Autolytic Mechanism for Spheroplast Formation in *Bacillus cereus* and *Escherichia coli*

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ABSTRACT

MOHAN, RAAM R. (Warner-Lambert Research Institute, Morris Plains, N.J.), DONALD P. KRONISH, ROLAND S. PIANOTTI, RAY L. EPSTEIN, AND BENJAMIN S. SCHWARTZ. Autolytic mechanism for spheroplast formation in *Bacillus cereus* and *Escherichia coli*. J. Bacteriol. 90:1355-1364. 1965.—Spheroplasts of *Bacillus cereus* strain T and *Escherichia coli* B were prepared by incubating early log-phase cells in appropriate buffers and stabilizers for 3 hr at 30 and 37 C, respectively. Upon incubation in 0.05 M tris(hydroxymethyl)aminomethane buffer osmotically stabilized with 16% polyethylene glycol at pH 7.5, 99% of the *B. cereus* cells formed spheroplasts; 90% of the *E. coli* cells were converted to spheroplasts in 0.4 M sodium acetate buffer osmotically stabilized with 1.6 M sucrose at pH 6.0. The extent of spheroplast formation was determined by phase-contrast microscopic examination, by measuring the rate of fall of optical density in the reaction mixture when subjected to osmotic shock, and by viable intact cell counts. The effect of a selected group of metabolic inhibitors on the autolytic system of *B. cereus* and *E. coli* has been examined. *B. cereus* and *E. coli* wall components comprising 26% of the dry weight of the original cellular material were recovered from dialyzed fractions by precipitation in 70% ethyl alcohol. Chemical and chromatographic analysis of cell-wall hydrolysates from *B. cereus* and *E. coli* indicated the presence of glucosamine, alanine, lysine, glycine, aspartic acid, diaminopimelic acid, glutamic acid, and muramic acid.

Spheroplast formation has been described in a wide variety of bacterial species (McQuillen, 1960), some fungi (Bachman and Bonner, 1959), and in at least one instance in higher plants (Cocking, 1960).

Methods for the physical, chemical, and enzymatic preparation of spheroplasts from both gram-positive and gram-negative bacteria have been discussed in reviews by Salton (1961) and Work (1961). Relatively little information, however, is available on an autolytic mechanism of spheroplast production. Nomura and Hosoda (1956) isolated an "autolysin" by $(\text{NH}_4)_2\text{SO}_4$ precipitation of autolysates of *Bacillus subtilis* strain H, which caused lysis of *B. subtilis* and *B. megaterium* but did not give rise to stable spheroplasts. Dark and Strange (1957) isolated an enzyme from sporulating cells of *B. cereus* which was capable of rapidly and quantitatively converting cells of the same species to spheroplasts. A lytic factor induced by ultraviolet irradiation of *B. cereus* 569 was reported by Csuzi and Kramer (1962) to induce lysis in *B.*

cereus 130. The material, a protein, was precipitated by $(\text{NH}_4)_2\text{SO}_4$, was inactivated by heat or trypsin treatment, and was irreversibly denatured at pH 4.0. In an osmotically stabilized system, Mitchell and Moyle (1957) showed that, under certain conditions in the absence of lysozyme, suspensions of *Staphylococcus aureus* became osmotically sensitive, presumably by autodigestion of the cell wall, but they did not examine the characteristics of the spheroplasts produced or the optimal conditions required for their production. Kato et al. (1962) isolated a lytic factor designated L₁₁ enzyme from culture supernatant liquids of *Flavobacterium* sp., which exhibited lytic activity against intact cells and isolated cell walls of *S. aureus* and *Micrococcus lysodeikticus*.

The principal advantage of an autolytic system for spheroplast production and recovery of degraded cell wall lies in the elimination of any requirements for extraneous supplements, with the concomitant contamination of isolates.

The present report describes an autolytic system operative in *B. cereus* strain T and *Escherichia coli* B, optimal conditions for the production of spheroplasts, and qualitative characterization of

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recovered degraded cell-wall material. The present work was reported in preliminary form by Kronish et al. (Bacteriol. Proc., p. 63, 1960).

MATERIALS AND METHODS

B. cereus T used in these studies was obtained from H. O. Halvorson, University of Wisconsin. *E. coli* B was obtained from R. McDonald, Cornell University.

Growth of cells. *B. cereus* was grown in the salts-glucose-yeast extract G medium of Stewart and Halvorson (1953). In later experiments, a water-dialyzed pancreatic digest of casein (Casitone, Difco) replaced yeast extract. Cells were routinely grown overnight on a water-bath rotary shaker at 30 C, washed once in sterile distilled water, and inoculated in fresh medium to give an optical density of 0.05 at 660 m μ (Bausch & Lomb Spectronic-20 colorimeter).

Cultures were incubated as above and grown to an optical density of 0.60 except where indicated. Cells were harvested by centrifugation at 5,000 $\times$ *g* and were washed once with distilled water. *E. coli* B was grown in the synthetic glucose-salts medium of Davis and Mingioli (1950) at 37 C on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) for 18 hr. A 3% inoculum was made to fresh medium and was incubated at 37 C on a rotary shaker. Cells were harvested at an optical density of 0.20 to 0.25 at 660 m μ and washed once in 0.2 M NaHCO₃. Cell pastes of both organisms were examined for lytic activity and spheroplast formation.

Lysis and spheroplast formation. *B. cereus* was suspended in 0.05 M tris(hydroxymethyl)amino-methane (Tris) buffer (Sigma Chemical Co., St. Louis, Mo.), pH 7.5, and was adjusted to an optical density of 0.70 at 660 m μ . Lysis of the cells was recorded during incubation at 30 C by measuring fall in optical density at 660 m μ . Spheroplasts were formed by suspending the cell paste in Tris buffer as above, containing 16% (w/v) polyethylene glycol 4000 (PEG; Union Carbide Chemicals Div., New York, N.Y.). Lagerwerff, Ogata, and Eagle (1961) have discussed the osmotic properties of PEG. *E. coli* was suspended in 0.4 M sodium acetate buffer at pH 6.0 and was incubated at 37 C. Lysis was determined by measuring fall in optical density at 660 m μ . The spheroplast-forming system contained 1.6 M sucrose and 1.0 mg/ml of MgSO₄, in addition to sodium acetate as a stabilizing agent against osmotic lysis.

Mechanical preparation of cell walls. *B. cereus* and *E. coli* were grown in 10-liter batches in a New Brunswick variable-drive fermentor (New Brunswick Scientific Co.). Cultures were agitated by horizontal impeller blades rotating at 250 rev/min and were aerated at 0.2 liters per min through a disc-type sintered stainless-steel sparger. The cells were harvested in a Lourdes continuous-flow centrifuge (Lourdes Instrument Corp., Brooklyn, N.Y.) at 10,000 $\times$ *g* with a flow rate of approximately 200 ml/min. Pellets were washed once,

suspended in distilled water (100 ml), and disintegrated at 10 C in a Raytheon 10-kc sonic oscillator for 15 min. The disintegrated cell mass was centrifuged at 10,000 $\times$ *g* for 90 min, and the top white fluffy layer was carefully removed from the pellet. After three additional washings in distilled water at 10,000 $\times$ *g*, the fluffy layer was treated with deoxynuclease and ribonuclease at 40 C for 90 min. The residual pellet was then washed eight times in distilled water. All operations except digestion were carried out at 4 C. The final washed pellet contained white fluffy cell-wall particulate material. In later experiments, equivalent yields of cell wall were obtained by placing the cell mass in an Eppenbach colloid mill (model QV-6; Gifford-Wood Co., Hudson, N.Y.) together with 150 ml of distilled water and 90 ml of acid-washed 120- μ Superbrite glass beads (Minnesota Mining and Manufacturing Co., St. Paul, Minn.). The colloid mill was run for 5 min at 17 C with a rotor-stator gap setting of 0.030 inches, according to the procedure of Garver and Epstein (1959). Subsequent washing procedures and deoxynuclease and ribonuclease treatments were identical to those described above. Isolated cell walls prepared by both methods were suspended in Tris or acetate buffers and adjusted to an optical density of approximately 0.40 at 660 m μ . Autolysis of the mechanically prepared cell walls from *B. cereus* and *E. coli* was determined by measuring the decrease in the optical density of the suspensions during incubation at 30 and 37 C, respectively.

Isolation of enzymatically degraded cell wall from the spheroplast-forming system. After incubation of whole cells (until spheroplast formation was complete), the spheroplast-forming system was centrifuged at 10,000 $\times$ *g* for 30 min to remove whole cells, undegraded cell wall, and debris. Cell-free supernatant fractions were made 70% with respect to ethyl alcohol at -10 C. (Ethyl alcohol was cooled to -76 C before addition to prevent local heating and denaturation.) After standing overnight at 4 C, precipitates were centrifuged at 5,000 $\times$ *g* and washed twice with cold 70% ethyl alcohol. The resultant pellet was water-soluble (with the exception of a very slight residue), and was hydrolyzed in sealed ampoules in 4 N HCl at 121 C for 4 hr. Acid was removed by repeated evaporation to dryness prior to chromatography. Glucosamine was estimated according to the method of Rondle and Morgan (1955). Muramic acid was separated from glucosamine by use of 80% pyridine as the first solvent, followed by *n*-butanol-acetic acid-water (68:15:30, v/v) in the second dimension; its identity was established by comparison with the *R_F* value of an authentic sample. Amino acid composition of hydrolysates was determined by two-dimensional paper chromatography on Whatman no. 1 filter paper in *n*-butanol-acetic acid-water (55:15:30, v/v) in the first dimension followed by 1 M ammonium acetate (pH 7.5)-ethyl alcohol (3:7, v/v) or 80% pyridine in the second direction. Amino acids and amino

sugars were detected by spraying papers with 0.2% ninhydrin aerosol in acetone.

RESULTS

Washed log-phase cells of *B. cereus* or *E. coli* suspended in the autolytic system underwent rapid lysis at optimal buffer molarity and pH. Figure 1 shows the effect of buffer concentrations on the rate of lysis of *B. cereus* in Tris buffer (pH 7.6) and *E. coli* in acetate buffers (pH 6.0.) The *K* values (decrease in optical density/time $\times 100$) used to express these data were calculated from curves which demonstrated the linear drop in optical density at 660 m μ . Optimal autolytic activity for *B. cereus* was between 0.10 and 0.05 M Tris buffer; for *E. coli*, the optimal autolytic ac-

FIG. 1. Rate of lysis of *Bacillus cereus* and *Escherichia coli* as a function of buffer concentration. Cell suspensions were prepared as described in the text. *K* values were calculated as described in the text from curves which showed linear decrease in optical density.

FIG. 2. Rate of lysis of *Bacillus cereus* in 0.05 M Tris buffer and *Escherichia coli* in 0.4 M sodium acetate buffer as a function of pH. Other conditions as in Fig. 1.

FIG. 3. Arrhenius plot of rate of lysis of *Bacillus cereus* and *Escherichia coli*. The numbers in parenthesis represent temperature in degrees centigrade. Deviation from linearity takes place only above 45 C.

tivity was between 0.2 and 0.4 M sodium acetate. At levels of sodium acetate below the optimum, lytic activity declined sharply; at 0.05 M acetate, autolytic activation was no greater than in controls.

The rate of lysis as a function of the pH of the lytic system is shown in Fig. 2. *B. cereus* suspended in 0.05 M Tris buffer showed lytic activity between pH 5.5 and 8.5, with optimal activity at pH 7.5. The *E. coli* lytic system showed optimal lytic activity at pH 6.0.

The effect of temperature on the rate of autolysis of *B. cereus* and *E. coli* was studied from 19 to 52 C. The results are plotted in Fig 3 in the conventional Arrhenius manner (log *K* versus 1/*T*), and show deviation from linearity only above 45 C.

The rate of lysis of both organisms was also dependent on the age of the cells at the time of harvest. Figure 4 shows the rate of decrease in optical density of *B. cereus* in the autolytic system after harvest at several different densities during logarithmic growth. Under optimal concentrations of buffer and pH, the maximal autolytic activity was observed after harvest of cells allowed to grow to an optical density of 0.30 (660 m μ). As growth density increased, the rate of autolysis decreased through an optical density of 0.80, although in every case lysis was eventually complete. The rate of lysis was also dependent on the medium in which cells were grown. *B. cereus* grown in nutrient broth (Difco) underwent more rapid lysis than did cells grown in G medium, although in the former spheroplast formation was irregular, as described below. Our strain of *B. cereus* grew poorly in Johnson's modification (personal communication) of the medium of Proom and Knight (1955), and ex-

FIG. 4. Rate of lysis of *Bacillus cereus* harvested at different phases of logarithmic growth. Numbers in parentheses represent optical density at 660 $m\mu$ of cells at harvest.

hibited no autolytic activity or spheroplast-forming ability. Attempts to separate factors required for growth of this organism and those required for the production of lytic activity have been only partially successful. An overnight water dialysate of Casitone (Difco) was fractionated by precipitation in cold ethyl alcohol prior to the addition of growth medium. Several graded ethyl alcohol fractions were examined for growth-promoting ability and autolytic activity. It was observed that the growth characteristics of *B. cereus* in G salts medium plus an 80 or 90% ethyl alcohol precipitate of Casitone dialysate were almost identical, but the autolytic activity of cells grown in the 80% ethyl alcohol cut was only one-fourth that of cells grown in a 90% ethyl alcohol cut. Similarly, cells adapted to grow in G salts medium with the addition of 100 $\mu\text{g}/\text{ml}$ of pantothenate, thiamine, aspartic acid, glycylglycine, and unsubstituted purine (Calbiochem) reached an optical density of 0.70 after 16 hr of incubation, but did not undergo lysis when suspended in Tris buffer.

The autolytic mechanism of *E. coli* was found to be independent of medium composition. Equivalent lysis was demonstrable in complex medium as well as in synthetic medium. After several transfers in synthetic medium, rate of lysis was decreased, although in every case it was eventually complete. Normal lytic rates were restored by growth of *E. coli* in complex medium.

The rate of lysis of *B. cereus* and *E. coli* was

FIG. 5. Rate of lysis of *Bacillus cereus* in the presence of 10^{-4} M concentrations of selected metabolic inhibitors.

examined in several different activating systems. Tris and sodium acetate buffers were not interchangeable as autolytic enzyme activators in *B. cereus* and *E. coli*. Citrate and succinate buffers were not as effective autolytic enzyme activators as acetate in *E. coli*. Tris-maleate, glycylglycine, Veronal, and phosphate buffers at several pH values and concentrations were less effective than Tris buffer in the activation of autolytic enzymes of *B. cereus*.

The autolytic systems of *B. cereus* and *E. coli* were not inhibited by 10^{-4} M sodium fluoride, iodoacetate, sodium azide, or 2,4-dinitrophenol. The autolytic system of *E. coli* was inhibited by 10^{-4} M Ag^+ and Hg^{++} . The lysis of *B. cereus* was partially inhibited by the same concentration of PCMB (*p*-chloromercuribenzoic acid, sodium), but that of *E. coli* was not (Fig. 5 and 6).

When 10^{-4} M colistin sulfate (lot CB7710; potency 660 μg of base per mg; 30 times minimal inhibitory concentration) was added to the *B. cereus* lytic system, lysis was completely inhibited. The same amount of colistin sulfate caused a rapid fall in the optical density of an *E. coli* lytic system as the result of release of cytoplasmic components without apparent lysis of cells (Mohan et al., 1963). However, examination

FIG. 6. Rate of lysis of *Escherichia coli* in the presence of 10^{-4} M concentrations of selected metabolic inhibitors.

FIG. 7. Rate of spheroplast formation of *Bacillus cereus* and *Escherichia coli* in osmotically protected systems. DW = distilled water, control. P = osmotically protected system; U = sample of cells from P diluted 1:1 in distilled water.

of this system by phase-contrast microscopy showed complete inhibition of spheroplast formation. This observation was confirmed by Chapman (1962), who observed an increase in cytoplasmic density accompanied by a loss in continuity of cell membrane and cell wall when *E. coli* or *Pseudomonas aeruginosa* was exposed to 10^{-4} M colistin sulfate.

To stabilize rapidly disintegrating spheroplasts in the autolytic system, a series of compounds of high osmotic density were added at zero-time to the incubation mixture; 20% (w/w) PEG was the only material which successfully stabilized forming *B. cereus* spheroplasts. *E. coli* spheroplasts were stabilized in 1.6 M sucrose. Sucrose and PEG were not interchangeable. The rate of spheroplast formation of *B. cereus* and *E. coli* is shown in Fig. 7. Since there is only a small difference in optical densities of spheroplasts and whole cells in osmotically protected media, the rate of spheroplast formation was measured in samples of cell suspension after osmotic shock in distilled water. A similar relationship was observed for *E. coli* in the acetate system protected with 1.6 M sucrose, except that some lysis was observed with whole cells suspended in distilled water. The rate and degree of spheroplast formation was confirmed by phase-microscopic observation. Figure 8 shows phase-contrast photomicrographs of *B. cereus* during spheroplast formation. Spheroplasts were always seen to emerge from the cell-wall casing at one end of the bacterial cell; initial wall breakdown did not take place along the length of the cell (Fig. 8B, C, and D).

In *B. cereus*, emergence of spheroplasts from the bacterial cell was essentially complete in 60 min. After 90 min of incubation, a sample of the spheroplast-forming system which initially contained 10^9 whole cells per milliliter was washed once in distilled water and plated on Tryptose Phosphate Agar (Difco). Approximately 10^2 colonies per milliliter developed after incubation at 37 C for 24 hr. Figure 8D shows an isolated segment of empty cell wall after the extrusion of the spheroplast. On continued incubation, empty cell walls underwent autolysis. Figure 8E shows completely extruded spheroplasts of *B. cereus* osmotically stabilized in PEG. Sequential phase-contrast photomicrographs of spheroplast formation in *E. coli* are shown in Fig. 9. In this organism, the spheroplast formation was somewhat different. After approximately 30 min of incubation in the spheroplast-forming system, *E. coli* appeared to shorten and formed a dumbbell shape at one end (Fig. 9B and C). Spheroplasts did not emerge from the cell-wall casing. The dumbbell increased in size, and, after approximately 1 hr, the cell became an osmotically sensitive sphere (Fig. 9D). These observed differences in the mechanism of spheroplast formation may be the result of differences in complexity of wall structure between gram-positive and gram-negative bacteria.

Lederberg (1956) and Lederberg and St. Clair (1957) reported that the addition of 0.1% MgSO_4 stabilized the "protoplasts" of *E. coli* produced in a medium containing sucrose and penicillin. The

FIG. 8. Phase-contrast photomicrographs of the formation of spheroplasts of *Bacillus cereus*. (A) Normal log-phase diad. (B, C, and D) Stages during spheroplast formation. (E) Empty cell wall. (F) Spheroplasts of *B. cereus*.

addition of MgSO_4 to the *E. coli* spheroplast-forming system resulted in production of stable spheroplasts. MgSO_4 at 4×10^{-3} M was optimal for stability, and gave minimal leakage of intracellular materials. The data in Fig. 10 indicate that higher concentrations (up to 2.4×10^{-2} M) resulted in the decreased stability of spheroplasts without apparent change in the loss of 260 mμ absorbing material.

Mechanically prepared cell walls of *E. coli* showed no autolytic activity when suspended in the activating system. The addition of cell-free supernatant liquid from the spheroplast-forming system did not induce the autolysis of mechanically prepared cell walls, nor did 10^{-4} M adenosine triphosphate or 10^{-4} M cysteine have any stimulatory effect. However, mechanically prepared cell walls of *B. cereus* did undergo lysis when suspended in the activating system. This lysis was followed by loss of turbidity and increase in solubilized glucosamine-containing material (Fig. 11). The autolytic activity was inhibited by 10^{-4} M PCMB, and the inhibition was reversed by the addition of cysteine (Fig. 12).

Autolyzed cell walls of *B. cereus* and *E. coli* were precipitated with ethyl alcohol. The ethyl alcohol precipitates were acid-hydrolyzed, and the chemical composition was determined by two-dimensional paper chromatography. Cell-wall material from *B. cereus* contained glucosamine,

muramic acid, alanine, glycine, lysine, glutamic acid, aspartic acid, and diaminopimelic acid. On the other hand, acid-hydrolyzed degraded cell wall from *E. coli* contained no aspartic acid but did contain serine, threonine, leucine, proline, and three unidentified ninhydrin-positive spots. These data are in accordance with the findings of Cummings and Harris (1956, 1958), Salton (1960), and Work (1957).

DISCUSSION

Autolytic enzymes have been found in a variety of gram-positive and gram-negative bacteria. The data presented in this paper suggest that the enzymatic processes are involved in the formation of spheroplasts of *B. cereus* and *E. coli*. Our attempts to isolate these enzymes have not been successful with either organism. However, spheroplast formation has the characteristics of an enzyme process, i.e., specific pH optima, molarity of buffers, temperature, age of cell, and the effect of SH-group inhibitors. In *B. cereus* there is a linear decrease in the turbidity of a cell suspension accompanied by an increase in free glucosamine from degraded cell wall. Although there was no apparent relationship between glucosamine and optical-density fall in *E. coli* lysis, detectable amounts of glucosamine were found in the soluble supernatant fluid when approximately 90% of the cells were converted to spheroplasts.

FIG. 9. Phase-contrast photomicrographs of the formation of spheroplasts of *Escherichia coli*. (A) Normal log-phase cells. (B and C) Stages during spheroplast formation—note characteristic dumbbell shapes. (D) Spheroplasts of *E. coli*.

FIG. 10. Effect of $MgSO_4$ on spheroplast formation in *Escherichia coli*. Cells were prepared as indicated in the text. Decrease in optical density (solid line) at 260 $m\mu$ is correlated with increased stability (broken line) of spheroplasts as described in the text.

FIG. 12. Inhibition of autolysis of mechanically prepared cell walls of *Bacillus cereus* by PCMB and reversal by cysteine. PCMB and cysteine were added as indicated by arrows.

FIG. 11. Increase in solubilized glucosamine during autolysis of mechanically prepared cell walls of *Bacillus cereus*. Symbols: O, turbidity; X, glucosamine.

The buffer systems described in this paper were designed primarily for the maximal yield of spheroplasts from midlog-phase cells of *B. cereus* and *E. coli*. Young and Spizizen (1963) reported that mechanically prepared cell walls of *B. subtilis* underwent lysis when suspended in phosphate, Tris, or ammonium formate buffers. Of the several buffer systems investigated, Tris buffer was the most effective for the production of spheroplasts of *B. cereus*. Mechanically prepared cell walls of *B. cereus* also underwent lysis when suspended in Tris buffer. However, we were unable to demon-

strate lysis of mechanically prepared cell walls of *B. cereus* when suspended in phosphate buffer. Neu and Heppel (1964) described for the production of *E. coli* spheroplasts a system consisting of sucrose-Tris buffer (pH 8.0), sodium ethylenediaminetetraacetate, and lysozyme. For the production of *E. coli* spheroplasts, our lytic enzyme(s) activating system contained sodium acetate buffer (pH 6.0) and sucrose. This system is free from extraneous supplements such as penicillin or lysozyme, which might interfere with subsequent analyses. We were unable to demonstrate the production of *E. coli* spheroplasts in Tris buffer.

Spheroplast formation in both these systems does not seem to be related to phage-associated lysis. No plaque formation was seen when heavy suspensions of cells were plated during or after the completion of spheroplast formation. No phage particles were observed in electron photomicrographs of isolated cell wall from either *B. cereus* or *E. coli*, and the addition of cell-free supernatant liquid from the spheroplast-forming system to a fresh lytic system containing intact cells did not increase the rate of lysis. Cell-wall breakdown with spheroplast formation occurred only when intact, log-phase cells were exposed to the inducing system. The failure of mechanically prepared *E. coli* cell walls to lyse when suspended in cell-free supernatant fractions of forming system suggests that cell-wall degrading enzyme(s) may be associated with either the membrane layer or with the cytoplasm.

However, it is interesting to note that the mechanically prepared cell walls of *B. cereus* underwent lysis when suspended in Tris buffer (pH 7.5). This observation suggests that the cell-

wall degrading enzyme(s) may be associated with the cell wall of *B. cereus*.

Phase-contrast microscopic examination of *B. cereus* and *E. coli* in the autolytic system revealed two distinct mechanisms for spheroplast formation. A possible mechanism for spheroplast formation in *B. cereus* was discussed by Kronish, Mohan, and Schwarz (1964). Spheroplast formation in *E. coli*, on the other hand, appears to be considerably more complex. Electron-microscopic studies have established that the cell walls of gram-negative bacteria are more complex than those of gram-positive cells. Kellenberger and Ryter (1958) were the first to establish the multi-layered nature of the outer component of *E. coli* cell wall. Independent studies by Weidel, Frank, and Martin (1960) suggest that the mucopeptide component represents the inner layer of the complex cell wall of *E. coli* strain B and that the outermost layer is composed of a protein-lipid-polysaccharide. The data have been reviewed in detail by Salton (1964). The fact that PCMB at 10^{-4} M did not inhibit spheroplast formation in *E. coli*, whereas identical concentrations of other sulfhydryl-binding agents, i.e., AgNO_3 or HgCl_2 , did inhibit spheroplast formation, may be significant. In *E. coli*, the autolytic enzyme(s) may selectively degrade the outer cell-wall layer which is bound through covalent and noncovalent bonds to the inner rigid mucopeptide component (Weibull, 1958; Salton, 1960; Weidel et al. 1960). It is probable, therefore, that the SH groups of the autolytic enzyme(s) are not readily accessible to PCMB, whereas they are to Hg^{++} and Ag^+ .

Our observations on the lysis of *E. coli* by autolytic enzyme(s) led us to speculate that initially the outer layer is separated from the inner layer through hydrolysis of weak covalent bonds, thus exposing the inner mucopeptide layer for further enzymatic degradation. Recent investigations of Weidel, Frank, and Leutgeb (1963) have shown that the inner mucopeptide layer is sensitive to lysozyme and is also degraded by autolytic enzyme(s). Our data on the appearance of detectable amounts of glucosamine in the late stages of *E. coli* spheroplast formation suggest that the cell-wall layers of *E. coli* are sequentially depolymerized by autolytic enzyme(s).

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