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Production and Ultrastructure of Lysozyme and Ethylenediaminetetraacetate-Lysozyme Spheroplasts of *Escherichia coli*[1]

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ABSTRACT

Spheroplast production by lysozyme and ethylenediaminetetraacetate (EDTA) was examined as a means of obtaining osmotically sensitive cells for studies of enzyme localization. Physiologically young cells plasmolyzed with 0.5 M sucrose in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7, 8, or 9) were quantitatively converted to plasmolyzed osmotically sensitive rods after lysozyme treatment. Although such cells were osmotically sensitive, a 1:1 dilution in Tris buffer was necessary for conversion of rods into spheroplasts. Addition of EDTA resulted in a rapid conversion of the plasmolyzed spheroplasts into spherical structures devoid of a plasmolysis vacuole. These structures, which we call EDTA-lysozyme spheroplasts, contained a number of attached membranes. We believe that this conversion results from a weakening of the outer trilaminar component of the cell wall by EDTA, resulting in the collapse of the plasmolysis vacuole. Dilution of sucrose below 0.15 M also resulted in the collapse of the plasmolysis vacuole. Both the lysozyme spheroplasts and the EDTA-lysozyme spheroplasts were osmotically sensitive. Thin sections of the EDTA-lysozyme spheroplasts demonstrated that the outer trilaminar component of the cell wall was broken, exposing large areas of the cytoplasmic membrane to the environment.

Spheroplasts of gram-negative bacteria have been and are being used in a variety of different investigations, for example, release of enzymes (5, 15, 19-21), accumulation of metabolites (25), isolation of polyribosomes (7), purification of enzymes (16), and assays of the biological activity of viral nucleic acids (9, 17). The standard technique which has evolved for production of spheroplasts involves treatment of exponential- or stationary-phase cultures with ethylenediaminetetraacetate (EDTA) and lysozyme at an alkaline *p*H in the presence of a stabilizing solute, usually sucrose. The resulting cells are spherical and sensitive to lysis by osmotic shock.

Our interest in spheroplasts has resulted from a desire to control their production prior to undertaking comprehensive studies of enzyme localization in cells of *Escherichia coli*. The present paper describes some parameters for the production of two types of spheroplasts from "physiologically young" *E. coli* B. The first type is produced by the addition of lysozyme alone. These cells are referred to in the text as "lysozyme spheroplasts." The second type are spheroplasts produced by the addition of EDTA to lysozyme spheroplasts. These are referred to as "EDTA-lysozyme spheroplasts." A further aspect of this report details the pattern of spheroplast production as determined by phase and electron microscopy. In this report, the cell wall of *E. coli* is considered to consist of an outer trilaminar component (outer membrane) plus a rigid mucocomplex. Our micrographs, as well as those of many others, seldom resolve the rigid mucocomplex. Murray, Steed, and Elson (18) found a structure, between the outer membrane and the cytoplasmic membrane in cells of *E. coli*, which they identified as the rigid layer. These workers feel that others do not resolve this structure primarily as a result of insufficient staining with a suitable metal salt.

[1] Portions of this report were presented at the 66th Annual Meeting of the American Society for Microbiology, Los Angeles, Calif., 1966.

MATERIALS AND METHODS

Spheroplast formation. E. coli cultured aerobically for 12 hr at 37 C in Fraser and Jerrel's glycerol medium (8) on a rotary shaker (500 rev/min) was inoculated into 500 ml of fresh medium in 2-liter flasks

(1%, v/v) and further incubated with shaking. At given times of incubation, cultures were harvested by centrifugation at room temperature, washed once with an equal volume of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride (*p*H 8.0), and suspended to a final cell density of approximately 5×10^8 cells per milliliter in the same buffer supplemented to contain 0.5 M sucrose. To prepare lysozyme spheroplasts, lysozyme (Sigma Chemical Co., St. Louis, Mo.) was added to a concentration of 20 μg/ml; the cells were incubated at room temperature for 5 to 10 min, and diluted 1:1 with Tris buffer. For preparation of EDTA-lysozyme spheroplasts, EDTA to a final concentration of 10^{-3} M was added to lysozyme spheroplast suspensions. Formation of spheroplasts was complete within 10 to 15 min as determined by osmotic sensitivity and phase-microscopic examination.

Electron microscopy. Cell suspensions were fixed 1 hr in 10% formalin followed by the standard Kellenberger and Ryter osmium fixation procedure (11) and were stained for 1 hr in uranyl acetate prior to dehydration through a graded acetone series and embedding in Vestopal W. Sections were cut with either glass or diamond knives on an LKB Ultrotome, stained with lead citrate (26), and examined in either an Hitachi HU11 or a Zeiss EM9 electron microscope.

Phase microscopy. Unfixed cell suspensions were examined and photographed in a Zeiss Standard GFL phase microscope. At least 500 cells were observed and counted to determine the percentage of spheroplast conversion after lysozyme treatment or the percentage of ghost formation after osmotic shock.

Osmotic sensitivity. Initially, osmotic sensitivity was measured by determining the decrease in absorbancy at 600 mμ after diluting the suspensions 1:5 in glass-distilled water and correcting for the dilution factor. In all later work, the cells were centrifuged from suspension and resuspended in the same volume of glass-distilled water.

Phage adsorption. After equilibration for 10 min in a water bath (37 C), 0.5 ml of cell suspension (approximately 10^8 cells) and 0.5 ml of coliphage T4 (5.7×10^8 per milliliter) were mixed and allowed to react for 10 min at 37 C. Portions (0.1 ml) were diluted into 9.9 ml of precooled buffer of the following composition (3.0 g of KH_2PO_4, 7.0 g of Na_2HPO_4, 4.0 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 10 ml of 1% solution of gelatin, and 1 liter of distilled water) and were immediately centrifuged at 10,000 $\times$ *g* for 5 min. Appropriate dilutions were made of the supernatant fraction and were assayed by the agar overlay technique (1) for enumeration of nonadsorbed phage.

RESULTS

Osmotic sensitivity. Figure 1 demonstrates the osmotic sensitivity of physiologically young *E. coli* B and K-12 W2244 after treatment with lysozyme alone, EDTA alone, or EDTA following lysozyme. Both strains became sensitive to lysis by osmotic shock upon lysozyme treatment and prior to the addition of EDTA, with little further increase in osmotic sensitivity after EDTA was added. EDTA treatment alone also rendered the cells osmotically sensitive; however, the resulting

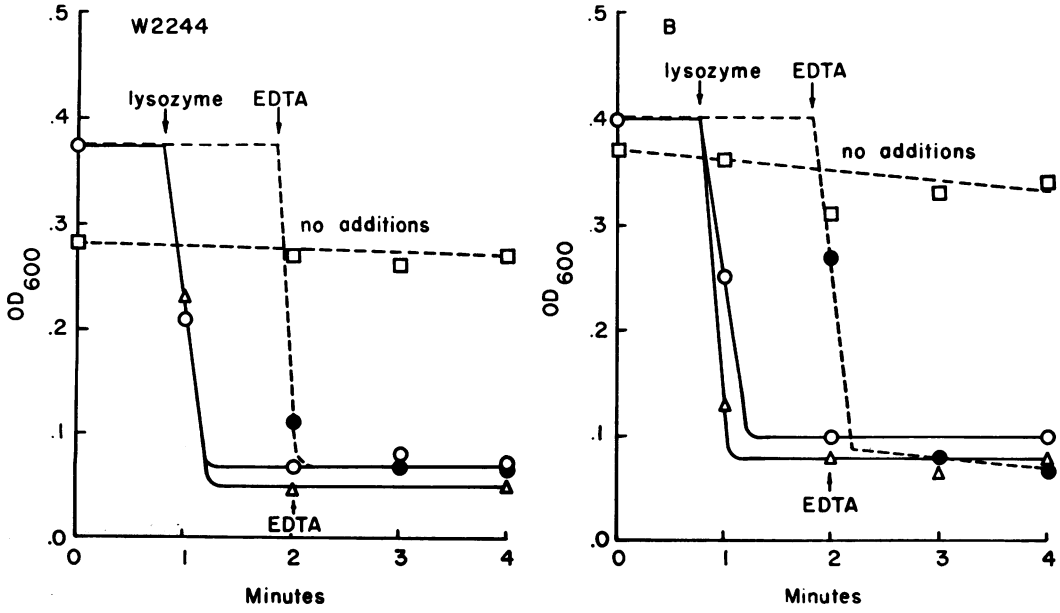

FIG. 1. *Osmotic sensitivity of Escherichia coli B and K-12 W2244. Symbols:* □, *no additions;* ○, *20 μg/ml of lysozyme;* ●, *10^{-3} M EDTA;* △, *EDTA after lysozyme treatment.*

"ghosts" retained the shape of the cell before treatment. Such "ghosts" of rods can also be obtained upon osmotic shock of plasmolyzed cells in the absence of EDTA. This phenomenon will be discussed in a future paper.

Lysozyme spheroplast production. The concentration of sucrose used regulated the extent of plasmolysis. Figure 2 shows that, within limits, spheroplast production and osmotic sensitivity are dependent upon the concentration of sucrose used in the suspending medium. Thus, spheroplast production and osmotic sensitivity are dependent upon the degree of plasmolysis of cells at the time of lysozyme treatment. This suggests that cells must be plasmolyzed before lysozyme can degrade the rigid layer. The relationship between final sucrose concentration and spheroplast production is shown in Fig. 3. As the suspension was diluted, an increasing proportion of the population became spherical until virtually all of the susceptible cells were typical lysozyme spheroplasts. The 1:1 dilution step routinely used was

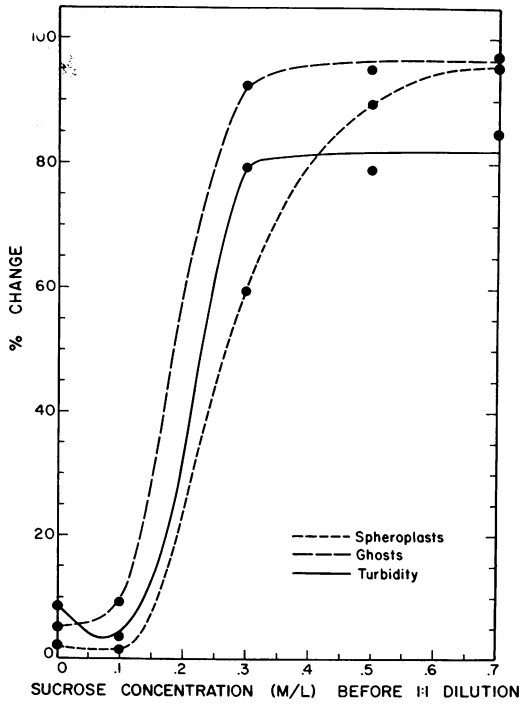

FIG. 2. *Effect of sucrose concentration on spheroplast production. After washing in Tris buffer, equal amounts of cells were suspended in Tris buffer supplemented to contain the sucrose concentrations given. Osmotic sensitivity is reported as per cent decrease in turbidity and per cent "ghost" formation. "Ghost" formation and spheroplast production were measured by phase-microscopic counts.*

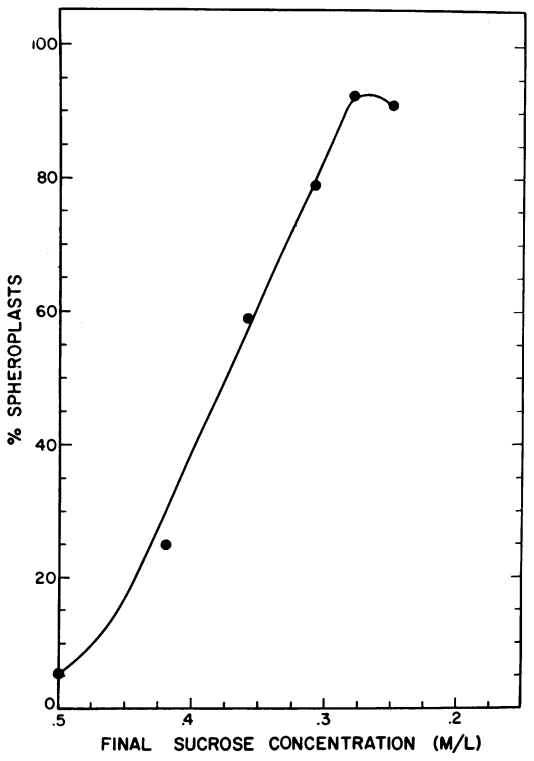

FIG. 3. *Effect of dilution on formation of spherical cells. Samples (5-ml) of lysozyme-treated cells were diluted with 1.0, 2.0, 3.0, 4.0, and 5.0 ml of Tris buffer. Formation of spherical cells was determined by phase-microscopic counts.*

necessary only for formation of spherical cells, not for osmotic sensitivity. Removing the sucrose from lysozyme-treated cells before or after sphering led to complete lysis and the formation of spherical ghosts. Quantitative conversion to lysozyme spheroplasts occurred at *p*H 7, 8, and 9.

Microscopic observations of normal cells. Normal cells of *E. coli* B fixed in the growth medium are shown in Fig. 4a and 4b. The fibrillar nucleoid, particulate cytoplasm, and triple-layered profiles of the outer unit membrane component of the cell wall and of the cytoplasmic membrane are well defined. Also present are membranous proliferations varying from simple vesicles (Fig. 4a) to more complex configurations (Fig. 4b). Internal membranes in cells of *E. coli* have been reported by Ryter and Jacob (24) and by Cota-Robles (J. Ultrastruct. Res., *in press*).

Microscopic observations of plasmolyzed cells. Upon plasmolysis in 0.5 M sucrose, the cells assumed the structural pattern shown in Fig. 5a. The cytoplasmic membrane limits the more condensed cytoplasm, and the convoluted outer

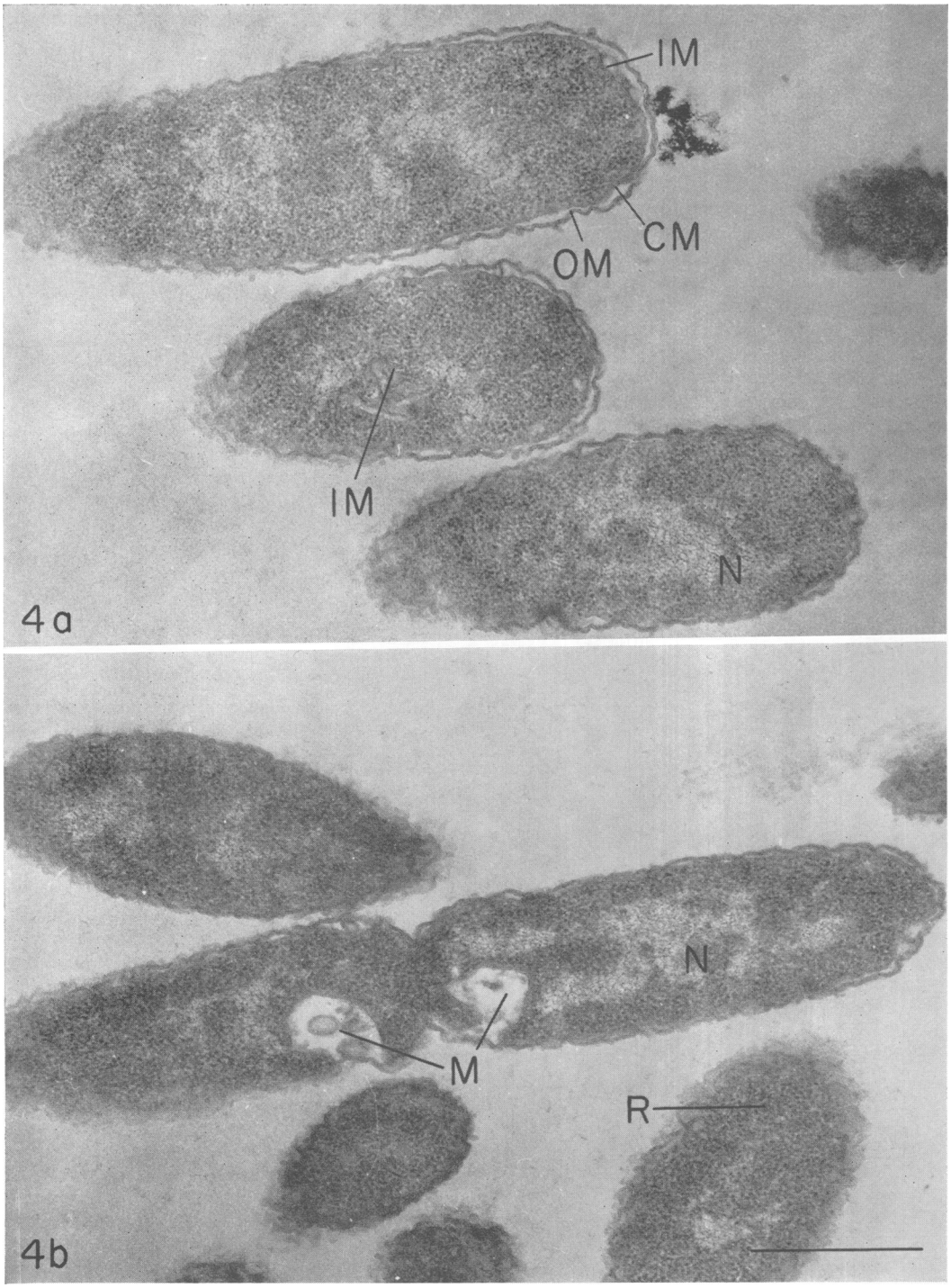

FIG. 4a and 4b. *Thin sections of normal Escherichia coli B. N, nucleoid; OM, outer unit membrane of the cell wall; CM, cytoplasmic membrane; IM, internal membranes; R, ribosomal particles; and M, membranous invaginations. The bar in all electron micrographs represents 0.5 μ. × 49,400.*

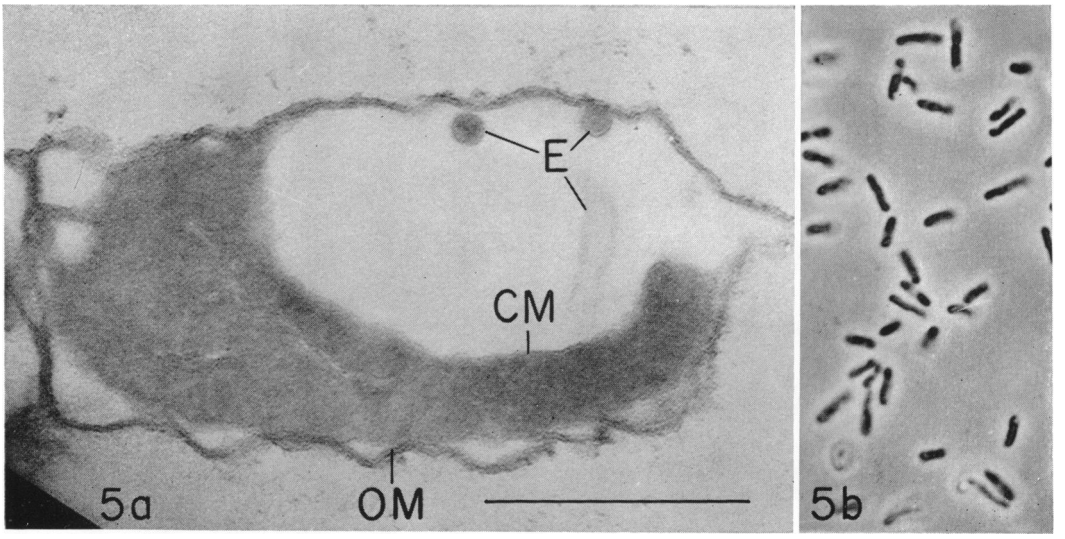

FIG. 5a. *Thin section of Escherichia coli B plasmolyzed in 0.5 M sucrose. E, extensions of the cytoplasm; CM cytoplasmic membrane.* × *67,500.*

FIG. 5b. *Phase micrograph of plasmolyzed E. coli B.* × *1,250.*

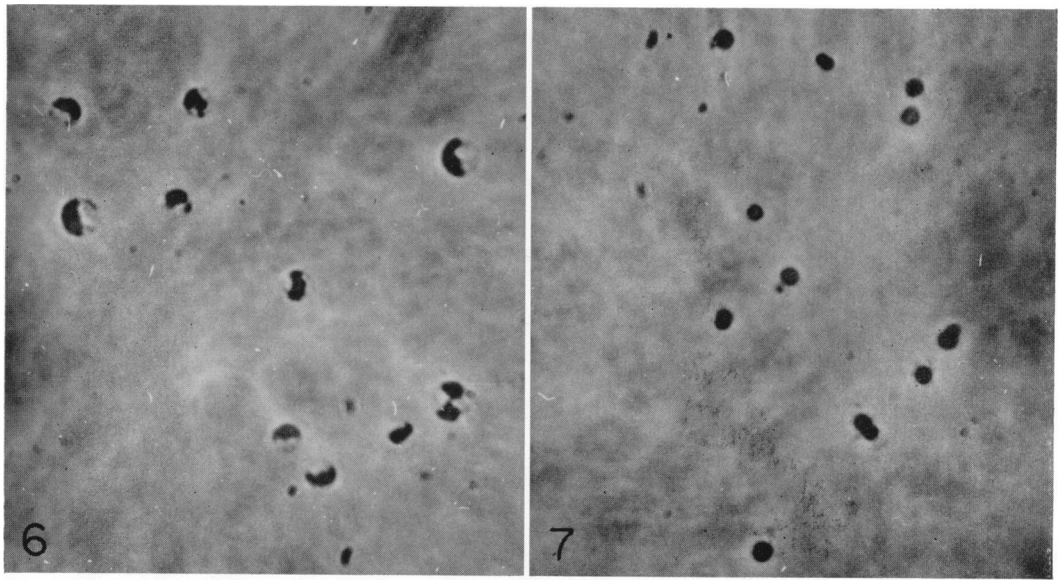

FIG. 6. *Phase micrograph of lysozyme spheroplasts of Escherichia coli B.* × *1,500.*

FIG. 7. *Phase micrograph of EDTA-lysozyme spheroplasts of Escherichia coli B.* × *1,500.*

membrane retains the shape of the cell prior to plasmolysis. The nucleoid has become quite compact, and ribosomes are no longer discernible as defined particles. In all of our sections of plasmolyzed cells, there appear to be areas where the cytoplasmic membrane adheres more firmly to the outer membrane-rigid layer complex. These were frequently observed as extensions of the cytoplasm, terminating at the cell wall. Both longitudinal and cross-sections through such extensions are shown in this micrograph. Figure 5b is a phase micrograph of unfixed plasmolyzed cells in which the plasmolysis vacuoles are clearly visible.

Microscopic observations of lysozyme spheroplasts. The appearance of lysozyme spheroplasts as observed by phase-contrast microscopy is shown in Fig. 6. The cytoplasm is crescent-shaped

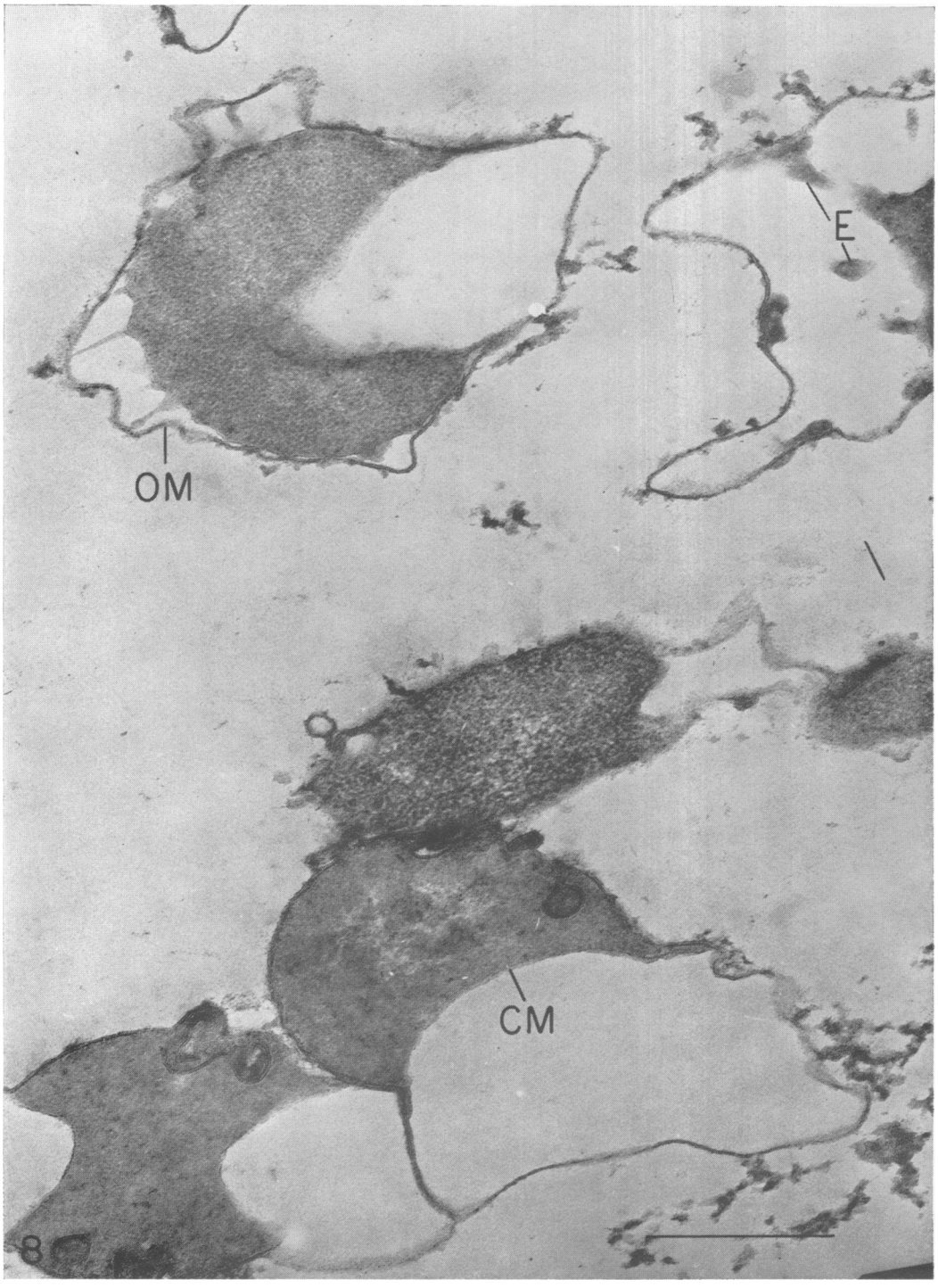

FIG. 8. *Thin sections of lysozyme spheroplasts of Escherichia coli B.* × *51,000.*

with the concavity facing the large plasmolysis vacuole. Within the vacuole are seen dense bodies which appear to be attached to the outer limiting structure and do not appear to be free within the vacuole. In some instances, the dense bodies can be seen to be extensions of the cytoplasm. Lysozyme spheroplasts (Fig. 6) differ from EDTA-lysozyme spheroplasts in that the latter are smaller and lack a plasmolysis vacuole (Fig. 7). Thin sections of lysozyme spheroplasts are shown in Fig. 8. The spheroplasts are bounded by both the outer and cytoplasmic membrane. The cytoplasmic membrane also limits extensions of the cytoplasm. Membranous proliferations are observed within the cytoplasm and may represent an internal membrane system. The nucleoid remains somewhat condensed although ribosomal particles can be resolved.

Microscopic observations of EDTA-lysozyme spheroplasts. Treatment of lysozyme spheroplasts with EDTA results in a rapid, dynamic conversion to EDTA-lysozyme spheroplasts. Phase-microscopic examination of this process reveals that it occurs instantaneously. It is possible to observe this conversion directly by viewing the diffusion boundary of EDTA as it travels through a population of lysozyme spheroplasts. As the EDTA strikes the spheroplast, there is a disappearance of the plasmolysis vacuole, and the cytoplasm assumes a spherical shape. EDTA-lysozyme spheroplasts can be seen to contain attached blebs (Fig. 7). The rapid conversion of lysozyme spheroplasts into EDTA-lysozyme spheroplasts results in a decrease in optical density, as shown in Fig. 9. It can be seen that this decrease in optical density is complete within 30 sec after the addition of EDTA. The spontaneous decrease in optical density in the absence of EDTA is slow, and would require more than 30 min to approach the same level observed after the addition of EDTA. We have designated the spherical structures formed after EDTA treatment of lysozyme spheroplasts as EDTA-lysozyme spheroplasts. However, EDTA is not an absolute requirement for this conversion. A similar change is observed upon mild dilution of lysozyme spheroplasts; however, the effect is not as uniform as with EDTA treatment. We believe that the outer membrane is weakened by EDTA and then ruptured by the osmotic pressure differential. A similar effect could be induced by mild dilution.

The cell shown in Fig. 10 was apparently fixed immediately after rupture of the outer membrane. The cytoplasm has assumed the spherical shape characteristic of EDTA-lysozyme spheroplasts. The ruptured outer membrane has begun to assume a curious configuration. The complexity of these configurations is emphasized in Fig. 11. The complex coils of the ruptured outer membrane would correspond to the blebs observable by phase microscopy (Fig. 7). It is abundantly clear that a single membrane, which must be the cytoplasmic membrane, is the outermost boundary between the cytoplasm and the environment over large areas of the spheroplast surface. The ruptured outer membrane remains attached to the cell, albeit to a small area of the surface.

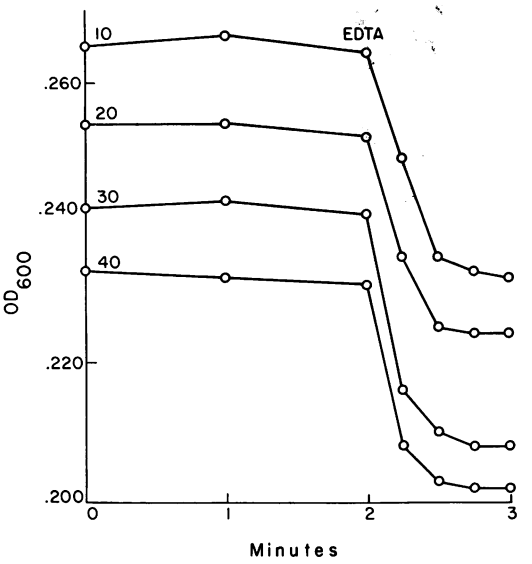

FIG. 9. *Effect of EDTA on lysozyme spheroplasts. EDTA to a final concentration of 10^{-3} M was added to a suspension of lysozyme spheroplasts at 2 min as indicated. The decrease in optical density was measured in a Zeiss PMQ II spectrophotometer at 600 mμ. The numbers above the curves represent minutes after the start of the experiment and demonstrate that a slow decrease in optical density occurs with time of incubation.*

Phage adsorption studies. The pronounced effect which the various treatments related to spheroplast production have on the adsorption of coliphage T4 to *E. coli* B is shown in Table 1. Lysozyme spheroplasts adsorb significantly less T4 than washed cells. However, the capacity of EDTA-lysozyme spheroplasts to adsorb T4 is virtually one-fifth of that of the untreated cell and only one-third of the capacity of lysozyme spheroplasts. We have shown that EDTA induces the rupture and subsequent coiling of the outer unit membrane component of the cell wall. The receptor sites for coliphage T4 are known to reside in this structure (28). Less effective adsorption of T4 to EDTA-lysozyme spheroplasts cannot be attributed to the presence of EDTA in the

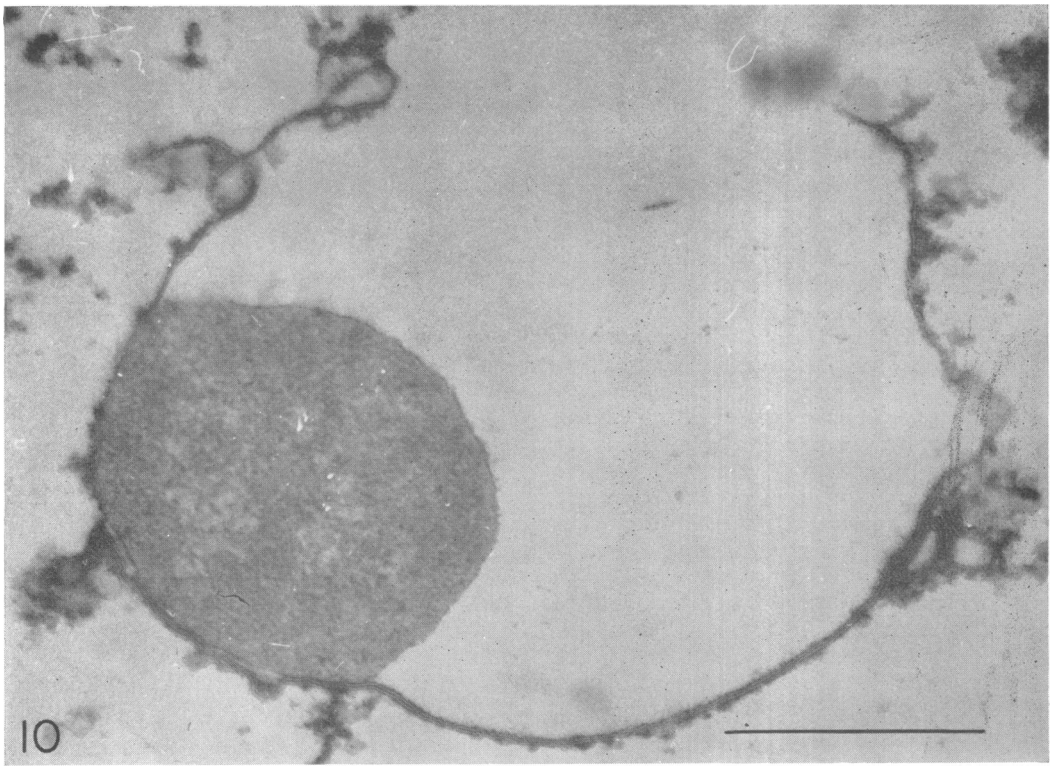

FIG. 10. *Thin section of EDTA-treated lysozyme spheroplast. Note the break in the outer membrane.* × *68,400.*

system, since EDTA alone does not influence adsorption of T4 (Table 1). Protass and Korn (23) recently reported that EDTA treatment of *E. coli* does not remove the receptor sites of T4 while removing the receptor sites of λ vir and phage 434. Thus, we must conclude that T4 cannot adsorb as effectively to EDTA-lysozyme spheroplasts as it does to whole cells, because the receptor sites are not available even though they are present. Coiling of the outer membrane must mask the receptor sites. The decreased ability of lysozyme spheroplasts to adsorb T4 is significant, but may merely be a reflection of the lysis of some spheroplasts.

DISCUSSION

A variety of techniques [enumerated by Noller and Hartsell (22)] have been utilized to render gram-negative bacteria susceptible to lysis by lysozyme. All of these alter either structurally or chemically the outer membrane exposing the rigid layer to enzymatic degradation. We find that plasmolysis of *E. coli*, harvested during exponential phase but particularly when "physiologically young" (late lag or early exponential phase), enables lysozyme to degrade the rigid layer at *p*H 7, 8, or 9. The resulting spheroplasts resemble the crescent-shaped cells produced by penicillin treatment (12), diaminopimelic acid deprivation (14), or D-amino acid treatment (6), without the increase in size observable in spheroplasts produced by metabolic manipulation.

Figure 12 is a diagrammatic representation of spheroplast formation and osmotic sensitivity of physiologically young cells of *E. coli* B. Cells harvested in early exponential phase, washed in Tris buffer, plasmolyzed in 0.5 M sucrose, and treated with lysozyme retain their rod shape, although they are susceptible to lysis by osmotic shock. The 1:1 dilution step appears necessary to decrease the external osmotic pressure below that within the cell to allow uptake of sufficient water to produce the spherical form. The increased pressure may be required to overcome residual binding of the rigid layer to adjacent polysaccharide chains, or to the membranes within which it is apparently sandwiched (18), or perhaps to overcome rigidity not attributable to the mucocomplex.

Evidence is accumulating which indicates that divalent cations have an important role in maintenance of the lipopolysaccharide of the outer

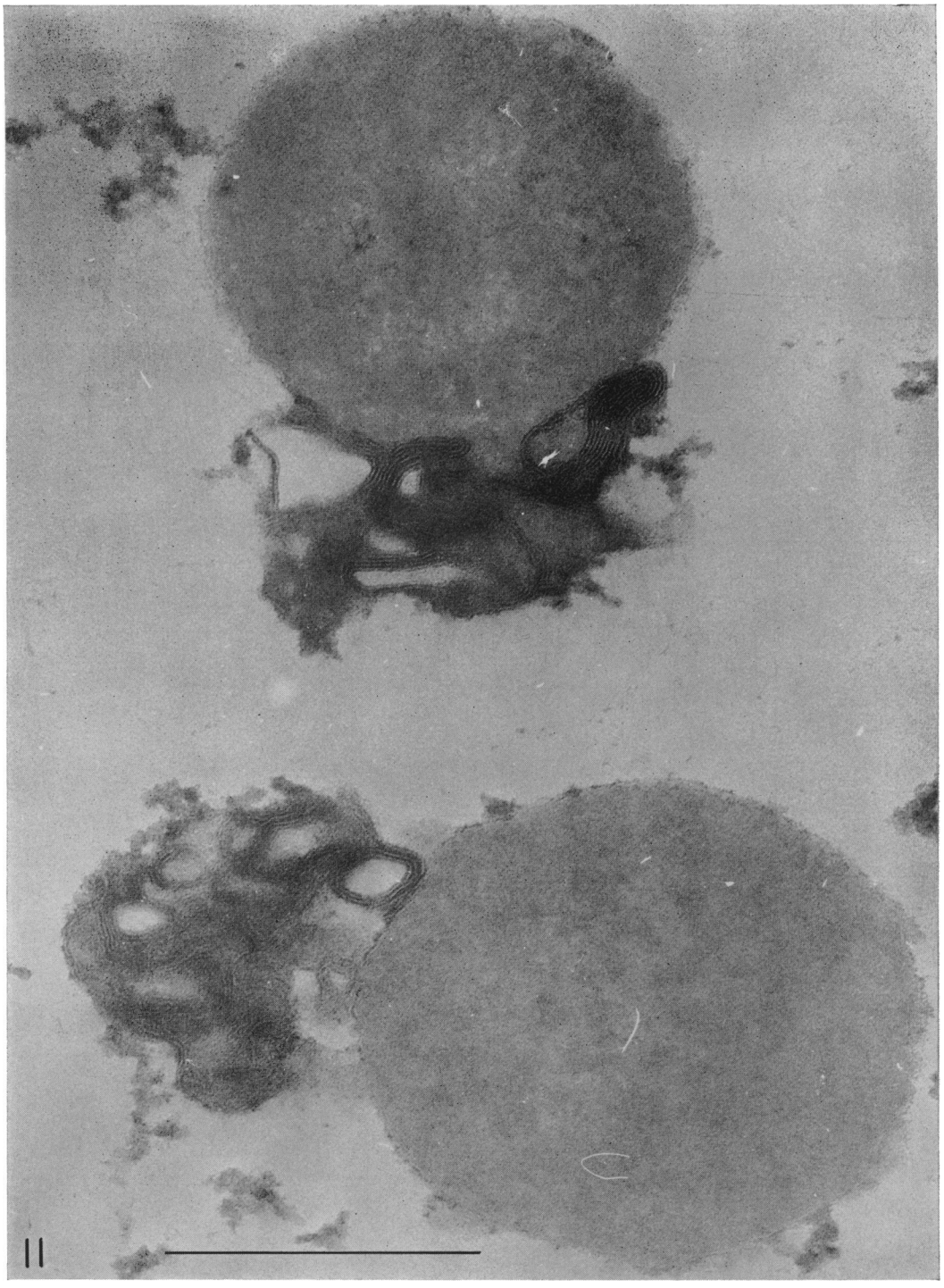

FIG. 11. *Thin section of EDTA-lysozyme spheroplasts. Note the coils of outer membrane and the presence of a single (cytoplasmic) membrane bounding the cytoplasm.* × *95,000.*

membrane of gram-negative bacteria (2, 13). Leive (13) in particular has clearly shown that EDTA treatment of *E. coli* results in a release of cell wall lipopolysaccharide. Carson and Eagon (4) reported that lysozyme-treated *Pseudomonas aeruginosa* retained their rod shape. Similar observations were reported by Voss (27) and by Asbell and Eagon (2). Asbell and Eagon proposed the term osmoplast to describe osmotically fragile rods such as those mentioned in this paper. It is conceivable that formation of spheroplasts after lysozyme treatment has been overlooked in some investigations as a result of the maintenance of rod morphology after lysozyme degradation. The conversion of osmotically sensitive rods into osmotically sensitive spheres after lysozyme treatment requires dilution of the stabilizing solute. Regardless of the sucrose concentration in which we have prepared lysozyme spheroplasts, dilution is necessary to obtain spherical forms. Thus, it seems likely that an abrupt osmotic imbalance must be introduced before the rod can be converted into a sphere.

TABLE 1. *Adsorption of coliphage T4 to spheroplasts of Escherichia coli*

Expt 1[a]		Expt 2[b]	
Sample	Phage removed	Sample	Phage removed
	%		%
Washed once	93.2	Washed once	98.9
		Washed once + EDTA	96.9
Lysozyme spheroplasts	68.2	Lysozyme spheroplasts	84.4
EDTA-lysozyme spheroplasts	18.0	EDTA-lysozyme spheroplasts	34.2

[a] Reaction carried out for 3 min.
[b] Reaction carried out for 10 min.

Treatment of lysozyme spheroplasts with EDTA weakens the outer membrane, permitting rupture of this membrane by the differential between internal and external pressure. The broken membrane coils upon itself and exposes the cytoplasmic membrane to the environment. Dilution of lysozyme spheroplast suspensions below a critical level could permit the pressure differential to induce the rupture of the outer membrane in the absence of EDTA. The resulting cells after either treatment have a spherical profile in which the outer membrane remains attached in a highly coiled configuration. Hofschneider (10) observed a single membrane limiting some of the cells in a suspension of *E. coli* treated with EDTA and lysozyme. He suggested that this was the cytoplasmic membrane. Our results substantiate his findings. Brenner et al. (3) suggested the restriction of the term "protoplast" to those cells in which there is good reason to believe that all cell wall components are absent. It must be recognized that our knowledge of what comprises the cell wall is limited. If the wall includes both the outer membrane and the rigid layer, then the EDTA-lysozyme treated cells described in this work are not protoplasts. On the other hand, if the wall refers only to the rigid layer, such cells are indeed protoplasts. In any case, our results indicate that the rupture of the outer membrane exposes large areas of the cytoplasmic membrane to the environment, and thus EDTA-lysozyme treated cells are truly "functional" protoplasts. The fact that the cytoplasmic membrane of EDTA-lysozyme spheroplasts is extensively ex-

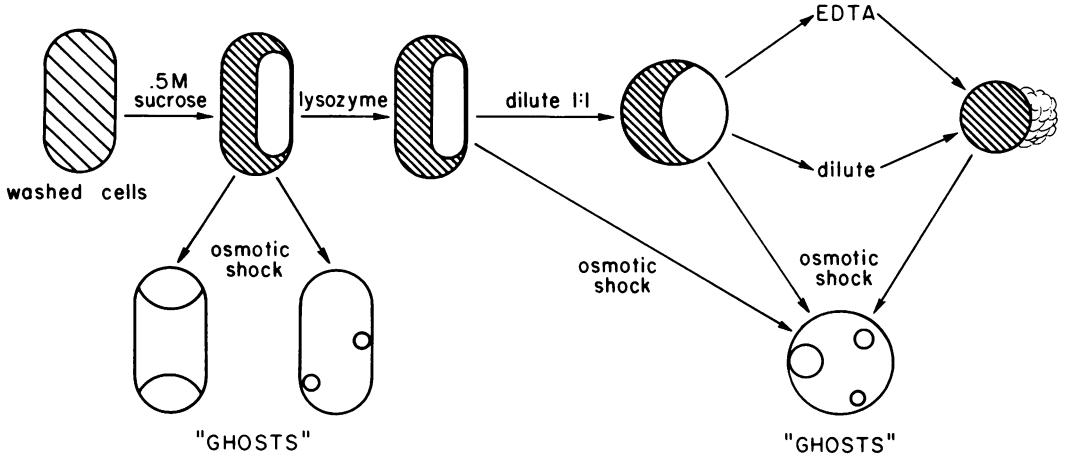

FIG. 12. *Schematic representation of spheroplast formation and osmotic sensitivity of Escherichia coli B.*

posed makes it less difficult to understand the utility of such spheroplasts as acceptors of free viral nucleic acids.

ACKNOWLEDGMENTS

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