THE MECHANISM OF ACTION OF A NEW HYDROXY-BENZINDAZOLE ON *PSEUDOMONAS FLUORESCENS*

By

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CHAPTER I

INTRODUCTION

Nitrogen containing compounds composed of fused heterocyclic ring systems exhibit a wide spectrum of biological activity. Compounds of this type have been isolated from natural sources as well as synthesized de novo (Elderfield, 1957). The importance of these molecules can be exemplified by the purine base adenine, an essential component of nucleic acid structure, the coenzymes, nicotinamide adenine dinucleotide (NAD) and adenosine triphosphate (ATP), which function in energy storage and transfer within the cell, as well as participating in numerous other cellular reactions.

Heterocyclic nitrogen compounds have been found to inhibit or to interfere with biological functions. The antibiotic, pyocyanine, produced by Pseudomonas aeruginosa, is a potent bacteriocidal agent against gram-positive organisms (Hays et al, 1945; Cavallito et al, 1949). In 1960, Newton proposed that antrycide inhibits the incorporation of purine into RNA thereby acting as a bacteriostatic agent. Another azaheterocycle, 6-chloro-7-methyl-9-isoalloxazine, inhibits riboflavin phosphorylation in Lactobacillus casei (Scala and Lambooy, 1958). Maxwell and Brody (1971) demonstrated the antifungal activity of a 2-benzimidazole carbamic acid methyl ester, but no attempt was made to elucidate the mechanism or site of action.

Nitrogen containing steroids have been reported to possess

antimicrobial activity. Barnett, Ryman, and Smithe (1946) noted the antibacterial action of epimeric-7-amino cholesterols. Smith and Shay (1965) demonstrated the ability of 5-azasteroids to lyse protoplasts of Sarcina Lutea possibly through direct action on the cell membrane. Silver and Levine (1968) have shown that steroidal mono- and diamines affect bacterial membranes by increasing leakage of intracellular material, and altering uptake ability. A new class of diazasteroids structurally related to equilenin have been reported to inhibit both gram-negative and gram-positive bacteria as well as to potentiate the actions of several antibiotics (Chesnut et al, 1971).

Indazole compounds, which may be used as precursor molecules for the synthesis of azasteroids have not been found in nature since the ring system results from the ortho condensation of a benzene ring with a pyrazol ring (Fig. 1). Since many positions on the compound are readily substituted, numerous examples of indazole derivatives have been reported (Elderfield, 1957). While literature reports on the chemistry of indazole compounds date to Fischer and Kuzel (1883), the biological activity of these compounds has only recently been reported.

Derivatives of indazole compounds exhibit a wide variety of actions in mammalian systems. Northover (1967) reported the anti-inflammatory and anti-vascular constriction activity of 1-H-indazole. Benzyl derivatives of 1-H-indazole have also been shown to possess anti-inflammatory properties (Fala and Silverstini, 1965). Indazole compounds also affect the central nervous system. Adler and Albert (1963) reported mixed stimulatory and depressant effects of a 1-H-indazole. Similarly, Batulin (1965) found that a 3-propoxy-1-H-indazole compound depressed polysynaptic, not monosynaptic reflexes, and depressed the

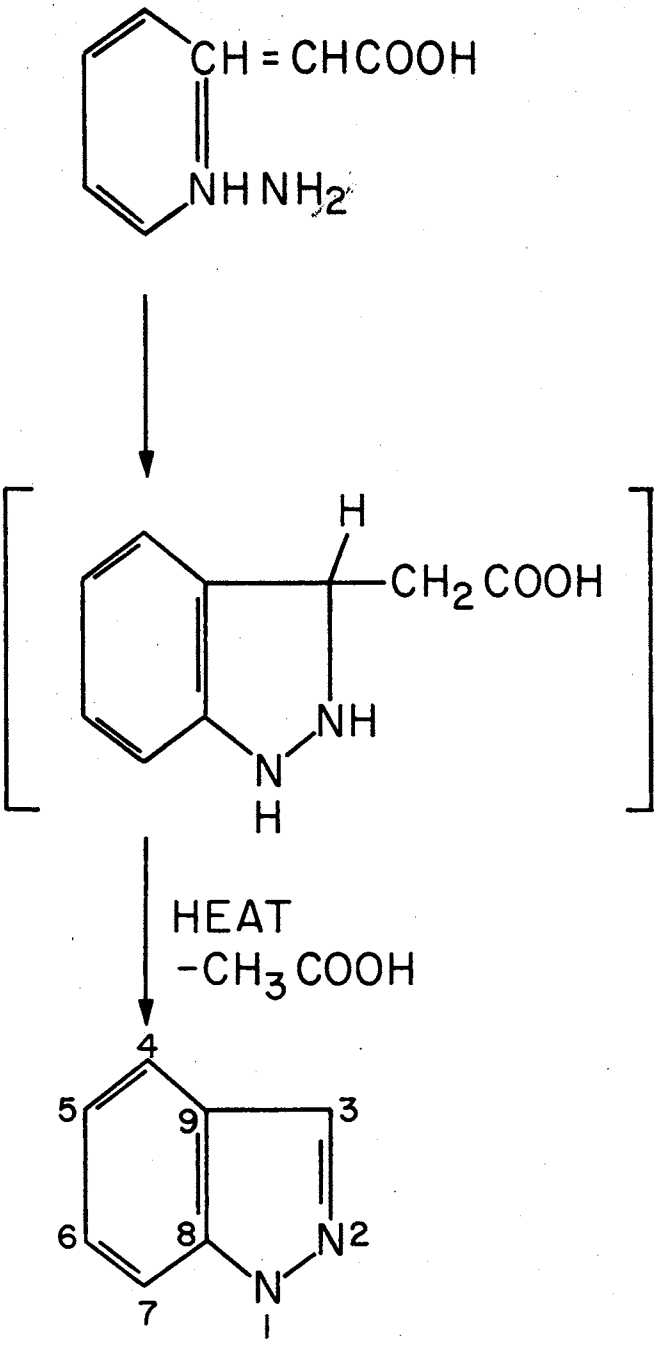

Figure 1. Synthesis of Indazole by Thermal Decomposition of O-hydrazinocinnamic Acid (Elderfield, 1957)

turnover reflexes an action which could be termed as a nephesin type central myorelaxant. Enzymatic activity is affected by 1-H-indazole which inhibits liver alcohol dehydrogenase by competitively blocking ethanol binding to the enzyme (Theorell and Yonetani, 1969).

In plants, 3-indazole acetic acid regulates plant growth by inhibiting avena coleoptile section growth and cucumber root growth (Hellman, Sell, and Witler, 1961). Silverstrini, Tagliopictra, and Angelini (1968) reported inhibition of Escherichia coli, Staphylococcus aureus, Bacillus subtilis, and Candida albicans by a 0.02 percent solution of benzydazime. More recently, this compound has been shown to potentiate the action of tetracycline against gram-negative bacteria (Ravagnan and Cipriani, 1969), although the precise mechanism or site of action has not been elucidated.

A new hydroxybenzindazole compound containing nitrogen in the 11 and 12 positions has been synthesized (Morgan et al, 1971). The unique chemical structure affords the possibility of studying several important biological properties (Fig. 2). The polar properties of the molecule are contributed by the diamine and hydroxyl groups, whereas the nonpolar moiety results from the fused benzene ring. At physiological pH the hydroxybenzindazole exhibits a net negative charge due to ionization of the hydroxyl group and as a result, the compound has the potential to participate in ionic bonding to positively charged cellular components. The compound is water soluble and stable for at least 72 hours at 37 C with constant shaking.

Preliminary tests of the biological activity of the hydroxybenzindazole showed the compound inhibited growth of B. subtilis W_{23} and E. coli B. These results differed from those reported for P. fluorescens

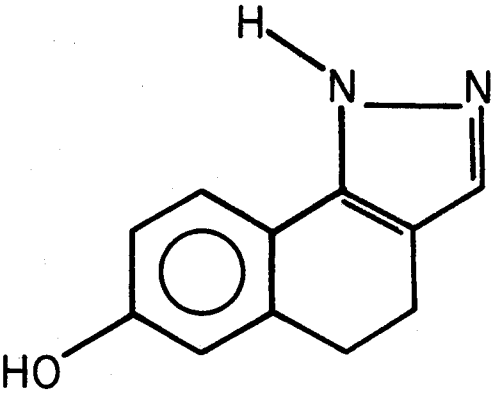

Figure 2. The Molecular Structure of the Hydroxybenzindazole

in which the hydroxybenzindazole did not show a pronounced effect on the growth of the organism but did increase the rate of lysis upon continued incubation (Chesnut et al, 1972). The enhanced lysis of P. fluorescens was of particular interest because studies have shown that members of the genus Pseudomonas produce fatal infections in hospitalized cancer patients (Dupont and Spink, 1969; Viola, 1967) and are generally unaffected by most antibacterial agents. The pseudomonad cell envelope, which acts as a permeability barrier, may play an important role in this resistance.

There are three layers involved in the structure of the cell envelope: the outer layer of lipopolysaccharide (LPS) and protein, the cell wall, and the cell membrane (Kellenberger and Ryter, 1958; Murray, Steed, and Edison, 1965; Weidel, Frank, and Martin, 1960).

The outer layer containing LPS is unique for gram-negative bacteria. Wardlaw (1964), as well as Bayer and Anderson (1965), have proposed a mosaic-type structural model in which LPS underlies a discontinuous layer of lipoprotein. In some organisms including E. coli, Salmonella typhimurium, and Serratia marcescens, the globular protein granules, in complex with the LPS and phospholipid, are covalently linked to the peptiodoglycan. This linkage may provide an attachment site for the complex (Braun, Rehn, and Wolff, 1970; Fishman and Weinbaum, 1967; Weidel, Frank, and Martin, 1960).

Structurally the LPS is a covalently linked macromolecule composed of the O-polysaccharide, the R core, and the lipid A. Components specifically identified in P. fluorescens are 2-keto-3-deoxyoctanate (KDO), galactosamine, glucosamine, galactose, glucose, rhamnose, and phosphatidylethanolamine (Wilkinson, 1971). The Pseudomonads contain

hydroxylauric and hydroxydecanoic acids in the lipid A moiety rather than the hydroxymyristic acid found in the enteric bacteria (Hancock, Humphreys, and Meadow, 1970). Two unusual sugars found in the envelope of P. fluorescens are 2-amino-2,6-dideoxyglucose and 3-amino-3,6-dideoxyglucose (Wilkinson and Carby, 1971).

Functionally there is little known about the LPS layer. The LPS macromolecule determines the O-antigen specificity, is responsible for endotoxic activity, acts as a receptor site for bacteriophage, and is implicated in certain cellular functions including permeability (Wright and Kanegasaki, 1971). The release of LPS from E. coli by various methods is accompanied by an increased sensitivity to actinomycin D (Leive, 1965; Roy and Mitra, 1970). The loss of LPS may be responsible for increased permeability to the antibiotic. Normally, the gram-negative bacteria are sensitive to lysozyme action only after the LPS has been altered or removed by EDTA treatment (Stolp and Starr, 1965). Mutants of E. coli and Salmonella which lack heptose in the LPS are susceptible to disruption by heat, detergents, and lysozyme (Tamaki, Sato, and Matsuhashi, 1971).

Therefore, it is possible that the LPS layer of the cell envelope may function both as a permeability barrier and as a protective stabilizing factor.

The murein layer or cell wall is found in all bacteria except Mycoplasma and some obligatory halophiles. Generally, the primary supporting structure of the cell wall, the peptidoglycan, consists of polysaccharide chains of alternating residues of two animo sugars N-acetyl-glucosamine and N-acetyl-nuramic acid, linked by 1-4 β bonds. Occasionally, the sugars have O-acetyl substitutions. The

polysaccharides are crosslinked by short peptides containing a limited number of amino acids among which D-glutamic acid and D-alanine are always present. In many cases, L-alanine and either L-lysine or $\alpha;\varepsilon$-diaminopimelic acid are found in the peptide. These peptides are crosslinked directly or by the insertion of various amino acids which serve as a bridge between the peptides (Ghuysen, Strominger, and Tipper, 1968; Roges and Perkins, 1968; Salton, 1964).

The cell wall conveys a definite shape to the microorganism and protects the cell. Bacteria with the murein layer survive in a hypotonic environment while loss of the cell wall renders cells osmotically sensitive. For example, penicillin specifically interferes with synthesis of the cell wall and converts bacteria to protoplasts resulting in lysis (Lederburg, 1957; Park and Strominger, 1957). Lysozyme acts at the murein layer breaking the amino sugar polymer at 1-4 linkages (Pelzer, Maass, and Weidel, 1963). Resistance to lysozyme can be conferred by increasing O-acetyl substitution which increases the complexity of the crosslinked peptidoglycan structure (Johnson and Campbell, 1972).

The third layer of the bacterial cell envelope, the cell membrane, was first demonstrated by Weibull (1953) who succeeded in stabilizing protoplasts by placing them in an osmotically protected environment. While the exact structure of the membrane is still subject to debate, the composition of bacterial membranes consists essentially of protein and phospholipid. Membrane proteins may exist in both the α-helical and β-sheet forms with some proteins appearing to exhibit amphipathic properties (Singer and Nicolson, 1972). The membrane phospholipids appear to be arranged in a bilayer structure created by hydrophobic

interactions of fatty acid chains and by ionic association of the polar heads with the water phase. However, there is little evidence to indicate if this bilayer is continuous or interrupted (Singer, 1970).

Functionally, the cell membrane is involved with permeability, electron transport, certain enzymatic activities, and DNA replication. The cell membrane also stabilizes the cell as changes or interactions with the membrane structure can lead to lysis. Alterations in lipid composition affect the elasticity of the membrane in responding to environment stress such as osmotic shock or sonification (Razin, 1969). Interactions with the hydrocarbon chains of phospholipids, which result in reduced fluidity of the chains have been implicated in the lytic action of the antibiotic chlorothricin (Pache and Chapman, 1972). Other antibiotics also exert bacteriocidal action through changes in the bacterial membrane. Polymyxim B and circulins, peptide antibiotics derived from species of Bacillus, combine with and disorganize the membrane which results in release of intracellular components and disruption of osmotic equilibrium (Newton, 1953; Newton, 1956). The action is similar to disruption of cell membranes by cationic detergents (Baker, Harrison, and Miller, 1941). Green, et al (1967) suggested that detergents are responsible for breaking bonds between the hypothesized membrane subunits.

The enhanced lysis of P. fluorescens by the hydroxybenzindazole compound could involve either the alteration of-or interaction with one or all of the layers of the cell envelope. The purpose of the research was to study in detail the effect of the hydroxybenzindazole on P. fluorescens, and to delineate macromolecular changes which could be associated with action of the compound.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The microorganism used throughout this study was a species of Pseudomonas which was tentatively identified as Psuedomonas fluorescens (Montgomery, 1966). Stock cultures were maintained on 0.2 percent succinate agar slants and stored at 4 C.

Media

A synthetic salts medium composed of 0.2 percent NaCl, 0.2 percent NH_4Cl, 0.32 percent KH_2PO_4, 0.42 percent K_2HPO_4, and 0.2 percent succinate was utilized in this study. The medium was adjusted to pH 7.0 with KOH, sterilized by autoclaving at 120 C with 15 lbs. pressure per square inch for 15 minutes, and cooled to room temperature. A sterile trace minerals solution (0.1 ml) was added to each 100 ml of the medium. The trace minerals solution consisted of 5.0 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $MnSO_4$, 1.0 g of $FeCl_3$, and 0.5 g of $CaCl_3$ in 100 ml of glass distilled water. When solid medium was desired, agar (Difco) was added to a final concentration of 2.0 percent. In this study, the term minimal salts medium refers to the basal salts medium lacking the carbon source. Succinate salts medium refers to the complete synthetic salts medium.

Growth of Cells

Tubes containing 5.0 ml of succinate salts medium were inoculated from a stock culture slant and incubated for 12-16 hours at 37 C on a reciprocal action shaker (100 movements per minute). These cells were used as the inoculum for growth studies. Growth was determined by measuring the increase in absorbance of the culture at 540 nm using a Coleman Junior II spectrophotometer. Growth assays were performed either in test tubes (18 mm light path) containing a total liquid volume of 7.0 ml or in 250 ml side arm flasks containing a total liquid volume of 35 ml. Fernbach flasks containing 500 ml of medium were utilized for larger cell volumes.

Hydroxybenzindazole

The hydroxybenzindazole was furnished by Dr. K. D. Berlin (Morgan, et al, 1971). The hydroxybenzindazole was dissolved with mild heating in 0.1 N HCl and sterile glass distilled water was added to the heated solution to give the desired final concentration. The solution was adjusted to pH 7.0 with KOH and added to the growth medium just prior to use. The hydroxybenzindazole was readily soluble at the concentrations used in this study. Purity of the compound was initially greater than 99.7 percent as indicated by mass spectral analyses. These data were substantiated by nuclear magnetic resonance (N. M. R.) and elemental analyses.

Antibiotics

Actinomycin D (Merk, Sharp, and Dohme Research Laboratory) and

Chloramphenical (Park, Davis, and Company) were dissolved in sterile glass distilled water to a concentration of 100 μg/ml and stored at 4 C.

Spectrophotometric Studies

The ultraviolet spectrum of an aqueous solution of either the hydroxybenzindazole (5×10^{-5}M), actinomycin D (1.03×10^{-5}M), or EDTA (5×10^{-5}M) was determined at room temperature in quartz cuvettes using a Cary 14 recording spectrophotometer. The absorbance of each compound and a mixture of either actinomycin D or EDTA with the hydroxybenzindazole was determined between the wavelengths of 185-500 nm.

Viability of Cells Grown in the Presence of the Hydroxybenzindazole

Cells were grown in 35.0 ml succinate salts medium containing 140 μg/ml of the hydroxybenzindazole or an equal amount of sterile glass distilled water. The cells were grown to late logarithmic phase as determined by absorbance at 540 nm, 1.0 ml samples were taken at various time intervals over a period of 12 hours. These samples were diluted and duplicate 0.1 ml aliquots of each dilution were spread with a sterile glass rod on plates containing 15 ml of 0.2 percent succinate salts agar. The plates were incubated at 37 C for 24 hours and counted.

Studies on Reversibility of the Hydroxybenzindazole Action

Cells were grown in succinate salts medium containing 140 μg/ml of the hydroxybenzindazole or an equal volume of sterile glass distilled water. At the indicated times, 5 ml samples were taken, centrifuged

(12100 x g), washed with potassium phosphate buffer (pH 7.2, 1 x 10^{-2}M), and suspended to the same absorbance in 5 ml succinate salts medium in the presence and absence of the hydroxybenzindazole. Growth of the cells was followed to determine if the hydroxybenzindazole effect on growth was reversible.

Binding of the Hydroxybenzindazole to Whole Cells

Absorbance of the hydroxybenzindazole at 270 nm increases linearly with the concentration of the compound. To quantitate binding, cells were grown overnight in 30.0 ml succinate salts medium, harvested, washed once with potassium phosphate buffer (1 x 10^{-2}M), and suspended to an A_{540} of 0.30 in tubes containing buffer and the various concentrations of hydroxybenzindazole. The absorbancy of the solution in each tube had previously been measured at 270 nm on a Beckman DU spectrophotometer. After incubation at 37 C for 15 minutes, the cells were centrifuged, the supernatant solution decanted, and the absorbancy measured at 270 nm. The amount of bound hydroxybenzindazole was determined by calculating the decrease in absorbance of the supernatant solution at 270 nm after incubation with the bacterial cells. A correction was made for any protein or nucleic acid material absorbing at this wavelength as determined from measurement of a control.

Uptake Studies

Cells were grown for 16 hours in 30 ml of succinate salts medium in the presence and absence of 140 μg/ml of the hydroxybenzindazole. The cells were harvested, washed once with potassium phosphate buffer

(1×10^{-2}M), and suspended to an A_{540} of 0.20 in 30 ml of succinate salts medium containing the same concentration of hydroxybenzindazole in which the cells were originally grown. The cells were incubated at 37 C for 30 minutes in a shaking water bath and a 5 ml sample from each flask was added to 1.0 ml of the following: 140 µg/ml of the hydroxybenzindazole or an equal volume of glass distilled water; 0.2 µc/ml labelled compound; 0.5 mg "unlabelled" carrier; 0.5 µg/ml chloramphenicol. At appropriate time intervals, a 0.5 ml sample was filtered through a millipore membrane filter (10 mm diameter; 0.45 micron pore size), washed twice with 1.0 ml potassium phosphate buffer at room temperature, and placed in scintillation counting vials. The filters were dried (room temperature, 6 hours) and 10 ml of Aquasol counting fluid (New England Nuclear) added to each vial. The radioactivity was determined using a Nuclear Chicago liquid scintillation spectrometer (Model No. 722). Under these conditions, the counting efficiency was 40 percent.

The radioactive compounds tested were L-serine-3-^{14}C (specific activity 24.3 mCi/mM), uracil-2-^{14}C (specific activity 40.6 mCi/mM), and succinic acid-2,5-^{14}C (specific activity 1.8 mCi/mM).

Potentiation of Actinomycin D Activity

The ability of the hydroxybenzindazole to potentiate the action of actinomycin D was determined by growing the cells in 5 ml succinate salts medium to which was added either 140 µg/ml hydroxybenzindazole, 5 µg/ml actinomycin D, the two in combination, or an equal volume of sterile glass distilled water. Growth was followed for 24 hours, then all samples were centrifuged, washed once with potassium phosphate

buffer (1×10^{-2}M), and suspended in 7 ml succinate salts medium. Growth was again observed for 24 hours to determine if the effects of the compounds were bacteriocidal or bacteriostatic.

Phospholipid Quantitation

Cells were grown in 250 ml side arm flasks containing 70 ml succinate salts medium in the presence and absence of 140 μg/ml of the hydroxybenzindazole. Sterile glass distilled water was used to bring the final liquid volume of all flasks to 100 ml. At approximately mid-logarithmic phase (A_{540} of 0.5), 25 μC of ^{14}C-sodium acetate (specific activity 0.25 mC per 0.373 mg) was added to each flask to permit labelling of the phospholipids. Growth was continued until early stationary phase (A_{540} of 0.80). The cells were harvested, washed once with minimal salts medium, and suspended in 100 ml minimal salts medium to an A_{540} of 0.75. The suspensions were centrifuged and the pellets extracted according to the procedure of Folch, Lees, and Sloane (1957). Methanol (20 ml of 100 percent) was added to each pellet and heated for 30 minutes at 55 C under an atmosphere of nitrogen. Chloroform (40 ml) was added and the nitrogen atmosphere re-established. After treatment of the suspension for 12 hours at room temperature, the cells were carefully filtered out. The solution was washed twice with equal volumes of KCl (2M), once with an equal volume of glass distilled water, and concentrated by rotary evaporation to a volume of 2 ml. This chloroform solution was stored under nitrogen at 4 C.

The extracted lipid material was spotted in microliter volumes on 5 x 20 silica gel G chromatography plates (Quantum Industries), and chromatographed in a solvent of chloroform; methanol; HOH (65: 25: 5).

The plates were developed with rhodamine G, ninhydrin, or in an iodine vapor chamber and the gel from the spots was scraped into scintillation vials. Aquasol (10 ml) was added to the vials, and radioactivity determined by counting in a liquid scintillation spectrometer.

Analysis of Total Protein

The total protein of whole cells was determined by the method of Koch and Putnam (1971). Cells were grown overnight in 30 ml succinate salts medium with various concentrations of the hydroxybenzindazole. The cells were harvested, washed once with sterile potassium phosphate buffer (1×10^{-2}M) and suspended in buffer to an A_{540} of 0.75. A 10 ml sample of each cell suspension was centrifuged, dispersed in 1 ml copperless reagent (115 ml of 10 N NaOH, 12.5 ml NH_4OH, and 125.5 ml H_2O), and heated at 100 C for 5 minutes. The samples were cooled to room temperature and 4 ml of the copper containing reagent (115 ml 10 N NaOH, 12.5 ml NH_4OH, 0.625 g $CuSO_4 \cdot 5\ H_2O$, and 125.5 ml H_2O) was added. After 30 minutes, the absorbances of the samples at 330 nm and 390 nm were read on the Beckman DU spectrophotometer. The total protein (mg) was calculated by subtracting the absorbance at 390 nm from the absorbance at 330 nm, and multiplying this figure by 4.02 (the constant used in determination of total protein by the 2 wavelength method).

Quantitation of 2-keto-3-deoxy-octosonic Acid (KDO)

KDO was determined by the method of Cynkin and Ashwell (1960). Cells were grown to the maximum stationary phase in 500 ml succinate salts containing the indicated concentrations of hydroxybenzindazole.

After the suspensions were adjusted to an A_{540} of 0.70, 200 ml samples were centrifuged, the supernatant solution decanted, and the cell pellet lyophilized. The decanted supernatant solution was filtered, dialyzed against glass distilled water for 48 hours at 4 C, and lyophilized. Both the lyophilized pellet and supernatant solution were treated with 1.0 ml of 0.1 N H_2SO_4 in a boiling water bath for 30 minutes and centrifuged. The solution was decanted and 0.25 ml assayed for KDO by oxidation with 0.25 ml periodic acid (0.025 N in 0.125 N H_2SO_4) at 55 C for 25 minutes. Addition of 0.5 ml sodium arsenite (2.0 percent in 0.5 N HCl) stopped the oxidation. The resultant cleavage products were reacted with 2.0 ml thiobarbituric acid (0.3 percent) in a boiling water bath for 12 minutes and the color read at 532 nm using a Beckman DU spectrophotometer.

The color from the β-formylpyruvate-thiobarbituric acid complex was faded with the addition of 0.1 ml saturated aqueous solution of NaOH since the complex is unstable in an alkaline environment. The remaining color was due to the malonaldehyde-thiobarbituric acid complex resulting from deoxyribose (Waravdeker and Saslaw, 1959). KDO content was calculated by correcting for the remaining deoxyribose color.

Levels of KDO During Reversal of Hydroxybenzindazole Inhibition of Growth

To determine if reversal of the hydroxybenzindazole growth inhibition was accompanied by an increase in thiobarbituric acid positive material, cells were grown to late logarithmic phase in 40 ml succinate salts medium in the presence and absence of 140 μg/ml of the hydroxybenzindazole. The cell suspensions were centrifuged at 4340 x g, washed

once with potassium phosphate buffer (pH 7.0, 1 x 10^{-2}M), suspended to an A_{540} of 0.55 in 50.0 ml of succinate salts medium, and incubated at 37 C. Growth was followed by measuring the change in absorbance at 540 nm until reversal of the hydroxybenzindazole inhibition was established. During this period, samples were taken at various time intervals and the KDO level quantitated by the method of Cynkin and Ashwell (1960) as previously described in the text.

Lysis of Cells by Ethylenediaminetetraacetic Acid (EDTA)

Cells were grown to maximum stationary phase in 5 ml succinate salts medium in various concentrations of hydroxybenzindazole. The cells were harvested and suspended to an A_{540} of 0.4 in 5 ml EDTA (pH 8.0, 1 x 10^{-3}M), or 5 ml of a mixture of the hydroxybenzindazole and EDTA in the indicated molar ratios. Lysis was followed for 2 hours by measuring the decrease in absorbance at 540 nm using a Coleman Junior II spectrophotometer.

Effect of a Low Mg^{++} Environment on KDO Level

A limited Mg^{++} environment was achieved by alteration of the Mg^{++} content of the trace mineral solution added to succinate salts medium. Normally the trace mineral solution contained 5.0 g of $MgSO_4 \cdot 7H_2O$ which resulted in a final Mg^{++} concentration of 50 µg/ml. A mineral solution containing 0.10 g of $MgSO_4 \cdot 7H_2O$ was prepared which reduced the final Mg^{++} concentration to 1 µg/ml. This concentration of Mg^{++} (1 µg/ml) has been reported to be limiting for Pseudomonas species (Brown and Melling, 1968).

Cells were grown to stationary phase in 20.0 ml of succinate salts medium containing 1 μg/ml Mg^{++} and 50 μg/ml Mg^{++}. The cell suspensions were centrifuged at 12100 x g, washed once with potassium phosphate buffer (pH 7.0, 1 x 10^{-2}M) and suspended to an A_{540} of 0.60. Samples (10 ml) of each suspension were assayed for KDO content according to the procedure of Cynkin and Ashwell (1960).

Effect of Limited Mg^{++} Environment on the Action of Actinomycin D

Cells were grown in 5 ml succinate salts medium containing either 1 μg/ml Mg^{++} or 50 μg/ml Mg^{++} in the presence and absence of 3 μg/ml actinomycin D. All samples were centrifuged, washed once with potassium phosphate buffer, suspended in 7 ml succinate salts medium, and growth was followed.

Effect of Mg^{++} on the Action of Hydroxybenzindazole

Cells were grown in 5 ml succinate salts medium containing 2.5 x 10^{-4} (60 μg/ml), 1 x 10^{-3} (250 μg/ml), 1.8 x 10^{-3}M (450 μg/ml), 2 x 10^{-3} (500 μg/ml), and 4 x 10^{-3} (1,000 μg/ml) M Mg^{++} in the presence and absence of 1 x 10^{-3}, 0.5 x 10^{-3}, or 2 x 10^{-3}M hydroxybenzindazole. Growth of the cells was followed to determine the effect of different Mg^{++} levels on the action of the hydroxybenzindazole.

CHAPTER III

RESULTS AND DISCUSSION

Characterization of Hydroxybenzindazole Action

Effect of the Hydroxybenzindazole on Growth of P. fluorescens

Experiments were conducted to determine the effect of the hydroxybenzindazole on the growth of P. fluorescens cells in succinate minimal salts medium supplemented with various concentrations of the hydroxybenzindazole.

The effect of the hydroxybenzindazole on growth of P. fluorescens was concentration dependent with the maximum effect occurring at a concentration of 280 µg/ml (Fig. 3). Growth in both the 280 and 140 µg/ml concentrations of the hydroxybenzindazole increased the growth lag and apparently decreased the total cell mass. Cells grown in 70 µg/ml of the compound also exhibited a lowered total cell mass. After 10 hours of growth, all three hydroxybenzindazole concentrations caused a significant drop in absorbance. When these cultures were observed microscopically, few if any intact cells could be seen. Growth in 35 µg/ml of the compound also resulted in an enhanced lysis, but the extent of cell damage appeared to be less extensive compared to higher concentrations of the hydroxybenzindazole.

The concentration dependent increased rate of lysis which resulted

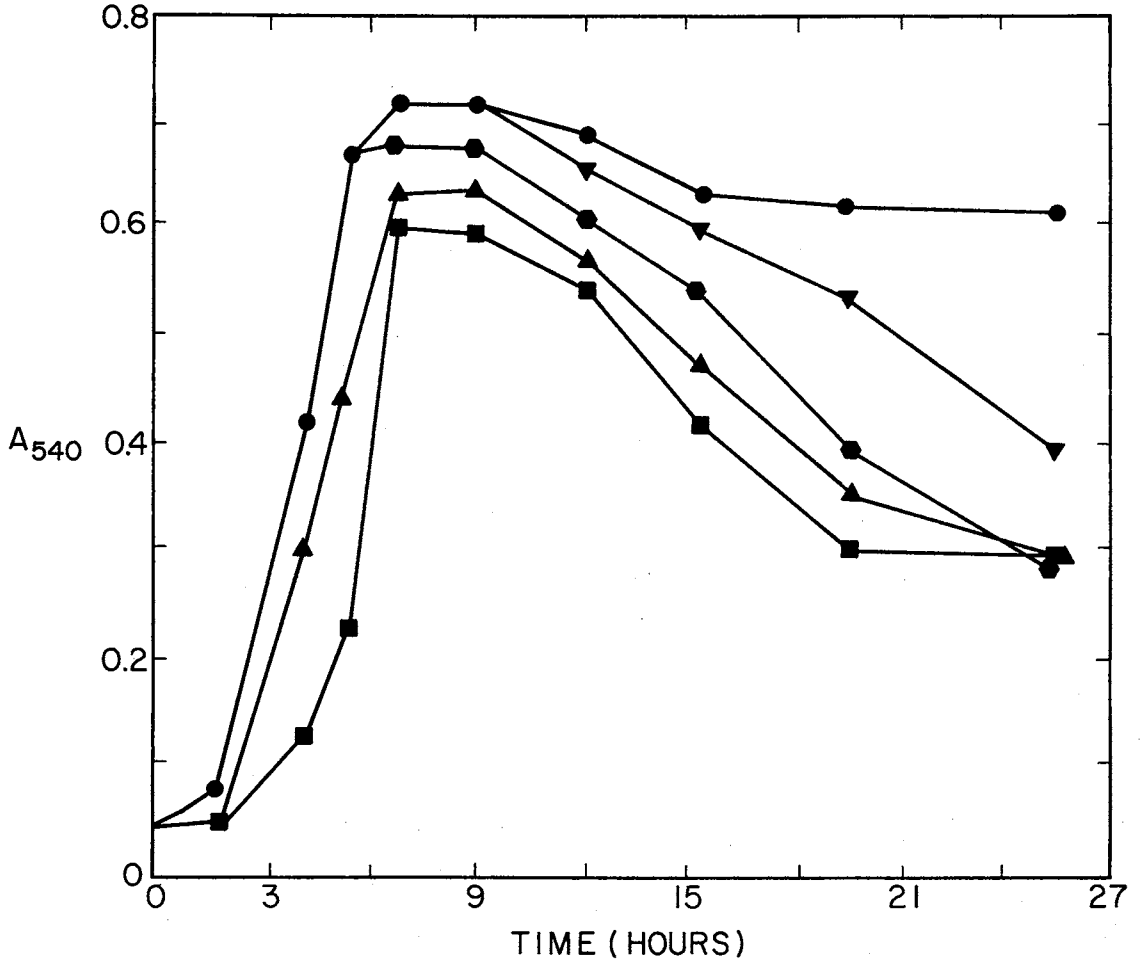

Figure 3. Effect of Growth in the Hydroxybenzindazole on P. fluorescens ●, control; ▼, 35 µg/ml hydroxybenzindazole; ⬢, 70 µg/ml hydroxybenzindazole; ▲, 140 µg/ml hydroxybenzindazole; ■, 280 µg/ml hydroxybenzindazole

from growth in the compound suggested that the action of the hydroxybenzindazole may involve an alteration of the cell surface.

Viability of Cells Grown in the Presence of the Hydroxybenzindazole

The effect of the hydroxybenzindazole on the viability of P. fluorescens was determined using cells grown in succinate salts medium in the presence and absence of the compound. At various times, aliquots were diluted, and duplicate 0.1 ml samples were plated on succinate salts agar. The plates were incubated at 37 C for 24 hours, and the colonies counted to determine the number of cells per ml.

Data demonstrated that cells grown in the hydroxybenzindazole showed a nearly complete loss of cell viability corresponding to an observed drop in culture absorbance (Fig. 4). In contrast, under the same conditions control cells exhibited only a slight drop in culture absorbance and cell viability.

Another important aspect of this experiment was the indication that, prior to the onset of lysis in hydroxybenzindazole grown cells, the absorbancy of the cell suspension was indicative of an actual cell viability very similar to that of control cells at the same absorbancy. After 12 hours of growth, the number of cells per ml grown in the compound at an absorbancy of 0.60, corresponded to the number of control cells per ml, 2.19×10^9, when measured at the same absorbancy. This information suggested that growth in the presence of the compound did not drastically alter cell viability prior to maximum stationary phase, and that the absorbancy measurement of cells growing in the hydroxybenzindazole, prior to the onset of lysis, was a reliable indicator of

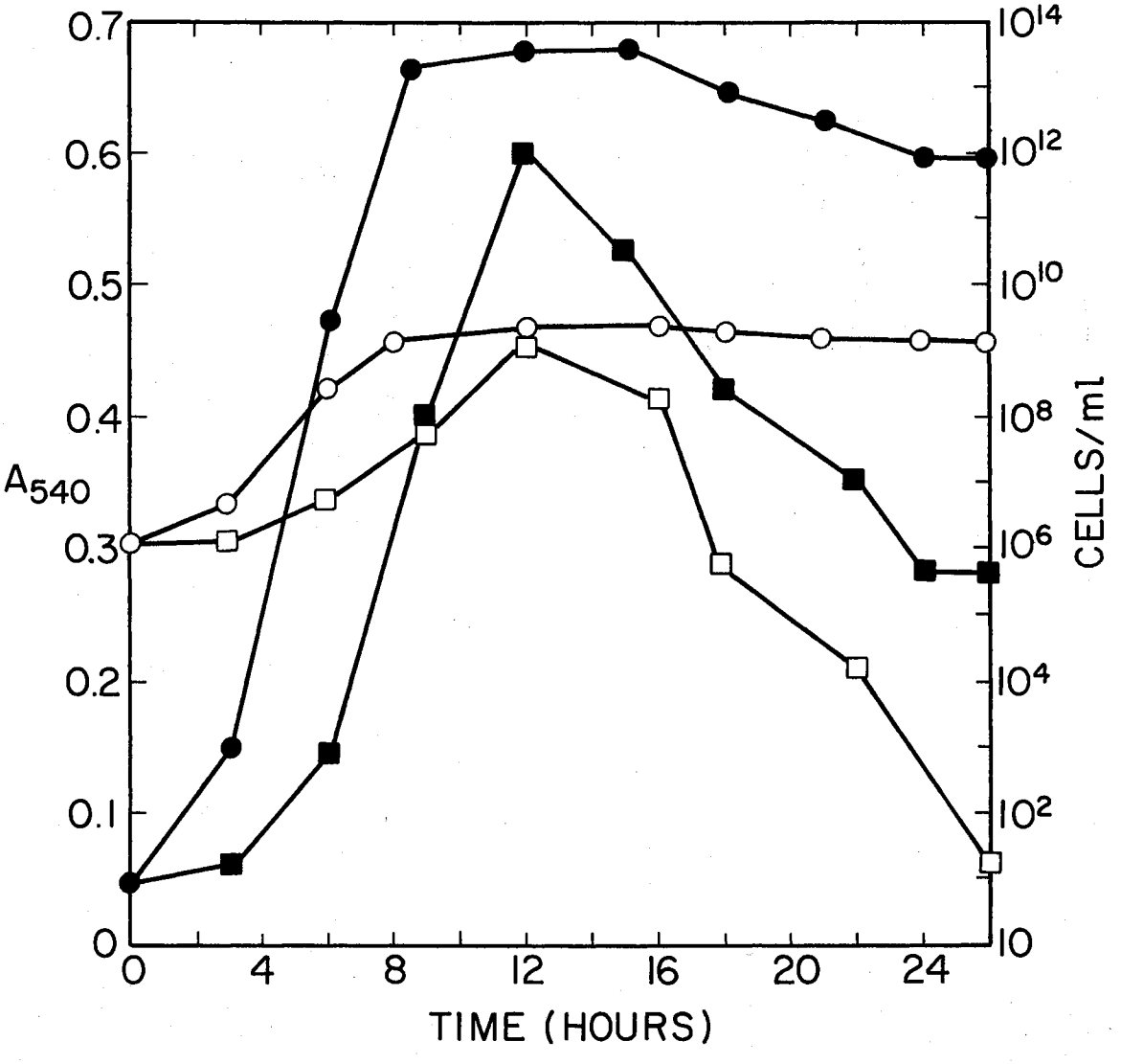

Figure 4. Effect of the Hydroxybenzindazole on the Viability of P. fluorescens Cells ●, A_{540} of control cells; ○, control viable cells per ml; ■, A_{540} of hydroxybenzindazole grown cells; □, hydroxybenzindazole grown viable cells per ml

actual viable cells comparable to control cells at the same absorbancy. Therefore during the first 10 to 12 hours of growth, the absorbancy measurement could be utilized to suspend equivalent numbers of both control and hydroxybenzindazole grown cells.

This experiment substantiated the hypothesis that the drop in absorbancy of hydroxybenzindazole grown cells, after maximum stationary phase was reached, corresponded to massive cell lysis resulting from growth in the compound.

Reversal of the Hydroxybenzindazole Inhibition of Growth

Studies were conducted to determine if the hydroxybenzindazole inhibition of growth could be reversed, cells were grown in the presence and absence of 140 μg/ml of the compound, harvested, and suspended to an A_{540} of either 0.10 or 0.75 in the presence and absence of the hydroxybenzindazole.

Cells grown in the hydroxybenzindazole and then placed in fresh medium in the absence of the compound exhibited a growth pattern almost identical to that of cells which had never been exposed to the compound (Fig. 5). Cells which have been grown in the absence of the hydroxybenzindazole and suspended in medium containing the compound showed the typical decreased total cell mass as well as enhanced lysis upon continued incubation. Similar results were obtained from cells grown in medium containing the hydroxybenzindazole during both phases of the experiment. Prior growth in the presence of the hydroxybenzindazole appeared to enhance the action of the compound. These results were observed for cultures suspended to an initial cell mass of an A_{540} of

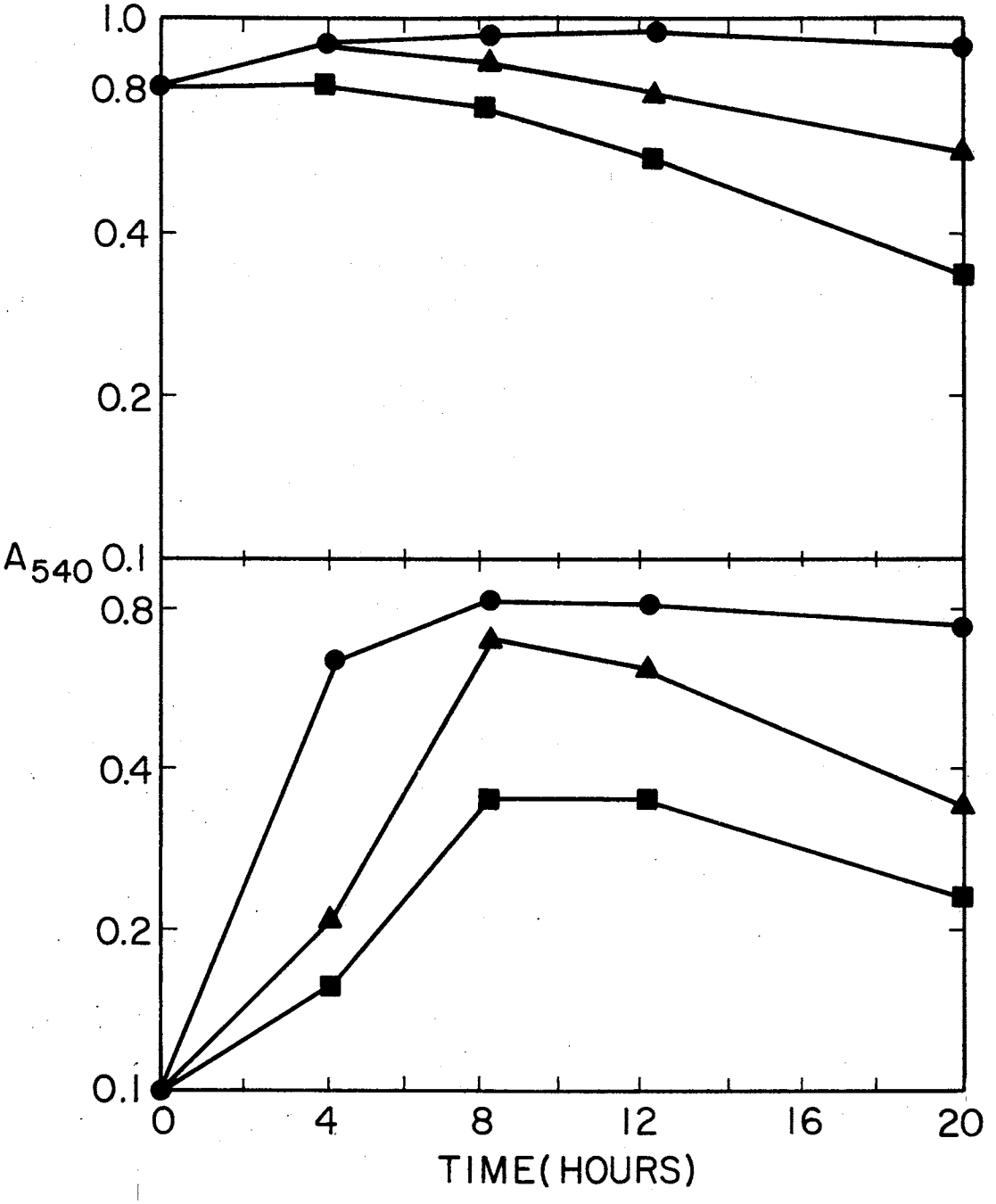

Figure 5. Reversal of the Hydroxybenzindazole Inhibition of Growth Cells were adjusted to an initial inoculum of either A_{540} = 0.1 or 0.80 ●, control cells suspended in control medium, and 140 µg/ml hydroxybenzindazole grown cells suspended in control medium; ▲, control cells suspended in 140 µg/ml hydroxybenzindazole; ■, 140 µg/ml hydroxybenzindazole grown cells suspended in 140 µg/ml hydroxybenzindazole

either 0.10 or 0.80.

The action of the hydroxybenzindazole was almost completely reversible which was evident almost immediately upon removal of the cells from the presence of the compound. Since no effect was observed when 140 μg/ml of the hydroxybenzindazole was added to maximum stationary phase cells grown in the absence of the compound, growth in the presence of the compound must be essential for the hydroxybenzindazole to affect the cell.

Spectrophotometry of the Hydroxybenzindazole

The ultraviolet spectrum of the hydroxybenzindazole at pH 7.0 revealed a single large peak with an absorption maximum at 271 nm (Fig. 6). A slight shoulder on the peak can be observed at 280 nm. The spectrum was not altered when the compound was incubated in succinate minimal medium at 37 C with constant shaking for 3 days, indicating that the hydroxybenzindazole was stable under these conditions.

The absorption at 271 nm of the hydroxybenzindazole increased linearly with increasing concentrations of the compound (Fig. 7).

Binding of the Hydroxybenzindazole
With Whole Cells of P. fluorescens

Cells grown in succinate minimal medium, harvested, suspended to an A_{540} of 0.2 in phosphate buffer containing known amounts of the hydroxybenzindazole, and incubated at 37 C for 10 minutes. The binding of the hydroxybenzindazole with whole cells of P. fluorescens was measured as the decrease in concentration of the compound after incubation with the cells. While it was difficult to separate binding of the

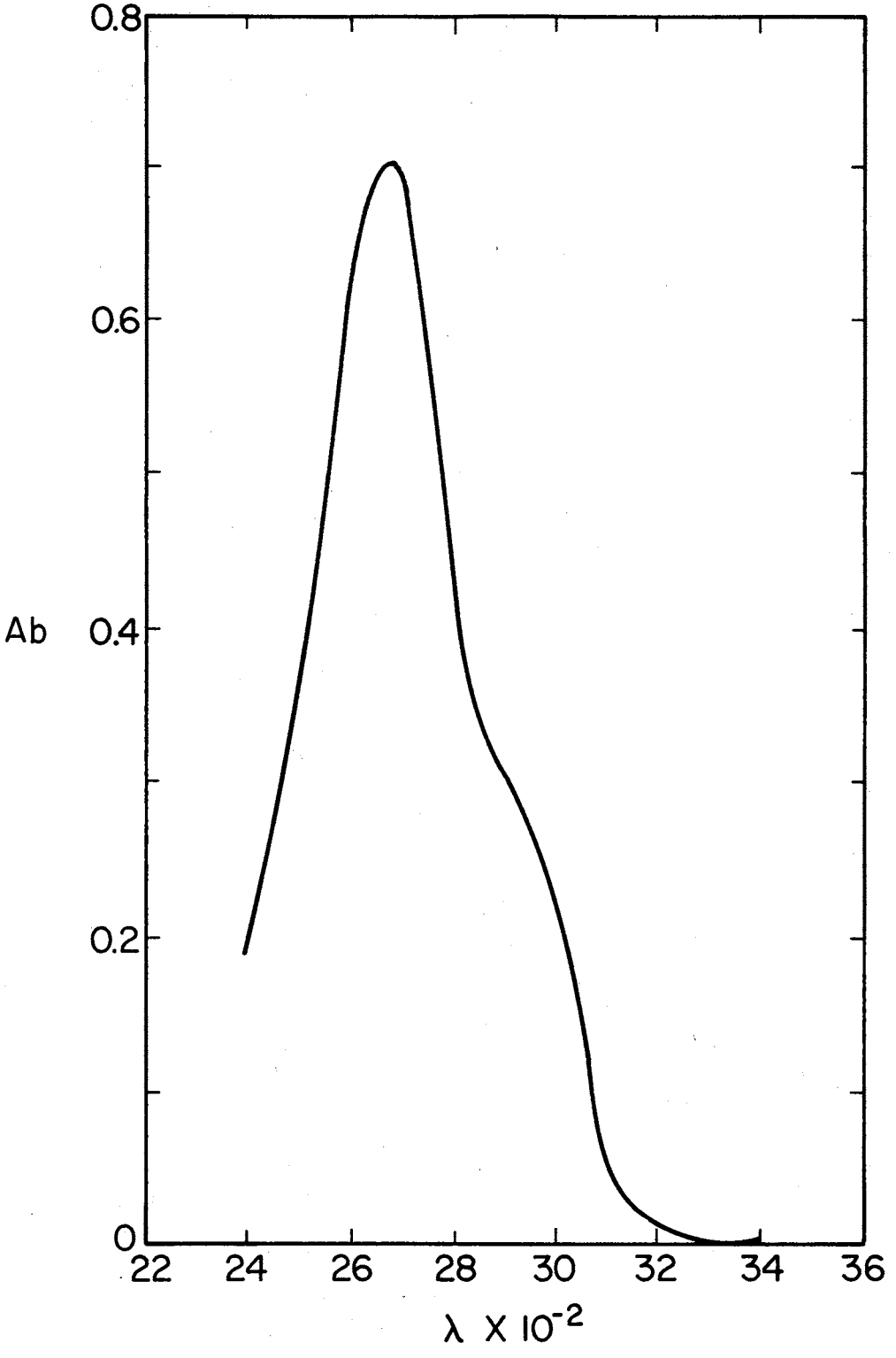

Figure 6. Ultraviolet Spectrum of an Aqueous Solution of the Hydroxybenzindazole 8 μg/ml concentration, pH 7.0

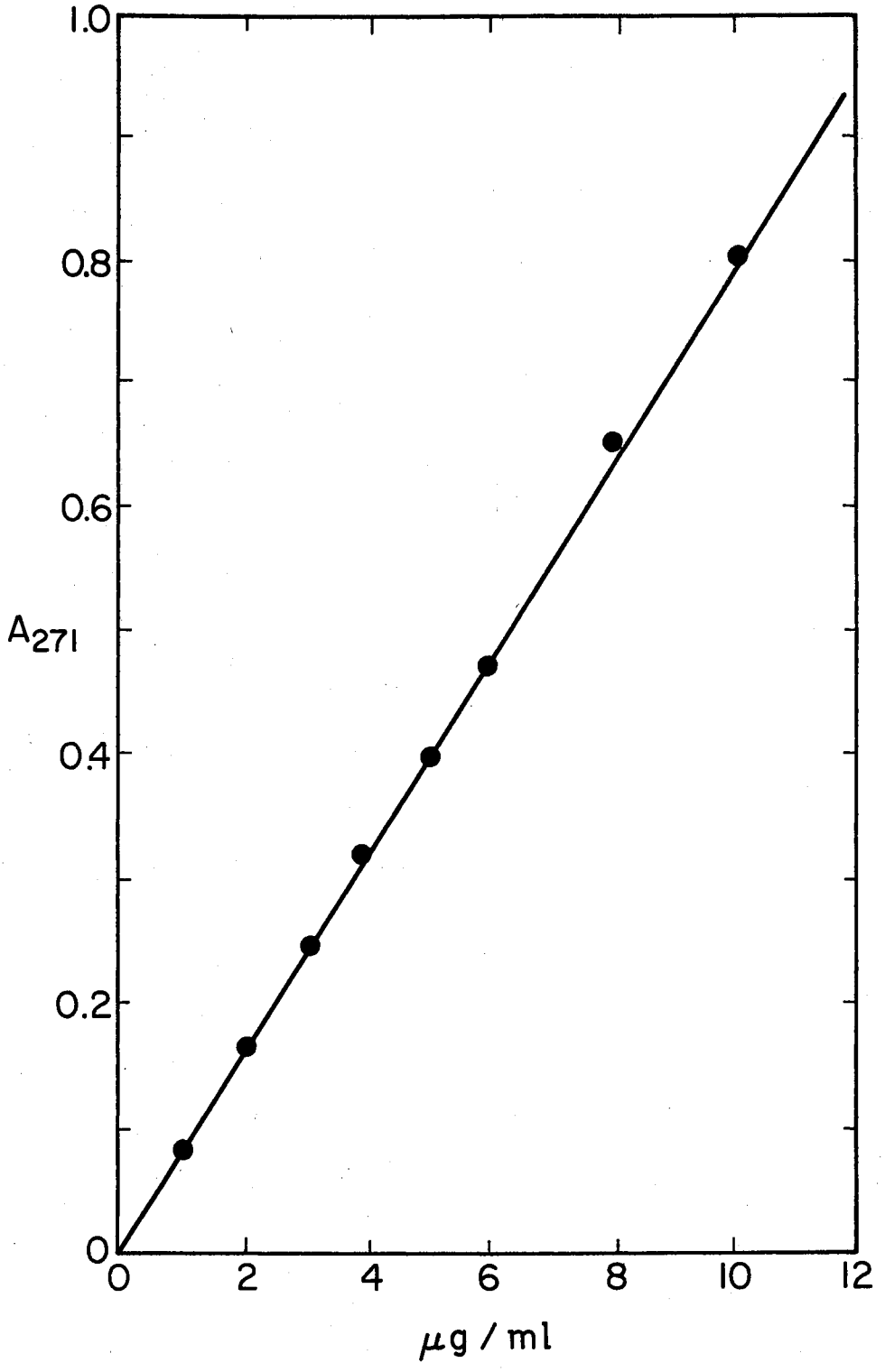

Figure 7. Standard Curve for the Absorption of the Hydroxybenzindazole at 271 nm

hydroxybenzindazole from uptake of the compound, the results indicated a definite association between the hydroxybenzindazole and the cells (Fig. 8). The binding increased with increasing concentrations of the compound. Approximately 40 percent of the total hydroxybenzindazole available was bound to the cells. Since the concentrations of compound used in this study were much less than the concentration of the compound required for inhibition in growth studies, 40 percent binding represents only a small amount of association between the compound and whole cells of P. fluorescens.

The low level of binding between the hydroxybenzindazole and cells, as well as the ease in reversal of the effects of growth in the compound, suggested that only limited amounts of the compound were directly binding or complexing with the cell periphery, or that the association between the compound and the cell periphery was unstable under the test conditions.

Effect of Growth in the Hydroxybenzindazole Upon Uptake of Radioactive Substrates

Since cells grown in the hydroxybenzindazole appeared to be structurally altered at the cell surface as indicated by an increased rate of lysis upon continued incubation, it was of interest to ascertain if these cells sustained peripheral damage prior to the onset of lysis. The uptake of substrates is a function of surface structure so a measure of substrate uptake should serve as an indicator of peripheral alteration.

Cells were grown to mid-logarithmic phase in the presence and absence of the compound, harvested, and suspended in fresh medium. The

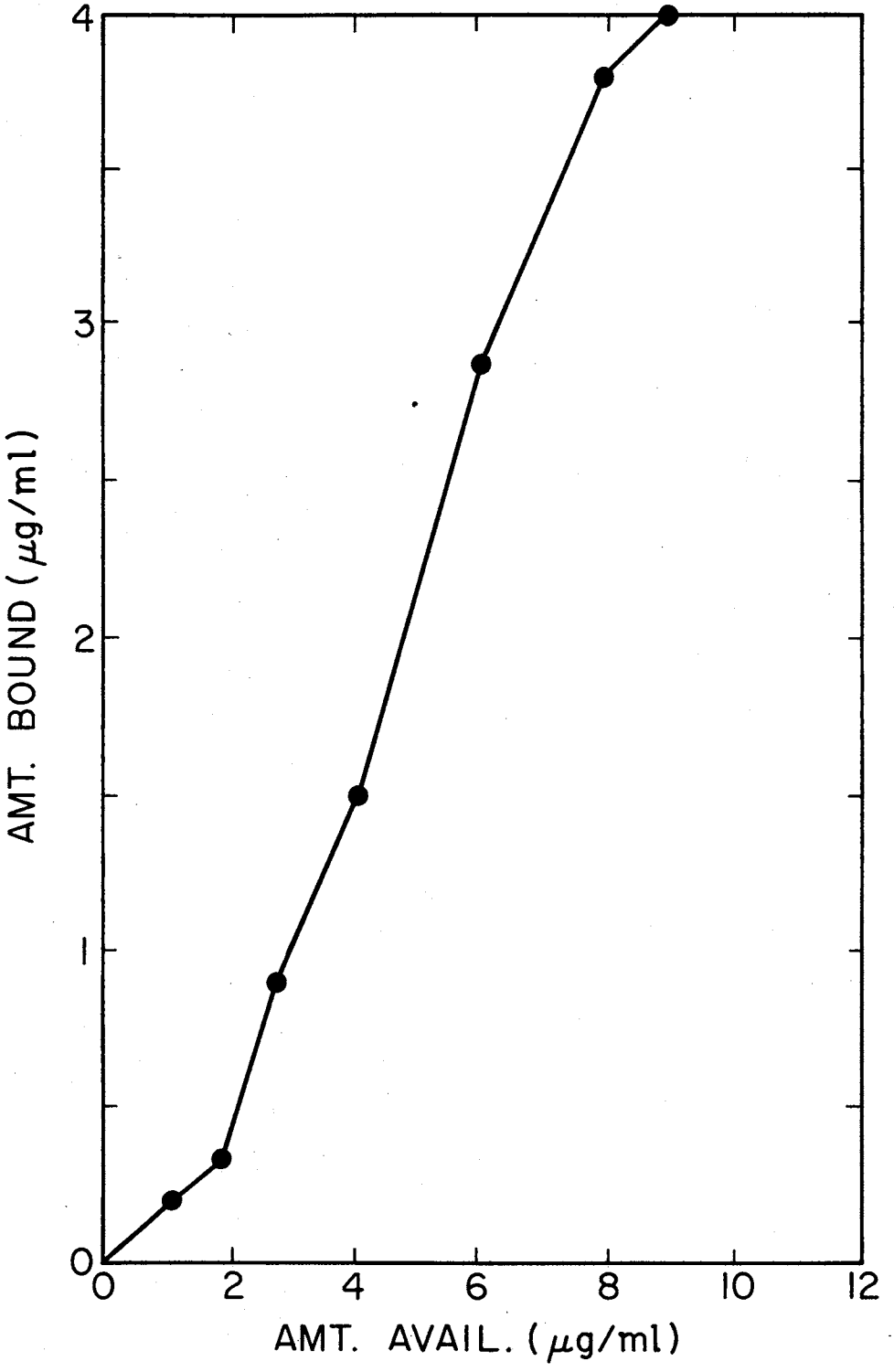

Figure 8. Binding of the Hydroxybenzindazole With Whole Cells of P. fluorescens

cells were incubated 30 minutes in the presence or absence of the hydroxybenzindazole prior to the uptake experiments. Radioactive substrates utilized in the uptake experiments included the carbon source succinate, the amino acid L-serine, and the pyrimidine base uracil. Results indicated that growth in the presence of the compound significantly altered the uptake patterns for all substrates.

Results from the succinate uptake studies (Fig. 9) indicated that growth in the presence of the hydroxybenzindazole decreased uptake of succinate by 30 percent. Addition of the compound simultaneously with the labelled substrate slightly increased the uptake of succinate. These data augment previous results which indicated the action of the compound was dependent upon growth of the cells in the presence of the compound. Short time exposure to the hydroxybenzindazole, as in the uptake experiment, was not sufficient to affect cellular processes.

Uptake studies with L-serine-3-^{14}C showed similar results (Fig. 10). Cells grown in the presence of the hydroxybenzindazole exhibited approximately a 40 percent inhibition of uptake. The simultaneous addition of the compound with the substrate did not significantly alter the uptake of serine by the cell.

Growth in the presence of the hydroxybenzindazole also produced dramatic alteration of uptake in the uracil system (Fig. 11). Cells grown in the hydroxybenzindazole showed a 66 percent inhibition of uracil uptake. There was no pronounced alteration in uptake in the cells when the hydroxybenzindazole was added simultaneously with uracil.

While all uptake systems were not affected by the hydroxybenzindazole to the same extent, in every case uptake was decreased by growth of the cells in the presence of the compound. If the cells were not

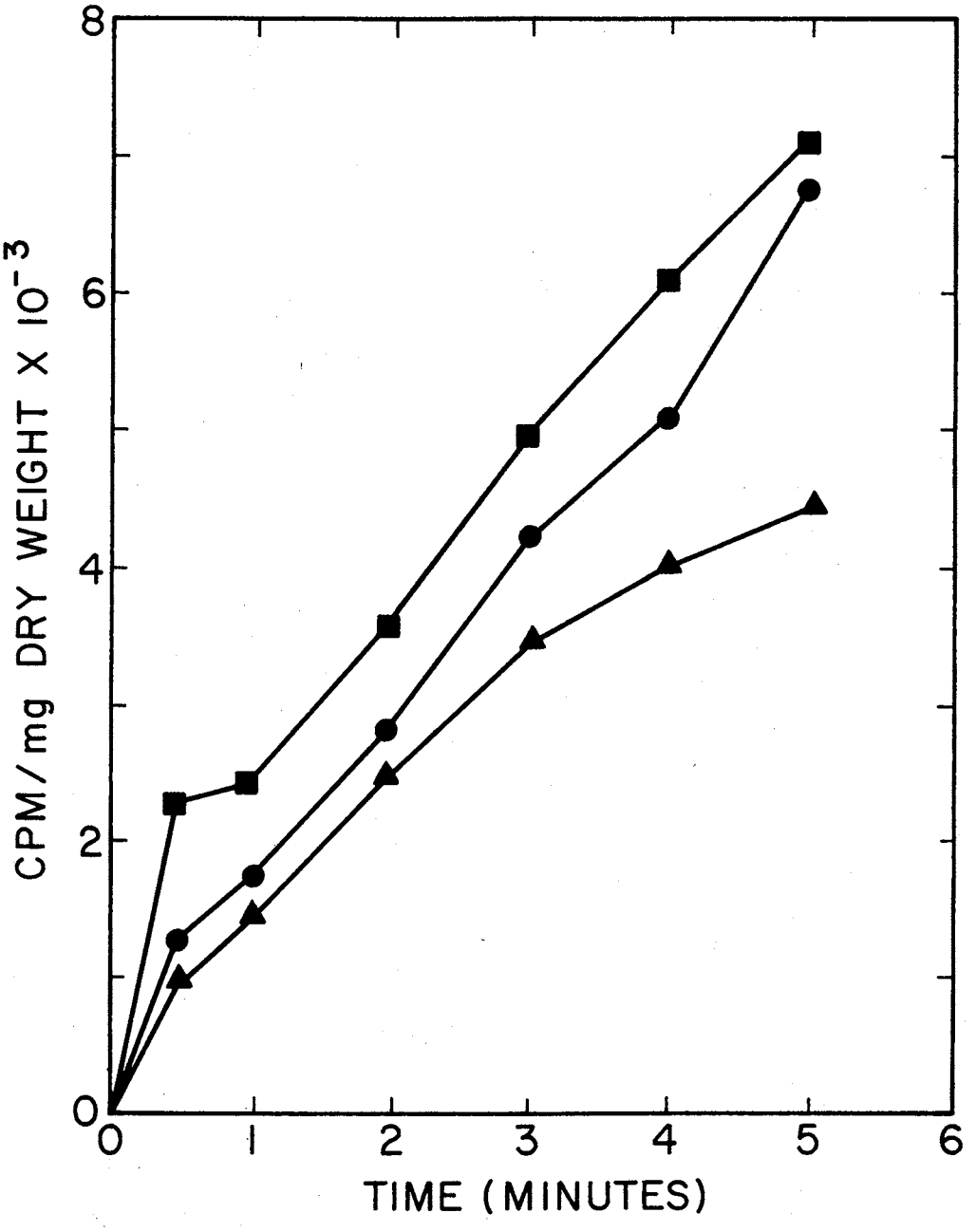

Figure 9. Effect of the Hydroxybenzindazole on Uptake of Succinic Acid-2,3-^{14}C in P. fluorescens ●, control; ■, 140 µg/ml hydroxybenzindazole added simultaneously with labelled substrate; ▲, cells grown in 140 µg/ml hydroxybenzindazole

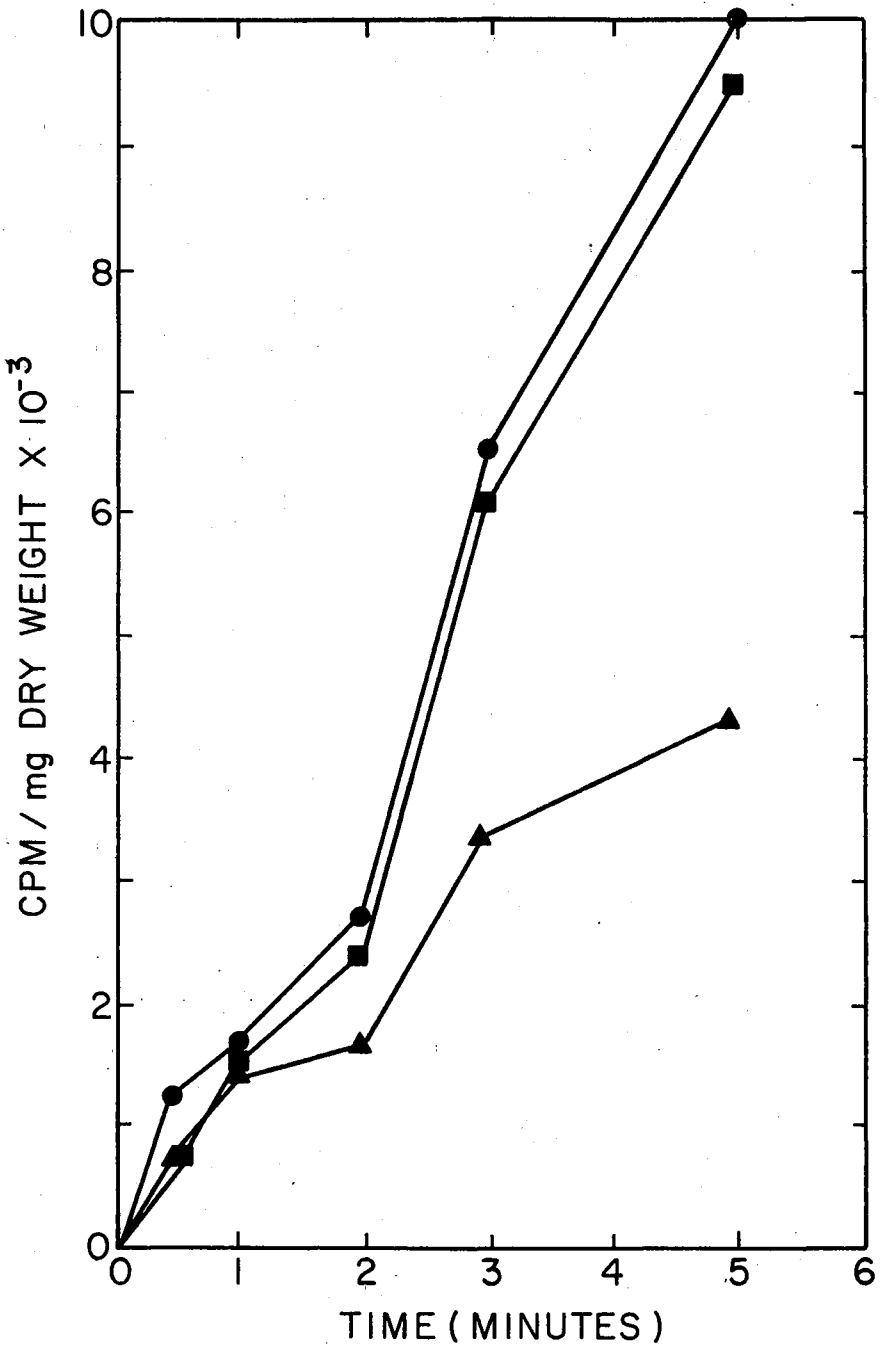

Figure 10. Effect of the Hydroxybenzindazole on Uptake of L-serine-3-^{14}C in P. fluorescens ●, control; ■, 140 µg/ml hydroxybenzindazole added simultaneously with labelled substrate; ▲, cells grown in 140 µg/ml hydroxybenzindazole

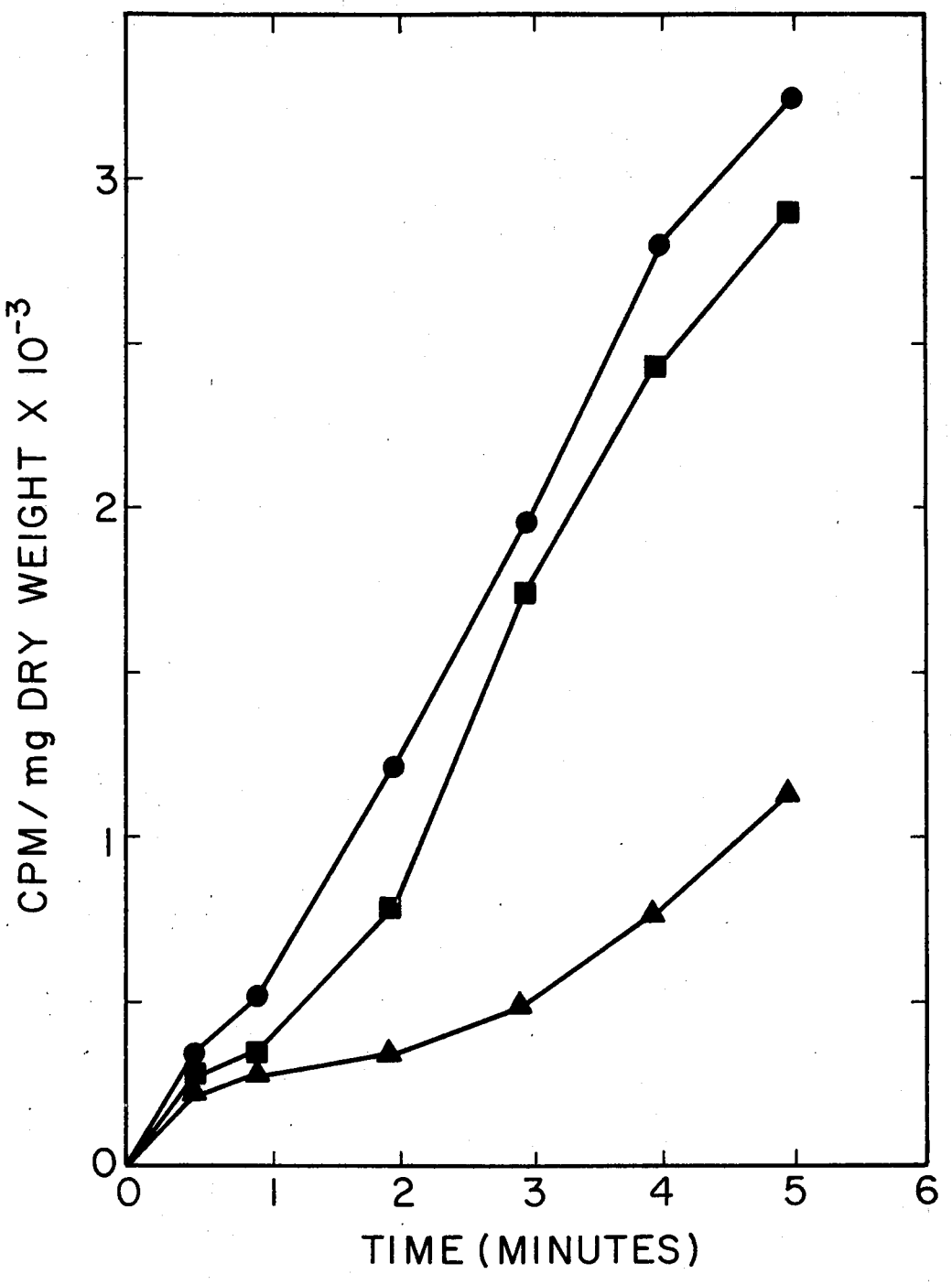

Figure 11. Effect of the Hydroxybenzindazole on Uptake of Uracil-2-^{14}C in P. fluorescens ●, control; ■, 140 µg/ml added simultaneously with the labelled substrate; ▲, cells grown in 140 µg/ml hydroxybenzindazole

previously grown in the compound, addition of the benzindazole did not influence the uptake ability of the cells.

These results eliminated the possibility that the hydroxybenzindazole was affecting uptake by competing for a binding site. Since growth in the compound is required, then the uptake system of each substrate may have been altered or the overall permeability of the cell may have been increased. In the case of increased overall permeability, the substrate would have been able to diffuse out of the cell almost as readily as it was transported inside the cell. The end result would be an apparent decrease in uptake ability. Regardless of the exact mechanism of alteration of uptake patterns, these results suggest that growth in the presence of the hydroxybenzindazole produced changes in the cell periphery prior to the onset of lysis.

Hydroxybenzindazole Potentiation of Actinomycin D Action Against P. fluorescens

Leive (1965) reported that gram-negative organisms are less sensitive to actinomycin D than gram-positive organisms because the gram-negative cells do not readily take up the antibiotic. To determine if growth in the hydroxybenzindazole alters permeability, potentiation of the action of actinomycin D against P. fluorescens was employed.

Cells were grown in succinate minimal medium containing the hydroxybenzindazole, actinomycin D, or a combination of the hydroxybenzindazole and actinomycin D. Growth of the cells was then followed for 18 hours to determine if growth in the hydroxybenzindazole could potentiate the action of actinomycin D.

Results (Fig. 12) indicated the hydroxybenzindazole potentiated the

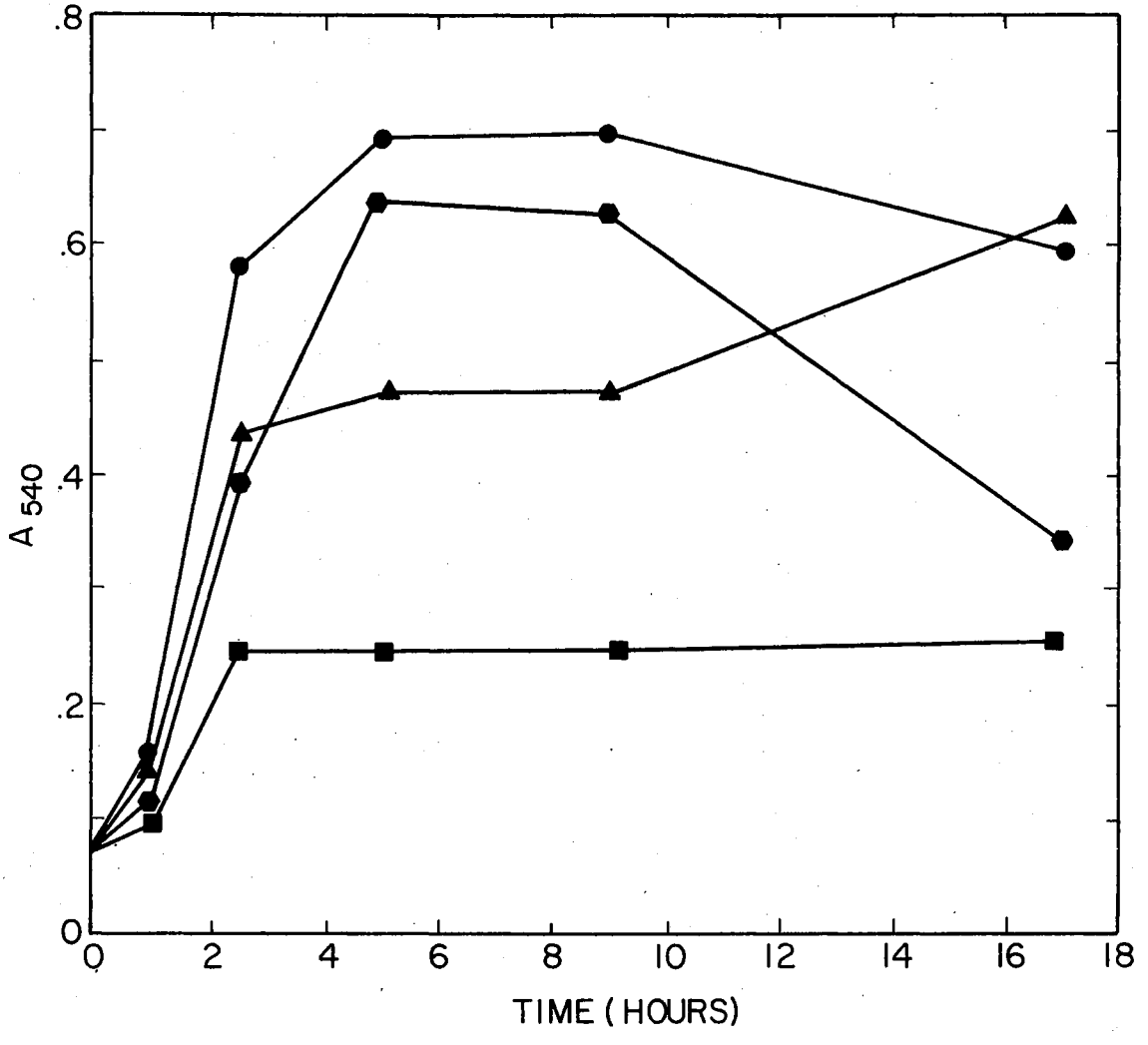

Figure 12. Potentiation of Actinomycin D Action by the Hydroxybenzindazole ●, control; ⬣, 140 μg/ml hydroxybenzindazole; ▲, 5 μg/ml actinomycin D; ■, 140 μg/ml hydroxybenzindazole plus 5 μg/ml actinomycin D

action of actinomycin D. The potentiation was established early during the growth cycle. Spectrophotometric data did not suggest any complex formation between the hydroxybenzindazole and actinomycin D. The spectrum of each compound remained unchanged in the presence of the other compound.

The potentiation of actinomycin D action by the hydroxybenzindazole could result from an overall increase in permeability due to growth in the presence of the hydroxybenzindazole. These results augment the finding obtained with radioactive substrate uptake studies.

This potentiation of actinomycin D by growth in the hydroxybenzindazole was a bacteriostatic effect. When cells were removed from the presence of the combination of hydroxybenzindazole and actinomycin D after 14 hours incubation, normal growth was resumed. This would appear reasonable since the action of the hydroxybenzindazole was readily reversible and actinomycin D had previously been demonstrated as a bacteriostatic agent (Waksman, 1954).

Macromolecular Changes Associated With the Action of the Hydroxybenzindazole

The Effect of Growth in the Hydroxybenzindazole on Isolated Phospholipids of P. fluorescens

It has been suggested that phospholipids contribute to the permeability characteristics of the cell membrane (Anderes, Sandine, and Elliker, 1971). Accordingly, the influence of growth in the presence of the hydroxybenzindazole on the phospholipids of P. fluorescens was

studied. Phospholipids were extracted from whole cells grown in the presence and absence of the hydroxybenzindazole (Folch, Lees, and Sloane, 1957). Iodine, rhodamine G, and ninhydrin were employed as detection agents. Identifications were made by comparison to known Rf values.

Two major phospholipids were extracted and identified as phosphatidylethanolamine and phosphatidylglycerol. An unidentified rhodamine G-positive spot that traveled with the solvent front was also detected. There were no apparent qualitative differences between the two sets of cells.

The incorporation of acetate-1-^{14}C during growth was used to quantitate the phospholipids. The labelled phospholipids were extracted as described, chromatographed, developed, the spots scraped from the plates, and counted to determine radioactivity.

Approximately 27 percent of the label was incorporated into phosphotidylglycerol, 73 percent into phosphotidylethanolamine, and 1 percent into the unidentified spot (Table I). Similar results were obtained for cells grown in the presence or absence of the hydroxybenzindazole, indicating that the phospholipids of P. fluorescens were not altered by growth in the hydroxybenzindazole.

When the total radioactivity incorporated was compared, cells grown in the presence of the compound incorporated approximately 10 percent more of the labelled acetate into the phospholipids. This difference is not sufficient to account for any appreciable surface structural change, especially since the ratios between the phospholipids remained constant in the two sets of cells. Therefore, influence of the hydroxybenzindazole during growth could not be readily explained by changes in

TABLE I

ANALYSIS OF PHOSPHOLIPIDS OF *P. FLUORESCENS* CELLS GROWN IN THE PRESENCE AND ABSENCE OF THE HYDROXYBENZINDAZOLE

Addition	Rf	rho G	ninhydrin	I_2	Cts/10 Min.	Percent Total
CONTROL	0.58 (PE)	+	+	+	65080	72
	0.47 (PG)	+	−	+	24253	27
	0.95 (UK)	+	−	+	689	1
140 µg/ml	0.59 (PE)	+	+	+	73125	73
HYDROXYBENZINDAZOLE	0.48 (PG)	+	−	+	25434	26
	0.95 (UK)	+	−	+	736	1

$$\frac{\text{Total Counts Incorporated into Control Cells}}{\text{Total Counts Incorporated into Cells Grown in the Hydroxybenzindazole}} = \frac{90022}{99295} = 0.91$$

Phosphatidylglycerol: PG
Phosphatidylethanolamine: PE
Unknown: UK

phospholipid components of the cell.

The Effect of Growth in the Hydroxybenzindazole on the Total Protein of P. fluorescens

In order to further characterize the effect of growth in the hydroxybenzindazole, total protein of P. fluorescens was quantitated by the microbiuret method of Koch and Putnam (1971). The results suggested that the total protein of P. fluorescens increased with growth in increasing concentrations of the hydroxybenzindazole (Table II). Hydroxybenzindazole concentrations of 70 µg/ml, 140 µg/ml, and 280 µg/ml increased the total protein of the cell by 19 percent, 32 percent, and 41 percent, respectively.

These results should be interpreted with caution. Growth in the presence of the hydroxybenzindazole could alter the solubility of some proteins without necessarily affecting the total amount of protein present in the cell. While the presence of the compound did not alter standard readings at 330 nm and 390 nm when added to the reaction solution, the possible effects of the compound and proteins present together have not been established. The results suggest that growth in the presence of the hydroxybenzindazole increased the total protein levels in the cells. It is not possible to determine if all proteins of the cell increased or if a particular protein increased. This increase in total protein was unusual since growth in the compound did not create a significant change in cell dry weight as would be expected by an increase in a major cell component.

TABLE II

TOTAL PROTEIN OF *P. FLUORESCENS* CELLS GROWN IN THE PRESENCE AND ABSENCE OF THE HYDROXYBENZINDAZOLE

µg/ml hydroxybenzindazole	mg Protein	mg Protein / mg Dry Weight	Percent Increase
0	2.79	0.37	0
70	3.24	0.44	19
140	3.66	0.49	32
280	3.85	0.52	41

The Effect of Growth in the Hydroxybenzindazole on Thiobarbituric Acid-Positive Material From P. fluorescens

The method of Cynkin and Ashwell (1960) was utilized to quantitate the KDO level of cells grown in the presence of various concentrations of the hydroxybenzindazole. Since the KDO molecule is found only in the backbone of the LPS macromolecule, the KDO level may be related to the amount of LPS present in a particular cell mass or supernatant solution.

Cells were grown to stationary phase in the presence and absence of various concentrations of the hydroxybenzindazole, centrifuged, both the pellet and supernatant solution were lyophilized, and hydrolyzed with 0.2 N H_2SO_4 (30 minutes, 100 C). A decrease in thiobarbituric acid-positive material was noted as the concentration of hydroxybenzindazole in the growth medium increased from 0 to 280 μg/ml (Table III). The μg/ml KDO/ mg dry weight decreased 35 percent for cells grown in 70 μg/ml, 42 percent for cells grown in 140 μg/ml, and 49 percent for cells grown in 280 μg/ml hydroxybenzindazole. The decrease in KDO levels was even more dramatic when expressed as μg KDO/ mg protein when the percentages were 44, 56, and 63 percent. The presence of hydroxybenzindazole did not alter the efficiency of the assay. These data established that growth in the presence of the hydroxybenzindazole decreased the total LPS present in the envelope of P. fluorescens.

The decrease in KDO, and thus the LPS, offers substantial evidence that growth in the presence of the compound altered a macromolecular structure of the cell which is associated with stability and permeability.

TABLE III

THE KDO LEVEL OF *P. FLUORESCENS* CELLS GROWN IN THE PRESENCE AND ABSENCE OF THE HYDROXYBENZINDAZOLE

µg/ml hydroxybenzindazole	µg KDO / mg Dry Weight			Percent Decrease	µg KDO mg Protein	Percent Decrease
	Pellet	Supernatant	Total			
0	14.46	2.90	17.36	0	6.22	0
70	9.41	1.89	11.35	35	3.50	44
140	8.45	1.69	10.14	42	2.77	56
280	7.41	1.48	8.89	49	2.30	63

Levels of Thiobarbituric Acid-Positive Material During Reversal of Hydroxybenzindazole Inhibition of Growth

Results indicated that growth of P. fluorescens cells in the presence of the hydroxybenzindazole decreased LPS synthesis, and that the growth inhibition caused by the action of the compound could be reversed if the cells were suspended in medium without the hydroxybenzindazole. An experiment was performed to measure LPS synthesis during reversal of the growth inhibition. Cells were grown to late logarithmic phase in succinate salts medium in the presence and absence of the hydroxybenzindazole, harvested, and suspended in medium without the compound. Growth of the cells was followed for 4 hours, and samples were taken at the indicated time intervals. The samples were assayed for thiobarbituric acid-positive material as was previously described, and the μg KDO/ mg dry weight for each was determined.

Results indicated that when cells were removed from the medium containing the hydroxybenzindazole, KDO synthesis and growth returned to normal levels (Fig. 13). At the initial sampling time, both sets of cells were suspended to an A_{540} of 0.55 and cells grown in the hydroxybenzindazole contained approximately 40 percent less KDO than did control cells. After 1 hour, the level of KDO in hydroxybenzindazole grown cells had risen to 94 percent that of the control cells. Throughout the remainder of the study, both the absorbancy and the thiobarbituric acid-positive material measurements for both sets of cells increased together to equal maximum values.

The apparent reversal of the inhibition of LPS upon removal of the

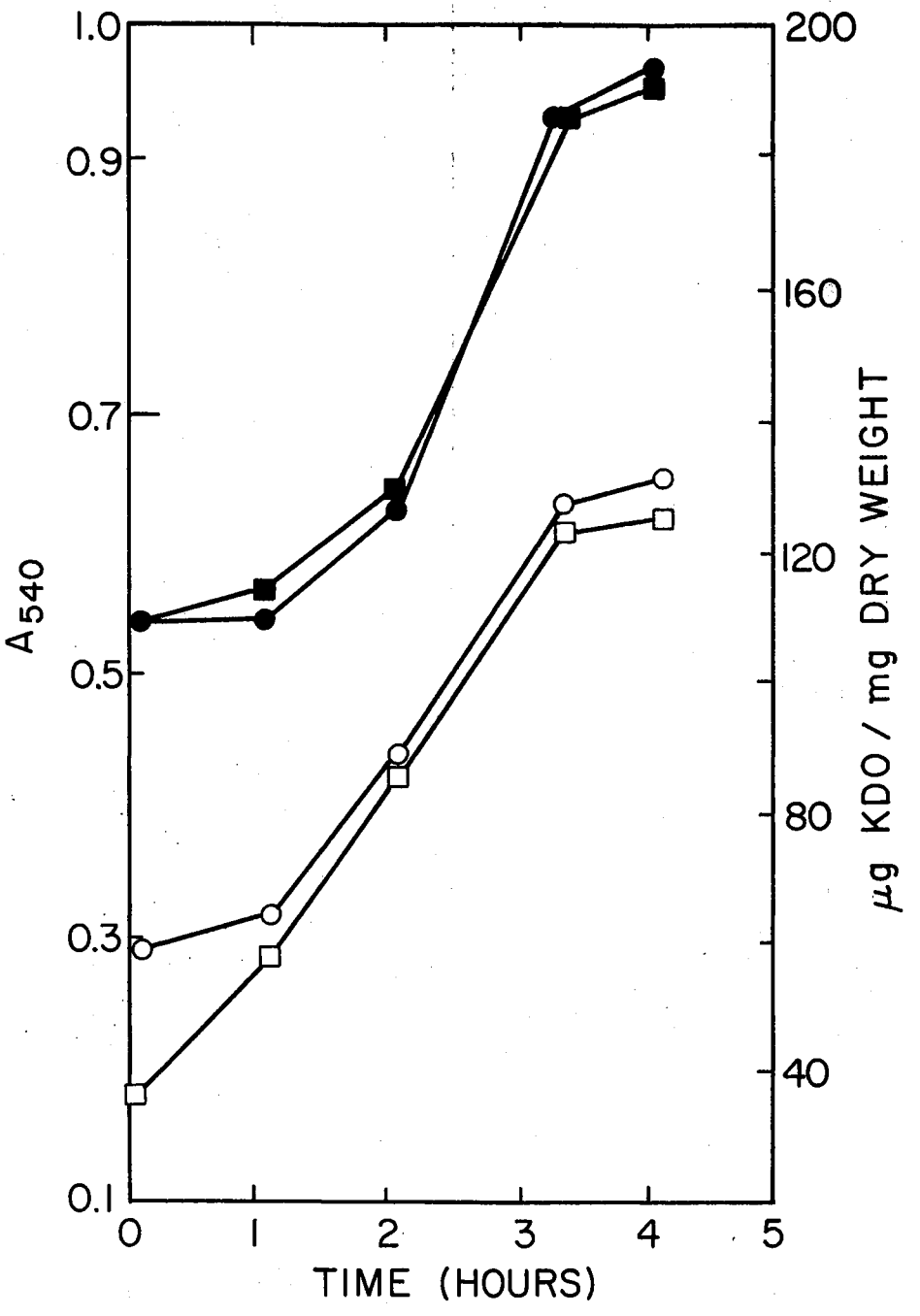

Figure 13. Growth and Synthesis of KDO During Reversal of Hydroxybenzindazole Inhibition ●, A540 of cells grown and resuspended in control medium; ■, A540 of cells grown in 140 μg/ml hydroxybenzindazole and resuspended in control medium; □, μg KDO/ mg dry weight of cells grown in 140 μg/ml hydroxybenzindazole and resuspended in control medium; ○, μg KDO/ mg dry weight of control cells

cells from the presence of the compound suggests that the diminished LPS levels were a direct result of hydroxybenzindazole action.

EDTA Lysis of P. fluorescens Cells Grown in the Presence and Absence of the Hydroxybenzindazole

Lysis of P. fluorescens by EDTA releases LPS from the cell envelope (Leive, 1965). It has also been reported that growth in a limited Mg^{++} concentration protects cells from EDTA promoted lysis (Brown and Melling, 1968). Studies were conducted to determine the effect of decreased LPS levels on the action of EDTA. Cells were grown for 8 hours in succinate salts medium containing either the hydroxybenzindazole (1×10^{-3}M), or a low Mg^{++} concentration (5×10^{-6}M), or an equal volume of glass distilled water. The cells were harvested and suspended in 1×10^{-3}M EDTA (pH 7.8) or both the EDTA and the hydroxybenzindazole (1×10^{-3}M, pH 7.0). Lysis was followed by measuring absorbance at 540 nm for 2 hours.

Cells grown in the hydroxybenzindazole and in a low Mg^{++} environment were not susceptible to EDTA promoted lysis (Fig. 14). However, normal control cells were protected from EDTA lysis by the addition of an equimolar concentration (1:1 ratio) of hydroxybenzindazole. This phenomenon is related to the molar ratios between the hydroxybenzindazole and EDTA (Fig. 15). Changing the ratio of EDTA to hydroxybenzindazole from 1:1 to 2:1 increased EDTA promoted lysis, and a 4:1 ratio produced lysis equal to that of the cells treated with EDTA alone. In order for cells grown in the presence of the hydroxybenzindazole to achieve a ratio of EDTA to the compound of 1:1, all of the

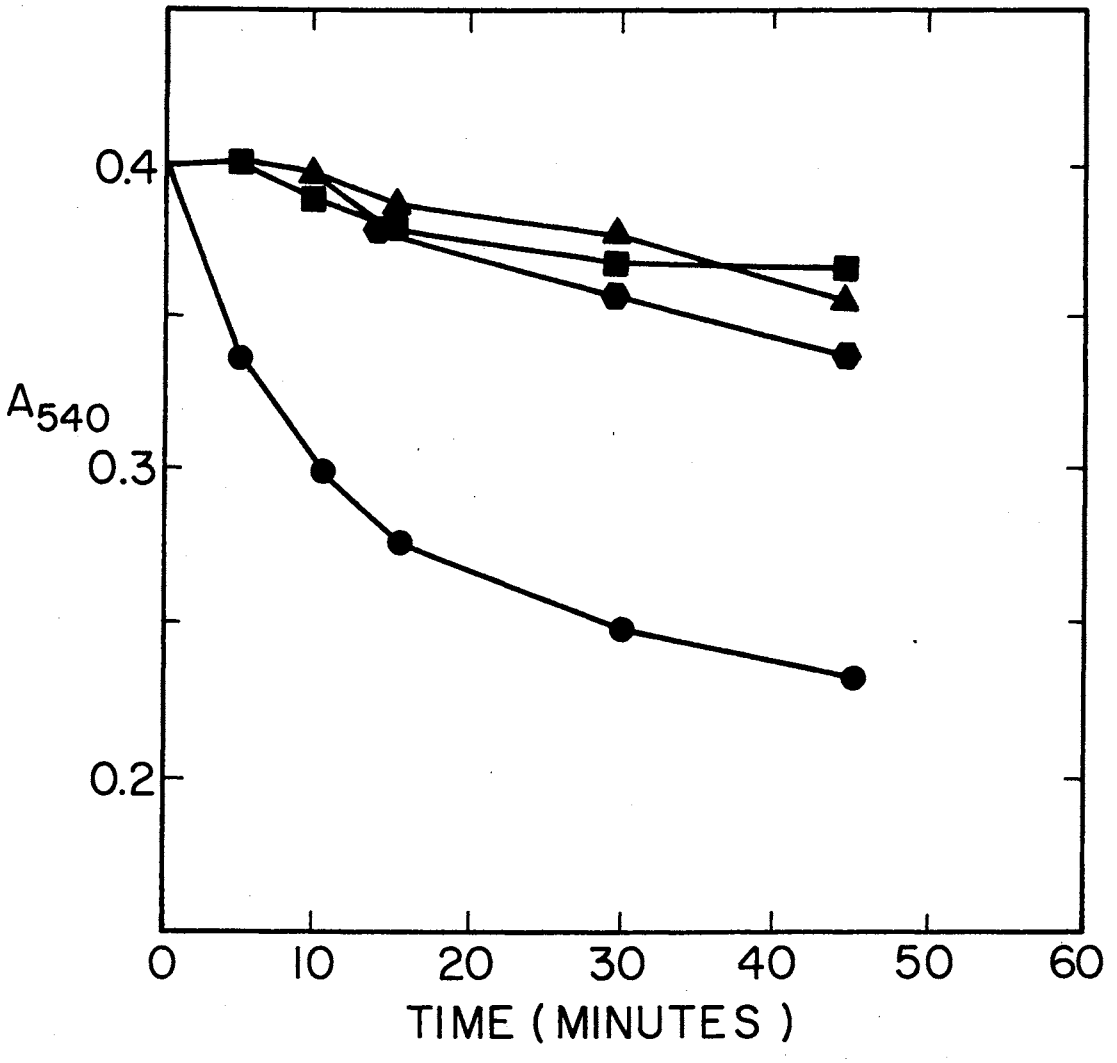

Figure 14. Effect of 1×10^{-3}M EDTA on P. fluorescens Cells ●, control cells; ■, cells grown in 140 µg/ml hydroxybenzindazole; ▲, 1×10^{-3}M hydroxybenzindazole added simultaneously with EDTA to control cells; ⬣, cells grown in 1 µg/ml Mg^{++}

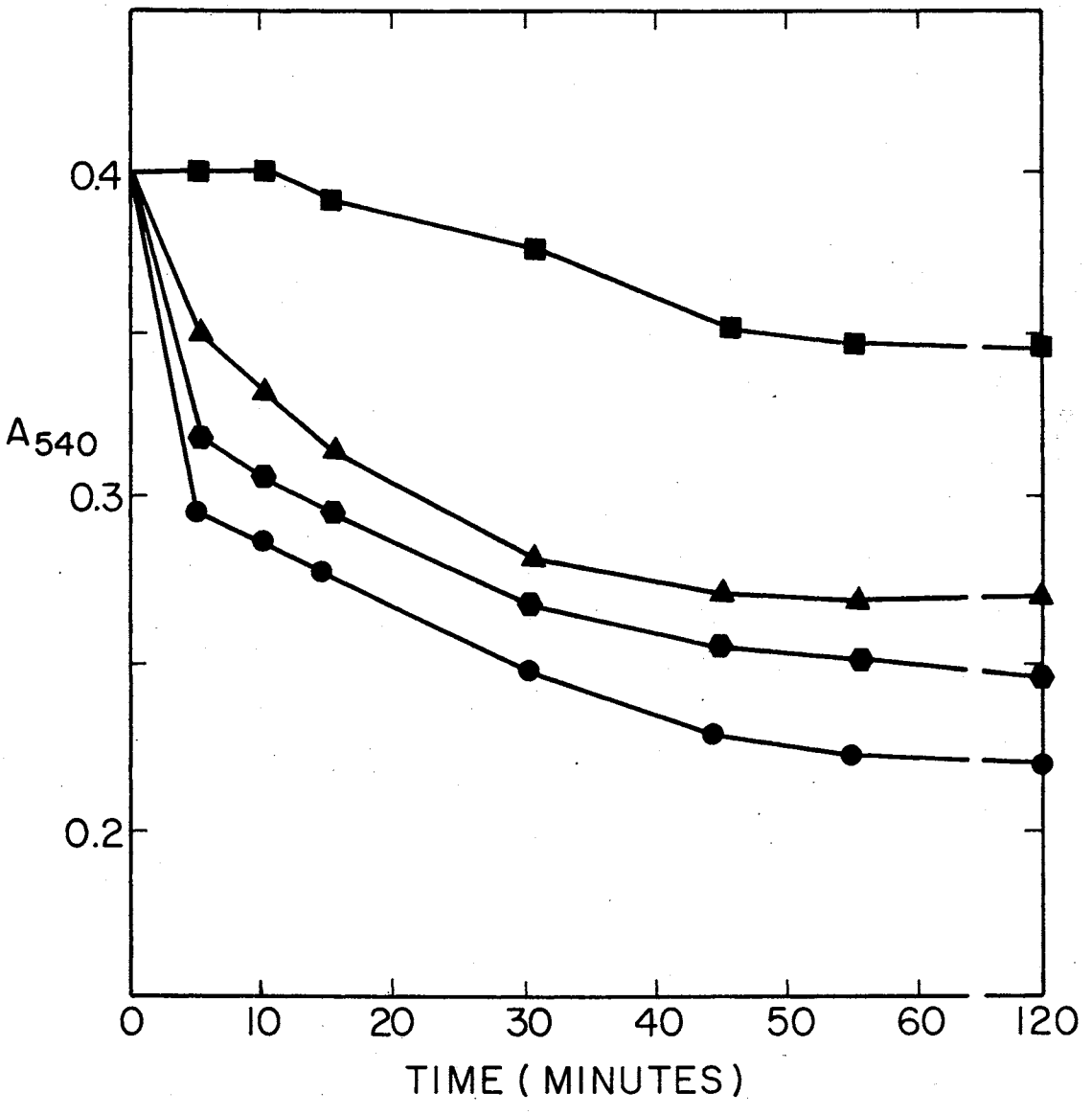

Figure 15. Effect of Different Molar Solutions of the Hydroxybenzindazole and EDTA on Lysis of P. fluorescens ■, EDTA: hydroxybenzindazole, 1:1; ▲, EDTA:hydroxybenzindazole, 2:1; ●, EDTA:hydroxybenzindazole, 3:1; ●, EDTA:hydroxybenzindazole, 4:1 and 1:0

hydroxybenzindazole present in the growth medium would have to have been associated with the cells. An association of half the available hydroxybenzindazole (0.5×10^{-3}) would have changed the EDTA to compound ratio to 2:1 and increased lysis almost 200 percent. The extensive association of the hydroxybenzindazole with whole cells necessary to achieve a 1:1 ratio with EDTA had not been demonstrated in previous experiments. Since only limited association of the compound with P. fluorescens cells could be shown, there was an indication that the protection from EDTA lysis due to growth in the hydroxybenzindazole was distinct from that afforded by addition of the hydroxybenzindazole with EDTA.

Further evidence for this possible difference in protection was obtained when the results from Fig. 14 were plotted as 1/percent lysis versus 1/time (Fig. 16). The graph of control cells treated with an equal molar mixture of hydroxybenzindazole and EDTA intersected that of the control cells treated with EDTA alone on the left side of the 1/percent lysis axis which indicated the possibility of competitive inhibition. On the other hand, the graphs of both the hydroxybenzindazole grown and low Mg^{++} grown cells treated with EDTA intersected that of the control cells treated with EDTA on the right side of the 1/percent lysis axis. Thus the protection afforded by growth in the hydroxybenzindazole appeared graphically different from that given by addition of the hydroxybenzindazole simultaneously with EDTA and similar to the protection given by growth in a low Mg^{++} environment.

Results of studies of EDTA promoted lysis of P. fluorescens cells suggested that protection from lysis was afforded by growth in either the hydroxybenzindazole or a limited Mg^{++} environment. The mechanism

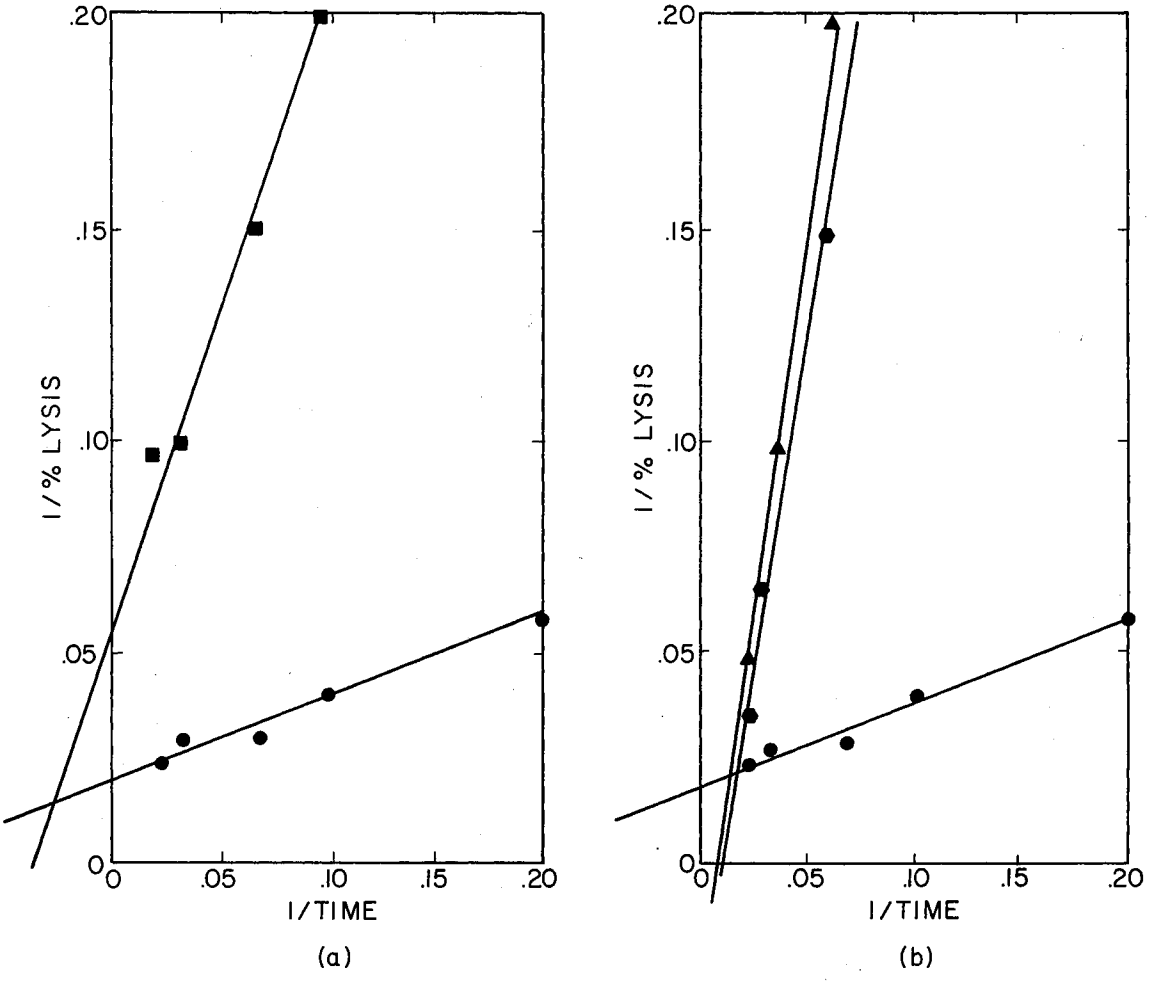

Figure 16. Reciprocal Plot of Effect of 1 x 10^{-3}M EDTA on P. fluorescens (a) ●, control cells; ■, addition of hydroxybenzindazole with EDTA (b) ●, control cells; ▲, cells grown in the hydroxybenzindazole; ●, cells grown in 1 μg/ml Mg^{++}

of this protection appeared to be different from the protection of control cells treated with an equimolar mixture of the hydroxybenzindazole and EDTA. This proposed difference in mechanism was based on graphic evidence and the evidence of only limited association between the hydroxybenzindazole and whole cells. In addition, spectrophotometric studies did not indicate formation of a complex between the hydroxybenzindazole and EDTA.

The Effect of Growth in a Limited Mg^{++} Environment on the Lipopolysaccharide Content of P. fluorescens

The LPS level of cells grown in a limited Mg^{++} environment was compared to the LPS level of cells grown in the presence of the hydroxybenzindazole and of control cells. Cells were grown in succinate salts medium containing either 1 μg/ml Mg^{++}, or 140 μg/ml hydroxybenzindazole, or an equal volume of glass distilled water, and were assayed for the NaOH-unstable complex of the oxidative product of KDO, β-formyl pyruvate, with thiobarbituric acid as previously described (Cynkin and Ashwell, 1960).

Data from this experiment indicated cells grown in both a limited Mg^{++} environment and in the hydroxybenzindazole contained decreased amounts of thiobarbituric acid-positive material (Fig. 17). In both cases, the μg KDO/ml decreased approximately 40 percent from the level observed for the control cells. The results obtained by limiting the Mg^{++} concentration of the medium indicated a possible connection between the availability of Mg^{++} and the synthesis of LPS, as a restriction of Mg^{++} appeared to be accompanied by a decreased level of LPS. There was

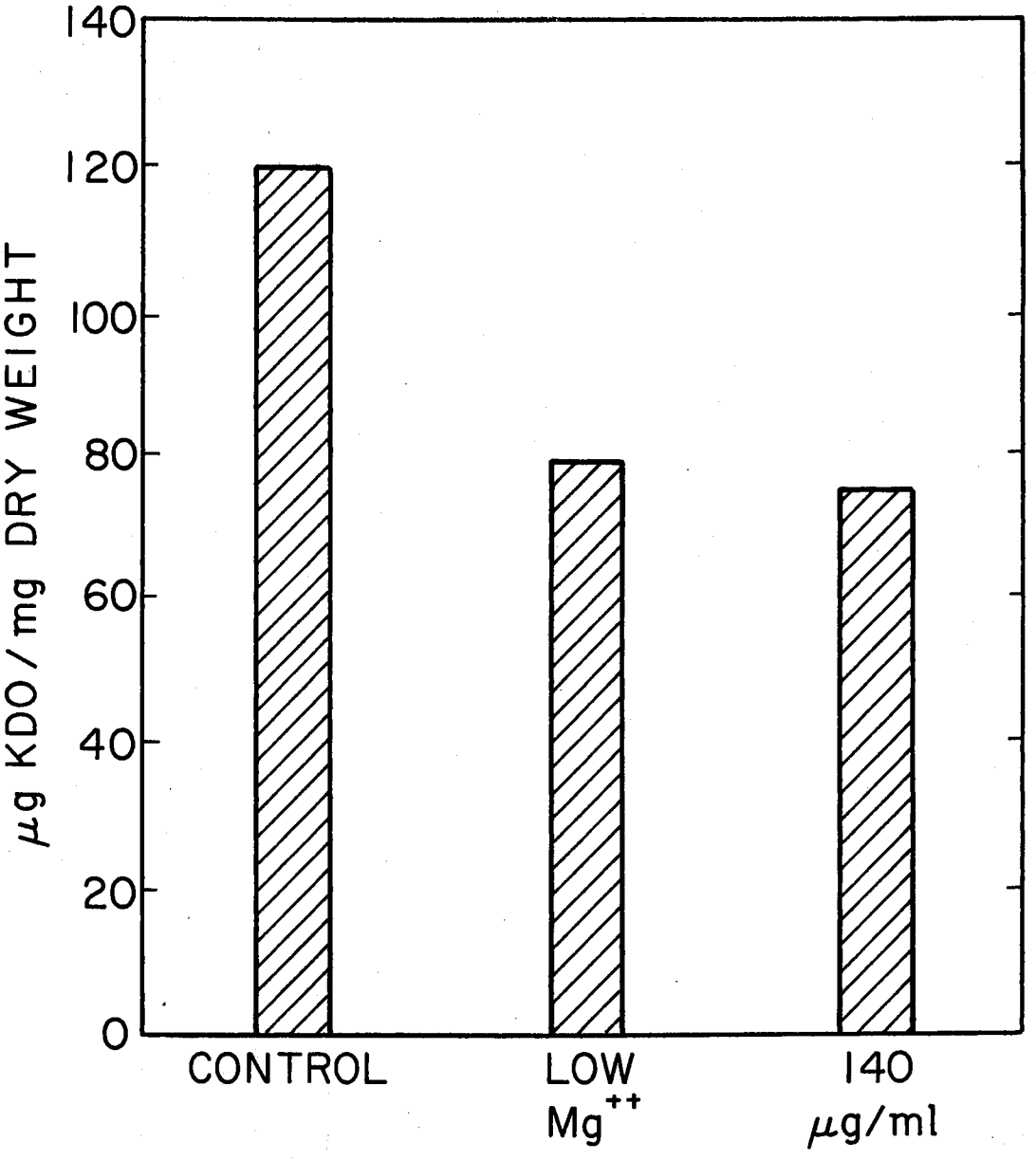

Figure 17. Quantitation of KDO in P. fluorescens Grown Under Conditions of Limited Mg^{++} and in the Presence of the Hydroxybenzindazole

also the implication of a similarity between the effect of growth in a low Mg^{++} environment and the effect of growth in the hydroxybenzindazole. Cells grown under both conditions contained decreased levels of LPS, were resistant to the action of EDTA, exhibited an increased lag phase of growth, and achieved a decreased total cell mass.

Potentiation of Actinomycin D Against P. fluorescens With a Limited Mg^{++} Environment

Potentiation of actinomycin D was employed as a measure of possible increased permeability in P. fluorescens created by growth in a low Mg^{++} environment. For this study, cells were grown in succinate salts medium containing either the normal Mg^{++} concentration (50 µg/ml), or a limiting Mg^{++} concentration (1 µg/ml), and either 3 µg/ml actinomycin D or an equal volume of glass distilled water. Growth of the cells was followed for 18 hours to determine the effect of growth in a low Mg^{++} environment on the sensitivity of P. fluorescens to actinomycin D.

Results indicated the action of actinomycin D against P. fluorescens was potentiated approximately 65 percent by the 1 µg/ml Mg^{++} concentration (Fig. 18). This level was established 4 to 5 hours after exposure to the antibiotic combined with the limited Mg^{++} which indicated a possible potentiation requirement for growth in the presence of low Mg^{++}. When cells were removed from the bacteriostatic combination of low Mg^{++} and actinomycin D, normal growth was resumed. Since potentiation of actinomycin D may be related to peripheral structural alterations and cells grown in limited Mg^{++} exhibited a decreased level of LPS, growth of P. fluorescens cells in a limited Mg^{++} environment appeared to

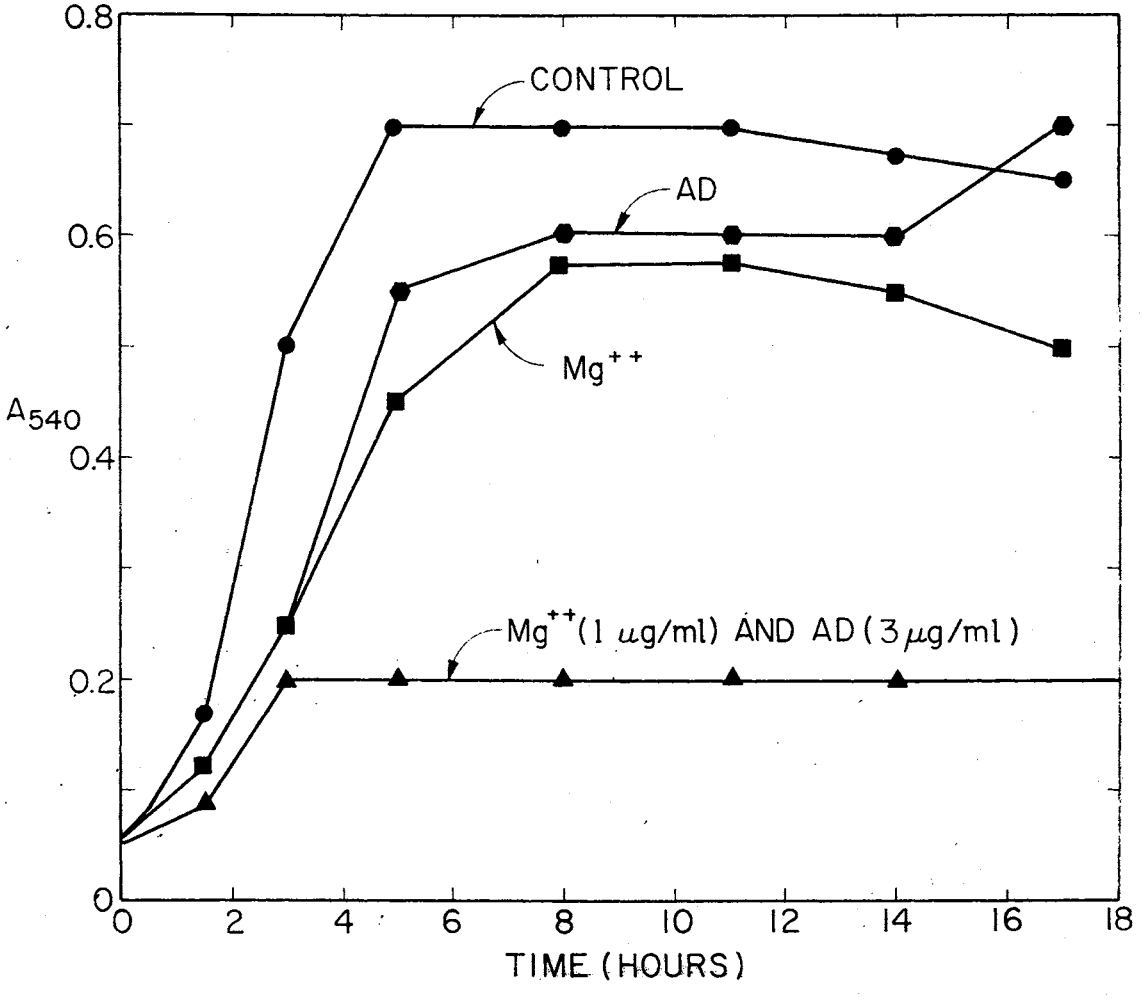

Figure 18. Potentiation of Actinomycin D for P. fluorescens Cells Growing in a Limited Mg^{++} Environment ●, control; ⬢, 3 μg/ml actinomycin D; ■, 1 μg/ml Mg^{++}; ▲, 3 μg/ml actinomycin D, 1 μg/ml Mg^{++}

result in an increased permeability of the cells. These results were consistent with those observed for potentiation of actinomycin D in hydroxybenzindazole grown cells. These cells contained decreased LPS levels as did low Mg^{++} grown cells. This again indicated the similarity between the effect of the hydroxybenzindazole and the effect of Mg^{++} limitation.

The Effect of Mg^{++} on the Action of the Hydroxybenzindazole on P. fluorescens

Cells grown in the hydroxybenzindazole or a limited Mg^{++} environment exhibited a decreased level of LPS, appeared to be resistant to EDTA promoted lysis, and potentiated the action of actinomycin D. Since the compound has been shown to carry a negative charge at pH 7.0, these results suggested that the hydroxybenzindazole anion could react with Mg^{++} to limit its availability to cells grown in the compound, possibly by association with Mg^{++} in the medium. If this were the case, addition of excess Mg^{++} would be expected to prevent the effect of growth in the hydroxybenzindazole. This assumption was tested by growing the cells in succinate salts medium which contained 2.5×10^{-4}M Mg^{++} (the normal concentration), and either 1.0×10^{-3}M Mg^{++}, 1.4×10^{-3}M Mg^{++}, 1.8×10^{-3}M Mg^{++}, or 2.2×10^{-3}M Mg^{++} with the addition of either 1.0×10^{-3}M hydroxybenzindazole or an equal volume of glass distilled water. Growth was followed for 24 hours to determine the effect of the various concentrations of Mg^{++} on the action of the hydroxybenzindazole on growing cells of P. fluorescens.

Results from this experiment showed that Mg^{++} was capable of preventing hydroxybenzindazole action if the molar concentration of Mg^{++}

was high enough (Fig. 19). The effect of growth in 1.0 x 10^{-3}M hydroxybenzindazole was only slightly changed by the addition of 1.0 x 10^{-3}M Mg^{++}, addition of 1.4 x 10^{-3}M Mg^{++} provided greater diminishment of the effect. Almost total protection was afforded by concentrations of 1.8 x 10^{-3}M and 2.2 x 10^{-3}M Mg^{++} in the growth medium containing 1.0 x 10^{-3}M hydroxybenzindazole. These data suggested that the presence of a molar concentration of Mg^{++} approximately twice that of the compound was sufficient for protection and that a concentration of Mg^{++} greater than this did not significantly alter the protection pattern. The possibility of a dose response was implicated by the partial protection of the 1.4 x 10^{-3}M Mg^{++} concentration.

The 2.1 ratio of Mg^{++} to hydroxybenzindazole necessary for protection was further demonstrated by addition of 4 x 10^{-3}M Mg^{++}, and 1.0 x 10^{-3}M Mg^{++} to succinate salts medium which contained 2.0 x 10^{-3}M and 0.5 x 10^{-3}M hydroxybenzindazole, respectively. As was shown previously, cells were almost totally protected by a molar concentration of Mg^{++} twice that of the hydroxybenzindazole. Since the excess Mg^{++} was added to medium containing the normal concentration of Mg^{++}, in systems containing an excess concentration of Mg^{++} twice that of the hydroxybenzindazole, a binding of Mg^{++} to the compound of 2:1 would leave unbound the normal concentration of Mg^{++} thus allowing normal growth. In addition, the growth pattern of cells grown in 1.0 x 10^{-3}M hydroxybenzindazole and 1.0 x 10^{-3}M Mg^{++} appeared to mimic that of cells grown in 0.5 x 10^{-3}M hydroxybenzindazole without Mg^{++}. It appeared that 1.0 x 10^{-3}M Mg^{++} lowered the effective concentration of hydroxybenzindazole from 1.0 x 10^{-3}, to 0.5 x 10^{-3}M.

The cumulative results from studies of the effect of Mg^{++} on the

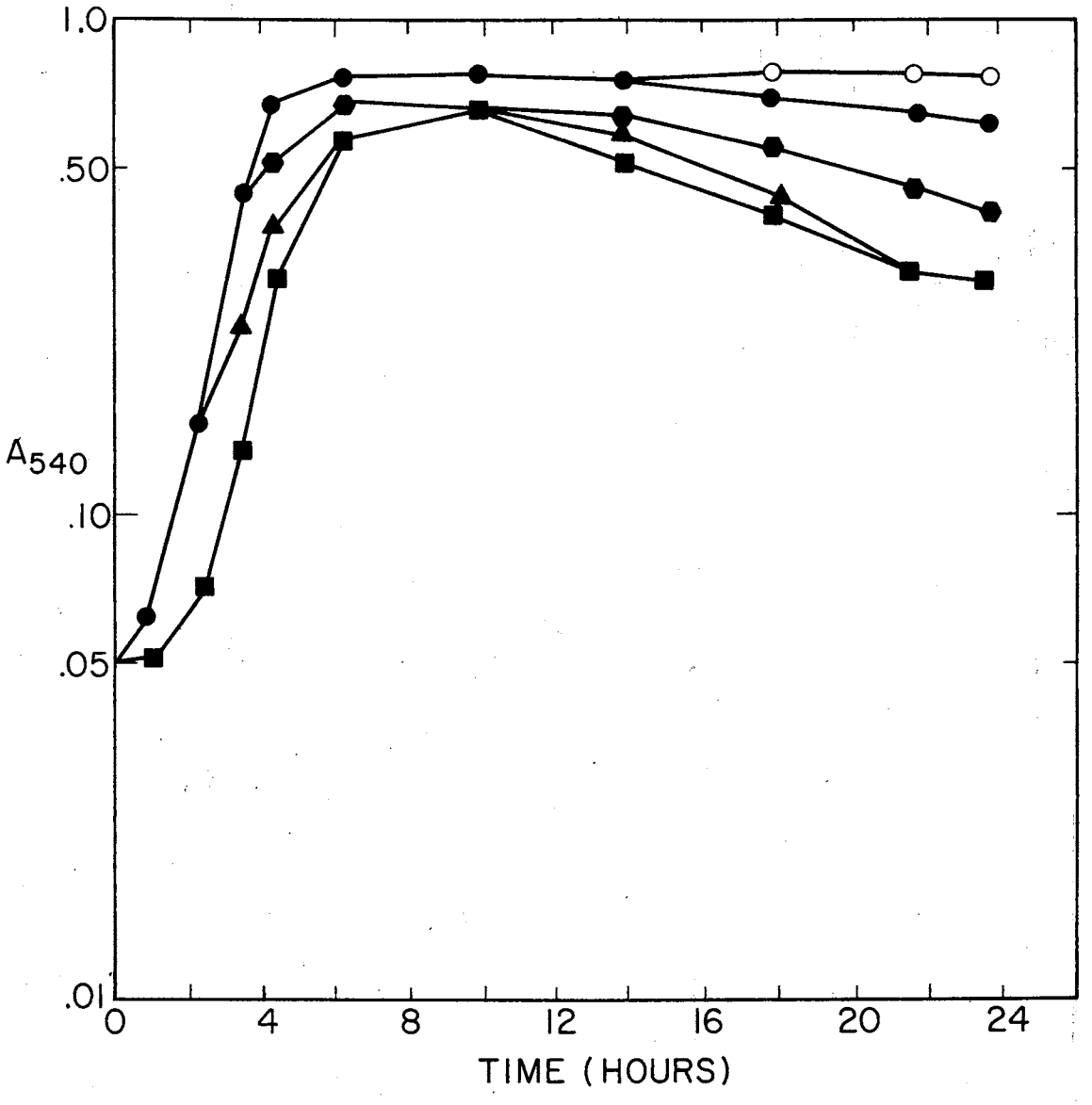

Figure 19. The Effect of Mg^{++} on the Action of Hydroxybenzindazole on P. fluorescens ●, control, 2.5 x 10^{-4} M Mg^{++}; ■, 1 x 10^{-3}M hydroxybenzindazole; ▲, 1 x 10^{-3}M hydroxybenzindazole and 1 x 10^{-3}M Mg^{++}; ⬢, 1 x 10^{-3}M hydroxybenzindazole and 1.4 x 10^{-3}M Mg^{++}; ○, 1 x 10^{-3}M hydroxybenzindazole and 1.8 x 10^{-3}M Mg^{++}, 1 x 10^{-3}M Mg^{++} and 0.4 x 10^{-3}M hydroxybenzindazole

action of the hydroxybenzindazole demonstrated an interaction between Mg^{++} and hydroxybenzindazole in a ratio of 2:1. If this association occurred in a succinate salts medium containing the normal Mg^{++} concentration, the effect of the interaction would be the establishment of a severely limited Mg^{++} environment by the hydroxybenzindazole. This possibility was further substantiated by the similarities in LPS levels, actinomycin D potentiation, and EDTA resistance between cells grown in an environment known to be limited in Mg^{++} and cells grown in the hydroxybenzindazole. In addition, the reversal of the hydroxybenzindazole effect could be seen as a replenishment of Mg^{++} supply accomplished by transfer of the hydroxybenzindazole grown cells to fresh medium without the compound. Therefore, the action of the hydroxybenzindazole on P. fluorescens cells grown in succinate salts medium could be directed through limitation of Mg^{++}, brought about by association of Mg^{++} with the compound.

CHAPTER IV

SUMMARY AND CONCLUSIONS

P. fluorescens grown in succinate salts medium containing a new hydroxybenzindazole compound exhibited an increased lag phase, a decreased total cell mass, and increased lysis upon continued incubation. Hydroxybenzindazole concentrations of 70-280 μg/ml promoted nearly complete lysis as determined by viable cell count and direct microscopic observation. A concentration of the compound of 35 μg/ml also produced lysis of maximum stationary phase cells, but in this case the lysis was less extensive than that observed for the higher concentrations. The effect of the compound on growing cells of P. fluorescens was shown to be concentration dependent.

This action of the hydroxybenzindazole appeared to result from the influence of the intact molecule since the ultraviolet absorption spectrum of the compound showed that the compound was stable at 37 C with constant shaking for 72 hours, and growth studies indicated that P. fluorescens was unable to degrade the compound for use as the sole carbon source for growth. The absorbance of the hydroxybenzindazole at 270 nm was also used to determine the extent of the compound's association with whole cells of P. fluorescens. It was not possible to separate binding from uptake of the hydroxybenzindazole by the cells using this technique. A maximum value of 40 percent binding of the compound to a constant cell mass, but a plateau value was not obtained in this study.

Two important aspects of the compound's action were pointed out by studies of the reversibility of the effects of growth in the hydroxybenzindazole. First, the effects of the compound were almost completely reversed by removal of the cells from the presence of the compound at any time prior to the onset of lysis. This reversal process appeared to be quite rapid as the cells exhibited only a short lag time before initiation of growth when placed in fresh medium. Second, the expression of the effect of the hydroxybenzindazole on P. fluorescens was dependent upon growth of the cells. When the compound was added to stationary cells, lysis did not ensue. However, the addition of the hydroxybenzindazole to actively growing cells produced a lytic effect independent of cell mass.

Possible alterations in the surface of hydroxybenzindazole grown cells prior to lysis were revealed by uptake of radioactive substrates. Growth in the presence of the compound was required before any significant change in uptake patterns could be observed. Competition for binding sites as a mechanism for decreased uptake was eliminated because the presence of the compound in the uptake solution did not interfere with substrate accumulation.

The uptake of uracil was decreased by approximately 66 percent by growth in the hydroxybenzindazole. A similar pattern of inhibition was observed for serine. Succinate uptake was also inhibited in hydroxybenzindazole grown cells, but not to the same extent as observed for the other substrates. In this case the uptake was reduced approximately 30 percent. This reduction in uptake could have been due to an inability to concentrate the substrates caused by an increased permeability.

Since the permeability barrier of gram-negative cells is thought to

be responsible for resistance to actinomycin D, potentiation of actinomycin D was employed as a measure of possible peripheral changes in cells grown in the compound. An actinomycin D potentiation of approximately 50 percent was observed in cells grown in medium containing both the antibiotic and the hydroxybenzindazole. This level of potentiation was established after 3 hours of incubation; a time sufficient to allow for growth of the cells in the presence of the compound. Ultraviolet spectral data did not reveal any evidence of a complex between the hydroxybenzindazole and actinomycin D.

Thus, the physiological evidence revealed that at least one action of the hydroxybenzindazole on growing cells of P. fluorescens was directed against the peripheral architecture, possibly affecting alteration in the overall permeability of the cells. The reversible nature of the growth effects of the compound suggested that this alteration did not produce the immediate cellular disruption or loss of viability usually seen in direct membrane damage, but rather a more delicate and gradual change in the surface structure. While many factors influence cellular permeability, the observed effect of growth in the hydroxybenzindazole could be explained by qualitative or quantitative modifications of the macromolecular components of the cell envelope.

A decrease in overall cellular permeability could be achieved by an increase in lipid components present in the cell envelope. In order to study this possibility, phospholipids were extracted from P. fluorescens and assayed quantitatively. The two major phospholipids of P. fluorescens, phosphatidyglycerol and phosphatidylethanolamine, were found to be present in approximately the same ratios in both control cells and cells grown in the hydroxybenzindazole. Overall, the total radioactive

acetate incorporated into hydroxybenzindazole grown cells was 10 percent greater than that incorporated into control cells. This quantitative difference was probably not significant. Since the phospholipids of both sets of cells were qualitatively identical, the action of the hydroxybenzindazole did not appear to be directed toward alteration of the phospholipid component of the cell envelope.

One of the identifying features of gram-negative cell envelopes is the presence of a lipopolysaccharide layer. This structure has been implicated in cellular permeability. Since the KDO molecule is found only in the backbone of LPS, quantitation of KDO can be utilized as a measure of the LPS present in the cell. When the LPS of hydroxybenzindazole grown cells was quantitated by KDO measurement, growth in the presence of the compound created a decrease in LPS synthesis which was dependent upon the concentration of hydroxybenzindazole present in the growth medium. The decrease in LPS was significant; ranging from 35 to 49 percent as the concentration of hydroxybenzindazole in the medium varied from 70 to 280 μg/ml. In addition, the LPS level was shown to increase to the level of control cells as the growth effects of the hydroxybenzindazole were reversed by suspension of the cells in fresh medium. All of this evidence indicated a quantitative modification of LPS as the result of action of the hydroxybenzindazole.

These results involving LPS are not unlike those obtained by Walker (1973) in studying the effect of carbon source on P. fluorescens sensitivity to actinomycin D. She found that cells grown in succinate salts medium were more sensitive to actinomycin D, were more permeable, and accumulated less LPS than did glucose grown cells. Similarly, in 1973, Smith observed a decreased level of LPS in asparagine grown cells as

opposed to succinate grown cells. Asparagine grown cells can be shown to enter death phase earlier and to lyse more extensively than succinate cells containing a higher level of LPS.

Since the action of EDTA has been reported to be directed against the LPS layer, EDTA promoted lysis of P. fluorescens was studied. It was found that hydroxybenzindazole grown cells were resistant to the action of EDTA as were control cells to which the hydroxybenzindazole was added simultaneously with EDTA. While the protection from lysis afforded by combination of the hydroxybenzindazole with EDTA appeared similar to that afforded by growth in the compound, simple combination of hydroxybenzindazole with EDTA was concentration dependent to such an extent that the 40 percent association of hydroxybenzindazole to P. fluorescens shown previously could not account for the degree of protection provided by growth in the compound. However, the protection from EDTA lysis by growth in the hydroxybenzindazole did appear similar to the EDTA resistance conferred by growth in a limited Mg^{++} environment as first reported by Brown and Melling (1968).

Again using a KDO measurement, LPS from cells grown in limited Mg^{++} was quantitated. These cells exhibited a decrease of approximately 40 percent in the level of LPS as compared to control cells. This decrease corresponded to that observed for hydroxybenzindazole grown cells and pointed to a similarity between the effect of Mg^{++} limitation and the effect of the compound.

It has been suggested that the action of EDTA involves the release of one of two different LPS moieties which can only be distinguished on the basis of density gradient centrifugation (Voll and Leive, 1970). While conclusive data has yet to be shown, it is possible the observed

resistance to EDTA in cells which contained decreased levels of LPS was due either to an absence or to a severe limitation of a specific LPS moiety, the release of which was necessary for the EDTA promoted lysis.

The similarity between cells grown in the hydroxybenzindazole and in low Mg^{++} was further substantiated by potentiation with actinomycin D. As with hydroxybenzindazole grown cells, cells in a limited Mg^{++} environment exhibited a markedly increased sensitivity to actinomycin D over that shown by control cells. In this case the action of actinomycin D was potentiated 65 percent after 4 hours of growth in a low Mg^{++} environment.

There was an apparent correlation between at least three parameters measured for hydroxybenzindazole grown cells and for limited Mg^{++} grown cells: increased EDTA resistance, increased actinomycin D sensitivity, and decreased LPS accumulation. Guided by these findings, the effect of excess Mg^{++} upon the action of the hydroxybenzindazole was studied. The presence of Mg^{++} in a molar concentration twice that of the hydroxybenzindazole provided protection from both the lytic and growth effects of the compound. Concentrations of Mg^{++} less than twice that of the compound afforded protection which was dependent upon the amount of Mg^{++} present. As the concentration of Mg^{++} to hydroxybenzindazole decreased from 2:1, the prevention of the hydroxybenzindazole effects also decreased.

All of this evidence suggested the compound acted in the normal succinate salts medium to create a limited Mg^{++} environment. Protection was achieved by the addition of excess Mg^{++} to the medium and reversal by resuspension in a fresh Mg^{++} supply without the compound. The molecular structure of the hydroxybenzindazole would allow for some

interaction with Mg^{++} (Fig. 20) which could be increased by configurational changes in the molecule due to environmental influences.

There is also evidence to suggest the limitation of Mg^{++} was responsible for a decreased level of LPS and thus in the observed physiological effects of growth in the hydroxybenzindazole. While no direct link between a Mg^{++} requirement and LPS biosynthesis has been established, there are steps in the biosynthetic pathway which could be regulated in part by Mg^{++}. For example, in the R core structure, phosphate groups appear to form phosphodiester linkages between heptose units on different LPS chains. The crosslinking mechanism is thought to involve activation of the heptose-linked phosphate group, perhaps by the creation of an adenylate derivative (Wright and Kanegasake, 1971). This action could involve Mg^{++} as the ion is an important requirement in most reactions involving adenylate and its phosphorylated derivatives. Polymerization of the O-polysaccharide has been proposed to occur in much the same manner as protein synthesis (Bray and Robbins, 1967), and the importance of Mg^{++} in protein synthesis has been well documented. In addition, Mg^{++} has been shown to be necessary for some ligase reactions and could be involved in the O-antigen-LPS ligase reaction which joins long polysaccharide chains in the final reaction of LPS formation (Wright and Kanegasaki, 1971).

Some advantage could be gained by the bacterial cell by the inhibition of LPS synthesis as a mechanism for conservation of Mg^{++}. Many functions necessary for immediate survival are dependent upon a supply of Mg^{++}. These functions include many of the energy producing reactions of the tricarboxylic acid cycle, the nucleic acid polymerases involved in replication and transcription, and oxidative phosphorylation.

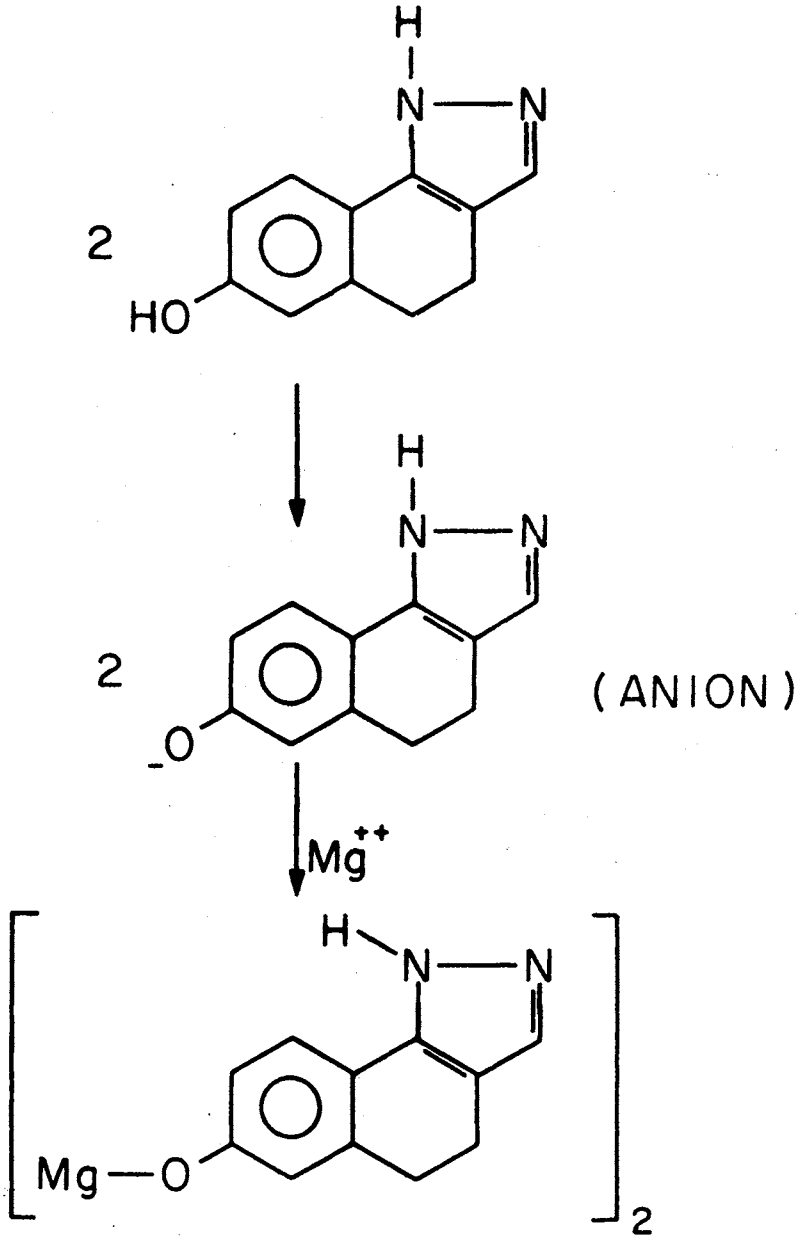

Figure 20. Model for the Possible Interaction of the Hydroxybenzindazole With Mg^{++}

Therefore, under conditions of Mg^{++} deficiency, a cell might restrict synthesis of LPS or LPS precursors in order to conserve Mg^{++} for more essential uses. This would be the opposite effect observed for cells growing in abundant, easily utilized nutrients in which large amounts of LPS are accumulated.

Growth of P. fluorescens in increasing concentrations of the hydroxybenzindazole was accompanied by decreasing levels of LPS. This pattern of diminished quantities of LPS was also observed in cells grown in a limited Mg^{++} environment. This information, in addition to similar reactions with actinomycin D and EDTA along with the fact that protection from the effect of growth in the hydroxybenzindazole can be achieved by the addition of excess Mg^{++} to the growth medium, suggested a direct link between the action of the hydroxybenzindazole and the limitation of Mg^{++}. This action resulted in a restricted synthesis of LPS thereby altering the molecular integrity of the cell peripheral structure, perhaps increasing the overall permeability of the cell.

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