EFFECTS OF PANTOYL LACTONE ON TRANSPORT AND FATTY ACID COMPOSITION IN *MICROCOCCUS LYSODEIKTICUS*

By

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AND FATTY ACID COMPOSITION IN

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Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Grula (1960b) observed that both growth and cell division in a species of Erwinia were profoundly inhibited by the D-forms of serine, methionine, phenylalanine, threonine, tryptophan or histidine. Durham and Milligan (1961) reported that D-serine was also able to inhibit growth and cell division in a Flavobacterium species. In addition to the six D-amino acids, Grula and Grula (1962a; 1964) reported that penicillin, mitomycin c, vancomycin, D-cycloserine, aminopterin, hydroxylamine, S-(dichlorovinyl)-L-cysteine, and ultraviolet light will inhibit both growth and cell division in Erwinia species.

Division inhibition caused by D-serine can be prevented by adding D- or L-alanine, para-aminobenzoic acid, calcium pantothenate, or inorganic ammonium salts (Grula, 1960a; Grula and Grula, 1962a). In addition, it was reported that addition of pantoyl lactone (PL) prevents the effects of all inhibitory agents tested on cell division in Erwinia species. It was also reported that addition of PL promotes division in preformed filamentous cells of Erwinia sp. either in the presence or absence of a division inhibitor (reversal action of PL).

Because it appeared improbable that synthesis of PL could be inhibited by all the chemically diverse agents that cause division inhibition, Grula and Grula (1962a) proposed that the cell division "system" undergoes varying degrees of physical damage in the presence

of all agents that inhibit cell division, and is extremely susceptible because it is located near the cell periphery.

Because hypertonic conditions also prevent division inhibition in Erwinia sp. it has been suggested that the cell membrane is intimately involved in the cell division process (Grula and Grula, 1964). Further evidence to substantiate this belief was obtained by observing that osmotic protective conditions significantly lower leakage of proteins and nucleic acids from cells of Erwinia sp. in the presence of division inhibiting agents. It has recently been concluded (Grula and Hopfer, 1972) that the release area of proteins is the cell periphery (cell wall plus periplasmic space). Observations of this type suggest that hypertonic conditions and PL probably stimulate division activity by preventing "secondary" cell membrane damage (Grula and Grula, 1964).

Grula and King (1970; 1972) reported that D-serine, D-cycloserine, mitomycin c, penicillin, hydroxylamine or suboptimal concentrations of magnesium inhibit division activity in a nutritional mutant of M. lysodeikticus (dis II or dis II p^+). These investigators also reported that PL is able to prevent but not reverse division inhibition caused by D-cycloserine or D-serine in these mutant organisms. Data were also presented which show that PL largely but not completely prevents the quantitative and conformational changes induced in cell membrane proteins during growth in the presence of D-cycloserine (Grula and King, 1971).

These investigators also observed that cells grown in the presence of D-cycloserine (non-dividing) possess an impaired uptake mechanism; this inhibition can be slightly overcome by PL. Because the restoration in uptake ability is not pronounced, they further investigated the

effects of PL on uptake of a variety of compounds. It was shown that presence of PL causes an immediate inhibition in uptake of D-alanine, L-phenylalanine, glycerol, or glycine in normal cells of M. lysodeikticus. In a concurrent study, Hopfer (1972) also demonstrated that PL inhibits uptake of a variety of compounds in normal cells of Erwinia species. Hopfer demonstrated additional effects of PL which appear to be membrane oriented. As examples: a) PL potentiates lysis of Erwinia species in the presence of sodium lauryl sulfate. b) At low concentrations, PL causes horse erythrocytes to undergo crenation; at higher concentrations, PL causes lysis. c) A significant reduction in agglutination of horse erythrocytes by specific antibodies occurs in the presence of PL. This effect is readily reversed by washing PL out of the system.

After having demonstrated that at least one major site of action of PL is the cell membrane (Hopfer, 1972), experiments were designed to aid in determining which type of molecules (lipids or proteins) associate or react with PL. No effect was observed on activity of lactate dehydrogenase (soluble form from Erwinia sp.), NADH dehydrogenase (whole membrane preparation from M. lysodeikticus), lysozyme (water soluble pure commercial product), aspartic acid "binding" protein (obtained from Erwinia species), or catalase (whole membrane preparation from M. lysodeikticus).

Although evidence could not be obtained which would indicate that PL associates or reacts with proteins in the cell membrane of bacteria, evidence for association with lipids was presented. For example, it was shown that PL is soluble to varying degrees in relatively nonpolar solvents such as hexane or chloroform. In equilibrium dialysis

type experiments, association of PL occurs only with membranes which have not had their lipids removed. Based on UV absorption spectra and data obtained from partitioning experiments, it was suggested that PL most probably alters structural and functional characteristics of membranes because of some type of weak association with phospholipids containing unsaturated fatty acid (uFA) side chains. Such a molecular association should decrease membrane fluidity, which could, in turn, allow activation of the cell division process (Hopfer, 1972).

Many reports have recently appeared in the literature which address themselves to a possible relationship of uFA to membrane structure and function. Marr and Ingraham (1962) reported that cells of Escherichia coli contain large amounts (40-50%) uFA. Kito et al. (1972) noted that uFA decrease in amount in cultures of Escherichia coli B as the cells enter the stationary growth phase, or as the temperature of growth is increased. Other investigators (Cullen, Phillips, and Shipley, 1971; Shen et al., 1970; Shaw and Ingraham, 1965) observed that the degree of unsaturation increases with decreasing growth temperature. Fox and Tsukagoshi (1972) demonstrated that fluidity of cellular membranes is related to degree of unsaturation of membrane lipids. Cullen, Phillips, and Shipley (1971) suggested that the degree of unsaturation within the fatty acids of cell membranes acts as a control mechanism which allows cells to maintain a "constant" membrane structure. Although confirmatory data have not yet appeared, Hunter et al. (1959) suggested that phospholipid-amino acid complexes may be involved in the transfer of amino acids from the site of amino acid activation to the final site of protein synthesis.

Many investigators find that the degree of unsaturation plays an important role in uptake of various compounds. Haining, Fukui, and Axelrod, (1960) working with mitochondria of rat tissues have shown that the extent of amino acid uptake by the lipid fraction of mitochondria varies with the type of compounds studied, but that they are taken up without degradation or alteration. Hendler (1959) concluded from his studies with hen oviduct that lipids appear to be an intermediate carrier of amino acids entering the cell. He characterized the lipid amino acid bond as being highly labile in contrast to the stable bond linking an amino acid component in a nucleic acid fraction. Hokin and Hokin (1959) have proposed, as a result of their studies, a mechanism of transport of enzymes across lipid membranes of the cell, with the phospholipids acting as some type of intermediate in the transfer.

Gale and Llewellin (1971) reported that uptake of aspartic acid in osmotically shocked cells of Staphylococcus aureus is stimulated by the addition of uFA to the uptake medium. Fox (1969) concluded that uFA are necessary for incorporation of the lactose transport system into the membrane of an auxotrophic strain of E. coli. Esfahani et al. (1971) have shown that proline uptake activity is also stimulated by uFA. The possibility that a combination between amino acids and lipids fulfills the postulated role of an intermediate in active transport has been proposed (Ames, 1968).

Romijn, et al., (1972) suggested that Acholeplasma laidlawii B is equipped with a mechanism to control the fluidity of its membrane lipids and, hence, permeability of the membrane by controlling the total degree of unsaturation of the membrane FA. Beebe (1972)

demonstrated that a phosphatidylethanolamine deficient mutant of Bacillus subtilis exhibits decreased uptake of several compounds when compared to the parent strain: (L-aspartic, L-tryptophan, L-serine, L-threonine, L-proline, L-methionine, glycine, pyruvate, uracil, thymine, uridine, and adenosine).

After considering the data which relate to membrane alterations by PL, it was felt that I should examine possible functional changes in the membrane by measuring uptake ability of cells. Therefore, the central theme of the experiments reported in this thesis has been to define the parameters involved in uptake activity in M. lysodeikticus and, further, to attempt definition of those components possibly altered or influenced by PL.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The organism used in this study is a mutant of the Purdue University strain of Micrococcus lysodeikticus. This mutant, which has been designated dis-IIp$^+$ (division inhibited by D-serine; purine not required for growth), was isolated by nutritional selection using aspartic acid as the major source of carbon, nitrogen, and energy in a chemically defined medium (Grula, 1962; Grula and King, 1970). Biochemical characteristics of this organism are identical to those described for the parent strain by Grula, (1962).

Stock cultures were maintained on slants of defined (basal) medium solidified by addition of 2.0 percent agar agar No. 3 (Oxoid-Consolidated Laboratories, Incorporated, Chicago Heights, Illinois). Stock cultures were transferred approximately every two months. Twenty-four hour old cells grown at 37 C were used as the source of inoculum for all experiments.

Medium and Growth

The basal medium utilized contained the following compounds per 100 ml: biotin (50 ug), L-glutamic acid (358 mg), L-aspartic acid (358 mg), L-phenylalanine (40 mg), L-tyrosine (30 mg), Na_2HPO_4 (200 mg), and $MgSO_4 \cdot 7H_2O$ (2 mg). The pH of the complete medium was

always adjusted to pH 7.6 to 7.8 using solid KOH prior to autoclaving (15 minutes at 250 F). A saturated solution of $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ was autoclaved separately and the resulting red precipitate removed by filtration before an aliquot (0.5 ml per 100 ml culture medium) was added aseptically to the medium.

Media were inoculated using cells grown on slants of defined media. The cells were washed twice in 0.85 percent sterile saline and adjusted to an optical density of approximately 0.5 (Coleman Junior Spectrophotometer). Five drops of this suspension were used to inoculate 100 ml of medium contained in 250 ml Erlenmeyer flasks. The cultures were incubated at 37 C on a Gyrotory shaker (New Brunswick Scientific Company, New Jersey, Model G-26) set at a shaking speed of 8.5. Cells grown under stationary conditions were grown in either 15 ml of basal medium contained in 250 ml Erlenmeyer flasks (with side arms) or 150 ml of basal medium contained in 1000 ml Erlenmeyer flasks and incubated at appropriate temperatures.

Uptake Studies

After 16 hours growth, cells were harvested, washed two times in "uptake buffer" (contains all salts present in the growth medium), and resuspended in the same buffer to an optical density of approximately 0.5 (measured at 540 nm). The suspensions were then incubated on a Burrell Wrist-Action shaker at a setting of 1.0 at room temperature, unless otherwise stated, for 20 minutes before addition of labeled compounds. Labeled compounds (0.15 ml of a stock solution containing 5.0 uC labeled compound and 100 ug "cold carrier" per 10 ml, unless otherwise stated) were added to 3 ml of cell suspension.

Compounds being investigated for inhibition of uptake activity were added 30 seconds prior to addition of the labeled compound. At appropriate time intervals, 0.5 ml of sample was withdrawn and passed through a 0.45 um size Millipore filter using vacuum suction. The filters containing the cells were then washed immediately (3 times) using 1 ml volumes of uptake buffer (25 C). The filters containing the washed bacterial cells were then placed in scintillation vials and allowed to dry (room temperature, overnight) before addition of 10 ml Aquasol. Samples were shaken at 37 C for 4 hours before counting to permit dissolution of the membrane filters.

The following labeled compounds were used: D-alanine-U-^{14}C (32 mC/mM), acetic acid-2-^{14}C (6 mC/mM), L-aspartic-U-^{14}C (154 mC/mM), 2-deoxy-D-glucose-1-^{14}C (60 mC/mM), L-glutamic-U-^{14}C (206 mC/mM), uracil-2-^{14}C (6.22 mC/mM), L-phenylalanine-U-^{14}C (135 mC/mM), glycerol-2-^{14}C (7.0 mC/mM), pyruvate-2-^{14}C (6.0 mC/mM), L-malic acid-U-^{14}C (26 mC/mM).

Isolation of Cell Membranes

Whole cell membranes of M. lysodeikticus dis-IIp^{+} were prepared according to the procedure described by Butler, Smith, and Grula (1967).

Surface Tension Measurements

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

Polyacrylamide Gel Electrophoresis

Sodium lauryl sulfate (SLS) gel electrophoresis was run according

to Weber and Osborn (1969) as modified by Grula and Savoy (1971).

Efflux Experiments

Sixteen hour old cells were labeled for 30 minutes in basal medium using either 2-deoxy-D-glucose-1-^{14}C or D-alanine-U-^{14}C. The cells were then passed through 0.45 um size Millipore filters using vacuum suction and resuspended in 40 ml of uptake buffer, uptake buffer plus pantoyl lactone (0.22M), or uptake buffer plus toluene (0.02 ml/ml of uptake buffer). Immediately following resuspension, 2.0 ml samples were removed and passed through 0.45 um size Millipore filters. Additional samples were taken at appropriate times after which the filters containing the bacteria were dissolved and radioactivity measured as described under Uptake Studies.

Shock Procedure

The procedure reported by Gale and Llewellin (1970) was utilized for release of binding fractions.

Division Inhibition

D-Cycloserine was dissolved in 0.1M K_2HPO_4, pH 7.2, and filter-sterilized. The antibiotic was added to growing cells after twelve hours incubation (approximately 0.15 O.D. at 540 nm) to a final concentration of 2×10^{-4}M. Compounds tested for prevention of division inhibition were also added at this time. Each compound was adjusted to pH 7.0 prior to sterilization by filtration (0.45 um size Millipore filters).

Lipid Extraction

After growth in a particular medium, 100 ml of cells (approximately 0.5 O.D. at 540 nm) were harvested, washed one time in saline and resuspended in 5 ml methanol in a screw cap tube. The samples were then sealed with teflon tape and capped under nitrogen. The sealed tubes were heated for 30 minutes in a 55 C water bath. After cooling to room temperature, 10 ml of chloroform was added and the extraction at 25 C continued for twenty-four hours under nitrogen. The following day, insoluble material was removed with suction using 0.45 um size Millipore filters. The liquid samples were then washed twice using 15 ml of 2M KCL followed by one wash using 10 ml of distilled water. The washed chloroform solution was then passed through fresh sodium sulfate columns (1/2 cm by 14 cm) and dried under nitrogen at 50 C (the last 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 (Na_2SO_4) methanol (v/v) was used to prepare methyl esters of the phospholipid fatty acids. Phospholipids (from a cell volume of 100 ml, 0.5 O.D. measured at 540 nm) were dissolved in 4.5 ml of the methanolic H_2SO_4 and sealed with teflon tape under nitrogen. After twenty-four hours at room temperature, 4.5 ml water was added and each sample was then extracted three times with 4 ml of hexane. The hexane extracts were pooled and washed one time with 4 ml distilled water, followed by passage through fresh columns of sodium sulfate (1/2 cm by 14 cm). The washed hexane

solutions were then dried under nitrogen at 50 C (except for approximately the last 1/2 ml, which was dried at room temperature to prevent possible oxidation). The resulting methyl esters were dissolved in 0.1 ml iso-octane immediately prior to injection into the gas-liquid chromatography unit.

Gas-Liquid Chromatography

Volume of sample injected --- 1 to 4 ul

Column conditions were as follows:

Column --- 6' X 1/4" glass

Column Packing --- Diethylene Glycol Succinate (5%)

Column Temperature --- 165 C

Injection Port Temperature --- 250 C

Detector Temperature --- 250 C

Carrier Gas --- Nitrogen

Flow Rate --- 40 ml/min.

Detector --- Hydrogen Flame

Hydrogen Pressure --- 15 PSI

Air Pressure --- 40 PSI

Instrument --- Perkin Elmer 990 Gas Chromatograph

Identification of Methyl Esters of Fatty Acids

The methyl esters of saturated and unsaturated fatty acids were tentatively identified by comparing retention times (relative to C 16:0) using known methyl ester standards. Branched-chain acids were tentatively identified by comparison with reported relative (C 16:0) retention times (Thorne and Kodicek, 1962). Further identifications

were obtained by analysis using the coupled mass spectrometer-gas chromatography unit in The Department of Biochemistry.

CHAPTER III

RESULTS

Uptake Studies

Characterization of the D-Alanine Uptake System in M. lysodeikticus

Grula and King (1971) have shown that pantoyl lactone (PL) inhibits the uptake of D-alanine, L-phenylalanine, and glycerol in M. lysodeikticus dis-IIp$^+$. The uptake ability of Erwinia sp. for D-glucose, L-aspartic acid, L-malic acid, D-alanine, thymidine, and 2-ketoglutaric acid was also shown to be inhibited in the presence of PL (Hopfer, 1972).

D-Alanine was selected as the compound of choice for uptake studies because sugar transport into cells of M. lysodeikticus is minimal. Also, it had previously been established (Grula and King, 1971) that D-alanine uptake activity is very good in this organism and inhibition by PL occurs immediately (within 30 seconds).

Within a four minute time period, uptake of D-alanine in the presence of 0.22M PL is inhibited approximately 50 percent (Figure 1). These data also reveal that the effect of PL on uptake of D-alanine is readily removed by washing cells free of PL. Data presented in Fig. 2 illustrate that the lactone structure is necessary to inhibit D-alanine uptake activity since pantoic acid has little if any effect

Figure 1. Uptake of D-Alanine-U-^{14}C in the Presence and Absence of PL. ○, control cells; ■, with PL (0.22M); ●, cells pre-treated (5 minutes) with PL (0.22M) and washed two times in uptake buffer prior to determination of uptake ability.

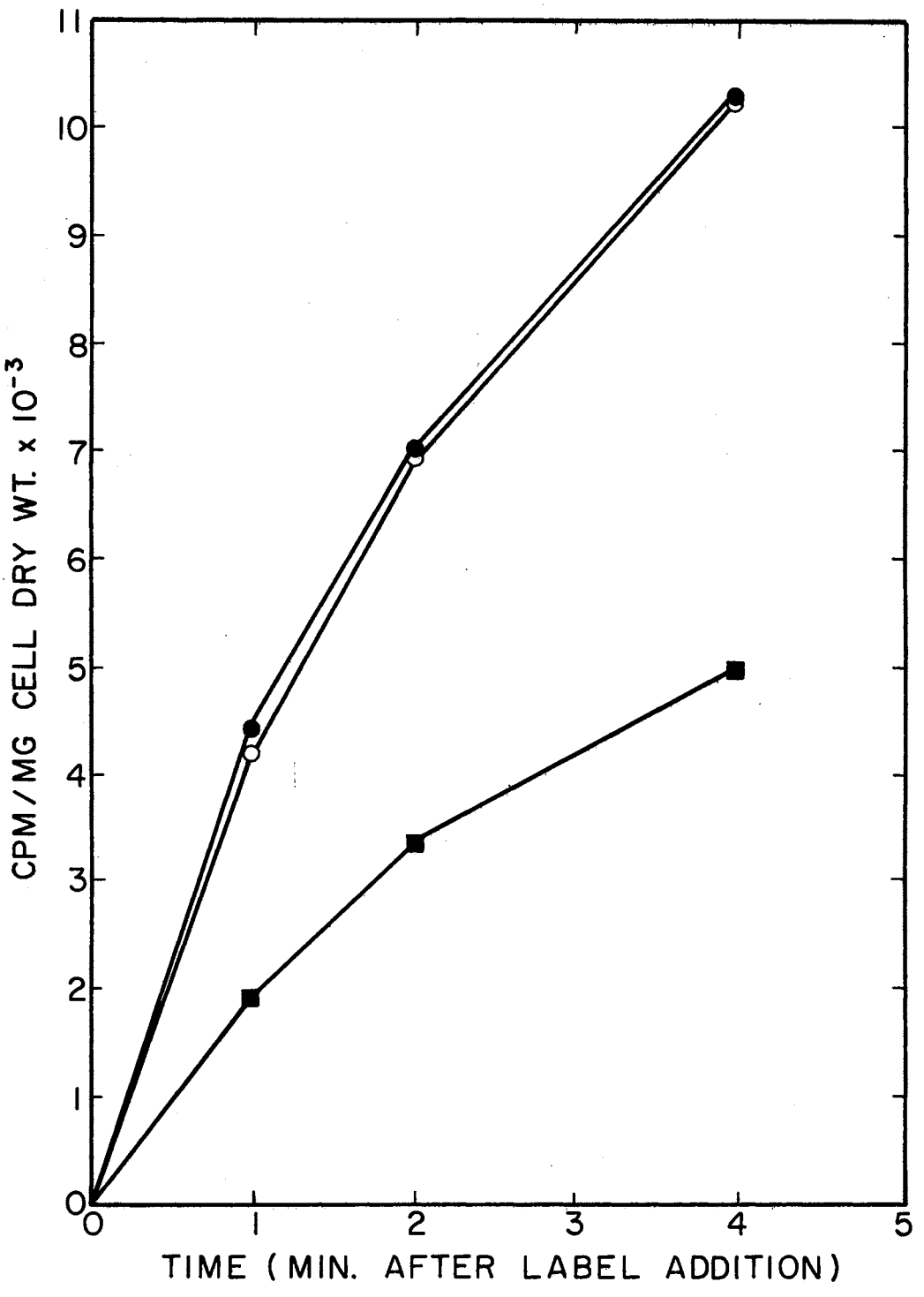
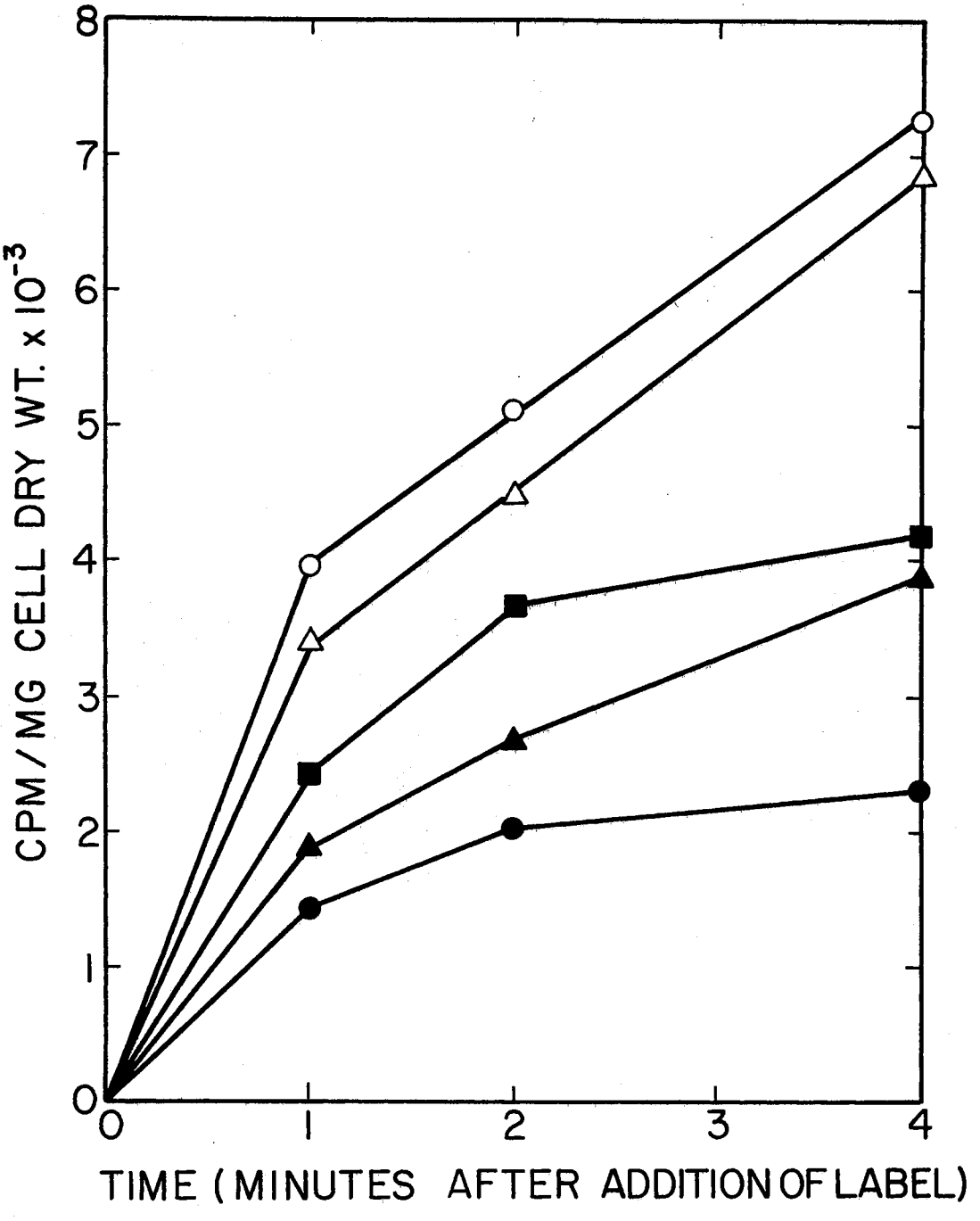

CPM/MG CELL DRY WT. x 10^{-3}
TIME (MIN. AFTER LABEL ADDITION)
0
1
2
3
4
5
6
7
8
9
10
11

Figure 2. Influence of Sodium Pantoic Acid and PL on Uptake of D-Alanine-U-^{14}C. ○, control cells; △, with sodium pantoic acid (0.1M); ■, with PL (0.1M); ●, with PL (0.2M); ▲, with sodium pantoic acid (0.1M) plus PL (0.1M).


CPM / MG CELL DRY WT. x 10^{-3}
0
1
2
3
4
5
6
7
8
0
1
2
3
4
TIME (MINUTES AFTER ADDITION OF LABEL)

on the system.

When cells are grown in the presence of PL and washed free of the compound (Fig. 3) less inhibition of uptake is evident when PL is added back to these cells (37% average decrease) than in control cells not grown in the presence of PL (average decrease of 57%).

Many investigators have studied transport systems for the different amino acids (Wilson and Holden, 1969; Rosen, 1971; Weiner, Furlong, and Heppel, 1971; Gale and Llewellin, 1971). Binding proteins which have varying degrees of affinity for substrate molecules are believed to be involved in both active and facilitated transport. Usually, binding proteins can be released from cells by the cold-osmotic shock procedure (Heppel, 1967) without complete loss of "binding" activity. In addition to the requirement for binding proteins, active transport requires energy to "drive" the transport system, whereas facilitated transport does not have an energy requirement.

Although, cold-osmotic shock will not decrease uptake activity of D-alanine in M. lysodeikticus, the shock procedure of Gale and Llewellin (1970) significantly reduces ability of cells to take up D-alanine (Figure 4). These data can be interpreted to mean the shock treatment causes release of the D-alanine binding fraction(s); thus, reducing uptake ability.

The need for energy in uptake of D-alanine is suggested by the data given in Fig. 5. An inhibitor of oxidative phosphorylation, 2,4-dinitrophenol (DNP), at a concentration of 10^{-2}M drastically reduces uptake ability. When PL is added to the uptake system in the presence of DNP (10^{-2}M), a further reduction in "uptake" activity

Figure 3. Influence of PL on Uptake of D-Alanine-U-^{14}C by Cells Grown in the Presence and Absence of PL. ○, control cells; △, PL (0.22M) present (6 hours) during growth (cells washed two times prior to determination of uptake ability); ●, control cells plus PL (0.22M); ▲, PL grown cells which had been washed two times before re-addition of PL (0.22M) to the uptake medium.

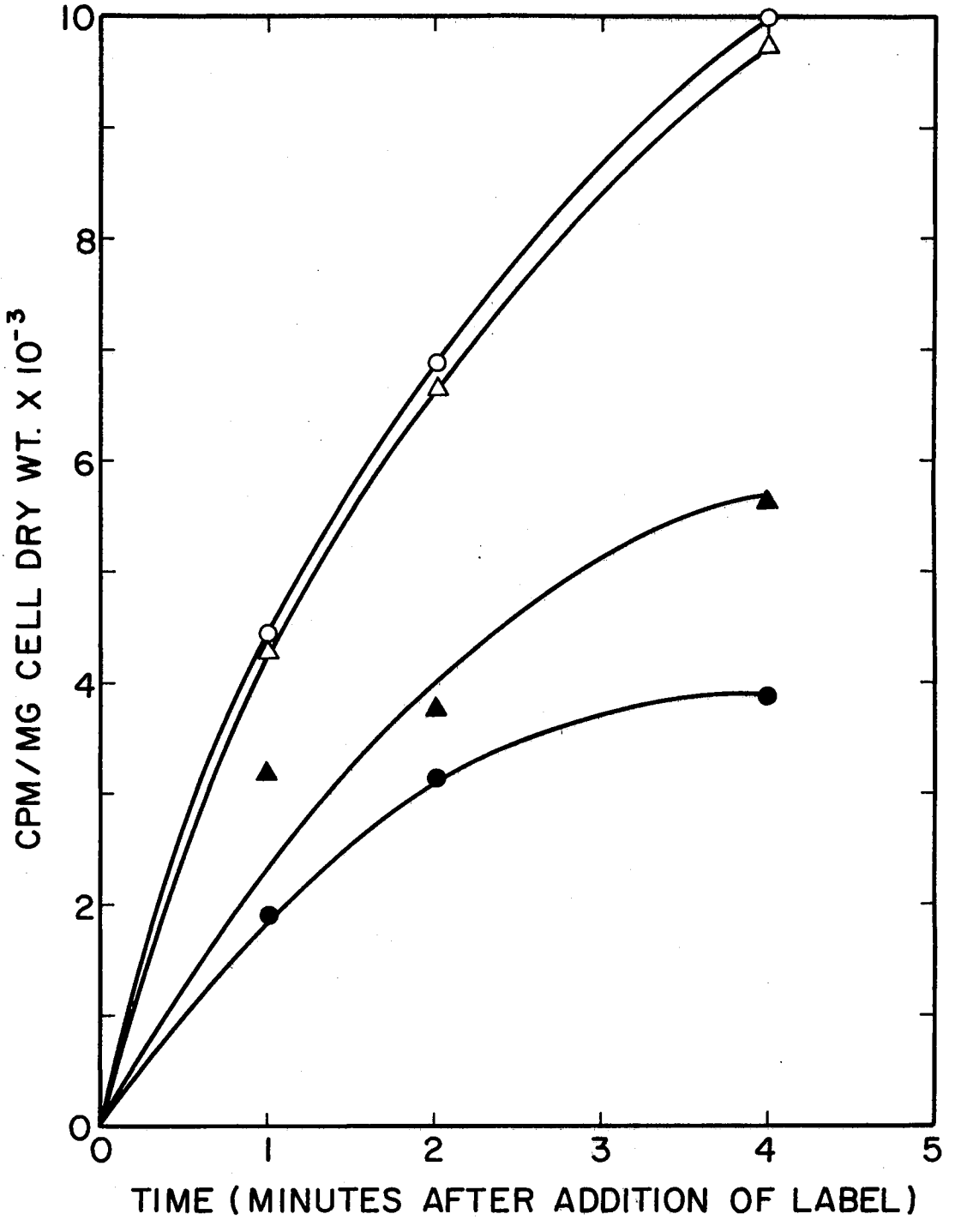

10
8
6
4
2
0
0
1
2
3
4
5
CPM/MG CELL DRY WT. X 10^{-3}
TIME (MINUTES AFTER ADDITION OF LABEL)

Figure 4. Uptake of D-Alanine-U-^{14}C by Control and Shocked Cells. ●, untreated control cells; ○, shocked cells.

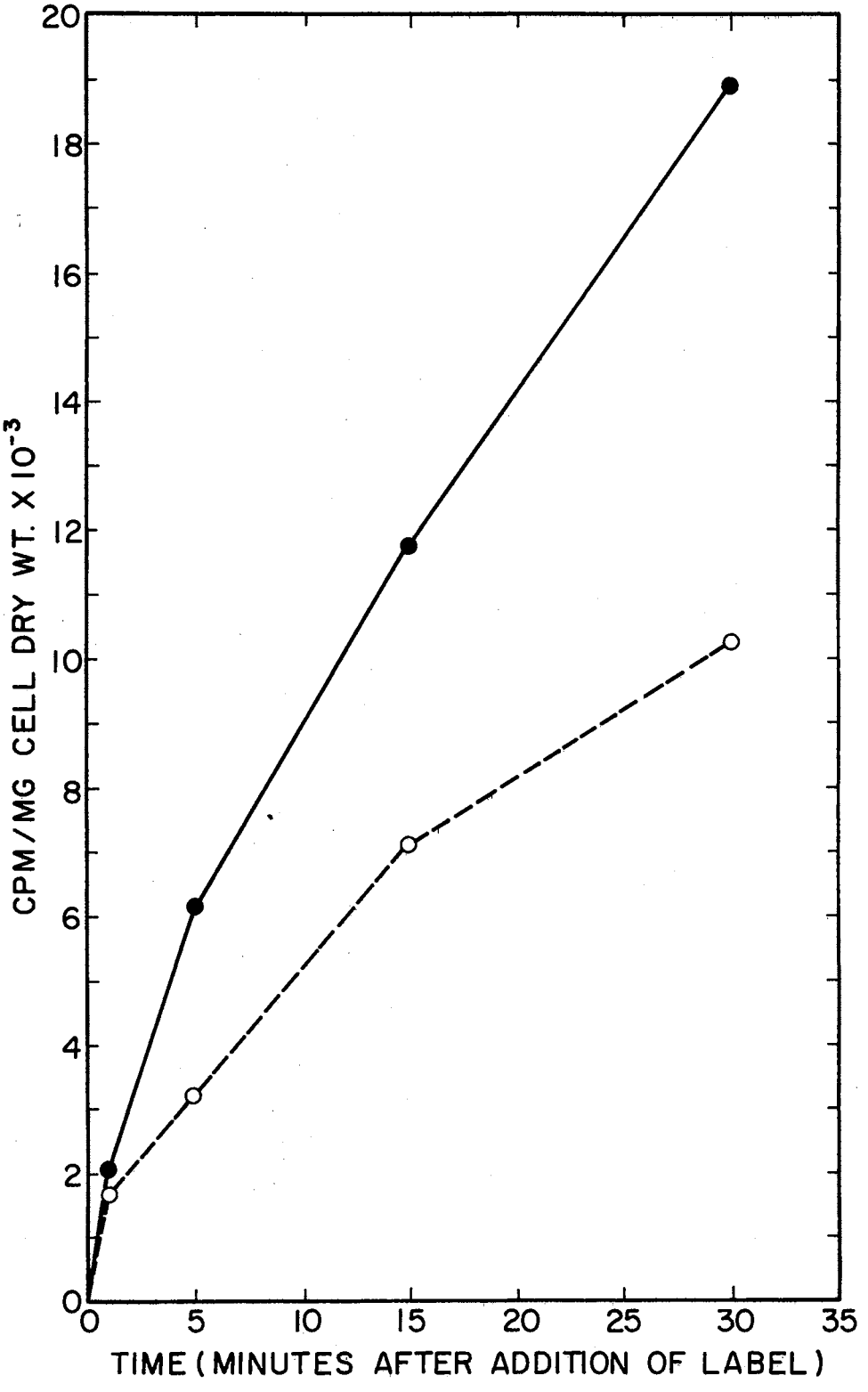
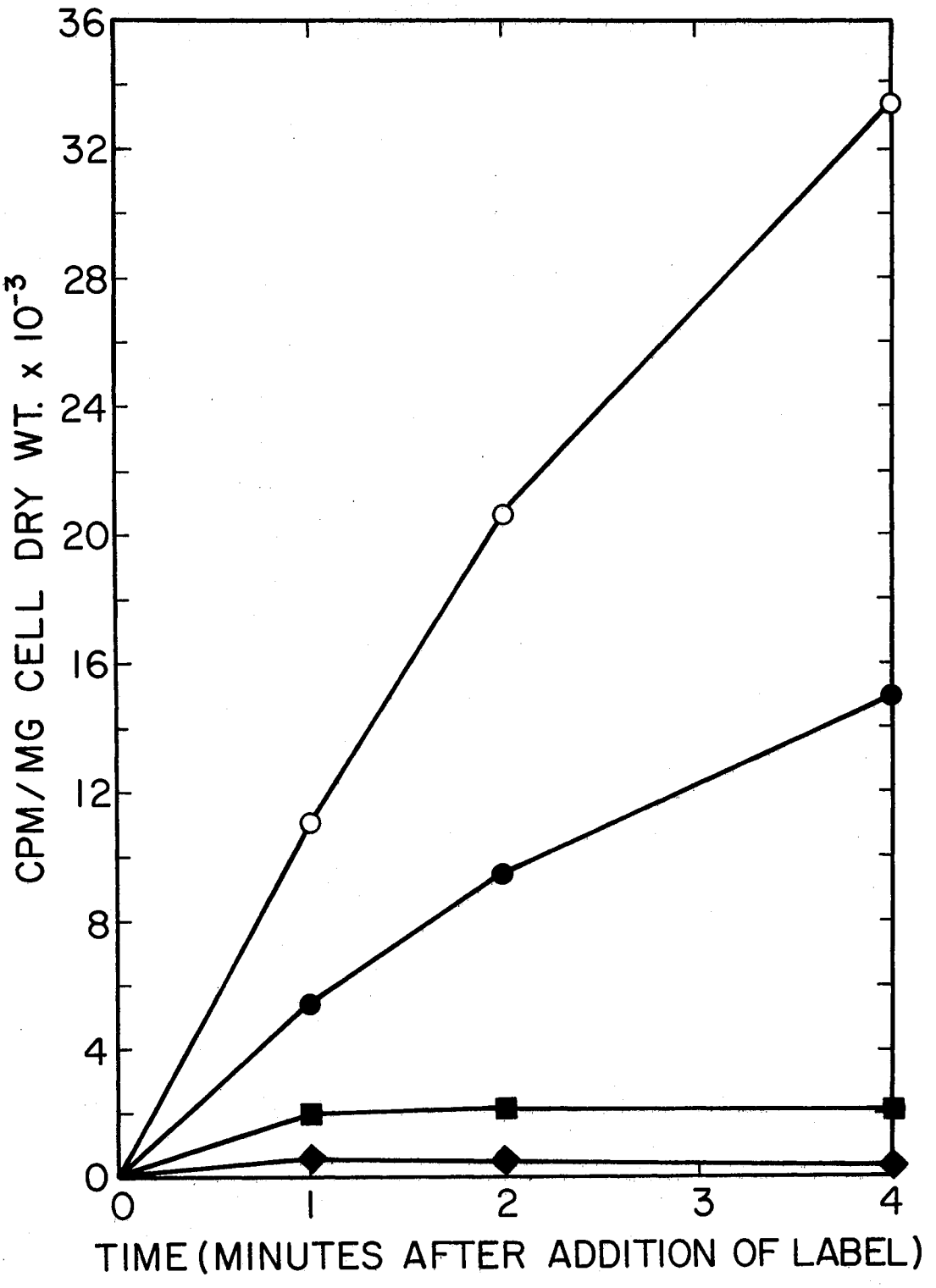

20
18
16
14
12
10
8
6
4
2
0
CPM/MG CELL DRY WT. X 10⁻³
0
5
10
15
20
25
30
35
TIME (MINUTES AFTER ADDITION OF LABEL)

Figure 5. Influence of PL and 2,4-Dinitrophenol on Uptake of D-Alanine-U-^{14}C in M. lysodeikticus. ○, control cells; ●, with PL (0.22M); ■, with 2,4-dinitrophenol (1.0×10^{-2} M); ◆, with 2,4-dinitrophenol (1.0×10^{-2} M) plus PL (0.22M).


36
32
28
24
20
16
12
8
4
0
CPM/MG CELL DRY WT. x 10^{-3}
0
1
2
3
4
TIME (MINUTES AFTER ADDITION OF LABEL)

occurs. This inhibition of residual uptake activity probably represents an inhibition in the ability of the substrate to bind to the binding protein, rather than a true inhibition of uptake.

The effects of several additional metabolic inhibitors on uptake of D-alanine are presented in Table I. Sodium azide, sodium arsenate, potassium cyanide, and iodoacetate, which inhibit electron transport, are also effective inhibitors of D-alanine uptake activity. Potassium cyanide and DNP cause the greatest amount of inhibition while sodium azide appears to be the least effective inhibitor investigated. These data suggest that PL is probably not acting as an uncoupler of oxidative phosphorylation or an inhibitor of the electron transport system since the effective concentration of the metabolic inhibitors needed to cause an equivalent reduction in D-alanine uptake ability is much smaller than the concentration of PL required.

An additional diagnostic tool to indicate uptake of D-alanine is of the active type can be obtained by observing for saturation kinetics (Eagon, 1971). As shown in Fig. 6, uptake of D-alanine both in the presence and absence of PL obeys saturation kinetics. Also, both the initial rate and concentration necessary for saturation of the uptake "system" are lowered in the presence of PL.

According to Schultz and Curran (1970) the spontaneous movement of Na^+, and possibly K^+, across the plasmalemna of the mammalian cell membrane provides the energy that is needed to transport amino acids against a concentration gradient.

Harold and Papineau (1972a, b) reported that cells of Streptococcus faecalis accumulate large quantities of dibenzyldimethylammonium (DDA^+) and triphenylmethylphosphonium ($TPMP^+$) and other lipid-soluble

TABLE I

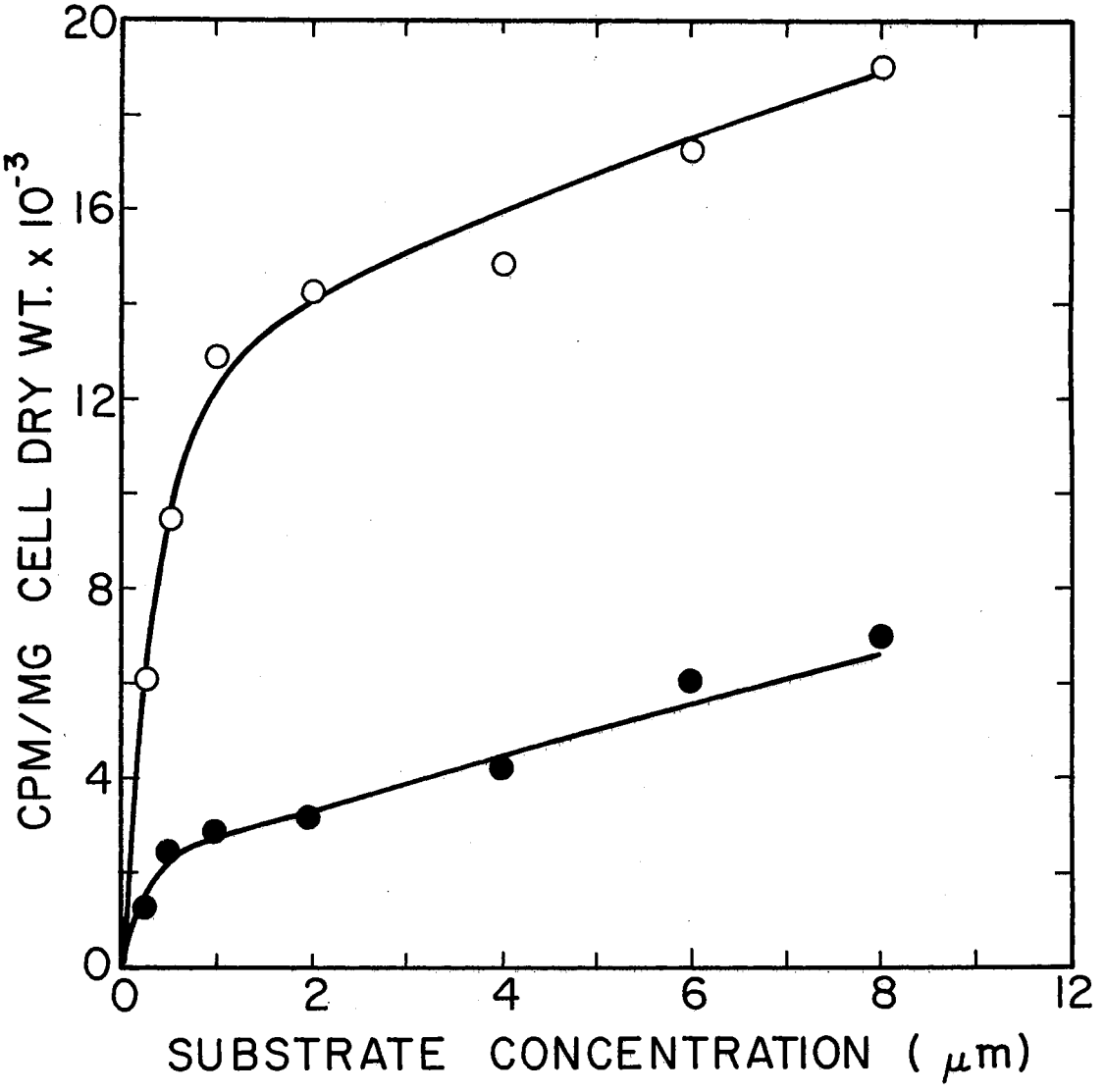
EFFECT OF METABOLIC INHIBITORS ON
D-ALANINE-U-^{14}C UPTAKE ACTIVITY

Inhibitor*	Final Concentration	% Inhibition**
Sodium azide	1×10^{-3}M	11%
	1×10^{-2}M	51%
Potassium cyanide	1×10^{-3}M	65%
	1×10^{-2}M	79%
Iodoacetate	1×10^{-3}M	45%
Sodium arsenate	1×10^{-3}M	44%
2,4-Dinitrophenol	1×10^{-3}M	65%

*Inhibitors were added 30 seconds prior to the addition of D-alanine-U-^{14}C.

**Values calculated from uptake readings made after 4 minutes exposure to D-alanine-U-^{14}C.

Figure 6. Influence of PL on Uptake of D-Alanine-U-^{14}C in M. lysodeikticus. ○, control cells; ●, with PL (0.22M). Data given are for 30 second uptake periods.


20
16
12
8
4
0
CPM/MG CELL DRY WT. x 10^{-3}
0
2
4
6
8
12
SUBSTRATE CONCENTRATION (μm)

cations by exchange for Na^+. It is their contention that glycolyzing cells extrude Na^+ in exchange for H^+(Na^+/H^+ antiport); the H^+ is then extruded by a membrane-located proton pump thus generating an electrical potential across the membrane (interior of the cell membrane becomes negative). Accumulation of the lipid-soluble cations thus becomes a thermodynamically passive response to the potential gradient.

A significant reduction in uptake of D-alanine results when potassium is substituted for sodium in the uptake medium (Figure 7). After one minute, uptake activity appears to be stopped when potassium is the major monovalent ion present. The initial uptake of D-alanine is very similar to that obtained in the presence of sodium and may be due to contaminating sodium since uptake activity levels off after one minute. Such peculiar kinetics suggest that the component necessary for active uptake is rapidly depleted. If this is true, then the accumulation of D-alanine in the presence of potassium is probably a result of passive diffusion rather than active uptake. As also shown in Fig. 7, inhibition by PL in the presence of sodium ions is much more severe than in the presence only of potassium ions or in distilled water.

To determine if transport of D-alanine is pH dependent, uptake ability was measured at three values; maximum activity occurs at pH 7.0 (Figure 8). Because a reduction in uptake activity occurs on either side of pH 7.0, uptake activity at additional pH values was not determined. Data also presented in Fig. 8 illustrate that transport of D-alanine in the presence of PL is inhibited very extensively whether measured at pH 6.0, 7.0, or 7.8.

Figure 7. Uptake of D-Alanine-U-^{14}C in the Presence of PL with Either Sodium Buffer, Potassium Buffer, or Distilled Water. ○, with sodium buffer; □, with potassium buffer; △, with distilled water; ●, with sodium buffer plus PL (0.22M); ■, with potassium buffer plus PL (0.22M); ▲, with distilled water plus PL (0.22M).

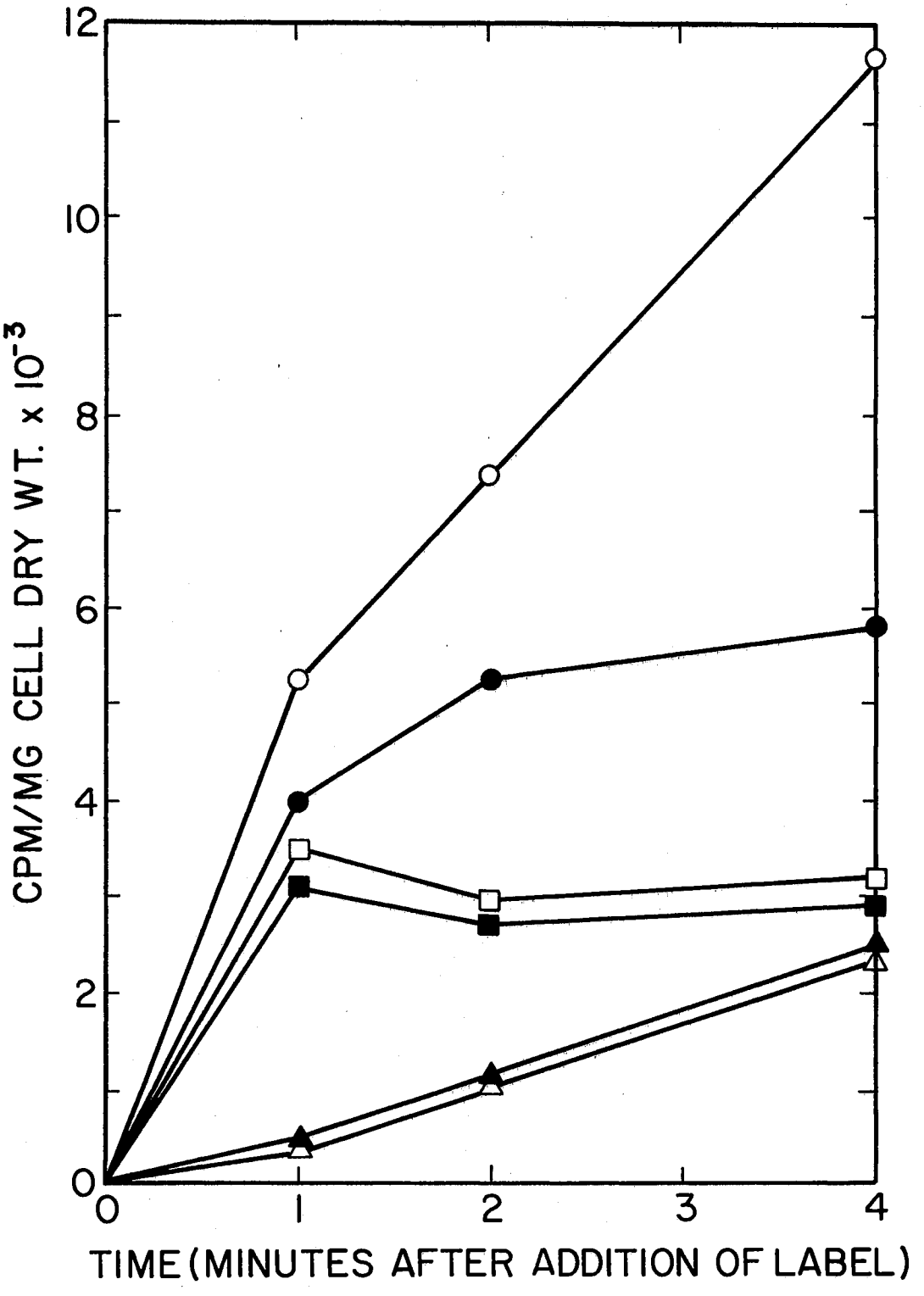
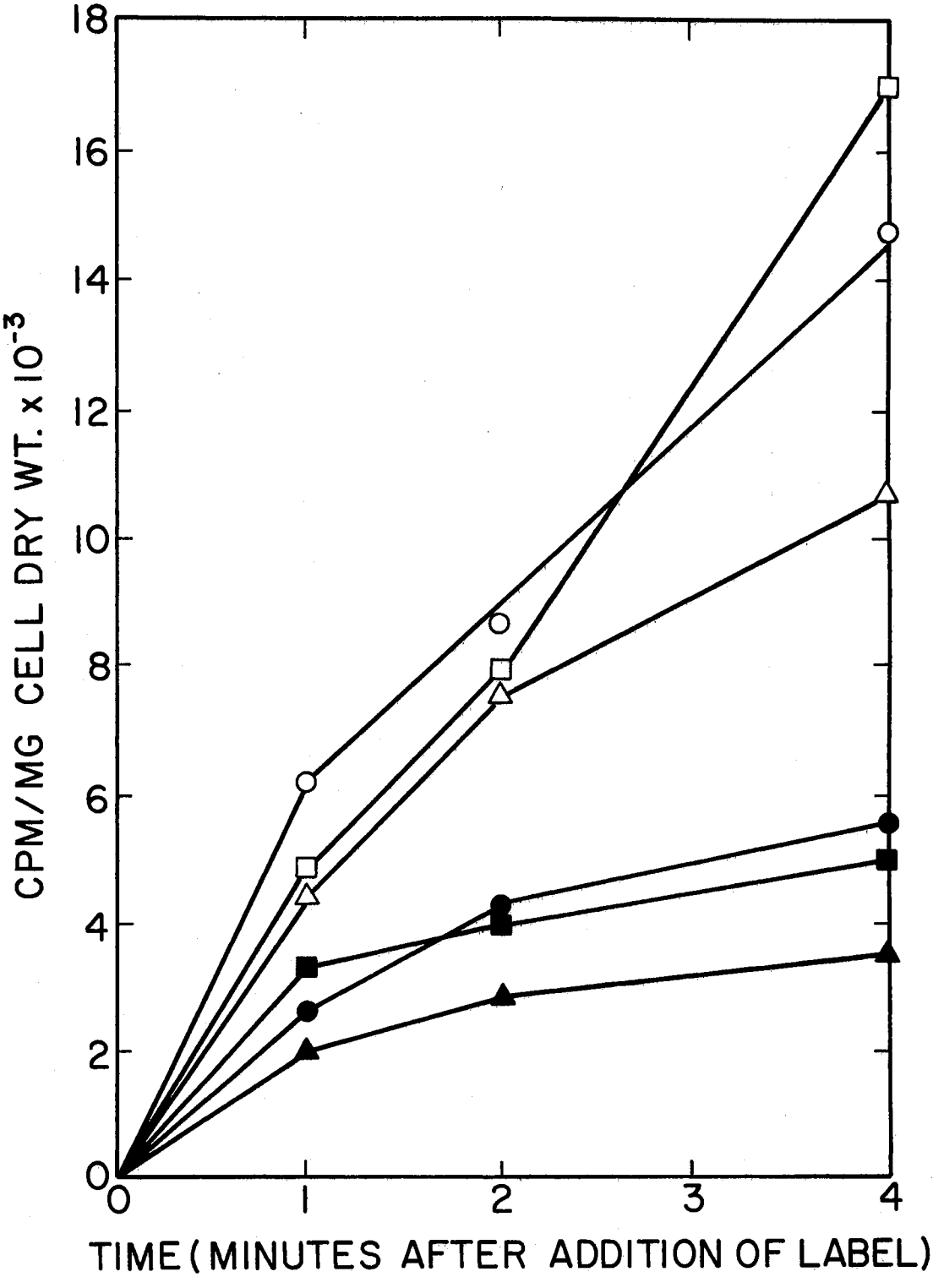

12
10
8
6
4
2
0
0
1
2
3
4
CPM/MG CELL DRY WT. x 10^{-3}
TIME (MINUTES AFTER ADDITION OF LABEL)

Figure 8. Influence of pH on D-Alanine-U-^{14}C Uptake Activity in *M. lysodeikticus*. △, pH 6.0; □, pH 7.0; ○, pH 7.8; ▲, pH 6.0 plus PL (0.22M); ■, pH 7.0 plus PL (0.22M); ●, pH 7.8 plus PL (0.22M).


18
16
14
12
10
8
6
4
2
0
0
1
2
3
4
CPM/MG CELL DRY WT. x 10⁻³
TIME (MINUTES AFTER ADDITION OF LABEL)

Kaback and Barnes (1971) suggested that carriers for the lactose transport system are transfer intermediates which undergo reversible oxidation-reduction. The carrier, in the oxidized state, is depicted as having a high affinity site for ligand which it binds on the exterior surface of the membrane. By some type of mechanism a critical disulfide in the carrier molecule becomes reduced; this, in turn, causes a conformational change. As a result of this conformational change, the affinity of the carrier for its ligand is decreased and ligand is then released on the interior surface of the membrane.

It was thought that PL could aid in causing a premature reduction in a critical disulfide group(s) resulting in decreased affinity of the carrier for its ligand; thus inhibiting uptake ability. As shown in Fig. 9, evidence could not be obtained to implicate reduced disulfide groups in D-alanine transport since inhibition of uptake does not occur in the presence of 2-mercaptoethanol (1.7×10^{-3}M).

The ability of M. lysodeikticus to take up D-alanine appears to be a function of both the temperature at which the determinations are performed as well as the temperature of growth (Figure 10). Of the four temperatures at which uptake ability was measured, 37 C appears to be optimal (uptake activity measured at 4 C may represent only external binding capacity). It is interesting that cells grown at 26 and 15 C exhibit greatest uptake ability; however, this ability is most apparent only when uptake is measured at 37 C. The decreased uptake at 4 C is a further indication that D-alanine is transported by an active process.

When uptake ability of cells grown at the different temperatures is measured in the presence of PL at different temperatures, inhibition

Figure 9. Influence of 2-Mercaptoethanol on D-Alanine Transport in the Presence and Absence of PL. ○, control cells; ●, with PL_3(0.22M); △, with 2-mercaptoethanol (1.7×10^{-3}M); □, with 2-mercaptoethanol (1.7×10^{-3}M) plus PL (0.22M).

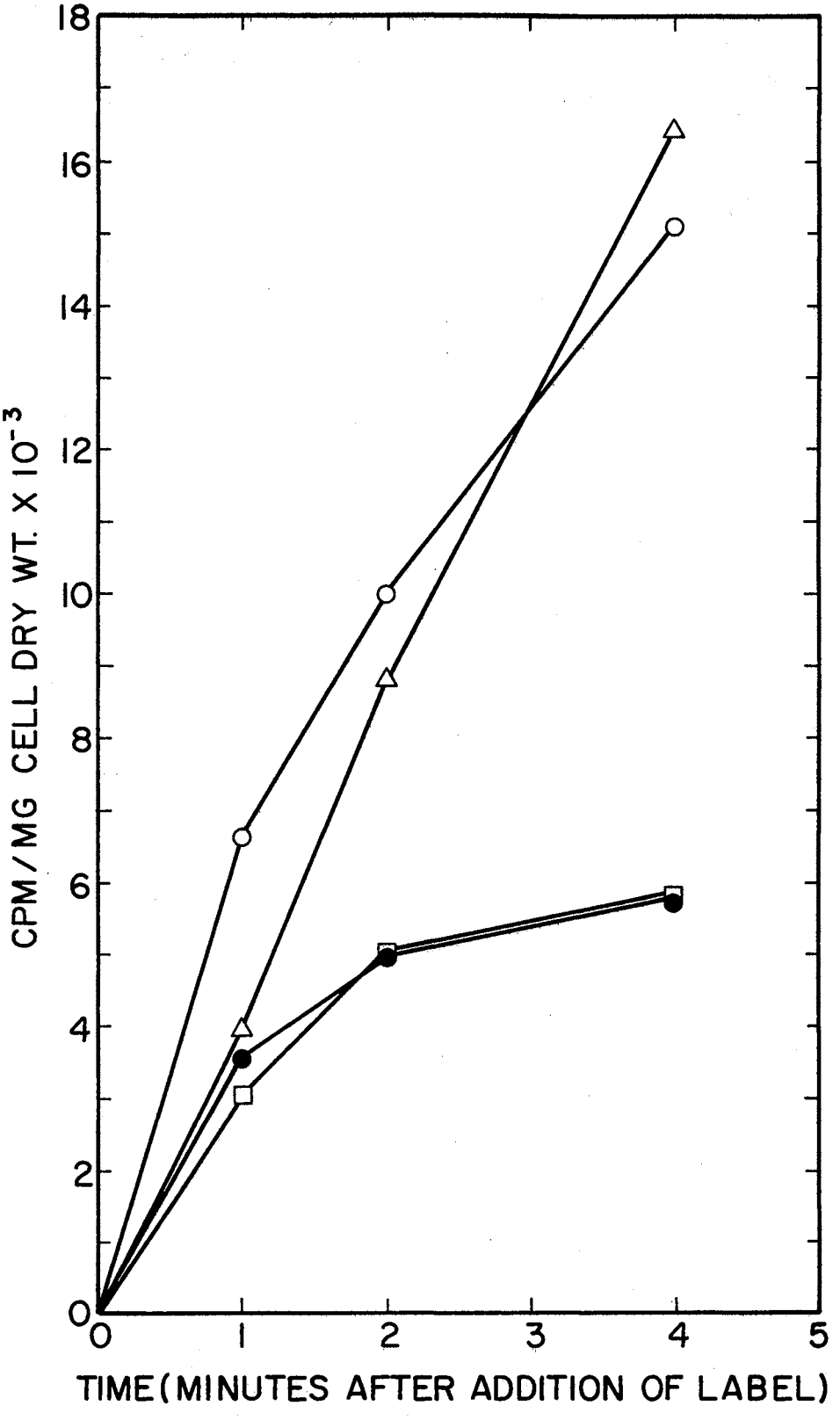
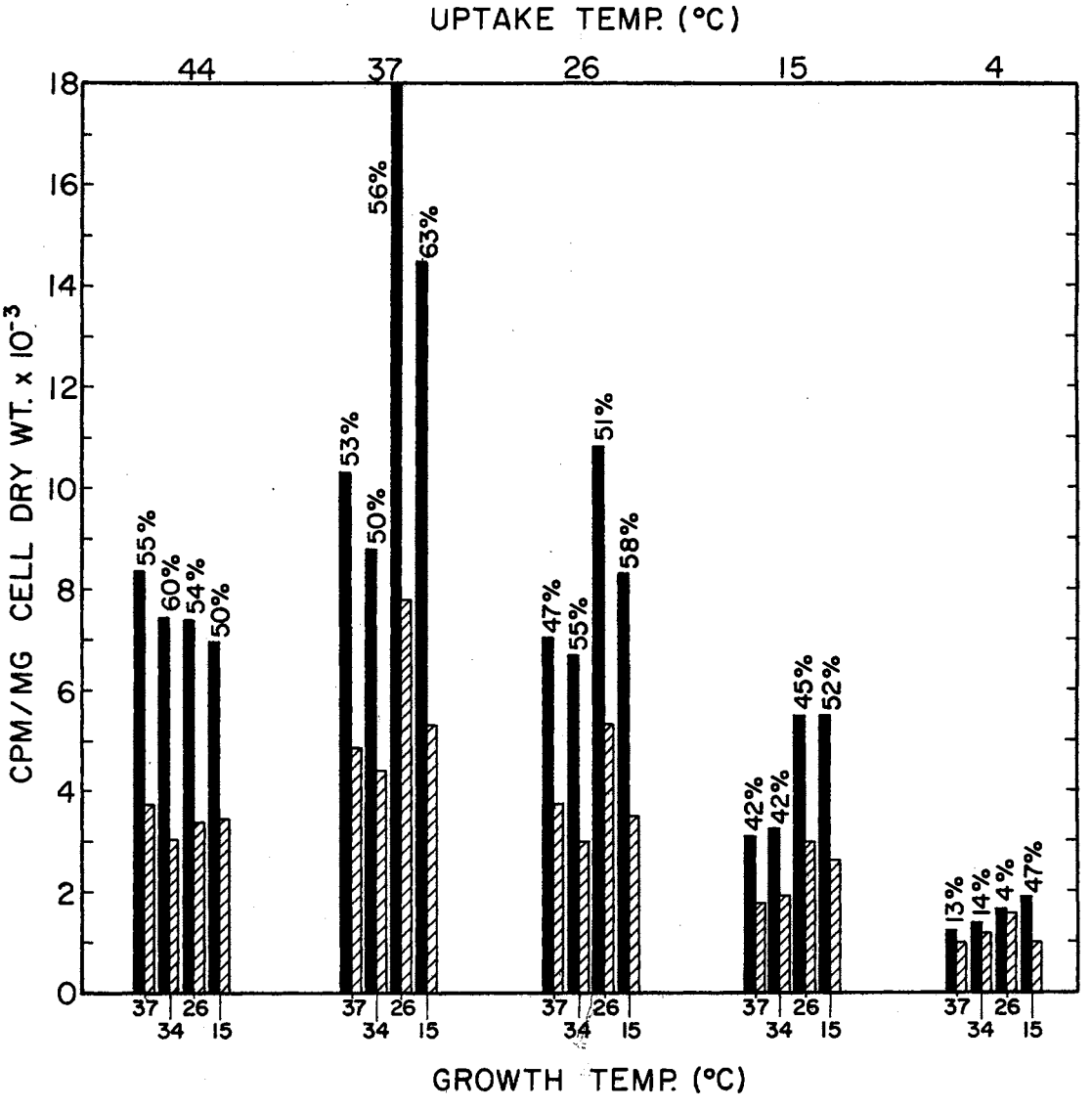

18
16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
CPM/MG CELL DRY WT. X 10⁻³
TIME(MINUTES AFTER ADDITION OF LABEL)

Figure 10. Influence of Temperature During Growth and Uptake on Transport of D-Alanine in the Presence and Absence of PL. All cells were grown under stationary conditions and radioactivity measured four minutes after addition of the label. The solid bars represent determinations made in the absence of PL while the striped bars represent determinations made in the presence of PL (0.22M).


UPTAKE TEMP. (°C)
44
37
26
15
4
CPM/MG CELL DRY WT. x 10^{-3}
0
2
4
6
8
10
12
14
16
18
55%
60%
54%
50%
53%
50%
56%
63%
47%
55%
51%
58%
42%
42%
45%
52%
13%
14%
4%
47%
37
34
26
15
GROWTH TEMP. (°C)

always occurs. Least inhibition occurs at 4 C where an active type transport is probably not occuring and uptake represents binding ability.

Considering the data presented on the uptake ability of M. lysodeikticus for D-alanine, it appears highly likely that D-alanine is taken up via an active process. This conclusion is in agreement with results reported by Ariel and Grossowicz (1972). It follows therefore that PL can inhibit active type transport processes.

Inhibition of Passive Uptake by PL

Since the active uptake of D-alanine is inhibited in the presence of PL, experiments were undertaken to determine whether PL also inhibits uptake of molecules that enter the cell by passive diffusion. Data presented in Table II demonstrate that PL inhibits the uptake of a variety of compounds in M. lysodeikticus dis-IIp^{+}. These experiments revealed that neither pyruvate-2-^{14}C nor 2-deoxy-D-glucose-1-^{14}C are taken up readily by M. lysodeikticus; thus their uptake is probably of the passive type. To obtain significant uptake of these two compounds, cold carrier molecules could not be added and much higher concentrations of the labeled compound were used.

The absence of a detectable inhibition of both 2-deoxy-D-glucose and pyruvate uptake activity in the presence of DNP (10^{-3}M) is additional evidence that these two compounds enter the cell by a passive type process (Figure 11). Data presented in Fig. 12 show that PL also inhibits uptake of these compounds. Similar results were reported by Hopfer (1972); he demonstrated the inhibition of uptake of several compounds thought to be taken up by a passive process into Erwinia

TABLE II

EFFECT OF PL ON UPTAKE OF VARIOUS COMPOUNDS BY CELLS OF MICROCOCCUS LYSODEIKTICUS dis-IIp+

Labeled Compound	CPM Taken up/mg Dry Wt. Cells		Percent Decrease Caused by PL*
	Control	Pantoyl Lactone (0.22M)	
L-Aspartic Acid	12,000	6,500	46
L-Phenylalanine	11,500	7,500	34
2-Deoxy-D-Glucose/	1,500	1,000	33
Uracil/	5,000	3,000	40
Glycerol	14,000	10,000	29
Pyruvate/	3,500	1,500	57
L-Malic Acid	7,500	5,000	33

*Values calculated from uptake readings made after 4 minutes exposure to the labeled substrate as given in Materials and Methods.

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

Figure 11. Uptake of Pyruvate-2-^{14}C and 2-Deoxy-D-Glucose-1-^{14}C by M. lysodeikticus in the Presence of 2,4-Dinitrophenol (DNP). △, 2-deoxy-D-glucose; ▲, 2-deoxy-D-glucose plus DNP (10^{-3} M); ○, pyruvate-2-^{14}C; ●, pyruvate-2-^{14}C plus DNP (10^{-3} M).

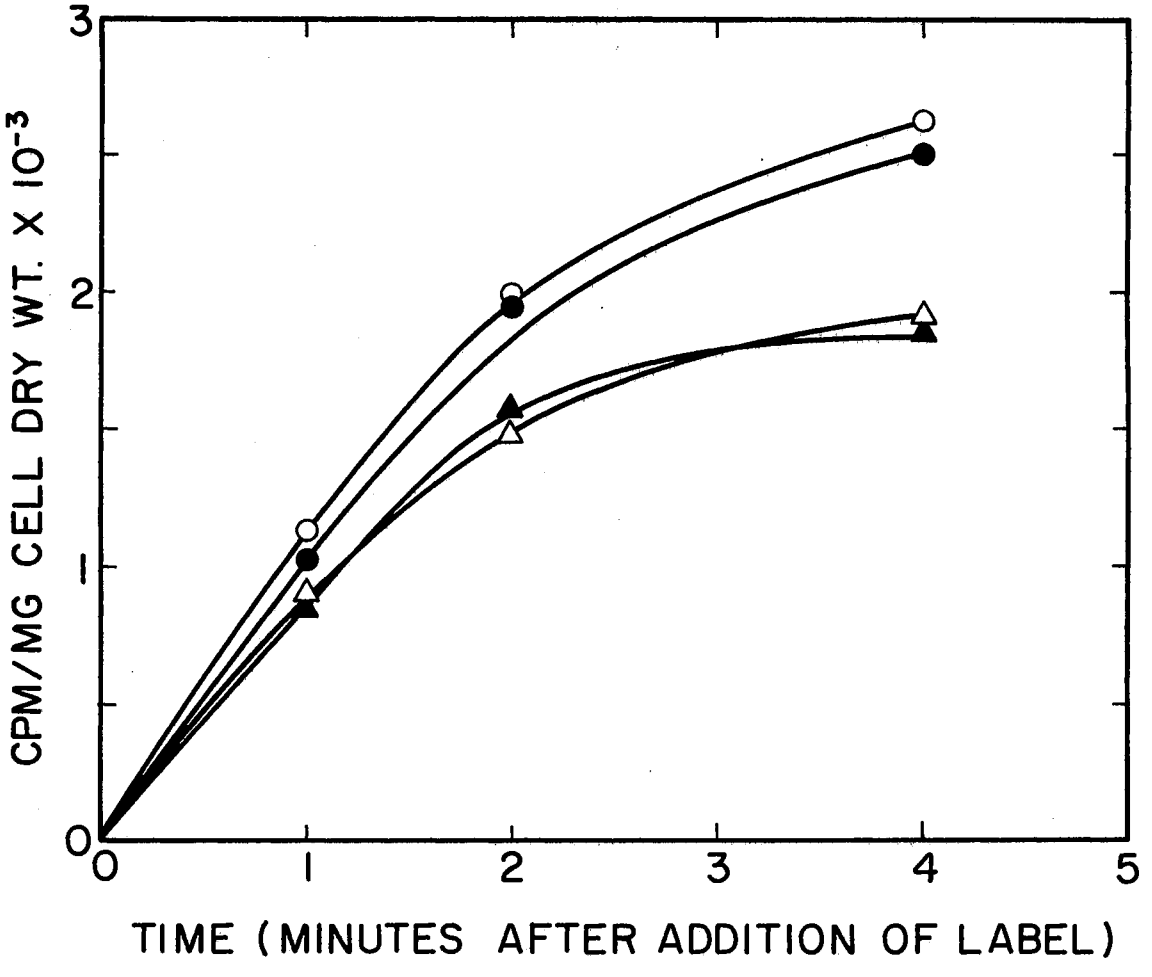
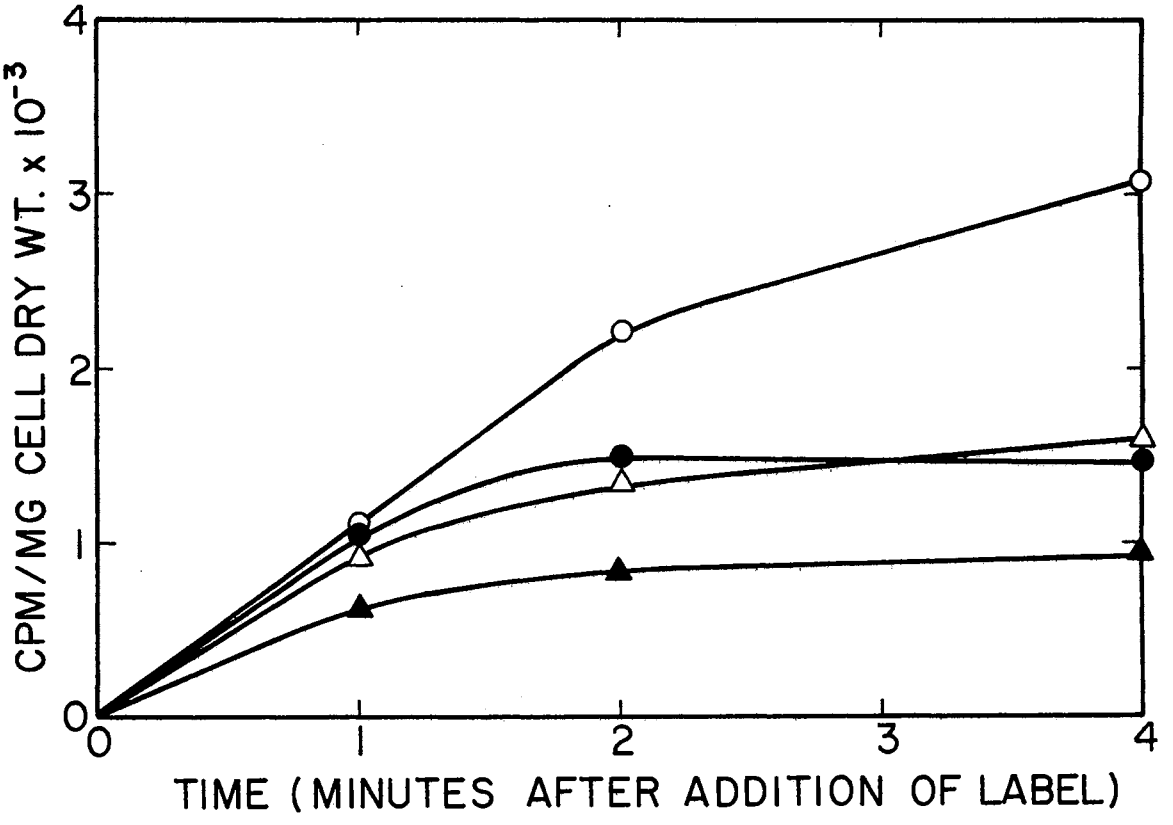

CPM/MG CELL DRY WT. X 10⁻³
3
2
1
0
0
1
2
3
4
5
TIME (MINUTES AFTER ADDITION OF LABEL)

Figure 12. Uptake of Pyruvate-2-^{14}C and 2-Deoxy-D-Glucose-1-^{14}C by M. lysodeiktigus in the Presence of PL. △, 2-deoxy-D-glucose; ▲, 2-deoxy-D-glucose plus PL (0.22M); ○, pyruvate-2-^{14}C; ●, pyruvate-2-^{14}C plus PL (0.22M).


CPM/MG CELL DRY WT. x 10^{-3}
0
1
2
3
4
TIME (MINUTES AFTER ADDITION OF LABEL)

species (thymine, thymidine, 2-ketoglutaric acid, and malic acid).

Efflux of 2-Deoxy-D-Glucose and D-Alanine in the Presence of PL

Several investigators have reported that with either active transport or facilitated diffusion the carrier functions in both directions; that is, in both efflux and influx. The efflux mechanism in a transport system has a twofold role. First, efflux may aid a control mechanism to prevent the concentration from building up in the cytoplasm to dangerously high levels. Second, the efflux mechanism could provide for return of the carrier (or a reorientation) to the location necessary for continued influx (Winkler, 1971).

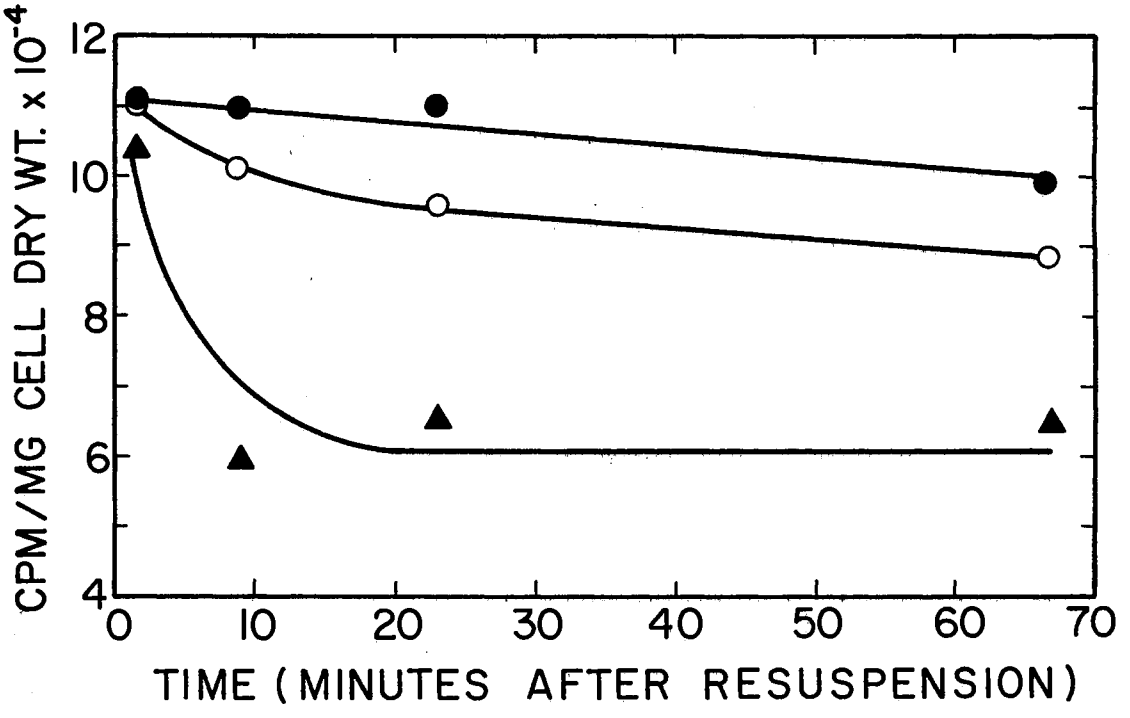
Konings and Freese (1972) working with Bacillus subtilis demonstrated that uptake of amino acids stops when the available energy source has been completely oxidized; the accumulated amino acids then leak out at a rate of about 8 percent per minute.

Because of the possibility that PL could be causing an increased efflux rate rather than an actual inhibition of uptake, the efflux of 2-deoxy-D-glucose, a non-metabolizable compound, was investigated.

Kirkland and Durham (1965) used toluene to facilitate enzyme assays in Pseudomonas fluorescens since it readily disrupts the permeability barrier of the cell. For this reason, toluene (0.02ml/ml sample) was selected as a positive control in these experiments. Data presented in Fig. 13, indicate that PL does not increase the efflux rate of 2-deoxy-D-glucose. Instead, it appears that PL not only prevents uptake but also decreases efflux.

Since an increase in the rate of efflux of 2-deoxy-D-glucose,

Figure 13. Influence of PL and Toluene on Efflux of 2-Deoxy-D-Glucose-1-^{14}C. ○, control cells; ▲, with toluene (0.02 ml/ml sample); ●, with PL (0.22M).


CPM/MG CELL DRY WT. x 10^{-4}
12
10
8
6
4
0
10
20
30
40
50
60
70
TIME (MINUTES AFTER RESUSPENSION)

could not be detected and because D-alanine was the compound of primary interest, a similar type of efflux experiment was performed using D-alanine instead of 2-deoxy-D-glucose. Again it can be seen (Fig. 14) that the rate of release of D-alanine is not increased; rather it is slowed in the presence of PL.

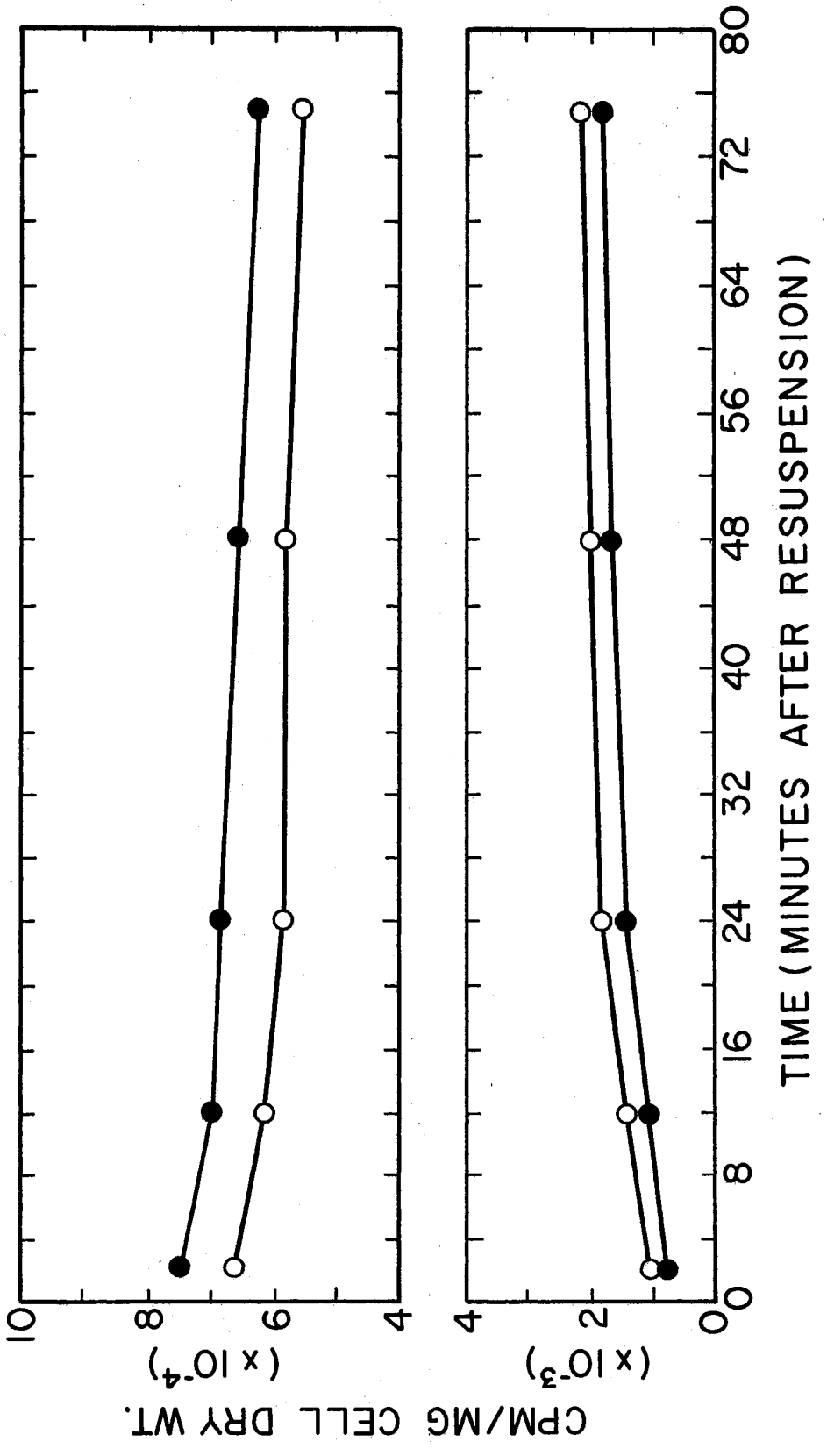
Effects of PL and Physical Properties of the Growth Medium on Growth and Uptake of D-Alanine-U-^{14}C

Division of M. lysodeikticus is definitely inhibited after six hours of incubation in the presence of D-cycloserine (DCS) as evidenced by the presence of large cells and inhibition of growth. Lysis of cells is initiated at about this time and many ghost cells can be detected throughout the remainder of the growth cycle. Grula and King (1971) have shown that PL (0.22M) will prevent this inhibition of division. Because of the high concentration of PL required to inhibit uptake activity or prevent division inhibition in the presence of DCS and the ease by which these effects of PL can be removed (simply washing the cells free of PL), it seemed possible that some or all of the effects of PL might result from changes in the physical properties of the growth medium.

Grula and Grula (1964) reported that either PL or osmotic protective conditions significantly lower leakage of proteins and nucleic acid from filamentous cells of Erwinia species, while stimulating significant amounts of division even in the absence of normal mucopeptide synthesis.

In view of their findings it was felt that PL might possibly be

Figure 14. Influence of PL on Efflux of D-Alanine-U-^{14}C. ○, control cells; ●, PL (0.22M) treated cells; ○, filtrate from control cells; ●, filtrate from PL (0.22M) treated cells. The top figure represents cells while the bottom figure represents the supernatant.


CPM/MG CELL DRY WT.
(x 10⁻⁴)
(x 10⁻³)
TIME (MINUTES AFTER RESUSPENSION)
0
8
16
24
32
40
48
56
64
72
80
4
2
0
10
8
6
4

acting by increasing osmotic pressure, thus causing the growth medium to become more hypertonic. To ascertain whether this is the manner by which PL is acting, the effects of several other compounds on growth and uptake activity were investigated. The compounds selected for study included sucrose, Carbowax-400 (Fisher Scientific Company), sodium chloride and spermine.

It was observed that sodium chloride at a concentration of 0.11M and sucrose (0.22M) do not prevent division inhibition caused by DCS, although this concentration of sodium chloride does reduce the extent of lysis. Carbowax-400 (0.22M) prevents large cell formation for eleven hours after DCS addition; however, after this time period many intermediate size cells can be seen. Although Carbowax-400 and PL are the only compounds which prevent division inhibition caused by DCS, growth in the presence and absence of all compounds was investigated. As shown in Fig. 15, sucrose at a concentration of 0.22M has no effect on growth of M. lysodeikticus. Also very little inhibition is observed using high levels (0.11M) of sodium chloride, whereas PL and Carbowax-400 at equivalent concentrations inhibit growth appreciably.

To determine if any of the compounds or conditions had lasting effects, the cells were treated with each compound for five minutes, followed by two washings with minimal salts and then checked for growth response by reinoculating the cells into medium free of the compound under investigation. As can be seen in Fig. 16, the inhibition of growth is completely removed in each case by the washing procedure.

Even though hypertonic conditions do not appear to be responsible for inhibition of growth by PL, effect of these compounds and conditions on uptake activity was investigated.

Figure 15. Growth Response of Cells to PL, Carbowax-400, Sodium Chloride, and Sucrose Added after Twelve Hours Incubation. ○, control cells; ●, with PL (0.22M); ■, with Carbowax-400 (0.22M); ▲, with sodium chloride (0.11M); ◆, with sucrose (0.22M).

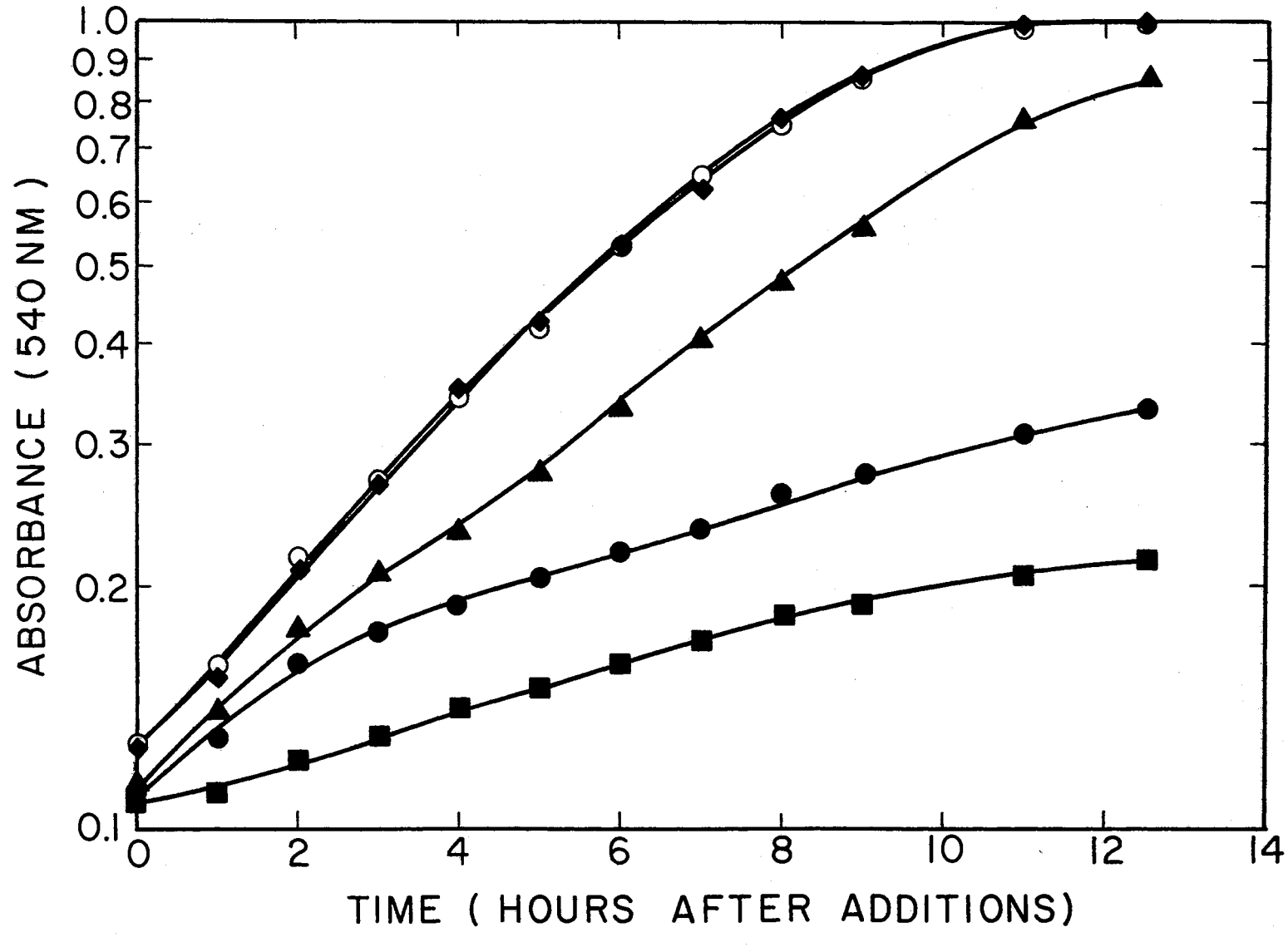
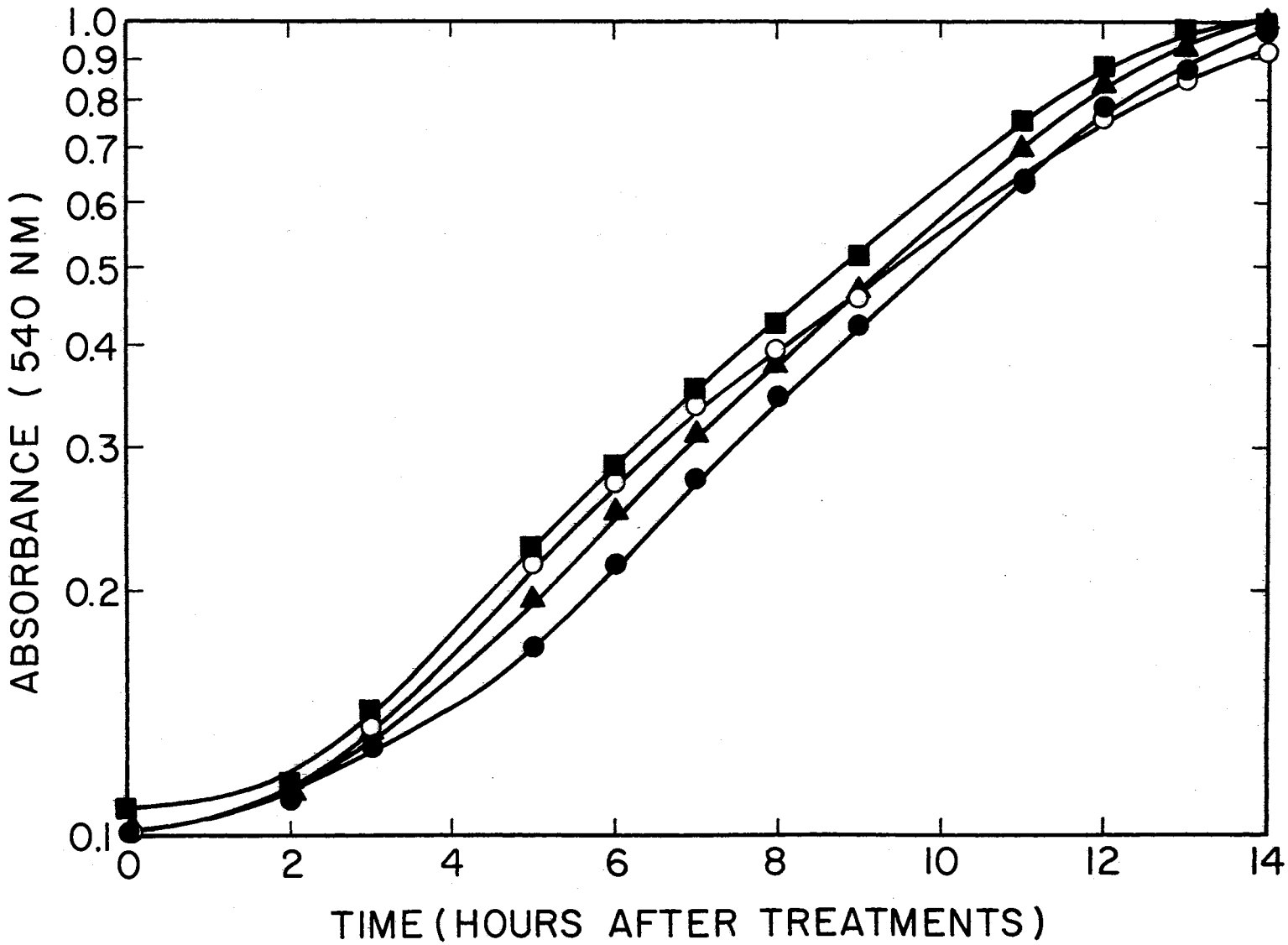

ABSORBANCE (540 NM)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
TIME (HOURS AFTER ADDITIONS)

Figure 16. Restoration of Growth by Washing Cells which had been Treated for Five Minutes with Either PL, Carbowax-400, or Sodium Chloride. ○, control cells; ●, with PL (0.22M); ■, with Carbowax-400 (0.22M); ▲, with sodium chloride (0.11M).


ABSORBANCE (540 NM)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
TIME (HOURS AFTER TREATMENTS)

As illustrated in Fig. 17, hypertonic levels of Carbowax-400, sucrose, and sodium chloride do not inhibit uptake of D-alanine to the same extent as PL (15-20% as opposed to about 50% for PL). Data presented in Fig. 18 reveal that all inhibitions on uptake activity are removed by washing.

The minimal inhibition of uptake observed in the presence of Carbowax-400 is interesting since Carbowax-400 caused the greatest amount of growth inhibition and, like PL, also prevents division inhibition by DCS. Wargel, Shadur, and Neuhaus (1970) reported using E. coli that uptake of D-alanine, glycine, and D-cycloserine is mediated by the same transport system. Since Carbowax-400 did not significantly inhibit uptake of D-alanine, it is improbable that the beneficial effects of this additive on cell division are due to exclusion of DCS. Nevertheless, since transport in M. lysodeikticus could be quite different from E. coli the effect of Carbowax-400 on uptake activity using L-glutamic-U-^{14}C and L-aspartic-U-^{14}C, which are the major carbon sources in the basal medium for M. lysodeikticus, was investigated. Although the data are not shown, it was observed that Carbowax-400 does not inhibit the uptake of these two compounds; instead, a slight stimulation may be effected.

Although the ease of restoration of both growth and uptake activity after treatment of cells with hypertonic levels of sucrose and sodium chloride closely resembles the situation seen using PL, equal molar concentrations of sodium chloride or sucrose do not inhibit growth or uptake of D-alanine to the same extent as PL. Also, neither prevents division inhibition caused by DCS. Thus, it appears reasonable to conclude that the ability of PL to stimulate division and

Figure 17. Effect of PL, Carbowax-400, Sodium Chloride, and Sucrose on Uptake Activity of D-Alanine-U-^{14}C. ●, control cells; ○, plus PL (0.22M); ■, plus Carbowax-400 (0.22M); ▲, plus sodium chloride (0.11M); ◆, plus sucrose (0.22M).

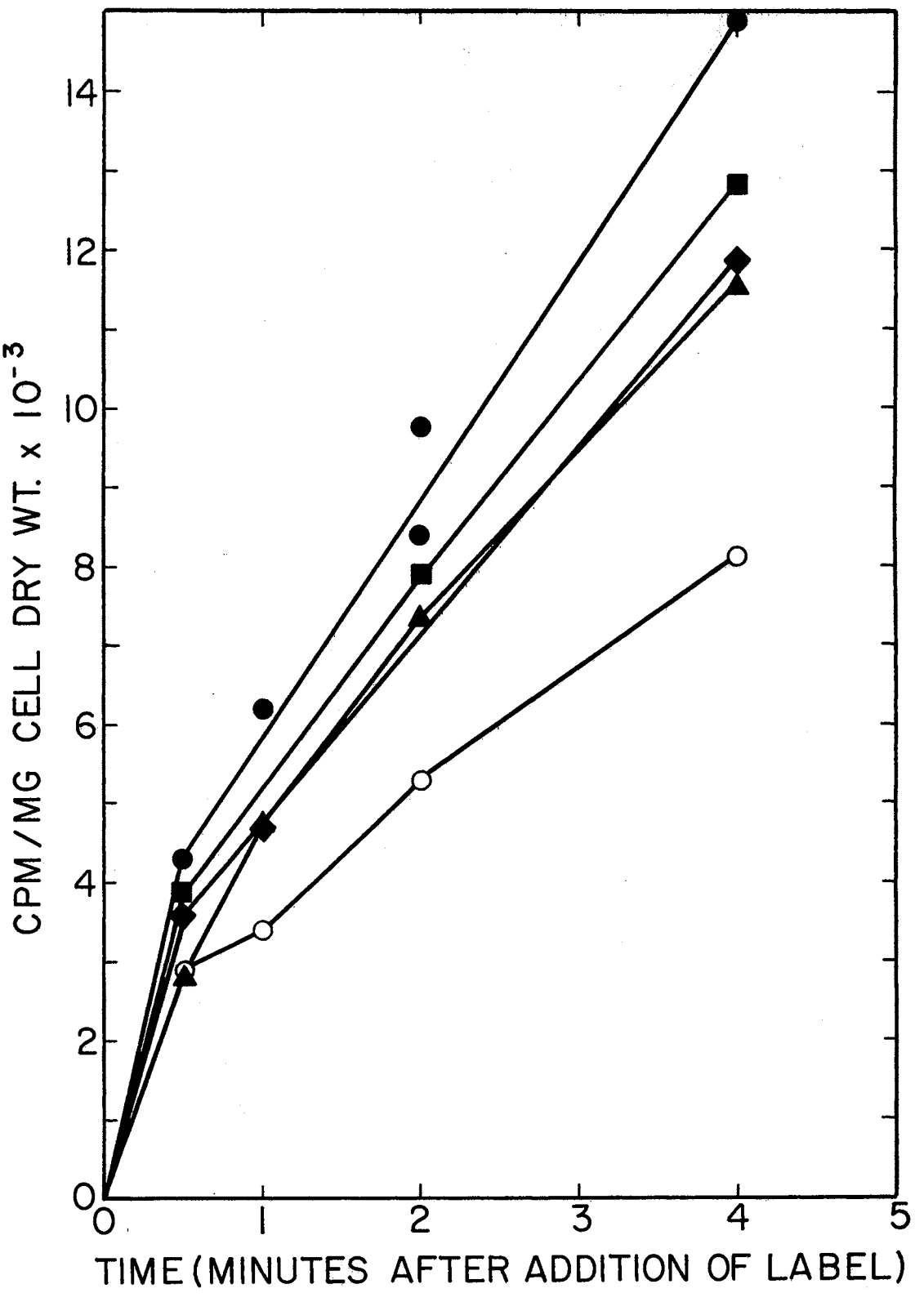
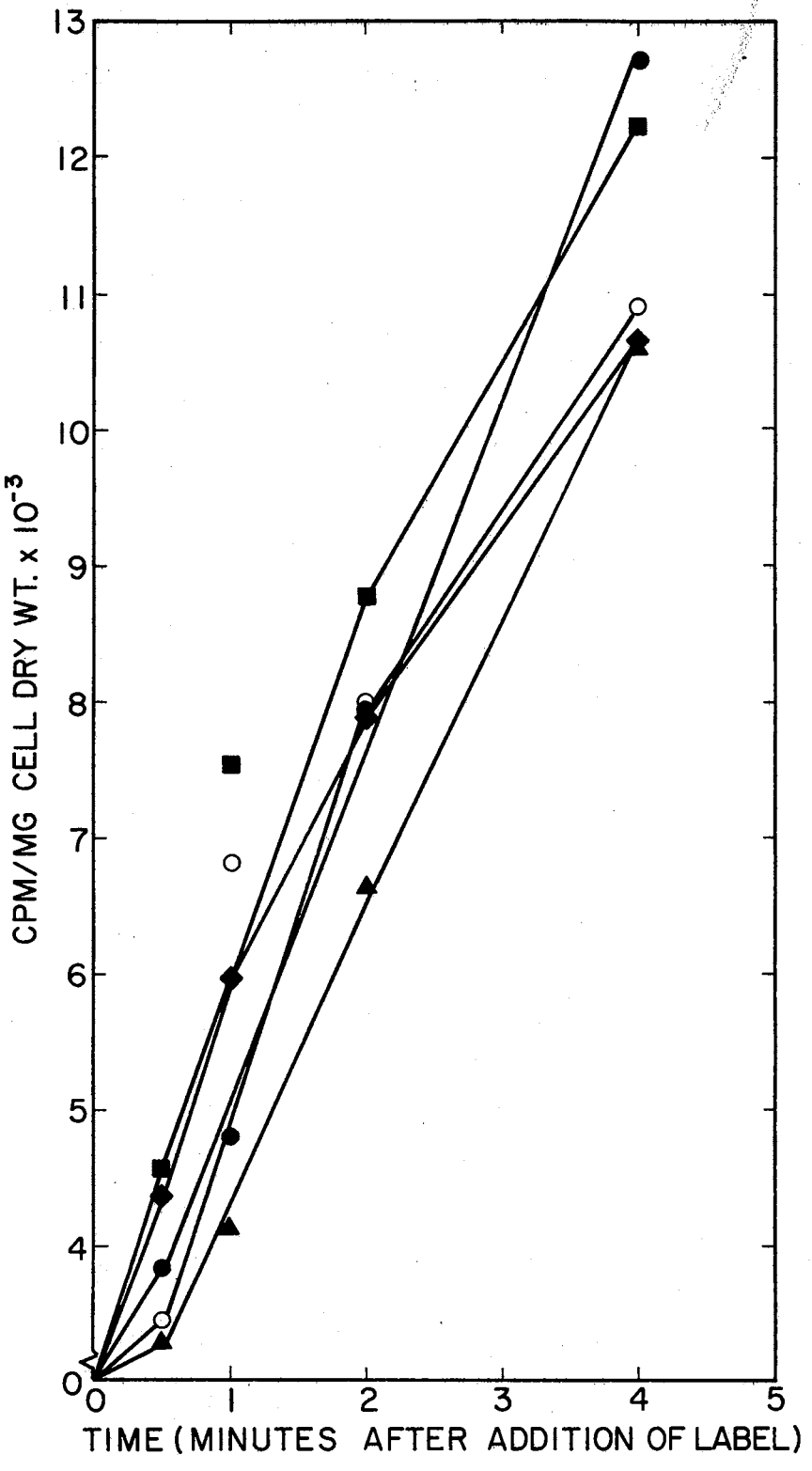

CPM/MG CELL DRY WT. x 10^{-3}
0
2
4
6
8
10
12
14
0
1
2
3
4
5
TIME (MINUTES AFTER ADDITION OF LABEL)

Figure 18. Reversal of D-Alanine Uptake Inhibition. Cells Pre-treated (5 minutes) with Either PL, Carbowax-400, Sodium Chloride, or Sucrose then Washed Two Times in Uptake Buffer prior to Determination of Uptake. ○, control cells; ●, PL (0.22M) treated cells; ■, Carbowax-400 (0.22M) treated cells; ▲, sodium chloride (0.11M) treated cells; ◆, sucrose (0.22M) treated cells.


13
12
11
10
9
8
7
6
5
4
0
0
1
2
3
4
5
CPM/MG CELL DRY WT. x 10^{-3}
TIME (MINUTES AFTER ADDITION OF LABEL)

inhibit uptake is not due to any effects by this compound on tonicity of the growth or uptake media.

Grossowicz and Ariel (1963) demonstrated that spermine can protect protoplasts of M. lysodeikticus against lysis under hypotonic conditions. They also reported (1972) that spermine inhibits uptake of D-alanine in M. lysodeikticus. Because Grula and King (1971, 1972) reported that spermine prevents inhibition of division caused by DCS in M. lysodeikticus, the effect of spermine on uptake of D-alanine was also studied.

Data presented in Fig. 19 show that spermine (6uM/ml) stimulates uptake activity in the dis-IIp$^+$ strain of M. lysodeikticus. The dissimilarity of these results from those reported by Ariel and Grossowicz may have occurred because of a difference in strains of organisms employed (dis-IIp$^+$ is a nutritional mutant of M. lysodeikticus). It is possible that the carrier molecule(s) for D-alanine are different in the two strains particularly since in later studies Ariel and Grossowicz (1972) have concluded that the inhibition by spermine is most likely due to a specific binding of spermine to the carrier molecule(s) for D-alanine.

It has often been observed in our laboratory that when making slides of cultures containing PL, the droplet containing the bacteria has a tendency to glide about on the slide rather than spreading out as normal. This observation suggests that PL causes an alteration in the physical properties of the growth medium. Since PL doesn't appear to be exerting its effects by increasing the hypertonicity of the growth medium, the possibility that PL alters surface tension was investigated.

As shown in Fig. 20, PL will cause a decrease in surface tension.

Figure 19. Uptake of D-Alanine-U-^{14}C in the Absence and Presence of Spermine. □, control cells; △, with spermine (6uM/ml).

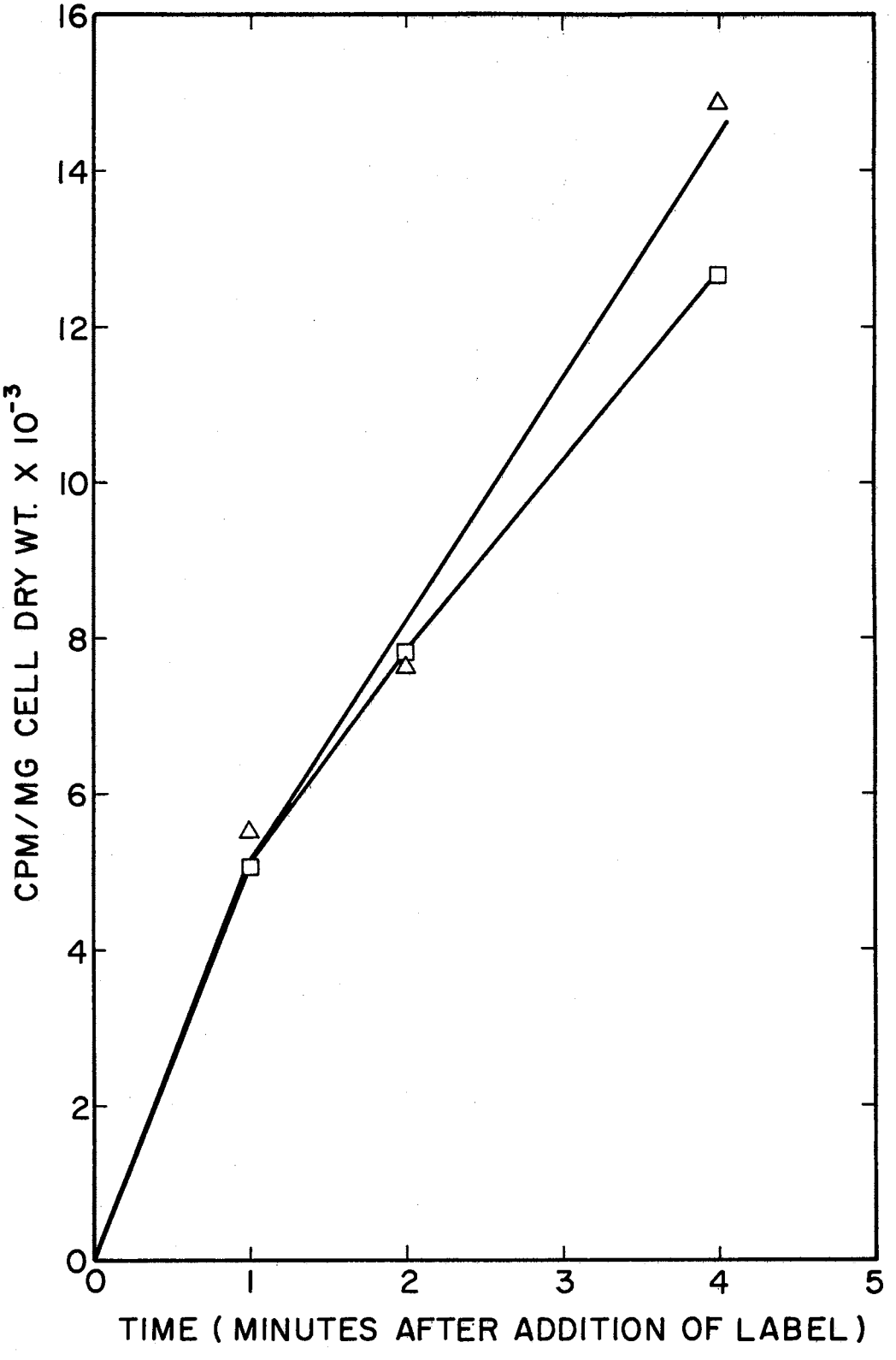
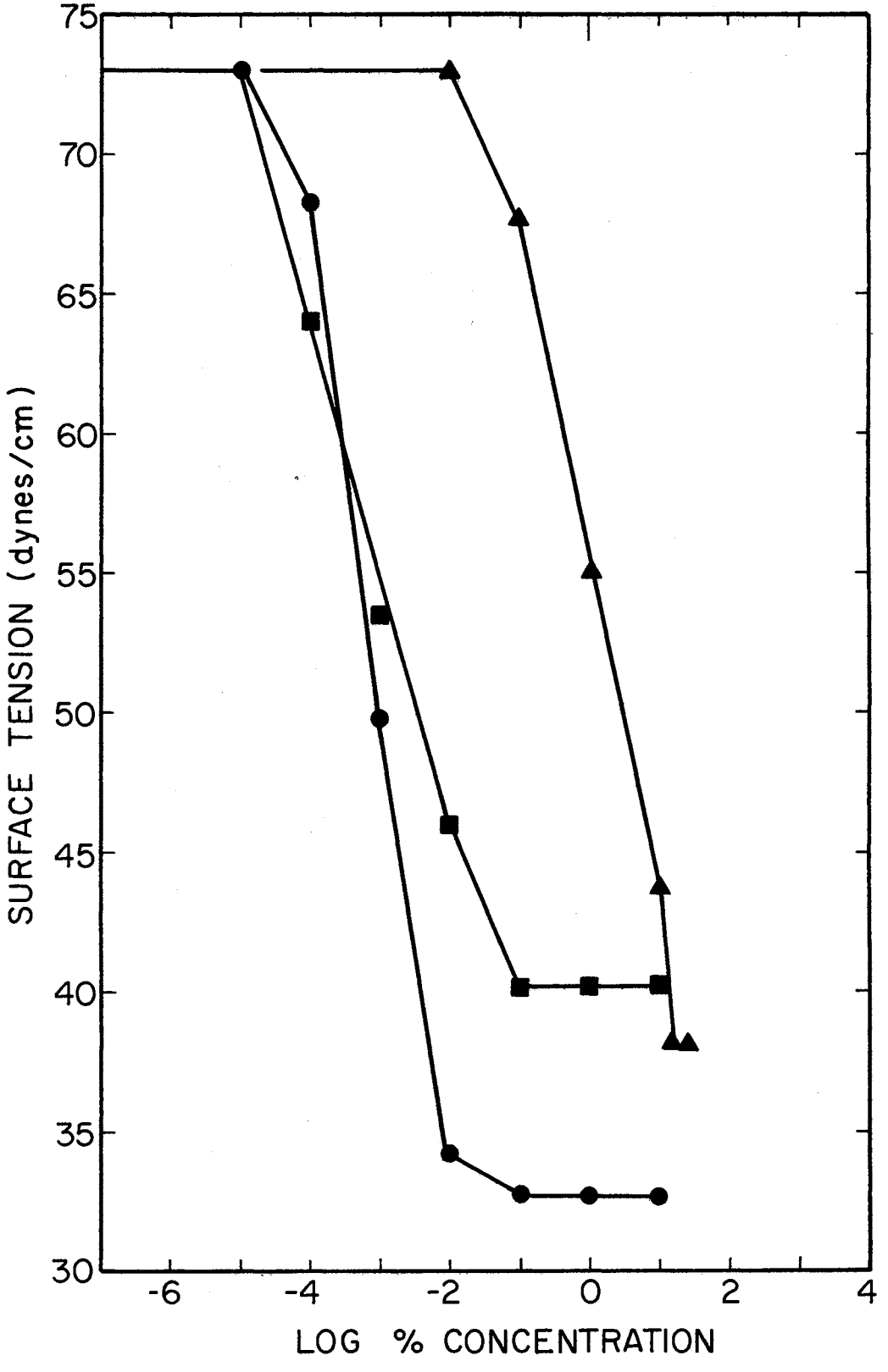

16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
CPM/MG CELL DRY WT. X 10^{-3}
TIME (MINUTES AFTER ADDITION OF LABEL)

Figure 20. Reduction in Surface Tension Caused by Sodium Lauryl Sulfate (SLS), Tween 80, and PL. ●, SLS; ■, Tween 80; ▲, PL.


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

Included in Fig. 20 are data relative to decrease in surface tension caused by an anionic detergent sodium lauryl sulfate (SLS) and a non-ionic detergent Tween 80. Compared to the detergents, much higher concentrations of PL are required to cause equivalent reductions in surface tension.

Data presented in Fig. 21, show that SLS at a concentration of 0.01% (33 dynes/cm) inhibits uptake activity approximately 55 percent, whereas Tween 80 at an equivalent surface tension value exhibits little if any effect on uptake activity of the cells for D-alanine. Since the molar concentration of Tween 80 cannot be calculated due to the impure nature of this detergent mixture, the effect of increasing concentrations of the mixture, as well as PL and SLS, was investigated. Results from this series of studies are shown in Fig. 22, wherein percent inhibition of uptake is plotted against surface tension. As the surface tension is lowered by both SLS and PL the percent inhibition of uptake increases to very near 90 percent. However, with Tween 80 no inhibition of uptake is detected even after the surface tension has been lowered to 40 dynes/cm. Since the concentration of PL required to lower surface tension to a value of 40 dynes/cm inhibits uptake of D-alanine approximately 85 percent, it appears that some effect other than a reduction of surface tension is responsible for inhibition of uptake activity by PL.

Ability of M. lysodeikticus to Transport Acetic Acid-2-^{14}C in the Presence of PL

Overath, et al. (1971) reported that only fatty acids with more than eight carbons are actively transported into E. coli. Data

Figure 21. Inhibition of D-Alanine-U-^{14}C Uptake Activity by Sodium Lauryl Sulfate (SLS), Tween 80, and PL. ○, control cells; ●, plus SLS (0.01%); ▲, plus Tween 80 (0.01%); ■, plus PL (0.01%).

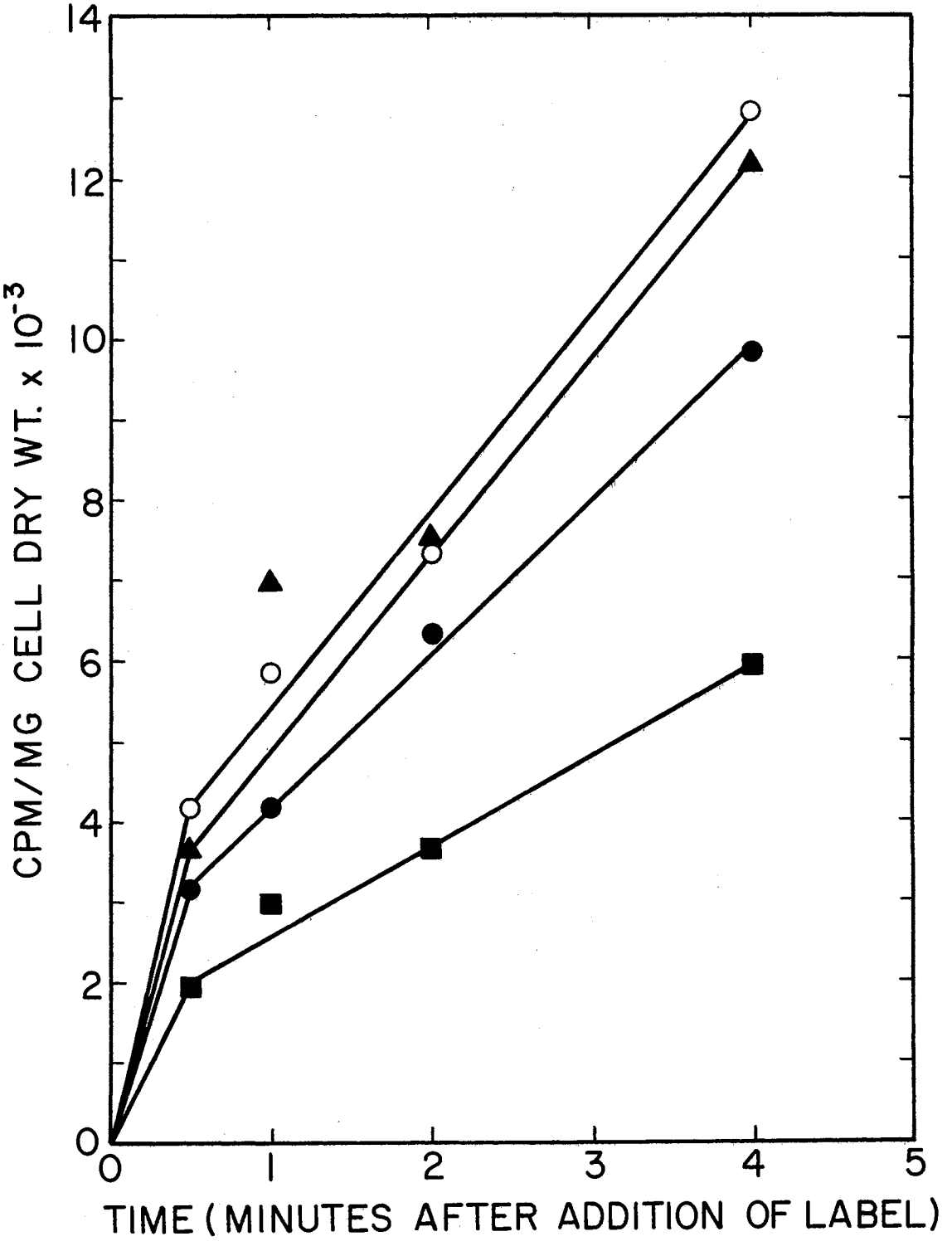
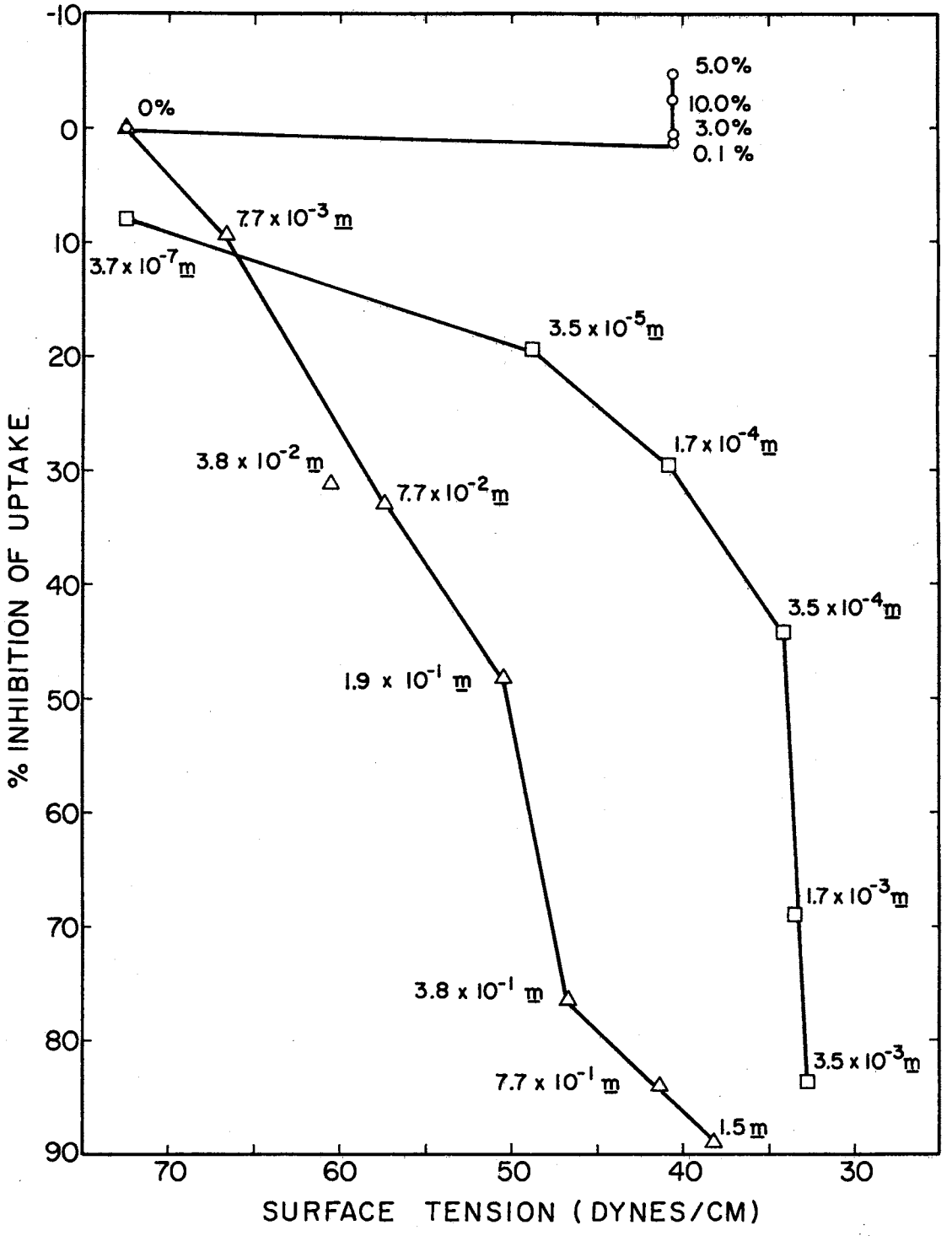

14
12
10
8
6
4
2
0
0
1
2
3
4
5
CPM/MG CELL DRY WT. x 10^{-3}
TIME (MINUTES AFTER ADDITION OF LABEL)

Figure 22. Percent Inhibition of D-Alanine-U-^{14}C Uptake Activity as a Function of PL Concentration, Sodium Lauryl Sulfate (SLS) Concentration, and Tween 80 Concentration and Surface Tension. △, PL; □, SLS; ○, Tween 80.


-10
0
10
20
30
40
50
60
70
80
90
% INHIBITION OF UPTAKE
70
60
50
40
30
SURFACE TENSION (DYNES/CM)
5.0%
10.0%
3.0%
0.1 %
0%
7.7 x 10-3 m
3.7 x 10-7 m
3.5 x 10-5 m
1.7 x 10-4 m
3.8 x 10-2 m
7.7 x 10-2 m
3.5 x 10-4 m
1.9 x 10-1 m
1.7 x 10-3 m
3.8 x 10-1 m
3.5 x 10-3 m
7.7 x 10-1 m
1.5 m

presented in Fig. 23 show that M. lysodeikticus is able to transport acetic acid by what appears to be an active type process (78 percent inhibition in the presence of 10^{-2}M DNP).

Also presented in Fig. 23 are data showing that PL may actually stimulate the uptake of acetate. This is the only compound tested, wherein an inhibition in uptake ability does not occur when PL is present. Also, if cells are grown in the presence of PL, they exhibit a much greater ability to transport acetate than cells grown in media without PL (Figure 24). Incorporation of PL into the uptake medium only slightly decreases uptake ability of such cells when acetate is the compound being transported. These data suggest that the transport system for acetate may be quite different; possibly located in an environment not common to the other compounds investigated.

Polyacrylamide Gel Electrophoresis

Polyacrylamide Gel Electrophoresis of Isolated Cell Membranes from Cells Grown at 37 C and 15 C

Data presented in Fig. 25 represent electropherogram patterns of proteins from membranes of cells grown at 37 C (shaking and standing cultures) and 15 C (standing cultures only). Cells grown at the lower temperature (15 C) have a greater ability to concentrate D-alanine than cells grown at 37 C under standing conditions (Fig. 10), and differences of a quantitative nature are apparent in the membrane proteins when these cells are compared. There is one band (starred) that is present in greater amounts in the cells grown at 15 C; however, it is

Figure 23. Influence of PL and 2,4-Dinitrophenol on the Ability of M. lysodeikticus to Transport Acetic Acid-2-^{14}C. ●, acetic acid-2-^{14}C; ○, acetic acid-2-^{14}C plus PL (0.44M); ■, acetic acid-2-^{14}C plus 2,4-dinitrophenol (10^{-2}M).

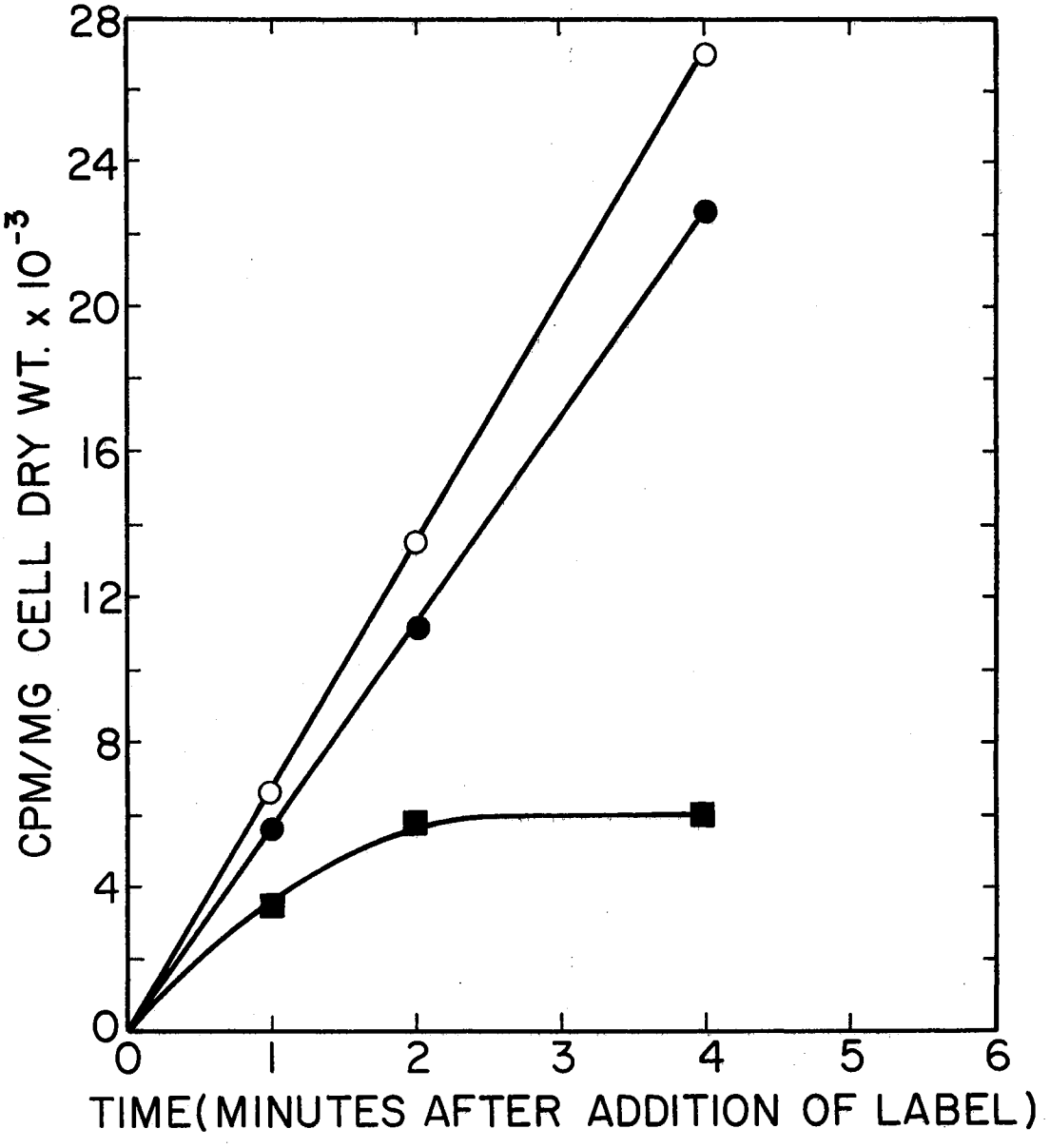

28
24
20
16
12
8
4
0
CPM/MG CELL DRY WT. x 10^{-3}
0
1
2
3
4
5
6
TIME(MINUTES AFTER ADDITION OF LABEL)

Figure 24. Influence of PL on Transport of Acetic Acid-2-^{14}C in Control Cells and PL Grown Cells. ○, control cells; △, cells grown in the presence of PL (0.22M) for 6 hours then washed twice with minimal salts and uptake ability determined; ●, control cells plus PL (0.22M); ▲, PL grown cells (6 hours) which were washed two times before readdition of PL (0.22M) to the uptake system.

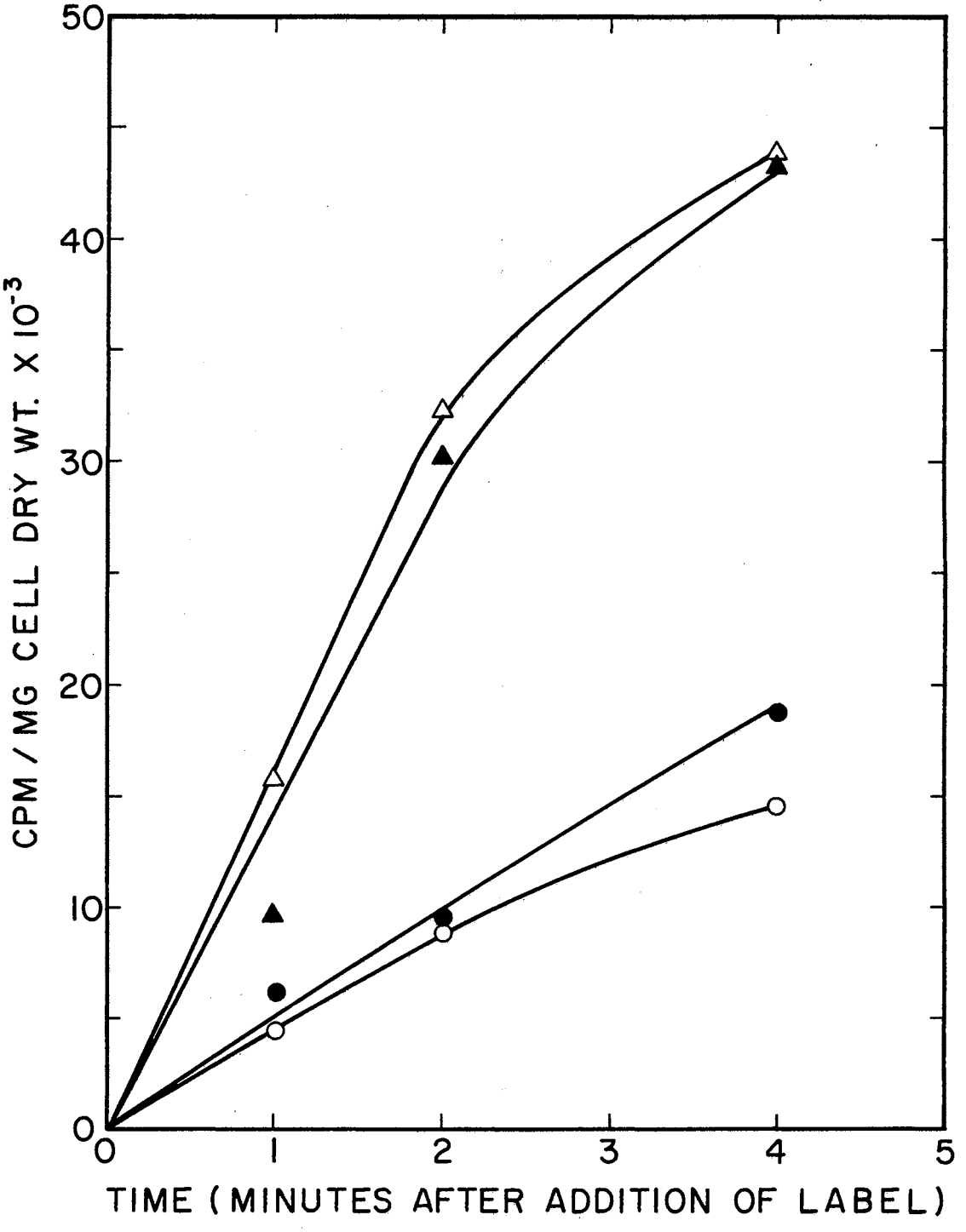
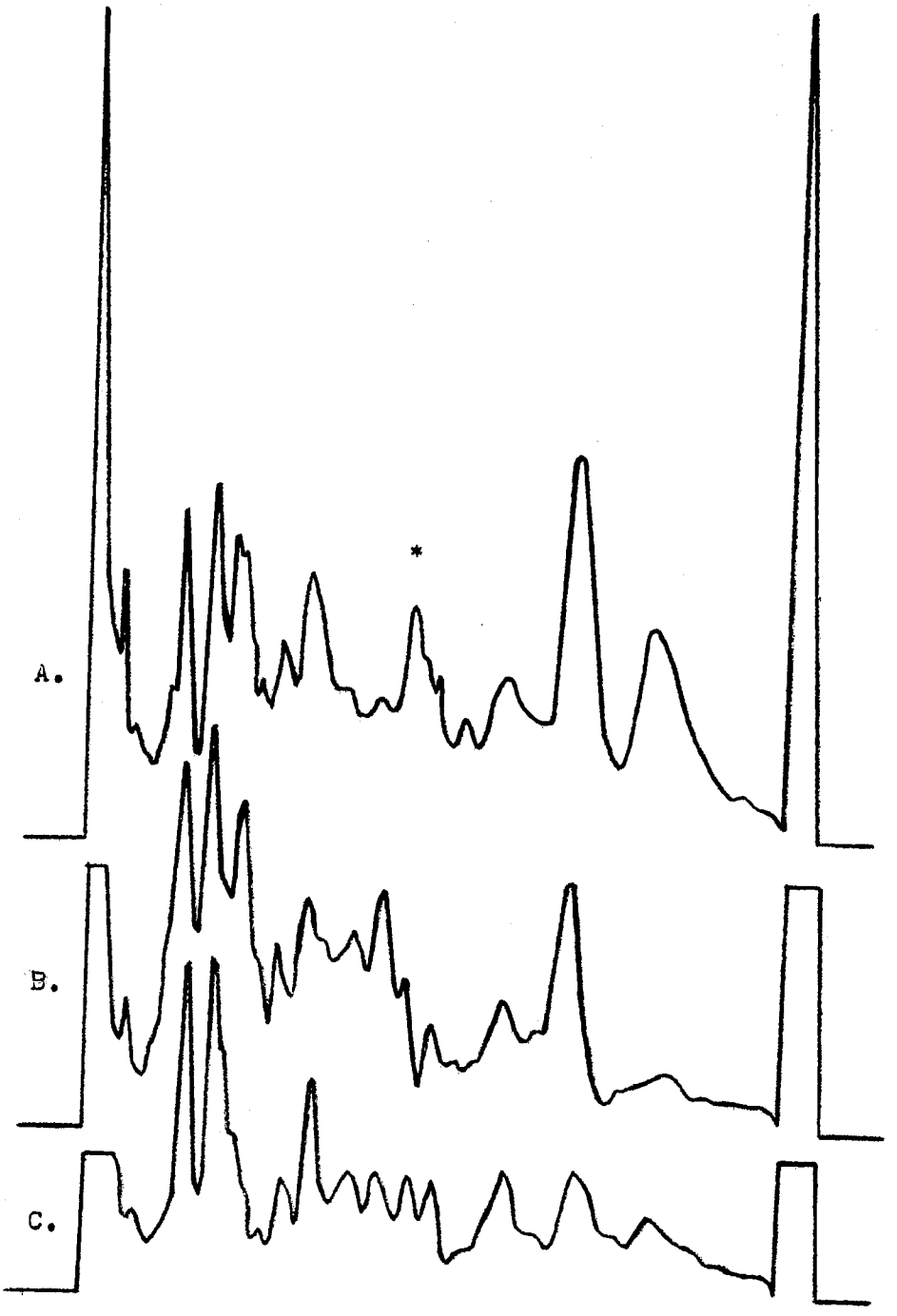

50
40
30
20
10
0
0
1
2
3
4
5
CPM / MG CELL DRY WT. X 10⁻³
TIME (MINUTES AFTER ADDITION OF LABEL)

Figure 25. Electropherogram Patterns (SLS gel system) of Isolated Membranes From Cells Grown at 37 C and 15 C.
A. Cells grown at 15 C under stationary conditions
B. Cells grown at 37 C under stationary conditions
C. Cells grown at 37 C under shaking conditions


A.
B.
C.

highly improbable that the protein(s) in this band is responsible for increased uptake ability of these cells since there is little or no increase in this band in cells grown under shaking conditions at 37 C. Although not shown, cells grown at 37 C with shaking have uptake ability which is equal to cells grown under stationary conditions at 15 C. Thus although it is entirely possible that increased uptake ability of cells grown at the lower temperature is due to increased synthesis of some critical protein(s), such synthesis could not be demonstrated utilizing the technique of polyacrylamide disc gel electrophoresis.

Fatty Acid Analyses

Fatty Acid Composition of M. lysodeikticus Whole Cells Grown Under Different Environmental Conditions

Cho and Salton (1966) reported that lipids in the membranes of aerobic Gram-positive bacteria contain a high proportion (over 50 percent) of branched-chain fatty acids. They further observed that the great majority of cellular lipids are localized exclusively in the membrane systems of Gram-positive organisms whereas these compounds are found in the "wall" or "envelope" fraction of Gram-negative bacteria.

Since it had been suggested that PL might be causing an alteration in membrane fluidity by associating with unsaturated fatty acids (Hopfer, 1972) a survey of the fatty acid composition of the lipids present in whole cells of M. lysodeikticus grown under different environmental conditions was performed.

The results of these analyses are shown in Table III. The data are in good agreement with those reported from other laboratories (Cho and Salton, 1966; Thorne and Kodicek, 1962). Both strains of M. lysodeikticus (dis-IIp$^+$ and Salton's strain) contain very large amounts of the C 15 branched-chain fatty acid. Although the quantitative differences in fatty acid composition between the two organisms are probably not significant, M. lysodeikticus dis-IIp$^+$ has a small quantity of one additional fatty acid which was not detected in Salton's strain (C 15).

A significant reduction in the percent of C 15:br(anteiso) fatty acid occurs when cells are grown in defined medium as opposed to cells grown on PWYE medium (14 percent reduction). This reduction is accompanied by an increase in several fatty acids /C 15:0, C 16:br, C 16:1, and C 17:br(anteiso)/. This quantitative variation in fatty acid composition is not surprising in view of the marked effect cultural conditions have on lipid composition in other organisms (Marr and Ingraham, 1962; Houtsmuller and VanDeenen, 1964).

Vorbeck and Marinetti (1965) reported that at least 95 percent of cellular phospholipids are found in the membrane fraction of Gram-positive bacteria and the remaining 5 percent, which are found in the cytoplasmic fraction, have a composition identical to membrane phospholipids. Data presented in Table IV reveal that the fatty acids extracted from whole cells and isolated cell membranes of M. lysodeikticus are very similar, if not identical. Because of this similarity, most data were obtained by extraction of whole cells. This decreased much of the tedium involved and permitted more extensive samplings to be made.

Many reports are available on the effect of environmental

TABLE III

FATTY ACID COMPOSITION OF LIPIDS EXTRACTED FROM WHOLE CELLS OF *MICROCOCCUS LYSODEIKTICUS*

Relative Retention Time**	Fatty Acid Designation	Average Percent Composition*		
		M. lysodeikticus dis-IIp+ Defined medium[a]	*M. lysodeikticus* dis-IIp+ PWYE Medium[b]	*M. lysodeikticus* (Salton's strain) PWYE medium
0.28	C 12:0	trace[c]	trace	trace
0.32	C 13:0	trace	trace	1
0.42	C 14:br	2	1	2
0.48	C 14:0	2	4	1
0.59	C 15:br(anteiso)	65	79	86
0.64	C 15:0	3	1	-
0.80	C 16:br	11	3	1
1.00	C 16:0	5	5	3
1.13	C 16:1	4	1	trace
1.24	C 17:br(anteiso)	6	4	4
1.57	A[d]	trace	trace	trace
1.81	C 18:0	trace	trace	trace
1.95	C 18:1	trace	trace	trace
2.27	C 19:br(anteiso)	trace	trace	trace

*The average number is based on two experimental determinations made at different times. The percentage of each acid was calculated after measurement of the total area under each peak (height of each peak times width at one-half peak height).

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

[a]Defined medium: as presented under Materials and Methods.

[b]PWYE medium: 1% w/v Bactopeptone, 0.1% w/v Difco yeast extract, 0.5% w/v NaCl, pH 7.5 (KOH).

[c]Percentages less than 1 are reported as trace amounts.

A[d]: unknown methyl ester.

All cell cultures were incubated at 30 C for 16 hours.

TABLE IV

A COMPARISON OF THE FATTY ACIDS EXTRACTED FROM WHOLE CELLS OR MEMBRANES OF MICROCOCCUS LYSODEIKTICUS dis-IIp+

Fatty Acid Designation	Percentage Fatty Acid Composition*	
	Whole Cells	Cell Membranes**
C 12:0	trace[a]	trace
C 13:0	trace	trace
C 14:br	2	2
C 14:0	3	3
C 15:br(anteiso)	61	60
C 15:0	3	4
C 16:br	11	15
C 16:0	8	6
C 16:1	6	5
C 17:br(anteiso)	5	1
A[b]	trace	trace
C 18:0	trace	1
C 18:1	trace	trace
C 19:br(anteiso)	trace	trace

*As given under Table III.

**Cell membranes were prepared as described under Materials and Methods.

[a]Percentages less than 1 are reported as trace amounts.

A[b]: unknown methyl ester.

All cell cultures were incubated at 37 C for 16 hours.

temperature on the fatty acid composition of bacterial lipids, showing that, in general, cells grown at low temperatures contain increased amounts of unsaturated fatty acids (Kates, 1964; Marr and Ingraham, 1962). Okuyama (1969) suggested that these changes enable cells in a cold environment to maintain fluid membranes since unsaturated fatty acids have lower melting points than saturated forms.

Although only a small percentage of unsaturated fatty acids are present in M. lysodeikticus, it appeared of interest to compare the fatty acid composition of cells grown at 15 C and 37 C. A slight increase in the two unsaturated fatty acids (C 16:1 and C 18:1) occurs when cells are grown at 15 C (Table V). Accompanying the increase in unsaturated fatty acids (at 15 C) is a decrease in percentage of C 14, C 14:br, and C 16:br. Although several differences in percent fatty acid composition are apparent at the two temperatures, the extent of each alteration is less than 5 percent, while the total of all the percent differences is only about 15 percent. Thus, it appears that only little change in percent fatty acid results when these cells are grown at 15 C as opposed to 37 C.

Because the fatty acid composition at the two different temperatures represent lipids extracted from cells grown under stationary conditions (non-shaking), data are also presented in Table V that represent aerated (shaking) cells, grown at 37 C. Again, there appears to be little overall difference in fatty acid composition between cells grown under stationary conditions and cells grown with shaking.

The possible involvement of phospholipids in the carrier-mediated transport of monosaccharides across erythrocyte membranes has been

TABLE V

THE EFFECT OF GROWTH TEMPERATURE ON THE FATTY ACID COMPOSITION OF LIPIDS FROM MICROCOCCUS LYSODEIKTICUS dis-IIp+

Fatty Acid Designation	Average Percent Composition* Cells Grown at 37 C (shaking)	Cells Grown at 37 C (stationary)	Cells Grown at 15 C (stationary)
C 12:0	trace	trace	trace
C 13:0	trace	1	trace
C 14:br	2	5	1
C 14:0	2	3	1
C 15:br(anteiso)	64	67	68
C 15:0	trace	trace	trace
C 16:br	12	7	4
C 16:0	7	7	7
C 16:1	1	trace	3
C 17:br(anteiso)	10	8	9
A	trace	trace	trace
C 18:0	trace	trace	2
C 18:1	trace	trace	1
C 19:br(anteiso)	trace	trace	trace

*The average number is based on two experimental determinations made at different times. All other conditions and calculations as given under Table III.

All cell cultures were harvested at an optical density of approximately 0.5.

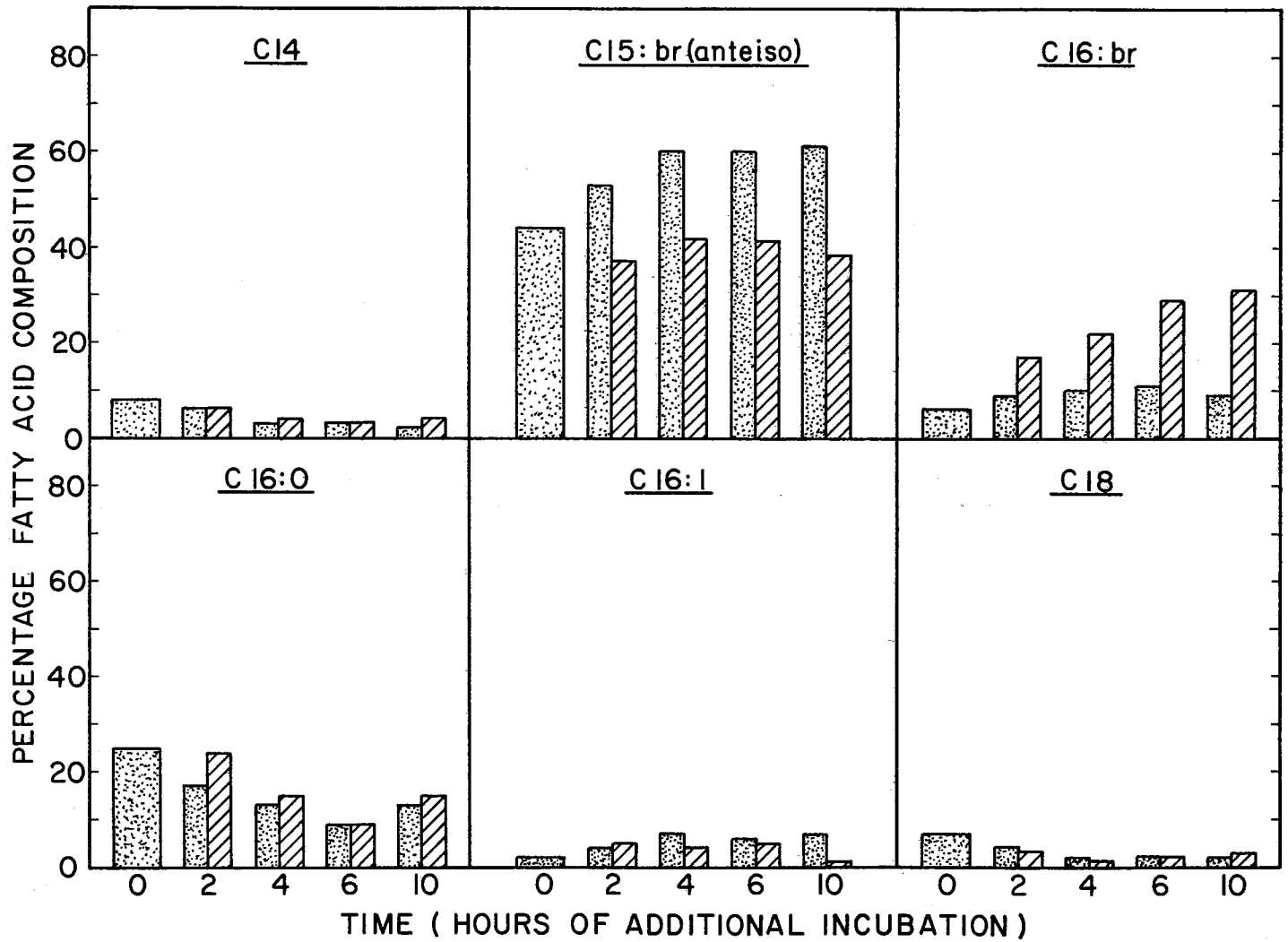
suggested by LeFevre et al. (1964) and Mawdsley and Widdas (1967), who found that phospholipids extracted from these membranes had the ability to bind various monosaccharides. Also data presented by Fox (1969) demonstrated that unsaturated fatty acids are required during the course of induction of the lac operon in E. coli to permit synthesis of a functional lactose transport system.

Since the transport system for various compounds in M. lysodeikticus is inhibited by PL, studies were initiated to determine what effect(s) PL has on fatty acid composition of lipids in M. lysodeikticus.

A shift in the percent of individual fatty acids occurs as normal (control) cells enter the logarithmic growth phase (Fig. 26). A gradual decrease in the percent of three fatty acids is shown: C 14, C 16, and C 18. This decrease appears to level off after about 18 hours at which time cells are entering the stationary growth phase. During this same time period an increase in the percentage of C 15:br (anteiso), C 16: br, and C 16:1 occurs.

When PL (0.22M) is present in the growth medium several alterations in fatty acid composition occur (Fig. 26). While there appears to be little difference in the percentage of saturated straight-chain fatty acids in normal and PL grown cells a significant difference can be seen in two branched-chain fatty acids. After 2 hours of incubation in the presence of PL the percent of C 15:br(anteiso) is only 37 percent of the total (53 percent in control cells). This level is maintained for the full incubation period (38 percent after 10 hours). The other branched-chain fatty acid affected by PL is designated C 16: br. It steadily increases in amount to a value of 31 percent after

Figure 26. Effects of PL on Fatty Acid Composition of Lipids in Micrococcus lysodeikticus. PL (0.22M) was added after twelve hours incubation at 37 C and percentage fatty acid composition of lipids was determined after 0, 2, 4, 6, and 10 hours additional incubation. Dotted bars represent control cells. Striped bars represent PL grown cells.


C14
C15: br(anteiso)
C 16: br
C 16:0
C 16:1
C 18
PERCENTAGE FATTY ACID COMPOSITION
0
20
40
60
80
0 2 4 6 10
TIME (HOURS OF ADDITIONAL INCUBATION)

10 hours. This is 22 percent above the value for control cells at this time and appears extremely significant.

Growth in the presence of PL can be termed "stress conditions" since amount of growth obtained is severly inhibited (Table VI). To determine if D-cycloserine (DCS), which inhibits growth as well as cell division, induces the same general type of effect, cells were grown in the presence of this compound for varying periods of time. Data which relate to growth and cell size are presented in Table VI. Although the effects of PL on growth are seen within 2 hours, inhibition of growth in the presence of DCS is not evident until 4 hours. At the end of this time, cells growing in the presence of DCS have begun to lyse (approximately 10 percent); however, lysis of cells never occurs in the presence of PL.

Data presented in Table VI also show that DCS-grown cells initiate enlargement (cells fail to divide) after 2 hours incubation as opposed to PL grown cells which never enlarge. By 10 hours, cells grown in medium containing DCS can be considered large (average size is 1.7 um which represents a volume increase of 9 times) and lysis of approximately 80 percent of the cells has occurred.

The effects of DCS on the fatty acid composition of membrane phospholipids occurs somewhere between the fourth and sixth hour of incubation since only slight changes can be measured in 4 hour cells; however definite changes can be seen 6 hours after addition of this cell wall and cell division inhibiting compound (Figure 27). After 6 hours, the percent of C 15:br(anteiso) is reduced by about 17 percent; however, by 10 hours the amount of this fatty acid is reduced 48 percent below the control value. The reduction in this C 15 branched-chain

TABLE VI

EFFECTS OF DCS AND PL ON GROWTH OF
MICROCOCCUS LYSODEIKTICUS

Time (Hours After Additions)	O. D.*			Percent lysis**		Av. Size (um)[a]	
	Control	DCS[b]	PL[b]	DCS	PL	DCS	PL
0	0.20	0.20	0.19	0	0	0.78	0.78
2	0.31	0.30	0.24	0	0	0.92	0.78
4	0.53	0.26	0.30	10	0	1.13	0.78
6	0.71	0.21	0.35	30	0	1.30	0.78
10	1.10	0.18	0.39	80	0	1.74	0.78

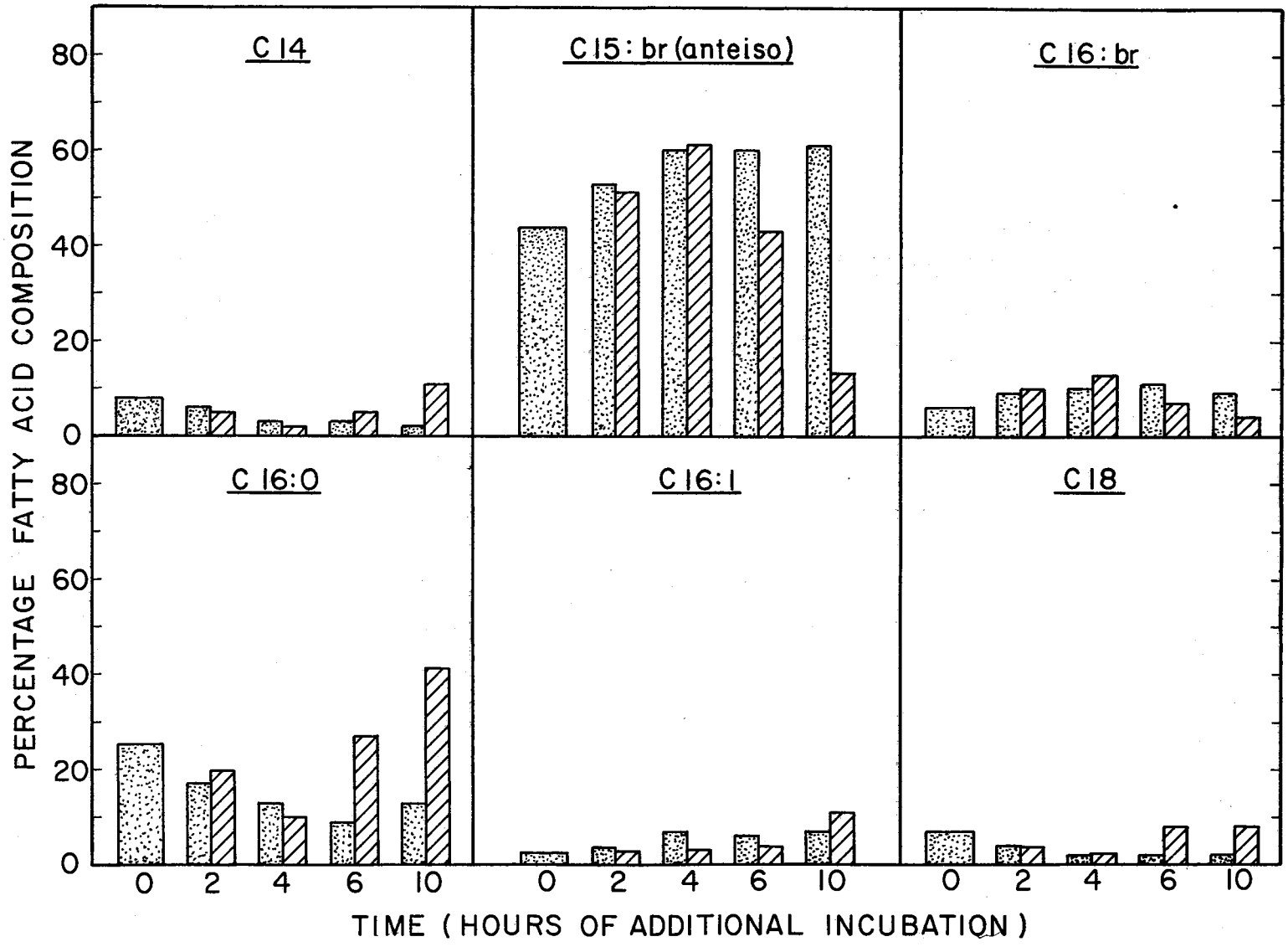
*O. D.: optical density measured at 540 nm.

**Percent lysis estimated by observation of cells using light microscopy.

[a]Cell size estimated by measurement of cells using electron microscopy.

[b]DCS: D-cycloserine, (2.0×10^{-4}M final concentration); PL, (0.22M final concentration)
Both compounds were added after incubation of cells for 12 hours at 37 C.

Figure 27. Effects of DCS on Fatty Acid Composition of Lipids in Micrococcus lysodeikticus. DCS (2×10^{-4} M) was added after twelve hours incubation at 37 C. Percentage fatty acid composition of lipids was determined after 0, 2, 4, 6, and 10 hours further incubation. Dotted bars represent control cells. Striped bars represent DCS grown cells.


C 14
C 15 : br (anteiso)
C 16 : br
C 16:0
C 16:1
C 18
PERCENTAGE FATTY ACID COMPOSITION
0
20
40
60
80
0 2 4 6 10
TIME (HOURS OF ADDITIONAL INCUBATION)

fatty acid is accompanied by an increase in four fatty acids, three of which are saturated straight-chain acids (C 14, C 16, and C 18) and one unsaturated fatty acid, C 16:1. The C 16 straight-chain fatty acid is increased to a much higher level than any of the other fatty acids (28 percent above the control value after 10 hours).

Lysis is always a problem and must be considered when a wall inhibiting compound such as DCS is present. At 6 hours, approximately 30 percent of the cells are lysed and this may have an effect on the percentage of fatty acids. This seems highly unlikely however since at 4 hours at least 10 percent of the cells are lysed and effects which appear to be due to DCS at this time are opposite those detected at 6 and 10 hours.

The effects of DCS and PL on the C 15:br(anteiso) fatty acid are of more than passing interest. This compound appears to be the "bellwether" fatty acid since it seems to undergo change and the large amounts present allow for ready detection of change. Amounts are decreased both by DCS and PL; however, the decrease is evident within 2 hours in cells grown in the presence of PL, but is not apparent until between the fourth and sixth hour when cells are grown in the presence of DCS. Further, in the presence of DCS, the amount continues to drop until a very low level is reached after 10 hours (from 60 to 13 percent of total lipid content); however, after the initial decrease caused by PL, which is small by comparison, further decreases are not evident. Also, cells grown in the presence of PL possess a significantly higher percentage of the C 16:br fatty acid whereas growth in the presence of DCS causes a significant increase in the level of the C 16:0 fatty acid. Thus, although both PL and DCS cause a decrease in

the amount of the C 15:br(anteiso) fatty acid, a different type of C 16 fatty acid is synthesized to compensate for this loss.

When lipids from cells grown in the presence of both PL and DCS (for 6 hours) are analyzed for their fatty acid composition the pattern obtained is very similar to cells grown only with PL present. Rather than a combination of the effects of PL and DCS occurring it appears that PL is able to prevent the alterations in fatty acid composition caused by DCS. However, after 10 hours of growth in the presence of both DCS and PL the effects of each compound are apparent.

CHAPTER IV

DISCUSSION

Pantoyl lactone (PL) has been shown to be an effective inhibitor of the D-alanine transport system in M. lysodeikticus dis-IIp$^+$. Inhibition in uptake ability occurs immediately after the addition of PL (within 30 seconds) and can be easily removed by aqueous washing of the cells. This effect of PL appears to depend on the lactone structure since pantoic acid has little or no deleterious effect on D-alanine uptake activity.

M. lysodeikticus is able to transport D-alanine by what appears to be an active process. A reduction in uptake occurs when cells are shocked by the procedure of Gale and Llewellin (1970) or when uptake activity is measured in the presence of 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation. Additional evidence for an active type process is the observation that uptake of D-alanine obeys saturation kinetics.

The direct energy donor and coupling mechanisms for the active transport of many metabolites across microbial membranes have yet to be clearly established. In the case of many sugar transport systems in bacteria, evidence has accumulated indicating that the high energy phosphate bond present in phosphoenolpyruvic acid drives the transport by way of a vectorial phosphorylation of the sugar (Roseman, 1969).

Other transport systems, those involving amino acids or lactose,

do not appear to require or utilize the phosphotransferase system. Recently it has been found that transport of these compounds is markedly stimulated, in cell-free bacterial membrane preparations (vesicles) from E. coli and B. subtilis, by D(-)lactate or succinate and the suggestion has been made that specific dehydrogenase activity in these preparations supplies the energy for transport via some mechanism other than the formation of high energy phosphate bonds (Kaback and Milner, 1970; Barnes and Kaback, 1970; Konings and Freese, 1972). Additional results reported by Konings and Freese (1972) indicate no involvement of a phosphorylated intermediate in such transport systems; however, the possibility of a rapid turnover of such a compound was not eliminated.

Although the energy-coupling mechanism for transport in aerobically grown cultures of organisms such as E. coli and B. subtilis seems to depend on dehydrogenase activity, the uptake of K^+ and cycloleucine in Streptococcus faecalis grown under microaerophilic conditions appears to be coupled to the activity of a membrane ATPase (Abrams, Smith, and Baron, 1972). It would appear therefore that organisms, such as S. faecalis, grown under conditions of limiting oxygen could require energy-coupling mechanisms for transport different from those utilized in aerobically grown organisms.

The drastic reduction in D-alanine uptake which occurs in M. lysodeikticus with the addition of DNP might be considered as evidence that the D-alanine transport carrier has become uncoupled from the electron transport chain; however, Konings and Freese (1972) have suggested that an additional effect of DNP may be to alter the permeability properties of the membrane.

Other metabolic inhibitors (cyanide, azide, arsenate, and iodoacetate) are also effective inhibitors of D-alanine uptake. The large degree of inhibition observed in the presence of cyanide and arsenate (65 percent and 44 percent inhibition respectively) suggests that both a high energy phosphate bond and electron transport are necessary for M. lysodeikticus to concentrate D-alanine. Since cyanide inhibits uptake ability to a greater degree than arsenate, it seems probable that the electron transport system is somewhat more important in coupling energy to D-alanine uptake than formation of high energy phosphate bonds.

When PL is added to the uptake system in the presence of DNP a further reduction in uptake results. This can be considered as indirect evidence that PL is altering the binding proteins for D-alanine or in some manner changing the permeability properties of the membrane, or both. This would be in agreement with data showing that saturation of D-alanine uptake occurs at a lower substrate concentration in the presence of PL. However, the additional reduction in uptake activity in the presence of DNP also might occur if PL were further uncoupling oxidative phosphorylation. This seems unlikely since the effective concentration of the metabolic inhibitors needed to cause an equivalent reduction in D-alanine uptake ability is much smaller than the concentration of PL required. Further evidence against PL acting as an inhibitor of energy generation by interfering with electron transport has been obtained in studies performed by Dr. Mary Grula (unpublished data). She has shown that in the presence of certain substrates, PL does not inhibit and may slightly stimulate oxygen uptake by cells of M. lysodeikticus.

One of the most significant attributes of the chemiosmotic hypothesis (Mitchell, 1966) is that it relates not only to oxidative phosphorylation, but to all of the many energetic and control functions associated with cellular membranes. In regard to transport, it could be predicted that both the major components of the protonmotive force, membrane potential and pH gradient, might be capable of driving the transport of ions and nutrients across membranes against their prevailing electrochemical gradients. A flux of cations might therefore be driven by the membrane potential (inside of the membrane becomes negative), while according to Niven, Jeacocke, and Hamilton (1973) anion transport might respond to the pH gradient through the action of a proton-symport (inside is alkaline).

A drastic reduction in uptake ability results when potassium is substituted for sodium in the uptake buffer of M. lysodeikticus or when uptake ability is measured in the absence of any ions (distilled water only). Thus, it would appear that sodium ions may be functioning in some type of exchange reaction to help concentrate D-alanine. Additional experiments, such as the monitoring of sodium ion efflux during uptake, will be necessary to resolve this problem.

As mentioned earlier the membrane potential may also be modified by varying the external pH. Addition of alkali could result in proton efflux and sodium influx with consequent increase in potential. Changing the pH from 6.0 to 7.0 results in an increase of D-alanine uptake activity; however, a further increase in pH to 7.8 reduces the ability of M. lysodeikticus to concentrate D-alanine (Figure 8). Therefore, it would appear that transport of D-alanine cannot be directly related to decreasing concentration either of H^+ or OH^- ions. It

should be cautioned that the binding protein(s) for D-alanine probably have different affinities for substrate at the various pH values; this would also result in altered uptake ability thus complicating any attempted interpretation. Whatever the cause for the variation in uptake activity, maximum uptake occurs at pH 7.0, and percent inhibition in the presence of PL is the same whether measured at pH 6.0, 7.0, or 7.8. Since a slightly acid pH does not stimulate transport of D-alanine, it is probable that movement of hydrogen protons through the cell membrane is not the driving force in uptake of this compound.

Because PL is able to inhibit the transport of a number of compounds (L-aspartic acid, L-phenylalanine, 2-deoxy-D-glucose, uracil, glycerol, pyruvate, and malic acid), it is highly unlikely that the inhibition is due to specific binding of PL to the active site of all the various binding proteins that could be involved (specific analog effect). Also the ease by which the effects of PL are removed by simple washing argues against a PL-protein interaction. Instead, it would appear that PL is causing some generalized alteration in membrane permeability. The observation that PL inhibits the uptake of 2-deoxy-D-glucose and pyruvate, which appear to be entering the cell by a passive diffusion process, supports this hypothesis.

A disruption in membrane permeability should result in loss of compounds through an efflux type mechanism. However, efflux experiments performed using two compounds (2-deoxy-D-glucose, a non-metabolizable compound, and D-alanine), showed that PL has a negative effect on efflux. These experiments help explain the observed increase in endogenous oxygen consumption which occurs in the presence of PL (Dr. M. Grula, unpublished). Oxygen uptake would be expected to continue as

long as substrates are present within the cell.

Experiments performed to determine whether PL is exerting its effects on uptake ability by altering a physical property of the uptake medium revealed that neither hypertonic conditions nor a lowering of surface tension inhibit transport of D-alanine.

It is extremely interesting that the uptake ability of M. lysodeikticus for acetate is not inhibited by PL. This observation tends to rule out a general type blockage of membrane "pores" by PL. Since acetate transport occurs by what appears to be an active process, the lack of inhibition in the presence of PL suggests that PL is also probably not influencing energy generating processes within the cell. It appears more likely that the transport proteins for acetate are located in some sort of different environment or one which is inaccessible to PL. It is also possible that transport of this particular molecule (in this organism) depends on a different set of membrane components and, for some unknown reason, these are not influenced by PL. The possibility that the transport of different molecules might depend on independent energy compartments also cannot be completely ruled out.

Several reports are available on the effect of temperature on the lipids of microorganisms showing that, in organisms grown at temperatures below their optimum membrane phospholipids contain higher proportions of unsaturated fatty acids than organisms grown at optimum temperatures (Pearson and Raper, 1927; Gaughran, 1947a; Marr and Ingraham, 1962). The converse effect of temperature, that organisms growing at temperatures above their optima have higher proportions of saturated acids and a lower degree of unsaturation, does not always apply (Bishop and Still, 1963b; Gaughran, 1947b).

Growing M. lysodeikticus at 15 C rather than 37 C results in little change in fatty acid composition. Thus, it would appear that there is not a direct relationship between fatty acid composition and growth temperature. Similar results were reported by Marr and Ingraham (1962) who demonstrated that growth of E. coli at a particular temperature does not always result in a unique fatty acid composition, since altering the nutrition or time of cell sampling, independent of temperature, also results in major changes in fatty acid composition.

A direct correlation between fatty acid composition of phospholipids and uptake of D-alanine in M. lysodeikticus does not appear to exist. Alterations (significant increase as shown in Fig. 10) in uptake ability can be observed in cells grown at a lower temperature (15 C), wherein only slight alterations in percentage of fatty acids occur. The converse, that is altering fatty acid composition of phospholipids without affecting uptake ability of M. lysodeikticus for D-alanine, is also true (cells grown in the presence of PL as shown in Fig. 3). Therefore, it appears that the transport ability of M. lysodeikticus for D-alanine probably is not directly related to a unique fatty acid composition within membrane phospholipids.

Data presented in Fig. 25 were obtained utilizing isolated membranes from cells grown at two temperatures (15 and 37 C) wherein changes in uptake ability were observed. The protein band patterns obtained were essentially the same from a qualitative standpoint. One band (molecular weight about 30,000), is significantly increased in cells grown at 15 C. The function of this and other proteins resolved utilizing this procedure are not known; however, it seems highly improbable that the increased uptake ability demonstrated by cells grown

at 15 C is a result of an increase in this particular protein band. Cells grown under shaking conditions (37 C) also are able to take up large amounts of D-alanine without a corresponding increase in this particular protein band.

Since it has not been possible to demonstrate any large and therefore possibly significant changes either in phospholipid fatty acids or proteins within the cell membrane which would account for the increased uptake ability of cells grown at 15 C, it is necessary to conclude that the increase probably occurs because of a more subtle type of change in these cells. Examples might be: (1) Increased synthesis of some small molecule specifically linked and required in transport such as ionophores, (2) Greater charging of the cell interior because of greater ability to maintain a higher intracellular Na^+ content, (3) Greater ability to synthesize components responsible for generation of energy, and (4) Some type of subtle change in association of those phospholipids and proteins involved in the active uptake process.

When phospholipids from cells grown in the presence of PL or DCS are analyzed for their fatty acid composition two significant changes can be seen. A significant reduction in the major fatty acid, C 15:br (anteiso), occurs in the presence of both compounds. However, in the presence of PL this reduction is accompanied by an increase in the fatty acid designated C 16:br (goes from 9 to 31 percent of the total), whereas with DCS an increase in the fatty acid designated C 16:0 (goes from 13 to 41 percent of the total) occurs.

Willecke and Pardee (1971) have recently shown that the methyl-branched portion of fatty acids in B. subtilis is derived from carbon skeltons of the three branched chain amino acids: valine, isoleucine,

and leucine. Oxidative decarboxylation of the corresponding 2-keto acids leads to the branched chain C_4 and C_5-coenzyme A derivatives, which are the "primer" molecules that are lengthened by successive addition of C_2 units derived from malonyl-CoA. Oxidative decarboxylation of 2-ketoisocaproate leads to formation of isovaleryl-CoA (as primer) and odd-numbered branched chain iso acid forms are produced (leucine serves as the precursor amino acid). Oxidative decarboxylation of 2-ketoisovalerate results in the production of isobutyryl-CoA as the primer molecule and even-numbered iso acid forms result (valine serves as the precursor amino acid). Synthesis of anteiso-branched odd-numbered fatty acids occurs by way of the 2-methylbutyryl CoA system which results from oxidative decarboxylation of 2-keto-3-methylvalerate with isoleucine serving as the precursor amino acid (Willecke and Pardee, 1971; Horning et al., 1961).

Evidence has also been reported by the above authors indicating that a single enzyme catalyzes the oxidative decarboxylation of all three branched chain 2-keto acids in B. subtilis. An inhibitory effect by PL or DCS on this decarboxylating enzyme could explain the decrease caused by both compounds in synthesis of the C 15:br(anteiso) fatty acid; however, the problem is more complex since a different C 16 fatty acid (branched- and straight-chain) is synthesized as a result of their presence. It appears more likely that both PL and DCS are exerting an influence on fatty acid composition by affecting synthesis of the primer fatty acids. This is the most likely possibility since chain elongation of all types of fatty acids occurs by way of 2-carbon additions involving the malonyl CoA system.

The metabolic basis for the shift from an odd-numbered branched

chain fatty acid to an even-numbered straight chain acid caused by DCS is not known. A reduction in the amount of available isoleucine would explain the decrease in C 15:br(anteiso) fatty acid; however, this would not explain the large increase in the C 16:0 form. For whatever reason the cell brings about this change it is worth noting that the greatest change in fatty acid composition is apparent after 10 hours of growth in the presence of DCS (Figure 27), at which time cells are very large (Table VI) and also quite old.

The changes effected in fatty acid composition by PL /C 15:br (anteiso) to C 16:br/ might possibly result because either more valine and/or 2-ketoisovaleric acid (would yield isobutyryl CoA after oxidative decarboxylation and reaction with CoA) is available.

As has been pointed out by Kodicek (1963), an ideal semipermeable membrane must have some means of remaining elastic and not becoming too rigid or brittle. This can be accomplished if the fatty acid chains in the phospholipids resist being packed very closely. Packing (or crystallization) of saturated forms of fatty acids is disrupted to varying degrees by short chain, branched chain (both iso and anteiso), cyclopropane, cis-forms and unsaturated fatty acids.

Since both DCS and PL induce shifts which result in decreased levels of the C 15:br(anteiso) fatty acid (to C 16:0 and C 16:br respectively), it is possible that such changes are beneficial and necessary to permit the cell to adapt itself to new requirements either of a more or less hostile environment. Increased synthesis of the C 16:0 fatty acid (DCS induced response) probably indicates that the bilayer portions of the cell membrane are more closely packed. Such a response could be beneficial and necessary because DCS causes a

decrease in synthesis of mucopeptide (King and Grula, 1972). This would bring about a closer association of the cell membrane with the external environment. The increased synthesis of C 16:br (PL induced response) could also indicate a general tightening of the cell membrane; most particularly if this fatty acid is of the iso rather than a anteiso form (data not available to make this distinction; however, no reports exist designating a possible primer molecule for an even-numbered branched anteiso fatty acid).

When uptake of D-alanine is measured in cells grown in the presence and absence of PL no measurable difference in activity is detected after washing cells free of PL. However, when PL is added back to the uptake system the percent inhibition in uptake by PL is significantly reduced (from 60 percent in control cells to 40 percent inhibition in PL grown cells as shown in Figure 3). This reduction in inhibition cannot be attributed to an increase in number of transport proteins since in the absence of PL, control cells and PL-grown cells possess equal ability to take up D-alanine (Figure 3). It appears instead that some adjustment in membrane phospholipids has occurred.

Because such a high concentration of PL is required to inhibit transport, it seems possible that some or all of the added PL could become partitioned into the phospholipid fatty acid residues within the bacterial membrane, thereby disrupting the natural state of the membrane lipids. Such a change in the physical state of the membrane phospholipids could result in a decreased permeability as well as uptake ability. Since iso-fatty acids in monolayer films have a smaller cross-sectional area than anteiso acids (Weitzel, Fretzdorff, and Heller, 1951) a shift from C 15:br(anteiso) to C 16:br(iso) could

result in less available space to accommodate PL, thereby reducing the effectiveness of added PL.

The decreased cross-sectional area (increased rigidity) caused by a shift from C 15:br(anteiso) to C 16:0 might also help explain why growth in the presence of DCS inhibits the division process. The ability of PL to prevent this change and increase synthesis of C 16:br fatty acid could be the basis for the prevention of division inhibition in M. lysodeikticus in the presence of DCS.

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