INTERACTIONS OF A HYDROXYPYRAZOLE AND PROTOCATECHUATE WITH ACTINOMYCIN D IN *PSEUDOMONAS FLUORESCENS* AND SELECTED *IN VITRO* SYSTEMS

By

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CHAPTER I

INTRODUCTION

Actinomycin D is a cyclic-polypeptide-containing antibiotic which has become an important tool in molecular and cellular biology because of its binding to deoxyribonucleic acid (DNA) and subsequent inhibition of ribonucleic acid (RNA) synthesis. As a result, a great amount of research has been directed towards elucidating the chemical and physical nature of its biological activity. Even though actinomycin D is highly toxic, this compound has been used in nontoxic dosages because of its antineoplastic effect. Actinomycin D has been proven to be a highly effective chemotherapeutic agent in the treatment of Wilms' tumor, trophoblastic tumors, rhabdomyosarcoma and testicular cancers (Meienhofer, 1970).

The name actinomycin was originated by Waksman, who discovered this group of antibiotics in cultures of Actinomyces antibioticus in 1940. Less than 10 years elapsed before the structure of the actinomycins began to be revealed by the investigations of Dagliesh et al. (1949), Brockman et al. (1949) and Dagliesh et al. (1950). Actinomycin D was characterized by Waksman (1954) as a brick red, crystalline substance that melted at approximately 250 C and absorbed light strongly at 230, 250 and 450 nm. Waksman observed that

actinomycin D was highly active against Gram-positive bacteria, less active against Gram-negative bacteria and almost totally ineffective against fungi. Manaker et al. (1955) crystallized actinomycin D from cultures of Streptomyces parvullus. The molecular weight was determined to be 1255 daltons and the molecular formula was deduced as $C_{60}H_{76}O_{15}N_{12} \cdot 3\ H_2O$.

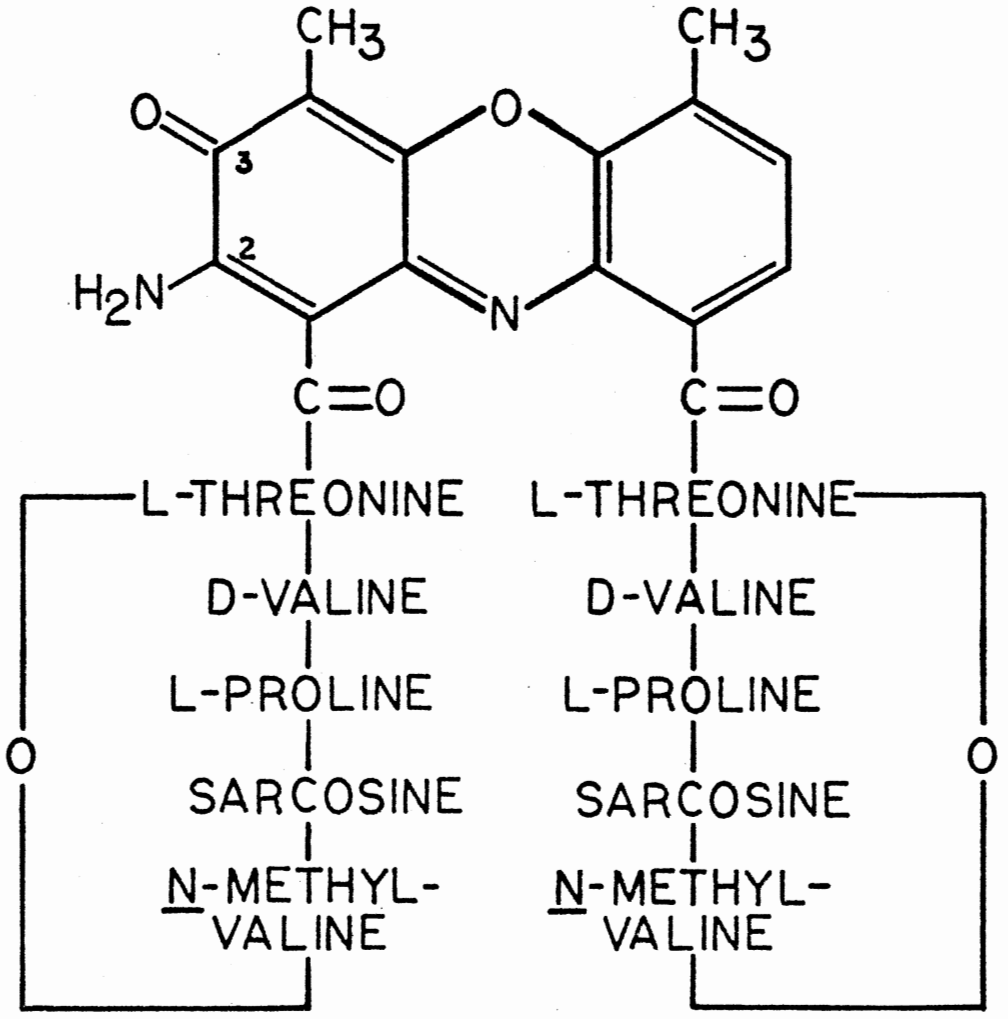
The actinomycins were recognized very early to contain a chromophore, which absorbed visible light, and a peptide linked to the chromophore. The actinomycin chromophore was isolated and assigned the structure, 3-amino-1,8-dimethyl-2-phenoxazone-4,5-dicarboxylic acid. Of interest is the fact that this same 2-amino-3-phenoxazone ring system is found in the ommochrome insect eye pigments and in several pigmented mold metabolites. Next, it was determined that attached to the two carboxyl groups of the chromophore were two pentapeptides, whose sequences were determined by various partial hydrolyses.

Isoactinomycin is used to describe an actinomycin with the same two pentapeptide residues, while those with different pentapeptides are referred to as anisoactinomycins. L-Threonine is always the amino acid linked by amide bonds to the 1- and 9-carboxyls of the chromophore and whose hydroxyl group always forms a lactone bond with the carboxyl of the fifth amino acid. The second amino acid can be either D-valine or D-allo-isoleucine. The third can be L-proline, L-3-hydroxyproline, L-3-ketoproline, pipecolic acid or

sarcosine. The fourth is always sarcosine and the fifth can be either L-N-methylvaline or L-N-methylisoleucine. Interestingly, the peptide groups contain free NH groups only in the two threonine residues since all other amino groups are methylated or contained in the ring of an imino acid. In addition, the second amino acid, valine or allo-isoleucine, has the D configuration. Actinomycin D is an isoactinomycin which contains the amino acids L-threonine, D-valine, L-proline, sarcosine and L-N-methylvaline (Figure 1).

Although the antimicrobial and antineoplastic activities of actinomycin D were recognized relatively soon after its discovery, it was the early 1960's before its complexation with DNA (Kersten et al., 1960; Kirk, 1960) and subsequent inhibition of RNA synthesis (Goldberg et al., 1962) became known. Actinomycin D binds tightly to double helical DNA, but poorly, if at all, to double helical RNA, RNA-DNA hybrids, or single stranded forms of DNA or RNA (Kaselkorn, 1964; Gellert et al., 1965). The complexation of actinomycin D with DNA has been studied using a variety of methods including spectrophotometric methods, buoyant density measurements, equilibrium dialysis, circular dichroism, optical rotatory dispersion, melting temperature and inhibition of DNA template controlled RNA synthesis. DNA controlled DNA synthesis can also be inhibited when much larger concentrations of actinomycin D are utilized. However, the mechanism appears to be quite different. Inhibition of RNA synthesis is a direct consequence of steric interference by the actinomycin

Figure 1. Structure of Actinomycin D. The functional groups of the antibiotic are the free chromophore amino group, the unreduced quinoidal ring system, and the pentapeptide rings.


CH3
CH3
O
O
3
2
H2N
N
C=O
C=O
L-THREONINE
L-THREONINE
D-VALINE
D-VALINE
L-PROLINE
L-PROLINE
O
O
SARCOSINE
SARCOSINE
N-METHYL-
VALINE
N-METHYL-
VALINE

D, while inhibition of DNA synthesis appears to be inhibited by stabilization of the DNA (Reich, 1964). Actinomycin D also interferes with reactions in which DNA is modified, such as methylation (Gold and Hurwitz, 1964).

Reich and Goldberg (1964) observed that complexation of actinomycin D with DNA required a double stranded helix containing guanine residues. Wells and Larson (1970) showed that the number of nucleotide pairs per bound actinomycin D molecule differs from 0 at 0% dG (deoxyguanosine) content to about 6 at 50% dG in poly-d(G-C). A relative constant number of binding sites was reported for DNA containing between 25-50% G+C. This suggests the possible involvement of more than one base pair. Furthermore, in crab poly-d(A-T)·poly-d(T-A) containing 97% A+T base pairs and 3% G+C base pairs, the number of sites available for binding was only 60% of the predicted value (Hyman and Davidson, 1971). This agreed with earlier studies by Cavalier and Nemchin (1964) who suggested that there were two types of binding sites on calf thymus and Escherichia coli DNA for actinomycin D, one of which has a binding constant about 50 times greater than the other. The distinction between the sites was thought to depend upon the helical structure of the DNA. Hyman and Davidson (1970) noted that actinomycin D binds to the "strong binding" sites on T7 DNA when the actinomycin D concentration is below 10^{-5}M. Above this concentration, more actinomycin D is bound at other weaker binding sites.

Goldberg et al. (1962) discovered that the C(2)-NH_2

group of dG was essential for actinomycin binding. However, there are two exceptions to the dG specificity. The presence of dG is not required for binding in the case of single stranded poly-dI. The unique conformation of this polymer is thought to be responsible for its ability to bind actinomycin D (Wells and Larson, 1970). Conversely, in the case of poly-d(A-T-C)·poly-d(G-A-T), Wells and Larson observed that actinomycin D did not bind to this polymer although dG was present. This may also be due to conformation, or the requirement by actinomycin D for a specific base sequence not found in this polynucleotide. It was also demonstrated in this investigation that actinomycin D would bind more tightly to a polydeoxyribonucleotide that contains both purines and pyrimidines on both strands than to a polydeoxyribonucleotide containing all purines or pyrimidines on complementary strands.

Krugh and Young (1977) reported that either daunomycin or adriamycin cooperatively facilitates the binding of actinomycin D to poly-d(A-T)·poly-d(A-T) as evidenced by circular dichroism. Normally, actinomycin D does not bind to this double stranded polynucleotide. Daunomycin and adriamycin intercalate into double stranded DNA, but in contrast to actinomycin D binding, neither of these compounds show any requirement for a particular base at the intercalation site. The results of this investigation demonstrated that when daunomycin or adriamycin bind to poly-d(A-T)·poly-d(A-T), a change occurs in the conformation of the polynucleotide

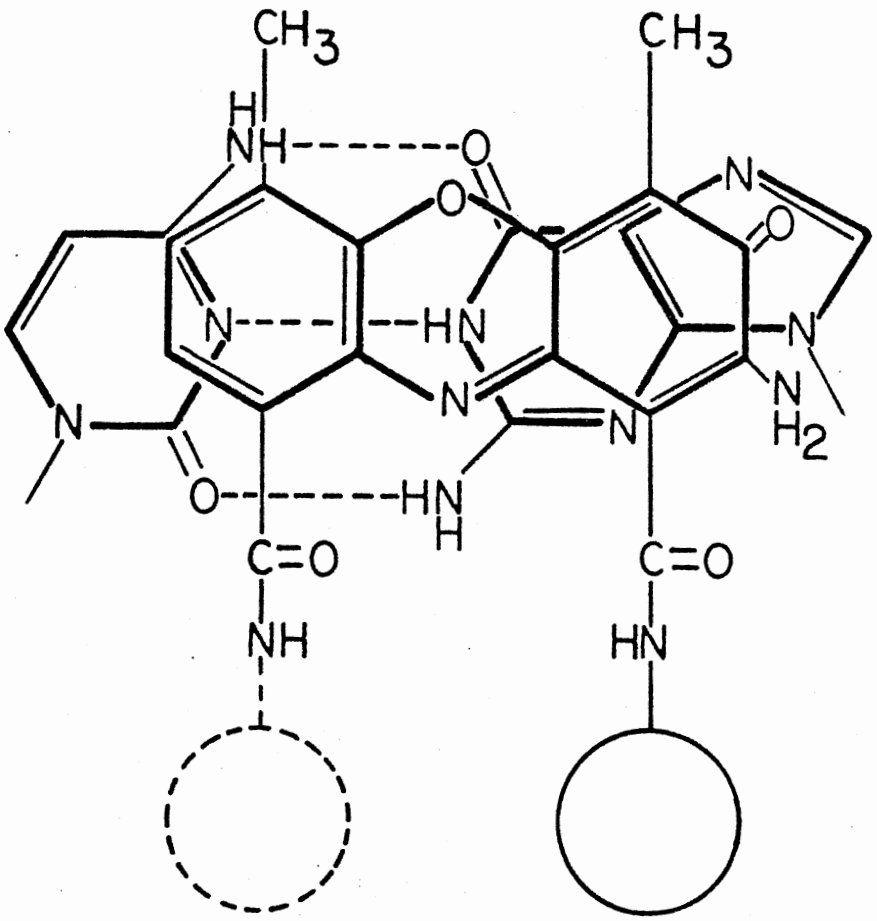
at an adjacent region of the double helix which results in an increase in the stability of the actinomycin D:poly-d(A-T)·poly-d(A-T) complex. The transmission of the distortions along the double helix may also be an important component in the selective recognition of nucleic acid sequences.

Since 1963, three models have been proposed for the complexation of actinomycin D and DNA. The earliest model by Hamilton et al. (1963) was an outside binding model in which the actinomycin D binds in the minor groove of DNA. The model involves hydrogen bonding between the actinomycin D C(3)=O and the C(2)-NH_2 group of guanine, the actinomycin D C(2)-NH_2 and the guanine C(3)-N, and the actinomycin D C(2)-NH_2 and the deoxyribose ring oxygen. The pentapeptides provide stabilization for the complex by forming four additional bonds between their four NH groups and the phosphodiester oxygens. This type of complex would occupy the minor groove over three nucleotide pairs.

A second model by Muller and Crothers (1968) involves the intercalation of the actinomycin D chromophore between two successive base pairs with the pentapeptides projecting into the minor groove. One of these base pairs is G-C. The proposed complex is stabilized by the electronic interaction of the chromophore π complex, hydrogen bonding between the actinomycin D carboxamide NH and the deoxyribose ring oxygen, and the interaction of the pentapeptide rings with each strand of the DNA in the minor groove.

The third and most recent model (Figure 2) by Sobell

Figure 2. Schematic Representation of Sobell's Model of the DNA:Actinomycin D Complex. The circles represent the pentapeptide rings of actinomycin D. (Adapted from Sobell, 1973).


CH3
CH3
H
NH
O
O
N
O
N
HN
N
N
N
N
H2
N
O
HN
H
C=O
C=O
NH
HN

(1973) combines the guanine C(2)-NH_2 group specificity of the outside binding model with the intercalative features of the second model. In this model, which is based on the crystalline dG_2:actinomycin D complex, the phenoxazone chromophore intercalates between adjacent G-C pairs when the guanine residues are on opposite strands. This model is stabilized by hydrogen bonds between the C(2)-NH_2 groups of guanine and the threonine C=O of the peptide rings which lie in the minor groove (trans with respect to the plane of the chromophore). Further stabilization results from hydrogen bonding between neighboring cyclic pentapeptide chains connecting the N-H of one D-valine residue with the carbonyl oxygen of the other D-valine residue.

A unique feature of Sobell's model is the presence of 2-fold symmetry in the actinomycin chromophore, as would be viewed on either side of a vector connecting the O and N bridging atoms of the phenoxazone ring. Although the symmetry is not exact because of the C(2)-NH_2 and the C(3)=O groups, the pentapeptide chains would closely approximate this non-crystallographic 2-fold symmetry. The conformations of the peptide linkages would be: L-threonine:D-valine, trans; D-valine:L-proline, cis; L-proline:sarcosine, cis; sarcosine:L-methylvaline, trans; L-threonine:carboxamide carbonyl oxygen and carbon of the chromophore, trans. Therefore, the DNA:actinomycin D complex, as described, would closely mimic the dG_2:actinomycin D crystalline complex (Jain and Sobell, 1972). Sobell's model predicts that

actinomycin D should bind most efficiently to poly-d(G-C)·poly-d(G-C) which contains alternating GpC sequences while other sequences would bind actinomycin D less effectively. This model also explains most of the binding data pertaining to actinomycin D and DNA. In addition, Sobell states that the binding of actinomycin D demonstrates a general principle that several classes of proteins may utilize in recognizing symmetrically arranged nucleotide sequences on the DNA helix. The binding of actinomycin D to DNA and its specificity in inhibiting the RNA polymerase reaction suggests a primitive repressor-operator character for this complex. If a repressor molecule has identical subunits related by 2-fold symmetry when it binds to DNA, in which the 2-fold axis coincides with the dyad axis of the DNA, then the base sequence in the operator must also have 2-fold symmetry.

In view of this currently accepted mechanism for actinomycin D binding, the potency of this compound results from a high degree of specificity requiring a precise and unique steric fit between the DNA and actinomycin D. Any modification of this sensitive geometry of the binding complex by introducing changes in either actinomycin D or the DNA will present different parameters to the mechanism. Although the activities of natural occurring actinomycins differ very little, changes in the different portions of the synthetic actinomycins may alter their respective activities to varying degrees. Several components of the actinomycin D molecule have been found to be indispensable for biological activity.

The unreduced quinoidal phenoxazone ring system, the unaltered C(2)-NH_2 and C(3)=0 groups, and the intact pentapeptide lactone rings are all significant. For example, the replacement of the two pentapeptides by the monocyclic peptide system of gramicidin S results in a molecule that does not bind to DNA or inhibit RNA synthesis (Mauger and Wade, 1966). Also, substitution of the C(2)-NH_2 group on the phenoxazone with a hydroxyl-, chloro- or dimethylene amino group inhibits the binding of actinomycin D to DNA (Reich et al., 1962; Muller and Crothers, 1968).

As a consequence of its binding to DNA, actinomycin D inhibits RNA chain elongation but not template site selection, RNA chain initiation or RNA chain termination. Kinetic studies performed by Hyman and Davidson (1970) have shown that actinomycin D inhibits the rates for the incorporation of CTP and GTP but not of ATP and UTP.

Three investigators, Weiss (1960), Hurwitz et al. (1960) and Stevens (1960), are credited with independently discovering a DNA dependent enzyme which was capable of forming a RNA polymer from ribonucleoside 5'-triphosphates which is complementary to the DNA template. Weiss isolated his enzyme from rat liver nuclei while Hurwitz and Stevens used cell free extracts of E. coli.

RNA polymerase, as defined by Burgess (1971), cannot only efficiently synthesize RNA but can also specifically initiate this synthesis on intact DNA templates. Bacterial RNA polymerase contains the following polypeptide subunits:

one beta prime, one beta, two alpha and one sigma. The enzyme is complete or holoenzyme when it has this makeup. Direct evidence that the major polypeptide components of the RNA polymerase holoenzyme are functional subunits is available for only the beta and sigma subunits. The holoenzyme can be separated into two functional parts: a core enzyme, consisting of the two alpha, the beta and the beta prime subunits, which is able to synthesize RNA but lacks the ability to initiate such synthesis with the same specificity as the intact holoenzyme; and a sigma factor which acts to allow the efficient initiation of RNA synthesis at a specific site. The sigma factor is responsible for the accurate and efficient initiation of RNA chains at specific sites on the DNA template and is not required after initiation has been completed. It is not known how the sigma factor functions in determining specificity of binding and facilitating initiation. The core enzyme is able to synthesize RNA and thus contains the catalytic site. However, the interactions of the separate subunits within the core are not fully understood.

The holoenzyme from E. coli RNA polymerase is the most widely studied RNA polymerase system among bacteria. Other bacterial RNA polymerases are closely related in structure to the E. coli holoenzyme. The subunits of other Gram-negative bacteria resemble the E. coli enzyme, although the molecular weight values for the sigma and alpha subunits are slightly higher.

The RNA polymerase reaction has the following four requirements. All four ribonucleoside 5'-triphosphates (ATP, UTP,GTP and CTP) are required simultaneously as substrates. The reaction requires a divalent metal ion. Magnesium or a 5:1 mixture of magnesium and manganese is usually employed. A template is required for the reaction since the nature of the template determines the substrate requirements. Usually, double stranded DNA is used although double stranded polynucleotides and single stranded DNA may be used. When the previous conditions are met, the RNA polymerase acts to add mononucleotide units to the hydroxyl end of the RNA chain with the elimination of pyrophosphate. As a result, the direction of RNA chain growth is 5' to 3'.

DNA directed synthesis of RNA by RNA polymerase may be separated into four steps (Burgess, 1971):

1. Template site selection and activation - The RNA polymerase holoenzyme attaches to the DNA template, locates a specific site at which RNA chain initiation can occur, and assumes an active conformation.
2. RNA chain initiation - The enzyme catalyzes the coupling of a purine ribonucleoside 5'-triphosphate to eliminate inorganic pyrophosphate and generate a diribonucleoside tetraphosphate which remains tightly bound to the RNA polymerase.
3. RNA chain elongation - Successive ribonucleoside monophosphate residues are added from the substrate ribonucleoside 5'-triphosphates to the initial

dinucleoside tetraphosphate at its 3' hydroxyl terminus to elongate the nascent RNA chain.

4. RNA chain termination and enzyme release - The newly formed RNA chain and the RNA polymerase are released from the template

A variety of compounds, utilizing a multiplicity of mechanisms, have been employed to block or inhibit the first three steps of the RNA polymerase reaction. Distamycin A, netropsin, pluramycin, anthramycin and phleomycin block template site selection by binding to the DNA to hinder RNA polymerase binding. Heparin and sodium polyethylene sulphonate bind to the RNA polymerase to prevent this same step. RNA chain initiation can be blocked by proflavine sulfate or kanchanomycin which bind to the DNA to interfere with initiation while streptovaricin, sodium polyethylene sulphonate, rifamycin derivatives and streptolydigin bind to the RNA polymerase to interfere with this step. Cordycepin 5'-triphosphate binds to the RNA chain to block RNA chain elongation. In addition to actinomycin D, several compounds including ethidium bromide, nogalomycin, daunomycin, adriamycin, echinomycin, olivomycin and hedamycin also bind to the DNA to inhibit RNA chain elongation. Streptolydigin inhibits RNA chain elongation by binding to the RNA polymerase.

Most attempts to potentiate actinomycin D action have been directed at creating permeability changes in the cell which ultimately allow actinomycin D to diffuse more readily through the permeability barrier. The decreased sensitivity

of the Gram-negative microorganisms is largely due to their outer membrane ultrastructure which was believed to possess an exclusion limit of about 600 daltons, as had previously been reported for the enterics. However, recent investigations by Hancock et al. (1979) have demonstrated that this value is not ubiquitous for all Gram-negatives since they observed that the exclusion limit of Pseudomonas aeruginosa is in the order of 6000 ± 3000 daltons. It was concluded that a single major outer membrane polypeptide, with an apparent molecular weight of 35,000 daltons, provides a hydrophilic pore which is responsible for the size-dependent permeability of the outer membrane in this bacterium. Similar proteins serve in this same role in the enterics.

Several different agents have been utilized to potentiate the action of actinomycin D by altering the permeability barrier of the cell such that entry of actinomycin D into the cell is increased. Leive (1965) reported that treatment of E. coli with EDTA increased susceptibility to actinomycin D and that lipopolysaccharide was released from the cell wall. Roy and Mitra (1970) were also able to release lipopolysaccharide by infecting E. coli K-12 cell with the small filamentous phage, M13. Consequently, an increase in actinomycin D inhibition was observed. The nonionic detergent, Tween 80, was successfully used in combination with actinomycin D and daunomycin to significantly increase the sensitivity of normally resistant Chinese hamster cells to these antibiotics (Riehm and Bieldler, 1972). This effect was observed at less

than toxic concentrations of Tween 80. A study by Marks and Venditti (1976) demonstrated the ability of DNA to potentiate the activity of actinomycin D with a concomitant negation of the cytotoxicity which accompanies treatment by this antibiotic.

Efforts have also been directed at alleviating inhibition resulting from actinomycin D. Foley (1956) noted the ability of pantothenate to compete with and reverse actinomycin D inhibition of growth. Reversal of actinomycin D inhibition of Neurospora crassa with p-aminobenzoic acid, tyrosine and phenylalanine was reported by Rauen and Hess (1959). The ability of B-vitamins, amino acids of caesin hydrolysate, purines, pyrimidines and nucleotides to prevent growth inhibition of Bacillus subtilis by actinomycin D was tested by Slotnick (1960). Addition of actinomycin D immediately inhibited protein and RNA synthesis but not DNA synthesis. In several organisms having an exogenous requirement for pantothenate, the inhibition of actinomycin D was competitively altered by the presence of pantothenate. However, efforts to restore balanced RNA and protein synthesis to the system proved unsuccessful. Kersten (1961) observed that deoxyguanosine and DNA could counteract growth inhibition of N. crassa and Streptococcus faecalis by actinomycin D. Prevention of the inhibition of protein synthesis by actinomycin D in Sarcoma-37 ascites cells was accomplished by adding glucose to the medium (Honig and Rabinovitz, 1965).

Previous investigation in this laboratory has

demonstrated the capacity of selected compounds to modify the biological activity of actinomycin D. Durham et al. (1974) reported that noninhibitory concentrations of a hydroxypyrazole (Figure 3), 10,11-dihydro-3H-napth(1,2g)indazol-7-ol, abets the inhibition of actinomycin D when the two were used in combination. A very pronounced increase in inhibition was observed with Pseudomonas fluorescens while a similar but lessened activity was noted with other microorganisms and L-M tissue culture cells. Conversely, Durham and Keudell (1969) observed that low concentrations of 3,4-dihydroxybenzoic acid (protocatechuate) (Figure 4) prevented or reversed the inhibition by actinomycin D of cell viability, uracil-2-^{14}C incorporation and leucine-2-^{14}C incorporation when P. fluorescens was the test organism. It was also noted that protocatechuate did not compete with or otherwise influence the uptake mechanism of ^{14}C-actinomycin D.

These data suggested that molecular complexation of the hydroxypyrazole or protocatechuate with actinomycin D may be an important facet of their respective mechanisms. Durham and Ferguson (1971) reported that the inhibition of the synthesis of amidase (acylamide amidohydrolase, EC 3.5.1.4) in P. fluorescens by actinomycin D could be prevented or reversed by protocatechuate. A number of selected benzoic acid derivatives were tested that influenced actinomycin D inhibition. The results revealed that the steric specificity and conformational integrity of the molecule were very important. The adjacent dihydroxyl structure, in addition to the occupation

Figure 3. Structure of 10,11-dihydro-3H-napth(1,2 g) indazol-7-ol. The molecule has both polar and non-polar properties. The aromatic rings constitute the non-polar portion of the molecule while the hydroxyl group and the nitrogens contribute polar properties to the molecule.

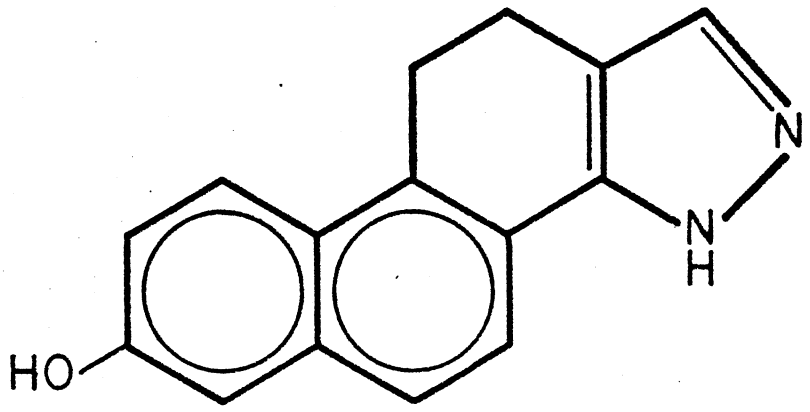
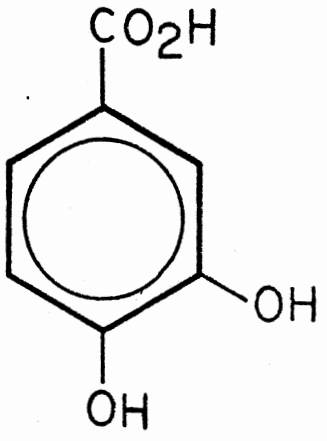

HO
N
N
H

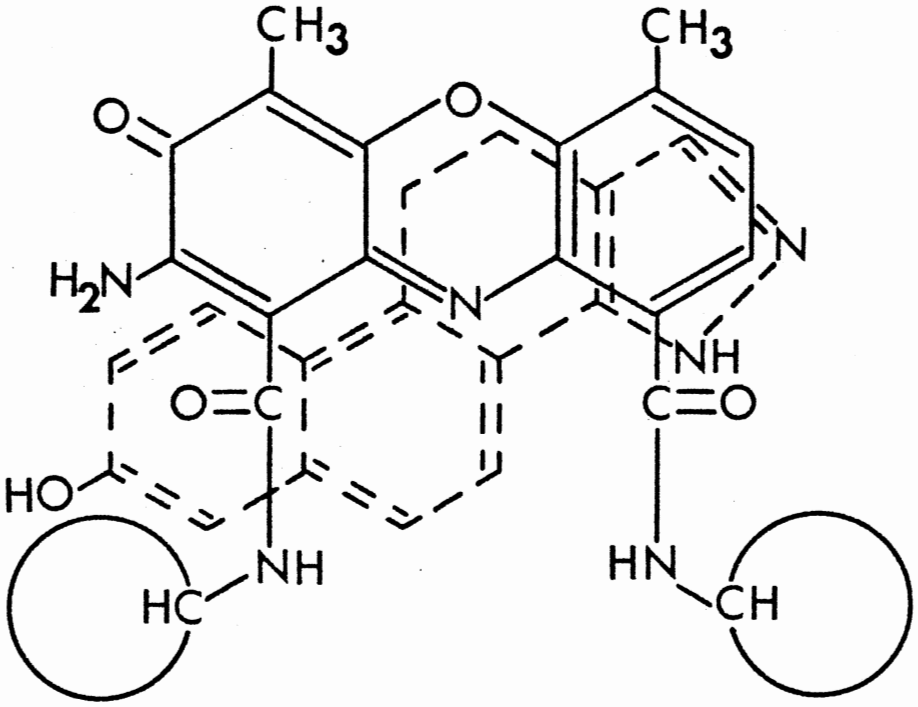
Figure 4. Structure of Protocatechuate (3,4-dihydroxybenzoic acid). The two hydroxyl groups and the carboxyl group which are attached to the aromatic ring give this compound great potential for molecular interaction.


CO2H
OH
OH

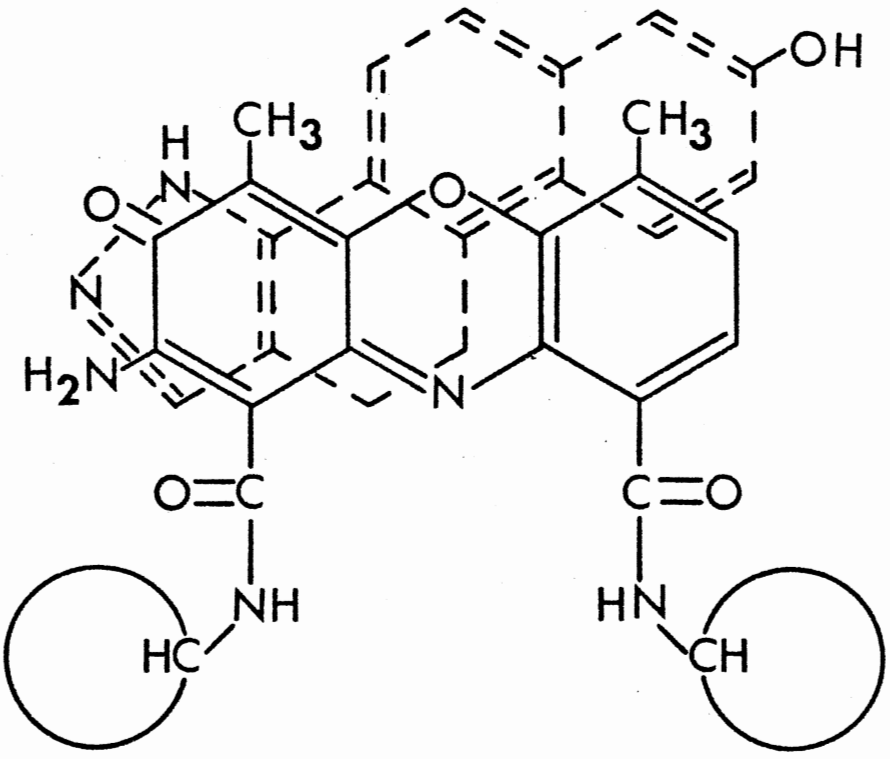
of the 3-position on the benzene ring, appears to be critical for the interaction of protocatechuate with actinomycin D. Molecular models depict that the benzenoid compounds may align themselves along the horizontal plane of the unreduced quinoidal phenoxazone ring structure of the actinomycin D. In this arrangement, a type of complex may be formed between the compounds in which there is π (orbital) overlap along with H-bonding so that a complex of the two molecules forms. These data suggested that the protocatechuate has a greater affinity than DNA for actinomycin D. Therefore, a complex between protocatechuate and actinomycin D may produce an equilibrium which no longer favors the interaction of the actinomycin D with the DNA.

Considerable evidence from UV-fluorescence, UV-absorption, DNA thermal denaturation and calorimetry studies have provided support for a complex between actinomycin D and the hydroxypyrazole (Haslam, 1973). Additional evidence for complexation between these two compounds was provided by ^{1}H NMR analysis in D_2O (Chestnut et al., 1974), and a later ^{1}H NMR and ^{13}C NMR analysis in D_3COD (O'Donnell et al., 1978). Of importance, is that all NMR data from these two investigations suggested that a molecular complex was formed between actinomycin D and the hydroxypyrazole regardless of the concentrations or solvents used. Furthermore, the preferred arrangement in both models was a 1:1 stacked arrangement rather than side by side (Figure 5), even though the proposed orientations of the two compounds were not identical.

Figure 5. Proposed Orientation of the Hydroxypyrazole:Actinomycin D Complex in D_2O (A Complex) and in D_3COD (B Complex).



A COMPLEX



B COMPLEX

Blunk (1977) reported that the inhibition of the synthesis of the amidase enzyme of P. fluorescens by actinomycin D could also be increased when the hydroxypyrazole was added in combination. It was also demonstrated in this study that protocatechuate could prevent or reverse the inhibition of enzyme synthesis resulting from the combination of these two compounds. DNA thermal denaturation studies yielded that all three compounds individually interact with the DNA to stabilize the DNA helix and shift the melting curve to higher temperatures. The combination of all three compounds almost completely stabilized the DNA until the melting temperature was elevated to 85 C. Equilibrium dialysis experiments illustrated that the combination of all three compounds was dialyzed from the DNA more quickly than the combination of actinomycin D and the hydroxypyrazole. From these data, it was concluded that protocatechuate may be interacting with actinomycin D and the hydroxypyrazole to form a three-way complex which does not interact as favorably with DNA as the proposed actinomycin D and hydroxypyrazole complex.

The hydroxypyrazole used in this investigation was synthesized by Dr. K. D. Berlin and Dr. J. G. Morgan at Oklahoma State University (Morgan et al., 1971). This compound has a molecular weight of 236 daltons, a melting point of 257-260 C and ultraviolet absorption peaks of 258, 266, 300 and 312 nm. The benzene rings of the hydroxypyrazole constitute a large nonpolar nucleus with the hydroxyl and the two nitrogen groups contributing polar properties to the molecule. B. subtilis,

a Gram-positive bacterium, was readily inhibited by the bacteriostatic action of the hydroxypyrazole while the Gram-negative bacterium, P. fluorescens, remained unaffected. Haslam (1973) utilized both sheep red blood cells and B. subtilis W23 protoplasts to demonstrate that the hydroxypyrazole exhibited only very subtle action against the membrane. Experiments were also conducted to determine if the hydroxypyrazole could increase the antibacterial action of selected antibiotics against P. fluorescens. With the exception of actinomycin D, only the two membrane active antibiotics, polymyxin and circulin, exhibited an increase in inhibition when used in combination with the hydroxypyrazole. No increase in the activity of vancomycin, penicillin, chloramphenicol, 5-fluorouracil or mitomycin C was demonstrated.

It is the purpose of this investigation to relate the possible mechanism(s) by which the hydroxypyrazole and protocatechuate influence actinomycin D action in selected in vitro model systems to those effects which are observed in P. fluorescens. Elucidation of these mechanisms will be useful for optimizing the antimicrobial and antitumor applications of actinomycin D. P. fluorescens provides a useful model system for determining the response of Gram-negative bacteria to these compounds because this bacterium is resistant to all but higher concentrations of most antibiotics and requires only an organic carbon source plus minimal salts for growth. Although actinomycin D is a potent antitumor agent, clinical usage has been severely limited by its

extreme toxicity. Compounds which increase actinomycin D action without contributing to its toxicity may allow smaller dosages of the antitumor agent to be given without decreasing its antitumor activity. Conversely, compounds which negate actinomycin D action may allow larger dosages of actinomycin D to be given because subsequential treatment with the appropriate compound may enhance recovery of normal cells and the immune system. Furthermore, if actinomycin D action can be affected by complexation with selected compounds, actinomycin D may be more efficiently utilized clinically. An understanding of molecular complexes formed between actinomycin D and compounds which modulate its biological activity would be invaluable for designing and synthesizing analogs of actinomycin D which may be more effective chemotherapeutic agents than the parent compound.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The microorganism used during the course of these studies was a strain of Pseudomonas obtained from the stock culture collection of Dr. N. N. Durham, Oklahoma State University. The organism was identified by Montgomery (1966) as Pseudomonas fluorescens. Stock cultures were maintained on 0.2% succinate-salts agar slants and stored at 4 C.

Deionized Water

Deionized water was obtained from a Barnstead Nanopure water system (model number D 1798) consisting of an organic removal cartridge, followed by two mixed resin deionization cartridges and one submicron filter. Water with an 18 meg-ohm-cm resistance was used to make all solutions except growth medium. Growth medium was made with distilled water which was passed through a Barnstead high capacity deionization column (model number D 8901).

Glassware Preparation

All glassware used in the RNA polymerase, DNA, amidase and vesicle investigations was cleaned with RBS 35 concentrate

(Pierce) before extensive rinsing with hot tap water followed by three rinses of deionized water from the Barnstead Nanopure water system. The glassware was then heat treated at 250 C for 3 hours to eliminate ribonuclease, deoxyribonuclease and protease contamination. Glassware utilized for phosphate determination was cleaned in a sulfuric acid:dichromate solution consisting of 125 g of sodium dichromate, 1000 ml of tap water and 1600 ml of concentrated sulfuric acid. All other glassware was cleaned with Alconox (Scientific Products) followed by extensive rinsing with hot tap water and three rinses with distilled deionized water from the Barnstead high capacity deionization column.

Media

The synthetic salts medium used in this investigation had the following composition: 0.2% sodium chloride, 0.2% ammonium chloride, 0.32% potassium dihydrogen phosphate, 0.42% dipotassium hydrogen phosphate and 0.2% succinate. The pH of the medium was adjusted to 6.8-7.0 with potassium hydroxide pellets prior to autoclaving at 121 C with 15 pounds pressure per square inch for 15 minutes. After cooling to room temperature, 0.1 ml of a sterile mineral salts solution was added to each 100 ml of medium. The mineral salts solution contained 5.0% magnesium sulfate, 0.1% manganese sulfate, 1.0% ferric chloride and 0.5% calcium chloride in distilled deionized water. This solution had been sterilized by autoclaving as described, and was used after the suspension was

allowed to settle (Durham, 1958). Agar (Difco) was added to give a final concentration of 2.0% when a solid medium was required. Acetamide was used as the inducer of amidase enzyme synthesis by adding acetamide to yield a final concentration of 0.08 M. The term mineral salts buffer was used to designate the basal salts medium in the absence of a carbon source.

Growth of Cells

Erlenmeyer flasks (250 ml) containing 50 ml of medium were inoculated from a freshly grown stock culture of P. fluorescens and incubated at 37 C on a reciprocal shaker (100 oscillations per minute). The cells were harvested by centrifugation (5000 x g for 10 minutes) and washed twice with minimal salts buffer. Sterile tubes (18 x 150 mm) containing growth medium and the appropriate compounds to be tested were inoculated with the washed cell suspension to give a final volume of 5.0 ml. Tube dilution assays were followed by measuring the increase in absorbency of the cultures at 540 nm on a Bausch and Lomb Spectronic 70 spectrophotometer. Amidase assays were performed in side-arm flasks (250 ml) containing a total volume of 25 ml. Larger quantities of cells were often needed for various isolations. These cells were grown in Fernbach flasks (2800 ml) containing 500 ml of growth medium.

Chemicals

Actinomycin D was generously supplied by the Merck, Sharp and Dohme Research Laboratory. The hydroxypyrazole was provided by Dr. K. D. Berlin, Oklahoma State University. Stock solutions of these two compounds were prepared in sterile deionized water and stored at 4 C in the dark. Stock solutions of protocatechuate (Aldrich Chemical Co., Inc.) were prepared and filter sterilized immediately prior to usage. All radioactive compounds were purchased from the Amersham Corporation and stored at -20 C in the dark. All other chemicals used in this investigation were of the highest purity available.

Amidase Enzyme Induction

Cultures of P. fluorescens were allowed to grow for 9 hours in succinate medium. At this time, the cells were harvested by centrifugation (5000 x g for 10 minutes) and washed twice with minimal salts buffer. The pellet was resuspended in minimal salts buffer and used to inoculate the growth medium containing succinate, acetamide and the compounds of interest. Sidearm flasks (250 ml) containing a total volume of 25 ml were inoculated to give an initial absorbance of 0.20 at 540 nm. Samples (0.25 ml) were removed from the flasks at the desired time intervals and immediately frozen at -20 C for later determination of enzyme activity. At this same time, a A_{540} nm reading was taken so that dry weight could be calculated.

Amidase Assay Method

The assay used in determining amidase enzyme synthesis was based on the ability of acyl phosphates to react with hydroxylamine at pH 6.5 to 7.0 to form hydroxamic acids (Lipmann and Tuttle, 1945). Quantitation of hydroxamic acid which reacts with ferric salts to produce red to violet colored complexes, was easily accomplished with a spectrophotometer.

Preparation of the Standard Curve

A stock solution of hydroxamic acid was prepared by dissolving 0.5 g of succinic anhydride in 20 ml of freshly neutralized 2.0 M hydroxylamine hydrochloride. After allowing the solution to stand for 10 minutes, deionized water was added to give a final volume of 50 ml. A 1:40 dilution of this stock solution with deionized water was used to produce the standard solution. Two ml of the standard solution was mixed with 1.0 ml of freshly neutralized hydroxylamine hydrochloride. After standing for 10 minutes, 2.0 ml of ferric chloride (6% w/v in 2% v/v hydrochloric acid) was added and the absorbance read in a Bausch and Lomb Spectronic 70 at 540 nm. The absorbance from this solution was equivalent to 4.0 micromoles of acetohydroxamic acid. Dilutions of the stock solution were prepared in a total volume of 40 ml to give the desired concentrations of acetohydroxamate. A standard curve for acetohydroxamate was plotted from the absorbencies of the different solutions.

Amidase Activity

The Brammer and Clarke (1964) modification of the Lipmann and Tuttle (1945) method for hydroxamic acid determination was used in this investigation. This modification utilizes the quantitation of acetohydroxamate which is one of the products of the translocase reaction catalyzed by the amidase enzyme. The amidase catalyzes the transfer of the acyl group of the substrate amides to hydroxylamine to form acylhydroxamates (Kelly and Kornberg, 1962). The nature of this reaction is described below.

$$CH_3\text{-}CO\text{-}NH_2 + NH_2\text{-}OH \dashrightarrow CH_3\text{-}CO\text{-}NH\text{-}OH + NH_3$$

The frozen samples were thawed and 0.75 ml of a substrate mixture containing equal volumes of 0.4 M acetamide, freshly neutralized 2.0 M hydroxylamine hydrochloride and 0.1 M 2-amino-2-(hydroxymethyl)-1-3-proanediol (Tris-HCl) buffer (pH 7.2) was added. The tubes containing the reaction mixture were incubated at 37 C for 15 minutes. Termination of the reaction was achieved by the addition of 2.0 ml of ferric chloride (6% w/v in 2% v/v hydrochloric acid). A red to violet color appears immediately and the absorbance at 540 nm was determined using a Bausch and Lomb Spectronic 70. The amount of acetohydroxamate was calculated from the acetohydroxamate standard curve. Specific activity was calculated by defining one unit of amidase as the amount of enzyme that would produce one micromole of acetohydroxamate per mg dry cell weight per 15 minutes.

Isolation and Purification of Amidase

Growth of P. fluorescens

Aliquots (25 ml) from a liquid culture which had been incubated for 9 hours on a reciprocal shaker were used to inoculate 6 Fernbach flasks (2800 ml) containing 475 ml of medium. These flasks were incubated for 9 hours before harvesting. At this time, the cells were in the late log phase of growth and had a cell density between 0.400 and 0.425 mg dry weight per ml. The cells were harvested and washed twice with minimal salts buffer by centrifugation at 4 C and 5000 x g for 10 minutes. The yield was between 25-30 g wet weight. Harvested cells were stored as a thick paste at -20 C without any significant loss of amidase activity.

Preparation of Ultrasonic Extracts

The method of Kelly and Kornberg (1964) was used to isolate and purify the amidase enzyme. Approximately 15 g of cells (wet weight) were suspended at 4 C in 200 ml of 0.5 M potassium phosphate buffer (pH 7.2). Twenty ml portions were exposed for 90 seconds (30 second bursts) to the output of a Branson Model S75 sonifier operating at 8.0 A. The suspensions of broken cells were combined and centrifuged at 4 C and 15,000 x g for 15 minutes. The supernatant was decanted and retained. The sediment was suspended in 200 ml of 0.2 M potassium phosphate buffer (pH 7.2) and the ultrasonic treatment and centrifugation procedures were repeated.

The supernatant from this treatment was combined with the initial supernatant. One or two crystals of bovine pancreas deoxyribonuclease I was added to the combined supernatants before allowing the suspension to incubate at 25 C for 15 minutes.

Heat Treatment

The combined supernatant solutions were divided between 2 thin-walled flasks (500 ml) and both flasks were incubated in a water bath at 60 C for 10 minutes. Then the flasks were rapidly cooled in an ice-water bath for 5 minutes. All subsequent operations were performed at 4 C. The 2 batches were combined and centrifuged at 4 C and 25,000 x g for 1 hour. The supernatant was decanted and the sediment was re-suspended in 50 ml of 0.1 M potassium phosphate buffer (pH 7.2) and centrifuged at 4 C and 25,000 x g for 1 hour.

Ammonium Sulfate Precipitation

The supernatant solutions were combined and the bulk of their protein content precipitated by the addition of ammonium sulfate at a concentration of 300 g per 450 ml of supernatant to give 90% (w/v) saturation. After standing for 30 minutes, the precipitate was collected by centrifugation at 4 C and 25,000 x g for 15 minutes. The precipitate was dissolved in a minimum volume of the initial column buffer (pH 7.2) consisting of 5 mM potassium phosphate, 1 mM disodium ethylenediaminetetraacetate (EDTA) and 1 mM cysteine.

This mixture was placed in dialysis tubing having an exclusion limit of 12,000 daltons and dialyzed against 4 changes of 2 liters of this buffer for 12 hours.

Column Chromatography

DEAE-cellulose (Sigma Chemical Company, fine mesh-0.89 meq/g) was prepared by suspending 10-20 g of the cellulose in 500 ml of the initial column buffer contained in a 1000 ml beaker. This mixture was allowed to settle for 15 minutes before decanting and discarding the supernatant. An additional 400 ml of buffer was added to the DEAE-cellulose, stirred gently and allowed to settle for 15 minutes. This process was repeated until the supernatant no longer became cloudy. After the final washing, the DEAE-cellulose was resuspended in a small amount of the buffer and adjusted to pH 7.2 with phosphoric acid. A 2x20 cm column was poured and packed by washing with 2 liters of the initial column buffer at a flow rate of 40 ml per hour. The dialyzed protein was applied to the column at the rate of 15 ml per hour. The loaded column was developed with the initial column buffer and subsequently with a linear phosphate gradient. A Pharmacia gradient mixer was used to form the gradient by allowing 300 ml of 0.4 M potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM cysteine to flow with constant stirring, into a vessel containing 300 ml of 5 mM potassium phosphate buffer (pH 7.2) containing 1mM EDTA and 1 mM cysteine. The gradient was allowed to flow through the column

at the rate of 20 ml per hour. Fractions (10 ml) were collected and analyzed for amidase activity and protein content (Lowry et al., 1951).

Storage of Purified Amidase

The fractions making up the central portion of the peak of enzyme activity were combined and precipitated by the addition of ammonium sulfate (5.2 g per 8.3 ml of eluate) to give 90% (w/v) saturation. This mixture was centrifuged at 25,000 x g for 15 minutes. The precipitate was collected and stored as a slurry at -20 C. For subsequent experiments, small aliquots of the enzyme slurry were dissolved in 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM cysteine and dialyzed extensively against this buffer prior to experimentation. All in vitro investigations were performed in this buffer.

Deoxyribonucleic Acid Isolation

Growth of P. fluorescens

Three Fernbach flasks (2800 ml) containing 500 ml of medium were inoculated from a liquid culture and incubated at 37 C on a reciprocal shaker. After 9 hours, the cells were harvested and washed twice with minimal salts buffer by centrifugation at 4 C and 5000 x g for 10 minutes. The wet weight yield was between 12-15 g. The cells were resuspended in 0.15 M sodium chloride and 0.1 M EDTA at a concentration of 1 g (wet weight) per ml. This suspension may

be used immediately or frozen at -20 C for later use.

Isolation of DNA

DNA from P. fluorescens was isolated utilizing the Saito and Miura (1963) modification of the method described by Marmur (1961). Lysozyme (egg white) was added to a 6 g of resuspended cells to give a final concentration of 2 mg per ml. Incubation of this mixture at 37 C was carried out for a period of 10-20 minutes with careful monitoring, by the addition of dilute sodium hydroxide, to maintain a pH of 8.0. Just as the cells began to lyse, they were quickly frozen in an acetone-dry ice bath. At this time, 50 ml of Tris-SDS buffer (pH 9.0) consisting of 0.1 M Tris-HCl, 1% (w/v) sodium dodecyl sulfate (SDS) and 0.1 M sodium chloride was added to the frozen cells. Resuspension of the cells was accomplished by stirring with a glass rod. While lysis was still incomplete, the freezing and thawing were repeated. The cell suspension was then mixed with an equal volume of redistilled phenol and the mixture was shaken by hand in a glass-stoppered round bottom flask for 20 minutes in an ice-water bath. The resulting emulsion was separated into 2 phases by low speed centrifugation at 4 C and 650 x g for 10 minutes. Clarification of the upper aqueous phase by high speed centrifugation at 4 C and 16,300 x g for 10 minutes was used to remove any remaining cell debris. The nucleic acids were precipitated by gently mixing the clarified suspension with 2 volumes of cold 95% (v/v) ethanol. The thread-like

precipitate was collected on glass rods and dissolved in 20-40 ml of dilute saline-citrate (pH 7.0) consisting of 15 mM sodium chloride and 1.5 mM trisodium citrate. Two to four ml of concentrated saline-citrate (pH 7.0) consisting of 1.5 M sodium chloride and 0.15 M trisodium citrate was then added to this solution. To remove RNA, ribonuclease I (bovine pancreas) which had been dissolved in 0.15 M sodium chloride and previously heated to 80 C for 10 minutes was added to the solution to give a final concentration of 50 μg per ml. After incubation for 30 minutes at 37 C, the digest was cooled and mixed with an equal volume of redistilled phenol. This was followed by shaking of the mixture for 10 minutes in an ice-water bath. The two phases were once again separated by centrifugation at 4 C and 650 x g for 10 minutes. As was done previously, the upper aqueous phase was clarified by centrifugation at 4 C and 16,000 x g for 10 minutes. The DNA was precipitated by the addition of 2 volumes of cold 95% (v/v) ethanol and collected on glass rods. Next, the DNA was dissolved in 20 ml of dilute saline-citrate. To this mixture, 2.2 ml of acetate-EDTA (pH 7.0) consisting of 3 M sodium acetate and 1 mM EDTA was added. While the solution was rapidly stirred, 0.54 volumes of cold isopropanol was slowly added to eliminate RNA. The precipitated DNA was subjected to the acetate-EDTA and isopropanol steps a second time using one-half the previous volumes. After this step, the DNA was washed by stirring successively in 70, 80 and 90% (v/v) aqueous ethanol before storage at 4 C in

95% (v/v) ethanol and 1% (v/v) chloroform.

Stock solutions of DNA were prepared by allowing the DNA to gently disperse in a small volume of the appropriate buffer over a period of 1-2 days at 4 C. Aliquots of this solution were removed and dialyzed against the experimental buffer to remove the remaining ethanol and chloroform. DNA concentration was determined by using either the diphenylamine reaction (Burton, 1955), or by using a nomograph distributed by the California Corporation for Biochemical Research, 3625 Medford St., Los Angeles 63, California. The 260/280 nm absorbencies were measured in a Beckman DU-2 spectrophotometer using quartz cuvettes with a 1 cm light path. RNA contamination was estimated using the orcinol method of Ogur and Rosen (1950).

RNA Polymerase Assays

Standard Assay of RNA Polymerase

RNA polymerase activity was determined by measuring the amount of radioactive precursor rendered acid insoluble in the test system. The standard assay procedure of McConnell and Bonner (1972) was used throughout the course of this investigation. RNA polymerase (EC No. 2.7.7.6), Type III from *E. coli* K-12 (Sigma Chemical Company) was assayed in a total volume of 0.25 ml. The components of the assay consisted of Tris-HCl buffer (pH 7.9), 50 mM; magnesium chloride, 8 mM; dithiothreitol, 0.1 mM; ATP, GTP and CTP, each 0.2 mM; UTP, 0.19 mM; 5-^{3}H-UTP (Amersham Corporation, specific

activity:2 curies per mmol), 0.01 mM; either calf thymus DNA (Sigma Chemical Company, Type I) or P. fluorescens DNA (isolated and purified as previously described), 30 μg; 3 units of RNA polymerase. As defined by Sigma, one unit of RNA polymerase will incorporate 1 nanomole of labeled ATP into acid insoluble product in 15 minutes at pH 7.9 at 37 C, using calf thymus DNA as the template. In this assay, actinomycin D, protocatechuate and/or the hydroxypyrazole were added to the combined salt and ribonucleoside 5'-triphosphate solution before the DNA and RNA polymerase. The reaction mixture was incubated at 37 C for 15 minutes. In some experiments, 0.05 ml aliquots of the reaction mixture were removed at the desired time intervals and used to plot the radioactivity incorporated with time. The reaction was stopped by rapid cooling of the reaction mixture in an ice-water bath followed by the addition of 75 μg of RNA (Sigma Chemical Company, Type III from yeast) as a carrier and 2 ml of 10% (w/v) trichloroacetic acid (TCA) in succession. After standing for 30 minutes in the ice-water bath, acid precipitable counts were collected on glass fiber filters (Whatman GF/C, 2.4 cm diameter) and washed 5 times with 10 ml of cold 5% (w/v) TCA containing 0.1 M sodium pyrophosphate and 0.05 mM ATP. The filters were placed in scintillation vials and dried under a heat lamp. After cooling, 10 ml of Quantofluor (Mallinckrodt) was added to the vials and the vials were counted for 20 minutes in a Beckman LS-3133P scintillation counter. Controls lacking either RNA

polymerase or DNA were routinely included in each experiment.

Time-Course Addition of Actinomycin D, Protocatechuate and the Hydroxypyrazole

Time-course addition of actinomycin D, protocatechuate and the hydroxypyrazole was studied by using a slight modification of the standard assay method. One of the compounds of interest was added to the complete reaction mixture lacking CTP and incubated for 15 minutes at 37 C to establish a binding equilibrium with the template. This incubation will allow an initiation complex to form. At the end of this incubation period, the other compound of interest was added and elongation of the RNA chain may be started by the addition of CTP, or the incubation of the reaction mixture and the two compounds may be extended for another 15 minutes to establish a binding equilibrium between the two compounds and the template. Then, CTP was added to start elongation. Controls incubated over these same periods were routinely included to insure that no loss of RNA polymerase activity was incurred.

Effect on RNA Chain Elongation

The high salt method of Hyman and Davidson (1970) was used to study the effect of the hydroxypyrazole on actinomycin D inhibition of RNA chain elongation. The complete assay mixture lacking CTP was mixed in the low salt buffer of the standard assay and incubated at 37 C for 15 minutes.

At this time, ammonium sulfate was added to give a final concentration of 0.4 M. This high salt concentration washes any non-initiated polymerase off the DNA, leaves the initiation complex intact and severely slows the rate of reinitiation. At this point, after initiation has occurred, actinomycin D and the hydroxypyrazole were added. Thus, only the effect of the compounds on RNA chain elongation was measured. CTP may be added at this time, or after incubation at 37 C for an additional 15 minutes to allow the compounds to establish a binding equilibrium with the template.

Treatment of Cells with Surface-Active Agents

Four surface-active agents, Triton X-100, Tween 80, 1-hexadecylpyridinium chloride and SDS, were investigated to determine the effect, if any, that these compounds might have on actinomycin D activity. Cultures of P. fluorescens were grown for 9 hours at 37 C. The cells were harvested, washed twice with minimal salts buffer and resuspended in minimal salts buffer to give an optical density of 0.4 at 540 nm. Two 20 ml aliquots were removed and placed in Erlenmeyer flasks (250 ml). Five ml of the surface-active agent was added to one flask while 5 ml of sterile deionized water was added to the second flask. The two flasks were then incubated on a slowly shaking water bath for 5 minutes at 37 C. At the end of this time period, the cells from the two flasks were harvested by low speed centrifugation at room

temperature, washed twice with minimal salts buffer and resuspended in minimal salts buffer to give an optical density of 0.2 at 540 nm. These two suspensions were then used to inoculate culture tubes containing the appropriate medium. Growth in the presence and absence of actinomycin D was followed spectrophotometrically.

Uptake of Radioactive Substrates

Cultures of P. fluorescens were grown for 9 hours at 37 C. One ml of this growth suspension was used to inoculate 24 ml of succinate medium contained in a side-arm flask (250 ml). At the end of 3 hours, these cells were harvested, washed twice with minimal salts buffer and resuspended in minimal salts buffer to give an optical density of 0.3 measured at 540 nm. This cell suspension was equilibrated with gentle shaking for 20 minutes at 37 C in a shaking water bath. Three ml of the cells were added to tubes containing the ^{14}C-labeled substrate (0.2 μCi per ml final concentration), unlabeled substrate (10:1 molar ratio relative to the radioactive substrate) and the appropriate compounds of interest. Samples (0.5 ml) were removed at the desired time intervals and immediately filtered through Millipore filters (type HA, 0.45 micron pore size, 1 cm diameter) and washed 3 times with 1 ml of cold minimal salts buffer. The filters were placed in scintillation vials containing 10 ml of Aquasol (New England Nuclear). After 5 hours to insure complete digestion of the Millipore filter, the samples were

counted in a Beckman LS-3133P scintillation counter for 20 minutes.

Isolation, Purification and Reconstitution of Vesicles from Outer Membrane Components of *P. fluorescens*

Extraction, Quantitation and Qualitation of Phospholipids

Phospholipids were prepared by the method of Folch et al. (1957). After growth in succinate medium for 9 hours, a 100 ml culture of *P. fluorescens* was harvested, washed twice with minimal salts buffer and resuspended in 5 ml of methanol contained in a screw-cap tube. The tube containing the suspension was flushed with nitrogen and capped with a teflon lined screw-cap. The sealed tube was incubated for 30 minutes in a 55 C water bath. After cooling to room temperature, 10 ml of chloroform was added and the extraction was continued at 25 C for 12 hours under a nitrogen atmosphere. Insoluble material was removed from the sample by passing the suspension through a Millipore filter (type HA, 0.45 micron pore size). The chloroform-methanol suspension was then washed twice with 15 ml of 2 M potassium chloride and once with 10 ml of deionized water. After the water wash, the chloroform and water layers were separated by low speed centrifugation at room temperature. The resulting chloroform solution was passed through a freshly prepared sodium sulfate column (0.5x14 cm) and dried down to about

0.5 ml under a steady stream of nitrogen. The phospholipid solution was maintained in chloroform at -20 C under a nitrogen atmosphere.

Since it is known that approximately 90% of the lipids of Pseudomonas aeruginosa and closely related species are phospholipids, the lipid phosphate content may be used as an assay of lipid levels (Hancock and Meadow, 1969). Lipid phosphate was determined by the method of Ames and Dubin (1960).

The phospholipids were qualitatively identified by spotting microliter volumes of the chloroform-phospholipid solution on 5x20 cm silica gel G chromatography plates (Quantum Industries) which had been activated at 100 C immediately before use. Chromatograms were developed in a solvent system of chloroform-methanol-water (65:25:4 by volume) after allowing the solvents to equilibrate for 1 hour. Rhodamine G spray reagent was used to detect lipid containing substances (Rouser et al., 1961). The detection of phosphate esters was determined using the ferric chloride-salicylsulphonic acid spray reagent (Wade and Morgan, 1953). Ninhydrin spray reagent was used to detect lipids containing free amino groups (Marinetti et al., 1962). Phosphatidylglycerol was detected by the Periodate-Schiff's test for vicinal hydroxyl groups (Shaw, 1968). Conclusive identification of the extracted phospholipids was accomplished by chromatography with known standards. These standards included phosphatidylethanolamine and phosphatidylglycerol

(Sigma Chemical Company).

Extraction of Lipopolysaccharide

Lipopolysaccharide (LPS) from P. fluorescens was extracted and purified by the method of Johnson and Perry (1976). Bacteria (5 g) were placed in 50 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA and 0.05% (w/v) sodium azide. This mixture was stirred with a magnetic stirrer at top speed for 1 minute. Lysozyme was added to give a final concentration of 100 μg per ml, and the suspension was slowly stirred at 4 C for 16 hours. After this incubation, the suspension was then incubated at 37 C for 20 minutes before stirring at top speed on a magnetic stirrer for 3 minutes. The volume of the suspension was adjusted to 100 ml with 20 mM magnesium chloride. Bovine pancreas ribonuclease I and deoxyribonuclease I were added to give a final concentration of 1 μg per ml. This mixture was incubated for 10 minutes at 37 C and then for 10 minutes at 60 C. If a gelatinous mixture was obtained, the suspension was stirred for 3 minutes at top speed on a magnetic stirrer before phenol extraction. Equal volumes of disrupted bacteria and redistilled 90% (w/v) phenol were added to a glass-stoppered round bottom flask which was shaken by hand for 15 minutes in a water bath at 70 C. Both the bacterial suspension and the phenol were preheated to 70 C. After extraction, the suspension was rapidly cooled by shaking in an ice-water bath. Centrifugation at 4 C and 18,000 x g for 15 minutes permits

sharp definition of aqueous, phenol and interfacial layers, and occasionally sediment. The aqueous and phenol phases were carefully removed. The aqueous phase was retained while the phenol phase was discarded. Material remaining after removal of the aqueous and phenol phases was resuspended in three volumes of deionized water and stirred on a magnetic stirrer for 5 minutes. This suspension was centrifuged at 4 C and 18,000 x g for 15 minutes, and the resultant supernatant fraction was removed and combined with the aqueous phase. The pooled aqueous phases were dialyzed against deionized water until no detectable phenol odor remained. The samples were lypholized and stored at -20 C.

Quantitation of LPS was determined by the L-glycero-D-mannoheptose assay of Osborn (1963) and the 2-keto-3-deoxyoctonate (KDO) assay of Weissbach and Hurwitz (1958). LPS concentrations were calculated by assuming that each mole of LPS contained 2 moles of heptose.

Outer Membrane Protein Isolation and Purification

The method of Hancock and Nikaido (1978) was used to prepare outer membranes of *P. fluorescens*. Nine liters of cells were grown in succinate medium for 9 hours and harvested by centrifugation at 4 C and 5000 x g for 10 minutes. The cells were washed with 30 mM Tris-HCl buffer (pH 8.0) and resuspended in 20 ml of 20% (w/v) sucrose in Tris-HCl buffer containing 1 mg each of bovine pancreatic

deoxyribonuclease I and ribonuclease I. The cells were passed twice through a French pressure cell at 15,000 pounds per square inch, after which 2 mg of egg white lysozyme were added. Ten minutes later, a protease inhibitor (alpha-toluenesulfonyl fluoride) was added to give a final concentration of 1 mM. Cell debris was removed by centrifugation at 4 C and 1000 x g for 10 minutes. The supernatant was decanted, diluted by the addition of Tris-HCl buffer and layered onto sucrose step gradients containing 1 ml of 70% (w/v) sucrose and 6 ml of 15% (w/v) sucrose in 30 mM Tris-HCl buffer. These tubes were centrifuged at 4 C and 183,000 x g for 1 hour in a Beckman SW 41 rotor. The bottom 2 ml of each gradient was removed, pooled, diluted and applied to sucrose density gradients containing steps of 5 ml 70%, 10 ml of 64%, 10 ml of 58%, and 10 ml of 52% (w/v) sucrose in 30 mM Tris-HCl buffer. These tubes were centrifuged at 4 C and 96,000 x g for 18 hours in a Beckman SW 27 rotor. The four bands which were observed were removed by dropwise collection from the bottom of the centrifuge tube and assayed for protein (Lowry et al., 1951), succinate dehydrogenase (Kasahara and Anraku, 1974), KDO (Weissbach and Hurwitz, 1958) and lipid phosphate (Ames and Dubin, 1960).

Fractionation of outer membrane proteins was performed according to the procedure of Hancock et al. (1979). The lower 2 bands were pooled, diluted with sterile deionized water and centrifuged at 4 C and 177,000 x g for 1 hour in a Beckman SW 41 rotor. The resulting pellet was solubilized

at a protein concentration of 10 mg per ml in 20 mM Tris-HCl buffer (pH 7.4) containing 2% (v/v) Triton X-100 with sonication for 2 minutes in a bath type sonicator (Laboratory Supplies Company, Inc.). Insoluble protein was sedimented by centrifugation at 4 C and 177,000 x g for 1 hour, and the supernatant was set aside as Triton X-100 soluble protein. Triton X-100 insoluble protein was resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing 2% (v/v) Triton X-100 and 10 mM EDTA at a protein concentration of 8 mg per ml with sonication as above to assist solubilization. Triton X-100/EDTA insoluble protein was removed by centrifugation at 4 C and 177,000 x g for 1 hour in a Beckman SW 41 rotor. The supernatant, containing Triton X-100/EDTA soluble outer membrane proteins was applied to a column (2.7x40 cm) of DEAE-Sephacel (Pharmacia Fine Chemicals) which had been equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 0.1% (w/v) Triton X-100 and 10 mM EDTA. The proteins were eluted at a linear flow rate of 8 cm per hour by using a Pharmacia gradient mixer to form a concentration gradient of sodium chloride (0.1 to 0.6 M). Fractions of 7 ml were collected, assayed for protein content (Lowry et al., 1951), and appropriate fractions were pooled. The pooled fractions were concentrated to about 3-5 ml by dialysis against a 30% (w/v) solution of polyethylene glycol 6000. Triton X-100 was removed from the protein samples by treatment with Bio-Gel SM-2 copolymer beads (Bio-Rad Laboratories) by the method of Holloway (1973). The assay procedure of Garewal (1973) was used to determine

Triton X-100 concentrations. After removal of Triton X-100, the protein fractions were divided into 0.5 ml aliquots and stored at -20 C in polypropylene micro test tubes.

Vesicle Preparation

Outer membrane vesicles were reconstituted by the technique described by Hancock and Nikaido (1978). Phospholipids (0.5 micromole) were dried down under a stream of nitrogen in disposable borosilicate tubes before placing the tubes in an evacuated dessicator for 30 minutes. LPS (0.08 micromole), with or without outer membrane protein (125-140 μg) derived from the outer membrane band material, was added to the tube and mixed on a Vortex mixer for 30 seconds. This mixture was then sonically disrupted in a bath type sonicator for 60 seconds to thoroughly randomize the components. The suspension was dried down under a stream of nitrogen at 45 C. The tubes were then placed in an evacuated dessicator for 30 minutes. A 100 microliter volume of a solution containing 1 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4), 100 mM sodium chloride, 10 mM magnesium chloride and 4.4×10^5 cpm of U-^{14}C-sucrose was added. The vesicle components were resuspended with a teflon spatula, followed by mixing for 30 seconds on a Vortex mixer and 30 seconds of sonic disruption in a sonicating water bath. The tubes were then incubated in a water bath at 45 C for 30 minutes. At the end of this time period, the water bath was turned off and allowed to come slowly, over the

course of approximately 2 hours, to room temperature. Aliquots of the vesicle solution were diluted 100 fold into a solution containing 1 mM HEPES buffer (pH 7.4), 100 mM sodium chloride, 10 mM magnesium chloride and 3 mM sodium azide. The effect of selected compounds on ^{14}C-sucrose retention was determined by exposing reconstituted vesicles to the compounds which were included at the desired concentration in this buffer. After 20 minutes at 25 C, the diluted vesicle suspension was filtered slowly, over the course of about 20 seconds, through a Millipore filter (type HA, 0.45 micron pore size, 1 cm diameter). The filter was washed six times with 1.5 ml of the buffer previously described. The washed filters were placed in scintillation vials containing 10 ml of Aquasol (New England Nuclear). After 5 hours to insure complete digestion of the Millipore filter, the amount of radioactivity retained in the vesicles was determined by liquid scintillation counting in a Beckman LS-3133P scintillation counter for 50 minutes. The amount of ^{14}C-sucrose retained in the vesicles was divided by the ^{14}C-sucrose content of the reconstitution medium to calculate the % ^{14}C-sucrose retained within the vesicles.

SDS Polyacrylamide Gel Electrophoresis of Outer Membrane Proteins

SDS polyacrylamide gel electrophoresis was performed by the method of Lugtenberg et al. (1975). The discontinuous, vertical slabs used in this investigation had a thickness of

1 mm and a running length of 9 cm. The following stock solutions were used for the preparation of the running and stacking gels. Stock solution I contained 44 g of acrylamide plus 0.8 g of methylene bisacrylamide while stock solution II contained 30 g of acrylamide plus 0.8 g of methylene bisacrylamide. The volumes of both solutions were adjusted to 100 ml with sterile deionized water, filtered through Whatman No. 1 paper and stored at 4 C in the dark. A fresh solution of ammonium persulphate was prepared for each slab gel. Polymerization was initiated by the addition of N,N,N',N'-tetramethylethylenediamine (TEMED) to yield a final concentration of 0.2% (v/v). The running and stacking gel solutions were degassed before the gel was poured and polymerized. The running gel contained 3.125 ml of stock solution I, 0.315 ml of ammonium persulphate (10 mg per ml), 0.25 ml of 10% (w/v) SDS, 6.25 ml of 0.75 M Tris-HCl buffer (pH 8.8), 2.56 ml deionized water and 0.025 ml of TEMED. The stacking gel contained 0.5 ml of stock solution II, 0.12 ml of ammonium persulphate (10 mg per ml), 0.05 ml of 10% (w/v) SDS, 2.5 ml of 0.25 M Tris-HCl buffer (pH 6.8), 1.83 ml of deionized water and 0.01 ml of TEMED. The buffer (pH 8.3) for both electrodes contained 0.025 M Tris-HCl, 0.19 M glycine and 0.1% (w/v) SDS.

Protein samples were prepared in 0.0625 M Tris-HCl buffer (pH 6.8) containing 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 5% (v/v) 2-mercaptoethanol. Protein was added to give a final concentration of 0.5-1 mg

per ml, and the samples were boiled for 5 minutes.

Routinely, 20 microliters (20 µg protein) of the samples were applied per slot. Samples containing a mixture of 1 µg each of standard proteins (Sigma Chemical Company) were prepared and applied as described. Electrophoresis was performed at room temperature using a constant current of 20 mA. The electrophoresis was stopped when the tracking dye had migrated to within 1 cm from the bottom of the gel. Gels were stained overnight in 0.1% (w/v) Coomassie Blue in 50% (v/v) methanol and 10% (v/v) acetic acid. The slabs were destained in 50% (v/v) methanol and 10% (v/v) acetic acid. In order to store the gels, they were soaked for additional 24 hours in a solution containing 50% (v/v) methanol and 5% (v/v) glycerol. After this treatment, the gels were spread on Whatman 3MM chromatography paper and dried in a glazing dryer.

CHAPTER III

RESULTS AND DISCUSSION

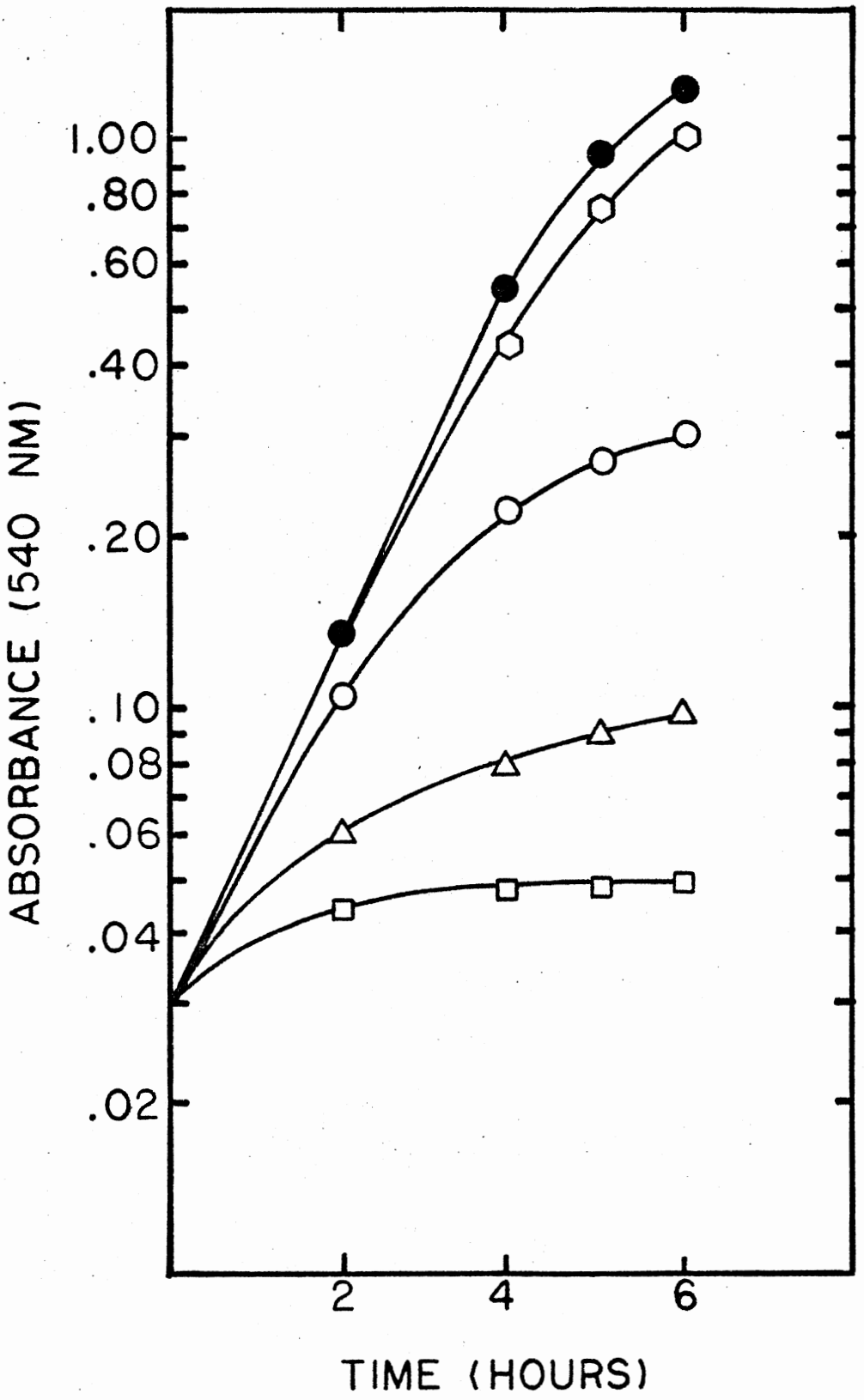
Influence of the Hydroxypyrazole and Protocatechuate on the Actinomycin D Inhibition of *P. fluorescens*

Effect of Actinomycin D on Growth of P. fluorescens

Waksman (1954) reported that Gram-positive microorganisms were very susceptible to actinomycin D action while Gram-negative microorganisms were less affected. The effect of the antibiotic can be both bacteriostatic and bactericidal, depending upon the concentrations utilized.

Tube dilution assays were performed in succinate medium to determine the sensitivity of *P. fluorescens* to actinomycin D. The response of this bacterium to actinomycin D concentrations of 3.2×10^{-6}, 8.0×10^{-6}, 1.6×10^{-5} and 2.4×10^{-5} M demonstrated that inhibition of growth was concentration dependent (Figure 6). Only slight inhibition of growth was observed when the concentration of actinomycin D was 3.2×10^{-6} M, while growth was completely inhibited when the concentration was increased to 2.4×10^{-5} M. Nearly 1-2 hours of growth in the presence of actinomycin D was required

Figure 6. Effect of Actinomycin D on Growth of P. fluorescens. ● , succinate control; ⬡ , actinomycin D, 3.2×10^{-6} M; ○ , actinomycin D, 8.0×10^{-6} M; △ , actinomycin D, 1.6×10^{-5} M; □ , actinomycin D, 2.4×10^{-5} M.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TIME (HOURS)

before any of the concentrations produced a discernible degree of inhibition. This period is assumed to represent the time required for actinomycin D to pass into the cell, reach its primary site of action (the DNA) and inhibit RNA synthesis.

Microscopic examination of cells exposed to the higher concentrations of actinomycin D revealed that these cells exhibited increases in both length and width.

Effect of the Hydroxypyrazole on Growth of P. fluorescens

Haslam (1973) reported that the hydroxypyrazole was a potent growth inhibitor of B. subtilis W23. A concentration of 8.5×10^{-6} M completely inhibited growth of this organism for 6 hours. LM tissue culture cells were also sensitive to the hydroxypyrazole. However, a concentration of 8.5×10^{-5} M was necessary to decrease the number of viable tissue culture cells by 60%.

In contrast, tube dilution assays demonstrated that P. fluorescens was not inhibited by hydroxypyrazole concentrations as high as 1.3×10^{-4} M. The inability of the hydroxypyrazole to inhibit growth of P. fluorescens is in agreement with the report by Smith et al. (1963) which noted that the spectrum of activity of steroids was almost totally confined to Gram-positive microorganisms.

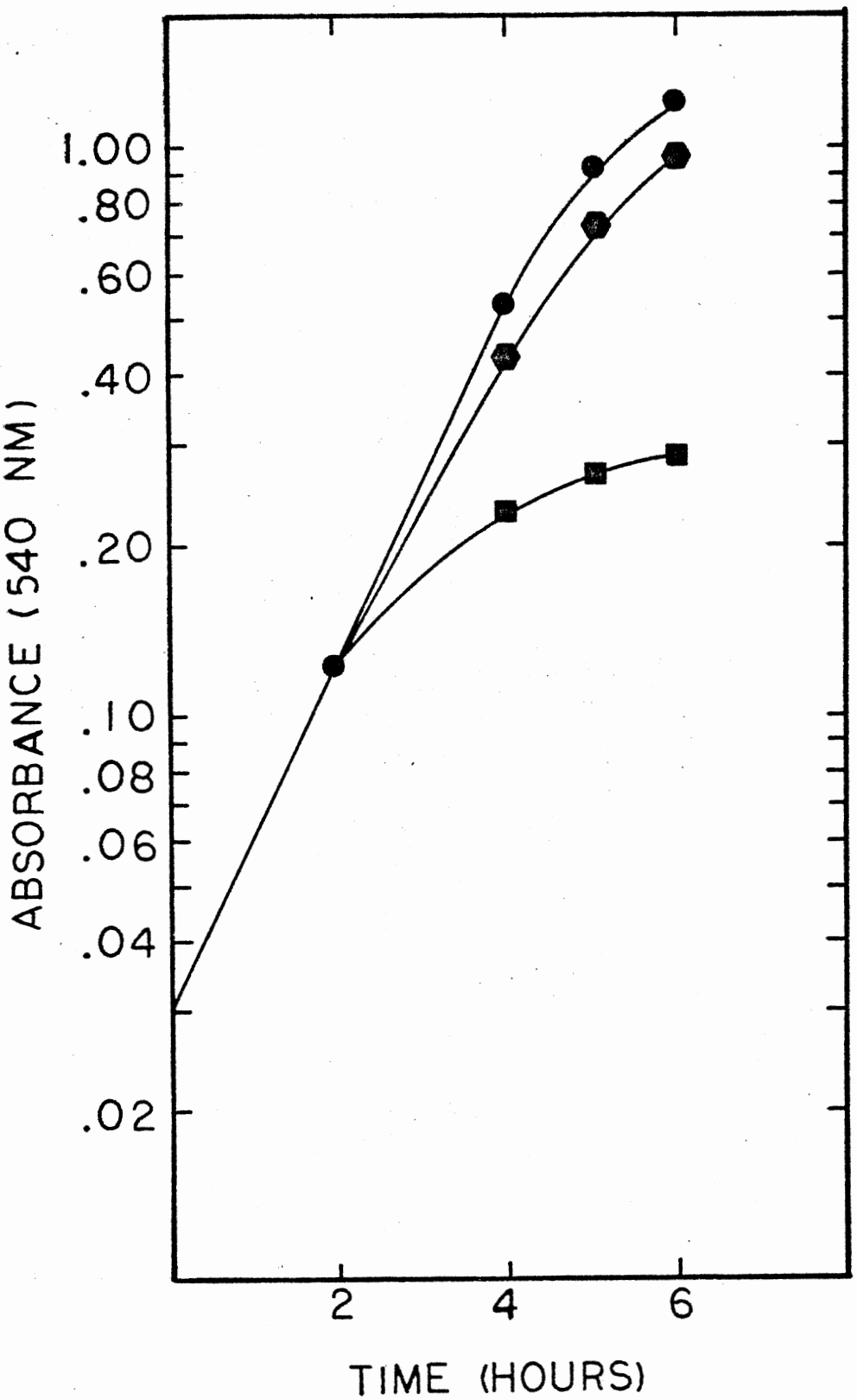
Effect of the Hydroxypyrazole on Actinomycin D Inhibition of P. fluorescens

Actinomycin D inhibition of P. fluorescens was increased by approximately 70% when the hydroxypyrazole was added in combination to the medium (Figure 7). The concentrations of actinomycin D and the hydroxypyrazole used in this study were 3.2×10^{-6} and 4.2×10^{-5} M, respectively. The very significant increase in actinomycin D inhibition resulting from the addition of the hydroxypyrazole appears to be bactericidal in nature. Neither prolonged incubation, nor washing and resuspension of the cells in fresh medium in the absence of actinomycin D was sufficient to reinitiate growth after exposure for 10 hours to the combination of these compounds. This is interesting since the concentration of actinomycin D used in this investigation was bacteriostatic. Furthermore, increases in cell length and width were observed microscopically which were customarily observed only for cells exposed to higher concentrations of the antibiotic.

Cell viability experiments utilizing the same concentrations which were used in the tube dilution assays revealed that there was a steady decline in the number of viable cells exposed to the combination of actinomycin D and the hydroxypyrazole, while the control cells and actinomycin D treated cells increased in viable cell number (Figure 8).

These data suggest that a molecular interaction between actinomycin D and the hydroxypyrazole may participate in the

Figure 7. Effect of the Hydroxypyrazole on Actinomycin D Inhibition of Growth of P. fluorescens. ● , succinate control and hydroxypyrazole, 4.2×10^{-5} M; ⬢ , actinomycin D, 3.2×10^{-6} M; ■ , hydroxypyrazole plus actinomycin D.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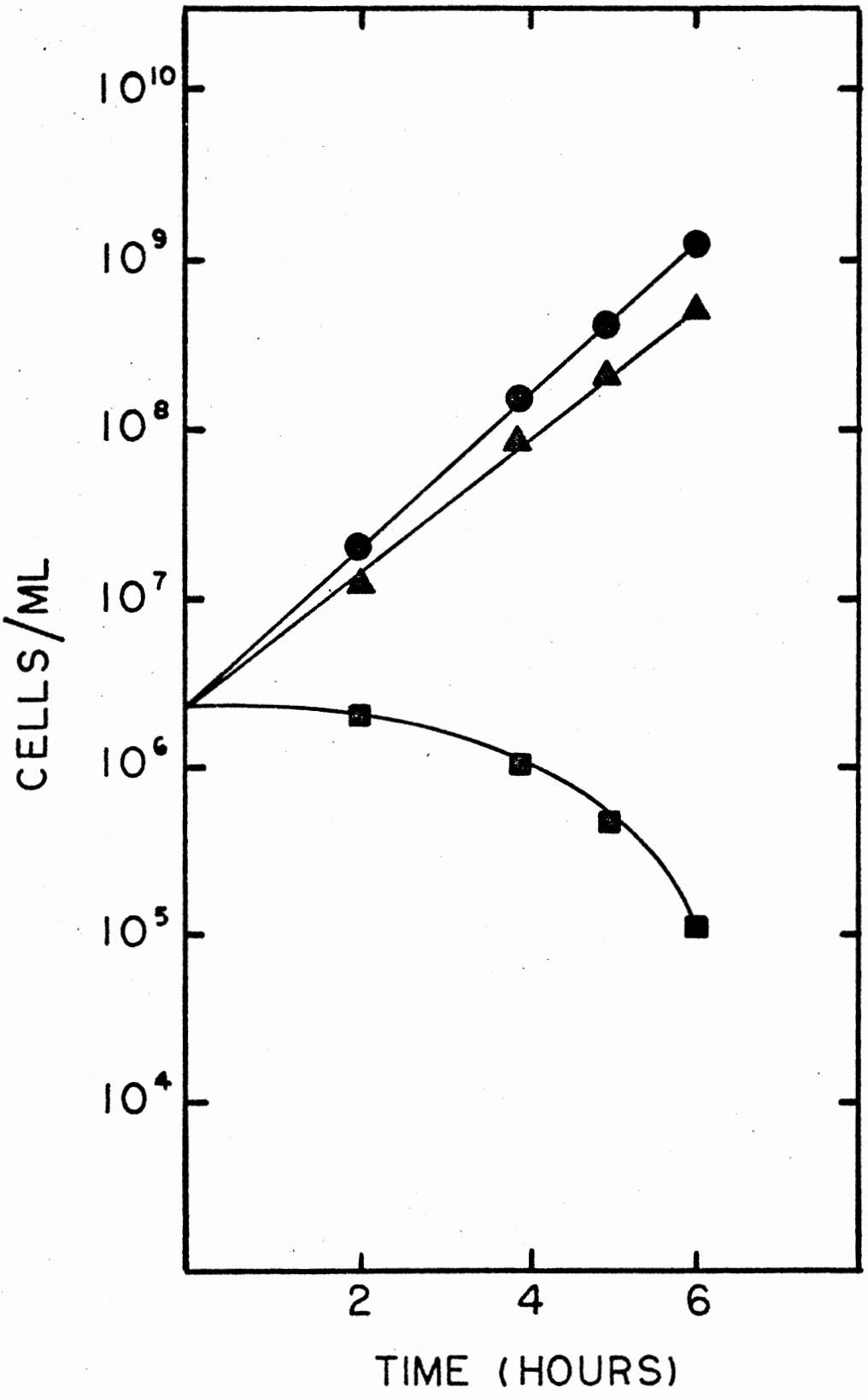
TIME (HOURS)

Figure 8. Effect of the Hydroxypyrazole on Actinomycin D Inhibition of P. fluorescens Cell Viability. ● , succinate control and hydroxypyrazole, 4.2×10^{-5} M; ▲ , actinomycin D, 3.2×10^{-6} M. ■ , hydroxypyrazole plus actinomycin D.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TIME (HOURS)

mechanism(s) by which actinomycin D action is increased. Previous investigation in this laboratory by Haslam (1973) initially provided evidence supporting the complexation of actinomycin D and the hydroxypyrazole. Further evidence from ^{1}H NMR analysis in D_2O (Chestnut et al., 1974), and ^{1}H NMR and ^{13}C NMR analysis in D_3COD (O'Donnell et al., 1978) augment this investigation.

Most attempts to potentiate actinomycin D action have utilized agents such as EDTA, detergents and bacteriophage to create permeability changes in the cell which allow more actinomycin D to diffuse into the cell. Although this type of action cannot be completely ruled out for the hydroxypyrazole, growth experiments have shown that the hydroxypyrazole appears to neither inhibit cell growth or cause aberrant changes in cell size at the concentrations used in this investigation. Furthermore, studies have shown that exposure of P. fluorescens to only the hydroxypyrazole does not result in an increase in actinomycin D action. P. fluorescens was grown for 8 hours in the presence of the hydroxypyrazole, washed twice with minimal salts buffer and resuspended in succinate medium containing actinomycin D. No difference was observed between the cells grown in the presence of the hydroxypyrazole and cells grown in succinate medium when both batches of cells were exposed to actinomycin D. Therefore, it appears from these data that the hydroxypyrazole and actinomycin D must both be present and act together, possibly via a molecular complex, to inhibit growth

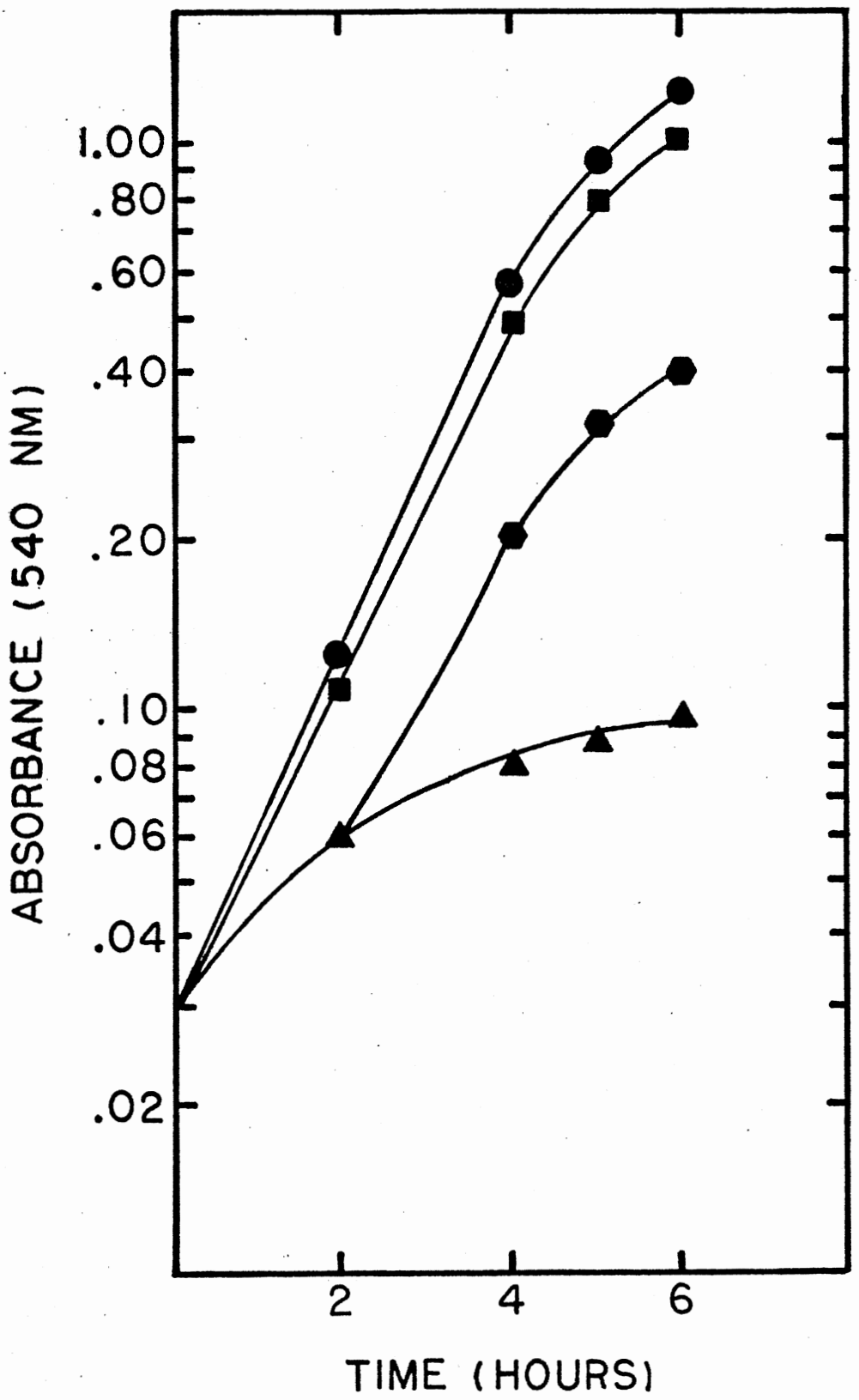
of P. fluorescens.

It is interesting to note that it was also approximately 1-2 hours after exposure to the combination of the hydroxypyrazole and actinomycin D before noticeable inhibition of growth was observed. This was not unlike the inhibition which was observed for a corresponding concentration of actinomycin D.

Prevention and Reversal of Actinomycin D Inhibition by Protocatechuate

Durham and Keudall (1969) observed that protocatechuate could prevent or reverse the inhibition by actinomycin D of P. fluorescens without interfering with the diffusion of ^{14}C-actinomycin D into the cell. Protocatechuate, at a concentration of 6.5×10^{-4} M, was observed to prevent or reverse the inhibition of growth of P. fluorescens resulting from an actinomycin D concentration of 1.6×10^{-5} M (Figure 9). Cell viability studies have shown that increases in viable cell number accompany the increases in absorbance which have been observed for P. fluorescens grown in the presence of protocatechuate and actinomycin D. The specificity that appears to be involved in the ability of the protocatechuate to alleviate actinomycin D action in this organism suggests that molecular complexation between these compounds probably plays an integral role in the mechanism(s) which may be employed. Also, it is important to note that the concentration of protocatechuate which was used in this study did not

Figure 9. Prevention and Reversal of Actinomycin D Inhibition of Growth of P. fluorescens by Protocatechuate. ● , succinate control and protocatechuate, 6.5×10^{-4} M; ▲ , actinomycin D, 1.6×10^{-5} M; ■ , actinomycin D plus protocatechuate added at 0 hours; ⬢ , actinomycin D plus protocatechuate added at 2 hours.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TIME (HOURS)

affect growth of the cells.

Prevention and Reversal of Inhibition Resulting from the Combination of Actinomycin D and the Hydroxypyrazole by Protocatechuate

Experiments were conducted to determine if protocatechuate would prevent or reverse the inhibition resulting from the combination of actinomycin D and the hydroxypyrazole. The results demonstrated that protocatechuate could prevent or reverse the increased inhibition which was observed when the hydroxypyrazole and actinomycin D were added in combination to the medium (Figure 10). The concentrations of protocatechuate, the hydroxypyrazole and actinomycin D were 6.5×10^{-4}, 4.2×10^{-5} and 3.2×10^{-6} M, respectively. The ability of protocatechuate to relieve this inhibition appears to be similar to its capacity to alleviate the inhibition resulting from only actinomycin D. The trends which were observed by following the growing cultures spectrophotometrically were supported by cell viability experiments which showed that prevention and reversal of the inhibition resulting from these two compounds was the consequence of increases in viable cell number (Figure 11). These data suggest that protocatechuate may be interacting with actinomycin D or the proposed hydroxypyrazole:actinomycin D complex to decrease its ability to inhibit the growth of *P. fluorescens*.

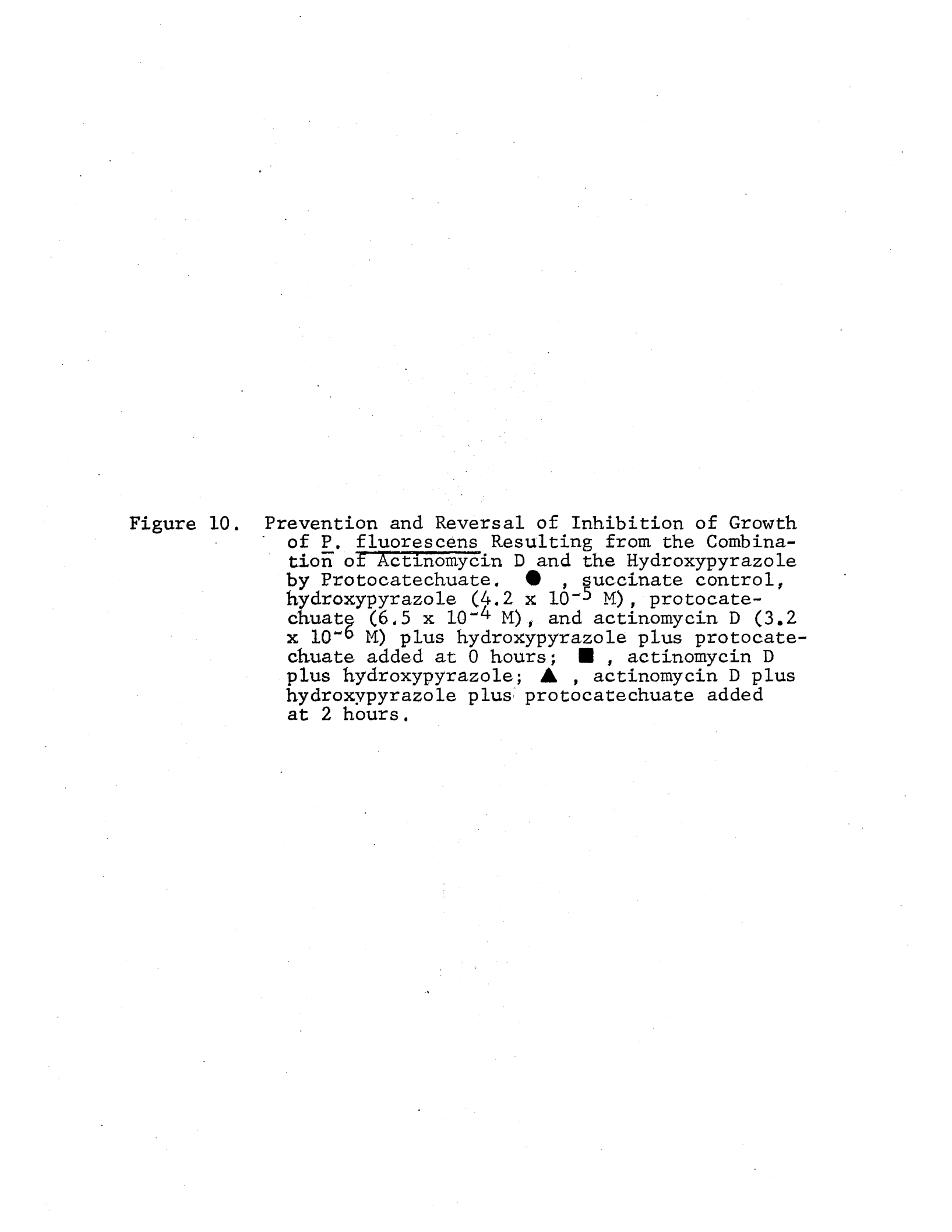
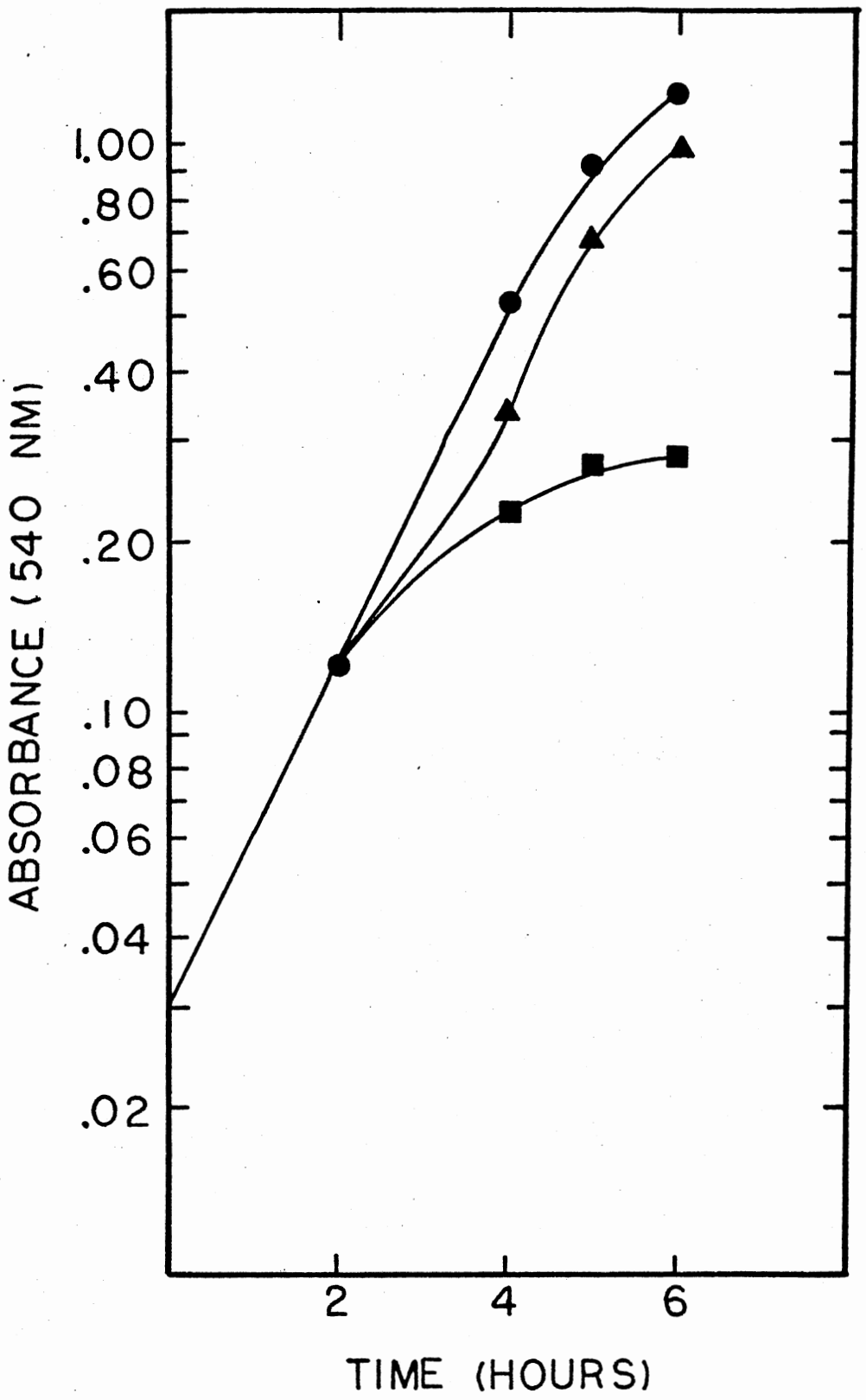


Figure 10. Prevention and Reversal of Inhibition of Growth of P. fluorescens Resulting from the Combination of Actinomycin D and the Hydroxypyrazole by Protocatechuate. ● , succinate control, hydroxypyrazole (4.2×10^{-5} M), protocatechuate (6.5×10^{-4} M), and actinomycin D (3.2×10^{-6} M) plus hydroxypyrazole plus protocatechuate added at 0 hours; ■ , actinomycin D plus hydroxypyrazole; ▲ , actinomycin D plus hydroxypyrazole plus protocatechuate added at 2 hours.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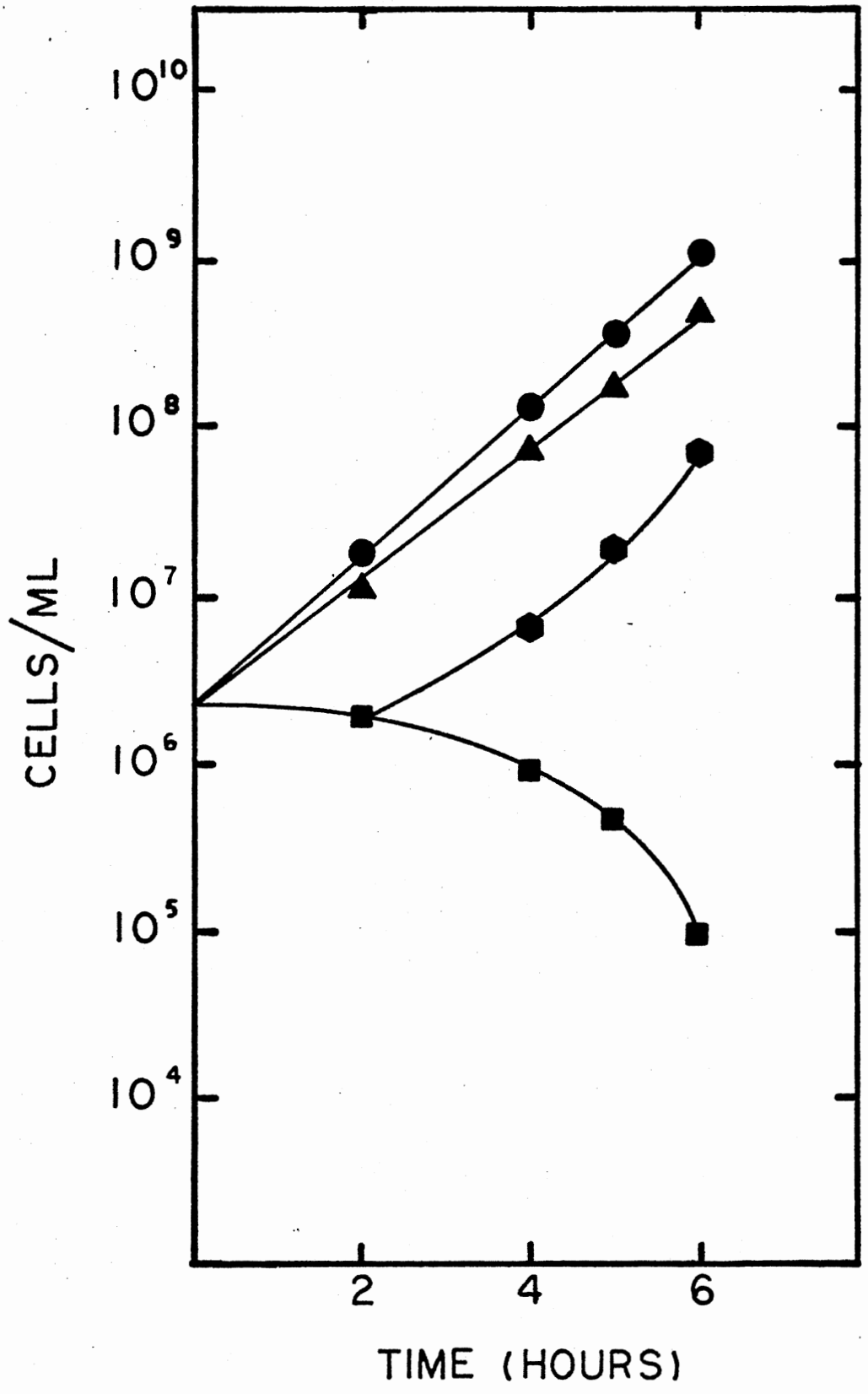
TIME (HOURS)

Figure 11. Prevention and Reversal of Inhibition of P. fluorescens Cell Viability Resulting from the Combination of Actinomycin D and the Hydroxypyrazole by Protocatechuate. ● , succinate control, hydroxypyrazole (4.2×10^{-5} M), protocatechuate (6.5×10^{-4} M), and actinomycin D plus hydroxypyrazole plus protocatechuate added at 0 hours; ▲ , actinomycin D (3.2×10^{-6} M); ■ , actinomycin D plus hydroxypyrazole; ⬢ , actinomycin D plus hydroxypyrazole plus protocatechuate added at 2 hours.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CELLS/ML
2
4
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TIME (HOURS)

Influence of the Hydroxypyrazole and Protocatechuate on Actinomycin D Inhibition of Amidase Synthesis by *P. fluorescens*

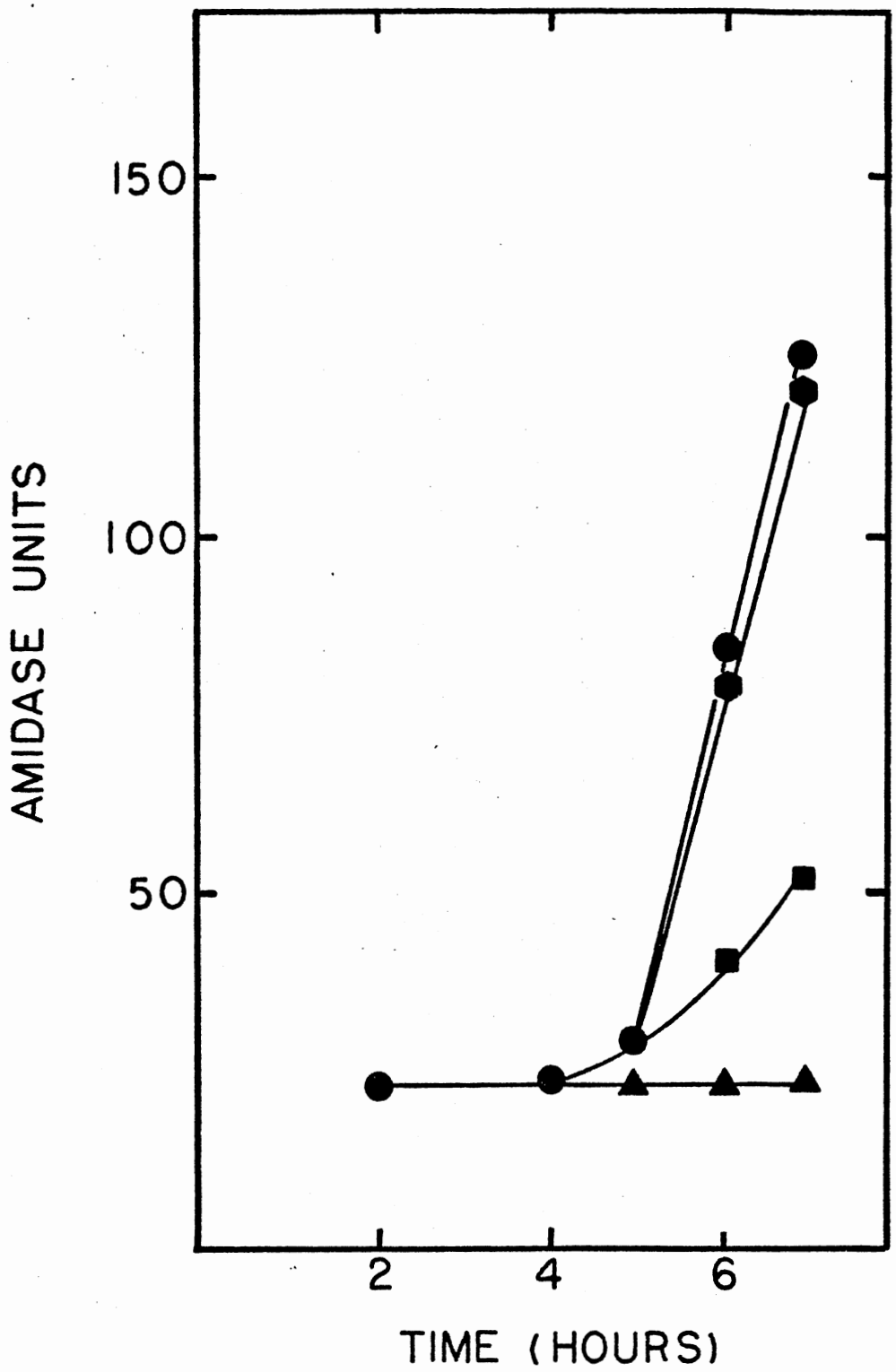
Effect of Actinomycin D on Amidase Synthesis

Actinomycin D inhibition of amidase synthesis was comparable to actinomycin D inhibition of growth in this organism since inhibition of both amidase synthesis and growth are concentration dependent. However, amidase synthesis is about 10 times more sensitive to the antibiotic. Actinomycin D at concentrations of 3.2×10^{-7} and 3.2×10^{-6} M inhibited amidase synthesis by approximately 5% and 100%, respectively (Figure 12). Thus, the monitoring of amidase synthesis provides a more sensitive and direct method for determining changes in actinomycin D action.

Effect of the Hydroxypyrazole on Amidase Synthesis

Several concentrations of the hydroxypyrazole were tested to determine if this compound could inhibit amidase synthesis by *P. fluorescens*. No inhibition of amidase synthesis was observed; even at concentrations as high as 1.3×10^{-4} M.

Figure 12. Effect of Actinomycin D on Amidase Synthesis. ● , succinate plus acetamide control; ⬢ , actinomycin D, 3.2×10^{-7} M; ■ , actinomycin D, 1.6×10^{-6} M; ▲ , actinomycin D, 3.2×10^{-6} M.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AMIDASE UNITS
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TIME (HOURS)

Effect of the Hydroxypyrazole on Actinomycin D Inhibition of Amidase Synthesis

The inhibition of amidase synthesis by actinomycin D, at a concentration of 3.2×10^{-7} M, was increased by nearly 50% when the hydroxypyrazole, at a concentration of 4.2×10^{-5} M, was added in combination to the medium (Figure 13). These results agree with those of Blunk (1977), although it was determined in this investigation that the hydroxypyrazole concentration required to achieve this degree of inhibition was one-half the amount which was previously reported. The increase in actinomycin D inhibition of amidase synthesis when the hydroxypyrazole was present in the medium provides further evidence that a molecular interaction between the hydroxypyrazole and actinomycin D may function to increase actinomycin D action. Regardless of the mechanism(s) involved, an important circumstance which results from growth in the presence of the hydroxypyrazole and actinomycin D is a decrease in protein synthesis.

Effect of the Hydroxypyrazole and Actinomycin D on Amidase Activity

In order to eliminate the possibility that the hydroxypyrazole, actinomycin D or combinations of these compounds inactivate the amidase enzyme rather than its synthesis, *in vitro* investigations were performed with amidase which was isolated from *P. fluorescens*. Amidase is a hexameric protein

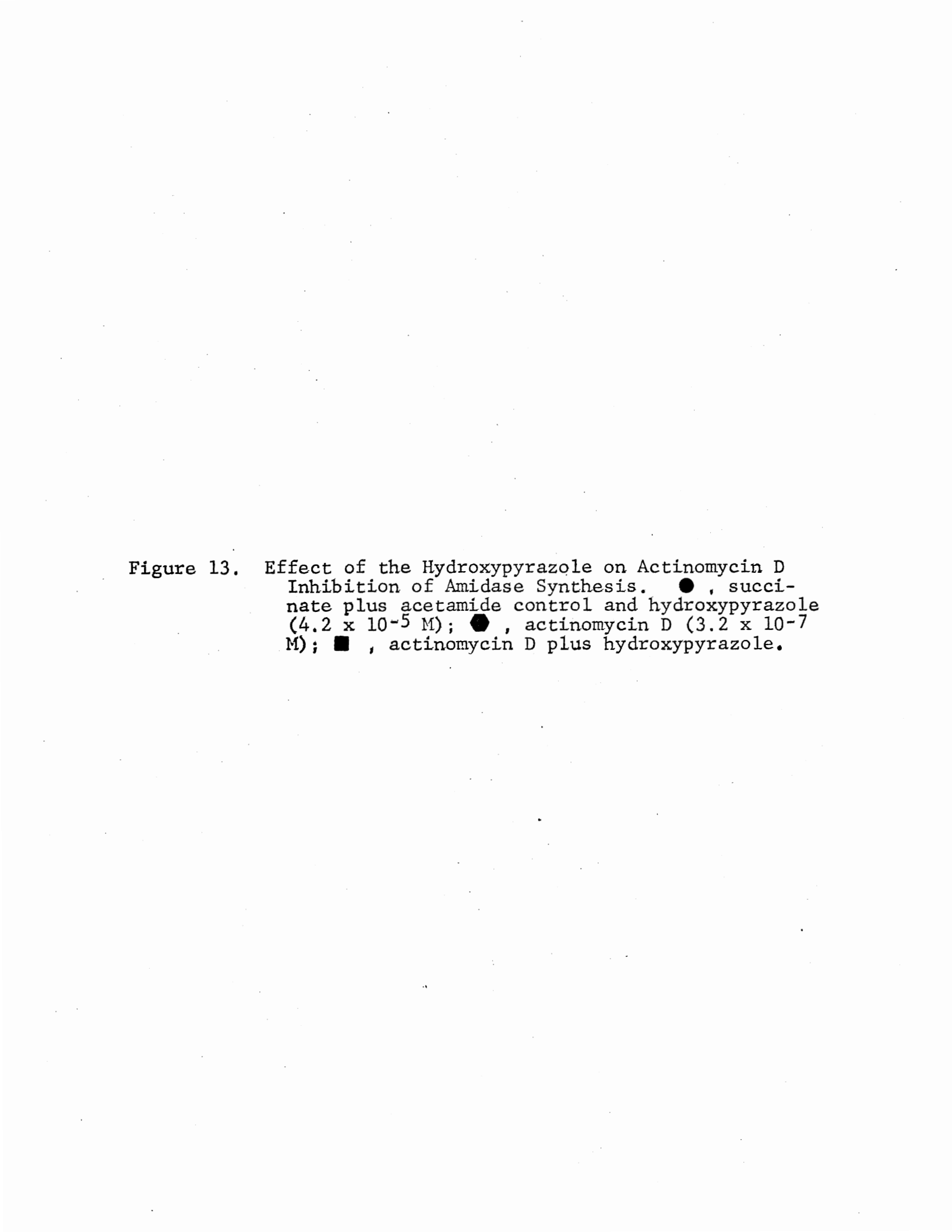
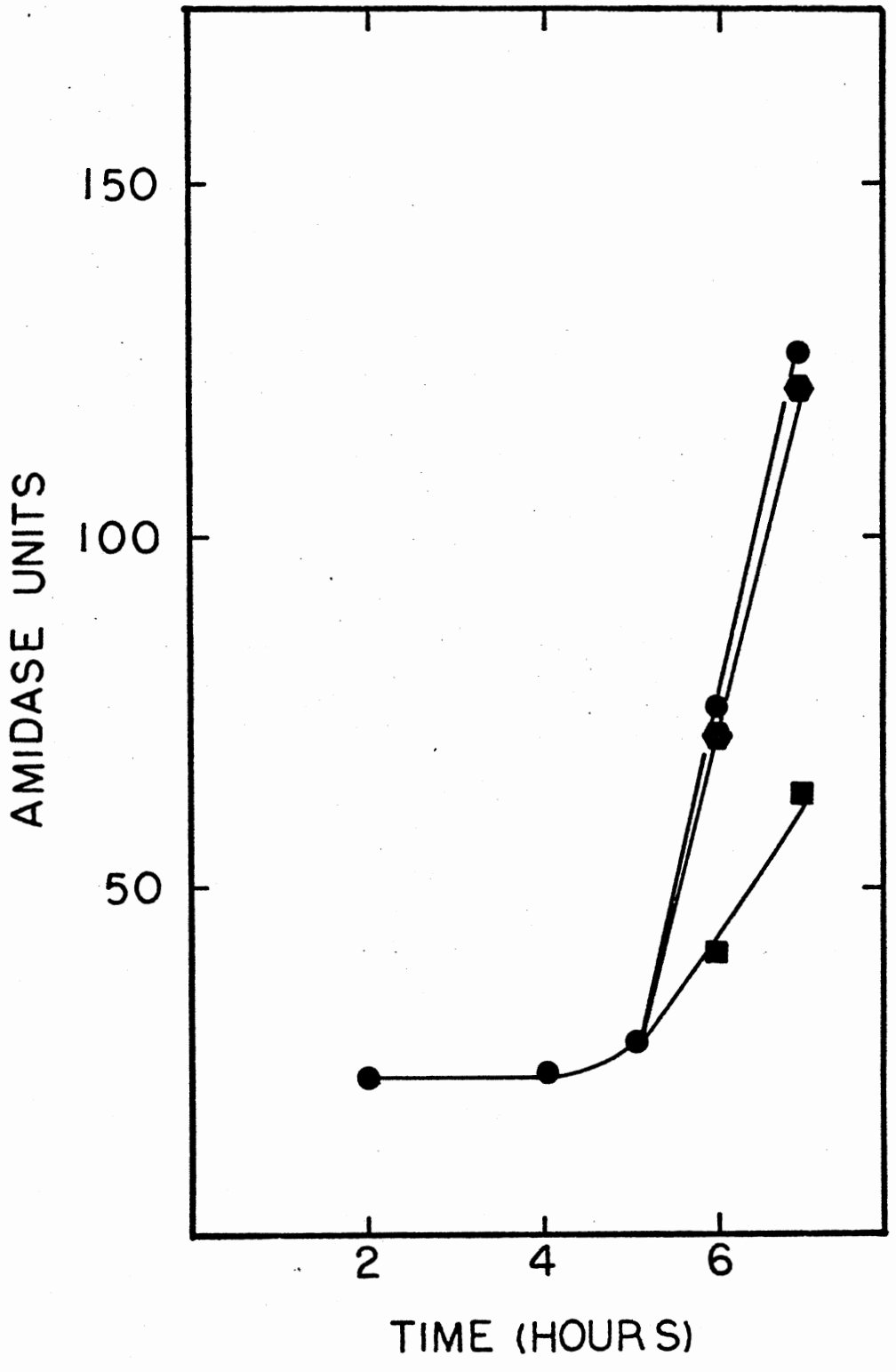


Figure 13. Effect of the Hydroxypyrazole on Actinomycin D Inhibition of Amidase Synthesis. ● , succinate plus acetamide control and hydroxypyrazole (4.2×10^{-5} M); ⬢ , actinomycin D (3.2×10^{-7} M); ■ , actinomycin D plus hydroxypyrazole.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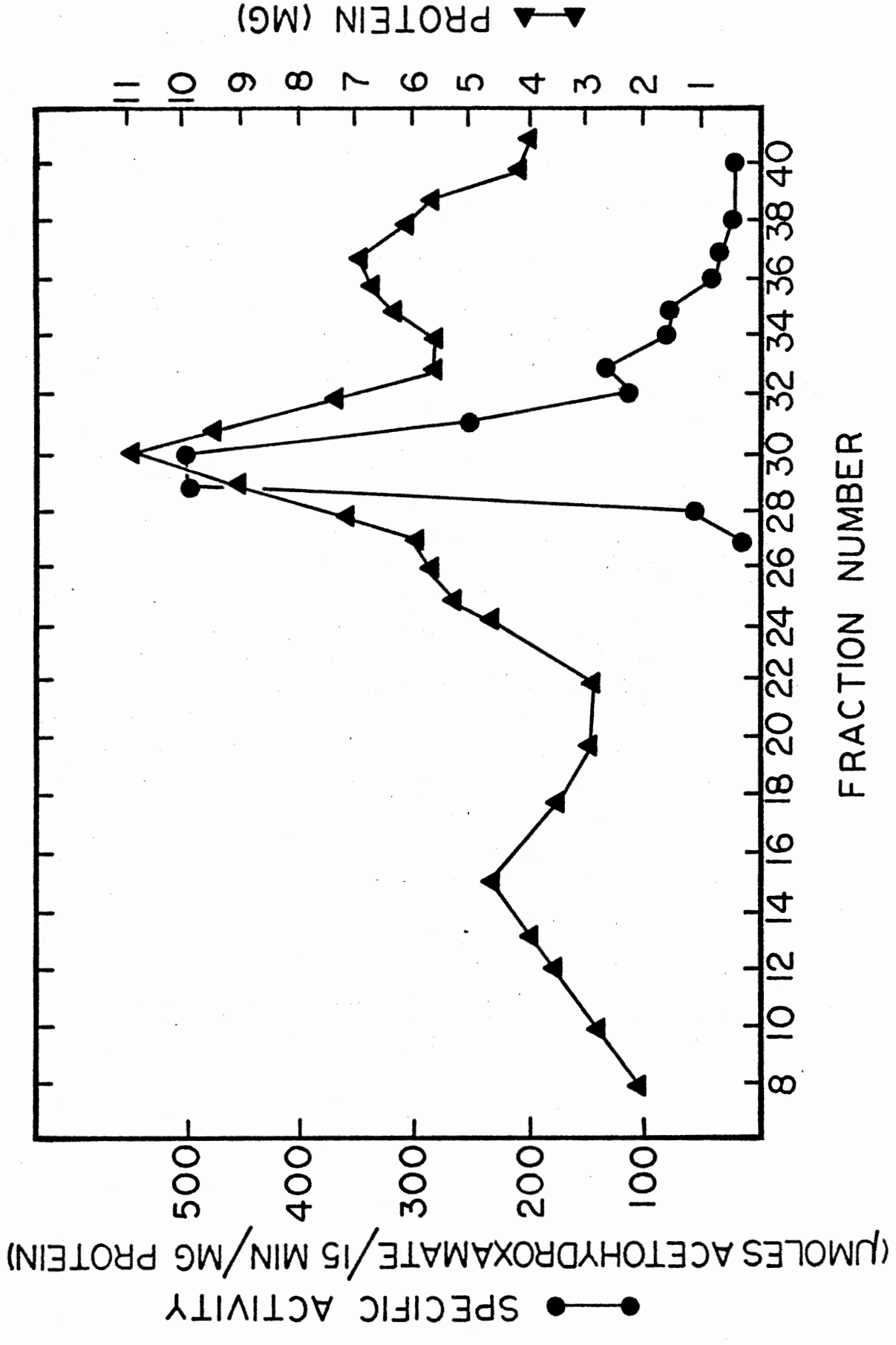
TIME (HOURS)

with identical subunits. The molecular weight of the oligomer was found to be 200,000 daltons while the subunits have molecular weights between 33,000 and 35,000 daltons (Brown et al., 1973).

The method of Kelly and Kornberg (1964) was used to isolate and purify amidase. Ultrasonic extracts of P. fluorescens were prepared and treated with deoxyribonuclease I before ammonium sulfate precipitation was used to recover the bulk of the protein content. After extensive dialysis, the dialyzed protein was applied to a DEAE-cellulose column and a phosphate gradient was used to elute the amidase from the DEAE-cellulose. Fractions (10 ml) were collected and analyzed for amidase activity and protein content (Figure 14). Most of the amidase activity was eluted between 40 and 80 mM phosphate. Fractions comprising the central portion of the peak of enzyme activity were combined and precipitated by addition of ammonium sulfate. The resulting slurry was stored at -20 C and prepared for in vitro investigation by dialysis to remove ammonium sulfate.

Experiments were designed to study the effect of the hydroxypyrazole, actinomycin D or combinations of these compounds on amidase activity. Amidase was added to tubes containing the appropriate compounds to yield a protein content of 0.233 mg per ml. The tubes were incubated at 37 C and 0.25 ml aliquots were removed at the designated time intervals. These aliquots were immediately assayed to determine enzyme activity. None of the concentrations or

Figure 14. Elution of Amidase of P. fluorescens from DEAE-Cellulose. ●—● , specific activity; ▲—▲ , protein.


PROTEIN (MG)
1 2 3 4 5 6 7 8 9 10 11
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
FRACTION NUMBER
100 200 300 400 500
SPECIFIC ACTIVITY
(UMOLES ACETOHYDROXAMATE/15 MIN/MG PROTEIN)

combinations of the hydroxypyrazole and actinomycin D affected amidase activity over this time-course (Table I). Identical experiments utilizing either higher or lower concentrations of amidase provided the same results. It is important to note that the concentrations of the hydroxypyrazole and actinomycin D (4.2×10^{-5} and 3.2×10^{-7} M respectively) which were responsible for the increase in actinomycin D inhibition of amidase synthesis (Figure 13) had no effect on amidase activity. Also, actinomycin D at a concentration of 3.2×10^{-6} M had no effect even though this concentration completely inhibited amidase synthesis. In addition, the proposed complex of the hydroxypyrazole and actinomycin D did not inactivate amidase since neither of the tubes containing 1:1 molar ratios of these compounds at 3.2×10^{-7} and 3.2×10^{-6} differed from the control.

Prevention and Reversal of Actinomycin D Inhibition of Amidase Synthesis by Protocatechuate

Prevention and reversal of actinomycin D inhibition of amidase synthesis by protocatechuate was first reported by Ferguson (1970). A study by Durham and Ferguson (1971) of selected benzoic acid derivatives reported that protocatechuate exhibited the greatest potential for mitigating actinomycin D inhibition of amidase synthesis. Protocatechuate at a concentration of 6.5×10^{-4} M can completely prevent and almost totally reverse the inhibition of amidase synthesis

TABLE I

EFFECT OF THE HYDROXYPYRAZOLE
AND ACTINOMYCIN D ON
AMIDASE ACTIVITY

Concentration of Compounds (Molarity)		Specific Activity umoles acetohydroxamate produced / 15 minutes/mg protein			
Actinomycin D	Hydroxypyrazole	0.0 hrs	1.0 hrs	2.0 hrs	3.0 hrs
0.0	0.0	3128	3117	3154	3138
3.2×10^{-7}	0.0	3117	3092	3097	3092
0.0	3.2×10^{-7}	3154	3143	3159	3164
0.0	3.2×10^{-6}	3148	3179	3169	3190
0.0	4.2×10^{-5}	3138	3097	3128	3128
3.2×10^{-7}	3.2×10^{-7}	3117	3169	3148	3128
3.2×10^{-7}	4.2×10^{-5}	3092	3086	3138	3086
3.2×10^{-6}	0.0	3117	3117	3107	3148
0.0	3.2×10^{-6}	3169	3092	3174	3107
0.0	4.2×10^{-5}	3076	3128	3117	3097
3.2×10^{-6}	3.2×10^{-6}	3107	3169	3148	3148
3.2×10^{-6}	4.2×10^{-5}	3154	3138	3128	3097

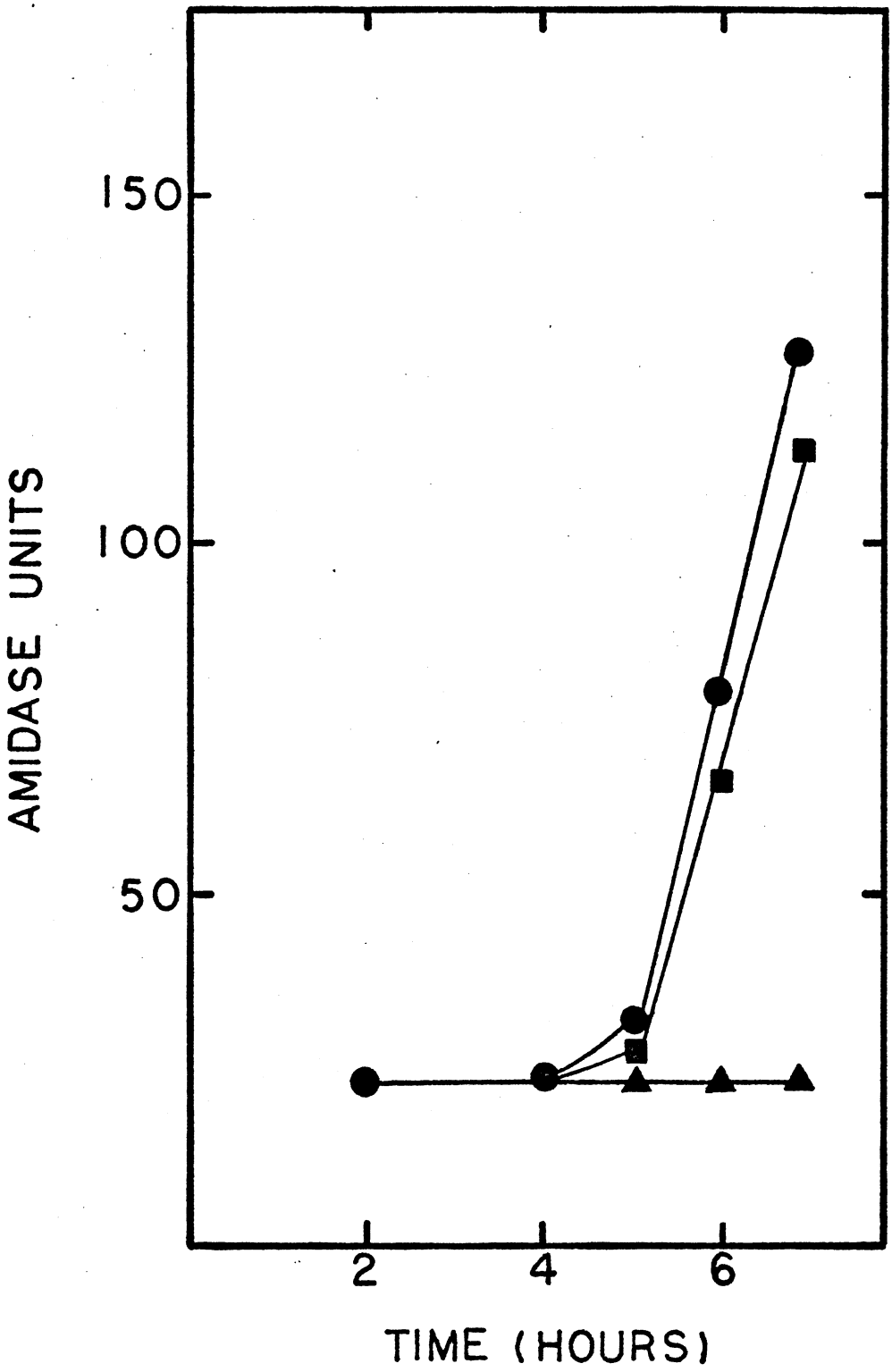
*The amidase concentration was 0.233 mg/ml.

resulting from 3.2×10^{-6} M actinomycin D (Figure 15). The ability of protocatechuate to relieve the inhibition of amidase synthesis by a concentration of actinomycin D which produced 100% inhibition provides additional support that the efficacy of this compound to diminish actinomycin D action may involve molecular interaction between these compounds.

Prevention and Reversal of Inhibition of Amidase Synthesis Resulting from the Combination of Actinomycin D and the Hydroxypyrazole by Protocatechuate

In a manner similar to that observed for growth inhibition of P. fluorescens, protocatechuate (6.5×10^{-4} M) prevents and reverses the inhibition of amidase synthesis resulting from the combination of the hydroxypyrazole (4.2×10^{-5} M) and actinomycin D (3.2×10^{-7} M) (Figure 16). These data confirm the earlier report by Blunk (1977) even though slightly different concentrations of the hydroxypyrazole and protocatechuate were used in this investigation. Therefore, it appears that an important element of protocatechuate's circumvention of either actinomycin D, or actinomycin D and hydroxypyrazole action may involve molecular complexations.

Figure 15. Prevention and Reversal of Actinomycin D Inhibition of Amidase Synthesis by Protocatechuate. ● , succinate plus acetamide control, protocatechuate (6.5×10^{-4} M), and actinomycin D (3.2×10^{-6} M) plus protocatechuate added at 0 hours; ▲ , actinomycin D; ■ , actinomycin D plus protocatechuate added at 2 hours.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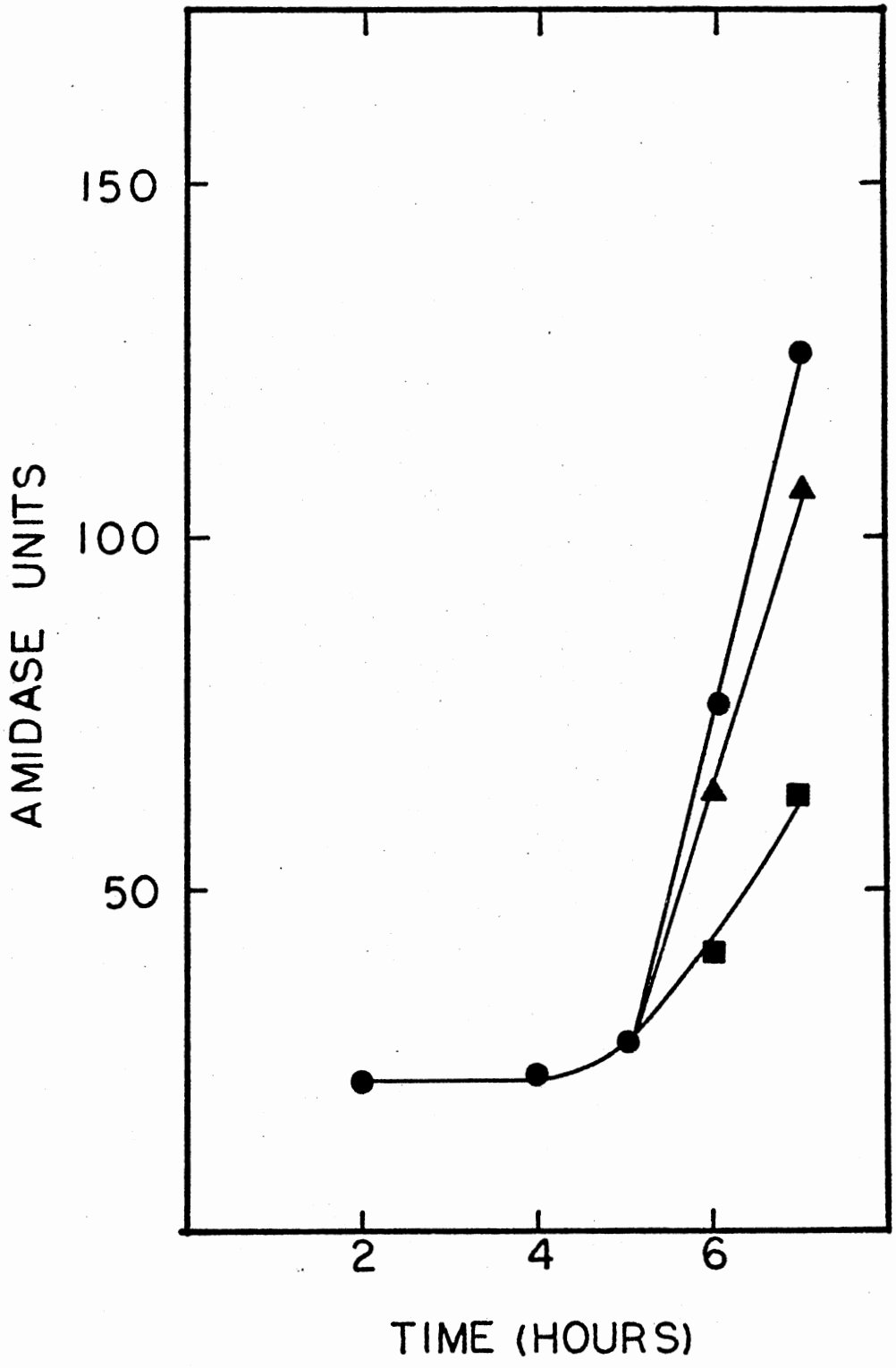
TIME (HOURS)

Figure 16. Prevention and Reversal of Inhibition of Amidase Synthesis Resulting from the Combination of Actinomycin D and the Hydroxypyrazole by Protocatechuate. ● , succinate plus acetamide control, hydroxypyrazole (4.2×10^{-5} M), protocatechuate (6.5×10^{-4} M), and actinomycin D (3.2×10^{-7} M) plus protocatechuate added at 0 hours; ■ , actinomycin D plus hydroxypyrazole; ▲ , actinomycin D plus hydroxypyrazole plus protocatechuate added at 2 hours.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TIME (HOURS)

Influence of the Hydroxypyrazole and Protocatechuate on Actinomycin D Inhibition of the RNA Polymerase Reaction

Actinomycin D binds to DNA and subsequently inhibits RNA chain elongation by affecting the rates at which CTP and GTP are incorporated (Hyman and Davidson, 1970). On the macroscopic level, the overall incorporation of the four ribonucleoside 5'-triphosphates is decreased equally. Transcription still proceeds linearly along the template, so that the base composition of the RNA is complementary to the transcribing strand. The steps at which CTP and GTP are incorporated are slowed down while the steps at which ATP and UTP are incorporated are not. A precise mechanism accounting for this inhibition has not been elucidated. In vitro RNA polymerase assays were designed and conducted to study the influence of the hydroxypyrazole and protocatechuate on actinomycin D inhibition of this reaction because it is the primary biological site of action for actinomycin D. In addition, the data from the amidase investigations have revealed that both the hydroxypyrazole and protocatechuate function to modulate actinomycin D inhibition of amidase synthesis. The sensitivity of the RNA polymerase reaction to actinomycin D provides a useful system for detecting subtle differences in actinomycin D binding.

Effect of Actinomycin D on the RNA Polymerase Reaction

Actinomycin D does not bind to the RNA polymerase, or interfere with its binding to the DNA template and subsequent initiation of RNA synthesis. For a given concentration of DNA, the actinomycin D inhibition of the RNA polymerase reaction is concentration dependent. This was observed in the course of this investigation. Utilizing the standard assay, increasing concentrations of actinomycin D were added to reaction mixtures containing 30 µg of calf thymus DNA (Figure 17). Very small increases in the concentration of actinomycin D resulted in very large increases in inhibition. This behavior was observed until approximately 70% inhibition was achieved, whereupon the degree of inhibition was only slightly increased with increasing concentrations of actinomycin D. Regardless of the actinomycin D concentration, some RNA was always synthesized.

Effect of the Hydroxypyrazole on the RNA Polymerase Reaction

The hydroxypyrazole was observed to inhibit the RNA polymerase reaction when assayed utilizing the same assay conditions as were used for actinomycin D (Figure 18). This result was not unexpected since Blunk (1977) demonstrated that the hydroxypyrazole binds to DNA to stabilize the DNA helix and shift the melting curve to higher temperatures. However, the concentration of the hydroxypyrazole which was

Figure 17. Effect of Actinomycin D on the RNA Polymerase Reaction. ● , actinomycin D.

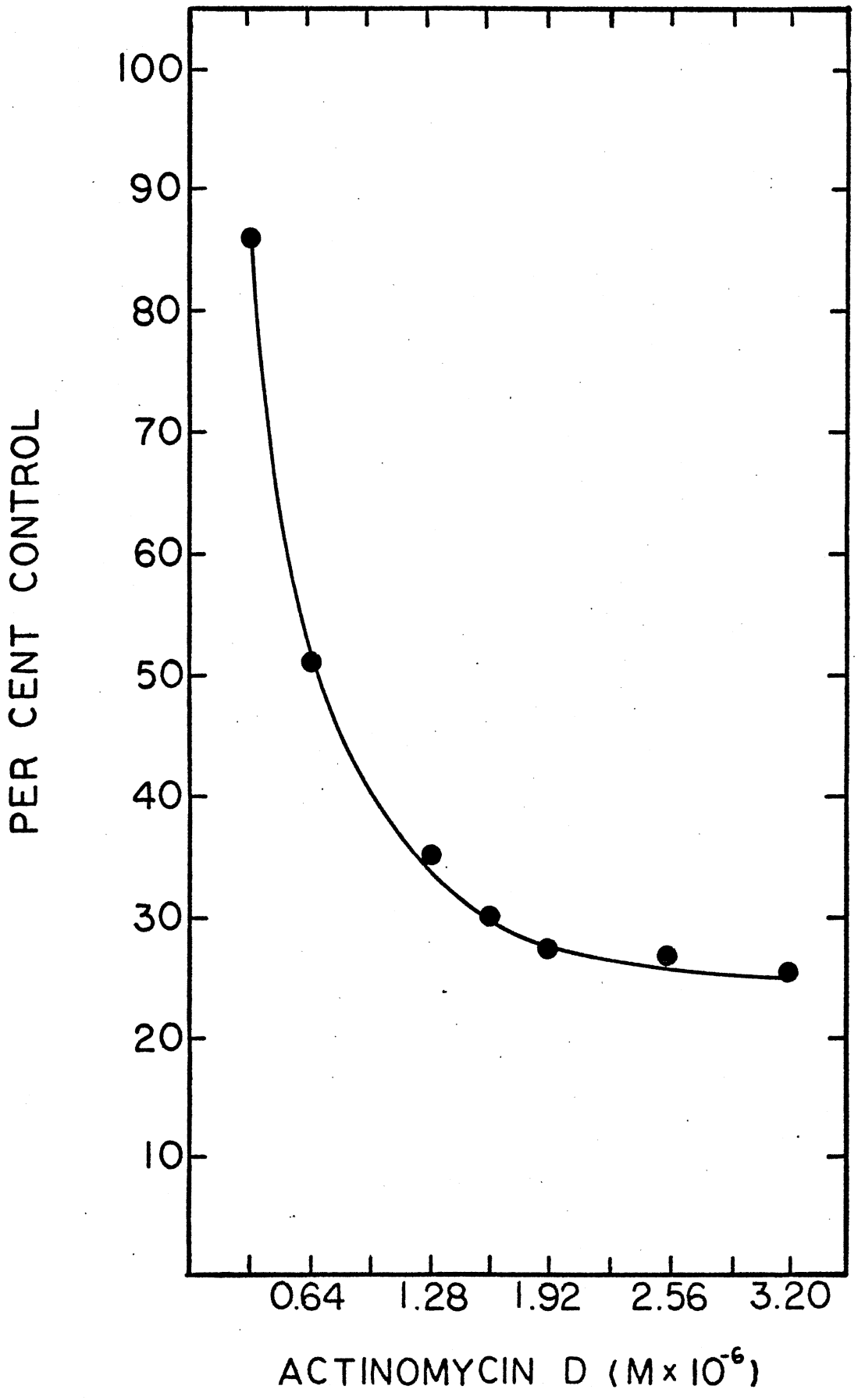
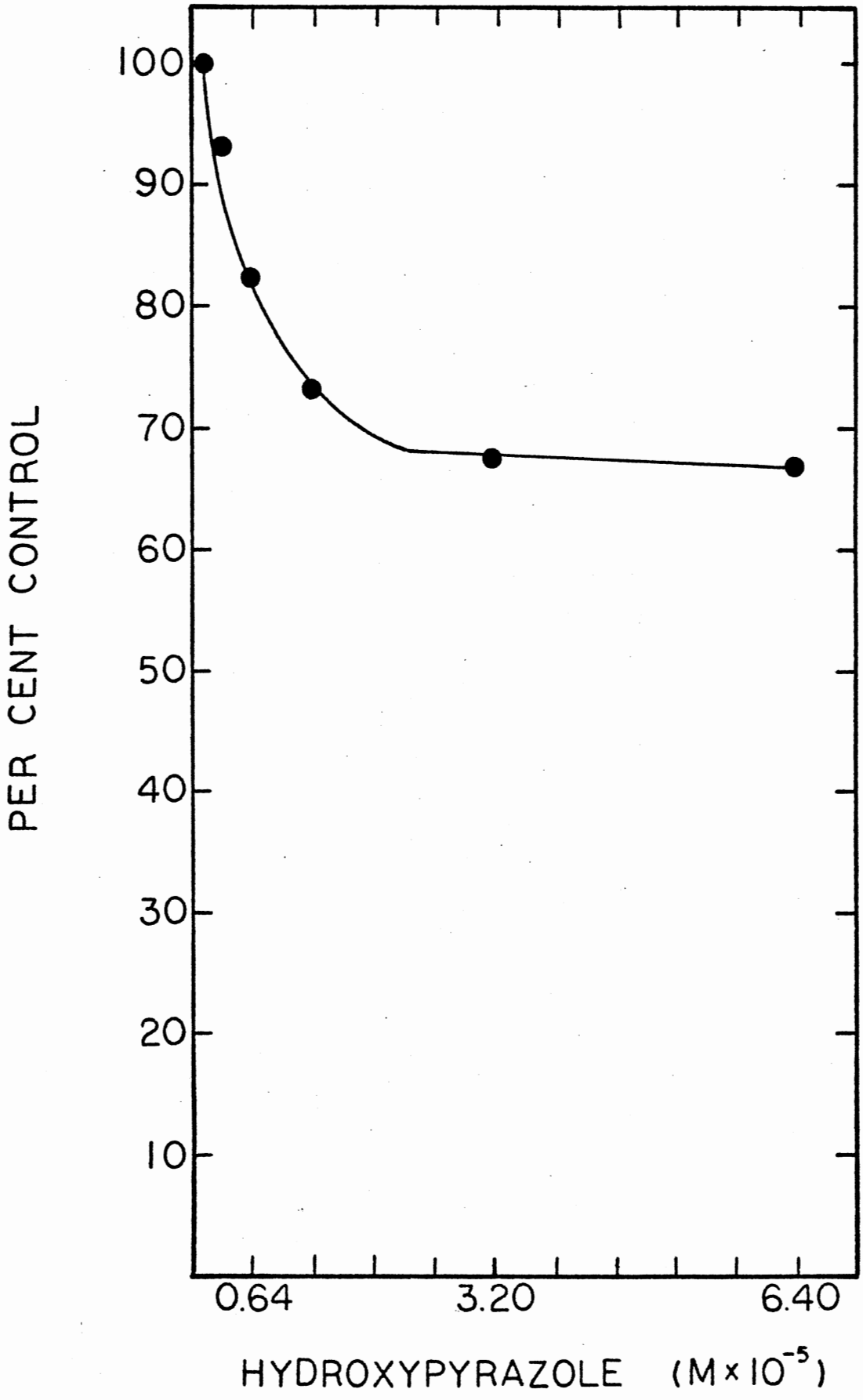

PER CENT CONTROL
100
90
80
70
60
50
40
30
20
10
0.64
1.28
1.92
2.56
3.20
ACTINOMYCIN D (M x 10⁻⁶)

Figure 18. Effect of the Hydroxypyrazole on the RNA Polymerase Reaction. ● , hydroxypyrazole.


100
90
80
70
60
50
40
30
20
10
PER CENT CONTROL
0.64
3.20
6.40
HYDROXYPYRAZOLE (M × 10⁻⁵)

necessary to establish inhibition was more than 10 fold greater than the corresponding concentration of actinomycin D. Moreover, only 30-35% inhibition was achieved with higher concentrations of the hydroxypyrazole.

The mechanism by which the hydroxypyrazole binds to DNA and inhibits the RNA polymerase reaction is not known. It is of interest that the steroidal diamine, irehdiamine A, also stabilizes DNA from thermal denaturation at low concentrations. Sobell (1973) reported that irehdiamine A may bind to DNA by an intercalative mechanism. In an intercalative model, the steroid nucleus fits between base pairs in the DNA helix resulting in local denaturation of base pairs immediately above and below the intercalation site. The binding may be stabilized by electrostatic interactions between the protonated amino groups of irehdiamine A and opposite DNA chains. Although the hydroxypyrazole has no protonated amino groups to stabilize its binding, an intercalative binding mechanism may explain its unusual effects on RNA synthesis.

A unique observation was noted when the hydroxypyrazole concentration was decreased to 3.2×10^{-7} M. This concentration, which was 10 fold less than the concentration required to establish inhibition, produced a significant stimulation of the RNA polymerase reaction. The rationality of this stimulation will be discussed in following sections.

Effect of the Hydroxypyrazole on Actinomycin D Inhibition of the RNA Polymerase Reaction

The effect of a 1:1 molar ratio (3.2×10^{-7} M) of the hydroxypyrazole and actinomycin D on RNA synthesis was investigated using the standard assay method. This combination of the hydroxypyrazole and actinomycin D produced approximately 21% more inhibition than the actinomycin D control when calf thymus DNA was used as the template (Table II). As was mentioned in the previous section, the hydroxypyrazole stimulated total RNA synthesis by nearly 13%. A time-course experiment demonstrated that both the hydroxypyrazole and complete controls synthesized RNA at a linear rate over this time period (Figure 19). These results also showed that both the actinomycin D control and the combination of the hydroxypyrazole and actinomycin D synthesized RNA at approximately the same rate as the complete control for the first few minutes of the reaction before declining with time.

Similar results were obtained when DNA isolated from *P. fluorescens* was used as the template (Table III). Therefore, the increase in actinomycin D inhibition of RNA synthesis by the hydroxypyrazole is not limited to one template. However, *P. fluorescens* DNA appears to be less efficient as a template than calf thymus DNA. This dissimilarity is probably the result of preparation rather than differences in base composition.

TABLE II

EFFECT OF THE HYDROXYPYRAZOLE ON ACTINOMYCIN D INHIBITION OF THE RNA POLYMERASE REACTION WHEN CALF THYMUS DNA WAS THE TEMPLATE

System	UMP Incorporation	
	pmoles	% Control
Complete	238.41	100.00
Plus Hydroxypyrazole	269.37	112.99
Plus Actinomycin D	202.96	85.13
Plus Hydroxypyrazole and Actinomycin D	151.92	63.72
Minus RNA Polymerase	1.80	0.76
Minus DNA	3.73	1.56

* A 1:1 molar ratio (3.2×10^{-7} M) of the hydroxypyrazole and actinomycin D was used in this investigation in which the results were determined by the standard assay method.

Figure 19. Time-Course: Effect of the Hydroxypyrazole on Actinomycin D Inhibition of the RNA Polymerase Reaction when Calf Thymus DNA Was the Template. ● , complete control; ⬢ , hydroxypyrazole, 3.2×10^{-7} M; ■ , actinomycin D, 3.2×10^{-7} M; ▲ , actinomycin D plus hydroxypyrazole.

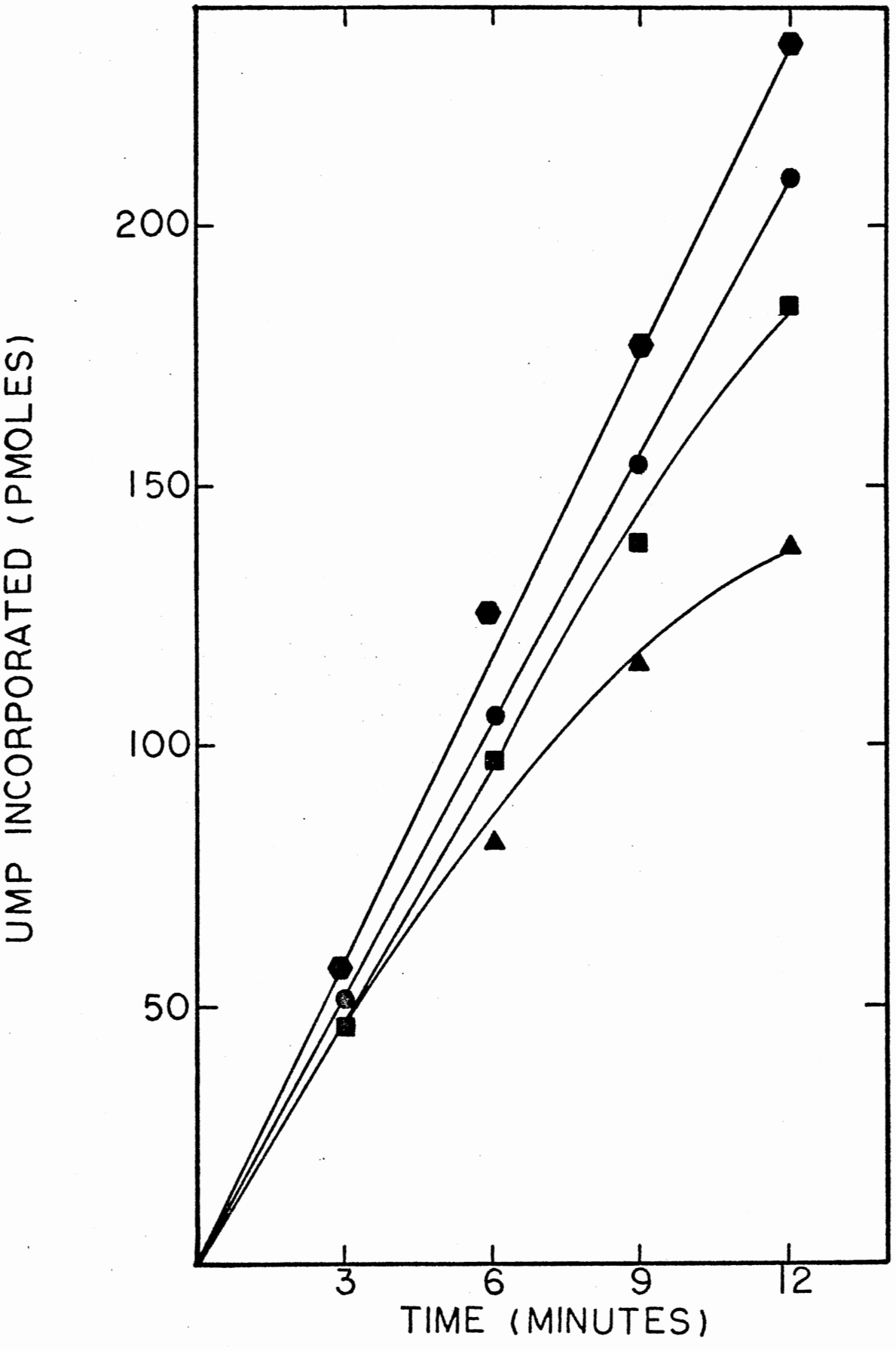

UMP INCORPORATED (PMOLES)
200
150
100
50
3
6
9
12
TIME (MINUTES)

TABLE III

EFFECT OF THE HYDROXYPYRAZOLE ON ACTINOMYCIN D INHIBITION OF THE RNA POLYMERASE WHEN *P. FLUORESCENS* DNA WAS THE TEMPLATE

System	UMP Incorporation pmoles	% Control
Complete	168.33	100.00
Plus Hydroxypyrazole	186.27	110.66
Plus Actinomycin D	146.63	87.11
Plus Hydroxypyrazole and Actinomycin D	116.91	69.45
Minus RNA Polymerase	0.92	0.55
Minus DNA	2.49	1.48

* A 1:1 molar ratio (3.2×10^{-7} M) of the hydroxypyrazole and actinomycin D was used in this investigation in which the results were determined by the standard assay method.

Effect of the Hydroxypyrazole on Actinomycin D Inhibition of RNA Chain Elongation

In order to study the effect of the hydroxypyrazole on actinomycin D inhibition of RNA chain elongation, experiments were performed using the high salt method of Hyman and Davidson (1970). Under these conditions, the 1:1 molar ratio (3.2×10^{-7} M) of the hydroxypyrazole and actinomycin D inhibited RNA chain elongation by an amount which was more than 15% greater than the actinomycin D control (Table IV). This suggests that the major effect of the hydroxypyrazole on actinomycin D action is at the level of RNA chain elongation. Of importance, is the observation that the hydroxypyrazole control exhibited no stimulation. Thus, the hydroxypyrazole most likely stimulates RNA synthesis by affecting template site selection and activation by the RNA polymerase holoenzyme.

Effects of Time-Course Addition on the Increase in Actinomycin D Inhibition of the RNA Polymerase Reaction by the Hydroxypyrazole

Time-course addition of the hydroxypyrazole and actinomycin D was investigated to determine if the hydroxypyrazole acts alone to increase actinomycin D inhibition of the RNA polymerase reaction, or if the proposed complex of the hydroxypyrazole and actinomycin D is responsible for this

TABLE IV

EFFECT OF THE HYDROXYPYRAZOLE ON ACTINOMYCIN D INHIBITION OF RNA CHAIN ELONGATION WHEN CALF THYMUS DNA WAS THE TEMPLATE

System	UMP Incorporation	
	pmoles	% Control
Complete	89.77	100.00
Plus Hydroxypyrazole	90.89	101.25
Plus Actinomycin D	74.95	83.49
Plus Hydroxypyrazole and Actinomycin D	61.27	68.25
Minus RNA Polymerase	1.15	1.28
Minus DNA	0.83	0.92

* A 1:1 molar ratio (3.2×10^{-7} M) of the hydroxypyrazole and actinomycin D was used in this investigation in which the results were determined by the RNA chain elongation assay.

action. The results reveal that the inhibition of actinomycin D was increased the greatest when the hydroxypyrazole was added first to the reaction mixture (Table V). When the actinomycin D was added first, a significant but slightly smaller increase was observed. Quite surprisingly, a 1:1 molar ratio (3.2×10^{-7} M) which had been preincubated for 15 minutes at 37 C failed to inhibit RNA synthesis when this mixture was added immediately before CTP was added to start the reaction. However, if this preincubated mixture was added to the reaction mixture and incubation was continued for another 15 minutes at 37 C before CTP was added to start the reaction, an increase in inhibition was observed which was comparable to the increase demonstrated by the addition of the hydroxypyrazole first. Therefore, it appears that the action of the proposed hydroxypyrazole:actinomycin D complex, which is readily formed in an aqueous environment, may not be completely responsible for the increase observed in actinomycin D inhibition of the RNA polymerase reaction. Two alternatives may be used to explain this. One possibility is that conformational changes may be required in the hydroxypyrazole:actinomycin D complex or the DNA before the hydroxypyrazole:actinomycin D complex can successfully bind to the DNA and inhibit RNA synthesis. For example, the unreduced quinoidal phenoxazone ring system of actinomycin D has an integral role in both the DNA:actinomycin D and hydroxypyrazole:actinomycin D models as they are presently depicted. Thus, it is reasonable to assume that some

TABLE V

EFFECTS OF TIME-COURSE ADDITION ON THE INCREASE IN ACTINOMYCIN D INHIBITION OF THE RNA POLYMERASE REACTION BY THE HYDROXYPYRAZOLE

System	UMP Incorporation pmoles	% Control
Complete	275.30	100.00
Plus Hydroxypyrazole	312.66	113.57
Plus Actinomycin D	234.39	85.14
Plus Hydroxypyrazole and Actinomycin D Added Simultaneously	188.64	68.52
Plus Hydroxypyrazole; Actinomycin D Added After Incubation for 15 Minutes at 37 C	175.48	63.74
Plus Actinomycin D; Hydroxypyrazole Added After Incubation for 15 Minutes at 37 C	199.26	72.38
Plus Hydroxypyrazole and Actinomycin D Which Have Been Preincubated for 15 Minutes at 37 C and Added just Prior to CTP	284.32	103.27
Plus Hydroxypyrazole and Actinomycin D Which Have Been Preincubated for 15 Minutes at 37 C	179.03	65.03
Minus RNA Polymerase	5.25	1.91
Minus DNA	7.65	2.78
Minus CTP	21.53	7.82

* A 1:1 molar ratio (3.2×10^{-7} M) of the hydroxypyrazole and actinomycin D was used in this investigation in which the results were determined by the time-course addition assay.

** Calf thymus DNA was used as the template.

conformational changes may be required before the hydroxypyrazole:actinomycin D complex effectively binds to DNA and inhibits RNA synthesis. In the second possibility, the hydroxypyrazole may bind to DNA in such a manner that subtle conformational changes are introduced in the DNA helix which allows actinomycin D to bind more efficiently. Regardless of the mechanisms employed, both alternatives may involve the binding of actinomycin D at secondary sites which normally bind actinomycin D only weakly. Several reports have noted the existence of at least two types of binding sites for actinomycin D, one of which has a binding constant that is many times greater than the other (Cavalier and Nemchin, 1964; Wells and Larson, 1970; Hyman and Davidson, 1971). The distinction between these sites is thought to depend on the helical structure of the DNA.

More recently, Krugh and Young (1977) reported that two intercalating agents, daunomycin and adriamycin, cooperatively facilitate the binding of actinomycin D to poly-d(A-T)·poly-d(A-T). Since actinomycin D does not normally bind to this double-stranded polynucleotide, the results of this investigation demonstrated that when daunomycin or adriamycin bind to poly-d(A-T)·poly-d(A-T), a change occurs in the conformation of the polynucleotide which results in an increase in the stability of the poly-d(A-T)·poly-d(A-T):actinomycin D complex.

The second alternative may also account for the stimulatory effect which was observed for the hydroxypyrazole.

Richardson (1966) reported that there are two kinds of association between RNA polymerase and the DNA. There is an interaction with a high binding affinity that keeps the enzyme bound to the DNA even though the concentration of free enzyme is very low, and there is an interaction with a much lower affinity that requires very high concentrations of free enzyme. If the hydroxypyrazole does produce conformational changes in the DNA helix, it would be logical to assume that the binding of RNA polymerase may be affected.

Effect of Protocatechuate on the Actinomycin D Inhibition of the RNA Polymerase Reaction

Data obtained utilizing the standard assay method revealed that 1:1 molar ratios (1.3×10^{-6} M) of protocatechuate and actinomycin D prevented actinomycin D inhibition of RNA synthesis by nearly 31%, as compared to the complete control when calf thymus DNA was the template (Table VI). Protocatechuate had no effect on RNA synthesis at this concentration. A time-course experiment demonstrated that protocatechuate began to alleviate actinomycin D inhibition shortly after the first few minutes of the reaction (Figure 20). Comparable results were obtained when *P. fluorescens* DNA was used as the template (Table VII).

TABLE VI

EFFECT OF PROTOCATECHUATE ON THE ACTINOMYCIN D INHIBITION OF THE RNA POLYMERASE REACTION WHEN CALF THYMUS DNA WAS THE TEMPLATE

System	UMP Incorporation	
	pmoles	% Control
Complete	229.24	100.00
Plus Protocatechuate	227.33	99.17
Plus Actinomycin D	83.76	36.54
Plus Protocatechuate and Actinomycin D	154.87	67.56
Minus RNA Polymerase	1.26	0.55
Minus DNA	3.15	1.37

* A 1:1 molar ratio (1.3×10^{-6} M) of protocatechuate and actinomycin D was used in this investigation in which the results were determined by the standard assay method.

Figure 20. Time-Course: Effect of Protocatechuate on the Actinomycin D Inhibition of the RNA Polymerase Reaction when Calf Thymus DNA Was the Template. ● , complete control and protocatechuate (1.3×10^{-6} M); ■ , actinomycin D (1.3×10^{-6} M); ▲ , actinomycin D plus protocatechuate.

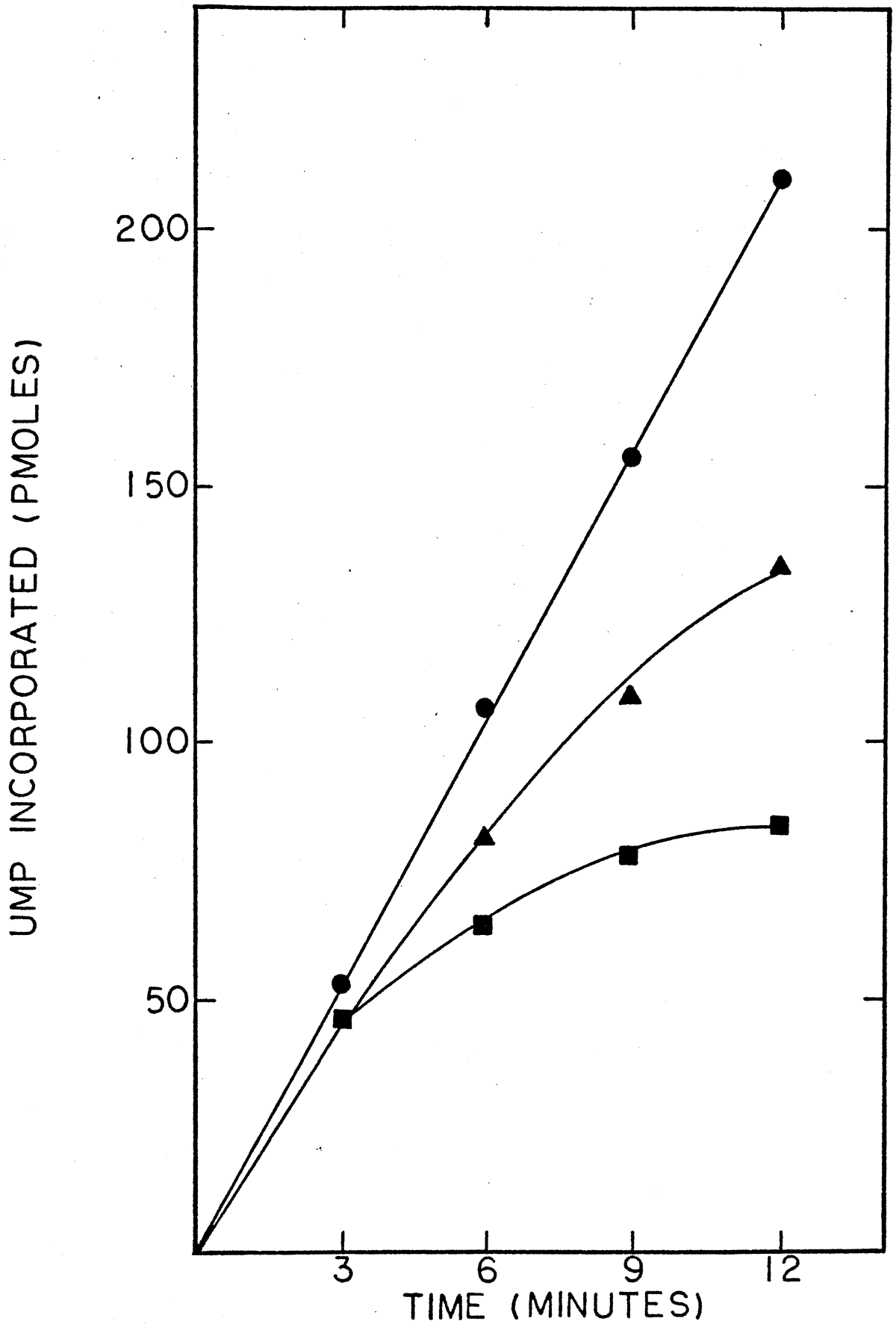

UMP INCORPORATED (PMOLES)
200
150
100
50
3
6
9
12
TIME (MINUTES)

TABLE VII

EFFECT OF PROTOCATECHUATE ON THE ACTINOMYCIN D INHIBITION OF THE RNA POLYMERASE REACTION WHEN *P. FLUORESCENS* DNA WAS THE TEMPLATE

System	UMP Incorporation	
	pmoles	% Control
Complete	176.73	100.00
Plus Protocatechuate	177.88	100.66
Plus Actinomycin D	69.35	39.24
Plus Protocatechuate and Actinomycin D	117.30	66.37
Minus RNA Polymerase	0.83	0.47
Minus DNA	2.24	1.27

* A 1:1 molar ratio (1.3×10^{-6} M) of protocatechuate and actinomycin D was used in this investigation in which the results were determined by the standard assay method.

Effects of Time-Course Addition on the Decrease in Actinomycin D Inhibition of the RNA Polymerase Reaction by Protocatechuate

Time-course addition of protocatechuate and actinomycin D was performed to determine if protocatechuate could reverse actinomycin D inhibition of RNA synthesis, in addition to its ability to partially prevent this inhibition. The results of this investigation revealed that a 1:1 molar ratio (1.3×10^{-6} M) of protocatechuate and actinomycin D which had been preincubated for 15 minutes at 37 C prevented actinomycin D inhibition of RNA synthesis by the greatest amount (Table VIII). When either protocatechuate or actinomycin D was added to the reaction mixture and incubated for 15 minutes at 37 C before the second compound and CTP were added to start the reaction, the inhibition which was observed was almost identical to the actinomycin D control. This suggests that protocatechuate, which binds to DNA (Blunk, 1977), is not readily available for interaction with the actinomycin D. However, if protocatechuate was added to the reaction mixture and incubation was continued for an additional 15 minutes at 37 C before CTP was added to start the reaction, a significant decrease in actinomycin D inhibition was demonstrated. Thus, the alleviation of actinomycin D inhibition of RNA synthesis by protocatechuate is a consequence of molecular interaction between these compounds. Both the prevention and reversal may be explained if the

TABLE VIII

EFFECTS OF TIME-COURSE ADDITION ON THE DECREASE IN ACTINOMYCIN D INHIBITION OF THE RNA POLYMERASE REACTION BY PROTOCATECHUATE

System	UMP Incorporation pmoles	% Control
Complete I	307.78	100.00
Plus Protocatechuate	303.31	98.55
Plus Actinomycin D	126.76	41.19
Plus Protocatechuate and Actinomycin D Added Simultaneously	204.30	66.38
Plus Protocatechuate and Actinomycin D Which Have Been Preincubated for 15 Minutes at 37 C	219.63	71.36
Plus Protocatechuate; Actinomycin D Added After Incubation for 15 Minutes at 37 C	120.23	39.06
Plus Actinomycin D; Protocatechuate Added After Incubation for 15 Minutes at 37 C	128.07	41.61
Complete II	312.85	101.64
Plus Actinomycin D; Protocatechuate Is Added and Another Incubation for 15 Minutes at 37 C Is Carried Out Before CTP Is Added	166.91	54.23
Minus RNA Polymerase	2.59	0.84
Minus DNA	6.34	2.06
Minus CTP	23.91	7.77

* A 1:1 molar ratio (1.3×10^{-6} M) of protocatechuate and actinomycin D was used in this investigation in which the results were determined by the time-course addition assay.

** Calf thymus DNA was used as the template.

equilibrium favors the protocatechuate:actinomycin D species rather than the DNA:actinomycin D complex. Because the binding of actinomycin D to DNA is reversible, protocatechuate may be interacting solely with unbound actinomycin D, or perhaps, protocatechuate may function to facilitate the release of actinomycin D from the DNA.

Influence of the Hydroxypyrazole on Membrane Permeability

Effect of Surface-Active Agents on Actinomycin D Inhibition of P. fluorescens

Haslam (1973) demonstrated that the hydroxypyrazole exhibited subtle membrane action against sheep red blood cells and B. subtilis W23 protoplasts. Further investigation revealed that the hydroxypyrazole potentiated the inhibition produced by two membrane active antibiotics, polymyxin and circulin, against P. fluorescens. Because previous investigation in this laboratory has revealed that the hydroxypyrazole may also have a site of action at the membrane, experiments were conducted with selected surface-active agents to compare increases in actinomycin D action produced by these compounds with the increase resulting from the hydroxypyrazole.

Triton X-100, Tween 80, 1-hexadecylpyridinium chloride and SDS were tested to determine if non-inhibitory concentrations of these compounds could increase the action of

actinomycin D against P. fluorescens. No increases in actinomycin D action were observed with either the non-ionic detergents, Triton X-100 and Tween 80, or the anionic detergent, SDS. Only cells treated with the cationic detergent, 1-hexadecylpyridinium chloride, exhibited moderate increases in susceptibility to actinomycin D (Table IX). The inability of the 1-hexadecylpyridinium chloride to produce large increases in actinomycin D inhibition provides added support that the hydroxypyrazole does not increase actinomycin D inhibition solely by its action on the membrane. For example, the hydroxypyrazole increases the inhibition of actinomycin D at a concentration of 3.2×10^{-6} M by approximately 70%.

The prevention of actinomycin D inhibition in both the 1-hexadecylpyridinium chloride treated and untreated cells by protocatechuate further demonstrates that molecular interaction between protocatechuate and actinomycin D may be responsible for this compounds action.

Effect of the Hydroxypyrazole and Actinomycin D on Uptake of Labeled Substrates

Uptake experiments with P. fluorescens were performed to measure the capacity of the hydroxypyrazole, actinomycin D, or combinations of these compounds to affect uptake of selected substrates. The substrates included ^{14}C-2,3-succinate, D-^{14}C-1-alanine, DL-^{14}C-3-phenylalanine, DL-^{14}C-3-aspartate and ^{14}C-2-uracil. Only the uptake of DL-aspartate

TABLE IX

EFFECT OF 1-HEXADECYLPYRIDINIUM CHLORIDE ON ACTINOMYCIN D INHIBITION OF P. FLUORESCENS AND PREVENTION OF THIS INHIBITION BY PROTOCATECHUATE

Concentration of Compounds (Molarity)			% Untreated Succinate Control at 6 hours
Actinomycin D	Protocatechuate	1-Hexadecylpyridinium Chloride	
3.2×10^{-6}	0.0	0.0	91.0
8.0×10^{-6}	0.0	0.0	72.5
0.0	6.5×10^{-4}	0.0	100.0
0.0	0.0	1.0×10^{-5}	98.5
3.2×10^{-6}	0.0	1.0×10^{-5}	82.5
8.0×10^{-6}	0.0	1.0×10^{-5}	54.0
3.2×10^{-6}	6.5×10^{-4}	0.0	100.0
8.0×10^{-6}	6.5×10^{-4}	0.0	99.5
3.2×10^{-6}	6.5×10^{-4}	1.0×10^{-5}	100.0
8.0×10^{-6}	6.5×10^{-4}	1.0×10^{-5}	98.0

was affected by any of the concentrations or combinations of the hydroxypyrazole and actinomycin D. Even so, the uptake of DL-aspartate was only slightly affected by the hydroxypyrazole and not at all by actinomycin D (Table X). Combinations of the hydroxypyrazole and actinomycin D inhibited DL-aspartate uptake to the same degree as the hydroxypyrazole. These results indicate that the proposed hydroxypyrazole: actinomycin D complex apparently does not alter membrane permeability of these substrates; and that the hydroxypyrazole inhibition of uptake is not comprehensive. This substantiates earlier reports that the hydroxypyrazole action at the membrane is slight.

Effect of the Hydroxypyrazole and Actinomycin D on Membrane Permeability of Reconstituted Outer Membrane Vesicles

The outer membrane of several Gram-negative bacteria constitutes a permeability barrier which allows only hydrophilic molecules below a given molecular weight to passively diffuse through the membrane. Outer membrane exclusion limits of 550-650 daltons have been reported for the enteric bacteria *E. coli* K-12 and B, *Salmonella typhimurium*, *Proteus mirabilis* and *Proteus morganii* (Nakae and Nikaido, 1975; Decad and Nikaido, 1976; Nixdorff et al., 1977). Outer membrane proteins, which have been named porins, have been demonstrated to be responsible for these exclusion limits. These porins are reported to have molecular weights between

TABLE X

EFFECT OF THE HYDROXYPYRAZOLE AND ACTINOMYCIN D ON UPTAKE OF DL-^{14}C-3-ASPARTATE BY P. FLUORESCENS

Concentration of Compounds (Molarity)		CPM/mg Cell Dry Weight x 10^{-3}				
Actinomycin D	Hydroxypyrazole	1 min	2 min	3 min	5 min	7 min
0.0	0.0	4.41	7.69	13.60	26.34	35.48
3.2×10^{-6}	0.0	4.47	8.07	13.16	27.23	36.88
0.0	3.2×10^{-6}	4.37	8.23	13.44	28.87	34.60
0.0	4.2×10^{-5}	4.28	7.45	12.73	21.48	29.89
3.2×10^{-6}	3.2×10^{-6}	4.59	8.11	14.25	27.54	37.31
3.2×10^{-6}	4.2×10^{-5}	4.33	7.34	12.97	21.02	30.56

35,000 to 40,000 daltons. In contrast to the enterics, Hancock et al. (1979) reported that the exclusion limit of P. aeruginosa was in the order of 6000 ± 3000 daltons; and that a major outer membrane polypeptide of approximately 35,000 daltons was responsible for the size-dependent permeability of the outer membrane in this bacterium.

Vesicles reconstituted from outer membrane components (including phospholipids, LPS and proteins) provide useful systems for detecting small changes in membrane permeability. Outer membrane components of P. fluorescens were isolated, purified and reconstituted into vesicles to further investigate any action which the hydroxypyrazole, or combinations of the hydroxypyrazole and actinomycin D may exert on membrane permeability.

Qualitative Analysis of Isolated Phospholipids from P. fluorescens. Phospholipids from P. fluorescens were prepared by the method of Folch et al. (1957). The two major phospholipids of P. fluorescens, phosphatidylethanolamine and phosphatidylglycerol, were conclusively identified by thin layer chromatography with known standards (Table XI). Detection was accomplished with the appropriate spray reagent. In addition to the phospholipids, two lipid containing compounds were included in the phospholipid preparation which were not identified.

Outer Membrane Protein Isolation and Purification. Outer membranes of P. fluorescens were prepared by the method

TABLE XI

QUALITATIVE ANALYSIS OF ISOLATED PHOSPHOLIPIDS FROM P. FLUORESCENS

Identification	Rf	Rhodamine	$FeCl_3$-Salicyl-sulphonic Acid	Ninhydrin	Periodate-Schiff's
Phosphatidyl-glycerol	0.456	+	+	-	+
Phosphatidyl-ethanolamine	0.563	+	+	+	-
Unknown	0.906	+	-	-	-
Unknown	0.969	+	-	-	-

of Hancock and Nikaido (1978). After two passages through a French pressure cell at 15,000 pounds per square inch, the disrupted cells were treated with deoxyribonuclease I, ribonuclease I and lysozyme. Cell debris was removed by low speed centrifugation, and the supernatant was decanted, diluted and layered onto sucrose gradients containing steps of 70 and 15% (w/v) sucrose in Tris-HCl buffer. These tubes were centrifuged at 4 C and 183,000 x g for 1 hour in a Beckman SW 41 rotor. The bottom 2 ml of each gradient was removed, pooled, diluted and applied to sucrose density gradients containing steps of 70, 64, 58 and 52% (w/v) sucrose in Tris-HCl buffer. These tubes were centrifuged at 4 C and 96,000 x g for 18 hours in a Beckman SW 27 rotor. The four bands which were observed (Figure 21) were removed by dropwise collection from the bottom of the centrifuge tube and assayed for succinate dehydrogenase, KDO, lipid phosphate and protein.

The results of the analysis of the four bands are shown in Table XII. Comparison of the relative amounts of succinate dehydrogenase and KDO contained in bands I and IV revealed that these bands consist of purified outer and inner membranes, respectively. The two most dense bands (I and II) were enriched with outer membrane components while the least dense band (IV) was purified inner membrane. Band II contained a small amount of the inner membrane marker, succinate dehydrogenase, while band III contained a slightly larger amount of this enzyme. Both these bands contained moderate

Figure 21. Relative Running Positions of the Four Visible Bands from the Second Sucrose Density Gradient. The size of the bands is represented by the width while the amount of protein is represented by the height of the bars.

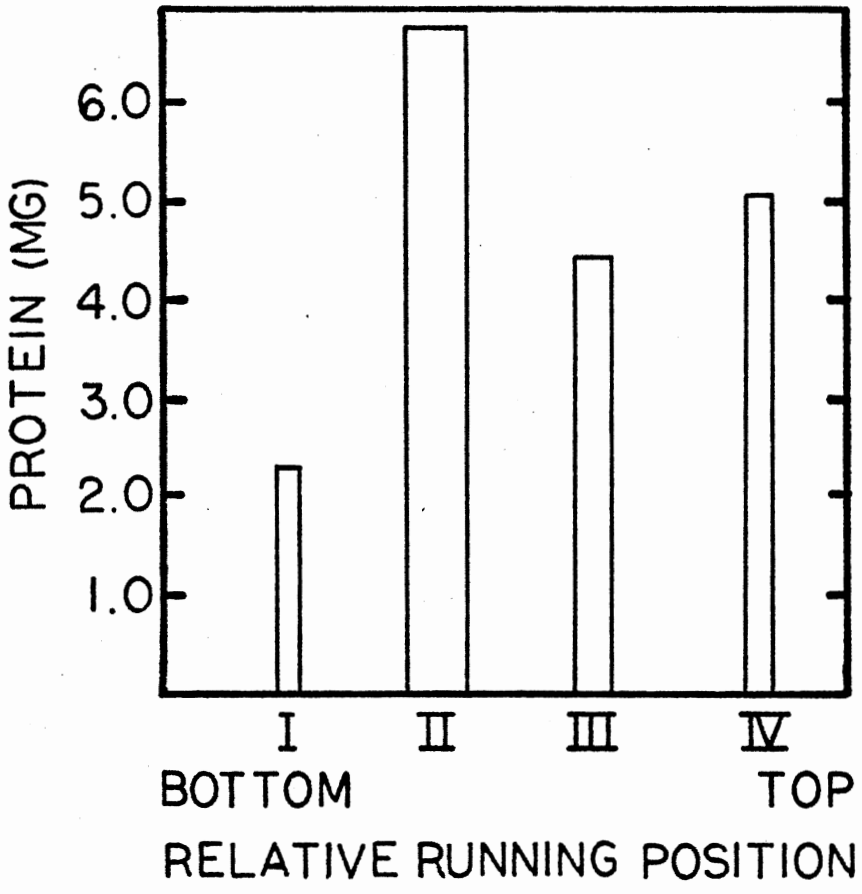

PROTEIN (MG)
6.0
5.0
4.0
3.0
2.0
1.0
I
II
III
IV
BOTTOM
TOP
RELATIVE RUNNING POSITION

TABLE XII

ANALYSIS OF THE OUTER MEMBRANE FRACTIONS

Fraction	% of Protein Recovered	SDH Activity*	KDO**	Lipid Phosphate***
I	12.3	10.24	213.37	0.203
II	36.5	27.45	59.14	0.393
III	23.9	50.51	93.75	0.717
IV	27.3	162.04	3.81	0.290

* SDH, Succinate dehydrogenase; Activity is expressed as micromoles of dichloroindolephenol reduced per minute per milligram of protein.

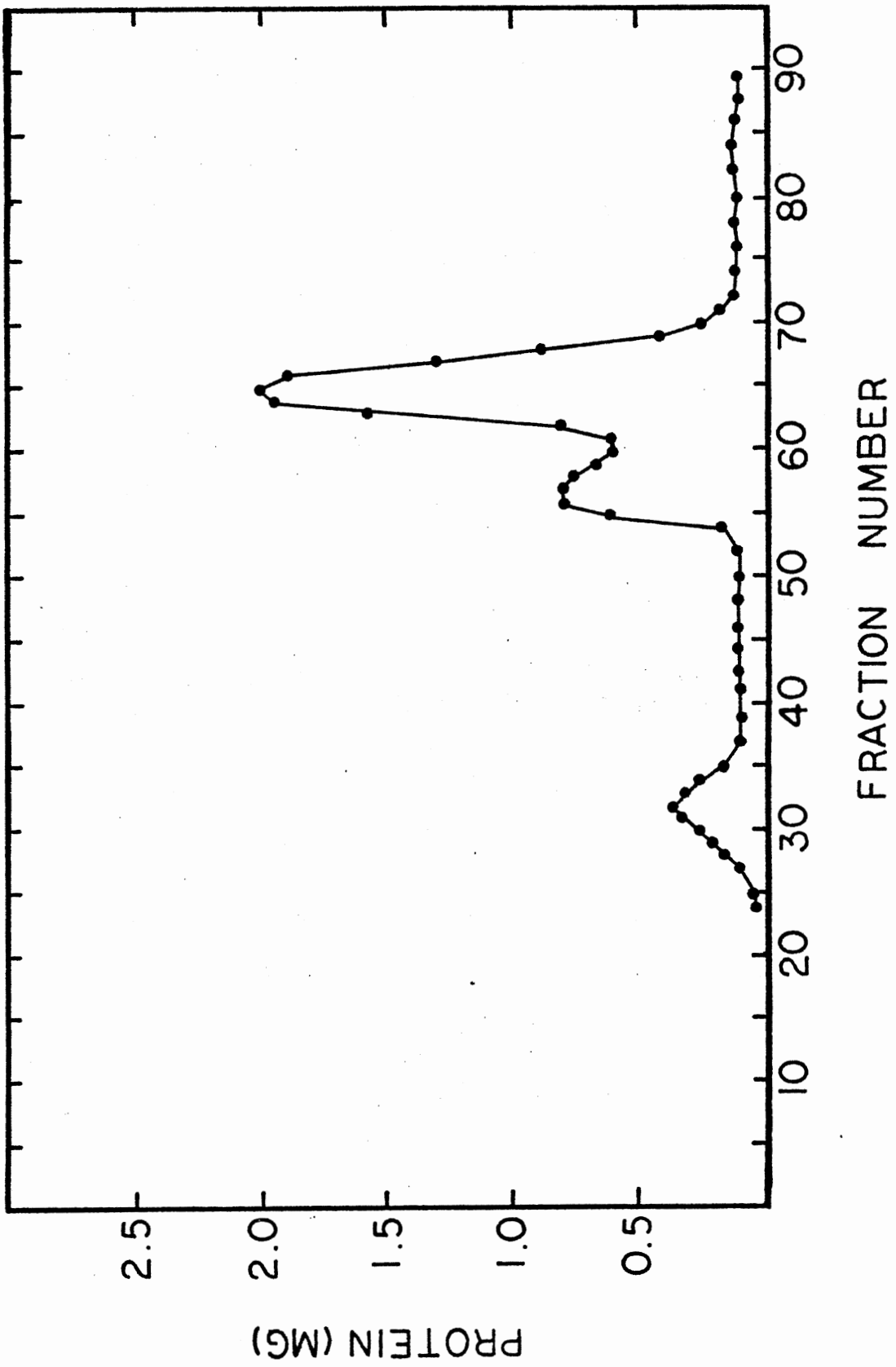
** KDO, 2-keto-3-deoxyoctonate; Amount is expressed as nanomoles per milligram of protein.

*** Expressed as micromoles of lipid phosphate per milligram of protein.

amounts of LPS. Band III contained both inner and outer membrane markers.

Outer membrane proteins were fractionated by the method of Hancock et al. (1979). The lower two bands were pooled, diluted with sterile deionized water and centrifuged at 4 C and 177,000 x g for 1 hour in a Beckman SW 41 rotor. As described, the resulting pellet was solubilized in Tris-HCl buffer containing 2% Triton X-100 and Triton X-100 insoluble protein was sedimented by ultracentrifugation. This Triton X-100 insoluble protein was then solubilized in Tris-HCl buffer containing 2% Triton X-100 and 10 mM EDTA. Triton X-100/EDTA insoluble protein was removed by ultracentrifugation and the supernatant containing Triton X-100/EDTA soluble outer membrane proteins was applied to a DEAE-Sephacel column. The proteins were eluted from the column with a sodium chloride concentration gradient (0.1 to 0.6 M) and fractions (7 ml) were collected and analyzed for protein content by the method of Lowry et al. (1951) (Figure 22). Appropriate fractions were pooled and concentrated to about 3-5 ml by dialysis in polyethylene glycol. Triton X-100 was removed from the protein samples with Bio-Gel SM-2 copolymer beads by by the method of Holloway (1973). This treatment removed Triton X-100 to a sufficiently low level (less than 0.05%) such that vesicle reconstitution was not affected. After Triton X-100 removal, the protein fractions were divided into 0.5 ml aliquots and stored at -20 C.

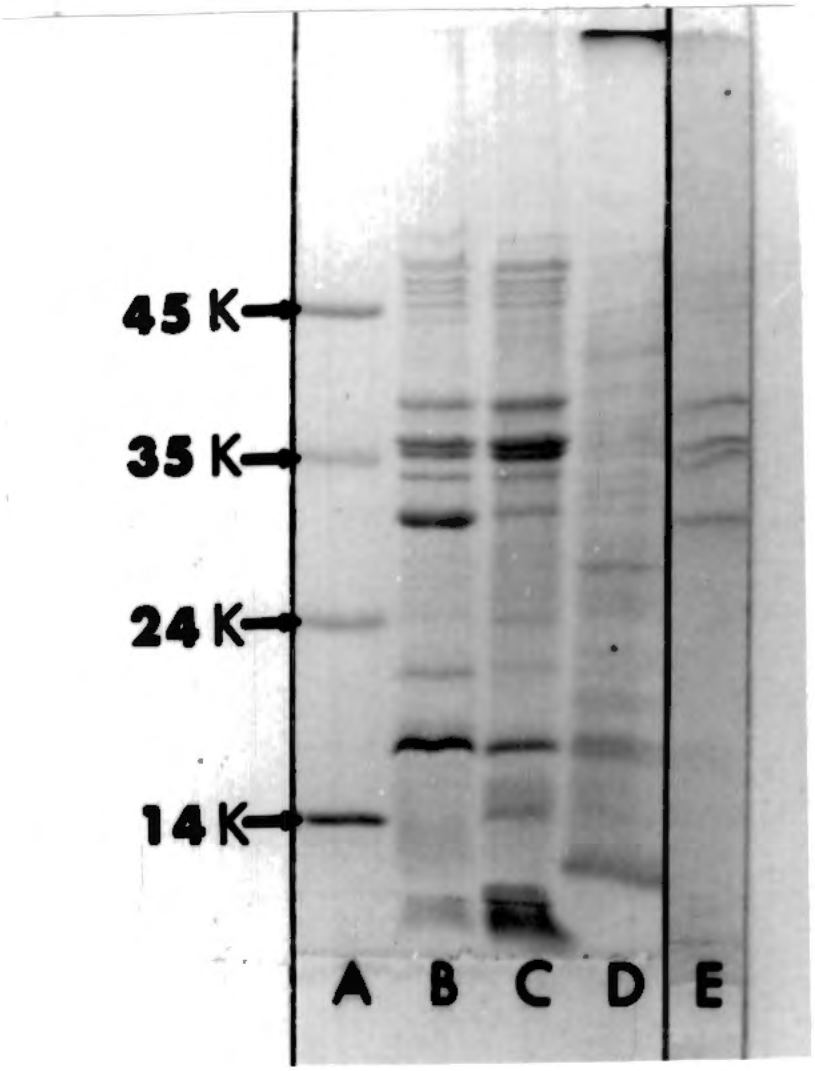
Figure 22. Elution of Outer Membrane Proteins from DEAE-Sephacel. ●—● , protein.


PROTEIN (MG)
2.5
2.0
1.5
1.0
0.5
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

SDS Polyacrylamide Gel Electrophoresis of Outer Membrane Proteins. SDS polyacrylamide gel electrophoresis of the pooled column fractions was performed by the method of Lugtenberg et al. (1975) to locate fractions containing outer membrane proteins having molecular weights of approximately 35,000 daltons. These gels were compared to gels of Triton X-100/EDTA soluble protein (which was loaded onto the column), Triton X-100/EDTA insoluble protein, Triton X-100 soluble protein and molecular weight standards (Figure 23).

Hancock and Nikaido (1978) reported that the outer membrane of P. aeruginosa PA01 contained four major outer membrane proteins having molecular weights of 17, 21, 35 and 37 K. The Triton X-100 soluble protein contained no significant amounts of any of the major outer membrane proteins. The Triton X-100/EDTA insoluble material contained significant amounts of the 17, 35 and 37 K proteins while the Triton X-100/EDTA soluble fraction contained these three proteins plus the 21 K protein. However, in both the Triton X-100/EDTA soluble and insoluble fractions, the 35 K protein appeared as two closely spaced bands. Whether this represents two very similar proteins or a partial breakdown of the 35 K protein is not known, since this pattern was observed in three separate outer membrane preparations. In addition, the Triton X-100/EDTA soluble material contained substantial amounts of two other proteins between the approximate molecular weights of 30 to 33 K. Comparison with the report by Hancock et al. (1979), indicates that these

Figure 23. SDS Polyacrylamide Gel Electrophoresis of Outer Membrane Proteins. A, protein standards: ovalbumin (45 K), pepsin (34.7 K), trypsinogen (24 K), and lysozyme (14.3 K), 1 μg of each; B, Triton X-100/EDTA soluble proteins (20 μg); C, Triton X-100/EDTA insoluble proteins (20 μg); D, Triton X-100 soluble proteins (20 μg); E, fractions 65-66 from DEAE-Sephacel column (5 μg).


45 K
35 K
24 K
14 K
A
B
C
D
E

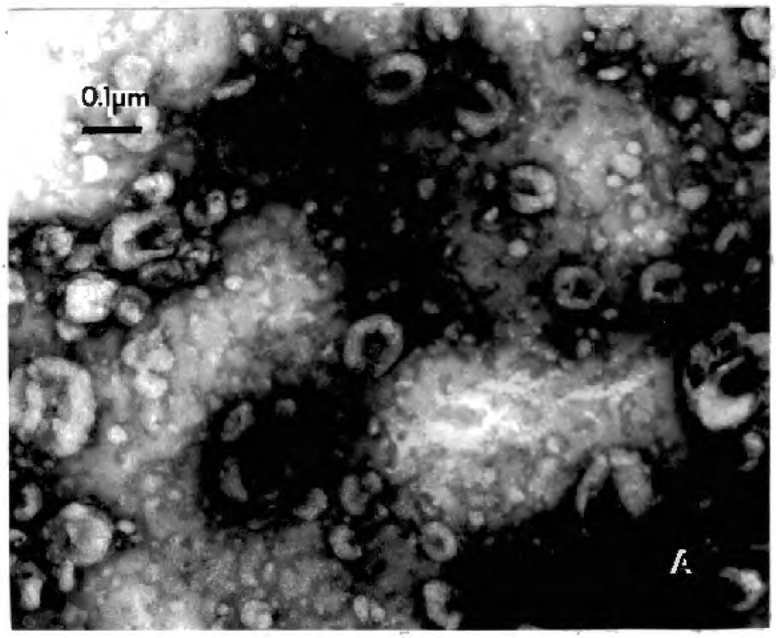
proteins remained in the Triton X-100/EDTA insoluble material in their preparations. Despite these differences, the Triton X-100 soluble fraction contained substantial amounts of the four major outer membrane proteins which were previously reported for P. aeruginosa PA01.

SDS electrophoresis of the column fractions revealed that fractions 65-66 contained substantial amounts of only those proteins between the molecular weights of 33-37 K. The gel from this fraction demonstrated a complete separation of the protein bands at approximately 35 K.

Effect of the Hydroxypyrazole and Actinomycin D on Membrane Permeability of Reconstituted Outer Vesicles from P. fluorescens. Outer membrane vesicles were reconstituted from outer membrane components of P. fluorescens by the technique of Hancock and Nikaido (1978). Electron microscopy was used to observe vesicles reconstituted from P. fluorescens phospholipids and LPS (Figure 24). Spherical vesicular structures were clearly demonstrated in all vesicle preparations.

Vesicles reconstituted from phospholipids and LPS, with or without outer membrane proteins, were used to investigate the effect of the hydroxypyrazole, actinomycin D, or combinations of these compounds on retention of ^{14}C-sucrose within closed vesicles. When vesicles reconstituted from phospholipids and LPS were exposed to the hydroxypyrazole for 20 minutes at 25 C, a slight decrease in ^{14}C-sucrose retention was demonstrated (Table XIII). However, the hydroxypyrazole

Figure 24. Electron Micrograph of Vesicles Reconstituted from P. fluorescens Phospholipids and LPS. The vesicle preparation was placed on a Formvar grid and negatively stained with uranyl acetate for 30-45 seconds. A, magnification, 76,000 X; B, magnification, 170,000 X.


0.1μm
A

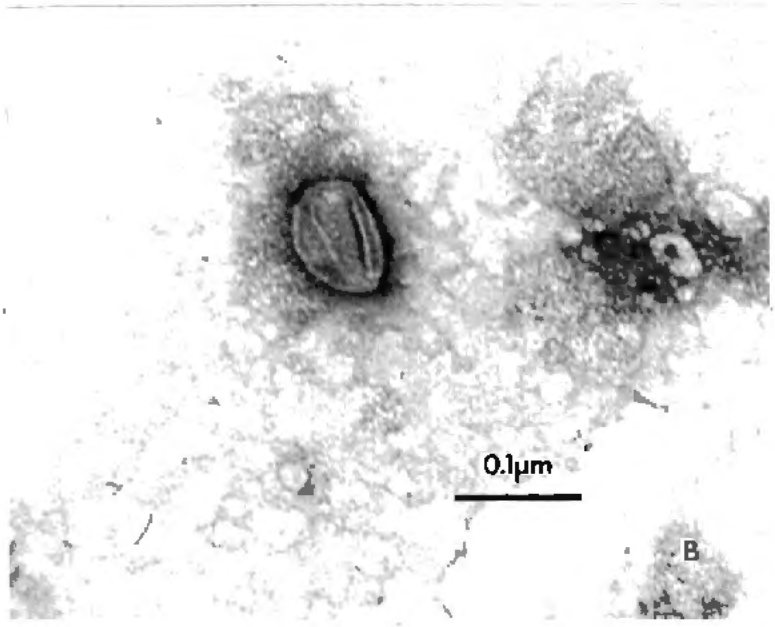

0.1μm
B

TABLE XIII

EFFECT OF THE HYDROXYPYRAZOLE ON THE RETENTION OF ^{14}C-SUCROSE BY RECONSTITUTED OUTER MEMBRANE VESICLES

Hydroxypyrazole (Molarity)	Vesicle Components*	% ^{14}C-Sucrose Retained
0.0	PL, LPS	1.644
2.1×10^{-5}	PL, LPS	1.542
4.2×10^{-5}	PL, LPS	1.411
8.5×10^{-5}	PL, LPS	1.396

* PL, phospholipid; LPS, lipopolysaccharide.

did not appear to perturb the vesicle components to any great extent since most of the ^{14}C-sucrose was still retained within the vesicles. Vesicles containing phospholipids, LPS and outer membrane proteins (either Triton X-100/EDTA soluble proteins or proteins from fractions 65-66) were not significantly affected by the hydroxypyrazole, actinomycin D, or combinations of these compounds (Table XIV). Apparently the proteins stabilize the phospholipid and LPS components of the vesicular membrane and negate the subtle effect of the hydroxypyrazole on these components. These results demonstrate that the hydroxypyrazole or the combination of the hydroxypyrazole and actinomycin D does not influence the permeability functions of the outer membrane proteins because the amount of ^{14}C-sucrose which diffused from the exposed vesicles was essentially the same as the controls. This substantiates other data which has demonstrated that the effect of these compounds, alone or in combination, on membrane permeability is very small.

It is important to note that vesicle preparations containing either Triton X-100/EDTA soluble proteins or proteins from fractions 65-66 exhibited significant decreases in ^{14}C-sucrose retention, as compared to vesicle preparations lacking outer membrane proteins. Therefore, both these protein sources appeared to possess porin activity.

TABLE XIV

EFFECT OF THE HYDROXYPYRAZOLE AND ACTINOMYCIN D ON THE RETENTION OF ^{14}C-SUCROSE BY RECONSTITUTED OUTER MEMBRANE VESICLES

Compounds (Molarity)			
Actinomycin D	Hydroxypyrazole	Components*	% ^{14}C-Sucrose Retained
0.0	0.0	PL,LPS	1.565
0.0	4.2×10^{-5}	PL,LPS	1.360
3.2×10^{-6}	0.0	PL,LPS	1.596
3.2×10^{-6}	4.2×10^{-5}	PL,LPS	1.487
0.0	0.0	PL,LPS,PR1	0.204
0.0	4.2×10^{-5}	PL,LPS,PR1	0.209
3.2×10^{-6}	0.0	PL,LPS,PR1	0.214
3.2×10^{-6}	4.2×10^{-5}	PL,LPS,PR1	0.217
0.0	0.0	PL,LPS,PR2	0.293
0.0	4.2×10^{-5}	PL,LPS,PR2	0.279
3.2×10^{-6}	0.0	PL,LPS,PR2	0.298
3.2×10^{-6}	4.2×10^{-5}	PL,LPS,PR2	0.274

* PL, phospholipid; LPS, lipopolysaccharide; PR1, Triton X-100/EDTA soluble protein; PR2, fraction 65-66 protein.

CHAPTER IV

CONCLUSIONS

The inhibition of P. fluorescens by actinomycin D was greatly increased when a non-inhibitory concentration of the hydroxypyrazole was added in combination to the medium. Decreases in viable cell number and alterations in cell size were demonstrated which were customarily observed only for cells treated with higher concentrations of the antibiotic. Furthermore, the inhibition of cells exposed for 10 hours to the combination of the hydroxypyrazole and actinomycin D was bactericidal, although this concentration of actinomycin D was bacteriostatic when administered alone. Since actinomycin D inhibition of P. fluorescens is concentration dependent, these results suggest that the hydroxypyrazole either facilitates entry of the antibiotic into the cell, or its ability to inhibit once it reaches a site of action within the cell. Investigations have revealed that exposure of growing cultures to the hydroxypyrazole was not sufficient to increase actinomycin D inhibition if the cells were washed and resuspended in fresh medium containing actinomycin D. This suggests that both the hydroxypyrazole and actinomycin D must be present and act together, possibly via a molecular complex, to inhibit growth of P. fluorescens.

Protocatechuate prevents and reverses the inhibition of P. fluorescens by actinomycin D without interfering with its diffusion into the cell (Durham and Keudell, 1969). Results have demonstrated that protocatechuate also prevents and reverses the increased inhibition which was observed when the hydroxypyrazole and actinomycin D were added in combination to the medium. The manner in which protocatechuate relieves this inhibition appears similar to the alleviation of inhibition resulting from only actinomycin D. These data augment previous investigations and provide further support that molecular complexations may have an important role in the mechanism(s) which may be involved.

The amidase system of P. fluorescens provided a more sensitive and direct method for determining changes in actinomycin D action since inhibition of amidase synthesis by actinomycin D is also concentration dependent. At actinomycin D concentrations which were 10 fold less than those used for growth studies, significant increases in actinomycin D inhibition of amidase synthesis were observed when the hydroxypyrazole was added in combination to the medium. Inactivation of the amidase was eliminated since none of the concentrations or combinations of the hydroxypyrazole and actinomycin D produced any changes in amidase activity when in vitro experiments were performed with purified amidase. Protocatechuate readily prevents and reverses inhibition of amidase synthesis by actinomycin D or the combination of the hydroxypyrazole and actinomycin D. Because both the

hydroxypyrazole and protocatechuate function to modulate actinomycin D inhibition of amidase synthesis, these data suggest that both compounds may affect the binding of actinomycin D to DNA. In view of the low concentration of actinomycin D used in combination with the hydroxypyrazole, this is particularly noteworthy.

The RNA polymerase reaction is the primary biological site of action for actinomycin D. For a given concentration of DNA, the actinomycin D inhibition of the RNA polymerase reaction was concentration dependent. Very small increases in actinomycin D concentration resulted in very large increases in inhibition. This behavior was observed until approximately 70% inhibition was achieved. Then the degree of inhibition was only slightly increased with increasing concentrations of actinomycin D.

Depending on the concentration, the hydroxypyrazole can both inhibit and stimulate the RNA polymerase reaction. The concentration which was necessary to establish inhibition was more than 10 fold greater than the corresponding concentration of actinomycin D. The maximum inhibition achieved by higher concentrations of the hydroxypyrazole was limited to 30-35%. Stimulation of RNA polymerase was observed when the concentration of the hydroxypyrazole was 10 fold less than the concentration required to establish inhibition. The hydroxypyrazole probably stimulates RNA synthesis by affecting template site selection and activation by the RNA polymerase holoenzyme. A stimulation of this nature may

involve low affinity binding sites which do not normally bind RNA polymerase effectively at low concentrations.

When this stimulatory concentration of the hydroxypyrazole was added in combination with a 1:1 molar ratio of actinomycin D, a significant increase in actinomycin D inhibition of RNA synthesis was observed. The major effect of the hydroxypyrazole on actinomycin D inhibition of RNA synthesis is at the level of RNA chain elongation.

The role that the proposed hydroxypyrazole:actinomycin D plays in increasing actinomycin D inhibition of RNA synthesis is uncertain. Conformational changes may be required in the complex or the DNA before the complex can effectively bind DNA and inhibit RNA synthesis. The hydroxypyrazole may bind to DNA and introduce subtle conformational changes in the DNA helix which allow actinomycin D to bind more efficiently. This alternative would help explain the stimulation of RNA polymerase by the hydroxypyrazole. The increase in actinomycin D inhibition of RNA synthesis by the hydroxypyrazole may involve the binding of actinomycin D at secondary sites which normally bind actinomycin D only weakly, regardless of the mechanism(s) utilized.

Protocatechuate partially prevented and reversed the inhibition of RNA synthesis resulting from a 1:1 molar ratio of actinomycin D. This provides further support that the alleviation of actinomycin D inhibition by protocatechuate is a consequence of molecular interactions between these compounds. Both the prevention and reversal may be explained

if a protocatechuate:actinomycin D complex is favored over the DNA:actinomycin D complex. Protocatechuate may be interacting solely with unbound actinomycin D or it may act to facilitate the release of actinomycin D from DNA.

Little or no effect on membrane permeability was observed with the hydroxypyrazole, actinomycin D, or combinations of these compounds during this investigation. This substantiates the report by Haslam (1973) that hydroxypyrazole action at the membrane is subtle.

A model depicting possible mechanisms by which the hydroxypyrazole and protocatechuate may influence actinomycin D action is shown in Figure 25.

In the simplest mechanism, the hydroxypyrazole acts alone to increase diffusion of actinomycin D into the cell. This is the least likely alternative because all investigations to date have revealed that membrane action by the hydroxypyrazole was minor. Furthermore, growth of cells in the presence of the hydroxypyrazole was not sufficient to increase actinomycin D action when the cells were washed and resuspended in medium containing actinomycin D.

The hydroxypyrazole and actinomycin D may enter the cell separately. Once inside the cell, the two compounds may complex before binding DNA or they may interact with the DNA individually. If the hydroxypyrazole binding to DNA introduces changes in the DNA helix, actinomycin D binding may be facilitated resulting in an increase in inhibition. This would be consistent with the RNA polymerase data; it would

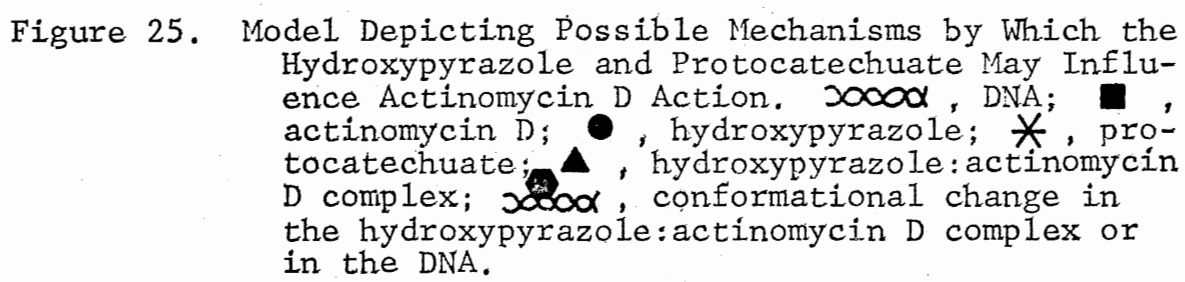
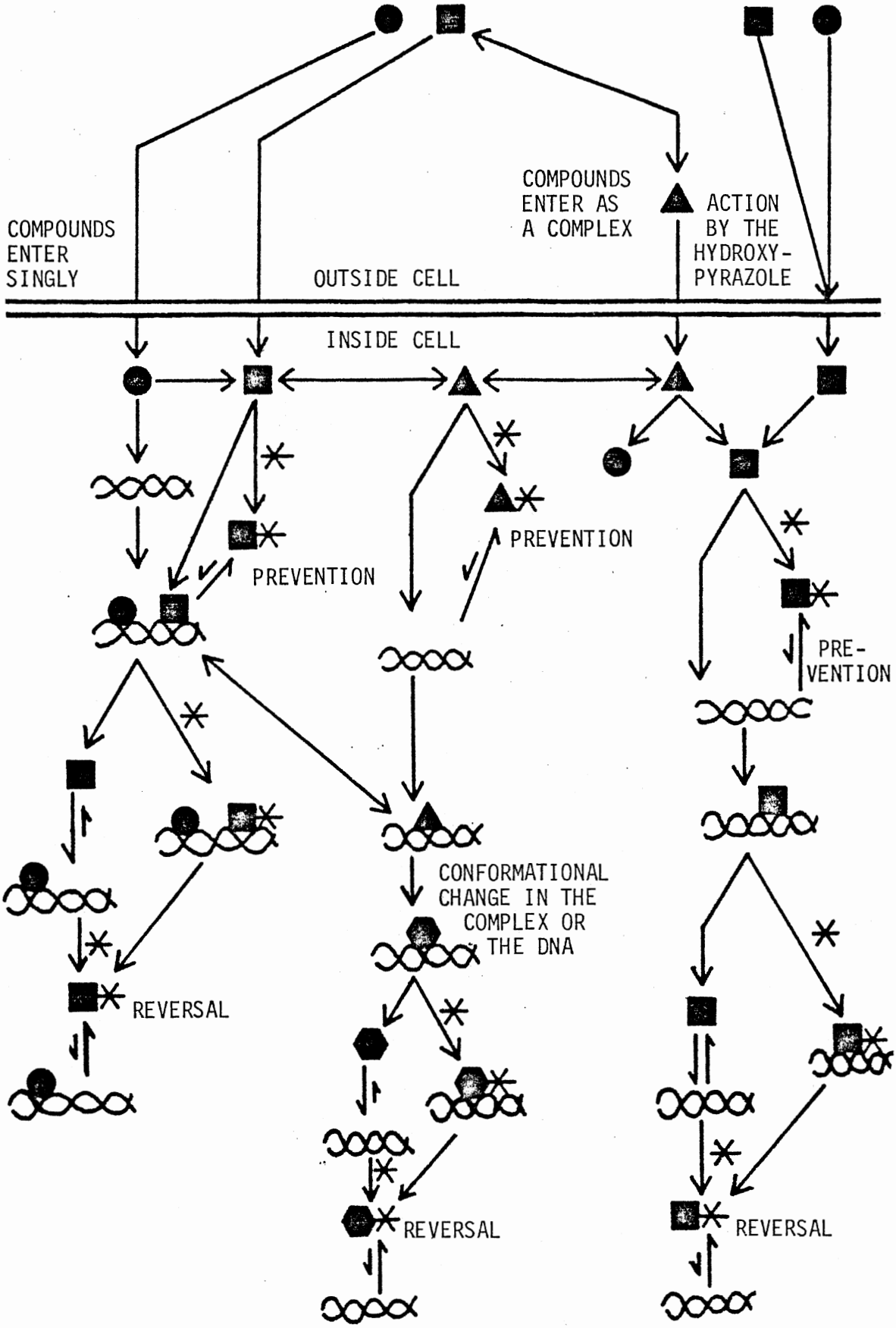


Figure 25. Model Depicting Possible Mechanisms by Which the Hydroxypyrazole and Protocatechuate May Influence Actinomycin D Action. ∽∽∽∽, DNA; ■, actinomycin D; ●, hydroxypyrazole; ✳, protocatechuate; ▲, hydroxypyrazole:actinomycin D complex; ∽∽∽∽, conformational change in the hydroxypyrazole:actinomycin D complex or in the DNA.


COMPOUNDS ENTER SINGLY
OUTSIDE CELL
COMPOUNDS ENTER AS A COMPLEX
ACTION BY THE HYDROXY-PYRAZOLE
INSIDE CELL
PREVENTION
PREVENTION
PRE-VENTION
CONFORMATIONAL CHANGE IN THE COMPLEX OR THE DNA
REVERSAL
REVERSAL
REVERSAL

explain both the increase in actinomycin D inhibition and the stimulation of RNA synthesis. Although this mode of entry cannot be ruled out, it also appears unlikely, since the cells grown in the presence of the hydroxypyrazole were not inhibited to a greater extent than control cells when both sets of cells were exposed to the antibiotic. A reverse of this situation yielded the same result when cells were grown in actinomycin D and then exposed to the hydroxypyrazole.

All data to date suggests that the hydroxypyrazole and actinomycin D must both be present and act together to increase actinomycin D action. A molecular complex of the two compounds, formed outside the cell, would explain most of the present data. The proposed hydroxypyrazole:actinomycin D complex may increase passage of these compounds into the cell, although it has not demonstrated any capacity for altering membrane permeability. A complex of the hydroxypyrazole and actinomycin D may be responsible for the increases observed in actinomycin D action, provided the complex has a greater affinity for DNA than does actinomycin D. If secondary sites are involved, a hydroxypyrazole: actinomycin D complex may bind effectively to DNA at sites which bind actinomycin D only weakly. This model is consistent with most of the present data. However, the RNA polymerase data indicated that conformational changes in the hydroxypyrazole:actinomycin D complex or the DNA may be necessary before RNA synthesis is inhibited.

Protocatechuate prevents and reverses actinomycin D action without interfering with its entry into the cell. Apparently, the ability of protocatechuate to alleviate inhibition produced by either actinomycin D or the combination of the hydroxypyrazole and actinomycin D is a consequence resulting from an interaction of protocatechuate with these compounds. In both cases, protocatechuate may be interacting with either the unbound molecules to decrease their affinity to bind DNA or with the bound molecules to facilitate their release from the DNA.

LITERATURE CITED

Ames, B. N., and D. T. Dubin. 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. 235: 769-775.

Blunk, J. D. 1977. Effects of hydroxypyrazole and protocatechuate on the biological activity of actinomycin D using Pseudomonas fluorescens. M.S. Thesis. Oklahoma State University.

Brammer, W. J. and P. H. Clarke. 1964. Induction and repression of Pseudomonas aeruginosa amidase. J. Gen. Microbiol. 37: 307-319.

Brockmann, H., and N. Grubfofer. 1949. Actinomycin C. Naturwissenschaften. 36: 376-377.

Brown, P. R., M. J. Smith, P. H. Clarke, and M. A. Rosemeyer. 1973. The subunit structure of the aliphatic amidase from Pseudomonas aeruginosa. Eur. J. Biochem. 34: 177-187.

Burgess, R. R. 1971. RNA polymerase. Ann. Rev. Biochem. 40: 711-740.

Burton, K. 1955. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 52: 315-323.

Cavalieri, L. F., and R. G. Nemchin. 1964. The mode of interaction of actinomycin D with deoxyribonucleic acid. Biochim. Biophys. Acta 87: 641-652.

Chestnut, R. W., N. N. Durham, E. A. Mawdsley, G. X. Thyvelikakath, K. D. Berlin, and B. M. Fung. 1974. Biological, proton-magnetic-resonance and ultraviolet-spectroscopic evidence for a complex of actinomycin D and 10, 11-dihydro-3H-napth(1,2 g)indaz-7-ol. Biochem. J. 143: 789-792.

Dagliesh, C. E., A. W. Johnson, A. R. Todd, and L. C. Vining. 1950. Actinomycin. Part I. Amino acid content. J. Chem. Soc. Part IV: 2946-2952.

Dagliesh, C. E., and A. R. Todd. 1949. Actinomycin. Nature (London). 164: 830.

Decad, G. M., and H. Nikaido. 1976. Outer membrane of gram-negative bacteria. XII. Molecular sieving function of cell wall. J. Bacteriol. 128: 325-326.

Durham, N. N. 1958. Studies on the metabolism of p-nitrobenzoic acid. Can. J. Microbiol. 4: 141-148.

Durham, N. N., R. W. Chestnut, D. F. Haslam, and K. D. Berlin. 1974. Hydropolar molecules as effectors in molecular complex-membrane interactions. Proc. Okla. Acad. Sci. pp. 77-86.

Durham, N. N., and D. V. Ferguson. 1971. Molecular specificity associated with alleviation of actinomycin D inhibition of protein synthesis. Biochem. J. 123: 291-292.

Durham, N. N., and K. C. Keudell. 1969. Synthesis of protocatechuate oxygenase by Pseudomonas fluorescens in the presence of actinomycin D. Antimicrob. Agents Chemother. p. 67.

Ferguson, D. V. 1970. The effect of actinomycin D on amidase induction in Pseudomonas fluorescens. Ph.D. thesis. Oklahoma State University.

Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226: 497-509.

Foley, G. E. 1956. Preliminary observations on the mechanism of action of actinomycin D in microbiological systems. Antibiotics Annual: 432-436.

Garewal, H. S. 1973. A procedure for the estimation of microgram quantities of Triton X-100. Anal. Biochem. 54: 319-324.

Gellert, M., C. E. Smith, D. Neville, and G. Felsenfeld. 1965. Actinomycin Binding to DNA: Mechanism and specificity. J. Mol. Biol. 11: 445-447.

Gold, M., and J. Hurwitz. 1964. The enzymatic methylation of ribonucleic acid and deoxyribonucleic acid. VI. Further studies on the properties of the deoxyribonucleic acid methylation reaction. J. Biol. Chem. 239: 3866-3874.

Goldberg, I. H., M. Rabinowitz, and E. Reich. 1962. Basis of actinomycin action. I. DNA binding and inhibition of RNA polymerase synthetic reaction by actinomycin. Proc. Natl. Acad. Sci. 48: 2094-2101.

Hamilton, L. D., W. Fuller, and E. Reich. 1963. X-ray diffraction and molecular model building studies of the interaction of actinomycin with nucleic acids. Nature 198: 538-540.

Hancock, I. C., and P. M. Meadow. 1969. The extractable lipids of Pseudomonas aeruginosa. Biochim. Biophys. Acta 187: 366-379.

Hancock, R. E. W., G. M. Decad, and H. Nikaido. 1979. Identification of the protein producing transmembrane diffusion pore in the outer membrane of Pseudomonas aeruginosa PAO1. Biochim. Biophys. Acta 554: 323-331.

Haslam, D. F. 1973. Studies on the biological action of a hydroxypyrazole compound. Ph.D. thesis. Oklahoma State University.

Holloway, P. W. 1973. A simple procedure for removal of Triton X-100 from protein samples. Anal. Biochem. 53: 304-308.

Honig, G. R., and M. Rabinowitz. 1965. Actinomycin D: Inhibition of protein synthesis unrelated to effect on template RNA synthesis. Science. 149: 1504-1505.

Hurwitz, J., A. Bresler, and R. Diringer. 1960. The enzymic incorporation of ribonucleotides in polyribonucleotides and the effect of DNA. Biochem. Biophys. Res. Commun. 3: 15-19.

Hyman, R. W., and N. Davidson. 1970. Kinetics of the in vitro inhibition of transcription by actinomycin. J. Mol. Biol. 50: 421-438.

Hyman, R. W., and N. Davidson. 1971. The binding of actinomycin D to crab poly d(A-T)·poly d(T-A). II. On the nature of the DNA binding site. Biochim. Biophys. Acta 228: 38-48.

Jain, S. C., and H. M. Sobell. 1972. Stereochemistry of actinomycin binding to DNA. I. Refinement and further structural details of the actinomycin-deoxyguanosine crystalline complex. J. Mol. Biol. 68: 1-20.

Johnson, K. G., and M. B. Perry. 1976. Improved techniques for the preparation of bacterial lipopolysaccharide. Can. J. Microbiol. 22: 29-34.

Kasahara, M., and Y. Anraku. 1974. Succinate dehydrogenase of Escherichia coli membrane vesicles : activation and properties of the enzyme. J. Biochem. 76: 959-966.

Kelly, M., and H. L. Kornberg. 1962. Amidase from Pseudomonas aeruginosa : A multi-headed enzyme. Biochim. Biophys. Acta 64: 190-196.

Kelly, M., and H. L. Kornberg. 1964. Purification and properties of acyltransferases from Pseudomonas aeruginosa. Biochem. J. 93: 557-566.

Kersten, W. 1961. Interaction of actinomycin D with constituents of nucleic acids. Biochim. Biophys. Acta 47: 610-611.

Kersten, W., H. Kersten, and H. M. Rauen. 1960. Action of nucleic acids on the inhibition of growth by actinomycin of Neurospora crassa. Nature (London).

Kirk, J. M. 1960. The mode of action of actinomycin D. Biochim. Biophys. Acta 42: 167-169.

Krugh, T. R., and M. A. Young. 1977. Daunorubicin and adriamycin facilitate actinomycin D binding to poly (dA-dT)·poly(dA-dT). Nature. 269: 627-628.

Leive, L. 1965. Release of lipopolysaccharide by EDTA treatment of E. coli. Biochem. Biophys. Res. Commun. 21: 290-296.

Lipmann, F., and L. C. Tuttle. 1945. A specific micro method for the determination of acyl phosphates. J. Biol Chem. 159: 21-28.

Lowry, O. H., N. J. Rosebrough, A. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen. 1975. Electrophoretic resolution of the major outer membrane protein of Escherichia coli K-12 into four bands. FEBS Letters. 58: 254-258.

Manaker, R. A., F. J. Gregory, L. C. Vining, and S. A. Waksman. 1955. Actinomycin. III. The production and properties of a new actinomycin. Antibiotics Annual. pp. 853-857.

Marinetti, G. V. 1962. Chromatographic separation, identification and analysis of phophatides. J. Lipid Res. 3: 1-20.

Marks, T. A., and J. M. Venditti. 1976. Potentiation of actinomycin D or adriamycin antitumor activity with DNA. Cancer Res. 36: 496-504.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3: 208-218.

Mauger, A. B., and R. Wade. 1966. The synthesis of actinomycin analogues. Part II. Actinocyl-gramicidin S. J. Chem. Soc. Sec. C: 1406-1408.

McConnell, D. J., and J. Bonner. 1972. Preparation of highly purified ribonucleic acid polymerase; separation from polynucleotide phosphorylase and polyphosphate kinase. Biochem. 11: 4329-4336

Meienhofer, J. 1970. Synthesis of actinomycin and analogs. III. A total synthesis of actinomycin D (C_1) via peptide cyclization between proline and sarcosine. J. Amer. Chem. Soc. 92: 3771-3777.

Montgomery, K. 1966. Influence of o-nitrobenzoic acid on the protocatechuate oxygenase system of Pseudomonas fluorescens. M.S. thesis. Oklahoma State University.

Morgan, J. G., K. D. Berlin, N. N. Durham, and R. W. Chestnut. 1971. Synthesis of some indazoles structurally related to equilenin. J. Heterocyclic Chem. 8: 61-63.

Muller, W., and D. M. Crothers. 1968. Studies of the binding of actinomycin and related compounds to DNA. J. Mol. Biol. 35: 251-290.

Nakae, T., and H. Nikaido. 1975. Outer membrane as a diffusion barrier in Salmonella typhimurium : penetration of oligo- and polysaccharides into isolated outer membrane vesicles and cells with degraded peptidoglycan layer. J. Biol. Chem. 250: 7359-7365.

Nixdorff, K., H. Fitzer, J. Gmeiner, and H. H. Martin. 1977. Reconstitution of model membranes from phospholipid and outer membrane proteins of Proteus mirabilis. Role of proteins in the formation of hydrophilic pores and protection of membranes against detergents. Eur. J. Biochem. 81: 63-69.

O'Donnell, D. J., K. Ramalingam, A. S. Radhakrishna, R. S. Fischer, N. N. Durham, and K. D. Berlin. 1978. Carbon-13 and proton nuclear magnetic resonance spectroscopic evidence for a molecular complex of actinomycin D and 10,11-dihydro-3H-napth(1,2g)indazol-7-ol. J. Org. Chem. 43: 4542-4545.

Ogur, M., and G. Rosen. 1950. The nucleic acids of plant tissues. I. The extraction of desoxypentose nucleic acid and pentose nucleic acid. Arch. Biochem. 25: 262-276.

Osburn, M. J. 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of Salmonella typhimurium. Proc. Natl. Acad. Sci. 50: 499-506.

Rauen, H. M., and G. Hess. 1959. The effect of actinomycin D and C on Neurospora crassa. Z. Physiol. Chem. 315: 70-71.

Reich, E. 1964. Actinomycin : Correlation of structure and function of its complexes with purines and DNA. Science. 143: 684-689.

Reich, E., and I. H. Goldberg. 1964. Actinomycin and nucleic acid function. Progr. Nucl. Acid Res. Mol. Biol. 3: 183-234.

Reihm, H., and J. L. Biedler. 1972. Potentiation of drug effect by Tween 80 in chinese hamster cells resistant to actinomycin D and daunomycin. Cancer Res. 32: 1195-1200.

Richardson, J. P. 1966. The binding of RNA polymerase to DNA. J. Mol. Biol. 21: 83-112.

Rouser, G., A. J. Bauman, N. Nicolaides, and D. Heller. 1961. Paper chromatography of lipids : methods, applications, and interpretations. J. Am. Oil Chem. Soc. 38: 565-581.

Roy, A., and S. Mitra. 1970. Susceptibility of E. coli K-12 to actinomycin D after infection with phage M 13. Nature (London). 228: 365-366.

Saito, H., and K. Miura. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta 72: 619-629.

Shaw, N. 1968. The detection of lipids on thin-layer chromatograms with the periodate-schiff reagents. Biochim. Biophys. Acta 164: 435-436.

Slotnick, I. J. 1960. Mechanism of action of actinomycin D in microbiological systems. Anal. N. Y. Acad. Sci. 89: 342-347.

Smith, R. F., D. E. Shay, and N. J. Doorenbos. 1963. Antimicrobial action of nitrogen-containing steroids. J. Bacteriol. 85: 1295-1299.

Sobell, H. M. 1973. The stereochemistry of actinomycin binding to DNA and its implications in molecular biology. Progr. Nucl. Acid Res. Mol. Biol. 13: 153-190.

Stevens, A. 1960. Incorporation of the adenine ribonucleotide into RNA by cell fractions from E. coli B. Biochem. Biophys. Res. Commun. 3: 92-96.

Wade, H. E., and D. E. Morgan. 1953. Detection of phosphate esters on paper chromatograms. Nature. 171: 529-530.

Waksman, S. A. 1954. Actinomycin I. Historical nature and cytostatic action. Antibiotics and Chemotherapy. 4: 502-510.

Waksman, S. A., and H. B. Woodruff. 1940. The soil as a source of microorganisms antagonistic to disease producing bacteria. J. Bacteriol. 40: 581-600.

Weiss, S. B. 1960. Enzymatic incorporation of ribonucleoside phosphate into the interpolynucleotide linkages of ribonucleic acid. Proc. Nat. Acad. Sci. 46: 1020-1030.

Weissbach, A., and J. Hurwitz. 1958. The formation of 2-keto-3-deoxyheptonic acid in extracts of Escherichia coli B. I. Identification. J. Biol. Chem. 234: 705-709.

Wells, R. D., and J. E. Larson. 1970. Binding of actinomycin D to deoxyribonucleic acid and deoxyribonucleic acid model polymers. J. Mol. Biol. 49: 319-342.

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Doctor of Philosophy

Thesis: INTERACTIONS OF A HYDROXYPYRAZOLE AND PROTOCATECHUATE WITH ACTINOMYCIN D IN PSEUDOMONAS FLUORESCENS AND SELECTED IN VITRO SYSTEMS

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