JOURNAL OF BACTERIOLOGY, Mar. 1972, p. 1181-1190

Vol. 109, No. 3
Printed in U.S.A.

Penicillin and Cell Wall Synthesis: A Study of *Bacillus cereus* by Electron Microscopy

PETER J. HIGHTON AND D. G. HOBBS
Department of Molecular Biology, King's Buildings, Edinburgh, Scotland

Received for publication 19 November 1971

The changes in wall structure of a penicillinase micro-constitutive strain of *Bacillus cereus* (569/H/24), on exposure to penicillin, and after its removal by addition of penicillinase, have suggested the following model for the growth of the walls of these cylindrical cells. Longitudinal extension is by addition of material to a large and continuously increasing number of growing points uniformly distributed over the cylindrical surface. Addition is only in the longitudinal direction so that the cell diameter remains constant. Cross walls grow by addition to their inner edge, and on completion the two new rounded ends of the daughter cells are formed by splitting at the outer edge and continued addition at the center. The ends are conserved.

The shape of a bacterial cell is determined by the mucopeptide in the wall, which in rods is effectively a hollow circular cylinder with rounded ends. The growth of such a cell involves two processes. One is the elongation of the cylinder and the other is the formation of a cross wall, in the form of a circular disc, which then splits in two, increasing in area to form the rounded ends of the daughter cells.

The step in bacterial cell wall mucopeptide formation that is inhibited by penicillin has been shown to be the transpeptidation reaction which forms links between peptide side chains on different sugar chains (20, 21, 23). This was first demonstrated in *Staphylococcus aureus* in which the linkage is via a penta-glycine cross bridge. However, interference with the reaction in *Escherichia coli*, in which the linkage is directly between alanine on one chain and diaminopimelic acid on the other, has subsequently been demonstrated, and the transpeptidase which is inhibited has been studied in a cell-free system (12).

Cell wall growth is thought to involve the action of mucopeptidases which break both the sugar chains and the peptide cross-links to permit the insertion of additional molecules (22). It is the continued action of these enzymes, after penicillin inhibition of transpeptidase, that is thought to result in a weakened wall which ultimately leads to lysis. The prevention of lysis by the further addition of chloramphenicol to suppress protein synthesis and hence mucopeptidase synthesis has been demonstrated in *S. aureus* (19). It has also been shown that in this species cessation of growth produced by penicillin could be reversed by penicillinase (16).

It is likely that the action of penicillin is similar in all cells, but whatever its precise action it certainly interferes with wall synthesis, and the observation of its effect and the reversal of it by addition of penicillinase can be used to study wall synthesis by electron microscopy. Such a study has already been performed on cells of *Bacillus licheniformis* and suggested that longitudinal growth was effected by addition of new material all over the cylindrical surface, whereas cross walls grew inwards from the cylindrical side walls.

One of the effects of penicillin, however, was to inhibit the separation of cells after cross wall formation, and this inhibition was not removed by addition of penicillinase, although the penicillin-induced inhibition of cell growth was relieved. As a result, the cells grew in long chains. Cell walls and cell shapes were also grossly distorted for several generations after the addition of penicillinase, and both of these effects limited analysis.

A similar study has now been made on a penicillinase micro-constitutive strain of *B. cereus* in which cell separation was not inhibited and gross distortion was not produced. This strain, 569/H/24, was a spontaneous mutation of the constitutive strain 569/H (13).

MATERIALS AND METHODS

Cultures. Cultures were grown from spores in S broth (17) with shaking at 37 C. They were diluted after 4 to 11 hr and regrown until the optical density at 675 nm had increased four- to eightfold before

penicillin was added. The time of addition was chosen so that all experiments could be completed before a control culture reached the end of exponential phase. Flasks with a volume five times that of the culture were used throughout.

Isolation of 569/H/24. Spores of 569/H were grown up in SS broth for 30 hr at 35 C until spores had formed again. The culture was spun, washed in water, heated to 60 C for 1 hr, and plated and replica plated on Andrade agar. Two penicillinase micro-constitutive mutants were found, one of which was 569/H/24. SS broth (*p*H 7.2) was developed by P. Hill in this laboratory and contained 1% potato extract (Difco), 1% Casitone (Difco), 0.2% yeast extract (Difco), and 0.2 ml of Collins salts (5) per 100 ml.

Penicillin. Crystalline sodium benzylpenicillin ("Solupen" from Dista Products Ltd.) was dissolved in water immediately before addition to the cultures at concentrations such as to allow 100× dilution in the cultures.

Penicillinase. Penicillinase was isolated from *B. licheniformis* 749C (18).

Electron microscopy. Cells were fixed with OsO_4, washed in uranyl acetate, dehydrated in acetone, and embedded in araldite as described previously (8). Sections were post-stained with lead citrate, and pictures were taken with a Siemens Elmiskop la.

RESULTS

Effect of penicillin. The effect of the addition of various concentrations of penicillin on the rate of increase of optical density of exponentially growing cultures is shown in Fig. 1. In 0.1 unit of penicillin per ml, the rate decreased after about 40 min (two doublings), became zero after about 60 min, and became negative after about 100 min, corresponding to the cessation of cell growth and the onset of lysis. As the concentration of penicillin was increased these effects occurred more rapidly.

The appearance of cells after exposure to penicillin is shown in Fig. 2, 3, and 4. The appearances produced by different concentrations of penicillin were distinct and reproducible. The effect of each concentration was analyzed on at least two separate cultures.

Figure 2a shows a cell from an exponentially growing culture in the absence of penicillin. It is in the process of separating from its sister cell, and the cross wall is splitting and increasing in area to form the rounded ends of the cell, which are thinner than the cylindrical side walls. Figure 2e, at higher magnification, shows that there is little density variation across the wall. The deoxyribonucleic acid [DNA (n)] is in quite distinct areas, and the mesosomes (m) are lamellar in structure.

In samples taken about 40 min after the addition of 0.1 unit of penicillin per ml, by which time the optical density had increased

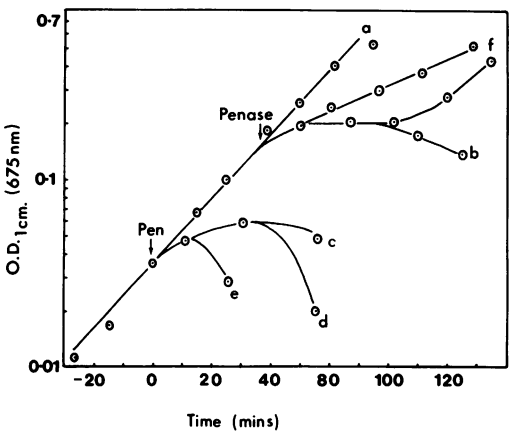

FIG. 1. *Changes in growth curves produced by penicillin, and on its hydrolysis by penicillinase. (a) No additions; (b, c, d, and e) 0.1, 1.0, 10, and 100 units of penicillin per ml, respectively, added at zero time; (f) as b but followed by addition of 200 units of penicillinase 37 min later. The two curves define the range of variation observed.*

fourfold, most cells were increased in diameter and less cylindrical (Fig. 2b). The side walls were uniformly thickened, as shown at higher magnification in Fig. 2f, and some cross walls were distorted (Fig. 2c and d, compare with 2a). The DNA was dispersed about the cell, and mesosomes were absent.

Cells such as in Fig. 2b could have been produced if the parent cell increased in diameter in the middle before division, which then proceeded normally. The distorted cross walls were probably not produced until towards the end of the period in penicillin.

These effects were more pronounced after addition of 1 unit of penicillin per ml (Fig. 3). Side walls were double-layered (Fig. 3e) as if a new layer had been formed inside that existing before the addition of penicillin. Cross walls were even more distorted (Fig. 3b, c, d).

At even higher concentrations of penicillin, gross distortion of cell shape did not occur (Fig. 4a). The side walls were uniformly thinner and some material of lower density was distributed irregularly along the sides of the cell between the cell membrane and wall (Fig. 4a, b, f). Developing cross walls were abnormally thick, and appeared continuous with this layer. Completed cross walls were either uniformly thick and distorted (Fig. 4d), or increased in thickness towards the middle. Penicillin at 100 units/ml produced a similar effect, but with much less deposition of material between plasma membrane and wall (Fig. 4e, g).

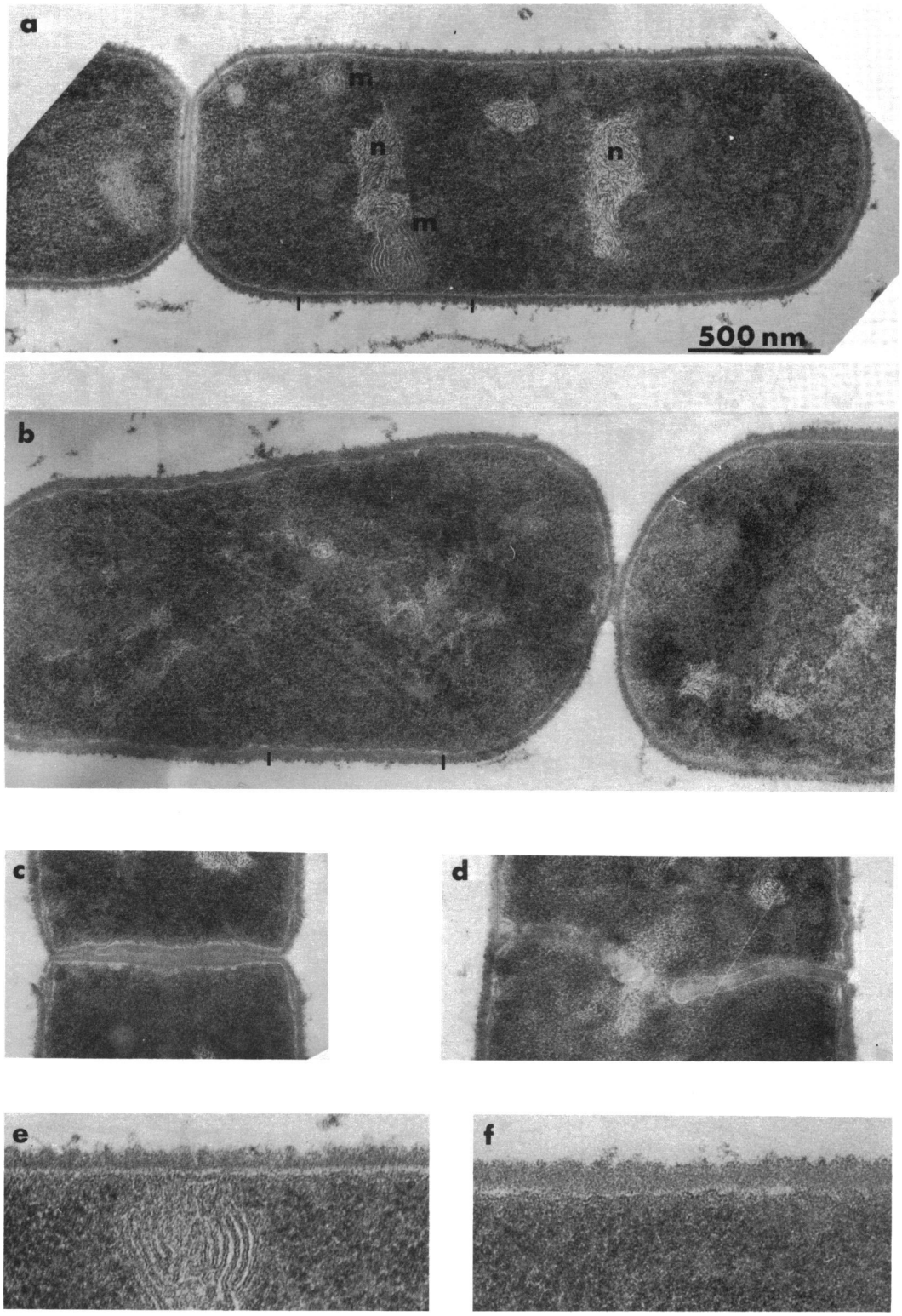

FIG. 2. *Changes in appearance produced by 0.1 unit of penicillin per ml. (a) Zero units per ml; n, DNA; m, mesosome; (b) 45 min in 0.1 unit per ml; (c and d) 37 min in 0.1 unit per ml; (e and f) 2× enlargements of the sections marked in a and b, respectively.*

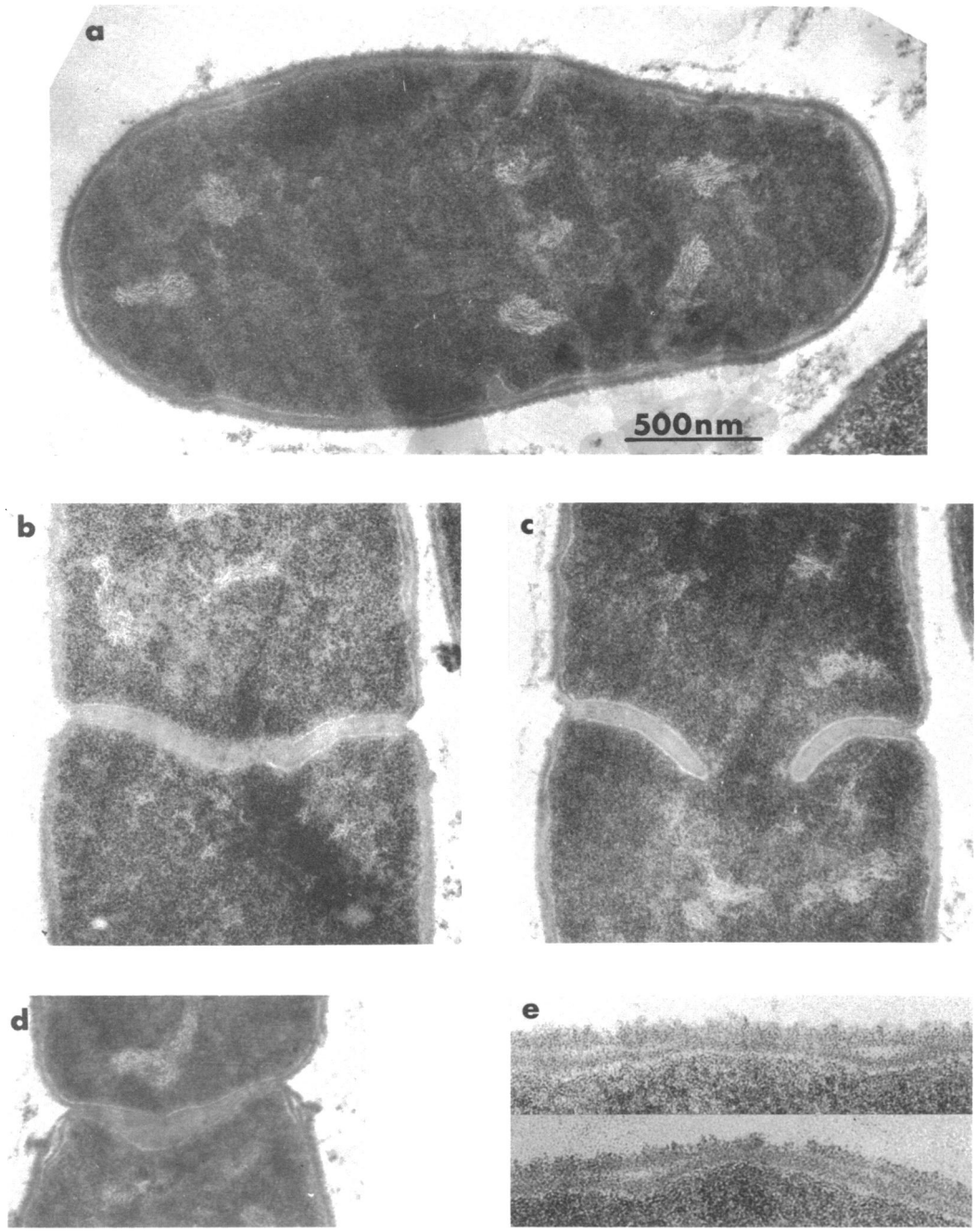

FIG. 3. *Changes in appearance produced by 1.0 unit of penicillin per ml. (a, b, c, d, and e) 30 min in 1.0 unit per ml; b and c are serial sections; e is at 2× higher magnification.*

A model for wall synthesis. To explain the above results the following model is proposed.

Longitudinal elongation is by addition of material to a large and continuously increasing number of growing points, uniformly distributed over the cylindrical surface. Addition is only in the longitudinal direction so that the cell diameter remains constant. Cross walls grow by addition to their inner edge, and on completion the two new rounded ends of the daughter cells are formed by splitting at the outer edge, and continued addition at the

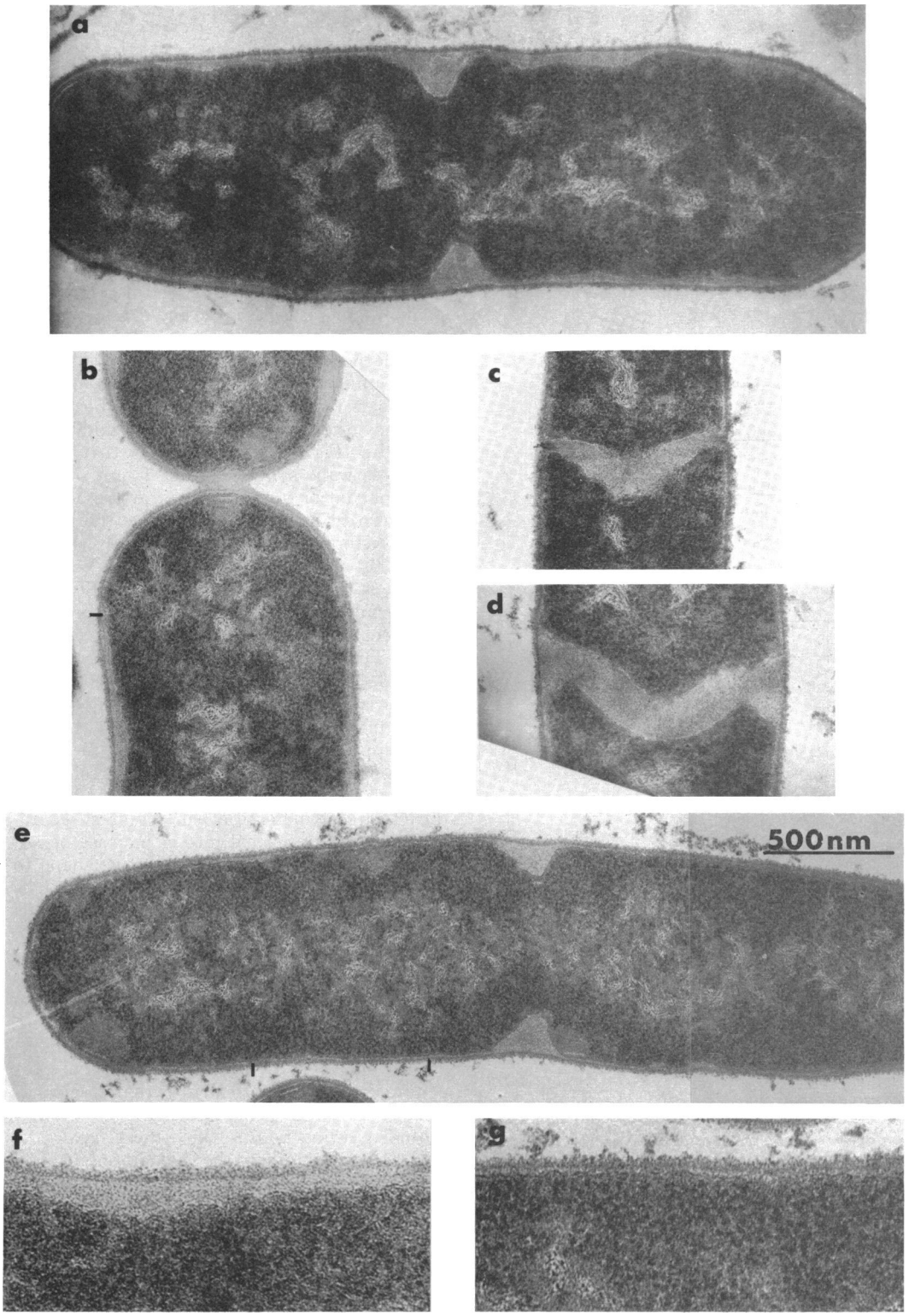

FIG. 4. *Changes in appearance produced by 10 and 100 units of penicillin per ml. (a, b, c, and d) 30, 30, 15, and 30 min in 10 units per ml, respectively; (e) 30 min in 100 units per ml; (f and g) 2× enlargements of sections marked in b and e, respectively.*

center (see Fig. 5a). Elongation is assumed to continue during this process since cells were seen with developing cross walls but of length less than twice that of the shortest cells. The ends are conserved. The way the model accounts for the results is summarized below.

(i) Penicillin would interfere with wall synthesis at all growing points and so produce abnormal wall all over the sides of the cell. This is seen at the lower penicillin concentrations (Fig. 2b, 3a). At the higher concentrations (Fig. 4a, e), even though the deposition of material between cell membrane and wall is not uniform, neither is it symmetrically distributed, and the wall is uniformly thinned.

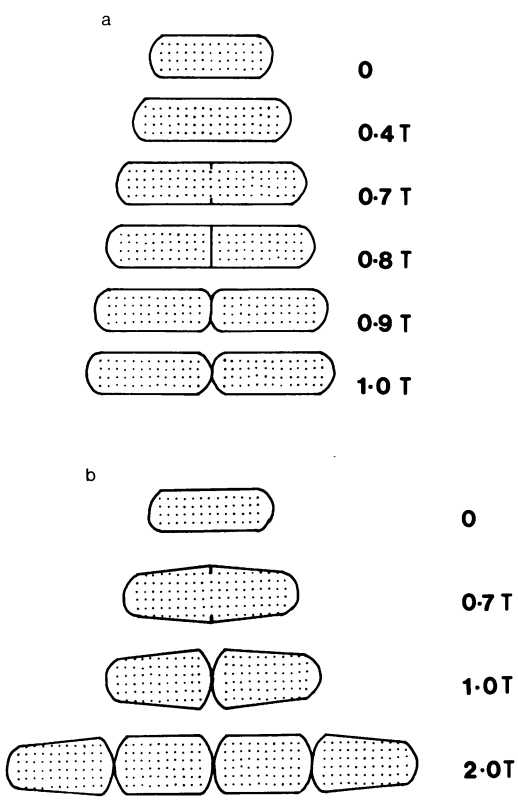

FIG. 5. *(a) Model for cell growth and division. Longitudinal extension is by addition of material to a logarithmically increasing number of growing points, represented schematically by dots, uniformly distributed over the cylindrical surface. Addition is only in the longitudinal direction, so that the cell diameter remains constant. Cross walls grow by addition to their inner edge, and on completion the rounded ends are formed by splitting at the outer edge and continued addition at the center. The duration of this process has not been estimated and has arbitrarily been chosen as about 30% of the generation time (T), for the purpose of illustration only. The ends are conserved. (b) Model for reduction of surface area to volume ratio by increase in diameter, and the resultant production of tapered cells as seen after growth in 0.1 or 1.0 unit of penicillin per ml (see Fig. 2b, c).*

(ii) Since the ends of the cell are conserved in the model, ends synthesized before the addition of penicillin would be unaffected by it (Fig. 3a, 4b).

(iii) Since cross walls are built inwards from the sides of the cell, distortion would increase towards the center as the effect of exposure to penicillin increased (Fig. 2c and d, 3b, c, and d). At higher concentrations, the effect seems to have been immediate so that cross walls were either uniformly distorted (Fig. 4d) or only increased in distortion towards the center where synthesis had begun before addition of penicillin (Fig. 4c). It is unlikely that any of the cross walls synthesized in 1 unit of penicillin per ml or higher concentrations actually separated to form cell ends, since the optical density stopped increasing so rapidly (Fig. 1), but in 0.1 unit per ml two doublings occurred and most ends looked fairly normal (Fig. 2b). Significant distortion of cross walls probably only occurred in the last doubling (Fig. 2c, d).

(iv) The increase in cell diameter producing tapered cells at the lower concentrations (Fig. 2b, 3a) is readily explained by the model. By partly inhibiting cell wall construction, but not the supply of components, penicillin might interfere with the normal process of cell elongation, producing thickened walls and a reduced rate of increase of surface area. To accommodate the normal rate of increase of cell mass, the cell would need to change shape to increase the volume to surface area ratio. This ratio increases as a cylinder with rounded ends increases in diameter and decreases in length, to become a sphere. Subject to the constraint that the ends of the cell are conserved, the best the cell can do is increase in diameter so that it is widest in the middle and tapers towards the ends (Fig. 5b). The increase in diameter is achieved by addition of material perpendicular to the normal direction of elongation, or in effect by the addition of new longitudinal growing points, since these have a finite size, possibly only the diameter of a single polymer.

An alternative explanation is that the wall synthesized is weaker than normal, and swelling occurs as on protoplast formation, but constrained by the conserved ends. However, this should then also occur at the higher concentrations in which the wall is thinned and presumably weakened by lytic enzymes. As it does not, it is assumed that at the lower con-

centrations control of cell shape is maintained, although the model would still be valid even if it were not maintained.

Interpretation of the variation of penicillin effect with concentration. In 0.1 unit of penicillin per ml, wall construction is assumed to have been partly inhibited, producing the effects described above and leading to cessation of growth after two doublings and eventual lysis from the continued action of lytic enzymes. One unit of penicillin per ml is assumed to have produced similar but stronger effects so that growth stopped much more rapidly, and wall material synthesized was not incorporated into the existing wall by the normal process of elongation but formed a new layer inside it, the original wall being stretched and possibly fragmented, although there may have been little increase in surface area.

In 10 units of penicillin per ml it is assumed that construction was so inhibited that no layer capable of determining the shape of the cell was formed. Some material accumulated inside the wall, which was stretched and thinned to accommodate some increase in mass, as some cross walls were synthesized, but lysis began rapidly. In 100 units of penicillin per ml, the effect was more extreme and even synthesis of wall material was prevented.

This interpretation is consistent with the observations made with *S. aureus* (21), that uncross-linked mucopeotide monomers accumulate at low penicillin concentrations, but not at concentrations significantly higher than that necessary to stop growth.

Reversal of the effect of penicillin with penicillinase. The effect of 0.1 unit of penicillin per ml on the rate of increase of optical density could be reversed by the addition of penicillinase (Fig. 1). The growth curves obtained varied in shape and slope between experiments, suggesting variable lysis, and probably masking the true growth rate of the recovering cells. However the changes in appearance of the cells were reproducible.

The side walls which were thickened in the presence of penicillin (Fig. 2b) became very irregular along their inner edge (Fig. 6a), and the appearance was consistent with the development of a new layer of wall inside the old, which then appeared to be fragmented and sloughed off (Fig. 6b). The excessively thick regions (arrow, Fig. 6b) could result from bits of rather firmly attached old wall which restricted the longitudinal expansion of the new. Cells under all conditions showed fragments of loosely packed material attached to the outside of the walls, but the material considered to be sloughing off here looked much more compact.

This reorganization appeared to be occurring all over the sides of the cell, as would be predicted by the model, and cross walls appeared to be synthesized normally and independently of the reorganizing side walls, growing inwards from them and separating to form normal ends (Fig. 6a, b). The appearance was very like that seen in *Arthrobacter crystallopoietes*, in which only the inner layer of the side walls extends in to form the cross wall (14). Eventually quite normal-looking cells were formed, and mesosomes reappeared (Fig. 6c). The details of these changes are shown at higher magnification in Fig. 7. In Fig. 8 the cells analyzed are summarized, showing the mean cell volume per sample and the variation in volume, which both increased on exposure to penicillin, presumably from interference with cross wall synthesis, and returned to normal after addition of penicillinase.

DISCUSSION

From direct observation by electron microscopy of the positions of raised bands associated with division sites on the surface of *Streptococcus faecalis*, Higgins and Shockman (7) proposed that the two new rounded halves of the daughter cells are fed out from the developing cross wall. The prediction of this model, that walls are conserved so that each daughter cell has one new and one old half, is supported by the observations of Cole (4) on *S. pyogenes* using fluorescent labeled antibody specific for M protein or C polysaccharide, and of Briles and Tomasz (1) on *Diplococcus pneumoniae* using ^{3}H-choline to label teichoic acid autoradiographically for light microscopy.

The model proposed above by us for the formation of the cross wall and its subsequent conversion to two rounded ends is basically the same as that proposed by Higgins and Shockman for the synthesis of two new rounded halves in the replication of coccal cells. Here material is added to the inner edge of the developing cross wall, which splits at its outer edge to form two new rounded halves for the daughter cells, while addition continues at the inner edge. In *B. cereus*, however, the cross wall grew across the cell before splitting, and its synthesis took only part of the replication cycle, whereas in the coccal cells the synthesis of cross walls in the future daughter cells began once doubling was complete before the completion of the cross wall of the parent.

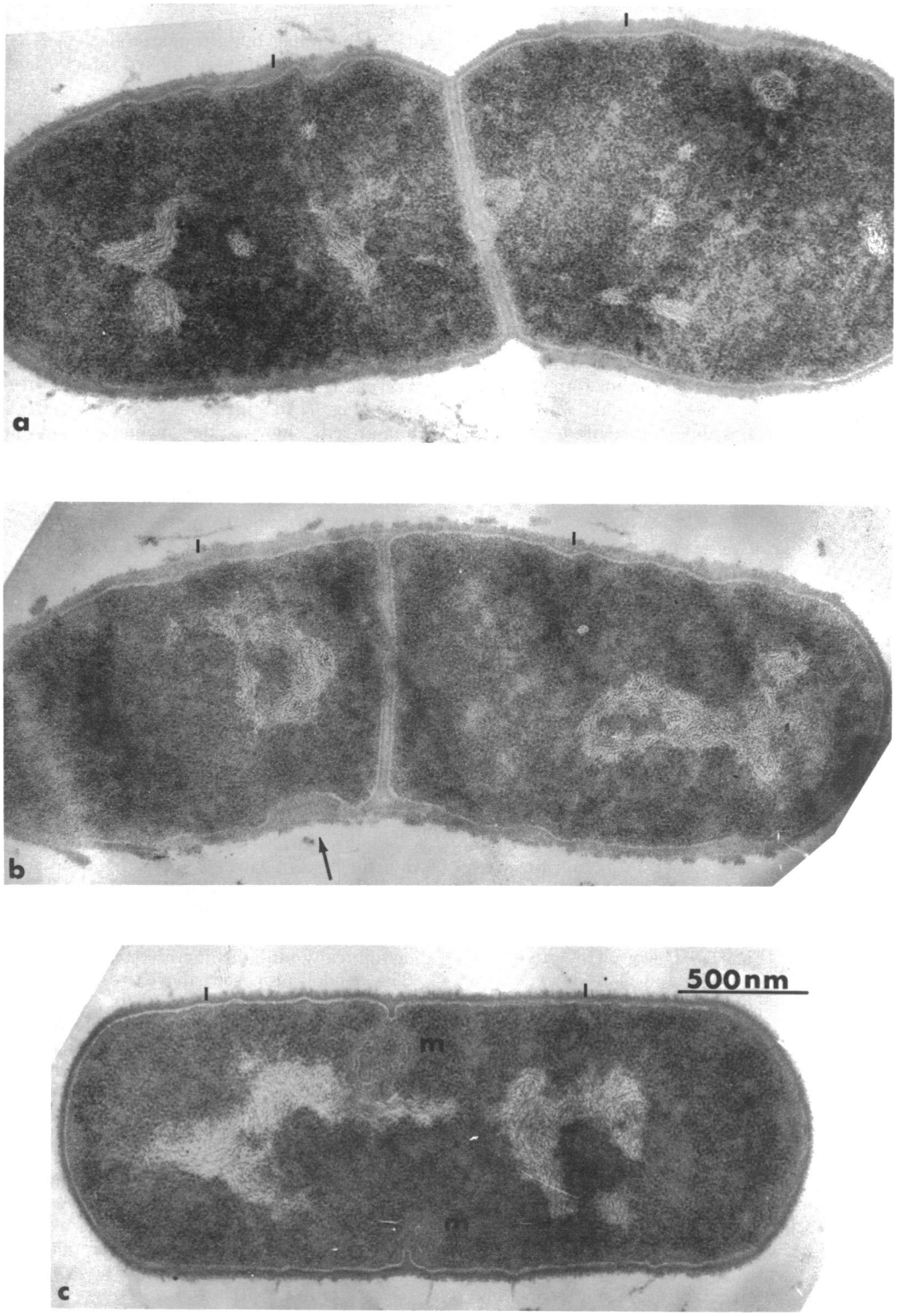

FIG. 6. *Reversal of the effect of 37 min in 0.1 unit of penicillin per ml. (a, b, and c) 15, 40, and 100 min, respectively, after the addition of 200 units of penicillinase per ml. The cells in b are considered to have derived from a cell such as one of the pair in a, and the cell in c from a cell such as in b, after several generations. m, Mesosome.*

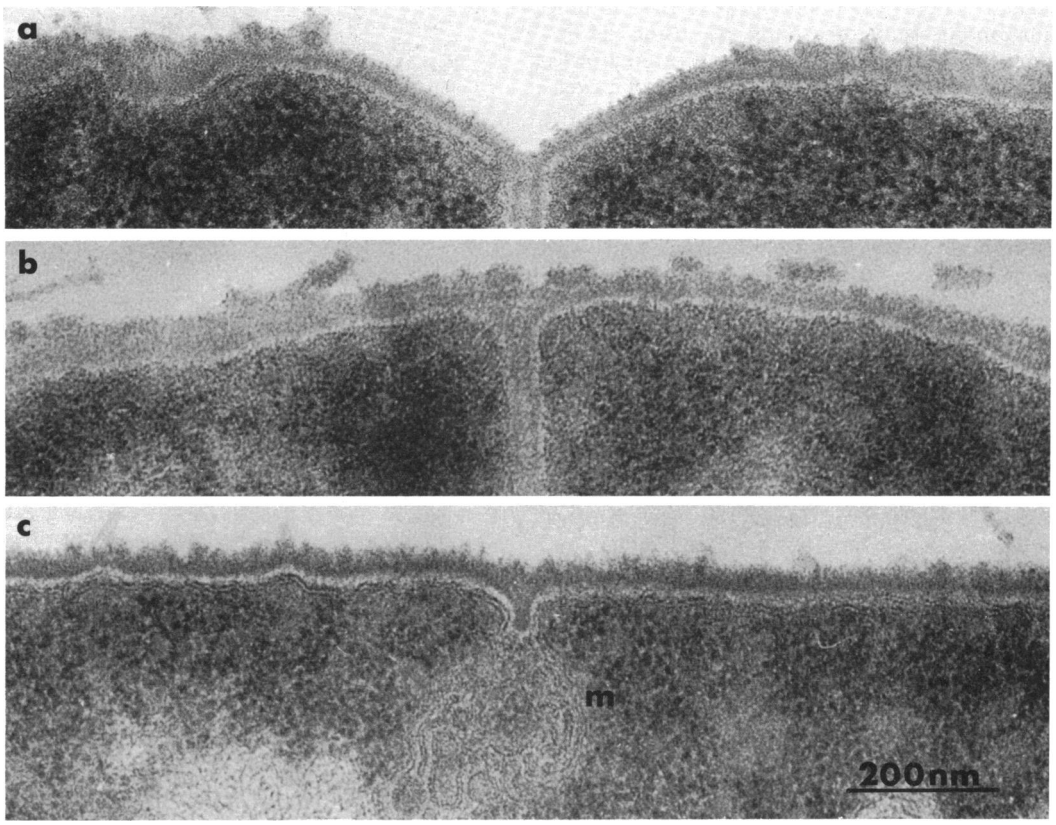

FIG. 7. *(a, b, and c) 2× enlargements of sections marked in Fig. 6a, b, and c, respectively. m, Mesosome.*

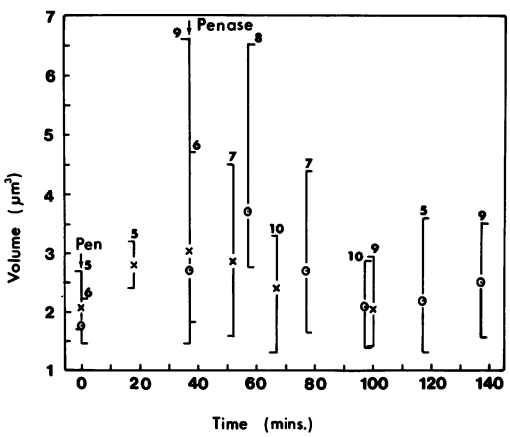

FIG. 8. *Effect of penicillin on cell volume, and its reversal with penicillinase. Penicillin (0.1 unit/ml) added at zero time and followed by 200 units of penicillinase per ml after 37 min. The number, range, and mean of the measurements are shown for each sample. ⊙ and × indicate means and ranges of two separate experiments. Volumes (as cylinders) were calculated from the width and length of central sections such as shown in Fig. 2 and 6.*

Addition of material to the inner edge of the cross wall would seem to be much more efficient than addition at the outer, as the growing cross wall is a circular disc with a central hole, and the latter method would involve constant reorganization of the part already built, as material was pushed towards the center and the hole decreased in diameter. The further development of the rounded ends by continued addition at the center of the cross wall was proposed in the model for simplicity and for lack of any data, but this would also involve considerable reorganization of existing structure. The conversion from flat disc to dome, however, might be more simply effected by addition of material at specific points distributed over the surface, if for instance the ends were built like geodesic domes.

The growth of the cylindrical side walls of gram-positive rods has previously been studied with *B. cereus* cells by Chung et al. (3) using fluorescent labeled antibody to whole cells. This suggested addition at discrete points along the cell length. The interpretation of their data has been strongly criticized by Cole

(4) on technical grounds, but a further autoradiographic study by Chung (2), using ^{3}H-DL-alanine and light microscopy, indicated growth at two to three sites at the beginning of a cycle of replication, increasing to six to eight sites before division. These cells however were recovering from the effects of chloramphenicol which was used to suppress protein synthesis and cell growth while the initial labeling was performed. Under these conditions alanine was only incorporated into the wall but at many unresolved sites along the cell.

A similar suppression of protein synthesis and cell growth, produced by removing tryptophan from a tryptophan-requiring strain of *B. subtilis*, was shown by Hughes et al. (11) to produce uniform thickening of the wall, and Landman et al. (15) showed that regrowth of wall on protoplasts of *B. subtilis* occurred all over the surface. However, recently Hughes and Stokes (10) have followed the redistribution of fluorescent labeled mucopeptide antibody on *B. licheniformis* and observed three newly synthesized regions along the cell after 120 min. Unfortunately densitometric analysis of only one cell was presented.

The changes in appearance of the side walls of *B. cereus* reported above, and of *B. licheniformis* (9), on exposure to penicillin and on its removal with penicillinase, indicated growth by addition of material at many points uniformly distributed over the cylindrical surface. Such a process, if the rate of increase of surface area per unit area is constant, produces in cells of fixed diameter a rate of increase of length proportional to existing length, i.e., exponential growth. A study by light microscopy (6) of cells in cultures of *B. cereus* 569/H growing exponentially, with a doubling time of 30 min, has shown that growth of individual cells is not exactly exponential, but that the rate of growth does increase continuously with length. Such a process is most simply achieved by a continuously increasing number of growth points.

ACKNOWLEDGMENTS

We are grateful to Joan Fleming for isolating and supplying spores of *B. cereus* 569/H/24, and to Len Kelly for a generous gift of purified penicillinase.

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