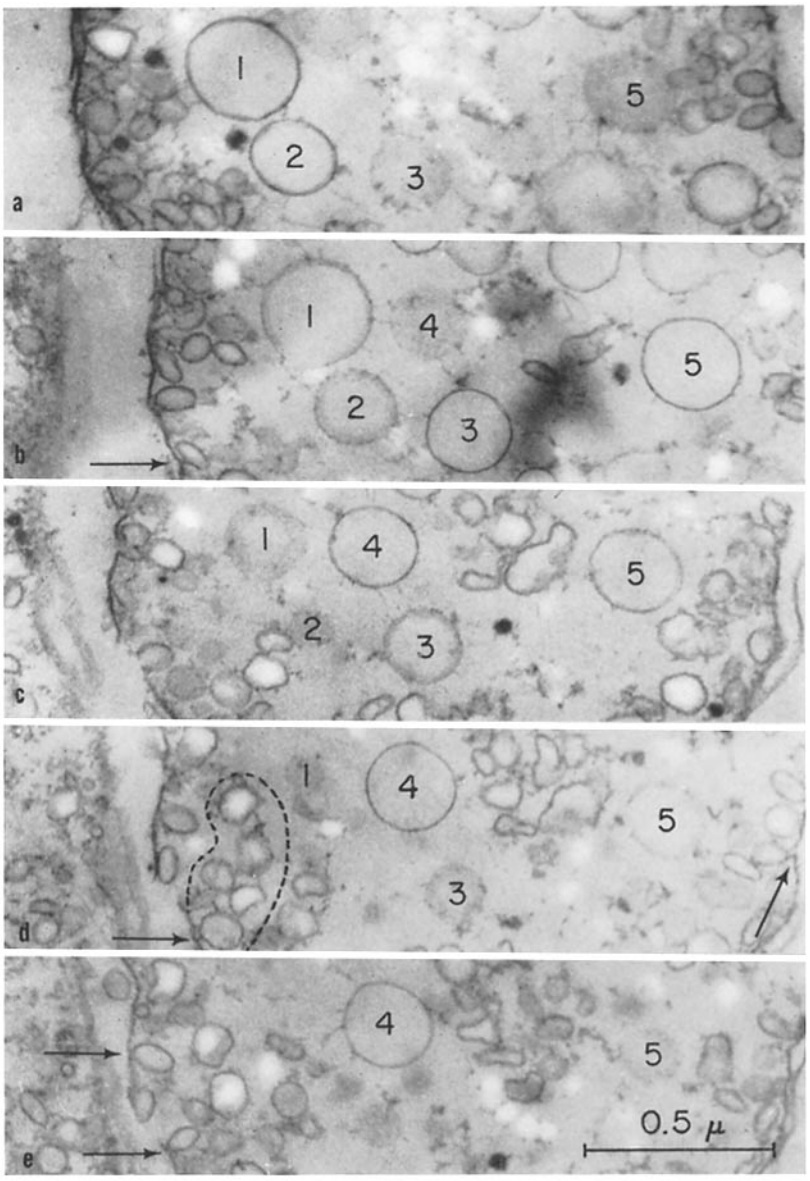
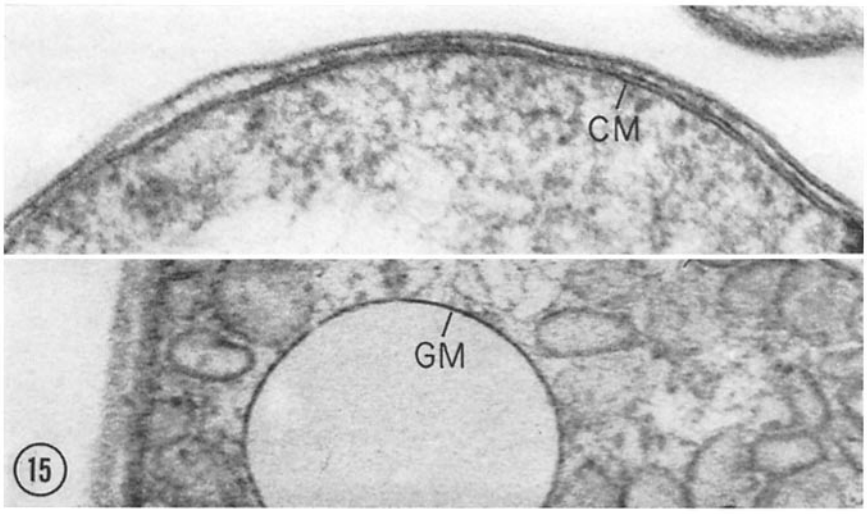


FIGURE 14 Serial sections of a portion of a spheroplast from light-grown cells. Granules numbered *1* to *5* can be followed individually through sections *a* to *e*. Note change in the appearance of the granule membrane as the level of the section is changed. Granules *3* and *5* show this particularly well. Arrows indicate obvious attachment of chromatophores to the cytoplasmic membrane. Dotted area suggestive of chromatophore interconnections. × 50,000.

membrane surrounding the granules is controversial. Murray (21) mentions that in a species of *Bacillus cereus* there was no evidence of a membranous border to the lipid granules, and Cohen-Bazire and Kunisawa (8) also concluded that poly-β-hydroxybutyrate granules of *R. rubrum* lack a limiting membrane. Knaysi (22), however, refers to lipid granules that appear to be surrounded by membranes of the dimensions of the cytoplasmic membrane. Such differences in the appearance of these granules can be explained on the basis of serial sectioning and the depth of



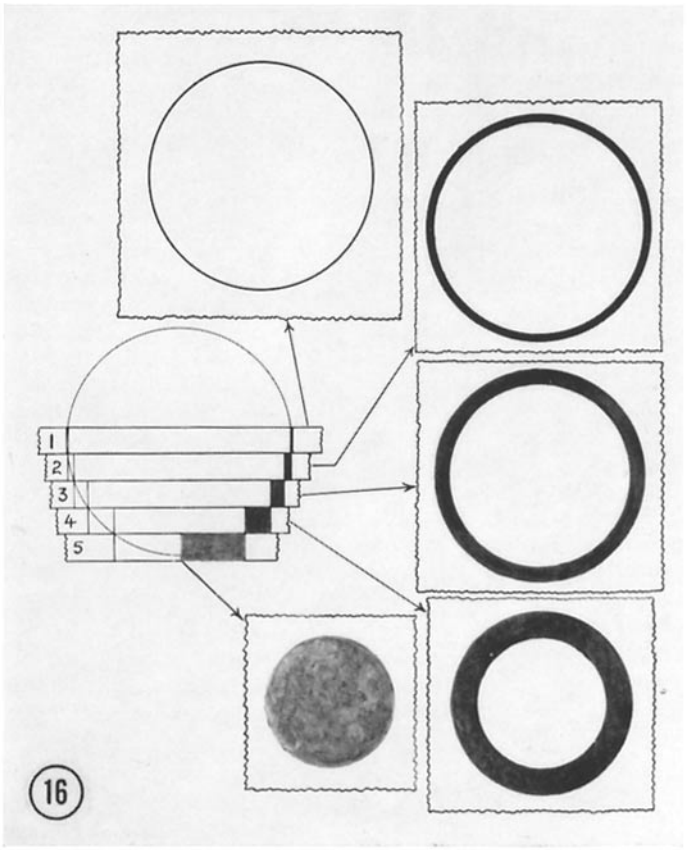


FIGURE 15 A comparison between the cytoplasmic unit membrane (*CM*) 85 A wide and the single granule membrane (*GM*) 40 A wide. × 121,000.

FIGURE 16 Diagram indicating the change in appearance of a granule membrane at five successive levels, starting from the center of the granule. Only at level *1* is the membrane sharp and of single membrane dimensions. At other levels the contrast is reduced and the membrane is increased in width due to the increasing slope of the curve of the sphere.

field of the electron microscope. Due in part to the thickness of the section, different planes above or below the center of a membrane-bounded sphere will include portions of a curve with an increasing slope giving rise consequently to a gradually increased blurring of the membrane which has distinctness only in the center plane of the sphere. Fig. 16 illustrates this point diagramatically, and it will be appreciated that the greatest electron opacity of the single granule membrane will occur at the center of the sphere where a length of membrane equal to the thickness of the section (600 A) is aligned parallel to the electron beam. In sections above or below the center, the density of the membrane amounts to the thickness of the membrane, *i.e.*, 85 A: this gives rise to a rapid drop in electron opacity and increased blurring as sections are removed farther from the center of the spherical granule. The rapidity of the decrease in electron opacity will be much more marked with spheres smaller than the one in the diagram. This effect is also observed in the significantly smaller chromatophores (Figs. 8, 11, and 12).

As a matter of interest, the different appearances of the membrane resulting from the sectioning of spherical granules have also been observed in this laboratory in sections of animal tissue containing numerous large vesicles.

It is to be expected that serial sectioning of other species of bacteria known to possess storage granules will clarify the problem concerning whether all such granules are membrane bounded. The problem of chromatophore connections is inherently more difficult because of the sectioning problems discussed, and the evidence for such interconnections may remain indirect for some time.

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BIBLIOGRAPHY

1. VATTER, A. E., and WOLFE, R. S., The structure of photosynthetic bacteria, *J. Bact.*, 1958, **75**, 480.
2. SCHACHMAN, H. K., PARDEE, A. B , and STANIER, R. Y., Studies on the macromolecular organization of microbial cells, *Arch. Biochem. and Biophys.*, 1952, **38**, 245.
3. HICKMAN, D. D., and FRENKEL, A. W., The structure of *Rhodospirillum rubrum*, *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 277.
4. STANIER, R. Y., DOUDOROFF, M., KUNISAWA, R., and CONTOPOULOU, R., The role of organic substrates in bacterial photosynthesis, *Proc. Nat. Acad. Sc.*, 1959, **45**, 1246.
5. FLEXER, A. S., SISTROM, W. R., and CHAPMAN, G. B., Chromatophore formation in *Rhodospirillum rubrum*, *Bacteriol. Proc.*, 1960.
6. TUTTLE, A. L., and GEST, H., Subcellular particulate systems and the photochemical apparatus of *Rhodospirillum rubrum*, *Proc. Nat. Acad. Sc.*, 1959, **45**, 1261.
7. NEWTON, J. W., Macromolecular variation in the chromatophores of the photosynthetic bacterium *Rhodospirillum rubrum*, *Biochim. et Biophysica Acta*, 1960, **42**, 34.
8. COHEN-BAZIRE, G., and KUNISAWA, R., The fine-structure of *Rhodospirillum rubrum*, *J. Cell Biol.*, 1963, **16**, 401.
9. BOATMAN, E. S., and DOUGLAS, H. C., Protoplast morphology and chromatophore formation in *Rhodospirillum rubrum*, Proceedings Fifth International Congress for Electron Microscopy, Philadelphia, 1962, New York, Academic Press, Inc.
10. BOATMAN, E. S., and DOUGLAS, H. C., Fine structure of the photosynthetic bacterium *Rhodomicrobium vannielii*, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 469.
11. BARER, R., Spectrophotometry of clarified cell suspensions, *Science*, 1955, **121**, 709.
12. KARUNAIRATNAM, M. C., SPIZIZEN, J., and GEST, H., Preparation and properties of protoplasts of *Rhodospirillum rubrum*, *Biochim. et Biophysica Acta*, 1958, **29**, 649.
13. KELLENBERGER, E., RYTER, A., and SÉCHAUD, J., Electron microscope study of DNA-containing plasms. II, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 671.
14. GIESBRECHT, P., and DREWS, G., Elektronenmikroskopische Untersuchungen über die Entwicklung der Chromatophoren von *Rhodospirillum molischianum* Giesberger, *Arch. Mikr.*, 1962, **43**, 152.
15. WEIBULL, C., The isolation of protoplasts from *B. megaterium* by controlled lysis with lysozyme, *J. Bact.*, 1953, **66**, 688.
16. SALTON, M. R. J., and WILLIAMS, R. C., Elec-

tron microscopy of the cell walls of *Bacillus megaterium* and *Rhodospirillum rubrum*, *Biochim. et Biophysica Acta*, 1954, **14**, 455.

17. LEE, S., Fixation of *E. coli* spheroplasts for electron microscopy, *Exp. Cell Research*, 1960, **21**, 252.
18. HOFSCHNEIDER, P. H., Zur Wandstruktur von *Escherichia coli B.* Sphaeroplasten, European Regional Conference Electron Microscopy, Delft, 1960, De Nederlandse Vereniging voor Electronenmicroscopie, **2**, 1028.
19. MITCHELL, P., and MOYLE, J., Autolytic release and osmotic properties of "protoplasts" from *Staphyloccus aureus*, *J. Gen. Microbiol.*, 1957, **16**, 184.
20. LASCELLES, J., The chromatophores of photosynthetic bacteria, *J. Gen. Microbiol.*, 1962, **29**, 47.
21. MURRAY, R. G. E., The Bacteria, New York, Academic Press, Inc., 1960, 48–50.
22. KNAYSI, G., Elements of Bacterial Cytology, 2nd edition, Ithaca, New York, Comstock, Publishing Co., Cornell University Press, 1951, 193.