

THE INITIAL STRUCTURAL LESION OF PENICILLIN ACTION IN *BACILLUS MEGATERIUM*

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ABSTRACT

The effect of penicillin on the structure of *Bacillus megaterium* cells was followed in media with and without osmotic stabilization. In peptone without osmotic support the cells showed a distortion of the normal membrane-wall relationship by 20 minutes. This appeared to be a combination of both membrane distortion and cytoplasmic leakage. Lytic changes quickly followed. With osmotic support a clean-cut lesion at the transverse-septal site developed by 10 minutes' growth in penicillin. The membrane lost its normal relationship to the cell wall and formed a pocket which was filled with a fibrous material which appeared to be unorganized wall mucopeptide. The pocket of fibers enlarged until the cell either lysed or formed a protoplast.

INTRODUCTION

A comparison of the effects of a number of antibiotics on the physiology of growing cells and protoplasts of *Bacillus megaterium* has provided useful information regarding the mode of action of these drugs (7). As part of a further investigation of the effects of these antibiotics on cell structure, the present paper describes the changes in fine structure occurring in *Bacillus megaterium* strain KM during the action of penicillin in normal medium and in medium affording osmotic stabilization.

MATERIALS AND METHODS

Structural changes induced by penicillin were studied using cells growing in one of three media. These were (a) 2 per cent peptone (Difco Laboratories, Inc., Detroit) (P), (b) 2 per cent peptone containing protoplast-stabilizing buffer, 0.3 M sucrose and 0.016 M MgSO₄ (SP), and (c) casein-hydrolysate-succinate broth medium (PP) originally developed by Dr. Spiegelman and used for rapid growth of protoplasts (2, 7). Growth was followed by observing

the optical density at 650 m μ in the Beckman DU spectrophotometer. A dense overnight culture of *Bacillus megaterium* in P medium was diluted (1/10 or 1/20) into the appropriate fresh warmed medium and aerated vigorously at 32°. When the rise in optical density indicated that the cells were in log growth, penicillin was added to an aliquot of the culture, and after continued aeration for required intervals samples of both control and penicillin-treated cultures were taken for electron microscopy.

Cells were fixed with osmium tetroxide and embedded in Vestopal by the method of Ryter and Kellenberger (10). Photographs were made both of random single sections, and of 8 or 9 serial sections of at least one cell in both control and treated embeddings, in order to establish the proper relationship of septa, membranous organelles, and nucleoids.

Penicillin G at doses of (100 to 1000 units/ml) and dimethoxyphenyl penicillin (methicillin sodium) at doses of 0.01 to 1.0 mg/ml were used and produced similar changes in fine structure.

RESULTS

Penicillin Effect in a Medium Without Osmotic Support

Micrographs of sections of cells of *B. megaterium* strain KM growing in peptone medium have already been published (Figs. 23, 24, reference 3). Of particular interest to this present comparison are the arrangements of the mesosomes and the profiles of the sectioned transverse septa. The somewhat fragile but prominent mesosomes of this species are usually situated at the transverse septa of dividing cells and attached to, or partially embedded in, the separating nucleoids. The transverse septal profile shown in Fig. 1 is typical of normal division in this organism.

Immediately following the addition of penicillin to cells growing in P medium the optical density rise became linear, rather than exponential, and continued for 20 to 30 minutes before leveling off. For the first 10 minutes the fine structure did not appear markedly altered. By 20 minutes, however, when an increase in cell length could be seen by phase-contrast microscopy, disorganization at the site of the transverse septum could be detected in thin sections (Fig. 2). Both leakage of cell contents and a partial displacement of membrane organelles could be seen in the sections. Mesosomes, which after 10 minutes of exposure to penicillin were still intracellular and nucleoid-associated, were found after 20 minutes of treatment to be more peripheral (Fig. 3). Often those at the septal sites ap-

peared to be partially spilling into wall-membrane vacuoles (Fig. 2). In a rare cell after 20 minutes of penicillin exposure, membrane pockets with fibrous contents of the type seen in cells undergoing penicillin treatment with osmotic support (see later) were found distorting the normal relationship of membrane to cell wall.

Sixty minutes after the addition of penicillin, the cells were short, bulbous rods. In sections, walls were now showing early signs of inadequacy and this was accompanied by a more severe state of membrane disorganization. Everywhere the normal unit membrane profile of dense outer-membrane line and lighter cytoplasmic border were altered to the lytic profile of equal, dense profiles 70 to 80 Å apart. In numerous places the profile of the plasma membrane could now be seen touching the cell wall (Fig. 4). This absence of the normal separation of cell wall from the outer dense profile of the membrane may be a late lytic effect rather than a specific effect of penicillin.

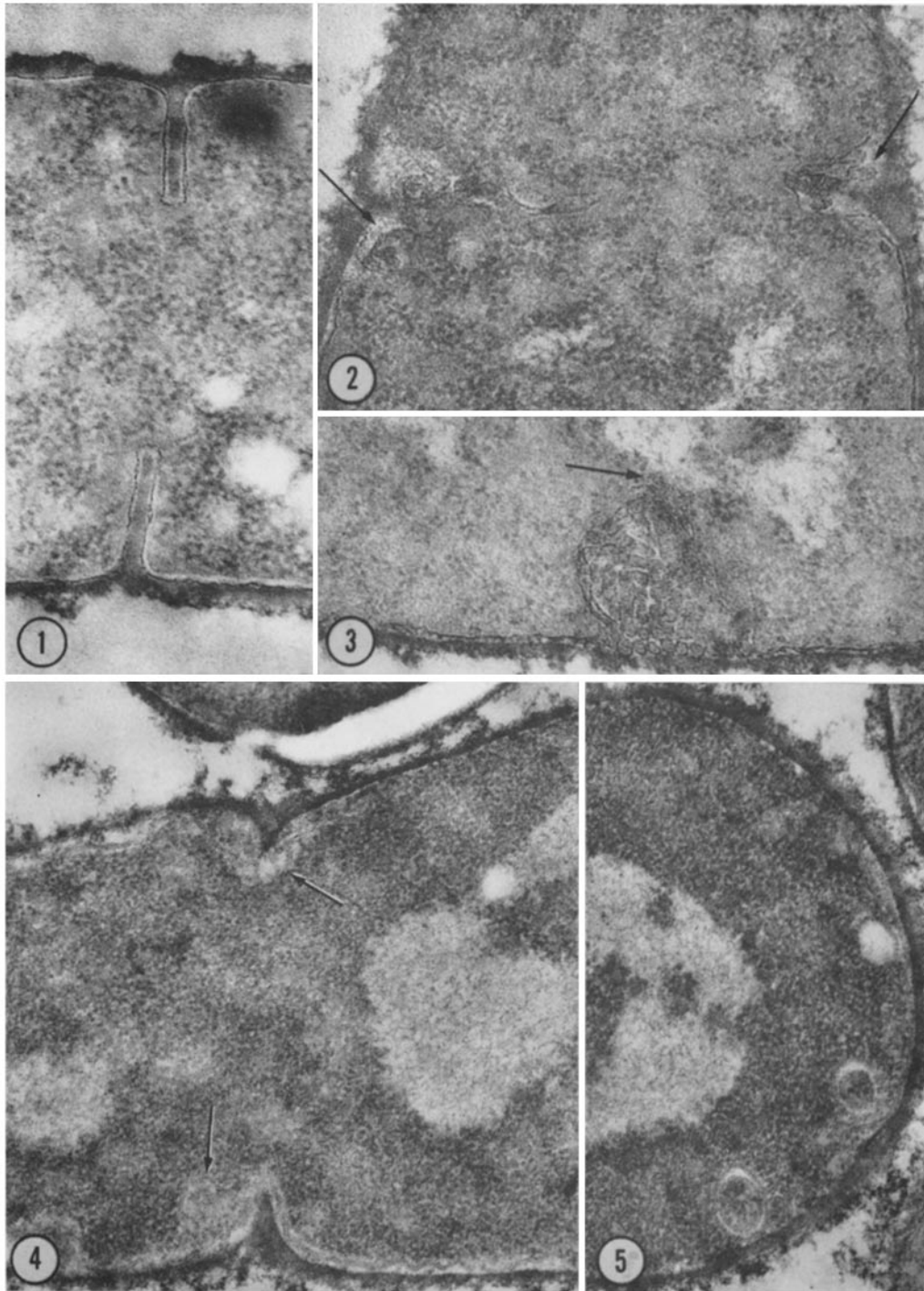
Besides the over-all change in membrane profile, a disorganization of arrangements was now very noticeable, particularly at the divisional sites. Mesosomes were largely absent, but membrane remnants at transverse septa (Fig. 4) and peripheral vacuoles (Fig. 5) were probable remnants of these formerly highly organized structures. In the cytoplasm the perinuclear and aggregated arrays of ribosomes seen in control cells and during early penicillin treatment were now replaced by a more dense, even arrangement.

FIGURE 1 A section through the center region of an untreated cell of *Bacillus megaterium* growing in P medium. The normal relationship of transverse wall and membrane at the septa is clearly shown at the bottom profile. $\times 99,000$.

FIGURE 2 Membrane distortion found at the septal sites after 20 minutes' growth with penicillin in P medium. Parts of mesosomes appear to have collapsed into the wall-membrane space around the transverse septa (arrows). The cytoplasm appears normal. $\times 77,000$.

FIGURE 3 A section from the same preparation shown in Fig. 2, showing a mesosome probably beginning the marginal dumping resulting from early loss of wall integrity induced by penicillin. Otherwise the mesosome is compact and still associated at its apex with the nucleoid (arrow). $\times 77,000$.

FIGURES 4 and 5 A cell after 60 minutes' growth with penicillin in P medium. Mesosomes are now generally lost to the wall-membrane interspace. Instead, membrane remnants are common at the septa (arrows, Fig. 4); a few remnant vesicles are also seen (Fig. 5). The double track lytic profile of membrane is now general. $\times 53,000$.



After 2 hours' exposure to penicillin nearly half the cells in these cultures had lysed. Those surviving lysis showed extensive degeneration of all structures.

Penicillin Effects in Osmotically Stabilized Media

Previous studies (6, 7) have already demonstrated the protection offered by osmotic stabilization against several secondary effects of penicillin action on growing cells. The early loss of cell organization seen at the site of transverse septa and the more serious loss of cell organization with prolonged penicillin treatment could be such secondary results of membrane distortion incidental to the loss of cell-wall integrity. To observe more directly the primary effect of penicillin, similar studies were carried out using cells growing in media providing osmotic stabilization against secondary changes. After a period of recovery from shock induced by the sudden transfer from P medium to either SP or PP (2, 4, 5), growth resumed at a normal rate. In cells which had only partially recovered from this shock the mesosomes were still seen in peripheral positions to which they had been displaced (Fig. 6), (5). In fully recovered, rapidly dividing cells, they were found, by study of serial sections, in the usual positions, extending further into the cell to the nucleoid (see also Fig. 14). Profiles of the transverse septa were also similar to those of cells dividing in P medium.

Ten minutes after adding penicillin to cells dividing in SP medium, a typical distortion of the membrane was found at the site of the transverse septum. Here, where new wall was being laid across the cell, a ring-like pocket developed, filled with a randomly organized array of fibrous material extending from wall to the dense profile line of the membrane (Fig. 7). Unlike the lesions seen

in cells not stabilized by sucrose, there was no evidence of leakage of cytoplasmic material. After 20 minutes, adjacent membranous organelles, although crowded around these penicillin lesions, were not yet displaced into these pockets (Fig. 8).¹

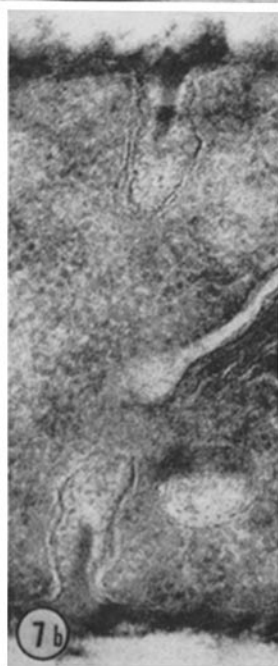
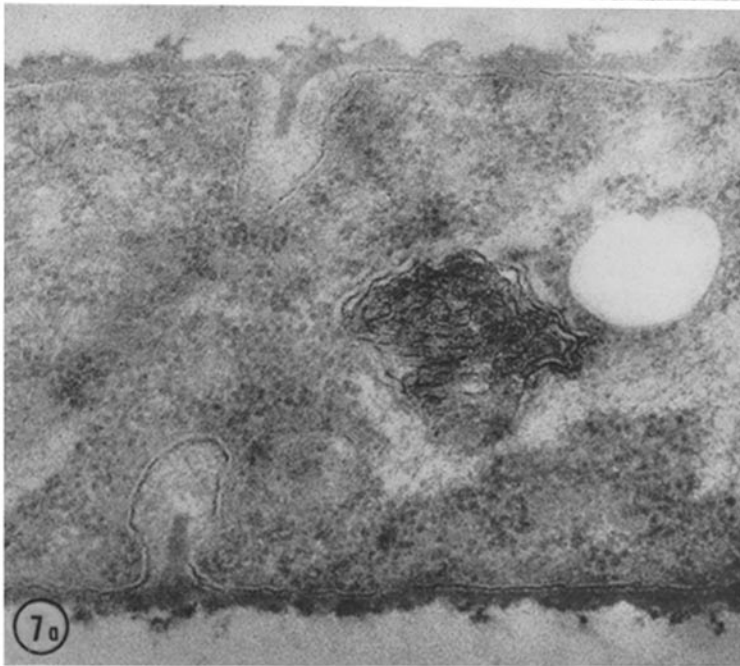
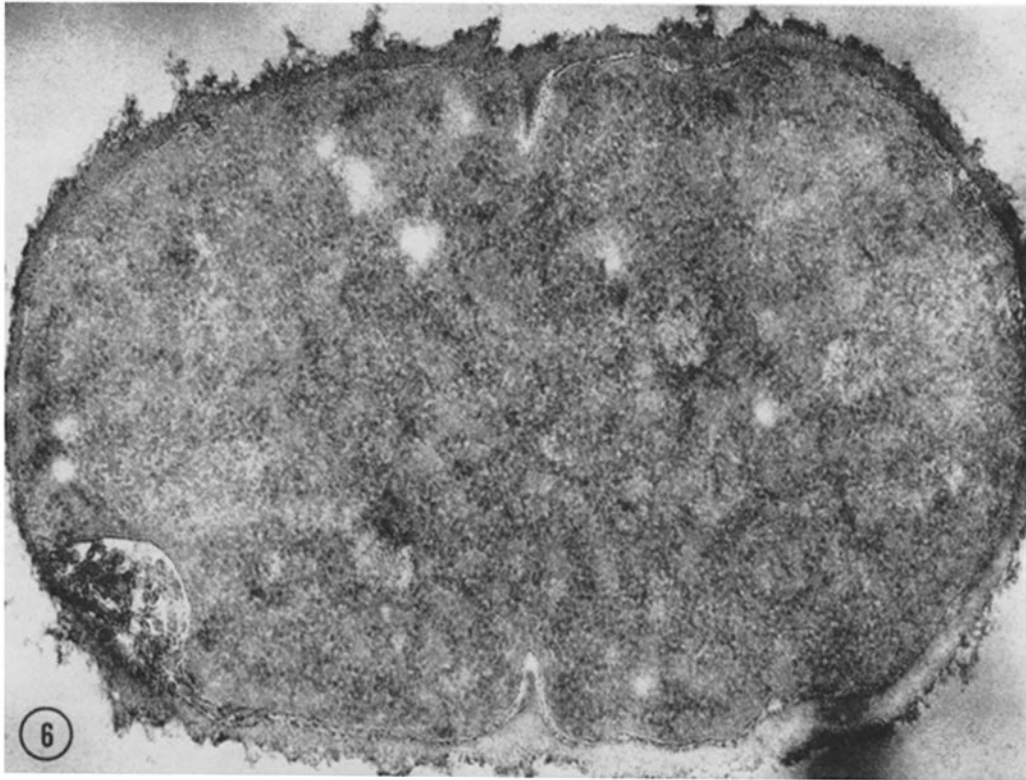
These clear-cut penicillin lesions developed markedly for about 60 minutes (Fig. 9 and 10). Then during the subsequent hour, evidence of cell-wall breakage (Fig. 11) was accompanied by collapse of membranous organelles (Fig. 12 and 13) similar to that seen during lysozyme treatment (5, 11). Prior to the complete loss of wall integrity, and in spite of their close proximity to the pockets, many mesosomes remained partially intact and still adherent by their boundary membranes to the nucleoids (Fig. 12). A similar persistent attachment of nucleoid to mesosomal membrane is seen after lysozyme displacement of mesosomes in both this organism (unpublished observations) and in *Bacillus subtilis* (11).

Penicillin action on cells growing in PP medium was similar to that described for cells growing in SP medium. Control growth, however, seemed more vigorous in the former medium. The cells were shorter, more bulbous, and richly endowed with mesosomes (Fig. 14). The more vigorous growth probably accounted for a better yield of penicillin protoplasts observed in this medium compared with SP. Prior to the loss of cell-wall integrity large pockets of penicillin-induced fibrous material developed (Fig. 15). After 45 minutes, mesosomes were seen in various stages of distortion and loss as already shown in Figs. 5 and 13. After 6 hours, when some 50 per cent of the cells had been converted into protoplasts or were

¹ A comparison of samples of SP culture taken 20 minutes after the addition of methicillin at 0.01, 0.1, and 1.0 mg/ml indicated a similar initial development of this septal lesion.

FIGURE 6 A section of an untreated cell aerated for some 90 minutes following transfer from P medium to SP medium, showing the normal transverse-septal profiles. A peripheral mesosome indicates partial recovery from the displacement induced by the transfer to SP. $\times 77,000$.

FIGURE 7 *a* and *b* Two adjacent sections showing the vacuolization at the site of division in *B. megaterium* after 10 minutes' growth in the presence of penicillin. The mesosome appears normal. Its association with the nucleoid is evident in Fig. 7 *a*, and its junction to the ballooned-out membrane at the transverse septum is partly evident in Fig. 7 *b*. $\times 77,000$.



emerging from broken cell walls, the fibrous material was not seen (Fig. 16). Presumably this material was able to disperse into the medium, not being structurally bound either to plasma membrane or to old cell wall.

DISCUSSION

Our observations show two main structural aspects of penicillin action which we believe have not been previously described in detail. The first of these is the very early distortion of the normal wall-membrane relationship at the developing septa, which appears under our conditions after 20 minutes in ordinary medium, and is already well developed by 10 minutes in medium with osmotic support. A general distortion of the developing septa in *Staphylococcus aureus* at this point has been observed earlier by Murray, Francombe, and Mayall (8). As pointed out by these authors, in view of the other evidence that penicillin rapidly inhibits formation of new cell-wall material, the specific location of this structural lesion indicates that cell-wall growth normally occurs at the transverse septa.

The second interesting finding concerns the fibrous accumulations seen developing at wall-membrane sites a few minutes after the addition of penicillin to cells growing in osmotically stabilized medium. The arrangement and location of this material strongly suggests that it represents unorganized wall material. If so, it is probably mucopeptide since the cell walls of *Bacillus megaterium* are very low in phosphorus (12) and apparently contain mucopeptide as their sole constituent. Moreover, as one would predict on this basis, lysozyme digestion of penicillin-treated cells growing in osmotically stabilized medium yields protoplasts free of both walls and the accumulated fibers. The

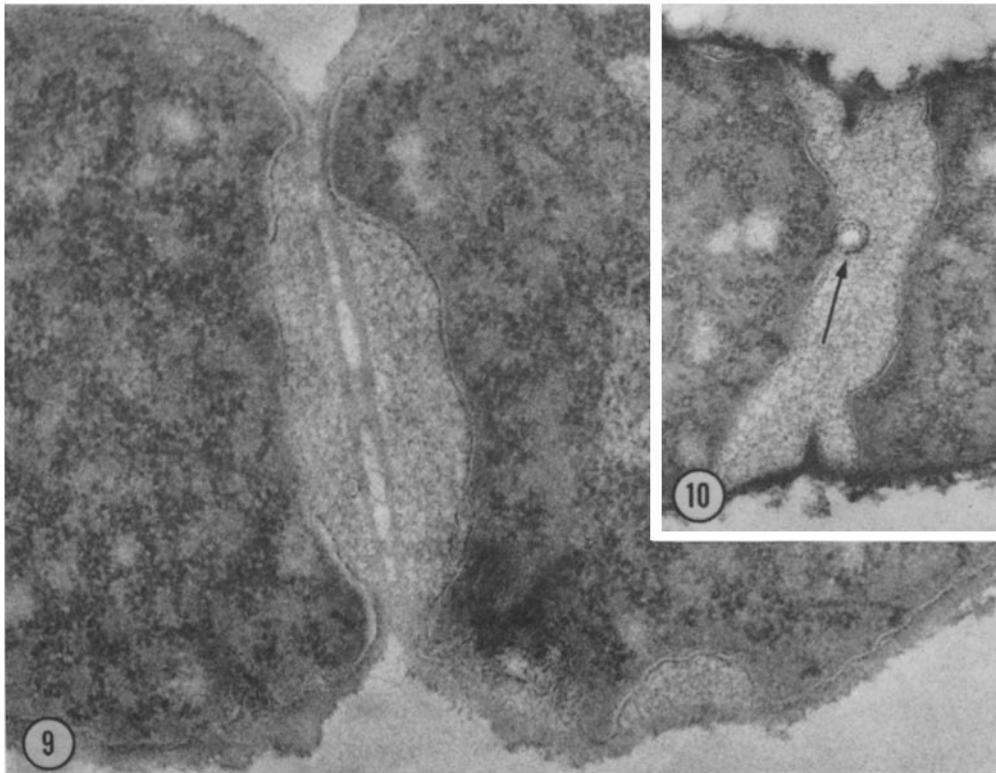
fibers seen here in thin sections are so extensive as to suggest that in the presence of penicillin and with osmotic support an extra-membranous wall mucopeptide of considerable size is formed.

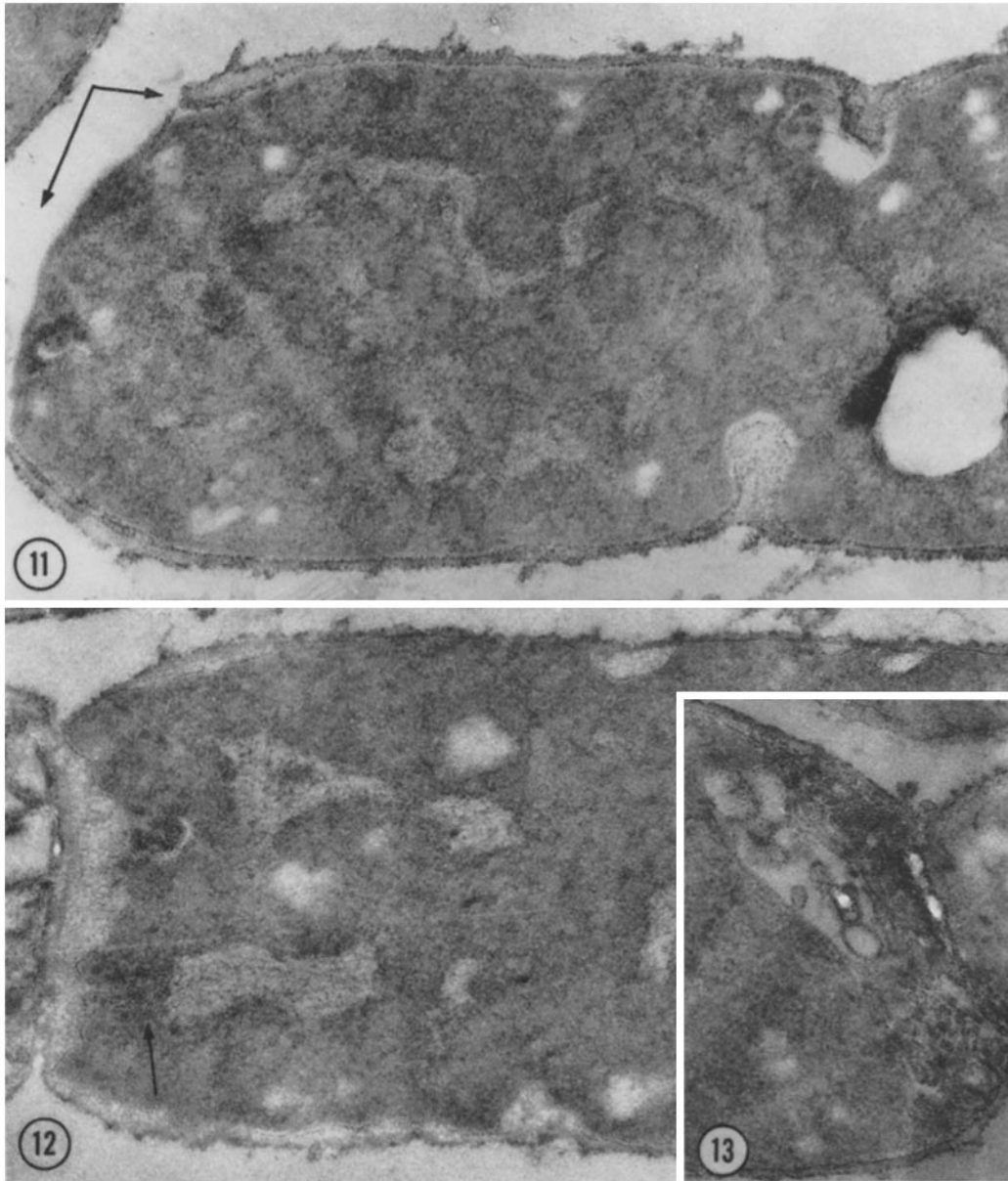
It should be recalled that earlier experiments on nucleotide accumulation induced by penicillin were done using cells without osmotic stabilization. Under such conditions, *B. megaterium* cells show only a relatively small accumulation of fibrous material and considerable early cytoplasmic leakage into these regions. Such osmotically unprotected cells suffer from early membrane damage, as shown by the early and rapid efflux of potassium (7). Thus it is possible that the uridine-linked mucopeptides found by Park (9) under osmotically unprotected conditions may represent only a part of the accumulating cell wall precursors; there may be other small molecules which leak from the cell and other larger polymers, which we observe as fibers. Should subsequent studies show that osmotically stabilized cells accumulate larger polymers free of carrier nucleotides as well as UDP-linked mucopeptides, then one might reason that penicillin action not only blocks acceptance of UDP-linked mucopeptides into a wall polymer (13) but also prevents secondary organization of this primary polymer into closely knit cell wall.

Studies by Collins and Richmond (1) suggest that, because of the structural resemblance of penicillin to the molecular topography of muramic acid, penicillin could compete with it for the active center of polymerizing enzymes. Substitution at C₁ and C₆ of the *N*-acetyl-muramic acid residue does not impair the similarity of the molecule with penicillin, so that by extension of this hypothesis penicillin could equally well inhibit the metabolism of both muramic acid-containing mucopep-

FIGURE 8 The degree of septal disorganization after 20 minutes' growth in the presence of penicillin in SP medium. The mesosomes are generally intact and nucleoid-associated (arrow). The plasma membrane and cytoplasm are free of lytic changes. Some of the fibers in the septal vacuole extend from the arrested wall profile to that of the membrane. $\times 132,000$.

FIGURES 9 and 10 Growth for 67 minutes in the presence of penicillin in SP medium. The cytoplasm and membrane are still those of healthy active cells. The penicillin-induced lesions are now larger and more heavily laden with fibrous material; some evidence of membrane proliferation or mesosomal collapse into these vacuoles is seen (Fig. 10, arrow). Fig. 9, $\times 72,000$; Fig. 10, $\times 53,000$.





FIGURES 11, 12, and 13 Sections of *B. megaterium* after 2 hours' growth in the presence of penicillin in SP. Wall loss is now seen (arrows, Fig. 11). Although some mesosomes are still associated with nuclear bodies (arrow, Fig. 12), many of them have now collapsed into the wall-membrane space (Fig. 13) or are invading the fibrous vesicles. Although major fiber accumulations occurred at cell ends and septal sites, smaller ones were also seen scattered along the cell periphery (Fig. 12). $\times 53,000$.

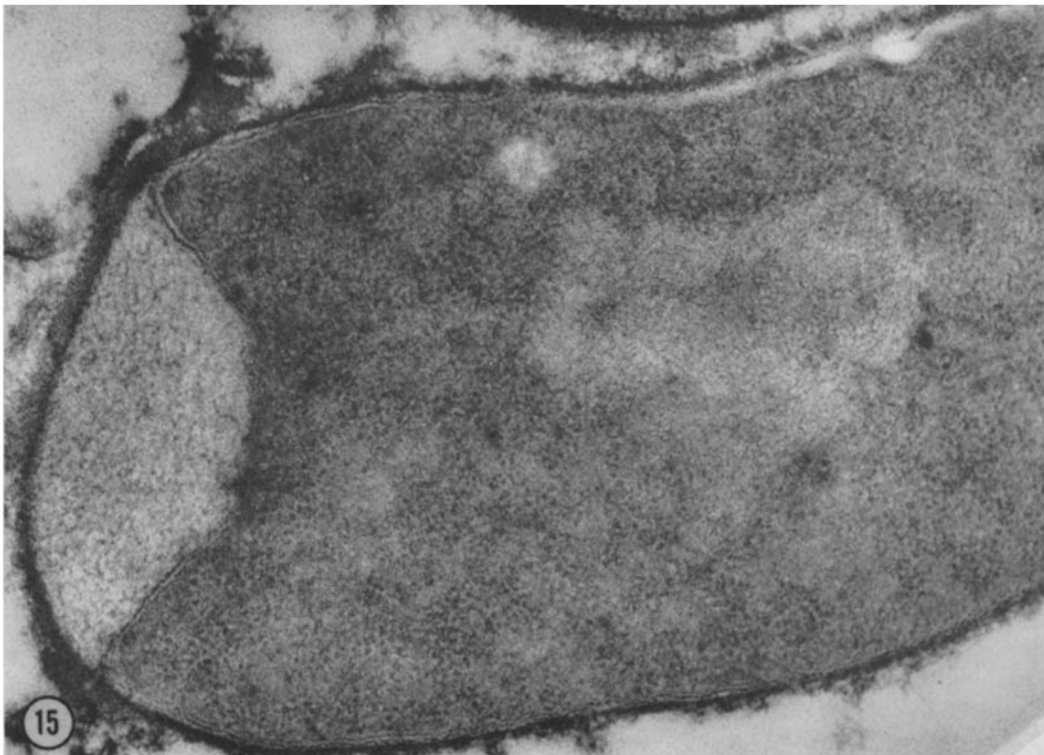
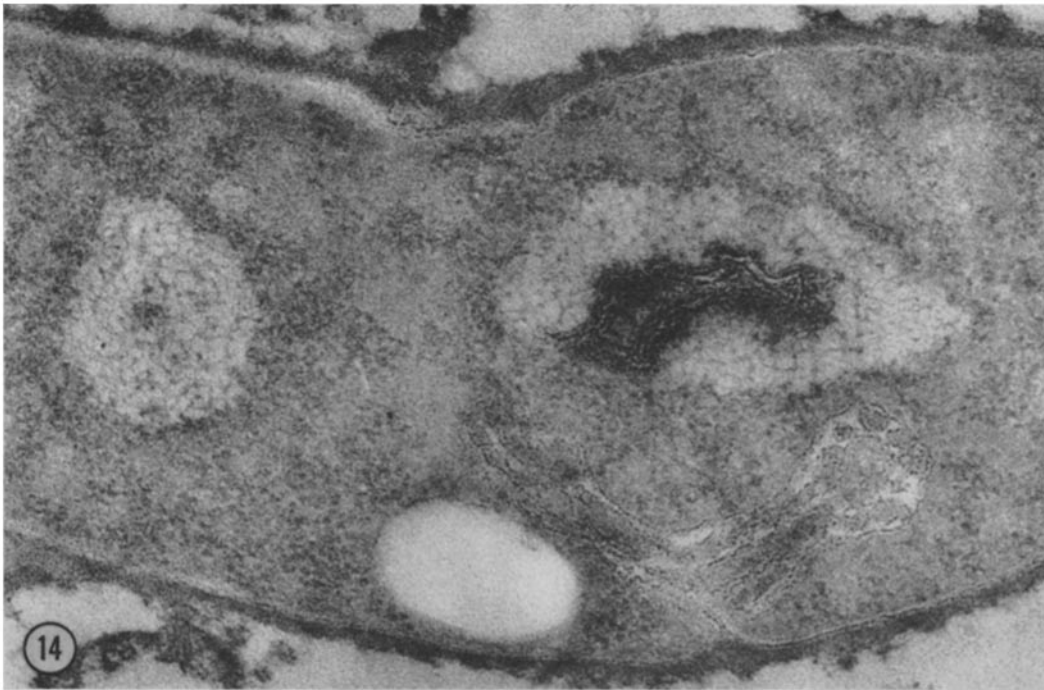


FIGURE 14 A section of an untreated cell adjusted to, and beginning division in, PP medium. $\times 91,000$.

FIGURE 15 After 45 minutes' growth in the presence of penicillin in PP medium, large pockets filled with fibrous material are found between membrane and wall. The formerly prominent mesosomes are generally absent. $\times 77,000$.



FIGURE 16 A protoplast of *Bacillus megaterium* forming as a result of some 6 hours' growth in the presence of penicillin in PP medium. In an adjacent section, the cell wall was ruptured at the site of the arrow. $\times 53,000$.

tides and muramic acid-containing polymers undergoing further organization.

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