THE INFLUENCE OF ACTINOMYCIN D ON THE SYNTHESIS

OF PROTOCATECHUATE OXYGENASE

IN PSEUDOMONAS FLUORESCENS

By

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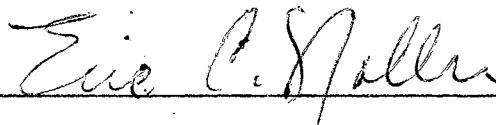for the degree of
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CHAPTER I

INTRODUCTION

The group of antibiotics known as the actinomycins were first
isolated by Waksman and Woodruff in 1940 from a culture of Actino-
myces (Streptomyces) antibioticus (Waksman, 1954). They found that
actinomycin A is characterized as a crystalline, brick-red colored
compound which melts at approximately 250 C and absorbs strongly at
230, 250, and 450 mu. It is very toxic to experimental animals, be-
ing fatal in doses of 0.5 to 1 mg per kilogram body weight.

Waksman, Katz, and Pugh (1956) characterized the various forms
of actinomycin as A, B, C. D, I, J, and X. The antibiotic contains
a chromophoric quinonoid moiety (an azanthraquinone) which is linked
to polypeptides containing the amino acids sarcosine, D-valine, L-
proline, L-threonine, and N-methylvaline (Figure 1). The forms of
the antibiotic differ in the relative proportion of the amino acids;
thus they are not chromatographically homogeneous but they are simi-
lar in their antimicrobial activities.

The various forms of actinomycin are extracted from different
species of Streptomyces. Actinomycin D is extracted from S. parvulus.
Actinomycin is highly active against Gram-positive bacteria but less
active against the Gram-negative bacteria. It is both bacteriostatic
and bactericidal and also demonstrates cytostatic and anti-tumor

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Figure 1.

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

CH$_3$ CH$_3$

O

O

N

NH$_2$

C=O

C=O

L-Threonine   L-Threonine

D-Valine   D-Valine

O   O

L-Proline   L-Proline

Sarcosine   N-Methylvaline   N-Methylvaline   Sarcosine
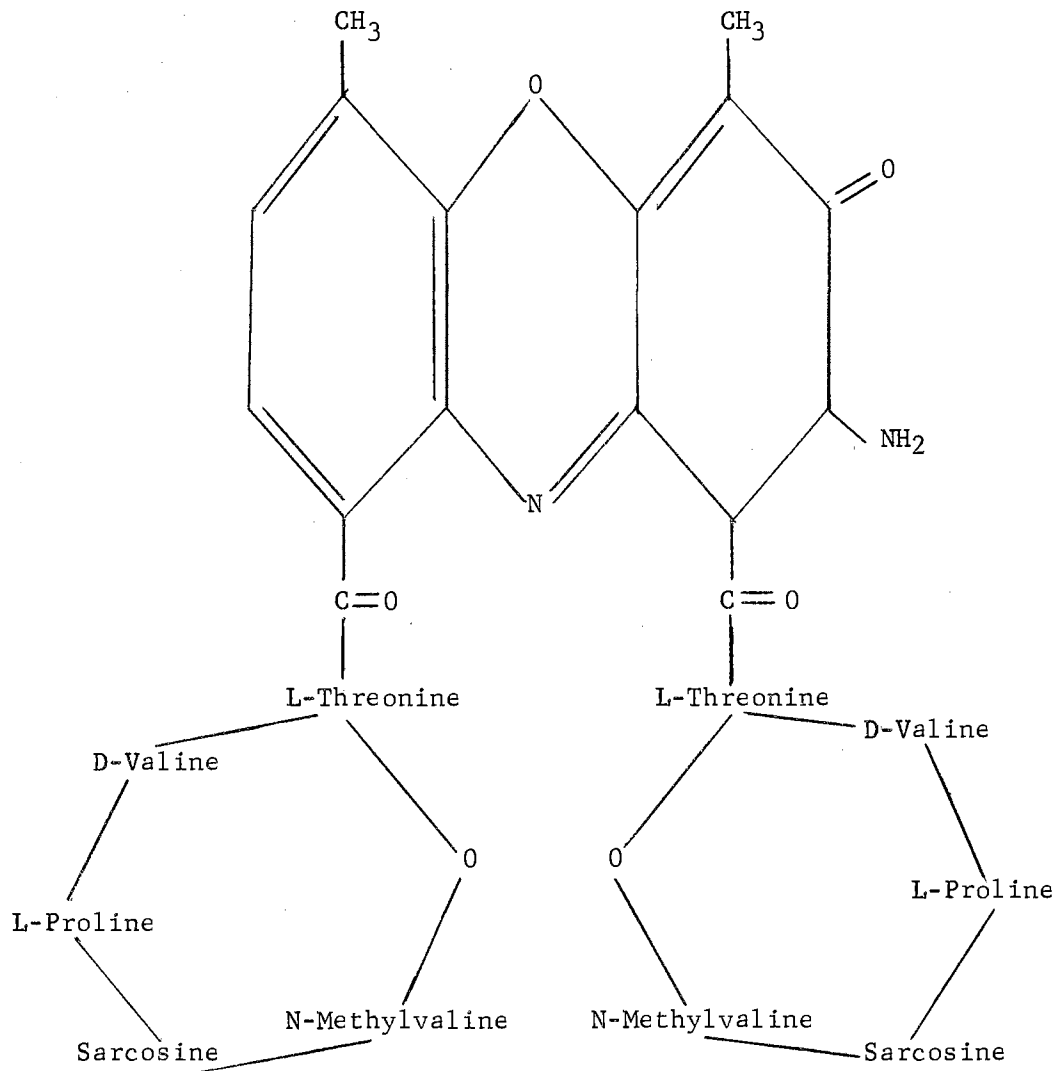
activity.

Kersten, Kersten, and Rauen (1960) found that the growth inhibitory effect of actinomycin D on Neurospora crassa could be reversed by deoxyribonucleic acid (DNA), oligonucleotides of DNA, and to a much lesser extent with ribonucleic acid (RNA). They suggested that actinomycin reacts directly with these compounds in the cell and that the reversal of growth inhibition was due to displacement of actinomycin from its site of action in the living cell.

When actinomycin D is added to an exponentially-growing culture of Staphylococcus aureus there is an immediate inhibition in the synthesis of RNA followed rapidly by an inhibition of protein synthesis (Kirk, 1960). Goldberg and Rabinovitz (1962) used an RNA-synthesizing system from HeLa cells and found that small amounts of actinomycin D inhibited the incorporation of ^{32}P-labeled uridine triphosphate into RNA. Furthermore, they could reverse this effect by the addition of DNA isolated from calf thymus or HeLa cells. This offered further support that actinomycin D interferes with DNA-dependent RNA synthesis.

Hurwitz et al. (1962) used various DNA primers (human bone marrow, heated thymus, bacteriophage O X174 and Micrococcus lysodeikticus) for the RNA polymerase and found that actinomycin D reduced RNA synthesis directed by these primers indicating that the DNA-linked RNA polymerase was being inhibited. Additional work with an in vivo system using Bacillus subtilis showed that actinomycin D (0.2 μM) completely blocked growth, inhibited RNA synthesis about 90-95 per cent, protein synthesis 50-75 per cent, and DNA synthesis only

approximately 25 per cent.

Investigations by Goldberg, Rabinovitz, and Reich (1962) led to the following conclusions: (1) guanine must be present in a polydeoxyribonucleotide for actinomycin D to bind to DNA and for the inhibition of RNA synthesis, (2) optimal binding is also favored by the native helical DNA conformation, (3) optimal binding is most likely influenced by the nucleotide sequences in the immediate vicinity of the guanine residues (possibly in the contralateral strands), and (4) the synthesis of polyribonucleotides from both strands is inhibited even though actinomycin D is bound to only one strand.

By using sedimentation analysis and radioisotope techniques it was found that messenger-RNA (m-RNA) decays in the presence of actinomycin D and that the m-RNA of B. subtilis has a life-time of about two minutes before it breaks down to acid soluble, low molecular weight material (Levinthal, Keynan, and Higa, 1962). It was also found that amino acid incorporation decreased and ^{14}C-uracil incorporated into the RNA characteristic of ribosomal and soluble RNA during prolonged preincubation remained stable upon further incubation in the presence of actinomycin D.

Data collected by Acs, Reich, and Valanju (1963) led them to believe that the breakdown of RNA in the presence of actinomycin D was not due merely to the inhibition of RNA synthesis but that actinomycin actually induced the breakdown of the normally stable RNA. Levinthal et al. (1963) suggested that the decay of RNA in the presence of actinomycin D was due only to the inhibition of synthesis.

It is now fairly well established that actinomycin D inhibits

DNA-dependent RNA synthesis, thus decreasing the uptake of labeled

uracil into the m-RNA fraction and labeled amino acids into protein.

The formation of alkaline phosphatase and α-amylase was strong-

ly inhibited by actinomycin D (0.5 µg/ml) in B. subtilis but ribo-

nuclease formation was not affected by the antibiotic; furthermore,

the incorporation of ^{32}P into RNA and labeled amino acids into pro-

tein was inhibited by actinomycin D (Kadowaki, Hosoda, and Maruo,

1965). Pollock (1963) showed that actinomycin D at a concentration

of 0.05 µg/ml only slightly inhibited α-glucosidase formation in

both constitutive and inducible strains of B. cereus, but the forma-

tion of the enzyme was decreased by 40 per cent at an actinomycin

concentration of 0.1 µg/ml. Induced cultures of B. cereus formed

penicillinase in the presence of actinomycin until 40 minutes after

addition of the antibiotic, at which time an inhibition was observed.

However, penicillinase was inhibited immediately when actinomycin was

added simultaneously with penicillin to noninduced cells. For the

constitutive strain there was no inhibition of penicillinase forma-

tion at an actinomycin concentration of 0.05 µg/ml but when the anti-

biotic concentration was increased they observed an inhibition of

penicillinase formation. In B. subtilis, 0.05 µg/ml of actinomycin D

almost completely suppressed α-glucosidase formation, slightly stim-

ulated penicillinase production, and inhibited β-galactosidase and

α-amylase to about the same extent as growth was inhibited. This

differential effect might be due to an unusually stable m-RNA or a

difference in the affinity of DNA sites to bind actinomycin. Pro-

duction of gramicidin S in B. brevis was not affected by actinomycin D

even though normal RNA synthesis and protein synthesis were inhibited. These results suggested that gramicidin S synthesis was independent of RNA synthesis or that a very stable form of m-RNA was involved in the synthesis (Eikhom and Laland, 1964).

Kennell (1964) prepared extracts from B. megaterium cells treated with actinomycin D, and isolated RNA that could stimulate peptide synthesis even though there was an inhibition of polypeptide synthesis in the intact cell. Yudkin and Davis (1965) found that the m-RNA associated with the membranes of B. megaterium was unusually stable in the presence of actinomycin D. Investigation of the amino acid incorporation in B. megaterium cells treated with actinomycin D led Yudkin (1965) to suggest that the long-lived m-RNA of the cell membrane synthesizes non-repressible proteins whereas the short-lived m-RNA synthesizes repressible proteins.

The quantitative binding of actinomycin D to DNA increases with increasing amounts of guanine in the DNA and is greatest when DNA is in the native helical B conformation (Hamilton, Fuller, and Reich, 1963; Reich, 1964; Gellert et al., 1965). Cavalieri and Nemchin (1964) found two binding sites for actinomycin D on the DNA molecule: one strong binding site and one weak binding site.

While RNA synthesis is inhibited by actinomycin D, DNA synthesis is only slightly affected; thus, it is possible that actinomycin lies in the minor groove of the helical DNA where RNA synthesis takes place and DNA synthesis proceeds in the major groove of DNA (Reich, 1964).

Hamilton, Fuller, and Reich (1963) proposed a model for actinomycin D bonding to DNA (Figure 2). Their work was augmented by

Figure 2.

Proposed model for the bonding of actinomycin D to DNA
(Hamilton, Fuller, and Reich, 1963). The actinomycin chromo-
phore group binds to guanine via three hydrogen bonds. The
long axis of the actinomycin chromophore is perpendicular
to the helix axis of the DNA and its plane is inclined ap-
proximately 70 degrees to the DNA helix axis. The actino-
mycin molecule may also be stabilized by the bonding of the
phosphate oxygen atoms of the DNA to the two peptide amino
groups in each chain of the actinomycin molecule.

2.71 Å

2.77 Å

2.70 Å

cyclic peptide
side chain

CH₃

CH₃
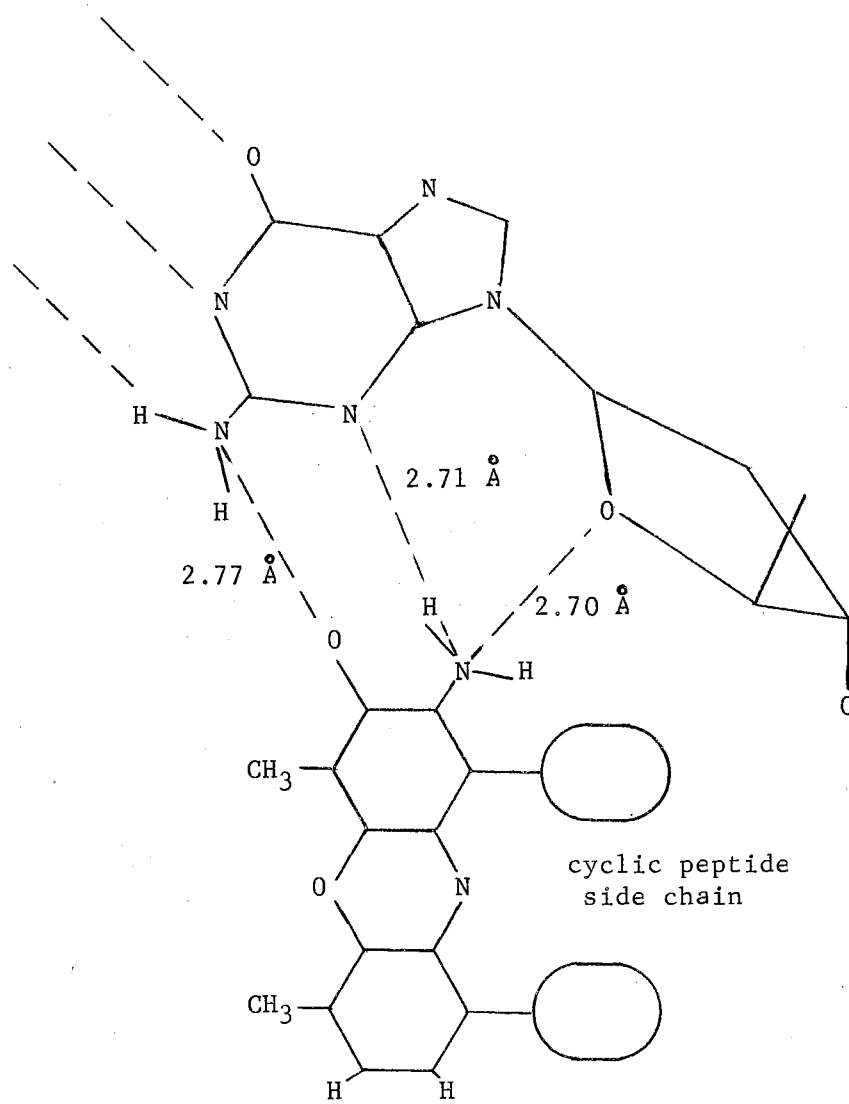
Gellert et al. (1965) who suggested that the binding site either in-
volved more than one base pair, one of which was guanine-cytosine
(G-C), or else actinomycin D was bound only to the G-C pairs but all
of the G-C sites were not bound because of steric interference be-
tween the actinomycin D molecules.

Actinomycin D does not affect the thermal dissociation of nu-
cleic acids having the A-helix conformation. Haselkorn (1964) found
that the thermal dissociation of a DNA-RNA hybrid was not affected by
actinomycin D; therefore, the hybrid was probably in the A-helix con-
formation. Jacoli and Zbarsky (1965) treated B. subtilis cells with
actinomycin D and detected the accumulation of guanine nucleotides
in the acid-soluble fraction. This could be explained by the degra-
dation of a guanine-rich RNA (not m-RNA) whose resynthesis was block-
ed by actinomycin, or secondly, an inhibition of the incorporation of
the nucleotides in the acid-soluble pools into RNA. Jurkowitz (1965)
has evidence to indicate that actinomycin D binds to only one half
of the DNA site in nucleoprotein. This could mean that the protein
in nucleoprotein is selectively blocking all the sites on one of the
DNA strands.

Escherichia coli is normally insensitive to actinomycin D. Leive
(1965b) sensitized E. coli cells to actinomycin D by treating the
cells with ethylenediaminetetraacetic acid (EDTA). The addition of
actinomycin D to the sensitized cells inhibited m-RNA synthesis for
2.5 minutes but after this time the antibiotic had no effect on m-RNA
synthesis. It was concluded that it takes 2.5 minutes after addition
of the inducer for synthesis of non-DNA bound m-RNA for B-galactosi-

dase. Enzyme production occurred during another 1.5 minutes. Studies with actinomycin D resistant strains of B. subtilis indicate that resistance might be due to a modification in the permeability of the cell wall (Polsinelli et al., 1964).

Revel, Hiat, and Revel (1964) observed that actinomycin D decreased the protein-synthesizing capacity of rat liver homogenates by a mechanism independent of its action on RNA synthesis. There was a decrease in amino acid incorporation and polyribosome content for liver homogenates, but there was no decrease in amino acid incorporation and polyribosome content for liver slices. This indicated to them that the bulk of m-RNA in liver is a stable RNA.

Honig and Rabinovitz (1965) observed that addition of glucose to the incubation medium prevented the inhibition of protein synthesis by actinomycin D in sarcoma-37 cells. A complete recovery from the inhibition of protein synthesis was observed when the addition of glucose was delayed until the end of the incubation period. This suggested that the inhibition was not due to the breakdown of m-RNA. This was substantiated by the observation that uridine and adenine were incorporated into RNA in the presence of actinomycin D at about the same rate as the control.

Laszlo et al. (1966) observed a significant inhibition of glycolysis and respiration in the presence of actinomycin D in human leukemic cells. The cells also showed decreased levels of adenosine triphosphate (ATP), and these authors speculated that actinomycin D may also cause an impairment of energy metabolism resulting in a decrease of ATP available for the formation of active amino acyl transfer-RNA.

Studies in this laboratory have established that the synthesis of protocatechuate oxygenase Pseudomonas fluorescens is not affected by actinomycin D. This investigation was conducted as an attempt to explain the apparent resistance of the synthesis of protocatechuate oxygenase to actinomycin D in P. fluorescens.

CHAPTER II

MATERIALS AND METHODS

Test organism.

The microorganism used in this investigation was a laboratory strain of Pseudomonas. It is a Gram-negative, motile rod which forms smooth, raised, colonies on nutrient agar. The organism gave a negative reaction for hydrogen sulfide production, indole production, nitrate reduction, and gas production in glucose; however, an acid reaction was observed in glucose. Litmus milk was peptonized within four days. This organism developed the pigmentation characteristic of both fluorescein and pyocyanin when grown on Bacto-Pseudomonas agar F and Bacto-Pseudomonas agar P, respectively. The organism has been tentatively identified as Pseudomonas fluorescens.

Stock cultures were maintained on nutrient agar slants and succinate-salts agar slants which were stored at 4 C.

Media.

The synthetic salts medium used in this study had the following composition: 0.42 per cent K_2HPO_4; 0.32 per cent KH_2PO_4; 0.2 per cent NH_4Cl; 0.2 per cent NaCl; and 0.2 per cent of the desired carbon source except for certain cases in which the concentration of the carbon source was varied as indicated in the text.

The pH of the medium was adjusted to 7.0. Agar (Difco) was added to give a final concentration of 2.0 per cent when a solid medium was desired. Sterilization was accomplished by autoclaving at 121 C for 15 minutes. The synthetic medium was cooled to 50 C and 0.1 ml of a sterile mineral salts solution was added to each 100 ml of medium. The mineral salts solution was composed of 5.0 g $MgSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4$, 1.0 g $FeCl_3$, and 0.5 g $CaCl_2$ in 100 ml of distilled water.

Glucose was sterilized by filtration through a Millipore membrane filter (47 mm diameter; 0.45 μ pore size).

Preparation of substrates and inhibitor.

Succinic acid, protocatechuic acid, and glucose were prepared fresh for each experiment. The compounds were dissolved in 0.01 M 2-amino-2-(hydroxymethyl)-1,3-propanediol buffer (Tris) and the pH adjusted to 7.0. Actinomycin D was dissolved in distilled water (4 C) and stored at 4 C.

Preparation of cell suspensions.

The cell suspensions used in manometric, radioisotopic, and growth studies were prepared as follows: nutrient agar or succinate agar slants were inoculated from the stock culture and incubated for 16-18 hours at 37 C. The cells were suspended in sterile 0.01 M Tris buffer and 0.5 ml of the suspension was spread over the surface of the appropriate agar plates with a sterile glass rod. The plates were incubated at 37 C for 16-18 hours, the cells harvested, washed twice, and suspended in sterile 0.01 M Tris buffer (pH 7.0) so that a 1:10 dilution gave the desired absorbancy reading at 540 mμ using a Bausch and Lomb "Spectronic 20" spectrophotometer. All absorbancy readings were

made using 18 mm diameter tubes.

Liquid scintillation counting fluid.

The counting fluid for uracil-2-^{14}C incorporation into RNA and leucine-2^{14}C incorporation into protein was prepared by adding 42 ml Liquifluor (50 g of 2,5-diphenyloxazole and 0.625 g of p-Bis $\sqrt{2}$-(5-phenyloxazolyl)$\overline{7}$ -benzene in 500 ml toluene) to one liter of toluene (sulfur-free).

The counting fluid for actinomycin D-^{14}C incorporation was prepared by adding 42 ml Liquifluor (Nuclear-Chicago) to 400 ml ethanol (absolute) and 600 ml toluene. The ethanol-toluene mixture was used instead of toluene to eliminate precipitation of the digested cell material.

Growth studies.

Growth studies were conducted to determine the effect of various concentrations of actinomycin D on growth and the influence of selected substrates on the inhibition of growth by actinomycin D. In these experiments a total volume of either 6 or 8 ml was used in the Spectronic tubes (150 mm x 18 mm) and the contents were incubated at 37 C on a reciprocal shaker. Constituents are described in the text. Absorbancy was measured at 540 mμ.

Experiments requiring the withdrawal of samples at various times were performed in 250 ml Erlenmeyer flask with side-arms. The flask contained a total liquid volume of 40 ml. Constituents are described in the text. The flask contents were incubated at 37 C on a reciprocal shaker and the absorbancy was read at 540 mμ.

Manometric studies.

Enzymic activity was measured by following oxygen uptake in the Warburg apparatus at 37 C with air as the gas phase. The carbon dioxide liberated during the reaction was absorbed by adding 0.2 ml of 20 per cent KOH and a strip of fluted filter paper to the center well of the Warburg flask.

The cell suspension was placed in the main chamber of the Warburg flask and the substrates and inhibitors were placed in the side arms. The total liquid volume was adjusted to 2.4 ml by adding 0.01 M Tris buffer (pH 7.0). Approximately 10-15 minutes were allowed for temperature equilibration following which the manometers were closed and readings were taken at indicated time intervals.

Radioactive isotope procedures.

The procedure for measuring uracil-2-^{14}C incorporation into the RNA of P. fluorescens varied according to the type of experiment being conducted.

The incorporation of uracil-2-^{14}C was performed in conjunction with the manometric investigations. Uracil-2-^{14}C (0.043 µC/ml final concentration) was placed in the sidearm of the Warburg flask and added to the cells simultaneously with the substrate. At 45, 90, and 135 minutes, the appropriate flasks were removed from the Warburg apparatus and the contents poured into test tubes. A one ml sample was then pipetted into a thick walled centrifuge tube and the sample was frozen in an acetone-dry ice bath to prevent further uptake of uracil-2-^{14}C by the cells. Four ml of cold 5.0 per cent trichloroacetic acid (TCA) were added to each frozen sample. The samples were allowed to

thaw at 4 C and centrifuged at 12,100 x g for 15 minutes at 4 C to re-
move the cells. The supernatant solutions from the first cold TCA ex-
traction were poured into counting vials. The cell pellets were then
suspended in 5 ml of cold 5.0 per cent TCA and permitted to stand for
10 minutes at 4 C. The samples were centrifuged and the supernatant
solutions from the second cold TCA extraction were poured into differ-
ent vials. The pellets were suspended in 5 ml of 5.0 per cent TCA
and the samples were heated in a 90 C water bath for 30 minutes. The
samples were cooled, centrifuged at 12,000 x g for 15 minutes, and
the supernatant solution was poured into counting vials. The contents
of the vials were dried at 55 C under vacuum in a desiccator contain-
ing anhydrous $CaCl_2$ until about 0.1 ml of fluid remained in the vials.
Ten ml of liquid scintillation counting fluid were added to each vial
and the samples were counted in a Nuclear Chicago liquid scintillation
spectrometer.

Uptake of actinomycin D-^{14}C.

The uptake of actinomycin D-^{14}C by succinate-grown P. fluores-
cens cells was measured in the presence of succinate, protocatechuic
acid, and glucose to determine if these substrates influenced the up-
take of the inhibitor.

Succinate-grown cells were suspended so that a 1:10 dilution
gave an absorbancy reading of 1.0 at 540 mu. Substrates for the ex-
periment were: 0.2 ml succinate (0.2 per cent), 0.2 ml protocatechuic
acid (0.2 per cent), 0.2 ml glucose (0.5 per cent), 0.2 ml succinate
(0.2 per cent) plus 0.05 ml protocatechuic acid (0.8 μmoles/ml final
concentration), 0.2 ml protocatechuic acid (6.8 μmoles/ml). Each

tube also contained 0.1 ml of actinomycin D (2.5 x 10^7 cpm/μmole),

0.065 ml of actinomycin D (30 μg/ml), and 0.01 M Tris buffer to bring

the total liquid volume to 0.5 ml.

The cell suspension (0.15 ml) was added to the substrates in a

Servall thick-walled centrifuge tube (100 mm x 15 mm), incubated at

37 C for 75 seconds, and frozen in a cellosolve-dry ice bath. Five

ml of cold Tris buffer were added to each tube, the pellet thawed at

4 C, and centrifuged at 12,100 x g for 15 minutes. The cell pellets

were washed twice by centrifugation. Five-tenths ml of NCS Reagent

(Nuclear-Chicago) was added to each tube and digestion of the cell

pellets was carried out overnight at room temperature. The digested

material was poured into a counting vial and the tube was rinsed with

9.5 ml counting fluid and placed into the counting vial. Vials were

then placed in the liquid scintillation counter to determine the a-

mount of actinomycin D-^{14}C uptake.

Incorporation of radioactive amino acid.

One ml of leucine-2-^{14}C (161,310 cpm/ml) was added to a 250 ml

Erlenmeyer side-arm flask which contained 0.2 per cent succinate, 0.8

μmoles/ml protocatechuic acid, and/or 30 μg/ml of actinomycin D as

indicated in the text.

Each flask was inoculated with 1.0 ml of a succinate-grown cell

suspension which had been previously adjusted to give an absorbancy

reading of 1.5 at 540 mμ. At the desired time, absorbancy readings

were taken at 540 mμ and 3 ml samples were removed and frozen in a

cellosolve-dry ice bath. Two ml of cold 10 per cent TCA were added,

the samples thawed at 4 C, centrifuged for 10 minutes at 12,100 x g,

and the supernatant solution was discarded. The pellets were suspend-
ed in 5 ml of 5.0 per cent cold TCA, incubated at 4 C for 15 minutes,
centrifuged at 12,100 x g for 15 minutes, and the supernatant solution
was poured into counting vials. The cells were suspended in 5 ml of
distilled water and centrifuged at 12,100 x g for 10 minutes. The
supernatant solution was discarded, the cell pellets were suspended
in 5 ml of 95 per cent ethyl alcohol, and incubated at room tempera-
ture for 15 minutes. The samples were centrifuged for 20 minutes at
27,000 x g, the ethyl alcohol was discarded, and the cells washed one
time with distilled water. The pellets were suspended in 5.0 per cent
TCA and heated at 90 C for 30 minutes. The samples were cooled, cen-
trifuged at 12,100 x g for 15 minutes, and the supernatant solution
was placed in counting vials and dried as previously described. Ten
ml counting fluid was added to each vial and then they were placed in
the liquid scintillation counter to determine the radioactivity. The
counting efficiency for ^{14}C was approximately 40 per cent.

CHAPTER III

RESULTS AND DISCUSSION

The effect of actinomycin D on growth of P. fluorescens in different media.

Experiments were conducted to determine if actinomycin D influenced the growth of P. fluorescens in nutrient broth or synthetic-salts media with different carbon and energy sources. The concentrations of actinomycin D used in nutrient broth and L-asparagine-salts medium were 0, 4, 8, 15, and 25 ug/ml.

Each tube contained 5 ml of medium plus the appropriate amount of inhibitor, and buffer was added to give a total liquid volume of 6 ml. One-tenth ml of a nutrient agar grown cell suspension, diluted so that a 1:10 dilution had an absorbancy of 0.3 at 500 mu, was added to each tube. Growth was followed by measuring absorbancy at the indicated time intervals.

The growth of P. fluorescens in nutrient broth was not significantly influenced by any of the concentrations of actinomycin D used in the experiment (Figure 3). Waksman et al. (1956) reported that P. aeruginosa was not inhibited by 100 ug/ml of actinomycin D when the cells were growing on nutrient agar plates; therefore, the data persented here are consistent with these results. Growth of the organism in 0.2 per cent L-asparagine-salts medium was inhibited by actinomycin

Figure 3.

The influence of actinomycin D on _P. fluorescens_ cells growing in nutrient broth. O, control; ●, actinomycin D (4 µg/ml); △, actinomycin D (8 µg/ml): ▲, actinomycin D (15 µg/ml); and □, actinomycin D (25 µg/ml).
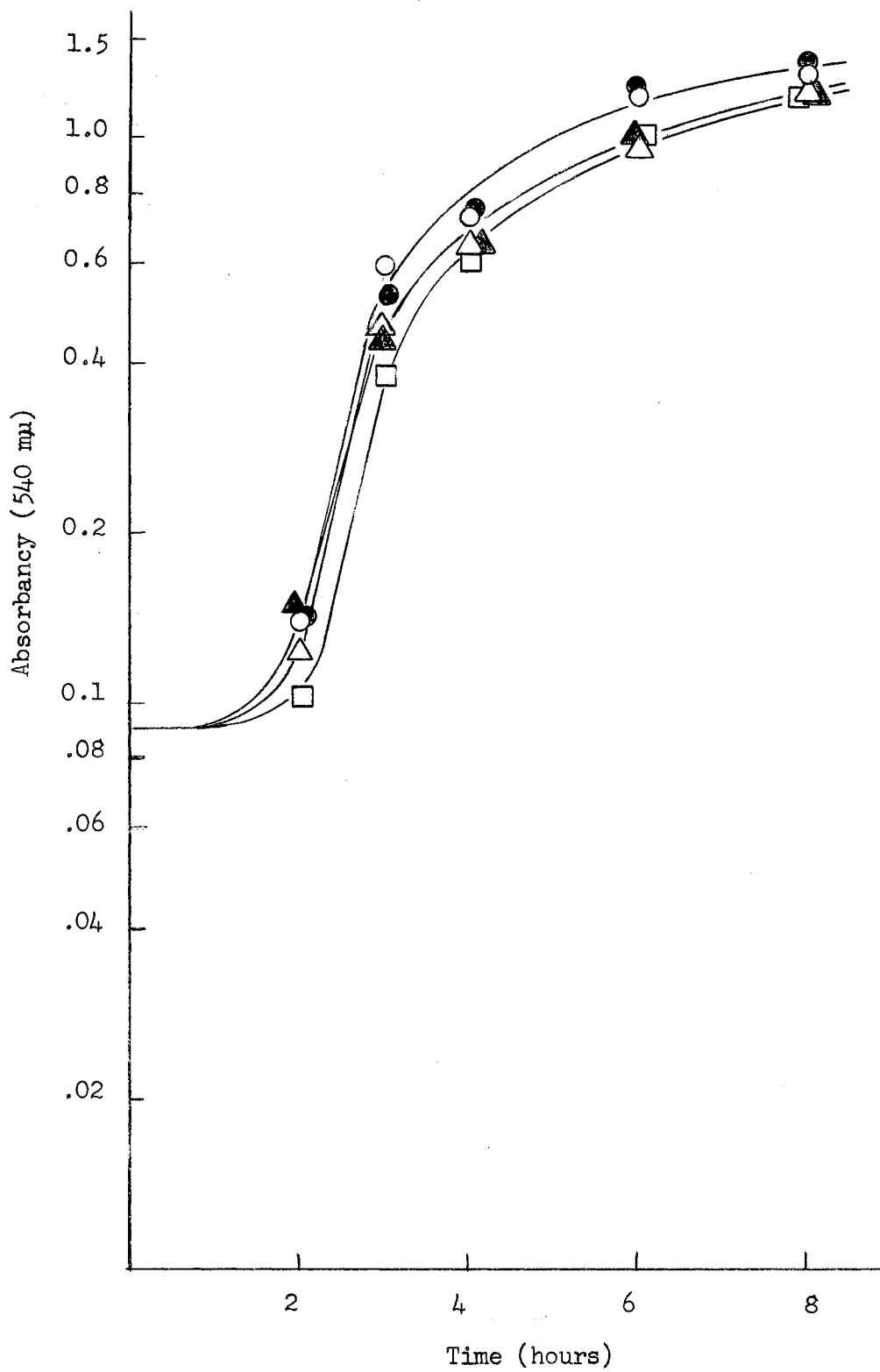
D and the inhibition increased with an increase in inhibitor concentration (Figure 4). Fifteen and twenty-five ug/ml of actinomycin D produced good inhibition of growth after approximately four hours. The absorbancy values of cells growing in the presence of actinomycin D was approximately the same as the control during the first 2-4 hours of growth indicating that actinomycin D may not readily penetrate the permeability barrier of the cell. When actinomycin D is added to a Gram-positive organism (Staphylococcus aureus) growth is rapidly inhibited (Kirk, 1960). The apparent difference in sensitivity to actinomycin D in a Gram-positive and a Gram-negative organism may be due to the difference in cell wall structure and composition. Four ug/ml only slightly decreased the rate of growth and 8 ug/ml inhibits by about one-half that of 25 ug/ml.

Actinomycin D also inhibited growth in succinate (0.2 per cent). Two-tenths ml of succinate-grown cells, having an absorbancy of 0.8 at 540 mu was inoculated into each tube containing 7 ml medium plus buffer and inhibitor to give a total liquid volume of 8 ml.

The results for cells growing in a succinate-salts medium showed that an increase in actinomycin D concentration increased the amount of growth inhibition (Figure 5). Thirty ug/ml of actinomycin D gave almost complete inhibition. Five and ten ug/ml of actinomycin D decreased the rate of growth as compared to the control and the cell population never reached that of the control.

Growth in a protocatechuate-salts medium was very different from that observed in a succinate-salts medium (Figure 6). Actino-

Figure 4.

The influence of actinomycin D on P. fluorescens cells grow-
ing in a L-asparagine-salts medium. O, control; ●, actinomycin
D (4 μg/ml); △, actinomycin D (8 μg/ml); ▲, actinomycin D
(15 μg/ml); and ⬡, actinomycin D (25 μg/ml).

Figure 5.

The influence of actinomycin D on succinate-grown P.
fluorescens cells growing in a succinate-salts medium. O, con-
trol; ●, actinomycin D (5 µg/ml); △, actinomycin D (10 µg/ml);
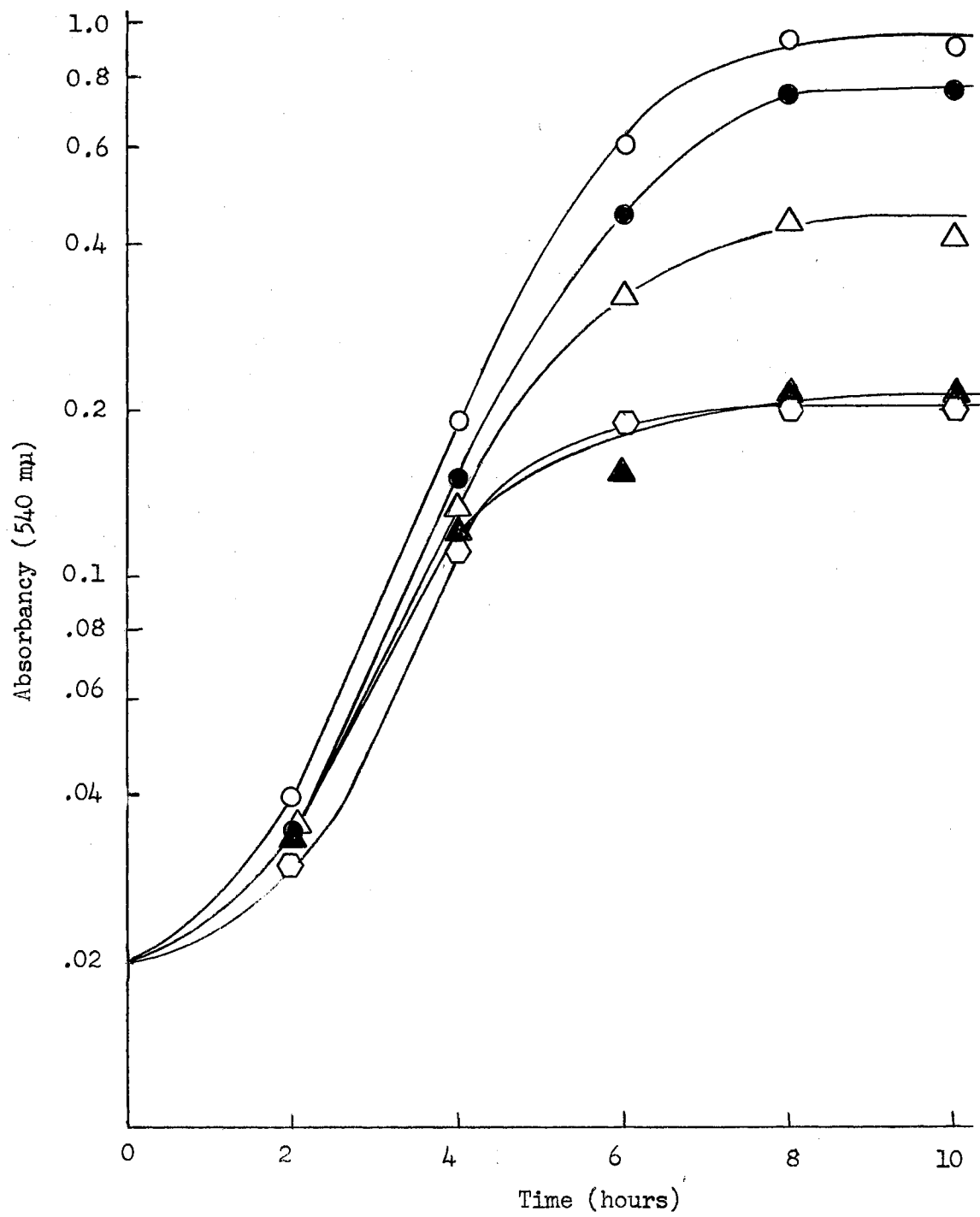▲ , actinomycin D (30 µg/ml).

Figure 6

The influence of actinomycin D on succinate-grown P. fluo-
rescens cells growing in a protocatechuate-salts medium. O,
control; ●, actinomycin D (5 µg/ml); △, actinomycin D (10
µg/ml); and ▲, actinomycin D (30 µg/ml).

Y-axis label: Absorbancy (540 mμ)

X-axis label: Time (hours)
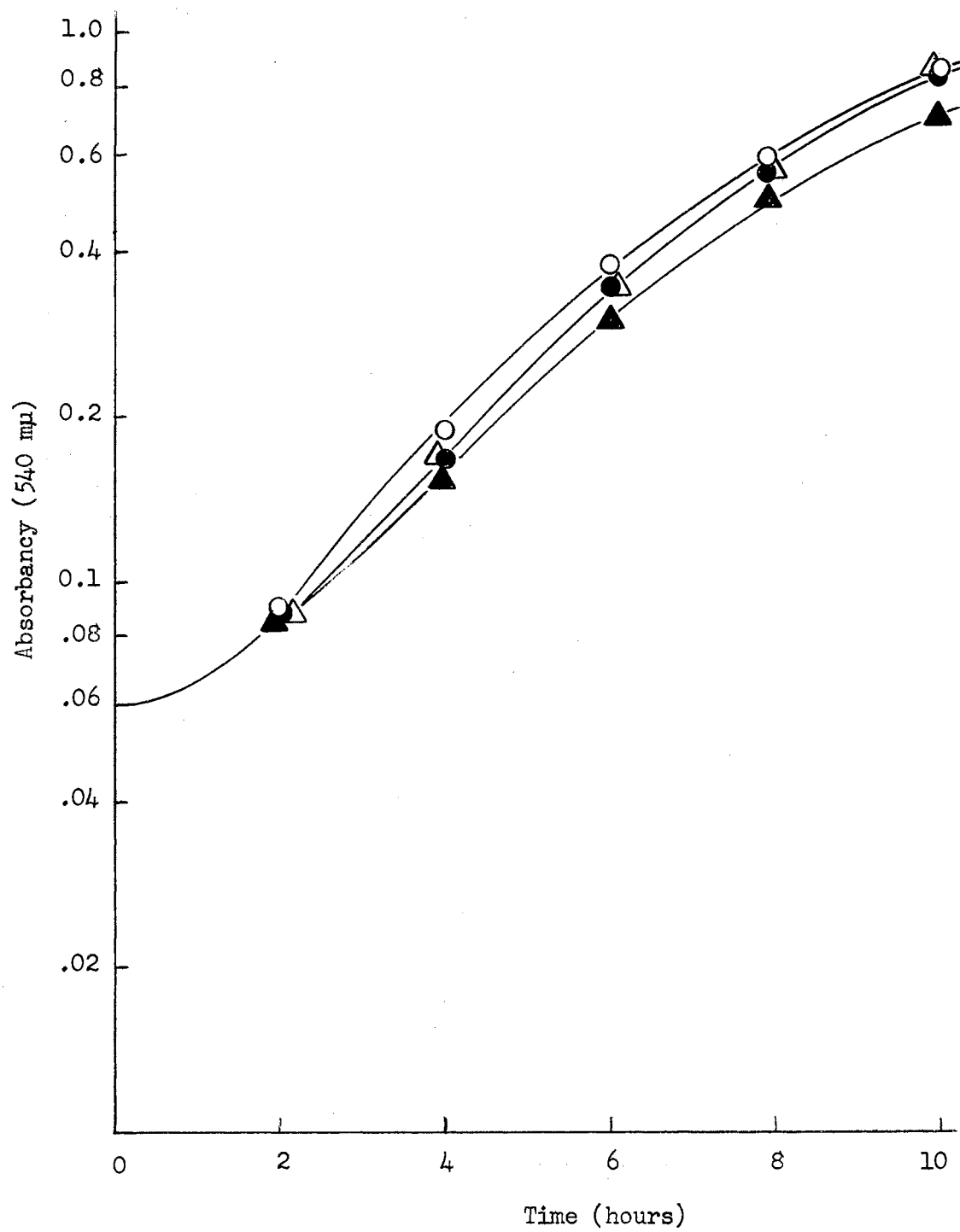
mycin D in the concentrations used in this experiment did not inhibit growth of the cells in protocatechuate-salts medium indicating that actinomycin D apparently did not effect induced enzyme synthesis for protocatechuic acid.

The effect of actinomycin D on protocatechuic acid-induced cells growing in a succinate and protocatechuate-salts medium.

An experiment identical to the one previously described was conducted using protocatechuic acid-induced cells instead of the non-induced cells. Induced cells growing in a succinate-salts medium in the presence of actinomycin D (Figure 7) were inhibited to the same extent as the non-induced cells. The absorbancy values for the cells in the presence of actinomycin D increased during the first four hours. This again illustrates the possibility of a permeability barrier. Cells induced to protocatechuic acid and grown in a protocatechuate-salts medium showed no inhibition of growth with actinomycin D (Figure 8).

Thus, cells using succinate as a sole source of carbon and energy are inhibited from growing by actinomycin D (30 µg/ml). Inhibition in succinate occurred even when the cells had been induced to protocatechuic acid. If actinomycin D, at the concentrations used in the experiment, had not caused growth inhibition, then it would be possible to say that this was entirely due to some type of cell permeability barrier; however, this was not the case. Protocatechuic acid-induced and non-induced cells did grow in the presence of actinomycin D when protocatechuic acid was the sole source of carbon and energy. This is in opposition to cells growing in the presence

Figure 7.

The influence of actinomycin D on P. fluorescens cells induced

to protocatechuic acid growing in a succinate-salts medium. O,

control; ●, actinomycin D (5 μg/ml); △, actinomycin D (10 μg/
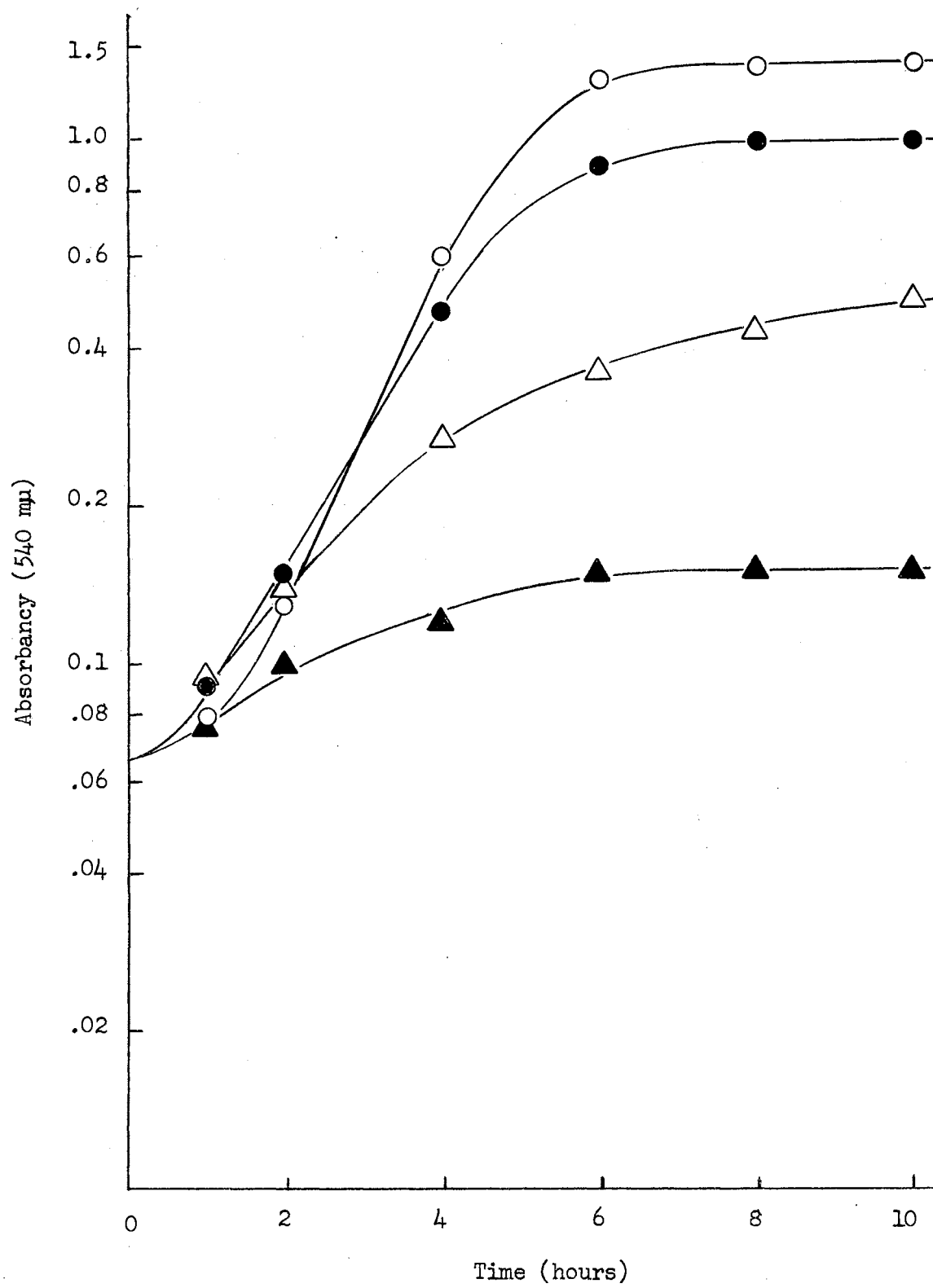
ml); and ▲, actinomycin D (30 μg/ml).
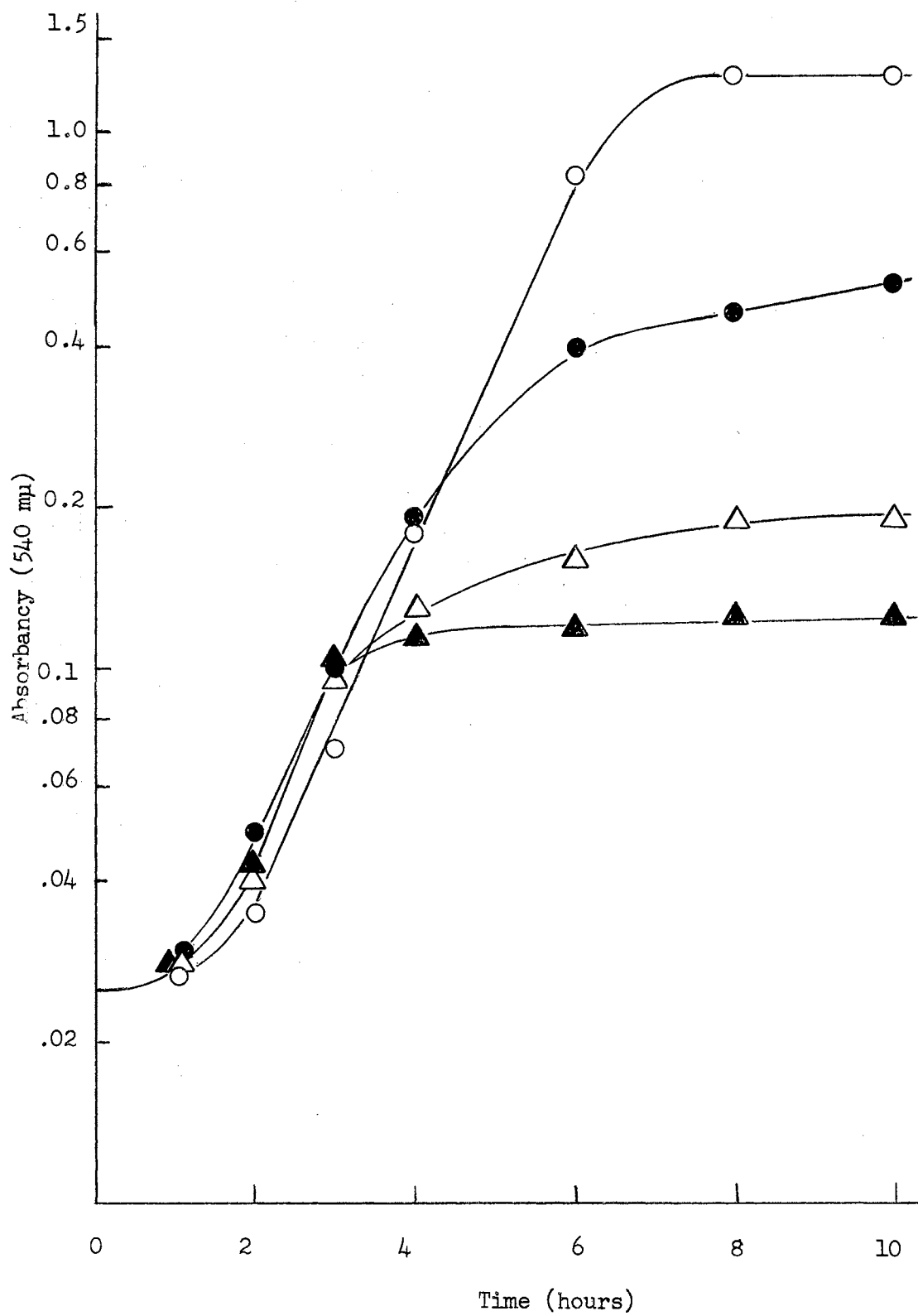
Figure 8.

The influence of actinomycin D on protocatechuic acid-induced

P. fluorescens cells growing in a protocatechuate-salts medium.

O, control; ◉, actinomycin D (5 µg/ml); △, actinomycin D (10 µg/

ml); and ▲, actinomycin D (30 µg/ml).
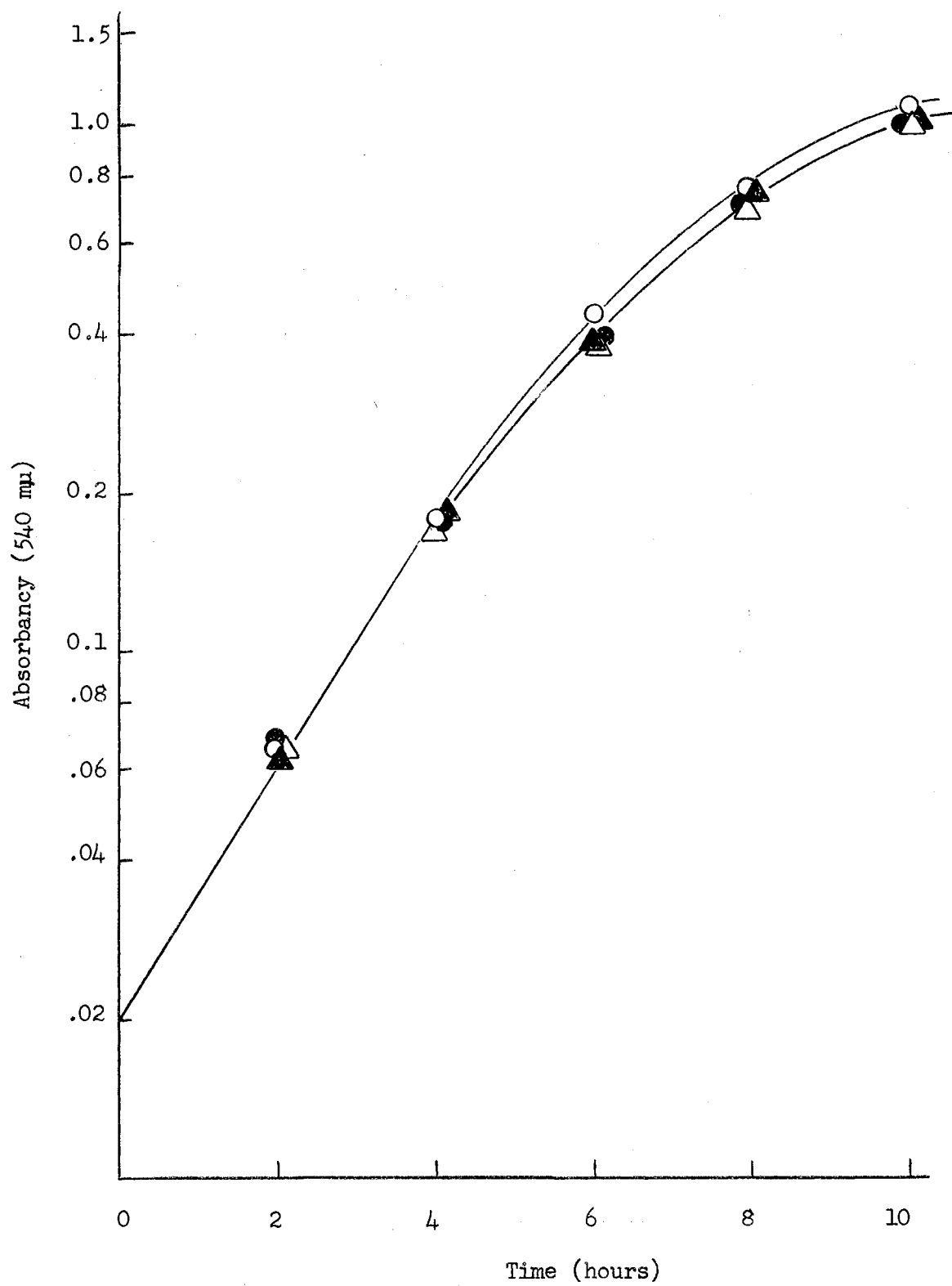
of succinate.

The effect of actinomycin D on growth in a glucose-salts medium.

Glucose was used as the sole source of carbon and energy to determine if actinomycin D inhibits growth of cells in this substrate.

A synthetic salts solution was prepared, autoclaved, and glucose, sterilized by filtration, was added aseptically. Glucose was used in concentrations of 0.2 per cent and 0.5 per cent. Seven ml of media were added to each tube and actinomycin D and buffer were added to give a total liquid volume of 8 ml. Two-tenths ml of a glucose-induced cell suspension, having an absorbancy of 0.5 at 540 mμ, were added to each tube. Absorbancy readings at 540 mμ were taken every hour.

Results (Figure 9) indicated that 5 and 10 μg/ml of actinomycin D did not show a very pronounced inhibition when 0.2 per cent glucose was used as the substrate. However, no inhibition was observed for 5 and 10 μg/ml of actinomycin D when 0.5 per cent glucose was used as the substrate (Figure 10). Thirty μg/ml of actinomycin D did produce inhibition in both concentrations of glucose but only from the standpoint that the rate of growth was reduced. This high concentration of inhibitor did not produce a growth inhibition curve which reached the stationary phase as was typical for actinomycin D inhibited cells growing in succinate, at least not in the time allowed for this experiment.

Influence of selected compounds on the actinomycin D inhibition of non-induced cells growing in a succinate-salts medium.

Since cells using protocatechuic acid and glucose as the sole

Figure 9.

The influence of actinomycin D on P. fluorescens cells grow-
ing in a glucose-salts medium (0.2 per cent glucose). ◯, control;
●, actinomycin D (5 μg/ml); △, actinomycin D (10 μg/ml); and ▲,
actinomycin D (30 μg/ml).
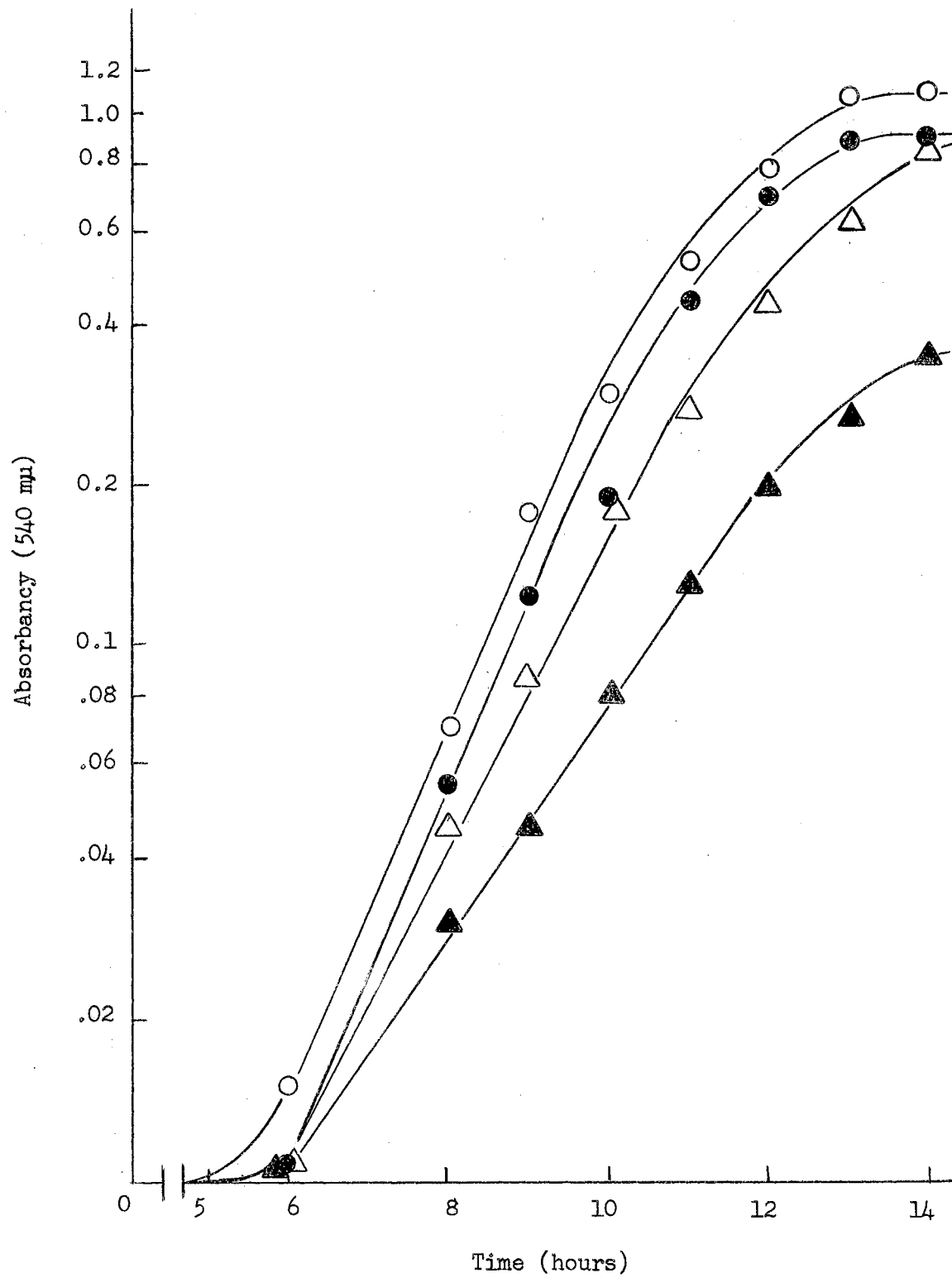
Figure 10.

The influence of actinomycin D on P. fluorescens cells growing in a glucose-salts medium (0.5 per cent glucose). ○, control; ●, actinomycin D (5 µg/ml); △, actinomycin D (10 µg/ml); and ▲, actinomycin D (30 µg/ml).
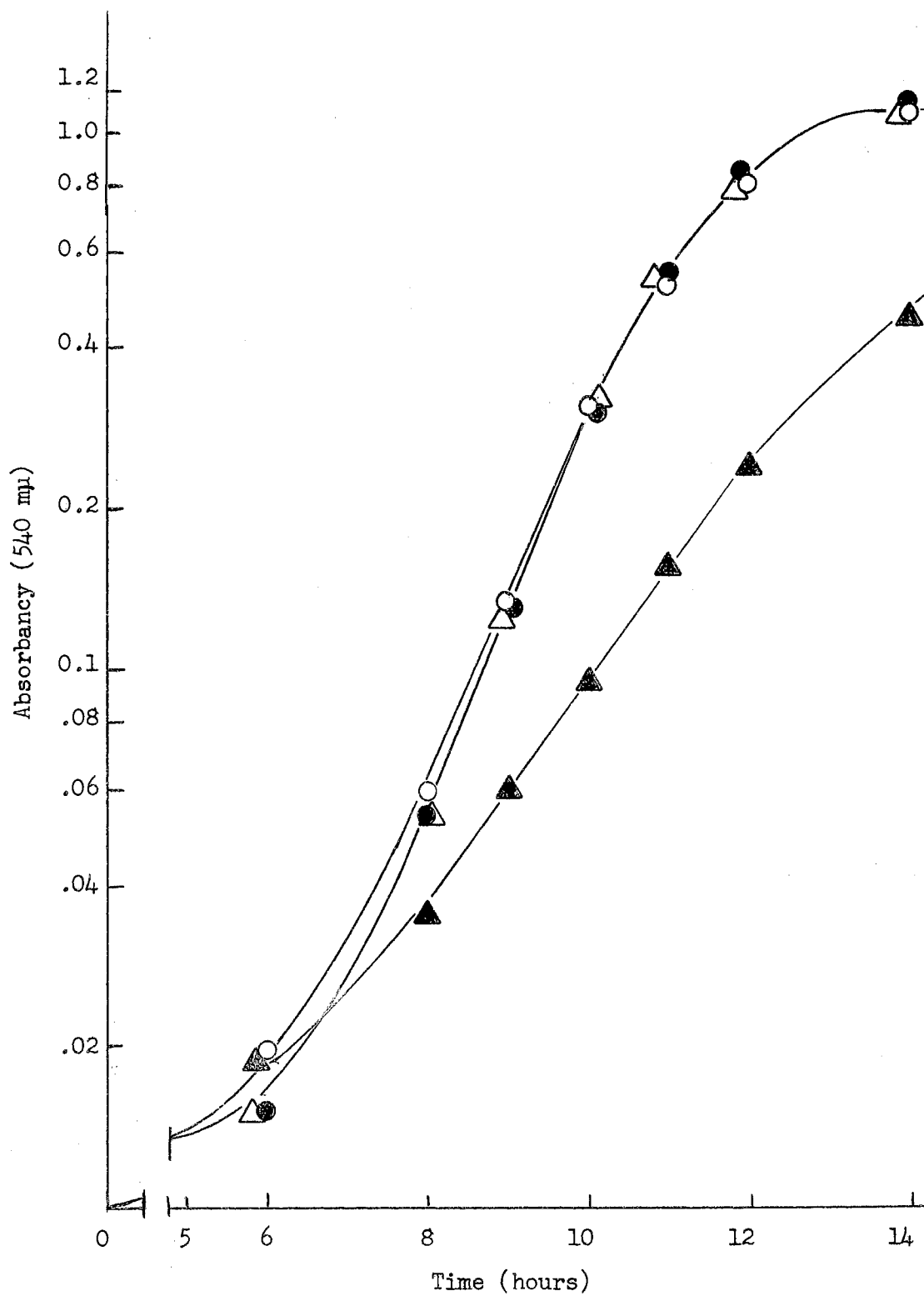
source of carbon and energy for growth apparently were not as sensitive to actinomycin D, an experiment was conducted to determine if these compounds would reverse the inhibition of cell growth when succinate was used as the primary carbon and energy source. Fructose and benzoic acid were also used to determine if they would reverse the inhibition by actinomycin D. Rauen and Hess (1959) have reported on the reversal of actinomycin C inhibition with p-aminobenzoic acid, tyrosine, and phenylalanine in some bacterial systems.

Previous experiments indicate that 30 ug/ml of actinomycin D gave almost complete inhibition of growth of cells growing in succinate; therefore, this concentration of antibiotic was used in these studies. The procedure for studying reversal of the inhibition by protocatechuic acid was as follows: seven ml of a succinate-salts medium were added aseptically to each of six tubes; three tubes contained 30 ug/ml of actinomycin D and three tubes were the controls. Two-tenths ml of succinate-grown cells, diluted so that a 1:10 dilution had an absorbancy reading of 0.8 at 540 mu, were inoculated into each tube. After 3.5 hours of incubation, protocatechuic acid (0.8 μmoles/ml final concentration) was added to one tube containing actinomycin D and to a control tube. One-tenth ml of protocatechuic acid (0.4 μmoles/ml final concentration) was added to a second tube containing actinomycin D and to a tube for the appropriate control. The results (Figure 11) indicated that protocatechuic acid reverses the inhibition of growth caused by actinomycin D, and the rate of growth of the cells was the same as the controls. The addition of protocatechuic acid to the controls did not effect the

Figure 11.

Protocatechuic acid (PA) reversal of actinomycin D in-
hibition of succinate-grown P. fluorescens cells growing in
a succinate-salts medium. Protocatechuic acid was added to
the cells at 3.5 hours. O, control; ▲, actinomycin D (30
µg/ml); △, actinomycin D (30 µg/ml) + PA (0.8 µmoles/ml);
●, actinomycin D (30 µg/ml + PA (0.4 µmoles/ml); ☐, PA
(0.8 µmoles/ml); and ■, PA (0.4 µmoles/ml).
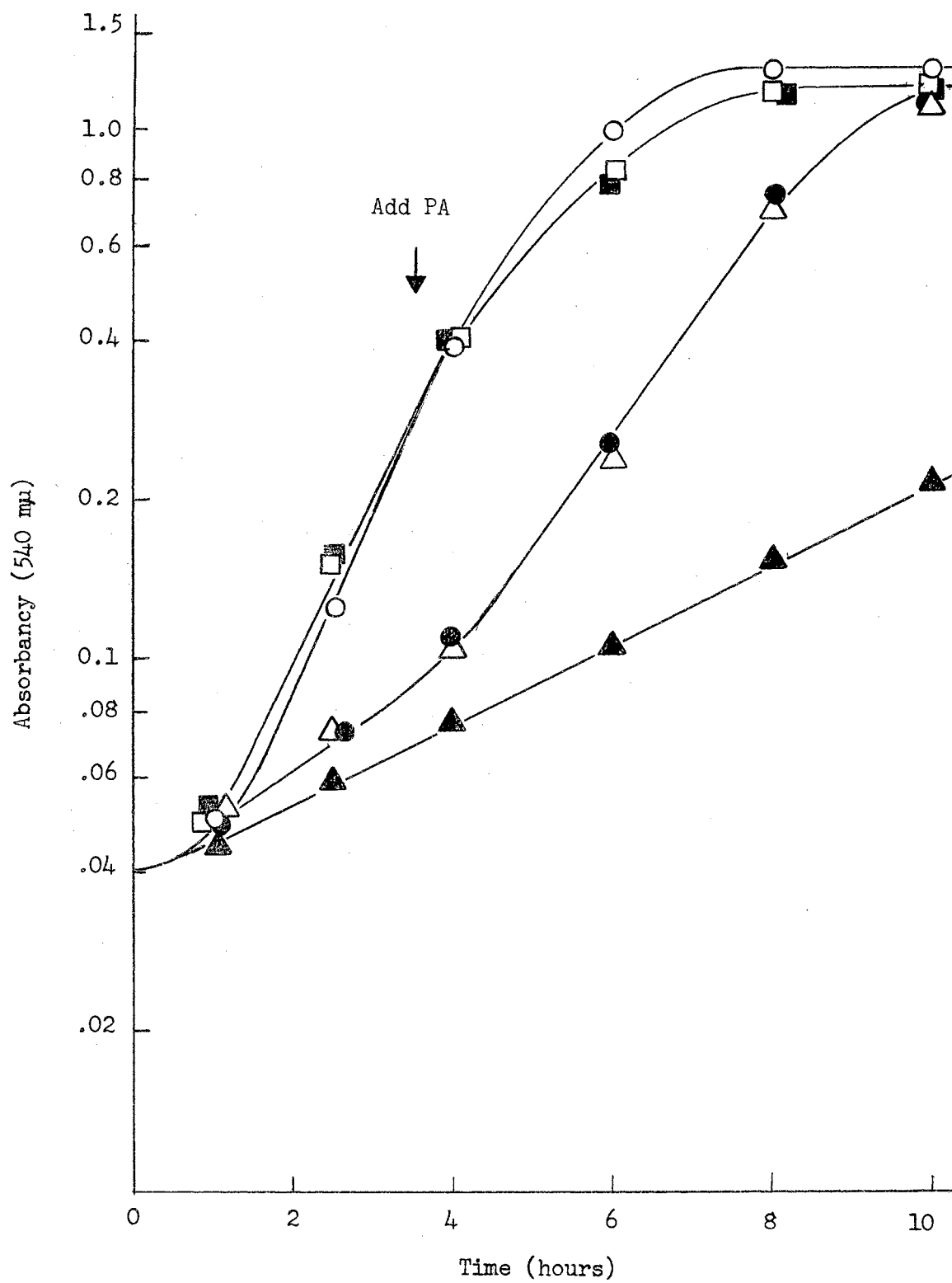
growth of the cells in the control tubes. All subsequent experiments contain protocatechuic acid in a final concentration of 0.8 μmoles/ml when used as a reversing compound.

Also investigated was the effect of adding protocatechuic acid and glucose simultaneously for reversing inhibition. If protocatechuic acid and glucose reversal is additive, then it would suggest that they reverse by acting on the same site; however, if they are not additive, then it would indicate that they act on different sites to cause reversal. Succinate-grown cells, protocatechuic acid-grown cells, and glucose-grown cells were used as the inocula for these growth studies. The cell suspensions were adjusted to have an absorbancy reading of 1.0 at 540 mμ and 0.2 ml of the suspension was inoculated into the appropriate tubes. At the end of 4 hours the following compounds were added to the tubes containing actinomycin D and to the control: 0.8 μmoles/ml of protocatechuic acid; 0.8 μmoles/ml of protocatechuic acid plus 0.8 μmoles/ml of glucose; 0.8 μmoles/ml of glucose.

The results (Figure 12, 13, and 14) suggested that protocatechuic acid reverses the inhibition of growth but glucose in the concentration used in this study did not reverse this inhibition. The simultaneous addition of both protocatechuic acid and glucose did not produce a more rapid rate (an additive effect) of reversal and this is to be expected since glucose alone did not reverse actinomycin D inhibition. Growth occurred at approximately the same rate in all the controls. It did not make any difference whether the cells were previously grown on succinate, protocatechuic acid, or glucose.

Figure 12.

The influence of protocatechuic acid and glucose on actino-
mycin D inhibition of succinate-grown P. fluorescens cells grow-
ing in a succinate-salts medium. Protocatechuic acid and glu-
cose were added to the cells at 4 hours. O, control; ●, actino-
mycin D (30 μg/ml); △, actinomycin D (30 μg/ml) + PA (0.8
μmoles/ml); ▲, actinomycin D (30 μg/ml) + glucose (0.8 μmoles
/ml); □, actinomycin D (30 μg/ml) + PA (0.8 μmoles/ml) + glu-
cose (0.8 μmoles/ml); ■, PA (0.8 μmoles/ml) + glucose (0.8
μmoles/ml); ◇, PA (0.8 μmoles/ml) and ⬡, glucose (0.8
μmoles/ml).
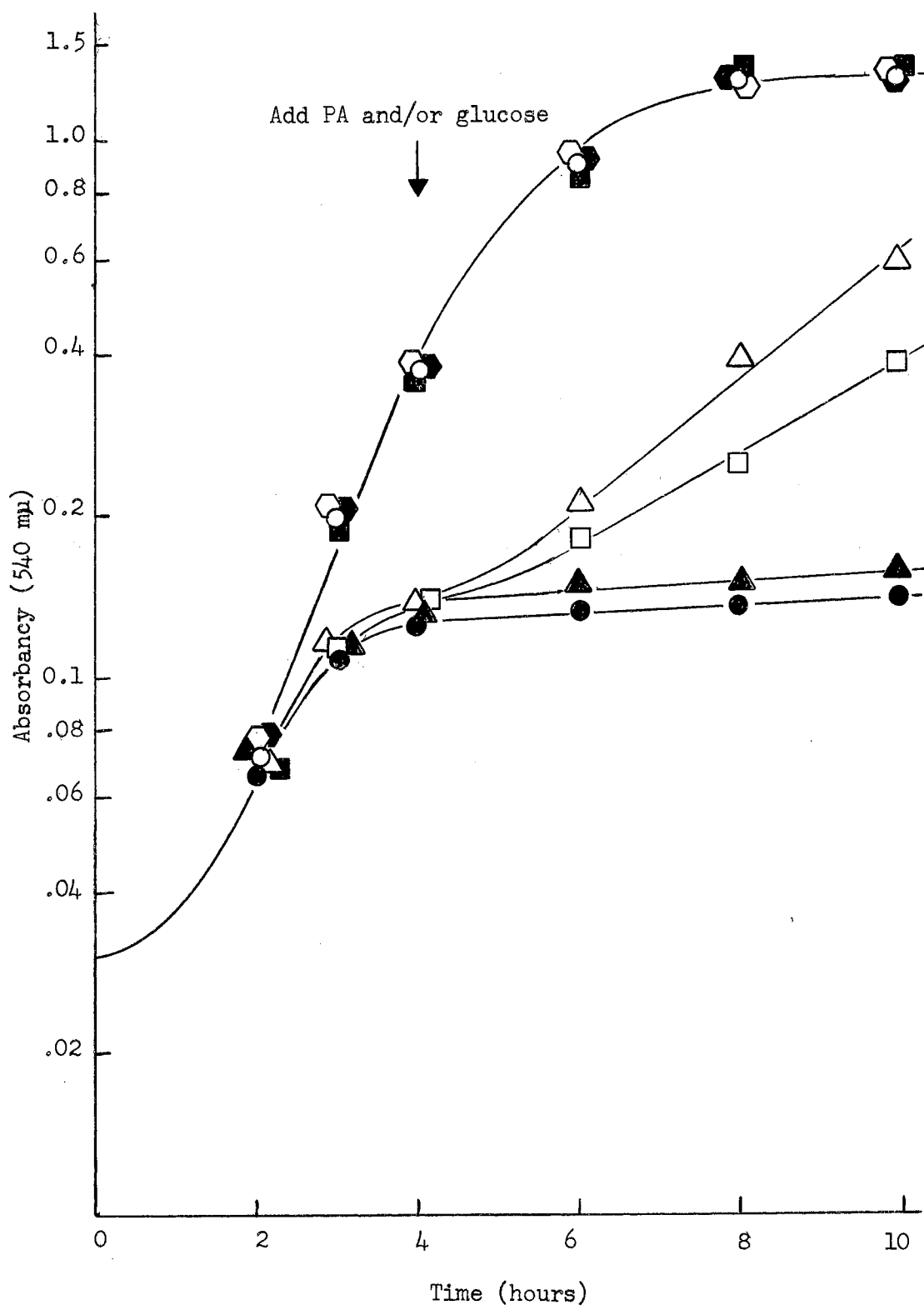
Figure 13.

The influence of protocatechuic acid and glucose on actino-
mycin D inhibition of protocatechuic acid-induced P. fluorescens
cells growing in a succinate-salts medium. Protocatechuic acid
and glucose were added to the cells at 4 hours. O, control; ●,
actinomycin D (30 µg/ml); △, actinomycin D (30 µg/ml) + PA
(0.8 µmoles/ml); ▲, actinomycin D (30 µg/ml) + glucose (0.8
µmoles/ml); □, actinomycin D (30 µg/ml) + PA (0.8 µmoles/ml)
+ glucose (0.8 µmoles/ml); ■, PA (0.8 µmoles.ml) + glucose
(0.8 µmoles/ml); ◯, PA (0.8 µmoles/ml); and ⬢, glucose (0.8
µmoles/ml).

Figure 14.

The influence of protocatechuic acid and glucose on actino-
mycin D inhibition of glucose-grown P. fluorescens cells growing
in a succinate-salts medium. Protocatechuic acid and glucose
were added to the cells at 4 hours. O, control; ●, actinomycin
D (30 µg/ml); △, actinomycin D (30 µg/ml) + PA (0.8 µmoles/
ml); ▲, actinomycin D (30 µg/ml) + glucose (0.8 µmoles/ml);
□, actinomycin D (30 µg/ml) + PA (0.8 µmoles/ml) + glucose
(0.8 µmoles/ml); ■ ,PA (0.8 µmoles/ml) + glucose (0.8 µmoles/
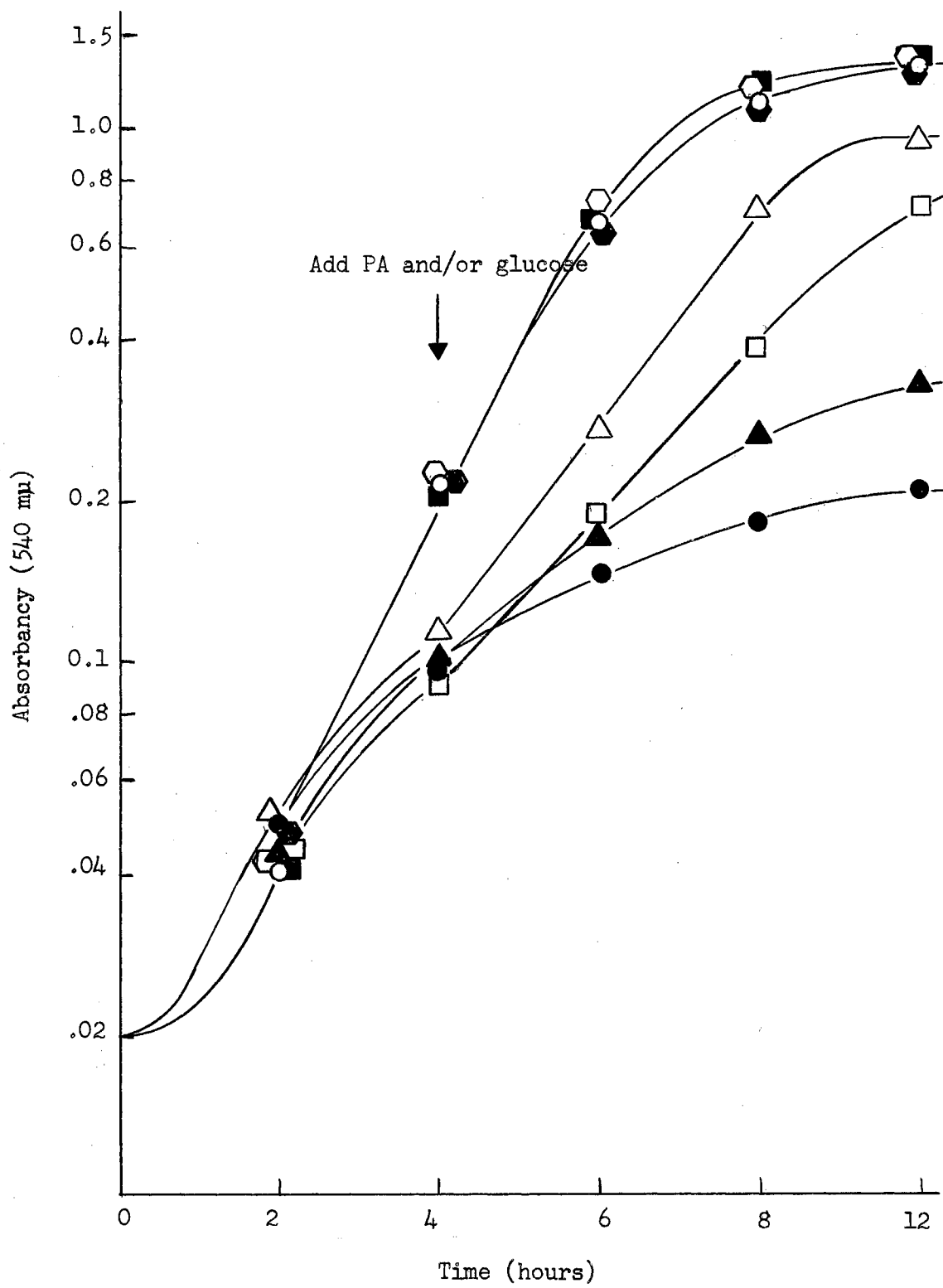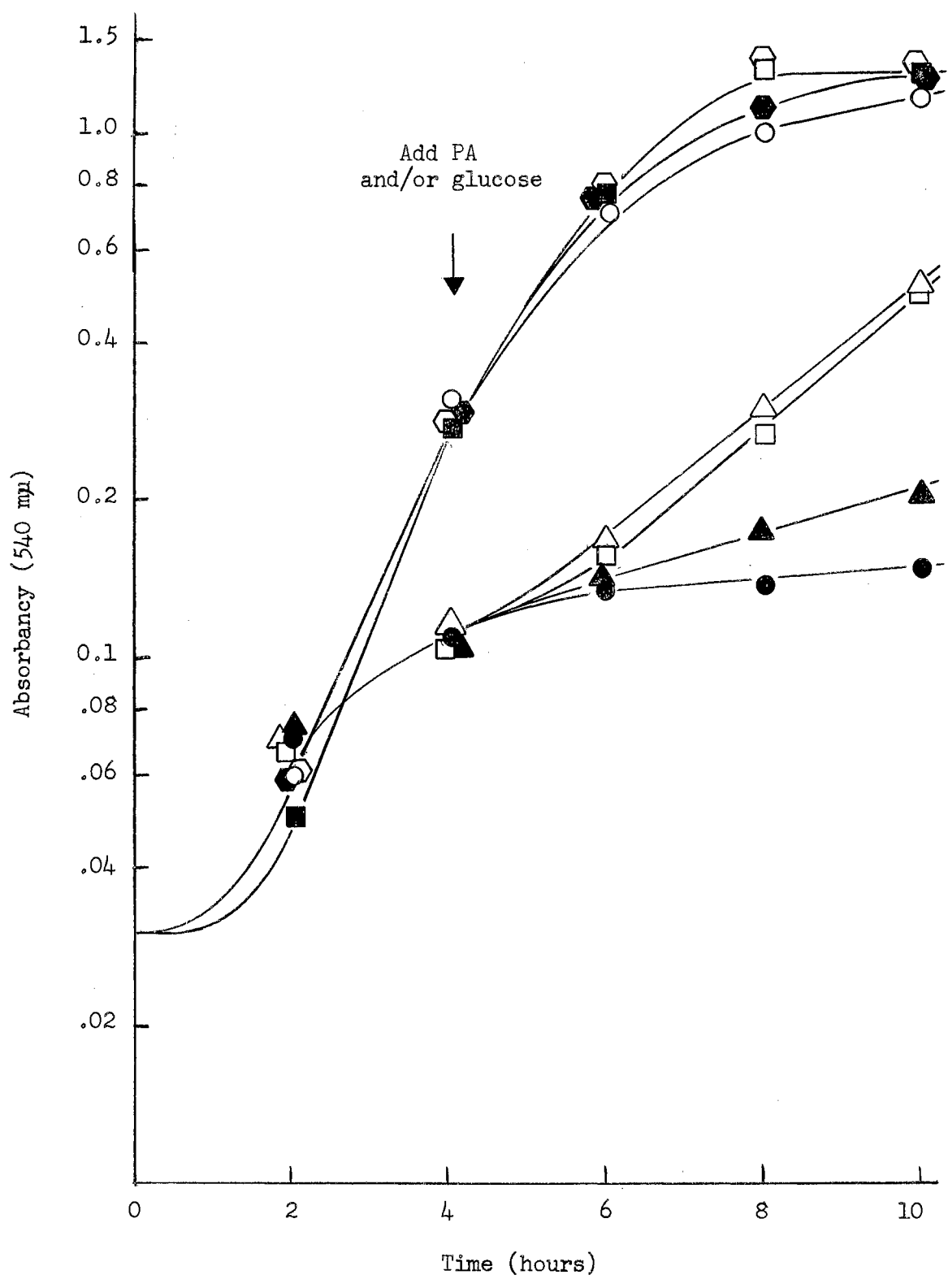ml); ◇,PA (0.8 µmoles/ml); and ⬡, glucose (0.8 µmoles/ml).

Fructose, benzoic acid, and another concentration of glucose were used to determine if they would reverse actinomycin D inhibition. The results (Figure 15) indicated that glucose (27 μmoles/ml; 2.7 μmoles/ml) did not act as a reversing compound. The results from Figure 16 and 17 indicated that fructose (27 μmoles/ml; 2.7 μmoles/ml) and benzoic acid (1.0 μmoles/ml; 0.5 μmoles/ml) did not act as reversing compounds.

Effect of actinomycin D and protocatechuic acid on cell viability.

An experiment was conducted to determine the cell viability of actinomycin D inhibited cells. The effect of protocatechuic acid on cell viability was also included in the experiment. If actinomycin D actually causes death of the cells, then there should be a large decrease in the viable cell count during the incubation period. Secondly, if protocatechuic acid does cause reversal of inhibition, then an increase in the number of viable cells should be observed. These findings should substantiate the results observed for the corresponding absorbancy readings.

Nutrient agar-grown cells were suspended in Tris buffer so that a 1:10 dilution had an absorbancy reading of 1.5 to 2.0 at 540 mμ. One ml of this suspension was added to a 250 ml Erlenmeyer side-arm flask. The flask contents were the same as for the amino acid-^{14}C incorporation experiment except for the deletion of the amino acid. At the indicated times, absorbancy readings (Figure 18) were taken at 540 mμ and a 1 ml sample was withdrawn from each flask for serial dilution and plating. The pour plate procedure was used and the cells were mixed with nutrient agar that had been cooled to 50 C. The

Figure 15.

The influence of glucose on actinomycin D inhibition of suc-
cinate-grown P. fluorescens cells growing in a succinate-salts
medium. Glucose was added to the cells at 6.5 hours. O, control;
⊛, actinomycin D (30 µg/ml); △, actinomycin D (30 µg/ml) + glu-
cose (27 µmoles/ml); ▲, glucose (27 µmoles/ml); □, actino-
mycin D (30 µg/ml) + glucose (2.7 µmoles/ml); and ▓, glucose
(2.7 µmoles/ml).
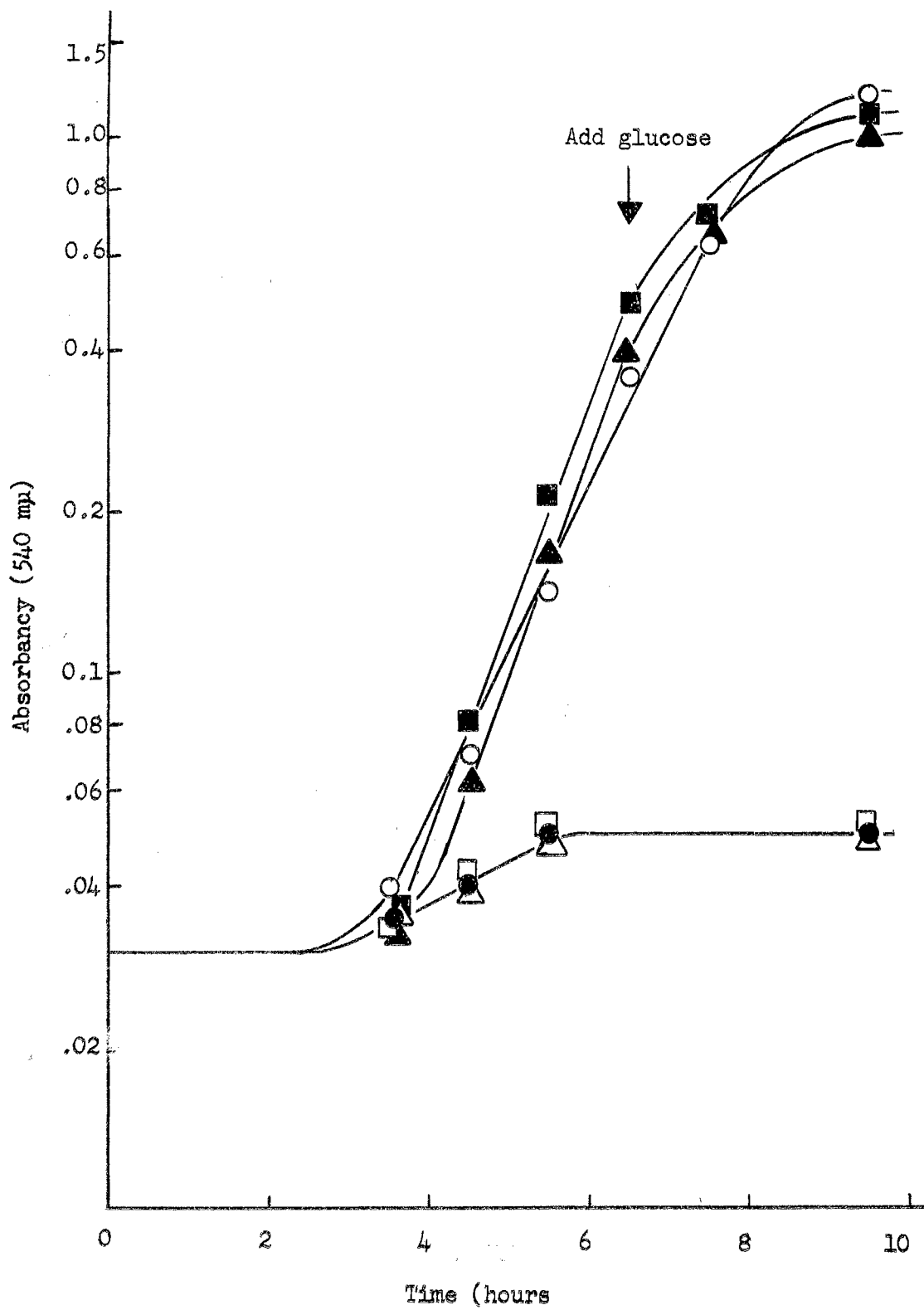
Figure 16.

The influence of fructose on actinomycin D inhibition of succinate-grown P. fluorescens cells growing in a succinate-salts medium. Fructose was added to the cells at 6.5 hours. O, control; ●, actinomycin D (30 µg/ml); △, actinomycin D (30 µg/ml) + fructose (27 µmoles/ml); ▲, fructose (27 µmoles /ml); □, actinomycin D (30 µg/ml) + fructose (2.7 µmoles/ ml); and ■, fructose (2.7 µmoles/ml).
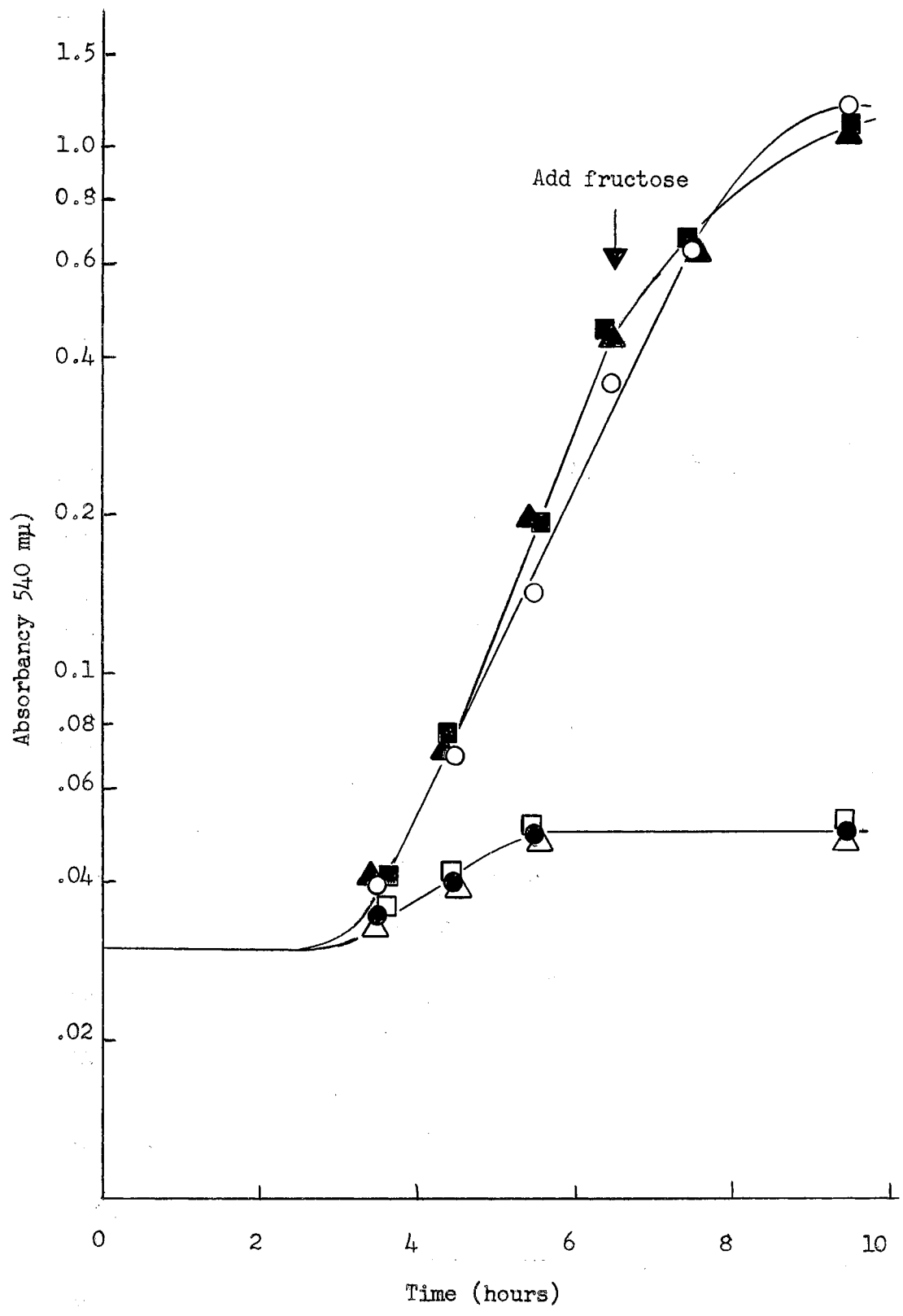
Figure 17.

The influence of benzoic acid on actinomycin D inhibition of
succinate-grown $\underline{P}$. fluorescens cells growing in a succinate-salts
medium. Benzoic acid was added to the cells at 6.5 hours. O,
control; ●, actinomycin D (30 μg/ml); △, actinomycin D (30 μg
/ml) + benzoic acid (1.0 μmoles/ml); ▲, benzoic acid (1.0
μmoles); □, actinomycin D (30 μg/ml) + benzoic acid (0.5
μmoles/ml) + benzoic acid (0.5 μmoles/ml); and ■, benzoic
acid (0.5 μmoles/ml).
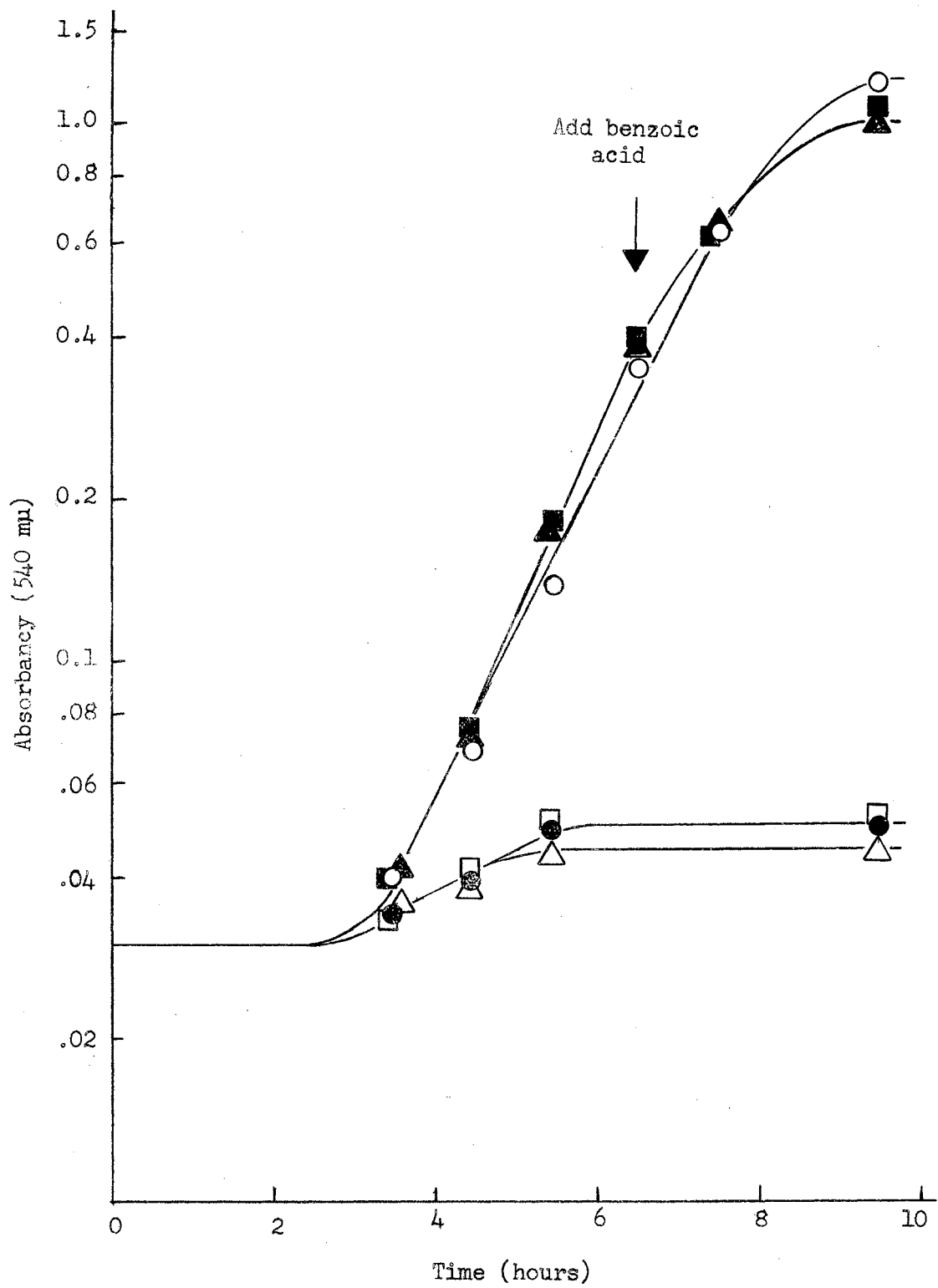
Figure 18.

The influence of protocatechuic acid on cell growth in the presence of actinomycin D. The cells were growing in a succinate-salts medium. The absorbancy readings were taken concurrently with the data presented in Table I. O, control; ◉, actinomycin D (30 µg/ml); ▲, actinomycin D (30 µg/ml) + PA (0.8 µmoles/ml) added at zero time; △, actinomycin D (30 µg/ml) + PA (0.8 µmoles/ml) added at 3 hours; and ☐, PA (0.8 µmoles/ml).
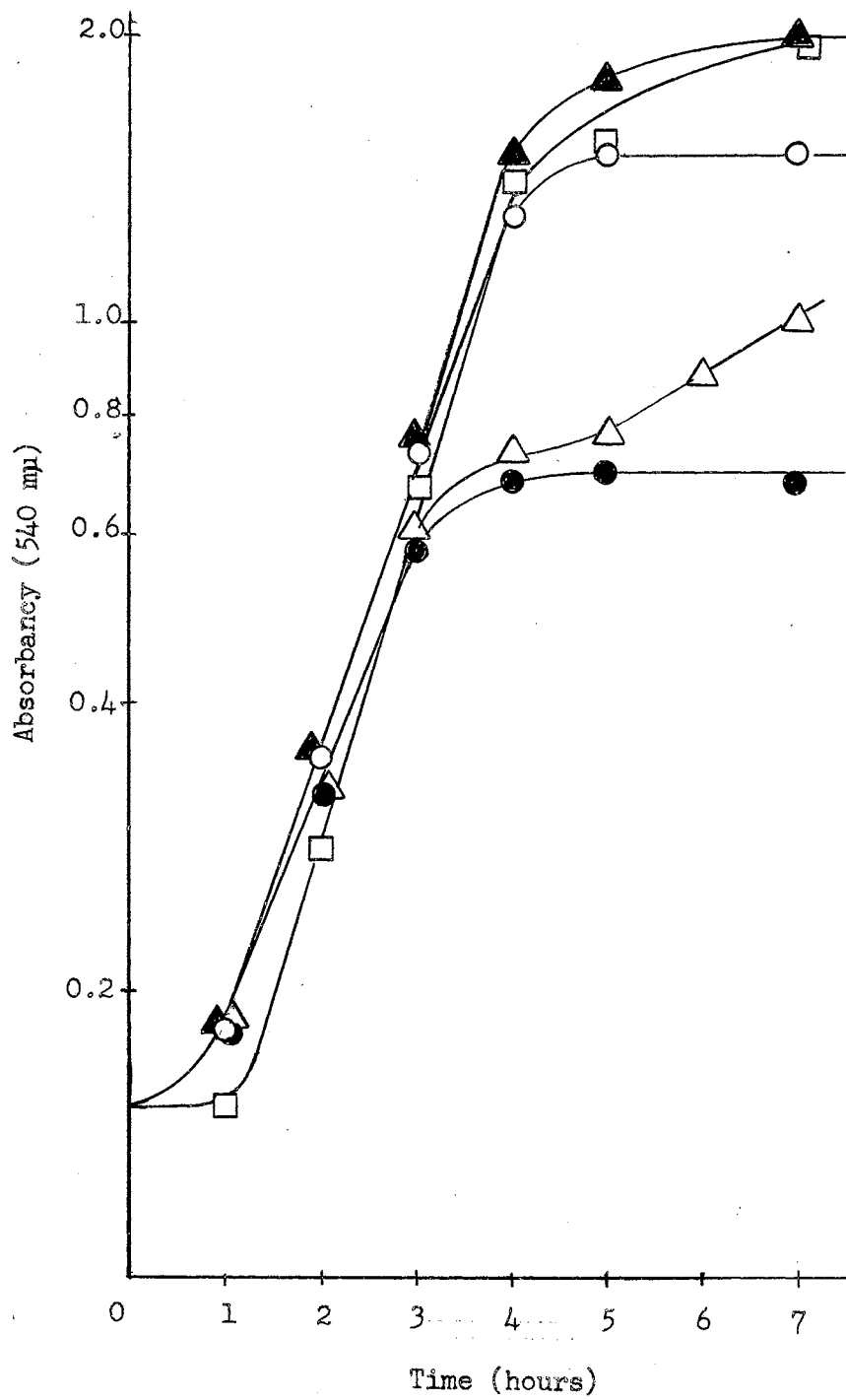
plates were incubated at 37 C for 24 hours and the colonies were counted using a Quebec Colony Counter.

Cell viability counts (Table I) show a steady decrease after approximately two hours in actinomycin D-inhibited cultures. This is good evidence that actinomycin D is actually killing P. fluorescens cells growing in a succinate-salts medium. Microscopic examination indicated that the cells in the presence of actinomycin D increased in size. This would explain the observation of an increase in absorbancy reading, although the viable cell count declined after approximately two hours of incubation in actinomycin D. Hurwitz et al. (1962) observed an increase in cell size of B. subtilis cells growing in the presence of actinomycin D. He stated that this was due to unbalanced growth resulting from the preferential inhibition of RNA synthesis. Cell counts for cells growing when actinomycin D and protocatechuic acid were added simultaneously at time 0 show that the cells grow out to about the same extent as the control. This would suggest that protocatechuic acid prevents actinomycin D from inhibiting cell growth. When protocatechuic acid was added to actinomycin D inhibited cells at 3 hours, inhibition ceased at 6 hours and viable cell counts began to increase. The small number of viable cells at 7 hours could be due to the fact that most of the cells were already killed from the action of the antibiotic when protocatechuic acid was added. The controls show a reasonable increase in cell numbers.

The influence of actinomycin D on uracil-2-^{14}C incorporation correlated with protocatechuate oxygenase synthesis.

Growth studies suggest that actinomycin D does not inhibit growth

Table I. <u>Viable cell count of cells growing in a succinate-salts medium in the presence and absence of actinomycin D and protocatechuic acid.</u>

			Viable cells X 10^6		
Time (hours)	Succinate	Succinate Act. D *	Succinate Act. D PA**	Succinate Act. D PA***	Succinate PA**
0	266	291	245	288	290
2	348	284	269	278	297
3	790	152	790	157	810
4	1980	17.2	1430	29.9	1720
6	2680	4.9	1660	16	2720
7	2560	2.3	1460	21	1980

*Actinomycin D (Act. D) 30 µg/ml.

**Protocatechuic acid (PA) 0.8 µmoles/ml (added at zero time).

***Protocatechuic acid (PA) 0.8 µmoles/ml (added at 3 hours).

The initial inoculum contained approximately 181 X 10^8 cells/ml and was diluted 1:40 in the test media.

of cells utilizing protocatechuic acid as the sole source of carbon and energy. Therefore, it should be possible to observe protocatechuate oxygenase synthesis manometrically and uracil-2-^{14}C incorporation into RNA. If actinomycin D is actually inhibiting protocatechuate oxygenase, then oxygen uptake would not occur in the Warburg apparatus and uracil-2-^{14}C should not be incorporated into the acid insoluble TCA fraction.

Nutrient agar-grown cells were harvested, washed twice with Tris buffer and suspended so that a 1:10 dilution gave an absorbancy reading of 1.0 at 540 mu. The cell suspension was placed into the main chamber of the Warburg flask and the substrate, inhibitor, and uracil-2-^{14}C were placed in the side arms.

Manometric results show that oxygen uptake occurs at approximately the same rate in the control and in the flask containing 18 ug/ml of actinomycin D. (Figure 19). The cells used were nutrient agar-grown; therefore, if actinomycin D had inhibited DNA dependent m-RNA for protocatechuate oxygenase it would have occurred within the time it takes for inducible enzyme synthesis by the control cells unless more time is required for actinomycin D uptake. This possibility is discussed later on in the text (p. 66). Uracil-2-^{14}C incorporation samples from these flasks show that uracil-2-^{14}C was incorporated into the RNA of cells incubated in the presence and absence of actinomycin D at approximately the same rate (Figure 20). These findings suggest that actinomycin D does not affect the inducible enzyme synthesis of protocatechuate oxygenase. Isotope data from cold TCA extracts were inconclusive. Regardless, the uracil-

Figure 19.

The influence of actinomycin D on protocatechuate oxygenase synthesis of P. fluorescens cells. Samples for uracil-2-^{14}C incorporation were removed at 45, 90, and 135 munutes (indicated by arrows). The concentration of protocatechuic acid was 4.2 μmoles/ml per flask. O, control; ⊕, actinomycin D (18 μg/ml); △, cells with actinomycin D (18 μg/ml) but lacking PA; ▲, cells lacking both actinomycin D and PA; and □, endogenous.
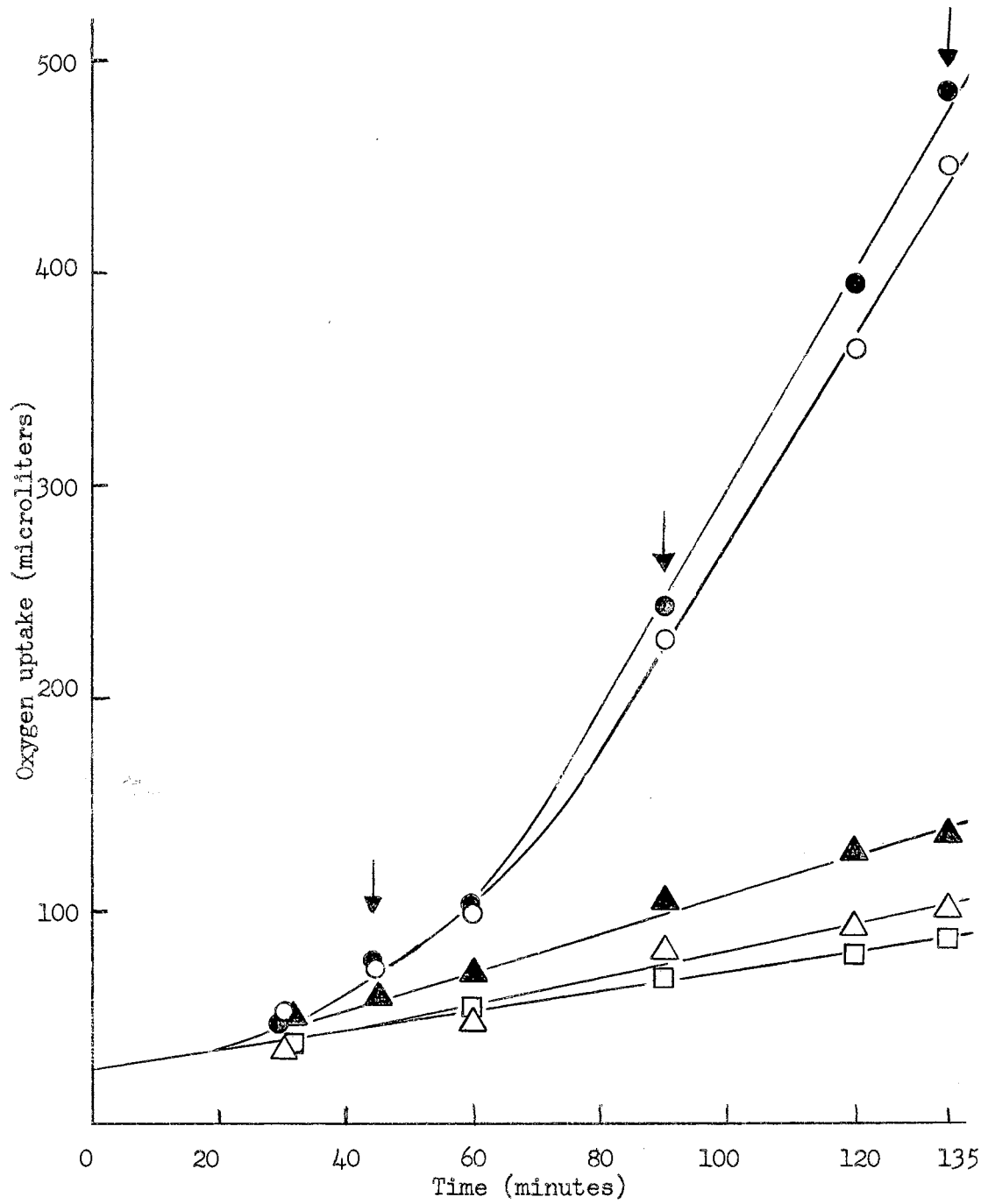
Figure 20.

The influence of actinomycin D on uracil-2-^{14}C incorporation into P. fluorescens cells with protocatechuic acid as the substrate. The concentration of protocatechuic acid was 4.2 μmoles/ml. per flask. ○, control; ◉, actinomycin D (18 μg/ml); ▲, cells with actinomycin D (18 μg/ml) but lacking PA; △, cells lacking actinomycin D and PA.
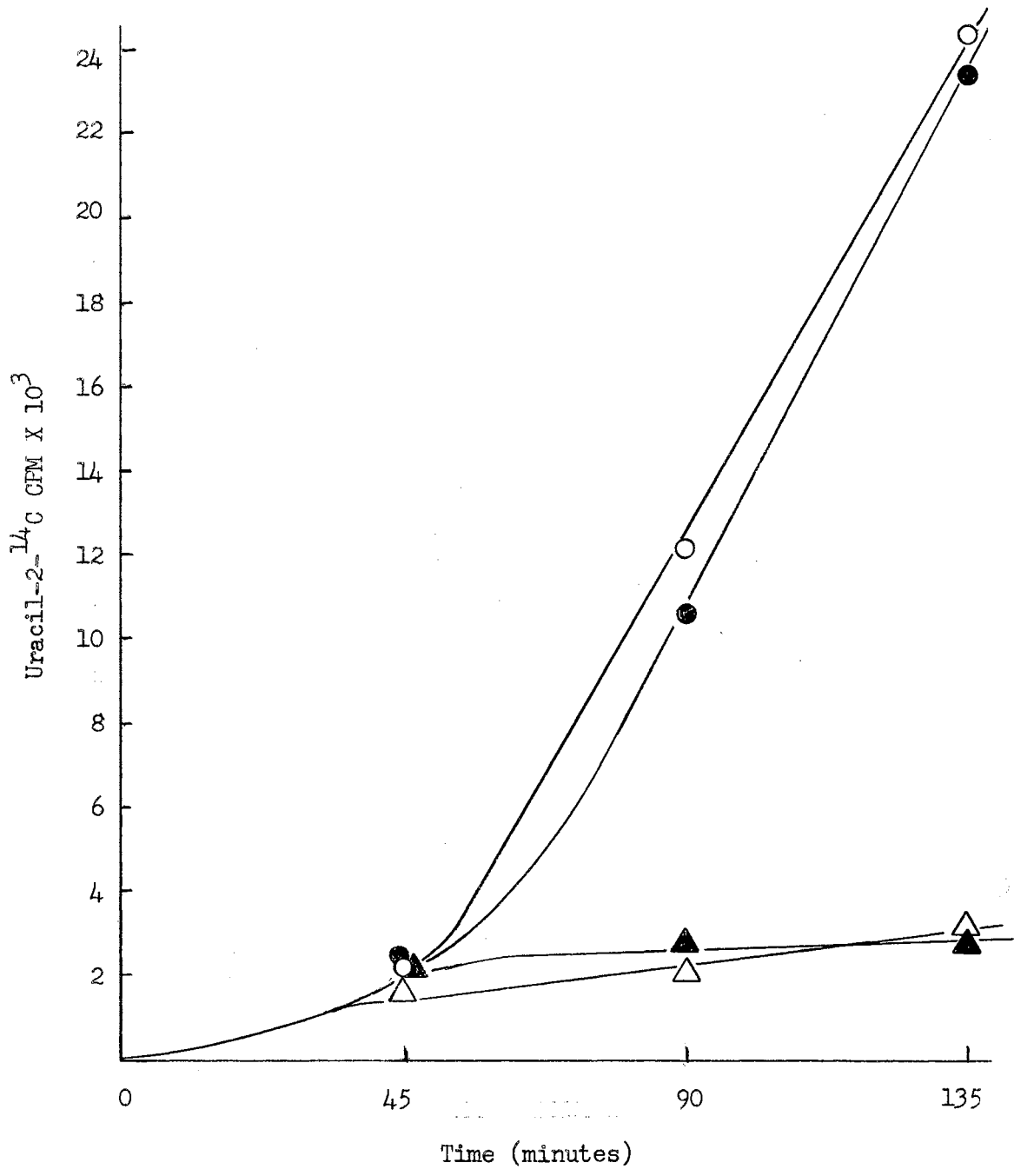
$2-^{14}C$ incorporation is significant.

An experiment was conducted to determine if preincubation in actinomycin D prior to protocatechuic acid addition would affect protocatechuate oxygenase synthesis. The cell suspension was prepared as described earlier. Actinomycin D was added to the cells and incubated for 60 minutes before the substrate was added. The results (Figure 21) indicated that protocatechuate oxygenase synthesis occurred in the presence of actinomycin D and was independent of prior incubation in the inhibitor. This would suggest that actinomycin D does not require a long period for uptake into the cells. This was further substantiated by a similar experiment in which the cells were incubated for 160 minutes before the addition of the inducer. Again, protocatechuate oxygenase synthesis occurred in the presence of actinomycin D. Thus, enough time was allowed for actinomycin D uptake and inhibition of protocatechuate oxygenase synthesis; however, the synthesis of protocatechuate oxygenase is apparently resistant to the inhibitor.

Treatment of P. fluorescens cells with disodium ethylenediaminetetraacetate.

Leive (1965a) has shown that treatment of E. coli cells with EDTA $(10^{-3}M)$ permits the uptake of actinomycin D with a subsequent inhibition of B-galactosidase synthesis. Previous results have indicated that actinomycin D enters P. fluorescens cells but did not inhibit the synthesis of protocatechuate oxygenase. This was further investigated by treating the cells with EDTA in the presence of actinomycin D and observing the synthesis of protocatechuate oxygenase. If

Figure 21.

The influence of preincubation of P. fluorescens cells with

actinomycin D on protocatechuate oxygenase synthesis. Each flask

contains 4.2 μmoles/ml of protocatechuic acid. O, control; ●,

actinomycin D (30 μg/ml); △, actinomycin D (30 μg/ml) in the

presence of the cells 60 minutes before substrate addition; and

▲, endogenous.
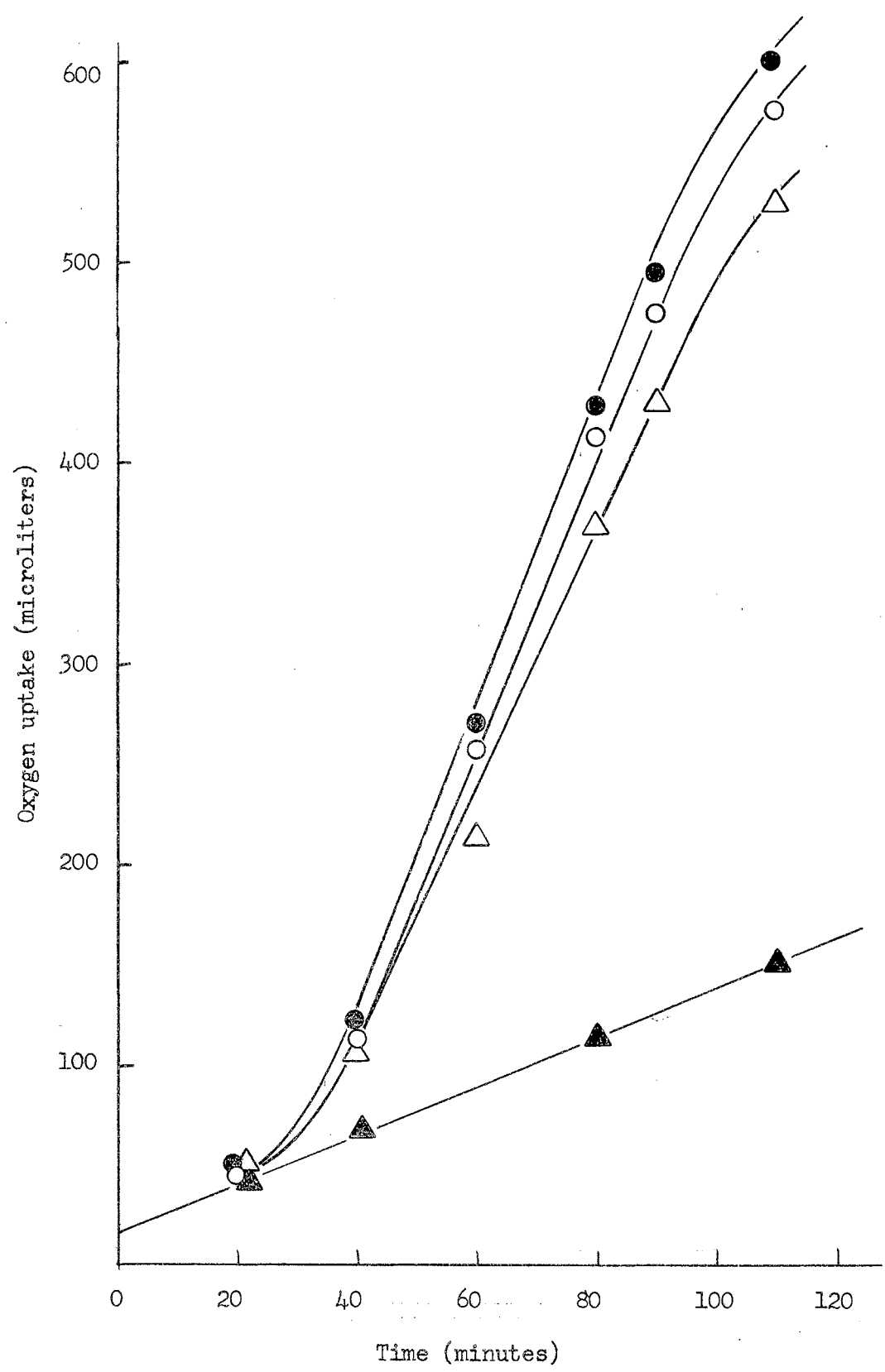
actinomycin D was not getting into the cells due to a permeability barrier, then EDTA treatment should eliminate this barrier and the true effect of actinomycin D on the synthesis of protocatechuate oxygenase should be observed.

Nutrient agar-grown cells were suspended so that a 1:10 dilution gave an absorbancy reading of 1.0 at 540 mμ. Twenty ml of the cell suspension were added to a 250 ml Erlenmeyer flask and one ml of EDTA (1 X 10^{-4} M final concentration) or Tris buffer was added to the flask. Higher EDTA concentrations were not used because they caused cell lysis. The contents were incubated for 30 minutes at 37 C on a reciprocal shaker. The cells were centrifuged, washed one time with Tris buffer, and 2 ml of cells were withdrawn for adding to the Warburg flask. Actinomycin D, EDTA, protocatechuic acid, and/or Tris buffer were added to the appropriate Warburg flask.

Manometric results (Figure 22) indicated that protocatechuate oxygenase synthesis occurred in the presence of actinomycin D in EDTA-treated P. fluorescens cells. The presence of EDTA appeared to increase the amount of oxygen uptake. Therefore, the conclusions drawn from these results are consistent with the earlier interpretations that the inducible enzyme synthesis of protocatechuate oxygenase is resistant to actinomycin D.

Uptake of actinomycin D-^{14}C into P. fluorescens cells.

By observing the uptake of actinomycin D-^{14}C into the cells (Table II), further proof could be obtained to indicate that actinomycin D is actually entering this Gram-negative organism.

Three controls were used in the first experiment. The first control contained all the constituents except the substrate. Actinomycin D uptake occurred in these cells. The second control contained all

Figure 22.

The influence of EDTA on the protocatechuate oxygenase syn-
thesis by P. fluorescens cells in the presence of actinomycin D.
Each flask contained 1.3 μmoles/ml of protocatechuic acid. O,
control; ◉, actinomycin D (30 μg/ml); △, actinomycin D (30
μg/ml) + EDTA (1 x 10^{-4}M); ▲, actinomycin D (30 μg/ml),
cells preincubated in EDTA (1 x 10^{-4}M) for 30 minutes; □,
control + EDTA (1 x 10^{-4}M); ▨, control, cells preincubated
in EDTA (1 x 10^{-4}M) for 30 minutes; and ⬡, endogenous.
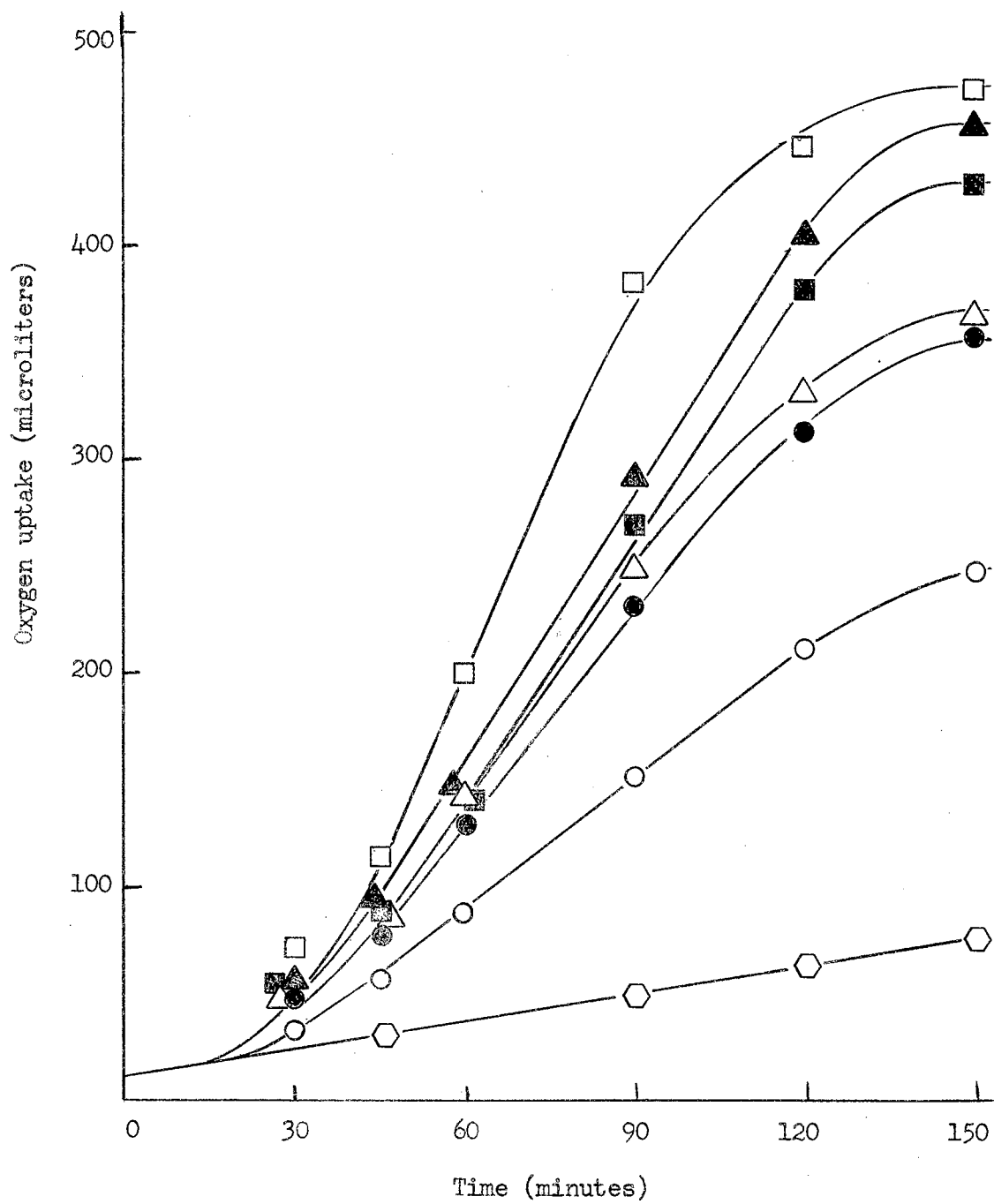
TABLE II. Uptake of radioactive actinomycin D by P. fluorescens.

Constituents	Exp. 1		Exp. 2	
	(CPM)	(Per cent)*	(CPM)	(Per cent)*
Succinate Actinomycin D	277	33.7	148	18
PA Actinomycin D	198	24.1	111	13.5
Glucose Actinomycin D	277	33.7	124	15.1
No substrate Actinomycin D (control)	287	34.9	Not tested	
Succinate PA (0.8 µmoles/ml) Actinomycin D	265	32.2	122	14.8
Succinate No labeled Actinomycin D (control)	0	0	Not tested	
PA (6.8 µmoles/ml) Actinomycin D	Not tested		111	13.5
No cells Succinate Actinomycin D (control)	0	0	Not tested	

*Per cent uptake based upon the total count rate for 0.1 ml of actinomycin D-^{14}C (821 CPM).

the constituents except actinomycin D-^{14}C, and the third control contained all the constituents except the cells. As was expected, the last two controls showed no radioactivity. Actinomycin D uptake occurred in the presence of all the substrates used in the experiment; however, there was a slight decrease in actinomycin D uptake in the presence of protocatechuic acid as compared to succinate, but it was not enough to state that it caused interference of actinomycin D uptake.

These studies suggest that none of the substrates are competing with or otherwise influencing the uptake of actinomycin D by the cells. Therefore, the growth of the cells in actinomycin D with protocatechuic acid as the substrate is not readily explained by the substrate competing with the inhibitor for uptake.

Incorporation of amino acid-^{14}C into protein of actinomycin D inhibited cells.

It is known that amino acid incorporation into protein is decreased in the presence of actinomycin D (Goldberg et al., 1962; Levinthal et al., 1962). An experiment was designed to determine if amino acid incorporation into proteins of cells growing in a succinate-salts medium was influenced by the presence of actinomycin D. Since protocatechuic acid reversed the inhibition of growth, the incorporation of amino acids into proteins was measured in the presence and absence of actinomycin D and protocatechuic acid.

Thirty-six ml of succinate-salts medium was added to a 250 ml Erlenmeyer side arm flask. One ml of leucine—2-^{14}C was added to the flask. While no information was available on the actual amount of

leucine present in the leucine-2-^{14}C, definite conclusions can be obtained if the isotope is incorporated into cell protein. Three flasks were prepared each containing 1 ml actinomycin D (30 ug/ml). Five-tenths ml of protocatechuic acid (0.8 μmoles/ml) was added to one flask containing actinomycin D and to another flask containing no inhibitor. After 150 minutes of incubation 0.5 ml of protocatechuic acid (0.8 μmoles/ml) was added to the other flask containing actinomycin D. Succinate-grown cells were suspended so that a 1:10 dilution had an adsorbancy reading of 1.5 at 540 mu, and 1 ml of this suspension was inoculated into each flask. Tris buffer was added to bring the total volume to 40 ml. A 3 ml sample was withdrawn from each flask at the same time that each absorbancy reading was taken.

Absorbancy readings (Figure 23) show that growth was inhibited in the presence of actinomycin D. No inhibition was observed in the presence of actinomycin D when protocatechuic acid was added at zero time. If protocatechuic acid was added after inhibition had begun, then this inhibition could be reversed with a resulting increase in growth. Leucine-2-^{14}C incorporation (Table III) into protein indicates that the presence of actinomycin D decreased the amount of incorporation as compared to the control. When protocatechuic acid was added simultaneously with actinomycin D at zero time, the rate of amino acid incorporation into protein was slightly inhibited for 2.5 hours. However, by 4 hours it began to approach the rate of amino acid incorporation of the controls. The data suggest that actinomycin D does not inhibit formation of protein in the presence of protocatechuic acid. Amino acid-^{14}C incorporation increased upon the addition

Figure 23.

The influence of protocatechuic acid on cell growth in the
presence of actinomycin D. The cells were growing in a suc-
cinate-salts medium. The absorbancy readings were taken con-
currently with the data presented in Table III. O, control;
●, actinomycin D (30 µg/ml); ▲, actinomycin D (30 µg/ml) +
PA (0.8 µmoles/ml) added at zero time; △, actinomycin D (30
µg/ml) + PA (0.8 µmoles) added at 150 minutes; and □, PA
(0.8 µmoles/ml).
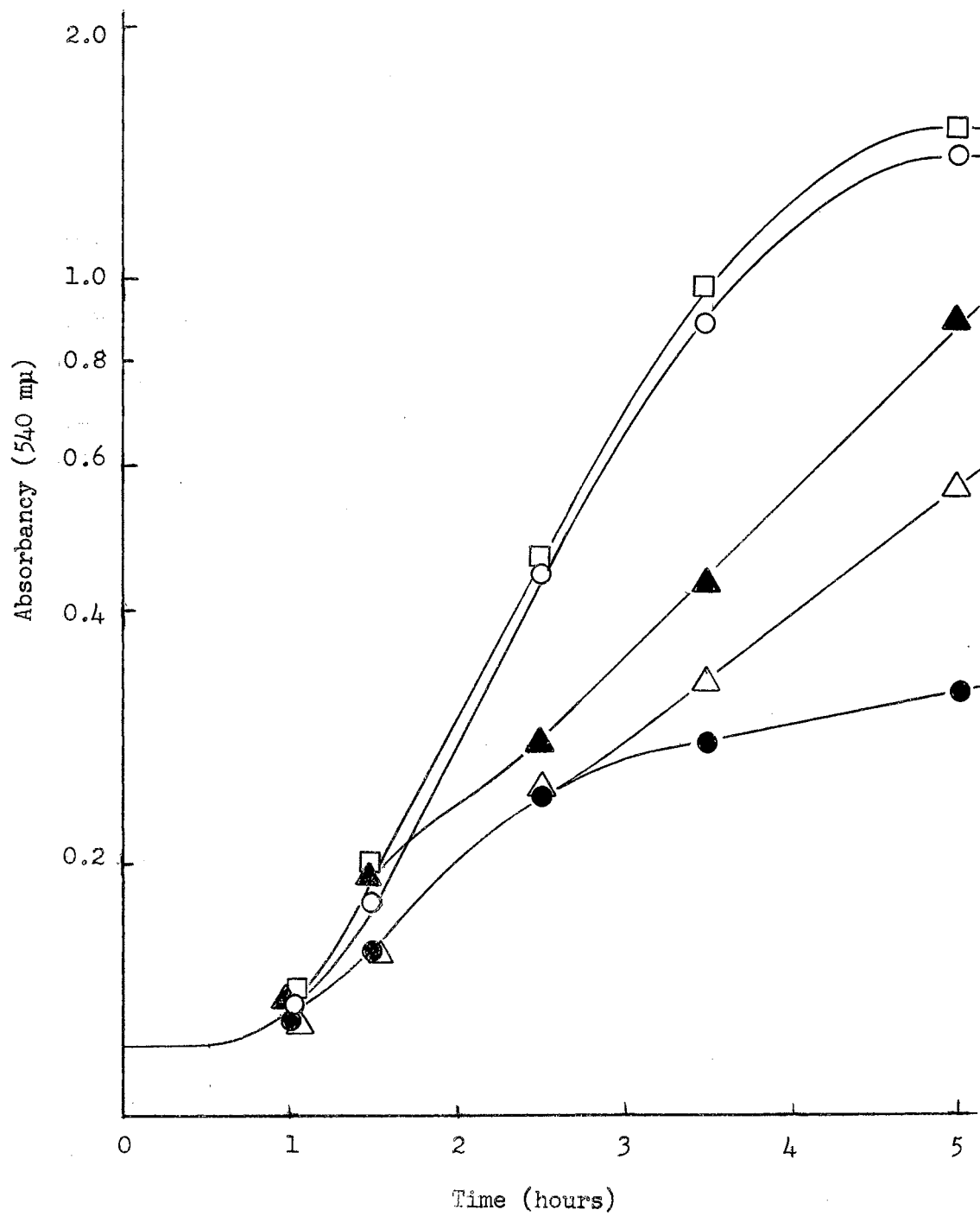
Table III. Incorporation of leucine -2-^{14}C into protein of P. fluorescens cells growing in the presence and absence of actinomycin D and protocatechuic acid.

Time (hours)	Succinate	Succinate Act. D*	Succinate Act. D PA**	Succinate Act. D PA***	Succinate PA**
			Counts per minute corrected for background		
0	0	0	0	0	0
0.5	5	8	7	10	14
1	24	23	13	18	11
1.5	42	31	40	27	47
2.5	132	40	55	36	82
3.5	191	36	72	45	111
4	199	44	147	104	205

*Actinomycin D (Act. D) 30 µg/ml.

**Protocatechuic acid (PA) 0.8 µmoles/ml (added at zero time).

***Protocatechuic acid (PA) 0.8 µmoles/ml (added at 2.5 hours).

of protocatechuic acid to actinomycin D inhibited cells. Apparently, protocatechuic acid released the actinomycin D inhibition and the cells were thus able to synthesis protein. While isotope data on cold TCA extracts were again inconclusive, the data showing incorporation of leucine-2-^{14}C are significant.

SUMMARY AND CONCLUSIONS

Actinomycin D is a known inhibitor of DNA-dependent RNA synthesis. The antibiotic produces a rapid and pronounced growth inhibition in Gram-positive organisms but its effectiveness varies in Gram-negative organisms.

This investigation has shown that growth of P. fluorescens was inhibited by actinomycin D when L-asparagine or succinate was used as the sole source of carbon and energy. Growth was not inhibited by the concentrations of actinomycin D used in the experiment when the cells were growing in nutrient broth. This could be due to the many nutrients present, some of which are probably utilized by constitutive enzymes that are long-lived or by the more stable m-RNA enzymes.

The results show that an increase in the inhibitor concentration produces an increase in the extent of inhibition. Growth studies revealed an increase in the absorbancy readings of actinomycin D-treated cells during the first 2-4 hours similar to the control system. While microscopic examination showed an increase in cell size, viable cell counts indicated that an increase in cell numbers was not occurring during this time. Therefore, actinomycin D was inhibiting the organism but the inhibition was not readily apparent using absorbancy measurements.

Actinomycin D did not produce a pronounced growth inhibition when glucose was used as the sole source of carbon and energy. This might possibly be due to the many metabolic pathways (long-lived enzymes) in which glucose could enter without encountering enzymes sensitive to actinomycin D. End-products of glucose metabolism may also be responsible for inactivation of actinomycin D. However, glucose, fructose, and benzoic acid failed to reverse the actinomycin D inhibition. Honig and Rabinovitz (1966) found that the inhibition of protein synthesis by actinomycin D in sarcoma 37 ascites cells could be relieved by the addition of glucose. These authors speculated that actinomycin D inhibits protein synthesis by interfering with a product of oxidative metabolism and this effect can be overcome under conditions of glycolysis.

Actinomycin D did not inhibit growth of the cells when protocatechuic acid was used as the sole source of carbon and energy. Protocatechuic acid induces the synthesis of protocatechuate oxygenase; therefore, this offers an example of an inducible enzyme that does not appear to be sensitive to actinomycin D. Results from manometric and growth experiments have confirmed the apparent resistance of protocatechuate oxygenase synthesis to actinomycin D inhibition.

Protocatechuic acid, in extremely low concentrations, could reverse the actinomycin D inhibition of growth when it was added after the inhibition became apparent but before the viable cell count had sharply declined. When protocatechuic acid and actinomycin D were added simultaneously with the inoculum, an inhibition did not occur. Actinomycin D inhibited the incorporation of a radioactive amino acid

into protein, but the incorporation of the amino acid was resumed, at least in part, when protocatechuic acid was added to the inhibited cells. Thus, the actinomycin D inhibition of protein synthesis was apparently reversed by the presence of protocatechuic acid.

This investigation has established that neither protocatechuate oxygenase synthesis nor growth is sensitive to actinomycin D when protocatechuic acid is present.

Guanine must be present in order for actinomycin D to bind to DNA (Goldberg et al., 1962). If guanine-cytosine bases were not present in the loci coding the m-RNA for protocatechuate oxygenase, then actinomycin D would not be able to bind to the specific loci and enzyme synthesis would occur. During the catabolism of protocatechuic acid to acetyl-CoA and succinate, succinate metabolism would be inhibited but acetyl-CoA might enter into a lipid pathway and other cell components without being inhibited by the antibiotic. This offers a plausible explanation for growth in a protocatechuate-salts medium when actinomycin D is present.

It is possible that actinomycin D binds to the regulator gene which codes for the repressor for protocatechuate oxygenase. This would mean that the regulator gene contains guanine-cytosine and other appropriately arranged bases in the immediate vicinity which would allow for binding of the antibiotic. Since actinomycin D does not inhibit protocatechuate oxygenase synthesis, the unaffected structural genes may contain guanine-cytosine bases but due to their arrangement in the genome or to the presence and sequences of other bases in the immediate vicinity may preclude binding of actinomycin

D. If actinomycin D acts on the repressor regulating locus, then the repressor may not be formed and the operator gene would permit the synthesis of protocatechuate oxygenase. Secondly, actinomycin D could possibly combine with the repressor (instead of with the regulator gene) which would also permit protocatechuate oxygenase synthesis.

Another possible explanation would be that actinomycin D does not readily penetrate the cell permeability barrier. For example, protocatechuic acid and actinomycin D might compete for a common uptake mechanism of the cell or the inhibitor simply may not readily penetrate the permeability barrier. However, this does not appear to be the case since (1) the incorporation of an amino acid into protein is inhibited in the presence of actinomycin D, (2) protocatechuic acid will reverse the inhibition by actinomycin D, and (3) the uptake of actinomycin D by the cells occurs in the presence of protocatechuic acid. These findings suggest that permeability is not a factor involved in the inhibition. Reversal of a pronounced inhibition would suggest the possibility of an inactivation of the inhibitor by some cell component. Leive (1965a) found that the inhibition of B-glactosidase by actinomycin D ceased after approximately 50 minutes.

It is possible that actinomycin D and protocatechuic acid form a complex inside the cell or protocatechuic acid may otherwise function to inactivate the antibiotic. If this is true, then it would indicate that actinomycin D may form a reversible complex with DNA and when the inhibitor detaches from the DNA it may then complex with

or otherwise be inactivated by protocatechuic acid. This would also offer an explanation for the reversal of actinomycin D inhibition. A possibility exists, though it is not very likely, that protocate-chuate oxygenase could be coded from a long-lived RNA, thus being resistant to actinomycin D inhibition.

Further investigation is necessary before the exact mechanism or mechanisms are known for the resistance of protocatechuate oxygenase to actinomycin D and the cause for reversal of actinomycin D inhibition by protocatechuic acid.

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