

# Improved Staining of Extracellular Polymer for Electron Microscopy: Examination of *Azotobacter*, *Zoogloea*, *Leuconostoc*, and *Bacillus*

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Phase contrast, ultraviolet microscopy, and freeze-etching were used to determine the amount of exocellular polymer surrounding unfixed cells of four genera of bacteria: *Azotobacter vinelandii*, *Zoogloea ramigera*, *Leuconostoc mesenteroides*, and an acid-tolerant, floc-forming *Bacillus* species. Thin-sectional electron microscopy was employed to measure the effectiveness of a modified ruthenium red staining method. The results obtained with this modification of ruthenium red staining technique were compared to results obtained when previously proposed ruthenium red methods of fixation were employed. The results of these relations were then compared to the amounts of exocellular material as determined with phase-contrast microscopy, ultraviolet microscopy, and freeze-etching. The data obtained indicate that improved fixation of exocellular polymer is achieved when cells are pretreated with ruthenium red as described herein. In addition, the modified methods also reveal cytological detail not apparent when other methods of ruthenium fixation are employed.

Early investigations of external layers of microorganisms revealed that certain components (e.g., slime, capsules, walls) could be demonstrated with the light microscope. Electron microscopy visualization of external bacterial polysaccharide has been achieved with the employment of negative staining (5) or shadow-casting. However, capsular material has been observed to crack, whereas slime layers sometimes absorb stains, decreasing the efficiency of negative staining. Although shadow-casting allows visualization of non-soluble extracellular polymer, internal cellular detail is not apparent. Attempts to adequately fix this material for ultrastructural study have not been very successful. Pate and Ordal (8) have achieved improved staining of *Chondrococcus columnaris* when ruthenium red was employed during prefixation with glutaraldehyde. Other workers have applied their methods to better visualize extracellular polymer in animal (11), plant (10), and microbial (1) thin sections. More recently, freeze-etching

techniques have been employed to study the structure and distribution of extracellular polysaccharide, as well as internal cellular detail (7).

This report describes a modification of the Luft ruthenium red staining technique, as reported by Pate and Ordal (8). The results of this study demonstrate improved staining qualities of extracellular polysaccharide, as well as improved internal cytological detail. The results obtained when these methods are used are compared to those obtained when Luft's methods are employed, as well as a comparison of these methods to light microscopy and frozen-etched preparations.

## MATERIALS AND METHODS

The four organisms used in this study are *Azotobacter vinelandii* ATCC 12837, *Zoogloea ramigera* I-115 (The Ohio State University, Culture Collection), *Leuconostoc mesenteroides* (OSU 553, The Ohio State University, Culture Collection), and an acid-tolerant, slime-forming *Bacillus* sp., previously characterized (4).

*A. vinelandii* ATCC 12837 was grown at 26 C in Burk's nitrogen-free salts (13) supplemented with 1%

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glucose. Vegetative forms were induced to encyst by transferring them to the same basal medium supplemented with 0.3% *n*-butanol and 2% agar. *Z. ramigera* I-115 was cultured in the following modification of the arginine medium of Crabtree et al. (2): arginine-hydrochloride, 0.5 g; alanine, 1.0 g;  $MgSO_4 \cdot 7H_2O$ , 0.2 g;  $K_2HPO_4$ , 2.0 g;  $KH_2PO_4$ , 1.0 g; glucose, 5.0 g; vitamin B12,  $1.5 \times 10^{-6}$  g; and distilled water to 1 liter. Cultures of *Z. ramigera* were grown for 18 to 24 hr at 26 C. *L. mesenteroides* was cultured in the medium of deMan et al. (3), with sucrose replacing glucose as the carbohydrate source, and was incubated for 18 hr at 25 C. The previously described (4) acid-tolerant, slime-producing *Bacillus* sp. was grown in the following modification of the medium of Thorne et al. (12): glycerol, 80 g; citric acid, 12 g; glutamic acid, 20 g;  $NH_4Cl$ , 7 g;  $MgSO_4 \cdot 7H_2O$ , 0.5 g;  $FeCl_3 \cdot 6H_2O$ , 0.04 g;  $K_2HPO_4$ , 0.5 g;  $CaCl_2$ , 0.15 g;  $MnSO_4 \cdot H_2O$ , 0.42 g; to 1 liter with distilled water, pH adjusted to 3.0. Cultures of this organism were grown for 24 hr at 24 C, at which time large flocs of the bacillus had formed.

**Phase-contrast and ultraviolet microscopy.** Aqueous solutions (0.1%) of Violet R monastral, Pontamine, and Paper White BP (diamine stilbene disulfonic acid dyes possessing high affinities for cellulose; E. I. DuPont de Nemours & Co., Inc., Chicago, Ill.) were used to stain cultures. Wet mounts of the cells, stained with fluorescent dye, were examined under phase-contrast and ultraviolet illumination with a Zeiss Universal microscope equipped for phase-fluorescence epi-illumination with ultraviolet light. Photomicrographs were taken on Kodak Tri-X film using a Nikon AFM microflex sensor.

**Freeze-etching.** Cells of each of the four organisms were prepared for freeze-etching by centrifuging into a pellet. The bacteria were placed on a copper disc (3 mm) that had been scratched to aid in adherence of the cells, quickly frozen in Freon 22 and rapidly transferred to liquid nitrogen. The copper disc was removed from the liquid nitrogen, placed on a precooled stage (-100 C) in a "Balzers"

apparatus (model 360 M), and frozen-etched (9). Following etching, the copper disc was removed and dipped in distilled water until the replica floated free. The replica was then transferred to 70%  $H_2SO_4$  and treated for a period of 1 hr, rinsed in distilled water, treated for another hour with Eau de Javell (9), and finally rinsed several times with distilled water. The replicas were then collected on an uncoated 400-mesh copper grid, allowed to dry, and examined with the electron microscope.

**Thin sections.** The methods of pretreatment, fixation, and staining are described below. All cells were dehydrated by passage through a graded alcohol series, followed by two rinses in propylene oxide. The bacteria were then embedded in Epon 812, and the resin was polymerized for 24 to 48 hr at 60 C. Thin sections were obtained on a Reichert Om-12 ultramicrotome, stained with uranyl acetate and then with lead citrate for 7 to 10 min. Thin sections were collected on 300-mesh copper grids and examined with a Phillips EM-300 electron microscope at an 80-kv accelerating voltage.

**Method 1.** The method employed when cells were prepared for electron microscopy was similar to that of Pate and Ordal (8). Cells were removed from the growth medium by centrifugation and were suspended in a solution containing 3.6% glutaraldehyde-0.15% ruthenium red (w/v) in 0.1 M cacodylate buffer for 1 hr at ambient temperature. The cells were washed twice with buffer and resuspended for an additional hour in a solution of 1%  $OsO_4$ -0.15% ruthenium red in 0.1 M cacodylate at 4 C. Following fixation, the bacteria were washed twice with buffer, dehydrated, and embedded as described above.

**Method 2.** Cells were collected from the growth medium by centrifugation and were suspended in a 0.15% solution (w/v) of ruthenium red for periods varying from 2 min to 1 hr prior to treatment with glutaraldehyde. The bacteria were centrifuged and treated as described in Method 1, except that all washings were performed with 0.15% ruthenium red in 0.1 M cacodylate buffer.

FIG. 1. Phase-contrast photomicrograph of *A. vinelandii* during the vegetative phase of growth. Marker = 10  $\mu m$ .  $\times 2,200$ .

FIG. 2. Same field as shown in Fig. 1, except that ultraviolet illumination has been employed. The fluorescent dye (Violet R monastral) employed acts as a negative stain, illustrating the extent of the exocellular polymer. Marker = 10  $\mu m$ .  $\times 2,200$ .

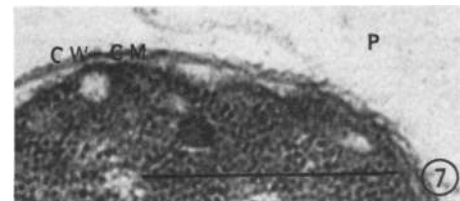
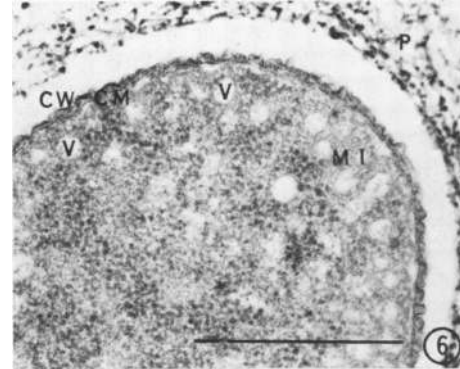
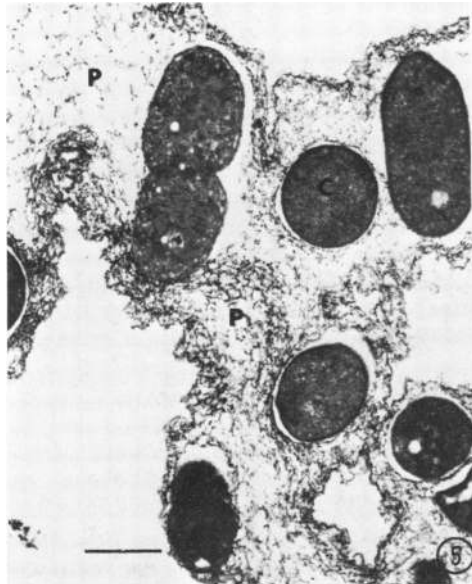
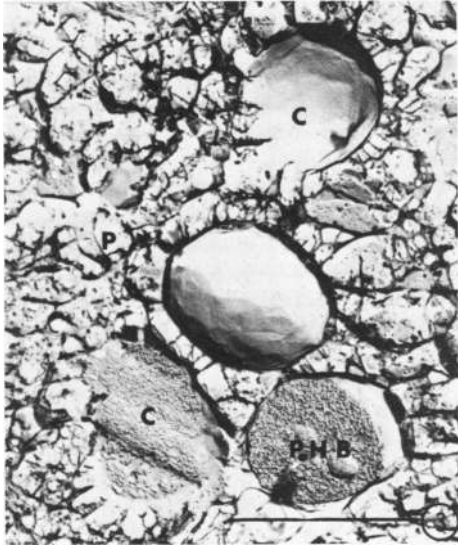
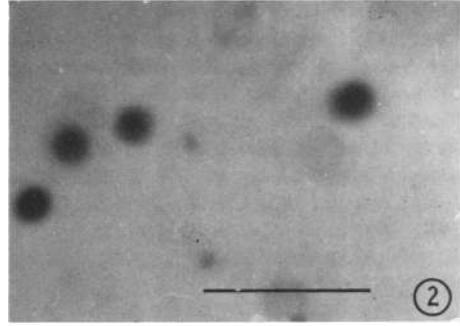
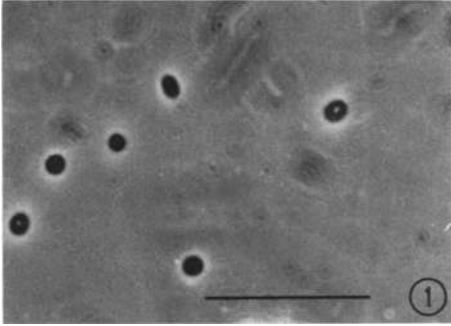
FIG. 3. Frozen-etched preparation of *A. vinelandii* showing polymer (P) distribution around the cells (C). One cell has been cleaved through the center to reveal several granules of poly- $\beta$ -hydroxybutyric acid (PHB). Marker = 1  $\mu m$ .  $\times 25,000$ .

FIG. 4. Thin sections of *A. vinelandii* fixed according to the methods of Pate and Ordal. Polymer (P) is distributed around the cells (C). Marker = 1  $\mu m$ .  $\times 17,000$ .

FIG. 5. Thin section of *A. vinelandii* as shown in Fig. 4 except that these cells have been pretreated with ruthenium red. Polymer (P) surrounding the cells (C) is much more evident than that in Fig. 4. Marker = 1  $\mu m$ .  $\times 13,400$ .

FIG. 6. Higher magnification of cells treated as in Fig. 5. The appearance of the cell wall-cell membrane (CW-CM) is much clearer and more defined than in Fig. 7. Polymer distribution (P) is also more distinct. Several membrane-bound vesicles (V) and membranous invaginations (MI) can be seen at the periphery of one cell. Marker = 1  $\mu m$ .  $\times 28,000$ .

FIG. 7. A portion of the cell wall-cell membrane (CW-CM) from the cells shown in Fig. 4. Polymer (P) surrounding the cells is also apparent. Marker = 1  $\mu m$ .  $\times 34,000$ .



## RESULTS

Individual cells and flocs of each of the four organisms were examined with phase-contrast and ultraviolet microscopy, and with the electron microscope, utilizing frozen-etched preparations and thin-sections prepared by the two previously described procedures. Initially, two gram-negative organisms, *A. vinelandii* and *Z. ramigera*, both observed to produce large amounts of extracellular polymer, were chosen for this study.

When *A. vinelandii* was examined with phase-contrast microscopy, after staining with Violet R monastral, a fluorescent dye, only faintly defined areas of exocellular material were observed surrounding the individual cells (Fig. 1). When these same cells were observed with ultraviolet illumination, the extent of the exocellular material became more evident, as the entire background fluoresced, whereas extracellular polymer or cells did not (Fig. 2). Aggregates of *A. vinelandii* also fluoresced when stained with other fluorescent dyes used in this study. The examination of frozen-etched replicas of *A. vinelandii* (Fig. 3) indicated that exocellular polymer (P) is present in large quantities around cells of the organism. We had previously employed ruthenium red, after Pate and Ordal (8), to stain polysaccharide surrounding the cells of *Azotobacter* (1) although the results (Fig. 4) obtained did not indicate the extent of polymer distribution as shown with fluorescent microscopy (Fig. 2) or with frozen-etched replicas (Fig. 3). The advantage of preincubation in ruthenium red prior to treatment with glutaraldehyde and ruthenium red is apparent in Fig. 5, which illustrates the relation and distribution of the

cells of *A. vinelandii* to the extracellular polymer. Cells of *A. vinelandii* fixed after Pate and Ordal (8) (Fig. 4) show the fibrous capsule polysaccharide (P), which extends beyond the cells, gradually decreasing in amount. On the other hand, when the same cells are prepared with pretreatment with ruthenium red, the bacteria appear embedded in a polysaccharide matrix (Fig. 5). Preincubations of as little as 3 min were observed to improve staining qualities in the bacteria studied. Periods longer than 30 min did not appear to improve staining qualities of exocellular polymer, or cytological detail. The material which surrounds the cells appears of the same fibrous consistency as when fixed with the previously described methods (8), although the quantity of polymer is evidently greater. The extracellular polymer appears to surround all of the cells, and, without exception, cells of *A. vinelandii* were not observed outside this matrix. Improved cytological staining was also observed in the cells preincubated in ruthenium red prior to prefixation with glutaraldehyde-ruthenium red, particularly in the region of the outer envelope of the organism (Fig. 6). In Fig. 6, the cell wall-cell membrane of bacteria pretreated with ruthenium red appears much more defined than those prepared by Luft's method as reported by Pate and Ordal (8) (Fig. 7). In addition to the increased resolution afforded by ruthenium red pretreatment, the amount of intracellular membrane is also observed to increase. Numerous membrane-bound vesicles, membranous invaginations around the periphery of the cell, are apparent (Fig. 6).

*Z. ramigera*, a pseudomonad, was also exam-

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Fig. 8. Phase-contrast photomicrograph of *Z. ramigera* stained with Pontamine and illuminated with visible light. Area of polymer is evident around cells. Marker = 10  $\mu$ m.  $\times 2,100$ .

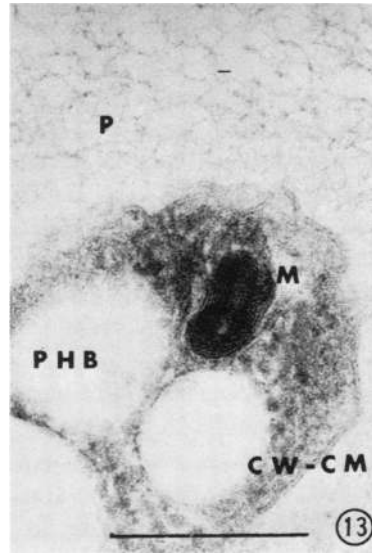
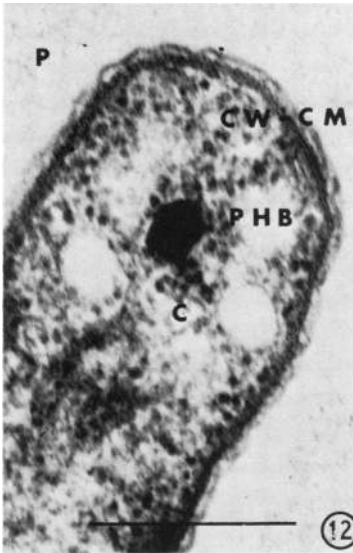
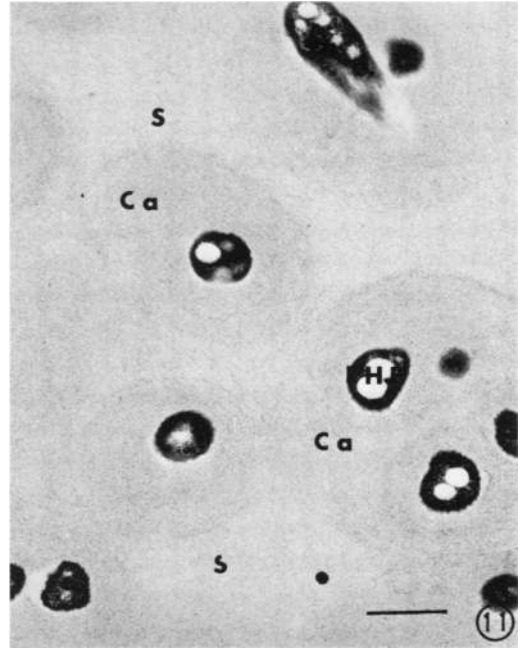
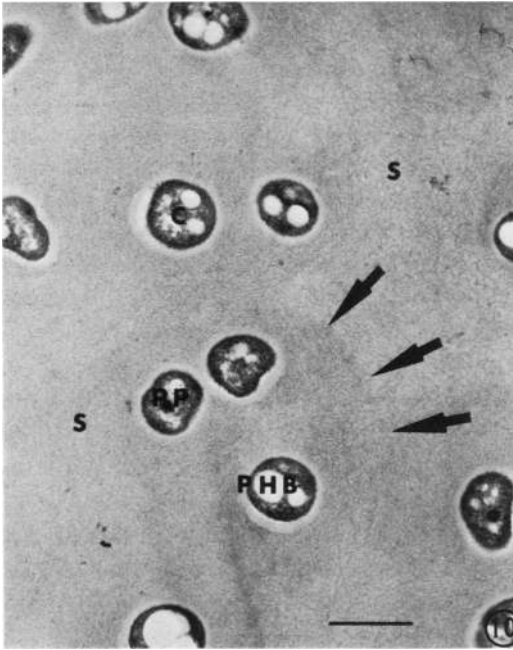
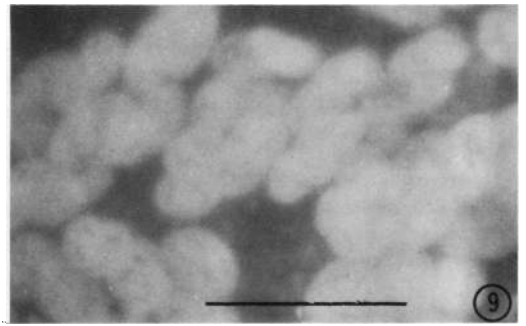
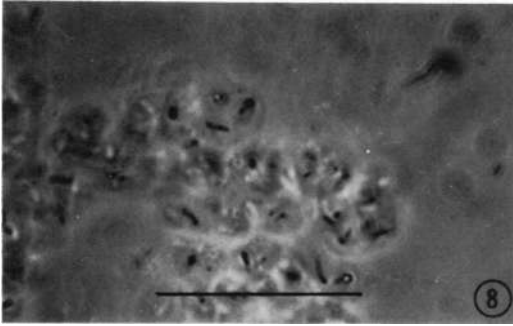
Fig. 9. Phase-contrast photomicrograph of the same cells shown in Fig. 8, except illumination is by ultraviolet light. Capsular areas are much more evident and extend much further around cells. Marker = 10  $\mu$ m.  $\times 2,100$ .

Fig. 10. Low-magnification electron micrograph of *Z. ramigera* fixed with the glutaraldehyde-ruthenium (Method 1). A nearly homogeneous slime layer (S) appears between cells (C). Faintly visible (arrows) is a capsular area, while inside the cells deposits of poly- $\beta$ -hydroxybutyric acid (PHB) and polyphosphate (PP) are visible. Marker = 1  $\mu$ m.  $\times 12,600$ .

Fig. 11. Cells are from the same culture as those in Fig. 10, except fixed with ruthenium red pretreatment (Method 2). Capsular areas (Ca) surround individual cells or cell groups, with slime (S) interconnecting cell groups. Intracellular deposits of PHB are also apparent. Marker = 1  $\mu$ m.  $\times 12,500$ .

Fig. 12. Electron micrograph of a single cell of *Z. ramigera* fixed according to Pate and Ordal (Method 1). Polysaccharide (P) surrounding the cell (C) appears indistinct, whereas the cell wall-cell membrane complex (CW-CM) appears very electron dense. Within the cell, PHB and a structure which appears to be a polyphosphate (PP) deposit are clear. Marker = 1  $\mu$ m.  $\times 29,000$ .

Fig. 13. Cell similar to that observed in Fig. 12, except treated, prior to treatment with glutaraldehyde, with ruthenium red (Method 2). The fine structure of the exocellular polymer (P), the cell wall-cell membrane (CW-CM), and a structure analogous to the polyphosphate deposit in Fig. 12, now apparent as a mesosome (M), are shown. Marker = 1  $\mu$ m.  $\times 27,500$ .



ined by using the procedures described above. When the organism was observed with phase-contrast microscopy, a distinct exocellular polymer was visible surrounding the cells (Fig. 8). Although this capsular matrix is evident with phase-contrast microscopy alone, the extent of this material beyond the cell is shown much more clearly in Fig. 9, which illustrates the same cells stained with the fluorescent dye, Pontamine, and photographed with ultraviolet illumination. When *Z. ramigera* was fixed for electron microscopy, as described by Pate and Ordal (8), large quantities of exocellular polymer were observed (Fig. 10). Faintly visible around the bacterium, a poorly defined capsular layer (arrows) was evident, with slime dispersed between the cell groups. Quantities of poly- $\beta$ -hydroxybutyric acid and structures which appear to be polyphosphate deposits are also observed in Fig. 10. When cells of the same culture are prepared for electron microscopy by the methods described in this study, notable improvement in general fixation quality is achieved. Improved capsular fixation is illustrated in Fig. 11; a discrete capsule surrounds each of the cell groups, with slime interdispersed only between cell group capsular areas. Improved fixation and staining qualities of cytoplasmic structures in *Z. ramigera* were also noted, when pretreatment with ruthenium red was employed. Structures previously described as polyphosphate bodies (Fig. 10), and enlarged in Fig. 12, demonstrate no distinct internal structure. In contrast, cells pretreated with ruthenium red (Fig. 13) appear to contain distinct mesosomes at sites corresponding to polyphosphate deposits in Fig. 12. During our observations when ruthenium red was used in conjunction with glutaraldehyde (8), inclusions in *Z. ramigera* appeared only as polyphosphate deposits (Fig. 12), but, when the bacteria were treated with ruthenium red prior to ruthenium red-glutaraldehyde fixation, the sites corresponding to a polyphosphate deposit appeared

as a distinct mesosome (Fig. 13). The results obtained with bacterial thin sections preincubated with ruthenium red correlate well with data obtained for polymer distribution as revealed by frozen-etched preparations (5).

Two gram-positive bacteria, *L. mesenteroides* and *B. cereus* were also used in these studies to test the applicability of these methods to other bacterial types. *L. mesenteroides* grown in media containing sucrose produces copious quantities of polysaccharide. Phase-contrast microscopy of cellular flocs illustrates the nature and distribution of this material (Fig. 14). These same cells (flocs) were observed to fluoresce when stained with the fluorescent dye, Paper White BP (Fig. 15), and observed with ultraviolet illumination. *L. mesenteroides* pretreated with ruthenium red, as described in this study, demonstrates a more clearly defined capsular area than in those bacterial cells fixed with ruthenium red-glutaraldehyde alone (Fig. 16). Although dispersed capsular polysaccharide can be observed in portions of Fig. 16, the amount and distribution of this material are clearly greater in Fig. 17, in which the cells were pretreated with ruthenium red. In the case of *L. mesenteroides*, mesosomes were observed with both methods of preparation of cells for electron microscopy. General cytological detail is, however, improved. Areas such as the cell wall-cell membrane, fine structure of mesosomes, and cell cytoplasm are more evident in the cells depicted in Fig. 17. When the results obtained with ruthenium red pretreatment are compared to those obtained with frozen-etched preparations, the amount of exocellular polymer demonstrable with each method appears quantitatively similar (Fig. 18).

The acid-tolerant, slime-forming *Bacillus* fluoresced differently when stained with the fluorescent dye Pontamine than did the other bacteria used in this study. In Fig. 19, a phase-contrast photomicrograph of the *Bacillus* sp.

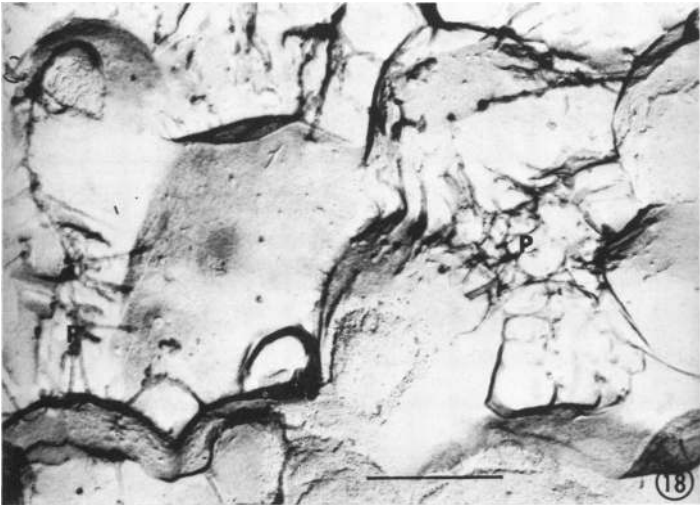
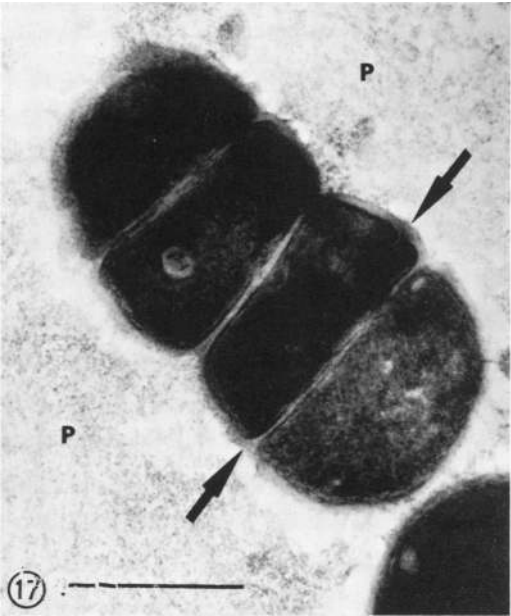
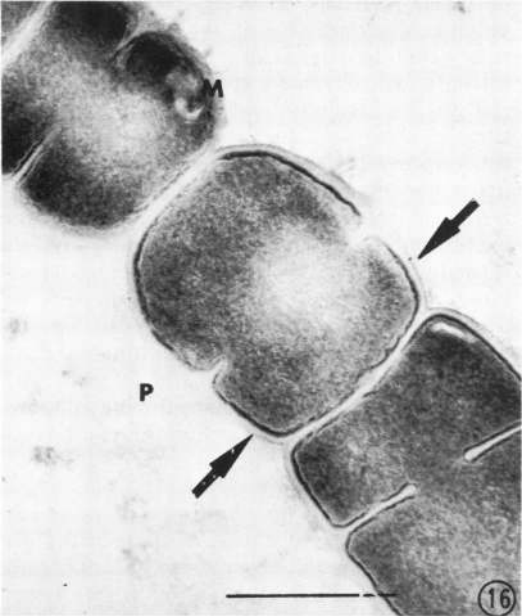
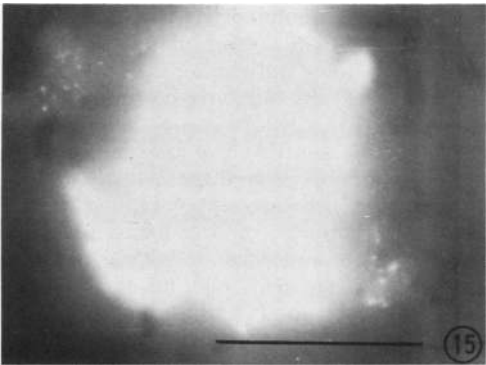
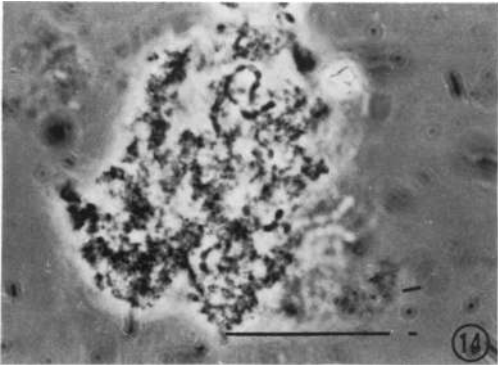
FIG. 14. Phase contrast of *L. mesenteroides* stained with Paper White BP and observed with visible light. Large quantities of extracellular polysaccharide are visible in Fig. 15 around chains of cells. Marker = 10  $\mu$ m.  $\times 2,100$ .

FIG. 15. Phase contrast of the same field shown in Fig. 14, except illuminated with ultraviolet light. The amount of polysaccharide surrounding the cells is evident. Marker = 10  $\mu$ m.  $\times 2,100$ .

FIG. 16. Electron micrograph of *L. mesenteroides* fixed with glutaraldehyde-ruthenium red, according to Pate and Ordal. Surrounding the chain of cells, a polysaccharide sheath (arrows) is evident, with dispersed polymer (P) also visible. A mesosome (M) is also visible in these cells. Marker = 1  $\mu$ m.  $\times 25,000$ .

FIG. 17. Electron micrograph of cells from the same culture as in Fig. 16, treated with ruthenium red prior to fixation. The polymeric sheath (arrows) surrounding the cells is apparent, although much more dispersed polysaccharide (P) is apparent. A mesosome (M) can be seen inside one of the cells comprising the chain. Marker = 1  $\mu$ m.  $\times 27,000$ .

FIG. 18. Frozen-etched preparation of *L. mesenteroides* showing the polysaccharide which encases the cells, as well as dispersed polymer (P). Marker = 1  $\mu$ m.  $\times 20,000$ .





(4), no exocellular polymer is evident about the cells. However, when examined with ultraviolet illumination, individual cells fluoresce, indicating a distinct slime coat surrounds each of the cells (Fig. 20). Frozen-etched preparations (Fig. 21) confirm the presence of this slime layer around each of the cells, as well as indicating the presence of exocellular fibrils. Thin-sectional studies of the bacterium (Fig. 22) support the presence of both exocellular fibrils and a slime coat. *Bacillus* sp. fixed according to the methods of Pate and Ordal (8) (Fig. 22) is observed to possess a thick, ruthenium-red-positive coat surrounding the cells. Several strands are apparent around each of the cells in Fig. 22 which appear to have become detached from the organism during the processes of preparation for electron microscopy. The employment of ruthenium red as a pretreatment prior to suspension of the cells in glutaraldehyde-ruthenium red improves the staining properties of the bacterial cells. The exocellular slime coat (Fig. 23) appears similar to that seen in Fig. 22, whereas exocellular fibrils (Fig. 24) appear much more defined when cells are pretreated with ruthenium red. The composition of the slime coat in this organism appears qualitatively different from those in *Azotobacter*, *Zoogloea*, and *Leuconostoc*. The sheath surrounding this acid-tolerant *Bacillus* may be composed of mucopeptide, rather than a polysaccharide, as in the other three bacteria. If this is the case, this would partially explain the presence of exocellular fibrils and the unique type of fluorescence demonstrated by this organism. Internal structure of this organism appeared to vary little regardless of the manner of fixation involved. Therefore, no statement can be made concerning the effect of these fixation methods on improved internal structure.

## DISCUSSION

Although ruthenium was developed and

implemented for electron microscopy staining of polysaccharide by Luft (8) approximately 8 years ago, no modifications of this staining method have been proposed. To demonstrate that the methods employed in this study do fix extracellular polymer better than those previously proposed, we had to demonstrate the amount of exocellular material present around cells used in this study. For this reason, we employed phase-contrast and ultraviolet microscopy to inspect the amount of material present surrounding the cells at the light microscopy level. In addition, we used freeze-etching procedures, which do not incorporate chemical fixatives, to compare the extent of polymer demonstrable at the electron microscopy level to chemically fixed thin sections. Initially, we observed that, when ruthenium red was preincubated with cells for periods of time as short as 2 to 3 min, capsular fixation was improved. Using *A. vinelandii* as a preliminary system, we observed a faint ruthenium-red-positive area around cell aggregates of the bacterium. By increasing the contact time of ruthenium red with the cells prior to treatment with glutaraldehyde-ruthenium red, we found that capsular fixation could be improved. Optimal preincubation time in ruthenium red was determined to be 30 min, with longer periods of pretreatment not notably improving fixation and staining qualities of exocellular polymer, or internal cytological detail.

In addition to *A. vinelandii*, we chose to test the applicability of these methods with another gram-negative bacterium, *Z. ramigera*, and two gram-positive bacteria, *L. mesenteroides* and an acid-tolerant variety of *Bacillus*. In each case, comparative studies of the organisms from the same cultures fixed by the methods of Pate and Ordal (8) and by our methods indicated that improved fixation and staining qualities were achieved with our modification. The most obvious improvement in

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FIG. 19. Phase-contrast photomicrograph of *B. cereus* stained with Pontamine and examined with visible light. No exocellular polymer is visible around cells of this organism. Marker = 10  $\mu$ m.  $\times 2,500$ .

FIG. 20. Phase-contrast photomicrograph with ultraviolet illumination of the same cells shown in Fig. 20. Only the cells in Fig. 20 are observed to fluoresce, indicating a distinct layer surrounds each cell. Marker = 10  $\mu$ m.  $\times 2,500$ .

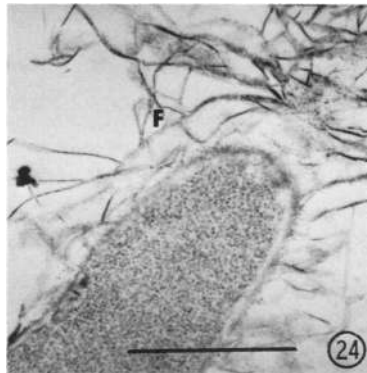
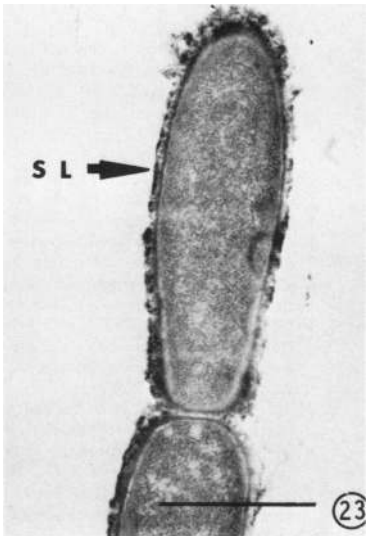
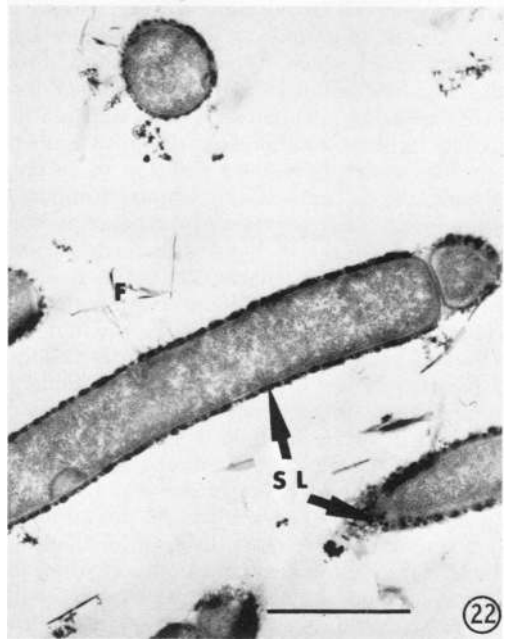
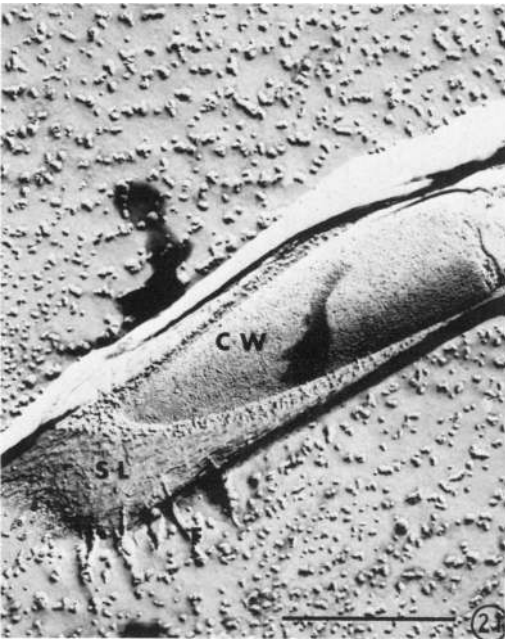
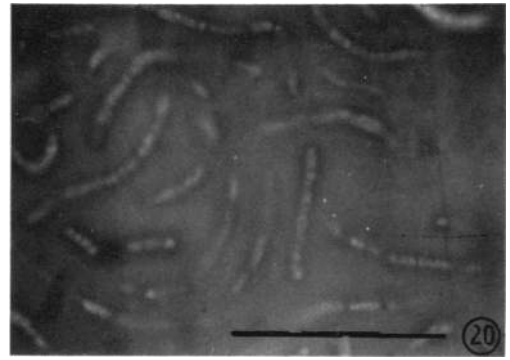
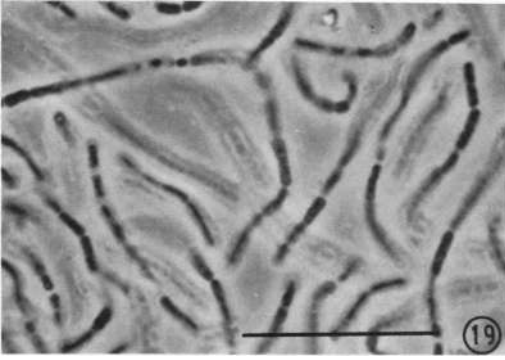
FIG. 21. Frozen-etched preparation of *B. cereus* showing the slime layer (SL) which surrounds the bacillus, as well as several fibrils (F) which radiate from it. The slime layer (L) has been cleaved away to reveal the cell wall (CW) of the organism. 24,000. Marker = 1  $\mu$ m.  $\times 21,000$ .

FIG. 22. Thin-sectional electron micrograph of *B. cereus* fixed as described by Pate and Ordal. Detached fibrils (F) are apparent around a cell which is encased in a ruthenium-red-positive slime layer (SL). Marker = 1  $\mu$ m.  $\times 21,500$ .

FIG. 23. Electron micrograph from the same culture as that seen in Fig. 23, except fixed according to the methods described here. A more defined slime layer (SL) surrounds these cells. Marker = 1  $\mu$ m.  $\times 22,500$ .

FIG. 24. Exocellular fibrils (F) surround the cell. Marker = 1  $\mu$ m.  $\times 25,000$ .





capsular fixation and staining was apparent in the gram-negative organisms. In addition to improved capsular and cytological detail notable in *A. vinelandii*, improved extracellular polymer appearance was observed with *Z. ramigera* (Fig. 10-13). When the two methods of preparation were compared (Fig. 10 versus 11 and 12 versus 13), differences were apparent. Improved cytological staining of internal organelles was most apparent in *Z. ramigera*, as evidenced by mesosomal structures (Fig. 12, 13). We propose that the presence of polyphosphate observed in normal thin sections as an electron-dense granule be reexamined in those organisms in which it has been found. From the data presented it is evident that, at least in *Z. ramigera*, adequate cellular fixation drastically improved cytological detail. The dense granules which have been referred to as polyphosphate (5) and which appear similar to granules in other bacteria also labeled as polyphosphate appear to be a series of concentric membranous layers that could just as easily be called a mesosome. We observed many sections during this study, and we did not find any dense nondefined granules in the Method 2 treatment. On the other hand, we found no well-defined membranous bodies as seen in Method 2 in the Method 1 samples. Furthermore, the location and distribution of the granules (Method 1) and mesosome-like structures (Method 2) appeared to be the same. We concluded that we were looking at the same structure in either case, but under greater resolution or more suitably stained in our modification (Method 2). In *A. vinelandii*, as well, we observed that the degree of detail discernable along the cell envelope indicates more membranous activity (Fig. 6 and 7).

With the gram-positive organisms, *L. mesenteroides* and the acid-tolerant *Bacillus*, the contrasted results of the two staining methods studied here are not as apparent as in the gram-negative organisms. Exocellular polymer in both organisms appears in greater quantities when our methods are employed, and this is particularly noticeable in *L. mesenteroides* (Fig. 17). Although mesosomes were observed when both methods of fixation were employed, we observed a generally improved appearance of cellular detail (Fig. 17). The *Bacillus* yielded results which revealed the fewest differences between the techniques employed. We proposed that this was due primarily to differences in the exocellular material surrounding the organism, which do not appear to be of a true polysaccharide nature.

The use of ruthenium red, as described by

Pate and Ordal (8), in cell preparation for electron microscopy has provided the best methods by which extracellular polysaccharide could be studied in thin sections. It has been shown that ruthenium red deposits itself randomly in polysaccharides containing two negatively charged sites, which possess an interchange distance of approximately 0.183 nm. The deposition of ruthenium red was first observed in pectins, which contain numerous charged sites at which ruthenium red is oxidized. We suggest that improved fixation of exocellular material, as well as improved cytological fine structure, is a result afforded by ruthenium red in that the materials which do oxidize ruthenium red become fixed prior to introduction of  $\text{OsO}_4$  into the system. The net effect of ruthenium red pretreatment is to increase the concentration of ruthenium red which can react. When ruthenium red is employed with glutaraldehyde, the action of the aldehyde is to partially mask the fixation of polysaccharides obtained when ruthenium red is incubated alone with bacterial cells. In addition, polysaccharide fixed with ruthenium red allows a more rapid and complete oxidation when  $\text{OsO}_4$  is added. Apparently these methods are less detrimental to cellular fine structure.

In light of the wide range of applicability which ruthenium red has had in the visualization of exocellular polymer, we feel that the methods proposed here will have a like effect, since it appears that these methods not only improve fixation and staining qualities of extracellular polymer, but also improve cytological detail as well.

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