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Organization of the Nucleoplasm in *Escherichia coli* Visualized by Phase-Contrast Light Microscopy, Freeze Fracturing, and Thin Sectioning

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The organization of the nucleoplasm in *Escherichia coli* was studied by comparing the results obtained by freeze fracturing and thin sectioning. In addition to exponentially growing cells, we used chloramphenicol-treated cells which show a well-defined nucleoplasm in the phase-contrast light microscope and can therefore function as a control for treatments necessary for electron microscopy. Two factors were found to determine the visibility of the nucleoplasm in freeze fractures: first, the state of lateral aggregation of deoxyribonucleic acid fibrils, which is enhanced by postfixation with OsO_4 according to the Ryter-Kellenberger technique; second, the presence of ice crystals. When their formation is prevented by the use of high concentrations of freeze-protecting agents, the nucleoplasm appears as a smooth region in cells that have been prefixed. In unfixed cells, however, the freeze-protecting agent causes disappearance of the nucleoplasm by rearrangement of structures within the cell. This observation makes it hard to determine whether the deoxyribonucleic acid in vivo occurs dispersed, as found after glutaraldehyde prefixation, or compact, as after OsO_4 prefixation.

The bacterial nucleoplasm comprises a deoxyribonucleic acid (DNA) thread of about 1 mm in length packed into a very limited space in such a way that DNA replication and DNA segregation are still possible. The problem of the way of packing the circular DNA molecule has two aspects. The first concerns the internal organization of the nucleoplasm, which is probably best studied by analyzing the structure of the isolated "folded genome" (23, 25). The second aspect refers to the in vivo external shape of the nucleoplasm, which also includes its spatial relationship to the cytoplasm and the cell membrane.

This paper concerns the external shape of the bacterial nucleoplasm and, to a large extent, this is a matter of interpretation of electron microscope observations. Two sets of observations appear relevant. (i) After freeze fracturing, the nucleoplasm cannot be demonstrated unambiguously in exponentially growing bacterial cells. However, if freeze fracturing is preceded by chemical fixation with OsO_4, the nucleoplasm becomes readily visible in *Pseudomonas aeruginosa* (9) and *Bacillus subtilis* (15, 16). (ii) In thin sections of *Escherichia coli* a compact shape of the nucleoplasm is observed after fixation with OsO_4, whereas glutaraldehyde fixation results in a dispersed nucleoplasm (10, 21, 24).

These observations have been interpreted in favor of a dispersed organization of the DNA in vivo, which would correspond best with both the freeze-fracture image of unfixed cells and the thin-section image after glutaraldehyde fixation (1, 26). Fixation with OsO_4 would cause an artificial contraction of the DNA into a more compact structure, as visualized by both techniques (9, 16).

Although freeze fracturing requires less manipulation of the cells than thin sectioning, partial dehydration with a freeze-protecting agent preceding physical fixation is still necessary. This partial dehydration may cause artifacts, especially in unfixed cells. Changes introduced by partial dehydration or by the fixation and dehydration necessary for embedding can only be judged by comparing the results of freeze fracturing and thin sectioning with the image of living bacteria obtained by phase-contrast light microscopy. From a cytological point of view, the three techniques should therefore be compared to determine the respective influences of preparation on the external shape of the nucleoplasm and to decide whether the dispersed or the contracted state is closer to reality.

MATERIALS AND METHODS

Strain and growth conditions. *E. coli* K-12 was used throughout this work. Cells were grown in broth medium composed of 1% tryptone (Difco), 0.5%

yeast extract, and 0.5% NaCl and harvested in the exponential phase of growth. Treatment with chloramphenicol (CAM) was carried out by growing the cells in the presence of 100 μg of CAM per ml (Brocades, The Netherlands) during one doubling time (35 to 40 min).

Fixation and embedding. Prefixation was done by adding to cells in their growth medium either OsO_4 to a final concentration of 0.1% or glutaraldehyde to a final concentration of 2.5%. After prefixation for 15 to 20 min at room temperature (20°C), cells were centrifuged at 3,000 $\times$ g for 7 min. Postfixation was done by suspending prefixed cells in 0.1% tryptone and 1% OsO_4 dissolved in acetate veronal buffer (pH 6) containing 0.12 M NaCl and 0.01 M $MgCl_2$, according to the Ryter-Kellenberger technique (19). For embedding, prefixed cells were first enmeshed in 2% agar, subsequently postfixed with OsO_4 and uranyl acetate, dehydrated in acetone, and embedded in Vestopal W as described by Ryter et al. (19). In some experiments cells were dehydrated with acetone directly after prefixation.

Freeze fracturing. Unless stated otherwise, unfixed or fixed cells were centrifuged in the presence of 20% glycerol, and small drops from the cell pellet were immediately frozen in liquid Freon 22 and fractured at −100°C in a Balzers BA 360 freeze-etch unit. Etching was allowed for 1 min at −100°C. Replicas were made by shadowing with platinum and carbon (13). In some experiments glycerol was replaced by ethylene glycol (30 to 100%) as the freeze-protecting agent, because cells suspended in ethylene glycol can be directly used for embedding in Vestopal W as well as for phase-contrast light microscopy.

Light microscopy. Microscope slides were coated with a thin layer containing 20% gelatin (Rousselot, France) in nutrient broth. After cooling, a small drop of the cell suspension was placed on the gelatin and spread between the layer and a cover slip.

RESULTS

The observation that the nucleoplasm of exponentially growing cells can be visualized if freeze fracturing is preceded by chemical fixation with OsO_4 (9, 15, 16) applies to *E. coli* as well (Fig. 1). However, as demonstrated by Fig. 2, prefixation by 0.1% OsO_4 alone is not sufficient to visualize the nucleoplasm. Only when prefixation is followed by postfixation with 1% OsO_4 according to the Ryter-Kellenberger technique (see above) does a roughly textured nucleoplasm become visible, i.e., either as a compact region in the case of prefixation with OsO_4 (Fig. 1) or as dispersed areas if the cells are first prefixed with 2.5% glutaraldehyde (results not shown).

As OsO_4 (20) and glutaraldehyde (4) do not react with DNA, prefixation alone is not sufficient to protect the DNA against collapse during the dehydration required for embedding. For this reason postfixation with OsO_4 according to the Ryter-Kellenberger technique is carried out, which causes lateral aggregation and cross-linking of the DNA fibrils (cf. 5). The structure is thereby protected against further collapse during dehydration. The increased visibility of the nucleoplasm in freeze fractures of postfixed cells can thus be explained as an aggregation of DNA fibrils into coarse structures. The reason why no nucleoplasm can be observed in unfixed cells or in merely prefixed cells could be the formation of small ice crystals, which blur the distinction between nucleoplasm and cytoplasm. This problem can be studied if cells are frozen in the presence of a high concentration of a freeze-protecting agent, which prevents the development of ice crystals. Due to the fact that in cells treated with CAM (see below) the shape of the nucleoplasm remains relatively unaffected by the conditions of fixation and dehydration, we used CAM-treated cells as a model system to study the problem further.

CAM-treated cells. The nucleoplasm contracts into a round body if *E. coli* cells are treated with 100 μg of CAM per ml during one doubling time (7). The nucleoplasm can then be easily observed with the phase-contrast light microscope. In contrast to exponentially growing cells (see below), the visualization of this rounded nucleoplasm does not change after prefixation with either glutaraldehyde (Fig. 3) or OsO_4. Furthermore, after prefixation with either fixative, the CAM-treated cells can be suspended in high concentrations of glycerol or ethylene glycol without any effect on the visualization of the nucleoplasm. This is shown in Fig. 7 (insert) for cells prefixed with OsO_4 and suspended in 100% ethylene glycol.

In spite of this similarity in overall shape of the nucleoplasm in CAM-treated cells prefixed with either OsO_4 or glutaraldehyde and its resistance to dehydration, thin sections showed the following differences in the internal structure of the nucleoplasm. (i) After OsO_4 prefixation (Fig. 4), the nucleoplasm assumes a more fibrillar appearance than after glutaraldehyde prefixation (Fig. 5). (ii) After glutaraldehyde prefixation, we found small cytoplasmic patches within the nucleoplasm (Fig. 5).

After freeze fracturing with 20% glycerol as freeze-protecting agent, i.e., under the conditions in which ice crystals are usually formed, the nucleoplasm is not readily visible in the CAM-treated cells, either unfixed or prefixed with OsO_4 or glutaraldehyde (results not shown). Only after postfixation with OsO_4 according to the Ryter-Kellenberger technique is the round nucleoplasm seen to contrast with

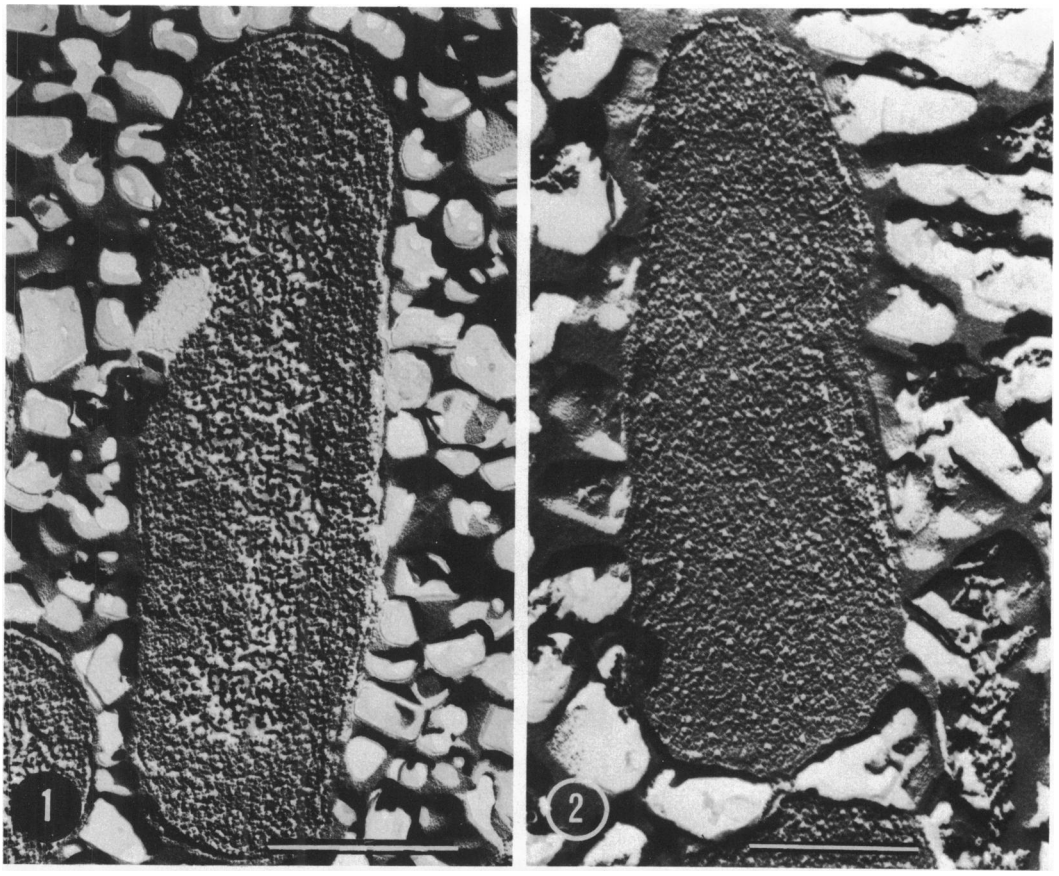

FIG. 1. *E. coli prefixed with 0.1% OsO_4 and postfixed with 1% OsO_4, according to the Ryter-Kellenberger technique, and frozen in the presence of 20% glycerol. The nucleoplasm can be distinguished by its rough texture. The bar in this and in all subsequent figures represents 0.5 μm.*

FIG. 2. *E. coli frozen in the presence of 20% glycerol after prefixation with 0.1 OsO_4 only. No distinction between nucleoplasm and cytoplasm can be observed.*

the cytoplasm on account of its rougher texture (Fig. 6), similarly to exponentially growing cells (Fig. 1).

However, in the presence of 100% ethylene glycol, freeze fracturing of cells prefixed with either OsO_4 (Fig. 7) or glutaraldehyde (Fig. 8) reveals the cytoplasm as a rough texture and the nucleoplasm as a smooth central area. The freeze-fracture images are similar to those obtained after thin sectioning: a more homogeneous nucleoplasm appears after OsO_4 fixation (cf. Fig. 4 and 7) and a rounded region interspersed with cytoplasmic material appears after glutaraldehyde fixation (cf. Fig. 5 and 8). Obviously, freezing without detectable ice crystal formation is sufficient to visualize the nucleoplasm in prefixed CAM-treated cells. The influence of ice crystals on the visualization of the nucleoplasm in freeze-fracture replicas has been further analyzed in exponentially growing cells.

Prefixation of exponentially growing cells. The obliteration of the nucleoplasm by ice crystals is clearly demonstrated in Fig. 9 and 10, which represent one and the same freeze-fracture replica displaying two areas of different local freezing rates. When proceeding inwards from the outer border of the replica, ice crystals were found to become more and more prominent, reflecting the lower freezing rate inside the drop. Figure 9 was taken about half-way along the radius of the frozen droplet; Fig. 10 represents the outer area. In the presence of ice crystals (Fig. 9) no nucleoplasm can be seen; in the absence of ice crystals the nucleoplasm appears as a centrally located smooth region. The compact shape of the nucleoplasm is confirmed by direct dehydration and embedding of cells

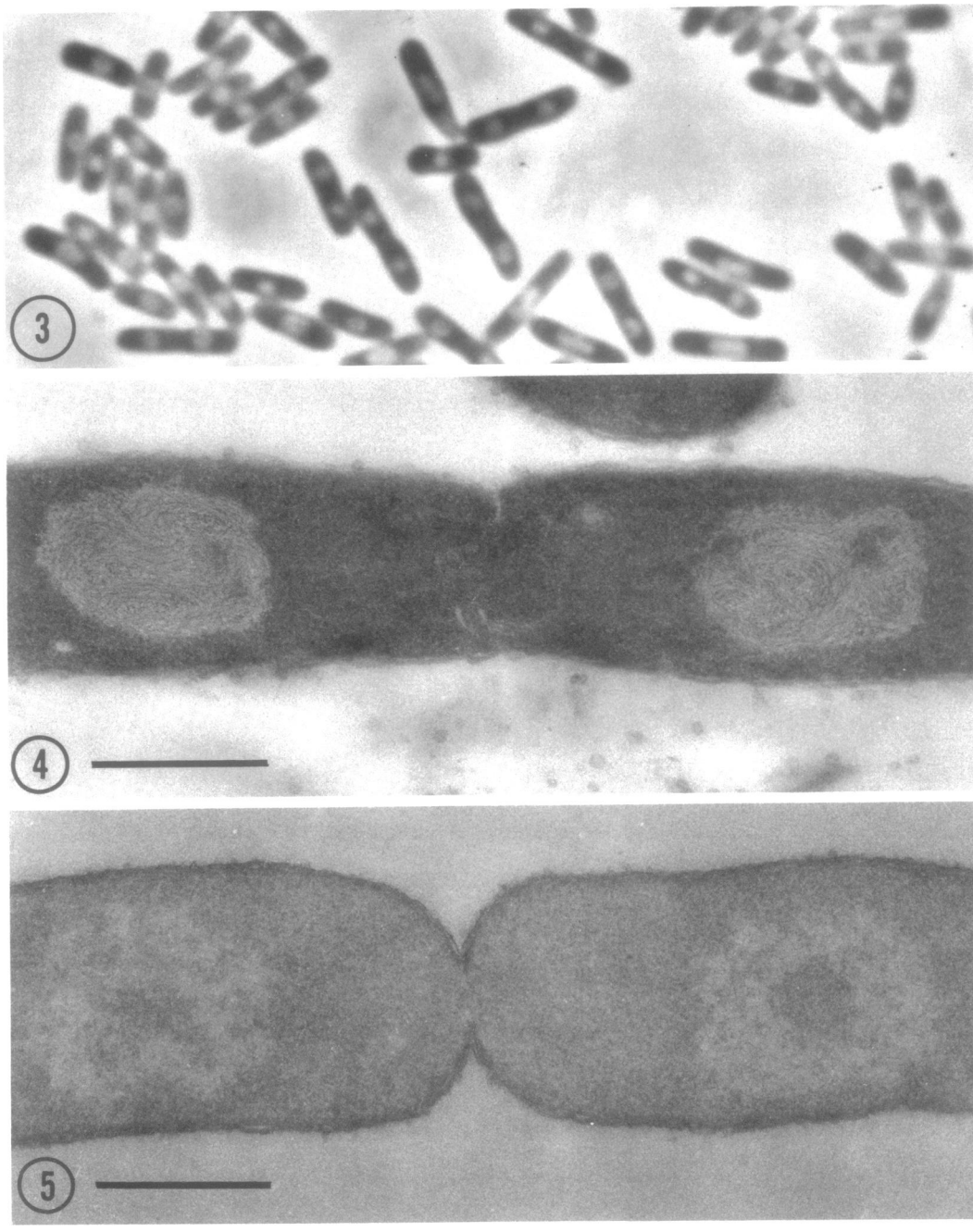

FIG. 3. *Phase-contrast light microscopy of E. coli cells treated with CAM and prefixed with 0.1% OsO_4. The cells show the round nucleoplasm characteristic for CAM-treated cells, whether unfixed or prefixed with OsO_4 or glutaraldehyde. All phase-contrast light micrographs are magnified ×4,000.*

FIG. 4. *E. coli treated with CAM, prefixed with 0.1% OsO_4, and postfixed with OsO_4 according to the Ryter-Kellenberger technique. DNA fibrils are clearly visible in the rounded nucleoplasm.*

FIG. 5. *E. coli cell from the same culture as in Fig. 4 but prefixed with 2.5% glutaraldehyde. The rounded nucleoplasm contains small patches of cytoplasmic material. The central core in the nucleoplasm represents a cytoplasmic invagination characteristic for CAM-treated cells.*

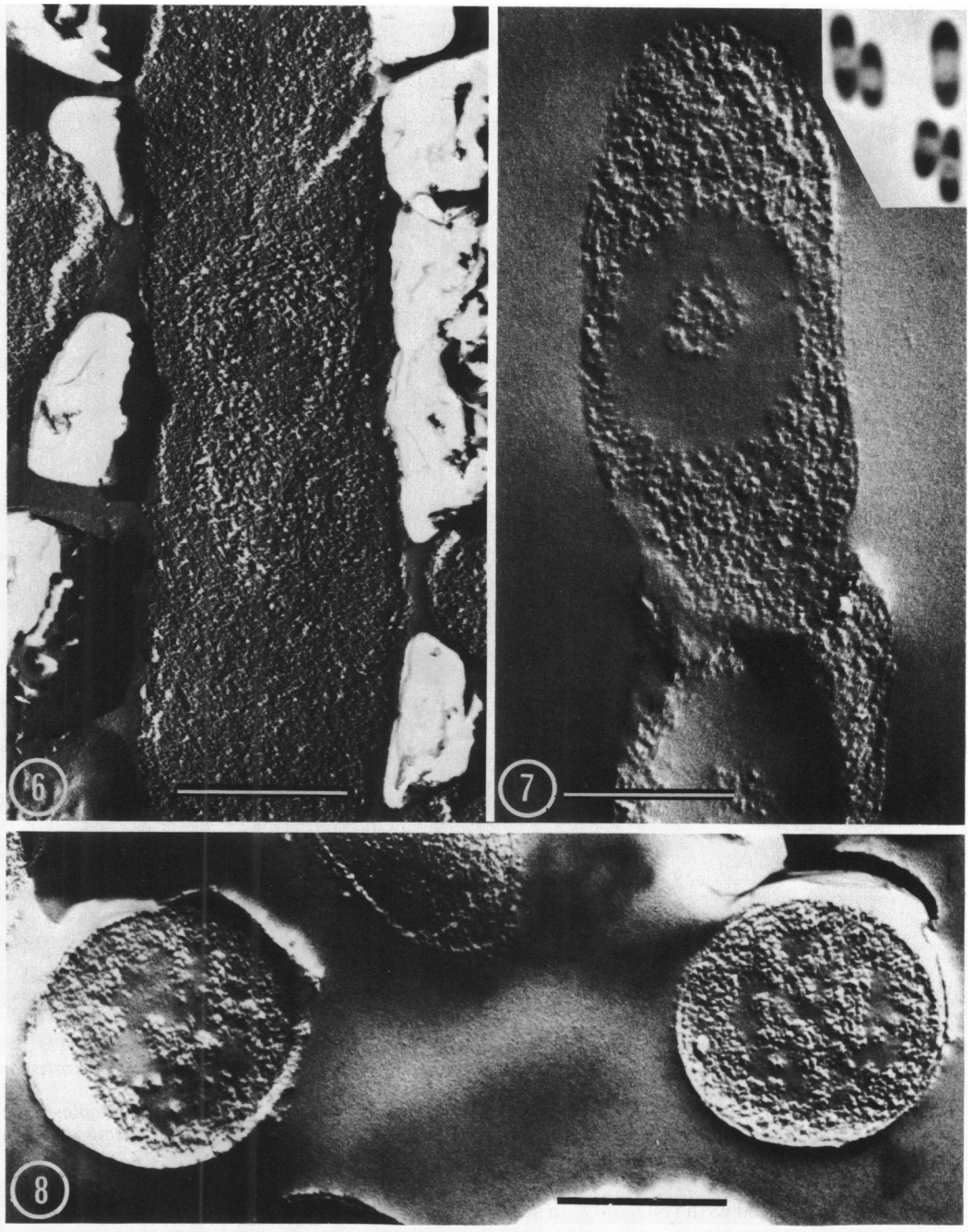

FIG. 6. *Freeze fracture of E. coli treated with CAM and prefixed with 0.1% OsO_4, followed by postfixation with 1% OsO_4 according to the Ryter-Kellenberger technique, and frozen in the presence of 20% glycerol. A well-defined, rounded nucleoplasm can be distinguished, comparable to the thin-sectioned cell of Fig. 4.*

FIG. 7. *Freeze fracture of E. coli treated with CAM and prefixed with 0.1% OsO_4 only. Ice crystal formation was prevented by freezing the cells in 100% ethylene glycol. The nucleoplasm is now visualized as a smooth round area with cytoplasmic inclusion. Insert shows a phase-contrast light microscope picture of cells from the same preparation.*

FIG. 8. *Freeze fracture of E. coli treated with CAM and prefixed with 2.5% glutaraldehyde without postfixation. Ice crystal formation was prevented by freezing in the presence of 30% glycerol. The nucleoplasm is visualized as a smooth area, interspersed with cytoplasmic material (cf. Fig. 5).*

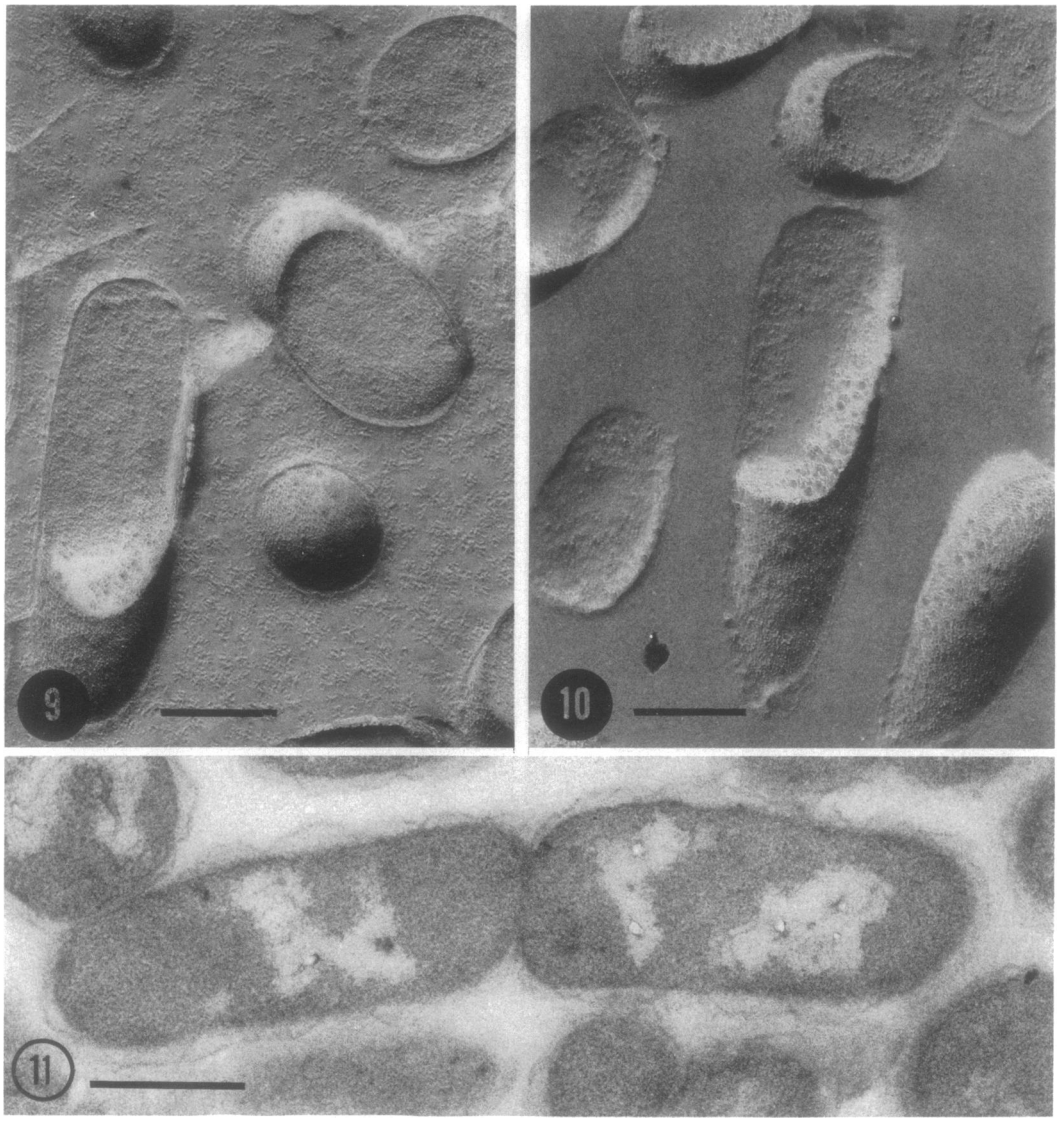

FIG. 9 and 10. *E. coli cells prefixed with 0.1% OsO_4 and frozen in the presence of 20% glycerol. The two pictures are from different regions of the same freeze-fracture replica. Ice crystals prevent the visualization of the nucleoplasm in Fig. 9, whereas in Fig. 10 the nucleoplasm is visible as a smooth region.*

FIG. 11. *E. coli, dehydrated for embedding directly after prefixation with 0.1% OsO_4. The cytoplasm has been fixed well enough to maintain the shape of the compact nucleoplasm. Within the nucleoplasmic areas the unfixed DNA has collapsed into coarse aggregates.*

that have been prefixed with OsO_4 only. In such cells (Fig. 11) the cytoplasm appears to have been fixed sufficiently well to maintain the nucleoplasmic region in a shape corresponding to that of cells in which prefixation has been followed by postfixation as well. Within this region, however, the unfixed DNA can be seen to have collapsed into electron-dense structures (Fig. 11).

In the case of CAM-treated cells, agreement exists between the light-microscope images of unfixed cells and cells prefixed with either OsO_4 (Fig. 3) or glutaraldehyde. For exponentially growing cells the situation is less satisfactory. The nucleoplasm of the OsO_4-prefixed cells (Fig. 13) appears somewhat more distinct than that of the unfixed control cells (Fig. 12) or of cells prefixed with glutaraldehyde (Fig. 14).

Freeze fracturing of cells prefixed with glutaraldehyde and frozen without formation of ice crystals, i.e., in the presence of 30% glycerol, allows the observation of smooth nucleoplasmic regions throughout the cell (Fig. 16). The image is compatible with the thin-section equivalent (24), and it contrasts with the compact nucleoplasm of cells prefixed with OsO_4 (Fig. 10

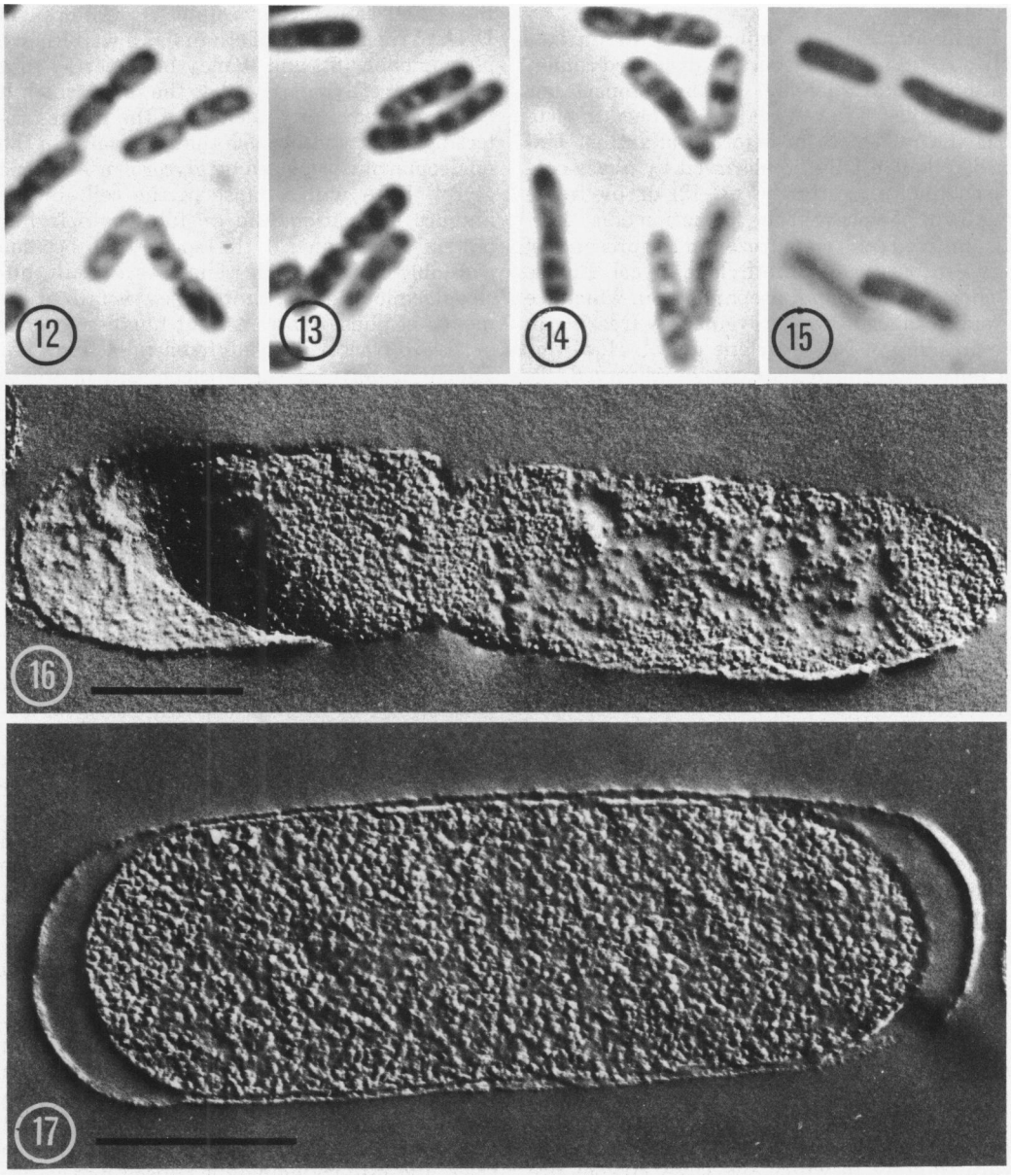

FIG. 12–15. *Phase-contrast light microscopy of exponentially growing E. coli cells. The nucleoplasm is visible as light areas in untreated cells (Fig. 12). The light areas appear smaller after prefixation with 0.1% OsO_4 (Fig. 13) than after prefixation with 2.5% glutaraldehyde (Fig. 14). Treatment of unfixed cells with 50% ethylene glycol causes disappearance of the nuclear areas (Fig. 15).*

FIG. 16. *Freeze fracture of E. coli prefixed with 2.5% glutaraldehyde and frozen in the presence of 100% ethylene glycol. Smooth nucleoplasmic areas occur dispersed throughout the cytoplasm.*

FIG. 17. *Freeze fracture of an unfixed E. coli cell frozen in the presence of 30% ethylene glycol. Although ice crystal formation has been prevented, no nucleoplasmic area can be distinguished.*

and 11). Thus, as in the case of CAM-treated cells, the differences between OsO_4 and glutaraldehyde as found in thin sections (24) already occur after mere prefixation and partial dehydration of the cells with the freeze-protecting agent.

Light and electron microscopy of unfixed cells. To find out whether the dispersed conformation (glutaraldehyde) or the compact one (OsO_4) comes closer to reality, we need to visualize the nucleoplasm before prefixation. Unfixed cells can only be observed by phase-contrast light microscopy (Fig. 12) or by freeze fracturing. To prevent the formation of ice crystals, unfixed cells were frozen in the presence of high concentrations of ethylene glycol. Figure 17 shows a cell from a preparation in which ice crystal formation was prevented by freezing in the presence of 30% ethylene glycol. However, no nucleoplasmic region can be observed. To further assess this phenomenon, infiltration of the cells with ethylene glycol was followed by phase-contrast light microscopy. In the presence of 50% ethylene glycol the nucleoplasm disappears as a distinct region. This disappearance is illustrated by the cells of Fig. 15, which were photographed within 30 min after suspension in 50% ethylene glycol. A possible interpretation is that the freeze-protecting agent causes rearrangement of nucleoplasmic and cytoplasmic material. Prefixation with either OsO_4 or glutaraldehyde would then prevent this rearrangement.

DISCUSSION

Freeze fracturing. From phase-contrast light microscopy we know (11) that in living cells the nucleoplasm occupies a distinct region in the cell. The invisibility of this nucleoplasmic region in freeze-fractured, exponentially growing cells (9, 15, 16, 18) can be attributed to the formation of ice crystals during freezing of the cells. On the other hand, freezing without detectable ice crystal formation, i.e., in the presence of an adequate concentration of glycerol or ethylene glycol, still prevents the demonstration of the nucleoplasm in unfixed cells (Fig. 17).

A possible interpretation is the following. The DNA in the bacterial cell occurs in a highly hydrated state in which the DNA strands are well separated from each other (cf. 5). Phage DNA, by contrast, can occur in a condensed state in which the fibrous nature of the DNA can no longer be observed. Both organizational states of the DNA, hydrated and condensed, can be seen in a thin section of one and the same phage T_4-infected cell (5, 21). When the conditions of Ryter-Kellenberger fixation are not met (for an extensive discussion, see 5 and 6), i.e., when the nucleoplasmic structure is not protected against dehydration with acetone or ethanol as required for embedding (cf. Fig. 11), the DNA can appear collapsed. Collapse of DNA in the presence of ethanol is a well-known phenomenon (8), and it may be expected that glycerol or ethylene glycol do the same (cf. 3). If it is assumed that the effect of the freeze-protecting agent will be such that the water in the nucleoplasm is replaced by glycerol or ethylene glycol, DNA will collapse in the cell. In the absence of fixation, the unstable cytoplasmic matrix will then fill the space that became available by DNA coalescence. As a result, nucleoplasmic regions cannot be visualized by means of phase-contrast light microscopy, nor by freeze fracturing of unfixed cells (Fig. 17). The invisibility of the nucleoplasm in freeze fractures of unfixed cells (9, 15, 16, 18) is therefore no proof that in vivo the DNA occurs dispersed throughout the cell. If our interpretation is correct, there arises the following dilemma: if freezing is not adequate, ice crystals will prevent distinction of nucleoplasm from cytoplasm (Fig. 2 and 9); if freezing is adequate, the freeze-protecting agent will cause, in unfixed cells, rearrangement of nucleoplasmic and cytoplasmic components, resulting in disappearance of the nucleoplasm (Fig. 17).

If, on the other hand, the cytoplasmic matrix is stabilized by prefixation with OsO_4, the external shape of the nucleoplasm (i.e., the boundary of the cytoplasm) remains intact despite DNA coalescence (Fig. 11). After proper freezing without formation of ice crystals, the nucleoplasm is then visible as a smooth region in a roughly textured cytoplasm. Because the high concentration of the freeze-protecting agent will prevent etching, no DNA structures are displayed within the smooth region (Fig. 7 and 10). Our view is schematically depicted in Fig. 18.

Chemical fixation. The role played by monovalent cations in determining the organization of the nucleoplasm of *E. coli* during prefixation with either OsO_4 or glutaraldehyde has been examined (24). It was inferred that the different shape of the nucleoplasm after prefixation with OsO_4 and glutaraldehyde is caused by the different effect of the two fixatives on the permeability of the cell membrane.

Prefixation with OsO_4 causes an immediate escape of K^+ ions and entrance of Na^+ ions. This re-establishment of ionic conditions in the cell presumably causes the contraction of the nucleoplasm observed by comparing unfixed

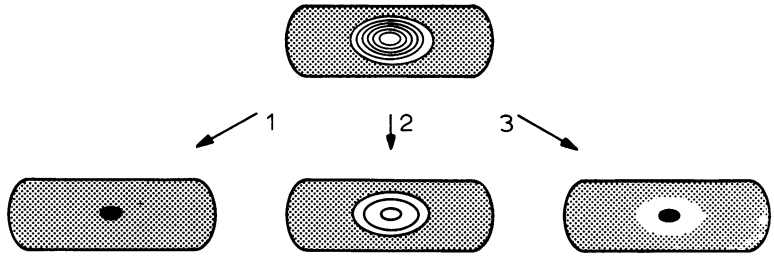

FIG. 18. *Alterations of nucleoplasmic structure after various treatments. The upper bacterium represents schematically the in vivo situation in which the nucleoplasm is highly hydrated with well-separated DNA fibrils. (1) Dehydration by high concentrations of freeze-protecting agents. As a result, DNA collapses into small aggregates. The cytoplasm moves into the space previously occupied by the hydrated nucleoplasm. Upon freeze fracturing, no distinction is observed between nucleoplasm and cytoplasm (Fig. 17). (2) Pre- and postfixation with OsO_4 according to the Ryter-Kellenberger technique (19). The cytoplasm is stabilized, and the nucleoplasmic outline is therefore preserved. Because of lateral aggregation of DNA fibrils, the nucleoplasmic region is easily visible after freeze fracturing, even in the presence of ice crystals (Fig. 1). (3) Prefixation with OsO_4 only. As in the previous case, the nucleoplasmic outline is preserved. The DNA, however, collapses because it is not fixed under these conditions (Fig. 11). In the presence of ice crystals the nucleoplasm is not visible (Fig. 2 and 9). In the absence of ice crystals the nucleoplasm is visible as a smooth region (Fig. 10).*

(Fig. 12) and prefixed (Fig. 13) cells with the phase-contrast light microscope.

In contrast to OsO_4, prefixation with glutaraldehyde has a less drastic effect on the permeability properties of the plasma membrane of *E. coli*, as deduced from the preservation of plasmolysis spaces during glutaraldehyde prefixation (24). This does not automatically imply that the glutaraldehyde image of the nucleoplasm (Fig. 16) is the correct one. A point of concern is the possibility of an artificial dispersion, i.e., the intermixing of nucleoplasmic with cytoplasmic material. A dispersed conformation could be induced by a net loss of cations from the cell resulting from a leakage of K^+ ions and exclusion of Na^+ ions (24). In addition, however, the fixation of the cytoplasm and of nucleoplasmic/cytoplasmic links should be considered. Relevant findings are that formaldehyde is used to stabilize the association of polysomes with *E. coli* DNA in the Miller spreading technique (12), and that the folded genome is likewise stabilized with formaldehyde (17) or with glutaraldehyde (2). This indicates that in the isolated state aldehyde fixation preserves the nucleoplasmic/cytoplasmic linkage, i.e., the transcriptional ribonucleic acid plus the relevant proteins that link DNA to the ribosomes (cf. 22). Experiments in progress, however, show that glutaraldehyde, unlike OsO_4, fixes the intact cell only very slowly. Phase-contrast light microscopy showed that dehydration of cells prefixed with glutaraldehyde caused a disappearance of the nucleoplasm similar to that observed during dehydration of unfixed cells (Fig. 15), whereas in OsO_4-prefixed cells this treatment did not change the appearance of the nucleoplasm. This observation suggests that after prefixation with glutaraldehyde the cytoplasm is still incompletely fixed, and that during dehydration it mixes with the nucleoplasm. Current research is concerned with the capacity of aldehydes to fix the cytoplasm.

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