

FACTORS INFLUENCING THE FREQUENCY OF MESOSOMES OBSERVED IN FIXED AND UNFIXED CELLS OF *STREPTOCOCCUS FAECALIS*

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ABSTRACT

Mesosomes of *Streptococcus faecalis* (American Type Culture Collection 9790) were seen about 92% less frequently in freeze fractures of unfixed cells than in freeze fractures and sections of fixed cells. This difference in frequency was not related to any period of unbalanced macromolecular synthesis induced by chemical fixation. All measured synthetic processes (DNA, RNA, and protein synthesis, and glycerol incorporation) were halted with either osmium tetroxide (OS) or glutaraldehyde fixation. That fewer mesosomes were seen in freeze fractures of unfixed cells was probably due to the difficulty of observing cross-fractured mesosomes in this organism in the unfixed state. Unfortunately, mesosomes probably preferentially cross fracture in the unfixed state and therefore are usually only observed, infrequently, in those cases where the freeze fracture follows the surface layer of a mesosomal membrane.

However, the addition of glycerol to unfixed cells, especially in the chilled state, greatly increased the frequency of observation of cytoplasmic mesosomes in freeze fractures. It is thought that glycerol, like chemical fixation, increases the number of surface-fractured mesosomes, which in turn increases the frequency of mesosome observation.

It was also observed that cellular autolysis occurring during OS fixation seemingly reduced the number of mesosomes observed in thin sections and freeze fractures of OS-fixed cells.

INTRODUCTION

Acting on a suggestion of Robertson, FitzJames introduced the term "mesosome" to describe an intracellular dense mass of membrane-like material attached to the bacterial cell membrane (1). In retrospect, Chapman and Hillier undoubtedly saw mesosomes in their early thin sections of *Bacillus cereus*, but were unable to resolve the membranous nature of their "peripheral bodies" (2). The close proximity of the peripheral bodies to the nascent

cross wall led these workers to speculate that they were involved in cross wall formation.

Since these early observations, the list of proposed mesosome functions has grown to include energy production, DNA replication and separation, secretion, photosynthesis, nitrogen fixation, nitrification, and spore formation (3). Most of these proposed functions have been assigned primarily on the basis of location and number of

mesosomes found in cells performing highly specialized functions or carrying out a particular phase of their cell cycle. However, because of the difficulty of purifying mesosomes, the biochemical evidence needed to confirm any of these proposed functions has not accumulated rapidly.

As pointed out by Salton, the difficulty in purification is that thus far no enzyme or chemical marker exists that has been unequivocally shown to be exclusively present in mesosomes (3).

The findings of Ghosh, Remsen, and Lampen complicate the picture even more, for these workers suggest that in addition to having "normal" cytoplasmic mesosomes, some bacteria may contain submural-supramembranal pockets of membrane vesicles presumably involved in secretion (4).

At present, not only are the morphology, location, number per cell, and function of the mesosomes debated (3, 5), but Nanninga has expressed some doubt as to whether they exist at all (6). Nanninga has based this doubt on the study of freeze fractures of *Bacillus subtilis*, where he saw only a few mesosomes in young, unfixed cells, whereas numerous mesosomes were seen in osmium tetroxide-fixed cells (6). He concluded that either (a) mesosomes are present in cells but require chemical fixation to stabilize their structure, or (b) mesosomes are not present in young cells and chemical fixation influences membrane metabolism so that mesosomes are formed.

We have recently observed that the size of mesosomes in thin sections of glutaraldehyde-osmium tetroxide-fixed *Streptococcus faecalis* can be varied by selective inhibition of macromolecular synthesis (7). When DNA synthesis was rapidly inhibited before fixation, the mesosomes appeared to decrease in size when compared to the untreated controls. On the other hand, when protein synthesis was inhibited but DNA synthesis continued, the mesosomes seemed to increase in size. This study suggests that mesosomes are real structures, since their size can be regulated by the manipulation of a physiological parameter, i.e., protein and/or DNA synthesis. Using these findings as a model, we have tested Nanninga's postulate that chemical fixation might result in a membrane metabolism which favors mesosome formation. To do this, we have measured the frequency with which mesosomes can be seen in fixed and unfixed cells, and have studied the possibility that the fewer mesosomes seen in the unfixed freeze fractures might result from a differential inhibition

of macromolecular synthesis induced by chemical fixatives.

MATERIALS AND METHODS

Growth and Labeling of Cells

Cells were grown in a defined medium as described previously (8). The incorporation of leucine, uracil, and thymine into 10% trichloroacetic acid (TCA)-precipitable material was used as an index of protein, RNA synthesis, and DNA synthesis, respectively. The specificity of the label and the labeling conditions have been described in detail (9). Label equilibrated for at least six generations of exponential growth was used, and experiments were performed at a cell density equivalent to 136 $\mu\text{g}/\text{ml}$ dry weight. [^{14}C]Glycerol incorporation into cold TCA-precipitable material was performed as described previously in reference 10. Under these experimental conditions, glycerol is not converted into either glucose or acetate (11), and the label incorporated appears to be restricted to lipids and to a membrane-associated glycerol teichoic acid.

Electron Microscopy

SOURCES AND CONDITIONS OF FIXATION: Freshly opened 8.0% solutions of glutaraldehyde, previously stored under nitrogen (Polysciences, Inc., Warrington, Pa.), and 1% osmium tetroxide, prepared in the dark in Veronal-acetate buffer (12, 13) 2 days before use, were used as sources of fixatives in this study.

Unless otherwise stated, prefixatives were added directly to the growth medium, pH 6.3–6.5, until a final concentration of 2–3% glutaraldehyde or 0.1% osmium tetroxide was reached. The cells were then allowed to equilibrate at 26°C for 60 min. After appropriate washing procedures (13), the main osmium tetroxide fixation was carried out at room temperature according to the method of Kellenberger et al. (14).

PREPARATION FOR THIN SECTIONING: The fixation, Epon 812 embedding, sectioning, and uranyl acetate-lead citrate staining methods used here have been given elsewhere (12, 13).

PREPARATION FOR FREEZE FRACTURE: Obviously the best procedure for preparing cells for freeze fracture would be to avoid concentration altogether. However, due to the paucity of cross-fractured cells that can be studied in unconcentrated samples, some method of cell concentra-

TABLE I
*Effect of Preparative Conditions on the Percent of Central, Longitudinal Cell Fractures
 Containing Cytoplasmic Mesosomes*

Treatment of cells before freezing			
Pretreatment conditions	20% Glycerol added	Centrifugation conditions	Percent of cell fractures containing mesosomes
		(°C)	
1. None	—	37	2 ± 2
2. None	+	37	27 ± 2
3. Growth tubes swirled in ice bath	—	5	13 ± 7
4. Cells poured over ice	—	5	6 ± 3
5. Cells poured over ice	+	5	50 ± 5
6. None	—	None—cell frozen directly	2
7. 10 µg/ml chloramphenicol for 15 min at 37°C	—	5	15
8. 2.0% GA for 60 min at 26°C	—	5	72 ± 2
9. 2.0% GA for 60 min at 26°C	+	5	63 ± 2
10. 0.1% OS for 30 min at 26°C	—	5	65
11. 0.1% OS for 30 min at 26°C	+	5	55

Cultures were allowed to go through six divisions (generation time 31–33 min at 37°C) before being used for experimentation. At this time, cells were exposed to the various treatments listed above before being centrifuged (1,500 *g* for 15 min). The percent of central, longitudinal fractures containing cytoplasmic mesosomes was measured by studying a minimum of 50 cells usually from two separate experiments. In each experiment, results were compiled from two separate fractures. The plus and minus figures refer to range of experiments from the combined average. The osmium-fixed cells rarely gave clean cytoplasmic fractures. The mesosome figures given for treatments nos. 10 and 11 are in the 60% range, however, in most experiments the mesosome frequency was in the 8–14% range.

tion was of practical necessity. The method adopted for most treatments shown in Table I was to pour the cells over an equal volume of ice (made from distilled water) before centrifuging the cells at 1,500 *g* for 15 min at 5°C.

When glycerol was added to cell pellets before freezing, it was done in a drop wise manner with constant vortex mixing at 5°C or 37°C until a concentration of 20% was reached.

QUANTITATIVE DETERMINATION OF THE FREQUENCY OF MESOSOMES OBSERVED AND AVERAGE MESOSOME AREA PER CELL SECTION: For quantitative purposes, central, longitudinal cell views were sought. Only silver-gray sections showing a tribanded wall profile around the entire cell perimeter were used for measurement (Fig. 1 *a*). Reconstruction of serial sections

of this organism showed that the tribanded wall profile is only observed when a section passes through the cell wall in an antitangential manner (12). Also, the tribanded profile indicates that section *a* was closely aligned with the cell's longitudinal axis, and *b* came from the central 15–25% portion of the cell.

The average area of mesosomal membrane per central, longitudinal cell section was obtained by superimposing a grid micrometer contained in the ocular of a Bausch and Lomb dissection microscope directly upon an electron image plate taken at about × 30,000 instrumental magnification (7, 12). The electron microscope was calibrated daily with a carbon grating replica (Ernest F. Fullam, Schenectady, N. Y.).

The cytological effect of each treatment was

quantitatively determined in at least two separate experiments. Every experimental point required the analysis of at least 50 sectioned cells, usually by two observers.

QUANTITATIVE DETERMINATION OF THE FREQUENCY OF MESOSOMES OBSERVED IN FREEZE-FRACTURED CELLS: For quantitation, central, longitudinal cell views of freeze-fractured cells were also sought. However, since the cell wall observed in such fractures has no distinctive characteristics (such as the tribandedness used in thin section studies), these fractures could be selected in only an approximate manner as compared to the thin-sectioned cells.

A cytoplasmic fracture was chosen when it (a)

appeared bilaterally symmetrical, and (b) had a maximum diameter of about 1,000–900 nm. We have found that this is the normal distribution of diameters for exponential-phase, central, longitudinal cell sections and fractures (M. L. Higgins, unpublished observations). In addition, only those fractures showing as little contamination as possible and a finely granular cytoplasm were analyzed, because mesosomes were rarely observed in fractures where the cytoplasm appeared smeared or streaked. The latter fractures were usually observed only at the edges and on one side of the replicas examined. The average frequency of mesosomes observed was derived from the measurement of at least 50 cells.

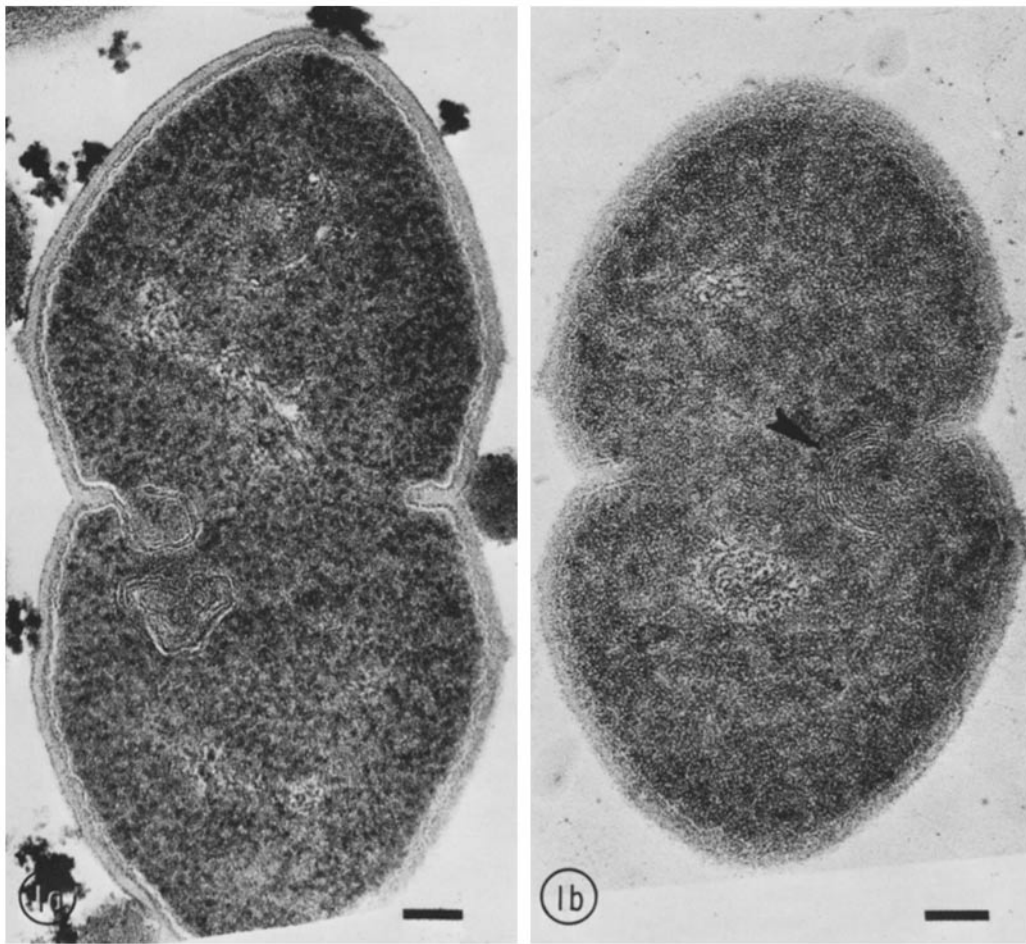


FIGURE 1 Mesosomes seen in longitudinal sections of exponential-phase cells prefixed for 60 min in 3% glutaraldehyde at 26°C (GA), (a), and 30 min in 0.1% osmium tetroxide (OS), (b). After both GA and OS prefixation, and counterfixation in 1.0% OS, the mesosomes appear to be predominantly attached to the septum. The OS-prefixed mesosomal membranes (arrow) stain much lighter than those prefixed in GA. Bars equal 100 nm. (a) $\times 77,000$. (b) $\times 85,000$.

RESULTS

Morphology and Frequency of Appearance of Mesosomes in Thin Sections after Glutaraldehyde-Osmium (GA-OS) and Osmium-Osmium (OS-OS) Fixation

In exponentially dividing cultures prefixed in glutaraldehyde (GA) and counterfixed in osmium tetroxide (OS), the mesosome appears to be a bag of membranes connected to the septal membrane by a stalk (Fig. 1 A), (12, 15). The membranes inside the bag are a complex mixture of tubular-vesicular configurations.

After 0.1% osmium tetroxide prefixation, followed by a 1.0% osmium tetroxide counterfixation (OS-OS), the mesosomes still appear septally attached (Fig. 1 b). However, they are much more difficult to see and have a tightly packed, "onion-like" appearance.

From an examination of Table II it can be seen that sections of OS-OS-fixed cells show mesosomes about 10–12% less frequently than do sections of GA-OS-fixed cells. Also, the area of mesosomal membrane in the OS-OS-fixed cells is 67–54% of

that of the GA-OS-fixed cells (Table II, Fig. 2). The 78% frequency of mesosomes observed in the GA-OS-fixed cells as shown in Table II is within the normal limits established from a previous analysis of seven separate fixation experiments. This study concluded that $75 \pm 5\%$ of all central, longitudinal cell sections taken from cells dividing every 31–33 min should contain mesosomes (7).

Morphology and Frequency of Appearance of Mesosomes in Freeze Fractures of GA- and OS-Fixed Cells

About 72% of the freeze fractures of GA-fixed cells showed mesosomes (Table I). This is also in good agreement with the $75 \pm 5\%$ range of frequencies observed in thin sections of the GA-OS-fixed cells (Table II).

The best freeze fractures of OS-fixed cells showed mesosomes in about 65% of the cells (Table I). However, in our hands, the OS-fixed cells usually did not fracture well, and most cytoplasmic views appeared smeared or streaked and lacked mesosomes. However, if fractures with fine cytoplasmic granularity could be found, the

TABLE II
Frequency of Observation and Average Mesosome Area per Central, Longitudinal Cell Section

Type of cell	Prefixation	Frequency (%)	Average area $\times 10^{+3}$ (nm ²)
1. Exponential phase	3.0% GA 60 min	78	18.2
2. Exponential phase	0.1% OS 30 min	68	12.2
3. Exponential phase	0.1% OS 90 min	66	9.8
4. CAP-treated	3.0% GA 60 min	74–75*	33.6*
5. CAP-treated	0.1% OS 30 min	74	24.0

* The results from treatment no. 4 were derived from past published work (7) where it was shown that mesosomal areas increased when cells were treated with chloramphenicol (CAP).

Cultures used for these experiments were kept in exponential-phase growth (doubling time 31–33 min) for six generations before being treated. The methods of measurement are given in Materials and Methods. In treatments nos. 4 and 5, 50 $\mu\text{g}/\text{ml}$ of CAP was added to exponential-phase cultures for 30 min. All cultures were counterfixed in 1.0% OS overnight at room temperature. Fig. 3 shows a histogram analysis of the area measurements from treatments nos. 1–3. The frequency and area measurements from treatments nos. 1–3 result from a single parallel fixation experiment. All treatments except no. 3 were studied in at least two separate experiments.

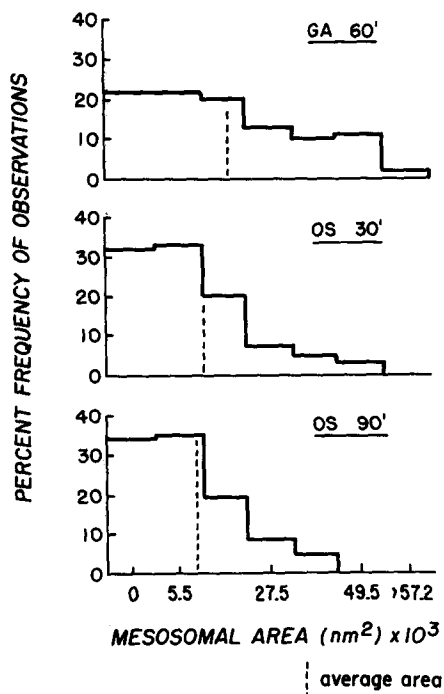


FIGURE 2 Histogram analysis of the frequency of mesosome area measurements from cells prefixed 60 min in 3.0% glutaraldehyde (GA), and 30 and 90 min in 0.1% osmium-tetroxide (OS), before being counter-fixed in 1.0% OS overnight. The measurements were taken from at least 50 central, longitudinal cell sections as described in Materials and Methods.

frequency of mesosomes observed was quite similar to that measured in thin sections of OS-OS-fixed cells (Table I). Therefore the freeze fracture and thin section techniques appear to yield the same answer: namely, that OS-fixed cells show fewer mesosomes than do GA-fixed cells (Tables I and II).

The addition of glycerol to cells before freezing does not seem to increase the number of mesosomes observed in freeze fractures of either OS- or GA-fixed cells (Table I).

As was the case with thin sections of GA-OS- and OS-OS-fixed cells, the mesosomes observed in freeze fractures of GA- and OS-fixed cells also appear to be attached predominantly to the septum, with the second most likely position being one of the cell poles. However, the GA-fixed mesosomes tend to surface fracture while the OS-fixed mesosomes are more likely to cross fracture (Fig. 3, *a* and *b*).

Autolysis and Mesosome Observation

From previous work, we know that cells of *S. faecalis* fixed in 0.1% OS can undergo autolysis not observed after 0.1% GA fixation (16).

The electron-transparent zone seen between the septal membrane and cross wall of Fig. 4 is interpreted as evidence of this autolysis, for such a zone has not been observed in GA-fixed cells unless a period of autolysis was induced before fixation (16). This suggests that the fewer mesosomes seen in the OS-OS-fixed cells might result from autolysis occurring during OS-OS fixation. To this point, we have observed that a small amount of cellular autolysis, i.e., a decrease of less than 5% of the initial turbidity (for method, see reference 16), can reduce the frequency of mesosomes observed in sections of GA-OS-fixed cells from about 76% to 25% (M. L. Higgins, unpublished observations).

Two types of evidence suggest that autolysis proceeding during OS-OS fixation could reduce the frequency of mesosomes observed: (*a*) mesosomes are observed at about the same frequency in sections of GA-OS- and OS-OS-fixed cells if cellular autolysis is inhibited by chloramphenicol (CAP) before fixation (references 17, 18, Table II, Fig. 5), i.e., previous studies have shown that reductions in protein synthesis induced by chloramphenicol produce a proportional reduction in whole cell autolysis (18); (*b*) the average area per section of mesosomal membranes is decreased as the 0.1% OS prefixation period (and supposedly the period of autolysis) is extended from 30 to 90 min (Table II).

However, when the average mesosome area per section of CAP-treated, OS-OS-fixed cells was compared to that of CAP-treated, GA-OS-fixed cells, the GA-OS-fixed samples still had about 30% more area per section. The smaller area observed in the OS-fixed cells may be due to the tighter, onion-like configuration of the OS-fixed mesosomes (Table II, Fig. 5).

Morphology and Frequencies of Mesosomes Observed in Unfixed, Freeze-Fractured Cells

When the frequency of cytoplasmic mesosomes observed was measured in unfixed cells, mesosomes were seen in only about 2-20% (average 5.3%) of the fractures (Table I; Fig. 6 *a*). However, when glycerol was added to a final concentration

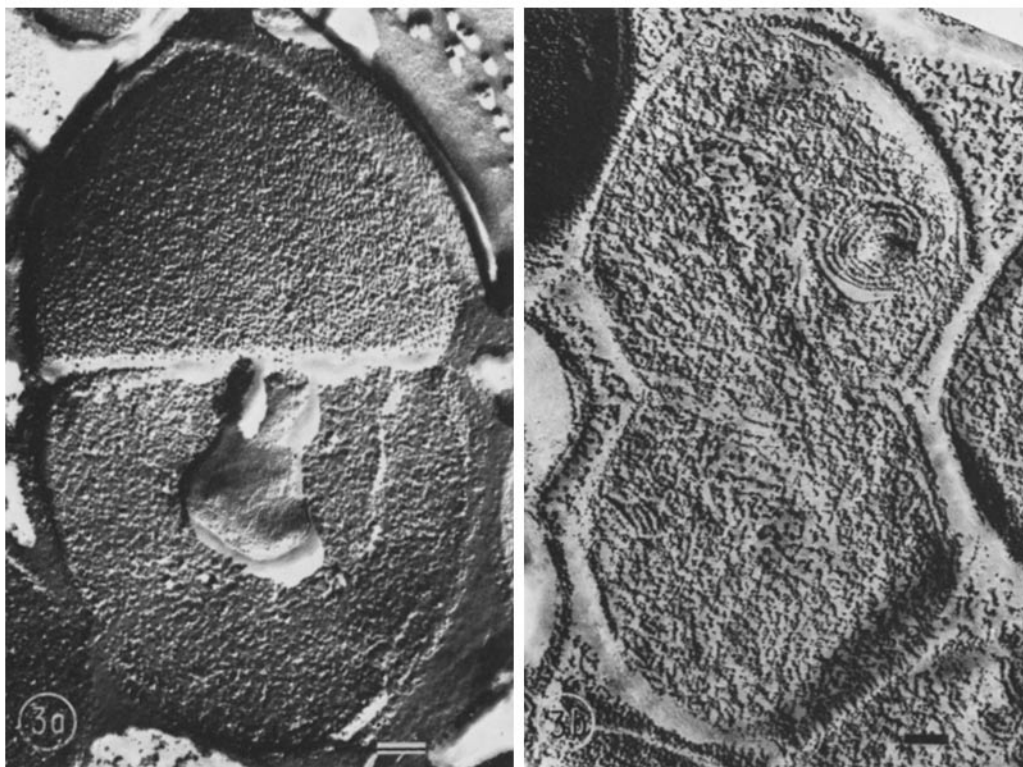


FIGURE 3 The cell membrane and mesosome freeze-fracture morphology of cells fixed in GA, (a) and OS, (b). The mesosomes seen in GA-fixed cells are predominantly surface fractured (a), while the OS-fixed mesosomes frequently show cross-fractured, onion-like configurations observed in thin sections (see Fig. 2 b). The mesosome in (b) is attached to a future cross wall site. Bars equal 100 nm. (a) $\times 63,000$. (b) $\times 59,000$.

of 20% to cells incubated at 37°C or at about 0°C before being frozen, the frequency of mesosomes observed increased above that observed for unfixed cells (Table I; Fig. 6 b, c, d). The addition of cold glycerol to chilled cells stimulated the frequency of mesosomes observed more ($50 \pm 5\%$) than when glycerol was added to cells incubated at 37°C ($27 \pm 2\%$).

Most of the mesosomes observed in unfixed cells in the presence of glycerol (Fig. 6 c, d) appeared to be attached to the septum; however, some everted mesosomes were also seen (Fig. 6 b). Eversion was especially common in the chilled preparations, and as described for *Bacillus megaterium*, may be a reaction to rapid reductions in temperature (19). Accordingly, we have observed in a parallel study of thin sections that the frequency of mesosomes observed was reduced from 70 to 50% by chilling the cells before GA-OS

fixation. From these observations it seems that in freeze fractures of unfixed cells there is, on the average, about a 92% decrease in the frequency of mesosomes observed as compared to fixed cells, and that this decrease can substantially be reversed by the addition of glycerol to unfixed cells before freezing (Table I).

*Possibility of Chemical Fixatives
Inducing Mesosome Formation as a
Result of Unbalanced Postfixation
Macromolecular Synthesis*

To account for fewer mesosomes being seen in the freeze fractures of unfixed cells, we explored Nanninga's hypothesis that chemical fixation might induce a membrane metabolism that would favor mesosome formation (6). We have previously ob-

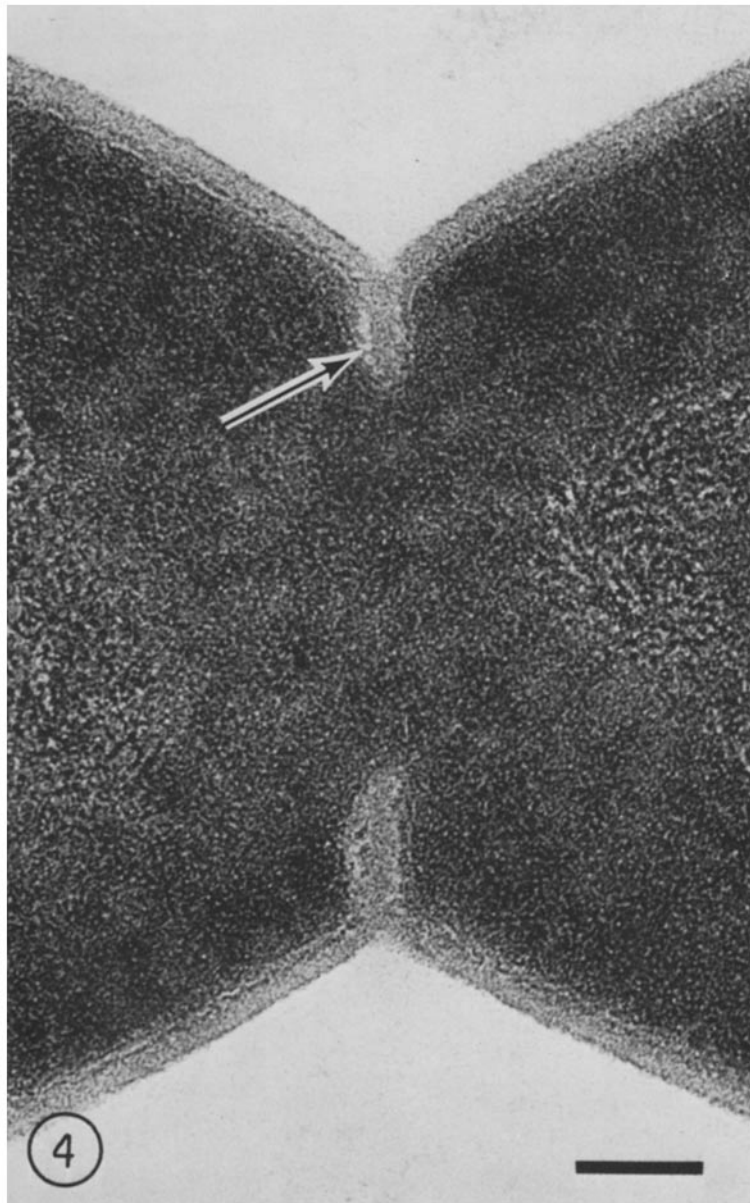


FIGURE 4 A thick longitudinal section of *S. faecalis* after 30 min of 0.1% OS prefixation. Many cells fixed in OS-OS show an electron-transparent zone between the septal membrane and the cross wall (see arrow). The appearance of such zones has been associated with early stages of cellular autolysis (16). $\times 170,000$.

served that unbalanced DNA and protein synthesis occurring in antibiotic-treated cells could dramatically affect the size of mesosomes found in these cells (7). Thus, on the basis of these studies, we asked if the mesosomes observed in fixed cells might result from an inability of the fixatives im-

mediately to inhibit the synthesis of all macromolecules associated with mesosome growth and regulation. However, we found that various concentrations of GA (0.125–2.5%) and OS (0.05–1.0%) inhibited the incorporation of specific precursors of DNA, RNA, and protein into cold

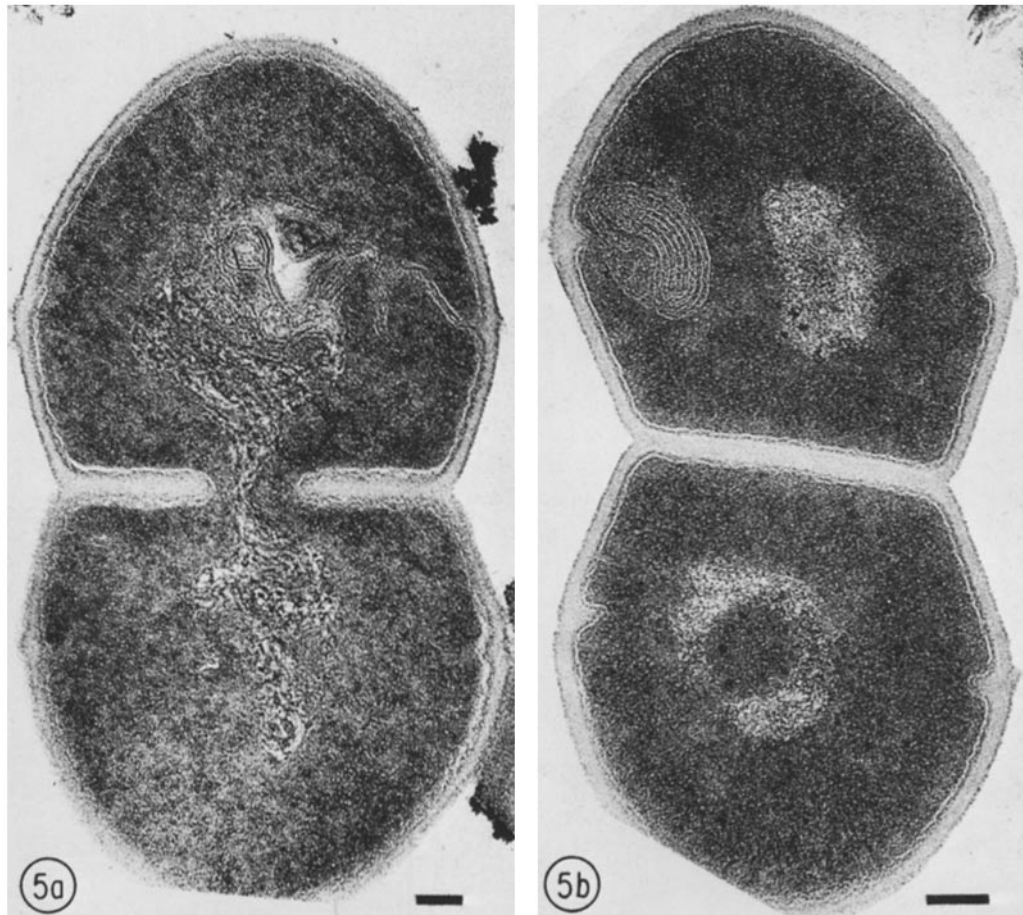


FIGURE 5 Mesosomes seen in chloramphenicol (CAP)-treated cell sections prefixed with GA (a) and OS (b). Cells were treated 30 min with 50 μg per ml of CAP before fixatives were added. (a) $\times 62,000$. (b) $\times 84,000$.

TCA-precipitable material (Table III). The results given in Table III are from cells grown at 37°C and fixed at 26°C; however, similar results were obtained by fixing at 37°C. Consequently, a differential inhibition of DNA, RNA, or protein synthesis by chemical fixation does not appear to explain the paucity of mesosomes seen in freeze fractures of unfixed cells as compared to fixed cells.

In addition, GA (Fig. 7 a, b) and OS (Fig. 7 c, d) rapidly inhibited the incorporation of glycerol label into TCA-precipitable material. The effects of fixation at 26°C and 37°C were similar, in that in certain concentration ranges both fixatives induced a subsequent loss of incorporated

glycerol (Fig. 7). The principal difference between fixation at 26°C and fixation at 37°C is that the observed losses on the addition of GA occurred more rapidly and more extensively at 37°C (Fig. 7 a) than at 26°C (Fig. 7 b). The apparent incorporation of [^{14}C]glycerol observed after the addition of OS (Fig. 7 c, d) probably is not due to true biosynthesis, but rather to the binding of [^{14}C]glycerol to OS-treated cells.

The loss of [^{14}C]glycerol was increased by raising the concentration of GA above 0.25%, and by decreasing the concentration of OS below 0.01%. The greater loss of lipids after glutaraldehyde fixation in comparison to osmium tetroxide fixation has been noted in other biological membranes

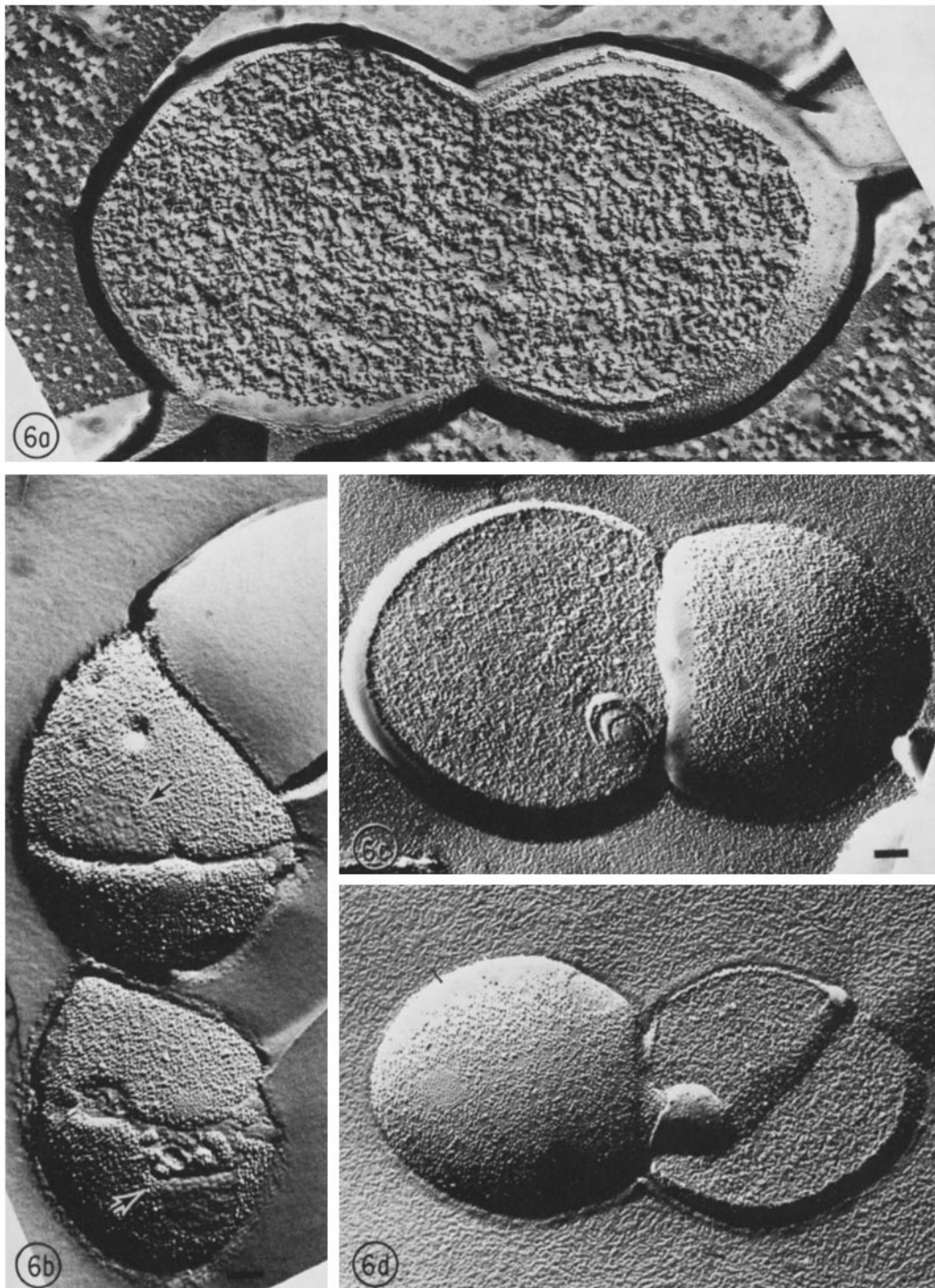


FIGURE 6 The internal freeze-fracture morphology of unfixed exponential-phase cells. Cells that are rapidly chilled before being frozen rarely show mesosomes (*a*). If glycerol is added to a final concentration of 20% before freezing, mesosomes are seen in both the submural-supramembranal zone (*b*, see arrows) and the cytoplasm (*c*, *d*). Bars equal 100 nm. The bar in (*c*) applies also to (*d*). The etch time for all samples was 1 min. (*a*, *b*) $\times 65,000$. (*c*, *d*) $\times 50,000$.

TABLE III
Relative Amount of Macromolecular Incorporation After
30 Min in the Presence of GA or OS

Fixative	Final Concentration (%)	DNA	RNA	Protein
GA	2.5	1.00	0.98	1.00
	1.25	1.00	*	1.00
	0.50	*	1.00	1.00
	0.25	1.00	1.00	1.00
	1.125	1.00	1.00	1.00
OS	0.1	1.08	0.86	1.00
	0.05	1.12	0.84	1.05
	0.01	*	0.66	1.05
	0.005	*	0.60	1.05

* Not done.

Cells in balanced exponential growth at 37°C were transferred to tubes containing various concentrations of fixatives and incubated for 30 min at 26°C. The values given represent the counts incorporated/0 time counts. The doubling time of counts in control cultures was 30–32 min at 37°C and 95–102 min at 26°C.

(20). However, the differential loss has usually not been noted until after the fixed cells had been treated with ethanol after fixation (21, 22).

Thus, the differences in the size and frequency of mesosomes observed between fixed and unfixed cells do not appear to be due to any early fixative-induced period of unbalanced synthesis of DNA, RNA, protein, or phospholipid. However, the loss of membrane components on fixation might, in part, be responsible for some of the conformational differences observed in the OS-OS- and GA-OS-fixed mesosomes (Figs. 1, 5).

DISCUSSION

An approximate 10% decrease in frequency of mesosomes observed was noted when exponential-phase cells were prefixed with osmium tetroxide instead of glutaraldehyde. Since no difference in the frequency of observation was noted with either fixation method if autolysis was blocked by the inhibition of protein synthesis (18) before fixation, the fewer OS-fixed mesosomes appeared to result from autolysis that occurs during osmium tetroxide but not glutaraldehyde prefixation. The reduction in the frequency of mesosomes observed on autolysis seems plausible, for it is known that lytic damage to the cell wall can result in the ejection of mesosomes from the cytoplasm of bacteria (23).

The freeze fracture method has been hailed as a “base-line” technique, as it permits examination of the cell with a minimum of preparative steps. It was therefore important to know how the frequencies of mesosomes observed as measured by this technique compared to the frequencies gathered from similarly treated thin-sectioned cells. When the two preparative techniques were used to study cells fixed in a like manner, similar quantitative results were obtained. Namely, mesosomes were seen in about 72–78% of the sections or freeze fractures of cells that had been fixed with glutaraldehyde, while 65–68% of the sections or fractures of osmium tetroxide-prefixed cells contained mesosomes.

Both preparative techniques also indicated that the mesosomes of *S. faecalis* are predominantly associated with the septum, with the second most likely location being one cell pole.

When these results, based on fixed material, were compared to those obtained with unfixed, freeze-fractured samples, the freeze fractures of unfixed cells contained, on the average, about 8% of the mesosomes found in sections or fractures of fixed cells. These observations were comparable to those obtained by Nanninga in a qualitative study of *B. subtilis* (6). However, unlike Nanninga, we were able to increase the frequency of cytoplasmic mesosome observation in *S. faecalis* from 0–20% to 45–55% by adding glycerol to the chilled cells before freezing.

As stated above, Nanninga suggests that: (a) mesosomes might exist in young cells but require stabilization by chemical fixation in order that they might be seen in freeze fractures; or (b) the structures are artifacts, created by chemical fixation which affects membrane metabolism in such a way that mesosomes are formed (6). Past work with *S. faecalis* has shown that the size of mesosomes increases in nongrowing cells only when DNA synthesis and glycerol incorporation continue in cells where protein synthesis is inhibited (7). Clearly, these synthetic conditions do not exist during either 0.1% osmium tetroxide or 2–3% glutaraldehyde fixation, for both rapidly inhibit DNA and protein synthesis, and glycerol incorporation. Therefore it appears that chemical fixation does not create a metabolic situation (at least in *S. faecalis*) favorable for mesosome formation and growth.

Mesosomes were only observed in freeze fractures of unfixed cells when the plane of the frac-

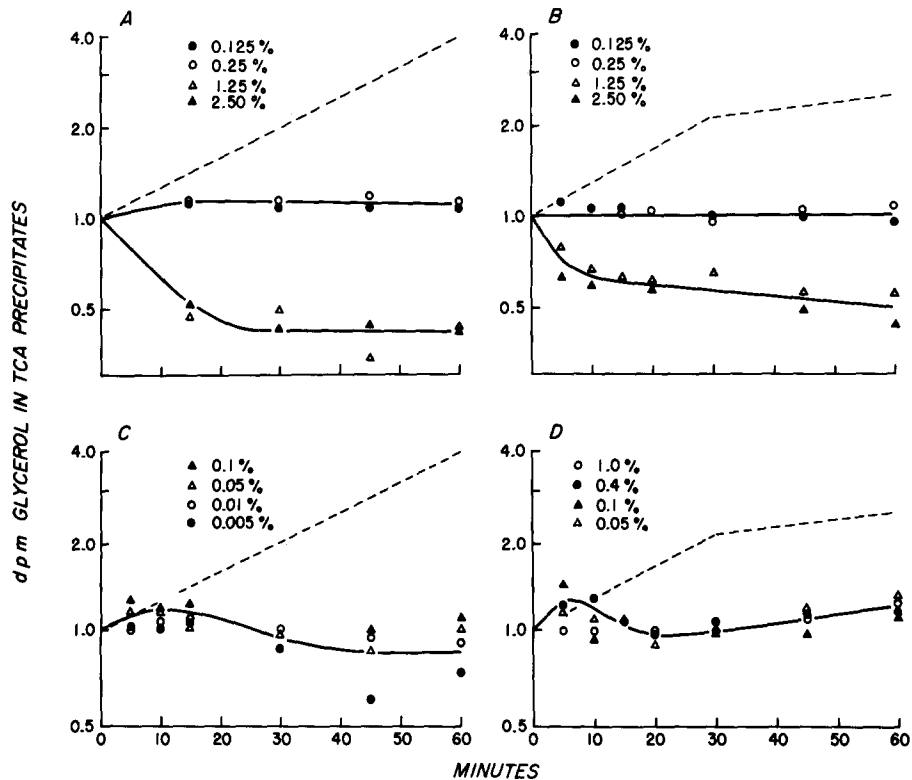


FIGURE 7 The incorporation of an equilibrated glycerol label into TCA precipitates was followed for various times after transfer of balanced exponential-phase cultures into GA (*a, b*), or OS (*c, d*), at 37°C (*a, c*), or 26°C (*b, d*). Label incorporation is expressed as the ratio of counts over the 0 time counts (3,100 dpm/0.5 ml). Cells were grown at 37°C and then incubated in fixatives at 37°C (*a, c*), or 26°C (*b, d*). The dashed line represents the incorporation of an untreated control culture maintained at 37°C (*a, c*) or shifted to 26°C (*b, d*). The control cultures moved to 26°C continued to incorporate radioactive glycerol at the old 37°C rate for an interval before shifting to the new 26°C rate of incorporation.

ture followed a surface layer of the mesosome. Thus mesosomes were seen only when a convex or concave membrane surface fracture differentiated the mesosome from the rest of the cytoplasm (Fig. 6 *c* and *d*). We suggest that in *S. faecalis* most unfixed mesosomes cross-fracture and are usually invisible in this condition. Chemical fixation would increase the frequency of visualizing mesosomes: (*a*) by stimulating mesosome surface fracturing, or (*b*), as in the case of OS fixation, by possibly reorganizing the membrane so that mesosomes can be observed in cross fracture (Fig. 3 *b*). In this regard, glutaraldehyde appears more effective than osmium tetroxide in producing surface fractures. This would be consistent with its known ability efficiently to cross link hydrophilic portions of proteins (24). The increased frequency of observed mesosomes on the addition of glycerol

to unfixed cells would also be explained in terms of promoting mesosome surface fractures.

Buckingham and Stachlin have observed increases in the thickness of artificial bimolecular leaflets on the addition of glycerol (25). They suggested that glycerol may increase the molecular order within the bilayers, and that the resultant straightening of the molecules may increase the bilayer thickness. It is possible that glycerol could produce some similar structural alteration in mesosomal membranes of *S. faecalis* and result in more fractures occurring in the surface layers of mesosomal membranes. That mesosomes are observed more frequently in freeze fractures of cells when glycerol has been added before freezing to chilled cells than when glycerol has been added to cells incubated at 37°C, may be due to the membrane lipid's being in a more crystal-

line state at the lower temperature (26). This may result in an additive effect of chilling and glycerol in stimulating surface fractures of mesosomal membranes.

Thus, according to our hypothesis, both chemical fixation of and glycerol addition to unfixed cells would produce a similar stimulation of surface fractures. However, when the ratio of cross fractures to surface fractures in unfixed cell membranes was compared with that in cell membranes treated with 20% glycerol and 2.0% glutaraldehyde, we observed no differences. This indicates that the cytoplasmic and mesosomal membranes react differently to these treatments. This is not inconceivable since (a) in at least one study, the mesosomal membrane has been shown to have a chemical composition different from that of the cytoplasmic membrane (27), and (b) the mesosome and cell membrane exist in different cellular environments. James and Branton have likewise observed that osmium tetroxide fixation does not stimulate surface fracturing of the mycoplasma cell membrane (28).

In summary, the mechanism by which fixation, or the addition of glycerol to unfixed cells apparently stimulates the surface fracture of the mesosomal membrane is not known. Possible differences in chemical composition and the frozen environments surrounding the mesosome and cytoplasmic membrane are suspected. However, since mesosomes are seen (a) in high frequency in glycerol-treated, unfixed cells, and (b) at approximately the same locations as in the fixed preparations, it seems that mesosomes do exist in unfixed cells and that the problem of seeing unfixed mesosomes probably lies in the difficulty of differentiating the cross-fractured mesosome from the surrounding cytoplasm. The glycerol studies also indicate that cytoplasmic mesosomes do not require stabilization by chemical fixation for observation in freeze fractures.

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