STUDIES ON THE BIOLOGICAL ACTION OF A HYDROXYPYRAZOLE COMPOUND

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CHAPTER I

INTRODUCTION

The broad spectrum of biological activity associated with steroids makes them a very intriguing class of compounds. In recent years much attention has been focused on the biological activities of steroids possessing nitrogen atoms (Alauddin and Martin-Smith, 1962a,b). The azasteroid class of compounds possess several desirable physical characteristics which make them useful as tools for elucidating the mechanisms of biological phenomenon: (1) a high degree of fluorescence; (2) increased water solubility at physiological pH; (3) hydrogen bonding potential; and, (4) the potential for molecular complex formation.

The nitrogenous steroids possess many pharmacological properties which make them suitable for use as therapeutic agents. The most notable of these properties are anti-inflammatory activity, anti-hypercholesterolaemic activity, digitalis-like activity, coronary dilatory activity and central nervous system activity. Several new azasteroid compounds have also been prepared that show weak androgenic, estrogenic and progestational activity. Several nitrogen containing steroids have been shown to possess anti-tumor activity, and the numbers of azasteroids shown to have antibacterial properties has also increased in recent years (Martin-Smith and Sugrue, 1964). Svoboda, Thompson and Robbins (1972) recently reported an interesting use of

azasteroid compounds as inhibitors of insect molting and metamorphosis.

Barnett, Ryman and Smith (1946a) were the first to report antibacterial activity of azasteroid compounds and found that both the α and β isomers of 7-aminocholesterol showed a high antibacterial activity *in vitro* against gram-positive organisms. These authors also reported that mono- and di-cholestane derivatives with nitrogen in the 3, 6 or 7 positions showed marked antimicrobial activity; however, only the diaminosteroids showed any appreciable bacteriostatic activity against gram-negative organisms. Both mono- and diaminosteroids were highly active *in vitro* against *Streptococci* and *Staphylococci*; however, the diaminosteroids consistently showed a higher antibacterial potency than the monoaminosteroids. Moreover, the 3:6, 3:7 and 6:7-diaminosteroids showed considerable bacteriostatic activity against gram-negative organisms, whereas, the monoaminosteroids showed activity only against gram-positive organisms (Barnett, Ryman and Smith, 1946b).

Kull, Castellano and Mayer (1953) and Tarbet, Oura and Sternberg (1953) also reported that certain steroids containing nitrogen in the form of amino groups attached to the steroid nucleus were effective against microorganisms. However, Smith, Shay and Doorenbos (1963a) were the first to report a group of steroids containing nitrogen in the nucleus that showed antimicrobial properties. Doorenbos and Bossle (1965) reported that derivatives of 4-aza-5α-cholestane were bacteriocidal against most gram-positive bacteria and fungicidal against most molds and yeasts.

The results of this work were in agreement with that of Lester and Hechter (1958) and Casas-Campillo et al. (1961), who found that

sensitivity to steroids was limited essentially to gram-positive microorganisms. The gram-positive bacteria were the most sensitive while the yeasts and molds were the least sensitive. With the exception of Brucella abortus, none of the gram-negative bacteria showed any sensitivity to the steroids. Although none of the steroids inhibited the gram-negative bacteria, the sixteen compounds tested by Smith, et al. (1963a) interfered with the production of the lipid-soluble pigment of Serratia marcescens. The water-soluble pigment of Pseudomonas aeruginosa was readily synthesized in the presence of the steroids.

Smith, Shay and Doorenbos (1963b) were among the first to devote attention to the mechanism of action of azasteroid compounds which do not occur in nature. Working with nitrogen-substituted cholestanes, they observed spontaneous hemolysis of sheep and human red blood cells. These same authors later reported (1964) that none of the biologically active azasteroid compounds caused complete disruption of sheep red blood cells. Azasteroid action caused marked extrusion of hemoglobin; however approximately 50 percent of the cells were still intact after 16 hours. A positive relationship between antimicrobial activity and hemolytic activity was not found; however, these authors postulated that the lethal effects of these azasteroids may be assisted by the surfactant properties.

Further work on the mechanism of action of synthetic steroids was done by Smith and Shay (1965). Working with nitrogen-substituted cholestanes, they observed that these compounds were inhibitory and lethal to cells of Sarcina lutea. From their work with S. lutea protoplasts, they suggested that direct action on cell membranes may

be chiefly responsible for the antimicrobial properties of the steroids. The work of Varricchio (1966) lends further evidence that azasteroid action may be at the membrane level. The same compounds used by Smith and Shay (1965) were found to be potent inhibitors of reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidation with membrane fragments from Bacillus subtilis. The relative effectiveness of the azasteroids in inhibiting $NADH_2$ oxidation paralleled their effectiveness in inhibiting the growth of cultures of B. subtilis or S. lutea. Varricchio (1966) suggested that since the azasteroids are potent inhibitors of $NADH_2$ oxidation, the effect of the azasteroids on membranes may be closely linked with inhibition of electron transport. Commager and Judis (1965) noted that the bacteriocidal action of phenols may be due to damage of permeability mechanisms, the repair of which is prevented by concomitant inhibition of energy-yielding metabolic reactions.

Bangham, Standish and Weissmann (1965) offered evidence for an interaction between steroid molecules and the lipids which compose the limiting membranes of biological material. They suggested that steroids may exert their pharmacological actions and physiological functions, at least in part, by regulating the permeability of cells and their organelles and that the membrane action of biologically active compounds may result from their direct interaction with lipid, which is independent of polysaccharide, proteins or active cell metabolism.

Research conducted using the steroidal diamine, irehdiamine A (IDA), indicated that it is a potent inhibitor of bacteriophage growth and macromolecular synthesis in Escherichia coli (Silver and Levine,

1968a). Their findings that IDA affected both the influx and efflux of ^{42}K at low steroid concentrations led them to conclude that the primary action of steroidal diamines is on the cell membrane, resulting in a generalized increase in cell permeability and an inactivation of cellular permeases. Reversal of IDA inhibition could be achieved by addition of 1 mM $MgCl_2$. The extent of the reversal depended upon the length of time between the addition of IDA and the addition of magnesium to the growth medium (Silver and Levine, 1968b).

The relationship between growth inhibition and the concentration of cells and steroid had been observed by many workers. Binding of steroids by protein was reported by Eik-Nes et al. (1954). This relationship prompted Varricchio, Doorenbos and Stevens (1967) to determine whether there was a relationship between antibacterial activity of the azasteroids and their ability to bind to the bacterial cell. Hartman and Holmlund (1962) had observed in earlier work that several microorganisms, especially fungi, were able to render β-sitosterol non-extractable by conventional methods; moreover, B. cereus was found to bind 6 percent of the sterol. Varricchio et al. (1967) found that the azasteroid compounds were readily bound to B. subtilis cells in amounts exceeding the growth inhibitory amount. Further studies using ^{14}C labeled cholestanone as a model compound indicated that approximately 50 percent of the label was bound to the membrane. They concluded that binding was probably nonspecific, and possibly on the surface of a lipid or phospholipid.

Other workers have observed action at the cell membrane level, but reported that deoxyribonucleic acid (DNA) is the primary target of steroidal diamine action. Mahler and Dutton (1964) were the first to

report that the alkaloid cyclobuxine, a steroidal diamine, exerted a profound effect on the stability of helical polynucleotides. Mahler et al. (1966) concluded that strong interaction between the azasteroid and DNA required the presence of two amino groups on the steroid nucleus--a single amino group in either ring A or D being completely ineffective. They postulated a stable but somewhat distorted helix with the bases tilted, or at least no longer aligned in the same manner as they were in the native Watson-Crick-Wilkins B conformation--a model in which the steroid molecules are bound but probably not intercalated into the DNA helix.

An agent capable of strong interaction with DNA might be expected to affect mutation rates *in vivo*. In work with IDA and malouetine, Mahler and Baylor (1967) demonstrated that steroidal diamines are mutagens and effective inhibitors of phage replication and postulated that DNA was the primary target of diamine action *in vivo*. IDA was found to effectively inhibit the reproduction of bacteriophages T2 and T4. Addition of IDA, either at zero minutes or after ten minutes, virtually stopped DNA synthesis, with effects on messenger RNA and protein synthesis being less pronounced. They concluded that transcription and translation are relatively insensitive to steroid action--the most sensitive event being the formation of the first few copies of parental DNA.

Binding to DNA was also found by Krey and Hahn (1969) in their work with the alkaloid, berberine. Berberine shifted the thermal strand separation profile of DNA to higher temperatures, thereby suggesting the formation of a complex between the alkaloid and DNA. Further evidence for binding to DNA was suggested by the addition of

increasing concentrations of DNA to berberine which progressively decreased the absorption intensity of the two bands of the berberine spectrum.

As the search for new and better antibiotics continues many workers have focused their efforts on enhancing the effect of known antibiotics. Recent work by Riehm and Biedler (1972) described the potentiation of the drug effect obtained when Tween 80, a nonionic detergent, is used in combination with actinomycin D or daunomycin. Chinese hamster cells resistant to actinomycin D and daunomycin were found to be much more sensitive to drug action when the antibiotics were used in combination with Tween 80. The synergistic effect was found to occur even at demonstrably nontoxic concentrations of Tween 80 for sensitive and resistant cells.

E. coli cells are normally impermeable to actinomycin D, which inhibits DNA-dependent RNA synthesis and the consequent inducible enzyme synthesis. Leive (1965) has shown that E. coli cells treated with EDTA are more susceptible to actinomycin D and that lipopolysaccharide is released from the cell wall. Roy and Mitra (1970) also reported lipopolysaccharide release with a small filamentous phage, M 13. E. coli K-12 cells infected with M 13 were shown to be permeable to actinomycin D and release lipopolysaccharide into the medium.

Since action at the membrane level would allow quicker entry of molecules into the cell, membrane active antibiotics should potentiate the action of other antibiotics with internal sites of action. Both polymyxin and circulin are surface active antibiotics thought to cause disorganization of the cell membrane leading to a loss of intracellular components (Coleman, 1969). Potentiation studies utilizing polymyxin,

circulin and actinomycin D were conducted during the course of this present investigation to determine the biological effect produced when these antibiotics were used in combination with a new azasteroid compound.

This thesis is concerned with the mode of biological action of a new hydroxypyrazole compound (Fig. 1) synthesized by Dr. K. D. Berlin and Dr. J. G. Morgan at Oklahoma State University (Morgan et al., 1971). The molecular weight of the compound is 236 with a melting point of 257-260 C. The structure of the compound suggests a potential for surfactant properties--the benzene rings would constitute the large non-polar nucleus with the hydroxyl group and two nitrogen groups contributing polar properties to the molecule.

The aromatic nucleus of the molecule gives the compound two properties which make it conducive to biological assay under test conditions. The first property is the ultraviolet (UV) absorption spectrum of the compound. Absorption scanning in the UV range indicates peaks of 258, 266, 300 and 312 nm (Fig. 2). The peak at 312 nm permits quantitation of the compound in growth medium under conditions in which 260 nm absorbing material may be released from the bacterial cell. The second property of the aromatic nucleus which can be used in assay procedures is its fluorescent characteristics. The hydroxypyrazole compound has fluorescent excitation peaks at 250, 268, 302 and 320 nm. The emission spectrum shows a single emission peak at 382 nm (Fig. 3).

It is the purpose of this paper to suggest the possible mode(s) of action of the hydroxypyrazole compound as well as illustrate potential use of the compound as a therapeutic agent in combination

Figure 1. The Hydroxypyrazole Compound. The molecule has a large non-polar nucleus with a polar hydroxy group attached to the number 3 carbon and polar nitrogens incorporated into the steroid nucleus at the 15 and 16 positions.

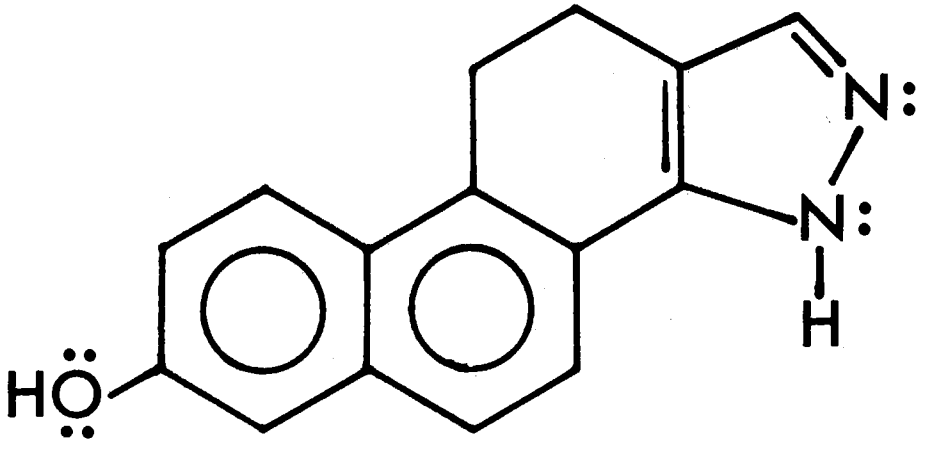

N:
N:
H
HO

Figure 2. UV Spectrum of the Hydroxypyrazole Compound. The compound demonstrates absorption maxima at 258, 266, 300 and 312 nm

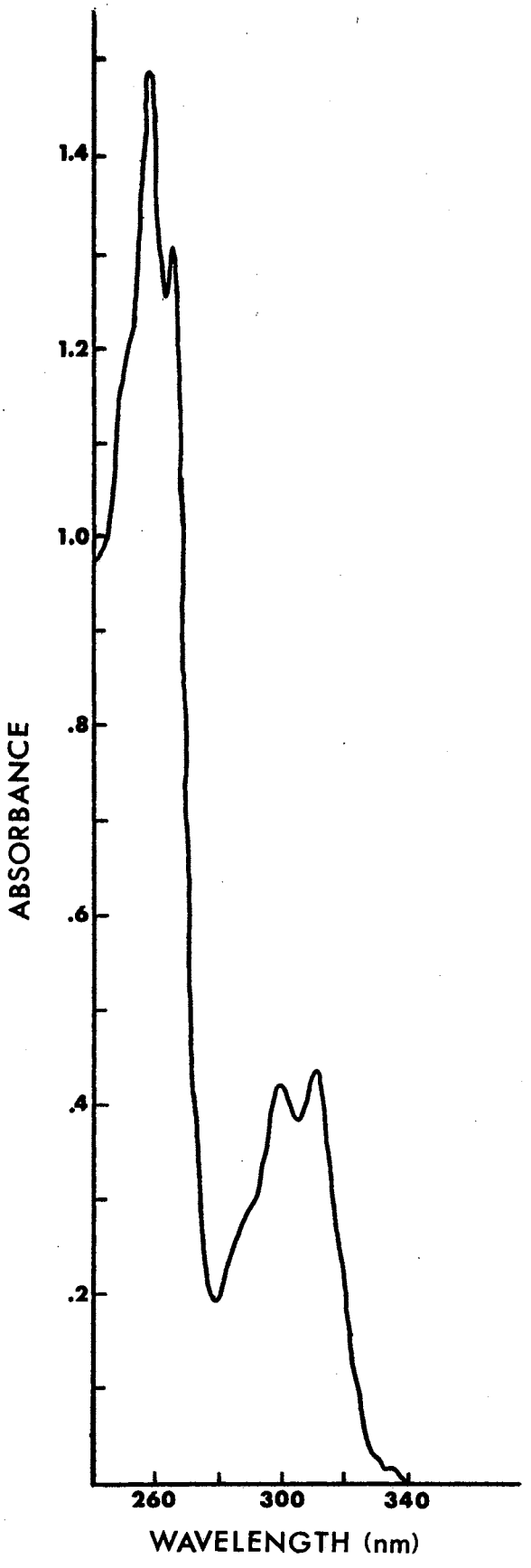
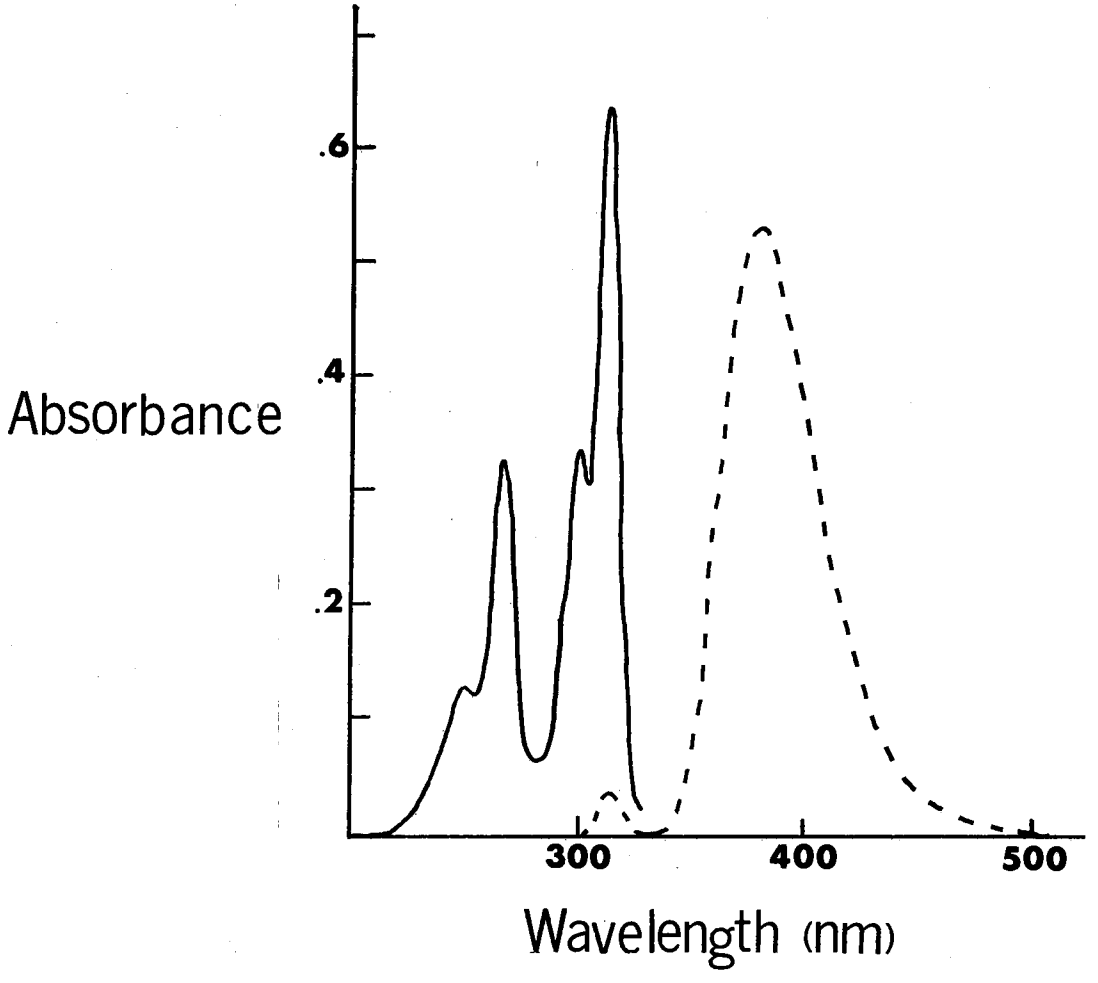

1.4
1.2
1.0
.8
.6
.4
.2
ABSORBANCE
260
300
340
WAVELENGTH (nm)

Figure 3. Fluorescent Excitation and Emission Spectra of the Hydroxypyrazole. The compound exhibits excitation maxima at 250, 268, 302 and 320 nm with a single emission peak at 382 nm. (——), excitation spectrum with emission wavelength of 382 nm; (---), emission spectrum with excitation wavelength of 320 nm.


Absorbance
.6
.4
.2
300
400
500
Wavelength (nm)

with various antibiotics. The compound has surfactant properties which are manifested by its action on sheep red blood cells and B. subtilis W23 protoplasts. Thermal melting curves of isolated P. fluorescens DNA and mutagenic studies utilizing B. subtilis 168, an indoleless strain, offer evidence that the compound may also have an action at the DNA level. Antibiotic potentiation studies using Pseudomonas organisms, a genus notorious for its resistance to antibiotics, indicate a potential use as a chemotherapeutic agent against Pseudomonas infections. The hydroxypyrazole greatly enhances the action of actinomycin D against P. fluorescens. Experiments using the UV and fluorescent characteristics of the compound indicate that it complexes with the actinomycin D molecule. The compound possesses no estrogenic properties and produces no toxic effect in treated animals which further emphasizes its potential as a therapeutic agent.

CHAPTER II

MATERIALS AND METHODS

Test Organisms

The microorganisms used in this study were obtained from the stock culture collection of Dr. N. N. Durham, Oklahoma State University. One of the organisms, Pseudomonas fluorescens, has been tentatively identified by Montogomery (1966). It is a gram-negative, motile rod which forms smooth, raised colonies on nutrient agar. The organism exhibits a negative reaction for hydrogen sulfide production, indole production and nitrate reduction, and produces acid but no gas in glucose. This organism also produces fluorescein and pyocyanin when grown on Bacto-Pseudomonas agar F and Bacto-Pseudomonas agar P, respectively. Bacillus subtilis W23 is a prototrophic strain initially obtained from Dr. W. C. McDonald, Tulane University. It is a gram-positive, motile organism with an optimal growth temperature of 37 C. B. subtilis 168, which has a growth requirement for indole or L-tryptophan (Anagnostopoulos and Spizizen, 1961), was used in the mutagenic phases of this study.

Stock cultures of P. fluorescens, B. subtilis W23 and B. subtilis 168 were maintained on slants of succinate-salts, glucose-salts and nutrient agar, respectively, and stored at 4 C.

Synthetic Media

The 0.2 percent succinate-salts synthetic medium used in this study had the following composition: 0.2 percent NaCl, 0.2 percent NH_4Cl, 0.32 percent KH_2PO_4 and 0.42 percent K_2HPO_4. Succinate was autoclaved separately and added aseptically to the sterile minimal salts base. The pH of the succinate was adjusted to 7.0 with KOH prior to sterilization. Sterilization was achieved by autoclaving for 15 minutes at 121 C. After cooling to room temperature, the medium was supplemented with 0.1 ml of a sterile mineral salts solution for each 100 ml of the medium. The mineral salts solution had the following composition: 5.0 percent $MgSO_4 \cdot 7H_2O$, 0.1 percent $MnSO_4$, 1.0 percent $FeCl_3$ and 0.5 percent $CaCl_2$. Two percent agar (Difco) was added when a solid medium was required.

The basal medium for the 0.5 percent glucose-salts had the following composition: 1.4 percent K_2HPO_4, 0.6 percent KH_2PO_4 and 0.2 percent $(NH_4)_2SO_4$. Glucose was autoclaved separately and added aseptically to the sterile minimal salts base. After cooling to room temperature following sterilization, the medium was supplemented with 0.1 ml of sterile mineral salts solution for each 100 ml of the medium.

Chemicals

Actinomycin D (Merck, Sharp and Dohme Research Laboratory), polymyxin (Pfizer Chemical Company), lysozyme (Worthington Biochemical Corporation), calf thymus DNA and guanosine 5'-monophosphoric acid (Sigma Chemical Company) were dissolved in sterile glass-distilled water, and stock solutions stored at 4 C. The concentrations of all

chemicals used in this study are recorded in the text.

Spectrophotometric Studies

The UV absorption spectra of aqueous solutions of the hydroxypyrazole, actinomycin D, calf thymus DNA, and guanosine 5'-monophosphoric acid (GMP) were determined using a Cary 14 recording spectrophotometer. Appropriate controls were used when the spectrum of a mixed solution (hydroxypyrazole plus actinomycin D, DNA or GMP) was determined. The spectrum of each compound or mixture of compounds was determined between the wavelengths of 220-340 nm using quartz cuvettes with a 1 cm light path.

The fluorescent excitation and emission spectra of the hydroxypyrazole compound in water was determined on an Aminco-Bowman scanning spectrophotofluorometer using quartz cuvettes with a 1 cm light path. The emission and excitation spectra were determined between the wavelengths of 200-500 nm. After the peak excitation and emission wavelengths were determined, further fluorescent determinations were made using a Hitachi photofluorometer. The hydroxypyrazole was excited at 320 nm, and the emission measured at 382 nm.

Growth Inhibition

Studies were conducted to determine the effect of the hydroxypyrazole on growth of P. fluorescens and B. subtilis W23 cells. The organisms were grown 10-12 hours in sterile tubes (18 x 150 mm) containing 6.0 ml of the appropriate medium. Aeration was provided by shaking on a reciprocal shaker (100 oscillations per minute) with an incubation temperature of 37 C. These cells were used as inocula

for growth studies which were performed in test tubes (18 x 150 mm) containing a total volume of 6.0 ml. Growth was followed by reading the absorbance at 540 nm on a Coleman Junior II spectrophotometer.

Dose response assays of the hydroxypyrazole and various antibiotics, used separately or in combination, were determined by addition of the appropriate test compound(s) in increasing concentrations to the culture at time sero. The inhibition was measured by following the absorbance of the culture at 540 nm.

Reversal of the hydroxypyrazole inhibition of growth was determined by growing B. subtilis W23 cells in glucose-salts minimal medium in the presence and absence of the compound. After the inhibition had been established and the control cells were well into log phase, the cells were centrifuged (6,000 x g) for 10 minutes under aseptic conditions and suspended in potassium phosphate buffer (pH 7.0; 10 mM) to the same absorbance. The cells were then inoculated into glucose-salts medium in the presence and absence of the compound. Growth was determined by following the absorbance at 540 nm.

Manometric Studies

The possible use of the hydroxypyrazole as a carbon source by P. fluorescens was determined by following oxygen uptake in a Warburg apparatus at 37 C. Cells were grown in the succinate-salts medium for 18 hours, transferred to fresh medium and grown for 6 additional hours. The cells were harvested by centrifugation (6,000 x g) for 10 minutes and suspended to an absorbance of 0.85 at 540 nm in minimal salts buffer with no carbon source. The Warburg flasks contained a total volume of 2.7 ml with 0.2 ml of a 20 percent solution of KOH placed

in the center well to absorb CO_2 released during the course of the experiment. Cells (2.3 ml) were placed in the flasks with 0.2 ml of either water, 0.05 M glucose or 20 µg/ml hydroxypyrazole to determine endogenous respiration, respiration in the presence of glucose, and respiration in the presence of the hydroxypyrazole, respectively. The amount of O_2 uptake (µℓ) was determined at 10 minute intervals.

Cell Wall Isolation

B. subtilis W23 cell walls were isolated by the procedure of Best and Durham (1965) except the isolated cell walls were further purified by twice heating to boiling in a water bath while suspended in 1.0 percent sodium laural sulfate in pH 9.0 Tris buffer (0.05 M Tris plus 0.005 M magnesium sulfate). The trypsinization and washing steps were then performed according to the procedure of Best and Durham (1965). The cell walls were lyophilized and stored at 4 C.

Binding Studies

Binding of the hydroxypyrazole compound to B. subtilis W23 cell walls was determined by exposing a fixed amount of cell walls to increasing concentrations of the compound. Lyophilized cell walls were prepared as described above and suspended to a concentration of 0.1 mg/ml in sterile glass-distilled water. Aliquots (1.0 ml) of this suspension were pipetted into 8 tubes and pelleted by centrifugation (27,000 x g for 15 minutes). The pellets were suspended in increasing concentrations of hydroxypyrazole (1.0-8.0 µg/ml final concentration) in a total volume of 5.0 ml. The samples were allowed to sit for 10 minutes at room temperature, centrifuged (27,000 x g for 15 minutes),

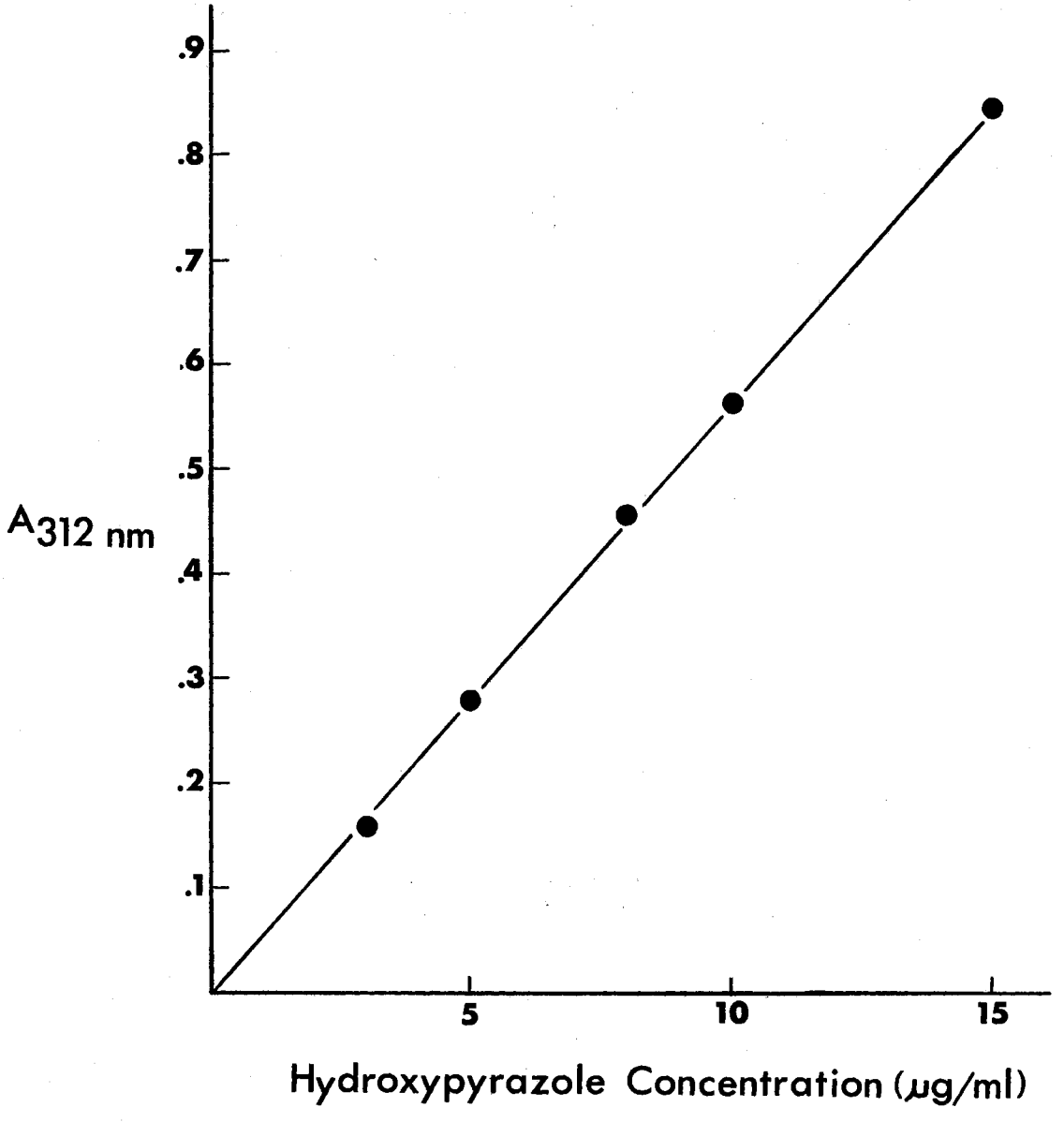
and the absorbance of the supernatant solution read at 312 nm. The amount of hydroxypyrazole adsorbed per sample was then calculated by comparing against a standard curve of the hydroxypyrazole measured at 312 nm (Fig. 4).

Adsorption to B. subtilis W23 whole cells was determined in a similar manner. Cells were grown 10-12 hours on glucose-salts medium, harvested by centrifugation (6,000 x g for 10 minutes), washed with potassium phosphate buffer (pH 7.0; 10 mM) and suspended to a concentration of 0.3 mg/ml. Aliquots (1.0 ml) of this suspension were pipetted into 6 tubes and pelleted by centrifugation (10,000 x g for 10 minutes). The pellets were suspended in increasing concentrations of the hydroxypyrazole (3.0-15.0 µg/ml final concentration) and allowed to sit for 10 minutes at room temperature. The cells were pelleted by centrifugation (10,000 x g for 10 minutes) and the absorbance of the supernatant solution determined at 312 nm. The number of binding sites per cell was determined by finding the number of cells present in the 0.3 mg pellet by viable cell count, then comparing the amount of hydroxypyrazole necessary for saturation. The cells were agitated vigorously with a Vortex mixer to help eliminate chained cells.

Protoplast Preparation

Protoplasts of B. subtilis W23 were prepared by growing the cells 10-12 hours on glucose-salts agar plates. The cells were harvested using potassium phosphate buffer (pH 7.0; 10 mM), pelleted, and washed twice by centrifugation (6,000 x g for 10 minutes) in buffer. The pellet was suspended in 30 ml of 0.6 M sucrose containing lysozyme

Figure 4. Standard Curve of the Hydroxypyrazole at 312 nm. Hydroxypyrazole absorption at 312 nm was measured by dissolving various concentrations of the compound in distilled water and reading the absorbance on a Beckman DU spectrophotometer using 1 cm quartz cuvettes.


.9
.8
.7
.6
.5
.4
.3
.2
.1
A312 nm
5
10
15
Hydroxypyrazole Concentration (µg/ml)

(400 µg/ml) and allowed to sit at room temperature under nitrogen gas for 2 hours. Protoplast formation was confirmed by phase-contrast microscopy. The protoplasts were further purified by a slow speed centrifugation (2,000 x g for 5 minutes) to eliminate whole cell or cell ghosts from the preparation.

Effect of Hydroxypyrazole on Membranes

The effect of the hydroxypyrazole compound on membranes of procaryotic and eucaryotic cells was determined using B. subtilis W23 protoplasts and sheep red blood cells. Protoplasts of B. subtilis W23 were prepared as described above. The amount of protoplasts necessary to give a final absorbance of 0.2 at 540 nm for a 5.0 ml sample was determined. The protoplasts were then pipetted into two tubes. Five ml of 0.6 M sucrose was added to one tube as a control, and 5.0 ml of the hydroxypyrazole (20 µg/ml) made up in 0.6 M sucrose was added to the second tube. The absorbance at 540 nm was followed to determine lysis or membrane leakage.

Three day old sheep red blood cells were used for determining the effect of the hydroxypyrazole on eucaryotic membranes. The red blood cells were washed 3 times in physiological saline solution (PSS) by centrifugation (6,000 x g for 5 minutes). The amount of cells necessary to give a final absorbance of 1.0 at 412 nm when lysed in 5.0 ml of water was determined. Aliquots were then pipetted into 3 tubes. Five ml of water, PSS or hydroxypyrazole (20 µg/ml) dissolved in PSS was added to the tubes to determine 100 percent lysis, leakage in PSS and the effect of the hydroxypyrazole on the cell membrane, respectively. The tubes were allowed to sit at room temperature for

24 hours. The intact cells were pelleted by centrifugation (10,000 x g for 10 minutes), and the absorbance of the supernatant solution read at 412 nm to determine leakage of hemoglobin from the cell.

Uptake of Radioactive Substrates

Glucose-salts grown B. subtilis W23 cells were used to assay the effect of the hydroxypyrazole on uptake of ^{14}C labeled substrates. Cells were grown 10-12 hours, and then inoculated into two 250 ml side arm flasks containing 15 ml of fresh medium to an absorbance of 0.1 at 540 nm. The cells were incubated at 37 C in a shaking water bath (100 oscillations per minute) until growth was initiated. Initiation of the uptake experiment was marked by the addition of the following compounds to the growing cultures: 0.2 ml of ^{14}C labeled substrate (5 μC/ml), 0.5 ml of cold carrier substrate (1 mg/ml) and either 2.0 ml of hydroxypyrazole (87 μg/ml) or water as a control. Samples (1.0 ml) were taken at the indicated time intervals and filtered through a Millipore filter (10 mm diameter, 0.45 micron pore size). The filters were washed 3 times with 1.0 ml volumes of potassium phosphate buffer (pH 7.0; 10 mM) and allowed to air dry. The filters were then placed in counting vials and 10 ml of Aquasol (New England Nuclear) added. Radioactivity was determined using a liquid scintillation counter (Nuclear Chicago) with a counting efficiency of 40 percent for ^{14}C under these condition.

Incorporation of Radioactive Substrates by Cells

The procedure for determining incorporation of ^{14}C labeled substrates was the same as for measuring uptake except that the cells

were washed 3 times with 1.0 ml volumes of cold 5 percent trichloroacetic acid (TCA) instead of using potassium phosphate buffer. Duration of the sampling time was 1 hour with samples taken at the indicated times.

Mutagenic Studies

B. subtilis 168, an indoleless strain, was used to determine if the hydroxypyrazole affected the mutation rate of bacteria. Cells, grown 10-12 hours in nutrient broth, were used as the inoculum for fresh nutrient broth with or without the hydroxypyrazole compound. Cells (0.1 ml) were inoculated into tubes (6.0 ml total liquid volume) containing nutrient broth plus hydroxypyrazole (12 μg/ml) or water as the control. The cells were incubated at 37 C on a reciprocal shaker (100 oscillation per minute), and the absorbance at 540 nm was followed. The cells were plated on nutrient agar and glucose-salts agar using the pour plate method when the cells attained an absorbance of 0.50 to determine the total number of cells present and the number of revertants to indole positive, respectively. A viable cell count of the initial inoculum (0.1 ml) was also performed using nutrient agar to determine the number of cells present initially. The plates were incubated 24-48 hours at 37 C to allow colony formation, then scored for colony forming units to determine the effect of the hydroxypyrazole on mutation rate.

DNA Isolation

P. fluorescens DNA was isolated using a modification of the procedure of Marmur (1961). P. fluorescens cells were grown 10-12

hours in a 2 liter Fernback flask containing 500 ml of succinate-salts broth. These cells were used to inoculate 7 liters of succinate-salts medium. Growth was accomplished by incubation at 37 C on a Microferm Model MF-214 Table Top Fermentor (New Brunswick Scientific Company). The culture was stirred at 400 rpm and aerated with sterile forced air (5 psig).

After the cells reached maximum stationary phase, they were harvested by centrifugation (6,000 x g for 10 minutes) and suspended in 10 ml of NaCl-EDTA (0.10 M NaCl-0.15 M EDTA, pH 8.0). Lysozyme (10 mg/ml) was added to give a final concentration of 100 µg/ml, and the suspension placed on a reciprocal shaker at 37 C for 1 hour. Duponal (30 percent) was added to a final concentration of 3 percent. After complete lysis was observed microscopically, 5 M sodium perchlorate was added to a final concentration of 1.0 M and the suspension permitted to stand at room temperature for 1 hour.

Deproteinization was accomplished by repeated shaking with an equal volume of chloroform-isoamyl alcohol (20:1) for 30 minutes. The aqueous and chloroform phases were separated by centrifugation at 3500 x g for 10 minutes. After pipetting off the top aqueous phase containing the nucleic acid, 2 volumes of cold 95 percent ethanol was gently layered on the aqueous phase to precipitate the nucleic acid strands. When these layers were gently mixed with a stirring rod, the nucleic acid strands could be spooled on the rod as a threadlike precipitate and removed. These strands were dissolved in 0.015 M sodium chloride-0.0015 M sodium citrate. Sufficient 1.5 M sodium chloride-0.15 M sodium citrate was added to give a final concentration of 0.15 M sodium chloride-0.015 M sodium citrate.

The deproteinization procedure was repeated until the protein interface was almost completely removed. Ribonuclease (1 mg/ml), previously heated to 100 C for 10 minutes, was added to the dissolved nucleic acid to give a final concentration of 50 μg/ml. The suspension was placed at 37 C for 1 hour. Deproteinization and alcohol precipitation was repeated until no protein appeared at the interface. The DNA strands were stored in cold 95 percent ethanol.

DNA Melting Studies

Melting studies on P. fluorescens DNA, isolated as described above, were performed using the procedure of Kerr (1963). The effect of the hydroxypyrazole compound (2.0 μg/ml) and actinomycin D (20.0 μg/ml), used separately and in combination, on DNA melting was determined.

Effect of the Hydroxypyrazole on Tissue Culture Cells

The effect of the hydroxypyrazole compound on growth of tissue culture cells was determined using KB and LM cell strains. These experiments were graciously performed by Dr. Louise Higgins. The experimental procedure is as described by Higgins et al. (1972).

CHAPTER III

RESULTS AND DISCUSSION

Effect of the Hydroxypyrazole on Growth of *B. subtilis* W23

Since many azasteroid compounds are known to possess antimicrobial activity, experiments were conducted to determine the effect of the hydroxypyrazole on microbial growth. The compound proved to be a potent growth inhibitor of *B. subtilis* W23 (Fig. 5). A concentration of 2 μg/ml completely inhibited growth for 6 hours, and 1 μg/ml produced a 2 hour lag in the initiation of growth and a 60 percent inhibition after 6 hours. Further experimentation, using different initial cell masses, indicated that the inhibition was dependent on both cell mass and the concentration of the hydroxypyrazole (Table I).

Growth inhibition experiments indicated that *P. fluorescens* cells were not sensitive to much higher concentrations (20 μg/ml) of the hydroxypyrazole. These results are consistent with the report of Smith, Shay and Doorenbos (1963a) that sensitivity to steroids was essentially limited to the gram-positive microorganisms.

It should be noted that *B. subtilis* W23 cells inhibited by 2 μg/ml of the hydroxypyrazole will initiate growth upon prolonged incubation (10-12 hours). This would indicate that resistant organisms are present and/or the compound is bacteriostatic rather than bacteriocidal. The cells might also undergo some physiological adaptation to the

Figure 5. Effect of the Hydroxypyrazole on Early Log Growth of B. subtilis W23. ●, control; ▲, 1 μg/ml hydroxypyrazole; ■, 2 μg/ml hydroxypyrazole.

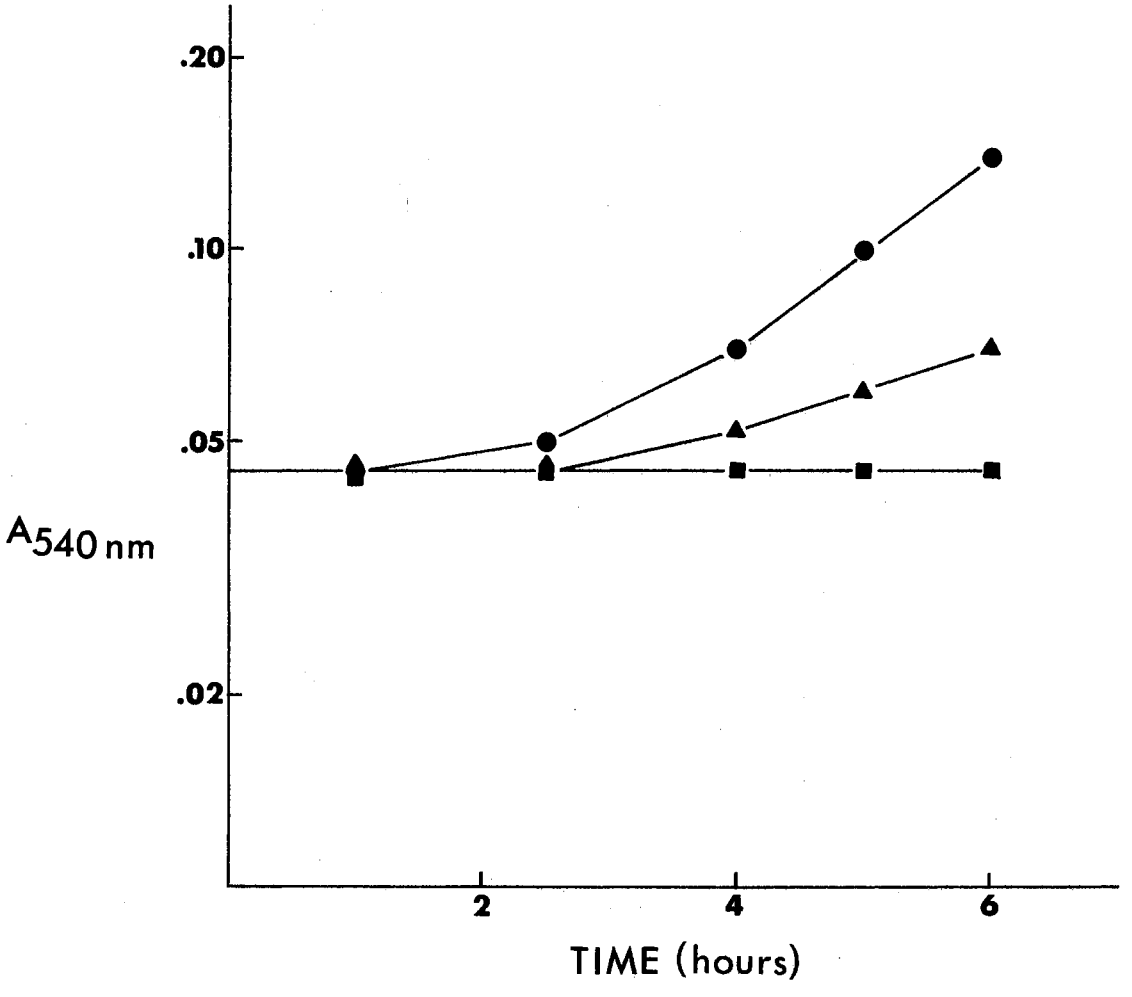

.20
.10
.05
.02
A540 nm
2
4
6
TIME (hours)

TABLE I

THE EFFECT OF CELL MASS ON THE HYDROXY-PYRAZOLE* PROMOTED GROWTH INHIBITION OF B. subtilis W23 CELLS

Initial Absorbance at 540 nm	Percent Inhibition after 3 Hours Incubation**
0.030	100
0.045	100
0.075	32
0.110	0
0.170	0
0.220	0

*Concentration of the hydroxypyrazole was 5.0 μg/ml.

**Incubation temperature was 37 C on a reciprocal shaker (100 oscillations per minute).

hydroxypyrazole. UV spectral analysis indicates that the compound is not broken down by shaking at 37 C.

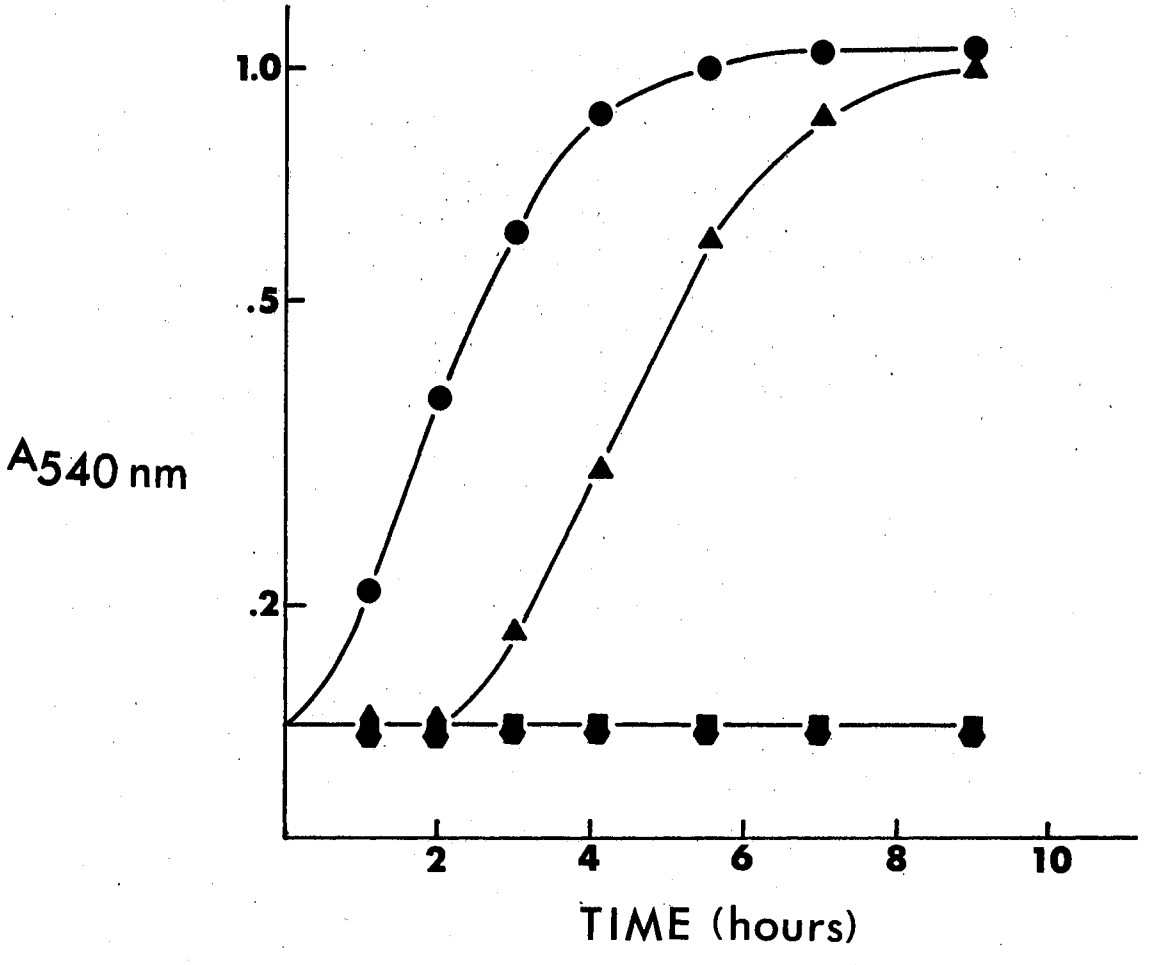
Reversal of Hydroxypyrazole Inhibition

Since the hydroxypyrazole inhibited B. subtilis W23 cells, studies were initiated to determine if the effect was bacteriostatic or bacteriocidal. Experiments were conducted to determine if inhibited cells would initiate growth when placed in fresh growth medium containing no hydroxypyrazole. The results indicated that growth inhibition was reversed after 2 hours incubation in fresh medium with the growth rate of the reversed cells being approximately the same as the control (Fig. 6). Cells suspended in medium containing the compound were completely inhibited for the full 9 hours of the experiment. These results established that the hydroxypyrazole is bacteriostatic rather than bacteriocidal.

Use of the Hydroxypyrazole as a Carbon Source by P. fluorescens

Mazur and Muir (1963) reported that Nocardia sp. A. T. C. C. 14558 was capable of dehydrogenating certain azasteroids with nitrogen in the C ring. Experiments were conducted to determine if P. fluorescens could oxidize the hydroxypyrazole as a source of carbon and energy. Oxygen uptake was measured in a Warburg apparatus and glucose was used as a substrate control. Cells incubated in the presence of the hydroxypyrazole demonstrated a respiration curve that coincided with endogenous respiration for the time examined, indicating that P. fluorescens did not oxidize the hydroxypyrazole (Fig. 7).

Figure 6. Reversal of Hydroxypyrazole Inhibition in B. subtilis W23 Cells. Hydroxypyrazole concentration was 10 µg/ml. ●, control; ▲, hydroxypyrazole inhibited then placed in fresh medium; ■, hydroxypyrazole inhibited then placed back in hydroxypyrazole; ⬢, control cells placed back in hydroxypyrazole.


1.0
.5
.2
A540 nm
2
4
6
8
10
TIME (hours)

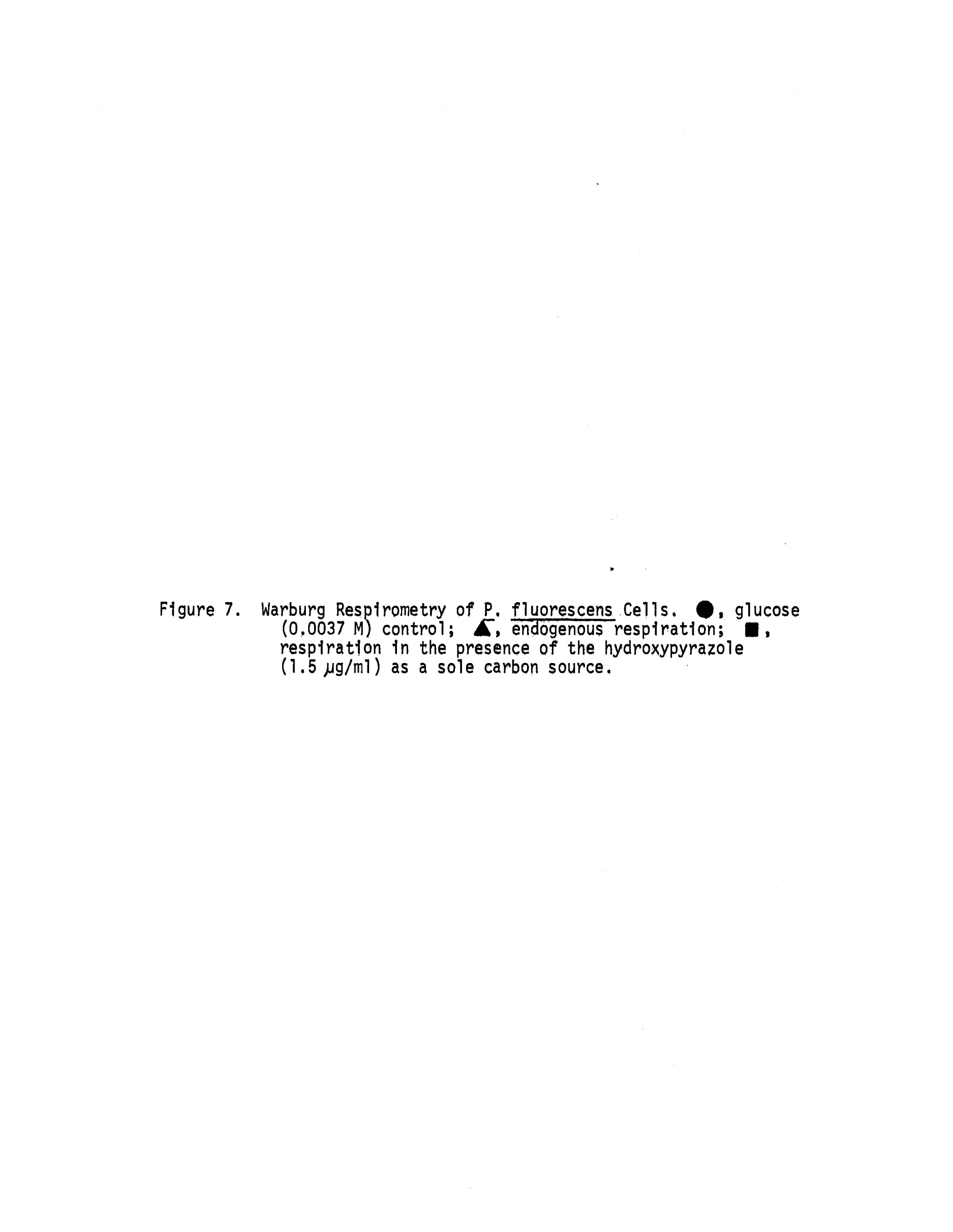
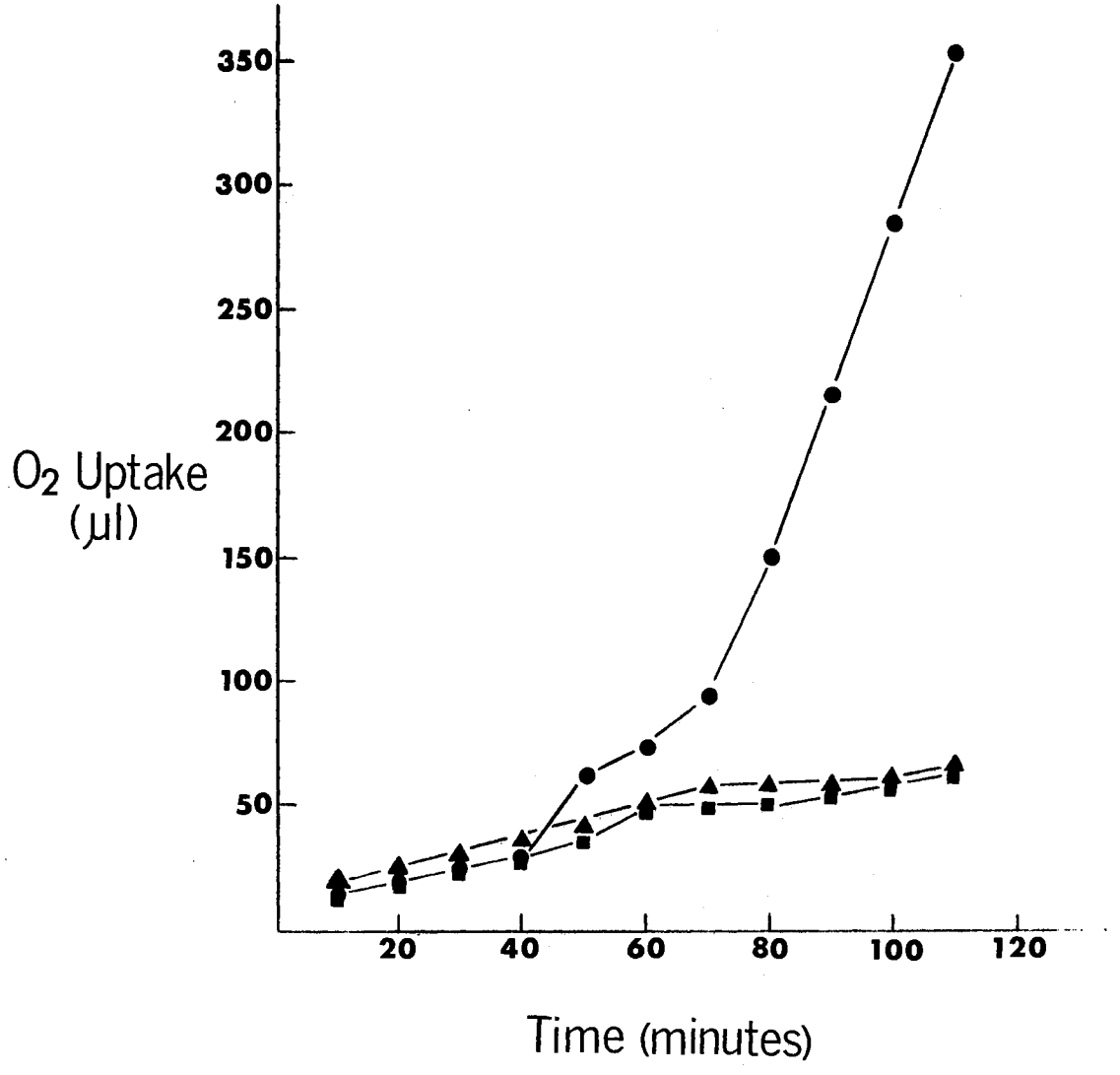


Figure 7. Warburg Respirometry of P. fluorescens Cells. ●, glucose (0.0037 M) control; ▲, endogenous respiration; ■, respiration in the presence of the hydroxypyrazole (1.5 μg/ml) as a sole carbon source.


350
300
250
200
150
100
50
O_2 Uptake
(µl)
20
40
60
80
100
120
Time (minutes)

These results are further substantiated by growth experiments in which P. fluorescens cells were incubated in basal salts medium supplemented with the hydroxypyrazole as the sole carbon source. No growth occurred upon extended incubation under these conditions

Binding of the Hydroxypyrazole to Isolated B. subtilis W23 Cell Walls

Varricchio et al. (1967) observed a relationship between the concentration of azasteroid necessary to inhibit growth and bacterial cell concentration and postulated that the azasteroid molecule may bind to the cell. Hartman and Holmlund (1962) had observed earlier that several microorganisms, especially fungi, were able to render certain steroids nonextractable by conventional means.

Experiments were conducted to determine if the hydroxypyrazole exhibited similar binding characteristics. Binding studies utilizing isolated cell walls of B. subtilis W23 were conducted using the UV absorption peak of the hydroxypyrazole at 312 nm to assay the amount bound. Using a constant amount of cell walls (0.1 mg dry weight), increasing concentrations of the azasteroid were added until saturation was achieved at the 30 μg level (Fig. 8).

The theoretical maximum adsorption can be determined by graphing these data in a double-reciprocal plot and taking the reciprocal of the point where the line crosses the y-axis (Fig. 9). It was determined that approximately 5×10^{16} molecules of the hydroxypyrazole are bound per 0.1 mg of cell wall. Washing of cell walls to which azasteroid had been bound indicated that 20 percent of the compound was released by washing with water, and an additional 5 percent was

Figure 8. Hydroxypyrazole Binding to *B. subtilis* W23 Cell Walls (0.1 mg dry weight).

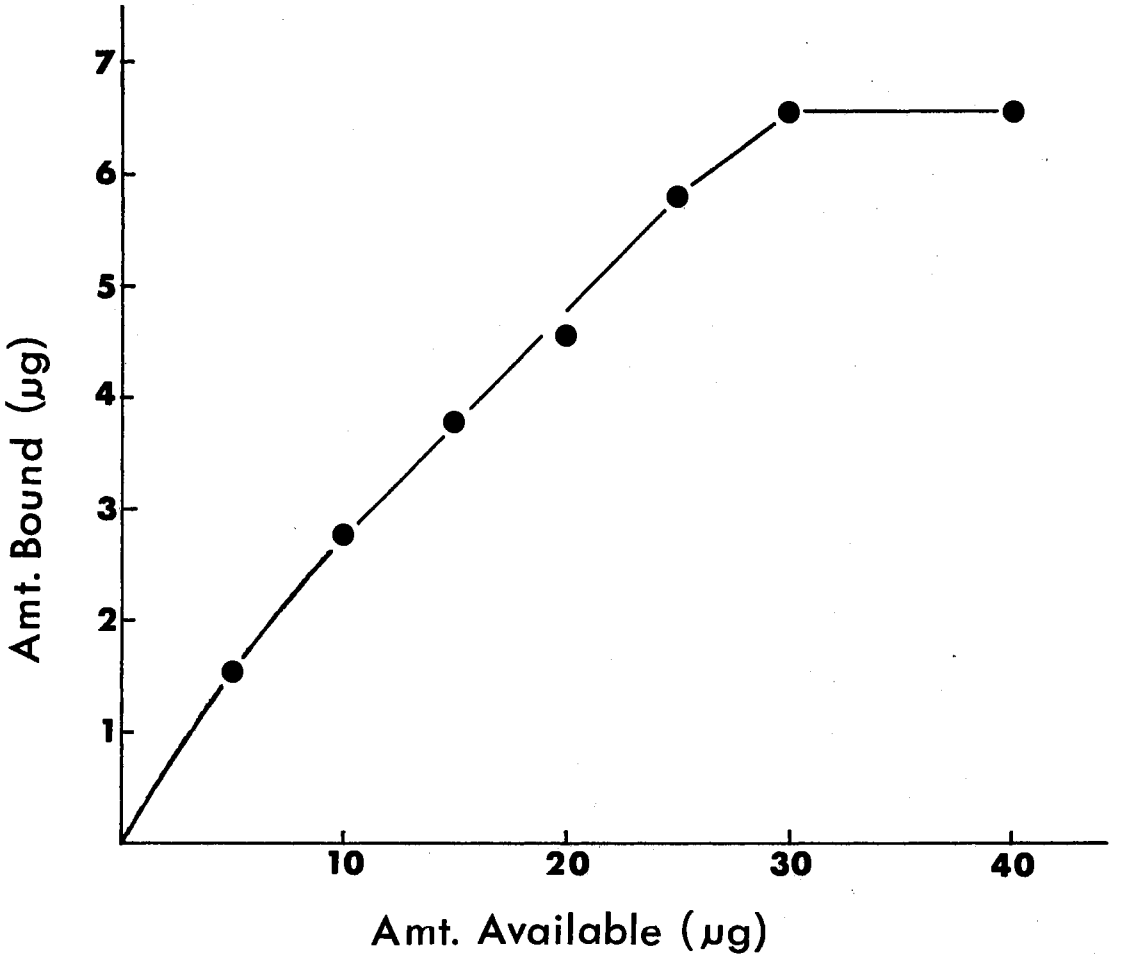
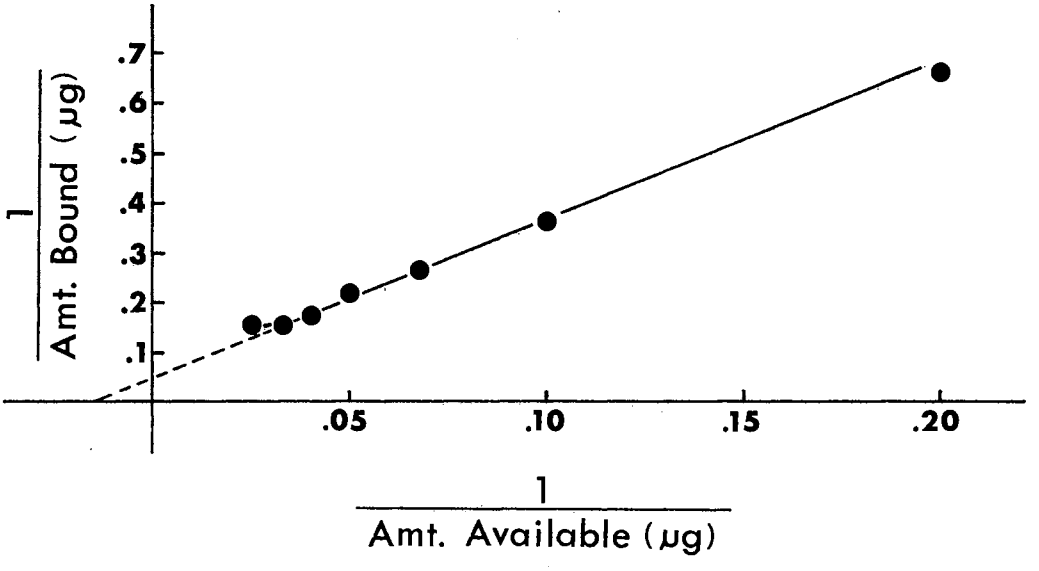

Amt. Bound (µg)
7
6
5
4
3
2
1
10
20
30
40
Amt. Available (µg)

Figure 9. Double-Reciprocal Plot of Hydroxypyrazole Binding to *B. subtilis* W23 Cell Walls (0.1 mg dry weight).


1
Amt. Bound (µg)
.7
.6
.5
.4
.3
.2
.1
.05
.10
.15
.20
1
Amt. Available (µg)

removed by further washing with 1 mM $MgCl_2$. Therefore, 75 percent of the hydroxypyrazole was tightly bound to the cell wall with the remaining 25 percent being somewhat more loosely bound.

Binding of the Hydroxypyrazole to *B. subtilis* W23 Whole Cells

Binding of the hydroxypyrazole to *B. subtilis* W23 whole cells was studied to determine the number of binding sites present on the cell. Using 0.3 mg dry weight of whole cells, saturation by the hydroxypyrazole was achieved at the 50 µg level (Fig. 10). By graphing in a double-reciprocal plot, it was determined that 3×10^{16} molecules bind per 0.3 mg dry weight of *B. subtilis* W23 whole cells or 1×10^{16} molecules per 0.1 mg of whole cells (Fig. 11). Using these data and the number of cells present in 0.3 mg dry weight of *B. subtilis* W23 whole cells, it was determined that approximately 1×10^{8} binding sites exist per cell.

Since the binding to isolated cell walls yielded a value of 5×10^{16} molecules per 0.1 mg, it would appear that the isolated cell wall will bind 5 times the amount bound by whole cells. However, if the fact that the cell wall is approximtely one-fifth of the total cell weight is considered, the two values are compatible.

Using cholestanone as a model steroid, Varricchio et al. (1967) determined that 16 percent of the steroid was bound by *B. subtilis* whole cells. Hartman and Holmlund (1962) reported a 6 percent binding of the sterol, β-sitosterol, to *B. cereus* cells. A value of 10 percent binding of the available hydroxypyrazole was obtained for *B. subtilis* W23 whole cells (Fig. 10). Therefore, the percent binding of the

Figure 10. Hydroxypyrazole Binding to B. subtilis W23 Whole Cells (0.3 mg dry weight).

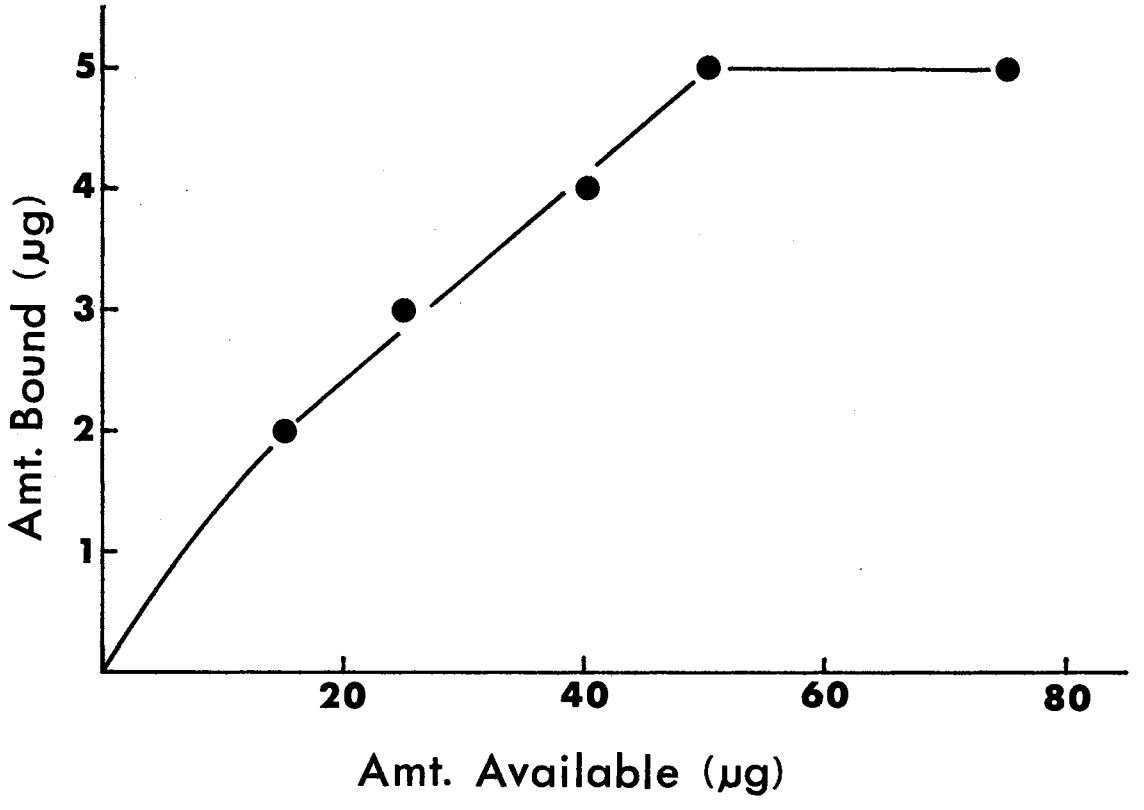
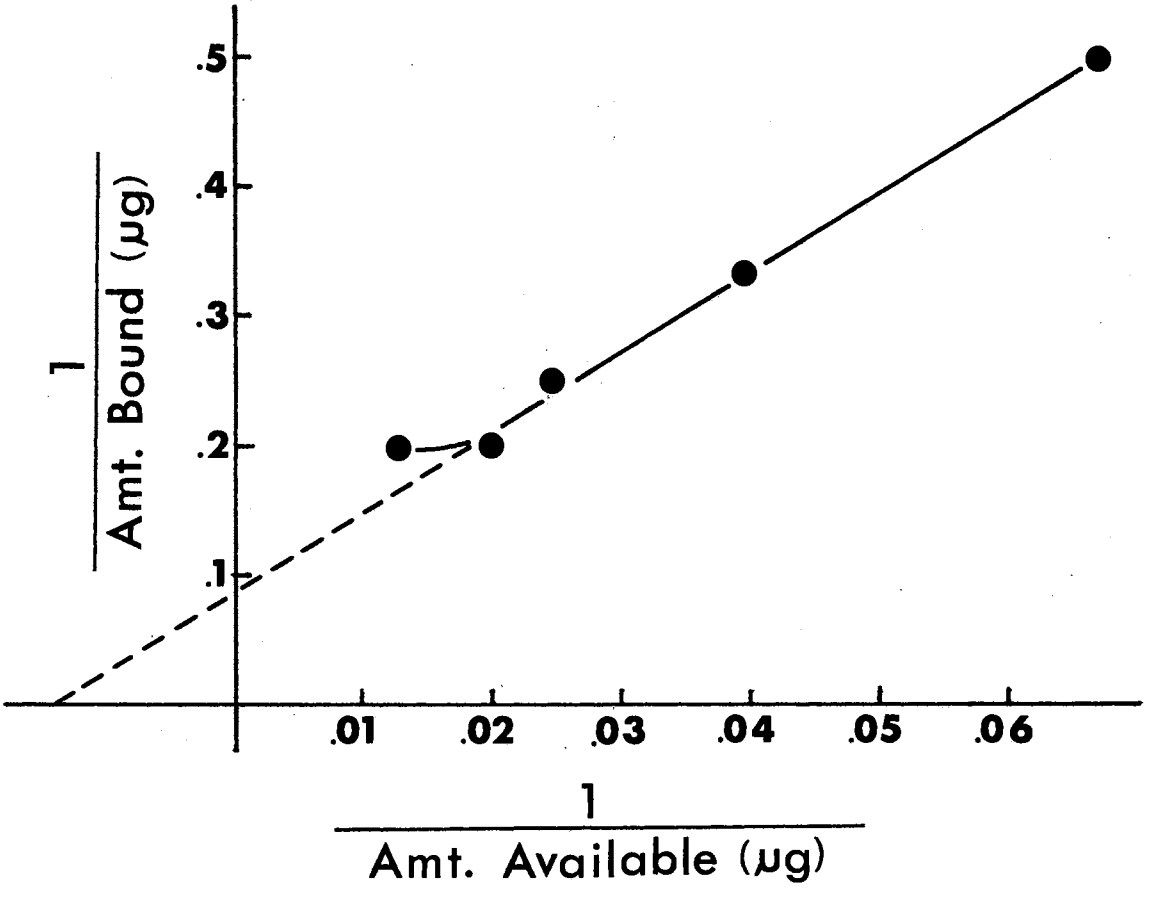

Amt. Bound (µg)
5
4
3
2
1
20
40
60
80
Amt. Available (µg)

Figure 11. Double-Reciprocal Plot of Hydroxypyrazole Binding to *B. subtilis* W23 Whole Cells (0.3 mg dry weight).


.5
.4
.3
.2
.1
1
Amt. Bound (µg)
.01
.02
.03
.04
.05
.06
1
Amt. Available (µg)

hydroxypyrazole to B. subtilis W23 generally corresponds with binding by cholestanone and β-sitosterol.

Effect of the Hydroxypyrazole on a Eucaryotic Membrane

Smith, Shay and Doorenbos (1963b) were the first to devote attention to the mechanism of action of azasteroid compounds. Working with nitrogen-substituted cholestanes, they observed spontaneous lysis of sheep and human red blood cells. Experiments utilizing 3 day old sheep red blood cells were conducted to ascertain if the hydroxypyrazole caused damage at the membrane level (Table II). The results indicated that the compound caused an increased leakage of the hemoglobin from the cell. Three times as much hemoglobin was released from treated cells when compared to the control after 24 hours incubation. Although a membrane effect may be evident, it should be noted that the action of the hydroxypyrazole was not as severe as that observed with detergents and membrane active antibiotics since a period of 24 hours was required to release approximately 30 percent of the total hemoglobin as compared to a 10 percent leakage by the control.

These results are in agreement with the work of Smith, Shay and Doorenbos (1964) who reported that 50 percent of the sheep red blood cells remained intact after 16 hours following treatment with a nitrogen-substituted cholestane.

TABLE II

EFFECT OF THE HYDROXYPYRAZOLE ON HEMOGLOBIN RELEASE FROM SHEEP RED BLOOD CELLS

	$A_{412\ nm}$ after 24 Hrs.	Percent Lysis
Lysis Control*	1.21	100
Control (PSS)	0.125	10
Hydroxypyrazole**	0.378	31

*Lysis was accomplished by suspending the red blood cells in distilled water.

**The hydroxypyrazole was dissolved in PSS to a concentration of 20 μg/ml.

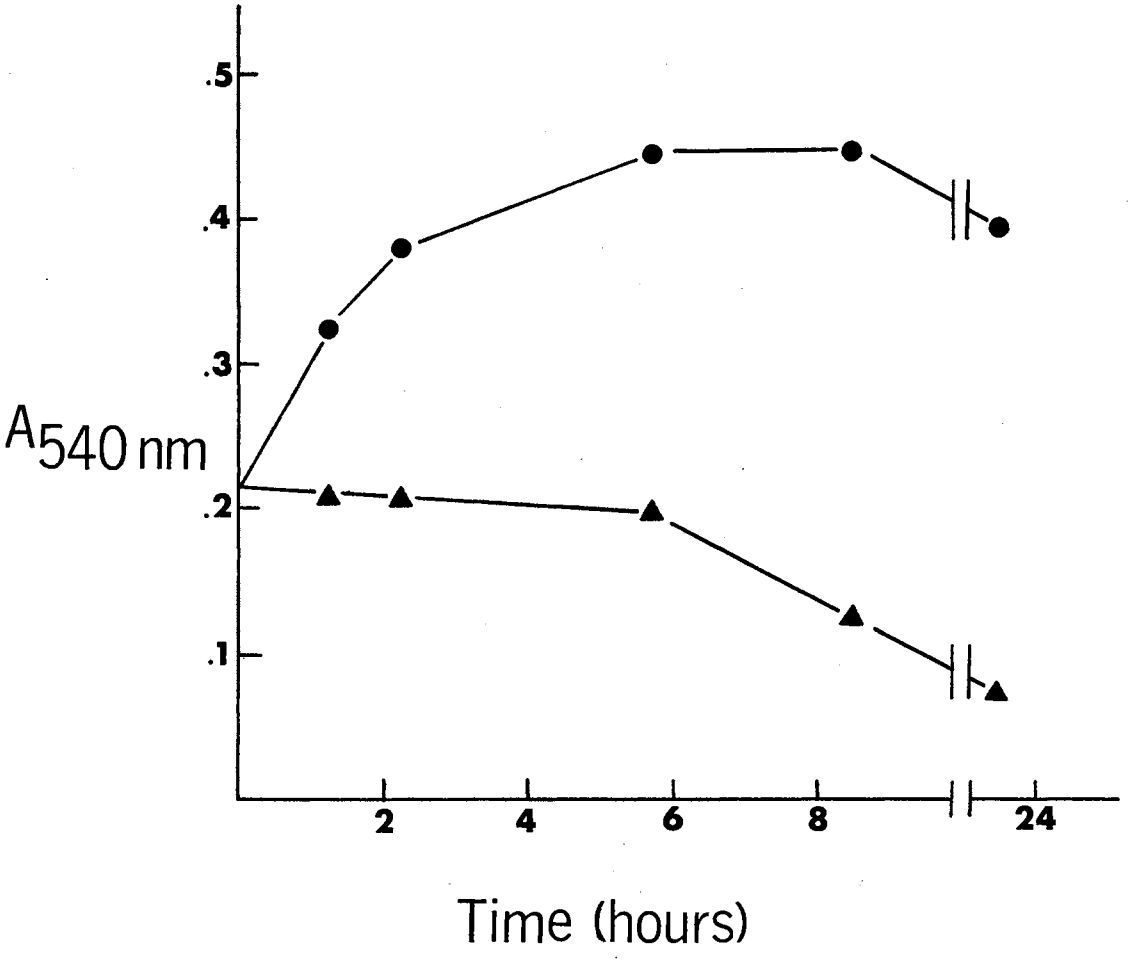
Effect of the Hydroxypyrazole on a Procaryotic Membrane

The effect of the hydroxypyrazole on B. subtilis W23 protoplasts was also examined. Whole cells were converted to protoplasts by treatment with lysozyme (400 µg/ml) in the presence of a stabilizing medium (0.6 M sucrose). As with the red blood cell experiment, the action of the compound on the membrane was slight, causing very little decrease in optical density for the first 6 hours (Fig. 12). A decrease in optical density is evident after 6 hours, which is indicative of lysis. Microscopic observation indicated that clumping did not occur and that some of the protoplasts were morphologically intact after 24 hours. However, the protoplasts in the presence of the hydroxypyrazole did not swell like the control protoplasts. This could indicate leakage, resulting in the protoplasts inability to maintain as high an internal pressure as the control.

Swelling of the control protoplasts results from the protoplasts being in a hypotonic environment (0.6 M sucrose). Approximately 2.5 M sucrose is required to completely stabilize B. subtilis protoplasts; however, significant lysis of protoplasts occurs only at sucrose concentrations below 0.5 M (Corner and Marquis, 1969). The bacterial cell is able to assimilate from the external medium soluble substances such as amino acids, purines, pyrimidines, nucleotides, phosphate and other ions. The cell can create a concentration gradient across the cell surface as high as 400 to 500 times. Consequently, a high osmotic pressure is set up within the cell that may be as high as 20-25 atmospheres for gram-positive bacteria (Gale, 1963).

Action of the hydroxypyrazole on B. subtilis W23 protoplasts is

Figure 12. Effect of the Hydroxypyrazole on B. subtilis W23 Protoplasts. ●, control in 0.6 M sucrose; ▲, hydroxypyrazole (20 µg/ml) treated in 0.6 M sucrose.


.5
.4
.3
.2
.1
A540 nm
2
4
6
8
24
Time (hours)

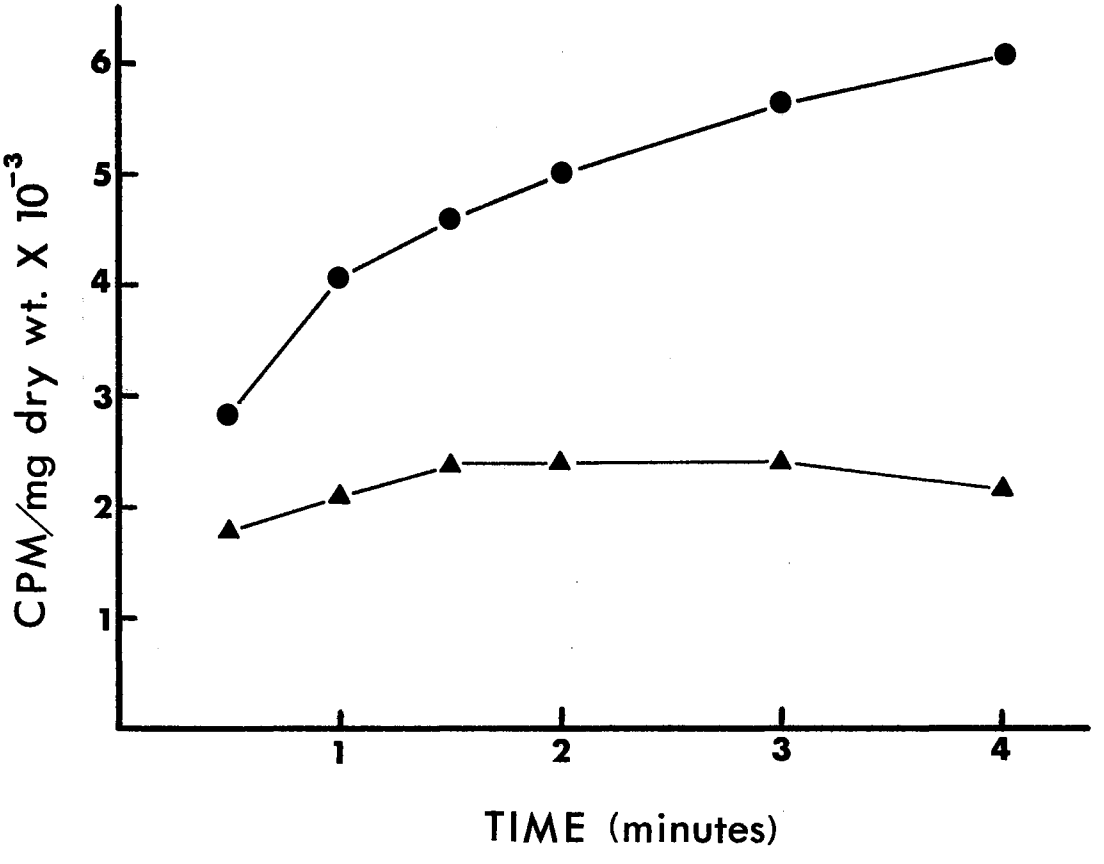
consistent with the work of Smith and Shay (1965) who observed that nitrogen-substituted cholestanes caused lysis of protoplasts of S. lutea and also with the work of Silver and Levine (1968a) who postulated that the action of IDA was at the membrane level.

Effect of the Hydroxypyrazole on Uptake of Labeled Substrates

Experiments utilizing B. subtilis W23 protoplasts and sheep red blood cells demonstrated that the hydroxypyrazole has an action at the membrane level; however, this action is slight, resulting in leakage of cellular constituents rather than lysis. Experiments were conducted to determine if the uptake of labeled substrates was affected by the hydroxypyrazole using B. subtilis W23 cells.

The substrates selected for the uptake experiments were uracil, serine and glucose. The uptake of ^{14}C-uracil (specific activity 40.6 mC/mM) was reduced by approximately 60 percent by the presence of the steroid (Fig. 13); however, the uptake of ^{14}C-serine (specific activity 87 mC/mM) and ^{14}C-glucose (specific activity 55 mC/mM) was not inhibited. This would indicate that inhibition of uptake is not of a generalized nature and that uracil is specifically inhibited by the hydroxypyrazole. These results would support the hypothesis that action at the membrane is slight; moreover, it is not sufficient to cause a generalized leakage of labeled substrates from the cell in the 4 minute uptake period.

Figure 13. Effect of the Hydroxypyrazole on ^{14}C-Uracil Uptake in *B. subtilis* W23. ●, control; ▲, hydroxypyrazole (10 µg/ml) treated.


CPM/mg dry wt. X 10^{-3}
6
5
4
3
2
1
1
2
3
4
TIME (minutes)

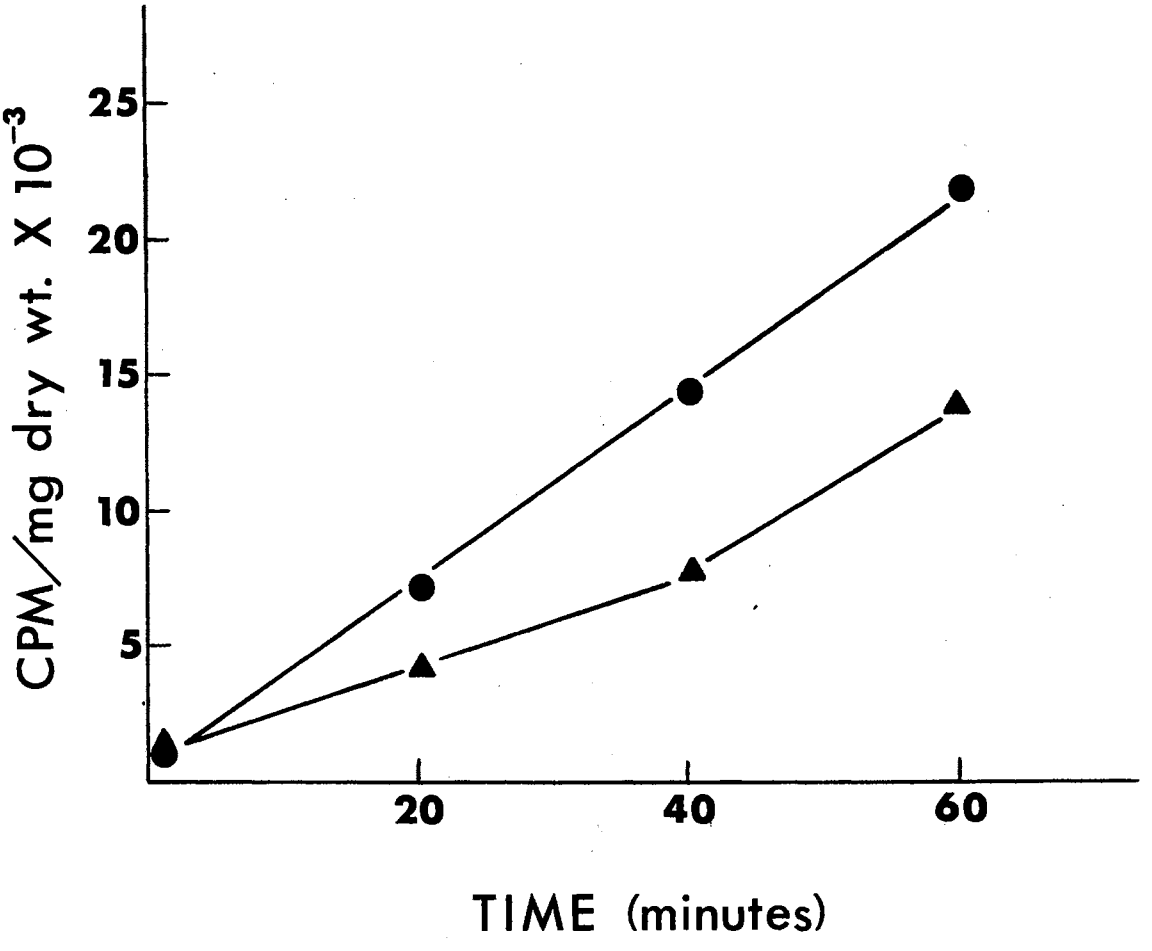
Effect of the Hydroxypyrazole on Incorporation of Labeled Substrates

Since only the uptake of uracil was inhibited by the hydroxypyrazole, experiments were conducted to determine the effect of the compound on incorporation of labeled substrates into cellular constituents. The concentration of hydroxypyrazole used was sufficient to cause only a slight inhibition of growth of the B. subtilis W23 cells. Results indicated that ^{14}C-serine (specific activity 87 mC/mM) incorporation into protein was not inhibited by the hydroxypyrazole. Both control and treated cells showed an identical incorporation curve during the 60 minute incorporation time. Incorporation of ^{14}C-uracil (specific activity 40.6 mC/mM) into messenger RNA was inhibited by the hydroxypyrazole (Fig. 14); however, since the uptake of uracil was inhibited by the hydroxypyrazole, it may be that uptake was the determinant. Inhibition of incorporation of uracil into cellular components can not be clearly separated from inhibition of uptake when both phenomena are occurring simultaneously.

Potentiation of Membrane Active Antibiotics

Since sheep red blood cell and B. subtilis W23 protoplast experiments demonstrated that the hydroxypyrazole had an action at the membrane level, potentiation experiments were conducted to determine if the compound would enhance the antibacterial action of selected antibiotics. Only membrane active antibiotics and actinomycin D showed any enhanced activity when used in combination with the hydroxypyrazole. The action of vancomycin, penicillin, chloramphenicol, 5-fluorouracil or mitomycin C was not potentiated by the hydroxypyrazole.

Figure 14. Effect of the Hydroxypyrazole on ^{14}C-Uracil Incorporation in B. subtilis W23. ●, control; ▲, hydroxypyrazole (10 μg/ml) treated.


CPM/mg dry wt. X 10^{-3}
25
20
15
10
5
20
40
60
TIME (minutes)

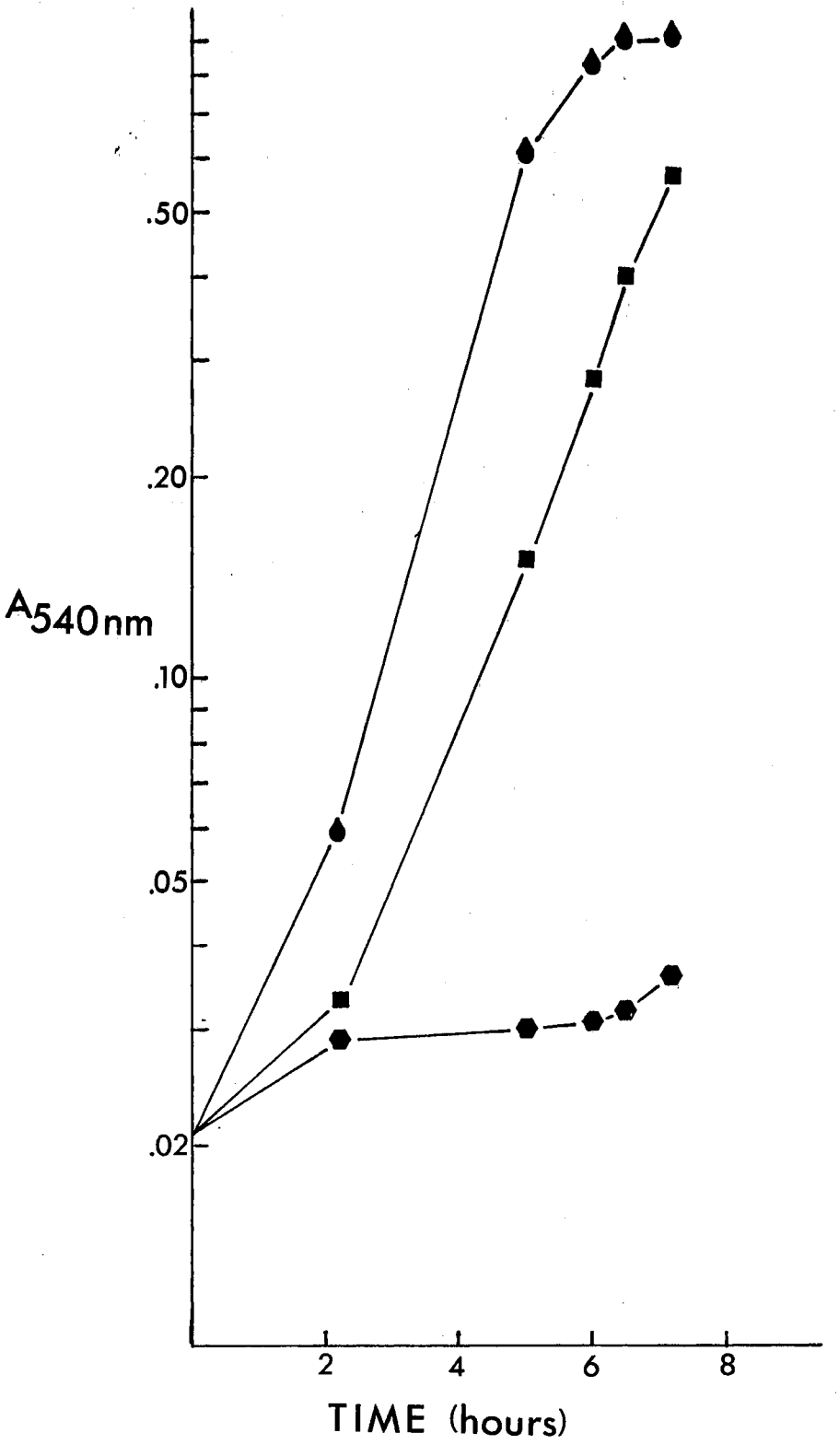
However, the action of both polymyxin and circulin, surface active antibiotics thought to cause disorganization of the cell membrane (Coleman, 1969), proved to be greatly enhanced when used in combination with the hydroxypyrazole. Sub-inhibitory concentrations of the azasteroid compound (13 μg/ml) which produced no growth inhibition in P. fluorescens almost completely inhibited growth when combined with 1 μg/ml polymyxin (Fig. 15). Similar results were also observed when circulin (2.5 μg/ml) was used in combination with the hydroxypyrazole.

Potentiation of polymyxin and circulin by the azasteroid was also observed using the gram-positive bacterium, B. subtilis W23. Polymyxin and the hydroxypyrazole used in combination showed a marked enhancement of inhibition as compared to either compound used alone (Fig. 16). Growth was inhibited for the entire 12 hours of the experiment.

Potentiation of Actinomycin D

A most interesting and potentially useful discovery was made when the action of actinomycin D was shown to be potentiated by the hydroxypyrazole. Two other anticancer drugs, 5-fluorouracil and mitomycin C, showed no enhanced activity when used in combination with the compound. Several authors have shown potentiation of actinomycin D by agents such as detergents, EDTA and phage, all of which have an action on the cell permeability barrier similar to the proposed action of the hydroxypyrazole. Riehm and Biedler (1972) recently reported that Tween 80, a nonionic detergent, will potentiate the drug effect of actinomycin D or daunomycin against resistant Chinese hamster cells. Leive (1965) noted that E. coli cells treated with EDTA are more susceptible to actinomycin D, and Roy and Mitra (1970) reported that E. coli K-12

Figure 15. Hydroxypyrazole Potentiation of Polymyxin Action Against *P. fluorescens*. ●, control; ▲, hydroxypyrazole (13 µg/ml) treated; ■, polymyxin (1 µg/ml) treated; ⬢, hydroxypyrazole (13 µg/ml) plus polymyxin (1 µg/ml) treated.


.50
.20
A_{540nm}
.10
.05
.02
2
4
6
8
TIME (hours)

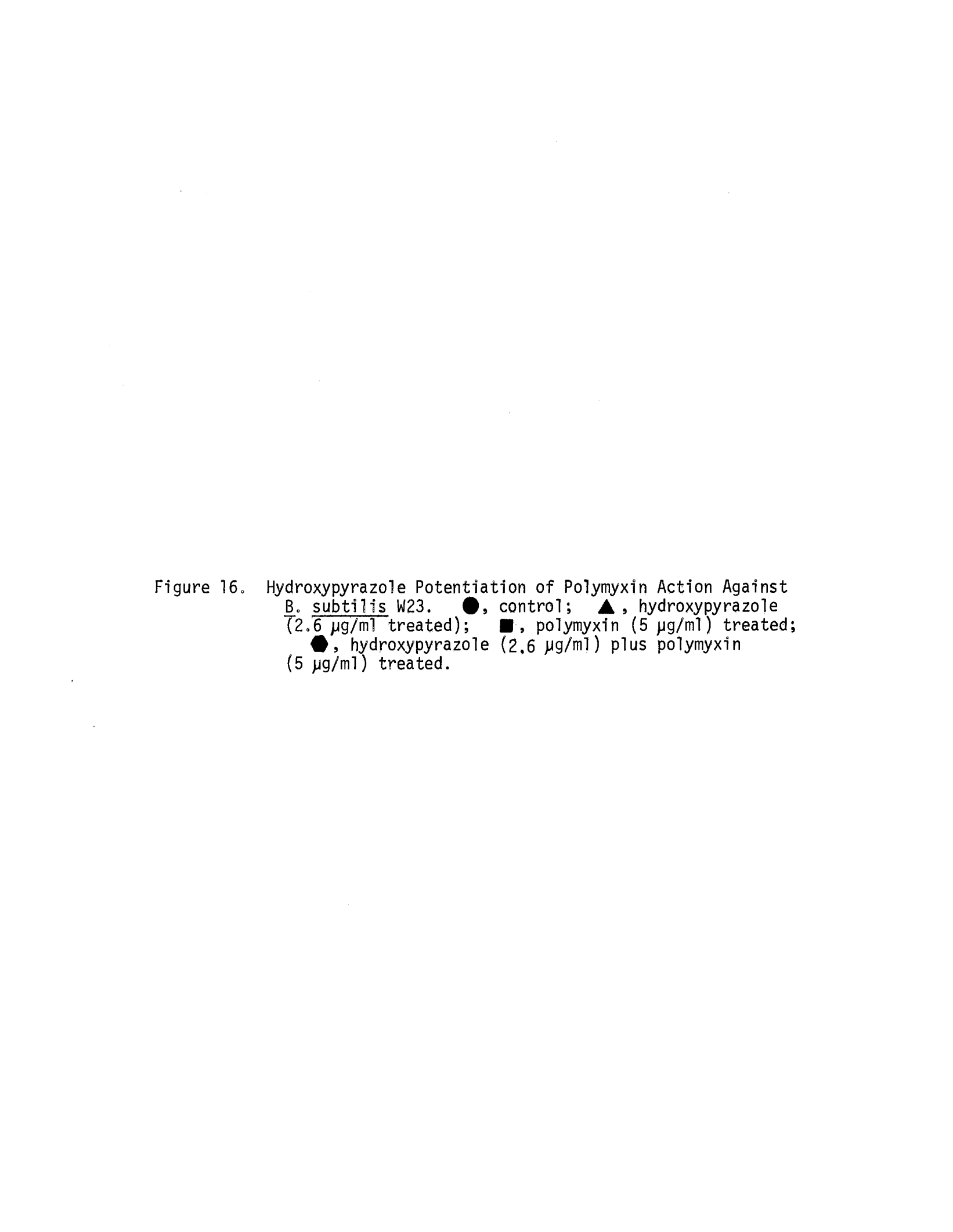
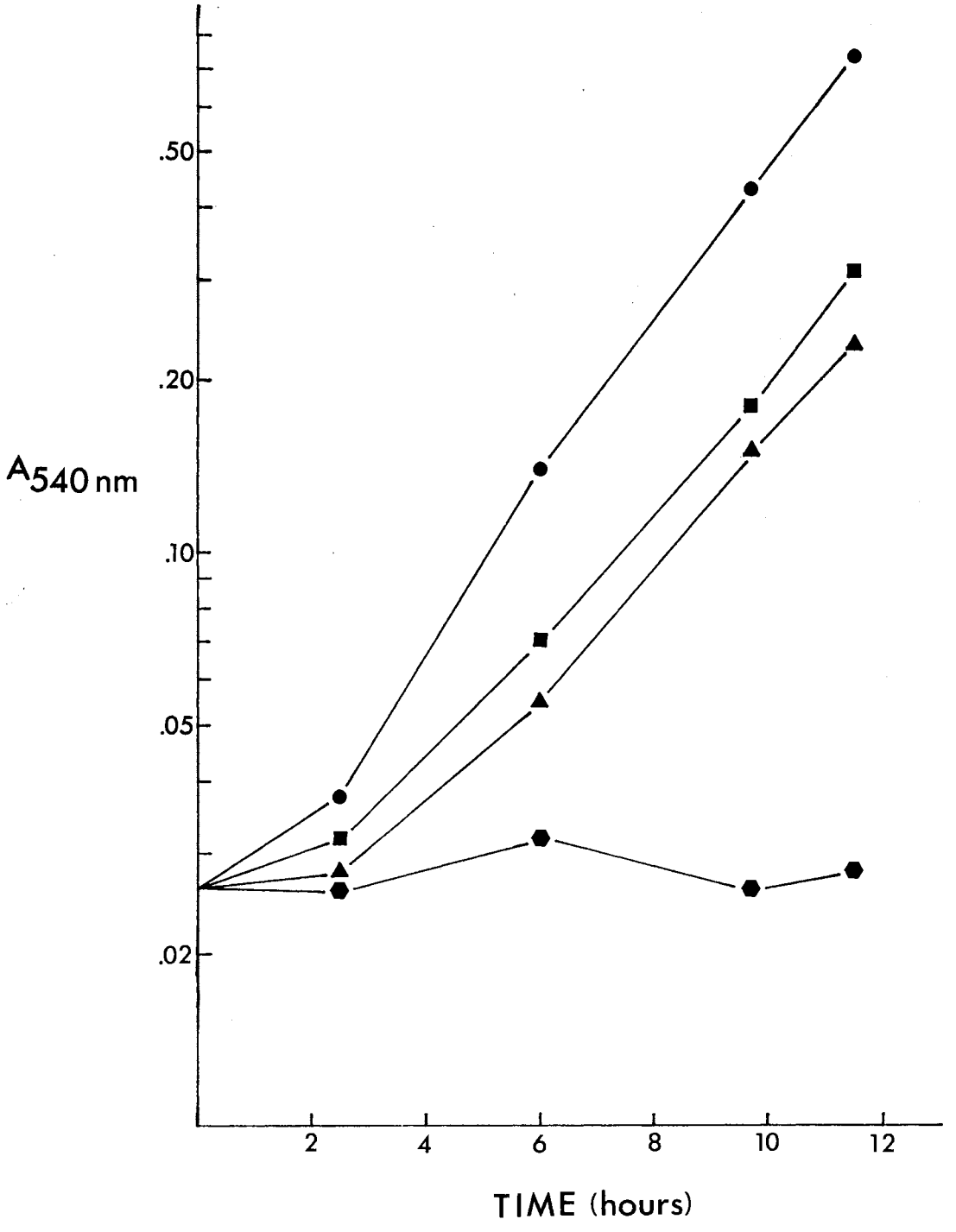


Figure 16. Hydroxypyrazole Potentiation of Polymyxin Action Against B. subtilis W23. ●, control; ▲, hydroxypyrazole (2.6 µg/ml treated); ■, polymyxin (5 µg/ml) treated; ⬢, hydroxypyrazole (2.6 µg/ml) plus polymyxin (5 µg/ml) treated.


$A_{540\,nm}$
.50
.20
.10
.05
.02
2
4
6
8
10
12
TIME (hours)

cells infected with the small filamentous phage, M 13, are more permeable to actinomycin D.

Results of the potentiation experiment using the hydroxypyrazole and actinomycin D in combination against P. fluorescens cells indicated that potentiation was occurring and was quite pronounced (Fig. 17). Subinhibitory concentrations of both the hydroxypyrazole and actinomycin D completely inhibited growth for 9 hours when used in combination. Prolonged incubation of these cells indicated that growth will initiate after 24 hours of incubation.

These results suggest that the hydroxypyrazole may possibly potentiate the action of actinomycin D against cancer cells. Potentiation experiments involving these compounds against tissue culture cells have proven to be inconclusive; however, results obtained with bacterial cells illustrate that the potential is present.

Molecular Complex Formation Between the Hydroxypyrazole and Actinomycin D

Actinomycin D binds strongly to double-stranded DNA and inhibits the DNA-dependent RNA sythesis (Arison and Hoogsteen, 1970). Of all the antibiotics tested, actinomycin D was the only antibiotic with a site of action other than the membrane which demonstrated potentiation with the hydroxypyrazole.

UV absorption spectra of the hydroxypyrazole compound and actinomycin D indicated that the two compounds may form a complex (Fig. 18). Actinomycin D has one peak in the UV range at 240 nm; however, when the two compounds are mixed, an additional peak appears at 270 nm. Additional evidence that the two compounds form a complex

Figure 17. Hydroxypyrazole Potentiation of Actinomycin D Action Against P. fluorescens. ●, control; ▲, hydroxypyrazole (6.7 µg/ml) treated; ■, actinomycin D (8 µg/ml) treated; ⬢, hydroxypyrazole (6.7 µg/ml) plus actinomycin D (8 µg/ml) treated.

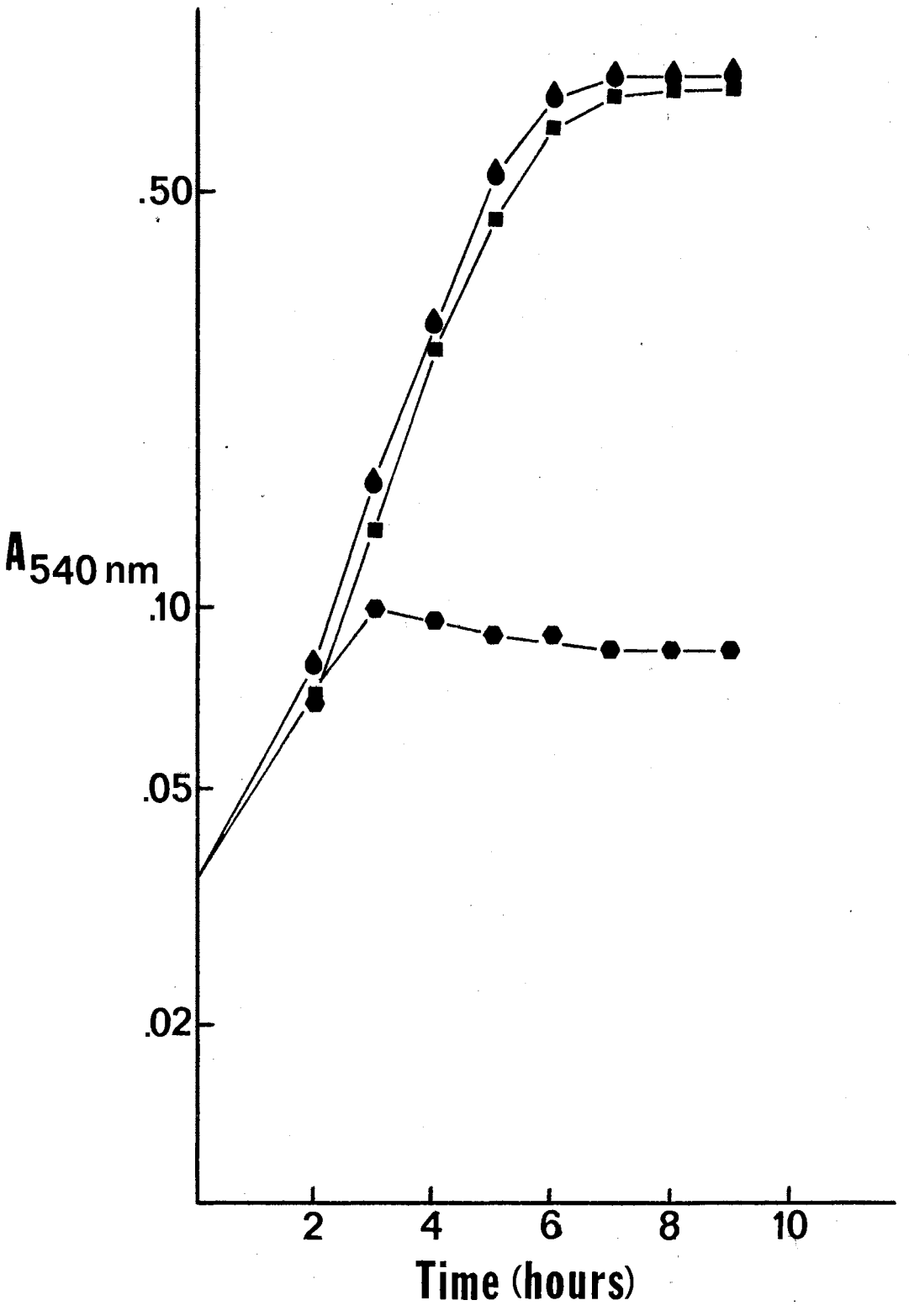
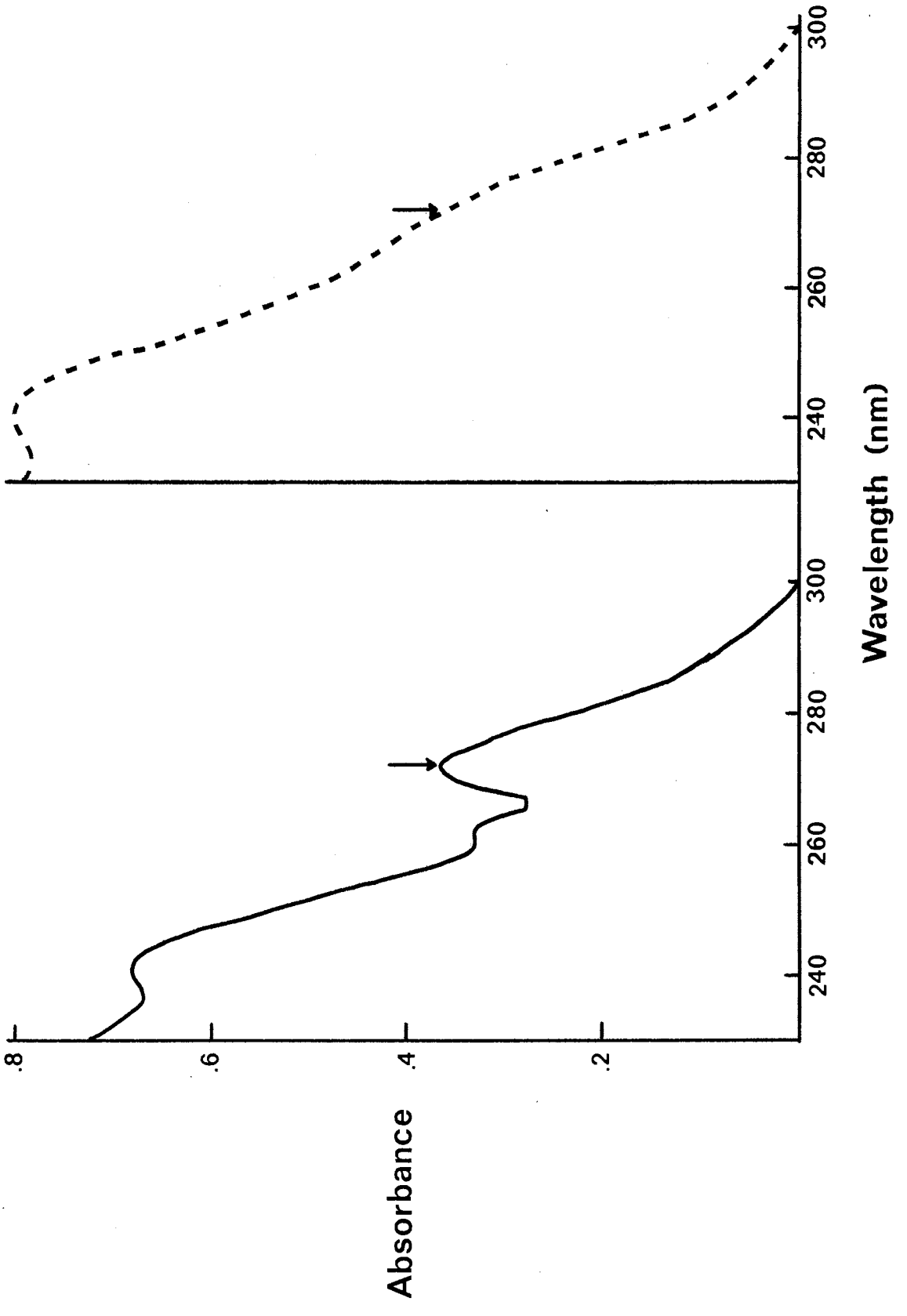

.50
.10
.05
.02
A540 nm
2
4
6
8
10
Time (hours)

Figure 18. UV Absorption Spectra of Actinomycin D and the Hydroxypyrazole Actinomycin D Complex. (——), hydroxypyrazole actinomycin D complex spectrum; (---), actinomycin D spectrum.


Absorbance
.8
.6
.4
.2
240
260
280
300
Wavelength (nm)
240
260
280
300

was furnished by work with two hydrogen bond breaking agents, dimethylsulfoxide (DMSO) and dimethylformamide (DMF). Addition of 2 M DMSO or DMF to the complex shifted the UV curve back to normal and prevented the appearance of the 270 nm absorbing peak if added prior to complex formation.

UV fluorescence experiments also substantiated the presence of a complex. The hydroxypyrazole has a fluorescent excitation maximum of 320 nm and an emission maximum of 382 nm (Fig. 3). Addition of actinomycin D to the hydroxypyrazole (1:1 molar ratio) quenched the emission peak at 382 nm by 35-40 percent. Addition of 2 M DMSO prior to complex formation yielded only a 5 percent quenching effect. Calorimetry experiments graciously performed by Wesley White, Oklahoma State University, indicated that the complex had a slightly exothermic heat of reaction of approximately 5 Kcal/mole. These results confirm other evidence of complex formation between the hydroxypyrazole and actinomycin D. These findings are in agreement with those of Durham and Keudell (1969) who postulated a complex between actinomycin D and protocatechuic acid which prevented interaction of actinomycin D with cellular DNA.

Binding of the Hydroxypyrazole to DNA

Mahler and Baylor (1967) reported mutagenic properties of IDA and postulated that the DNA was the primary site of action. Experiments utilizing B. subtilis 168, an indoleless strain, indicated that the hydroxypyrazole compound may also function as a mutagen. Reversion to indole positive was approximately 8 times higher in cells grown in the presence of the hydroxypyrazole.

Since evidence indicated that the hydroxypyrazole may also act at the DNA level, thermal melting experiments utilizing isolated P. fluorescens DNA were run to determine if the hydroxypyrazole would bind to DNA. The thermal melting curves of DNA supplemented with actinomycin D alone and in combination with the hydroxypyrazole were also run to determine the binding characteristics of the complex. Melting was determined by reading the increase in absorbance at 260 nm as compared to DNA. Results indicated that the hydroxypyrazole would bind to DNA as evidenced by the shift of the melting curve to higher temperatures (Fig. 19). The hydroxypyrazole and actinomycin D used singly demonstrated the same melting curve, while the complex shifted the melting curve to a slightly higher temperature. Similar results were also obtained using commercially prepared calf thymus DNA. These results are in agreement with the work of Mahler and Dutton (1964) and Mahler et al. (1966) who observed that the three azasteroids, cyclobuxine, IDA and malouetine, bound to DNA and shifted the melting curves to higher temperatures.

Further indication of binding of the hydroxypyrazole to DNA was furnished by observing the effect of adding DNA to a solution of the compound. The UV absorption peaks of the hydroxypyrazole at 312 and 300 nm were decreased by addition of calf thymus DNA to an aqueous solution of the compound (Fig. 20). The absorbance at 312 nm was lowered by approximately 30 percent and the peak at 300 nm virtually disappeared. These results are in agreement with the observations of Krey and Hahn (1969) in their work with the alkaloid, berberine. They found that increasing concentrations of DNA progressively decreased the absorption intensity of the two bands of the berberine spectrum

Figure 19. Effect of the Hydroxypyrazole and Actinomycin D on Thermal Melting of P. fluorescens DNA. ●, control; ▲, hydroxypyrazole (2 µg/ml) treated; ■, actinomycin D (20 µg/ml) treated; ⬢, hydroxypyrazole (2 µg/ml) plus actinomycin D (20 µg/ml) treated.

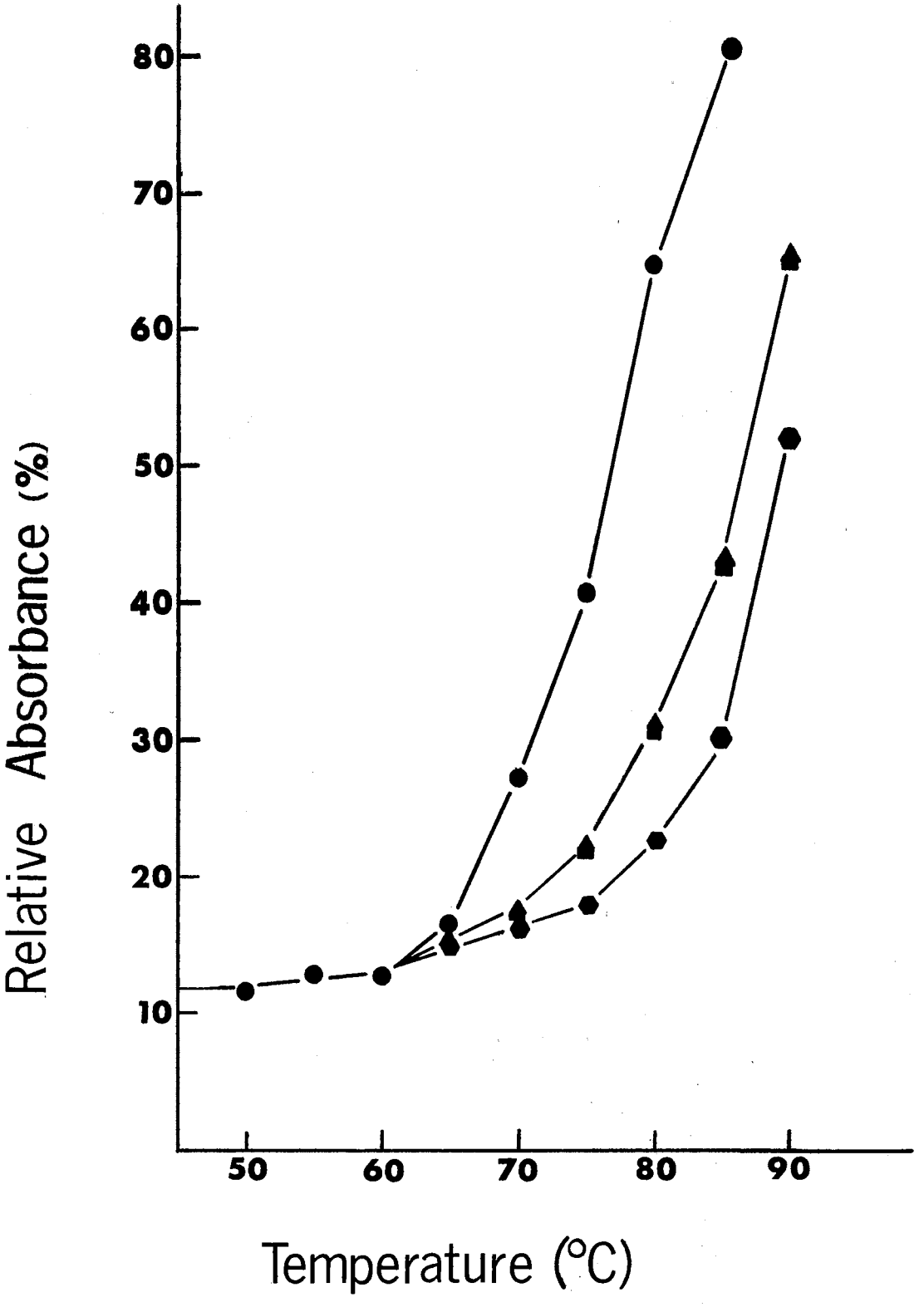
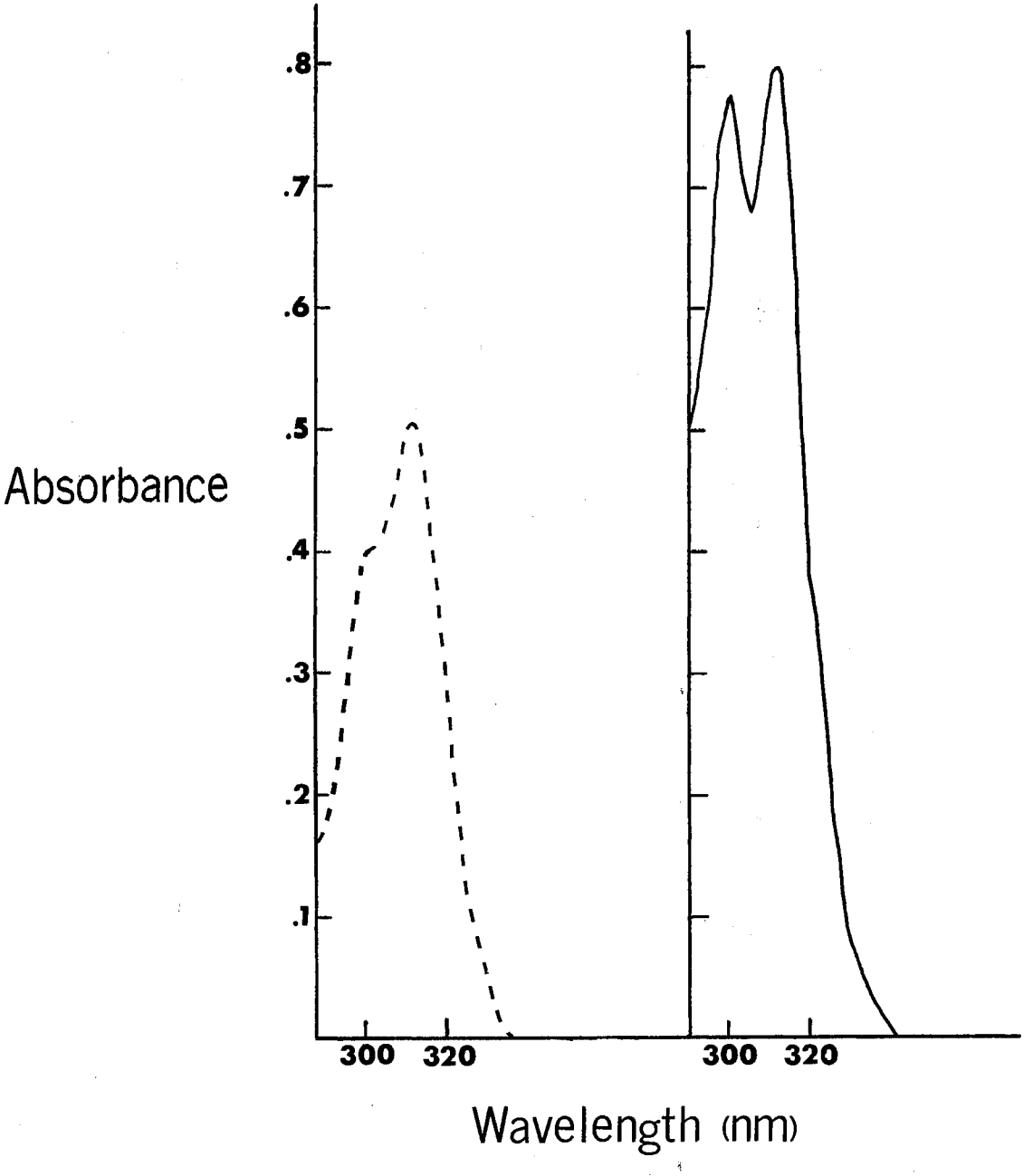

Relative Absorbance (%)
80
70
60
50
40
30
20
10
50
60
70
80
90
Temperature (°C)

Figure 20. Effect of Calf Thymus DNA on the UV Absorption Spectrum of the Hydroxypyrazole Compound. (——), hydroxypyrazole absorption peaks at 300 and 312 nm; (---), hydroxypyrazole absorption peaks at 300 and 312 nm after addition of calf thymus DNA (1 mg/ml).


Absorbance
.8
.7
.6
.5
.4
.3
.2
.1
300
320
300
320
Wavelength (nm)

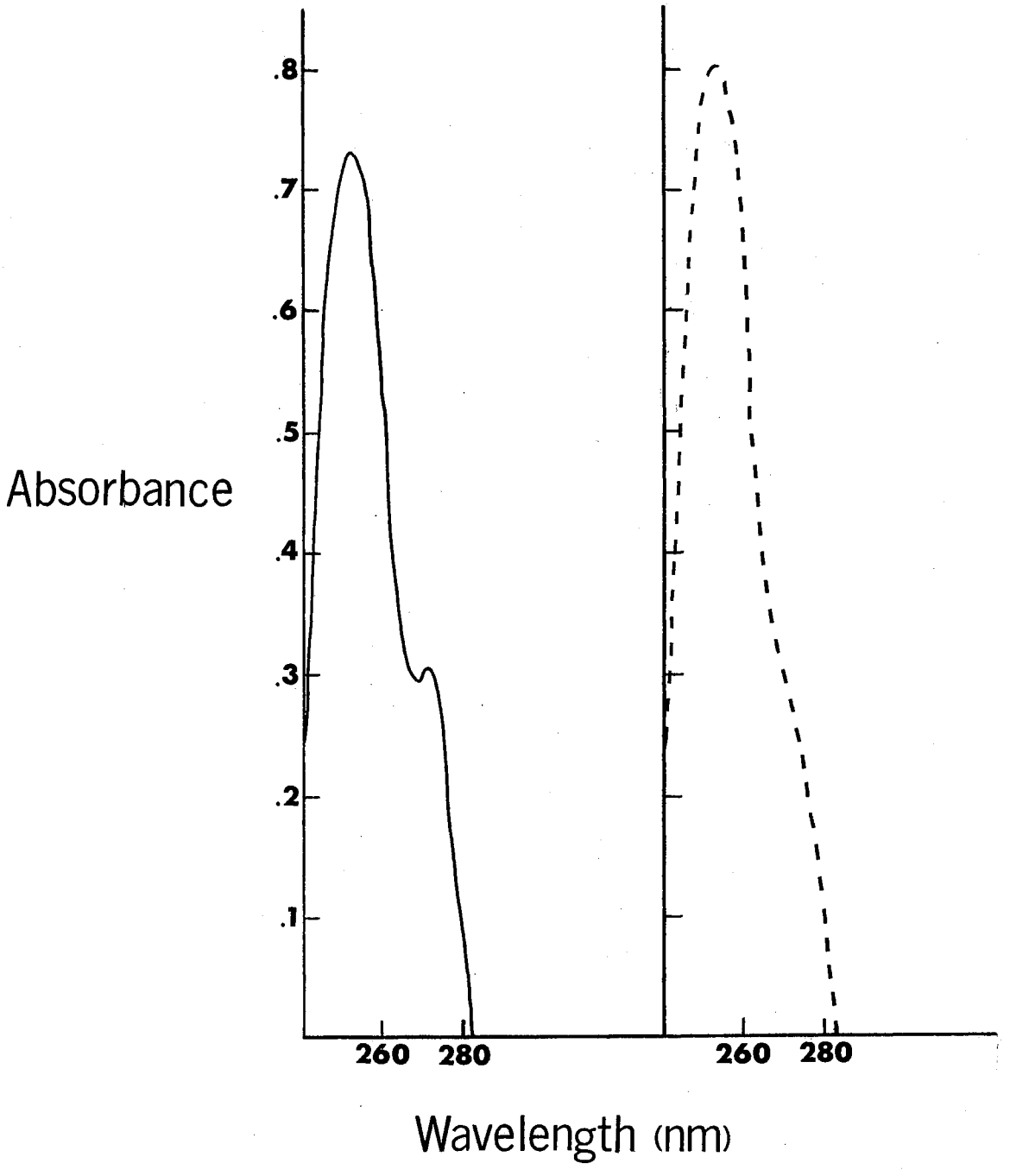
and concluded that the berberine was binding to the DNA.

Possible Binding to Guanosine 5'-Monophosphoric Acid

Since the hydroxypyrazole compound binds to DNA as evidenced by the shift of thermal melting to higher temperatures, experiments were conducted to determine the site of binding on the DNA strand. Golberg, Rabinovitz and Reich (1962) found that guanine was required for binding of actinomycin to DNA. UV absorption spectra of uracil, thymine, thymidine, cytosine, adenine and guanosine 5'-monophosphoric acid (GMP) were run in the presence and absence of the hydroxypyrazole to determine if the compound caused a shift in the UV absorption spectra. GMP was the only nucleic acid base which demonstrated any shift in the UV absorption spectrum in the presence of the hydroxypyrazole (Fig. 21). Aside from the normal UV peak of GMP at 253 nm, a slight peak at 270 nm appeared when the hydroxypyrazole was combined with GMP. It should be noted, however, that this peak was only evident in the acidic pH range. Only a slight shoulder at 270 nm was observed at neutral pH.

Addition of GMP to the hydroxypyrazole (1:1 molar ratio) caused a slight quenching (6 percent) of the hydroxypyrazole emission peak at 382 nm. Although this is a small quenching effect, none of the other nucleic acid bases were observed to cause any quenching. The UV and fluorescent spectral changes caused by the addition of the hydroxypyrazole to GMP offer evidence for possible binding of the compound to guanine molecules in the DNA strand.

Figure 21. UV Absorption Spectrum of GMP and GMP Plus the Hydroxypyrazole Compound. (---), GMP spectrum; (——), GMP plus the hydroxypyrazole spectrum.


Absorbance
.8
.7
.6
.5
.4
.3
.2
.1
260
280
260
280
Wavelength (nm)

Effect of the Hydroxypyrazole on Tissue Culture Cells

Since the hydroxypyrazole compound exhibited some potential for possible therapeutic value, tissue culture experiments were performed on KB and LM cell strains to determine its effect on viability of tissue culture cells. These experiments were graciously performed by Dr. Louise Higgins and Robert Chesnut, Oklahoma State University. The results indicated that the plating efficiency of the KB cell strain was not decreased significantly by the hydroxypyrazole at the concentrations used in this study (Table III). Plating efficiency of the KB cells was decreased by 15 percent by a concentration of 10 μg/ml of the hydroxypyrazole.

Viability experiments of tissue culture cells were also performed by exposing LM cells to increasing concentrations of the hydroxypyrazole (Fig. 22). A concentration of 20 μg/ml of the hydroxypyrazole caused approximately a 60 percent reduction in the number of viable cells; however, this effect was not as pronounced at the 1-10 μg/ml range. These results indicate that the hydroxypyrazole will affect tissue culture cells; however, this effect is small at the concentrations used to inhibit bacterial cells in this study.

Effect of the Hydroxypyrazole on Whole Animals

The effect of the hydroxypyrazole compound on whole animals was tested by Dr. Claude Desjardins, Oklahoma State University. Since the hydroxypyrazole was modeled after the female hormone, equilenin, experiments were conducted to determine if the compound possessed estrogenic activity. Female mice were injected subcutaneously with

TABLE III

EFFECT OF THE HYDROXYPYRAZOLE COMPOUND ON PLATING EFFICIENCY OF KB CELL STRAIN GROWN IN STATIONARY CULTURES AND COUNTED AFTER SIX DAYS

	Concentration (μg/ml)	Plating Efficiency
Control	--	100
Hydroxypyrazole	10	85

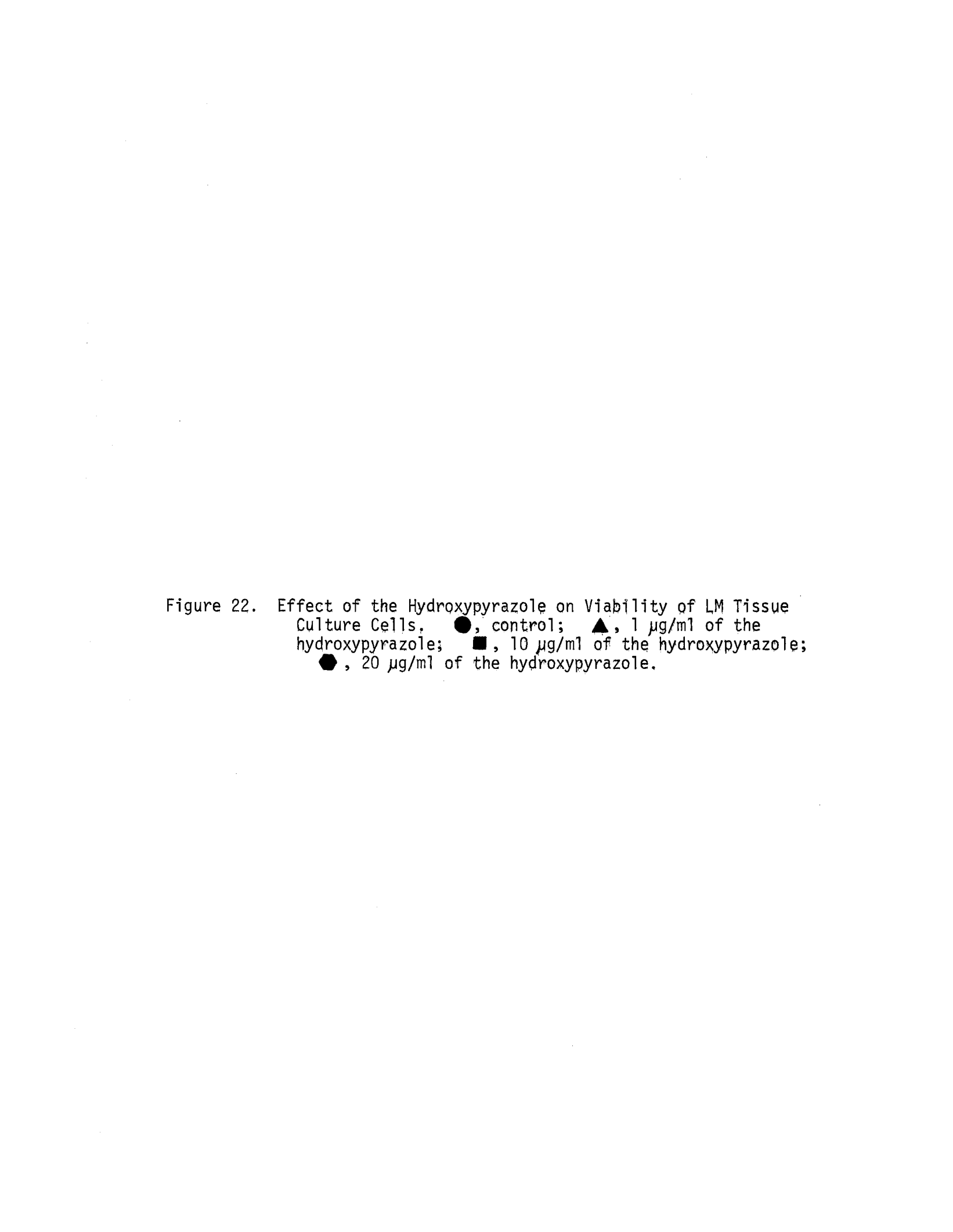
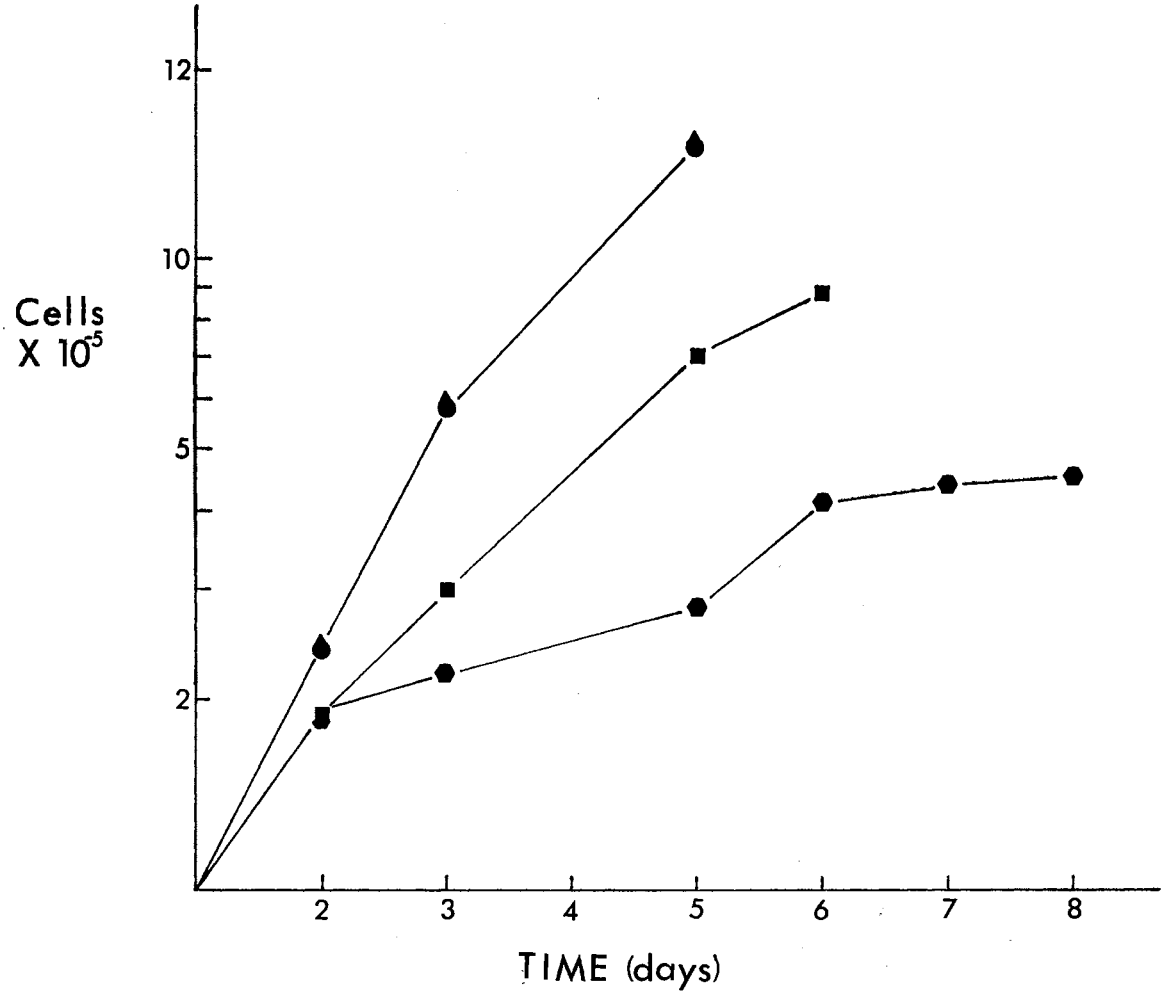


Figure 22. Effect of the Hydroxypyrazole on Viability of LM Tissue Culture Cells. ●, control; ▲, 1 µg/ml of the hydroxypyrazole; ■, 10 µg/ml of the hydroxypyrazole; ⬢, 20 µg/ml of the hydroxypyrazole.


Cells
X 10⁻⁵
12
10
5
2
2
3
4
5
6
7
8
TIME (days)

the hydroxypyrazole over a 7 day period. Measurements were made to ascertain the effect on total body weight, uterine weight and gross effect on major organs.

Results indicated that the hydroxypyrazole caused no effect on either total body weight or uterine weight. Subjective inspection of the lungs, liver, spleen, intestines, kidney and mammary glands for gross lesions did not yield any detectable or striking differences between the control and treated animals. Therefore, it appears that the hydroxypyrazole possesses no estrogenic properties and causes no toxic effect in at least one type of whole animal.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The hydroxypyrazole compound proved to be a potent inhibitor of gram-positive bacteria. Action against gram-negative bacteria was diminished; however, the compound did potentiate the action of membrane active antibiotics and actinomycin D against both gram-positive and gram-negative bacteria. These results are in agreement with the work of Smith, Shay and Doorenbos (1963a) who found that sensitivity to steroids was essentially limited to the gram-positive microorganisms. Growth inhibition by the hydroxypyrazole was found to be reversible by incubating the cells in fresh medium in the absence of the compound. Inhibition was dependent upon concentration of the hydroxypyrazole and the nature and cell mass of the microorganism.

The hydroxypyrazole was capable of binding to both whole cells and isolated cell walls of B. subtilis W23. Binding to the cell could account for the pronounced inhibition of gram-positive cells by a low concentration of the hydroxypyrazole. B. subtilis W23 possessed approximately 1×10^8 binding sites per cell for the hydroxypyrazole. Twenty percent of the hydroxypyrazole could be removed from B. subtilis W23 cell walls by washing with water and an additional 5 percent could be removed by washing in 1 mM $MgCl_2$. Therefore, 75 percent of the steroid was tightly bound to the cell wall with the remaining 25 percent being somewhat more loosely bound.

Experiments utilizing both sheep red blood cells and B. subtilis W23 protoplasts demonstrated that the hydroxypyrazole was membrane active. This action at the membrane was somewhat gentle in nature in that it caused leakage rather than lysis of the membrane in contrast to the action of detergents and membrane active antibiotics. A period of 24 hours was necessary to release 30 percent of the hemoglobin from sheep red blood cells, and the optical density of B. subtilis W23 protoplasts did not decrease significantly until 6 hours after treatment with 20 μg/ml of the hydroxypyrazole. These results are in agreement with those of Smith, Shay and Doorenbos (1964) who reported that 50 percent of the sheep red blood cells remained intact after 16 hours following treatment with a nitrogen-substituted cholestane.

Experiments concerning the effect of the hydroxypyrazole on uptake of labeled substrates substantiated the theory that action at the membrane was gentle in nature. A generalized type of inhibition was not observed. On the contrary, of the substrates tested only the uptake of ^{14}C-uracil was shown to be affected by the hydroxypyrazole. Therefore, uptake inhibition was specific and not indicative of an extensive disorganization of the cell membrane.

Further investigation indicated that the hydroxypyrazole compound was also active at the DNA level. Experiments utilizing B. subtilis 168, an indoleless strain, indicated that reversion to indole positive was 8 times higher in cells grown in the presence of the hydroxypyrazole as compared to a control. Thermal melting experiments using DNA isolated from P. fluorescens showed a shift to higher melting temperatures in the presence of the hydroxypyrazole which is indicative of binding to DNA. Further evidence for binding of the hydroxypyrazole

to DNA was indicated by addition of calf thymus DNA to the compound. The UV absorption peaks of the hydroxypyrazole at 300 and 312 nm were decreased by at least 30 percent which indicated that the compound was binding to DNA. These observations are in agreement with the work of Mahler and Baylor (1967) who postulated that the primary site of action of the azasteroid, IDA, was at the DNA level. Krey and Hahn (1969) observed a decrease in the UV absorbance peaks of berberine, similar to that observed with the hydroxypyrazole, when calf thymus DNA was added to a solution of the compound.

The site of binding of the hydroxypyrazole on the DNA molecule was not definitely determined; however, UV absorption data indicated that the compound may bind to guanine. Mixing of the hydroxypyrazole and GMP under acid conditions produced a slight change in the UV spectrum of GMP which was visible to a lesser extent at neutral pH.

Experiments involving the use of the hydroxypyrazole in combination with various antibiotics indicated that potentiation of antibiotic action was present with two membrane active antibiotics, polymyxin and circulin, and also with actinomycin D. UV absorption spectra of the hydroxypyrazole and actinomycin D indicated that the two compounds form a complex. An additional absorption peak at 270 nm in the actinomycin D UV absorption spectrum was observed when the hydroxypyrazole was added. Addition of either DMSO or DMF, two hydrogen bond breaking agents, either prevented formation of the complex or shifted the UV spectrum back to normal after complex formation. This evidence was substantiated by UV fluorescence experiments which indicated that actinomycin D would quench the fluorescent spectrum of the hydroxypyrazole. As with the UV absorp-

tion experiments, addition of DMSO prevented the quenching phenomenon. Calorimetry studies indicated that the heat of formation of the complex was slightly exothermic.

Tissue culture experiments using KB and LM cell strains indicated that the effect of the hydroxypyrazole was small at the concentrations used to inhibit bacterial cells. Whole animal experiments using female mice indicated that the compound had no estrogenic properties and produced no toxic effect in treated animals.

Therefore, it is proposed that the hydroxypyrazole compound has at least two sites of action--one at the membrane and the other at the DNA level. Potentiation experiments with membrane active antibiotics indicate that the compound may have therapeutic value in treatment of Pseudomonas and related infections. Potentiation of actinomycin D action also demonstrates a potential use of the hydroxypyrazole as an aid in the treatment of cancer. It is plausible that much lower concentrations of actinomycin D would be necessary for treatment when used in combination with the hydroxypyrazole. Complex formation between the hydroxypyrazole and actinomycin D could allow quicker passage of actinomycin D through the membrane by virtue of the membrane action of the hydroxypyrazole. The fact that the hydroxypyrazole possesses no estrogenic activity and produces no toxic effect in whole animals further emphasizes its potential as a therapeutic agent.

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