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Shape and Fine Structure of Nucleoids Observed on Sections of Ultrarapidly Frozen and Cryosubstituted Bacteria

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Very rapidly frozen cells of *Escherichia coli* and *Bacillus subtilis* were substituted at low temperature into acetone with 1% OsO_4 and embedded in Epon. They showed ribosome-free spaces filled with globular and fibrillar material of up to 15 nm. The sizes of structures seen do not exclude DNA superstructures such as supercoils, aggregates, and nucleosomes. With the Feulgen analog osmium-ammines stain, DNA was localized within the ribosome-free space. The bulk of DNA, the nucleoid, is therefore a major part of, or identical to, the main ribosome-free space. The ribosome-free space would correspond directly to the light microscopy phase-contrast image of nucleoids in living bacteria. The shape of the ribosome-free space does not reflect intracellular salt concentrations, nor do the Feulgen-positive areas. The previously observed dependency on the salt concentration of the growth medium seems to be due to permeabilization induced by the chemical fixative at room temperature. The ribosome-free space is more cleft in appearance than the nucleoid obtained by fixation with OsO_4 but more confined than its very dispersed form found after aldehyde fixation.

In the last decade, enormous progress has been made in elucidating the organization of eucaryotic chromatin. The contribution of electron microscopy to solving these problems has been mainly through observation of isolated and extracted material and in vitro reassemblies; thin sections have still not contributed very much to a deeper understanding of the important in vivo-in situ situations. During the same time, our gain in knowledge of the organization of procaryotic DNA has been very much slower. The existence of procaryotic nucleosomes (13, 36) which would be organized like those of the eucaryotes is still questionable and experimentally not well documented; the stoichiometry of protein(s) per nucleosome is still not known. Only data obtained by trimethylpsoralen cross-linking have provided relatively clear evidence for a DNA supercoiling (48). Even our results, obtained through the study of thin sections of bacteria, become contradictory in the sense that some fixatives (aldehydes) produced micrographs of dispersed DNA, which fits with the expressed theoretical views (see below), whereas OsO_4 fixation produced more localized, confined nuclei. Additional experimental facts tended to increase the confusion. Among them are the following two recent findings. (i) Fixation with OsO_4 leads not only to the desired cross-links between proteins but also to fragmentation of polypeptides and therefore to production of fragments detectable by polyacrylamide gel electrophoresis (1, 7). Although with this method, the detected separation of the fragments occurs in general only after heating in detergent (1), it cannot be excluded that, for example, natural, protein-mediated cross-links between DNA strands might be ruptured by OsO_4 or that DNA-binding proteins might become separated from DNA (C. L. Woldringh, personal communication). (ii) Aldehydes and OsO_4 induce a rapid leakage of cellular small solutes (54; M. L. J. Moncany, Thesis Docteur d'Etat, l'Universite de Paris VII, Paris, France). Of particular interest for us is potassium, which, with its counterion, is responsible for maintaining the cellular turgor pressure in response to the outside osmotic pressure. Consequently, the ionic strength of the cellular sap is modified. Magnesium also leaks, but more slowly, because 80 to 90% of it is bound to nucleic acids (28; Moncany, Thesis Docteur d'Etat). The change of the intracellular ion concentration might influence the configuration of the phase separations between the ribosome-containing cytoplasm and what we call the DNA-containing plasm (DNA plasm).

The possible consequences of these two new facts on both the gross morphology of the nucleoids and on the fine structural organization of the DNA are easy to imagine and are certainly related to several of the following well-known experimental facts. (i) Very different distributions of the DNA plasms are produced after aldehyde and OsO_4 fixation, as has been observed in many laboratories. (ii) The degree of dispersion is dependent on the salt concentration of the growth medium when nucleoids are fixed with OsO_4 but not when they are fixed with aldehyde (41, 53, 55). With OsO_4, the nucleoids are more confined with higher salt concentrations in the growth medium. With aldehydes, the nucleoids are always maximally dispersed. (iii) Different specific shapes of the nucleoids are induced by particular actions: exclusive cessation of protein synthesis, e.g., as mediated by chloramphenicol, leads with *Escherichia coli* to the formation of very characteristic spherical nucleoids (mostly with a central core) which are independent of fixation (23). Conversely, UV radiation (even at sublethal doses) produces a very strong dispersion of nucleoids (19, 21, 24, 37).

Data obtained by a new approach, namely rapid immobilization by quick freezing, should provide the possibility of overcoming the present barriers. We report here our results obtained by freeze-substitution of a gram-negative bacterium, *E. coli*, and a gram-positive bacterium, *Bacillus subtilis*. Our results will be compared with those of Dubochet et al. (4) obtained by observing frozen-hydrated cryosections.

Terminology. It is our experience that the study of DNA plasms of any origin is suffering from an unclear understand-

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ing of terminologies, particularly when used in relation to electron microscopic observations. Compaction and condensation both mean actions by which a structure is moved into occupying less space. We propose to reserve both terms for any process that leads to an increased local concentration of DNA. Condensation occurs when part of the replicating DNA of a bacteriophage becomes packaged into the head precursor. By this process, the concentration of DNA increases more than 30 times. The term compaction is used currently for eucaryotic chromatin to describe supercoiling at all levels. As for nucleoid shape description, we propose to use the terms dispersion and confinement, by which we describe the scattering of patches of DNA plasm all over a bacterium (dispersion) versus the containment of DNA plasm within a clearly delimited, confined area (confinement). Confinement or dispersion are thus not accompanied by a local increase of the DNA concentration. To avoid misinterpretations, confinement should not be described as condensation, as is frequently done.

MATERIALS AND METHODS

Culture conditions. *E. coli* B was grown in liquid media that had the following basic compositions. Low salt medium (LS) contained tryptone (10 g liter^{-1}; Difco Laboratories, Detroit, Mich.). Normal salt medium (NS) was LS supplemented with 6 g of KCl liter^{-1}; high salt medium (HS) was LS plus 21 g of KCl liter^{-1}. For tryptone agar slants, 20 g of Difco agar liter^{-1} was added. In some cases, sucrose (46.2 or 147 g liter^{-1}) was added to the liquid media in place of KCl at the same osmolality. Osmolality was determined on a Halbmikro osmometer (Knauer & Co., Berlin). NaCl could also be used instead of KCl with similar results. Cells were harvested or prefixed in the medium during exponential growth at a concentration of approximately 2×10^8 cells ml^{-1} after overnight growth and subculturing.

B. subtilis SMY was grown in nutrient broth (38) either in liquid medium or on solid agar medium.

Electron microscopic procedures: (i) conventional fixation. Conventional fixation was performed by the Ryter-Kellenberger (RK) procedure (24, 41) with prefixation directly in the culture medium with either OsO_4 (final concentration, 0.1% [vol/vol]) or gutaraldehyde (final concentration, 1% [vol/vol]) for 30 min. Cells were then centrifuged ($2,000 \times g$ for 5 min at 20°C) and taken up into agar blocks (25) (2% [wt/vol] Difco agar and 1% [wt/vol] tryptone in Michaelis veronal-acetate buffer containing 10 mM $CaCl_2$ [pH 6.2] to satisfy the RK conditions of fixation [41]). Fixation was then continued in the fixative (concentration, 1% for both in Michaelis veronal-acetate buffer) overnight. Cells were then washed in Michaelis veronal-acetate buffer, postfixed with 0.5% (wt/vol) uranyl acetate in Michaelis veronal-acetate buffer (41) for 2 h, washed again with buffer, dehydrated in a graded series of ethanol solutions, and embedded in Epon.

(ii) PLT procedure. For progressive lowering of temperature (PLT), cells were fixed directly in the culture medium (37°C) with 1% glutaraldehyde (final concentration) and collected into agar blocks as described above, followed by dehydration, infiltration in either Lowicryl HM20 or K4M resin, and UV polymerization at low temperature (−35°C) as reported earlier (16, 17). In this novel PLT procedure, the temperature during dehydration was lowered stepwise to −35°C, and the concentration of organic solvent was simultaneously increased to a final concentration of 100% (vol/vol).

(iii) Freeze-substitution. Cells were harvested directly from liquid culture by filtration for 3 to 4 min (plus aeration) on a nucleopore filter (diameter, 13 mm; pore size, 0.2 μm), after which the cells were collected immediately on a small piece of Whatman no. 111 filter paper or Job no. 807S cigarette paper (punched out to a diameter of 5.5 mm) and then mounted and immediately frozen by projection onto a copper block cooled via liquid helium (Cryoblock, Reichert-Jung, Paris, France) by the method of Escaig (9). Samples were stored in liquid nitrogen before the substitution step.

In some experiments, liquid cultures of *B. subtilis* were deposited directly onto a small piece of filter paper to avoid centrifugation. However, since the number of bacteria found in thin sections was very low, a drop of culture was also spread on solid medium, and the bacteria were collected with a spatula 2 to 4 h after inoculation for exponential-phase cells and 20 h after inoculation for sporulating cells. The dense bacterial suspension was immediately deposited onto filter paper and frozen by the same procedure (9) as described above for *E. coli* B.

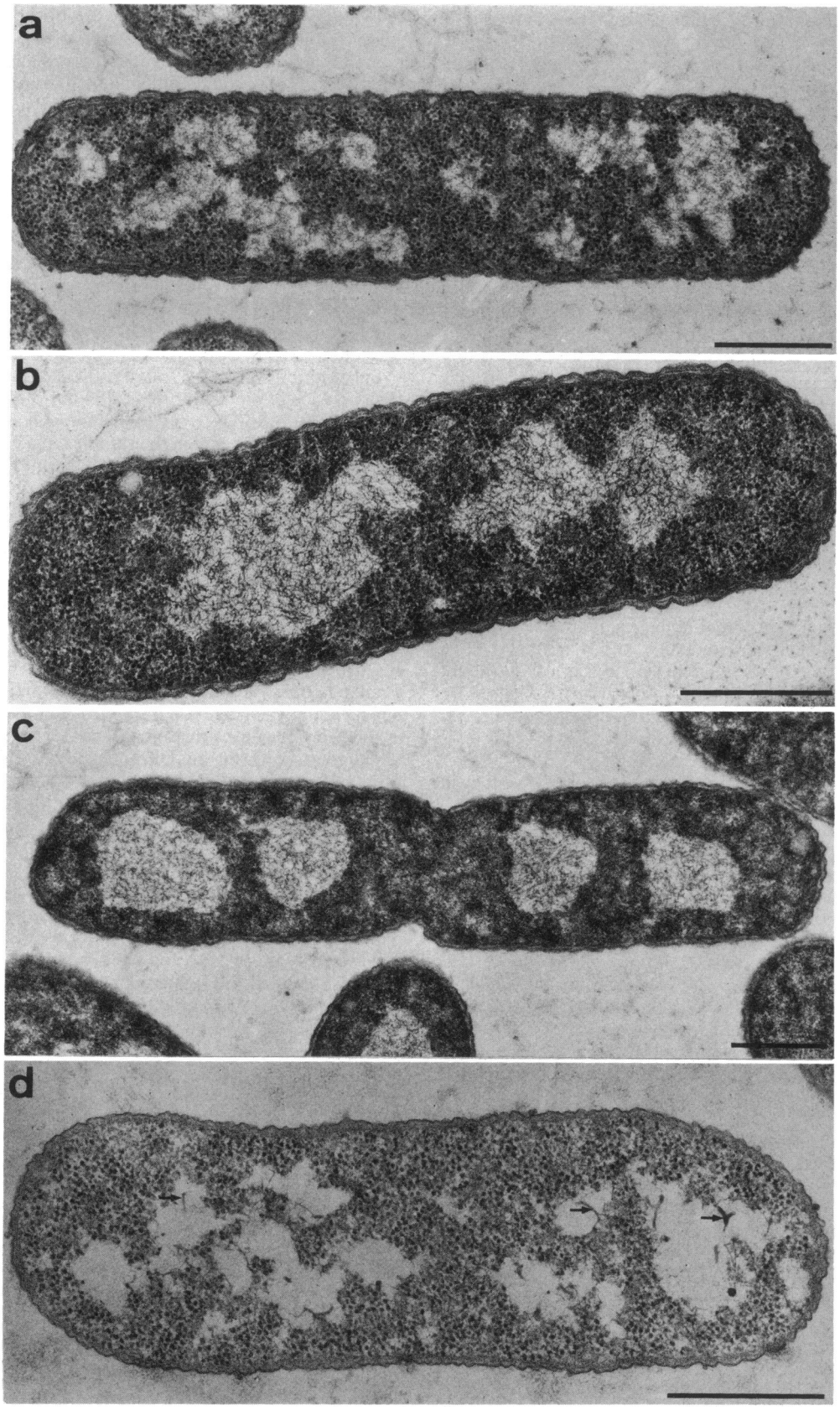
Substitution was in 2.5% (wt/vol) OsO_4 in pure acetone in the presence of molecular sieve (0.4 nm; Perlform; Merck & Co., Inc., Rahway, N.J.) at −90°C for 64 h. The temperature was controlled by keeping the samples in a Cryocool CC-100 (Neslab; distributor, Bender & Hobein, Zürich, Switzerland). After 64 h at −90°C, the temperature was raised to −35°C, and the samples were kept at −35°C for 2 h. The temperature was then raised to 4°C, and the samples were kept at 4°C for 2 h, after which the samples were left at room temperature for 2.5 h, washed three times in acetone, and embedded in the following graded series of Epon-acetone mixtures: 1:2 for 1 h, 1:1 for 1 h, 2:1 overnight with vial tops removed to allow acetone to evaporate, and pure Epon for 8 h. All samples were then polymerized at 65°C for 3 days. Prefixed cells were treated in the same manner except that substitution was carried out in pure acetone.

(iv) Microscopic examination. Thin sections were cut with a diamond knife on an Ultramicrotome III (LKB Instruments, Inc., Rockville, Md.), stained with uranyl acetate and lead citrate (33, 52), and examined in either a Philips 300 or a Zeiss 10 electron microscope operating at 80 kV. Osmium-ammines sections were observed in a Hitachi HU 12 electron microscope operating at 75 kV.

(v) Osmium-ammines staining. Osmium-ammines staining was performed by the method of Cogliati and Gautier (2). Thin sections of untreated cells were incubated with 2% H_2O_2 for 20 min at 20°C, followed by acid hydrolysis on 5 N HCl for 15 min at 20°C. Staining with 0.1% osmium-ammines complex previously bubbled with SO_2 was performed for 90 min at 36°C (12).

RESULTS

***E. coli*: (i) conventional fixation.** Under RK conditions of OsO_4 fixation, the shape of the nucleoid changed as the external salt concentration in the medium was varied (Fig. 1a, b, and c). In cells grown in LS, the nucleoid was semilocalized (Fig. 1a); in cells grown in NS, the nucleoid was localized (Fig. 1b); and in cells grown in HS, the nucleoid was confined (Fig. 1c). With glutaraldehyde fixation, the nucleoid was dispersed irrespective of the external salt or sucrose concentration used (Fig. 1d). Variation of the external osmolality with sucrose in the absence of salt always gave the nucleoid a semilocalized appearance under RK conditions with OsO_4 and a dispersed appearance in all cases with glutaraldehyde fixation (results not shown). The DNA plasm appears finely fibrillar under RK conditions (Fig. 1a, b, and c). With glutaraldehyde fixation alone without postfixation in uranyl acetate, coarse aggregates of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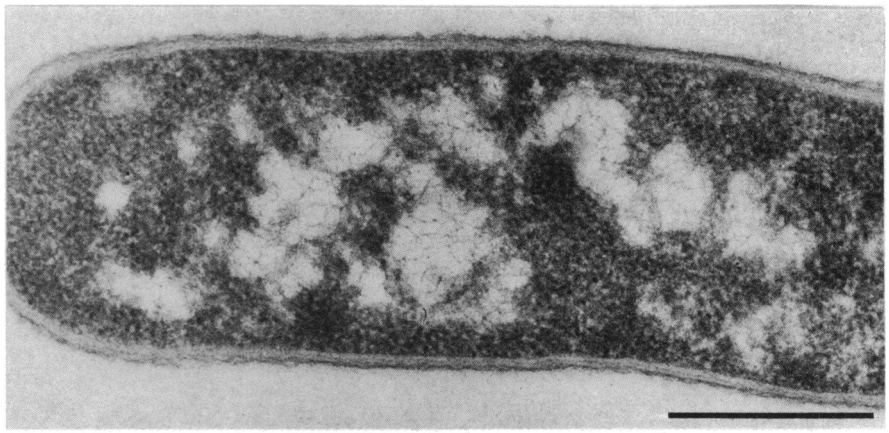


FIG. 2. *E. coli* B fixation with glutaraldehyde PLT in Lowicryl K4M. Cell was grown in NS. The nucleoid is dispersed with random strands crisscrossing the nucleoid areas. Bar marker, 0.5 μm.

DNA were present within the seemingly empty dispersed nucleoid (Fig. 1d, arrowed).

(ii) PLT. Low-temperature embedding of solely glutaraldehyde-fixed *E. coli* with either Lowicryl HM20 or K4M gave nucleoids a dispersed morphology (Fig. 2). The DNA plasm appeared as random strands crisscrossing the dispersed nucleoid areas (Fig. 2). However, with Lowicryl embeddings, the DNA plasm had a tendency to vary in appearance from coarse aggregates to fine strands, depending upon the type of aldehyde fixation used and on whether uranyl acetate postfixation (47) was followed before the dehydration steps.

(iii) Freeze-substitution. With freeze-substitution, *E. coli* grown in NS showed visible ribosome-free areas containing rather grainy structures intermingled with fibrous elements (Fig. 3a, b, and c). The ribosome-free regions could be clearly distinguished and had generally more indentations than did the nucleoids of bacteria fixed under the standard RK conditions (Fig. 1b). It also appeared less dispersed than did the nucleoid observed after glutaraldehyde fixation (Fig. 1d and 2). The ribosome-free area is therefore localized within the cytoplasm as a specific region having a cleft appearance (indented leaf shape). No change in the ribosome-free shape occurred in cultures grown with high external salt concentrations (Fig. 4a, b, and c) or with low salt concentrations or in media containing sucrose with the same osmolality as NS or HS (results not shown). Harvesting of the cells by centrifugation before freezing caused the nucleoid to be totally dispersed in the cytoplasm, whereas collecting the cells by filtration or by direct deposition of liquid culture onto filter paper gave the above-described results. Centrifugation, as opposed to filtration, can cause leakage of ions due to oxygen starvation (28) and so influence the shape of the ribosome-free space or nucleoid.

Regarding the fine structure of the DNA plasm within the ribosome-free space, freeze-substitution (Fig. 3 and 4) produced a new finding. DNA appeared more evenly distributed throughout the entire ribosome-free area (Fig. 5a) and had a grainy appearance with a finer, fibrillar structure than was seen after either RK fixation (Fig. 5b) or PLT embedding in Lowicryl K4M (Fig. 2).

The treatment of bacteria with chloramphenicol (40 μg ml^{-1}) for 2 h before freezing and substitution led to the typical spherical nucleoid containing very often a typical homogeneous central core (Fig. 6) (21, 23, 24).

The cell envelope after freeze-substitution did not have the wavy appearance of cells fixed under RK conditions (Fig. 1) but was of uniform width and delimited by a fairly straight outer electron-dense line (Fig. 3 and 4). This is discussed in more detail by Hobot et al. (16). Another feature that was observed fairly frequently is a tendency for the ribosomes to appear in ordered patterns. These might be related to polysomes.

(iv) Prefixation studies. After growth in HS, cells prefixed in liquid culture at 37°C for 20 to 30 min by 0.1% OsO_4 and then treated by the freeze-substitution method (with the substitution medium containing only acetone) showed a confined nucleoid shape (Fig. 7a). The DNA plasm seemed to be slightly arranged into fibrillar strands. Cells prefixed with only 1% glutaraldehyde by the conditions just mentioned had a dispersed nucleoid in which DNA plasm fibrils appeared more disordered (Fig. 7b). In both cases, the DNA was not so finely distributed throughout the nucleoid as it was in the ribosome-free space of untreated freeze-substituted bacteria. These prefixation experiments demonstrate the influence of permeabilization (one of the effects of chemical fixation together with cross-linking) on the shape of the ribosome-free area and on DNA plasm structures. It could be suggested that during the fixation-induced permeabilization, ionic leakage could influence not only the overall nucleoid shape but also the fine structures of the DNA plasm.

(v) Osmium-ammines stain. The osmium-ammines stain for electron microscopy is a chemical analog of the Feulgen stain. The RNA, not the DNA, is hydrolyzed preferentially, and, thus, only DNA is stained. Two different regions were

FIG. 1. (a) Standard OsO_4 fixation of *E. coli* B under RK conditions. The cell was grown in LS. The nucleoid is dispersed. Bar marker, 0.5 μm. (b) Same as (a), except that the cell was grown in NS. The nucleoid is more confined and localized. Bar marker, 0.5 μm. (c) Same as (a), except that the cell was grown in HS. The nucleoid is still more confined. Bar marker, 0.5 μm. (d) Fixation of *E. coli* B with glutaraldehyde, embedded in Epon. The cell was grown in HS. The nucleoid is dispersed into small patches which contain DNA precipitates (arrows). Bar marker, 0.5 μm.

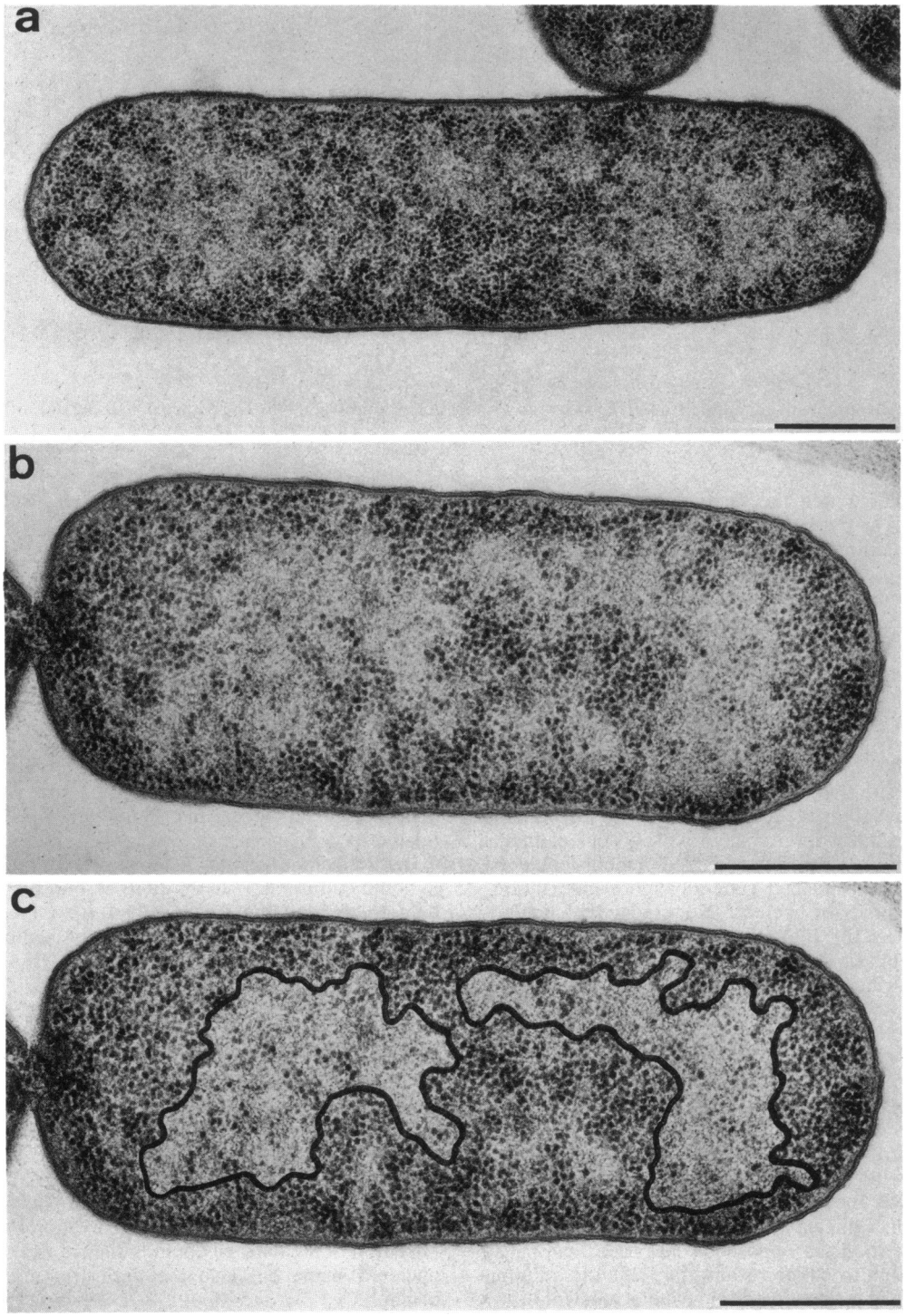


FIG. 3. (a) *E. coli* B freeze-substitution with no pretreatments. The cell was grown in NS. The ribosome-free space is localized and filled completely with a fine-grained plasm. Note the uniform appearance of the cell envelope in contrast to its appearance under conventional fixation procedures in Fig. 1. Bar marker, 0.5 μm. (b) Same as (a). Bar marker, 0.5 μm. (c) Same as (a), but with the outline of the ribosome-free area drawn in. Bar marker, 0.5 μm.

clearly discernible (Fig. 8): a white, bleached cytoplasm and a darker region which corresponds to the ribosome-free area of untreated cells (cf. Fig. 3, 4, and 8). Only within this ribosome-free area were the black deposits of osmium-ammines visible, signifying that DNA is concentrated within the ribosome-free space. This technique is not sensitive enough to reveal DNA fibrils which could be dispersed among the ribosomes. The same distribution of stain deposits was obtained whatever the growth conditions (LS, NS, or HS) used.

***B. subtilis*: (i) conventional fixation.** As shown in many studies, the nucleoid of *B. subtilis*, like those of other

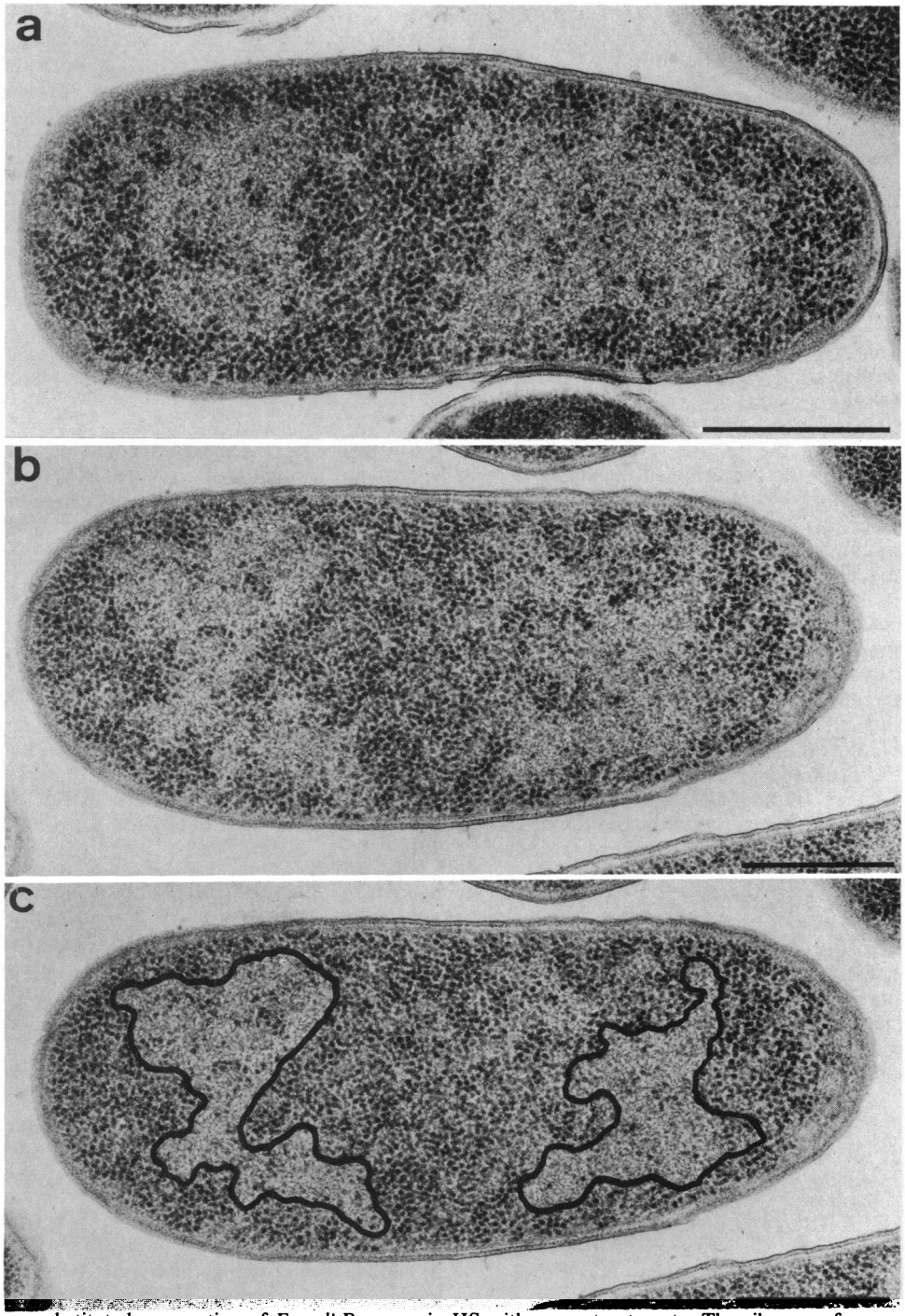


FIG. 4. (a) Freeze-substituted preparation of *E. coli* B grown in HS with no pretreatments. The ribosome-free space is localized and contains a fine-grained plasm. Bar marker, 0.5 μm. (b) Same as (a). Bar marker, 0.5 μm. (c) Same as (b), but with the outline of the ribosome-free space drawn in. Bar marker, 0.5 μm.

gram-positive bacteria, has a more confined shape and a more compact texture than does *E. coli* when it has been fixed with OsO_4 under RK conditions (35, 39, 51). Its boundary is generally more delimited, and the DNA plasm has fibrils that are often organized into twisted bundles (Fig. 9c). After glutaraldehyde fixation, the nucleoid was more dispersed in the cytoplasm but to a lower extent than in *E. coli* (Fig. 9d).

(ii) Freeze-substitution. After freeze-substitution, the ribosome-free area was more dispersed than after RK fixation but still rather localized (Fig. 9a). However, its shape was quite similar to that of the *E. coli* ribosome-free space (Fig. 3 and 4). As seen in Fig. 9b no bundles of filaments were apparent in the DNA plasm within the ribosome-free space after freeze-substitution (Fig. 9b), in contrast to the DNA plasm after RK fixation (Fig. 9c). The freeze-substituted ribosome-free space of *B. subtilis* was directly comparable to that of *E. coli* (Fig. 5a).

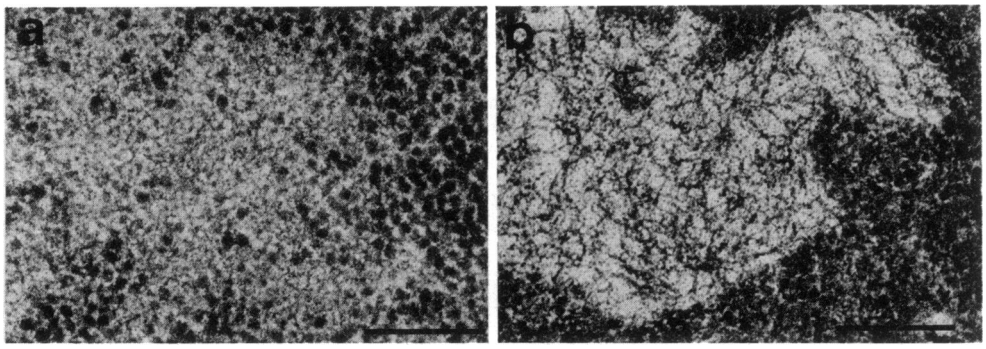


FIG. 5. (a) Higher magnification of *E. coli* B ribosome-free space in freeze-substituted preparation of cell grown in NS. The fine-grained plasm is finely distributed throughout the ribosome-free region, filling it completely. Bar marker, 0.2 μm. (b) Higher magnification of nucleoid area of *E. coli* B in conventional RK fixation with OsO_4. The DNA appears as fine fibrils which fill the nucleoid area but not so evenly as in the freeze-substituted cell. Bar marker, 0.2 μm.

No mesosomes were visible in the region of septum formation nor elsewhere in the bacterium. Ribosomes were clearly visible, and the cell wall had the normal electron-dense outer appearance (cf. Fig. 9a and d). The cytoplasmic membrane was sometimes difficult to discern and was not separated from the cell wall by an electron-transparent space.

Studies carried out on sporulating cells have shown that cells at all stages of sporulation present their usual features (38). It is noteworthy to observe that the sporal nucleoid presented the same characteristic features during spore maturation that it showed after RK fixation (43). The sporal nucleoid was small and spherical at stage II and more dispersed at stage III. From the beginning of the appearance of the spore wall (stages III and IV), it took the typical ribbon-like shape, showing a clearcut boundary with the cytoplasm. The mother cell nucleoid was large and dispersed at stage II, as after RK fixation, and remained quite dispersed throughout spore maturation. Freeze-substitution also demonstrated the absence of mesosomes associated with the forespore or the mother cell (Fig. 9e and f) as observed previously (5).

Freeze-substitution presented two other new observations: (i) the cortex had a spongy structure, and (ii) the ovoid forespore was surrounded by homogeneous material which totally filled the space between the sporangium wall and the forespore. This material could be detected along the sporulation septum as soon as it started to bulge (Fig. 9e). At the end of stage IV, this material began to take on a stratified appearance, and at stage V and VI, it was replaced by the inner and outer coats.

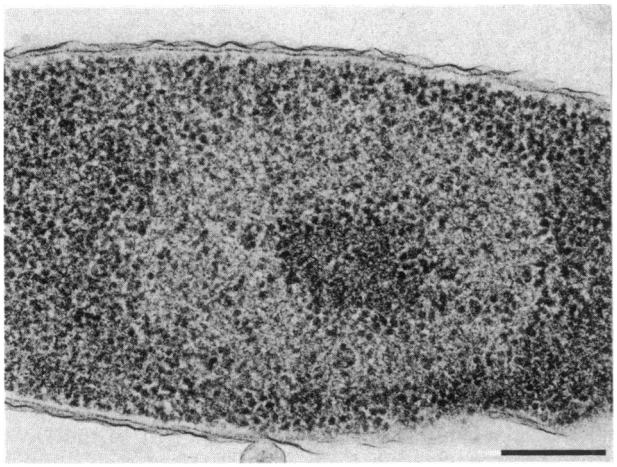


FIG. 6. Chloramphenicol-induced nucleoid from freeze-substituted *E. coli*. Bar marker, 0.2 μm.

DISCUSSION

When judging the preservation of thin-sectioned cells, we must consider two levels. The first concerns the nucleoid shape, which is related to the immobilization of the protoplasmic elements during fixation. In other words, we have to be sure whether the DNA and cytoplasmic materials are able to rearrange before their immobilization by the fixative. On the second level, we must consider the preservation of finer structural details of the bacterial DNA organization (e.g. supercoiled DNA and nucleosomes [reviewed in reference 30]).

Nucleoid shape. The observation of metabolically active DNA by Miller and Hamkalo (27) has revealed a fundamental, experimentally ascertained difference between procaryotes and eucaryotes: the "Christmas trees" formed by transcribing genes are naked in eucaryotes, but the mRNA branches are studded with ribosomes in procaryotes. This means that intracellularly the metabolically very active bacterial DNA of growing cells has to be in the close neighborhood of the ribosomes, as is shown very well in Fig. 10. It is clear that a complete intermixing between ribosomes and DNA would provide theoretically for an optimal efficiency in translation if all the genes are in activity. The distribution of DNA over the whole cell (i.e., complete dispersion) would be the extreme consequence of this concept. How far is such a theoretical view compatible with the experimental facts? From phase-contrast light microscopy it is known that growing cells show two to several areas of lower density, which divide in synchrony with cell division (26, 34). This technique, however, needs experimental skill to achieve comparably good results, especially with the aerobic *E. coli* and *B. subtilis*. It is therefore not easy to observe very actively metabolizing bacteria. In our laboratory and in that of Woldringh, we made similar observations supporting the view that the *E. coli* nucleoids are distinct areas but tend to be less precisely and sharply confined than those obtained by OsO_4 fixation and observed on unsectioned entire bacteria by the agar filtration technique (18, 20). This conclusion is similar to the one reached by Daneo-Moore et al. (3) and Edelstein et al. (6).

The pictures obtained with *E. coli* and *B. subtilis* after freeze-substitution are thus in good agreement with the light

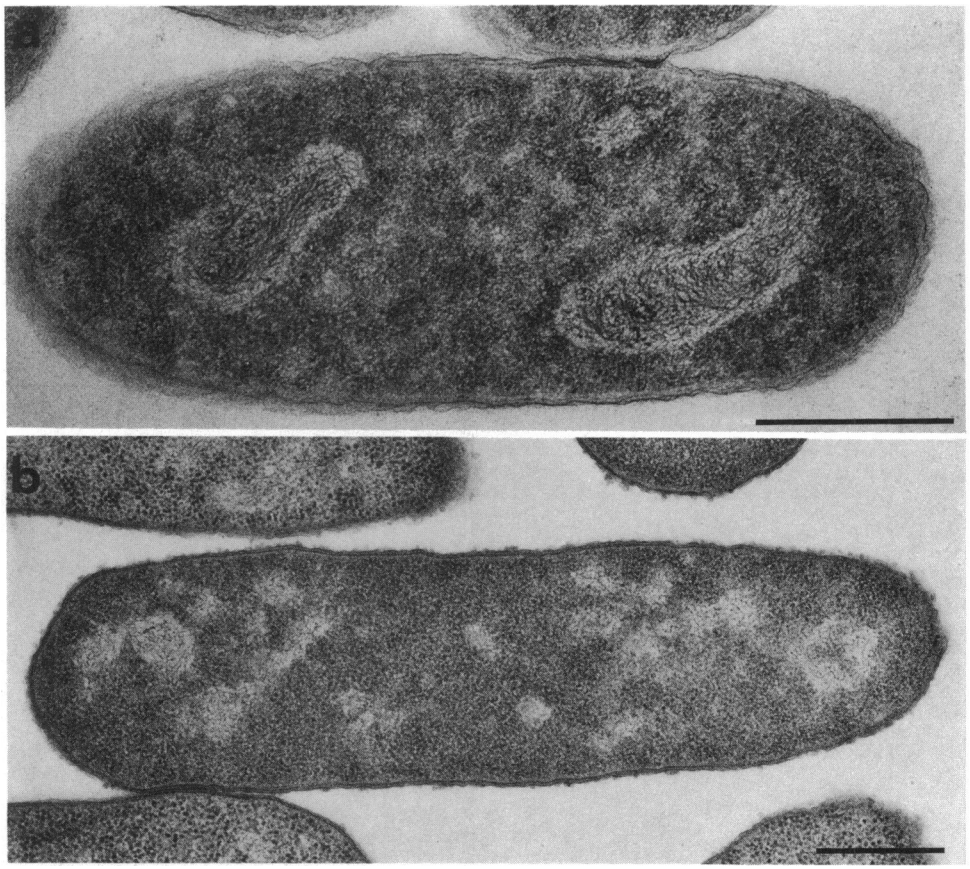


FIG. 7. (a) *E. coli* B, prefixed with 0.1% OsO_4 directly in liquid culture (HS) and freeze-substituted. The nucleoid is confined and contains DNA in a fibrillar form. Bar marker, 0.5 μm. (b) Same as (a), except that the cell was prefixed with 1% glutaraldehyde. The nucleoid is dispersed, and the DNA is in a fibrillar form. Bar marker, 0.5 μm.

microscopic results. In both species, the ribosome-free space or nucleoids had a more dispersed shape than after osmium RK fixation, and the boundary between DNA plasm and cytoplasm was not clearcut. Our results are thus in good agreement with those obtained by Dubochet et al. on frozen-hydrated cryosections (4). This means that OsO_4 fixation leads to an increased confinement of the nucleoid. Conversely, glutaraldehyde tends to disperse DNA plasms, especially in *E. coli*, even with the PLT technique.

The results obtained in freeze-substitution with *E. coli* growth with HS and LS clearly show that the different nucleoid shapes observed after RK fixation are artifactual. In the Basel laboratory it has been found that OsO_4 and aldehydes rapidly induce leakage of small cellular solutes, particularly of potassium (Moncany, Thesis Docteur d'Etat). Was it possible, therefore, that the change in internal ionic conditions associated with the external salt concentration might induce a rearrangement of the cellular content before the cytoplasm became cross-linked and gelled, and that this would consequently influence the distribution of the areas containing the DNA plasm? It was known for procaryotes that a gellation of the DNA was not possible with either

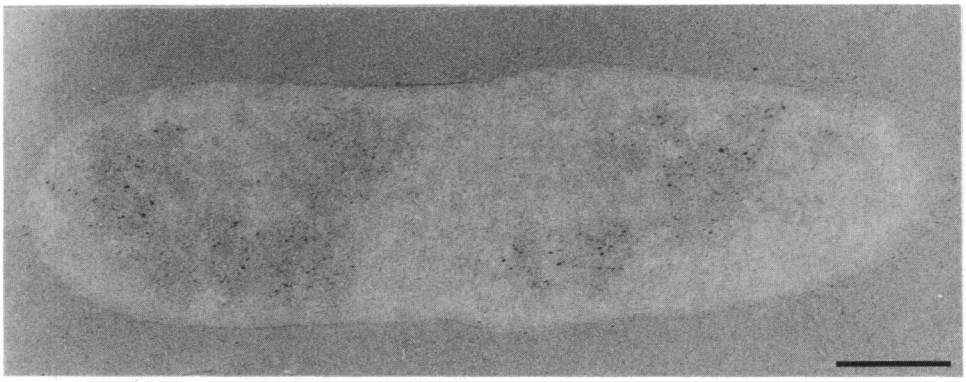
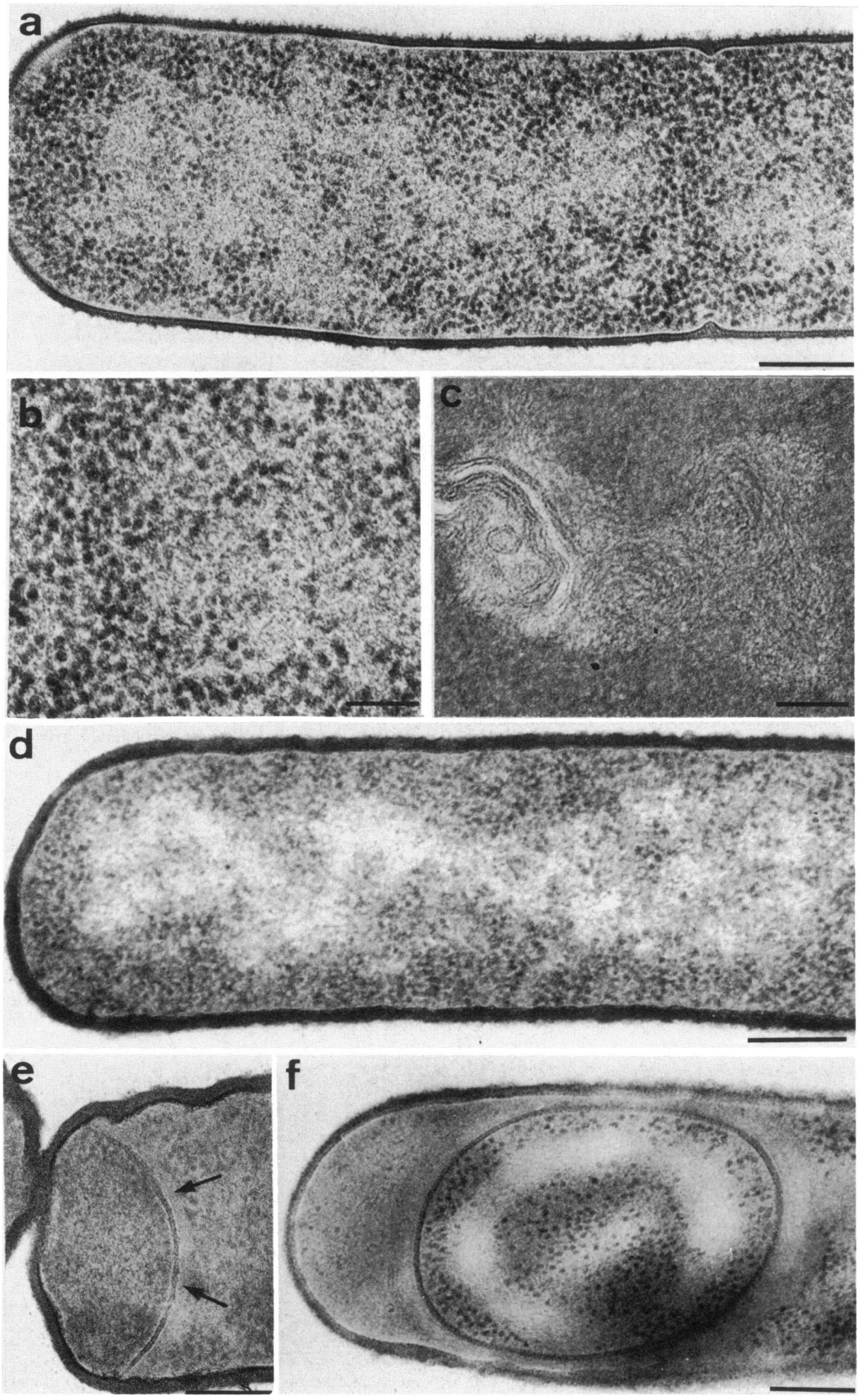


FIG. 8. Freeze-substituted preparation of *E. coli* B grown in NS with no pretreatments. Thin sections were hydrolyzed and stained by the osmium-ammines method. The darker ribosome-free area stands out from the bleached cytoplasm and contains the black osmium-ammines deposits. Bar marker, 0.5 μm.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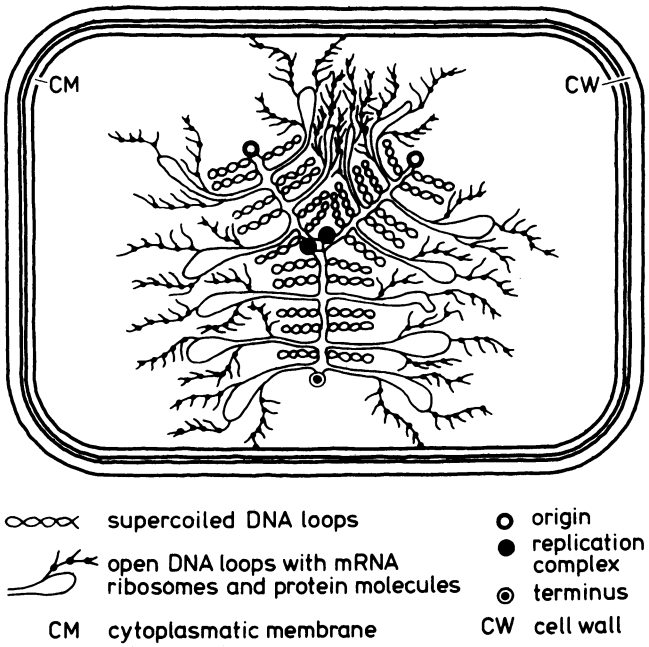

FIG. 10. Schematic concept of the flow of information from the genome to proteins. This drawing is reproduced by permission of J. Gumpert (Jena) and shows transcription of a segment of DNA into mRNA that binds immediately to the ribosomes and translates the information into protein. This scheme takes into account the results of Miller and Hamkalo (27) and the accumulated biochemical knowledge. It has now to be compared with the new structural observations.

fixative, except in the case of OsO_4, but only in the presence of amino acids or peptide fragments (22, 41, 44, 45). It is certain that the rapid freezing used in our experiments (20 to −263°C, approximately 10 to 30 ms [9]) is much faster than anything related to induced leakage (2 min, OsO_4; 3 min, glutaraldehyde [Moncany, Thesis Docteur d'Etat]) by an estimated factor of 4,000 to 12,000.

In all our experiments, we have concentrated the bacteria by a rapid filtration of still oxygenated cultures of cells. Earlier work has shown that filtration causes a lower ionic leakage than does centrifugation (8). By maintaining the oxygenation and temperature, better conditions for reducing ionic leakage during filtration are achieved (Moncany, Thesis Docteur d'Etat). It is therefore clear that filtration is better than centrifugation. Higgins et al. (15) have suggested, however, that filtration can already cause a "physical insult." We had to compromise between a reasonable population statistic, which requires concentration of the cells, and such possible insults. Knowing that exponentially rapidly growing cells represent a very heterogeneous population in respect to nucleoid shapes, we chose the alternative of using a number of cells high enough to exclude any statistical inbalance in our observations, due to low numbers, which could lead to wrong conclusions.

The typical aspect of the spherical nucleoid with a central core of chloramphenicol-treated *E. coli* cells is maintained in freeze-substitution. It therefore represents something real, deserving further studies. Such nuclei with *E. coli* are induced in all situations leading to arrest of protein synthesis: antibiotics (chloramphenicol, puromycin and aureomycin [21, 23]) and growth of amino-requiring strains in the absence of the amino acid (unpublished data). For chloramphenicol, one of us, with Moncany (Thesis Docteur d'Etat) have found experimentally that no leakage of ions occurs.

The question immediately arose about the relation between DNA and the ribosome-free space. Through the theoretical concept of Fig. 10, no stringent requirement would impose an identity or even only an overlap between ribosome-free space and DNA-containing nucleoids. Gautier osmium-ammines stain (11), which is specific for DNA, gave good evidence that the DNA is largely concentrated in an area which lies within the ribosome-free space.

Fine structure of the DNA plasm. In the conventional RK procedure, the DNA plasm has a characteristic texture of a system of fibrils (Fig. 5b and 9c). This is in contrast to the texture observed after freeze-substitution, in which we see rather grainy structures intermingled with fibrous elements (Fig. 5a and 9b). Grains of up to 15 nm and segments of fibers in the range of 4 to 6 nm can be seen easily. This is true not only for the large, ribosome-free areas but also for anywhere else in the smaller spaces between the ribosomes. Our results neither exclude nor prove the existence of supercoiled structures (as revealed with trimethylpsoralen cross-linking [48]) which, among other forms, could take that of nucleosomes possibly comparable to those in eucaryotes as postulated by Griffith (13). The rather grainy background of the ribosome-free space in freeze-substituted bacteria suggests that during this preparation procedure for electron microscopy, the contents (DNA, RNA, and proteins) are not removed, whereas with OsO_4 fixation under standard RK conditions, the DNA fibrils remain and stand out against a lighter background due to a possibly reduced protein content. This is also true for glutaraldehyde-fixed bacteria, in which the procaryotic DNA is not fixed and precipitates out as aggregates during dehydration after classical or PLT procedures. Such an interpretation would be in agreement with the observations of Dubochet et al. (4), who found little density differences between cytoplasm and nucleoid in the frozen-hydrated bacterial sections.

Other structural features observed after freeze-substitution. After immobilization of the cell protoplasm by freezing, we find no mesosomes. This is the case with cryosections (4) as well as the subjects of a previous freeze-substitution study (5). There is now, therefore, better experimental evidence for the nonexistence of mesosomes than the earlier results, obtained by cryofracturing, which had led to a similar result (14, 15, 29). In cryofractures, the fracture surface follows the

FIG. 9. (a) Freeze-substituted preparation of *B. subtilis* with no pretreatments. Note the ribosome-free area within the cytoplasm, and the fine grainy texture of its contents similar to that of *E. coli* (cf. Fig. 3 and 4). Bar marker, 0.2 μm. (b) Enlargement of a ribosome-free area in (a), showing the fine-grained plasm filling this region completely. Bar marker, 0.1 μm. (c) Higher magnification of a nucleoid area of *B. subtilis* after RK fixation. The DNA is arranged into bundles of fibrils. Bar marker, 0.1 μm. (d) *B. subtilis* was fixed overnight in 2% glutaraldehyde and postfixed with OsO_4, followed by embedding in Epon. Bar marker, 0.2 μm. (e) Stage II of sporulation of *B. subtilis* after freeze-substitution with no pretreatments. A thin layer of material lies along the septum (arrows). Bar marker, 0.2 μm. (f) Stage III of sporulation, prepared as in (e). The forespore is surrounded by a homogeneous material filling the whole space between the cell wall of the sporangium and the forespore. Bar marker, 0.2 μm.

plane of least tensile strength. Because of the selectivity of this way of producing fracture surfaces, some structures are not revealed. For example, tubular variants of phage T4, which were present inside well-preserved, metabolizing infected cells, were never revealed in cryofractures. They were always easily visible after partial or complete lysis of the cell (46). This phenomenon could have occurred equally well with mesosomes, which would not be seen in fractures of well-preserved, metabolizing cells but are observed in physically insulted ones (15). After decades of research, no functions have been appointed to the mesosomes (cf. references reviewed in reference 50). It is therefore not so sad to contemplate that whether it is pleasant or not, mesosomes (as we are used to seeing them in beautiful sections of gram-positive cells) are artificially produced. How this comes about we do not understand at all. Higgins et al. (15) have suggested that they may be formed when the cells receive any physical insult such as centrifugation, fixation, cryoprotectants, or filtrations. This could explain why Remsen (31, 32) saw mesosomes in unfixed *B. subtilis* cryofractures, as they had been harvested before freezing by centrifugation. Since we do not find mesosomes by our technique, filtration would therefore not present a physical insult. The absence of mesosomes shows, of course, that the role of these artifactual structures in DNA-membrane attachment does not exist. This means that chromosome attachment corresponds probably to a direct binding with the cytoplasmic membrane, as observed in the cells devoid of mesosomes such as protoplasts (40) and reverting protoplasts (42) and during growth resumption of chloramphenicol-treated bacilli (10).

Other new data obtained by freeze-substitution concern *B. subtilis* sporulation. (i) We saw that, contrary to nucleoids of growing cells, the sporal nucleoid presented the different shapes found during spore maturation after RK fixation. It can be concluded that pictures obtained in the past correspond to true nucleoid appearance. In addition, it was observed that the ovoid forespore is surrounded by a material devoid of ribosomes. This suggests that the forespore is not surrounded by the mother cell cytoplasm (38) and that the whole polar end of the sporangium corresponds to the sporal region. (ii) The fact that the spore coats emerge from the homogeneous material surrounding the forespore strongly suggests that the coat precursors which were shown to be synthesized soon after the end of exponential growth (49) are probably located at a very early time around the forespore.

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