THE MESOSOME OF *BACILLUS SUBTILIS* AS AFFECTED BY CHEMICAL AND PHYSICAL FIXATION

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The reliability of structures observed with electron microscope techniques represents a general problem in ultrastructure research. Obviously, every technique employed will have its limitations. Unfortunately the limitations are incompletely understood, and the eventual image obtained by electron microscopy deviates to an unknown extent from reality. The experimental approach to this problem is not an easy one. However, the effects of chemical fixation on the preservation of ultrastructure can, within limits (9), be studied by means of freeze-fracturing.

In previous studies (8, 9) some of these effects were studied on young cells of *Bacillus subtilis*, strain Marburg. Freeze-fracturing after chemical fixation according to the Ryter-Kellenberger technique (9, 12) showed a good preservation of the plasma membrane and visible changes in the native state of the nucleoplasm (9). The present study deals with the effect of chemical fixation with osmium tetroxide on the mesosome.

MATERIALS AND METHODS

Culture and Medium

B. subtilis, strain Marburg, was grown aerobically in heart infusion broth (Difco Laboratories, Detroit, Mich.) on shaker at about 37°C. From an overnight culture 4 drops were inoculated into 70 ml of prewarmed broth. Growth was measured as optical density at 620 nm. Cells were collected after 3.5–4.5 hr in the exponential phase of growth. Centrifugation

was carried out at room temperature for 15 min at 7000 *g*.

Freeze-Fracturing

Sediments of bacteria were freeze-fractured either directly or after overnight fixation in the cold as previously described (9). No posttreatment with uranyl acetate was applied. Two types of freeze-fracture equipment were used: (*a*) the Balzers freeze-etch apparatus (Balzers AG, Liechtenstein) according to Moor et al. (7), and (*b*) a workshop-made device according to the basic design of Bullivant and Ames (1). With equipment (*a*), unfixed and fixed bacteria were frozen in the presence of 20% (v/v) glycerol in liquid Freon 22 (E. I. duPont de Nemours & Co., Inc., Wilmington Del.), in growth medium or fixation buffer (9) respectively. With equipment (*b*), unfixed and fixed cells were frozen in liquid Freon 22 or liquid nitrogen, and glycerol was omitted. In the Bullivant-Ames type of device, shadow-casting with platinum-carbon and replicating with carbon were carried out with pre-aligned evaporation sources through performed holes in the cover of the cold block at the appropriate angles (reference 15 and Fig. 1 *a*). A specimen holder was used which enables easy freezing of the object in liquid Freon and easy collecting of the replica after thawing. The specimen holder in our device (Figs. 1 *a* and 1 *b*) is a simple copper rivet in the head of which a small hole has been made for the object. Freeze-fracturing was carried out under liquid nitrogen (1). No etching facility was introduced. Well-preserved specimens could be obtained in the absence of glycerol as a freeze-protecting agent. We have included the description of this inexpensive, workshop-made device to confirm its applicability for freeze-fracturing as originally demonstrated by Bullivant and Ames (1).

Thin Sectioning

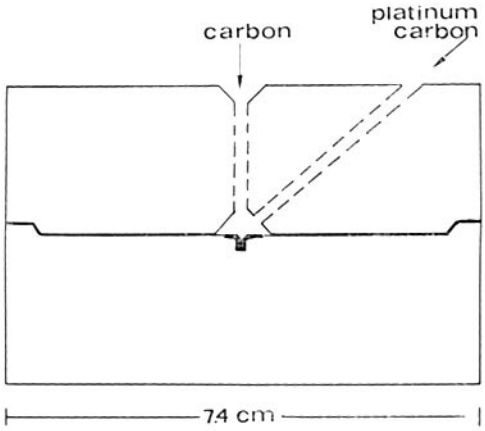
Fixation in osmium tetroxide, posttreatment with uranyl acetate, dehydration in a graded series of acetone, and embedding in Vestopal W (Martin Jaeger, Geneva, Switzerland) were done as described previously (9, 12).

Electron Microscopy

Electron micrographs were taken with a Philips EM 300 electron microscope. The micrographs of shadowed objects are printed in negative in order to produce a "natural" black shadow which facilitates their interpretation.

RESULTS

Preparations of exponentially growing *B. subtilis* were made in the presence or absence of glycerol. For each case several hundred cells were scanned in the electron microscope. Only a very limited number of mesosomes were encountered in preparations of freeze-fractured young cells. In contrast, in cells fixed in osmium tetroxide before freeze-fracturing, mesosomes were readily detected. The location and structure of these mesosomes were quite comparable to those in chemically fixed and sectioned cells (see reference 8).



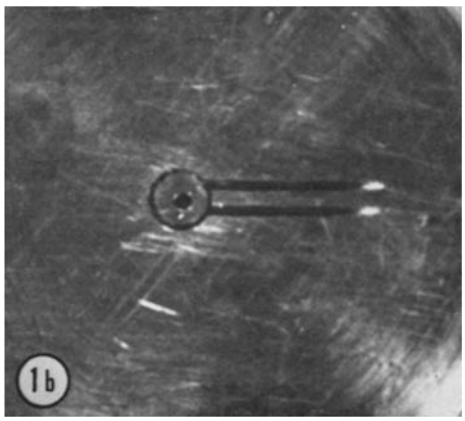
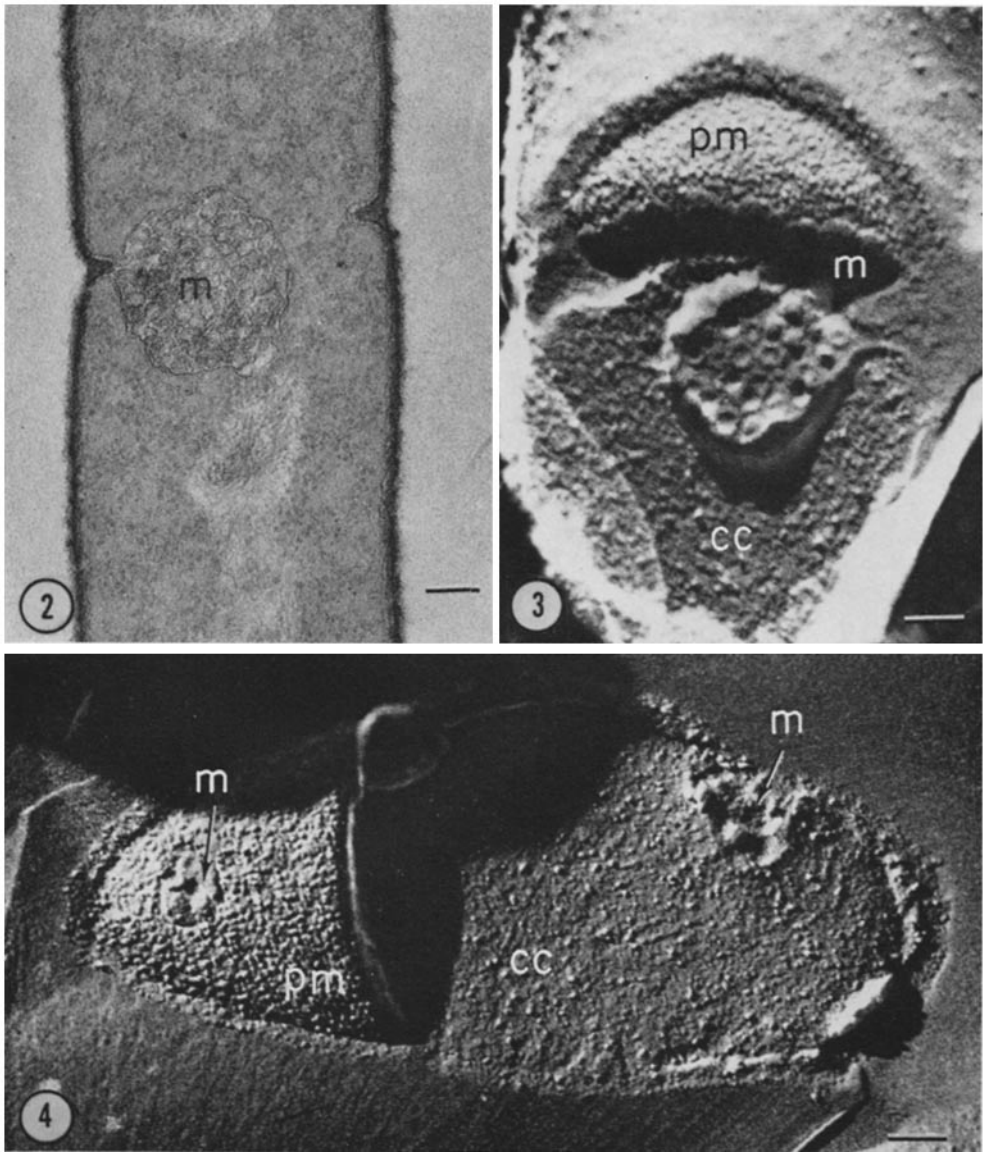


FIGURE 1 *a* Schematic representation of the two cylindrical brass blocks constituting the Bullivant-Ames type of apparatus.

FIGURE 1 *b* Top view of the specimen holder (a rivet) in the lower brass block. The diameter of the rivet is 0.5 cm. Two grooves are present to facilitate handling of the rivet with tweezers. The basic principles of the apparatus are to be found in references 1 and 15.

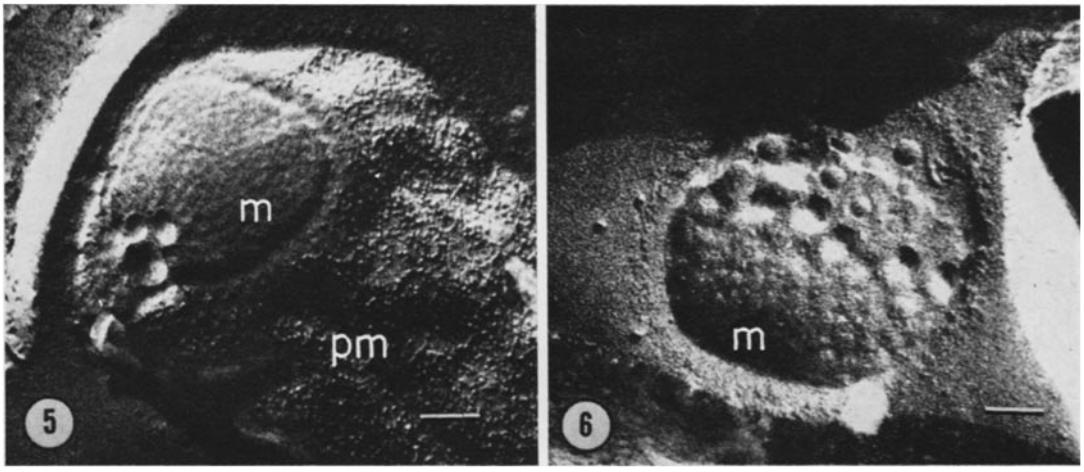


FIGURES 2–4 Mesosomes after chemical fixation. *m*, mesosome; *cc*, cell content; *pm*, convex fracture face of the plasma membrane. (scale mark equals 0.1 μ in Figs. 2–4.)

FIGURE 2 Thin section showing vesicular mesosome near ingrowing septum. × 65,000.

FIGURE 3 Mesosome freeze-fractured after chemical fixation. The mesosome seems to be packed with vesicles. Preparation made with the Balzers apparatus. × 75,000.

FIGURE 4 Similar situation as in Fig. 3 except that the preparation was made with the Bullivant-Ames type of apparatus. No etching. × 75,000.



FIGURES 5 and 6 Mesosomes visible in cells of a sediment stored overnight in the cold. No fixation was applied. Note some separate vesicles and complex larger structures. Both mesosomes are thought to be located at the tip of the cell. *m*, mesosome; *pm*, convex fracture face of the plasma membrane. × 75,000. Scale mark equals 0.1 μ.

Fig. 2 shows an example of a conventionally fixed and sectioned cell, and Figs. 3 and 4 show fixed and subsequently freeze-fractured cells. We also observed that in unfixed and freeze-fractured cells mesosomes, if present, never reached the size and complexity that they did in freeze fractured fixed cells. In neither case were mesosomes observed in the periplasm.

When a sediment of *B. subtilis* was stored overnight in the cold, we could find mesosomal structures in the periplasm after freeze-fracturing without chemical prefixation (Figs. 5 and 6). In these figures some separate vesicles can be seen together with larger structures. Thin sectioning of such a stored sediment showed, besides cells with normal appearance, a number of cells that were slightly autolyzed concomitant with plasmolysis. In these particular cells vesicles could be found in the infrawall space. The freeze-fracture pictures possibly refer to such cells. In normal young cells such mesosomal conformations have not been observed.

DISCUSSION

This investigation was initiated to study the effect of chemical fixation on mesosome structure, and is therefore a direct continuation of previous work concerning the plasma membrane, the cytoplasm, and the nucleoplasm (9). Together, these observations are summarized in Fig. 7.

A number of reports stress the influence of conditions of fixation on mesosome morphology. Mesosomes were found to be poorly preserved after thin sectioning if fixation according to Ryter and Kellenberger (12) was not followed by post-treatment with uranyl acetate (13). Variations in conditions preceding or during osmium tetroxide fixation were reported to influence mesosomal conformation too (2, 5, 11).

In the present work an attempt has been made to study the effect of the chemical fixation proper by fracturing frozen, fixed cells. The results obtained by direct freeze-fracturing are used as a standard, although one should realize that the artifacts of freeze-fracturing are insufficiently understood (see Discussion in reference 9). The observation that mesosomal membranes (in contrast to the plasma membrane) cannot be clearly demonstrated in young *B. subtilis* cells unless chemical fixation is applied before freeze-fracturing is rather unexpected. Two interpretations suggest themselves: (*a*) chemical fixation with osmium tetroxide stabilizes mesosomal structure with respect to the freeze-fracturing process, and although invisible in unfixed freeze-fractured cells, they are present; or (*b*) mesosomes are absent (or very inconspicuous) in young cells. The unfavorable conditions of chemical fixation influence membrane metabolism in such a way that mesosomes are formed. In fact, a comparable situation exists in the case of the nucleoplasm (reference 9 and Fig. 7). The nucleoplasmic region has, how-

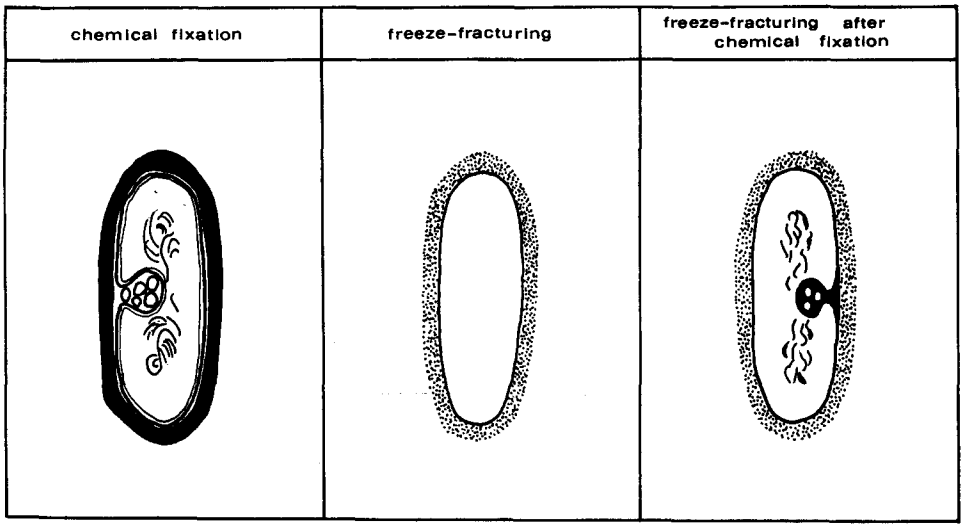


FIGURE 7 Schematic representation of results obtained by freeze-fracturing log phase cells of *B. subtilis*, strain Marburg, chemically fixed with osmium tetroxide. The effects of chemical fixation on the nucleoplasm (references 8 and 9) and the mesosome (this paper) are depicted. The cytoplasm has been left out of consideration because we are not yet able to analyze its structure in freeze-fractured cells (see Discussion in reference 9).

ever, been demonstrated by phase-contrast light microscopy in living cells, whereas this is less clear for the mesosome (except on or near the surface of protoplasts [3]). Nevertheless, mesosomes have been observed after freeze-fracturing without chemical prefixation under differing conditions (6, 8, 10). This applies especially to mesosomes located in the infrawall space (8). Our earlier observations (8) were confined to mesosomes in cells cultivated for 4–5 hr in broth supplemented with 20% (v/v) glycerol, or to cells grown in the absence of glycerol and fixed with osmium tetroxide prior to freeze-fracturing. In later experiments (9) glycerol was omitted from the broth. It was then observed that, after chemical fixation before freeze-fracturing, mesosomes could be found more easily than when no fixation was applied. We have also observed that keeping the cells as a thick suspension in the cold before freeze-fracturing can result in expelling mesosomes into the periplasm. This possibly applies to slightly deteriorated cells (see Results). As soon as mesosomes occupy part of the periplasm, they seem to become more visible upon freeze-fracturing than mesosomes located more internally (see Figs. 5 and 6). Two circumstances seem to favor mesosome visibility after freeze-fracturing: (*a*) chemical fixation with osmium tetroxide, and (*b*) their presence in the infrawall space. Of interest in this connection are the observations of Gosh et al. (4), who studied the occurrence of periplasmic vesicles in relation to penicillinase formation in *Bacillus licheniformis* 749/C. They noticed "some lack of correspondence between freeze-etching, negative staining, and thin sectioning."

Several functions have been ascribed to the mesosome in the actively dividing cell (11, 14). None of these functions is, however, unequivocally established. Since the present paper deals with membrane structure, it follows that, in order to understand our observations, more information will be needed on bacterial membrane metabolism. We would like to go still one step further and suggest that the basic problem in research on mesosomes is not to assign a defined function to these structures, but to determine the control of their formation.

Finally, it can be said that some care is needed in drawing conclusions concerning the structure of mesosomes in chemically fixed material.

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REFERENCES

1. BULLIVANT, S., and A. AMES. 1966. *J. Cell Biol.* **29**:435.
2. BURDETT, I. D. J., and H. J. ROGERS. 1970. *J. Ultrastruct. Res.* **30**:354.
3. FITZ-JAMES, P. C. 1964. *J. Bacteriol.* **87**:1483.
4. GOSH, B. K., J. O. LAMPEN, and C. C. REMSEN. 1969. *J. Bacteriol.* **100**:1002.
5. HIGHTON, P. 1969. *J. Ultrastruct. Res.* **26**:130.
6. HOLT, S. C., and E. R. LEADBETTER. 1969. *Bacteriol. Rev.* **33**:346.
7. MOOR, H., K. MÜHLETHALER, H. WALDNER, and A. FREY-WYSSLING. 1961. *J. Biophys. Biochem. Cytol.* **10**:1.
8. NANNINGA, N. 1968. *J. Cell Biol.* **39**:251.
9. NANNINGA, N. 1969. *J. Cell Biol.* **42**:733.
10. REMSEN, C. C. 1968. *Arch. Mikrobiol.* **61**:40.
11. RYTER, A. 1969. *Curr. Top. Microbiol. Immunol.* **49**:151.
12. RYTER, A., and E. KELLENBERGER. 1958. *Z. Naturforsch. B.* **13**:597.
13. SILVA, M. T. 1966. *Int. Congr. Electron Microsc.* 6th. **2**:275.
14. VAN ITERSON, W. 1969. *In* Handbook of Molecular Cytology. A. Lima-de-Faria, editor. North Holland Publishing Co. Amsterdam. 174.
15. WEINSTEIN, R. S., and K. SOMEDA. 1967. *Cryobiology.* **4**:116.