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# Morphology and Round Body Formation in *Vibrio marinus*

R. A. FELTER, R. R. COLWELL, AND G. B. CHAPMAN
*Department of Biology, Georgetown University, Washington, D.C. 20007*

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The morphology of *Vibrio marinus* MP-1 was studied by phase and electron microscopy. The ultrastructure of the vibrio form of *V. marinus* was found to be typically gram-negative with a trilaminar plasma membrane and cell wall. The coccoid or round bodies noted in otherwise pure cultures of *V. marinus* were frequently found in early and late stationary phase of growth. The round bodies in ultrathin section were found to contain at least one, and often three or four, cell units. Three types of round bodies were observed in ultrathin section, each differing in size and behavior: "spherules," "spheres" or the "round body," and "giant cells" or "macrospheres." The round bodies appeared to be associated with, or to result from, the constrictive cell division of *V. marinus*.

Interest in the *Vibrio* group of organisms has increased in recent years, principally because of their economic and public health importance and relationship to marine enteropathogens (21). Diagnosis of *Vibrio* has been difficult, and only recently has the separation of *Aeromonas* and *Vibrio* spp. been clarified. Morphologically, the *Vibrio* spp. are interesting and have received much attention since the classical description (in 1884) of *V. cholerae* by Koch (13), who noted the curved and occasionally spiral shape of these organisms.

After Koch's publication, many outstanding bacteriologists carried out research on *V. cholerae*, reporting the frequent appearance and disappearance of coccoid and other nonvibrioid bodies in otherwise pure cultures of the vibrio (4, 9, 10). Thus, round body or coccoid forms have long been associated with the *Vibrio* and *Spirillum* spp. A life cycle was proposed for both *Vibrio* and *Spirillum* with round bodies considered a possible stage in development. During the course of fine-structure studies of the effect of salts and nutrients on the morphology of *V. marinus*, the frequent appearance in older cultures of unusual forms, in particular, the round body, attracted our attention. Thus, a study of the formation of round bodies in *V. marinus* was undertaken.

## MATERIALS AND METHODS

**Organism.** *V. marinus* MP-1, a seawater-requiring psychrophile, was isolated from a water sample (ca. 500 ml) raised from a depth of 1,200 m in the north Pacific Ocean (19). The temperature of the water at the time of sampling was 3.24 C. Isolation procedures and description of the strain have been published (3). The cells were grown, unless otherwise stated, at 15 C for 12 to 48 hr in a medium of the following composition (grams per liter): proteose peptone (Difco), 10 g; yeast extract (Difco), 3 g; NaCl, 29.25 g; and $MgCl_2$, 5.3 g. The medium was adjusted to *p*H 7.4 with 0.1 N NaOH.

**Growth studies.** Cells were inoculated into the growth medium and were examined for morphological changes by phase-contrast and electron microscopy at 12, 18, 36, and 48 hr. Growth curves were prepared from optical density measurements taken on a Bausch & Lomb Spectronic 20 colorimeter at 620 nm. Cultures were inoculated into 10 ml of the culture medium in screw-cap test tubes, 12 by 150 mm (Kimax; Owens Illinois Inc., Toledo, Ohio). Temperature was controlled to 25 $\pm$ 0.25 C in a Metabolyte Water Bath Shaker (New Brunswick Scientific Co., New Brunswick, N.J.), with the shaker speed set at 300 rev/min.

**Light microscopy.** To monitor the morphological changes occurring during the growth of the organism, samples (0.05 ml) were taken at various times and examined under oil immersion by phase-contrast microscopy (Zeiss research microscope with aristophot camera attachment). Because of the psychrophilic nature of the organism, the observations with the phase-contrast microscope were time-limited and cultures were retained at 15 C during observation to protect the organism from undue exposure to heat. Both Polaroid (Polapan, type 52) and plate film (Kodak Panatomic-X) were used to record the morphological observations. The Kodak Pan-X film was developed in D-11 and prints were made on Kodabromide paper (Eastman Kodak Co., Rochester, N.Y.).

**Ultrathin sectioning.** Whole cells were prefixed in 0.1% osmium tetroxide for 10 min, centrifuged at 1,800 rev/min at 0 C, and then fixed in 1.0% osmium tetroxide by the Kellenberger-Ryter method (12), ex-

cept that the duration of fixation was extended from 16 to 24 hr and carried out at 15 C. After fixation, the cells were washed (2 hr) in 0.5% uranyl acetate in the Kellenberger buffer. Dehydration was performed in the following sequence: ethyl alcohol, 50, 70, 85, 95, 100, and 100%, each 15 min; propylene oxide, two changes each for 15 min. The cells were infiltrated and embedded in Epon by the method of Luft (17). No. 0 gelatin capsules were used as embedding molds and polymerization was carried out at a temperature of 60 C for 24 hr. Ultrathin sections were cut with an LKB Ultratome, collected on Formvar-coated 200-mesh copper grids, and stained with saturated aqueous uranyl acetate and 0.2 to 0.4% lead citrate, following the method of Venable and Coggeshall (22).

**Electron microscopy.** Specimens were examined in an RCA EMU-2D electron microscope fitted with an 0.04-cm externally centrable (Canalco) condenser aperture and an approximately 50-$\mu$m aperture in the standard objective pole piece. Electron micrographs were taken at instrumental magnifications of 7,900 to 13,500 × on Kodak projector slide medium plates (5 by 25 cm).

## RESULTS

**Growth studies.** The growth curves obtained from monitoring optical density increase over the period of examination at 15 C are shown in Fig. 1. The logarithmic phase of growth under the conditions tested was ca. 12 to 28 hr after inoculation. The morphological changes occurring during the growth cycle experiments were followed by phase microscopy. At 12 hr, the cells demonstrated a generally uniform size (Fig. 2a) and slightly curved rod shape. Short chains of three to eight cells were occasionally seen. At 18 hr, cell size was not greatly increased. After 24 hr, however, some giant cells were seen (Fig. 2b, c). Serpentine forms (Fig. 2e) and short chains (Fig. 2d) also appeared at this time. Long chains of relatively small cells (Fig. 3) were observed. Round bodies were commonly seen after 24 hr (Fig. 4a). The round bodies seen at 24 and 48 hr, when examined under higher magnification, revealed considerable internal detail (Fig. 4b). The cytoplasm appeared to be peripherally disposed with a clear area in the central portions of the round body.

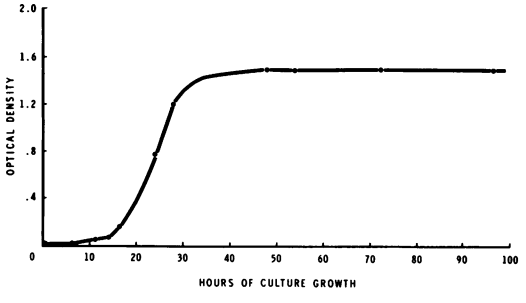



FIG. 1. *V. marinus MP-1 growth curve.*

**Ultrathin sections of whole cells of V. marinus MP-1.** Ultrathin sections prepared from cells of *V. marinus* MP-1 in the logarithmic phase of growth revealed a typically gram-negative plasma membrane and cell wall. Each was composed of a tripartite structure approximately 7.5 nm thick. The cell wall is usually disposed parallel to the plasma membrane but may be lifted away from it in some regions (Fig. 6, arrow). The general structural features of these cells can be seen in longitudinal and transverse sections (Fig. 5–7). The nuclear material may be axially (Fig. 5) or terminally (Fig. 6) disposed, and distinct ribonucleoprotein particles (Fig. 6) and polyribosomes (Fig. 7) are visible in the cytoplasm. The tripartite nature of the plasma membrane and cell wall may also be seen (Fig. 7). A short length of duplicated plasma membrane may be seen (Fig. 5, arrow). This is reminiscent of a similar situation observed in an unidentified streptomycete by Moore and Chapman (18). This phenomenon was rarely observed in the present study, in contrast to its frequent occurrence in the streptomycete.

Cellular division in *V. marinus* appears to be accomplished by an initial constriction of the plasma membrane, unaccompanied by the cell wall, to septate the cytoplasm (Fig. 8–10). Subsequently, cell wall constriction follows (Fig. 11) to produce two separate daughter cells. Vesicular structures have been seen (Fig. 11) in this plane of cell wall constriction. It is not clear whether they represent derivatives of the cell wall or merely continuations of it which only appear separate owing to their orientation in the plane of section.

The unusual cell morphologies observed by phase-contrast microscopy during the 24 to 36 hr stage of the growth period were also seen in ultrathin sections in the electron microscope. Giant cells (Fig. 12), which were packed with ribosomes and nuclear material, as well as with membranous structures, were found more frequently in the 24 to 36 hr period. The serpentine forms, also prevalent at this stage, showed nuclear material disposed axially for most of the length of the cell, and ribonucleoprotein particles were disposed throughout the entire cytoplasmic regions. Round bodies (Fig. 13) were seen occasionally in all preparations. They were, however, more common in the older cultures, i.e., older than 24 hr. The cell wall of the round body enclosed two or more cell units, each of which was bounded by a separate and intact plasma membrane (Fig. 14). The cell wall showed a uniform width throughout, with no indication of "stretching" or "unraveling." The plasma membrane remained attached to the cell wall at only one side. In many cases, three or

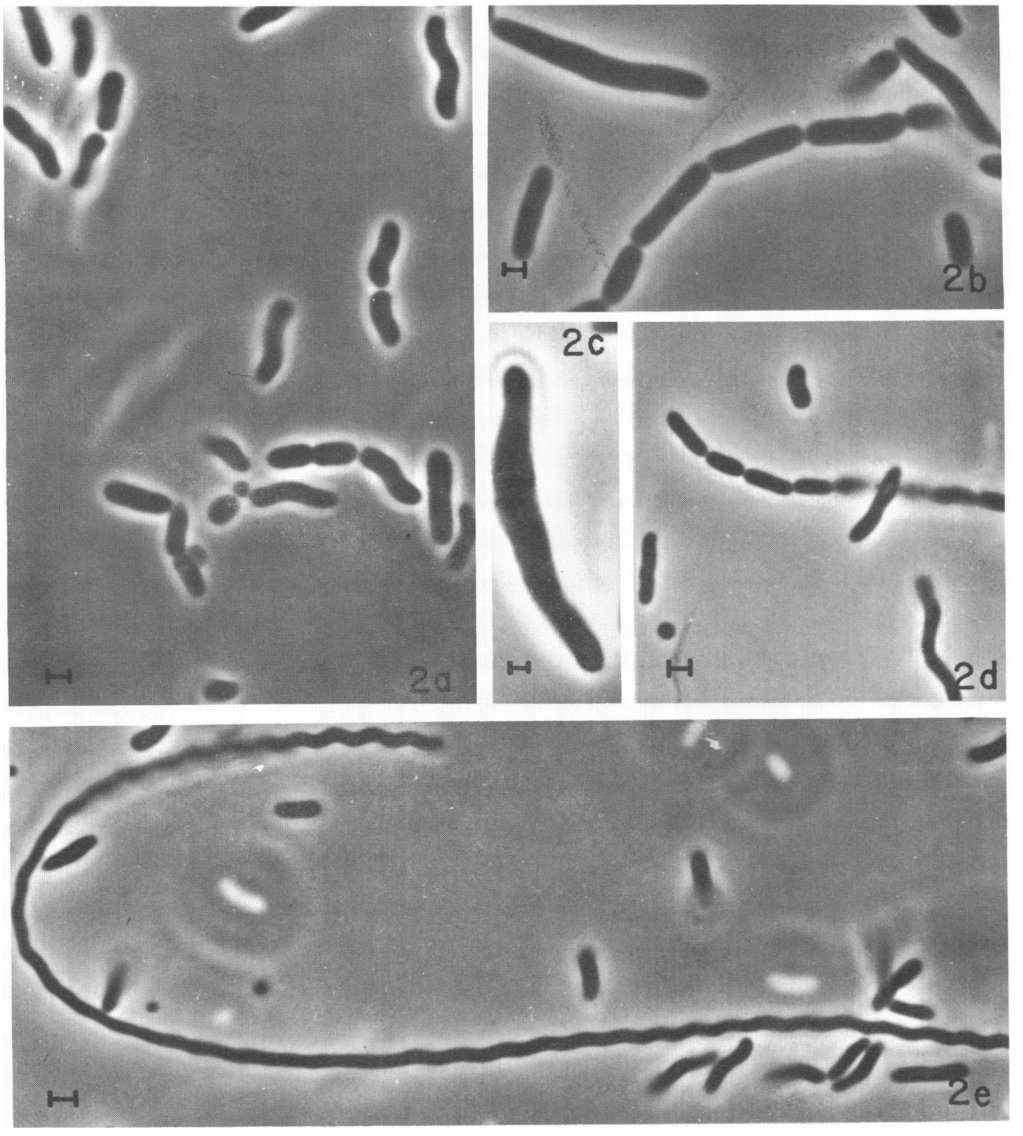
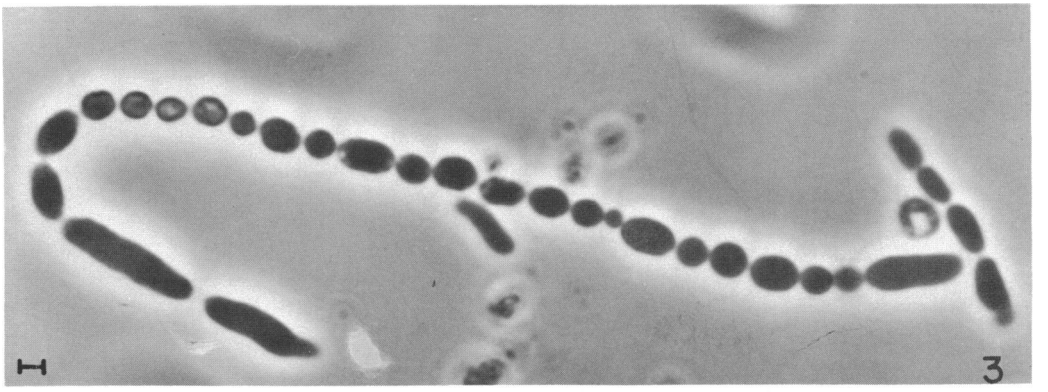


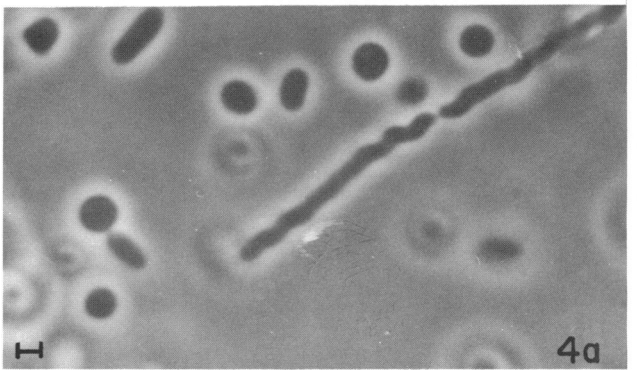

FIG. 2. *Phase-contrast micrographs of V. marinus. Cells were grown in maintenance medium at 15 C for (a) 12 hr, (b) 24 hr, (c) 48 hr, (d) 24 hr, (e) 24 hr. Note giant cells (b, c), short chains (d), and serpentine form (e). (a, b, d, e) × 3,000; (c) × 2,600.*

four cell units were seen within the single cell wall. Division of the membrane-limited cell units was seen occasionally within the cell wall of the round body. In several instances, forms were observed in which the cell units appeared to be in the process of separating from the round body structure (Fig. 15).

## DISCUSSION

Coccoid forms, or round bodies, have long been observed in *Spirillum* and *Vibrio* spp. (4, 7, 9, 10). The coccoid structures of vibrios and spirillae have been suggested as representing a stage in the life cycle of these organisms (7, 23). Also, the round bodies, characteristically appearing in later stages of growth (ca. 18 to 24 hr or later), have been employed as a diagnostic characteristic for the genus *Vibrio* by many investigators. The "gut group" vibrios found in North Sea flatfish demonstrated the round body forms and were postulated to be commensals in the flatfish gut (15). Buttiaux (1) referred to the "extreme polymorphism" of vibrios isolated from good-quality ham brines.



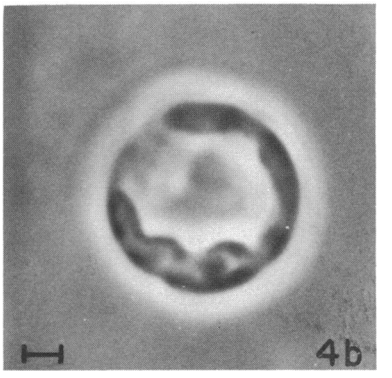




FIG. 3. *Phase-contrast micrograph of V. marinus showing long chain of cells of varied morphology; 48-hr culture.* × *3,000.*

FIG. 4. (*a*) *Culture at 48 hr showing numerous round bodies.* × *3,000.* (*b*) *Round body showing internal structure.* × *5,000.*

The morphological changes observed in *V. marinus* MP-1 during growth parallel those described by Williams and Rittenberg for *Spirillum lunatum* (23). The swellings and formation of crescent-shaped forms, termed "microcysts" by these workers, are remarkably similar to the forms shown in Fig. 4b. Williams and Rittenberg concluded that the round bodies observed by them in *S. lunatum* were apparently the same as the "coccoid" bodies reported by earlier investigators. The ultrathin sections of the round bodies from *V. marinus* (Fig. 12–15) permit the interpretation that these forms result from aberrant cell division. A clarification of the situation in *S. lunatum* appears in order.

The carefully recorded observations of Hallock (7, 8) on the morphological changes occurring in vibrios during growth are strikingly similar to those reported here. As Hallock emphasized, observations reported by most of the bacteriologists studying vibrios were made on 24-hr cultures. On the second, third, and fourth days after transfer, the nature of the vibrio cell changes dramatically. As noted by Hallock (7), stock cultures of vibrios, 10 days to 6 weeks old, demonstrate predominantly cells of the coccoid body or spherical morphology. Four types of coccoid bodies can be distinguished: large spheres, with a diameter of 1.5 to 3 μm; "spheres" (7); minute spheres, fairly uniform in size, with a diameter of ca. 0.2 μm; occasional huge globular cells, "macrospheres"; and "spherules" formed within a mother cell. In the course of the present study, three of the four types of coccoid bodies have been observed; the fourth type was described by Kennedy, Colwell, and Chapman (Limnol. Oceanogr., *in press*) and occurs in *V. marinus* PS-207 cultures. Hallock observed that "life sequences vary," with the "mother cell/spherule" formation not necessarily occurring in all species or strains of *Vibrio*. Our observations are in accord with this conclusion, since the mother cell/spherule morphology was not found in *V. marinus* MP-1.

When transferred from a culture in logarithmic phase of growth, the vibrio cells, up to 18 hr, were fairly uniform in size and shape (short, curved cells ca. 1 by 3 to 4 μm). After this time, the appearance of distorted "giant" or "monster" cells

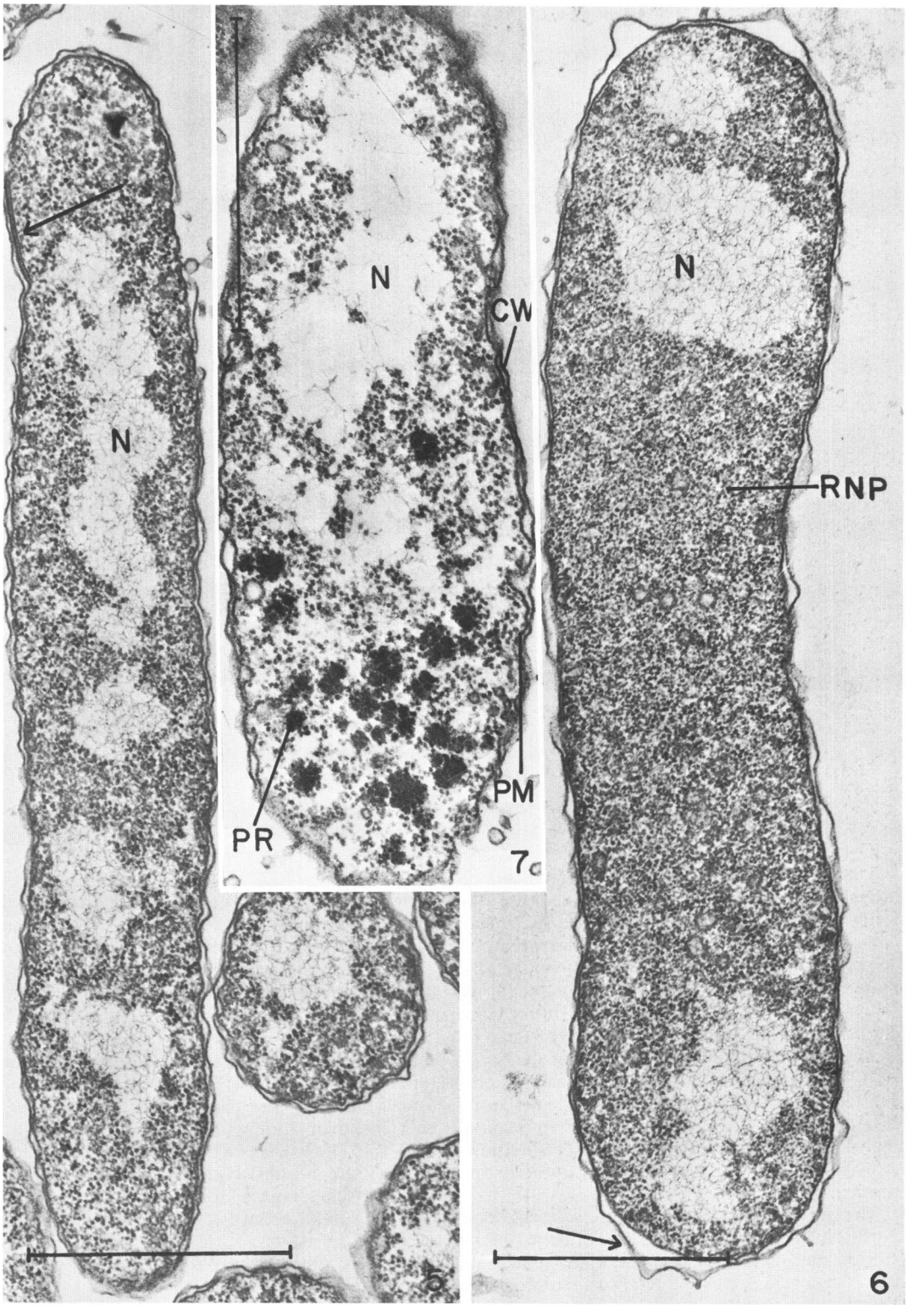



FIG. 5. *Longitudinal and cross sections of V. marinus MP-1. Arrow indicates area of plasma membrane duplications.* × *40,300. In all electron micrographs, the magnification bar represents 1 μm.*

FIG. 6. *Longitudinal section showing terminally distributed nuclear material (N) and densely packed ribosomes (RNP). Arrow indicates area of separation of cell wall and plasma membrane.* × *36,500.*

FIG. 7. *Longitudinal section of V. marinus. The tripartite nature of the cell wall (CW) and plasma membrane (PM) may be seen. Nuclear material (N) is axially disposed. Polyribosomal aggregates (PR) may be seen.* × *48,500.*

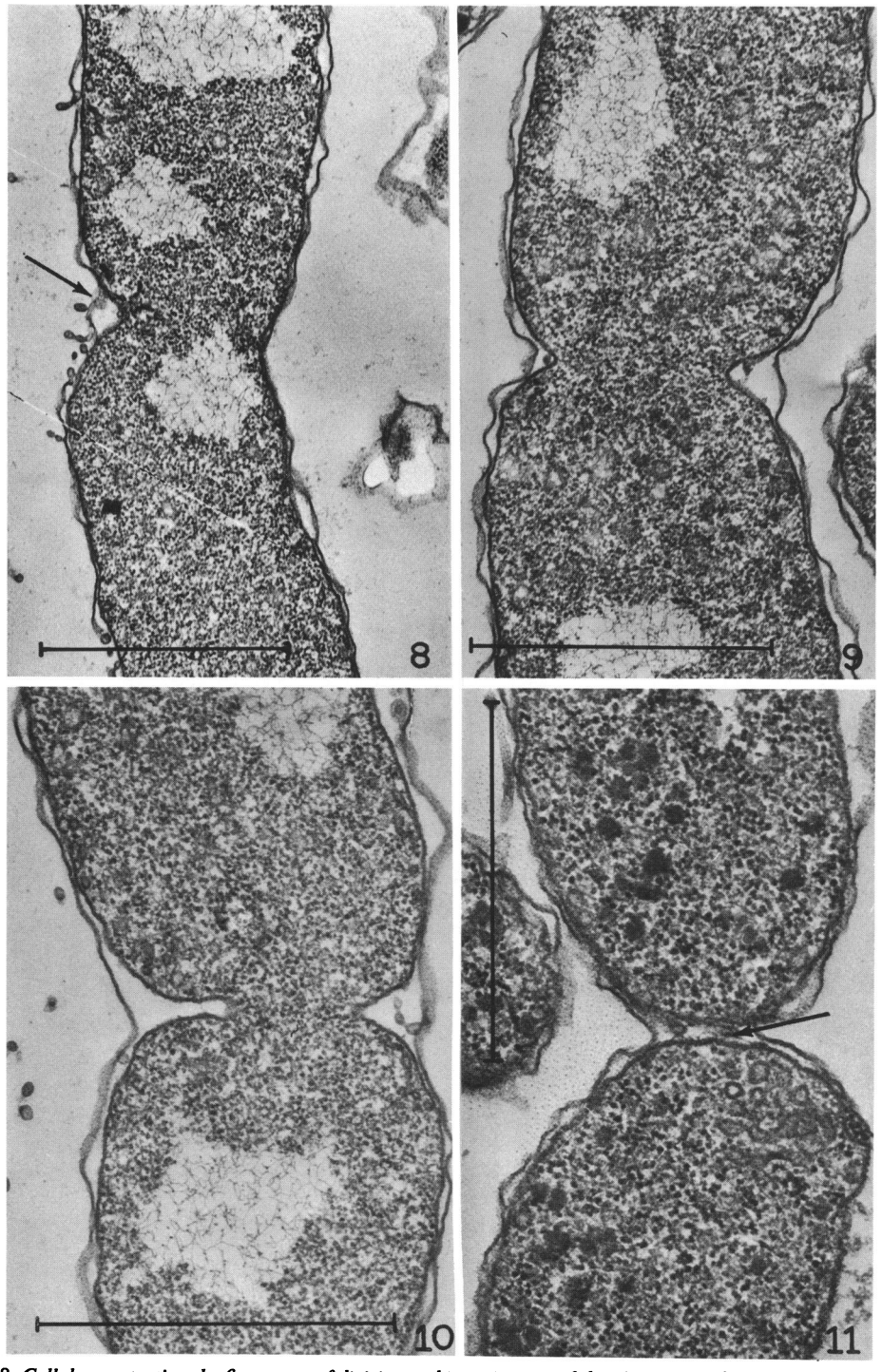



FIG. 8. *Cell demonstrating the first stage of division and invagination of the plasma membrane (arrow).* × *35,000.*
FIG. 9. *Invagination of the plasma membrane, without cell wall involvement, may be seen.* × *41,300.*
FIG. 10. *Constriction of the dividing cell, involving the plasma membrane.* × *50,300.*
FIG. 11. *Completed division of the cell into two daughter cells. Invagination of the cell wall can be seen. Vesicular elements (arrow) may be seen near the site of division.* × *49,000.*

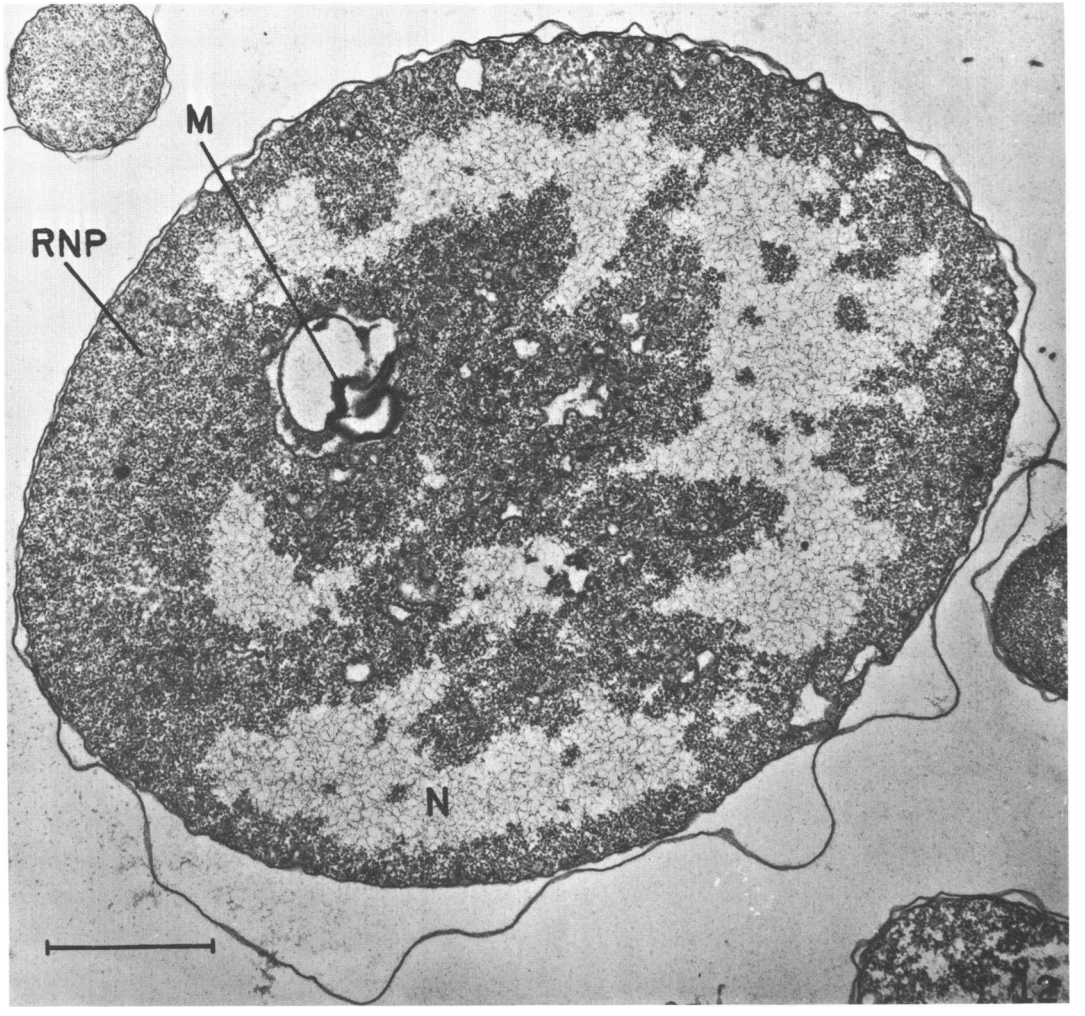



FIG. 12. *Electron micrograph of a "giant" cell. The diffuse nuclear material (N), ribosomes (RNP), and membrane structures (M) are evident.* × *21,500.*

[termed "involution forms" by early workers (4, 16) but probably our "giant cells"] and serpentine forms, reported here for *V. marinus* MP-1, were noted. These forms may account for the fact that no decrease in optical density was noted after 72 hr (Fig. 1). Slight increase in the number of coccoid forms ("spheres," "spherules," and "macrospheres") was observed after 24 hr of growth of *V. marinus* MP-1.

The nature of the consecutive stages in development from spheres to "previbrios" and ultimately to vibrios was presented by Hallock (8) as one of the manifestations of the "life cycle" of vibrios. From our observations, these stages appear either to be a function of aberrant cell wall synthesis or to be due to the unusual mode of cell division of *V. marinus* MP-1. The ring cell inside a sphere wall, which was carefully traced by Hallock (8), appears to be nearly identical to those shown in Fig. 4b and 13. Unbalanced growth of cultures, occurring 24 hr after transfer to fresh medium, might be interpreted as the cause of detachment of the cell wall from the dividing cells. Cell wall, failing to reform around the daughter cells, would result in a quasi-spheroplastic state of the daughter cells.

Cell division in *V. marinus* appears to be somewhat unique. From the sequence of illustrations in Fig. 8–11, cellular division progresses via a constrictive process involving the plasma membrane, followed, subsequently, by the cell wall. The emphasis appears to be on membrane constriction rather than on membrane deposition. The latter clearly predominates in the case of

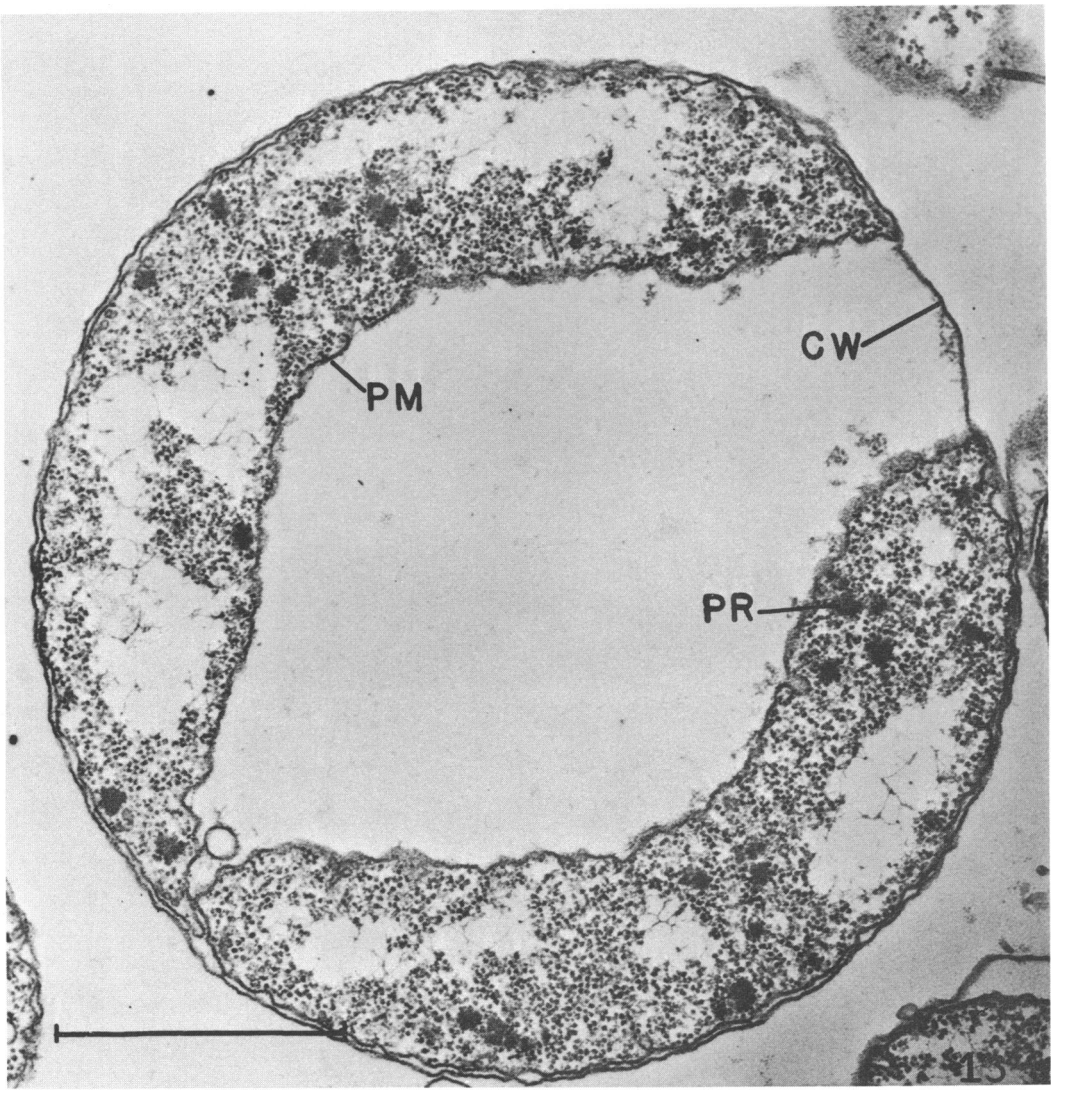
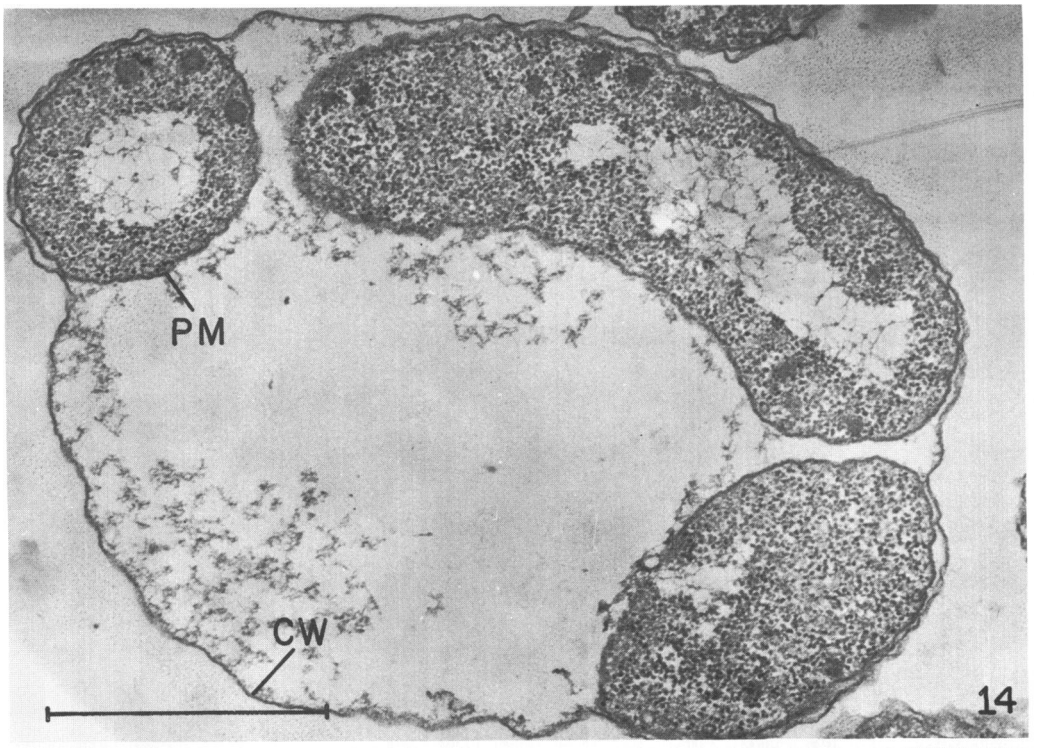



FIG. 13. *Round body in cross section. This figure should be compared with Fig. 5b. The cell wall (CW) encloses the entire structure and both cell units are bound by a plasma membrane (PM). Polyribosomal aggregates (PR) may also be seen.* × *38,400.*

membrane septation described by Chapman (2), in the only other instance of which we are aware wherein membrane septation clearly proceeds to completion (with septum formation) before the cell wall begins to participate in the division process.

The round bodies, seen in phase contrast as coccoid forms with internal "crescent shapes" (Fig. 4b), in the electron microscope were found to be paired cells bounded by a single outer wall structure (Fig. 13). The spontaneous spheroplast formation observed by Levin and Vaughn (14) in *Desulfovibrio aestuarii* appears to be a very similar process. The formation of "blebs" at the concave side of the vibrio, observed in the later stage of spheroplast formation in *D. aestuarii*, resembles that shown in Fig. 13. Das and Chatterjee (5) have shown similar morphological changes in *V. cholerae* grown in glucose-saline solution. Multinucleated cells would result in the formation of several rods from each spheroplast, as was concluded by Hirokawa (11) in studies of penicillin-induced *Escherichia coli* spheroplasts. Thus, three or four cells could arise from the outgrowth of a single protoplasted cell. The "germination" of microcysts (round bodies) as described by



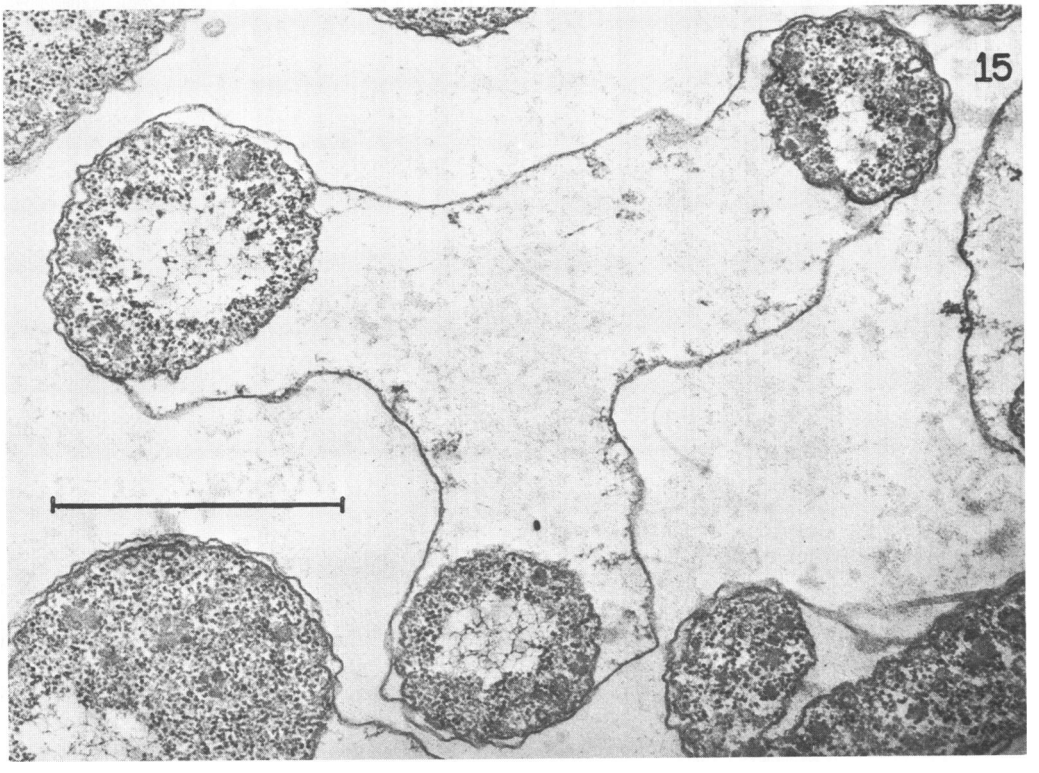




FIG. 14. *Section showing a round body containing three cell units. Note cell wall (CW) and plasma membrane (PM).* × *36,500.*

FIG. 15. *Round body showing cell units pulled away from each other. The cell units are cross sections, probably of rod forms.* × *38,400.*

Williams and Rittenberg (23) could be interpreted as an outgrowth of the dividing cells.

Other features of the fine structure of *V. marinus* that may be noted are the polyribosomal aggregates (Fig. 7, 13) and the "giant cells" (Fig. 12) which contained internal membrane structures and vacuoles. A discussion of the membrane formations and their relation to culture age and growth conditions will be provided in a later communication (Felter et al., *in preparation*).

In conclusion, the *V. marinus* ultrastructure is not unlike that described for gram-negative bacteria. However, the round bodies of *V. marinus* possess some striking features and appear to be associated with aberrations in cell division and cell wall formation. Because of their obvious differences in behavior during cell division, the plasma membrane and the cell wall of the marine vibrios possess quite different and separate functions, although both structures superficially appear identical, i.e., trilaminar (20).

## ACKNOWLEDGMENTS

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