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SOME DIFFERENCES IN THE ACTION OF PENICILLIN, BACITRACIN, AND VANCOMYCIN ON *BACILLUS MEGATERIUM*

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ABSTRACT

HANCOCK, R. (Harvard Medical School, Boston, Mass.), AND P. C. FITZ-JAMES. Some differences in the action of penicillin, bacitracin, and vancomycin on *Bacillus megaterium*. J. Bacteriol. 87:1044-1050. 1964.—Penicillin and cycloserine do not inhibit the growth of protoplasts of *Bacillus megaterium*, indicating that inhibition of cell-wall synthesis is the only significant process by which they inhibit growth of bacteria. In contrast, bacitracin and vancomycin inhibit growth of protoplasts and bacteria at similar concentrations, indicating that they have important sites of action other than their known inhibition of cell-wall synthesis. At concentrations which inhibit mucopeptide synthesis, penicillin, bacitracin, and vancomycin each cause an increased rate of efflux of K ions from growing bacteria. This effect of penicillin is prevented by chloramphenicol or hypertonic sucrose, whereas the effects of bacitracin and vancomycin are unchanged under these conditions. It is concluded that bacitracin and vancomycin have direct effects on the cytoplasmic membrane, and it is proposed that their inhibition of cell-wall synthesis could be a consequence of these effects. Bacitracin and vancomycin do not compete with penicillin for binding to cells of *B. megaterium*, a further indication that they have a different primary site of action.

A number of antibiotics, including penicillin, bacitracin, cycloserine, vancomycin, and novobiocin, inhibit synthesis of the mucopeptide polymer of bacterial cell walls (reviewed by Davis and Feingold, 1963). Because these agents appear to have little structural resemblance to each other, except for the thiazoline rings of penicillin and bacitracin, their effects on mucopeptide synthesis may well involve different mechanisms. They each have additional effects on sensitive bacteria (Prestidge and Pardee, 1957; Smith and Weinberg, 1962; Reynolds, 1962; Brock, 1962), and it is therefore possible that inhibition of cell-wall synthesis is not their primary or most important action.

To determine whether inhibition of mucopeptide synthesis can account completely for the growth-inhibitory action of these antibiotics, we compared their effects on the growth of bacteria and protoplasts under similar conditions. Because the growth of protoplasts does not depend on the integrity of a cell wall, it should not be affected by agents which only block cell-wall synthesis. We describe here the results of experiments with *Bacillus megaterium;* during this work, Shockman and Lampen (1962) reported similar results with *Streptococcus faecalis*. Our observations suggest that bacitracin and vancomycin affect the cytoplasmic membrane directly, and we confirmed this finding by measuring their effects on the rate of efflux of K ions from cells. We also investigated the effect of these antibiotics on the binding of penicillin.

MATERIALS AND METHODS

Organism and growth. B. megaterium strain KM was used. To grow bacteria for preparation of protoplasts, the medium and conditions of Fitz-James (1958) were used; for all other purposes, bacteria were grown in medium A (Davis and Mingioli, 1950) containing glucose (0.2%) and other supplements as indicated. Cultures of up to 30-ml volume were grown in 150-ml Erlenmeyer flasks on a Gyrotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 34 C. Growth was followed by determining optical density at 490 mμ in a Lumetron colorimeter; antibiotics were added, after at least two generations of exponential growth, at an optical density of 1.0 to 1.2, corresponding to ca. 2×10^7 bacteria per ml.

The methods and media described by Fitz-James (1958) were used for preparation and growth of protoplasts. Protoplasts were formed in sucrose-phosphate medium [0.3 M sucrose, 0.1 M phosphate (pH 6.1), 0.016 M $MgSO_4$] by addition of lysozyme (200 μg/ml), and were washed and resuspended in the same medium.

Samples of the suspension were added to 10 ml of growth medium (0.5 M Na_2 succinate, 0.1 M KCl, 10^{-3} M $MgSO_4$, 10^{-4} M $MnSO_4$, 10 mg/ml of enzymatic casein hydrolysate, 0.6 mg/ml of hexose diphosphate; pH 6.5) in 150-ml Erlenmeyer flasks, and were shaken at ca. 100 rev/min on a Gyrotary shaker at 34 C. The initial optical density was ca. 0.4, and antibiotics were added when the optical density reached 0.6. Because antibiotic-treated protoplasts occasionally agglutinated, optical density was not a completely reliable index of growth; hence, the incorporation of C^{14}-leucine into protein was measured, as described below, as an independent estimate of protoplast growth. To compare the effects of antibiotics on the growth of bacteria, the same media and conditions were used.

Inhibition of growth was expressed by an arbitrary parameter representing the increase in optical density after 2 hr in an antibiotic-treated culture relative to that in the control culture; for protoplasts, a similar parameter was also derived from the amount of C^{14}-leucine incorporated.

To separate bacteria from growth medium and to filter material precipitated with trichloroacetic acid, Millipore filters (type HA, 0.45-μ pores) were used; 24-mm diameter filters were used for small samples and for radioactivity determinations, and 60-mm diameter filters for rapid filtration of larger volumes.

Measurement of efflux of K. The procedure was similar to that described by Dubin and Davis (1961). Bacteria were grown for at least two generations in medium containing K^{42} (ca. 0.25 μc/ml). At an optical density of about 1.2, the cells were filtered and washed twice with 5 ml of medium; the filter was transferred to a petri dish, and the bacteria were rapidly resuspended by pipetting prewarmed unlabeled growth medium onto the filter. Samples of the suspension were distributed into flasks containing additions as required and shaken at 37 C. Samples (1 ml) were filtered immediately and at intervals of 4 to 5 min subsequently; each filter was immediately washed twice with 2.5 ml of medium and dried for radioactivity determinations.

Measurement of mucopeptide and protein synthesis. To measure synthesis of cell-wall mucopeptide, the incorporation of C^{14}-diaminopimelic acid (DAP) into cold trichloroacetic acid-precipitable material was determined. Bacteria were grown in medium supplemented with DAP (20 μg/ml); lysine (100 μg/ml) was added to suppress incorporation of C^{14}-lysine formed from C^{14}-DAP. 2-C^{14}-DAP (Calbiochem; 0.01 μc/ml) was added immediately before addition of antibiotic. Under these conditions, over 95% of the incorporated radioactivity was found in the mucopeptide fraction prepared by the procedure of Park and Hancock (1960).

To measure protein synthesis in protoplast cultures, C^{14}-leucine (New England Nuclear Corp., Boston, Mass.) was added to the culture to a concentration of 0.05 μc/ml immediately before addition of antibiotic. (Excess unlabeled leucine was present in the medium.)

To determine incorporated radioactivity, 1-ml samples of culture were pipetted at intervals into an equal volume of 10% trichloroacetic acid on ice. The precipitates were filtered and washed once with 5% trichloroacetic acid containing unlabeled DAP or leucine (100 μg/ml); the dried filters were glued to planchets and their radioactivity was determined in a thin-window gas-flow counter.

When K^{42} and C^{14} were present in the same samples, they were differentiated by determining radioactivity before and after decay of the K^{42}.

Measurement of binding of penicillin. H^3-benzyl penicillin (Radiochemical Centre, Amersham, England; 3 μc/μg) was added to 25-ml portions of an exponentially growing culture at an optical density of 1.3. After growth for 5 min at 37 C, an excess of unlabeled benzyl penicillin (2.5 mg/ml) was added to stop further binding and displace reversibly bound penicillin; the bacteria were immediately harvested by centrifugation and washed once by resuspension for 5 min in medium containing unlabeled benzyl penicillin (1 mg/ml). After centrifugation, the pellet was suspended in 0.5 ml of phosphate buffer (0.05 M, pH 7) containing lysozyme (1 mg/ml) and deoxyribonuclease (20 μg/ml) for 1 hr at 37 C; 2 drops of 1 M $NaHCO_3$ and 500 μg of trypsin were then added, and the protein was digested for 1 hr at 37 C. The sample was incubated for 2 hr at 50 C with 1 ml of methanolic "Hyamine" hydroxide (Packard Instrument Co., LaGrange, Ill.); 10 ml of scintillant solution (Bray, 1960) were added, and, after transfer to counting vials and cooling, radioactivity was determined in a liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill).

Antibiotics. The preparations used were penicillin G (Squibb 2D 73720), vancomycin HCl (Lilly 1112-792298 UD), bacitracin (Pfizer

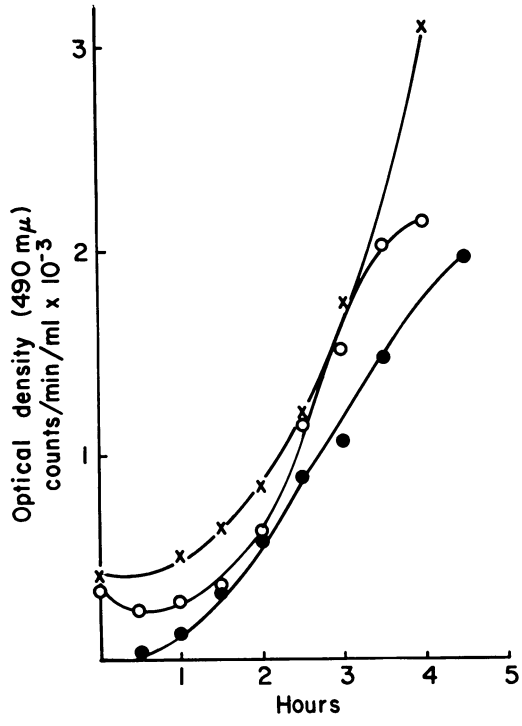

FIG. 1. *Growth of bacteria* (×) *and protoplasts* (○) *measured by optical density, and incorporation of* C^{14}*-leucine* (●) *into growing protoplasts.*

18081), cycloserine (Calbiochem), and novobiocin (Upjohn LM-443).

RESULTS

Some characteristics of the growth of protoplasts under the conditions used here are shown in Fig. 1. There was an initial decrease in optical density on transfer of protoplasts to growth medium, followed by an increase of ca. tenfold, which was usually linear but more approximately exponential in some experiments; this growth is not accompanied by division (Fitz-James, 1958).

Effects of antibiotics on growth of bacteria and protoplasts. Penicillin and cycloserine had no inhibitory effect on the growth of protoplasts at concentrations up to 30 times those which inhibit growth of bacteria (Fig. 2). By contrast, the inhibitory concentrations of bacitracin, vancomycin, and novobiocin for protoplasts were similar to those for bacteria (Fig. 3). Protoplasts treated with bacitracin and vancomycin at inhibitory concentrations lysed after 2 to 3 hr, and phase-contrast microscopy showed many fragments of protoplast membranes.

Effects of antibiotics on K efflux. The lytic action of bacitracin and vancomycin on protoplasts indicates that they eventually cause major

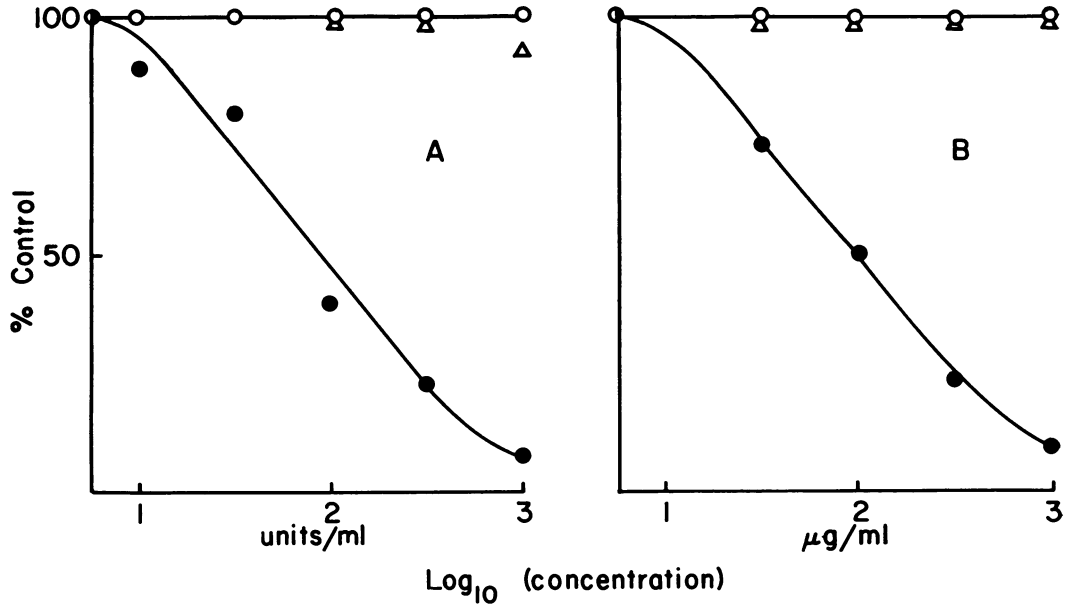

FIG. 2. *Effects of penicillin* (*A*) *and cycloserine* (*B*) *on optical density increase of bacteria* (●) *and of protoplasts* (○) *under the same conditions, and on incorporation of* C^{14}*-leucine by protoplasts* (△). *Conditions are described in the text.*

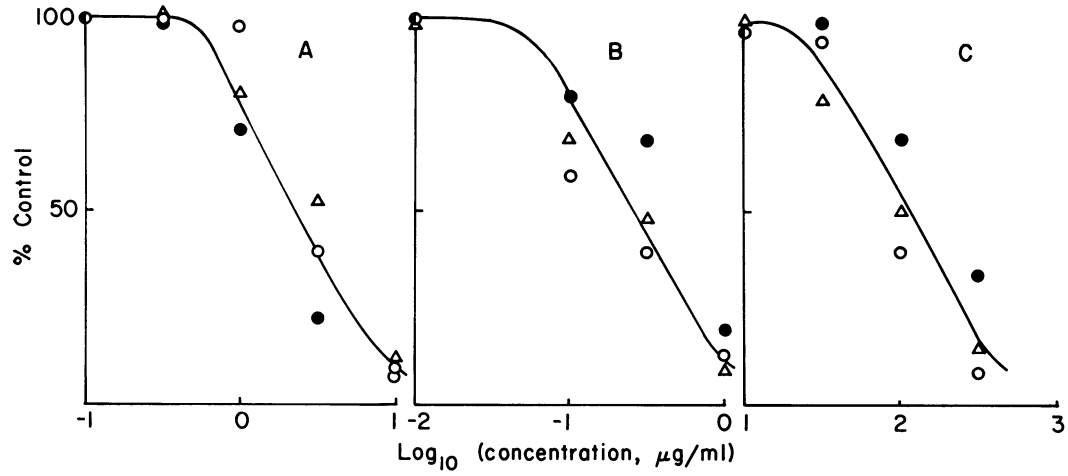

FIG. 3. *Effects of vancomycin (A), novobiocin (B), and bacitracin (C) on bacteria and protoplasts; the symbols used are the same as those in Fig. 2.*

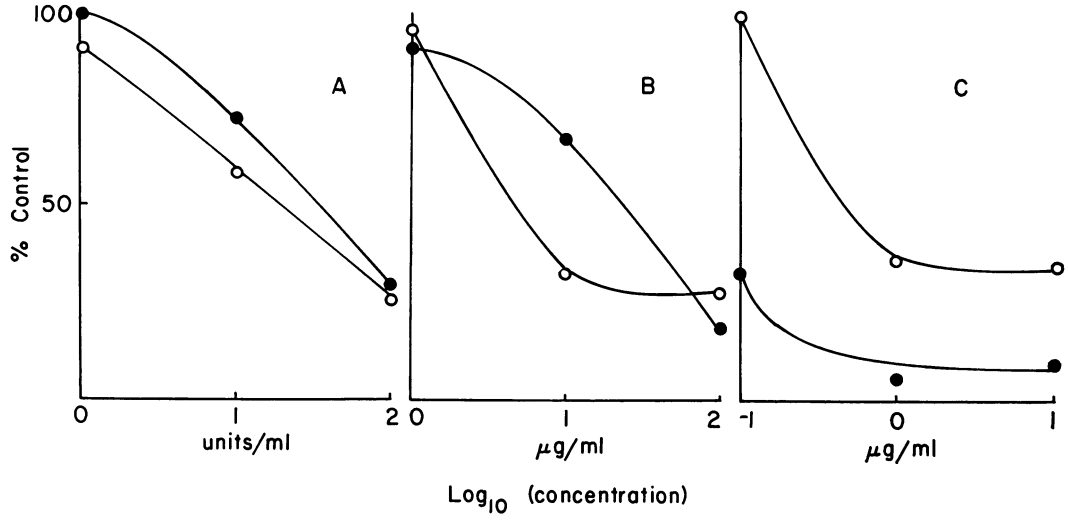

FIG. 4. *Effects of penicillin (A), bacitracin (B), and vancomycin (C) on efflux of K^{42} and incorporation of C^{14}-DAP in growing bacteria. Antibiotics were added at an optical density of 1.05. The residual cellular K^{42} (●) and the amount of C^{14}-DAP incorporated (○) in the same culture 15 min after addition of antibiotic are expressed relative to the values for a control culture.*

changes in the protoplast membrane. To see whether earlier and more subtle changes in membrane function could be detected, we investigated the effect of these drugs on the efflux of K ions from bacteria prelabeled by growth in medium containing K^{42}; their effect on mucopeptide synthesis was measured simultaneously. Although the nature of the two parameters studied (residual K^{42} and C^{14}-DAP incorporation) does not allow precise quantitative comparisons, penicillin, bacitracin, and vancomycin each caused an increased K efflux from growing bacteria at drug concentrations within the range inhibiting mucopeptide synthesis (Fig. 4). K efflux appeared to be more sensitive to vancomycin than was mucopeptide synthesis.

One might expect that such an effect on K efflux could be due either to a direct effect on the membrane or to secondary membrane changes caused by lesions in the supporting cell wall. To

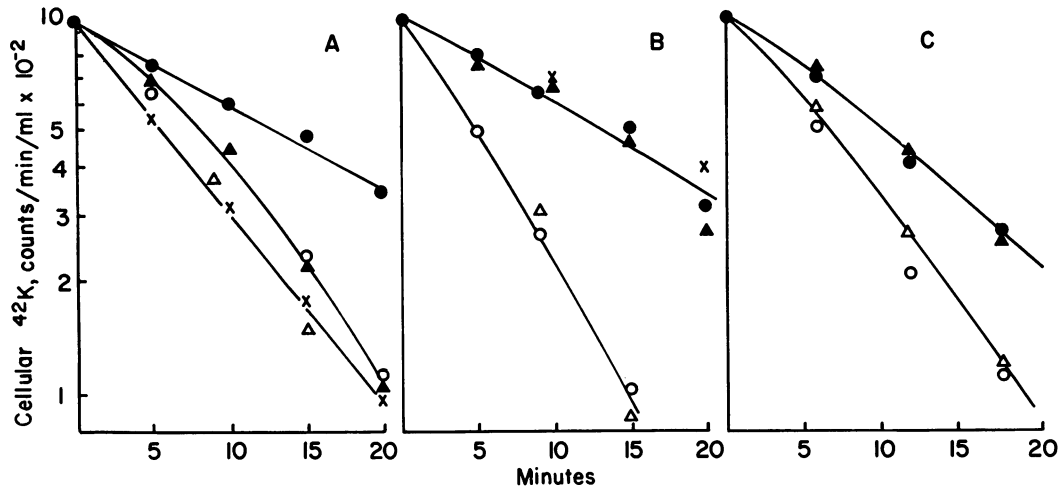

FIG. 5. *Efflux of K^{42} from bacteria after addition at zero time of penicillin (100 units per ml, ▲); cyclo serine (100 μg/ml, ×); bacitracin (30 μg/ml, △); or vancomycin (30 μg/ml, ○); and from cells in contro cultures without antibiotic (●). In A, the bacteria were in normal medium. In B, chloramphenicol (100 μg/ml) was added 15 min before addition of antibiotic and was present during the experiment. In C, the medium contained sucrose (0.3 M) before and after addition of antibiotic.*

distinguish between these possibilities, the effect of each antibiotic on K efflux was measured in media containing chloramphenicol or hypertonic sucrose. Because chloramphenicol would prevent differential increase of cell protein relative to cell wall, it would be expected to protect against membrane effects secondary to wall lesions, but not against a direct action on the nongrowing membrane. Sucrose would give similar protection by providing osmotic support to replace the mechanical protection provided by the cell wall. Such experiments showed that prior addition of chloramphenicol or inclusion of sucrose in the medium completely prevented the effect of penicillin on K efflux (Fig. 5). (Chloramphenicol also prevented the effect of cycloserine on K efflux.) By contrast, the effects of bacitracin and vancomycin remained essentially the same when chloramphenicol or sucrose was present (Fig. 5).

TABLE 1. *Binding of H^3-penicillin by Bacillus megaterium**

Additions	Penicillin bound (mμg/mg of cells)
None	0.87
Penicillin	0.09
Bacitracin	1.11
Vancomycin	0.86

* Penicillin, bacitracin, or vancomycin (200 μg/ml) was added 5 min before addition of H^3-benzyl penicillin (20 μg/ml) to each flask. The bacteria were washed and prepared for counting as described in the text. The values are the mean of duplicate determinations.

Effects of bacitracin and vancomycin on binding of penicillin. Because one would expect some competition between these antibiotics for a common cellular binding site if they have the same site of action, we investigated the effects of bacitracin and vancomycin on the binding of radioactive penicillin. We found that *B. megaterium* binds about 500 molecules of penicillin per cell, a value similar to that reported for *Staphylococcus aureus* under similar experimental conditions (short exposure to a saturating concentration of penicillin; Cooper, 1956). Prior addition of the other antibiotics in tenfold excess did not significantly affect this value (Table 1), showing that they are not bound at the penicillin-binding sites.

DISCUSSION

The observations described here with *B. megaterium* and those of Shockman and Lampen (1962) with *Streptococcus faecalis* show directly that penicillin and cycloserine have no inhibitory effect on the growth of gram-positive bacteria, except that caused by inhibition of cell-wall

synthesis. The other biochemical effects ascribed to penicillin must, therefore, be secondary consequences of this primary effect. The same primary action may be responsible for inhibition by penicillin of cortex formation during sporulation of bacilli (Fitz-James, *unpublished data*).

By contrast, the inhibition of growth of protoplasts by bacitracin, vancomycin, and novobiocin observed here and by Shockman and Lampen (1962) shows that these antibiotics have other important effects in addition to inhibition of mucopeptide synthesis. Novobiocin has a number of effects which were ascribed to induction of a general deficiency of Mg^{++} (Brock, 1962); we have not considered it further here. Because bacitracin and vancomycin cause eventual lysis of protoplasts and an increased rate of K efflux in growing bacteria which is not prevented by chloramphenicol or sucrose, they must affect the membrane directly and independently of their action on wall synthesis. Penicillin also increases K efflux, but this action is prevented by chloramphenicol or sucrose and, hence, is probably due to secondary distortion of the membrane following cessation of cell-wall synthesis. A similar explanation was proposed for some effects of penicillin on membrane functions in staphylococci, which can be prevented by inclusion of osmotic stabilizers in the medium (Hancock, 1958).

The actions of bacitracin and vancomycin on the membrane and on wall synthesis may be independent; however, the cessation of wall synthesis might well be secondary to an effect on the membrane and caused by relatively nonspecific interference with the transfer of mucopeptide precursors across the membrane, rather than primary and caused by a specific reaction with mucopeptide-polymerizing enzymes, as discussed for penicillin (Park and Strominger, 1957; Collins and Richmond, 1962); this possibility is open to further experimentation. The absence of competition by bacitracin or vancomycin at the penicillin-binding sites, and the absence of cross-resistance among penicillin, bacitracin, and vancomycin (Dangerfield et al., 1960; Stone, 1949; Szybalski and Bryson, 1952) also provide strong evidence that these antibiotics have different primary sites of action.

Our results suggest that accumulation of cell-wall precursors in bacteria treated with an antibiotic is not sufficient evidence to establish that inhibition of wall synthesis is its primary mechanism of action. Although bacitracin and vancomycin inhibit cell-wall synthesis, they also have significant effects on the protoplast membrane which are concurrent with, and may cause, the inhibition of wall synthesis. We hope to publish separately morphological studies of the effects of these antibiotics on the structure of bacteria and protoplasts.

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