APPLIED MICROBIOLOGY, Mar. 1972, p. 637-648

Vol. 23, No. 3
Printed in U.S.A.

Microflora of Soil as Viewed by Transmission Electron Microscopy[1]

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Received for publication 28 October 1971

Several procedures were evaluated for separating and concentrating indigenous microorganisms from soil without the occurrence of growth. Electron microscopy of nontangential, thin sections through these cells revealed that all of the cells examined were less than 0.9 μm in diameter, and up to 72% were "dwarf" cells less than 0.3 μm in diameter. Some were small enough that they should not be resolved with the light microscope. Approximately 27% had a fine structure bearing some resemblance to that of a bacterial cyst or microcyst, but this value may be low because cells having their outer layers partially stripped off were not included in the count. Approximately 25% showed a distinct periplasmic space, which often contained stainable material. Other fine structure features are presented together with frequencies of occurrence for the populations examined.

Many types of soil microorganisms have been cultivated in the laboratory, and their morphology and physiology have been examined extensively. Generally, it has been assumed that these same microorganisms, as they naturally reside in soil, would have a morphology and physiology similar to that of their laboratory-cultivated counterparts. There are some indications however, that this may not be the case (2, 3, 5, 7, 10, 12, 20-22, 24, 34, 35), but it has been difficult to obtain evidence to support this hypothesis. The chief problem is lack of good experimental techniques. Most of the available techniques require that growth be initiated on laboratory media or by adding nutrients to the soil, or that in situ microflora be examined by various forms of bright-field or ultraviolet-fluorescence microscopy. The use of medium or nutrient additions usually upsets the climax community balance and causes a relatively quick enrichment for certain segments of the population, although much of the population may not respond at all. In addition, those cells that do respond by germination or multiplication may no longer be in their natural soil state. The various forms of light and ultraviolet-fluorescence microscopy often are hampered by the apparently small size of many of the indigenous cells (7, 16, 33, 34) and, at times, by the difficulty in distinguishing the microbial cells from other components of the habitat (7). Use of transmission electron microscopy of whole and sectioned cells would circumvent some of these problems, if the cells could be physically separated from the habitat materials and concentrated so that whole cells could be recognized and sections could be made without encountering sand or mineral fragments. The present study presents an evaluation of several possible methods for accomplishing this. The fine structure, with frequency of occurrence, and the sizes of the cells obtained by these methods are also described.

[1] This research was authorized for publication as paper no. 4085 in the journal series of the Pennsylvania Agricultural Experiment Station on 25 October 1971.

MATERIALS AND METHODS

Organism and media. *Agromyces ramosus* (ATCC 25173) was grown in Difco heart infusion broth for 4 days on a rotary shaker at 28 C. Plate counts of microorganisms in soil samples and in the various soil fractions were made on dilute, heart infusion agar (2.5 g of heart infusion broth and 20 g of Difco Noble agar per liter).

Soil samples. Soils, obtained at a depth of approximately 6 cm below the surface vegetation, were stored without drying in sealed polyethylene bags. The use of stored samples allowed reproducibility of the soil populations examined. A local Hagerstown silty clay loam (*p*H 5.4; plate count 4.6×10^6), which probably had not been exposed to pesticides, was used for most of this work, although five other soil samples from central Pennsylvania and two from New Jersey were also studied. The *p*H values of these samples ranged from 3.4 to 7.1, and the microbial counts from 8.2×10^3 to 5.7×10^7 per g of

soil. Thus, slightly acidic soils were chosen so that spores of streptomycetes would not be prevalent.

Release of cells. Soil suspensions, at a concentration of one part soil to four parts deflocculating agent solution, were prepared with distilled water, 0.9% NaCl, saturated NaCl (35 g per 100 ml of distilled water), 0.1% sodium pyrophosphate, or 0.1% sodium hexametaphosphate. These suspensions were either hand-shaken for 5 to 10 min, mixed in a Waring Blendor for 1 to 2 min, or treated sonically in an ice bath for 1 to 2 min (Biosonic oscillator, Brownwill Scientific Inc. operating at 31 to 125 watts acoustic energy at probe tip). They were then subjected to various cell-washing, fractionation, and concentration procedures.

Concentration and purification. Sucrose gradients were prepared by layering 5-ml portions of 50% (at the top), 55%, 60%, and 65% sucrose in 30-ml cellulose nitrate tubes. The Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) gradients utilized 5-ml layers of 14% (at the top), 26%, 38%, and 50% distilled water solutions. The Ludox solution was 20 ml of either Ludox AM 30 or Ludox HS 40 (E. I. Dupont DeNemours and Co.) placed in similar tubes. A 10-ml soil suspension that had been treated as above for release of microbial cells from soil particles was overlaid on each of these systems and centrifuged for 30 min at 63,581 $\times$ *g* in a Beckman L ultracentrifuge with a SW 25.1 rotor. The resulting supernatant fluids, including all of the pooled materials above the packed sediments, were removed with syringes fitted with a Becton, Dickinson and Co. 4-inch cannula. These supernatant fluids then were centrifuged at 12,100 $\times$ *g*, and the resulting sediments were washed free of gradient materials by further centrifugation in distilled water.

The two-phase polyethylene glycol (PEG) system (30) was composed of 11.18 g of PEG 4,000 (Union Carbide) and 34.1 ml of 3 M potassium phosphate buffer (*p*H 7.1). The total volume, including sample, was 100 ml. This was mixed for 5 min with a Vortex mixer and then centrifuged at 463 $\times$ *g* for 2 min. The resulting upper-phase, lower-phase, and interface materials each were washed three to four times by centrifugation at 12,100 $\times$ *g* for 5 to 10 min in distilled water to remove residual PEG, and then were concentrated at 12,100 $\times$ *g* for 10 min.

For separation and concentration of cells from soil particles by "simple centrifugal washing," a mixture of one part soil and two parts deflocculating agent or distilled water was hand-shaken for 5 min and then centrifuged for 5 min at 665 $\times$ *g*. The supernatant fluid, with suspended microbial cells, was separated and saved, and the soil sediment was resuspended in two parts of fresh distilled water for centrifugation at 821 $\times$ *g* for 5 min. The supernatant fluid was again separated and saved, and the soil sediment resuspended in fresh, distilled water. This procedure was then repeated with centrifugations at 1,850 and 5,061 $\times$ *g*, with all supernatant fluids being saved. These fluids then were combined and centrifuged for 10 min at 12,100 $\times$ *g*. The resulting supernatant fluid was discarded, and the cell sediment was processed for electron microscopy observations.

For the "exhaustive centrifugal washing" concentration of the soil organisms, the final soil sediment from the above was used, the entire procedure was repeated three additional times, and all supernatant fluids were saved. The residual soil sediment then was resuspended in distilled water, subjected to sonic treatment for 1 min in a Biosonic oscillator operating at 31-watt acoustic energy, and then manually shaken for 5 min. This suspension was subjected to sequential washings at increments of centrifugal force as given above, and all supernatant fluids were saved. The final soil sediment then was treated further in a similar manner, except that sonic treatment power levels of 62-, 94-, or 125-watt acoustic energy were applied before each manual shaking and centrifugation series.

The final soil sediment was discarded, and all supernatant fluids from the above procedure and the simple centrifugal washing were pooled for centrifugation at 12,100 $\times$ *g* for 10 min. The resulting supernatant fluid was discarded, and the cell sediment was processed for electron microscopy observations.

Evaluation of fractionation techniques. The effectiveness of the above procedures in separating and concentrating microorganisms from the soil samples was assessed by using (i) light microscopy in conjunction with a Petroff-Hausser bacteria counter to determine the number of microbial cells present and the degree of contamination by soil materials, (ii) light-diffraction microscopy (6), (iii) plate count, (iv) the ease of sectioning of preparations for electron microscopy, and (v) the numbers of cell sections observed per field during electron microscopy observations.

Electron microscopy. Cell fractions viewed as whole-cell preparations were stained with a 1% (w/v) aqueous solution of potassium phosphotungstate (KPT, *p*H 7.0 to 7.3) for 10 sec to 1 min, or with a 0.5 to 1% (w/v) aqueous solution of uranyl acetate (UAc, *p*H 4.5 to 4.6) for 5 to 10 min. For sectioning, approximately 0.1 ml of cell sediment resulting from the above procedures was fixed overnight (10 to 15 hr) in either 1% (w/v) OsO_4 [the method of Kellenberger, Ryter, and Sechaud (15)], neutral Veronal-buffered OsO_4 (4), or 2.5% (w/v) glutaraldehyde in phosphate buffer (0.15 M, *p*H 7.3) for 1.5 hr. The latter preparation was postfixed by the method of Kellenberger, Ryter, and Sechaud (15). The fixed soil fractions were dehydrated in a graded acetone series, embedded in either Epon 812 (17), Vestopal W (29), or Spurr's low-viscosity resin (32), and sectioned with a glass knife or a Dupont diamond knife. The sections were poststained with lead citrate (26) and observed with a Hitachi HU 11E or a Philips EM 300 electron microscope operating at 60 kv.

Estimation of cell numbers and size in sections. The number of cells occurring in sections of microbial preparations from soil was estimated by comparing similar preparations containing known cell numbers of *A. ramosus*. Thus, a final cell fraction from soil was concentrated to approximately 0.1 ml by centrifugation at 12,100 $\times$ *g* for 10 min, mixed with an equal volume of 2% melted Noble agar (45 C), and formed as an agar block of approximately 1

mm^3. Sections were compared with those of *A. ramosus* prepared from 0.2 ml of a 1:1 agar-cell suspension containing 10^9 cells. In this procedure, it was assumed that the cells were randomly distributed in the agar.

The size of the cells was measured from the negatives.

RESULTS

Release and concentration of cells. Most of the methods for releasing indigenous cells from soil and for concentrating and purifying the cells gave approximately similar cell recoveries and a similar range of types of cells observed. Notable exceptions were the Ludox gradient and the exhaustive centrifugal washing of soil with distilled water. The high alkalinity required for the Ludox apparently caused death and destruction of cells; plate counts were only 1% of those for the other methods, and fewer small cells were noted by electron microscopy. The exhaustive centrifugal washing of soil provided the greatest numbers of sectioned cells for viewing, a point of considerable importance in the present study. This method provided an average of four cells per field at 25,500-fold scope magnification; use of only the first part of the centrifugal force sequence gave an average of one cell per field. Use of the other methods provided one cell section per several fields. Magnifications of less than 25,500 were rarely used because of the difficulty in distinguishing the sections of small cells from the soil debris still present in the various preparations. The use of distilled water for certain of these procedures apparently had no adverse effect on cell structure, because soil fixed with glutaraldehyde before the centrifugal washing of soil provided cell fine structure comparable with similar preparations from non-prefixed soil.

Electron microscopy. Only a few cells were detected with certainty in KPT or UAc negatively stained, whole-cell preparations of the cell concentrates from soil. Most of these were small cocco-bacilli, with more than half of those observed less than 0.35 μm in diameter (Fig. 1a–c), while the rest ranged from 0.36 to 1.1 μm in diameter. Apparently, the small size of many of the cells, combined with the fact that residual soil debris tended to cling to their surfaces, made definite recognition of the cells difficult. Particles similar in size and shape to microorganisms were occasionally observed (Fig. 2).

Cell sections, in general, showed a similar array of structural features for the cells from the various types of cell concentrates. The various fixation procedures and embedding resins provided similar results with respect to the range and clarity of structural details observed. The ease of sectioning, however, was significantly improved with the use of Spurr's low-viscosity embedding resin (32).

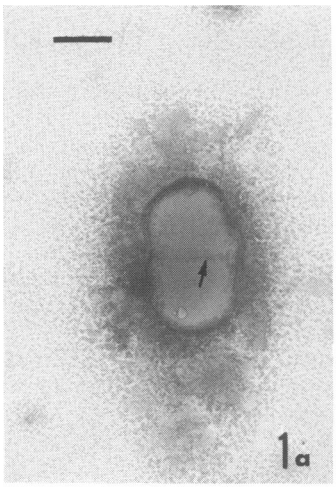
A comparison of sections for soil cell concentrates, obtained by exhaustive centrifugal washing of soil, with sections for known cell numbers of *A. ramosus* prepared in a similar manner, revealed that there were approximately 10^8 to 10^9 soil cells in the 0.2 ml of agar used to make the blocks.

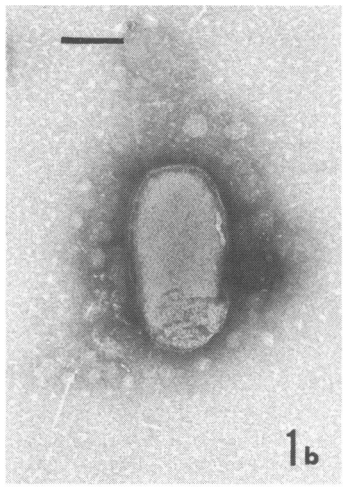
Approximately 75% of the cell sections of recognizable microbial cells from the various soil cell concentrates were selected at random and photographed. Of these, 330 (approximately 75% of the total cells photographed) showed enough fine structural detail to be critically evaluated. Size-distribution determinations were made for 306 sections which appeared to be nontangential. Of these cells, 63% were less than 0.3 μm in diameter (Fig. 3a–c), 31% were between 0.31 and 0.5 μm, and only 6% were between 0.51 and 0.9 μm in diameter; none had a diameter greater than 0.9. These values, however, were 72%, 26%, and 2%, respectively, if the 55 cell sections obtained by Ludox density gradient centrifugation were not included. This method apparently either destroyed some of the smaller cells or did not separate them from the soil particles.

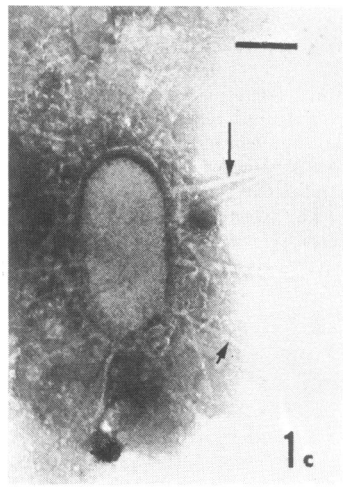
The frequencies of occurrence of various cell structural characteristics are presented in Table 1. Examples of these structures are shown in Fig. 4 and Fig. 6 to 14. Because of the greater numbers of sections which were obtained, 73% of the cell sections listed in Table 1 resulted from the simple and exhaustive centrifugal washing procedures. The occurrence of specific structural features, however, did not seem to be related to the cell concentration procedure used. In most cases, the initial suspension fluid for the soil was distilled water, and the cells were fixed in 1% OsO_4 in Kellenberger buffer. All preparations for Table 1 were embedded in Spurr's low-viscosity resin, because of its greater ease in sectioning.

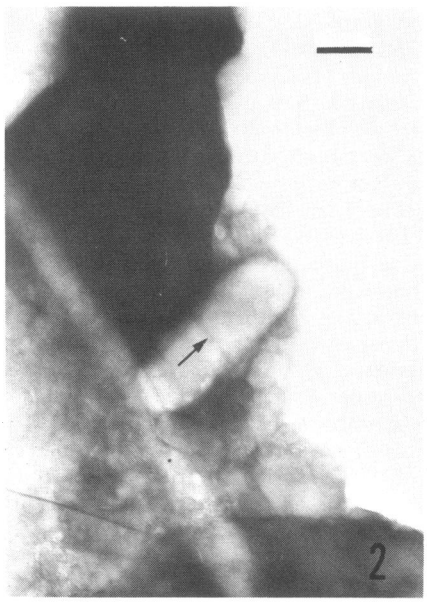
DISCUSSION

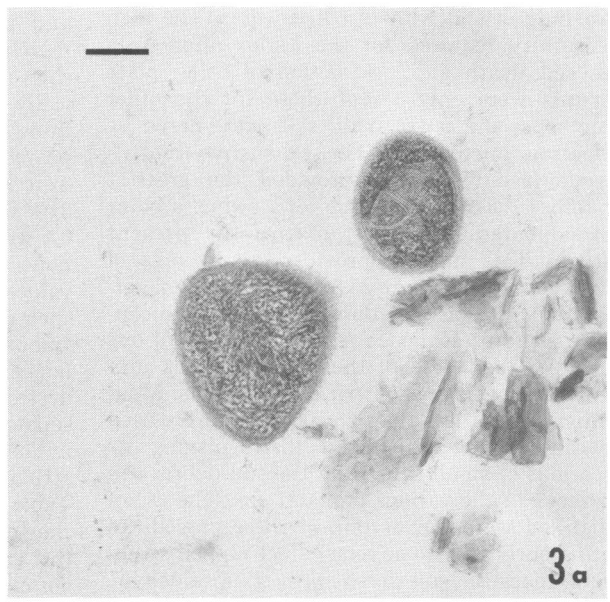
Procedures are presented which allowed separation and concentration from soil of indigenous microbial cells in numbers great enough to be processed and viewed as thin sections by electron microscopy. The percentage of cells that remained with the discarded soil debris

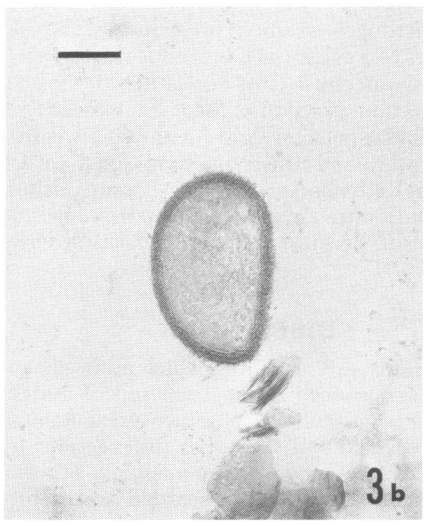


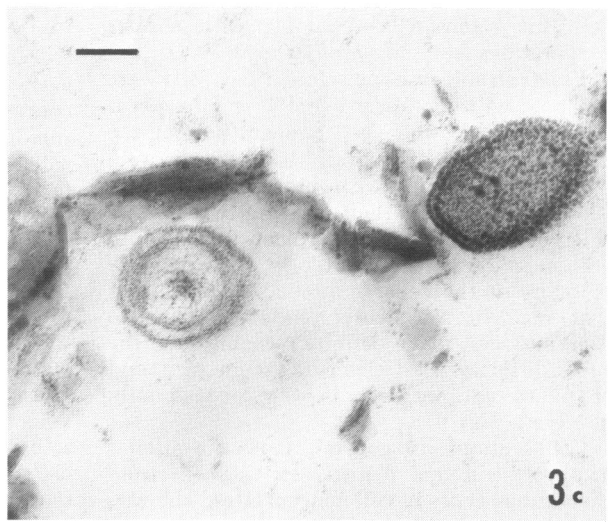












FIGS. 1 TO 3

could not be accurately determined, however. Estimates, by comparing plating results with the frequency of occurrence of cell sections during electron microscopy observation of cell concentrates for the simple centrifugal washing procedure, indicated that approximately 80% remained with the soil debris. In contrast, approximately 15 to 20% were not separated from soil by using the exhaustive centrifugal washing procedure. Light-diffraction microscopy (6, 7), however, indicated that the simple centrifugal washing of soil separated most of the dormant cells from the soil debris. It is possible, therefore, that the platable nondormant soil microflora was not being truly represented in these studies.

Although time-consuming, the exhaustive centrifugal washing of soil yielded greater numbers of sectioned cells than the other methods. In contrast, Ludox gradients, which were quite alkaline, provided plate counts that were two orders of magnitude less than for the other procedures. Electron microscopy showed that the smaller cells tended to be discarded, or possibly destroyed, by this technique. Attempts to lower the *p*H of Ludox were unsuccessful because of loss of its colloidal state at *p*H values below 8.5

Observations of sectioned and whole-cell preparations revealed that soil microorganisms residing naturally in this habitat differed from laboratory-grown cultures in size and fine structure. Although fine structural comparisons of specific cells, as they resided in soil (designation of species cannot be made in this case), with organisms of similar classification but growing in laboratory culture was not possible, it was apparent that the majority of the in situ soil microorganisms (at least for those separated from soil and concentrated by these procedures) were in a "dwarf" state. Thus, 72% of a total of 251 sectioned cells examined (disregarding cell sections from Ludox gradients) were less than 0.3 μm in diameter; similar results were obtained by whole-cell measurements. The possibility that dwarf cells (or small cells) might occur in soil has been noted by others (1, 28, 33, 34).

TABLE 1. *Frequencies of occurrence of fine structure characteristics for indigenous cells separated from soil*

Structural characteristics	No. of cells with the structure	% Occurrence among total cells examined[a]	Fig no.
Cyst-like forms	88	26.7	4a–c
Cells with distinct periplasmic space	81	24.6	9a–d
Cells with mesosomes	27	8.2	6a, b
Cells with capsules	25	7.6	12
Cells with congealed nuclear region	20	6.7	11
Electron-dense granules	19	5.8	7
Blebs on cell wall	13	3.9	7
Cells with electron-transparent area	12	3.6	8
Star-shaped cells	11	3.3	10
Spores	6	1.8	13
Cells resembling soil *Mycobacterium*	2	0.6	14
Normal-appearing cells without unique characteristics	91	27.6	

[a] Some cells presented more than one feature.

Recognition of microbial cells in the whole-cell preparations was difficult, because they often were obscured by contaminating soil particles on their surfaces. Consequently, whole-cell preparations gave rise to only a few cells which could be identified with certainty as being cells and, of these, a few large cells (up to 1.1 μm in diameter) were observed, as compared to a maximum diameter of 0.9 μm for sectioned cells. Most of the cells from whole-cell preparations were not as large, however, as most laboratory-grown cells where the diameters usually are greater than 0.5 μm. Large cells, if present, should have been easily observed. There was a possibility, however, that

FIG. 1. *KPT negatively stained dwarf cells from a fraction obtained by centrifugation washing of soil. (a) Electron-dense line which appears to be a division septum (arrow); (b) division septum or appendages not present; (c) appendages which do not appear to be bacterial flagella (arrows). Bar markers represent 0.2 μm.*

FIG. 2. *KPT negatively stained, microorganism-like particle from a fraction obtained by centrifugation washing of a soil initially suspended in 0.1% sodium pyrophosphate. Note the electron-dense line in the center of the particle (arrow). Bar marker represents 0.2 μm.*

FIG. 3. *Sections of dwarf cells; embedding in Spurr's medium. (a) From a fraction obtained by centrifugation washing of soil; fixation in neutral Veronal-buffered OsO_4; (b) from a fraction obtained by centrifugation washing of soil; fixation in 1% OsO_4 in Kellenberger buffer; (c) from a fraction obtained by sucrose density gradient centrifugation of soil; fixation in 1% OsO_4 in Kellenberger buffer. Bar markers represent 0.1 μm.*

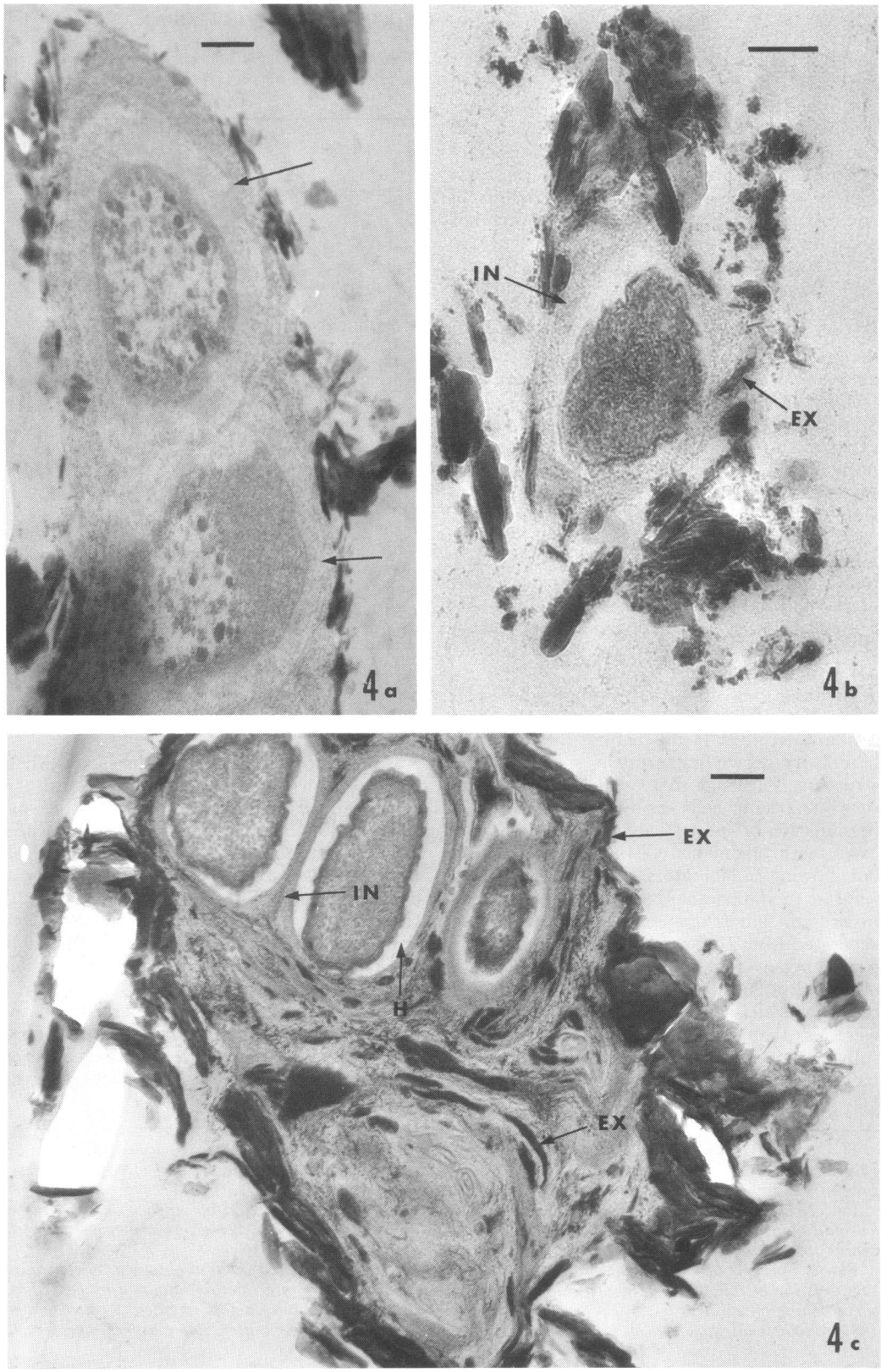
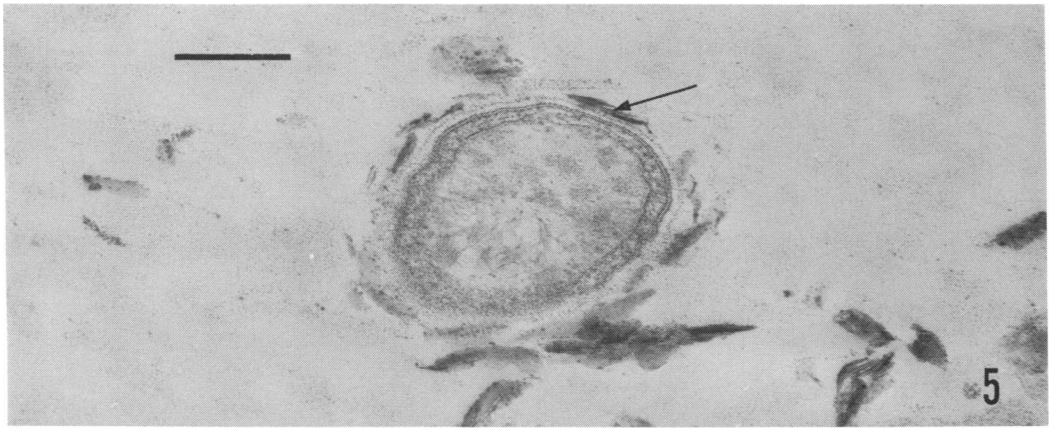
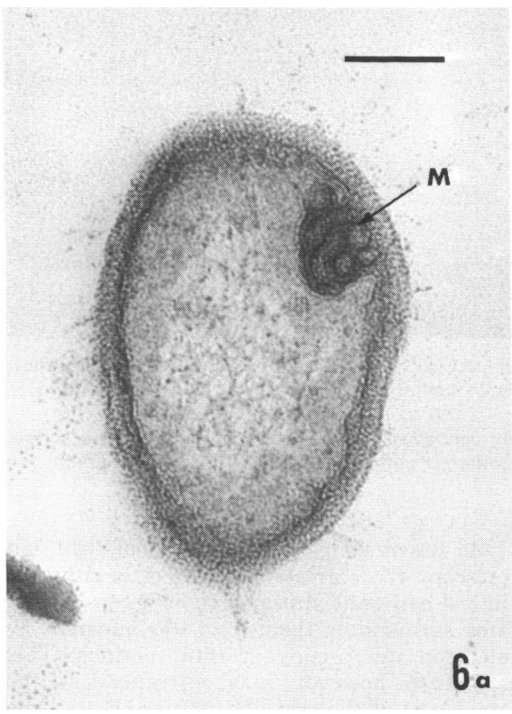


FIG. 4





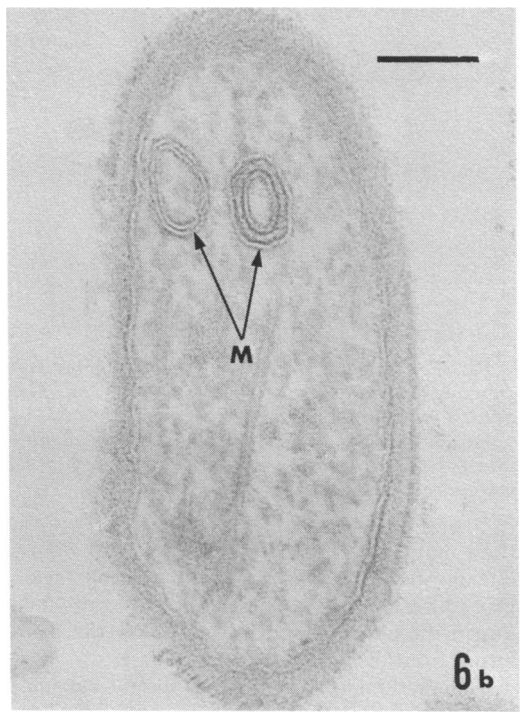
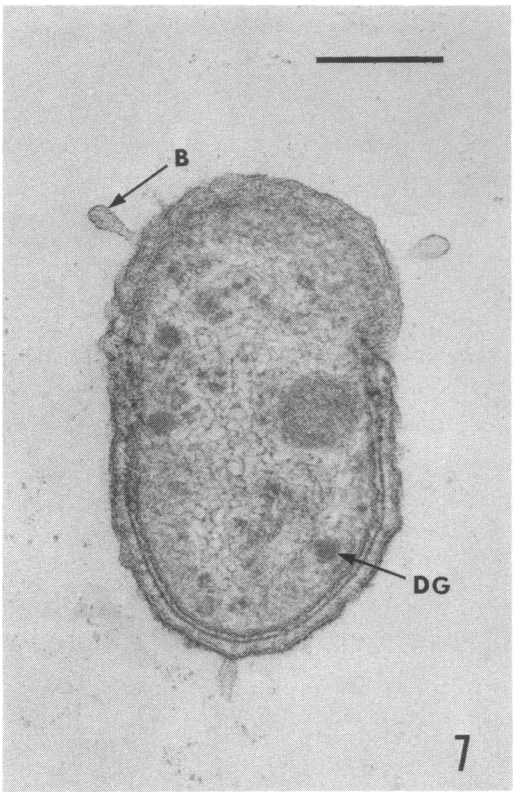


FIG. 5. *Section of a cell showing fragments of ruptured or incomplete exine-like structures (arrow). From a fraction obtained by exhaustive centrifugation washing of soil; fixation in 1% OsO_4 in Kellenberger buffer; embedding in Spurr's medium. Bar marker represents 0.2 μm.*

FIG. 6. *Sections of cells from fractions obtained by centrifugation washing of soil, showing mesosomes (M). (a) A tubular mesosome; fixation in 1% OsO_4 in Kellenberger buffer; (b) laminar mesosomes; fixation in neutral Veronal-buffered OsO_4. Embedding in Spurr's medium. Bar markers represent 0.1 μm.*

FIG. 4. *Sections of cyst-like and microcyst-like cells in soil fractions. Fixation in 1% OsO_4 in Kellenberger buffer; embedding in Spurr's medium. (a) Myxobacteria microcyst-like cells from a fraction obtained by centrifugation washing of soil; note the thick capsule-like area (arrow); (b) Azotobacter cyst-like cell from a fraction obtained by PEG two-phase partition of soil; note the cell surrounded by the intine-like (IN) and ruptured, exine-like (EX) materials; (c) Azotobacter cyst-like cells from a fraction obtained by exhaustive centrifugation washing of soil; note the intine-like (IN) and exine-like (EX) materials, and halo-like area (H). Bar markers represent 0.2 μm.*



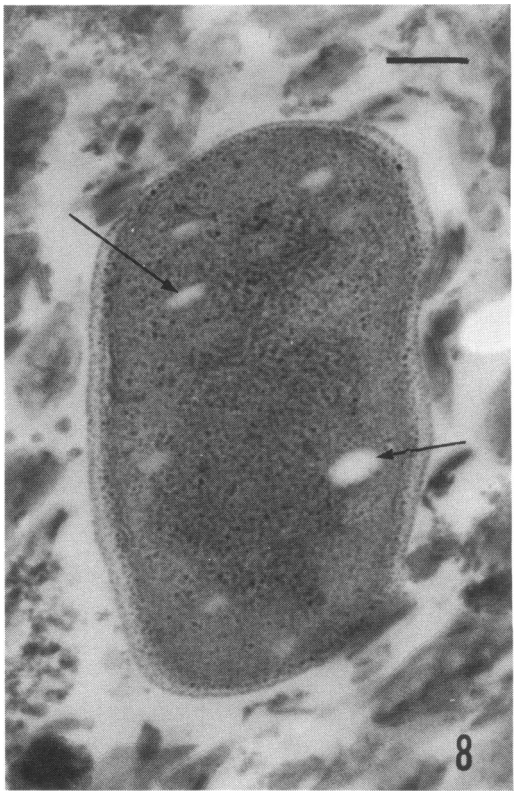


FIG. 7. *Section of a cell from a fraction obtained by centrifugation washing of soil, showing electron-dense granules (DG) and blebs (B) on the cell wall. Fixation in neutral Veronal-buffered OsO_4; embedding in Spurr's medium. Bar marker represents 0.1 μm.*

FIG. 8. *Section of a cell from a fraction obtained by centrifugation washing of soil, showing electron-transparent areas (arrows). Fixation in 1% OsO_4 in Kellenberger buffer; embedding in Spurr's medium. Bar marker represents 0.1 μm.*

they may have been lost with the soil debris during the cell separation and concentration procedures. A few of the dwarf cells were 0.08 μm in diameter, which is well below the resolution limit of light microscopy.

Cell sections showing a cyst-like or capsule-like structure comprised 26.7% and 7.6%, respectively, of the cells examined, whereas cysts are rarely encountered in laboratory-grown cultures of soil isolates. Some of the cyst-like structures (Fig. 4a) resembled the microcyst of myxobacteria, while others had features of the cyst of *Azotobacter* (Fig. 4b, c). The halo-like area observed around some of these cells (Fig. 4c) might have been caused by shrinkage of the cells during preparation for electron microscopy. However, it also is possible that this area may be an inherent structural part of the cyst-like organism, or that some unknown material may have been lost from this area during preparation.

As observed by various forms of light microscopy (7), a greater number of in situ cells in soil had what appeared to be a capsule-like area surrounding them than was apparent by electron microscopy of thin sections. This structure, however, may correspond to the outer coat of the cyst-like cells as observed by electron microscopy, particularly since light microscopy showed that this structure probably was not a capsule (7). It is also possible that capsules were present (including a capsule beyond the exine-like layer), but that they often were lost during cell preparation for electron microscopy. In studies on peat cultures of *Rhizobium trifolii*, Dart, Roughley, and Chandler (8) noted a matrix of fine, fibrous materials connecting the cells with the peat surface, but the nature of this material was not determined.

The number of cells in the various soil fractions having certain of the features of an *Azo-*

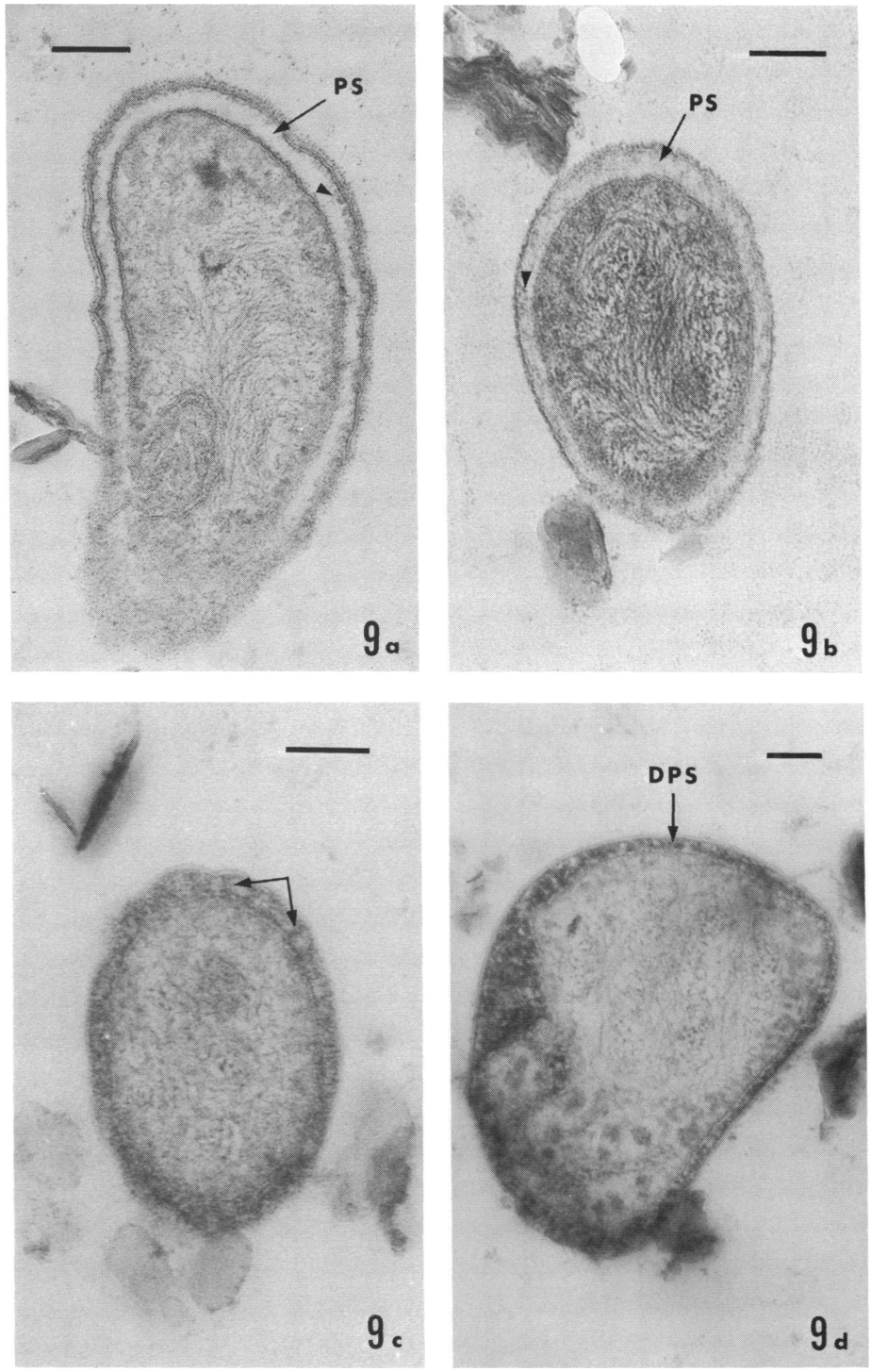
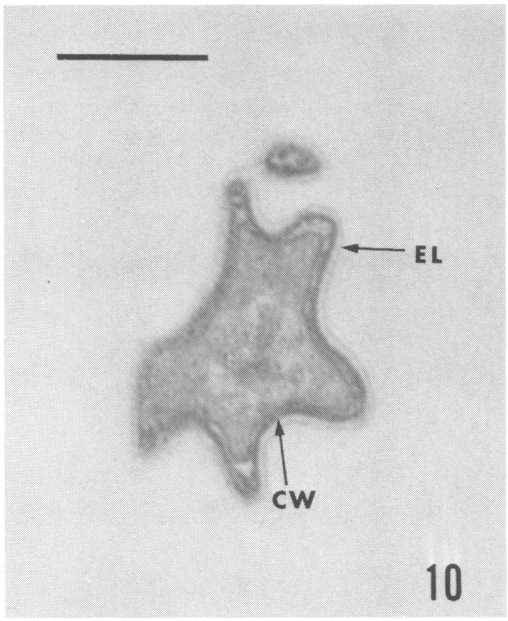
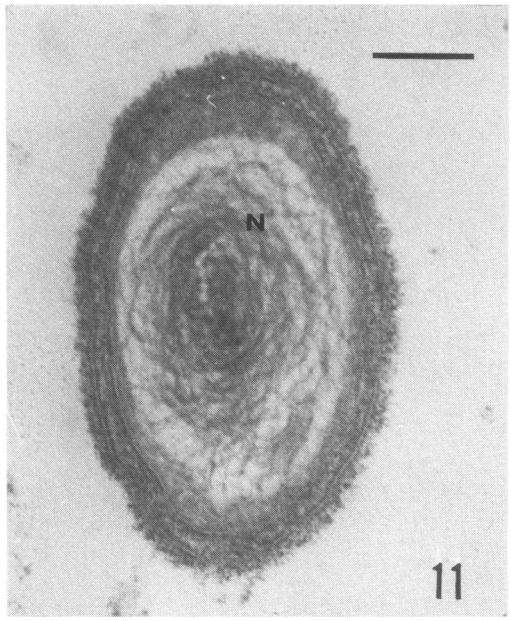
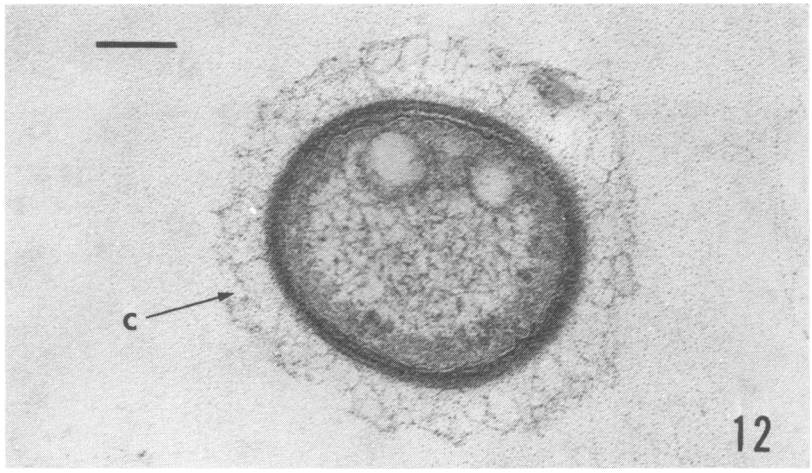
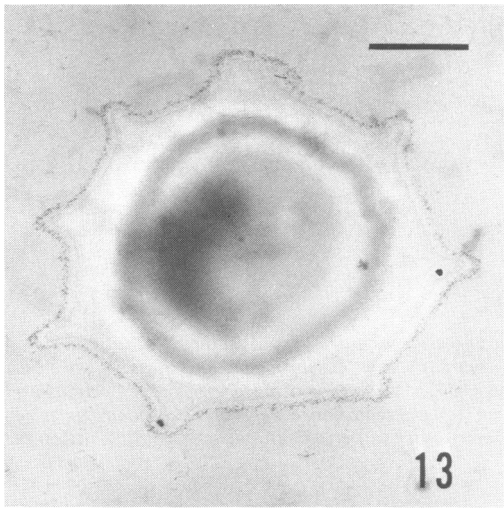


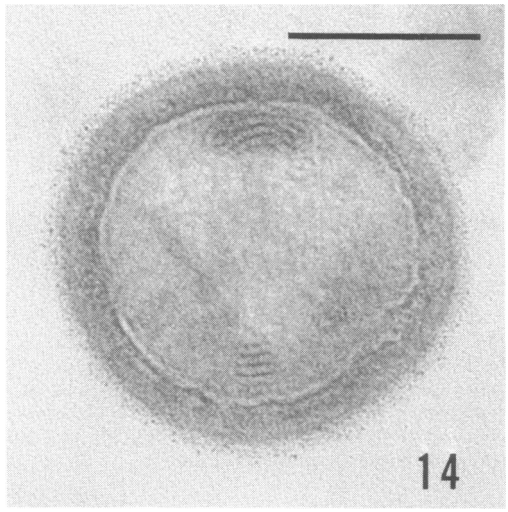
FIG. 9. *Sections of cells from fractions obtained by centrifugation washing of soil, showing a distinct periplasmic space (PS). Embedding in Spurr's medium. (a) Remnants of some unknown materials in the periplasmic space (arrow); fixation in neutral Veronal-buffered OsO_4; (b) unknown materials in the periplasmic space (arrow); fixation in 1% OsO_4 in Kellenberger buffer; (c) electron-dense pegs or bridges in the periplasmic space (arrow); fixation as for (b); (d) unusually electron-dense periplasmic space (DPS); fixation as for (b). Bar markers represent 0.1 μm.*











FIGS. 10 TO 14

tobacter cyst actually may have been considerably greater than reported herein, because cells possessing only residual fragments of a structure resembling an *Azotobacter* exine (Fig. 5) were not counted. Thus, layers resembling intine or exine associated with these cells may have been partially removed during preparation for electron microscopy; the exine of the *Azotobacter* cyst is easily removed by a number of chemicals (11, 23, 31).

The mesosomes (Fig. 6a, b) found in cell sections from soil fractions were not as extensive as the membranous organelles observed in the nitrifying autotrophs (19, 25). The latter, however, were observed only in laboratory-grown cultures. The electron-dense granules (Fig. 7) and electron-transparent areas (Fig. 8) may be polyphosphate granules and poly β-hydroxybutyrate, or glycogen, respectively. However, it is also possible that the electron-dense area is a metabolic response of the organism to its soil environment, and that the electron-transparent area may be the result of degradation of cellular components.

The function of the rather extensive periplasmic space observed in some of the soil microorganisms is not known. This space does not appear to be a result of shrinkage during preparation for electron microscopy because, in some cases, the entire plasma membrane is uniformly separated from the cell wall by the periplasmic space (Fig. 9a–c). An unknown material appears to be present in the periplasmic space of some of these cells (Fig. 9b). The unusually electron-dense periplasmic space observed for certain cells (Fig. 9c, d), and also observed in rhizobia peat cultures (8), might be related to the presence in this space of enzymes such as alkaline phosphatase (13) and glucose-6-phosphatase (18). This electron opacity also might represent "pegs" or "bridges" (Fig. 9c) connecting the outer plasma membrane to the inner cell wall (9, 14, 27).

The blebs on the cell walls of some of the organisms (Fig. 7) may have been the result of sloughing of the cell walls. The star-shaped cells (Fig. 10) may represent an artifact of cell preparation for electron microscopy, but cannot at present be dismissed as being an authentic form for certain soil microorganisms.

Some of the cells of the in situ soil microorganisms appeared to have large and somewhat congealed nuclear regions (Fig. 11), and they also appeared to lack ribosomes. The latter could suggest that a turnover of internal polymers actually is occurring under the nutritionally poor conditions of soil, although this also might represent a problem in fixation.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant GB-14487 and contract NGR 39-009-180 with the National Aeronautics and Space Administration.

We thank C. L. Jeffries for able technical assistance.

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FIG. 10. *Section of a star-shaped cell from a fraction obtained by exhaustive centrifugation washing of soil. Prepared as for Fig. 8. Note the enveloping layer (EL) outside the cell wall (CW). Bar marker represents 0.1 μm.*

FIG. 11. *Section of a cell from a fraction obtained by exhaustive centrifugation washing of soil, showing congealed and large nuclear region (N). Prepared as for Fig. 8. Note the apparent lack of ribosomes. Bar marker represents 0.1 μm.*

FIG. 12. *Section of a cell from a fraction obtained by exhaustive centrifugation washing of soil, showing a capsule (C). Prepared as for Fig. 8. Bar marker represents 0.1 μm.*

FIG. 13. *Section of a bacterial spore from a fraction obtained by Ludox density gradient centrifugation of soil. Prepared as for Fig. 8. Bar marker represents 0.2 μm.*

FIG. 14. *Section of a mycobacteria-like cell from a fraction obtained by exhaustive centrifugation washing of soil. Prepared as for Fig. 8. Bar marker represents 0.1 μm.*

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