NUTRITIONAL MODIFICATION OF THE CELL WALL MUCOPEPTIDE OF *MICROCOCCUS LYSODEIKTICUS*

By

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CHAPTER I

INTRODUCTION

Because of the large amount of published material concerning the bacterial cell wall and the fact that this study was confined to a single bacterium, Micrococcus lysodeikticus, the following discussion will deal only with pertinent literature concerning Gram-positive bacteria. Reference to specific work with Gram-negative bacteria will be made however, when necessary for clarification and completeness.

During studies spanning the years 1930 to 1941 Knaysi (1, 2), observed in stained preparations of Bacillus subtilis, two peripheral structures which appeared to have a role in cell division. For these structures he coined the terms cell wall and cytoplasmic membrane. Later workers, employing the electron microscope, were able to show that the cell wall was a well-defined, rigid structure since it retained its form upon removal of the protoplasm from the cell (3).

In 1951 Salton and Horne (4) physically ruptured Gram-positive bacterial cells and isolated an insoluble material, which was shown by electron microscopic examination to retain the form of the cells from which it had been isolated. These authors concluded that they had isolated the cell wall.

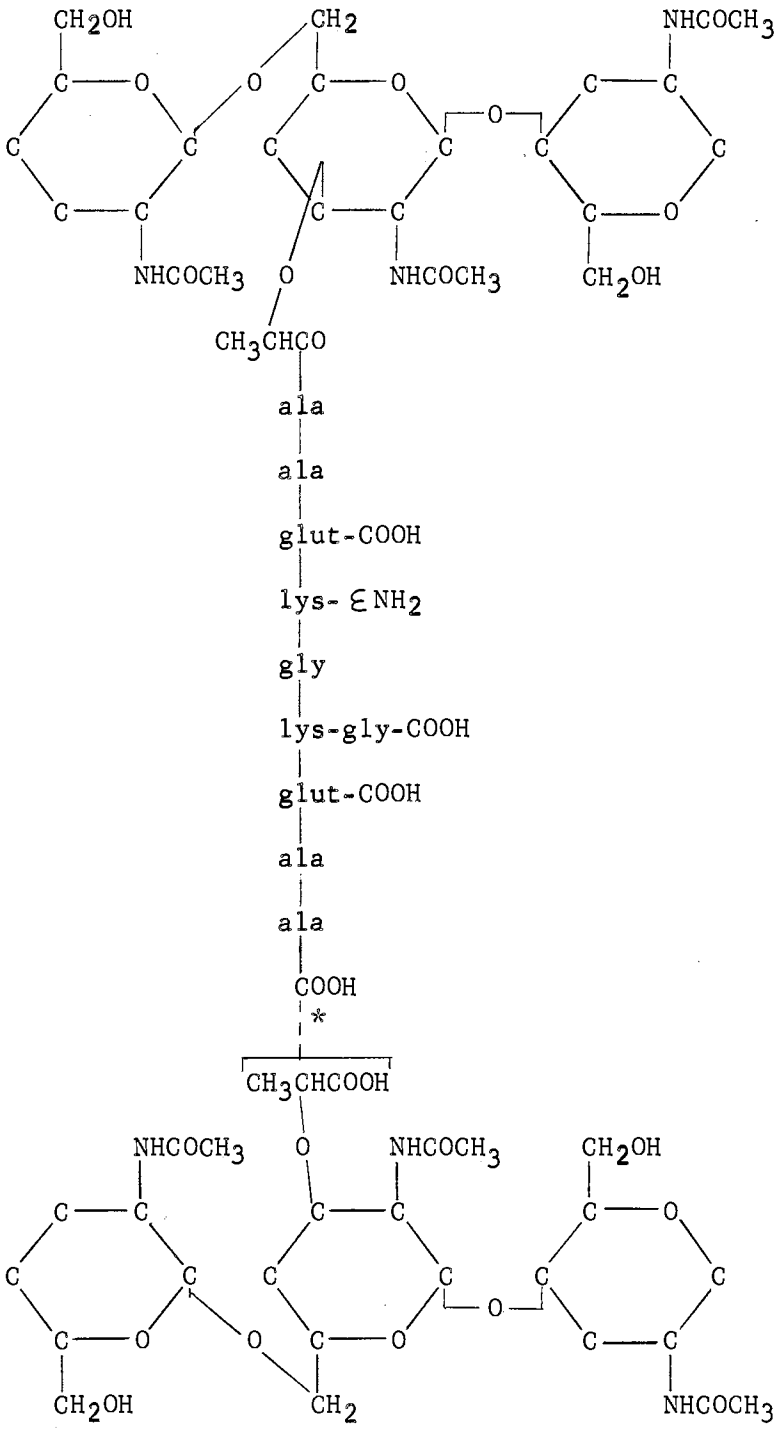
Later a somewhat different method for preparing cell wall material involving the use of the enzymes trypsin, ribonuclease, and pepsin was described by Cummins and Harris (5). Qualitative chemical examination

of acid hydrolysates of cell wall material isolated by either method revealed that the cell wall had a rather simple chemical composition (5, 6). Walls obtained from most species contained only a few amino acids (glutamic acid, glycine, lysine or diaminopimelic acid, alanine, and aspartic acid), some in the D-configuration, one or two hexoses, and two hexosamines (7-10). The hexosamines usually present were N-acetylglucosamine and N-acetylmuramic acid, the 3-O-alpha-carboxyethyl derivative of N-acetylglucosamine. Muramic acid has been crystallized by Strange and Dark (11) and synthesized by Kent (12). The polymer built from these molecules is the material which confers rigidity to the cell wall, and has been called mucopeptide or glycopeptide. In some cases the cell wall consists of nothing but mucopeptide, however, in certain Gram-positive species the mucopeptide is associated with a polymer of either polyribitol or polyglycerol phosphate and the amino acid alanine (13-15). These compounds have been designated as teichoic acids. Thus, the composition of the cell wall mucopeptide has been found to vary widely from one organism to another, although in some instances very similar composition have been observed.

At the present time the exact structure of the intact cell wall mucopeptide of M. lysodeikticus has not been ascertained. Rather sound inferences concerning its structure have been made however, using data obtained from chemical characterization studies of compounds released from purified cell wall mucopeptide by lysozyme and the F_1 and F_2B enzymes produced (exocellularly) by Streptomyces. A second source of useful information has been the excellent studies on the mechanism of cell wall mucopeptide synthesis using a cell-free enzyme system obtained from Staphlococcus aureus.

In 1922 Fleming (16) reported the discovery of lysozyme, an enzyme-like material which, when added to a thick suspension of M. lysodeikticus, caused rapid lysis. Following the finding by Myer et al. (17) that muco-polysaccharide served as the substrate for lysozyme, Salton (6, 18, 19) established its direct action on the isolated cell wall mucopeptide of M. lysodeikticus and confirmed the mucocomplex as the cell wall substrate. Using lysozyme and the F_1 enzymes of Streptomyces it was determined that both enzymes were specific for a $\beta(1\rightarrow4)$ linkage which occured in a proposed amino sugar polymer of the cell wall (20, 21). Following chemical characterization of the di- and tetra-saccharides thus obtained, the structure for the amino sugar polymer of the cell wall mucopeptide of M. lysodeikticus as shown in Figure 1 has been proposed (20-22). More recently Bouille and co-workers (23) have obtained data which indicates that all glycosidic linkages within the amino sugar backbone are $\beta(1\rightarrow4)$. In addition Ghuysen, Salton, and Ingram (24-28) chemically analysed the peptide moiety of the mucopeptide and proposed its structure and site of attachment to the amino sugar polymer (Figure 1).

Additional information concerning mucopeptide structure has been obtained from the studies of Strominger and co-workers (29-36) and others (37, 38) who have demonstrated the participation of a uridine nucleotide, sRNA, and phospholipid membrane transport intermediates in cell wall synthesis of S. aureus (Figures 2, 3, and 4). The involvement of sRNA seems to indicate that the mechanism for synthesis of the penta-glycine bridge for cross-linking may be similar to normal protein synthesis. The ramainder of the peptide, however, is synthesized by another mechanism, perhaps specific for the cell wall, since Hancock and Park (39) and Whitney and Grula (40) have shown that cell wall mucopeptide



*Exact bonding not known.

Figure 1. Proposed Cell Wall Mucopeptide Structure in M. lysodeikticus

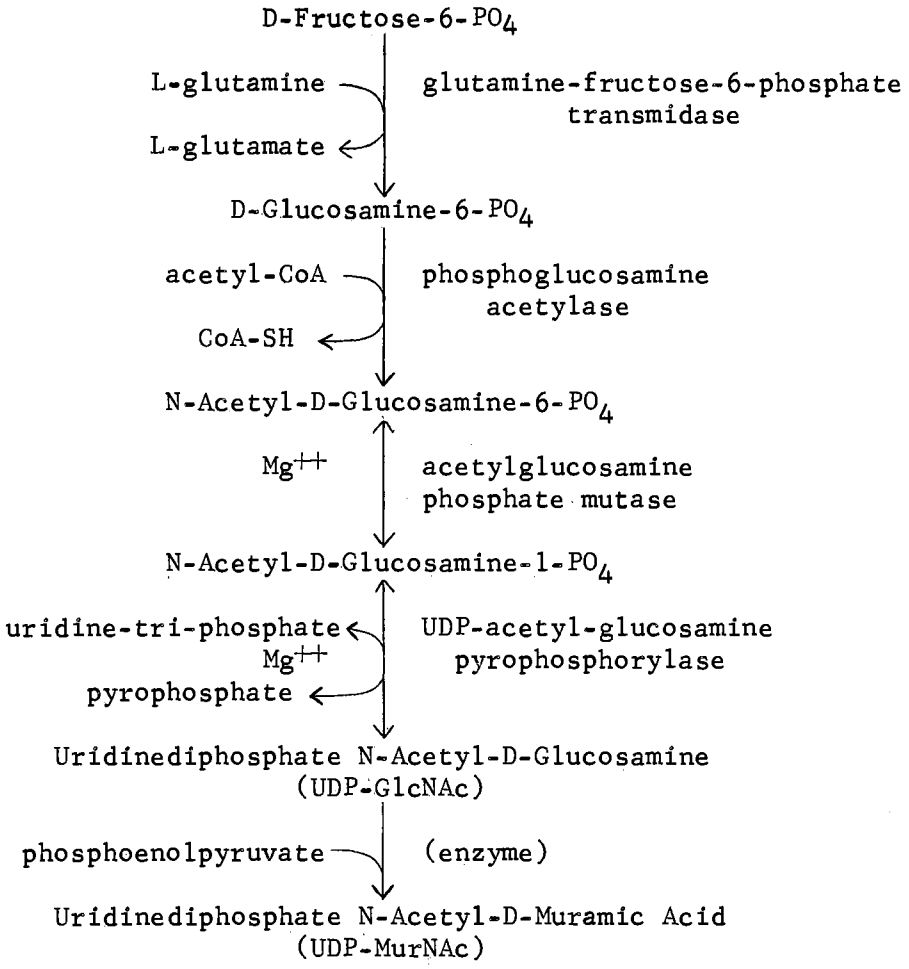


Figure 2. Cell Wall Synthesis in S. aureus

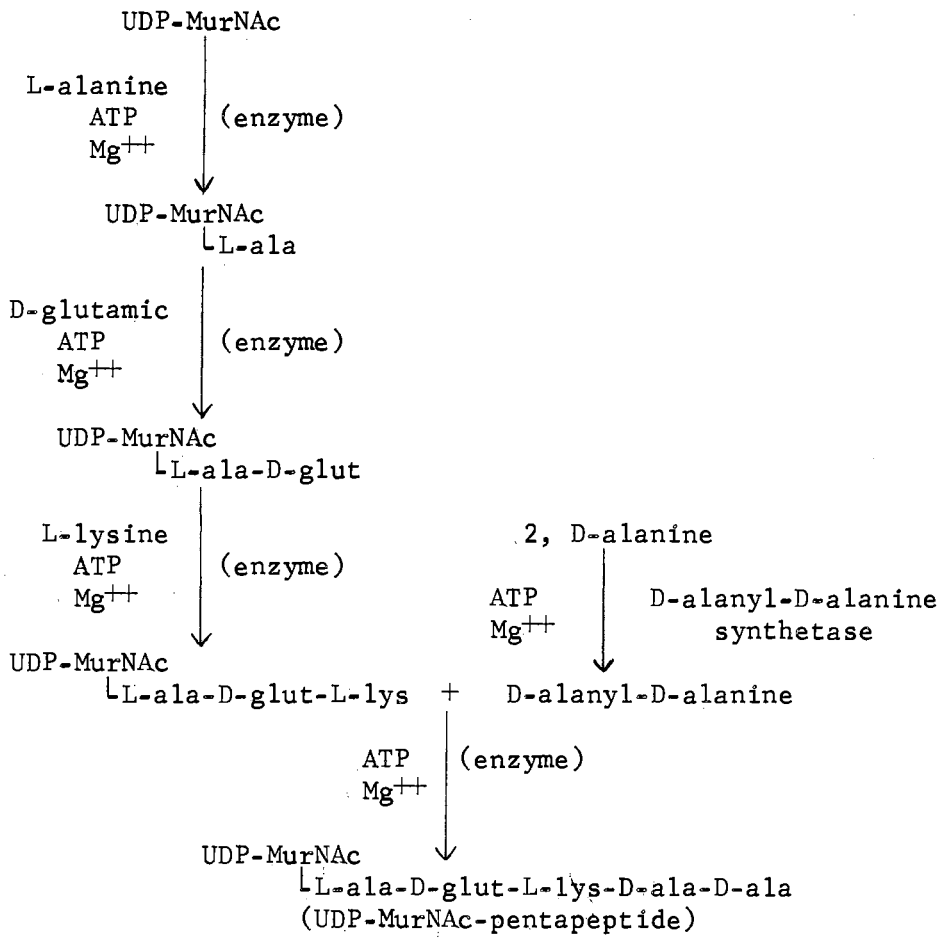


Figure 3. Cell Wall Synthesis in S. aureus

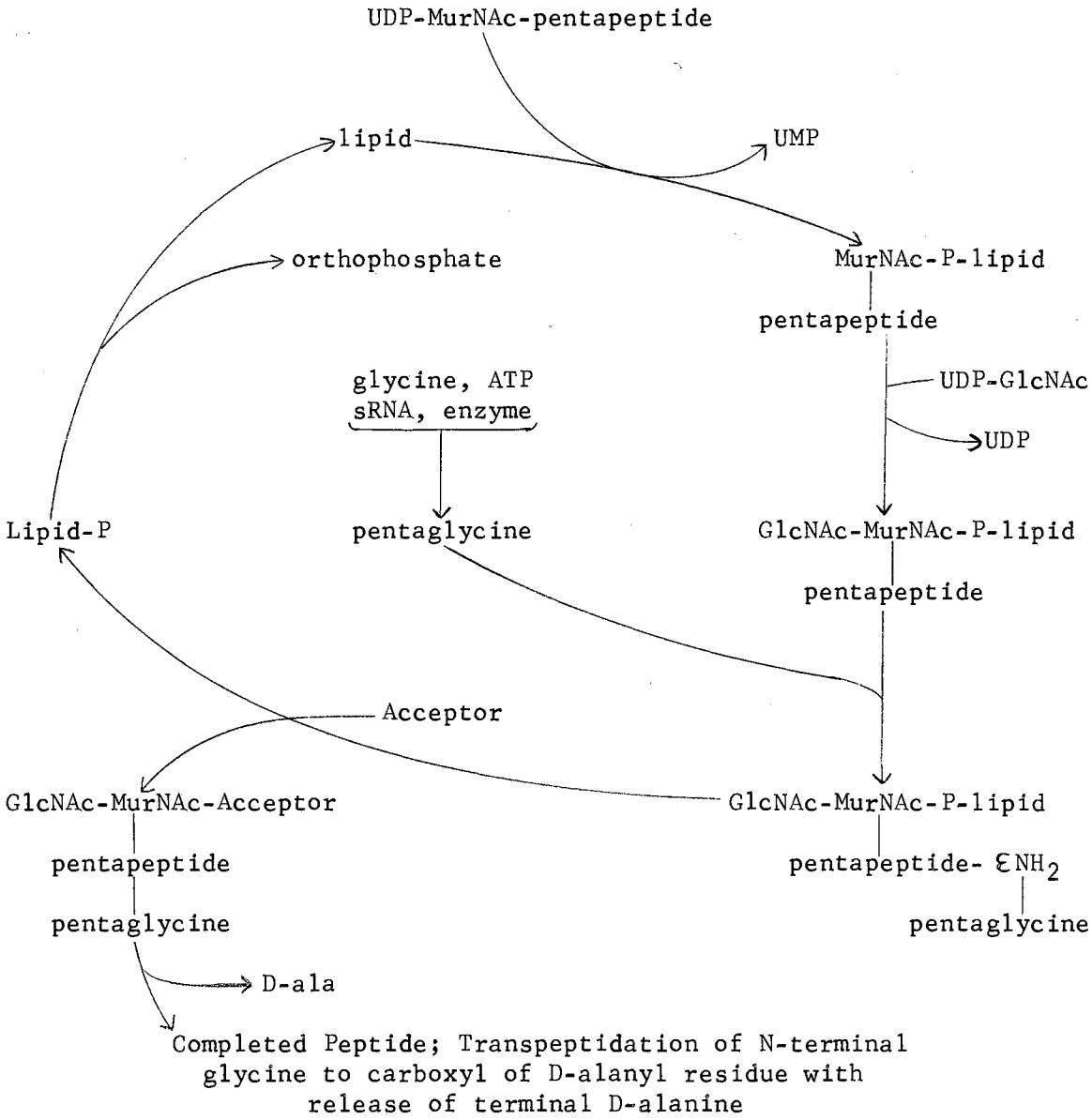


Figure 4. Cell Wall Synthesis in S. aureus

synthesis continues in the presence of actinomycin-D, puromycin, and D-chloramphenicol, all of which are known inhibitors of normal protein synthesis.

In contrast with the earlier proposed structure for the mucopeptide of M. lysodeikticus, (Figure 1), the latest information supports the structure as given in Figure 5 (41, 42). Some information which is being used to formulate possible sites of cross-linking has been published by Petit and co-workers (41) and substantiated in this study. The molar ratio of the mucopeptide components is found to be as follows: N-acetylglucosamine, N-acetylmuramic acid, alanine, glutamic acid, glycine, and lysine (1:1:2:1:1:1). The alanine attached to the muramic acid is the L-isomer while the terminal alanine is the D form. Twenty percent of the terminal alanine is bonded, via its carboxyl group, to the epsilon-amino group of lysine, twenty percent is C-terminal, and the carboxyl of the remaining sixty percent cannot be detected in either a free or bonded state. The remainder of the lysine possess a free epsilon-amino group. All of the glycine is C-terminal, being attached via its amino group to glutamic acid.

In 1963 Perkins (10) isolated a glucose rich polymer from the mucopeptide of M. lysodeikticus which had associated with it an equal molar quantity of acetamidomannuronic acid. The mode of linkage between this polymer and the cell wall mucopeptide is not known, nor has any further report of this polymer been published.

Autolysis and the optimum conditions for its occurance have been studied in a variety of Gram-positive and Gram-negative organisms (43-52). Most findings thus far indicate that autolytic systems are most active near the end of the exponential phase of growth and are highly specific

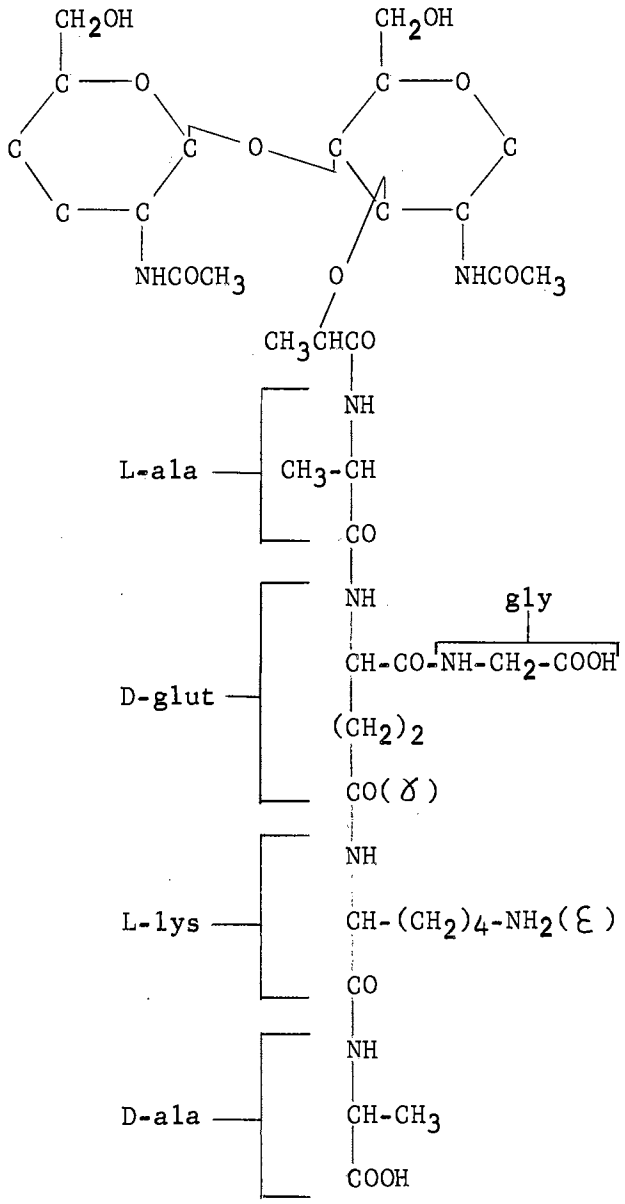


Figure 5. Proposed Cell Wall Mucopeptide Structure in M. lysodeikticus

(43, 44, 48, 49). Extracts of Streptococcus faecalis cultures in the exponential phase increased the rate of lysis of S. faecalis cells taken from the exponential phase of growth and of isolated cell walls from such cells. Cells or isolated cell walls from stationary phase cultures, however, were resistant to the same extracts. Autolysis has been observed in several suspending media including phosphate, sodium acetate, and tris hydroxymethylaminomethane buffers and sodium chloride solution (49, 50). In most studies, pH ranges near neutrality have been employed, since acid conditions appear to be inhibitory (49). The extract activities (enzymes) are nondialyzable, heat-labile, and do not appear to be a lysozyme. This latter point is borne-out by their failure to lyse M. lysodeikticus (50). Young (52) has shown that the autolytic enzyme from B. subtilis is an N-acyl muramyl-L-alanine amidase which solubilizes the cell wall by hydrolyzing the amide bond between N-acetyl muramic acid and L-alanine.

Most workers agree that these enzymes are involved in wall growth and cell division (44, 46, 51). Perhaps the enzyme breaks existing bonds such that a piece of new wall polymer can be inserted, thus allowing for the growth of the cell wall (intercalary growth?).

An excellent review of autolysis has been recently published by Shockman (53).

Cummins and Harris (5), having found that the cell wall composition of several bacteria was unchanged after growth on different complex media, proposed the use of bacterial cell wall composition as a taxonomic criterion. Since their published studies, several laboratories have reported nutritional modifications of the cell wall mucopeptide.

When hydroxylysine is present in the growth medium, it can be in-

corporated into the cell wall mucopeptide by S. faecalis, Leuconostoc mesenteroides, and S. aureus (54-56). In S. faecalis this uptake and incorporation can be inhibited by the addition of lysine to the growth medium. Incorporation of hydroxylysine in S. faecalis resulted in an increased resistance to autolysis, decreased penicillin susceptibility (when postexponentially incorporated), and no change in lysozyme susceptibility (57). Lark and Lark (58) have reported the incorporation of D-methionine into the cell wall mucopeptide of Alcaligenes faecalis after growth in the presence of C^{14}-labeled D-methionine. This modification resulted in defective wall synthesis leading to spheroplasting of the cells. Snell, Radin, and Ikawa (59) have shown that S. faecalis, when grown in the presence of D-alpha-aminobutyric acid, incorporates this compound into its cell wall in place of the naturally occurring component D-alanine. Rhuland and Hamilton (60) found that Escherichia coli could replace the alpha, epsilon-diaminopimelic acid of its mucopeptide with gamma-methyldiaminopimelic acid when the latter was present in the growth medium with lysine. Also working with E. coli, Sundharadas and Gilvarg (61) showed the incorporation of beta-hydroxy-alpha, epsilon-diaminopimelic acid in place of alpha, epsilon-diaminopimelic acid, a natural component of the cell wall layer. Here too, lysine was required for the alteration. Whitney and Grula (62) reported the incorporation of D-serine into the mucopeptide of M. lysodeikticus at the expense of glycine. The details of this alteration will be discussed in this study.

The experiments reported in this thesis were undertaken to determine if the cell wall mucopeptide of M. lysodeikticus could be qualitatively and/or quantitatively modified by nutritional alteration of the growth

medium. If accomplished, the physiological effect(s) of such modifications, and the mechanism(s) by which they occurred were to be investigated.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The organism used throughout this study was a stock culture of M. lysodeikticus, strain PU (Purdue University). Though not listed in the 7th edition of Bergey's Manual of Determinative Bacteriology, a rather complete characterization has been published by Grula (63). The organism is a Gram-positive, non-motile coccus which is easily lysed by lysozyme. Optimum temperature for growth is 30 C, although growth is obtained at 37 and 25 C.

Stock cultures were maintained on nutrient agar slants with transfers made daily. To insure purity the culture was periodically streaked on nutrient agar plates and isolated colonies checked for characteristic pigment production and Gram reaction.

Media

The basal medium used throughout this study was that utilized by Grula, Luk, and Chu (64), and Grula (63) which was modified to contain the following per 100 ml: biotin (50 ug), inosine (6 mg), L-glutamic acid (358 mg), L-phenylalanine (40 mg), L-tyrosine (30 mg), NH_4CL (100 mg), Na_2HPO_4 (200 mg), $MgSO_4 \cdot 7HOH$ (2 mg), and $FeSO_4(NH_4)_2SO_4 \cdot 6HOH$ (0.25 ug). The medium was adjusted to pH 7.6 to 7.8 using solid potassium

hydroxide and sterilized by autoclaving 12 minutes at 10 pounds pressure. The ferrous ammonium sulfate solution and solutions of desired test compounds were sterilized separately either by filtration (millipore) or autoclaving (12 minutes at 10 pounds pressure) and added aseptically.

Growth of Cells

Cells used for inoculation purposes were grown for 22 to 24 hours on nutrient agar slants at 30 C, washed twice in 5.0 ml of 0.85% sterile saline solution, and resuspended in sterile saline to an optical density of 0.22 to 0.26 (Spectronic 20). One drop of the resulting cell suspension was used to inoculate 5.0 ml of medium. Five ml volumes of medium were incubated in tubes (17 mm I.D.) while volumes of 25 ml or more were incubated in 250 ml Erlenmeyer side-arm flasks. Because aeration is necessary for optimum growth cells were incubated at 30 C with shaking. Cells were harvested by centrifugation after the appropriate incubation period.

Determination of Lysozyme Susceptibility

Cells to be checked for their susceptibility to the action of lysozyme were harvested by centrifugation, washed twice in 5.0 ml of 0.85% saline solution, and resuspended in saline to an optical density of 0.6 to 0.8 (Spectronic 20). Two ml of the resulting cell suspension was mixed with two ml of lysozyme solution (10 mg per 100 ml 0.85% saline) and the change in optical density observed for 150 seconds at 540 mu using the Spectronic 20. In order to compare cells from various growth situations the percent lysis was calculated using the following equation:

$$\frac{\text{Initial Optical Density - Optical Density at 150 Seconds}}{\text{Initial Optical Density}} \times 100 = \% \text{ lysis}$$

Cell Fractionation Procedure

All cell fractionations were made using the chemical procedure of Park and Hancock (65).

Hydrolysis Procedures

Complete hydrolysis of protein or cell wall was performed by placing 0.5 ml of sample in an 8x100 mm test tube. An equal volume of 12 N hydrochloric acid was added and the tube sealed *in vacuo*. Hydrolysis was allowed to proceed for 18 hours at 105 C.

The hydrolytic technique of Ellwood, Keleman, and Baddiley (66) was used for detecting O-ester linked amino acids.

Release of amino sugars from cell wall was accomplished by hydrolysis in 4 N hydrochloric acid for 4 hours at 105 C *in vacuo*.

Hydrolysis of the cell wall mucopeptide for the purpose of studying rates of release of amino acids and obtaining partial cell wall peptides was carried out in 6 N hydrochloric acid for one to 10 hours at 55 C.

The hydrolysates were taken to dryness twice under a stream of hot air and the residues dissolved in deionized water for subsequent analyses.

Titration of Test Compounds

All titrations were made by adding various concentrations of the compounds under study to 5.0 ml (final volume) of basal medium. Following 40 hours of incubation optical density readings were taken at 540 mu

using either the Spectronic 20 or the Coleman Junior Spectrophotometer. Concentrations which gave either a stimulation or an inhibition of growth were used in subsequent experiments.

Chromatography

For amino acid analysis hydrolysates were spotted on 8x8 inch sheets of Whatman #1 filter paper and developed (ascending) in the two-dimensional system of either Redfield (67) or Roberts et al. (68). After development, chromatograms from the Redfield system were autoclaved at 121 C for 30 minutes to remove residual diethylamine. Amino acids were detected by spraying with a solution of 0.5% ninhydrin in 95% acetone containing 5% deionized water (V/V). After spraying, the chromatograms were heated at 100 C for 5 minutes. Amino acids appear as blue, yellow, or reddish-brown spots on a white background.

Hydrolysates used for the isolation of serine were band spotted on 1.5x22 inch strips of Whatman #1 chromatography paper and developed (descending) in n-butanol:pyridine:water (6:4:3) for 60 hours at 25 C.

Hydrolysates for amino sugar analysis were spotted on 8x8 inch sheets of Whatman #1 filter paper and developed (ascending) in the two-dimensional system of Redfield (67). After development, chromatograms were autoclaved at 121 C for 30 minutes to remove residual diethylamine. Amino sugars were detected using the spray technique of Partridge and Westall (69).

The thin-layer chromatographic technique of Morse and Horecker (70) was used to separate the 1-dimethylaminophythalene-5-sulfonyl (DNS) amino acid derivatives.

Amino Acid Quantitation

Amino acids were quantitated according to the procedure of Giri, Radhakrishnan, and Vaidyanathan (71). Standard curves were prepared for each amino acid after chromatography in each of the solvent systems. The standard curve was determined using a concentration of 10 to 200 mumoles of amino acid per spot.

Radioautography

The presence or absence of radioactivity in various compounds was determined by exposing developed chromatograms to Blue Brand X-ray film for 21 days and developing in Diafine. This procedure permitted good detection of 100 counts per minute as determined by a Picker gas-flow automatic planchet counter operated windowless.

Counting Procedure

All samples were plated at infinite thinness on stainless steel planchets and counted using a Picker automatic gas-flow planchet counter operated windowless. A counting efficiency of approximately 40% can be obtained with this unit. All planchets were discarded after one use.

Dry Weight Determination

The relation between dry weight and absorbancy at 540 mu was determined for cells grown in basal medium on both a Coleman Junior and a Spectronic 20 Spectrophotometer. Cells which had grown for 25 hours were washed twice in 0.85% saline solution and once with deionized water. Serial dilutions were made in water from the resulting cell

suspension and the absorbancy of each sample measured. Aliquots were placed into dried, pre-weighed aluminum dishes and heated at 100 C in a dry air oven to a constant weight. Results were plotted as mg dry weight of cells versus absorbancy at 540 mu for both instruments.

Serine Isolation and Configuration Studies

Cells were grown in the presence of $5x10^{-4}$ M D-serine in 50 ml of basal medium for 40 hours, harvested, and fractionated. The trypsin digest, used as representative cellular protein, and the cell wall mucopeptide were subjected to amino acid hydrolysis and chromatographed for serine isolation. For control purposes, a sample of crystalline trypsin was carried through the same hydrolytic and chromatographic procedures. Areas containing serine were determined through the use of control strips spotted with known amino acids. These areas were removed and eluted with deionized water for 40 hours at 4 C. The samples were taken to dryness under a stream of warm air, redissolved in deionized water, and checked for purity in the chromatographic system of Redfield (67). These samples were used for configuration studies.

The D-amino acid oxidase (Worthington Biochemical Company, Freehold, New Jersey) preparation consisted of 50 ml of a 1% solution in 0.05 M pyrophosphate buffer (pH 8.5), dialysed against 1000 ml of the above buffer at 4 C for 6 hours. The assay was performed by mixing equal volumes (0.5 ml) of the enzyme preparation and the amino acid solution and incubating for 16 hours at 37 C under toluene.

The assay system for L-serine consisted of 0.22 ml amino acid solution (substrate), 0.30 ml Tris-hydrochloric acid buffer (pH 7.8), 0.20

ml L-amino acid oxidase (Worthington Biochemical Company, Freehold, New Jersey, 0.5 mg per ml of 0.1 M potassium chloride), and 0.50 ml potassium chloride (0.1 M). The mixture was incubated at 37 C for 3 hours.

All keto acid determinations were performed using the technique of Haidle and Knight (72).

Serine isolated from cellular protein and cell wall mucopeptide was also assayed by Dr. Mary M. Grula using an unpublished technique.

Uptake and Incorporation of D-Serine-3-C^{14}

Cells were grown for 25 hours in 50 ml of basal medium. At that time 19.5 ml of cells were added to flasks containing various amino acids and 0.05 uC of D-serine-3-C^{14}. The final concentration of each amino acid was $5x10^{-4}$ M. Incubation was continued for 30 minutes in a Dubnoff water bath shaker. After measuring absorbancy at 540 mu on a Coleman Junior Spectrophotometer the cells were washed two times in 0.85% saline solution and fractionated. The radioactivity of the cell pools, protein, and cell walls was determined. Results are expressed as counts per minute per mg dry weight of cells fractionated.

Labeled Sulfur Experiments

Cells were grown for 25 hours in two 50 ml volumes of basal medium. At this time 0.2 ml of sodium sulfate (S^{35}) was added to each, such that the final concentration was 0.2 uC per ml medium. To one flask D-serine was added to a concentration of $5x10^{-4}$ M. Incubation was continued for 15 hours. After measuring the absorbancy at 540 mu on a Coleman Junior Spectrophotometer the cells were washed twice in 0.85% saline solution and fractionated. The protein fraction was subjected to acid hydrolysis

for amino acids and chromatographed in the systems of Redfield (67) and Roberts et al. (68). Radioautography was employed to identify labeled compounds.

Amino Acid Labeling Experiments

Cells were grown in 100 ml of basal medium for 40 hours in the presence of 10 uC of various C^{14}-labeled amino acids. Following optical density readings at 540 mu on a Coleman Junior Spectrophotometer the cells were washed twice in 0.85% saline solution and fractionated. The protein and cell wall mucopeptide fractions were subjected to acid hydrolysis for amino acids and analysed for radioactive compounds by radioautography. A sample of cell wall mucopeptide was also hydrolysed for amino sugars and analysed by radioautography.

Induction Experiments

The test system consisted of cells grown in basal medium in a Dubnoff water bath shaker. At an optical density of 0.5 (540 mu, Spectronic 20), D-glucose was added to a final concentration of 0.2%. After 30 minutes either actinomycin-D (10 ug per ml medium), puromycin (45 ug per ml medium), or D-chloramphenicol (100 ug per ml medium) was added. Following an additional 30 minute incubation D-serine-3-C^{14} (0.1 uC per ml medium) or glycine-2-C^{14} (0.04 uC per ml medium) was added. Unlabeled D-serine or glycine was also added such that the final concentration was 5×10^{-4} M. After one hour, optical density readings were made (540 mu, Spectronic 20), the cells immersed in an ice bath, washed in cold deionized water, and fractionated. The cell wall mucopeptide was hydrolysed, counted for radioactivity, and the labeled

compounds identified by radioautography. The radioactivity of the protein fraction was also determined. Results are expressed as counts per minute per mg dry weight of cells fractionated.

Autolytic Studies

Cells were grown in 50 ml basal medium for 25 hours giving an optical density of 0.30 on a Coleman Junior Spectrophotometer. Five ml aliquots were washed twice in either phosphate buffer (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} M, pH 7.0), 0.85% saline solution, or deionized water. After resuspension in the above solutions one-half of the samples were heated at 100 C for 15 minutes. Incubation was then con tinued, with and without shaking, at 30 C. Periodic optical density readings were made on a Coleman Junior Spectrophotometer. After 24 hours samples were taken for crystal violet staining and observation using the electron microscope.

Cell Wall Turnover Studies

Cells were grown in the presence of 10 uC of the desired isotope in 50 ml basal medium to an optical density reading (Spectronic 20) of 0.50. Cells were spun out, washed twice in 0.85% saline solution, resuspended in 50 ml basal medium, and reincubated for 4 hours. Following an optical density reading using a Spectronic 20, one-half of the cell suspension was harvested by centrifugation (27,000 X G for 15 minutes) and fractionated for cell wall mucopeptide. The remaining cells were washed in 0.85% saline solution, resuspended in 100 ml basal medium, and reincubated. Following a several fold increase in mass an optical density reading (Spectronic 20) was taken and the cells fractionated for

cell wall mucopeptide. Specific activities are expressed as counts per minute per mg dry weight of cell walls isolated or counts per minute per ug of cell wall amino acid. Changes in mass and specific activity are represented by "fold" increases or decreases. For example, a two-fold increase.

Formation of Dansyl Derivatives

The technique of Gray and Hartley (73) was used to form all dansyl derivatives.

Hydrazinolysis Procedure

The procedure of Akabori, Ohno, and Narita (74) was used throughout this study.

Electron Microscopy

Cells were grown in 5.0 ml basal medium for 40 hours. Following growth, cells were prefixed directly in the growth medium by use of a final concentration of 0.10% osmium tetroxide for 30 minutes at 25 C. After centrifugation, the cells were fixed for an additional one hour in 2 ml of 1.0% osmium tetroxide in Veronal buffer (pH 6.1) under R-K conditions at 25 C (75). Cells were then washed three times in Veronal buffer and postfixed in 0.5% uranyl acetate in Veronal buffer (pH 3.5) for 24 hours at 25 C (76). The cells were again washed three times in Veronal buffer and embedded in agar blocks. The agar blocks were dehydrated through an ethyl alcohol series (25 to 100%), treated with three changes of propylene oxide (10 minute treatments), and placed in an equal mixture of propylene oxide and complete Araldite resin monomer

for 2 hours. Another equal part of complete resin monomer was then added, mixed, and allowed to infiltrate overnight. Araldite (100%) was then placed on the blocks for 24 hours at room temperature. The Araldite monomer was prepared according to the method of Luft (77). Gelatin capsules (size 00) were filled with fresh monomer, and the blocks were placed on the surface and allowed to settle to the bottom of the capsules. The blocks were then polymerized for 72 hours at 62 C.

Sections were cut on a Porter-Blum MT-2 microtome and transferred to uncoated 400-mesh copper grids. They were then stained 5 minutes with 2.0% aqueous uranyl acetate and 15 minutes with 0.4% lead citrate, washed in distilled water, dried on filter paper, and examined in an RCA EMU 3-G electron microscope using 100 KV accelerating voltage (78).

Cells and cell wall preparations were placed on 200-mesh copper grids, washed three times in distilled water, and dried on filter paper. Specimens were shadowed with chromium at a 15 degree angle and observed using 100 KV of accelerating voltage.

CHAPTER III

RESULTS AND DISCUSSION

Medium Alterations

Shortly after the initiation of this investigation difficulty was encountered in obtaining reproducible growth responses by M. lysodeikticus in the defined medium as published by Grula, Luk, and Chu (64). Since a great portion of this study would involve nutritional modifications it seemed advisable to reassess the medium and make necessary alterations to maximize and stabilize the growth response.

First, the effect of the addition of various vitamins, alone and in combinations, to the existing medium, which already contained biotin, was checked. The results of this study are presented in Table I. No vitamin, or combination thereof, significantly increased growth over that of the control situation. As a result no change was made in the vitamin content of the medium.

Consideration was next given to the possibility that during sterilization by autoclaving toxic breakdown products of medium components were formed. To check this point two samples of medium were compounded; one was sterilized by autoclaving; the other by filtration. The results are shown in Table II. The method of sterilization has no effect on growth of the organism and sterilization by autoclaving was continued.

Because of the frequent appearance of a white precipitate on the

TABLE I

VITAMIN ASSAY: GROWTH RESPONSE OF M. LYSODEIKTICUS

Vitamin addition*	Optical density at 540 mu** at 40 hours
none (control)	0.225
folic acid	0.223
p-aminobenzoic acid (PABA)	0.230
calcium pantothenate	0.200
pyridoxine-HCl	0.253
riboflavin	0.170
pyridoxal-5-PO_4	0.264
pyridoxamine-di-HCl	0.188
thiamine-HCl	0.161
niacin	0.217
folic acid + PABA	0.215
pyridoxine-HCl + riboflavin	0.135
pyridoxal-5-PO_4 + thiamine-HCl	0.238

*Vitamin concentration of 0.5 ug per ml of medium.
**Coleman Junior Spectrophotometer (tube size 10x85 mm I.D.).

TABLE II

METHOD OF STERILIZATION: EFFECT ON GROWTH RESPONSE OF M. LYSODEIKTICUS

Method of sterilization	Optical density at 540 mu* at 40 hours
autoclave (12 minutes at 10 pounds pressure)	0.07
filtration (millipore)	0.04

*Coleman Junior Spectrophotometer.

walls of the incubation tubes the next factors taken into consideration were the concentrations of tyrosine, sodium and potassium ions, and the temperature of incubation. Prior to this, potassium phosphate had been added to the medium for buffering, the pH of the medium had always been adjusted with potassium hydroxide, and incubation carried out at 37 C. Tyrosine had been added at a concentration of one mg per ml of medium. The alterations that were made and results obtained are presented in Table III. The greatest effect on growth, of the three factors checked, was the lowered incubation temperature. Also indicated, was the need for some balance between the sodium and potassium ions. Because the increased level of tyrosine seemed to have no effect on growth, its concentration was reduced to 0.30 mg per ml of medium. The temperature of incubation was lowered to 30 C and the sodium phosphate was incorporated into the medium. Following this series of experiments the growth medium as indicated in the Materials and Methods section was used. A typical growth curve in this medium is given in Figure 6.

Nutritional Studies: Effects on Growth and Lysis by Lysozyme

Micrococcus lysodeikticus is extremely sensitive to lysis by the enzyme lysozyme, which cleaves the $\beta(1\rightarrow4)$ linkage present in cell wall mucopeptide. This fact served as the basis for the assay used to detect nutritional modifications of the cell wall. Since lysozyme causes hydrolysis of the mucopeptide we felt that an alteration of this structure might be reflected in a change in susceptibility of the organism to the enzyme.

Growth of the organism in the presence of various compounds and the

TABLE III

EFFECT OF CHANGES OF VARIOUS MEDIUM COMPONENTS AND INCUBATION TEMPERATURE ON THE GROWTH RESPONSE OF M. LYSODEIKTICUS

Tyrosine concentration (mg per ml medium)	Phosphate added Na_2HPO_4	Phosphate added K_2HPO_4	Base for pH adjustment NaOH	Base for pH adjustment KOH	Incubation temperature 37 C	Incubation temperature 30 C	Optical density at 540 mu* at 40 hours
1.0	-	+	-	+	+	-	0.065
1.0	-	+	-	+	-	+	0.670
1.0	-	+	+	-	+	-	0.099
1.0	-	+	+	-	-	+	0.710
1.0	+	-	-	+	+	-	0.072
1.0	+	-	-	+	-	+	0.712
1.0	+	-	+	-	+	-	0.076
1.0	+	-	+	-	-	+	0.413
0.75	-	+	-	+	+	-	0.071
0.75	-	+	-	+	-	+	0.660
0.50	-	+	-	+	+	-	0.076
0.50	-	+	-	+	-	+	0.610
0.50	+	-	-	+	+	-	0.076
0.50	+	-	-	+	-	+	0.620
0.50	+	-	+	-	+	-	0.059
0.50	+	-	+	-	-	+	0.445
0.50	-	+	+	-	+	-	0.043
0.50	-	+	+	-	-	+	0.620
0.20	-	+	-	+	+	-	0.071
0.20	-	+	-	+	-	+	0.565

*Coleman Junior Spectrophotometer.

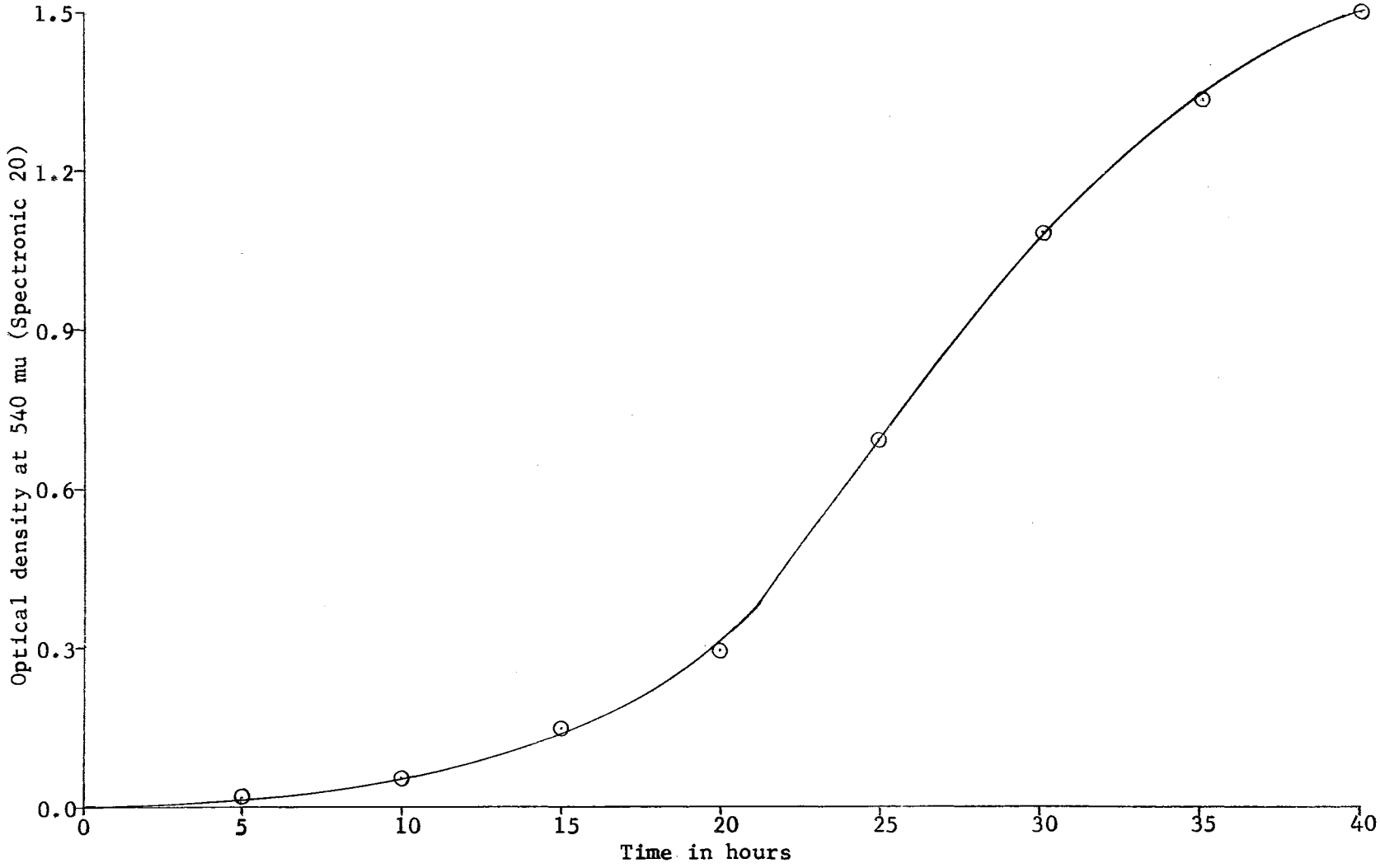


Figure 6. Growth of M. lysodeikticus in Defined Medium

assay of the cells thus obtained were performed as previously described. Representative data are given in Table IV. As indicated, little or no change in lysozyme susceptibility was found, although several of the compounds markedly inhibited growth.

Nutritional Studies: Qualitative and Quantitative Effects on the Amino Acids of the Mucopeptide

Since the lysozyme assay would measure an alteration in the rate of enzymic cleavage of only one bond in the cell wall, it was surmised that a modification could be occurring which would go undetected. As a result, experiments were initiated in which the amino acid portion of the mucopeptide of cells from various growth situations was qualitatively and quantitatively analysed. The results of these studies are presented in Table V. Of the amino acids tested only D-serine, which appears to be incorporated at the expense of glycine, produced a modification. As shown in Table IV this alteration does not change the lysozyme susceptibility, indicating that the amino acid composition of the peptide portion of the cell wall has little or no effect on the action of this enzyme. In addition, no morphological abnormalities such as protoplast or filament formation have been observed. The greatest variation in the cell wall caused by D-serine involves the amount of glycine, which is reduced to about one-half of normal. Also, the amount of serine incorporated exceeds the amount required only to replace the missing glycine and therefore, the possibility exists that the dimensions of the wall peptide may have been increased.

TABLE IV

EFFECT OF VARIOUS COMPOUNDS ON GROWTH AND LYSOZYME SUSCEPTIBILITY OF M. LYSODEIKTICUS

Medium addition	Concentration*	Optical density at 540 mu** at 40 hours	Percent lysis
none	-	1.50	87.5
adonitol	$3x10^{-1}$	1.00	87.7
D-mannitol	$3x10^{-1}$	1.20	89.3
D-sorbitol	$3x10^{-1}$	1.20	86.9
sodium formate	$7x10^{-1}$	1.10	86.4
D-mannose	$3x10^{-1}$	1.10	85.9
rhamnose	$3x10^{-1}$	0.90	85.6
sodium acetate	$6x10^{-1}$	1.40	89.7
sodium pyruvate	$5x10^{-1}$	1.50	86.6
glucose	$3x10^{-1}$	1.50	91.3
D-glucosamine	$5x10^{-4}$	0.49	88.0
N-acetylglucosamine	$2x10^{-2}$	1.47	87.0
raffinose	$8x10^{-2}$	1.50	89.0
D-aspartic acid	$2x10^{-3}$	1.50	89.0
D-valine	$2x10^{-3}$	1.30	90.0
D-methionine	$2x10^{-3}$	0.78	80.0
D-serine	$5x10^{-4}$	0.58	89.0
beta-alanine	$1x10^{-1}$	0.16	89.0
D-alanine	$2x10^{-3}$	1.49	88.0
D-threonine	$1x10^{-1}$	0.34	90.0
D-malic acid	$2x10^{-3}$	1.22	89.0
D-histidine	$6x10^{-3}$	0.94	91.0
D-phenylalanine	$6x10^{-3}$	1.02	89.0
D-glutamic acid	$5x10^{-3}$	1.50	87.0
D-tryptophan	$8x10^{-3}$	0.82	88.0
N-acetyl-D-alanine	$2x10^{-3}$	1.50	89.0
DL-alanyl-DL-serine	$2x10^{-3}$	1.50	86.0

*Molar concentration in growth medium.
**Spectronic 20 Spectrophotometer.

TABLE V

AMINO ACID COMPOSITION OF THE CELL WALL MUCOPEPTIDE FROM M. LYSODEIKTICUS GROWN IN THE PRESENCE OF VARIOUS AMINO ACIDS

Medium addition*	Amino acid molar ratios**									
	Alanine	Glycine	Glutamic acid	Lysine	Serine	Threonine	Histidine	Valine	Methionine	Aspartic acid
none	2.1	1.1	1.0	1.0	-	-	-	-	-	-
D-serine	2.1	0.4	1.0	1.0	0.9	-	-	-	-	-
L-serine	2.3	1.2	1.0	1.0	0.0	-	-	-	-	-
D-threonine	2.0	1.1	1.0	1.0	-	0.0	-	-	-	-
D-histidine	2.1	1.2	1.0	1.0	-	-	0.0	-	-	-
D-valine	2.1	1.1	1.0	1.0	-	-	-	0.0	-	-
D-methionine	1.9	1.1	1.0	1.0	-	-	-	-	0.0	-
D-aspartic acid	2.0	1.2	1.0	1.0	-	-	-	-	-	0.0

*Amino acids added to concentration of 5×10^{-4} M.
**Glutamic acid taken as 1.0. Amino acids not listed were not present.

Compounds Reversing the Effects of D-Serine

Attention was next given to compounds which might aid in reversing or preventing the growth inhibition and cell wall modification caused by D-serine. Tables VI and VII contain the results of these experiments. Glycine and L-serine almost completely prevent the growth inhibition caused by D-serine. Addition of L-alanine results in some reversal, however, restoration of growth is better using D-alanine. The other compounds examined had little or no effect. Incorporation of serine into the mucopeptide is decreased in the presence of D- or L-alanine, L-serine, and glycine. Where serine incorporation is reduced by glycine or D-alanine, glycine levels increase to what may be considered normal. In no case, however, is serine incorporation completely inhibited.

Studies using D-serine-3-C^{14} (Table VIII) revealed that all four amino acids which decrease incorporation of serine into cell wall (D- and L-alanine, L-serine, and glycine) decrease the amount of label from D-serine in intracellular pools. Therefore at least part of the reversal of the effects of D-serine by these amino acids appears to be due to their competition with D-serine at the cell entry level.

Incorporated Serine: Configuration and Site of Attachment

Having established the incorporation of serine, it next became of interest to attempt to determine the optical configuration and site of attachment of the serine in the cell wall.

Serine obtained from the mucopeptide of cells grown in the presence of D-serine was reacted with L- and D-amino acid oxidase enzymes. The

TABLE VI

EFFECT OF VARIOUS COMPOUNDS ON GROWTH INHIBITION BY D-SERINE IN M. LYSODEIKTICUS

Medium additions	Concentration of reversing agents*	Optical density** at 40 hours
none	-	0.59
D-serine***	-	0.22
D-serine + D-alanine	5×10^{-4}	0.48
D-serine + L-alanine	5×10^{-4}	0.39
D-serine + glycine	5×10^{-4}	0.55
D-serine + pantoic acid	5×10^{-4}	0.19
D-serine + L-serine	5×10^{-4}	0.57
D-serine + $CaCl_2$	2×10^{-4}	0.26
D-serine + beta-alanine	2×10^{-2}	0.29
D-serine + Na-pantothenate	2×10^{-4}	0.22

*Molar concentration in growth medium.
**Coleman Junior Spectrophotometer at 540 mu.
***5×10^{-4} M concentration in all tubes.

TABLE VII

AMINO ACID COMPOSITION OF THE CELL WALL MUCOPEPTIDE FROM M. LYSODEIKTICUS GROWN IN THE PRESENCE OF VARIOUS COMBINATIONS OF AMINO ACIDS

Medium additions*	Amino acid molar ratios**				
	Alanine	Glycine	Serine	Glutamic acid	Lysine
none	2.1	1.1	0.0	1.0	1.0
D-serine	2.1	0.4	0.9	1.0	1.0
D-serine + L-alanine	2.0	0.3	0.8	1.0	1.0
D-serine + D-alanine	2.0	1.0	0.4	1.0	1.0
D-serine + glycine	2.0	1.0	0.3	1.0	1.0
D-serine + L-serine	1.9	0.5	0.6	1.0	1.0

*All amino acids added to concentration of 5×10^{-4} M.
**Glutamic acid taken as 1.0.

TABLE VIII

EFFECT OF VARIOUS AMINO ACIDS UPON THE UPTAKE AND INCORPORATION OF LABEL FROM D-SERINE-3-C^{14}

Medium additions	Cell fraction (counts per minute per mg cells fractionated)					
	Cell pools	% Inhibition	Protein	% Inhibition	Cell wall	% Inhibition
D-serine-3-C^{14}	3917	-	625	-	363	-
D-serine-3-C^{14} + D-alanine	576	85	206	67	108	70
D-serine-3-C^{14} + L-alanine	1441	63	262	58	120	66
D-serine-3-C^{14} + L-serine	1567	60	81	94	158	56
D-serine-3-C^{14} + glycine	1919	51	136	78	177	51

results of these experiments are shown in Table IX. The incorporated serine appears to be the D-isomer since treatment with D-amino acid oxidase results in the formation of keto acid whereas treatment with L-amino acid oxidase does not. Additional data obtained by Dr. Mary M. Grula, using an unpublished enzyme technique, which is quite specific for the D-isomer, also support this finding. It should be pointed out however, that the sensitivity of the L-amino acid oxidase enzyme preparation is not good as indicated by the control in Table IX. Therefore, the possibility exists that a small portion of the serine in the mucopeptide could be in the L configuration and go undetected by this technique.

Having a free hydroxyl group it seemed possible that serine might be attaching to the mucopeptide via an O-ester linkage. No evidence for such bonding was found since treatment with ammonium hydroxide caused no release of serine (66).

Detection of free amino groups by formation of dansyl derivatives was undertaken to determine if serine might be attached through its amino group. The technique of Gray and Hartley (73) was employed in all determinations. The derivatives obtained from both normal and serine-containing cell walls were identified using thin-layer chromatography in three different solvent systems (70). The Rf values obtained are presented in Table X. In all three solvent systems the Rf value which most closely matches the derivative obtained from our cell wall preparations is that of lysine. The two values given for lysine are those for the beta and epsilon amino groups, since lysine exists as a di-amino acid. A survey of the literature reveals that the dansyl derivative of the epsilon amino group generally gives the higher Rf

TABLE IX

EFFECT OF D- AND L-AMINO ACID OXIDASE ENZYMES ON THE SERINE ISOLATED FROM THE CELL WALL MUCOPEPTIDE OF M. LYSODEIKTICUS

Substrate	Optical density at 540 mu*	
	L-oxidase	D-oxidase
none	0.00	0.00
D-serine**	0.00	0.35
D-alanine**	0.00	0.50
L-serine**	0.04	0.00
serine from cell wall	0.00	0.05

*Coleman Junior Spectrophotometer.
**Concentration of amino acid was 500 ug.

TABLE X

THIN-LAYER CHROMATOGRAPHY OF AMINO ACID DANSYL DERIVATIVES: Rf VALUES IN VARIOUS SOLVENT SYSTEMS

Source of derivative	Rf values		
	Solvent*		
	A	B	C
glutamic acid	0.10	0.23	0.75
glycine	0.21	0.53	0.80
lysine	0.24, 0.61	0.79, 0.96	0.65, 0.91
purchased dansyl-serine	0.07	0.09	0.23
purchased dansyl-alanine	0.39	0.69	0.82
serine cell wall	0.64	0.96	0.92
normal cell wall	0.61	0.96	0.91

*A - Benzene:pyridine:acetic acid (80:20:2).
B - Chloroform:tert-amylalcohol:acetic acid (70:30:3).
C - Chloroform:tert-amylalcohol:formic acid (70:30:1).

value in most solvent systems (70, 73). This holds true for the solvent systems used here. These experiments thus indicated that serine, present in the mucopeptide, is bonded in such a way that its amino group is not free.

Since serine is incorporated at the expense of glycine it became of interest to determine the status of its carboxyl group. If it is incorporated in place of glycine it should be attached to the alpha carboxyl of glutamic acid via its amino group and thus have its carboxyl group free and unattached (42).

The determination of free carboxyl groups was performed using the technique of Akabori, Ohno, and Narita (74). Table XI shows the results of these determinations. Glycine and serine are the two major C-terminal amino acids. These data indicate that serine is replacing glycine and is attached through its amino group while its carboxyl remains free. In addition, no C-terminal glutamic acid is found in either the normal or serine-containing cell wall. This would be expected if serine is truly replacing glycine, since Mirelman and Sharon (42) have shown that glycine is bonded via its amino group to the alpha carboxyl of glutamic acid. The overall significance of these findings will be considered in more detail when they are related to cellular integrity as studied using the electron microscope.

In order to obtain more direct evidence relative to the site of attachment of serine in the mucopeptide, incomplete hydrolysates of cell walls were analysed for serine-containing peptides. In addition, the rates of release of cell wall amino acids by mild acid hydrolysis were also studied. It was believed that the rate of release might give an indication as to the number of bonds which must be cleaved in order to

TABLE XI

HYDRAZINOLYSIS: C-TERMINAL AMINO ACIDS IN NORMAL AND SERINE CONTAINING CELL WALLS OF M. LYSODEIKTICUS

Source	Percent of total amino acid as C-terminal				
	Alanine	Glutamic acid	Lysine	Glycine	Serine
normal cell wall	6.3	0.0	3.3	37.0	0.0
serine cell wall	3.7	0.0	3.3	59.0	75.0

release free serine from the mucopeptide. The rates of release of three cell wall amino acids are given in Table XII and Figures 7 and 8. Glycine, known to be linked to the mucopeptide by a single peptide bond, is released from both cell walls more quickly than alanine (42). This is to be expected since all evidence indicates that most of the alanine is linked by two bonds, and therefore would be more slowly released by acid hydrolysis (41, 42). Serine, on the other hand, is released rapidly and almost completely in eight hours. The significance of this result when compared to the glycine release is not clear, but may indicate a difference in bond strength or accessibility of the serine.

Table XIII shows a comparison of the percent C-terminal amino acids (10 hours reaction time, see Materials and Methods section) and the percent of amino acids released after ten hours mild hydrolysis from both normal and serine-containing cell walls. Fairly good correlation exists in almost all cases. These data further substantiate our hypothesis that serine is C-terminal and replaces glycine in the cell wall, by bonding to the alpha carboxyl of glutamic acid via its amino group.

No partial peptides containing serine have been obtained. A possible explanation could be the rapid and almost complete release of serine as shown in Table XII.

Inducible Nature of Serine-Adding Enzyme(s): Effect of Inhibitors of Protein Synthesis on Protein and Cell Wall Mucopeptide Synthesis

Incorporation of serine into the cell wall of M. lysodeikticus could be mediated by the glycine-adding enzyme or by an enzyme specific

TABLE XII

RELEASE OF AMINO ACIDS FROM NORMAL AND SERINE CONTAINING CELL WALLS OF M. LYSODEIKTICUS

Time in hours	Percent of total amino acid released				
	Normal cell wall		Serine cell wall		
	Alanine	Glycine	Alanine	Glycine	Serine
0	0.0	0.0	0.0	0.0	0.0
2	2.1	4.3	2.4	9.4	20.0
4	2.9	9.8	3.6	21.1	35.0
6	4.4	17.4	7.5	37.6	78.3
10	8.0	25.1	9.0	47.0	78.3
% per hour (avg.)	0.8	2.5	0.9	4.7	7.8

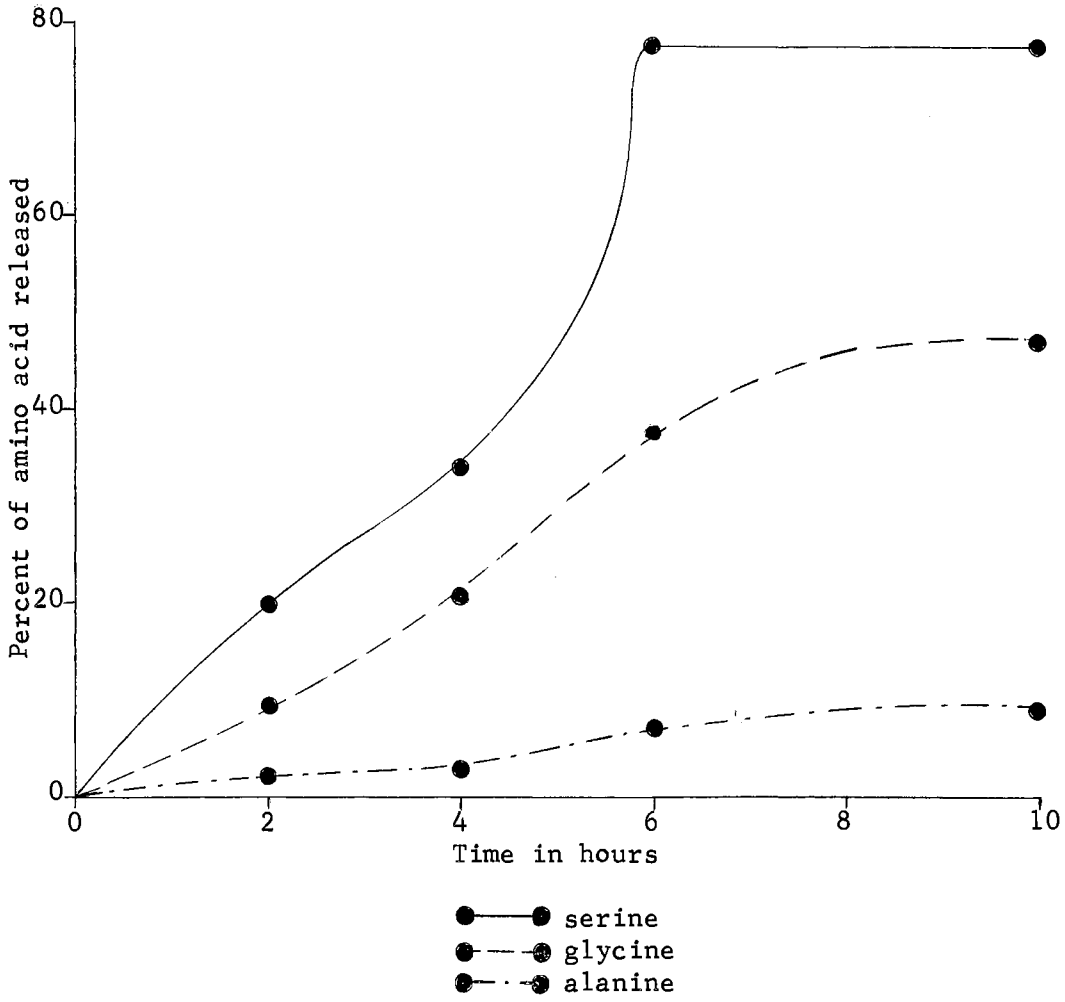


Figure 7. Rates of Release of Serine, Glycine, and Alanine From Serine-Containing Cell Walls of M. lysodeikticus

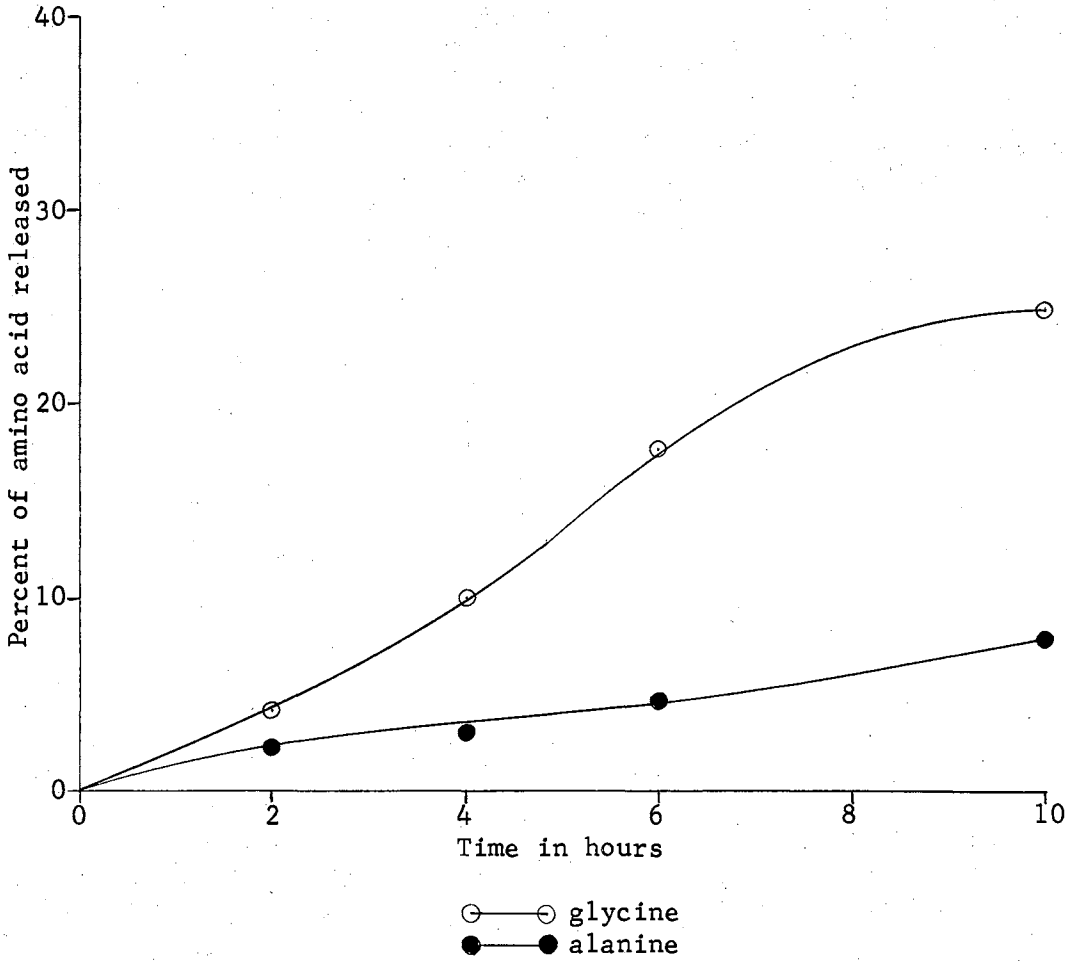


Figure 8. Rates of Release of Glycine and Alanine From Normal Cell Walls of M. lysodeikticus

TABLE XIII

PERCENT CELL WALL AMINO ACID RELEASE VERSUS
PERCENT C-TERMINAL CELL WALL AMINO ACID

Source	% C-terminal amino acid			% amino acid release in 10 hours		
	Alanine	Glycine	Serine	Alanine	Glycine	Serine
normal cell wall	6.3	37.0	0.0	8.0	25.1	0.0
serine cell wall	3.7	59.0	75.0	9.0	47.0	78.3

for serine, which could be either constitutive or induced.

One approach for obtaining data relative to the inducible nature of the serine-adding enzyme would involve incorporation of serine into the cell wall in the absence of protein synthesis. Incorporation could be accepted as evidence for a constitutive enzyme (glycine- or serine-adding), whereas lack of incorporation could indicate the need for synthesis of an induced enzyme for serine addition.

Three known inhibitors of protein synthesis, actinomycin-D, puromycin, and D-chloramphenicol (CAP) were selected for study.

To determine if the three antibiotics inhibited protein synthesis while allowing synthesis of mucopeptide in M. lysodeikticus, incorporation of glycine-2-C^{14} into protein and mucopeptide was examined in the presence and absence of the antibiotics. This amino acid was chosen because it is a normally occurring component of the cell wall and protein of this organism. Representative data are given in Table XIV. All antibiotics drastically inhibit the incorporation of glycine into protein. However, incorporation of glycine into mucopeptide is not inhibited; instead a stimulation in the presence of all antibiotics occurs. A possible explanation for the stimulation could be a sparing effect due to inhibition in protein synthesis. It is also possible that inhibition of protein synthesis does not allow formation or activation of autolytic enzymes which would normally remove amino acids from the mucopeptide (cell wall turnover).

Having established that all of the antibiotics inhibit protein synthesis while allowing cell wall mucopeptide synthesis to continue, their effect on the incorporation of D-serine-3-C^{14} was next determined. Data in Table XV reveal that incorporation of D-serine into both protein

TABLE XIV

EFFECT OF CHLORAMPHENICOL, ACTINOMYCIN-D, AND PUROMYCIN ON THE SYNTHESIS OF CELL WALL MUCOPEPTIDE AND PROTEIN IN *M. LYSODEIKTICUS*

Medium addition	Incorporation of C^{14} from glycine-2-C^{14}			
	Cell wall mucopeptide	% change	Protein	% change
none	10996*	-	35484*	-
D-chloramphenicol	14247	+29	2195	-93
actinomycin-D	16158	+46	1452	-95
puromycin	15412	+40	4690	-87

*Counts per minute per mg dry weight of cells fractionated.

TABLE XV

EFFECT OF CHLORAMPHENICOL, ACTINOMYCIN-D, AND PUROMYCIN ON THE INCORPORATION OF D-SERINE-3-C^{14} INTO THE PROTEIN AND MUCOPEPTIDE FRACTIONS OF M. LYSODEIKTICUS

Medium addition	Incorporation of C^{14} from D-serine-3-C^{14}			
	Cell wall mucopeptide	% change	Protein	% change
none	970*	-	1330*	-
D-chloramphenicol	126	-87	168	-87
actinomycin-D	563	-41	168	-87
puromycin	222	-77	260	-80

*Counts per minute per mg dry weight of cells fractionated.

and mucopeptide is greatly inhibited, particularly in the presence of puromycin and CAP.

Since lack of incorporation of D-serine into the cell wall in the presence of these antibiotics could have been due to inhibition in synthesis of an enzyme necessary for entry of the molecule into the cell, the C^{14}-content of the intracellular pools was determined after growth in the presence of either D-serine-3-C^{14} or glycine-2-C^{14} and the antibiotics. Table XVI reveals that the cell pools contain appreciable amounts of label from D-serine-3-C^{14}, and therefore, lack of incorporation into the mucopeptide is not due to inhibition in synthesis of a permease necessary for entry of the amino acid into the cells.

Because all data are based on the counting of radioactivity in pure cell walls, it was possible that glycine and particularly D-serine were being metabolized and their carbon entering the cell wall as other compounds. Data in Table XVII reveal that the cell wall preparations were pure, in that no contaminating amino acids were present and also that glycine and D-serine were incorporated essentially unchanged.

As a check on the reproducibility of the specific activity figures, obtained from our chemically isolated cell wall preparations, five cell wall preparations were made from the same flask culture. The greatest deviation from an average specific activity of 10,996 was $\pm$600 counts per minute.

Isotope Studies: Labeling Patterns of Various Radioactive Compounds

In order to gain a better insight into the metabolism and to obtain specific methods for isotopic labeling of various cellular components of

TABLE XVI

EFFECT OF ACTINOMYCIN-D, PUROMYCIN, AND D-CHLORAMPHENICOL ON THE INTRACELLULAR POOL CONTENT OF LABEL FROM D-SERINE-3-C^{14} AND GLYCINE-2-C^{14}

Inhibitor added	D-Serine-3-C^{14}	Glycine-2-C^{14}
none	5700*	1658
D-chloramphenicol	4111	3838
actinomycin-D	5033	1203
puromycin	7075	1772

*Counts per minute per mg dry weight of cells fractionated.

TABLE XVII

CELL WALL AMINO ACID LABELING PATTERNS FROM D-SERINE-3-C^{14} AND GLYCINE-2-C^{14} IN THE PRESENCE AND ABSENCE OF PUROMYCIN, D-CHLORAMPHENICOL, AND ACTINOMYCIN-D

Medium additions	Cell wall amino acids detected with ninhydrin in 60 minute samples					Cell wall amino acids C^{14} labeled* in 60 minute samples				
	Alanine	Glycine	Glutamic acid	Lysine	Serine	Alanine	Glycine	Glutamic acid	Lysine	Serine
none	+	+	+	+	-	-	-	-	-	-
glycine-2-C^{14}	+	+	+	+	-	V.S.**	+	-	-	-
glycine-2-C^{14} + D-chloramphenicol	+	+	+	+	-	-	+	-	-	-
glycine-2-C^{14} + puromycin	+	+	+	+	-	V.S.	+	-	-	-
glycine-2-C^{14} + actinomycin-D	+	+	+	+	-	V.S.	+	-	-	-
D-serine-3-C^{14}	+	+	+	+	+	-	-	-	-	+
D-serine-3-C^{14} + D-chloramphenicol	+	+	+	+	-	-	-	-	-	V.S.
D-serine-3-C^{14} + puromycin	+	+	+	+	-	-	-	-	-	V.S.
D-serine-3-C^{14} + actinomycin-D	+	+	+	+	-	-	-	-	-	V.S.

*Label determined by radioautography.
**Very slight labeling observed.

M. lysodeikticus, the labeling pattern of various radioactive compounds was determined for the protein and mucopeptide fractions. The results of these determinations are presented in Tables XVIII and XIX.

Tyrosine and phenylalanine label primarily the pyruvate family of amino acids. It appears that the aromatic ring of these compounds is split off giving rise to alanine which is converted to pyruvate, thus also giving rise to leucine and valine. Some randomization of both compounds seems to occur (probably via pyruvic acid) since a small amount of label is found in all amino acids. There is obviously no interconversion between tyrosine and phenylalanine, thus helping to explain the growth requirement for these two amino acids. Carbon from glutamic acid is completely randomized. D-Serine is metabolized very little, while L-serine is readily interconverted, particularly to amino acids other than those arising from Krebs cycle intermediates (glutamic and aspartic acid families). This is probably due to dilution by glutamic acid carbon since this amino acid was present in the growth medium. Glycine gives rise to serine, but except for a trace of label from D-serine, serine is not metabolized to glycine. Since serine was labeled in the three position, glycine would only be labeled if there were extensive randomization of the hydroxymethyl group of serine. Carbon from threonine is poorly utilized except for the synthesis of glycine and isoleucine. No deviations from these patterns were obtained when the labeling patterns for synthesis of cell wall amino acids were studied (Table XIX).

Effect of D-Serine on Sulfur Metabolism

In an attempt to explain the growth inhibition caused by D-serine,

TABLE XVIII

LABELING PATTERNS OF VARIOUS RADIOACTIVE COMPOUNDS IN THE PROTEIN FRACTION OF M. LYSODEIKTICUS

Amino acid*	Radioactive compound added						
	L-Glutamic -U-C^{14}	L-Tyrosine -U-C^{14}	L-Phenylalanine -U-C^{14}	D-Serine -3-C^{14}**	L-Serine -3-C^{14}	Glycine -2-C^{14}	DL-Threonine -2-C^{14}
alanine	+++	++	++	+	++++	++	-
aspartic acid	++++	+	+	-	-	-	-
arginine	++	+	+	-	++	-	-
glutamic acid	++++	+	+	+	++	-	-
glycine	++	+	+	+	-	++++	++
histidine	++	+	+	-	+++	-	-
isoleucine + leucine	++	++	++	+	+++	+	+++
lysine	++	+	+	-	-	-	-
methionine	+++	++	+	-	+	-	-
phenylalanine	-	-	++	-	-	-	-
serine	+++	+	+	++	++++	+++	-
threonine	++	+	+	-	+	-	++++
tyrosine	+	++	-	-	-	-	-
valine	+++	++	+	-	++	+	-
proline	++++	+	+	-	+	-	-

*Tryptophan and cysteine destroyed during hydrolysis.
**Contains small amount of labeled alanine.
- no label
+ very slight label
++ light label
+++ moderate label
++++ heavy label

TABLE XIX

LABELING PATTERNS OF VARIOUS RADIOACTIVE COMPOUNDS IN THE MUCOPEPTIDE FRACTION OF M. LYSODEIKTICUS

Medium addition	Labeled cell wall amino acids				
	Alanine	Glutamic acid	Lysine	Glycine	Serine*
L-glutamic-U-C^{14}	++	++	++	++	
L-tyrosine-U-C^{14}	++	+	+	+	
L-phenylalanine-U-C^{14}	+	+	+	+	
D-serine-3-C^{14}**	+	-	-	+	++++
L-serine-3-C^{14}	++	++	+	-	
glycine-2-C^{14}	+	-	-	+++	
DL-threonine-2-C^{14}	-	-	-	++	

*Serine present in cell wall only when D-serine is added to medium.
**Contains small amount of labeled alanine
- no label
+ light label
++ moderate label
+++ heavy label

studies were made concerning the effect of D-serine on sulfur metabolism using $Na_2S^{35}O_4$. The results are shown in Table XX. The amount of label from $Na_2S^{35}O_4$ incorporated into the sulfur containing compounds of cell protein is reduced by approximately 50% when D-serine is present in the growth medium. D-Serine appears to inhibit the uptake of sulfate, since counts of washed, whole cells and intracellular pools from cells grown in the presence of D-serine and $Na_2S^{35}O_4$ also show a 50% reduction in the amount of radioactive sulfur present. The reduction in uptake of sulfate however, doesn't account for the inhibition in growth caused by D-serine since no growth inhibition is obtained when the normal sulfate level of the growth medium is reduced to 25% of normal.

Cell Wall Turnover Studies

Studies were initiated to determine if existing cell wall mucopeptide is broken down during growth and new cell wall mucopeptide inserted in its place. This could be termed cell wall turnover, analogous to the turnover known to occur in the protein of the cell. In addition, data were sought which would aid in determining if serine-containing walls were either more or less susceptible to autolytic digestion and subsequent replacement.

The approach used was one in which a known amount of radioactive label was incorporated into a known mass of cell wall mucopeptide, removal of the source of the label from the medium, allowing the cell wall mucopeptide mass to increase a known amount, and determination of the amount of label present in the cell wall mucopeptide after the increase in mass. Following the initial labeling, a specific activity for the mucopeptide could be determined or predicted. This figure could then be

TABLE XX

EFFECT OF D-SERINE ON THE METABOLISM OF SULFUR IN M. LYSODEIKTICUS

Medium additions	Labeled cellular compounds*		
	Methionine sulfoxide	Cysteine or homocysteine	Methionine
$Na_2S^{35}O_4$	619	539	1222
$Na_2S^{35}O_4$ + D-serine	251	255	547

*Compounds located using radioautography. Spots were eluted and the eluant counted using a Picker Gas-Flow Automatic Planchet Counter. Protein was adjusted to equal amounts in both systems prior to hydrolysis and chromatography. Numbers are given as counts per minute per spot.

compared to a similar figure obtained after the increase in mass of the cell wall. If no radioactivity was removed during the increase in mass, the magnitude of the decrease in specific activity should be proportional to the magnitude of the increase in mass. However, if the magnitude of the decrease in specific activity is greater than the magnitude of the increase in mass, a net loss of radioactivity had to occur; this was interpreted as indicating cell wall turnover.

Experiments were run in which both the specific activity of the cell wall mucopeptide and cell wall amino acids was determined. Representative data are given in Tables XXI and XXII. As determined by this method it would appear that cell wall mucopeptide does turn over at approximately equal rates in both normal and serine containing cell walls. Furthermore, the rate of turnover compares favorably with accepted values for protein turnover (79-81).

Having obtained data which supported cell wall turnover it next became necessary to determine if this organism possessed enzyme(s) which could cleave bonds in existing mucopeptide. Examination was made for an autolytic enzyme system in growing cells which, if found, would support cell wall turnover. Tables XXIII and XXIV contain results which indicate that the organism contains an autolytic enzyme system which functions under standing, but not shaking conditions, in 0.01 M phosphate buffer at pH 7.0. Failure to obtain lysis under shaking conditions may indicate a requirement for a low oxygen tension and activation of enzymes similar to the cathepsins, where reduced sulfhydryl groups are required for activity (82). In addition, electron microscopic examination of the cells before and after action of the autolytic enzyme(s) showed that gross damage to the structural integrity of the cells had

TABLE XXI

CELL WALL TURNOVER DURING GROWTH OF M. LYSODEIKTICUS

Source of label	Mass* increase	Specific activity* decrease	Turnover factor**	% turnover per hour (avg.)
L-glutamic-U-C^{14}	3.00	5.60	1.86	5.3
DL-glutamic-5-C^{14}	3.45	4.35	1.26	3.7
glycine-2-C^{14}	3.20	3.90	1.22	3.6
D-serine-3-C^{14}	5.60	6.50	1.16	3.5

*Specific activity given as counts per minute per mg cell walls. Both increase in mass and decrease in specific activity are given as multiples of initial values. For example: a mass increase of 3.00 is a three-fold increase in cell dry weight.

**Decrease in specific activity/Mass increase

where: 1.0 = no turnover

>1.0 = turnover

<1.0 = incorporation of additional label

TABLE XXII

CELL WALL AMINO ACID TURNOVER DURING GROWTH OF M. LYSODEIKTICUS

Source of label	Mass increase of cell wall amino acids*	Cell wall amino acids specific* activity decrease	Turnover factor**	% turnover per hour (avg.)
L-glutamic-U-C^{14}	6.8	10.6	1.56	4.0

*Specific activity given as counts per minute per mg cell wall amino acids. Both increase in mass and decrease in specific activity are given as multiples of initial values. For example: a mass increase of 3.00 is a three-fold increase in cell dry weight.

**Decrease in specific activity/Mass increase

where: 1.0 = no turnover

>1.0 = turnover

<1.0 = incorporation of additional label

TABLE XXIII

AUTOLYSIS OF LOG-PHASE CELLS OF M. LYSODEIKTICUS

Medium	Shaking	Standing	Pre-heated	Optical density at 540 mu*		% lysis
				0 hours	24 hours	
phosphate buffer**	+	-	-	0.48	0.35	27
phosphate buffer	-	+	-	0.48	0.11	77
phosphate buffer	+	-	+	0.50	0.45	30
phosphate buffer	-	+	+	0.48	0.46	4
saline solution	+	-	-	0.19	0.16	15
saline solution	-	+	-	0.24	0.23	4
saline solution	+	-	+	0.22	0.21	4
saline solution	-	+	+	0.20	0.20	0
water	+	-	-	0.44	0.43	2
water	-	+	-	0.40	0.39	2
water	+	-	+	0.60	0.58	3
water	-	+	+	0.61	0.58	5

*Spectronic 20 Spectrophotometer.
**0.01 M, pH 7.0.

TABLE XXIV

AUTOLYSIS OF LOG-PHASE CELLS OF M. LYSODEIKTICUS IN PHOSPHATE BUFFER*

Molarity	Optical density at 540 mu**		% lysis
	0 hours	24 hours	
$1x10^{-1}$	0.40	0.33	17
$1x10^{-2}$	0.40	0.10	75
$1x10^{-3}$	0.40	0.12	70
$1x10^{-4}$	0.40	0.13	67

*Incubated under standing conditions at 30 C.
**Spectronic 20 Spectrophotometer.

occurred (Figures 9 and 10).

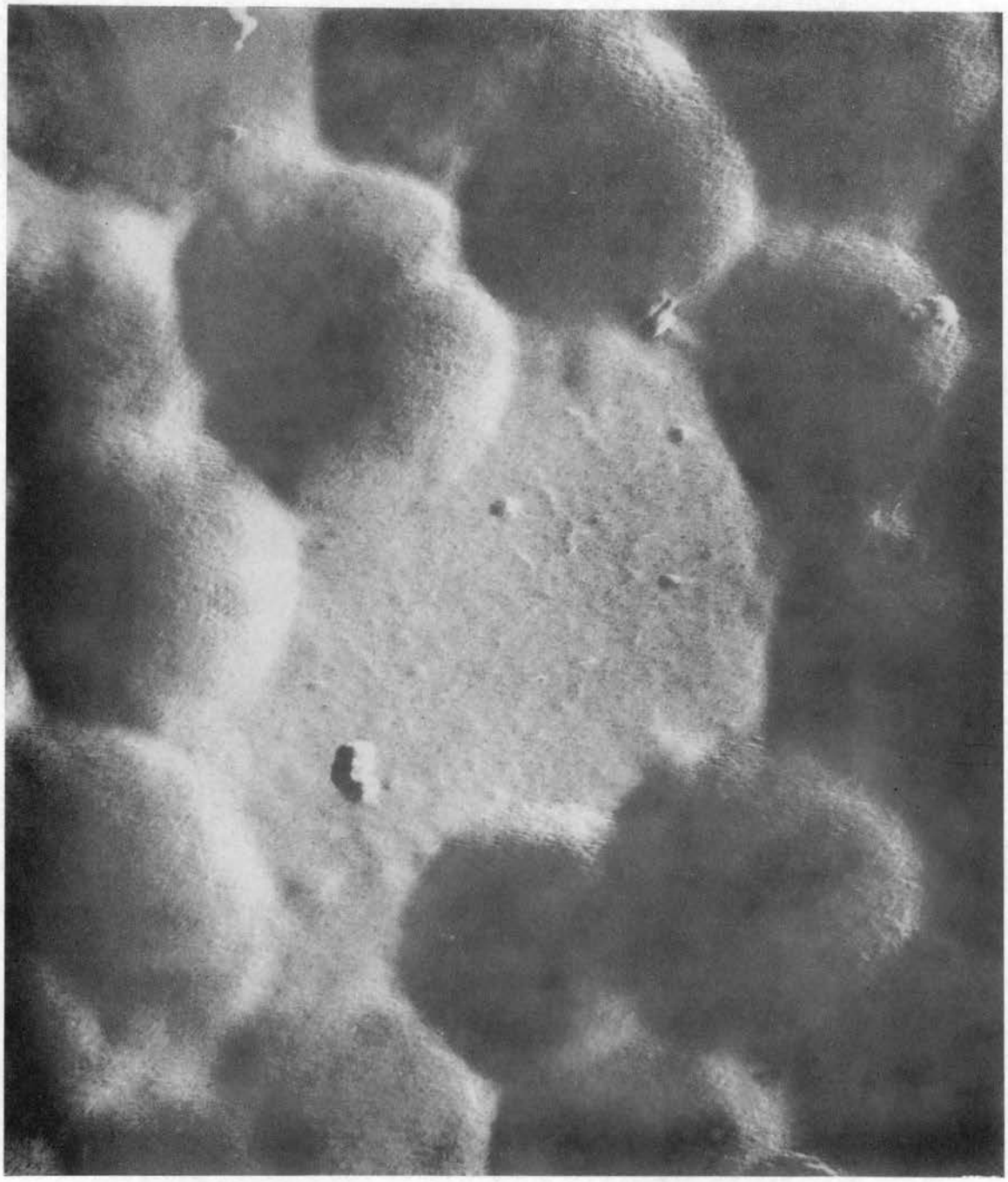


Figure 9. Whole Cells of M. lysodeikticus
(magnification 45,000 X)

Figure 10. Autolysed Cells of M. lysodeikticus
(magnification 45,000 X)

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Rather conclusive chemical data have been obtained concerning two major areas in Gram-positive bacterial cell wall research: (1) an organism in the proper growth situation can alter the composition of its cell wall mucopeptide; and (2) an organism can break down existing mucopeptide and replace it with new cell wall material (cell wall turnover). Though not directly shown, replacement of excised cell wall material had to occur because the resulting cells possess normal mucopeptide layers and are osmotically stable.

The amino acid D-serine, which does not normally occur in the cell wall mucopeptide of M. lysodeikticus, can be incorporated (as the D-isomer) into the mucopeptide when it is present in the growth medium. In addition to incorporation into wall material, this amino acid drastically inhibits both the rate, and final amount of growth obtained. Cells which have incorporated D-serine into their mucopeptide show no morphological abnormalities such as protoplast or filament formation or any change in staining properties and susceptibility to the action of lysozyme. This latter point would seem to indicate that the structure of the cell wall peptide has little or no effect on the action of this enzyme. Thus, one would anticipate that the attachment sites, necessary for proper steric register of the enzyme and substrate, exist primarily in the amino sugar backbone of the mucopeptide.

When D-serine is incorporated into cell wall mucopeptide, amounts of the normally occurring amino acid glycine are reduced to about 40 percent of normal, while the amount of serine incorporated is about equal to the amount of lysine and glutamic acid normally present. Therefore, the possibility exists that the dimensions of the mucopeptide may be larger. One could visualize that serine incorporation could lead to the formation of a cell wall which was less dense or more diffuse than the same structure in normal cells. Thin-slicing of normal and D-serine grown cells (Figures 11 and 12) indicate that this well may be the case. The mucopeptide from cells grown in the presence of D-serine exhibits a more loose network of cell wall material, while the cell wall from normal cells is a much more compact appearing structure. Chemical data obtained from hydrazinolysis determinations reveal that 4.3 percent of the amino acids in the normal cell wall are C-terminal while 8.0 percent in the serine-containing cell wall are C-terminal. This would indicate more free carboxyl groups in the serine-containing wall mainly due to the preponderance of C-terminal serine. However, the amount of C-terminal D-alanine in the serine-containing wall, which is one of the amino acids involved in cross-linking in this organism, is reduced to about 50 percent of that present in the normal cell wall. This result does not necessarily indicate more cross-linking in the altered wall since some of the terminal D-alanine residues which would normally be C-terminal could have D-serine attached. In this condition they would not be detected as C-terminal and yet not be involved in cross-linking. An additional factor which could help account for a less compact, yet stable serine-containing mucopeptide would be cross-linking in which serine served as the linking amino acid between the terminal

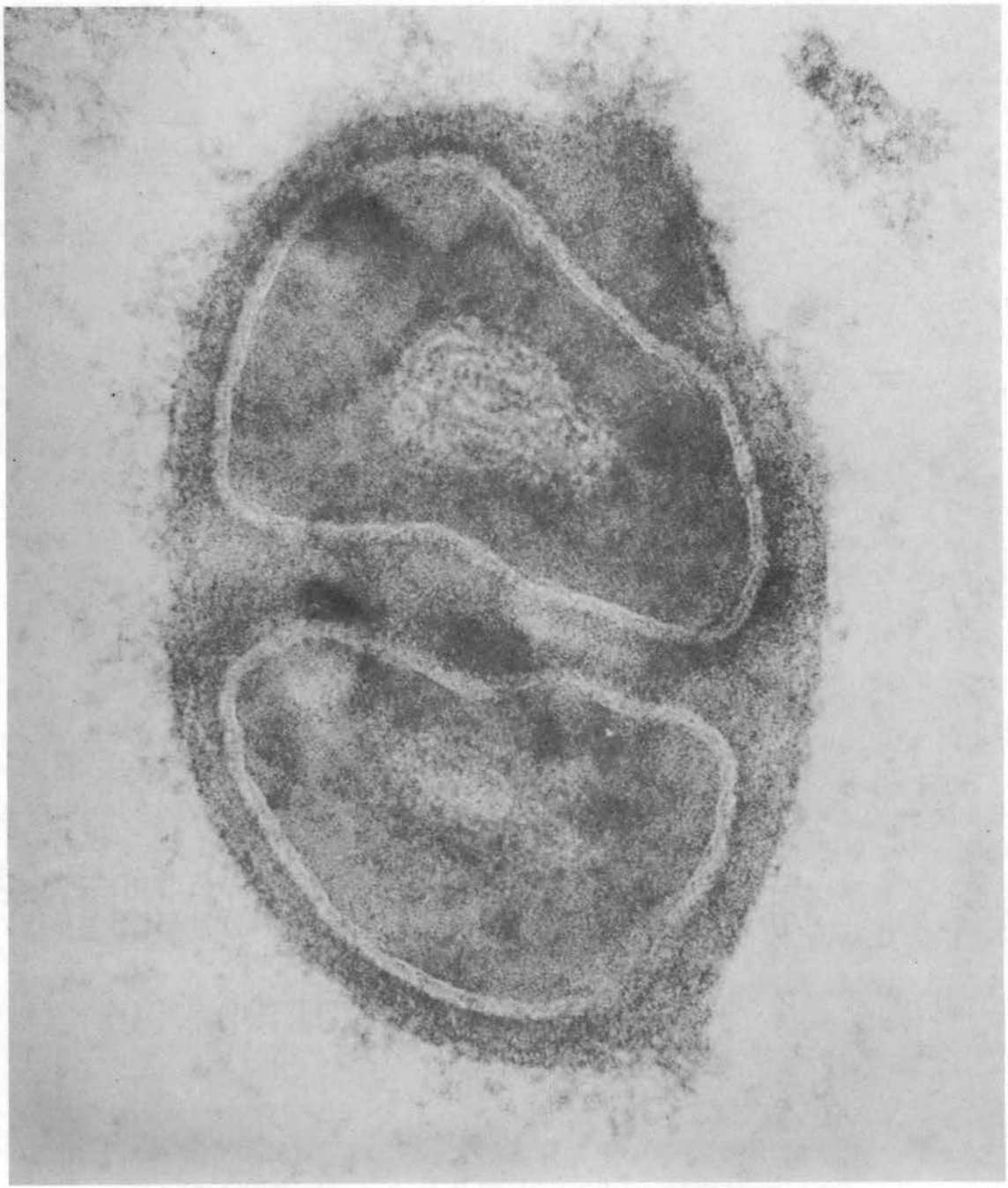


Figure 11. Thin-Section of Normal Cell of M. lysodeikticus (magnification 163,000 X)

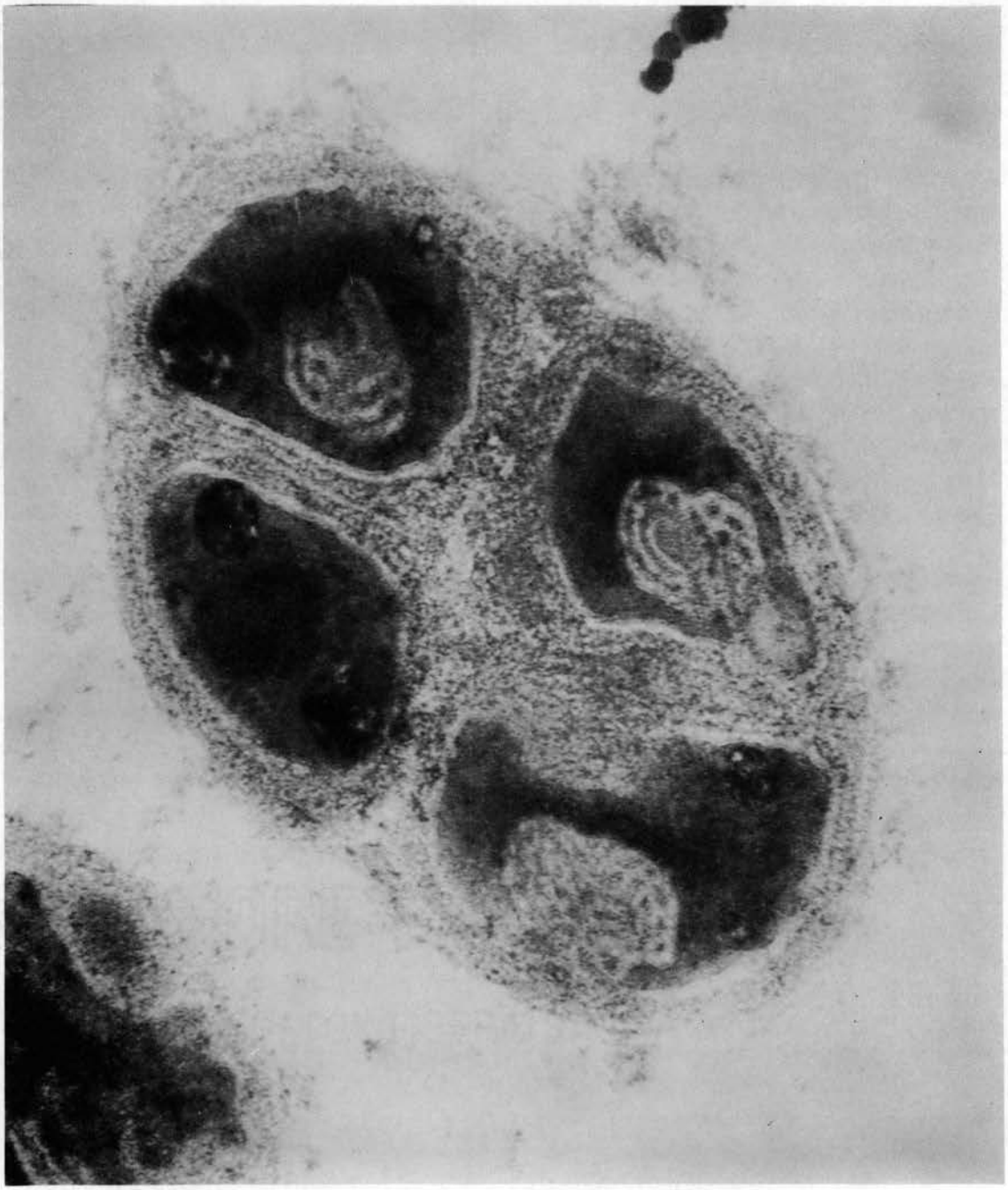


Figure 12. Thin-Section of D-Serine-Containing Cell of M. lysodeikticus (magnification 123,000 X)

D-alanine and the epsilon amino group of lysine. In this position the distance between interlinking peptides could be greatly increased yielding a more diffuse, yet stable cell wall. One must keep in mind however, that any serine and alanine thus attached, would involve only a small fraction of the total, since the majority of the D-serine is attached in another position to be discussed presently. These hypotheses are brought forth to help correlate the chemical analyses with observations made using the electron microscope.

Incorporation of D-serine into mucopeptide is decreased in the presence of four amino acids, D- or L-alanine, L-serine, or glycine; these amino acids inhibit, to varying degrees, the uptake of D-serine into the cell. Where D-serine incorporation is lowered by D-alanine or glycine, glycine levels increase to what may be considered normal. L-Alanine is not as effective as D-alanine or glycine. In no case is incorporation of D-serine completely inhibited.

Two of these amino acids, glycine and L-serine, also reverse the growth inhibition caused by D-serine. Addition of L-alanine to the D-serine-containing medium also results in some reversal of growth inhibition; however, restoration is better with D-alanine. Pantoic acid, which partially reverses growth inhibition caused by D-serine in a species of Erwinia does not reverse growth inhibition in this organism (83).

Apparently, the wall modification caused by D-serine is only partially responsible for the inhibition in growth caused by this amino acid. However, although growth is restored to very near normal by glycine, incorporation of D-serine is still approximately 30 percent that found in the absence of exogenous glycine. This amount of incor-

poration should have a greater effect on growth than is apparent if D-serine incorporation were to be accepted as the only reason for growth inhibition. A good correlation also exists between the reversal of growth inhibition by these amino acids and the inhibition in incorporation of label from D-serine into the protein fraction. L-Serine, which is most effective in overcoming growth inhibition caused by D-serine, is also the most effective inhibitor of incorporation of label from D-serine into protein. This relationship also holds true for the other three amino acids. These data indicate that synthesis of fraudulent proteins may occur in the presence of D-serine since growth inhibition cannot be directly correlated with incorporation of D-serine into the cell wall of this organism. Further information to support this view comes from data showing that the three antibiotics inhibiting protein synthesis significantly decrease incorporation of label from D-serine into protein. Also, labeled serine is present in cell protein fractions from cells incubated with D-serine-3-C^{14}, and labeling patterns of L- and D-serine are different in this organism.

Addition of D-chloramphenicol, actinomycin-D, or puromycin to growing cells of M. lysodeikticus causes an inhibition in protein synthesis while mucopeptide synthesis continues. Also, during exposure to the antibiotics the optical density of the cell suspensions increases slightly. This would be expected since growth or increase in cell mass is not completely stopped due to continued cell wall synthesis. Incorporation of D-serine into the cell wall mucopeptide in the presence of these antibiotics is greatly reduced during a one hour incubation indicating a need for protein synthesis in order for D-serine addition to occur. In the absence of these antibiotics low levels of D-serine can

be detected, both isotopically and chemically, in the mucopeptide after 15 minutes of exposure. Therefore, we interpret our results to mean that the major incorporation of D-serine into the mucopeptide of this organism occurs because of enzyme induction by D-serine. The enzyme(s) thus induced are quite specific for D-serine since cells induced to D-serine will not incorporate L-serine or D-threonine (the higher homolog of D-serine).

Actinomycin-D, which gave the least inhibition in D-serine, incorporation into the cell wall (41%) was also tested at increased concentrations. When the antibiotic was present at 40 ug per ml of medium, inhibition in protein synthesis was increased to 98 percent and inhibition of D-serine incorporation into mucopeptide was increased to 59 percent. These data may indicate a concentration effect by this antibiotic on synthesis of RNA involved in protein fabrication.

Pollock (84) reported that actinomycin-D manifests a difference in affinity for genetic sites depending on the incidence of guanine residues. It is possible that the genetic site for synthesis of the D-serine-adding enzyme is low in guanine and is therefore less susceptible to actinomycin-D.

It appears that cell wall turnover occurs in growing cultures of this organism. The rates of this turnover very closely approach the published rates for protein turnover which have been studied in a variety of bacteria (79-81). Supporting this finding are the data relative to autolysis, which indicate that growing cells of M. lysodeikticus also possess enzyme(s) capable of breaking down existing cell wall mucopeptide leading to loss of all cellular integrity. Shockman and coworkers (48, 49) have shown that the autolytic system from growing cells

of S. faecalis is highly specific in that it will not lyse stationary phase cells or cell walls isolated from stationary phase cells. This would indicate that the mucopeptide of stationary phase cells is different from that found in log-phase cells. If such differences are present they have not been, or cannot be detected chemically. Perhaps the difference lies in the fact that some log-phase cells have growing points for cell wall synthesis while stationary phase cells would not possess such areas (85-90). In such cases the autolytic enzymes might function by breaking existing bonds so that a new piece of completed cell wall precusor can be inserted, thus allowing for lengthening of the cell wall polymer. Perhaps the growing points for new wall synthesis are initiated by the autolytic enzymes and correspond to the only areas of wall that are susceptible to these enzymes. Such areas may be absent or modified in stationary phase cells. Mitchell and Moyle (42) have observed hemispherical wall fragments after the action of an autolytic enzyme on S. aureus. On occasions during this study, similar structures have been observed in autolysed preparations of M. lysodeikticus.

These same autolytic enzymes may have a role in intercalary cell wall growth, however, the absence of discrete growing points for new cell wall synthesis in this type of growth, may dictate a different enzyme system.

Chemical data obtained employing the techniques of hydrazinolysis, amino acid dansyl derivative formation, and partial hydrolysis (rates of release of cell wall amino acids) support the hypothetical structure of the D-serine-containing cell wall mucopeptide presented in Figure 13. In this proposed structure, D-serine is attached via its amino group to the alpha carboxyl of glutamic acid, thus occupying a position in the

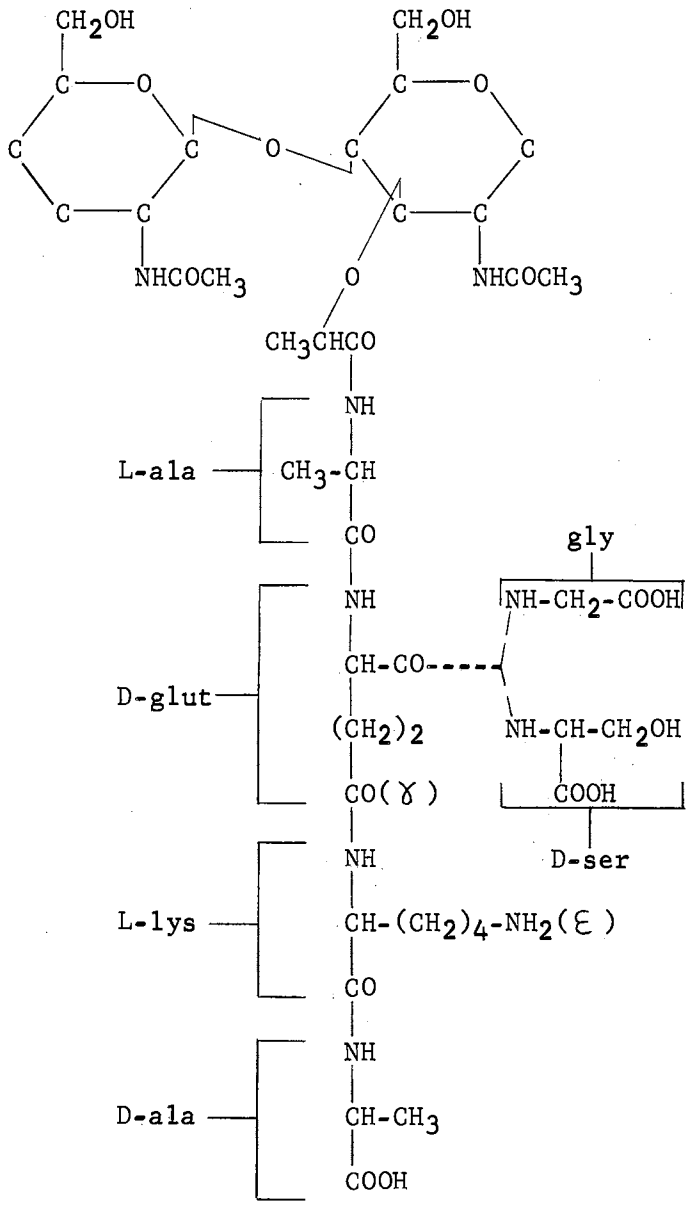


Figure 13. Proposed Cell Wall Mucopeptide Structure in M. lysodeikticus

peptide chain equivalent to that occupied by glycine in the normal cell wall. Attached in such a way serine would be C-terminal and have no free amino group available to form a dansyl derivative. Glutamic acid, which has no reactive carboxyl or amino group in the normal cell wall due to its central position in the cell wall peptide (Figure 5), also is non-reactive in the serine-containing wall indicating that none of its functional groups (carboxyl or amino) are free. Chemical data on lysine are identical in both normal and serine-containing cell walls, where the only free group is the epsilon amino group. This would strongly indicate that lysine occupies a similar position in both structures and further eliminates the possibility for attachment of serine to the epsilon amino group of this amino acid. No dansyl derivative for alanine was formed from either cell wall. Hydrazinolysis data revealed small amounts of C-terminal alanine in both walls, and in addition, that there was approximately 43 percent less C-terminal alanine in the serine-containing wall. This could indicate that some serine might be linked to the terminal D-alanine. If so, the amount would be quite small, approximately 8 percent of the total. As pointed out in the Introduction of this study, there is a paucity of C-terminal alanine in the mucopeptide of this organism which cannot, as yet, be accounted for. Very likely the carboxyl of this amino acid is involved in bonding, other than that to the epsilon amino group of lysine, which adds stability to the cell wall.

The low percentages of C-terminal amino acids obtained here is due to the fact that during hydrazinolysis as much as 50 percent of the amino acids are destroyed (27). If the percent C-terminal figures presented in this study are corrected for the loss of amino acids during

hydrazinolysis, they agree favorably with those obtained by other workers (27, 41).

In further support of this proposed structure are the data concerning the rates of release of various cell wall amino acids. In the normal cell wall, glycine, which is known to be attached to the cell wall peptide by a single bond, is released quite rapidly during mild acid hydrolysis. Serine, which in the modified wall as proposed here is also attached by a single bond, is also released very rapidly under the same conditions of hydrolysis. All other cell wall amino acids, being attached by at least two bonds, are released much more slowly. It should be noted that the same amount of glycine is released per unit time from both cell wall preparations. The apparent difference in the rates of release (Figures 7 and 8) is due to the fact that there is less glycine in the serine-containing wall. Therefore, when the results are expressed as percent of cell wall amino acid released per unit time it appears that glycine is released more quickly and completely from the altered mucopeptide.

The structure as proposed in this study (Figure 13) will account for approximately 75 to 80 percent of the D-serine incorporated into the mucopeptide. An additional 20 to 25 percent is not released during mild acid hydrolysis (Figure 7) therefore indicating a site of attachment, other than that proposed. Since glycine is known to be attached to glutamic acid (Figure 5) and is C-terminal in the serine-containing wall, it must be assumed that the small amount of glycine present in the altered cell wall is attached to glutamic acid. The combined molar ratios of glycine and serine (altered wall) amount to 1.3 molar proportions and this amount exceeds the molar proportion of glutamic acid

(taken as 1.0). This type of calculation indicates that about 25 percent of the serine must be attached in another position. The location of this attachment is not known, however three possibilities can be envisioned.

(1) Approximately 8 percent of the serine could be attached to the terminal D-alanine as discussed previously. Such a linkage, though not accounting for all of the remaining serine, would help explain the paucity of C-terminal alanine in the altered cell wall.

(2) If one-third of the D-serine, as bonded in Figure 13 (one-fourth of the total), is actually attached and presented as a serine dipeptide (D-seryl-D-serine), it would not alter the chemical data obtained with respect to free carboxyl or amino groups. Results concerning the rate of release of serine could, however, be misleading. Since the rate of release curve (Figure 7) plateaus at about 78 percent serine released, this could indicate that one-fourth of the serine is released as a dipeptide. Quantitation of this peptide (without further hydrolysis to monomeric serine molecules) by color development using ninhydrin would measure the dipeptide as only one serine residue because the additional amino group is not free, but, rather, involved in peptide bonding.

(3) D-Serine could be bonded into the backbone of the peptide chain wherein both the amino and carboxyl groups would be tied up and unavailable for further reaction. In such a position, rate of release during mild acid hydrolysis would be slower than for serine attached by a single bond. This type of

positioning of serine could account for the plateau observed in Figure 7.

Cells which have D-serine incorporated into their mucopeptide are osmotically stable, indicating that the attachment of D-serine, as proposed here, has little or no effect on cross-linking. Since D-serine is primarily C-terminal, its involvement in cross-linking would be limited to hydrogen bonding or salt linkages.

An area which the author feels warrants further investigation is more direct proof of structure of the D-serine-containing cell wall mucopeptide. This could be accomplished by isolation of a partial cell wall peptide containing glutamyl-serine or some other serine-containing peptide. Attempts during this study to isolate such peptides were unsuccessful due to inadequate separation of partial peptides released in the chromatographic systems employed. Three bands of ninhydrin positive material were discernible in a region which migrated approximately one inch from the origin after 100 hours of continuous irrigation with solvent. An excessive amount of trailing made separation of these bands very difficult, thus giving rise to a large amount of cross-contamination which made identification of the composition of the three bands impossible. It is believed that a successful isolation could be achieved employing column chromatographic techniques or electrophoresis.

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