BACTERIOLYSIS OF ENTEROBACTERIACEAE

II. Pre- AND CO-LYTIC TREATMENTS POTENTIATING THE ACTION OF LYSOZYME\textsuperscript{1, 2}

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Most gram-negative bacteria are refractory to the lytic action of lysozyme unless the cells have been conditioned for lysis by certain pre-lytic or co-lytic treatments. There are suggestions that the resistance of gram-negative bacteria is conferred by protein which protects the mucopolysaccharide lysozyme substrate (Becker and Hartsell, 1954, 1955; Salton, 1958; Weidel and Primosigh, 1958; Kellenberger and Ryter, 1958) from lysozyme action. It is also possible that the substrate exists in a state of interdispersion with lipoprotein and that it is the lipoprotein-mucopolysaccharide complex that must be dissociated before lysozyme can reach its substrate. The function of pre- and co-lytic treatments which potentiate lysozyme action appears to be one of releasing the lysozyme substrate by degradation of the protective lipoprotein moiety of the cell wall.

The mucopolysaccharide is presently considered to be the wall component that lends rigidity to the gram-negative cell wall (Salton, 1955, 1958). However, Grula and Hartsell (1957) observed that lysozyme degradation of gram-negative bacteria by Nakamura’s technique (Nakamura, 1923) resulted in ghosts possessing a general rodlike appearance that would shrink and swell after alternate addition of acid and alkali. Since lysozyme substrate was presumably absent, it appeared likely that some other type of reticulum (protein?) may also be important in conferring shape upon gram-negative cells. This possibility has also been suggested by Tuttle and Gest (1959).

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During a comparative study of the lytic response of Enterobacteriaceae to four lytic systems utilizing lysozyme (Noller and Hartsell, 1961), there appeared to be a similarity between the mode of action of the pre- and co-lytic treatments that potentiated lysozyme action. The present report is concerned with a comparison of the effects of these treatments and offers evidence for the mechanism by which heat, low pH, butanol, and Versene potentiate lysozyme action. Additional evidence to that of Grula and Hartsell (1957) will also be presented indicating that some component of the cell wall other than mucopolysaccharide is also important in lending rigidity to the gram-negative cell wall.

MATERIALS AND METHODS

Bacterial strains. The following bacterial strains were used in these investigations: Escherichia coli strains 19, U-2, and 4157; Aerobacter aerogenes strain PU-2.

Comparison of heat and acid pretreatments. One suspension of E. coli strain 19 in water at pH 7 was heated for 15 min at 70 C and then cooled to 45 C. A second cell suspension was incubated at 45 C at pH 3.5 for 1 hr. A control suspension was incubated in water at pH 7 for 1 hr at 45 C. After these pretreatments, each of the cell suspensions was tested for its sensitivity to 10 \mu g/ml lysozyme, 20 \mu g/ml trypsin, and 10 \mu g/ml lysozyme plus 20 \mu g/ml trypsin (all at 45 C) in 0.0067 M phosphate buffer (pH 7). The extent of lytic sensitivity of each cell suspension was determined turbidimetrically (Coleman model 14 Universal spectrophotometer).

In a second experiment, a suspension of E. coli strain 19 cells was incubated at 45 C for 30 min at pH 3.5. Control suspensions were incubated under the same conditions except at pH 7. Each suspension was then adjusted to pH 10 with 0.1 \% NaOH. The extent of lysis was determined before and after addition of lysozyme, circulin, Hyamine 1622, or salmine to a concen-
tation of 10 µg/ml. The suspensions that were not acid pretreated were adjusted to pH 3.5 with HCl for 5 sec and readjusted back to pH 10 with alkali. Lysis was noted and followed by addition of lysozyme (10 µg/ml) to those tubes containing circulin, Hyamine, or salmine, and final observations for the degree of lysis were made.

Butanol pretreatment. A suspension of E. coli strain U-2 cells was treated with n-butanol as indicated in Fig. 1. The extent of lysis was noted after alkali addition to pH 10 and after addition of lysozyme at pH 10.

Versene cotreatment. A. aerogenes strain PU-2 and E. coli strain U-2 were used as test species and were exposed in 0.03 M tris [tris(hydroxymethyl)aminomethane] buffer (pH 8) to 135 µg/ml Versene, 20 µg/ml lysozyme, or 10 µg/ml trypsin as single and as combined reagents. The extent of lysis was determined spectrophotometrically during incubation of the system at 45 C.

Butanol, heat, or acid treatment. To evaluate the cell wall disorganizing effects of butanol, heat, or acid treatments by some means other than macroscopic lysis, hydrogen ion uptake studies on control and treated cells were made by a method similar to that described by Gilby and Few (1958). Washed cells of E. coli strains U-2 and 19 and A. aerogenes strain PU-2 were suspended in distilled water at a concentration of 5 mg cells per ml (dry weight basis). Ten-milliliter portions were heated at 70 C for 15 min and immediately cooled to room temperature, treated with 5% v/v n-butanol, or left untreated. These 10-ml portions were titrated potentiometrically at 26 C with 0.5 N HCl. Acid was added to the constantly stirred samples in 0.01-ml aliquots with an Agla micrometer syringe and pH of the suspension was measured 3 min after each addition. Milliequivalents of hydrogen ion bound per gram dry weight of cells were calculated from pH values for distilled water and cell suspensions resulting from each addition of acid.

Cell structures remaining after lysis. The lysis of acid or butanol pretreated cells by alkaline lysozyme was observed with the phase and electron microscope. E. coli strain U-2 suspensions were pretreated by incubation at pH 3.5 for 30 min at 45 C or in the presence of 5% v/v n-butanol at pH 7 for 30 min at 45 C. Cells from these suspensions were dried on cover glasses and observed by phase microscopy during sequential treatment with pH 10.5 alkali, 10 µg/ml lysozyme at pH 10.5, and pH 3.5 acid. Cells were also air-dried to collodion-covered grids and then treated on the grids by the same reagents, followed by observation of the preparations with the electron microscope.

In a second series of experiments, the effect of trypsin digestion of the ghosts remaining following alkaline-lysozyme digestion of acid pretreated cells of E. coli strain 4157 was determined. Residues remaining after treatment of E. coli strain 4157 cells with lysozyme-trypsin-butanol, lysozyme-Versene, or lysozyme-circulin were also subjected to alternate treatment with acid and alkali to determine whether shrinking or swelling of these ghosts occurred.

**RESULTS**

Comparison of heat and acid pretreatments. Cells incubated at 45 C at neutral pH were not lysed by lysozyme or trypsin individually or combined. Heated cells, however, exhibited trypsin sensitivity (Fig. 1) and synergistic lysis by the combination of lysozyme and trypsin. This verifies reports of Becker and Hartsell (1954, 1955). Acid pretreated cells were also lysed markedly by lysozyme or trypsin and were more sensitive to the action of these enzymes than were heated cells. No synergistic action with
Fig. 2. Lysis of *Escherichia coli* strain 19 by lysozyme at pH 10.

Fig. 3. Lysozyme action at pH 10 after pretreatment of *Escherichia coli* strain U-2 cells with 5% n-butanol.

Lysozyme plus trypsin is apparent with acid treated cells. This is undoubtedly due to the high activity of the individual enzymes. These data suggest that neutral heat treatment (70 C) and pH 3.5 treatment (45 C) accomplish similar disorganization of lipoprotein.

From the results presented in Fig 2, it is apparent that the presence of lysozyme at pH 3.5 is not necessary for good lysing (clearing) by alkali. Addition of lysozyme after raising acid treated cells to pH 10 with alkali resulted in immediate clearing comparable in extent to cells treated by Nakamura's technique. Lysozyme appears quite capable of substrate degradation at a pH as high as 10. This lytic effect of lysozyme at pH 10 appears specific since other basic peptides such as salmine or circulin do not produce the effect.

**Butanol pretreatment.** Pretreatment of cells with 5% v/v n-butanol at pH 7 appeared to have
Fig. 6. Effect of alkali plus lysozyme and acid on pH 3.5 pretreated *Escherichia coli* strain U-2. a) pH 3.5 pretreated cells (30 min at 45°C); b and c) same cells at pH 10.5 plus lysozyme for 5 min; d and e) followed by pH 3.5 treatment; f and g) followed by pH 10.5 treatment; h and i) by pH 3.5 treatment.
a comparable effect with acid pretreatment (Fig. 3). Addition of lysozyme following adjustment of the suspension of pretreated cells to pH 10 resulted in immediate and extensive lysis (clearing). Hence, butanol effects appear similar to acid effects and perhaps to those of heat. Similarity between the effects of butanol and heat pretreatments is supported by the fact that lysis curves after heat treatment (Fig. 1) are comparable to those obtained with butanol.
cotreated cells observed in an earlier report (Noller and Hartsell, 1961).

*Versene cotreatment.* The presence of Versene was found to increase both trypsin and lysozyme activity (Fig. 4). While *E. coli* strain U-2 was more sensitive than *A. aerogenes* strain PU-2 to the action of Versene and trypsin or Versene and lysozyme, both organisms were comparably lysed by a combination of all three compounds. The lysis curves again appear similar to those obtained with lysozyme, trypsin, and butanol (Noller and Hartsell, 1961). While metal chelation may influence lytic sensitivity, it appears that the major role of Versene in lysozyme or trypsin potentiation could be due to lipoprotein dissociation.

*Butanol, heat, or acid treatment.* The hydrogen ion uptake curves presented in Fig. 5 were obtained using *A. aerogenes* strain PU-2 cells and are typical of the results obtained with all three organisms. Control cells were observed to bind hydrogen ion slowly until a critical acidity was reached in the vicinity of pH 3. At lower pH, hydrogen ion was bound rapidly with each addition of acid. The low initial uptake represents titration of cell wall groups prior to complete disorganization of the wall and plasma membrane. At pH 3 or less, wall and membrane damage allows penetration of the cytoplasm by protons. Heat treated cells and cells titrated in the presence of n-butanol exhibited marked proton uptake from the beginning of acid titration. The most logical explanation of this result is that heat and butanol cause prior disorganization of the wall and membrane allowing proton uptake by both wall and cytoplasm.

*Cell structures remaining after lysis.* The alternate addition of alkali and acid to acid or butanol...
pretreated cells had no effect on cell morphology. When lysozyme (10 \( \mu g/ml \)) was added to acid pretreated (Fig. 6) or \( n \)-butanol pretreated (Fig. 7) cells under alkaline conditions (pH 10.5), marked swelling occurred with a concomitant loss of refractility. Moreover, the swelling and refractility loss could be reversed by lowering the pH to 3.5 with hydrochloric acid. Alternate addition of alkali and acid caused alternate reversal of the cell residues from a swollen to a shrunken state. This effect was first noted by Grula and Hartsell (1957) with acid pretreated cells. The same effect was observed with butanol pretreated cells. The series of electron micrographs (Fig. 6) indicates that acid treatment confers definite rigidity to the cells since shadows are cast by cells only under acid conditions. Residues under alkaline conditions dried flat to the grid and no shadow was cast.

When the swollen residues from alkaline-lysozyme treatment of acid pretreated cells (Fig. 8) were further treated with trypsin at pH 8, the shrinking and swelling reaction no longer occurred. This effect provides further evidence that the residual structures first noted by Grula and Hartsell (1957) that undergo shrinking and swelling are truly proteinaceous (lipoprotein?).

Cell residues remaining after lysozyme-trypsin-butanol or lysozyme-Versene action also remain largely unchanged by acid or alkali treatment. Residues from lysozyme-circulin action shrink and swell somewhat during alternate changes from acid to alkaline conditions but not to the extent of acid or \( n \)-butanol pretreated cells. Tuttle and Gest (1959) observed that \textit{Rhodospirillum rubrum} loses its spiral form following polymyxin treatment. They suggested that lipoprotein was conferring structural rigidity in the untreated cell. Degradation of lipoprotein by circulin should likewise preclude the shrink-swell effect. Residues from lysozyme and circulin, acid, or \( n \)-butanol treatments were of such low refractibility that photomicrography by phase microscopy was impossible.

**DISCUSSION**

All of the pre- and co-lytic treatments tested appear to be capable of degrading the protective lipoprotein complex to an extent that permits lysozyme penetration. These treatments also potentiate the action of trypsin on the protein components of the cell.

Comparison of the effects of acid and butanol pretreatment indicated that exposure to low pH is not the only cell treatment that permits good lysing of cells by alkali after lysozyme exposure. The prerequisite for alkali lysing is not acid pretreatment alone, but probably any treatment that disorganizes the lipoprotein component of the cell wall. Lysozyme then degrades the unbound substrate and alkali aids in solubilization of the partially degraded cell structures.

It is also apparent that lysozyme is enzymatically active over a wide pH range. Lysozymic action at pH 10 was apparent with acid or butanol pretreated cells. Optimal lysing of \textit{Micrococcus lysodeikticus} cells by lysozyme appears to occur optimally at pH 6.2 (Smolelis and Hartsell, 1952) and some activity on cell walls of \textit{M. lysodeikticus} has been observed at pH 3.5 (Grula and Hartsell, 1954). Hence, lysozyme appears capable of activity over a range of at least pH 3.5 to 10. A relatively broad pH range for lysozyme action has also been reported by Salton (1956). Since lysis of gram-negative cells at pH 10 to 10.5 is as rapid when lysozyme is added after rendering the system alkaline as when lysozyme was present during acid pretreatment, it is possible that lysozyme incubated with cells at pH 3.5 does not have activity until the pH is raised by addition of alkali.

The effect of Versene in lysozyme-potentiation is quite comparable to that of butanol, and, hence, would appear to unmask lysozyme substrate through lipoprotein dissociation rather than through chelation of metals. Repaske (1958) stated that other chelating agents (8-hydroxyquinoline, \( o \)-phenanthroline, or other Versene) were ineffective as co-lytic agents with lysozyme. This observation would appear to support some action of Versene other than that of metal chelation at the cell surface.

The use of hydrogen ion uptake studies on butanol and heat treated cells lends further support to the cell wall-dissociating effects of these treatments. It has been frequently suggested that such disorganization of cell structure could conceivably unmask the lysozyme substrate and allow lysozyme action. While wall disorganization by acid alone does not occur until approximately pH 3 at 26 C, the pH values at which disorganization occurs would probably be higher at 45 C.

None of the lytic treatments studied resulted in spheroplasts when carried out in the presence of sucrose as a stabilizer. It may be presumed that the lytic systems caused marked dissociation
of the plasma membrane. Since the plasma membrane is believed to consist largely of lipoprotein (McQuillen, 1958), it could hardly be expected to withstand the action of pH extremes, lipid solvents, heat, or a highly active surfactant such as Hyamine 1622 or cirkulin. If lysozyme has degraded the mucopolysaccharide in the cell wall during treatment in the presence of alkali, butanol and trypsin, cirkulin, or Versene, then it is extremely interesting to conjecture as to the structure remaining that continues to retain the cell in its usual rodlike morphology. If the mucopolysaccharide has not been degraded to the extent that it no longer confers rigidity to the cell wall, then swelling of the residues would be impossible under alkaline conditions. The implications of the shrinking and swelling phenomenon are important since they indicate that some component of the cell wall other than mucopolysaccharide is also conferring shape to the gram-negative cell. Since shrinking and swelling do not occur following tryptic hydrolysis of the residue, it is apparent that the protein (or lipoprotein) moiety of the cell wall (along with the muco-complex) also contributes to maintenance of cell morphology.

**SUMMARY**

Pretreatment of gram-negative bacteria by heat, low pH, or n-butanol, or cotreatment with butanol or Versene, sensitizes the cells to the lytic action of lysozyme. These treatments also increase subsequent tryptic activity on proteinaceous components of the cell. Turbidimetric and microscopic observation of lytic patterns after such pre- or cotreatments indicate that the mechanism by which sensitization occurs is through dissociation of the lipoprotein components of the cell wall which protect the lysozyme substrate. This conclusion has been further supported by hydrogen ion uptake studies on heat or butanol treated cells. The evidence suggests that Versene potentiates lysozyme and trypsin activity through lipoprotein dissociation although chelation may also be important.

Studies on residual cell structures remaining after lysis of gram-negative bacteria indicate that some cell wall or cytoplasmic component (probably lipoprotein) also confers rigidity to the cell along with mucopolysaccharide.

**REFERENCES**


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