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Response of Cell Walls of *Escherichia coli* to a Sudden Reduction of the Environmental Osmotic Pressure

M. E. BAYER

Department of Molecular Biology, The Institute for Cancer Research, Philadelphia, Pennsylvania

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The rate of survival after osmotic shocks was found to be dependent on the state of growth. When growing logarithmically, *Escherichia coli* was about 20 to 100 times more sensitive to an abrupt decrease of the environmental osmotic pressure than when it was in the stationary phase. Subjecting rapidly growing cells to such a treatment caused fingerlike extrusions to emerge from the bacterial wall. Our results suggest that underneath these extrusions the rigid layer of the wall contains weak areas which appear as discontinuities or gaps when viewed in an electron microscope. After exposure to osmotic shock, the gaps became wider. We concluded that the gaps represent sites of mucopolymer synthesis where the rigid structure has temporarily been opened by hydrolytic enzymes to allow for the insertion of new wall material into the older portions of the wall.

Abrupt changes in the osmotic pressure of bacterial cultures cause mechanical injury and death of the cells (6, 7, 12, 17, 23), whereas slow changes permit many cell systems to adjust to a wide range in the conditions of the milieu (5). The osmotic regulation of a cell depends upon an intact cytoplasmic membrane (6, 14). Gram-negative bacteria show plasmolysis in solutions of high osmotic pressure and survive in media of an osmolarity much lower than that of their protoplasm. In the latter case, the protoplasmic contents are protected from mechanical disruption by a surrounding rigid wall. Walls of gram-negative bacteria appear as multilayered structures when viewed in an electron microscope (13, 16).

It has been shown that the innermost layer of the wall of *Escherichia coli* is almost entirely responsible for the mechanical strength of the wall (16).

In a recent study (3), structural differences were found in the "rigid layer" (21) of *E. coli* strains, depending on the state of growth; the rigid layer of logarithmically growing cells showed abundant discontinuities and gaps in the distribution of its proteinaceous component. It had been suggested that these gaps might represent the sites where the rigid structure was opened enzymatically to allow for introduction of new building blocks in the older portions of the wall. As a consequence of this, the rigid layer should be mechanically weakened at these locations.

In the present study, a mechanical stress was exerted on the cell walls of *E. coli* by means of abrupt changes of the osmolarity of the medium from high to low concentrations. Differences in the sensitivity of the bacteria to osmotic shock are correlated with growth-dependent structural differences of the wall.

MATERIALS AND METHODS

E. coli B was obtained from W. Six, Iowa State University, and has been grown for a number of years in this laboratory. The cells were cultured in amounts of 10 ml of L-broth with aeration of about 150 ml of air per min at 37 C. Under these conditions, the generation time was 25 to 30 min. The L-medium consists of 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, and 0.1% glucose; the *p*H was adjusted to 7.0 with 1 N NaOH.

For measurement of the susceptibility of the cells to osmotic shock, bacteria in the logarithmic state were suspended in L-medium containing 50% (w/v) sucrose. With stationary cell cultures, crystalline sucrose had to be added to prevent the bacteria from starting to grow logarithmically should they become diluted in L-broth. The sucrose was quickly dissolved by bubbling air through the cultures. The cells suspended in the sucrose medium were centrifuged at 7,700 × *g* for 15 min at 4 C. The number of viable cells was determined in the same way as in the unshocked control cultures. For shock treatment, the

pellet obtained in sucrose was resuspended in 0.5 ml of sucrose in L-broth and then was quickly diluted in 10 ml of distilled water of the same temperature. Control cultures, exposed to the same sucrose concentrations, were gradually diluted by addition of water at 4 C and at a rate of 0.5 ml/min until a sucrose concentration of 3% was obtained. Colonies on L-broth agar plates were counted after incubation for 12 hr at 37 C.

For electron microscopy, the sucrose contained in the specimen had to be removed before negative staining and observation; otherwise, the drying of the specimen in the vacuum was slowed down considerably, and artifacts could easily be caused by the electron beam. To keep the cells in the state of plasmolysis in spite of the removal of sucrose, the following procedure was applied. The cultures were centrifuged twice and the pellets were resuspended in 0.015 M maleate buffer (*p*H 6.8) containing 50% sucrose; they were then exposed to 10^{-3} M spermine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) in sucrose maleate buffer. This treatment prevented the protoplasts from expanding to their original volume when the osmotic pressure was reduced (11). To prepare the bacteria for electron microscopy after osmotic shock, the cells were first centrifuged in the sucrose solutions at 4,500 × *g* for 20 min. They were then observed after one of two treatments. (i) Whole cells were subjected to brief washing with water on the electron microscopic grid (which served as osmotic shock), and were negatively stained in 4% silicotungstate at *p*H 6.8. (ii) Alternatively, frozen-state microtomy (2) was used to investigate walls and membranes of the cells without the disturbance of larger amounts of cytoplasmic contents. For this procedure, portions of the pellets were quickly frozen in liquid nitrogen and were cut open in a cryostat at −30 C; they were then briefly washed in water and negatively stained in silicotungstate.

When the lipoid layers from the walls had to be removed, the opened cells were treated on the electron microscopic grid with 0.5% sodium dodecylsulfate for 5 to 20 min at 20 C; they were then washed in water and finally were stained negatively. Egg white lysozyme (Worthington Biochemical Corp., Freehold, N.J.; twice crystallized), when applied, was used in concentrations of 0.1 to 0.2 mg/ml on the grid before staining. All negatively stained specimens were dried in the vacuum of a Siemens Elmiskop I electron microscope. For ultrathin sections of plasmolyzed cells, the bacteria were fixed with 2% formaldehyde in sucrose medium (30%, w/v) at 20 C for 4 hr, centrifuged at 3,000 × *g*, and resuspended in sucrose medium which contained 1% osmium tetroxide. After fixation for 8 hr at 4 C, the material was dehydrated in acetone and embedded in Vestopal. The thin sections were stained in aqueous saturated uranyl acetate for 1 hr at room temperature. Photographs were taken at magnifications of 20,000 to 40,000 times.

RESULTS

Viability. About 90% of logarithmically growing cells survived exposure for 10 min to 50% sucrose followed by a slow dilution, whereas about 70% of the cells in stationary cultures survived this treatment.

When cultures were subjected to an osmotic shock produced by a sudden reduction of the osmolarity of the medium, the rate of survival was dependent on the state of growth. The viability of logarithmically growing cells decreased to values of 0.1 to 1% after shocking. However, 20 to 67% of the stationary cells survived the shock (Table 1).

Morphology. When plasmolyzed cells of *E. coli* were fixed, dehydrated, and embedded in polyester, they showed a retraction of their protoplasts from the wall in ultrathin sections (Fig. 1a). For observation of unfixed bacteria in the state of plasmolysis, the cells had to be stabilized with spermine in 50% sucrose, followed by brief washing in water, and negative staining; the bacteria then showed dense regions which corresponded to the mass distribution of their protoplasts (Fig. 1b). However, there was no detailed structure visible inside the wrinkled wall because of an insufficient permeation of the staining fluid through the wall.

Frozen-state microtomy resulted in a considerable increase in resolution (Fig. 2 to 4), and a clear morphological separation of wall and protoplast was obtained (Fig. 2a). The wall unfolded and showed the channel-like structures described earlier (2). The protoplast was outlined by the contour of the protoplasmic membrane. The rigid nature of the wall was indicated by the fact that, in spite of the unfolding during the preparation, it still retained the general shape of the cell (*see also* 16). Treatment of the cell walls with sodium dodecylsulfate removed the lipoid surface layers and exposed the rigid layer of the wall with its well defined edges and the coarse surface pattern produced by the proteinaceous material covering the mucopolymer. The shape of the wall was well preserved. In the proteinaceous component of wall residues of logarithmically growing cells,

TABLE 1. *Viability of Escherichia coli B after osmotic shock*

Growth phase	No. of viable cells per ml		Per cent survival
	Before shock	After shock	
Log.....	3.5×10^7	5.5×10^5	1.5
	4.0×10^7	5.0×10^4	0.1
	2.0×10^8	$<2.0 \times 10^6$	<1.0
Stationary...	4.0×10^8	8.0×10^7	20.0
	1.3×10^8	2.5×10^7	19.0
	4.0×10^8	2.7×10^8	67.0

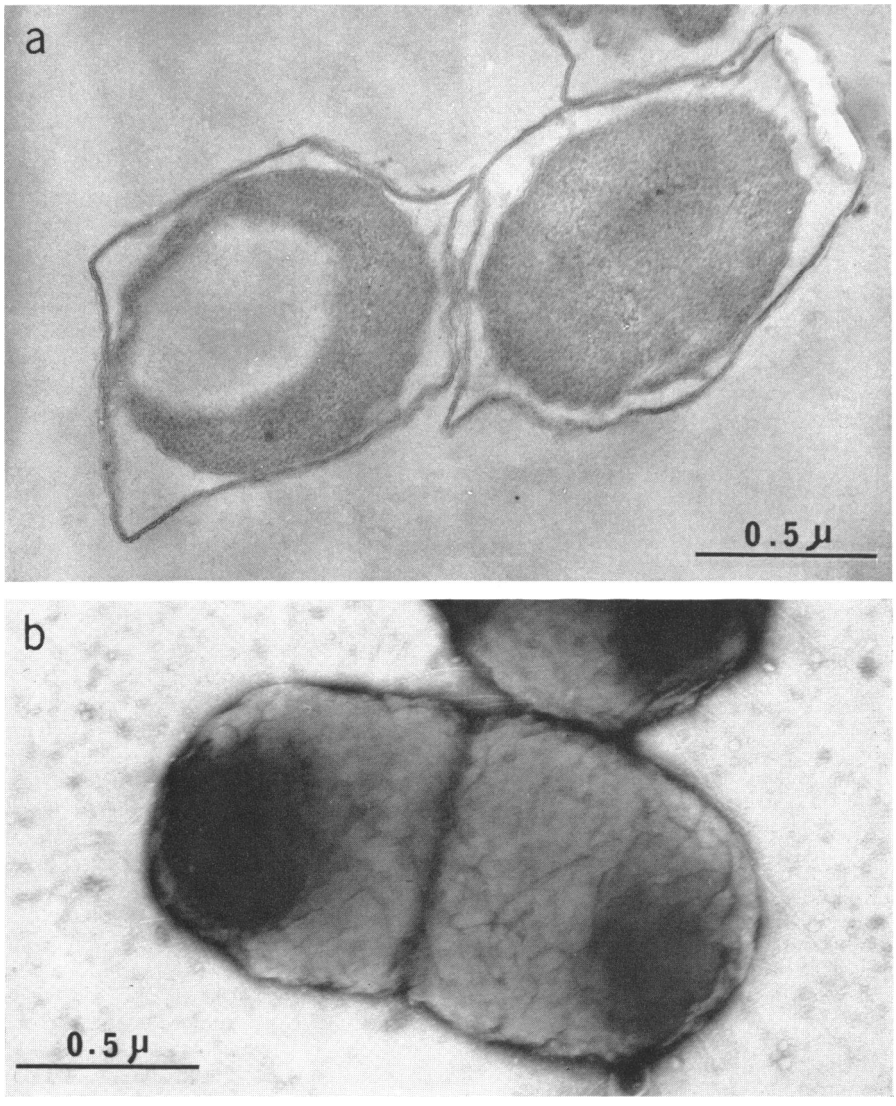

FIG. 1. *(a) Ultrathin section of Escherichia coli B after plasmolysis, fixation in 2% formaldehyde, postfixation in OsO_4, and embedding in Vestopal. The section has been stained with uranyl acetate. × 48,000. (b) E. coli B after plasmolysis and stabilization of the protoplast with spermine. Electron micrograph of complete cells; negatively stained. × 48,000.*

many scattered gaps 100 to 200 A wide were visible (Fig. 2b). In the wall residue of stationary cells, such gaps were rather scarce. Treatment of such a preparation with lysozyme for 1 min at 20 C disintegrated the wall to a large number of more or less spherical elements about 100 to 120 A in diameter, many of which contained a hollow center; though smaller, these are probably similar to particles found in lysozyme digests of "S-B" membranes of *E. coli* (21).

When whole cells, without being opened in a previous step of preparation, were shocked osmotically, a large amount of material was released. Frequently, an "explosion" of the cell with disruption of the wall could be observed. Cutting open of cells in the frozen sucrose solution and subsequent exposure to distilled water revealed more detailed pictures (Fig. 3 and 4) in many of the cells.

The formerly smooth contour of the wall was

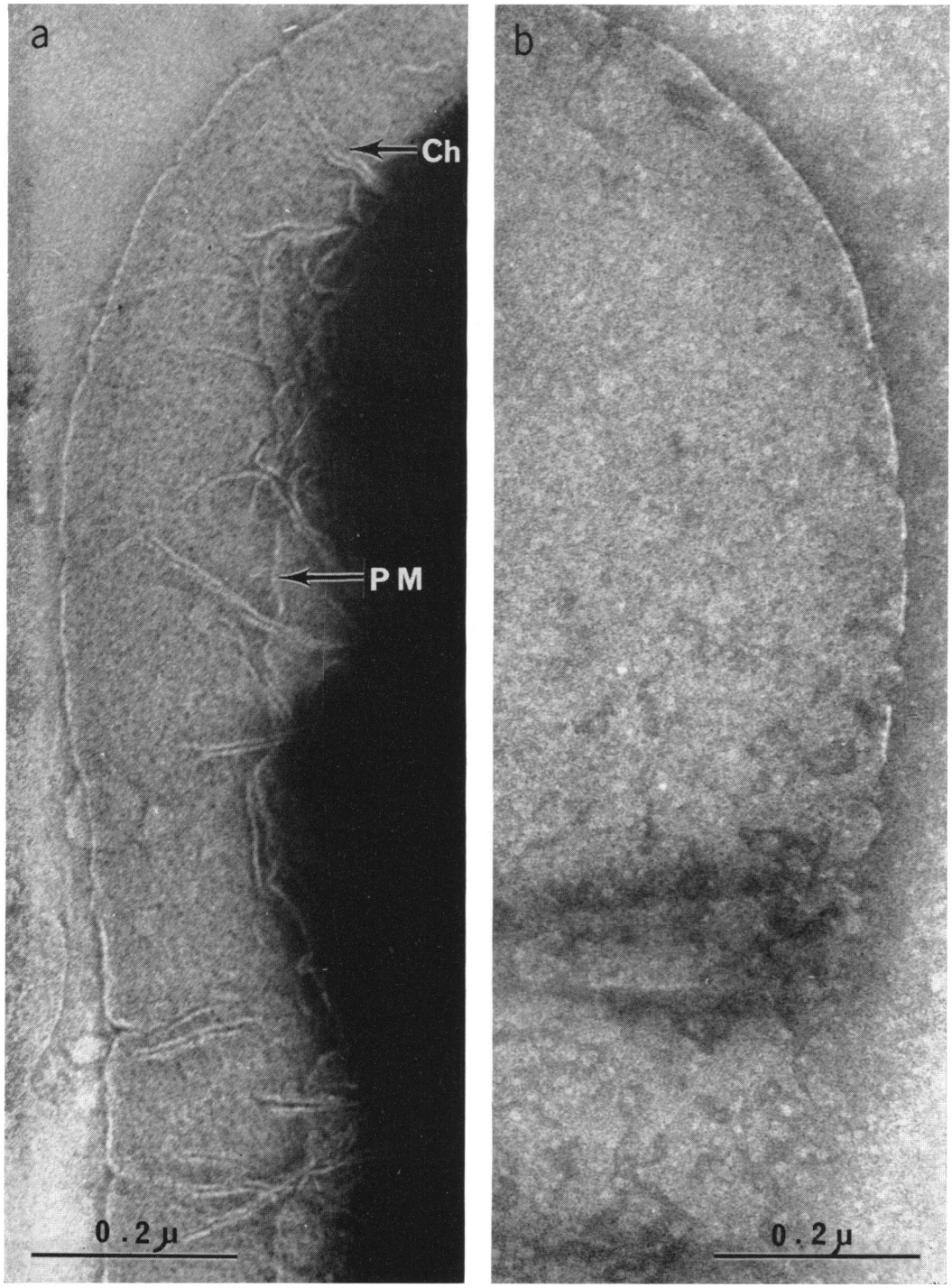

FIG. 2. *Escherichia coli after frozen-state microtomy; negatively stained.* × *150,000.* (*a*) *Portion of a plasmolyzed cell after spermine stabilization. The wall shows channel-like structures* (*Ch*); *the protoplasmic membrane* (*PM*) *and the dense protoplasmic contents are clearly separated from the wall.* (*b*) *Rigid layer of the wall of a rapidly growing cell, after treatment with sodium dodecylsulfate. Note the discontinuities in the layer.*

interrupted by extruding elongated elements (Fig. 3a). The shape of the extrusions was finger- or drumstick-like, with the protoplasmic membrane occasionally driven into the inner space of such a structure. The diameter of an extrusion near its base at the wall measured 200 to 300 A. The effect was visible, almost exclusively, in logarithmically growing cells, whereas extrusions

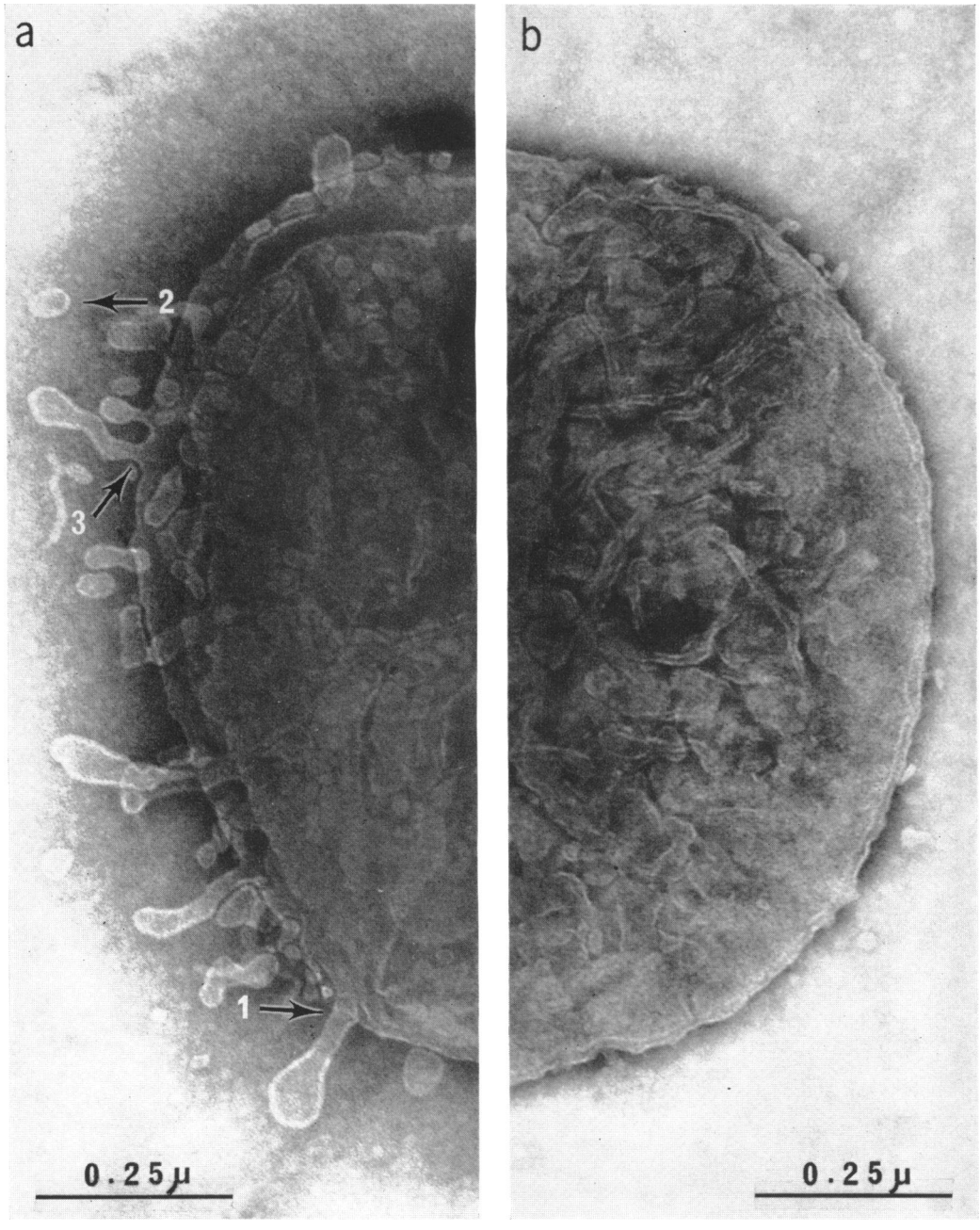

FIG. 3. *Surface of Escherichia coli after frozen-state microtomy and osmotic shock; negatively stained.* × *110,000.* (*a*) *Wall of a logarithmically growing cell. Fingerlike extrusions of plastic wall material have developed.* (*b*) *Wall of a stationary cell. Only a few, very small extrusions are visible.*

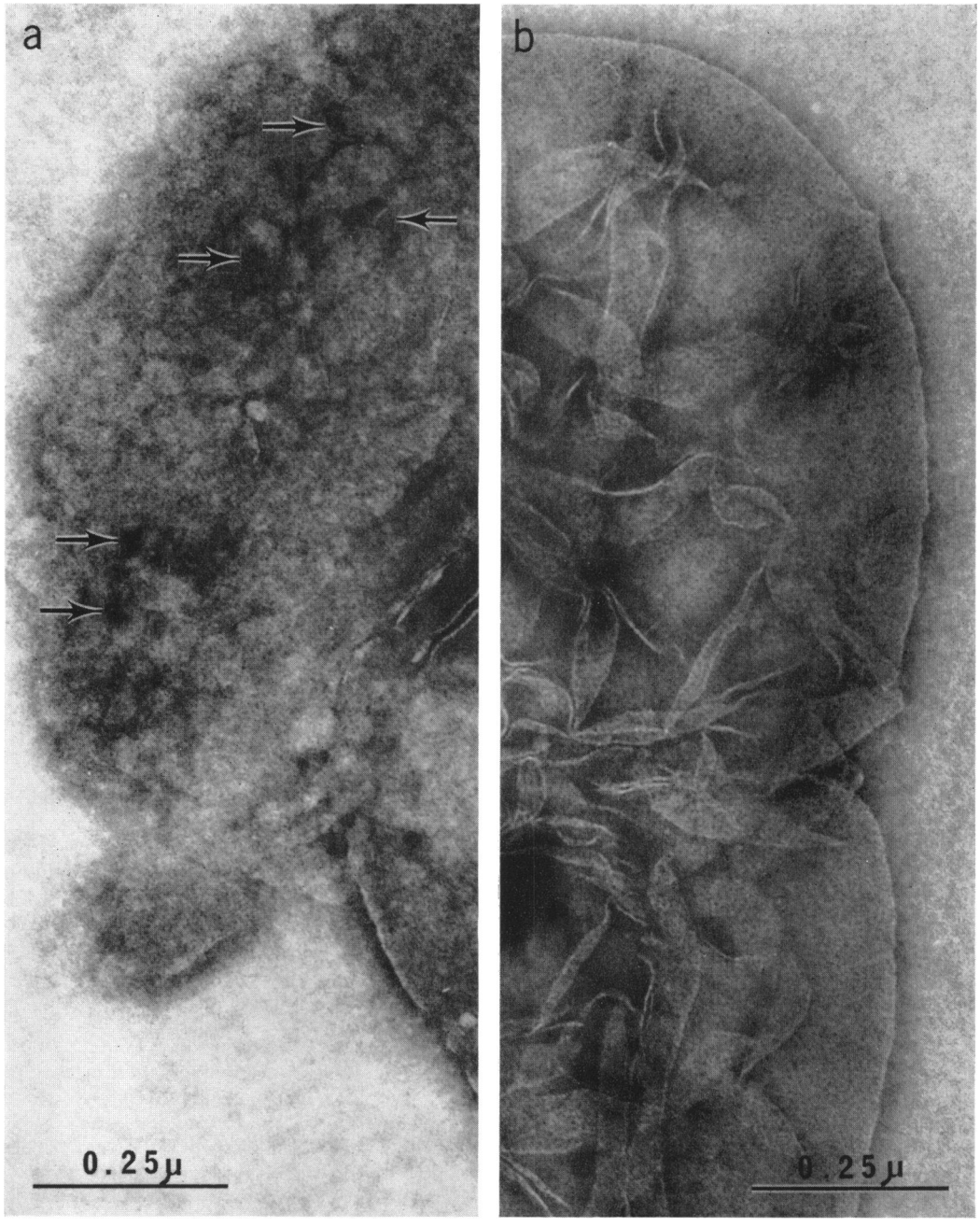

FIG. 4. *Rigid layer of Escherichia coli after frozen-state microtomy and osmotic shock; negatively stained.* × *110,000.* (*a*) *A logarithmically growing cell showing signs of disorganization and an irregular widening of the discontinuities* (*gaps*) *in the wall residue* (*arrows*); *compare with Fig. 2b.* (*b*) *A stationary cell showing very little visible damage of the wall residue. It resembles completely the picture of a rigid layer of an unshocked stationary cell.*

in stationary cells (Fig. 3b) were found less frequently and measured less than 100 A at their bases. It was not possible to distinguish between these extrusions and the small protrusions normally found in various numbers on the surface of the bacterial wall after application of frozen-state microtomy and negative staining (2).

The distribution of the extrusions seemed to be random over the entire cell wall. The protoplasmic membrane appeared as a rather flexible but uniform layer with a slightly grained surface. No holes or pits (4) were visible after the osmotic shock.

When, after an osmotic shock, the lipoid layers of the walls of cells in logarithmic growth were removed with sodium dodecylsulfate, the remaining rigid layer appeared to be structurally disorganized, and the shape of the cell wall was barely recognizable (Fig. 4a). Gaps and holes measuring 200 to 300 A in diameter were abundantly visible. The contour of the rigid layer was not so distinctive as in the unshocked controls, and a much lower negative contrast was obtained, possibly indicating a stretching and extension of the total structure. The rigid layers of stationary cells did not show any visible damage attributable to osmotic shock. The shape of the cell wall was well retained. There were no widened gaps visible, and the proteinaceous particles were evenly distributed over the wall residue (Fig. 4b). The wall was usually collapsed to a structure with many folds. These effects of osmotic shock indicated that the walls of logarithmically growing cells are mechanically weaker than walls of stationary cells. Killing of plasmolized *E. coli* by sudden reduction of the osmolarity of the medium seemed to be caused by disruption of the wall under the increased pressure of the cytoplasmic contents. The discontinuities in the structure of the rigid layer showed an increase in width after the shock, which suggests that bursting of the wall might take place at these locations.

DISCUSSION

Exposure of bacteria to a slowly changing environment allows time for adaptation to the differences in osmotic pressure inside and outside the protoplasm, and complete survival of the culture can be achieved. Sudden changes of the osmotic pressure of the medium cause a loss in viability, even without going beyond the conditions which are suitable for growth (8, 17). It has been demonstrated that sudden exposure of cultures to high solute concentrations of the medium have an effect on the viability (17). The unexpected observation, however, that cells in the stationary phase were somewhat more susceptible to an increase in the osmolarity of the medium than were cells in log phase might be spurious, i.e., caused by a difference in the techniques of increasing osmolarity applied at the two growth stages. Since log-phase cells were suspended directly in the sucrose solution, they could not have been subjected to concentrations above 50%. On the other hand, solid sucrose was added to cell suspensions in the stationary phase, and the "local" osmolarity in the vicinity of dissolving crystals could have greatly exceeded 50% for a short time.

The cultures are most drastically affected in their viability when subjected to an abrupt decrease in osmotic pressure. Our values of 0.1 to 1% survivors in logarithmically growing cultures and of 20 to 60% in stationary cultures are in agreement with other reports (7, 17). According to our results, the killing of *E. coli* after an osmotic shock can mainly be attributed to a bursting of cells, with the following sequence of events. When the cells before the shock treatment are exposed to a concentrated sucrose solution, water flows from inside the protoplast through the protoplasmic membrane to the extraprotoplasmic space. Sucrose, however, cannot pass the "semipermeable" plasma membrane (12); if small amounts should pass the membrane, the rate of uptake would be slow compared with that of water. Because of the initial loss of water, the protoplast will plasmolyze (6). In the subsequent period, the concentration of protoplasmic solutes will gradually increase, as has been shown for various solute systems by Britten (5). A sudden exposure to a medium of very low osmotic pressure causes a rapid inflow of water into the protoplasm and a swelling of the protoplast. Without a mechanical hindrance, this expansion would lead to a larger protoplasmic volume than the rigid cell wall is able to contain; furthermore, the osmotic strength of the highly diluted medium in our shock experiment would not prevent an isolated protoplast from bursting. Thus, the protection against bursting resides entirely in the rigid wall. Depending on the wall's mechanical resistance, it might withstand the pressure, it might swell, or it might be torn apart by the expanding protoplast. [In the following portion of this report, the term "osmotic shock" will be used only for a sudden reduction of the environmental osmolarity from 50% sucrose to less than 3% sucrose (w/v).] It has also been suggested that, during shock, the membrane develops temporary pores through which it releases enough internal solutes to reduce the protoplasmic pressure and eventually "heal" (5).

The release of material from bacteria after

osmotic shock has been used to liberate enzymes from *E. coli*. The cells were osmotically shocked by a transfer from a sucrose concentration of 20% to water. The supernatant fraction then contained most of the cellular alkaline and acid phosphatase, and some of the deoxyribonuclease and acid-soluble nucleotides. The rate of survival after this comparatively "mild" shock was found to be 60 to 90% by C. F. Neu and L. A. Heppel (Federation Proc. **24**:349, 1965); these investigators suggested that the released enzymes are located "near the cell surface."

The actual release of material, as suggested above, seems to be visible in an electron microscope. If one looks at Fig. 3a, one can see a few extrusions which apparently originate from the contour of the protoplasmic membrane (arrow 1). When completely separated from the cell surface, this material forms more or less spherical bodies (Fig. 3a, arrow 2). A cell with only these alterations would probably survive the shock, and the "temporary pores" of the protoplasmic membrane would close up.

When larger extrusions are found, they show the protoplasmic membrane sometimes buckled out into the cavity of the extruding wall, a picture probably resembling the first stage of an explosion of a cell. Further stress on such a site will lead to an irreparable blow-out of cell contents into the surrounding medium.

At other locations in Fig. 3a (arrow 3), it can be noted that portions of the wall are extruded without any apparent participation of material associated with the protoplasmic membrane. In these instances, it can be assumed that increasing pressure in the space between wall and swelling protoplast has pushed out the softer portions of the wall. Whatever type of extrusion and substance release is observed, any expansion or disruption of the wall will occur at zones of the least rigidity. It can be assumed that such zones are to be found in the wall during synthesis, when autolytic enzymes have opened the structure to allow for introduction of new building blocks (10). It has been found that rapidly growing cells with their intense wall synthesis are more sensitive to mechanical injury such as freezing and thawing (19) and high hydrostatic pressure (23). A complication of this interpretation could arise from the possibility that cells in the log phase have a higher osmolarity of their protoplasm than do stationary cells. Reports in this respect are conflicting (5, 9). It has been established that most of the mechanical stability of the wall resides in its rigid layer (16, 21). Larger discontinuities in this layer were observed after autolytic processes had been allowed to develop (20).

In using our technique, we avoided the action of autolytic enzymes, but we could still observe gaps in the rigid layer of logarithmically growing *E. coli*. We regarded the gaps tentatively as sites of wall synthesis (2) where the building blocks were to be inserted. The older structure would prime this reaction and provide for proper orientation and integration of the new molecules; eventually, the gap would be closed, proceeding from its rim to the central portions. Supporting these assumptions were results of studies on the action of penicillin (3), a drug which appears to interfere specifically with the final steps of polymerization of the rigid mucopolymer (18, 22) without affecting previous steps in the wall synthesis (1). When growing cells are being treated with penicillin, the sites of wall synthesis remain uncompleted and contain an increasing amount of mechanically "weak" mucopolymer (1), which will eventually give way to the pressure of the protoplast. Our electron microscopic observations indicated that the weakening of the wall in penicillin-treated cells is accompanied by a considerable increase in the width of the gaps of the rigid layer. The decreasing stability of the walls seemed therefore to be caused by the rapid development of gaps, with incomplete and mechanically less resistant mucopolymer.

The total area of the rigid layer which is covered with "weak" gaps is considerably smaller in logarithmically growing cells than in penicillin-treated cells. Consequently, a considerably greater force has to be applied for disruption "from within" of an untreated growing cell than of a cell after penicillin treatment. Osmotic shocks provide a sufficient pressure gradient to produce this effect in *E. coli*.

In several of our experiments, an osmotic shock was applied to cells that had been cut open and picked up on the electron microscope grid. Many of the cell walls responded to such treatment by forming extrusions (Fig. 3a). This indicates that a pressure is built up between the "interior" of the cell and the environment during osmotic shock, in spite of the fact that the wall and membrane had been cut open by the microtome knife. It seems possible that a rather small opening in the wall would restrict the outflow of the swelling protoplasmic contents, so that, for a short moment, a pressure could build up and extrusions could be formed. It is also possible that the disrupted area of wall and membrane might have adhered to the supporting Formvar film to form a pressure seal.

The viability tests, together with the morphology of bursting walls, seem to support the assumption of mechanically less resistant locations at the

sites of the gaps in the rigid layer. Further evidence in this direction is derived from the behavior of the lipoid layers of the wall, which cover the rigid layer. In walls weakened by penicillin as well as in walls disrupted by osmotic shock, we found portions of the outer layers being pushed into the environment of the cell, forming hernialike or fingerlike extrusions. In either experiment, the diameters at the places of extrusion correspond to the dimensions of the widened gaps and holes in the rigid layer underneath. The appearance of extrusions of the outer layers seems to indicate that the extruded wall material is extremely plastic, a fact which is in agreement with reports on the properties of lipopolysaccharides and lipoproteins of gram-negative bacteria (15, 21).

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