Studies on Antibiotic Synergism Against Enterococci

II. EFFECT OF VARIOUS ANTIBIOTICS ON THE UPTAKE OF
14C-LABELED STREPTOMYCIN BY ENTEROCOCCI

ROBERT C. MOELLERING, JR. and ARNOLD N. WEINBERG

*From the Infectious Disease Unit and Department of Medicine, Massachusetts
General Hospital and Harvard Medical School, Boston, Massachusetts 02114*

A B S T R A C T The mechanism by which agents that in-
hibit bacterial cell wall synthesis produce a synergistic
effect against enterococci when combined with amino-
glycoside antibiotics has not been elucidated. Using 14C-
labeled streptomycin, it could be shown that uptake of
this aminoglycoside antibiotic was markedly enhanced
in enterococci growing in the presence of penicillin or
other agents which inhibit the synthesis of bacterial cell
walls. There was no enhancement of streptomycin uptake
when the cells were incubated with antibiotics which pri-
marily affect the bacterial cell membrane or inhibit pro-
tein synthesis. Increased streptomycin uptake was pro-
duced by penicillin only in actively growing bacteria.
These observations are consistent with the hypothesis
that enterococci exhibit a natural barrier to the entry
of streptomycin which can be overcome by agents which
inhibit cell wall synthesis, thus producing a synergistic
effect.

INTRODUCTION

The fact that penicillin and streptomycin can act syner-
gistically against enterococci is well known (1, 2). As
a result, this combination has been extensively used in
the treatment of enterococcal infections (3). The mecha-
nism of penicillin-streptomycin synergism, however, has
not been elucidated (4). Our previous studies have shown
that antibiotics which inhibit bacterial cell wall forma-
tion (irrespective of the synthetic step blocked) pro-
duce synergism against enterococci when used in com-
bination with streptomycin or other aminoglycoside anti-

biotics (5). On the basis of these studies we suggested
that enterococci are relatively impermeable to amino-
glycoside antibiotics, and that the permeability barrier
can be breached by agents which inhibit bacterial cell
wall synthesis. To test the validity of that hypothesis,
we have studied the uptake of 14C-labeled streptomycin by
enterococci in the presence of various antibiotics.

METHODS

Organisms. The organism used for the initial studies
was an enterococcus (*Streptococcus faecalis var. liquefaciens,*
designated strain EI) obtained from a patient with endo-
carditis, and has been described in detail elsewhere (5).
The minimal inhibitory concentration (MIC)[1] of penicillin
for this organism was 1 U/ml and the minimal bactericidal
concentration (MBC) was > 1000 U/ml. The MIC of
streptomycin was 400 μg/ml and the MBC was 800 μg/ml.
Penicillin and streptomycin in combination were synergistic
against this organism (5). A spontaneous mutant to very
high level streptomycin resistance (MIC, MBC > 5000
μg/ml, designated strain EI^R) was also studied. Penicillin
and streptomycin failed to act synergistically against this
mutant (5).

Antibiotics. 14C-labeled streptomycin (Streptomycin-Ca-
Cl$_2$, specific activity 0.054 μCi/mg) was a gift from Dr. C.
Rosenblum of Merck Sharp & Dohme, West Point, Pa.
Unlabeled antibiotics were supplied to us as follows: cy-
closerine, erythromycin (Eli Lilly and Company); chlor-
amphenicol (Parke, Davis & Company); penicillin (Pfizer
Laboratories Division, Pfizer, Inc.); bacitracin (Pfizer
Laboratories Division and The Upjohn Company); strep-
tomycin (E. R. Squibb & Sons); colistin (Warner-Chilcott
Laboratories).

Determination of streptomycin uptake. 1 ml of an over-
night nutrient broth culture of the test organism was added
to 14 ml of dextrose-phosphate broth (Albimini) in a 250
ml flask and preincubated at 37°C for 90 min to allow the
organisms to reestablish rapid ("log phase") growth. At
the end of this period, appropriate antibiotics were added;

[1] *Abbreviations used in this paper:* MBC, minimal bac-
tericidal concentration; MIC, minimal inhibitory concen-
tration.

and after mixing, 1 ml samples were removed for counting and for protein determination. The flasks were then reincubated at 37°C and additional 1 ml samples were removed for counting and protein determination at varying intervals (usually 15, 30, 60, 90, 120, and 180 min for counting and 90 and 180 min for protein determinations).

The samples for counting were filtered through a 25 mm membrane filter (Millipore, HA 0.45 μ, Millipore Corp.). The samples were washed with 15 ml of distilled H_2O (or in certain instances as noted in the Results, 15 ml of unlabeled streptomycin solution, 2 mg/ml) and the filters dried. Filters were then placed in counting vials, with 0.2 ml distilled H_2O, and 10 ml Buhlers solution (6) added; and the vials were counted for at least 30 min (1000–4300 counts) on a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co.). The data were corrected for background counts and for blank values obtained by filtering and washing medium containing labeled streptomycin but no bacteria. All counts (except an occasional time 0 determination) were twice background or more. Samples for protein determination were washed three times in 5 ml volumes of distilled water and protein concentrations measured using the Lowry micromethod (7).

Streptomycin uptake by bacteria was expressed as micrograms streptomycin per milligram protein.

RESULTS

When streptomycin-^{14}C was added to a culture of growing bacteria, there was a rapid initial uptake of antibiotic by the cells, which was present at the time of first sampling, immediately after adding streptomycin. This is shown in Fig. 1. After the initial uptake, the amount of streptomycin-^{14}C bound to the bacteria remained approximately constant for the duration of the experiment. When penicillin and streptomycin were added simultaneously, the rapid initial uptake also oc-
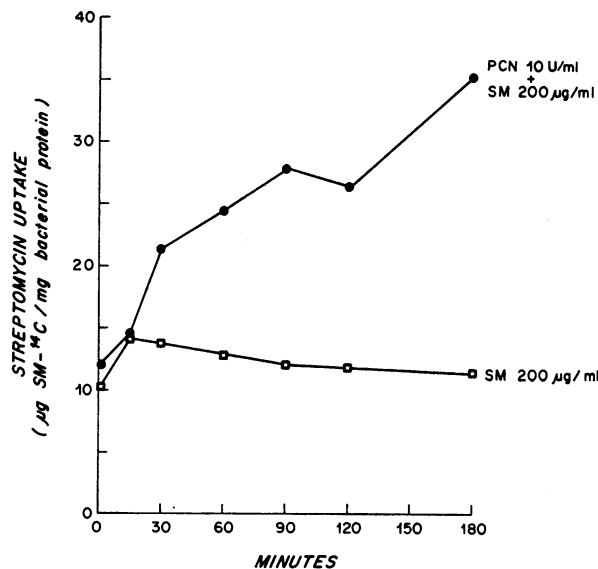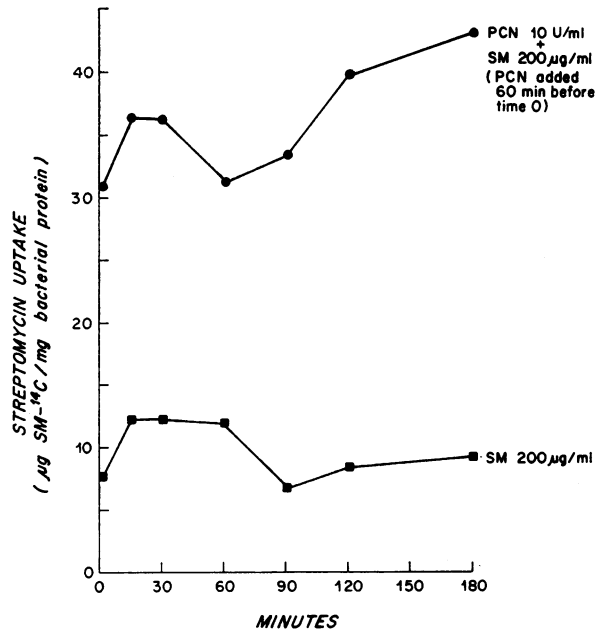
FIGURE 2 Effect of pretreatment with penicillin (PCN) on uptake of streptomycin-^{14}C (SM) by *S. faecalis* strain EI.

curred, but, in addition, a marked secondary uptake of streptomycin was noted. This began approximately 30 min after the addition of the antibiotics and progressed so that after 180 min the amount of streptomycin-^{14}C taken up by the bacteria in the presence of penicillin was more than three times that bound in the absence of penicillin (Fig. 1).

FIGURE 1 Effect of penicillin (PCN) on uptake of streptomycin-^{14}C (SM) by *S. faecalis* strain EI.
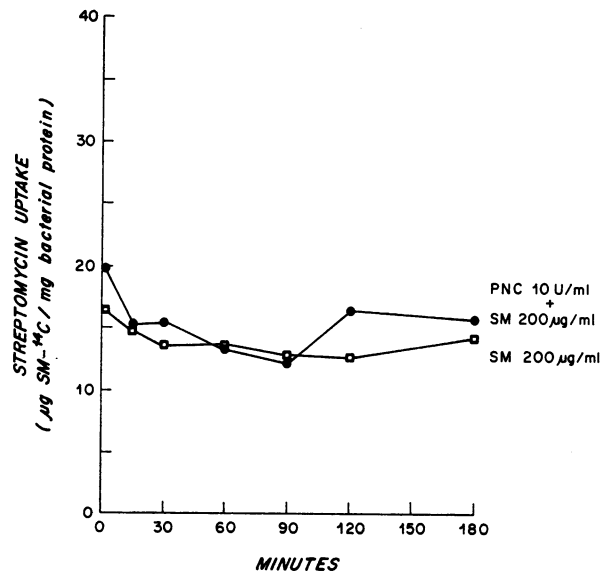
FIGURE 3 Effect of penicillin (PNC) on uptake of streptomycin-^{14}C (SM) by nondividing (post "log phase") *S. faecalis* strain EI.
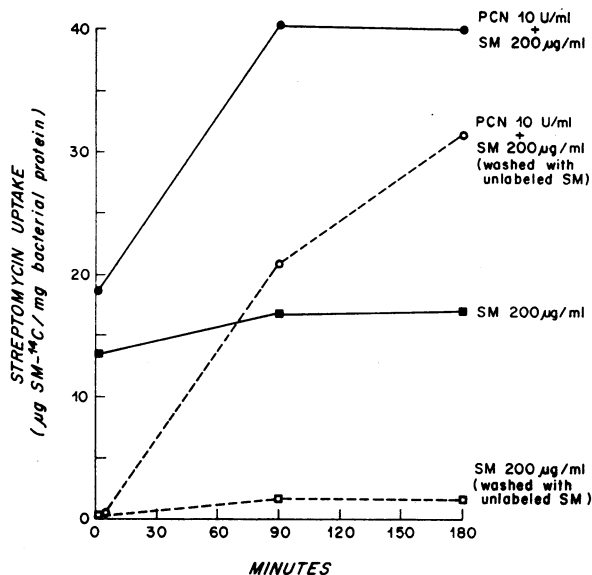
FIGURE 4 Uptake of streptomycin-^{14}C (SM) by *S. faecalis* strain EI in the presence and absence of penicillin (PCN): effect of washing with unlabeled streptomycin.

If the growing bacteria were pretreated with penicillin by adding this antibiotic 1 hr before adding streptomycin-^{14}C, there was no delay in "secondary uptake" of streptomycin-^{14}C. Indeed, there was a marked increase in uptake at the time of initial sampling, as shown in Fig. 2.

To insure that the increased uptake of streptomycin-^{14}C in the presence of penicillin was not due to a nonspecific increase in streptomycin affinity of the cells
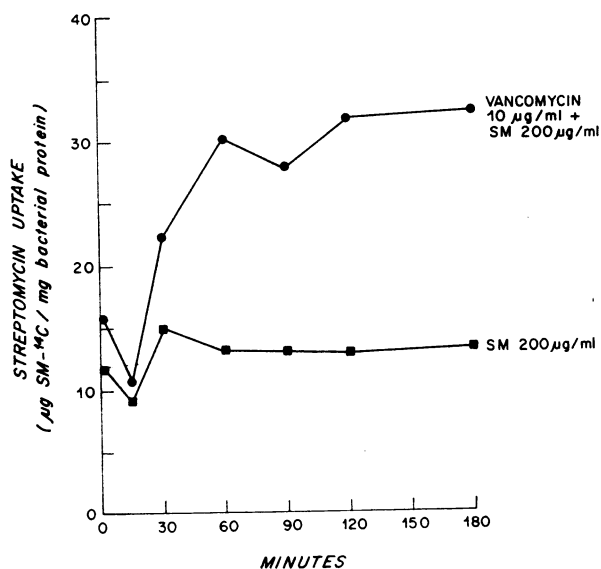
caused merely by the physical presence of penicillin, the experiments were repeated using nondividing ("post log phase") enterococci. As shown in Fig. 3, penicillin produced no increase in streptomycin uptake in nondividing cells.

To learn the reason for the rapid initial uptake of streptomycin by enterococci, irrespective of the presence of penicillin, unlabeled streptomycin was used to wash the cells. When the filtered bacteria were washed with unlabeled streptomycin instead of distilled water, virtually all of the streptomycin-^{14}C bound in the absence of penicillin could be removed as shown in Fig. 4. There was also a decrease in amount of labeled aminoglycoside bound in the presence of penicillin; but as can also be noted in Fig. 4, a significant amount of the drug remained associated with the penicillin-treated cells despite the streptomycin wash.

Our previous studies (5) have shown that other antibiotics which inhibit cell wall synthesis, including cycloserine, bacitracin, and vancomycin, produced synergism when each was used in combination with streptomycin against enterococcus strain EI. Each of these antibiotics produced an increased uptake of streptomycin-^{14}C when substituted for penicillin in the present test system. Fig. 5 shows the results of an experiment utilizing vancomycin. Cycloserine and bacitracin (not shown) yielded similar findings.

Neither chloramphenicol, erythromycin, nor colistin caused an increased uptake of labeled drug when used in combination with streptomycin-^{14}C.

Penicillin and streptomycin did not demonstrate synergism against a mutant enterococcal strain (EI^R)

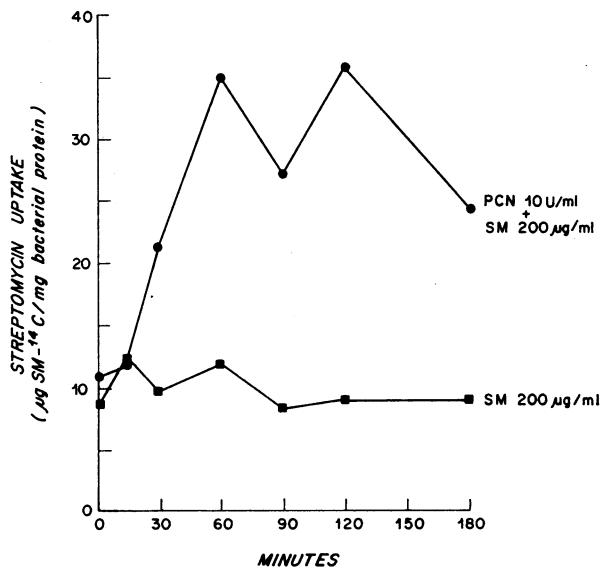FIGURE 5 Effect of vancomycin on uptake of streptomycin-^{14}C (SM) by *S. faecalis* strain EI.

FIGURE 6 Effect of penicillin (PCN) on uptake of streptomycin-^{14}C (SM) by *S. faecalis* strain EI^R, a mutant exhibiting very high level streptomycin resistance.

2582   R. C. Moellering, Jr. and A. N. Weinberg

which exhibited very high level resistance to streptomycin (MIC, MBC > 5000 μg/ml) (5). When this strain was substituted for the parent strain in the test system (Fig. 6), penicillin again caused an increase in streptomycin-^{14}C uptake, despite the fact that increased killing did not occur.

DISCUSSION

Although penicillin and streptomycin have been widely used to treat infections caused by enterococci (1–3), the mechanism whereby these antibiotics produce a synergistic effect has not been definitely worked out (4). After an extensive investigation of this phenomenon, Hewitt, Seligman, and Deigh suggested that penicillin treatment resulted in the formation of enterococcal L forms which were more sensitive to streptomycin than their parent forms (8). Several other investigators have likewise shown that enterococcal L forms show enhanced susceptibility to aminoglycoside antibiotics (9,10). However, there is no concrete evidence that a significant number of L forms are produced in vivo when enterococci are treated with penicillin. Furthermore, synergism occurs when growth experiments are performed in routine liquid media which do not contain the osmotic protection necessary for the survival of L forms; and neither erythromycin nor tetracycline (to which L forms are usually sensitive in vitro) produces synergism with penicillin against enterococci (5). Therefore our data suggest that synergism can occur in the absence of L form production.

Our previous studies (5) have suggested that the resistance of enterococci to streptomycin may be related to the fact that these organisms exhibit a natural permeability barrier to aminoglycoside antibiotics. This barrier can be overcome by using very high concentrations of streptomycin (5). We have also shown that all antibiotics which inhibit bacterial cell wall synthesis, irrespective of the synthetic step blocked, produce synergism with streptomycin against enterococci. Antibiotics which affect the bacterial cell membrane or inhibit protein synthesis fail to produce synergism with streptomycin. These studies therefore suggested that synergism occurs when penicillin or other agents which inhibit bacterial cell wall synthesis breach a natural barrier (presumably the cell wall), allowing streptomycin to enter and kill the bacteria.

The experiments reported here confirm this hypothesis. The rapid, initial uptake of streptomycin-^{14}C by enterococci in the absence of other agents almost certainly represented nonspecific adsorption to the cell surface since it was easily and completely removed by washing with unlabeled streptomycin. The lack of a spontaneous secondary uptake of streptomycin such

as has been shown to occur in *Escherichia coli* in the absence of penicillin (11) further suggests that enterococci are relatively impermeable to streptomycin. Penicillin and other agents which inhibit bacterial cell wall synthesis (cycloserine, bacitracin, vancomycin) clearly overcame that permeability barrier and allowed a marked secondary uptake of streptomycin. The fact that much of this streptomycin-^{14}C could not be washed out with unlabeled streptomycin suggests that it was in an intracellular location or was bound in such a way that it was not easily accessible to displacement by the streptomycin wash.

The increased uptake of streptomycin in the presence of penicillin occurred only in actively dividing cells. This is further indirect evidence that formation of cell walls and inhibition thereof by penicillin is necessary for increased uptake of streptomycin to occur. It also shows that the increased uptake is not simply due to a nonspecific increase in binding of streptomycin by cells due to the mere physical presence of penicillin. As predicted from our previous experiments (5), agents which do not affect cell wall synthesis failed to enhance the uptake of streptomycin by enterococci.

The increased uptake of streptomycin-^{14}C in the presence of penicillin by a highly streptomycin resistant ("nonsynergistic") enterococcal strain is of interest. This suggests that failure of synergism in this strain was not due to failure of increased streptomycin uptake, but rather to inability of the streptomycin to act once it entered the cell. Recent studies in our laboratory have shown that streptomycin failed to cause misreading or to inhibit amino acid incorporation when ribosomes from the "nonsynergistic" strain were tested in an in vitro system (12). This explains the failure of synergism of penicillin and streptomycin against the above, and other strains of enterococci which exhibit very high level resistance to streptomycin (13).

On the basis of these experiments we cannot state with absolute certainty that the formation of L forms plays no role in antibiotic synergism against enterococci. However, if such forms are produced, it seems likely that they too will be more permeable to streptomycin. This almost certainly accounts for the previous observations that enterococcal L forms are more sensitive to aminoglycoside antibiotics than their parent strains (9, 10).

It is not yet clear whether the phenomenon we have described is limited to enterococci. However, Plotz and Davis showed in 1962 that penicillin could increase the rate of uptake of streptomycin-^{14}C in a strain of E. coli, an effect that they attributed to damage of the cell membrane by penicillin (14). This suggests that enhanced uptake of aminoglycoside antibiotics may be induced in bacteria other than enterococci by agents

which inhibit cell wall synthesis. If this proves to be the case, it could have significant application in the treatment of infections caused by organisms which are resistant to a large number of antibiotics when used alone.

ACKNOWLEDGMENTS

REFERENCES

1. Dowling, H. F. 1965. Present status of therapy with combinations of antibiotics. *Amer. J. Med.* **39**: 796.
2. Jawetz, E. 1968. Combined antibiotic action: some definitions and correlations between laboratory and clinical results. *Antimicrob. Agents Chemother.* 203.
3. Mandell, G. L., D. Kaye, M. E. Levison, and E. W. Hook. 1970. Enterococcal endocarditis.. *Arch. Intern. Med.* **125**: 258.
4. Sabath, L. D. 1968. Synergy of antibacterial substances by apparently known mechanisms. *Antimicrob. Agents Chemother.* 210.
5. Moellering, R. C., Jr., C. Wennersten, and A. N. Weinberg. 1971. Studies on antibiotic synergism against enterococci. I. Bacteriologic studies. *J. Lab. Clin. Med.* **77**: 821.
6. Buhler, D. R. 1962. A simple scintillation counting technique for assaying $C^{14}O_2$ in a Warburg flask. *Anal. Biochem.* **4**: 413.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265.
8. Hewitt, W. L., S. J. Seligman, and R. A. Deigh. 1966. Kinetics of the synergism of penicillin-streptomycin and penicillin-kanamycin for enterococci and its relationship to L-phase variants. *J. Lab. Clin. Med.* **67**: 792.
9. Shockman, G. D., and J. O. Lampen. 1962. Inhibition by antibiotics of the growth of bacterial and yeast protoplasts. *J. Bacteriol.* **84**: 508.
10. Montgomerie, J. Z., G. M. Kalmanson, and L. B. Guze. 1968. The susceptibility of protoplast and bacterial forms of *Streptococcus faecalis* to antibiotics. *In* Microbial Protoplasts, Spheroplasts, and L-forms. L. B. Guze, editor. Williams & Wilkins Co., Baltimore. 306.
11. Anand, N., B. D. Davis, and A. K. Armitage. 1960. Uptake of streptomycin by *Escherichia coli. Nature (London).* **185**: 23.
12. Zimmermann, R. A., R. C. Moellering, Jr., and A. N. Weinberg. 1971. Mechanism of resistance to antibiotic synergism in enterococci. *J. Bacteriol.* **105**: 873.
13. Standiford, H. D., J. B. deMaine, and W. M. M. Kirby. 1970. Antibiotic Synergism of Enterococci. *Arch. Intern. Med.* **126**: 255.
14. Plotz, P. H., and B. D. Davis. 1962. Synergism between streptomycin and penicillin: a proposed mechanism. *Science (Washington).* **135**: 1067.