Photosynthetic Apparatus in the Green Bacterium
Chloropseudomonas ethylicum

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ABSTRACT

HOLT, STANLEY C. (Dartmouth Medical School, Hanover, N. H.), S. F. CONTI, AND R. C. FULLER. Photosynthetic apparatus in the green bacterium Chloropseudomonas ethylicum. J. Bacteriol. 91:311–323. 1966.—When cells of Chloropseudomonas ethylicum were broken by ballistic disruption and examined by electron microscopy, vesicles 1,300 to 1,500 A long and 300 to 500 A wide were found to rim the periphery of the cell. Examination of these vesicles obtained by disruption with a French pressure cell and purified by density gradient centrifugation revealed interconnections between the vesicles. During sonic and Mickle disruption of the cells, chlorophyll was released at a lower rate than soluble cytoplasmic components, but faster than the membrane-bound enzyme succinic dehydrogenase. Unlike the situation that exists in the purple photosynthetic bacteria, it appears that the chlorophyll in the green bacteria is contained as part of a structure which may be differentiated both structurally and functionally from the bacterial cytoplasmic membrane.

Schachman, Pardee, and Stanier (20) demonstrated that the photosynthetic pigments of Rhodospirillum rubrum were sedimentable in the ultracentrifuge. This fraction, which contained particles 600 to 1,000 A in diameter, had a sedimentation constant of 190S and contained the entire pigment complement of the cell.

Vatter and Wolfe (24), studying the ultrastructure of R. rubrum, Rhodopseudomonas spheroides, and Chromatium in thin sections, found the cytoplasm to contain circular profiles approximately 500 to 1,000 A in diameter. It was concluded that the circular profiles observed in sections were similar to the isolated chromatophores of Schachman and co-workers (20). Bergeron (1) also observed that the cytoplasm of Chromatium grown at low light intensity contains annular images approximately 300 A in diameter. These structures were assumed to be similar to the chromatophores, as observed by Vatter and Wolfe (24).

Marr (15), Tuttle and Gest (23), Stanier (22), and Holt and Marr (10), by use of the techniques of osmotic shock and sonic and ballistic disruption, presented evidence that the chromatophores of R. rubrum, as isolated by Schachman and co-workers (20), are merely the result of mechanical disruption of an extensive intracytoplasmic membrane. The presence of a highly differentiated photochemical membrane system has also been observed by Fuller, Conti, and Mellin (7) in Chromatium, strain D. At low light intensity, the cytoplasm of Chromatium is packed with the classical circular profiles similar in appearance to the chromatophore observed in thin sections of the nonsulfur purple bacteria grown under low-intensity light.

Holt and Marr (10) isolated and purified the membrane fraction from R. rubrum by the technique of gradient centrifugation and electrophoresis. They showed that there is a relationship between membrane content and chlorophyll synthesis as the light intensity is varied, which indicates a constant composition of photochemical structure.

The green sulfur bacteria, represented by two genera, Chlorobium and Chloropseudomonas, were examined by Bergeron and Fuller (2) and Cohen-Bazire, Pfennig, and Kunisawa (4). Cohen-Bazire and associates (4) suggested that these green bacteria contain a photosynthetic structure quite different from that reported for other photosynthetic forms. Bergeron and Fuller (2) isolated a functional particle from Chlorobium which is approximately 150 A in diameter, and which contained all of the pigment of the cell. These particles were able to catalyze the light-induced
formation of adenosine triphosphate from adeno-
sine diphosphate and inorganic phosphate. It is
probable that this 150-A particle is either a frag-
mentation product of a more extensive structure
or a macromolecular component associated with
a larger structure. In *Chlorobium*, the photochem-
ical pigment system is thought to be housed in an
ellipsoidal structure, 1,000 to 1,500 A long and
300 to 500 A wide, rimming the periphery of the
cell. These structures, which were isolated by
Cohen-Bazire and co-workers (4), have been
named "Chlorobium vesicles."

This paper presents evidence of the location of the
photosynthetic pigments in the green bacteri-
un, *Chloropsis pseudonas ethylicum*, in a vesicu-
lar structure similar to that reported by Cohen-
Bazire et al. (4) in *Chlorobium*. The kinetics of
release of cellular components during sonic and
Mickle disruption was used to investigate the
location of the photosynthetic vesicles. The phis-
ical separation of the vesicular structure contain-
ing the photosynthetic pigments of *C. ethyl-
icum* from cells grown at different light intensities is
described.

**Materials and Methods**

*C. ethylicum* (strain 2-K) was cultured in the liquid
medium of Konrat'eva (12) with 0.2% Na2S-9H2O,
4% NaHCO3, and 3 ml of a 70% sterile ethyl alcohol
solution per liter. The pH was adjusted to 7.2 to 7.4
with sterile HCl after autoclaving.

Cultures were grown phototrophically in screw-cap
bottles (4-oz) in a water bath at 30 C. Illumination
was provided by 375-w General Electric photoflood
lights adjusted to give uniform light intensity at
the entrance window of the culture vessel. The voltage was
stabilized with a constant voltage transformer. The
light intensity was varied by adjustment of the distance
from the lamp to the culture vessel or by interposing
wire screen rather than by varying the voltage. Light
incident on the culture vessel was measured with a
Gossen Tri-Lux model C light meter calibrated in foot
candles.

All cultures were determined to be growing expon-
entially at the time of harvest. As evidence of the
presence of a steady state, the differential rate of
chlorophyll synthesis was determined to be constant
prior to harvest.

The cells were harvested by centrifugation at 4 C
and washed by three successive centrifugations at
3,000 × g for 20 min from cold 0.02 M phosphate
buffer (pH 7.0) containing 0.001 M MgSO4.
Chlorophyll was extracted in absolute methanol and
quantitatively determined by use of the absorption
coefficients reported by Stanier and Smith (21).

Turbidity was estimated by measuring the optical
density in a 1-cm absorption cell with a Zeiss PMQ
spectrophotometer at 600 μm, at which wavelength
there is minimal absorption by the photosynthetic
pigments.

Protein was determined by the Folin-Ciocalteau
method (13) either after alkaline digestion of intact
cells at 40 C for 2 hr in 2 M NaOH or directly on
samples of isolated membranes.

**Electron microscopy.** Samples were fixed, dehy-
drated, and embedded, as described by Ryter and
Kellenberger (19). The agar blocks were dehydrated
in a graded aqueous ethyl alcohol series according to
the following schedule: 20, 50, 75, 95% ethyl alcohol
10 min each, and two successive dehydrations in
absolute ethyl alcohol for 15 min. Alcohol was re-
placed with propylene oxide, and the samples were
embedded in the epoxy mixture, 812 according to
the procedure of Luft (14). Sections were cut on an
LKB ultratome with a diamond knife, mounted on
Formvar-coated 150- or 200-mesh copper grids, and
stained with lead hydroxide, according to the pro-
cedure of Millonig (17).

Samples were negatively stained with 2% phospho-
tungstic acid and adjusted to pH 7.0 according to the
procedure of Huxley and Zubay (11).

All samples for electron microscopy were examined
in a Phillips EM 200 electron microscope operating
at 60 kv.

**Sonic treatment.** A pellet of washed cells, harvested
from a culture grown at 10 ft-c was suspended in cold
0.02 M phosphate (pH 7.0) buffer containing 0.001 M
MgSO4 to give a concentration of 5 mg (dry weight)
per ml. A 20-ml amount was transferred to a frozen
metal beaker, and the cells were broken with a Branson
Sonifier operating at a setting of 5 with a maximal
output of 5.5 d-c amps. The temperature was main-
tained below 10 C during treatment. At intervals,
1.0-ml samples were removed and diluted with cold
phosphate buffer. After each sample was taken, cold
phosphate buffer was added to the cup, and an approp-
riate correction was made for the resulting progres-
sive dilution. A portion of each sample was cen-
trifuged at 3,000 × g for 30 min. The absorbancy at
260 μm, the release of succinic dehydrogenase, and
the chlorophyll content of the supernatant fluid were
measured. From the sample volume, the amount of
chlorophyll was calculated and the decrease in optical
density at 600 μm.

**Ballistic disintegration.** Exponentially growing cells of
*C. ethylicum* were suspended in 0.02 M phosphate
buffer (pH 7.0) containing 0.001 M MgSO4 to a
concentration of 5 mg (dry weight) per ml. A 5-ml amount
of this suspension, together with 3 ml of glass beads
(Ballotini, no. 10), was added to the sample vessel and
while running the ultrasonic apparatus. The cups were shaken with a 9-mm peak-
to-peak displacement and were cooled to 4 C every
30 sec, which maintained the temperature below 20 C
during the entire treatment. At intervals, samples were
removed; turbidity, sedimentable chlorophyll,
260-μm absorbing material, and the release of succinic
dehydrogenase were determined as for sonic treatment.

**Succinic dehydrogenase.** Succinic dehydrogenase
was measured in both the supernatant fluid and pellet
after sonic and Mickle treatment. The activity of the
enzyme was measured by the reduction of cytochrome
C in the presence of sodium succinate by use of the
procedure of Massey (16).

**Isolation and purification of vesicle fraction.** Washed
cells of *C. ethylicum* were suspended in 0.02 M phos-
phate buffer (pH 7.0) containing 0.001 M MgSO4 to a
concentration of 5 mg (dry weight) of cells per ml. The cells were broken in a French pressure cell at 20,000 psi. The extract was treated with 0.5 μg of deoxyribonuclease per ml for 30 min and was centrifuged at 18,000 × g for 30 min to remove whole cells and debris. The green-pigmented supernatant fluid was then centrifuged at 104,000 × g for 1 hr, and the supernatant liquid was discarded. The pellet was suspended in cold 0.02 M phosphate buffer (pH 7.0) containing 0.001 M MgSO₄.

Preparation of sucrose gradients. A linear sucrose gradient was prepared as described by Holt and Marr (10). The sample was layered on the surface of the gradient as a 1- to 2-mm band, and the tubes were centrifuged in a swinging-bucket rotor (Spinco SW 39L) at 25,000 rev/min for 120 min.

Sucrose-gradient electrophoresis. The procedure as outlined by Holt and Marr (10) for the preparation of the sucrose-gradient electrophoretic cell was followed with the following modification: to prevent the oxidation of chlorophyll, 0.1 M L-cysteine was added to the sucrose—0.02 M tris(hydroxymethyl)aminomethane-Cl buffer in the electrophoretic cell. A potential of 250 v at 25 ma was applied to platinum-stainless steel electrodes producing a current of 25 ma through the cell.

RESULTS

To observe the relationship between the vesicular component and the cytoplasmic membrane in C. ethylicum, cells were subjected to ballistic disintegration and examined by electron microscopy. Figure 1 is a thin section of a cell of C. ethylicum grown at 10 ft-c. The cell contains a normal gram-negative cell wall, peripheral membrane, and nuclear region. Rimming the interior surface of the envelope of the cell are ellipsoidal vesicles 1,000 to 1,500 A long. Figure 2 is a section of C. ethylicum grown at 10 ft-c and subjected to ballistic disintegration for 15 min. At this low light intensity, the vesicles line the interior surface of the cell, even after most of the cytoplasmic contents have been removed. The vesicles which appear to be located in the center of one cell represent a different plane of section. This micrograph suggests apical interconnections of the vesicles. Figure 3 is part of a section of C. ethylicum which has been treated for 15 min in the Mickle apparatus. The possible interconnections between the inner portion of the peripheral membrane and the single-membraned vesicle are indicated. Figure 4 is a thin section of C. ethylicum grown at 10,000 ft-c and subjected to Mickle treatment for 15 min. At this light intensity, there are very few vesicles. Those that are present lie very close to the peripheral membrane. Possible attachments of the vesicles to the membrane are indicated.

The vesicular structures of C. ethylicum were further examined by the technique of phosphotungstic acid negative staining. Figures 5 to 8 are examples of cells grown at low light intensity and opened by ballistic disintegration for 30 min. The ellipsoidal 1,300 to 1,500 A vesicles are clearly evident in these preparations. Note the difference in electron density of the vesicles as compared with the surrounding cell wall and cytoplasmic membrane.

C. ethylicum was subjected to disruption in a French pressure cell to investigate further possible interconnections between the vesicular elements as observed in thin sections. Centrifugation of the resulting extracts in a density gradient gave two pigmented bands. The upper band, which was located 4.1 cm from the origin, was removed from the upper portion of the gradient with a syringe, and the lower band, which was located 5.6 cm from the origin, was removed by draining the remaining liquid through a small hole punctured in the bottom of the centrifuge tube.

Both bands were examined by electron microscopy after phosphotungstic acid negative staining. The lower band was found to contain membrane and cell-wall fragments. The upper band (Fig. 9–11) contained ellipsoidal vesicles approximately 1,300 to 1,500 A long and 300 to 500 A in width. Possible interconnections between some of the vesicles are indicated by the arrows. The presence of a possible macromolecular fine structure is also indicated. Contaminating the field are ribosomal and membrane fragments. Since ribosomes have a much greater surface charge than membranes, as demonstrated by Robrish and Marr (18), gradient electrophoresis was used to remove most of the contaminating material. Figure 12 shows the results of density-gradient electrophoresis. There is a significant reduction in the contamination by wall and membrane fragments.

The kinetics of release of chlorophyll from the cell by means of sonic and ballistic treatment were determined to see if the vesicles as observed in thin sections of C. ethylicum are a part of an extensive structure within the cell or occur as discrete cytoplasmic components of the cell. Substances which are contained within the cell as discrete cytoplasmic components should be released at a rate equal to the disruption of the cells. A component which is part of a more extensive structure of the cell should be released at a rate lower than the disruption of the cells.

The results of sonic treatment are shown in Fig. 13. The rate of release of solutes absorbing at 260 μm is greater than the decrease in turbidity (optical density, 600 μm) or the rate of release of the membrane-bound enzyme, succinic dehydrogenase. This indicates that the soluble cytoplasmic components are released from the cell at a much
FIG. 1. Thin section of Chloropseudomonas ethylicum grown at a light of 10 ft-c. The single membrane-bound vesicles (V) rim the periphery of the cell. The cell wall (CW), peripheral membrane (PM), and nucleoplasm (N) are evident in this micrograph. Main fixation, 12 hr; poststained with lead hydroxide. × 110,000.

FIG. 2. Section of Chloropseudomonas ethylicum grown at 10 ft-c and ballistically disintegrated for 15 min. The cytoplasmic contents of the cell have been removed, leaving the 1,300 to 1,500 Å long vesicles (V) rimming the periphery. The arrow indicates a possible interconnection between two vesicles. Main fixation, 12 hr; poststained with lead hydroxide. × 132,000.
FIG. 3. Portion of a section of Chloropseudomonas ethylicum grown at 10 ft-c and ballistically treated for 15 min. The arrows indicate possible interconnections of the vesicles to the inner part of the peripheral membrane. Main fixation, 12 hr; poststained with lead hydroxide. × 400,000.

FIG. 4. Section of Chloropseudomonas ethylicum grown at 10,000 ft-c and ballistically disintegrated for 15 min. Note the sparsity of vesicle structure in this micrograph. The arrows indicate possible interconnections between the vesicle and the peripheral membrane. Main fixation, 12 hr; poststained with lead hydroxide. × 199,500.
Fig. 5 and 6. Cells of Chloropseudomonas ethylicum (10 ft-c) opened by 30 min of Mickle treatment. The vesicles still remain in the opened cells. Note the differences in electron density between the vesicles and the enclosing envelope. Negatively stained with phosphotungstic acid. X 88,400.
higher rate than the comminution of the cell and the release of succinic dehydrogenase from the intracytoplasmic membrane. The rate of loss of sedimentable chlorophyll appears to be exponential, but does not correspond to the rate of decrease in turbidity, to the release of 260 μm-absorbing material, or to the release of succinic dehydrogenase. Several interpretations of this are possible: the vesicles could be attached to the peripheral membrane by fragile interconnections, the vesicles could be interconnected, or both situations could exist.

A second method of cell disruption, ballistic disintegration in a Mickle apparatus, was also employed. In this procedure, cell comminution (decrease in turbidity at 600 μm) occurs more slowly and with less physical fragmentation of the cell envelope.

The results of ballistic disintegration are shown in Fig. 14. The rate of release of chlorophyll and
Fig. 8. High-magnification micrograph similar to Fig. 7. The single arrow indicates a possible connection of the vesicle to the internal membrane. The double arrow indicates a possible fine structure to the vesicle. Negatively stained with phosphotungstic acid. $\times$ 264,000.
succinic dehydrogenase is lower than the decrease in turbidity or the release of substances absorbing at 260 m\(\mu\). During ballistic disintegration, the attached vesicular structures may be sheared from the peripheral membrane or from each other, but the breach produced in the cell is not sufficient to allow the large (1,300 to 1,500 \(\AA\) long) vesicular elements to escape. This hypothesis was tested by examining the kinetics of release after combined ballistic-sonic experiment. \textit{C. ethylicum} was ballistically disrupted as previously described, and then subjected to a short period of sonic treatment. Figure 15 shows the results of this study. After 15 min of ballistic disintegration, approximately 4\% of the chlorophyll is released. Brief sonic treatment at the end of the 15 min of

**Fig. 9, 10, and 11.** Vesicles of Chloropseudomonas ethylicum recovered from the upper band after density-gradient centrifugation. Both a fine structure and interconnections are apparent. The arrows indicate possible vesicle interconnections. Negatively stained with phosphotungstic acid. \(\times\) 392,000. (Figure 11 is on p. 320.)
FIG. 12. Vesicles of Chloropseudomonas ethylicum purified by density-gradient electrophoresis. Note the absence of contaminating wall and membrane. The background material appears to be phosphotungstic acid. Negatively stained with phosphotungstic acid. × 264,000.
bacterial cell is part of an extensive structure which includes the bacterial cytoplasmic membrane.

Observations of Cohen-Bazire, Pfennig, and Kunisawa (4) on the green sulfur bacterium Chlorobium thiosulfatophilum indicated that the photosynthetic pigment of this organism is located in large peripheral vesicles. The earlier observations of Bergeron and Fuller (2) and Hughes, Conti, and Fuller (10a) of functional photophosphorylating pigmented macromolecules from this organism can be perhaps best explained as being macromolecular complexes derived by comminution of the described vesicles during the isolation procedure. It must be remembered that these structures from Chlorobium capable of photophosphorylation contain not only the chlorophyll but the quinones, cytochromes, etc. associated with photochemical electron transport. These functional photochemical systems might well be correlated with the apparent substructure of the vesicles described by Cohen-Bazire et al. (4).

Substances which are contained in independent structures of the cell should be released at a rate equal to the rate of disruption of that cell, pro-

**FIG. 13.** Sonic disruption of Chloropseudomonas ethylicum. The logarithms of the per cent of the initial values of turbidity, release of material absorbing at 260 mp, loss of sedimentable chlorophyll, and release of succinic dehydrogenase are shown as a function of the time of sonic treatment.

Mickle disintegration releases most of the chlorophyll remaining in the cells. This supports the hypothesis that the vesicular elements in C. ethylicum are unable to be released from the "opened" cell because of an insufficient breach in the cell wall. Whether the vesicles are attached to the peripheral membrane or interconnected has not been clearly established.

**DISCUSSION**

It is now quite clear that the photosynthetic apparatus of the purple bacteria consists of either a tubular or lamellar membranous system derived from the bacterial cytoplasmic membrane (5, 7, 8, 9). These data are not in agreement with the concept that the bacterial chromatophore exists as an independent vesicular structure within the cell. The enzymes associated with oxidative electron transport, which are localized in the bacterial cytoplasmic membrane, are present in the purified chromatophore fraction of both the nonsulfur and the sulfur purple bacteria (3; Bennett and Fuller, unpublished data). These observations indicate that the photosynthetic apparatus of the

**FIG. 14.** Disruption of Chloropseudomonas ethylicum by ballistic disintegration. Release of material absorbing at 260 mp, loss of sedimentable chlorophyll and succinic dehydrogenase, and the decrease in turbidity are shown as a function of the time of shaking in the Mickle apparatus.
bound enzyme, succinic dehydrogenase, from the chlorophyll-containing vesicle indicates a difference between the green and purple photosynthetic bacteria. A similar separation has been described by Fuller (6), who was able to separate the pigmented component probably produced by comminution of these vesicles from succinic dehydrogenase in *C. thiosulfatophilum*.

This degree of functional separation and observed structural integrity indicates that the photosynthetic vesicles of the green bacteria may be more highly differentiated than the photosynthetic membrane system of the purple bacteria. Whether this extensive structural unit exists attached to the inner portion of the peripheral membrane has not been conclusively determined.

The kinetics of release do not indicate complete independence of these structures within the cell. These results support the concept that the breach in the cell wall produced by ballistic action in the Mickle apparatus is not large enough to release the vesicular structures and may suggest an extensive chlorophyll-containing structure within the cell, since sonic treatment of these "opened" cells immediately releases all the remaining chlorophyll.

The examination of electron micrographs of both thin sections and negative-stained cells of *C. ethylicum* shows both interconnected (Fig. 2) and free vesicles. It is quite possible that ballistic treatment of the cells causes disruption of the vesicles at their apparent interconnections. In addition, the vesicle fraction isolated by comminution in the French pressure cell also suggests that the vesicles are attached to one another at the apex (Fig. 9, 10).

Further work is required to establish firmly the relationship between the cytoplasmic (peripheral) membrane and the vesicular structure as it exists in the green bacteria. In any event, the photosynthetic vesicles present in the green bacteria suggest a functional and structural differentiation not previously known in the procaryotic bacterial cell.

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**Literature Cited**


PHOTOSYNTHETIC APPARATUS IN C. ETYLYCUM


