# Synergy of Vancomycin with Penicillins and Cephalosporins Against Pseudomonas, Klebsiella, and Serratia

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A model of antibiotic synergy based on a molecular mechanism of action which blocked sequential steps in a single metabolic pathway was tested. Twenty-five strains each of Pseudomonas, Klebsiella, and Serratia were tested *in vitro* against *three different two drug* combinations of vancomycin, carbenicillin, or cephalothin. Synergy was observed when vancomycin was combined with either carbenicillin or cephalothin against isolates of Pseudomonas or Serratia, whereas the combination of carbenicillin and cephalothin did not result in significant synergy against these isolates. The presence of synergy was not related to the sensitivity or resistance of the isolates to the drugs in the combination. Synergy was also observed with all three antibiotic combinations against Klebsiella isolates which may be related to enzyme inactivation by one of the drugs in the combination. These observations support the hypothetical model of antibiotic synergy based on sequential blocking of one biochemical pathway.

## INTRODUCTION

The principles of antibiotic synergy were discussed over twenty years ago [1], but the development of synergistic combinations was often based on experimental trials rather than theoretical grounds. Much has been learned during the past twenty years about the molecular mechanisms of antibiotic action. These earlier studies have provided an opportunity to possibly predict and to develop, on theoretical grounds, synergistic combinations of antimicrobial agents.

The present investigation was undertaken in an attempt to test a theoretical model of antibiotic synergy by selecting combinations of antibiotics that theoretically have the potential for synergistic activity and comparing these combinations with other drug combinations that theoretically should not act synergistically. The theoretical model tested was that of sequential antibiotic action. Specifically, the model postulates that synergy can be obtained by using two different antibiotics with molecular mechanisms of action which block different steps in a single metabolic pathway of the organism. The activity of such antibiotic combinations was compared with a two drug antibiotic combination with molecular mechanisms of action which potentially blocked the same site in the same metabolic pathway of the organism.

The antibiotics used were vancomycin, carbenicillin, and cephalothin which are all cell wall synthesis inhibitors. These antibiotics were tested *in vitro,* singly and in combination with each other, against *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Serratia marcescens.* The combination of vancomycin and carbenicillin, as well as that of vancomycin and cephalothin theoretically act sequentially, whereas the combination of carbenicillin and cephalothin theoretically acts at the same metabolic site (Fig. 1). The use of these antibiotics and bacterial strains permitted an extensive test of the synergy model since vancomycin alone has little, if any, antibacterial



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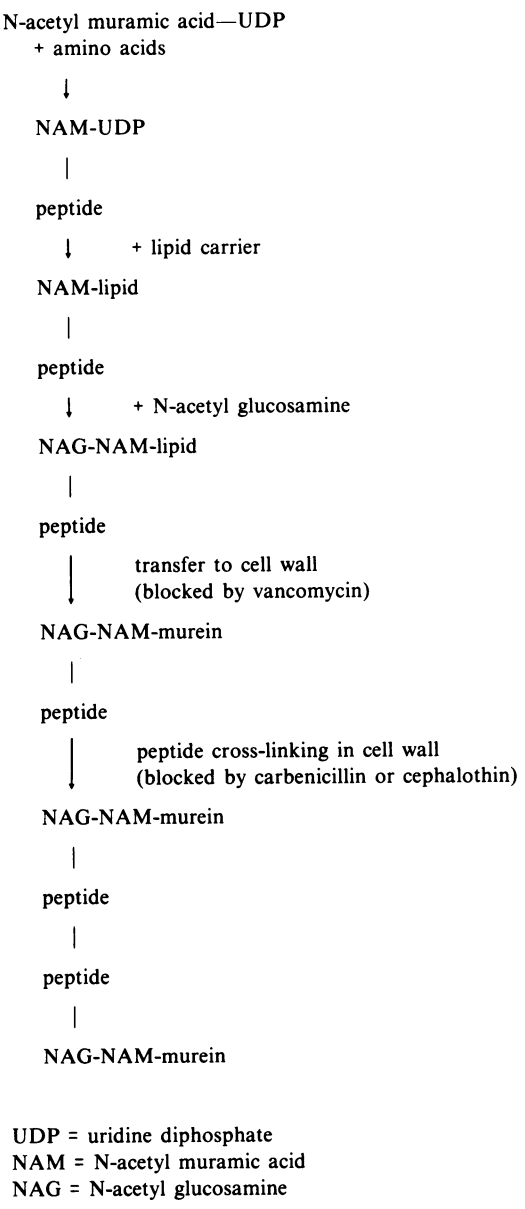


FIG. 1. Cell Wall Synthesis Scheme

activity against the gram-negative bacilli used in the present study [2], carbenicillin, though effective against strains of pseudomonas [3], has intermediate activity against *Serratia marcescens* [4] and virtually no activity against klebsiella species [5], and cephalothin, though effective against klebsiella species, has no activity against strains of pseudomonas or *Serratia marcescens* [6].

## MATERIALS AND METHODS

*Bacterial Strains.* Twenty-five strains each of *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Serratia marcescens* were obtained from clinical isolates. These bacterial strains were identified by the Yale-New Haven Hospital Clinical Microbiol-

ogy Laboratory and were subcultured and reidentified in our Research Laboratory as previously described [3].

*Antibiotics.* All antibiotics were kindly supplied as laboratory standards by their manufacturers. Vancomycin as vancomycin sulfate and cephalothin as the sodium salt were supplied by Eli Lilly and Company, Indianapolis, Indiana, and carbenicillin was supplied by Roerig, New York, New York. Stock solutions of vancomycin and carbenicillin were made up in normal saline and distilled water respectively. The cephalothin laboratory standard was used for concentrations less than 1000 ug/ml and sodium cephalothin intravenous injection grade was used for concentrations greater than 1000 ug/ml. Cephalothin stock solutions were made up in pH 6.0 potassium-phosphate buffer and were used on the same day. Stock solutions of vancomycin and carbenicillin were stored at -4°C for up to one month.

*In Vitro Testing.* All studies were done using Mueller-Hinton Broth (BBL Inc., Cockeysville, Md.). This broth was chosen because of its use with agar in disc sensitivity testing and because of its reproducibility from batch to batch [7]. All tests of synergy were done *in vitro* using an Autotiter III microdilution system (Canalco Inc., Rockville, Md.) and plastic trays with grids of wells in a manner previously described [3]. The inoculum used was between 1–5 x $10^5$ organisms/ml and the incubation time was 22–24 hours at 37°C and maximum humidity. At the end of the incubation period, each tray was read visually for the pattern of bacterial growth (as turbidity) in the wells. Each bacterial strain was tested in triplicate for each antibiotic combination stocked and the results were recorded only if at least two trays had identical patterns.

*Definitions.* As previously described [3] the patterns of growth and inhibition of growth were interpreted for each test as follows:

*Synergy.* No growth of bacteria in the presence of two antibiotics, each of which is present at a concentration which is less than ½ of its minimal inhibitory concentration (MIC).

*Partial Synergy.* No growth of bacteria in the presence of two antibiotics, one of which is present at a concentration less than ½ of its MIC and the other of which is present at a concentration which is ½ of its MIC.

*Additive.* No growth of bacteria in the presence of two antibiotics, each of which is present at a concentration which is ½ of its MIC.

*Indifferent.* No inhibition of growth in the presence of two antibiotics, each of which is present at ½ of its MIC.

*Partial Antagonism.* No inhibition of growth in the presence of two antibiotics when one of the antibiotics is present in a concentration *at least* that of its MIC and the other is present at a concentration *less* than its MIC.

*Analysis.* The significance of differences in the incidence of synergy (including partial synergy) between two antibiotic combinations was measured using Fishers Test [8]. Due to the distribution of results over several categories, synergistic and partially synergistic results were combined in the analysis and compared together against additive and indifferent results.

## RESULTS

The minimum inhibitory concentrations of vancomycin, carbenicillin and cephalothin when tested alone against twenty-five strains each of *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Serratia marcescens* are listed in Table 1. None of the organisms tested could be inhibited by vancomycin in concentrations less than 1250

TABLE 1
Minimum Inhibitory Concentrations (MIC)
of Vancomycin, Carbenicillin and Cephalothin for
Pseudomonas, Klebsiella and Serratia Isolates

| Antibiotic (μg(ml) | Pseudomonas (No. strains) | Klebsiella (No. strains) | Serratia (No. strains) |
|---|---|---|---|
| Vancomycin | | | |
| 1250 | 6 | 25 | 4 |
| 2500 | 19 | — | 16 |
| >5000 | — | — | 5 |
| Carbenicillin | | | |
| 0.625 | — | — | 2 |
| 2.5 | — | — | 4 |
| 5.0 | — | — | 2 |
| 7.8 | — | — | 3 |
| 15.6 | 14 | — | 1 |
| 31.2 | 7 | — | — |
| 62.5 | 4 | — | — |
| 125 | — | 4 | — |
| 250 | — | 10 | — |
| 500 | — | 1 | — |
| 2,000 | — | 4 | — |
| 2,500 | — | — | 2 |
| 5,000 | — | — | 4 |
| 8,000 | — | 6 | — |
| 10,000 | — | — | 7 |
| Cephalothin | | | |
| 3.12 | — | 9 | — |
| 6.25 | — | 8 | — |
| 12.5 | — | 5 | — |
| >1,600 | — | 3 | — |
| 5,000 | 21 | — | 2 |
| >5,000 | 4 | — | — |
| 10,000 | — | — | 23 |

ug/ml. Carbenicillin inhibited the strains of pseudomonas at concentrations between 15.6 and 62.5 ug/ml. Klebsiella isolates were more resistant and required concentrations of carbenicillin between 125 and 8000 ug/ml. Isolates of *Serratia marcescens* fell into two groups, those which were inhibited by concentrations of carbenicillin between 0.625 and 15.6 ug/ml and a resistant group which required between 2500 and 10,000 ug/ml. Cephalothin inhibited most Klebsiella isolates at concentrations of 12.5 ug/ml or less whereas isolates of Pseudomonas and Serratia were highly resistant and were only inhibited by concentrations of 5000 to 10,000 ug/ml.

A synergistic effect between vancomycin and carbenicillin was observed in the majority (19 of 25) of isolates of *Pseudomonas aeruginosa* (Fig. 2). Similarly, the combination of vancomycin and cephalothin was synergistic for 12 of the 25 Pseudomonas isolates. In contrast, the combination of carbenicillin and cephalothin produced a synergistic effect in only 4 of the 25 Pseudomonas isolates. The combination of vancomycin and carbenicillin, each of which alone had little effect against Klebsiella isolates, was synergistic against 12 of 25 of these isolates (Fig. 2). Furthermore, the combination of vancomycin and cephalothin was synergistic against 19 of these 25 isolates. However, the combination of carbenicillin and cephalothin produced unexpected results. This antibiotic combination resulted in synergistic activity

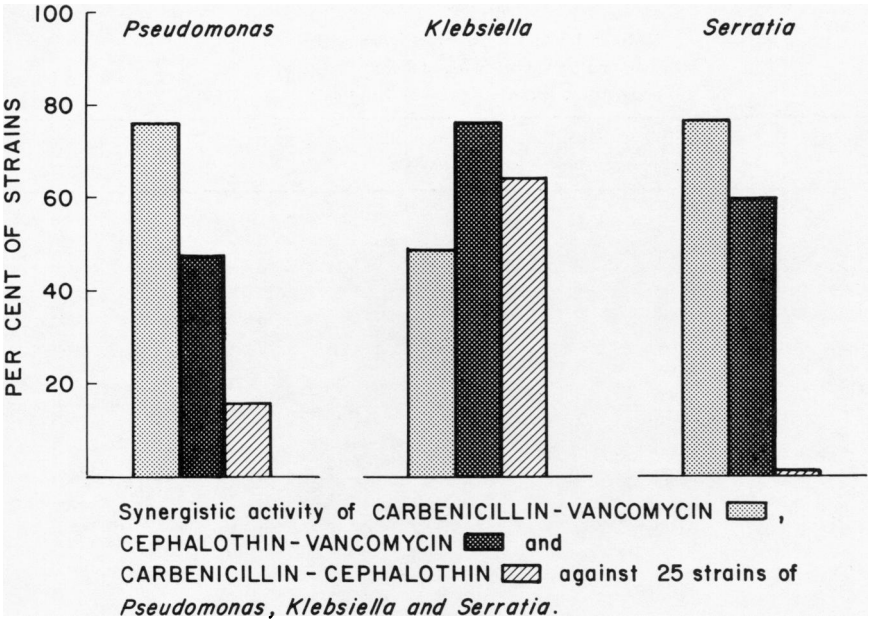


FIG. 2.

against 16 of the 25 isolates studied (Fig. 2). The combination of vancomycin and carbenicillin was synergistic against 19 of the 25 isolates of *Serratia marcescens* (Table 2 and Fig. 2). Similar results were observed with the combination of vancomycin and cephalothin which produced a synergistic effect in 15 of 25 Serratia isolates (Fig. 2). In contrast, the combination of carbenicillin and cephalothin, two antibiotics which inhibit the same metabolic site of the bacterial cell, not only produced no synergy but resulted in a partially antagonistic effect in 14 of the 25 isolates of Serratia studied (Table 3 and Fig. 2). A summary of the results of each of the three antibiotic combinations against the twenty-five isolates each of Pseudomonas, Klebsiella, and Serratia is listed in Table 4. The carbenicillin-vancomycin and cephalothin-vancomycin combinations had significantly more synergistic and partial synergistic activity against Pseudomonas isolates than the combination of carbenicillin and cephalothin, and the incidence of synergy and partial synergy was not significantly higher when vancomycin was combined with carbenicillin rather than cephalothin (Table 4). In contrast, the incidence of synergy and partial synergy was not significantly different among these three combinations of antibiotics when tested against Klebsiella isolates (Table 4). However, the carbenicillin-vancomycin and cephalothin-vancomycin combinations were also significantly more synergistic and partially synergistic against Serratia isolates than the combination of carbenicillin-cephalothin. In addition, the incidence of synergy and partial synergy was not significantly higher when vancomycin was combined with carbenicillin rather than cephalothin (Table 4). Further analysis comparing the carbenicillin-sensitive (0.625 to 15.6 ug/ml) and carbenicillin-resistant (2500 to 10,000 ug/ml) isolates of Serratia (Table 1) revealed no significant difference in the incidence of synergy and partial synergy for the carbenicillin-vancomycin combination. Thus, vancomycin and carbenicillin produced a similar incidence of synergy and partial synergy regardless of whether the Serratia isolates were sensitive or resistant to carbenicillin (Table 2). A significant difference ($p < 0.05$) however, was observed in the incidence of partial antagonism in the carbenicillin-cephalothin experiments (Table 3). The results show

TABLE 2
Minimal Inhibitory Concentrations of
Carbenicillin and Vancomycin Alone and in Combination
Against Twenty-five Strains of Serratia

| Strain No. | Carbenicillin ($\mu$g/ml) | Carbenicillin + Vancomycin Lowest MIC* | ($\mu$g/ml) | Vancomycin ($\mu$g/ml) |
|---|---|---|---|---|
| Synergy | | | | |
| 1009 | 2.5 | 0.312 | 2500 | >5000 |
| 1011 | 7.8 | 1.95 | 1250 | >5000 |
| 1122 | 15.6 | 1.95 | 625 | >5000 |
| 782 | 5000 | 156 | 1250 | 5000 |
| 798 | 10,000 | 156 | 1250 | 5000 |
| 800 | 10,000 | 156 | 625 | 5000 |
| 802 | 10,000 | 156 | 625 | 5000 |
| 980 | 5000 | 156 | 312 | >5000 |
| 996 | 5000 | 156 | 625 | 5000 |
| 1115 | 10,000 | 156 | 2500 | >5000 |
| 1117 | 10,000 | 156 | 2500 | >5000 |
| 1120 | 10,000 | 156 | 1250 | 5000 |
| Partial Synergy | | | | |
| 1125 | 5 | 2.5 | 312 | 2500 |
| 1126 | 5 | 2.5 | 78 | 1250 |
| 1127 | 7.8 | 3.9 | 625 | 2500 |
| 1130 | 7.8 | 1.95 | 1250 | 2500 |
| 698 | 2500 | 312 | 625 | 1250 |
| 720 | 5000 | 156 | 1250 | 2500 |
| 1121 | 10,000 | 156 | 2500 | 5000 |
| Additive | | | | |
| 756 | 2.5 | — | — | 2500 |
| 759 | 0.625 | — | — | 2500 |
| Indifferent | | | | |
| 758 | 0.625 | — | — | 1250 |
| 1124 | 2.5 | — | — | 1250 |
| 1128 | 2.5 | — | — | 2500 |
| 1123 | 2500 | — | — | 2500 |

*Lowest MIC listed is the lowest concentration of each antibiotic at which synergy or partial synergy can be seen. Carbenicillin-sensitive strains of Serratia are listed first in each category, followed by the carbenicillin-resistant strains.

that the carbenicillin-sensitive strains are significantly more likely to be antagonized by cephalothin than are the carbenicillin-resistant strains. This may simply reflect the marked differences in the concentration of carbenicillin in the two groups whereas the concentration of cephalothin in both groups was similar, that is, the concentration of cephalothin relative to the concentration of carbenicillin was much greater for the carbenicillin-sensitive strains than for the carbenicillin-resistant isolates of Serratia. This concentration difference may well facilitate any postulated interference of carbenicillin by cephalothin.

## DISCUSSION

The results of the present study demonstrate that the addition of vancomycin to either carbenicillin or cephalothin results in significant synergistic activity against isolates of Pseudomonas or Serratia as compared to the activity of the combination of carbenicillin and cephalothin. These observations would appear to support the theory that the potential for synergistic activity can be predicted for antibiotic

TABLE 3
Minimal Inhibitory Concentrations of
Carbenicillin and Cephalothin Alone and in Combination
Against Twenty-five Strains of Serratia

| Strain No. | Carbenicillin (μg/ml) | Carbenicillin + Cephalothin Highest MIC* | (μg/ml) | Cephalothin (μg/ml) |
|---|---|---|---|---|
| Partial Antagonism | | | | |
| 756 | 6.25 | 12.5 | 5000 | 10,000 |
| 758 | 1.56 | 12.5 | 5000 | 10,000 |
| 759 | 0.78 | 12.5 | 5000 | 10,000 |
| 1009 | 6.25 | 12.5 | 5000 | 10,000 |
| 1011 | 6.25 | 12.5 | 5000 | 10,000 |
| 1122 | 12.5 | 12.5 | 5000 | 10,000 |
| 1124 | 6.25 | 12.5 | 5000 | 10,000 |
| 1125 | 6.25 | 12.5 | 5000 | 10,000 |
| 1126 | 3.12 | 12.5 | 5000 | 10,000 |
| 1127 | 12.5 | 12.5 | 5000 | 10,000 |
| 1128 | 6.25 | 12.5 | 5000 | 10,000 |
| 698 | 1250 | 5000 | 2500 | 10,000 |
| 720 | 2500 | 5000 | 5000 | 10,000 |
| 782 | 2500 | 5000 | 5000 | 10,000 |
| Indifferent | | | | |
| 1130 | 25 | — | — | 10,000 |
| 798 | 10,000 | — | — | 10,000 |
| 800 | 10,000 | — | — | 5000 |
| 802 | 10,000 | — | — | 10,000 |
| 980 | 10,000 | — | — | 10,000 |
| 996 | 10,000 | — | — | 10,000 |
| 1115 | 10,000 | — | — | 10,000 |
| 1117 | 10,000 | — | — | 10,000 |
| 1120 | 10,000 | — | — | 10,000 |
| 1121 | 10,000 | — | — | 10,000 |
| 1123 | 10,000 | — | — | 10,000 |

*Highest concentration listed is the highest concentration of each antibiotic at which there is partial antagonism. For example, strain #756 has no inhibition of growth at 12.5 ug/ml of carbenicillin even though the minimal inhibitory concentration of carbenicillin alone is 6.25 ug/ml. Carbenicillin-sensitive strains are listed first in each category, followed by the carbenicillin-resistant strains.

combinations which block sequential sites, i.e., successive stages of the bacterial biosynthetic pathway [9]. The use of sequentially acting antibiotics to produce synergy has been observed previously with the combination of trimethoprim-sulfamethoxazole which blocks two successive stages of bacterial folic acid synthesis [10]. The present study was designed to evaluate sequential blockage of murein cell wall synthesis of some gram-negative bacilli although other biosynthetic pathways such as ribosomal protein synthesis, could have been investigated by other antibiotic combinations.

Each species of bacteria has its own particular cell wall composition. All gram-negative cell walls are complex in their composition, but all contain a murein cell wall component. In gram-negative bacteria, the murein component makes up about 10% of the dry weight of the cell wall. The murein component in all bacteria is made up of polymers of alternating N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) in a beta 1,4 linkage (Fig. 1). Attached to each NAM residue is a chain of a few (usually four) amino acids. These amino acid chains are cross-linked by transpeptidation to form the lattice work of the polymer chains that is the murein cell wall.

TABLE 4
The Effect of Antibiotic Combinations on
Twenty-five Isolates each of Pseudomonas, Klebsiella and Serratia

| Antibiotic Combination* | Synergy | Partial Synergy | Additive | Indifferent |
|---|---|---|---|---|
| Pseudomonas | | | | |
| C + V | 7† (28%) | 12 (48%) | 5 (20%) | 1 (4%) |
| K + V | 0 | 12 (48%) | 6 (24%) | 7 (28%) |
| C + K | 0 | 4 (16%) | 5 (20%) | 16 (64%) |
| Carbenicillin-vancomycin vs carbenicillin-cephalothin | | p<0.01 | | |
| Cephalothin-vancomycin vs carbenicillin-cephalothin | | p<0.05 | | |
| Carbenicillin-vancomycin vs cephalothin-vancomycin | | NS§ | | |
| Klebsiella | | | | |
| C + V | 0 | 12 (48%) | 7 (28%) | 6 (24%) |
| K + V | 2 (8%) | 17 (68%) | 6 (24%) | 0 |
| C + K | 2 (8%) | 14 (56%) | 7 (28%) | 2 (8%) |
| Carbenicillin-vancomycin vs carbenicillin-cephalothin | | NS | | |
| Cephalothin-vancomycin vs carbenicillin-cephalothin | | NS | | |
| Carbenicillin-vancomycin vs cephalothin-vancomycin | | NS | | |
| Serratia | | | | |
| C + V | 12 (48%) | 7 (28%) | 2 (8%) | 4 (16%) |
| K + V | 11 (44%) | 4 (16%) | 5 (20%) | 5 (20%) |
| | | Partial antagonism | | Indifferent |
| C + K | | 14 (56%) | | 11 (44%) |
| Carbenicillin-vancomycin vs carbenicillin-cephalothin | | p<0.01 | | |
| Cephalothin-vancomycin vs carbenicillin-cephalothin | | p<0.01 | | |
| Carbenicillin-vancomycin vs cephalothin-vancomycin | | NS | | |

*V = Vancomycin C = Carbenicillin K= Cephalothin
†Number of isolates
§NS = no significant difference

The general scheme of murein synthesis includes the following steps (Fig. 1): (1) N-acetyl muramic acid is attached to a uridine diphosphate carrier; (2) the amino acid chain is built upon the NAM residue to form the UDP-NAM-peptide unit. The peptide chain invariably has as its two terminal amino acids D alanine and D alanine; (3) the NAM-peptide unit is transferred from the UDP to a lipid diphosphate carrier; (4) an N-acetyl glucosamine unit is added to the NAM to form the lipid-NAG-NAM-peptide peptidoglycan subunit; (5) the peptidoglycan subunit is transferred from the lipid carrier inside the cell to the cell wall outside the cell; (6) the free amino acid side chains are cross-linked to each other through transpeptidation to form a network. In cross-linking, the D alanine at the end of the amino acid chain is hydrolyzed off the peptide. There is variation in the number and types of amino acids in the peptide side chains and the method of transpeptidation depending on the bacterial species. However, all the species studied have two D alanine residues as the terminal amino acids in the peptidoglycan subunits. These two D alanines are the key to the action of vancomycin.

There is good evidence that the penicillins and the cephalosporins both act at the same site in cell wall synthesis [11–13]. That site is the final transpeptidation of the peptide side chains. The site of vancomycin's action is not absolutely defined, but there is good evidence that vancomycin binds to the D-alanine configuration of cell wall subunit precursors [14–16] and inhibits the incorporation of fully formed

subunits into the cell wall [15] of both gram-negative and gram-positive species. A series of studies using isolated intermediates and artificially prepared peptides showed that vancomycin combined specifically with intermediates and peptides with the terminal D alanine D alanine configuration [14–16]. Artificially synthesized peptides with terminal D alanine D alanine will bind vancomycin very effectively and block vancomycin's *in vivo* effect on several bacterial species. The binding can be followed spectrophotometrically and is quite strong. There is detectable binding in 8 M urea, 4 M KCl, and 1% sodium dodecyl sulfate. The binding occurs at a pH range of four to nine. The binding also occurs with UDP-NAM-peptide, NAM-peptide, and the peptide alone. A precursor UDP-NAM-peptide without the terminal D alanine D alanine amino acids had no vancomycin binding. Vancomycin was also shown to be almost completely bound to the cell wall, with very little entering the cell itself [17]. In these studies, Nieto and Perkins elucidated the binding of vancomycin to both UDP-NAM-peptide and cell walls. Labelled vancomycin $I^{125}$ was used to study its binding activity with various substrates. Whole cells of *M. lysodeikticus* were used in 10 ug/ml vancomycin. Within three minutes 75% of all the labelled antibiotic was recoverable on the cell walls. When cell wall-free protoplasts were used, less than 2% of the vancomycin was taken up by the cells [17]. Further work [18] indicated that vancomycin does not act effectively at the penicillins or the cephalosporins site of action. Thus, there is good evidence that vancomycin acts *in vivo* to prevent the transfer of the NAG-NAM-peptide subunit from the lipid carrier to the cell wall and that this is the primary site of vancomycin action. Therefore, vancomycin is a reasonable choice for studies of sequential blockage of steps in a single pathway since it is known that penicillins and cephalosporins act by inhibiting the last step of cell wall synthesis, that of cross-linking the peptides, whereas vancomycin acts at a step prior to the action of both the penicillins and cephalosporins.

In the present studies, a representative penicillin, carbenicillin, was chosen because it is effective against most strains of Pseudomonas and some strains of Serratia, but usually ineffective against strains of Klebsiella. Cephalothin was chosen as the cephalosporin because it is usually effective against strains of Klebsiella and usually ineffective against strains of Pseudomonas and Serratia. Vancomycin was selected because it is ineffective against strains of Pseudomonas, Klebsiella, and Serratia. Furthermore, the experiments described in the present study used gram-negative bacteria in order to see whether the synergy achieved would be sufficient to clinically extend the use of vancomycin to gram-negative bacillary infections. The results of the present experiments support the model of sequential blockage as a basis for synergy. The incidence of synergy and partial synergy, observed when vancomycin and carbenicillin or vancomycin or cephalothin were combined against isolates of Pseudomonas and Serratia, was significantly higher than when carbenicillin and cephalothin were combined against these isolates. These observations suggest that under the conditions of the experiments, only those antibiotics which act sequentially produced a significant incidence of synergy and partial synergy. It is particularly interesting to note that there was no significant difference in the incidence of synergy and partial synergy in the Pseudomonas and Serratia studies regardless of whether the bacteria were sensitive or resistant to the drug added to vancomycin. The Pseudomonas isolates studied had essentially the same incidence of synergy and partial synergy with the combination of vancomycin and carbenicillin as with the combination of vancomycin and cephalothin. This was observed even though these Pseudomonas isolates were sensitive to carbenicillin and highly resistant to cephalothin. Further-

more, when the carbenicillin-sensitive and carbenicillin-resistant strains of Serratia were analyzed separately, there was no significant difference in the incidence of synergy between these two groups of organisms when tested against the carbenicillin-vancomycin combination. These data further support the model of sequential action regardless of whether the organisms are sensitive or resistant to one of the antibiotics. These observations are in contrast to those of Baltimore and colleagues [19] who noted that synergy against Klebsiella isolates was related to the susceptibility of the isolates to one of the antibiotics in the combination studied. We have no obvious explanation for this difference except to suggest that it may be related to the bacterial species.

In contrast to the results observed in the present study with isolates of Pseudomonas and Serratia, there was no significant difference in the incidence of synergy and partial synergy among the three combinations of antibiotics when tested against isolates of Klebsiella species. The incidence of synergy observed against Klebsiella with the combination of vancomycin and carbenicillin as well as with vancomycin and cephalothin was not significantly greater than that observed with the combination of carbenicillin and cephalothin. Since carbenicillin and cephalothin act at the same site in the pathway of cell wall biosynthesis, the model does not predict this degree of synergy with the combination. We have no clear explanation for this observation. Further work is needed to explain why there should be synergy and partial synergy with the carbenicillin-cephalothin combination when these antibiotics act at the same site. One possible explanation may lie in the prevalence of penicillin-degrading enzymes produced by gram-negative bacteria. Rollinson [20] showed that many enterobacteria produce various types of enzymes which degrade penicillin and would presumably degrade carbenicillin. If carbenicillin resistance in Klebsiella is conferred by an enzyme which degrades carbenicillin and if cephalothin interferes with this carbenicillin degradation, then the presence of cephalothin might increase the efficacy of carbenicillin and thus lead to synergy. Several studies [21–25] have shown that beta lactamase inhibitors such as oxacillin can inhibit enzymes that degrade penicillins and cephalosporins. These studies indicate that synergy could be obtained in many cases of gram-negative organisms which are resistant to penicillins or cephalosporins by combination of cephalosporins or penicillins with such beta lactamase inhibitors as oxacillin and methicillin. Since cephalothin can inhibit some lactamases, it is not unreasonable to suppose that it might inhibit certain enzymes produced by Klebsiella which degrade carbenicillin. Another possible explanation for the synergy observed against Klebsiella isolates, in the present study, with the carbenicillin-cephalothin combination may be the mechanism recently described by Poe [26]. In these latter studies sulfonamides were shown to be moderately potent inhibitors of bacterial dihydrofolate reductase and provide some basis for a new theory of synergy. Specifically, the mechanism for the synergistic activity observed with the combination of trimethoprim and sulfamethoxazole has been thought to be based on sequential inhibition of the bacterial cell with trimethoprim inhibiting dihydrofolate reductase and sulfamethoxazole competing with para-aminobenzoic acid. Poe's [26] experiments, however, suggest that synergistic dose-response curves can be obtained in vitro by the action of two inhibitors on a single enzyme, i.e., multiple simultaneous inhibitors of bacterial dihydrofolate reductase by the sulfonamides and trimethoprim acting together. The possibility exists that carbenicillin and cephalothin may produce a synergistic effect against Klebsiella by multiple simultaneous inhibition of transpeptidation. However, we did not see a similar effect with this

combination when tested against Pseudomonas or Serratia isolates, nor did we do any experiments to investigate this possible mechanism of action against Klebsiella isolates.

Although the present observations support, in general, the hypothetical model of antibiotic synergy based on sequential blocking of one biochemical pathway, another mechanism of synergy must also be considered. Namely, one of the antibiotics in the combination might increase the penetration into the cell of the other antibiotic. This mechanism has been shown to be responsible for the antibiotic synergy observed with the combination of penicillin and streptomycin against enterococci [26]. However, this explanation is not likely to be responsible for the present observations because earlier studies by Perkins and Nieto [17], demonstrated that the uptake of vancomycin into the cytoplasm of bacteria without cell walls is only slightly greater than the uptake of vancomycin by bacteria with complete cell walls. Finally, although the synergistic and partially synergistic combinations developed in the present study require concentrations of vancomycin which are not clinically achievable in man, the results observed do support the model of sequential action. This theoretical basis of action can predict synergy and therefore can be used to investigate both old and new antibiotics in an attempt to develop synergistic combinations which may in the future be clinically useful.

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