MECHANISM OF ACTION OF VANCOMYCIN

By

GARY KEITH BEST

Bachelor of Science

Southwestern State College

Weatherford, Oklahoma

1960

Submitted to the Faculty of the Graduate School of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY
May, 1965

MECHANISM OF ACTION OF VANCOMYCIN

Thesis Approved:

Thesis Adviser

Dean of the Graduate School

ACKNOWLEDGMENTS

The guidance and encouragement of Dr. N. N. Durham, throughout this study, is most gratefully acknowledged.

Appreciation is also expressed to Dr. L. L. Gee, Dr. E. C. Noller, Dr. E. N. Hodnett, Dr. R. E. Koeppe, and Dr. F. R. Leach for contributing valuable discussions during various phases of this investigation.

The facilities and financial support furnished by the Department of Microbiology, Oklahoma State University, are deeply appreciated.

A special gratitude is expressed to my wife, Norma, who made invaluable contributions to this endeavor.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Vancomycin, generic designation for Vancocin, was originally extracted from fermentation filtrates of Streptomyces orientalis. The workers at Eli Lilly and Company isolated this streptomycete from an Indonesian soil sample and developed the techniques for producing this antibiotic on a commercial scale (McCormick et al., 1956).

The efficacy of vancomycin is based on its effectiveness at low concentrations, low toxicity for humans, activity against microorganisms resistant to other antibiotics, and its propensity against development of resistance in vitro (McCormick et al., 1956).

Original studies with vancomycin showed that it was effective against a wide range of microorganisms, but Gram-positive bacteria were more sensitive to vancomycin than Gram-negative organisms (Ziegler, Wolfe, and McGuire, 1956). In addition, these workers showed vancomycin to be bactericidal, but only when the bacteria were actively multiplying.

The present study was initiated to supplement the limited information available on the mechanism by which vancomycin affects Gram-positive bacteria.

CHAPTER II

LITERATURE REVIEW

Chemical characterization of vancomycin.

Vancomycin is a complex molecule of unknown structure. The molecular weight of this antibiotic was estimated to be 3200-3500 (±200) from titration data and 3300 on the basis of ultracentrifugation studies (McCormick et al., 1956). However, Johnson (1962) reported that the molecular weight of vancomycin was possibly 1560, and corresponded to an empirical formula of $C_{70}H_{90}Cl_2N_9O_{27}$. Estimations of the molecular weight by the different research groups would be reconcilable if vancomycin contains four instead of two chlorine atoms per molecule.

A chemical analysis of vancomycin by Johnson (1962) revealed the following components:

1 mole L-aspartic acid
1 mole N-methyl-D-leucine
2 moles glycine
1 mole alanine
3 groups that released NH_3 upon hydrolysis
1 group that evolves methylamine upon hydrolysis
1 mole glucose
2 groups 2-chloro-4-hydroxyphenol
2 groups 2-hydroxyphenol
6 groups periodate-labile (one yielding acetaldehyde)
1 system giving 2-methyl-4-ketohexanoic acid upon strong acid hydrolysis

There is no indication of how these entities are linked in vancomycin, and the possibility exists that vancomycin is comprised of two

separate fragments in an approximately 80:20 ratio (J. M. McGuire, private communication).

Biological activity of vancomycin.

Vancomycin was originally investigated by the workers at Eli Lilly and Company as a potential agent for use in treatment of penicillin-resistant Staphylococcus aureus infections. The preliminary studies by Ziegler, Wolfe, and McGuire (1956) on the in vitro antibacterial activity of vancomycin revealed that pH had little effect on the bactericidal effects of this antibiotic, and several inorganic salts, amino acids, vitamins, and growth factors were found to have no effect on vancomycin action.

Jordan and Inniss (1959) reported that vancomycin selectively inhibited ribonucleic acid synthesis in S. aureus. No reversal of the action was noted when purines, pyrimidines, peptides, or metallic ions were added to the growth medium prior to addition of vancomycin. However, in later studies, Jordan and Inniss (1961) reported that vancomycin did not selectively inhibit ribonucleic acid synthesis, but primarily inhibited cell wall mucopeptide synthesis. Ribonucleic acid synthesis was inhibited, but only after a delay of about 30 minutes. Deoxyribonucleic acid and protein synthesis were not affected by vancomycin. These workers also stated that vancomycin did not cause permeability changes or lysis of the organism.

Reynolds (1961) noted that vancomycin-treated S. aureus accumulated hexosamine-containing nucleotides and concluded that vancomycin and penicillin acted similarily since both caused the accumulation of this cell wall material. In a subsequent report, Reynolds (1962) contrasted

the effects of penicillin and vancomycin on the basis of cellular binding of radioactive penicillin. Since vancomycin had no effect on binding of penicillin, it was suggested that the two antibiotics had different sites of action. However, bacitracin competed with penicillin for binding sites on the cell surface of both S. aureus and Bacillus megaterium (Reynolds, 1962).

Shockman and Lampen (1962) compared the effects of several antibiotics on whole cells and protoplasts of Streptococcus faecalis. Vancomycin was reported to inhibit the growth of whole cells by at least 50 per cent at a concentration of 1 µg per ml. Growth (increase in cell mass) of protoplasts of this organism was inhibited by 50 per cent at a concentration of 0.5 µg per ml. The possible significance of these findings was not discussed by these workers.

Yudkin (1963) compared the activity of several antibiotics on the synthesis of cell membrane by protoplasts of B. megaterium, and noted that vancomycin, but not penicillin, inhibited the incorporation of both 1-^{14}C-glycerol and U-^{14}C-tyrosine into the cell membrane to the same extent. This finding was interpreted by Yudkin as an indication that vancomycin was inhibiting membrane synthesis rather than causing a disproportionate synthesis of its two major components (lipid and protein).

Hancock and Fitz-James (1964) compared penicillin, vancomycin, and bacitracin using whole cells and protoplasts of B. megaterium. Vancomycin caused an efflux of ^{42}K from whole cells labeled with this cation. Also, these workers challenged the earlier report by Reynolds (1962) stating that neither bacitracin nor vancomycin competed with penicillin for binding sites on the surface of this organism.

In summary, the results of several recent studies have largely substantiated the initial observations by Jordan and Inniss (1961) that vancomycin inhibits the synthesis of cell wall mucopeptide, but no evidence of how this might occur has been offered. Also, the evidence that vancomycin causes defects in the cell membrane (Best and Durham, 1964; Hancock and Fitz-James, 1964) makes the relationship between the vancomycin effect on the cell wall and the cell membrane of primary importance in considering the mode of action of vancomycin.

CHAPTER III

MATERIALS AND METHODS

Test organism.

The organism used throughout this study was B. subtilis W23, a prototrophic strain supplied by Dr. W. C. McDonald, Washington State University. This organism is Gram-positive, motile, grows optimally at 37 C, and, in general, the biochemical and morphological characteristics agree with those described for B. subtilis in the 7th edition of Bergey's Manual of Determinative Bacteriology. The threonine auxotroph, B. subtilis 23, was used in one phase of this study. Stock cultures of both organisms were maintained on either nutrient agar or glucose salts slants at 4 C. L-Threonine (50 mg per liter) was added to the glucose salts medium for growth of the auxotroph.

Media.

A synthetic medium was used for all growth studies. This medium is referred to as "glucose salts" and consists of: K_2HPO_4, 1.4 per cent; KH_2PO_4, 0.6 per cent; $(NH_4)_2SO_4$, 0.2 per cent; and glucose, 0.5 per cent. Agar, 2.0 per cent, was used for slants and plates. One ml of a mineral salts solution composed of $MgSO_4 \cdot 7H_2O$, 5.0 per cent; $MnSO_4 \cdot H_2O$, 0.1 per cent; $FeCl_3$, 1.0 per cent; and $CaCl_2$, 0.5 per cent was added per liter of medium. The mineral salts were sterilized separately by autoclaving and glucose was sterilized by filtration. These components

were added aseptically to the medium just prior to use. In some experiments, the "glucose salts" medium was used without adding the mineral salts, and this medium is referred to as "buffered glucose."

The "incorporation medium," used for measuring incorporation of radioactivity from ^{14}C-labeled amino acids into the cell wall mucopeptide, consisted of 500 ml of the glucose salts medium supplemented with 0.4 g each of the following amino acids: DL-alanine, DL-glutamic acid, DL-lysine, and glycine. All media were adjusted to pH 7.0 prior to sterilization.

In some instances, large masses of cells were obtained using nutrient agar plates. The nutrient agar was a dehydrated, commercial preparation obtained from Difco Laboratories, Incorporated. It was fortified with Difco agar to give a final agar concentration of 2.0 per cent. All media were sterilized by autoclaving for 15-20 minutes at 121 C.

Growth experiments.

The inoculum for growth studies was prepared by suspending cells grown for 12-16 hours on glucose salts agar in 0.01 M potassium phosphate buffer (pH 7.0) to an absorbance of 0.5-0.6 at 540 mμ using a Bausch and Lomb Spectronic 20 spectrophotometer. One-tenth ml inoculum was added to each tube (18 x 150 mm) used in the growth studies (final liquid volume of 6.0 ml). Unless otherwise noted, all tubes were incubated at 37 C on a reciprocal shaker.

Influence of magnesium on growth inhibition.

Cells were grown to an absorbance of 0.5-0.6 at 540 mμ in glucose salts medium. These cells were washed, diluted, and transferred to tubes

containing sufficient vancomycin (3.3 µg per mg dry weight of cells) to produce a complete inhibition of growth, but insufficient to cause lysis. An absorbance of 0.15 at 540 mµ was established in each tube at time zero. Magnesium (1.66 µmoles per ml) was added to the vancomycin-containing tubes (total liquid volume of 6.0 ml) and to appropriate controls at 0, 15, and 30 minutes. The effect of magnesium on growth inhibition when added at the various times was determined by measuring the absorbance of the tubes at 540 mµ at 30 minute intervals.

Radioactive compounds.

DL-Alanine-2-^{14}C (15.5 mc per mmole) and D-alanine-1-^{14}C (7.5 mc per mmole) were purchased from the California Corporation for Biochemical Research. DL-Glutamic-2-^{14}C acid (1 mc per 272 mg) was a gift of Dr. Roger E. Koeppe, Department of Biochemistry, Oklahoma State University. D-Glucose-U-^{14}C (6.45 mc per mmole) and DL-alanine-1-^{14}C (4.42 mc per mmole) were purchased from Nuclear Chicago, Incorporated.

Radioactivity measurements.

Radioactivity measurements were made in a Packard Tricarb or Nuclear Chicago scintillation spectrometer using the following phosphor solution: diphenyloxozole, 4.0 g; 1.4-bis-2(5-phenyloxoyl)benzene, 0.2 g; ethanol (absolute), 400 ml; and toluene,(sulfur-free), 600 ml.

Effect of vancomycin and magnesium on cell wall mucopeptide synthesis.

The incorporation of radioactivity from ^{14}C-labeled amino acids into the mucopeptide of B. subtilis was studied. Cells were grown for 16 hours on glucose salts slants, harvested in phosphate buffer, inoculated into 25-50 ml of fresh incorporation medium, and incubated at 37 C

with constant shaking. When the cell density reached an absorbance of 0.8-1.0 at 540 mμ, the cells were harvested by centrifugation, washed three times with potassium phosphate buffer (0.01 M, pH 7.0) and suspended in the buffer. The cell suspension was adjusted so that a 1:25 dilution gave an absorbance of 0.3-0.4 at 540 mμ. One ml of this suspension was added to 250 ml flasks containing 20 ml incorporation medium, 1 ml chloramphenicol (1.75 mg per ml), 1 ml ^{14}C-labeled amino acid (3 μc per ml), and either 1 ml vancomycin (10 μg per ml) or 1 ml $MgSO_4 \cdot 7H_2O$ (0.1 M) or both. The contents were adjusted to a final liquid volume of 25 ml with 0.01 M potassium phosphate buffer (pH 7.0). A final cell concentration of about 3 mg dry weight per 10 ml was present in each flask.

The flask contents were incubated at 37 C with constant shaking for 90 minutes at which time a 10 ml sample was withdrawn from each flask. The samples were immediately frozen in a dry ice-acetone bath and placed in a deep freeze. The cells were chemically fractionated by the procedure of Park and Hancock (1960) to obtain the mucopeptide. The cell wall mucopeptide fraction was solubilized for isotope counting in 1 mmolar Hyamine (Rhome and Haas, Incorporated) at room temperature. Ten ml of scintillation fluid was added to each vial. When samples were concentrated prior to counting, they were either lyophilized or dried in a vacuum desiccator at 55 C.

Essentially the same procedure was used to follow the time course of vancomycin inhibition of cell wall synthesis. B. subtilis cells were grown to an absorbance of 0.8-1.0 at 540 mμ in incorporation medium and harvested by centrifugation. The cells were washed three times with distilled water, suspended in distilled water, and the cell concentration

adjusted so that a 1:25 dilution gave an absorbance of 0.35 at 540 mμ. One ml of this cell suspension was used to inoculate 250 ml flasks containing 20 ml incorporation medium, 1 ml DL-alanine-1-^{14}C (5 μc per ml), 1 ml chloramphenicol (1.75 mg per ml), and either 1 ml vancomycin (15 μg per ml) or 1 ml water. Distilled water was added to give a final liquid volume of 25 ml in each flask. Five ml samples were removed from the two flasks at 15, 30, 45, and 60 minutes and immediately frozen in a dry ice-acetone bath. All samples were fractionated by the Park and Hancock (1960) procedure to obtain the mucopeptide. The radioactivity was counted as previously described.

Effect of chloramphenicol on mucopeptide synthesis.

B. subtilis 23, a threonine auxotroph of B. subtilis W23, was used in this study. The cells were grown to an absorbance of 0.8-0.9 at 540 mμ in glucose salts containing L-threonine (50 milligrams per liter). After centrifugation, the cells were suspended in incorporation medium and incubated with shaking at 37 C until the absorbance remained constant for 60 minutes. At this time, the incorporation of DL-alanine-1-^{14}C was followed in the presence and absence of chloramphenicol (70 μg per ml). The procedure as described for studies with vancomycin was followed to determine the effect of chloramphenicol on cell wall synthesis. Since threonine was limiting, there was no increase in cell mass during the course of the incubation (as measured by absorbance).

Leakage of cell metabolites.

Cells were grown at 37 C with constant shaking to an absorbance of 0.5-0.6 in 25 ml buffered glucose containing $MgSO_4 \cdot 7H_2O$ (0.2 μmoles per ml) and D-glucose-U-^{14}C (10 μc). The cells were harvested by centrifugation

at 4 C, washed three times with distilled water, and suspended in buffer so that a 1:25 dilution gave an absorbance of 0.1-0.2 at 540 mμ. One ml of the cell suspension was inoculated into flasks containing buffered glucose, $MgSO_4 \cdot 7H_2O$ (0.2 μmoles per ml), and various levels of vancomycin. When chloramphenicol was employed, this antibiotic was added to give a final concentration of 70 μg per ml. The total volume in each flask was adjusted to 25 ml with 0.01 M potassium phosphate buffer (pH 7.0). At 30 minute intervals, a 5.0 ml sample was removed, the absorbance measured at 540 mμ, and the samples frozen in a dry ice-acetone bath. The frozen samples were usually thawed and centrifuged, but in some experiments the samples were not frozen, but centrifuged immediately. In either case, the supernatant solution (after centrifugation) was transferred to counting vials, evaporated to dryness under vacuum at 55 C, and the radioactivity counted as previously described. The presence of radioactivity in the supernatant solution indicated the release of ^{14}C-metabolites from the cell.

Precipitation of vancomycin by divalent cations.

Higgins et al., (1957) showed that vancomycin could be precipitated from an alkaline solution by high concentrations of several divalent cations, but magnesium was not included in this study. Vancomycin has a characteristic absorption peak at about 280 mμ at pH 7.0 (Fig. 10). To determine if vancomycin is precipitated from solution by various metals, vancomycin (200 μg per ml) was prepared in 0.1 M tris buffer at pH 7.0 and 9.0. One per cent solutions of magnesium sulfate, manganous sulfate, and nickel(ous) chloride were prepared in distilled water. Nine ml of the vancomycin solutions at each pH were mixed with 1 ml of

each of the metals and the tubes were incubated at 37 C for 2 hours. The solutions were filtered through Millipore filters (0.45 μ pore size) and the absorbance at 280 mμ was determined using the Beckman DU spectrophotometer. A significant decrease in absorbance at this wavelength indicated precipitation of the antibiotic since controls showed that the metals did not shift the absorption peak of vancomycin.

Preparation of cell walls.

Cell walls were originally prepared by disrupting a heavy cell suspension by sonic rupture. The cells were grown 12-14 hours on nutrient agar plates at 37 C, harvested with distilled water, washed three times with water, and concentrated into a heavy suspension. Three 30 second exposures to a sonic probe (Branson Instruments, Incorporated) were used to rupture the cells. After sonic rupture, the suspension was immediately centrifuged at 12,100 x g for 15 minutes at 4 C. The cell walls were easily visible as a white layer upon a brownish pellet of unbroken cells. The wall layer was separated from the whole cells by adding a small amount of water and gently shaking the tube to dislodge the walls. By repeatedly decanting the walls which were suspended in the wash water, most of the walls could be separated from the unbroken cells. Repeated washing with water eventually yielded a cell wall suspension devoid of whole cells. The cell walls were suspended in buffer composed of 0.05 M NH_4HCO_3 and 0.005 N NH_4OH (pH 7.8) and trypsin (500 μg per ml) was added. After 2 hours at 37 C, the walls were removed by centrifugation, and the trypsinization process repeated. After trypsinization, the cell walls were repeatedly washed with distilled water. The washing was continued until no 280 mμ absorbing material remained in the wash supernatant.

The cells walls were hydrolyzed at 100 C in 6 N HCl for 18 hours in a sealed tube. The HCl was partially removed by evaporating the hydrolysate to dryness with a warm stream of air and dissolving the hydrolysate in distilled water. This material was spotted over NH_3 onto Whatman No. 1 chromatography paper, and developed by the two-dimensional system of Redfield (1953). The amino acids were detected by spraying the chromatograms with 0.25 per cent ninhydrin in acetone and heating at 100 C for 5 minutes. The chromatograms revealed traces of several amino acids in the cell wall preparation, but alanine, α,ϵ-diaminopimelic acid, and glutamic acid were predominate. These three amino acids were identified by co-chromatography using the acid hydrolyzed cell walls and known amino acids from commercial sources.

Since cell walls prepared by sonic disruption retained traces of several amino acids in addition to the three previously reported to comprise the cell wall mucopeptide of B. subtilis (Salton, 1960), a modification of the chemical fractionation procedure of Park and Hancock (1960) was used to obtain cell wall material for adsorption studies. This modification consisted of using about 20 mg dry weight of nutrient agar grown cells per tube and 5 ml of each solvent. The extraction time was increased from 5 minutes to 2 hours and each fractionation step was performed twice. All centrifugations were made for 10 minutes at 12,100 x g in a Servall RC-2 superspeed centrifuge at 4 C. Trypsinization was considered complete when the sediment was essentially translucent. Usually 6-8 hours were required for complete protein solubilization, and both buffer and enzyme were changed 2-3 times during this period. After trypsinization, the remaining material was washed, hydrolyzed, and chromatographed as previously described. Only the three amino acids

which comprise the cell wall mucopeptide of this organism could be detected upon chromatography of these preparations.

Both preparations of cell walls could be used in the various adsorption experiments since all comparisons of the two preparations did not reveal any differences in ability to adsorb vancomycin. This would indicate that the small amount of protein remaining on the cell walls prepared by sonic disruption did not influence the adsorption of vancomycin.

Adsorption of vancomycin to cell walls.

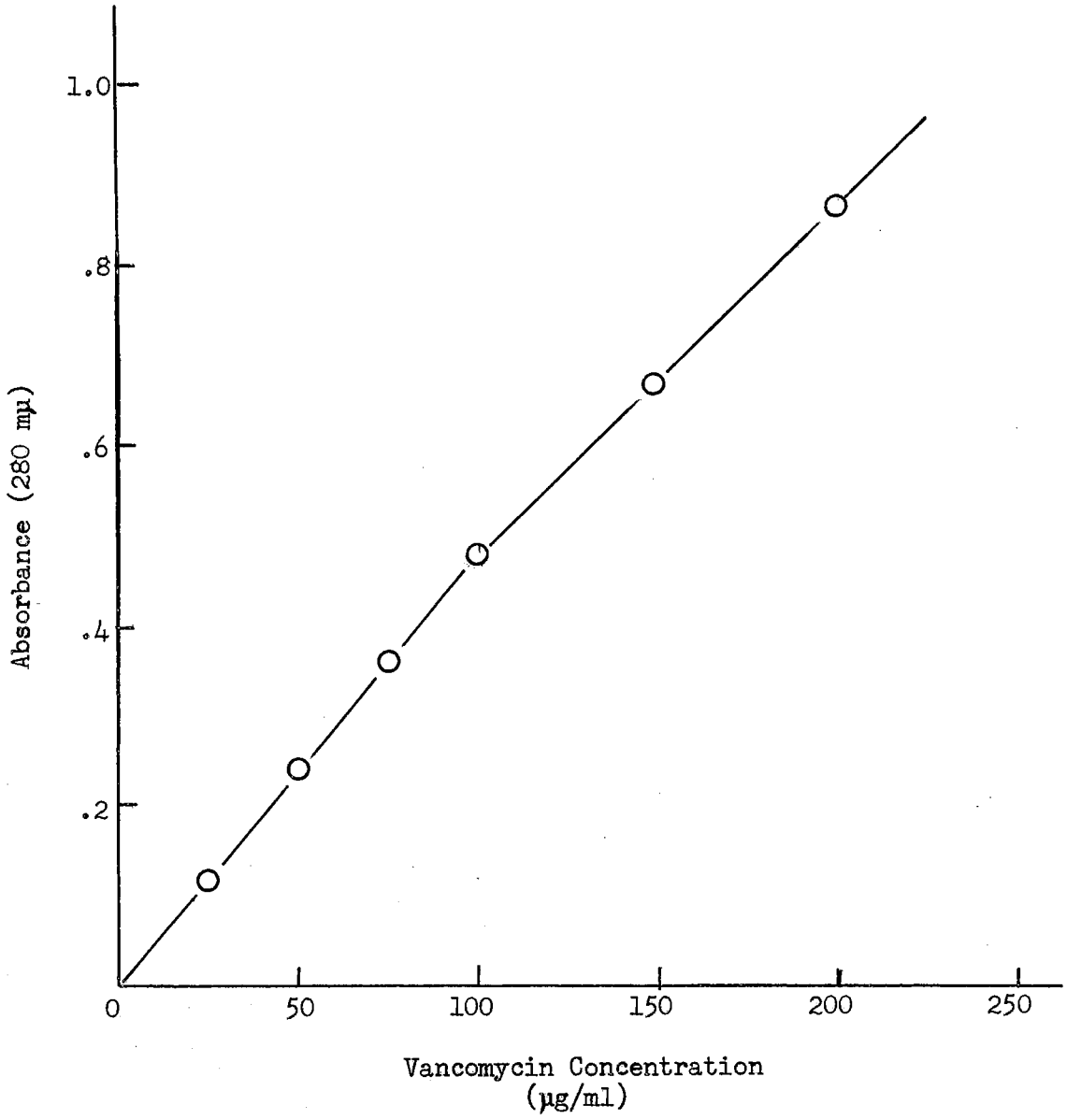
Vancomycin absorbs ultraviolet light characteristically at approximately 280 mμ in neutral or slightly acidic solutions due to phenolic chromaphores (Johnson, 1962). A standard curve for the free base of this antibiotic dissolved in triple distilled water (pH 7.0) at 280 mμ is presented in Fig. 1.

Adsorption of vancomycin to cell wall material of B. subtilis was determined by mixing 2.5 ml of the cell walls (adjusted to an absorbance of 0.30-0.40 at 540 mμ) with sufficient vancomycin to give a final antibiotic concentration of 150 μg per ml in a total liquid volume of 6.0 ml. After adsorption for about 10 minutes at room temperature, the walls were removed by centrifugation at 12,100 x g for 10 minutes or by Millipore filtration (0.45 μ pore size). The quantity of vancomycin adsorbed to the cell walls was determined from the standard curve (Fig. 1) by measuring the absorbance at 280 mμ of the supernatant solution after centrifugation, or of the filtrate after Millipore filtration. Controls indicated that vancomycin did not adsorb to the Millipore membrane.

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 using a distilled water blank.


1.0
.8
.6
.4
.2
Absorbance (280 mμ)
0
50
100
150
200
250
Vancomycin Concentration
(μg/ml)

Preparation of protoplasts.

B. subtilis W23 was grown 12-14 hours on nutrient agar plates, harvested, washed twice with distilled water, and suspended in 100 ml 0.01 M tris buffer (pH 7.8) containing 20 mg ethylenediaminetetra-acetic acid. The cells were centrifuged and suspended in 0.58 M sucrose containing 0.01 M $MgSO_4 \cdot 7H_2O$. Muramidase (E.C. 3.2.1.17) was added to a final concentration of 200 µg per ml and the flasks placed in a 37 C water bath shaker for gentle agitation. Formation of protoplasts was usually complete in 90-120 minutes as judged by phase contrast microscopy.

Comparison of lysis of B. subtilis by vancomycin and penicillin.

B. subtilis W23 was grown to an absorbance of 0.5-0.6 in 100 ml glucose salts at 37 C with constant shaking. After centrifugation, the cells were suspended in one of two chemically defined growth media. One medium was glucose salts which has been described previously. The second medium was glucose salts supplemented with sucrose (0.58 M) and $MgSO_4 \cdot 7H_2O$ (0.01 M). Both cell suspensions were adjusted so that 4 ml in 25 ml of the respective medium gave an absorbance of 0.10-0.20 at 540 mµ. These cells (4.0 ml) were then used to inoculate flasks containing the corresponding medium and either vancomycin, penicillin, or neither. Triple distilled water was used to bring the total liquid volume in each flask to 25 ml. The growth response was followed at 30 minute intervals for 120 minutes by taking 5 ml samples and measuring the absorbance at 540 mµ.

Much the same procedure was used to determine if chloramphenicol-treated cells lysed in the presence of vancomycin. The cells were grown

in glucose salts to an absorbance of about 0.50 at 540 mμ. These cells were then used to inoculate flasks containing glucose salts (20 ml), chloramphenicol (70 μg per ml), vancomycin (1.2 μg per ml), and buffer was used to bring the total volume to 25 ml. Appropriate control flasks containing only one of these antibiotics were also included. Absorbance was followed in the manner described previously.

CHAPTER IV

RESULTS AND DISCUSSION

Influence of magnesium on growth inhibition by vancomycin.

Vancomycin is a potent growth inhibitor of B. subtilis W23. A final concentration of 0.056 µg per ml of the antibiotic inhibited growth by approximately 45 per cent (Fig. 2). Complete alleviation of this inhibition was obtained by the addition of 1.66 µmoles per ml of magnesium (either as the chloride or sulfate) to the glucose salts medium. Magnesium did not influence the growth rate of cells in the control system. When a slightly higher vancomycin concentration was employed (Fig. 3), growth inhibition was complete, and the same concentration of magnesium only partially alleviated this inhibition. The temperature of growth did not influence either the inhibition or the effect of magnesium on the inhibition since similar results were obtained when cells were grown at 25, 30, or 37 C.

Magnesium must be present soon after the addition of vancomycin in order to influence the inhibition (Fig. 4). The cells showed a slight growth response when magnesium was added after the cells had been in contact with vancomycin for 15 minutes. However, when magnesium was added 30 minutes after vancomycin, the inhibition was well established and the cation had no effect on the growth of the cells during the course of the experiment. Magnesium was most effective when added simultaneously with vancomycin. No added growth response was

Figure 2.

Influence of magnesium on partial growth inhibition of B. subtilis by vancomycin.

All systems contained 5.0 ml glucose salts and were supplemented as follows: ●, none; ▲, 1.66 µmoles per ml $MgSO_4 \cdot 7H_2O$; ○, 0.056 µg per ml vancomycin; ▼, 0.056 µg per ml vancomycin and 1.66 µmoles per ml $MgSO_4 \cdot 7H_2O$.

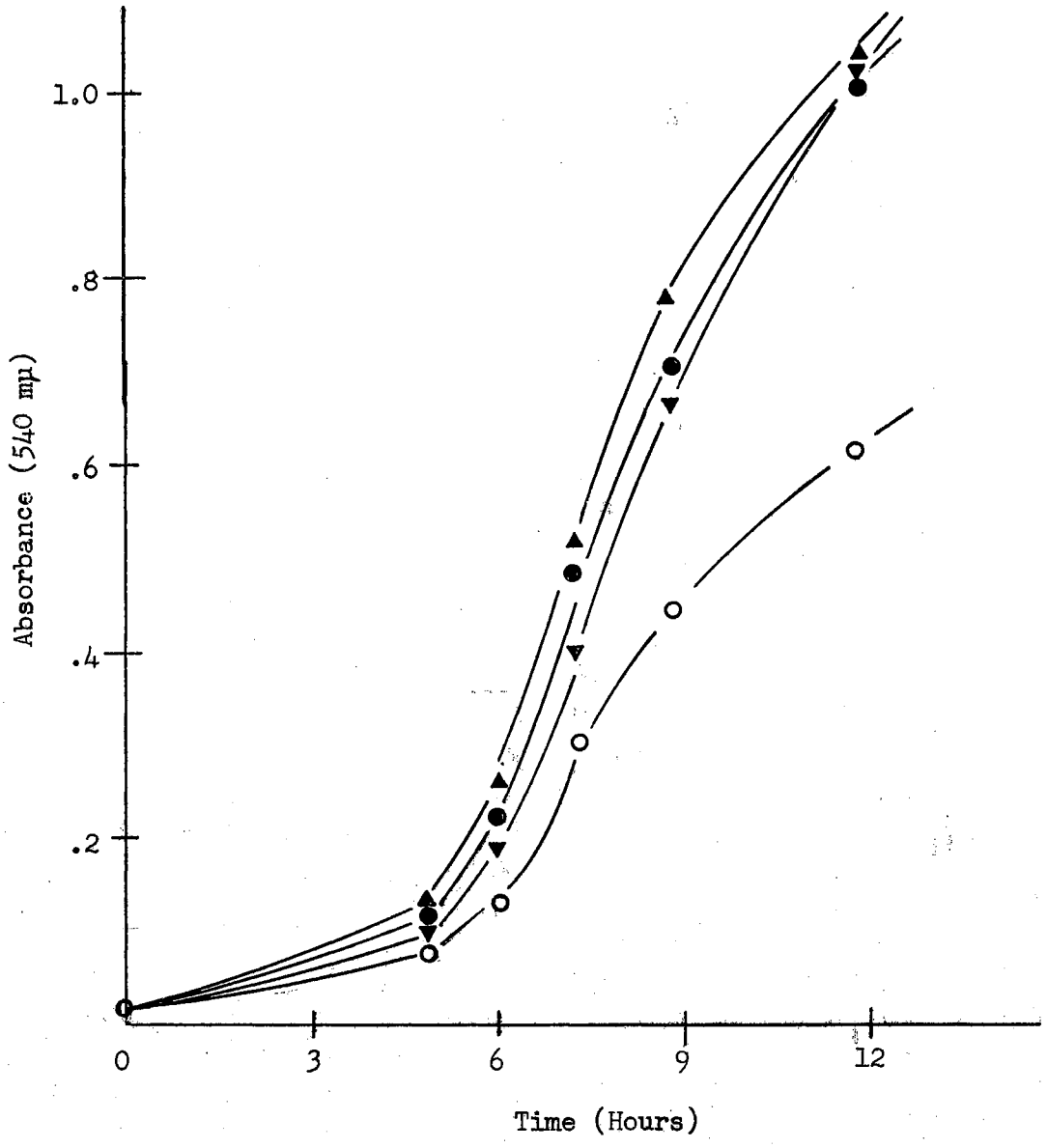

1.0
.8
.6
.4
.2
Absorbance (540 mμ)
0
3
6
9
12
Time (Hours)

Figure 3.

Influence of magnesium on complete growth inhibition by vancomycin.

All systems contained 5.0 ml glucose salts and were supplemented as follows: ●, none; ▲, 1.66 µmoles per ml $MgSO_4 \cdot 7H_2O$; ○, 0.066 µg per ml vancomycin; ▼, 0.066 µg per ml vancomycin and 1.66 µmoles per ml $MgSO_4 \cdot 7H_2O$. The tubes were incubated at 30 C with shaking.

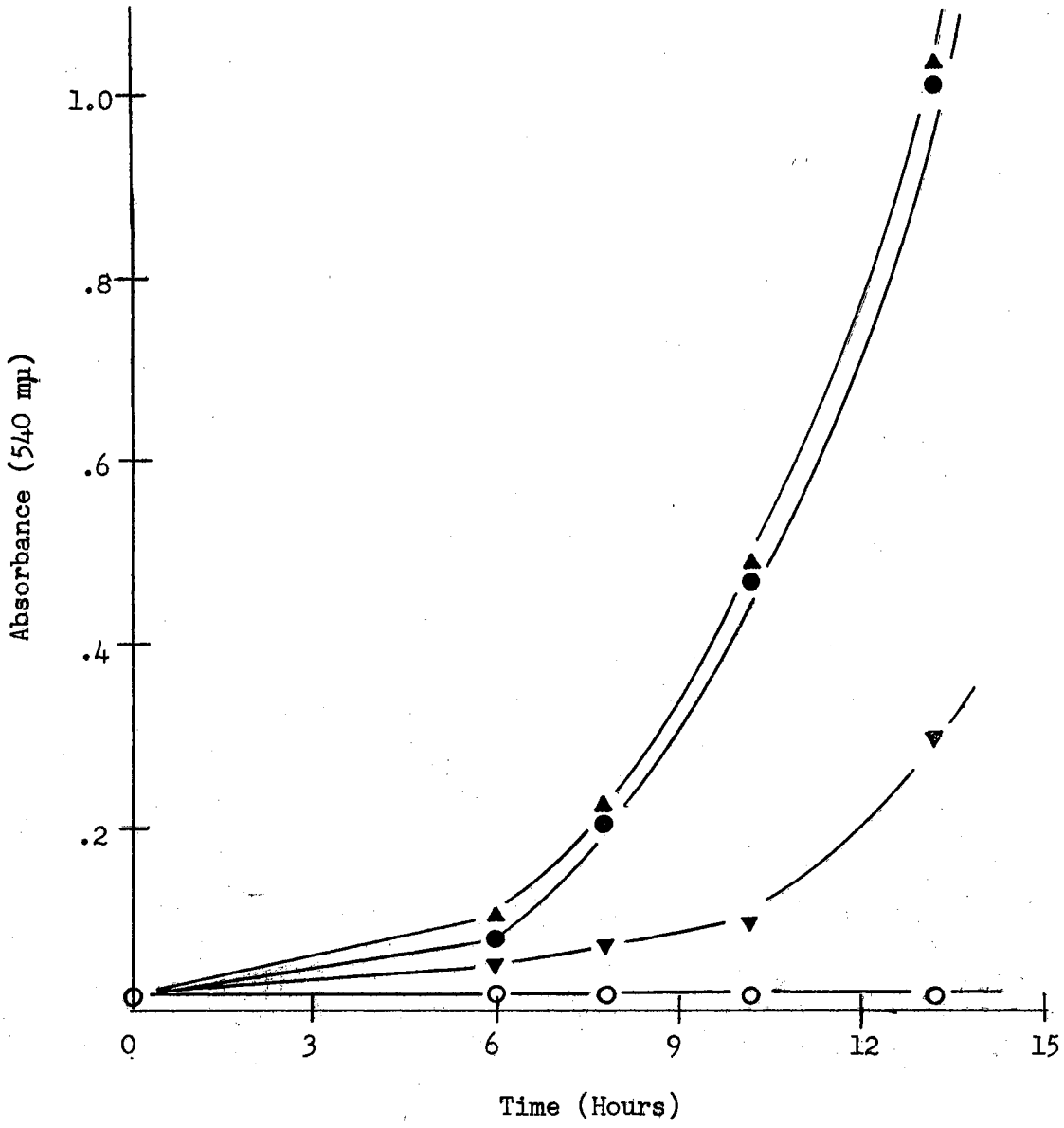
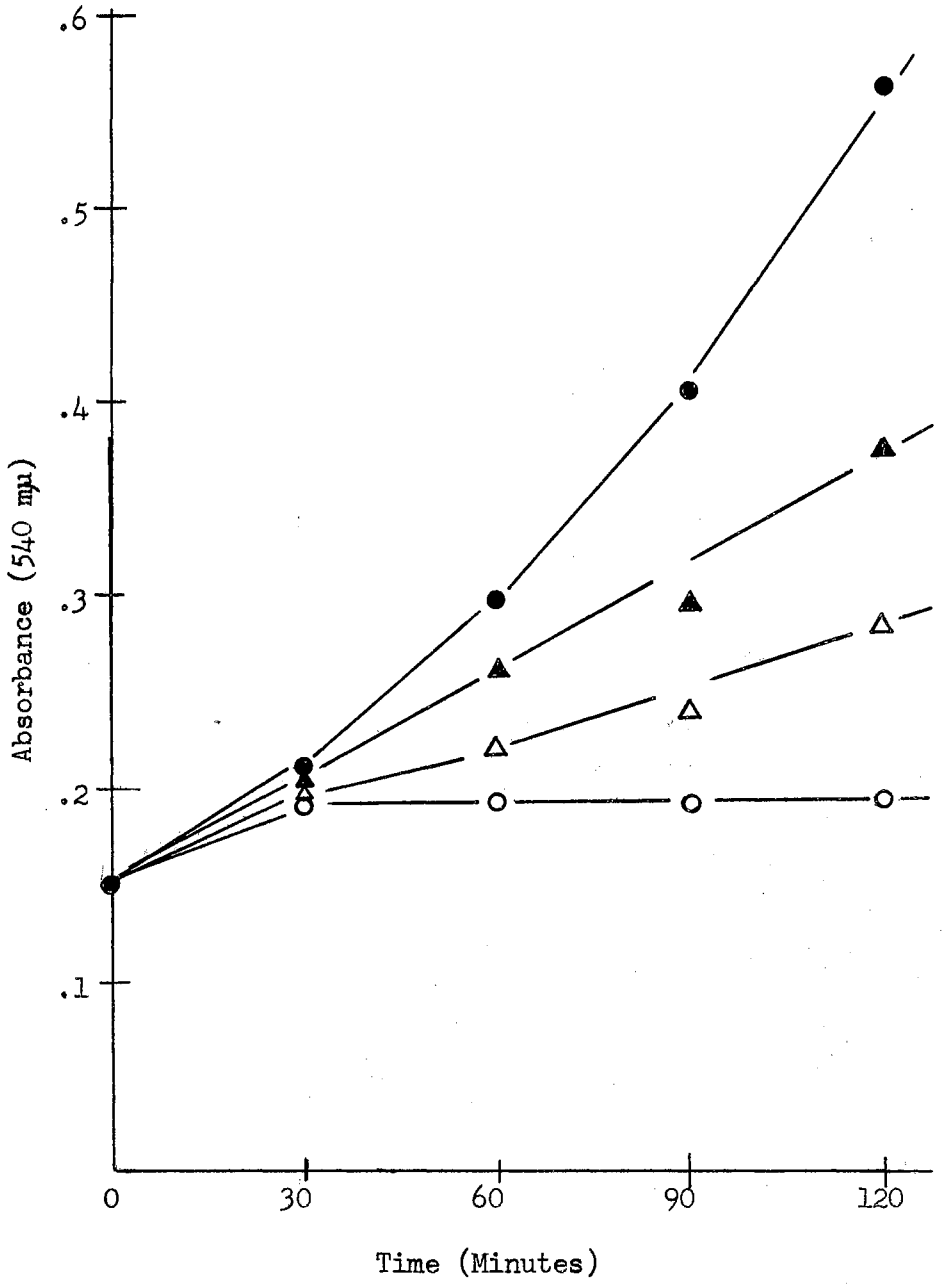

1.0
.8
.6
.4
.2
0
3
6
9
12
15
Absorbance (540 mμ)
Time (Hours)

Figure 4.

Addition of magnesium to vancomycin-treated cells at different time intervals.

Magnesium (1.66 µmoles per ml) was added to cells growing in glucose salts containing vancomycin (3.3 µg per mg dry weight of cells) as follows: ●, no vancomycin and magnesium added at 0, 15, or 30 minutes; ▲, magnesium added to inhibited cells at 0 minutes; △, magnesium added at 15 minutes; ○, magnesium added at 30 minutes and no magnesium added.


.6
.5
.4
.3
.2
.1
Absorbance (540 mμ)
0
30
60
90
120
Time (Minutes)

noted when magnesium was added to the cells 30 or 60 minutes prior to the addition of vancomycin, and controls indicated that, in the absence of vancomycin, magnesium had no effect on growth of the cells when added either before or after growth ensued. These results suggest that magnesium does not truly reverse vancomycin inhibition. Since magnesium was effective only when added to the cells before the vancomycin inhibition was established, the cation appeared to exert a protective effect.

Inhibition of cell wall synthesis by vancomycin.

Jordan and Inniss (1961) reported that vancomycin inhibited mucopeptide synthesis in S. aureus. Studies were conducted to determine the influence of vancomycin on mucopeptide synthesis in B. subtilis. Table I presents results which establish that vancomycin inhibits the incorporation of radioactivity from DL-alanine-2-^{14}C and DL-glutamic-2-^{14}C acid into the mucopeptide of the cell wall. The incorporation of radioactivity from DL-alanine-2-^{14}C was inhibited by 43.5 per cent, but that from DL-glutamic-2-^{14}C acid was inhibited by 76.9 per cent. In both instances, the addition of magnesium significantly decreased the inhibition of incorporation of radioactivity but the disparity between the effects of vancomycin on cell wall synthesis as measured using ^{14}C-alanine and ^{14}C-glutamic acid requires comment.

The specific activity of radioactivity from labeled alanine incorporated into the cell wall mucopeptide should be greater than that from labeled glutamic acid since Bacillus species in general (Salton, 1960) and B. subtilis in particular (Roberts and Johnson, 1962) have been shown to contain about 3 moles of alanine per mole of glutamic

TABLE I

THE INHIBITION OF CELL WALL SYNTHESIS BY VANCOMYCIN AND THE EFFECT OF MAGNESIUM ON THIS INHIBITION

	Addition to Incorporation Medium	Specific Activity (Counts/min/mg dry wt. of cells extracted) DL-Alanine-2-C^{14} (.12 μc/ml)	Specific Activity (Counts/min/mg dry wt. of cells extracted) DL-Glutamic-2-C^{14} Acid (.11 μc/ml)	Per Cent Inhibition DL-Alanine-2-C^{14}	Per Cent Inhibition DL-Glutamic-2-C^{14} Acid
1)	None	2,438	307	----	----
2)	4.0 μmoles/ml magnesium	2,573	352	----	----
3)	1.3 μg/mg dry wt. vancomycin	1,377	71	43.5	76.9
4)	1.3 μg/mg dry wt. vancomycin and 4.0 μmoles/ml magnesium	2,121	202	17.5	42.5

Incorporation of each labeled amino acid was stopped at 90 minutes by freezing 10 ml samples in a dry ice-acetone bath.

acid in the mucopeptide. This, however, would not explain why vancomycin appears to inhibit the incorporation of radioactivity from DL-glutamic-2-^{14}C to a greater extent than the radioactivity from DL-alanine-2-^{14}C. However, if alanine were incorporated into two different cell wall components, and if the synthesis of one of these components was more sensitive to vancomycin than the other, a difference in inhibition as measured by incorporation of radioactivity from alanine and glutamic acid would be understandable.

The cell walls of B. subtilis have been shown to contain large amounts of a ribitylphosphate polymer that contains D-alanine esterified to a free hydroxyl group on the carbohydrate moiety (Salton, 1960). These polymers are called teichoic acids and can be removed from cell wall preparations by prolonged (several days) extraction with 5-10 per cent trichloroacetic acid at 4 C (Sanderson, Strominger, and Nathenson, 1962). The Park and Hancock (1960) procedure calls for only 10 minutes total extraction with cold trichloroacetic acid. Therefore, some teichoic acid would be expected to escape extraction and be carried into the mucopeptide fraction. To determine if vancomycin had an effect on the esterification of D-alanine into the teichoic acid of the B. subtilis cell wall, the extraction with cold trichloroacetic acid was extended to 70 hours and the radioactivity removed by this extraction was determined as previously described. The results of an incorporation experiment using D-alanine-1-^{14}C are presented in Table II. These results indicate that vancomycin had no effect on the synthesis of teichoic acid. However, the incorporation of radioactivity into the mucopeptide was inhibited 30 per cent by vancomycin. These results would indicate that vancomycin acts primarily to inhibit

TABLE II

EFFECT OF VANCOMYCIN ON THE SYNTHESIS OF TEICHOIC ACID AND MUCOPEPTIDE BY B. SUBTILIS*

Additions	Specific Activity	
	Teichoic Acid	Mucopeptide
None	373	1957
Vancomycin (1.3 µg per milligram dry weight)	396	1370

*D-Alanine-1-^{14}C (0.4 µc per ml) was incubated with cells (0.3 milligram dry weight per ml) for 90 minutes in incorporation medium. The cell "pool" was extracted with boiling water for 5 minutes. The teichoic acid fraction was obtained by extraction of the cells with 70 per cent trichloroacetic acid for 70 hours at 4 C. The mucopeptide fraction was obtained by the procedure of Park and Hancock (1960). Chloramphenicol (70 µg per ml) was present in both flasks. Specific activity is expressed as counts per minute per milligram dry weight of cells extracted.

polymerization of the mucopeptide and has no appreciable effect on the esterification of alanine to the ribitylphosphate of teichoic acid.

It should perhaps be noted in connection with these studies on cell wall synthesis that chloramphenicol (70 µg per ml, final concentration) was added to all flasks. This antibiotic allows the measurement of mucopeptide synthesis by following the incorporation of radioactivity from a labeled amino acid that is a constituent of both the cell wall and cell protein. Chloramphenicol inhibits protein synthesis (Gale and Folkes, 1953), but Mandalstam and Rogers (1959) reported it had no effect on the synthesis of cell wall mucopeptide by S. aureus. The presence of chloramphenicol, therefore, would permit a study of the incorporation of an amino acid into cell wall mucopeptide without concomitant incorporation into protein. However, it was noticed during the course of this study that chloramphenicol does inhibit the synthesis of mucopeptide by B. subtilis (Table III). This inhibition differed from that observed with vancomycin in that very little inhibition was evident until 45-60 minutes (Jordan and Inniss, 1961).

Various explanations for the influence of magnesium on vancomycin-mediated inhibition of growth and mucopeptide synthesis are possible from a priori considerations. Magnesium and vancomycin could form an insoluble complex, which would precipitate both from solution, or vancomycin could conceivably form a soluble complex with magnesium (chelate). Even though soluble, magnesium might be made unavailable to the cells as the result of this complex. Webb (1952) showed that magnesium is required for growth of bacilli, and Chatterjee and Park (1964) have shown mucopeptide synthesis to be dependent on magnesium. Therefore, a magnesium deficiency could cause an inhibition of both

TABLE III

EFFECT OF CHLORAMPHENICOL ON THE INCORPORATION OF RADIOACTIVITY INTO THE MUCOPEPTIDE OF B. SUBTILIS 23

	Specific Activity*		
	Minutes of Incubation		
	25	45	75
Control	122	190	284
Chloramphenicol (70 µg per ml)	129	181	183

*Mucopeptide synthesis was measured as previously described for Table II. The specific activity expressed is based on the counts per minute per milligram dry weight of cells extracted.

growth and mucopeptide synthesis. However, a complex need not be responsible for this deficiency. Magnesium and vancomycin could compete with each other for binding sites on the cell surface. The net effect of such a competition could be either the creation of a magnesium deficiency or simply a reduction in the amount of vancomycin binding to the cell surface. That is, vancomycin could 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.

All of these possibilities except the last one would involve the creation of magnesium deficiency, but excess magnesium would alleviate the inhibition caused by any of these conditions. The following experiments were designed to choose between these possibilities.

Vancomycin precipitation by divalent cations.

Studies were conducted to determine if vancomycin and magnesium form an insoluble complex which would function to remove both from solution. Manganese, nickel, and magnesium were compared for their ability to precipitate vancomycin from solution at pH 7.0 and 9.0. None of the metals precipitated vancomycin at pH 7.0 (Fig. 5). However, manganese and nickel (as reported by Higgins et al., 1958) precipitated vancomycin at pH 9.0. Magnesium had no effect on the ultraviolet absorption by vancomycin at this pH. These results suggest that vancomycin and magnesium do not form an insoluble complex, and that magnesium does not remove the antibiotic from solution by precipitation.

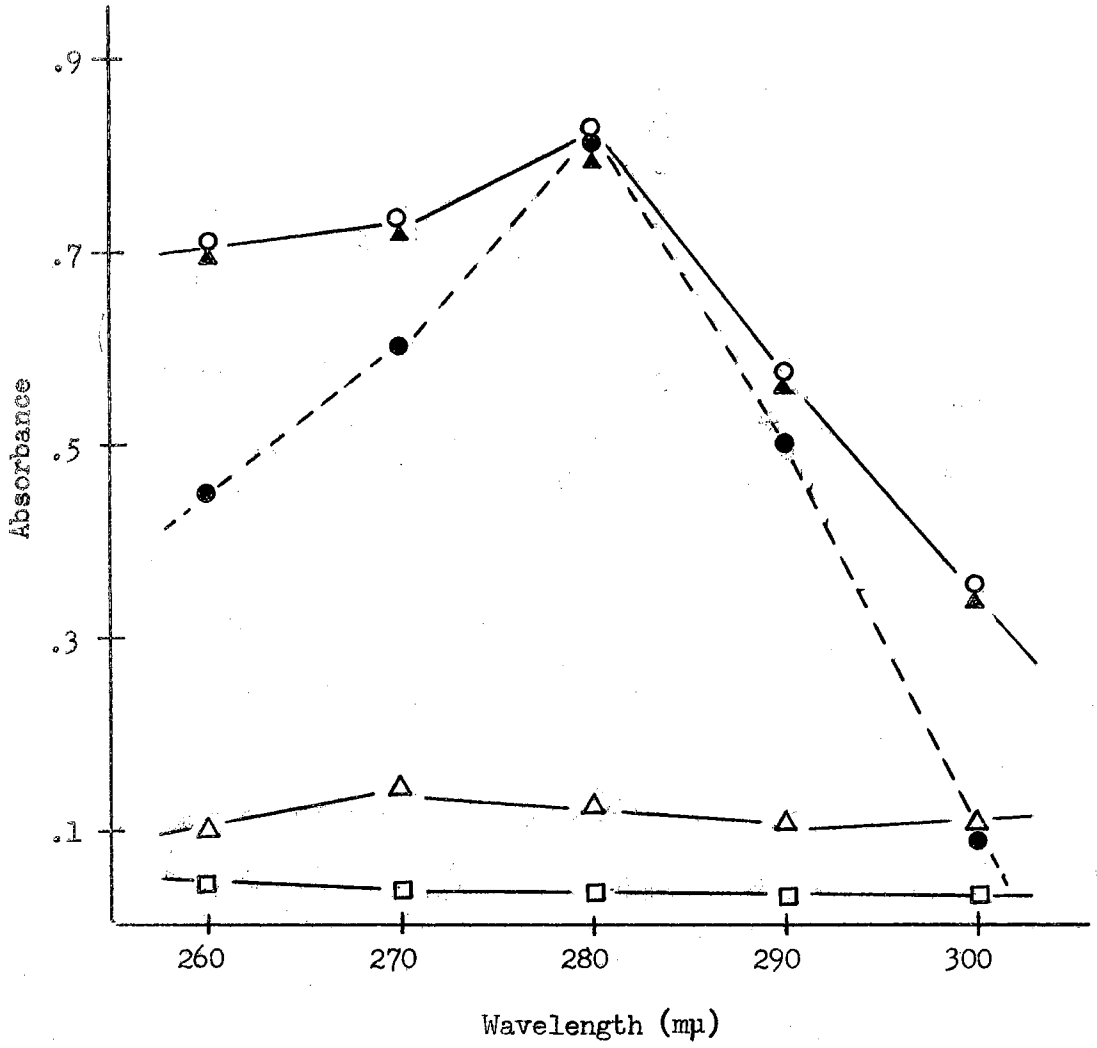
Effect of vancomycin on cell permeability.

Jordan and Inniss (1961) stated that vancomycin caused no permeability alterations in S. aureus and that these cells growing in vancomycin did

Figure 5.

Precipitation of vancomycin by divalent cations.

The absorption spectrum of 180 µg per ml vancomycin at pH 9.0 (solid line) when treated with three divalent cations (0.1 per cent) is indicated as follows: ○, none; △, $NiCl_2$; ▲, $MgSO_4 \cdot 7H_2O$; □, $MnSO_4$. At pH 7.0 (dashed line) none of these metals had any effect on the vancomycin absorption spectrum and all systems are indicated by: ●.


.9
.7
.5
.3
.1
Absorbance
260
270
280
290
300
Wavelength (mμ)

not lyse. However, it would seem reasonable to expect that a defective cell wall, as evidenced by an inhibition of mucopeptide synthesis, could lead to an alteration of cellular integrity since the cell wall is responsible for cell rigidity. Thus, vancomycin-treated cells could lyse in the absence of osmotic stabilizers for the membrane if the cell wall is defective due to an inhibition of its synthesis. The results presented in Fig. 6 show that B. subtilis cells growing in buffered glucose are lysed by vancomycin. A complete growth medium is required to demonstrate lysis since cells suspended in 0.01 M potassium phosphate buffer did not lyse in 90 minutes when treated with vancomycin at the same concentration. Higher concentrations of the antibiotic were required to demonstrate lysis than were employed to inhibit mucopeptide synthesis. By way of comparison, 1.3 µg vancomycin per milligram dry weight of cells was used to inhibit (by 43 per cent) the incorporation of DL-alanine-2-^{14}C into the mucopeptide (Table I) and 8.5 µg vancomycin per milligram dry weight of cells was employed to lyse this organism (Fig. 6).

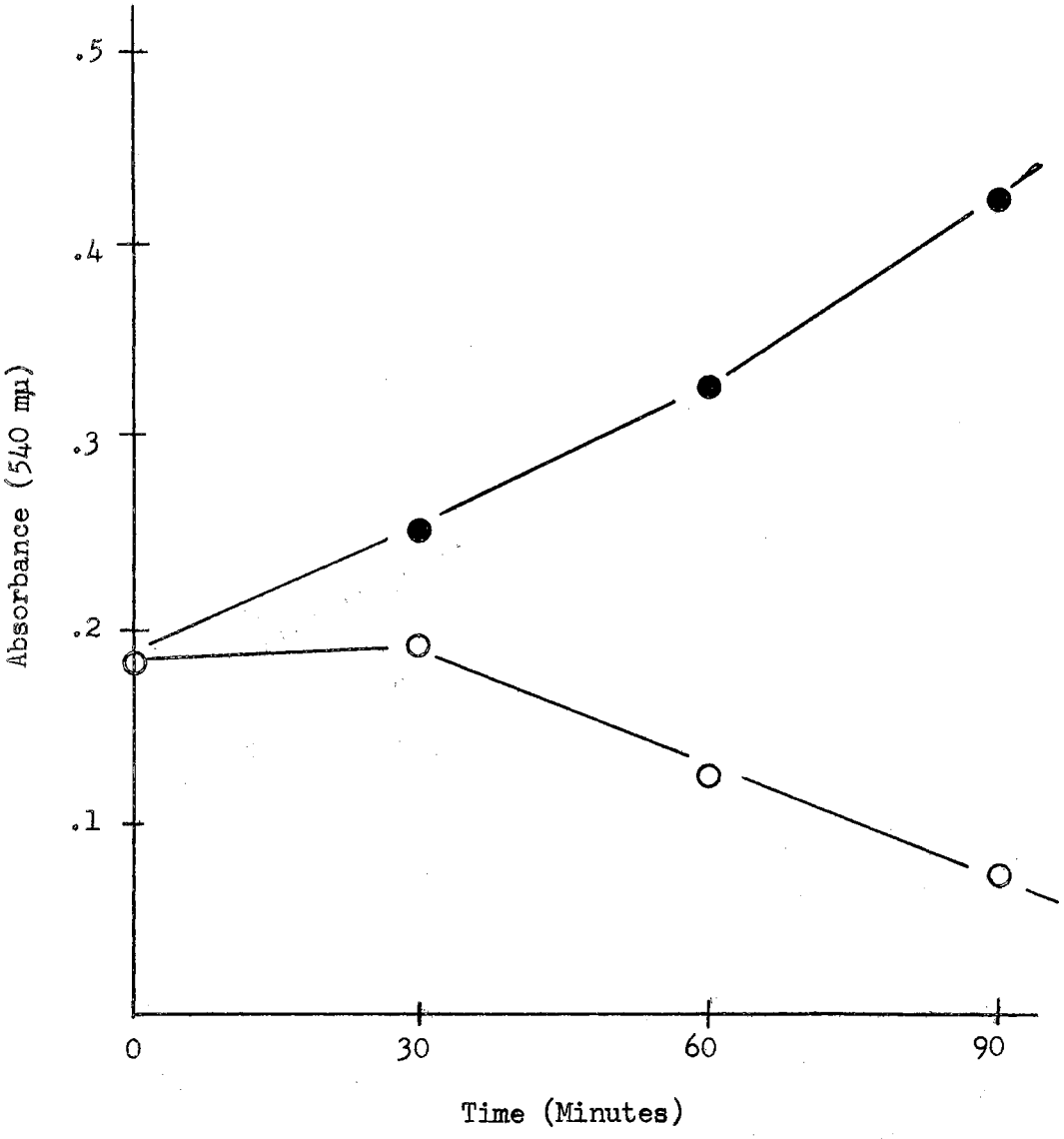
These results establish that vancomycin does alter the permeability barrier of B. subtilis. However, these results do not show whether vancomycin is causing lysis by a direct or by an indirect effect on the membrane. That is, vancomycin could cause lysis of the cells by directly inhibiting membrane synthesis and/or function, or it could inhibit cell wall synthesis to such an extent that the cells lyse from osmotic shock.

To determine if vancomycin caused permeability damage that was not severe enough to produce lysis, B. subtilis cells were labeled by growing them in glucose salts medium supplemented with D-glucose-U-^{14}C. When these labeled cells were incubated in a sub-lytic vancomycin concentration

Figure 6.

Lysis of Bacillus subtilis by vancomycin.

Exponentially growing cells were added to buffered glucose containing 0.2 μmoles per ml $MgSO_4$ and the following levels of vancomycin: ●, none; ○, 1.2 μg per ml (8.5 μg vancomycin per milligram dry weight of cells).


.5
.4
.3
.2
.1
Absorbance (540 mμ)
0
30
60
90
Time (Minutes)

(4.6 µg per mg dry weight of cells), there was a progressive release of ^{14}C-labeled metabolites by the inhibited cells that was not accompanied by lysis (Fig. 7). However, when chloramphenicol was present with vancomycin, the amount of radioactivity released by the labeled cells was significantly reduced (Fig. 8).

Hancock and Fitz-James (1964) found that chloramphenicol had no effect on the efflux of ^{42}K from B. megaterium labeled with this ion. They assumed that chloramphenicol inhibited the "growth" of the membrane and concluded that vancomycin acted directly on existing membrane to disturb permeability.

The results of this study and others (McCormick et al., 1956) had indicated that vancomycin affects growing cells only. To determine if vancomycin might act directly on a non-growing membrane, protoplasts of B. subtilis W23 were prepared as described and suspended in 0.58 M sucrose and 0.01 M $MgSO_4 \cdot 7H_2O$. The suspension of protoplasts was divided into two 20 ml portions. To one fraction, 5 ml distilled water was added, and to the other fraction, 5 ml vancomycin (50 µg per ml) was added. The two suspensions were incubated with shaking at 37 C and the absorbance at 540 mµ was followed at 30 minute intervals. The results presented in Table IV show that 10 µg per ml vancomycin did not lyse the protoplasts. Since this concentration of vancomycin is about 5 times the effective level required to lyse whole, growing cells of this organism, it does not appear likely that vancomycin acts directly on a non-growing membrane to cause lysis. Hancock and Fitz-James (1964) reported that growing protoplasts of B. megaterium were lysed by vancomycin, but these workers did not mention the effect of vancomycin on non-growing protoplasts.

Figure 7.

Effect of vancomycin on cellular permeability.

Cells were grown in buffered glucose supplemented with 0.2 μmoles per ml $MgSO_4$ and 10 μc D-glucose-U-^{14}C. After washing, the cells were inoculated into the following systems: ▲, buffered glucose and 0.2 μmoles per ml $MgSO_4$; ○, buffered glucose, 0.2 μmoles per ml $MgSO_4$, and 4.6 μg vancomycin per mg dry weight of cells. Samples (5 ml) were taken at the indicated time intervals, the absorbance measured at 540 mμ (solid lines), the cells removed by centrifugation, and the ^{14}C in the supernatant solution was measured (dashed lines).

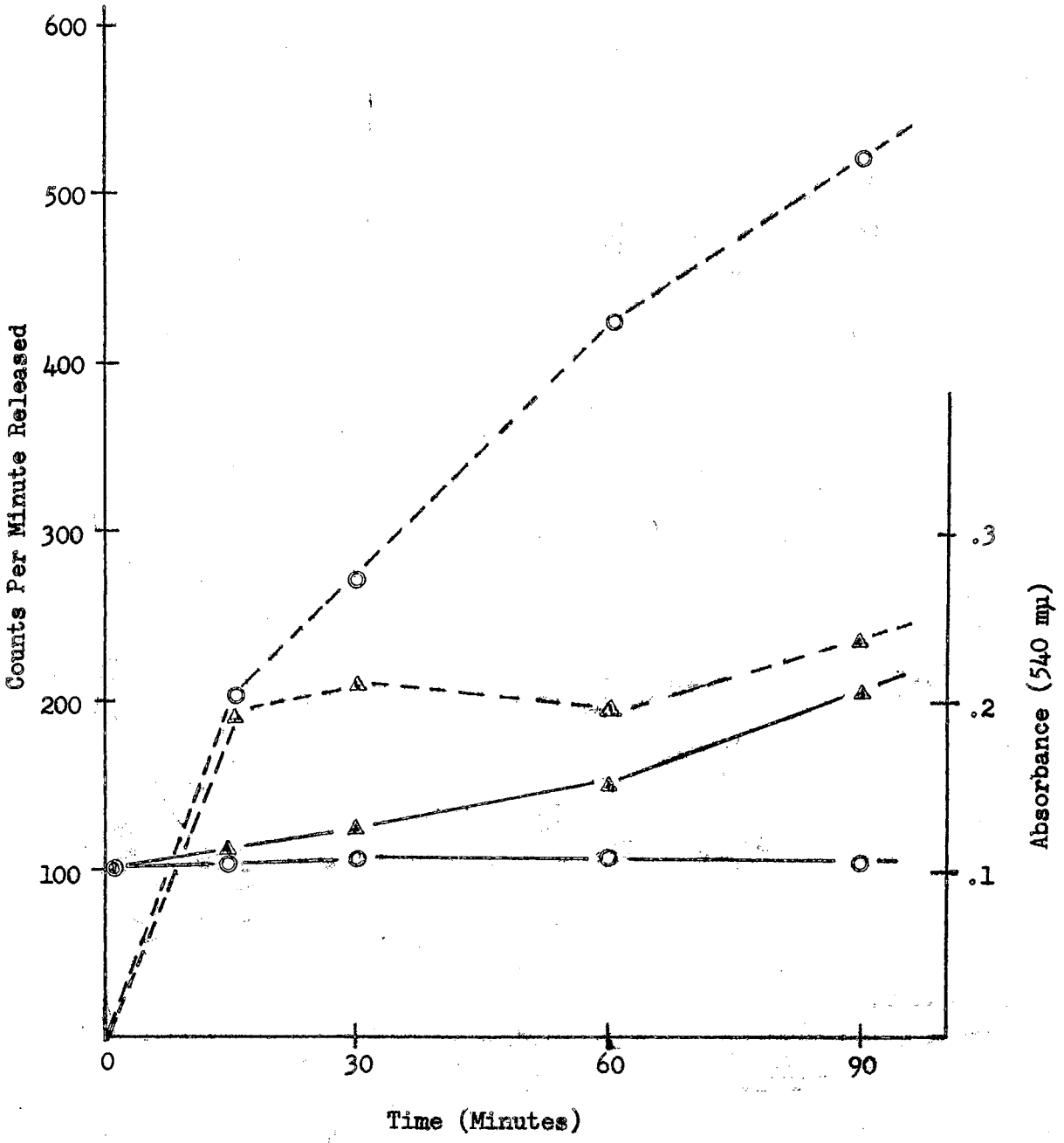

600
500
400
300
200
100
Counts Per Minute Released
.3
.2
.1
Absorbance (540 mμ)
0
30
60
90
Time (Minutes)

Figure 8.

Effect of chloramphenicol on vancomycin-mediated leakage.

Cells were grown in buffered glucose supplemented with 0.2 μmoles per ml $MgSO_4$ and 10 μc D-glucose-U-^{14}C. After washing, the cells were inoculated into flasks containing buffered glucose, 0.2 μmoles per ml $MgSO_4$, and the following antibiotics: ●, none; △, 4.6 μg vancomycin per mg dry weight; ○, 70 μg chloramphenicol per ml; ▲, 4.6 μg vancomycin per mg dry weight and 70 μg chloramphenicol per ml. Samples (5 ml) were taken at the indicated time intervals, the absorbance measured at 540 mμ (dashed line), the cells removed by centrifugation, and the ^{14}C in the supernatant solution was measured (solid line).

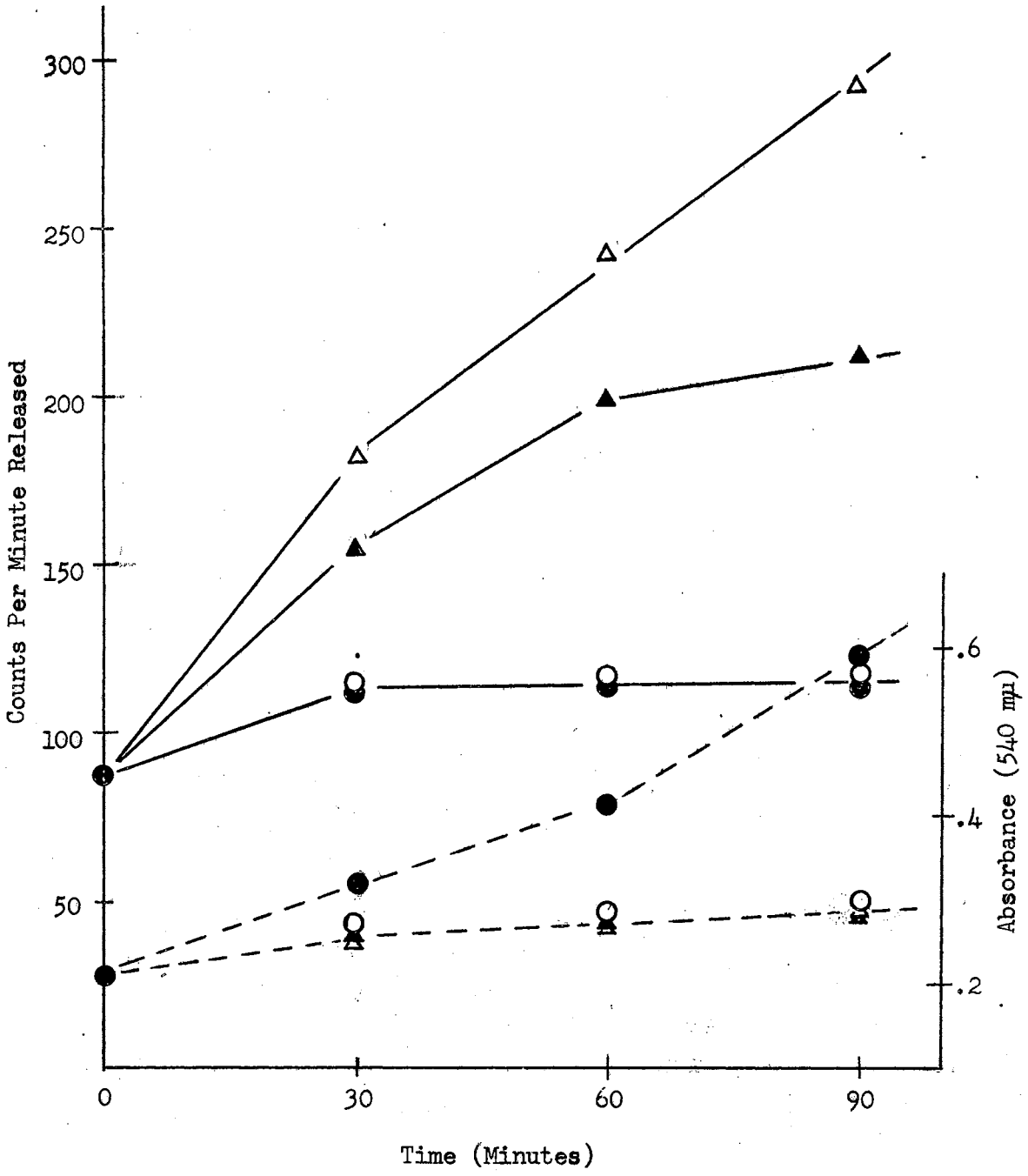

300
250
200
150
100
50
Counts Per Minute Released
0
30
60
90
Time (Minutes)
.6
.4
.2
Absorbance (540 mμ)

TABLE IV

EFFECT OF VANCOMYCIN ON NON-GROWING PROTOPLASTS*

	Absorbance (540 mu)			
	Time (minutes)			
	0	30	60	90
Control	.58	.52	.52	.50
Vancomycin	.60	.52	.50	.51

*Protoplasts of B. subtilis W23 were suspended in 40 ml 0.58 M sucrose containing 0.01 M $MgSO_4 \cdot 7H_2O$. This suspension was divided equally into two 250 ml flasks and at time zero, 5 ml water was added to the control flask and 5 ml vancomycin (50 µg per ml) to the other flask. Five ml samples were taken at the indicated times to determine the absorbance of the suspension.

Vancomycin adsorption to cell walls.

All attempts to alleviate either lysis or leakage with excess magnesium failed. These findings make unlikely the possibility that vancomycin acts on the membrane by any mechanism which ultimately results in a magnesium deficiency. Since vancomycin did not form an insoluble complex with magnesium and did not appear to create a magnesium deficiency, studies were conducted to determine if vancomycin and magnesium competed for binding sites on the cell surface.

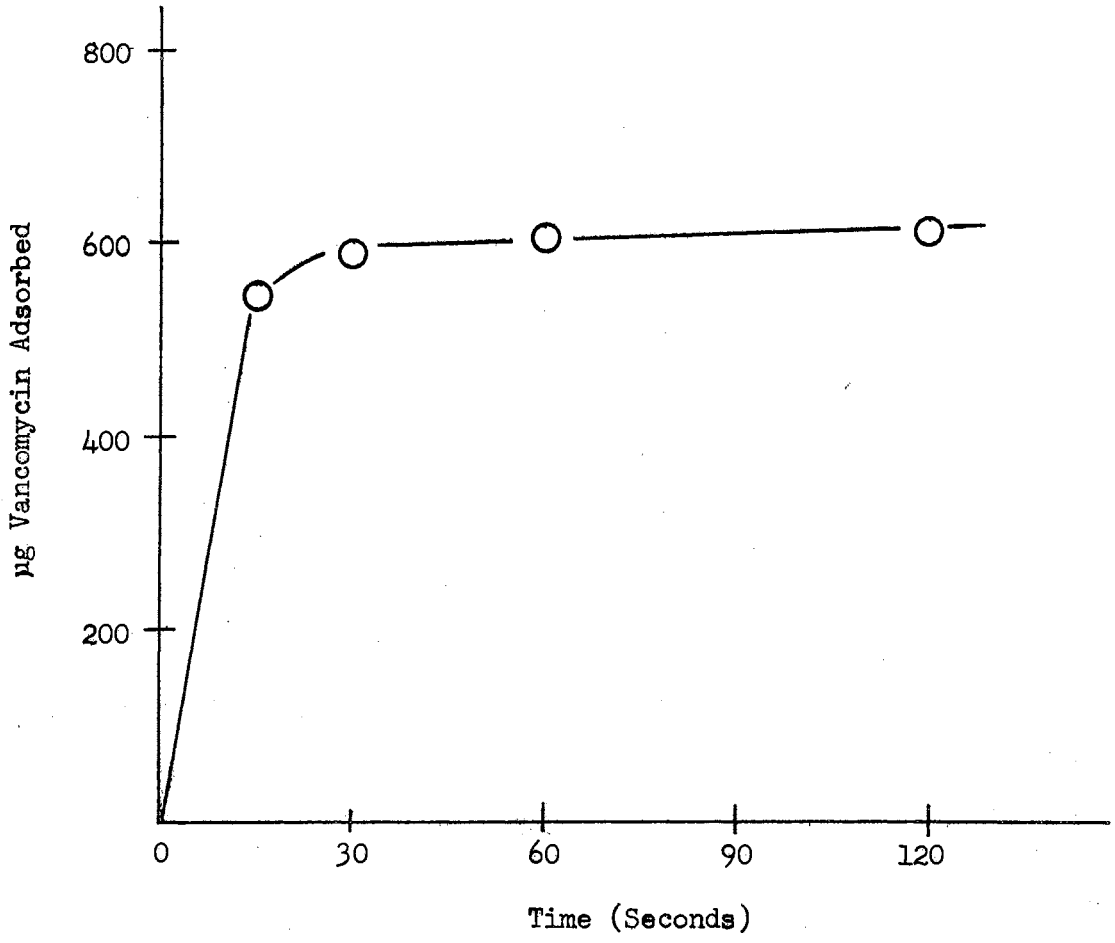
Johnson (1962) found that vancomycin had pKa values at pH 5.1, 6.0, 6.7, 7.7, 9.1, 10.1, 11.5, and three others between 12.0 and 13.5. Therefore, vancomycin is a basic molecule with a large number of ionizable groups which could allow binding to the cell surface by electrostatic or ionic linkages. In addition, vancomycin could adhere to the cell surface by van der Waals forces or a lyophobic complex. McCormick et al., (1956) stated that part of the vancomycin molecule must be lyophobic since it complexed with paraffin oil. Adsorption by any of these processes could disrupt the synthesis of the structure to which the molecule was bound.

Cell walls were prepared as described in Materials and Methods to determine if vancomycin complexed with this structure. The results in Fig. 9 show that vancomycin is rapidly adsorbed to cell walls of B. subtilis. Approximately 620 µg or 69 per cent of the total vancomycin (900 µg) in each tube was adsorbed in 2 minutes while 550 µg or 62 per cent was adsorbed to the cell walls in the first 15 seconds. Temperature had little effect on adsorption of vancomycin since a comparison showed that 600 and 588 µg of vancomycin were adsorbed in 10 minutes at 4 and 37 C respectively. Subsequent adsorption studies were conducted

Figure 9.

Adsorption of vancomycin to cell wall material as a function of time.

Three ml vancomycin (300 µg/ml) and 0.5 ml distilled water were added to 2.5 ml cell walls (absorbance of 0.30 at 540 mµ). The tube contents were thoroughly mixed and the walls removed by Millipore filtration at the indicated times. The filtrate was collected and the absorbance at 280 mµ was determined.


800
600
400
200
µg Vancomycin Adsorbed
0
30
60
90
120
Time (Seconds)

at room temperature, and 5-10 minutes were allowed for adsorption to occur.

The effect of magnesium on the adsorption of vancomycin was determined by adding increasing concentrations of magnesium to a series of tubes containing cell walls and vancomycin. As shown in Fig. 10, increasing concentrations of magnesium decreased adsorption of vancomycin to cell walls. At a magnesium concentration of 4.0 μmoles per ml, essentially no vancomycin adsorbed to the cell walls. This finding demonstrates that the magnesium concentrations which alleviate growth and mucopeptide inhibition by vancomycin also reduce vancomycin adsorption to isolated cell walls.

These studies were modified to determine the effect of the order of addition of cell walls, vancomycin, and magnesium on adsorption. Regardless of the order in which these materials were added to the adsorption system, only the concentration of magnesium had any effect on the extent of vancomycin adsorption.

Other divalent cations such as manganese, calcium, and ferrous ions were tested to determine if vancomycin binding was affected by magnesium alone, or if other cations could also compete with the antibiotic for binding sites on the cell walls. As depicted in Table V, each of these ions was effective in reducing vancomycin adsorption to cell walls. However, an equivalent concentration of NaCl had no effect on vancomycin binding, and very high levels of NaCl (0.2-0.3 M) only slightly decreased the extent of vancomycin adsorption to cell walls. This indicates that divalent cations are most effective in competing for binding sites and the anions of the salts employed must have little effect on adsorption.

Figure 10.

Effect of magnesium on vancomycin adsorption to cell walls of B. subtilis.

Three ml of vancomycin (300 µg per ml) and increasing amounts of $MgSO_4$ were added in 0.5 ml volumes to 2.5 ml of cell walls (absorbance of 0.30 at 540 mµ). Triple distilled water was added to bring the total volume in the control tube to 6.0 ml. Adsorption was conducted for 10 minutes at 26 C and all tubes were centrifuged at 12,100 x g for 10 minutes. The supernatant solution was decanted and the absorbance at 280 mµ was measured to quantitate the extent of vancomycin adsorption.

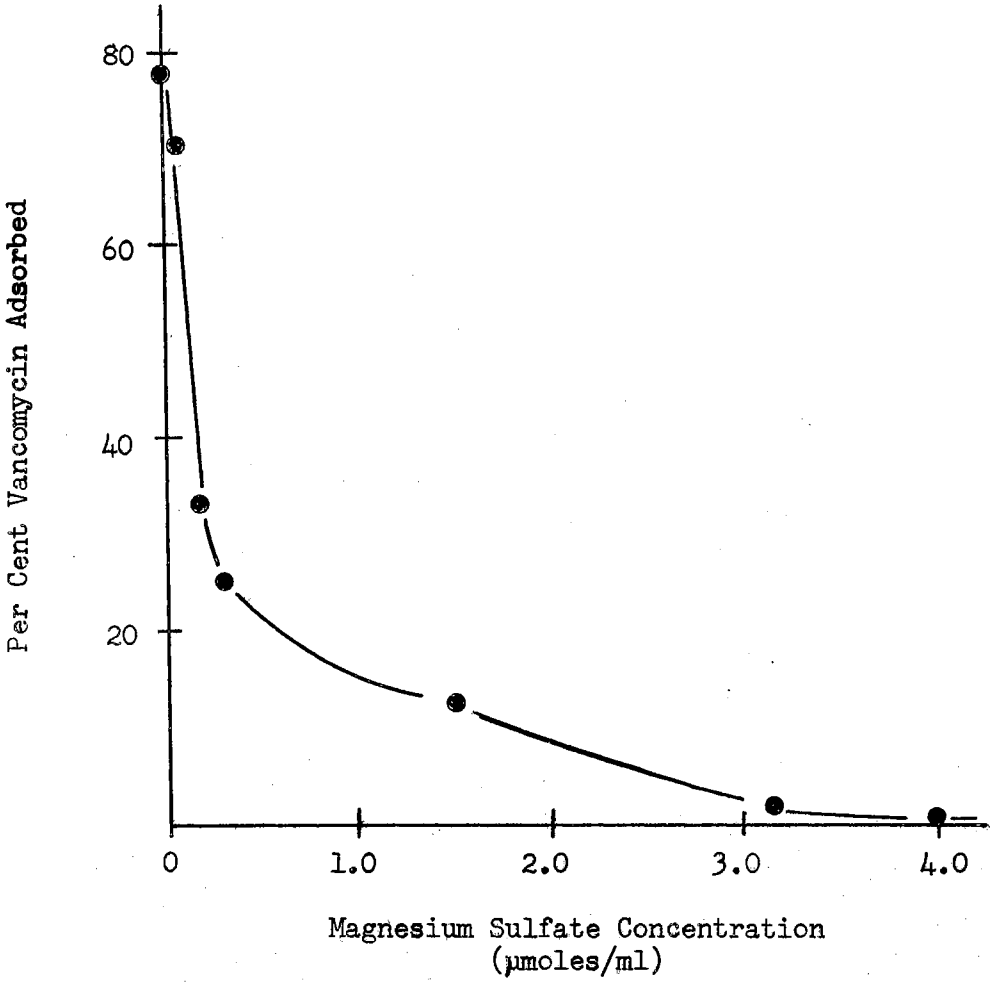

Per Cent Vancomycin Adsorbed
80
60
40
20
0
1.0
2.0
3.0
4.0
Magnesium Sulfate Concentration
(μmoles/ml)

TABLE V

EFFECT OF METALS ON VANCOMYCIN ADSORPTION TO CELL WALLS*

Salt		µg vancomycin adsorbed	per cent vancomycin adsorbed	final pH of supernatant
1)	none	750	83.3	7.0
2)	$MgSO_4$	490	54.5	7.0
3)	$MnSO_4$	480	53.4	6.4
4)	$CaSO_4$	480	53.4	6.5
5)	$FeSO_4$	540	60.0	6.3

*Cell walls were suspended in triple distilled water to an absorbance of 0.32 at 540 mµ and 2.5 ml were mixed with 3.0 ml vancomycin (300 µg per ml). One micromole of each of the above salts was added in a 0.1 ml volume. Triple distilled water was used to bring the total liquid volume to 6.0 ml. After adsorption for 10 minutes at 26 C, the tubes were centrifuged as described previously. The amount of antibiotic adsorbed by the wall preparation was determined by reading the absorbance of the supernatant solution at 280 mµ.

Magnesium, therefore, is not specific in its effect on vancomycin adsorption. Other divalent cations such as the manganous and ferrous ions were toxic for B. subtilis at levels which were employed with magnesium to demonstrate an alleviation of the growth inhibition caused by vancomycin. Therefore, an alleviation of vancomycin inhibition of growth by magnesium was possible only because this cation is tolerated in relatively high levels by this organism.

Binding of vancomycin to the cell wall is reversible. This was shown by adsorbing the antibiotic to cell walls and washing the complex with water and then with magnesium or one of the other divalent cations. The results in Table VI show that very little vancomycin is removed by washing the complex with water. However, when magnesium is added to the wash solution, vancomycin is readily eluted from the cell walls. As indicated in Table VI, the extent of elution is dependent on the amount of magnesium present in the wash solution.

Since the assay for vancomycin adsorption depends entirely on a decrease in ultraviolet absorption at 280 mμ, an absorption spectrum was obtained between 240 and 320 mμ (using a Bausch and Lomb 505 recording spectrophotometer) to eliminate the possibility that the absorption peak of vancomycin at 280 mμ was not merely shifted under the various conditions. It is apparent from these spectra that vancomycin absorbs ultraviolet light characteristically at about 280 mμ in distilled water, after adsorption to cell walls, and after elution from cell walls by magnesium (Fig. 11). These results suggest that magnesium is competing for binding sites and not merely shifting the absorption peak of vancomycin at 280 mμ.

TABLE VI

ELUTION OF ADSORBED VANCOMYCIN BY MAGNESIUM*

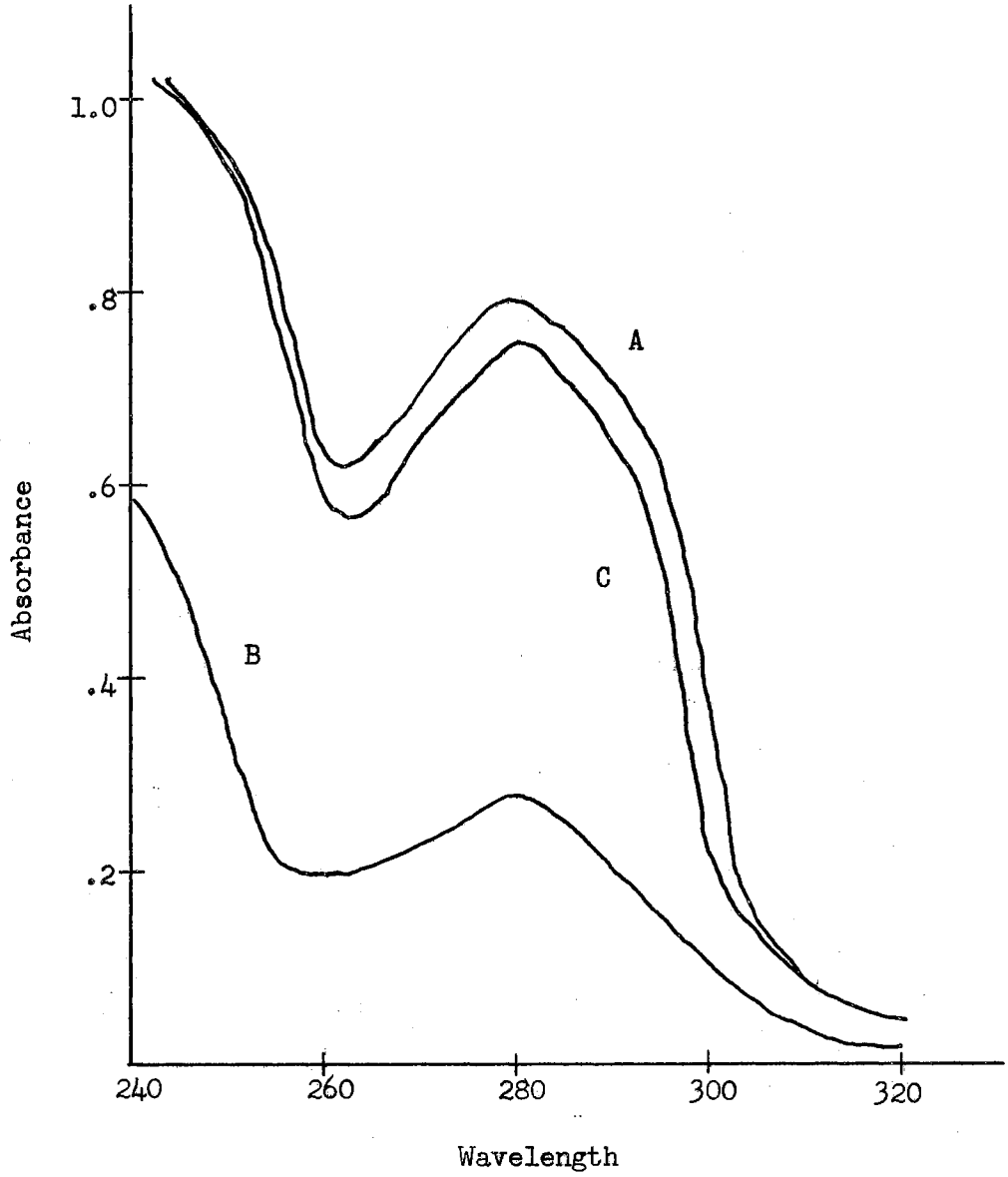
	Cell wall absorbance (540 mμ)	μg Vancomycin			
		Adsorbed	Eluted by H_2O	Eluted by magnesium	Magnesium concentration
1)	0.32	735	72	612	8.33 μmoles/ml
2)	0.30	675	75	225	1.66 μmoles/ml

*A total of 900 μg of vancomycin was present in each tube. Adsorption was conducted for 10 minutes at 26 C and the cell walls were removed by centrifugation.

Figure 11.

Vancomycin spectra after adsorption to and elution from cell walls.

Absorption spectra between 240 mμ and 320 mμ are indicated as follows: line A, vancomycin dissolved in distilled water at 180 μg per ml; line B, vancomycin remaining after adsorption to cell walls; line C, vancomycin eluted by 8.3 μmoles per ml $MgSO_4$.


1.0
.8
.6
.4
.2
Absorbance
A
B
C
240
260
280
300
320
Wavelength

Adsorption of vancomycin to the cell membrane.

The finding that magnesium alleviated the effect of vancomycin on cell wall mucopeptide synthesis but not on vancomycin-induced lysis, suggested that this antibiotic had separate effects on the two structures.

To determine if vancomycin is adsorbed to the cell membrane of this organism, protoplasts were prepared as previously described. When these bodies were lysed in distilled water, the membranes could be sedimented by high speed centrifugation (20,000 x g for 20 minutes). However, no vancomycin adsorption could be demonstrated with these preparations. There may be too few binding sites on the membrane to allow detection of adsorbed vancomycin by this assay. It is equally likely, however, that vancomycin inhibits membrane synthesis (Yudkin, 1963) by some mechanism totally unrelated to adsorption. These data would indicate, therefore, that vancomycin inhibits mucopeptide and membrane synthesis by different mechanisms.

Lysis of B. subtilis by vancomycin.

The lysis of B. subtilis could occur by a direct inhibition of membrane synthesis, or indirectly by osmotic lysis resulting from the inhibition of cell wall synthesis. The results presented in Figs. 12 and 13 distinguish between these possibilities. In Fig. 12, vancomycin and penicillin were added to suspensions of this organism growing in glucose salts. Both antibiotics rapidly lysed this organism. In Fig. 13, however, the glucose was supplemented with a hypertonic concentration of sucrose (0.58 M) and 0.01 M $MgSO_4 \cdot 7H_2O$. In this medium, protoplasts of this organism did not lyse. Consequently, when a suspension of this organism was inhibited by a lytic concentration of

Figure 12.

Lysis of B. subtilis growing in glucose salts by vancomycin and penicillin.

Actively dividing cells of B. subtilis were inoculated into flasks containing 20 ml glucose salts and the following antibiotics: ●, none; ○, vancomycin (2 µg per ml); △, penicillin (75 µg per ml). Growth was followed by measuring the absorbance at 540 mµ of 5 ml samples at the indicated times.

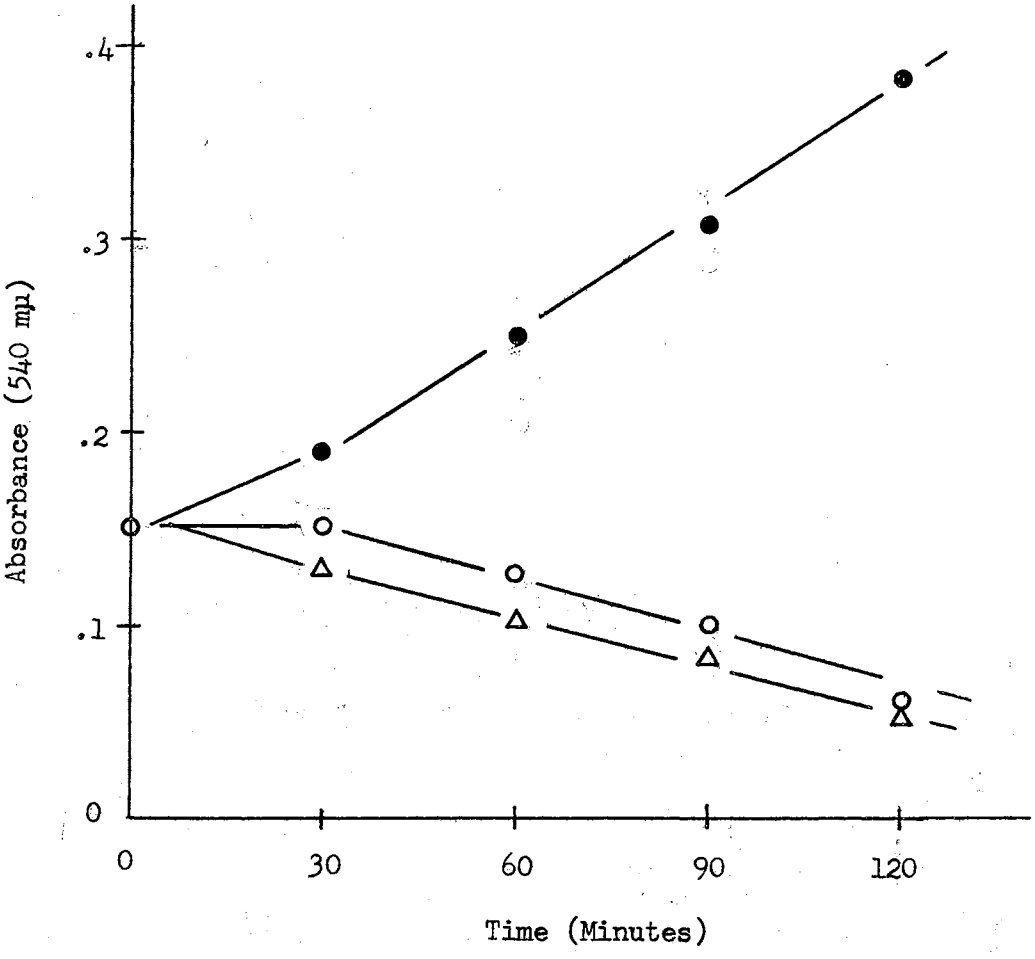
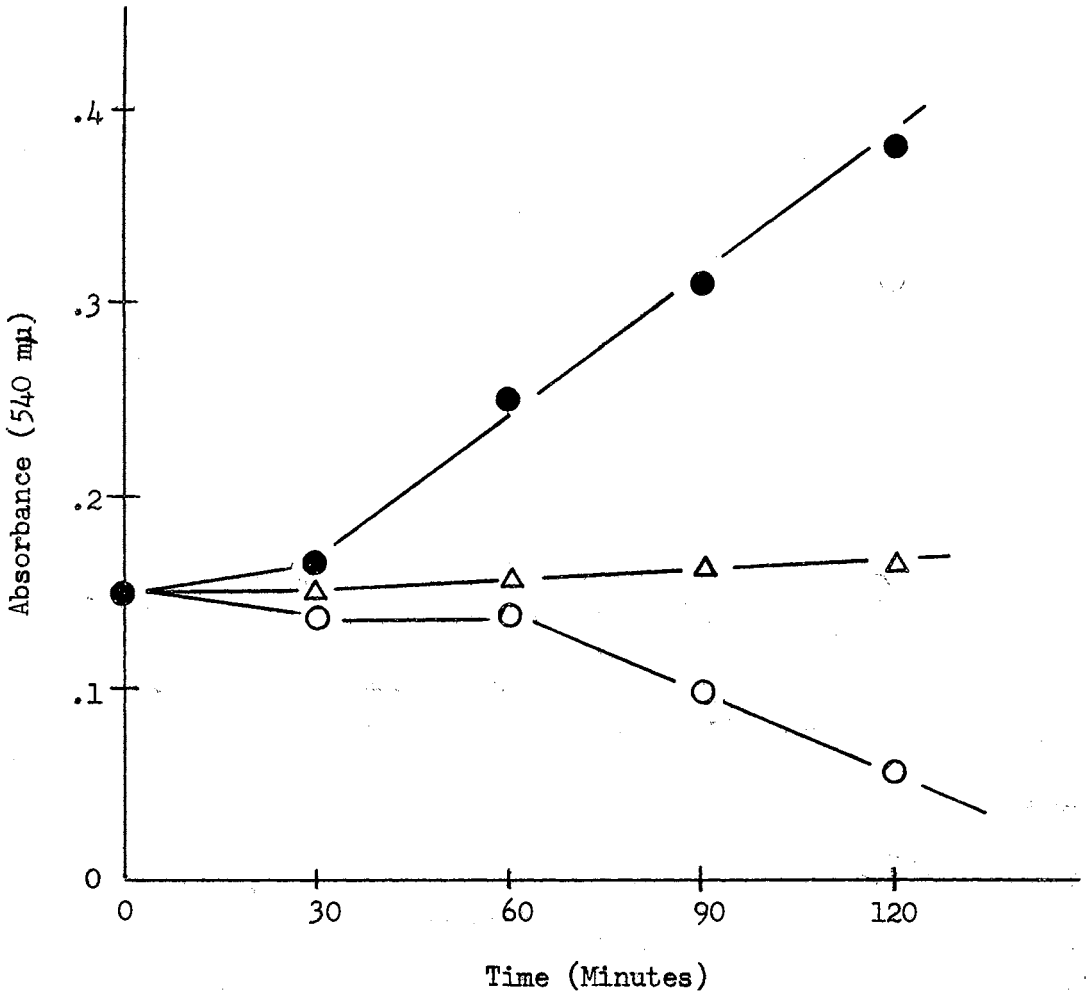

.4
.3
.2
.1
0
Absorbance (540 mμ)
0
30
60
90
120
Time (Minutes)

Figure 13.

Effect of lytic concentrations of vancomycin and penicillin on B. subtilis cells growing in a hypertonic medium.

Actively dividing cells (4.0 ml) were added to flasks containing 20 ml glucose salts supplemented with 0.58 M sucrose and 0.01 M $MgSO_4$ and antibiotics as follows: ●, none; ○, vancomycin (2 µg per ml); △, penicillin (75 µg per ml). Growth was followed as described in Fig. 12.


.4
.3
.2
.1
0
Absorbance (540 mμ)
0
30
60
90
120
Time (Minutes)

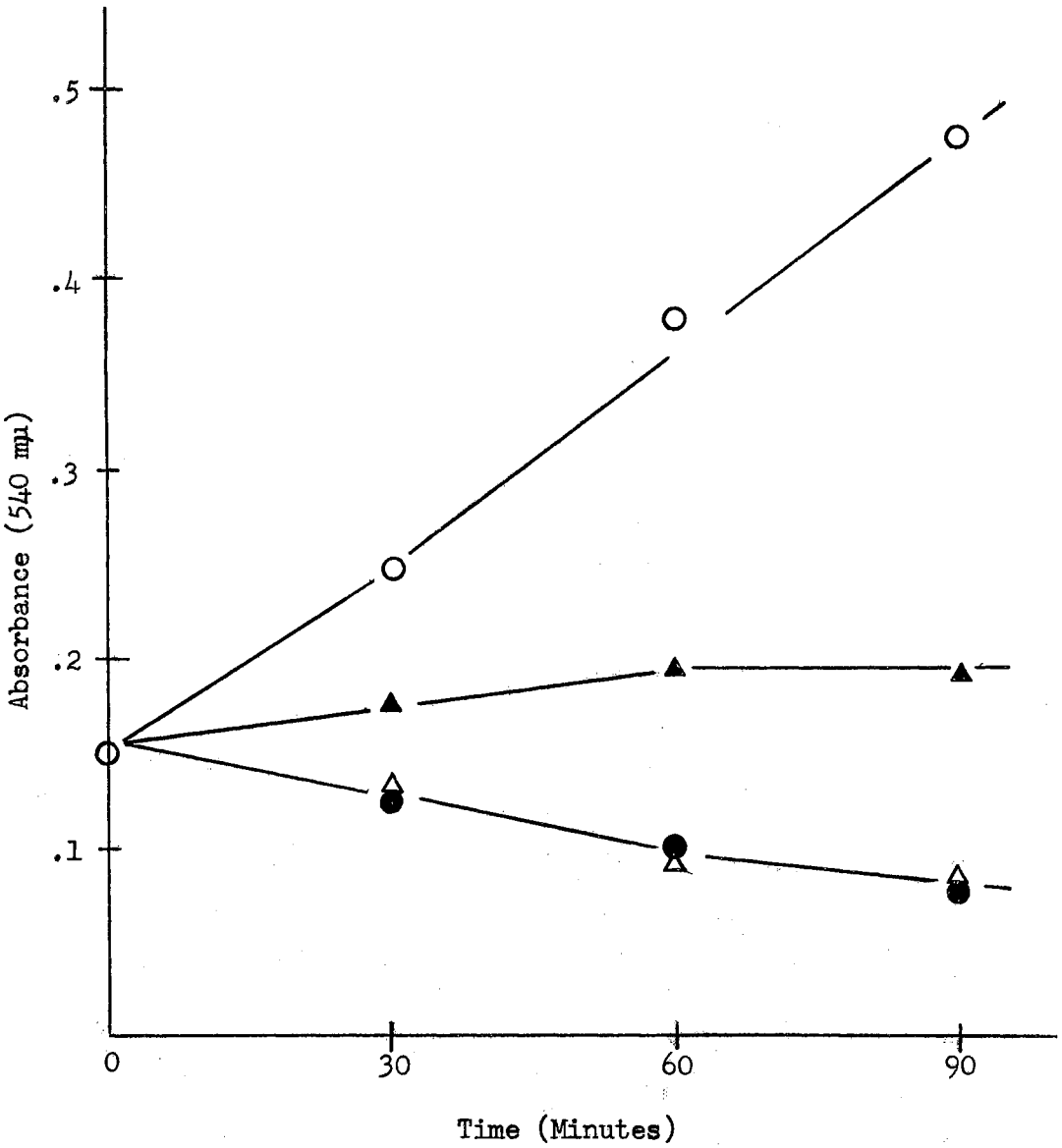
penicillin, the cells did not lyse. However, when vancomycin was added to cells growing in this medium, lysis occurred in spite of the osmotic protection. This finding would indicate that vancomycin acts directly on the membrane to cause lysis.

This conclusion is supported by the results presented in Fig. 14. Chloramphenicol prevents synthesis of cell protein (Gale and Folkes, 1953), thereby precluding a substantial increase in cell mass. In the presence of chloramphenicol, an inhibitor of cell wall synthesis would not cause lysis, but an inhibitor of the synthesis of the permeability membrane would still lyse a cell suspension. When vancomycin was added to chloramphenicol-treated cells, a rapid lysis ensued. This would support the previous conclusion that vancomycin acts on the cell membrane directly to inhibit its synthesis.

Figure 14.

Lysis of B. subtilis by vancomycin in the presence of chloramphenicol.

Cells were grown to the mid-log phase in glucose salts and used to inoculate flasks containing 20 ml glucose salts, and other additions as follows: ○, none: ▲, 70.0 µg/ml chloramphenicol; ●, 0.40 µg/ml vancomycin; △, 0.40 µg/ml vancomycin and 70.0 µg/ml chloramphenicol. Growth was followed by measuring the absorbance of 5.0 ml samples at 540 mµ.


.5
.4
.3
.2
.1
Absorbance (540 mμ)
0
30
60
90
Time (Minutes)

CHAPTER V

SUMMARY AND CONCLUSIONS

Vancomycin is a potent inhibitor of B. subtilis W23. Growth, mucopeptide synthesis, and cell permeability are affected by vancomycin. The extent of inhibition was related to the amount of vancomycin present with respect to the cell mass. Vancomycin at 1.3, 4.6, and 8.5 µg per mg dry weight of cells respectively inhibited mucopeptide synthesis, initiated leakage of cell metabolites, and caused lysis of the cells. These vancomycin concentrations do not, of course, represent the absolute levels necessary to attain a given inhibition, but the response of the cells to the different vancomycin concentrations indicates that the mechanism of inhibition becomes more complicated as the concentration of the antibiotic is increased.

The results with magnesium suggest that vancomycin inhibits B. subtilis by at least two different mechanisms. Magnesium ions alleviate the inhibition of mucopeptide synthesis by vancomycin, but this cation had no effect on the inhibition when the membrane appeared to be involved (lysis and leakage).

The finding that vancomycin adsorbs to cell walls furnished a possible mechanism of inhibition of cell wall synthesis. It is readily apparent that adsorption of a large molecular weight compound could inhibit the synthesis of a polymer, such as the cell wall, by physically

blocking extension of the polymer. This would be a reasonable speculation if it was based solely on the finding that vancomycin adsorbs to cell walls. However, the fact that magnesium and other divalent cations reduce this adsorption as well as alleviate the inhibition of cell wall synthesis makes the suggested mechanism more attractive.

The chemical basis for vancomycin adsorption to cell walls is probably related to the basic nature of vancomycin. Seven pKa values above pH 7.0 have been reported for vancomycin (Johnson, 1962). Thus, the molecule could complex with free anions present in the cell wall. Both muramic acid and C-terminal amino acids in the peptide of the cell wall could contribute an ionized carboxyl group for adsorption, while the phosphoryl linkages of the teichoic acids would be expected to carry negative charges that might also be involved in adsorption of vancomycin. Therefore, vancomycin could complex with cell walls via electrostatic or ionic linkages. Other types of linkages such as hydrogen bonding might supplement the ionic linkages, but cation competition would be expected only with electrostatic bonds.

Since vancomycin is adsorbed to cell walls, it is not possible to state whether the synthesis of cell wall or cytoplasmic membrane is the more sensitive to vancomycin. Higher levels of vancomycin are required to demonstrate an effect on the membrane, but it is not yet possible to determine how much vancomycin adsorbs to the cell wall before the antibiotic is able to reach the membrane. Also, the manner in which vancomycin inhibits the synthesis of the cytoplasmic membrane (Yudkin, 1963) is not understood. It is conceivable, however, that vancomycin blocks the polymerization of new membrane by forming a lyophobic complex with existing membrane. No such complex was demonstrated in this study, but

McCormick et al. (1956) stated that such a complex was formed between vancomycin and paraffin oil.

Chloramphenicol was employed during this investigation to facilitate the study of cell wall synthesis and to determine whether vancomycin acts directly on a non-growing membrane. The observed inhibition by chloramphenicol of cell wall mucopeptide synthesis (Table III) has probably been correctly interpreted by Chatterjee and Park (1964). They also found that chloramphenicol had a delayed effect on cell wall synthesis by S. aureus and suggested that this was due to an inhibition of the synthesis of new enzymes required for cell wall synthesis. That is, the cell wall-synthesizing enzymes presumably must be replaced after a period of time--perhaps because the cell wall-synthesizing sites constantly shift.

As mentioned previously, Hancock and Fitz-James (1964) found that chloramphenicol had no effect on the vancomycin-induced leakage of ^{42}K from B. megaterium. The results of this study (Fig. 8) indicate that chloramphenicol reduces the extent of leakage from B. subtilis labeled by growth in a medium containing D-glucose-U-^{14}C. The different results obtained in the presence of chloramphenicol is thought to represent a difference in the technique used to measure leakage. Urbá (1959) reported that non-growing cells of B. cereus degrade cytoplasmic protein and release amino acids into the medium. Chloramphenicol essentially prevents this protein degradation. The metabolites released from vancomycin-treated cells in all probability contain some amino acids which are liberated following protein breakdown. When chloramphenicol is present, the extent of leakage caused by vancomycin is reduced since protein is not being degraded.

In summary, it appears from these data that vancomycin affects B. subtilis in at least two ways. At low vancomycin to cell mass ratios, vancomycin is adsorbed to the cell wall and physically blocks the polymerization of the cell wall. Magnesium protects the cell from this inhibition by reducing vancomycin adsorption. At higher levels of vancomycin, the antibiotic also inhibits the synthesis of the cytoplasmic membrane (Yudkin, 1963). The manner in which vancomycin inhibits the synthesis of the membrane is not understood. It is conceivable, however, that vancomycin interferes with the polymerization of membrane material by forming a lyophobic complex with existing membrane.

LITERATURE CITED

1. Best, G. K. and N. N. Durham. 1964. Effect of vancomycin on Bacillus subtilis. Arch. Biochem. Biophys., 105:120-125.

2. Chatterjee, A. N. and J. T. Park. 1964. Biosynthesis of cell wall mucopeptide by a particulate fraction from Staphylococcus aureus. Proc. Natl. Acad. Sci., 51:9-16.

3. Gale, E. F. and J. P. Folkes. 1953. The assimilation of amino acids by bacteria. 18. The incorporation of glutamic acid into the protein fraction of Staphylococcus aureus. Biochem. J., 55:721-729.

4. Hancock, R. and P. C. Fitz-James. 1964. Some differences in the action of penicillin, bacitracin, and vancomycin on Bacillus megaterium. J. Bacteriol., 87:1044-1049.

5. Higgins, H. M., W. H. Harrison, G. M. Wild, H. R. Bungay, and M. H. McCormick. 1958. Vancomycin, a new antibiotic. IV. Purification and properties of vancomycin, p. 906-914. In H. Welch and F. Marti-Ibañez (ed.), Antibiotics annual. Medical Encyclopedia, Inc., New York.

6. Johnson, C. R. 1962. A prospectus of the vancomycin structure. Ph.D. Thesis, Part II. University of Illinois.

7. Jordan, D. C. and W. E. Inniss. 1959. Selective inhibition of ribonucleic acid synthesis in Staphylococcus aureus by vancomycin. Nature, 184:1894-1895.

8. Jordan, D. C. and W. E. Inniss. 1961. Mode of action of vancomycin on Staphylococcus aureus, p. 218-225. In, M. Finland and G. M. Savage, (ed.), Antimicrobial agents and chemotherapy-1961. American Society for Microbiology, Detroit.

9. Jordan, D. C. 1961. Effect of vancomycin on the cell wall mucopeptide of Staphylococcus aureus. Biochem. Biophys. Res. Commun., 6:167-170.

10. Mandalstam, J. and H. J. Rogers. 1959. The incorporation of amino acids into the cell-wall mucopeptide of staphylococci and the effect of antibiotics on the process. Biochem. J., 72:654-662.

11. McCormick, M. H., W. M. Stark, G. E. Pittenger, R. C. Pittenger, and J. M. McGuire. 1956. Vancomycin, a new antibiotic. I. Chemical and biologic properties, p. 606-611. In, H. Welch and F. Marti-Ibañez, (ed.), Antibiotics annual. Medical Encyclopedia, Inc., New York.

12. Park, J. T. and R. Hancock. 1960. A fractionation procedure for studies on the synthesis of cell wall mucopeptide and other polymers in cells of Staphylococcus aureus. J. Gen. Microbiol., 22:249-258.

13. Redfield, R. R. 1953. Two-dimensional chromatographic systems with high resolving power for amino acids. Biochim. Biophys. Acta, 10:344-345.

14. Reynolds, P. E. 1961. Studies on the mode of action of vancomycin. Biochim. Biophys. Acta, 52:403-505.

15. Reynolds, P. E. 1962. A comparative study of the effects of penicillin and vancomycin. Biochem. J., 84:99-100p.

16. Roberts, J. and M. J. Johnson. 1962. Effect of penicillin on the cell wall of Bacillus subtilis. Biochim. Biophys. Acta, 59:458-466.

17. Salton, M. R. J. 1960. Surface layers of the bacterial cell. p. 97-151. In, I. C. Gunsalus and R. Y. Stanier, (ed.), The bacteria, Vol. I. Academic Press, Inc., New York.

18. Sanderson, A. R., J. L. Strominger, S. G. Nathenson. 1962. Chemical structure of teichoic acid from Staphylococcus aureus, strain Copenhagen. J. Biol. Chem., 237:3603-3613.

19. Shockman, G. D. and J. O. Lampen. 1962. Inhibition by antibiotics of the growth of bacterial and yeast protoplasts. J. Bacteriol., 84:508-512.

20. Urbá, R. C. 1959. Protein breakdown in Bacillus cereus. Biochem. J., 71:513-518.

21. Webb, M. 1949. The influence of magnesium on cell division. II. The effect of magnesium on the growth and cell division of various bacterial species in complex media. III. The effect of magnesium on the growth of bacteria in simple, chemically defined media. J. Gen. Microbiol., 5:485-495.

22. Yudkin, M. D. 1963. The effect of penicillin, novobiocin, streptomycin, and vancomycin on membrane synthesis by protoplasts of Bacillus megaterium. Biochem. J., 89:290-296.

23. Ziegler, D. W., R. N. Wolfe, and J. M. McGuire. 1956. Vancomycin, a new antibiotic. II. In vitro antibacterial studies, p. 612-618. In, H. Welch and F. Marti-Ibañez, (ed.), Antibiotics annual. Medical Encyclopedia, Inc., New York.

VITA

Gary Keith Best

Candidate for the Degree of

Doctor of Philosophy

Thesis: MECHANISM OF ACTION OF VANCOMYCIN

Major Field: Microbiology

Biographical:

Personal Data: Born at Weatherford, Oklahoma, October 8, 1938, the son of Coy Thomas and Laura Mae Best; married Norma Jone Hefley on January 19, 1962.

Education: Graduated from Odessa High School, Odessa, Texas in 1956. Received the Bachelor of Science degree, with a major in chemistry and biology, from Southwestern State College, Weatherford, Oklahoma. Completed the requirements for the Doctor of Philosophy degree in May, 1965.

Organizations: Member of Phi Lambda Upsilon, Missouri Valley Branch of American Society for Microbiology, and Society of the Sigma Xi.