EFFECT OF PANTOYL LACTONE ON BACTERIAL TRANSPORT AND ENERGETICS

By

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TRANSPORT AND ENERGETICS

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CHAPTER I

INTRODUCTION

Pantoyl lactone (PL), first synthesized by Glassen in 1904, was brought to the attention of the biological community by R. J. Williams et al. (1933, 1938, 1939) who demonstrated that PL is a component of the hydrolysis products of pantothenic acid (Mitchell et al., 1940; Stiller et al., 1940b). Several biological systems were also capable of synthesizing their pantothenate requirements by utilizing PL as a substrate. These include yeast (Weiland and Moler, 1941; Kuhn and Weiland, 1942), Neurospora (Wagner et al., 1948), Clostridium speticum (Ryan et al., 1945), Brucella abortus (Altenbern and Ginoza, 1954), Escherichia coli (Kawachi, 1960), and Acetobacter suboxydans (Hall et al., 1945; Sarett and Cheldelin, 1945). However, some yeast are not able to grow by utilizing PL as a pantothenate precursor (Hartelus and Johnson, 1946) and alkaline hydrolysis of PL to pantoic acid will increase its growth stimulating activity for some organisms (Sarett and Cheldelin, 1945; Stansly and Schlosser, 1945).

In view of these data it has been suggested that PL is not a natural intermediate in pantothenic acid anabolism

(Stansly and Schlosser, 1945). However, the ability to utilize exogenous PL in pantothenate synthesis is fairly widespread among microorganisms and PL has a fairly wide distribution in nature. In addition to being a hydrolytic breakdown product of pantothenic acid (Williams et al., 1933, 1938, 1939; Mitchell et al., 1940; Stiller et al., 1940a), which is ubiquitously distributed in nature, PL is believed to be in part responsible for the aroma of sherry (Webb et al., 1967), tobacco (Fujimore et al., 1976; Lloyd et al., 1976), cigarette smoke (Schumacher et al., 1977), molasses (Ito, 1976), cooked meat (Flament et al., 1978), and soy hydrolyzate (Liardon and Philipossian, 1978).

PL utilization as a growth factor has not been demonstrated in any vertebrates. Conversely, PL shows many inhibitory activities in these systems. The d-isomer produces convulsions or related symptoms in rats, pigeons, rabbit ears and dog heart (Chan et al., 1960; Benda and Peres, 1961) and the l-isomer elicits aggressive behavior from rats (Chan et al., 1960). The acute intra-peritoneal LD_{50} of PL for mice is 0.59 g/kg (Dorofeev, 1975). PL is apparently not metabolized by man (Sarett, 1945) but will inhibit K^+ transport into cold stored human erythrocytes (Kahn and Cohen, 1957). In rabbits maintained on a high cholesterol diet, PL (185 mg/kg/6 days) lowered serum cholesterol levels.

One of the most intriguing aspects of PL's physiological effects is its abililty to induce cell

division in division-inhibited bacteria (Grula and Grula, 1962; Adler and Hardigree, 1965; 1972; Swenson et al., 1972; Kirby et al., 1972). Division inhibition and subsequent reversion to normal sized cells is a complex and much studied phenomenon. Many investigators (see Grula and Grula, 1964) have demonstrated that division inhibition by a wide variety of compounds results in reduced levels of mucopeptide in the cell walls of the affected cells. However, reversion to normal sized cells initiated by an equally diverse group of compounds does not result in increased levels of mucopeptide incorporation (Grula and Grula, 1964; King and Grula, 1972). The cell membranes of division-inhibited cells are also altered. Alterations include proteins (Grula and King, 1971), density (Grula and King, 1972), and phospholipids (Johnson and Grula, 1980). These cells also leak periplasmic proteins and phospholipids (Grula and Hopfer, 1972; Johnson, 1978). Treatment with PL will prevent or reverse many of these effects. However, growth in the presence of PL induces further alterations in the phospholipids (Johnson et al., 1980).

Several hypotheses have been advanced to explain the effect PL has upon division. The Grula group has favored explanations that implicate the cell membrane as being central to division with PL exerting its effects through this medium (Grula and Grula, 1962; Grula and Grula, 1964; Grula and King, 1971; Johnson and Grula, 1980). Yoshiyama (1972) suggests that PL induces filaments to divide by the

selective inhibition of the initiation of a new round of DNA synthesis. Others (Van de Putt et al., 1963; Adler and Hardigree, 1965) suggest that PL stimulates septum formation. Clearly, confusion between cause and effect has resulted in these divergent models.

One way to distinguish between cause and effect is to determine the time course of the various reactions being considered. Alterations in membrane phospholipids and melting point are slow. Generations are required before PL exerts its full effect (Johnson et al., 1980). Therefore these changes probably represent an adaptation to PL. This is supported by the fact that one of the immediate effects of PL is a 'tightening' of the membrane (Grula and King, 1971; Johnson et al., 1979) and phospholipid changes observed result in a more fluid membrane thereby counterbalancing the tightening due to PL. Another immediate effect of PL is transport inhibition (Grula and King, 1971).

Since one of the objectives of this work was to describe a mechanism for the inhibition of transport, a review of the current concepts of active transport is included.

Classically, transport is divided into three major categories; simple diffusion, facilitated diffusion and active transport. Of these, only simple diffusion is not mediated by a membrane-bound permease and is therefore the only one that does not demonstrate saturation kinetics. Substrates transported in this manner are lipid soluble and are not accumulated against a concentration gradient in a freely

soluble form.

Facilitated diffusion does not result in movement of a solute against its concentration gradient either. Hallmarks of this system are rates of transport across the hydrophobic membrane that are greater than would be predicted by simple diffusion, susceptibility to mutation, stereospecificity, and, as already mentioned, saturation kinetics.

The last category is distinguished by solutes that are accumulated against their concentration gradient and require energy. Active transport is divided into two types based upon whether or not the solute is chemically modified during transport. Those that are modified are accumulated by group translocation and those that are not modified are accumulated by active transport. This results in some confusion; therefore, alternative terminology is being adopted.

Mitchell's (1963) terminology starts from the concept of vectorial metabolism. Simply speaking, vectorial metabolism results from enzymes embedded in an impermeable membrane that catalyze the cleavage of a substrate with the subsequent separation of the resulting products across the membrane. As an example, when water is the substrate a H^+ ion is extruded and an OH^- ion is retained resulting in the generation of a membrane potential that is both alkaline and electrically negative inside. This potential is mathematically expressed by equation 1.

$$\Delta_{\mu}H^{+} = \Delta\Psi - 2.3 \frac{RT}{F} \Delta pH \quad (1)$$

Where μH^{+} is the proton motive force in millivolts and is a measure of the combined electrical and chemical forces acting on the protons; $\Delta\Psi$ is the electrical potential difference across the membrane; R is the gas constant, T is temperature in degrees Kelvin; F is the Faraday constant and ΔpH is the pH difference that exists across the membrane.

Mitchell's terminology divides active transport into two major groups--primary translocations and secondary translocations. Primary translocations are directly linked to a biochemical reaction and may be of two kinds--group translocation and enzyme-linked solute translocation. In the latter class, the substrate is not altered as the result of the biochemical reaction but is obligately translocated as a result. H^{+}- and Na^{+}- pumping ATPases are examples of enzyme-linked solute translocators. Secondary translocators are not directly coupled to a biochemical reaction and are of three basic types: uniport, in which only the solute interacts with the permease corresponding to facilitated diffusion; symport or co-transport, in which the translocation of one solute is linked to the translocation of a second solute in the same direction; and antiport, in which the translocation of two solutes is linked but each moves in opposite directions.

In this thesis, group translocation and facililtated diffusion are used as described and the term active

transport refers to processes that are catalyzed by chemiosmotic symport mechanisms.

CHAPTER II

MATERIALS AND METHODS

Test Organisms and Growth Media

The two organisms used during the course of this study were *Micrococcus lysodeikticus* dis-IIp$^+$ and *Streptococcus faecalis*. *M. lysodeikticus* is a highly aerobic bacterium and its ATPase is isolated in a cryptic form (Carreira et al., 1975) while *S. faecalis* is a strictly fermentative organism. The ATPase of *S. faecalis* is not cryptic, ie. it is not isolated in a form that is stimulated by trypsin treatment (Abrams and Baron, 1967; Schnebli and Abrams, 1970; Schnebli et al., 1970). These facts lend credence to the hypothesis that *M. lysodeikticus* ATPase is physiologically employed in the synthesis of ATP utilizing the membrane potential formed by electron transport for energy while the ATPase of *S. faecalis* operates primarily in the reverse direction, hydrolyzing ATP produced by substrate level phosphorylation to generate the membrane potential.

Both organisms were maintained on trypticase-soy agar slants (TSA, Difco). *M. lysodeikticus* was grown in a defined medium (Grula et al., 1961) for transport studies

while S. faecalis was transferred to trypticase soy broth (TSB, Difco) for transport experiments. S. faecalis was cultured in 1% glucose, 1% tryptone (Difco), 0.5% yeast extract (Difco) and 1% K_2HPO_4 (Abrams et al., 1974) for ATPase isolations.

Uptake Studies

Cells cultured in their respective media were harvested by centrifugation (5,000 x g, 10 min), washed twice in uptake buffer (salts of the M. lysodeikticus defined medium) and suspended to an absorbance of 0.5 at 540 nm (A_{540} = 0.5). This suspension was allowed to equilibrate at room temperature for 30 min. At this time 2.3 ml samples of the cell suspension were transferred to 30 x 180 test tubes and shaken on a Burrell wrist action shaker. Pantoyl lactone was added 30 s prior to the addition of radioactive substrates. PL and substrate additions totaled 0.7 ml to bring the total volume to 3 ml. Samples (0.5 ml) were taken at 0.5, 1.0, 2.0, and 3.0 min, vacuum filtered (13 mm diameter, 0.45μm pore size Millipore nitrocellulose filters), and washed 3 times with cold uptake buffer. The filter pads were then placed in 10 ml of scintillation cocktail (Aquasol, Amersham) and left overnight to dissolve the filter pad. The vials were then counted and the results reported as counts per min (CPM) or converted to disintegrations per min (DPM) or nmoles substrate accumulated.

Isolation of ATPases

Solubilized ATPase from S. faecalis was isolated by the method of Abrams et al. (1974). Isolation of membrane bound ATPase utilized the same procedure except that the low ionic strength osmotic washes were omitted. Membranes isolated in this manner were resuspended in 10 mM $MgCl_2$, 0.1% p-amino-benzamidine, 10 mM TRIS-HCl pH 7.5, centrifuged at 50,000 x g for 30 min at 4° C, frozen, lyophilized and stored at -20° C. The lyophilized membranes were resuspended in 10 mM $MgCl_2$, 10 mM TRIS-HCl pH 7.5, and sonicated to optical clarity with 15 s bursts in a bath-type sonicator (Laboratory Supplies, Inc.). The suspension was then centrifuged at 20,000 x g for 15 min at 4° C and resuspended in the same buffer.

Porcine cerebral cortex Na^+/K^+-activated ATPase (PCA), Na^+/K^+-activated dog kidney ATPase, apyrase and firefly luciferin-luciferase (L-lase) were purchased from Sigma Chemical Co. PCA and apyrase were solubilized in 10 mM $MgCl_2$, 30 mM HEPES pH 7.5 and used as such. L-Lase was suspended in the same buffer then centrifuged at 35,000 x g for 15 min at 4° C. The supernatant was layered onto a 1.5 x 7 cm Sephadex G-75 column and eluted with the same buffer. Five ml fractions were collected. Luciferase activity was near the void volume (fraction 3). Fractions containing luciferin (5 and 6) were pooled and used for assays.

Turkey heart mitochondria (THM, Chance and Hagihara,

1963) and electron transport particles (ETP) from beef heart mitochondria (Crane et al., 1956) were the generous gift of Dr. H. J. Harmon, Oklahoma State University, Department of Physics, Stillwater, Oklahoma.

ATPase Assay

ATPase activity was monitored by measuring the inorganic phosphate liberated using the method of Adolphsen and Moudrianakas (1971). Buffers and ATP concentrations were varied according to the experiment and are given in the legends to figures and tables.

Measurement of Proton Pumping and Respiration

Proton extrusion and respiration were measured by the method of Wickstrom and Krab, 1978; Wickstrom, 1977). Respiration was monitored using a Clark type electrode amplified by a Johnson Foundation oxygen meter. The output was recorded on a Linear flat bed stripchart recorder. Respiration was driven by malate + glutamate and started by the addition of 2.5 mM ADP. Proton pumping was monitored using a Corning combination electrode and pH meter. Output was recorded on a Linear flat bead strip-chart recorder. The instrument was calibrated and changes in buffering capacity were monitored by the addition of 2µl of 0.100 N HCl following each experiment. Proton extrusion was driven by the addition of 2.04 mM ATP in the presence of 81.8 mM

TRIS-sulfate pH 7.1. Where indicated, oligomycin was present at 0.163 mM.

Luciferin-Luciferase Assay

ATP-dependent light production was measured in a Lab-Line ATP photometer in peak height mode and set for a 6 s interval. The sensitivity setting was 300 and the zero setting was 180.

Protein Determination

Protein was estimated by the method of Lowry et al. (1951).

Chemicals

Carbonylcyanide-m-chlorophenylhydrazone (CCCP) and dicyclohexylcarbodiimide (DCCD) were the gifts of Drs. H. J. Harmon and R. Essenberg respectively. All other chemicals were reagent grade and commercially available.

CHAPTER III

RESULTS

Studies on Transport

Pantoyl lactone inhibits the transport of D-alanine, L-aspartate, L-glutamate, and glycerol in both division-inhibited and normal cells. (Grula and King, 1971; Johnson and Grula, 1980; Johnson et al., 1980). This inhibition is over and above the reduced rates of transport reported in filamentous cells (Grula et al., 1968). PL also inhibits K^+ accumulation by cold-stored human erythrocytes (Kahn and Cohen, 1957). Figures 1-3 confirm that PL inhibits D-alanine, L-aspartate, and L-glutamate transport in normal M. lysodeikticus cells. The pH profile of aspartate transport is given in data presented in Figure 4. The data are similar to those for filament induction. D-Glucose transport is also inhibited (Fig. 5) even though D-glucose is a poorly utilized substrate and is not accumulated at the same rate as the amino acids. D-Glucose transport demonstrates saturation kinetics (Fig. 5), indicating that M. lysodeikticus possesses a functioning D-glucose permease even though it is a poorly utilized substrate.

A physical interaction with the membrane has been

Figure 1. Effect of pantoyl lactone on D-alanine transport by M. lysodeikticus (dose response curve). D-Alanine (232 Ci/mole) was present at 31.3 uM.

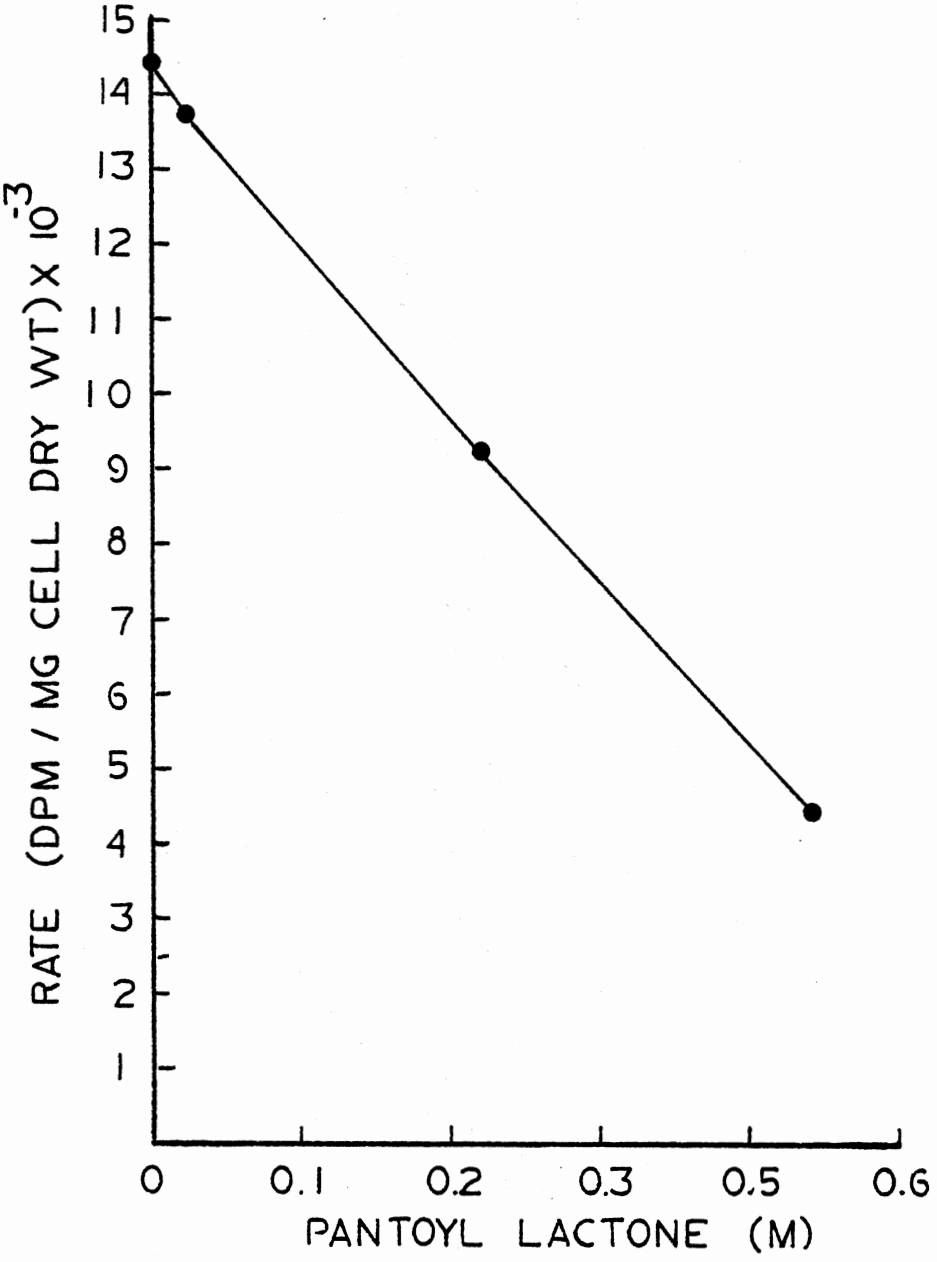

RATE (DPM / MG CELL DRY WT) X 10^{-3}
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0.1
0.2
0.3
0.5
0.6
PANTOYL LACTONE (M)

Figure 2. Effect of pantoyl lactone on L- aspartate transport by M. lysodeikticus (initial velocity curve). L-aspartate (232 Ci/mole). ○ , control cells; ● 0.22 M Pl.

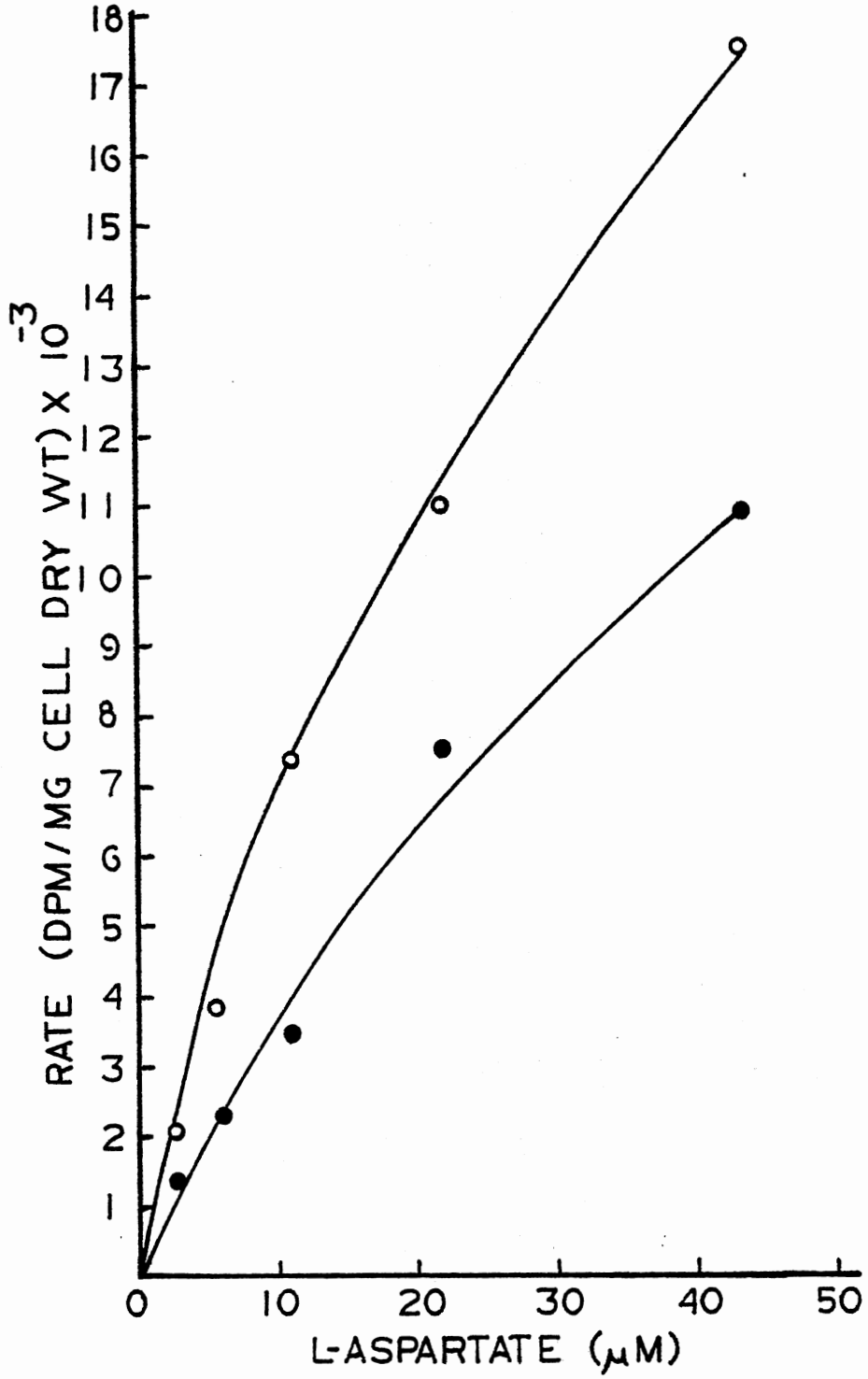

RATE (DPM/MG CELL DRY WT) X 10^{-3}
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
10
20
30
40
50
L-ASPARTATE (μM)

Figure 3. Effect of pantoyl lactone on L-glutamate transport by M. lysodeikticus (inital velocity curve). O , control cells; ● , 0.22 M Pl.

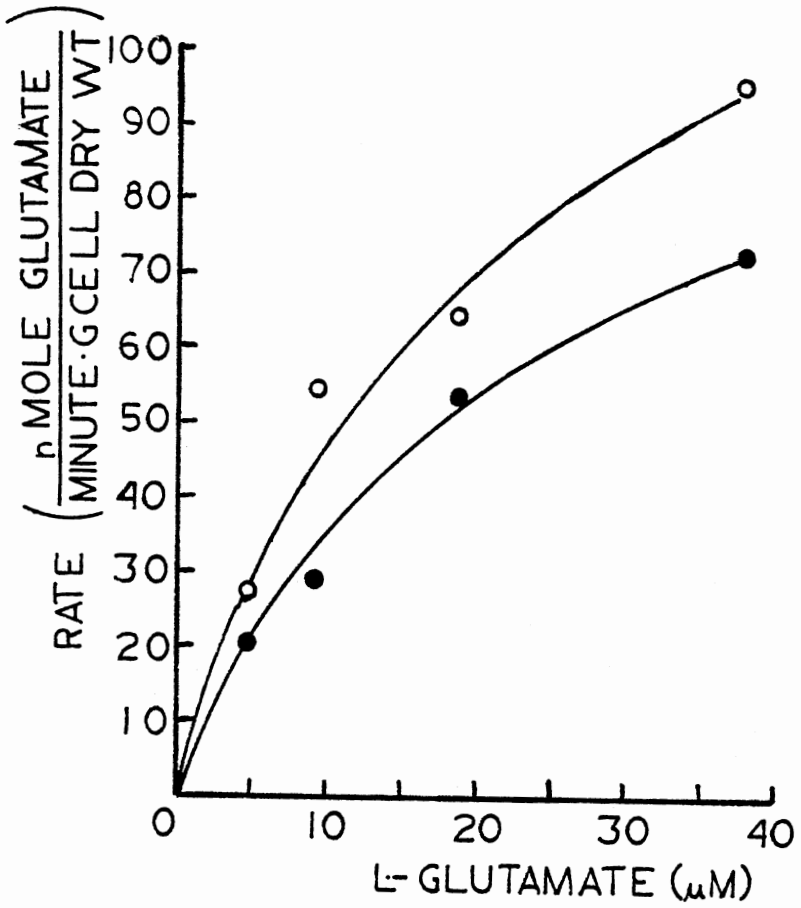

RATE (n MOLE GLUTAMATE / MINUTE·G CELL DRY WT)
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
L- GLUTAMATE (μM)

Figure 4. Effect of pH on L-aspartate transport by M. lysodeikticus. L-Aspartate (232 Ci/mole) was present at 50μM.

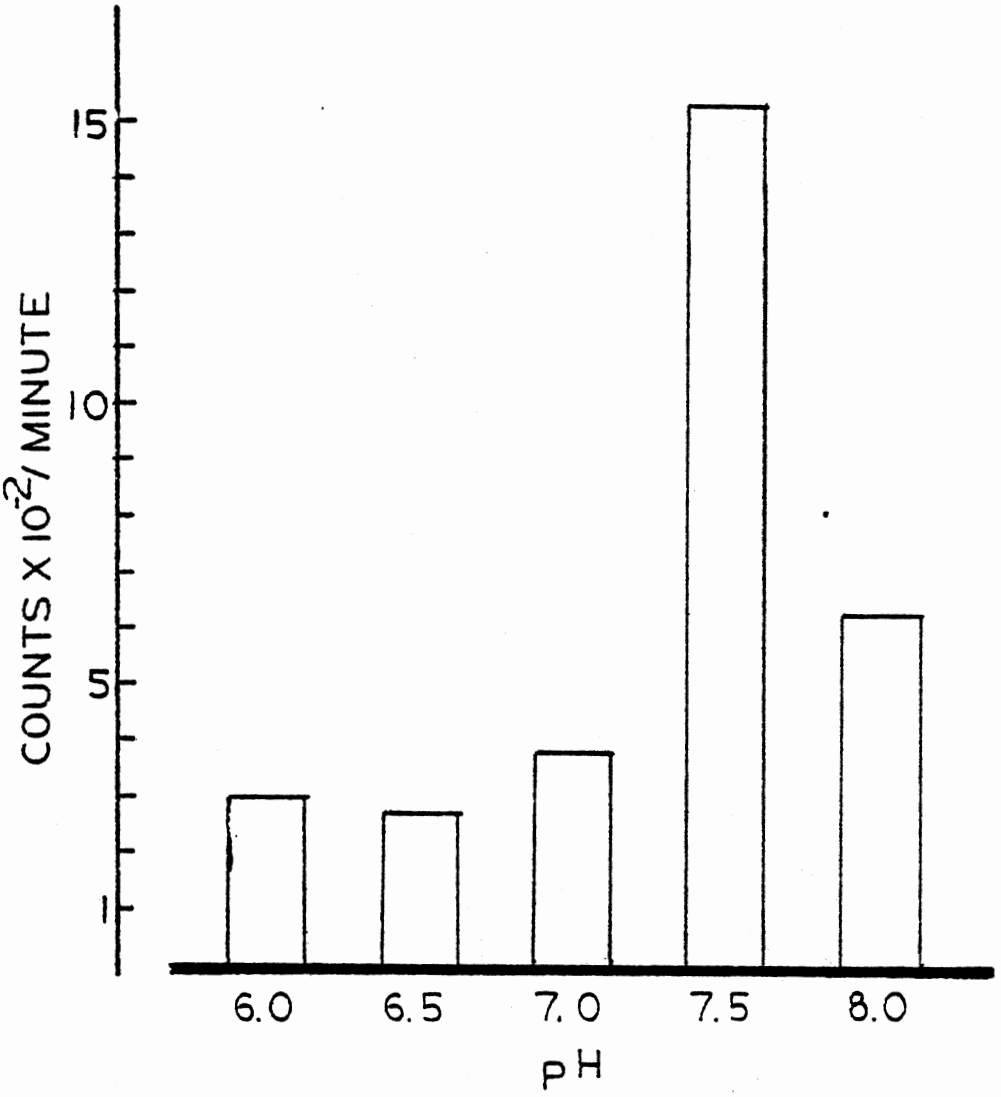
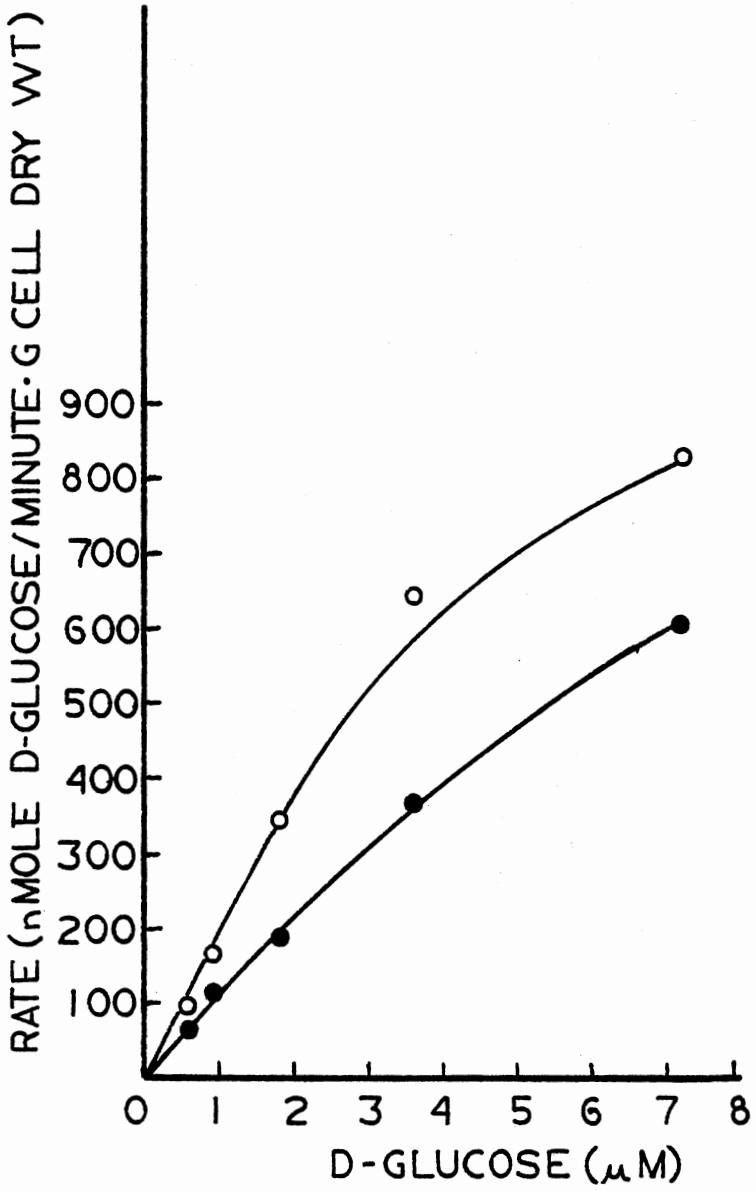

COUNTS X 10^{-2}/ MINUTE
15
10
5
1
6.0
6.5
7.0
7.5
8.0
pH

Figure 5. Effect of pantoyl lactone on D-glucose transport by M. lysodeikticus (initial velocity curve). O, control cells; ●, 0.22 M PL.


RATE (nMOLE D-GLUCOSE/MINUTE·G CELL DRY WT)
900
800
700
600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
D-GLUCOSE (μM)

proposed (Johnson et al., 1980) since it was deemed unlikely that PL could be conpetitively interacting with so many diverse transport systems. Further studies on M. lysodeikticus transport indicate that PL is a non-competitive inhibitor of D-alanine, L-aspartate, L-glutamate and D-glucose transport (Figs. 6-9). These data lend credence to the physical interaction hypothesis proposed by Johnson et al.(1978), especially since examples of three of the four major transport mechanisms are inhibited. Glycerol uptake (Grula and King, 1971) is considered an example of simple diffusion whereas D-glucose transport appears to occur via facilitated diffusion (Fig. 5). The amino acids tested are accumulated by active transport.

Since S. faecalis was to be used in studying the effect of PL on bacterial energetics, the effect on active transport was first determined since it is a sensitive indicator of this physiological activity. PL inhibits the transport of L-aspartate and L-glutamate but not that of D-glucose into starved S. faecalis cells (Figs. 10-12) In these cells, glucose accumulation is considered to be a group translocation process (Harold, 1972). This may be the reason S. faecalis is relatively resistant to PL concentrations up to at least 0.48 M (Fig. 13). However, L-aspartate and L-glutamate transport seem to be non-competitively inhibited by PL (Figs. 14-15). These data indicate that PL can have a differential effect on transport depending upon how the process is energized.

Figure 6. Effect of pantoyl lactone on D-alanine transport by M. lysodeikticus (double reciprocal plot). O, control cells; ▲, 0.02 M PL; ●, 0.22 M PL; ■, 0.44 M PL.

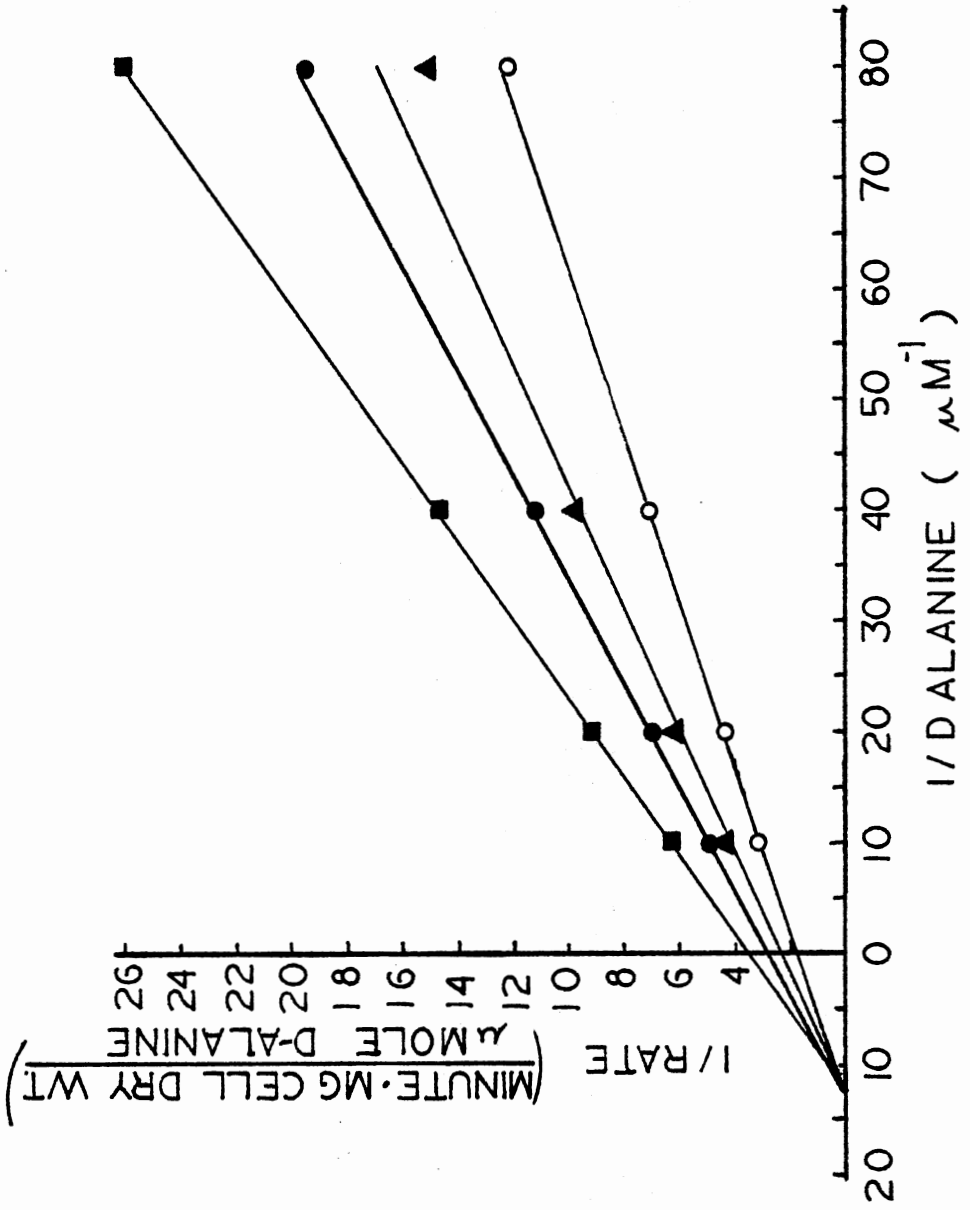

1/RATE
(MINUTE·MG CELL DRY WT / μMOLE D-ALANINE)
26
24
22
20
18
16
14
12
10
8
6
4
20
10
0
10
20
30
40
50
60
70
80
1/D ALANINE (μM⁻¹)

Figure 7. Effect of pantoyl lactone on L-aspartate transport by M. lysodeikticus (double reciprocal plot). O , control cells; ●, 0.22 M PL.

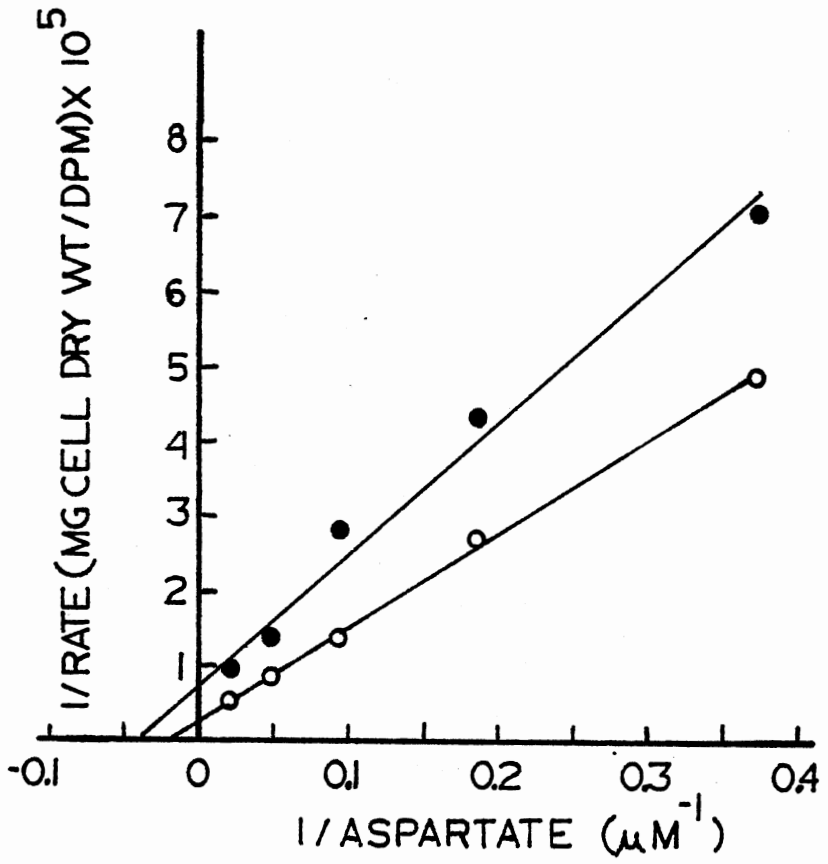

1/RATE (MG CELL DRY WT/DPM) X 10^5
8
7
6
5
4
3
2
1
-0.1
0
0.1
0.2
0.3
0.4
1/ASPARTATE (μM^-1)

Figure 8. Effect of pantoyl lactone on L-glutamate transport by M. lysodeikticus (double reciprocal plot). o , control cells; ●, 0.22 M PL.

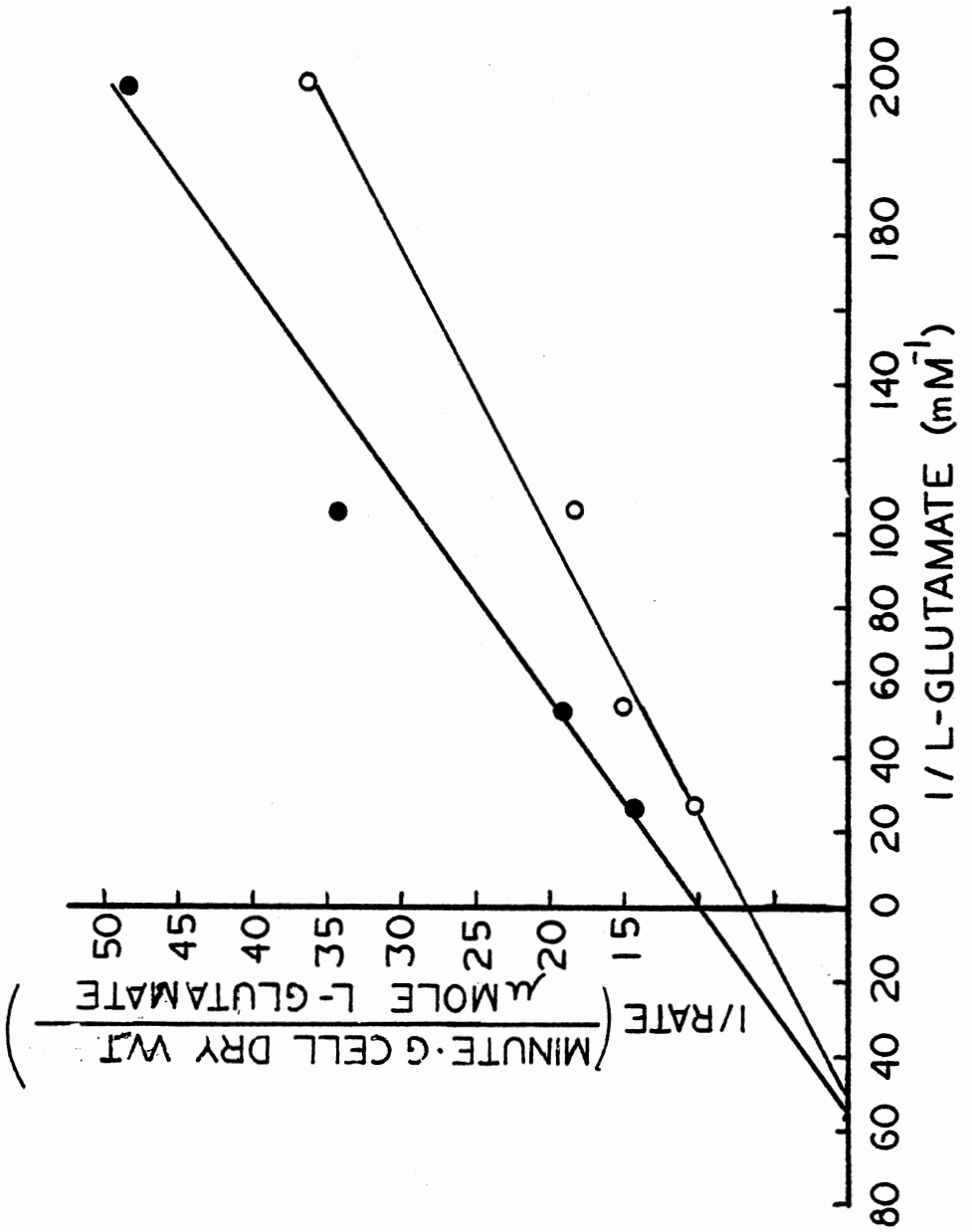

1/ L-GLUTAMATE (mM^{-1})
80 60 40 20 0 20 40 60 80 100 140 180 200
1/RATE $\left(\frac{\text{MINUTE}\cdot\text{G CELL DRY WT}}{\mu\text{MOLE L-GLUTAMATE}}\right)$
50 45 40 35 30 25 20 15

Figure 9. Effect of pantoyl lactone on D-glucose transport by M. lysodeikticus (double reciprocal plot). o , control cells; ● , 0.22 M PL.

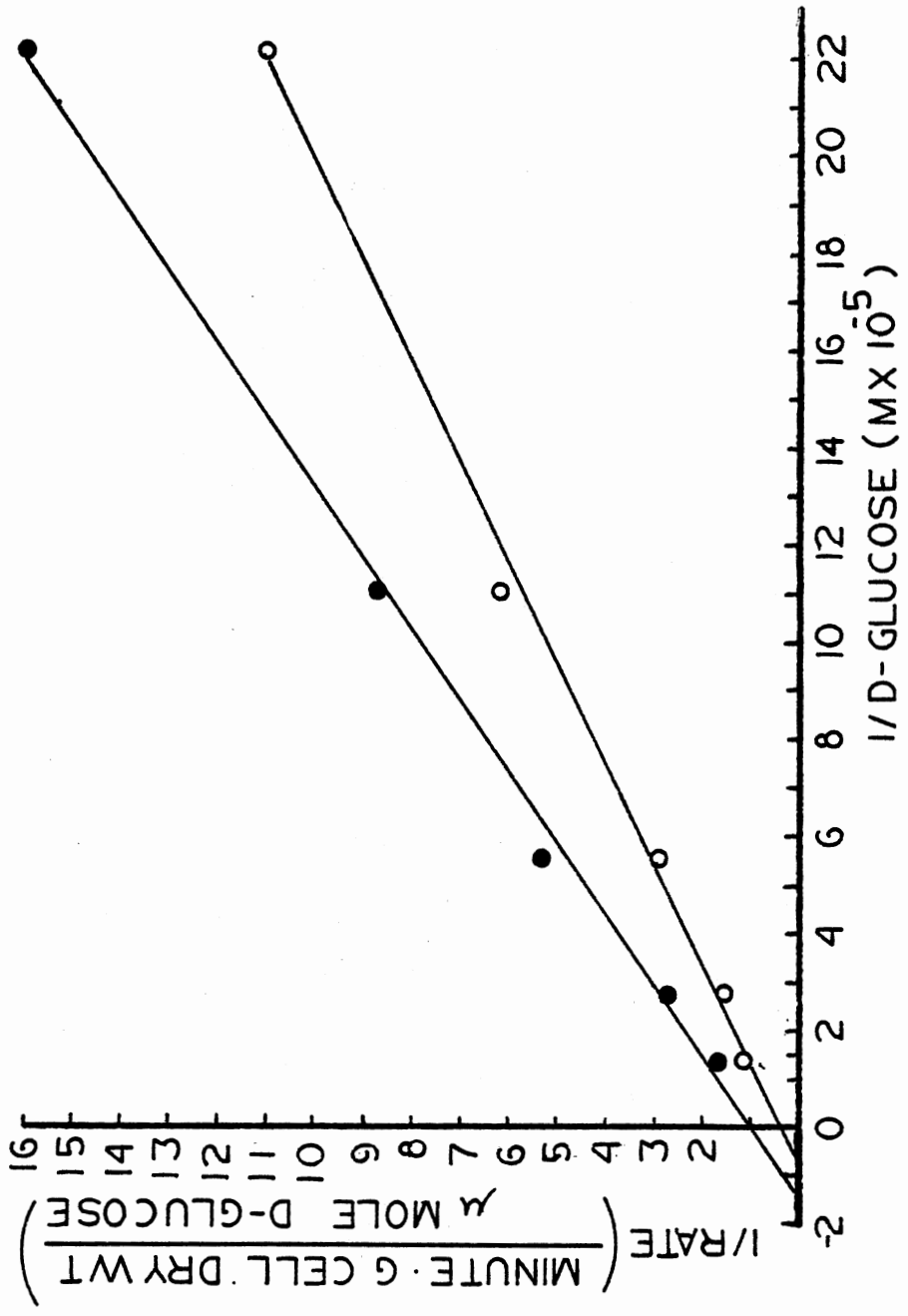

1/RATE (MINUTE · G CELL. DRY WT / μ MOLE D-GLUCOSE)
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2
-2 0 2 4 6 8 10 12 14 16 18 20 22
1/D-GLUCOSE (M X 10^-5)

Figure 10. Effect of pantoyl lactone on L-aspartate transport by S. faecalis (initial velocity curve). L-Aspartate (232 Ci/mole) was present at 50μM. O , control cells; ●, 0.22 M Pl.

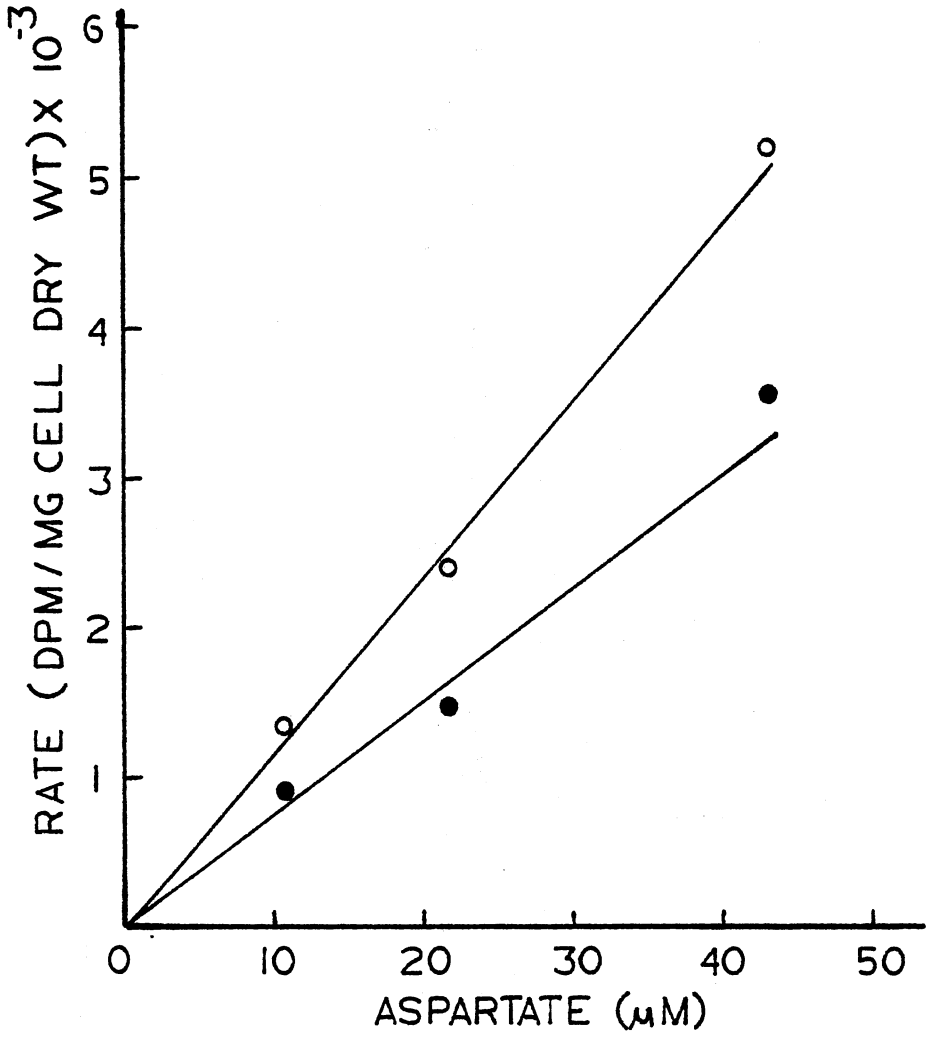

RATE (DPM/MG CELL DRY WT) X 10^{-3}
6
5
4
3
2
1
0
10
20
30
40
50
ASPARTATE (μM)

Figure 11. Effect of pantoyl lactone on L-glutamate transport by S. faecalis (initial velocity curve). L-Glutamate (206 Ci/mole). O , control cells; ● , 0.22 M PL.

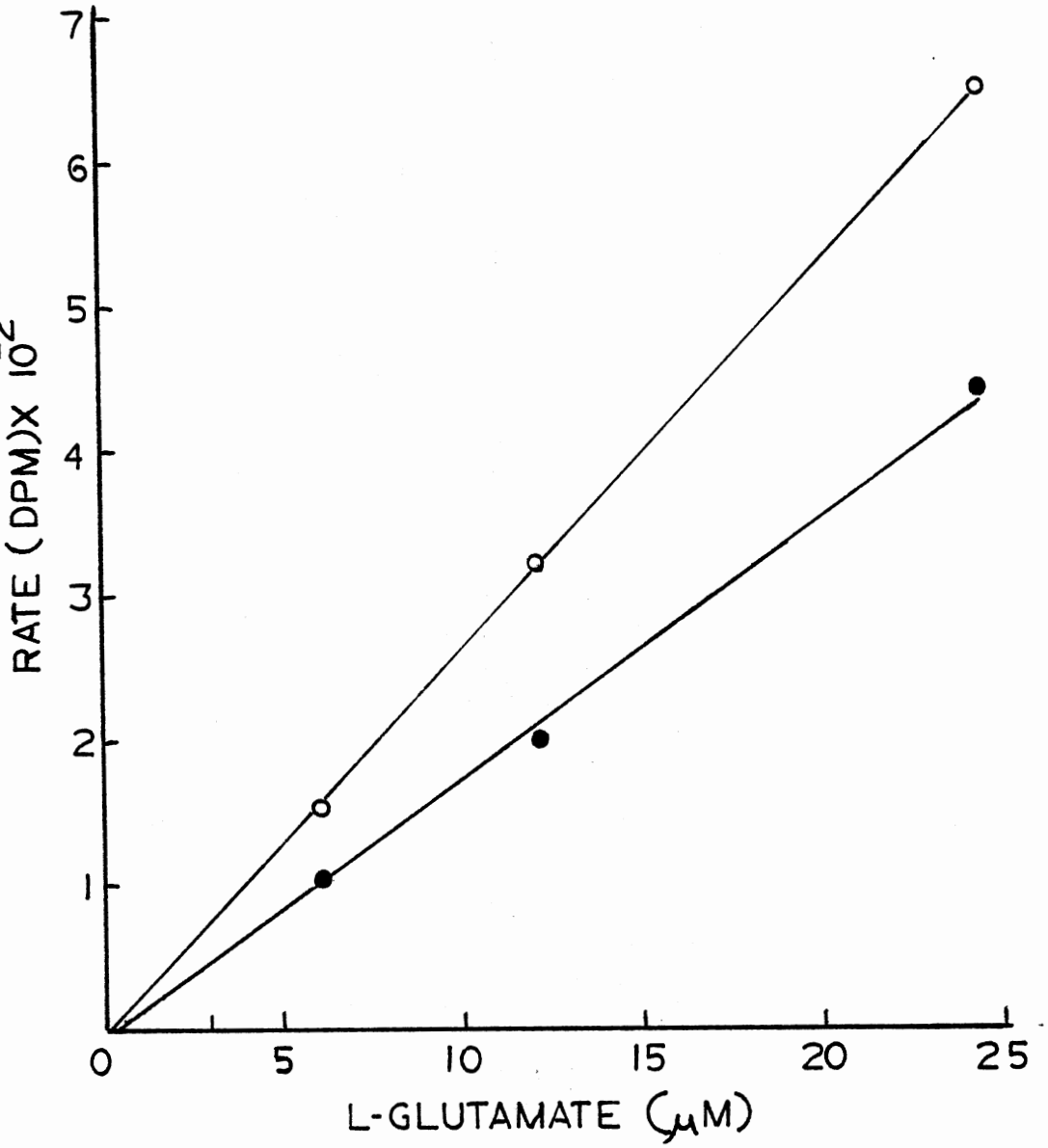

RATE (DPM) X 10^{-2}
7
6
5
4
3
2
1
0
5
10
15
20
25
L-GLUTAMATE (μM)

Figure 12. Effect of pantoyl lactone on D-glucose transport by *S. faecalis* (dose response curve).

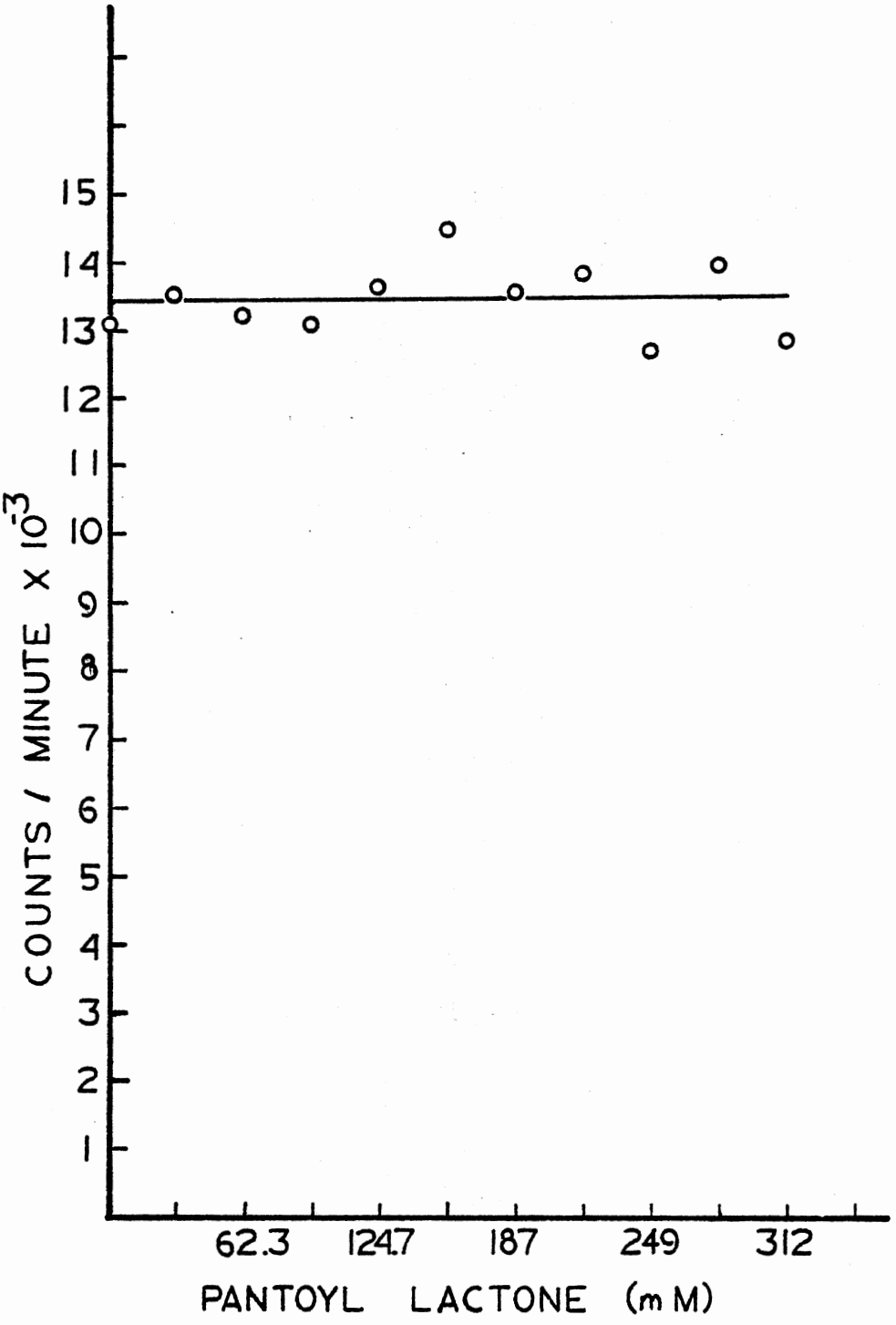
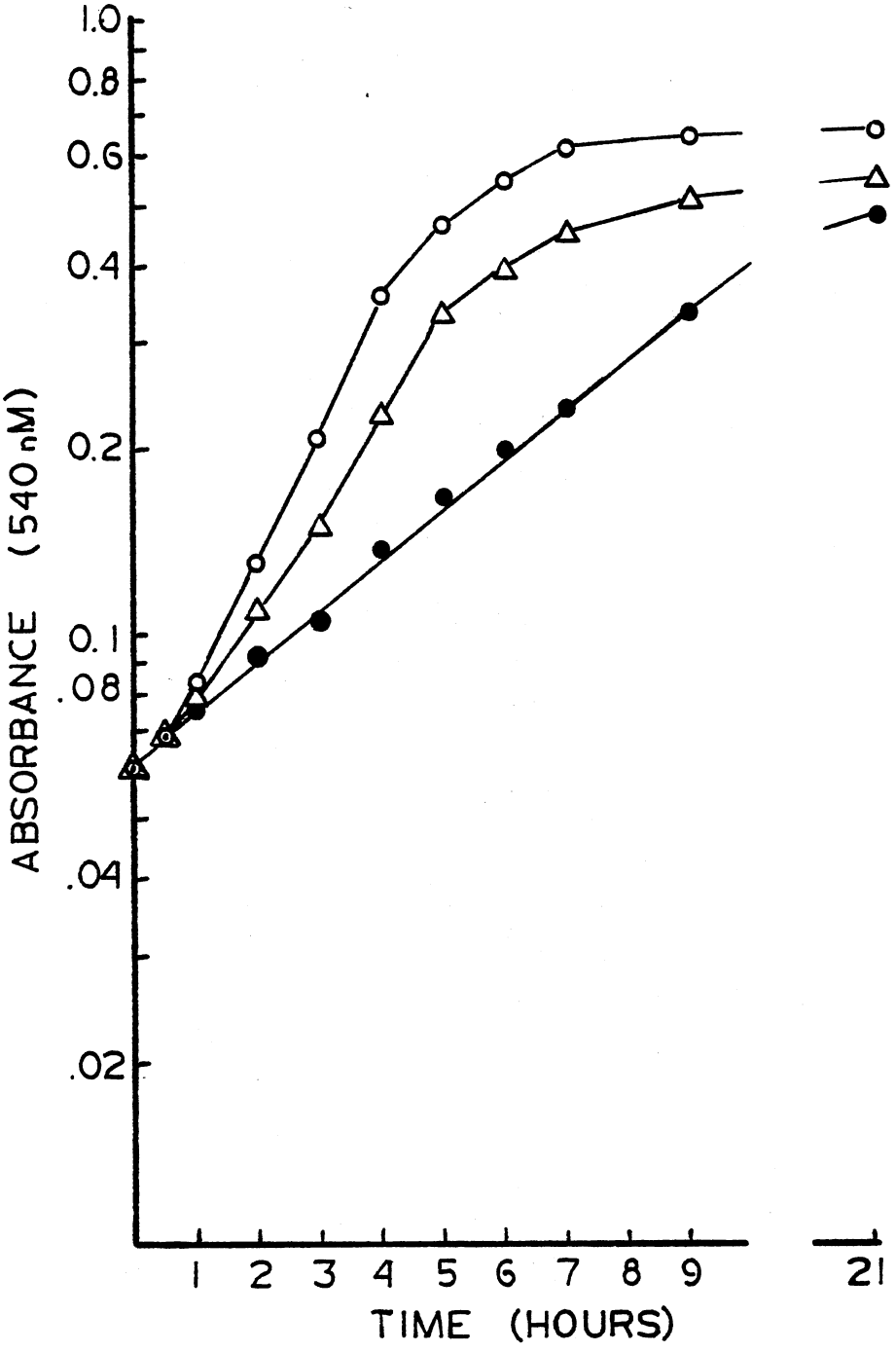

15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
COUNTS / MINUTE X 10^{-3}
62.3
124.7
187
249
312
PANTOYL LACTONE (m M)

Figure 13. Effect of pantoyl lactone on growth of S. faecalis. Side-arm flasks of nutrient broth + 1% glucose + 0.1% yeast extract were inoculated with cells from trypicase-soy agar slants and incubated at 25° C on a rotary shaker. PL was added at zero time. The absorbance at 540 nm was periodically measured using a Bausch and Lomb Spectronic 20. O, control cells; Δ, 0.273 M PL; ●, 0.48 M PL.


1.0
0.8
0.6
0.4
0.2
0.1
.08
.04
.02
ABSORBANCE (540 nM)
1 2 3 4 5 6 7 8 9 21
TIME (HOURS)

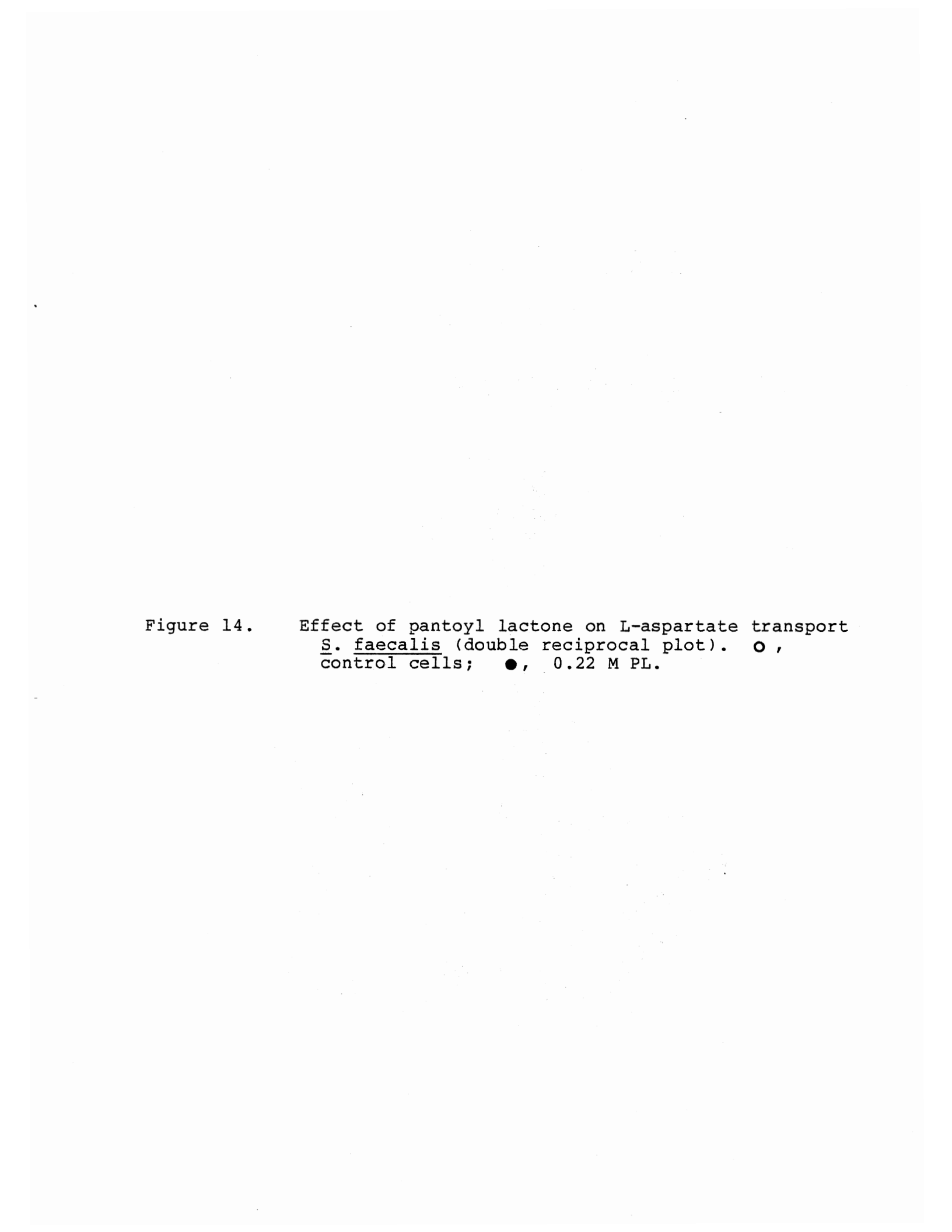


Figure 14. Effect of pantoyl lactone on L-aspartate transport S. faecalis (double reciprocal plot). o, control cells; ●, 0.22 M PL.

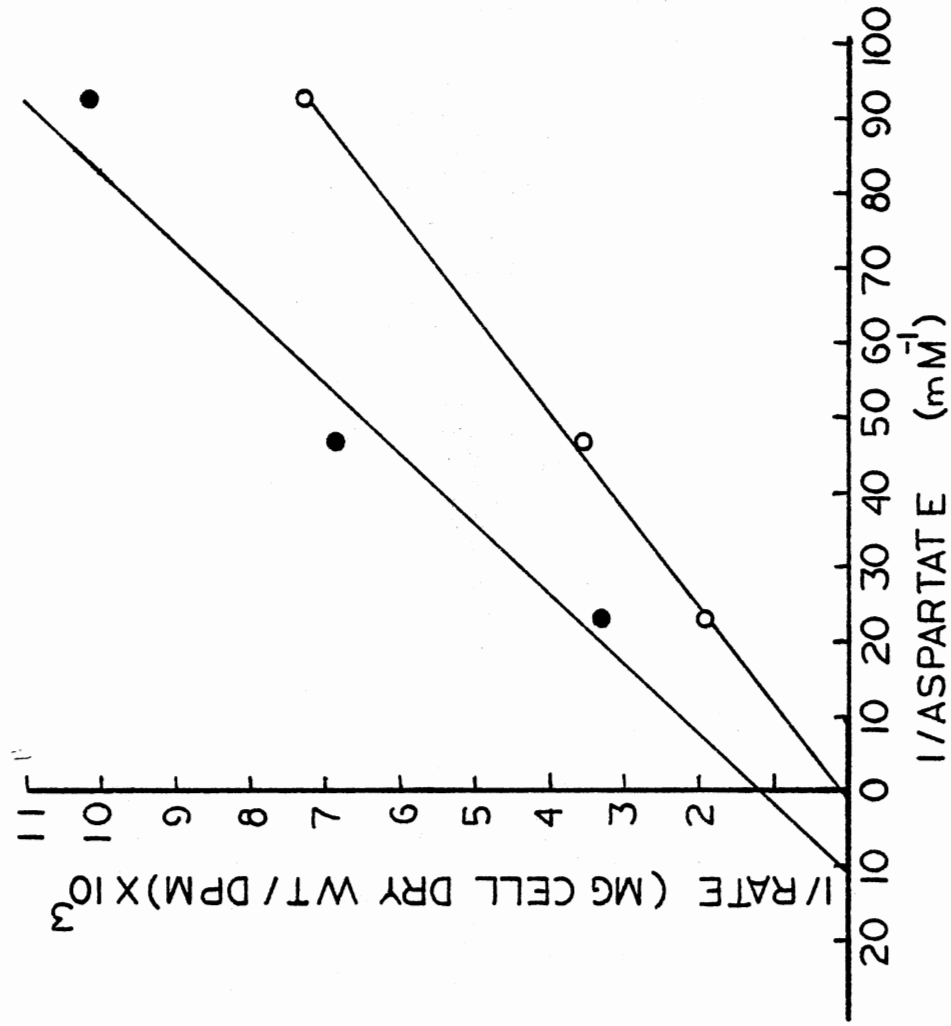
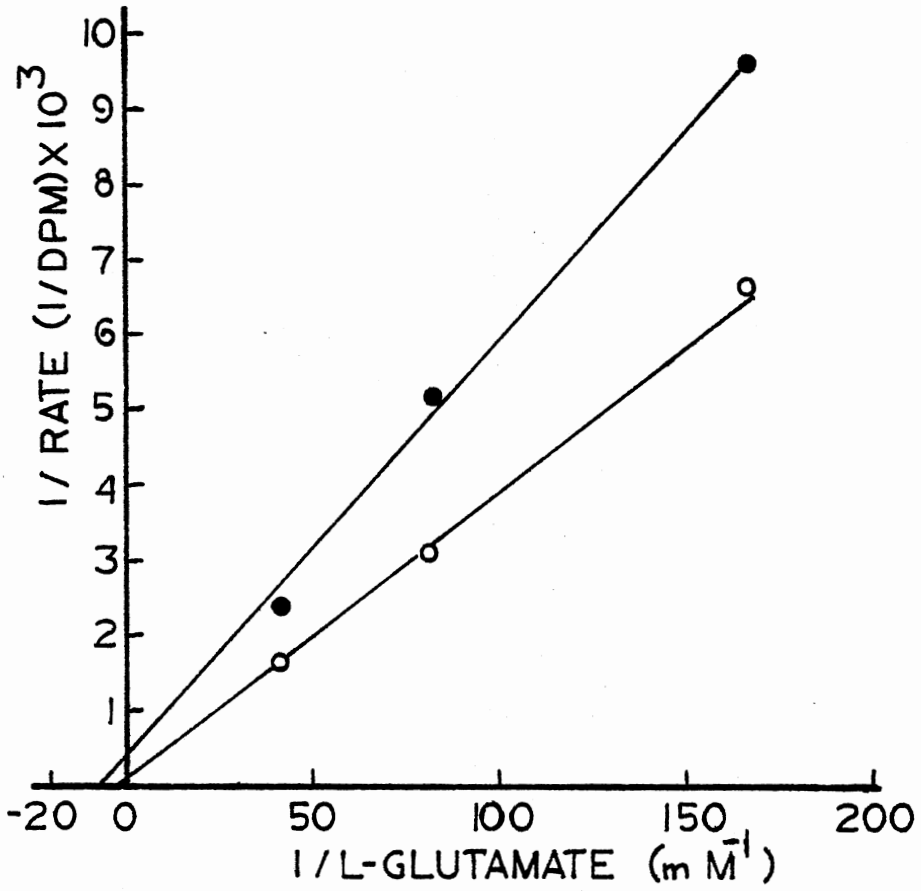

1/RATE (MG CELL DRY WT/DPM) X 10^3
11
10
9
8
7
6
5
4
3
2
0
20 10 0 10 20 30 40 50 60 70 80 90 100
1/ASPARTATE (mM^{-1})

Figure 15. Effect of pantoyl lactone on L-glutamate transport by S. faecalis (double reciprocal plot). o , controls cells; ● , 0.22 M PL.


1/ RATE (1/DPM) X 10^3
10
9
8
7
6
5
4
3
2
1
-20
0
50
100
150
200
1/L-GLUTAMATE (m M^-1)

Studies on Energetics

S. faecalis ATPase

Since S. faecalis is a strictly fermentative organism, the only way it has of chemiosmotically energizing its membrane (Mitchell, 1963) is via a H^+-pumping ATPase (Harold, 1972). Since the energy for aspartate and glutamate transport is generated by this enzyme, it should be inhibited by PL if the energizing system and not the individual permeases is the site of action. The solubilized form was isolated and tested for PL sensitivity. It is not inhibited by PL (Fig. 16) however, if the enzyme is assayed at an earlier stage during the isolation procedure (when it is still bound to the membrane) it is sensitive to PL (Fig. 17). PL is a non-competitive inhibitor of membrane-bound S. faecalis ATPase (Fig. 18). These data indicate that a membrane-protein interaction is an important factor in PL inhibition. The similar apparent K_I of PL inhibition of membrane-bound ATPase and amino acid transport suggests that the inhibition of the energizing system (ATPase) is sufficient to account for the transport inhibition.

Although interactions with other membrane proteins (permeases) cannot be ruled out, the differential effects on dissimilar types of transport systems is evidence against this. The inhibition by PL of S. faecalis membrane-bound ATPase does not seem to be reversed by increasing the Mg^{++} concentration (Fig. 19) as is the case for PL induced

Figure 16. Effect of pantoyl lactone on solubilized by S. faecalis ATPase (initial velocity curve). The buffer used was 30 mM HEPES, 20 mM $MgCl_2$, pH 7.8. O , control; ● , 0.22 M PL

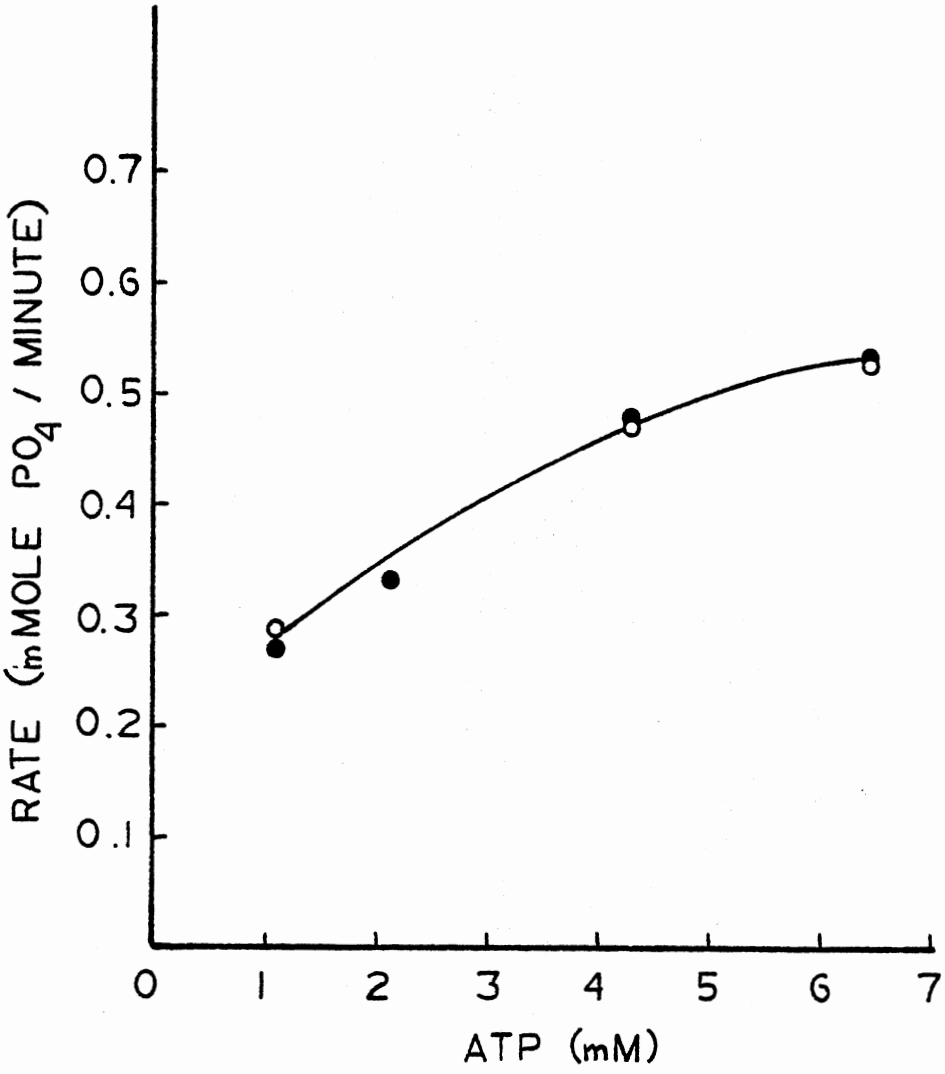

RATE (mMOLE PO4 / MINUTE)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
ATP (mM)

Figure 17. Effect of pantoyl lactone on membrane-bound ATPase from S. faecalis (initial velocity curve). The buffer used was 30 mM HEPES, 20 mM $MgCl_2$, pH 7.8. ○, control; ●, 0.165 M PL.

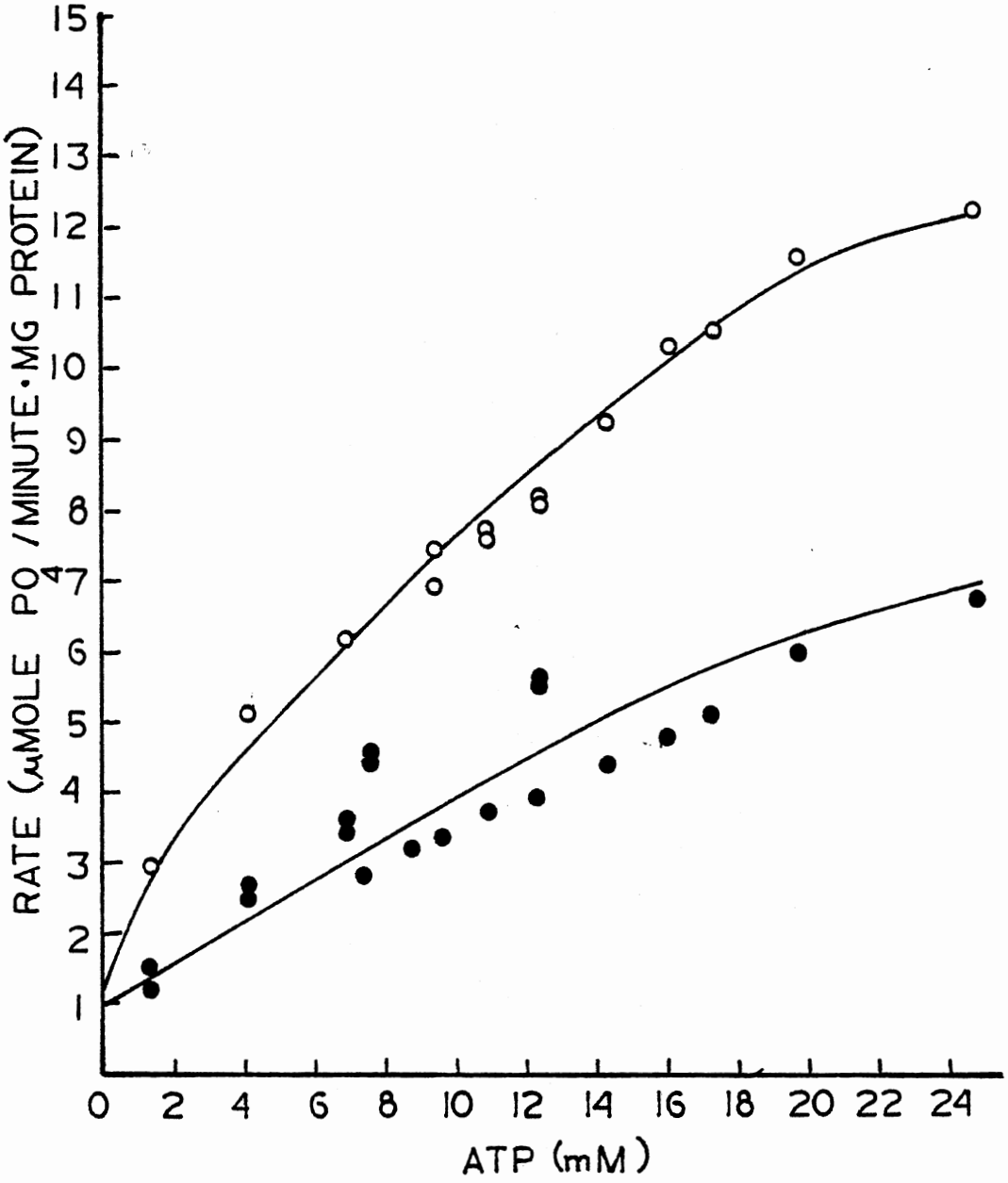

RATE (μMOLE PO4 /MINUTE•MG PROTEIN)
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
16
18
20
22
24
ATP (mM)

Figure 18. Effect of pantoyl lactone on membrane-bound ATPase from S. faecalis (double reciprocal plot). The buffer used was 30 mM HEPES, 20 mM $MgCl_2$, pH 78. O, control; ● , 0.165 M PL.

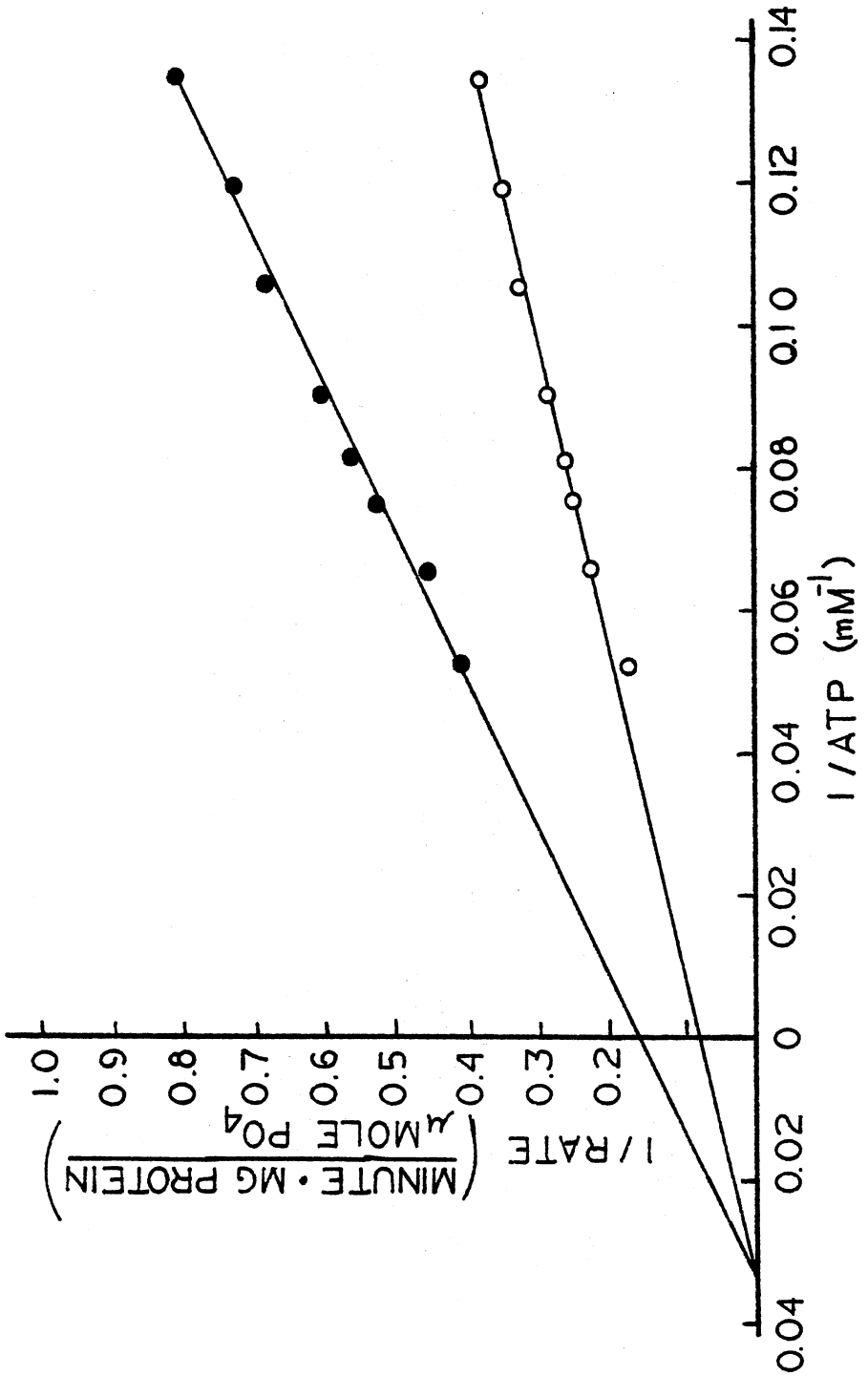
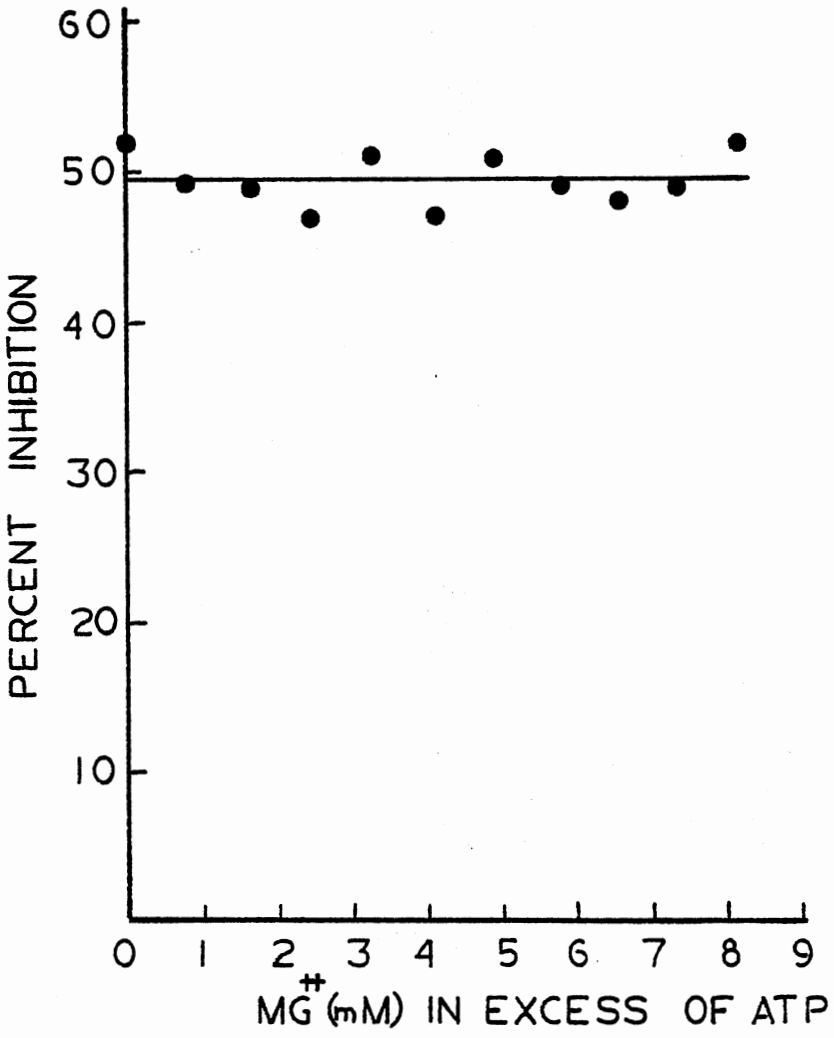

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.04
0.02
1/RATE (MINUTE • MG PROTEIN / μMOLE PO4)
1/ATP (mM-1)

Figure 19. Influence of Mg^{++} on pantoyl lactone inhibition of S. faecalis membrane-bound ATPase. Each assay contained 4.44 mM ATP, 4.44 mM $MgCl_2$, 30Mm HEPES pH 7.8, 0.136 M PL and excess $MgCl_2$ as indicated on the ordinate. Percent inhibition was calculated using results from samples without PL or excess $MgCl_2$ as the control value.


60
50
40
30
20
10
PERCENT INHIBITION
0 1 2 3 4 5 6 7 8 9
MG++ (mM) IN EXCESS OF ATP

alterations in the laser Raman spectra of isolated membrane vesicles (Johnson et al., 1978).

The inhibition pattern with respect to the solubilized enzyme versus the membrane-bound form is similar to the inhibition that occurs in the presence of DCCD (Figs. 20, 21). DCCD and PL inhibition are additive (Table I) and data presented in Figure 22 indicate that even though PL and DCCD affect the same portion of the enzyme (F_0), they have different sites of action.

These data indicate that PL inhibits S. faecalis ATPase through interactions with the membrane-bound (F_0) portion of the enzyme even though PL and DCCD have different specific sites of action. This is consistent with the membrane lipid-protein interaction hypothesis for the mechanism of action of PL.

Other ATPases

To see if inhibition by PL was limited to S. faecalis membrane-bound ATPase or if it is more universal, other ATPases were assayed for sensitivity to PL. The membrane-bound Na^+/K^+-activated ATPase from porcine cerebral cortex cytoplasmic membrane is inhibited by either PL (Fig. 23), DCCD or oligomycin (Fig. 24). The Na^+/K^+-activated ATPase from dog kidney is also sensitive to PL (Fig. 25). Apyrase, a solubilized ATPase isolated from potatoes, is not senstitve to either PL or DCCD (Figs. 26-27). The membrane-bound Mg^{++}-activated ATPase of beef

Figure 20. Effect of DCCD on solubilized S. faecalis ATPase. Assays were done using 7.5 mM ATP in 10 mM $MgCl_2$, 30 mM HEPES pH 7.8 with additions as indicated. O, controls; Δ, 0.8% ethanol; ▲, 8 uM DCCD.

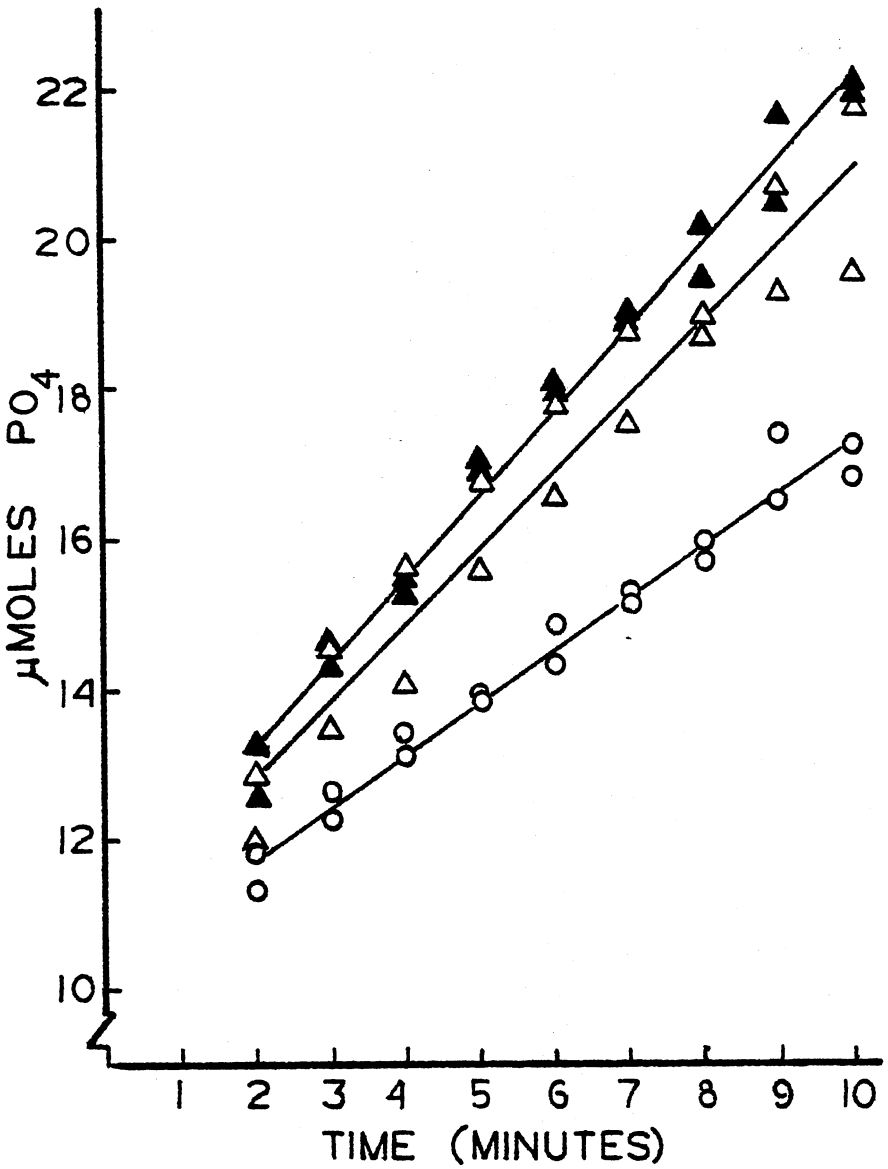

22
20
18
16
14
12
10
μMOLES PO4
1
2
3
4
5
6
7
8
9
10
TIME (MINUTES)

Figure 21. Effect of DCCD on membrane-bound S. faecalis ATPase. Assays were done using 7.5 mM ATP, 10 mM $MgCl_2$, 30 mM HEPES pH 7.8 with additions as indicated. o , control; Δ , 0.8% ethanol; ▲ , 8 mM DCCD.

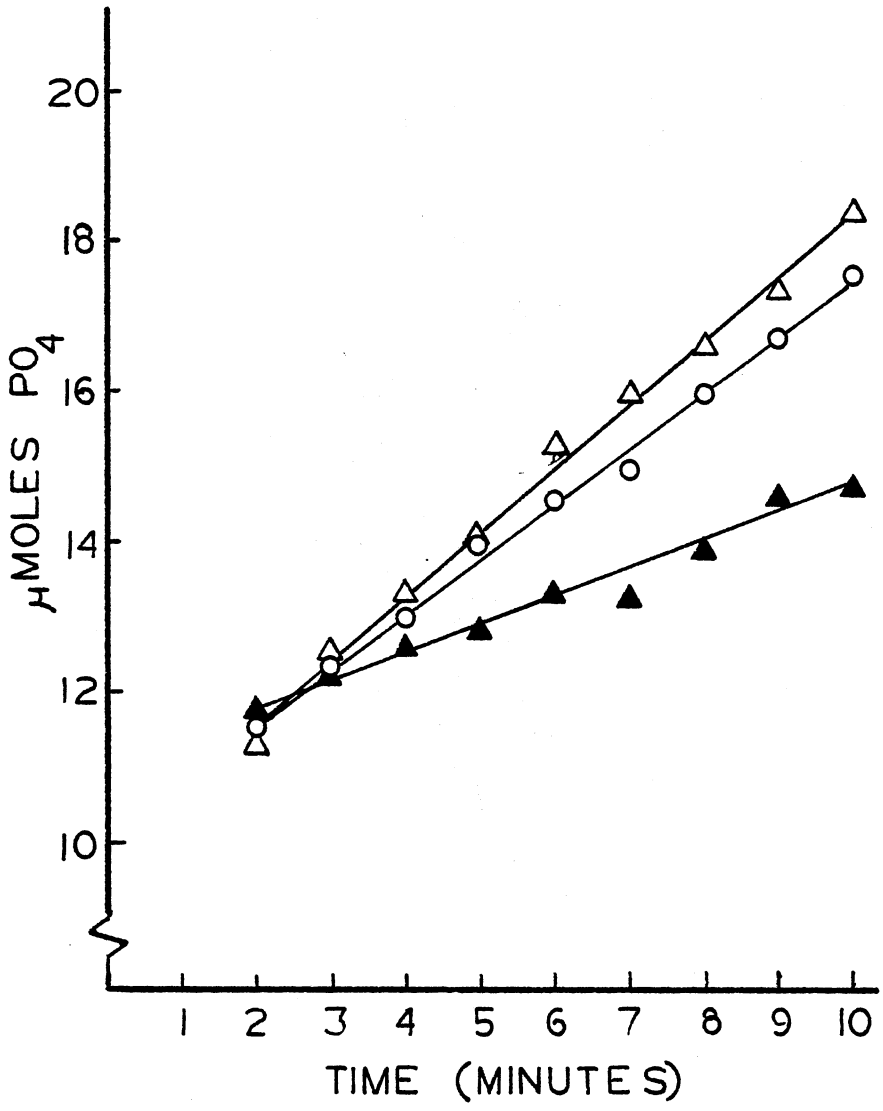

20
18
16
14
12
10
μMOLES PO4
1
2
3
4
5
6
7
8
9
10
TIME (MINUTES)

TABLE I

ADDITIVE NATURE OF PANTOYL LACTONE AND DCCD INHIBITION OF S. FAECALIS MEMBRANE-BOUND ATPase

umole PO_4/minute·mg protein*			
Control	2.11 uM DCCD	144 mM PL	2.11 uM DCCD + 144 mM PL
8.828	5.67	4.00	2.005
8.965	5.19	4.18	2.167
8.654	4.73	3.50	2.067
9.463	4.67		1.918
9.165			1.768
9.015			1.519
			2.430
			2.34
			2.43
			2.67
			3.08
			1.76
			1.95
			1.72
			1.81
			2.05
			2.79
			1.74
$\bar{x}$=9.015	$\bar{x}$=5.065	$\bar{x}$=3.893	$\bar{x}$=2.123
s=0.280	s=0.465	s=0.352	s=0.422

* Assays were done using 9.9 mM ATP, 10 mM $MgCl_2$, 30 mM HEPES pH 7.8.

Figure 22. Dixon plot of the effect of DCCD + pantoyl lactone on S. faecalis membrane-bound ATPase. Assays were done using 9 mM ATP, 9 mM $MgCl_2$, 30 mM HEPES pH 7.8. ○, control; □, 0.058 M PL. ●, 0.145 M PL; ■, 0.290 M PL.

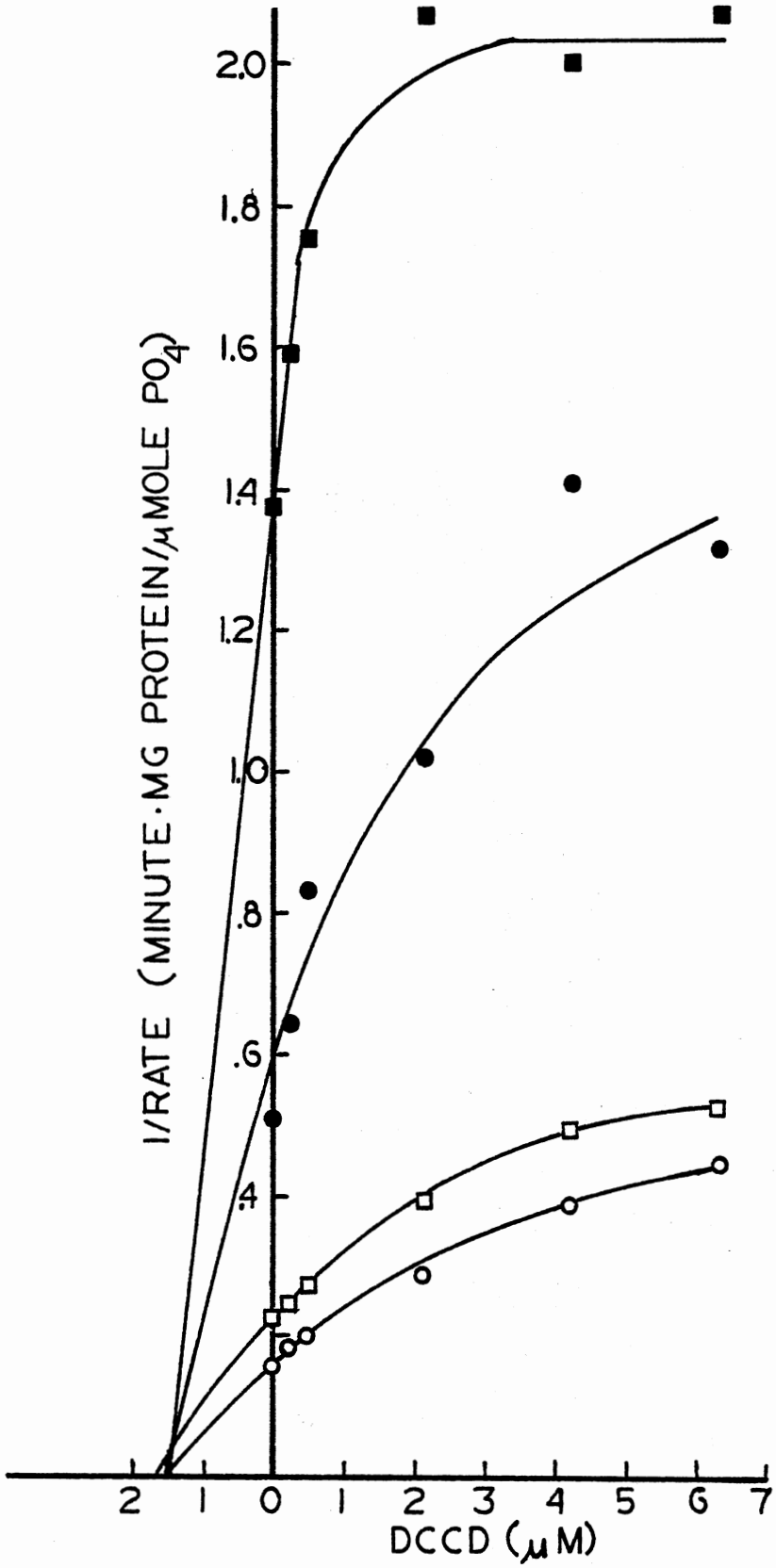

2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
I/RATE (MINUTE·MG PROTEIN/μMOLE PO4)
2 1 0 1 2 3 4 5 6 7
DCCD (μM)

Figure 23. Effect of pantoyl lactone on Na^+/K^+-activated ATPase from porcine cerebral cortex. Assays were done using 5.94 mM ATP, 28 mM $MgCl_2$, 25 mM KCL, 100 mM NaCl, and 300 mM TRIS pH 7.8. O , control; ● , 0.165 M PL.

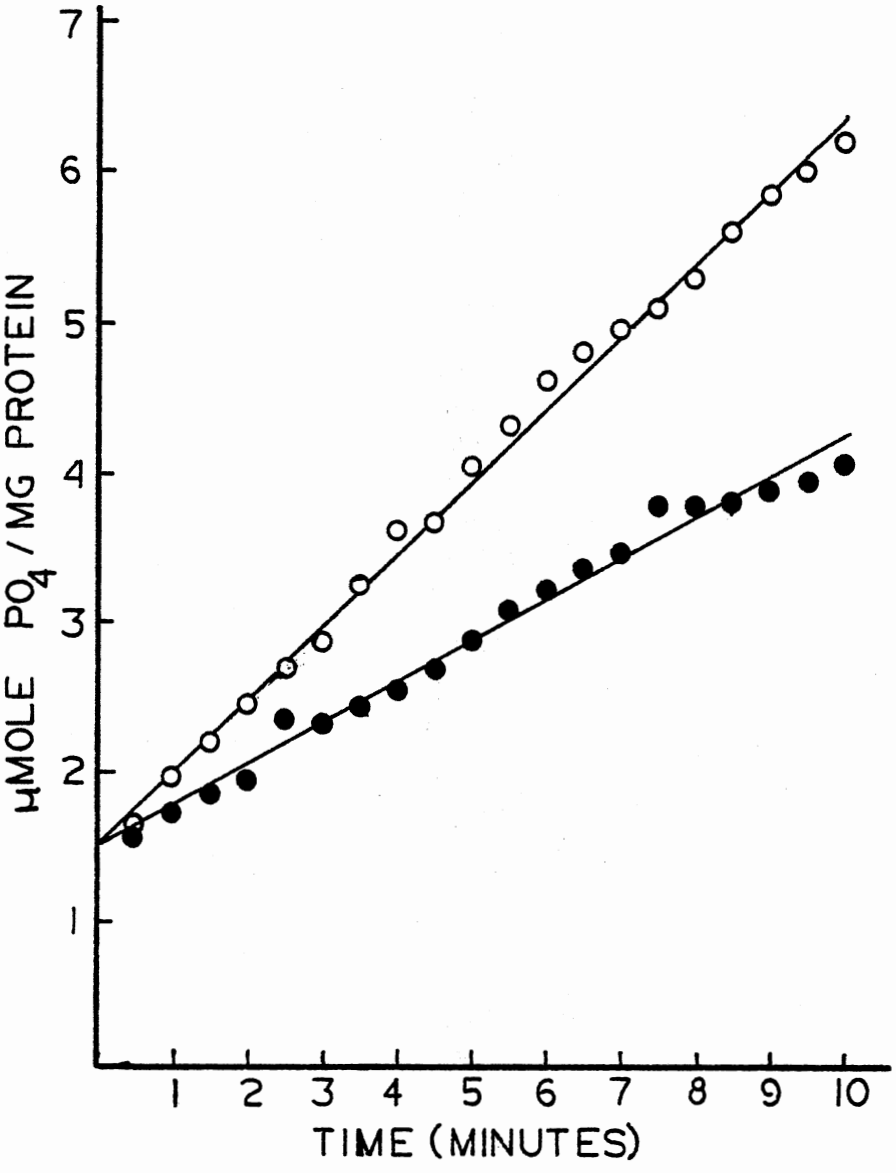
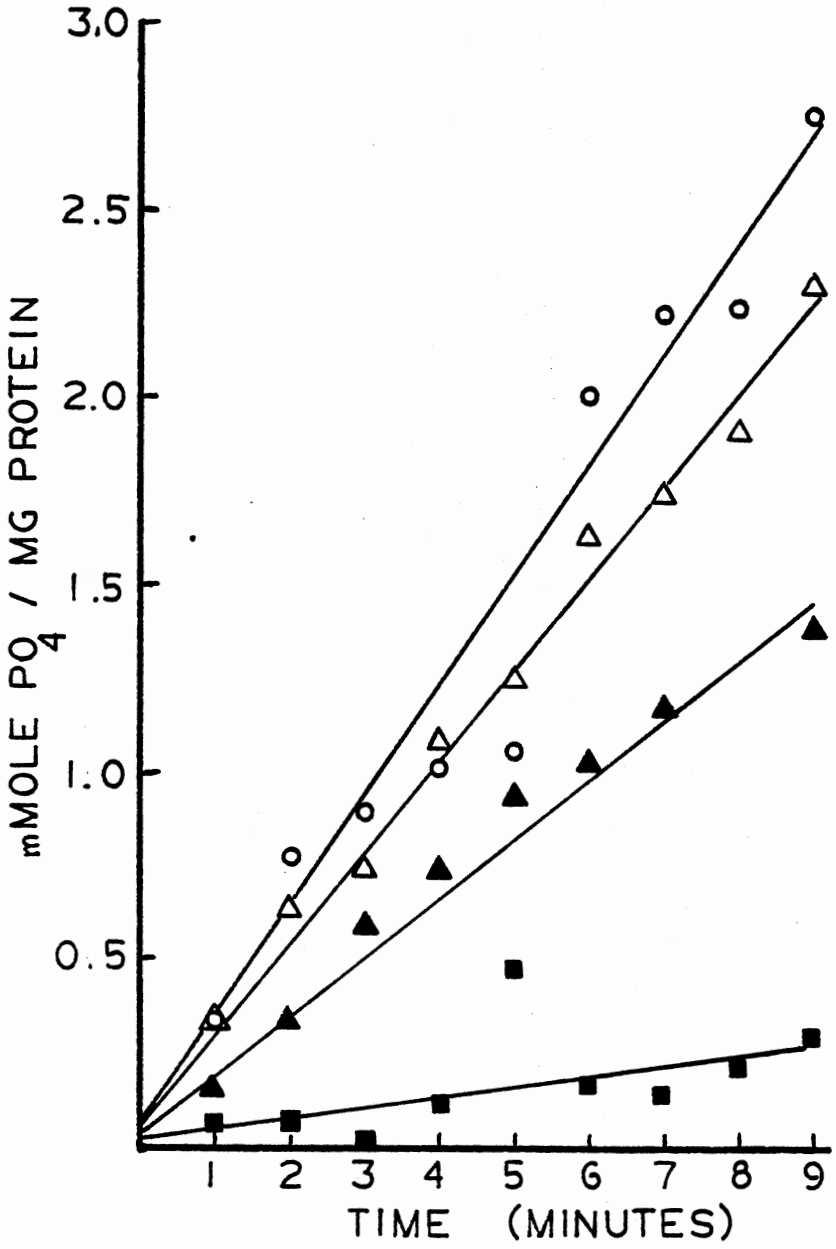

μMOLE PO4 / MG PROTEIN
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
9
10
TIME (MINUTES)

Figure 24. Effect of DCCD and oligomycin on Na^+/K^+-activated porcine cerebral ATPase. Assays were done using 4.5 mM ATP, 28 mM $MgCl_2$, 25 mM KCL, 100 mM NaCl, and 300 mM TRIS pH 7.8. O, control; Δ, 0.9% ethanol; ▲, 9µg/ml DCCD; ■, 9µg/ml oligomycin.


3.0
2.5
2.0
1.5
1.0
0.5
mMOLE PO4 / MG PROTEIN
1
2
3
4
5
6
7
8
9
TIME (MINUTES)

heart mitochondria (electron transport particles) is also inhibited by PL (Fig. 28). These data further emphasize a need for membrane association in PL mediated inhibition. However, luciferase, a solubilized ATPase (McElroy and Seliger, 1962; Cromier et al., 1975) isolated from fire-fly (Photonius pyralis) lanterns is sensitive to PL (Fig. 29). This may be due to competition between PL and luminol (luciferin) (Figs. 29, 30). A clear interpretation of the data is not possible because standard type kinetics are not demonstrated.

Respiration and $H^{\pm}$ Pumping

Based on data given above it appeared that PL inhibits ATPases by interacting with the membrane-bound (F_o) portion of this enzyme. As a further test of this hypothesis, ATP driven H^+ extrusion by isolated turkey heart mitochondria was measured as a function of PL concentration. The data presented in Figure 31 show that this process is also inhibited. Since many cells can also energize their membranes through reactions catalyzed by the electron transport chain, the effect of PL on respiration was also measured. A compound that inhibits ATPase will also inhibit respiration when measured as increased O_2 consumption in response to added ADP. This inhibition can be prevented by uncouplers while decreased respiration due to inhibition of the electron transport system is insensitive to added uncouplers. PL inhibition of respiration of turkey heart mitochondria

Figure 25. Effect of pantoyl lactone on Na^+/K^+-activated ATPase from dog kidney. Assays were done using 3 mM ATP, 28 mM $MgCl_2$, 25 mM KCl, 100 mM NaCl, and 300 mM TRIS pH 7.8. O , control; ● , 0.2 M PL.

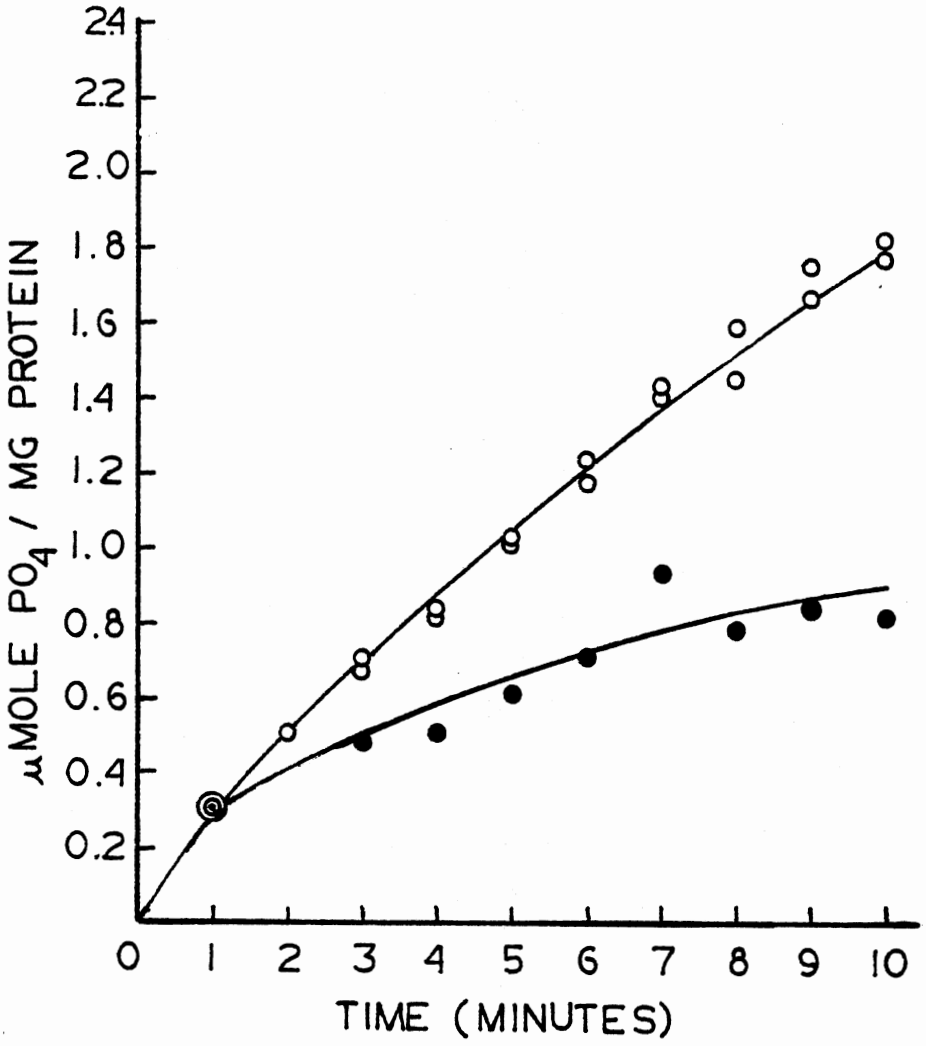

µMOLE PO4 / MG PROTEIN
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
TIME (MINUTES)

Figure 26. Effect of pantoyl lactone on apyrase. Assays were done using 5 mM ATP, 28 mM $MgCl_2$, 25 mM KCl, 100 mM NaCl, 300 mM TRIS pH 7.0. O, control; ●, 0.2 M PL.

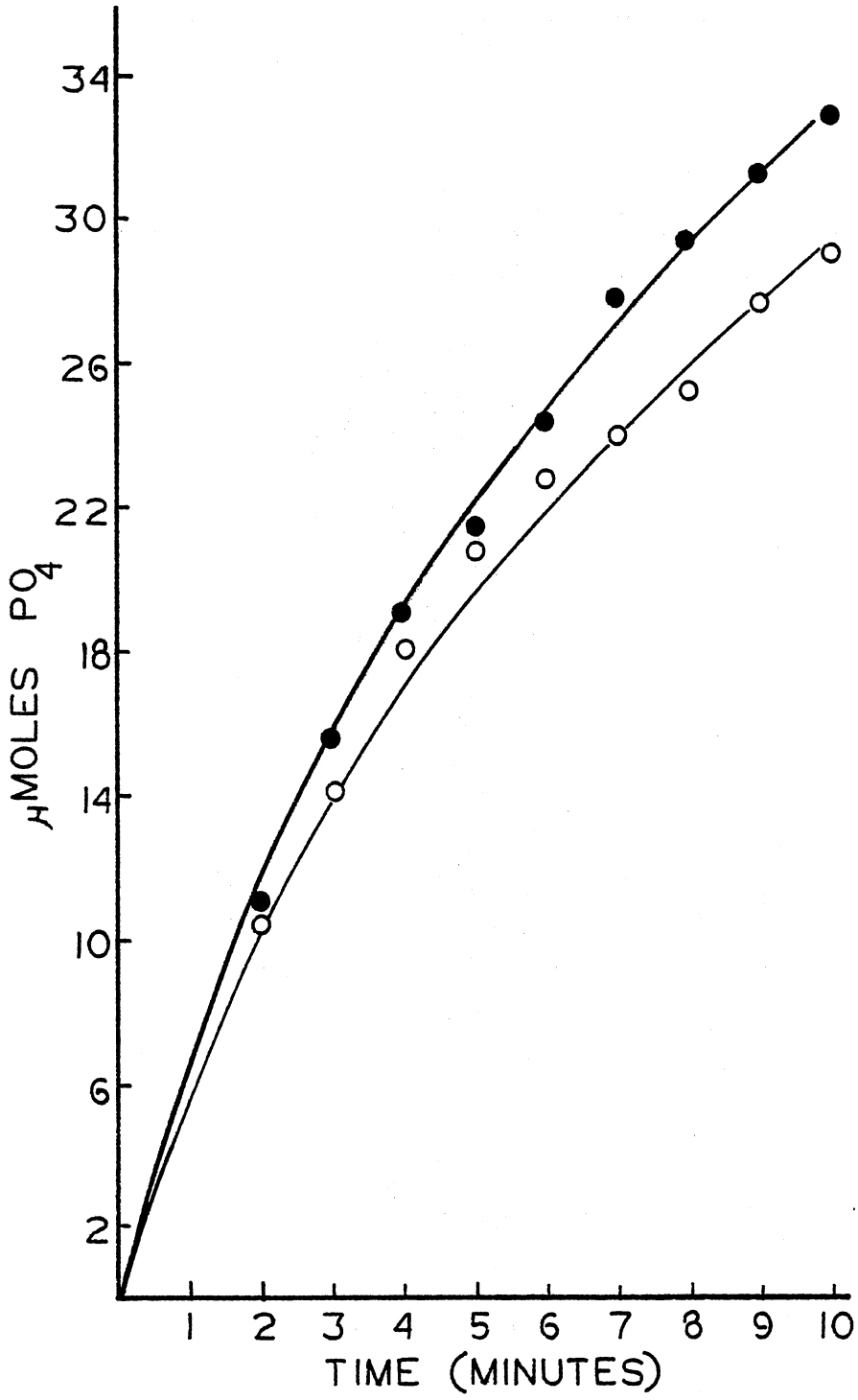

34
30
26
22
18
14
10
6
2
μMOLES PO4
1
2
3
4
5
6
7
8
9
10
TIME (MINUTES)

Figure 27. Effect of DCCD on apyrase. Assays were done using 9.9 mM ATP, 28 mM $MgCl_2$, 25 mM KCl, 100 mM NaCl, and 300 mM TRIS pH 7.0. ○, control; ▲, 44 uM DCCD.

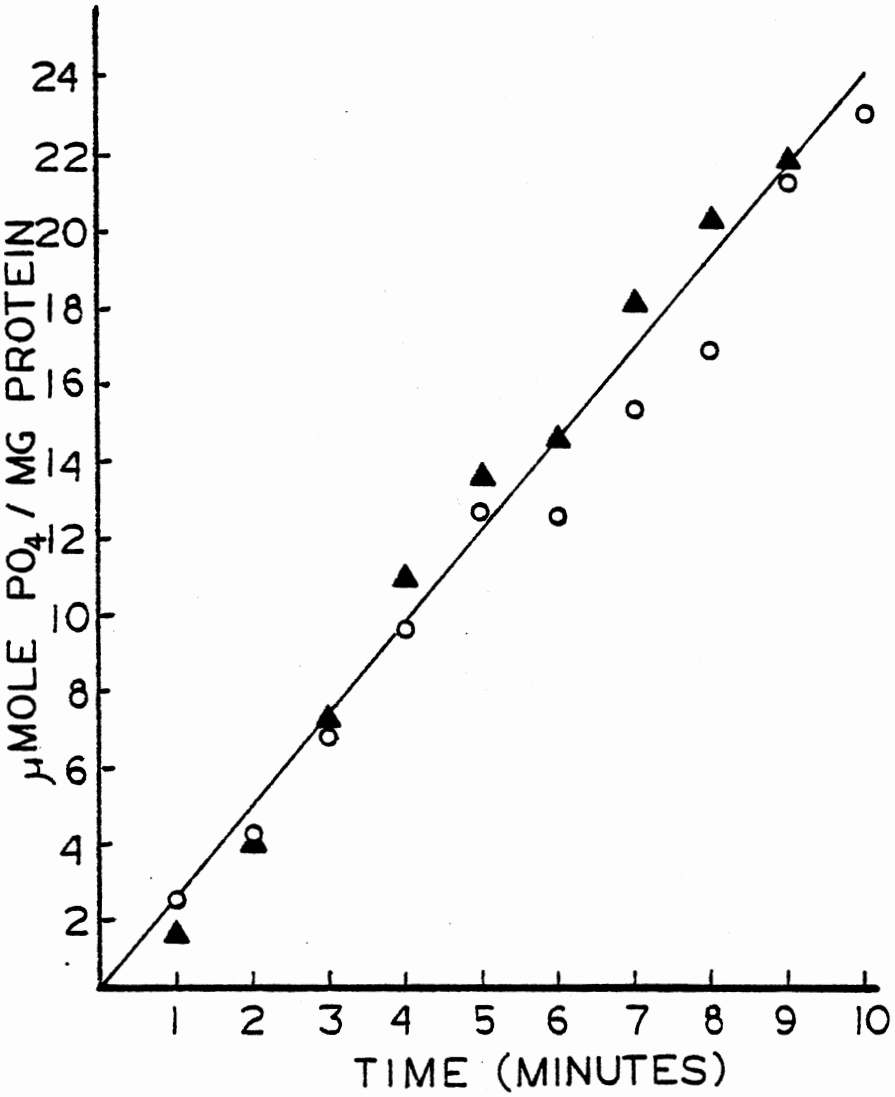

µMOLE PO4 / MG PROTEIN
24
22
20
18
16
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8
9
10
TIME (MINUTES)

Figure 28. Effect of pantoyl lactone on ATPase activity of beef heart mitochondrial electron transport particles (Dixon plot). Assays were done using 9 mM ATP, 10 mM $MgCl_2$, 30 mM HEPES pH 7.8. K_{PL} = 50 mM.

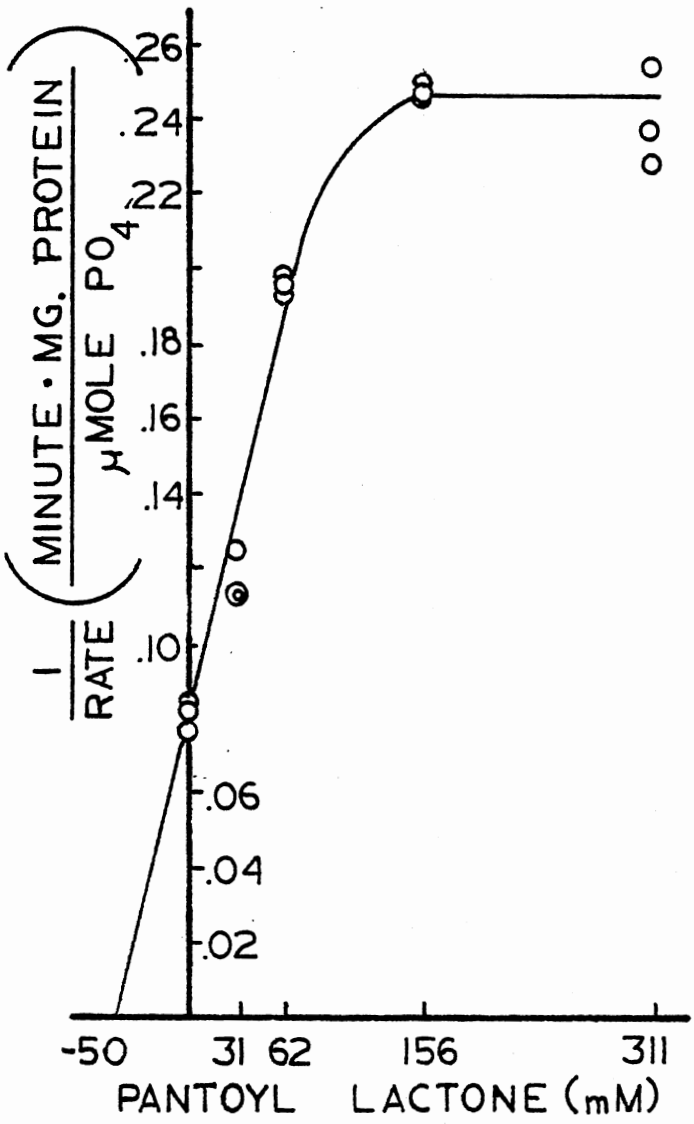
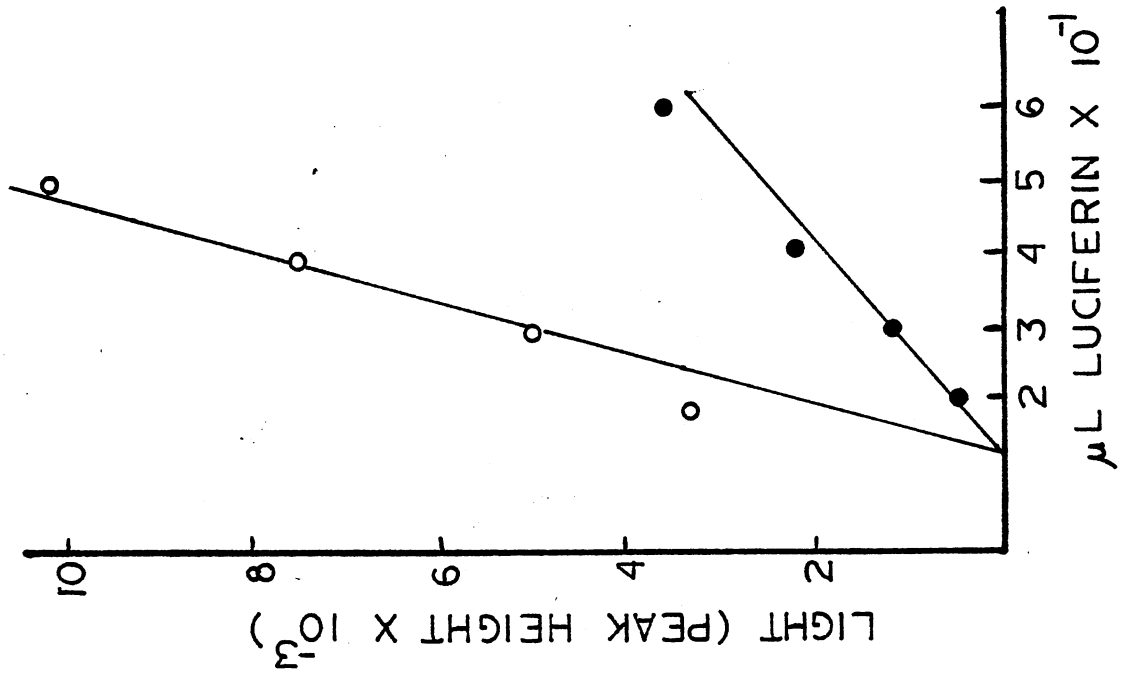

1/RATE (MINUTE • MG. PROTEIN / μMOLE PO4)
.26
.24
.22
.18
.16
.14
.10
.06
.04
.02
-50
31
62
156
311
PANTOYL LACTONE (mM)

Figure 29. Effect of pantoyl lactone on luciferin-luciferase light production (initial velocity curve). Peak height was measured using 80μm ATP, 10 mM $MgCl_2$, 30 mM HEPES, pH 7.2. O , controls; ● , 55 mM PL.

Figure 30. Effect of pantoyl lactone on luciferin-luciferase light production (double reciprocal plot). Peak height was measured using 80μM ATP, 10 mM $MgCl_2$, 30 mM HEPES, pH 7.2. O , controls; ● , 55 mM PL.


LIGHT (PEAK HEIGHT X 10^{-3})
2
4
6
8
10
μL LUCIFERIN X 10^{-1}
2
3
4
5
6

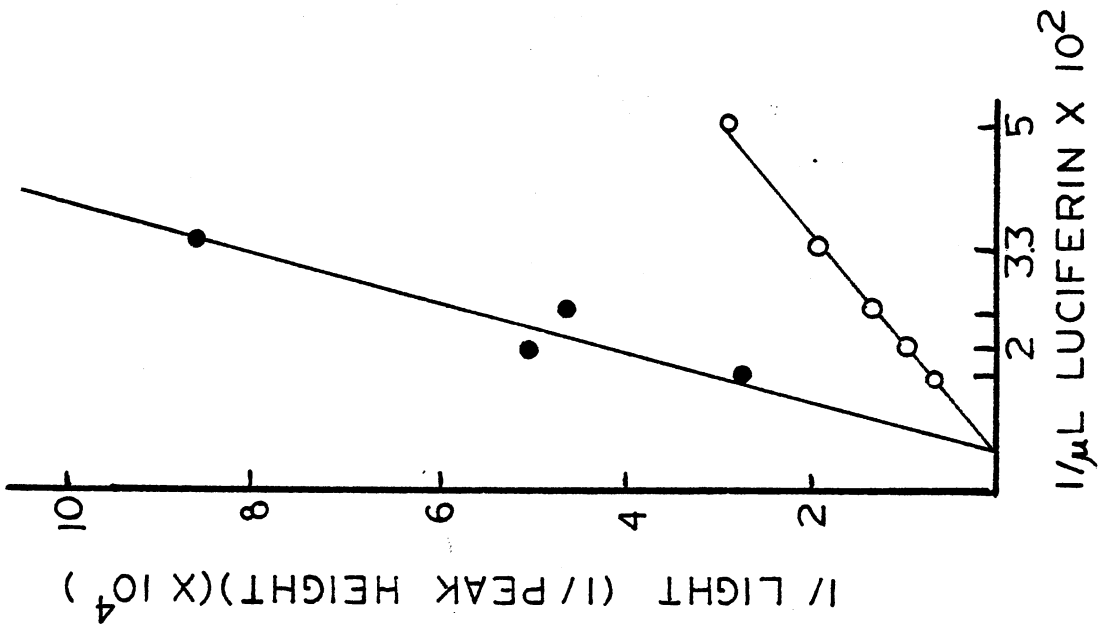
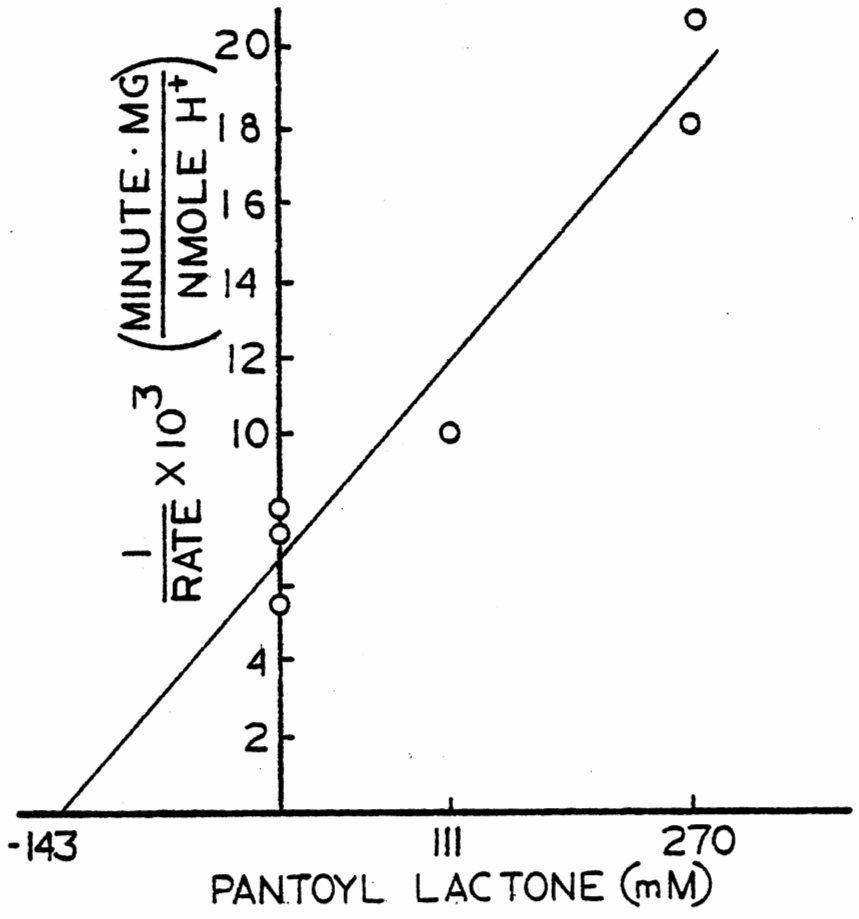

1/ LIGHT (1/PEAK HEIGHT)(X 10^{4})
2
4
6
8
10
1/μL LUCIFERIN X 10^{2}
2
3.3
5

Figure 31. Effect of pantoyl lactone on H^+ pumping by turkey heart mitochondria (Dixon plot). K_{PL} = 143 mM.


$\frac{1}{\text{RATE}} \times 10^3 \left(\frac{\text{MINUTE} \cdot \text{MG}}{\text{NMOLE } H^+}\right)$
20
18
16
14
12
10
4
2
-143
111
270
PANTOYL LACTONE (mM)

(Fig. 32), unlike that due to the mitochondrial ATPase inhibitor oligomycin, is not relieved by the uncoupler CCCP (Fig. 33). These data suggest that PL also inhibits an element(s) of the electron transport chain. This is further evidence for a generalized physical interaction by PL with membrane-bound or associated proteins.

Figure 32. Effect of pantoyl lactone on respiration by turkey heart mitochondria (Dixon plot). K_{PL} = 47 mM.

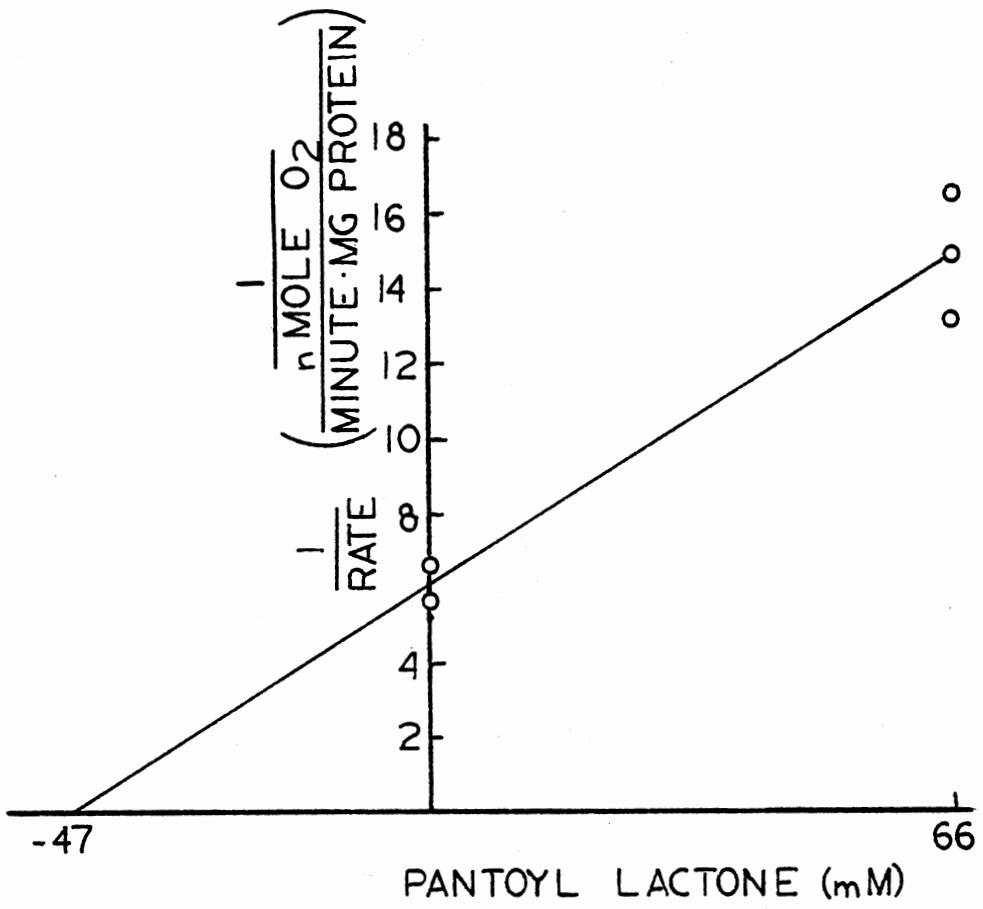
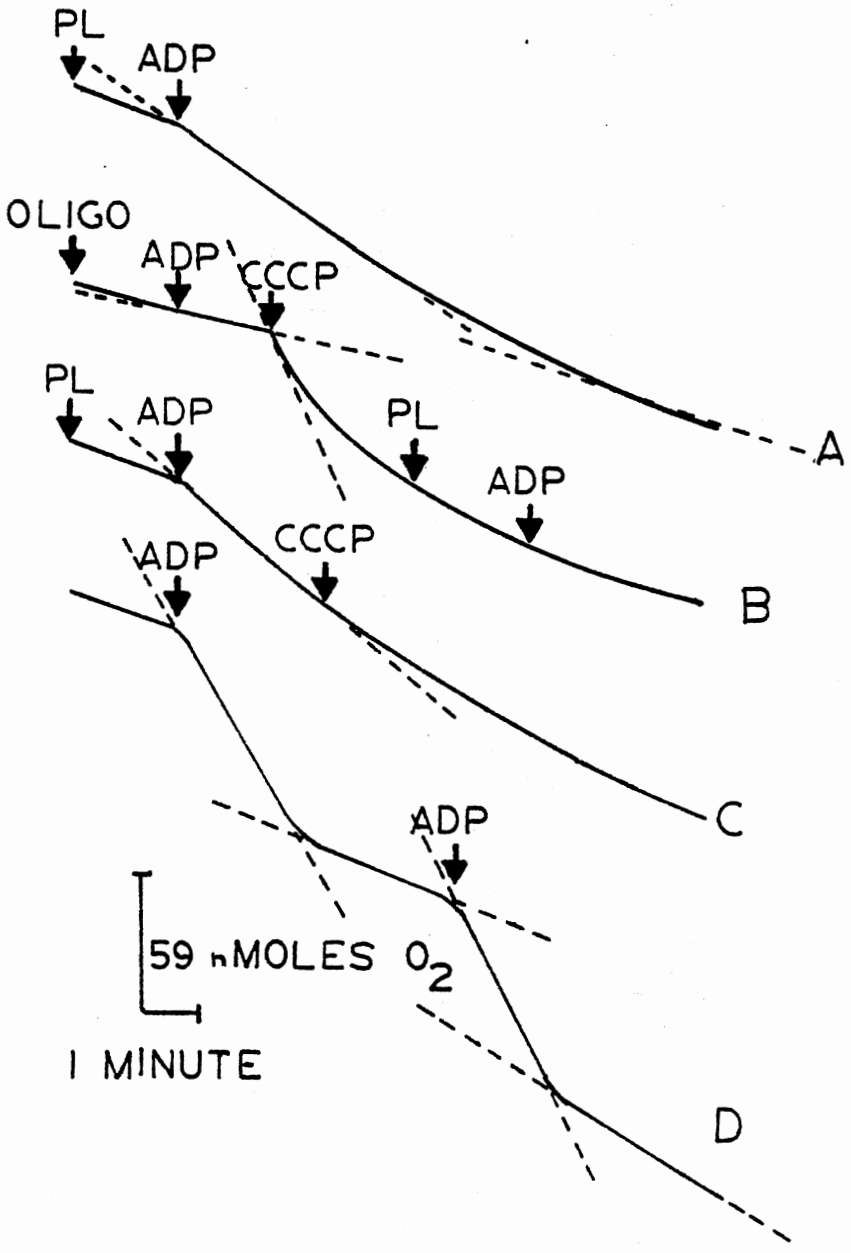

1/RATE (1/n MOLE O2 / MINUTE·MG PROTEIN)
18
16
14
12
10
8
4
2
-47
66
PANTOYL LACTONE (mM)

Figure 33. Effect of CCCP on oligomycin and pantoyl lactone induced inhibition of respiration (rate tracings) Concentration of reagents added at arrows are given in Materials and Methods. D, control tracings; A and C, PL mediated inhibition of respiration; B, reversal of oligomycin (oligo) inhibition of respiration by CCCP; C, non-reversable nature of PL inhibition.


PL
ADP
OLIGO
ADP
CCCP
A
PL
ADP
PL
ADP
B
ADP
CCCP
C
ADP
59 nMOLES O_2
1 MINUTE
D

CHAPTER IV

DISCUSSION

The data reported clearly indicate that PL (in the concentrations used to revert filaments) has profound effects upon the energetics of a cell. Transport, H^+ extrusion, respiration, and membrane-bound forms of both Mg^{++} and Na^+/K^+-activated ATPases are inhibited. The concentration of PL needed to inhibit these vectorial reactions varies over an approximately three to five-fold range. This variation can be correlated with the sideness of the particular enzymatic system and probably indicates that PL is not readily transported across the cytoplasmic membrane. For example, transport, H^+ extrusion and ATPase activity of isolated membranes indicate K_{PL} values in the 150-250 mM range while enzymatic systems that are trans-membranous or everted (electron transport chain and the ATPase of electron transport particles) indicate K_{PL} values in the 40-50 mM range. The only solubilized system that has been determined to be sensitive to PL (luciferase), has an even lower K_{PL} (20mM). In addition, equilibrium dialysis experiments and the uptake of labelled PL have been uniformly negative in attempts to demonstrate accumulation of PL by cells. Such data indicate a permeability problem

and, in part, explain the non-physiological concentrations of PL required.

All transport systems that demonstrated saturation kinetics are mediated by a permease. Permeases can be described as membrane associated proteins that catalyze the movement of solutes across biological membranes and are an integral part of all proposed models of active transport. Of all transport systems tested, D-glucose accumulation by S. faecalis is the only one not inhibited by PL. It is also the only one that is energized by substrate level phosphorylation as opposed to a membrane potential which is the driving force behind the other transporters. This information leads one to postulate that PL is inhibiting transport by interfering with those enzymatic systems that generate the membrane potential. As reported in this thesis, PL does inhibit such enzymes (ATPase and electron transport chain). However, the non-competitive kinetics of transport inhibition indicate that specific permeases are probably also affected.

My results support generalized physical interactions with membranes as a mechanism of action for PL (Johnson et al., 1980). In addition, a profound effect on cellular energetics is indicated. Correlation of these results with PL's effect on growth and division indicate an involvement of the membrane potential with these processes. Inhibition of growth by PL can be directly attributed to the inhibition of transport which can, in turn, be at least partly linked to

inhibition of membrane-bound ATPase and the respiratory system. Stimulation of division by PL suggests an involvement of membrane potential in this process. However, there is, as yet, no clear relation between division and the membrane potential. Cell division is intimately associated with the cell membrane (Grula and King, 1971; Harold, 1972) thus implying that vectorial reactions may be associated with cell division. Filament formation induced by diverse agents results in altered cell membranes (Grula and King, 1971; Grula and Hopfer, 1972; Johnson, 1978) which could easily alter a cell's ability to maintain or respond to the membrane potential. In addition to PL, several other compounds which either potentiate or retard filament formation can also alter the membrane potential. For example, inclusion of high levels of NH_4^+ in media will often result in shorter cells (Grula, 1970). Ammonia is accumulated in response to the membrane potential and can act as a mobile cation thereby depleting the membrane potential. Inclusion of glucose on the other hand often results in longer cells (E. Grula, 1960; M. Grula, 1970). Glucose can be viewed as an excellent substrate for growth and energy production or as a repressor of oxidative phosphorylation (Cavari et al., 1968; Hempfling, 1970; Sanwal, 1970). The effect of glucose on membrane potential would depend upon which of the above effects predominated in the particular organism being utilized. Aeration which would certainly favor formation of larger membrane potentials is important for good filament

formation (E. Grula, 1960). The medium pH has a distinct effect on filament formation (Grula and Grula, 1961). Raising the pH to 7.5 which is equivalent to the internal pH maintained by most cells (Harold, 1972), results in smaller cells and ΔpH.

Using the above information, it would seem that an increase in the membrane potential is associated with increased cell length. However, some lines of evidence exist which are contrary to such an association. Primarily, 2,4-dinitrophenol, which destroys the membrane potential, does not cause filaments to divide and, in fact, prevents PL-induced division (Grula and Grula, 1962). Also, Felle et al. (1978) used microelectrodes and fluorescent dyes to measure the membrane potential of normal and penicillin-induced giant cells of Escherichia coli and found that no substantial differences exist. Finally, Harold and Van Brunt (1977) using enriched media in addition to elevated K^+ levels and pH demonstrated that S. faecalis grew normally in the presence of gramicidin and other ionophores under conditions wherein there was no membrane potential.

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VITA 2

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