EFFECTS OF LYSOZYME-POTENTIATING TREATMENTS ON ISOLATED CELL WALL MATERIAL OF *ESCHERICHIA COLI*

By

CHARLES DWAYNE EDWARDS

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Thesis Approved:

Eric C. Mallette
Thesis Adviser

Lynn P. Lee

J. H. Boggs
Dean of the Graduate School

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CHAPTER I

INTRODUCTION

The bacterial cell wall is the rigid, water-insoluble structure which shapes and protects the organism. Although the cell walls of gram-negative bacteria represent approximately 15-20% of the dry weight of the whole cell, chemical analyses indicate that it is chemically more complex than the walls of gram-positive bacteria (Salton, 1953, 1960). Walls from gram-positive organisms contain more hexosamine and considerably less total lipid than the walls of gram-negative organisms. A significant difference is also noted in the amino acid content of the two types of organisms. Walls of gram-positive organisms contain hexosamine, alanine, glutamic acid, lysine, and diaminopimelic acid (Work, 1957). Many of the gram-positive bacteria also contain polymers of ribitol phosphate, glycerol phosphate, or ribitol teichoic acids. The teichoic acids are polymers of ribitol phosphate which usually contain a sugar moiety to which alanine is linked as an ester (Armstrong, Baddiley, and Buchanan, 1961). Walls of gram-negative organisms have a full complement of amino acids including aromatic and sulfur containing amino acids (Work, 1961). Both types of organisms contain N-acetylglucosamine and N-acetylmuramic acid (Strange and Dark, 1956).

Kellenberger and Ryter (1958) were able to clearly show that Escherichia coli possessed a multi-layered cell wall. The wall appeared to consist of three layers, two of which were electron dense and

one which was electron transparent. Each layer was approximately 20-30 Å in thickness. Weidel, Frank, and Martin (1960) reported that many of the gram-negative organisms appear to have multi-layered walls. An outer layer can be separated as a pliable lipoprotein coat (phenol-soluble). Just under the outer layer lies the lipopolysaccharide layer (less soluble in phenol) and it is thought to house cellular antigens. The innermost layer is termed the mucopeptide (rigid and insoluble in phenol). The mucopeptide is made up of polymers of N-acetylglucosamine and N-acetylmuramic acid connected by alternating $\beta(1\rightarrow 4)$ and $\beta(1\rightarrow 6$ or $1\rightarrow 3)$ linkages with a pentapeptide attached to the muramic acid. The mucopeptide polymer comprises at least 4% of the cell wall by dry weight with the remainder of the gram-negative cell wall comprised of lipids (1-20%), protein (60-80%), and sugar polymers (Salton, 1961). The mucopeptide forms the rigid backbone component with covalently bonded amino acids and amino sugars. The outer cell wall components appear to be attached to the mucopeptide by weaker linkages (hydrogen-bonding).

In a study using Aerobacter cloacae cell walls to determine the composition of purified mucopeptide (lysozyme substrate), Schocher, Bayley, and Watson (1962) reported molar ratios of glucosamine, muramic acid, alanine, glutamic acid, and diaminopimelic acid (DAP) of 2:2:3:2:2 respectively. It was postulated that two types of peptides existed: one containing two moles of alanine along with glucose and DAP, and one containing only a single alanine residue plus glucose and DAP.

Evidence for the direct action of lysozyme on the cell wall was first reported by Weibull (1953) who observed formation of spherical protoplasts, appreciably devoid of cell wall, following treatment of Bacillus megaterium with lysozyme in sucrose media. Further, Grula and

Hartsell (1954) and Salton (1956) observed that isolated cell wall suspensions were readily degraded by lysozyme. Colobert and Dirheimer (1960) isolated a chromatographically pure hexosamine-muramic acid peptide polymer from Micrococcus lysodeikticus that was rapidly depolymerized by lysozyme.

The use of synthetic substrates for lysozyme by Hamaguchi et al. (1960) revealed that lysozyme only acts on polymers of N-acetyl derivatives of glucosamine and not on deacetylated polymers.

The correlation of lysozyme action and the appearance of reducing sugar was confirmed by Epstein and Chain (1940). Lysozyme appears to catalyse depolymerization before liberation of reducing groups.

Lysozyme action is characterized by display of the following: (a) rupture of $\beta(1\rightarrow 4)$ hexosamine linkages, (b) turbidity reduction of insoluble cell wall structure (or lysis where the wall is in situ), and (c) liberation of reducing groups.

Isolation of the lysozyme substrate was described by Salton and Ghuysen (1960) and by Perkins (1960). Purified di- and tetra- saccharide from the dialysable fraction of M. lysodeikticus cell walls were released following the action of lysozyme and Streptomyces F_1 peptidase. Lysozyme cleaved the $\beta(1\rightarrow 4)$ linkage between the N-acetylglucosamine and the N-acetylmuramic acid resulting in depolymerization of the mucopeptide complex. When lysozyme acts on whole cells in hypotonic media, lysis of the cell results.

Lysozyme, because of its high isoelectric point, complexes readily with acid groups on the cell surface. The presence of salts appear necessary to prevent neutralization of surface charge by lysozyme which tends to cause agglutination of cell material.

Several investigators have reported the release of cell wall components following lysozyme digestion of gram-negative organisms (Weidel et al., 1960; Primosigh et al., 1961; Schocher et al., 1962; Lark, Bradley, and Lark, 1963). The components normally released from isolated mucopeptide are: alanine, glutamic acid, diaminopimelic acid or lysine, muramic acid, and glucosamine.

While most gram-positive bacteria are sensitive to the lytic action of lysozyme, the majority of the gram-negative cells are unaffected by this enzyme.

Lysozyme lysis of gram-negative and gram-positive bacteria involves the same substrate, the difference being the inferior location of the substrate in the gram-negative cell wall. It is conceivable that agents which sensitize gram-negative bacteria to the action of lysozyme have the ability to create "holes" in the outer lipoprotein layer, thus exposing the lysozyme substrate.

Mandelstam (1962) isolated and characterized a lysozyme-soluble mucopeptide fraction from Escherichia coli cell walls. Separation of mucopeptide from protein was accomplished by ethanol-ether (3/1 v/v) extraction. Analysis of the soluble fragments from walls of E. coli following treatments of lysozyme revealed mucopeptide material containing diaminopimelic acid, muramic acid, glucosamine, alanine, and glutamic acid. This observation is consistent with that of Weidel et al. (1960). The material accounted for approximately 75-80% of the theoretical dry weight of the wall. The same technique of mucopeptide isolation was attempted on gram-positive bacterial walls, in which case almost identical components were obtained. Further, the mucopeptides isolated from five strains of E. coli had compositions that were virtually identical to

that found in the mucopeptide of Staphylococcus aureus 524. Also, four other strains of micrococci gave similar results. The prepared mucopeptide was examined for sensitivity to lysozyme. An inhibitor of lysozyme action appeared to be present which was possibly introduced during preparation. Increased concentration of lysozyme was found to overcome the inhibition. The author concluded that cell walls of E. coli contained mucopeptide as an integral structure (not separated sub-units) and that its sensitivity to lysozyme resembled that of gram-positive bacteria. Gram-positive cell walls usually contain either diaminopimelic acid or lysine, whereas the coliform mucopeptide contains both. The ratio of lysine with respect to diaminopimelic acid increased at each successive stage of purification.

Differences in lysozyme susceptibility cannot yet be ascribed to any given chemical component. No generalized explanation has yet been offered to account for differences between those species which show varied sensitivity to lysozyme.

Resistance is not due to the absence of lysozyme substrate in gram-negative bacterial cell walls, since a lysozyme-sensitive fraction has been obtained from the cell walls of Salmonella typhosa (Colobert and Creach, 1960), Aerobacter cloacae (Schocher et al., 1962), and E. coli (Mandelstam, 1961; Primosigh et al., 1961), and others (Mandelstam, 1962). A mechanism explaining the lysozyme-resistance of gram-negative organisms is even more difficult due to the more complex chemical composition of the cell wall.

Brumfitt, Wardlaw, and Park (1958), using a mutant strain of M. lysodeikticus resistant to lysozyme up to 4000 µg/ml, observed a higher ratio of O-acetyl groups attached to the muramic acid. It was further

observed that chemical manipulation of the O-acetyl content correspondingly altered the sensitivity to lysozyme. It was concluded that the relative proportion of O-acetyl groups had a direct effect upon the susceptibility to lysozyme. Salton and Pavlik (1960) suggested that lysozyme sensitivity is dependent upon the existing differences in the mode of linkage of the acetylamino groups to one another, inferring that sensitivity of any species depends upon the available number of specific bonds sensitive to lysozyme.

Biosynthetic alteration of lysozyme sensitivity has been accomplished by several investigators. Smith et al. (1962) reported that cells of Streptococcus faecalis grown in the presence of hydroxylysine were more resistant to rupture by sonic oscillation and to lysis by lysozyme or penicillin than were control cells. Salton (1953) reported cell walls of lysozyme-resistant S. faecalis differed from M. lysodeikticus and B. subtilis in rhamnose content. Later, Abrams (1958) reported that purified cell walls of susceptible cells of S. faecalis did contain rhamnose, but no obvious correlation could be established between O-acetyl content of typical gram-positive and gram-negative organisms and their resistance to lysozyme.

Current research indicates that lysozyme-resistance in gram-negative cells may possibly be due to the presence of the external lysozyme-impermeable layer of lipid, protein, and/or lipoprotein which could serve to mask the enzyme substrate in the cell wall. Several investigators have reported evidence that certain treatments enhance or potentiate the action of lysozyme on the gram-negative bacteria. Grula and Hartsell (1954), Becker and Hartsell (1954), Noller and Hartsell (1961b), Kohn (1960), and Repaske (1958) have reported that certain treatments of the gram-negative cell wall such as low pH, heat, solvents, poly-

basic antibiotics, freezing and thawing, or EDTA (versene) are capable of enhancing lysozyme action on whole cells. In 1923, Nakamura observed a lysozyme-potentiating treatment in which suspensions of gram-negative cells, if incubated in 0.0033 N HCl plus lysozyme, cleared following addition of alkali to a pH greater than neutrality. The bacteria could be made alkali-sensitive by pre-exposure to acid and lysozyme, but not as alkali-sensitive when exposed to either alone. The mechanism has since been clarified by Grula and Hartsell (1957b) by demonstrating that alkaline clearing of acid-lysozyme treated cells resulted from swelling and hydration of residual structures of partially degraded cells. Re-acidification of the test suspensions caused shrinkage of these "ghosts" and a return of turbidity to the suspensions. It was concluded that pH 3.5 pre-treatment released substrate from a "bound state" and permitted lysozyme digestion.

Becker and Hartsell (1954, 1955) reported that lysozyme sensitivity of gram-negative bacteria may also be increased by heat pre-treatment of cells at 70 C for 15 minutes. Heat pre-treatment alone does not permit marked sensitization to lysozyme, but cell lysis greater than 90% is observed when lysozyme and trypsin act in combination. The conclusion here presumes that heat treatment allows unfolding either by melting or disaggregation of the protein components sufficiently to allow tryptic digestion. The tryptic action attacks the protein, and conceivably uncovers the lysozyme substrate.

Noller and Hartsell (1961b) made an extensive study to relate the function of lysozyme-potentiating action of selected pre- and co-treatments including heat, acid, butanol, circulin, and EDTA using members of the Enterobacteriaceae. Agents of known lipoprotein-dissociating

properties were shown to elicit marked effects. Ethyl acetate and n-butanol appeared to cause similar lysis patterns in the presence of lysozyme, trypsin, or lysozyme plus trypsin at pH 7 compared to those using heated cells. The action of both n-butanol and ethyl acetate conceivably dissociate much of the lipoprotein permitting trypsin digestion and subsequent unmasking of the lysozyme substrate.

Increased sensitivity of gram-negative bacterial cells to lysozyme was observed by Warren, Gray, and Yurchenco (1957) following exposure of cells to polymyxin. Noller and Hartsell (1961b) observed rapid lysis if lysozyme and polymyxin were added simultaneously. Newton (1956) has shown that polymyxin possesses specific affinity for the cell wall and cell membrane. Polymyxin-sensitive bacteria appear to have more phospholipid than polymyxin-resistant bacteria. The action of polymyxin in potentiating lysozyme action seems best explained by its action as a strong cationic surfactant which disorganizes the substrate-protecting lipoprotein (Noller and Hartsell, 1961b; Goldberg, 1959). Clearing of whole cell suspensions follows lysozyme-polymyxin action due to loss of cytoplasm through the disorganized wall and plasma membrane.

Sensitization of gram-negative bacteria to lysozyme has been demonstrated using ethylenediaminetetraacetic acid (EDTA) in pH 8.0 tris (hydroxymethyl) aminomethane buffer (Repaske, 1958). Repaske concluded that EDTA functions as a chelating agent by splitting coordinate bonds between metals and sulfhydryl groups at the cell surface which normally block enzyme-substrate formation. In a comparison of EDTA action with that of synthetic detergents, Colobert (1958) reported that EDTA appeared to function more as a lipid-dissociant rather than as a chelating agent in potentiating lysozyme action. The function of versene as a

chelating agent is supported by the finding that exposure of Dowex 50 (H+)-treated Azotobacter cells to a variety of divalent metals in the presence of lysozyme and tris buffer did not cause their lysis. Versene, when added to the reaction mixture, caused complete lysis (Repaske, 1960). It is proposed that versene complexes with the metals causing exposure of lysozyme substrate. Grula and Hartsell (1957b) also reported Zn^{++} inhibition in their lytic systems.

Salton and Shafa (1958) observed disaggregation of isolated S. gallinarum cell walls using sodium dodecyl sulfate. The action of sodium dodecyl sulfate appeared to be a combination of protein denaturation and disaggregation of the oriented lipoprotein complex in the cell wall. Addition of phospholipid to the reaction mixture protected the cell walls from the germicidal action of cationic and anionic detergents. Protein denaturation was evidenced by the liberation of sulfhydryl groups into the supernatant material. It was concluded that the muco-complex does not form a continuous layer of wall material, but rather a reinforcing network extending across a multi-layered wall. The later conclusion is compatible with the hypothesis of Weidel et al. (1960), who proposed that the cell wall of gram-negative bacteria is made of three distinct layers.

Certain treatments of the gram-negative cell such as low pH, heat, solvents, polybasic antibiotics, freezing and thawing, and EDTA are capable of potentiating concurrent or subsequent lysozyme action resulting in lysis of the cell. While it is presently presumed that these treatments dissociate the lysozyme-protecting components of the cell wall (lipoprotein), the exact nature of their potentiating effects remains unknown.

The primary aim of this study was to compare the effects of selected lysozyme-potentiating treatments on whole cells and cell wall material of E. coli. This was carried out by determining the composition and amount of material released from isolated E. coli wall material following selected lysozyme-potentiating treatments. Study of isolated cell wall material, rather than whole cells limits the effects of lysozyme-potentiating treatments to the wall proper. Thus, chemical analyses of materials released from the wall will not be obscured by protoplast components other than the protoplast membrane.

Much of the stimulus for this study was provided by the fact that little is known about the mode of action of these lysozyme-potentiating cell treatments. Do they actually "strip off" wall components from the cell or is there merely dissociation of the wall components to permit lysozyme to reach its substrate without actual disaggregation of the wall? This study was conducted to answer these questions by determining the nature of the components released, if any, during treatment of isolated cell wall material with the indicated lysozyme-potentiating treatments. This study anticipated insight into the understanding of the nature of the various components that comprise the gram-negative cell wall, and the mechanism of lysozyme resistance.

CHAPTER II

MATERIALS AND METHODS

Organism

Selection and maintenance.

Escherichia coli (ATCC 8739) was selected as test culture on the basis of typical biochemical characteristics and of whole cell lytic response to lysozyme-potentiating systems. Master stock cultures were maintained as refrigerated (4 C) stab cultures in nutrient agar overlayered with 3 ml of sterile mineral oil after incubation. Working stock cultures were transferred monthly on nutrient agar slants containing 0.2% dextrose and stored at 4 C. Master stock cultures under oil were not disturbed unless lytic or biochemical characteristics changed in the working stock cultures as a result of repeated transfer. In such cases, new working stock cultures were prepared from the oiled cultures. Oiled cultures have maintained their original lytic and biochemical characteristics for 2-1/2 years. All cultures were incubated at 37 C.

Preparation of whole cells for lytic testing.

Whole cells for lytic testing were grown on nutrient agar slants for 24 hours. Cell growth was suspended in sterile saline and used as spread inoculum for 10-12 petri dishes containing nutrient agar plus 0.2% dextrose. Following incubation for 18 hours, the cells were har-

vested and washed twice with distilled water. Cell suspensions were standardized prior to use by diluting with water so that an addition of 0.5 ml of the suspension to 4.5 ml of the lytic system would give an initial optical density in the vicinity of 0.5. Washed cells were stored at 4 C and used on the same day that they were prepared.

Preparation of cell wall material.

For preparation of large quantities of cells, the test organism was grown in 10 liter quantities of nutrient broth plus 0.2% dextrose at 37 C for 18 hour using forced aeration. Antifoam A (aerosol spray) was added prior to sterilization to control foaming. Cells were harvested using a Sharples centrifuge at approximately 45,000 rpm, resuspended in 0.067 M phosphate buffer (pH 7), and standardized for rupture.

Cell rupture was accomplished by using a 10 K. C. Raytheon Sonic oscillator on 75 ml aliquots of cell suspension for 11 min. Breed counts revealed that approximately 90% rupture was achieved when initial cell concentration was in the range of 2-4 x 10^9 cells/ml (optical density of approximately 0.4-0.8). Higher or lower concentrations decreased rupture efficiency. Cell wall material was purified by the differential centrifugation method of Schocher et al. (1962), lyophilized, and stored in a vacuum desiccator.

Lysozyme-Sensitivity of Escherichia coli Whole Cells

Lysozyme-potentiating treatments.

General. Lysozyme potentiation was determined by measuring the

lytic effect at 45 C of lysozyme[1], or a combination of lysozyme and trypsin on cells with and without the treatments indicated in the following paragraphs.

All water for preparation of reagents, enzymes, lytic systems, and for analyses was glass-distilled from single-distilled tap water. Enzyme solutions were stored at 4 C and prepared fresh at least monthly.

Lytic reactions were generally carried out at 45 C in 14 mm Pyrex culture tubes standardized for spectrophotometric use. Lysis was determined by measuring the decrease in optical density of the test suspension during exposure of the cells to the test systems. Optical density was measured with a Klett-Summerson (Model 800-3) colorimeter at a nominal wave-length of 600 mμ. The tubes were read for turbidity change at time intervals of 0, 5, 15, 30, and 60 minutes.

The terms pre-treatment and co-treatment will be used extensively throughout the text. They are defined respectively as exposure of cells or cell walls to conditions such as heat, low pH, etc., prior to the addition of lytic reagents and as exposure of cells or cell walls to these various conditions simultaneously with lytic reagents.

Heat pre-treatment. Standardized cells were heated for 15 min at 70 C and then cooled to 45 C in a water bath. One-half ml volumes of heated cells were added to the lytic systems contained in 4.5 ml phosphate buffer (pH 7) pre-warmed to 45 C. Final concentrations of enzymes, whether alone or in combination, were 20 μg/ml lysozyme and 10 μg/ml try-

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[1]The term lysozyme will be used as the trivial name for N-acetylmuramide glycanohydrolase (Jollès et al., 1963). While the term muramidase has been suggested as the preferred trivial name, the term lysozyme has adequate precedence to merit its continued use.

psin. Final buffer concentration was 0.0067 M. Controls using buffer alone were included.

Butanol co-treatment. Unheated standardized cells were exposed to lysozyme, trypsin, or lysozyme and trypsin as indicated above except that 5% v/v n-butanol was added to each lytic system. Controls of n-butanol in buffer without enzymes were also included.

Polymyxin co-treatment. The procedure used was the same as for butanol co-treatment except that butanol was replaced by 70 U/ml polymyxin B-SO_4.

Versene co-treatment. Unheated standardized cells were used. Lysozyme and trypsin remained as the lytic agents, but were prepared in 0.034 M tris buffer [tris (hydroxymethyl) aminomethane], pH 8. Versene (disodium ethylenediaminetetraacetate), at a level of 133 µg/ml, was used as the lysozyme-potentiating co-lytic agent.

Modified Nakamura treatment. To determine the effects of acid co-treatment, cells were incubated in water adjusted to pH 3.5 using 0.01 N HCl. In addition to a tube containing lysozyme (20 µg/ml), a control tube (absence of lysozyme) was also included. Optical density was recorded initially and after incubation for varying intervals up to and including 60 minutes at 45 C. After each incubation interval, 0.1 N alkali (NaOH) was added to each tube to give a final pH of 10.5 and the optical density again noted after 5 minutes.

Effect of electrolytes on lysis.

In a preliminary study, it was found that paper chromatographic analyses of dried supernatant samples containing residual salts of the 0.0067 M phosphate buffer (100 ml systems) prevented amino acid resolution. Experiments were conducted to determine whether salts could

be reduced or eliminated from the reaction mixture. At the same time the general need for the presence of an electrolyte was also investigated. For this study, water, 0.015 M NaCl, and 0.0067 M ammonium acetate (pH 7) were substituted for phosphate buffer. All systems except water were of the same ionic strength (0.015). The lysozyme-potentiating components of the LTB system were used as the basis for comparison. Preparation of the test systems was the same as indicated for whole cell lytic studies. The resulting lytic patterns were used to determine the necessity for electrolytes. All systems were incubated for 1 hour at 45 C with the extent of lysis being determined turbidimetrically.

Effect of lyophilization on cell lysis.

An experiment was conducted to determine whether or not lyophilization would alter the lysozyme sensitivity of whole cells. A suspension of lyophilized and non-lyophilized whole cells was standardized for testing using the same procedure as outlined for whole cell lytic studies. The potentiating treatments of heat, butanol, polymyxin, and versene were used as the basis for comparison of lyophilized versus non-lyophilized cells.

Lysozyme-Sensitivity of Isolated Cell Wall Material

Turbidimetric analysis.

Turbidimetric analyses were conducted using isolated cell wall material in an attempt to determine whether or not the lysozyme-potentiating treatments for whole cells caused similar clearing of cell wall suspensions. Except for the use of cell wall material in the place of whole cells, all treatments and lytic systems were identical to those used for whole cell experiments.

Analyses for release of cell wall components.

Experiments were conducted to determine the type and amount of components released from cell wall material following lysozyme-potentiating treatments. One-hundred ml systems containing 50 mg samples of cell wall material pre-heated for 15 min at 70 C were exposed to lysozyme, trypsin, and lysozyme plus trypsin in the presence and absence of n-butanol, polymyxin, or versene at 45 C for 1 hour. All reagent concentrations were the same as used for turbidimetric analysis. The effect of acid co-treatment was determined by use of Nakamura treatment as shown in Figure 1.

Following the 1 hour incubation, all systems were centrifuged at 18,000 x G to remove wall residues and the supernatant liquid was analyzed as indicated in Figure 2.

Amino acid chromatography. One-hundred mg of cell wall material was suspended in 2 ml of distilled water and divided into two 1 ml aliquots. One of the aliquots was hydrolysed in 6 N HCl at 100 C for 18 hours. The hydrolysate was dried at 80 C in a dry air oven and resuspended to a volume of 1 ml. The second aliquot was left untreated. Ten microliter aliquots from both the hydrolysed and unhydrolysed samples were spotted onto separate Whatman #1 chromatographic papers and irrigated in two dimensions using the Redfield (1953) solvent system (methanol/water/pyridine, 80/20/4, v/v, in the first dimension; tertiary butanol/methylethyl ketone/water/diethylamine, 40/40/20/4, v/v in the second dimension). The chromatograms were developed by spraying with 0.5% ninhydrin in acetone followed by heating in a dry air oven at 100 C for 5 min. The unhydrolysed chromatograms were examined for evidence of isolated ninhydrin positive spots corresponding to free amino acids

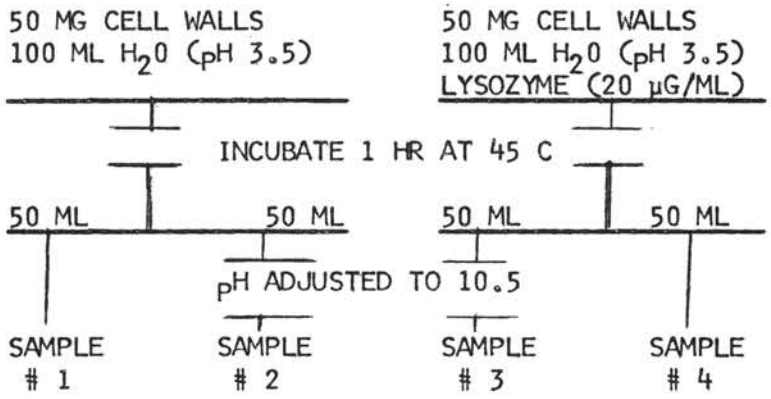

FIG. 1. PROCEDURE FOR THE NAKAMURA TREATMENT OF CELL WALLS

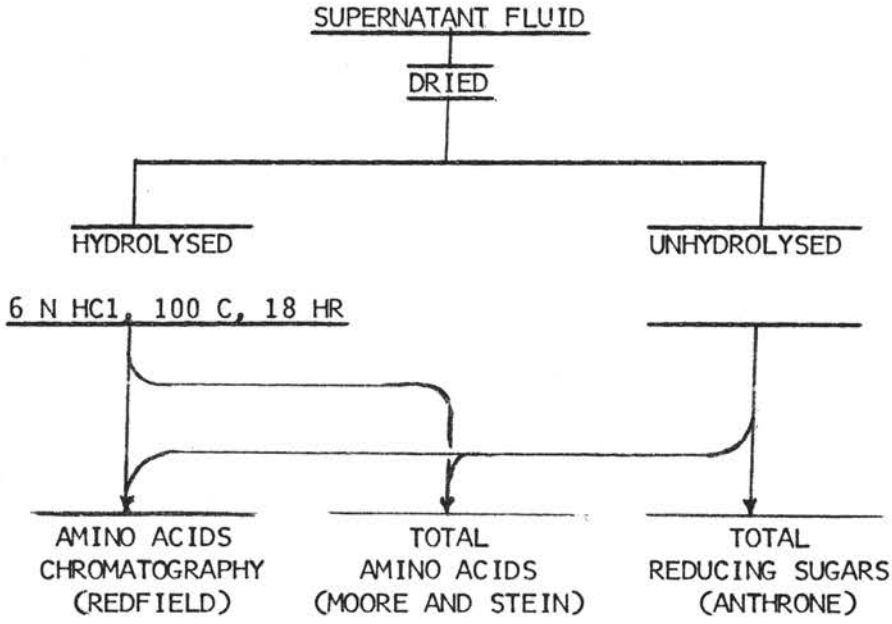

FIG. 2. ANALYSES PERFORMED ON SUPERNATANT FLUID FROM CELL WALL FOLLOWING SELECTED PRE- AND CO-LYTIC TREATMENTS

present in hydrolysed samples. The same procedure was followed for analysis of the supernatant fluid for amino acids released following the action of each of the selected lysozyme-potentiating treatments.

Total amino acids. Total amino acids were determined by a modified Moore and Stein procedure for quantitative amino acid analysis (Spies, 1957) using 0.1 ml aliquots from the same samples employed in the chromatographic analysis for amino acids.

A comparison was also made between hydrolysed and unhydrolysed samples to aid in understanding the nature (i.e. free amino acids or peptides) of the wall components released during a particular pre- or co-lytic treatment.

Total reducing sugars. Reducing sugars released following each of the lysozyme-potentiating treatments were detected using the anthrone test described by Gaudy, Komolrit, and Bhatla (1963). Each of the unhydrolysed samples prepared for the qualitative amino acid determination (paper chromatographic analysis) was diluted 1-5 in distilled water. Nine ml of anthrone reagent (0.2% anthrone in 95% sulfuric acid) was added to 1 ml aliquots of the respective diluted supernatant samples. The units of measure for reducing sugars (reducing equivalents) were expressed as milligrams of glucose with glucose used as the control. Determination of reducing sugars was not conducted on the hydrolysed samples (6 N HCl, 100 C, 18 hr) since a pilot study revealed that the reducing capacity of each of the samples had been completely destroyed by the indicated hydrolytic treatment.

Extractable lipids. One-hundred mg samples of cell wall material were treated with lysozyme-potentiating agents for 1 hour at 45 C. Each reaction system was centrifuged at 18,000 x G for 20 min to remove the

cell-wall residue. The resulting supernatant fluid was taken to dryness, resuspended in 1 ml of distilled water, transferred to small aluminum-foil boats, and evaporated to dryness. The dried samples in the boats were placed into extraction thimbles and extracted with chloroform-methanol (4/1, v/v) for 4 hours. The thimbles plus samples were then dried and weight loss due to extraction was calculated. Controls permitted calculation of total chloroform-methanol extractable material present in untreated cell wall material.

CHAPTER III

RESULTS

Lysozyme-Sensitivity of Escherichia coli Whole Cells

Lysozyme-potentiating treatments.

General. The lytic response of the selected test culture, Escherichia coli (ATCC 8739), approximated that of E. coli 19 used by Noller and Hartsell (1961a,b). The effects of the first four lysozyme-potentiating treatments listed below are shown in Fig. 3 (broken lines in Figs. 3, 5, 6, and 8 represent unpotentiated cell response).

Heat pre-treatment. The action of lysozyme on heat pre-treated cells (70 C for 15 min, Fig. 3A) caused somewhat more lysis than its action on unheated whole cells (Fig. 3B,C,D). Becker and Hartsell (1955) have observed electron dense areas in the cytoplasm of heat-treated cells (70 C for 15 min) and proposed that it was coagulated protein. The action of heat treatment, as supported by these studies, is in agreement with Becker and Hartsell (1955), Noller and Hartsell (1961a,b) in that the effect of heat is to denature the constituent protein and concurrently melt the lipoidal components in both the cell wall and cell membrane. The effect of trypsin on heat-treated whole cells in these studies was not as extensive as that reported for E. coli 19 by Noller and Hartsell (1961a,b). The greater trypsin response observed by Noller and Hartsell may be related to differences in enzyme concentration (20 μg/ml trypsin, 10 μg/ml lysozyme) compared to that used in this study, wherein

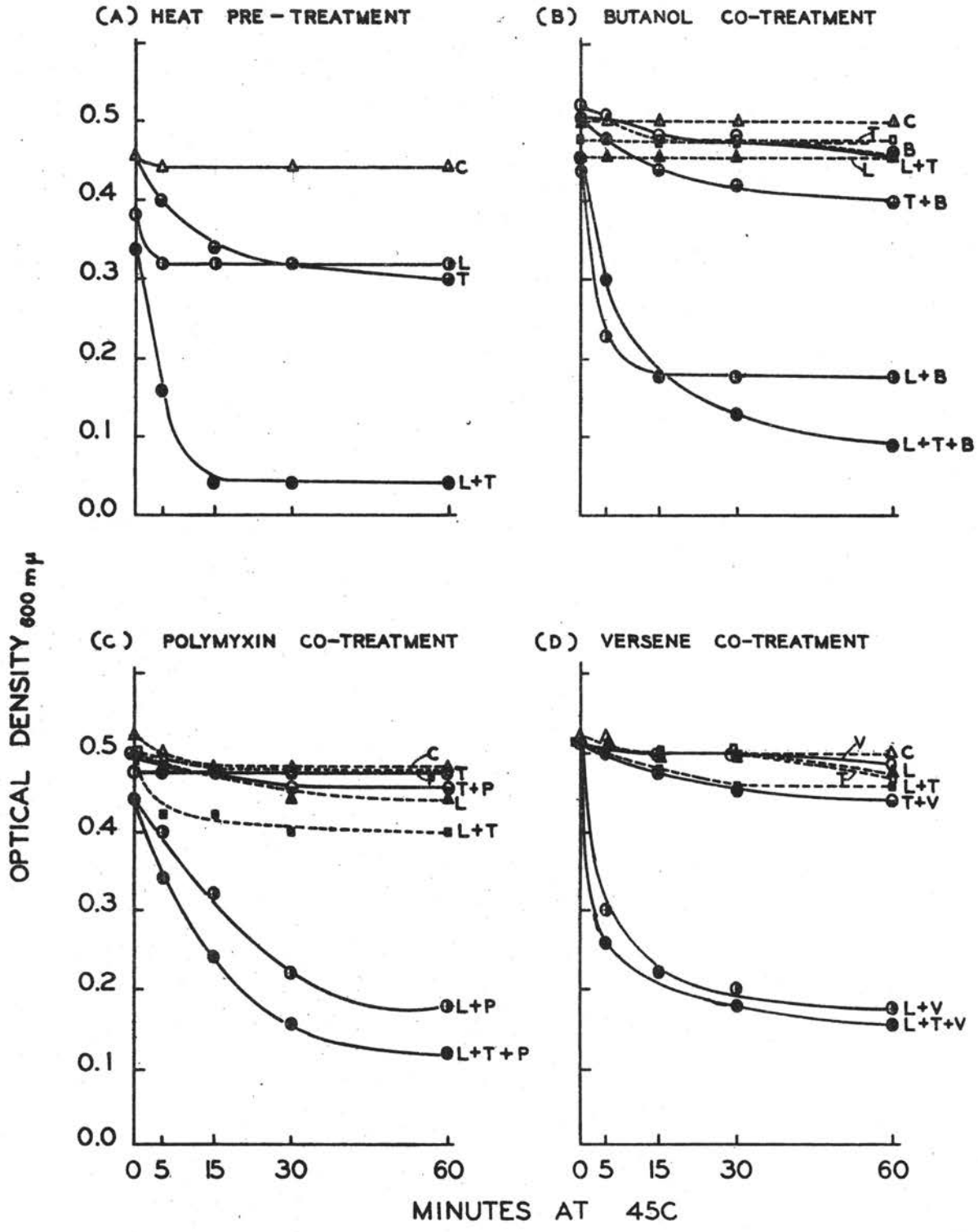

FIG. 3. EFFECT OF LYSOZYME-POTENTIATING TREATMENT ON ESCHERICHIA COLI WHOLE CELLS

T- 10 μg/ml. TRYPSIN
L- 20 μg/ml. LYSOZYME
B- 5% n-BUTANOL
P- 70 U/ml. POLYMYXIN
V- 133 μg/ml. VERSENE

the enzyme levels were respectively reversed. The enzyme level indicated for heat treatment was maintained to allow a comparison with the other lysozyme-potentiating treatments used in this study.

Butanol co-treatment. Exposure of cells to 5% v/v n-butanol at pH 7 (Fig. 3B) appears to have a comparable effect to that of heat pre-treatment (Fig. 3A, Control). The limited action of lysozyme observed on untreated control cells has been attributed to the masking effect of lipoprotein which prevents access of lysozyme to its substrate. Butanol co-treatment of cells potentiated the action of lysozyme (Fig. 3B) with a greater lytic response than observed when lysozyme was allowed to act in combination with butanol and trypsin. Trypsin was also observed to be relatively inactive in lysing whole cells unless they are sensitized by some treatment or agent. Butanol conceivably has as its action that of disrupting tertiary and secondary structural bonds of protein with concurrent lipid dissociation such to facilitate lysozyme action. Optimal lysis for this system was observed when lysozyme-trypsin-butanol (LTB) acted in combination causing approximately 83% lysis.

Polymyxin co-treatment. A lytic response similar to that caused by butanol co-treatment of whole cells is observed when polymyxin is substituted for butanol (Fig. 3C). Polymyxin potentiated the action of lysozyme and, to a lesser extent, the action of trypsin. The amount of lysis resulting from lysozyme-polymyxin action compared to that of lysozyme-trypsin-polymyxin (LTP) suggests that trypsin has a limited effect in the triple system. At a concentration of 70 U/ml (9.6 μg/ml), polymyxin appears also to unmask the lysozyme substrate by lipoprotein dissociation. Newton (1956) observed a strong affinity of polymyxin for both gram-negative cell walls and cell membranes. This antibiotic acts

as a strong cationic surfactant causing disorganization of plasma-membrane protein and/or lipids plus cell wall lipoprotein.

Versene co-treatment. Treatment of E. coli cells with versene and lysozyme in tris buffer (pH 8) caused about 60% lysis which was equal to that caused by the combined treatments of lysozyme-trypsin-versene (LTV). The effect of trypsin in this system again appears negligible.

Modified Nakamura treatment. Escherichia coli whole cells were incubated at pH 3.5 at 45 C for 1 hour to determine the effect of low pH alone and in combination with lysozyme on the lysis of whole cell suspensions (Fig. 4). Addition of alkali to a pH of 10.5 caused rapid lysis of both acid and acid-lysozyme co-treated whole cells, with greater lysis observed in the acid-lysozyme co-treated systems. Following 5 minutes of acid co-treatment, whole cells are significantly sensitized to alkaline lysis in the presence or absence of lysozyme. Also, whole-cell suspensions incubated under acid conditions increased in turbidity. This appears comparable to the increased light scattering properties of partially denatured heat pre-treated cells. Low pH induces cellular fragility by breaking hydrogen bonds by hydrolysis to an extent sufficient to unmask the mucopeptide and allow lysozyme action.

Effect of electrolytes on lysis.

Systems in which distilled water was substituted for 0.0067 M phosphate buffer gave restricted lytic activity for each of the potentiating components of the lysozyme-trypsin-butanol system (Fig. 5). The limited lysis resulting from lysozyme-trypsin-butanol action in water demonstrates that an electrolyte is required for optimal effect of the lytic agents. Such a charge-stabilizing medium is conceivably required to prevent subsequent clumping of the bacterial cells. Lysozyme-trypsin-

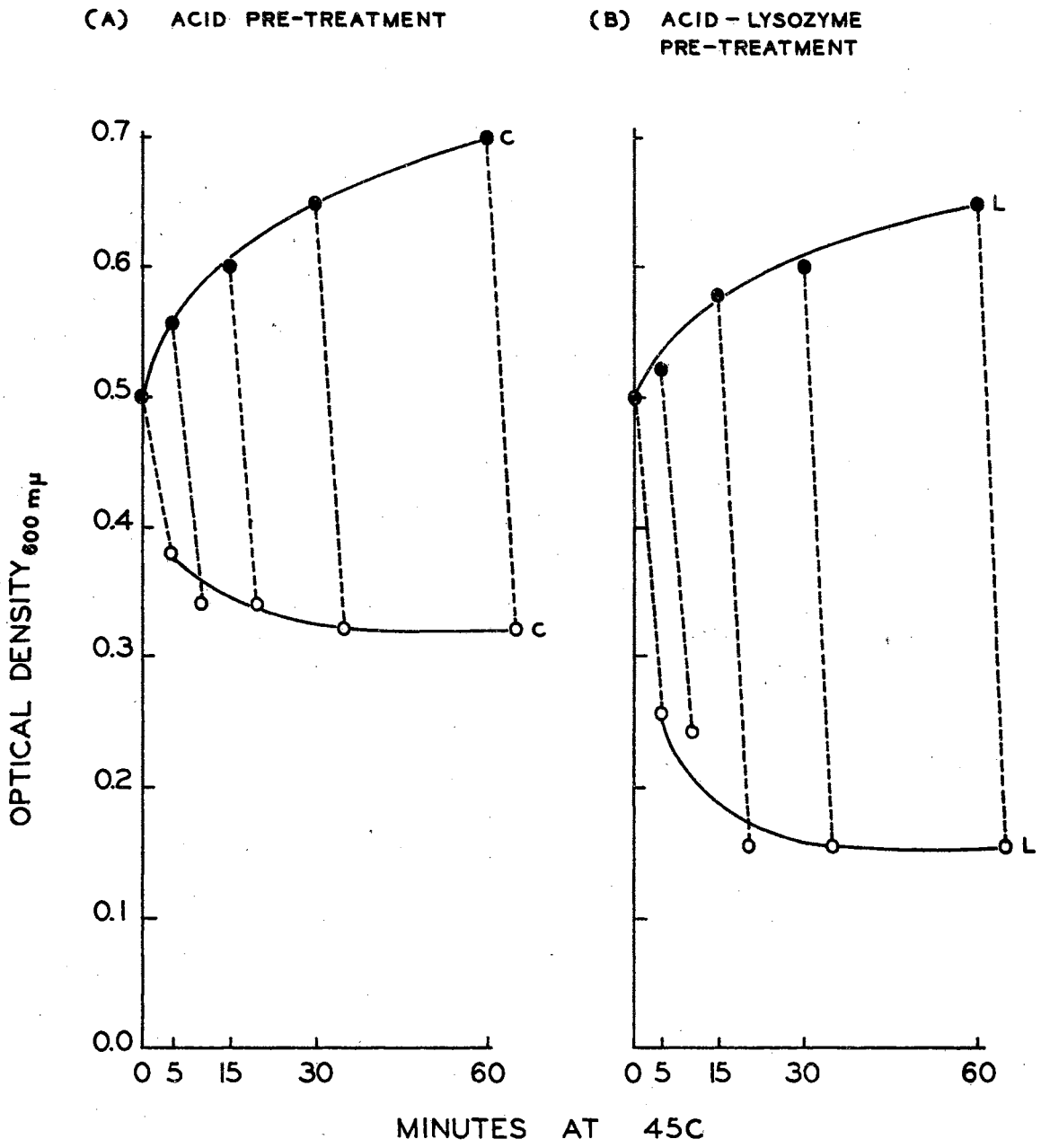

FIG. 4. EFFECT OF MODIFIED NAKAMURA TREATMENT ON E. COLI WHOLE CELLS

●——● ACID pH 3.5

○——○ ACID pH 3.5 FOLLOWED BY ALKALI TO pH 10.5

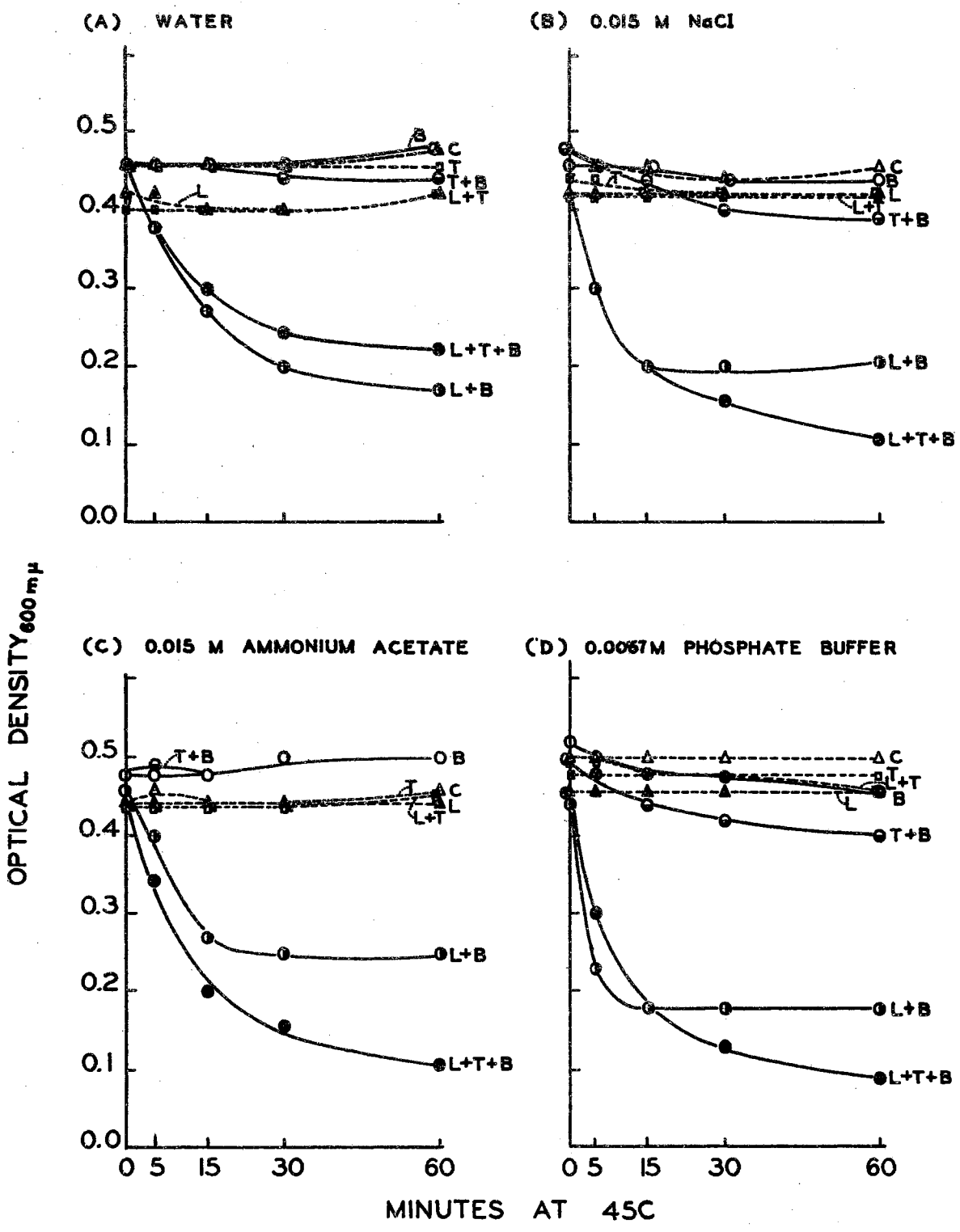

FIG. 5. EFFECT OF ELECTROLYTES ON LYSIS OF E. COLI WHOLE CELLS

L – 20 μg/ml. LYSOZYME
T – 10 μg /ml. TRYPSIN
B – 5% n-BUTANOL

butanol in systems containing 0.015 M NaCl gave a similar lytic response compared to a reaction menstruum containing ammonium acetate. Butanol potentiated the action of both lysozyme and trypsin regardless of the presence of electrolytes although the NaCl, ammonium acetate, and phosphate-buffered media allowed greatest expression of the lysozyme potentiating components.

Effect of lyophilization on lysis.

Lyophilization of whole cells (Fig. 6 and 7) in all cases, sensitized the cells to the action of both lysozyme and trypsin. This observation was evident regardless of the potentiating treatment employed. Since lyophilization of whole cells altered the cellular lytic characteristics, it is probable that a similar response will be observed with lyophilized cell wall material. Trypsin activity was noticeably enhanced (probably the result of lyophilization) when acting in combination with lysozyme and versene. This is in contrast to trypsin action on unlyophilized whole cells (Fig. 3D). Lysozyme, potentiated by versene, was equally as active on unlyophilized whole cells as was the lysozyme-trypsin-versene system. As shown in Fig. 7, lyophilization also significantly increased the effect of alkali on acid-lysozyme co-treated cells.

Lysozyme-Sensitivity of Isolated Cell Wall Material

Turbidimetric analysis.

A convenient evaluation of the lysozyme-potentiating treatments on isolated cell wall material was conducted by turbidimetric analyses (Fig. 8). The most obvious difference in the effect of the selected lysozyme-potentiating agents acting on whole cells and on cell wall

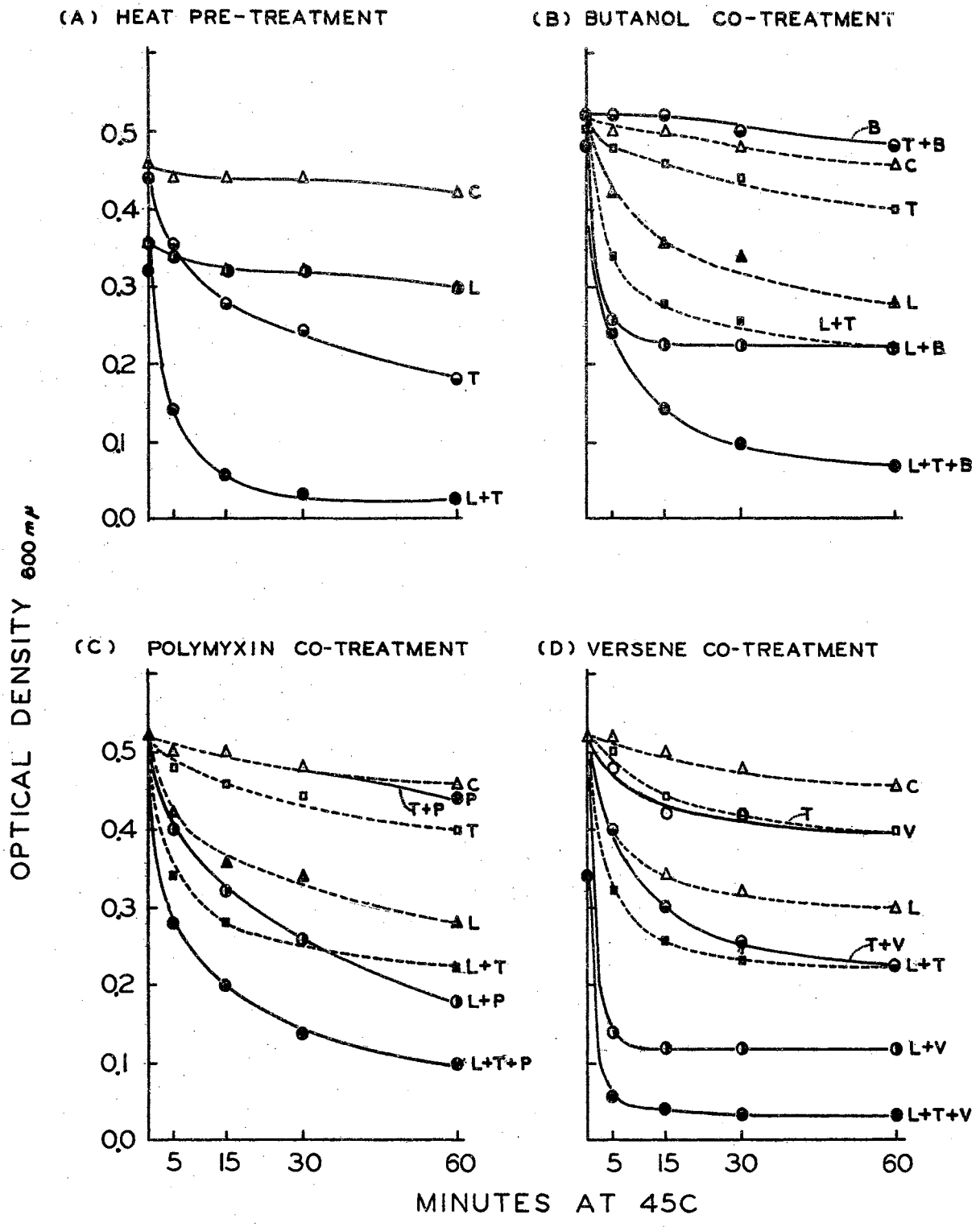

FIG. 6. EFFECT OF LYSOZYME-POTENTIATING TREATMENTS ON LYSIS OF LYOPHILIZED E. COLI WHOLE CELLS

L = 20 μg/ml LYSOZYME
T = 10 μg/ml TRYPSIN
B = 5% n-BUTANOL
P = 70 U/ml POLYMYXIN
V = 133 μg/ml VERSENE

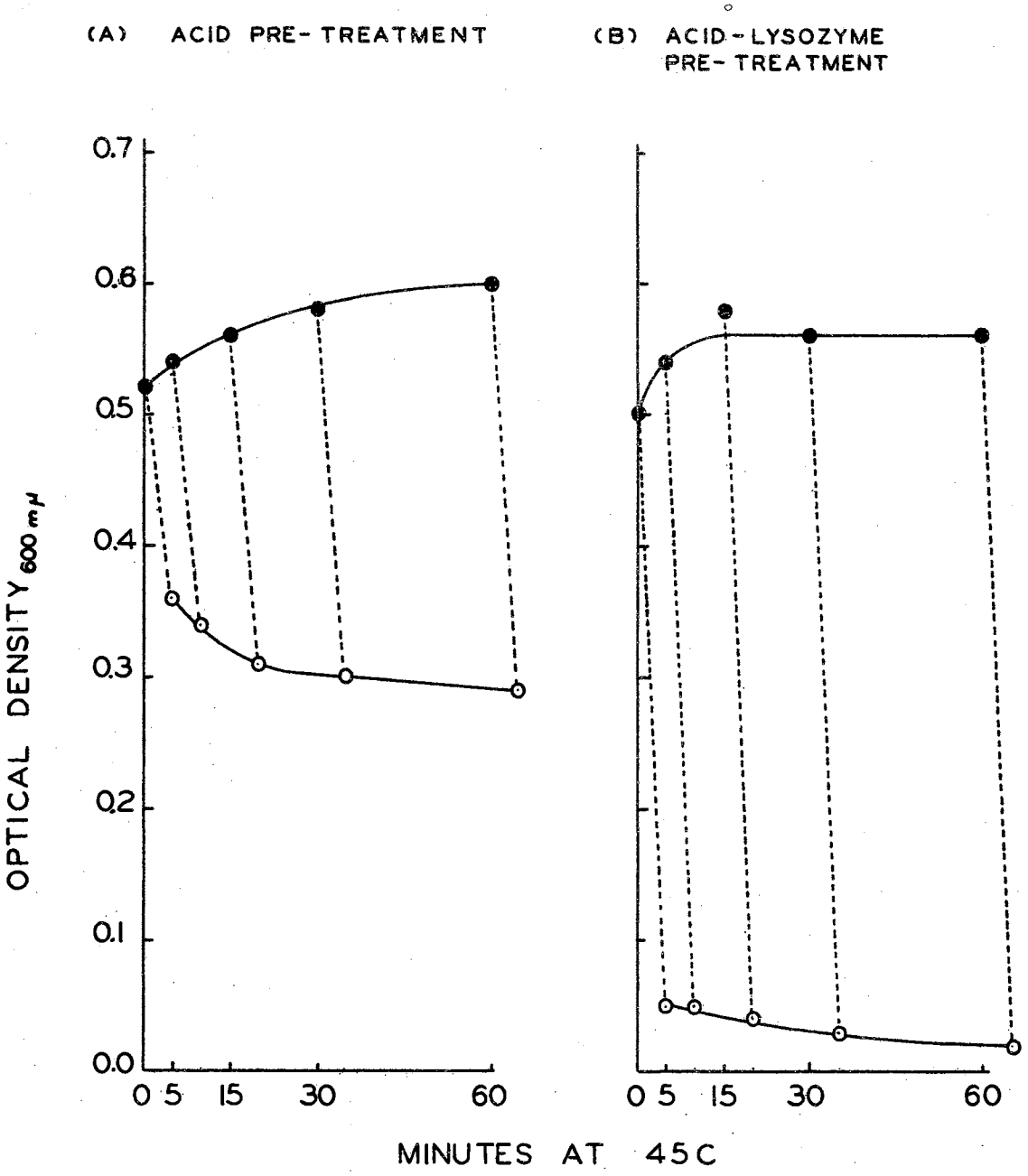

FIG. 7. EFFECT OF MODIFIED NAKAMURA TREATMENT ON LYOPHILIZED E. COLI WHOLE CELLS

C - CONTROL (WATER ADJUSTED TO pH 3.5 @ 0.01 N HCL)
L - LYSOZYME (20 μg/ml PLUS pH 3.5)

●—● pH 3.5
○—○ pH 3.5 FOLLOWED BY ALKALI TO pH 10.5

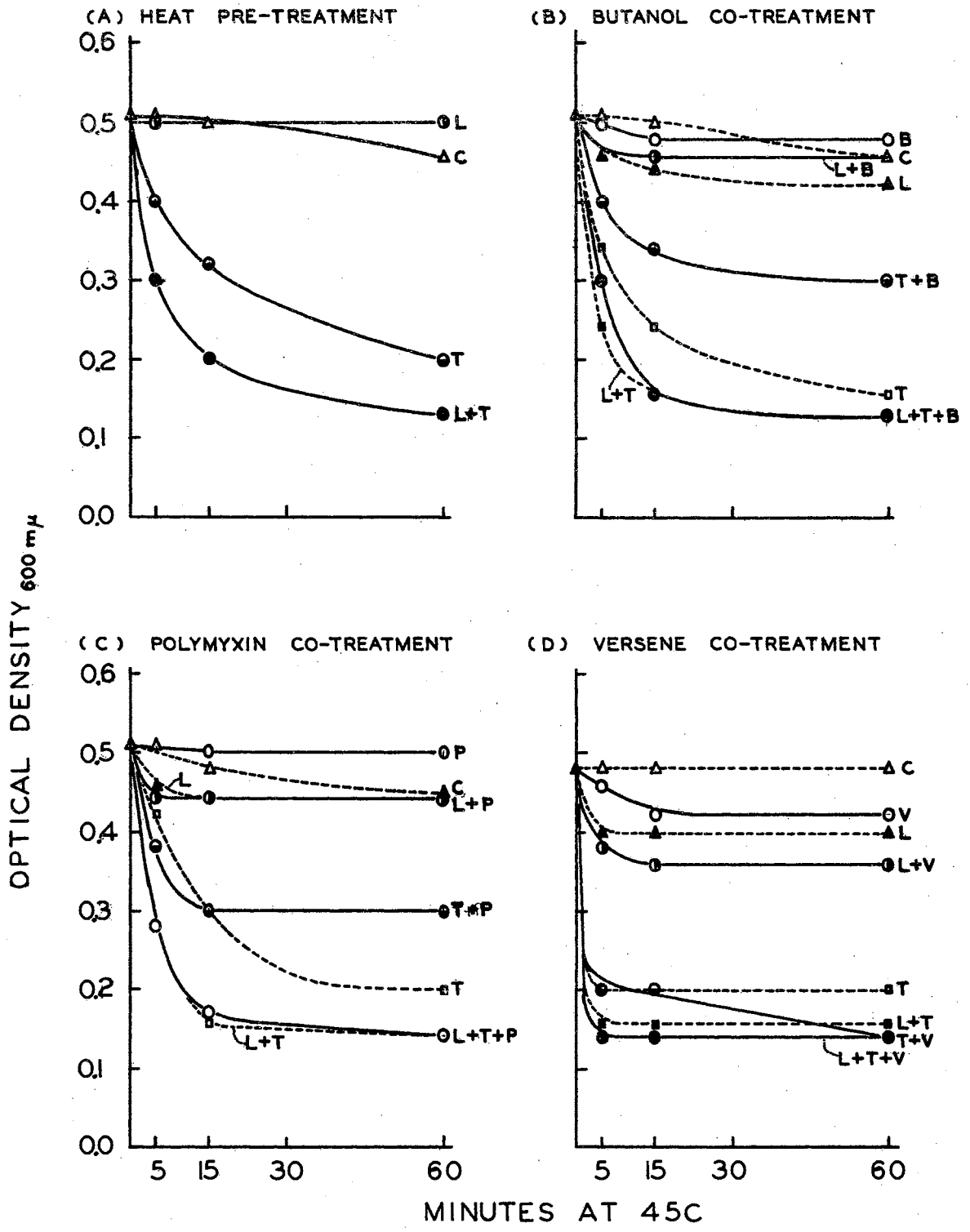

FIG. 8. EFFECT OF LYSOZYME-POTENTIATING TREATMENT ON ISOLATED E. COLI CELL WALL

L – 20 μg/ml. LYSOZYME
T – 10 μg/ml. TRYPSIN
B – 5 % n-BUTANOL
P – 70 U/ml. POLYMYXIN
V – 133 μg/ml. VERSENE

material was the increased susceptibility of wall material to trypsin digestion. Increased sensitivity of wall material to trypsin action was expected, since both lysozyme and trypsin action is enhanced following lyophilization of whole cells. The effect of lyophilization plus the action of intracellular-catabolic enzymes released during cell wall preparation (probably minimal since preparation was conducted at 4 C) is likely to affect enzymatic sensitivity of wall material. The action of lysozyme on wall material was similar to that observed using whole cells. A final optical density of 0.13-0.15 (75% clearing) was common for most of the combined lysozyme-sensitizing systems (heat-LT, LTB, LTP, and LTV). This common clearing plateau is in part explained by the fact that under optimal conditions, only 40-50% of the wall material is solubilized; the remaining 50% maintains light scattering properties. Additional clearing by lysozyme action beyond a certain point would not be anticipated, since the mucopeptide represents approximately 4% of the dry weight of the cell wall.

Acid-treated and acid-lysozyme co-treated wall material (Fig. 9) was extremely sensitive to alkali clearing, but to a lesser extent than lyophilized whole cells (Fig. 7). An incubation period of 4 min for either acid or acid-lysozyme co-treatment is adequate to sensitize the wall material to alkali clearing. The control wall material (absence of lysozyme) displayed a characteristic increase in optical density with time. Acid-lysozyme pre-treated wall material, unmasked the lysozyme substrate to allow some clearing without alkali addition as indicated by a decrease in turbidity. The increase in suspension turbidity during acid co-treatment, like that of heat or butanol pre- or co-treatment, may be correlated with protein denaturation.

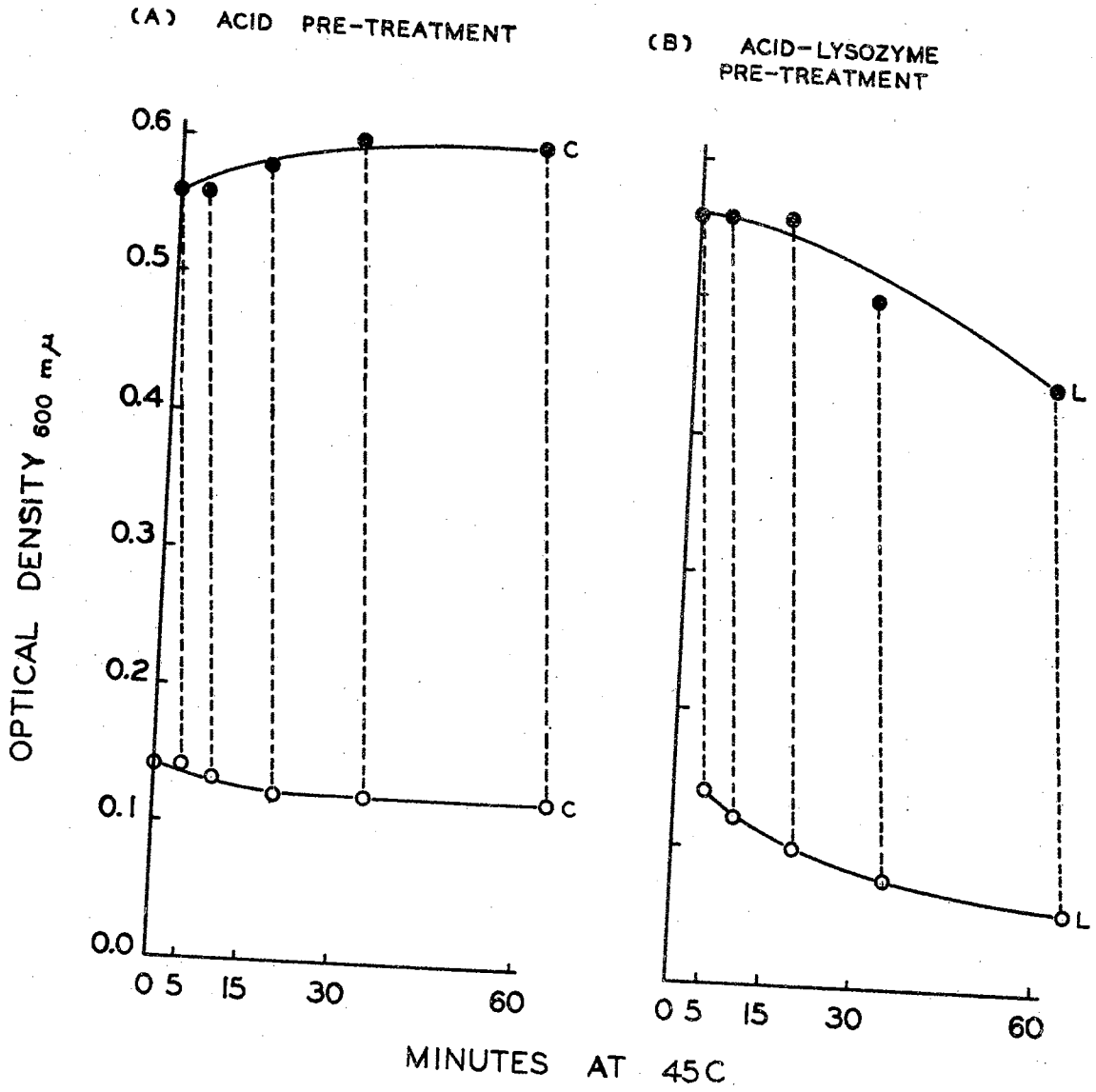

FIG. 9. EFFECT OF MODIFIED NAKAMURA TREATMENT ON ISOLATED E. COLI CELL WALL

C - CONTROL (WATER ADJUSTED TO pH 3.5 @ 0.01 N HCl)
L - LYSOZYME (20 μg/ml PLUS pH 3.5)

●—● pH 3.5
○—○ pH 3.5 FOLLOWED BY ALKALI TO pH 10.5

Analyses for release of cell wall components.

Samples of supernatant fluid from each of the lysozyme-potentiating systems were analysed for the presence and amount of amino acids, the amount of total reducing sugars, and the amount of total chloroform-methanol-extractable lipid material. A correlation between treatments and their probable mode of action will be presented.

Amino acid chromatography. Two-dimensional paper chromatographic analysis of unhydrolysed isolated wall material (not exposed to lysozyme-potentiating treatments) revealed an unresolved mass of ninhydrin positive material with respective R_f values of 0.4 and 0.2. Within the ninhydrin-positive area, no individual amino acids could be identified. This suggests that the bulk of the released material was of peptide nature. However, the hydrolysed samples yielded fourteen of the sixteen amino acids reported for *E. coli* by Salton (1960). They are as follows: alanine, arginine, aspartic acid, lysine, glycine, glutamic acid, proline, hydroxyproline, serine, tyrosine, valine, methionine, leucine, isoleucine, and possibly threonine. Histidine and phenylalanine were not observed.

Preliminary studies using paper chromatography for amino acid analysis of supernatant material following selected pre- and co-treatments were unsatisfactory due to the high concentration of salt present in the supernatant samples. The residual salts from those reaction media containing 0.0067 M phosphate buffer caused distorted migration of the ninhydrin-positive material from both the hydrolysed and the unhydrolysed supernatant samples. This difficulty was in part overcome by substituting a reaction medium containing 0.0067 M ammonium acetate instead of 0.0067 M phosphate. Since the whole cell lytic response using the

ammonium acetate menstruum (pH 7) reported earlier (Fig. 5C) indicated that the lysozyme-potentiating treatments were still functional, volatile electrolyte substitution seemed to be a good remedy for the problem of residual salts (phosphates). Attempts to quantitate the total amino acid material released following each of the lysis-inducing agents by paper chromatography were unsuccessful. Difficulty using paper chromatography was also encountered in determining the effect(s) of the different treatments on the release of particular amino acids. Specific amino acid release could not be correlated with any one treatment. This was a common characteristic of both hydrolysed and unhydrolysed samples, though most pronounced using the unhydrolysed sample material.

The hydrolysates of the control supernatant samples from cell wall material suspended in ammonium acetate revealed four unidentifiable amino acid spots. Six separate spots were observed from the system containing lysozyme, while trypsin acting singly gave twelve separate spots of medium-to-heavy intensity. Fourteen individual spots were observed with the lysozyme-trypsin (LT) and lysozyme-trypsin-butanol (LTB) system. The supernatant LT and LTB samples gave the same number of spots (of equal intensity) as were observed using the corresponding sedimented wall residues. This indicated that approximately 50% of the cell wall material was solubilized by these treatments.

Paper chromatography of hydrolysed samples was more revealing due to good resolution of individual amino acids. However, chromatography of both hydrolysed and unhydrolysed samples was relatively ineffective in determining the amount of ninhydrin-positive material released attributable to a particular treatment. As was anticipated, those systems containing trypsin were most effective in the release of protein material.

The chromatograms of hydrolysates from lysozyme-trypsin, lysozyme-trypsin-butanol, and lysozyme-trypsin-polymyxin supernatant samples also indicated spots of equal intensity compared to their respective sedimented wall-residue samples. A difference in the intensity of the ninhydrin-positive spots from material released by each of the various treatments was the only noticeable effect that can be related to specific treatments. Paper chromatographic analysis was not conducted on supernatant samples following Nakamura treatment.

Total amino acids. The unhydrolysed control samples of each of the pre- and co-lytic treatments (Tables I, II, and III) were similar (3-5 μM expressed as leucine). The action of lysozyme plus trypsin on heat pre-treated cell wall material (Table I) resulted in greater release of wall protein than the additive effects of the enzymes acting singly. The action of lysozyme on cell wall material was less effective than trypsin in releasing protein (hydrolysed and unhydrolysed samples).

In systems containing unheated cell walls (Tables II and III), trypsin caused a significant release of protein material. This observation correlates with the action of trypsin on wall material determined by turbidimetric analyses (Fig. 8). Synergistic amino acid release by systems containing lysozyme and trypsin where cells were butanol or polymyxin co-treated (Table II) is probably due to release of additional protein material following digestion of the mucopeptide by lysozyme.

The synergistic response was not observed in the lysozyme-trypsin-versene (LTV) system (Table III). Versene had a limited effect upon release of protein material from cell walls (probably due to the physical state of the wall itself). The combined effects of LTV appear more attributable to the action of the lysozyme and trypsin than to that of

TABLE I

Release of Amino Acids and Reducing Sugars From Heat Pre-Treated Cell Walls by Lysozyme, Trypsin, and Lysozyme Plus Trypsin

Treatment 50 Mg Sample 0.0067M PO_4, pH 7	Total Amino Acids* (as µM Leucine)		Total Reducing Sugars* (as Mg Glucose)
	Unhydrolysed	Hydrolysed	Unhydrolysed
Control	-- (3)	-- (16)	-- (1.8)
Lysozyme (20 µg/ml)	--	10	1.2
Trypsin (10 µg/ml)	13	78	0.4
Lysozyme + Trypsin	17 [L+T=13]**	96 [L+T=88]	1.6

* Corrected for control values shown in parentheses.
** Values in brackets represent the additive effect of individual components.

TABLE II

Release of Amino Acids and Reducing Sugars From Unheated Cell Walls by Lysozyme and/or Trypsin in the Presence of n-Butanol or Polymyxin

Treatment 50 Mg Sample 0.0067M PO_4, pH 7	Total Amino Acids* (as µM Leucine)		Total Reducing Sugars* (as Mg Glucose)
	Unhydrolysed	Hydrolysed	Unhydrolysed
Control	-- (5)	-- (22)	-- (1.2)
Lysozyme (20 µg/ml)	1	34	1.0
Trypsin (10 µg/ml)	11	64	0.8
Lysozyme + Trypsin	16 [L+T=12]**	82 [L+T=98]	1.8
n-Butanol (5% v/v)	0	2	0
Polymyxin (70 U/ml)	0	6	0.4
Lysozyme + Butanol	11 [L+B=1]	34 [L+B=36]	0.9
Lysozyme + Polymyxin	6 [L+P=1]	40 [L+P=40]	0.8
Trypsin + Butanol	10 [T+B=11]	128 [T+B=64]	0.8
Trypsin + Polymyxin	12 [T+P=11]	64 [T+P=70]	1.2
Lysozyme + Trypsin + Butanol	20 [L+T+B=12]	122 [L+T+B=100]	1.2
Lysozyme + Trypsin + Polymyxin	17 [L+T+P=12]	110 [L+T+P=104]	1.3

* Corrected for control values shown in parentheses.
** Values in brackets represent the additive effect of individual components.

TABLE III

Effect of Versene Alone or in Combination With Lysozyme and/or Trypsin on Unheated Cell Walls in Tris Buffer

Treatment 50 Mg Sample 0.033 M Tris, pH 8	Total Amino Acids* (as µM Leucine) Unhydrolysed	 Hydrolysed	Total Reducing Sugars* (as Mg Glucose) Unhydrolysed
Control	-- (5)	-- (28)	-- (3.0)
Lysozyme (20 µg/ml)	2	12	0.0
Trypsin (10 µg/ml)	4	58	0.2
Lysozyme + Trypsin	16	76 [L+T=70]**	0.5
Versene (133 µg/ml)	1	8	0.0
Lysozyme + Versene	1	16 [L+V=28]	0.0
Trypsin + Versene	6	46 [T+V=66]	0.2
Lysozyme + Trypsin + Versene	19	76 [L+T+V=78]	0.3

* Corrected for control values shown in parentheses.
** Values in brackets represent the additive effect of individual components.

TABLE IV

Release of Amino Acids and Reducing Sugars Following Modified Nakamura Treatment of Cell Walls

Treatment 50 Mg Sample Nakamura Treatment	Total Amino Acids (as µM Leucine) Unhydrolysed	 Hydrolysed	Total Reducing Sugars (as Mg Glucose) Unhydrolysed
Acid Only (pH 3.5)	4	4	1.0
Acid--> Alkali (pH 10.5)	2	10	0.7
Acid + Lysozyme (20 µg/ml)	3	17	0.9
Acid + Lysozyme--> Alkali (pH 10.5)	3	17	1.5

the versene. This observation tends to support the chelation mechanism of action of versene (Repaske, 1958) since little activity would be expected using pre-ruptured cells.

Analysis of the hydrolysed amino acid components released from wall material gave values approximately five times larger than those recorded for unhydrolysed material. This indicates that the action of the various pre- and co-treatments solubilize fragments of the walls rather than causing release of individual amino acids. Trypsin-butanol co-treatment of wall material (Table II) had a pronounced effect on release of protein material (hydrolysed sample). Since the bulk of the amino acids in the wall are components of the lipoprotein, it is conceivable that the lipoprotein is the source of amino acids released by trypsin. Yet, it is difficult to explain why the trypsin-butanol activity exceeded that of the LTB system.

The addition of alkali to acid co-treated wall material appears capable of initiating protein release equal to acid-lysozyme wall pretreatment (hydrolysed sample, Table IV). The protein material of the cell wall in the presence of acid is conceivably denatured to an extent which allows easy access of the mucopeptide to lysozyme. The action of trypsin on acid co-treated wall material was not evaluated.

Total reducing sugars. The action of lysozyme on heat pre-treated wall material was observed to cause a significant release of reducing sugars, with lysozyme plus trypsin giving a value equal to the action of lysozyme and trypsin acting singly (Table I). Both polymyxin and butanol failed to assist lysozyme in causing the release of reducing sugars from unheated wall material. With the exception of the trypsin-polymyxin (T+P, Table II) system and systems in tris buffer, those sys-

tems containing lysozyme gave optimal release of reducing sugars (Table I-IV). This observation was predictable in that the action of trypsin is restricted to proteinaceous material. However, trypsin may digest protein very near the sensitive mucopeptide bonds such that physical hydrolysis may release some reducing groups. The action of versene in potentiating enzymatic release of reducing sugar appears rather limited. The values recorded for reducing-sugars released in the tris buffered system (pH 8) correlates poorly with the observations made using turbidimetric analyses (Fig. 8D). However, control values for reducing sugars in tris systems (Table III) are higher than for phosphate-buffered systems (Table I and II).

Extractable lipids. The results obtained from these studies are in strong support of the idea that lipoprotein serves to mask the lysozyme substrate. A preliminary determination of cell wall lipid revealed that 18% of the dry weight of the wall is extractable using methanol-chloroform (1/4, v/v).

To determine the presence of protein released by extraction, extracted lipid material was hydrolysed (6 N HCl, 100 C, 18 hr), and analysed by paper chromatography. The same fourteen amino acids were found as were obtained from cell wall hydrolysates. The effect of each of the components of the lysozyme-trypsin-butanol system on extractable lipid from the supernatant material released from walls is presented in Table V.

Butanol has the effect of releasing material containing 8% lipid which accounts for 0.8% of the total dry weight of the wall. This indicates that butanol action does not "leach away" a major portion of lipid material, but rather causes "holes" or "disorganizes" the lipo-

TABLE V

Release of Extractable Lipids From Unheated Cell Walls by Lysozyme and/or Trypsin in the Presence of n-Butanol

Treatment 100 Mg Sample 0.0067M PO_4, pH 7	Weight of Released Wall Material*	Extractable Lipid From Total Wall Material Released	
		Weight**	Percent
Control	-- mg	-- (2.1)	-- %
n-Butanol	9.8	0.8	8
Lysozyme (20 µg/ml)	18.2	2.3	13
Trypsin (10 µg/ml)	44.9	6.5	14
Lysozyme + Trypsin	41.6	4.4	11
Lysozyme + Butanol	22.2	6.0	27
Trypsin + Butanol	19.8	5.7	29
Lysozyme + Trypsin + Butanol	42.8	6.7	15

* Control corrected for buffer and released wall material; other values corrected for control values plus any other non-volatile components of the treatment system.

** Corrected for control values in parthenses. Since 100 mg samples of wall material were treated, these values also represent percent of the lipid in untreated walls that was released by the treatments.

protein layer to allow penetration of lysozyme to the vicinity of the mucopeptide. The action of lysozyme released 18.2 mg of wall material of which 13% was lipid-extractable.

Lysozyme-butanol treatment released material containing 27% extractable lipid. This high value is best explained by the fact that butanol allows the penetration of lysozyme with subsequent cleavage of the mucopeptide. This further disorganization of the wall by lysozyme permits additional butanol leaching of the lipid material within the walls.

The combined action of lysozyme and trypsin solubilized 41.6 mg of wall material of which only 11% was extractable lipid. The combined treatment (LTB) solubilized approximately 43% of the cell wall material of which only 15% was lipid-extractable. This value of 15% approximates the total extractable lipid of untreated wall. Also, the amount of total material released by each treatment closely correlates with the turbidimetric data obtained for the respective treatments on unheated walls.

Comparative Effects of the Lysozyme-Trypsin-Butanol Systems on Isolated Cell Wall Material

Of the components of the lysozyme-trypsin-butanol system acting singly, the effect of trypsin was most pronounced in its ability to release wall material (40-50% of initial wall material). These total solids released included extractable lipids, protein (as amino acids following hydrolysis), and reducing sugars. Lysozyme was instrumental in solubilizing approximately 18% of the total wall material accompanied by release of lipid, amino acids, and a relatively high amount of reducing sugar. Butanol alone caused release of only a small amount of lipid, negligible amounts of reducing sugars and amino acids, and caused only slight reduction in turbidity of the test suspensions.

Lysozyme and trypsin in combination had a pronounced effect on release of total wall material (40%) which correlates directly with a large decrease in turbidity of wall suspensions. Moderate amounts of extractable lipids, total amino acids, and large amounts of reducing sugar were found in the released wall material.

Trypsin combined with butanol released large amounts of extractable lipid, some amino acids, and moderate amounts of reducing sugars. The low amount of total wall material released does not conform to the trend set by the other trypsin-containing treatments of the lysozyme-trypsin-butanol system.

When the three components of the lysozyme-trypsin-butanol system act together, optimal release of all previously mentioned wall components occurs. The amount of amino acids and reducing sugars released compares favorably to that observed using the lysozyme-trypsin-polymyxin system.

The increased sensitivity of cell wall material to trypsin often overshadows the effect of other components present in the combined systems (lysozyme and/or butanol). Systems containing trypsin were characterized by large amounts of released total wall material and amino acids, high percent clearing of the test suspensions, but low release of reducing sugar and extractable lipid.

Those systems containing lysozyme were often unable to elicit release of additional material over that released by trypsin, except in the case of reducing sugars.

The action of butanol as a lysozyme-potentiating treatment is not expressed as dramatically using isolated wall material as when using whole cells.

Although slight differences in effect were observed among the various treatments employed, the proposed mode of action of the indicated agents (components of the LTB system) on whole cells appears applicable to isolated cell wall material.

CHAPTER IV

DISCUSSION AND CONCLUSION

One of the primary objectives of this study has been to compare the effects of selected lysozyme-potentiating treatments on whole cells and isolated cell wall material of Escherichia coli. Prior to these investigations, fragmentary information was available regarding the nature and amount of material released following selected lysozyme-potentiating treatments. For this reason, various analyses of the material released from walls were conducted to: (1) render possible insight into the mechanism of action of each treatment, and (2) offer an explanation for the general resistance of the enteric bacilli to the action of lysozyme.

Treatments used in this study were largely refined by Becker and Hartsell (1955), Noller (1961), and Noller and Hartsell (1961a,b). The observed effects of the selected lysozyme-potentiating treatments on E. coli (ATCC 8739) whole cells were similar to those observed by Noller and Hartsell (1961a,b) using E. coli strain 19. Whole cells were again observed to be refractory to the lytic action of lysozyme and/or trypsin unless conditioned for lysis by certain pre- or co-lytic treatments.

Each of the treatments for gram-negative cells (acid co-treatment at pH 3.5 for 1 hour; heat pre-treatment at 70 C for 15 minutes; co-treatment with 5% n-butanol; co-treatment with 70 U/ml polymyxin; and co-treatment by 133 µg/ml versene in tris buffer, pH 8) was capable of

potentiating the action of both lysozyme and trypsin (apparently by causing dissociation of the protein and/or lipoprotein layer). Optimal lysis with all systems resulted when each of the pre- or co-treatments was used in combination with lysozyme and trypsin. When lysozyme and trypsin acted in combination with the pre- or co-lytic treatments mentioned, the lytic response of whole cells is greater than the additive effect of lysozyme or trypsin acting singly (synergism).

A close evaluation of the lytic response, and especially of the synergistic response, suggests the following sequential mode of action of the agents acting in combination. The action of heat, butanol, or polymyxin upon the protein or lipoprotein layer causes lipid disorganization sufficient to expose the protein to the action of trypsin (Salton and Horne, 1951; Becker and Hartsell, 1955; Noller and Hartsell, 1961a,b). Trypsin digestion of protein and the continued effect of butanol or polymyxin eventually disorganize the lipoprotein layer to unmask the lysozyme substrate. Following lysozyme action, ensuing lysis of the fragile cell occurs due to osmotic imbalance.

After firmly establishing the lytic characteristics of the whole cells, studies were made to determine the effect of the same potentiating agents on isolated cell wall material. Isolated cell wall material of the test organism provided a convenient means of studying the localized effects of the selected lysozyme-potentiating treatments. Much of the wall preparation maintained typical rod-like morphology characteristic of whole cells (determined by phase microscopy), though fragmentation of the prepared wall was not uncommon. Should further studies be conducted using isolated wall material, examination of the preparation by electron microscopy is recommended to determine the

adequacy of the preparation (as nearly intact as possible and devoid of attached protoplast membrane). However, it is doubtful that current techniques of cell wall preparation will yield such material from gram-negative cells.

Turbidimetric analysis of isolated cell wall material provided a convenient method of comparing the effects of the selected lysozyme-potentiating treatments on lyophilized and non-lyophilized whole cells and cell wall material. Isolated cell wall suspensions generally gave a lower final percent clearing than lyophilized whole cells. Isolated wall material was observed to have an increased sensitivity to trypsin. To account for this increased sensitivity of wall material to trypsin, consideration should be given to the fact that protein constitutes the bulk of the gram-negative cell wall (approximately 60-80% of the dry weight of cell wall). Cell wall sensitivity to enzymes is probably due to denaturation of the constitutive protein or lipoprotein during the brief period of rupture, exposure to released catabolic enzymes, and additional physical harassment during further preparation (lyophilization, etc.).

The observed plateau of 0.13-0.15 optical density (73% clearing) using wall material is a common characteristic of the combined treatments and probably represents the presence of "limit material" possessing light scattering properties. This limit material accounts for approximately 50-60% of the wall material with the remaining portion being solubilized as revealed by paper chromatographic analysis of hydrolysed supernatant samples.

Prasad and Litwack (1963) cautioned against interpretation of lysis by turbidimetric measurements on the basis that lysis curves

using whole cells show far greater clearing than with cell walls. Brumfitt (1959) has also shown that the rate of lysis of whole cells of M. lysodeikticus is nearly twice as rapid as that of cell wall material. The apparent difference in the action of lysozyme on whole cells and cell wall material is directly related to the ability of whole cells to produce a greater turbidimetric change when they collapse and undergo solubilization. A consideration of equal importance is the fact that larger amounts of cell wall material are necessary to give an optical density of 0.5 compared to the amount of whole cells to give the same optical density value.

The results obtained by analyses of the components released following selected lysozyme-potentiating treatments augment those observed using turbidimetric analysis. A reasonably close correlation was found to exist between release of cell wall components and turbidimetric clearing. Systems containing lysozyme are characterized by release of reducing sugar while those containing trypsin are characterized by the release of ninhydrin-positive material (paper chromatography and total amino acid determinations). In those systems in which a synergistic response is noted using turbidimetry, a related synergistic response also exists for the release of ninhydrin-positive material. However, supernatant samples analysed for either reducing sugars or extractable lipids failed to elicit the synergistic response.

Chromatographic analysis of the protein material released into the supernatant fluid was effectively improved by substituting 0.0067 M ammonium acetate for phosphate buffer in the reaction media; however, release of specific amino acids could not be correlated with a particular treatment.

Since intensity differences of ninhydrin-positive material were the only noticeable effect relatable to specific treatments, it is suggested that more sophisticated methods of chromatographic analysis be employed to more accurately determine which amino acids are released by a given treatment. For a more accurate method of determining the effects of lysozyme action on wall material plus the effects of the potentiating treatment employed, it is suggested that analyses be made to determine the release of hexosamines by the method outlined by Salton and Ghuysen (1960).

The mode of action of n-butanol as a lysozyme-potentiating agent is more evident when consideration is given to the thermodynamic properties of alcohols in water solutions containing lipoidal material in the presence of inorganic salts. An exothermic reaction is believed to occur causing an increase in molecular bombardment of the n-butanol molecules such that the hydrocarbon moiety of the molecule is forced into contact with the cell wall lipid material of like composition (polar aggregation) to cause increased hydration at the periphery of the lipoprotein layer (Knight, 1964). This increased hydration is sufficient to "leach away" areas in the lipoprotein layer allowing penetration of lysozyme and concurrent potentiation of trypsin action. Reaction systems containing n-butanol at 45 C conceivably are capable of destroying native lipophilic, hydrophilic, and hydrogen bonds.

Since protein material was found to be released by chloroform-methanol extraction, it is conceivable that protein is interlaced with the lipid material and attached by relatively weak linkages. As indicated, trypsin and butanol had a pronounced effect on lipid release because of its localized effect on the peripheral lipoprotein coat.

Further, any lipoidal or proteinaceous material inferior to the external lipoprotein layer would also be susceptible to both trypsin and butanol. The addition of lysozyme to the trypsin-butanol systems caused an increased release of wall material due to cleavage of the mucopeptide; but in doing so, the percent extractable lipid was decreased. This is observed when one compares the 15% extractable lipid resulting from the lysozyme-trypsin-butanol systems to the 29% lipid material released by the trypsin-butanol system (Table V). More critical analyses should be made on the released lipids. Such lipid determinations should be extended to include the other lysozyme-potentiating treatments studied.

The interested investigator is encouraged to determine the effects of polymyxin on lipid release and to evaluate its ability to disorganize the cell wall phospholipids in combination with lysozyme and/or trypsin.

This study failed to answer the question regarding the role of versene as a lysozyme-potentiating agent (i.e. whether versene functions as a chelating agent or as a lipoprotein-dissociating agent). Since versene action on isolated wall material did potentiate lysozyme and/or trypsin action (as did butanol or polymyxin), the lipid-dissociating properties of versene may still be as important as those of chelation.

Variations of lysozyme sensitivity among different species of gram-negative organisms is assumed to be due to variation in peripheral deposit of lipoprotein material in the outer layer which is the result of the metabolic patterns of the particular organism.

This is insufficient evidence to be certain of the precise structure and arrangement of gram-negative cell walls, but it seems prob-

able that the amino sugars are joined together forming polysaccharide chains which are linked together by peptides attached to the carboxyl groups of the muramic acid.

A possible macromolecular arrangement of the mucopeptides in the wall (Rogers, 1963) is as sheets of polysaccharide fibers linked together by peptides attached to the carboxyl groups of muramic acid. Such an arrangement would have the advantage of great strength and rigidity combined with an open mesh-work structure through which molecules could move with relative ease. The minimum intervals between the peptide chains, assuming a cross-linkage of the type proposed by Ghuysen and Salton (1960) to occur on every muramic acid residue, would be the length of a di-saccharide unit (15-20 Å). Mucopeptides are considered to be the principle structure of mechanical support in both gram-positive and gram-negative microorganisms. While the mucopeptide forms the major portion of the cell wall of gram-positive organisms and attached protein plays a secondary structural role, in the gram-negative organisms, protein assumes a more important structural role. The lipoidal material is conceived to be attached to polymers of glucosamine phosphate in which all the available amino and hydroxy groups are esterified by long chain fatty acids lying normal to the wall itself.

The results of this study, plus those cited in the text, gives assurance that any combination of treatments which have as their action the capacity of digesting or hydrolysing the three major components of the wall (i.e. lipid, protein, mucopeptide) has the capacity of causing rupture of intact bacterial cells.

Until techniques are developed that are capable of isolating the various wall components without changing their structure and/or com-

position from that which exists *in situ*, the actual architecture of the cell wall and the structural function of the wall components will remain in question.

These results suggest that when conditions for lysis exist [as outlined by Becker and Hartsell (1955) and Noller and Hartsell (1961a,b)], lysozyme in combination with the selected pre- and co-lytic treatments causes complete or partial removal of the peripheral wall material of gram-negative whole cells. Lysozyme hydrolyses the inner mucopeptide and the weakened cell loses cytoplasmic contents.

SUMMARY

The effects of selected lysozyme-potentiating agents were studied using Escherichia coli (ATCC 8739). A correlation was established between the ability of these agents to lyse whole cells and to solubilize isolated cell wall material. Analyses of the material released by the treatments provided a means of determining their mode of action and permitted further speculation on the composition of the gram-negative cell wall.

That lysozyme has little or no effect upon the lytic sensitivity of untreated whole cells has been supported. Optimal lysis of whole cells due to lysozyme action demands the presence of ions, and it is preferable that they possess buffering capacity to prevent change in the reaction of the menstruum during cellular rupture.

Isolated cell wall material was found to be extremely sensitive to the action of trypsin which suggests that constituent cell wall protein was partially denatured during wall preparation.

Versene had little or no effect upon potentiating lysozyme or trypsin action on cell wall material. Additional experimentation on this phase is suggested to further establish the role of versene as a lysozyme-potentiating treatment. For a more precise means of following lysozyme action, analyses for the release of hexosamines following each lysozyme-potentiating treatment are suggested.

Electron microscopy should be employed to examine the cell wall

preparation to determine its purity. Further study aiding in the understanding of this problem is encouraged in the area of determining the enzyme sensitivity of cell wall material prior to lyophilization to evaluate the effect of rupture and lyophilization during the preparation of wall material.

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APPENDIX

Materials and Sources

Antifoam A (aerosol spray), Dow Corning Corporation

Ammonium Acetate, Fisher Certified Reagent

Anthrone, Fisher Certified Reagent

n-Butanol, Fisher Certified Reagent

t-Butanol, Fisher Certified Reagent

Chloroform, Fisher Certified Reagent

Citric Acid, Fisher Certified Reagent

Diethylamine, Eastman Organic Chemicals

Disodium Ethylenediaminetetraacetate, Fisher Certified Reagent

Dextrose, Fisher Certified Reagent

Hydrochloric Acid, Baker Analysed Reagent

Lysozyme (twice crystallized and lyophilized), Worthington Biochemical Corporation

Methyl Cellosolve, Fisher Certified Reagent

Methyl Ethyl Ketone (2-butanone), Matheson Coleman & Bell

Methanol, Fisher Certified Reagent

Mineral Oil, E. R. Squibb & Sons

Ninhydrin (triketohydrindene hydrate), Fisher Certified Reagent

Nutrient Agar, Difco Laboratories

Nutrient Broth, Difco Laboratories

Polymyxin B-SO_4 (7400 U/ml), Burroughs Wellcome & Company

Potassium Phosphate (monobasic crystal), Baker Analysed Reagent

APPENDIX (CONTINUED)

Propanol, Fisher Certified Reagent

Pyridine, Fisher Certified Reagent

Sodium Hydroxide, Baker Analysed Reagent

Sodium Phosphate (dibasic dodecahydrate crystal), Baker Analysed Reagent

Stannous Chloride, Mallinckrodt Chemical Works

Sulfuric Acid, Baker Analysed Reagent

Tris (hydroxymethyl) aminomethane, Eastman Organic Chemicals

Trypsin (twice crystallized and lyophilized), Worthington Biochemical Corporation.

VITA

Charles Dwayne Edwards

Candidate for the Degree of

Master of Science

Thesis: EFFECTS OF LYSOZYME-POTENTIATING TREATMENTS ON ESCHERICHIA COLI

Major Field: Microbiology

Biographical:

Personal Data: Born in Pawnee, Oklahoma, December 21, 1938, the third son of Glen Franklin and Mary Lela Edwards.

Education: Attended grade school and high school in Pawnee, Oklahoma. Graduated from Pawnee High School in 1957; received the Bachelor of Science degree from Oklahoma State University at Stillwater, Oklahoma in May 1961; completed the requirements for the Master of Science degree in May, 1964.

Professional experience: Graduate research and teaching assistant, Department of Microbiology, Oklahoma State University.