INFECTION AND IMMUNITY, Dec. 1977, p. 717-725

Vol. 18, No. 3
Printed in U.S.A.

Cell Envelope of *Neisseria gonorrhoeae*: Penicillin Enhancement of Peptidoglycan Hydrolysis

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Received for publication 28 June 1977

The addition of 10 μg of penicillin G per ml to log-phase cultures of *Neisseria gonorrhoeae* JW-31 (minimum inhibitory concentration for penicillin G, <0.007 μg/ml) resulted in cellular lysis after a lag of 30 min. Penicillin markedly decreased the rate of peptidoglycan synthesis and enhanced the rate of hydrolysis of existing peptidoglycan. Hydrolysis was initiated immediately after addition of penicillin; cellular lysis did not occur until a considerable percentage of the peptidoglycan had been degraded. Cellular lysis was not due to penicillin per se but resulted from inhibition of cell wall synthesis. When cells were grown in media buffered with *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid at pH 6, penicillin did not cause lysis; however, at this pH, peptidoglycan hydrolysis occurred and cells lost viability at the same rate as in the control (pH 7.2). We suggest that the stability of gonococci grown at pH 6 is related to increased stability of the outer membrane. The penicillin-enhanced rate of peptidoglycan hydrolysis decreased approximately 50% at pH 6.0. Penicillin-enhanced lysis, peptidoglycan hydrolysis, and loss of viability were also markedly reduced in cells grown at 28°C.

Unlike the majority of gram-negative bacteria, gonococci are sensitive to low concentrations (0.1- to 1-μg/ml range) of penicillin G (PEN). Recently, PEN-resistant strains possessing a plasmid that codes for β-lactamase have been isolated (1). Other strains that exhibit either increased resistance or hypersensitivity to a wide spectrum of antibiotics, including PEN, have also been described (19). These strains appear to be altered with respect to outer membrane permeability (3, 17). In gram-negative enteric bacteria, one of the functions of the outer membrane may be a nonselective permeability barrier to large molecules (10). The specific contributions of phospholipids, proteins, and lipopolysaccharide in determining the permeability properties of the gonococcal outer membrane have not been resolved.

The fragility of gonococci is well known; however, the basis for the poor survival of these bacteria outside the host under nongrowth conditions (22) is not understood. Fragility and antibiotic sensitivity are important aspects of the pathophysiology of gonococci. We have previously shown that autolysis in buffer is accompanied by peptidoglycan (PG) hydrolysis (23). The available data suggest that the stability of the outer membrane may be a factor in this, since PG hydrolysis occurs under conditions (e.g., in the presence of divalent cations or at an acid pH) under which cells do not autolyse. This communication reports that hydrolysis of existing PG precedes PEN-enhanced lysis of gonococci and that growth conditions influence gonococcal sensitivity to PEN-enhanced lysis by altering the rate of existing PG hydrolysis.

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MATERIALS AND METHODS

Organism and growth conditions. *Neisseria gonorrhoeae* JW-31 colonial type 4 was used in these studies. The minimum inhibitory concentration of PEN for strain JW-31 was <0.007 μg/ml. Cultures were maintained as previously described (12).

The basal medium (LGCB) contained (per liter of distilled water): proteose peptone no. 3, 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 5 g; and soluble starch, 1 g. The unadjusted pH of the medium was 7.2 and, unless indicated, pH was not adjusted. To determine the effect of growth pH on the properties of strain JW-31, *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) was added to the medium at a concentration of 50 mM, and pH was adjusted with NaOH (pH range of 5.5 to 8.5). $NaHCO_3$ (420 mg/liter) and growth factor supplement similar to IsoVitaleX but lacking glucose (1%, vol/vol) were added after autoclaving. Glucose (0.5%, wt/vol) or sodium pyruvate (0.5%, wt/vol) was added as the energy source, as indicated. Cultures were inoculated from frozen-cell suspensions as previously described (4) and incubated in a gyratory shaker at 37 or 28°C. Turbidity was measured by Klett-Summerson colorimetry at 540 nm.

Measurement of autolysis and PEN-enhanced lysis. For autolysis studies, log-phase cultures in

LGCB-glucose medium (100 to 150 Klett units) were centrifuged (4,100 × *g* for 5 min at 20°C) and suspended in 50 mM HEPES buffer (pH 8.5 or 6.0). Where indicated, $MgCl_2$ was added to a final concentration of 10 mM. Autolysis was measured as the initial rate of decrease in turbidity at 540 nm. The autolytic rate constant (k) was calculated as $k = \log_{10} C_0/C_1 \times 2.303 \times \text{min}^{-1}$, where C_0 and C_1 indicate turbidity at T_0 and T_1, respectively. To determine response to PEN, potassium penicillin G (Eli Lilly & Co., Indianapolis, Ind.) was added to a final concentration of 10 μg/ml to log-phase cultures (90 to 100 Klett units) in LGCB-glucose medium, and turbidity was monitored as described above. Viability was determined by plating serial dilutions on GC agar (Difco Laboratories, Detroit, Mich.) as previously described (12). The stability of PEN in solution buffered over a pH range of 5.5 to 8.0 was determined by the procedure of Sabath et al. (16).

Preparation of labeled PG. To radioactively label PG, cells were grown to mid-log phase (100 Klett units) in LGCB-pyruvate medium, then [6-^{3}H]glucose (New England Nuclear Corp., Boston, Mass.; specific activity, 33.9 Ci/mmol) was added (1 to 2 μCi/ml of medium), and the culture was incubated for an additional 20 min. Under these conditions, ^{3}H incorporation stopped after 10 to 15 min (23). Unlabeled glucose (0.2%, wt/vol) was added, and the culture was incubated for an additional 10 min to facilitate incorporation of any residual intracellular [^{3}H]glucose; then cells were harvested by centrifugation (4,100 × *g* for 5 min at 20°C). These conditions label newly synthesized PG. To obtain old ^{3}H-labeled PG, the cell pellet was suspended in four to five times its original volume in LGCB-glucose medium and incubated for an additional two generations. Where indicated, PG also was radioactively labeled with [1-^{14}C]glucose (New England Nuclear Corp.; specific activity, 9.6 mCi/mmol). Of the labeled glucose incorporated, approximately 2.5% (2.1 to 2.7%) was located in the PG.

Measurement of PG hydrolysis. To measure turnover or hydrolysis of PG, 10-ml portions of ^{3}H- or ^{14}C-labeled cell suspensions were pipetted into centrifuge tubes, held at 4°C, containing 1 ml of unlabeled carrier cells of strain JW-31 (10 to 20 mg [dry weight] of cells). The tubes were immediately centrifuged (3,400 × *g* for 3 min) and the supernatant fluids were decanted. PG was isolated from the cell pellets by sodium dodecyl sulfate (4%, wt/vol) digestion at 100°C, followed by ultracentrifugation at 90,000 × *g* for 40 min as previously described (5, 23). The resultant clear gel-like pellets were dissolved in water and placed in vials containing 15 ml of Aquasol (New England Nuclear Corp.), and radioactivity was determined in a liquid scintillation spectrometer.

Incorporation of labeled substrates. To determine incorporation of ^{3}H or ^{14}C into cell material, 0.5- to 1.0-ml portions of labeled cell suspensions were pipetted into an equal volume of cold 20% (wt/vol) trichloroacetic acid and incubated at 4°C for 30 to 60 min. The suspensions were drawn by suction through Whatman GF/C filter disks, and the filters were sequentially washed with cold 10% (wt/vol) trichloroacetic acid and cold 95% ethanol. The filters were placed in vials and incubated for 1 to 2 h with 0.5 ml of NCS solubilizer (Amersham/Searle, Arlington Heights, Ill.), and radioactivity was determined after addition of a toluene-based scintillation fluid (13).

Determination of binding of [^{14}C]PEN. PEN binding to strain JW-31 was determined by a modification of the procedure of Rodriguez and Saz (14). Log-phase cells were suspended to a concentration of 10^{10} colony-forming units per ml in 2 ml of LGCB lacking an energy source. [^{14}C]benzyl potassium penicillin (Amersham/Searle; specific activity, 0.043 μCi/μg) was added at concentrations ranging from 0.01 to 5.0 μg/ml of medium, and the suspensions were incubated for 30 min at 37°C. To terminate binding, suspensions were diluted with 10 ml of cold LGCB and immediately centrifuged (4,100 × *g* for 5 min). The cell pellets were washed twice with 10 ml of LGCB and resuspended in 2 ml of LGCB. Portions (1 ml) of the cell suspensions were diluted to 5 ml with LGCB or with LGCB containing unlabeled PEN (2.5 mg/ml). After incubation for 10 min at 4°C, the tubes were centrifuged (4,100 × *g* for 5 min), the cell pellets were suspended in 1 ml of water, and 0.2-ml portions were placed in vials containing 15 ml of Aquasol. Radioactivity was determined as described above.

RESULTS

Effect of Mg^{2+} on PEN-enhanced lysis. We have previously reported (23) that the autolysis of *N. gonorrhoeae* JW-31 suspended in HEPES buffer (pH 8.5) was accompanied by PG hydrolysis. The addition of 10 mM $MgCl_2$ to the buffer prevented autolysis but not PG hydrolysis (Table 1). Addition of 20 mM $MgCl_2$ to LGCB-glucose medium also reduced the rate of lysis resulting from the addition of PEN to the medium (Fig. 1). In the absence of Mg^{2+}, turbidity increased for approximately 30 min after the addition of PEN (10 μg/ml) and then abruptly decreased, indicating cell lysis. Loss of viability occurred before the initiation of lysis (see Fig. 4). In the presence of Mg^{2+}, turbidity increased for 30 min after addition of PEN, and cells then lysed at a slow rate. Mg^{2+} did not

TABLE 1. *Autolysis and PG hydrolysis in N. gonorrhoeae JW-31 suspended in HEPES buffer (pH 8.5)*[a]

Addition to buffer	Autolysis[b] ($k \times 10^{-3}$)	PG hydrolysis[c] (%)
None	17	19
$MgCl_2$	<0.5	20

[a] Log-phase cells in LGCB-glucose medium were harvested by centrifugation and suspended in 50 mM HEPES buffer (pH 8.5) with or without addition of 10 mM $MgCl_2$.

[b] Autolytic rate constant calculated as described in the text.

[c] PG was labeled during growth in the presence of [6-^{3}H]glucose. After 40 min of incubation in buffer, PG was isolated and the percent loss of radioactivity was determined relative to a time-zero control.

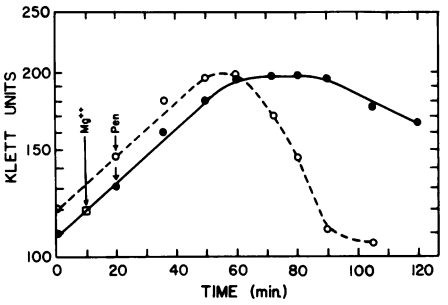

FIG. 1. *Effect of Mg^{2+} on PEN-enhanced lysis in N. gonorrhoeae JW-31. Conditions are as described in the text.*

prevent loss of viability either in autolysis buffer or in growth medium containing PEN (data not shown). These results are consistent with the hypothesis (23) that cell lysis involves both PG hydrolysis and loss of outer membrane integrity. Loss of viability appears to result from PG hydrolysis, since Mg^{2+} inhibits cell lysis (presumably by stabilizing the outer membrane) but does not prevent PG hydrolysis (23) or loss of viability. Alternatively, the stabilization of the cytoplasmic membrane and/or the change in the nature of PG hydrolysis should be considered mechanisms that regulate cell lysis.

Effect of growth conditions on the sensitivity of gonococci to PEN-enhanced lysis. Growth pH influenced the rate and extent of PEN-enhanced lysis (Fig. 2). Cells grown in LGCB-glucose medium buffered with HEPES at an acid pH (pH 5.5 to 6.0) exhibited both a reduced growth rate and a reduced rate of lysis after addition of 10 μg of PEN per ml. In contrast, cells grown in media buffered with HEPES at a neutral or alkaline pH exhibited a rapid rate of PEN-enhanced lysis. This rate was maximal in cells cultured at pH 8.0. Gonococci did not grow in LGCB medium at pH values below 5.5 or above 8.0. No decrease was observed in concentration when PEN was suspended in growth media buffered over a pH range of 5.5 to 8.0 and incubated for 3 h. Only a minimal change in pH (<0.2 pH unit) occurred during growth in HEPES-buffered media. Although growth pH altered the rate of PEN-enhanced lysis, cells grown over a pH range of 5.5 to 8.0 had similar rates of autolysis when suspended in HEPES buffer (pH 8.5; data not shown), suggesting that growth at acid pH values did not repress the synthesis of a PG hydrolase.

Growth of *N. gonorrhoeae* JW-31 at a suboptimal temperature (28°C) also resulted in a decreased rate of PEN-enhanced lysis (Fig. 3). Cells that were initially grown at 28°C and then were shifted to 37°C immediately before addition of PEN lysed at the same rate as those grown at 37°C, indicating that the low rate of PEN-enhanced lysis at 28°C was not the result of an altered cell wall composition. This conclusion is also supported by the observation that cells grown at 28 or 37°C were similar with respect to the binding of [^{14}C]PEN (Table 2). Most of the [^{14}C]PEN was tightly bound and

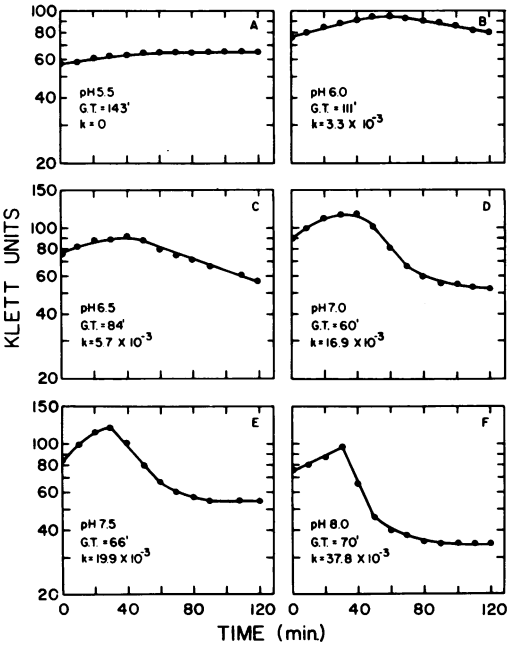

FIG. 2. *Effect of growth pH on rate of PEN-enhanced lysis. Cells were grown in LGCB-glucose medium buffered at the indicated pH with 50 mM HEPES buffer. PEN (10 μg/ml) was added to log-phase cultures and turbidity was monitored as described. G.T., Generation time in minutes; k, the rate constant for PEN-enhanced lysis.*

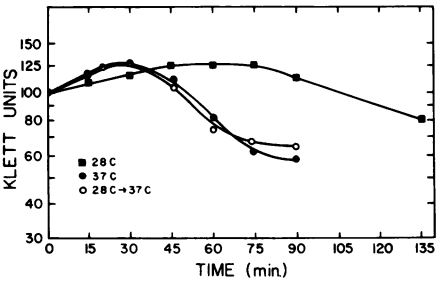

FIG. 3. *Effect of growth temperature on rate of PEN-enhanced lysis. PEN (10 μg/ml) was added to log-phase cultures in LGCB-glucose medium (pH 7.2) during growth at 28°C or 37°C. The generation time of strain JW-31 at 28°C was 150 to 160 min as compared with 70 min at 37°C.*

was not released after incubation with unlabeled PEN.

Figure 4 compares rates of viability loss after addition of PEN to cells growing in LGCB-glucose medium at 37 and 28°C and in LGCB-glucose medium buffered with HEPES (pH 6.0) at 37°C. Although cells grown in medium at pH 6 exhibited little lysis upon addition of PEN, viability decreased at the same rate as in the control (LGCB-glucose, pH 7.2 at 37°C). In contrast, loss of viability occurred at a significantly reduced rate in cells cultured at 28°C after PEN addition.

Relationship between PEN-enhanced cell lysis and PG hydrolysis. In the experiments shown in Fig. 5, old PG was labeled during growth with [6-^{3}H]glucose as described above. The hydrolysis rate of old PG was assayed in intact cells growing in the presence or absence of 10 μg of PEN per ml by determining the rate of ^{3}H loss from PG purified from cell pellets. During growth in LGCB-glucose medium at 37°C (Fig. 5A), addition of PEN resulted in cell lysis after a lag of 30 min. However, there was immediate and rapid hydrolysis of old PG. The effect of PEN on cells growing in LGCB-glucose medium at 28°C is shown in Fig. 5B. There was a lag of 30 to 40 min before cell lysis began; the rate of lysis was much less than in cells incubated at 37°C. PG hydrolysis preceded lysis but was not as extensive as in cells grown at 37°C. Figure 5C shows the effect of PEN on cells growing at 37°C in LGCB-glucose medium buffered with HEPES (pH 6.0). PEN-enhanced lysis occurred after a lag of 40 min but at a much reduced rate. PG hydrolysis preceded cell lysis; the rate of PG hydrolysis was approximately 50% of the control (cells cultured in LGCB-glucose [pH 7.2] at 37°C).

Figure 5 also demonstrates that PG turns over during growth. The rate of PG turnover (10 to 20% generation) is lower than the value of 50% per generation reported by Hebeler and Young (5). The latter study used a different strain (RD5) and medium, and labeled PG by growth in the presence of [^{3}H]diaminopimelic acid.

TABLE 2. *Binding of [^{14}C]PEN to N. gonorrhoeae JW-31*

[C^{14}]PEN added (μg/ml)	PEN bound (ng/10^{10} CFU)[a] by cultures grown at:	
	37°C	28°C
0.01	3.6 (0)	3.2 (0)
0.10	4.9 (0)	4.6 (0.1)
1.0	7.0 (0.6)	7.6 (1.0)
5.0	14.3 (1.7)	14.8 (2.1)

[a] Binding of [^{14}C]PEN to cells grown at 37°C or 28°C in LGCB-glucose was determined as described in the text. Parentheses indicate [^{14}C]PEN lost after incubation of cells with 2.5 mg of unlabeled PEN per ml. CFU, Colony-forming units.

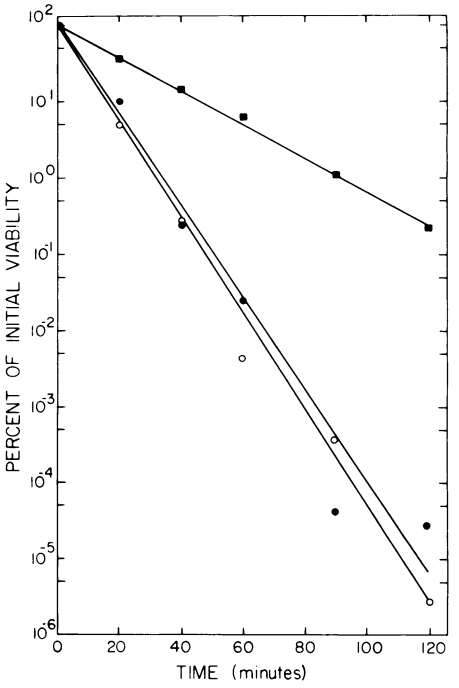

FIG. 4. *PEN-enhanced loss of viability. PEN (10 μg/ml) was added to log-phase cultures in LGCB-glucose medium at 37°C (○) or 28°C (■), or at 37°C in HEPES-buffered medium at pH 6 (●). Viability was measured as described in the text.*

To compare the PEN-enhanced hydrolysis rate of old and newly synthesized PG, old PG was labeled with [6-^{3}H]glucose and newly synthesized PG was labeled with [1-^{14}C]glucose, as described above. The ratio of ^{3}H (old)/^{14}C (new) in purified PG remained relatively constant over a 60-min incubation period in growth medium with or without addition of PEN and in autolysis buffer (data not shown), suggesting that, under each of these conditions, old and newly synthesized PG are hydrolyzed at similar rates.

Figure 6 provides additional evidence that the PEN-enhanced lysis is a consequence of PG hydrolysis. In these experiments, PEN (10 μg/ml) was added to a culture growing in HEPES-buffered medium (pH 6.0). After incubation for 45 min, the culture was centrifuged and cells were suspended either in HEPES-buffered medium (pH 6.0 or 8.0), or in LGCB-glucose (pH 7.2; no HEPES) with or without chloramphenicol (CAP, 50 μg/ml). PEN-sensitized cells did not lyse in pH 6.0 medium (lysis-nonpermissive conditions; Fig. 6A), but ex-

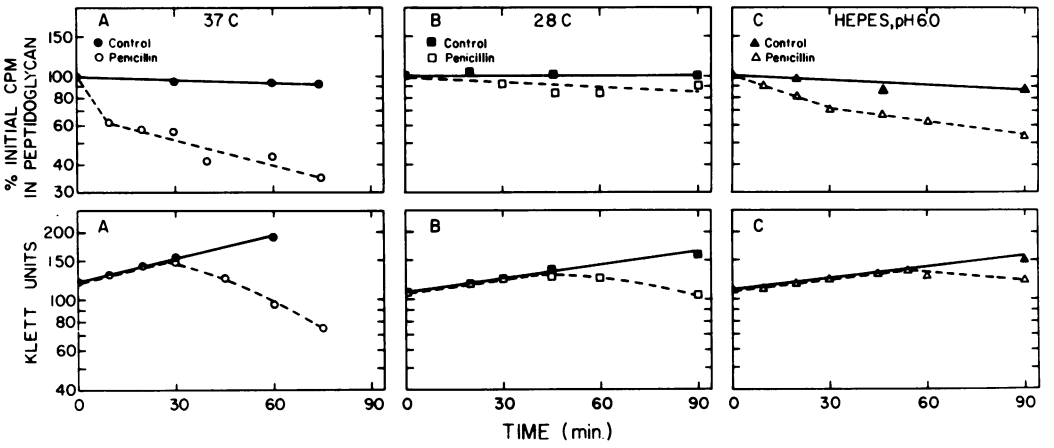

FIG. 5. *PEN-enhanced hydrolysis of old PG. Cells were grown in LGCB-glucose medium at 37°C (A) or 28°C (B), or in HEPES-buffered medium (pH 6) at 37°C (C). The hydrolysis of old PG labeled with [6-^{3}H]-glucose in log-phase cultures incubated with or without PEN (10 μg/ml) was determined relative to a time-zero control as described in the text.*

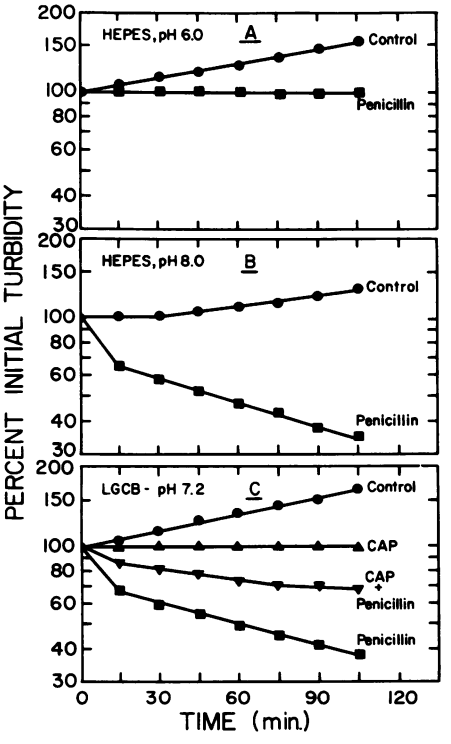

FIG. 6. *PEN-tolerant and lysis-permissive growth conditions. Log-phase cultures in LGCB-glucose medium buffered with HEPES (pH 6) were incubated at 37°C with or without PEN (10 μg/ml) for 45 min. The cultures were then centrifuged and the cells were suspended at 37°C in HEPES-buffered medium at pH 6 (A) or pH 8 (B), or in LGCB-glucose at pH 7.2 (C) with or without addition of CAP (50 μg/ml). Turbidity was monitored, and data are expressed as percent initial turbidity.*

hibited immediate and rapid lysis in pH 8.0 medium (lysis-permissive conditions; Fig. 6B). Experiments of similar design have given rise to identical conclusions concerning the role of murein hydrolases in the PEN-induced lysis of *Bacillus subtilis, Escherichia coli,* and several other bacteria (2, 11). The results obtained when PEN-sensitized cells were transferred to LGCB-glucose (pH 7.2; lysis-permissive conditions) in the presence of CAP (Fig. 6C) are of special interest. In strain JW-31, CAP inhibits growth, protein synthesis, and lysis upon subsequent addition of PEN (see Table 3). When cells growing at pH 6.0 were first sensitized to PEN and then transferred to medium at pH 7.2 containing CAP, lysis still occurred but at a reduced rate. These data suggest that CAP does not prevent PEN-enhanced lysis by inhibiting the synthesis or activity of a PG hydrolase. Identical conclusions have been reached for the pneumococcal system (21).

To determine the effect of CAP on glucose incorporation and PG synthesis, cultures were incubated with CAP (50 μg/ml) for various time periods, then labeled with [6-^{3}H]glucose (2 μCi/ml) for 10 min. Portions (0.5 ml) were removed to determine incorporation of ^{3}H into trichloroacetic acid-insoluble material. The remainder of the suspension was centrifuged, and PG was isolated from the pellet. Rate of PG synthesis (or glucose incorporation) is expressed as the percentage of ^{3}H incorporated into PG (or trichloroacetic acid-insoluble material) at time T relative to ^{3}H incorporated into PG (or trichloroacetic acid-insoluble material) at time zero. The results (Fig. 7) show that PG synthesis continues but at a reduced rate when cells are

incubated in the presence of CAP.

The effect of PEN on the PG synthesis rate in cells cultured under lysis-nonpermissive (LGCB-glucose medium at 28°C and HEPES [pH 6] medium at 37°C) and lysis-permissive (LGCB-glucose at 37°C) conditions was examined. PEN inhibited PG synthesis to a similar extent under each of these conditions (Fig. 8). Similar results were obtained when D-cycloserine (200 μg/ml; Sigma Chemical Co., St. Louis, Mo.) was substituted for PEN (data not shown).

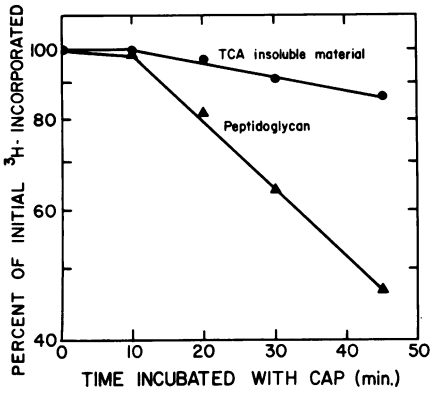

FIG. 7. *Effect of CAP on glucose incorporation and PG synthesis. Log-phase cultures in LGCB-pyruvate medium at 37°C were incubated with CAP (50 μg/ml). After the times indicated, 10-ml portions of the culture were withdrawn and incubated for 10 min with [6-^{3}H]glucose (2 μCi/ml). Portions (0.5 ml) were removed to determine incorporation of ^{3}H into trichloroacetic acid-insoluble material. The remainder of the suspension was centrifuged, and PG was isolated from the pellet. The rate of PG synthesis was calculated as percent ^{3}H incorporated into PG (in 10 min) at time T relative to ^{3}H incorporated into PG at time zero (immediately preceding addition of CAP).*

The effects of PEN, D-cycloserine, and CAP on cell lysis and hydrolysis of old PG under growth conditions are shown in Table 3. Hydrolysis of old PG was measured after a 40-min incubation period. In the presence of PEN, both cell lysis and PG hydrolysis occurred; addition of Mg^{2+} prevented cell lysis but not PG hydrolysis. The addition of D-cycloserine resulted in both cell lysis and PG hydrolysis, demonstrating that PG hydrolysis was not a specific effect of PEN but may result from a general inhibition of new wall synthesis. CAP inhibited both growth and PG turnover. Although PEN-enhanced lysis was prevented by preincubation of gonococci with CAP, CAP reduced but did not prevent the PEN-enhanced hydrolysis of PG. This finding agrees with previous results (23) demonstrating that incubation of log-phase cultures with CAP, followed by suspension in autolysis buffer, reduced but did not prevent PG hydrolysis.

DISCUSSION

Although it had been speculated (15, 24) that PEN-induced lysis resulted from an unregulated activity of cell autolytic enzymes, this concept has only recently been demonstrated experimentally. The relationship between PEN-enhanced lysis and autolytic enzymes has been most clearly defined in *Streptococcus pneumoniae* by

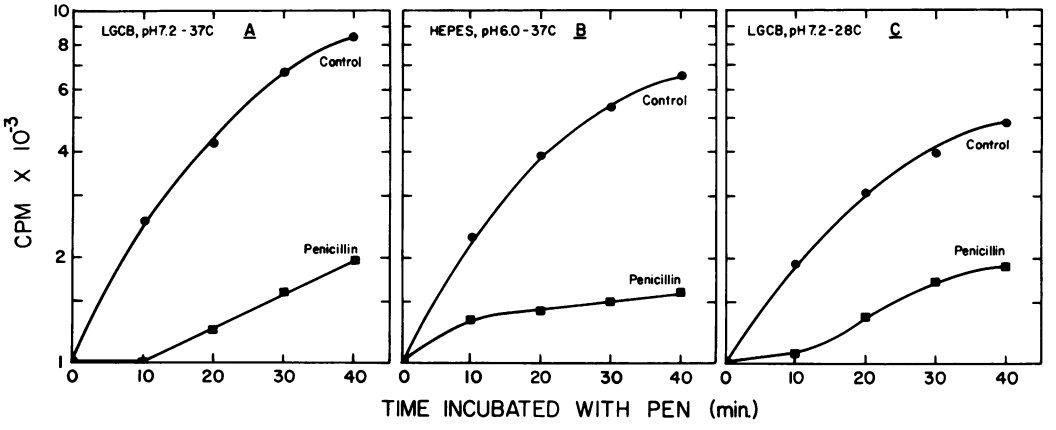

FIG. 8. *Inhibition of PG synthesis by PEN under tolerant and lysis-permissive conditions. Log-phase cultures in LGCB-pyruvate medium containing 0.02% glucose were incubated with or without addition of PEN (10 μg/ml). PG synthesis was measured as rate of incorporation of [6-^{3}H]glucose (2 μg/ml) into PG. The growth conditions employed were LGCB (pH 7.2) at 37°C (A), or 28°C (B), or HEPES-buffered medium (pH 6.0) at 37°C (C).*

TABLE 3. *Effect of selected antibiotics on cell lysis and hydrolysis of old PG under growing conditions*

Additions to medium[a]	Change (%) in turbidity[b]		Hydrolysis (%) of PG[c] (40 min)
	30 min	60 min	
None	+18	+45	7
PEN	+17	−32	39
PEN, Mg^{2+}	+26	+32	44
D-Cycloserine	+17	−43	65
CAP	0	0	0
CAP, PEN	0	0	0

[a] Antibiotics were added to log-phase cultures growing at 37°C in LGCB-glucose medium at the following concentrations per ml: PEN, 10 μg; D-cycloserine, 200 μg; and CAP, 50 μg. $MgCl_2$ was added at a concentration of 20 mM.

[b] Values preceded by + indicate the percent increase in turbidity (turbidity at time T/initial turbidity); values preceded by − indicate percent loss in turbidity between 30 and 60 min.

[c] Old PG was labeled during growth in the presence of [6-^{3}H]glucose as described in the text. PG hydrolysis was measured after purification of PG as loss of ^{3}H relative to a time-zero control.

Tomasz and co-workers. In *S. pneumoniae* (8), as in *N. gonorrhoeae* (6), the major PG hydrolase is believed to be an *N*-acetylmuramyl-L-alanine amidase. When pneumococci are grown under conditions in which the choline in the cell wall is replaced by ethanolamine, the cell wall becomes resistant to the action of the amidase (20). In these cells, PEN inhibits growth but does not cause lysis. Similarly, in mutants that lack the amidase and do not lyse in the presence of deoxycholate, PEN inhibits growth but does not cause lysis. Similar results have been obtained with other antibiotics that exert their inhibitory effect on cell wall synthesis at other points in its biosynthetic pathway (20). When inhibition of cell wall synthesis was not accompanied by cell lysis, the rate of loss in viability was markedly reduced. These data suggest that the primary effect of wall synthesis inhibition by these antibiotics is inhibition of growth, and that cell lysis and loss in viability are secondary events resulting from PG hydrolase activity. The stimulation of PG hydrolase activity after inhibition of wall synthesis may involve dissociation of an autolysin-inhibitor complex. PEN causes release into the medium of a lipoteichoic acid-like polymer in wild-type strains of *S. pneumoniae* grown in the presence of choline or ethanolamine and in DOC^- mutants (7, 21). Höltje and Tomasz (7) demonstrated that purified lipoteichoic acid can inhibit the activity of the amidase in vitro.

Inhibition of PG synthesis in gonococci by PEN or cycloserine results in hydrolysis of both old and newly synthesized PG. Although cultures continued to increase in turbidity for approximately 30 min after PEN addition, inhibition of PG synthesis, hydrolysis of old PG, and loss of cell viability began almost immediately. PEN-enhanced cell lysis occurred only after a considerable fraction of old PG had been solubilized. The mechanism by which the inhibition of PG synthesis results in activation of PG hydrolysis (presumably by an *N*-acetylmuramyl-L-alanine amidase) is not known. Lipoteichoic acid, which has been proposed as an inhibitor of autolysin activity in gram-positive bacteria (7), is not generally present in gram-negative bacteria (26) and could not be detected in gonococci (W. S. Wegener, unpublished data).

In gonococci, cell lysis is not necessarily a consequence of an inhibition of PG synthesis. When strain JW-31 was grown at a neutral or alkaline pH (pH 7.0 to 8.0), the addition of PEN resulted in extensive cell lysis and loss of viability after a lag of 30 min. In contrast, addition of PEN (500 to 1,000 times the minimum inhibitory concentration) to cultures growing at an acid pH (pH 5.5 to 6.0) prevented further growth but caused minimal lysis. Although PEN-enhanced lysis was markedly reduced in cells cultured at pH 6.0, PG hydrolysis still occurred (at approximately 50% of the rate at pH 7.2), and viability decreased at the same rate as under lysis-permissive conditions. The decreased rate of PEN-induced PG hydrolysis at pH 6.0 may result from decreased amidase activity at acid pH values. In intact cells suspended in HEPES buffer, the PG hydrolysis rate was highest at pH 8.5 and was 60% of maximum at pH 5.5 to 6.0 (23). The increased rate of PEN-enhanced lysis at pH 8.0, as compared with that at pH 7.2, can also be correlated with increased amidase activity at alkaline pH values. The observation that gonococci grown at pH 5.5 to 6.0 do not lyse in the presence of PEN may reflect increased stabilization of the outer membrane at an acid pH. When suspended in HEPES buffer, cells undergo rapid autolysis at pH 8.0 to 8.5 but do not autolyze at pH 5.5 to 6.0. Mg^{2+} protects against autolysis in HEPES (pH 8.5) buffer and partially prevents PEN-enhanced lysis under growth conditions at pH 7.2. Mg^{2+} does not alter the rate of PG hydrolysis either in cells suspended in buffer or in growth media containing PEN. The mechanism(s) by which acid pH and divalent cations protect against autolysis is not known. It is possible that these conditions may exert a physical effect on membrane fluidity or on interactions between membrane components. Alternatively, an acid pH may inhibit PEN-enhanced lysis by affecting the hydrolysis of membrane phospholipids. The activity of gonococcal membrane-associated

phospholipase A is greatest at pH 8.5 but is markedly reduced at pH 6.0 (18). However, phospholipid hydrolysis does not appear to be a major factor in autolysis, since the activity of phospholipase A and lysophospholipase in intact cells is stimulated by Ca^{2+} and, to a lesser extent, by Mg^{2+} (Wegener, unpublished data); both ions prevent cell autolysis in pH 8.5 buffer.

In addition to low pH, growth at a suboptimum temperature also influenced the response of gonococci to PEN. Addition of PEN to cultures growing at 28°C resulted in minimal lysis and decreased rates of viability loss and of PG hydrolysis. These data further suggest that the rate of PG hydrolysis determines rate of viability loss. Although the generation time of strain JW-31 grown at 28°C was 3.0 to 3.5 times that when grown at 37°C, the synthesis of PG was inhibited by PEN to approximately the same extent at both temperatures. Since cultures grown at 28°C exhibited a rapid rate of PEN-enhanced lysis when transferred to 37°C, it is probable that the low rate of PG hydrolysis at 28°C merely reflects a decreased amidase activity at this low temperature.

The response of bacteria to PEN as a function of growth pH has also been studied by Tomasz and co-workers (2, 11). In *B. subtilis,* PEN was bacteriostatic when added to cultures growing at pH 6.0, but was bacteriolytic when added to cultures growing at pH 8.0 (11). Similar results were observed with *S. pneumoniae* with other antibiotics that inhibit at early or late stages of cell wall biosynthesis (2). The pH-dependent PEN tolerance was attributed to reduced PG hydrolase (amidase) activity at lysis-nonpermissive pH values. Growth pH has also been shown to modify the response to PEN by various other bacteria including *E. coli, Staphylococcus aureus,* and *S. faecium* (2). *S. sanguis* appears to represent a naturally occurring PEN-tolerant species (9), since low concentrations of PEN inhibit growth but are not bacteriolytic or bactericidal. As previously observed in *S. pneumoniae* (7, 21), inhibition of cell wall synthesis by a variety of antibiotics results in the release of lipoteichoic acid-like material into the medium. The absence of a bacteriolytic or bactericidal response to PEN by *S. sanguis* can be correlated with the observation that neither PG hydrolase activity nor PG turnover could be demonstrated (9).

A hypothetical model depicting the relationship between PEN-enhanced cell lysis and PG hydrolysis in gonococci is presented in Fig. 9. Westling-Häggström et al. (25) suggested that gonococci divide in two planes at right angles to each other, and that a second site of septum formation is initiated before completion of the first. This division sequence gives rise to transient tetrads. We also have observed tetrad formation in *N. gonorrhoeae* (23). In several strains, including JW-31, tetrads rather than diplococci are the predominant cell type in agar-grown cultures. Exposure of gonococci to PEN results in bulging of the cell envelope at the sites of septum formation (25). This suggests that sites of septum formation are areas of new wall growth and that inhibition of PG synthesis by PEN occurs at these sites. In the model, growth results in the release of old and newly synthesized PG fragments in areas of new wall synthesis, i.e., sites of septum formation. Our data support previous findings (4) that PG does

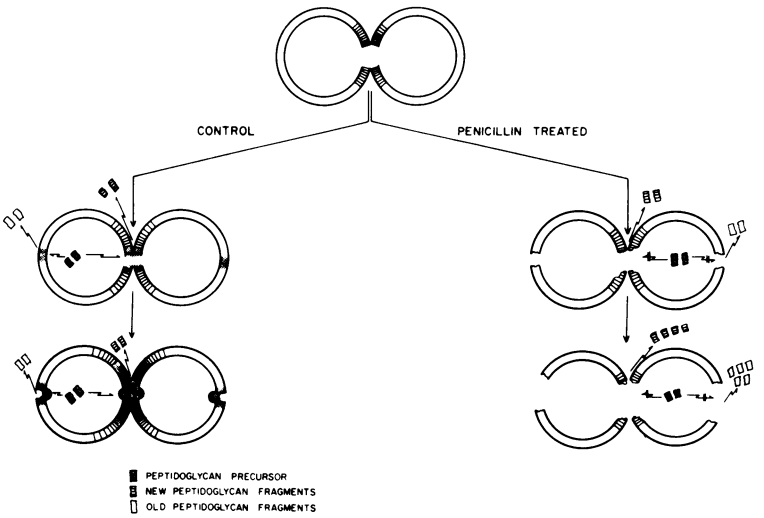

FIG. 9. *Hypothetical model depicting the relationship between PEN-enhanced cell lysis and PG hydrolysis.*

turn over during active growth; moreover, the rates of release of old and newly synthesized PG fragments appear to be similar. It is expected that insertion of new PG fragments into existing PG would require cleavage of the PG backbone. To date, the only PG hydrolase activity confirmed in gonococci is an amidase; however, previous studies (23) showed that both the peptide and glycan moieties of old PG are released when cells are suspended in buffer, suggesting the presence of additional PG hydrolases. In the presence of PEN, inhibition of new PG synthesis, coupled with turnover of existing PG, results in weakening of the cell envelope and in subsequent lysis.

In gonococci, the addition of PEN resulted in PG hydrolysis and an immediate loss of viability. In contrast, after PEN addition, turbidity increased at the same rate as that of the control before lysis occurred. Loss of viability was directly related to PG hydrolysis. Cells in which the envelope was weakened by PG hydrolysis lost the ability to form a colony under conditions (dilution and plating) used to quantitate viability. The increase in turbidity for 30 min after the addition of PEN suggests that cell division occurred in part of the population in the presence of PEN. This could be explained if cells in different stages of cell division were differentially sensitive to PEN. Cells in which septum formation had reached a critical point may complete septum formation and separate, resulting in the formation of two daughter cells and a concomitant increase in turbidity. However, such daughter cells would not be capable of further cell division and would lyse as a result of PG hydrolysis.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-12928 and AI-13571 from the National Institute of Allergy and Infectious Diseases. S.A.M. is the recipient of Public Health Service Research Career Development Award AI-00140 from the same institute.

LITERATURE CITED

1. **Elwell, L. P., M. Roberts, L. W. Mayer, and S. Falkow.** 1977. Plasmid-mediated beta-lactamase production in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **11**:528–533.
2. **Goodell, E. W., R. Lopez, and A. Tomasz.** 1976. Suppression of the lytic effects of beta lactams on *Escherichia coli* and other bacteria. Proc. Natl. Acad. Sci. U.S.A. **73**:3293–3297.
3. **Guymon, L. F., and P. F. Sparling.** 1975. Altered crystal violet permeability and lytic behavior in antibiotic-resistant and -sensitive mutants of *Neisseria gonorrhoeae*. J. Bacteriol. **124**:757–763.
4. **Hebeler, B. H., and S. A. Morse.** 1976. Physiology and metabolism of pathogenic *Neisseria*: tricarboxylic acid cycle activity in *Neisseria gonorrhoeae*. J. Bacteriol. **128**:192–201.
5. **Hebeler, B. H., and F. E. Young.** 1976. Chemical composition and turnover of peptidoglycan in *Neisseria gonorrhoeae*. J. Bacteriol. **126**:1180–1185.
6. **Hebeler, B. H., and F. E. Young.** 1976. Mechanism of autolysis of *Neisseria gonorrhoeae*. J. Bacteriol. **126**:1186–1193.
7. **Höltje, J. V., and A. Tomasz.** 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in pneumococcus. Proc. Natl. Acad. Sci. U.S.A. **72**:1690–1694.
8. **Höltje, J. V., and A. Tomasz.** 1976. Purification of the pneumococcal *N*-actylmuramyl-L-alanine amidase to biochemical heterogeneity. J. Biol. Chem. **251**:4199–4207.
9. **Horne, D., and A. Tomasz.** 1977. Tolerant response of *Streptococcus sanguis* to beta-lactams and other cell wall inhibitors. Antimicrob. Agents Chemother. **11**:888–896.
10. **Leive, L.** 1974. The barrier function of the gram-negative envelope. Ann. N.Y. Acad. Sci. **235**:109–129.
11. **Lopez, R., C. Ronda-Lain, A. Tapia, S. B. Waks, and A. Tomász.** 1976. Suppression of the lytic and bactericidal effects of cell wall-inhibitory antibiotics. Antimicrob. Agents Chemother. **10**:697–706.
12. **Morse, S. A., and L. Bartenstein.** 1974. Factors affecting autolysis of *Neisseria gonorrhoeae*. Proc. Soc. Exp. Biol. Med. **145**:1418–1421.
13. **Morse, S. A., S. Stein, and J. Hines.** 1974. Glucose metabolism in *Neisseria gonorrhoeae*. J. Bacteriol. **120**:702–714.
14. **Rodriguez, W., and A. K. Saz.** 1975. Possible mechanism of decreased susceptibility of *Neisseria gonorrhoeae* to penicillin. Antimicrob. Agents Chemother. **7**:788–792.
15. **Rogers, H. J.** 1967. Killing of *Staphylococci* by penicillins. Nature (London) **213**:31–33.
16. **Sabath, L. D., J. I. Casey, P. A. Ruch, L. L. Stumpf, and M. Finland.** 1971. Rapid microassay for circulating nephrotoxic antibiotics, p. 83–90. Antimicrob. Agents Chemother. 1970.
17. **Sarubbi, F. A., Jr., P. F. Sparling, E. Blackman, and E. Lewis.** 1975. Loss of low-level antibiotic resistance in *Neisseria gonorrhoeae* due to *env* mutations. J. Bacteriol. **124**:750–756.
18. **Senff, L. M., W. S. Wegener, G. F. Brooks, W. R. Finnerty, and R. A. Makula.** 1976. Phospholipid composition and phospholipase A activity of *Neisseria gonorrhoeae*. J. Bacteriol. **127**:874–880.
19. **Sparling, P. F., F. A. Sarubbi, Jr., and E. Blackman.** 1975. Inheritance of low-level resistance to penicillin, tetracycline, and chloramphenicol in *Neisseria gonorrhoeae*. J. Bacteriol. **124**:740–749.
20. **Tomasz, A., A. Albino, and E. Zanati.** 1970. Multiple antibiotic resistance in a bacterium with suppressed autolytic system. Nature (London) **227**:5254–5256.
21. **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.
22. **U.S. Department of Health, Education and Welfare.** 1962. Gonococcus—procedures for isolation and identification. Public Health Service Publication 499. United States Government Printing Office, Washington, D.C.
23. **Wegener, W. S., B. H. Hebeler, and S. A. Morse.** 1977. Cell envelope of *Neisseria gonorrhoeae*: relationship between autolysis in buffer and the hydrolysis of peptidoglycan. Infect. Immun. **18**:210–219.
24. **Weidel, W., and H. Pelzer.** 1964. Bagshaped macromolecules—a new outlook on bacterial cell walls. Adv. Enzymol. Relat. Areas Mol. Biol. **26**:193–232.
25. **Westling-Häggström, B., T. Elmros, S. Normark, and B. Winblad.** 1977. Growth pattern and cell division in *Neisseria gonorrhoeae*. J. Bacteriol. **129**:333–342.
26. **Wicken, A. J., and K. W. Knox.** 1975. Lipoteichoic acids. Science **187**:1161–1167.