THE EFFECT OF VANCOMYCIN ON *PSEUDOMONAS FLUORESCENS*

by

DALE VERNON FERGUSON

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Thesis Approved:

Norman D Durham
Thesis Adviser

Eric C Stallen

Norman D Durham
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Vancomycin, the generic name for vancocin, was isolated from Streptomyces orientalis which was obtained from an Indonesian soil sample by McCormick et al., (1956). Eli Lilly and Company isolated and purified the antibiotic and developed techniques for the commercial production.

Vancomycin has special properties such as low toxicity to humans, bactericidal action at low concentrations and is effective against penicillin-resistant microorganisms. The antibiotic was found to be especially active against gram-positive bacteria and the spirochetes (McCormick et al., 1956).

Ziegler, Wolfe, and McGuire (1956) determined that vancomycin was bactericidal for actively growing cells, but did not affect either resting or respiring cells. The activity of the antibiotic was not significantly altered by large changes in the pH of the medium. Inorganic salts, reducing agents, amino acids, vitamins and growth factors had no pronounced effect on vancomycin activity. One characteristic of vancomycin was that an immediate killing effect was evident in all bactericidal concentrations tested.

Considerable research has been conducted concerning the effect of vancomycin on gram-positive organisms, therefore, it is the purpose of this investigation to give some insight into the effect of vancomycin

on gram-negative organisms.

CHAPTER II

LITERATURE REVIEW

Chemical Characterization of Vancomycin

McCormick et al. (1956) reported that data obtained from titration studies indicated the molecular weight of vancomycin to be 3200-3500 ± 200 while the data obtained from ultracentrifugation studies suggested a molecular weight of 3300. Johnson (1962) reported that the molecular weight of vancomycin was either 1560 or 3120. The higher molecular weight could result from an aggregation of two "1560" components. A tentative formula of $C_{70}H_{90}Cl_2N_9O_{27}$ and a chemical analysis, based on a molecular weight of 1560, revealed the following components;

1 mole L-aspartic acid

1 mole N-methyl-D-leucine

2 moles glycine

1 mole alanine

3 groups releasing NH_3 upon hydrolysis

1 group evolving methylamine upon hydrolysis

1 mole glucose

2 moles 3-chloro-4-hydroxyphenyl groups

2 moles 2-hydroxyphenyl moieties

1 system which provides 3-methyl-4-ketohexanoic acid on strong acid hydrolysis

6 periodate-labile groups (one yielding acetaldehyde).

There is no indication as to how these component parts are linked together to form the complete vancomycin molecule.

Biological Activity of Vancomycin.

Vancomycin was originally investigated by workers at Eli Lilly and Company as a potential agent for controlling penicillin-resistant, coagulase positive Staphylococcus aureus (Mc Cormick et al., 1956).

Jordon and Inniss (1959) reported that vancomycin inhibited division of S. aureus but permitted protoplasmic synthesis to continue. Washing the vancomycin-treated cells with water or different buffers did not influence the inhibition and no ameliorative effect was found when various concentrations of certain purines, pyrimidines, peptides or metallic ions were added to the growth medium prior to the addition of the vancomycin. Treatment of cells with the antibiotic did not influence the rate of synthesis of either proteins or deoxyribonucleic acid. There was, however, a complete inhibition of synthesis of ribonucleic acid. Later studies by Jordon (1961) suggested that the primary effect of vancomycin on S. aureus was an inhibition of the synthesis of cell wall mucopeptide, and the inhibition of ribonucleic acid production reported previously was a secondary effect and could have been caused by the initial defect in mucopeptide synthesis.

Reynolds (1961) reported that the net synthesis of ribonucleic acid was inhibited and that hexosamine-containing nucleotides accumulated in vancomycin-treated S. aureus. He concluded that vancomycin and penicillin acted in a similar manner since both caused the accumulation of the cell wall precursors. However, since there is no cross-resistance exhibited by bacteria to the two antibiotics, it is unlikely

that they have precisely the same mode of action. Later, Reynolds (1962) compared the effects of penicillin and vancomycin using the cellular binding of radioactive penicillin and noted that the prior treatment of S. aureus with either penicillin or bacitracin prevented the uptake of radioactive penicillin. Prior treatment of the cells with vancomycin did not reduce the uptake of radioactive penicillin so it was concluded that the sites of action of penicillin and vancomycin were different.

Shockman and Lampen (1962) compared the effects of several antibiotics on protoplasts and whole cells of Streptococcus faecalis. A concentration of 1.0 μg per ml of vancomycin inhibited growth (increase in cell mass) of S. faecalis protoplasts. Growth of S. faecalis cells was inhibited at least 50 per cent by a vancomycin concentration of 0.5 μg per ml. The significance of these results was not discussed.

Yudkin (1963) compared the effect of penicillin, novobiocin, streptomycin and vancomycin on the incorporation of 1-14C-glycerol and U-14C-tyrosine into the membrane fraction of Bacillus megaterium. He noted that vancomycin inhibited the synthesis of proteins and lipids to approximately the same extent and suggested that vancomycin inhibited membrane synthesis rather than causing a disproportionate synthesis of proteins and lipids.

Hancock and Fitz-James (1964) compared the effects of penicillin, bacitracin and vancomycin on whole cells and protoplasts of B. megaterium. They concluded that bacitracin and vancomycin directly affected the membrane since the antibiotics caused an increased rate of efflux of 42K in growing cells and eventual lysis of protoplasts. They suggested that the action of the antibiotics on the

cell wall may be secondary to the effect on the cell membrane.

Best and Durham (1964) reported that vancomycin inhibited growth and mucopeptide synthesis of B. subtilis cells. Magnesium ions partially alleviated the inhibition of growth and mucopeptide synthesis when added simultaneously with the antibiotic, but did not prevent lysis or leaching of cell metabolites at higher concentrations of vancomycin. Later studies (Best and Durham, 1965) indicated that magnesium and other cations apparently competed with vancomycin for attachment sites on the cells of B. subtilis and it was suggested that the initial bonding of vancomycin to cell walls may occur by ionic bonding between the basic groups of the antibiotic and acidic groups on the cell wall.

Burger and Glaser (1964) studied the influence of vancomycin on the synthesis of polyglycerolphosphate by cells of B. licheniformis and B. subtilis. They noted that the enzyme required the presence of high concentrations of calcium and magnesium ions for activity and was inhibited by a vancomycin concentration of 2 mg per ml. The degree of antibiotic inhibition was a function of the ratio of the enzyme to inhibitor. In addition, the enzyme involved in synthesis of polyribitolphosphate, which was closely associated with the cell wall, was also sensitive to vancomycin (0.03 mg per mg dry weight of enzyme) and showed a requirement for calcium and magnesium.

Anderson et al. (1965) noted that the enzymes necessary for glycopeptide synthesis in S. aureus and Micrococcus lysodeikticus were inhibited approximately 50 per cent by either vancomycin or ristocetin at a concentration of 20 μg per ml. This concentration of either antibiotic also inhibited growth by 50 per cent. These workers concluded that the antibiotics do not interfere with formation of the

lipid intermediates, but do inhibit the utilization of the lipid-phosphodissacharide-pentapeptide for cell wall synthesis.

Chatterjee and Perkins (1966) found that in the presence of vancomycin, certain corynebacteria accumulate not only a uridine diphosphate-nucleopeptide but also a compound of uridine diphosphate-nucleopeptide complexed with a molecule of vancomycin. Similar substances accumulate in S. aureus and M. lysodeikticus. Using paper chromatography, they isolated a "fast moving" and a "slow moving" nucleotide in the vancomycin-treated cells, and their results suggested that there is one mole of vancomycin (molecular weight 1560) for each mole of the "slow moving" amino sugar nucleotide.

Struve, Sinha and Neuhaus (1966) studied cell-free enzymes prepared from S. aureus and noted that low concentrations of the ristocetins or vancomycin caused the enhanced formation of acceptor-phospho-NAc-muramyl-pentapeptide. Also, the enzyme catalyzing the exchange of (3H) uridine monophosphate with the UMP moiety of the UDP-NAc-muramyl-pentapeptide was inhibited by these antibiotics.

Since recent reports suggest that the antibiotic acts primarily on the cell wall then the difference in the cell walls of gram-positive and gram-negative organisms is of importance in the study of the mechanism of action of vancomycin.

CHAPTER III

MATERIALS AND METHODS

Test Organism

The principle microorganism used throughout this investigation has been tentatively identified as Pseudomonas fluorescens. It is a gram-negative, motile rod which forms smooth, raised colonies on nutrient agar. The organism exhibits a negative reaction for hydrogen sulfide, indole production, nitrate reduction, and produces acid but no gas in glucose. This organism produced both fluorescin and pyocyanin when grown on Bacto-Pseudomonas agar F and Bacto-Pseudomonas agar P respectively. In certain phases of this study a culture isolated from the soil and tentatively identified as belonging to the genus Flavobacterium was used. The gram-positive microorganism, Micrococcus lysodeikticus, was used in one aspect of this investigation.

Stock cultures of P. fluorescens and the species of Flavobacterium were maintained on slants of either nutrient agar or succinate-salts agar. M. lysodeikticus was maintained on slants of brain heart infusion (Difco) containing two per cent agar. All slants were stored at 4 C.

Synthetic Medium

A synthetic succinate-salts medium was used to measure growth of the organisms in some aspects of the study. The medium had the following composition: NaCl, 0.2g; NH_4Cl, 0.2g; KH_2PO_4, 0.32g;

K_2HPO_4, 0.42 g; and succinic acid, 0.2 g in 100 ml of distilled water. The pH was adjusted to 6.8-7.0 with KOH and the medium sterilized by autoclaving for 15 minutes at 121 C. Each 100 ml of the synthetic medium was supplemented with 0.1 ml of a mineral salts solution that was autoclaved separately and added aseptically just prior to use. The mineral salts contained; $MgSO_4 \cdot 7\ H_2O$, 5.0 g; $MnSO_4$, 0.1 g; $FeCl_3$, 1.0 g; and $CaCl_2$, 0.5 g in 100 ml of distilled water. Two per cent agar was added when a solid medium was needed.

Growth Studies

Growth studies were conducted to determine the effect of various concentrations of vancomycin on the growth of P. fluorescens and the Flavobacterium. The organisms were grown 12-15 hours on succinate agar slants, harvested, washed and suspended in 0.01 M 2-amino-2-(hydroxymethyl)-1, 3-propanediol (tris) buffer (pH 7.0) to an absorbance of 0.6 at 540 mμ using a Bausch and Lomb "Spectronic 20" colorimeter. One-tenth ml of this cell suspension was used to inoculate each tube (18 mm x 150 mm) in the growth studies (final liquid volume of 6.0 ml). The absorbance of the tubes immediately following inoculation was approximately 0.01 at 540 mμ. All tubes were incubated at 37 C on a reciprocal shaker. Growth of the cultures was determined by measuring the absorbance at 540 mμ with a Bausch and Lomb "Spectronic 20" colorimeter. An uninoculated tube of medium was used as a standard.

Influence of Magnesium on Growth Inhibition

Cells of P. fluorescens were grown 12-15 hours on succinate agar slants, harvested, washed and suspended in 0.01 M tris buffer (pH 7.0)

to an absorbance of 0.6 at 540 mμ. The inoculum (0.5 ml) was added to the 250 ml Erlenmeyer side-arm flasks used in the growth studies (final liquid volume of 30.0 ml). A vancomycin concentration, as noted in the text, was added to the appropriate flasks. The initial absorbance of the flasks was 0.03 at 540 mμ. Magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$), in the indicated concentration, was added to the appropriate vancomycin-containing flasks. Controls were run concurrently. The growth of the cells was determined by measuring the change in absorbance at 540 mμ.

Adsorption of Vancomycin to Cells

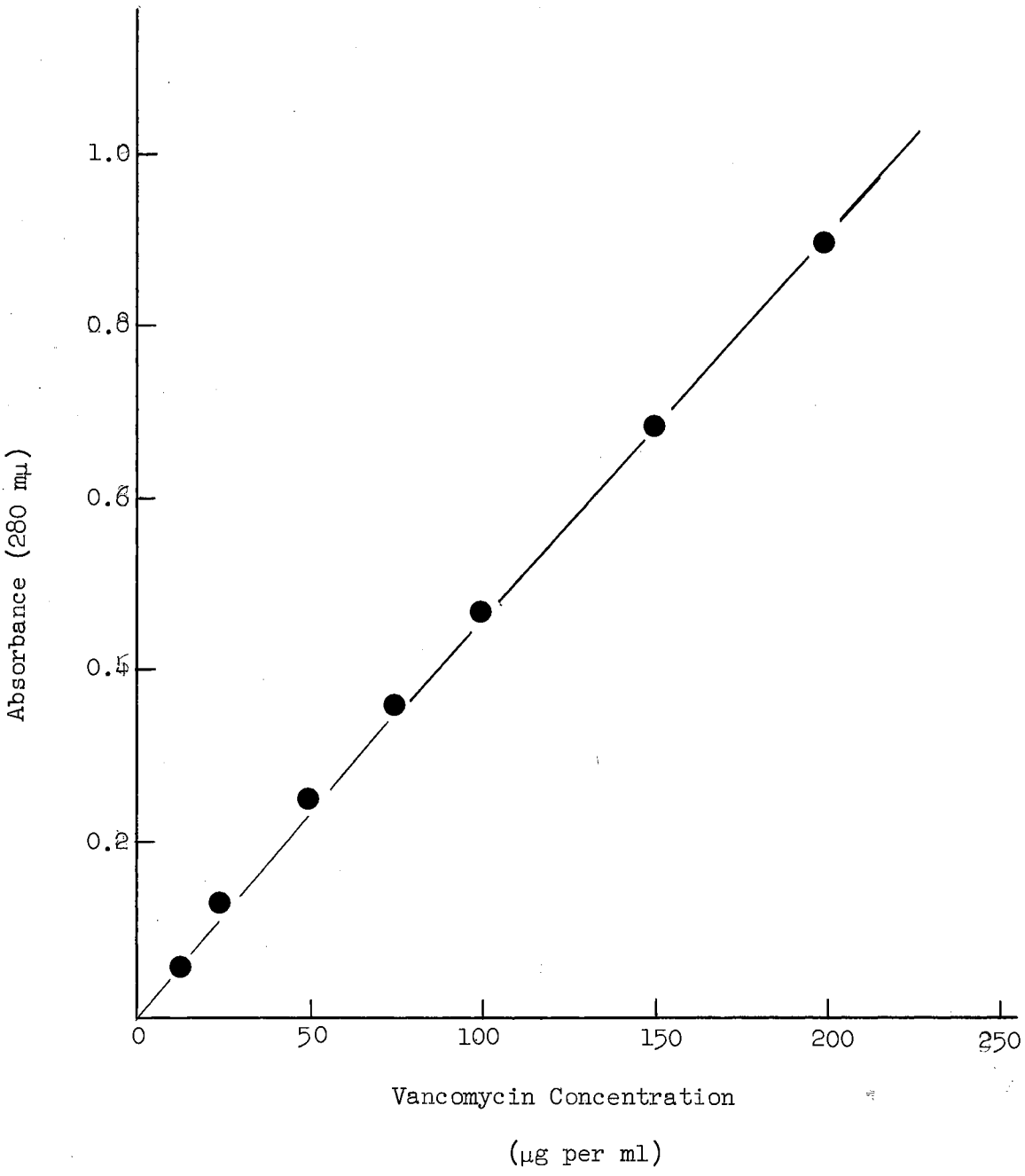
The adsorption of vancomycin to the cells of P. fluorescens or the species of Flavobacterium was determined by mixing cells (diluted in triple distilled water so that a 1:2 dilution gave an absorbance of 1.0 at 540 mμ) with sufficient vancomycin to give a final concentration of 150 μg per ml. The antibiotic was permitted to adsorb to the cells for 10 minutes at room temperature. The cells were removed by centrifugation at 12,000 x g for 10 minutes or by Millipore filtration (0.45 μ pore size). The quantity of vancomycin adsorbed to the cells was determined from a standard curve by measuring the absorbance of the supernatant solution or filtrate at 280 mμ using a Beckman DU spectrophotometer. Controls indicated that vancomycin did not adsorb to the Millipore filter membrane.

Vancomycin absorbs ultraviolet light maximally at approximately 280 mμ in neutral or slightly acidic solution due to the presence of phenolic chromophores (Johnson, 1962). A standard curve for the free base of this antibiotic dissolved in triple distilled water (pH 7.0) as measured at 280 mμ is presented in Figure 1.

Figure 1

Standard Curve for Vancomycin Absorption at 280 mμ

Vancomycin (free base) was dissolved in distilled water. The absorbance of various concentrations of the antibiotic was measured at 280 mμ.


1.0
0.8
0.6
0.4
0.2
Absorbance (280 mμ)
0
50
100
150
200
250
Vancomycin Concentration
(μg per ml)

Effect of Ethylenediaminetetraacetate (EDTA) and Ethanol on Adsorption

P. fluorescens was grown 12-15 hours on nutrient agar plates, harvested, washed (two times) and suspended in 0.01 M tris buffer (pH 7.0) to give an absorbance of 1.0 at 540 mμ. Seven ml of the diluted cell suspension was added to each of six tubes and centrifuged. The supernatant solution was discarded and 7.0 ml of different concentrations of ethanol was added to three tubes. Seven ml of EDTA (10^{-4} M) solution was added to one tube. Triple distilled water (7.0 ml) was added to each of two tubes which served as controls. The cells were suspended by mixing and incubated for 20 minutes at 37 C with constant shaking. One ml of each tube was removed, diluted and plated on nutrient agar to determine viable counts. The tubes were centrifuged and the supernatant solutions discarded. The cell pellets were washed once and suspended in 3.0 ml of triple distilled water. Three ml of a vancomycin solution (300 μg per ml) were added to all tubes except the control tube, which received water. Triple distilled water was added to all tubes to bring the total liquid volume to 6.0 ml. The tubes were mixed and incubated 10 minutes at room temperature to permit adsorption of the antibiotic to the cells. The tubes were centrifuged and the absorbance of the supernatant solutions measured at 280 mμ. All absorbance values were corrected for the 280 mμ absorbing materials released by the control cells during the incubation. The corrected values were then used to calculate the amount of antibiotic adsorbed.

Effect of Metals on the Adsorption of Vancomycin to Cells

P. fluorescens cells were grown 12-15 hours on nutrient agar

plates, harvested and washed two times with 0.01 M tris buffer (pH 7.0). The cells were diluted in triple distilled water to an absorbance of 1.0 at 540 mμ. Six ml of the diluted cell suspension were added to each of seven tubes, centrifuged and the supernatant solution discarded. The cell pellets were suspended in 1.5 ml of the appropriate ion solution (0.05M), except that the control tubes were suspended in 1.5 ml of triple distilled water. Three ml of a vancomycin solution (300 μg per ml) were added to all tubes. Triple distilled water was added to bring the total liquid volume to 6.0 ml. The tubes were incubated 10 minutes at room temperature, centrifuged, and the absorbance of the supernatant solution from each tube was measured at 280 mμ. The absorbance of the control system, which did not contain vancomycin, was subtracted from the absorbance values of the vancomycin-treated tubes to correct for 280 mμ absorbing material released from the cells as a result of the incubation. The corrected values were used to calculate the amount of antibiotic adsorbed to the cells.

Effect of Magnesium on Adsorption and Elution of Vancomycin

Cells of P. fluorescens were grown 12-15 hours on nutrient agar and suspended as previously described. Six ml of the diluted cell suspension were added to two tubes, centrifuged, and the supernatant solutions discarded. The cell pellet of one tube was suspended in 6.0 ml of a vancomycin solution containing 150 μg per ml. The cell pellet of the second tube was suspended in 6.0 ml of a 0.05 M magnesium sulfate solution. Both tubes were incubated for 10 minutes at room temperature, centrifuged, and the supernatant solution was saved for analysis. The vancomycin-treated pellet was then suspended in 6.0 ml of a magnesium sulfate solution (0.05 M). The magnesium sulfate-

treated pellet was suspended in 6.0 ml of vancomycin containing 150 μg per ml. The tubes were incubated at room temperature, centrifuged, and the supernatant solutions from each tube saved. The supernatant solutions were filtered and the absorbance measured at 280 mμ. to quantitate the amount of antibiotic adsorbed to the cells or eluted from the cells by magnesium.

Effect of Growth Medium on Adsorption of Vancomycin

Cells of P. fluorescens were grown 12-15 hours on nutrient agar slants. The cells were harvested and used to inoculate plates of nutrient agar and succinate-salts agar. All plates were incubated for 12 hours at 37 C. The cells were harvested, washed two times with triple distilled water and suspended in triple distilled water so that 1:2 dilution gave an absorbance of 1.0 at 540 mμ. Three ml of the diluted cell suspension were added to tubes. Three ml of a vancomycin solution (300 μg per ml) were added to all tubes except the control tube which received 3.0 ml of water. Triple distilled water (3.0 ml) was added to all tubes to bring the total liquid volume to 6.0 ml. Adsorption was permitted to take place for ten minutes at room temperature. The tubes were centrifuged and the absorbance of the supernatant solutions measured at 280 mμ. The absorbance values were corrected for the 280 mμ absorbing material released by the water-treated cells during the incubation and the amount of antibiotic adsorbed to the cells was determined.

Adsorption of Vancomycin by cells Grown on Nutrient Agar Poised at Different pH Values

Studies were conducted to determine if P. fluorescens cells grown

on nutrient agar adjusted to different pH values showed quantitative differences in the adsorption of vancomycin. Nutrient agar was prepared and adjusted to the desired pH by adding either HCl or NaOH. P. fluorescens cells were grown on nutrient agar slants for 12-15 hours, harvested and used as the inoculum. The plates were incubated for 12 hours at 37 C, harvested and diluted in triple distilled water so that a 1:2 dilution gave an absorbance of 1.0 at 540 mμ. Adsorption studies were conducted as previously described.

CHAPTER IV

RESULTS AND DISCUSSION

The Effect of Vancomycin on Growth of P. fluorescens and a Species of Flavobacterium

Experiments were conducted to determine if vancomycin influenced the growth of P. fluorescens or the Flavobacterium in a succinate-salts medium. The concentrations of vancomycin were 25 and 50 μg per ml.

Each tube contained 5.0 ml of medium plus the appropriate amount of inhibitor and 0.01 M tris buffer to give a total liquid volume of 6.0 ml per tube. The tubes were inoculated with a suspension of nutrient agar-grown cells. The initial absorbance was 0.03 at 540 mμ. Small aliquots were removed from each tube periodically for microscopic observation. Growth was determined by measuring absorbance at the indicated time intervals.

The growth of the species of Flavobacterium was not significantly influenced by either concentration of vancomycin (Figure 2). The lower concentration of vancomycin (25 μg per ml) did not influence growth of P. fluorescens but the higher concentration of the antibiotic did show some inhibition of growth (Figure 3). Microscopic observations made during the early stages of the growth study indicated that the lower concentration of vancomycin (25 μg per ml) did not appear to influence the morphology of the Flavobacterium cells, but the

Figure 2

The influence of vancomycin on a species of Flavobacterium growing in a succinate-salts medium (0.2 per cent succinic acid). ● , control: □ , vancomycin (25 μg per ml): and ■ , vancomycin (50 μg per ml).

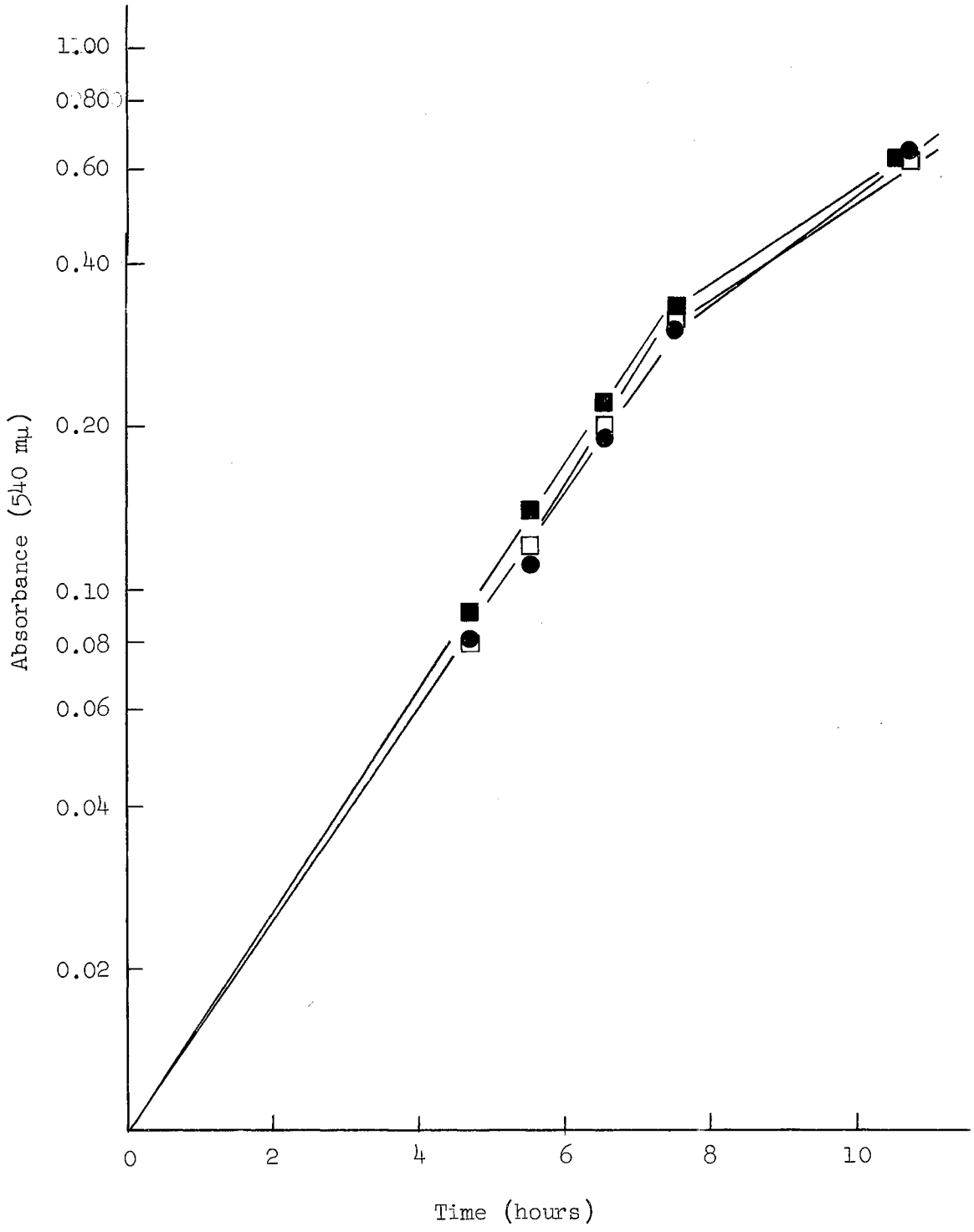
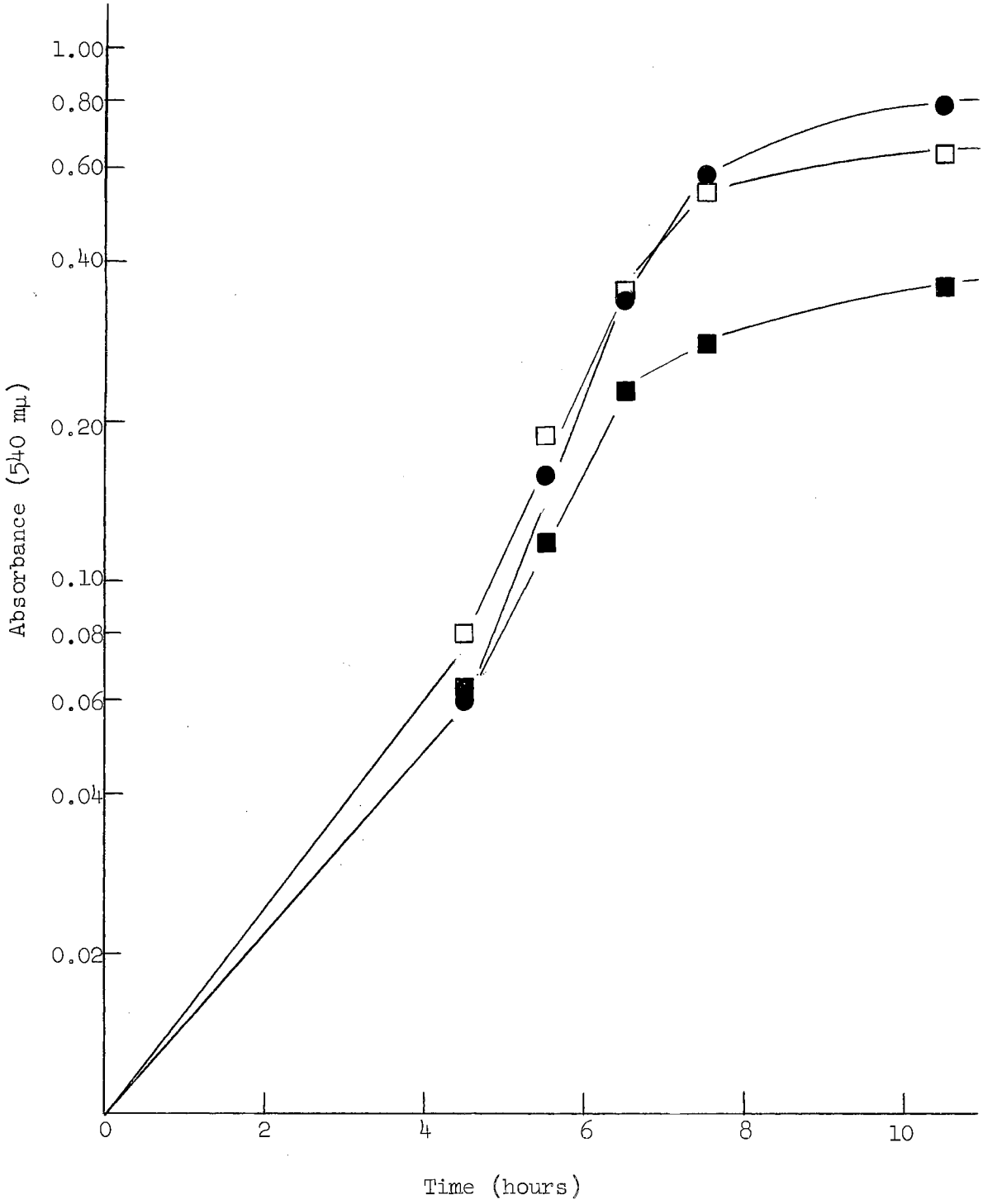

1.00
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
Absorbance (540 mμ)
0
2
4
6
8
10
Time (hours)

Figure 3

The influence of vancomycin on growth of P. fluorescens in succinate-salts medium. ● , control: □ , vancomycin (25 μg per ml); and ■ , vancomycin (50 μg per ml).


1.00
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
Absorbance (540 mμ)
0
2
4
6
8
10
Time (hours)

formation of short chains (3-5 cells) was evident in an antibiotic concentration of 50 μg per ml. P. fluorescens did form short chains in the presence of 25 μg per ml of vancomycin and chaining, abberant forms, and remnants of lysed cells were observed in the higher concentration.

Best and Durham (1964) noted a pronounced inhibition of growth of B. subtilis at a vancomycin concentration of 0.056 μg per ml. Lysis of B. subtilis occurred when the cells were incubated at 37 C at a vancomycin concentration of 8.5 μg per milligram dry cell weight. A vancomycin concentration of 50 μg per ml was required to inhibit growth of P. fluorescens cells. Thus, the difference in the concentration of antibiotic required to inhibit growth of B. subtilis and P. fluorescens emphasizes the difference in sensitivity of these two organisms to vancomycin. Ziegler, Wolfe, and McGuire (1956) reported that low concentrations of vancomycin inhibited growth of gram-positive bacteria, but had little effect on the growth of gram-negative bacteria. A difference in sensitivity was also noted with the two gram-negative organisms. The sensitivity of the gram-positive and gram-negative organisms to vancomycin may be due to the structure and composition of the cell wall of the two organisms which would result in a difference in the quantitative adsorption of the antibiotic to the cell. This explanation might also explain the variation in sensitivity of the gram-negative organisms. However, if the antibiotic acts at an intracellular site then there may be a difference in the permeability of the gram-negative and gram-positive organisms to vancomycin.

The Influence of Magnesium and EDTA on the Inhibition of Growth by Vancomycin

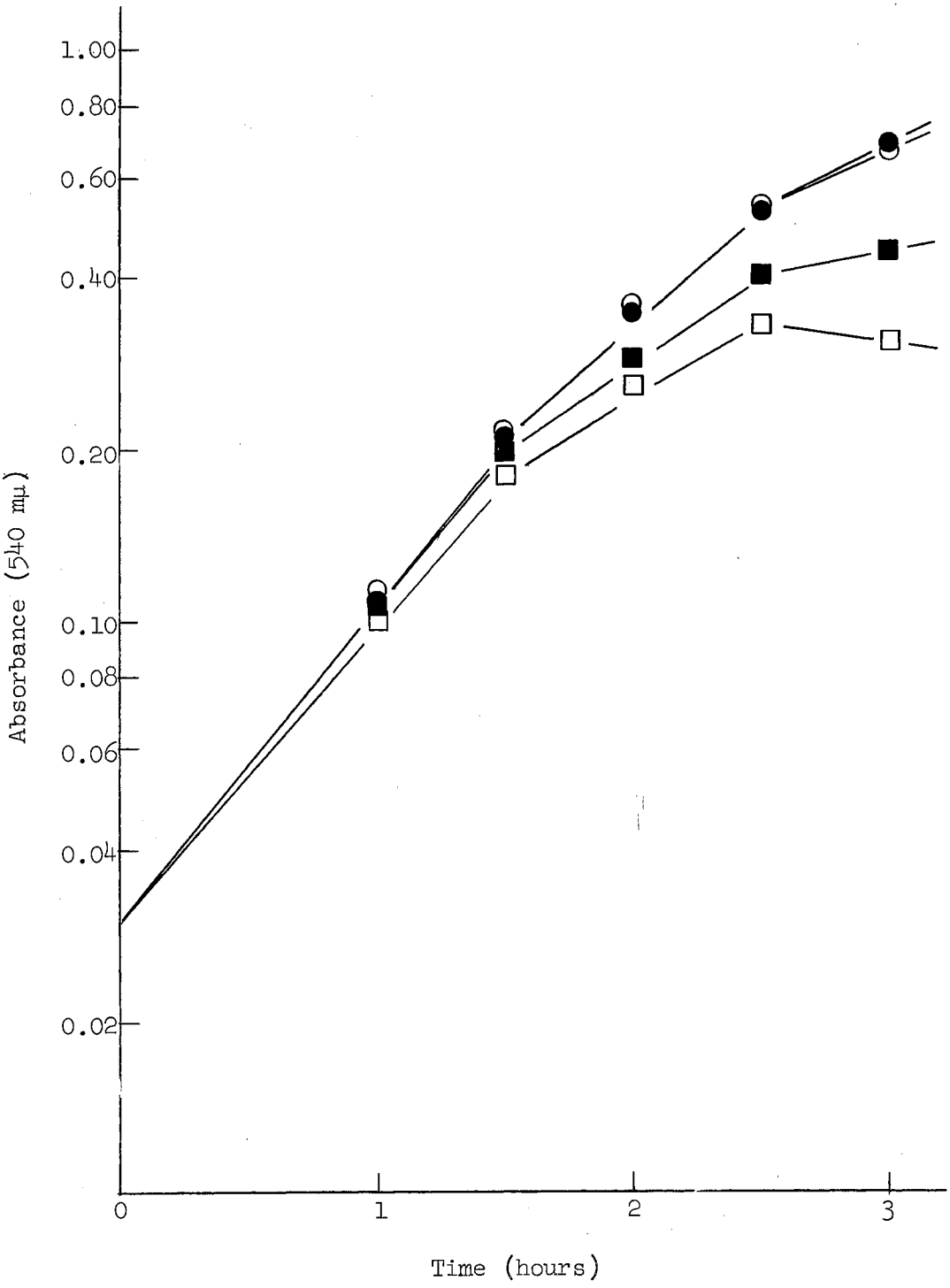
Best and Durham (1964) noted that magnesium partially reversed the inhibition of growth of B. subtilis by vancomycin. Studies were conducted to ascertain if magnesium showed a similar effect with P. fluorescens.

P. fluorescens cells were grown on succinate agar slants, harvested, and used to inoculate tubes of succinate-salts medium. The initial absorbance was 0.02 at 540 mμ. The tubes were incubated for two and one-half hours with constant shaking. The cells were centrifuged and suspended in 7.0 ml of 0.01 M tris buffer. The absorbance of the cell suspension was approximately 0.30 at 540 mμ. One-half ml of the cell suspension was used to inoculate 250 ml Erlenmeyer sidearm flasks containing 25.0 ml of succinate-salts medium. The temperature of the medium at the time of inoculation was 37 C. Two and one-half ml of vancomycin (900 μg per ml) were added to three of the flasks. One and one-half ml of a magnesium sulfate solution (0.05 M) were added to one of the vancomycin-containing flasks and to a flask that did not contain vancomycin to serve as a magnesium control. Two ml of EDTA (10^{-4} M) were added to a vancomycin-containing flask and to an appropriate flask to serve as an EDTA control. Triple distilled water was added to all flasks to bring the total liquid volume of each flask to 29.5 ml. The final liquid volume of each flask after inoculation was 30.0 ml.

Vancomycin in a concentration of 77 μg per ml inhibited the growth and continued incubation indicated some lysis of the cells (Figure 4). The addition of magnesium (2.5 μmoles per ml) partially reversed the

Figure 4

The influence of magnesium on the inhibition of growth of P. fluorescens by vancomycin. ● , control: ○ , control plus magnesium (2.5 μmoles per ml): ■ , vancomycin (77 μg per ml) plus magnesium (2.5 μmoles per ml): and □ , vancomycin (77 μg per ml).


1.00
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
Absorbance (540 mμ)
0
1
2
3
Time (hours)

inhibition of growth and delayed but did not overcome the lysis. The data obtained from the study with the gram-negative organism are consistent with the results obtained by Best and Durham (1964) using a gram-positive organism.

The presence of 6.6 μmoles per ml of EDTA did not influence either the inhibition of growth or the initiation of lysis of the P. fluorescens cells by vancomycin (Figure 5).

Adsorption of Vancomycin to Gram-negative Organisms

Studies were made to quantitate the amount of vancomycin adsorbed to cells of P. fluorescens and Flavobacterium since these organisms showed a difference in sensitivity to the antibiotic.

Cells of P. fluorescens and the Flavobacterium were grown for 12-15 hours on nutrient agar plates, harvested and suspended in 0.01 M tris buffer to an absorbance of 1.0 at 540 mμ. Two tubes containing 6.0 ml of the cell suspension were prepared for each culture. The cells were centrifuged and the cell pellets suspended in 3.0 ml of triple distilled water. Triple distilled water (3.0 ml) was added to one tube of each culture (total liquid volume of 6.0 ml) to serve as a control. Three ml of vancomycin (300 μg per ml) were added to the remaining tubes. The tubes were incubated at room temperature for 10 minutes, centrifuged, and the absorbance of the supernatant solutions measured at 280 mμ. The values were corrected for the control and the amount of antibiotic adsorbed was calculated.

The results (Table I) establish that approximately five times as much vancomycin is adsorbed to cells of P. fluorescens as is adsorbed to the cells of the Flavobacterium. These results might partially explain the difference in the sensitivity of the organisms as

Figure 5

The influence of EDTA on the inhibition of growth of P. fluorescens by vancomycin. ● , control; ○ , control plus EDTA (6.6 μmoles per ml); ■ , vancomycin (77 μg per ml) plus EDTA (6.6 μmoles per ml); and □ , vancomycin (77 μg per ml).

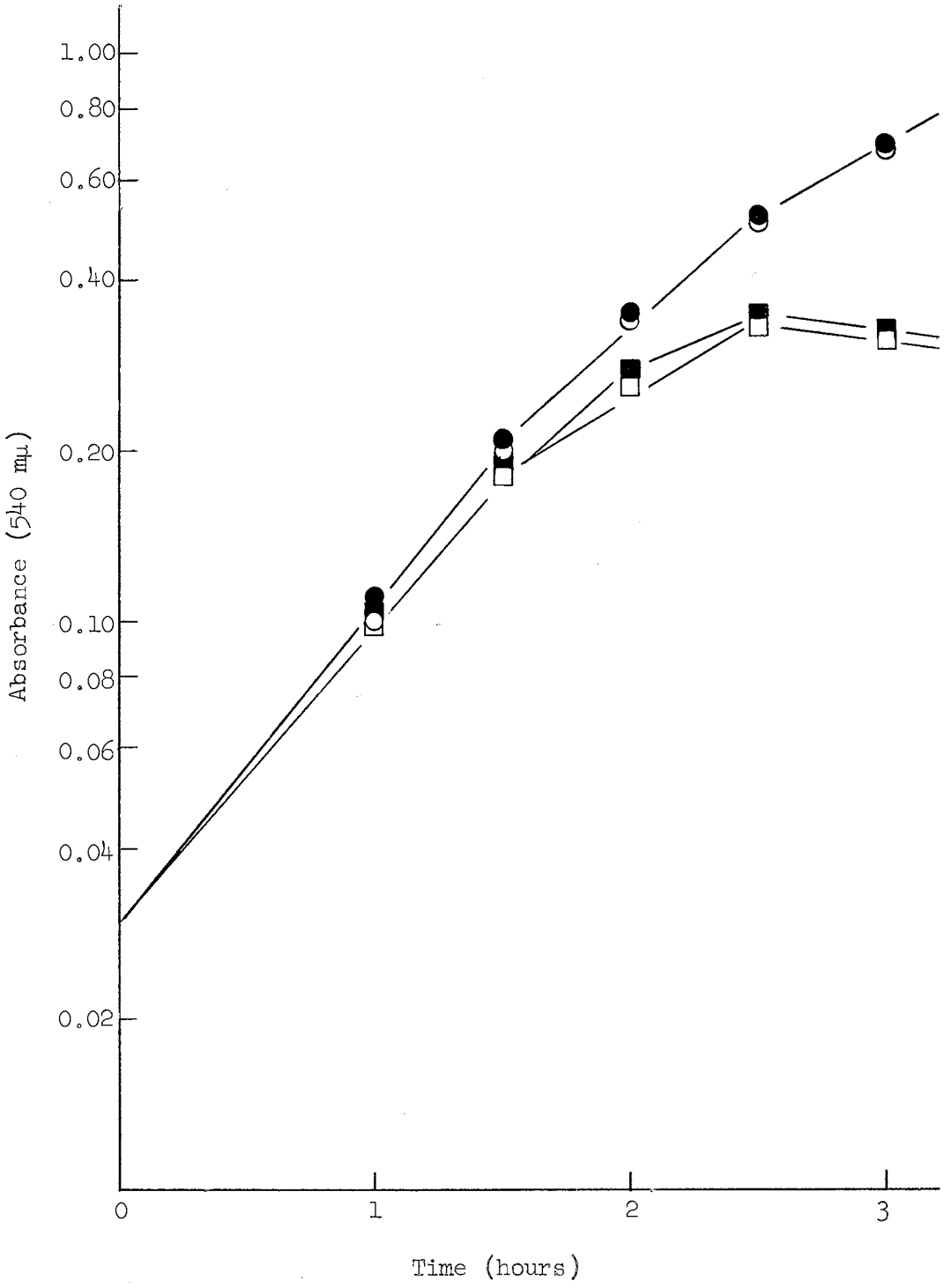

1.00
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
Absorbance (540 mμ)
0
1
2
3
Time (hours)

TABLE I

Adsorption of Vancomycin to Gram-negative Organisms

Organism	Vancomycin Adsorbed (μg)*
Flavobacterium species	26
P. fluorescens	180

*The total quantity of vancomycin available for adsorption to the cells was 900 μg.

suggested by the growth studies, in which the Flavobacterium was less sensitive to vancomycin than was P. fluorescens. Since less of the antibiotic is adsorbed to the Flavobacterium, then this finding would support the hypothesis that there is a correlation between adsorption and susceptibility of an organism to vancomycin.

Effect of Magnesium on Adsorption and Elution of Vancomycin

Since magnesium influenced the inhibition of growth by vancomycin, studies were conducted to determine if the cation affected the adsorption of the antibiotic to the cells.

Cells of P. fluorescens were grown 12-15 hours on nutrient agar plates, harvested and suspended in triple distilled water to an absorbance of 1.0 at 540 mμ. Six ml of the cell suspension were added to test tubes, centrifuged, the supernatant solutions were discarded, and the experiment conducted as previously described.

The results indicated that prior treatment of the cells with magnesium decreased the amount of vancomycin adsorbed (Table II). The cation could also elute vancomycin which had been adsorbed to cells. Similar results were reported by Best (1965) using B. subtilis, and it was proposed that vancomycin might lessen the binding of magnesium to some essential site on the cell surface, or magnesium could be acting to reduce the binding of vancomycin to the cell surface. The results suggest that binding of vancomycin to the cell is a reversable process since the antibiotic adsorbed to the cells could be readily eluted by the magnesium.

Effect of Metals on Adsorption of Vancomycin

Since magnesium influenced adsorption of the antibiotic to the

TABLE II

Effect of Magnesium on Adsorption and Elution of Vancomycin

Additions	Vancomycin Absorbed* (μg)	Vancomycin Eluted by Magnesium (μg)	Per Cent Eluted
None	228	210	92
Magnesium	138	-	-

*The total quantity of vancomycin available for adsorption to the cells was 900 μg.

cells, experiments were conducted to determine the effect of other divalent and monovalent cations on the adsorption of vancomycin to P. fluorescens.

P. fluorescens cells were prepared as described in the previous experiment and the cells were suspended in triple distilled water to an absorbance of 1.0 at 540 mμ. Six ml of the cell suspension were added to seven tubes, centrifuged and the supernatant solutions discarded. The cell pellets were suspended in 1.5 ml of the appropriate ion solution (12.5 mmoles per ml). Two tubes were used as controls and the pellets were suspended in 1.5 ml of triple distilled water. Three ml of vancomycin solution (300 μg per ml) were added to all tubes except one of the control tubes. Triple distilled water was added to each tube to bring the final liquid volume to 6.0 ml. All tubes were incubated 10 minutes at room temperature to permit adsorption of the antibiotic. The tubes were centrifuged and the absorbance of the supernatant solutions measured at 280 mμ.

As depicted in Table III, several other cations interferred with the binding of vancomycin to the cells. The greatest effect was observed with the divalent cations, but an equivalent concentration of the monovalent cations also influenced adsorption of the antibiotic to the cells. Best and Durham (1964) noted similar results with divalent cations (0.83 mM), but observed that monovalent cations did not influence the adsorption of vancomycin to cells of B. subtilis. The divalent cations are much more efficient in preventing adsorption of vancomycin than the monovalent cations with the gram-negative organism. The difference in the data may be resolved in differences in the concentrations of the cations used in the studies.

TABLE III

Effect of Metals on Vancomycin Adsorption to P. fluorescens*

Salt	Vancomycin Absorbed (µg)	Per Cent Adsorbed
1) Control	108	100.0
2) $MgSO_4$	15	13.9
3) $MnSO_4$	18	16.7
4) $CaCl_2$	30	27.8
5) NaCl	48	44.4
6) KCl	48	44.4

*Each salt was added to final concentration of 12.5 mM. A total of 900 µg of vancomycin was present in each tube.

Effect of Ethanol and EDTA on Adsorption of Vancomycin

Salton (1960) and Perkins (1963) reported that the external structure of gram-negative bacteria was composed of a three-layered complex, consisting of lipoprotein, lipopolysaccharide and mucopeptide. Since vancomycin adsorbs to the cell, studies were conducted using ethanol and EDTA to alter the structural arrangement of the wall complex to ascertain if this influenced adsorption of the antibiotic. Ethanol was used since it is reported to solubilize the lipid material and EDTA was used since it is a chelating agent. Lieve (1965), Gray, and Wilkinson (1965) and Ashbell and Eagon (1966) have noted the release of lipopolysaccharide material following the treatment of gram-negative organisms with EDTA. Gray and Wilkinson (1965) and Ashbell and Eagon (1966) theorized that multivalent cations, primarily calcium and magnesium, were present in the outer layer of gram-negative bacteria and acted as cross-links for the lipopolysaccharide layer to other components of the cell wall. If the adsorption of vancomycin is primarily by an ionic type bond then it could compete with cations for the adsorption sites and removal of these stabilizing cations might increase the number of adsorption sites for vancomycin.

P. fluorescens cells were grown on nutrient agar, harvested as previously described and suspended to an absorbance of 1.0 at 540 mμ. Seven ml of the cell suspension were added to each of 6 tubes. The cells were centrifuged and 7.0 ml of the indicated additive were added (Table IV). Triple distilled water (7.0 ml) was added to two of the tubes to serve as controls. The tubes were incubated 20 minutes at room temperature with frequent mixing. One ml was removed, diluted and plated on nutrient agar for viable counts. The cells were

TABLE IV

Adsorption of Vancomycin by Cells Previously Treated with Ethanol and EDTA

Additive	Vancomycin Adsorbed* (μg)	Viable Count
Control	266	7.6×10^8
Ethanol 15%	364	5.6×10^8
Ethanol 35%	455	4.0×10^4
Ethanol 70%	714	4.0×10^4
EDTA 10^{-4} M	441	8.4×10^6

*The total quantity of vancomycin available for adsorption to the cells was 900 μg.

centrifuged, washed once and suspended in 3.0 ml of triple distilled water. Three ml of vancomycin (300 μg per ml) were added to all tubes except one of the control tubes. The tubes were incubated at room temperature for 10 minutes to permit adsorption, centrifuged and the absorbance of the supernatant solutions was measured.

The absorbance values were corrected for the 280 mμ absorbing material released by the cells during incubation and the amount of antibiotic adsorbed to the cells was determined.

The results (Table IV) indicate that treatment of the cells with either ethanol or EDTA increased the adsorption of the antibiotic. An EDTA concentration of 10^{-4} was used since preliminary studies indicated that higher concentrations, such as 10^{-3} M, produced lysis of the cells. The viable cell count decreased as the concentration of ethanol was increased, and the adsorption of vancomycin to the cells was greater in the higher ethanol concentrations. EDTA showed some decrease in viable counts, but no lysis was evident. Therefore, treatment of the cells with either ethanol or EDTA apparently increased the number of adsorption sites available for vancomycin interaction.

Since treatment of cells with ethanol or EDTA increased the adsorption of vancomycin to P. fluorescens, studies were conducted to determine if ethanol enhanced the adsorption of vancomycin to cells of the Flavobacterium.

Cells of P. fluorescens and the Flavobacterium were grown 12-15 hours on nutrient agar plates, harvested and suspended to an absorbance of 1.0 at 540 mμ. Six ml of the cell suspensions were added to tubes, centrifuged, and the supernatant solutions discarded. Two tubes of each culture received 6.0 ml of triple distilled water. These

tubes served as the controls. The cell pellet in a third tube was suspended in 6.0 ml of 35 per cent ethanol. The tubes were incubated at room temperature with frequent mixing for 15 minutes, centrifuged, washed once and suspended in 3.0 ml of triple distilled water. Three ml of vancomycin (300 μg per ml) was added to a water-treated and an ethanol-treated cell pellet. Three ml of water was added to the remaining tube to serve as a control. All tubes were incubated 10 minutes at room temperature to permit adsorption. The tubes were centrifuged and the absorbance of the supernatant solution measured.

The results (Table V) show that ethanol treatment of both organisms increased adsorption of the antibiotic to the cells. These results would be expected since the general structure of the outer layer of all gram-negative organisms is thought to be similar (Salton, 1960; Perkins, 1963). Therefore, if ethanol is able to effect some reorientation of the outer layer of one gram-negative organism, it should produce a similar effect with other gram-negative organisms.

The Adsorption of Vancomycin by P. fluorescens Following Treatment with Both Ethanol and EDTA

Treatment of cells with EDTA or ethanol increased the adsorption of vancomycin, so studies were conducted to determine if these agents had a similar mode of action. Lieve (1965) and Gray and Wilkinson (1965) reported that 0.0068 M EDTA was responsible for the release of lipid material from P. aeruginosa cells. This phenomenon was reported (Gray and Wilkinson, 1965; Ashbell and Eagon, 1966) to most probably be a secondary feature of the chelation of stabilizing multivalent cations present in the cell wall of gram-negative organisms. Hamilton-Miller (1966) reported EDTA damaged permeability barriers

TABLE V

The Adsorption of Vancomycin Following Ethanol Treatment of *P. fluorescens* and Flavobacterium

Organism	Vancomycin Adsorbed (μg)*
P. fluorescens	
Control	180
Ethanol-treated	390
Flavobacterium	
Control	36
Ethanol-treated	150

*The total quantity of vancomycin available for adsorption to the cells was 900 μg.

in Klebsiella aerogenes and Escherichia coli. If ethanol and EDTA act by different mechanisms, then treatment of the cells with both reagents should produce an additive adsorption effect. If the reagents have a similar mode of action, then the adsorption would not be additive. The experimental procedure was the same as that followed for previous extraction studies. The cell pellet that was treated with both additives was treated first with ethanol, centrifuged and then treated with EDTA.

The results (Table VI) indicate that cells treated with both reagents did not adsorb more antibiotic than cells treated only with EDTA. These results would indicate that EDTA and ethanol probably have a similar effect on the gram-negative cells as measured by vancomycin adsorption.

The Influence of Magnesium on the Vancomycin Adsorbed to Control and Ethanol-treated Cells.

Studies with both the gram-positive B. subtilis (Best and Durham, 1964) and the gram-negative P. fluorescens indicated that magnesium eluted vancomycin that was adsorbed to bacterial cells. Experiments were conducted to determine if the cation exerted a similar effect on vancomycin adsorption to ethanol-treated cells.

Six ml of a suspension of P. fluorescens cells was added to three tubes, centrifuged and the supernatant solutions discarded. Triple distilled water (6.0 ml) was added to the pellets in two tubes to serve as controls. The third tube received 6.0 ml of 35 per cent ethanol. All tubes were incubated for 15 minutes at room temperature with frequent mixing. The cells were centrifuged, washed once and suspended in 3.0 ml of triple distilled water. Three ml of vancomycin (300 μg per ml) were added to the cells treated with ethanol and to one of the water-

TABLE VI

The Adsorption of Vancomycin by P. fluorescens Following Treatment with Both Ethanol and EDTA

Additive	Vancomycin Adsorbed* (μg)
H_2O	270
Ethanol 15%	288
EDTA 10^{-4} M	558
Ethanol (15%) and EDTA (10^{-4} M)	558

*The total quantity of vancomycin available for adsorption to the cells was 900 μg.

treated controls. Triple distilled water (3.0 ml) was added to the third tube to serve as a control. Adsorption was permitted for 10 minutes at room temperature. The tubes were centrifuged and the absorbance of the supernatant solutions was measured. The vancomycin-treated pellets were washed once with deionize water and suspended in 6.0 ml of magnesium sulfate (0.05 M). These tubes were centrifuged immediately and the vancomycin in the supernatant solutions was determined.

The results (Table VII) augument the earlier studies that treatment of cells with ethanol increased the amount of antibiotic adsorbed to P. fluorescens cells. This finding would suggest that the bond between vancomycin and the cell is not primarily a lyophobic bond, since removal of the lipid by ethanol would be expected to decrease the amount of antibiotic adsorbed to the cells, not increase it. However, if removal of the lipid components created more negatively-charged groups, then the positively-charged vancomycin molecule could complex with these groups and the increased adsorption of vancomycin would be evident. If the bonding between vancomycin and the negative-charged groups on the wall is an ionic type rather than a lyophobic bond, then magnesium should be able to substitute for vancomycin. Magnesium does elute the vancomycin adsorbed to ethanol-treated cells (Table VII). These data support the proposed mechanism of an ionic type bond for vancomycin attachment to the cell since ethanol-treated cells adsorbed more antibiotic than the control cells and the antibiotic could be eluted by the positively charged ion, magnesium.

TABLE VII

The Elution by Magnesium of Vancomycin Adsorbed to Control and Ethanol-treated Cells

Additive	Vancomycin		
	Adsorbed* (μg)	Eluted by Magnesium (μg)	Per Cent Eluted
Control	228	210	92
Ethanol-treated	612	450	74

*The total quantity of vancomycin available for adsorption to the cells was 900 μg.

Adsorption of Vancomycin to Control and Ethanol-treated cells of Micrococcus lysodeikticus

Perkins (1963) reported that the outer layer (cell wall) of M. lysodeikticus was composed of alanine, glutamic acid, lysine, glycine, glucose, glucosamine and muramic acid. Teichoic acid was thought to be absent since there was no phosphorus detected in the cell wall. The presence of lipid in the cell wall of M. lysodeikticus has not been reported.

If the cells of this organism have no lipid in the cell wall, adsorption studies should contribute to elucidating binding sites for the antibiotic.

M. lysodeikticus cells were grown 12 hours on brain heart infusion medium (Difco), harvested and suspended to an absorbance of 1.0 at 540 mμ. Treatment of the cells was similar to the procedure used with P. fluorescens. The results (Table VIII) indicate that treatment of M. lysodeikticus cells with ethanol may have produced a slight increase in the antibiotic adsorbed. However, the effect is not nearly so pronounced as was observed in the gram-negative organisms. These results suggest that ethanol may be effecting some structural orientation of the mucopeptide layer. Since M. lysodeikticus does not contain teichoic acid in the cell wall, then these results would indicate that teichoic acid does not play a critical role in the adsorption of vancomycin to the cells. Also, the adsorption of vancomycin to cell walls containing little if any lipid would suggest that lipids do not play an important role in the interaction of the antibiotic and wall.

TABLE VIII

Adsorption of Vancomycin to Control and Ethanol-treated Cells of *Micrococcus lysodeikticus*

Additive	Vancomycin Adsorbed (μg)*
Water-treated	462
Ethanol-treated	540

*The total quantity of vancomycin available for adsorption to the cells was 900 μg.

Influence of EDTA on Adsorption of Vancomycin to P. fluorescens Cells Grown on Succinate and Nutrient Agar.

The results indicated that more vancomycin was adsorbed to succinate-grown cells than to nutrient agar-grown cells of P. fluorescens. Studies were conducted to determine if EDTA treatment influenced the adsorption of vancomycin by cells grown on the different media.

P. fluorescens cells were grown 12-15 hours at 37 C on nutrient agar slants, harvested, suspended in 0.01 M tris buffer and used to inoculate plates of nutrient and succinate agar. The plates were incubated 12-15 hours at 37 C, harvested, the cells washed once and suspended in triple distilled water to an absorbance of 1.0 at 540 mμ. Six ml of the appropriate cell suspension were added to three tubes. The tubes were centrifuged and the supernatant solutions discarded. Triple distilled water (6.0 ml) was added to two tubes of each set. The third tube received 6.0 ml of 10^{-4} M EDTA. All tubes were incubated 15 minutes at room temperature, centrifuged, the supernatant solution discarded.and the pellets suspended in 3.0 ml of triple distilled water. Three ml of vancomycin (300 μg per ml) were added to the EDTA-treated cells and to one of the water-treated controls. Triple distilled water (3.0 ml) was added to the remaining tube to serve as a control. The tubes were incubated 10 minutes at room temperature, centrifuged and the absorbance of the supernatant solutions measured at 280 mμ.

The results (Table IX) confirm the earlier observation that cells grown on succinate agar adsorb more vancomycin than cells grown on nutrient agar. Treatment of the cells with EDTA increases the adsorption of vancomycin to both groups of cells. The nutrient agar-grown cells appear to be more responsive to the EDTA treatment than

TABLE IX

Influence of EDTA on Adsorption of Vancomycin to P. fluorescens Cells Grown on Succinate and Nutrient Agar

Growth Medium	Treatment	Vancomycin Adsorbed* (μg)
Succinate agar	H_2O	300
Succinate agar	EDTA (10^{-4} M)	492
Nutrient agar	H_2O	228
Nutrient agar	EDTA (10^{-4} M)	552

*The total quantity of vancomycin available for adsorpt on to the cells was 900 μg.

the succinate-grown cells which suggests that the two groups of cells may differ in the cell wall structure or composition, at least in those groups functional in vancomycin adsorption.

Influence of Age of the Culture on Adsorption of Vancomycin

Experiments were conducted to determine if the age of the cells affected the adsorption of vancomycin. P. fluorescens cells were grown on plates of nutrient and succinate agar. The plates were incubated 12-15 hours at 37 C, harvested and the cells inoculated into the respective liquid medium. Two liter flasks were prepared containing 500 ml of succinate-salts medium or 500 ml of nutrient broth. The flasks were incubated at 37 C with constant shaking and a 50 ml sample was removed at the indicated time intervals. The samples were centrifuged, suspended to an absorbance of 1.0 at 540 mμ, 6.0 ml of the cell suspension was removed centrifuged, and the adsorption of vancomycin determined.

The results (Table X) indicate that the age of the culture apparently does not have a profound effect on the adsorption of vancomycin.

Adsorption of Vancomycin by Cells Grown on Nutrient Agar Poised at Different pH Values

Studies were conducted to determine if the pH of the medium on which the cells were grown might effect vancomycin adsorption. Nutrient agar plates were prepared at pH 6.0, 6.8, and 7.5 and inoculated with P. fluorescens. The plates were incubated at 37 C for 12-15 hours, the cells harvested and suspended to an absorbance of 1.0 at 540 mμ in triple distilled water. Two test tubes received 6.0 ml of each culture making a total of six tubes. All tubes were centrifuged and the

TABLE X

Influence of the Age of the Culture on Adsorption of Vancomycin

Age of Culture (Hours)	Vancomycin Adsorbed*	
	Succinate-grown	Nutrient broth-grown
1	46	43
6	55	49
12	50	45
24	49	51
48	59	54

*The quantity of vancomycin adsorbed is expressed as μg per ml. The final concentration of vancomycin in each tube was 150 μg per ml.

supernatant solutions discarded. The cell pellet of one tube of each culture received 6.0 ml of triple distilled water and the other tube received 6.0 ml of vancomycin containing 150 μg per ml. Adsorption was permitted for 10 minutes at room temperature. The tubes were centrifuged and the absorbance of the supernatant solutions was measured.

The results (Table XI) of this experiment indicate that the pH of the medium on which the cells were grown did not influence the amount of antibiotic adsorbed to the cells.

The Influence of Different Media on the Adsorption and Sensitivity of P. fluorescens to Vancomycin.

Previous results indicated that cells of P. fluorescens grown on succinate agar adsorbed more vancomycin than cells grown on nutrient agar. An experiment was proposed to ascertain if these cells showed a difference in growth when inoculated into either nutrient broth or succinate broth that reflected the sensitivity to vancomycin.

P. fluorescens cells were grown on nutrient agar slants for 12-15 hours, harvested, suspended in triple distilled water and used to inoculate plates of nutrient and succinate agar. All plates were incubated for 12-15 hours at 37 C. The cells were harvested, washed once and suspended in triple distilled water to an absorbance of 0.5 at 540 mμ. Five ml of the cell suspension was placed into three tubes. The first tube served as the control, the second tube received 0.2 ml of vancomycin solution (900 μg per ml), and the third tube received 0.5 ml of vancomycin solution (900 μg per ml). Triple distilled water was added to all tubes to give a final liquid volume of 6.0 ml. The tubes were incubated 15 minutes at room temperature, centrifuged and the supernatant solutions assayed for vancomycin. The cell pellets were

TABLE XI

Adsorption of Vancomycin by Cells Grown on Nutrient Agar Poised at Different pH Values

pH of Growth Medium	Vancomycin Adsorbed (μg)*
6.0	156
6.8	162
7.5	162

*The total quantity of vancomycin available for adsorption to the cells was 900 μg.

suspended in 6.0 ml of tris buffer. One ml was removed from each tube and plated on nutrient agar to determine the number of viable cells. One tenth ml from each tube was added to a tube containing 6.0 ml of nutrient broth or 6.0 ml of succinate-salts. The growth of the organisms in these media was determined as indicated previously.

The results (Table XII) support the previous findings that cells grown on succinate agar adsorbed more antibiotic than cells grown on nutrient agar. There did not appear to be a profound decrease in the number of viable cells at these antibiotic concentrations. Growth studies of the vancomycin-treated cells in nutrient and succinate broth are presented in Figures 6, 7, 8, and 9. Vancomycin treatment did not appear to influence the rate of growth of P. fluorescens in either medium. This is not unexpected since a vancomycin concentration of 75 μg per ml did not show a profound reduction in the number of viable cells.

An antibiotic concentration of 75 μg per ml inhibits growth when the organism is cultivated in the presence of vancomycin. However, when the organism is placed in an antibiotic-free environment, growth ensues, indicating that vancomycin attachment to the cell appears to be a reversible process under certain conditions.

TABLE XII

The Influence of Different Media on the Adsorption and Sensitivity of *P. fluorescens* to Vancomycin

Growth Medium	Vancomycin Concentration (μg per ml)	Vancomycin Adsorbed (μg per ml)	Viable Cells x 10^6
Nutrient agar	0	-	236
	30	1	238
	75	2	220
Succinate	0	-	254
	30	4	237
	75	20	217

Figure 6

The growth of vancomycin-treated P. fluorescens in different media. The cells were grown on nutrient agar, harvested, incubated with vancomycin, and inoculated into nutrient broth. ● , control; □ , incubated with 30 μg per ml of vancomycin; and ■ , incubated with 75 μg per ml of vancomycin.

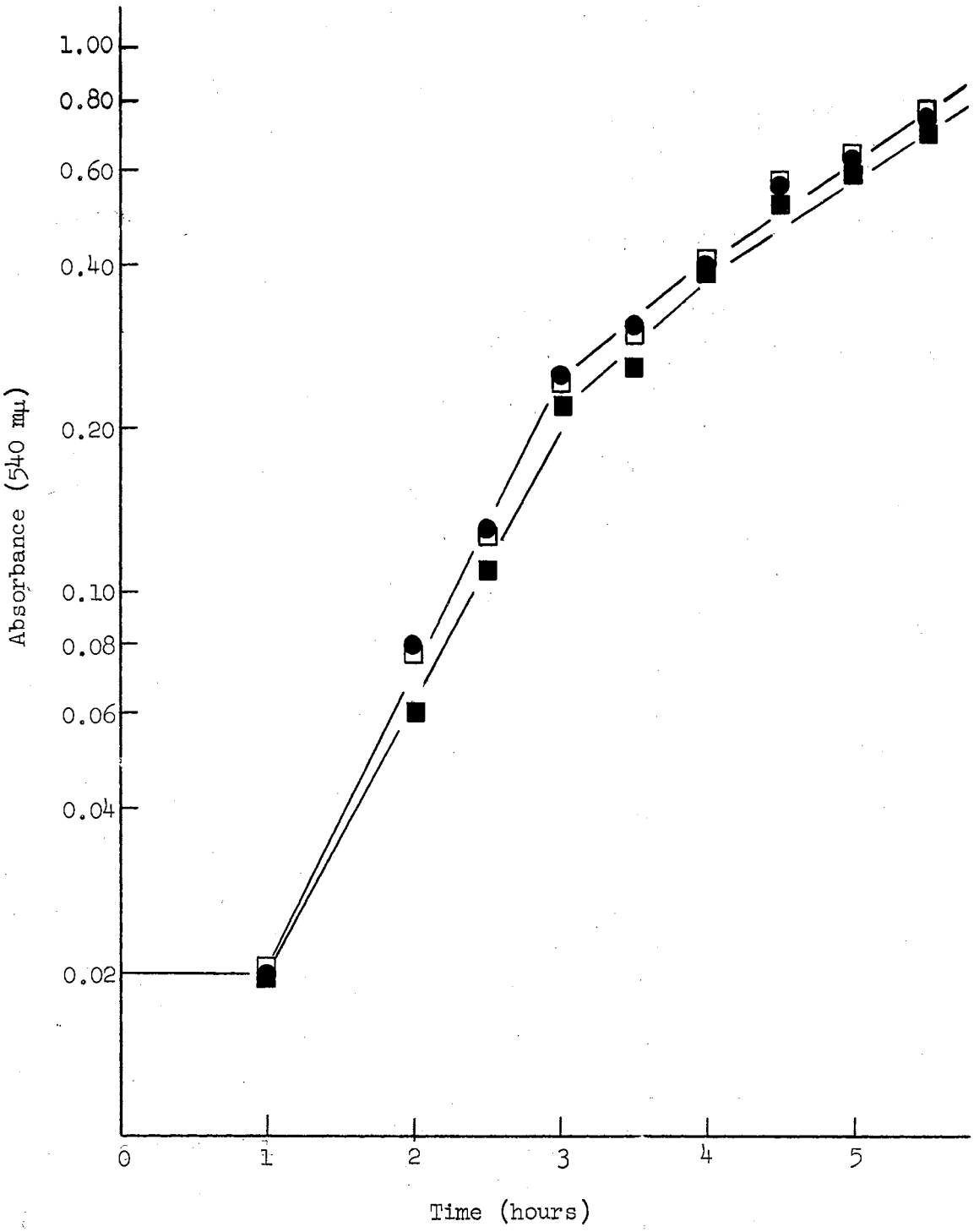

1.00
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
Absorbance (540 mμ)
0
1
2
3
4
5
Time (hours)

Figure 7

The growth of vancomycin-treated P. fluorescens in different media. The cells were grown on nutrient agar, harvested, incubated with vancomycin, and inoculated into succinate salts. ● , control; □ , incubated with 30 μg per ml of vancomycin; and ■ , incubated with 75 μg per ml of vancomycin.

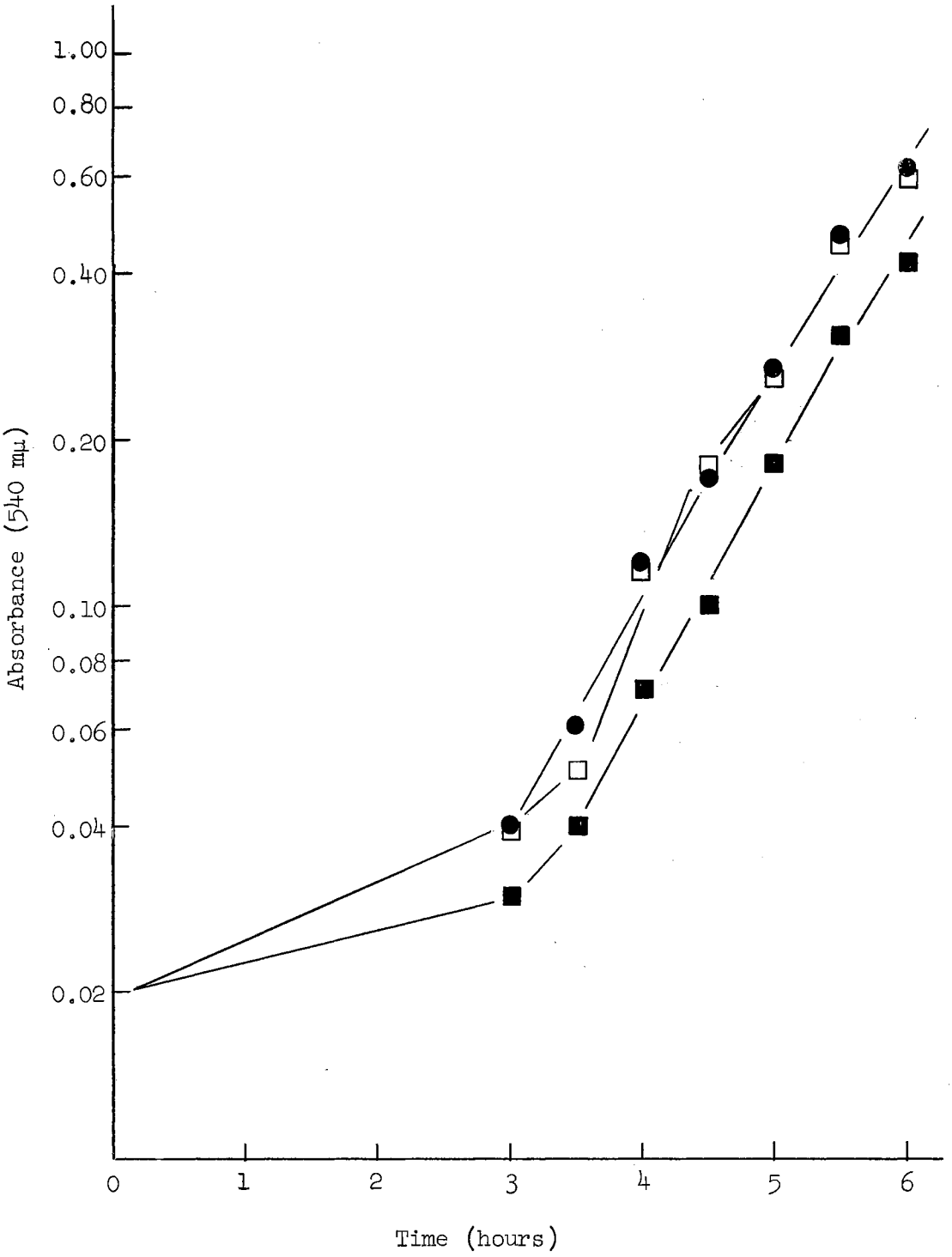
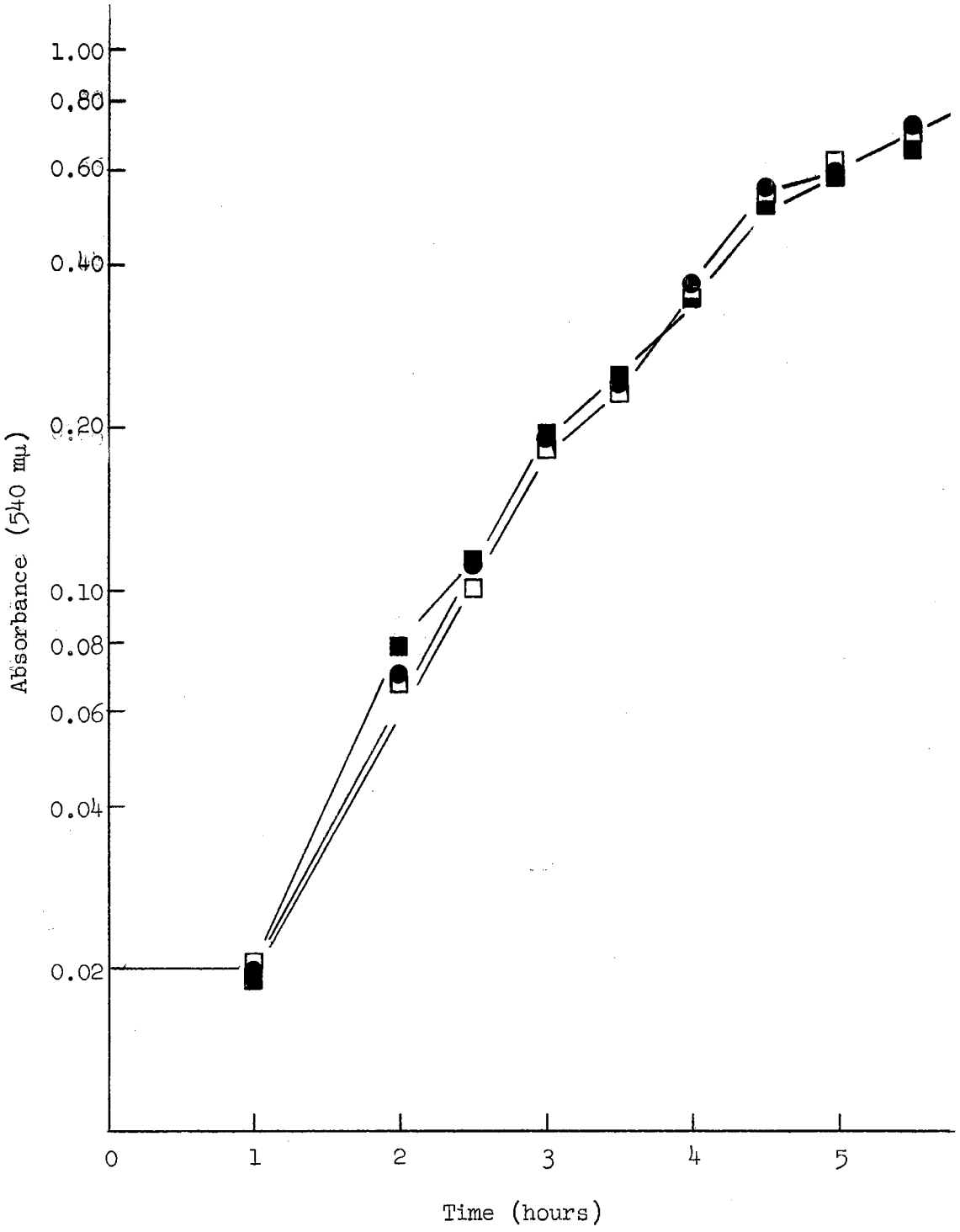

1.00
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
Absorbance (540 mμ)
0
1
2
3
4
5
6
Time (hours)

Figure 8

The growth of vancomycin-treated P. fluorescens in different media. The cells were grown on succinate-salts agar, harvested, incubated with vancomycin, and inoculated into nutrient broth. ●, control; □, incubated with 30 μg per ml of vancomycin; and ■, incubated with 75 μg per ml of vancomycin.


1.00
0.80
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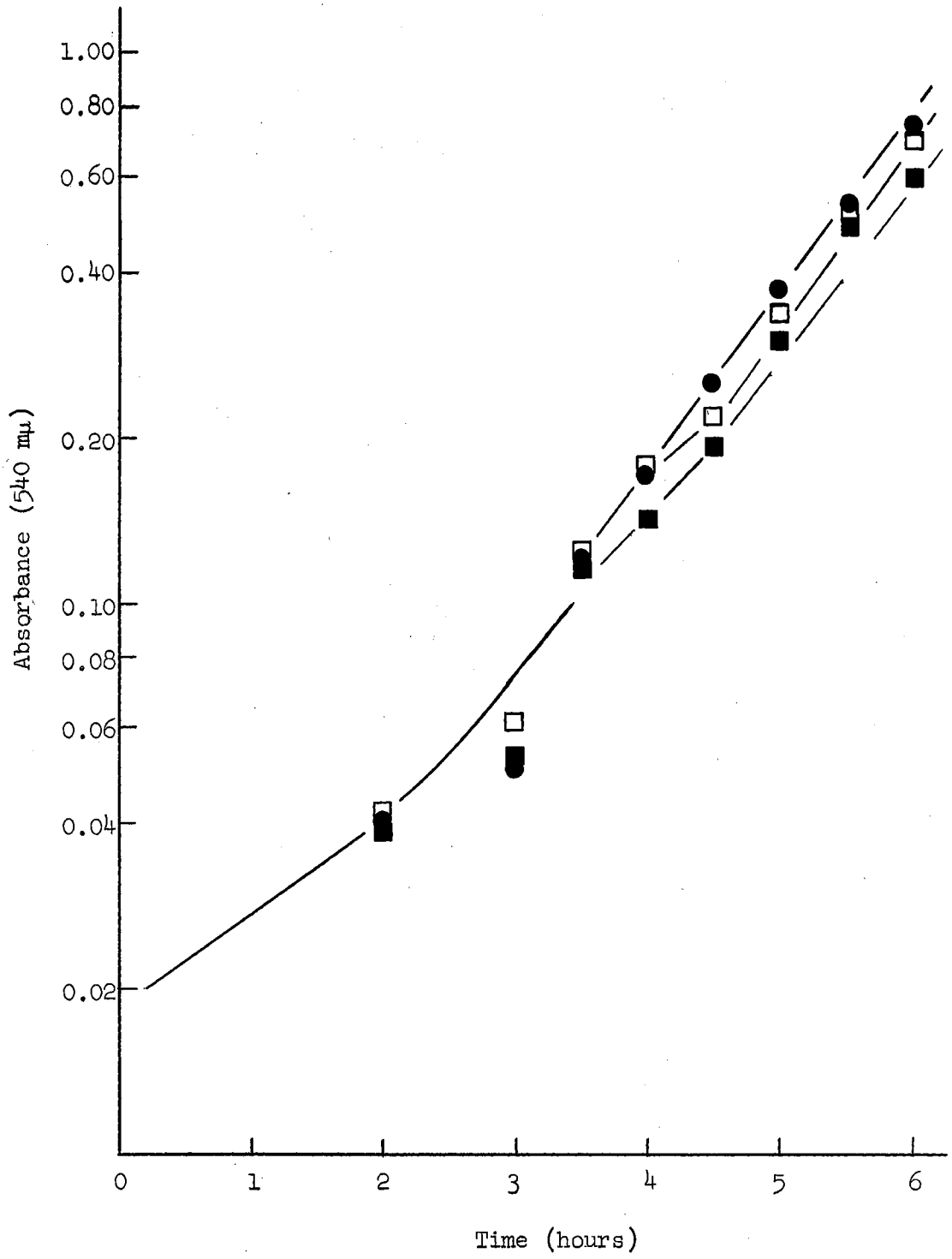
0.40
0.20
0.10
0.08
0.06
0.04
0.02
Absorbance (540 mμ)
0
1
2
3
4
5
Time (hours)

Figure 9

The growth of vancomycin-treated P. fluorescens in different media. The cells were grown on succinate-salts agar, harvested, incubated with vancomycin, and inoculated into succinate salts. ●, control; □, incubated with 30 μg per ml of vancomycin; and ■, incubated with 75 μg per ml of vancomycin.


1.00
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
Absorbance (540 mμ)
0
1
2
3
4
5
6
Time (hours)

CHAPTER V

SUMMARY AND CONCLUSIONS

Vancomycin inhibits the growth of P. fluorescens but does not influence the growth of a species of Flavobacterium at the antibiotic concentrations tested. A vancomycin concentration of 77 μg per ml caused a pronounced inhibition of growth of P. fluorescens, and lysis of the cells was evident with continued incubation. These vancomycin concentrations do not represent the absolute levels necessary to attain a given inhibition, but the differences in response of the cells as a function of the vancomycin concentration indicate that the mechanism of inhibition becomes more intricate as the antibiotic concentration is increased.

Magnesium ions partially reversed the inhibition of growth of P. fluorescens by vancomycin. The results support the concept that magnesium was influential at the active sites on the cell that functioned in the attachment of vancomycin.

Best and Durham (1964) reported that vancomycin adsorbed to cell walls of B. subtilis and proposed that one mechanism of the antibiotic was an inhibition of cell wall synthesis. These researchers theorized that adsorption of the vancomycin molecule might inhibit synthesis of the cell wall polymer by physically blocking the additional extension of the polymer. The observation that magnesium reversed the inhibition of growth and reduced the adsorption of the antibiotic to the cell makes

the suggested mechanism more attractive.

Best (1965) noted that adsorption of vancomycin to cell walls of B. subtilis might be related to the basic nature of the antibiotic. Johnson (1962) reported that the vancomycin molecule had pKa values above 7.0 of 7.7, 9.1, 10.1, 11.5 as well as three other values between 12.0 and 13.5 and these would be positively charged at a pH of 7.0. Thus, the molecule could complex with free anionic groups present in the cell wall of microorganisms. Muramic acid and the free C-terminal amino acids in the cell wall could possess an ionizable carboxyl group that would function in adsorption. The interaction of vancomycin with the cells could occur via electrostatic or ionic linkages.

McCormick et al. (1956) reported that vancomycin was capable of forming a lyophobic complex with paraffin. Such a complex could exist between the lipid fractions of the cell wall of gram-negative organisms and the antibiotic. The data presented in this study using the ethanol and EDTA-treated cells would suggest that this type of bonding does not exist in P. fluorescens. Yudkin (1963) reported inhibition of the synthesis of the cytoplasmic membrane by vancomycin. It is possible that vancomycin could block the polymerization of new membrane by forming a lyophobic complex with the existing membrane. Chatterjee and Perkins (1966) noted the accumulation of "Park nucleotides" associated with vancomycin in a species of Corynebacterium. Therefore, the portion of the antibiotic associated with the membrane might also complex with the intracellular cell wall precursors.

An increased adsorption of vancomycin to P. fluorescens cells was noted when the organism was grown on succinate-salts medium, as compared to the quantity of antibiotic adsorbed to cells grown on

nutrient agar. One explanation for this observation would be that cells grown on nutrient agar may produce some nonionizable exocellular product, perhaps capsular material, which would interfere with the adsorption of the antibiotic. This product would not be formed in great quantity when the cells were grown on succinate-salts medium.

In summary, the results indicate that the concentration of vancomycin is important in determining how the antibiotic affects the cell. Low concentrations of vancomycin adsorbed to cells of P. fluorescens and inhibited growth. At higher concentrations of the antibiotic, lysis of cells occurred with continued incubation. Magnesium partially alleviated the growth inhibition presumably by reducing the amount of antibiotic adsorbed to the cells. It seems logical that vancomycin may complex with the existing cell wall, interfere with the polymerization process, and produce an inhibition of growth.

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VITA

Dale Vernon Ferguson

Candidate for the Degree of

Master of Science

Thesis: THE EFFECT OF VANCOMYCIN ON PSEUDOMONAS FLUORESCENS

Major Field: Microbiology

Biographical:

Personal Data: Born at Tulsa, Oklahoma, November 29, 1943, son of Jack and Goldie Ferguson; married to Barbara Davalt on August 18, 1963; Daughter, Barbara Denise, born December 2, 1966.

Education: Graduated from Thomas A. Edison High School, Tulsa, Oklahoma, in 1961. Received the Bachelor of Science degree with a major in Medical Technology from Oklahoma State University, Stillwater, Oklahoma, May, 1966. Completed requirements for the Master of Science degree in May, 1967.

Experience: Teaching Assistant and Graduate Research Assistant, Department of Microbiology, Oklahoma State University, 1965-1967.