ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1976, p. 697-706

Vol. 10, No. 4
Printed in U.S.A.

Suppression of the Lytic and Bactericidal Effects of Cell Wall-Inhibitory Antibiotics

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Received for publication 10 June 1976

The bacteriolytic effect of beta-lactam antibiotics on *Bacillus subtilis* and on *Streptococcus pneumoniae* was found to be a function of the pH; lysis was suppressed if the pH of the pneumococcal culture was below 6.0 during penicillin treatment. In the case of *B. subtilis*, growth at pH 6.6 prevented penicillin-induced lysis. In pneumococci, the addition of trypsin to the growth medium also protected against lysis. The pH-dependent protection phenomenon resembled in several respects the antibiotic "tolerance" of pneumococci with a defective autolytic system. (i) At the pH nonpermissive for lysis, the bacteria retained their normal sensitivity to beta-lactam and to other cell wall inhibitors; however, instead of lysis, the drug-treated bacteria simply stopped growing. Loss of viability of the cells was also greatly reduced. (ii) Protection against lysis was independent of the dose and chemical nature of the cell wall inhibitors. (iii) The protection effect was reversible; lysis and loss of viability could be triggered by a postincubation of the drug-treated bacteria at the pH permissive for lysis.

The beta-lactams, D-cycloserine and beta-halogeno-D-alanine, can inhibit specific enzymatic reactions in the cell wall metabolism of bacteria. In most bacteria treatment with these antibiotics results in a rapid loss of viability, and in many species the treated cells actually disintegrate (lyse).

How inhibition of the antibiotic-sensitive enzymatic reactions may lead to the important antimicrobial effects of these drugs (namely, to the killing and lysis of the bacteria) is not well understood. The possible involvement of cell wall-hydrolyzing enzymes (autolysins) in penicillin-induced lysis has been considered repeatedly in the literature (12, 14, 21). More recently, direct experimental evidence for such a role has been obtained in pneumococci. In studies reported previously (17, 18), suppression of the activity of the *N*-acetylmuramic acid-L-alanine amidase resulted in a striking change in the response of this bacterium to penicillin and other cell wall inhibitors; although the drug-treated bacteria stopped growing, they no longer lysed and the rate of loss of cellular viability was greatly reduced. Such a "tolerant" response to cell wall inhibitors was subsequently also observed in lysis-defective mutants of *Bacillus licheniformis* (13).

In this communication, we describe growth conditions that can produce antibiotic tolerance in pneumococci and in *Bacillus subtilis*. We have observed that a suitable choice of pH in the growth media can dramatically change the response of these bacteria to cell wall inhibitors from lysis to simple growth inhibition; the same conditions also cause a substantial reduction in the rate of loss of viability. In pneumococci, addition of trypsin to the growth medium also has a similar effect. We suggest that the mechanism of these protective phenomena involves the suppression of the activity (or inactivation) of an autolysin that catalyzes the antibiotic-induced lysis of the bacterial cells.

MATERIALS AND METHODS

Bacterial strains. Two bacterial strains were used: *Streptococcus pneumoniae* strain R36A (Rockefeller University laboratory stock) and *B. subtilis* 168 (obtained from Leonard Mindich of the Public Health Research Institute, New York, N.Y.).

Antibiotics. Amidino penicillin FL 1060 (mecillinam) was obtained from Leo Co., Ballerup, Denmark. D-Cycloserine (Merck) and benzylpenicillin (Squibb) were commercial products; sodium dicloxacillin was obtained from Wyeth Labs, Inc., Philadelphia, Pa.; cephalothin was a gift of Kenneth Price of Bristol Laboratories, Syracuse, N.Y.; and beta-chloro-D-alanine was kindly supplied by James Manning of The Rockefeller University, New York, N.Y. Trypsin (2× recrystallized, Worthington Biochemicals Corp., Freehold, N.J.) and all other reagents and medium components were reagent-grade, commercially available products.

Growth media. A synthetic medium containing

acid-hydrolyzed casein (7a, 16; C-medium) was used with both pneumococci and *B. subtilis* in all the experiments illustrated in the figures. In the case of *B. subtilis*, the pH of the medium was set by potassium phosphate buffers (at pH values of 8.0 or 6.6) at a final concentration of 0.1 M (3). Cultures of *B. subtilis* were grown aerobically in a shaker bath at about 60 strokes per min (Gyrotory water bath shaker model G76, New Brunswick Corp.).

Pneumococci were cultured in glass test tubes, without shaking, in C-medium containing potassium phosphate buffers at a final concentration of 0.05 M and with initial pH values of 8.0 or 6.6.

Occasionally, *B. subtilis* cultures were also grown in the medium of Anagnostopoulos and Spizizen (1) buffered at pH 8.0 or 6.6. In a few experiments with *B. subtilis*, the pH 8 phosphate buffer of the C-medium was replaced by 0.1 M tris(hydroxymethyl)aminomethane (Tris), pH 8 (potassium phosphate was added to such media to give a concentration of 0.02 M). The effects of culture pH on lysis sensitivity were reproduced with these media. However, only experiments with phosphate-buffered media are illustrated in figures.

The sharp pH dependence of some of thephenomena to be described in this paper made it necessary to monitor changes in the pH of cultures as a function of bacterial growth. In the case of *B. subtilis* cultures grown with aeration and with buffer concentrations of 0.1 M, there was relatively little change in the culture pH during the experiments. As the cell concentration of the cultures reached the range of 10^7 to 10^8 viable cells per ml, the pH of the phosphate-buffered media was found to shift from the initial 8.0 to about 7.5 and from the initial pH of 6.6 to about 6.0; with the Tris buffer the pH changed from 8.0 to about 7.8 only.

In contrast to the *B. subtilis* cultures, the phosphate-buffered pneumococcal cultures underwent more substantial changes in the medium pH during growth, presumably due to the production of lactic acid. With buffer concentrations of 0.05 M, the pH values of cultures were found to change in a characteristic manner during bacterial growth (for details, see Fig. 9). At a cell concentration of 5×10^7 viable units per ml, the pH of cultures containing pH 6.6 buffers had shifted to about 5.8 and the pH had further dropped to 4.5 to 4.8 in the stationary phase of growth. The pH of cultures with pH 8 buffers had dropped to about 6.9 when the cell concentration reached 5×10^7 viable units per ml (corresponding to the light-scattering value of N = 200 on the Coleman nephocolorimeter used to monitor bacterial growth), and the pH value stabilized at 6.5 to 6.6 in the stationary phase of growth.

Growth of the cultures was measured by monitoring the light scattering of the suspensions using a Coleman nephocolorimeter (16). The arbitrary "nephelometric (N) units" of the instrument were calibrated by determining the corresponding colony-forming units of the bacteria. Viability was determined by the routine agar plating methods (1, 16). Antibiotics were "removed" before plating by dilution to levels that had no detectable effect on bacterial growth in liquid culture or in agar medium. Growth media were used as diluents.

To avoid the activation of the lytic response on the agar plates, the antibiotic-treated bacteria (such as the ones titrated in Fig. 4 through 6 and Table 1) were plated on agar media whose pH had been adjusted to values that protect against lysis in liquid cultures (i.e., 0.1 M phosphate at pH 6.6 for *B. subtilis* and 0.05 M phosphate for pneumococci).

Assay of autolysis. The bacterial suspensions or debris were incubated in the appropriate buffers at 37°C and at intervals the turbidities were determined using a Zeiss spectrophotometer (optical density at 660 nm) (22).

Assay of radioactive material secreted into the medium. Radioactive choline ([*methyl*-^{3}H]choline, 0.5 Ci/mmol, 2 μCi and 5μg/ml of medium) was added to the medium of exponentially growing pneumococci at a cell concentration of about 5×10^7 viable units per ml. Incorporation of the isotope into total macromolecular material secreted into the medium was determined by a published procedure (20), the essential features of which are as follows: 100-μl portions of the suspension were removed and treated with cold 10% trichloroacetic acid for 15 min; the precipitated material was collected on to glass-fiber filter disks (GFA) under vacuum. After drying at 100°C for 10 min, the disks were transferred to scintillation vials and the amounts of radioactivity were determined by using toluene-based cocktail with 2,5-diphenyl-oxazole (PPO) and a scintillation spectrometer (Mark II, Nuclear-Chicago Corp.).

Material secreted into the medium was first separated from the bacteria by centrifugation: 500-μl portions of the cultures were centrifuged for 10 min in an Eppendorf microcentrifuge (Brinkman Co.), and 100-μl portions of the supernatant solutions were assayed as described above.

RESULTS

Protection against antibiotic-induced lysis in *B. subtilis* cultures grown at pH 6.6. Figure 1 demonstrates the strikingly different response of *B. subtilis* cultures to four cell wall-inhibiting antibiotics, dependent upon the pH of the growth medium. In contrast to the behavior of cultures at pH 8.0, the low-pH cultures were not lysed but only inhibited by the antibiotic treatments. Several additional conclusions may also be drawn from the figure: (i) the dose responses of the high- and low-pH cultures to the various antibiotics were similar in spite of the fact that the antibiotics had a bacteriolytic effect at the high pH, whereas at the lower pH they only acted as bacteriostatic agents; (ii) the antibiotic tolerance at low pH did not seem to depend on the antibiotic dose, since lysis did not occur even at very high doses of the antibiotics (e.g., treatment with 500 times the minimal inhibitory concentration of cloxacillin for 5 h); and (iii) the antibiotic tolerance was independent of the chemical nature of the drug and did not depend on the inhibition of a specific enzymatic reaction, since a low pH was found to

protect equally well against lysis by beta-lactams and by D-cycloserine.

Reversibility of the tolerant response. Figures 2 and 3 demonstrate the reversibility of the pH-dependent antibiotic tolerance. In these experiments, bacterial cultures were first treated with the four different antibiotics at the low pH. After the cultures had stopped growing, the bacteria were centrifuged, washed, and resuspended in fresh, antibiotic-free growth media at two different pH values corresponding to the lysis-permissive (pH 8.0) and lysis-nonpermissive (pH 6.6) conditions. The lytic response of the bacteria could be reactivated by the postincubation at the permissive pH. However, the degree of clearing of the cultures (as registered by the light scattering) seemed less extensive than the lysis observed during incubation in a single, lysis-permissive medium. The causes of this discrepancy are not known.

These experiments suggest that: (i) the primary (growth-inhibitory) effect of the antibiotics may be experimentally separated, in time, from the secondary (lytic) effect; and (ii) during treatment at the low pH, the antibiotics reach their normal specific inhibitory targets, since the "normal" (i.e., lytic) response can be reactivated in the absence of added antibiotics after pretreatment at the low pH.

Protection against the cytocidal effect of antibiotics. The low pH also gave a dramatic protection against the killing effects of cloxacillin and mecillinam (Fig. 4 and 5). This protection effect was reversible; after pretreatment of the bacteria with cloxacillin at the low pH, one could trigger the killing effect by a postincubation at the high pH in the absence of the antibiotic (Fig. 6).

pH dependence of autolysis in *B. subtilis*. In an attempt to better characterize the biochemical nature of the process inactivated at the low pH, we determined the pH dependence of autolysis in *B. subtilis*. Figure 7 shows the effect of pH on the rate of lysis of mechanically disrupted bacteria (i.e., the washed, crude debris obtained after brief [2-min] sonication of the cells in a Raytheon sonicator) (22). In Fig. 8 intact exponentially growing bacteria were suspended in 0.1 M Tris-maleate buffers of various pH values and were incubated at 37°C. Apparently, cells were unstable under these conditions and autolyzed. With both the disrupted and the intact cells, the rate of autolysis increased with the pH up to about pH 8.5. Autolysis was substantially slower below pH 7.0. These findings suggest that the autolytic processes are catalyzed by the *B. subtilis* amidase (*N*-acetylmuramic acid-L-alanine amidase); the purified amidase has a pH optimum at 8.5 (4).

Antibiotic tolerance of pneumococci grown at low pH. It was important to test the possible applicability of the pH-dependent antibiotic tolerance in pneumococci, since the mechanism of antibiotic-induced lysis and killing has been most extensively studied in this microorganism (17–20). The tolerant response to benzylpenicillin, mecillinam, and cephalothin could also be invoked in pneumococci by the suitable choice of pH. Pneumococcal cultures grew with comparable growth rates in media with initial pH values of 8.0 and 6.6. On the other hand, lysis by benzylpenicillin only occurred at the higher pH (Fig. 9). The pH changed in a reproducible manner during culture growth (as a function of cell concentration) in these media (see also Materials and Methods). Lysis occurred if the culture pH was in the vicinity of 7 during penicillin treatment (see figure). Lysis was inhibited if the pH was below 6.0 during drug treatment (see figure) or if it was above 8.0 (not shown). The rapid loss of viability observable during beta-lactam treatment of cultures growing at the higher pH was also dramatically slowed down at the lower pH (Table 1).

The tolerant response of pneumococci could also be reversed (by shifting the pH of the antibiotic-treated culture to the pH permissive for lysis; data not shown) in a manner exactly analogous to that of the *B. subtilis* cultures.

Secretion of a choline-containing autolysin inhibitor during treatment of pneumococci with penicillin under conditions nonpermissive for lysis. Leakage of a choline-containing macromolecular autolysin inhibitor into the growth medium has been observed during treatment of autolysin-defective mutant pneumococci with cell wall inhibitors (20). A similar leakage of macromolecular choline compounds occurred during penicillin treatment of a wild-type pneumococcus growing under conditions nonpermissive for lysis (pH 5.8) (Fig. 10).

Protection against lysis by trypsin. Lysis of pneumococci (growing in the medium with initial pH of 8.0) by D-cycloserine or benzylpenicillin could also be inhibited by the addition of trypsin or Pronase to the growth medium (Fig. 11). Heat-denatured trypsin or serum albumin (bovine serum albumin, Armour fraction V) added at the same concentration (500 μg/ml) had no protective effect. Even the spontaneous lysis characteristic of late-stationary-phase cultures of pneumococci was inhibited in the presence of the proteolytic enzyme (Fig. 11).

DISCUSSION

Disintegration (lysis) of bacteria upon treatment with antibiotics involves damage to both structural components of the cells, the cell

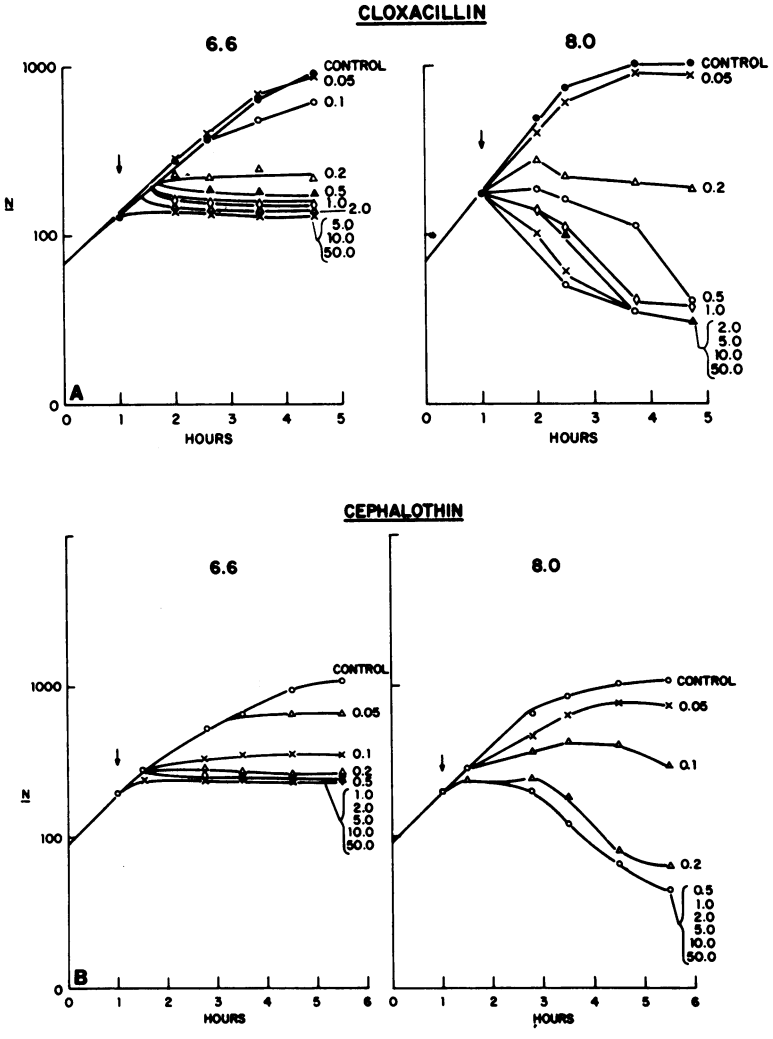

FIG. 1. *Effects of four cell wall-inhibitory antibiotics on cultures of Bacillus subtilis 168 growing at pH 6.6 and pH 8.0. Bacterial cultures were grown in C-medium (7a) either at pH 6.6 or at pH 8.0 buffered with potassium phosphate (0.1 M) from overnight inocula. In the exponential phase of growth at cell concentrations of about 5 × 10⁷ viable cells per ml (arrow), the cultures received antibiotics at the concentrations (micrograms per milliliter of growth medium) indicated by the numbers, and the growth response of the bacterial cultures was followed by nephelometry (see Materials and Methods).*

walls and the plasma membrane (8, 11). Data in the literature suggest that lysis is initiated by damage to the cell wall. In pneumococci, the agent responsible for this damage is the *N*-acetylmuramic acid-L-alanine amidase (17). Participation of autolysins in penicillin-induced lysis has also been confirmed in *B. licheniformis* (13).

Until now, the mechanism of lytic phenomena could only be investigated through the use of lytic mutants or, in the case of pneumococci, by the use of ethanolamine-grown bacteria (15). In this communication we describe a simple experimental technique that produces an alteration in the bacterial response to lytic antibiotics exactly analogous to that of the lytic-defective mutants. Both in pneumococci and in *B. subtilis*, appropriate modulation of the pH of the growth medium produces the typical features of antibiotic tolerance. (i) The cellular response to lytic antibiotics changes from lysis to bacteriostasis, while the cells retain their normal sensitivity (dose response) to the drugs. (ii) The cells become tolerant not only to penicillin but also against drugs inhibiting "early" steps of wall synthesis. However, one should add at

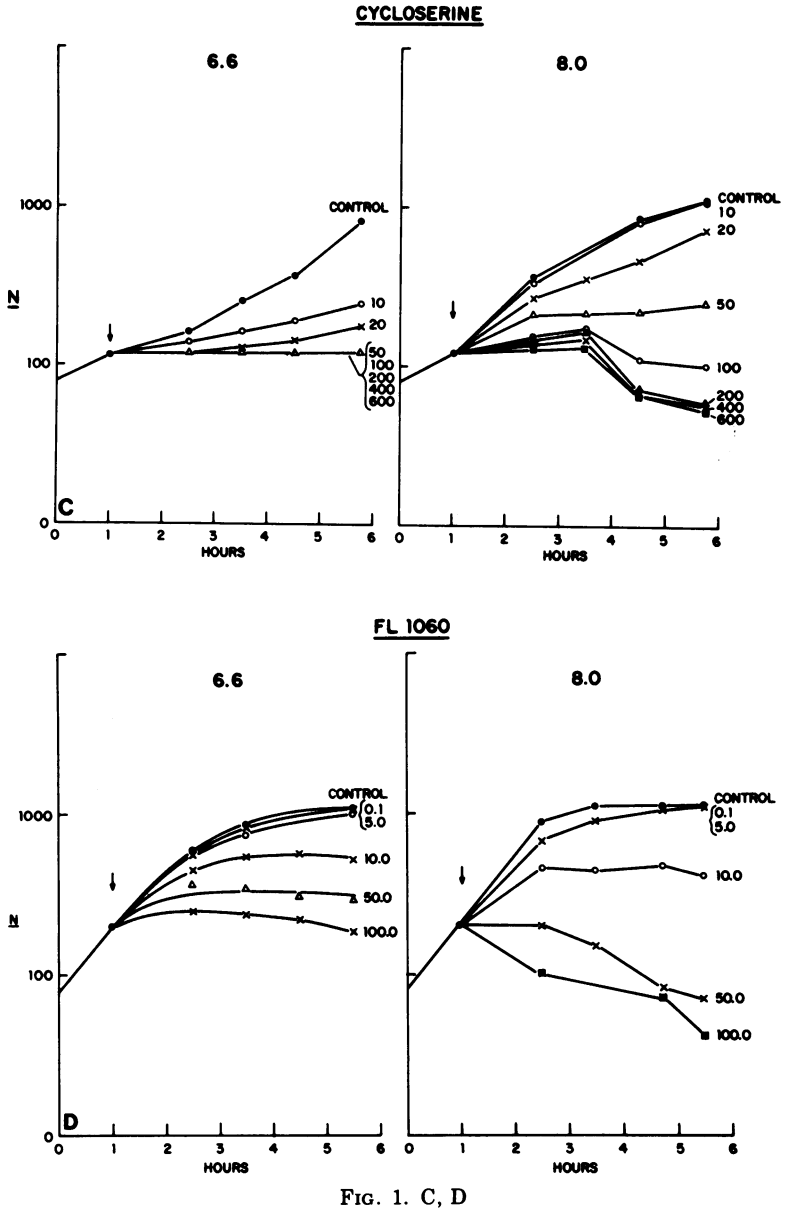

FIG. 1. C, D

this point that D-cycloserine-induced lysis (as determined by the light-scattering method; see Fig. 1C and 3) has always been less extensive than lysis by the beta-lactams. The reasons for this discrepancy are not understood at the present time. (iii) The tolerant phenotype is reversible. (iv) In both species, the conditions nonpermissive for lysis also provide a substantial protection against loss of viability.

The growth rates of cultures are similar if not identical under the lytic and tolerant conditions. Furthermore, the antibiotics seem to reach their "normal" enzymatic targets in the cells growing under the tolerant condition, since (i) the dose responses under lytic and tolerant conditions are similar and (ii) it is possible to invoke the lytic response by the following sequence of treatments: exposure of cells to penicillin under tolerant conditions, followed by removal of penicillin (by penicillinase) and, finally, transfer of cells to fresh growth medium with the pH optimal for lysis.

It therefore appears that there exist two kinds of growth conditions for these bacteria:

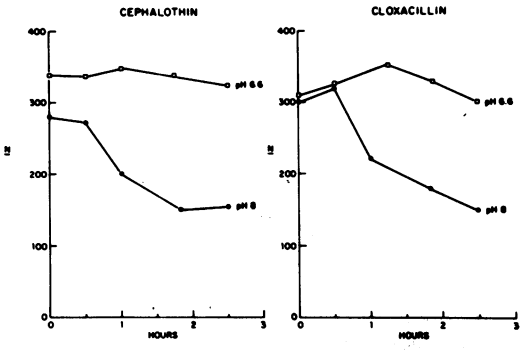

FIG. 2. *Reactivation of the lytic response of B. subtilis to cephalothin and cloxacillin. Bacterial cultures were treated with cephalothin (0.5 μg/ml) and cloxacillin (0.2 μg/ml), respectively, for 2 h in growth media at pH 6.6. The cultures were next centrifuged, washed with buffer-free medium, and resuspended each in two different media, one at pH 6.6 and the other at pH 8.0. Both media were free of antibiotics The change of light scattering of these suspensions was followed (nephelometry) during incubation at 37°C. Note that the light-scattering data are plotted on a linear scale (unlike the log scales in Fig. 1).*

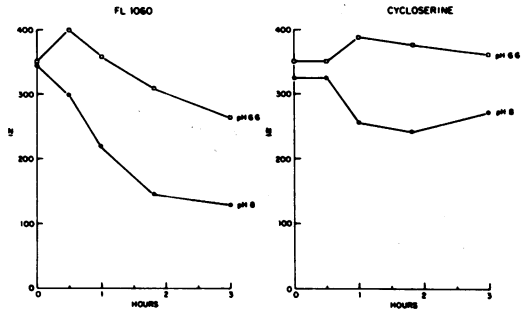

FIG. 3. *Reactivation of the lytic response of B. subtilis to D-cycloserine and mecillinam (FL 1060). Bacterial cultures were treated at pH 6.6 with D-cycloserine (100 μg/ml) and mecillinam (50 μg/ml), respectively, for 2 h and were subsequently transferred to the antibiotic-free postincubation media, as described in the legend to Fig. 2. Light-scattering data are plotted on a linear scale.*

one permissive and another nonpermissive for lysis. In pneumococci the permissive pH was found to be in the vicinity of pH 7.0; cultures kept at a pH less than 6.0 showed inhibition of lysis, and lysis was also suppressed at pH 8 and above (data not shown). In *B. subtilis* the permissive pH was around and above 8; pH values below 6.6 were inhibitory for lysis. Although the absolute pH values permissive for lysis are different in the two organisms, in each case they correspond to the in vitro pH optima of the activity of the major murein hydrolases (*N*-acetyl-muramic acid-L-alanine amidase) (4, 6). The pneumococcal enzyme has about 30% of its maximal activity (at the pH optimum of 6.9) at pH 6.0 and about 20% of maximal activity at pH 8 (6). These observations suggest that in *B. subtilis* as in pneumococci, the murein hydrolase responsible for the antibiotic-induced cell lysis is the amidase (rather than the muramidase [2] that has also been detected in this bacterium).

Although bacteria are known to regulate their intracellular pH, it seems likely that the activity of enzymes located on the outer surface of the plasma membrane or in the periplasmic

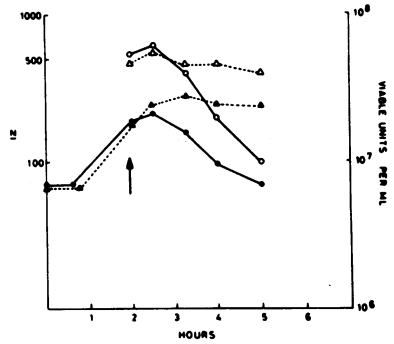

FIG. 4. *Protection against cloxacillin-induced loss of viability at low pH. Bacillus subtilis cultures, growing at pH 6.6 and pH 8.0, respectively, received cloxacillin (0.2 μg/ml) at the time indicated by the arrow, and the rate of loss of viable bacteria in the cultures was followed by agar plating, during incubation with the antibiotic. Open symbols indicate viable units per milliliter; closed symbols represent growth measured by nephelometry. Viability at pH 6.6 (△, dashed line); growth at pH 6.6 (▲, dashed line); viability at pH 8.0 (○, solid line); growth at pH 8.0 (●, solid line).*

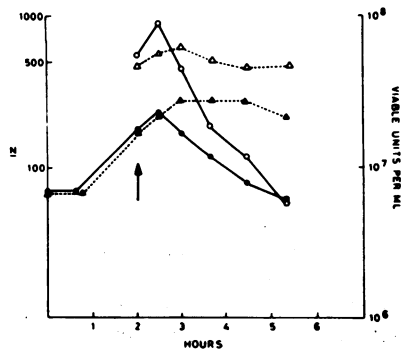

FIG. 5. *Protection against mecillinam (FL 1060)-induced loss of viability at low pH. A culture of B. subtilis was treated with mecillinam (50 μg/ml), and the rates of loss of viability and growth were assayed as described in the legend to Fig. 4. Symbols are the same as in Fig. 4.*

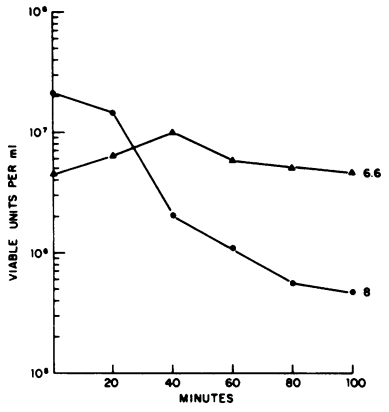

FIG. 6. *Reactivation of the killing effect of cloxacillin by postincubation at pH 8.0. A culture of B. subtilis was pretreated with cloxacillin (0.2 μg/ml) for 2 h at pH 6.6. The culture was divided into two parts; both were centrifuged, and the cells were washed with buffer-free media and resuspended in fresh, antibiotic-free media at pH 6.6 and pH 8.0, respectively. The two suspensions were incubated at 37°C, and samples were removed at intervals for the determination of the viable titer.*

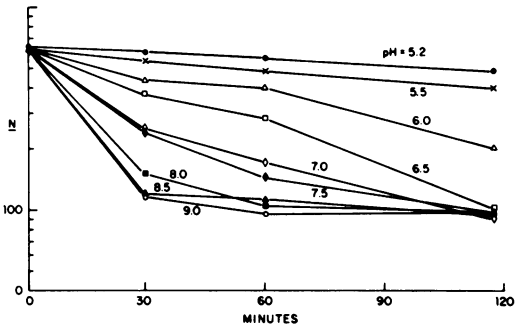

FIG. 8. *Autolysis of Bacillus subtilis suspensions in buffers of various pH values. An exponentially growing culture of B. subtilis (2 × 10⁸ viable cells per ml) in pH 8.0 medium was centrifuged; the pelleted cells were rinsed with saline (0.15 M NaCl) and resuspended in saline at 10 times the original cell concentration. This stock suspension was then quickly diluted 10-fold into a series of test tubes, each containing 0.1 M Tris-maleate buffer at the pH values indicated by the numbers on the figure. The suspensions were incubated at 37°C, and the turbidity (optical density at 660 nm) change was monitored at intervals, using a Zeiss spectrophotometer.*

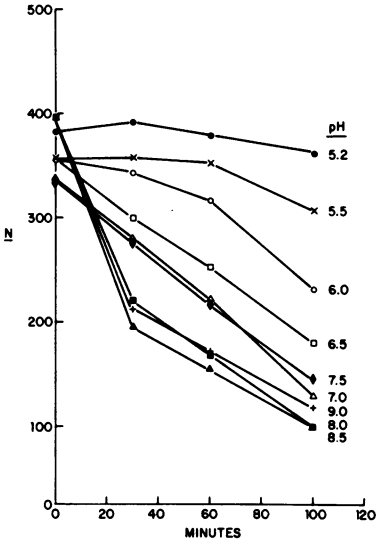

FIG. 7. *Autolysis of sonically disrupted Bacillus subtilis at different pH values. A 100-ml culture of B. subtilis growing exponentially at pH 8.0 was harvested by centrifugation at a cell concentration of 2 × 10⁸ viable cells per ml. The cells were resuspended in 10 ml of ice-cold 0.15 M saline and sonicated for 2 min in a Raytheon sonicator. Examination of the sonicated suspension by phase-contrast microscopy showed that over 90% of the cells had become "empty." The suspension was centrifuged, resuspended in 10 ml of fresh saline, and diluted 10-fold into test tubes containing Tris-maleate buffers (0.1 M) ranging in pH from 5.2 to 9.0. The rate of loss of turbidity (optical density at 660 nm) was followed during incubation at 37°C.*

space would be accessible to manipulation by changes in the ionic milieu of the growth medium. The molecular sieve properties of the bacterial outer envelopes are known to allow the free penetration of molecules of substantial molecular weights (9). We suggest that pH values nonpermissive for antibiotic-induced lysis inhibit the endogenous activity of murein hydrolases which, under the permissive conditions, are responsible for the antibiotic-induced destruction of the bacteria.

The pH value of the bacterial growth medium is, of course, a major parameter of bacterial growth, and different pH values may cause complex physiological differences in the cells. For instance, bacteria are known to produce different phospholipids when shifted to subphysiological pH (7), and the plasma membrane of such bacteria may be more stable to osmotic lysis (10). We cannot exclude with certainty that such indirect changes in cellular physiology may not also contribute to the pH-dependent drug tolerance. However, the quick reversibility of bacteriostatic to lytic response and the remarkable similarity of the phenomenon to the well-documented cases of drug tolerance in lytic mutants suggest that in this case we may be observing direct and relatively simple consequences of the suppression of a surface-located enzyme activity. Additional evidence supporting this interpretation comes from the observation that pneumococci growing in a medium with a pH permissive for lysis can nevertheless be protected against lysis by the

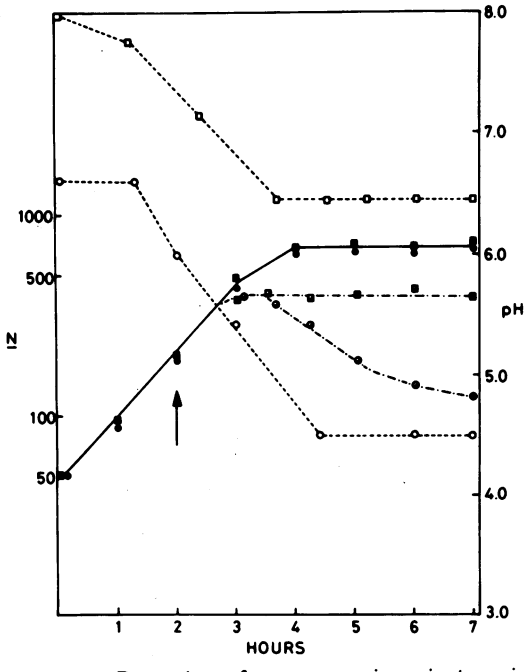

FIG. 9. *Protection of pneumococci against penicillin-induced lysis by growth at low pH. Two test tube cultures (A and B) of Streptococcus pneumoniae (20 ml each) were grown from small inocula (2 × 10⁵ to 5 × 10⁵ viable cells per ml) in C-medium (7a) containing potassium phosphate buffers (0.05 M) at pH 8.0 and pH 6.6. When the cell concentration had reached 5 × 10⁷ viable cells per ml, each culture was divided in two (A1, A2, and B1, B2), and one of the cultures (A1 and B1) received benzylpenicillin (0.1 μg/ml) (see arrow in figure); the other two cultures (A2 and B2) served as controls. Growth and lysis were followed by nephelometry (N). Periodically, samples were removed for the determination of the culture pH, using a Beckman automatic pH meter equipped with class microelectrodes (Beckman model Zeromatic SS-3). The changes in the culture pH of culture A1 and A2 were found to be virtually identical. No significant differences were observed between the pH values of cultures B1 versus B2. Symbols: (——) Growth of the control cultures; (■) A2; (●) B2; (----) pH of the cultures; (□) A1 and A2; (○) B1 and B2; (-·-·) growth and lysis of penicillin-treated cultures; (◑) A1; (◪) B1.*

simple addition of trypsin to the growth medium. We interpret this observation as the proteolytic destruction of amidase molecules that are liberated into the periplasm or onto the surface of the cell wall and thus made accessible to trypsin. The pneumococcal autolysin is extremely sensitive to proteolysis. The relative resistance of the *B. subtilis* amidase to proteolytic enzymes (4) may be the reason for the failure of trypsin to protect *B. subtilis* against penicillin lysis.

The pH-dependent antibiotic tolerance described in this communication may have a general applicability, since closely similar effects have been observed in a number of species, such as *Escherichia coli*, staphylococci, and *Streptococcus faecium* (W. Goodell, R. Lopez, and A. Tomasz, submitted for publication). It is possible that in some species of bacteria additional unique properties of the murine hydrolases (such as salt sensitivity) could also be exploited for the selective suppression of the activity of these enzymes. Such studies may

TABLE 1. *Protection against the killing action of beta-lactams in the lysis-nonpermissive growth medium*[a]

Antibiotic (μg/ml)	Duration of treatment (min)	Antibiotic treatment in:[b]	
		Lysis-permissive medium	Lysis-nonpermissive medium
Benzylpenicillin (0.1)	0	5.0	5.0
	20	4.5	4.8
	40	0.8	2.0
	60	0.1	1.0
	120	0.0003	0.2
Cephalothin (5)	0	5.2	5.2
	20	0.5	1.0
	40	0.07	0.8
	60	0.04	0.6
	120	0.0001	0.3
Mecillinam (50)	0	5.4	5.4
	20	0.6	2.0
	40	0.4	1.0
	60	0.08	0.5
	120	0.0002	0.2

[a] Two pneumococcal cultures (A and B, 30 ml each) were grown from small inocula (5 × 10⁵ viable cells per ml) in C-medium containing potassium phosphate buffers (0.05 M) at pH 8.0 (culture A, lysis-permissive medium) and pH 6.6 (culture B, lysis-nonpermissive medium). At a cell concentration of 5 × 10⁷ viable bacteria per ml (corresponding to the light-scattering value of N = 200 on the nephelometer scale), each culture was divided into three portions; one pair of cultures (A1 and B1) received benzylpenicillin (0.1 μg/ml), and the second pair (A2, B2) received cephalothin (5 μg/ml); mecillinam (50 μg/ml) was added to the third pair (A3, B3). Just before the addition of drugs, samples were removed from A and B to determine the initial (0 min) titer of viable bacteria and to measure the pH. The pH values were 7.1 in culture A and 6.0 in culture B. The cultures were incubated at 37°C, and samples were removed at intervals to determine the viable titers. The pH values of the cultures were also determined after 60 and 120 min of incubation with the antibiotics. The pH of the A cultures was 6.8 at 60 min and 6.5 at 120 min. In the B cultures, the pH dropped to 5.6 at 60 min and to 5.0 at 120 min. There was virtually no difference between the pH values of cultures A1, A2, or A3. Similarly, the pH values of cultures B1, B2, and B3 were practically identical. Samples for the viable-titer determination were diluted in lysis-nonpermissive C-medium and plated in 1.0% agar-containing lysis-nonpermissive C-medium.

[b] Number of surviving viable cell per milliliter (×10⁷).

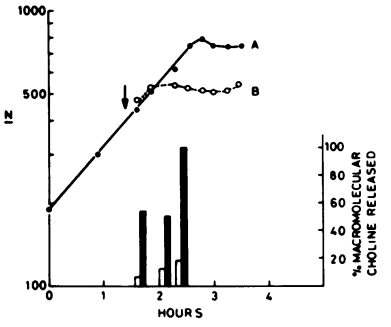

FIG. 10. *Secretion of choline-containing macromolecules during penicillin treatment of pneumococci at low pH. A pneumococcal culture was grown at pH 6.6. At the time indicated by the arrow, the culture was divided; one of the two cultures received benzylpenicillin (0.1 μg/ml), and the other served as control. Immediately after penicillin addition, both cultures received radioactive choline (1 μCi and 0.1 μg of [methyl-^{3}H]choline per ml; specific radioactivity in the medium; 5.1 μg and 0.5 μCi/ml). The cultures were incubated at 37°C, growth was monitored by nephelometry, and at intervals aliquots were removed for the determination of the total isotope incorporated into macromolecular choline compounds and also for the quantitation of macromolecular choline label that had escaped into the culture medium (see text). (Curve A) Control; (curve B) penicillin-treated culture. The histogram indicates the percentage of macromolecular choline label that has escaped into the growth medium in the control (empty bars) and in the penicillin-treated (solid bars) cultures.*

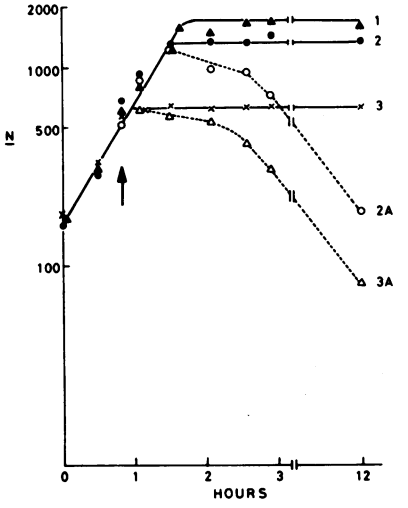

FIG. 11. *Protection of pneumococci against penicillin-induced lysis by trypsin added to the growth medium. Pneumococci growing in pH 8.0 culture medium were distributed to a number of culture tubes at the time indicated by the arrow. Cultures 1, 2, and 3 received trypsin (500 μg/ml); cultures 2 and 2A received D-cycloserine (100 μg/ml), and cultures 3 and 3A received benzylpenicillin (0.1 μg/ml). Incubation at 37°C continued, and growth and lysis were measured by nephelometry.*

provide simple experimental systems for the investigation of the mechanism of action of cell wall-inhibitory antibiotics.

An important conclusion emerging from these observations and from findings described earlier in the pneumococcal system concern the mechanism of action of penicillin and other cell wall inhibitors. We suggest that, contrary to the common notion, the direct primary effect of penicillins and cell wall inhibitors on bacteria is inhibition of growth. However, this primary effect can only be observed in bacteria with a suppressed autolytic system. The most important antimicrobial effects of these antibiotics (namely, lysis and killing) are indirect, secondary consequences of the inhibition of cell wall synthesis. Lysis and, at least in part, killing are caused by the uncontrolled activity of bacterial autolytic enzymes (murein hydrolases). Recent studies with pneumococci indicated that interruption of murein synthesis by cell wall inhibitors can cause a loss into the medium of a lipoteichoic acid-containing autolysin inhibitor (20). It has been suggested that such a loss of an endogenous inhibitor constitutes the direct cause of the triggering of the self-destructive activity of autolytic enzymes (19, 20).

The possible involvement of bacterial autolysins in the mode of action of penicillin and the indirect nature of the cytocidal and cytolytic action of this drug have been considered repeatedly in the literature (5, 8, 11, 12, 21), and the present studies provide further experimental evidence for these views. We are presently testing the possibility that, as in pneumococci and other bacteria too, the lytic and killing process triggered by cell wall inhibitors is accompanied by the leakage to the medium of lipopolysaccharide-like material.

ACKNOWLEDGMENTS

This investigation was supported in part by a grant from the National Institutes of Health (Biomedical Support Grant to The Rockefeller University) and by a grant from the National Science Foundation.

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