PRESERVATION OF THE ULTRASTRUCTURE OF *BACILLUS SUBTILIS* BY CHEMICAL FIXATION AS VERIFIED BY FREEZE-ETCHING

N. NANNINGA

From the Laboratory of Electron Microscopy, the University of Amsterdam, Amsterdam, The Netherlands

ABSTRACT

The present study on the ultrastructure of *Bacillus subtilis* was undertaken in order to examine by means of the freeze-etching technique possible structural changes occurring during the chemical fixation procedure (Ryter-Kellenberger (R-K) fixation). Three stages were followed by freeze-etching, viz.: (*a*) fixation in osmium tetroxide, (*b*) fixation in osmium tetroxide and posttreatment with uranyl acetate, and (*c*) fixation in osmium tetroxide, posttreatment in uranyl acetate, and dehydration in a graded series of acetone. Preparations were made after each stage in the presence of 20% glycerol. Good preservation of ultrastructure was observed, after any of the three treatments, of the outer surface of the plasma membrane, and the inner surface of the plasma membrane. No alteration in fracturing properties could be observed. However, if we are to judge by the results of freeze-etching, any of the successive steps of the chemical fixation procedure achieve strong contrast between the nucleoplasmic region and the cytoplasm. Dependent on the quality of fixation, very delicately preserved DNA fibrils or strongly aggregated ones were seen. It appears that R-K fixation is capable of producing more or less distinctly visible changes in the native state of the nucleoplasm in young cells of *B. subtilis*.

The interpretation of the freeze-etch image of bacterial cells is largely influenced by previous knowledge obtained with thin sections, though direct comparison of sectioned material with freeze-etched preparations is not always easy to perform. For instance, *young* cells of *Bacillus subtilis* (17, 20), when freeze-etched, failed to show a clear differentiation in nucleoplasm and cytoplasm. But, if prior to freeze-etching a chemical fixation with osmium tetroxide is applied, the above differentiation becomes visible (15, 17). As a result of these observations we have investigated in greater detail the possible ultrastructural changes introduced by chemical fixation. To this end, freeze-etch preparations were made of specimens at different stages of the chemical fixation and embedding procedure. This type of approach has been anticipated by Steere (26) in his pioneer work on the technique of freeze-etching.

The chemical fixation and embedding procedure employed (R-K technique) here is the one introduced by Ryter and Kellenberger in 1958 (9, 23), which up till now has been used most widely for the study of bacterial fine structure. 6 yr later this technique was critically reviewed by its originators, who concluded that proper fixation can be achieved if the following requirements are met: (*a*) application of the acetate-Veronal buffer of Michaelis, pH 6.0; (*b*) a concentration of osmium tetroxide ranging from 1 to 3%; (*c*) presence of Ca^{++} or Mg^{++} ions in the fixative; (*d*) presence of tryptone or casaminic acids in the fixative; (*e*) fixation for 4–16 hr; and (*f*) posttreatment with uranyl acetate.

After the posttreatment, the specimens are dehydrated in acetone and subsequently embedded in Vestopal W (9, 23).

When this fixation technique is applied, the specific appearances of the nucleoplasm "vary with the authors as much as with the species" (8). Nevertheless, when the fixed nucleoplasm is well preserved, one observes fine fibrils either arranged in bundles or constituting a network (4, 8, 10, 29).

It is clear that with the R-K technique stress is put on the preservation of the nucleoplasm rather than on other cell constituents. The interpretation of the bacterial cytoplasm, for instance, continues to represent a serious problem (28). It is difficult to arrive at criteria for a reliable fixation of cytoplasmic ultrastructure, especially as regards its ribonucleoprotein component.

In the present work we have examined by means of freeze-etching the following steps in order to be able to make a comparison with sectioned material and to estimate the structural changes occurring during chemical fixation, viz.: (*a*) fixation in osmium tetroxide; (*b*) fixation in osmium tetroxide followed by posttreatment with uranyl acetate; and (*c*) fixation in osmium tetroxide, posttreatment with uranyl acetate, and dehydration in a graded series of acetone. The following (combinations of) cell components have been studied: cell wall and outer surface of the plasma membrane, inner surface of the plasma membrane, nucleoplasm, and cytoplasm.

Part of this work has been presented at the Fourth Regional European Conference on Electron Microscopy held in Rome in 1968 (15).

MATERIALS AND METHODS

Culture and Medium

B. subtilis, strain Marburg, was grown aerobically in heart infusion broth (Difco Laboratories, Detroit, Mich.) on a shaker at 35°C for about 4 hr. In contrast to previous experiments (17), the bacteria were not cultivated in the presence of glycerol.

Freeze-Etching after Chemical Fixation

The freeze-etching was performed essentially as described by Moor and Mühlethaler (12). Preparations for freeze-etching were made after the three stages of the fixation and embedding procedure (see below) and were completed once thick suspensions had been made in 20% (v/v) glycerol (Merck, Darmstadt, West Germany) in the acetate-Veronal buffer of Michaelis, pH 6.0, containing 0.01 M $MgCl_2$ (fixation buffer).

(*a*) FIXATION: After 4 hr of growth the cells were collected by centrifugation and resuspended in 1% (w/v) OsO_4 in fixation buffer plus tryptone (9 volumes of fixative and 1 volume of tryptone). 10 min thereafter the cells were once more sedimented and resuspended in fresh fixative plus tryptone, and they were left overnight at about 4°C.

(*b*) POSTTREATMENT: The fixation in OsO_4 was followed by two washes in fixation buffer. The washed cells were kept for 90 min in 0.5% (w/v) uranyl acetate in the fixation buffer. The posttreatment was done at about 4°C.

(*c*) DEHYDRATION: The dehydration was performed in acetone (30, 50, 70, 90, and 100% (v/v) for 15 min, and 100% for 1 hr). Control specimens (no chemical fixation) were made of sediments obtained from broth to which glycerol had been added to a final concentration of 20% (v/v) after 4 hr of growth, just before harvesting. In addition, preparations were made without glycerol, in 10 or 25% glycerol, and in 10% dimethylsulfoxyde (Serva, Heidelberg, West Germany).

Thin-Sectioning after Chemical Fixation

For thin sectioning the same procedure was followed as for fixation prior to freeze-etching, except that agar blocks (23) were made during stage *a* (see above). Dehydration in acetone was followed by embedding in Vestopal W (9, 23). Sections were cut with glass knives on an LKB ultratome III. No poststaining of sections was applied.

Electron Microscopy

Electron micrographs were taken with either a Philips EM 200 or EM 300 electron microscope.

The micrographs are printed in negative in order to produce a "natural" black shadow. The direction of shadowing is indicated by an arrow in the lower right-hand corner of each micrograph.

RESULTS

The general outline of the fine structure of freeze-etched *B. subtilis* has been described previously (17); complications arising in the spatial interpretation of electron micrographs of freeze-etched preparations were then also discussed.

The effects of the different treatments on the fine structure of *B. subtilis* as revealed with freeze-etching will be dealt with in the following four sections: cell wall and outer surface of the plasma membrane, inner surface of the plasma membrane, nucleoplasm, and cytoplasm. Some freeze-etch micrographs are accompanied by micrographs of thin sections showing a comparable situation.

Cell Wall and Outer Surface of the Plasma Membrane

Figs. 1–3 demonstrate the freeze-etch image of the cell envelope as compared with the profile of this envelope in a sectioned cell. The surface of the cell wall (*cw*, Fig. 1) is not always observed in our freeze-etch preparations (cf. references 3 and 17). It does not reveal a special substructure. Fig. 1 further shows the outer surface of the plasma membrane (*opm*) where the cell wall has been removed by fracturing. The outer surface of the plasma membrane is characterized by its numerous granules and short strands, each measuring about 50 A in diameter (cf. references 17 and 20). After the cell content is removed, a large area of the inner surface of the plasma membrane (*ipm*) can be seen as in Fig. 2. The inner surface is sparsely dotted with small particles (17, 20) and is quite different in appearance from the outer surface of the plasma membrane. A difference can also be noticed in the sectioned profile of the plasma membrane (Fig. 3); the inner profile (*ipm*) appears less dense than the outer one (*opm*). The sectioned profile of the cell wall in Fig. 3 resembles the cross-fractured profile in Fig. 2 (*cw*).

The outer surface of the cell wall after freeze-etching is visible in preparations fixed in osmium tetroxide (Fig. 5, treatment *a*; see Materials and Methods) and in preparations fixed in osmium tetroxide, posttreated with uranyl acetate, and dehydrated in acetone (Fig. 7, treatment *c*). No obvious changes in surface structure could be detected.

Likewise, the outer surface of the plasma membrane (*opm*) appears almost unchanged (Figs. 4–7). The good preservation of the surface of the plasma membrane after thorough dehydration in acetone is remarkable (Fig. 7).

Inner Surface of the Plasma Membrane

The inner surface of the plasma membrane (*ipm*) also withstands the different treatments very well (cf. Fig. 8 with Figs. 9–11), even dehydration in acetone (Fig.11).

Nucleoplasm

As found earlier, nucleoplasm and cytoplasm are not clearly set apart from each other, regardless of whether they have been cultivated in the presence (17) or absence (20) of glycerol after freeze-etching of young cells of *B. subtilis*. In the present work, cells were grown without glycerol in the growth medium. Freeze-etch preparations have been made after growth in (*a*) the absence of glycerol, (*b*) the presence of 10, 20, or 25% glycerol, and (*c*) the presence of 10% dimethylsulfoxide. In none of these cases could the nucleoplasmic region be recognized as convincingly as when the cells were fixed in osmium tetroxide before freeze-etching (see also reference 17). Fig. 12 represents a control specimen prepared in the presence of 20% glycerol. Occasionally the nucleoplasmic region could be faintly recognized (arrows in Fig. 12). After fixation in osmium tetroxide the nucleoplasm exhibits fiber patterns of varying degrees of coarseness (Figs. 13 and 14). In Fig. 13 very delicate fibrils are observed, whereas in Fig. 14 the nucleoplasm appears rather coarse. Fig. 15 shows a similar situation of poorly preserved nucleoplasm in a thin section.

When osmium tetroxide fixation is followed by a posttreatment with uranyl acetate (treatment *b*, see Materials and Methods), the nucleoplasmic fibrils (*N*) are also observed in neat patterns (Figs. 16 and 18) resembling the organized bundles of fibrils in the sections (Figs. 17 and 19, respectively).

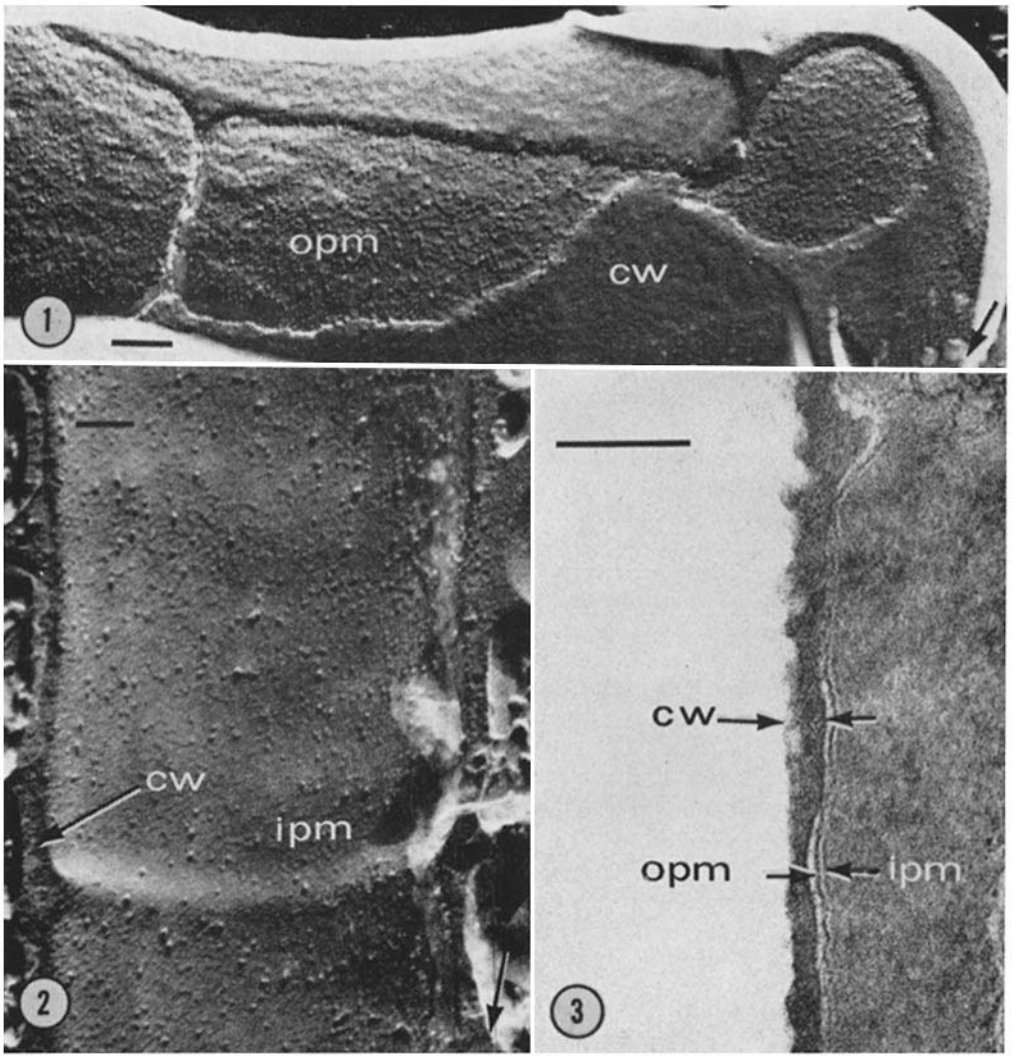
An additional dehydration in acetone (treatment *c*) again reveals the nucleoplasmic region (Fig. 20). A corresponding similar condition in a thin section is indicated in Fig. 21.

Cytoplasm

The cytoplasmic region appears to be the most complicated structure of all to interpret. Whereas the nucleoplasm can be clearly distinguished from the cytoplasmic region when the cells are fixed in osmium tetroxide before freeze-etching, this treatment does not clarify the architecture of the cytoplasm. Also, dependent on the amount of platinum-carbon deposited on the fractured cells, the cytoplasm appears composed of particles and fibrils of variable sizes. So far we have not been able to demonstrate ribosomes in freeze-etched cells (see Discussion).

DISCUSSION

There are two ways in which freeze-etching of *fixed specimens* can contribute to the evaluation of bacterial fine structure. (*a*) This method makes it possible to judge, within limits, artifacts that may be introduced by chemical fixation. (*b*) It facilitates the comparison of the complicated freeze-etch images with images obtained from thin



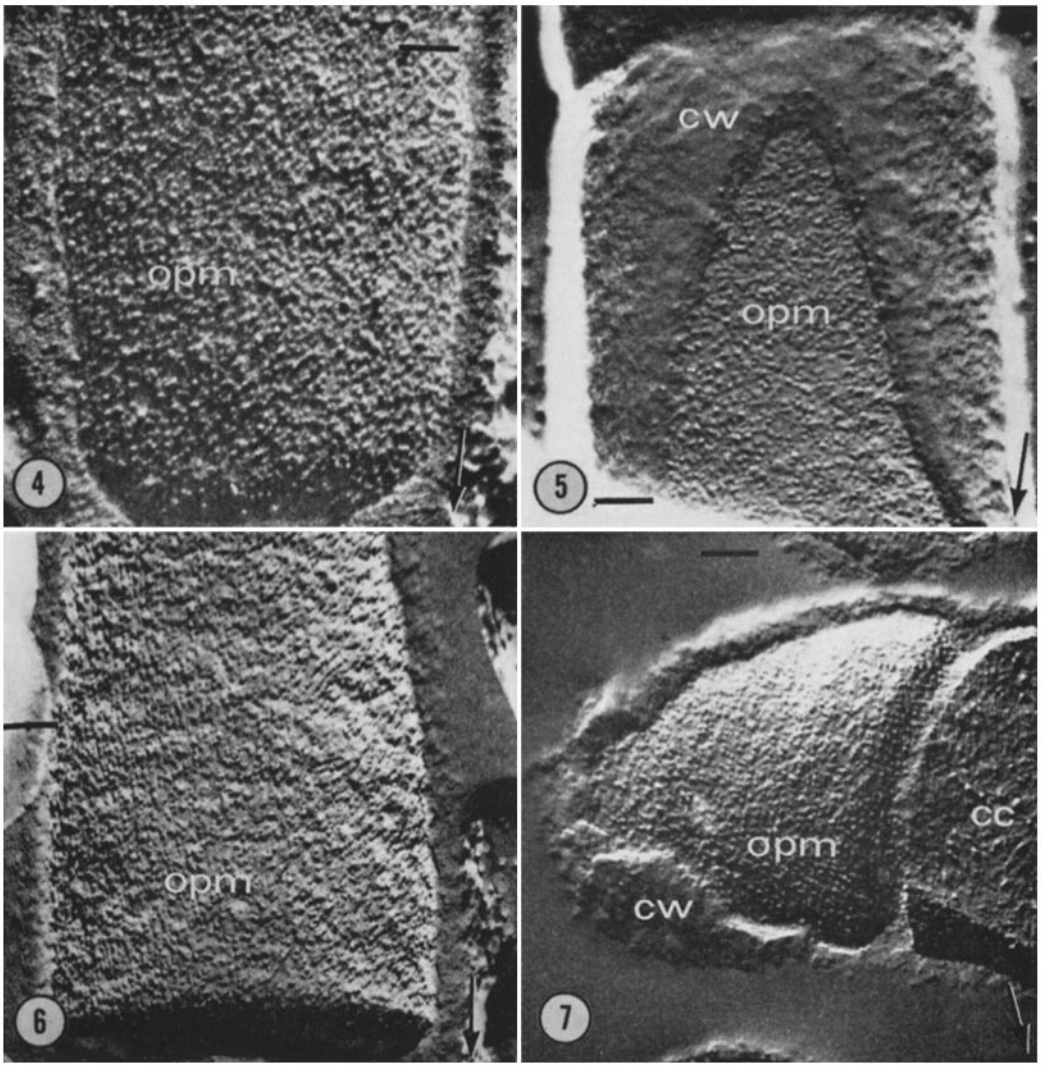
Figs. 1–3 show the general outline of the cell envelope of *B. subtilis* after freeze-etching and thin-sectioning. Bar represents 0.1 μ. Arrows in Figs. 1 and 2 indicate direction of shadowing.

FIGURE 1 A cell showing the cell wall surface (*cw*) and the outer surface of the plasma membrane (*opm*). This preparation was frozen in the absence of glycerol. × 80,000.

FIGURE 2 *B. subtilis* cell from which the cell content has been removed, showing inner surface of the plasma membrane (*ipm*). The cell wall (*cw*) has been cross-fractured. × 80,000.

FIGURE 3 Thin section of *B. subtilis*, showing the profile of the cell envelope. *cw*, cell wall; *opm*, outer profile of the plasma membrane; *ipm*, inner profile of the plasma membrane. No poststaining was applied. × 180,000.

sections. It thus reduces the interpretation gap between the two techniques. However, when using the results obtained with the freeze-etching technique as a standard by which to judge the image of a thin section, one should, of course, not overlook possible artifacts introduced by the freeze-etching technique proper. For example, Giesbrecht (6) made interesting observations on the deformation



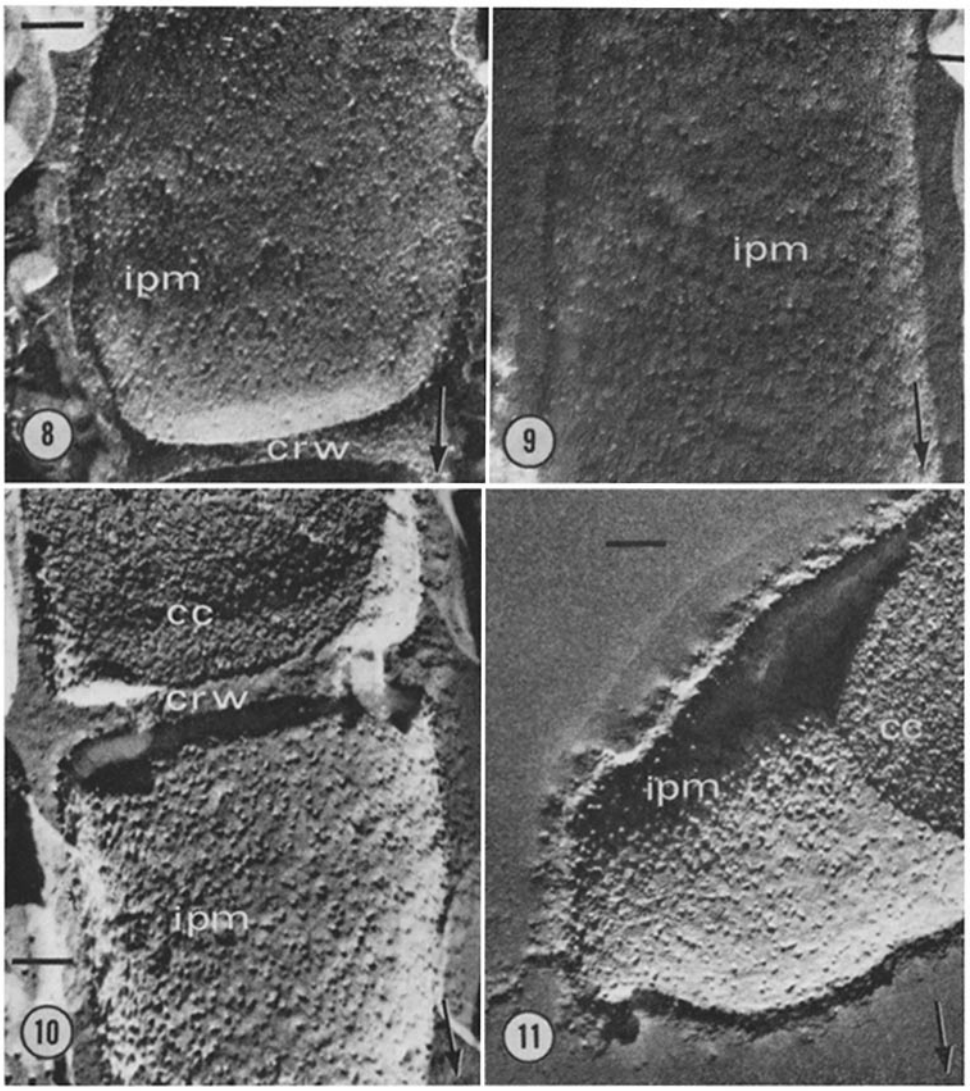
Figs. 4–7 show the preservation of the cell wall (*cw*) and outer surface of the plasma membrane (*opm*). Bar represents 0.1 μ. Arrows indicate direction of shadowing.

FIGURE 4 Control preparation. The cells were directly frozen. *opm*, outer surface of the plasma membrane. × 80,000.

FIGURE 5 Cell fixed in osmium tetroxide (treatment *a*, see Materials and Methods) prior to freeze-etching. Note good preservation of the outer surface of the plasma membrane (*opm*) and cell wall (*cw*). × 80,000.

FIGURE 6 Cell fixed in osmium tetroxide and posttreated with uranyl acetate (treatment *b*) before freeze-etchng. Note good preservation of the outer surface of the plasma membrane (*opm*). × 80,000.

FIGURE 7 Cell fixed in osmium tetroxide, posttreated with uranyl acetate, and dehydrated in a graded series of acetone (treatment *c*). Note good preservation of the surfaces of membrane and cell wall. *cw*, cell wall; *opm*, outer surface of the plasma membrane; *cc*, cell content. × 80,000.



Figs. 8–11 show the preservation of the inner surface of the plasma membrane (*ipm*) after different treatments prior to freeze-etching. In all cases the surface appears almost unchanged. Bar represents 0.1 μ. Arrows indicate direction of shadowing.

FIGURE 8 Control cell. *ipm*, inner surface of the plasma membrane; *crw*, cross wall. × 80,000.

FIGURE 9 Cell fixed in osmium tetroxide (treatment *a*, see Materials and Methods) before freeze-etching. *ipm*, inner surface of the plasma membrane. × 35,000.

FIGURE 10 Cell fixed in osmium tetroxide and posttreated with uranyl acetate (treatment *b*) before freeze-etching. *ipm*, inner surface of the plasma membrane; *cc*, cell content; *crw*, cross wall. ×80,000.

FIGURE 11 Cell of a preparation fixed in osmium tetroxide, posttreated with uranyl acetate, and dehydrated in a graded series of acetone. *ipm*, inner surface of the plasma membrane; *cc*, cell content. × 80,000.

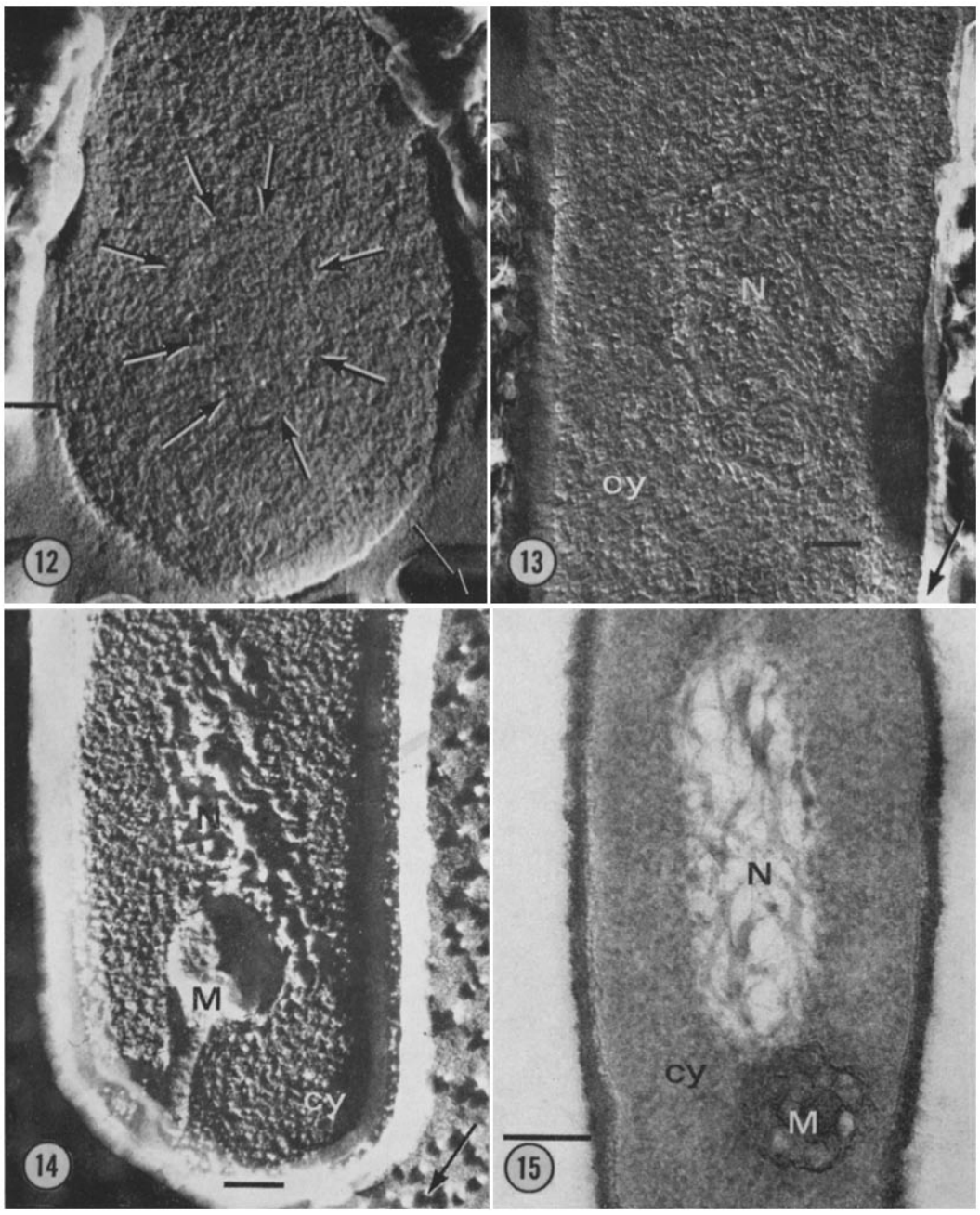
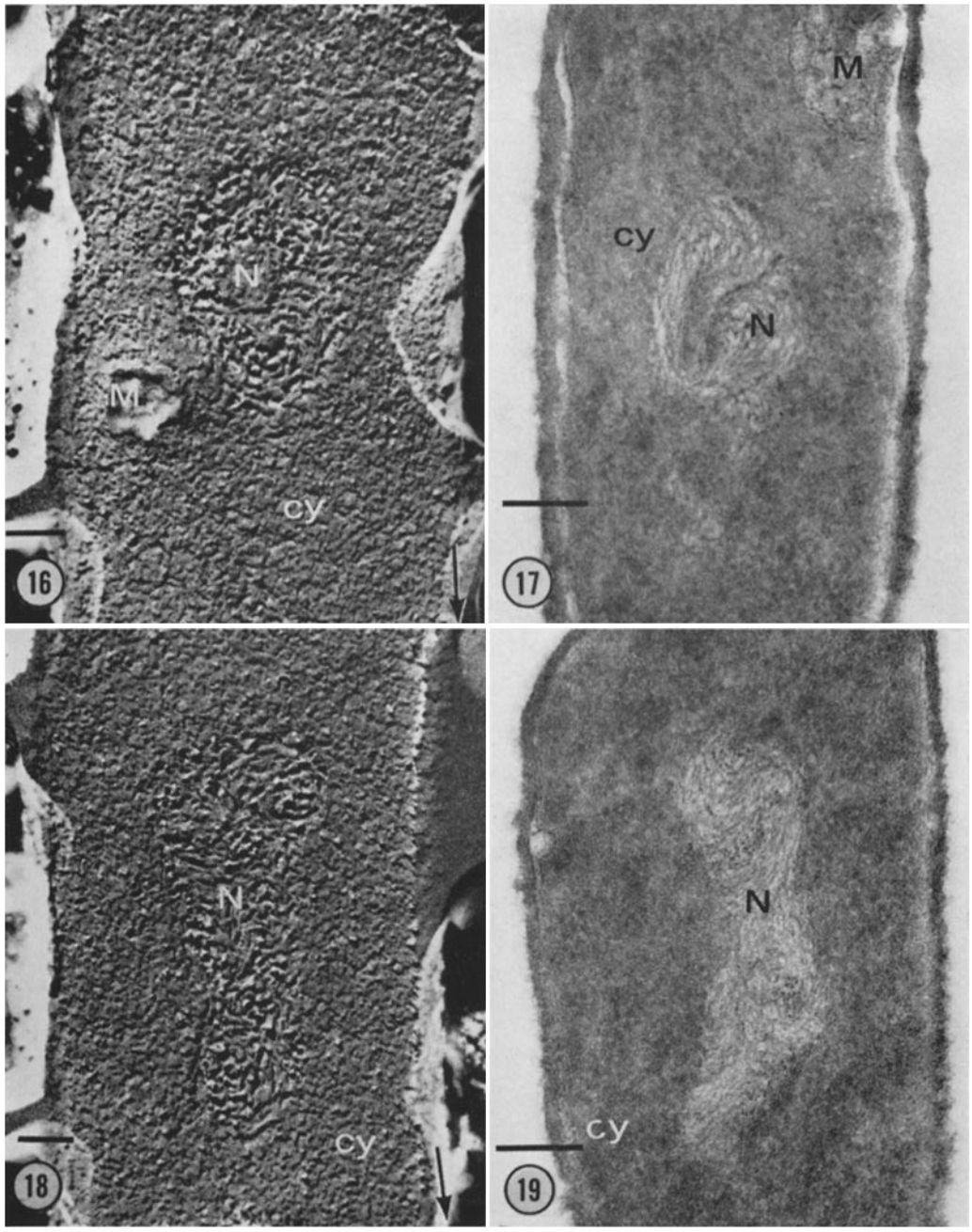


FIGURE 12 Control cell. Nucleoplasmic region (arrows) barely differentiated from the cytoplasm. Bar (Figs. 12–15) represents 0.1 μ. Arrow at lower right indicates direction of shadowing. × 30,000.

FIGURE 13 *B. subtilis* cell fixed in osmium tetroxide (treatment *a*, see Materials and Methods) before freeze-etching. The nucleoplasm (*N*) appears as very delicate fibrils. *cy*, cytoplasm. Arrow indicates direction of shadowing. × 80,000.

FIGURE 14 Preparation treated as in Fig. 13. Note coarse appearance of the nucleoplasm (*N*), which is due to inadequate fixation. *cy*, cytoplasm; *M*, mesosome. Arrow indicates direction of shadowing. × 80,000.

FIGURE 15 Thin section of *B. subtilis*. The nucleoplasmic fibrils (*N*) are strongly aggregated, which resembles the situation in Fig. 14. No poststaining was applied. *cy*, cytoplasm; *M*, mesosome. × 120,000.



FIGURES 16 and 18 *B. subtilis* fixed in osmium tetroxide and posttreated with uranyl acetate (treatment *b*, see Materials and Methods). The nucleoplasm (*N*) seems to be composed of coiled bundle(s) of fibrils. *cy*, cytoplasm; *M*, mesosome. Bar represents 0.1 μ. Arrows indicate direction of shadowing. × 80,000.

FIGURES 17 and 19 Thin sections showing comparable situations as in Figs. 16 and 18. No poststaining of sections was applied. *N*, nucleoplasm; *cy*, cytoplasm; *M*, mesosome. Bar represents 0.1 μ. × 120,000.

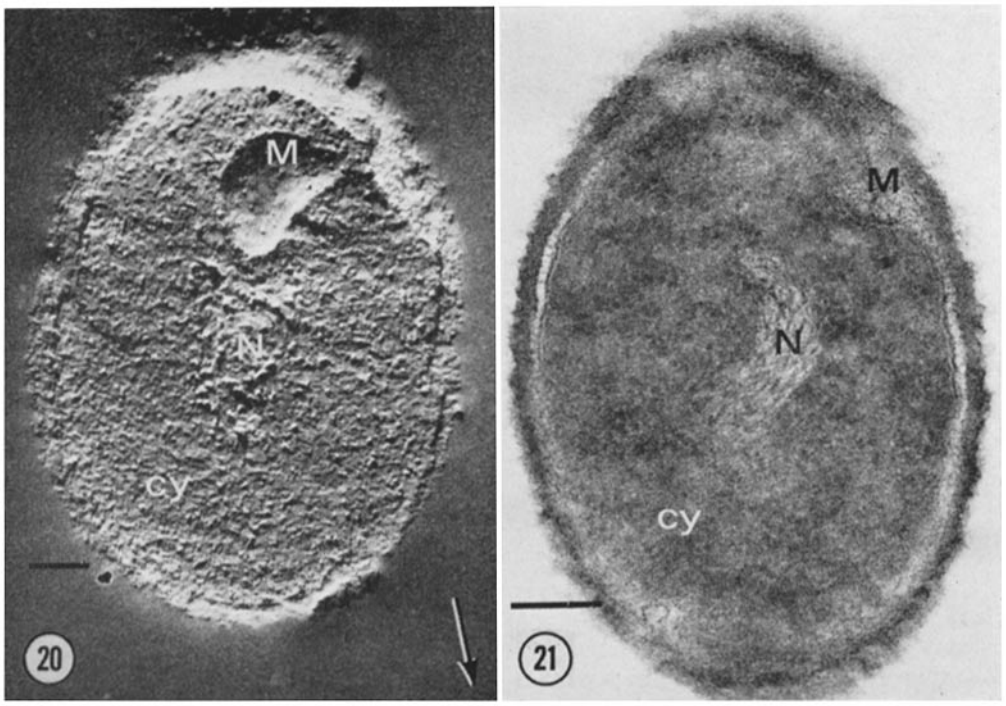


FIGURE 20 *B. subtilis* fixed in osmium tetroxide, posttreated with uranyl acetate, and dehydrated in a graded series of acetone (treatment *c*, see Materials and Methods). The nucleoplasmic region (*N*) appears clearly differentiated from the surrounding cytoplasm (*cy*). *M*, mesosome. Bar represents 0.1 μ. Arrow indicates direction of shadowing. × 80,000.

FIGURE 21 A situation comparable to Fig. 20 in a thin section. *N*, nucleoplasm; *cy*, cytoplasm; *M*, mesosome. Bar represents 0.1 μ. × 120,000.

of what presumably were poly-beta-hydroxy butyrate bodies (19) in *Rhodopseudomonas viridis* at temperatures as low as −150°C. He observed hornlike structures pointing in the direction of fracturing. Yet, an alternative explanation could be that the bending (not the creation) of the hornlike structures could be caused by exposure to heat during shadowing (cf. Hannay, reference 7). In our preparations deformation of the cell components of *B. subtilis* in the same direction as that of the surrounding knife scratches, and as such recognizable, was found to occur occasionally.

Cell Envelope

The different treatments to which the bacteria were subjected prior to freeze-etching revealed good preservation of the surface structure of plasma membrane and cell wall, as far as this could be detected by means of electron microscopy of shadowed specimens. Notably the thorough dehydration in acetone following osmium tetroxide and posttreatment with uranyl acetate appeared to cause but little visible damage. Moreover, the treatments applied did not affect the fracturing properties of the plasma membrane. In *B. subtilis*, fracturing seems to occur along existing surfaces (17, 20; and cf. reference 1, 3, 25). A change in fracturing properties of membranes was, on the other hand, observed when chloroplasts prefixed for 1 hr in 6% glutaraldehyde, pH 7.0, at room temperature were extracted with acetone for a short period (2). "Normal" surfaces were seen (2) when the glutaraldehyde prefixation was not followed by acetone extraction. Our experiments with *B. subtilis* membranes seem to indicate that osmium tetroxide is a good fixative for membranes from the point of view that it protects them against subsequent dehydration in acetone.

Nucleoplasm

A complication in the interpretation of the fine structure of the cell content is presented by the observation that *young* unfixed cells of *B. subtilis* fail to show a prominent differentiation in nucleoplasm and cytoplasm (17, 20). In the present work however, a faint indication of the existence of a nucleoplasmic region was occasionally found (Fig. 12). As has been pointed out before (17), no clear-cut separation of nucleoplasm and cytoplasm can be readily expected when the freeze-etching technique is used, due to the lack of selectivity of this technique in revealing definite chemical compounds. This result is in contrast to that in heavy metal-stained sections which possess in particular the property of showing nucleic acid-containing elements (DNA and ribosomes). Moreover, as has been shown by Van Iterson (27, 28), the nucleoplasm seems to be structurally continuous with the cytoplasm; fibrils from the nucleoplasmic region were seen to penetrate deeply into the cytoplasm, presumably towards the plasma membrane (28).

After osmium tetroxide fixation prior to freeze-etching, however, the nucleoplasmic region can be better distinguished from its surroundings in *B. subtilis* than is possible in unfixed cells (17). The same observation was recently made by Lickfeld (11) with *Pseudomonas aeruginosa* cultivated for 2.5 hr in broth. Giesbrecht, on the other hand, observed the nucleoplasmic region in 2–25-day-old cells of *Rhodopseudomonas palustris* without previous fixation (6). The visibility of the nucleoplasm after freeze-etching would depend on a reduction of the glycerol content (from 20 to 10%) or on the use of different freeze-protecting agents such as dimethylsulfoxide (6). Further, according to Giesbrecht (6) the presence of 20% glycerol in the freezing medium may hide macromolecular structures in *R. palustris*. The observation on *B. subtilis* seems, however, to point in a different direction. Firstly, no clear delineated nucleoplasmic region is found in *young* cells of *B. subtilis* when freeze-etched in the absence (see also reference 20) of glycerol, or in the presence of 10, 20, or 25% glycerol, or of 10% dimethylsulfoxide. Only occasionally a faint distinction between nucleoplasmic region and cytoplasmic region could be discerned (Fig. 12). Secondly, the visibility of the nucleoplasm after freeze-etching (in the presence of 20% glycerol) is enhanced by a chemical prefixation with any of the three treatments (see Materials and Methods and Figs. 12–14, 16, 18, and 20). Here the presence of 20% glycerol does not cancel the visibility of the nucleoplasmic fibrils, whether coarse (Fig. 14) or very delicately preserved (Fig. 13). Thirdly, the nucleoplasmic region can be observed directly without previous fixation in *old* (36 hr) cells of *B. subtilis* in a stage just prior to sporulation (20). As described by Young and Fitz-James (30), in this stage the nucleoplasm can be found in the shape of a so-called axial filament. The arrangement of the nucleoplasm in such an axial filament was shown to be accompanied by a cessation of DNA synthesis (30). At the level of light microscopy the variation in appearance of the nucleoplasm in relation to age and different environmental circumstances has been amply pointed out in the reviews of Robinow (21) and Murray (13). The visibility of the nucleoplasm after freeze-etching might therefore not depend primarily on whether fine structural detail is hidden by glycerol, but on its actual physiological condition and, presumably, in particular on its state of hydration affecting the degree of aggregation of the DNA fibrils. The degree of aggregation may be influenced by the age of the organism (cf. reference 20) as well as by agents such as osmium tetroxide, glycerol, or dimethylsulfoxide, and it also may depend on the organism studied. Further experiments are needed to clarify these interesting problems.

Whether or not R-K fixation can be regarded as achieving satisfactory preservation of bacterial nucleoplasm depends basically on one's readiness to accept the freeze-etch image as a standard. Very little is known about the plasticity of biological structures at low temperatures (cf. reference 6). Also, the detailed effect of the embedding material (Vestopal W) on nucleoplasmic fine structure remains to be studied. Apart from these reservations, our results seem to indicate that the Ryter-Kellenberger fixation, as applied in the present study, does affect the native organization of the nucleoplasmic fibrils. However, the R-K fixation technique appears quite satisfactory for the chromosomes of dinoflagellates, since direct freeze-etching revealed a similar DNA configuration (5). It should also be emphasized that the aim of this paper is not to disprove the visibility of the nucleoplasmic region, but rather is to show the increase in visibility of this region when freeze-etching is preceded by chemical fixation.

The comparison of the electron microscope image of the nucleoplasm after chemical fixation before freeze-etching with the image produced

after direct freeze-etching, strikingly resembles a similar situation in light microscopy, namely when the image of the nucleoplasm in stained cells is compared with that obtained with phase-contrast microscopy of living cells (22). Both techniques, freeze-etching and phase-contrast light microscopy, suggest a more diffuse, and probably less static, nucleoplasm than is observed in fixed cells (see also reference 11).

Cytoplasm

In unfixed or osmium tetroxide-fixed cells no cytoplasmic components such as ribosomes could be visualized after freeze-etching. At first sight this is rather surprising in view of, for instance, the polyhedral shape of the 50S ribosomal subunit (14, 16). The visibility of the ribosomes in the fractured cell content may, however, depend on the following factors: (*a*) during the fracturing process the fracture shows no tendency to follow the ribosomal surface, (*b*) the visibility is obscured because of other components intervening, and (*c*) ribosomes or ribosomal subunits do not exist as compact particles in vivo.

Gentle fixation and dehydration techniques have led some authors to express the opinion that the ribosomes attached to the endoplasmic reticulum of animal cells are in a flattened condition (18, 24), whereas those free in the cytoplasm have more isodiametric shapes (18). Since as yet it has proved difficult to ascertain whether the point raised under *c* above can be applied to *B. subtilis*, the conformations of ribosomes are currently being examined in our laboratory.

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