THE EFFECT OF CARBON SOURCE ON THE SENSITIVITY OF *PSEUDOMONAS FLUORESCENS* TO ACTINOMYCIN D

By

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Bachelor of Science

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1969

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
May, 1973

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ACKNOWLEDGMENTS

I wish to express sincere appreciation to Dr. Norman N. Durham for his insight, patient guidance and counsel, and his friendship throughout the course of this investigation.

Appreciation is also extended to Drs. Lynn L. Gee, Edward A. Grula, and Franklin R. Leach for their service as committee members, and to Dr. Billy Hudson for his invaluable technical assistance.

Financial assistance in the form of a National Science Foundation graduate traineeship and an American Cancer Institute grant is acknowledged and appreciated.

Special gratitude is extended to my husband, Joe, for his infinite understanding and love.

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CHAPTER I

INTRODUCTION

The pseudomonads comprise a unique group of microorganisms demonstrating a high degree of adaptability to their environment. They possess great versatility in their metabolic system and can utilize a wide variety of unusual substrates as carbon and nitrogen sources. Because they can rapidly mutate under harsh conditions and are known to carry transducing viruses, they have been singularly effective as a biological group.

Members of the genus Pseudomonas are short, gram negative rods. They grow well on unusual substrates such as protocatechuic acid and p-nitrobenzene and a variety of the more common sugars and acids. They require only an organic carbon source and minimal salts for growth, and growth temperatures range from 0 C up to 45 C. They possess an inherent resistance to most antibiotics, and this is thought to be principally a function of permeability.

Permeability may be defined as the capacity of a semipermeable barrier to allow passage of materials across it. The barrier in the case of microorganisms would be the cell envelope. The phenomenon of permeability may be categorized into two basic types: active transport and simple diffusion. It is not logical to conclude that a cell would possess a transport system for an antibiotic, so it must be assumed that the passage of such a molecule would be by diffusion. The cell

envelope is comprised of several layers through which a molecule must pass to enter the cell. They are the layer composed of lipopolysaccharide and protein, the murein layer or cell wall, and the cell membrane, with the membrane being the major effector of active entry into the cell (Kellenberger and Ryter, 1958; Murray, Steed, and Elson, 1965; Ogura, 1963; Weidel, Frank, and Martin, 1960).

Of the three, the layer which has drawn considerable attention of late is the outer, lipopolysaccharide-containing layer. Lipopolysaccharide, which is specific to gram negative cells, determines the O-antigen specificity, acts as a receptor site for various bacteriophages, and is responsible for endotoxic activity, as well as being strongly implicated in the permeability properties of the cell (Wright and Kanegasaki, 1971).

Based on electron microscopy and biochemical observations, a model has been proposed in which lipopolysaccharide underlies a discontinuous layer of lipoprotein in a mosaic-type structure (Wardlaw, 1964; Bayer and Anderson, 1965). A major portion of the molecule must be exposed as it is responsible for the O-somatic antigen determinants and for receptor sites for bacteriophage. The nature of attachment of lipopolysaccharide to the cell is uncertain. The inhibition of protein synthesis, either by withholding a required amino acid (Knox, Cullen, and Work, 1967) or by chloramphenicol treatment (Rothfield and Pearlman-Kothencz, 1969) liberates lipopolysaccharide. Leive, Shovlin, and Mergenhagen (1968) have shown that treatment with ethylenediaminetetraacetic acid (EDTA) releases up to 50 percent of the lipopolysaccharide, along with phospholipid and protein, from the surface of Escherichia coli. There is a limit to the amount of lipopolysaccharide

that can be released by EDTA, and the releasable and non-releasable fractions are brought rapidly into equilibrium (Levy and Leive, 1968). This suggests that there are two forms of lipopolysaccharide in the cell wall.

The current data suggest that lipopolysaccharide is present in the outer layer of the cell as a complex with phospholipid and protein. The globular proteins, which are covalently linked to the peptidoglycan, may be the attachment site for this complex (Braun, Rehn, and Wolff, 1970; Fishman and Weinbaum, 1967; Weidel, Frank, and Martin, 1960).

The molecular structure of the lipopolysaccharide molecule may be considered in three separate parts: the O-polysaccharide, the R core, and lipid A. These three units are linked covalently to form a single macromolecule. The structure of lipid A and the R core seem to remain constant for a given genus (Luderitz, Jann, and Wheat, 1968; Luderitz, Staub, and Westphal, 1966). Even in different genera, there does not seem to be a significant variation in these two components. In contrast, the O-polysaccharide shows a high degree of variation in structure and composition in different organisms. The synthesis of O-polysaccharide and the R core are under separate genetic control and are joined in covalent linkage only after they are completely formed (Wright and Kanegasaki, 1971).

The basic structural unit of lipid A is a diglucosamine unit (Burton and Carter, 1964) that is substituted with fatty acids and phosphate groups. Glucosamine molecules are linked glycosidically and carry both N- and O-acyl substituents. The free amino and hydroxyl groups are substituted with fatty acids. The major acid is

β-hydroxymyristic acid (Lunderitz, Jann, and Wheat, 1968).

The sugar components of the lipopolysaccharide are thought to be linked to lipid A through ketodeoxyoctanate (Gmeiner, Luderitz, and Westphal, 1969), which is the innermost component of the R core. Other components of the R core are the amino sugar N-acetylglucosamine; the hexoses, glucose and galactose; a seven carbon sugar, L-glycero-D-mannoheptose; phosphate; and O-phosphorylethanolamine.

Although the O-polysaccharide is variable, the common components include a number of hexoses, N-acetylated hexosamines, deoxyhexoses, and dideoxyhexoses. Many also contain ester-linked acetyl groups. All O-polysaccharides examined so far consist of regularly repeating sequences of their constituent sugars, and these may be either linear arrangements or structures with one or more side branches.

No detailed structure for the lipopolysaccharide of Pseudomonas fluorescens has been elucidated. The components which have been identified are 2-keto-3-deoxyoctanate (KDO), galactosamine, glucosamine, galactose, glucose, and rhamnose, along with phosphatidylethanolamine (Wilkinson, 1970). Two unusual sugars have also been reported in the envelope of P. fluorescens; these are quinovosamine (2-amino-2,6-dideoxyglucose) and 3-amino-3,6-dideoxyglucose (Wilkinson and Carby, 1971). Rather than the hydroxymyristic acid found in the lipid A of the enteric bacteria, the pseudomonads contain hydroxylauric and hydroxydecanoic acids (Hancock, Humphreys, and Meadow, 1970).

The physical properties of lipopolysaccharide in solution are similar to those of phospholipids; however, the large polysaccharide region of the molecule makes it more hydrophilic. In the outer membrane of the cell the molecules are thought to form micellar structures

with protein and phospholipid.

Although there is a considerable amount of information regarding structure and composition, there is little known about the functional role of lipopolysaccharide. The most clearly defined functions are the interactions with the host animal and with bacteriophages, but the cellular functions are also important. Heptoseless mutants of E. coli and Salmonella are sensitive to heat, detergents, and lysozyme. Antibiotic sensitivity of these organisms may also be related to lipopolysaccharide structure. For example, mutants of E. coli K12 that lack heptose-linked phosphodiester bridges show increased sensitivity to several antibiotics, including actinomycin D (Tamaki, Sato, and Matsuhashi, 1971).

The release of lipopolysaccharide from the wall of E. coli by treatment with EDTA increases the sensitivity of the organism to actinomycin D (Leive, 1965), indicating that a loss of lipopolysaccharide may cause increased permeability. Roy and Mitra (1970) reported that the release of lipopolysaccharide which accompanied infection of E. coli K12 with phage M13 increased permeability to actinomycin D. Winshell and Neu (1970) postulated that release of lipopolysaccharide is necessary for the release of periplasmic enzymes in the Enterobacteriaceae. A group of E. coli mutants that fail to show permeability increase upon treatment with EDTA have been isolated by Voll and Leive (1970). The mutants released only 20 to 40 percent less lipopolysaccharide than the wild-type cells. However, when the lipopolysaccharide was fractionated by density gradient centrifugation it was found that the mutant released only one-third as much of a slower moving component. The suggestion was made that it may be this slower moving

component which is responsible for the permeability increase; it differs only quantitatively from the faster moving component.

From the information now available, it seems logical that intact lipopolysaccharide plays a protective role, not only stabilizing the outer layer of the cell, but acting as a permeability barrier as well.

It was noted earlier that the O-polysaccharide was variable among genera, but to date, there is only a paucity of information regarding variation within a single species in response to environmental changes. An increased production of somatic antigens by Pasteurella pestis is noted when the organism is grown in an enriched medium (Veinblat and Bakhrakh, 1970). Also, there is a direct relationship between growth rate and KDO level in Aerobacter aerogenes (Ellwood and Tempest, 1967).

There are, however, numerous reports in the literature regarding changes in cellular components in response to different media or environments, and it would not be unreasonable to assume that a similar response could occur in the lipopolysaccharide. A cell grown in one medium can differ greatly from the genetically identical cell grown in a different medium; the protein, ribonucleic acid, deoxyribonucleic acid, lipid, and carbohydrate content, as well as the size, may differ greatly (Neidhart, 1963).

Differences in cell size were probably among the first noted changes due to environment and growth conditions. Growth rate is thought to be the primary effector in determination of cell size. It was observed that during balanced exponential growth in a given medium, cell size is an exponential function of the growth rate (Schaechter, Maaloe, and Kjeldgaard, 1958). Grula (1970) reported that cell size may also vary with carbon source, and the variance was not directly

related to growth rate as in the case of growth in one carbon source with variation in concentration of a single nutrient. It is interesting to note that both studies were conducted with organisms that have lipopolysaccharide in their envelope.

The cell wall differs quantitatively under various growth conditions. When Bacillus subtilis was examined at intervals in the growth cycle, the concentration of galactosamine in the wall varied, and reached a maximum level when the organism was competent for transformation. The galactosamine content also decreased in media not conducive to competence (Young, 1965). The suggestion is made that a change in wall structure might be the result of the increase in galactosamine. Johnson and Campbell (1972) reported that the amount of O-acetyl substitution and the complexity of cross-linked structure in the wall of Micrococcus sodonensis changed when a synthetic medium was used for growth as opposed to a complex medium. A decrease in both characteristics resulted from growth in the synthetic medium, and enhanced susceptibility to lysozyme was the physical result. In a study conducted with P. aeruginosa, Wilkinson (1970) found that the envelope of cells grown on nutrient agar had a lower phosphate and a higher carbohydrate content than cells grown on tryptone glucose extract agar, and cells grown on the latter exhibited the greatest sensitivity to EDTA.

The membrane structure is not clearly understood, but there have been some changes noted in response to the environment. Marr and Ingraham (1962) analyzed the fatty acids of E. coli cells grown at different temperatures and in different media. They found that as the temperature of incubation was lowered, the proportion of unsaturated fatty acids increased. The variation occurred in media of different

composition, as well. When cells grown in a chemostat with limiting nitrogen were examined, they were found to have a higher content of saturated fatty acids than cells grown in batch cultures. When glucose was the limiting factor, the cells had a higher concentration of unsaturated fatty acids. Both lipid groups were attributed to the membrane. Kabach (1970) reported a similar finding in membranes isolated from E. coli. Membranes from cells grown in glucose contained less diphosphatidylglycerol than membranes isolated from cells grown in succinate. The change was believed to contribute to the difference in permeability of the two membranes to the sugar substrates.

Many bacteria accumulate significant amounts of polysaccharide material within and around the cell under certain environmental conditions. This accumulation may occur for various reasons, but usually it is the result of the carbon source remaining in excess after growth of the culture has been halted by the depletion of some other nutrient in the medium. Accumulation of polysaccharides is evidenced as storage granules in the case of E. coli B growing in a chemostat in glucose medium with nitrogen as the limiting factor (Holme, 1957). Klebsiella aerogenes (Wilkinson, 1958) produces a complex capsular and extracellular slime material, and the presence of the capsular material usually confers a degree of resistance to antibiotics and harsh environmental conditions.

Many of the above changes are the result of an adaptive shift in the cells' regulatory mechanisms in response to an environmental change. In most cases, the environment offers a condition of stress for the cell. The antibiotic, actinomycin D, is particularly of interest in the study of the gram negative cell's response to environment.

It is only partially effective, and high dosages are required for complete inhibition. This makes it a sensitive measure of the slight changes that may occur in the cell which make it increasingly or decreasingly subject to inhibition by the antibiotic.

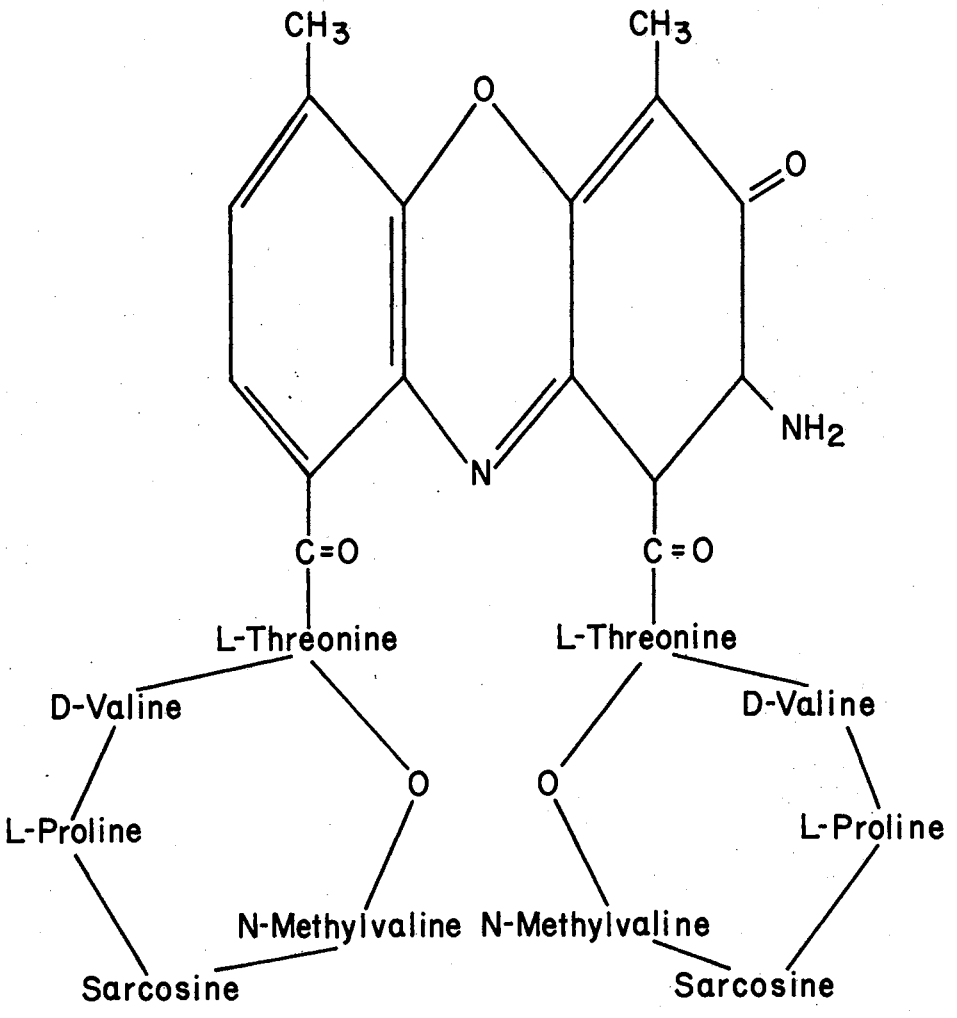
Actinomycin D was isolated and identified by Waksman and Woodruff (1940) from a soil organism isolated by enrichment techniques. The organism isolated was a species of Actinomyces. Waksman (1954) later characterized the compound as a brick red, crystalline substance that melted at approximately 250 C and absorbed light strongly at 230, 250, and 450 nm.

Manaker et al. (1955) isolated the crystalline form of actinomycin D either by solvent extraction or by charcoal adsorption, and the molecular formula was deduced as $C_{60}H_{76}O_{15}N_{12} \cdot 3\ H_2O$. Structural studies revealed the actinomycin molecule consisted of a chromophoric quinoid ring, 2-aminophenoxazine-3-one, which is linked to two peptides, each containing the amino acids sarcosine, D-valine, L-proline, L-threonine and N-methylvaline (Fig. 1).

A pronounced growth inhibition resulted when either gram negative or gram positive bacteria were grown in the presence of actinomycin. It was also observed that the antibiotic was bacteriostatic for pathogenic organisms in vitro (Waksman and Woodruff, 1940). Waksman and Robinson (1942) noted that a dose of 1.0 mg or more of actinomycin per kilogram of body weight was lethal in laboratory animals. The antibiotic was swiftly removed from the blood stream and deposited in all the organs of the body.

Anticancer activity of the antibiotic in laboratory animals was reported by Pugh, Katz, and Waksman (1956). Actinomycin was tested

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


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

against Wilmsis tumor of the kidney and was used to successfully treat patients for three years (Farber, 1961).

Since actinomycin was effective in inhibiting both bacterial growth and tumor growth, a molecular mechanism was sought. In the course of investigations, it was found that the antibiotic interacted specifically with deoxyribonucleic acid (DNA) (Kawamata and Imanishi, 1960). Results from starch and zone electrophoresis showed that actinomycin distribution in the cell corresponded to the DNA fraction. The inhibition of bacterial growth was abolished in the presence of calf thymus DNA, and the proposal was made that the interaction of actinomycin with the DNA played an important role in the mechanism of inhibition.

From the results of studies done with calf thymus and E. coli DNA, Cavalieri and Nemchin (1964) suggested that there were two types of binding sites for actinomycin, one having approximately 50 times greater affinity than the other. Binding of actinomycin to the sites with the greatest affinity resulted in "lateral" dimerization of the DNA; removal of the drug resulted in return of the DNA to the original molecular weight. The conclusion was reached that actinomycin inhibited ribonucleic acid polymerase by competing with it for binding sites on the DNA molecule.

Reich (1964) noted that DNA synthesis continued at normal rates following substantial inhibition of RNA formation and postulated that the enzymes catalyzing DNA and DNA-dependent RNA synthesis differed in their steric relationship to the DNA molecule. He proposed that each nucleic acid polymer normally "sees" the DNA from only one groove, and that RNA polymerase was displaced from the DNA by actinomycin. If

actinomycin is assumed to lie in the minor groove, then this must also be the site for RNA transcription. Since the DNA polymerase functioned on surfaces of the DNA molecule without obstruction by actinomycin, it was postulated that this enzyme proceded in the major groove (Goldberg and Reich, 1964).

Further studies of the molecular interaction of actinomycin with DNA led to the discovery that guanine residues were apparently indispensable for binding to occur (Goldberg, Rabinovitz, and Reich, 1962). When deoxyadenosine deoxythymidine primed RNA synthesis was tested, it was completely resistant to inhibition by the antibiotic, while deoxyguanosine deoxycytidine primed RNA synthesis was inhibited. However, because the latter inhibition was less than that observed with native DNA isolated from M. lysodeikticus, these researchers concluded that optimal binding occurred when native helical DNA was present. Polyuridine and polyadenine directed RNA synthesis was also found to be partially sensitive to actinomycin, which suggested that these regions might be the source of the low affinity binding sites previously observed. Gellert et al. (1965) suggested that the binding site of actinomycin either involved more than one base pair, one of which was guanine-cytosine, or else only guanine-cytosine pairs, with steric hindrance between antibiotic molecules preventing saturation of all the binding sites.

Two major models have emerged from the investigations for a mechanism of binding of actinomycin to DNA. The first of these was proposed by Hamilton, Fuller, and Reich (1963). They postulated that the actinomycin chromophore was hydrogen bonded to the outside of the DNA helix. Stabilization for the complex was provided by a hydrogen bond

between the actinomycin quinone oxygen and the 2-amino group of guanine, as well as hydrogen bonds from the actinomycin amino group to the nitrogen (3) of guanine and to the deoxyribose ring oxygen. The peptide lactones were considered to provide additional hydrogen bonding with the phosphodiester oxygens. The major justifications for this proposed mechanism of binding were the apparent specificity of actinomycin for deoxyguanosine residues, the importance of an unsubstituted amino group, and the inability to detect intercalation with x-ray diffraction studies.

The second major model was proposed by Muller and Crothers (1968). They suggested that the actinomycin chromophore was intercalated into the DNA chain with the peptide lactones projecting into the minor groove. They based their proposal on the marked decrease in the rate of complex formation with actinomycin analogs having bulky substitutions in the seven position of the chromophore, and a careful examination of the hydro-dynamic properties of the DNA of various molecular weights when in the presence of actinomycin.

There has yet to be a definite resolution of the two hypotheses. Inagaki and Kageyama (1970) used the elution patterns of actinomycin from a DNA-cellulose column to identify two types of binding. Actinomycin was eluted in a single peak by 2 M NaCl, which indicated the probability of the involvement of electrostatic bonding. Elution also occurred with urea between 0.5 and 5.0 M. The latter indicates that hydrogen bonding was involved and supports the model proposed by Hamilton, Fuller, and Reich (1963).

Hyman and Davidson (1970) studied the kinetics of <u>in vitro</u> inhibition of transcription by actinomycin and concluded that the main effect

of the antibiotic was to inhibit the rate of chain formation, although a small effect on termination was also noted. Actinomycin inhibited the propagation of RNA synthesis as evidenced by the rate terms of both guanosine triphosphate and cytidine triphosphate. Since only the two terms were altered, it was suggested that the actinomycin must be acting more strongly with the 'guanosine-cytidine pairs than with the other base pairs. Although this does not eliminate intercalation, it does support the conclusion that actinomycin would only intercalate where a guanosine-cytosine pair occurred, and would interact more strongly with this base pair than the other base pair in the intercalated structure. In a later article, Hyman and Davidson (1971) suggest that some combination of steric restrictions and sequence restrictions are operative in the formation of the actinomycin-DNA complex.

Nuclear magnetic resonance spectrometry studies suggested formation of a complex between actinomycin D and 5'-deoxyguanylic acid (Arison and Hoogsteen, 1970). This would require that the base be positioned above the chromophore group and be oriented so that the planes of the two systems are parallel. The nuclear magnetic resonance data clearly favor a base-stacking or intercalation model for the complex formed by actinomycin and DNA.

One of the most extensive studies was conducted by Wells and Larson (1970). By observing complex formation between actinomycin D and both native and model DNA polymers by a number of techniques, they reached several conclusions. Deoxyguanylic acid was not sufficient, by itself, for bonding; polydeoxyinosine bound 25 percent as much actinomycin as did DNA containing 50 percent guanosine-cytosine, and the equilibrium constant observed for polydeoxyinosine and actinomycin was

as large as that observed for DNA containing 50 percent guanosine-cytosine. Poly d(A-T-C)·poly d(G-A-T), a model polymer which contains 33 percent guanosine-cytosine, binds little or no actinomycin. Rather than a specificity for a single base, there is a marked preference for a specific nucleotide sequence. These authors suggested that the essential factor for complex formation is the proper configuration of DNA.

Despite the controversy over the absolute mechanism of complex formation, most agree that the primary level of actinomycin inhibition involves interaction with the cell's DNA. Thus, it is essential that the antibiotic reach the genome of the cell, which requires passage of the molecule through the cell's permeability barrier. Consequently, one of the major mechanisms of resistance in bacterial and tissue culture cells is effected through a change in the permeability.

Tamaki, Sato, and Matsuhashi (1970) investigated mutants of E. coli that showed increased sensitivity to actinomycin and found the cells possessed an incomplete lipopolysaccharide. The macromolecular structure lacked phosphate diester bridges, and consequently the outer core polysaccharides. As a result, the cell surface was more easily penetrated by the antibiotic. Wild type E. coli cells can be treated with EDTA to increase their sensitivity to actinomycin. Leive (1965) indicated that a permeability barrier must be broken down to allow the antibiotic to enter the cells in great enough quantities to be effective, and noted that lipopolysaccharide was released from the cell surface by EDTA.

Totten and Howe (1971) found that the inhibition of DNA-directed RNA synthesis in Neurospora crassa by actinomycin was temperature

dependent, the greatest amount of inhibition occurring at the higher temperatures. They proposed that increased temperatures gave rise to greater permeability. An antibiotic-resistant strain of P. aeruginosa isolated by Anderes, Sandine, and Elliker (1971) showed an increase in the lipid content of the cell envelope. Since the lipid content was inversely related to the permeability, an increase in lipids would accompany a decrease in permeability.

Chinese hamster cells resistant to actinomycin showed a marked potentiation of the antibiotic effect when treated with Tween 80 (Riehm and Biedler, 1972). Treatment with Tween 80 increased uptake of ^{3}H-actinomycin D, and the uptake was directly related to the concentration of the detergent. This evidence strongly supports the concept that resistance may be related to an acquired alteration manifested in the cell periphery which causes a decrease in permeability. Kessel and Bosmann (1970) reported that mouse L cells which were resistant to actinomycin D had an impaired capacity for uptake of the antibiotic in vivo. When the cells were examined for molecular differences, reduced levels of glycosidases were found (Bosmann and Kessel, 1970), and markedly different levels of carbohydrate were bound to the cell surfaces. The change in the membrane layer was thought to be responsible for the decreased drug permeability.

Keudell (1967) observed that P. fluorescens shows a reduced degree of sensitivity to actinomycin D when grown in glucose minimal medium when compared to the same organism grown in succinate minimal medium. The purpose of this research was to characterize the degree of difference in sensitivity to actinomycin D which is produced when P. fluorescens is grown on the two carbon sources, succinate and glucose;

and to elucidate any macromolecular changes which might be contributing to the observed differences in antibiotic sensitivity.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The microorganism used through the course of these studies was a strain of Pseudomonas which has been tentatively identified as Pseudomonas fluorescens (Montgomery, 1966). Cultures were maintained on 0.2 percent succinate-agar slants and stored at 4 C.

Media

The synthetic minimal salts medium used in this study had the following composition: 0.2 percent NaCl, 0.2 percent NH_4Cl, 0.32 percent KH_2PO_4, and 0.42 percent K_2HPO_4. Carbon sources were used in concentrations of 1.7×10^{-2} M or 2.78×10^{-2} M. Succinate was added directly to the minimal salts base; glucose was autoclaved separately and added aseptically to the sterile minimal salts base. The pH was adjusted to 7.0 with KOH prior to sterilization unless otherwise indicated. Sterilization was achieved by autoclaving at 121 C with 15 pounds pressure per square inch for 15 minutes. After the medium had cooled to room temperature, 0.1 ml of a sterile mineral salts solution was added for 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 concentration of 2.0 percent when a solid medium was desired. Throughout this study, the term minimal salts buffer was used to designate the basal salts medium lacking a carbon source.

Growth of Cells

Sterile tubes (18 x 150 mm) containing 6.0 ml of medium were inoculated from the appropriate stock culture and incubated 10-12 hours at 37 C on a reciprocal shaker (100 excursions per minute). These cultures were then used as inocula for growth studies. Growth was determined by following the increase in absorbency of the cultures at 540 nm on a Coleman Junior II spectrophotometer. Growth assays were performed either in test tubes (18 mm light path) containing a total volume of 6.0 ml or in 250 ml side-arm flasks containing 30.0 ml total volume. When a larger quantity of cells was needed for assay, the cells were grown in Fernbach flasks containing 500 ml of medium.

Antibiotics

Actinomycin D (Merck, Sharp, and Dohme Research Laboratory) was dissolved in sterile glass-distilled water to give a concentration of 300 μg/ml and stored at 4 C in the dark. D-Chloramphenicol (Parke, Davis, and Company) was made up to a concentration of 600 μg/ml in sterile glass-distilled water and stored at 4 C.

Uptake of Radioactive Substrates

The cells were grown in test tubes of minimal salts medium containing the appropriate carbon source (glucose or succinate). One ml

of the suspension was used to inoculate 26.0 ml of medium in a 250 ml side-arm flask, and the cells were incubated for 2-3 hours. At that time, either actinomycin D (final concentration, 30 μg/ml) or an equal volume of sterile minimal salts buffer was added to each flask, and the cells incubated for two additional hours. The cells were harvested and suspended in minimal salts buffer to $A_{540\ nm} = 0.3$. The cell suspensions were equilibrated to 37 C in a shaking water bath, and 5.0 ml pipetted into tubes containing 1.0 ml of the following: actinomycin D (30 μg/ml, final concentration), or an equal volume of minimal salts buffer; unlabeled carrier (0.2 mg, final concentration); and labeled substrate (0.2 μc/ml final concentration). Samples (0.5 ml) were removed at the indicated time intervals, immediately filtered on Millipore membrane filters (10 mm diameter, 0.45 micron pore size) and washed with 2.0 ml of minimal salts buffer. Membrane filters were subsequently placed in counting vials and 10 ml of Aquasol counting fluid (New England Nuclear) was added. Radioactivity was determined using a Nuclear Chicago liquid scintillation spectrometer (Model no. 722). The counting efficiency under these conditions was 40 percent.

Incorporation of ^{14}C-Uracil

The incorporation of ^{14}C-uracil (specific activity 40.6 mc/mM) was measured using cells that had been grown in 100 ml of medium for 12 hours. The cells were harvested and suspended in 0.01 M potassium phosphate buffer (pH 7.0) so that addition of 0.5 ml of the cell suspension to 19.5 ml of uptake medium gave an $A_{540\ nm} = 0.5$. The uptake medium contained uracil (0.05 mg per ml), uracil-2-^{14}C (0.05 μc/ml) and either actinomycin D (30 μg/ml, final concentration) or an equal amount

of sterile glass distilled water. Samples (0.5 ml) were removed at the specified intervals, placed in 2.0 ml of ice-cold 5.0 percent trichloroacetic acid and allowed to stand in an ice bath 15 minutes. The suspension was filtered through Millipore filters (10 mm diameter, 0.22 micron pore size), and washed twice with 1.0 ml of cold 5.0 percent trichloroacetic acid.

The filters were placed in counting vials, dried overnight at room temperature, and 1.0 ml of 3,4-dioxane was added to each vial. 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 (POPOP) in 237 ml of 3,4-dioxane. Counting fluid (9.0 ml) was added to each vial, and the radioactivity determined using the liquid scintillation spectrometer.

Maintenance of Constant pH in Growing Cell Cultures

Cells were grown for 10 hours in the appropriate medium and 5.0 ml of the culture used to inoculate 125 ml of fresh medium containing the same carbon source and adjusted to pH 6.5, 7.0, or 7.8. The growing cultures were supplied oxygen by means of a sintered glass aereator which was placed in the mouth of the flask alongside the electrodes connected to the pH meter. Air was heated by passing it through a warm water trap before reaching the flask. A Metrohm-Herisau pH stat was used to maintain the initial pH. A solution of 5.0×10^{-1} N HCl was used to maintain the desired pH when succinate was the carbon source, and a solution of 5.0×10^{-1} N NaOH was used when glucose was the carbon source. Cells were maintained under these conditions for 12

hours, during which time the addition of acid or alkaline solution was monitored automatically by a Metrohm-Herisau recording unit attached to the pH stat. At the end of 12 hours, the cells were harvested, suspended in saline, and used to determine whether the pH at which the cells were grown influenced the amount of actinomycin D bound/taken up by the cells.

Spectrophotometric Studies

The ultraviolet adsorption spectra of an aqueous solution of actinomycin D (1.03×10^{-5} M), glucose (2.78×10^{-2} M), and succinate (2.78×10^{-2} M) were determined using a Cary 14 recording spectrophotometer at room temperature in quartz cuvettes. The adsorption spectrum of each compound and a mixture of each carbon source with the antibiotic was determined between the wave lengths of 185-500 nm.

Manometric Studies

The effect of actinomycin D on cell respiration was measured by following oxygen uptake in a Warburg apparatus at 37 C with air as the gas phase. Cells were grown 2 hours in 27.0 ml of minimal salts medium with the appropriate carbon source. Either actinomycin D (30 μg/ml, final concentration) or an equal volume of sterile potassium phosphate buffer (0.01 M, pH 7.0), was added, and growth continued for 2 additional hours. Cells were harvested, washed once with minimal salts buffer, and suspended in minimal salts buffer to $A_{540\ nm} = 0.8$. Substrates were dissolved in glass distilled water to a concentration of 0.05 M or 0.025 M; actinomycin D was made up to a concentration of 405 μg/ml, and D-chloramphenicol to a concentration of 810 μg/ml. The

appropriate concentration of mineral salts solution was added to the minimal salts buffer in which the cells were suspended.

The Warburg flasks contained a total volume of 2.7 ml of liquid; 0.2 ml of a 20 percent solution of KOH was used in the center well to absorb any CO_2 released during the course of the experiment. All readings were taken at 10 minute intervals, and the basal metabolic rate (endogenous) was determined over the initial 30 minute time period. Induction is designated as the first 10 minute interval in which a departure from the basal metabolic rate was detected. The oxidative rate is designated as the average number of microliters of oxygen taken up during a 10 minute time period as calculated from the linear portion of a curve obtained by plotting time against the total microliters of oxygen taken up.

Treatment of Cells With Ethylenediaminetetra-acetic Acid (EDTA)

Cells were grown for 12 hours in minimal medium plus glucose or succinate, harvested, washed once in 0.12 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0, and suspended in Tris buffer $A_{540\ nm}$ = 0.5. The cells were treated with an equal volume of EDTA (2×10^{-4} M, pH 7.0; final concentration 1×10^{-4} M) for 2 minutes at 37 C. The action of EDTA was stopped by making a 1:10 dilution into fresh medium. The suspensions were used to inoculate culture tubes containing the appropriate medium and growth in the presence and absence of actinomycin C followed spectrophotometrically. Control cells were treated with Tris buffer. This procedure is based on a similar study conducted by Leive (1968).

Cold Osmotic Shock of Cells

The procedure reported by Heppel (1967) was used in this study. Cell pellets were standardized by suspending the cells in saline to a given cell density and centrifuged. Ten ml of a shock solution containing 34.23 g of sucrose, 24.22 mg of Tris, and 5.8 mg of EDTA (7.8×10^{-5} M) per 200 ml distilled water was added to the pellets, and the cell suspension placed on a wrist-action shaker for 15 minutes at room temperature. The cell suspensions were centrifuged, 10 ml of ice-cold $MgCl_2$ (5×10^{-4} M) was added to the pellets, and the tubes shaken vigorously. The suspensions were immediately centrifuged and the pellets retained for analysis of actinomycin D binding capacity and lipopolysaccharide content.

Phospholipid Extraction and Qualitative Analysis

The extraction procedure was previously described by Folch, Lees, and Sloane (1957). The cells were grown in glucose or succinate minimal salts medium, harvested, suspended in 100 ml of saline to $A_{540} = 0.4$, and centrifuged. The pellet was treated with 20 ml of methanol at 55 C for 30 minutes under a nitrogen atmosphere. Chloroform (40 ml) was added, the nitrogen atmosphere replaced, and the suspension remained at room temperature for 24 hours. The suspension was filtered to remove the cells, washed twice with equivolumes of 2 M KCl and once with an equivolume of glass-distilled water, concentrated under rotary evaporation to a volume of 2.0 ml, and stored under nitrogen at 4 C.

Microliter volumes of the chloroform solution were spotted on 5 x 20 cm silica gel G chromatography plates (Quantum Industries). Solvents used for development were as follows: (1) chloroform:methanol:HOH (65:25:5); and (2) chloroform:methanol:NH_4OH (75:25:2). Detection of lipids was by iodine vapor chamber or rhodamine G spray. The presence of phosphorous was determined by the phosphomolybdate spray reagent described by Hanes and Isherwood (1949). Ninhydrin spray reagent was used for the detection of amines.

Known standards used for identification of the extracted phospholipids were made up in chloroform and maintained under a nitrogen atmosphere. These included phosphatidylethanolamine (Pierce Chemical Co.), and phosphatidylserine and L-α-lecithin (both of which were graciously supplied by Dr. Kermit Carraway).

Phospholipid Quantitation

To quantitate the phospholipid present, cells were grown to A_{540nm} = 0.5 in 100 ml of succinate or glucose minimal salts medium, and 25 μc of ^{14}C-acetate (New England Nuclear, specific activity 0.25 mc per 0.373 mg) added to the growing culture. The cells were incubated until logarithmic phase was reached, harvested, washed once with minimal salts buffer, and suspended in 100 ml of minimal salts buffer to A_{540nm} = 0.8. The suspension was centrifuged and the pellet subjected to the Folch et al. (1957) extraction as described previously.

The extracted lipid material was spotted on 5 x 20 silica gel G plates and chromatographed in chloroform:methanol:HOH (65:25:5). Spots were developed in an iodine vapor chamber or with rhodamine G. After the lipids were located, the gel was scraped from the plates into

scintillation vials containing 10.0 ml of Aquasol counting solution, and radioactivity measured using the liquid scintillation spectrometer.

Binding of ^{3}H-Actinomycin D

To examine the actinomycin D binding properties of cells grown under various nutritional and environmental conditions, the following procedure was developed. Cells were suspended to the desired absorbency in saline, and 5.0 ml of the cell suspension added to 0.5 ml of ^{3}H-actinomycin D (Schwarz Bioresearch, specific activity 8.4 c/mM; 5.0 μc/ml) in thick walled centrifuge tubes. The cells were kept at room temperature for 15 minutes and centrifuged at 4 C at 12,000 rpm in a Sorval centrifuge, Model RC-2. Pellets were washed twice with physiological saline and digested overnight in 0.25 ml of NCS reagent (Nuclear Chicago) at room temperature. The digested material was placed in counting vials and the tubes rinsed with 10.0 ml of counting fluid (2.0 g of PPO, 25 mg of POPOP, 237 ml 3,4-dioxane), which was added to the digested material, and the vials counted.

Envelope Isolation

After growth in a defined nutritional condition, cells were harvested, suspended to a very high density in 10.0 ml of saline, and frozen overnight as small pellets. The cells were mechanically broken by one pass in a prechilled X-PRESS. Phase-contrast microscopy revealed approximately 95-99 percent of the cells were broken. The broken cells were diluted in 100 ml of cold saline and centrifuged at 4,000 rpm at 4 C in a Sorval centrifuge (Model RC-2) to remove the large debris. The supernatant solution was centrifuged at 15,000 rpm

at 4 C for 20 minutes, and envelope material collected as the pellet. The isolated envelope material was washed repeatedly with cold saline, centrifuging alternately at 15,000 rpm and 4,000 rpm until a homogeneous pellet was obtained. If not used immediately, envelope material was frozen in a minimal volume of saline for storage.

Isolation of an Actinomycin D-Resistant Mutant

An antibiotic gradient plating technique was used to isolate an actinomycin D-resistant mutant of P. fluorescens. A gradient from 0-50 μg/ml of actinomycin D in succinate agar was prepared, streaked with the wild type organism, and incubated at 37 C until growth appeared. The cells were streaked up the gradient until growth was achieved at the highest concentration of the antibiotic. The resultant growth was subsequently transferred to succinate agar containing 50 μg/ml of actinomycin D and then to succinate agar as a stock culture. The resistant organism was designated P. fluorescens AD+.

Butanol Extraction of ^{3}H-Actinomycin D From Cell Suspensions

The procedure was an adaptation of the method reported by Greenhouse, Hynes, and Gross (1971) for separating the antibiotic from products resulting from metabolic activity. Mutant (AD+) and wild type cells (AD-) were grown in glucose or succinate minimal salts medium for 12 hours, harvested, and suspended in 5.0 ml of complete medium containing the carbon source in which the cells had grown. The antibiotic (5.0 μc of ^{3}H-actinomycin D) was added, the cells incubated at 37 C for 5 hours, and then broken by sonication using three treatments at 15

second intervals. During sonication the cells were kept in an ice bath to prevent heating. Carrier actinomycin D (60 μg) was added, and the lysates were extracted twice with 2.5 ml of n-butanol. A control containing 5.0 ml of medium and 5.0 μc of ^{3}H-actinomycin D was treated in the same manner to determine if the procedure resulted in breakdown of the antibiotic.

The butanol extracts and the remaining aqueous phases were chromatographed by descending chromatography on Whatman #1 paper using a butanol:acetic acid:HOH (60:15:25) solvent. Strips were allowed to dry and then counted on a Packard gas flow strip counter.

Isolation of Lipopolysaccharide

The cell pellets used in lipopolysaccharide isolation were standardized to the same absorbency by suspension in saline and then centrifuged. The pellets were treated with cold acetone (4 C) for 30 minutes, the cells removed by centrifugation, and washed two additional times with cold acetone. The defatted cells were suspended in 45 percent phenol and heated at 55 C for 30 minutes with occasional stirring. The cell suspensions were centrifuged at 4000 rpm for 60 minutes, thereby allowing the mixture to separate into three phases. The top, aqueous phase (containing the lipopolysaccharide) was removed by aspiration, and the entire procedure repeated two additional times. The combined aqueous phases were then dialyzed against distilled water at 4 C for 64 hours using three changes of water. The dialyzed material was lyophilized, weighed, and dispersed in glass distilled water at a concentration of 1.0 mg/ml for further analysis. The isolation procedure is similar to the one used by Westphal and Jann (1965).

Quantitative Analysis of Neutral Monosaccharides Present in Isolated Lipopolysaccharide

Lipopolysaccharide samples (approximately 2.0 mg) were hydrolyzed in 0.8 ml of 2 N H_2SO_4 in sealed tubes for 4 hours at 100 C. Mannose (2.0 ml of 0.25 M solution) was added to the hydrolysate as the internal standard. The hydrolysate was passed through a column of Dowex 50-X4 (H^+) (200-400 mesh) coupled to a column of Dowex 1-X8 (formate) (200-400 mesh) (Spiro, 1966). The effluent and wash fractions were combined and taken to dryness by lyophilization and dissolved in 10.0 ml of water. Xylose (0.5 ml of 0.25 μM solution) was added as a standard to 4.0-5.0 ml aliquots of the suspensions, and the mixtures taken to dryness. Neutral sugars were determined by the Technicon automatic sugar chromatography system as described by Lee (1969).

Quantitative Analysis of Aminosugars Present in Isolated Lipopolysaccharide

Lipopolysaccharide samples (3 mg) were hydrolyzed in 1.0 ml of 4 N HCl in sealed tubes for 6 hours at 100 C. To the hydrolysate was added 1.0 ml of 0.25 μM guanidinoalanine to serve as an internal standard, and the mixture taken to dryness on a rotary evapomix at 50 C. The aminosugars were determined on the short column of a Beckman 120C amino acid analyzer (Spackman, et al., 1958).

Quantitation of KDO

The method of Cynkin and Ashwell (1960) was used to determine the KDO present in whole cells. A 10.0 ml suspension of cells was pelleted

by centrifugation, the pellet treated with 1.0 ml of 0.1 N H_2SO_4 in a boiling water bath for 30 minutes and centrifuged. The supernatant solution was decanted and 0.25 ml assayed for KDO by oxidation with 0.25 ml periodate (0.025 N in 0.125 N H_2SO_4) at 55 C for 25 minutes. The oxidation was stopped by the addition of 0.5 ml sodium arsenite (2.0 percent in 0.5 N HCl). The products of the cleavage were reacted with 2.0 ml of a 0.3 percent solution of thiobarbituric acid at 95 C for 12 minutes, and the color read at 532 nm on a Beckman DU spectrophotometer.

The addition of 0.1 ml of a saturated aqueous solution of NaOH was made to fade the color resulting from the β-formylpyruvate-thiobarbituric acid complex, which is broken down by an alkaline environment. This leaves only the malonaldehyde-thiobarbituric acid complex produced from deoxyribose (Waravdeker and Saslaw, 1959). An estimation of the KDO content was made by correcting for the remaining color product.

Determination of Inorganic Phosphate

The sample of cell suspension (0.01-0.05 ml of a $A_{540\ nm}$=0.6 suspension) to be assayed was placed in a pyrex tube and mixed with a 10 percent solution of $Mg(NO_3)_2 \cdot 6\ H_2O$ in ethanol (Ames and Dubin, 1960). The mixture was evaporated to dryness with rapid shaking over a strong flame and heating was continued until the brown fumes had disappeared. After the tube had cooled, 1.0 N HCl (0.3 ml) was added, the tube was capped with a spherical glass condenser and heated in a boiling water bath for 15 minutes to hydrolyze any pyrophosphate formed. Ascorbic-molybdate mixture (0.7 ml) was added to each tube, heated for 60 minutes at 37 C, and the solutions were read against a

reagent blank at 820 nm. Phosphate concentration was then determined by comparison to a standard curve.

The ascorbic-molybdate mixture was made up daily using one part of 10 percent ascorbic acid to six parts of 0.42 percent ammonium molybdate·4 H_2O dissolved in 1.0 N H_2SO_4.

CHAPTER III

RESULTS AND DISCUSSION

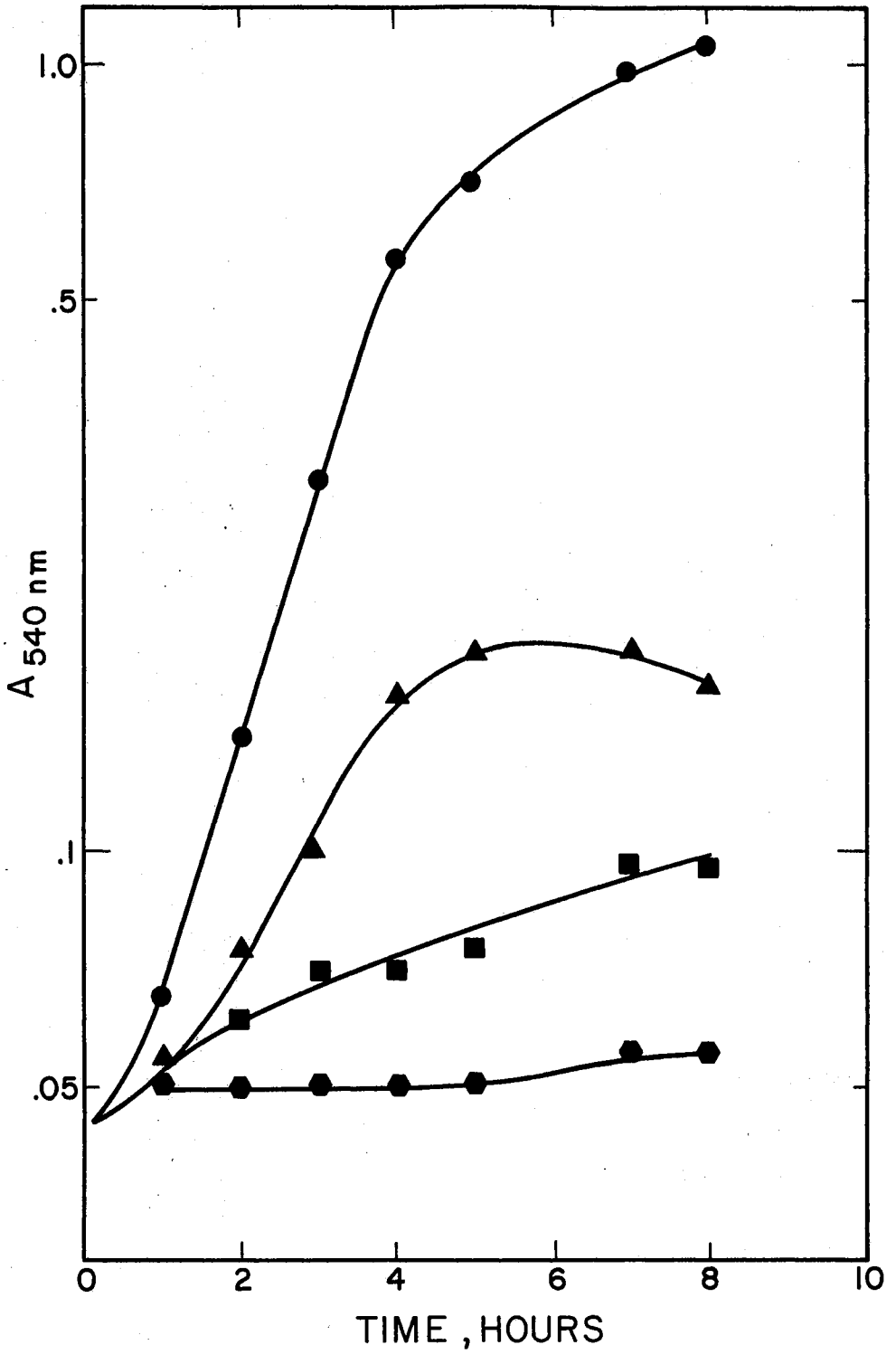
Characterization of Antibiotic Sensitivity

Effect of Actinomycin D on Growth of P. fluorescens

Experiments were conducted to determine the effect of actinomycin D on the growth of P. fluorescens in glucose and succinate minimal media. The concentrations of the antibiotic used in these studies were 0, 10, 20, and 30 μg/ml. Growth studies were conducted in 18 x 150 mm tubes containing 5.0 ml of medium plus the appropriate amount of actinomycin. Sterile buffer was added to give a final liquid volume of 6.0 ml.

The inoculum was grown in 6.0 ml of the appropriate medium for 10 to 12 hours, and 0.1 ml used to inoculate the growth tubes. Growth was followed by measuring absorbency at 540 nm at the indicated time intervals. The effect of actinomycin on growth in the succinate medium was concentration dependent with a maximum inhibition occurring at 30 μg/ml (Fig. 2). Growth for 1-2 hours in the presence of the antibiotic was necessary to establish a visible level of inhibition. The delay in the establishment of growth inhibition probably indicates the amount of time required for the antibiotic to penetrate the cell's permeability barrier, as P. fluorescens does not readily take up actinomycin D.

Figure 2. Effect of Actinomycin D on Growth of P. fluorescens in Succinate (1.7×10^{-2} M) Minimal Salts Medium ● , control; ▲ , 10 µg/ml actinomycin D; ■ , 20 µg/ml actinomycin D; ⬢ , 30 µg/ml actinomycin D.


1.0
.5
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.05
A 540 nm
0
2
4
6
8
10
TIME , HOURS

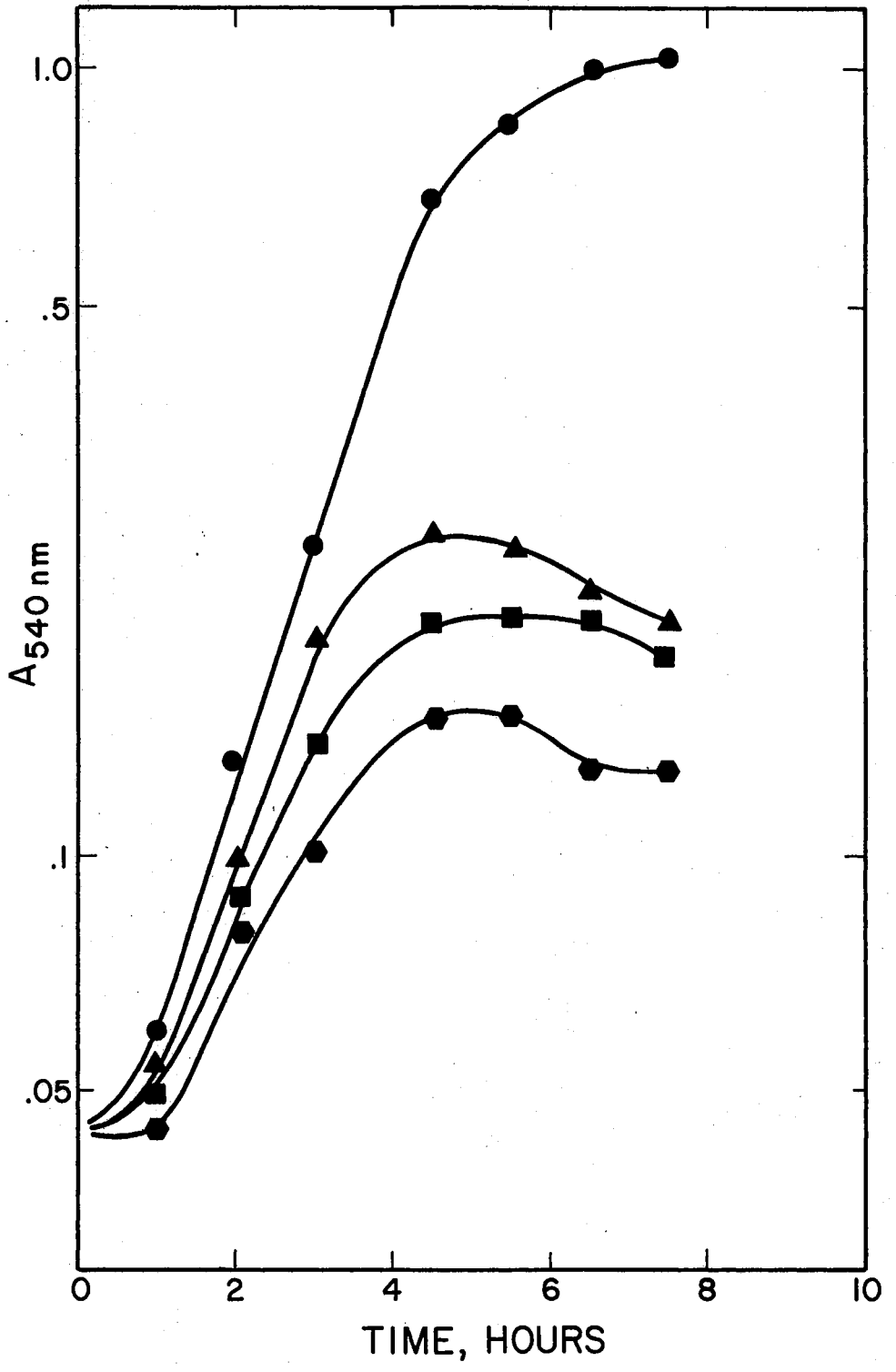
Gram positive organisms are much more sensitive to actinomycin D (Kirk, 1960), and this difference is believed to be related to the structure of the respective cell envelopes.

The actinomycin D inhibition of growth in succinate was also dependent on the concentration of the carbon source in the medium. When the molarity was increased from 1.7×10^{-2} M to 2.78×10^{-2} M (Fig. 3), the cells were slightly less sensitive to the antibiotic, and a concentration of 25-30 μg/ml actinomycin was necessary to establish an 80 percent inhibition of total growth, as compared to 10-15 μg/ml actinomycin in the lower concentration of succinate. Rate of growth was also decreased less drastically by actinomycin D at the higher concentration of succinate.

Microscopic examination of the cells revealed that exposure to the antibiotic increased the average length and width, suggesting that the division mechanism of the cells had been disrupted. Few if any normal cells were seen in concentrations of 25 μg/ml or higher.

In contrast, the cells grown in glucose exhibited a marked decrease in sensitivity. Growth inhibition was also dependent on the concentrations of the antibiotic and of the carbon source, but an increased antibiotic concentration was required in the glucose medium to establish a similar level of inhibition to that in succinate minimal medium (Fig. 4, 5). When the glucose concentration was 1.7×10^{-2} M, 30 μg/ml actinomycin inhibited growth by less than 20 percent. It is interesting to note that the delay in establishment of visible growth inhibition was 1-2 hours longer in glucose minimal medium than in succinate minimal medium.

Figure 3. Effect of Actinomycin D on Growth of P. fluorescens in Succinate (2.78×10^{-2} M) Minimal Salts Medium ● , control; ▲, 10 μg/ml actinomycin D; ■, 20 μg/ml actinomycin D; ⬢, 30 μg/ml actinomycin D.


1.0
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$A_{540\,nm}$
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8
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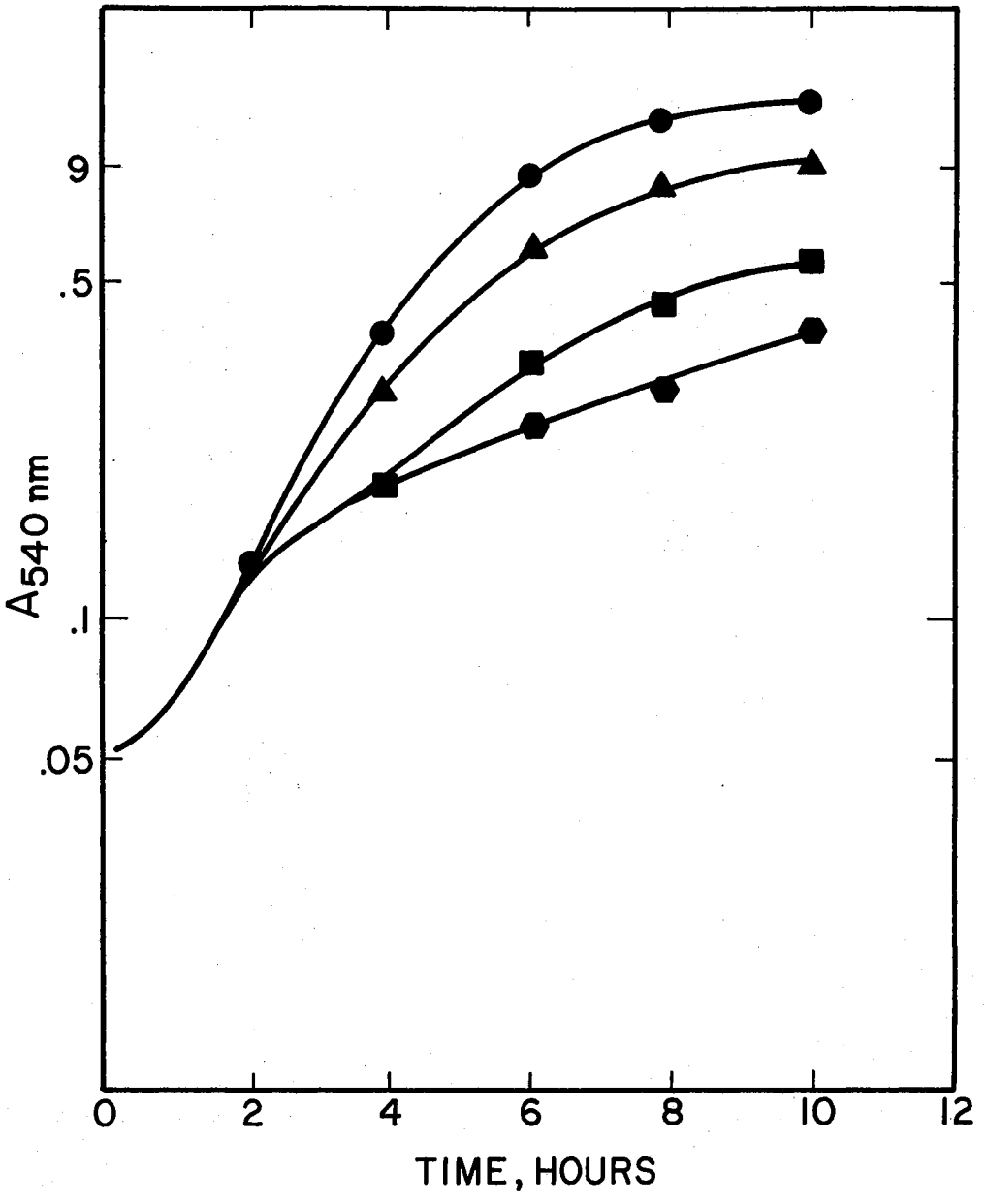
TIME, HOURS

Figure 4. Effect of Actinomycin D on Growth of P. fluorescens in Glucose (1.7×10^{-2} M) Minimal Salts Medium ● , control; ▲ , 10 μg/ml actinomycin D; ■ , 20 μg/ml actinomycin D; ⬢ , 30 μg/ml actinomycin D.


9
.5
.1
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A540 nm
0
2
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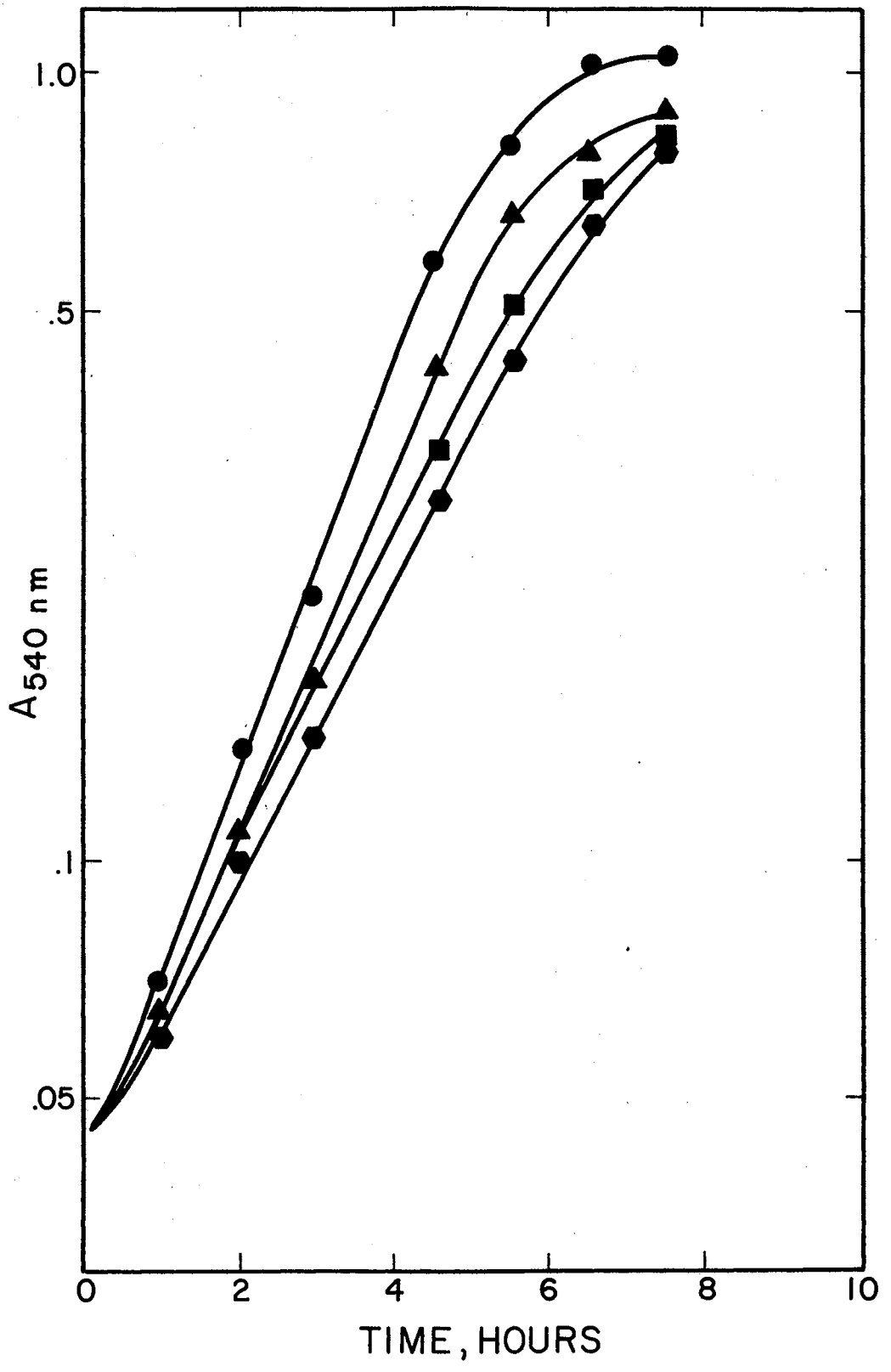
10
12
TIME, HOURS

Figure 5. Effect of Actinomycin D on Growth of P. fluorescens in Glucose (2.78×10^{-2} M) Minimal Salts Medium. ●, control; ▲, 10 μg/ml actinomycin D; ■, 20 μg/ml actinomycin D; ⬢, 30 μg/ml actinomycin D.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$A_{540\ nm}$
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8
10
TIME, HOURS

Microscopic examination of the cells exposed to the antibiotic revealed the increase in over-all size, but the percentage of glucose grown cells resembling the control was much higher than observed with the succinate grown cells, at all concentrations of actinomycin, and the higher the concentration of glucose, the less aberrant were the cells.

These results augment those obtained by Keudell (1967) and suggest that some differences do exist between cells grown in succinate and cells grown in glucose, and this difference is manifested in the level of cellular sensitivity to actinomycin D.

Influence of Glucose Addition on Actinomycin D Inhibition of P. fluorescens Growing in Succinate

Ferguson (1970) demonstrated the formation of a complex between actinomycin D and protocatechuic acid, which in turn alleviated the inhibitory properties of the antibiotic. Reversal of the inhibition could be effected by addition of small concentrations of protocatechuic acid (0.08 μmole) to the growth medium. Since cells growing in glucose showed much greater tolerance to actinomycin D, studies were conducted to determine if the addition of glucose immediately reversed the actinomycin D inhibition of cells growing in succinate.

The addition of small amounts (0.08 μmole) of glucose at different times did not affect the inhibition pattern of cells growing in succinate. This concentration of glucose is not sufficient to produce any demonstrable growth. The addition of glucose prior to the establishment of visible inhibition (0-1 hour post inoculation) did not change either the time necessary for the inhibition to become perceptible or

the degree of inhibition established by the different concentrations of the antibiotic. Addition of glucose after the inhibition had become obvious (2-4 hours post inoculation) likewise showed no decrease in the level of inhibition. These results indicate that this concentration of glucose does not protect the cells from inhibition nor reverse the inhibition once it has been established in cells growing in succinate and suggest that glucose does not interact with the actinomycin D molecule, thereby altering its biological activity.

Spectrophotometry of Actinomycin D

Some further studies were conducted to insure the absence of complex formation between glucose and actinomycin D and to determine if a metabolic product in the glucose medium was inactivating the antibiotic. The method employed for detection of complex formation was the comparison of spectrophotometric patterns of the combined and isolated molecules; the ultraviolet spectra of actinomycin, actinomycin and glucose, and actinomycin and succinate were run. The position of the major peaks (230 nm, 250 nm, and 450 nm) and the intensity remained unchanged when actinomycin was suspended in either glucose or succinate minimal medium. This would further substantiate the results obtained in the reversal experiments, indicating that no complex formation occurred, and actinomycin was not inactivated by glucose.

Since actinomycin might be complexing with some metabolic breakdown product of glucose resulting from cell growth, the ultraviolet spectrum of actinomycin was measured when suspended in spent glucose minimal medium. The spent medium was prepared by growing P. fluorescens cells in glucose minimal medium to the stationary phase, and then

removing the cells by centrifugation. A very slight decrease (approximately 8-10 percent) resulted in the intensity of absorption, but no shift was evident in the position of the three peaks. A similar decrease in absorption was also recorded when actinomycin was suspended in spent succinate medium. The decrease probably was the result of some of the actinomycin molecules complexing with either free DNA or with cell fragments, both of which would be present in spent medium. The decrease apparently has no relevance to the sensitivity levels of cells grown in the two carbon sources.

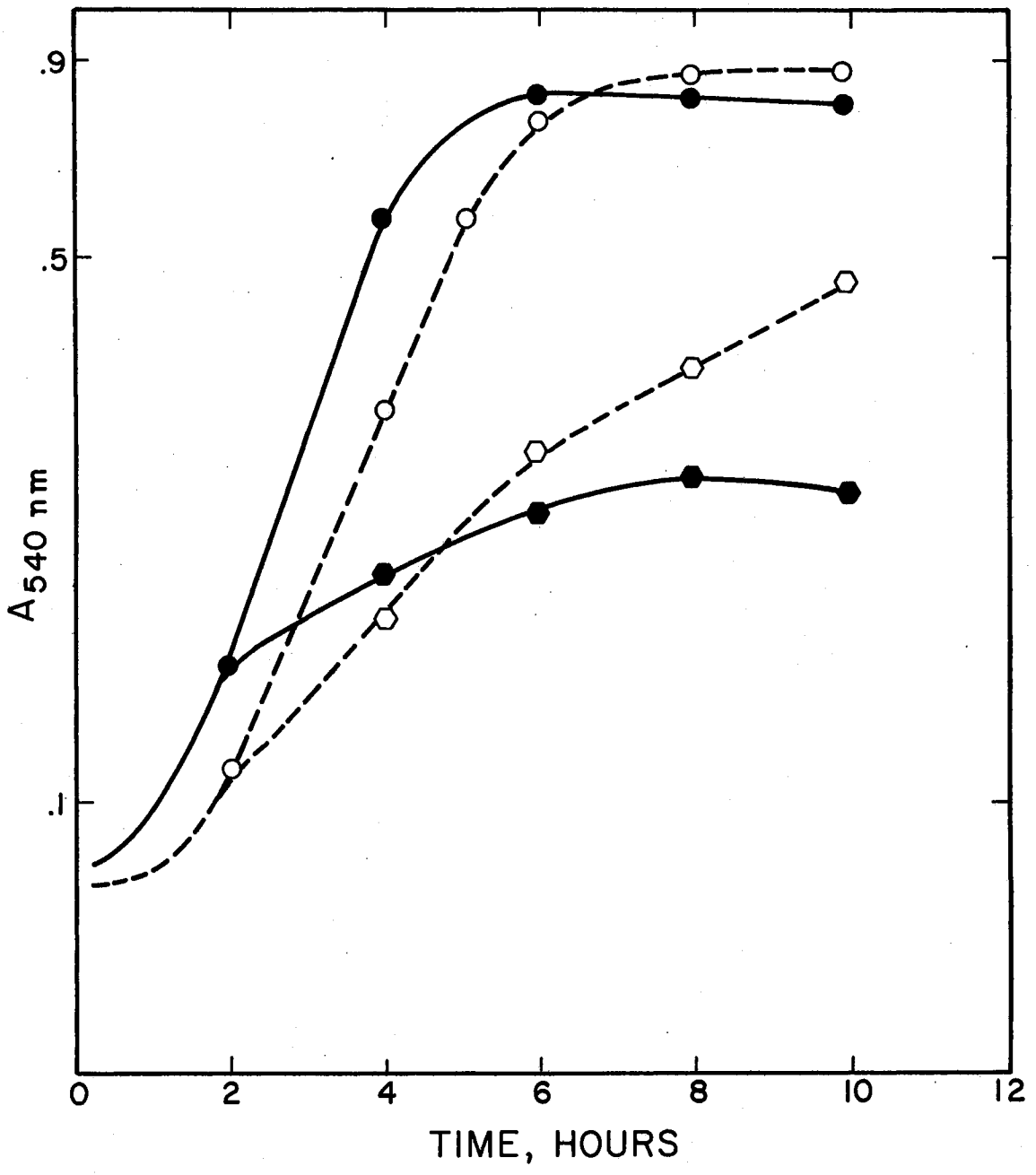
Effect of Growth Rate on Sensitivity to Actinomycin D

The growth rate of P. fluorescens in glucose is slightly slower than that in succinate. An experiment was thus conducted to observe whether a controlled decrease in growth rate would occasion a subsequent reduction in sensitivity to actinomycin D. Growth rate can be effectively slowed by lowering the temperature of incubation, so one set of tubes was placed at 37 C and another set at 30 C. The resultant lengthening of generation time produced a decrease in sensitivity of approximately the same magnitude in both the glucose and succinate grown cells (Fig. 6, 7). Thus, the decreased growth rate imparts sufficient time for the cells to effect some change which causes a decreased level of sensitivity.

Study of a Change in Sensitivity of Cells to Actinomycin D During Growth in Glucose

Since cells exposed to actinomycin in glucose medium exhibited a

Figure 6. Effect of Actinomycin D on Growth of P. fluorescens in Succinate (1.7×10^{-2} M) Minimal Salts Medium at 30 C and 37 C. ● , control, 37 C; O , control, 30 C; ● , 30 μg/ml actinomycin D, 30 C; ⬡ , 30 μg/ml actinomycin D, 37 C.


.9
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.1
A 540 nm
0
2
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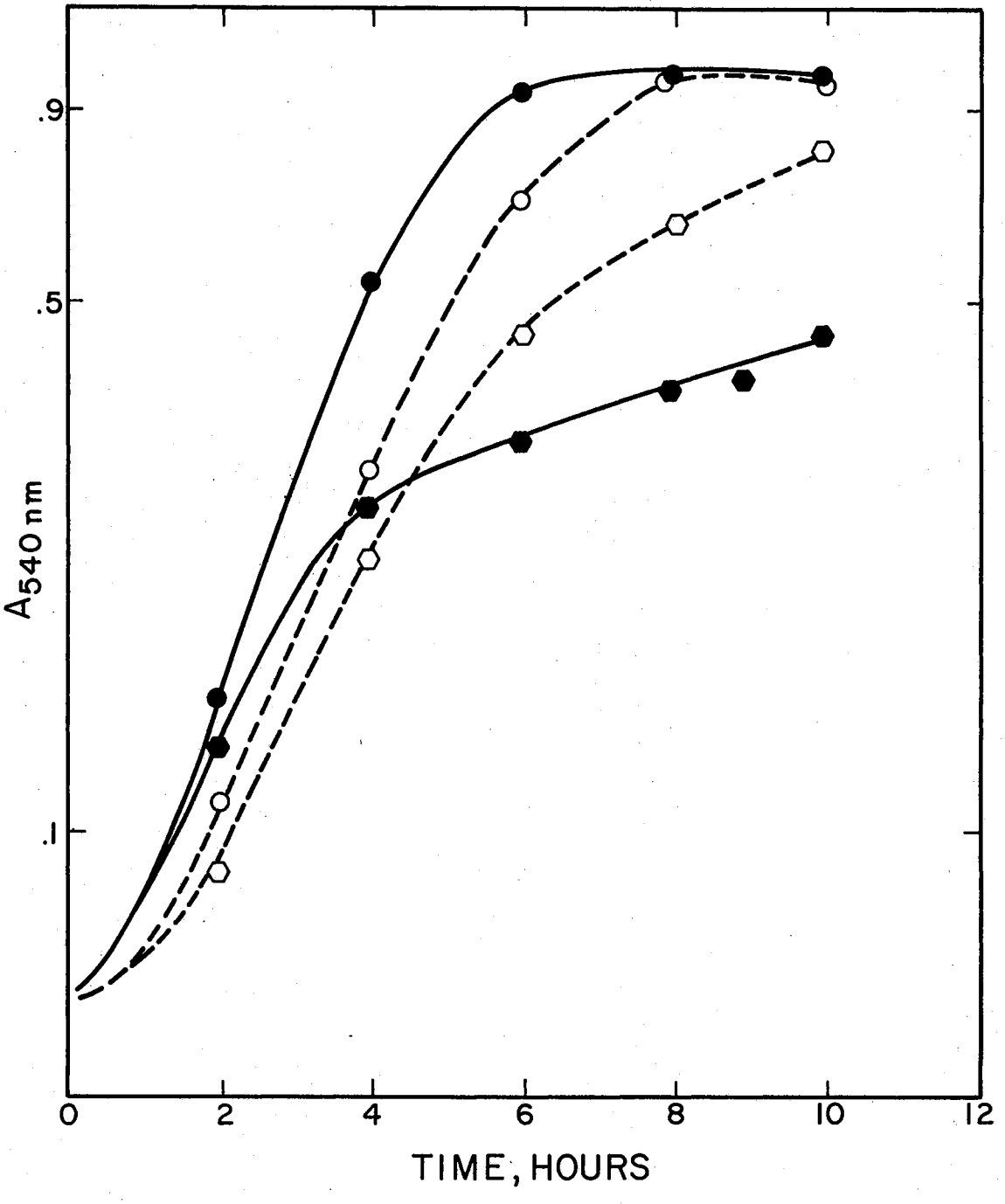
6
8
10
12
TIME, HOURS

Figure 7. Effect of Actinomycin D on Growth of P. fluorescens in Glucose (1.7×10^{-2} M) Minimal Salts Medium at 30 C and 37 C ●, control, 37 C; ○, control, 30 C; ⬢, 30 μg/ml actinomycin D, 37 C; ⬡, 30 μg/ml actinomycin D, 30 C.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TIME, HOURS

drug-induced lag and then grew out to the same final density as the control, the glucose grown cells were studied to ascertain if the medium was selecting for resistant cells. P. fluorescens cells were grown in glucose medium containing 40 μg/ml actinomycin to an absorbency of 1.0. The cells were harvested, used to inoculate fresh growth medium with and without actinomycin D, and their response to the antibiotic was observed.

The cells previously grown in actinomycin D showed no increased resistance to the antibiotic, regardless of whether they were grown in succinate or glucose. In fact, there seemed to be a slight increase in sensitivity, indicating that the cells were damaged as a result of their growth in the presence of actinomycin. These results eliminate the selection of resistant cells as being responsible for the decreased sensitivity in P. fluorescens grown in glucose.

Effect of Actinomycin D on Uptake of Labeled Substrates

In order to study the effect of actinomycin D on uptake in P. fluorescens, cells were grown to mid-logarithmic stage, at which time 30 μg/ml of actinomycin was added to half of the cells, and buffer to the remaining half. Cells were incubated for an additional two hours in the presence of the antibiotic before conducting the uptake experiments.

The substrates selected for use in the uptake experiments included the two carbon sources, succinate and glucose; two wall amino acids, alanine and lysine; one non-wall amino acid, phenylalanine; and the pyrimidine base, uracil. Results indicated that growth of the cells

in succinate plus actinomycin D either drastically reduced or completely eliminated uptake of all substrates tested, while growth of cells in glucose plus actinomycin D either did not affect or caused only moderate reduction of uptake capacity for the substrates.

Results from the phenylalanine uptake studies (Fig. 8, 9) indicated that growth in succinate plus actinomycin D completely blocks all but a minimal level of uptake. Although the glucose grown cells take up a lower level of the substrate, there was no appreciable effect when cells were grown in the presence or absence of the antibiotic. The presence or absence of actinomycin D in the uptake solution did not change the pattern of label accumulation in either set of cells. The fact that growth of the cells in the presence of actinomycin was necessary before any inhibition of uptake occurred emphasizes the permeability factor involved with gram negative cells. Short periods of exposure to the antibiotic, as in the case of an uptake experiment, are not sufficient to allow penetration of the antibiotic to the interior of the cell.

The succinate grown cells do not actively transport lysine, so the substrate did not provide a basis for comparison. However, the glucose grown cells manifested no inhibition of uptake of lysine upon previous growth in actinomycin D.

Uptake of succinate in cells grown in succinate plus actinomycin D (Fig. 10) was eliminated except for a basal level of activity which was probably due to free diffusion into the cell. In contrast, there was no inhibition of glucose uptake in cells grown in glucose plus actinomycin D (Fig. 11). There appears to be a slight stimulation of uptake, which may simply be the result of increased permeability due to growth

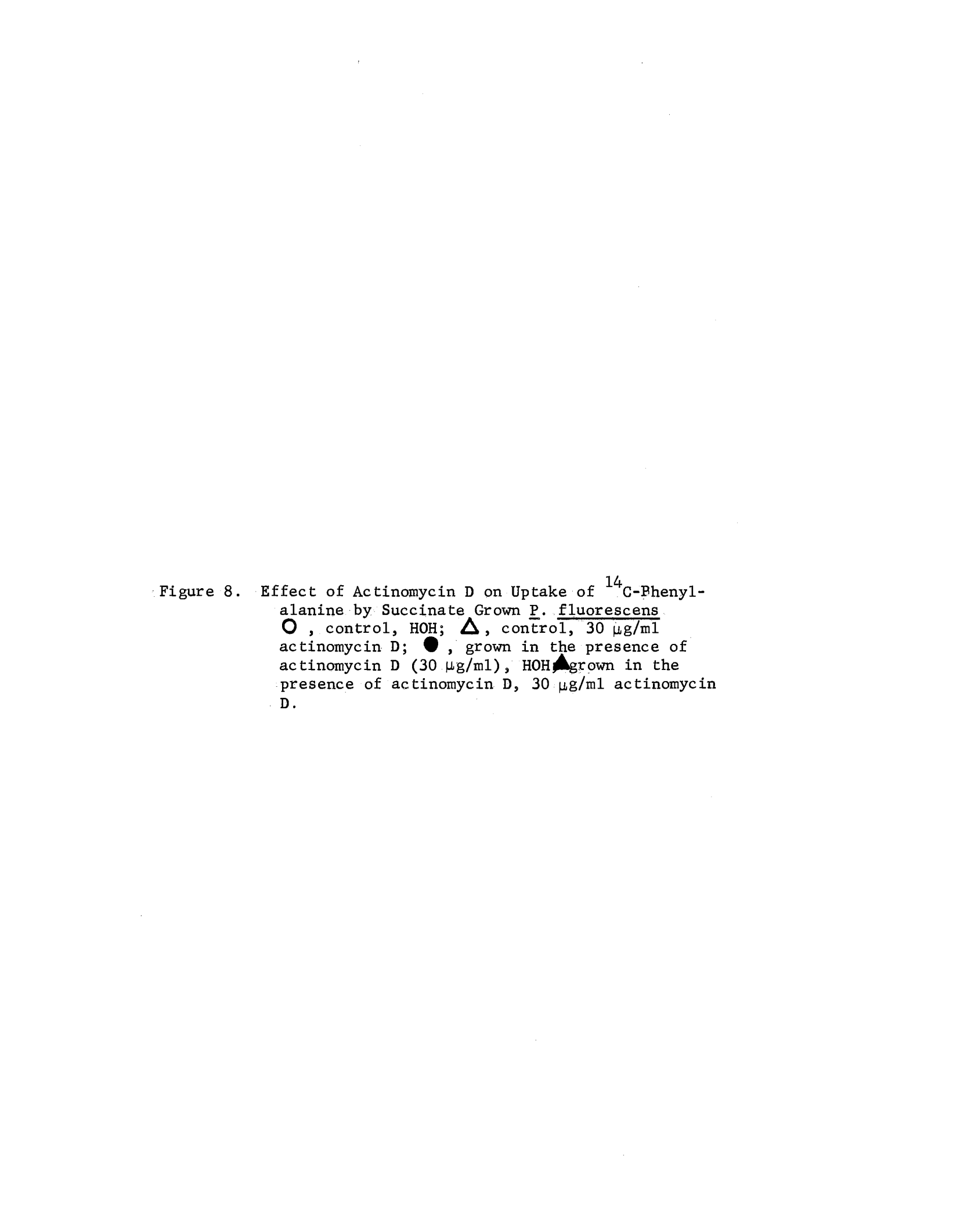


Figure 8. Effect of Actinomycin D on Uptake of ^{14}C-Phenylalanine by Succinate Grown P. fluorescens ○, control, HOH; △, control, 30 μg/ml actinomycin D; ●, grown in the presence of actinomycin D (30 μg/ml), HOH; ▲, grown in the presence of actinomycin D, 30 μg/ml actinomycin D.

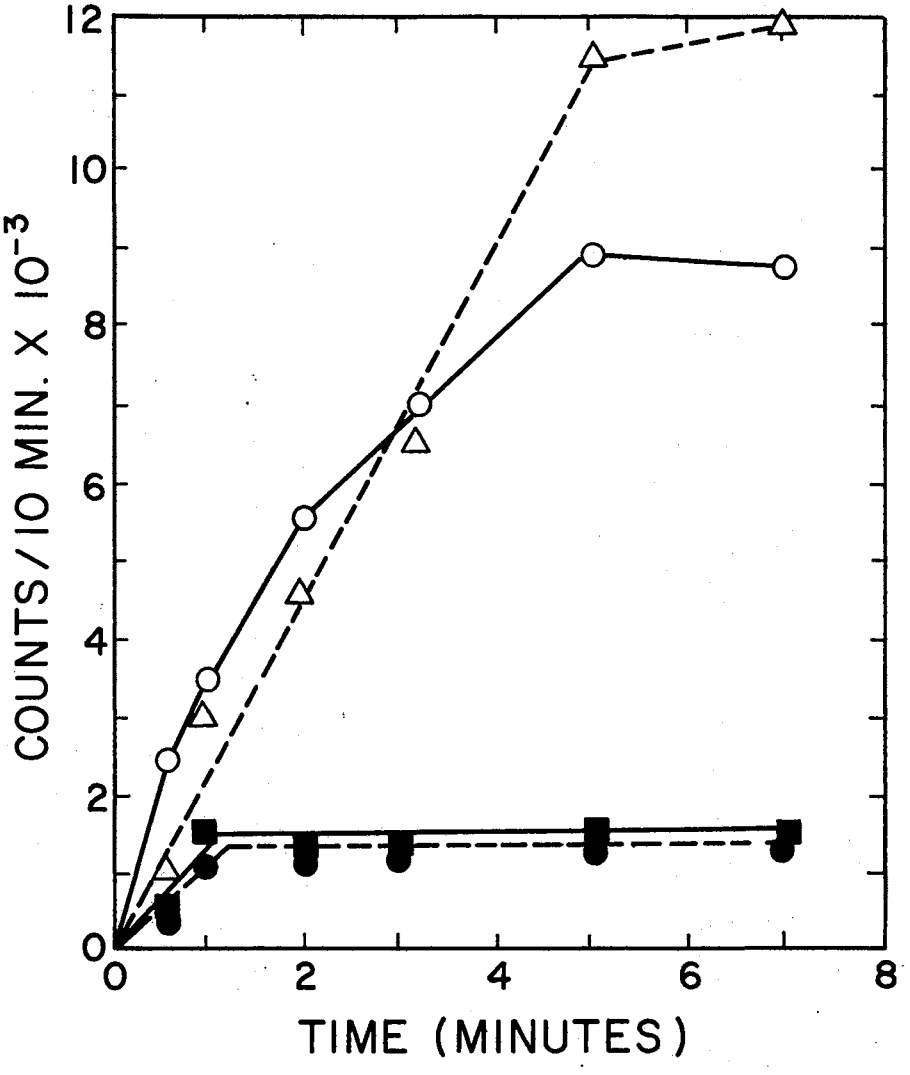

12
10
8
6
4
2
0
0
2
4
6
8
COUNTS / 10 MIN. X 10^{-3}
TIME (MINUTES)

Figure 9. Effect of Actinomycin D on Uptake of ^{14}C-Phenylalanine by Glucose Grown P. fluorescens. O , control, HOH; Δ , control, 30 μg/ml actinomycin D; ● , grown in the presence of actinomycin D (30 μg/ml), HOH; ■ , grown in the presence of actinomycin D, 30 μg/ml actinomycin D.

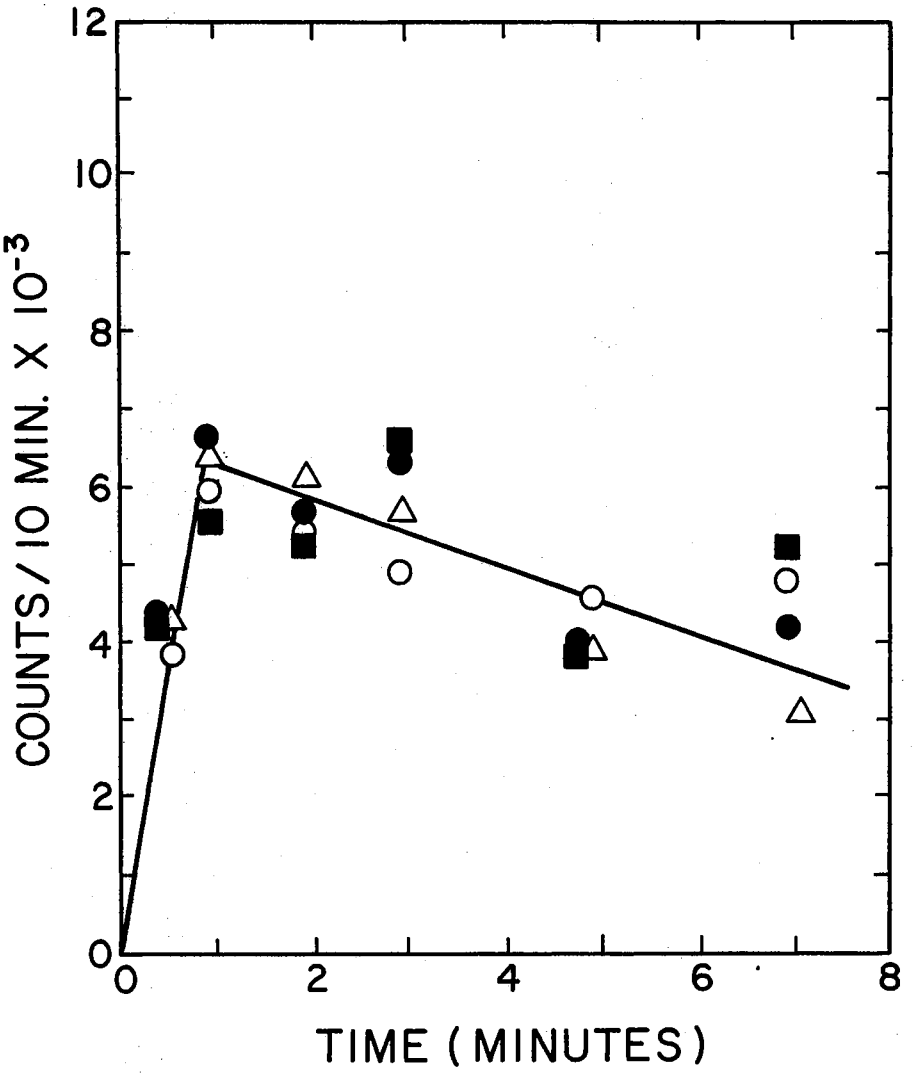
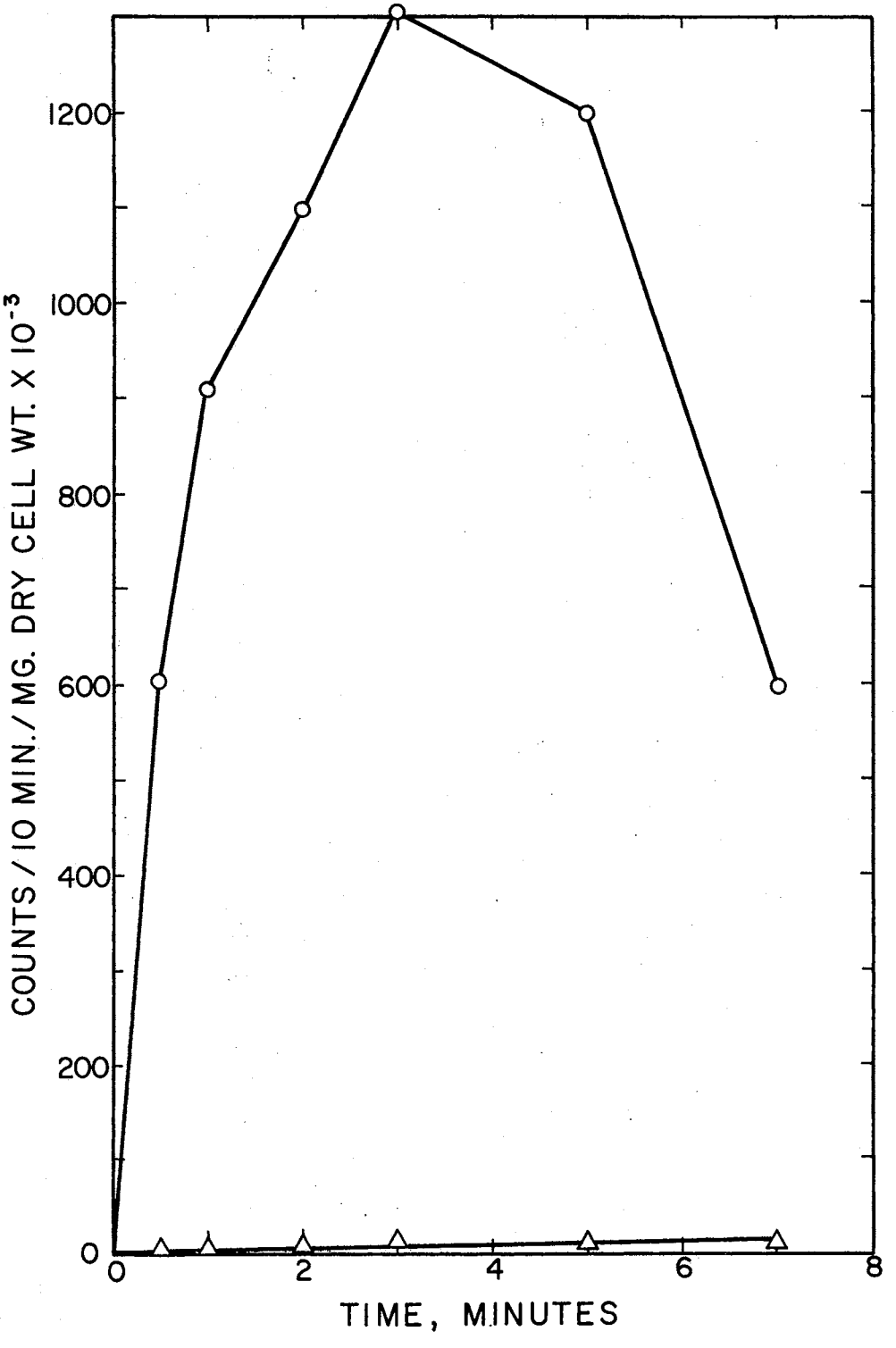

12
10
8
6
4
2
0
0
2
4
6
8
COUNTS / 10 MIN. X 10^{-3}
TIME (MINUTES)

Figure 10. Effect of Actinomycin D on Uptake of ^{14}C-Succinate by Succinate Grown P. fluorescens ○, control; △, pre-exposed.


COUNTS / 10 MIN. / MG. DRY CELL WT. X 10^{-3}
1200
1000
800
600
400
200
0
0
2
4
6
8
TIME, MINUTES

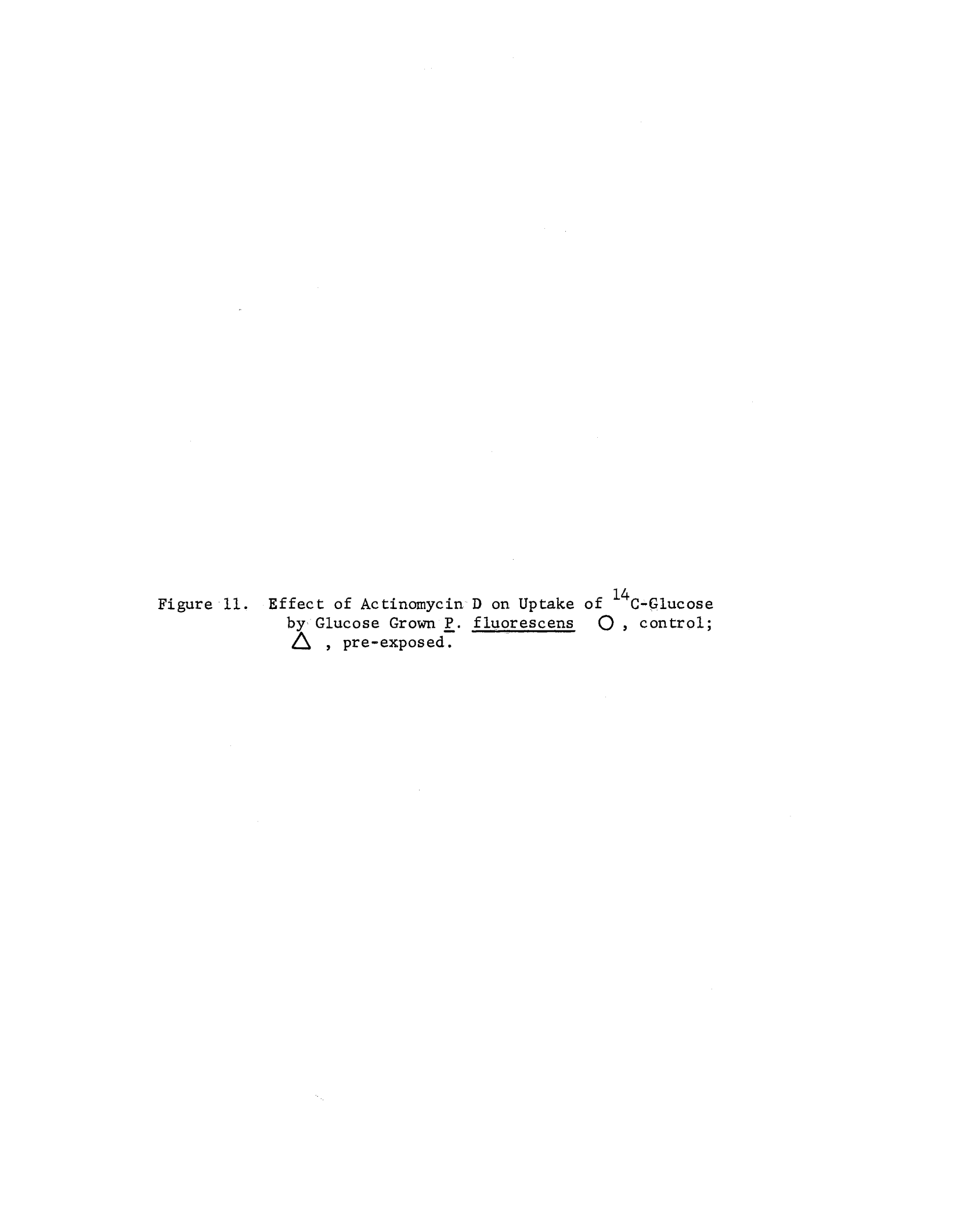
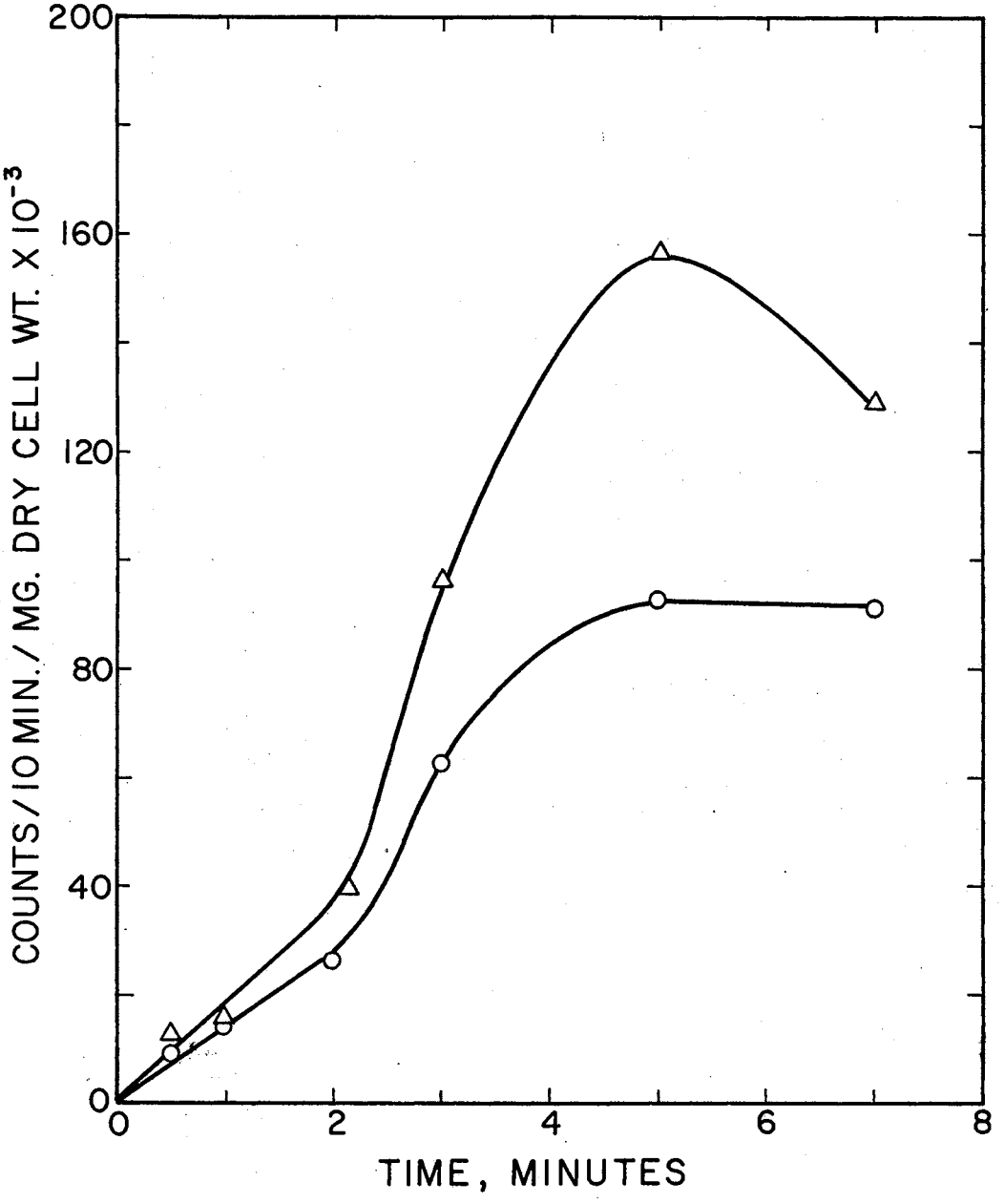


Figure 11. Effect of Actinomycin D on Uptake of ^{14}C-Glucose by Glucose Grown P. fluorescens ○, control; △, pre-exposed.


200
160
120
80
40
0
0
2
4
6
8
COUNTS/10 MIN./ MG. DRY CELL WT. X 10^{-3}
TIME, MINUTES

in the antibiotic. Glucose grown cells are not as permeable to the actinomycin molecule, and therefore the uptake system was not eliminated as it was with succinate grown cells.

It was also found that cells grown in succinate would not take up glucose, and that cells grown in glucose would not take up succinate. The addition of chloramphenicol to the systems blocked induction of transport for glucose and for succinate, indicating that neither uptake system was constituitive to the cells.

Two interesting conclusions follow from the preceding results. The first is that all uptake systems are not affected by actinomycin D to the same degree, perhaps indicating that some difference may exist in the DNA-RNA mediation of protein synthesis for each system. The second, and obvious, conclusion is that the glucose grown cells are affected by growth in actinomycin D to a much lesser degree than are the succinate grown cells. This could indicate several possibilities, but the most likely seems to be a rather significant difference in the permeability properties of the cells for the antibiotic molecule. In order to observe any significant effect of the antibiotic on the uptake systems of either the glucose grown or the succinate grown cells, growth in the presence of actinomycin D for at least an hour was necessary. If the cells were not grown previously in the presence of actinomycin D, addition of the antibiotic did not decrease the uptake capacity of the cells during the time they were observed, eliminating the possibility that the antibiotic was limiting uptake by competing for a binding site. The results of the uptake studies augment previous results and further substantiate that the glucose grown cells are less permeable to actinomycin D.

Effect of Actinomycin D on Uracil Incorporation

Since actinomycin D purportedly binds to the DNA in such a way as to block messenger (m)-RNA synthesis, the classic method for determining if actinomycin enters the cell and interacts with DNA is to measure the incorporation of uracil into the RNA fraction of the cell. Cells were grown in succinate or glucose minimal medium containing ^{14}C-uracil, and a cold trichloroacetic acid precipitation used to remove unincorporated label. A measure of the inhibition of RNA synthesis was determined by the amount of radioactivity incorporated into control cells and cells growing in the presence of actinomycin D.

The results indicated that up to a time of 60 minutes, actinomycin did not inhibit RNA synthesis in either the cells growing in glucose or in the cells growing in succinate (Fig. 12, 13). The amount of uracil incorporated into the cells growing in the presence of the antibiotic is the same as the amount of label incorporated into the control. However, if incorporation is followed beyond 60 minutes, the cells growing in succinate and exposed to actinomycin begin to show a marked depression in the amount of label incorporated as compared to the control. The cells growing in glucose do not exhibit a depression of incorporation within the time course of the experiment.

There are several possibilities which would explain the decrease in uracil incorporation in the cells growing in succinate and exposed to actinomycin D. There may actually be enough actinomycin reaching the interior of the cell to effectively block RNA synthesis. This would in turn mean that the succinate grown cells were more permeable to the antibiotic than the glucose grown cells, which did not exhibit

Figure 12. Effect of Actinomycin D on Incorporation of ^{14}C-Uracil by Succinate Grown P. fluorescens ▲, $A_{540\ nm}$, control; ●, $A_{540\ nm}$, actinomycin D (30 μg/ml); △, radioactivity incorporated, control; ○, radioactivity incorporated, actinomycin D (30 μg/ml).

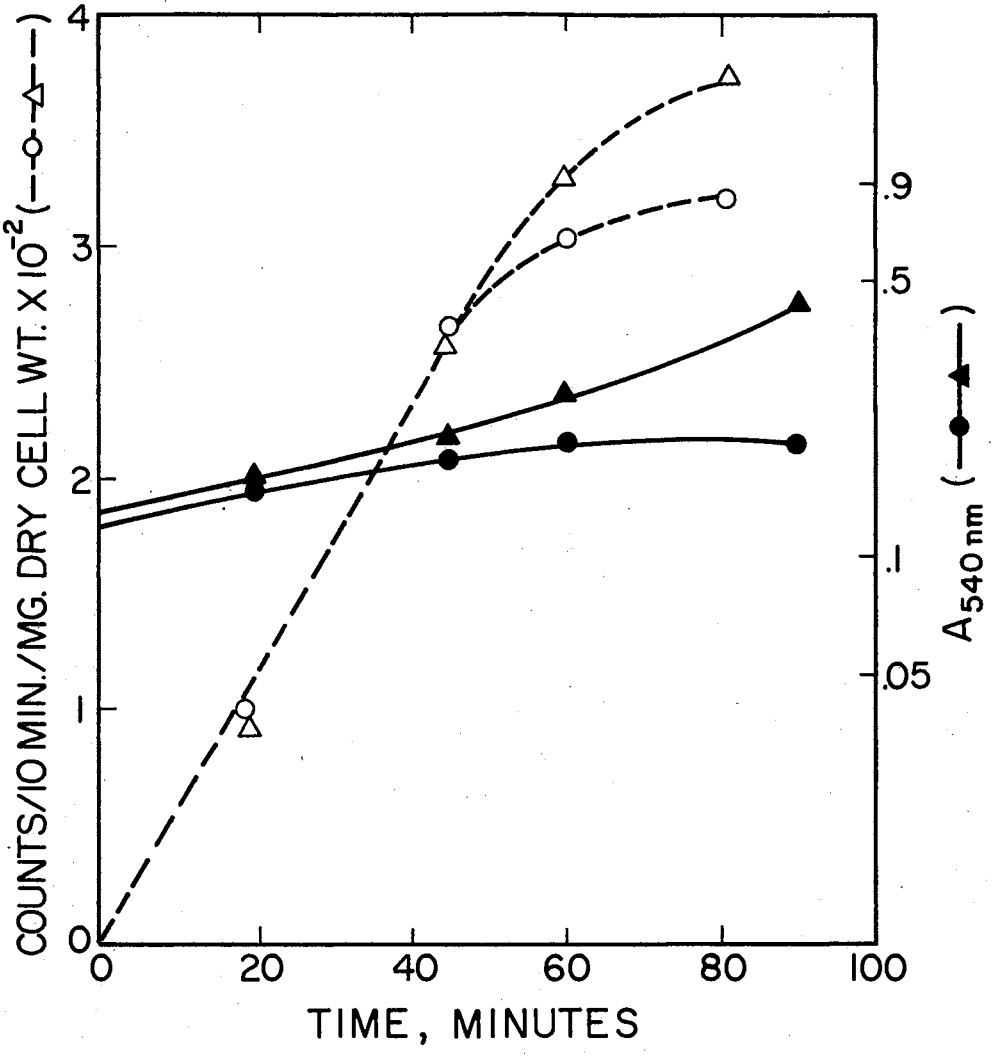
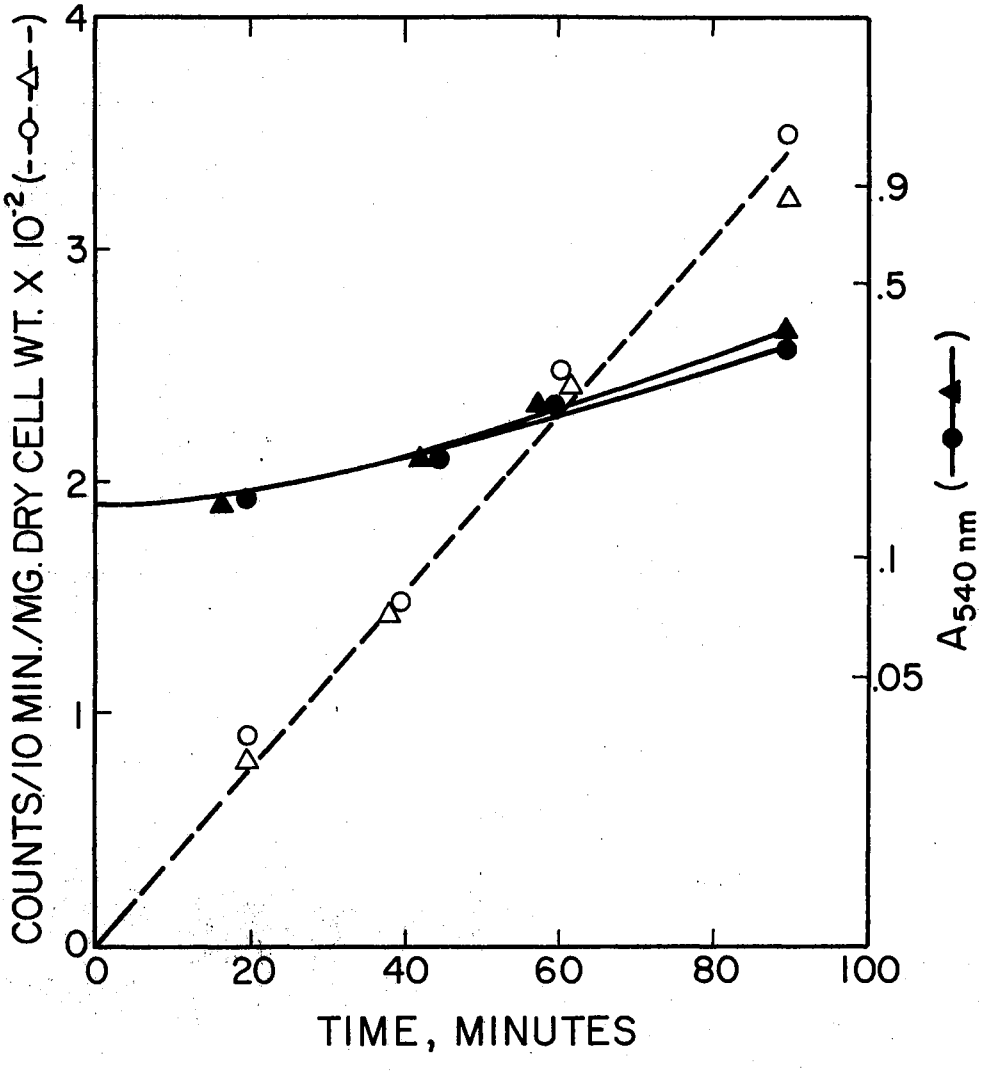

COUNTS/10 MIN./MG. DRY CELL WT. X 10^{-2} (—o—△—)
$A_{540\,nm}$ (—●—)
TIME, MINUTES
0
1
2
3
4
0
20
40
60
80
100
.05
.1
.5
.9

Figure 13. Effect of Actinomycin D on Incorporation of ^{14}C-Uracil by Glucose Grown P. fluorescens ▲, $A_{540\ nm}$, control; ●, $A_{540\ nm}$, actinomycin D (30 μg/ml); △, radioactivity incorporated, control; ○, radioactivity incorporated, actinomycin D (30 μg/ml).


COUNTS/10 MIN./MG. DRY CELL WT. X 10^{-2} (--o--Δ--)
$A_{540\,nm}$ (—●—)
TIME, MINUTES
0
1
2
3
4
.05
.1
.5
.9
0
20
40
60
80
100

decreased levels of incorporation. Apparently the actinomycin molecule is not able to penetrate the envelope of the glucose grown cells within the time course of the experiment. However, the cells growing in succinate minimal medium had reached maximum stationary phase before the inhibition of RNA synthesis became apparent, and there is the possibility that RNA synthesis becomes more susceptible to actinomycin inhibition as the growth rate is slowed.

Another facet must also be mentioned, as it has a direct bearing on the level of incorporation. Results from the uracil uptake experiments established that growth in actinomycin decreased the uptake of uracil in succinate grown cells, but not in glucose grown cells. Thus, the lowered level of incorporation in the succinate grown cells may merely be a reflection of a decreased level of uracil in the cell's pools, rather than an actual blockage of RNA synthesis.

Either way, it is still apparent that the succinate grown cells are more sensitive to actinomycin than are the glucose grown cells, and permeability seems to be the most plausible answer. These data support previous observations of the difference in sensitivity.

Effect of Actinomycin D on Cellular Respiration

Manometric studies were performed to determine whether exposure to the antibiotic would decrease the rate of oxygen consumption and/or alter induction to succinate and glucose. The studies were also conducted to determine whether the sensitive cells, those grown in succinate minimal medium, would exhibit greater sensitivity in their respiratory response to actinomycin D than would the less sensitive glucose grown cells.

Trace minerals aided in the induction of the transport and metabolic systems for glucose and succinate, particularly when the cells were grown in succinate and the metabolism of glucose was observed. Additional investigations indicated that Mg^{+2} was the most effective of those cations in the mineral salts solution used in the growth medium.

Incubation of the cells in glucose or succinate medium containing the antibiotic was necessary to produce a significant change in respiratory patterns (Table I). This requirement for incubation in the presence of the antibiotic was previously seen in the uptake experiments. Succinate grown cells oxidized the substrates at the same rate in the presence or absence of the antibiotic, and the total amount of oxygen consumed was the same in both situations. However, the presence of actinomycin D in the flask decreased the rate of glucose oxidation by succinate grown cells by approximately 25 percent of the control value.

Based on previous results with uptake, growth, and incorporation experiments, the time interval required for induction to glucose was sufficient for the antibiotic to penetrate the succinate grown cells, and this could explain the decrease in rate when actinomycin D was present in the flask. Although the rate of oxygen consumption was decreased, the total oxygen consumed was increased, which might indicate that glucose was being oxidized almost completely, with less activity directed toward synthesis.

The cells grown in succinate plus actinomycin D showed a 50-55 percent decrease in the rate of oxygen consumption when compared to succinate grown control cells when the oxidation of succinate was

TABLE I

WARBURG RESPIROMETRY OF SUCCINATE AND GLUCOSE GROWN P. FLUORESCENS--
THE EFFECT OF ACTINOMYCIN D ON THE METABOLISM
OF GLUCOSE AND SUCCINATE

Growth Medium	Addition to Flask	Rate of Oxidation (μl O_2/min.)		Total O_2 Consumed (μl)		Time for Induction (Min.)	
		Succinate	Glucose	Succinate	Glucose	Succinate	Glucose
Succinate	HOH	9.5	3.6	272	190	IG	75
	AD	9.5	2.8	272	283	IG	60
Succinate plus Actinomycin D (AD)	HOH	4.4	1.9	275	146	IG	180
	AD	5.0	0.0	257	0	IG	DNI
Glucose	HOH	8.2	8.8	326	624	20	IG
	AD	8.6	9.5	337	694	20	IG
Glucose plus Actinomycin D (AD)	HOH	8.8	7.5	319	464	30	IG
	AD	7.6	4.8	327	500	30	IG

IG = induced as the result of growth on the substrate
DNI = did not induce

followed. This was true whether the cells were in the presence or absence of actinomycin D. Total oxygen consumption was not changed. When the oxidation of glucose was observed, the cells grown in succinate plus actinomycin D showed a 50 percent reduction in rate as compared to the control system (succinate grown cells with glucose as the substrate). Oxygen consumption was reduced slightly, and the time required for induction was increased from 75 to 180 minutes. In the presence of actinomycin D, these cells were not able to induce to glucose. This inability to induce would strongly suggest that the cell's synthetic mechanisms were completely inhibited. The complete blockage of protein synthesis might be expected if a sufficient amount of actinomycin had entered the cell and interacted with the DNA.

The oxidation of glucose and succinate by the glucose grown cells was not affected by the presence of actinomycin. The rate of oxidation remained the same, as did the total amount of oxygen consumed. However, induction time to succinate metabolism was within 20-30 minutes after addition of the carbon source, and previous data from growth, uptake, and incorporation studies establish that this would not allow time for penetration of the antibiotic into the glucose grown cells.

Growth of cells in glucose plus actinomycin D caused a 10 percent decrease in the rate of oxidation as compared to the control when succinate was the substrate. This decrease was the same whether or not the antibiotic was present in the flask. The cells exhibited a slightly decreased total oxygen consumption, and the time required for induction to succinate was increased. However, the increase was only an additional 10 minutes as compared to the control. With glucose as substrate, only a slight decrease in rate was apparent, and when the

antibiotic was present in the flask, the rate of oxygen consumption was decreased by approximately 40 percent. In all instances, the endogenous metabolic rate was not altered by growth in the carbon source plus the antibiotic.

These results support the hypothesis that actinomycin is crossing the permeability barrier of the succinate grown cells with greater expediency than that of the less sensitive glucose grown cells. Since the oxidative enzymes as well as the protein synthesizing machinery are functionally dependent upon the DNA to RNA mediated transfer of information, the disruption of the two systems provides a criterion for determining if the antibiotic is reaching the active center of the cell. The results of the respirometry studies further substantiate the evidence that the succinate grown cells are more susceptible to the inhibitory effects of actinomycin D. This would, in turn, signify that more of the antibiotic is reaching the active site in the succinate grown cells than in the glucose grown cells.

Detection of Actinomycin D Alteration

Although evidence indicates that a difference in permeability exists between glucose grown and succinate grown cells of P. fluorescens, it was important to determine if the cells grown in glucose were capable of oxidizing or in some way altering the actinomycin molecule to an inactive form.

Warburg respirometry studies of glucose grown and succinate grown cells with actinomycin D present as the only possible source of carbon revealed that neither set of cells utilized the molecule for oxidative purposes during the 120 minute incubation study. There was no increase

in the basal metabolic activity of the cells in the flasks containing actinomycin D as compared to cells incubated in minimal salts buffer.

The intact actinomycin molecule may be readily extracted from cells with butanol, while the breakdown products, should any be formed, are left in the water phase of the extraction (Greenhouse, Hynes, and Gross, 1971). Cells which had been incubated in the presence of ^{3}H-actinomycin D for 5 hours at 37 C in a shaking water bath were sonicated and extracted with butanol. The water and butanol extracts were chromatographed, and the chromatograms scanned with a Packard strip counter. A single peak appeared when the butanol extraction was scanned, and there was no significant activity present in the water phase. The extracted actinomycin D migrated at a similar rate to the control in several solvent systems.

Thus, actinomycin D was not being altered to an inactive form, nor was it being utilized as a substrate by either glucose or succinate grown cells.

Effect of Ethylenediaminetetraacetic Acid (EDTA) Treatment on Cellular Sensitivity to Actinomycin D

Leive (1965) reported that brief treatment of *E. coli* with low concentrations of EDTA increased the permeability, but this was associated with little or no change in viability, growth rate, or normal RNA and protein synthesis. The increase in permeability was manifested by an increase in sensitivity to actinomycin D.

A modification of Leive's procedure was used to treat glucose grown and succinate grown *P. fluorescens*. If the succinate grown cells

do have a different permeability barrier from glucose grown cells, then there should be a difference in cellular response when treated with EDTA, and sensitivity to actinomycin D would act as an indicator.

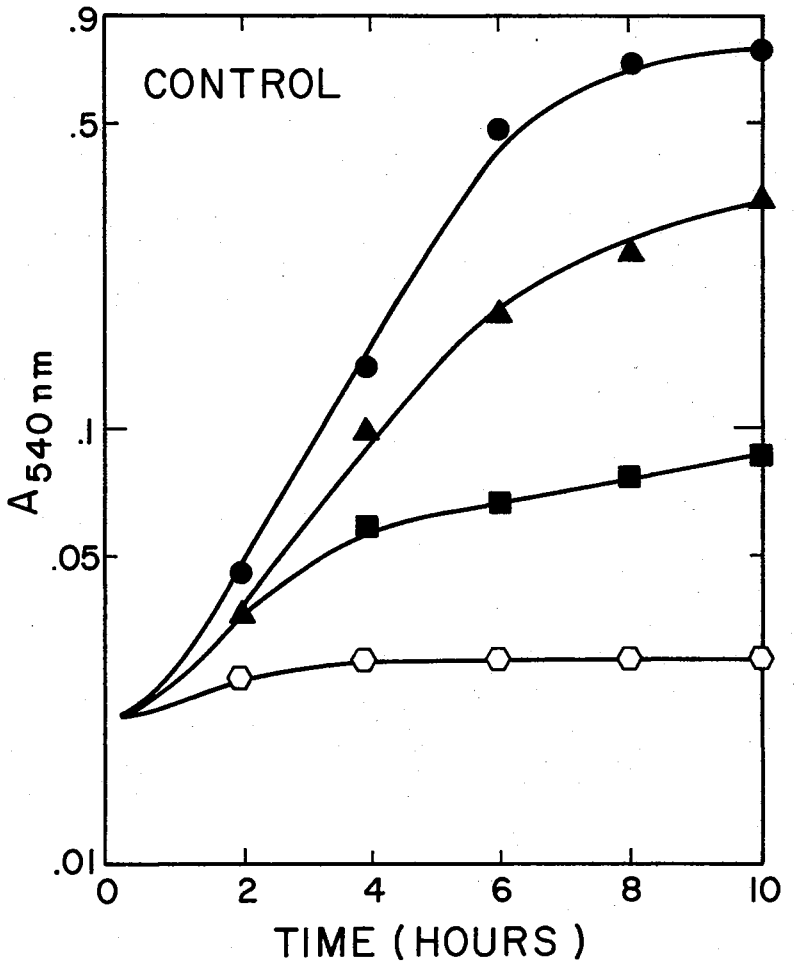
EDTA treatment of succinate grown cells caused a pronounced increase in sensitivity to actinomycin D (Fig. 14). Even the lowest concentration of antibiotic (5 μg/ml) caused complete inhibition of growth in the EDTA-treated cells. The same treatment in glucose grown cells increased the lag time, but the level of inhibition was similar to that exhibited by the control cells (Fig. 15). These results suggest that the permeability barrier of the succinate grown cells is not only more easily penetrated, but also more easily altered than the supposedly similar barrier in the glucose grown cells. Therefore, the barriers of the cells must be different; similar barriers would have shown a similar response.

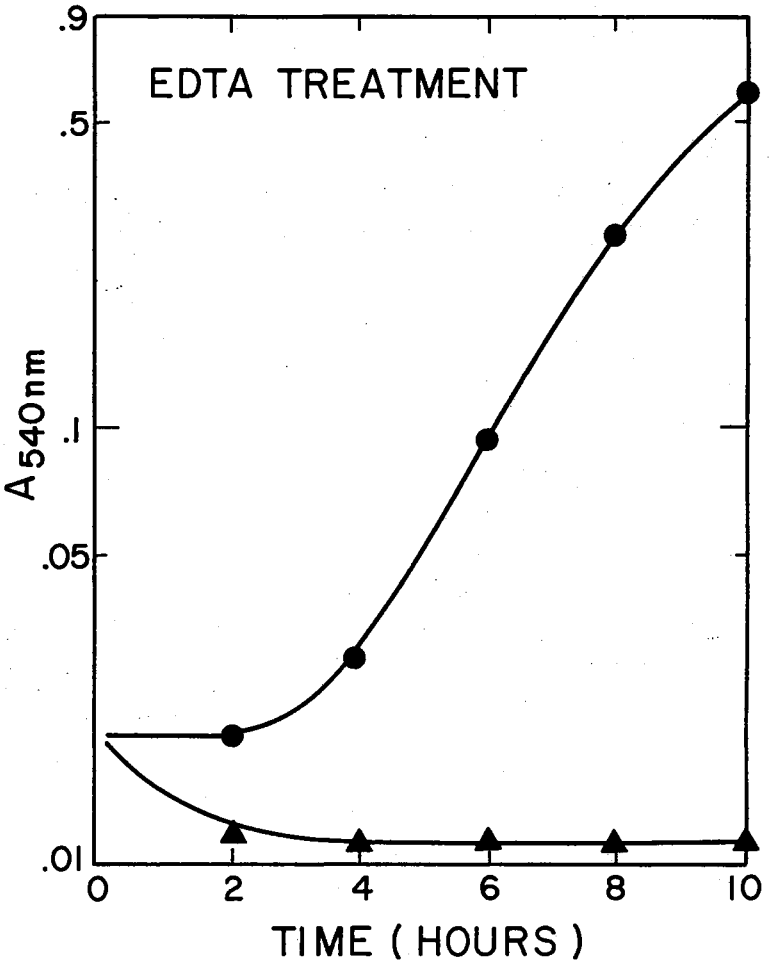
Binding of ^{3}H-Actinomycin D to Whole Cells and Isolated Envelopes

Because of the peculiar binding properties of the actinomycin molecule, it is difficult to differentiate between binding to the cell surface and actual uptake by the cells. However, by performing a series of binding studies using ^{3}H-actinomycin D and cell pellets, it was shown that cells grown in succinate bind 2-3 times the amount of activity as the same cell mass of glucose grown cells (Fig. 16). Adsorption is linear with cell density in both sets of cells and is independent of temperature of incubation.

It is not possible from this type of study to determine how much of the bound antibiotic is inside the cell and how much is merely bound

Figure 14. Effect of EDTA on the Sensitivity of Succinate Grown P. fluorescens to Actinomycin D. Control: ●, HOH; ▲, 5 μg/ml actinomycin D; ■, 10 μg/ml actinomycin D; ⬡, 20 μg/ml actinomycin D. EDTA-treated: ●, HOH; ▲, 5, 10, and 20 μg/ml actinomycin D.


CONTROL
.9
.5
.1
.05
.01
$A_{540\,nm}$
0
2
4
6
8
10
TIME (HOURS)


EDTA TREATMENT
.9
.5
.1
.05
.01
A_{540nm}
0
2
4
6
8
10
TIME (HOURS)

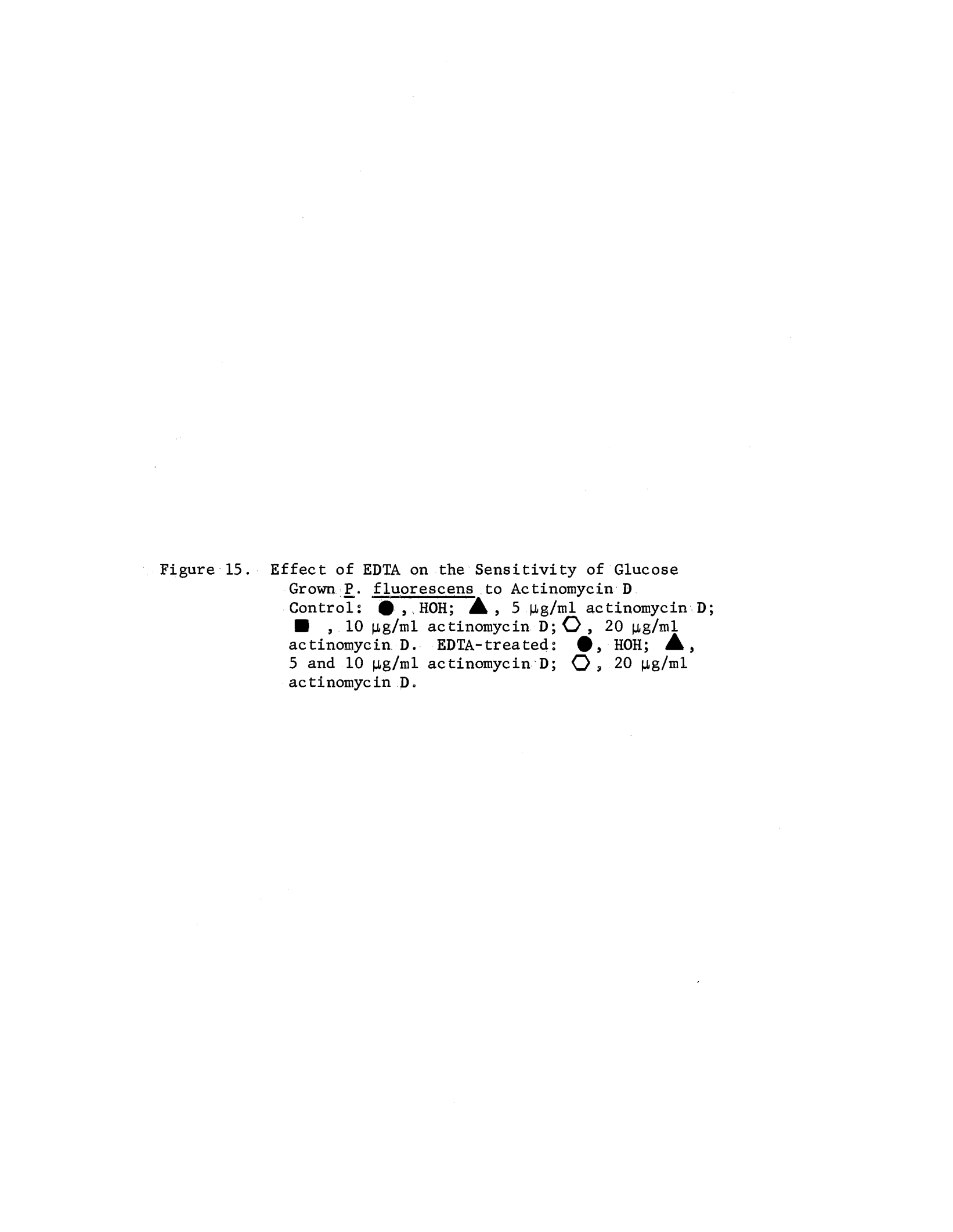
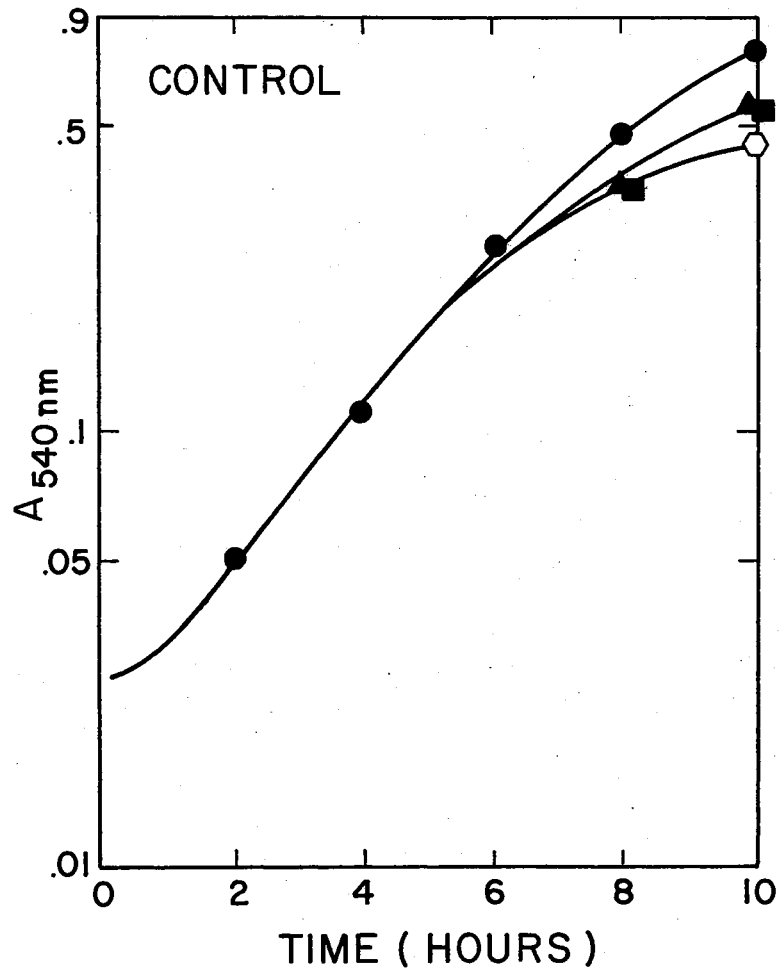


Figure 15. Effect of EDTA on the Sensitivity of Glucose Grown P. fluorescens to Actinomycin D
Control: ●, HOH; ▲, 5 μg/ml actinomycin D; ■, 10 μg/ml actinomycin D; ⬡, 20 μg/ml actinomycin D. EDTA-treated: ●, HOH; ▲, 5 and 10 μg/ml actinomycin D; ⬡, 20 μg/ml actinomycin D.


CONTROL
.9
.5
.1
.05
.01
$A_{540\,nm}$
0
2
4
6
8
10
TIME (HOURS)

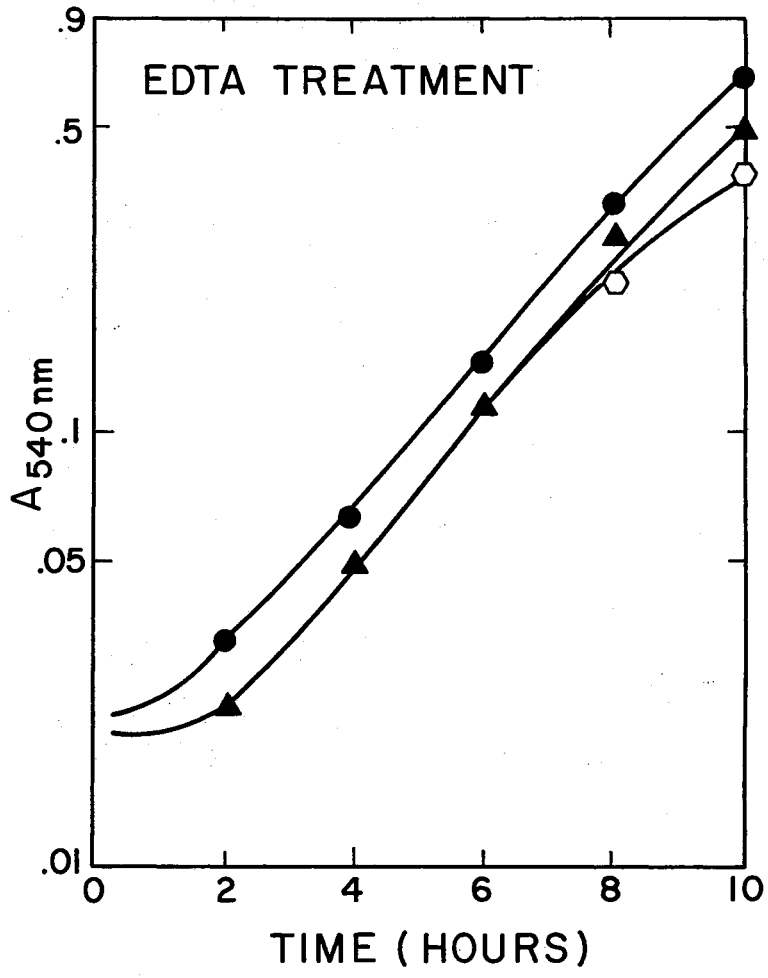
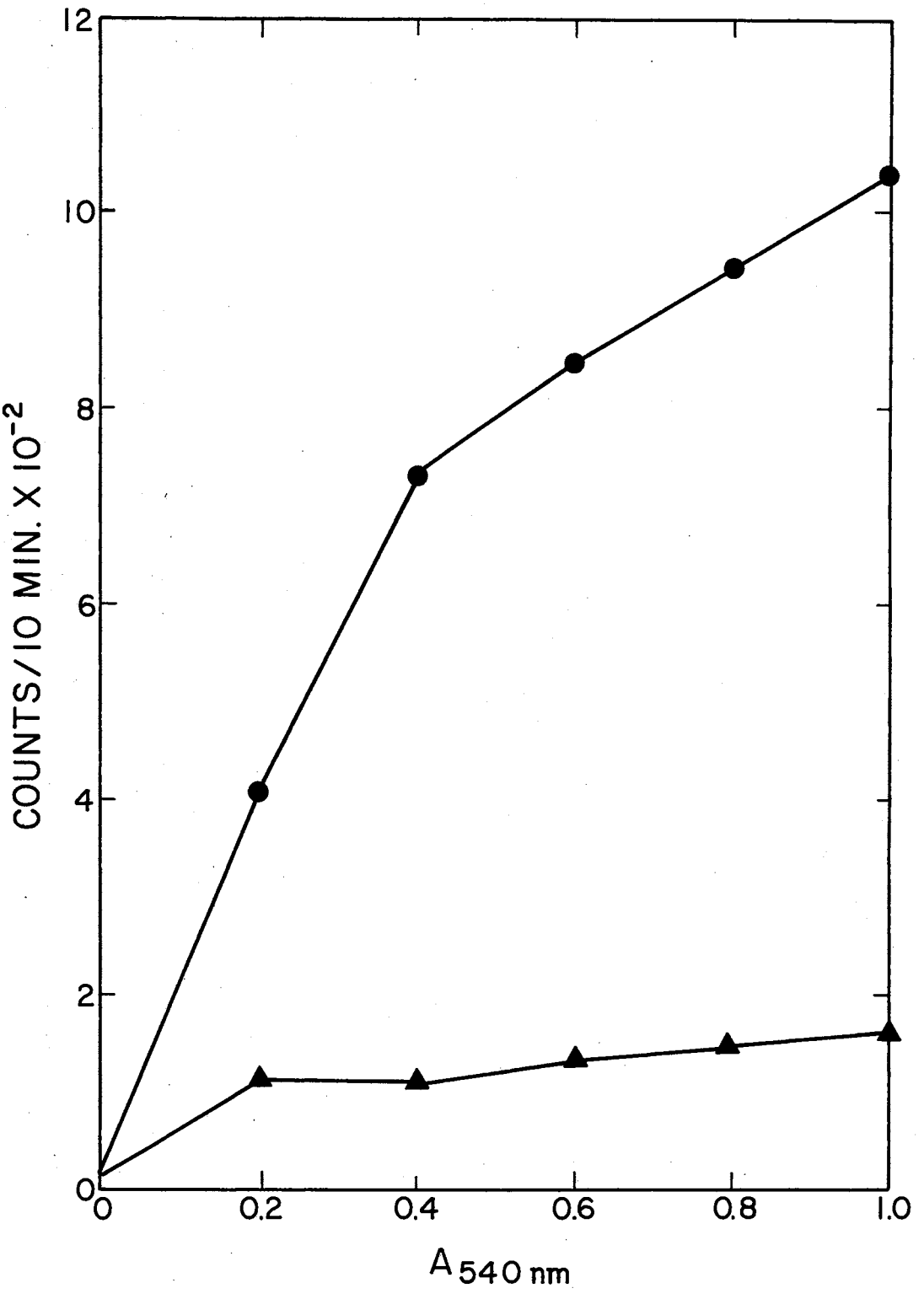

EDTA TREATMENT
.9
.5
.1
.05
.01
$A_{540\,nm}$
0
2
4
6
8
10
TIME (HOURS)

Figure 16. Binding of ^{3}H-Actinomycin D. ●, succinate grown *P. fluorescens*; ▲, glucose grown *P. fluorescens*


12
10
8
6
4
2
0
COUNTS/10 MIN. X 10⁻²
0
0.2
0.4
0.6
0.8
1.0
A 540 nm

to the periphery, but if the assumption is made that the cells have approximately the same surface area, then the difference in binding capacity would have to be explained by (1) a greater permeability of the succinate grown cells, or (2) a difference in the binding properties of the envelope of the two sets of cells. Electron micrographs of the cells revealed only slight differences in cell size, with the glucose grown cells being the larger of the two.

To determine if the difference in the amount of actinomycin D bound was a property of the envelope, similar studies were conducted using isolated envelope material from glucose grown and succinate grown cells. Envelopes prepared from cells grown under both nutritional conditions adsorb closer to the same level of activity when exposed to the labeled antibiotic. Envelopes from succinate grown cells adsorbed 29,129 counts/10 minutes into a pellet obtained by centrifuging 5.0 ml of an envelope suspension having an A_{540}=0.40, while a similar pellet of envelope material from glucose grown cells adsorbed 23,512 counts/10 minutes. This represents a difference of 20 percent compared to 200-300 percent obtained with whole cells.

A study was made to determine the effects of extensive washing on the binding capacity and sensitivity of the two sets of cells. It was found that washing with saline four times often increased the binding capacity of the cells by 10 to 20 percent with a general correlation between increased binding and increased sensitivity to the antibiotic. It was also noted that the preceding responses were accompanied by a decrease of 20 percent or less in the total amount of lipopolysaccharide present as judged by KDO content. Thus, the lipopolysaccharide may be actually masking a quantitatively equivalent number of "binding

sites," with the difference in adsorptive properties in whole cells dependent upon the accessibility of the sites to the antibiotic molecule.

Effect of pH on the Binding of ^{3}H-Actinomycin D

In the course of this investigation it was found that a pH change occurred in the medium during growth of the cells, presumably as a result of metabolic end products released into the medium. When the cells were growing in succinate, the pH increased from 7.0 to 7.8-8.2 by the end of a growth cycle. However, when glucose was the substrate, the pH decreased from 7.0 to 5.8-6.0. This shift in pH might be expected to change the surface charge of the two sets of cells. At an alkaline pH the net negative charge at the surface would be increased.

Cerbón (1970) found in studies done with Nocardia asteroides and Mycobacterium smegmatis that pH increments from 5.0 to 8.5 decreased the line width at half peak height of the lipid signal in the nuclear magnetic resonance spectrum made with whole cells. This decrease signifies an increase in lipid mobility, and there is a concomitant increment in water outflow and leakage of cell constituents. Cerbón interprets these results to mean that an increase in pH will create an increase in permeability.

Since the increment in which the major change in line width occurred (between 7.0 and 8.5) was within the same range as the pH shift in the succinate grown cells, experiments were undertaken to determine whether growth at controlled pH levels would significantly alter the binding properties of the two sets of cells. If the pH shift

was responsible for the increased sensitivity of the succinate grown cells, growing the cells at a lower pH would be expected to decrease their capacity to adsorb actinomycin. The opposite should be true for the glucose grown cells - raising the pH at which they were growing should increase their capacity to adsorb the antibiotic.

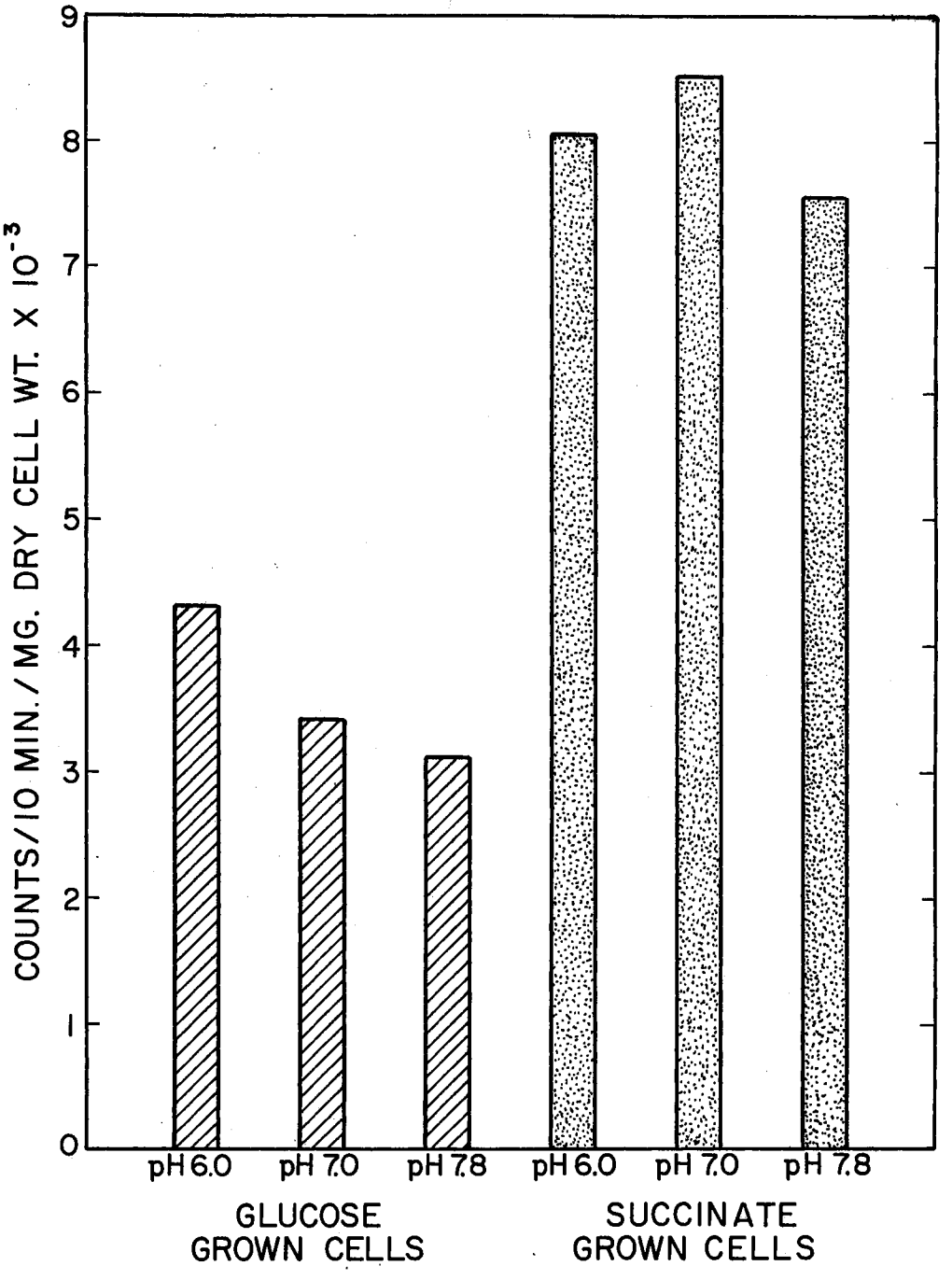
To maintain the pH of the growing cultures at the desired value, a pH-stat was adapted so that addition of 0.5 N HCl to the succinate medium, or 0.5 N NaOH to the glucose medium could be made automatically. The cells were grown in both media at pH 6.0, 7.0, and 7.8, and the amount of base or acid that was added recorded. After the cells had reached late logarithmic stage, they were harvested, and the amount of ^{3}H-actinomycin that the cells adsorbed was determined.

The results (Fig. 17) demonstrate that while there is a small change in the amount of ^{3}H-actinomycin D adsorbed as the pH is increased from 6.0 to 7.8, the succinate grown cells adsorb 2-3 times the amount of antibiotic adsorbed by the glucose grown cells regardless of the pH at which they are grown. Increasing the pH of the growth medium actually decreased the binding capacity of the glucose grown cells, while the succinate grown cells bound the greatest amount of actinomycin D at pH 7.0. Thus the difference in sensitivity cannot be explained on the basis of a pH shift resulting from metabolic products from the carbon sources.

^{3}H-Actinomycin D Binding to Osmotically Shocked Cells

Treatment with EDTA increased the sensitivity of the succinate grown cells, but did not affect the glucose grown cells. Studies were

Figure 17. Binding of ^{3}H-Actinomycin D by P. fluorescens Grown in Succinate or Glucose Minimal Salts Medium at pH 6.0, 7.0, and 7.8


COUNTS/10 MIN./MG. DRY CELL WT. X 10^{-3}
0
1
2
3
4
5
6
7
8
9
pH 6.0
pH 7.0
pH 7.8
pH 6.0
pH 7.0
pH 7.8
GLUCOSE GROWN CELLS
SUCCINATE GROWN CELLS

conducted using osmotic shocking to determine if this difference in sensitivity was related to the removal of essential cellular components. Cells were osmotically shocked by the Heppel (1967) cold shock procedure, using EDTA-sucrose solution followed by an ice cold $MgCl_2$ solution. After the shock treatment, the amount of ^3H-actinomycin D which the cells bound was determined.

Osmotic shocking decreased the amount of ^3H-actinomycin bound by the cells (Fig. 18). Attempts to restore the binding capacity of the cells by addition of shock fluid were not successful. When milligram quantities of lyophilized supernatant from the EDTA treatment were assayed by the diphenylamine test for DNA (Clarke, 1964), it was found that 90-100 percent of the lyophilized material was DNA. The remainder was protein and RNA.

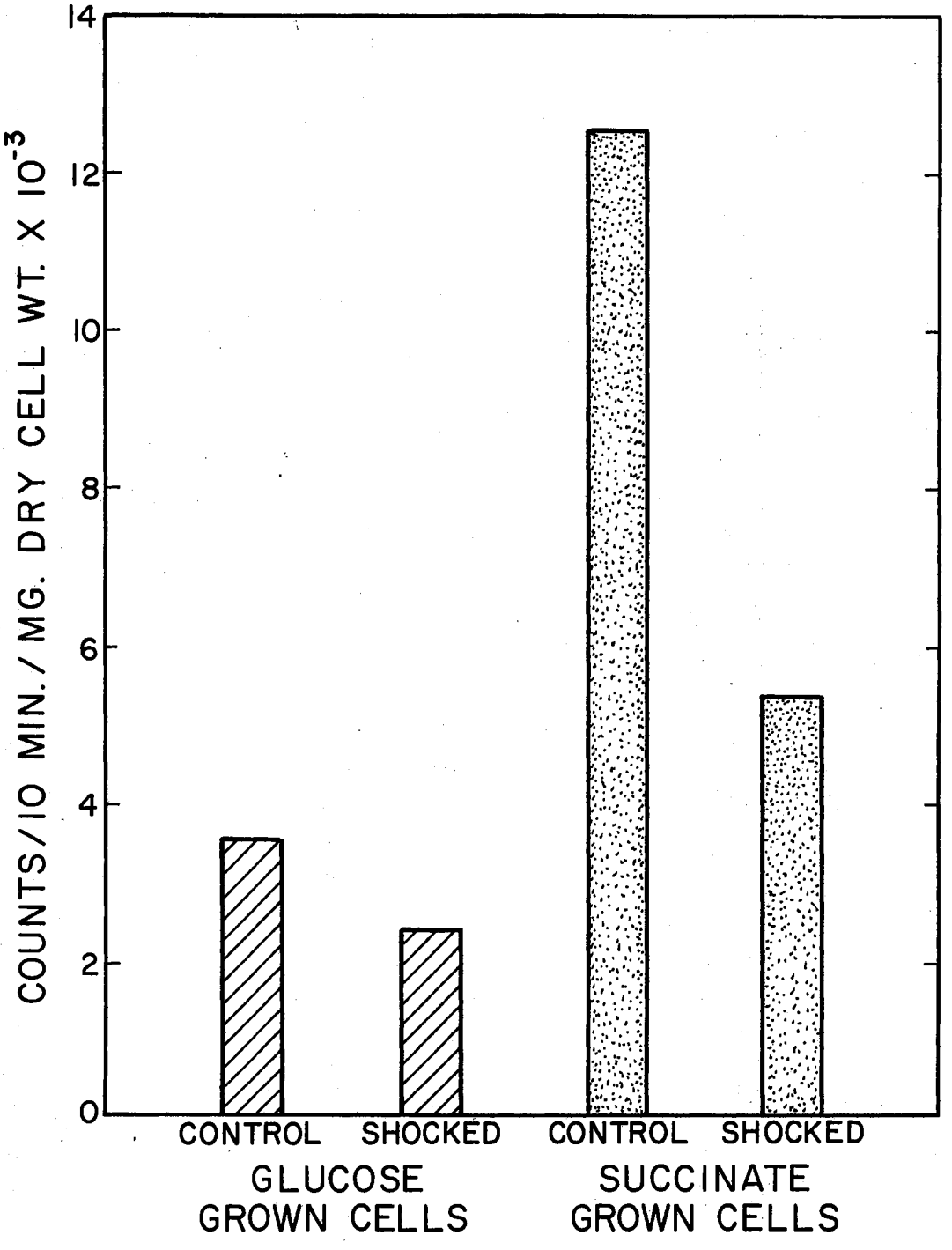
The binding capacity of the succinate grown cells drops almost 60 percent while that of the glucose grown cells drops only 30 percent. These data augment the finding that envelopes isolated from glucose and succinate grown cells approach each other in binding capacity.

Macromolecular Changes Associated With Sensitivity Differences

Qualitative and Quantitative Analyses of Isolated Phospholipids of P. fluorescens Grown in Different Carbon Sources

Lipids, and particularly phospholipids, have been implicated as contributing to the permeability function of the membrane. Accordingly, the phospholipid components of the glucose grown and succinate grown

Figure 18. Effect of Osmotic Shock on ^{3}H-Actinomycin D Binding by P. fluorescens


14
12
10
8
6
4
2
0
COUNTS/10 MIN./MG. DRY CELL WT. X 10^{-3}
CONTROL
SHOCKED
CONTROL
SHOCKED
GLUCOSE GROWN CELLS
SUCCINATE GROWN CELLS

cells were assayed. Phospholipids were extracted from whole cells by the Folch, Lees, and Sloane (1957) procedure and analyzed qualitatively and quantitatively. The major phospholipids extracted and identified were phosphatidylethanolamine and phosphatidylglycerol, along with a small amount of free glycerol. Detection reagents were iodine, rhodamine G, ninhydrin, and phosphomolybdate. Identifications were made by comparison to known Rf values, and in the case of phosphatidylethanolamine, comparison with a known standard.

Both glucose and succinate grown P. fluorescens cells contained these components, along with a large rhodamine G-positive spot that traveled to the front in all the solvent systems employed. There were no apparent qualitative differences between the two sets of cells.

Quantitative analyses were performed through the use of acetate-1-^{14}C, which was incorporated during growth into the phospholipid portion of the cell. The phospholipids were extracted from the labeled cells as described above, and the components separated by chromatography, the identified spots scraped from the plates, and radioactivity determined.

Approximately 20-30 percent of the label was incorporated as phosphatidylglycerol, 70-80 percent as phosphatidylethanolamine, and 1 percent remained as free glycerol (Table II). Less than 1 percent of the total activity was incorporated into the unidentified front-moving spot. These results were constant whether the cells were grown in glucose or in succinate, the ratios between components remaining very close to the same in both sets.

If the total activity incorporated into the two sets of cells is compared, the cells growing in glucose incorporated approximately 30

TABLE II

QUALITATIVE AND QUANTITATIVE ANALYSES OF ISOLATED PHOSPHOLIPIDS FROM GLUCOSE AND SUCCINATE GROWN *P. FLUORESCENS*

	Rf	Rho G	PO_4	Ninhydrin	I_2	Counts/10 Min.	% Total
Succinate grown *P. fluorescens*	.471 (PG)	+	+	-	+	24,970	29
	.606 (PE)	+	+	+	+	59,975	69
	.677 (UK)	+	-	-	+	1,560	2
	.971 (UK)	+	-	-	+	698	1
Glucose grown *P. fluorescens*	.477 (PG)	+	+	-	+	31,243	24
	.589 (PE)	+	+	+	+	92,904	72
	.656 (UK)	+	-	-	+	3,534	3
	.971 (UK)	+	-	-	+	836	1

$$\frac{\text{Total counts incorporated into succinate grown cells}}{\text{Total counts incorporated into glucose grown cells}} =$$

$$\frac{24,970 + 59,975 + 1,560 + 698}{31,243 + 92,904 + 3,534 + 836} = 0.6785$$

Phosphatidylglycerol: PG
Phosphatidylethanolamine: PE
Unknown: UK

percent more of the labeled acetate into their phospholipid. This could imply either that the turnover rate in the phospholipid of the glucose grown cells is much higher than the turnover rate in succinate grown cells, or that the cells growing in glucose deposit a greater amount of phospholipid in their outer layers. However, results must be interpreted with caution since the dilution factor for acetate in the two sets of cells is not known, and the difference observed may be a result of a greater dilution of the ^{14}C-acetate when succinate is the carbon source. If glucose grown cells do synthesize phospholipids more readily, this elevated phospholipid content could be related to the decreased level of sensitivity in the glucose grown cells by preventing the penetration of actinomycin D into the cell.

Binding of ^{3}H-Actinomycin D to Isolated Phospholipid

Results from the adsorption experiments indicated that some actinomycin D was binding to the peripheral area of the cell, and the phospholipid component would be a logical candidate for the binding site. The actinomycin molecule has a large cyclic ring complex, and would be expected to have a hydrophobic center as well as the hydrophilic center associated with the peptide side chains. Thus it could form a complex with the similarly amphipathic phospholipid molecules present in the membrane.

Experiments were conducted to determine whether the actinomycin molecule would bind to isolated phospholipid. ^{3}H-Actinomycin was overspotted on the extracted phospholipids from both sets of cells and a number of solvent systems employed to separate the components. These

included (1) chloroform:methanol:HOH (65:25:5), (2) chloroform: methanol:NH_4OH (75:25:2), and (3) butanol:acetic acid:HOH (60:15:25). The migration of the isotope was measured on the Packard strip counter adapted to counting chromatography plates.

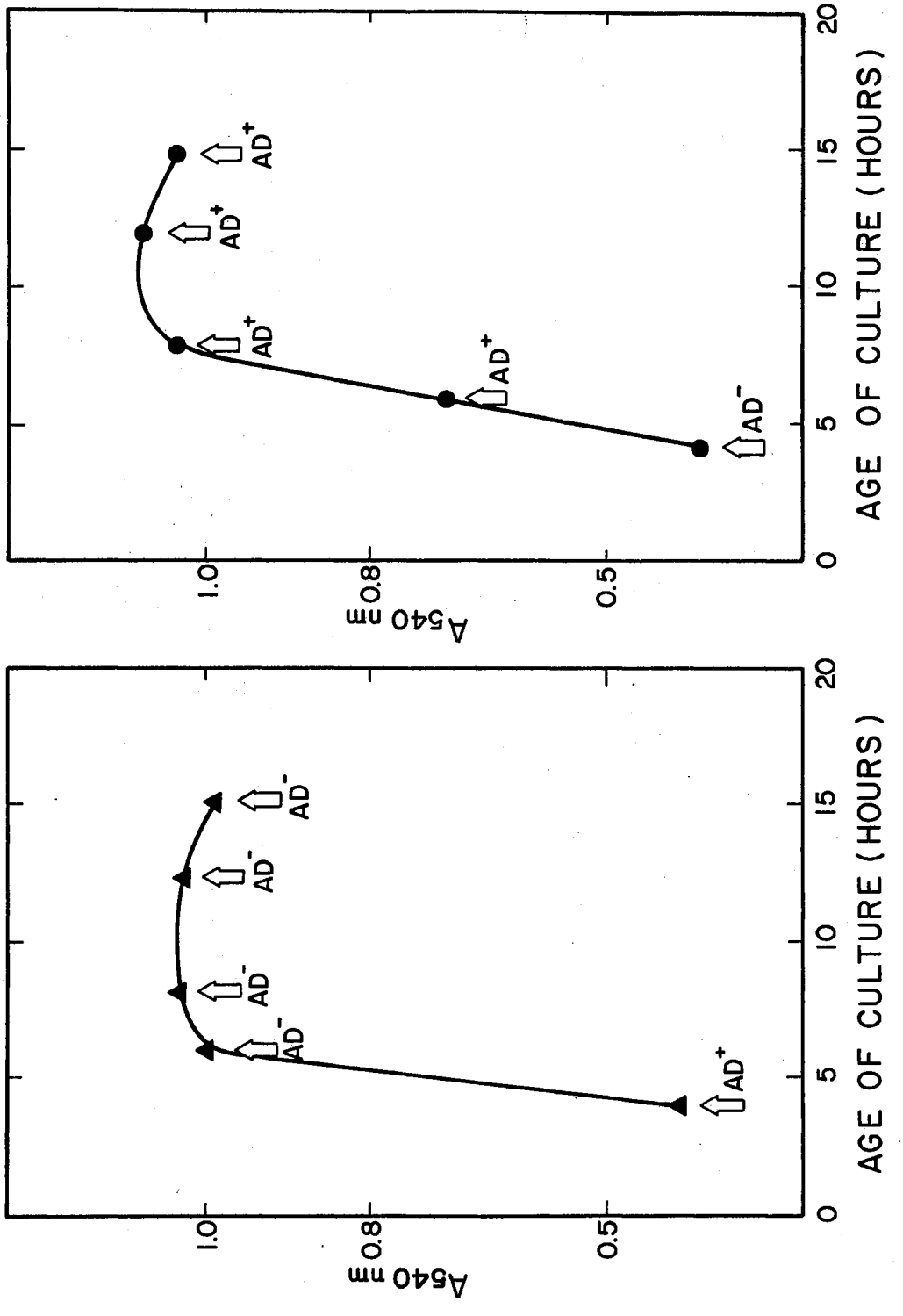
There was no evidence to support any form of binding between the antibiotic and the different phospholipid components; the radioactivity traveled to the front in all the solvents used, and the Rf values of the phospholipids were identical on control and experimental chromatograms.

Antibiotic Sensitivity Versus Time During the Course of a Growth Cycle

Because the cells demonstrated a variability in their sensitivity to actinomycin D within the two nutritional groups, studies were performed to determine at what point in the growth cycle each set of cells was the most sensitive to the antibiotic. The inoculum was grown in side-arm flasks, the absorbency monitored, and samples were removed aseptically at various intervals during the growth cycle. The samples were centrifuged, suspended in sterile buffer to A_{540}=0.40, and used as the inoculum for growth experiments employing the appropriate carbon source and the antibiotic.

Glucose grown cells showed the greatest degree of sensitivity during the early logarithmic phase (Fig. 19). The early logarithmic phase cells exhibited an extremely long lag (8-12 hrs) before initiation of growth in the presence of actinomycin D and did not reach the same final density as did the control cells and cells from the other phases of the growth cycle. Cells used as the inoculum which were

Figure 19. Sensitivity to Actinomycin D as a Function of Culture Age ▲, glucose grown cells; ●, succinate grown cell. AD^+ denotes increased sensitivity; AD^- denotes decreased sensitivity.


A540 nm
1.0
0.8
0.5
0
5
10
15
20
AGE OF CULTURE (HOURS)
AD+
AD-

taken at the time intervals later in the growth cycle became increasingly less sensitive to the inhibitory effects of actinomycin D, with the cells from stationary phase showing almost no sensitivity.

Succinate grown cells showed a completely opposite picture. The least degree of sensitivity occurred when cells taken from the early logarithmic phase were used as the inoculum, and the sensitivity increased as the culture aged. In stationary phase, the cells were extremely sensitive to the antibiotic; there was approximately 90 percent inhibition of growth in the tubes containing 30 μg/ml actinomycin D, and the cells appeared extremely abnormal when examined microscopically.

Quantitation of Thiobarbituric Acid-Positive Material From P. fluorescens

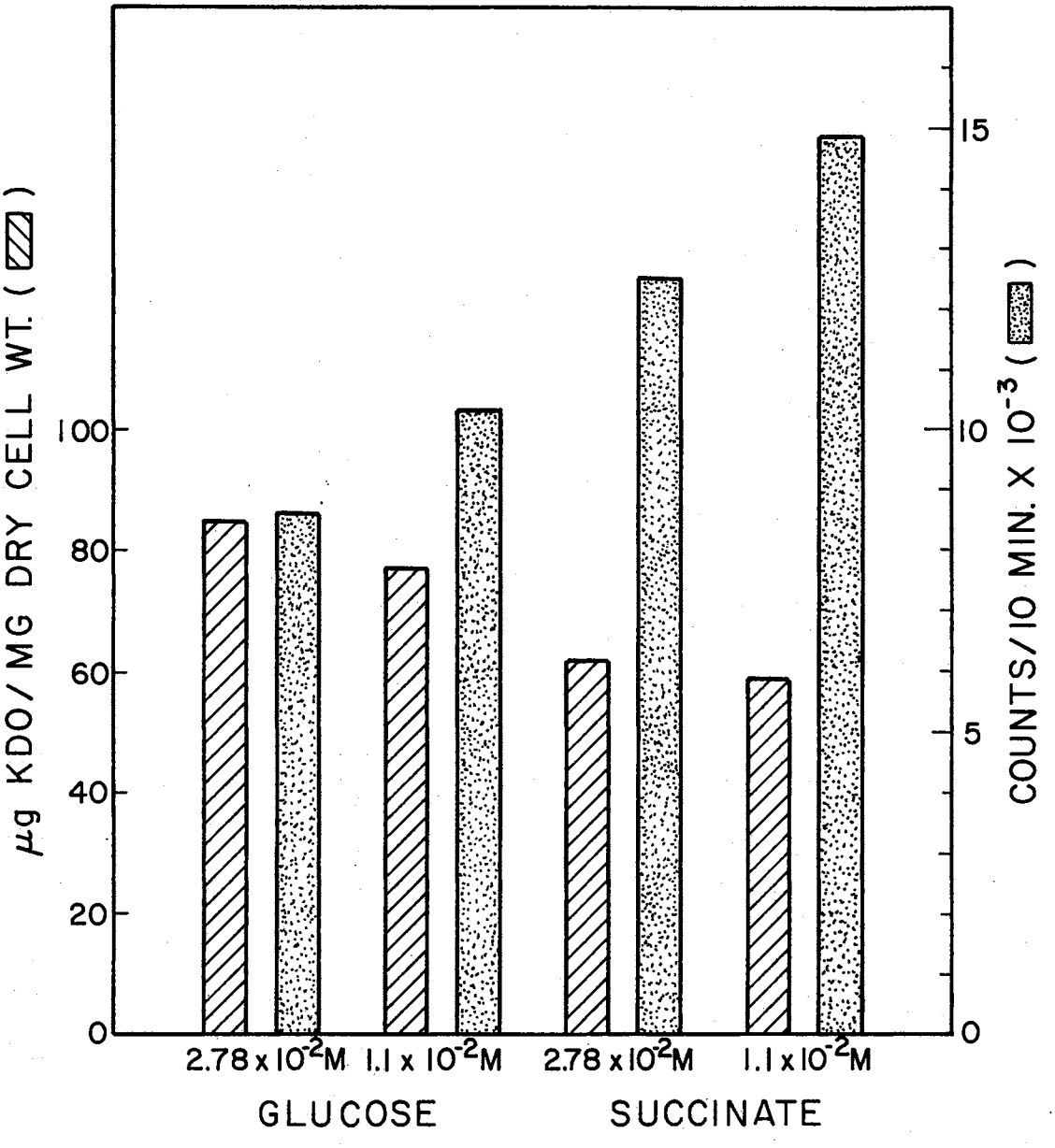
The thiobarbituric acid assay (Cynkin and Ashwell, 1959) can be used to measure deoxysugars present in the cell. The reactive compounds include 2-keto-3-deoxyoctosonic acid (KDO), which forms the backbone of the R core of the lipopolysaccharide; colitose, peritose, and similar dideoxysugars which are found attached to the O-polysaccharide, and the deoxyribose of DNA. The assay involves the formation of a colored product between thiobarbituric acid and the cleavage products formed during periodate oxidation of the deoxysugars. In the case of KDO, the oxidative product is β-formylpyruvate, and the colored product with thiobarbituric acid is readily broken down by the addition of saturated NaOH to the assay. The cleavage product formed during periodate oxidation of the deoxysugars is malonaldehyde, and the colored product formed with thiobarbituric acid is stable in the presence

of the NaOH. This difference in stability of the colored products to saturated NaOH allows a gross measurement of the deoxysugars of the cell along with a technique for determining the KDO level, which in turn can be related to the amount of lipopolysaccharide present in a given cell mass.

Cells grown to the maximum stationary phase in glucose or succinate (1.1×10^{-2} M and 2.78×10^{-2} M) were suspended to the desired absorbency in saline. A 10 ml sample of the cell suspension was centrifuged, and the pellet hydrolyzed in 1.0 ml of 0.1 N H_2SO_4 for 30 minutes. The results (Fig. 20) revealed a stepwise increase in the amount of thiobarbituric acid-positive material from the most sensitive cells (those grown in 1.1×10^{-2} M succinate) to the least sensitive cells (those grown in 2.78×10^{-2} M glucose). The cells grown in 1.1×10^{-2} M succinate had 59.0 μg KDO/mg dry cell weight, while cells grown in 2.78×10^{-2} M succinate showed an increase in KDO content to 61.4 μg/mg dry cell weight. When the cells were grown in 1.1×10^{-2} M glucose, the concentration of KDO was 77.0 μg/mg dry cell weight, and if 2.78×10^{-2} M glucose was the substrate, the cells show a level of 84.5 μg KDO/mg dry cell weight. The cells grown in glucose (1.1×10^{-2} M) showed an increase in KDO of approximately 30 percent over the cells grown in an equimolar concentration of succinate. Similar results were observed with the higher substrate concentration. These data establish that growth in glucose increases the total amount of lipopolysaccharide in the envelope.

A ^{3}H-actinomycin D binding study was performed simultaneously with the above KDO assay, and the results correlate with an increase in lipopolysaccharide resulting in decreased permeability. The cells

Figure 20. Thiobarbituric Acid and ^{3}H-Actinomycin D Assays on P. fluorescens Grown in Different Concentrations of the Carbon Source. Cells were grown in glucose (1.1×10^{-2} M, 2.78×10^{-2} M) and succinate (1.1×10^{-2} M, 2.78×10^{-2} M).


μg KDO/MG DRY CELL WT. (▨)
COUNTS/10 MIN. X 10⁻³ (▩)
0
20
40
60
80
100
0
5
10
15
2.78 x 10⁻²M
1.1 x 10⁻²M
2.78 x 10⁻²M
1.1 x 10⁻²M
GLUCOSE
SUCCINATE

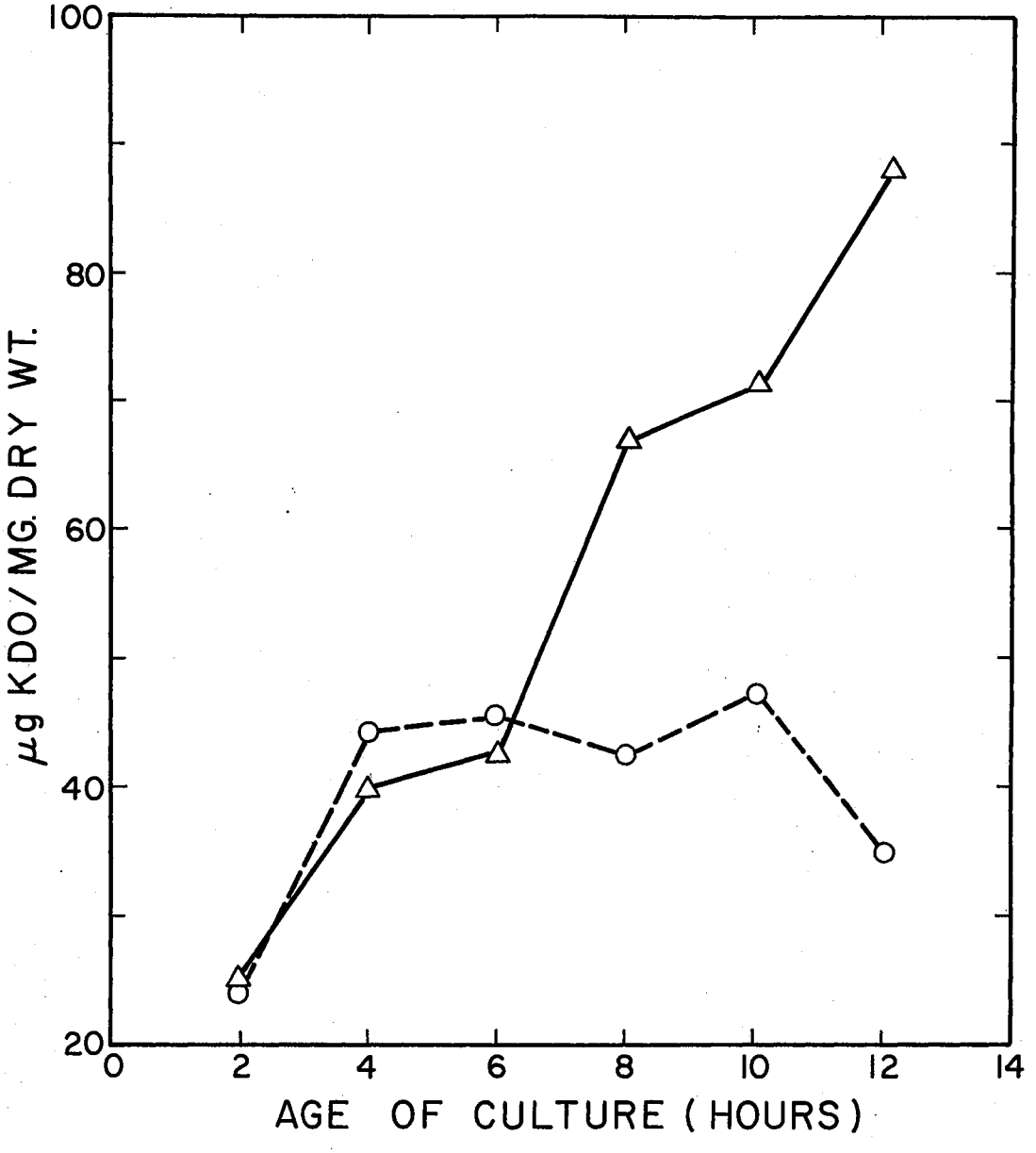
grown in the lower concentration of succinate bound the largest amount of ^{3}H-actinomycin D. A consecutive decrease in the bound antibiotic occurred from the higher concentration of succinate to the lower concentration of glucose, and finally to the higher concentration of glucose. The glucose (2.78×10^{-2} M) grown cells had the highest lipopolysaccharide concentration and adsorbed the lowest concentration of ^{3}H-actinomycin D. This inverse relationship between KDO and binding capacity would result if the lipopolysaccharide concentrations of the cell were affecting the permeability of the cells for the antibiotic. Lipopolysaccharide has been repeatedly linked to cellular permeability, but only indirect evidence exists to suggest that it is actually involved in the phenomenon. However, the increase in KDO, and thus lipopolysaccharide, in the glucose grown cells as compared to the succinate grown cells is the first definite evidence that a macromolecular change is occurring which can be directly related to permeability.

Lipopolysaccharide Synthesis During the Growth Cycle and Its Relation to Actinomycin D Sensitivity

Since sensitivity to actinomycin D varies according to the phase of the cell growth cycle, a determination of KDO concentration was made in the cells taken at similar stages to those in the antibiotic sensitivity studies.

P. fluorescens cells growing in either glucose or succinate showed an increase in the concentration of KDO as the cells progressed from early logarithmic stage into stationary stage (Fig. 21). However, the increase in KDO in the cells growing in glucose continued far into

Figure 21. Thiobarbituric Acid Assay of P. fluorescens Sampled at Intervals Along a Growth Cycle ○, μg KDO/mg dry cell weight, succinate grown cells; △, μg KDO/mg dry cell weight, glucose grown cells.


100
80
60
40
20
μg KDO/MG. DRY WT.
0
2
4
6
8
10
12
14
AGE OF CULTURE (HOURS)

stationary phase and reached a much higher final concentration than the KDO in cells growing in succinate. The succinate grown cells increased in KDO concentration at a similar rate during the early phase of growth, but never reached the level of the cells grown in glucose. There is often even a drop in the level of KDO concentration in the succinate grown cells after they have reached the late logarithmic stage of growth. The only time when the level of KDO in the succinate grown cells approached that of the glucose grown cells was in the early logarithmic stage, and this was soon reversed by the more extensive accumulation in the glucose grown cells. If the KDO content of the cells were proportional to the amount of lipopolysaccharide, the increase in that component implies that there is an increase in the amount of lipopolysaccharide in the cell envelope.

When these results are compared to the results from the antibiotic sensitivity studies (Fig. 19, page 92), there is a high degree of correlation between sensitivity to actinomycin D and the apparent lipopolysaccharide concentration in the cell envelope. The only time in the growth cycle of P. fluorescens when the succinate grown cells were less sensitive to actinomycin D than the glucose grown cells was in the early logarithmic stage. This is also the time period in which the level of lipopolysaccharide in the succinate grown cells approaches or exceeds the level in the glucose grown cells. Otherwise, the glucose grown cells are less sensitive and contain higher levels of lipopolysaccharide in their envelope. Also, the succinate grown cells became increasingly sensitive as they moved from late logarithmic into stationary phase, and this increase corresponds to the drop in the level of cellular lipopolysaccharide.

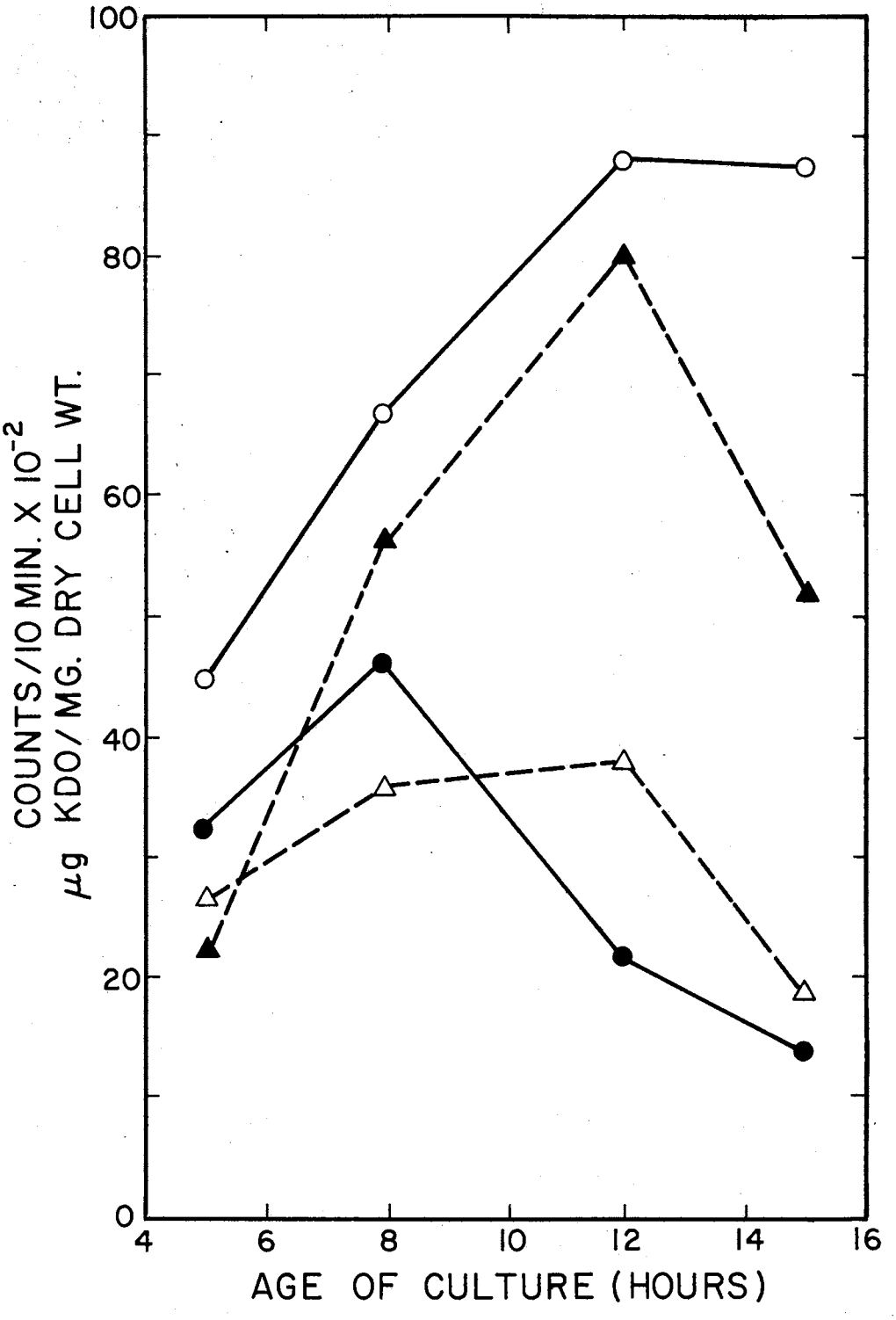
Lipopolysaccharide Levels and ^{3}H-Actinomycin Binding Capacities of Glucose and Succinate Grown Cells

If an increase in lipopolysaccharide were the antecedent of a decrease in sensitivity, then a similar decrease in the amount of ^{3}H-actinomycin D bound into the cells could be expected. In order to test this hypothesis, a thiobarbituric acid assay and a ^{3}H-actinomycin D binding assay were run simultaneously on cells sampled at the appropriate intervals along the growth curve.

Although there does not seem to be absolute correlation between KDO concentration and binding capacity, results of this experiment indicated that a loose relationship does exist between the two characteristics (Fig. 22). The succinate grown cells bind the least amount of ^{3}H-actinomycin D when they are in early logarithmic phase, and this is the time that the lipopolysaccharide level is often higher than in the glucose grown cells, and the only time at which the succinate grown cells do exhibit reduced sensitivity. At this point in the growth cycle, the glucose grown cells bind a higher level of ^{3}H-actinomycin D and show an increased sensitivity to the antibiotic as well.

As the binding capacity increases in the cells growing in succinate, there is still a slow increase in the lipopolysaccharide level. However, by the time that late logarithmic stage is reached, the lipopolysaccharide level either has stabilized or, as more often is the case, has begun to drop, while the binding capacity of the cells continues to increase.

Figure 22. Thiobarbituric Acid and ^{3}H-Actinomycin D Binding Assays on P. fluorescens Sampled at Intervals Along a Growth Cycle. ●, μg KDO/mg dry cell weight, succinate grown cells; ○, μg KDO/mg dry weight, glucose grown cells; ▲, radioactivity bound, succinate grown cells; △, radioactivity bound, glucose grown cells.


100
80
60
40
20
0
4
6
8
10
12
14
16
COUNTS/10 MIN. X 10^{-2}
μg KDO/MG. DRY CELL WT.
AGE OF CULTURE (HOURS)

In the glucose grown cells, the amount of activity bound remains reasonably constant, with only a slight increase as the cells progress toward late logarithmic stage. At this point, the lipopolysaccharide level increases to its maximum, and the binding capacity of the cells for ^{3}H-actinomycin D drops to its lowest level.

The fact that there is not a direct correspondence between sensitivity, lipopolysaccharide content and binding capacities may be partially explained by the fact that the simple binding of the antibiotic to the cell does not mean that it will reach the interior of the cell and act as an effective inhibitor. Evidence supports the conclusion that much of the antibiotic bound by the cell never crosses the permeability barrier to bind with DNA, and although a specific binding site has not been identified, it is reasonable to assume that changes occurring in the periphery of the cell could influence the amount of antibiotic which would bind to it. In general, an increase in lipopolysaccharide of the cell decreases both sensitivity to actinomycin D and binding of the antibiotic to the cells.

Release of KDO From the Surface of *P. fluorescens* by EDTA Treatment

Treatment with EDTA increased the sensitivity of succinate grown cells to actinomycin D but did not affect the sensitivity of glucose grown cells to any significant extent. EDTA releases lipopolysaccharide from the surface of many of the enterobacteria. *P. fluorescens* cells were examined to determine if EDTA treatment effected a similar release of lipopolysaccharide.

The cells were grown to late logarithmic stage in succinate and in glucose minimal medium and were treated with either 2×10^{-4} M EDTA or saline for a period of 3 minutes at 37 C. The treated and control cells were centrifuged, suspended in saline, and the KDO level determined. EDTA treatment released KDO from both glucose and succinate grown cells. However, the succinate grown cells released 35 percent of their total KDO, while the glucose grown cells released only 17 percent. Since the glucose grown cells have a higher lipopolysaccharide content than the succinate grown cells, the difference in the amount of release between the two sets of cells could well account for the notable difference in their response to EDTA treatment as manifested by their sensitivity to actinomycin D. The EDTA treated succinate grown cells have a greatly decreased level of lipopolysaccharide when compared to the EDTA treated glucose grown cells, making them more readily permeated by the antibiotic. Even though release of lipopolysaccharide does occur in the glucose grown cells, it is not as extensive, and the amount remaining is sufficient to form a functional barrier to the antibiotic molecule.

Effect of Incubation Temperature on KDO Levels of Cells Growing in Succinate or Glucose Minimal Medium

Since P. fluorescens has a variable sensitivity to actinomycin D depending upon the temperature (and consequently the growth rate) at which the cells were incubated, assays were performed to establish the KDO level of cells growing at 30 C and at 37 C. Samples were removed at the early logarithmic stage, the late logarithmic stage, and at the

early stationary stage and assayed for KDO by the thiobarbituric acid procedure.

Results (Fig. 23) indicate that there was a slight increase in the amount of KDO per milligram dry cell weight in the cells grown at 30 C as compared to those grown at 37 C. The greatest difference occurred in stationary phase, and the increase was present whether the cells were growing in glucose or succinate minimal medium. The cells growing at 30 C were shown to be less sensitive to actinomycin D, and the slight increase in KDO may be related to that reduced sensitivity.

Isolation and Characterization of P. fluorescens AD+

A gradient plating technique was employed in an effort to isolate a mutant resistant to actinomycin D. Cells were streaked repeatedly on a gradient of the antibiotic ranging from 0-50 μg/ml in succinate agar medium, and those cells which grew at the highest concentration were transferred to a second plate containing 75 μg/ml actinomycin D. The colonies that developed in the highest actinomycin D concentration were transferred to succinate agar slants, and grown in the absence of the antibiotic through three transfers. When these cells were tested for sensitivity to actinomycin D (Fig. 24, 25), they were less sensitive than the parent culture to the antibiotic whether they were growing in succinate or glucose minimal salts, and the strain was designated *P. fluorescens* AD+. A concentration of 30 μg/ml actinomycin D decreased the total cell mass by less than 20 percent as compared to almost a 90 percent decrease in the wild type organism when succinate was the carbon source. A similar concentration of antibiotic in the glucose

Figure 23. Thiobarbituric Acid Assay for KDO of P. fluorescens Grown in Glucose or Succinate Minimal Salts Medium at 30 C or at 37 C
(1) sample removed at early logarithmic phase;
(2) sample removed at mid logarithmic phase;
(3) sample removed at late logarithmic phase.

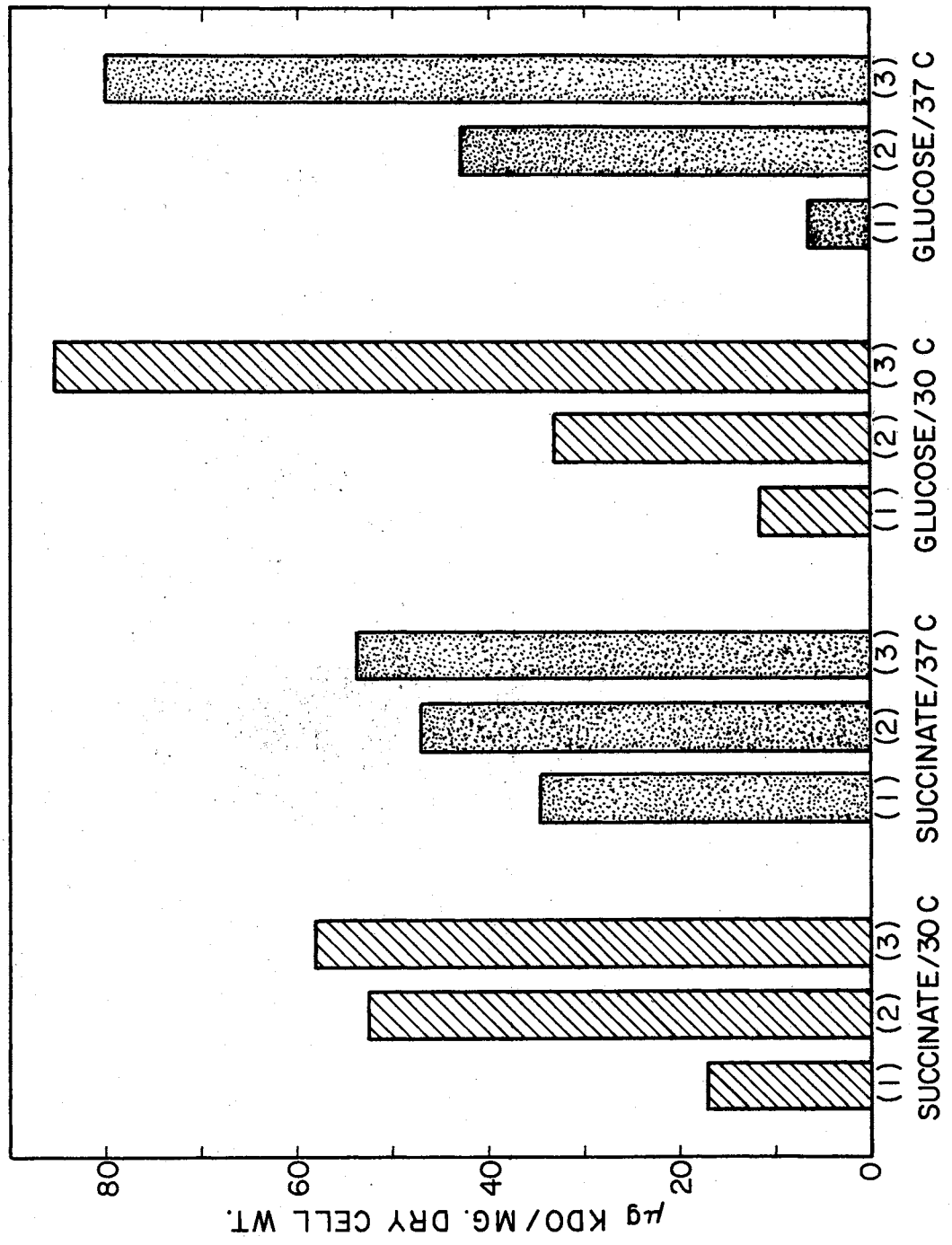

μg KDO/MG. DRY CELL WT.
0
20
40
60
80
SUCCINATE/30 C
(1) (2) (3)
SUCCINATE/37 C
(1) (2) (3)
GLUCOSE/30 C
(1) (2) (3)
GLUCOSE/37 C
(1) (2) (3)

Figure 24. Effect of Actinomycin D on Growth of P. fluorescens AD+ in Succinate Minimal Salts Medium ●, control; ▲, 10 μg/ml actinomycin D; ■, 20 μg/ml actinomycin D; ⬡, 30 μg/ml actinomycin D.

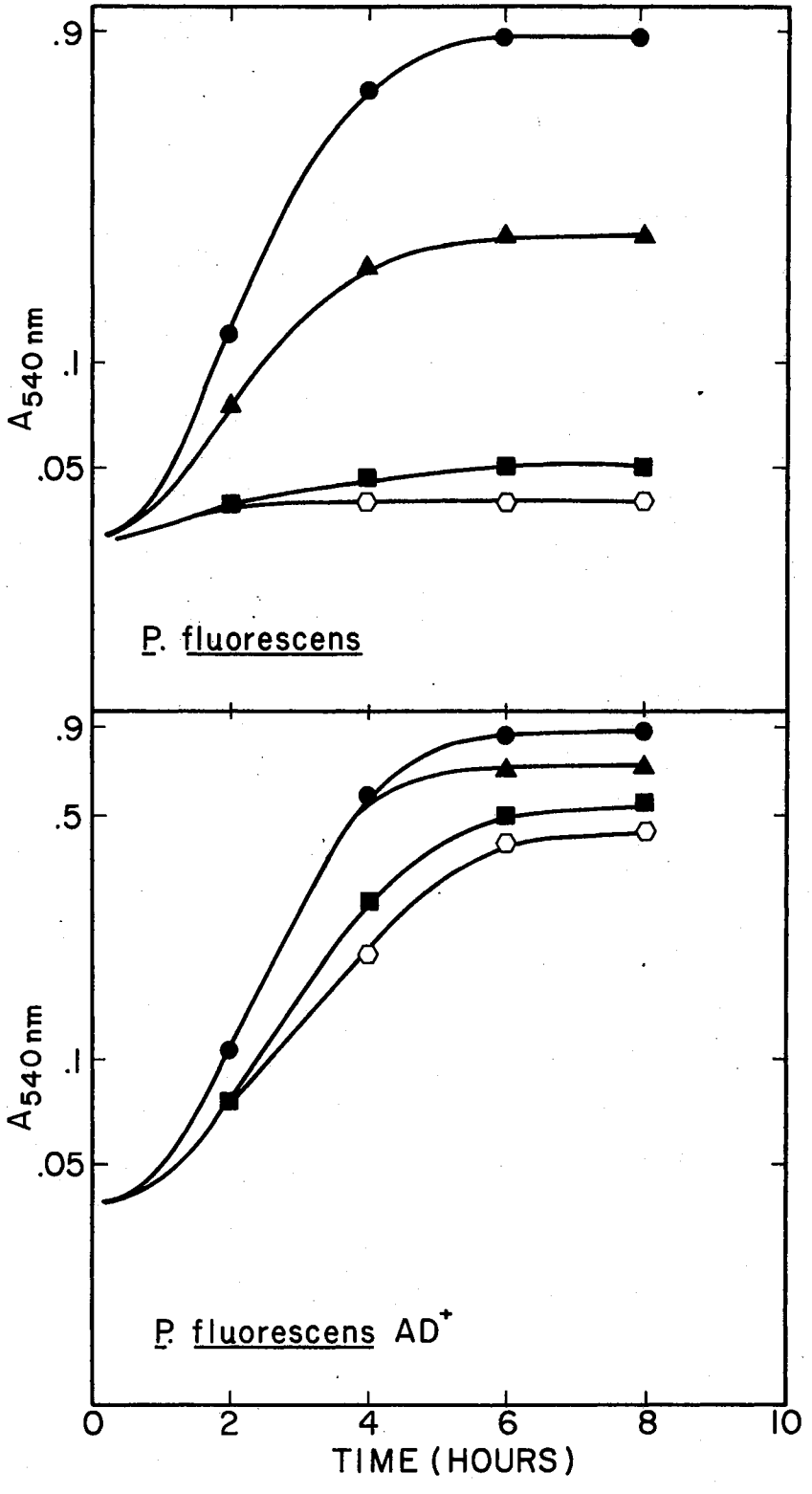
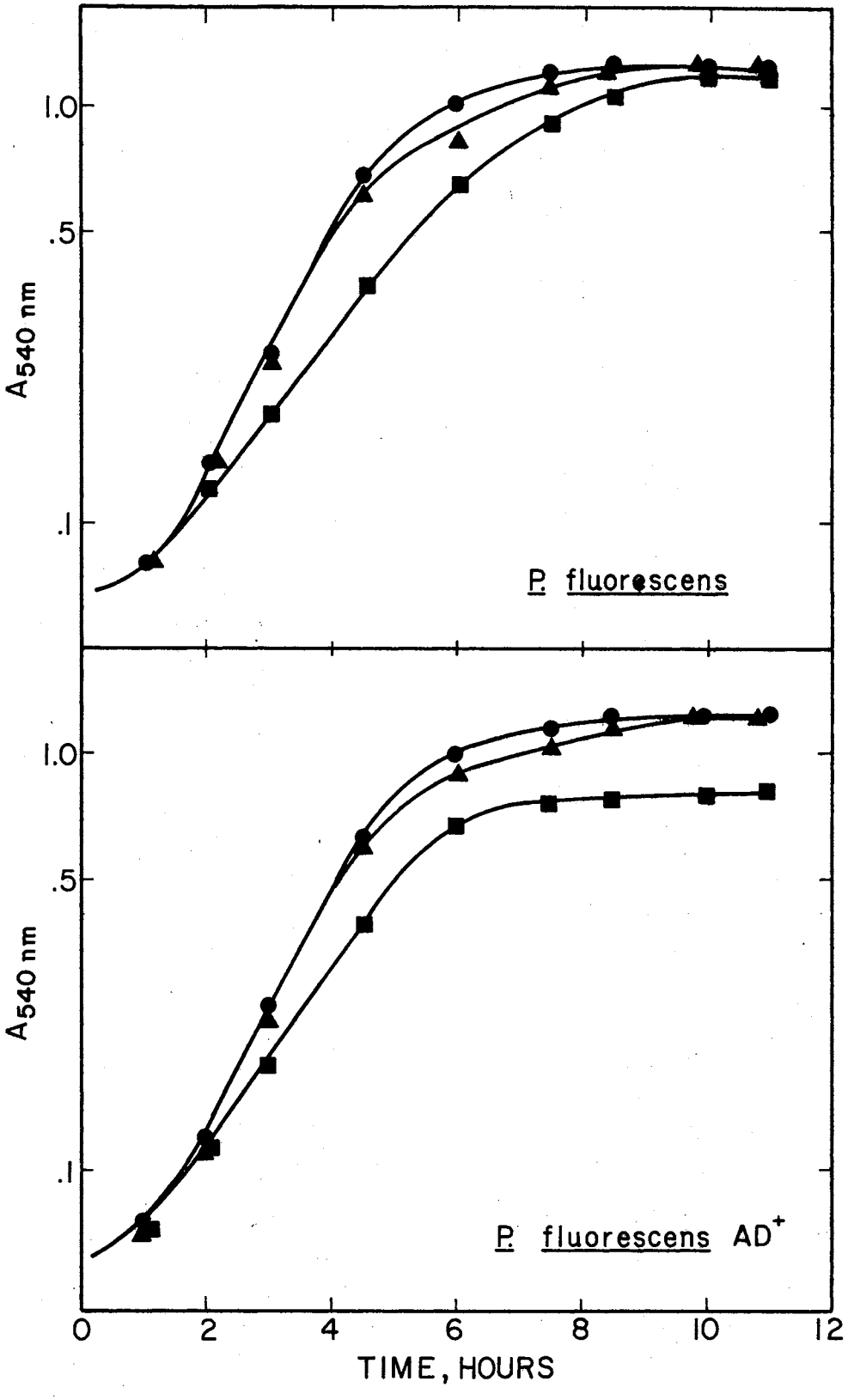

.9
.1
.05
$A_{540\,nm}$
P. fluorescens
.9
.5
.1
.05
$A_{540\,nm}$
P. fluorescens AD+
0
2
4
6
8
10
TIME (HOURS)

Figure 25. Effect of Actinomycin D on Growth of P. fluorescens AD+ in Glucose Minimal Salts Medium ●, control; ▲, 10 and 20 μg/ml actinomycin D; ■, 30 μg/ml actinomycin D.


1.0
.5
.1
A540 nm
P. fluorescens
1.0
.5
.1
A540 nm
P. fluorescens AD+
0
2
4
6
8
10
12
TIME, HOURS

medium decreased total cell mass by only 4.5 percent in the P. fluorescens AD+ cells as compared to 35 percent in the parental strain. These data establish that P. fluorescens AD+ possessed genetic resistance to actinomycin D. Although the mutation causes reduced sensitivity, P. fluorescens AD+ responds to the two carbon sources in a manner similar to the parental strain, since growth in glucose still promotes less sensitivity to the antibiotic than does growth in succinate.

Acridine orange is a dye used to destroy episomal structures which often are responsible for drug resistance in bacteria. To determine if the mutation to actinomycin D resistance might be associated with an episome, P. fluorescens AD+ cells were grown in succinate medium containing 50 μg/ml acridine orange and then transferred to a succinate agar slant. Growth studies with the two minimal media and actinomycin D indicated that no change had occurred in the resistant organism's sensitivity level to the antibiotic after growth in acridine orange. The cells grown in the presence of acridine orange exhibited the same degree of sensitivity as the untreated cells. The P. fluorescens AD+ cells growing in glucose and succinate medium were inhibited by the acridine orange to a considerably less degree than the wild type, indicating that the mutation produced a generalized resistance that might be associated with permeability.

P. fluorescens AD+ cells show normal size and staining characteristics when examined microscopically. When streaked on Pseudomonas agar pyocyanin and Pseudomonas agar fluorescin (Difco), both the wild type and mutant produced fluorescin and pyocyanin. Cultural characteristics remained the same. When the colonial morphology of P. fluorescens AD+ was compared to that of the parent strain, the colonies

appeared to be more mucoid, particularly on succinate agar.

Tests were made to determine whether P. fluorescens AD+ was altering the antibiotic to a non-inhibitory form. A butanol extraction was performed on P. fluorescens AD+ grown in glucose or succinate and incubated in the labeled antibiotic (1 μc/ml) at 37 C for 5 hours. When the extracts were chromatographed and scanned for radioactivity, there was no evidence that an alteration was occurring in the actinomycin moledule. An examination of the basal metabolic rate in the presence of actinomycin D by Warburg respirometry confirmed that the antibiotic was not being oxidized by the resistant mutant.

Uptake experiments conducted with P. fluorescens AD+ revealed a striking dissimilarity from the parental strain when succinate grown cells were examined. A significant stimulation in uptake occurred when P. fluorescens AD+ cells were grown in antibiotic-containing succinate medium and the uptake of succinate and phenylalanine observed, as opposed to the complete inhibition established by growth of the parent strain in the presence of the antibiotic. Also, the presence of actinomycin D in the uptake solution enhanced uptake in control cells and cells grown in the presence of actinomycin D above the level of uptake when no actinomycin D was present. Glucose grown P. fluorescens AD+ cells did not exhibit this stimulatory effect, but rather appeared to behave much as the glucose grown P. fluorescens cells. Stimulation of uptake in the succinate grown P. fluorescens AD+ cells did not prove to be a general phenomenon, for uptake of alanine was similar to that of the parental strain.

Manometric analyses of the oxidation of glucose and succinate by P. fluorescens AD+ cells were performed in a similar manner to the

previous experiments with the parent strain. There were only slight differences from the results presented for P. fluorescens. The mutant did appear to be less sensitive to the effects of actinomycin D, particularly when the cells were grown in the presence of the antibiotic, but in general there were no outstanding or significant differences. The major factor controlling respiratory response to actinomycin D seemed to be the influence of the carbon source on which the cells were grown.

The same situation proved to be true when phospholipids from P. fluorescens AD+ cells were examined; the carbon source was still the most influential factor regarding any changes in the cells (Table III). The two phospholipids phosphatidylethanolamine and phosphatidylglycerol were present in the same ratio as in the parental strain. There was a slight increase in total activity incorporated into the phospholipid as compared to the level in the parental strain. This increase was particularly notable when succinate was the carbon source.

The binding capacities of P. fluorescens AD+ cells for ^{3}H-actinomycin D are surprisingly close to those of the parent strain; occasionally the cells will even bind more actinomycin than do the wild type cells. These results indicate that the binding of the antibiotic does not necessarily correspond to the amount that actually crosses the cell's permeability barrier. Although an equivalent amount of actinomycin D may bind to the periphery of wild type and mutant cells, there may not be as much antibiotic reaching the interior of the mutant cells, and thus less inhibition established.

Thiobarbituric acid assays were performed on the mutant to determine the level of KDO in the glucose and succinate grown cells. When

TABLE III

QUALITATIVE AND QUANTITATIVE ANALYSES OF ISOLATED PHOSPHOLIPIDS FROM GLUCOSE AND SUCCINATE GROWN *P. FLUORESCENS* AD+

	Rf	Rho G	PO_4	Ninhydrin	I_2	Counts/10 Min.	% Total
Succinate grown *P. fluorescens* AD+	.535 (PG)	+	+	-	+	17,651	19
	.628 (PE)	+	+	+	+	71,062	78
	.697 (UK)	+	-	-	+	1,342	1
	.907 (UK)	+	-	-	+	1,637	2
Glucose grown *P. fluorescens* AD+	.535 (PG)	+	+	-	+	19,049	16
	.629 (PE)	+	+	+	+	94,432	81
	.695 (UK)	+	-	-	+	2,270	2
	.907 (UK)	+	-	-	+	1,350	1

$$\frac{\text{Total counts incorporated into succinate grown cells}}{\text{Total counts incorporated into glucose grown cells}} =$$

$$\frac{17,651 + 71,062 + 1,342 + 1,637}{19,049 + 94,432 + 2,270 + 1,350} = 0.783$$

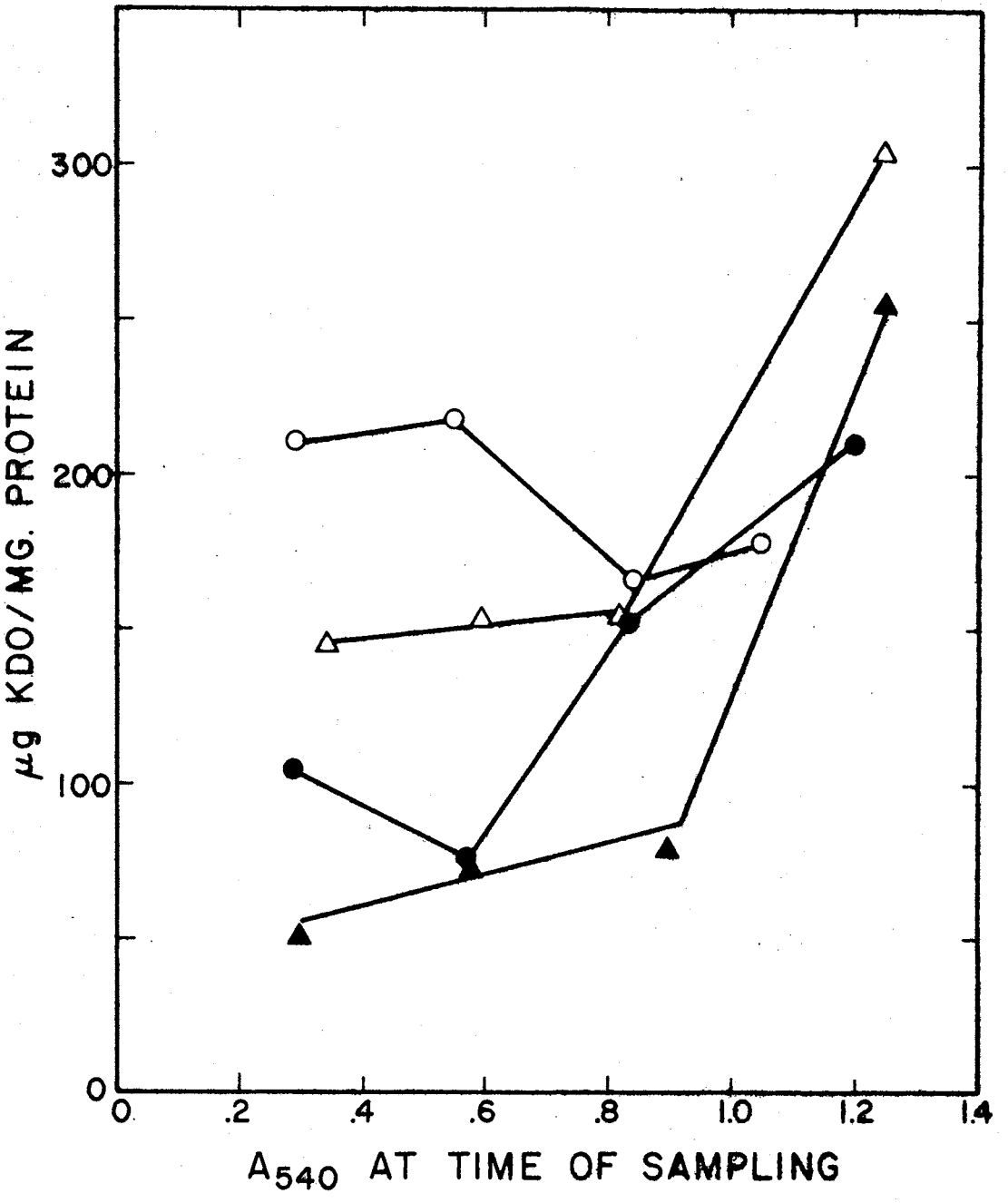
Phosphatidylgylcerol: PG
Phosphatidylethanolamine: PE
Unknown: UK

the values obtained were compared to the KDO level in the wild type counterpart, there was little difference observed. However, if the KDO level is based on a milligram protein ratio as determined by the method of Koch and Putnam (1971) rather than a milligram dry cell weight ratio, the P. fluorescens AD+ cells have a much higher ratio, particularly in the earlier stages of the growth cycle (Fig. 26). Dry weight determinations were performed on P. fluorescens AD+ cells, and cell density represented the same dry cell weight as the parental strain. One explanation may be in the relative proportions of various cellular components. P. fluorescens AD+ cells grown in succinate minimal medium had a decreased protein content of 12 percent, while P. fluorescens AD+ grown in glucose minimal medium had a 35 percent decrease in protein content when compared to the parental strain. This along with an elevated lipopolysaccharide content would suffice to keep their dry weight consistent with that of the wild type. If this is the case, the lipopolysaccharide level could again be related to the apparent decrease in permeability associated with the resistance of the mutant.

The increase in the level of lipopolysaccharide is supported by the level of inorganic phosphate in the cells. Phosphodiester bridges form the cross-linkage in the lipopolysaccharide molecule, and an increase in inorganic phosphate would accompany any increase in lipopolysaccharide. When cells grown in the two carbon sources were examined for inorganic phosphate content by the procedure of Ames and Dubin (1960), the following results were obtained:

Succinate grown P. fluorescens	1.47 μmole PO_4/μg dry wt.
Succinate grown P. fluorescens AD+	1.58 μmole PO_4/μg dry wt.

Figure 26. Thiobarbituric Assay of P. fluorescens and P. fluorescens AD+ Grown in Succinate Minimal Salts and Glucose Minimal Salts Medium. ●, succinate grown wild type, μg KDO/mg protein; ▲, glucose grown wild type, μg KDO/mg protein; ○, succinate grown mutant, μg KDO/mg protein; △, glucose grown mutant, μg KDO/mg protein.


300
200
100
0
μg KDO/MG. PROTEIN
0
.2
.4
.6
.8
1.0
1.2
1.4
A540 AT TIME OF SAMPLING

Glucose grown P. fluorescens 1.76 μmole PO_4/μg dry wt.

Glucose grown P. fluorescens AD+ 2.08 μmole PO_4/μg dry wt.

The inorganic phosphate content increased in accordance with the lipopolysaccharide, with the lowest phosphate level evident in the most sensitive cells and the highest phosphate level evident in the least sensitive cells.

Analysis of Isolated Lipopolysaccharide for Neutral and Amino Sugar Content

Lipopolysaccharide was isolated from P. fluorescens and P. fluorescens AD+ cells grown on succinate and on glucose (Westphal and Jann, 1965). Both qualitative and quantitative analyses for neutral sugars and for amino sugars were performed on the isolated material by Dr. B. Hudson. Results are expressed as μmole of sugar/10 mg of isolated lipopolysaccharide.

Results (Table IV) show that definite differences do exist between the material isolated from glucose and succinate grown cells in both the sugars present and the relative amount of the component sugars. Lipopolysaccharide from P. fluorescens AD+ did not differ in the sugars present when compared to that of the parental strain. The major differences were apparently more dependent on the carbon source in which the cells were grown rather than the genetic alteration. The ribose found in the samples was thought to be a contaminant; RNA is also extracted by the treatment with phenol, and treatment with RNase removed most of the ribose present in the samples.

The most notable difference in chemical composition of the lipopolysaccharide is the absence of fucose and galactose in the samples

TABLE IV

QUANTITATIVE AND QUALITATIVE ANALYSES OF ISOLATED LIPOPOLYSACCHARIDE FROM GLUCOSE AND SUCCINATE GROWN P. FLUORESCENS AND P. FLUORESCENS AD+

	Succinate Grown		Glucose Grown	
	P. fluorescens	P. fluorescens AD+	P. fluorescens	P. fluorescens AD+
NEUTRAL SUGARS (μmoles/10 mg lipopolysaccharide)				
Rhamnose	1.615	1.260	1.414	1.934
Glucose	1.042	1.117	1.861	1.590
Ribose	3.606	3.140	2.996	3.025
Fucose	--	--	0.092	0.123
Galactose	--	--	0.029	0.059
AMINO SUGARS (μmoles/10 μg lipopolysaccharide)				
Glucosamine	0.5130	0.4260	0.3212	0.2237
Galactosamine	0.4270	0.3155	0.2238	0.1387
Ratio: GLUCOSAMINE/GALACTOSAMINE	1.20	1.35	1.43	1.61

Figures represent the average values for at least two separate determinations.

taken from succinate grown cells. Both of the sugars are present in glucose grown cells, although in comparatively small amounts. These two neutral sugars would probably be located in the O-polysaccharide portion of the molecule which contains the majority of the neutral sugars. This portion is the most variable of the lipopolysaccharide components. A slight change in the sugar components in this portion of the lipopolysaccharide could be expected to alter the stereochemical alignment of the outer structuring of the macromolecule.

A second, and equally significant, difference is the increase in glucose that is incorporated into the glucose grown cells. When glucose grown P. fluorescens was compared to succinate grown P. fluorescens, the amount of glucose in isolated lipopolysaccharide increases by 44 percent. While the increase is not as great when lipopolysaccharide from P. fluorescens AD+ is examined, there is a 30 percent increase in glucose grown cells over succinate grown cells.

In the glucose grown cells, the amount of galactose and fucose increases in the lipopolysaccharide from P. fluorescens AD+ cells when compared to the lipopolysaccharide from the parent strain. There is an increase of approximately 25 percent in fucose content and 50 percent in galactose content.

When the glucosamine/galactosamine ratios of each lipopolysaccharide sample were compared, there was an increase of 16 percent in the glucose grown P. fluorescens over the succinate grown, and a 35 percent increase in the glucose grown P. fluorescens AD+ over its succinate grown counterpart. The amino sugars are believed to be located in the R core of the lipopolysaccharide, where they are substituted into the heptose backbone. When glucosamine and galactosamine are calculated on

the basis of the amount of amino sugar per milligram of isolated lipopolysaccharide, that from succinate grown P. fluorescens cells had almost twice the amount incorporated as did that from glucose grown cells. Additionally, there is a decrease in the amount of glucosamine and galactosamine when the lipopolysaccharide from the parent strain is compared to the lipopolysaccharide from P. fluorescens AD+ cells.

CHAPTER IV

CONCLUSIONS

P. fluorescens cells grown in glucose minimal medium showed a much lower level of sensitivity to actinomycin D than did cells grown in succinate minimal medium. A concentration of 30 μg/ml actinomycin D produced a 20-30 percent inhibition of growth in glucose and an 80-90 percent inhibition of growth in succinate. Sensitivity of the cells was also decreased by increasing the concentration of the carbon source in the medium or by decreasing the growth rate by lowering the temperature of incubation. Under all conditions the inhibition of growth was dependent on the antibiotic concentration.

The inhibition by actinomycin D was not reversible on the addition of low levels of glucose to an inhibited system. Ultraviolet spectral data revealed no evidence for complex formation between the antibiotic molecule and glucose or succinate, or between the antibiotic and any metabolite present in glucose or succinate spent medium.

Warburg respirometry studies revealed that neither set of cells was oxidizing the actinomycin D. A butanol extraction for actinomycin D indicated that the molecule was not being altered to a non-inhibitory form by cells grown in either nutritional condition.

There was no evidence that a selective process for resistance was occurring in the glucose minimal medium. Cells grown in glucose minimal medium containing 30 μg/ml actinomycin D exhibited the same degree

of sensitivity as cells grown in the absence of the antibiotic when inoculated into tubes containing fresh medium and actinomycin D.

Uptake studies offered evidence which augmented the difference in sensitivity levels. Competition for a common binding site between actinomycin D and the substrate was eliminated as the presence of the antibiotic in the uptake solution did not inhibit accumulation of labeled substrates. There was a requirement for growth in the presence of the antibiotic before any significant inhibition of uptake could be observed in either set of cells.

Glucose uptake by cells grown in glucose plus actinomycin D was not altered significantly, but the uptake of labeled succinate by cells grown in succinate plus actinomycin D was completely inhibited. The uptake of phenylalanine and alanine showed a similar inhibition pattern. Cells grown in glucose plus actinomycin D were not affected in their uptake of the two amino acids, whereas uptake of the amino acids in the cells grown in succinate plus actinomycin D was completely inhibited. The uptake of uracil was reduced approximately 50 percent in the cells grown in succinate plus actinomycin D. Uracil uptake in cells grown in glucose plus actinomycin D was not inhibited.

Because actinomycin D inhibits the incorporation of uracil during DNA-directed RNA synthesis, the amount of activity incorporated into the cell fraction which had been extracted with cold trichloroacetic acid was used to monitor the relative amount of inhibition occurring at the molecular level. When succinate grown cells were compared to glucose grown cells, no inhibition of uracil incorporation occurred up to 60 minutes in either set. However, after 60 minutes, the succinate grown cells did show very slight inhibition of uracil incorporation,

while the glucose grown cells did not. This difference may simply reflect the decrease in uptake of uracil. Such a decrease occurs in cells grown in succinate plus the antibiotic. The results of growth and uptake experiments indicate an exposure time of 60 minutes should be sufficient to allow penetration of actinomycin D to the interior of P. fluorescens cells growing in succinate. Thus the decrease in uracil incorporation may be a valid measure of RNA synthetic inhibition. However, either explanation indicates that the succinate grown cells are much more susceptible to the antibiotic than are the glucose grown cells.

Warburg respirometry provided a means of comparing the effect of actinomycin D on the oxidative systems for glucose and succinate metabolism, both of which are inducible. The presence of the antibiotic in the flask with glucose grown cells did not influence oxygen consumption or rate of oxidation using either glucose or succinate as the substrate. When the cells were grown in glucose plus actinomycin D, a 10 percent decrease in the oxidation rate was noted when succinate was the substrate. A slightly increased lag time for induction to succinate was also evident. Glucose metabolism was altered only slightly.

Succinate grown cells exhibited a 25 percent decrease in oxidation rate in the presence of actinomycin D when glucose was the substrate. Cells grown in succinate plus actinomycin D showed a 50-55 percent decrease in rate of oxidation with glucose as the substrate. If actinomycin D was present in the flask, glucose oxidation was completely inhibited in the cells grown in succinate plus the antibiotic.

Treatment of glucose grown cells with EDTA caused only a short extension of the growth lag time when sensitivity to actinomycin D was

observed. The same treatment of succinate grown cells increased sensitivity toward the antibiotic drastically; a concentration of 5 μg/ml actinomycin D completely inhibited growth of the EDTA treated succinate grown cells.

It was not possible to dissociate completely binding or adsorption from uptake of the antibiotic by the cells. Succinate grown cells generally bound 2-3 times more ^{3}H-actinomycin D than glucose grown cells. Examination of the binding properties of isolated envelope material showed that envelopes from both glucose and succinate grown cells bound equivalent amounts of radioactivity. Repeated washing of the succinate grown cells generally increased the binding capacity which caused an increased sensitivity to the antibiotic. These effects were often associated with a decreased lipopolysaccharide content. Washing the glucose grown cells produced a similar reduction in lipopolysaccharide with increased binding, but the sensitivity of the cells to actinomycin D remained approximately the same.

The pH at which the cells were grown did not significantly influence the adsorption of ^{3}H-actinomycin D, and the binding capacity for the antibiotic varied according to the stage of the growth cycle in which the cells were examined. Osmotic shock decreased the amount of actinomycin D bound by both succinate and glucose grown cells.

All of the physiological evidence indicates that the permeability barrier of the two sets of cells must differ. There are a multitude of physical parameters which directly or indirectly affect the non-selective movement of materials through the cell surface. Several of the probable changes influencing the macromolecular dynamism of the

cell surface were likely candidates for the glucose-succinate sensitivity differences.

Phospholipids are involved in a change in sensitivity to certain bacteriocidal agents (Anderes, Sandine, and Elliker, 1971). A buildup of lipid in the envelope of the cell may decrease the permeability. In this study, phospholipids were extracted from P. fluorescens grown under the two nutritional conditions, and the quantitative and qualitative assays for major phospholipids were made. Two major phospholipids, phosphatidylglycerol and phosphatidylethanolamine, were present in approximately the same ratio in both sets of cells. There was an increase in the amount of total radioactive acetate incorporated into the extractable phospholipids of glucose grown cells, but it is not known whether the increase was due to a buildup of lipid, an increased rate of turnover in the lipid, or a decreased dilution factor when glucose was the carbon source. If an increase is occurring, this might decrease the over-all permeability of the cells. Actinomycin D did not bind directly to any of the phospholipids present during chromatography studies.

The lipopolysaccharide of the gram negative bacterium has been associated with permeability. KDO is found only in the backbone of the lipopolysaccharide molecule, and the quantitative determination of KDO content provides an indication of the level of the lipopolysaccharide in the cell envelope. Cells grown in different media displayed significant differences in the KDO levels, and an increase in KDO corresponded, in general, with decreased sensitivity to actinomycin D. As growth approached late logarithmic phase, glucose grown cells had a higher concentration of KDO than succinate grown cells, and the concentration

of KDO increased in both sets of cells with increasing concentration of the carbon source. The KDO level was somewhat dependent on the growth rate, and release of KDO by treatment with EDTA was greater in succinate grown cells than in glucose grown cells.

A mutant was isolated which showed a slightly increased level of phospholipid as judged by incorporation of ^{14}C-acetate. The cells have an increased level of KDO if the ratio of KDO to protein was compared to that of the wild type cells.

Qualitative analyses of isolated lipopolysaccharide revealed further differences between the two sets of cells. Lipopolysaccharide from cells grown in glucose contained two additional sugars, galactose and fucose, which were not detected in lipopolysaccharide from succinate grown cells. The lipopolysaccharide from glucose grown cells also had a higher level of glucose, and the glucosamine/galactosamine ratio was higher by approximately 20 percent. However, succinate grown cells incorporated almost twice the amount of glucosamine and galactosamine per milligram lipopolysaccharide.

These data suggest two conclusions regarding permeability and its relationship to the lipopolysaccharide fraction of cells grown on the two carbon sources. First, the succinate grown cells are more sensitive to actinomycin D apparently because of increased permeability. They do not accumulate as high a level of lipopolysaccharide in the cell envelope as do the glucose grown cells, nor are they capable of maintaining a static level of lipopolysaccharide once logarithmic growth has ceased.

Second, the quantitative and qualitative data from the analysis of isolated lipopolysaccharide establishes that cells grown in succinate,

whether wild type or mutant, do not synthesize as complex a lipopolysaccharide molecule as do the cells grown in glucose. Neither galactose nor fucose is present in the lipopolysaccharide from succinate grown cells, and the level of glucose is almost one-half of that found in the lipopolysaccharide from glucose grown cells. The fucose, galactose, and additional glucose in the lipopolysaccharide from glucose grown cells are probably located in branching side chains on the O-polysaccharide. This portion of the molecule contains the majority of the neutral sugars and is known to be the most variable. Any increase in the amount of branch structure could be associated with decreased cellular permeability in the glucose grown cells.

Further evidence for the increased complexity in the macromolecule in the glucose grown cells is the apparent decrease in the amounts of glucosamine and galactosamine per mg lipopolysaccharide. If the molecular weight of the lipopolysaccharide were decreased as a result of decreased molecular complexity, the ratio of the aminosugars per mg lipopolysaccharide would be expected to increase. An increase is reflected in the aminosugar analysis of lipopolysaccharide from succinate grown cells.

These observations are not unlike those of Schlecht and Westphal (1968, 1970), who reported that sensitivity to erythromycin, rifamycin, actinomycin D, and bacitracin increased progressively with the loss of sugar moieties from the outside of the R core of lipopolysaccharide isolated from R mutants of Salmonella minnesota. The loss of neutral sugars from the lipopolysaccharide occurs in mutants of E. coli which are supersensitive to various antibiotics, including actinomycin (Tamaki, Sato, and Matsuhashi, 1971).

From a strictly teleological standpoint, utilization of the two carbon sources could produce the differences in the lipopolysaccharide. Glucose is a much better source of energy and synthetic precursors than is succinate. It is located at a hub in the metabolic pathways by which any number of synthetic products can be reached. Succinate, on the other hand, does not provide direct access to the synthesis of five and six carbon sugars. Any synthetic activity must involve a reversal of the glycolytic pathway, which in turn requires energy beyond that necessary for the conversion of glucose. Thus it would be much more difficult for the cells to synthesize the neutral sugars required for lipopolysaccharide when they are growing on succinate. Additionally, there is much less total energy derived from utilization of succinate since it must enter the tricarboxylic acid cycle as pyruvate.

The mutant cells grown in succinate do seem to have a higher glucose level in their lipopolysaccharide than the wild type grown in succinate, and the amount of glucosamine and galactosamine more closely approaches the level found in the glucose grown cells. It is tempting to suggest that the mutation involves a step in the synthesis of the O-polysaccharide, leading to increasing complexity in the macromolecule, and thus decreasing the permeability of the cell.

The lipopolysaccharide molecule is believed to be positioned in a mosaic-like structure in the envelope of the cell (Wright and Kanegasaki, 1971). Evidence for this model comes from electron microscopy of cells treated with ferritin antibody conjugate directed against O-polysaccharide (Shands, 1965) and the release of a lipopolysaccharide-phospholipid-protein complex from cells by amino acid starvation (Knox, Cullen, and Work, 1967), chloramphenicol treatment (Rothfield and

Pearlman-Kothencz, 1969), and EDTA treatment (Leive, Shovlin and Mergenhagen, 1968). The same complex is also released by cells growing under normal conditions, but the amounts released are much less.

The apparent increase in phospholipid in cells grown in glucose and the increase in phospholipid in the mutant is thus consistent with the increased level of lipopolysaccharide. Since these two molecular species are found in a stable complex, an increase in one might necessitate an increase in the other. The increase in phosphate in the cells also lends support to this hypothesis. Phosphate is associated with the lipopolysaccharide through phosphodiester bridges, and the increase corresponds to the decrease in sensitivity as well as the increased levels of phospholipids and lipopolysaccharide.

Throughout these investigations where there was a decrease in sensitivity to actinomycin D, there was a concomitant increase in the lipopolysaccharide concentration of the cells. Evidence for the decreased accumulation of lipopolysaccharide along with evidence for integration of a less complex macromolecule into the cell envelope of succinate grown P. fluorescens provides a feasible link between the increased permeability of the cells and their peripheral architecture. The more complex structure and the increased amount of lipopolysaccharide in the envelope of glucose grown cells may then be associated with reduced movement of the antibiotic into the cell, and thus the decreased sensitivity to the antibiotic.

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