PANTOYL LACTONE-INDUCED ALTERATIONS IN BIOLOGICAL SYSTEMS

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CHAPTER I

INTRODUCTION

Because cell division is by its nature a very complex process, there are many different aspects of the phenomenon that could be investigated. This study was limited to observing structural changes occurring at the cell periphery (cell wall and cell membrane) during cell division as well as cell division inhibition. Therefore, the literature survey that follows will deal only with those studies pertinent to such peripheral changes.

In order to discern what specific structure is primarily responsible for cell division, most workers have used organisms in which division can be drastically inhibited. The reasons for this are as follows: (1) With an understanding of the events leading to a "block" in division (filament formation), one can better understand the mechanism(s) in operation during "normal" cell division. (2) The changes are likely to be very pronounced in filamentous cells, thus making the block easier to detect. (3) The alternative to studying filamentous cell growth is to study synchronously growing cultures of cells; however, not only is synchrony hard to achieve and maintain for any length of time, but an imbalance in some aspect of cellular metabolism is usually required.

Grula (1960) reported that the D-isomers of serine, threonine, histidine, tryptophan, phenylalanine, and methionine inhibited both

growth and cell division in a species of Erwinia. He also reported that spheroplast formation was evident and indicated that some inhibition of cell wall synthesis was therefore likely.

Strominger (1960) reported that various treatments which lead to filament formation (growth in the presence of crystal violet, novobiocin, or penicillin) will inhibit cell wall mucopeptide synthesis in bacteria.

Grula and Grula (1964) were able to show that non-dividing cells of Erwinia sp., (produced by growing cultures in the presence of D-serine, vancomycin, penicillin, mitomycin c, or D-cycloserine, or treatment with ultraviolet light), contain 30 to 40% less cell wall mucopeptide. They also reported that compounds, such a pantoyl lactone or spermine, which allow cells to divide in the presence of division inhibiting agents, do not restore mucopeptide content to control levels. Additional evidence for peripheral damage was their observation that 260/280 nm absorbing materials "leak" from filamentous cells; however, due to an inherent lysis problem this leakage could not be accurately resolved.

From these and other data, the authors suggested the decreased mucopeptide content of filamentous cells may allow some type of "secondary" damage to the cell membrane. Because reversal agents such as pantoyl lactone, spermine, and hypertonic conditions do not correct the inhibition of mucopeptide synthesis, it appears that these agents probably function by preventing the suspected secondary damage to the cell membrane. Although their study firmly established that filamentous cells contain decreased amounts of cell wall mucopeptide, it was also demonstrated that a simple and direct relationship between

inhibition of mucopeptide synthesis and lack of cell division activity does not exist.

It should be pointed out that compounds such as penicillin, vancomycin, or D-amino acids which inhibit division activity and cell wall synthesis do not impair DNA synthesis (Grula et al., 1968).

Other investigators, (Weidel, Frank, and Martin, 1964) have equated inhibition of cell division, in the presence of penicillin, to enhanced "autolytic" enzyme activity. They suggested that a muramidase-like enzyme activity is responsible for opening new sites in the wall mucopeptide. This creates a site for new monomeric mucopeptide units to be inserted into pre-existing wall (intercalary insertion). Another enzyme with transpeptidase activity, which is inhibited by penicillin, is responsible for "resealing" the newly inserted mucopeptide unit to the pre-existing wall. When muramidase activity is operating at a normal rate, but transpeptidase activity is inhibited (penicillin present), "autolytic" activity is disproportionate and lysis can be the end result when the imbalance becomes too great.

Because penicillin inhibits transpeptidase (cross-linking) activity (Wise and Park, 1965; Tipper and Strominger, 1965), Bazill (1967) has presented the hypothesis that division inhibition may be the result of an imbalance between cell wall and cell membrane synthesis. He argues that defective cell wall synthesis, in some strains, may be the primary cause for death by ultraviolet irradiation as well as thymine starvation. Most workers have assumed that death by these two treatments is wholly a DNA related phenomenon even though Grula and Grula (1964) have shown that ultraviolet irradiation causes a decrease in mucopeptide content. According to Bazill, for proper division to

occur, a rigid wall must be present to give support to the membrane, otherwise the invagination process will not occur. If the wall is weak, whatever "triggers" the inward movement of the membrane (synthesis or sudden conformational change) will proceed as usual; however, division will not occur because, due to the weakened wall, the membrane is permanently distended by the internal osmotic pressure.

Lending support for the importance of membrane involvement in cell division are the data presented by Landman and co-workers (Landman, Ryter, and Frehel, 1968; Landman and Forman, 1969). They studied reversion of Bacillus subtilis protoplasts to the bacillary form. The fact that protoplasts cannot divide in liquid media supports the idea of wall involvement; however, they can divide in a solid medium. This suggests that the solid agar replaces the "rigidity" normally furnished by the cell wall, thereby giving the membrane support for its inward movement. The authors state that invagination of the membrane is not only necessary but sufficient to trigger septum and cross-wall formation; however, direct wall involvement cannot be completely ruled out because, as the authors point out, the agar may be preventing cell wall enzymes and precursors from diffusing away from the exterior surface of the membrane.

Extensive studies of cell wall alterations of non-dividing cells of Micrococcus lysodeikticus by King and Grula (1971) further indicate that the primary function of the cell wall is to provide "protection" for the membrane. This conclusion is supported by their findings which reveal that changes in membrane protein composition, conformation, and uptake activity occur in non-dividing cells.

Based on electron micrographs of Ryter and Jacob (1964), which

show possible nuclear association with mesosomal structures, Jacob, Brenner, and Cuzin (1963) have suggested that membrane synthesis is coordinated with chromosomal replication. They suggest (replicon hypothesis) that after completion of a cycle of DNA replication, growth of the membrane between the points of attachment of the new pair of chromosomes is triggered, thus segregating the nuclear bodies. Many publications lend support to the replicon model of Jacob; however, many are in disagreement with his concept. The replicon model requires that at least the two following conditions be met: (1) The DNA is bound either directly or indirectly to the cell membrane. (2) Segregation of nuclear bodies depends on intercalary growth of the cell membrane between the attachment sites.

Ganesan and Lederberg (1965) presented chemical evidence for a DNA-membrane attachment; however, a fortuitous association could not be completely ruled out. Using a detergent (sodium lauryl sarcosinate), whose crystals form a complex with membrane but not free DNA, Tremblay, Daniels, and Schaechter (1969) extracted a membrane-DNA-detergent complex from Escherichia coli. Woldringh (1970) presented data (including electron micrographs) showing that sodium lauryl sarcosinate "dissolves" the plasma membrane of E. coli. As this investigator points out, his results make the conclusions of Ganesan and Lederberg (1965) and Tremblay, Daniels, and Schaechter (1969) somewhat suspect.

Morrison and Morowitz (1970) reported that membrane synthesis, as measured by incorporation of labeled glycerol, is primarily end-localized in Bacillus megaterium KM. These data along with those of Ryter (1967) support the second requirement of the replicon model,

namely, that membrane growth occurs by local intercalation.

Tsukagoshi, Fielding, and Fox (1971) observed that no differences exist in membranes from synchronized cells of a fatty acid auxotroph of E. coli after initial growth in oleate (light) medium followed by growth in stearate (heavy) medium. Their data, obtained using linear sucrose gradients, indicate that during membrane growth, lipids are inserted by diffuse intercalation. Even Jacob, the strongest proponent of the replicon model (Lin, Hirota, and Jacob, 1970), has now reported that a direct DNA-membrane association cannot be demonstrated. Further, growth of both the cell wall and membrane occurs by diffuse rather than local intercalary insertion. The data were obtained by following incorporation of specific labeled compounds in cells of E. coli growing in methocel, a compound which allows cell division, but not cell separation.

If there is coordination between the cell membrane and DNA replication during cell division, it is possible that such coordination is effected via membrane proteins that might be associated with DNA synthesis. Studies of this type were initiated when Inouye and Guthrie (1969) isolated a mutant of E. coli which could not synthesize DNA at temperatures above 40 C but could continue to divide. Utilizing polyacrylamide gel analysis and double label techniques (^{3}H and ^{14}C), envelope protein patterns of cells grown normally at 28 C were compared to envelopes prepared from cells grown at the restrictive temperature (41 C). These investigators found a decrease in one and an increase in another envelope protein when DNA synthesis was inhibited. Their data suggest that these particular envelope proteins are related to DNA synthesis.

Inouye and Pardee (1970) extended the work using E. coli K12. Using five different agents to inhibit DNA synthesis and/or cell division, they found, in general, that conditions which inhibit DNA synthesis also inhibit synthesis of an envelope protein which they labeled "Y" (mw 34,000). In addition, conditions which inhibit cell division increase synthesis of another envelope protein labeled "X" (mw 39,000).

Similar findings relating to coordination between envelope proteins, cell division, and DNA synthesis were reported by Shapiro et al. (1970). Using a temperature sensitive DNA "A" (initiator) mutant, this group found an increase in an envelope protein component (mw 32,000) when cells were grown under permissive conditions (30 C) and a decrease in another envelope protein (mw 60,000) when cells were grown under restrictive conditions (41 C). In a related study, Siccardi et al. (1971), using a temperature sensitive DNA "B" (replication) mutant, adds the further complication that the changes in envelope proteins found in this mutant are not due to presence or absence of the proteins per se; but, these cells lack whatever is responsible for holding subunits of this particular envelope protein together.

Other data relevant to changes in composition of cell wall and cell membrane have been presented by Weinbaum and co-workers. Weinbaum (1966) reported that filamentous cell forms of E. coli, which are nutritionally produced in complex media, are deficient (20 to 30%) in cell wall mucopeptide. In addition, the cell membrane of these filaments is deficient in cyclopropane fatty acids (Weinbaum and Panos,

1966) as well as glycoprotein components (Weinbaum, Fischman, and Okuda, 1970).

Another possible type of change occurring at the periphery of a non-dividing cell, is the "loss" of surface macromolecules which could be important in the division process. As mentioned earlier, Grula and Grula (1964) observed "leakage" of 260/280 nm absorbing materials from filamentous cells.

Heppel (1967) has been able to demonstrate that certain proteins are contained in what he has termed the periplasmic space (area between the cell wall and cell membrane) of Gram-negative bacteria. A cold osmotic-shock procedure causes the proteins to be released; however, cells, when re-inoculated into fresh medium, continue to divide after a 30 minute lag in growth. It was pointed out that possible rapid re-synthesis of the proteins, during the lag period, might mask any requirement for these particular proteins in cell division. In a related study, Fisher et al. (1969) observed other evidence demonstrating possible involvement of the peripheral area of the cell in cell division. These investigators reported that normally dividing cells of E. coli excrete a large (100S) lipoprotein complex which aids division in irradiated cells of the same organism.

As can be seen, there are three possible types of alterations that can be studied in following peripheral changes relating to cell division. These include the cell wall, the cell membrane, and related peripheral molecules that may be lost during division inhibition.

When this study was initiated, a new technique for growing filaments without concurrent lysis had just been developed. Based on observations (Grula et al., 1968) that either pantothenate, or

beta-alanine, increases elongation in D-serine-induced filaments, it was observed that if cells are grown in beta-alanine for 13 hours, before the addition of D-serine, good growth of filamentous cells can be obtained with only minimal lysis. Using this growth system, it appeared that we could clarify the previously reported "leakage" by determining the origin of the molecules and their possible involvement in cell division.

We have been able to show (Grula and Hopfer, 1972) that at least 5 proteins are released from growing filamentous cells; however, detectable amounts of the proteins are not released from normally growing cells or cells growing in the presence of pantoyl lactone. Origin of these proteins appears to be the periplasmic space area since it was also shown that these plus a few additional proteins can be released from normally grown cells by the Heppel (1967) cold osmotic-shock procedure.

Addition of released proteins, or proteins obtained by cold osmotic-shocking of control cells, to UV-irradiated cells, which are destined to become filamentous, neither protects nor activates the division mechanism. Also, we reported that antibodies to these released proteins will not inhibit the division mechanism from functioning in normally growing cells; however, our data in this area must be accepted with certain reservations. The proteins could be involved in the division process but their involvement might never be expressed under conditions of the experiment, due to inactivation, inability to be properly re-inserted, or indeed inaccessibility to the proper location. Similar arguments can be made against our antibody experiments.

Regardless of how the antibody experiments are interpreted, two

other observations which we reported also suggest that protein release is the result, not the cause, of division inhibition. First, study of rate of release of proteins indicates that the proteins are not released until after a significant amount of cell elongation has occurred. Secondly, pantoyl lactone, but not spermine, prevents protein release, yet both compounds allow good division activity. These data imply that while protein release from the periplasmic space area during division inhibition is an indication of wall and possible membrane damage, the proteins, as such, probably are not directly involved in the division process.

We were also able to demonstrate that, in this particular growth system, either D-serine or UV-irradiation causes decreased synthesis of cell wall mucopeptide in filamentous cells. Further, reversal agents, such as pantoyl lactone, spermine, and hypertonic conditions, do not restore mucopeptide synthesis to control levels. It was also reported that D-serine-induced filaments release thiobarbituric acid-positive material into the growth medium. Such data tend to reinforce our conclusion that extensive wall damage occurs in filamentous cells.

In the same study, several quantitative changes in protein composition of envelopes prepared from filamentous cells were observed. Pantoyl lactone prevention of division inhibition allows a more normal protein composition to be maintained. Furthermore, Grula et al. (1968) have shown that filaments have a decreased ability to take up aspartic acid. Decreased uptake of aspartic acid by filamentous cells is probably due to release by such cells of binding protein into the growth medium. Since pantoyl lactone causes retention of all proteins (including binding proteins), it is difficult to explain the decreased

uptake activity that occurs in normal cells treated with pantoyl lactone. Nevertheless, because pantoyl lactone influences protein composition of the cell envelope in Erwinia sp. and of the cell membrane in Micrococcus lysodeikticus and also inhibits uptake of various compounds in both organisms without repair of defective mucopeptide synthesis, it permits us to identify the cell membrane as the most probable site of action of pantoyl lactone (Grula and Hopfer, 1972; Grula and King, 1971).

Considering the direction and known information relating to cell division, it seemed most logical that I should concentrate my efforts in the following areas: (1) The mechanism by which pantoyl lactone causes retention of peripheral proteins. (2) Evidence for secondary damage to the membrane and/or its reversal by pantoyl lactone. (3) Mechanism of action of pantoyl lactone on the transport ability of normally grown cells. (4) Determine in general, the specific molecular structure in the cell membrane that is affected by pantoyl lactone.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The organism used throughout this study was a species of Erwinia. It is a Gram-negative, motile, rod-shaped organism that most nearly resembles Erwinia carotovora (Grula, 1960).

Stock cultures were maintained alternately (24 hour intervals) on nutrient agar slants containing 0.5% sodium chloride with and without 1.0% dextrose. To ensure against possible contamination of the culture, it was occasionally streaked on nutrient agar.

Medium and Growth

The basal medium used in this study contained the following compounds per 100 ml: D-glucose, 250 mg; NH_4Cl, 480 mg; K_2HPO_4, 174 mg; KH_2PO_4, 136 mg; $MgSO_4 \cdot 7H_2O$, 3 mg; and trace minerals.

The trace mineral solution was compounded separately and added to give the following amounts per 100 ml: H_3BO_3, 0.5 ug; $CaCO_3$, 10.0 ug; $CuSO_4 \cdot 5H_2O$, 1.0 ug; $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 50.0 ug; KI, 1.0 ug; MoO_3, 1.0 ug; $MnSO_4 \cdot H_2O$, 2.0 ug; and $ZnSO_4 \cdot 7H_2O$, 5.0 ug (Grula, 1960). When desired, DL-serine (441 mg) or D-serine (220 mg), beta-alanine (0.484 mg), and DL-pantoyl lactone (1.0 g) were added. All stock solutions were adjusted to near pH 6.8 prior to addition to the medium.

D-Glucose was autoclaved separately (10 minutes at 12 pounds pressure); beta-alanine and pantoyl lactone were filter sterilized and added aseptically to the medium. All other components were sterilized by autoclaving 15 minutes at 15 pounds pressure.

Media were inoculated with cells grown on nutrient agar slants containing 0.5% sodium chloride (without dextrose). The cells were washed twice in 0.85% sterile saline and adjusted to an optical density of approximately 0.1 (Coleman Junior Spectrophotometer). Five drops of this suspension were used to inoculate 100 ml of medium contained in 250 ml Erlenmeyer flasks. The cultures were incubated on a rotary shaker at 25 C for approximately 17½ hours.

All components were present in the basal medium from time of inoculation except serine and/or pantoyl lactone. Serine was added aseptically after 13 hours growth (logarithmetic phase) and pantoyl lactone was added either at that time (for prevention of division inhibition) or 3 to 4 hours later (for reversal of division inhibition).

Isolation of Envelope

After growth in a particular medium, cells were harvested, washed two times in saline, and resuspended at a very high density in approximately 5 ml deionized water. This suspension was frozen overnight and the cells were mechanically broken in one pass in a prechilled X-PRESS the following day. Breakage occurs at about 15,000 to 20,000 pounds of pressure per square inch. Approximately 99% of the cells are broken using this procedure (based on observation of cells using dark-field microscopy). The particulate fraction (envelope) was collected by centrifugation at 65,000 x g for 15 minutes (Spinco Model

L centrifuge). The envelope pellet was washed twice in 2.5 x 10^{-3}M Tris buffer (pH 8.0) followed by three washes in deionized water. Unless otherwise stated, the washed envelopes were frozen at -20 C overnight and lyophilized.

Preparation of Wall Fraction

To obtain a "wall" fraction, washed envelope material was extracted in 2% Triton X-100 for 45 minutes at 37 C, then collected by centrifugation and washed as given above.

Isolation of Lipopolysaccharide

Cells were harvested, washed twice, and resuspended in cold acetone (4 C). After 30 minutes the cells were removed and washed two additional times using cold acetone. The resulting cell pellet was resuspended in 50% phenol and heated at 55 C for 30 minutes with occasional stirring. The mixture was allowed to separate into two phases and the aqueous layer (containing lipopolysaccharide) then was carefully removed. This suspension was dialyzed against 20 volumes of 0.01 M phosphate buffer (pH 7.4) for 24 hours at 4 C. The material was then concentrated to about 1/10th of the original volume by dialysis against 10% Carbowax-6000 and kept frozen (-20 C) until needed.

Uptake Studies

After about 17 hours growth, cells were harvested, washed two times in "uptake buffer" (contains all salts present in the growth medium excepting nitrogen), and resuspended in the same salts-buffer to an optical density of about 0.2 (measured at 540 nm). The

suspensions were then incubated on a Burrell Wrist-Action shaker at a setting of 1.0 at room temperature for 20 minutes before addition of labeled compounds. Unless otherwise stated, 0.1 ml of a stock solution (containing 1.0 uC labeled compound and 100 ug "cold carrier" per 10 ml) was added to 3 ml of cell suspension. At appropriate time intervals, 0.5 ml of sample was filtered through a 0.45 um size Millipore filter using vacuum suction. The filters containing the cells were then immediately washed 3 times using 1 ml volumes of uptake buffer. Filters were then placed in scintillation vials and allowed to dry (room temperature, overnight) before addition of 10 ml Aquasol. Samples were then shaken at 37 C for 4 hours before counting to permit dissolution of the membrane filters.

The following labeled compounds were used: L-aspartic acid-U-^{14}C (154 mC/mM), D-alanine-U-^{14}C (32.2 mC/mM), D-glucose-U-^{14}C (3.1 mC/mM), thymidine-2-^{14}C (67 mC/mM), L-malic acid-U-^{14}C (26 mC/mM), 2-ketoglutarate-5-^{14}C (17.1 mC/mM), and D-pantoyl lactone-U-^{3}H (107 mC/mM).

Infrared Spectroscopy

A Beckman Spectrophotometer Model IR-7 located in the Department of Chemistry, Oklahoma State University, was utilized throughout this study. Approximately 2 mg of lyophilized envelope was mixed thoroughly with 20 mg 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.

Surface Tension Measurements

All measurements were made using a Cenco Tensiometer equipped with a 4 cm ring. All compounds were tested in growth media to ensure conditions were equivalent to those found during growth of the organism.

Cell Lysis Experiments

After harvesting, cells were washed 2 times and resuspended in uptake media at an optical density of approximately 0.6 at 540 nm. After addition of pantoyl lactone (7.7×10^{-3}M) and/or a specific detergent (3.5×10^{-4}M), decrease in optical density was followed at hourly intervals.

Spectrophotofluorimetry

An Amicon-Bowman Spectrophotofluorimeter located in the Department of Biochemistry, Oklahoma State University, was utilized. All samples were freshly prepared (not lyophilized) and contained approximately 1.5 mg protein in 1 ml of 0.01 M phosphate buffer (pH 7.4). 1-Anilinonaphthalene-8-sulfonate (ANS) was added to give a final concentration of 8.0×10^{-5}M. Excitation wavelength was set at 305 nm and the peak at 475 nm in the emission spectrum was used to measure fluorescence. Temperature was 25 C.

Chromatographic Identification of Pantoyl Lactone

After spotting samples, thin-layer plates containing Silica Gel G were run in either of two solvent systems. One system consisted of chloroform/methanol/water (65:25:4, v/v/v). The other was a

one-directional double-solvent system described by Skipski (1965). In the latter system, plates were allowed to run 13-14 cm in a solvent which consisted of isopropyl ether/acetic acid (96:4, v/v). After drying, the plates were re-run 19-20 cm in light petroleum/ethyl ether/acetic acid (90:10:1, v/v/v).

Pantoyl lactone is easily identifiable as light brown material after exposing the plate to I_2 vapors (Rf of 0.85 in the first solvent and Rf of 0.48 using the procedure of Skipski).

Purification of Labeled ^{3}H-Pantoyl Lactone

Labeled ^{3}H-D-pantoyl lactone was obtained from New England Nuclear Corporation. Before use the labeled material was extracted with heptane and further purified using thin-layer chromatography (Skipski system). Pantoyl lactone "spots" were scraped off the plates and eluted from the gel using 0.01 M phosphate buffer. To ensure purity, the eluted material was re-chromatographed and sequential 1 cm "cuts" made from the plates were eluted and counted. From these data, a "scan" curve was constructed to locate the position (Rf value) of radioactive material.

Equilibrium Dialysis Studies

Unless otherwise stated, approximately 1 ml (0.1 O.D. at 540 nm) of material was placed inside dialysis tubing suspended in 10 ml 0.01 M phosphate buffer containing approximately 1 uC ^{3}H-pantoyl lactone at 4 C. At regular time intervals, 0.1 ml samples were withdrawn from the dialysis tubing and from the surrounding buffer and counted. Evidence for molecular association was assumed when the Distribution Ratio (DR)

in the following formula exceeded 1.1.

$$DR = \frac{\text{CPM inside bag/unit volume}}{\text{CPM outside bag/unit volume}}$$

Hemagglutination and Lysis of Red Blood Cells

Equine red blood cells (RBC's) and sera from rabbits inoculated with horse RBC's were used in the hemagglutination studies. The RBC's were washed 3 times in physiologic saline (P/SS) and resuspended in P/SS to make a 2% cell suspension. All compounds tested were contained in P/SS. Agglutination was determined 2 hours after mixing the cells with sera.

Lysis studies were also made using P/SS as the suspending medium for RBC's and the different additives.

Cold Osmotic Shock

The procedure reported by Heppel (1967) was utilized for release of proteins from the periplasmic space of control cells. When various compounds were substituted, equimolar concentrations were utilized.

Preparation of Released Proteins

The method reported by Grula and Hopfer (1972) was utilized.

Polyacrylamide Gel Electrophoresis

For anionic (pH 9.3) gels, the procedure used was that given in the Buchler Instructions for Operation of the Polyanalyst unit. Sodium lauryl sulfate gel electrophoresis was run according to Weber and Osborn (1969) as modified by Grula and Savoy (1971).

Detection of Thiobarbituric Acid-Positive Material

After collecting 1 ml samples, 0.1 ml of 2.2 N H_2SO_4 was added and the tubes were placed in a boiling water bath (covered with glass marbles) for 15 minutes. After centrifugation, 0.25 ml was withdrawn and added to 0.25 ml of 0.025 M periodic acid. This solution was heated 25 minutes at 55 C, after which, 0.5 ml of sodium arsenite (2%) was added. The mixture was then vigorously shaken before addition of 2 ml of 0.3% thiobarbituric acid. The resulting solution was boiled 12 minutes and the pink color which is indicative of thiobarbituric acid-positive material (2-keto-3-deoxyoctanate, normally found in the lipopolysaccharide of Gram-negative bacteria, is an example) was read at 532 nm.

CHAPTER III

RESULTS AND DISCUSSION

Effect of Pantoyl Lactone on Uptake of Labeled Compounds

Grula and King (1971) have shown that pantoyl lactone inhibits the ability of Micrococcus lysodeikticus to take up D-alanine, L-phenylalanine, and glycerol. Data presented in Table I demonstrates that pantoyl lactone also inhibits, to varying degrees, the uptake of a variety of compounds in Erwinia species.

Many investigators have studied the transport systems for various amino acids (Wilson and Holden, 1969; Rosen, 1971; Weiner, Furlong, and Heppel, 1971; Gale and Llewellin, 1971), carbohydrates (Fox and Kennedy, 1965; Jones and Kennedy, 1969; Eagon, 1971; Phibbs and Eagon, 1970), and ions (Pardee et al., 1966; Medveczky and Rosenberg, 1969 and 1970). To date, all active and/or facilitated transport processes are believed to involve binding proteins which have varying degrees of specificity for substrate molecules. Generally, binding proteins are released from cells by the cold-osmotic shock procedure without complete loss of "binding" activity. In addition to the need for binding proteins, in many instances, energy must be furnished to "drive" the transport system. Because pantoyl lactone inhibits uptake of all compounds tested, we thought it important to determine if pantoyl lactone inhibits

TABLE I

EFFECT OF PANTOYL LACTONE ON UPTAKE OF VARIOUS COMPOUNDS BY CELLS OF ERWINIA SPECIES

Labeled Compound	Average CPM Taken up/mg Dry Wt. Cells*		Average % Decrease Caused by Pantoyl Lactone**
	Control	Pantoyl Lactone (1%)	
D-Glucose†	17,000	9,500	45
L-Aspartic Acid†	9,000	5,000	45
L-Malic Acid†	19,000	9,000	55
D-Alanine‡	2,000	1,350	30
Thymidine‡	10,500	3,000	70
2-Ketoglutaric Acid‡	2,500	900	55

*The average number is based on at least two experimental determinations made at different times.

**Values calculated from uptake readings made after 4 minutes exposure to the labeled substrate as given in materials and methods

†Cells were grown in the presence of these compounds prior to determination of uptake ability.

‡Cold carrier was not present during the uptake of these compounds.

active transport, passive transport, or both.

Since the active uptake of various carbohydrates is well documented (Eagon, 1971), it was decided to obtain information relevant to the effect of pantoyl lactone on D-glucose (glucose) uptake. To demonstrate active transport, the following four criteria should be satisfied: 1) A specific binding protein for the substrate should be present. 2) An energy requirement must be demonstrable since energy is needed to accumulate a compound against a concentration gradient via "carrier" protein. 3) Active transport should follow saturation kinetics, whereas, linear kinetics will exist during passive influx of a compound. 4) Active uptake requires a carrier molecule the presence or absence of which is inducible.

As can be seen in Figure 1, cold-osmotic shocking of cells inhibits their ability to take up glucose. This is indirect evidence to support the need for a binding protein. The data can be interpreted to mean the cold-osmotic shock treatment causes the release of the glucose binding protein(s) into the medium; therefore, uptake ability is reduced.

Regardless of glucose concentration, 0.03 M 2-4-dinitrophenyl (DNP), an inhibitor of oxidative phosphorylation, drastically reduces uptake (Figure 2). This suggests there is an energy requirement for glucose uptake; thus the type of uptake is probably of the active type. The residual "uptake" in the presence of DNP is probably indicative of binding, rather than uptake. Although DNP inhibits transport, recognition and binding are not inhibited.

As further shown in Figure 2, glucose uptake obeys saturation kinetics which is also indicative of active transport (Eagon, 1971).

Figure 1. Uptake of D-Glucose-U-^{14}C ($10^{-4}M$) by Control and Osmotically-Shocked Cells. ● , untreated control cells; ■ , osmotically-shocked cells.

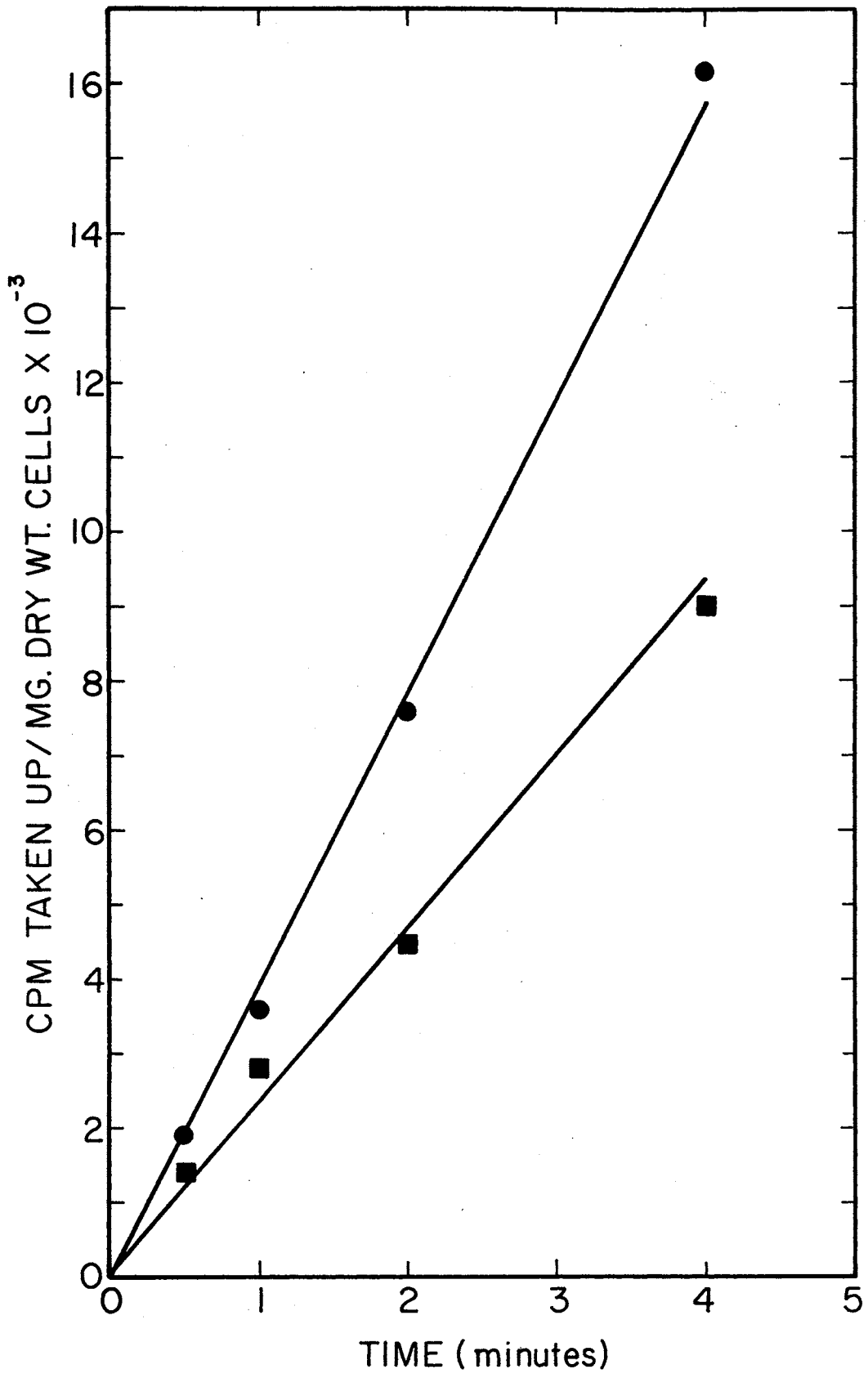
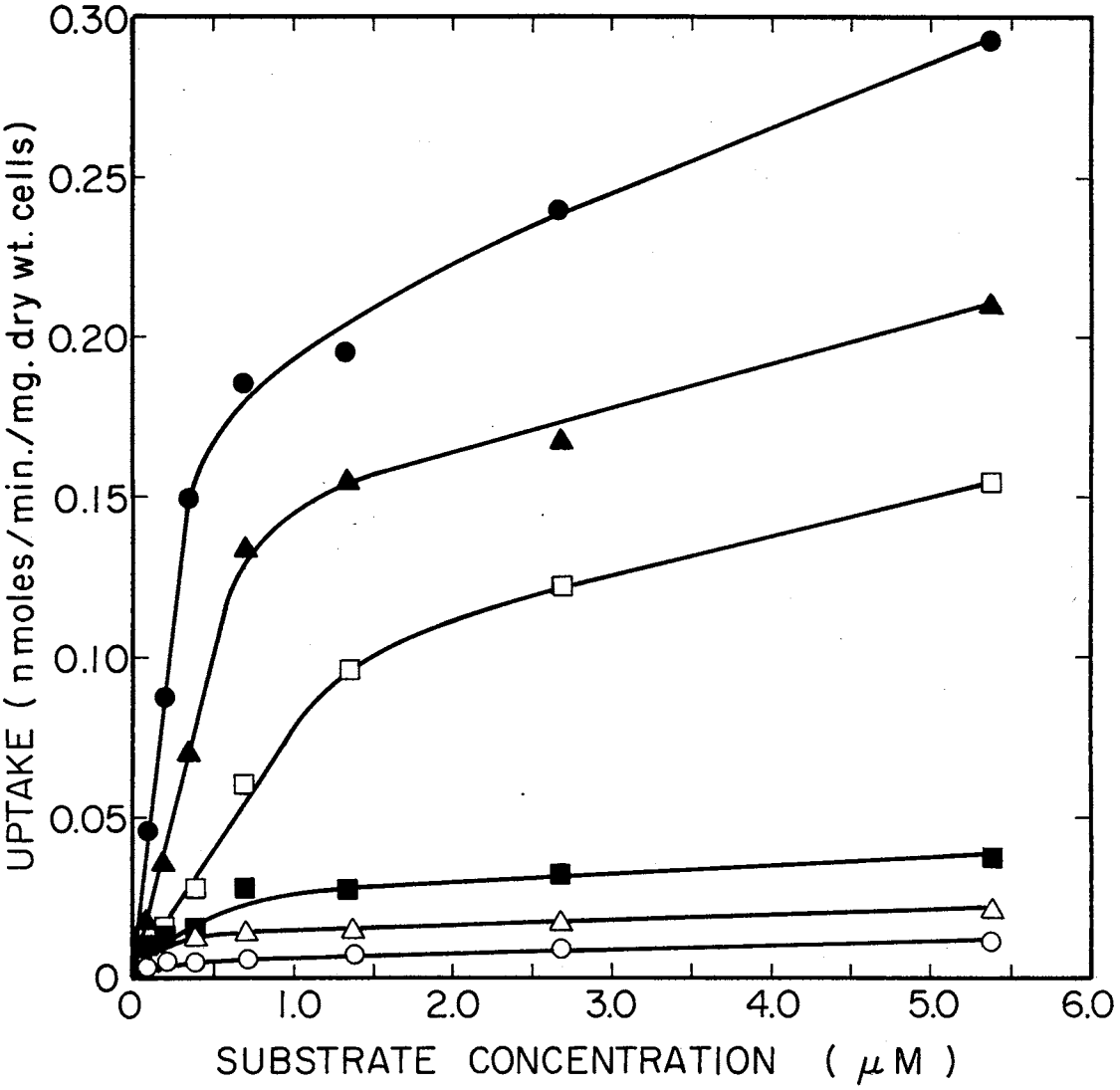

CPM TAKEN UP/MG. DRY WT. CELLS X 10^{-3}
0
2
4
6
8
10
12
14
16
0
1
2
3
4
5
TIME (minutes)

Figure 2. Influence of Pantoyl Lactone and 2-4-Dinitrophenol on Uptake of D-Glucose-U-^{14}C in Erwinia Species. ●, control cells; ▲, with pantoyl lactone ($7.7 \times 10^{-2}M$); □, with 2-4-dinitrophenol ($1.0 \times 10^{-3}M$) at pH 7.0; ■, with 2-4-dinitrophenol ($3.0 \times 10^{-2}M$) at pH 7.0; △, with 2-4-dinitrophenol ($3.0 \times 10^{-2}M$) plus pantoyl lactone; ○, with 2-4-dinitrophenol ($3.0 \times 10^{-2}M$) at pH 4.0.


0.30
0.25
0.20
0.15
0.10
0.05
0
UPTAKE (n moles/min./mg. dry wt. cells)
0
1.0
2.0
3.0
4.0
5.0
6.0
SUBSTRATE CONCENTRATION (μM)

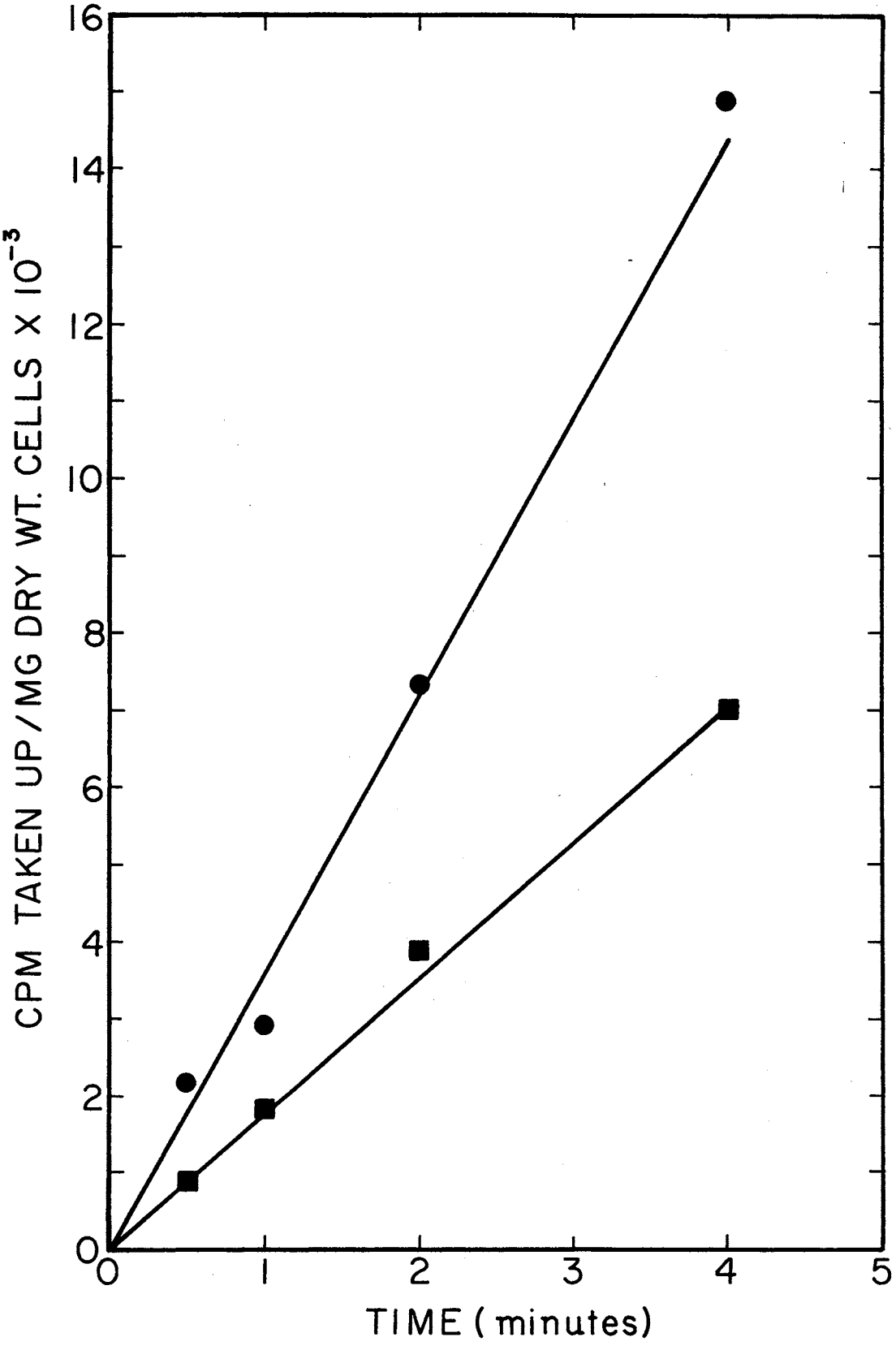
In these experiments, cells were exposed to the label for short periods of time (30 seconds). This ensures that the data do not reflect depletion of substrate at the lower concentrations. At higher concentrations, where the saturation phenomenon is expressed, the short exposure time makes it less likely for a labeled product to be excreted back into the medium.

The uptake of glucose by glucose-grown cells, compared to aspartic-grown cells, demonstrates the inducible nature of the glucose uptake "permease" system (Figure 3). Although not shown, aspartic acid permease is also inducible. Considering all these data, it appears highly likely that glucose is taken up via an active transport process.

At the level of 1% (7.7×10^{-2}M), pantoyl lactone inhibits the uptake of glucose regardless of concentration of glucose utilized. Both initial rate and level necessary for substrate saturation are lowered (Figure 2). Because data obtained using pantoyl lactone and DNP (1.0×10^{-3}M) are similar (Figure 2), it could indicate that pantoyl lactone exerts its effects at some point in the energy producing process. Such a conclusion would be in agreement with the observation that pantoyl lactone inhibits growth of the organism and exerts a primary effect on the cell membrane. It has been reported however (Grula and Grula, 1962), that cell division requires energy and pantoyl lactone is a potent stimulator of cell division activity.

When pantoyl lactone is added to the uptake system in the presence of DNP, there is an additional reduction in "uptake" activity (Figure 2). Actually, as mentioned earlier, this probably represents an inhibition in the ability of the substrate to bind to the binding protein, rather than a true inhibition of uptake. Such data could imply that

Figure 3. Uptake of D-Glucose-U-^{14}C (10^{-4}M) by Glucose- or Aspartic Acid-Grown Cells. ● , glucose-grown cells; ■ , aspartic acid-grown cells.

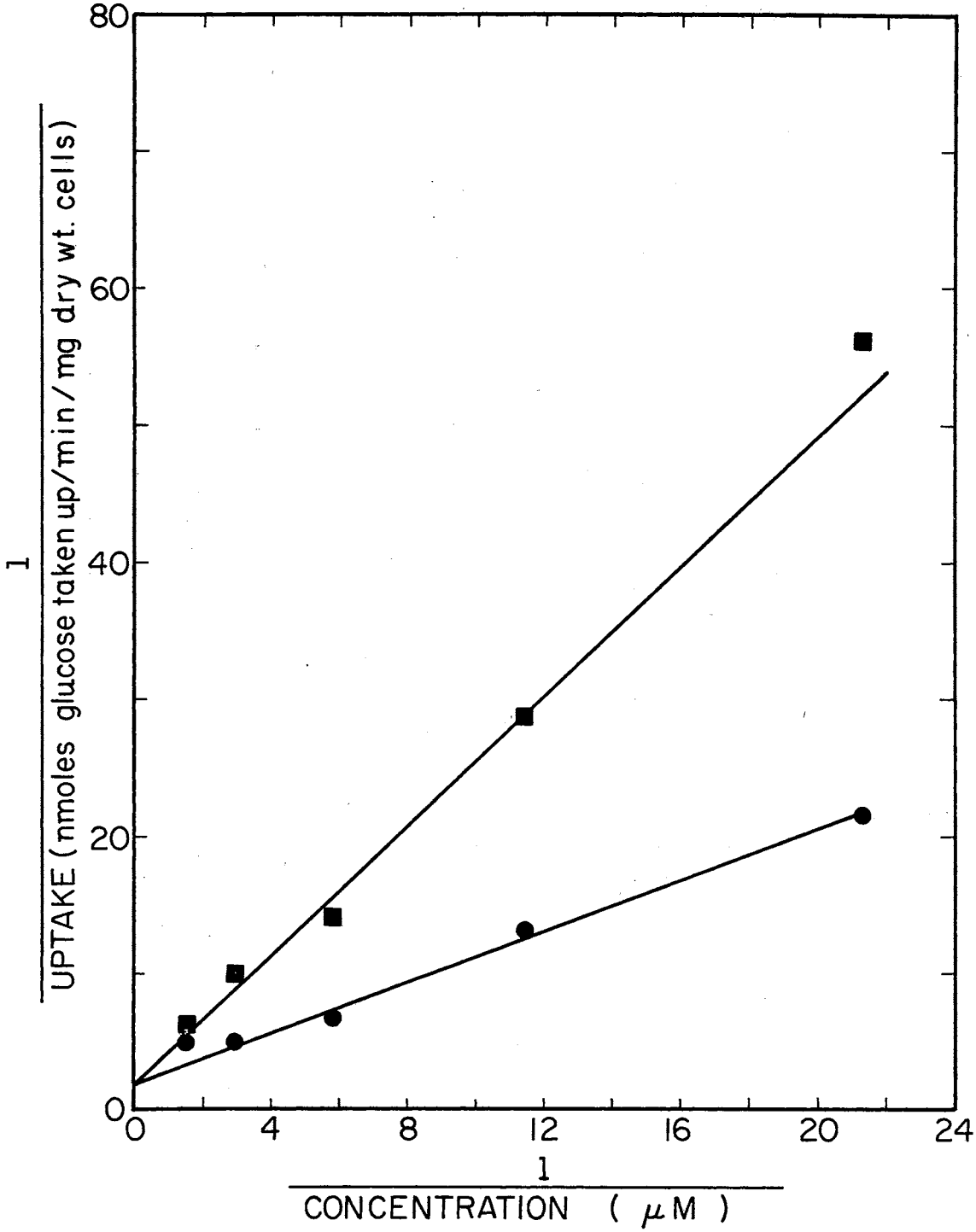

16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
CPM TAKEN UP / MG DRY WT. CELLS X 10^{-3}
TIME (minutes)

pantoyl lactone competes with substrate for the active (binding) site of the binding protein. This view is somewhat strengthened by the finding that acidic conditions (pH 4.0) also inhibit "binding" in the presence of DNP (Figure 2). This suggests that proper linkages between binding protein and substrate cannot occur at the low pH, due perhaps to an acid induced conformational change in the binding protein.

Kinetics resembling competitive inhibition are observed when Lineweaver-Burke plots of pantoyl lactone-caused inhibition are constructed (Figure 4). It does not seem possible, however, that pantoyl lactone can compete with the wide variety of compounds tested for their specific binding sites. Therefore, since competitive type inhibition implies competition for the same form of enzyme (free enzyme or enzyme complex) we feel a structural change of the cell membrane might give rise to these type kinetics. Pantoyl lactone could cause a structural change which would, by a folding in (involution) of portions of the membrane, "smother" or hide some of the binding molecules. Alternatively, pantoyl lactone could directly affect the binding proteins, causing them to undergo a conformational change, resulting in loss of binding activity. Either of these conditions could effectively inhibit functionality of a certain percentage of the binding sites, or reduce their affinity for the substrate, thereby giving a type of pseudo-competitive kinetics.

Since pantoyl lactone inhibits active uptake of glucose, possibly by some membrane alteration, we thought pantoyl lactone might also inhibit uptake of molecules that enter the cell via passive diffusion. Preliminary experiments revealed that neither thymine nor thymidine are taken up readily by our cells or other type cells (Kammen, 1967). To

Figure 4. Lineweaver-Burke Plot of D-Glucose-U-^{14}C Uptake in the Presence of Pantoyl Lactone. ● , control; ■ , plus pantoyl lactone (7.7×10^{-2} M).


80
60
40
20
0
0
4
8
12
16
20
24
1
UPTAKE (nmoles glucose taken up/min/mg dry wt. cells)
1
CONCENTRATION (μM)

obtain significant uptake of labeled thymidine, cold carrier could not be added and much higher concentrations of labeled compound were used. The linear kinetics of thymidine uptake, shown in Figure 5, are indicative of passive diffusion (Eagon, 1971). As can be seen, pantoyl lactone also inhibits uptake of compounds which apparently enter by passive diffusion. Although not shown, similar results were obtained using other compounds that appear to be taken up via passive diffusion in uninduced cells (2-ketoglutaric and malic acid).

When cells are pre-treated with pantoyl lactone, washed, and then tested for uptake ability, in the absence of pantoyl lactone, transport activity is completely restored (Figure 6). These data strongly suggest the inhibitory action of pantoyl lactone on uptake activity is most probably a surface related phenomenon which causes "moderate" but reversible alterations.

When cells are grown in the presence of pantoyl lactone washed and exposed to substrates in the absence of pantoyl lactone, uptake data similar to those shown in Figure 7 are obtained. As shown, such cells exhibit a significantly higher ability to take up glucose.

Scarborough (1970) reported glucose uptake by Neurospora crassa changes from a facilitated to an active uptake mechanism when cells are grown at low glucose concentrations. We found that cells of Erwinia species, when grown for 17½ hours at low glucose concentration (5 mg/100 ml), also exhibit an increased ability to take up glucose (Figure 8). As shown, the increased uptake does not appear to be the result of using cells that are physiologically younger (17½ vs 12 hour cells). The increased synthesis under "starvation" conditions (low level of substrate or presence of pantoyl lactone) may reflect a lack

Figure 5. Uptake of Thymidine-2-^{14}C by Erwinia Species in the Presence of Pantoyl Lactone. ● , control; ■ , plus pantoyl lactone (7.7 x 10^{-2}M).

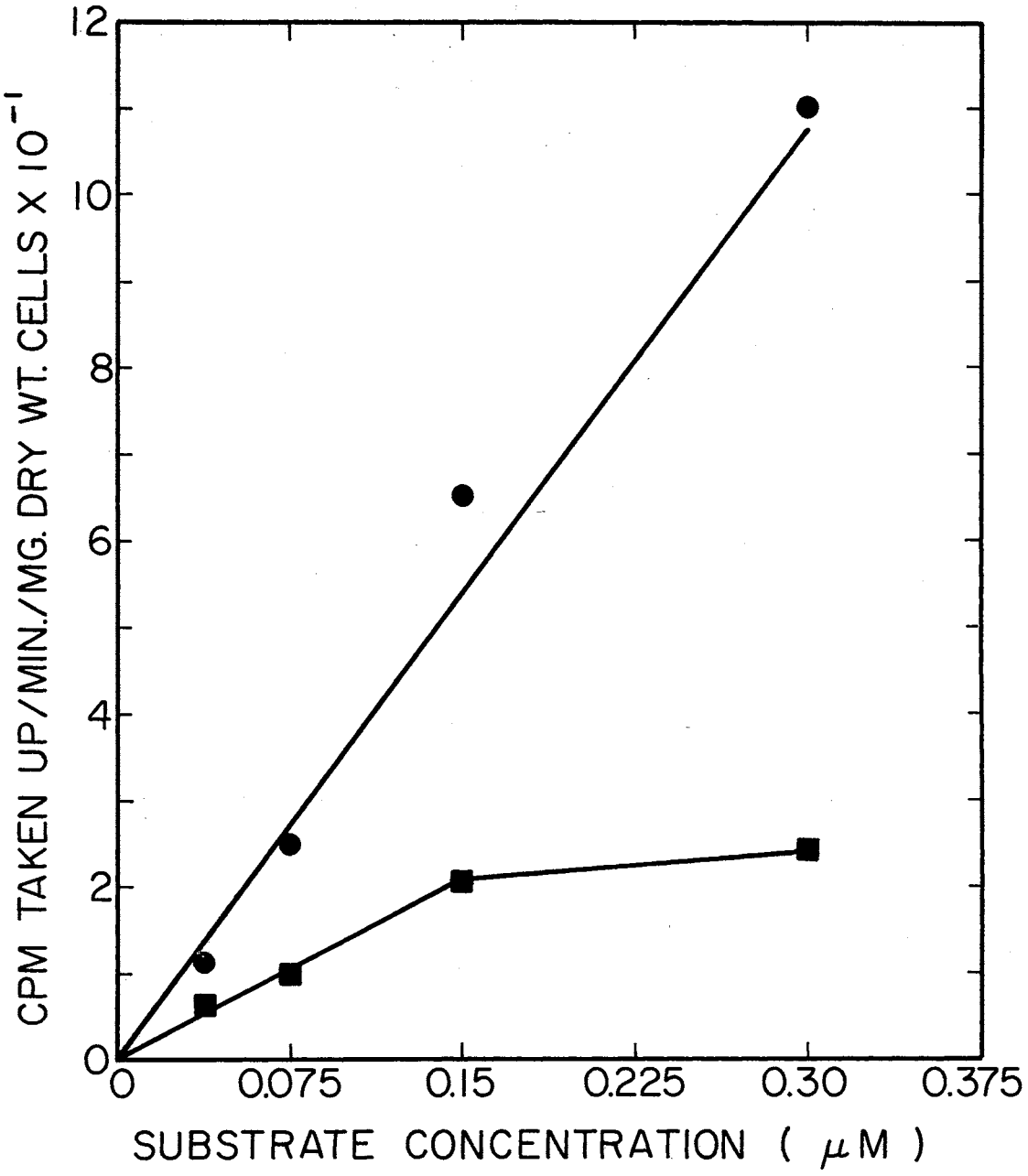

12
10
8
6
4
2
0
CPM TAKEN UP/MIN./MG. DRY WT. CELLS X 10^{-1}
0
0.075
0.15
0.225
0.30
0.375
SUBSTRATE CONCENTRATION (μM)

Figure 6. Reversal of the Inhibition of Glucose Uptake Activity Caused by Pantoyl Lactone. ● , control using 10^{-4}M D-glucose-U-^{14}C, ■ , plus pantoyl lactone (7.7×10^{-2}M); □,cells pre-treated (5 minutes) with pantoyl lactone (7.7×10^{-2}M) and washed two times in mineral uptake solution prior to determination of uptake ability.

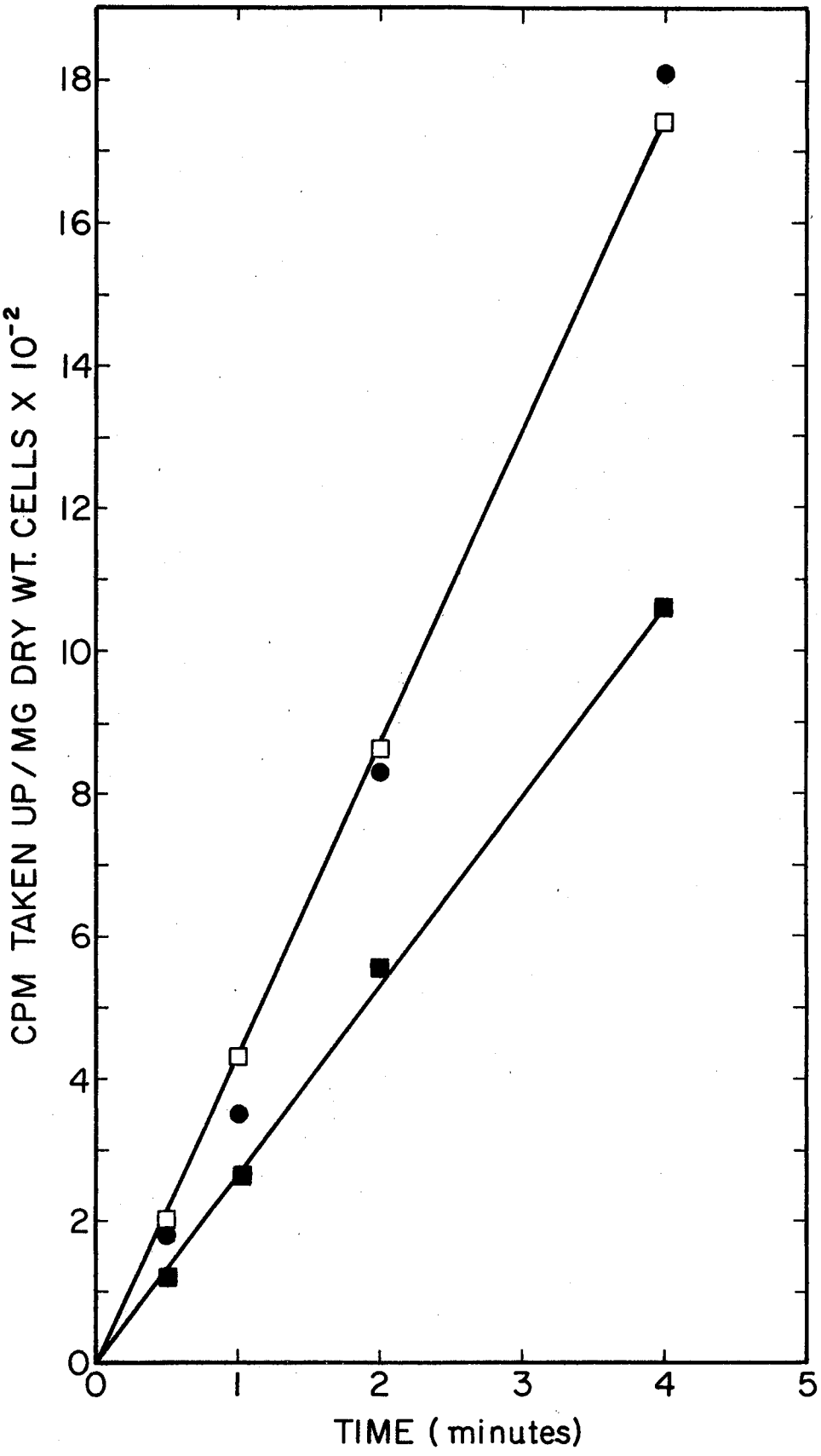

CPM TAKEN UP / MG DRY WT. CELLS X 10^{-2}
0
2
4
6
8
10
12
14
16
18
0
1
2
3
4
5
TIME (minutes)

Figure 7. Uptake of D-Glucose-U-^{14}C ($10^{-4}M$) by Cells Grown in the Presence and Absence of Pantoyl Lactone. ●, control; ■, pantoyl lactone ($7.7 \times 10^{-2}M$) present (4½ hours) during growth.

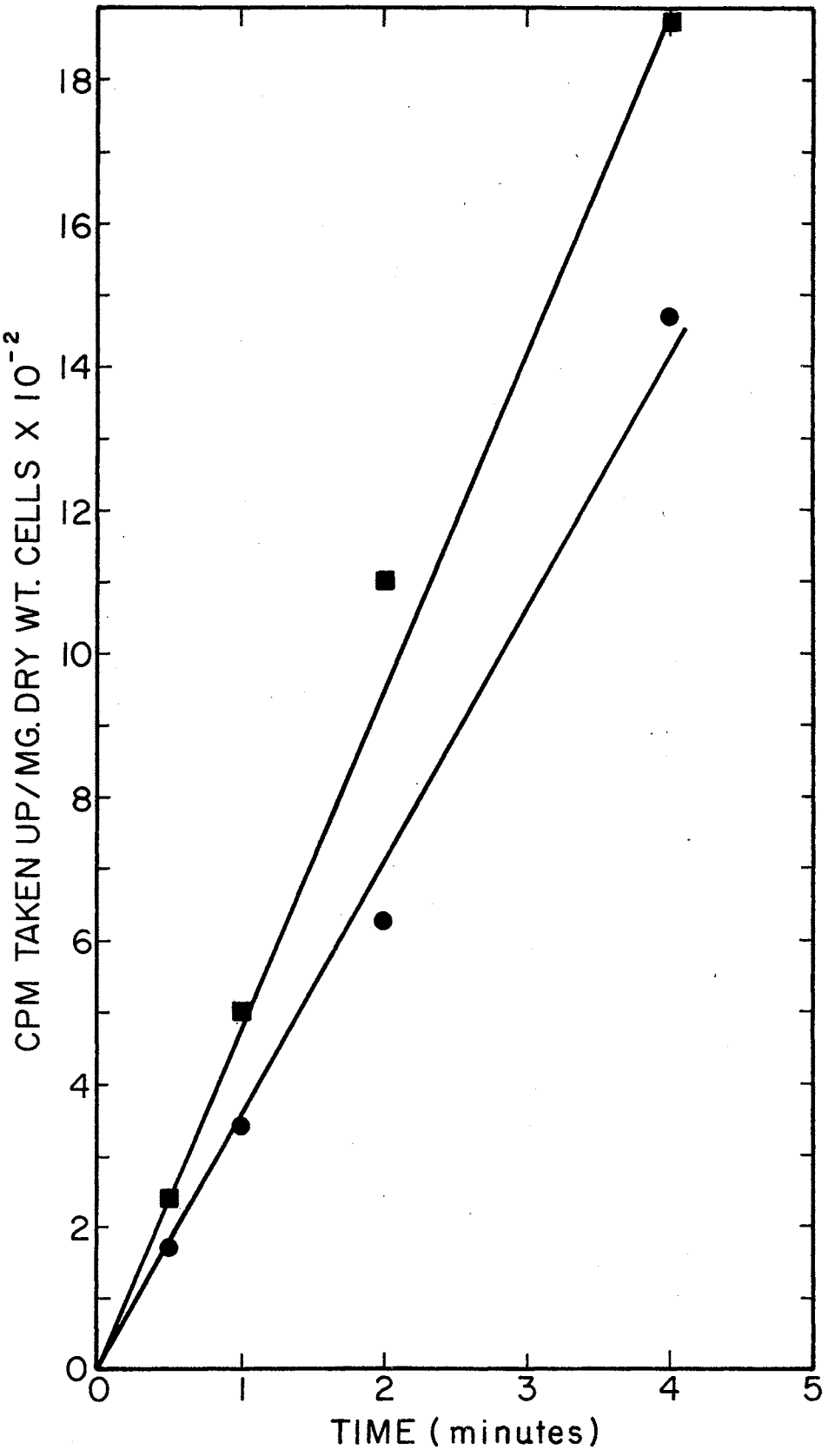
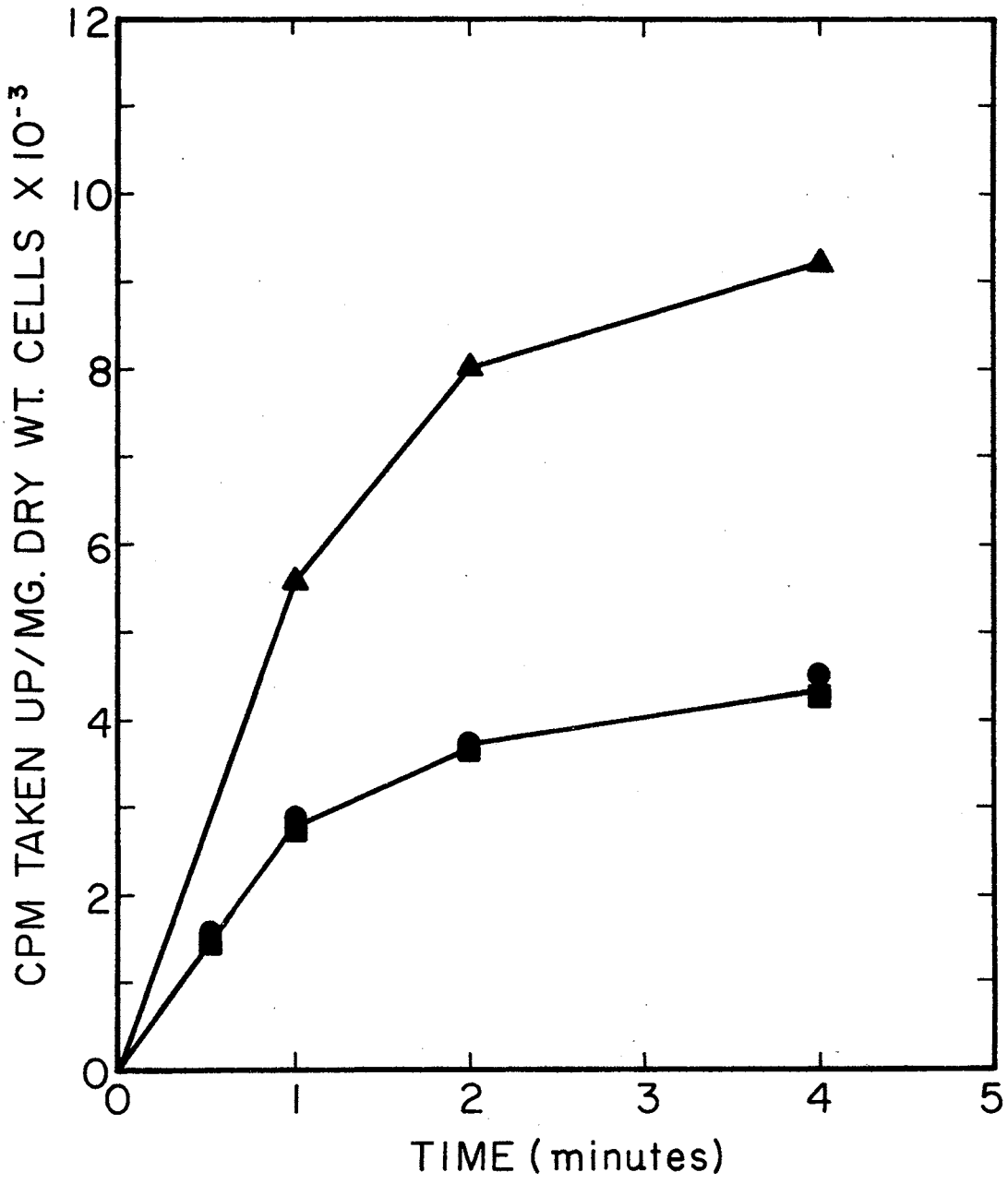

CPM TAKEN UP/MG. DRY WT. CELLS X 10^{-2}
0
2
4
6
8
10
12
14
16
18
0
1
2
3
4
5
TIME (minutes)

Figure 8. Uptake of D-Glucose-U-^{14}C ($10^{-5}M$) by "Young", "Old", and "Starved" Cells. ● , 17½ hour cells; ■ , 12½ hour cells; ▲, 17½ hour cells grown in "limited" (5 mg/100 ml medium) glucose concentration (normal level employed is 250 mg/100 ml medium).


CPM TAKEN UP/MG. DRY WT. CELLS X 10⁻³
12
10
8
6
4
2
0
0
1
2
3
4
5
TIME (minutes)

of glucose repression due to the low level of glucose entering the cells. This seeming "repression-derepression" appears to regulate uptake of other compounds since either pantoyl lactone or "limited" substrate (7 vs 280 mg/100 ml) during growth also gives rise to increased transport ability for aspartic acid.

Some organisms can take up certain substrates via more than one transport system. Leucine can be taken up by a leucine specific permease (Furlong and Weiner, 1970) or by a leucine-isoleucine-valine transport system (Anraku, 1968). Similarly, lysine is transported either by a lysine specific or a lysine-ornithine-arginine (LOA) transport system (Rosen, 1971). Rosen postulated that the LOA system is a transport system for arginine precursors and it is regulated by repression of the permease and by inhibition of the carrier by exogenous arginine. Our data suggest that cells of Erwinia species may possess more than one transport system for uptake of both glucose and aspartic acid. Also, these suspected transport systems may be regulated (repressed or derepressed) by substrate concentration.

It is quite apparent that pantoyl lactone changes the physical properties of aqueous solutions. When making slides of cultures containing pantoyl lactone, the droplet containing the bacteria has a tendency to glide about on the slide but does not spread out or stay in a spread condition as does water. It seems possible that some or all of the effects of pantoyl lactone might be the result of changes in the physical properties of the growth medium. We, therefore, decided to check the surface tension of growth media in the presence and absence of pantoyl lactone.

As can be seen in Figure 9, pantoyl lactone causes a decrease in surface tension. Included in Figure 9 are data relevant to decrease in surface tension caused by an anionic detergent (sodium lauryl sulfate) and a non-ionic detergent (Tween 80). Compared to the detergents, much higher concentrations of pantoyl lactone are required to cause equivalent reductions in surface tension.

Although sodium lauryl sulfate (SLS) is capable of reducing surface tension to a greater extent than pantoyl lactone, pantoyl lactone can rather readily reduce surface tension to a level comparable to Tween 80. Using data presented in Figure 9, levels of SLS and Tween 80 required to lower surface tension to approximately 55 dynes/cm were determined. This surface tension was chosen becuase it is equivalent to that found to exist in a 1% solution of pantoyl lactone and is the preferred level used to prevent D-serine-induced division inhibition.

To ensure the decreased uptake ability caused by pantoyl lactone was not the result of lowered surface tension, uptake of glucose was studied in the presence of SLS as well as Tween 80. As can be seen in Figure 10, neither of these two detergents, at surface tension values equivalent to that caused by a 1% solution of pantoyl lactone, inhibits uptake of glucose (uptake is actually stimulated by Tween 80). Therefore, it is apparent that inhibition of uptake caused by pantoyl lactone is not due to a decrease in surface tension.

Because uptake is a membrane-associated phenomenon, inhibition of uptake ability by pantoyl lactone probably involves a reversible structural or conformational change in or on the cell membrane. This change could involve some type of physical alteration of lipids or proteins or both; however, pantoyl lactone could also affect the outer wall

Figure 9. Reduction in Surface Tension Caused by Sodium Lauryl Sulfate (SLS), Tween 80, or Pantoyl Lactone. ● , SLS; ■ , Tween 80; ▲ , Pantoyl Lactone.

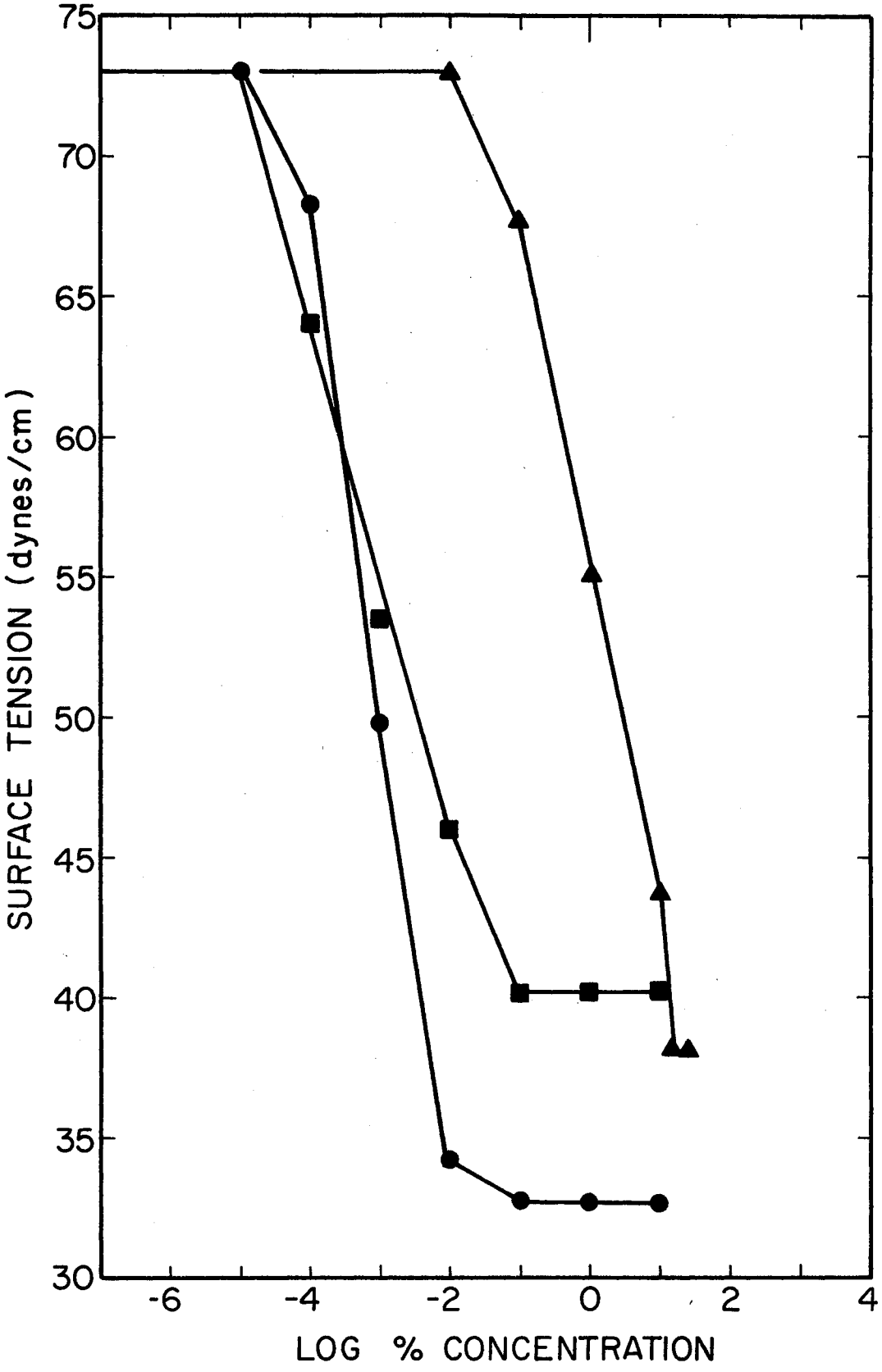
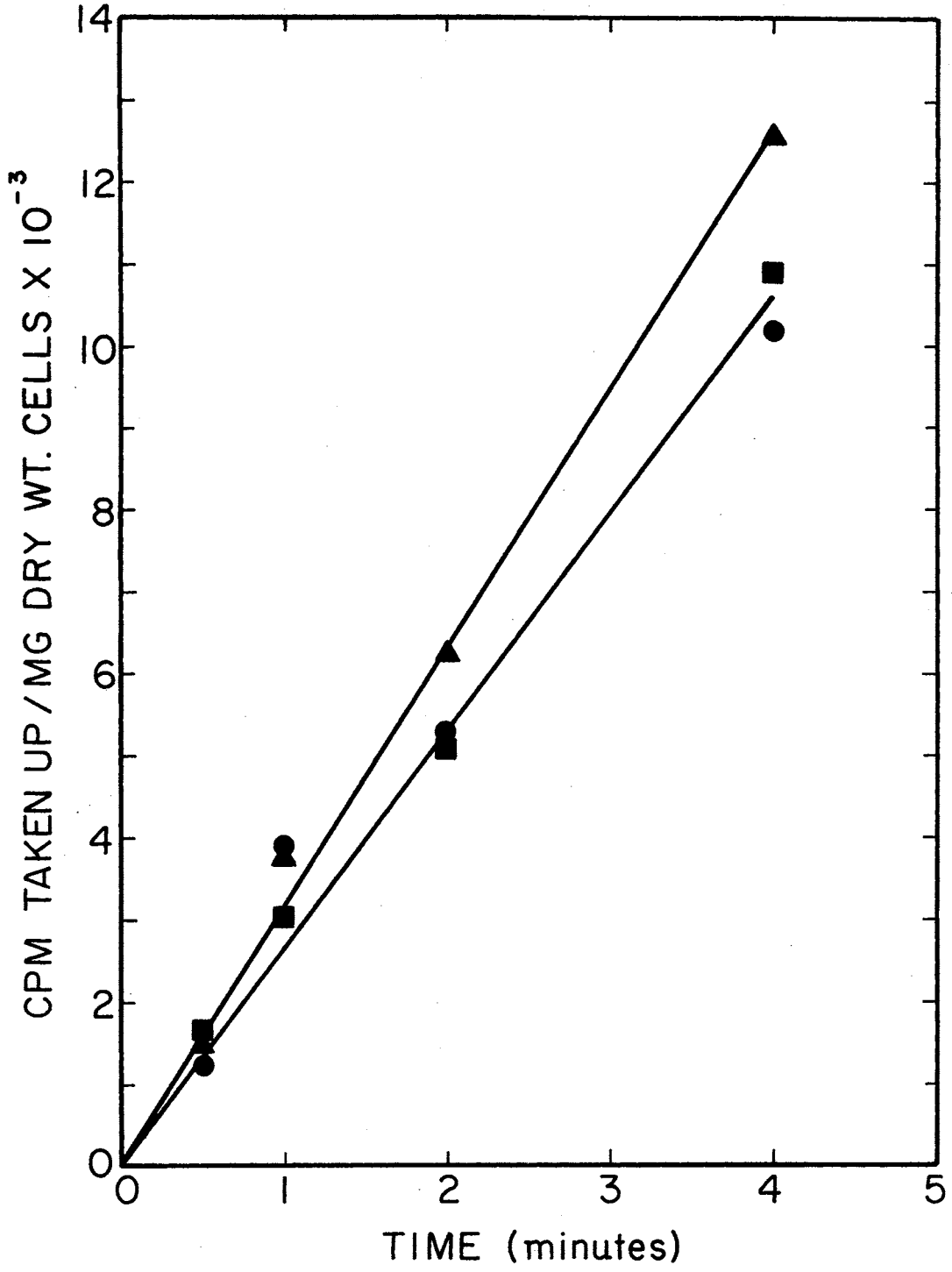

75
70
65
60
55
50
45
40
35
30
SURFACE TENSION (dynes/cm)
-6
-4
-2
0
2
4
LOG % CONCENTRATION

Figure 10. Uptake of D-Glucose-U-^{14}C ($10^{-4}M$) in the Presence of SLS or Tween 80. ● , control; ■ , plus SLS (0.0008%); ▲ , plus Tween 80 (0.001%).


CPM TAKEN UP / MG DRY WT. CELLS X 10^{-3}
14
12
10
8
6
4
2
0
0
1
2
3
4
5
TIME (minutes)

layers (outer membrane). To aid in determining whether or not the outer lipopolysaccharide (LPS) membrane is being affected by pantoyl lactone, the following experiment was done.

Leive (1965) has demonstrated that entry of Actnomycin D into Escherichia coli depends on structural integrity of the outer cell membrane. This can be readily demonstrated by pre-treating cells with mixtures containing ethylenediamine tetra-acetic acid (EDTA) which causes removal of at least one-half of the LPS thus permitting entry of Actinomycin D.

As shown in Figure 11, growth of Erwinia species is significantly inhibited by Actinomycin D at a concentration of 20 ug/ml. Although addition of pantoyl lactone causes a further inhibition in growth, the effect seems to be additive; therefore, it appears that pantoyl lactone does not affect the outer membrane. To further determine possible alteration of LPS by pantoyl lactone, cells were pre-treated with pantoyl lactone; then grown in the presence and absence of Actinomycin D. As shown in Figure 12, such pre-treatment does not alter growth response to Actinomycin D.

Evidence for Membrane Alterations

Infrared Analysis

Infrared spectra were obtained using envelopes isolated from control, division-inhibited, and pantoyl lactone reversed cells of Erwinia species to determine presence or absence of conformational changes in envelope proteins of the different type cells.

Figure 11. Effect of Pantoyl Lactone and Actinomycin D on Growth of Erwinia Species. ●, control; ○, plus pantoyl lactone (7.7×10^{-2}M); ■, plus Actinomycin D (20 ug/ml); □, plus pantoyl lactone and Actinomycin D.

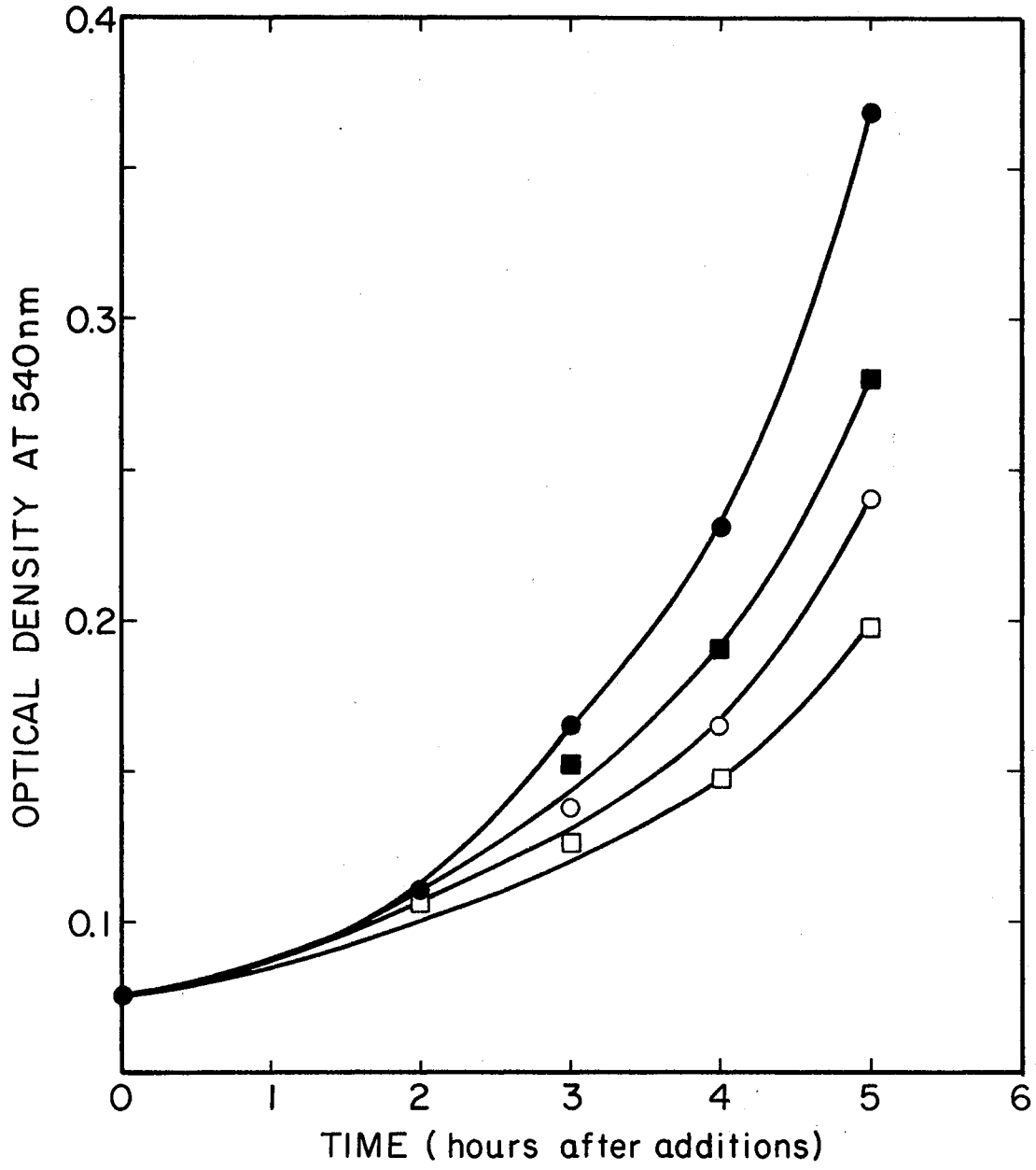
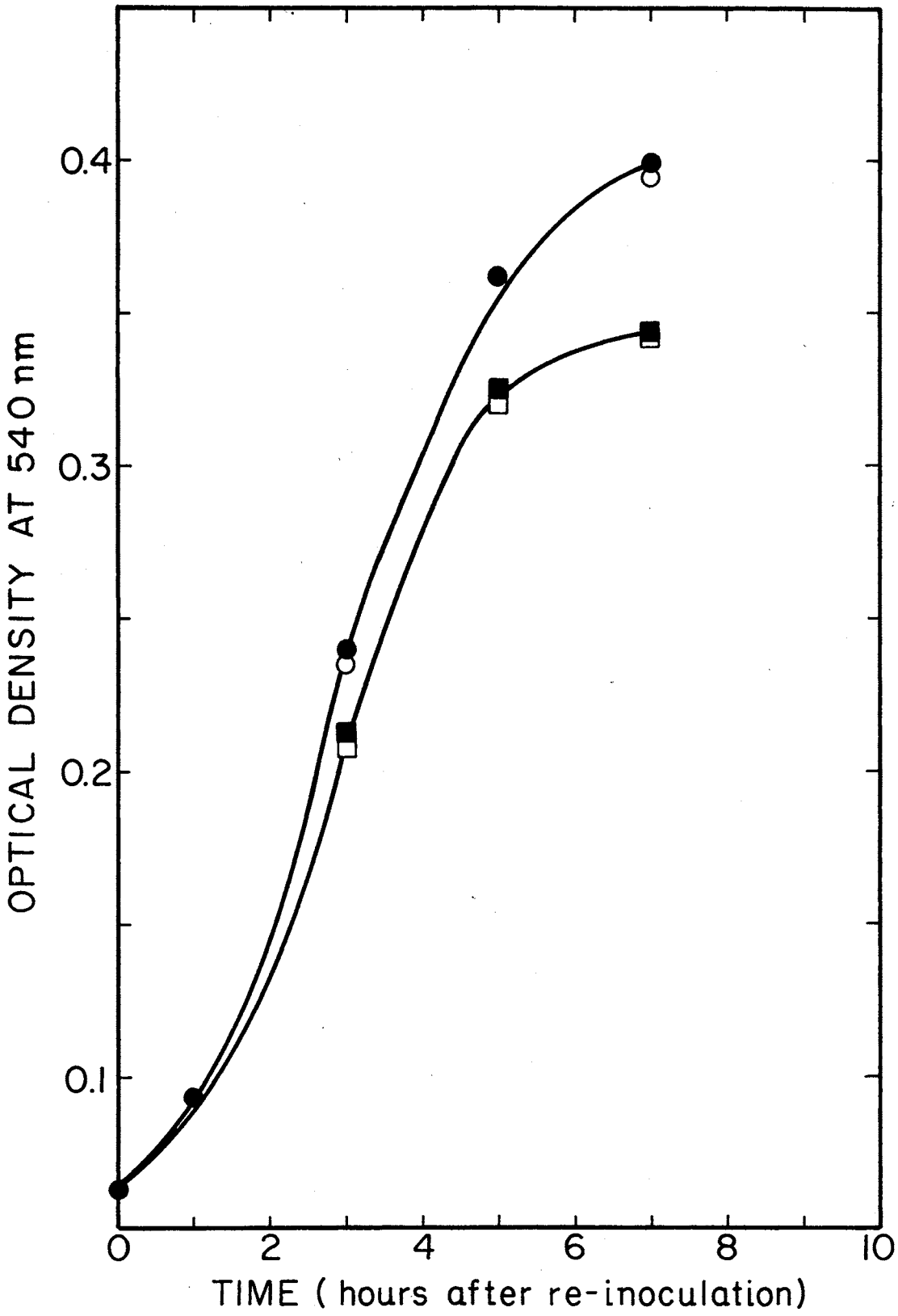

0.4
0.3
0.2
0.1
OPTICAL DENSITY AT 540nm
0
1
2
3
4
5
6
TIME (hours after additions)

Figure 12. Growth of Erwinia Species in the Presence and Absence of Actinomycin D After Pre-treatment (5 minutes) in Pantoyl Lactone. ●, control cells; ○, pantoyl lactone (7.7×10^{-2}M) pre-treated cells; ■, plus Actinomycin D (10 ug/ml); □, pantoyl lactone pre-treated cells plus Actinomycin D.


OPTICAL DENSITY AT 540 nm
0.4
0.3
0.2
0.1
0
2
4
6
8
10
TIME (hours after re-inoculation)

Green and Salton (1970), using membranes isolated from Micrococcus lysodeikticus, reported absorption maxima in the infrared for the Amide I and II bands of the proteins, as well as the areas for vibrations from the OH, C-H, C=O, P=O, C-O-C, and P-O-C groups. They reported that proteins from isolated membranes contain more alpha-helical and/or "un-ordered" than beta-conformational (anti-parallel pleated sheet) structure.

Grula and King (1971) confirmed the findings of Green and Salton. They were also able to show that membranes isolated from division-inhibited cells of M. lysodeikticus (grown in the presence of D-cycloserine), contain increased amounts or portions of proteins in the beta-conformational state. In addition, prevention of division inhibition by pantoyl lactone is associated with a decrease in the observed "shift".

As shown in Figure 13, the infrared spectra obtained using isolated envelope material from control and division-inhibited cells of Erwinia species are nearly identical. Because these data are not in agreement with those of Grula and King, we thought additional control preparations should be analyzed. Also, in order to obtain semi-quantitative measurements, peak height ratios were calculated according to Grula and King (1971).

These data are given in Table II and further demonstrate the close similarities in spectra obtained using envelopes from control and division inhibited cells. An increase in either the 1630/1655 or the 1700/1655 ratio indicates a relative increase in the beta-conformational structure. As shown there appear to be no significant differences in these ratios. The slight decrease in the 1735/1655

Figure 13. Infrared Spectra of Envelopes From Dividing and Non-Dividing Cells of Erwinia. Solid line (——), envelope from normally dividing cells; dashed line (-----), envelope from non-dividing cells.

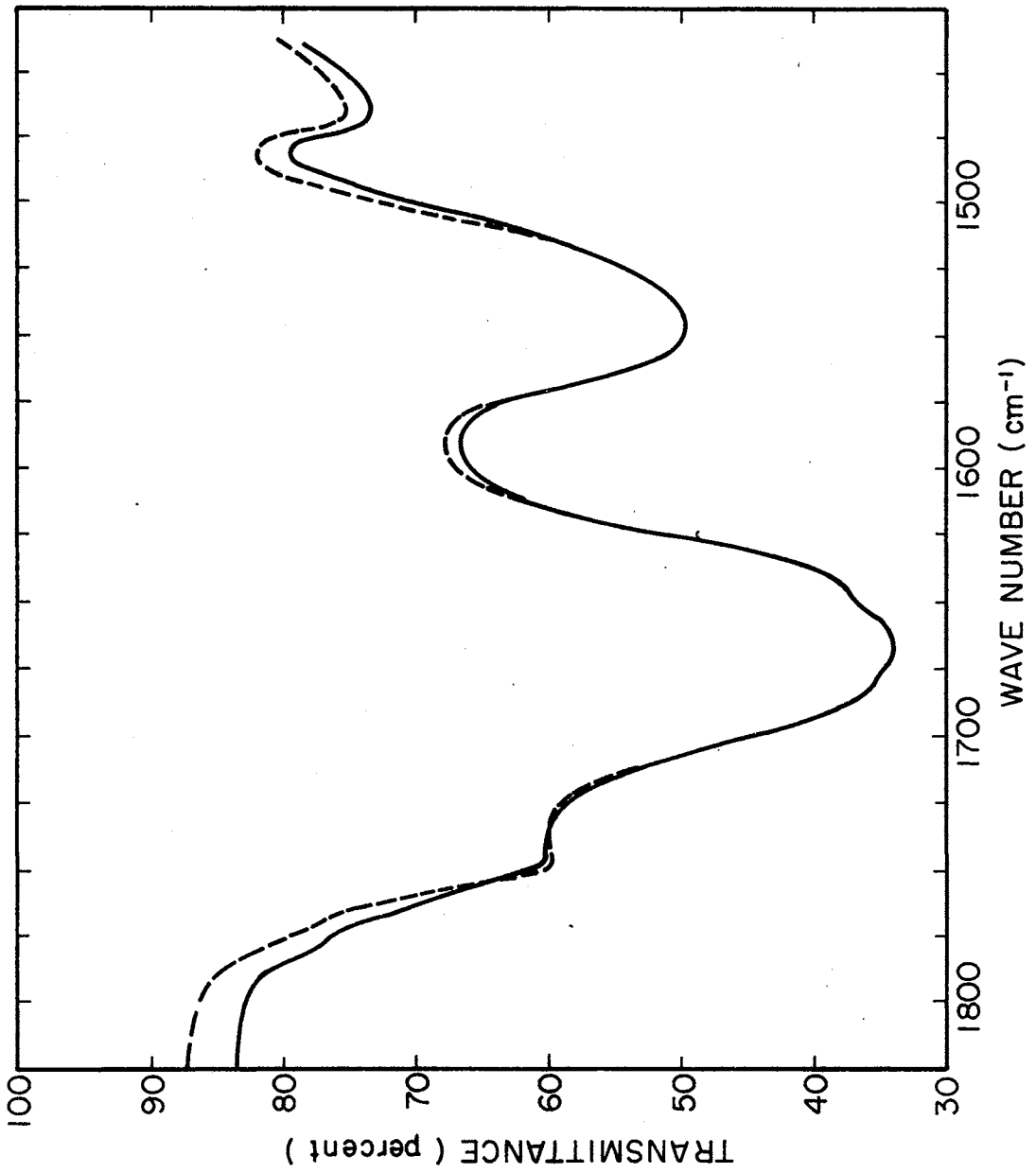

TRANSMITTANCE (percent)
100
90
80
70
60
50
40
30
1800
1700
1600
1500
WAVE NUMBER (cm⁻¹)

TABLE II

RATIOS OF PEAK HEIGHTS CALCULATED USING INFRARED SPECTRA OF ENVELOPES FROM DIVIDING, NON-DIVIDING, AND PANTOYL LACTONE-REVERSED CELLS OF ERWINIA SPECIES

Growth Conditions	Ratios of Peak Heights (cm^{-1})			
	$\frac{1630}{1655}$	$\frac{1700}{1655}$	$\frac{1735}{1655}$	$\frac{1700}{1735}$
Basal Medium*# (pH 6.0)	0.8072	0.7226	0.4395	1.6413
+ D-Serine Beta-Alanine*# (pH 6.2)	0.8051	0.7192	0.4122	1.7550
+ D-Serine Beta Alanine Pantoyl Lactone*	0.7930	0.7299	0.4427	1.7350
Basal Medium** (pH 4.5)	0.8053	1.0338	0.6779	1.5251
+ D-Serine Beta Alanine** (pH 5.5)	0.7836	1.0142	0.5998	1.6907

*Cells harvested after 17½ hours incubation.
**Cells harvested after 21 hours incubation.
#These values represent the average values from six different envelope preparations.

ratio found in division inhibited cells suggests a decreased amount of lipid; however, because I could not show any significant quantitative changes in lipids from the envelopes of these two types of cells, I think this change, as well as the 1700/1735 change, falls within experimental error for this type of determination. Also, as can be seen, presence of pantoyl lactone does not alter the ratios to any significant extent.

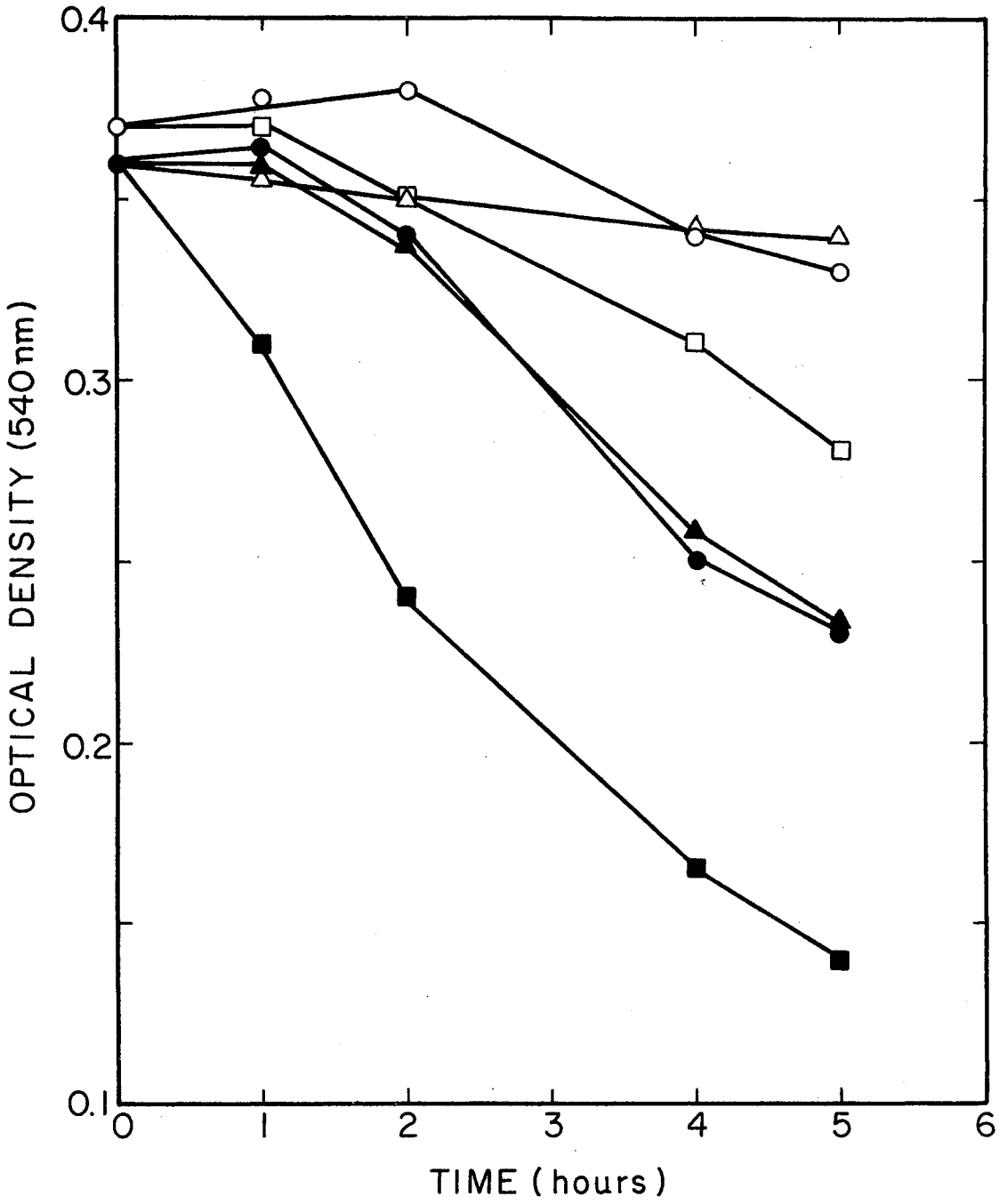
Although the large increases found in both the 1630/1655 and 1700/1655 ratios obtained using membranes from division inhibited cells of M. lysodeikticus may be a direct manifestation of division inhibition, they may also reflect extreme ageing or death of these cells. Division inhibition in this organism cannot be reversed; therefore, it can be argued that such cells have progressed beyond some irreversible step(s) which leads ultimately to cellular death. If this is true, alterations in membrane proteins in M. lysodeikticus may only be casually related to inhibition of cell division. To determine if a possible relationship to ageing could be established in Erwinia species, both control and division-inhibited cells were allowed to grow "old" (21 hour cultures) prior to analysis. As shown in Table II, there are large increases in the 1700/1655 and 1735/1655 ratios when envelopes from such aged cells are analyzed. Therefore, it appears that shifts from alpha-helical or "un-ordered" to the beta-sheet conformational state in membrane proteins may be related more to cell ageing than inhibition of division particularly since the aged cells of Erwinia species are not dead. The relatively high acidity of the 21 hour spent growth media may be a contributing factor to the changed conformational states observed.

It is technically impossible to make infrared analysis of envelope material in the presence of pantoyl lactone using the procedure I employed. Infrared spectra are highly distorted if any moisture is present and pantoyl lactone is a very hygroscopic substance. When potassium bromide pellets of envelopes were pressed directly in the presence of pantoyl lactone, the result was a "gooey" mess. Envelopes which had been pre-treated with pantoyl lactone and washed before lyophilization (for drying) give spectra similar to untreated control envelopes. Such data indicate that pantoyl lactone does not bring about conformational changes in envelope proteins; however, it seems reasonable that the complex chemical nature of the envelope from Gram-negative cells could "mask" any changes thereby making them undetectable. Also since the inhibitory effect of pantoyl lactone on uptake activity is readily removed after washing, it is necessary to assume that other changes induced by pantoyl lactone probably also return to normal after such washing.

SLS Lysis of Erwinia Species in the Presence and Absence of Pantoyl Lactone

To further study effects of pantoyl lactone at the cell periphery, resting cells were permitted to undergo lysis in the presence and absence of SLS and pantoyl lactone. As shown in Figure 14, filamentous cells are more susceptible to lysis by SLS than control cells. It was thought that pantoyl lactone would inhibit SLS-induced lysis by altering the wall portion of the envelope or by keeping SLS away from the cell membrane. As shown however, SLS-induced lysis proceeds more rapidly and to a greater extent in the presence of pantoyl lactone.

Figure 14. SLS Lysis of Normal and Filamentous Cell Forms of Erwinia Species in the Presence of Pantoyl Lactone. ○, control cells in SLS (0.01%); ●, filamentous cells in SLS; △, filamentous cells in pantoyl lactone (0.077 M); □, control cells in pantoyl lactone and SLS; ■, filamentous cells in pantoyl lactone and SLS; ▲, pantoyl lactone pre-treated filamentous cells in SLS.


0.4
0.3
0.2
0.1
OPTICAL DENSITY (540nm)
0
1
2
3
4
5
6
TIME (hours)

Because SLS is thought to act by disrupting hydrophobic bonding in cell membranes, these data indicate that pantoyl lactone either aids SLS in getting to the membrane or the membrane is altered by pantoyl lactone exposing critical hydrophobic areas to SLS.

If pantoyl lactone aids SLS to penetrate the outer wall layers, then pantoyl lactone should enhance susceptibility of cells to Actinomycin D. As shown earlier, pantoyl lactone neither enhances nor inhibits entry of Actinomycin D. Also if pantoyl lactone alters the wall layers in a significant and lasting manner then enhanced lysis should occur in filaments pre-treated with pantoyl lactone. As also shown in Figure 14, lysis profiles are the same for both non-treated and pre-treated cells.

Although data are not shown, pantoyl lactone also aids SLS-solubilization of isolated envelopes. Theoretically, the membrane is directly exposed to SLS in envelope preparations; therefore, more rapid solubilization in such isolated structures indicates that pantoyl lactone does not aid SLS to penetrate to the membrane but instead directly alters the membrane allowing for faster solubilization. Further, since filaments have a "porous" wall (Grula and Hopfer, 1972), SLS should have little difficulty in penetrating the outer wall layers. It may also be pointed out that addition of pantoyl lactone to SLS-containing solutions (0.01%) does not result in a further lowering of surface tension.

Effect of Pantoyl Lactone on Horse Erythrocytes

Because erythrocytes have no wall or structure external to the membrane, we thought any surface effects of pantoyl lactone on such

cells should be more readily amenable to direct interpretations. We observed that three distinct effects by pantoyl lactone can be demonstrated. These involve crenation, agglutination, and osmotic protection.

Examination using dark field microscopy reveals that pantoyl lactone (0.077 M in 0.146 M NaCl) causes horse erythrocytes to undergo almost immediate crenation. High concentrations of pantoyl lactone (0.77 M or greater) cause very rapid lysis of erythrocytes. Tween 80 does not lyse red cells at surface tension values below those wherein pantoyl lactone-induced lysis of erythrocytes occurs. Therefore, lysis by pantoyl lactone must entail more than a reduction in surface tension.

Generally the crenation phenomenon is caused by amphipathic molecules (compounds having both hydrophobic and hydrophilic properties) which are either anionic or non-ionic (Deuticke, 1968). The amphipathic nature of pantoyl lactone can be established by observation of its solubility in either water or chloroform (data presented in following section). Crenation using anionic compounds probably results from interaction with cationic groups within the membrane. Both anionic- and non-ionic-induced crenation are thought to result either from changes in interfacial tensions or conformational changes induced in membrane lipoproteins (Deuticke, 1968). Regardless of the mechanism, the physical manifestation of erythrocyte crenation by pantoyl lactone is evidence that this compound can directly affect cell membranes.

For agglutination of red blood cells, antibodies are thought to be directed against and interact with various sugar residues (antigens) at the cell surface. Agglutination may occur by the cross-linking

"lattice" mechanism (Heidelberger, 1939) or as a result of reduced surface charge (Boyd, 1947). Sialic acid residues are thought to be involved in the reduction of surface charge (Pollack et al., 1965). Regardless of the actual mechanism, the interaction is most certainly a surface-linked and therefore a membrane phenomenon.

Data given in Table III demonstrate that pantoyl lactone causes a significant reduction in agglutination of horse erythrocytes. Also, as demonstrated previously, this effect of pantoyl lactone is reversible. These data again strongly suggest a direct effect by pantoyl lactone against membrane components.

A structural or conformational change (including the observed crenation) in the membrane should decrease the number of antibody "binding" sites available at the surface. The titer might not be decreased to zero however, since some of the antigenic sites could be left or positioned near or in the tip of the crenation spikes. Also, my data might suggest a degree of "selectivity" in the ability of pantoyl lactone to "mask" only certain antigenic groups, otherwise there should be no detectable titer in the presence of pantoyl lactone. A membrane, rather than antibody, alteration is suggested by the finding that pre-treatment of antibody with pantoyl lactone does not cause a reduction in titer. This suggestion must be tempered by our knowledge that <u>all</u> effects of pantoyl lactone thus far measured are negated when pantoyl lactone is removed from a given system.

Recently Machleidt, Roth, and Seeman (1972) have shown that nineteen anesthetics protect human erythrocytes from hypotonic hemolysis. By measuring mean cell volumes, Seeman, Kwant, and Sauks (1969) computed membrane areas. At lower drug concentrations, the

TABLE III

EFFECT OF PANTOYL LACTONE ON AGGLUTINATION
OF HORSE ERYTHROCYTES

Additions	Titer
0.146 M NaCl and 0.01 M PO_4 Buffer	1/5,000
+ 0.077 M Pantoyl Lactone	1/1,250
Cells Pre-treated with Pantoyl Lactone*	1/5,000
Antibody Pre-treated with Pantoyl Lactone**	1/5,000

*Cells were exposed to pantoyl lactone (5 min.), removed, washed twice in the saline buffer, and titered in the absence of pantoyl lactone.

**Antibody was exposed to pantoyl lactone in saline buffer (1 hour), placed in dialysis tubing, and dialyzed 3 days (against 4 changes of 0.01 M phosphate buffer, pH 6.8) before being titered in the absence of pantoyl lactone.

cells crenate (less volume) before later regaining their spherical shape in which condition they have from 1.3 to 5% larger membrane areas. The coefficient of correlation between the amount of drug necessary to give 50% anti-hemolysis ($AH_{50\%}$) and the octanol/water partition coefficients of the drugs is very high (0.963). Their data indicate that a hydrophobic interaction occurs between the membrane and drugs used as anesthetics.

As shown in Table IV, when horse erythrocytes are treated with 0.077 M pantoyl lactone, there is a reduction in lysis (about 36%) in hypotonic media. The protection is not a result of an increase in osmolarity of the suspending medium by pantoyl lactone because no protection is afforded by equi-osmolar increases using sodium chloride. As will be shown later, significant amounts of pantoyl lactone partition from water into octanol.

Although molecular interpretations cannot be given, all the data obtained using horse erythrocytes indicate that pantoyl lactone can cause membrane alterations. The morphological change (crenation) is a visual demonstration; the agglutination studies suggest the alteration may be of considerable magnitude (enough to somehow "cover" or "hide" surface antigenic groups); whereas the osmotic protection data indicate pantoyl lactone may be acting through hydrophobic association with membrane components.

Spectrophotofluorimetric Studies

The fluorescent probe, 1-anilinonaphthalene-8-sulfonate (ANS), fluoresces only when in a hydrophobic environment. Many investigators, most notably Rubalcava, de Munoz, and Gitler (1969) and Wallach et al.

TABLE IV

OSMOTIC PROTECTION OF HORSE ERYTHROCYTES
BY PANTOYL LACTONE

Additions	% Lysis*
0.015 M Tris-HCl (pH 7)	100
+ 0.077 M Pantoyl Lactone	64
+ 0.038 M NaCl	100

*The procedure followed was the same as that given by Machleidt, Roth, and Seeman (1972). After equilibrating (5 min) 1 ml of a 2% erythrocyte suspension in saline (containing pantoyl lactone or increased NaCl), a final aliquot of 1.5 ml of either pantoyl lactone or NaCl in 15 mM Tris-HCl, pH 7 was added while mixing on a vortex mixer. After setting an additional 15 min, the erythrocytes were sedimented using a clinical centrifuge and absorbance of the resulting supernatant read at 540 nm. The absorbance for 0% hemolysis (control value) was obtained by adding physiologic saline to the erythrocyte suspension.

(1970), have studied the fluorescence of ANS in erythrocyte membranes. Apparently all that is required is that a hydrophobic area be available regardless of whether the area occurs in membrane proteins, protein-lipid interfaces, or lipid regions (Wallach et al., 1969).

As shown in Figure 15, there is no difference in level of fluorescence of ANS in envelopes from control versus filamentous cells. Unfortunately we have no precise way of determining if the lack of variation in response occurs as a result of the rupturing and washing procedures which are necessary prior to examination of the structures. Because of the essentially negative data obtained using isolated envelopes from normal and filamentous cells, we reasoned that if pantoyl lactone could alter fluorescence of ANS in isolated envelope or membrane material, then by removing various components from such structures and re-checking fluorescence with and without pantoyl lactone present, an indication of the type compounds reacting with pantoyl lactone could be obtained.

As shown in Figure 15, pantoyl lactone does bring about a decrease in fluorescence in envelopes from both control and division-inhibited cells. Since the decrease occurs in both types of preparations, it could indicate that the negative effect caused by pantoyl lactone on fluorescence is not important for the division process and therefore further study would be irrelevant. On the other hand there is no reason to suppose that the influence of pantoyl lactone on fluorescence of ANS in envelopes from control and filamentous cells should differ, particularly since no significant quantitative differences in lipids from the two types of cells can be demonstrated (Table V).

The data in Table VI show relative decreases in fluorescent

Figure 15. Spectrophotofluorimetric Scan Using ANS to Determine Fluorescence in the Presence and Absence of Pantoyl Lactone. (———), envelope from control as well as division inhibited cells; (-----), envelope from control as well as division inhibited cells plus pantoyl lactone (0.077 M).

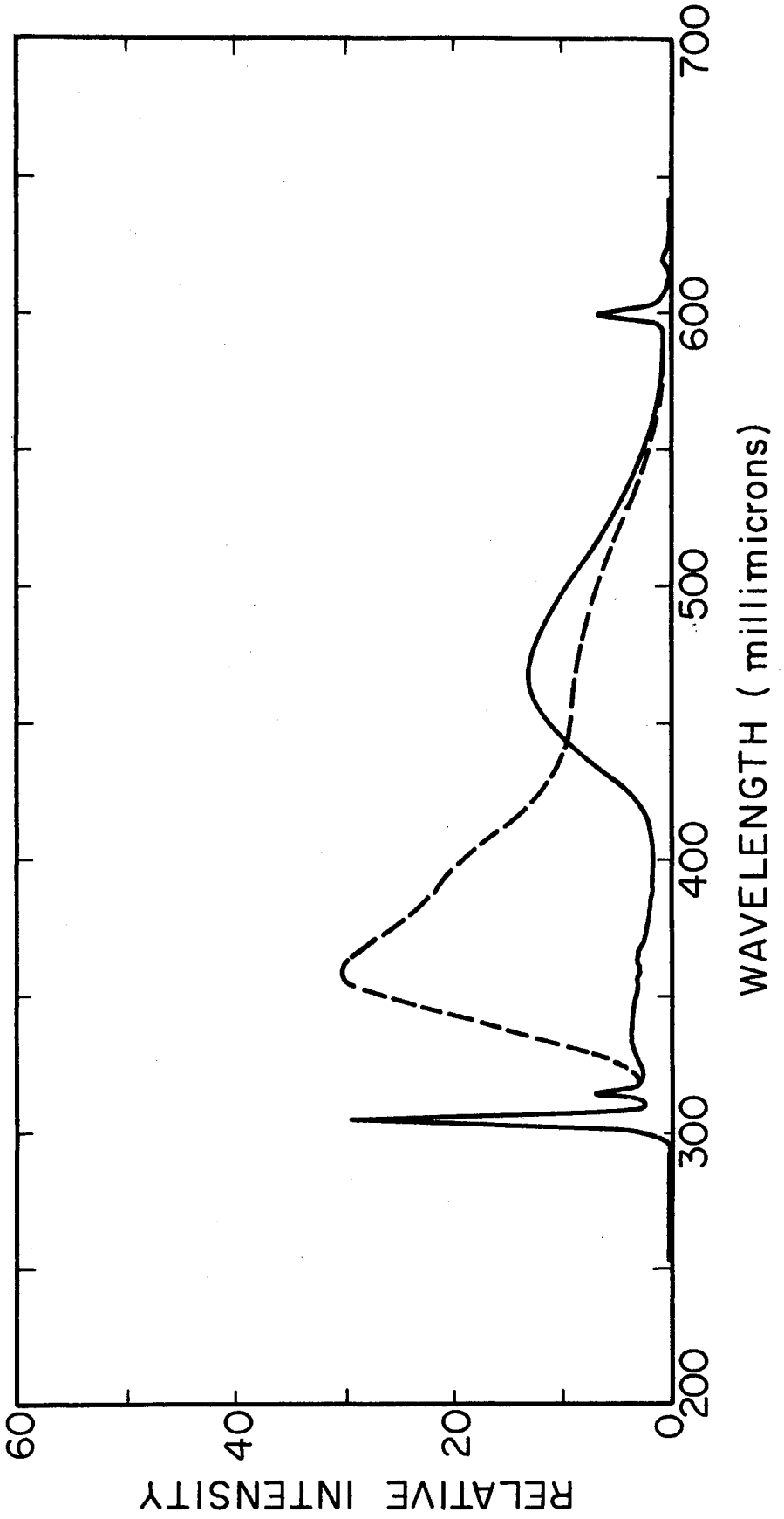

RELATIVE INTENSITY
0
20
40
60
200
300
400
500
600
700
WAVELENGTH (millimicrons)

TABLE V

AMOUNT OF EXTRACTABLE LIPIDS IN ENVELOPES FROM NORMAL AND FILAMENTOUS CELLS OF *ERWINIA* SPECIES

Cells	Lipid Extracted by Chloroform-Methanol (2:1, v/v) (CPM/mg dry wt)*
Control	61.9
Filamentous	58.8

*Cells were grown in D-Glucose-U-^{14}C (0.01 uC/ml) for 4 hours before harvesting and subsequent lipid extraction.

TABLE VI

FLUORESCENCE OF 1-ANILINONAPHTHALENE-8-SULFONATE (ANS) IN VARIOUS COMPONENTS IN THE PRESENCE OF PANTOYL LACTONE

Material	Relative Fluorescent Intensity in Presence of Pantoyl Lactone*
Envelopes from:	
Control Cells	70-80
Division Inhibited Cells	70-80
Lipid Extracted Envelope	92
LPS from *Erwinia* species	100
Membrane from *M. lysodeikticus*	70-80

*Fluorescence was measured at 475 nm; excitation wavelength was 305 nm. Concentration of pantoyl lactone was 0.077 M. All samples were freshly prepared and contained approximately 1.5 mg protein in 1 ml of 0.01 M potassium phosphate buffer (pH 7.4).

intensity in various preparations in the presence of pantoyl lactone. In each case, fluorescence of the same preparation in the absence of pantoyl lactone was set at an intensity of 100. As can be seen, the decrease caused by pantoyl lactone is greatest using either whole envolopes of Erwinia species or membranes from M. lysodeikticus. When lipids are extracted using the acetone-ammonia procedure (Grula et al., 1967), the resulting stripped envelope still fluoresces (actually increases over the control specimens), but the effect of pantoyl lactone is not nearly as evident. Also, pantoyl lactone causes no decrease in fluorescence using isolated LPS.

These data indicate the decreased fluorescence of ANS caused by pantoyl lactone in isolated envelopes or membranes is due either to a pantoyl lactone-induced decrease in hydrophobicity or because pantoyl lactone keeps ANS from reaching the hydrophobic areas of the cell membrane. At present, data are not available to distinguish between these possibilities.

Overall, my results could indicate that pantoyl lactone associates more with lipids than proteins since decrease in fluorescence is lower in stripped envelopes in the presence or pantoyl lactone. In this connection, it can be pointed out that addition of ANS to whole cells of Erwinia species causes no significant decrease in uptake ability using glucose. This would suggest that pantoyl lactone is doing more than merely associating with hydrophobic (lipid) areas of the cell membrane in order to decrease uptake activity; however, ANS-treated whole cells fluoresce very poorly. This latter observation could indicate that penetration by ANS into hydrophobic areas of the cell envelope (cell membrane?) does not readily occur in live whole cells.

Effect of Pantoyl Lactone on Proteins and Lipids

After having demonstrated that at least one major site of action of pantoyl lactone is the cell membrane, experiments were designed to show possible associations of pantoyl lactone with either proteins, lipids, or a combination of both. When doing work with membrane associated proteins, caution should be exercised since the absence of a demonstrable effect by pantoyl lactone on one or more of the proteins chosen for study may not reflect a possible action of pantoyl lactone on other membrane proteins. In addition, a demonstrable lipid association in an *in vitro* situation may reflect the possibility of a lipid-protein interaction *in vivo*.

Proteins

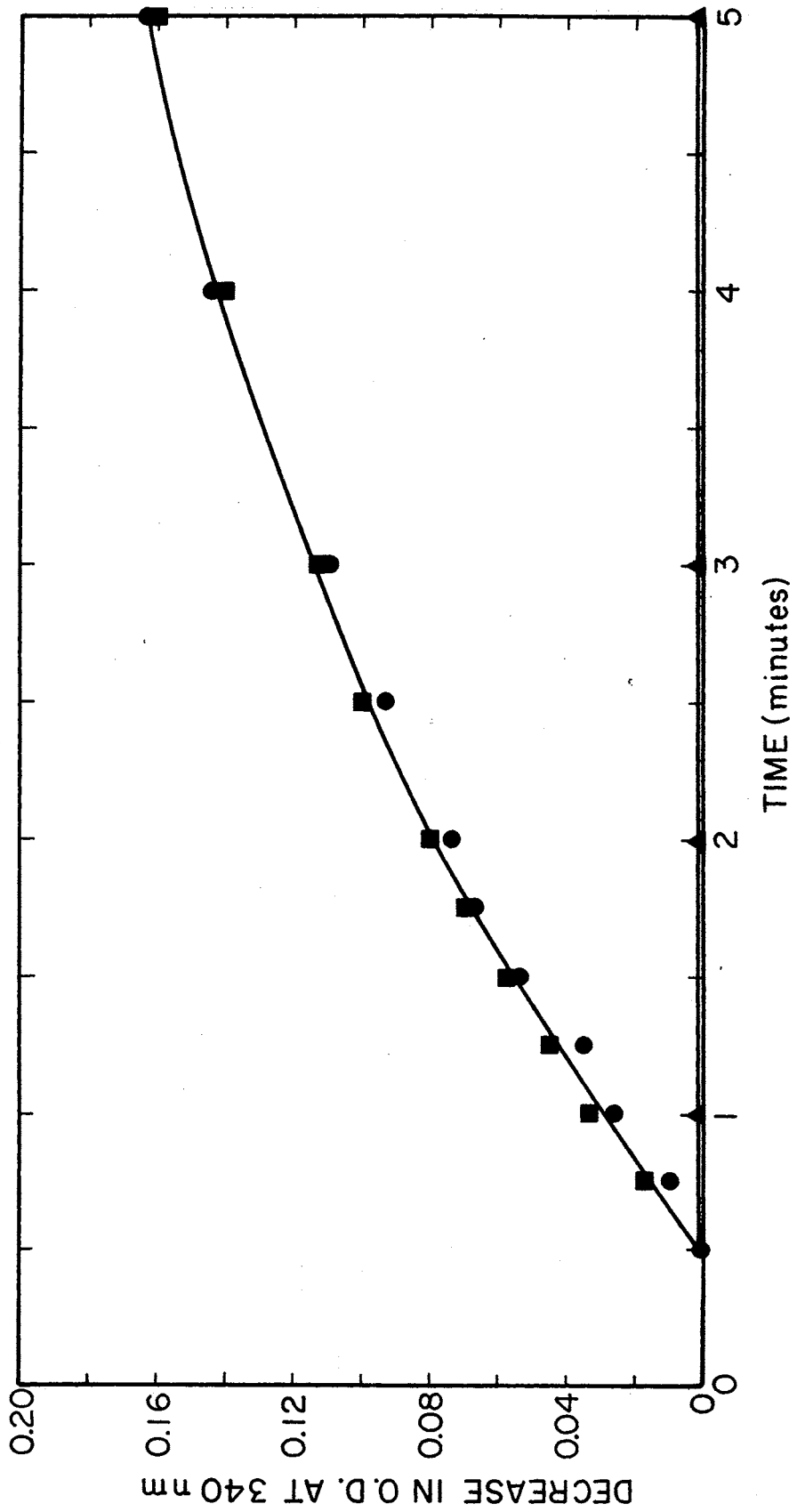
The activities of four protein enzymes were studied in the presence and absence of pantoyl lactone. Lactate dehydrogenase (EC 1.1.1.27), a cytoplasmic (water soluble) enzyme protein present in *Erwinia* species, and lysozyme (EC 3.2.1.17), another water soluble enzyme protein, were tested to determine whether or not pantoyl lactone could decrease activity of proteins that are known not to be intimately associated with the cell membrane. NADH dehydrogenase (EC 1.6.99.3) was tested because it is a protein activity tightly bound to the cell membrane and also associated with membrane lipids in *M. lysodeikticus* (Nachbar and Salton, 1970). As source of enzyme activity, we used whole membranes isolated from our wild-type strain of *M. lysodeikticus* grown in defined medium (Grula, Luk, and Chu, 1961). The fourth protein studied was an aspartic acid "binding" protein

isolated from Erwinia species. This protein is of particular interest because, as previously documented, pantoyl lactone inhibits uptake of aspartic acid by normally grown cells. Thus, inhibition of binding activity by pantoyl lactone would indicate that inhibition of uptake activity caused by pantoyl lactone may be the result of a direct effect on binding protein.

Although quantitation is extremely difficult, because of the very rapid evolution of oxygen, I also assayed isolated membranes of M. lysodeikticus and envelopes of Erwinia species for membrane-associated catalase activity (EC 1.11.1.6) in the presence and absence of pantoyl lactone. Although precise data relating to rate of evolution of oxygen could not be obtained, pantoyl lactone did not appear to inhibit evolution of oxygen from hydrogen peroxide by the membrane-associated enzyme. It may or may not be significant that presence of pantoyl lactone appeared to delay for a few seconds the evolution of oxygen and the bubbles that subsequently moved to the surface of the assay medium were observed to be smaller than those seen in the absence of pantoyl lactone. Presence of the smaller type oxygen bubbles is probably a reflection of the lowered surface tension caused by pantoyl lactone.

The enzyme assay for lactic acid dehydrogenase was performed as given in The Worthington Enzyme Manual (pages 7-10) using a soluble cell-free preparation from Erwinia species which was obtained by breaking the cells in an X-PRESS followed by sedimentation of the insoluble debris (75,000 x g for 30 min at 4 C). As shown in Figure 16, pantoyl lactone neither enhances nor inhibits the rate of disappearance of NADH in such a system. Similarly, pantoyl lactone does not affect the ability of lysozyme to solubilize the isolated cell wall mucopeptide

Figure 16. Effect of Pantoyl Lactone on Lactate Dehydrogenase Activity of Erwinia Species. ▲ , control containing buffer, NADH, and pyruvic acid; ● , plus enzyme extract; ■ , plus enzyme extract and pantoyl lactone (0.077 M).


DECREASE IN O.D. AT 340 nm
0.20
0.16
0.12
0.08
0.04
0
0
1
2
3
4
5
TIME (minutes)

from M. lysodeikticus (Figure 17). Judging from these data, pantoyl lactone does not appear to appreciably alter activity of water soluble enzymes.

The assay for NADH dehydrogenase as given by Nachbar and Salton (1970) was followed utilizing membranes prepared according to the procedure of Butler et al. (1967). As can be seen in Figure 18, pantoyl lactone has no effect on the reaction rate of this membrane-localized enzyme. These data constitute evidence that not all membrane proteins (including those in a lipid environment) are affected by pantoyl lactone, or at least in such a manner as to alter reaction rates.

The aspartic acid binding protein was obtained after release from aspartic acid-grown cells using the cold-osmotic shock procedure of Heppel. After concentrating the material approximately 40-fold by dialysis against Carbowax-6000, equilibrium dialysis studies served as a check for binding activity. A major problem in this study was the continued presence of bacterial contamination in the "binding" side of the dialysis unit. High levels of chloramphenicol (1 mg/ml) or penicillin (1000 U/ml) inhibited growth; however, after the dialysis experiments were run, colonies would usually form when the solutions were plated on nutrient agar (when antibiotics were present growth would not appear until 5-7 days after plating). We tried filtering the shock fluid and keeping all buffers and equipment as free from contamination as possible. Our procedures also included UV-irradiation (30 min) of the dialysis unit and steam-sterilization of all solutions not containing proteins. Representative data presented in Table VII indicate that pantoyl lactone does not inhibit uptake of aspartic acid by directly affecting binding protein (2 colonies grew out after

Figure 17. Effect of Pantoyl Lactone on Lysozyme Digestion of Mucopeptide Isolated from M. lysodeikticus. ▲, control containing buffer and mucopeptide; ●, plus lysozyme (3.3 ug/ml); ■, plus lysozyme and pantoyl lactone (0.077 M).

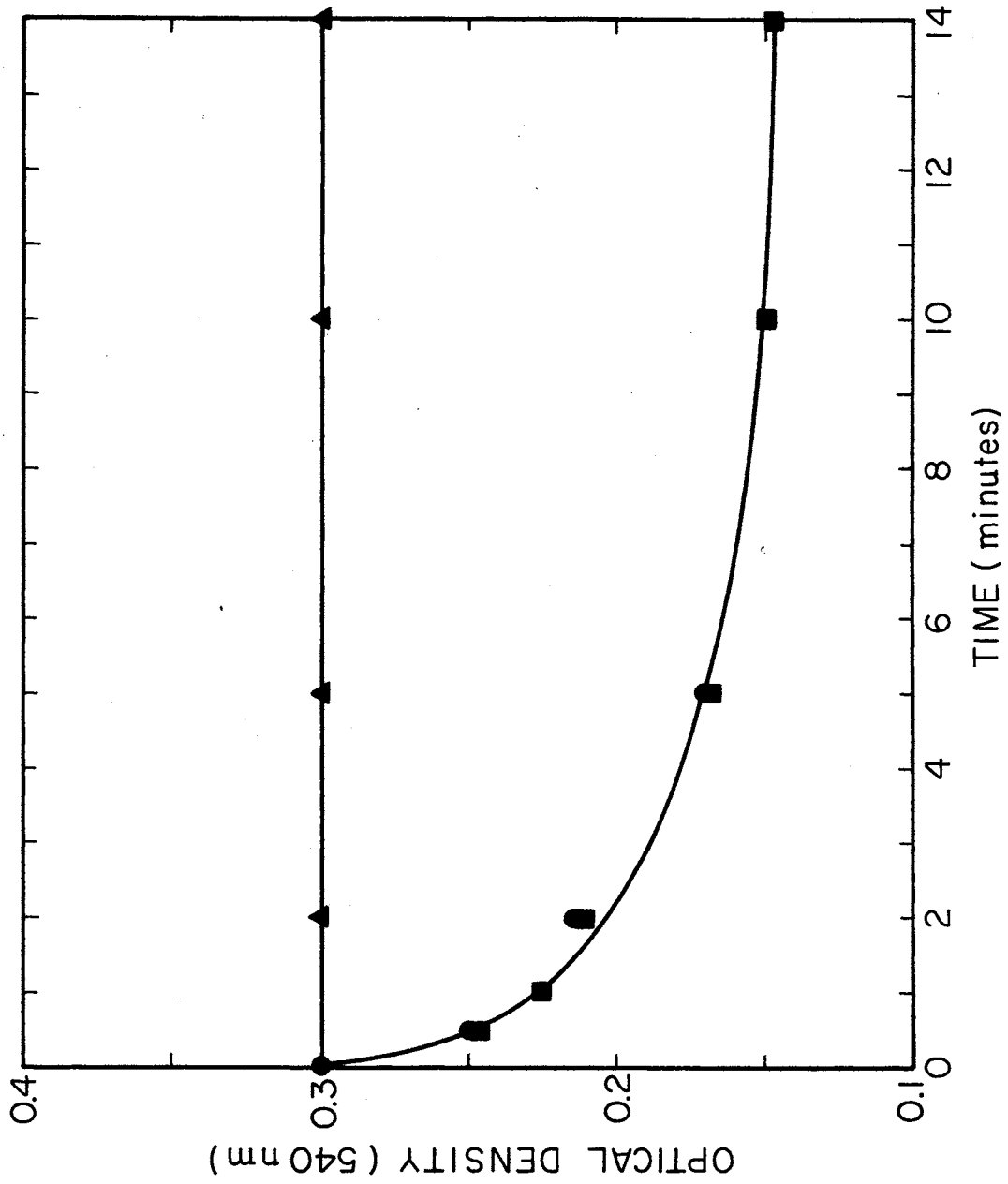

OPTICAL DENSITY (540 nm)
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
TIME (minutes)

Figure 18. Effect of Pantoyl Lactone on NADH Dehydrogenase Activity in Membranes Isolated from M. lysodeikticus. ▲ , control containing buffer, DCIP, and NADH; ● , plus membrane (0.4 mg/ml); ■ , plus membrane and pantoyl lactone (0.077 M).

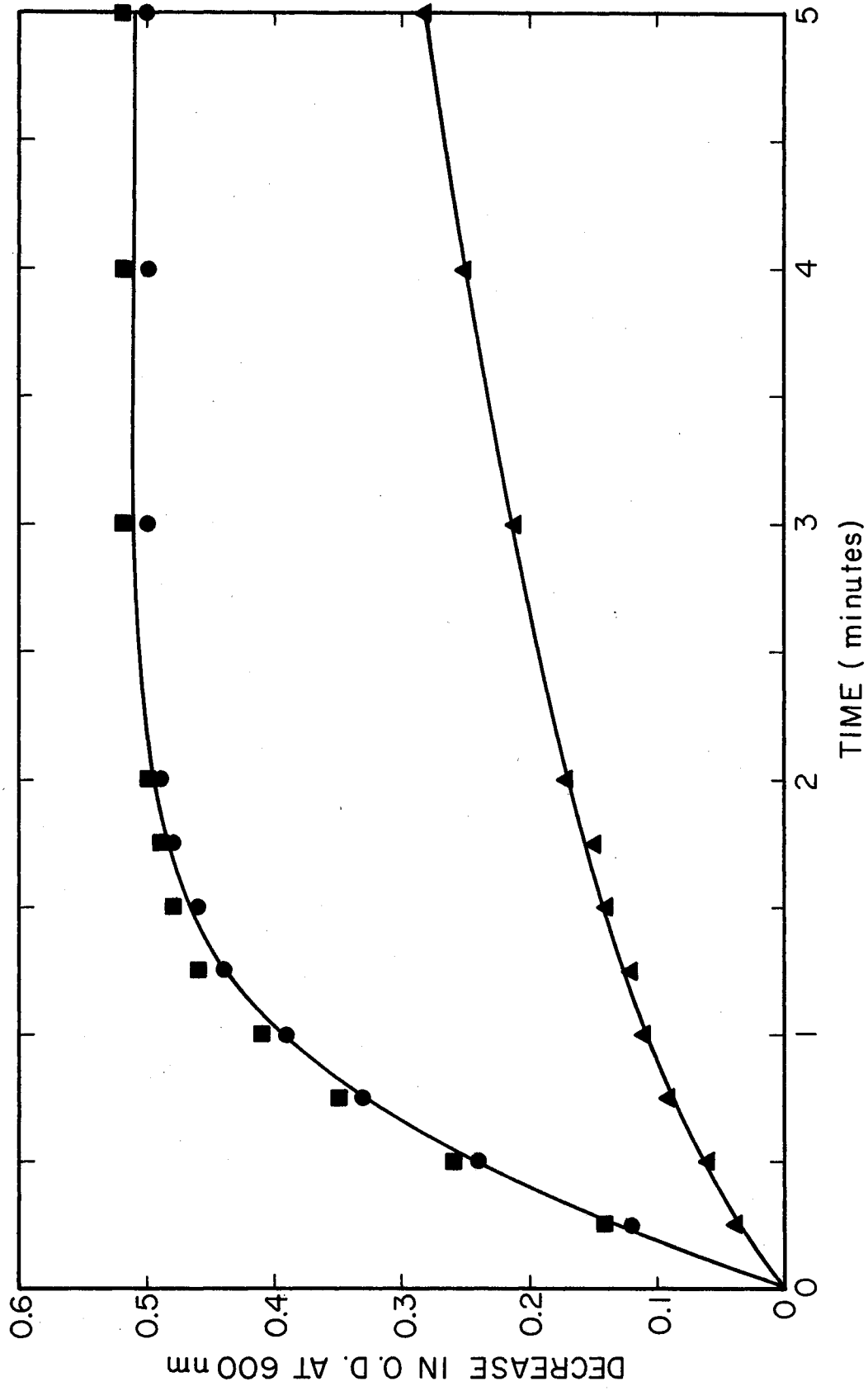

DECREASE IN O.D. AT 600 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
TIME (minutes)

TABLE VII

EFFECT OF PANTOYL LACTONE ON ASPARTIC ACID BINDING PROTEIN

Test Situation	Distribution Ratio
Tris Buffer (0.01 M, pH 5.0)	0.97
+ Binding Protein*	1.39
+ Binding Protein and Pantoyl Lactone (0.077 M)	1.88

*Cells from which binding proteins had been removed were inhibited in uptake activity on aspartic acid by approximately 30 per cent (based on 4 min count). Approximately 350 mg/ml of solubilized protein were used in this binding study.

several days incubation from the binding protein solution used in this experiment; therefore, this was considered to represent a "sterile" experimental run). Unfortunately, we have no direct way of testing if pantoyl lactone can inhibit activity of binding protein when the protein is present in its normal environment (hydrophobic?) in the cell membrane.

In summary, my data indicate that pantoyl lactone has no effect on activity of hydrophilic or hydrophobic enzyme proteins.

Lipids

If pantoyl lactone reacts with lipids in the cell membrane, we thought a molecular association could be demonstrated using labeled pantoyl lactone. D-Pantoyl lactone was tritiated by New England Nuclear Company using the Wilzbach gas exposure method and returned to us as a solution containing from 1-5% pure pantoyl lactone. Even extensive extractions with heptane and chloroform both before and after thin-layer chromatography did not give a chromatographically-pure product. After eluting the pantoyl lactone area from thin-layer plates, the material could be re-chromatographed and the tritium label would always be found running from the origin to the pantoyl lactone area (trailing effect). In addition, heptane/water partition chromatography using such relatively "pure" labeled pantoyl lactone indicated that approximately 10 times more pantoyl lactone (radioactivity) was entering the heptane than could be demonstrated utilizing unlabeled pantoyl lactone and a colorimetric procedure. Therefore, the Hestrin (1949) colorimetric test for ester linkages was utilized to obtain the following data using unlabeled DL-pantoyl lactone.

As shown in Table VIII, pantoyl lactone is soluble in numerous solvents having varying degrees of polarity. These data are given to demonstrate the amphipathic nature of the molecule. We also observed that pantoyl lactone is only slightly soluble in heptane; however, most lipids are quite soluble in this solvent. Based on these solubility characteristics, we were able to design a liquid partition method for study of possible pantoyl lactone-lipid associations. Basically the method utilizes the solubilization of various lipids in heptane and mixing this solution against water containing pantoyl lactone. Theoretically, since pantoyl lactone is highly insoluble in heptane, little or no pantoyl lactone should migrate into the heptane unless the lipid present somehow associates with and therefore "holds" pantoyl lactone molecules in the heptane layer. Prior to the liquid partition studies, I had established that pantoyl lactone associates with envelopes (both control and lipid depleted); however, as can be seen in Table IX, the association is very "weak" since pantoyl lactone is readily removed by simple washing procedures.

Heptane/water partition coefficients increase using lipids extracted from the envelopes of Erwinia species or membranes of M. lysodeikticus (Table X). Unfortunately, it is very difficult to accurately weigh the small amounts of lipids used and, therefore, the partition coefficients cannot be compared. It is significant that these membrane lipids do "encourage" migration of pantoyl lactone into heptane. We, therefore, did further studies to determine if all lipids or only a particular class of lipids can interact (extract) pantoyl lactone and these data are also included in Table X.

TABLE VIII

SOLUBILITY OF DL-PANTOYL LACTONE IN SOLVENTS HAVING VARYING DEGREES OF POLARITY

Solvent	Solubility**
Water	+
Chloroform	+
n-Propanol	+
n-Butanol	+
n-Amyl Alcohol	+
sec-Butyl Alcohol	+
3-Methyl-1-Butanol	+
n-Octanol	+
Ethyl Acetate	+
Methyl Ethyl Ketone	+
iso-Butyric Acid	+
Ethyl Ether*	+
Toluene*	+
Benzene*	+
Carbon Tetrachloride*	+
Hexane*	-
Heptane*	-

*Considered relatively non-polar.

**Solubility was judged by either the disappearance of solid pantoyl lactone in the solvent or, in the case of the letter two solvents, by a negative Hestrin test.

TABLE IX

ASSOCIATION OF PANTOYL LACTONE WITH ENVELOPES FROM ERWINIA SPECIES (CONTROL AND LIPID DEPLETED) AND ITS REMOVAL BY WASHING WITH POTASSIUM PHOSPHATE BUFFER

Type of Envelope	Amount (mg) Pantoyl Lactone Associated with 1 mg Envelope*
Control (whole envelope)	
Initial	8
After 1st Wash	1
After 2nd Wash	0.05
Lipid Depleted (stripped**)	
Initial	8
After 1st Wash	0.5
After 2nd Wash	0.05

*Initial amounts (approximately 8 mg) represent the amount of pantoyl lactone remaining with the first envelope pellet after prior exposure to 50 mg pantoyl lactone for 30 min at 23 C.

**Stripping was accomplished utilizing the acetone-ammonia procedure of Grula et al. (1967).

TABLE X

PANTOYL LACTONE HEPTANE/WATER PARTITION COEFFICIENTS USING LIPIDS EXTRACTED FROM ENVELOPES OF ERWINIA SPECIES, MEMBRANES FROM M. LYSODEIKTICUS AND VARIOUS LIPID MOLECULES

Addition to Heptane	Partition Coefficient*
None	0 (blank)
Lipids from Erwinia species**	0.0044
Lipids from M. lysodeikticus**	0.0075
Palmitic Acid	0.0025
Lauric Acid	0.0008
Tripalmitin	0.0022
Cephalin	0.0242
Linoleic Acid	0.0116
Linolenic Acid	0.0205
Trilinolenin	0.0098

*Data for fatty acids and triglycerides were obtained using 20 mg/ml of each lipid and 100 mg/ml pantoyl lactone. To insure complete solubilization of all lipids, the lipid-heptane solution was heated 10 sec at 95 C. The heptane/water phases (1 ml each) were mixed 10 sec on a Vortex mixer and allowed to set 30 min at room temperature before initiation of the Hestrin test for pantoyl lactone using the heptane layer. Partition coefficient equals the mg pantoyl lactone migrating into the heptane layer divided by the mg pantoyl lactone remaining in the water layer.

**Due to the scarcity and the hygroscopic nature of these lipids, accurate weighings were impossible; however, probably between 1-2 mg were used.

As shown in both Table X and Figure 19, on a weight basis, cephalin extracts (associates with) pantoyl lactone better than several other lipids. The cephalin used is an unpurified commercial extract (chloroform-methanol) of sheep brain obtained from Sigma Chemical Company and has at least three phospholipid components when checked for purity by thin-layer chromatography. Washing of the cephalin-heptane mixture two times using 2 M KCl to remove water soluble components (primarily proteins) did not appreciably change the partition coefficient. Pure (synthetic) phosphatidylethanolamine (dipalmitoyl form) is not soluble in heptane even after mild heating; therefore, this form of the compound could not be directly studied. Presumably, the natural (and impure) cephalin that possesses the highest partition coefficient has a variety of both saturated and unsaturated fatty acid side groups. Apparently the high partition coefficient of pantoyl lactone in the presence of natural cephalin must occur as a result of the presence of unsaturated fatty acids since, as shown in Table X, pantoyl lactone partitions poorly in the presence of saturated fatty acids.

The data shown in Table XI were obtained by re-extraction of pantoyl lactone which had previously been partitioned into heptane (which contained cephalin). As can be seen, pantoyl lactone is also easily removed from the cephalin (heptane) layer, thus demonstrating the "weak" nature of the pantoyl lactone-heptane association. An indication of the type of association involved (other than "weak") cannot be suggested since water, SLS, KCl, Urea, and Tris buffer extract pantoyl lactone to about the same extent.

Figure 19. Extraction of Pantoyl Lactone into Heptane by Various Concentrations of Different Lipids. ■ , Cephalin; □ , Linolenic Acid; ▲ , Linoleic Acid; △ , Trilinolenin; ● , Tripalmitin; O—O, Palmitic Acid; O---O, Lauric Acid. Only one concentration of Linolenic acid and Trilinolenin (20 mg) was used due to availability of these compounds. Procedure for partitioning was the same as given in Table X.

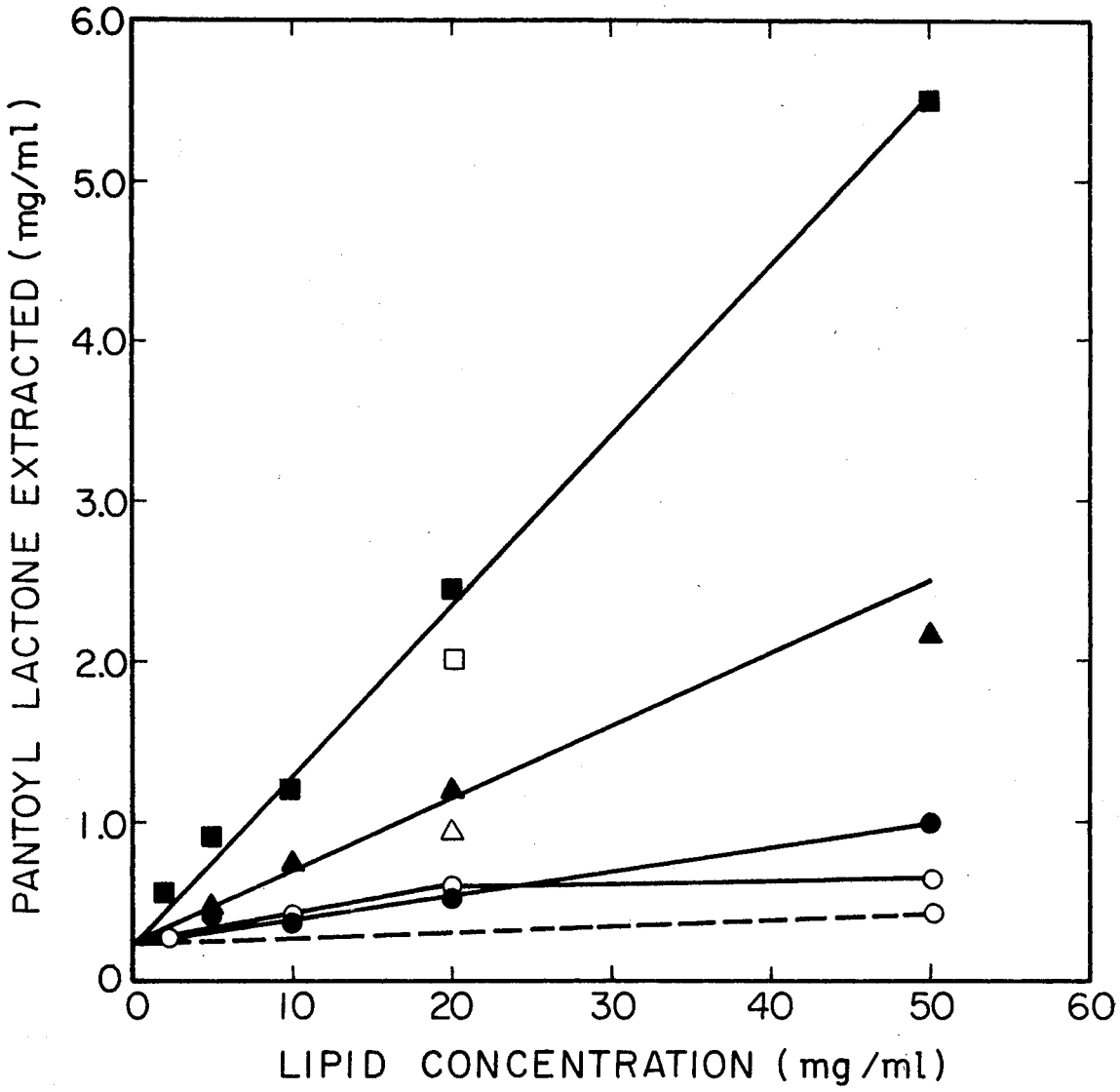

6.0
5.0
4.0
3.0
2.0
1.0
0
0
10
20
30
40
50
60
PANTOYL LACTONE EXTRACTED (mg/ml)
LIPID CONCENTRATION (mg/ml)

TABLE XI

RE-EXTRACTION OF PANTOYL LACTONE FROM HEPTANE CONTAINING CEPHALIN

Extraction Solvent	mg Pantoyl Lactone Remaining in Heptane*	mg Pantoyl Lactone Extracted
Water	1.02	5.50
KCL (2 M)	0.88	5.64
SLS (0.1%)	1.16	5.36
Urea (8 M)	0.44	6.08
Tris (0.0025 M pH 7.4)	1.12	5.60

*Amount of pantoyl lactone partitioned into the heptane layer prior to extraction was 6.52 mg. The procedure for partitioning was the same as given in Table X.

The glyceride moiety does not seem to play an important role (as a reactant with pantoyl lactone) because neither tripalmitin nor tributyrin (not shown) increase partitioning of pantoyl lactone to any appreciable degree. In this connection it should be pointed out that for some unknown reason the alkali-hydroxylamine (Hestrin) test gives a positive reaction with tributyrin but not tripalmitin. For this reason, tubes containing proper controls of each lipid tested were always run along with the experimental tubes.

It seems that some degree of unsaturation in the fatty acid moiety is all that is necessary to cause increased partitioning of pantoyl lactone. Linolenic acid (3 unsaturated carbon-carbon bonds) extracts pantoyl lactone better than linoleic acid (2 unsaturated bonds); however, its triglyceride (trilinolenin) is less effective than linoleic acid.

These data indicate that pantoyl lactone most probably alters structural and functional characteristics of membranes because of some type of weak association with phospholipids containing unsaturated fatty acid side chains.

It should also be mentioned that similar data can be obtained using chloroform/water as the partitioning system. However, due to the high solubility of pantoyl lactone in chloroform (partition coefficient equals 0.388), the results are not as clear-cut as those obtained using heptane. Nevertheless, representative data are given in Table XII.

TABLE XII

PANTOYL LACTONE CHLOROFORM/WATER PARTITION COEFFICIENTS IN THE PRESENCE AND ABSENCE OF CEPHALIN AND PALMITIC ACID

Addition to Chloroform	Partition Coefficient*
None	0.388
Cephalin	0.478
Palmitic Acid	0.388

*Data were obtained using 20 mg/ml of each lipid and 10 mg/ml pantoyl lactone and the procedure for partitioning was the same as given in Table X.

CHAPTER IV

CONCLUSIONS

Grula and Grula (1964) first suggested that division inhibition might result from "secondary" damage to the cell membrane which can occur after a decrease in wall mucopeptide synthesis. These investigators also suggested that various agents, such as pantoyl lactone, spermine, and Carbowax-400 (hypertonic conditions), reverse and/or prevent division inhibition by somehow protecting the membrane from damage since these compounds do not restore mucopeptide synthesis to control levels. Although a few investigators have reported that changes occur in membrane proteins (Siccardi et al., 1971; Inouye and Pardee, 1970; Grula and King, 1971; Grula and Hopfer, 1972) or lipids (Weinbaum and Panos, 1966) in non-dividing cells, no one has yet directly demonstrated the involvement of any type of membrane molecule in the bacterial cell division process. Therefore, the main goal of my research was to document such a possible involvement. In order to achieve this goal, we decided to study the effects of pantoyl lactone on membrane-associated phenomena in Erwinia species, M. lysodeikticus, and horse erythrocytes and determine if a specific molecular association between a membrane component and pantoyl lactone could be demonstrated.

The inability of pantoyl lactone to alter the sensitivity of cells to Actinomycin D suggests a membrane rather than wall site of action.

The data I have presented which relate to uptake activity of whole cells of Erwinia species strongly indicate that pantoyl lactone induces membrane alterations. It is generally believed that transport "carrier molecules" are membrane localized; therefore, a membrane alteration should have either a positive or negative effect on transport ability.

The enhancement by pantoyl lactone of SLS-lysis of whole cells of Erwinia species as well as SLS-solubilization of isolated envelopes from this organism indicates a membrane alteration. This conclusion is also supported by the data showing that pantoyl lactone causes a decrease in fluorescence of ANS in envelopes and membranes isolated from bacteria. In addition, the crenation, agglutination, and osmotic protection studies using horse erythrocytes further indicate a pantoyl lactone-membrane interaction. The crenation and osmotic protection phenomena have been associated with and believed to occur as a direct result of membrane lipid interactions (Deuticke, 1968; Livne, Kuiper, and Meyerstein, 1972). Most of my data suggest that pantoyl lactone causes membrane alterations by some type of association with unsaturated fatty acids which are present in the bacterial membrane.

Many reports have recently appeared in the literature which address themselves to the importance of unsaturated fatty acids in membrane structure and function. Marr and Ingraham (1962) reported that cells of Escherichia coli contain large amounts (40-50%) of unsaturated fatty acids. Other investigators (Cullen, Phillips, and Shipley, 1971; Shen et al., 1970; Shaw and Ingraham, 1965) have found that, in general, the degree of unsaturation increases with decreasing growth temperature. Also, fluidity of cellular membranes is directly related to degree of unsaturation of membrane lipids (Fox, 1972);

therefore, it seems reasonable that the activity of membrane-localized proteins may increase with fluidity, and thus also be related to degree of membrane lipid unsaturation. Cullen, Phillips, and Shipley (1971), using X-ray diffraction methods have compared lipid structures (both lamellar and hexagonal) formed in water by phosphatidylethanolamine isolated from Pseudomonas fluorescens grown at either 5 or 22 C. They find that the physical states of such lipid structures are identical if compared at the same temperature at which the cells were grown. Therefore, they suggest that the degree of unsaturation within the fatty acids of the cell membrane acts as a control mechanism which allows cells to maintain a "constant" membrane structure (with regard to physical states of the phospholipids). Fox (1969), using an auxotrophic strain of E. coli which requires unsaturated fatty acids for growth, concluded that unsaturated fatty acids are necessary for incorporation of the lactose transport system into the membrane.

Hubbard and Hall (1968) have shown that an increase in branched-chain fatty acids in the cell membrane of Bacillus cereus 14B22 does not affect the ability of this organism to take up glutamic acid. However, many investigators find that the degree of unsaturation plays an important role in uptake of various compounds. Gale and Llewellin (1971) reported that addition of unsaturated fatty acids to the uptake medium stimulates uptake of aspartic acid (to control levels) in osmotically shocked cells of Staphylococcus aureus. Esfahani et al. (1971) have shown that proline uptake activity is stimulated by unsaturated fatty acids present in the membrane of E. coli; however, at the same time, succinic dehydrogenase activity is not affected. Kundig and Roseman (1971) have found that a minor lipid component

(phosphatidylglycerol) in E. coli is necessary for activity of constitutive membrane-bound Enzymes II of the phosphotransferase system when tested in vitro. Analysis of isolated phosphatidylglycerol from this organism reveals that approximately 60% of the fatty acid side chains are present in the unsaturated form. Further, Fox (1972) reports that membranes of bacterial cells rich in unsaturated fatty acids are capable of transporting molecules up to 20 times faster than cells having relatively more saturated fatty acids.

Weinbaum and Panos (1966) demonstrated that filamentous cells of E. coli not only have decreased amounts of phospholipids, but, also, that division inhibited cells exhibit large relative decreases in cyclopropane (unsaturated) fatty acids. However, closer examination of their data reveals a slight (3%) increase in the amount of unsaturated (palmitoleic, oleic, and cis-vaccenic) fatty acids.

Because penicillin and other antibiotics known to inhibit mucopeptide synthesis also inhibit bacterial cell division (Grula and Grula, 1964; Grula and King, 1970; King and Grula, 1972; Grula and Hopfer, 1972), the data of Exterkate, Vrensen, and Veerkamp (1970) become quite important. These investigators find that inhibition of wall synthesis in Bifidobacterium bifidum, occurring as a result of the withdrawal of milk from the medium, causes a concomitant increase in unsaturated fatty acids in the cell membrane of this organism. It might be argued therefore that division-inhibited cells which contain decreased amounts of cell wall mucopeptide have a "secondary" type of damage; that is, they contain larger amounts of unsaturated fatty acids in their membranes than do normally dividing cells.

Such a generalization becomes somewhat doubtful when temperature

sensitive mutants are considered. Any cells grown at a temperature above their optimum should, in order to maintain a constant degree of fluidity in their membranes, have less unsaturated fatty acids in their membranes than cells grown at their optimum temperature. However, since such temperature-sensitive mutants are division-inhibited, they may actually possess a higher degree of unsaturation than cells of the same organism which divide normally at an elevated temperature. Unfortunately lipid analyses of such cells have yet to be reported. At any rate, there is no reason to suspect that increased fludity (increase in unsaturation) of the cell membrane does not play a key role in bacterial cell division.

Because I have been able to show that pantoyl lactone associates with unsaturated fatty acids, it can be proposed that such a molecular association decreases membrane fluidity and therefore allows activation of the cell division process. Using this as a working hypothesis, some of the previously reported findings will be re-examined.

Decreased membrane fluidity in the presence of pantoyl lactone could account for inhibition of both active and passive transport. The pantoyl lactone-induced decrease in fluidity could physically slow entry of molecules via a passive diffusion mechanism. At the same time, entry of molecules via a carrier-mediated process could be reduced due to a "tightening" effect in the immediate or near environment of the "permease" system. However, decreased uptake activity might also occur because pantoyl lactone causes some untoward effect on the needed energy-generating components of the cell membrane due to association with unsaturated fatty acids. It might also interfere with the needed concomitant efflux of Na^+, K^+, or H^+ recently reported to be

necessary and therefore implicated in active transport (Gale, 1971; Eddy et al., 1970; Eagon and Wilkerson, 1972). As shown earlier, pantoyl lactone does not affect binding activity in vitro; however, data presented in Figure 2 could be interpreted to indicate a possible inhibition of binding in vivo. Therefore, the decreased fluidity might affect both binding and subsequent transport across or through the membrane.

The data I presented which relate to a possible effect of pantoyl lactone on various proteins also agree with the need for a fluid state of the cell membrane since a direct effect by pantoyl lactone on certain membrane-localized enzyme proteins could not be demonstrated. However, the supposed tightening caused by pantoyl lactone might only affect proteins whose function necessitates a large conformational change as, for example, carrier-protein.

The finding that unsaturated fatty acids stimulate proline uptake but do not alter succinic dehydrogenase activity led Esfahani et al. (1971) to propose two possible explanations. Either the lipid environment is identical for both proline permease and succinic dehydrogenase, but different lipid-protein interactions are involved, or there may be areas of heterogeneity within the molecular organization of the cell membrane and unsaturated fatty acids associate more with the proline uptake components. Regardless of which of the two explanations is correct (both may be correct) the proposed decrease in fluidity (caused by pantoyl lactone) need not necessarily inhibit functioning of all membrane proteins.

The decrease in ANS fluorescence of envelopes and the increase in osmotic protection of erythrocytes caused by pantoyl lactone could also

be envisioned to occur as a result of decreased membrane fluidity. This change in fluidity (tightening) could make it more difficult for ANS to enter the hydrophobic areas of the membrane and, in a similar manner, give added strength to erythrocyte membranes.

Other phenomena known to be associated with pantoyl lactone might be explained if it is accepted that pantoyl lactone causes a decrease in fluidity of the cell membrane. As reported by Grula and Hopfer (1972), pantoyl lactone prevents release of periplasmic proteins from filamentous cells and the retention of proteins occurs in the absence of any appreciable restoration of wall mucopeptide. It was concluded that a porous wall aids escape of periplasmic space proteins. It is possible however that the tightening of the cell membrane caused by pantoyl lactone may serve to hold the protein molecules better than would be the case in a fluid or loose membrane.

In summary, it is quite possible that the proposed decrease in fluidity of the cell membrane caused by pantoyl lactone plays a very important role in cell division, transport, and protein retention, but a direct demonstration of such involvement will be difficult to obtain. It is significant that I have been able to show that pantoyl lactone interacts with unsaturated fatty acids; however, pantoyl lactone may also associate with other types of molecules in the cell membrane which would account for some of its effects on cellular activities.

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