EFFECTS OF HYDROXYPYRAZOLE AND PROTOCATECHUATE ON THE BIOLOGICAL ACTIVITY OF ACTINOMYCIN D USING *PSEUDOMONAS FLUORESCENS*

By

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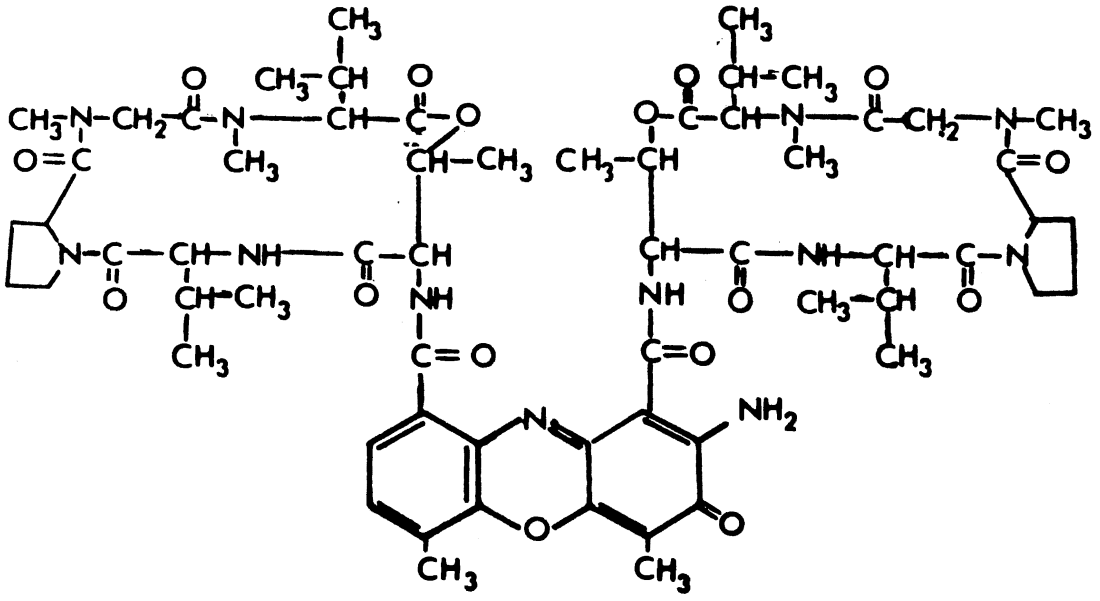
CHAPTER 1

INTRODUCTION

Using enrichment techniques, Waksman and Woodruff (1940), isolated from soil a species of actinomycetes. They found the ether soluble fraction of the culture filtrate to be inhibitory towards growth of select organisms. The ether soluble compound was named actinomycin. Waksman (1954) investigated the antibiotic's spectrum of activity. It was found to be highly active against gram positive bacteria and actinomycetes, with less activity towards gram-negative bacteria and no activity upon fungi. The crystallized compound was brick red in color with a melting temperature of approximately 250°C, it absorbed light at 230, 250, and 450 nm, and the molecular weight was 1255.

Manaker, et al. (1955) crystallized actinomycin D from cultures of Streptomyces parvullus by both solvent (ether) and charcoal adsorption procedures. The molecular formula of the crystalline compound was determined to be $C_{60}H_{76}O_{15}N_{12}\cdot 3H_2O$ and the molecule contained a chromophoric moiety with two peptide chains linked to it. The peptide chains each contained the amino acids sarcosine, D-valine, L-proline, L-threonine, and N-methyl-valine (Fig. 1).

Figure 1. Structure of Actinomycin D. The functional groups of the actinomycin molecule are the free chromophore amino groups, the unreduced quinoidal ring system, and the lactone rings.



Toxicity of actinomycin was shown in mice, rats, and rabbits by Robinson and Waksman (1942). Doses as small as 1 mg per kilogram of body weight were lethal in the test animals. Actinomycin injected intravenously rapidly left the blood stream and could be found in various quantities throughout all organs of the test animals. Pugh, Katz, and Waksman (1956) demonstrated the antitumor potential of the actinomycins in laboratory animals. However, they found the drug had a high toxicity in the test animals, with a marked decrease in spleen size occurring in many cases.

Actinomycin D is currently used against treatment of disseminated neoplasia. It has proven of value against testicular tumors, trophoblastic malignancies, soft tissue sarcomas, and Wilm's tumor. Actinomycin D may be the most effective agent available for testicular cancer (Livingston and Carter, 1970).

Barnett, Ryman, and Smith (1946a) were the first to report antimicrobial activity of azasteroid compounds. They found that two isomers of 7-amino-cholesterol showed a high antibacterial activity <u>in vitro</u> against gram-positive organisms. Barnett, Ryman, and Smith (1946b) also reported that mono- and di-cholestane derivatives with nitrogen in the 3,6 or 7 position showed antimicrobial activity. Chesnut, et al. (1972) demonstrated that ring structures containing nitrogen and related compounds exhibit a broad spectrum of biological activities which makes them useful as therapeutic agents.

Agents such as detergents, EDTA, and bacteriophage have the capability to potentiate the action of actinomycin D by altering the permeability barrier of the cell, thus enhancing the entry of the antibiotic into the cell (Leive, 1965; Roy and Mitra, 1970; Reihm and Biedler, 1972). Marks and Venditte (1976) demonstrated the abilityof DNA, which is the primary site which actinomycin D acts upon in the cell, to form a complex with the antibiotic and potentiate it's activity while at the same time negate the cytotoxicity of the drug. Chesnut, et al. (1974) have provided proton-magnetic-resonance and ultraviolet spectroscopic evidence which suggests a molecular complex is formed between a novel azasteroid and actinomycin D. This complexation of the azasteroid with actinomycin D gives much greater biological activity against Pseudomonas fluorescens. The new azasteroid compound, 10,11,-dihydro-3H-napth(1,2 g)indazol-7-ol, was synthesized by Dr. K.D. Berlin and Dr. J.G. Morgan (Morgan, et al. 1971). The azasteroid, which will be referred to as hydroxypyrazole in this thesis, possesses antimicrobial activity against a gram-positive bacterium Bacillus subtilis but shows no activity against the gram-negative bacterium Pseudomonas fluorescens. The molecular weight of the hydroxypyrazole is 236 with a melting point of 257-260^{o}C. The benzene rings constitute a large non-polar nucleus with the hydroxyl group and two nitrogen groups contributing polar properties to the molecule. The molecule exhibits absorption peaks in the UV range at 258,

266, 300, and 312 nm (Fig. 2).

Earlier work in this laboratory has shown that select aromatic compounds prevent the inhibition of protein synthesis and growth caused by actinomycin D (Durham and Ferguson, 1971). Their results indicated that 3,4-dihydroxybenzoate (protocatechuate) was the most effective in preventing the inhibition caused by actinomycin D (Fig. 3).

Actinomycin D prevents protein synthesis by inhibiting DNA-directed RNA synthesis. Durham and Keudall (1969) reported that the inhibition of synthesis of amidase (acylamide aminohydrolase, E C 3.5.1.4) by actinomycin D could be prevented or alleviated by protocatechuate. It was proposed that protocatechuate complexes with actinomycin D to negate the effect of the antibiotic, by preventing the interaction of it with cellular DNA.

This investigation was conducted to investigate the biological activity of actinomycin D-hydroxypyrazole complex and the ability of protocatechuate to interact with actinomycin D or the complex to negate their biological effects.

Figure 2. 10,11,-dihydro-3H-napth(1,2 g)indazol-7-ol. The molecule has a large non-polar hydroxy group attached to the number 3 carbon and polar nitrogens incorporated into the steroid nucleus at the 15 and 16 positions.

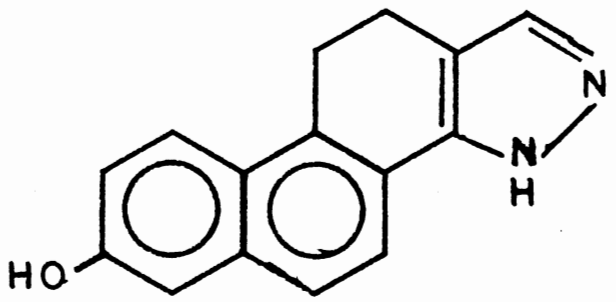
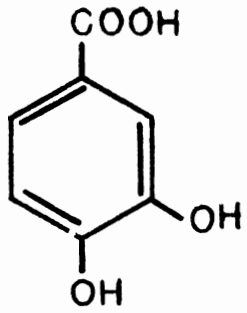

HO
N
N
H

Figure 3. Structure of Protocatechuate. 3,4-Dihydroxybenzoate has a high potential for hydrogen bonding via the two hydroxy groups and the carboxyl group.


COOH
OH
OH

CHAPTER II

MATERIALS AND METHODS

Test Organism

The microorganism used in this study was obtained from the stock culture collection of Dr. N.N. Durham, Oklahoma State University. This organism was tentatively identified by Montgomery (1966) as Pseudomonas fluorescens. This organism is a gram-negative, motile rod which forms smooth, raised colonies on nutrient agar. P. fluorescens exhibits a negative reaction for hydrogen sulfide production, indole production and nitrate reduction, and produces acid but no gas in glucose. The pigments fluorescein and pyocyanin are produced when the organism is grown on Bacto-Pseudomonas agar F and Bacto-Pseudomonas agar P, respectively.

Stock cultures of P. fluorescens were maintained on slants of succinate-salts synthetic medium, and stored at 4°C.

Media

The synthetic salts medium used in this investigation contained: 0.2 percent sodium chloride, 0.2 percent ammonium chloride, 0.32 percent potassium dihydrogen phosphate,

0.42 percent dipotassium hydrogen phosphate and 0.2 percent of the desired carbon source (succinate, acetamide, or glucose). The pH of the medium was adjusted to 6.8-7.0 with KOH prior to sterilization by autoclaving at 121°C with 15 lbs. 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. In studies where acetamide was used as the inducer of enzyme synthesis, a concentration of 0.08 M was used. Two percent agar (Difco) was added when a solid medium was required.

The mineral salts solution used to supplement the medium contained: 5.0 percent $MgSO_4 \cdot 7\ H_2O$, 0.1 percent $MnSO_4$, 1.0 percent $FeCl_3$ and 0.5 percent $CaCl_2$ in distilled deionized water. This solution was then sterilized by autoclaving as described above, and used after the suspension of minerals was allowed to settle (Durham, 1958).

Chemicals

Actinomycin D was obtained from Merk, Sharp and Dohme Research Laboratory, the hydroxypyrazole was provided by Dr. K.D. Berlin, Oklahoma State University, and mitomycin C was obtained from Sigma Chemical Company. Stock solutions of these three compounds were prepared in sterile distilled water and stored at 4°C. Protocatechuate (Aldrich Chemical Co., Inc.) stock solutions were prepared and filter sterilized prior to usage. The concentrations of all chemicals used in this study are given in the text.

Enzyme Induction

P. fluorescens cells were grown in succinate-salts medium for approximately 12 hours. The cells were harvested by centrifugation (5,900 X g for 10 minutes) and washed twice with 0.01M potassium phosphate buffer, pH 7.0 (0.068 percent potassium dihydrogen phosphate, 0.088 percent dipotassium hydrogen phosphate). The washed cells were then suspended in 0.01 M phosphate buffer and used to inoculate the growth medium (containing succinate plus acetamide), and the appropriate combinations of the compounds to be tested, to an initial O.D. of 0.2 at 540 nm. Samples (0.25 ml) were removed at the specified time intervals and immediately placed in the freezer (-14°C) for subsequent enzyme determinations.

Assay Method

The assay for the determination of enzyme synthesis was based on the reaction of acyl phosphates with hydroxylamine at pH 6.5 to 7.0 to form hydroxamic acids (Lipmann and Tuttle, 1945). Spectrophotometric procedures can then be used to quantitate the hydroxamic acids which will react with ferric salts to produce red to violet color complexes.

Preparation of the Standard Curve

A hydroxamic acid stock solution was prepared by dissolving 0.5 g of succinic anhydride in 20 ml of freshly neutralized 2.0 M hydroxylamine hydrochloride. This solu-

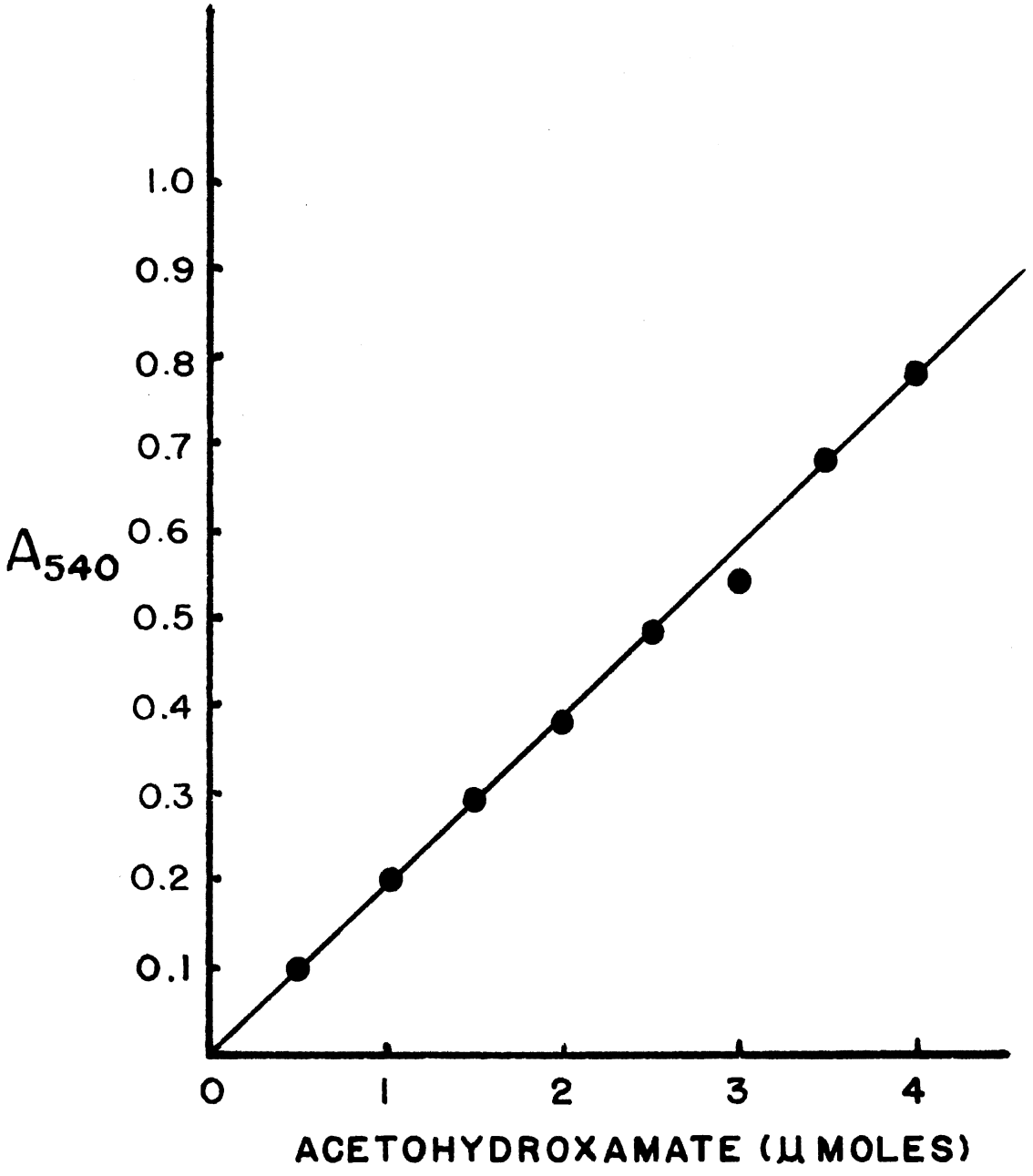
tion was allowed to stand 10 minutes. Thirty ml of distilled water was then added to give a final volume of 50 ml. One ml of this stock solution was diluted to a final liquid volume of 40 ml with distilled water to produce the standard solution. Two ml of the standard solution was added to 1.0 ml of freshly neutralized hydroxylamine hydrochloride and allowed to stand for 10 minutes. Three ml of ferric chloride ($FeCl_3$) reagent (6.0 percent w/v in 2.0 percent HCl v/v) was added and the absorbance read in a Bausch and Lomb Spectronic 70 at 540 nm. The reading obtained from this solution was equivalent to 4.0 micromoles of acetohydroxamic acid. Different dilutions of the stock solution were prepared in a total volume of 40 ml to give varying concentrations of the acetohydroxamate. The absorbance of each dilution was measured, and a standard curve for acetohydroxamate was prepared (Fig. 4).

Amidase Activity

The Brammer and Clarke (1964) modification of the Lipmann and Tuttle (1945) method for hydroxamic acid determination was used in this study. This modification is based on quantitative determination of acetohydroxamate, which is the end product of the translocase reaction of the amidase enzyme. The amidase transfers the acyl group of the substrate amides to hydroxylamine to form acylhydroxamates (Kelly and Kornberg, 1962). This reaction is shown below.

$$CH_3C(O)NH_2 + NH_2OH \text{ ----- } CH_3C(O)NHOH + NH_3$$

Figure 4. Standard curve. Used to determine the micro-moles of acetohydroxamate produced by the amidase enzyme.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A_{540}
0
1
2
3
4
ACETOHYDROXAMATE (μ MOLES)

The substrate mixture was prepared by mixing equal volumes of 0.4 M acetamide solution, 2.0 M freshly neutralized hydroxylamine hydrochloride, and 0.1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (tris) buffer (pH 7.2). The frozen samples which were taken from the culture flasks were thawed and 0.75 ml of the substrate mixture was added to each. The tubes were incubated at 37°C for 15 minutes, and the reaction was stopped by adding 2.0 ml of $FeCl_3$ (6.0 percent w/v in 2.0 percent HCl v/v). The absorbance was then read at 540 nm using a Bausch and Lomb Spectronic 70, and the amount of acetohydroxamate produced was calculated using the standard curve. The specific activity was measured, and one unit of amidase was defined as the amount of enzyme that would produce one micromole of acetohydroxamate per milligram dry cell weight per 15 minutes.

Deoxyribonucleic Acid Isolation

Growth of Cells

P. fluorescens deoxyribonucleic acid (DNA) was isolated using the Saito and Miura (1963) modification of the procedure outlined by Marmur (1961). *P. fluorescens* cells were grown approximately 12 hours on a reciprocal shaker in a 2800 ml Fernback flask containing 500 ml of succinate-salts medium. These cells were then used to inoculate 3 liters of succinate-salts medium distributed in 6 Fernback flasks. The flasks were incubated at 37°C on a reciprocal shaker for 5-6 hours before harvesting the cells by centri-

fugation (8,000 X g for 10 minutes). Usually 12 g of packed cells were obtained by this method. The cells were divided into two 6 g masses and stored at (-14°C) until needed for DNA extraction.

Extraction of DNA

Six grams of packed cells were suspended in 6 ml of saline-disodium ethylene diamine tetracetate (EDTA) (0.15 M NaCl-0.10 M EDTA). Lysozyme was added giving a final concentration of 2 mg/ml. This suspension was then incubated at 37°C. After 45 minutes of incubation, 50 ml of tris-SDS buffer (0.10 M tris-buffer-1.0 percent SDS-0.10 M NaCl, pH 9.0) was added, followed by the addition of an equal volume of redistilled phenol saturated with water prior to use. This mixture was placed in a glass stoppered round bottom flask and shaken for 20 minutes in an ice bath (below 4°C). The emulsion was separated into two layers by slow speed centrifugation (650 X g for 10 minutes). After the slow speed separation, the upper phase was clarified by centrifugation (16,300 X g for 10 minutes) to remove any remaining cell debris. The nucleic acids were precipitated by gently mixing the clarified suspension with two volumes of cold ethanol (95 percent). The thread-like precipitate was collected on a glass rod and dissolved in 20 ml of dilute saline-citrate (1:10 dilution of 0.15 M NaCl-0.015 M trisodium citrate, pH 7.0), followed by the addition of 2.2 ml of acetate-EDTA (3.0 M sodium acetate-0.001 M EDTA, pH

7.0). Ribonuclease (600 µg/ml), previously heated for 10 minutes at 80°C, was added to the dissolved nucleic acid to give a final concentration of 50 µg/ml. The suspension was placed at 37°C for 30 minutes. The digest was cooled and mixed with an equal volume of water-saturated phenol and shaken for 10 minutes in an ice bath (below 4°C). The suspension was again separated by centrifugation (650 X g for 10 minutes) and the upper phase clarified (16,300 X g for 10 minutes). DNA was again precipitated by gently mixing two volumes of cold ethanol (95 percent) with the suspension; the DNA was collected on a glass rod and dissolved in 20 ml of the dilute saline-citrate and 2.2 ml of acetate-EDTA for the complete elimination of RNA. While the solution was rapidly stirred, 0.54 volumes of isopropanol was added slowly, and the DNA precipitate was collected with a glass rod. The RNA elimination procedure was repeated once more using only one-half of the required volumes in the precipitation. DNA was then stored in ethanol (95 percent) at 4°C until used.

Stock solutions of DNA were prepared by dissolving DNA in saline-citrate and storing at 4°C. DNA concentration was determined by using a nomograph (distributed by California Corporation for Biochemical Research 3625 Medford St., Los Angeles 63, California) and the DNA absorbance ratio at 260-280 nm.

DNA Thermal Denaturation Studies

Thermal denaturation studies of P. fluorescens DNA, isolated as described above, were performed using the procedure of Kerr (1963). Tubes containing DNA and DNA plus the test compounds were prepared in a total volume of 2.5 ml of saline-citrate (0.15 M NaCl-0.015 M trisodium citrate, pH 7.0). These mixtures were incubated 30 minutes at 37°C. An equal volume of methanol (2.5 ml) was added to each tube which was shaken thoroughly; the tube was plugged with a rubber stopper. Thermal denaturation temperatures were reached by heating the DNA-containing mixtures and the appropriate controls in a laboratory water bath. When the desired temperature was attained, a 7 minute temperature equilibration period was observed. The tubes were removed from the water bath and placed in an ice bath for 5 minutes. Absorbancies were read on the Beckman DU Spectrophotometer at 260 nm and an absorbance ratio was calculated by dividing the absorbance at 260 nm of each temperature by the initial absorbance 260 nm at 37°C.

DNA Dialysis Procedure

DNA plus the appropriate combinations of compounds to be tested were prepared in saline-citrate buffer. These mixtures were then placed in dialysis tubing and dialyzed against 4 liters of saline-citrate buffer for the prescribed time intervals at 4°C with constant stirring and with the buffer changed at 24 hour intervals. After the dialy-

sis was complete, the samples were removed from the tubing for use in denaturation studies or in UV spectrophotometric studies.

Spectrophotometric Studies

The ultraviolet absorption spectra of aqueous solutions containing the prescribed concentrations and combinations of compounds were conducted using a Cary 14 recording spectrophotometer at room temperature in 1 centimeter light path quartz cuvettes.

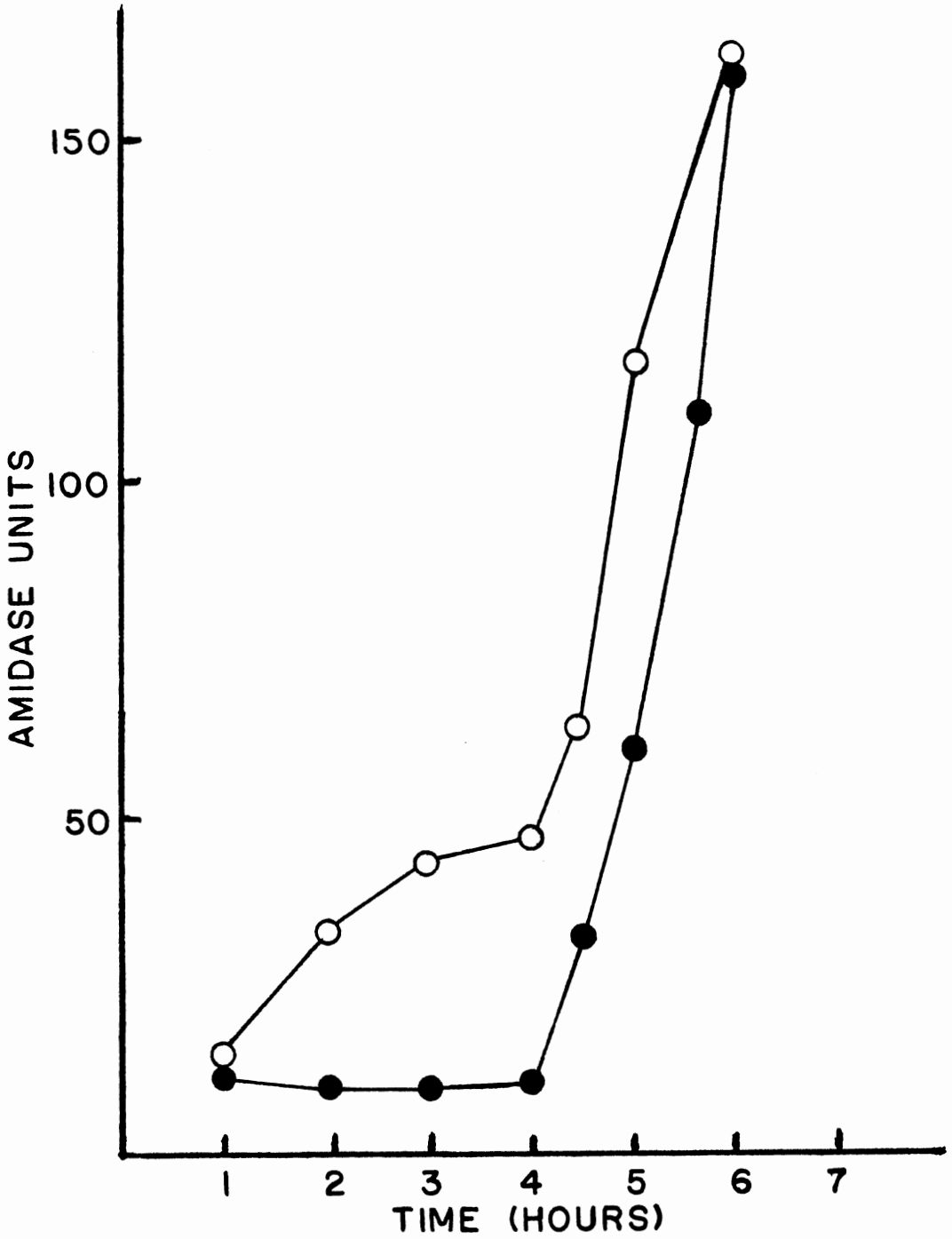
CHAPTER III

RESULTS AND DISCUSSION

Amidase Synthesis by Cells Grown in Different Carbon Sources

Cells were grown in succinate-salts medium plus acetamide and acetamide-salts medium, and enzyme production was measured in the two media (Fig. 5). The enzyme production pattern of cells grown in the acetamide-salts medium was characteristic of that described by Ferguson (1970). A diphasic induction pattern for amidase synthesis was observed. Kelly and Kornberg (1962) also reported a similar induction pattern in acetamide-salts medium. They proposed that phase I of the enzyme synthesis prior to the plateau was due to induction by acetamide. This phase was complete when the acetamide content of the medium dropped to a low level. Phase II, the rapid initiation of enzyme synthesis following the plateau, occurred during subsequent growth on the acetate that was formed during the hydrolysis of acetamide. The induction pattern of cells grown in the succinate-salts plus acetamide medium, compared to the cells with acetamide as the sole carbon source, did not exhibit the diphasic induction pattern.

Figure 5. Comparison of Amidase Synthesis by P. fluorescens Grown in Media with Two Different Carbon Sources. ●, succinate-salts plus acetamide; ○, acetamide-salts medium.


150
100
50
AMIDASE UNITS
1
2
3
4
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6
7
TIME (HOURS)

The quicker production of enzyme by cells grown in acetamide as the carbon source probably resulted from the cells immediate need for enzyme to utilize the acetamide for growth. Cells grown with succinate as carbon source and acetamide as inducer had no immediate need for the acetamide, and thus did not begin synthesizing the enzyme until the succinate carbon source was exhausted. These results are consistent with those reported by Brammer and Clarke (1964), that some tricarboxylic acid intermediates acted as repressors of amidase synthesis.

Comparison of Actinomycin D Inhibition of Amidase Synthesis by Cells Grown in Two Different Carbon Sources

The actinomycin D inhibition of amidase synthesis of cells grown in succinate-salts plus acetamide medium and acetamide-salts medium was followed to compare the sensitivity of enzyme synthesis to actinomycin D in cells grown in the presence of different carbon sources. Actinomycin D (in concentrations of 0.004 μg/ml, 0.04 μg/ml, 0.4 μg/ml, and 4.0 μg/ml) was added to cells growing in the two different media. In the succinate-salts medium, amidase synthesis was inhibited only 3% by a concentration of 0.4 μg/ml of actinomycin D, while in the acetamide-salts medium, actinomycin D (at a concentration of 0.04 μg/ml) gave 6% inhibition of amidase synthesis. This may occur because of the additional carbon source in the succinate-salts plus

acetamide medium, resulting in the requirement for higher concentrations of actinomycin D to inhibit amidase synthesis (Table I). Actinomycin D at much smaller concentrations inhibited amidase synthesis in the acetamide-salts medium, possibly due to only one carbon source being available or a change in the cells permeability to actinomycin D, allowing smaller concentrations of actinomycin D to inhibit the cells. Thus, higher concentrations of actinomycin D were required to inhibit amidase synthesis in succinate-salts plus acetamide than acetamide-salts medium.

TABLE I

PERCENT INHIBITION OF ENZYME SYNTHESIS
BY DIFFERENT CONCENTRATIONS
OF ACTINOMYCIN D

Concentration of Actinomycin D	Succinate-Salts Medium	Acetamide-Salts Medium
0.004 μg/ml	0%	0%
0.04 μg/ml	0%	6%
0.4 μg/ml	3%	32%
4.0 μg/ml	100%	100%

In the following experiments involving the amidase system, succinate-salts plus acetamide medium will be used because a diphasic induction pattern does not occur, making it easier to interpret the results. Also higher concentrations of actinomycin D can be used in the experiments. In experiments requiring a non-inhibitory concentration of

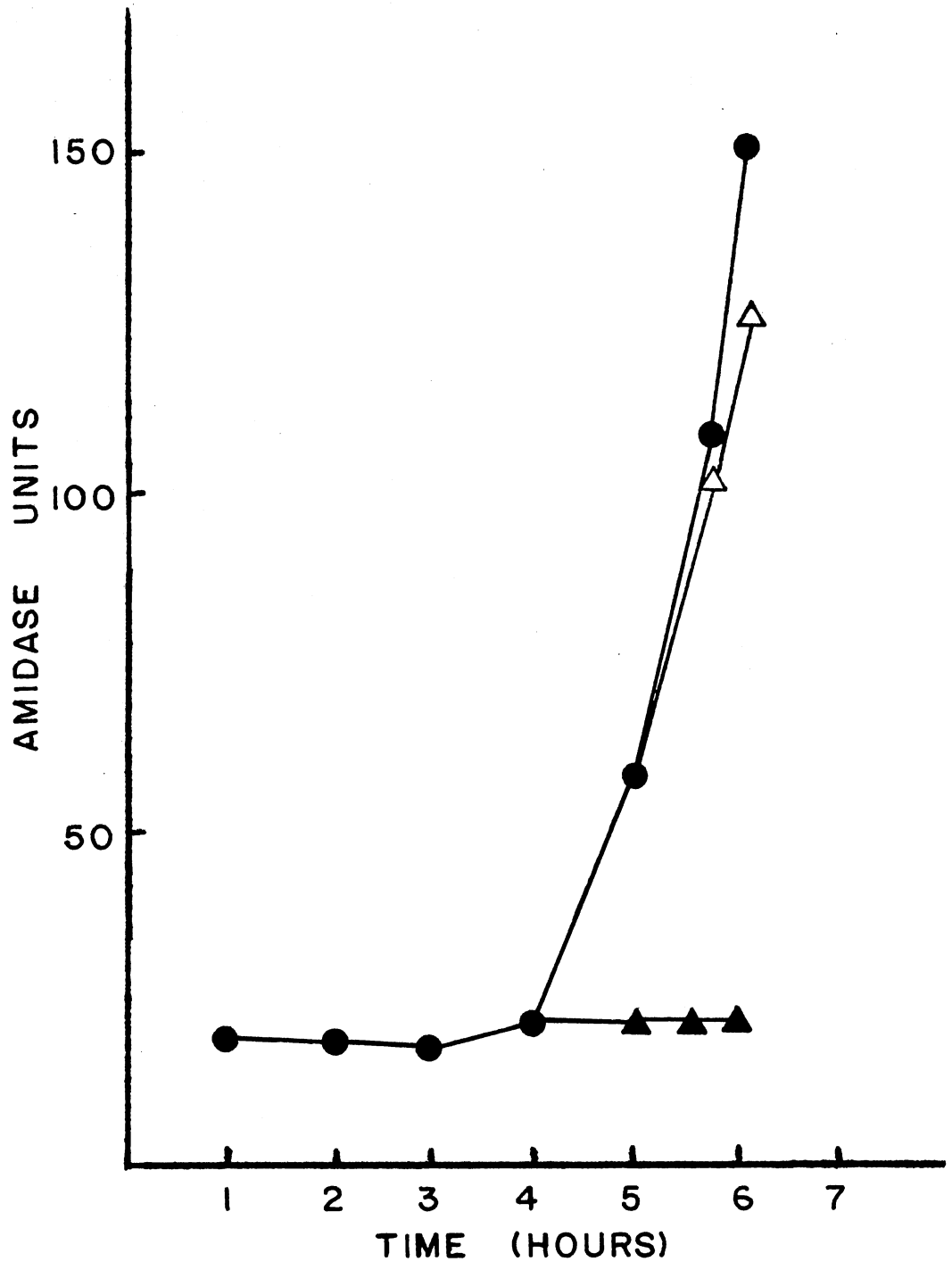
actinomycin D, 0.4 μg/ml was used, and a concentration of 4.0 μg/ml was used as an inhibitory concentration.

An experiment was conducted to determine if actinomycin D was inhibiting the actual synthesis of the enzyme itself or possibly acting on the amidase enzyme to inactivate it. The latter would make it impossible to assay for the enzyme even if it was synthesized by the cells. Cells were allowed to grow in succinate-salts plus acetamide medium, and, at 5 hours, actinomycin D at a concentration of 4.0 μg/ml was added. Actinomycin D added after 5 hours inhibited enzyme synthesis only a small amount. Actinomycin D added at time zero completely inhibited enzyme synthesis (Figure 6). The cells in which actinomycin D was added at 5 hours had begun to synthesize the enzyme for which an assay was made. The results indicated that the presence of actinomycin D did not inactivate the enzyme already present, but had begun to inhibit the synthesis of it.

Potentiation of Actinomycin D by the Hydroxypyrazole in Succinate-Salts Plus Acetamide Medium

The effect of the hydroxypyrazole on amidase synthesis was studied by adding different concentrations of the hydroxypyrazole to the cells. Amidase synthesis by P. fluorescens in (succinate, glucose, or acetamide)-salts medium was not affected by the hydroxypyrazole at a maximum concentration of 30 μg/ml.

Figure 6. Effects of Actinomycin D on the Activity of the Amidase Enzyme. ●, succinate-salts plus acetamide control; ▲, actinomycin D, 4.0 µg/ml; Δ, actinomycin D, 4 µg/ml added at 5 hours.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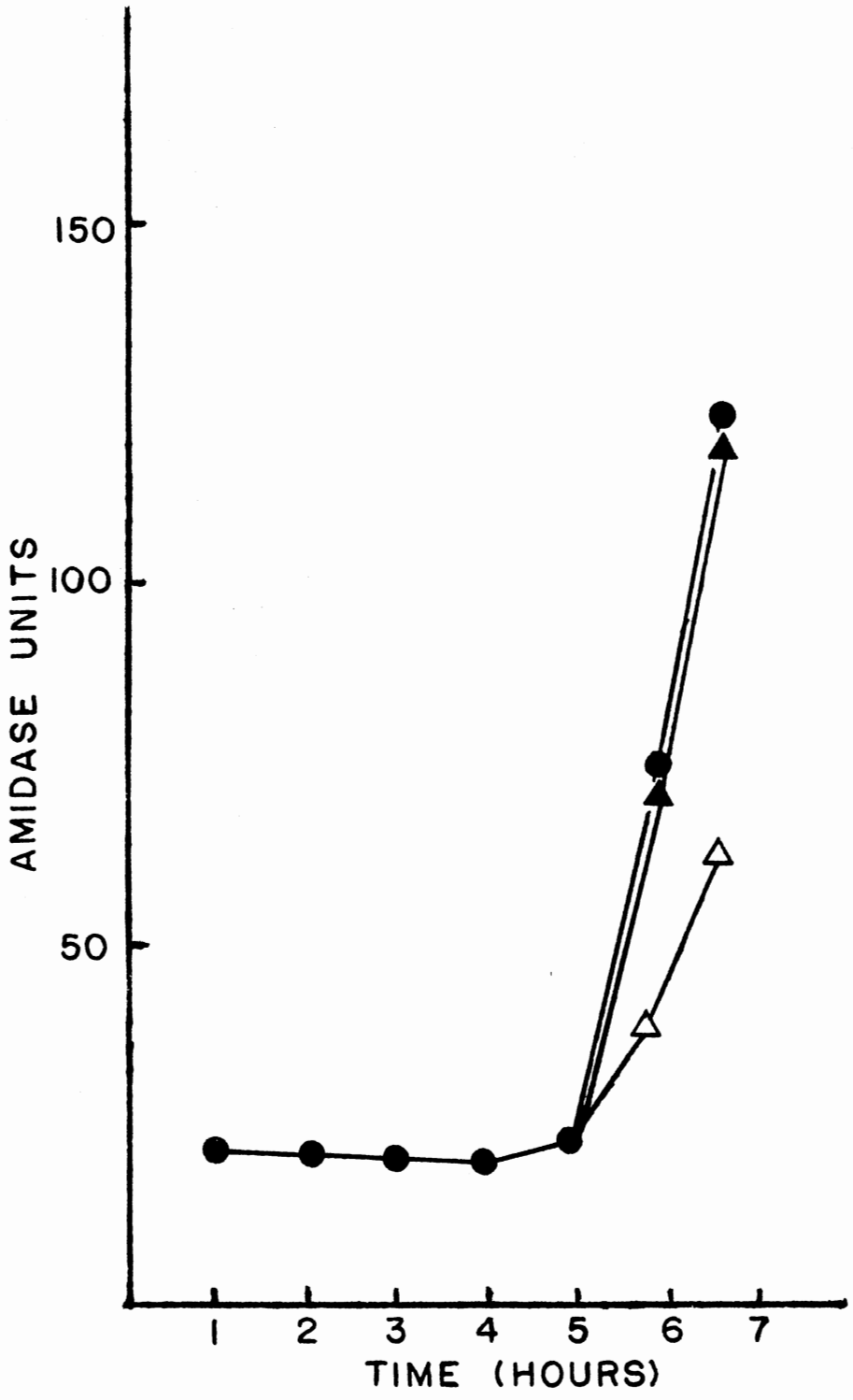
TIME (HOURS)

An interesting phenomenon occurred when the hydroxypyrazole and actinomycin D were combined. The action of actinomycin D, at 0.4 μg/ml (a subinhibitory concentration) was potentiated by the hydroxypyrazole at 20 μg/ml. The compounds separately at these concentrations had no effect upon amidase synthesis, but in combination, a 50% inhibition of amidase synthesis was observed (Fig. 7). Chesnut et al. (1974) provided proton-magnetic-resonance and ultraviolet spectroscopic evidence for a molecular complex between actinomycin D and the hydroxypyrazole. The potentiation of actinomycin D by the hydroxypyrazole was postulated by Chesnut et al. (1974) to occur via a complex between the two compounds rather than the hydroxypyrazole potentiating actinomycin D by acting at the membrane and allowing quicker entry of actinomycin D into the cells.

Experiments were next conducted to investigate the hydroxypyrazole's mechanism of potentiating actinomycin D. Cells were grown in the presence of the hydroxypyrazole at a concentration of 20 μg/ml, which is the same concentration used in the potentiation experiment. The cells were allowed to grow for 12 hours, were then washed in 0.01M phosphate buffer (pH 7.0) and were resuspended in succinate-salts plus acetamide medium. Actinomycin D was added to give a final concentration of 0.4 μg/ml. Amidase synthesis by these cells was followed to determine if potentiation of a subinhibitory concentration of actinomycin D occurred. Potentiation did not occur, indicating that the ability of

Figure 7. Potentiation of Actinomycin D by the Hydroxypyrazole in Succinate-Salts plus Acetamide Medium. ●, succinate-salts plus acetamide control and hydroxypyrazole, 20 μg/ml; ▲, actinomycin D, 0.4 μg/ml; △, actinomycin D, 0.4 μg/ml plus hydroxypyrazole, 20 μg/ml.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AMIDASE UNITS
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TIME (HOURS)

the hydroxypyrazole to potentiate actinomycin D action does not result from the hydroxypyrazole alone (Table II). Potentiation of actinomycin D most likely occurred when the two compounds combined, perhaps as the complex proposed by Chesnut et al. (1974).

TABLE II

INHIBITION OF ENZYME SYNTHESIS RESULTING FROM ALTERNATING GROWTH OF CELLS IN ACTINOMYCIN D AND HYDROXYPYRAZOLE

Compound Added Initial Time	Compound Added After 12 Hours	Percent of Enzyme Inhibition
Hydroxypyrazole (20 μg/ml)	Actinomycin D (0.4 μg/ml)	0%
Actinomycin D (0.4 μg/ml)	Hydroxypyrazole (20 μg/ml)	44%

The previous experiment was conducted to examine the action of the hydroxypyrazole and actinomycin D by adding actinomycin D to hydroxypyrazole grown cells. This procedure was reversed, and the hydroxypyrazole was added to cells grown in the presence of 0.4 μg/ml of actinomycin D. The cells were allowed to grow for 12 hours in the presence of actinomycin D, were washed in 0.01M phosphate buffer and were suspended in succinate-salts plus acetamide medium. The hydroxypyrazole was added to give a final concentration of 20 μg/ml. Amidase synthesis was again followed. However, potentiation did occur when the cells were exposed to to the hydroxypyrazole (Table II). This lends support to

the hypothesis that the hydroxypyrazole is not potentiating actinomycin D by altering the cell permeability to allow quicker passage of actinomycin D into the cell, but instead it may occur via a complex between the two compounds.

Potentiation of Actinomycin D Activity by the Hydroxypyrazole in Glucose-Salts Plus Acetamide Medium

The degree of sensitivity of P. fluorescens to actinomycin D varies significantly depending on the carbon source in which the organism is growing. Cells grown in succinate exhibit pronounced sensitivity to actinomycin D when compared with glucose grown cells (Walker and Durham, 1975). Experiments were conducted to determine if the cells ability to synthesize amidase in the presence of actinomycin D or the combination of actinomycin D and the hydroxypyrazole was altered when the cells were grown in glucose-salts plus acetamide medium.

Amidase synthesis was followed by the same procedure as in previous experiments except the carbon source in the growth medium was changed. Enzyme production of cells with glucose as their carbon source was much quicker than with cells with succinate as the carbon source. That is, enzyme formation began after 2 hours of growth with glucose as compared to 4 hours required when succinate grown cells synthesized amidase. The overall quantity of enzyme produced by glucose grown cells was smaller, however, than that pro-

duced by succinate grown cells (Fig. 8).

Potentiation of actinomycin D at a concentration of 0.4 μg/ml by the hydroxypyrazole and with a concentration of 20 μg/ml for glucose as the carbon source could not be demonstrated. An experiment was conducted with actinomycin D concentrations increased from 0.4 μg/ml to 1.2 and 1.6 μg/ml and the hydroxypyrazole concentration was 20 μg/ml (Fig. 9). At these concentrations, potentiation of actinomycin D could be demonstrated in cells grown with glucose as the carbon source.

Walker and Durham (1975) established that glucose-grown cells have a higher concentration of lipopolysaccharide than succinate-grown cells, which may influence the permeability of the cells to actinomycin D. The higher concentration of actinomycin D required to demonstrate potentiation may possibly be explained by a decrease in permeability of the glucose grown cells to actinomycin D due to an increased amount of lipopolysaccharide.

Time Requirement for Actinomycin D-Hydroxypyrazole Complex Formation

The proposed interaction of actinomycin D and the hydroxypyrazole was investigated to determine the time requirement for complex formation. Amidase synthesis by P. fluorescens was followed as previously described. Actinomycin D at a concentration of 0.4 μg/ml and hydroxypyrazole at 20 μg/ml were combined and incubated (at room temperature) for

Figure 8. Comparison of Amidase Synthesis by *P. fluorescens* Grown in Succinate-Salts plus acetamide and Glucose-Salts plus Acetamide. ●, succinate-salts plus acetamide; ○, glucose-salts plus acetamide.

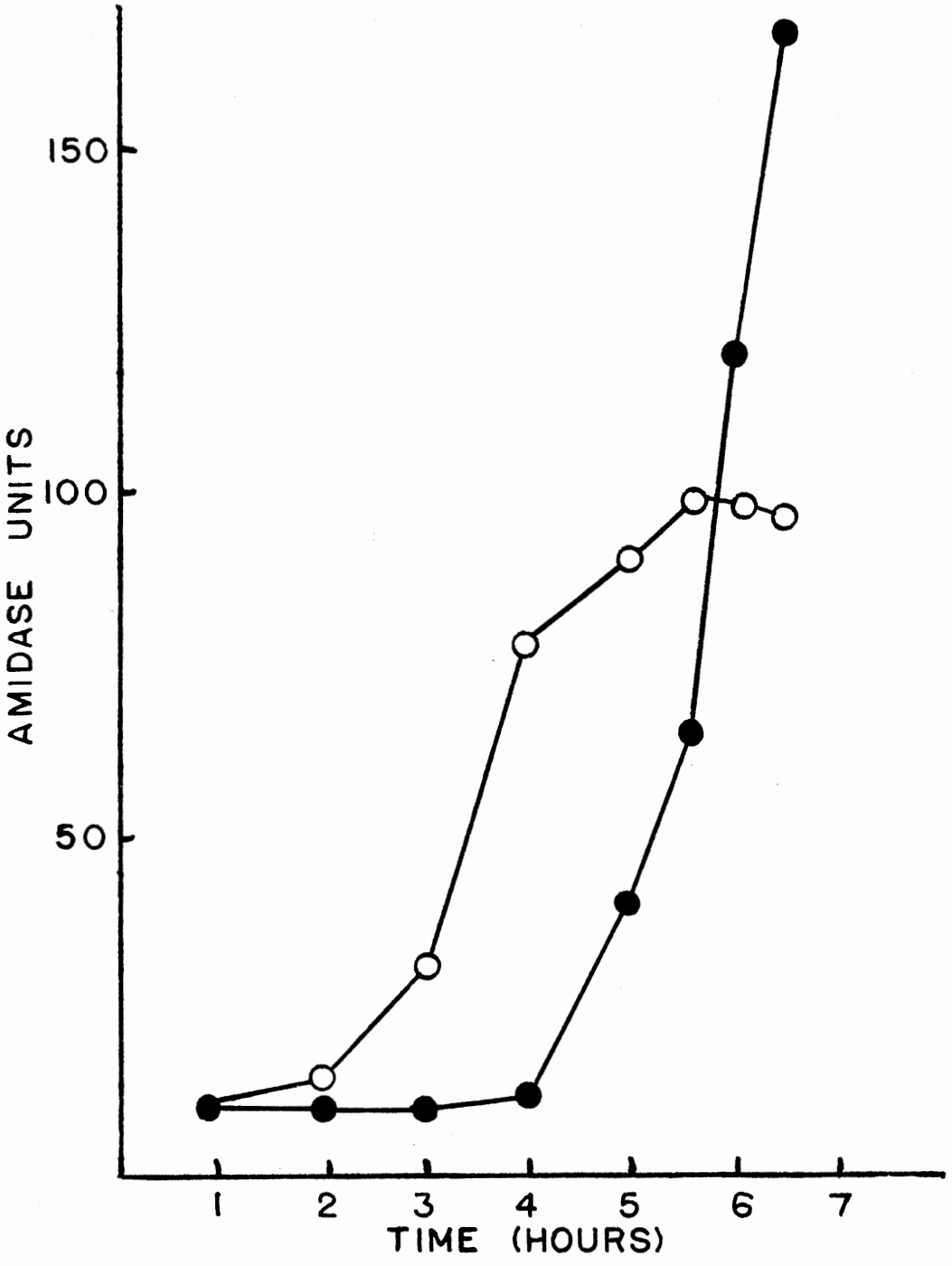
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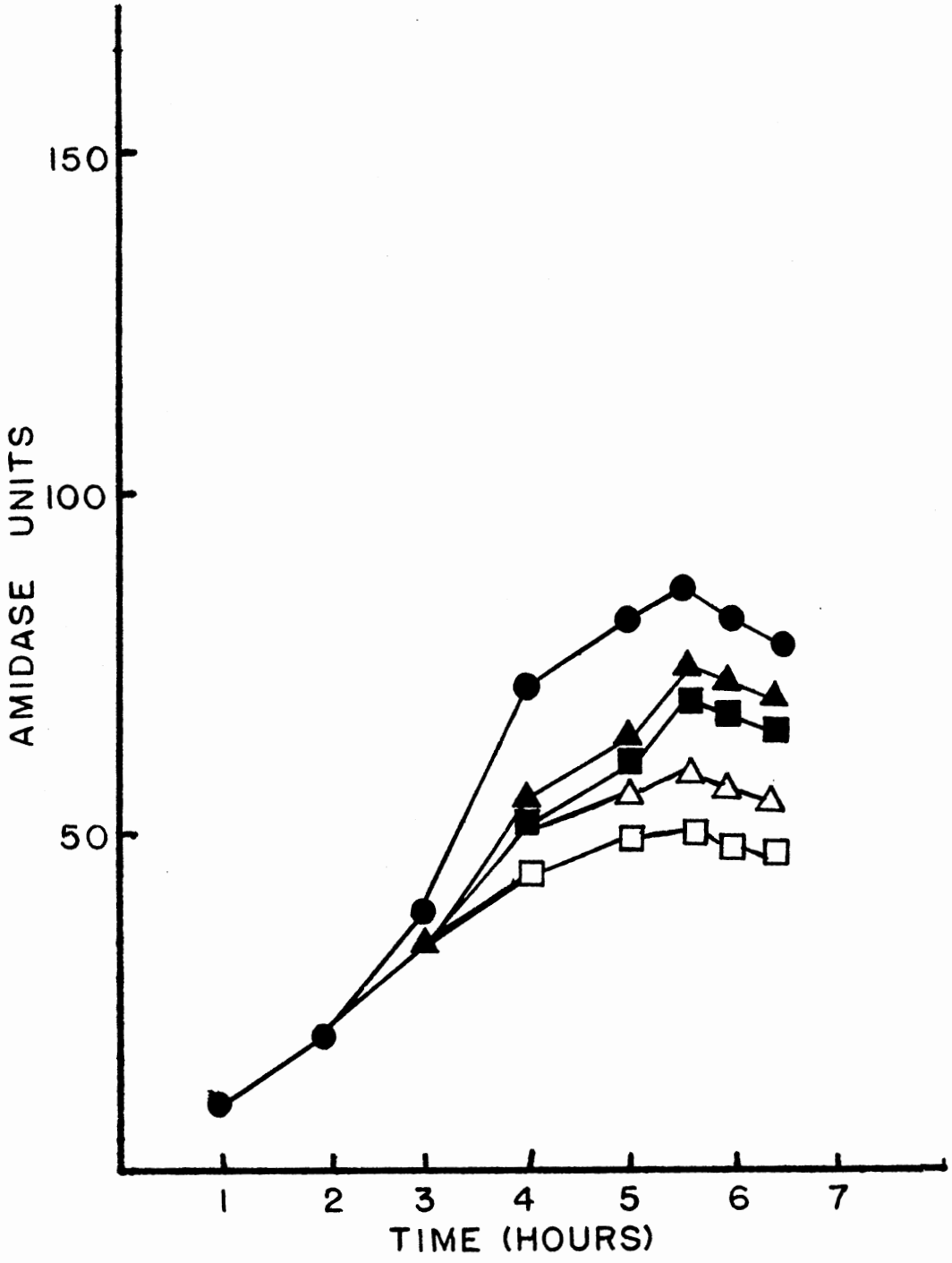
Figure 9. Potentiation of Actinomycin D by the Hydroxypyrazole in Glucose-Salts plus Acetamide Medium. ●, glucose-salts plus acetamide control and hydroxypyrazole, 20 μg/ml; ▲, actinomycin D, 1.2 μg/ml; ■, actinomycin D, 1.6 μg/ml; △, actinomycin D, 1.2 μg/ml plus hydroxypyrazole, 20 μg/ml; □, actinomycin D, 1.6 μg/ml plus hydroxypyrazole, 20 μg/ml.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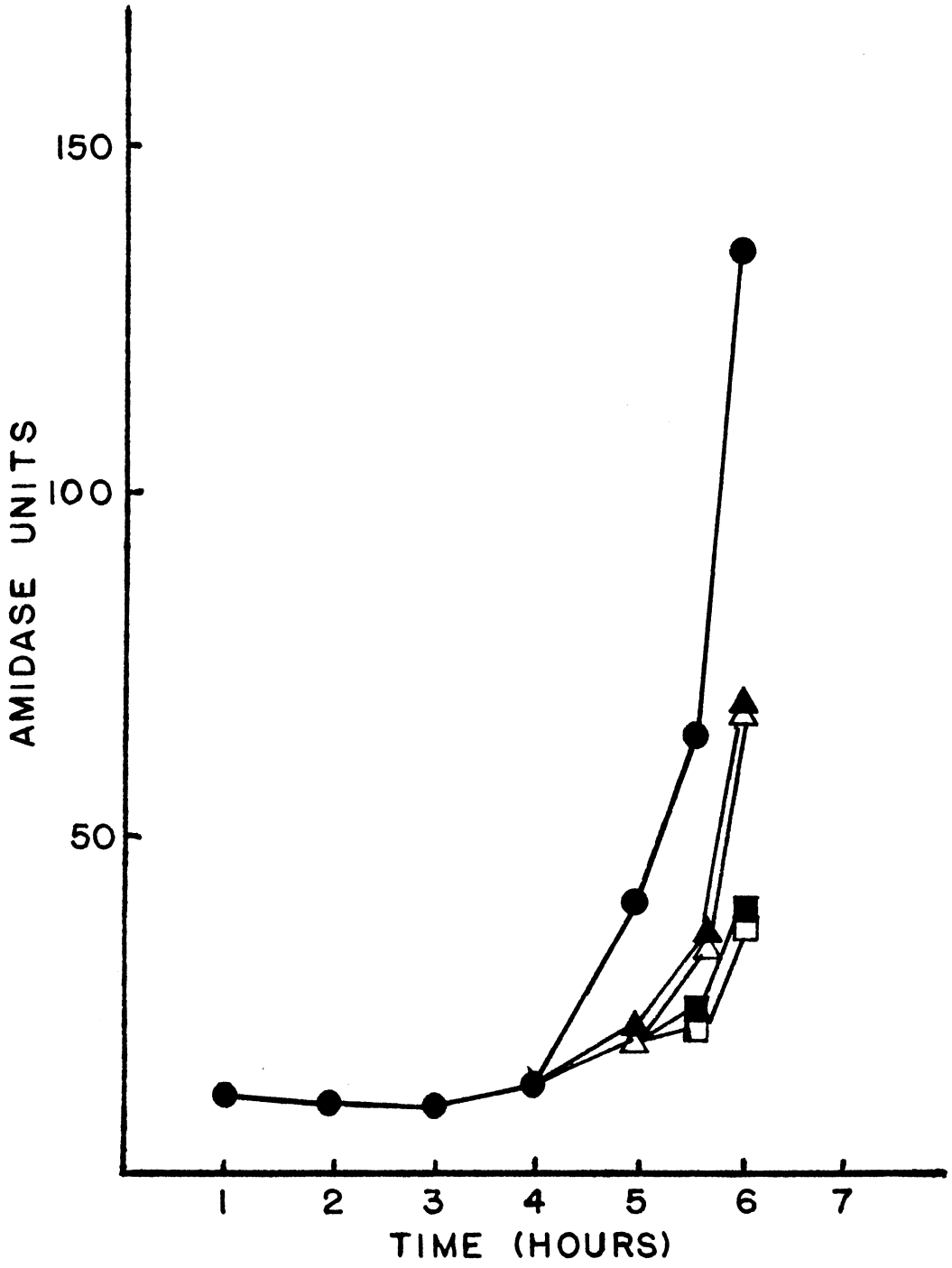
1 hour before addition to the culture flasks. The combination of the compounds incubated for 1 hour did not demonstrate an increased potentiation effect of actinomycin D as might be expected if the complexation of the compounds was not immediate. The same amount of amidase inhibition occurred if the compounds were incubated for 1 hour or added simultaneously to the culture flask at the beginning of the experiment. Results from this type of experimentation indicated that the complex formation probably occurred immediately between the two compounds.

Effect of the Hydroxypyrazole on Mitomycin C

It has been reported that the hydroxypyrazole demonstrates an action on sheep red blood cells and Bacillus subtilis protoplasts. The hydroxypyrazole also potentiates polymyxin and circulin, which are membrane active antibiotics. The action of mitomycin C was not potentiated by the hydroxypyrazole in experiments using sheep red blood cells and B. subtilis protoplasts (Haslam, 1973).

The lack of potentiation of mitomycin C by the hydroxypyrazole reported by Haslam (1973) was investigated using the inducible enzyme system. Concentrations of 0.025 μg/ml and 0.05 μg/ml of mitomycin C were used with a hydroxypyrazole concentration of 20 μg/ml. Enzyme production was followed as before to check for a possible potentiation of mitomycin C by the hydroxypyrazole. Figure (10) illustrates

Figure 10. Effects of the Hydroxypyrazole on Mitomycin C Inhibition of Amidase Synthesis. ●, succinate-salts plus acetamide control and hydroxypyrazole, 20 μg/ml; ▲, mitomycin C, 0.025 μg/ml; △, mitomycin C, 0.025 μg/ml plus hydroxypyrazole, 20 μg/ml; ■, mitomycin C, 0.05 μg/ml; □, mitomycin C, 0.05 μg/ml plus hydroxypyrazole, 20 μg/ml.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the results from the experiment. The combinations of mitomycin C and the hydroxypyrazole gave the same amount of enzyme inhibition as mitomycin C alone.

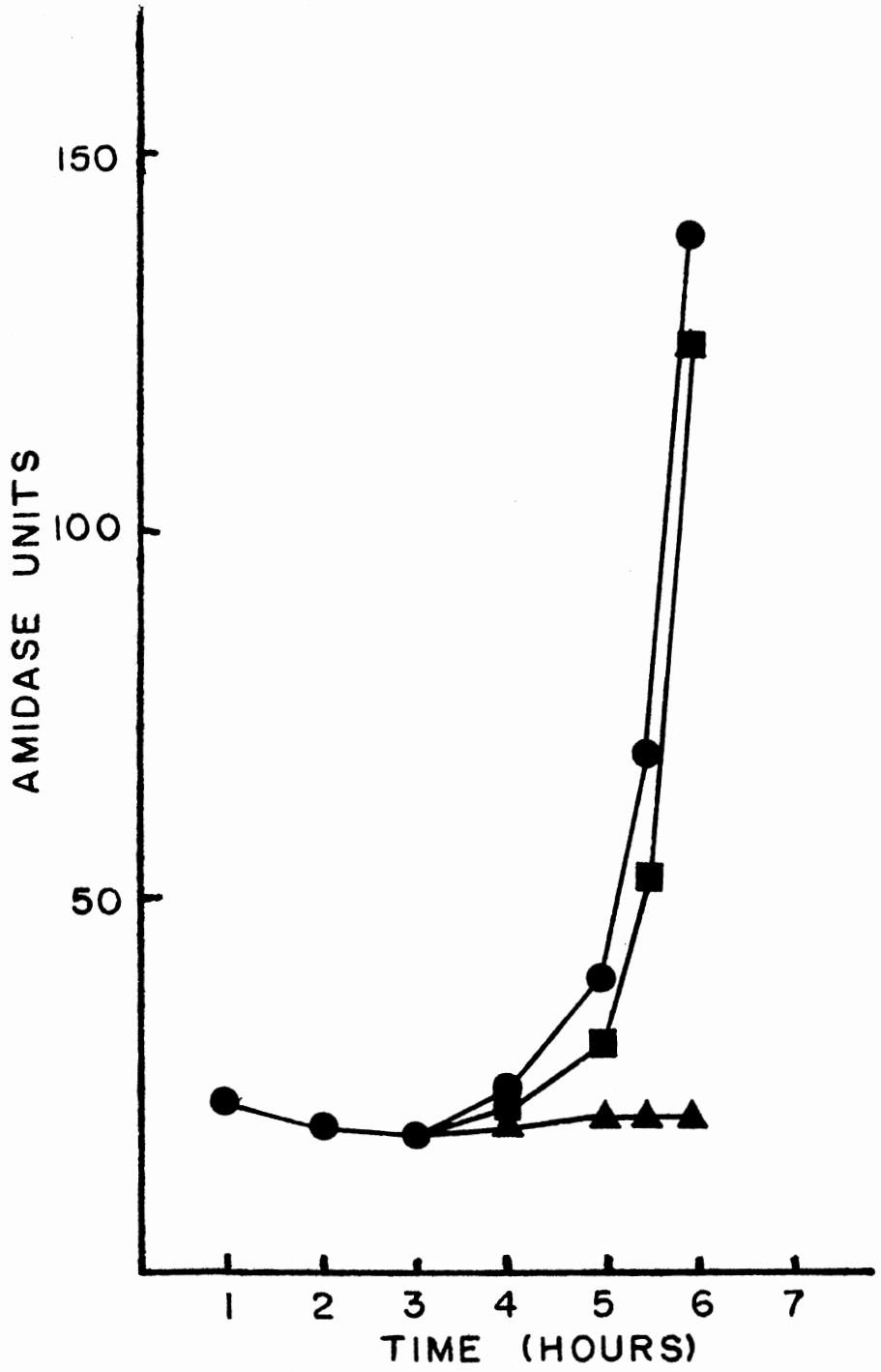
Results from this experiment support the finding of Haslam (1973) that the action of mitomycin C was not potentiated by the hydroxypyrazole. The lack of potentiation of mitomycin C may result from the lack of adequate complexation of the antibiotic with the hydroxypyrazole.

Reversal of Enzyme Inhibition by Protocatechuate

Ferguson (1970) established that protocatechuate would prevent and/or reverse actinomycin D inhibition of growth and amidase synthesis by P. fluorescens. Durham and Ferguson (1971) established that 3,4-dihydroxybenzoate (protocatechuate) was the most effective of the dihydroxybenzoate analogs in preventing and/or reversing the actinomycin D inhibition of amidase synthesis. Experiments were conducted to determine if protocatechuate had the potential to also reverse the potentiation of actinomycin D by the hydroxypyrazole.

The prevention of actinomycin inhibition was reestablished by following amidase synthesis of cells in the presence of an inhibitory concentration of actinomycin D and of cells in the presence of the inhibitory concentration of actinomycin D plus protocatechuate all added simultaneously to the culture flask. The results indicate (Fig. 11)

Figure 11. Reversal of Actinomycin D Inhibition of Amidase Synthesis by Protocatechuate. ●, succinate-salts plus acetamide control and protocatechuate, 120 µg/ml; ■, actinomycin D, 4.0 µg/ml plus protocatechuate, 120 µg/ml; ▲, actinomycin D, 4.0 µg/ml.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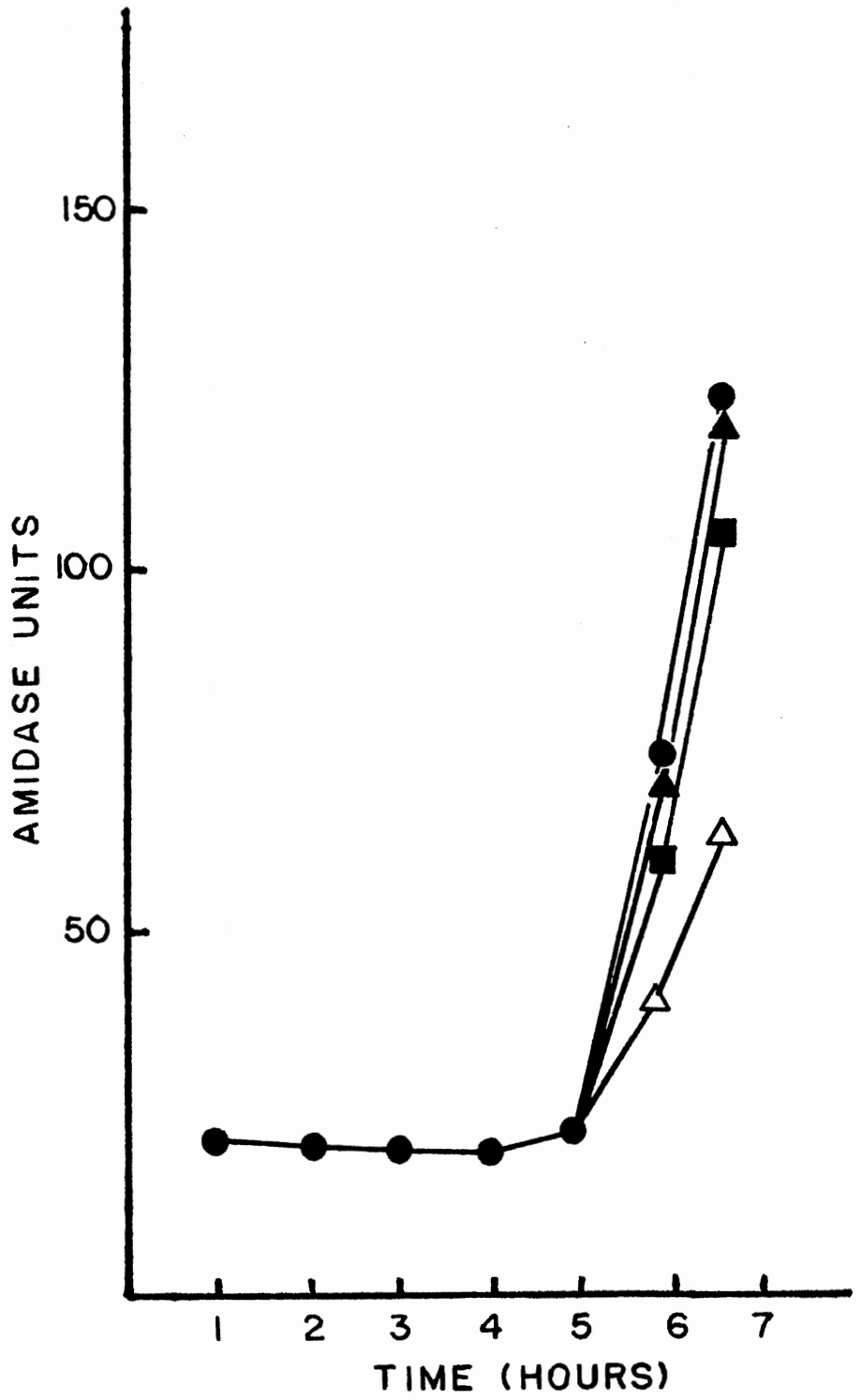
that protocatechuate does possess the ability to prevent the inhibition of amidase synthesis.

Actinomycin D was combined with the hydroxypyrazole and protocatechuate to determine if protocatechuate was able to prevent the potentiation effect of the hydroxypyrazole on actinomycin D. The appropriate controls and combination of the compounds were added to the cultures simultaneously and amidase synthesis was followed (Fig. 12). The results from this experiment indicate protocatechuate has the ability to prevent inhibition of actinomycin D which is potentiated by the hydroxypyrazole.

Experiments were also conducted which indicated that protocatechuate could not only prevent, but also reverse the inhibition of actinomycin D potentiated by the hydroxypyrazole. This was done by allowing the cell cultures to incubate with the combination of actinomycin D and the hydroxypyrazole for 2 hours before adding protocatechuate to the culture flasks. After addition of protocatechuate to the cultures, enzyme synthesis resumed at a normal level with about a 2 hour lag in the synthesis.

A possible interaction occurring between the three compounds via a trimember complex was next investigated by examining thermal denaturation of P. fluorescens DNA.

Figure 12. Reversal of Actinomycin D-Hydroxypyrazole Inhibition of Amidase Synthesis by Protocatechuate. ●, succinate-salts plus acetamide control and hydroxypyrazole, 20 μg/ml, and protocatechuate, 120 μg/ml; ▲, actinomycin D, 0.4 μg/ml; △, actinomycin D, 0.4 μg/ml plus hydroxypyrazole, 20 μg/ml; ■, actinomycin D, 0.4 μg/ml plus hydroxypyrazole, 20 μg/ml plus protocatechuate, 102 μg/ml.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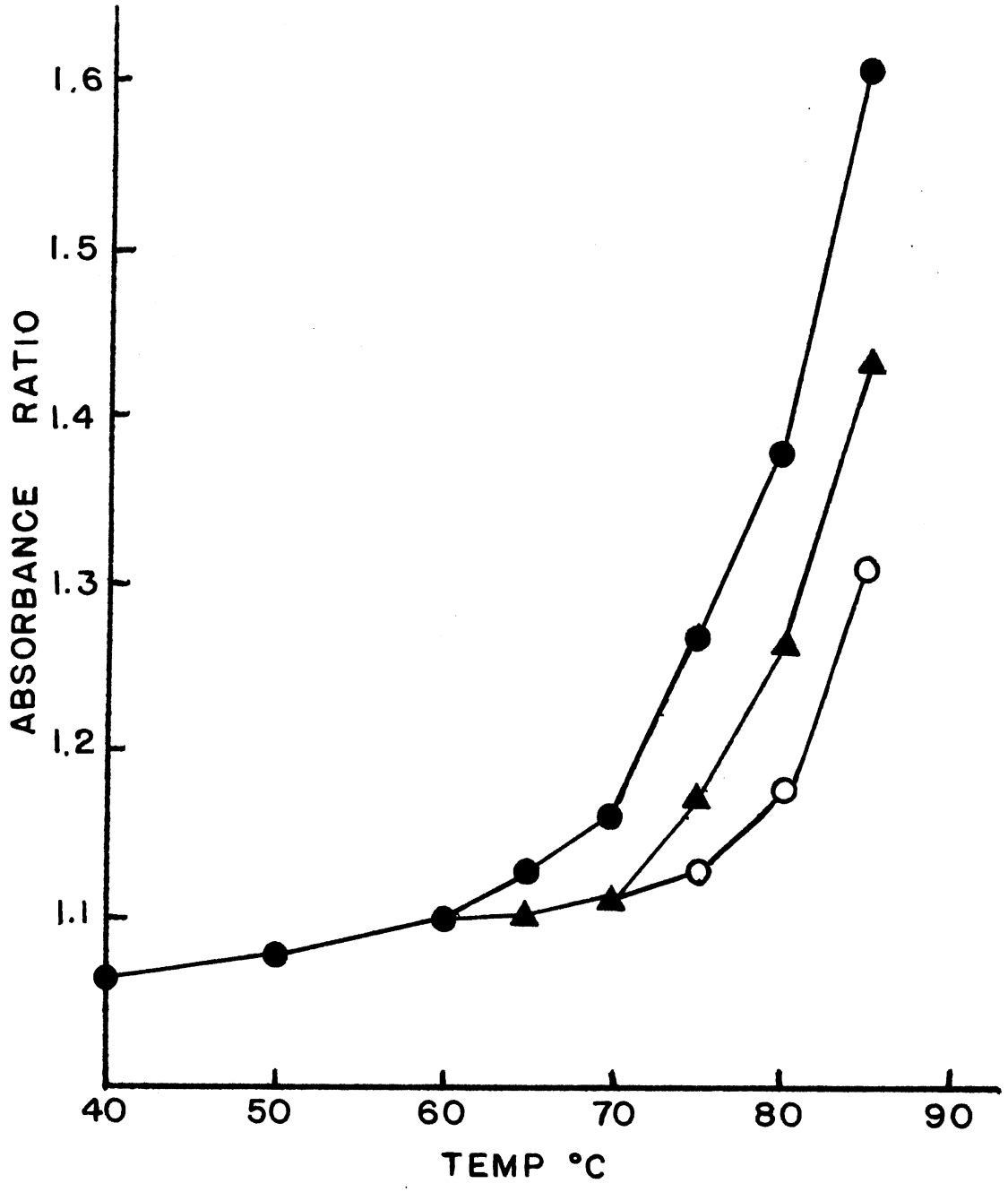
Interactions of Actinomycin D, Hydroxypyrazole, and Protocatechuate with DNA

It has been well established that actinomycin D has a primary site of action at the DNA level within a cell (Kirk, 1960). Evidence from earlier work (Haslam, 1973; Ferguson, 1970) indicated that possible complexation occurred between the hydroxypyrazole and actinomycin D, and between protocatechuate and actinomycin D. If a complexation of hydroxypyrazole and protocatechuate occurred with actinomycin D, which has a site of action at the DNA level, the hydroxypyrazole and protocatechuate may also possess a site of action at the DNA level.

A thermal denaturation experiment was conducted using P. fluorescens DNA as a control, and with DNA, at a concentration of 20 μg/ml, singly combined with actinomycin D at 20 μg/ml, hydroxypyrazole at 4 μg/ml, and protocatechuate at 20 μg/ml, to determine if the compounds individually would bind to or otherwise influence the thermal melting of DNA. Individually, all three compounds caused a shift in the DNA melting curve to higher temperatures. Actinomycin D and the hydroxypyrazole alone demonstrated approximately the same melting curve with a 5°C increase in thermal denaturation temperature. Protocatechuate demonstrated the greatest shift in the melting curve with a 10°C increase in thermal denaturation temperature (Fig. 13). Results from the experiment indicated that actinomycin D binds to DNA,

Figure 13. Effect of Actinomycin D, Hydroxypyrazole, and Protocatechuate Upon Thermal Denaturation of P. fluorescens DNA. ●, control DNA, 20 µg/ml; ▲, DNA and actinomycin D, 20 µg/ml, DNA and hydroxypyrazole, 4.0 µg/ml; ○, DNA and protocatechuate, 20 µg/ml.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and both the hydroxypyrazole and protocatechuate also have the potential to bind or interact with DNA.

Effects of Combinations of Compounds Upon DNA

The combination of the hydroxypyrazole with actinomycin D potentiated the inhibitory effect of actinomycin D as demonstrated in the amidase experiments. Protocatechuate was shown by the same method to reverse or prevent the inhibition of the combination of actinomycin D and the hydroxypyrazole, or the inhibition caused by actinomycin D alone.

The effects of the three combinations of compounds upon DNA was next investigated. The thermal denaturation of P. fluorescens DNA was used as the test system. Combinations used to react with DNA were: hydroxypyrazole and actinomycin D; actinomycin D, protocatechuate, and hydroxypyrazole. Both combinations of compounds produced a shift in the melting curve. Actinomycin D and hydroxypyrazole in combination increased the thermal denaturation end point 9°C. The thermal denaturation end point for the combination of actinomycin D, hydroxypyrazole and protocatechuate could not be determined since only a small change was observed at temperatures as high as 85°C (Fig. 14).

The shifts in melting curves of DNA with the trimember combination of compounds was greater than the shifts in the curve caused by each compound separately (Fig. 15). The combinations of all three compounds shifted the DNA melting

Figure 14. Effects of Combinations of Compounds Upon Thermal Denaturation of P. fluorescens DNA. ●, control DNA, 20 μg/ml; ■, DNA and actinomycin D, 20 μg/ml plus hydroxypyrazole, 4.0 μg/ml; Δ, DNA and actinomycin D, 20 μg/ml plus hydroxypyrazole, 4.0 μg/ml plus protocatechuate, 20 μg/ml.

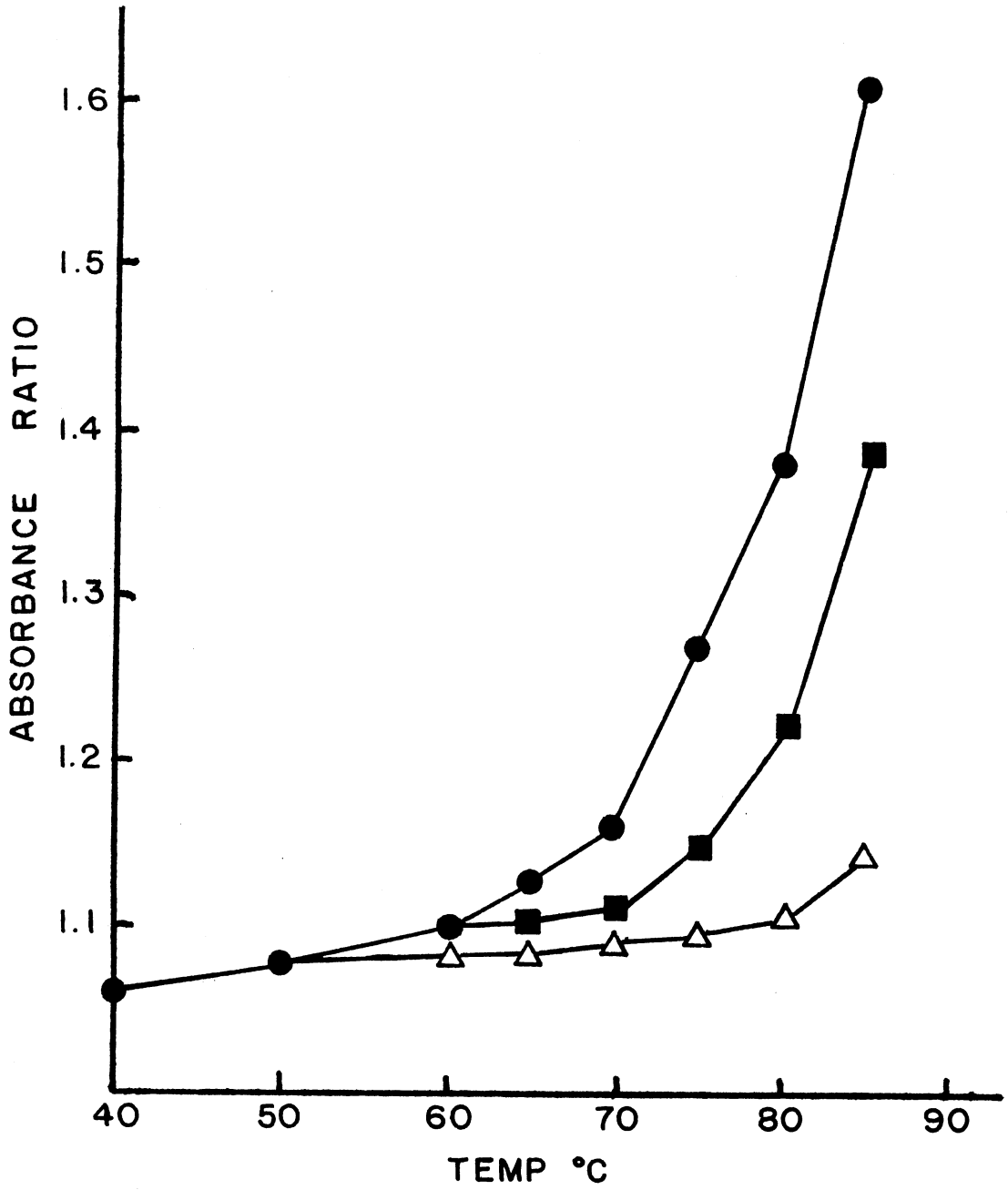
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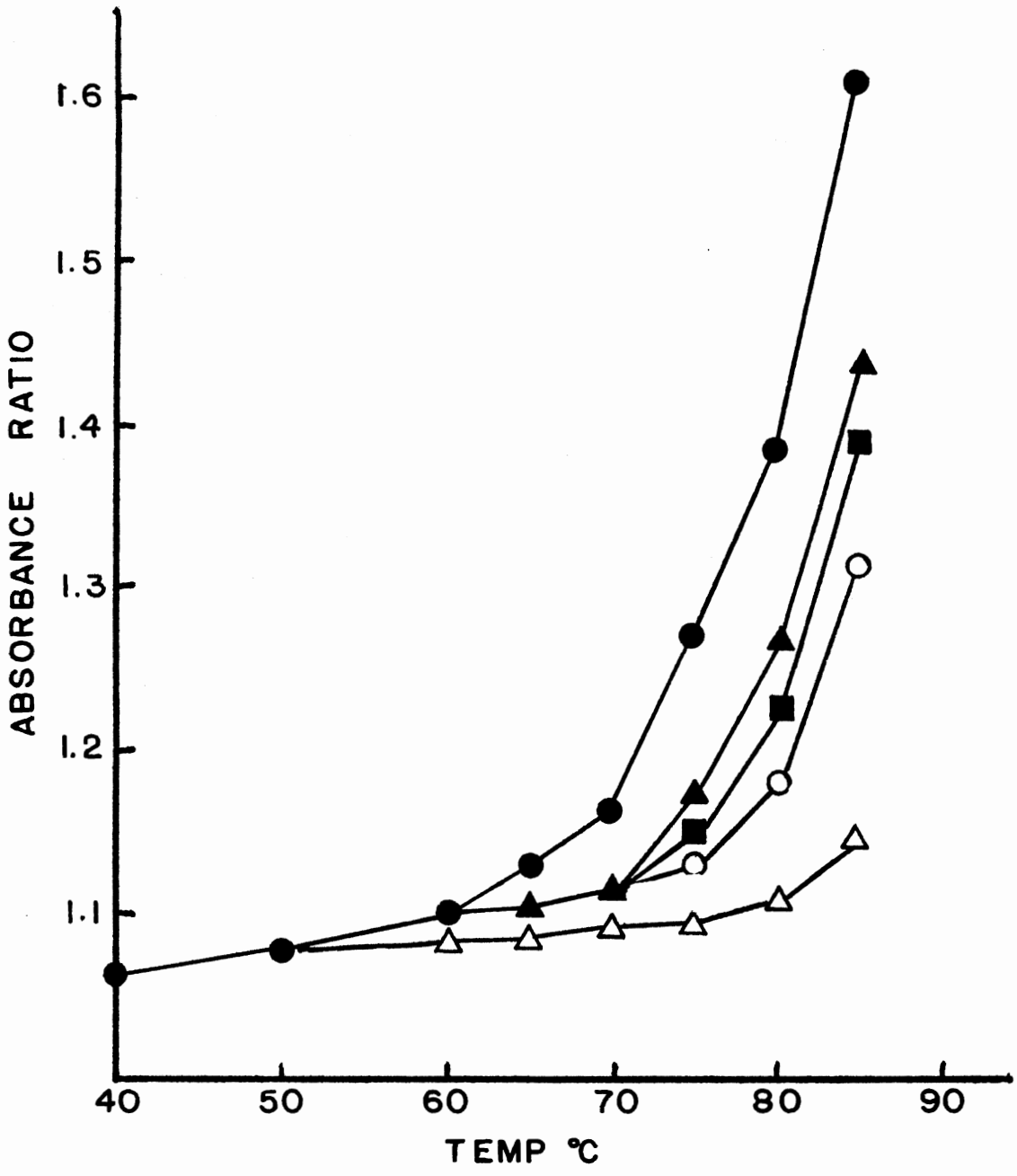
Figure 15. Combination of Figure 13 and 14. ●, control DNA 20 μg/ml; ▲, DNA and actinomycin D, 20 μg/ml, also DNA and hydroxypyrazole, 4.0 μg/ml; ○, DNA and protocatechuate, 20 μg/ml; ■, DNA and actinomycin D, 20 μg/ml plus hydroxypyrazole, 4.0 μg/ml; △, DNA and actinomycin D, 20 μg/ml plus hydroxypyrazole, 4.0 μg/ml plus protocatechuate, 20 μg/ml.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curve the greatest. The combination of the three compounds almost completely stabilized the DNA helix since only a small amount of thermal denaturation occurred. A 4°C increase in thermal denaturation temperature occurred with the combination of actinomycin D and hydroxypyrazole compared to the two compounds used individually. This increase in temperature and the complete stabilization of the DNA helix by all three compounds may support the hypothesis that the compounds are complexing and demonstrating new properties.

Several experiments were conducted in which the order of addition of the compounds to DNA was changed. This type of experiment was conducted to determine if there was any type of competition for the same binding site on DNA. One of the compounds was added to DNA and allowed to incubate (at room temperature) for ten minutes; the other compounds were added to the mixture, and thermal denaturation experiments were then conducted (Table III). Different combinations of addition sequences were tested and no correlations could be found between the addition sequence of the compounds and any effects upon thermal denaturation of DNA. The results seem to indicate that competition between the compounds for a specific binding site on the DNA helix was not occurring. This may support the hypothesis that the compounds were complexing. However, at this time, interpretation of these results remains unclear.

Earlier it was noted that P. fluorescens was more resistant to actinomycin D when grown in a medium containing

glucose as the carbon source. The reason for this resistance of the cells grown in glucose medium was believed to be due to more lipopolysaccharide being synthesized by the cells, which would decrease the cells permeability to actinomycin D. However, to determine if there might be some difference in DNA of glucose and succinate-grown cells, the following study was conducted.

TABLE III

THERMAL DENATURATION RESULTS FROM SEQUENCING THE COMPOUNDS ADDITION TO DNA

Compounds Added Initially	Compound Added After 10 Minutes	Thermal Denaturation Endpoint
Actinomycin D (20 μg/ml) Hydroxypyrazole (4 μg/ml)	None	74°C
Actinomycin D (20 μg/ml)	Hydroxypyrazole (4 μg/ml)	74°C
Hydroxypyrazole (4 μg/ml)	Actinomycin D (20 μg/ml)	74°C
Actinomycin D (20 μg/ml) Protocatechuate (20 μg/ml)	None	76°C
Actinomycin D (20 μg/ml)	Protocatechuate (20 μg/ml)	76°C
Protocatechuate (20 μg/ml)	Actinomycin D (20 μg/ml)	76°C

A thermal denaturation experiment was performed with

DNA isolated from P. fluorescens cells grown in glucose-salts medium to explore the possibility of a change in DNA which might alter the compounds binding. The same experimental procedure was followed as in earlier experiments only using DNA from glucose grown cells. The same melting curves were obtained in this experiment with DNA from glucose grown cells as those of DNA from succinate grown cells. The results obtained indicated that the resistance to actinomycin D was not a result of changes occurring at the DNA level but probably at the permeability barrier.

Dialysis of Compounds and DNA Prior to Thermal Denaturation

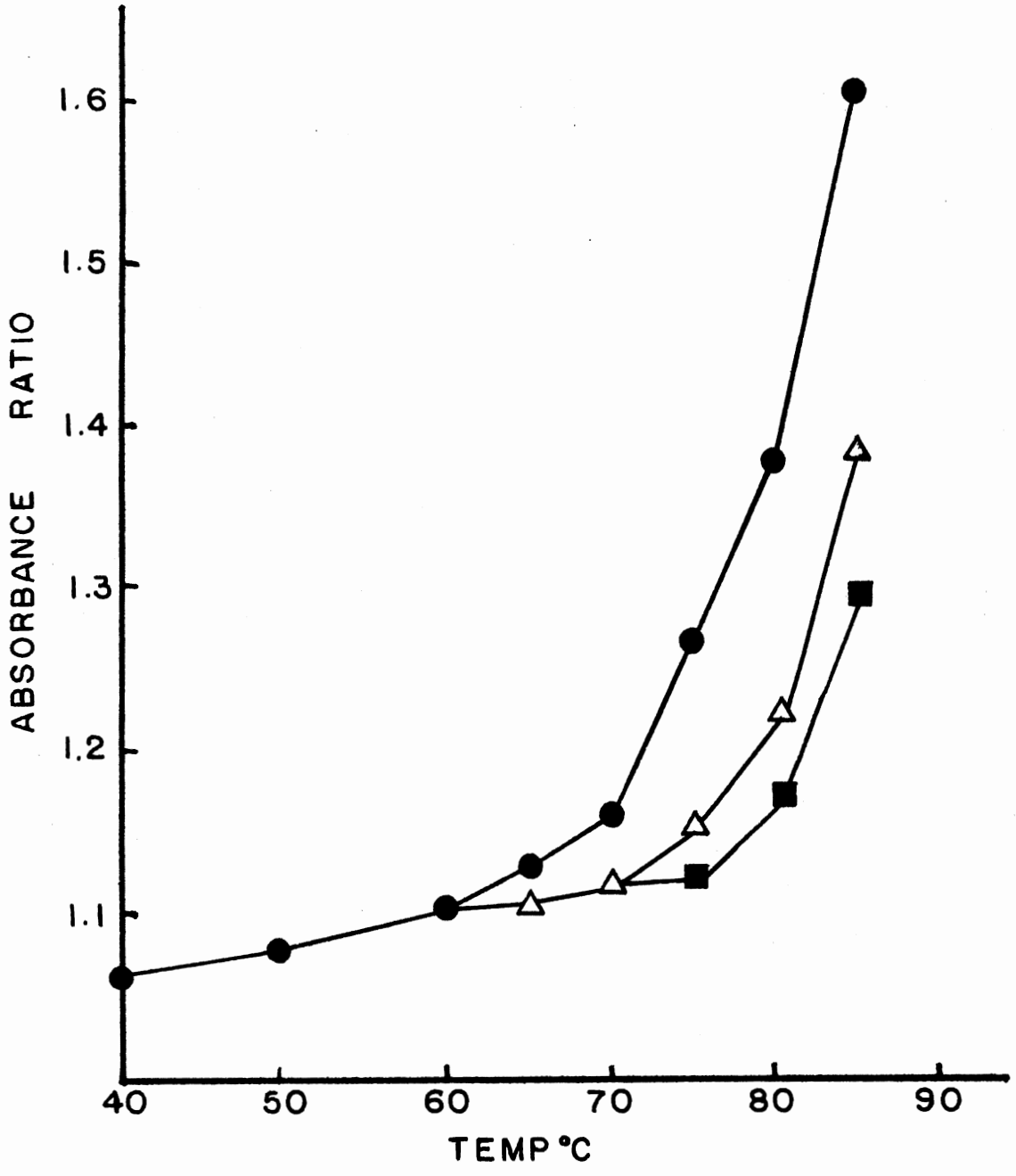
Protocatechuate has demonstrated the ability to prevent and/or reverse inhibition of amidase by actinomycin D or a combination of actinomycin D and the hydroxypyrazole. Durham and Ferguson (1971) proposed that actinomycin D complexes with DNA and is reversed by protocatechuate. That is, actinomycin D has a greater affinity for the protocatechuate molecule than the DNA helix: thus protocatechuate may act by combining with actinomycin D at such time as the antibiotic comes off the DNA helix. An experiment was conducted to investigate the possibility of protocatechuate decreasing the affinity of actinomycin D and the complex of actinomycin D-hydroxypyrazole for the DNA helix.

The same volumes and concentrations of the compounds used in the thermal denaturation studies were combined with

DNA, which will not pass through the dialysis tubing, and each combination placed in separate dialysis tubes. The samples were allowed to dialize for 72 hours in saline-citrate buffer, pH 7.0, and then removed from the dialysis tubing and used in thermal denaturation experiments. Actinomycin D, hydroxypyrazole, and protocatechuate combined singly with DNA exhibited the same DNA melting curve as the control, indicating that they were removed sufficiently by the dialysis. The combination of actinomycin D and protocatechuate also exhibited the same DNA melting curve as the control, again indicating any complex they form with DNA is reversible. Actinomycin D and the hydroxypyrazole after dialysis gave about the same DNA melting curve, which was demonstrated before dialysis, indicating the complex which formed with DNA was not reversible. The combination of all three compounds increased the thermal denaturation end point 7°C above the control, but the increase in thermal denaturation was not as great as before dialysis at which time the three compounds individually almost completely inhibited DNA denaturation (Fig. 16).

This experiment attempted to show a correlation between the compounds movement out of the dialysis tube and their affinity for the DNA helix. Results from the experiment indicate that the combination of actinomycin D and the hydroxypyrazole together had a greater affinity for DNA than the combination of the two compounds plus protocatechuate. The ability of protocatechuate to prevent and/or reverse

Figure 16. Effect of the Compounds Upon Thermal Denaturation of P. fluorescens DNA after 72 Hours of Dialysis. ●, control DNA, 20 µg/ml, also DNA and actinomycin D, 20 µg/ml, also DNA and hydroxypyrazole, 4.0 µg/ml, also DNA and protocatechuate, 20 µg/ml, also DNA and actinomycin D, 20 µg/ml plus protocatechuate, 20 µg/ml; Δ, DNA and actinomycin D, 20 µg/ml plus hydroxypyrazole, 4.0 µg/ml plus protocatechuate, 20 µg/ml; ■, DNA and actinomycin D, 20 µg/ml plus hydroxypyrazole, 4.0 µg/ml.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the inhibition effects of the combination of actinomycin D-hydroxypyrazole may possibly be occurring by protocatechuate interacting with actinomycin D and the hydroxypyrazole to form a complex with DNA which is reversible.

Further dialysis experimentation was conducted with the compounds and combinations used at the same concentrations as in the previous experiment but in the absence of DNA. This type of experiment attempted to show that complexes of these compounds would pass through dialysis tubing slower than the individual compounds. The compounds were placed in dialysis tubing and allowed to dialyze up to 24 hours. Samples were taken at 2 hour intervals and scanned in the spectrophotometer. The shorter time period was used because the compounds passed out of the dialysis tubing much quicker when DNA was not present. The interpretation of the spectral results from this experiment was somewhat unclear and no conclusion could be drawn.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The enzyme induction pattern of P. fluorescens was shown to be distinctly different when cells were grown in media containing different carbon sources. The sensitivity of the enzyme system to actinomycin D also changed depending on the carbon source in which the cells were grown. Inhibition of enzyme synthesis was greatest in cells grown with acetamide as the carbon source followed by less inhibition with succinate grown cells. The least inhibition was with glucose grown cells being the most resistant to actinomycin D.

The reasons for these differences in sensitivity to actinomycin D were probably not the results of changes at the DNA level within the cells. This conclusion was based on the lack of a difference in temperatures of denaturation for DNA isolated from glucose-grown cells or succinate-grown cells when the test compounds were present. Thus the difference in sensitivity likely resulted from changes in the lipopolysaccharide of the cells grown in media with a different carbon source which in turn changes the cells permeability to actinomycin D.

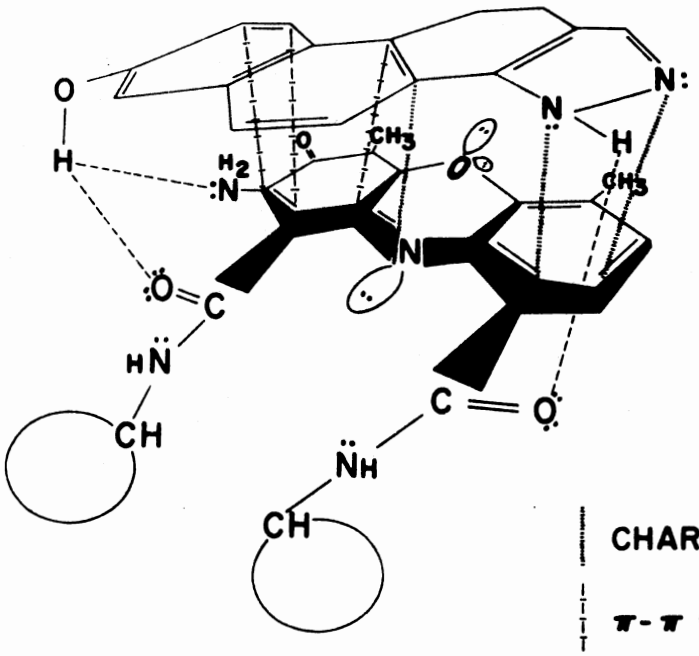
A complexation between actinomycin D and the hydroxy-

pyrazole which probably occurred instantly was observed in this investigation. When these two compounds were mixed together, the proposed complex was allowed to form, and a definite potentiation effect upon actinomycin D occurred, which was demonstrated by the amidase system. Experiments conducted by growing the cells in the presence of one component of the complex, and then adding the other after thorough washing of the cells, gives support to a complexation occurring between actinomycin D and the hydroxypyrazole, causing the potentiation effect (Fig. 17).

The ability of the hydroxypyrazole to potentiate actinomycin D was demonstrated in both succinate-salts plus acetamide and glucose-salts plus acetamide media. The hydroxypyrazole however was not capable of potentiating mitomycin C. The lack of an active complex formation could be the possible reason for this, since there is a definite difference in mitomycin C and the structure of actinomycin D. This inability of the hydroxypyrazole to potentiate mitomycin C indicated that the potentiation of actinomycin D is not coming from some type of action at the surface of the cell for if this was true it should have potentiated mitomycin C.

It was demonstrated in the amidase system that protocatechuate could be added simultaneously with actinomycin D or the combination of actinomycin D and the hydroxypyrazole or after inhibition was partially established to prevent and/or reverse the inhibitory effects.

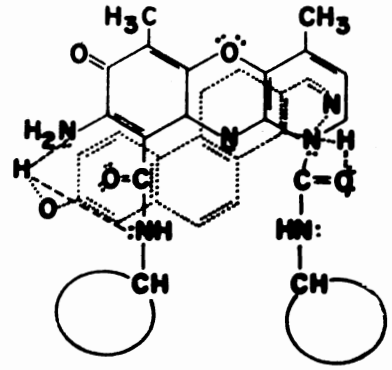
Figure 17. Proposed Model of Actinomycin D-Hydroxypyrazole Complexation. This is an illustrative model of the complex formation between actinomycin D and the hydroxypyrazole. Chesnut et al. (1974) provided evidence that the complex is stabilized by charge transfer, π-π overlap and H-Bonding.



CHARGE TRANSFER

π-π OVERLAP

H-BONDING



Thermal denaturation conducted with P. fluorescens DNA and the compounds demonstrated that all three compounds interact with DNA to stabilize the DNA helix, resulting in a shift of the DNA melting curve to high temperatures. When the compounds are combined together the combination of actinomycin D and the hydroxypyrazole shifts the DNA melting curve a greater amount than either compound alone. The combination of all three compounds almost completely stabilized the DNA up to thermal denaturation temperatures of 85°C. The ability of the three compounds, combined together, to cause an increased amount of DNA stabilization may be the result of a three-member complex between actinomycin D, hydroxypyrazole, and protocatechuate.

A possible mechanism partially explaining the ability of protocatechuate to reverse the inhibition effects of actinomycin D combined with the hydroxypyrazole in the amidase system was postulated. Protocatechuate may be interacting with actinomycin D and the hydroxypyrazole to cause a complexation of three compounds with DNA which is reversible. This was supported by the dialysis experiment in which the combination of all three compounds was dialyzed off of DNA quicker than the combination of actinomycin D and the hydroxypyrazole. This would indicate that protocatechuate is altering the compounds affinity for the DNA helix by possibly forming a three-member complex with actinomycin D and the hydroxypyrazole.

In conclusion, this investigation demonstrates that

some type of interaction is occurring between the three compounds. The results from the enzyme system and thermal denaturation studies point to a three-member complexation between actinomycin D, hydroxypyrazole and protocatechuate which may allow protocatechuate to prevent and/or reverse inhibition of amidase synthesis by actinomycin D potentiated by the hydroxypyrazole.

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Master of Science

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