MEMBRANE LIPIDS OF DIVIDING AND NON-DIVIDING CELLS OF MICROCOCCUS LYSODEIKTICUS AND INTERACTIONS OF PANTOYL LACTONE WITH MODEL AND ISOLATED CELL MEMBRANES

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

Previous work from this laboratory has established that cell division inhibition in Erwinia carotovora can be induced by agents as diverse as D-amino acids, ultraviolet light, vancomycin, penicillin, aminopterin, 5-fluorouracil, S-(dichlorovinyl)-L-cysteine, mitomycin c, and D-cycloserine (Grula, 1960; Grula and Grula, 1962, 1964). Grula and King (1970, 1972) have further shown that D-serine, mitomycin c, penicillin, hydroxylamine, suboptimal concentrations of magnesium, and D-cycloserine inhibit division activity in a nutritional mutant of Micrococcus lysodeikticus (disII p^+). Many of these compounds, particularly penicillin, D-amino acids, and D-cycloserine, share the characteristic of decreasing levels of mucopeptide synthesis in addition to inhibiting cell division (Grula and Grula, 1964; King and Grula, 1972). These studies demonstrated that the inhibition of mucopeptide formation can lead to spheroplast formation in E. carotovora or protoplast formation in M. lysodeikticus culminating in lysis of the cell.

In addition to the reports of division inhibition in E. carotovora, Grula (1960) reported that a group of agents, which were equally as diverse as their division inhibiting counterparts, were capable of preventing or reversing filament formation. Two of these agents, pantoyl lactone (PL) and ω-methyl pantoyl lactone, were shown to induce preformed filaments of E. carotovora to revert to cells of normal size

regardless of the agent used to inhibit the division process (Grula and Grula, 1962). PL was subsequently shown to promote cell division without providing relief from the arrest of mucopeptide synthesis which suggested that a lesion, other than a mucopeptide deficiency, may be ultimately responsible for the inhibition of cell division. This finding led to the proposal that a lack of mucopeptide might create conditions favorable for secondary damage to the cell membrane (Grula and Grula, 1964). Grula, et al. (1968) and Grula and King (1971) later demonstrated that a functional parameter of the membrane, specifically transport ability, is altered in division inhibited cells of E. carotovora and M. lysodeikticus. Other observed membrane alterations include changes in the composition (quantitative) and conformation of membrane proteins in M. lysodeikticus (Grula and King, 1971), and leakage of several periplasmic enzymes, one of which has ATPase activity, in E. carotovora (Grula and Hopfer, 1972). The presence of PL was shown to prevent the loss of periplasmic enzymes, the conformational changes in membrane proteins, and largely, but not completely, the quantitative changes in protein. These data, coupled with the fact that we have never been able to demonstrate pantoyl lactone synthesis, uptake, or utilization in E. carotovora, provide indirect evidence that cell division inhibition as well as its prevention may be closely associated with the maintenance of cell membrane integrity.

Since current concepts of the structure of biomembranes place emphasis on the intimate relationship between cell membrane proteins and lipids, conformation changes in proteins having an obligate association with lipids, as well as leakage of peripheral proteins, might be accounted for by alterations in the phospholipid or phospholipid fatty acid compo-

nents of cell membranes. Many reports have recently appeared in the literature which relate membrane architecture and function with phospholipid and phospholipid fatty acid composition.

Although interactions between phospholipid head groups and proteins have been largely unexplored, Beebe (1972) reported that the uptake of several amino acids, uracil, thymine, uridine, adenosine, and pyruvic acid is inhibited in a mutant of Bacillus subtilis deficient in the synthesis of phosphatidylethanolamine. In reconstitution experiments with mitochondrial cytochrome oxidase and QH_2 - cytochrome c reductase, Eytan, et al. (1976) demonstrated that the activities of these enzymes are markedly stimulated when incorporated into liposomes containing acidic phospholipids. Further, Eytan and Racker (1977) have shown that cytochrome oxidase will incorporate exclusively into phosphatidylserine liposomes when a population of phosphatidylserine liposomes is mixed with a population of phosphatidylethanolamine liposomes. These authors suggest that the phospholipid composition of a cell membrane might dictate the correct insertion point in the membrane for a newly synthesized protein.

Fatty acid involvement in membrane structure and function has been more extensively investigated. Kito, et al. (1972) have reported that the unsaturated fatty acid composition of Escherichia coli increases with the age of the culture, and there has been extensive documentation of the decrease of the unsaturated fatty acid composition with increasing growth temperature for several organisms (Marr and Ingraham, 1962; Shaw and Ingraham, 1965; Shen, et al., 1970; and Cullen, et al., 1972). Similar observations have led Romijn, et al. (1972) to propose that the membrane fluidity and, therefore, the permeability of Acholeplasma laidlawii B is

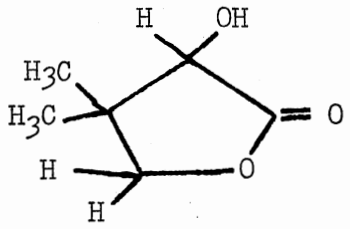
controlled by the regulation of the levels of unsaturated fatty acids in the membrane. Fox (1969) has observed that unsaturated fatty acids are necessary for the induction of a functional lactose transport system in a fatty acid auxotroph of E. coli. The addition of unsaturated fatty acids to the uptake medium of osmotically shocked cells of Staphylococcus aureus has been reported to stimulate the uptake of aspartic acid (Gale and Llewellin, 1970). Reconstituted cytochrome c oxidase has been shown to require the fluid environment provided by unsaturated fatty acids and highest activities for this enzyme are obtained when it is reconstituted into liposomes containing lysophospholipids (Vik and Capaldi, 1977). In a study relating the fatty acid composition of E. coli to cell division, Weinbaum and Panos (1966) reported that filamentous cells contain decreased levels of cyclopropane fatty acids.

Because of these considerations, division inhibitors such as D-cycloserine, which act at the level of mucopeptide synthesis, might exert a secondary effect on other membrane components, such as phospholipids and phospholipid fatty acids, through a loss of the insulating (and protective) effects of the cell wall. Furthermore, since PL has a 0.5 partition coefficient into n-octanol (Hopfer, 1972) and is able to prevent cell division inhibition without restoring mucopeptide synthesis; it is plausible that PL is able to exert its effects on the hydrophobic domains of the cell membrane. One phase of this research has been a test of this hypothesis.

A second phase of this research has been a direct study of the interaction of PL with isolated membranes, model membrane systems, and isolated proteins. Most of the techniques used in this study were physical methods capable of probing the interactions of individual groups

or atoms in a molecule and yielding information on small, local changes due to the interaction of the molecule with its environment. One of the important obstacles in defining the mechanism of action of PL is that a binding of PL to membrane components cannot be demonstrated. Furthermore, all of the biologically measureable effects of PL are readily reversed by simply washing the cells out of the medium containing the compound; therefore measurements capable of monitoring environmental changes in individual molecules were needed to detect the seemingly weak interaction of PL with the cell surface. The two pertinent considerations in this approach are the definition of the groups of the PL molecule that are capable of interacting with membrane components and the changes that occur in these components as a result of this interaction.

Perusal of the structure of the PL molecule reveals the potential for a wide variety of interactions



ranging from hydrogen bonding through the carbonyl oxygen and α-hydroxyl group to hydrophobic interactions through the β, β-dimethyl group. Two techniques which allow the monitoring of the environments of these groups are laser Raman spectroscopy and nuclear magnetic resonance spectroscopy.

The laser Raman spectrum of a molecule gives detailed information about the vibrational transitions of its substituents. For a molecular vibration to be Raman active, there must be a change in its induced dipole moment which results in a change in the polarizability of the

molecule (Colthup, et al., 1964). The basic quantitative expression of this effect will be summarized in the ensuing discussion while more detailed treatments are given by Peticolas (1972) and Chantry (1971).

Light transmitted through space has fluctuating electrical and magnetic fields at right angles to each other. The varying electrical field E, of a light wave with frequency ν' can be described by the expression:

$$E = E_o \cos 2\pi\nu' t \qquad (1)$$

where $\cos 2\pi\nu'$ describes the geometry of the electrical field, E_o is the maximum value of the electrical field and t is time. When this light is passed through a molecule, an oscillating dipole, μ , can be induced in a group of the molecule described by the expression

$$\mu = \alpha E \qquad (2)$$

where α is the polarizability of the substituent. By substituting the description of the electrical field (equation 1) into equation 2, it can be seen that:

$$\mu = \alpha E_o \cos 2\pi \nu' t \qquad (3)$$

which states that an incident light wave will induce a dipole moment in the molecular substituent which oscillates at the frequency as that of the incident light wave. The molecule can now emit radiation at the same frequency as the incident light giving Rayleigh scattering.

The polarizability, α , of the group is not a constant value because vibrations and rotations cause the molecular shape to become compressed and extended. The polarizability of a polyatomic molecule is given by:

$$\alpha = \alpha_o + \frac{\partial \alpha}{\partial Q} Q \qquad (4)$$

where α_o is the equilibrium polarizability and $\partial\alpha/\partial Q$ is the rate at

which α changes during a normal mode of vibration, Q, as the atoms pass through their equilibrium positions. The normal mode of vibration, Q, is considered simple harmonic motion and is described by:

$$Q = Q_o \cos 2\pi \nu_v t \tag{5}$$

where ν_v is the vibrational frequency. This equation can be substituted into the description of the polarizability of the polyatomic molecule (equation 4) resulting in the expression:

$$\alpha = \alpha_o + \frac{\partial \alpha}{\partial Q} Q_o \cos 2\pi \nu_v t. \tag{6}$$

The description of the oscillating induced dipole can now be completed by substituting equation six into equation three yielding:

$$\mu = \alpha_o + \frac{\partial \alpha}{\partial Q} Q_o \cos 2\pi \nu_v t \, (E_o \cos 2\pi \nu' t). \tag{7}$$

This expression can be rearranged to give:

$$\mu = \alpha_o E_o \cos 2\pi \nu' t + \frac{E_o Q_o}{2} \frac{\partial \alpha}{\partial Q} (\cos 2\pi \nu' t)(\cos 2\pi \nu_v t) \tag{8}$$

and expanded to:

$$\mu = \alpha_o E_o \cos 2\pi \nu' t + \frac{E_o Q_o}{2} \frac{\partial \alpha}{\partial Q} (\cos 2\pi (\nu' + \nu_v) t + \cos 2 (\nu' - \nu_v) t) \tag{9}$$

Equation 9 shows that the dipole moment induced in the components of a molecule by a beam of incident light can give rise to three types of scattering:

a. Rayleigh scattering described by

$$\alpha_o E_o \cos 2\pi \nu' t$$

b. Stokes scattering described by:

$$\frac{E_o Q_o}{2} \frac{\partial \alpha}{\partial Q} \cos 2\pi (\nu' - \nu_v) t$$

c. Anti-Stokes scattering described by:

$$\frac{E_o Q_o}{2} \frac{\partial \alpha}{\partial Q} \cos 2\pi (\nu' + \nu_v) t \quad .$$

Rayleigh scattering is dependent upon the equilibrium polarability (α) of the groups while the Raman lines (or Stokes and anti-Stokes lines) are dependent upon a change in the polarizability of the substituents of the polyatomic species caused by the vibrations of these groups ($\partial\alpha / \partial Q$). Since Stokes lines originate from components in the vibrational ground state and anti-Stokes lines arise from components in an excited vibrational state, Raman spectra are usually reported as Stokes lines because at ambient temperatures, most of the groups of a population of molecules are in the vibrational ground state: conditions which make Stokes lines significantly stronger than anti-Stokes lines.

As pointed out by Spiro and Gaber (1977), the Raman effect is a low probability event which makes it necessary for large amounts of incident radiation to be applied to the sample. The application of laser technology to Raman spectrometers has made the technique available for study of biological samples since the laser overcomes this disadvantage. The advantage of the technique in studying biological samples is that the detection of Raman scattering ($\nu' - \nu_v$) discriminates against Rayleigh scattering (ν') which allows the study of environmentally sensitive vibrations of biological samples in a variety of physical states (ie. solids, mixtures of molecules, and tissues).

Laser Raman spectra have been obtained for a wide variety of

biological materials. Freeman (1974) and Spiro and Gaber (1977) have reviewed work done with isolated proteins. The bulk of the information obtained with protein samples has pertained to conformation, conformational changes and the environments of amino acid residues within the protein. Verma, et al. (1975) have extended these finding to the measurement of protein conformation in the plasma membrane and endoplasmic reticulum of thymocyte membranes. These authors also compared carbon skeleton stretching modes of synthetic phospholipid liposomes with thymocyte plasma membrane and endoplasmic reticulum lipid stretching modes to monitor phase transitions within these membranes. Spiker and Levin (1976) have shown that cholesterol incorporated into phosphatidylcholine vesicular liposomes causes an increase in vesicle size and preferentially distributes within the inner face of the bilayer.

In samples containing chromophores with $\pi \rightarrow \pi^*$ transitions, the laser frequency can be tuned to a frequency close to(or coincident with) the electronic absorption of the chromophore. The Raman spectrum obtained under these conditions is resonance enhanced by a factor of up to 10^6 (Freeman, 1974). This results in a large sensitivity and selectivity advantage in the study of chromophoric groups. This technique has been used to monitor changes in the iron states of oxidized and reduced cytochrome c (Strekas and Spiro, 1972), conformational changes in the retinal substituent of bacteriorhodopsin during the absorption of light (Campion, et al. 1977), and the distribution of antenna chlorophyll a and chlorophyll b in chloroplasts of green plants and unicellular algae (Lutz, 1977). Gill, et al. (1970) have shown that membrane carotenoids are sensitive to changes in environmental polarity and Szalontai, et al. (1977) have used the resonance

enhancement of these carotenoids to monitor action potentials in frog sciatic nerve.

As can be seen, the technique of Raman spectroscopy not only lends itself to the study of the various interactions of the substituents of PL, but also is useful in the assessment of the effects of the PL interaction on a variety of membrane components.

A second method of studying these interactions is nuclear magnetic resonance (NMR) spectroscopy. Most of the work with biological samples has employed the chemical shift of the proton continuous wave spectrum for monitoring changes in molecular structure, but the application of Fourier transform technology to pulsed systems has expanded the use of NMR to include chemical shift information of low natural abundance nuclei (ie. carbon-13, deuterium, fluorine-19, and phosphorous-31). Also, spin-lattice (T_1) and spin-spin (T_2) relaxation of nuclei in biological systems have provided useful information on the local environment surrounding the nucleus.

A voluminous amount of material has been published on the study of proteins using this technique and these works have been reviewed by Wüthrich (1976), Roberts and Jardetzky (1970), and Komoroski, et al. (1976), Recently, high resolution spectra have been obtained with biological tissues. Enrichment of E. coli membranes with ^{13}C-tyrosine and m-fluorotyrosine by growth of the organism in the presence of these compounds has allowed Lee, et al. (1977) to show that carboxy-terminal region of membrane lipoprotein is relatively mobile in situ. Cramer and Prestegard (1977) have shown that transmembrane pH gradients affect the transport of carboxylic acids across phosphatidylcholine-cholesterol vesicular liposomes and that these acids cross in a protonated form.

Matthews, et al.(1977) have used high resolution proton NMR to demonstrate changes in the E. coli lactose operon repressor upon binding isopropyl-β -D-thiogalactoside.

In studies with phospholipid liposomes, Fung and Martin (1975) reported that the polar head groups of phospholipids contained a bound layer of water. Lysophospholipids have been shown to be distributed toward the outer monolayer of vesicular phospholipid liposomes, but the addition of cholesterol disrupts this distribution (De Kruyff, et al., 1977. Interactions of small moleucles with outer monolayer phospholipid polar head groups in vesicular liposome preparations have been demonstrated because the head group signals of the inner and outer monolayers can be resolved when lanthanide ions are added to the suspension (Bystrov, et al., 1972).

NMR has also been applied to the study of metabolic functions. Spin-lattice and spin-spin relaxation times of water have been shown to be dependent upon the physiological state of tissue samples (Fung, 1977). Navon, et al. (1977) have reported that the intracellular pH of E. coli is raised during respiration creating an outward directed pH gradient, and the ATPase inhibitor, dicyclocarbohexadiimide, prevented the formation of this gradient. In an elegant study utilizing the phosphorous resonance of nucleotides, Sehr, et al. (1977) have monitored metabolic degeneration and recovery of rat kidneys during transplant operations.

One of the experimental advantages of both laser Raman and NMR spectroscopy is that they provide the versatility needed to observe a wide variety of molecular interactions and the consequences of these interactions without the use of potentially disruptive probe. For this reason, these two techniques have provided the bulk of the information

we have obtained about the interaction of PL with isolated and model membrane systems.

Although a study of the interaction of PL with isolated and model membrane systems may not by itself yield a molecular explanation for cell division, such a study is essential for the understanding of how molecules such as PL influence membrane processes, wherein triggering of the process occurs (Grula and Smith, 1965). A possible understanding of this key event has been the focal point for this investigation.

CHAPTER II

MATERIALS AND METHODS

Test Organism

Micrococcus lysodeikticus dis-II p^+ is a mutant of the Purdue University strain which is cell division inhibited by D-serine and has the ability to synthesize purines. The mutant was isolated as a nutritional mutant according to the procedures outline by Grula and King (1970). The biochemical characteristics of the organism are identical to those described for the parent strain by Grula (1962).

Twenty-four hour old cells, grown at 37°C on slants of the defined medium solidified with 0.8 percent agar agar No. 3 (Oxoid-Consolidated Laboratories, Incorporated, Chicago Heights, Illinois), were used as the inoculum for all experiments.

Medium and Growth

The defined medium utilized in the growth of this organism contained the following compounds per 100 ml liquid: biotin (50 mg), L-glutamic acid (358 mg), L-aspartic acid (358 mg), L-phenylalanine (40 mg), L-tyrosine (30 mg), Na_2HPO_4 (200 mg), $MgSO_4 \cdot 7H_2O$ (2mg). The pH of the medium was adjusted to between 7.6 and 7.8 using solid KOH, to preserve the delicate sodium-potassium balance required by this organism, before autoclaving for 15 minutes at 250°F. A saturated solution of $FeSO_4$ $(NH_4)_2SO_4 \cdot 6H_2O$) was autoclaved separately and the

resulting precipitate was removed by filtration before an aliquot (0.5 ml per 100 ml culture medium) was added aseptically to the medium.

Cells taken from the defined medium slants were washed twice with 0.85 percent sterile saline and adjusted to an optical density of approximately 0.5 (Bausch and Lomb Spectronic 20) before the addition of five drops of the suspension to a 250 ml Erlenmeyer flask containing 100 ml of medium. Cultures were then incubated at 37°C on a Gyrotory shaker (New Brunswick Scientific Company, New Jersey, Model G-26) set at a shaking speed of 8.5(200 rpm).

Division inhibition was chemically induced by adding D-Cycloserine (DCS) to the growth medium to a final concentration of 2×10^{-4} M. DCS was dissolved in 0.1 M K_2HPO_4, pH 7.2 before filter sterilization. L-Cycloserine (LCS) was treated in a similar manner and was added to a final concentration of 2×10^{-4} M. In studies where cell division inhibition was prevented with D-alanine (DA), the DA was dissolved in the same buffer and added to the growth medium in a 5×10^{-5} M final concentration. Pantoyl lactone (PL) was added to a final concentration of 0.15 M.

The compounds used in this study were filter sterilized and added aseptically to the growth medium after the cultures had been allowed 12 hours growth in the defined medium.

Isolation of Cell Membranes

Cell membranes from M. lysodeikticus were prepared according to the procedures of Butler, Smith and Grula (1967).

Lipid Extraction

Lipid extractions were carried out according to the procedures of

Folch, et al. (1957). After growth in the appropriate medium, a 100 ml culture was harvested by centrifugation, washed one time in saline and resuspended in 5 ml of methanol in a screw cap tube. The samples were flushed with nitrogen and capped with a teflon-lined screw cap. The sealed tubes were incubated for 30 minutes in a 55 C water bath. After cooling to room temperature, 10 ml of chloroform was added and the extraction was continued at 25°C for twelve hours under a nitrogen atmosphere. The insoluble material was removed from the sample by passing the suspension through a 0.45 u size Millipore filter. The chloroform-methanol suspension was then washed twice with 15 ml of 2 M KCl followed by one wash with 10 ml of distilled water. The resulting chloroform solution was then passed through fresh sodium sulfate columns ($\frac{1}{2}$ cm by 14 cm) and dried under nitrogen at 45°C. The last $\frac{1}{2}$ ml was dried at room temperature to prevent possible oxidation.

Preparation of Methyl Esters

A solution of 2% H_2SO_4 in column dried (sodium sulfate) methanol (v/v) was used to prepare the methyl esters of the phospholipid fatty acids. Phospholipids prepared as described above were dissolved in 4.5 ml of the methanolic H_2SO_4 and sealed in screw capped tubes under nitrogen. After twelve hours at 25°C, 4.5 ml of water was added and each sample was extracted three times with 3 ml of hexane. The hexane extracts were pooled and washed one time with 4 ml of distilled water before passage through fresh columns of sodium sulfate ($\frac{1}{2}$ cm by 14 cm). The resulting hexane solutions were dried under a stream of nitrogen at 45°C (except for the last $\frac{1}{2}$ ml, which was dried at room temperature to prevent possible oxidation). The resulting methyl esters were stored under a nitrogen atmosphere at -20°C until the time of analysis.

Lipid Leakage Determinations

Experiments to determine lipid leakage into the growth medium were performed by growing M. lysodeikticus for twelve hours in the defined medium supplemented with 1 μC of L-aspartic acid-U-^{14}C (154 mC/mM) and 1 μC of L-glutamic acid-U-^{14}C (206 mC/mM). The cells were harvested by centrifugation, washed three times in 0.85 percent sterile saline, and reinoculated into 100 ml unlabeled medium containing the test compounds.

At various times in the growth cycle, 10 ml of the medium was withdrawn and the cells, as well as membranes from lysed cells, were removed by centrifugation at 78,000 x g in a Spinco Model L ultracentrifuge. The medium was then lyophilized and the lipids were extracted as outlined above.

Gas-Liquid Chromatography

The gas-liquid chromatography unit used to separate the methyl esters of the fatty acids was a Perkin Elmer 990 Gas Chromatograph equipped with a hydrogen flame detector. Column and instrument conditions were as follows:

Air Pressure ... 40 PSI

Hydrogen Pressure ... 15 PSI

Detector Temperature ... 250°C

Injection Port Temperature ... 225°C

Carrier Gas ... Nitrogen

Carrier Gas Flow Rate ... 40 ml/min.

Column Specifications ... 6' x $\frac{1}{4}$" glass

Column Packing ... 14% Diethylene Glycol Succinate on a Chromosorb Support

Column Temperature ... 165°C

Sample Specifications ... 1 to 4 ul in Isooctane

The methyl esters of sample fatty acids were identified by comparing retention times with known fatty acid methyl ester standards (obtained from Sigma Chemical Company, St. Louis). Further analyses, utilizing coupled gas chromatography and mass spectrometry, confirmed the identifications. Mass spectra were evaluated by the criteria outlined by Ryhage and Stenhagen (1960). Both the gas-liquid chromatography and mass spectrometer units are located in the Department of Biochemistry, Oklahoma State University.

Quantitative determinations of fatty acids from gas-liquid chromatography were made in the following manner. Extractions were made from whole cells, membranes, or fatty acid standards of known dry weight. Before each gas-liquid chromatographic analysis of samples, detector responses to known amounts of fatty acid methyl ester standards were determined by measuring the area under the curves of five standard methyl ester preparations. Detector responses to each methyl ester of the sample was then determined by computing the area under each curve. These areas were then assigned a weight value based on the detector response to the standards. The weight was then compared to the total dry weight of the extracted cell material.

Thin-Layer Chromatography

Phospholipids were identified using thin-layer chromatography as described by Stahl (1960). Glass plates, 20 x 20 cm square, were layered with Silica Gel G (E.M. Reagents, Westbury, N. Y.) 250 microns thick and dried at room temperature. Plates were activated at 100°C for one

hour immediately before use. Chromatograms were developed in a solvent system of chloroform-methanol-water (65:25:4 by volume) after allowing the solvent to equilibrate for one hour.

In situations where separation of neutral lipids and β-carotene from phospholipid was desired, the thin-layer chromatographic procedure of Overath, Pauli, and Schairer (1969) was used. This procedure consists of layering the 20 X 20 cm glass plates with a 250 micron thick layer of silicic acid (100 mesh from Mallinckrodt Chemical Works, St. Louis) and allowing the plates to dry at room temperature. After a one hour activation period at 100°C, the chromatograms are developed in a solvent system of ether-acetic acid (100:1 by volume). In this system, neutral lipids move with the solvent front, β-carotene moves to an Rf of 0.8, and phospholipids remain at the origin.

After development, the chromatograms were first observed under ultraviolet light and then sprayed with the appropriate spray reagent. Phosphate-containing compounds were detected using the $FeCl_3$-sulfosalicylic acid spray indicator of Wade and Morgan (1953). The identifications of phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylinositol were made on the basis of comparing Rf values to known standards (Sigma Chemical Company, St. Louis) and on the Periodate-Schiff's test for vicinal hydroxyl groups (Shaw 1968). Lysophospholipids were identified in essentially the same manner except that they were prepared by treating known standards, as well as lipids from control cells, with phospholipase-A_2 (Type I, Sigma Chemical Company, St. Louis).

Phospholipid quantitation was achieved by two methods. First, each phospholipid was taken from a developed chromatogram, hydrolyzed to form the fatty acid methyl esters, and quantitation of the amount

of fatty acid hydrolyzed per phospholipid was determined. Secondly, lipids were labeled by growth in the presence of 1 μc of L-aspartic acid-U-^{14}C (173 mC/mM), 1 μc of L-glutamic acid-U-^{14}C (195 mC/mM, or 1 μc of sodium acetic acid-^{3}H (350 mC/mM). Lipids from these cells were then extracted and separated using thin-layer chromatography. Each spot was scraped from the developed plate, eluted from the support, and counted with a Beckman LS-3133 liquid scintillation counter.

Uptake Studies

Cells were harvested after each growth situation indicated in the text, washed twice in a suspension of the salts from the growth medium, and resuspended to an optical density of 0.5 measured at 540 nm. These suspensions were then incubated on a Burrell Wrist-Action shaker at a setting of 5 for 20 minutes before the addition of labeled compounds. Labeled compounds (100 ul of a stock solution containing 5.0 μC labeled compound and 100 μg "cold carrier" per 10 ml) were added to 2.6 ml cells. Compounds being investigated for inhibitory activity were added 30 seconds prior to the addition of label. These compounds were added in 300 μl volumes bringing the total volume of the suspension to 3 ml. When no inhibitory compound was added, 300 ul of the medium salt solution was added, At appropriate time intervals, 0.5 ml aliquots were withdrawn and passed through a 0.45 μm pore size Millipore prewetted filter using vacuum suction. The filters were immediately washed three times with 1 ml of chilled (4° C) medium salt solution. The filters containing the washed bacterial cells were immediately placed in scintillation vials containing 10 ml Aquasol counting cocktail. Samples were then incubated at room temperature for 2 hours to permit dissolution of the membrane

filters before counting. This procedure proved satisfactory for measuring the uptake of readily metabolized substrates.

The following labeled compounds were used: D-alanine-U-^{14}C (51 mC/mM), L-aspartic acid-U-^{14}C (173 mC/mM), L-glutamic acid-U-^{14}C (195 mC/mM).

Sucrose and Glycerol Density Gradient Sedimentation

Density gradient sedimentation analyses of isolated cell membranes were performed by layering a 5 ml suspension of isolated cell membrane on discontinuous sucrose gradients from 30 to 70% sucrose (weight: volume) solutions in 215 nM tris pH 7.4. These gradients were placed in an SW-27 rotor and centrifuged at 18,000 rpm for two hours at 4° C in a Beckman L3-50 ultracentrifuge. Longer centrifugation times did not result in any further change in sedimentation patterns.

Sedimentations in discontinuous glycerol gradients from 50% to 90% glycerol (volume: volume) were performed under the same conditions except that a centrifugation time of 4 hours was required for equilibrium to be reached.

Gradients were fractionated by mounting the tubes on a ring stand and puncturing the bottom of the tube with a fixed 10 gauge needle. Fractions were collected in 3 ml volumes and optical density at 540 nm was monitored. For lipid analyses, the fractions were diluted with 20 mls of 2.5 mM tris pH 7.4 and harvested by centrifugation. The lipid analyses were performed as described above.

Liposome Preparation

The method for preparation of vesicular liposomes was a modification

of the procedure of Barenholz et al. (1977). All phospholipids used, except for those isolated from M. lysodeikticus, were obtained from Sigma Chemical Company, St. Louis, and were checked for purity by thin-layer chromatography. Phospholipids were suspended in a 5 ml chloroform-methanol (2:1 by volume) in the desired concentrations (indicated in the text) and dried in a 250 Erlenmeyer flask under a stream of nitrogen. The dried phospholipid was suspended in a 10 ml solutions of 10 mM tris buffer containing 50 mM KCl at pH 7.2 by adding five (2 mm diameter) glass beads to the flask and shaking at 45° C on a Burrell Wrist action shaker at a setting of 7 under a stream of nitrogen. Shaking for 30 minutes resulted in a "milky" white suspension which was then passed through a 75 mm long 24 gauge needle one hundred times. The resultant suspension was then sonicated until optical clarity was reached. This routinely required twelve to fifteen 30 second bursts at maximum power with a Branson ultra-sonicator. For nuclear magnetic resonance experiments, 10 mM NaCl was substituted for tris and deuterium oxide which was 99.96% deuterium enriched (Norell Chemical Company, Landisville, New Jersey) was substituted for water. The water used in these preparations was triple glass-distilled water which had been passed through activated charcoal.

A homogenous vesicle suspension was obtained by centrifuging the suspension at 100,000 X G for 30 minutes (Type 40 fixed angle rotor) to remove multilamellar and large vesicular liposomes. The time course of vesicle formation can be monitored by observing the resolution of the $-N^{+}-(CH_3)_3$ proton signal occuring at 3.2 ppm downfield from tetramethylsilane in the proton magnetic resonance spectrum of phosphatidylcholine.

Phospholipids used in preparing vesicular liposomes were: synthetic dipalmitoyl phosphatidylcholine, soybean phosphatidylcholine, egg-yolk

phosphatidylcholine, and the phospholipids purified from M. lysodeikticus.

Equilibrium Dialysis

Equilibrium dialysis measurements of the binding of PL to lysozyme and isolated cell membranes from M. lysodeikticus were carried out by suspending the appropriate sample in 2.5 mM tris (pH 7.4) containing 0.15 M PL and placing 10 ml of this suspension inside dialysis tubing having an exclusion limit of 10,000 daltons. The suspension was then dialyzed against 90 ml of the 2.5 mM tris pH 7.4 solution for 24 hours at 4° C. After the incubation period, 1 ml aliquots were taken from the inside of the tubing and the surrounding medium and the concentration of PL in each compartment was determined using the method of Hestrin (1949).

Lasar-Raman Spectroscopy

Raman spectra were obtained on a Raman spectrometer located in the Department of Chemistry, Oklahoma State University. The laser source was a Coherent Radiation-52 Argon laser coupled with a Jarrell-Ash double monochromater. Detection of scattered radiation was accomplished using a Hamner photon counting system. Samples of about 20 µl were placed in a sealed glass capillary cell having a 1 mm path length and the incident laser beam was focused at a grazing angle from below the sample. "Dark counts", or the background scatter created by the cell, were less than 200 photons per second. The laser power was 28 Amps.

Solvents used in these studies were at least reagent grade and were spectroscopic grade where possible. Each solvent was scanned in

the Raman unit for purity before use. Because of the fluorescent properties of contaminating molecular species in water, the water used in this study was triple glass distilled, charcoal and Millipore (0.45 M) filtered. This water was not treated with ion exchangers because of their tendency to contribute to the fluorescence background of the water. Deuterium oxide (Norell Chemical Company, Landisville, New Jersey) did not have an extensive fluorescence background and was therefore used withour further purification. When phospholipid liposomes were studied using this technique, a period of about 30 minutes exposure to the laser was required before fluorescence levels were low enough to give satisfactory spectra. This preincubation time was not required in resonance spectra of the β-carotene molecule because the resonance enhancement dominates the fluorescence levels. Fluorescence levels were also minimized when the laser frequency was 5145 Å, rather than 4880 Å, which had no effect on the Raman scattering intensities of the sample. Because of these considerations, the 5145 Å frequency was used exclusively.

Nuclear Magnetic Resonance Spectroscopy

Continuous wave proton magnetic resonance spectra were obtained on a Varian XL-100 spectrometer equipped with 15 inch magnets which is housed in the Department of Chemistry, Oklahoma State University. Solvents and experimental conditions were varied according to the type of experiment and are outlined in the text. All proton magnetic resonance spectra we obtained at a 100 MHz frequency. Analysis of the chemical shift (δ) and the spin coupling constant (J) for the AB splitting pattern seen in the methylene protons of PL were according to the procedure of Pople,

et al. (1959). All measurements were made in 5 mm tubes.

Spin-lattice (T_1) and spin-spin (T_2) relaxation times for deuterobenzene (99.99%, Merck and Company, Rahway, N. J.) in phosphatidylcholine liposomes, M. lysodeikticus membranes, and liposomes prepared from lipids isolated from M. lysodeikticus were obtained at 9.2 MHz on a home built, pulsed NMR spectrometer in the laboratory of Dr. Bing Fung, Department of Chemistry, The University of Oklahoma. This spectrometer is equipped with 12 inch high-resolution magnets and a Bruker variable temperature probe.

Values for T_1 relaxation times were determined by the $180^\circ - \tau - 90^\circ$ technique. The rotational correlation time, τ, was varied from $\tau = 0$ to $\tau = \infty$ and the signal intensity was measured as a function of each τ value. For the $\tau = \infty$ measurement, the 180° pulse is eliminated resulting in conditions where every nucleus is in thermal equilibrium. The intensities of the signals as a function of τ are related to T_1 by the expression:

$$V_{\tau=\infty} - V_{t=\tau} = 2M_o e^{-t/T_1}$$

Therefore:

$$\ln (V_{\tau=\infty} - V_{t=\tau}) = \ln M_o^{-\tau/T_1}$$

The slope of the line obtained from plotting $\ln (V_{\tau=\infty} - V_{t=\tau})$ as a function of τ is $1/T_1$.

Measurements of T_2 relaxation times were made by the $90^\circ - \tau - 180^\circ$ technique. This method consists of pulsing the sample with a 90° radiofrequency pulse, allowing τ relaxation time, and pulsing the sample with a 180° radiofrequency pulse. The 180° pulse is applied at time

τ, 3τ, 5τ ... $n\tau$ and the Hahn spin echo amplitude is measured at 2τ, 4τ, 6τ ... $(n + 1)\tau$. The Hahn spin echo amplitude is proportional to $e^{-\tau/T_2}$ and T_2 is calculated from the exponential decay of the spin echo amplitude. In experiments measuring deuterobenzene T_2, the rotational correlation time, τ, used was six seconds. All relaxation experiments were done in a 12 mm tube at 25°C and signals were accumulated with a Nicolet 1070 signal averager.

Fluorescence Spectroscopy

Fluorescence spectroscopic measurements were made with an Amicon-Bowman Spectrophotofluorometer located in the Department of Biochemistry, Oklahoma State University. Recrystallized 8-anilinonapthalene - 1 - sulfonic acid (ANS) at a final concentration of 2×10^{-3}M was used as the fluorescent probe. ANS was recrystallized by saturating an aqueous solution containing a molar excess of $MgCl_2$ with ANS at 70°C. A small amount of decolorizing carbon (Norit-A) was added and the solution was boiled for five minutes. After filtration, crystallization was accomplished by allowing the suspension to cool to room temperature. The crystals were then collected by filtration. Procedures for measuring fluorescence intensity as a function of temperature were essentially those outlined by Overath and Trauble (1973). Samples of membrane from M. lysodeikticus were placed in the cuvette, ANS was added, and a thermophile was positioned in the cuvette and connected to the X-axis of an X-Y recorder. Excitation of ANS was at 340 nm while emission intensity, measured at 480 nm, was monitored on the Y axis of the recorder.

Circular Dichroism Spectroscopy

Circular dichroism spectroscopy measurements on the lysozyme molecule were made on a 7% lysozyme solution in 0.01 M NaCl. Measurements made in the presence of PL were made on a sample of similar composition using a 0.15 M concentration of PL. Measurements were made by scanning between 350 nm and 190 nm on a Cary Model 61 spectrometer located in the Department of Biochemistry, Oklahoma State University. Calculations of the helical content of the molecule were made according to the method of Chen and Yang (1971).

Electron Paramagnetic Resonance

Electron paramagnetic resonance spectra were obtained using an electron paramagnetic resonance spectrometer constructed in the Physics Department at Oklahoma State University. The instrument is composed of the following units:

Varian 100 KHz field modulation and control unit V4560

Varian V 4531 rectangular cavity

Varian V153/6315 reflex klystron tube (90 Mwatts)

Varian 4007-1 electromagnet

Varian V2200 regulated power supply

Varian E-248-1 aqueous solution sample cell

Samples were placed in a sealed pasteur pipet after labeling with 2 - (10-carboxydecyl) - 2 - hexyl - 4, 4' dimethyl - 3 - oxizolidinyloxyl (5NS) which was obtained from Syva Associates, Palo Alto, California. The label was dissolved in CCl_4 at a concentration of 1 mg/ml and 1 ml of this solution was evaporated under nitrogen which leaves a thin film of the label on the sides of the test tube. An aqueous membrane suspen-

sion was added to the test tube and allowed to stand for two hours at room temperature. Spectra of the spin labels were always run prior to obtaining the sample spectra.

CHAPTER III

RESULTS

Lipid Studies

The Fatty Acid Composition of Whole Cells and Isolated Membranes from Dividing and Non-dividing Cells of M. lysodeikticus dis II-p^+

D-Cycloserine (DCS) is able to inhibit cell wall mucopeptide synthesis by competing with D-alanine (DA) for binding sites on the alanine racemase and D-alanyl-D-alanine synthetase enzymes (Strominger, et al., 1960; Neuhaus, 1967). In addition to the inhibition of mucopeptide synthesis, King and Grula (1972) demonstrated that DCS induces cell division inhibition in M. lysodeikticus dis II-p^+. Addition of DA to the culture medium containing DCS relieves both mucopeptide synthesis and cell division inhibition. These investigators further showed that the addition of pantoyl lactone (PL) to the growth medium was able to overcome cell division inhibition without the restoration of mucopeptide synthesis. Because mucopeptide synthesis remains suppressed and because PL is needed at non-enzymatic concentrations (0.15 M) for prevention of the effects of DCS on division, it is likely that PL and DA act via different mechanisms to allow division under otherwise non-permissive conditions.

The growth of M. lysodeikticus in the presence of DCS, DCS with DA,

DCS with PL, and L-cycloserine (LCS), which inhibits growth but has no effect on cell division, is shown in Figure 1. As can be seen, DA and PL also affect growth differently. Cultures grown in the presence of DCS and DA exhibit growth rates quite similar to control cultures, but cells grown in the presence of DCS and PL show approximately 50% inhibition of growth. Microscopic examination of these cultures reveals cells of normal size and lysis does not occur with either treatment. LCS induces growth inhibition (Fig. 1) but does not cause cell division inhibition. Cells grown in the presence of DCS (2×10^{-4} M) show little evidence of either growth or cell division inhibition through the first four hours; but, six hours exposure to DCS results in growth inhibition and microscopic examination of the culture reveals the presence of large cells and evidence of lysis. By the eighth hour, lysis becomes evident in the culture and continues throughout the duration of exposure. Therefore, cultures harvested at six hours (before extensive lysis occurs) show optimal effects of DCS on cell division. Because of this consideration, lipid analyses were routinely performed on cultures harvested after six hours exposure to the test compounds.

Cho and Salton (1966) have reported that aerobic Gram-positive bacteria have large amounts of branched-chain fatty acids (FA), and the mass spectra of the FA from M. lysodeikticus reveal that this organism is no exception. The mass spectrum of the most abundant FA in M. lysodeikticus is shown in Fig. 2. It is 12 - methyltetradecanoic acid [C 15:br (anteiso)] and has been identified by use of the criteria outlined by Rhyage and Stenhagen (1960) for distinguishing FA isomers. The base peak of the spectrum at m/e = 74 is characteristic of FA methyl

Fig. 1. Growth Response of M. lysodeikticus to DCS, DCS with DA, DCS with PL, and LCS Added After Twelve Hours Incubation. ⬢, control cells; ▲, with DCS (2×10^{-4} M) and DA (4×10^{-5} M); ●, with LCS (2×10^{-4} M); □, with DCS (2×10^{-4} M) and PL (0.15 M); △, with DCS (2×10^{-4} M).

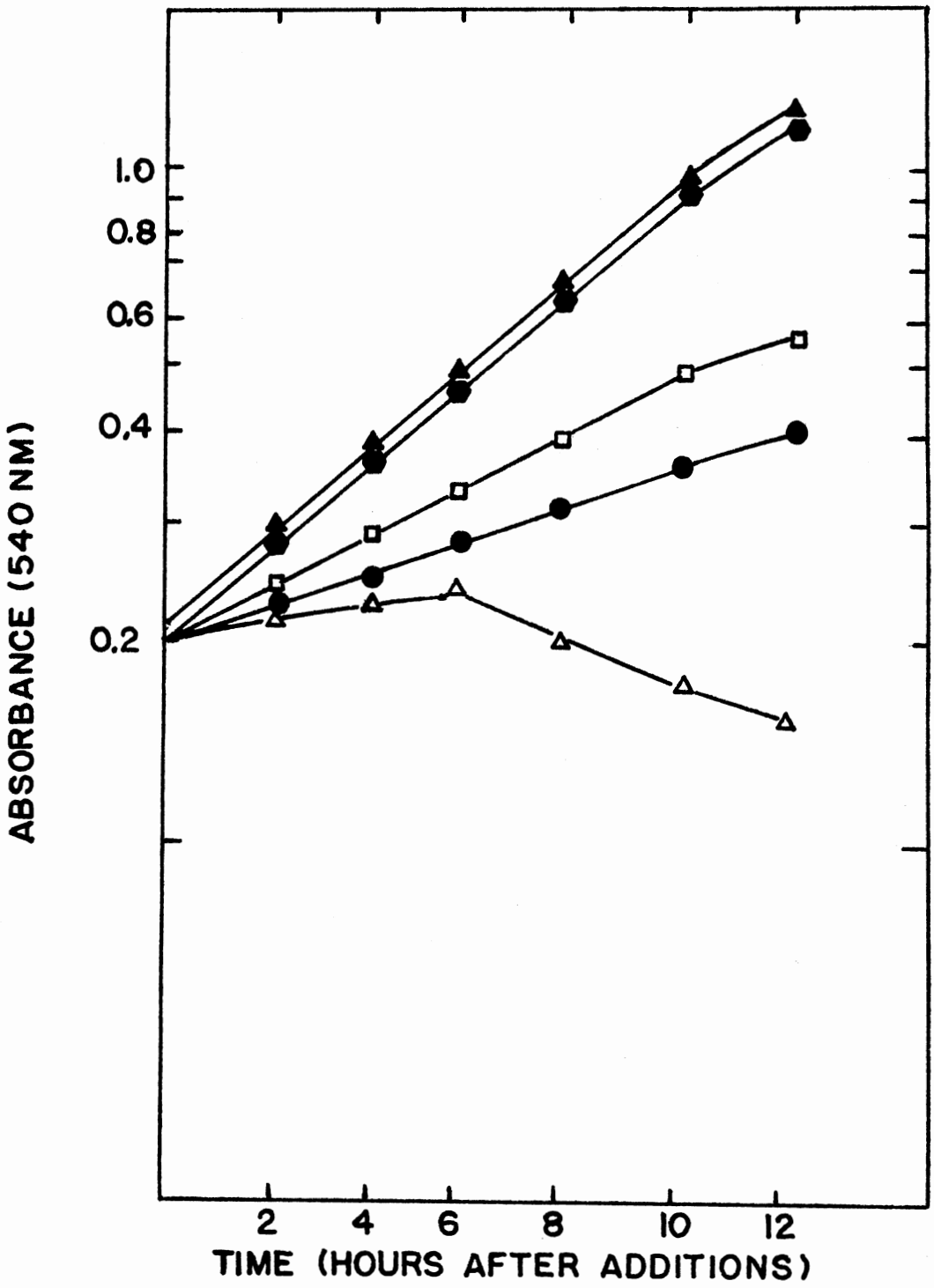
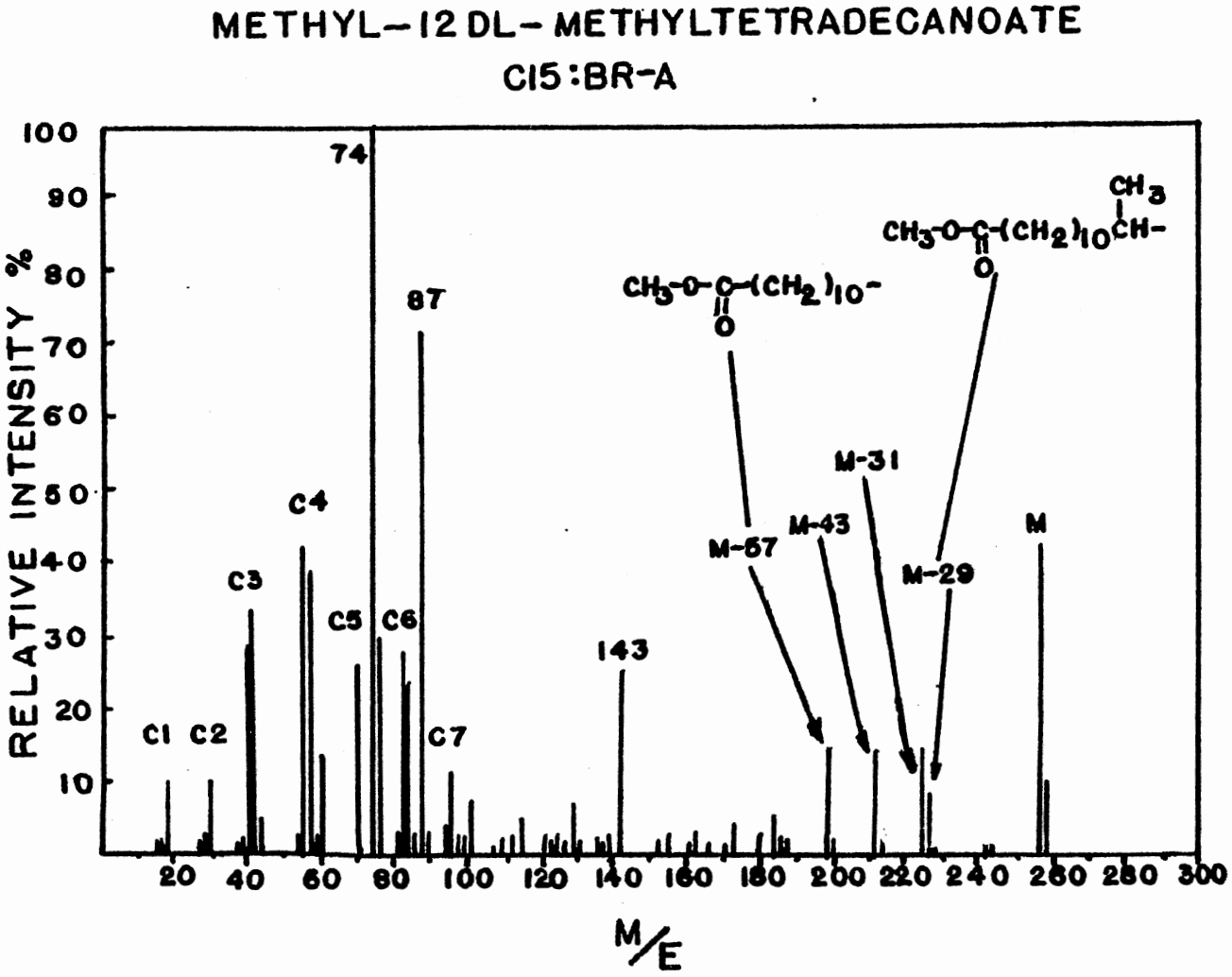

ABSORBANCE (540 NM)
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
12
TIME (HOURS AFTER ADDITIONS)

Fig. 2. Mass Spectrum of 12-Methyltetradecanoic Acid Methyl Ester.


METHYL-12 DL-METHYLTETRADECANOATE
C15:BR-A
RELATIVE INTENSITY %
100
90
80
70
60
50
40
30
20
10
74
87
C1
C2
C3
C4
C5
C6
C7
143
M-57
M-43
M-31
M-29
M
CH3-O-C-(CH2)10-
CH3-O-C-(CH2)10CH-
CH3
O
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
M/E

esters and represents the loss of the CH_3-O-C(OH) = CH_2^+ fragment from the methylated carboxyl group of the molecule. The anteiso character of this FA is confirmed by peaks occurring at M-29 and M-57 which represent the loss of the acyl chain terminus two-carbon fragment and four-carbon fragment (containing the branched CH_3 group) respectively. The mass spectrum of the second most abundant FA, 14-methylpentadecanoic acid [C 16:br (iso)], is shown in Fig. 3. The spectrum of this molecule very closely resembles that of a straight chain FA except that it has a characteristic weak peak at M-65 instead of an intermediate strength peak at M-63.

Perusal of Table I reveals that 83% of the total FA found in whole cells of M. lysodeikticus dis II-p^+ are branched chain. The C 15:br (anteiso) FA comprises about 64% of the total FA of the control cell while the C 16:br (iso) FA represents about 12% of the total. Palmitic acid (C 16:0) and 14-methylhexadecanoic acid [C 17:br (anteiso)] are about 8 and 5% of the total respectively. Quantitative measurements show that FA accounts for approximately 7% of the dry weight of the control cell.

Also contained in Table I are the FA compositions of cells grown in the presence of DCS and LCS. A comparison of the FA from these cultures with control cultures fails to show any qualitative or quantitative effect of these compounds on the whole cell FA composition.

The FA composition of cells grown in the presence of DCS supplemented with DA or PL are given in Table II. Since DCS has little effect on the FA composition of M. lysodeikticus, it would be expected that prevention of DCS effects by the addition of DA would also have no effect on FA and data given in Table II confirm this predection. Rice (1973) has shown that the

Fig. 3. Mass Spectrum of 14-Methylpentadecanoic Acid Methyl Ester.

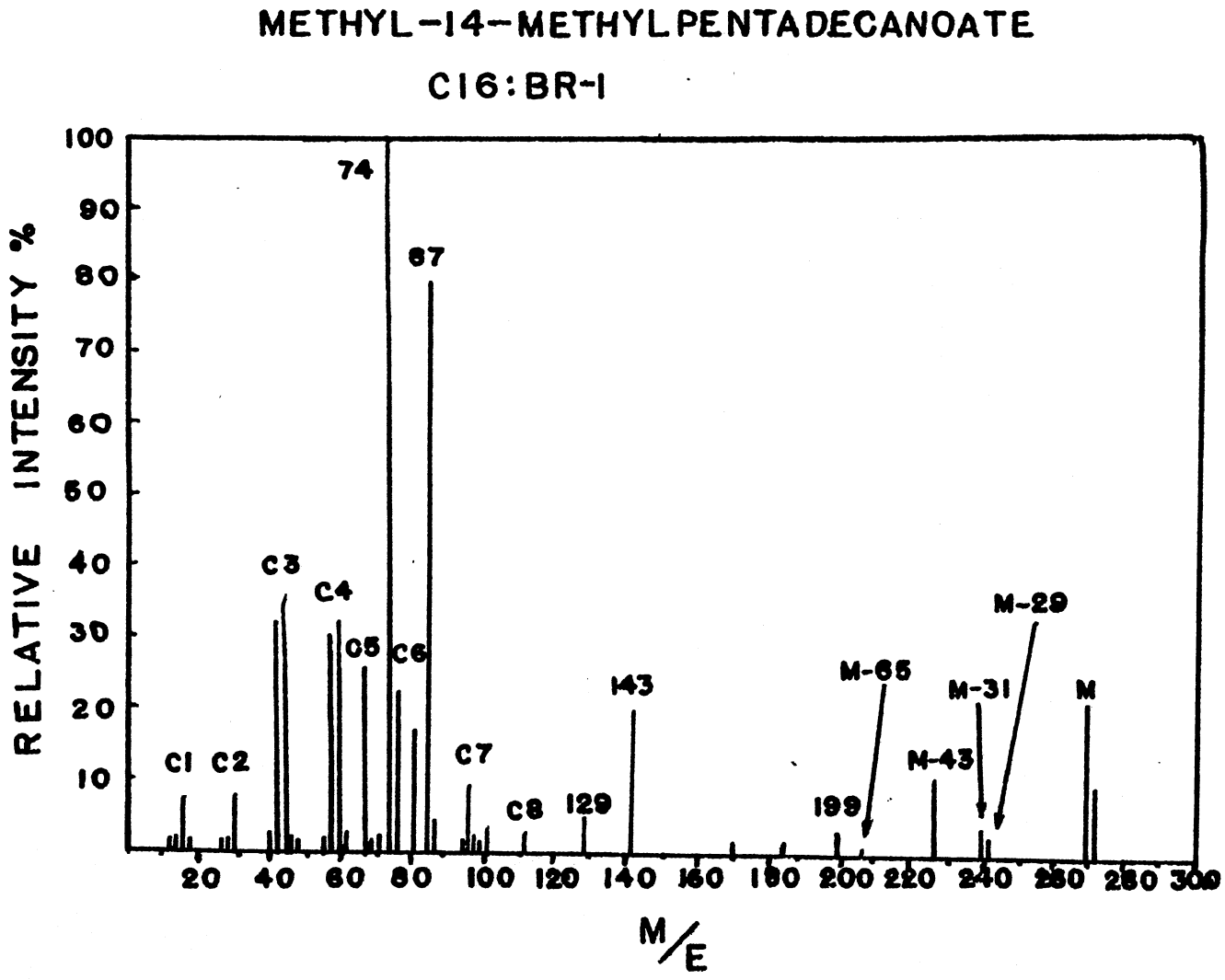

METHYL-14-METHYLPENTADECANOATE
C16:BR-1
RELATIVE INTENSITY %
100
90
80
70
60
50
40
30
20
10
74
87
C3
C4
C5
C6
C1
C2
C7
C8
129
143
M-65
M-31
M-29
M-43
199
M
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
M/E

TABLE I

EFFECTS OF GROWTH IN THE PRESENCE OF DCS AND LCS ON THE FATTY ACID COMPOSITION OF LIPIDS EXTRACTED FROM WHOLE CELLS OF *MICROCOCCUS LYSODEIKTICUS* dis II-p[+]

Relative Retention Time	Fatty Acid Designation[a]	Control Cells		DCS Grown Cells[d]		LCS Grown Cells[d]	
		% of Total*	µg/mg Cell Dry Wt.*	% of Total*	µg/mg Cell Dry Wt.*	% of Total*	µg/mg Cell Dry Wt.*
0.28	C 12:0	trace[b]	trace	trace	trace	trace	trace
0.32	C 13:0	trace	trace	trace	trace	trace	trace
0.42	C 14:br (iso)	1.7	1.3	1.2	0.9	1.6	1.4
0.48	C 14:0	2.0	1.5	2.2	1.6	1.9	1.5
0.59	C 15:br (anteiso)	64.0	47.3	65.7	48.0	64.6	47.6
0.64	C 15:0	1.0	0.7	1.1	0.8	1.0	0.8
0.80	C 16:br (iso)	12.0	8.9	11.4	8.4	11.9	8.9
1.00	C 16:0	7.9	5.9	8.3	7.1	8.1	6.1
1.13	C 16:1	1.0	0.8	1.1	0.8	1.0	0.8
1.24	C 17:br (anteiso)	5.0	3.7	4.9	3.6	4.1	3.1
1.57	U[c]	trace	trace	trace	trace	trace	trace
1.81	C 18:0	trace	trace	trace	trace	trace	trace
1.95	C 18:1	trace	trace	trace	trace	trace	trace
2.27	C 19:br (anteiso)	trace	trace	trace	trace	trace	trace
	Total		70.1		71.2		70.2

* Figures represent the mean of five determinations. Calculations of both percentages of total FA present and fatty acid weights were made using the procedures outlined in Materials and Methods.

** Retention times were calculated relative to C 16:0.

[a] The number on the left of the colon refers to the number of carbons in the FA. The symbol to the right of the colon refers to the degree of saturation (0, saturated; 1, double bond), or chain branching (br).

[b] Percentages less than 1 are designated as trace amounts.

[c] Unknown methyl ester.

[d] DCS (2×10^{-4}M) and LCS (2×10^{-4}M) were added after 12 hrs. growth at 37°C in the defined medium. All cultures were harvested after 18 hrs. growth at 37° C.

TABLE II

EFFECTS OF GROWTH IN THE PRESENCE OF VARIOUS COMPOUNDS CAPABLE OF PREVENTING CELL DIVISION INHIBITION ON THE FATTY ACID COMPOSITION OF WHOLE CELLS OF MICROCOCCUS LYSODEIKTICUS dis II-p+

Fatty Acid	Control Cells		DCS+DA Grown Cells[a]		DCS+PL Grown Cells[b]		PL Grown Cells[c]	
	% of Total*	µg/mg Cell Dry Wt.*	% of Total*	µg/mg Cell Dry Wt.*	% of Total*	µg/mg Cell Dry Wt.*	% of Total*	µg/mg Cell Dry Wt.*
C 12:0	trace	trace	trace	trace	trace	trace	trace	trace
C 13:0	trace	trace	trace	trace	trace	trace	trace	trace
C 14:br (iso)	1.7	1.3	1.7	1.3	2.2	1.3	2.0	1.0
C 14:0	2.0	1.5	2.0	1.5	2.4	1.5	1.0	0.5
C 15:br (anteiso)	64.0	47.3	64.4	47.6	45.2	27.2	47.7	27.9
C 15:0	1.0	0.7	1.0	0.8	1.0	0.6	1.0	0.6
C 16:br (iso)	12.0	8.9	12.0	8.9	26.2	15.8	26.8	15.7
C 16:0	7.9	5.9	8.1	6.0	10.4	6.3	10.3	6.0
C 16:1	1.0	0.8	1.0	0.7	7.2	4.3	4.3	2.5
C 17:br (anteiso)	5.0	3.7	4.1	3.1	4.8	2.9	5.2	3.0
U	trace	trace	trace	trace	trace	trace	trace	trace
C 18:0	trace	trace	trace	trace	trace	trace	trace	trace
C 18:1	trace	trace	trace	trace	trace	trace	trace	trace
C 19:br (anteiso)	trace	trace	trace	trace	trace	trace	trace	trace
Total		70.1		69.9		60.0		57.2

* Figures represent the mean of five determinations. Designations and calculations are outlined in Table I.

a DCS (2×10^{-4}M) and DA (4×10^{-5}M) were added after 12 hrs. incubation at 37° C in the defined medium.

b DCS (2×10^{-4}M) and Pl (0.15M) were added after 12 hrs. incubation at 37° C in the defined medium.

c PL (0.15M) was added after 12 hrs. incubation in the defined medium.

All cultures were harvested after 18 hrs. growth at 37° C.

TABLE III

FATTY ACIDS FROM CELLS GROWN FOR VARYING TIMES IN THE PRESENCE OF PL

Fatty Acid	Time (hrs. after addition of PL)[a] 6		10		12	
	% of Total*	µg/mg Cell Dry Wt.*	% of Total*	µg/mg Cell Dry Wt.*	% of Total*	µg/mg Cell Dry Wt.*
C 12:0	trace	trace	trace	trace	trace	trace
C 13:0	trace	trace	trace	trace	trace	trace
C 14:br (iso)	2.0	1.0	4.6	2.4	3.6	1.2
C 14:0	1.0	0.5	2.5	1.3	2.0	0.6
C 15:br (anteiso)	47.7	27.9	38.5	19.3	38.5	13.2
C 15:0	1.0	0.6	1.0	0.4	1.0	0.3
C 16:br (iso)	26.8	15.7	30.2	15.1	33.2	11.4
C 16:0	10.3	6.0	11.2	5.6	11.7	4.0
C 16:1	4.3	2.5	5.7	2.9	2.7	0.9
C 17:br	5.2	3.0	4.4	2.2	3.9	1.3
U	trace	trace	trace	trace	trace	trace
C 18:0	trace	trace	trace	trace	trace	trace
C 18:1	trace	trace	trace	trace	trace	trace
C 19:br (anteiso)	trace	trace	trace	trace	trace	trace
Total		57.2		49.2		32.9

* Figures represent the mean of three determinations. Designations and calculations are outlined in Table I.

[a] Cultures were grown at 37° C for 12 hrs. in the defined medium before addition of 0.15 M PL. Cultures were harvested at the times indicated above.

presence of PL in the growth medium of Erwinia carotovora results in a time-dependent reduction in the unsaturated FA content of this organism. Table II shows that cells of M. lysodeikticus treated with both DCS and PL also show an altered FA composition. The C 15:br (anteiso) FA content of these cultures is lowered from 64% to 45% of the total FA. The C 16:br (iso) FA shows a concomitant increase from 12% to 26% of the total while the C 16:0 FA increases from 8% to about 10%. Cells treated with PL alone show a similar shift in FA content. A second change induced by the presence of PL is in the total amount of FA present in these cells. The FA content of these cells is lowered from 7% of the total cell dry weight to 5.7% in the presence of PL alone and 6.0% in the presence of DCS and PL.

Data in Table III demonstrate that the PL-induced alteration of FA is a time dependent process. As the time of exposure to PL increases through 12 hours, the C 15: br (anteiso) composition decreases while the C 16: br (iso) composition increases. Smaller changes in other FA [C 16:0, C 17:br (anteiso), C 14:br (iso), and C 14:0] are also noted. Accompanying these FA composition changes is a striking decrease in the amount of FA present in these cells. The FA content of cells exposed to PL for 12 hours is about 3.3%, or about half the content of control cells which remains at about 7% during this time period.

An experiment to determine how extensively PL is able to modify the FA composition of M. lysodeikticus is shown in Table IV. In these experiments, cultures were grown in the defined medium for 12 hours before the addition of PL. After growth in the presence of PL for 12 hours, cells were reinoculated into fresh medium containing PL every 24 hours until harvest at the times indicated in Table IV. Although

TABLE IV

EFFECTS OF PROLONGED INCUBATION IN THE PRESENCE OF PL ON THE FATTY ACID COMPOSITION OF MICROCOCCUS LYSODEIKTICUS dis II-p+

Fatty Acid	Control Cells		Time (hrs. after the addition of PL)[a] 12		56		92	
	% of Total*	µg/mg Cells Dry Wt.*	% of Total*	µg/mg Cell Dry Wt.*	% of Total*	µg/mg Cell Dry Wt.*	% of Total*	µg/mg Cell Dry Wt.*
C 12:0	trace	trace	trace	trace	trace	trace	trace	trace
C 13:0	trace	trace	trace	trace	trace	trace	trace	trace
C 14:br (iso)	1.7	1.3	3.6	1.2	2.7	1.6	3.0	1.9
C 14:0	2.0	1.5	2.0	0.6	2.4	1.5	2.2	1.4
C 15:br (anteiso)	64.0	47.3	38.5	13.2	48.8	24.1	51.1	31.7
C 15:0	1.0	0.7	1.0	0.3	1.0	0.5	1.0	0.6
C 16:br (iso)	12.0	8.9	33.2	11.4	23.0	11.3	20.1	12.5
C 16:0	7.9	5.9	11.7	4.0	10.9	5.0	10.1	6.3
C 16:1	1.0	0.8	2.7	0.9	3.8	2.2	4.2	2.7
C 17:br	5.0	3.7	3.9	1.3	5.0	3.0	5.0	3.1
U	trace	trace	trace	trace	trace	trace	trace	trace
C 18:0	trace	trace	trace	trace	trace	trace	trace	trace
C 18:1	trace	trace	trace	trace	trace	trace	trace	trace
C 19:br	trace	trace	trace	trace	trace	trace	trace	trace
Total		70.1		32.9		49.2		61.1

* Figures reported are the mean of two determinations. Designations and calculations ate outlined in Table I.

[a] PL (0.15 M) was added after 12 hrs. growth at 37° C in the defined medium. Measurements made at 56 hrs. and 92 hrs. were from cultures which were transferred every 24 hrs. into fresh medium containing 0.15 M PL.

these cells do not fully regain the FA composition of control cells, it can be seen that both the qualitative and quantitative FA composition of cells continually exposed to PL for 96 hours more closely resembles the control cell FA composition than cells treated with PL for 12 hours. These data indicate that the effects of PL on the M. lysodeikticus FA composition are not permanent and continual exposure to PL does not result in a cell with a FA composition of predominantly C 16:br (iso) FA.

In summarizing the changes in FA seen with growth in the presence of PL, the general trend in FA composition is a shift from predominantly odd carbon number branched-chain FA to even carbon number branched-chain FA. Chapman (1969) has reported that odd carbon numbered anteiso branched-chain FA and even carbon numbered iso branched-chain FA are synthesized from isoleucine and valine precursors, respectively. Since M. lysodeikticus dis II-p^+ is grown in a defined medium containing aspartic acid and glutamic acid as sources of carbon and energy, it is possible that PL preferentially inhibits the utilization of one of these compounds for FA synthesis. Therefore, an attempt to duplicate the action of PL on the FA composition of these cells was made by growing cultures in the defined medium with either glutamic acid or aspartic acid as the source of carbon and energy. Results are shown in Table V. One important point that should be made before considering these data is that M. lysodeikticus cannot use aspartic acid as efficiently as glutamic acid for growth. Cultures grown in the defined medium with glutamic acid as the carbon and energy source give a growth yield of about 550 µg cell dry weight per ml of medium as opposed to 140 µg cell dry weight per ml of medium when aspartic acid is used. Data in Table V reveal that glutamic acid is a suitable carbon source for both iso and

anteiso branched-chain FA. Cells harvested from this medium contain a FA spectrum which quantitatively and qualitatively resembles control cultures. Cells grown in the presence of aspartic acid also contain both iso and anteiso branched-chain FA, but the total branched-chain FA content is decreased while the straight chain forms (specifically the C 16:0 and C 18:0) are increased. This is in contrast to the results obtained with cells grown in the presence of PL (Table III) where the relative amounts of C 16:br (iso) FA increase. A second notable difference is that cells treated with PL for 12 hours (Table III) contain only half the FA found in control cells, whereas cells grown in the medium containing aspartic acid for a comparable period of time contain amounts of FA similar to control cells (Table V). These data indicate that PL does not exert its effect by preferentially limiting the utilization of one of the carbon and energy sources for FA synthesis.

One further question that must be considered in assessing the PL-induced FA change is whether or not the lactone ring is required for the FA effect. PL is hydrolyzed in the presence of base to form pantoic acid. M. lysodeikticus has a very delicate Na^+ - K^+ balance that must be preserved when compounding the basal medium, hence the effects of growth in both Na^+- pantoic acid and K^+- pantoic acid were monitored. As can be seen in the growth curves shown in Fig. 4, M. lysodeikticus growth is inhibited in the presence of excess Na^+ ion while K^+ ion has little effect on growth. The data presented in Table VI reveal that both Na^+- pantoic acid and K^+- pantoic acid have effects on the FA composition of this organism. However, these effects are not comparable to the effects noted with PL. These data indicate the γ- lactone form of the molecule is more effective in altering the FA composition of M. lysodeik-

TABLE V

EFFECTS OF ASPARTIC ACID AND GLUTAMIC ACID AS CARBON AND ENERGY SOURCES ON THE FATTY ACID COMPOSITION OF *MICROCOCCUS LYSODEIKTICUS* dis II-p+

Fatty Acid	Control Cells		PL Grown Cells		Glutamate Grown Cells[a]		Aspartate Grown Cells[a]	
	% of Total*	ug/mg Cell Dry Wt.*	% of Total*	ug/mg Cell Dry Wt.*	% of Total*	ug/mg Cell Dry Wt.*	% of Total*	ug/mg Cell Dry Wt.*
C 12:0	trace	trace	trace	trace	trace	trace	trace	trace
C 13:0	trace	trace	trace	trace	trace	trace	trace	trace
C 14:br (iso)	1.7	1.3	3.6	1.2	2.3	1.7	2.1	1.3
C 14:0	2.0	1.5	2.0	0.6	2.8	2.1	6.9	4.1
C 15:br (anteiso)	64.0	47.3	38.5	13.2	63.2	48.0	49.9	31.9
C 15:0	1.0	0.7	1.0	0.3	1.0	0.7	2.0	1.2
C 16:br (iso)	12.0	8.9	33.2	11.4	12.5	9.1	4.8	3.1
C 16:0	7.9	5.9	11.7	4.0	7.5	5.3	15.1	9.7
C 16:1	1.0	0.8	2.7	0.9	3.7	2.8	3.1	2.0
C 17:br (anteiso)	5.0	3.7	3.9	1.3	5.5	4.2	5.8	3.7
U	trace	trace	trace	trace	trace	trace	trace	trace
C 18:0	trace	trace	trace	trace	trace	trace	10.4	6.7
C 18:1	trace	trace	trace	trace	trace	trace	trace	trace
C 19:br	trace	trace	trace	trace	trace	trace	trace	trace
Total		70.1		32.9		76.0		64.3

* Figures represent the mean of two determinations. Designations and calculations are outlined in Table I.

[a] See text for a discussion of the composition of these media.

All cells were harvested after 24 hrs. growth at 37°C in the appropriate medium

Fig. 4. Growth Response of M. lysodeikticus to Sodium Pantoic Acid, Potassium Pantoic Acid, Sodium Chloride, Potassium Chloride, and PL Added After Twelve Hours Growth. ⬢, control cells; ▲, with potassium pantoic acid (0.15 M); Δ, with potassium chloride (0.15 M); □, with pantoyl lactone (0.15 M); ○, with sodium pantoic acid (0.15 M); ●, with sodium chloride.

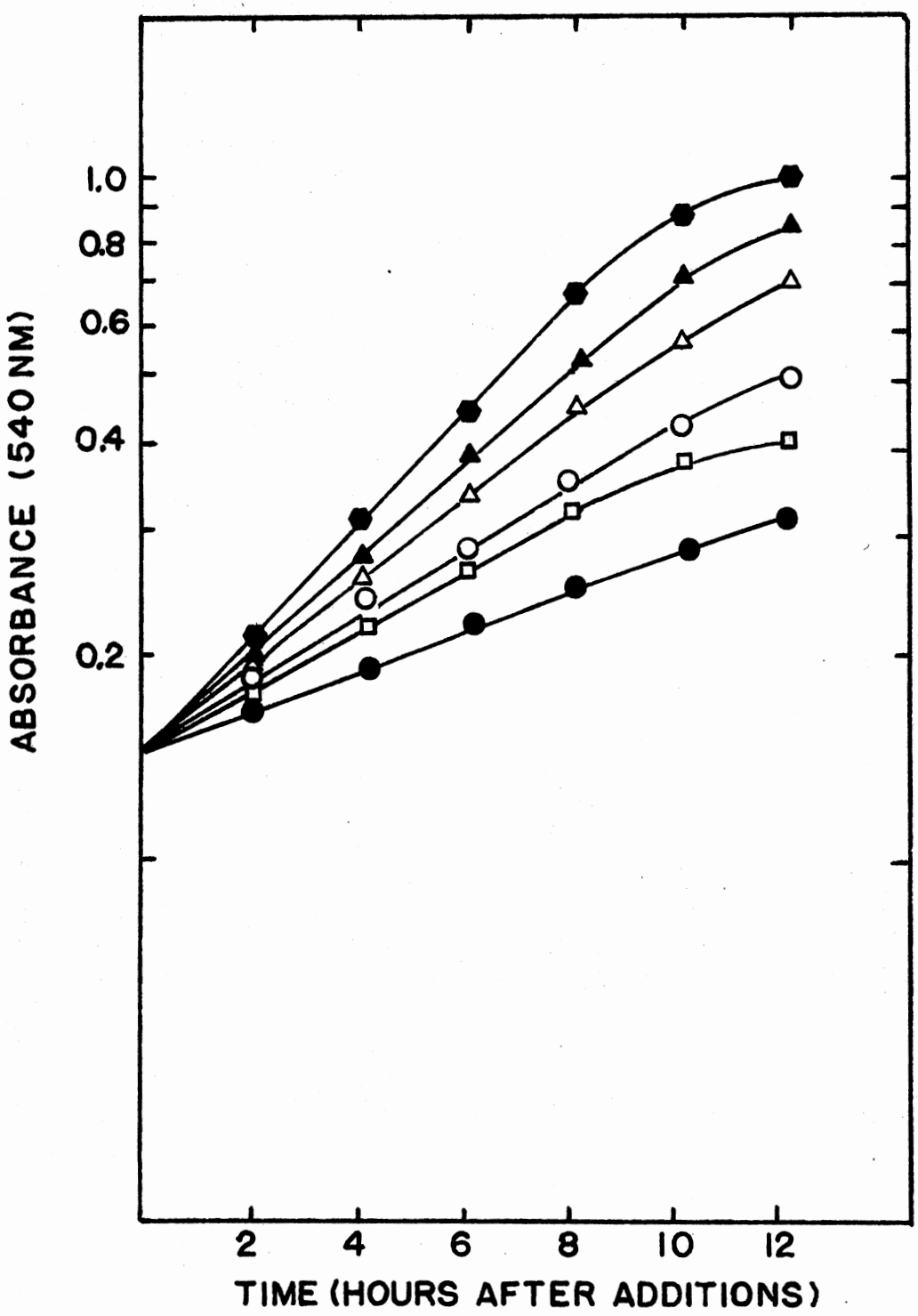

ABSORBANCE (540 NM)
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
12
TIME (HOURS AFTER ADDITIONS)

TABLE VI

EFFECTS OF GROWTH IN THE PRESENCE OF SODIUM AND POTASSIUM PANTOIC ACID ON THE FATTY ACID COMPOSITION OF WHOLE CELLS OF MICROCOCCUS LYSODEIKTICUS dis II-p^+

Fatty Acid	PL Grown Cells[a] % of Total*	Na^+-Pantoic Acid Grown Cells[a] % of Total*	K^+-Pantoic Acid Grown Cells[a] % of Total*
C 12:0	trace	trace	trace
C 13:0	trace	trace	trace
C 14:br (iso)	3.6	1.0	1.0
C 14:0	2.0	2.6	2.2
C 15:br (anteiso)	38.5	56.1	55.8
C 15:0	1.0	1.0	1.0
C 16:br (iso)	33.2	18.3	19.2
C 16:0	11.7	9.5	10.1
C 16:1	2.7	4.4	4.6
C 17:br (anteiso)	3.9	5.3	5.7
U	trace	trace	trace
C 18:0	trace	trace	trace
C 18:1	trace	trace	trace
C 19:br (anteiso)	trace	trace	trace

* Figures represent the mean of three determinations. Designations and calculations are outlined in Table I.

a PL, Na^+-pantoic acid, and K^+-pantoic acid were added to a final concentration of 0.15 M after 12 hrs. growth at 37° C in the defined medium, The subsequent incubation period was 12 hrs.

ticus than the corresponding acid.

Vorbeck and Marinetti (1965) have reported that 95% of the cellular phospholipids of Gram-positive bacteria are found in the cell membrane and those which are found in the cytoplasm are identical to the membrane phospholipids. Data given in Table VII show that the membrane FA composition of M. lysodeikticus is very similar to the FA composition of whole cells (see Table I). The control cell membrane shows differences of less than 2% in all cases. Quantitative measures of the membrane FA content reveal that the membrane isolated from control cells is about 19% FA by weight. Again, the FA composition of membranes from cells grown in the presence of DCS is similar to the FA composition of the control membrane with the possible exception of the C 15:br (anteiso) FA which is reduced about 4%. The FA content of membranes from DCS-treated cells accounts for about 19.6% of the total membrane weight as opposed to 19.1% in the control membrane. Membranes from cells treated with LCS do not appear to differ either quantitatively or qualitatively from control membranes.

Table VIII demonstrates that treatment of M. lysodeikticus with DA in combination with DCS results in the restoration of the levels of the C 15:br (anteiso) FA to levels found in the control membrane. Although it is difficult to consider the 4% decrease in the C 15:br (anteiso) FA observed in the membranes of DCS-treated cells significant, the simultaneous treatment of cultures with DCS and DA does result in an approximate 4% increase in the relative composition of this FA and, thus, the decrease seen in DCS-treated cells cannot be overlooked. Quantitatively, membranes from DCS and DA treated cells are very similar to control membranes. As was seen in whole cell extracts, membranes from cells

TABLE VII

EFFECTS OF GROWTH IN THE PRESENCE OF DCS AND LCS ON THE FATTY ACID COMPOSITION OF ISOLATED CELL MEMBRANES OF *MICROCOCCUS LYSODEIKTICUS* dis II-p+

Fatty Acid	Control Cells		DCS Grown Cells[a]		LCS Grown Cells[a]	
	% of Total*	µg/mg CM Dry Wt.*	% of Total*	µg/mg CM Dry Wt.*	% of Total*	µg/mg CM Dry Wt.*
C 12:0	trace	trace	trace	trace	trace	trace
C 13:0	trace	trace	trace	trace	trace	trace
C 14:br (iso)	2.0	3.9	2.3	4.7	2.0	4.1
C 14:0	2.0	3.9	2.0	4.2	2.1	4.1
C 15:br (anteiso)	65.4	126.8	61.9	126.8	64.4	127.1
C 15:0	2.0	3.9	1.0	2.0	2.0	4.1
C 16:br (iso)	10.8	21.0	12.1	24.9	11.0	22.1
C 16:0	6.9	13.4	7.8	16.1	6.8	13.9
C 16:1	4.9	9.5	1.0	4.0	4.7	10.1
C 17:br (anteiso)	4.9	9.5	6.5	13.4	5.9	11.7
U	trace	trace	trace	trace	trace	trace
C 18:0	trace	trace	trace	trace	trace	trace
C 18:1	trace	trace	trace	trace	trace	trace
C 19:br (anteiso)	trace	trace	trace	trace	trace	trace
Total		191.9		196.2		193.2

* Figures represent the mean of five determinations. Designations and calculations are outlined in Table I.

[a] Conditions for treatment with DCS and LCS are outlined in Table I.

All cultures were harvested after 18 hrs. growth at 37° C and membranes were prepared as outlined in the Materials and Methods.

TABLE VIII

EFFECTS OF GROWTH IN THE PRESENCE OF VARIOUS COMPOUNDS CAPABLE OF PREVENTING CELL DIVISION INHIBITION ON THE FATTY ACID COMPOSITION OF ISOLATED CELL MEMBRANES OF *MICROCOCCUS LYSODEIKTICUS* dis II-p+

Fatty Acid	Control Cells		DCS+DA Grown Cells[a]		DCS+PL Grown Cells[a]		PL Grown Cells	
	% of Total*	µg/mg CM Dry Wt.*	% of Total*	µg/mg CM Dry Wt.*	% of Total*	µg/mg CM Dry Wt.*	% of Total*	µg/mg CM Dry Wt.*
C 12:0	trace	trace	trace	trace	trace	trace	trace	trace
C 13:0	trace	trace	trace	trace	trace	trace	trace	trace
C 14:br (iso)	2.0	3.9	2.0	3.9	2.0	2.4	1.8	2.0
C 14:0	2.0	3.9	2.1	4.0	1.0	1.2	1.0	1.1
C 15:br (anteiso)	65.4	126.8	66.1	126.8	52.2	61.5	49.3	55.8
C 15:0	2.0	3.9	2.1	4.1	1.0	1.2	1.0	1.1
C 16:br (iso)	10.8	21.0	11.8	22.6	23.1	26.6	25.0	28.4
C 16:0	6.9	13.4	7.5	14.4	8.0	9.4	7.1	8.0
C 16:1	4.9	9.5	1.1	6.2	3.0	3.9	4.5	5.1
C 17:br (anteiso)	4.9	9.5	4.5	8.7	8.1	9.4	9.4	10.6
U	trace	trace	trace	trace	trace	trace	trace	trace
C 18:0	trace	trace	trace	trace	trace	trace	trace	trace
C 18:1	trace	trace	trace	trace	trace	trace	trace	trace
C 19:br (anteiso)	trace	trace	trace	trace	trace	trace	trace	trace
Total		191.9		190.7		113.7		112.0

* Figures represent the mean of five determinations. Designations and calculations are outlined in Table I.

[a] Conditions for treatment with DCS+DA, DCS+PL, and PL are outlined in Table II.

All cultures were harvested after 18 hrs. growth at 37° C and membranes were prepared as outlined in the Materials and Methods.

treated with DCS and PL, or PL alone, show altered FA profiles. Again, the C 15:br (anteiso) content of these membranes is lowered with a concomitant rise in the C 16:br (iso) content. FA represent about 11.3% of the total weight of the membranes from cells grown in the presence of PL as opposed to the 19.1% composition of control membrane.

The Phospholipid Composition and Fatty Acid Specificities of Phospholipids from Membranes of Dividing and Non-Dividing Cells of M. lysodeikticus dis II-p$^+$

Butler, et al. (1967) have reported that the phospholipids found in M. lysodeikticus are diphosphatidylglycerol (DPTG), phosphatidylglycerol (PTG), and phosphatidylinositol (PTI). Table IX outlines the results of thin-layer chromatography of the phospholipids isolated from membranes of control cells, DCS treated cells, and LCS treated cells on Silica gel G coated plates in a solvent system of chloroform-methanol-water (65:25:4 by volume). Visual inspection of the thin-layer plates reveals two spots which appear yellow due to the membrane chromophore β-carotene. Examination of the plates under ultraviolet light and after spraying with the $FeCl_3$-sulfosalicylic spray indicator discloses the presence of three phospholipids. Until recently when synthetic standards became available, one of the most reliable methods for distinguishing DPTG, PTG, and PTI was based on the Periodate-Schiff's reaction as outlined by Shaw (1968). This reaction is able to distinguish between these compounds on the basis of their vicinal hydroxyl group content. DPTG has no vicinal hydroxyl groups by virtue of its dimerization and, therefore, gives no reaction after treatment with the Periodate-Schiff reagents. PTG has one vicinal

TABLE IX

EFFECTS OF GROWTH IN THE PRESENCE OF DCS AND LCS ON THE PHOSPHOLIPIDS EXTRACTED FROM MEMBRANES OF *MICROCOCCUS LYSODEIKTICUS* dis II-p+

Phospholipid Source*	Rf Value[a]	% of Total Lipid[b]	Periodate-Schiff's Reaction[c]	Identification[d]
Membranes from Control Cells	0.81	38.7	no reaction	Diphosphatidylglycerol
	0.54	40.7	purple	Phosphatidylglycerol
	0.26	13.6	yellow	Phosphatidylinositol
Membranes from DCS grown cells[e]	0.81	56.0	no reaction	Diphosphatidylglycerol
	0.54	31.1	purple	Phosphatidylglycerol
	0.26	8.4	yellow	Phosphatidylinositol
Membranes from LCS grown cells[e]	0.81	39.0	no reaction	Diphosphatidylglycerol
	0.54	41.2	purple	Phosphatidylglycerol
	0.26	12.5	yellow	Phosphatidylinositol

* Membranes were isolated as described in the Materials and Methods Section from cultures frown for 16 hrs. at 37° C.

a Thin-layer chromatographic procedures for phospholipid separations are outlined in the Materials and Methods Section.

b Quantitative measures of the percentage of the total lipid were based on both labeling patterns and fatty acid weight determinations.

c Periodate-Schiff's reactions for vicinal hydroxyl groups.

d Identifications were also made by comparing Rf values with known standards as outlined in the Materials and Methods Section.

e DCS and LCS treatments are given in Table I.

hydroxyl group and shows a characteristic purple color after the treatment. PTI can be distinguished from PTG on the basis of its characteristic yellow color after treatment with The Periodate-Schiff reagent. These criteria were used in identifying the phospholipids isolated from M. lysodeikticus membranes. Confirmation of the identifications was later achieved by comparing the Rf values of these phospholipids with those of synthetic standards.

Quantitative determinations of the amount of fatty acid released from these phospholipids, as well as labeling patterns obtained from phospholipids isolated from cells grown in the defined medium supplemented with labeled aspartic and glutamic acid, have shown that DPTG, PTG, and PTI represent approximately 39%, 41%, and 13%, respectively, of the phospholipid found in membranes isolated from control cells (Table IX). Phospholipids isolated from membranes of cells grown in the presence of LCS have a relative composition quite similar to the control membrane. As can be seen in Table IX, membranes from cells treated with DCS contain decreased levels of PTG and increased levels of DPTG. The DPTG to PTG ratio in these membranes is 1.80 as compared to 0.95 in control membranes. Similar increases in the DPTG to PTG ratio have recently been reported in division inhibited cells of Bifidobacterium bifidum (Veerkamp, 1976) and E. coli (Michel, et al., 1977). There are several interesting aspects to these reports. B. bifidum division inhibition was induced with a variety of chemical agents while the E. coli changes were observed in a mutant which was a non-conditional chain former. Although studies of the effects of reversing cell division inhibition were not done with B. bifidum, Michel, et al. (1977) showed that growing the mutant of E. coli in a variety of high

osmolarity media resulted in both short cells and a return of DPTG to PTG ratios to those found in the control cells. They further showed that the alterations were due to increased PTG turnover rates which were slowed under conditions favorable for the completion of cell division.

Examination of Table X reveals that the DPTG to PTG ratio returns to 1.0 when cells are treated with both DCS and DA. These data indicate that the alterations in phospholipids observed in division inhibited cells of M. lysodeikticus are a secondary alteration induced by the presence of DCS since DA competition with DCS corrects this modification. M. lysodeikticus is a particularly useful organism for assessing the structural implications of this alteration. Barsukov, et al. (1976) have shown that the membrane of M. lysodeikticus possesses a phospholipid asymmetry with at least 80% of the PTG being found in the outer half of the bilayer. Therefore, it appears that the DCS induced phospholipid alteration is localized in the outer half of the membrane bilayer where the greatest physical (environmental) stress would be placed as a result of the loss of the insulating (and protective) effect of the cell wall. Support for this concept is that the restoration of mucopeptide synthesis by treatment of these cells with DA eliminates this stress and corrects the phospholipid alteration.

Treatment with DCS and PL also results in an alteration of membrane phospholipid (Table X). As was noted for cells grown in the presence of DCS, DPTG is the major phospholipid isolated from cells treated with DCS and PL and, in addition, significant amounts of lysoDPTG are present. The amounts of PTG are significantly decreased and the Periodate-Schiff's reaction shows that most, if not all, of the PTG is present as lysoPTG.

TABLE X

EFFECTS OF GROWTH IN THE PRESENCE OF VARIOUS COMPOUNDS CAPABLE OF PREVENTING CELL DIVISION INHIBITION ON THE PHOSPHOLIPIDS EXTRACTED FROM MEMBRANES OF *MICROCOCCUS LYSODEIKTICUS* dis II-p+

Phospholipid Source*	Rf Value	% of Total Lipid	Periodate-Schiff's Reaction	Identification
Membranes from Control cells	0.81	38.7	no reaction	Diphosphatidylglycerol
	0.54	40.7	purple	Phosphatidylglycerol
	0.26	13.6	yellow	Phosphatidylinositol
Membranes from DCS-DA grown cells	0.81	39.2	no reaction	Diphosphatidylglycerol
	0.54	39.0	purple	Phosphatidylglycerol
	0.26	14.6	yellow	Phosphatidylinositol
Membranes from DCS-PL and PL grown cells	0.81	52.7	no reaction	Diphosphatidylglycerol
	0.70**	not determined**	no reaction**	Unknown**
	0.33	20.4	no reaction	Lysodiphosphatidylglycerol
	0.28	~8.2[a]	purple	Lysophosphatidylglycerol
	0.24	~12.4[a]	yellow	Phosphatidylinositol

* Phospholipid analyses were performed as outlined in Table V.

** See text for discussion

a These values are designated as approximate because complete separation of these two phospholipids was not possible under the chromatographic conditions used.

Quantitative values for lysoPTG and PTI are labeled as approximate because the thin-layer chromatography system used in these studies does not completely separate the two compounds. The lysophospholipid identifications shown in Table X were further confirmed by comparing the Rf values of these phospholipids with both known standards and phospholipids from control membranes after treatment with phospholipase A_2. Table X further reveals the presence of a new compound found in cultures treated with PL which migrates to a slightly lower Rf value (0.70) than DPTG. This molecule contains no FA, no carbohydrate, no free amino groups, no phosphate, and gives a blue-white fluorescence emmission under ultraviolet irradiation. This compound is not PL as judged by fluorescence properties, lactone indicator reagents and Rf values. At the present time, this compound has not been identified.

Although the phospholipid profiles of DCS and PL treated membranes show the same general trend for DPTG to PTG ratios found in DCS treated cells, none of the effects noted in DCS and PL treated cultures are attributable to the presence of DCS since cultures treated with PL alone show identical profiles to those obtained for DCS and PL treated cells. Further, the phospholipid profiles obtained from these membranes indicate that the effects of PL are membrane surface oriented.

Data shown in Tables XI and XII give the FA composition of the individual phospholipids from dividing and non-dividing cells of M. lysodeikticus. The FA spectrum observed in phospholipids from control cells indicates that there is little FA specificity in these phospholipids (Table XI). If any specificity can be said to exist in control membrane phospholipid, it is in PTI where the relative content of the C 15:br (anteiso) FA is decreased and the C 16:0 FA shows a concomitant increase.

TABLE XI

FATTY ACID COMPOSITION OF ISOLATED PHOSPHOLIPIDS FROM MEMBRANES OF DIVIDING AND NON-DIVIDING CELLS OF *MICROCOCCUS LYSODEIKTICUS* dis II-p+

Fatty Acid[a]	DPTG[b] Control Cells % of Total*	DPTG[b] DCS[c] Cells % of Total*	DPTG[b] DCS+DA[c] Cells % of Total*	PTG[b] Control Cells % of Total*	PTG[b] DCS[c] Cells % of Total*	PTG[b] DCS+DA[c] Cells % of Total*	PTI[b] Control Cells % of Total*	PTI[b] DCS[c] Cells % of Total*	PTI[b] DCS+DA[c] Cells % of Total*
C 14:br (iso)	1.0	3.0	3.0	1.0	2.0	1.0	2.0	2.0	2.0
C 14:0	2.0	3.0	3.0	3.0	4.3	3.2	4.0	4.0	4.0
C 15:br (anteiso)	65.8	65.2	65.2	61.2	60.1	62.5	56.3	40.1	59.1
C 15:0	2.0	1.0	1.0	1.0	2.0	1.0	1.0	trace	trace
C 16:br (iso)	12.1	11.4	12.1	11.2	14.2	11.8	8.2	8.5	8.3
C 16:0	8.0	9.3	10.0	10.1	11.2	9.4	17.1	36.1	16.6
C 16:1	2.9	2.9	2.9	5.1	2.1	5.7	5.5	4.1	3.5
C 17:br (anteiso)	5.6	4.7	5.6	4.7	4.3	4.7	6.8	trace	2.9

* Figures represent the mean of three determinations. Designations and calculations are outlined in Table I.

[a] Fatty acids found in trace amounts are not included.

[b] Phospholipid abbreviations are DPTG, diphosphatidylglycerol; PTG, phosphatidylglycerol; PTI, Phosphatidylinositol.

[c] DCS and DA treatments are outlined in Tables I and II.

All cultures were harvested after 18 hrs. growth at 37° C in the defined medium.

TABLE XII

FATTY ACID COMPOSITION OF ISOLATED PHOSPHOLIPIDS FROM MEMBRANES OF *MICROCOCCUS LYSODEIKTICUS* dis II-p+ GROWN IN THE PRESENCE OF PL

Fatty Acid[a]	DPTG[b] Control Cells % of Total*	DPTG[b] PL Grown Cells[c] % of Total*	PTG[b] Control Cells % of Total*	Lyso DPTG[b] PL Grown Cells[c] % of Total*	Lyso PTG[b] PL Grown Cells[c] % of Total*	PTI[b] Control Cells % of Total*	PTI[b] PL Grown Cells[c] % of Total*
C 14:br (iso)	1.0	2.0	1.0	1.0	1.0	2.0	1.0
C 14:0	2.0	4.0	3.0	2.0	2.0	4.0	2.0
C 15:br (anteiso)	65.8	46.3	61.2	43.0	34.1	56.3	32.8
C 15:0	2.0	2.0	1.0	1.0	1.0	1.0	1.0
C 16:br (iso)	12.1	21.1	11.2	16.3	22.3	8.2	16.2
C 16:0	8.1	16.1	10.1	26.1	29.7	17.1	37.7
C 16:1	2.9	4.8	5.7	4.9	5.1	5.5	5.2
C 17:br (anteiso)	5.6	2.1	4.7	3.2	2.8	4.8	2.8

* Figures represent the mean of three determinations. Designations and calculations are outlined in Table I.

[a] Fatty acids found in trace amounts are not included.

[b] Phospholipid abbreviations are given in Table X. LysoDPTG is lysodiphosphatidylglycerol and LysoPTG is lysophosphatidylglycerol.

[c] PL (0.15 M) treatments are described in Table II.

All cultures were harvested after 18 hrs. growth at 37°C in the defined medium.

Cells treated simultaneously with DCS and DA show phospholipid FA profiles which closely parallel the values found in control cells, but cultures treated with DCS show an elevated C 16:0 and decreased C 15:br (anteiso) content in PTI. At first glance, these data for DCS treated cultures seem to contradict the data presented in Tables I and VII where no differences were found in the FA composition of dividing and non-dividing cells. However, when a calculation of the total FA spectrum is made from the results of the phospholipid FA analyses by considering the relative amounts of each phospholipid, a spectrum very close to the spectrum presented in Table VII is obtained. In other words, although cells treated with DCS contain altered ratios of phospholipid and a FA difference <u>within</u> PTI, these changes are only observed after a detailed investigation of each phospholipid is carried out.

Table XII shows the constituent FA of the phospholipids from cells grown in the presence of PL. When these data are compared with the membrane FA spectrum of PL treated cells shown in Table VIII, a greater content of the C 16:0 FA is noted in every phospholipid. Another notable difference is that no phospholipid contains the level of the C 16:br (iso) FA observed in whole cells or isolated membranes from cells treated with PL. The C 16:br (iso) FA is only a small fraction of the free FA and of the FA associated with the carotenoids. One possible explanation for these results is that the C 16:br (iso) FA is a structural component of a molecule which is chloroform-methanol extractable but is not detected by the phospholipid fractionation procedures used in this study.

Sucrose Density Gradient Sedimentation Patterns and Lipid Composition of Membranes from Dividing and Non-Dividng Cells

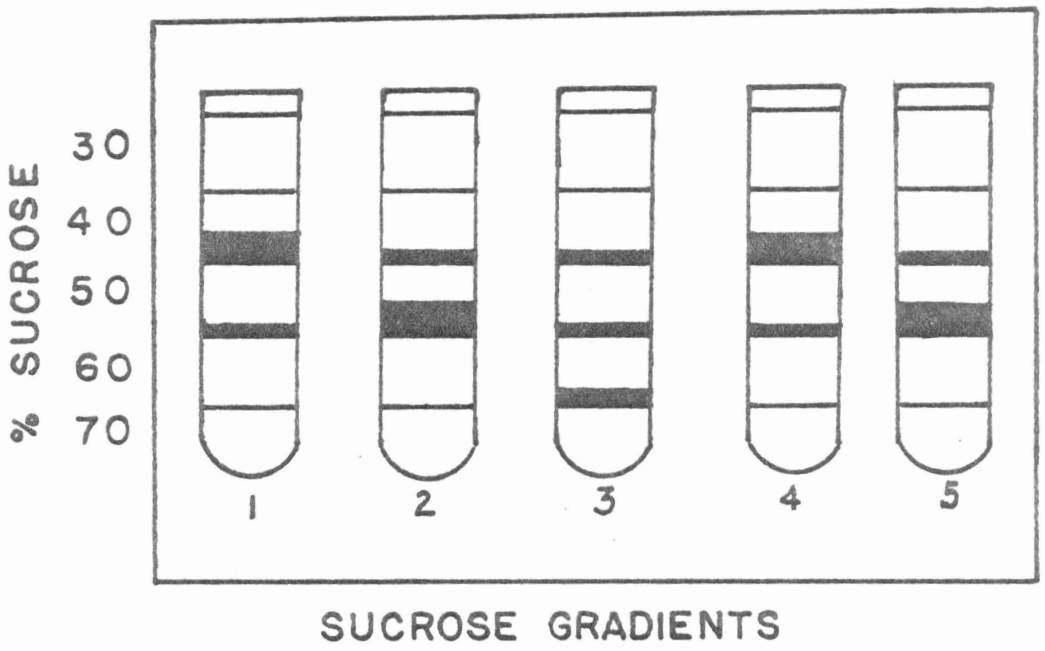
Since phospholipid changes observed in non-dividing cells and both fatty acid and phospholipid changes observed in cells treated with PL could result in changes in the physical properties of these membranes, isolated membranes were fractionated on sucrose density gradients. The results of sedimentation of these membranes in sucrose gradients is shown in Fig. 5. Surprisingly, membranes from control cells possess two bouyant densities. The largest fraction of membrane sediments to the 40-50% (ρ = 1.176) sucrose interface with a second band appearing at the 50-60% (ρ = 1.230) interface. Similar results have been reported by Salton, et al. (1968).

It was first thought that cell wall contamination or nucleic acid associations could account for the multiple banding patterns; however, retreatment of the individual bands with lysozyme and DNase failed to alter the patterns shown. These density differences cannot be attributed to the presence of mesosomes since these structures have never been observed in this strain after growth in the defined medium. Further, direct electron microscopic examination fails to reveal any significant morphological variations within the bands.

Membranes from cells grown in the presence of DCS exhibit three bands with the additional fraction sedimenting to the 60-70% (ρ = 1.289) sucrose interface, while treatment with DCS and DA returns this sedimentation pattern to a pattern like that of control membrane. Membranes from DCS and PL treated cultures, or cultures treated with PL alone, sediment to the 40-50% and 50-60% interface. However, the largest fraction of mem-

Fig. 5. Sucrose Density Gradient Sedimentation Patterns of *M. lysodeikticus* Membranes.

(1) Membranes isolated from control cells.
(2) Membranes isolated from cells grown in the presence of PL (0.15 M) for six hours.
(3) Membranes isolated from cells grown in the presence of DCS (2×10^{-4} M) for six hours.
(4) Membranes isolated from cells grown in the presence of DCS (2×10^{-4} M) and DA (5×10^{-5}M) for six hours.
(5) Membranes isolated from cells grown in the DCS (2×10^{-4} M) and PL (0.15 M) for six hours.


% SUCROSE
30
40
50
60
70
1
2
3
4
5
SUCROSE GRADIENTS

brane is found at the 50-60% interface. This result could be predicted from the lipid analyses since these membranes contain less lipid than control membranes.

To determine if qualitative lipid differences exist in the sucrose gradient fractions obtained from each membrane preparation, the total chloroform-methanol extractable lipids were analyzed. The fatty acid content of each fraction is given in Tables XIII and XV while the phospholipid content of each fraction is shown in Tables XIV and XVI.

Each of the two sucrose gradient fractions shown in Fig. 5 for both control membranes and membranes from cells treated with DCS and DA have similar FA and phospholipid contents. These FA and phospholipid patterns also closely correspond to those observed for isolated membranes before sucrose gradient fractionation (Tables VII and VIII); thus, qualitative lipid differences cannot account for the multiple bands observed after sedimentation.

When the FA and phospholipids of the three membrane fractions from cells grown in the presence of DCS are analyzed, both the phospholipid and FA content of the fraction sedimenting to the 50-60% sucrose interface are altered (Tables XIII and XIV). This fraction contains decreased levels of PTG (Table XIII) making the predominant phospholipids DPTG and PTI. An analysis of the FA content of this fraction (Table XIV) reflects the phospholipid alteration in that it contains elevated levels of C 16:0 which is consistent with an increase in the relative PTI content (Table XI). Considering the data of Barsukov, et al (1976), it is tempting to speculate that the 50-60% sucrose fraction is enriched with membrane materials from the inner half of the bilayer; but an explanation for how this is physically possible cannot be offered.

Data presented in Tables XV and XVI give the phospholipid and FA

content of cells grown in the presence of PL and DCS plus PL. No differences in the sedimentation patterns of the membranes, FA content, or phospholipid content can be attributed to the presence of DCS, hence the results of the two growth situations are not separated in these tables. The phospholipid profiles in Table XV reveal that the lysophospholipids found in this membrane are exclusively localized in the band sedimenting to the 50-60% sucrose interface. The FA profiles show that the C 16:br (iso) FA content of this fraction is increased while the C 16:0 content of the 40-50% sucrose interface fraction is elevated (Table XVI).

Although the presence of lysophospholipid in the membranes of cells treated with PL could provide a basis for the multiple banding observed in these membrane preparations, no such evidence can be found in the lipid patterns of the fractions of control, DCS, or DCS and DA membrane preparations. Quantitative determinations of the protein (Lowry procedure) content of each fraction showed small differences between bands, but not on the level required for the density differences being reported (data not shown). Further, chromatography of acid hydrolyzates of these fractions did not reveal the presence of carbohydrate; a fact which supports the conclusion that cell wall contamination is not responsible for the sedimentation patterns observed.

Another possible explanation is that the isolation procedures employed result in the fragmentation and resealing of membrane vesicles of various sizes. If this is true, the sedimentation of these vesicles in sucrose, which is a poorly permeant molecule, would depend on variables such as vesicle size, shape, and degree of hydration. Sedimentation in a gradient composed of a freely permeant molecule, such as glycerol, would

TABLE XIII

PHOSPHOLIPIDS FROM MEMBRANES OF DIVIDING AND NON-DIVIDING CELLS AFTER FRACTIONATION ON SUCROSE GRADIENTS

Phospholipid Source*	Sucrose Interface	Rf Value	Periodate-Schiff's Reaction	Identification
Control Cells and DCS-DA grown cells**	40-50%	0.81	no reaction	DPTG
		0.55	purple	PTG
		0.23	yellow	PTI
	50-60%	0.81	no reaction	DPTG
		0.53	purple	PTG
		0.24	yellow	PTI
DCS grown cells	40-50%	0.81	no reaction	DPTG
		0.55	purple	PTG
		0.23	yellow	PTI
	50-60%	0.81	no reaction	DPTG
		0.55	no reaction[a]	PTG[a]
		0.25	yellow	PTI
	60-70%	0.81	no reaction	DPTG
		0.55	purple	PTG
		0.23	yellow	PTI

* Conditions and procedures for phospholipid analyses are outlined in Table VIII. Sucrose gradient conditions are outlined in the Materials and Methods Section.

** Neither the gradient fractionation patterns nor the phospholipid separation patterns exhibited a difference from control cells or PL cells attributable to the presence of DCS, hence DCS-DA membrane fractions are grouped with Control membrane fractions and DCS-PL membranes are grouped with PL membrane fractions.

XIII (Continued)

[a] A component migrating to the same rf as phosphatidylglycerol was faintly visible with ultraviolet light and iodine vapors. Other reactions were negative. We tentatively identify this compound as phosphatidylglycerol and assume that it is present in amounts below the sensitivity of the spray reagents.

TABLE XIV

FATTY ACID COMPOSITION OF MEMBRANES FROM DIVIDING AND NON-DIVIDING CELLS AFTER FRACTIONATION ON SUCROSE DENSITY GRADIENTS

Fatty Acid	40-50% Interface[a] Control Cells % of Total*	40-50% Interface[a] DCS Grown Cells % of Total*	40-50% Interface[a] DCS+DA Grown Cells % of Total*	50-60% Interface[a] Control Cells % of Total*	50-60% Interface[a] DCS Grown Cells % of Total*	50-60% Interface[a] DCS+DA Grown Cells % of Total*	60-70% Interface[a] DCS Grown Cells % of Total*
C 14:br (iso)	1.3	2.1	1.6	1.0	1.0	1.0	1.4
C 14:0	1.3	2.3	1.6	1.8	1.8	1.5	1.4
C 15:br (anteiso)	64.6	62.5	64.2	63.2	54.0	64.1	65.4
C 15:0	1.3	1.0	1.2	1.0	2.0	1.2	1.2
C 16:br (anteiso)	13.5	8.0	12.9	10.1	12.1	10.3	12.8
C 16:0	9.0	9.6	10.1	12.1	20.8	11.1	7.8
C 16:1	4.1	4.6	5.3	4.3	4.0	5.9	5.3
C 17:br (anteiso)	4.8	5.3	4.6	4.5	1.5	4.9	5.4

* Figures represent the mean of three determinations. Designations and calculations are given in Table I.

[a] Growth and treatment of membranes are outlined in The Materials and Methods Section.

All cultures were harvested after 18 hrs. growth at 37° C in the defined medium.

TABLE XV

PHOSPHOLIPIDS FROM MEMBRANES OF CELLS GROWN IN THE PRESENCE OF PL AFTER FRACTIONATION ON SUCROSE GRADIENTS

Phospholipid Source*	Sucrose Interface	Rf Value	Periodate-Schiff's Reaction	Identification
PL and DCS-PL grown cells*	40-50%	0.80	no reaction	DPTG
		0.71	no reaction	Unknown
		0.54	purple (faint)	PTG
		0.21	yellow	PTI
	50-60%	0.81	no reaction	DPTG
		0.70	no reaction	Unknown
		0.33	no reaction	LysoDPTG
		0.28	purple	LysoPTG
		0.26	yellow	PTI

* Neither the sucrose gradient fractionation patterns nor the phospholipid separation patterns revealed a difference attributable to the presence of DCS, hence DCS-PL results are not listed separately.

TABLE XVI

FATTY ACID COMPOSITION OF MEMBRANES FROM CELLS GROWN IN THE PRESENCE OF PL AFTER FRACTIONATION ON SUCROSE GRADIENTS

Fatty Acid	40-50% Interface[a] Control Cells % of Total*	40-50% Interface[a] PL Grown Cells[b] % of Total*	50-60% Interface[a] Control Cells % of Total*	50-60% Interface[a] PL Grown Cells[b] % of Total*
C 14:br (iso)	1.3	2.6	1.0	1.6
C 14:0	1.3	2.7	1.8	2.0
C 15:br (anteiso)	64.6	42.7	63.2	46.7
C 15:0	1.3	1.0	1.0	1.0
C 16:br (iso)	13.5	17.1	10.1	26.6
C 16:0	9.0	23.1	12.1	10.1
C 16:1	4.1	3.4	4.3	5.7
C 17:br (anteiso)	4.8	5.7	4.5	5.7

* Figures represent the mean of three determinations. Designations and calculations are outlined in Table I.

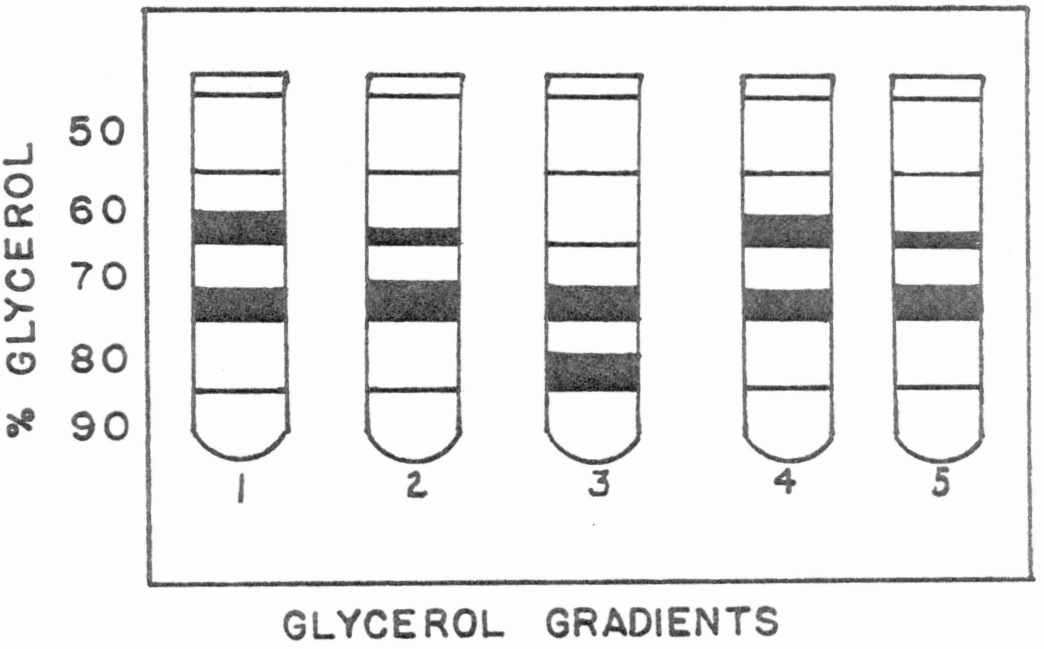
[a] Growth conditions and treatment of membranes are outlined in the Materials and Methods Section.

[b] Neither the sucrose gradient sedimentation patterns nor the fatty acid composition revealed a difference attributable to the presence of DCS hence DCS-PL results are not listed separately.

All cultures were isolated after 18 hrs. growth at 37° C in the defined medium.

Fig. 6. Glycerol Density Gradient Sedimentation Patterns of *M. lysodeikticus* Membranes.

(1) Membranes isolated from control cells.
(2) Membranes isolated from cells grown in the presence of PL (0.15 M) for six hours.
(3) Membranes isolated from cells grown in the presence of DCS (2×10^{-4} M) for six hours.
(4) Membranes isolated from cells grown in the presence of DCS (2×10^{-4} M) and DA (5×10^{-5}M) for six hours.
(5) Membranes isolated from cells grown in the presence of DCS (2×10^{-4} M) and PL (0.15 M) for six hours.


% GLYCEROL
50
60
70
80
90
1
2
3
4
5
GLYCEROL GRADIENTS

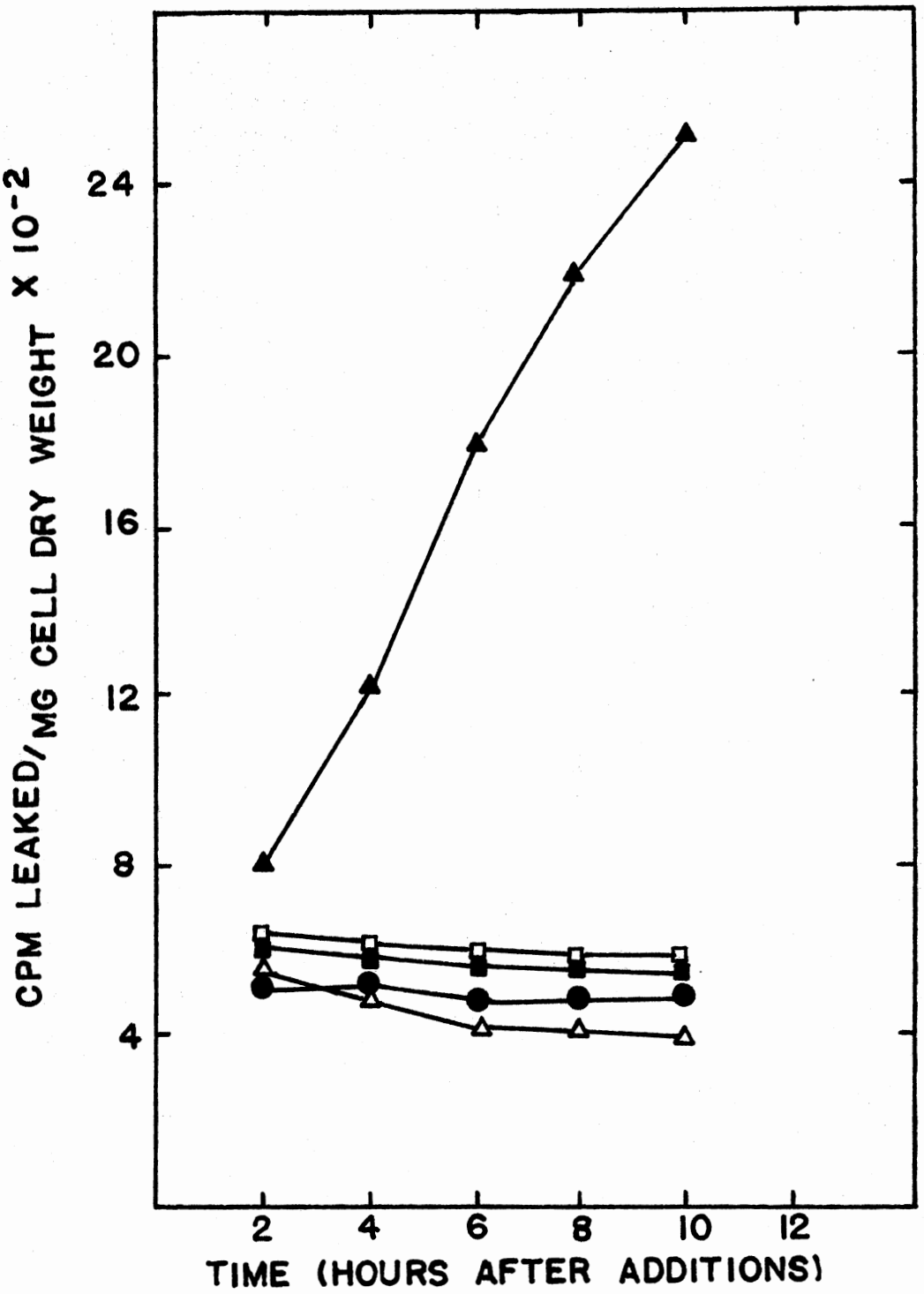
eliminate these variables since the inside of the vesicle would be able to assume the same density as the surrounding medium. In this case, the final equilibrium density would reflect the true membrane density. Multiple banding is clearly seen in glycerol gradients and membranes from cells grown in the presence of DCS or PL still show a difference from control cells (Fig. 6).

Lipid Leakage of Dividing and Non-Dividing Cells

Veerkamp (1976) and Horne, et al. (1977) have shown that lipids are leaked into the growth medium when several strains of bacteria are grown in the presence of inhibitors of mucopeptide synthesis. This leakage was not due to growth inhibition since leakage was not observed when cells were grown in the presence of antibiotics which did not inhibit mucopeptide synthesis. In an attempt to determine if the changes in the lipids of M. lysodeikticus after growth in the presence of DCS are due to leakage, cells were labeled by growth in the presence of labeled aspartic and glutamic acid and reinoculated into unlabeled media containing the test compounds.

Cells exposed to DCS show a significant increase in the amount of leakage of chloroform-methanol extractable substances after two hours exposure to DCS and leakage increases throughout the course of exposure (Fig. 7). About 50% of the isotope found in the medium after removal of these cells is not extractable into chloroform and methanol. Lysis does not account for this leakage since lysis is minimal throughout the first six hours exposure to DCS and the 78,000 x G centrifugation procedure for the removal of cells will also remove membranes from lysed cells. Electron microscopic observation of the spent medium reveals

Fig. 7. Leakage of Chloroform-Methanol Extractable Substances from Cells Grown in the Presence of DCS, DCS with DA, DCS with PL, and PL. ●, control cells; ▲, with DCS (2×10^{-4} M); △, with DCS (2×10^{-4} M) and DA (5×10^{-5} M); ■, with DCS (2×10^{-4} M) and PL (0.15 M); □, with PL (0.15 M).


CPM LEAKED/MG CELL DRY WEIGHT X 10⁻²
24
20
16
12
8
4
2
4
6
8
10
12
TIME (HOURS AFTER ADDITIONS)

the presence of small vesicular structures (Fig. 8) similar to those observed by Horne, et al. (1977). Although these structures cannot be sedimented under centrifugation conditions which remove membranes, some indirect evidence that these structures contain membrane material is available since the membrane carotenoids can be seen in the medium when these structures are present.

When M. lysodeikticus is grown in the presence of PL, DCS plus PL, or DCS plus DA, the amount of leakage of chloroform-methanol extractable material is similar to that released in control cultures. The vesicular structures and carotenoids cannot be observed in the growth medium when these treatments are employed. Although PL probably exerts a physical effect on the membrane, Pl does not confer osmotic stability to mucopeptide deficient cells (Grula, et al., 1968) and hence, osmotic protection cannot explain the PL-induced cessation of lipid leakage. DA, on the other hand, is able to overcome every consequence of growth in the presence of DCS thus far studied by restoring mucopeptide synthesis; however, PL does not restore mucopeptide synthesis (King and Grula, 1972). Although it is not yet possible to implicate the leakage of membrane components as the important alteration induced by the presence of DCS which results in cell division inhibition, it is of interest that PL and DA prevent cell division inhibition via two different mechanisms and the one membrane effect that they can be shown to have in common is a prevention of such leakage.

Is Lysophospholipid Formation the Mechanism of Action of PL?

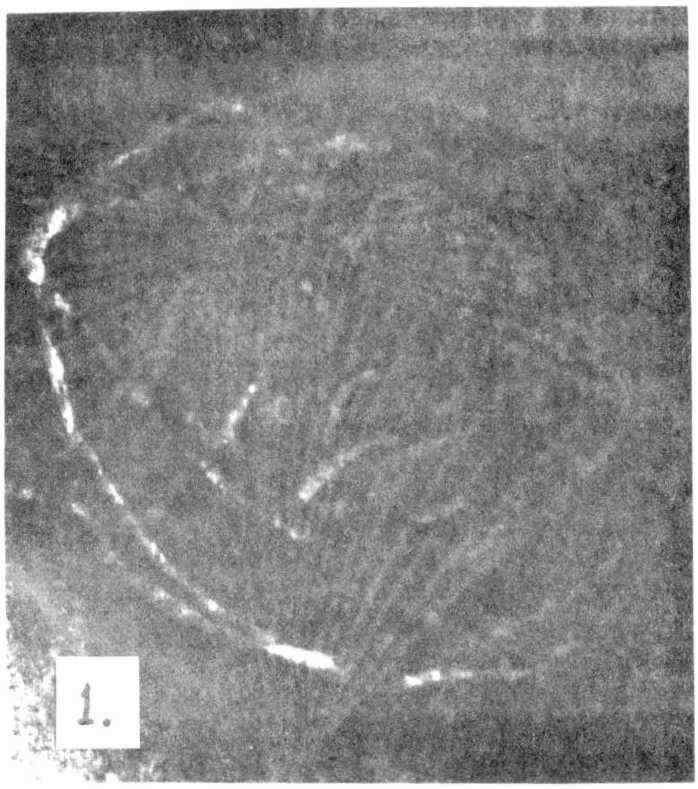
In an earlier section (see Table X), it was shown that PL can promote

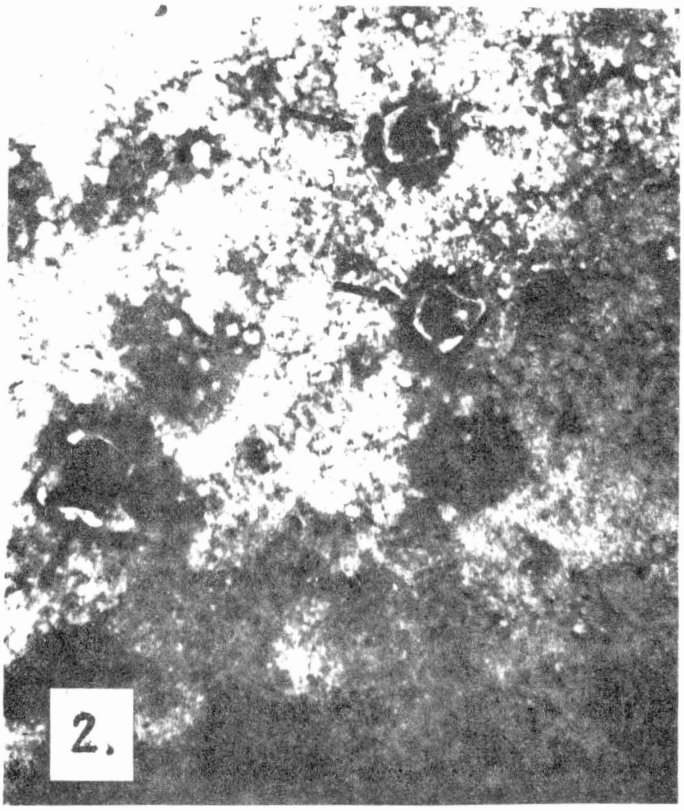
Fig. 8. Electron Micrographs of Membranes Isolated from Cells Grown in the Presence of DCS and Vesicular Structures Found in the Spent Medium After Growth of Cells in the Presence of DCS.

(1) Membranes isolated from cells grown in the presence of DCS (2×10^{-4} M) for six hours. Magnification, 46,000 X.

(2) Vesicular structures found in the spent medium after harvest of cells grown in the presence of DCS (2×10^{-4} M) for six hours. Magnification, 46,000 X.

Preparations were negatively stained with uranyl acetate.


1.

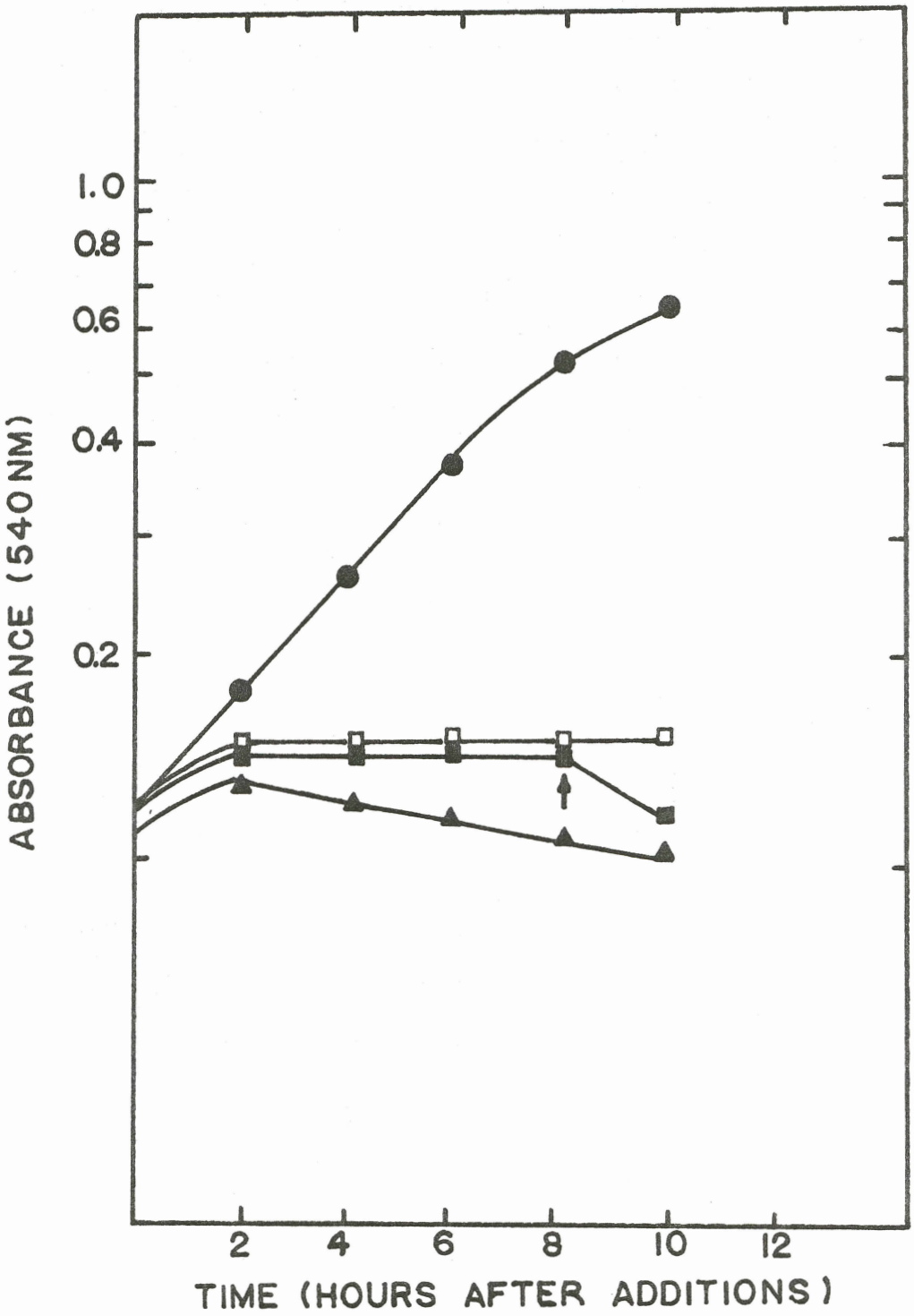

2.

the formation of significant amounts of lysophospholipid in M. lysodeikticus. Lysophospholipids and their free FA products have been shown to be "fusogenic" agents (Poole, et al., 1970; Ahkong, et al., 1973). If lipid leakage is an obligate step in cell division inhibition induced by inhibitors of mucopeptide synthesis, then PL might alleviate cell division inhibition through the formation of lysophospholipids which prevent this leakage. As a test of this hypothesis, three types of experiments were performed.

The first experiment was a test for the activation of phospholipase A_2 activities by PL in isolated membrane preparations. In these experiments, isolated membranes were incubated at 37°C in the presence of PL for up to 10 hours. These membranes were then harvested and their phospholipids analyzed using thin-layer chromatography. Buffers with differing ionic compositions (salts of the defined medium; salts of the defined medium plus 1 x 10^{-3}M Ca^{2+}; 0.0025M tris, pH 7.4; and 0.0025M tris, pH 7.4 plus 1 x 10^{-3}M Ca^{2+}) were used in anticipation of the fact that the phospholipase A_2 might have unusual ion requirements. The only sample where lysophospholipid formation was noted was a control preparation (no PL added) resuspended in the salt solution of the defined medium for 10 hours.

The second experiment was an attempt to simulate the action of PL on membrane lipids by adding phospholipase A_2 to a culture exposed to DCS for four hours. The four hour preincubation time was chosen so that sufficient mucopeptide synthesis inhibition had occurred to ensure that the enzyme was able to penetrate the cell wall and come into contact with the cell membrane. Results of these experiments are shown in Fig. 9. These cultures did not show an increase in optical density characteristic

Fig. 9. Growth Response of M. lysodeikticus to DCS and DCS with Phospholipase A_2. ●, control cell; □, cells treated with DCS (2×10^{-4} M) for four hours and then treated with phospholipase A_2 (200 ug) at time zero of curve; ■, cells treated with DCS (2×10^{-4} M) for four hours and then treated with phospholipase A_2 (200 ug) at time zero and again at eight hours (arrow); ▲ cells treated with DCS (2×10^{-4} M).


ABSORBANCE (540 NM)
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
12
TIME (HOURS AFTER ADDITIONS)

of PL treated cultures and lysis was evident by the tenth hour of exposure to DCS. A second addition of phospholipase A_2 was made at eight hours in anticipation of decreased enzyme activities by 10 hours (see arrow at eight hours). This second treatment did not confer lytic protection but actually potentiated lysis.

The third experiment involved growth of M. lysodeikticus in the presence of DCS, DCS with lysoDPTG (the major lysophospholipid found in PL treated cells), and DCS with the deacylation products of lysoDPTG formation. Fig. 10 shows the results of this experiment. Both lysoDPTG and the deacylation products were formed by treating control cell DPTG with phospholipase A_2. Neither lysoDPTG nor the FA products prevented DCS-induced lysis of these cultures (Fig. 10).

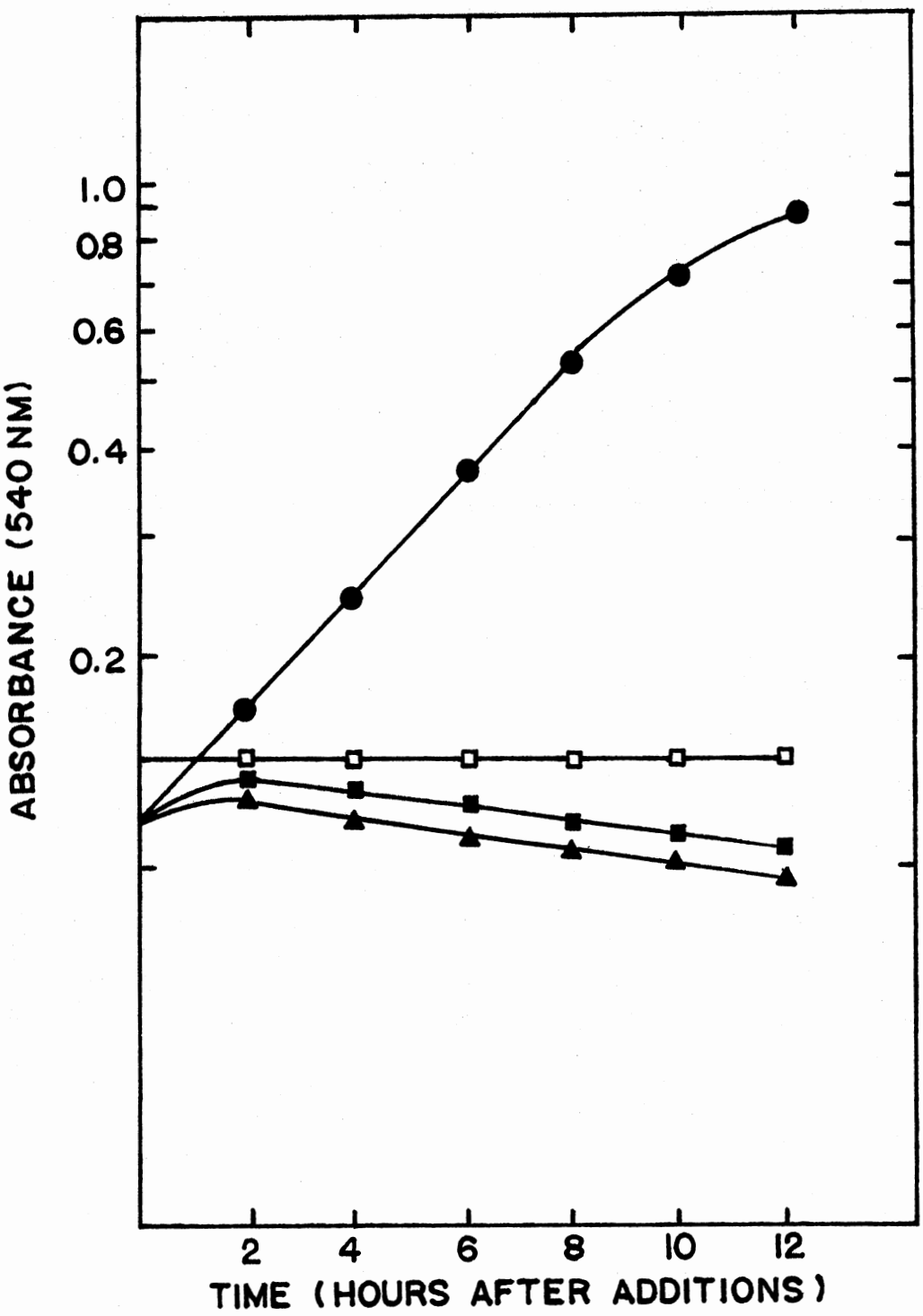
The above data and the time dependence of the PL-induced lipid effect indicate that lysophospholipid formation is probably due to an inhibition of lipid synthesis, rather than phospholipase A activation, resulting from growth in the presence of PL. It must also be concluded that this consequence is probably not the mechanism by which PL prevents cell division inhibition, although prevention of damage to the cell membrane by prevention of the leakage of membrane components cannot be overlooked.

Transport Studies

D-Alanine Transport in M. lysodeikticus dis II-p^+

One of the more direct measurements of the relationship between membrane structure and function is the measurement of the transport ability of a cell after structural alteration. Grula and King (1971) have demonstrated that the transport of DA, L-phenylalanine, and

Fig. 10. Growth Response of M. lysodeikticus to DCS, DCS with Lysodiphosphatidylglycerol, and DCS with the Deacylation Products of Diphosphatidylglycerol. ●, control cells; □, with DCS (2×10^{-4} M) and deacylation products of lysodiphosphatidylglycerol formation; ■, with DCS (2×10^{-4} M) and lysodiphosphatidylglycerol; ▲, with DCS (2×10^{-4} M).


ABSORBANCE (540 NM)
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
12
TIME (HOURS AFTER ADDITIONS)

glycerol are inhibited by PL in M. lysodeikticus dis II-p^+ within 30 seconds of the addition of PL. Amino acid uptake in M. lysodeikticus is the best system for study since carbohydrate transport in this organism is minimal.

The energy dependence of DA uptake in M. lysodeikticus is shown in Fig. 11. The addition of the uncoupler 2,4-dinitrophenol at a concentration of 10^{-2}M has drastic consequences upon the uptake of DA within one minute. Further, oxygen is required for DA transport as evidenced by the inhibition of uptake after five minutes under a stream of N_2. The ionophore valinomycin, which collapses K^+ gradients across cell membranes, can inhibit DA uptake within 30 seconds at concentrations of 1 x 10^{-8}M.

The curves in Fig. 12 demonstrate that PL at 0.15M concentration is able to inhibit the uptake of DA approximately 50% during the time course of a three minute experiment. One of the more curious properties of PL is shown in Fig. 12. When M. lysodeikticus is grown in the presence of PL for 12 hours and washed twice in the salts solution of the defined medium prior to the measurement of uptake, none of the inhibitory effects on DA transport are observed. Readdition of PL to these cells results in the same inhibition pattern seen in control cells after the addition of PL (Fig. 12). These data indicate that the physical presence of PL is required for the transport effects on DA. Further, DA transport appears to be unaffected by the profound alterations in membrane lipids resulting from 12 hours growth in the presence of PL (Tables II and X).

The lactone linkage is required for the inhibition of DA uptake since K^+- pantoic acid has no effect on DA transport (Fig. 13). Again, M. lysodeikticus is adversely affected by excess concentrations of sodium ion as

Fig. 11. Influence of 2,4 - Dinitrophenol, Anaerobic Conditions, and Valinomycin on the Uptake of D-Alanine - U - ^{14}C in M. lysodeikticus. ● , control cells; ▲ , with 2,4 - dinitrophenol (1 x 10^{-2} M) added one minute prior to the addition of label; ○, with Valinomycin (1 x 10^{-8} M) added one minute prior to the addition of label; □ , after 20 minutes under a stream of nitrogen.

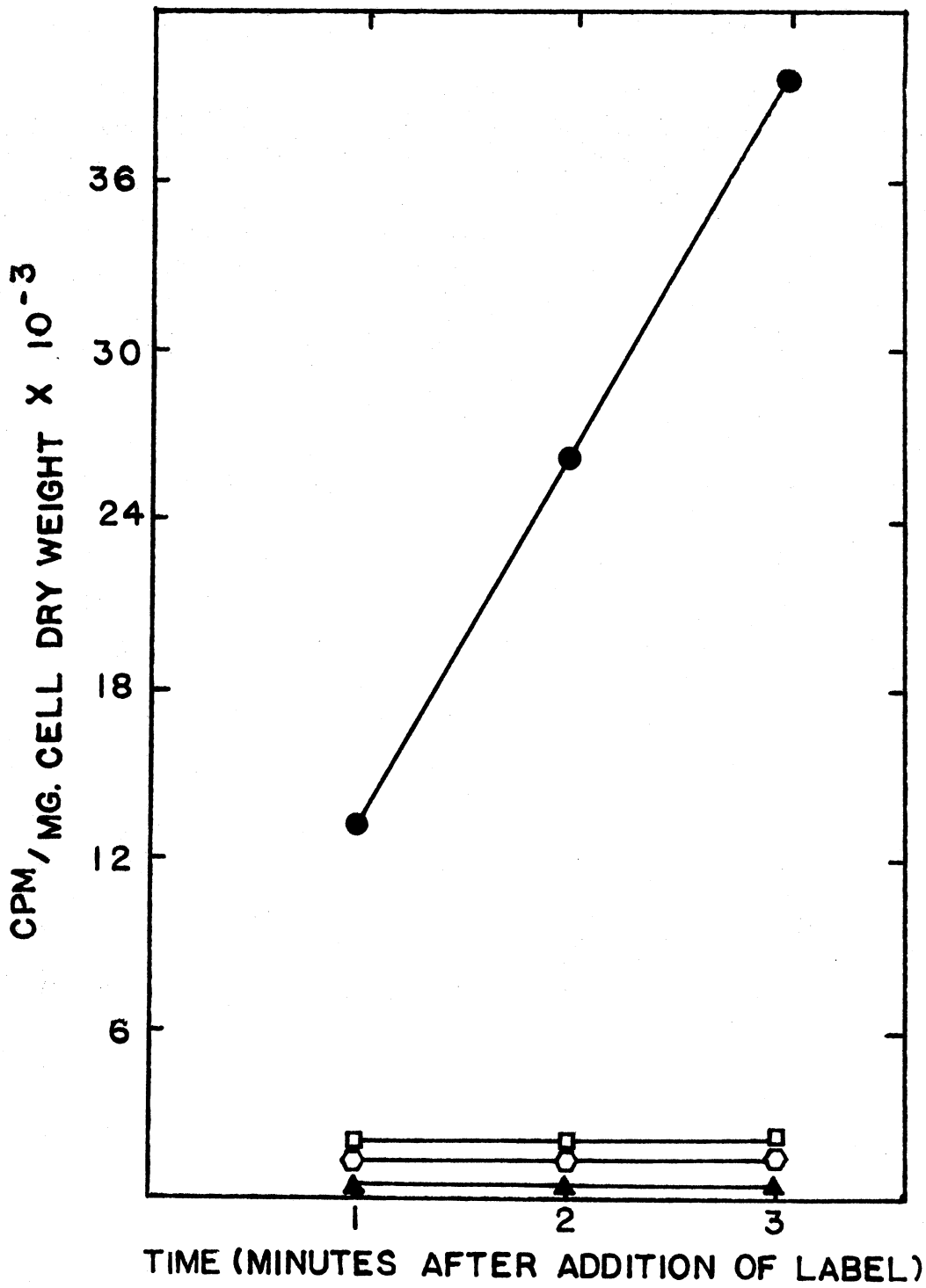
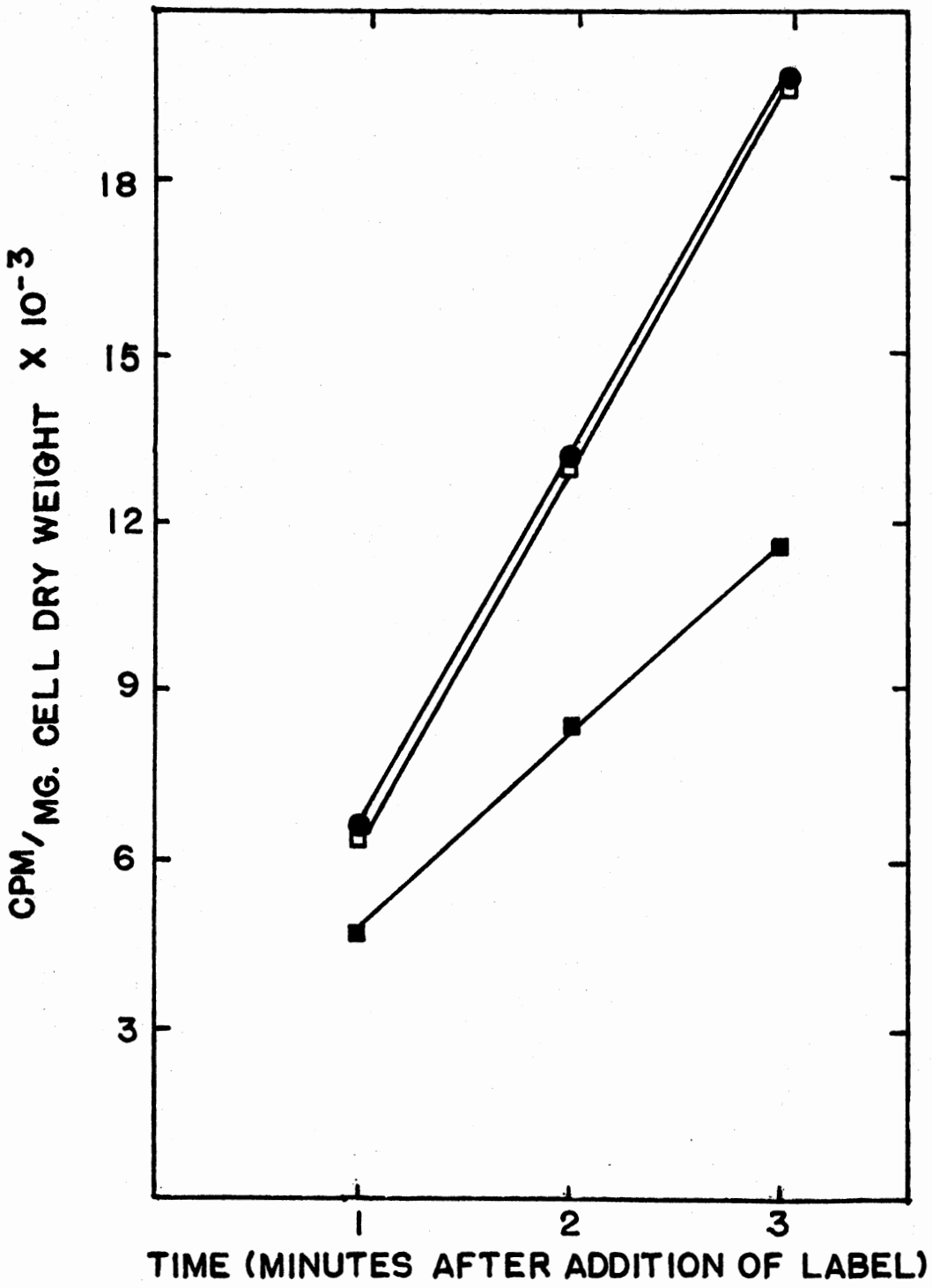

CPM / MG. CELL DRY WEIGHT X 10^{-3}
36
30
24
18
12
6
1
2
3
TIME (MINUTES AFTER ADDITION OF LABEL)

Fig. 12. Influence of PL on the Uptake of D-Alanine - U - ^{14}C. ●, control cells; □, cells grown in the presence of PL (0.15 M) for 12 hours and then washed three times; ■, with PL (0.15 M) added 30 seconds prior to the addition of label.


CPM/MG. CELL DRY WEIGHT X 10^{-3}
18
15
12
9
6
3
1
2
3
TIME (MINUTES AFTER ADDITION OF LABEL)

evidenced by DA transport inhibition induced by Na^+- pantoic acid. This effect can be shown to be due to the sodium ion since NaCl has a larger inhibitory effect than Na^+- pantoic acid (Fig. 13). KCl has little effect on DA transport.

Division inhibited cells show DA transport rates that are slightly higher than control rates through the first six hours of exposure to DCS (Fig. 14). However, at the eighth hour, cultures exposed to DCS begin to exhibit extensive lysis (Fig. 1) and, at this time, DA transport rates fall off sharply (Fig. 14) and continue to do so through 10 hours of DCS exposure. Simultaneous treatment of cultures with DCS and DA results in cells with DA transport rates very similar to control cultures. Cells treated with DCS and PL exhibit two unusual characteristics. The first is that these cells transport DA at slower rates than control cells through the first six hours of exposure to DCS and PL. This trend is not observed when these cells are treated with either DCS or PL alone. The second characteristic is that DA transport decreases after eight hours growth in the presence of DCS and PL in a manner similar to the decrease observed in DCS treated cultures even though DCS and PL treated cultures do not lyse and have the ability to divide. These data lend futher support to the cencept that PL does not prevent cell division inhibition by preventing DCS binding.

L-Glutamic Acid and L-Aspartic Acid Uptake in M. lysodeikticus

The uptake of L-glutamic acid and L-aspartic acid; the carbon, nitrogen and energy sources of the defined medium; was measured in dividing and non-dividing cells. Results of the uptake of L-aspartic acid in control,

Fig. 13. Influence of Potassium Pantoic Acid, Sodium Pantoic Acid, Potassium Chloride, Sodium Chloride, and PL on the Uptake of D-Alanine - U - ^{14}C. ●, control cells; ▲, with potassium pantoic acid (0.15 M) added 30 seconds prior to the addition of label; △, potassium chloride (0.15 M) added 30 seconds prior to the addition of label; ⬢, sodium pantoic acid (0.15 M) added 30 seconds prior to the addition of label; ⬡, sodium chloride (0.15 M) added 30 seconds prior to the addition of label; ■, with PL (0.15 M) added 30 seconds prior to the addition of label.

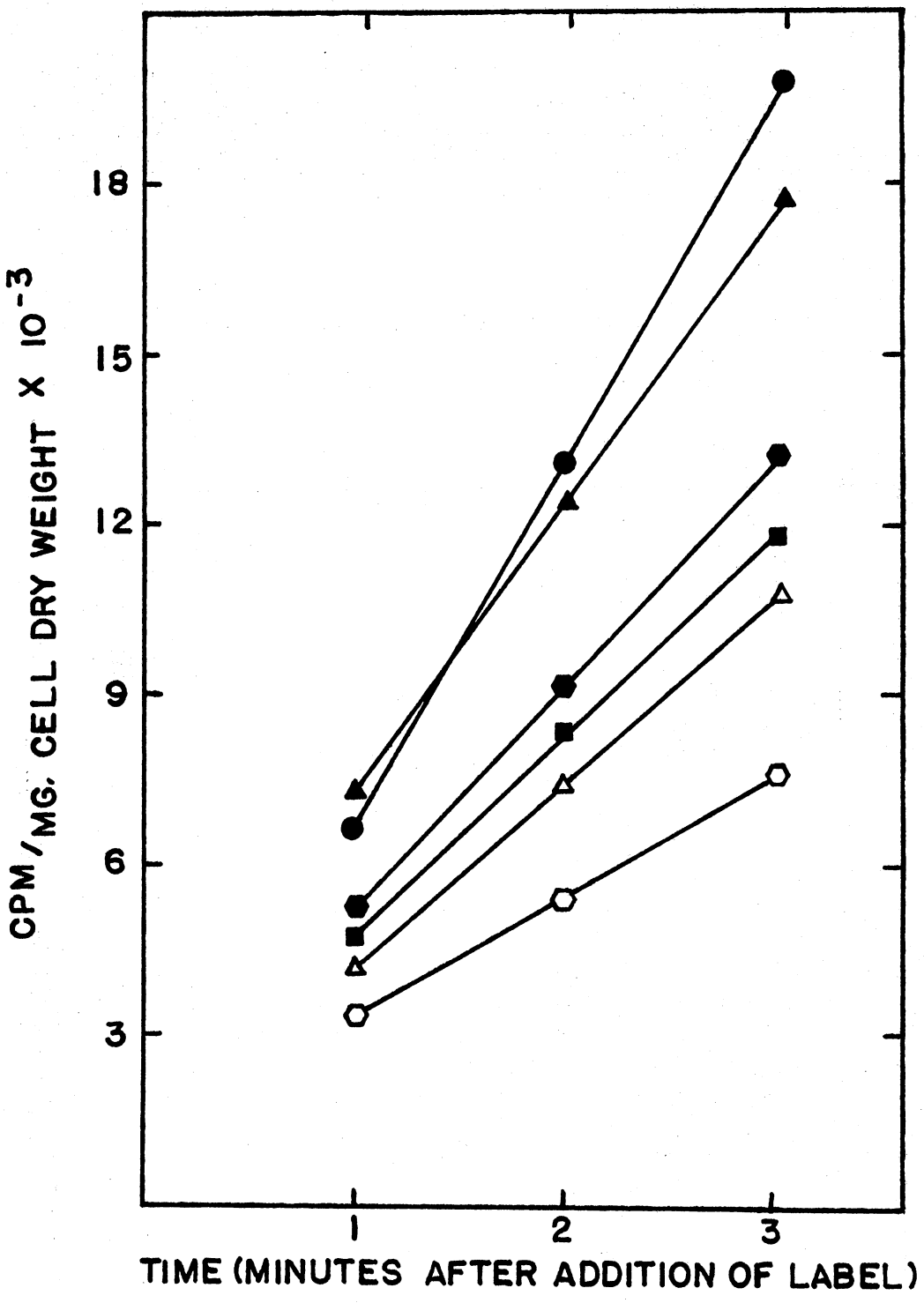
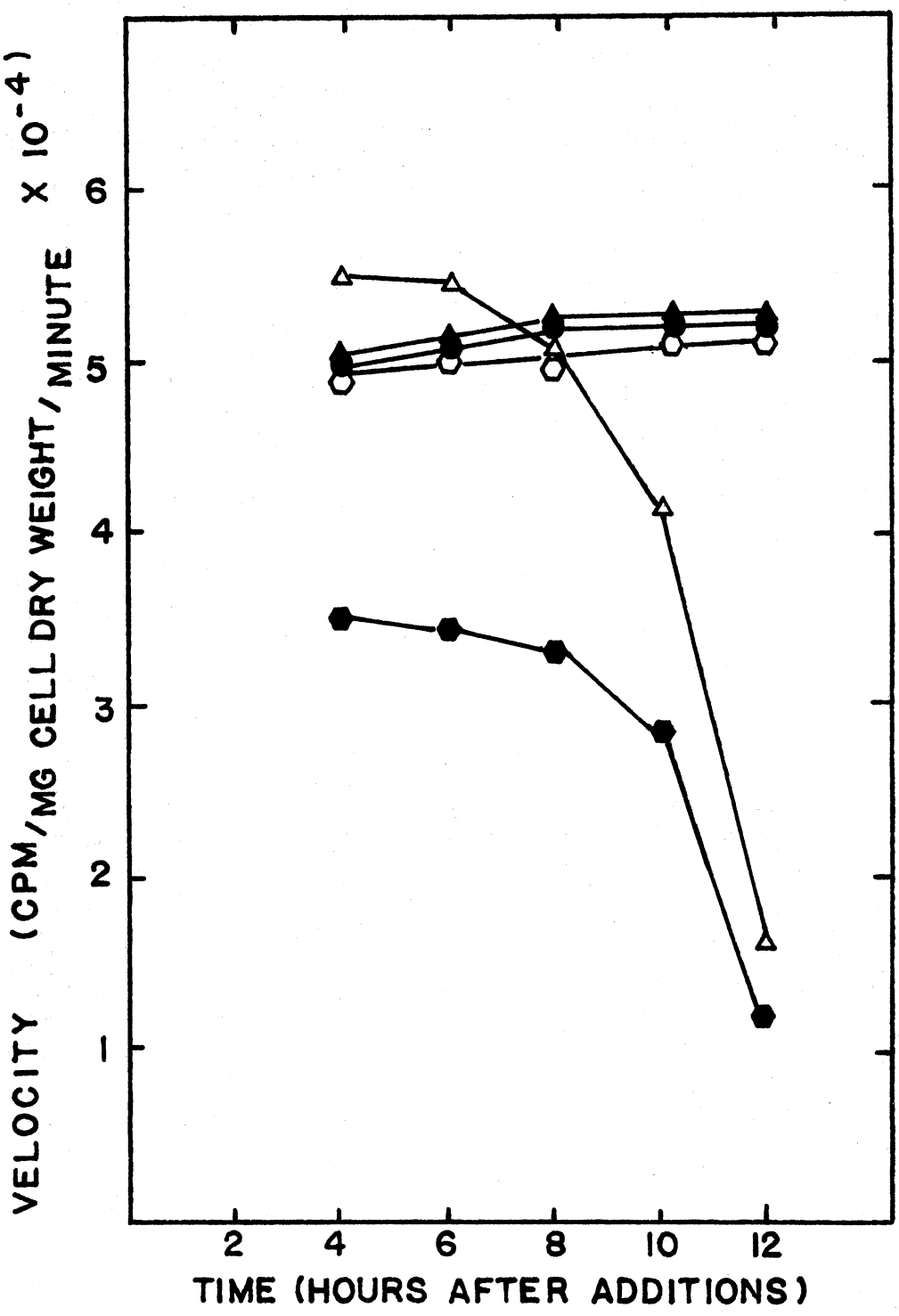

CPM/MG. CELL DRY WEIGHT X 10^{-3}
18
15
12
9
6
3
1
2
3
TIME (MINUTES AFTER ADDITION OF LABEL)

Fig. 14. Influence of Growth in the Presence of DCS, DCS with DA, DCS with PL, and PL on the Uptake of D-Alanine - U - ^{14}C. Cells were harvested at the times indicated in the figure and D-alanine uptake velocities were determined. ●, control cells; ▲, treated with DCS (2 x 10^{-4} M) and DA (5 x 10^{-5} M); ⬡, treated with PL (0.15 M); ⬢, treated with DCS (2 x 10^{-4} M) and PL (0.15 M); Δ, treated with DCS (2 x 10^{-4} M).


VELOCITY (CPM/MG CELL DRY WEIGHT/MINUTE X 10-4)
1
2
3
4
5
6
2
4
6
8
10
12
TIME (HOURS AFTER ADDITIONS)

DCS, DCS plus DA, and PL-treated cultures are shown in Fig. 15. Control, DCS and DA, and PL-grown cultures show nearly identical L-aspartic acid uptake rates. When PL is added to control cells one minute prior to the addition of aspartic acid, uptake is inhibited to a greater degree than is noted with DA uptake (80% as opposed to 50%). Cultures treated with DCS for eight hours show uptake rates similar to those observed in DA uptake.

L-Glutamic acid uptake rates show similar trends except that PL addition one minute prior to the addition of label results in rates that are slightly higher than those observed with DA uptake (Fig. 16). These data are plotted as u moles of substrate taken up per minute per mg cell dry weight to allow a comparison of aspartic acid uptake and glutamic acid uptake. Comparison of rates reveals that control cells of M. lysodeikticus accumulate glutamic acid more rapidly than aspartic acid. Further, the addition of PL to the uptake medium affects aspartic acid uptake to a greater degree than glutamic acid. These results do not account for the FA alterations induced by growth in the presence of PL because cells grown in the defined medium with glutamic acid as the only carbon and energy source have FA compositions similar to control cells (Table V).

Physical Interactions of Pantoyl Lactone with Model and Isolated Membrane Systems

Interactions of Pantoyl Lactone with Phospholipid Liposomes

In summarizing the effects of PL upon bacterial cells, it has been shown that PL is able to reverse or prevent cell division inhibition

Fig. 15. Influence of Growth in the Presence of DCS, DCS with DA, and PL for Six Hours on the Uptake of L-Aspartic Acid - U- ^{14}C. ●, control cells; △, with DCS (2×10^{-4} M) and DA (5×10^{-5} M); □, with PL (0.15 M); ▲, with DCS (2×10^{-4} M); ■, control cells with PL (0.15 M) added 30 seconds prior to the addition of label.

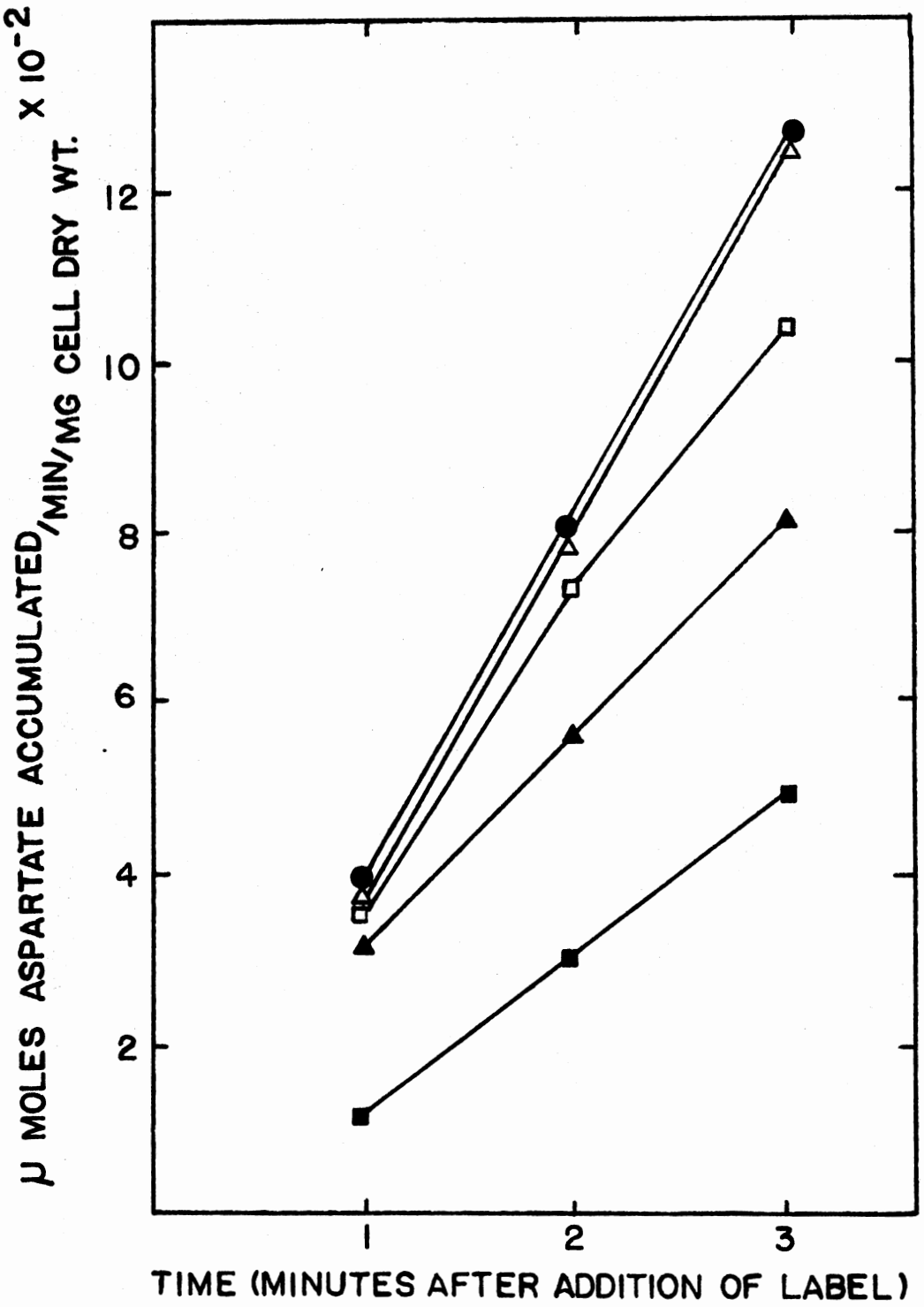

μ MOLES ASPARTATE ACCUMULATED/MIN/MG CELL DRY WT. X 10⁻²
2
4
6
8
10
12
1
2
3
TIME (MINUTES AFTER ADDITION OF LABEL)

Fig. 16. Influence of Growth in the Presence of DCS, DCS with DA, and PL for Six Hours on the Uptake of L-Glutamic Acid - U - ^{14}C. ●, control cells; Δ, with DCS (2×10^{-4} M) and DA (5×10^{-5} M); □, with PL (0.15 M); ▲, with DCS (2×10^{-4} M); ■, control cells with PL (0.15 M) added 30 seconds prior to the addition of label.

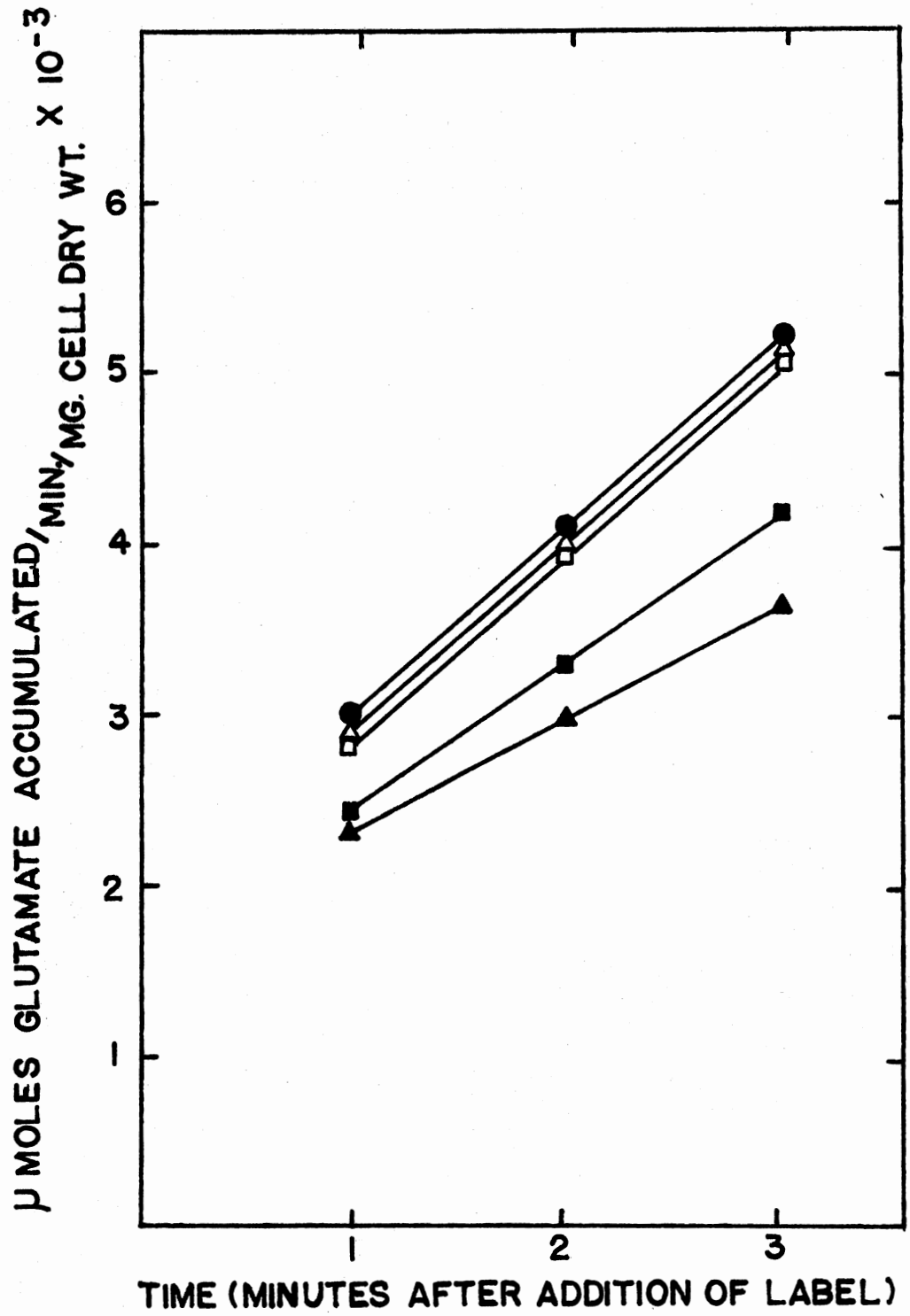
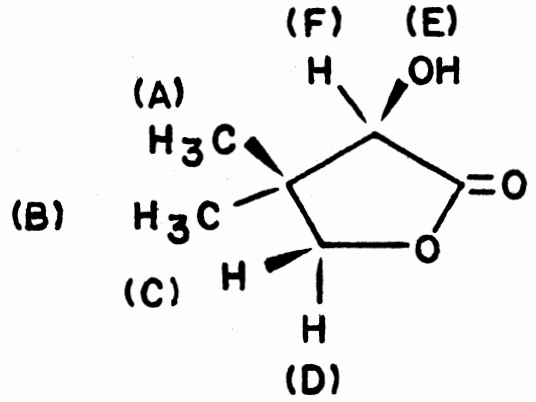

μ MOLES GLUTAMATE ACCUMULATED/MIN/MG. CELL DRY WT. X 10⁻³
1
2
3
4
5
6
1
2
3
TIME (MINUTES AFTER ADDITION OF LABEL)

Fig. 17. The Structure of Pantoyl Lactone.



PANTOYL LACTONE

regardless of the agent used to inhibit the process (Grula and Grula, 1962). When cell division is induced through the use of inhibitors of mucopeptide synthesis, PL overcomes division inhibition without restoring mucopeptide synthesis (Grula and Grula, 1964; Grula, et al., 1968; King and Grula, 1972). PL must be used at non-enzymatic concentrations for effects on cell division inhibition (Grula and Grula, 1962; King and Grula, 1972). PL prevents the leakage of periplasmic proteins (Grula and Hopfer, 1972), conformational changes in membrane proteins, and quantitative changes in membrane proteins (Grula and King, 1971) which accompany cell division inhibition. Growth in the presence of PL results in a time dependent alteration of both the phospholipid and phospholipid fatty acid composition of the cell membrane. Leakage of cell membrane components induced by the treatment of M. lysodeikticus with DCS is prevented by the addition of PL to the growth medium. PL synthesis, utilization, or strong banding has never been demonstrated in E. carotovora or M. lysodeikticus, and all of the biologically measureable effects of PL are readily reversed by simply washing the cells out of the medium containing the compound.

This type of information strongly indicates that the cell membrane is the primary site of action for PL and, further, that PL exerts a direct physical effect on the cell surface. To assess the physical effects of the presence of PL on the cell membrane, measurements of the interactions of PL with model membrane systems were initiated.

The structure of the PL molecule is shown in Fig. 17 and the letters assigned to the protons of the molecule will be used in the subsequent proton magnetic resonance (PMR) spectra to identify the peaks of the PL molecule. Proton assignments were made after observing the PMR spectrum

of PL in dichloromethane at low temperatures (Fig. 18). This spectrum is an enlargement of the -3.0 to -6.0 parts per million (ppm) region downfield from tetramethylsilane (TMS). The intense peak at -4.0 ppm on the room temperature scan shows an AB splitting pattern and represents the methylene protons (C and D in Fig. 17) at the γ-position of the lactone ring. The next most intense peak (-4.2 ppm) represents the α -proton adjacent to the hydroxyl group (E in Fig. 17), while the broad peak at -4.3 ppm is the proton of the hydroxyl group (F in Fig. 17). As the temperature is lowered, the hydroxyl group proton (F) moves downfield. The proton adjacent to the hydroxyl group (F) also moves downfield and, at -50°C, these two protons show magnetic coupling. When a lanthanide series metal shift reagent, in this case Eu^{3+}, is added to the sample; complexation of the Eu^{3+} with the hydroxyl group abolishes the magnetic coupling and the hydroxyl group proton peak (F) is no longer observable. The peak due to the proton adjacent to the hydroxyl group (E) becomes sharper and moves back upfield to its position at room temperature. The coupling not only indicates that the peak assignments above are correct, but it also indicates that the PL used in this study has a high degree of purity.

The entire 500 Hz scan of the spectrum of the PL molecule in deuterochloroform ($CDCl_3$) is shown in Fig. 19. The methylene protons (C and D) again show an AB splitting pattern with a chemical shift δ= 4Hz and a coupling constant J = 9 Hz. The addition of soybean phosphatidylcholine to this solution at equimolar concentrations results in a 16Hz upfield shift of the proton adjacent to the hydroxyl group (E). The chemical shift in the AB splitting pattern for the methylene protons (C and D) increases to δ = 8Hz.

Fig. 18. Proton Magnetic Resonance Spectra of PL in Dichloromethane.
(1) At room temperature.
(2) At 0°C.
(3) At -50°C.
(4) At -50°C in the presence of EuFOD (15 mg) shift reagent.

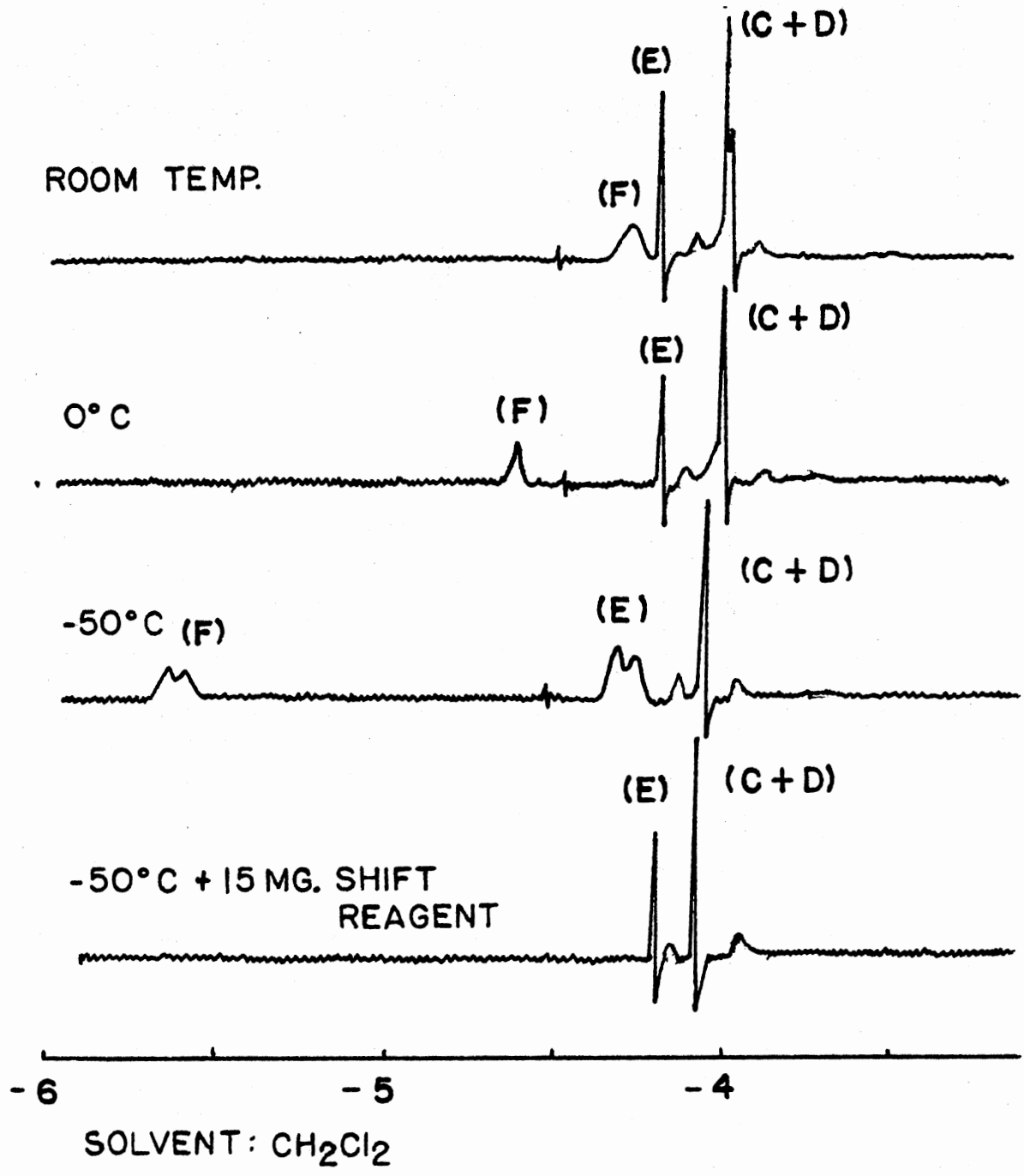
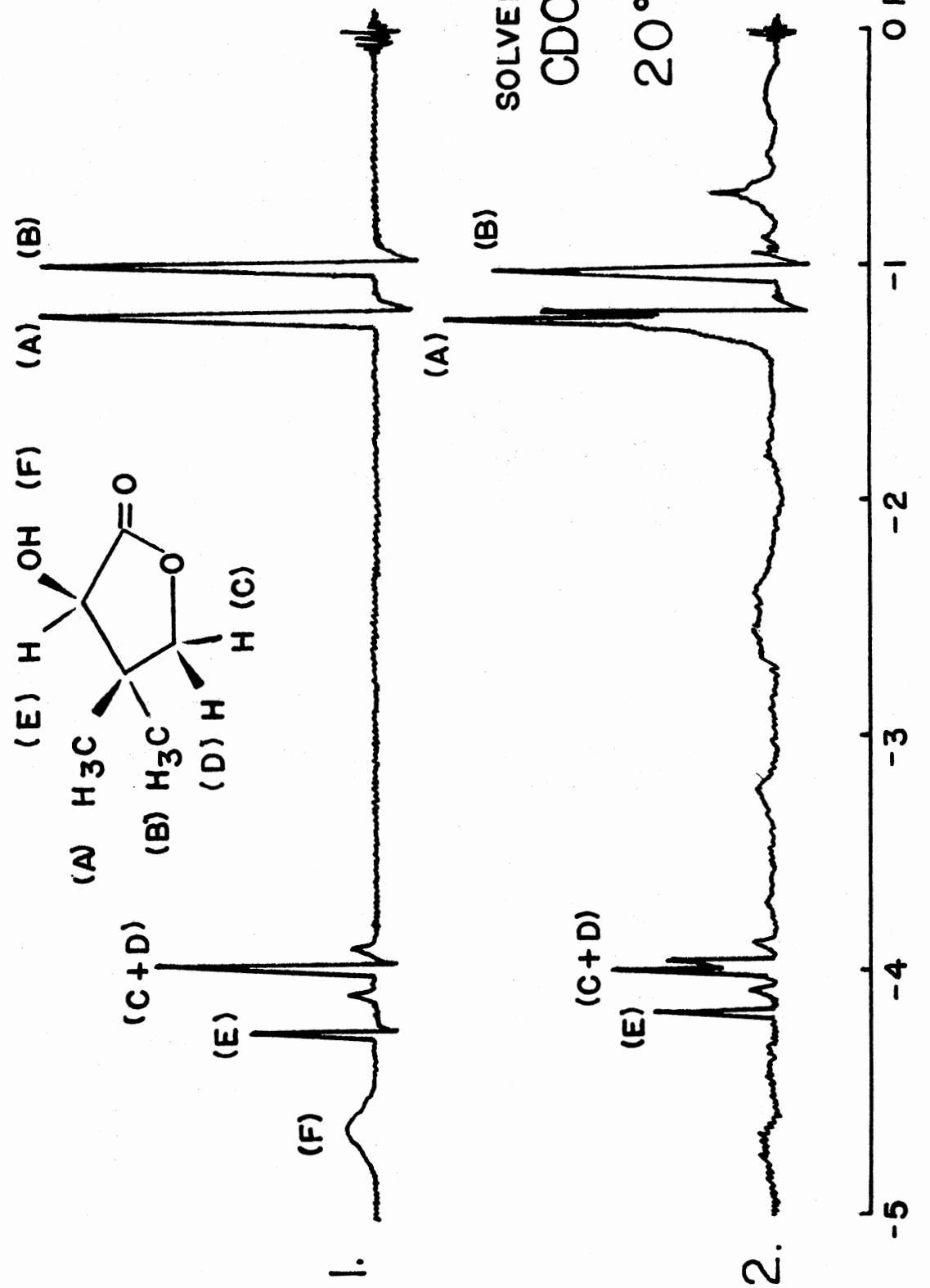

(C + D)
(E)
ROOM TEMP.
(F)
(C + D)
(E)
0° C
(F)
(C + D)
-50° C
(F)
(E)
(C + D)
(E)
-50° C + 15 MG. SHIFT
REAGENT
-6
-5
-4
SOLVENT: CH_2Cl_2

Fig. 19. Proton Magnetic Resonance Spectra of PL and PL with Phospholipids in Deuterochloroform.
(1) PL (1×10^{-2} M).
(2) PL (1×10^{-2} M) with soybean lecithin (1×10^{-2} M).


(A) H3C
(B) H3C
(C) H
(D) H
(E) H
OH
(F)
O
O
(A)
(B)
(C+D)
(E)
(F)
1.
SOLVENT:
CDCl3
20°C
(A)
(B)
(C+D)
(E)
2.
0 PPM
-1
-2
-3
-4
-5

The same experiment was performed in a deuterium oxide (D_2O) solvent as shown in Fig. 20. The proton adjacent to the hydroxyl group (E) moves upfield 10Hz, while the chemical shift of the AB splitting pattern for the methylene protons increases from δ = 4Hz to δ =6Hz in the presence of equimolar concentrations of phosphatidylcholine vesicular liposomes.

The data in both the D_2O and $CDCl_3$ solvents demonstrate that a weak interaction of the hydroxyl group proton is occurring with the phospholipid. The increase in chemical shift between the methylene protons could be the result of a decrease of the ring puckering rate which allows these two protons to become more magnetically distinguishable. This effect could also occur because the interactions of the hydroxyl group exerts a larger effect upon the methylene proton in the "cis" position relative to the hydroxyl group. It should also be noted that the dimethyl group peaks (A and B) are unaffected by the presence of phospholipid.

An interaction that would result in a slowing of PL ring puckering would require an interaction of the molecule through more than one functional group. The next best candidate for such an interaction is the carbonyl group. Since this group has no adjacent protons, the carbonyl stretching mode was studied using laser Raman spectroscopy.

The spectra of the 1300 - 1800 cm^{-1} region of the Raman spectrum of PL in a variety of solvents is shown in Fig. 21. The carbonyl symmetrical stretching frequency for γ-lactones occurs at about 1780 - 1785 cm^{-1} in organic solvents such as chloroform (top curve). When the PL molecule is put into polar solvents such as H_2O or D_2O, the carbonyl

Fig. 20. Proton Magnetic Resonance Spectra of PL and PL with Phospholipids in Deuterium Oxide.
(1) PL (1×10^{-2} M).
(2) PL (1×10^{-2} M) with soybean lecithin (1×10^{-2} M).

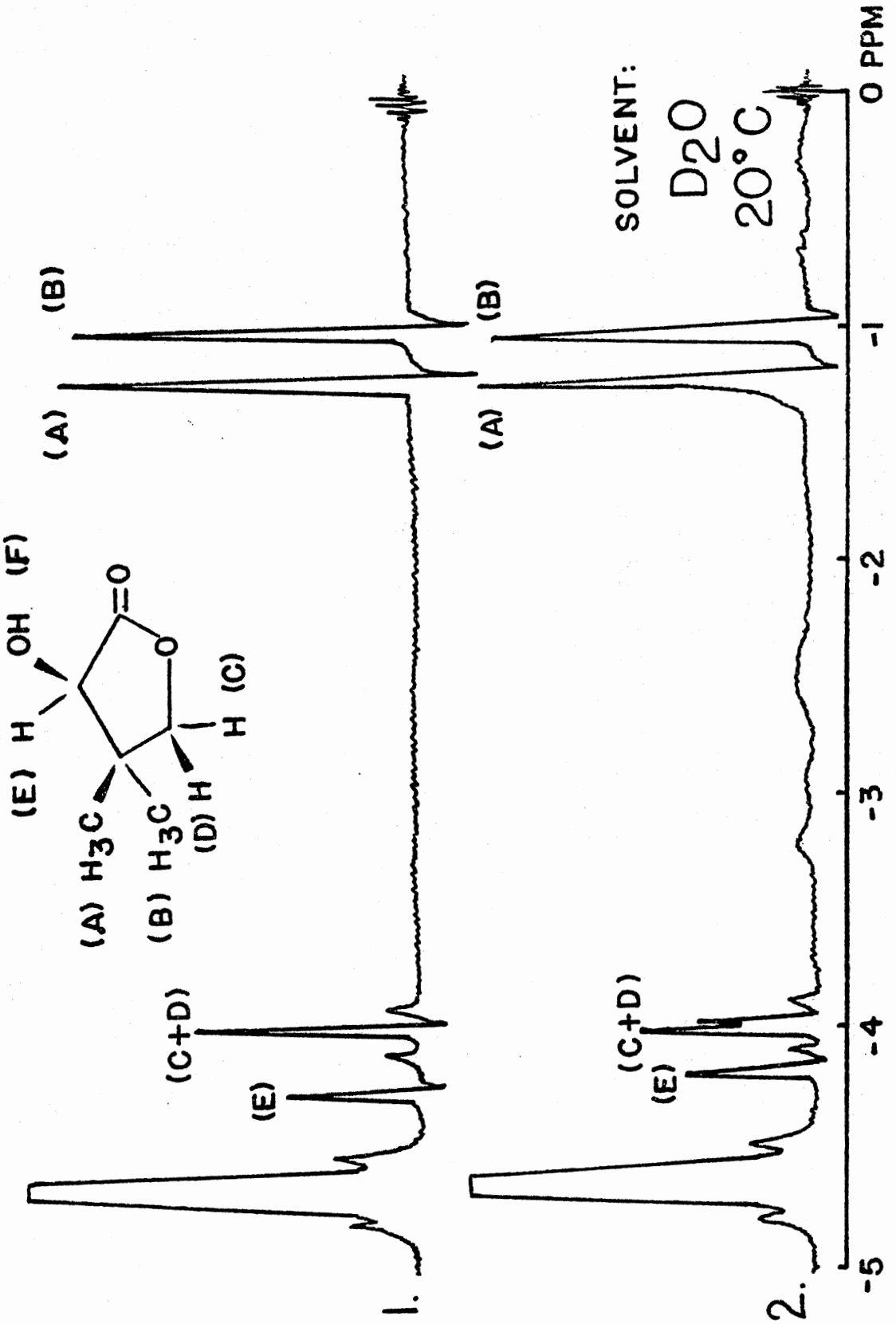
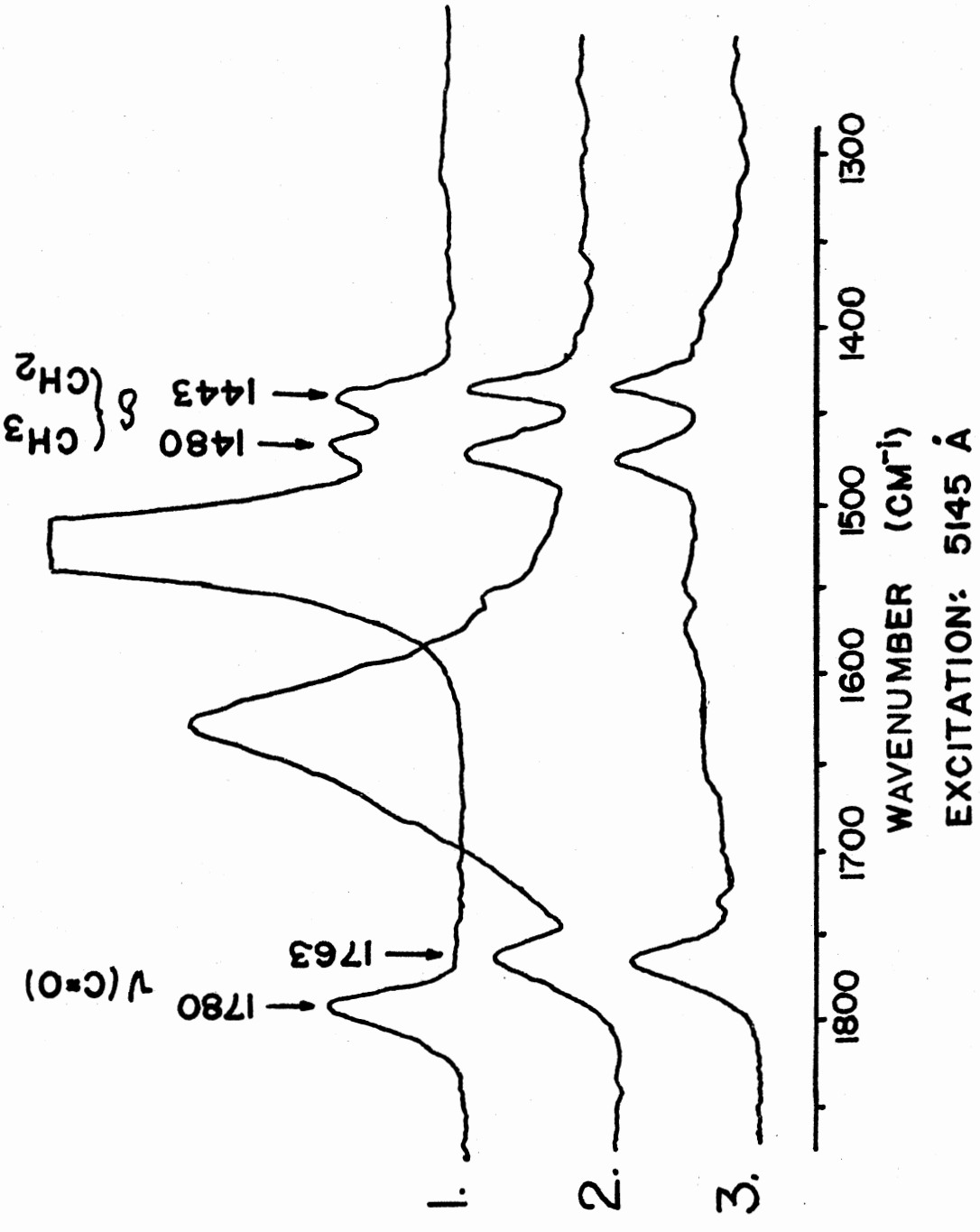

(A) H3C
(B) H3C
(C) H
(D) H
(E) H
OH (F)
O
=O
(A)
(B)
(C+D)
(E)
1.
SOLVENT:
D2O
20°C
2.
0 PPM
-1
-2
-3
-4
-5

Fig. 21. Laser Raman Spectra of PL in Chloroform, Water and Deuterium Oxide Solvents.
(1) PL (0.15 M) in $CHCl_3$.
(2) PL (0.15 M) in H_2O.
(3) PL (0.15 M) in D_2O.


1.
2.
3.
1780
1763
ν(C=O)
1480
1443
δ (CH3 CH2)
1800
1700
1600
1500
1400
1300
WAVENUMBER (CM-1)
EXCITATION: 5145 Å

stretching frequency shifts to 1763 cm^{-1} due to the ability of the molecule to hydrogen bond with the solvent and/or itself. The peaks observed at 1480 and 1440 cm^{-1} are deformation modes of the methyl and methylene groups of the molecule. These three peaks are of approximately equal intensity.

A detailed laser Raman spectrum of the PL molecule is given in Fig. 22. As can be seen, the molecule has ring "breathing" modes at 719 and 800 cm^{-1}, while the ring vibration modes occur at 1050 and 1167 cm^{-1}. Symmetrical and asymmetrical stretching modes of the methyl and methylene groups occur in the 2800 to 3000 cm^{-1} region. Measurement of the hydroxyl group stretching frequencies were not possible in the H_2O solvent because H_2O stretching modes above the 3200 cm^{-1} frequency dominate the spectrum.

When phosphatidylcholine liposomes are added to the PL solution at an equimolar concentration, the carbonyl stretching intensity is decreased and the peak is broadened. Ring breathing and vibration modes appear unaltered in both intensity and frequency, but the asymmetrical methyl group stretching intensity is decreased. None of the changes in the spectrum were due to the phospholipid. Liposome preparations must be used in concentrations of greater than 20% by weight before adequate Raman spectra are obtained (Spiker and Levin, 1976). The concentrations used in this study were 1.2% - 1.5%; and, therefore, these data indicate that there is both a carbonyl and methyl group interaction with phospholipid liposomes.

Since a primary site of interaction of the polar groups of the PL molecule with phospholipids is the phosphate group, spin-lattice (T_1) relaxation times of the ^{31}P nucleus of egg yolk phosphatidylcholine

Fig. 22. Laser Raman Spectra of PL and PL with Phospholipids in Deuterium Oxide.
(1) PL (0.15 M).
(2) PL (0.15 M) with soybean lecithin (0.15 M).

2.
1.
3000
2800
1800
1500
1000
500
100
WAVENUMBER CM⁻¹
2960 ASYM. ν CH3
2900 ν CH2
2850 SYM. ν CH2 AND CH3
1763 ν C=O
1481
1443
δ CH2 AND CH3
1167
1050
RING VIBRATION
800
719 "RING BREATHING"

liposomes in water were measured. The addition of PL lowers the T_1 of the ^{31}P nucleus from 0.75 to 0.67 sec. Although the lowering of T_1 can sometimes be caused by an increase in the viscosity of the medium (Bloembergen, et al., 1948), the addition of PL to water actually lowers its viscosity. Therefore, the increase in the T_1 may be due to the slowing down of the motion of the phosphate group caused by the binding of PL.

The phosphatidylcholine liposomes used in the above measurement were in the lyotropic liquid crystalline state and are, therefore, anisotropic. The ordering of these liposomes can be studied by observing the orientation factors of rigid, small molecules with three-fold (or higher) axes of symmetry. Benzene is such a molecule and it orients with its C_6 axis perpendicular to the aliphatic chains of phospholipids. Although the partitioning of a molecule such as deuterobenzene (C_6D_6) into liposomes is analogous to the use of EPR probes, perturbations induced in the aliphatic chains by the flat, planar C_6D_6 molecule are likely to be much smaller than for the bulkier nitroxide groups of spin labels in spite of the larger concentration requirements.

Data presented in Table XVII show that in an isotropic hydrocarbon medium, such as hexadecane, the spin-lattice (T_1) relaxation time is equal to the spin-spin relaxation (T_2) time which is 0.72 seconds at 25° C. In liposomes prepared from purified M. lysodeikticus phospholipids, $T_1 \simeq 8T_2$ which indicates that benzene is able to tumble in the plane of its C_6 axis more freely than out of the plane of the C_6 axis. The addition of varying amounts of PL to this system sequentially lowers the T_1 and raises the T_2 relaxation time indicating that a higher degree of disorder within the aliphatic chains of the phospholipids accompanies the interaction of PL with these lipsomes since the mobility of benzene

TABLE XVII

DEUTERON RELAXATION TIMES[a] OF DEUTEROBENZENE[b] IN LIPOSOMES[c] PREPARED FROM PHOSPHOLIPIDS ISOLATED FROM M. LYSODEIKTICUS

	PL Concentration			
	0	120 mg	230 mg	400 mg
T_1 (sec.)	0.680	0.650	0.610	0.590
T_2 (sec.)	0.080	0.090	0.110	0.130
T_2/T_1	0.120	0.140	0.180	0.220

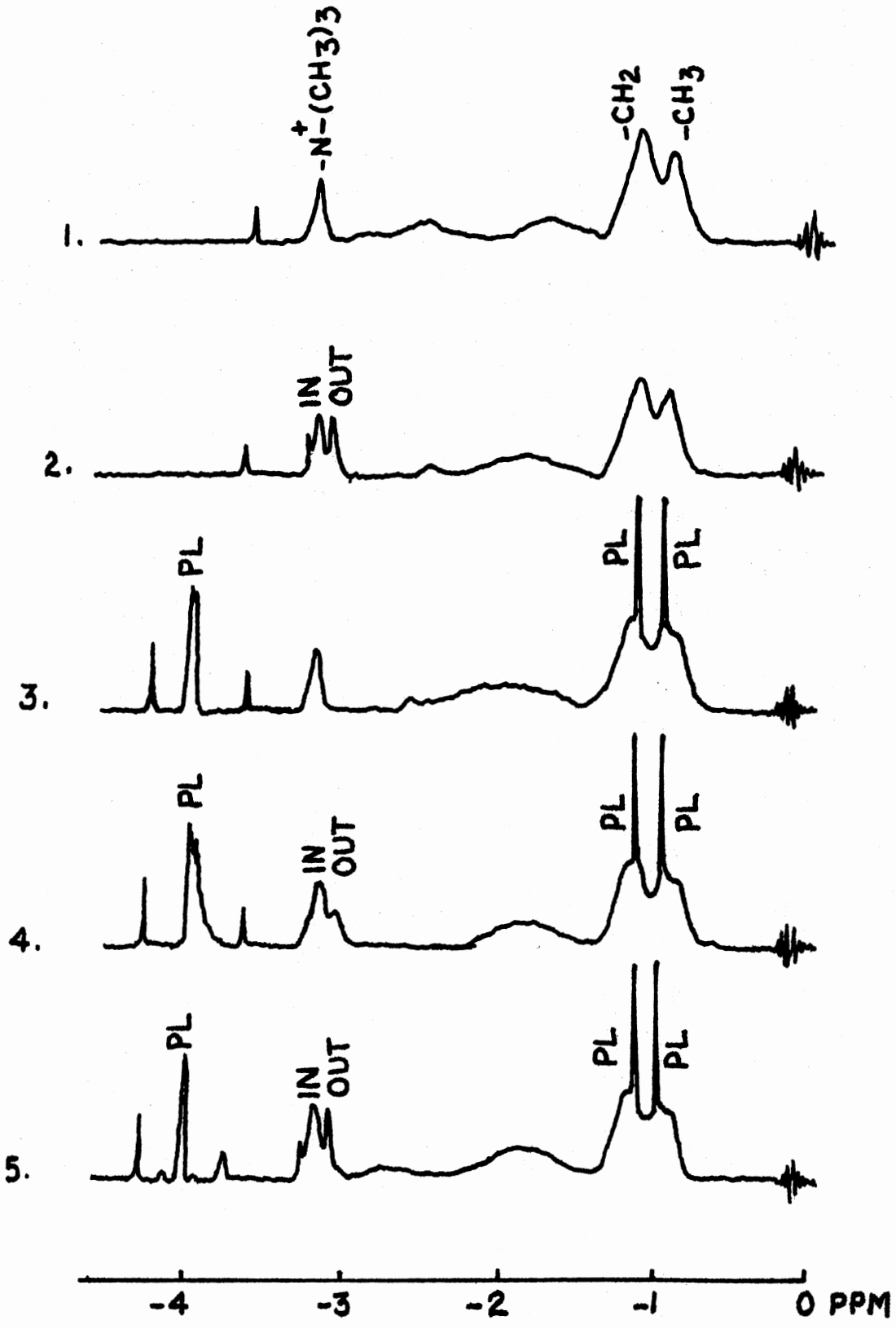
[a] Experiments were performed at 9.21 MHz NMR and 25°C
[b] Deuterobenzene (0.040 g) was partitioned into a phospholipid liposomes (1.350 g) suspension in H_2O.
[c] In hexadecane, $T_1 = T_2 = 0.72$ sec.

out of the plane of its C_6 axis is increased. These results are not unique to phospholipid liposomes from M. lysodeikticus membranes because similar data were obtained using liposomes prepared from dipalmitoyl phosphatidylcholine liposomes.

In addition to these relaxation measurements, the chemical shift of the $N^+ - (CH_3)_3$ proton signal of phosphatidylcholine lipsomes was studied. Bystrov, et al. (1972) have shown that lanthanide ions can induce chemical shifts in these protons and, since these ions cannot penetrate the liposomes, the chemical shifts induced are in the N^+-$(CH_3)_3$ protons of the outer phospholipid layer. Spectra are shown in Fig. 23. As can be seen in the top spectrum, 10% (by weight) suspension of soybean phosphatidylcholine has a well resolved $N^+ - (CH_3)_3$ proton signal at -3.2 ppm downfield from TMS. The addition of $EuCl_3$ to the suspension resolves the inner and outer signals of the choline methyl protons. When PL is added to this suspension after the addition of $EuCl_3$, no change is observed in the signal indicating that Eu^{3+} has tied up the ionic sites to which PL weakly binds. When PL is added before $EuCl_3$, a broadening of the outer signal is observed while PL alone has no effect on the proton signals of any of the peaks of the phospholipid. These data indicate that the interaction of PL is weak and the molecule cannot displace previously associated ions. Also, ions such as Eu^{3+} cannot displace PL when it has associated with the phospholipid, but Eu^{3+} may also bind to PL. One explanation for these data is that PL is able to associate with phospholipid at a site different from Eu^{3+}. If Eu^{3+} could not bind, no differences between the inner and outer choline methyl proton signal would be observed. Unfortunately, PL alone had no effect on any peak of the phospholipid spectrum and, hence, the pene-

Fig. 23. Proton Magnetic Resonance Spectra of Soybean Lecithin, Soybean Lecithin with Europium Chloride, Soybean Lecithin with PL, and Soybean Lecithin with Europium Chloride and PL.

(1) Soybean lecithin (0.13 M).
(2) Soybean lecithin (0.13 M) with $EuCl_3$ (1×10^{-3} M).
(3) Soybean lecithin (0.13 M) with PL (0.15 M).
(4) Soybean lecithin (0.13 M) with $EuCl_3$ (1×10^{-3} M) added after the addition of PL (0.15 M).
(5) Soybean lecithin (0.13 M) with $EuCl_3$ (1×10^{-3} M) added before the addition of PL (0.15 M).


-N+-(CH3)3
-CH2
-CH3
1.
IN
OUT
2.
PL
PL
PL
3.
PL
IN
OUT
PL
PL
4.
PL
IN
OUT
PL
PL
5.
-4
-3
-2
-1
0 PPM

tration ability of PL could not be monitored.

The data discussed in the above sections are consistent with a hydrogen bonding mechanism of the carbonyl and hydroxyl groups of PL with the phosphate group and solvent, or constituent head group, of phospholipids (Fig. 24). However, because the T_1 and T_2 parameters of C_6D_6 in the liposome hydrocarbon region and the Raman spectrum of the methyl and methylene groups of PL are affected by the binding of PL to liposomes, the partitioning and hydrogen bonding interaction representation of PL and liposome interaction cannot be overlooked.

Interactions of Pantoyl Lactone with Lysozyme

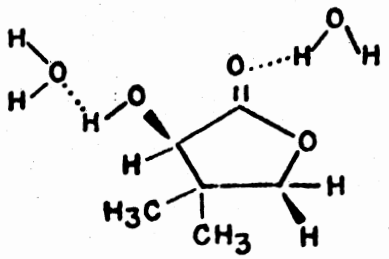
It will be shown later (next section) that PL exerts a different effect on the T_1 and T_2 relaxation times of C_6D_6 in isolated cell membranes from the effect seen in phospholipid liposomes. The data obtained in these studies suggest that PL does not interact exclusively with phospholipid and, when the uptake results are considered, an interaction of PL with proteins is suggested. It, therefore, appeared important to determine if PL has any effects on protein molecules.

Three major criteria were considered in choosing lysozyme as a candidate for such studies. Although lysozyme is not a membrane protein, this molecule has a measureable function, a great deal is known about its structure (which allows an assessment of structure and function relationships), and lysozyme can serve as a model for measuring the effects of the presence of PL on periplasmic proteins.

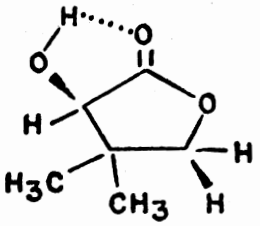
The laser Raman spectra of lysozyme in a NaCl buffer with and without PL are shown in Fig. 25. Arrows in the spectrum of lysozyme in the presence of PL (bottom curve) identify the various vibrations contributed

Fig. 24. Possible Hydrogen Bonding Interactions of PL with Phospholipids.

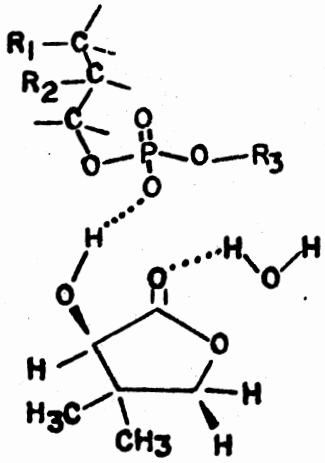
POSSIBLE H-BOND INTERACTIONS FOR PANTOYL LACTONE



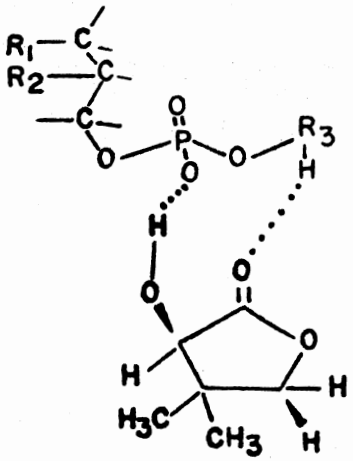
1. SOLVENT INTERACTION



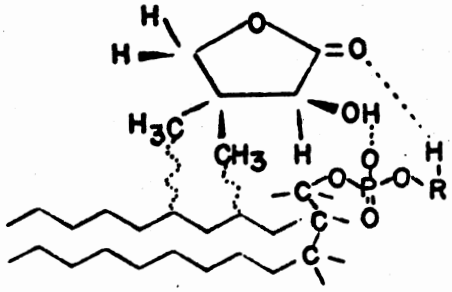
2. INTRAMOLECULAR INTERACTION



3. PHOSPHOLIPID AND SOLVENT INTERACTION

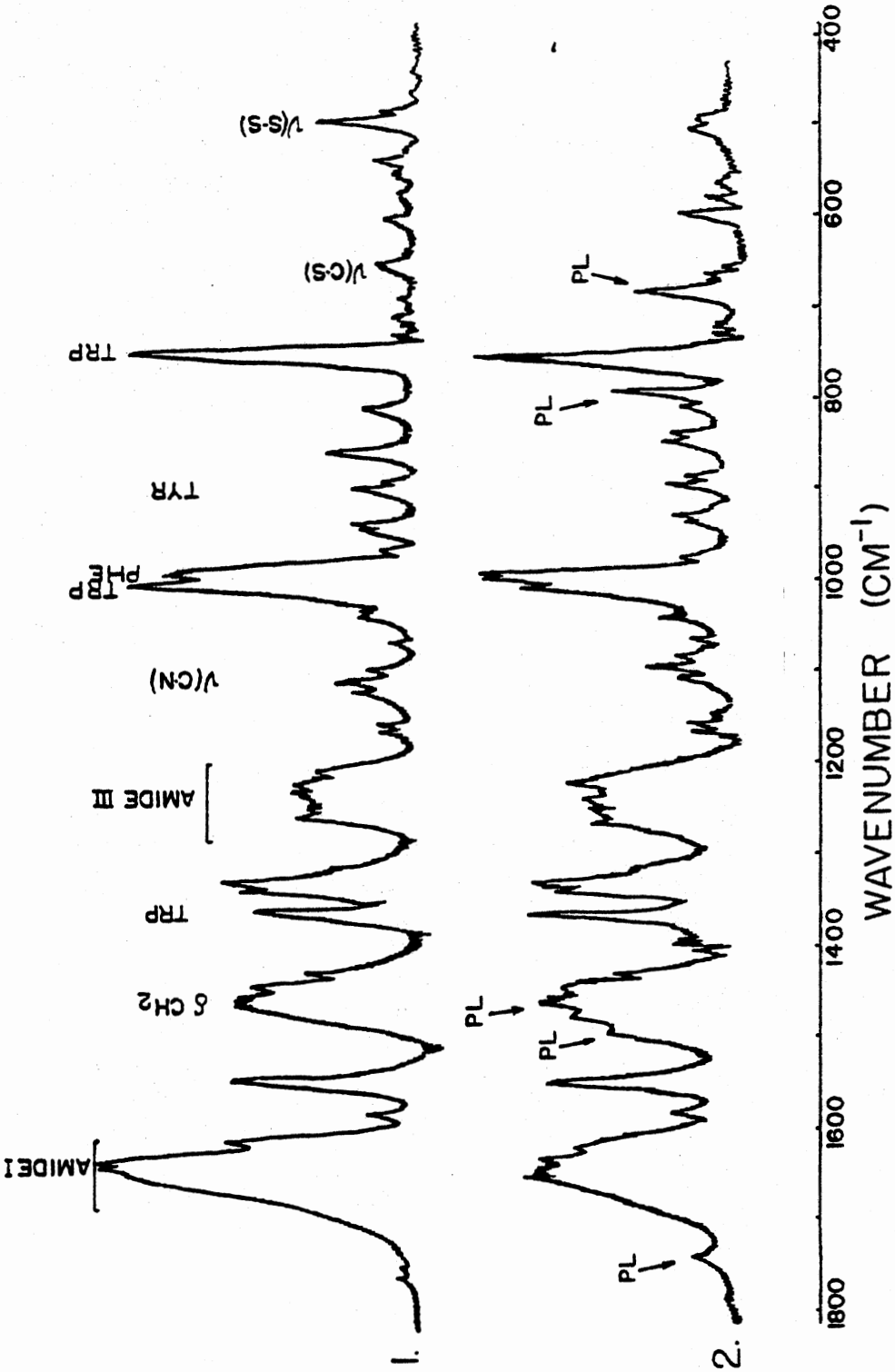


4. PHOSPHOLIPID INTERACTION



5. PHOSPHOLIPID PARTITIONING INTERACTION

Fig. 25. Influence of PL on the Laser Raman Spectrum of Lysozyme.
(1) Lysozyme (7% solution) in 0.01 M NaCl, pH 7.0.
(2) Lysozyme (7% solution) in 0.01 M NaCl, pH 7.0 with PL (0.15 M).


AMIDE I
δ CH2
TRP
AMIDE III
ν(C-N)
TRP
PHE
TYR
TRP
ν(C-S)
ν(S-S)
1.
PL
PL
PL
PL
2.
1800
1600
1400
1200
1000
800
600
400
WAVENUMBER (CM-1)

by PL. There are three major differences in these spectra and peaks were identified by using the criteria of Chen, et al. (1973). The relative intensity of the S-S stretching mode at ν = 511 cm^{-1} is decreased in the presence of PL and, unfortunately, a concomitant change in the C-S stretching mode at ν= 698 cm^{-1} cannot be observed because one of the major ring breathing modes of the PL molecule occurs at ν = 701 cm^{-1} and obscures the resolution of the C-S stretch. The second major difference in the two spectra is the enhanced intensity of the phenylalanine ring C-N stretch at ν = 1005 cm^{-1}. Although this increase could be accounted for by a decrease in the tryptophan ring C-N stretch at ν = 1011 cm^{-1}; this explanation is unlikely since the other tryptophan stretching modes at ν= 761 cm^{-1} and ν= 1361 cm^{-1} remain unchanged. The third major difference observed is in the amide I and amide III regions which are indicators of conformational state.

As can be seen, PL induces a reduction in the peak intensity at ν= 1655 cm^{-1} and a shoulder appears at 1665 cm^{-1}. There is also an increase in the intensity of the ν= 1245 cm^{-1} peak. These data indicate a decrease in the helical content and an increase in the amount of disordered structure in the molecule.

Quantitative measurement of the change in helical content can be obtained using circular dichroism (CD) spectroscopy. The CD spectra of lysozyme obtained under the conditions used for the Raman measurements are shown in Fig. 26. Using the procedures of Chen and Yang (1971), the fraction of helix in lysozyme can be calculated in the following manner:

For lysozyme in 0.1M NaCl, pH 7.0 buffer:

$$[\theta]_{220} = -27{,}700\ F_h - 3380$$

Fig. 26. Influence of PL on the Circular Dichroism Spectra of Lysozyme.
——— Lysozyme (7% solution) in 0.01 M NaCl, pH 7.0.
---- Lysozyme (7% solution) in 0.01 M NaCl, pH 7.0 with PL (0.15 M).

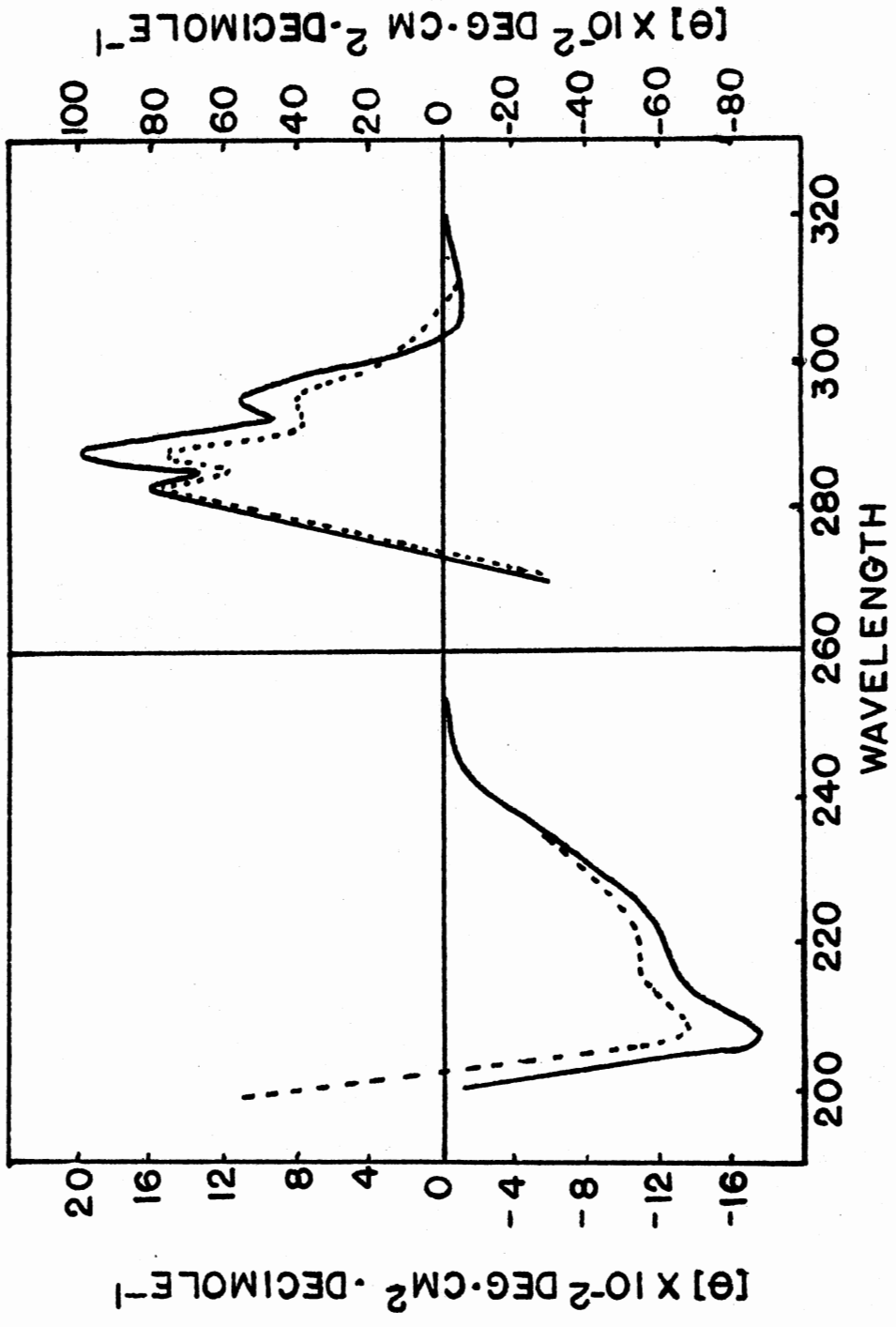
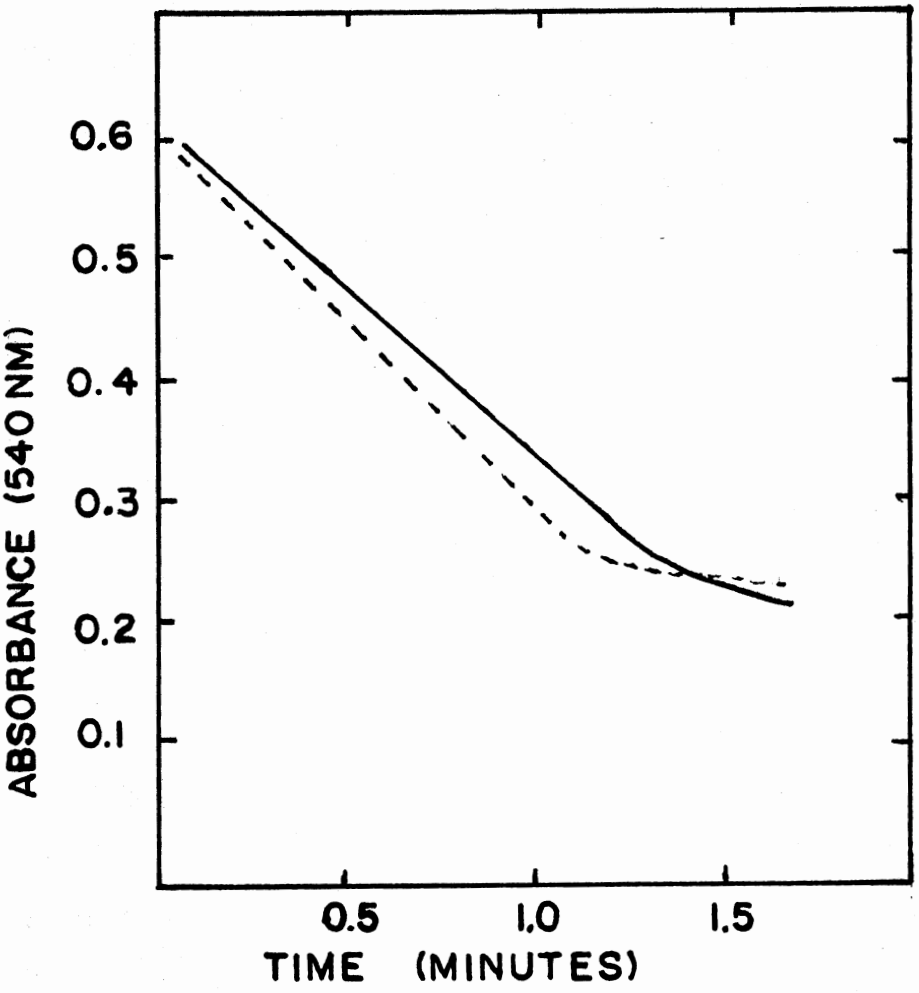

[θ] X 10⁻² DEG·CM² ·DECIMOLE⁻¹
20
16
12
8
4
0
-4
-8
-12
-16
200
220
240
260
280
300
320
WAVELENGTH
[θ] X 10⁻² DEG·CM ²·DECIMOLE⁻¹
100
80
60
40
20
0
-20
-40
-60
-80

Fig. 27. Influence of PL on the Enzymic Activity of Lysozyme.
—— Lysozyme (1 mg/ml) in tris buffer (0.0025 M) pH 7.2.
---- Lysozyme (1 mg/ml) in tris buffer (0.0025 M) pH 7.2 with PL (0.15 M).


ABSORBANCE (540 NM)
0.6
0.5
0.4
0.3
0.2
0.1
0.5
1.0
1.5
TIME (MINUTES)

where $[\theta]_{220}$ is the mean residue elipticity of the lysozyme molecule at 220 nm F_h is the fraction of helix.

$$F_h = 31\%$$

which is in good agreement with the figure reported by Chen and Yang (1971).

For lysozyme in 0.1 M NaCl, pH 7.0 buffer with 0.15 M PL

$$F_h = 25\%$$

which is consistent with the Raman observation that the helical content of the molecules is reduced.

The decrease in helical content observed in the above experiments does not indicate complete denaturation of lysozyme; indeed, measurement of the lytic activity of the molecule using M. lysodeikticus demonstrates that lysozyme is functionally unimpaired in the presence of 0.15 M PL (Fig. 27). The above observations suggest that PL may be capable of competing with peptide carbonyl groups for hydrogen bonding with peptide bonded amino group protons. Since less than 20% of the total helical structure of the molecule is affected, it can be speculated that areas such as the region of amino acid residues from 88-100 and 24-34 are inaccessible to PL. These areas are surrounded by charged amino acid residues and also are close to the crevice of the active site (Dickerson and Geis, 1969); therefore, an extensive change in these regions would be expected to result in a change in the enzymic activity of the molecule (Fig. 28).

One curious facet of the Raman spectra is the reduction of the S-S stretch in the presence of PL. It is difficult to conceive of how such an intensity reduction could occur without the breaking of a disulfide bond. Equilibrium dialysis studies (Table XVIII) with lysozyme show

Fig. 28. Helical Regions of Lysozyme. (Adapted from Dickerson and Geis, 1969)

●—● Amino acid residues 1 - 16.
▲·····▲ Amino acid residues 24 - 34.
■·····■ Amino acid residues 88 - 100.
⬡—⬡ Amino acid residues 101 - 129.

NAG-NAM substrate.

●●●● Boundary of the molecule.

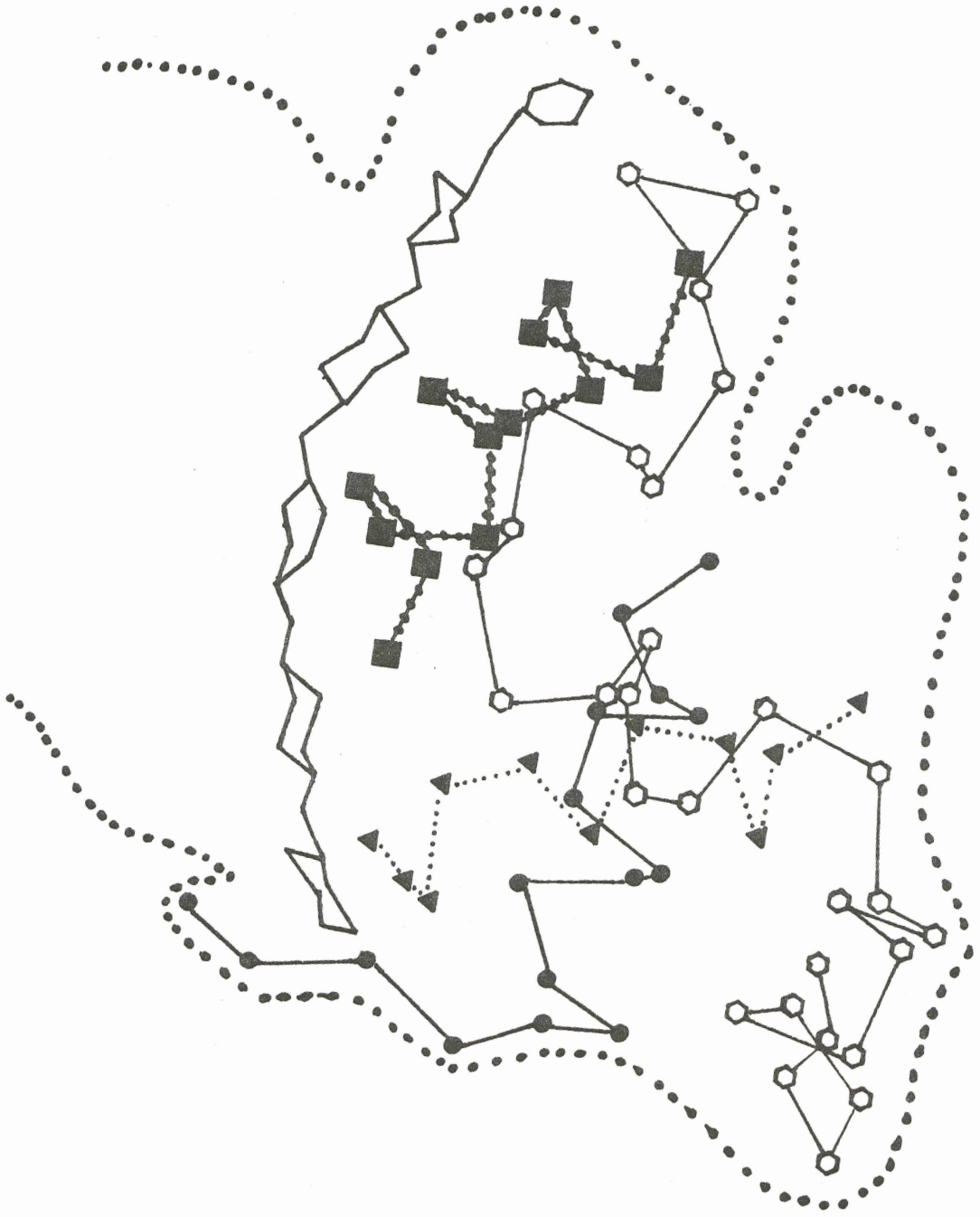


TABLE XVIII

BINDING[a] OF PL TO LYSOZYME[b]

Total PL Concentration	PL Concentration Inside the Dialysis Tubing	PL Concentration Outside the Dialysis Tubing
.015 M	0.015 M	0.015 M
0.007 M	0.007 M	0.007 M

[a] Binding was determined by equilibrium dialysis

[b] Lysozyme was suspended in 2.5 mM tris, pH 7.4 at a concentration of 4×10^{-3} M in the presence of the PL concentrations listed above. This solution was dialyzed against 2.4 mM tris, pH 7.4 for 24 hours at 4 C.

that PL does not irreversibly bind the molecule and, hence, the effects noted above are probably solvation effects. At the present time, no explanation can be offered for the reduction in the S-S stretch as it is highly unlikely that a disruption in helix due to the hydrogen bonding of PL would be enough to cause a S-S bond disruption.

Interactions of Pantoyl Lactone with Isolated Cell Membranes

Model membrane systems provided the information which made it possible to postulate that a hydrogen bonding interaction occurs between PL and polar groups on proteins and lipids. Comparisons of these results with those obtained with isolated cell membranes will now be made.

Data presented in Table XIX show the effects of the addition of PL to isolated cell membranes on the T_1 and T_2 relaxation times of C_6D_6. As can be seen, $T_1 \simeq 8T_2$ as was found with phosphatidylcholine liposomes, but the addition of PL to this suspension decreases both T_1 and T_2, however, the ratio of T_1 to T_2 is almost unchanged. These data indicate that C_6D_6 in the isolated cell membrane of M. lysodeikticus is in an anisotropic (ie. the molecule cannot tumble freely in all directions) environment and the addition of PL to the system seriously restricts the rotation of the molecule about its six-fold axis. This can only be interpreted as an ordering of the environment surrounding the C_6D_6 molecule, which is in opposition to the results observed with phosphatidylcholine liposomes. Also, these data do not reflect the difference between the lipid polar head groups of M. lysodeikticus lipids as shown earlier; therfore, it is reasonable to

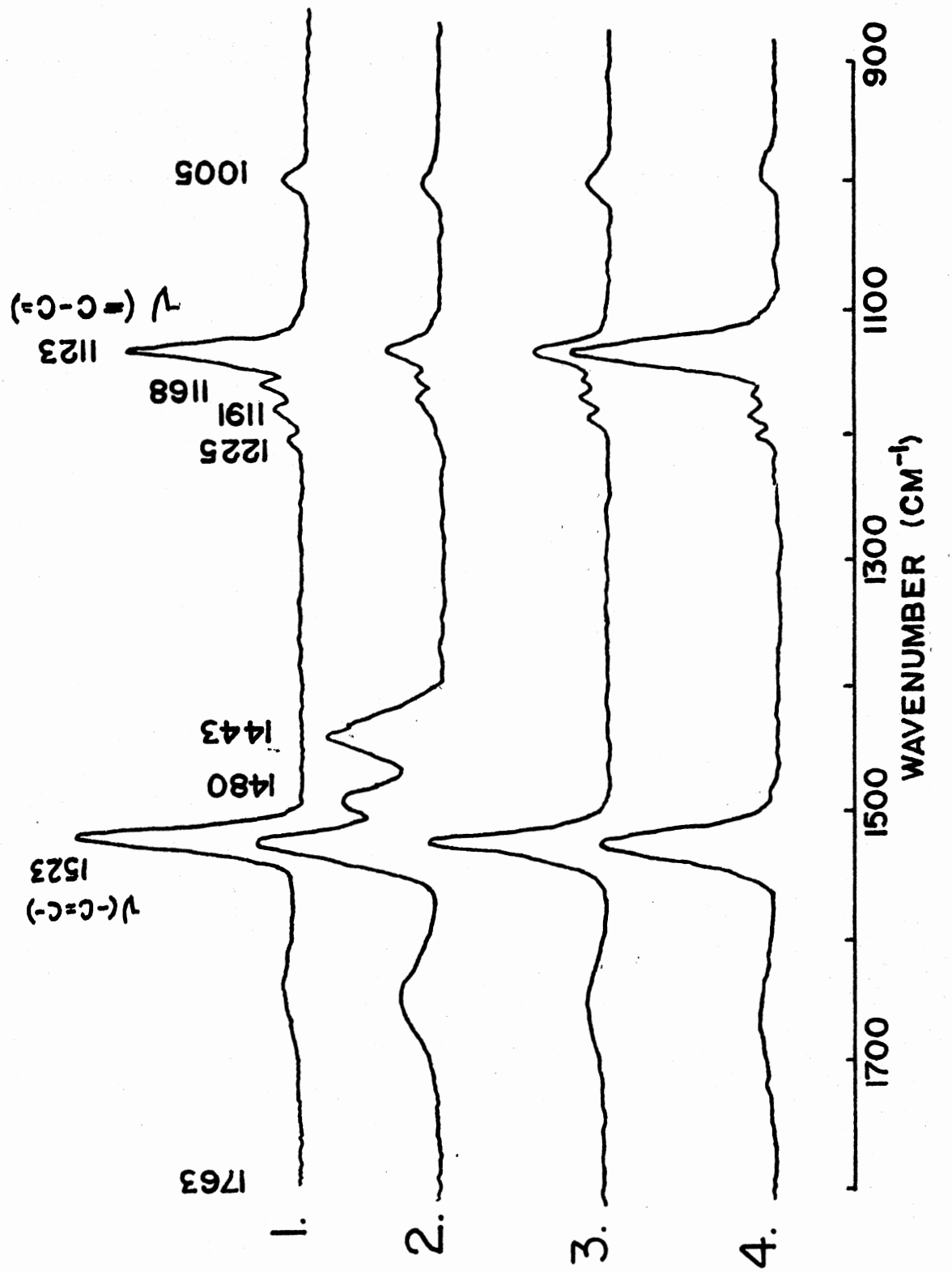
TABLE XIX

DEUTERON RELAXATION TIMES[a] OF DEUTEROBENZENE[b] IN ISOLATED CELL MEMBRANES FROM M. LYSODEIKTICUS

	PL Concentration			
	0	90 mg	210 mg	320 mg
T_1 (sec.)	0.170	0.150	0.120	0.090
T_2 (sec.)	0.020	0.013	0.011	0.008
T_2/T_1	0.084	0.081	0.091	0.090

[a] Experiments were performed at 9.21 MHz NMR and 25°C.
[b] Deuterobenzene (0.024 g) was partitioned into M. lysodeikticus membranes (1.36 g) suspended in H_2O.

Fig. 29. Influence of the Presence of PL, Growth in the Presence of PL and Growth in the Presence of DCS on the Resonance Raman Spectrum of β - Carotene.
(1) Control membranes.
(2) Control membranes with PL (0.15 M).
(3) Membranes from cells grown in the presence of PL (0.15 M).
(4) Membranes from cells grown in the presence of DCS (2×10^{-4} M).


1763
1523
ν(-C=C-)
1480
1443
1225
1191
1168
1123
ν (≡C-C≡)
1005
1.
2.
3.
4.
1700
1500
1300
1100
900
WAVENUMBER (CM⁻¹)

suggest that the effects of PL are exerted through interactions with both proteins and lipids.

The Raman spectrum of the cell membrane of M. lysodeikticus is dominated by the resonance enhancement of the β - carotene molecule within the membrane (Fig. 29). This resonance Raman effect arises from the $\pi \rightarrow \pi^*$ transitions within the chromophore and Gill, et al. (1970) have shown that changes in the local environment of the molecule can be easily monitored through changes in its resonance Raman spectrum.

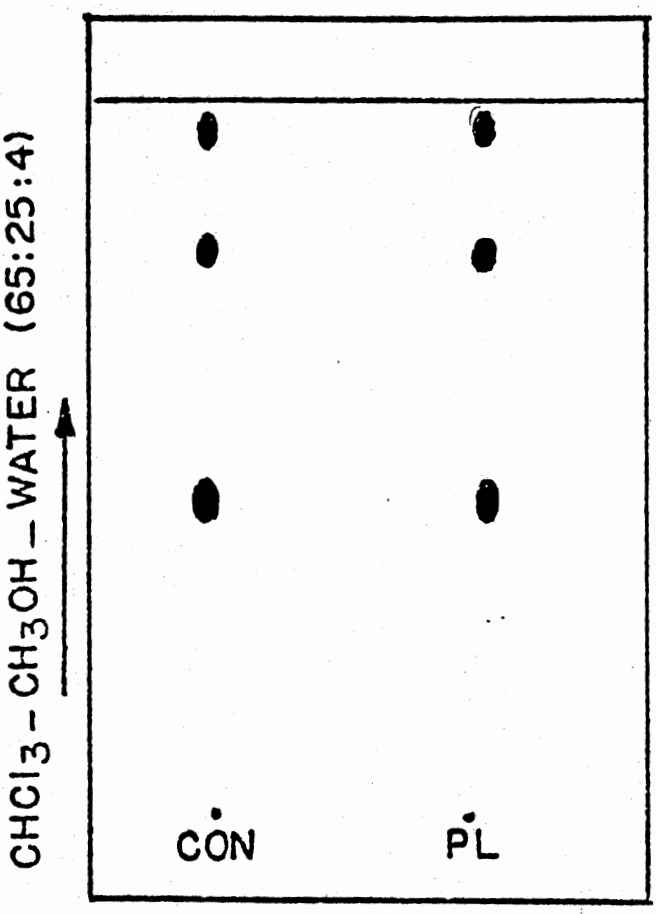
As can be seen in Fig. 29, there are two major bands due to the conjugated carbon skeleton at $\nu = 1523\ cm^{-1}$ (- C = C - stretch) and $\nu = 1123\ cm^{-1}$ (= C - C = stretch). The addition of PL to a suspension of membranes from control cells results in a change of the intensity ratio of these two bands from 1.30 to 2.50. The additional peaks seen in the spectrum of control membrane with PL added are due to the methylene deformation ($\nu = 1443\ cm^{-1}$) and methyl deformation ($\nu = 1482\ cm^{-1}$). One interesting aspect is the absence of a peak at $\nu = 1763\ cm^{-1}$. This peak is the carbonyl stretching mode of the PL molecule and exhibits an intensity which is equal to the intensity of the methylene deformation at $\nu = 1443\ cm^{-1}$. The absence of this peak indicates that the carbonyl group is able to completely interact with cell membranes which is in contrast to the result observed with liposomes. Carbonyl interactions with liposomes do occur as judged by the broadening and loss of intensity of this peak, but evidently not to the same extent as with cell membranes.

The above experiments were performed with suspensions of 10 mg of cell membrane material in 0.15 M PL, hence, small concentrations

of PL cannot account for this observation. A second result shown in Fig. 29 is that the environment of the β - carotene molecule from both cells grown in the presence of PL and cells exposed to DCS is different from control membrane. The I 1523/ I 1123 of membranes from PL treated cells is 1.95 as compared to 1.30 for control membrane. The ratio for membranes from cells treated with DCS is 0.95. These differences can be shown to be the result of environmental differences rather than structural alterations in the β - carotene molecule as shown in Fig. 30 and Fig. 31. When β - carotene is isolated and purified, both the thin-layer chromatographic separation patterns and the visible absorption spectra of the molecule are identical regardless of the membrane from which the molecule was obtained. Gill, et al. (1970) have shown that the resonance Raman spectrum of β - carotene reflects an increase in the I 1523/ I 1123 with decreasing environmental polarity and, therefore, it seems reasonable to suggest that binding of PL to cell membranes results in an environmental polarity decrease.

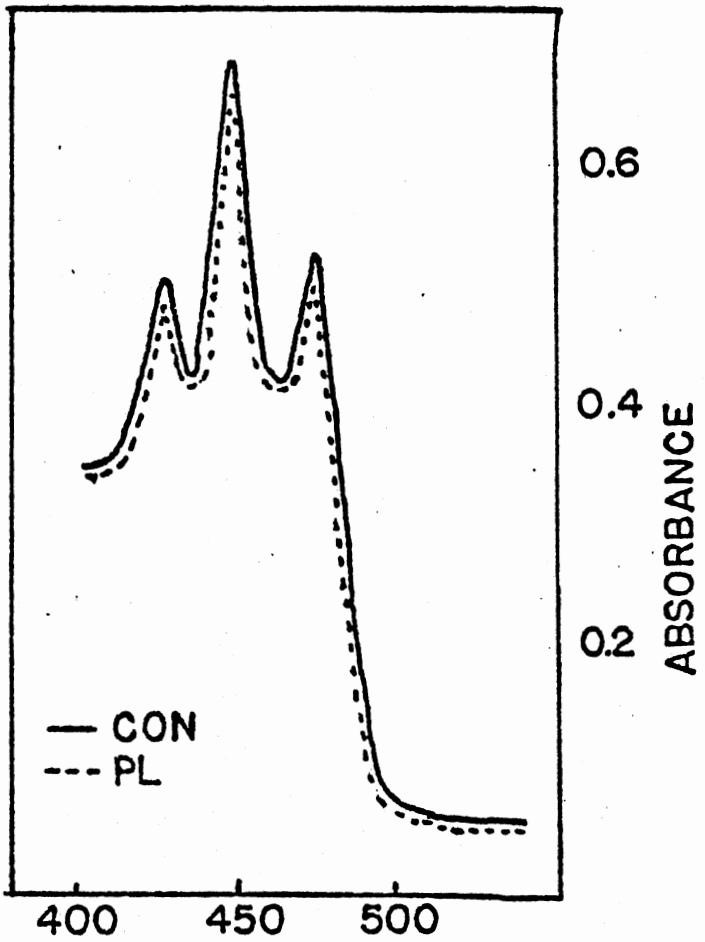
Results which relate to the above postulation are shown in Fig. 31. These results represent the EPR spectra of the 2 - (10 - carboxydecyl) - 2 - hexyl - 4, 4 - dimethyl - 3 - oxazolidinyl - oxyl, (5-NS) spin label incorporated into M. lysodeikticus membranes. The top curve is the spectrum of the 5 NS label in $CHCl_3$. Incorporation of 5-NS into control membrane results in an increase in the anisotropy of the spin label. The addition of PL to this membrane suspension results in an increase in the isotropy of the 5 NS label (the peaks marked with arrows indicate that some of the label moves with little or no restriction) and close examination of this spectrum reveals that it is the superposition of the curves of the spin label incorporated into control

Fig. 30. Thin-Layer Chromatographic Separation of β - Carotene Isolated from Control Cells and Cells Grown in the Presence of PL.



T.L.C. OF ISOLATED
β - CAROTENE

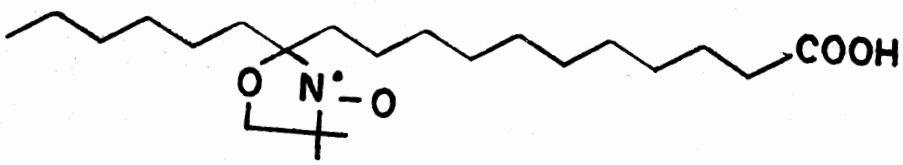
Fig. 31. Visible Absoprtion Spectra of β- Carotene Isolated from Control Cells and Cells Grown in the Presence of PL.
——— β - Carotene from control cells.
---- β - Carotene from cells grown in the presence of PL (0.15 M).

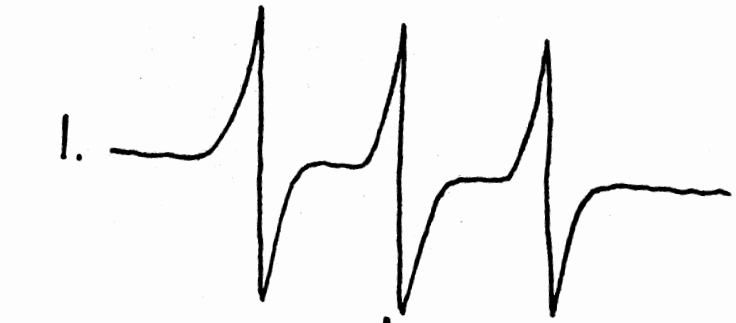


ABSORPTION SPECTRA OF ISOLATED β-CAROTENE

Fig. 32. Influence of PL on the Electron Paramagnetic Resonance Spectrum of 2 - (10 - Carboxydecyl) - 2 - Hexyl - 4, 4 - Dimethyl - 3 - Oxazolidinyl - Oxyl Fatty Acid Spin Label.

(1) The 5-NS probe in $CHCl_3$.
(2) The 5-NS probe incorporated into membranes from control cells.
(3) The 5-NS probe incorporated into membranes from control cells after the addition of PL (0.15 M).
(4) The 5-NS probe found in the supernatant after the removal of membranes by centrifugation of the sample treated with PL (curve 3).


COOH
O
N
O


1.


2.


3.


4.

membrane and the spin label in $CHCl_3$. The last spectrum in this figure represents the signal remaining in the supernatant after the removal of membranes by ultracentrifugation. These data demonstrate that the presence of PL re-extracts the 5-NS spin label from these membranes.

Similar experiments were performed on membranes isolated from M. lysodeikticus after growth in the presence of DCS and PL in an attempt to determine if the lipid alterations noted in these cultures results in a measurable change in the physical state of the hydrophobic domains of these membranes. The spectra of the 5-NS FA spin label incorporated into these membranes are shown in Fig. 33. Membranes from control cells and cells grown in the presence of DCS provide very similar environments for the 5-NS spin label despite the phospholipid alterations in DCS membranes. Membranes from cells grown in the presence of PL reflect the lipid alterations discussed in earlier sections as fluid lines (see arrows in curve 3) appear in this spectrum. Unlike the EPR spectra in Fig. 32, these lines reflect increased probe mobility within the membrane because these membranes have been sedimented after label incorporation.

A second measurement of the physical differences in membranes from dividing and non-dividing cells is shown in Fig. 34. These spectra show the temperature dependence of 8 - anilino, 1- napthalene-sulfonic acid (ANS) fluorescence in the presence of membranes from control, DCS, and PL treated cells. Again, control membranes and membranes from cells grown in the presence of DCS appear to be very similar. Both spectra show a biphasic decrease in fluorescence intensity with the steepest break occurring between 22°C and 24°C and a second

Fig. 33. Electron Paramagnetic Resonance Spectra of 2 - (10 - Carboxydecyl) - 2 - Hexyl - 4, 4 - Dimethyl - 3 - Oxazolidinyl - Oxyl Fatty Acid Spin Label Incorporated into Membranes Isolated from Control Cells, Cells Grown in the Presence of PL, and Cells Grown in the Presence of DCS.

(1) The 5-NS probe in $CHCl_3$.

(2) The 5-NS probe incorporated into membranes from control cells.

(3) The 5-NS probe incorporated into membranes from cells grown in the presence of PL (0.15 M) for 12 hours. Arrows indicate isotropic lines.

(4) The 5-NS probe incorporated into membranes from cells grown in the presence of DCS (2×10^{-4} M).

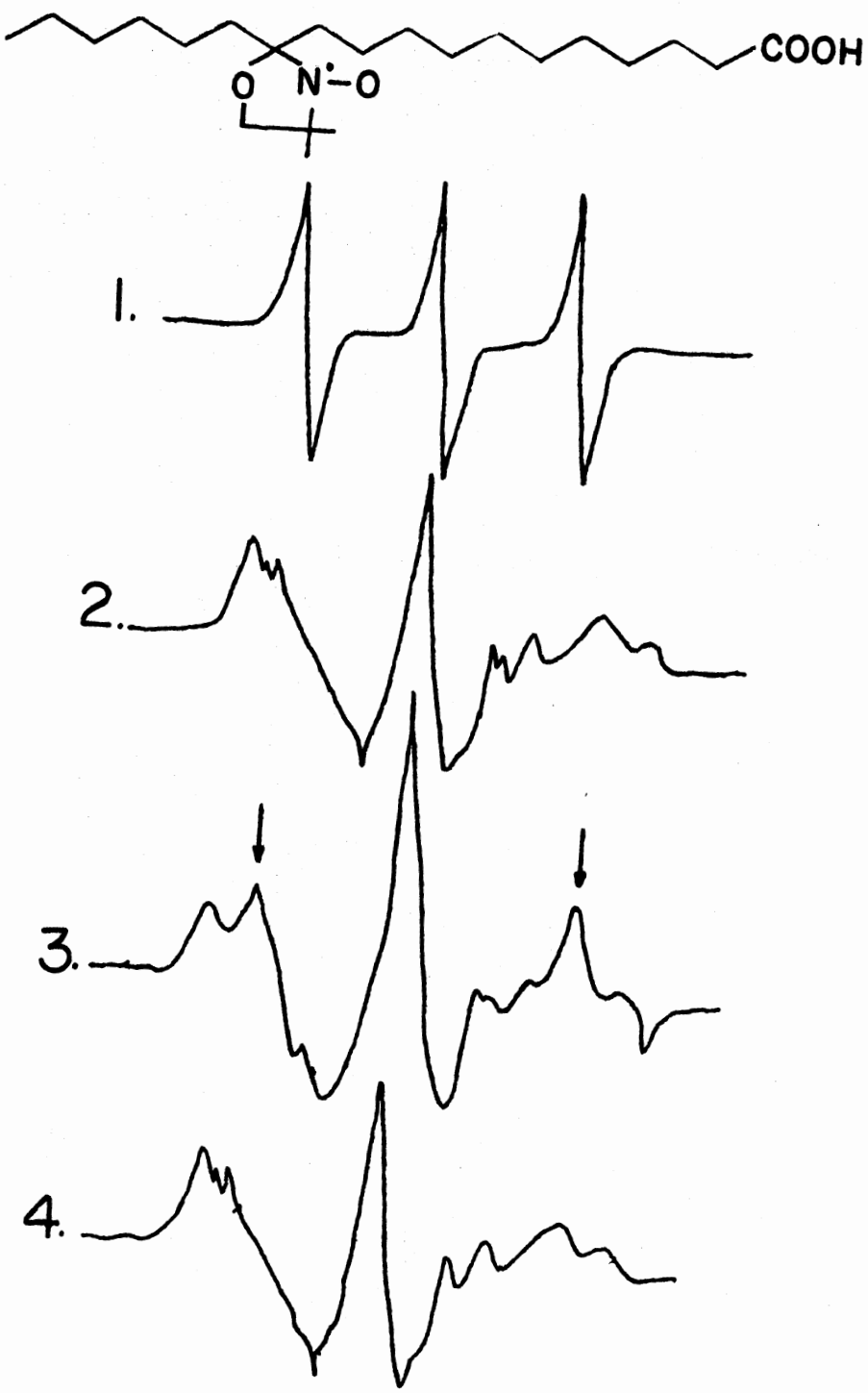
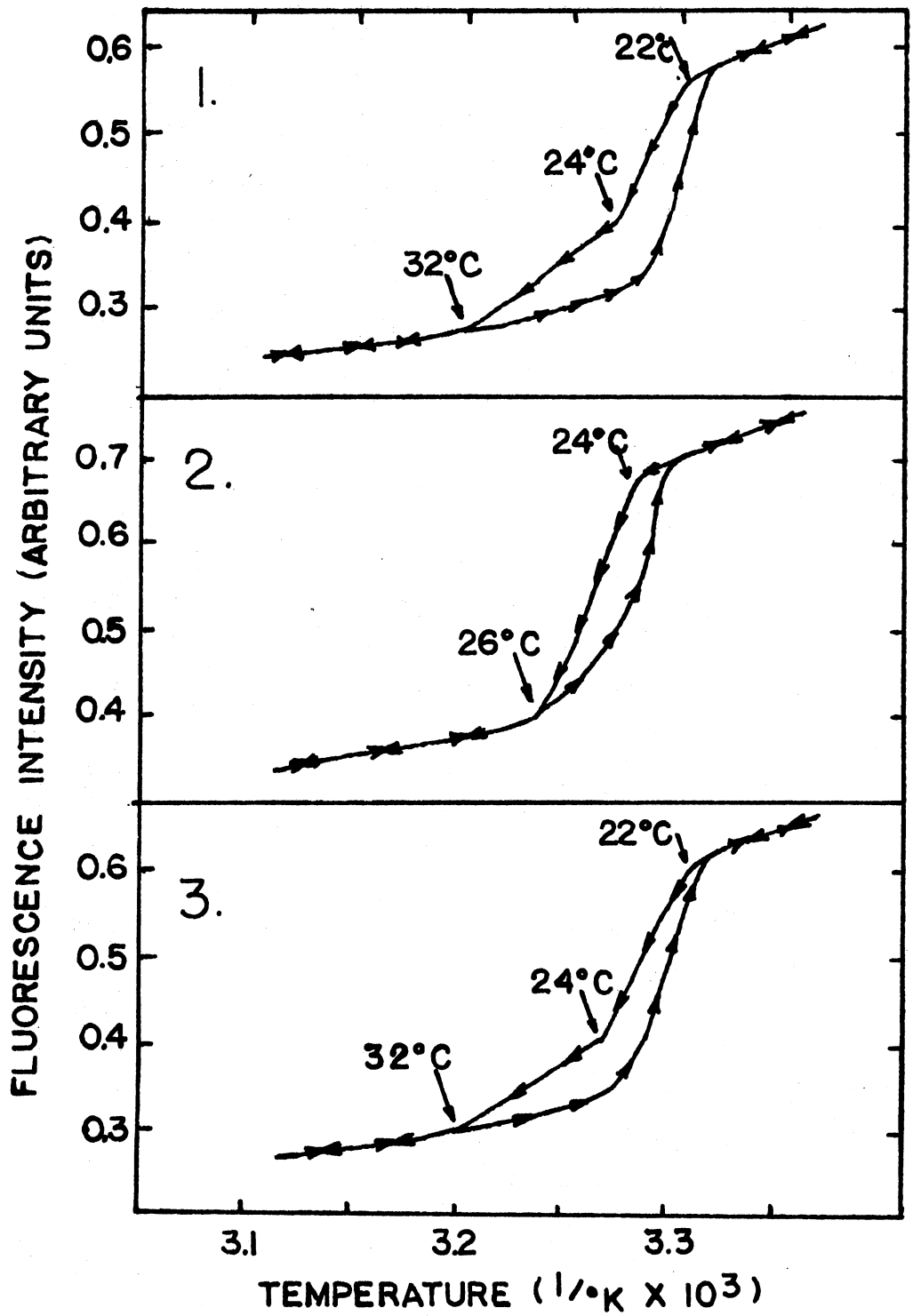

COOH
O N·–O
1.
2.
3.
4.

Fig. 34. Temperature Dependence of 8 - Anilino, 1 - Napthalene-sulfonic Acid Fluorescence Associated with Membranes from Control Cells, Cells Grown in the Presence of PL, and Cells Grown in the Presence of DCS.
(1) Membranes from control cells
(2) Membranes from cells grown in the presence of PL
(3) Membranes from cells grown in the presence of DCS (2×10^{-4} M).


1.
22°C
24°C
32°C
2.
24°C
26°C
3.
22°C
24°C
32°C
0.6
0.5
0.4
0.3
0.7
0.6
0.5
0.4
0.6
0.5
0.4
0.3
FLUORESCENCE INTENSITY (ARBITRARY UNITS)
3.1
3.2
3.3
TEMPERATURE (1/°K X 10³)

break between 24° C and 32° C. Membranes from cells grown in the presence of PL do not have a biphasic decrease in fluorescence intensity. Instead, a single decrease is noted between 24° C and 26° C.

Lively debates have centered around whether or not experiments such as those shown in Figs. 33 and 34 actually represent lipid phase transitions. The data presented here are subject to question in this regard since the only instance where differences are observed is in membranes from cells treated with PL. It has been shown (see lipid studies) that these membranes contain decreased amounts of fatty acids and significant amounts of lysophospholipid. Therefore, the increased "freedom" of mobility of the 5-NS FA spin label and ANS may simply reflect less lipid in the hydrocarbon regions of the lipid bilayer.

CHAPTER IV

DISCUSSION

The first section of this study has dealt with perturbations of the lipid components of the cell membrane of M. lysodeikticus in various states of cell division activity; primarily because, many of the previously reported effects of cell division inhibition upon membrane components could be accounted for by an alteration in the lipid fraction of the cell membrane.

This study has demonstrated that cell division inhibition, induced by the deleterious effects of DCS upon mucopeptide synthesis, is accompanied by an alteration in the phospholipid profiles of the cell membrane. Interestingly, the phospholipids affected are found on the cell surface (Barsukov, et al.,1976) which is the area of the cell membrane which would be subjected to the greatest environmental stress due to the loss of mucopeptide. It has also been shown that leakage of materials found in the cell membrane occurs in cells treated with DCS because lipid material and membrane-associated carotenoids are present in the medium after four hours treatment with DCS. Data of others, utilizing several organisms and inhibitors of mucopeptide synthesis, suggest that lipid leakage and phospholipid alterations may be a generalized phenomenon accompanying mucopeptide synthesis inhibition (Horne, et al., 1977; Veerkamp, 1976).

King (1971) has suggested that a correlation appears to exist

between the conformation of membrane proteins and cell division activity, since agents such as PL which are capable of preventing division inhibition repair the conformational alterations induced by division inhibition. Other agents show little, if any, restoration of the original protein conformation states. It is quite plausible that lipid alterations, particularly leakage, could result in conformational alterations in membrane proteins which require an obligate association with lipids. Further, proteins which have phospholipid polar head group specificities can be expected to reflect the changes in phospholipid content of the cell membrane found in cells treated with DCS. The data presented in this study indicate that the FA effect may not be as important as the prevention of lipid leakage since PL is able to prevent cell division inhibition and lipid leakage but alters the membrane content of PTG and DPTG (by lowering PTG and raising DPTG levels) in a manner similar to DCS.

The FA content of the phospholipids of non-dividing cells is only slightly altered (the C16:0 FA content of PTI is slightly elevated) while PL extensively alters the membrane FA profiles of M. lysodeikticus. Although the possibility cannot be negated, the above observations make it difficult to conceive of how such a small change in the membrane FA content of non-dividing cells could culminate in cell division inhibition. If this change is significant, it can be postulated that a specific fatty acid requirement by a molecule involved in the division process is the critical lesion, rather than a change in the physical state of the FA domains of the cell membrane, since both the EPR spectrum of the 5-NS FA spin label and the ANS fluorescence spectra are very similar in membranes from control and non-dividing

cells.

It is now evident that the inhibition of cell wall mucopeptide synthesis causes a cascading effect that results in changes to both proteins and lipids in the cell membrane (Grula and King, 1971; Grula and Hopfer, 1972). These changes can truly be viewed as secondary damage to the cell membrane because the one agent that prevents the inhibition of cell wall mucopeptide synthesis by DCS (DA) allows for correction of all membrane alterations in M. lysodeikticus.

Data presented in this study have shown that growth in the presence of PL induces a wide variety of membrane lipid alterations. Lysophospholipids account for a large portion (29%) of the total phospholipid in these membranes and the FA of the phospholipids have altered profiles. All of the physical parameters of these membranes tested (resonance Raman, EPR, fluorescence, and sucrose density gradient data) reflect these changes. It is tempting to speculate that the EPR data using the 5-NS FA spin label and the results of the ANS fluorescence studies using membranes from cells grown in the presence of PL indicate a greater fluidity of the FA hydrocarbon regions of the membrane bilayer. However, caution must be exercised in this interpretation since the phospholipids spectrum of this membrane contains significant amounts of lysophospholipids. The changes observed in these experiments may simply be due to a greater freedom of probe mobility, because of the lack of lipid, rather than an actual increase in membrane fluidity.

Several lines of evidence indicate that the PL-induced alterations of membrane lipids play a minimal role in the beneficial effects of PL on cell division. The lipid effects observed in cells grown in the

presence of PL are time dependent over a twelve hour period while some reversion of E. carotovora filaments can be observed after a one hour exposure to PL (Grula and Grula, 1962). Efforts to mimic the effects of PL on cell division by promoting lysophospholipid formation and supplementing the growth medium of cells treated with DCS with lysophospholipids and free fatty acids have been uniformly negative. Also, the effects of PL-induced lipid alterations on functional parameters of the cell membrane, such as transport, appear to be minimal since M. lysodeikticus is able to transport DA, L-glutamic acid, and L-aspartic acid at near normal rates after growth in the presence of PL and after being washed free of the compound.

The second part of this work has been an assessment of the nature of the interaction of the PL molecule using model and isolated cell membranes and the effects of the interaction. It has been shown that Pl is able to interact with phospholipid liposomes involving a weak association of the α- hydroxyl and carbonyl groups of PL with the polar head groups of phospholipids. The presence of PL has further been shown to decrease the α- helical content of the lysozyme molecule without affecting its enzymic activity. This effect is probably a solvation effect since equilibrium dialysis failed to demonstrate a tight association of PL to the enzyme. These data are consistent with a hydrogen bonding type of interaction. The laser Raman spectrum of PL reveals that the asymmetrical stretching intensity of the dimethyl group of the molecule is altered in the presence of phospholipid liposomes, which indicates that hydrophobic types of interaction between PL. and phospholipid FA probably also occur.

When results of similar experiments with isolated cell membranes

are compared with those obtained with model systems, differences in physical properties due to the interaction of PL are observed. The spin-lattice (T_1) and spin-spin (T_2) relaxation times of deuterobenzene partitioned into cell membranes are lowered indicating that PL is able to induce a restriction of motion of the deuterobenzene molecule. In liposomes, however, the T_1 relaxation time of deuterobenzene is lowered while the T_2 relaxation time remains constant in the presence of PL indicating that the motion of the deuterobenzene molecule is less restricted. Further, the Raman spectrum of PL in the presence of liposomes exhibits a decreased carbonyl stretching intensity indicative of a weak interaction of the carbonyl group with phospholipid. When the spectrum is observed with isolated cell membranes, no carbonyl stretch is detected which indicates a stronger interaction. Finally, lysozyme conformation is altered while its function is unimpaired in the presence of PL, but inhibition of the transport of a wide variety of compounds occurs within 30 seconds of exposure of whole cells to PL.

The fact that PL inhibits the transport of several compounds has led to the postulation that Pl may have a general rather than specific effect on the cell membrane. King (1971) has suggested that transport inhibition may be due to a "tightening" of the cell membrane thus changing its permeability properties. Hopfer (1972) has suggested a similar mechanism to explain the retention of membrane associated (periplasmic) proteins of filamentous cells of E. carotovora in the presence of PL. A direct demonstration of this type of effect is seen in the restriction of deuterobenzene motion when PL is added to a membrane suspension. Also, if the 5-NS FA spin label is not partitioned

into the hydrophobic domains of the cell membrane in a position parallel to the phospholipid-associated FA, the PL-induced leakage of probe back into the medium may indicate that the probe has been squeezed out of the membrane.

In summary, although it is quite possible that PL is able to influence cell division through a specific action upon a molecule(s) of the cell membrane; the evidence obtained to date indicates a physical influence is exerted on the membrane. This investigation has centered on the consequences of cell division inhibition, and secondary membrane damage as proposed by Grula and Grula (1964) has been demonstrated to accompany cell division inhibition induced by DCS. The two agents used to relieve division inhibition (DA and PL) share one common characteristic; they prevent leakage of membrane components induced by a cessation of mucopeptide synthesis. The leakage of lipid can explain the disruption of membrane protein conformation seen in the cells grown in the presence of DCS (Grula and King, 1971) as well as the leakage of periplasmic enzymes (Grula and Hopfer, 1972). As part of a cascade effect, these changes could be viewed as epiphenomena. If however, they are responsible for cell division inhibition, the action of PL may be the prevention of leakage by "tightening" the cell membrane as proposed by Grula and Grula (1964).

Such a proposal for the mechanism of action of PL implicates a physical influence of PL upon the cell membrane. Support for this implication can be found in the observation by Grula, et al. (1968) that the continual physical presence of the PL molecule is required for beneficial effects to cell division. Data presented herein reinforce this idea since the inhibitory effects of PL on transport are

readily reversed by simply washing the cells free from PL.

The relaxation times of deuterobenzene partitioned into cell membranes are suggestive of a "tightening" effect on the hydrophobic domains of the cell membrane. Two observed interactions of PL with cell membranes could account for this effect; but which is more important, a hydrogen bonding type of interaction or a hydrophobic type of interaction (or are both required)?

Grula and Grula (1962) have reported that preformed filaments of Erwinia carotovora can revert to cells of normal size under hypertonic conditions or in the presence of spermine which argues for the importance of a polar type of interaction. These investigators further demonstrated that Mg^{2+} was the most effective ion tested. Such an interaction could produce a "tightening" effect through a neutralization of phospholipid polar head groups. In addition, because some ions are more effective than others, it indicates that steric considerations are probably involved (See Fig. 24).

Although Raman and EPR data indicate that PL can physically influence hydrophobic domains of the cell membrane, as assessment of the importance of this type of interaction in cell division is difficult. It is also difficult to conceive of how a hydrophobic interaction within the hydrocarbon regions of the bilayer could result in membrane "tightening". The most compelling line of evidence for the importance of such an interaction is that ω - methyl pantoyl lactone promotes cell division in a manner similar to PL but at ten-fold lower concentrations.

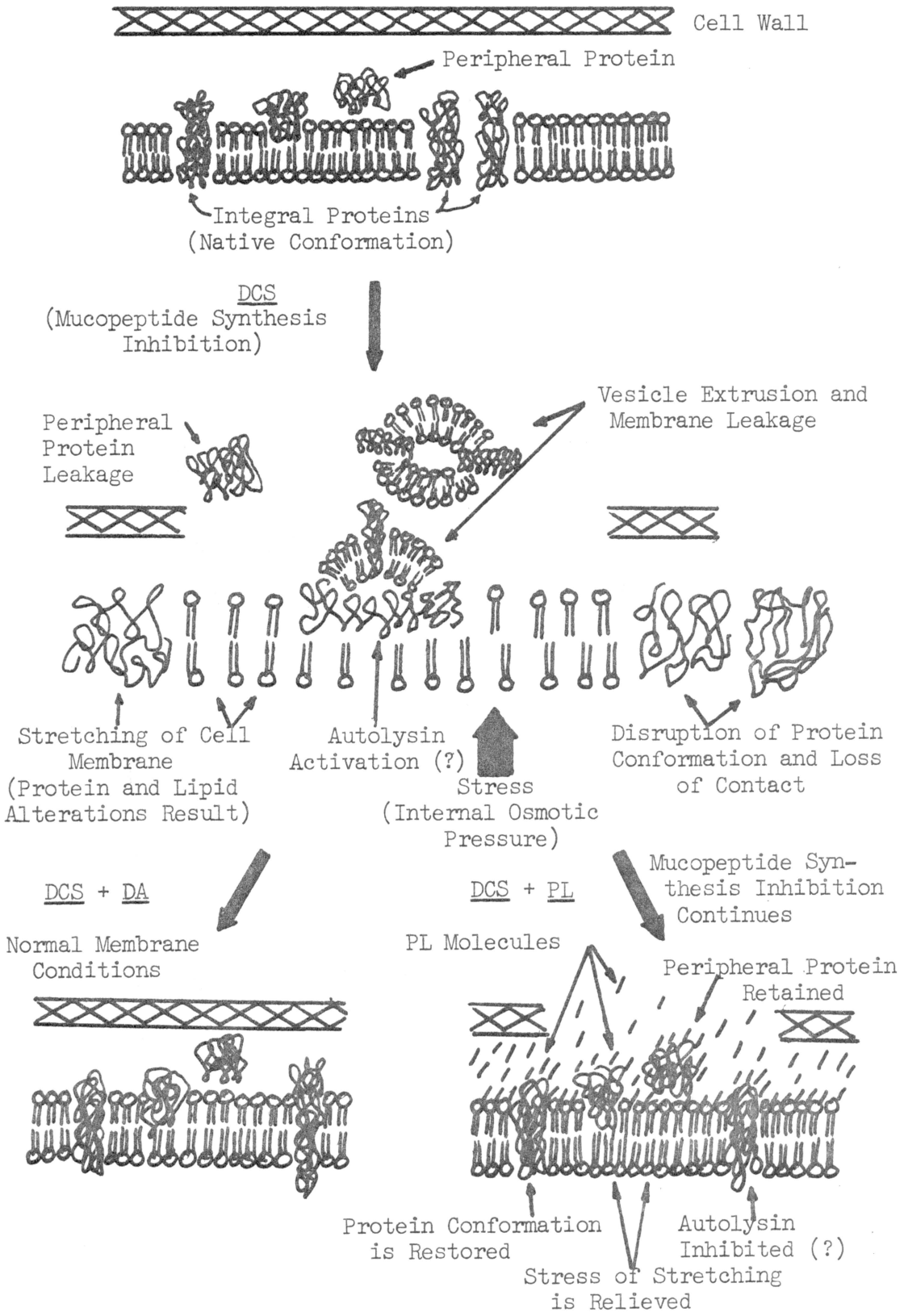
From these considerations, it is entirely possible that both types of interactions are involved in producing a "tightening" effect. Grula and Grula (1962) observed that PL affects cell division at ten-fold

lower concentrations than the concentrations of agents used to produce hypertonic conditions. Therefore, it appears that hydrogen bonding is the more important interaction for membrane "tightening", however, hydrogen or ionic bonding is enhanced by hydrophobic interactions (Fig. 24, number 5).

A model for cell membrane damage arising from a cascade effect induced by inhibitors of mucopeptide synthesis and the prevention (or repair) of this damage through a membrane "tightening" type of mechanism is shown in Fig. 35. In this model, cell wall synthesis inhibition, induced by compounds such as DCS, results in the loss of insulation and protection at points along the cell membrane where cell wall is no longer present. At these points, several types of alterations are envisioned to occur.

The first type of damage would be due to physical stress applied to the cell membrane from factors such as internal osmotic pressure. This stress could result in membrane stretching and the extrusion of membrane components such as the vesicular structures observed in the spent medium of division inhibited cells (see Fig. 8; Grula and Grula, 1964; and Horne et al., 1977), the leakage of lipid (see Fig. 7; Horne et al., 1977; and Veerkamp, 1976), conformational changes in membrane proteins (Grula and King, 1971), and the loss of peripheral proteins (Grula and Hopfer, 1972). Conformational changes in membrane proteins and the loss of peripheral proteins required for cell division would then account for the functional impairment of the cell division apparatus via loss of enzymic activity or loss of physical contact. Further, if cooperativity of lipids in a phase transition is required for membrane invagination; membrane stretching would result in force being

Fig. 35. A Model for Cell Membrane Damage Arising from a Cascade Effect Induced by Inhibitors of Mucopeptide Synthesis and the Prevention (or Repair) of this Damage Through a Membrane "Tightening" Type of Mechanism.


Cell Wall
Peripheral Protein
Integral Proteins
(Native Conformation)
DCS
(Mucopeptide Synthesis
Inhibition)
Peripheral
Protein
Leakage
Vesicle Extrusion and
Membrane Leakage
Stretching of Cell
Membrane
(Protein and Lipid
Alterations Result)
Autolysin
Activation (?)
Stress
(Internal Osmotic
Pressure)
Disruption of Protein
Conformation and Loss
of Contact
DCS + DA
Normal Membrane
Conditions
DCS + PL
Mucopeptide Syn-
thesis Inhibition
Continues
PL Molecules
Peripheral Protein
Retained
Protein Conformation
is Restored
Stress of Stretching
is Relieved
Autolysin
Inhibited (?)

applied to these lipids in a direction opposite to the direction of invagination.

Another type of lesion which could account for these observations is the activation of an autolysin at points along the membrane where cell wall is no longer present. Autolytic activity could account for the extrusion of membrane components. This type of mechanism is particularly attractive in view of the phospholipid alterations noted in the membranes of cells grown in the presence of DCS, where a decrease in phosphatidylglycerol (localized on the outer half of the bilayer) is observed. It is difficult to conceive of how a separation of the membrane bilayer could occur without some type of mediation. The model presented in Fig. 35 does not attempt to distinguish between alterations induced by physical stress and alterations induced by autolytic activity because one or both of these mechanisms could be operable. Indeed, autolytic activity could be dependent upon physical stress.

Agents which relieve cell division inhibition, such as PL, are envisioned to overcome membrane stretching by "tightening" the cell membrane through polar interactions. This effect would then prevent the leakage of membrane components (see Fig. 7 and Grula and Grula, 1964), allow membrane proteins to return to their native conformational states (Grula and King, 1971), and prevent the loss of peripheral proteins (Grula and Hopfer, 1972). Retention of peripheral proteins and the restoration of the native conformation of integral proteins required for cell division would then result in the repair of enzymic function or the return of required physical contact. Also, physical stress upon lipids participating in an obligate phase transition could

be explained by this mechanism since a "tightening" of the membrane would relieve the force opposing invagination.

Agents which "tighten" the cell membrane would also inhibit autolysin activity if the activation of such a molecule is dependent upon physical stress (see Fig. 35). However, autolysin inhibition could also be explained by a direct interaction of the autolysin with the agent. Experiments discussed on page 78 revealed that when M. lysodeikticus membranes were incubated for 10 hours with and without PL present, only control membranes showed lysophospholipid formation. This indicates that PL is capable of inhibiting autolytic activity. Thus, although a direct interaction of PL with the autolytic enzyme cannot be negated, the proposed "tightening" (ordering) of the cell membrane as its mechanism of action is more attractive because of its consistency with the wide variety of effects attributable to the presence of PL.

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