

Peptidoglycan Biosynthesis in *Neisseria gonorrhoeae* Strains Sensitive and Intrinsically Resistant to β -Lactam Antibiotics

THOMAS J. DOUGHERTY

Laboratory of Microbiology, The Rockefeller University, New York, New York 10021

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Treatment of penicillin-sensitive and intrinsically resistant *Neisseria gonorrhoeae* strains with their respective inhibitory concentrations of penicillin caused rapid cell death. When the peptidoglycan syntheses of these two strains were examined in the presence of penicillin, the sensitive strain continued to make this cell wall polymer for an extended time, whereas the resistant strain underwent a rapid and marked depression in synthesis. Examination of the labeled sodium dodecyl sulfate-insoluble peptidoglycan made in the presence of inhibitory concentrations of penicillin revealed further differences. The primary effect on the penicillin-sensitive gonococcus was a slight change in peptide cross-linking and a sharp decline in the degree of *O*-acetylation. In contrast, the resistant strain exhibited a substantial decline in cross-linking, with a very moderate change in *O*-acetylation. The degree of saturation of the individual penicillin-binding proteins (PBPs) was assessed under these conditions. PBP 2, which exhibits a reduced affinity for penicillin in the resistant strain, appeared to be related to *O*-acetylation, whereas PBP 1 was implicated in the transpeptidation reaction.

Treatment of *Neisseria gonorrhoeae* with β -lactam antibiotics causes a series of changes in cell wall metabolism. Prior work has established the presence of penicillin-induced disturbances in peptidoglycan synthesis (2, 4), wall hydrolase activation (11, 18, 25), and secretion of cell surface components (11). Clearly, the response of the gonococcus to inhibition by penicillin of cell wall synthesis is complex.

The targets for penicillin action are the penicillin-binding proteins (PBPs), a group of enzymes located on the cytoplasmic membrane and involved with the terminal stages of peptidoglycan assembly (20, 21). The gonococcus has been shown to possess three major PBPs, and acquisition of intrinsic resistance to β -lactam antibiotics is accompanied by stepwise changes in the penicillin affinity of two of the PBPs (7). A natural extension of this finding was to examine peptidoglycan synthesis in sensitive and intrinsically resistant gonococci.

Recent investigations have revealed that, similar to the situation found in *Proteus mirabilis* (15), the gonococcus possesses *O*-acetyl groups on its peptidoglycan (3). Treatment of gonococci with β -lactam antibiotics is reported to cause a marked decline in the percentage of *O*-acetyl groups present, with little change in peptidoglycan cross-linkage (2). In the present study, we have examined the synthetic rates and structure of peptidoglycan made in the presence of penicillin by sensitive and isogenic resistant gono-

cocci. The extent of penicillin binding to the individual PBPs was assayed along with peptidoglycan biosynthesis. In contrast to results reported previously by Blundell and Perkins, who used continuously labeled peptidoglycan (2), we found that newly made cell wall in the presence of penicillin was deficient in cross-linking. In addition, the decrease in *O*-acetylation was found to be more extensive in the penicillin-sensitive strain.

MATERIALS AND METHODS

Strains and growth conditions. The *N. gonorrhoeae* strains used in this study were FA19 (obtained from P. F. Sparling, University of North Carolina, Chapel Hill) and the resistant isogenic transformant FA19tr3, which was constructed by genetic transformation and has been previously described (7, 19). The resistant strain FA19tr3 has a modified PBP 2, with a reduced affinity for β -lactam antibiotics and relatively high resistance to penicillin. The minimum inhibitory concentration (MIC) was determined by spreading $\sim 5 \times 10^4$ cells on a series of gonococcal broth agar plates containing twofold dilutions of penicillin. After 24 h of incubation in 5% CO₂ at 37°C, the lowest concentration allowing no growth was designated the MIC (7). MICs of penicillin for these strains are 0.01 and 0.5 μ g/ml, respectively.

The strains were grown at 37°C with aeration in gonococcal broth plus 420 μ g of NaHCO₃ per ml and a supplement identical in composition to IsoVitaleX in which glucose was replaced with 0.2% pyruvate (23). Bacterial growth was monitored by a Coleman Nephelometer, and experiments started at the same Neph-

los values. The viability of β -lactam antibiotic-treated cultures was measured by diluting them in brain heart infusion broth and plating appropriate dilutions on gonococcal broth agar plates. Plates were incubated for 24 h at 37°C in 5% CO₂, and colonies were counted.

Rate of peptidoglycan synthesis. Rates of peptidoglycan biosynthesis were determined by labeling mid-logarithmic phase cells with [³H]glucosamine (20.2 Ci/mmol, New England Nuclear Corp., Boston, Mass.) at 1 μ Ci/ml (24). Samples of 0.5 ml were added to ice cold 5% (final concentration) trichloroacetic acid, and after centrifugation (10 min, 6,000 \times g), 1 ml of 4% sodium dodecyl sulfate (SDS) was added to the pellet, and the sample was placed in a boiling water bath for 20 min. After cooling, the SDS-insoluble fraction was collected on Millipore filters (0.45 μ m), washed three times with distilled water, placed in a vial, and dried at 100°C for 1 h; a toluene-based fluor was added. Radioactivity was determined in a Nuclear-Chicago Mark II scintillation counter. Quench corrections were made by the channels-ratio method.

Peptidoglycan structure analysis. Gonococci were grown in gonococcal broth with supplements to mid-logarithmic phase ($\sim 2 \times 10^8$ colony forming units/ml). The culture was divided into 10-ml subcultures, and the desired concentration of penicillin (unlabeled) was added for 15 min (initial rapid binding of antibiotics occurs during this time [7]). Control cultures received no antibiotic. At the end of the 15-min period, 1 ml was removed for a PBP assay (see below). The remainder of the culture received 0.25 μ Ci of [¹⁴C]glucosamine (285 mCi/mmol, New England Nuclear) per ml for a 20-min period. In some experiments, indicated in the text, the period of preexposure to penicillin was longer.

Labeling was terminated by adding the culture to 5% (final concentration) ice cold trichloroacetic acid and holding at 0°C for at least 30 min. The cells were collected by centrifugation (10,000 \times g, 5 min), washed once, and suspended in 4% SDS in a boiling water bath for 20 min. Both the wash and the SDS were buffered with 20 mM sodium acetate, pH 6.4, to prevent loss of *O*-acetylation (3). After cooling, the SDS-insoluble peptidoglycan was collected at 100,000 \times g for 30 min. The pellet was washed with distilled water three times by ultracentrifugation. Samples were suspended in 200 μ l of 25 mM sodium acetate buffer (pH 5.0); 10 μ l (20 μ g) of *Chalaropsis* muramidase (12) was added, and the samples were incubated overnight. Digestion was >95%, determined by ultracentrifuging the suspension and testing the distribution of radioactivity in the supernatant fluid and pellet.

Thin-layer chromatography. Solubilized peptidoglycan ($\sim 15,000$ dpm) was applied to a 0.25-mm-thick silica gel-coated plastic sheet (20 by 20 cm, Eastman Organic Chemicals, Rochester, N.Y.). The thin-layer chromatography plate was activated before sample application at 100°C for 30 min. The thin-layer chromatograph was eluted twice in isobutyric acid-1 M ammonia (5:3, vol/vol) (15). The plates were dried, sprayed with En³Hance (New England Nuclear), and fluorographed for 30 to 36 h. The fluorograph was used as a template to locate the radioactive spots. These were cut out, placed in scintillation vials, and counted in a toluene-based fluor. The samples were counted for an appropriate length of time necessary to achieve statistical significance (14). Individual spots were in the range of 800 to 3,000 dpm.

PBP assay. Volumes (1 ml) of gonococci (both control and penicillin treated) from the experiments described above were removed at the indicated times and immediately exposed *in vivo* to saturating [³H]benzylpenicillin (2 μ g/ml, 25 Ci/mmol, Merck & Co., Inc., Rahway, N.J.) and incubated for 15 min. The cultures were then rapidly chilled and centrifuged, and the supernatant fluid was removed. The pellets were suspended in 50 μ l of phosphate buffer; 25 μ l of sample buffer was added (5), and they were boiled for 5 min.

The samples were subjected to polyacrylamide gel electrophoresis and fluorographed as described previously (7). Quantitation of the amount of PBP available to bind the [³H]penicillin was achieved by scanning the fluorograph with a Gelman ACD-15 computing densitometer. The percentage of unlabeled penicillin bound was estimated by comparison with control samples which received only saturating [³H]penicillin. The values are expressed as percentage of unlabeled penicillin bound to PBPs.

In initial experiments, an additional sample for estimation of PBP binding was taken at the end of the [¹⁴C]glucosamine treatment. However, incorporation of the labeled glucosamine into cell components other than peptidoglycan, most notably proteins, interfered with the quantitation of the PBP fluorograph. All PBP estimates herein were sampled at the initiation of peptidoglycan labeling.

Media and reagents. Growth media components were products of Difco Laboratories, Detroit, Mich. Reagent chemicals were obtained from Fisher Scientific, Fair Lawn, N.J., and Sigma Chemical Co., St. Louis, Mo. All electrophoresis reagents were from Bio-Rad Laboratories, Richmond, Calif.

RESULTS

Effect of β -lactam antibiotics on growth. Growing cultures of *N. gonorrhoeae* FA19 and FA19tr3 were treated with their respective MICs of benzylpenicillin (Fig. 1). The two cultures underwent loss of colony-forming ability with very similar kinetics. Treatment with the respective 0.1 \times MIC did not lead to decreased viability (data not shown).

Peptidoglycan metabolism was followed by assaying the incorporation of [³H]glucosamine into the SDS-insoluble fraction (Fig. 2). Treatment of FA19 with 1 \times MIC of penicillin resulted in only a slight change in incorporation for the first 60 min. In contrast, treatment of the intrinsically resistant strain FA19tr3 resulted in a marked and rapid depression in the rate of synthesis of SDS-insoluble material. Both strains were severely affected by the addition of 10 \times MIC of penicillin, with [³H]glucosamine incorporation sharply curtailed. The results of these experiments were confirmed by a similar experiment in which 10-min pulse-labels were substituted for the continuous labeling regime (data not shown).

Structural analysis of peptidoglycan synthesized during penicillin treatment. Cultures (10 ml) of FA19 and FA19tr3 were exposed to 0.1

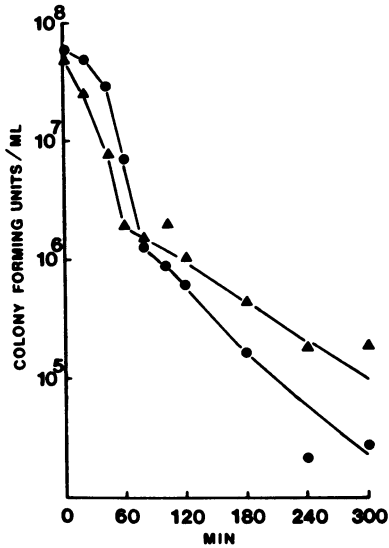


FIG. 1. Loss of viability of penicillin-treated *N. gonorrhoeae*. The penicillin-sensitive strain FA19 (MIC 0.01 $\mu\text{g/ml}$, ●) and the resistant transformant strain FA19tr3 (MIC 0.5 $\mu\text{g/ml}$, ▲) were treated with their respective inhibitory concentrations of penicillin, and the number of colony-forming units was assayed at intervals.

and 1.0 \times their respective MICs of penicillin for 15 min. A 1-ml sample was then removed from each for PBP assay, and the remaining cultures were labeled for an additional 20 min with

[¹⁴C]glucosamine in the presence of penicillin. The SDS-insoluble fractions of the cultures were isolated, digested with muramidase, and subjected to thin-layer chromatography. A typical autoradiograph of a muramidase digest is shown in Fig. 3. The relative composition of the labeled peptidoglycan is presented in Table 1. The results show that for FA19, the newly made material in the presence of 1 \times MIC of penicillin underwent a small (~3%) drop in percent cross-linkage. At the same time, the total content of *O*-acetylated components declined from 39.3 to 30.4%. When FA19tr3 was exposed under identical conditions to 1 \times MIC of penicillin, the newly synthesized peptidoglycan exhibited a decline in the degree of cross-linking of about 10%, and *O*-acetylation went from 38.6 to 35.5%.

Table 2 contains the penicillin binding data derived from the same cultures used for the peptidoglycan assays shown in Table 1. At 1 \times MIC of penicillin against FA19, virtually all of PBP 3, more than half of PBP 2, and less than 20% of PBP 1 were bound with penicillin. This can be compared with 0.1 \times MIC against the resistant strain (which is 5 \times the MIC against the sensitive strain), where the percent binding was very similar, with the exception of PBP 2, as would be expected because of the documented low affinity of the mutant PBP 2 for penicillin (7, 8). At the MIC of penicillin against FA19tr3, very little of PBPs 1 and 2 were available for biosynthesis of peptidoglycan.

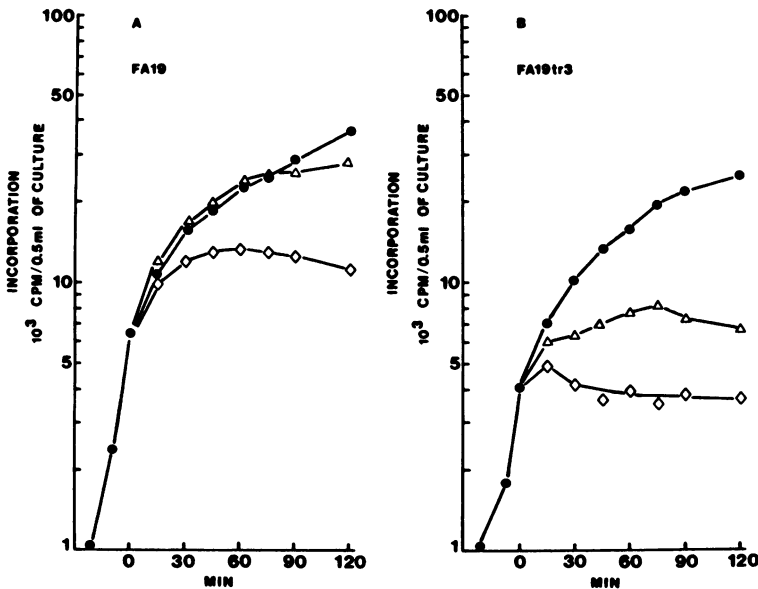


FIG. 2. Rate of peptidoglycan synthesis in penicillin-sensitive and -resistant gonococci. Incorporation of [³H]glucosamine into the hot SDS-insoluble fraction was measured in FA19 (A) and FA19tr3 (B). Penicillin was added at 0 min. Symbols: ●, control cells; ▲, cells exposed to 1 \times respective MIC; ◇, cells exposed to 10 \times respective MIC.

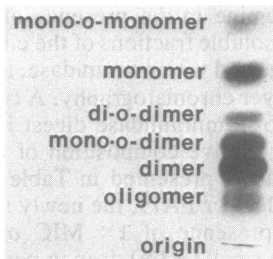


FIG. 3. Fluorograph of a thin-layer chromatograph of FA19 [^{14}C]labeled peptidoglycan fragments after muramidase digestion. The bis-disaccharide dimers give three species: di-*O*-acetylated, mono-*O*-acetylated, and nonacetylated. There are two disaccharide monomers: mono-*O*-acetylated and nonacetylated. Higher-molecular-weight species (oligomers) are unresolved by this system (2, 15).

Antibiotic concentration and time of exposure. Since peptidoglycan synthetic rates declined rapidly when FA19tr3 was treated with $1\times$ MIC of penicillin, it was technically difficult to carry out further studies with this strain. However, a number of additional experiments were performed with FA19. In the first set of experiments, subcultures were exposed to 1, 5, and $10\times$ the MIC of penicillin for 15 min and then were labeled in the presence of the antibiotic for an additional 20 min. As before, PBP saturation levels were also determined (Fig. 4A and B). The percentage of cross-linking declined with increasing antibiotic concentration, reaching a value of 26% at $10\times$ MIC. The PBP saturation levels increased from 45% at $1\times$ MIC to 100% at $10\times$ MIC for PBP 2 and 14 to 70% for PBP 1 at 1 and $10\times$ MIC, respectively. Interestingly, as the

concentration of penicillin was increased, the shift in percent *O*-acetylation declined. At $1\times$ MIC, the decrease in the *O*-acetylation value over the control value was 10.8%, whereas at $10\times$ MIC, the overall decrease in *O*-acetyl groups was 1% (data not shown).

It was also possible to follow the progress of cross-linking and PBP saturation in FA19 exposed to penicillin for increasing periods of time (Fig. 4C and D). In this case, cells received $1\times$ MIC of penicillin for 15 min before the start of the experiment (0 min in Fig. 4C and D), and then a portion of this culture was labeled from 0 to 20 min (designated "20 min") with [^{14}C]glucosamine. A second aliquot was labeled from 20 to 40 min ("40 min"), and a third aliquot was labeled from 40 to 60 min ("60 min"). PBPs were assayed at the beginning of each labeling period.

Cross-linking declined in this experiment during the 60-min incubation with penicillin. The saturation values for PBP 1 and 2 increased to 75 to 80%. The values for *O*-acetylation dropped from 36 in the control to 23% by the end of the 60-min incubation with penicillin (data not shown).

DISCUSSION

The physiological responses of penicillin-sensitive and an isogenic intrinsically resistant *N. gonorrhoeae* strain to β -lactam antibiotics were examined. The resistant strain used was constructed by genetic transformation, using purified DNA from a highly resistant clinical isolate obtained from the Centers for Disease Control (CDC77-124615) and FA19 as the recipient (7). Since MIC determinations are performed at cell

TABLE 1. Distribution of peptidoglycan fragments after muramidase digestion and thin-layer chromatography

Strain and conditions ^a	% Radioactivity for the following peptidoglycan fragment ^b :							
	Oligomer ^c	Dimer	Mono- <i>O</i> -acetylated dimer	Di- <i>O</i> -acetylated dimer	Monomer	<i>O</i> -acetylated monomer	% Cross-linkage ^d	% <i>O</i> -acetyl groups
FA19 (MIC 0.01 $\mu\text{g/ml}$)								
Control	21.7	23.5	15.5	11.0	15.5	12.8	40.2	39.3
$0.1\times$ MIC	20.0	23.6	16.6	10.4	16.3	12.9	39.3	39.9
$1\times$ MIC	18.2	26.8	13.4	7.9	24.5	9.1	36.8	30.4
FA19tr3 (MIC 0.5 $\mu\text{g/ml}$)								
Control	15.8	26.0	18.1	9.8	19.5	10.7	38.0	38.6
$0.1\times$ MIC	14.6	25.3	15.6	9.9	23.4	11.2	35.6	36.7
$1\times$ MIC	9.3	19.8	13.4	9.2	35.5	12.9	27.7	35.5

^a MIC refers to that of penicillin against each strain.

^b Results are from representative experiment. Standard deviations for percent cross-linkage and percent *O*-acetylation from triplicate set of experiments were all within ± 2.4 .

^c Oligomers were slow-running material that moved from the origin. This system does not adequately resolve the *O*-acetylated multimers, and these species are therefore not represented in the tabulation of *O*-acetyl groups.

^d Calculated as $0.7 \times$ oligomer plus $0.5 \times$ dimers.

TABLE 2. Penicillin-binding proteins in FA19 and FA19tr3

Strain and conditions ^a	% of the following PBP bound ^b		
	PBP 1	PBP 2	PBP 3
FA19 (MIC 0.01 $\mu\text{g/ml}$)			
0.1 \times MIC	0	0	39
1 \times MIC	16	60	97
FA19tr3 (MIC 0.5 $\mu\text{g/ml}$)			
0.1 \times MIC	21	20	98
1 \times MIC	78	92	100

^a MIC refers to that of penicillin against each strain.

^b PBPs were measured simultaneously with the peptidoglycan labeling experiment in Table 1. Values are expressed as the percent of individual PBP bound with unlabeled penicillin. This was estimated by subsequent incubation of samples with saturating concentrations of [³H]penicillin and, after electrophoresis and fluorography, scanning with a computing densitometer. The percent of a PBP bound by the unlabeled penicillin in the antibiotic-treated cultures was calculated by using the control cells, which receive only [³H]penicillin, to calibrate the maximum binding levels.

densities that are much lower than those used for physiological studies, it was necessary to establish the lethal effect of these antibiotic concentrations under the conditions used in the present work. The isogenic sensitive and resistant strains exhibited very similar rates of loss of viability when exposed to their respective MICs of penicillin. The rate of peptidoglycan synthesis after the addition of 1 \times MIC penicillin in the two strains, however, was markedly different. Synthesis continued in the sensitive strain after penicillin addition, as reported previously by Blundell and Perkins (2). The resistant strain, on the other hand, rapidly decreased peptidoglycan synthesis under the same conditions. This suggested that there was more than one physiological response leading to cell inhibition and loss of viability.

Examination of the structural properties of the peptidoglycan incorporated into the preexisting sacculus during penicillin treatment demonstrated further differences between the sensitive and resistant strains. The initial effect observable in sensitive strains exposed to 1 \times MIC of penicillin was a decrease in the number of *O*-acetyl groups on the peptidoglycan. It is unknown exactly how, during biosynthesis, the *O*-acetyl groups are added to the peptidoglycan of the gonococcus. However, there is evidence in *Proteus mirabilis* that the acetylation occurs after the nascent peptidoglycan has been attached to the preexisting sacculus (10). A similar situation occurs in the gonococcus (T. J. Dougherty, FEMS Microbiol. Lett., in press), and levels of

penicillin sufficient to inhibit the sensitive strain may interfere with this process.

Examination of the PBP binding profile revealed that at 1 \times MIC of penicillin against the sensitive strain, less than 20% of PBP 1 was bound, PBP 2 was bound to 60%, and PBP 3 was virtually 100% bound by penicillin. When compared with the results for the resistant strain at 0.1 \times MIC (which is 5 \times MIC of penicillin against the sensitive strain), there was less penicillin bound to PBP 2, as expected for a strain with the *penA* gene (7), and there was little change in *O*-acetylation. This is suggestive of a role for PBP 2 in controlling the degree of *O*-acetylation.

Further differences between the sensitive and resistant strains became evident when the latter was exposed to inhibitory concentrations (1 \times MIC) of penicillin. Much less peptidoglycan was made, and what was synthesized was deficient in peptide cross-links. Here the majority of all PBPs were bound with penicillin at the MIC. I believe that the shift in *O*-acetylation is not seen in this case possibly because the shutdown in peptidoglycan biosynthesis is rapid, and *O*-acetylation can be completed on this smaller population of labeled peptidoglycan molecules. A similar situation arises when the sensitive strain is treated with high levels of penicillin, levels approaching the MIC for the resistant strain.

It appears that PBP 1 is associated with the cross-linkage (transpeptidation) of gonococcal

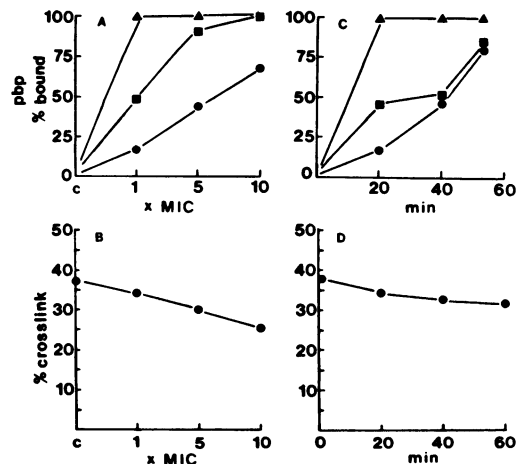


FIG. 4. PBP profiles and percent cross-linking in FA19. The PBP saturations (A) and cross-linkage of peptidoglycan (B) are shown for FA19 samples exposed to increasing concentrations of penicillin, as described in the text. The penicillin-binding profiles (C) and cross-linkage of peptidoglycan (D) for FA19 samples exposed to 0.01 μg of penicillin per ml (1 \times MIC) for increasing intervals of time are illustrated. Symbols: ●, PBP 1; ■, PBP 2; ▲, PBP 3. Peptidoglycan cross-linkage was calculated as described in Table 1.

peptidoglycan, as has been reported for high-molecular-weight PBPs in other systems (21, 22). A reasonably quantitative relationship appears to exist between PBP 1 saturation and percent cross-links in Tables 1 and 2 and in Fig. 4A and B. This relationship is not, however, as straightforward in the extended incubation in Fig. 4C and D. In this case, the decline in cross-linking is somewhat less than might be predicted from the PBP saturation values. It is unclear at present why this occurred. It should be noted in this regard that recent investigations have revealed the presence of a secondary transpeptidase in gonococci that appears to be insensitive to penicillin inhibition (Dougherty, in press). The function of this system during prolonged penicillin exposure needs to be investigated.

With respect to PBP 3, data from this laboratory (7, 8) and others (1) have suggested that this binding protein performs a dispensable function analogous to other low-molecular-weight PBPs (6, 13, 16). No additional information on the role of PBP 3 was obtained in the present study.

The above-mentioned conclusions concerning peptidoglycan assembly and PBPs are tentative, and it is clear that additional experimentation will be essential to further delineate the *in vivo* roles of the gonococcal PBPs. β -Lactam antibiotics that exhibit high selective affinities for PBP 2 or PBP 3 or both substantially below their MICs have been described (8), and the assay of *O*-acetylation and transpeptidation under these conditions should further resolve this problem. Among various questions, these experiments should clarify the role of PBP 2 in *O*-acetylation and explore the possibility of intercompensating roles for gonococcal PBPs, as seen in several other systems (9, 22). It is also unclear what the relationship is between loss of viability and *O*-acetylation levels of peptidoglycan.

It is important to realize that there are other classes of peptidoglycan molecules that were not assayed in the present study. These include trichloroacetic acid-precipitable, SDS-soluble material (putative precursor molecules) and the unique 1,6-anhydro-muramyl compounds released during cell wall hydrolysis and cell wall turnover (4, 17). These molecules must also be tested for modification of biosynthesis by β -lactam antibiotics. Indeed, changes have already been noted in the structure of penicillin-induced autolysis products (18).

The differences between the results of Blundell and Perkins (2) and those described here are most likely due to the different radioactive labeling regimes. Whereas in the former study, the major portion of labeled peptidoglycan represented biosynthetically "old" molecules, our labeling method has primarily resolved the

"new" peptidoglycan synthesized during antibiotic treatment. These newly made molecules may represent the specialized subpopulation of peptidoglycan referred to by Blundell and Perkins as the molecules that may have suffered a more substantial decrease in peptidoglycan cross-linking than the overall bulk of cell wall material.

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