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Tolerant Response of *Streptococcus sanguis* to Beta-Lactams and Other Cell Wall Inhibitors

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In contrast to group A streptococci or *Streptococcus pneumoniae*, cells of *Streptococcus sanguis* (group H) do not exhibit the irreversible effects of penicillin treatment, such as loss of viability or lysis. On the other hand, the same bacteria show typical effects of penicillin, such as morphological alterations, reduction in the rate of cell wall synthesis, and secretion of murein and lipoteichoic acid polymers into the medium. A novel effect of cell wall inhibitors was also noted: treatment with beta-lactams or with fosfomycin, D-cycloserine, or beta-halogeno-D-alanine caused the release of substantial amounts of glycerol lipids into the growth medium. The antibiotic "tolerance" of *S. sanguis* is interpreted in terms of the hypothesis that the activity of bacterial murein hydrolases is essential for the irreversible effects of cell wall inhibitors.

In spite of the major advances made in the understanding of the nature of penicillin-susceptible bacterial enzymes, the mechanism by which antibiotic-treated bacteria lose their viability and disintegrate has remained obscure. Recent studies with pneumococci and with several other species of bacteria have produced strong evidence implicating the central role of bacterial murein hydrolases and their cellular control in the irreversible effects of penicillin and other cell wall inhibitors (3, 9, 15-17). Bacteria with suppressed hydrolase systems were shown to retain their normal susceptibility to penicillin; on the other hand, such cells have become resistant to the lytic effect of penicillin, and their rate of loss of colony-forming ability during drug treatment was also substantially reduced. In an attempt to gain a better understanding of these phenomena, we examined the response of several species of streptococci to penicillin. In the present communication, we describe what appears to be a natural penicillin-tolerant bacterial species: cells of *Streptococcus sanguis* are extremely susceptible to penicillin (minimal growth-inhibitory concentration of benzylpenicillin is 0.013 μg/ml), yet they do not lyse and there is only a minor loss in the colony-forming ability of the cells during treatment with cell wall inhibitors.

MATERIALS AND METHODS

Bacterial strains. Three strains of streptococci were used in these experiments: *Streptococcus pneumoniae* strain R36A (Rockefeller University Laboratory stock); *S. sanguis* strain Wicky, Lancefield group H (obtained from Dennis Perry of Northwestern University Medical School, Chicago); and *Streptococcus pyogenes* strain T4/56, Lancefield group A (obtained from Maclyn McCarty, Rockefeller University).

Antibiotics. Benzylpenicillin (E. R. Squibb & Sons, New York, N.Y.), mecillinam (Leo Pharmaceutical Products, Ballerup, Denmark), and sodium dicloxacillin (Wyeth Laboratories, Philadelphia, Pa.) were commercial products. Cephalexin and cephaloridine were kindly provided by Kenneth Price of Bristol Laboratories, Syracuse, N.Y., and beta-chloro-D-alanine was a gift from James Manning of the Rockefeller University. D-Cycloserine, fosfomycin, 3-fluoro-2-deutero-D-alanine, and D-4-[(2-oxo-3-pentene-4-yl)amino]-3-isoxazolidinone sodium salt (a D-cycloserine derivative) were obtained from Merck Sharp & Dohme Research Laboratories, Rahway, N.J. Mitomycin C was kindly donated by Maria Tomasz of Hunter College, New York, N.Y. All other antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo.

Growth media. The growth media (C medium, containing acid-hydrolyzed casein, and Cden, chemically defined [8; A. Tomasz, Bacteriol. Proc., p. 29, 1964]) were used for both pneumococci and *S. sanguis* strain Wicky. Stock cultures of strain Wicky were, however, maintained in brain heart infusion (Difco Laboratories, Detroit, Mich.) medium. *S. pyogenes* strain T4/56 was routinely grown in Todd Hewitt broth (Difco). All bacteria were grown (10-ml cultures in standard, 18-mm-wide test tubes) at 37°C without aeration.

Growth of the bacteria was determined by measuring the light scattering of the cultures (Coleman nephelometer; 14). Viable titers were determined by routine plating procedures (4, 14). Antibiotics were removed by dilution to concentrations having no detectable effect on bacterial growth in liquid culture or agar-containing medium. With benzylpenicillin, the cells were diluted 1:10 into medium con-

taining a 1,000-fold (wt/wt) excess of penicillinase (Calbiochem, Los Angeles, Calif.) and incubated for 10 min at 37°C.

Assay of incorporation of radioactive precursors into *S. sanguis* and release of radioactively labeled material into the medium. The radioactively labeled precursors used were: [U-^{14}C]glycerol (0.1 Ci/mmol, 1 μCi and 10 μg/ml of medium); [2-^{3}H]glycerol (5 Ci/mmol, 1 μCi and 10 μg/ml of medium); [6-^{3}H]uracil (20 Ci/mmol, 2.5 μCi and 10 μg/ml of medium); L-[5-^{3}H]lysine-4, (60 Ci/mmol, 1 μCi and 9 μg/ml of medium); 1-[G-^{3}H]phenylalanine (10 Ci/mmol, 2.5 μCi and 6 μg/ml of medium); and *N*-acetyl-D-[1-^{3}H]glucosamine (4 Ci/mmol, 1 μCi and 0.05 μg/ml of medium).

Three types of labeling regimes were used in these experiments. (i) In the "old label" type, radioactively labeled precursors were added to the cells at a concentration of approximately 5 × 10^6 colony-forming units/ml and incubated for 90 min (1.5 generations). The bacteria were then collected and washed with isotope-free medium on membrane filters (0.45 μm; Millipore Corp., Bedford, Mass.). After resuspension in fresh, unlabeled medium, the bacteria were allowed to grow for 30 min before the addition of the antibiotic at a cell density of about 2 × 10^7 colony-forming units/ml. (ii) In the "nascent label" type of experiment, the label was added at the same time as the antibiotic. (iii) In "continuous labeling," the precursors were added at the same cell density as in (i), but the cells were kept in the isotope-containing medium throughout the time of antibiotic treatment.

Incorporation into macromolecular components was measured as described earlier (18), with some modifications. Amounts of 100 μl of the cell suspension were precipitated with 10% cold trichloroacetic acid and 80 μg of carrier bovine serum albumin (BSA; Armour) for 15 min. The precipitates were collected onto glass fiber filters (GFA; Whatman, Inc., Clifton, N.J.) and washed with 10% trichloroacetic acid. After drying at 100°C for 10 min, the disks were placed in vials containing toluene scintillation fluid with 2,5-diphenyloxazole and counted in a Mark II (Nuclear-Chicago Corp., Des Plaines, Ill.) scintillation counter.

The amount of macromolecular components released into the medium was measured after the removal of the bacteria (0.5 ml) in an Eppendorf microcentrifuge (Brinkmann Instruments Inc., Westbury, N.Y.), and the acid-insoluble material was determined in 100 μl of supernatant as described above.

Polyacrylamide gel electrophoresis. Supernatant fluids from penicillin-treated *S. sanguis*, dialyzed against distilled water and concentrated by lyophilization, were layered on top of 10% acrylamide gels. Electrophoresis in the presence of sodium dodecyl sulfate was done according to the method of Weber and Osborn (20). The gels were sliced (1.2-mm slices), and the slices were added to vials and solubilized in 0.5 ml of 90% (vol/vol) NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) at 50°C for 2 h. A 10-ml amount of toluene scintillation fluid was added, and the samples were counted as described above.

Sucrose density gradient centrifugation. Supernatant fluids of penicillin-treated *S. sanguis*, di-

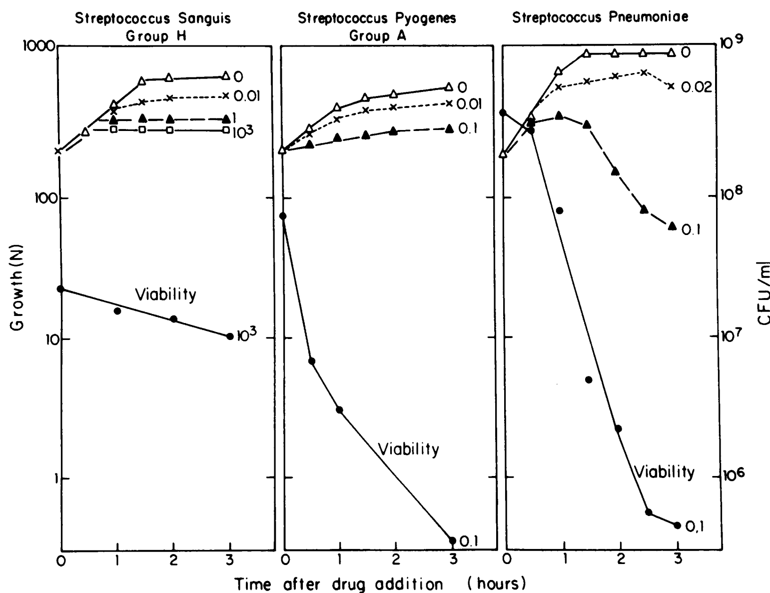

FIG. 1. *Effect of benzylpenicillin on the growth, viability, and lysis of three species of streptococci. Exponentially growing cultures received benzylpenicillin at the concentrations (units per milliliter) indicated. Growth and lysis were monitored by nephelometry (Coleman nephelometer). Viability was determined as described in the text. CFU, Colony-forming units.*

alyzed and concentrated as for electrophoresis, were layered on top of linear sucrose gradients (5 to 25% in saline) with or without the presence of 0.04% sodium dodecyl sulfate throughout the gradient. Centrifugation, collection of fractions, and determination of radioactivity were carried out as described in a recent publication (17).

Lipid extraction and thin-layer chromatography. Lipids were extracted from *S. sanguis* (cells or supernatant fluids) with chloroform and methanol by the method of Arbogast and Henderson (1). Two-dimensional thin-layer chromatography was performed with commercially available Silica Gel G plates (E. Merck, 0.25-mm thickness). The plates were developed in the first dimension with chloroform-methanol-water (65:25:4) and in the second with chloroform-methanol-acetic acid (80:15:8) (13). The lipids located by autoradiography with Cronex 2_C^D X-ray film (E. I. du Pont de Nemours & Co., Wilmington, Del.) were quantitated by scraping the appropriate areas of gel into vials and counting directly in toluene scintillation fluid. Tentative identification of phospholipids was done by comparison of the R_f values with those of authentic phospholipid standards (Sigma).

RESULTS

Inhibition of growth of *S. sanguis* by penicillin. Figure 1 documents our basic observation: treatment of *S. sanguis* (group H) with benzylpenicillin causes growth inhibition unaccompanied by either lysis or any major loss of viability. For a comparison, the figure also illustrates the response of two other streptococcal species to penicillin treatment: *S. pyogenes* (group A) rapidly loses viability but shows no culture lysis, whereas *S. pneumoniae* loses viability and undergoes culture lysis as well. It is important to note that each one of the three strains is extremely susceptible to benzyl-penicillin, as judged by growth inhibition: the minimum inhibitory concentrations (MICs) are between 0.007 and 0.13 μg/ml for each of the strains.

TABLE 1. *Susceptibility of S. sanguis strain Wicky (group H) to antibiotics*

Antibiotic	MIC (μg/ml)	Survival[a] at: MIC	10× MIC
Benzylpenicillin	0.013	100	63
Cloxacillin	2.5	100	ND[b]
Oxacillin	1.0	ND	67
Mecillinam	10.0	80	ND
Cephalexin	1.0	100	100
Cephaloridine	0.1	93	79
β-Chloro-D-alanine	100.0	100	ND
D-Cycloserine	100.0	ND	95
Fosfomycin	50.0	100	ND
Fluoro-D-alanine[c]	5.0[d]		
+ D-cycloserine derivative[e]	10.0[d]	ND	80
Mitomycin C	0.2	ND	0.007

[a] Percentage of surviving cells after 2 h of antibiotic treatment at 37°C.
[b] ND, Not determined.
[c] 3-Fluoro-2-deutero-D-alanine.
[d] The ratio of the antibiotics' concentrations recommended by the Merck Co.
[e] D-4-[(2-Oxo-3-pentene-4-yl)amino]-3-isoxazolidinone, sodium salt.

TABLE 2. *Susceptibility of S. pyogenes strain T4/56 (group A) to antibiotics*

Antibiotic	MIC (μg/ml)	Survival[a] at: MIC	10× MIC
Benzylpenicillin	0.0066	11.0	0.5
Cephalexin	0.5	ND[b]	2.2
Cephaloridine	0.02	ND	2.0
Fluoro-D-alanine[c]	2.0[d]	10.3	4.5
+ D-cycloserine derivative[e]	4.0[d]		

[a] Percentage of surviving cells after 2 h of antibiotic treatment at 37°C.
[b] ND, Not determined.
[c] 3-Fluoro-2-deutero-D-alanine.
[d] The ratio of the antibiotics' concentrations recommended by the Merck Co.
[e] D-4-[(2-Oxo-3-pentene-4-yl)amino]-3-isoxazolidinone, sodium salt.

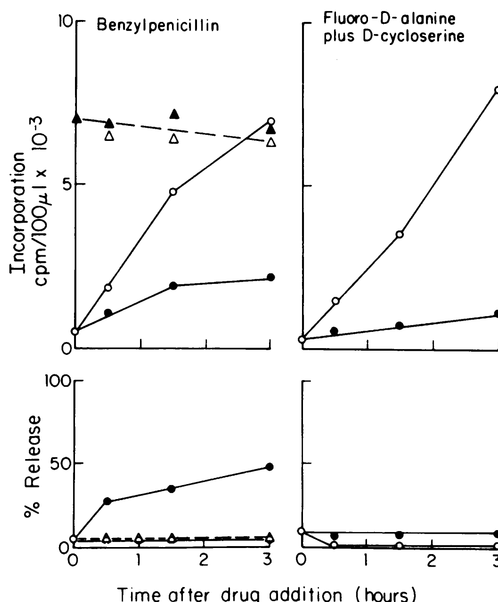

FIG. 2. *Release of N-acetylglucosamine-labeled material from antibiotic-treated S. sanguis. The concentration of benzylpenicillin was 0.66 μg/ml, and those of 3-fluoro-2-deutero-D-alanine and the D-cycloserine derivative were 5 and 10 μg/ml, respectively. Symbols: (○) control (nascently labeled cells); (●) plus antibiotic(s) (nascently labeled cells); (△) control (prelabeled cells); and (▲) plus antibiotic (prelabeled cells).*

The virtually complete lack of irreversible effects in penicillin-treated *S. sanguis* was also found true for other beta-lactams and cell wall inhibitors. Treatment with high doses of these drugs (2 h with 10 times the MICs) gave only a marginal killing effect (loss of viability in a maximum of 40% of the cells) (Table 1). In contrast, treatment of group A streptococci with beta-lactams as well as with inhibitors of early stages in cell wall synthesis (e.g., D-cycloserine) caused extensive loss of viability (Table 2).

Both group A and group H streptococci grow in chains of up to 5 to 15 cells, and this makes a precise quantitation of changes in colony-forming ability difficult. However, the low mortality of *S. sanguis* is not likely to be grossly influenced by chain formation, since the breaking-up of chains (by mechanical shear applied after penicillin treatment) did not substantially decrease the frequency of colony formers, although the average chain size was reduced to 1 to 4. Rapid and extensive loss of viability in mitomycin C-treated *S. sanguis* may be detected without difficulty (see Table 1).

Inhibition of cell wall synthesis. Treatment with benzylpenicillin (0.66 μg/ml) caused an immediate and substantial reduction in the rate of incorporation of radioactive *N*-acetylglucosamine (GlcNAc) into macromolecular material (Fig. 2). The selective use of this label for cell wall synthesis has been documented earlier (4). A significant portion (up to 50%) of the cell wall material synthesized after the addition of penicillin was found to be released into the growth medium in the form of material precipitable with cold trichloroacetic acid and carrier albumin. There was no solubilization of biosynthetically old cell wall label during the penicillin treatment (Fig. 2).

Treatment of the bacteria with a combination of D-cycloserine (10 μg/ml) and fluoro-D-alanine (5 μg/ml) caused about 90% inhibition of cell wall synthesis.

Release of glycerol-labeled material during treatment with cell wall inhibitors. Treatment with benzylpenicillin (0.66 μg/ml) and with fluoro-D-alanine (5 μg/ml) plus D-cycloserine (10 μg/ml) both caused inhibition of glycerol incorporation into macromolecular material; the inhibition was virtually immediate in the case of penicillin, whereas the halt in glycerol incorporation occurred about 1.5 h after the addition of the other drugs (Fig. 3). The drug treatments greatly stimulated the release (into the medium) of glycerol-labeled material that was precipitable with cold trichloroacetic acid in the presence of albumin carrier. A substantial fraction of both biosynthetically new and old

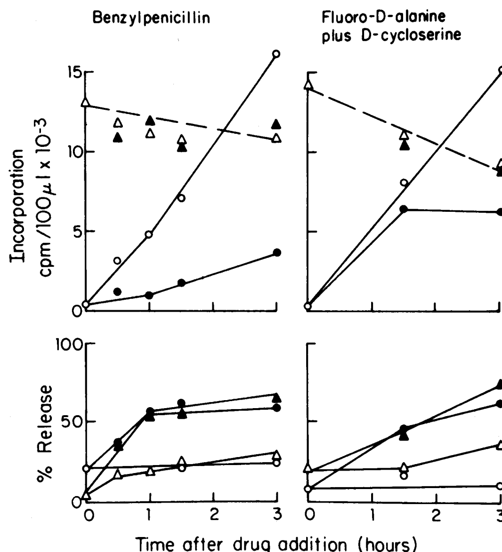

FIG. 3. *Release of glycerol-labeled material from antibiotic-treated S. sanguis. The concentration of benzylpenicillin was 0.66 μg/ml, and those of 3-fluoro-2-deutero-D-alanine and of the D-cycloserine derivative were 5 and 10 μg/ml, respectively. Symbols: (○) control (no antibiotic, nascently labeled cells); (●) plus antibiotic (nascently labeled cells); (△) control (prelabeled cells); and (▲) plus antibiotic (prelabeled cells).*

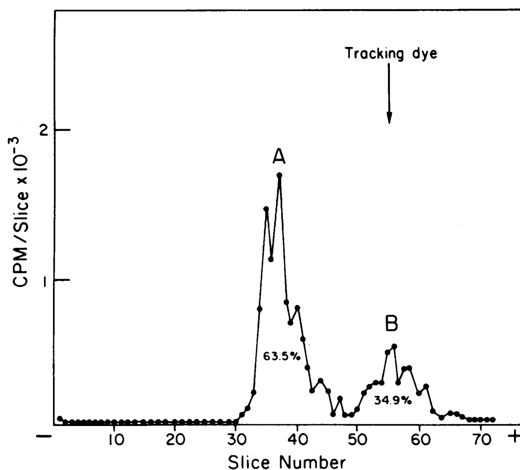

FIG. 4. *Electrophoresis of dialyzed and concentrated supernatant fluids from S. sanguis treated with benzylpenicillin. The sample was derived from the supernatant of a 10-ml streptococcal culture that received 0.66 μg of benzylpenicillin per ml in the exponential phase of growth (2 × 10^7 colony-forming units/ml) and was incubated with the drug for 90 min. The cell-free supernatant was dialyzed against water, freeze-dried, and redissolved in water. A 200-μl sample (containing 3 × 10^5 cpm) was subjected to electrophoresis on 10% acrylamide gels at pH 7.4 in the presence of sodium dodecyl sulfate, according to the method of Weber and Osborn (20).*

glycerol label (i.e., glycerol label incorporated either before or after the drug addition) was found to be released into the growth medium (Fig. 3).

Nature of the glycerol-labeled material released into the medium. The supernatant of penicillin-treated and glycerol-labeled bacteria was dialyzed, concentrated by freeze-drying, and analyzed by several biochemical methods. Gel electrophoresis in the presence of sodium dodecyl sulfate separated the glycerol-labeled materials into two major fractions (Fig. 4A and B). Fraction B, running near the tracking dye and representing about 30% of the radioactivity, was composed of lipids. Extraction of the supernatant of penicillin-treated bacteria with lipid solvents quantitatively removed this fraction of glycerol label. Analysis by thin-layer chromatography showed the presence in the supernatants of each one of the major cellular glycerol-lipids. Although an increase in the relative amount of phosphatidylglycerol was accompanied by a decrease in cardiolipin, all other glycerol-lipids were in virtually the same concentrations as found in normally growing cells (Table 3). Fraction A of the sodium dodecyl sulfate-gel electrophoresis was tentatively identified as lipoteichoic acid-containing material on the basis of the following properties: formation of micellar aggregates, disaggregation by detergents, insolubility in lipid solvents and distribution into the water phase during phenol-water extraction, and positive reaction with antiserum against polyglycerol phosphate (7).

The release of macromolecular cell surface material shows considerable specificity. (i) Release is only caused by inhibitors of cell wall synthesis. (ii) Release is restricted to material labeled with glycerol (and, in the case of cells treated with beta-lactams, to GlcNAc-labeled material), but it does not occur with protein and

TABLE 3. *Lipids extracted from [^{14}C]glycerol-labeled strain Wicky cells and supernatant fluids of penicillin-treated cells*

Lipid[a]	Before addition of antibiotic (% in cells)[b]	After 90 min of incubation (37°C) with:[c]			
		No addition, % in:		Benzylpenicillin (1 U/ml), % in:	
		Cells	Supernatant	Cells	Supernatant
Glycolipid A	17.7	30.8	36.8	18.6	28.8
PG	29.1	18.8	35.9	17.0	36.6
CL	19.4	25.3	6.1	39.0	10.1
Glycolipid B	13.8	9.8	10.1	8.4	10.9
Neutral lipids	19.9	14.6	10.9	16.0	11.7

[a] The tentative identification of the phospholipids made by comparison with authentic standards, which include: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL).

[b] The values listed are the percentages of the total counts chromatographed.

[c] The percent release of total acid-insoluble [^{14}C]glycerol counts per minute into the supernatant was 7.0 before drug addition, 10.0 in the control at 90 min, and 63 in the penicillin-treated sample. Lipid-extractable counts per minute accounted for 65 to 75% of the total counts per minute in the cells and 40 to 50% of that in the supernatants.

TABLE 4. *Selectivity of the antibiotic-induced release of macromolecular material from S. sanguis*

Antibiotic	Concn (μg/ml)	Total incorporation[a] (% of control)				Release[b] (% of total)			
		Lysine	Phenylalanine	Uracil	Glycerol	Lysine	Phenylalanine	Uracil	Glycerol
None		100	100	100	100	1.0	2.9	2.8	10.0
Benzylpenicillin	0.066	65	75.7	69.6	36.7	7.0	5.1	4.0	38.5
Fluoro-D-alanine[c]	10.0[d]								
+ D-cycloserine derivative[e]	20.0[d]	80	80.1	85.3	70.3	4.7	4.2	4.9	56.1

[a] Bacteria were labeled with tritiated precursors (nascent labeling, see legend to Fig. 3) plus or minus the drugs for 90 min at 37°C. Incorporation into trichloroacetic acid-precipitable material was determined with BSA carrier as described in the text.

[b] Percentage of total acid-insoluble counts per minute (in the presence of BSA) remaining in the supernatant after centrifugation.

[c] 3-Fluoro-2-deutero-D-alanine.

[d] The ratio of the antibiotics' concentrations recommended by the Merck Co.

[e] D-4-[(2-Oxo-3-pentene-4-yl)amino]-3-isoxazolidinone, sodium salt.

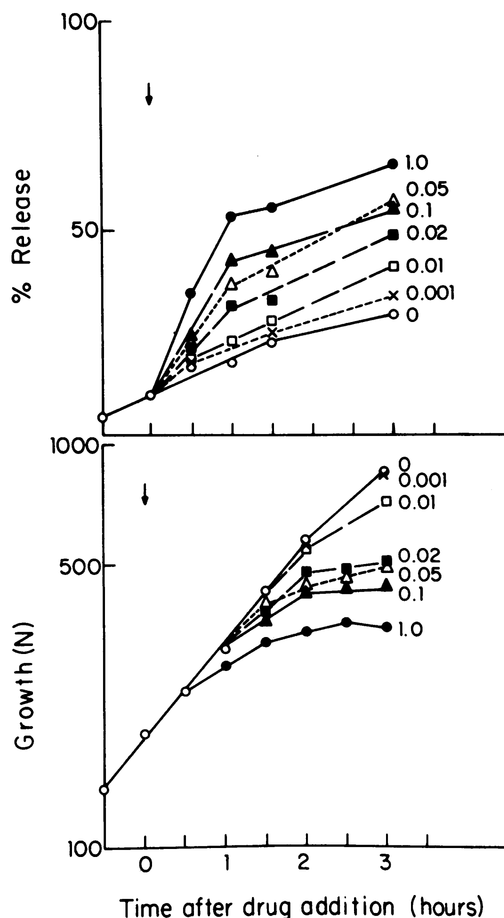

FIG. 5. *Release of prelabeled, macromolecular [^{3}H]glycerol from S. sanguis treated with low doses of benzylpenicillin. The cells were labeled by growth in [^{3}H]glycerol-containing medium (2.5 h), harvested, and allowed to grow in isotope-free medium for an additional 30 min, at which time benzylpenicillin was added (shown by the arrow). The numbers indicate the concentrations of penicillin in units per milliliter. The lower portion of the figure illustrates the effects of these concentrations of penicillin on the growth of the cells.*

nucleic acid material (Table 4). (iii) Stimulation of the release of glycerol label is proportional to the growth inhibition caused by penicillin and is already observable at the minimum growth-inhibitory concentration of the drug (Fig. 5). (iv) In contrast with the case of cell wall inhibitors, inhibition of growth by mitomycin C, rifampin, or chloramphenicol does not cause stimulation of the release of cell surface material (Fig. 6). (v) Release of glycerol label requires both the treatment with cell wall inhibitor and conditions appropriate for growth. Incubation in buffer or at low temperature or exposure of the cells to mechanical shear did not stimulate the release of glycerol-labeled macromolecules (Table 5).

Table 6 summarizes the effects of a large number of inhibitors on the synthesis and release of glycerol- and GlcNAc-labeled material. Inhibitors of both early and late biosynthetic stages of cell wall synthesis greatly stimulated the release of glycerol label into the medium, irrespective of the mode of isotope labeling (nascent, old, or continuous); beta-lactams (but not "early" cell wall inhibitors, such as D-cycloserine and the beta-halogeno alanines) also caused release of nascent GlcNAc-labeled material. Inhibitors of ribonucleic acid (rifampin), deoxyribonucleic acid (mitomycin C), and protein (chloramphenicol) syntheses caused no release of any of the isotope label.

DISCUSSION

S. sanguis is very susceptible to penicillin: 0.013 μg of benzylpenicillin added per ml of culture medium already caused inhibition of growth. In addition, penicillin treatment caused several typical effects of this antibiotic: (i) the rate of cell wall synthesis was reduced; (ii) a substantial fraction of peptidoglycan synthesized after penicillin addition was released into the growth medium, similarly to the case of penicillin-treated bacilli (2, 18); (iii) typical morphological effects of penicillin (formation of "bulges" at the cellular equator and selective inhibition of cell division) could be observed by electron microscopy; and (iv) the bacteria released into the medium lipoteichoic acid-containing macromolecules, similarly to the case described earlier for pneumococci (17). Yet in spite of the presence of these typical phenomena, penicillin had virtually no irreversible antimicrobial effects on this bacterium.

The response of *S. sanguis* to penicillin is reminiscent of that of ethanolamine-grown pneumococci and the mutant pneumococcus defective in murein hydrolase (16). Suppression of the activity of the pneumococcal autolytic system in mutants or physiological variants has been shown to cause resistance to penicillin-induced lysis and a greatly reduced rate in the loss of bacterial viability. Such bacteria nevertheless retain their high susceptibility to penicillin, and their entire dose response to penicillin remains identical to that of the wild-type bacterium (15). The term "antibiotic tolerance" has been suggested for this phenomenon (16). These findings were confirmed in autolysin-defective mutants of *Bacillus licheniformis* (12). More recently, physiological conditions were found for several bacterial species (including

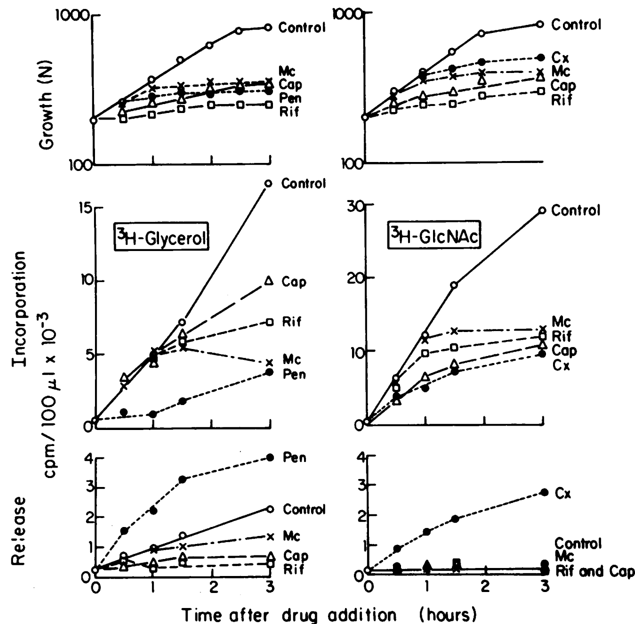

FIG. 6. *Effects of various antibiotics on growth and incorporation and release of macromolecular [³H]glycerol and ³H-labeled GlcNAc in S. sanguis. The isotopic compounds were added at the same time as the antibiotics to exponentially growing cultures. The concentrations used were as follows: benzylpenicillin (Pen), 0.66 μg/ml; mitomycin C (Mc), 1 μg/ml; rifampin (Rif), 1 μg/ml; chloramphenicol (Cap), 20 μg/ml; and cephalexin (Cx), 5 μg/ml.*

TABLE 5. *Effect of experimental conditions on the release of glycerol-labeled macromolecules.*

Experimental conditions during 90 min of incubation	Release of macromolecular glycerol (% of total) with:[a]	
	No antibiotic	Benzylpenicillin (0.66 μg/ml)
In growth medium at:		
37°C	13.4	54.7
30°C	11.2	43.2
0°C	4.0	11.4
37°C, followed by vigorous vortex mixing for 30 s	14.1	60.3
37°C, followed by freezing and thawing[b]	14.4	57.8
In SP buffer at 37°C[c]	1.0	3.7

[a] Bacteria were grown to mid-log phase in medium containing [³H]glycerol, adjusted to the appropriate temperature, and held for 90 min plus or minus penicillin. Total and supernatant trichloroacetic acid-insoluble counts per minute with BSA as carrier was determined as described in the text.

[b] The culture was frozen by immersion into a dry ice-acetone bath (−40°C) for 5 min. Melting was at 37°C.

[c] The labeled bacteria were harvested by centrifugation and resuspended in SP buffer (0.15 M NaCl plus 0.01 M K_2HPO_4).

staphylococci, streptococci, *Bacillus subtilis,* and *Escherichia coli*) that could also reduce or eliminate the lytic and bactericidal effects of penicillin without substantially affecting the MIC and dose response (3, 9). The tolerant response of *S. sanguis* (group H) to penicillin and to other cell wall inhibitors mimics all the typical features of autolysin-defective bacteria. It is important in this respect that extensive attempts to detect murein hydrolase activity (using isologous cell walls as substrates) in *S. sanguis* have been negative so far (unpublished observations). No cell wall turnover could be detected in normally growing (5) or penicillin-treated cultures (see Fig. 2) of this bacterium. On the other hand, it has been reported that *S. sanguis* made "competent" (to undergo genetic transformation with deoxyribonucleic acid) may undergo "lysis" upon incubation in a certain buffer (11). The same incubation conditions did not cause lysis of penicillin-pretreated "incompetent" streptococci (unpublished observation).

It has been suggested recently that the primary physiological response to penicillin and cell wall inhibitors may be a simple, reversible inhibition of growth in all bacteria (A. Tomasz and J. V. Höltje, *Microbiology—1977,* American Society for Microbiology, Washington, D.C., in press). However, this primary response

TABLE 6. *Release of macromolecular material into the growth medium from S. sanguis during treatment with certain antibiotics*

Antibiotic	Concn (μg/ml)	Total incorporation (% of control)[a]		Release (% of total incorporation)[b]	
		Glycerol	GlcNAc	Glycerol	GlcNAc
Nascent label					
None		100.0	100.0	17.7	1.2
Benzylpenicillin	0.66	46.5	48.0	71.0	42.2
Mecillinam	100.0	46.0	61.0	43.5	13.0
Oxacillin	100.0	55.9	26.0	53.0	51.8
Keflin	100.0	58.0	14.5	52.0	45.9
Cephalexin	5.0	51.0	47.9	65.0	17.8
Cephaloridine	1.0	52.0	ND[c]	68.7	ND
Fosfomycin	100.0	69.0	66.0	42.2	4.6
β-Chloro-D-alanine	200.0	70.4	54.8	43.6	3.2
D-Cycloserine	100.0	65.2	42.5	48.3	3.8
Fluoro-D-alanine[d] +	20.0[e]				
D-cycloserine derivative[f]	40.0[e]	55.4	20.8	79.0	2.7
Mitomycin C	1.0	82.9	69.1	11.4	2.1
Rifampin	1.0	87.8	59.3	5.5	2.8
Chloramphenicol	20.0	92.4	48.4	6.3	1.7
Old label					
None		100.0	100.0	23.4	1.2
Benzylpenicillin	0.66	97.0	103.0	55.7	0.7
Fluoro-D-alanine[d]	20.0[e]	93.0	ND	65.5	ND
+ D-cycloserine derivative[f]	40.0[e]				
Continuous label					
None		100.0	100.0	22.9	1.7
Benzylpenicillin	0.66	76.0	86.9	70.6	4.8
Cloxacillin	2.5	84.0	ND	64.6	ND

[a] Bacteria were labeled with radioactive isotopes added to the growth medium at concentrations indicated in the text. Three types of labeling regimes were used. (i) The culture received radioactive isotope at a cell concentration of approximately 5×10^6 colony-forming units/ml; after 90 min (corresponding to 1.5 doublings of cell mass) the cultures were harvested, resuspended in isotope-free growth medium, and incubated at 37°C. Drugs were added to such cultures 30 min after resuspension (old label). (ii) Isotopes and drugs were added simultaneously (nascent label). (iii) In continuous labeling, the regime was similar to that in (i), except the cells were left in the isotope-containing medium during drug treatment. Incorporation into trichloroacetic acid-precipitable material was determined with BSA carrier added, as described in the text.

[b] Percentage of total acid-insoluble counts per minute (plus BSA) remaining in the supernatant after centrifugation.

[c] ND, Not determined.

[d] 3-Fluoro-2-deutero-D-alanine.

[e] The ratio of the antibiotics' concentration recommended by the Merck Co.

[f] D-4[(2-Oxo-3-pentene-4-yl)amino]-3-isoxazolidinone, sodium salt.

can only be observed in autolysin-defective cells, since in most wild-type species the primary response is rapidly followed by secondary effects (triggering of murein hydrolases and, possibly, a separate "killing" event) that constitute the irreversible aspects of penicillin action. In this context *S. sanguis* may represent a bacterial species that is autolysin-defective in the wild type. Alternatively, this bacterium may have an unusually well-controlled (set of) murein hydrolase(s) that would become inhibited whenever cell wall synthesis comes to a halt (e.g., it may require a peptidoglycan precursor for catalytic activity).

Studies by Ward and co-workers (2, 18, 19) have shown that penicillin-treated bacteria may continue peptidoglycan synthesis in such a manner that the products end up in the outside medium. Penicillin-treated penumococci continue synthesis of cell wall teichoic acid and lipid-containing teichoic acid material (Forssman antigen) and secrete these polymers into the growth medium (17). *S. sanguis* seems to exhibit both peptidoglycan release and release of lipoteichoic acid-containing material. In addition, a substantial amount of lipid material also appears in the medium. The exact chemical nature of the polymer containing the lipoteichoic acid-like material is not known yet. Spontaneous release of lipoteichoic acids has been reported in some strains of oral streptococci and lactobacilli (6, 10). It is not clear what the rela-

tionship of this phenomenon may be to the antibiotic-induced lipoteichoic acid release described in this communication. Neither is it obvious why and via what exact mechanism lipid and lipoteichoic acid materials are released from this bacterium during inhibition of cell wall synthesis. Release of a lipid-containing teichoic acid (Forssman antigen) with autolysin-inhibitory activity has already been described in pneumococci (17). On the other hand, release of lipid material to the medium during treatment with cell wall inhibitors as it is described in the present communication seems to represent a novel biochemical effect of these antibiotics. The rather limited bactericidal effect of cell wall inhibitors in this bacterium indicates that the release of the glycerol-labeled macromolecules, though specifically stimulated by these drugs, is not in itself sufficient to cause irreversible damage to the bacteria.

S. sanguis, *S. pyogenes*, and *S. pneumoniae* represent a whole spectrum in physiological responses to penicillin treatment. Parallel studies on these three species may provide useful further information concerning the mechanism of the cytocidal and lytic effects of antibiotics.

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LITERATURE CITED

1. **Arbogast, L. Y., and T. O. Henderson.** 1975. Effect of inhibition of protein synthesis on lipid metabolism in *Lactobacillus plantarum*. J. Bacteriol. 123:962-971.
2. **Elliott, T. S. J., J. B. Ward, and H. J. Rogers.** 1975. Formation of cell wall polymers by reverting protoplasts of *Bacillus licheniformis*. J. Bacteriol. 124:623-632.
3. **Goodell, E. W., R. Lopez, and A. Tomasz.** 1976. Suppression of lytic effect of beta lactams on *Escherichia coli* and other bacteria. Proc. Natl. Acad. Sci. U.S.A. 73:3293-3297.
4. **Horne, D. S., and D. Perry.** 1974. Effect of competence induction on macromolecular synthesis in a group H streptococcus. J. Bacteriol. 118:830-836.
5. **Horne, D. S., and D. Perry.** 1975. Relationship of macromolecular synthesis to competence induction in a group H streptococcus. J. Bacteriol. 121:1014-1021.
6. **Joseph, R., and G. D. Shockman.** 1975. Synthesis and excretion of glycerol teichoic acid during growth of two streptococcal species. Infect. Immun. 12:333-338.
7. **Knox, K. W., and A. J. Wicken.** 1973. Immunological properties of teichoic acids. Bacteriol. Rev. 37:215-257.
8. **Lacks, S., and R. D. Hotchkiss.** 1960. A study of the genetic material determining an enzyme activity in Pneumococcus. Biochim. Biophys. Acta 39:508-517.
9. **Lopez, R., C. Ronda-Lain, A. Tapia, S. B. Waks, and A. Tomasz.** 1976. Suppression of the lytic and bactericidal effects of cell wall-inhibitory antibiotics. Antimicrob. Agents Chemother. 10:697-706.
10. **Markham, J. L., K. W. Knox, A. J. Wicken, and M. J. Hewett.** 1975. Formation of extracellular lipoteichoic acid by oral streptococci and lactobacilli. Infect. Immun. 12:378-386.
11. **Ranhand, J. M., C. G. Leonard, and R. M. Cole.** 1971. Autolytic activity associated with competent group H streptococci. J. Bacteriol. 106:257-268.
12. **Rogers, H. J., and C. W. Forsberg.** 1971. Role of autolysins in killing of bacteria by some bactericidal antibiotics. J. Bacteriol. 108:1235-1243.
13. **Skipski, V. P., and M. Barclay.** 1969. Thin-layer chromatography of lipids, p. 530-597. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press Inc., New York.
14. **Tomasz, A.** 1970. Cellular metabolism in genetic transformation of pneumococci: requirement for protein synthesis during induction of competence. J. Bacteriol. 101:860-871.
15. **Tomasz, A.** 1974. The role of autolysins in cell death. Ann. N.Y. Acad. Sci. 235:439-447.
16. **Tomasz, A., A. Albino, and E. Zanati.** 1970. Multiple antibiotic resistance in a bacterium with suppressed autolytic system. Nature (London) 227:138-140.
17. **Tomasz, A., and S. Waks.** 1975. Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. Proc. Natl. Acad. Sci. U.S.A. 72:4162-4166.
18. **Tynecka, Z., and J. B. Ward.** 1975. Peptidoglycan synthesis in *Bacillus licheniformis*. The inhibition of cross-linking by benzyl penicillin and cephaloridin *in vivo* accompanied by the formation of soluble peptidoglycan. Biochem. J. 146:253-267.
19. **Ward, J. B.** 1974. The synthesis of peptidoglycan in an autolysin-deficient mutant of *Bacillus licheniformis* N.C.T.C. 6346 and the effect of beta-lactam antibiotics, bacitracin and vancomycin. Biochem. J. 141:227-241.
20. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecylsulfate. J. Biol. Chem. 244:4406-4412.