THE EFFECT OF ACTINOMYCIN D ON AMIDASE INDUCTION IN *PSEUDOMONAS FLUORESCENS*

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1966

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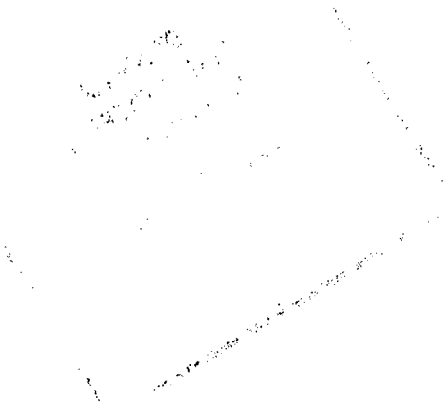
1967

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
July , 1970



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ACKNOWLEDGEMENT

I wish to express sincere appreciation to Dr. Norman N. Durham for his inspiration, patient guidance, counsel, and encouragement throughout this investigation.

Appreciation is also extended to Drs. Lynn L. Gee, Edward A. Grula and Kurt E. Ebner for suggestions and selected technical contributions utilized in this program.

Financial assistance in the form of a Title IV National Defense Education Act Fellowship is acknowledged and appreciated.

Special gratitude is extended to my wife, Barbara, and my daughter, Denise, for their help and understanding.

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CHAPTER I

INTRODUCTION

Pathogenic bacteria do not survive for extended periods of time in the soil. Waksman and Woodruff (1940) suggested that disappearance of the pathogens was due to the presence of other soil-inhabiting microbes. Using enrichment techniques, a species of Actinomyces was isolated from the soil and an ether-soluble fraction of the culture filtrate was found to completely inhibit growth of selected organisms. The authors designated the compound present in the ether fraction as actinomycin. Waksman (1954) characterized actinomycin A as a crystalline, brick-red colored compound which melted at approximately 250 C and absorbed light strongly at 230, 250 and 450 nm.

Katz, Pienta and Sivak (1958) determined that the nutritional environment of the culture had a marked effect on the synthesis of actinomycin produced. Their data clearly demonstrated that D-galactose and the nitrogen source were important for actinomycin formation.

Actinomycin D in crystalline form was isolated by Manaker et al. (1955) from cultures of Streptomyces parvullus by either solvent extraction or charcoal adsorption. Analytical values corresponded to a molecular formula of $C_{60}H_{76}O_{15}N_{12}.3\ H_2O$. Structural studies revealed the actinomycin molecule consisted of a chromophoric quinoid moiety, 2-aminophenoxazine-3-one, which was linked to two peptides each of which contains the amino acids sarcosine, D-valine, L-proline,

L-threonine, and N-methylvaline (Figure 1). Different forms of the antibiotic (A,B,C,D,I,J, and X) may differ in the relative proportion of the amino acids present in the molecule (Bullock and Johnson, 1957).

The actinomycins are not chromatographically homogenous because of their difference in amino acid composition. Vining, Gregory, and Waksman (1955) used this characteristic to separate the forms of actinomycin by cellulose partition chromatography.

Robinson and Waksman (1942) studied the toxicity of actinomycin in laboratory animals and noted that a dose of 1.0 mg or more of actinomycin per kg of body weight was lethal for mice, rats and rabbits. The antibiotic was rapidly removed from the blood following intravenous injection and was found in various quantities in all organs of the body. Robinson and Waksman (1942) observed a shrinkage in spleen size in test animals and an impairment of liver and kidney function following daily administration of actinomycin. Manaker et al. (1955) observed that the antibiotic reduced spleen weight in mice and had a LD_{50} of 0.67 mg to 0.74 mg per kg of body weight.

The anticancer activity of actinomycin C in laboratory animals was reported by Pugh, Katz, and Waksman (1956). Farber (1961) measured the activity of actinomycin D against Wilmsis tumor of the kidney and successfully treated patients for periods of three years. The treatment was most effective when actinomycin was administered simultaneously with radiotherapy.

Waksman and Woodruff (1940) observed a pronounced inhibition of growth of both gram-positive and gram-negative bacteria by actinomycin. They observed that actinomycin was bacteriostatic for pathogenic organisms *in vitro*.

To elucidate the actinomycin inhibition of tumor production and bacterial growth, researchers studied the inhibition at the molecular level using protein, enzyme and phage systems. Korn, Protass and Leive (1965) observed that actinomycin D inhibited T_4 bacteriophage production in Escherichia coli under conditions where there was no detectable interference with macromolecular synthesis in the infected cells. Korn (1967) subsequently reported that the drug blocked a process or processes required for the normal packaging of phage deoxyribonucleic acid (DNA) within the head membrane. Electron microscopy has demonstrated the presence of large numbers of empty phage heads, unattached tails and virtually no intact progeny in lysates of actinomycin-treated, T_4-infected cells (Korn, 1967).

Reich et al. (1961;1962) reported that concentrations of actinomycin, which inhibited incorporation of precursors into ribonucleic acid (RNA) by more than 90 percent, permitted normal DNA and protein synthesis for extended periods of time. Growth of Mengovirus, an RNA virus, was not affected under conditions in which host cell DNA and RNA biosynthesis was no longer detected. Poliovirus production was also resistant to actinomycin. Reich et al. (1962) concluded that actinomycin D inhibited the synthesis of all fractions of cellular RNA (nuclear, ribosomal and soluble). Data from studies using RNA viruses suggested a developmental process fundamentally different from the biosynthesis of RNA in the normal cell.

Kadowaki, Yamaguchi and Maruo (1966) studied the synthesis of α-amylase and found that 0.5 μg per ml of actinomycin inhibited protein synthesis but not ribonuclease synthesis. The addition of calf thymus DNA to actinomycin-treated cells induced recovery of enzyme synthesis.

Pollack (1963) determined that a concentration of the drug which produced a 30 percent inhibition of Bacillus subtilis growth almost completely inhibited induction of α-glucosidase without affecting penicillinase induction. Pollack proposed that these findings supported the messenger-RNA (m-RNA) hypothesis as related to enzyme synthesis and that the m-RNA for penicillinase formation in B. cereus may be metabolically stable for time periods up to 40 minutes. It is also possible that the different effect of actinomycin on enzyme synthesis may be due to differences in the binding affinity for different genes. The slow-de-adaptation of penicillinase synthesis was explained on the basis of a long-lived m-RNA molecule. Davies (1969) discovered that de-adaptation of penicillinase synthesis by washed cells suspended in a fresh medium did not indicate removal of the bound inducer from the cells and, thus, the slow de-adaptation could be explained without involving long-lived m-RNA. When actinomycin D was used to inhibit transcription of the enzyme-forming system, the half-life of the penicillinase m-RNA was established to be 4.7 minutes in both induced and cells constitutive for penicillinase production.

Synthesis of gramicidin S in cells of Bacillus brevis (Eikhorn and Laland; 1965) was resistant to actinomycin for as long as one hour following addition of the antibiotic to the culture. They studied de novo synthesis by measuring incorporation of radioactive amino acids into purified gramicidin S and suggested that the synthesis of gramicidin S was independent of RNA synthesis, since RNA synthesis was inhibited but antibiotic production was not influenced. Eikhorn and Laland did not exclude the possibility that a stable form of RNA was responsible for gramicidin S synthesis.

Laszlo et al. (1966) noted an inhibition of respiration and anaerobic glycolysis in human leukemia leukocytes and determined that actinomycin D decreased the adenosine triphosphate (ATP) content of the cells. The inhibition of respiration and glycolysis could not be dissociated over a wide range of drug concentrations. Actinomycin D also impaired protein synthesis which was explained on the basis that the antibiotic decreased the availability of ATP and inhibited m-RNA synthesis.

Leive (1965) investigated the effects of inducer (lactose) on the synthesis and utilization of β-galactosidase m-RNA in an actinomycin-sensitive E. coli. The data supported two postulates: a) the inducer, by some mechanism, stimulated formation of a specific m-RNA for β-galactosidase production, and b) actinomycin prevented formation of a new m-RNA, but did not affect activity of performed m-RNA. One would predict from these postulates that addition of actinomycin, after the inducer has catalyzed the synthesis of a m-RNA molecule, but before the molecule was completely formed and released from the DNA, should prevent formation of functional m-RNA and thus prevent subsequent synthesis of active enzyme. If actinomycin was added after formation of the m-RNA was complete, some enzyme synthesis would occur prior to degradation of the m-RNA by the cell and de novo synthesis of m-RNA would be inhibited by the antibiotic.

RNA prepared from cell extracts of actinomycin-treated Bacillus megaterium (Kennell; 1964) stimulated peptide synthesis even though polypeptide synthesis in the intact cell was inhibited. Kennell (1964) concluded that, in the presence of agents which block DNA-dependent RNA synthesis, certain fractions of newly synthesized RNA, which would

normally remain stable, are probably degraded. Acs, Reich and Valanju (1963) in a similar study found that newly-formed RNA of high molecular weight was rapidly degraded in B. subtilis cells exposed to actinomycin. The degradation process initiated by actinomycin was probably not merely an inhibition of RNA synthesis per se, but was a separate, direct effect of the antibiotic. Ribosomal RNA was very susceptible to actinomycin-induced degradation prior to acquiring the protective protein component. However, Levinthal et al.(1963) suggested that the decay of RNA in the presence of actinomycin D was due entirely to the inhibition of RNA synthesis. Yudkin and Davis (1965) studied RNA associated with the protoplast membrane of B. megaterium. When cells were incubated for 10 minutes with actinomycin, the proportion of cellular RNA found in the membrane fraction (approximately one-quarter of the total cellular RNA) remained unchanged. When bacteria, pulse-labeled with $^{32}PO_4$ for five minutes, were incubated with actinomycin an additional 10 minutes, the specific activity of the RNA in the cytoplasmic fraction did not increase, and actually decreased, indicating newly synthesized RNA flowed from the cytoplasm to the membrane during treatment with actinomycin.

In the course of investigating the mechanisms of action of actinomycin, evidence was obtained that the antibiotic showed a specific interaction with DNA (Kawamata and Imanishi, 1960). Results from studies using starch and zone electrophoresis determined that actinomycin distribution corresponded to the fraction of DNA. Also, the inhibition of bacterial growth was virtually abolished in the presence of calf thymus DNA. They proposed that an interaction between DNA and actinomycin played an important role in the mechanism of action of the

antibiotic.

Cavalieri and Nemchin (1964) suggested two types of binding sites on calf thymus or E. coli DNA, one of which had a binding affinity approximately 50 times greater than the other. The binding of actinomycin to the strong sites resulted in a "lateral" dimerization of the DNA. Upon removal of the drug, the molecular weight of DNA reverted to its original value. It was concluded that actinomycin inhibited RNA polymerase by competing with it for strong sites on the DNA molecule.

Reich (1962) proposed that action of the antibiotic presumably resulted from actinomycin binding to DNA; and, since normal rates of DNA biosynthesis persisted following substantial inhibition of RNA, it seemed likely that the enzymes catalyzing DNA and DNA-dependent RNA biosyntheses differed significantly in their stereochemical relationship to the DNA molecule.

Goldberg, Rabinovitz and Reich (1962) suggested that the base composition of DNA primers influenced the extent of actinomycin binding and thereby the degree of antibiotic inhibition of the different reactions catalyzed by RNA polymerase. Guanine residues appeared to be indispensible for actinomycin binding to DNA. When deoxyadenine-deoxythymine primed RNA synthesis was tested, the RNA synthesis was totally resistant to actinomycin inhibition. Deoxyguanine-deoxycytosine primed RNA synthesis was inhibited by actinomycin but to a lesser extent than DNA directed RNA synthesis when the DNA was isolated from Micrococcus lysodeikticus. Goldberg, Rabinovitz and Reich (1966) concluded that optimal binding occurred when native helical DNA was present and that guanine must be present for binding. Partial sensitivity to actinomycin of polyuracil and polyadenine directed RNA synthesis

suggested that these regions may contain an occasional binding site of low affinity. Gellert et al. (1965) suggested that the binding site of actinomycin to DNA either involved more than one base pair, one of which was guanine-cytosine (G-C), or else actinomycin D was bound only to the G-C pairs. Steric interference between antibiotic molecules prevented saturation of all G-C binding sites within the DNA molecule. The structure of actinomycin D (Figure 1) was studied by Hamilton, Fuller and Reich (1963) as well as the proposed model for interaction with guanine (Figure 2). The hydrogen bonding is critically dependent on the relative positions of the sugar oxygen atom and the guanine hydrogen-bonding groups. Additional studies (Reich, Cerami and Ward, 1967) indicated that only guanine furnished the hydrogen in the DNA minor groove for which the quinoidal oxygen of actinomycin served as an acceptor. If the hydrogen was removed, the DNA-actinomycin complex was not formed. Reich, Cerami and Ward (1967) conducted dissociation studies and determined that the binding of actinomycin to guanine did not lend sufficient energy to account for the dissociation constant value obtained. They postulated that the cyclic peptides of actinomycin also interacted with DNA and made a major contribution to the stability of the complex. Accumulation of the antibiotic occurred in membranes, but only after saturation of the binding sites on DNA. Reich (1964) and Goldberg and Reich (1964) proposed that actinomycin bound to guanine-containing sites on the helical DNA and directly inhibited RNA-polymerase by blocking the surfaces on the DNA-template which participated in enzyme activity. Since the quantity of bond actinomycin, which almost completely suppressed DNA-dependent RNA synthesis, did not affect DNA polymerase activity, it was concluded

Figure 1

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

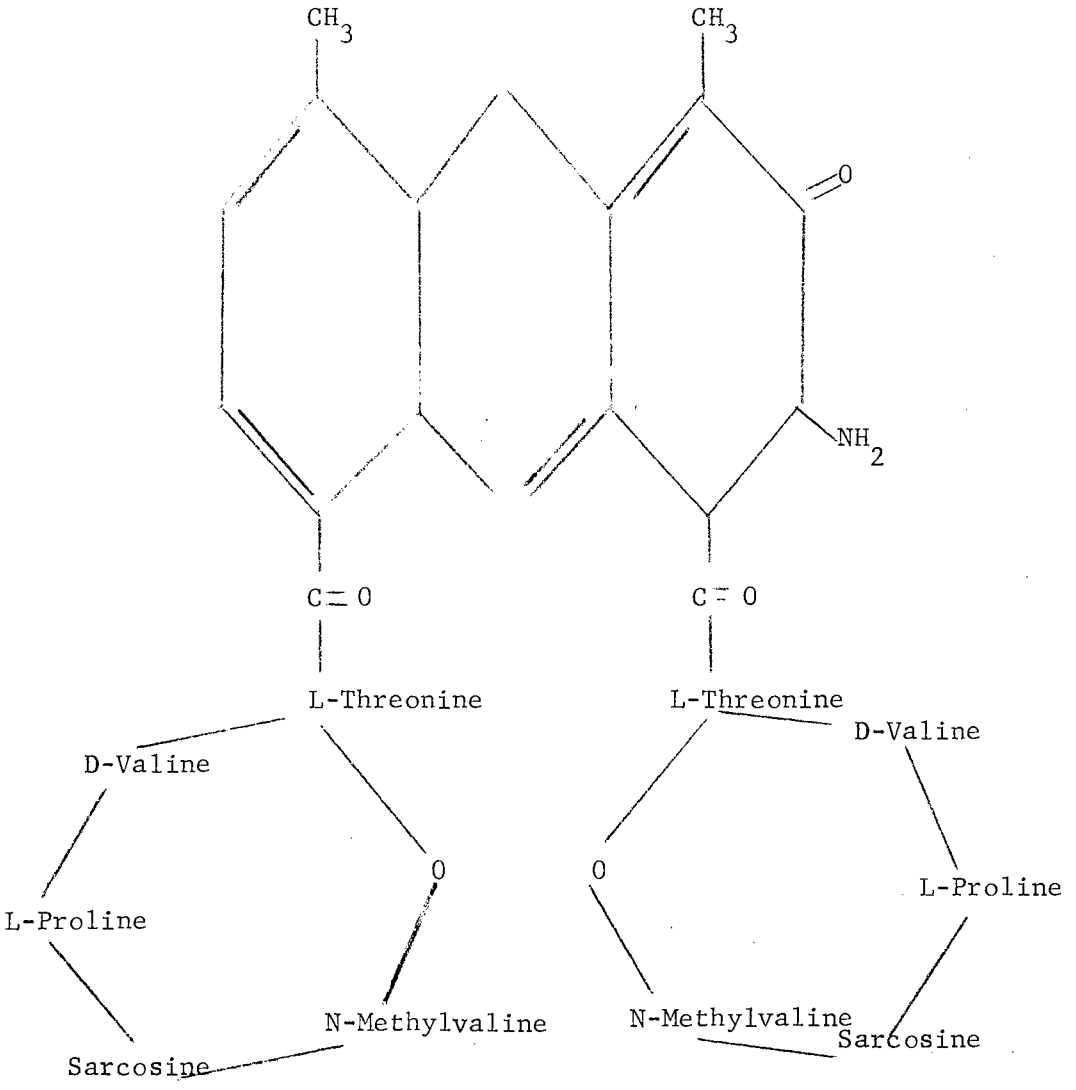
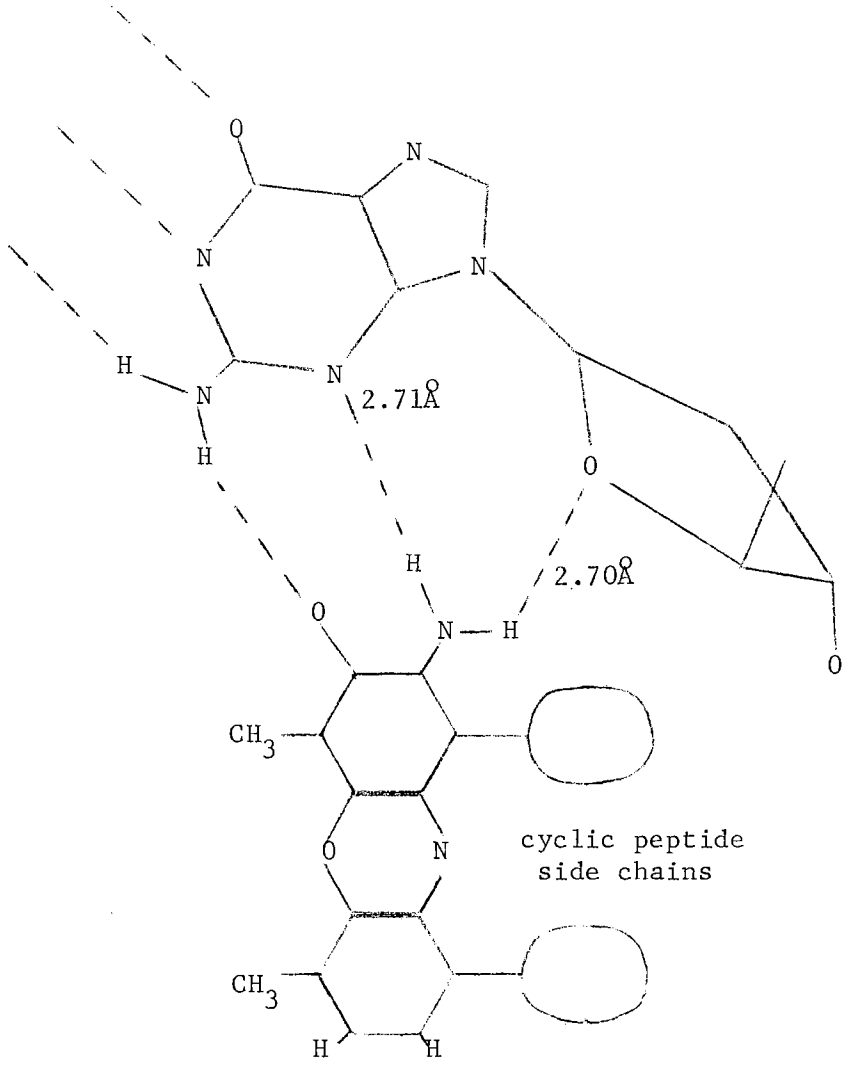

CH3
CH3
O
NH2
C=O
C=O
L-Threonine
L-Threonine
D-Valine
D-Valine
O
O
L-Proline
L-Proline
N-Methylvaline
N-Methylvaline
Sarcosine
Sarcosine

Figure 2

Proposed model for the binding of actinomycin D to DNA (Hamilton, Fuller, and Reich, 1963). The actinomycin chromophore group binds to guanine via three hydrogen bonds. The actinomycin molecule may also be stabilized by the bonding of the phosphate oxygen atoms of the DNA to the peptide chains of the antibiotic.


O
N
N
N
H
N
N
H
2.71Å
O
H
2.70Å
O
N — H
O
CH3
O
N
cyclic peptide
side chains
CH3
H
H

that during DNA replication, the DNA polymerase functioned on surfaces of the template which were not obstructed by the antibiotic. Reich (1964) proposed that each nucleic acid polymer normally "sees" the DNA base sequence from only one groove. RNA polymerase is displaced from DNA by actinomycin. If actinomycin is assumed to lie in the minor groove, then this groove was the specific template site for RNA polymerase and thus the site of RNA synthesis. DNA replication would then be postulated to proceed in the major groove.

In a recent study, Wells (1969) determined that the ability of a synthetically synthesized double-stranded DNA containing 33 percent guanine-cytosine (G-C) to bind actinomycin D was negligible and proposed that, contrary to previous predictions, the presence of guanine in DNA was not a sufficient requisite for binding. He noted that binding of the antibiotic required a specific base sequence or DNA configuration, and the presence of guanine, in most instances, induced a suitable configuration to permit binding of actinomycin.

Kirk (1960) reported that incorporation of glutamic acid-^{14}C into the cell wall of Staphylococcus aureus was not affected by antibiotic concentrations as high as 20 μg per ml. Kirk further noted that inhibition of the incorporation of ^{14}C-labeled metabolites into protein and nucleic acid fractions by actinomycin D was alleviated by the addition of nucleic acid. Hurwitz et al.(1962) observed actinomycin-induced inhibition of RNA synthesis could be reversed by increasing the concentration of DNA present in the medium. Hurwitz et al. (1962) also observed that addition of the antibiotic to B. subtilis resulted in the production of long "snake-like" cells. The aberrant cell forms appeared to

be related to unbalanced growth resulting from the preferential inhibition of RNA synthesis. Holden and Utech (1967) suggested that the system for uptake of aminoisobutyric acid and glutamic acid by Streptococcus faecalis cells was inhibited by actinomycin D. The results indicated that inhibition of the uptake system depended on the simultaneous presence of amino acids and antibiotic and it was not dependent on blockage of protein synthesis since puromycin did not affect the system. These researchers proposed that actinomycin D prevented synthesis of ribonucleotide or polyribonucleotide-dependent catalysts required for operation of the uptake systems, caused an accumulation of inhibitory nucleotides, or interfered with the utilization of high-energy substances.

Kersten (1961) noted that DNA, RNA and certain of their degradative products counteract the growth inhibition due to actinomycin in Neurospora crassa and St. faecalis. Deoxyguanosine and DNA were most effective for reversal of the growth inhibition. The observation that breakdown products of nucleic acids reversed growth inhibition led Foley (1956) to study the ability of additional compounds to alleviate actinomycin inhibition. Pantothenate appeared to compete with actinomycin and readily reversed actinomycin-induced growth inhibition. Prevention of growth inhibition was obtained in different bioassay systems using acid hydrolyzed casein. Rauen and Hess (1959) reported reversal of actinomycin inhibition with p-aminobenzoic acid, tyrosine and phenylalanine in N. crassa.

Slotnick (1960) tested the ability of several compounds (b-vitamins, amino acids of casein hydrolysate, purines, pyrimidines and nucleotides) to prevent growth inhibition of B. subtilis by actinomycin D.

Immediately after addition of the antibiotic, protein and RNA synthesis were completely inhibited, while DNA synthesis proceeded at the normal rate. The growth inhibition, caused by actinomycin D, was competitively altered by the presence of pantothenic acid in several organisms having an exogenous requirement for this vitamin, but not in organisms capable of pantothenate-independent growth. Efforts to restore a balanced DNA, RNA and protein synthesis by additional of several compounds to the system have proven unsuccessful.

Honig and Rabinovitz (1965) determined that addition of glucose to the medium prevented the inhibition of protein synthesis in Sarcoma-37 ascites cells by actinomycin D and concluded that the inhibition was not related to breakdown of template RNA.

Studies in this laboratory have shown that growth of Pseudomonas fluorescens was not inhibited by actinomycin D when cells were incubated in a protocatechuate-salts medium (Keudell, 1967). Further studies revealed that actinomycin D did not inhibit synthesis of the inducible enzyme, protocatechuate oxygenase, nor incorporation of uracil-2-^{14}C into RNA in this organism. The results suggested the following: 1) the genetic loci for coding protocatechuate oxygenase m-RNA may not contain residues of guanine-cytosine; or 2) the bases may not be appropriately arranged within the DNA molecule for actinomycin D binding; or 3) actinomycin D may be inactivated under certain physiological conditions.

This investigation was conducted to describe the synthesis of the inducible enzyme, acyltransferase (amidase-E.C. 3.5.1.4.). and to elucidate the effect of actinomycin D on synthesis of the enzyme. The second

phase of this study delineates the ability of protocatechuate (3,4-dihydroxybenzoic acid) to reverse the inhibition of growth and enzyme synthesis by actinomycin D.

CHAPTER II

MATERIALS AND METHODS

Test organisms

The principal microorganism used during this study was a strain of Pseudomonas which was tentatively identified as Pseudomonas fluorescens (Montgomery, 1966). A species of Flavobacterium was also used in one phase of this investigation. This organism was obtained from the stock culture collection of Dr. N. N. Durham. Bacillus subtilis W23 was obtained from the stock culture collection of Dr. Franklin Leach, Oklahoma State University. All stock cultures were maintained on nutrient agar slants and stored at 4 C.

Media

The synthetic salts medium used in this study was composed of 0.2 percent NaCl, 0.2 percent NH_4Cl, 0.32 percent KH_2PO_4, 0.42 percent K_2HPO_4, and 0.2 percent of the desired carbon source (succinate, acetate, or glucose). In studies where acetamide was used as a carbon source or inducer, a concentration of 0.08 M was used, unless otherwise specified. The medium was adjusted to pH 7.0 with KOH, sterilized by autoclaving at 121 C with 15 lbs. pressure per square inch for 15 minutes, cooled to room temperature and 0.1 ml of a sterile mineral salts solution was added to each 100 ml of the medium. The mineral salts solution had the

following composition: 5.0 g. of $MgSO_4 \cdot 7\ H_2O$; 0.1 g. of $MnSO_4$; 1.0 g. of $FeCl_3$; and 0.5 g. of $CaCl_3$ in 100 ml of distilled water. Agar (Difco) was added to give a final concentration of 2.0 percent when a solid medium was desired. In the context of this study, the term minimal salts was used to refer to the basal salts medium lacking a carbon source.

Nutrient agar was prepared from the dehydrated medium (Difco) and additional agar (Difco) was added to give a final concentration of 2.0 percent.

Growth of cells

Nutrient agar or succinate agar slants were inoculated from the appropriate stock cultures and incubated for 12-16 hours at 37 C. The cells were harvested in sterile distilled water and 250 ml flasks containing 39.0 ml of the appropriate medium were inoculated with 1.0 ml of the cell suspension to give an initial absorbance of 0.1 at 540 nm. The flasks were incubated at 37 C for 12-16 hours on a reciprocal shaker (100 revolutions per minute). The cells were harvested by centrifugation, washed twice and suspended in sterile distilled water. The washed cells were used as the inoculum for growth studies. Growth was determined by measuring the increase in absorbance of the culture at 540 nm using a Coleman Junior II spectrophotometer. Growth assays were performed either in test tubes (18 mm light path) containing 6.0 ml of medium or in 250 ml side-arm flasks containing 40.0 ml of liquid medium.

Inhibitors

The compounds used as inhibitors were purchased from commercial sources. D-chloramphenicol was purchased from Parke, Davis and Company, Detroit, Michigan, and actinomycin D was obtained from Merck, Sharp and Dohme Research Lab., division of Merck and Co., West Point, Pennsylvania.

Enzyme induction

Pseudomonas fluorescens cells were grown in succinate-salts medium for approximately 12 hours. The cells were harvested by centrifugation (10 minutes at 4500 x g), washed once with sterile triple distilled water and were used to inoculate the appropriate medium (succinate-salts plus acetamide or acetamide-salts). One ml samples were removed at the specified time intervals and immediately placed in the freezer (-14C) for subsequent enzyme determinations.

Enzyme 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). Hydroxamic acids react with ferric salts to produce red to violet color complexes which may be quantitated by spectrophotometric procedures.

A. Preparation of the standard curve.

A hydroxamic acid standard solution was prepared from succinic anhydride as follows: One gram of succinic anhydride was dissolved in

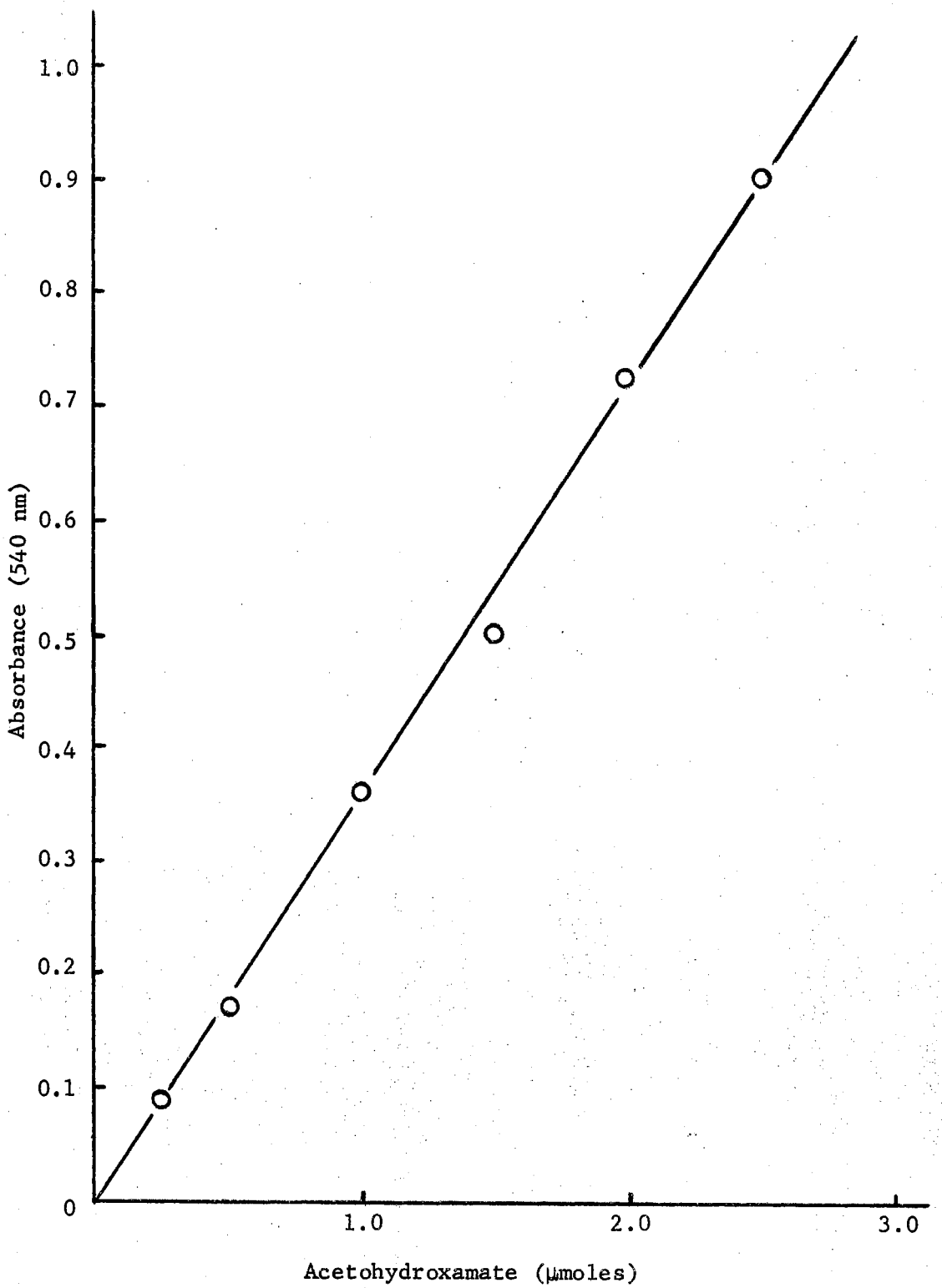
40.0 ml of freshly neutralized hydroxylamine hydrochloride solution (2.0 M) and diluted to 100 ml with water. One ml of this stock solution was diluted to 40.0 ml with water to produce the standard solution. One ml of freshly neutralized hydroxylamine reagent was added to 2.0 ml of the standard solution. After 10 minutes at room temperature, 3.0 ml of ferric chloride reagent (6 percent w/v in 2 percent HCl v/v) were added, the color permitted to develop, then read in a Coleman Junior II spectrophotometer at 540 nm. The color of the resulting solution was equivalent to 4.0 micromoles of acetohydroxamic acid. The solution was diluted with water to give varying concentrations of acetohydroxamate, the absorbance of each dilution was measured, and a standard curve for acetohydroxamate was determined (Figure 3).

B. Determination of cellular enzymatic 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. Kelly and Clarke (1962) measured the hydrolase activity of amidase by estimating ammonia production during hydrolysis of acetamide. Studies with partially purified enzyme preparations suggested that the amidase enzyme has both hydrolytic activity and acyltransferase activity. Kelly and Kornberg (1964) suggested that the enzyme should be designated "aliphatic acyltransferase" rather than "amidase." Throughout this investigation the term amidase will be used to denote the inducible "aliphatic acyltransferase" enzyme. The

Figure 3

A standard curve for the colorimetric assay of acetohydroxamic acid.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance (540 nm)
1.0
2.0
3.0
Acetohydroxamate (µmoles)

acyltransferase transfers the acyl group of the substrate amides to hydroxylamine to form acylhydroxamates (Kelly, 1961).

Amidase activities

1) Hydrolase

$$CH_3CONH_2 + H_2O \rightleftarrows CH_3COOH + NH_3$$

2) Transferase

$$CH_3CONH_2 + NH_2OH \rightleftarrows CH_3CONHOH + NH_3$$

The transferase reaction was used during this study for the enzyme assay which was performed as follows: frozen samples were thawed and 0.25 ml was added to 0.75 ml of the substrate mixture, which was prepared by mixing equal volumes of 0.4 M acetamide solution, 2.0 M freshly neutralized hydroxylamine hydrochloride, and 0.1 M Tris buffer (pH 7.2). After incubation at 37 C for 15 minutes, the reaction was stopped by adding 2.0 ml of ferric chloride (6 percent w/v in 2 percent HCl v/v). The absorbance was read immediately at 540 nm using a coleman Junior II spectrophotometer and the acetohydroxamate concentration was calculated using the standard curve. The specific activity was measured and one unit of amidase is the amount of enzyme that will produce one micromole of acetohydroxamate per milligram dry cell weight per 15 minutes.

Recrystallization of acetamide

Commercial (Baker Chemical Co.) acetamide was added to 100 ml of a 70 percent ethanol solution equilibrated to 50 C in a waterbath until a saturated solution was obtained. The supernatant solution was filtered through a millipore membrane filter (47 mm diameter; 0.25 micron pore size). The filtrate was placed in the refrigerator at 4 C to enhance

further precipitation. The precipitate was collected on a millipore filter and dried at 37 C in a dessicator containing $CaCl_2$ crystals. The precipitate was dissolved in 70 percent ethanol and the recrystallization process repeated. The resultant precipitate was dried at 37 C in a dessicator and used in the experiments as indicated.

Uptake of radioactive substrate

Pseudomonos fluorescens cells were grown on acetamide-salts (0.08 M) medium for 9 hours to measure the uptake of acetamide-1-^{14}C (specific activity 4.13 μC per mmole) by the cells. The cells were harvested by centrifugation, washed one time and suspended in triple distilled water. One ml of the cell suspension (a 1:10 dilution giving an absorbance of 0.50 at 540 nm) was incubated at 25 C with 0.4 ml of acetamide (0.08 M) as carrier, 0.4 ml acetamide-^{14}C (1.0 μC per ml) and 0.4 ml of a chloramphenicol (CAP) solution (3.7×10^{-3}M). Four-tenths ml of acetate (0.1 M) or succinate (0.1 M) was added to the appropriate tube and distilled water was used to bring the liquid volume to 4.0 ml in all the tubes. One ml samples were withdrawn at 10 and 90 seconds, immediately filtered on a Millipore membrane filter (47 mm diameter; 0.45 micron pore size) and washed two times with 5.0 ml of distilled water. The membrane filter was placed in a counting vial containing 1.0 ml of 3,4-dioxane. Liquid scintillation counting fluid was prepared by dissolving 2.0 g of 2,5-diphenyloxazole (PPO) and 25 mg of p-bis-(2-(5-phenyloxazolyl))-benzene in 237 ml of 3,4-dioxane. Counting fluid (9.0 ml) was added to each vial and the radioactivity was determined using a Nuclear Chicago liquid scintillation spectrometer (Model No. 722). The counting efficiency for ^{14}C under these conditions was 40 percent.

Spectrophotometric studies

The ultraviolet absorption spectra of an aqueous solution of protocatechuate (3.8×10^{-4} M) and actinomycin D (1.03×10^{-5} M) were determined using a Cary 14 recording spectrophotometer at room temperature in silicon cuvettes. The absorbance of each compound and a mixture of the two compounds was determined between the wavelengths of 185-320 nm.

Spectrophotofluorometric studies

Fluorescent measurements were conducted using an Aminco-Bowman spectrophotofluorometer. An excitation spectrum was determined for protocatechuate (8.0×10^{-3} M dissolved in 1.0×10^{-2} M Tris buffer, pH 7.0) and the emission spectrum for protocatechuate was determined using the wavelength of maximum excitation of the compound. The excitation and emission spectra for actinomycin (8.0×10^{-5} M dissolved in 1.0×10^{-2} M Tris buffer, pH 7.0) were determined in the same manner. A mixture of protocatechuate and actinomycin was made and the emission and excitation spectra were determined.

CHAPTER III

RESULTS AND DISCUSSIONS

Inhibition of growth of P. fluorescens by actinomycin D

Studies were performed to ascertain the concentration of actinomycin D that would inhibit growth of P. fluorescens in a synthetic salts medium with different carbon and energy sources. These experiments were carried out in tubes containing five and a half ml of medium with either succinate or acetamide as the carbon source and the inhibitor was added as indicated. Sterile triple distilled water was used to bring the volume to 5.9 ml. Controls were prepared of each actinomycin concentration to compensate for any color attributable to the antibiotic. All of the tubes, except the antibiotic controls, were inoculated with 0.1 m of a suspension of washed succinate-salts grown cells to give an initial absorbance of 0.1 at 540 nm.

Pseudomonas fluorescens cells growing in succinate-salts medium showed a very short lag time (Figure 4) while cells growing in acetamide-salts required a lag time of approximately 2.5 hours (Figure 5). The lag indicates that synthesis of an inducible enzyme was required for utilization of acetamide by the organism. Growth of P. fluorescens in either medium was inhibited by actinomycin D (Figures 4 and 5) and the inhibition was concentration dependent. The rate or total growth of the cells was not significantly altered by an antibiotic concentration of 8.0×10^{-7} but concentrations of 4.0×10^{-6}M, 8.0×10^{-6}M and 1.04×10^{-5} M inhibited

Figure 4

The influence of actinomycin D on P. fluorescens cells growing in a succinate-salts medium. ○, succinate-salts (control); Δ, actinomycin D (8.0×10^{-7}M); □ , actinomycin D (4.0×10^{-6}M); ■ , actinomycin D (8.0×10^{-6}M); ●, actinomycin D (1.04×10^{-5}M).

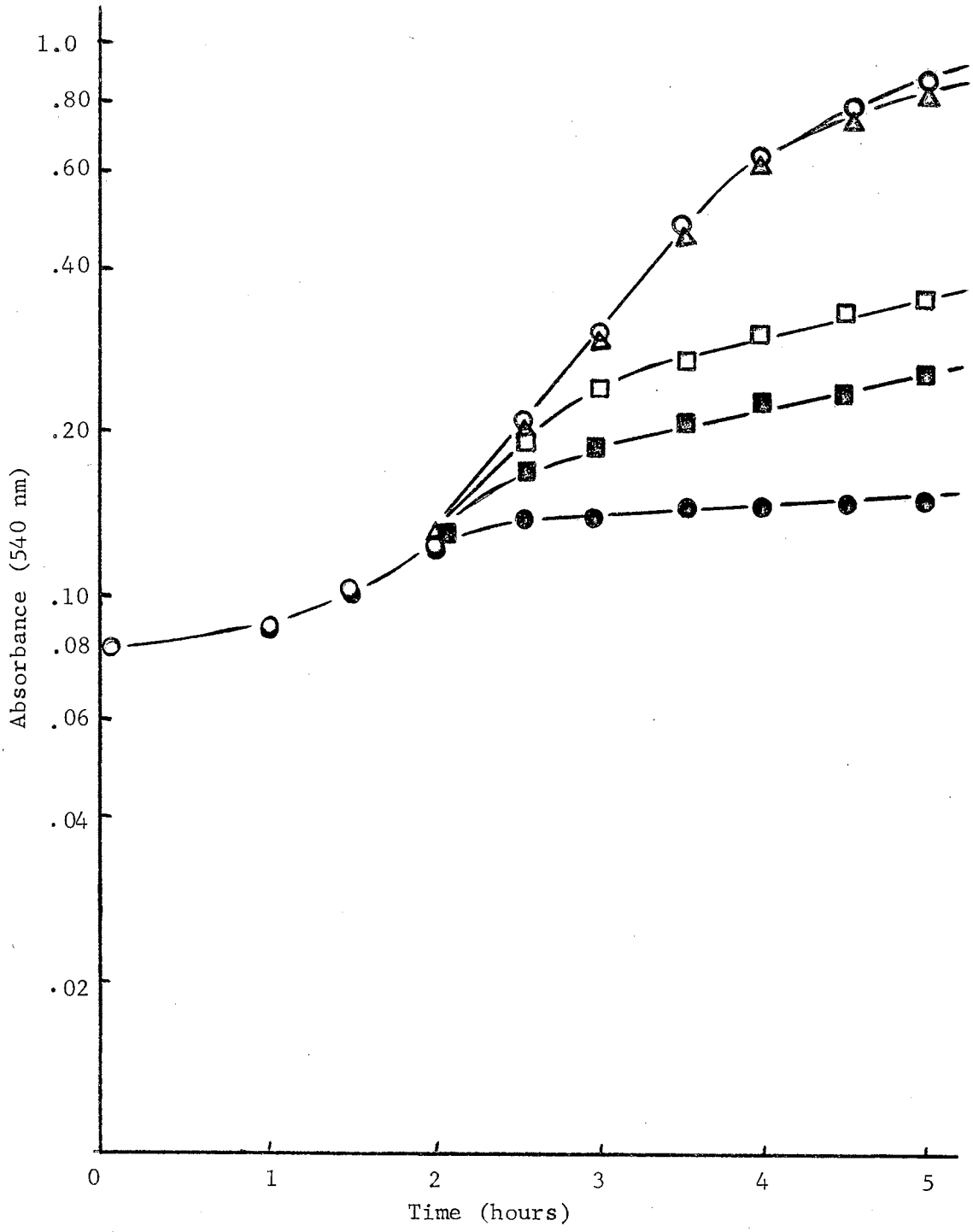
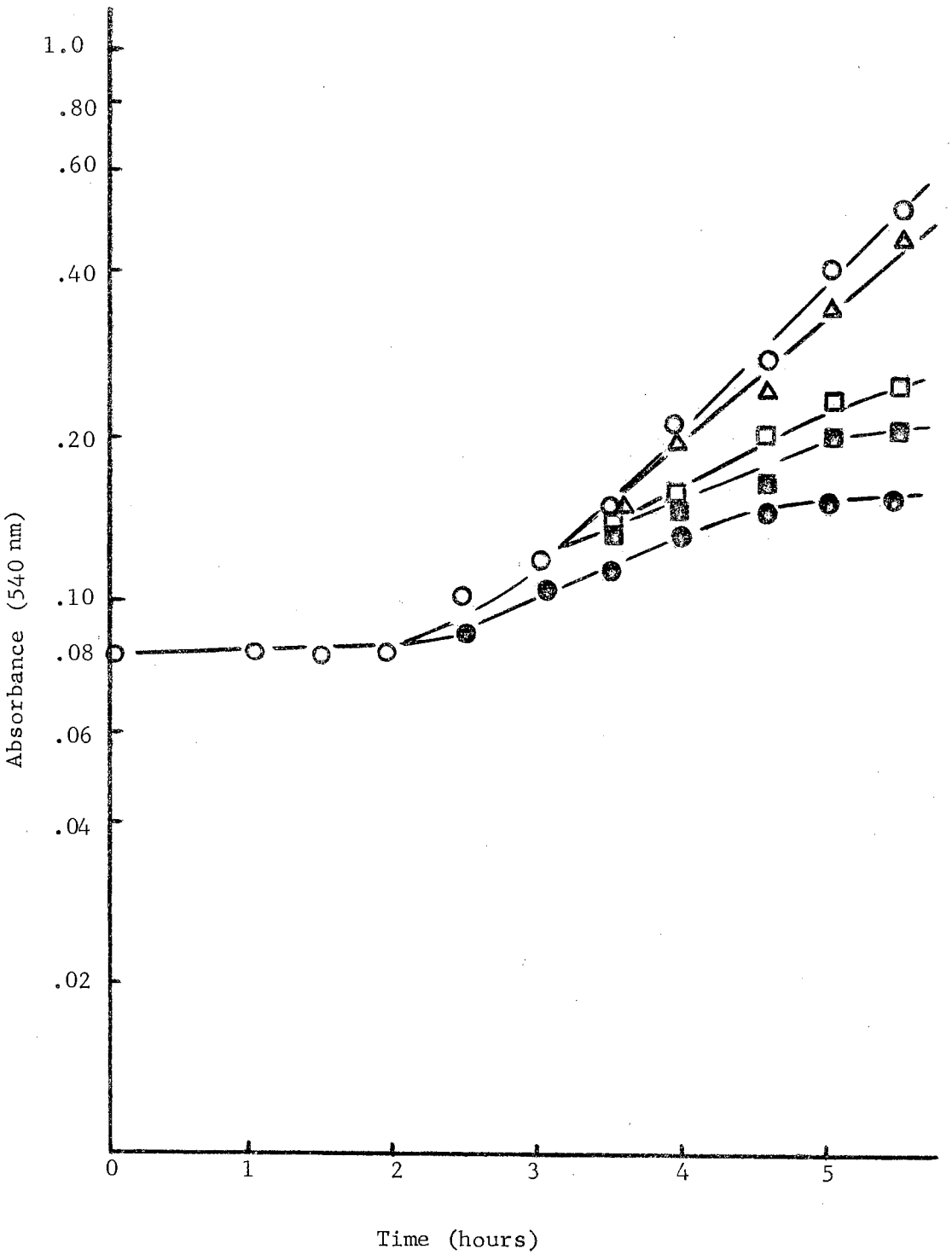

1.0
.80
.60
.40
.20
.10
.08
.06
.04
.02
Absorbance (540 nm)
0
1
2
3
4
5
Time (hours)

Figure 5

The influence of actinomycin D on P. fluorescens cells growing in 0.08 M acetamide-salts medium. ○, acetamide-salts (control); Δ, actinomycin D (8.0×10^{-7} M); □, actinomycin D (4.0×10^{-6} M); ■, actinomycin D (8.0×10^{-6} M); and ●, actinomycin D (1.04×10^{-5} M).


1.0
.80
.60
.40
.20
.10
.08
.06
.04
.02
Absorbance (540 nm)
0
1
2
3
4
5
Time (hours)

both the rate and total growth of the cells (Figures 4 and 5) in the succinate and acetamide medium.

Growth studies were conducted to compare the inhibition of growth by actinomycin D in succinate-salts and protocatechuate-salts media. Tubes containing either protocatechuate-salts or succinate-salts medium were inoculated with succinate-grown P. fluorescens cells and the growth was determined. Actinomycin D (8.0×10^{-6} M) inhibited growth in the succinate-salts medium, but did not inhibit cells growing in a protocatechuate-salts medium (Figure 6). These results support the observations of Keudell (1967) who reported that actinomycin D did not inhibit P. fluorescens cells growing in a protocatechuate-salts medium. He suggested that actinomycin did not inhibit induction of the enzyme required for utilization of protocatechuate.

Prevention and reversal of actinomycin D inhibition by protocatechuate

Studies were conducted to determine if protocatechuate could prevent the actinomycin D inhibition of P. fluorescens growing in succinate-salts medium. Tubes containing succinate-salts medium were inoculated with P. fluorescens to an initial absorbance of 0.1 at 540 nm and growth was determined in the presence of actinomycin D and actinomycin D plus protocatechuate. The concentration of protocatechuate used in this experiment was not sufficient to effect the succinate control or provide sufficient carbon for demonstrable growth of the cells (Figure 7). Results indicated that protocatechuate (8.0×10^{-4}M) partially prevented the growth inhibition produced by an actinomycin D concentration of 8.0×10^{-6}M (Figure 7). Pseudomonas fluorescens cells growing in the acetamide-salts medium where acetamide is utilized as

Figure 6

The inhibition of growth by actinomycin D of P. fluorescens cells growing in either a succinate-salts or protocatechuate-salts medium. ○, succinate-salts (control); ⊛, succinate-salts plus actinomycin D (8.0×10^{-6} M); □, protocatechuate-salts (control); ■, protocatechuate-salts plus actinomycin D (8.0×10^{-6}M).

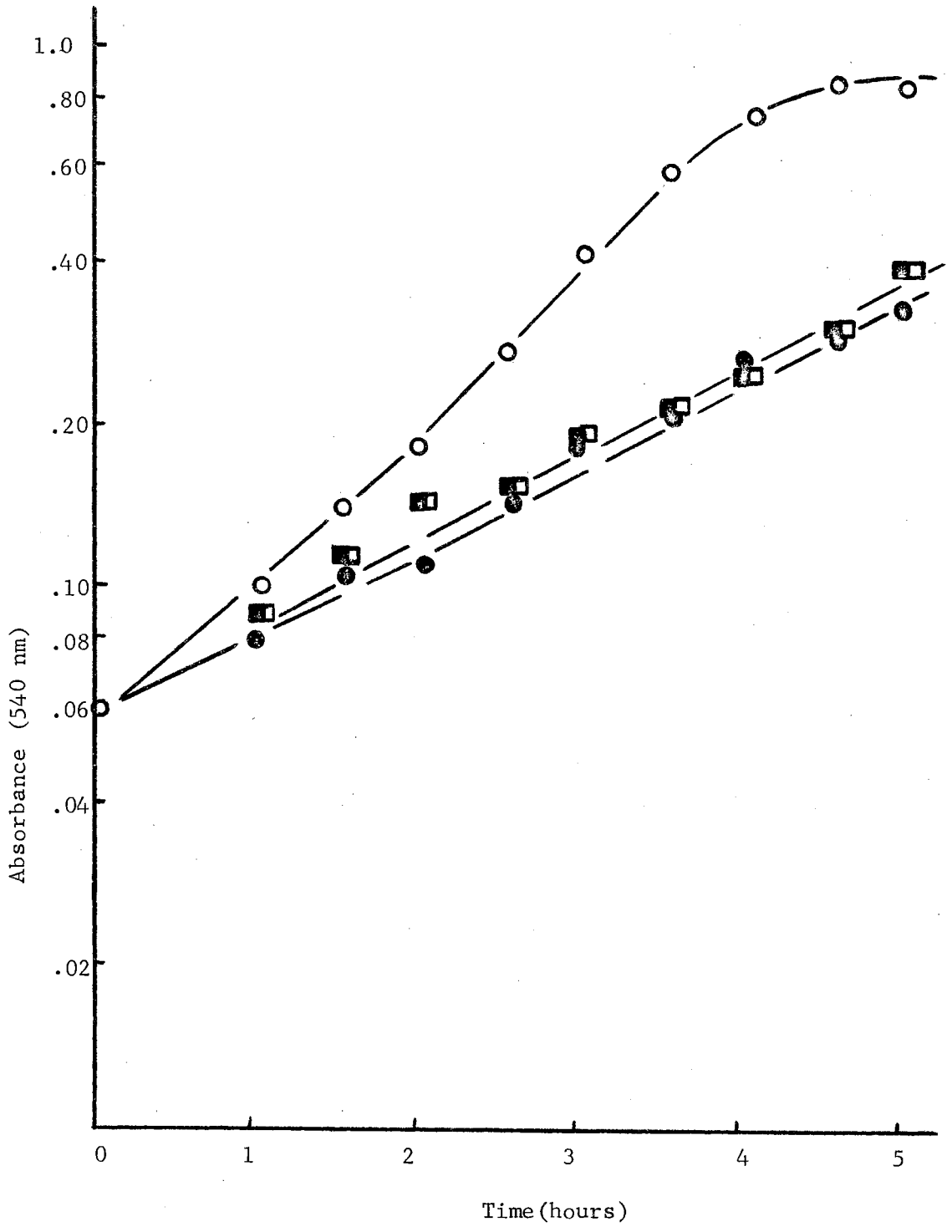
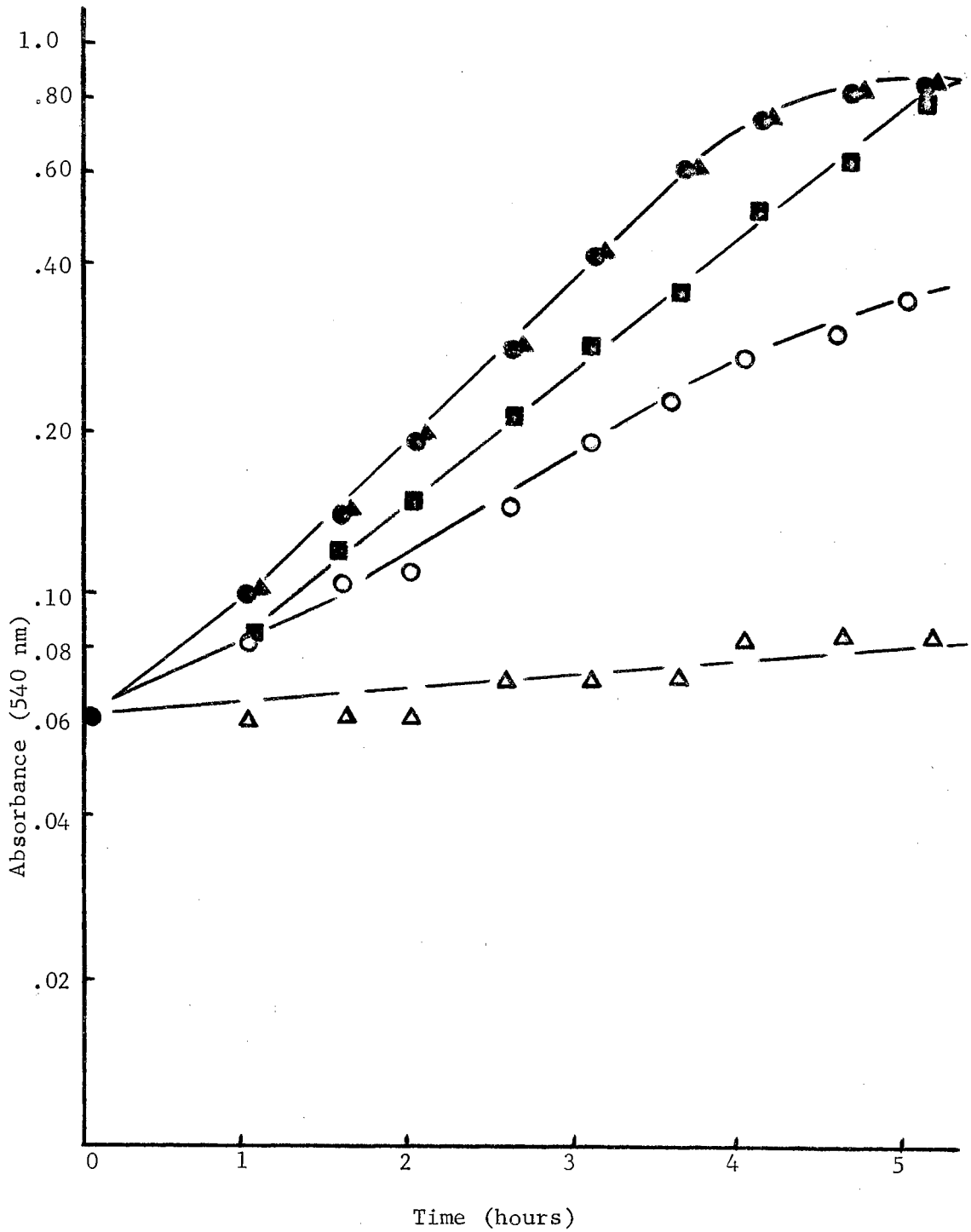

1.0
.80
.60
.40
.20
.10
.08
.06
.04
.02
Absorbance (540 nm)
0
1
2
3
4
5
Time (hours)

Figure 7

The influence of protocatechuate (8.0×10^{-4} M) on actinomycin D-inhibited P. fluorescens cells growing in succinate-salts medium. ●, succinate-salts (control); ▲, succinate-salts plus protocatechuate; △, protocatechuate; ○, actinomycin D (8.0×10^{-6} M); and ■, actinomycin D (8.0×10^{-6} M) plus protocatechuate.


1.0
.80
.60
.40
.20
.10
.08
.06
.04
.02
Absorbance (540 nm)
0
1
2
3
4
5
Time (hours)

the carbon source showed a growth lag of approximately 2.5 hours which was followed by rapid growth (Figure 5). The rate of growth was inhibited by the addition of actinomycin D. The addition of protocatechuate (8.0×10^{-4} M) to the inhibited system partially alleviated the growth inhibition as was shown for cells growing in succinate-salts medium.

Since the results establish that protocatechuate will prevent the inhibition, if added simultaneously with actinomycin D, studies were conducted to determine if protocatechuate could reverse the growth inhibition produced by actinomycin D.

Pseudomonas fluorescens cells were inoculated into succinate-salts medium containing actinomycin D (8.0×10^{-6}M). Protocatechuate was added to one set of flasks at zero time and a second set of flasks after two hours. Actinomycin D inhibited the total growth of the cells. Addition of protocatechuate, at either zero time or after the cells were incubated for two hours with actinomycin, reversed and prevented the growth inhibition by actinomycin D (Figure 8). These results established that protocatechuate can both prevent and reverse the inhibition of growth by actinomycin D.

Kirk (1960) reported that addition of actinomycin (2.0×10^{-7}M) to the gram-positive organism, Staphylococcus aureus, inhibited growth. Studies were conducted to ascertain the ability of protocatechuate to prevent the actinomycin D inhibition of growth in microorganisms other that P. fluorescens. The succinate-salts medium was used for growth of the species of Flavobacterium (Figure 9) and glucose salts was used for growth of B. subtilis (Figure 10). The results show that the

Figure 8

The prevention and reversal by protocatechuate (8.0×10^{-4}M) of actinomycin D inhibition of growth of P. fluorescens in succinate-salts medium. ●, succinate-salts (control); ■, succinate-salts plus protocatechuate; ○, actinomycin D (8.0×10^{-6} M); □, actinomycin D (8.0×10^{-6}M) plus protocatechuate (added at zero time); and Δ, actinomycin D (8.0×10^{-6}M) plus protocatechuate (added at the two hour time interval).

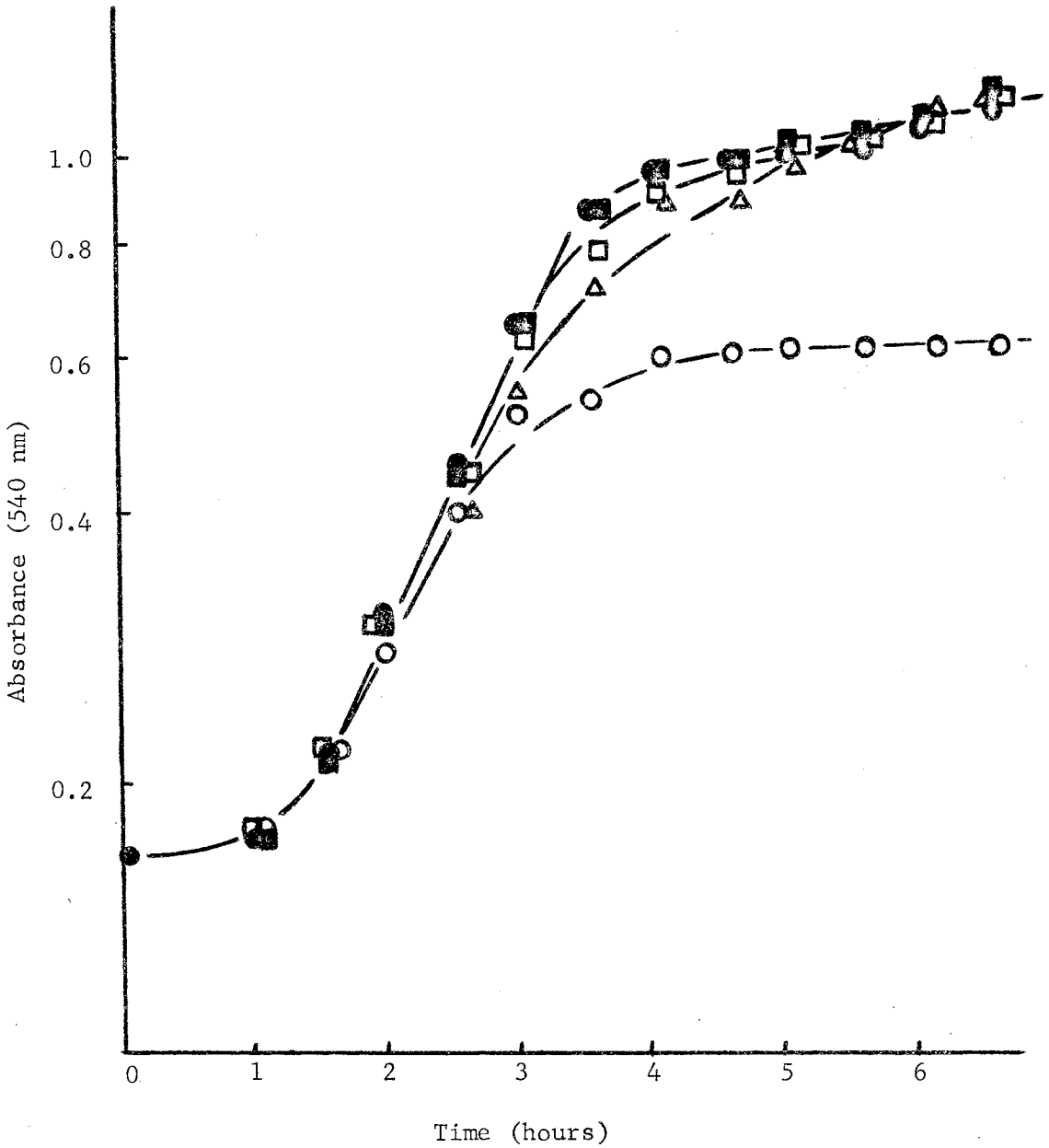

1.0
0.8
0.6
0.4
0.2
Absorbance (540 nm)
0
1
2
3
4
5
6
Time (hours)

Figure 9

The influence of protocatechuate (8.0×10^{-4} M) on actinomycin D inhibition of growth of B. subtilis. The cells were growing in a glucose-salts medium. ●, glucose-salts (control); ○, glucose-salts plus protocatechuate; ▲, actinomycin D (8.0×10^{-8} M); and □, actinomycin D (8.0×10^{-8} M) plus protocatechuate.

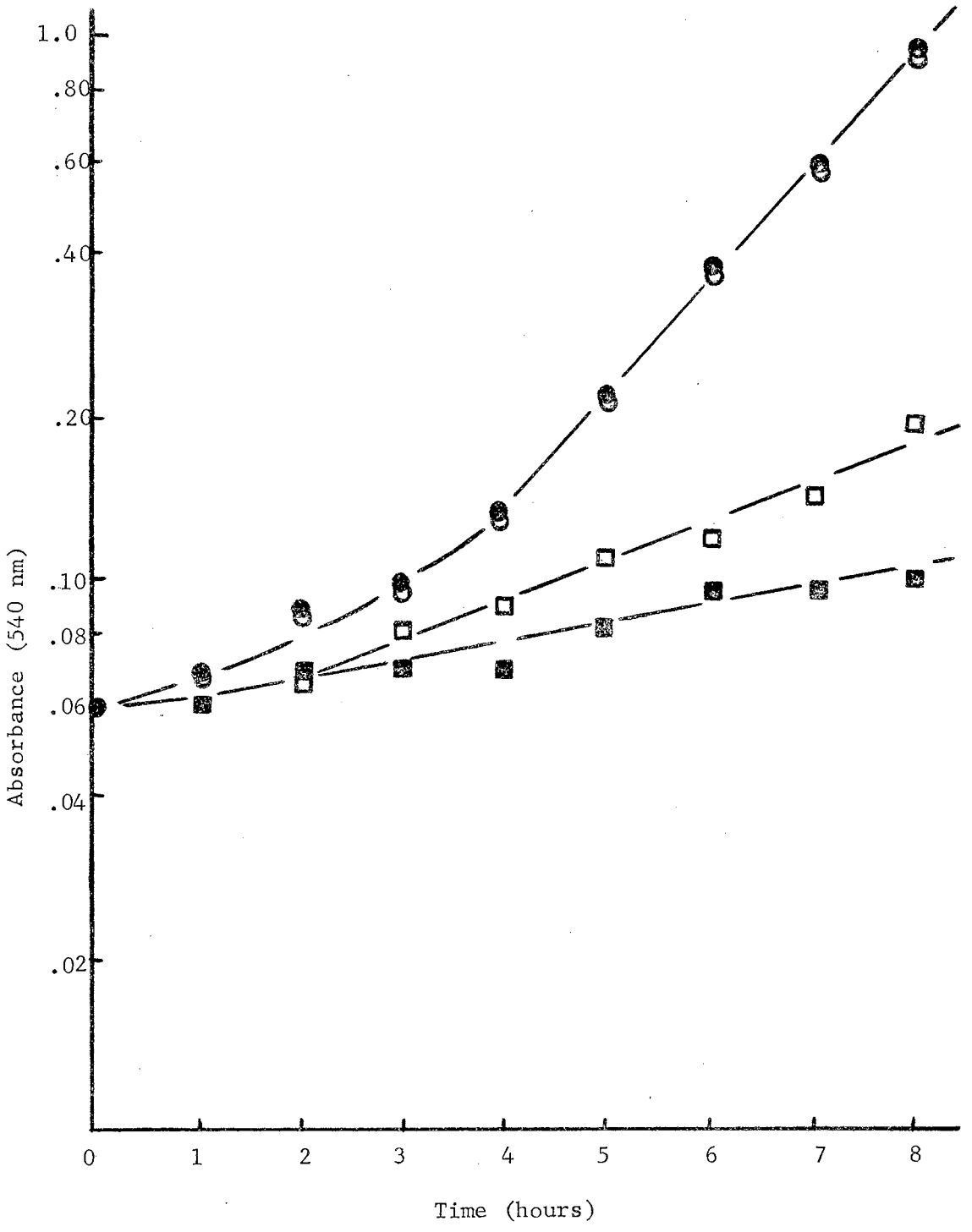
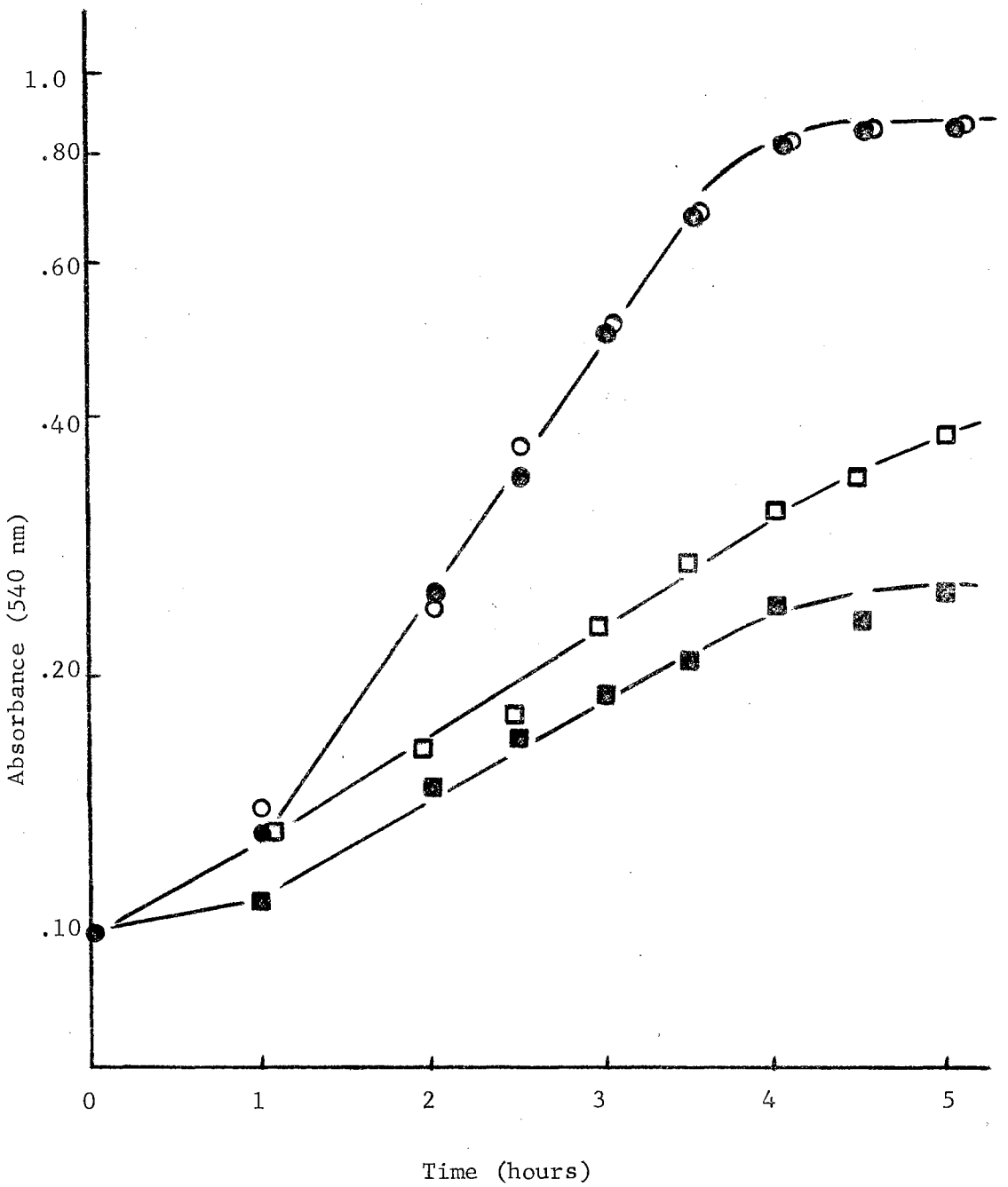

1.0
.80
.60
.40
.20
.10
.08
.06
.04
.02
Absorbance (540 nm)
0
1
2
3
4
5
6
7
8
Time (hours)

Figure 10

The influence of protocatechuate (8.0×10^{-4} M) on actinomycin D inhibition of growth of a species of Flavobacterium. The cells were growing in succinate-salts medium. ◉, succinate-salts (control); ○, succinate-salts plus protocatechuate; ■, actinomycin D (8.0×10^{-6} M); and □, actinomycin D (8.0×10^{-6} M) plus protocatechuate.


1.0
.80
.60
.40
.20
.10
Absorbance (540 nm)
0
1
2
3
4
5
Time (hours)

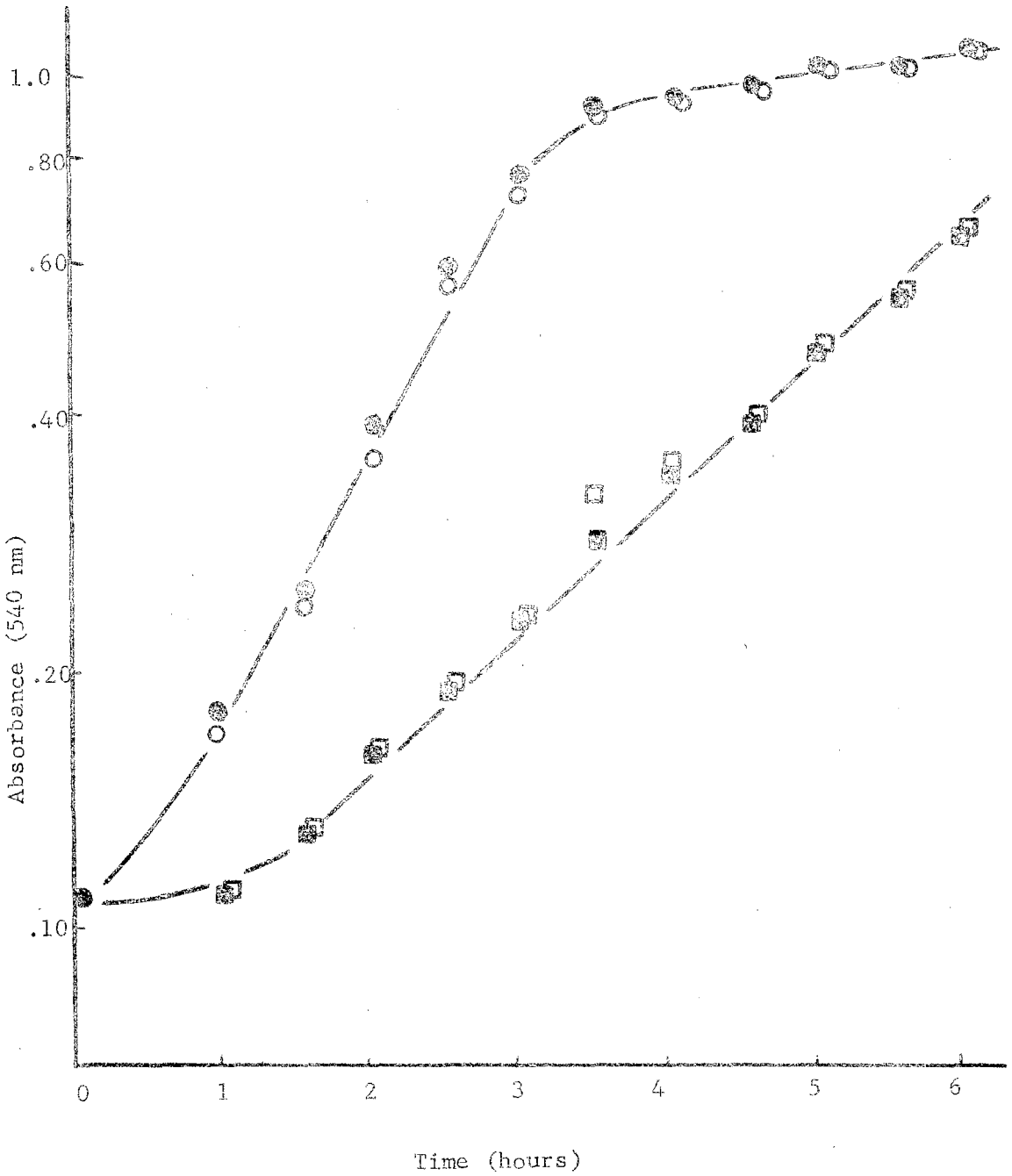
gram-positive B. subtilis was more sensitive to actinomycin D than the two gram-negative organisms, P. fluorescens and the species of Flavobacterium. Bacillus subtilis was sensitive to 8.0×10^{-8} M actinomycin D while the gram-negative organisms were sensitive to 8.0×10^{-6} M actinomycin D. The apparent difference in sensitivity to actinomycin D between the gram-positive and the gram-negative organisms may be due to a difference in the cell wall structure and composition or permeability to actinomycin D. Addition of protocatechuate resulted in partial alleviation of the growth-inhibition by actinomycin D in all of the organisms tested. The prevention of growth inhibition by protocatechuate was more pronounced in P. fluorescens than in either B. subtilis or the species of Flavobacterium. These results show that the prevention of actinomycin D inhibition by protocatechuate is not limited to a single bacterial species but may, in fact, be a general phenomenon.

Inhibition of growth of P. fluorescens by chloramphenicol

Chloramphenicol (CAP) inhibits growth and protein synthesis and may act as a competitive inhibitor of aminoacyl-tRNA (Pestka, 1970). The precise reaction affected by CAP may be either the peptidyl-transferase reaction or the binding of the aminoacyl-adenyl terminus of t-RNA to the ribosome. Studies were conducted to determine if CAP inhibited growth of P. fluorescens and if protocatechuate had any effect on the inhibition. The results (Figure 11) showed that CAP (5.0×10^{-5}M) inhibited growth of P. fluorescens. Protocatechuate, at the concentration tested (8.0×10^{-4} M), did not affect the inhibition of growth by CAP.

Figure 11

The influence of protocatechuate (8.0×10^{-4} M) on chloramphenicol inhibition of growth of P. fluorescens. The cells were growing in a succinate-salts medium. ●, succinate-salts (control); ○, succinate-salts plus protocatechuate; ■, chloramphenicol (5.0×10^{-5} M); and □, chloramphenicol (5.0×10^{-5} M) plus protocatechuate.


1.0
.80
.60
.40
.20
.10
Absorbance (540 nm)
0
1
2
3
4
5
6
Time (hours)

Induction of amidase by acetamide

Actinomycin D and CAP inhibited growth of P. fluorescens. Since both CAP and actinomycin D inhibit protein synthesis by different mechanisms, (Pestka, 1970; Gross, Malkin and Moyer, 1964), the effect of these antibiotics on the synthesis of amidase was studied.

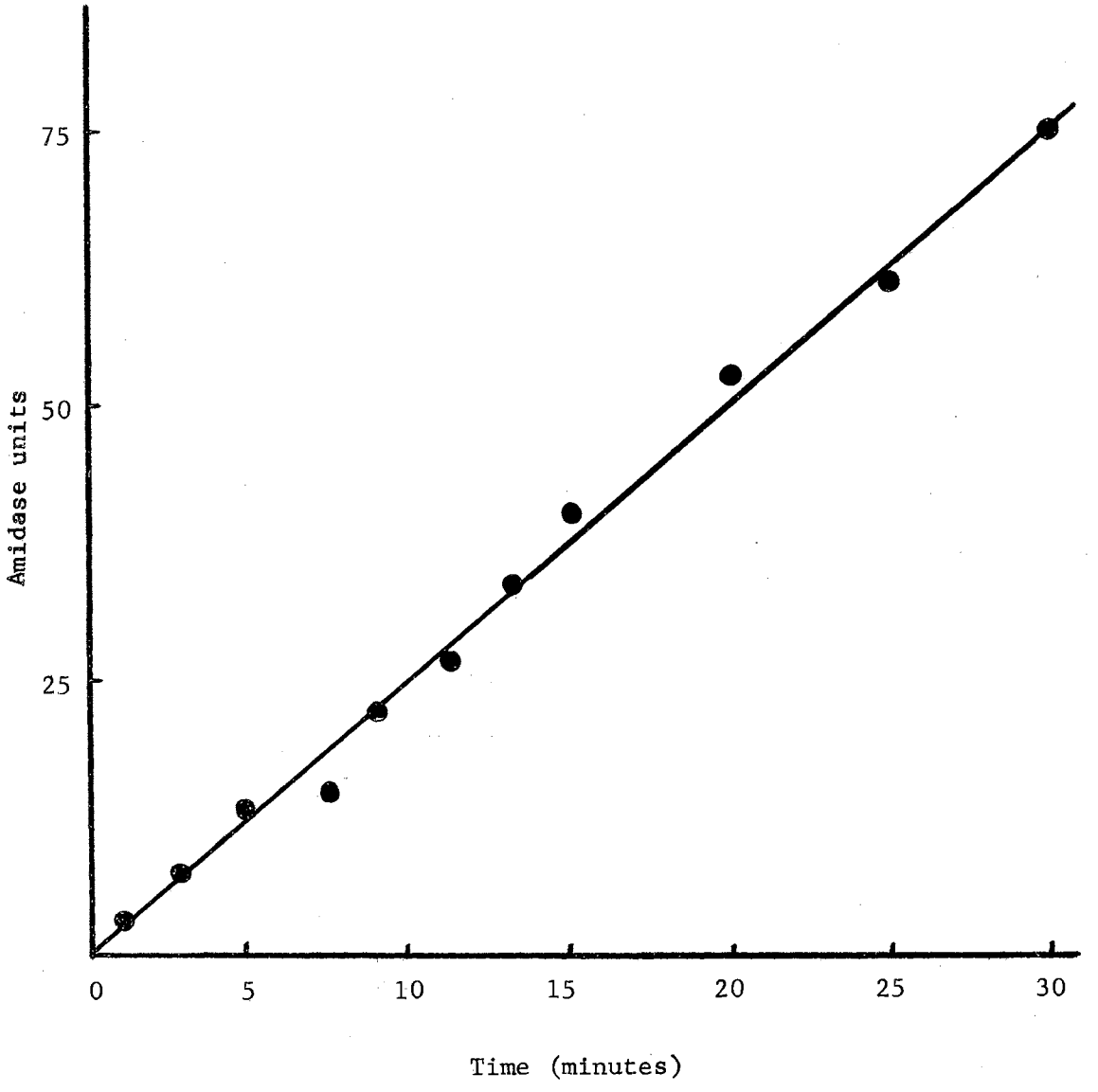
Kelly and Clarke (1962) observed that P. aeurginosa 8602/A produced an inducible enzyme, amidase, which hydrolyzed acetamide. The enzyme could be induced by acetamide or N-methylacetamide, a nonsubstrate inducer. Experiments were conducted to determine if enzyme activity was a linear function with respect to time under the conditions of the assay. Acetamide-salts medium was inoculated with P. fluorescens and the cells allowed to grow for 9 hours at 37 C with constant shaking. Enzyme assays were conducted using the induced cells as described except that the assay reactions were stopped at the specified time intervals by addition of ferric chloride solution. The results (Figure 12) show that enzyme activity is linear with respect to time for at least 30 minutes. This indicates that the enzyme present was saturated with substrate during the assay period. In all of the subsequent experiments, the enzyme assays were stopped after 15 minutes of incubation at 37 C.

Effect of acetamide concentration on amidase synthesis

Studies were conducted to determine the optimum concentration of acetamide required for maximum induction of amidase. Minimal-salts medium was supplemented with varying concentrations of acetamide and inoculated with P. fluorescens cells grown on succinate-salts. The

Figure 12

The measure of enzyme activity and its relationship to time. The cells for this assay were induced to acetamide and the reaction was stopped at the indicated time intervals.


75
50
25
Amidase units
0
5
10
15
20
25
30
Time (minutes)

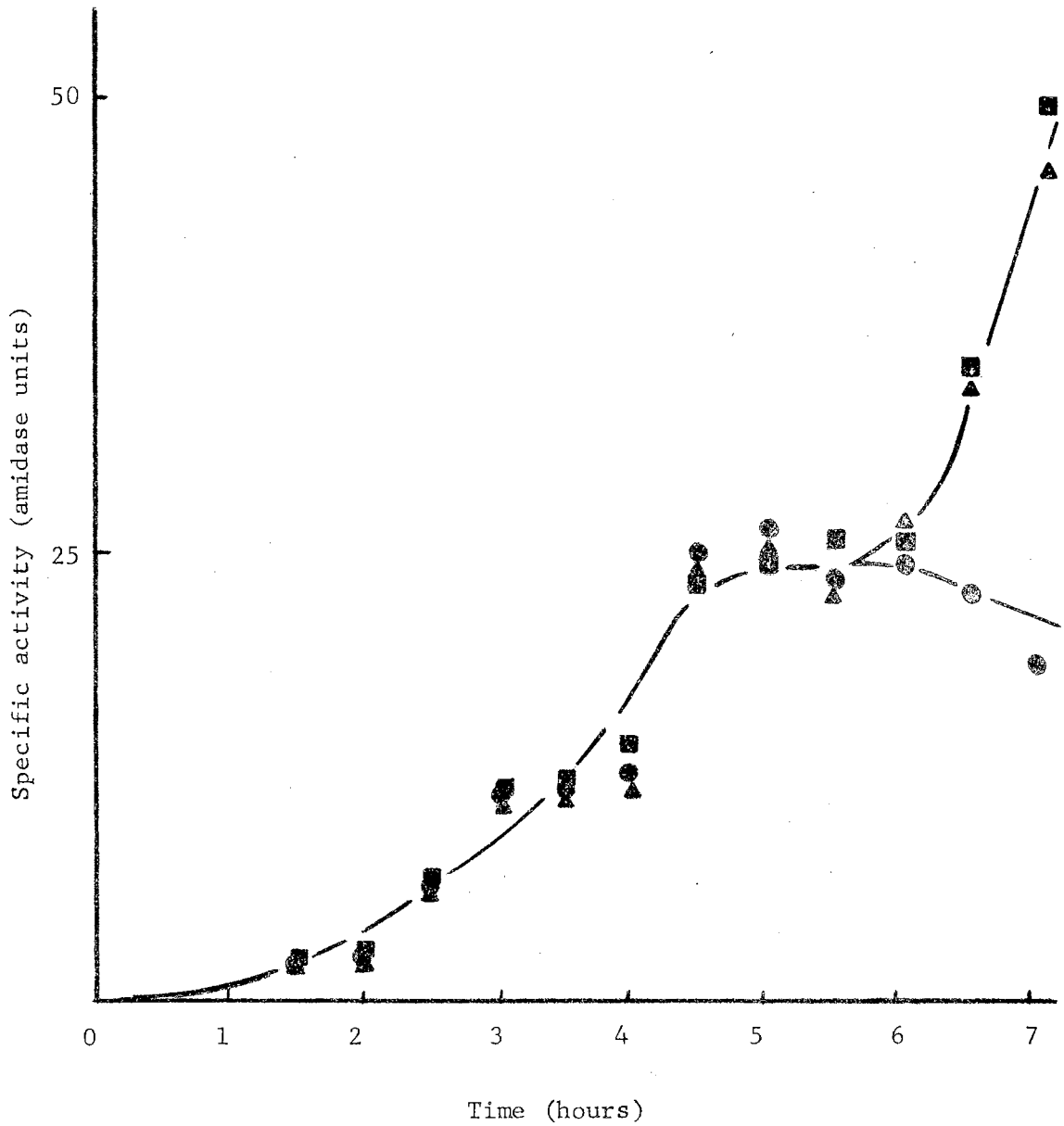
cells were incubated for six hours during which time enzyme synthesis was determined. The results (Figure 13) show that acetamide concentrations of 0.08 M and 0.16 M are sufficient for maximum induction for the duration of this experient. There was no apparent difference between the induction patterns obtained with these two concentrations. An inducer concentration of 0.08 M was used in all subsequent enzyme studies. An acetamide concentration of 0.04 M was insufficient for maximum induction. A diphasic induction pattern of amidase synthesis was observed with the optimum inducer concentration in which amidase synthesis occurred for a period of time, then plateaued, and was followed by a very rapid initiation of enzyme synthesis. Kelly and Kornberg (1962) had previously noted 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 and 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, was gratuitous and occurred during subsequent growth on the acetate that was formed during the hydrolysis of acetamide.

Induction of amidase using recrystallized acetamide as inducer

Studies were conducted to determine if the diphasic induction pattern, obtained when cells were grown in acetamide-salts, could be duplicated using recrystallized acetamide as the inducer. The results indicated that recrystallization of the inducer did not influence the induction pattern of amidase since the diphasic pattern, similar to the induction curve obtained in Figure 13 was obtained. These results

Figure 13

Synthesis of amidase in minimal-salts medium supplemented with varying concentrations of acetamide. ●, 0.04 M acetamide; ■, 0.08 M acetamide; and ▲, 0.16 M acetamide.


50
25
Specific activity (amidase units)
0
1
2
3
4
5
6
7
Time (hours)

suggest that enzyme synthesis during both phase I and phase II are due to the original presence of acetamide.

Influence of cell inoculum on induction and synthesis of amidase

Experiments were conducted to determine if the mass of cells used as the inoculum would affect the induction and synthesis of amidase (Figure 14). Acetamide-salts medium was inoculated with P. fluorescens to an initial absorbance of 0.1 or 0.35 at 540 nm. The results indicated that as the inoculum size was increased, the rate of initial enzyme synthesis was increased and the lag period preceeding phase II was shortened. Increasing the inoculum size did not eliminate the diphasic induction pattern.

Effect of cell toluenization on amidase activity

Toluene disrupts the permeability barrier of the cell and has been used to facilitate enzyme assays in this organism (Kirkland and Durham, 1965). Experiments were conducted to determine if toluenization of amidase-induced cells increased the measurable enzymatic activity, Pseudomonas fluorescens cells were grown on acetamide-salts medium. Two (1.0 ml) samples were removed at the selected time intervals and placed in the freezer. Immediately prior to thawing the samples, 0.02 ml of toluene was added to one set of the samples and 0.02 ml of triple distilled water was added to the second set of samples. The samples were thawed at 37 C and the enzymatic activity was determined. The results showed (Figure 15) that toluenization of induced cells did not influence the measurable enzymatic activity.

Figure 14

The influence of inoculum size on induction and synthesis of amidase. P. fluorescens cells were growing in acetamide-salts medium. ●, initial absorbance of 0.10 at 540 nm; and ◐, initial absorbance of 0.35 at 540 nm.

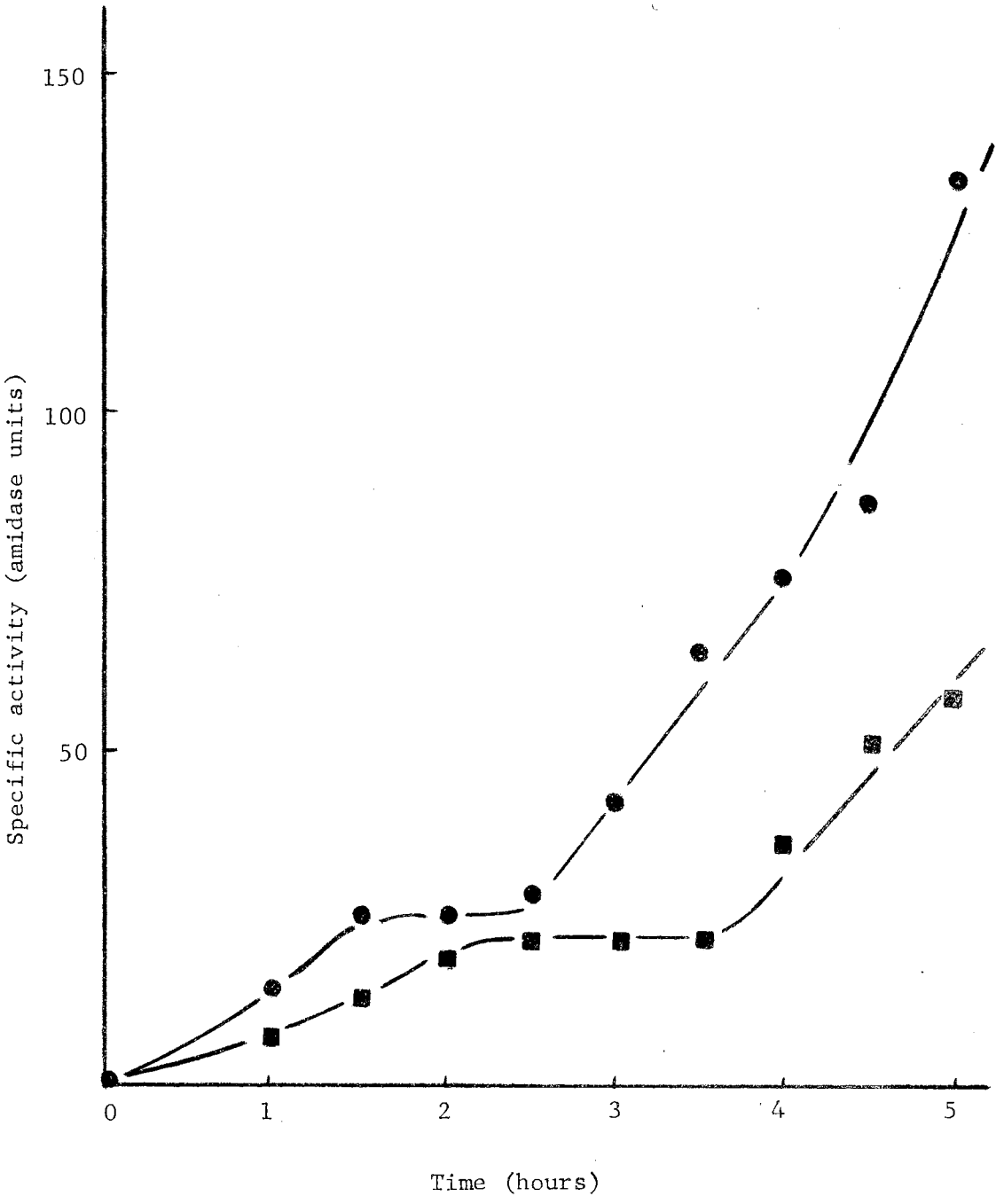
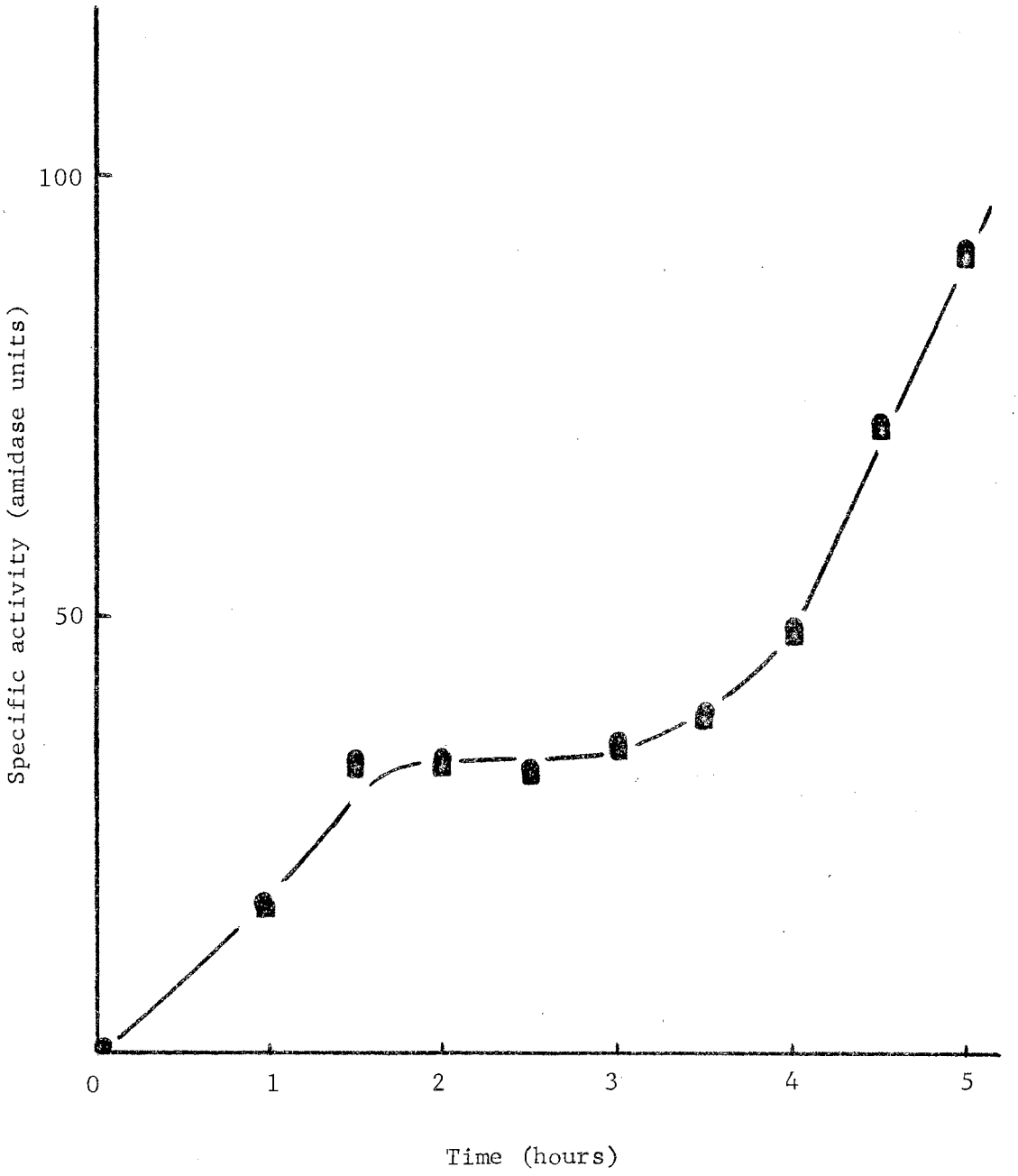

150
100
50
Specific activity (amidase units)
0
1
2
3
4
5
Time (hours)

Figure 15

The effect of toluenization of on the enzyme activity of P. fluorescens cells. ●, water-treated cells; and ■, toluene-treated cells.


100
50
Specific activity (amidase units)
0
1
2
3
4
5
Time (hours)

Effect of carbon source on amidase synthesis

Because of the unusual induction pattern obtained in acetamide-salts medium, experiments were conducted to compare enzyme synthesis in different media. Pseudomonos fluorescens cells were grown on succinate-salts medium and inoculated into the following media: succinate-salts, succinate-salts plus acetamide, and acetamide-salts medium. Enzyme synthesis was determined and the results are presented in figure 16. The diphasic induction pattern for amidase was observed in the acetamide-salts medium. The induction pattern of cells growing in succinate-salts plus acetamide did not show the diphasic pattern, characteristic of enzyme induction in acetamide-salts. Amidase synthesis did not occur in the succinate-salts medium without inducer.

Brammar and Clarke (1964) reported induction of amidase synthesis by the nonsubstrate inducer N-methylacetamide. Studies were made to compare the induction pattern obtained when either acetamide or N-methylacetamide was used as the inducer. Pseudomanas fluorescens cells, grown on succinate-salts, were inoculated into the following media: a) acetamide-salts, b) succinate-salts plus acetamide, c) succinate-salts plus N-methylacetamide, and d) minimal-salts plus N-methylacetamide. The results indicated that enzyme synthesis using N-methylacetamide or acetamide as the inducer in a succinate-salts medium gave similar induction patterns (Figure 17). The diphasic induction pattern was observed only in the acetamide-salts medium. Only a small amount of enzyme synthesis occurred in the flask containing minimal-salts plus N-methylacetamide, since there was no other readily available carbon source. Brammar and Clarke (1964) reported that a diphasic induction

Figure 16

Amidase synthesis by P. fluorescens cells growing in succinate-salts and acetamide-salts medium. ●, acetamide-salts medium; ■, succinate-salts plus acetamide; and ▲, succinate-salts.

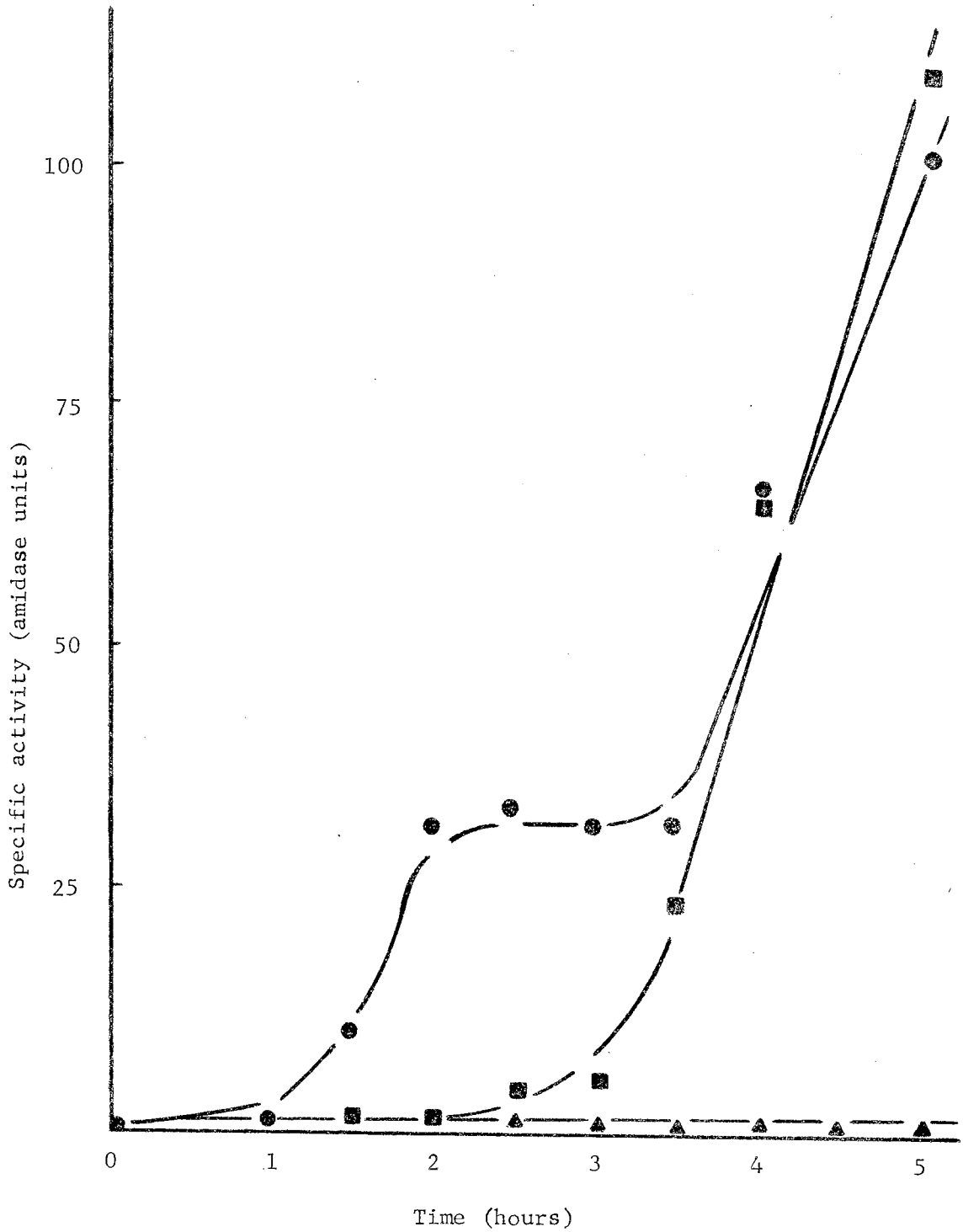
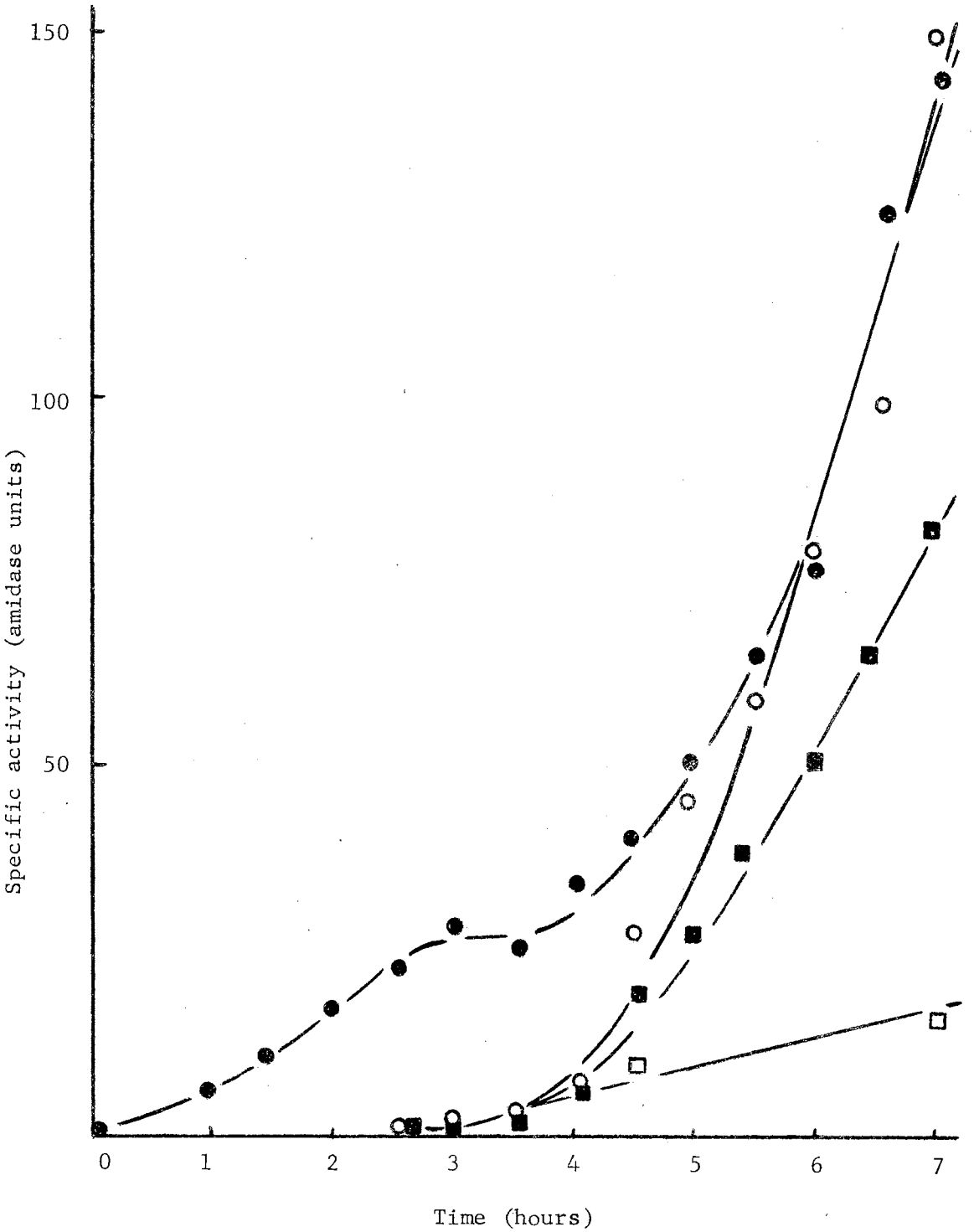

100
75
50
25
Specific activity (amidase units)
0
1
2
3
4
5
Time (hours)

Figure 17

A comparison of the amidase induction pattern in P. fluorescens cells using acetamide or N-methylacetamide as the inducer. ●, acetamide-salts medium; ○, succinate-salts plus acetamide; ◒, succinate-salts plus N-methylacetamide (0.05 M); and □, minimal-salts plus N-methylacetamide (0.05 M).


150
100
50
Specific activity (amidase units)
0
1
2
3
4
5
6
7
Time (hours)

pattern was obtained when acetate plus acetamide was used as the growth medium. We have confirmed these results in our laboratory (Figure 18). Pseudomonas fluorescens cells growing in a medium in which acetate was the sole carbon and energy source did not synthesize any enzyme. If the acetate-salts medium was supplemented with acetamide, a diphasic induction pattern for amidase synthesis was observed. Either acetamide or N-methylacetamide may serve to induce the synthesis of amidase. The induction pattern of amidase depends on the carbon source of the medium in which the cells are growing.

Inhibition of amidase synthesis by actinomycin D and chloramphenicol

Since results from this study have established that actinomycin D and CAP inhibit growth of P. fluorescens, the influence of these antibiotics on amidase synthesis was studied. A diphasic induction of amidase was observed in the absence of the antibiotics (control) (Figure 19). CAP inhibited both phase I and phase II of amidase synthesis. Actinomycin D did not inhibit phase I of amidase synthesis, but did inhibit the synthesis of enzyme during phase II. The results obtained with actinomycin D could indicate that a) the antibiotic may not be taken into the cell in an adequate concentration to inhibit amidase synthesis during phase I which lasted approximately 2 hours, or b) if the m-RNA which codes for amidase synthesis preexisted within the cell, then amidase synthesis would not be inhibited by actinomycin D until a new m-RNA molecule was required for coding, such as might be the case in phase II. CAP was added to acetamide-salts medium at two different times (0 and 4½ hours) and results indicated the antibiotic inhibited enzyme synthesis whenever added to the system (Figure 20).

Figure 18

Induction of amidase in P. fluorescens cells growing in acetate-salts medium. ▣, acetate-salts medium and ●, acetate-salts medium plus acetamide (0.08 M).

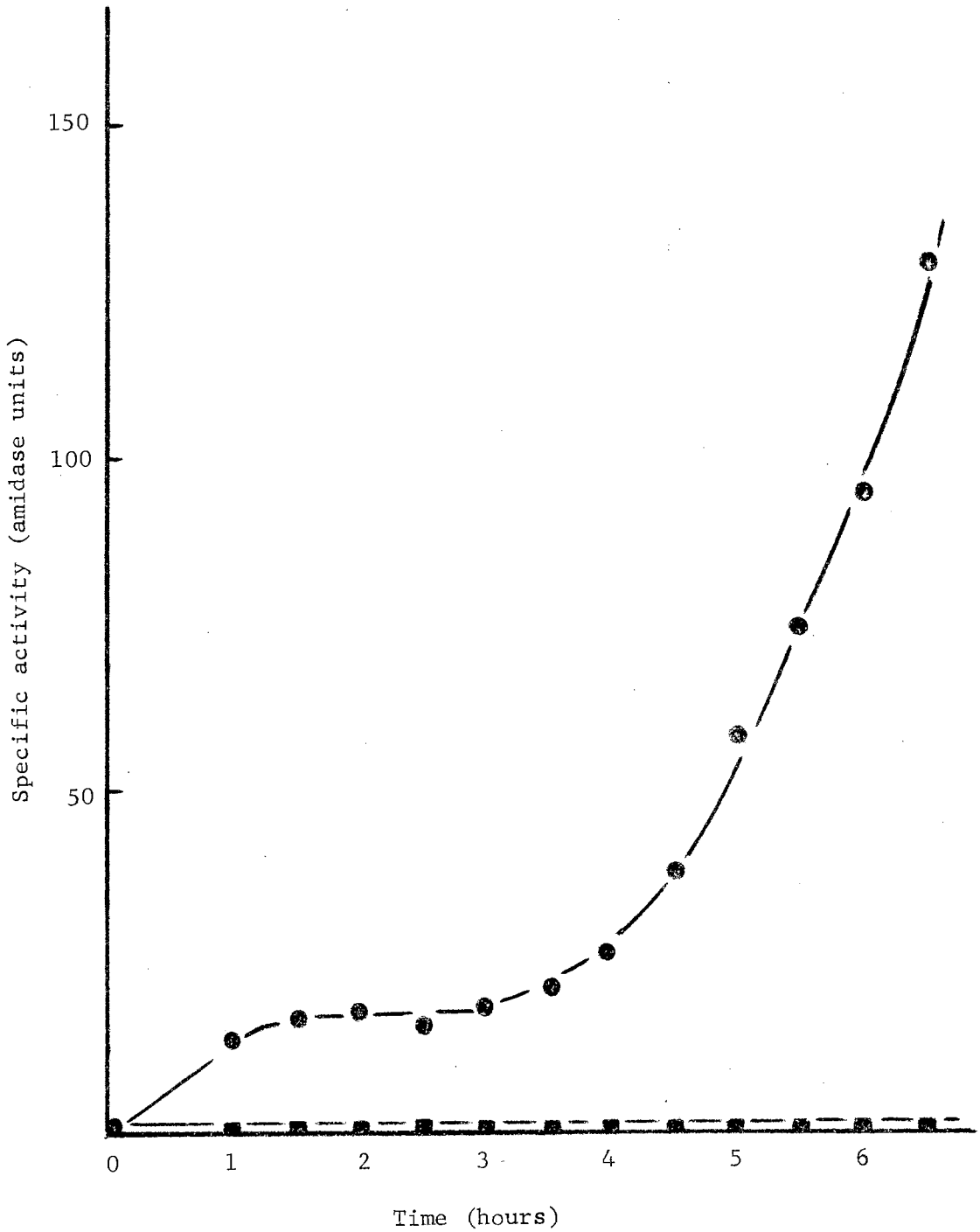

150
100
50
Specific activity (amidase units)
0
1
2
3
4
5
6
Time (hours)

Figure 19

The effect of chloramphenicol and actinomycin D on amidase synthesis in acetamide-salts medium. ●, acetamide-salts (control); ■, acetamide-salts plus actinomycin D (8.0×10^{-6}M); and ▲, acetamide-salts plus chloramphenicol ($5.0 \times .0^{-5}$M).

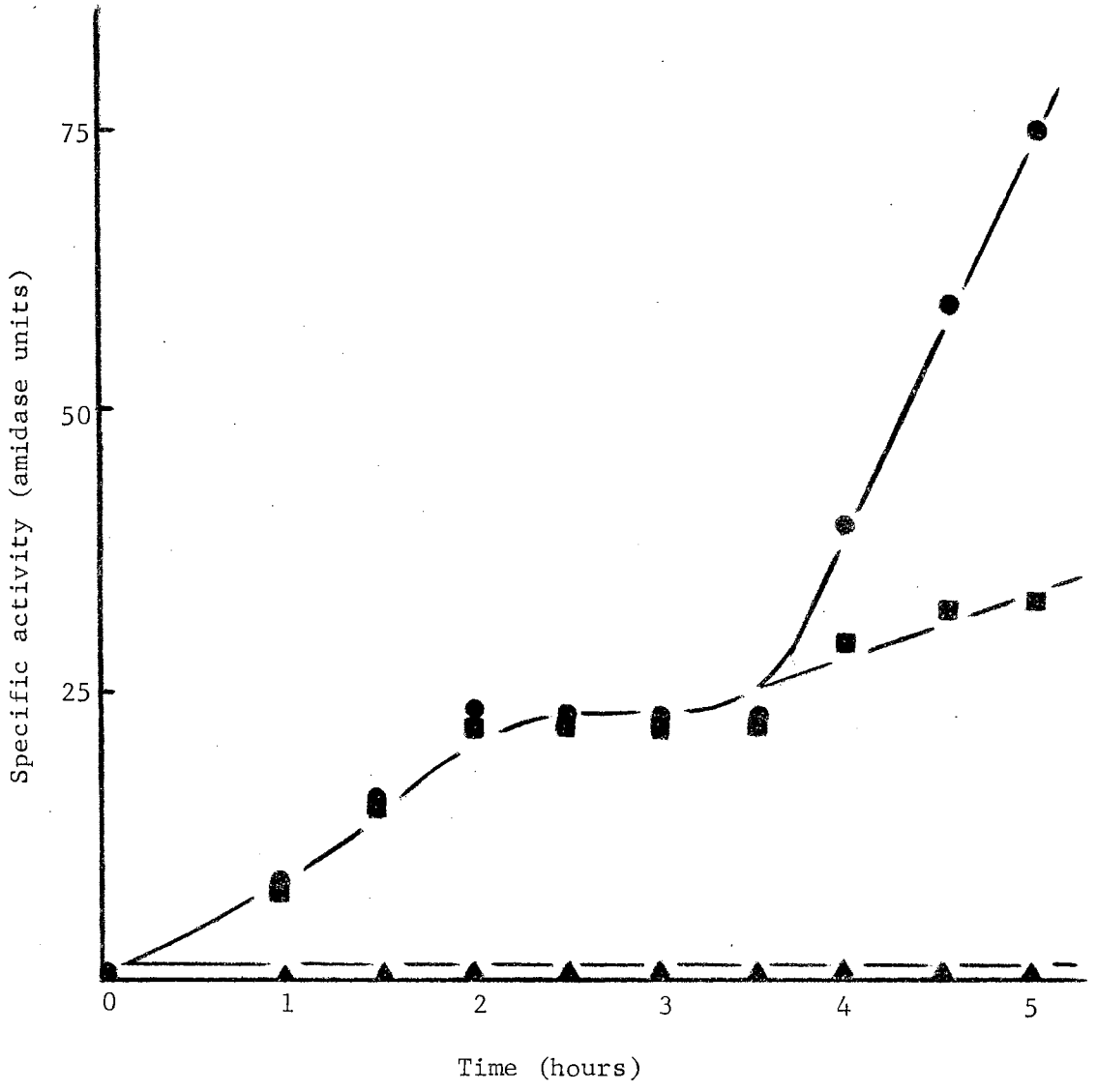
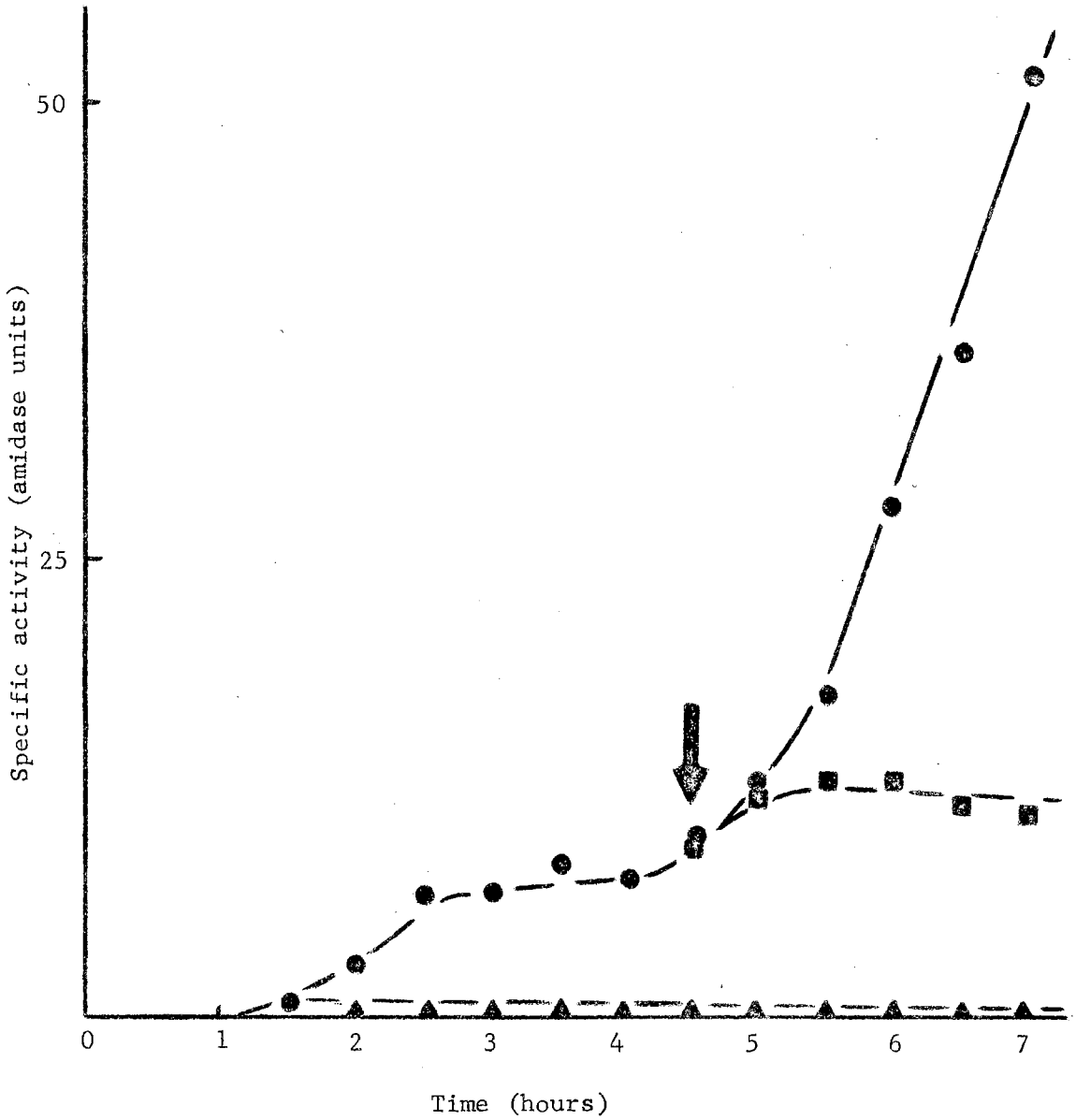

Specific activity (amidase units)
75
50
25
0
1
2
3
4
5
Time (hours)

Figure 20

The effect of chloramphenicol on amidase synthesis in cells growing in an acetamide-salts medium. ●, acetamide-salts (control); ▲, acetamide-salts plus chloramphenicol (5.0×10^{-5}M) (added at zero time); ■, acetamide-salts plus chloramphenicol (5.0×10^{-5}M) (added at 4½ hours).


50
25
Specific activity (amidase units)
0
1
2
3
4
5
6
7
Time (hours)

Since CAP inhibits enzyme synthesis by acting as a competitive inhibitor of aminoacyl-t-RNA, then it should inhibit amidase synthesis whether the m-RNA molecule which codes for amidase preexisted or was newly-synthesized.

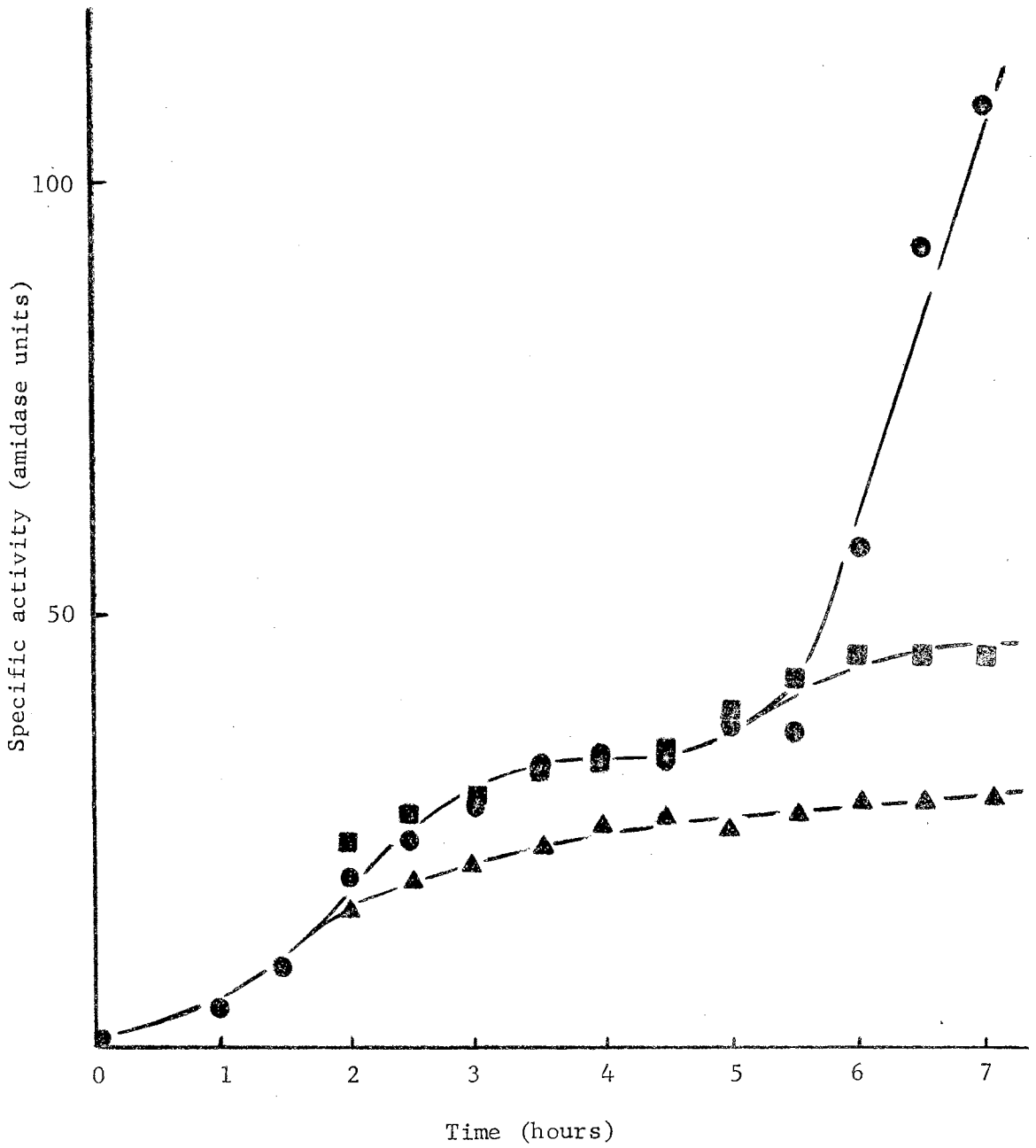
Experiments were conducted to study the effect of a higher concentration of actinomycin D on amidase synthesis. Actinomycin D (8.0×10^{-6} M) did not inhibit the synthesis of amidase during phase I, but did prevent enzyme synthesis during phase II (Figure 21). An actinomycin concentration of 2.4×10^{-5} M partially inhibited phase I and phase II of amidase synthesis. The inhibition of phase I of amidase synthesis by 2.4×10^{-5} M actinomycin D could be explained if an increased cellular concentration of the antibiotic was observed at the higher antibiotic concentration. A second explanation may be based on the theory of the preexisting m-RNA which codes for phase I of amidase synthesis. If the preformed m-RNA molecule were degraded in the presence of increased concentrations of actinomycin, one should observe a decrease in amidase synthesis during phase I. Acs, Reich and Valanju (1963) reported that newly-formed RNA of high molecular weight was rapidly degraded in bacteria exposed to actinomycin.

Repression of amidase synthesis

Brammar and Clarke (1964) reported that acetate and some tricarboxylic acid intermediates acted as repressors of amidase synthesis. If acetate is produced in sufficient concentration during the hydrolysis of acetamide to acetate and ammonia, then the plateau observed during enzyme synthesis in the acetamide-salts medium could be due to

Figure 21

The effect of actinomycin D on amidase synthesis. These cells were growing in acetamide-salts medium. ●, acetamide-salts (control); ▩, acetamide-salts plus actinomycin (8.0×10^{-6}M); and ▲, acetamide salts plus actinomycin D (2.4×10^{-5}M).


Specific activity (amidase units)
100
50
0
1
2
3
4
5
6
7
Time (hours)

repression of amidase synthesis by acetate. Studies were conducted to elucidate the repression of amidase synthesis by acetate and succinate. Pseudomonas fluorescens cells were grown on acetate-salts or succinate-salts medium. The acetate-grown cells should utilize acetate more readily than cells grown on succinate. Therefore, in acetate-grown cells there would be faster utilization of acetate and less repression of amidase synthesis. The results indicate that acetate-grown cells have an increased rate of amidase synthesis as compared to succinate-grown cells (Figure 22). Initiation of phase II of amidase synthesis occurred at approximately the same time in both cultures. Pseudomonas fluorescens cells, which were grown on acetate-salts, showed a decreased growth lag compared to succinate-grown cells when inoculated into acetamide-salts medium (Figure 23). These results did not establish the role of acetate as a repressor of amidase synthesis.

Additional studies were conducted in which succinate grown cells of Pseudomonas fluorescens were inoculated into flasks containing acetamide salts medium supplemented with 0.01 M acetate or 0.01 M succinate. Enzyme synthesis was determined and the results are presented in figure 24. The results showed that during the initial stages of enzyme synthesis in phase I, succinate and acetate repressed amidase synthesis. At equal molar concentrations, succinate was more effective as a repression than acetate.

Additional experiments were conducted to illustrate succinate repression during phase I and phase II of amidase synthesis. Pseudomonas fluorescens cells were grown in flasks of acetamide-salts medium to which succinate (0.01 M) was added to one flask at zero time and to a second flask at 4½ hours. The results (Figure 25) indicated

Figure 22

A comparison of enzyme synthesis between acetate salts-grown and succinate salts-grown P. fluorescens cells growing in acetamide-salts medium. ◔, acetate-grown cells; and ◉, succinate-grown cells.

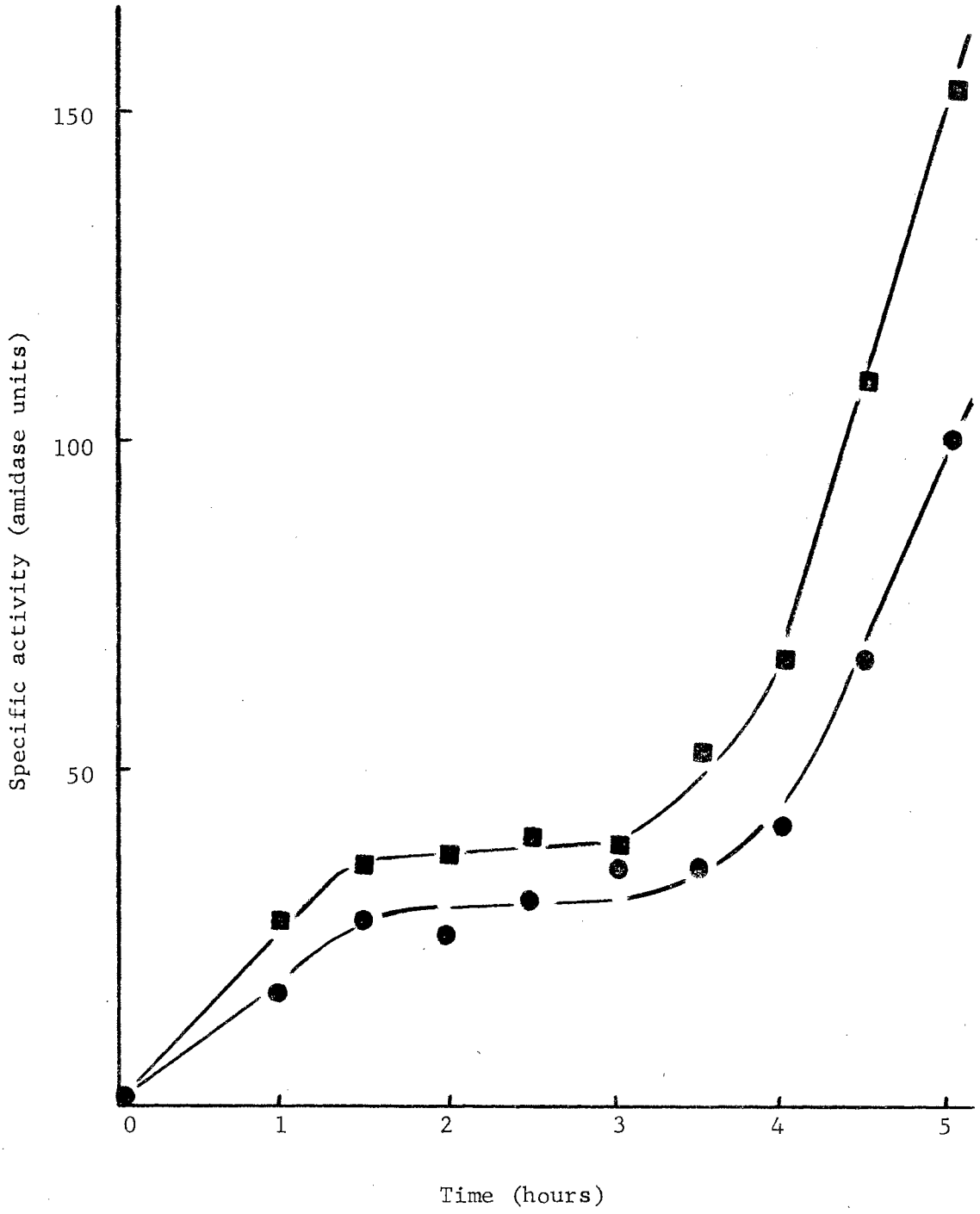

Specific activity (amidase units)
150
100
50
0
1
2
3
4
5
Time (hours)

Figure 23

A comparison of the rate of growth between acetate salts-grown and succinate salts-grown P. fluorescens cells growing in acetamide-salts medium. □, acetate-grown cells; and ○, succinate-grown cells.

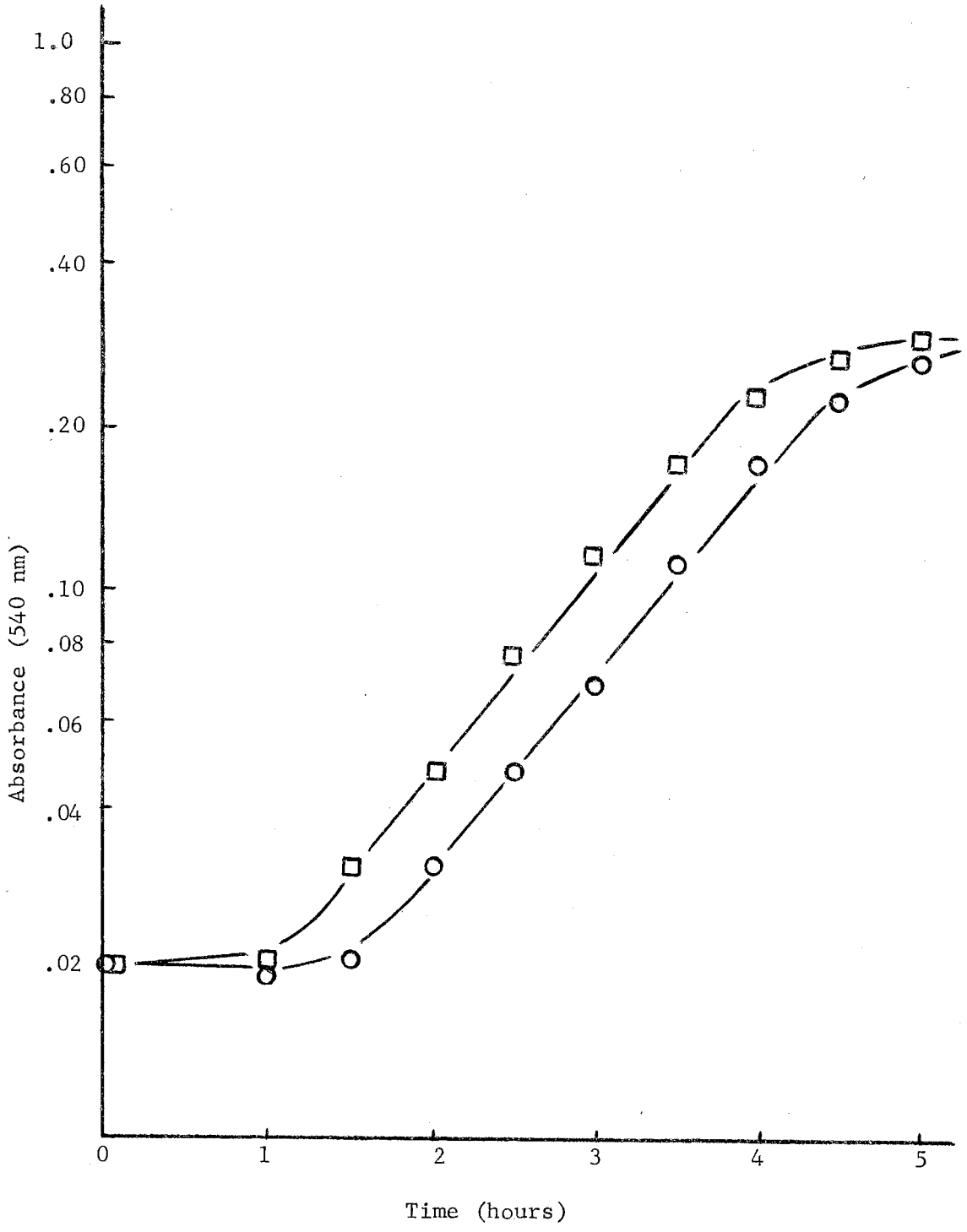

1.0
.80
.60
.40
.20
.10
.08
.06
.04
.02
Absorbance (540 nm)
0
1
2
3
4
5
Time (hours)

Figure 24

Repression by succinate and acetate of amidase synthesis in P. fluorescens. The cells were growing in acetamide salts medium. ●, acetamide-salts (control); ◉, acetamide-salts plus succinate (0.01 M); and ▲, acetamide-salts plus acetate (0.01 M).

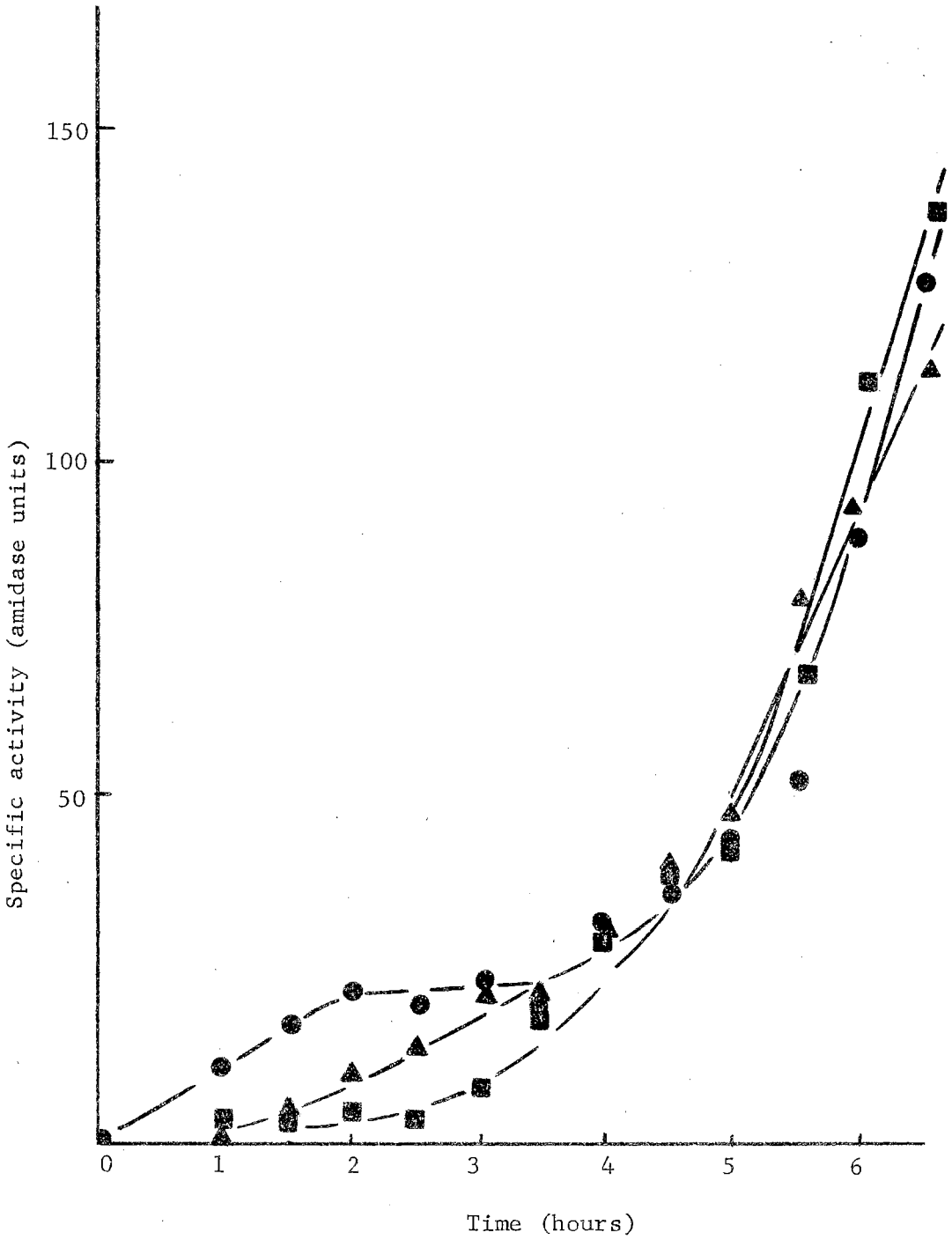
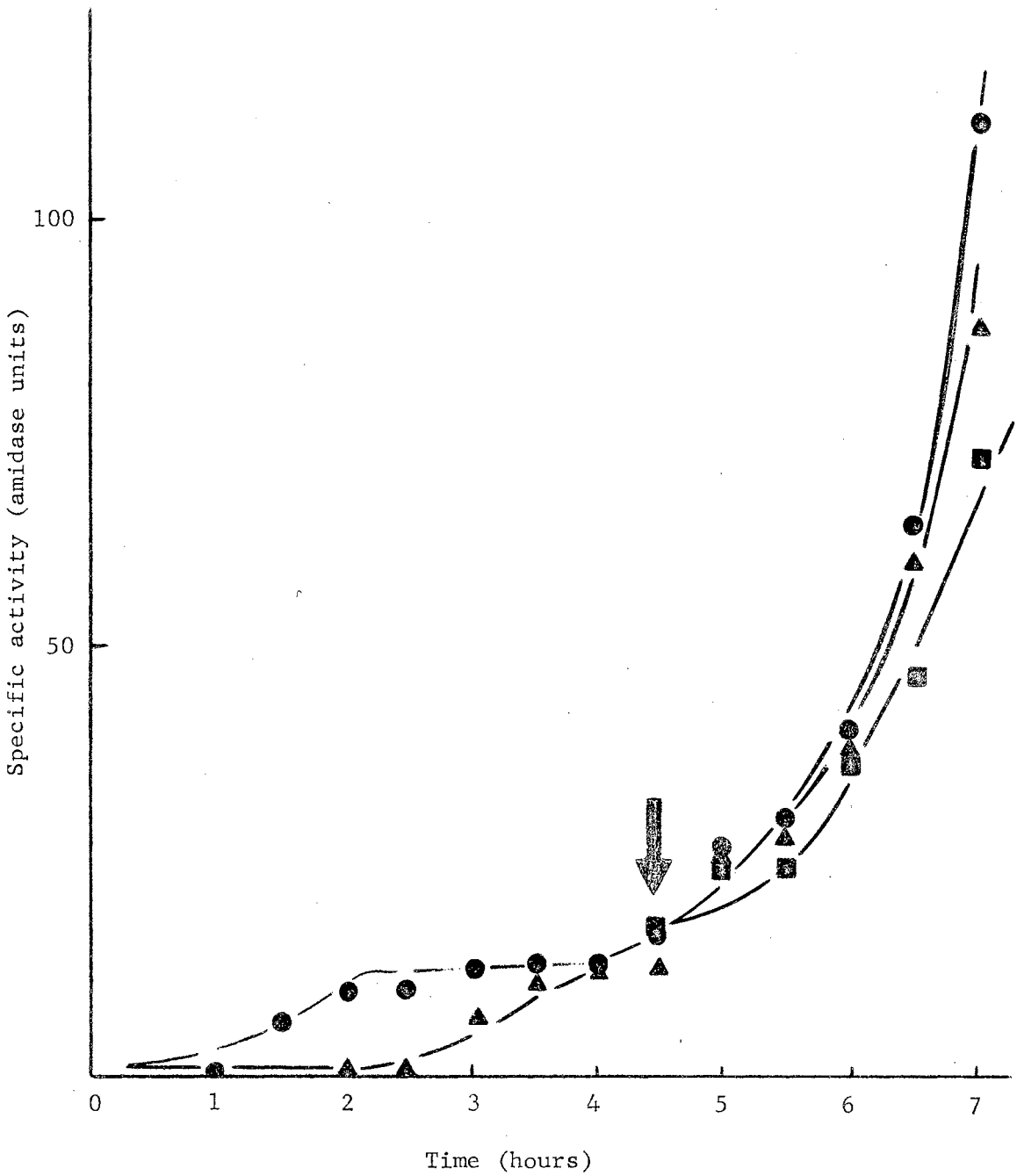

150
100
50
Specific activity (amidase units)
0
1
2
3
4
5
6
Time (hours)

Figure 25

Repression of amidase synthesis in P. fluorescens by succinate. The cells were growing in acetamide-salts medium. ●, acetamide-salts (control); ▲, acetamide-salts plus succinate (0.01 M) (added at zero time); and ■, acetamide-salts plus succinate (0.01 M) (added at 4½ hours).

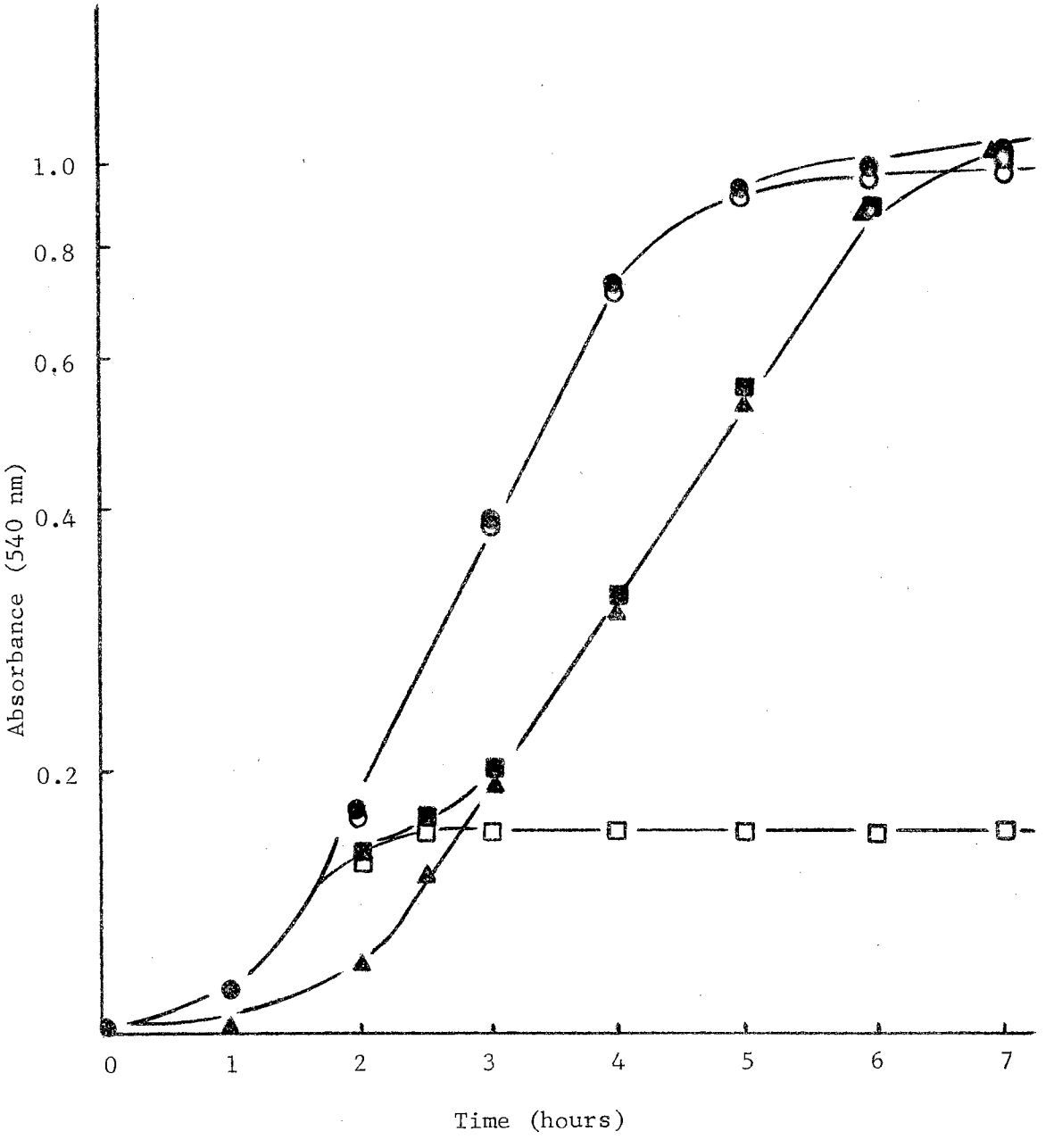

100
50
Specific activity (amidase units)
0
1
2
3
4
5
6
7
Time (hours)

that addition of succinate at zero time repressed phase I of amidase synthesis while the addition of succinate at 4½ hours partially repressed synthesis during phase II. A pronounced repression was not observed when succinate was added after 4½ hours, which may have been due, as measured by appropriate growth controls, to the rapid depletion of the succinate by the large number of cells present in the flask.

The metabolite repression observed in the amidase system has been noted with other enzymes and referred to by the general term "glucose effect" (Monod, 1942). This type of repression has been observed with metabolites other than glucose and in recent years the term metabolite repression has replaced "glucose effect." According to Spiegelman (1955), growth on glucose preempts the cells supply of pools to produce glucose degrading enzymes and this presupposes that glucose is a more readily utilizable substrate than the inducer. Growth of cells in a medium containing two carbon sources at concentrations insufficient to support full growth is often diphasic, depending on the specific carbon sources supplied. This phenomenon has been termed diauxie (Monod, 1942). Our results (Figure 26) showed that when cells are growing in the succinate-acetamide medium, succinate is utilized during the initial growth phase and, following the apparent depletion of succinate, acetamide is utilized during the second growth phase. When the succinate was present in sufficient concentration to support full growth of the bacteria, the diauxie growth pattern was not observed. Similar results have been reported with Escherichia coli grown in a glucose-lactose medium. The cells utilized glucose exclusively prior to the utilization of lactose (Monod, 1942). Neidhart and Magasanik (1956) reported that all glucose-sensitive enzymes were capable of converting their

Figure 26

Diauxie response of P. fluorescens growing in succinate-acetamide medium. ○, succinate (0.01 M); ●, succinate (0.01 M) plus acetamide (0.08 M); □, succinate (0.001 M); ■, succinate (0.001 M) plus acetamide (0.08 M); and ▲, acetamide (0.08 M).


1.0
0.8
0.6
0.4
0.2
Absorbance (540 nm)
0
1
2
3
4
5
6
7
Time (hours)

substrates to metabolites which the cell can also obtain independently and more readily by the metabolism of glucose. In this situation, it is disadvantageous for a cell to produce enzymes to degrade a compound if it can get faster results by degrading glucose.

Cohn and Monod (1953) proposed that the mechanism of "glucose effect" or metabolite repression was possibly an interference by the metabolite with the transport mechanism for the inducer. Experiments were conducted to determine if succinate and acetate influenced the uptake of acetamine-1-^{14}C by cells of P. fluorescens (Table I).

TABLE I.

UPTAKE OF ACETAMIDE-1-^{14}C BY CELLS OF P. FLUORESCENS

Constituents	counts per minute		percent of control	
	10 sec	90 sec	10 sec	90 sec
control	90	349	100	100
control + succinate	78	66	86	19
control + acetate	61	119	68	34

These results show that the presence of either succinate or acetate in the system decreased the uptake of radioactive acetamide by the cells. These experiments did not unequivocally show whether succinate or

acetate was the most effective inhibitor of uptake of ^{14}C-labeled acetamide.

Reversal of actinomycin D inhibition of enzyme synthesis by protocatechuate

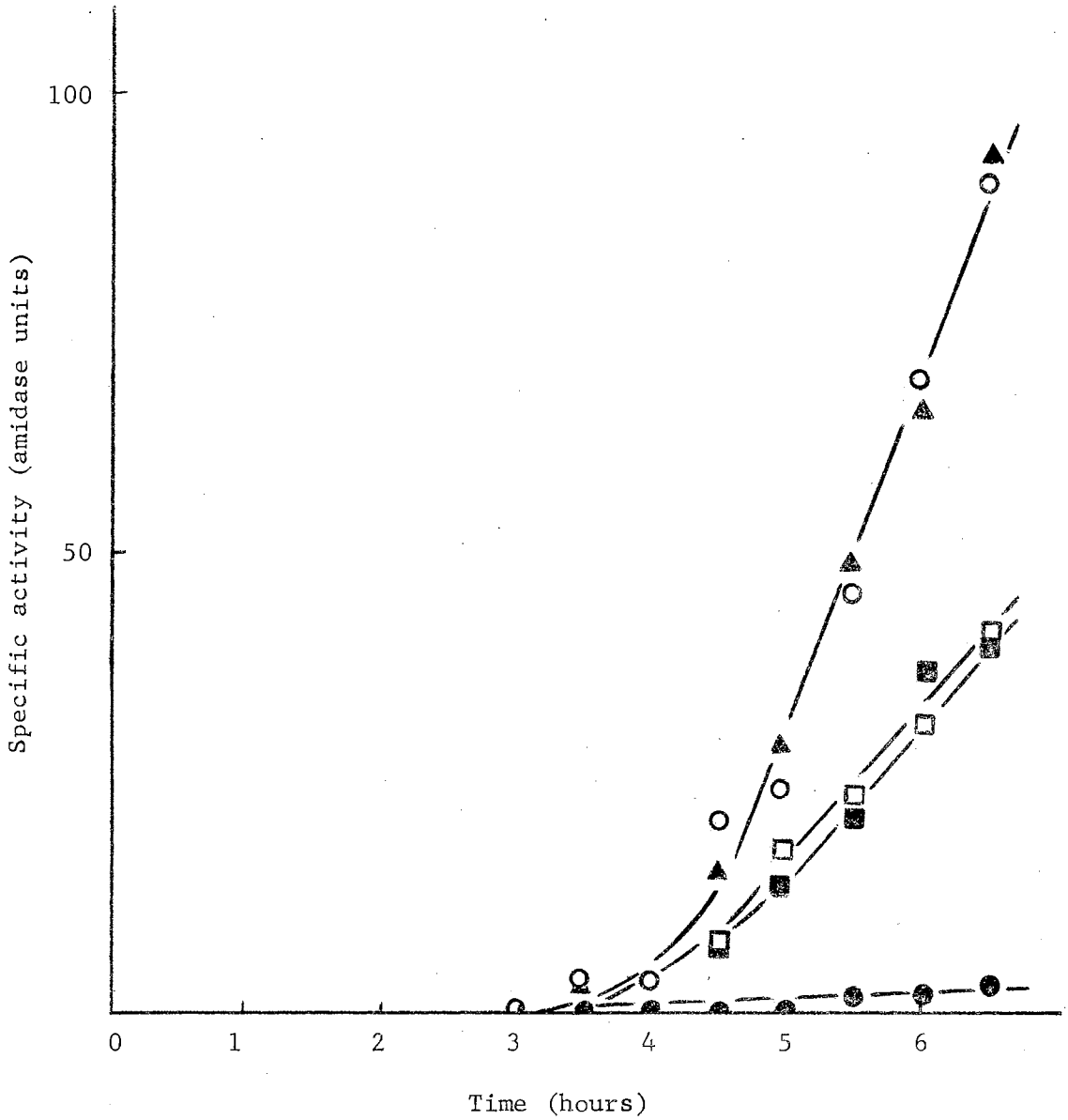
The results had established that protocatechuate prevented and reversed growth inhibition of P. fluorescens by actinomycin D. Experiments were conducted to determine if protocatechuate prevented and/or reversed the actinomycin D inhibition of amidase synthesis. Protocatechuate was added simultaneously with actinomycin in one system to determine if protocatechuate could prevent actinomycin D inhibition of amidase synthesis. In a second system, the cells were incubated with the antibiotic for two hours to allow an inhibition of amidase synthesis to become established and protocatechuate was added to test the ability of protocatechuate to reverse the actinomycin D inhibition of amidase synthesis. Enzyme studies (Figure 27) indicate that protocatechuate could both prevent and reverse the actinomycin D inhibition of amidase synthesis. These results also indicate that the binding of actinomycin D to DNA could be viewed as reversible since protocatechuate was able to reverse the inhibition of amidase synthesis after the antibiotic supposedly was bound to the DNA.

Attempted reversal of CAP inhibition of amidase synthesis with protocatechuate

Protocatechuate did not reverse CAP inhibition of growth of P. fluorescens (Figure 11). Since amidase synthesis was inhibited by CAP,

Figure 27

Protocatechuate (8.0×10^{-4}M) reversal of actinomycin D inhibition of amidase synthesis. The cells were growing in succinate-salts medium. ○, succinate-salts (control); ▲, succinate-salts plus protocatechuate; ●, actinomycin D (8.0×10^{-6}M); □, actinomycin D (8.0×10^{-6}M) plus protocatechuate (added at zero time); and ■, actinomycin D (8.0×10^{-6}M) plus protocatechuate (added at 2 hours).


100
50
Specific activity (amidase units)
0
1
2
3
4
5
6
Time (hours)

studies were made to determine if protocatechuate influenced the CAP inhibition of enzyme synthesis. The results (Figure 28) established that CAP inhibited the synthesis of amidase and the addition of protocatechuate did not affect the inhibition.

Prevention of actinomycin D inhibition of amidase synthesis by selected compounds and analogues of protocatechuate

The ability of selected compounds and structural analogues of protocatechuate to prevent the actinomycin D inhibition of amidase synthesis was studied. The purpose of these experiments was to determine if a specific site or functional group of the protocatechuate molecule was involved in the reversal phenomenon. The results (Table II) do not indicate that a specific site or functional group of protocatechuate was involved in the reversal phenomenon. There was no correlation between compounds which have the same functional groups located in the same or different positions as protocatechuate. Anthranilic acid, 3,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid were found, in addition to protocatechuate, to prevent actinomycin D inhibition of amidase synthesis.

Studies were conducted to determine if the reversal of actinomycin D inhibition was dependent on the ratio of protocatechuate to the antibiotic. Varying concentrations of protocatechuate were added to actinomycin D-containing flasks and amidase synthesis was determined. The results (Figure 29) indicate that the extent of reversal of actinomycin D inhibition of amidase synthesis was dependent on the ratio of protocatechuate to antibiotic. As the molar ratio was decreased

Figure 28

Inhibition of amidase synthesis by chloramphenicol. The cells were growing in succinate-salts plus acetamide medium. ○, succinate-salts (control); □, succinate-salts plus protocatechuate (8.0×10^{-4} M); ●, chloramphenicol (5.0×10^{-5} M); and ■, chloramphenicol (5.0×10^{-5} M) plus protocatechuate (8.0×10^{-4} M).

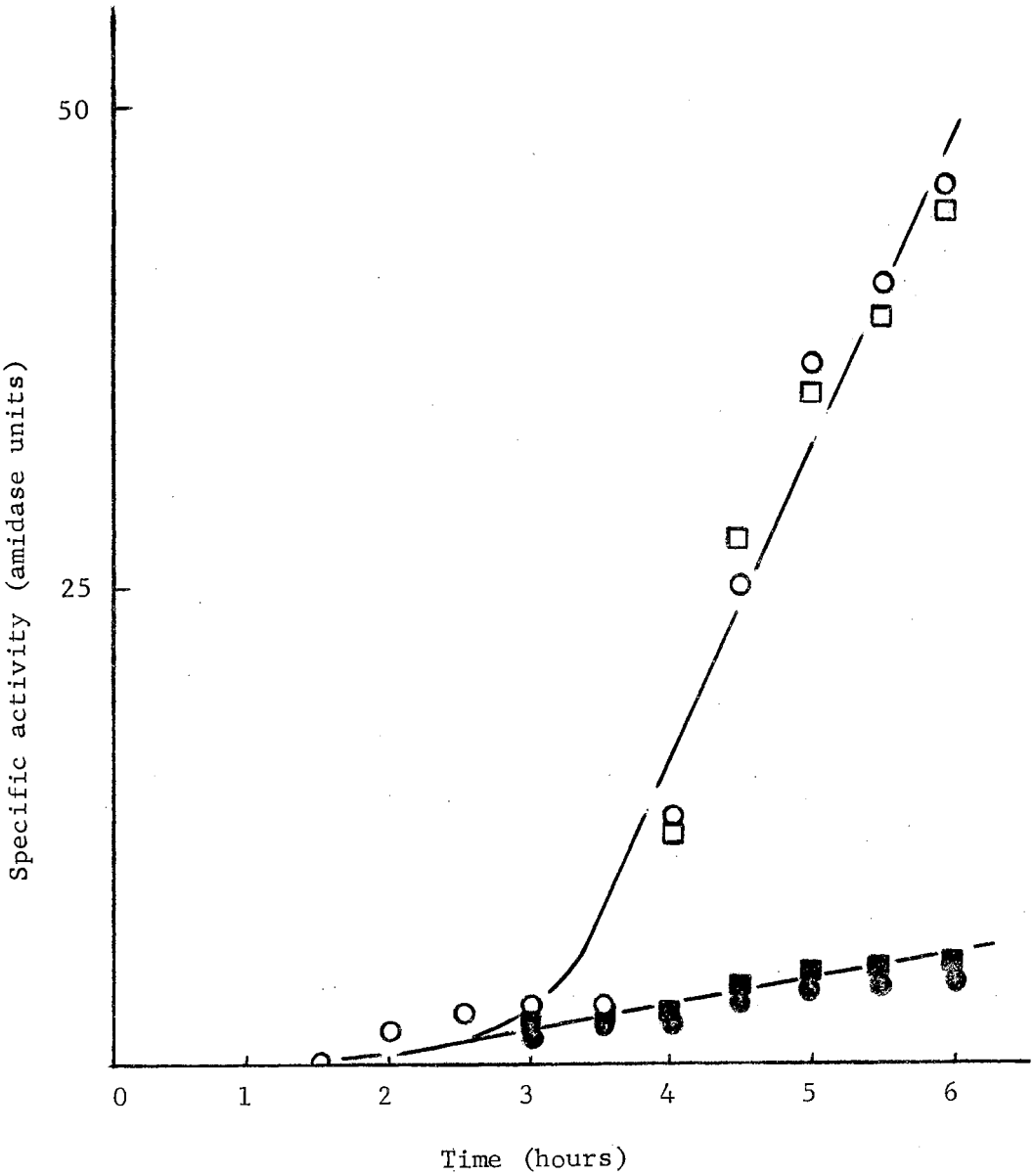

50
25
Specific activity (amidase units)
0
1
2
3
4
5
6
Time (hours)

TABLE II

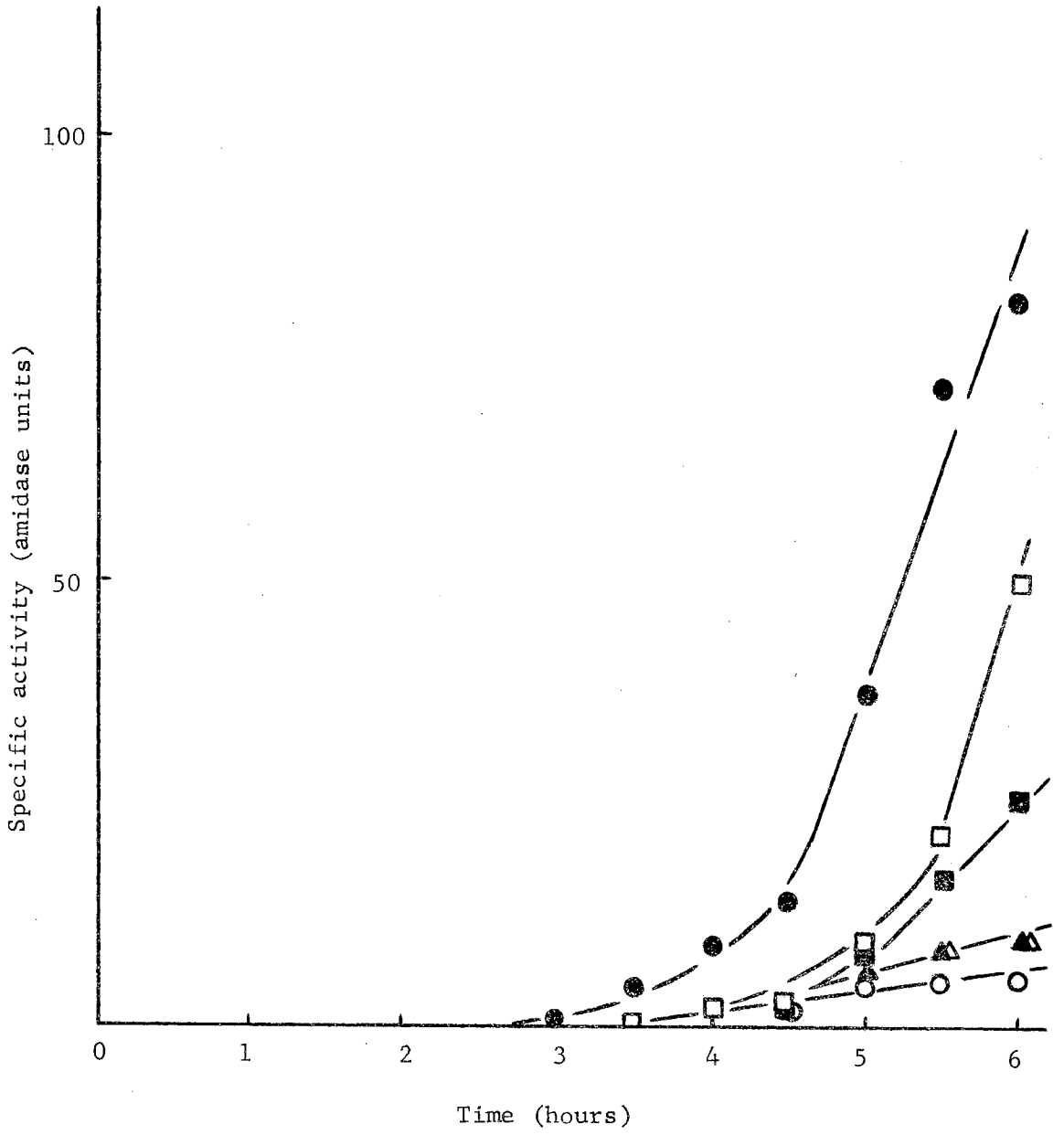
REVERSAL OF AMIDASE INHIBITION BY ACTINOMYCIN D WITH SELECTED COMPOUNDS

Additive	Experiment 1	Experiment 2
succinate + acetamide (control)	100	100
ortho-hydroxybenzoic acid	0	-
meta-hydroxybenzoic acid	5	-
para-hydroxybenzoic acid	0	-
2,3-dihydroxybenzoic acid	74	-
2,4-dihydroxybenzoic acid	20	-
2,5-dihydroxybenzoic acid	31	-
3,4-dihydroxybenzoic acid (protocatechuate)	82	69
3,5-dihydroxybenzoic acid	37	-
3,4,5-trihydroxybenzoic acid	0	-
anthranilic acid	-	29
para-aminobenzoic acid	-	4
para-hydroxybenzoic acid	-	18
benzoic acid	-	7
B-alanine	-	6
pantothenate	-	6
glucose	-	2
actinomycin D (control)	0	0

Cells of P. fluorescens were inoculated into succinate-salts plus acetamide medium. The concentration of all compounds was 8.0×10^{-4} M except glucose which was 2.7×10^{-2} M. The experiment was terminated at the 6 hour time interval and the amount of enzyme activity was determined. All values are expressed as percent of the acetamide control which did not contain actinomycin D.

Figure 29

The reversal of actinomycin D inhibition of amidase synthesis by different concentrations of protocatechuate. The cells were growing in succinate-salts medium supplemented with acetamide (0.08 M). The actinomycin D concentration used in this experiment was 8.0×10^{-6}M. ●, succinate-salts (control); ○, actinomycin D; ▽, actinomycin D plus protocatechuate (8.0×10^{-4}M); ■, actinomycin D plus protocatechuate (8.0×10^{-5}M); △, actinomycin D plus protocatechuate (8.0×10^{-6}M); and ▲, actinomycin D plus protocatechuate (8.0×10^{-7}M).


100
50
Specific activity (amidase units)
0
1
2
3
4
5
6
Time (hours)

from 100:1 to 0.1:1, the extent of reversal of the actinomycin D inhibition of amidase synthesis was decreased. Since the ability of protocatechuate to reverse the inhibition of enzyme synthesis is concentration dependent, the results suggest an interaction between protocatechuate and actinomycin D.

Spectrophotometric studies of an interaction between protocatechuate and actinomycin D

Spectrophotometric studies were performed using the Cary 14 spectrophotometer to determine if an interaction between protocatechuate and actinomycin occurred as indicated by a change in their light absorbing properties. The results do not explicitely show an interaction, but the possibility was suggested since, when the absorbance of the mixture was determined using protocatechuate as a reference, the spectrum for actinomycin was different from the spectrum of the actinomycin control (Figures 30 and 31). Additional tests for an interaction were conducted using the Aminco-Bowman spectrophotofluorometer. This instrument measures the fluorescence of a compound at different wavelengths. If a fluorescent compound is excited at a particular wavelength, it will emit light at another wavelength. These light emissions are then measured. The results (Figure 32) indicated that actinomycin D was excited with a peak at 325 nm and 360 nm. When actinomycin D was excited at 325 nm and the emission spectrum measured, a peak was observed at 500 nm. Protocatechuate was excited with a peak at 325 nm. When protocatechuate was excited at 325 nm, the emission spectrum showed a peak of 438 nm. The excitation and emission spectrum of a mixture of the two compounds was determined. The point of maximum excitation of actinomycin D in the

Figure 30

The ultraviolet absorption spectrum of a mixture of actinomycin D (1.03×10^{-5} M) and protocatechuate (3.8×10^{-4} M). Protocatechuate (3.8×10^{-4} M) was used as the blank.

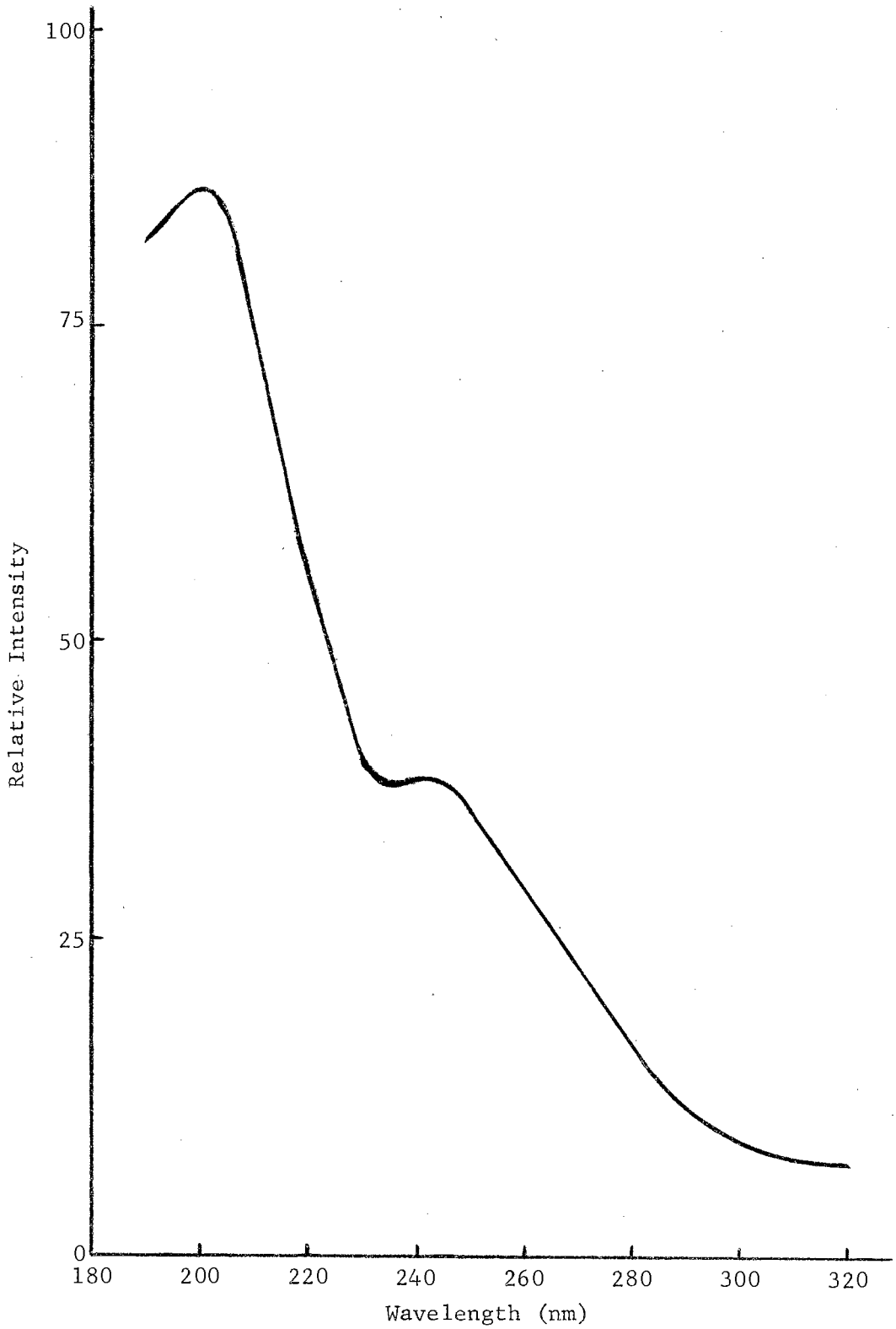

100
75
50
25
0
Relative Intensity
180
200
220
240
260
280
300
320
Wavelength (nm)

Figure 31

The ultraviolet absorption spectrum of actinomycin D (1.03×10^{-5} M) when water was used as the blank.

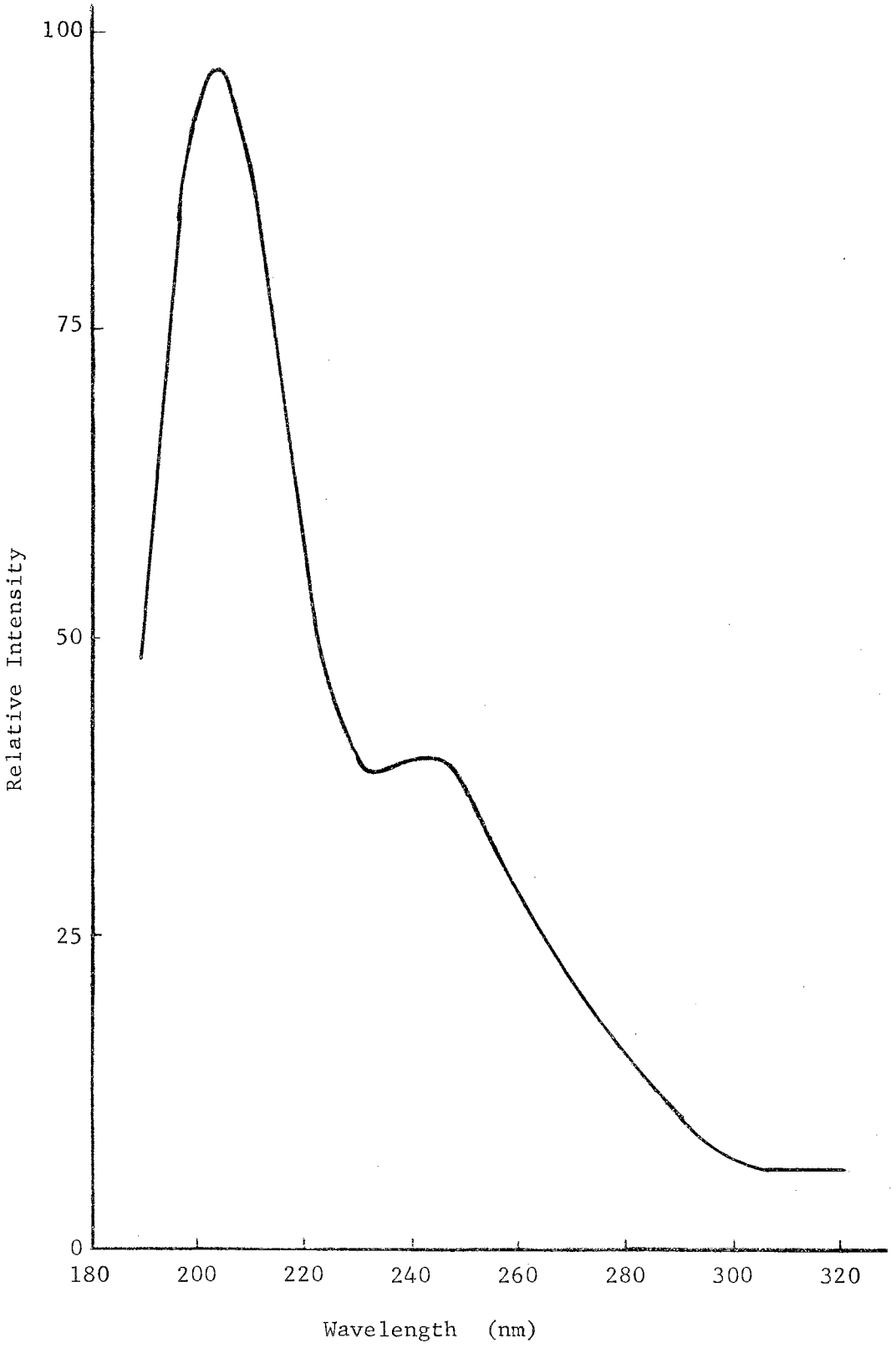
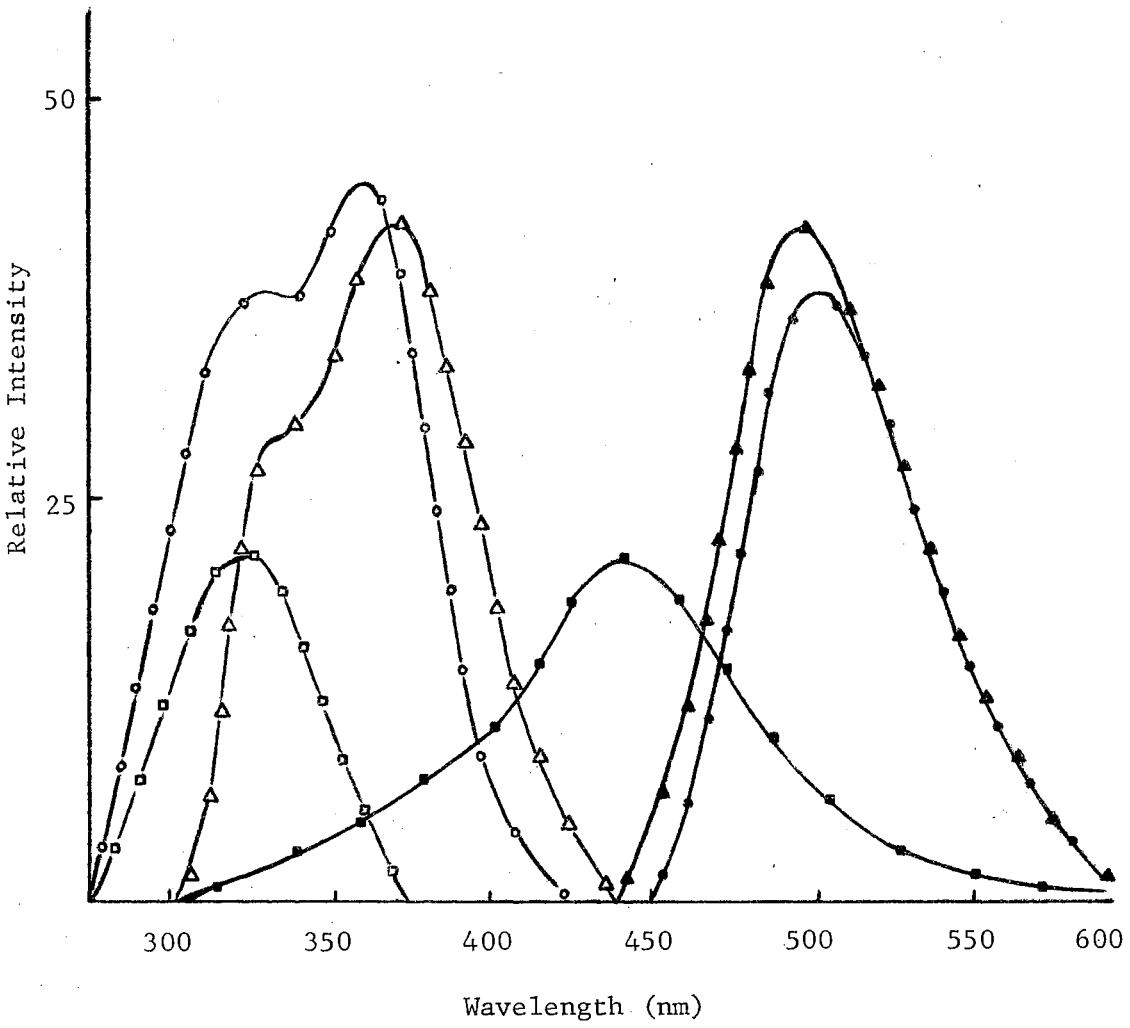

100
75
50
25
0
Relative Intensity
180
200
220
240
260
280
300
320
Wavelength (nm)

Figure 32

Spectrophotofluorometric studies of protocatechuate and actinomycin D. ·, excitation spectrum of actinomycin (8.3×10^{-5} M); ·, excitation spectrum of protocatechuate (8.0×10^{-3} M); ·, emission spectrum of actinomycin when excited at 325 nm; ·, emission spectrum of protocatechuate when excited at 325 nm; △, excitation spectrum of actinomycin in the presence of protocatechuate; and ▲, emission spectrum of actinomycin excited at 325 nm in the presence of protocatechuate.


50
25
Relative Intensity
300
350
400
450
500
550
600
Wavelength (nm)

mixture shifted from 360 nm to 380 nm. The point of maximum emission of actinomycin in the mixture shifted from 500 nm to 485 nm. A complete quenching of the emission spectrum of protecatechuate in the mixture was noted. These results support the proposed interaction between protocatechuate and actinomycin D and could explain the reversal of inhibition of amidase synthesis by protocatechuate. This interaction also offers an explanation for the results observed by Keudell (1967) that actinomycin D did not inhibit the synthesis of the inducible enzyme, protocatechuate oxygenase, in P. fluorescens cells growing in a protocatechuate-salts medium. Protocatechuate may combine with the antibiotic to retard entry of the antibiotic into the cell or, once the antibiotic was in the cell, protocatechuate could prevent the attachment of actinomycin D to the genome.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Actinomycin D inhibited the rate and total growth of P. fluorescens in an acetamide-salts or succinate-salts medium. The inhibition of growth was concentration dependent in both media. Growth of P. fluorescens in protocatechuate-salts medium was not inhibited by actinomycin D at a concentration of 8.0×10^{-6}M. Pseudomonas fluorescens cells grown on succinate showed a growth lag of approximately 2.5 hours when inoculated into acetamide-salts medium. The lag was due to synthesis of an inducible enzyme, acyltrasferase (amidase E.C.3.5.1.4), which was required for utilization of acetamide by the organism.

The addition of protocatechuate (3,4-dihydroxybenzoic acid) to the actinomycin-containing systems at zero time or after a two hour exposure of the cells to the antibiotic reversed the inhibition of total growth by actinomycin D. Inhibition of growth of B. subtilis was obtained at a lower concentration of actinomycin D than was necessary for inhibition of two gram-negative organisms, P. fluorescens or a species of Flavobacterium. Prevention of growth inhibition by protocatechuate was more pronounced in P. fluorescens than in the species of Flavobacterium or B. subtilis. The results showed that the prevention of actinomycin D inhibition was not limited to a single bacterial species. Chloramphenicol inhibition of growth of P. fluorescens was not affected by the

addition of protocatechuate to the system.

Acyltrasferase (amidase) synthesis was induced by acetamide or N-methylacetamide, a nonsubstrate inducer. A diphasic induction pattern was observed in the acetamide-salts medium with an acetamide concentration of 0.08 M or 0.16 M. Kelly and Kornberg (1962) proposed that phase I of the enzyme synthesis, the initial synthesis followed by the plateau, was due to induction by acetamide and 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, was thought to be gratuitous since it occurred during subsequent growth on the acetate that was formed through the hydrolysis of acetamide.

Recrystallization of acetamide or an increased cell inoculum did not affect the induction pattern of amidase. However, results indicated that a larger initial inoculum increased the rate of amidase synthesis of phase I and shortened the lag of phase II. Studies to determine the effect of different carbon sources and inducers on amidase synthesis showed that the diphasic induction was not demonstrated by cells growing in succinate-salts plus acetamide or N-methylacetamide.

Chloramphenicol inhibited both phase I and phase II of amidase synthesis in the acetamide-salts medium while actinomycin D did not inhibit synthesis of phase I, but did prevent the synthesis of enzyme during phase II. The failure of actinomycin D to inhibit enzyme synthesis during phase I might be due to: a) a sufficient concentration of the antibiotic did not accumulate within the cell during the 2 hour incubation period to inhibit enzyme synthesis or b) a pre-existing m-RNA, coding for phase I of amidase synthesis, was present and, since

actinomycin D would have little if any effect on preformed m-RNA, an inhibition of amidase synthesis during phase I was not observed.

Brammar and Clarke (1964) reported that acetate and some tricarboxylic acid intermediates repressed amidase synthesis. Acetate and succinate repress synthesis of amidase in P. fluorescens. At equimolar concentrations, succinate appeared to be the more effective repressor. Growth studies using succinate-grown cells of P. fluorescens inoculated into succinate-acetamide medium showed that succinate was utilized initially as a substrate and then acetamide was utilized. Spiegelman (1965) proposed that, in a metabolite-sensitive enzyme system, the "glucose effect" or "metabolite repression" resulted when the metabolite (glucose) preempted the cells supply of pools to produce metabolite (glucose) degrading enzymes. Cohn and Monod (1953) proposed that the mechanism of the "glucose effect" was a possible interference by the metabolite with the transport of the inducer. Studies measuring the rate of substrate uptake using cells of P. fluorescens showed that both acetate or succinate interfered with the uptake of radioactive acetamide.

Both chloramphenicol and actinomycin D inhibited growth and amidase synthesis. Protocatechuate not only prevented, but reversed actinomycin D inhibition of growth and enzyme synthesis. Addition of protocatechuate to the chloramphenicol-inhibited cells had no affect on growth or enzyme inhibition. The extent of reversal of the actinomycin D inhibition was dependent on the ratio (100:1 to 0.1:1) of protocatechuate to actinomycin D. Spectrophotofluorometric studies indicated that an interaction occurred between protocatechuate and actinomycin D.

Protocatechuate and actinomycin D may form a complex to retard entry of the antibiotic into the cell or, once the antibiotic had penetrated the permeability barrier, the protocatechuate could interact with the antibiotic to prevent or alleviate actinomycin D attachment to the cell genome.

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VITA

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