CELL DIVISION IN *MICROCOCCUS LYSODEIKTICUS* DIS-II PUR^{+}

By

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CHAPTER I

INTRODUCTION

In what appears to be the first recorded observation, Coleman (1959) reported that growth and cell division of Rhodospirillum rubrum were inhibited when the organism was grown in the presence of D-glutamic acid. Shortly thereafter, Tuttle and Gest (1960) reported that a variety of morphological changes occurred when R. rubrum was grown in the presence of the D-isomers of glutamic or aspartic acid, isoleucine, alanine, serine, valine, histidine, lysine, leucine, or methionine. Grula (1960) observed that the D-forms of serine, methionine, phenylalanine, threonine, tryptophan, or histidine either alone or in combination with glucose profoundly inhibit growth and cell division in a species of Erwinia under chemically defined conditions. The ability of D-serine to also inhibit both growth and cell division in a Flavobacterium species was reported by Durham and Milligan (1961). In addition to the six D-amino acids, Grula and Grula (1962a; 1964) observed that several other agents or treatments (penicillin, mitomycin c, vancomycin, D-cycloserine, aminopterin, hydroxylamine, S-(dichlorovinyl)-L-cysteine, and ultraviolet light) will inhibit growth and cell division in Erwinia species.

Prevention of division inhibition caused by D-serine can be effected by adding to the growth medium D- or L-alanine, para-aminobenzoic, but not folic, acid, calcium pantothenate, or inorganic

ammonium salts (Grula, 1960; Grula and Grula, 1962a). Durham and Milligan (1961; 1962) reported that the inhibitions of both growth and cell division caused by D-serine in a Flavobacterium species could be prevented by p-amino-benzoic, but not folic acid. They also noted that L-aspartic acid, L-alanine, and pantothenic acid prevented growth inhibition in this organism in the presence of D-serine.

In further studies, Grula and Grula (1962a) reported that addition of certain divalent cations such as zinc, calcium, or manganese or the precursors of pantothenic acid (pantoic acid or pantoyl lactone) prevented the inhibitory effects of D-serine, ultraviolet light, or penicillin on cell division in Erwinia species.

Regardless of the division inhibiting agent used, pantoyl lactone was able not only to prevent, but also to reverse the inhibition of division (Grula and Grula, 1962a). Thus, it appeared that pantoyl lactone occupies a key role in bacterial cell division; however, it was difficult for the authors to believe that synthesis of this compound could be inhibited by all the chemically diverse agents that caused division inhibition. For this reason, Grula and Grula (1962a) proposed (1) that the cell division "system" undergoes varying degrees of physical damage in the presence of all agents that inhibit cell division, and (2) that the cell division mechanism is extremely susceptible because it is located near the cell periphery.

Involvement of surface structures in division inhibition of Erwinia sp. was reported by Grula (1960) who observed the formation of spheroplasts from some filamentous cells when this organism was grown in the presence of high concentrations of D-serine. This observation and the apparent ability of D- or L-alanine to prevent

the deleterious action of D-serine indicated that inhibition of mucopeptide synthesis was occurring. Direct measurement of the amount of glucosamine and muramic acid present in the envelope of dividing and non-dividing cells of Erwinia sp. was done in order to obtain data relative to this point. It was found that several agents or treatments (D-serine, vancomycin, D-cycloserine, penicillin, mitomycin c, or ultraviolet light) decrease the amount of both amino sugars in the envelope of filamentous cells 33 to 42 percent (Grula and Grula, 1964).

Other investigators also have noted possible inhibition in synthesis of cell wall mucopeptide in the presence of D-amino acids. Lark and Lark (1959) reported that D-methionine induced the formation of crescent-like protoplasts in Alcaligenes faecalis. This D-methionine effect was said to resemble the action of penicillin in that both compounds caused a decrease in cell wall mucopeptide (Lark and Lark, 1959; 1961; Lark, Bradley, and Lark, 1963).

Wise and Park (1965) postulated that a transpeptidation reaction involving elimination of D-alanine from a pentapeptide unit (cell wall precursor) during mucopeptide synthesis (cross-linking) could explain the observation that several D-amino acids induce formation of spheroplasts. They stated it was possible that several different D-amino acids might compete, to varying degrees, for a site on the cross-linking enzyme and thus interfere with its function. Izaki, Matsuhashi, and Strominger (1968), using a particulate enzyme system obtained from strains of Escherichia coli, showed that this was probably the case by demonstrating that D-amino acids can act as reversible inhibitors of the transpeptidation reaction. Thus,

mucopeptide synthesis would be decreased by D-amino acids in much the same way as in the presence of penicillin; however, penicillin inhibits the transpeptidation reaction irreversibly by behaving as an analog to the D-alanyl-D-alanine end of a pentapeptide and thus complexing directly with the transpeptidase.

Other aspects of cell wall synthesis reported by Grula, Smith, and Grula (1965) include the following. (1) Erwinia sp. possesses a discrete mucopeptide layer in the cell wall which contains muramic, diaminopimelic, glutamic, and aspartic acids, glucosamine and alanine in the molecular proportions of 1-1-1-0.2-1-2. (2) Cells grown in the presence of D-serine incorporate this amino acid into their mucopeptide (about 0.4 molecular proportions). It was further reported that cells grown in the presence of glycine incorporate glycine into the mucopeptide (about 0.4 molecular proportions); also, cells grown in the presence of glycine and D-serine contain decreased amounts of glycine (about 0.24 molecular proportions) but "normal" levels of D-serine (about 0.4 molecular proportions). This latter relationship between serine and glycine was also reported by Whitney and Grula (1964) who established that D-serine is incorporated into mucopeptide, in place of glycine, when cells of Micrococcus lysodeikticus are grown in the presence of this D-amino acid. (3) Pantoyl lactone and high concentrations of ammonium chloride, which allow excellent division activity in the presence of D-serine, do not prevent incorporation of serine into the mucopeptide.

Since it was shown that the amount of mucopeptide in non-dividing cells of Erwinia sp. is decreased, these cells can be considered to be partial spheroplasts even though they do not lyse readily in

distilled water. For this reason, osmotic protective conditions were utilized to determine if hypertonic conditions could prevent division inhibition and, if so, could restoration of division activity be correlated with increased or normal cell wall synthesis. Data relative to this point revealed that hypertonic conditions (obtained using propylene glycol, alpha-methyl-D-glucoside, or NaCl) or pantoyl lactone restore active cell division; however, in all instances amino sugar content of the cell wall continues to be lowered (Grula and Grula, 1964). Although the effects of pantoyl lactone on cell division and cell wall synthesis are similar to those that occur during growth under hypertonic conditions, pantoyl lactone cannot be acting osmotically since its effective concentration is only one-tenth that of osmotic protective agents. From these studies, Grula and Grula concluded that hypertonic conditions and pantoyl lactone probably prevent secondary cell membrane damage since, under conditions which allow either cell wall alterations and/or incorporation of D-serine, hypertonic conditions and pantoyl lactone permit both the invagination and separation phases of cell division to proceed in what appears to be a normal manner.

Prevention of division inhibition by hypertonic conditions strongly suggests involvement of the cell membrane in the cellular division process and further evidence to substantiate this belief can be obtained by observing that "leakage" of protein and nucleic acid occurs from filamentous cells of Erwinia species. In nearly all cases, pantoyl lactone and osmotic protective conditions significantly lower leakage of these cellular materials while causing good cell division activity. As a result of these observations, and the

direct demonstration that the cell membrane rather than the cell wall initiates the invagination process, it has been concluded that the cell membrane is probably the key structural entity in cell division in Erwinia species (Grula and Grula, 1964; Grula, Smith, and Grula, 1965).

Additional studies have revealed that D-serine causes the following metabolic lesions in Erwinia species. (a) Inhibition in synthesis of alpha-ketopantoic from alpha-ketoisovaleric acid and the subsequent reduction of alpha-ketopantoic to pantoic acid. (b) Inhibition of the alpha-decarboxylation of aspartic acid which would allow formation of beta-alanine (Grula and Grula, 1962a; 1962b). These inhibitions in the synthesis of pantothenic acid presursors, which differ from that reported by Maas and Davis (1950) in E. coli, are responsible for the diminished synthesis of coenzyme-A caused by D-serine which, in turn, leads to a decreased growth response. Although pantoyl lactone, L-alanine, or ammonium chloride can overcome division inhibition caused by D-serine, these compounds cannot, in all cases, completely restore normal levels of pantothenic acid within the cells (Grula and Grula, 1962b). Because of these findings, it was concluded that the inhibition in synthesis of pantothenic acid by D-serine does not account for inhibition of cell division in this organism (Grula and Grula, 1962b).

Most of the information presented suggests that both the cell wall and cell membrane are involved in bacterial cell division. Because pantoyl lactone or hypertonic conditions will allow division to proceed in the absence of normal mucopeptide synthesis while causing "repair" of membrane "leakage," it appears that the cell

membrane is the more important of the two structures. Evidence has not been presented however which would clearly establish that some type of damage can occur to the cell membrane in the absence of inhibition or alteration in mucopeptide synthesis which would, in turn, result in inhibition of cell division. For completeness, it should be pointed out that no one has yet clearly established the reverse set of conditions; i.e., inhibition in synthesis of mucopeptide without concomitant damage to the cell membrane which results in inhibition of cell division.

Grula and Grula (1962c) were able to demonstrate that known cell wall inhibitors, such as penicillin or vancomycin, do not inhibit division induced by pantoyl lactone in filamentous cells produced by growth in the presence of D-serine. Also, data were presented which indicate the need for both a carbon and nitrogen source in order for terminal division to occur. They postulated that this dual requirement is necessary for mucopeptide synthesis since D-chloramphenicol, although inhibiting protein synthesis, does not inhibit synthesis of cell wall mucopeptide (Hancock and Park, 1958) or terminal cell division. Recently Reeve, Groves, and Clark (1970), using a temperature-sensitive division mutant of E. coli, indicated that the block in cell division of this mutant was probably associated with a terminal step in cell wall synthesis.

In order to more fully evaluate possible functions of the cell wall and membrane during cell division in bacteria, isolation of each structure in a relatively pure form (95 to 100 percent) is necessary. These studies cannot be done at present using Gram-negative bacteria such as Erwinia sp. because the cell wall is made

up of heterogenous polymers which cannot be isolated in pure form for the needed rigorous qualitative and quantitative studies. Further, it is not possible to satisfactorily isolate the cell membrane from Gram-negative bacteria. Therefore, to obtain the needed information, it appeared necessary to isolate a Gram-positive organism in which division could be inhibited and from which the necessary structures could be isolated in a high state of purity.

In the early part of this study we were successful in isolating a nutritional mutant of M. lysodeikticus in which division was inhibited by growth in the presence of D-serine, D-cycloserine, mitomycin c, penicillin, or sub-optimal concentrations of magnesium (Grula and King, 1970). This organism was chosen for these studies for the following reasons. (a) A defined medium is available for growth (Grula, 1962); this medium permits the extensive chemical manipulations necessary during growth and development of non-dividing cells. (b) The cell wall can be isolated easily in a pure form and it is one of the least complex known. (c) Extensive study of the cell membrane of this organism has been done by Butler, Smith, and Grula (1967) who developed a method by which membranes of this organism can be isolated in a high state of purity. Also, as reported by Grula, Butler, King, and Smith (1967), proteins making up the cell membrane of this organism can be obtained relatively free of associated lipids. (d) Lysozyme-produced protoplasts of this organism are easy to prepare thus making it possible to isolate and study any and all internal macromolecules in a high state of purity and with minimum "damage" during preparation.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The organism used in this study is a mutant of the Purdue University strain of Micrococcus lysodeikticus, a Gram-positive non-motile coccus believed to be a transplant from the original strain isolated by Fleming (Grula, Luk, and Chu, 1961). This mutant, which has been designated dis-II (division inhibited by serine), was isolated by nutritional selection using aspartic acid as the major source of carbon, nitrogen, and energy in a chemically defined medium (Grula, 1962). Techniques for isolation and several characteristics of the organism have been reported (Grula and King, 1970). Further nutritional selection has been accomplished using the dis-II strain to obtain a mutant not requiring the addition of a purine (inosine) for growth. This mutant was isolated by growth in the absence of inosine, designated dis-II pur^+, and used for the studies reported in this thesis. This organism grows rapidly and responds to all the compounds reported previously (Grula and King, 1970) in the same manner as the dis-II strain. Biochemical characteristics of this organism are identical to those described for the parent strain (Grula, 1962).

Stock cultures of the organism were maintained on slants of

defined medium solidified by addition of 1.5 percent Agar Agar No. 3 (Oxoid-Consolidated Laboratories, Incorporated, Chicago Heights, Illinois, USA). Cultures (stocks) were transferred approximately every two months. Twenty-four hour old cells grown at 30° C were used as source of inoculum for all experiments. The streak plate method was occasionally employed to aid in possible detection of contamination in the culture.

Medium and Growth

The defined medium used has the same formulation as described by Grula and King (1970) except for the omission of inosine. This medium is composed of the following compounds per 100 ml: biotin (50 ug), L-glutamic acid (358 mg), L-aspartic acid (358 mg), L-phenylalanine (40 mg), L-tyrosine (30 mg), Na_2HPO_4 (200 mg), and $MgSO_4 \cdot 7H_2O$ (2.0 mg). The pH of the complete medium was always adjusted to pH 7.6 to 7.8 using solid KOH prior to autoclaving (15 minutes at 250° F). A saturated solution of $FeSO_4$ $(NH_4)_2SO_4 \cdot 6H_2O$ was autoclaved separately and the resulting red precipitate removed by filtration before a trace aliquot (1 drop per 5 ml culture medium) was added aseptically to the medium (approximately 25 ug per 100 ml medium).

Inoculation of all media was accomplished using cells grown on defined medium slants as given above. The inoculum was washed one time using centrifugation (3000 rpm for 15 minutes) in 0.85 percent sterile saline and the optical density at 540 nm (Coleman Junior Spectrophotometer) adjusted to approximately 0.40. One drop of this suspension was added per 5.0 ml of medium (tube experiments).

To obtain larger amounts of cells, 100 ml aliquots of defined medium were placed in 250 ml Erlenmeyer flasks and 20 drops (approximately 1 ml) of inoculum were added. Incubation was done at 30° C using a Gyrotory shaker (New Brunswick Scientific Company, New Jersey, Model G-26) set at a shaking speed of 9.5.

Cell Fractionation Procedures

All chemical fractionations of cells were accomplished utilizing the procedure of Park and Hancock (1960) except that pepsin was substituted for trypsin in the enzymatic treatment.

Isolation of Cell Membranes

Whole cell membranes of M. lysodeikticus dis-II pur^+ were prepared according to the procedure described by Butler et. al. (1967).

Cell Wall Mucopeptide Isolation

Cell wall mucopeptide was obtained using the chemical procedure of Park and Hancock (1960) except that pepsin was used in place of trypsin because the cell wall of the dis-II strain appears to undergo some type of degradation by trypsin (Grula and King, 1970).

Titration of Test Compounds

All titrations were made by adding various concentrations of the compounds under study to 5.0 ml (final volume) of the basal defined medium. Growth was monitored by measuring optical density of cultures at 540 nm using a Coleman Junior Spectrophotometer after

various times of incubation.

Hydrolysis Procedures

Complete hydrolysis of protein or cell wall was accomplished by placing a weighed and dry (lyophilized) sample in 6N hydrochloric acid in a soft glass tube (8 x 100 mm) and the tube sealed *in vacuo*. Liquid samples were diluted with an equal volume of concentrated hydrochloric acid and placed in soft glass tubes and subsequently sealed *in vacuo*. Sealed tubes were kept at 100 to 105° C for 18 hours.

Partial hydrolysis was used to release the amino sugars or to obtain peptides from cell walls. For these studies, samples were hydrolyzed in 4N hydrochloric acid for 4 hours at 105° C *in vacuo*.

After hydrolysis, samples were always taken to dryness by placing them in an evacuated desiccator containing concentrated sulfuric acid and potassium hydroxide pellets in separate containers. Dried residues were dissolved in deionized water for subsequent analyses unless otherwise specified.

Chromatography and Detection of Amino Acids

Hydrolyzed samples were spotted on thin-layer chromatograms (MN300 Cellulose obtained from Brinkmann Instruments, Westbury, New York) and developed in the two-dimensional system of Heathcote and Jones (1965). Samples of 10 to 500 ul were spotted using a stream of either cold or warm air for drying. Amino acids were detected by spraying with a solution of 0.5 percent ninhydrin in 95 percent

acetone, then heating at 100° C for approximately 5 minutes. Amino acids appear as blue, yellow, or reddish-brown spots on a white background.

Chromatography and Detection of Carbohydrates

Hydrolyzed samples were spotted on Whatman No. 1 paper and developed in either a one-dimensional system containing 1-butanol-pyridine-deionized H_2O-(6.0:1.8:1.8), or in the two-dimensional system of Redfield (1953). Detection of carbohydrates was accomplished by spraying chromatograms with aniline hydrogen oxalate reagent (100 ml 0.1M oxalic acid containing 0.9 ml aniline) then heating to 100 to 105° C for approximately 15 minutes. This spray gives a bright red color with pentoses or aldopentoses in 5 minutes and a brown color with hexoses in 10 minutes. Ketohexoses and dihydroxyacetone do not produce either a red or brown color reaction. Analysis for amino sugars (glucosamine and muramic acid) was performed utilizing a sensitive colorimetric technique (Rondle and Morgan, 1955).

Quantitative Ninhydrin Determination of Free Amino Groups

Ninhydrin reacts with free amino groups yielding a purple product which can be determined colorimetrically. To a 1.0 ml sample containing between 0.02 and 0.2 uM of free amino groups, 0.5 ml of 3 percent ninhydrin in methycellosolve was added. The samples were mixed thoroughly, the tubes stoppered with marbles, and placed

in a boiling water bath for 15 minutes. After removal from the bath, 5.0 ml of diluent (50 percent isopropanol) was added and the tube contents mixed vigorously. After cooling to room temperature, the absorbance was read at 570 nm using a Coleman Junior Spectrophotometer. The amino acid L-leucine was used to prepare a standard curve.

Quantitative Amino Acid Determination

This work was done by Dr. P. Guire using a Beckman 120° C amino acid analyzer stationed in the Biochemistry Department at Oklahoma State University. Dried samples of known weight were utilized in all of these determinations.

Glucose Oxidase

Glucostat reagent was obtained from Worthington Biochemical Company, Freehold, New Jersey. Preparation of the reagent was done according to the following procedure: The chromogen was placed in 60 ml of water, the glucose reagent was dissolved in the chromogen solution, and the volume then brought to 90 ml using deionized water. To 1 ml sample and 1 ml of a glucose standard (0.2 mg), 9 ml of the above reagent was added and mixed thoroughly. After incubation (10 minutes at room temperature), 1 drop of 4N hydrochloric acid was added, mixed, and incubation continued an additional 5 minutes for stabilization of color. Readout was accomplished at 400 nm using a Coleman Junior Spectrophotometer.

Calculation of glucose present in unknown samples was done in the following manner:

$$\frac{\text{Absorbance of Sample}}{\text{Absorbance of Glucose Standard}} \times \text{Concentration of Glucose Standard} =$$

Concentration of the Unknown

Radioautography

Radioautography was performed by placing thin-layer chromatograms next to Blue Brand x-ray film and allowing them to remain in close contact in the dark for 1 month at 25° C. This length of time will allow detection of about 100 counts per minute of ^{14}C-labeled compound. Films were developed in Diafine or Edwals x-ray developer.

Isotope Labeling

Cells were grown in defined medium and various additions made at time of inoculation or after 12 hours of growth. Labeled compounds were added to a final concentration of 0.01 uC per ml of medium. These compounds were always diluted with non-labeled carrier to obtain correct concentrations during growth. The following radioisotopes were used: L-aspartic-U-^{14}C (154 mC/mM), L-glutamic-U-^{14}C (148 mC/mM), D-alanine-1-^{14}C (13.45 mC/mM), glycine-2-^{14}C (5.0 mC/mM), glycerol-2-^{14}C (7.0 mC/mM), L-lysine-U-^{14}C (100 mC/mM), D-serine-3-^{14}C (5.2 mC/mM), D-cycloserine-^{14}C (0.09 uC/uM), L-phenylalanine-U-^{14}C (135 mC/mM).

Dry Weight Determination

The relation between dry weight and absorbancy at 540 nm was determined for cells grown in basal medium using a Coleman Junior Spectrophotometer. Cells which had grown 24 hours were washed twice

in 0.85 percent saline solution and once with deionized water. Dilutions of washed cells were made in water and the absorbance of each sample measured. Samples were then placed into dried, pre-weighed aluminum dishes and heated at 100° C in a dry air oven to a constant weight (usually 24 hours). The curves were constructed by plotting absorbancy at 540 nm versus mg dry weight of cells.

Preparation of 2,4-Dinitrophenyl Derivatives

Weighed samples (preferably less than 2.0 mg) of cell wall were suspended in 0.80 ml of deionized water and 0.2 ml of 10 percent $K_2B_4O_7 \cdot 5H_2O$. To this suspension 0.2 ml of a 10 percent 1-fluoro-2,4-dinitrobenzene (FDNB) solution was added (FDNB reagent is diluted to 10 percent with 100 percent ethanol) and the suspension then incubated at 60° C for 2 hours (Ghuysen and Strominger, 1963). At the end of the incubation period the solutions were centrifuged for 15 minutes at 15,000 rpm. The resulting pellet was washed two times with 100 percent ethanol, placed in 6N hydrochloric acid and sealed *in vacuo* (Whittle and Anderson, 1969). Hydrolysis was accomplished by placing the samples at 100 to 105° C for 15 hours. The 2,4-dinitrophenyl (DNP) derivatives of possible N-terminal amino acids were then extracted with ether, leaving mono-DNP lysine in the aqueous phase. The ether extracts were dried by evaporation at 37° C and the residue redissolved in 0.05M ammonium hydroxide. Aliquots were chromatographed on thin-layer plates of silica gel G in chloroform-methanol-acetic acid (85:14:1; v/v/v) as described by Ghuysen, Tipper, Birge, and Strominger (1965). The mono-DNP lysine remaining in the aqueous HCl was dried *in vacuo* in the presence of

concentrated sulfuric acid and potassium hydroxide pellets and redissolved in water-saturated n-butanol. The aqueous phase derivatives were separated by thin-layer chromatography (silica gel G) in benzyl alcohol-chloroform-methanol-water-concentrated ammonia (30:30:30:6:2; v/v/v/v/v) as described by Ghuysen and Strominger (1963). An additional chromatographic system utilized for separation of possible DNP derivatives contained n-butanol-pyridine-acetic acid-H_2O (60:45:4:30; v/v/v/v). The mono-DNP lysine (epsilon-DNP-lysine) was eluted from plate scrapings using 0.01M ammonium hydroxide-methanol (1:1; v/v). Amount of this DNP derivative was estimated by reading color present at 360 nm and relating this to a standard curve prepared using N-epsilon-DNP-L-lysine obtained from Sigma Chemical Company, Saint Louis, Missouri.

Electron Microscopy

Cells were grown in 5.0 ml of basal medium plus the appropriate concentration of test compound. After incubation, cells were prefixed directly in the growth medium by adding glutaraldehyde to a final concentration of 2.5 percent and permitting contact for 1 hour at 25° C. After centrifugation, the cells were washed three times in Veronal buffer (pH 6.1) under RK conditions (Ryter and Kellenberger, 1958). The cells were then post-fixed using 1 percent osmium tetroxide in Veronal buffer for 24 hours at 4° C. The cells were again washed three times in Veronal buffer after which they were embedded in 3 percent Bacto-Agar (Difco Laboratories, Detroit, Michigan). After solidification, the agar was then cut into small blocks and the blocks treated with 0.5 percent uranyl acetate in Veronal buffer

(pH 6.1) for 5 hours at 25° C. The agar blocks were then dehydrated by passing them through a 25 to 100 percent ethyl alcohol series (15 minute treatments), treated with three changes of propylene oxide (15 minute treatments), and placed in a mixture containing equal parts of propylene oxide and complete Araldite resin monomer for 2 hours at 25° C. Another equal part of complete resin monomer was then added, mixed, and allowed to infiltrate overnight at 25° C. The Araldite monomer was prepared according to the method of Luft (1961). Gelatin capsules (size 00) were filled with fresh monomeric form of resin, the agar blocks placed on the surface and then allowed to settle to the bottom of the capsules (agar blocks settled to the bottom in 24 hours). The resin was polymerized by heating for 72 hours at 62° C.

Thin-sections of the bacteria were cut using a Porter-Blum MT-2 microtome, picked up on uncoated 400-mesh copper grids and stained according to the procedure described by Grula and Smith (1965). Observation of sections was accomplished utilizing an RCA EMU-3G electron microscope.

Hydrazinolysis Procedure

The procedure of Akrabori, Ohno, and Narita (1952) was used throughout this study. Carboxy-terminal amino acids were quantitatively analyzed using an amino acid analyzer as mentioned previously.

Liquid Scintillation Counting

The counting fluid for ^{14}C-labeled materials was prepared by dissolving 2.0 gm of 2,5-diphenyloxazole (PPO) and 25 mg of 1,4-

bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 238 ml of 1,4-diethylene dioxide. Also, ^{14}C-labeled materials were counted using Aquasol (New England Nuclear Company, Boston, Massachusetts). To a 1 ml sample, 9 ml of either of the counting solutions was added.

Preparation and Detection of Released Proteins

Cells were grown in 100 ml of defined medium for the appropriate incubation time and under proper test conditions. The supernatant from sedimented cells was placed in dialysis tubing and dialyzed against two changes of tris-hydrochloric acid buffer (pH 8.5; 0.14M) to allow escape of small molecules. The supernatant was then concentrated (to about 1 ml) by placing the dialysis bag in 10 percent Carbowax-6000. The concentrated supernatant was analyzed for proteins using polyacrylamide gel electrophoresis.

Cold Osmotic Shock

The technique reported by Heppel (1967) was utilized.

Polyacrylamide Gel Electrophoresis

The procedure is the same as the one described in the Buchler Instructions for Operation of the Polyanalyst for an anionic gel system (pH 9.3) or the procedure described by Weber and Osborn (1969) utilizing sodium lauryl sulfate polyacrylamide gel electrophoresis.

Infrared Spectroscopy

A Beckman spectrophotometer Model IR-7 located in the Department of Chemistry, Oklahoma State University, was utilized throughout this

study. Whole membranes of M. lysodeikticus dis-II pur^+ were prepared according to the procedure of Butler et. al. (1967). The whole membranes were washed three times in cold (4° C) deionized water and then lyophilized. Approximately 10 mg of lyophilized membrane was mixed thoroughly with 200 mg of potassium bromide. Pellets were prepared by compressing this mixture in a Beckman pellet press. The pellets were dried in vacuo over P_2O_5 for 24 hours before spectra were taken.

Preparation of Samples for Sucrose Density Gradients

Cells were harvested by centrifugation and resuspended in 0.01M Tris buffer (pH = 8.0) after growth for 30 hours at 30° C on a reciprocal shaker. Lysozyme was added to a final concentration of 0.05 mg/ml and the mixture was allowed to incubate 30 minutes at room temperature. After incubation, two volumes of cold (4° C) distilled H_2O were added dropwise to the mixture. The resulting lysate was then layered on top of discontinuous sucrose gradients (20 to 50 percent). A Spinco Model-2 Ultracentrifuge was employed for centrifugation using the SW-39 head at 37,000 rpm for 4 hours at 4° C. After centrifugation, the bottom of the tubes were pierced with a 24-gauge needle and samples were collected manually.

CHAPTER III

RESULTS

Development of Division Inhibition System

Grula and King (1970) described the isolation of a strain of Micrococcus lysodeikticus (designated dis-II) by nutritional selection using aspartic acid as the major source of carbon, nitrogen, and energy. As reported, this strain of M. lysodeikticus does not divide when grown in the presence of D-serine, penicillin, mitomycin c, D-cycloserine or sub-optimal concentrations of magnesium. It was also reported that all of these division inhibiting compounds decrease growth yields and induce varying amounts of lysis. Addition of L-lysine to the D-serine-containing medium allows some increased growth and more pronounced inhibition of division but does not significantly reduce lysis. Pantoyl lactone, D- and L-alanine, and to a lesser degree Carbowax-400 (hypertonic conditions), or glycine will significantly prevent but not reverse inhibition of division in a medium containing D-serine plus L-lysine. Spermine, a compound known to prevent inhibition of division in Erwinia sp. (Grula, Smith, and Grula, 1968), will neither prevent nor reverse inhibition of division of M. lysodeikticus dis-II in a D-serine plus L-lysine medium.

The large cells produced in the presence of the various division-inhibiting compounds are not considered to be protoplasts since they

do not lyse in distilled water, are easily lysed by losyzyme, stain readily with the cell wall stain Alcian Blue, and remain Gram-positive. Also, thin-slicing reveals that a prominent cell wall is present.

None of these initial division inhibiting systems was completely adequate for detailed structural analyses since poor growth yields and extensive lysis are obtained with all compounds tested for division inhibiting activity (mitomycin c is an exception) even when using the dis-II pur^+ strain. It should also be noted that of the division inhibiting agents tested, only penicillin appears to have a specific site of action. Therefore, development of a system was needed wherein good growth (with minimal lysis) and a high percentage (greater than 95 percent) of non-dividing cells could be obtained. It was also reasoned that it would be helpful if the division inhibiting compound had a specific site of attack. Of the compounds originally tested, D-cycloserine seemed to be the most promising since a great percentage of non-dividing cells can be obtained and the mechanism of action of D-cycloserine has been extensively studied and documented.

Strominger, Ito, and Threnn (1960) have shown that either D- or L-cycloserine inhibits several transaminases having pyridoxal phosphate as cofactor. Inhibition of alanine racemase and D-alanyl-D-alanine synthetase are most profoundly affected by D-, but not L-cycloserine, exhibiting Ki values of 6.0×10^{-5} and 9.0×10^{-3}M respectively for D-cycloserine. Since both enzymes are important for proper cell wall synthesis, inhibition of either would lead to decreased mucopeptide synthesis and probably lysis of the cell.

In order to utilize D-cycloserine, an optimal concentration had to be ascertained which permitted the most growth and greatest

percentage of large cells. Data given in Figure 1 show that the dose versus growth curve is very steep and much like the one previously reported for Erwinia sp. (Grula and Grula, 1965). At a concentration of 4.0×10^{-5}M the population consists primarily of non-dividing cells and growth is inhibited approximately 87 percent on a dry weight basis. Although a large amount of lysis occurs, particularly at higher concentrations, D-cycloserine is a potent inhibitor of cell division.

Except for pantoyl lactone and D-alanine, most compounds which were tested for their ability to prevent either growth or division inhibition by D-cycloserine proved to be ineffective (Table I). Addition of pantoyl lactone to the medium prevents inhibition of division but does not restore growth to the control level. Although D-alanine does, for all practical purposes, completely prevent inhibition of both growth and division by D-cycloserine, this effect of D-alanine is, to a large measure, negated when pantoyl lactone is also added to the same medium. At this point in my study, it appeared possible that pantoyl lactone can interfere with entry of D-alanine into the cell thus blocking its effect. Data which relate to this point will be presented later in this chapter.

Since Strominger, Ito, and Threnn (1960) have shown that the mechanism of action of D- but not L-cycloserine involves inhibition of cell wall synthesizing enzymes (D-alanine racemase and D-alanyl-D-alanine synthetase), it was of interest to determine if the L-isomer was also an effective inhibitor of division. Data relative to these compounds are shown in Figure 2. At lower concentrations, L-cycloserine is actually more toxic to growth than D-cycloserine, but of greater importance is the fact that toxicity due to the L-isomer

Figure 1. Inhibition of Growth by D-Cycloserine of *M. lysodeikticus* Dis-II Pur^+.

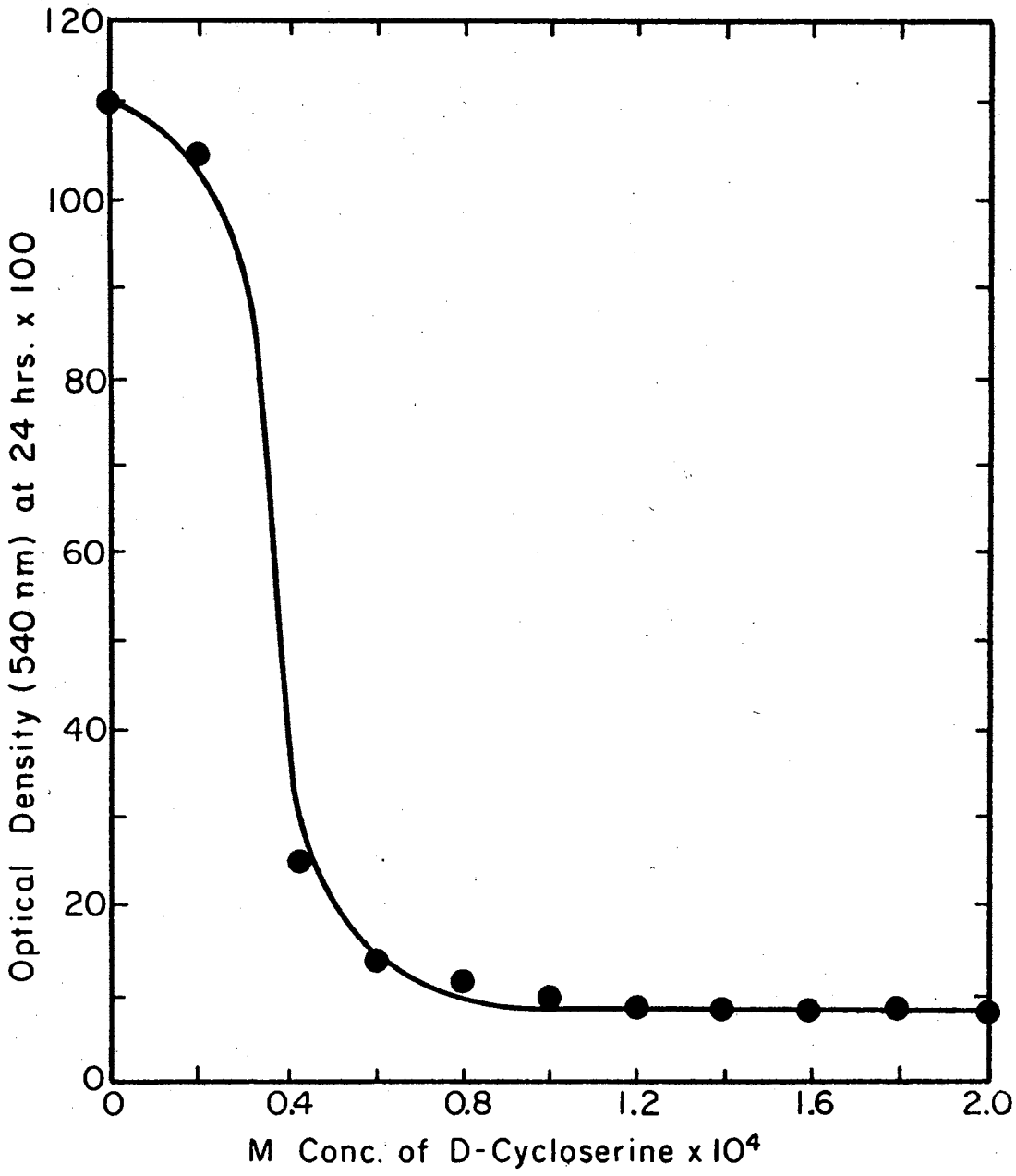

120
100
80
60
40
20
0
Optical Density (540 nm) at 24 hrs. x 100
0
0.4
0.8
1.2
1.6
2.0
M Conc. of D-Cycloserine x 10⁴

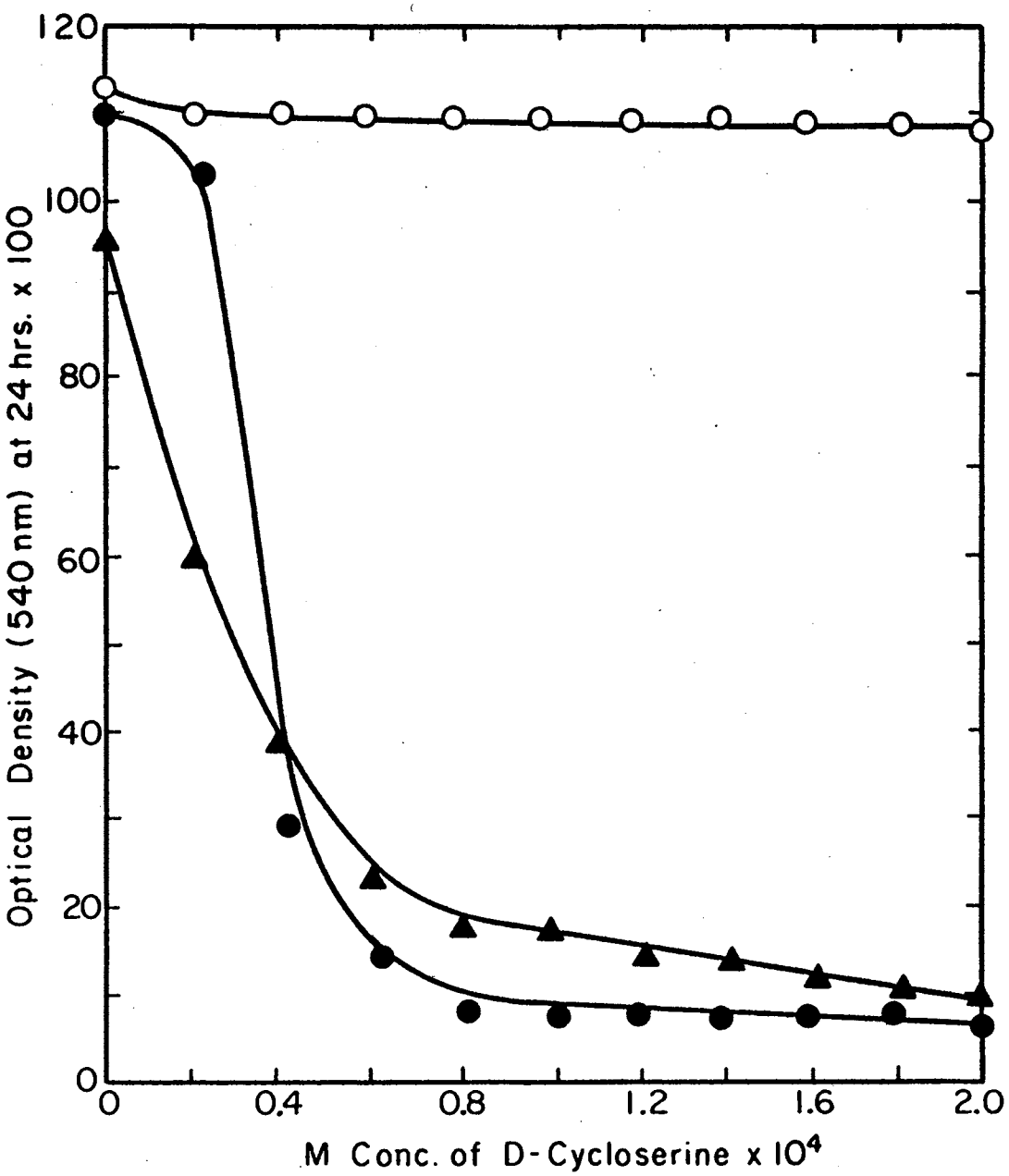
TABLE I

EFFECT OF VARIOUS COMPOUNDS ON GROWTH AND DIVISION IN THE PRESENCE OF D-CYCLOSERINE*

Compound Added	Final Concentration	O.D. (24 hour)	Cell Size in Microns (24 hour)
Basal Medium	---	0.95	1.0
+ D-Cycloserine	4.0×10^{-5}M	0.20	4.0-5.0
+ Pantoyl lactone	8.0×10^{-2}M	0.55	1.0-2.0
+ D-Alanine	6.0×10^{-5}M	0.90	1.0
+ L-Alanine	6.0×10^{-5}M	0.26	4.0-5.0
+ Pyridoxal	6.0×10^{-5}M	0.16	4.0-5.0
+ DL-Alanyl-DL-Alanine	6.0×10^{-5}M	0.23	4.0-5.0
+ Pantoyl lactone + D-alanine	8.0×10^{-2}M 6.0×10^{-5}M	0.40	2.0-4.0

*All cultures except #1 contained D-cycloserine (4.0×10^{-5}M).

Figure 2. Antagonism of L- or D-Cycloserine Growth Inhibition by D-Alanine. ●, D-cycloserine; ○, D-cycloserine plus D-alanine (6.0×10^{-5}M); and ▲, L-cycloserine plus D-alanine (6.0×10^{-5}M).


120
100
80
60
40
20
0
Optical Density (540 nm) at 24 hrs. x 100
0
0.4
0.8
1.2
1.6
2.0
M Conc. of D-Cycloserine x 10⁴

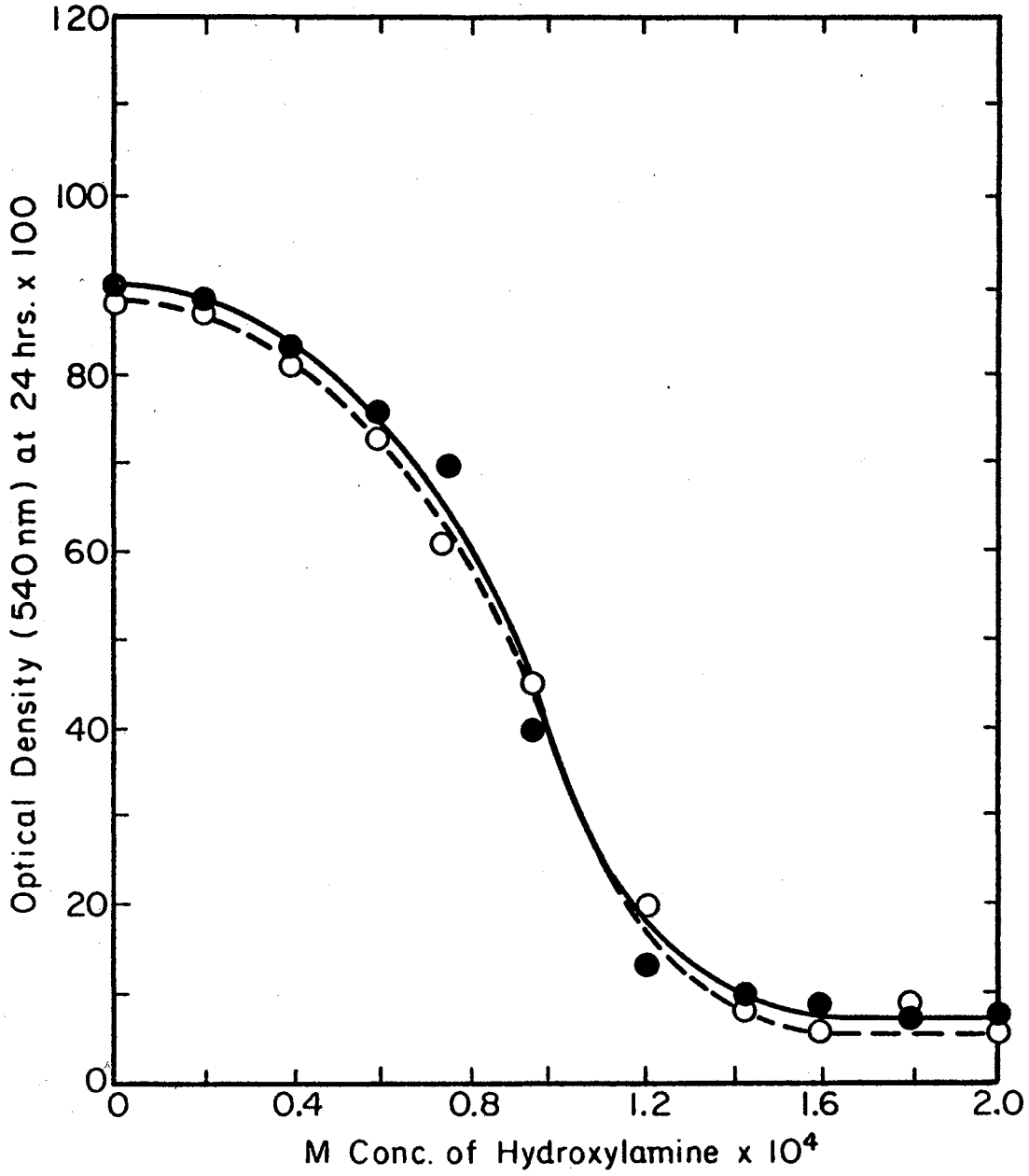
cannot be prevented by D-alanine. Also, regardless of concentration, L-cycloserine will not inhibit division of the dis-II pur^+ strain of M. lysodeikticus.

Preliminary data of this type made it appear likely that the two wall synthesizing enzymes (alanine racemase and alanine synthetase) are intimately involved in division behavior of this organism. To test further possible involvement of these two enzymes, various concentrations of hydroxylamine were added to the growth medium since Neuhaus (1967) reported that hydroxylamine also has a strong affinity for alanine racemase (Ki is 1.2×10^{-5}M). Data presented in Figure 3 show that hydroxylamine is very toxic to growth. At a concentration of 1.0×10^{-4}M or higher, extensive development of non-dividing cells occurs and lysis, indicative of cell wall damage, is very evident.

Grula and Grula (1962c) also reported that hydroxylamine is a potent inhibitor of cell division and causes lysis in Erwinia species. Although hydroxylamine inhibits division and appears to be causing a decrease in mucopeptide synthesis, neither growth nor division inhibition can be overcome by D-alanine. Because of these observations, it is necessary to conclude that hydroxylamine causes inhibition of growth and division by a mechanism(s) other than simply by inhibition of alanine racemase. Thus, inhibition of cell division by hydroxylamine does not help in proving that inhibition of alanine racemase is a sufficient condition to bring about inhibition of division in this organism.

Detailed cell wall analyses require a substantial quantity of mucopeptide in a high state of purity; therefore, to obtain better growth and an even greater percentage of division inhibited cells,

Figure 3. Growth as a Function of Hydroxylamine Concentration. ●, hydroxylamine; and ○, hydroxylamine plus D-alanine (6.0×10^{-5}M).


120
100
80
60
40
20
0
Optical Density (540nm) at 24 hrs. x 100
0
0.4
0.8
1.2
1.6
2.0
M Conc. of Hydroxylamine x 10^4

D-cycloserine was added to logarithmically growing cells (12 hours) rather than at time of inoculation. Growth of this organism after such addition of D-cycloserine is shown in Figure 4. It appears that, unlike D-serine which will not inhibit either division or growth when added to logarithmically growing cells (Grula and King, 1970), addition of D-cycloserine to cells in this stage of growth will cause formation of large cells (nearly 100 percent) in approximately 6 hours. Although some lysis also occurs (none is evident within 4 hours after addition of D-cycloserine), this procedure was felt to be adequate for analysis of mucopeptide and other components of the cell since amount of growth, in particular, is substantially increased.

As shown in Table II, addition of D-alanine, pantoyl lactone, or spermine with D-cycloserine prevents division inhibition and, depending upon which agent is added, allows various amounts of growth stimulation. Carbowax-400 prevents division inhibition to some extent but it is not nearly as effective as the other three compounds.

To study possible reversal of division inhibition, I added D-cycloserine at 12 hours, allowed 4 and 8 additional hours incubation to permit the cells to be committed to division inhibition, then added the compounds shown in Table II to the growth medium. D-Alanine was able to cause some "reversal" only of 4-hour cells, but the effects were not great. Extensive growth of normal sized cells eventually dominates in all cultures (after 24 hours) but these are judged to represent preventions rather than reversals of division inhibition. Thus, we have again concluded that prevention of division inhibition in this organism is possible, but no method yet exists to reverse division inhibition once the cells are committed to this state.

Figure 4. Growth Response of Cells to D-Cycloserine Added at Twelve Hours. ●, basal medium (control); and ▲, basal medium plus D-cycloserine (7.0×10^{-5}M).

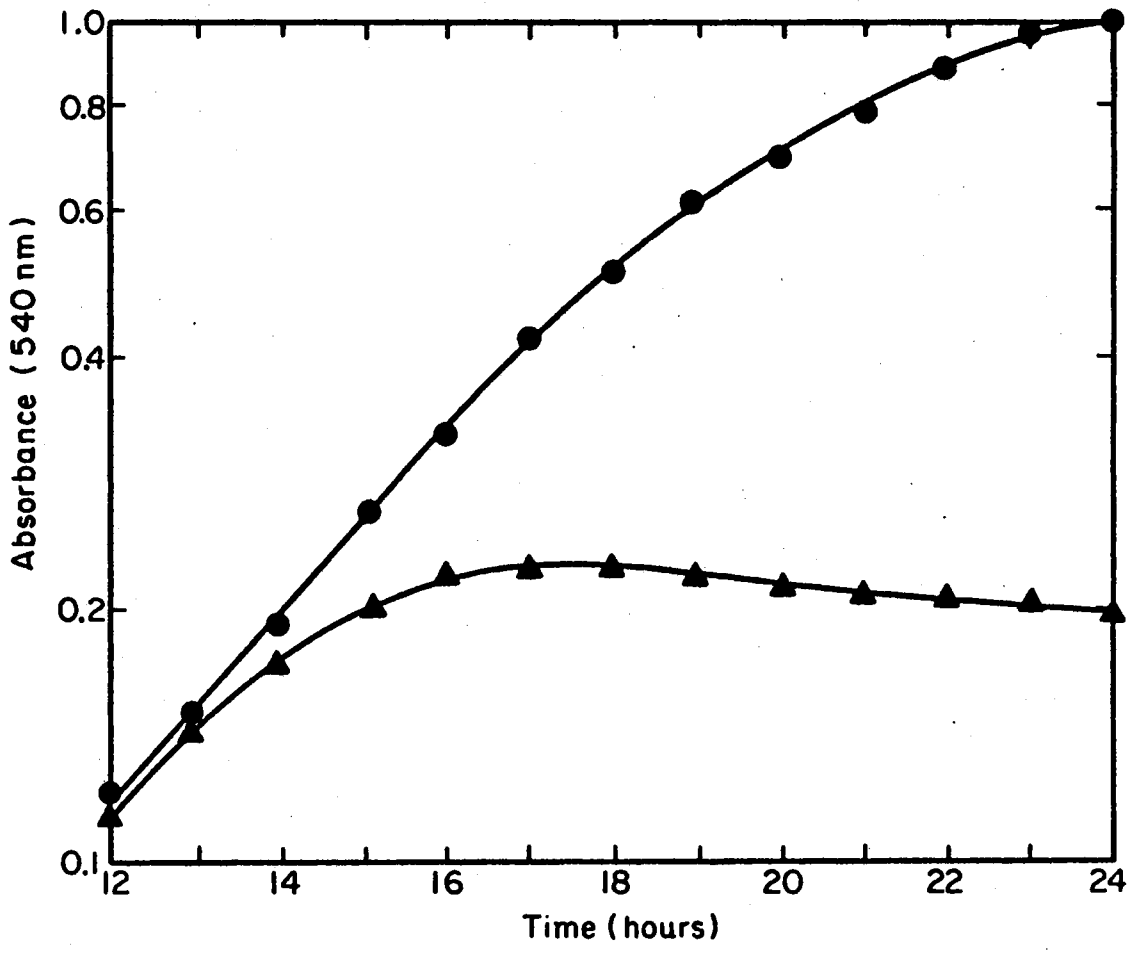

1.0
0.8
0.6
0.4
0.2
0.1
Absorbance (540 nm)
12
14
16
18
20
22
24
Time (hours)

TABLE II

PREVENTION OF THE EFFECTS OF D-CYCLOSERINE*

Compound Added at 12 hour	Final Concentration	O.D. (20 hour)	Cell Size in Microns (20 hour)
Basal Medium	---	0.75	1.0
+ D-Cycloserine	7.0×10^{-5}M	0.18	4.0-5.0
+ Pantoyl lactone	8.0×10^{-2}M	0.29	1.0-2.0
+ Spermine	1.9×10^{-2}M	0.22	1.0-2.0
+ Carbowax-400	12%	0.21	3.0-5.0
+ D-Alanine	6.0×10^{-5}M	0.73	1.0

*All cultures except #1 contained D-cycloserine (7.0×10^{-5}M) which was added after 12 hours of growth had occurred.

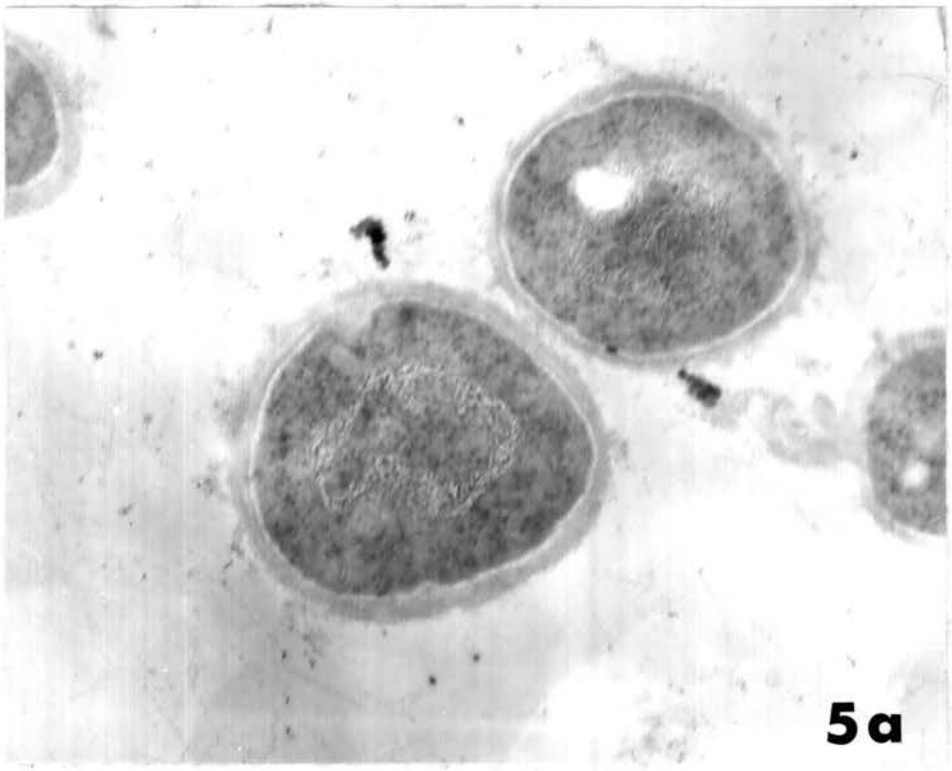
Morphological Effects of D-Cycloserine

A comparison of thin-sections of typical dividing and non-dividing cells is shown in Figure 5a and 5b. It is apparent from these pictures that cells grown in the presence of D-cycloserine possess a discrete cell wall. In Figure 6 cells are shown that have been protected from D-cycloserine action by pantoyl lactone (6a), spermine (6b), or Carbowax-400 (6c). Cells grown in the presence of any one of these three compounds appear to possess a decreased amount of cell wall even though either pantoyl lactone or spermine permits cells to be normal in size.

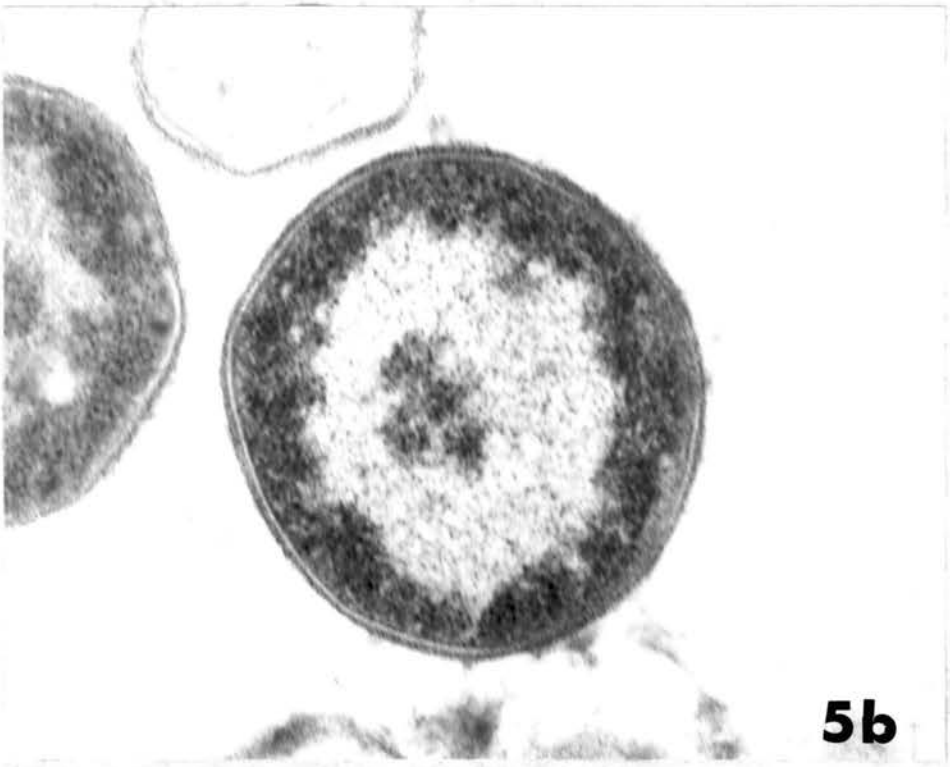
In order to determine if the amount of cell wall per unit of cell volume in each situation was actually decreased, the following calculations were made using electron micrographs all enlarged to the same extent: total cell volume (diameter measured completely across the cell), inner cell volume (diameter measured from just inside the cell wall), and volume of the cell wall (outer cell volume minus inner cell volume). All volume calculations were made using the formula $4/3\ \pi r^3$ (volume formula for a sphere) and only round cells (5 to 10 of each) were used for measurements. In order to calculate various ratios of cell or cell wall volumes, the cell and cell wall volumes calculated for normal cells were set as one. To have the same amount of cell wall per unit cell volume as in control cells, the ratios of the cell and cell wall volume should increase equally. In other words, if the cell volume increases 5 times, then an equal increase of cell wall volume should occur. Percent inhibition of mucopeptide was calculated using the following formula:

Figure 5a. Thin-Section of a Normally Dividing Cell (55,940X).

Figure 5b. Thin-Section of a Non-Dividing Cell (55,940X). Cells were grown in the presence of D-cycloserine (7.0×10^{-5}M).



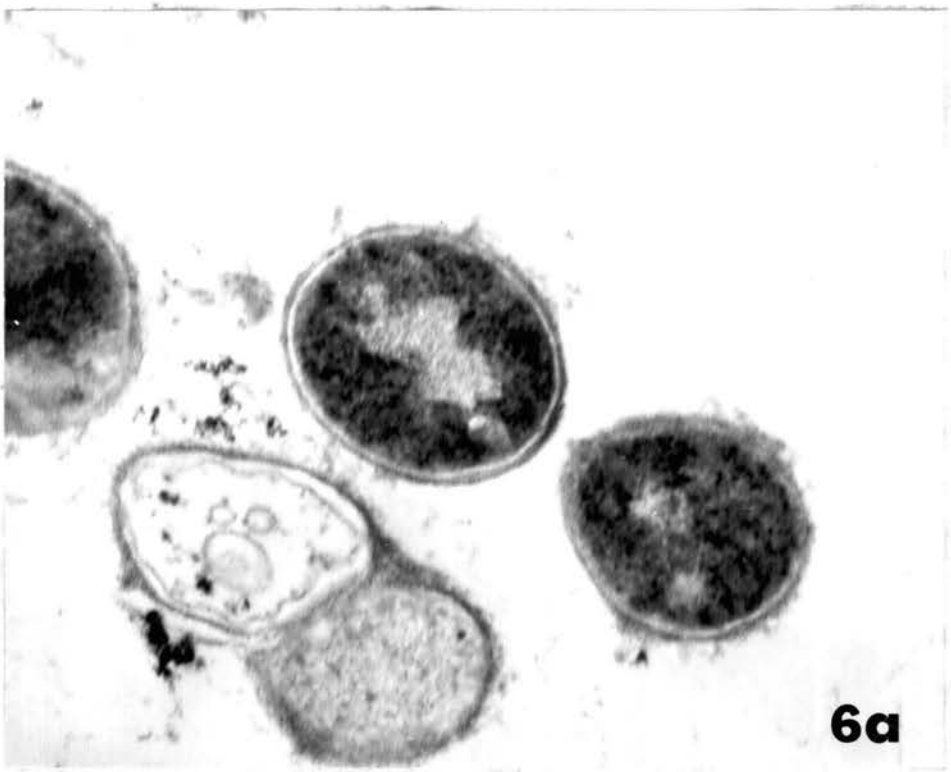
5a



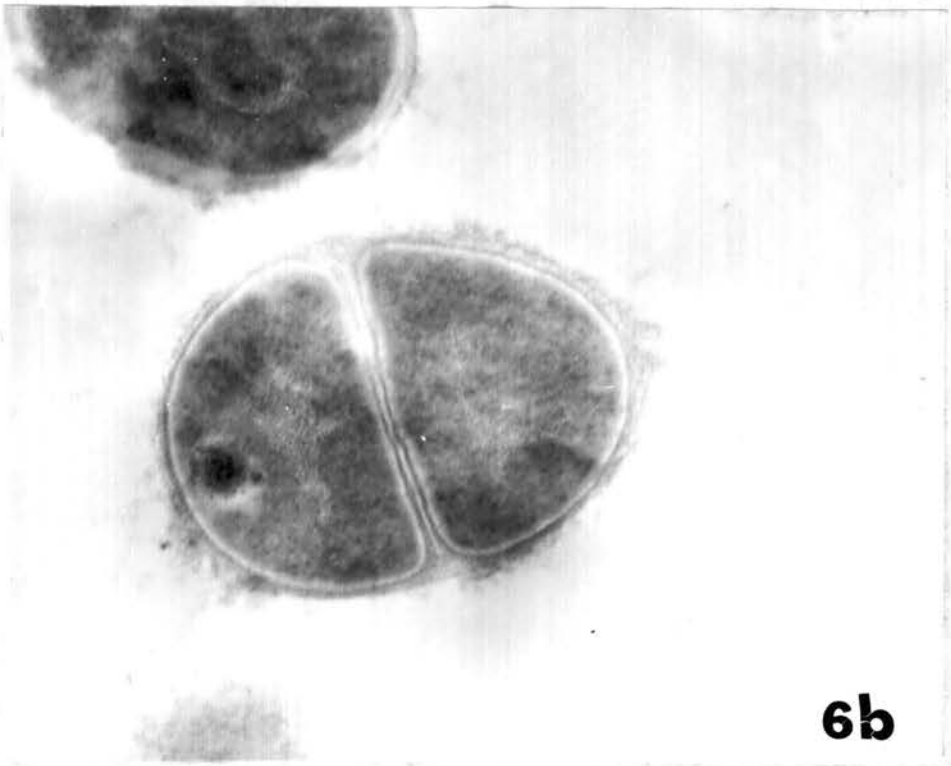
5b

Figure 6a. Cells Grown in the Presence of D-Cycloserine Plus Pantoyl Lactone (55,940X). All concentrations were as given in Table V.

Figure 6b. Cells Grown in the Presence of D-Cycloserine Plus Spermine (55,940X). All concentrations were as given in Table V.

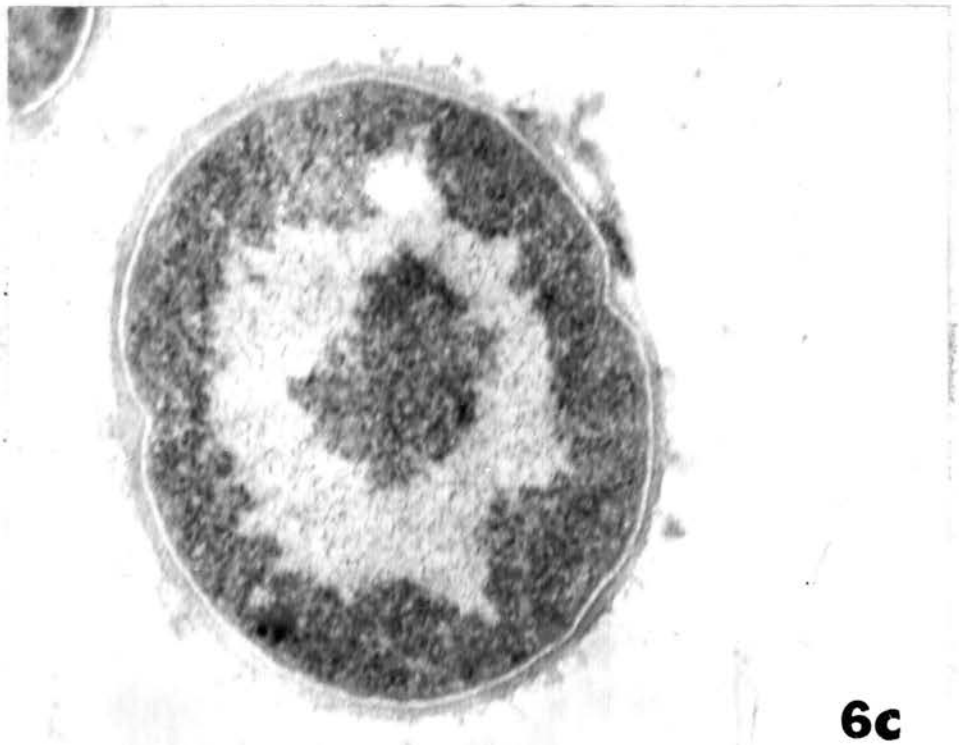


6a



6b

Figure 6c. Cells Grown in the Presence of D-Cycloserine Plus Carbowax-400 (55,940X). All concentrations were as given in Table V.



6c

$$\frac{\text{Cell volume ratio (outside) - cell wall volume ratio}}{\text{Cell volume ratio (outside)}} \times 100$$

Our data (Table III) reveal that the volume of cell wall in D-cycloserine grown cells is diminished about 50 percent. Although pantoyl lactone partially restores this deficit, it is not complete and no restoration occurs in cells grown in the presence either of spermine or Carbowax-400. It can also be seen from observation of these cells (either normal or non-dividing) that only one nuclear body is present in each cell.

Cell Wall Analyses

Electron microscopic examination of cells as well as lysis of growing cultures of non-dividing cells indicated that growth in the presence of D-cycloserine brings about a decrease in cell wall synthesis. In order to ascertain the extent and type of damage occurring to the cell wall, various chemical analyses were performed. The objective of these studies was to determine if a correlation could be established between inhibition of cell division and some specific type of wall damage.

In one type of study cell walls were isolated from a known weight of whole cells, lyophilized, and weighed. Since the dry weight of both the cells and isolated mucopeptide were known, percentage of mucopeptide present could be calculated. Representative data presented in Table IV reveal that, on a weight basis, non-dividing cells possess less mucopeptide. It can again be seen that compounds preventing division inhibition by D-cycloserine (pantoyl lactone, spermine, and to a much lesser degree Carbowax-400) do not restore normal cell wall

TABLE III

CELL AND CELL WALL VOLUMES IN DIVIDING AND NON-DIVIDING CELLS*

Growth Conditions**	Volume (outside)	Volume (inside)	Volume (cell wall)	Ratio Cell Volumes (outside)	Ratio Cell Wall Volumes	Percent Decrease of Mucopeptide
Basal Medium	189	139	50	1.0	1.0	0
+ D-Cycloserine	814	703	111	4.3	2.2	49
+ D-Cycloserine + pantoyl lactone	166	133	33	0.9	0.7	22
+ D-Cycloserine + spermine	246	210	36	1.3	0.7	46
+ D-Cycloserine + Carbowax-400	1057	913	144	5.6	2.9	48

*Cell volume calculations were made from electron micrographs enlarged 120,000 diameters. All volume calculations were performed using the formula $4/3\ \pi r^3$. Measurements of diameters were made in cm; therefore, volumes are expressed as cm^3.

**All concentrations were as given in Table V.

TABLE IV

AMOUNT OF MUCOPEPTIDE OBTAINED FROM
DIVIDING AND NON-DIVIDING CELLS

Growth Conditions*	Percent Mucopeptide**	Percent Decrease
Basal Medium	14.16	
+ D-Cycloserine	6.90	52
+ D-Cycloserine + pantoyl lactone	8.60	39
+ D-Cycloserine + spermine	8.85	38
+ D-Cycloserine + Carbowax-400	7.75	45

*All concentrations were as given in Table V.

**Calculated from formula: $\frac{\text{mg mucopeptide}}{\text{mg cell dry weight}} \times 100$

synthesis.

Several chemical analyses involving mucopeptide were done to detect qualitative and/or quantitative modifications that might occur to the mucopeptide during division inhibition. First, cell walls were hydrolyzed and analyzed using an amino acid analyzer; qualitative data are given in Table V. These data are expressed as the molar ratio of each component (glutamic acid was set as 1.0).

Analysis of normal mucopeptide reveals that for every molecule of glutamic acid present there are two molecules of alanine and one each of lysine and glycine. It can be seen that D-cycloserine does little or nothing to change this ratio. Further, other agents (D-serine is an exception) that cause inhibition of division in this organism (penicillin, hydroxylamine, mitomycin c) do not significantly alter these ratios. Also of importance is the finding that all compounds that prevent inhibition of division caused by D-cycloserine have little or no significant effect on amino acid or amino sugar ratios.

As shown in Table V, cells of the dis-II pur^+ strain grown in the presence of D-serine incorporate this amino acid into their mucopeptide in place of glycine. This modification of mucopeptide was also reported for the parent strain of this organism (Whitney and Grula, 1964) and confirmatory data are included in Table V. Since replacement of glycine by D-serine does not cause inhibition of cell division in the parent strain, it would appear logical to conclude that replacement of glycine by D-serine in the dis-II pur^+ strain is not the primary lesion leading to division inhibition. In order to determine if D-serine is incorporated differently in the dis-II pur^+

TABLE V

RATIOS OF CELL WALL COMPONENTS IN MUCOPEPTIDE OF THE DIS-II PUR$^+$ STRAIN OR PARENT CELLS GROWN UNDER VARIOUS CONDITIONS

Growth Conditions and Concentrations	Molar Ratios						
	Glucosamine	Muramic Acid	Alanine	Glutamic Acid	Lysine	Glycine	Serine
Basal Medium	0.98	0.98	1.92	1.00	1.09	0.99	0.00
+ D-Cycloserine*	1.02	1.04	1.87	1.00	0.98	0.96	0.00
+ D-Cycloserine + pantoyl lactone (0.15M)	-	-	1.92	1.00	1.05	1.00	0.00
+ D-Cycloserine + spermine (7.0×10^{-3}M)	-	-	1.94	1.00	0.97	0.98	0.00
+ D-Cycloserine + Carbowax-400 (12%)	-	-	1.92	1.00	0.98	0.96	0.00
+ D-Cycloserine + D-alanine (1.0×10^{-5}M)	-	-	1.94	1.00	1.09	0.97	0.00
+ D-Cycloserine + pantoyl lactone (0.15M) + D-alanine (1.0×10^{-5}M)	-	-	1.99	1.00	1.10	0.98	0.00
+ Penicillin (0.14 ug/ml)	0.95	1.03	2.05	1.00	1.03	1.09	0.00
+ D-Serine (3.43×10^{-3}M)	1.09	1.12	1.88	1.00	1.06	0.40	0.64
+ Hydroxylamine (1.1×10^{-4}M)	-	-	2.00	1.00	1.06	1.02	0.00
+ Hydroxylamine (1.1×10^{-4}M) + D-alanine (1.0×10^{-4}M)	-	-	1.93	1.00	1.04	1.02	0.00
+ Vancomycin (0.32 ug/ml)	-	-	1.97	1.00	1.01	1.02	0.00
+ Mitomycin c (0.14 ug/ml)	1.02	1.03	2.00	1.00	1.00	1.31	0.00
Basal Medium**	-	-	1.87	1.00	0.96	1.03	0.00
+ D-Serine** (3.43×10^{-3}M)	-	-	1.65	1.00	0.95	0.35	0.67

*Concentration of D-cycloserine was 7.0×10^{-5}M throughout.
**Parent strain of M. lysodeikticus; all other analyses apply to the dis-II pur$^+$ strain.

strain, however, and for that reason induces inhibition of division, both organisms were grown in the presence of D-serine-3-^{14}C. Mucopeptide was isolated from both organisms, subjected to partial hydrolysis and all peptides containing D-serine-3-^{14}C were located utilizing radioautographic analysis of two-dimensional thin-layer chromatograms. Quantitatively equal amounts were chromatographed so that both quantitative and qualitative determinations could be made. Results of this type of study indicated that there was no essential difference in the incorporation of D-serine into the two mucopeptides (Figure 7).

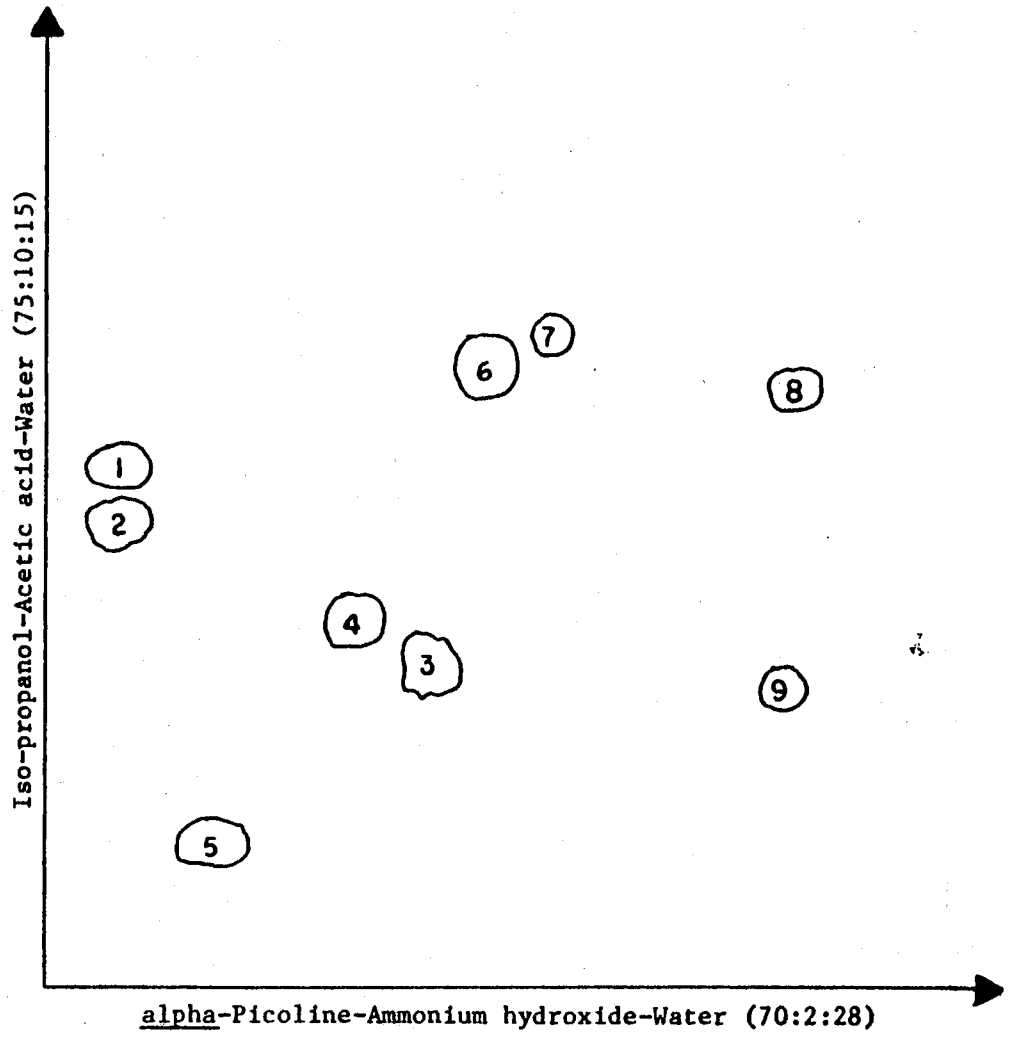
Based on the chromatography templates published by Schleifer and Kandler (1967) and Miller, Plapp, and Kandler (1966) we could detect peptide #2 which consists of glutamic acid and serine. Also, free serine was easy to detect (spot #3). In addition, I was definitely able to rule out the existence of an alanine-serine peptide since no radioactive spot was present in this area on the chromatograms that yielded serine after hydrolysis. This latter observation is significant since Neuhaus (1962) reported that cell-free extracts from Streptococcus faecalis catalyze the formation of D-seryl-D-alanine as well as of D-alanyl-D-alanine. Further, he was also able to demonstrate that the D-seryl-D-alanine peptide is a competitive inhibitor of D-alanyl-D-alanine synthetase. Unfortunately, he did not report whether or not the D-seryl-D-alanine peptide could be incorporated in place of D-alanyl-D-alanine in which case it could interfere with transpeptidation reactions essential for cross-linking within the mucopeptide.

Because the incorporation of D-serine appears not to differ in the parent or dis-II pur^{+} strain of M. lysodeikticus, and definitive evidence for the existence of a D-seryl-D-alanine peptide could not

Figure 7. Composite Template of the Relative Chromatographic Migration of Certain Amino Acids and Peptides. Template was based on data presented by Schleifer and Kandler (1967) and Miller, Plapp, and Kandler (1966).

Identification:

1 = glutamic acid
2 = glutamyl-serine
3 = serine
4 = glycine
5 = lysine
6 = alanine
7 = alanyl-alanine
8 = muramic acid
9 = glucosamine


Iso-propanol-Acetic acid-Water (75:10:15)
alpha-Picoline-Ammonium hydroxide-Water (70:2:28)
1
2
3
4
5
6
7
8
9

be obtained, it appears reasonable to conclude that incorporation of D-serine into mucopeptide is not responsible for the division inhibition obtained by growing the dis-II pur^+ strain in the presence of D-serine. This conclusion is consistent with the qualitative data obtained using D-cycloserine, penicillin, and mitomycin c, which also inhibit division, but do not add to or cause addition of other possible mucopeptide components.

Although data are not shown for all situations, mucopeptide was isolated from several of our division-inhibiting growth situations (mitomycin c, D-cycloserine, penicillin, or D-serine), partially hydrolyzed, and analyzed for muramic acid and glucosamine content using the amino acid analyzer. As shown in Table V, for every molecule of glutamic acid, one molecule each of muramic acid and glucosamine is present. Because ratios are not altered in any of the division inhibited situations studied, each component is being incorporated into the mucopeptide in its normal proportion. Further, no deletion or preferential incorporation of any mucopeptide component occurs in any situation I have tested except when cells are grown in the presence of D-serine.

Quantitative data on mucopeptide components are given in Table VI and expressed as umoles of each component per mg of mucopeptide. These data also reveal that in every situation wherein division is inhibited (D-cycloserine, mitomycin c, D-serine, penicillin, or hydroxylamine), an overall decrease in umoles of each component is noted when compared to control.

In order to express decreases in mucopeptide synthesis, addition of umoles of all components present in each sample was done and a

TABLE VI

AMOUNT OF CELL WALL AMINO ACIDS AND AMINO SUGARS PRESENT IN MUCOPEPTIDE OF THE DIS-II PUR$^+$ STRAIN OR PARENT CELLS GROWN UNDER VARIOUS CONDITIONS

	uM/mg Mucopeptide								
Growth Conditions*	Glucosamine	Muramic Acid	Alanine	Glutamic Acid	Lysine	Glycine	Serine	Total uMoles	Percent Inhibition
Basal Medium	0.680	0.676	1.328	0.691	0.750	0.683	0.000	3.452	0
+ D-Cycloserine	0.571	0.584	1.049	0.560	0.546	0.536	0.000	2.691	22
+ D-Cycloserine + pantoyl lactone	-	-	1.134	0.591	0.622	0.589	0.000	2.936	15
+ D-Cycloserine + spermine	-	-	1.150	0.594	0.576	0.581	0.000	2.901	16
+ D-Cycloserine + Carbowax-400	-	-	1.086	0.565	0.555	0.544	0.000	2.750	20
+ D-Cycloserine + D-alanine	-	-	1.236	0.638	0.693	0.618	0.000	3.185	8
+ D-Cycloserine + pantoyl lactone + D-alanine	-	-	1.184	0.595	0.655	0.585	0.000	3.019	12
+ Penicillin	0.420	0.451	0.900	0.440	0.453	0.479	0.000	2.272	34
+ D-Serine	0.565	0.583	0.980	0.519	0.551	0.210	0.330	2.590	25
+ Hydroxylamine	-	-	1.001	0.501	0.531	0.511	0.000	2.554	26
+ Hydroxylamine + D-alanine	-	-	1.042	0.540	0.562	0.550	0.000	2.694	22
+ Vancomycin	-	-	1.170	0.595	0.600	0.605	0.000	2.970	14
+ Mitomycin c	0.379	0.382	0.741	0.370	0.370	0.486	0.000	1.967	43
Basal Medium**	-	-	1.063	0.568	0.544	0.583	0.000	2.758	0
+ D-Serine**	-	-	0.878	0.531	0.505	0.185	0.356	2.455	11

*All concentrations were as given in Table V.

**Parent strain of M. lysodeikticus; all other analyses apply to the dis-II pur$^+$ strain.

percent decrease was calculated using the total umoles present in control cells as 100 percent. These data are included in Table VI and again it can be observed (see also Tables III and IV) that there is a decrease in synthesis of mucopeptide in all division inhibited situations. D-Cycloserine causes a 22 percent decrease and in two situations, wherein cells are significantly protected from division inhibition caused by D-cycloserine (pantoyl lactone and spermine), only a partial restoration of the umole decrease is observed. Carbowax-400, the most inefficient compound tested for protection against D-cycloserine, also does not significantly restore the umole deficit. D-Alanine, which can restore growth and prevent division inhibition caused by D-cycloserine, permits a substantial restoration of mucopeptide synthesis in the presence of D-cycloserine but does not restore mucopeptide synthesis in the presence of hydroxylamine.

Restoration of the umoles decrease obtained by growth in the presence of D-alanine could occur because the functional need for alanine racemase is by-passed; therefore, inhibition of this enzyme by D-cycloserine would be of little consequence. Another possibility could be that D-alanine prevents the uptake of D-cycloserine into the cell. The latter is a particularly attractive hypothesis since Wargel, Shadur, and Neuhaus (1970) have shown, using E. coli, that D-cycloserine is carried into the cell by the same system that transports D-alanine and glycine.

Pantoyl lactone and spermine which also allow partial restoration of mucopeptide synthesis may also inhibit uptake of D-cycloserine since it is doubtful that pantoyl lactone or spermine have any ability to replace the need for or repair defective alanine racemase activity

(neither compound permits normal mucopeptide synthesis as shown in Table VI). The possibility that these compounds prevent uptake of D-cycloserine will be explored more fully in the next section of this chapter.

Vancomycin inhibits mucopeptide synthesis 14 percent, but non-dividing cells were never observed when this compound was added to the growth medium. The inability of this compound, which inhibits cell division in Erwinia sp. (Grula and Grula, 1962c) and is a known inhibitor of mucopeptide synthesis (Best and Durham, 1964; 1965; Chatterjee and Perkins, 1966), to inhibit cell division in this organism was quite surprising and unexpected. To insure that I was not incorrectly assessing effects of this compound, several titrations involving vancomycin were made (from 0.100 ug/ml to 0.600 ug/ml) within a relatively wide range of pH (6.0 to 8.5). Representative data given in Figure 8 show that sufficient amounts of vancomycin were used to inhibit growth significantly. In most cultures where growth inhibition was relatively extensive, lysis was often observed. The latter observation, indirectly at least, also indicates that vancomycin causes significant inhibition of mucopeptide synthesis in this organism.

In order to obtain further evidence for inhibition of mucopeptide synthesis in key growth situations, several different types of measurements were made. Mucopeptide, which had been extensively hydrolyzed (24 hours in 6N HCl at 105^{o} C), was subjected to quantitative ninhydrin determination. Data given in Table VII reveal that D-cycloserine causes a 30 percent decrease in ninhydrin-positive components. Using this procedure, it can again be demonstrated that

Figure 8. Growth as a Function of Vancomycin Concentration (pH 7.1) and Growth Response at Various pH's With and Without Vancomycin (0.22 ug/ml). ●, vancomycin (at pH 7.1); △, basal medium (control); and ▲, basal medium plus vancomycin (0.22 ug/ml).

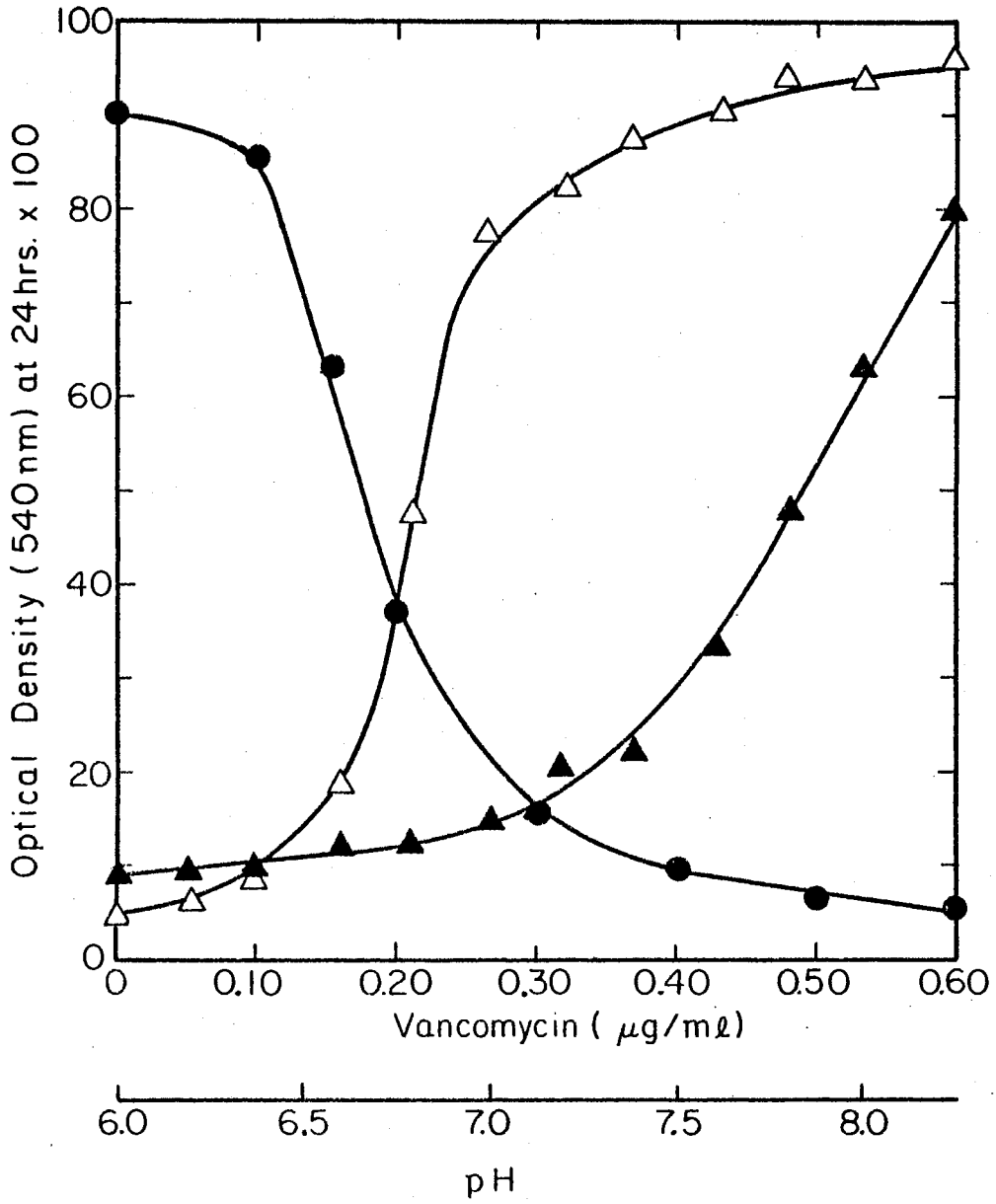

Optical Density (540 nm) at 24 hrs. x 100
100
80
60
40
20
0
0
0.10
0.20
0.30
0.40
0.50
0.60
Vancomycin (μg/mℓ)
6.0
6.5
7.0
7.5
8.0
pH

TABLE VII

AMOUNT OF NINHYDRIN-POSITIVE MATERIALS PRESENT IN MUCOPEPTIDE OF DIVIDING AND NON-DIVIDING CELLS

Growth Conditions*	uMoles of Ninhydrin-Positive Materials/ mg Mucopeptide**	Percent Decrease
Basal Medium	2.52	0
+ D-Cycloserine	1.73	30
+ D-Cycloserine + pantoyl lactone	1.92	24

*All concentrations were as given in Table V. Additions were made at 12 hours and incubation continued for an additional 6 hours before isolation of mucopeptide.

**Estimations made using a leucine standard curve.

pantoyl lactone does not permit significant repair of the D-cycloserine-induced inhibition of mucopeptide synthesis.

Quantitative analysis of amino sugar residues was also performed using the method of Rondle and Morgan (1955). A representative analysis is given in Table VIII wherein it can again be seen that less mucopeptide is present in cells grown in the presence of D-cycloserine, and pantoyl lactone does not effect repair of the D-cycloserine-induced lesion.

Another method used to monitor cell wall synthesis involved incorporation of L-lysine-U-^{14}C into mucopeptide of growing cultures. Representative data are shown in Table IX wherein it can be seen that D-cycloserine inhibits mucopeptide synthesis by 27 percent and pantoyl lactone does not substantially restore this decrease. It can be pointed out that radioautography of hydrolyzed mucopeptide revealed that the great majority of lysine enters the wall in an unaltered state; therefore, incorporation of counts from L-lysine-U-^{14}C is a good measure of actual presence of lysine in the mucopeptide of this organism.

Because penicillin, a known inhibitor of the transpeptidation reaction, inhibited division of the dis-II pur^{+} strain it was felt that a determination of the extent of cross-linking in mucopeptide from dividing and non-dividing cells could yield valuable information about a specific type of wall lesion. Schleifer and Kandler (1967) have shown, by analysis of peptides from mucopeptide of M. lysodeikticus, the probable mode of cross-linking and stated that the extent of cross-linking can be monitored by measurement of free epsilon amino groups of lysine. A decrease in cross-linking should

TABLE VIII

AMOUNT OF AMINO SUGARS PRESENT IN
DIVIDING AND NON-DIVIDING CELLS

Growth Conditions*	Total ug of Amino Sugar/ mg of Mucopeptide**	Percent Decrease
Basal Medium	108	0
+ D-Cycloserine	79	26
+ D-Cycloserine + pantoyl lactone	85	22

*All concentrations were as given in Table V. Additions were made at 12 hours and incubation continued for an additional 6 hours before isolation of mucopeptide.

**Estimations made using a glucosamine standard curve.

TABLE IX

DECREASE IN MUCOPEPTIDE SYNTHESIS AS MEASURED BY L-LYSINE-U-^{14}C INCORPORATION*

Growth Conditions**	Counts Per Minute/ mg Mucopeptide	Percent Decrease
Basal Medium	8345	0
+ D-Cycloserine	6090	27
+ D-Cycloserine + pantoyl lactone	6200	26

*L-Lysine-U-^{14}C (0.01 uC/ml) was added at 12 hours. After 4 hours growth, mucopeptide was isolated and counted.
**All concentrations were as given in Table V.

result in an increased amount of free epsilon amino groups of lysine. Schleifer and Kandler (1967) also reported that in a normal mucopeptide of M. lysodeikticus about 32 to 40 percent of the epsilon amino groups of lysine are free; therefore, the mucopeptide is 60 to 68 percent cross-linked.

Isolated mucopeptide from various situations was analyzed quantitatively for the amount of free epsilon amino groups of lysine and data are given in Table X. In all situations wherein inhibition of division occurs (D-cycloserine, penicillin, mitomycin c, D-serine, hydroxylamine), inhibition of cross-linking is greater than in control cultures or situations wherein inhibition of division has been prevented (pantoyl lactone, D-alanine, and spermine). It should also be noted that the amount of free epsilon amino groups of lysine in control mucopeptide (parent as well as the dis-II pur^+ strain) was within the range of that reported by Schleifer and Kandler.

Penicillin, the only one of the division inhibiting agents used with a known ability to inhibit the transpeptidation reaction, gave the largest decrease in mucopeptide cross-linking being only 44 percent cross-linked as opposed to D-cycloserine which permits 58 percent of the lysine residues to remain cross-linked. It should also be noted that parent type cells, wherein cell division is not inhibited by D-serine, showed only a 7 percent decrease (63 percent remains cross-linked as opposed to 60 percent in the dis-II pur^+ strain).

Schleifer (1969) reported that cells of Staphylococcus aureus, grown in the presence of D-serine, contain mucopeptide with decreased cross-linking (data on percent of inhibition were not presented).

TABLE X

N-TERMINAL ANALYSIS OF MUCOPEPTIDE FROM DIVIDING AND NON-DIVIDING CELLS

Growth Conditions*	Percent as N-Terminal				
	Alanine	Glutamic Acid	Lysine	Glycine	Serine
Basal Medium	0	0	30	0	0
+ D-Cycloserine	0	0	42	0	0
+ D-Cycloserine + pantoyl lactone	0	0	35	0	0
+ D-Cycloserine + spermine	0	0	36	0	0
+ D-Cycloserine + Carbowax-400	0	0	40	0	0
+ D-Cycloserine + D-alanine	0	0	32	0	0
+ D-Cycloserine + pantoyl lactone + D-alanine	0	0	36	0	0
+ Penicillin	0	0	66	0	0
+ D-Serine	0	0	40	0	0
+ Hydroxylamine	0	0	46	0	0
+ Hydroxylamine + D-alanine	0	0	43	0	0
+ Vancomycin	0	0	39	0	0
+ Mitomycin c	0	0	45	0	0
Basal Medium**	0	0	33	0	0
+ D-Serine**	0	0	37	0	0

*All concentrations were as given in Table V.
**Parent strain of M. lysodeikticus; all other analyses apply to the dis-II pur+ strain.

He also reported that no morphological changes were evident in such cells. Since the difference between the parent organism and the dis-II pur^+ strain is only 3 percent, it is not felt that this difference is sufficient to cause division inhibition. Therefore, it is difficult to reach the conclusion that a direct correlation exists between the amount of cross-linking in mucopeptide and cell division.

The significance of the N-terminal data is that in situations wherein division is inhibited, a decreased amount of cross-linking can be demonstrated and compounds able to prevent the inhibitory action of D-cycloserine on division, such as pantoyl lactone and spermine (D-alanine appears to be an exception), do not restore cross-linking completely to normal.

As mentioned previously, Whitney and Grula (1964) were able to show that the great bulk of D-serine incorporated into the mucopeptide of the parent organism, is C-terminal and replaces glycine. Data shown in Table XI reveal that D-serine is incorporated into the dis-II pur^+ strain in much the same position since approximately 98 percent can be measured as C-terminal. Mucopeptide was also isolated from cells grown in D-serine plus L-lysine and analyzed since lysine permits an increase in the inhibition of division. The amount of C-terminal D-serine remains very high and the small difference shown in Table XI is probably not significant. It should be noted that an increase in C-terminal lysine occurs when cells are grown in the presence of D-serine or D-serine plus L-lysine. This could possibly indicate that D-serine may be interfering with normal incorporation of the D-alanyl-D-alanine dipeptide which would, in turn, lead to an alteration in cross-linking. As stated previously,

TABLE XI

C-TERMINAL ANALYSIS OF MUCOPEPTIDE FROM DIVIDING AND NON-DIVIDING CELLS

Growth Conditions*	Percent of Component as C-Terminal						
	Glucosamine	Muramic Acid	Alanine	Glutamic Acid	Lysine	Glycine	Serine
Basal Medium	0	0	6.0	0	9.5	55	0
+ D-Serine	0	0	8.7	0	15.0	43	98
+ D-Serine + L-lysine	0	0	6.7	0	14.5	53	93

*All concentrations were as given in Table V.

studies using partial hydrolysis for analysis of peptides reveal that a serine-alanine peptide is not present in mucopeptide of cells grown in the presence of D-serine.

Since a umole decrease of mucopeptide components occurs in all division inhibited situations, some component(s) of the cell wall must be increasing or apparently increasing (it may stay the same but since there is less mucopeptide it would make it appear larger). It would be helpful to determine what component(s) is increasing since this increase could be viewed as a unique response by division inhibited cells. The component(s) cannot be an amino acid or amino sugar since quantitative ninhydrin data already presented (Table VII) reveal that mucopeptide from non-dividing cells contains less ninhydrin-positive materials. Also, cell wall analyses (Tables V and VI) obtained using the amino acid analyzer were very carefully checked in that lyophilized samples were carefully weighed and all data re-calculated in our laboratory. In addition, duplicate as well as different wall samples were analyzed as a check on the analytical procedures employed.

Qualitative and quantitative analysis of mineral as well as glucose content (glucose oxidase test) also failed to reveal any increase or decrease in these particular components, but the amounts present are so small that their presence or absence is not quantitatively significant.

In a final attempt to identify the elusive and "new" component(s), the following experiment was performed. Cells were grown in the presence of both aspartic and L-glutamic-U-^{14}C with and without D-cycloserine and mucopeptide isolated by the normal chemical procedure.

Weighed aliquots of the isolated mucopeptides were then subjected to partial hydrolysis as follows: (a) 6N HCl for 18 hours at 100° C, (b) 4N HCl for 4 hours at 100° C, (c) 1N HCl for 1 hour at 100° C, (d) 0.1N HCl for 20 minutes at 100° C. After drying and re-solubilization in distilled water, the partial hydrolyzates were chromatographed in two directions on thin-layer plates and analyzed using radioautography. It was felt that the new compound had to be carbon-containing and we would be able to analyze for it in this manner. Development of the x-ray films revealed that no new compound(s) was present in the mucopeptide of division inhibited cells; also, there did not appear to be a qualitative increase or decrease in any of the compounds present. Therefore, the question remains open as to what component(s) is increased in the mucopeptide of non-dividing cells, or, indeed, if such a component(s) exists. All of my quantitative data reveal that the decrease in amino sugars and amino acids is occurring, but, as stated, investigation of the situation using several different techniques yielded uniformly negative data.

It appears to me that the analyses which I performed on mucopeptide isolated from dividing and non-dividing cells permit three major conclusions: (1) Situations that cause division inhibition do indeed cause cell wall damage in the form of decreased mucopeptide synthesis and cross-linking. (2) Pantoyl lactone and spermine, which prevent D-cycloserine action, do not significantly restore the amount of mucopeptide synthesis or cross-linking even though the cells are normal in size. (3) Incorporation of D-serine into the mucopeptide of the dis-II pur^+ strain is not responsible for division inhibition in this organism.

A summary of pertinent information relating to cell wall mucopeptide is included to aid my reader to view significant data in capsule form (Table XII). If one ignores the data which relate to cell size and percent inhibition in mucopeptide synthesis in the presence of cycloserine plus both D-alanine and pantoyl lactone, my data appear to indicate that if the percent inhibition in mucopeptide synthesis is 20 percent or greater and the amount of cross-linking is 60 percent or less, then growing cells will not divide.

Rather than ignore data that did not fit into the total pattern that appeared to be emerging, I turned my attentions to study of events taking place within the cell membrane.

Membrane Alterations Occurring During Inhibition of Cell Division

Grula and Grula (1964) proposed that inhibition of division caused by D-serine in a species of Erwinia was due to secondary damage to the cell membrane. This proposal was the outcome of studies which showed that mucopeptide fabrication in filamentous cells is decreased 30 to 40 percent and the cells are "leaky." Because hypertonic conditions or compounds such as spermine or pantoyl lactone induce division activity without repair of the mucopeptide lesion while greatly decreasing leakage, some type of damage to the cell membrane appears to be involved. Involvement of the cell membrane in the division process is also indicated by pictures which show that spermine is able to stabilize spheroplasts produced by D-serine (Grula and Noller, unpublished).

Cell wall analyses presented in this thesis indicate that the

TABLE XII

SUMMARY OF THE CONDITION OF CELL WALL MUCOPEPTIDE PRODUCED IN DIVIDING AND NON-DIVIDING ENVIRONMENTS

Growth Conditions*	Cell Size in Microns	Percent Inhibition of Mucopeptide Synthesis**	Percent Cross-linked***
Basal Medium	1.0	0	70
+ Vancomycin	1.0	14	61
+ D-Cycloserine + D-alanine	1.0	8	68
+ D-Cycloserine + pantoyl lactone	1.0-2.0	15	65
+ D-Cycloserine + spermine	1.0-2.0	16	64
+ D-Cycloserine + pantoyl lactone + D-alanine	2.0-4.0	12	64
+ D-Cycloserine + Carbowax-400	3.0-5.0	20	60
+ D-Cycloserine	4.0-5.0	22	58
+ D-Serine	2.0-4.0	25	60
+ Hydroxylamine	2.0-4.0	26	59
+ Hydroxylamine + D-alanine	2.0-4.0	22	57
+ Penicillin	3.0-5.0	34	34
+ Mitomycin c	3.0-5.0	43	55

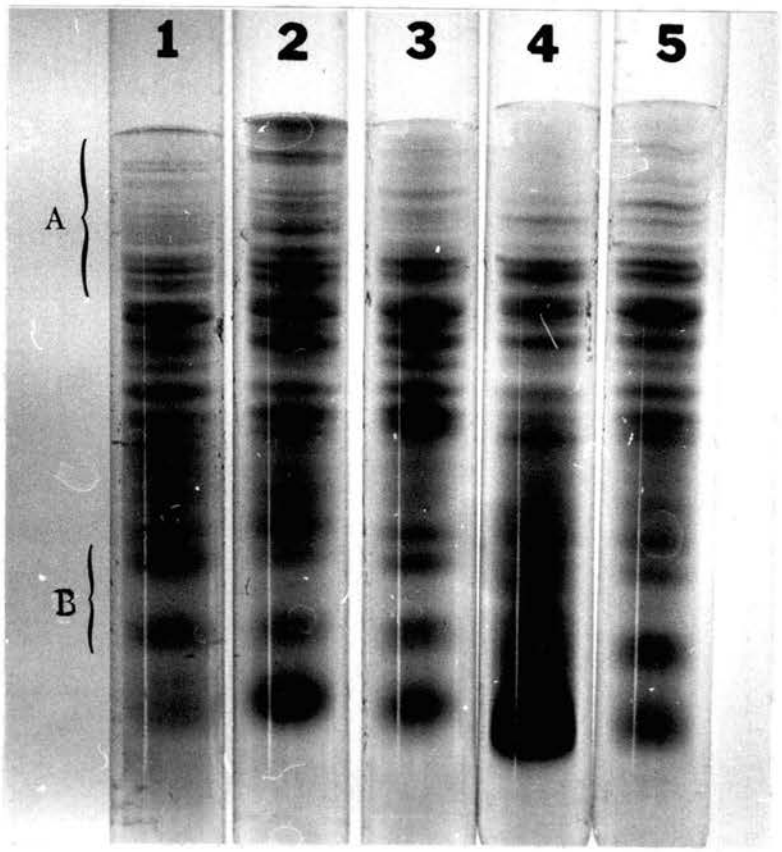
*All concentrations were as given in Table V.
**Based on data obtained using the amino acid analyzer.
***Based on free epsilon amino lysine content.

same general pattern observed using Erwinia sp. exists in the dis-II pur$^+$ strain of M. lysodeikticus; i.e., division inhibited cells have less mucopeptide and compounds such as spermine or pantoyl lactone which prevent inhibition of division do not restore mucopeptide synthesis back to normal. Because of these important similar findings, it was felt that division inhibition in the dis-II pur$^+$ strain could also be due to secondary damage to the cell membrane.

Possible alterations in protein content of the cell membrane were studied using sodium lauryl sulfate polyacrylamide gel electrophoresis. Electrophoresis was performed utilizing 10 percent acrylamide gels in the basic system of Weber and Osborn (1969). The gels shown in Figure 9 represent patterns obtained from disaggregated whole cell membranes isolated from dividing and non-dividing cells.

Using this technique, it can be seen that the whole cell membrane of the dis-II pur$^+$ strain contains at least 27 species of protein (each band is assumed to represent one species) when the cells are grown in the basal medium. When cell membranes isolated from cells grown in the presence of D-cycloserine are compared to the normal membranes (#1 versus #2), it can be seen that several differences exist. As shown at the top of gel #2, in the area denoted as "A" (area of highest molecular weight proteins), the most striking feature of "D-cycloserine membranes" is that D-cycloserine somehow causes a significant increase in several of these proteins. Also, at least three additional bands can be resolved in gel #2. As shown, these quantitative and qualitative changes are striking and permit immediate identification of membranes obtained from D-cycloserine-grown cells. Other changes are primarily quantitative in nature

Figure 9. Representative Electrophoretic Protein Profiles Obtained From Disaggregated Whole Cell Membranes Isolated From Dividing and Non-Dividing Cells. Whole cell membranes isolated from cells grown in the following situations: (1) basal medium (control), (2) basal medium plus D-cycloserine, (3) basal medium plus D-cycloserine and pantoyl lactone, (4) basal medium plus D-cycloserine and spermine, and (5) basal medium plus D-cycloserine and Carbowax-400. Concentrations were as given in Table V.


1
2
3
4
5
A
B

except for a possible new band in gel #2 in the area designated "B."

The membrane profile from cells grown in the presence of D-cycloserine plus pantoyl lactone (gel #3) resembles most gel #1 (control). On the other hand, the membrane profiles from cells grown in the presence of D-cycloserine plus either spermine (gel #4) or Carbowax-400 (gel #5) appear to bear greatest resemblance to gel #2 (D-cycloserine alone). In no instance do any of the profiles return to or appear to be "normal."

Although some changes appear at the bottom of all gels, these are not considered to be changes in protein since phospholipids migrate to this area and are also lightly stained with Coomassie Blue. Therefore, any differences in this area indicate possible differences in lipids and should be studied using other types of technology.

In summation, it is obvious that the cell membrane from non-dividing cells grown in the presence of D-cycloserine differs quantitatively and qualitatively from normal cell membranes. Rather than losses or decreases only (decreases do occur), it was very surprising to observe that growth in the presence of D-cycloserine induces large increases in several higher molecular weight proteins and, possibly, formation of at least three new proteins.

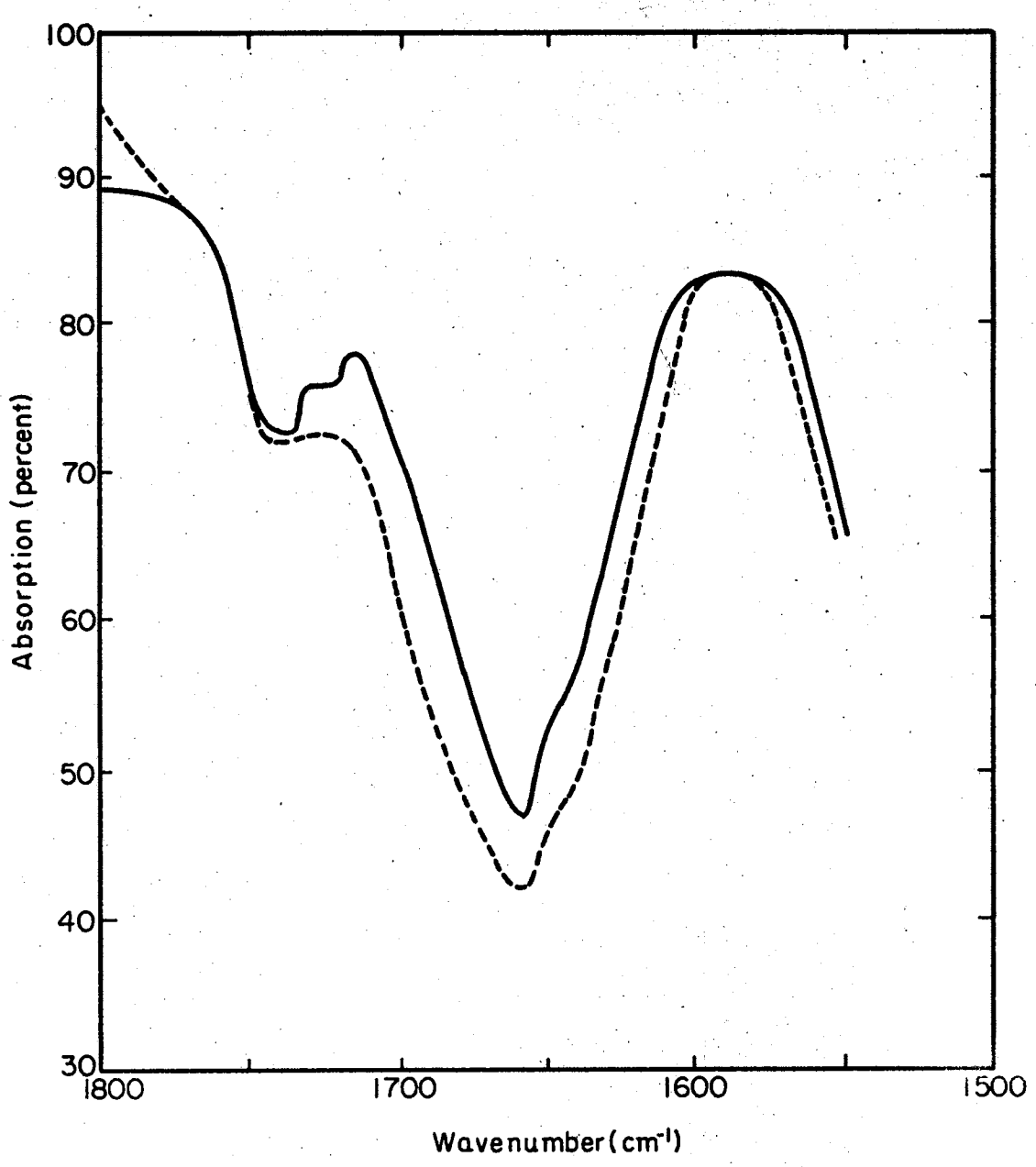
Because neither pantoyl lactone nor spermine return the membrane protein profile completely back to normal, it is difficult to conclude that a direct correlation exists between protein composition of the cell membrane and cell division activity. These data do show, however, that a structure other than cell wall mucopeptide is affected by D-cycloserine.

Graham and Wallach (1969) have shown that energy dependent protein conformational transitions occur in mitochondrial membranes isolated from rat liver mitochondria. Also, it was shown that an accurate measurement of these transitions in membrane proteins could be made using infrared analysis. Green and Salton (1970) recently reported that the principal peaks of the infrared spectrum of membranes from M. lysodeikticus, originate from the Amide I and II bands of the proteins present as well as vibrations from the OH, C-H, C=O, P=O, C-O-C, and P-O-C groups of lipids. They were also able to show that proteins in the membrane of this organism are present mostly in the "un-ordered" or alpha-helical conformations, however, some proteins (or portions thereof) also exist in the beta-conformation.

We feel that a possible role for the cell membrane in the cell division process involves or necessitates a constriction or pinching in the area of membrane invagination. A convenient mechanism by which constriction could be accomplished is that some or all membrane proteins undergo a conformational change. In order to evaluate this possibility, infrared data were obtained using membranes isolated from dividing and non-dividing cells. The assignment of spectral peaks and positions of the main bands follows that reported by Wallach and Zahler (1968).

Figure 10 shows the infrared spectra obtained for cell membranes of normal and non-dividing cells. The band near 1655 cm^{-1} reflects the presence of protein in the "un-ordered" or alpha-helical conformation or both. The shoulder between 1630 and 1645 cm^{-1} and the inflection near 1690 cm^{-1} arise from peptides in the anti-parallel beta-conformation. Vibrations at 1735 cm^{-1} are due primarily to

Figure 10. Infrared Spectra of Membranes From Dividing and Non-Dividing Cells. Solid line (——), membranes from normally dividing cells; and dashed line (----), membranes from non-dividing cells.


100
90
80
70
60
50
40
30
Absorption (percent)
1800
1700
1600
1500
Wavenumber (cm⁻¹)

ester absorptions indicative of lipids (Green and Salton, 1970). It should be pointed out that no attempt was made to determine the absolute absorbance of any of the bands in the spectra shown. The difficulties involved in a quantitative analysis would require computer programming and thus be beyond the scope of my study. Therefore, a semi-quantitative analysis was accomplished by calculating ratios from a particular band height ratio within a given portion of the spectra.

Increases in the ratios of 1630/1655, 1700/1655, and 1700/1735 indicate that there is a shift toward the 1630 and 1700 cm^{-1} areas which, in turn, indicates that there is a shift toward the anti-parallel beta-conformation. The ratio of 1735/1655 is used to monitor the protein to lipid ratio and increases in this ratio indicate an increase in the amount of lipid. A summary of calculations of these ratios is shown in Table XIII.

These data indicate that membranes isolated from cells grown in the presence of D-cycloserine have a substantial increase in proteins having the anti-parallel beta-conformation since increases were observed in the 1630/1655, 1700/1655, and 1700/1735 ratios. The increased 1735/1655 ratio indicates that more lipid may be present in the cell membrane of cells grown in the presence of D-cycloserine; however, because of experiments relating to synthesis of lipids, to be reported later in this thesis, I feel that this increase is due primarily to expansion toward the 1700 cm^{-1} region which occurs as a result of an increase in the anti-parallel beta-conformational state of these proteins. It can also be observed that growth of cells in the presence of D-cycloserine plus pantoyl lactone causes

TABLE XIII

RATIOS OF PEAK HEIGHTS CALCULATED USING INFRARED SPECTRA OF MEMBRANES FROM DIVIDING AND NON-DIVIDING CELLS

Growth Conditions*	Ratios of Peak Heights (cm^{-1})			
	1630/1655	1700/1655	1735/1655	1700/1735
Basal Medium	0.483	0.343	0.324	1.06
+ D-Cycloserine	0.706	0.646	0.426	1.51
+ D-Cycloserine + pantoyl lactone	0.570	0.425	0.356	1.19
+ D-Cycloserine + spermine	0.820	0.507	0.460	1.48
+ D-Cycloserine + Carbowax-400	0.630	0.760	0.342	1.65

*All concentrations were as given in Table V. Cells were grown for 12 hours after which all additions were made; growth was then continued for an additional 6 hours.

a decrease in all ratios thus bringing (or allowing) membrane proteins significantly nearer to the conformational state that exists in control cells.

Carbowax-400 does not prevent inhibition of division by D-cycloserine and, as shown in Table XIII, does little to antagonize its effects on conformational state of the membrane proteins. The only ratios that are decreased are the 1630/1655 and 1735/1655 ratios and the 1630/1655 decrease is not great. The other two ratios are actually greater than those seen in cells grown in the presence of D-cycloserine alone.

Data obtained with spermine and shown in Table XIII appear to be erroneous since large distortions in all vibrations were recorded. It was observed, during this phase of the study, that cells grown in the presence of D-cycloserine plus spermine are not as susceptible to lysozyme treatment as those obtained from other growth situations. Since lysozyme appears to function very poorly on spermine-grown cells, it was felt that cell wall material was not being completely removed. Therefore, isolated mucopeptide was subjected to infrared analysis and it was observed that very strong vibrations are evident at 1700 and 1630 cm^{-1}. In fact, the whole area between 1600 and 1800 cm^{-1} is excessively distorted toward what would appear to be proteins having the anti-parallel beta-conformation. Therefore, data obtained utilizing membranes from spermine-grown cells are probably in error and a better method for isolation of these membranes must be developed before valid conclusions can be reached.

My results relating to the conformational state(s) of membrane proteins as observed using infrared analysis, although not as

quantitative as desired, illustrate three major points: (1) Membranes from control cells of M. lysodeikticus dis-II pur$^+$ contain a large number of protein molecules (or specific portions of some or all protein molecules) in the "un-ordered" or alpha-helical conformation. (2) Proteins present in the membrane of non-dividing cells show a definite shift toward the anti-parallel beta-conformation. (3) Pantoyl lactone, which prevents the deleterious effects of D-cycloserine on cell division and also prevents a change in the membrane protein profile, but does not prevent inhibition in synthesis of mucopeptide, keeps (or induces) membrane proteins in the conformational state found under normal conditions of growth and division.

Up to this point in my study, direct evidence was available to show that compounds inhibiting or stimulating cell division affected protein composition of the cell membrane as well as conformational states of those proteins. We had assumed early in this study that D-alanine prevented the deleterious effects of D-cycloserine simply by supplying the end-product of the inhibited enzyme, alanine racemase. Recently, however, Wargel et. al. (1970) reported, using E. coli, that uptake of D-alanine, glycine, and D-cycloserine is mediated by the same transport system. This conclusion is based on data which show that D-cycloserine is an effective inhibitor of glycine or D-alanine uptake. Because much of our data which relate to membrane alterations by pantoyl lactone could be explained by assuming that pantoyl lactone alters the membrane and thus keeps D-cycloserine from getting into the cells, I examined possible functional changes in the membrane by measurement of uptake of compounds involved in this study.

Uptake studies were performed using normal log-phase cells (16 hours) that were first rested for depletion of pool components by shaking (wrist-action shaker set at 2.0) them for 30 minutes in 0.05M phosphate buffer (pH = 7.8). All compounds tested for inhibiting activity were added 25 seconds before addition of the labeled compound to be taken up.

Data relative to the uptake of D-alanine-1-^{14}C (Figure 11) show that D-cycloserine inhibits the uptake of D-alanine probably in a competitive manner, but is not as effective an inhibitor as glycine (D-cycloserine inhibits 34 percent of normal uptake at 4 minutes at a molar ratio of 1.7 D-cycloserine to 1.0 D-alanine, whereas glycine inhibits 48 percent of normal uptake at the same time interval at a molar ratio of 1.0 to 1.0). It is also shown that pantoyl lactone inhibits uptake of D-alanine by what must be a non-competitive mechanism (67 percent with a molar ratio of 1,500 pantoyl lactone to 1.0 D-alanine).

To further substantiate these findings, uptake of glycine-2-^{14}C was studied. Data relative to glycine uptake (Figure 12) show that D-cycloserine also inhibits uptake of glycine but inhibition is not as great (13 percent at a molar ratio of 1.7 D-cycloserine to 1.0 glycine) as that obtained using D-alanine (47 percent at a molar ratio of 1.0 D-alanine to 1.0 glycine). These data demonstrate that pantoyl lactone can also inhibit uptake of glycine (69 percent at a molar ratio of 1,500 pantoyl lactone to 1.0 glycine).

Because of these results, it is apparent that pantoyl lactone has a drastic effect on uptake of at least two compounds that share the same transport system with D-cycloserine. This indirectly implies

Figure 11. Uptake of D-Alanine-1-^{14}C in the Presence of D-Cycloserine, Glycine, or Pantoyl Lactone. ●, D-alanine-1-^{14}C ($10^{-4}M$); ■, D-alanine-1-^{14}C ($10^{-4}M$) plus D-cycloserine ($1.7 \times 10^{-4}M$); ▲, D-alanine-1-^{14}C ($10^{-4}M$) plus glycine ($10^{-4}M$); and ⬢, D-alanine-1-^{14}C ($10^{-4}M$) plus pantoyl lactone (0.15M).

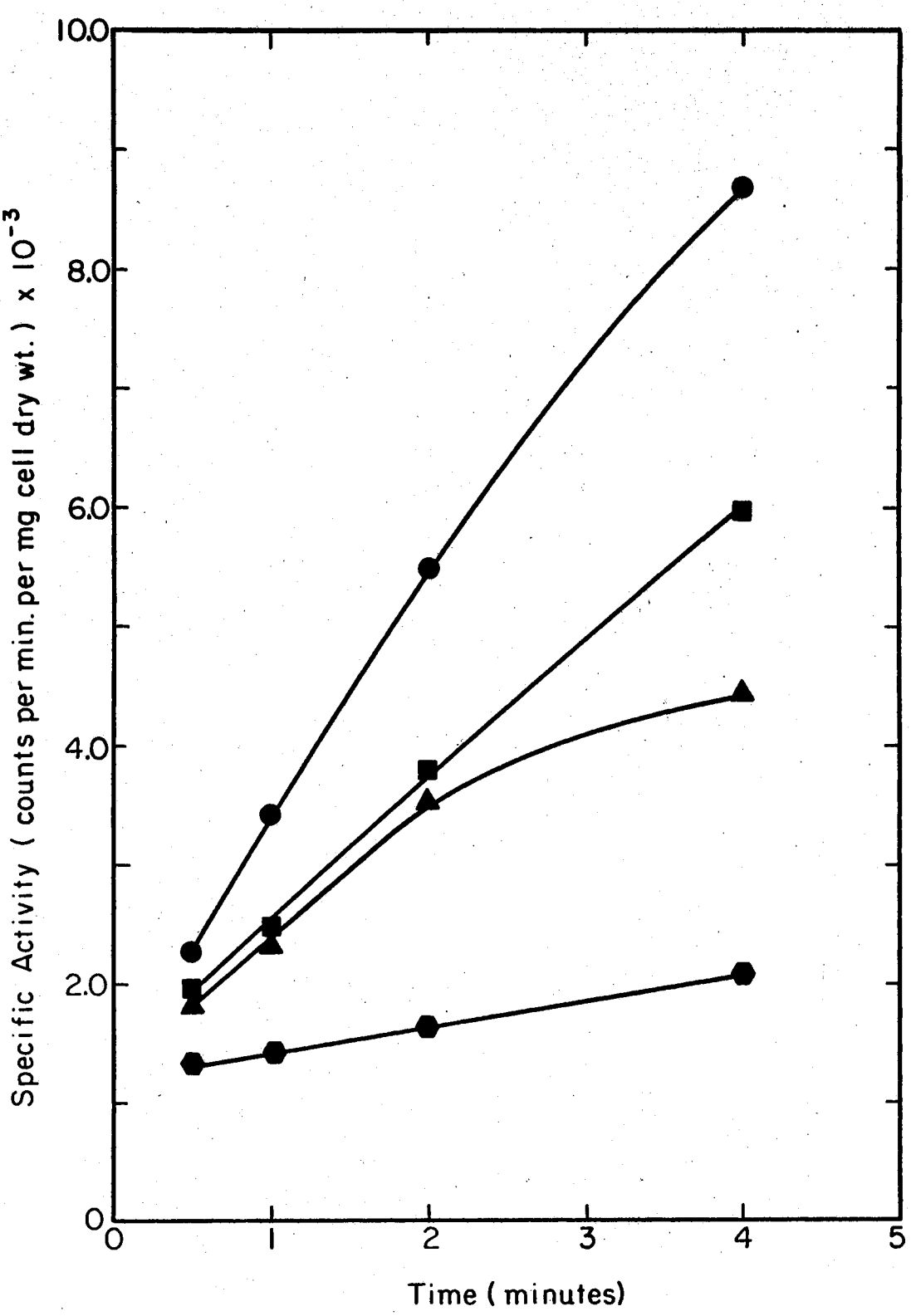
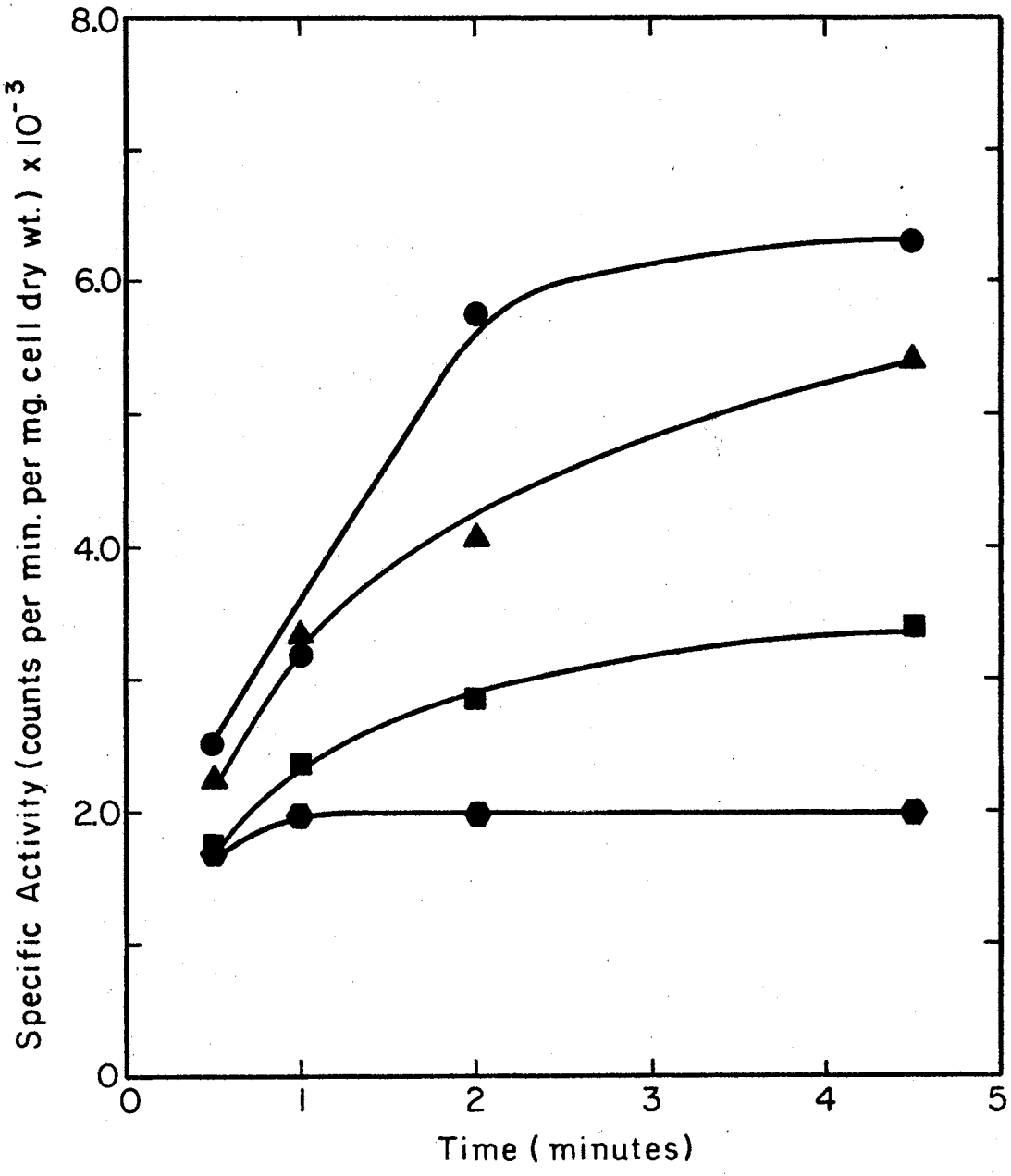

10.0
8.0
6.0
4.0
2.0
0
0
1
2
3
4
5
Specific Activity (counts per min. per mg cell dry wt.) x 10^{-3}
Time (minutes)

Figure 12. Uptake of Glycine-2-^{14}C in the Presence of D-Cycloserine, D-Alanine, or Pantoyl Lactone. ●, glycine-2-^{14}C (10^{-4}M); ▲, glycine-2-^{14}C (10^{-4}M) plus D-cycloserine (1.7×10^{-4}M); ■, glycine-2-^{14}C (10^{-4}M) plus D-alanine (10^{-4}M); and ⬢, glycine-2-^{14}C plus pantoyl lactone (0.15M).

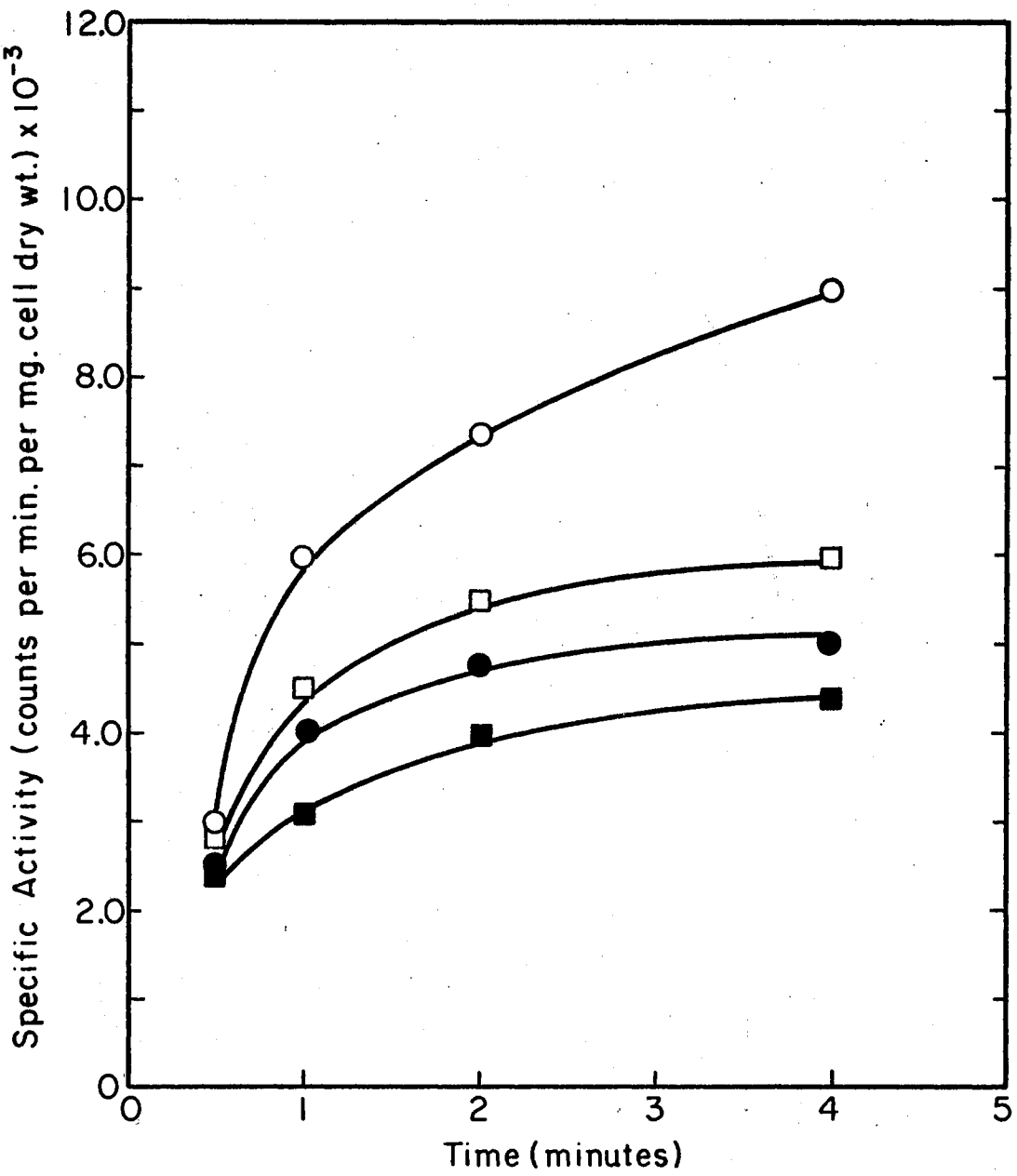

8.0
6.0
4.0
2.0
0
0
1
2
3
4
5
Specific Activity (counts per min. per mg. cell dry wt.) x 10^{-3}
Time (minutes)

that D-cycloserine uptake would also be inhibited by pantoyl lactone.

In order to show a direct inhibition by pantoyl lactone on D-cycloserine uptake, investigation of this possibility was initiated utilizing D-cycloserine-^{14}C. These studies proved to be very inconclusive since an accurate uptake curve could not be obtained. Many attempts to measure uptake were made at several time intervals and all were inconclusive since only a very small amount of uptake (actually may only represent absorption of D-cycloserine to some portion of the cell surface) occurred. It should also be pointed out that radioautography of D-cycloserine-^{14}C used in these uptake studies revealed that our sample of radioactive D-cycloserine contains four additional compounds in substantial amounts. Besides these impurities, a large amount of radioactive material remains at the origin and additional amounts of some type(s) of compound migrate to the solvent fronts. Therefore, since the amount of uptake is very small, it is felt that interpretation of our data would not be valid.

If it could be shown that pantoyl lactone decreases uptake of several types of compounds then such data, along with data obtained using D-alanine and glycine, could help substantiate the fact that uptake of D-cycloserine is inhibited by pantoyl lactone. As shown in Figure 13, testing, using other compounds, such as L-phenylalanine-U-^{14}C or glycerol-2-^{14}C, showed that their entry into the cell is also inhibited by pantoyl lactone (42 and 36 percent for phenylalanine and glycerol respectively). Therefore, it appears reasonable to conclude that another effect of pantoyl lactone at the level of the cell membrane is related to a general "tightening" of the structure which leads to a decrease in entry of D-cycloserine (or several other

Figure 13. Uptake of L-Phenylalanine-U-^{14}C and Glycerol-2-^{14}C in the Presence of Pantoyl Lactone. ○, L-phenylalanine-U-^{14}C (10^{-4}M); ●, L-phenylalanine-U-^{14}C (10^{-4}M) plus pantoyl lactone (0.15M); □, glycerol-2-^{14}C (10^{-4}M); and ■, glycerol-2-^{14}C (10^{-4}M) plus pantoyl lactone (0.15M).


12.0
10.0
8.0
6.0
4.0
2.0
0
0
1
2
3
4
5
Specific Activity (counts per min. per mg. cell dry wt.) x 10^{-3}
Time (minutes)

compounds) into the cell. Inhibition in entry of D-cycloserine during growth cannot be complete since mucopeptide synthesis is still significantly decreased in cells grown in the presence of pantoyl lactone and D-cycloserine.

Miscellaneous Experiments

Possible Release of Protein Material From Non-Dividing Cells

Grula and Grula (1964) reported that "leakage" of protein and nucleic acid material (measured by 260/280 absorption) occurs from filamentous cells of Erwinia species. Also, it was reported that leakage was probably the result of damage incurred by the cell membrane during inhibition of division since either pantoyl lactone or hypertonic conditions allow division to proceed in the absence of normal mucopeptide synthesis while causing "repair" of membrane leakage.

It was felt that possible leakage of protein and nucleic acid should be studied using the dis-II pur^+ strain since such measurements could possibly reveal damage occurring to the cell membrane during inhibition of division. Because our defined growth medium contains L-tyrosine, measurement of absorbance at 260/280 is not a good quantitative estimation of leakage of protein or nucleic acid. For this reason, polyacrylamide gel electrophoresis was utilized to analyze for proteins that might be released during growth in the presence of D-cycloserine.

Because lysis of logarithmically growing cells (12 hour) begins to occur 4 hours after addition of D-cycloserine, cells were removed

(by centrifugation) 3 hours after addition of D-cycloserine and the supernatant collected, dialyzed, and concentrated. Although the cells are not very large (indicative of division inhibition) at this time, other experiments have shown that such cells are committed to the division inhibited "state" since removal of such cells from D-cycloserine-containing medium and subsequent resuspension in fresh medium without D-cycloserine will not permit normal growth or division to occur.

Electrophoresis of concentrated growth media indicated that there is no release of proteins from either normal cells or cells grown in the presence of D-cycloserine.

Grula and Hopfer (unpublished) have shown that the leakage of proteins from non-dividing cells of Erwinia sp. is probably related to cell wall damage since leaked proteins originate from the periplasmic space. The periplasmic space as defined by Heppel (1967) is an area located between the cell wall and cell membrane and applies essentially to Gram-negative bacteria. Proteins contained in this area can be released by a cold osmotic shock treatment described by Heppel.

Since release of proteins could not be demonstrated in non-dividing cells of the dis-II pur^+ strain, it could be concluded that damage was not sufficient to cause their release or such proteins simply are not available for release from this Gram-positive organism. To study this latter possibility, cells were subjected to the cold osmotic shock procedure of Heppel and concentrated "shockates" analyzed using polyacrylamide gel electrophoresis. In no instance could I demonstrate the presence of proteins released from the cells

using the cold osmotic shock procedure. Therefore, based on my rather limited data (conditions of the Heppel procedure were never varied), it appears either that the dis-II pur^+ strain does not have periplasmic space proteins or any proteins that might be present in this area are more tightly bound than in Gram-negative bacteria. Regardless of what the situation actually is, non-dividing cells of the dis-II pur^+ strain do not release proteins into the growth medium.

Search for an Autolytic Enzyme System

Throughout this investigation lysis of the dis-II pur^+ strain was observed after 4 to 6 hours growth in the presence of D-cycloserine. It was felt that this lysis was due either to decreased mucopeptide fabrication, with plasmoptysis of the resulting protoplast form, or induction of an autolytic enzyme(s). Considerable amounts of data have already been presented which indicate that mucopeptide synthesis has been significantly altered and inhibited. Tomasz, Albino, and Zanati (1970) have suggested, using Diplococcus pneumoniae, that inhibition of cell wall synthesis and lysis of cells are separate effects and that inhibition of wall synthesis is not enough to cause lysis of cells. In order to determine if we could relate the activity of D-cycloserine to activation of an autolytic enzyme, studies were initiated to determine conditions wherein autolytic activity could be expressed in our dis-II pur^+ strain.

Several attempts were made using either normal or non-dividing whole cells. Also, different types of buffers, at different pH values and molarities were used. A summary of the data obtained from these experiments (Table XIV) reveals that little, if any, lysis

TABLE XIV

SUMMARY OF CONDITIONS EMPLOYED TO PERMIT EXPRESSION OF AUTOLYTIC ACTIVITY IN DIVIDING AND NON-DIVIDING CELLS

Incubation Conditions*	Type of Cell**		pH	O.D.		Percent Lysis
	Normal	Non-Dividing		0 hours	24 hours	
phosphate buffer*** (0.100M)	+	-	7.0	0.53	0.50	6
phosphate buffer (0.010M)	+	-	7.0	0.51	0.49	4
phosphate buffer (0.100M)	-	+	7.0	0.52	0.50	4
phosphate buffer (0.010M)	-	+	7.0	0.49	0.47	4
phosphate buffer (0.100M)	+	-	8.0	0.50	0.48	4
phosphate buffer (0.010M)	+	-	8.0	0.49	0.48	4
phosphate buffer (0.100M)	-	+	8.0	0.51	0.48	4
phosphate buffer (0.010M)	-	+	8.0	0.49	0.48	2
citrate buffer**** (0.100M)	+	-	6.0	0.56	0.54	4
citrate buffer (0.010M)	+	-	6.0	0.53	0.52	2
citrate buffer (0.001M)	+	-	6.0	0.54	0.51	6
citrate buffer (0.100M)	-	+	6.0	0.57	0.55	4
citrate buffer (0.010M)	-	+	6.0	0.52	0.50	4
citrate buffer (0.001M)	-	+	6.0	0.51	0.49	4
saline (0.148M)	+	-	7.0	0.52	0.50	4
saline (0.148M)	-	+	7.0	0.50	0.48	4

*All incubations were done at 30° C without shaking.

**Cells (16 hours) harvested by centrifugation and resuspended in appropriate buffer. Non-dividing cells obtained by growth in the presence of D-cycloserine (7.0×10^{-5}M).

***Potassium phosphate buffers

****Sodium citrate buffers

(autolysis) was obtained regardless of the situation tested.

In addition to studies using whole cells, cells were sonically disrupted in the presence of glass beads (0.0037 to 0.0042 inch in diameter from Prismo Safety Corporation, Huntingdon, Pennsylvania) and the resulting isolated mucopeptide incubated for varying periods of time at pH 7.0 and 8.0 in 0.05M potassium phosphate buffer at room temperature. Again, no evidence for presence of an autolytic enzyme could be obtained. Therefore, all attempts to define conditions for activation of an autolytic enzyme(s) were unsuccessful.

Sucrose Density Gradient Studies on Membrane Bound Deoxyribonucleic Acid (DNA)

In their replicon model for bacterial DNA replication, Jacob, Brenner, and Cuzin (1963) proposed a membrane attachment site for DNA which permits orderly segregation of daughter chromosomes. Shortly thereafter, data were presented to demonstrate that DNA is attached to a rapidly sedimenting structure, possibly membrane, in E. coli (Smith and Hanawalt, 1967) and Bacillus subtilis (Ganesan and Lederberg, 1965).

Jacob et. al. (1963) have also suggested that cell membrane synthesis is coordinated with chromosome replication so that, on completion of a cycle of DNA replication, growth of the cell membrane between the sites of attachment of the new pair of chromosomes is triggered (intercalary growth), which, in turn, then permits segregation of the chromosomes.

Although direct data have not been presented in the literature, it is possible that failure of chromosomes to segregate could lead

to failure of a cell to divide. Since we had been able to demonstrate that the cell wall and membrane of the dis-II pur^+ strain are both altered during inhibition of division, it seemed feasible that this damage could lead to irreversible dissociation of chromosomes from possible attachment points on the cell membrane thus leading to inhibition of division. In order to gather data relative to this possibility, studies utilizing sucrose density gradient centrifugation were initiated.

Data shown in Figure 14 indicate that normal and non-dividing cells have essentially the same sucrose density gradient profiles. These profiles reveal that 260/280 absorbing materials (nucleic acid and protein) are located in the first fraction and all fractions 8 through 11. It should also be pointed out that a rapidly sedimenting yellow insoluble pellet is located in the bottom of the centrifuge tube. This rapidly sedimenting material undoubtedly consists of membrane components since it has the characteristic color (yellow) associated with membranes isolated from M. lysodeikticus. The large absorption at 260 nm indicates some type of nucleic acid (DNA or RNA) is associated with this material.

To determine if the nucleic acid could be dissociated from the sedimented membrane fragments, sodium lauryl sulfate (0.30M) was added and the solubilized material again layered on sucrose gradients. As shown in Figure 15, only one peak is obtained from both types of membrane preparations and it is positioned in the same place as non-sedimenting nucleic acid previously seen (Figure 14) for each cell type. In addition, the yellow insoluble material is not present which indicates that it is membrane material since it is completely

Figure 14. Discontinuous Sucrose Density Gradients of Lysates From Dividing and Non-Dividing Cells. ○ , protein (mg) from normally dividing cell lysate; ●, nucleic acid (mg) from normally dividing cell lysate; △, protein (mg) from non-dividing cell lysate; and ▲, nucleic acid (mg) from non-dividing cell lysate.

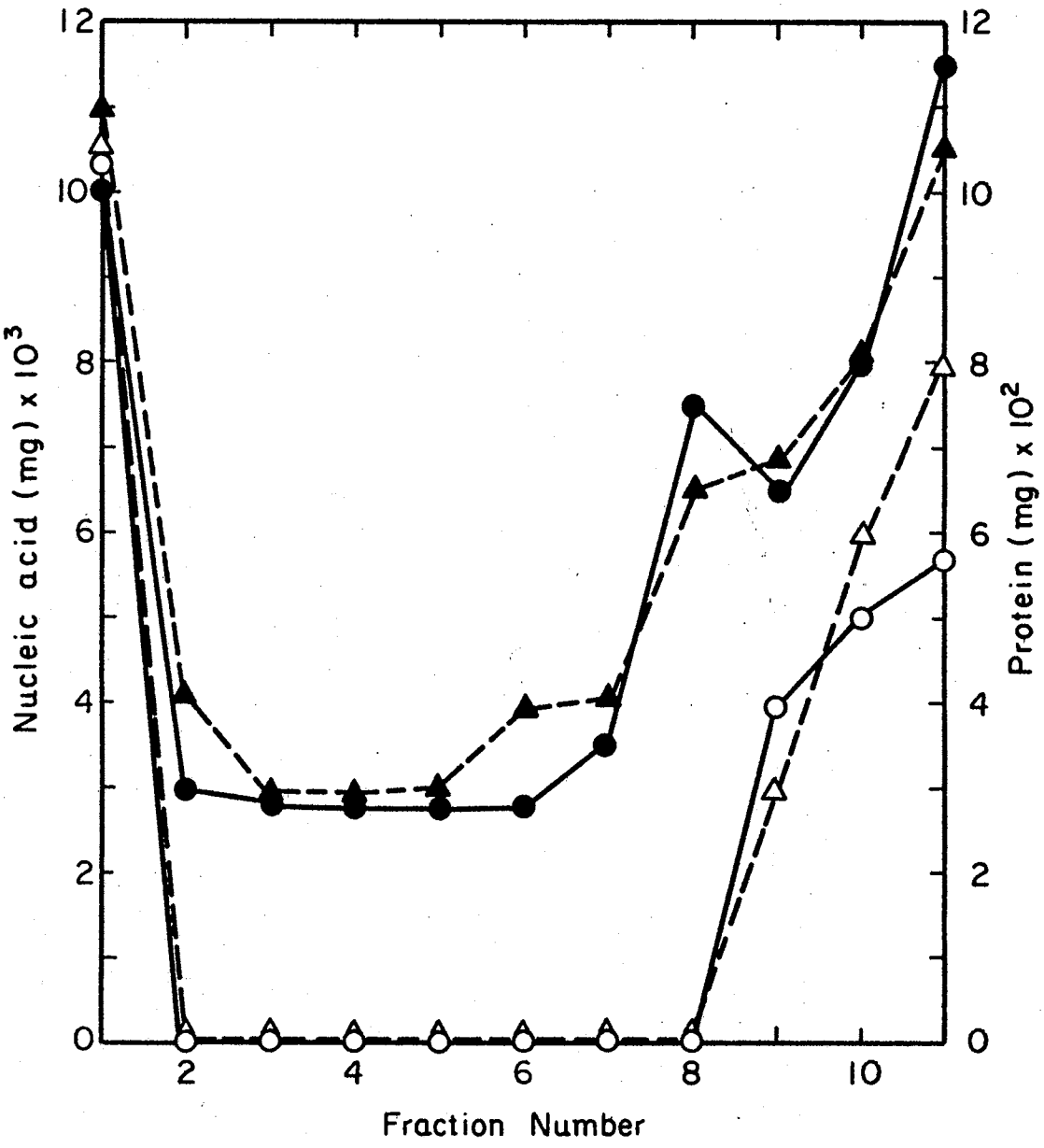
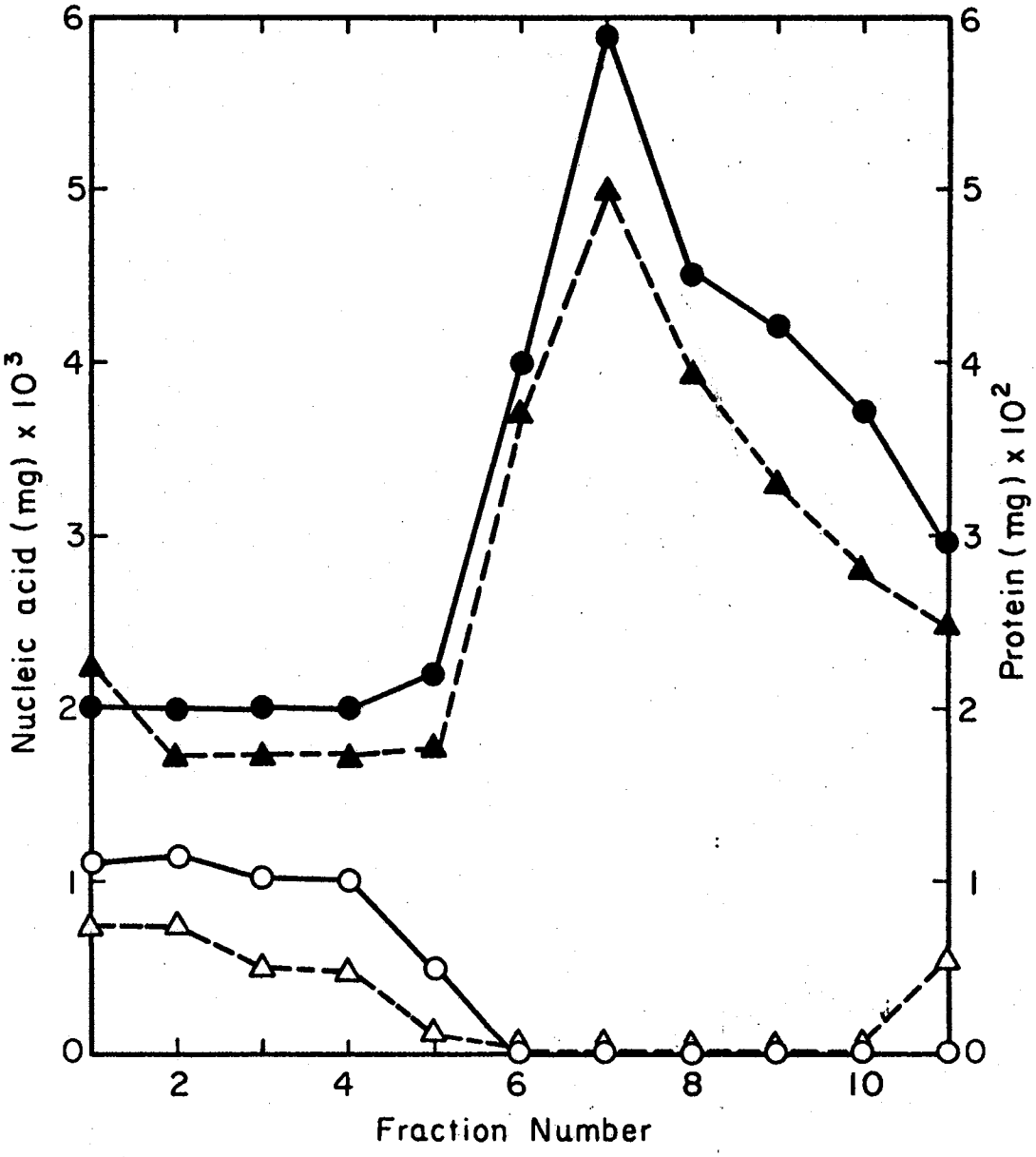

Nucleic acid (mg) x 10³
Protein (mg) x 10²
Fraction Number
0
2
4
6
8
10
12

Figure 15. Discontinuous Sucrose Density Gradients of Solubilized Membrane Pellets Obtained From Dividing and Non-Dividing Cells. ○, protein (mg) from normally dividing cell membrane pellet; ●, nucleic acid (mg) from normally dividing cell membrane pellet; △, protein (mg) from non-dividing cell membrane pellet; and ▲, nucleic acid (mg) from non-dividing cell membrane pellet.


Nucleic acid (mg) x 10³
Protein (mg) x 10²
Fraction Number
0
1
2
3
4
5
6
2
4
6
8
10

solubilized by the detergent. It may also be significant that little or no protein is present with the nucleic acid after detergent treatment.

These data indicate in a presumptive manner that nucleic acid material is associated with some portion(s) of the cell membrane and is released after solubilization of the membrane using detergent. Further chemical analyses on the rapidly sedimenting material must be done in order to determine if this material is DNA or RNA or both. It can be concluded at this time, however, that the sucrose density gradient profiles of dividing and non-dividing cells are not qualitatively different. Also, if the rapidly sedimenting material is assumed to be membrane bound DNA, then it can be stated that DNA is still bound to the membrane in non-dividing cells and possible dissociation due to the membrane alterations occurring during division inhibition does not occur.

Membrane Synthesis

Although all of the present published information indicates that D-cycloserine action is directed against mucopeptide synthesis, data presented in this thesis also demonstrate that cell membrane alterations occur during inhibition of division of the dis-II pur^+ strain by D-cycloserine. Because the 1735/1655 ratio (infrared spectra of D-cycloserine membranes) is increased, the possibility existed that lipid deposition is increased in the cell membrane of cells grown in the presence of D-cycloserine. In order to directly determine if D-cycloserine had such an effect on synthesis of cell membrane lipids, incorporation of glycerol-2-^{14}C into the cell membrane was

measured in the presence and absence of D-cycloserine.

Data presented in Table XV indicate that D-cycloserine has very little effect on incorporation of glycerol-2-^{14}C into the cell membrane. However, in the presence of D-cycloserine and pantoyl lactone it is noted that incorporation of glycerol-2-^{14}C is inhibited 24 percent. A possible explanation for this observation could be that pantoyl lactone has the ability to solubilize or elute lipid components away from the membrane surface. In order to check this possibility, pre-labeled (glycerol-2-^{14}C) membranes were treated with 0.15M pantoyl lactone for 2 hours at room temperature. After incubation, the membranes were removed (by centrifugation) and the supernatant counted. This count was then subtracted from the initial specific activity of the membrane sample and the percent decrease calculated. As shown in Table XVI, pantoyl lactone has little, if any, ability to elute material from the cell membrane.

Based on glycerol incorporation, it can be concluded that D-cycloserine does not cause a significant decrease in synthesis of lipids in the cell membrane. Further, it could also be concluded that pantoyl lactone brings about a significant decrease in synthesis of membrane lipids. Although I was not able to perform the necessary experiments, due to the lack of labeled pantoyl lactone, it is very possible that some carbon from pantoyl lactone or pantoic acid is also used for lipid synthesis. If this does occur then the decrease shown in glycerol incorporation represents a dilution of glycerol carbon into lipids rather than a true decrease in synthesis of lipids.

To insure that glycerol-2-^{14}C incorporation represents a genuine

TABLE XV

MEMBRANE LIPID SYNTHESIS MEASURED BY GLYCEROL-2-^{14}C INCORPORATION*

Growth Conditions**	Specific Activity of Membrane Samples***	Percent Decrease
Basal Medium	537	0
+ D-Cycloserine	516	4
+ D-Cycloserine + pantoyl lactone	410	24

*Glycerol-2-^{14}C (0.01 uC/ml) was added at 12 hours. After 4 hours growth, whole cell membranes were isolated and counted.
**All concentrations were as given in Table V.
***Expressed as: Counts per minute/mg membrane/mg cell dry weight

TABLE XVI

MEASUREMENT OF POSSIBLE ELUTION BY PANTOYL LACTONE OF LIPID MATERIAL FROM GLYCEROL-2-^{14}C-LABELED MEMBRANES*

Treatment**	Specific Activity of Membrane Sample***		Percent Decrease
	Before Treatment	After Treatment	
Distilled Water	830	815	2
+ Pantoyl lactone (0.15M)	920	913	1

*Glycerol-2-^{14}C (0.01 uC/ml) labeling was performed as described in Table XV.

**Glycerol-2-^{14}C-labeled membranes treated for 2 hours at room temperature.

***Expressed as: Counts per minute/mg membrane/mg cell dry weight

measure of membrane lipid synthesis, lipids were extracted from whole cells using chloroform and methanol (2:1), washed and then counted. Data shown in Table XVII indicate that the more extensive extraction and washing procedures are not necessary and measurement of glycerol-2-^{14}C incorporation into the isolated membrane is a reliable measurement of lipid synthesis.

TABLE XVII

MEMBRANE LIPID SYNTHESIS MEASURED BY ISOLATION OF GLYCEROL-2-^{14}C-LABELED LIPIDS FROM WHOLE CELLS*

Growth Conditions**	Specific Activity of Isolated Lipids***	Percent Decrease
Basal Medium	5050	0
+ D-Cycloserine	4940	2
+ D-Cycloserine + pantoyl lactone	4132	18

*Glycerol-2-^{14}C (0.01 uC/ml) was added at 12 hours. After 4 hours growth, lipids were isolated using chloroform methanol (2:1) and counted.

**All concentrations were as given in Table V.

***Expressed as: Counts per minute/mg cell dry weight

CHAPTER IV

DISCUSSION

Experiments in this thesis were initially designed to determine whether or not a direct correlation exists between inhibition of mucopeptide synthesis and inhibition of cell division. It was hoped that data obtained from these experiments would help provide an answer to the critical question regarding the relative importance of the cell wall in the process of bacterial cell division.

As shown in this thesis, every situation wherein division is inhibited a concomitant decrease in mucopeptide synthesis is also observed. Because most of the agents tested for division inhibiting activity are known inhibitors of mucopeptide synthesis, the apparent relationship between wall synthesis and cell division is not surprising. The inability of vancomycin, a known inhibitor of mucopeptide synthesis, to significantly inhibit division while causing partial wall damage suggests that a critical amount or kind of cell wall damage must occur before division is inhibited. Such a conclusion is reinforced by data obtained using pantoyl lactone and spermine. As shown in Table XII, division is inhibited when cell wall synthesis and cross-linking are decreased by at least 20 and 10 percent respectively. Overall, these findings suggest that cell wall synthesis is important for and somehow involved in the division process and cannot be negated.

Data of others also imply or suggest that the cell wall could be important in the division process. Cole and Hahn (1962) have shown, using Streptococcus pyogenes, that new cell wall is not diffusely intercalated with old, but its formation is instead initiated equatorially such that centripetal growth (invagination) occurs until the cell divides into two daughter cells. Also, their data indicate that cell wall synthesis occurs during the division process; such growth could imply that the wall is responsible for initiation and thereafter sustains the invagination process. Unfortunately, their methodology (fluorescent-antibody staining and optical microscopy) gave no information relative to possible activities of the cell membrane during the division process.

Kusaka (1970) recently reported that protoplasts of Bacillus megaterium grow and divide in 1 percent casein hydrolyzate medium containing 0.5M sodium chloride as an osmotic stabilizer. It was also noted that a fibrous layer or a partially complete coat, which he identified as mucopeptide, develops around the growing and dividing protoplast; division of the protoplasts is completely inhibited in the presence of penicillin which totally inhibits mucopeptide synthesis under his conditions. Unfortunately, his study was not quantitative and nothing more than electron micrographs (thin-slices) were presented.

Although the concept of having the cell wall provide centripetal pinching (through synthesis) is attractive, it should be pointed out that two agents which protect the dis-II pur^+ strain from inhibition of division (pantoyl lactone and spermine) by D-cycloserine do not significantly correct the cell wall lesion. This finding suggests

that cell wall synthesis does not have to occur at normal rates in order for a cell to divide. It could be argued, however, that although these protective agents (pantoyl lactone and spermine) do not restore cell wall synthesis to normal they do allow enough synthesis for cell division to occur. Grula and Grula (1964), using a species of Erwinia, have also shown that hypertonic conditions or pantoyl lactone, although allowing substantial division activity to occur in the presence of a variety of division inhibiting agents, do not significantly restore cell wall synthesis. Therefore, it appears that inhibition in synthesis of the cell wall is not in itself a sufficient condition for inhibition of cell division.

Since pantoyl lactone and spermine do not restore mucopeptide synthesis in the presence of D-cycloserine, they must be acting at some other site in order to allow cell division to occur and it is this site which holds the key to cell division. As discussed by Ryter and Landman (1964) and Landman and Halle (1963), the stimulus for division of B. subtilis protoplasts seems to be invagination of the protoplast membrane. Also, Martin (1964) has reported that a stable L-strain of Proteus mirabilis devoid of cell wall material (protoplasts) can grow and divide indefinitely. Grula and Grula (1964) suggested that inhibition of division is the result of secondary damages incurred by the cell membrane as a result of inhibition of mucopeptide synthesis and that pantoyl lactone and spermine prevent these secondary membrane damages from occurring. Grula et. al. (1965) also presented evidence obtained using the electron microscope to demonstrate that the cell membrane initiates and sustains the invagination process of cell division.

As shown in this thesis, alterations in the cell membrane do occur during inhibition of division by D-cycloserine. Data from my membrane studies indicate the following: (1) Membranes isolated from cells grown in the presence of D-cycloserine have an increased amount of protein(s) in the beta-conformation (infrared analysis) and also have an altered electrophoretic profile (polyacrylamide gel electrophoresis of proteins). (2) Membranes isolated from cells protected from division inhibition by D-cycloserine particularly in the presence of pantoyl lactone appear to be near normal both in conformation and content of protein. Although a return to near normal appearance was consistently noted, restorations were never complete. (3) Membranes isolated from cells grown in the presence of D-cycloserine plus either spermine or Carbowax-400 showed little, if any, restoration in electrophoretic patterns or conformation of proteins. Accurate infrared spectra could not be obtained using spermine but membranes from cells grown in the presence of Carbowax-400 always showed a substantial shift toward the beta-conformation.

For the above reasons, it does not appear that a direct correlation can be made between protein composition of the cell membrane, as monitored by electrophoresis, and cell division. A correlation does appear to exist, however, between cell division activity and the "conformational state" of membrane proteins. Such a correlation is suggested because of the restoration and/or maintenance of the alpha-helical conformation to membrane proteins by pantoyl lactone during growth in the presence of D-cycloserine. It appears entirely logical that the conformation of cell membrane proteins is important for cell division and a shift in conformation might result in a stretching or

constricting (invagination) of the cell membrane. This latter activity could cause initiation of the division process.

The above concept has some merit since Morrison and Morowitz (1970) have shown, using B. subtilis, that the cell membrane grows only at the "tips" of the cell and that synthetic activity does not occur in the division area. Their data suggest that the membrane would have to "stretch" rather than grow in order to initiate cell division. A probable mechanism by which this stretching could be accomplished is through a conformational change in the cell membrane.

All of my data suggest that pantoyl lactone (and possibly spermine) act at the level of the cell membrane in order to prevent division inhibition by D-cycloserine. Pantoyl lactone was shown to have a direct effect on functionality of the cell membrane by also demonstrating that it could inhibit uptake of a variety of compounds. Because uptake of several compounds was retarded, my data suggest that pantoyl lactone may have a general rather than a specific effect on the cell membrane structure. The retardation of uptake may be due to a "tightening" of the membrane structure as envisioned by Grula and Grula (1964) who were able to show that pantoyl lactone "repairs membrane leakage." It could also be due to a change in conformational state of the "permease" proteins.

Although investigations into the involvement of DNA and the division process were only preliminary, it appears the membrane damage that occurs during division inhibition does not cause a dissociation of DNA or any of its associated proteins or lipids from the membrane (if they are really attached to the cell membrane). Therefore, it does not appear that damage to a possible DNA membrane attachment

site has any correlation to the inhibition of division by D-cycloserine in the dis-II pur^+ strain of M. lysodeikticus.

In summary, it can be suggested that cell wall damage is not a sufficient condition to cause inhibition of division. Division inhibition is probably due to some type of secondary damage to the cell membrane caused by inhibition of cell wall synthesis. Also, it is felt that pantoyl lactone (and possibly spermine) directly affect the cell membrane in order to prevent division inhibition. It appears that primary functions of the cell wall are to provide rigidity to the cell and protect the cell membrane from external environmental influences so that it may carry out its functions, one of which is to initiate the cell division process.

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