Reactivation of Peptidoglycan Synthesis in Ether-Permeabilized *Escherichia coli* after Inhibition by β-Lactam Antibiotics

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The recovery of peptidoglycan-synthesizing activity after inhibition by β-lactam antibiotics was investigated in ether-permeabilized cells of *Escherichia coli* B. Such cells synthesize sodium dodecyl sulfate-insoluble peptidoglycan when provided with UDP-linked precursors and Mg++. The ability of β-lactam antibiotics to inhibit the synthesis of peptidoglycan was correlated with their affinity for penicillin-binding proteins 1A and 1B. Penicillin-binding protein 1B is thought to be the major peptidoglycan synthetase in *E. coli* and is a major lethal target for β-lactam antibiotics. Ether-treated bacteria were preincubated with concentrations of β-lactams sufficient to completely inhibit peptidoglycan synthesis and then treated with β-lactamas to inactivate free antibiotic prior to measurement of peptidoglycan synthesis. At 40 min after β-lactamase treatment, the rate of peptidoglycan synthesis was about 74% of the control rate in cells pretreated with ampicillin, but only 15% of the control in cells pretreated with penicillin G or azlocillin. Reversal of inhibition by several other antibiotics fell between these extremes. When cross-linking of peptidoglycan was measured specifically, reversal of inhibition by ampicillin also occurred more rapidly than that by penicillin G. Reactivation of peptidoglycan synthesis was not due to de novo synthesis of penicillin-binding proteins since it occurred under conditions that did not allow incorporation of [3H]leucine. We conclude that there is considerable variation in the stability of the inactive acyl enzymes formed between various β-lactams and penicillin-binding protein 1Bs, with those formed by penicillin G being relatively long-lived.

Progress in the understanding of enzyme-catalyzed reactions has resulted largely from kinetic studies of highly purified enzymes in aqueous solution. The conditions that have supported this progress, however, do not prevail for the study of the final stages of peptidoglycan synthesis. In this case, the role of supramolecular structure seems inseparable from enzyme function: the enzymes are integrally membrane-bound proteins, the immediate substrates are also membrane bound, and the ultimate product is not soluble but exists as a separate phase of complex structure. Peptidoglycan-synthesizing enzymes have been purified, but the purified enzymes are usually inactive (see, for example, reference 6) or somehow deranged in their activity (as evidenced, for example, by a requirement for filter paper for optimal activity [21]). In searching for a system in which to study the inhibition of peptidoglycan synthesis by antibiotics, we were struck by the advantages of the ether-permeabilized cells described by Mirelman et al. (13). This system seemed to resemble closely the in vivo situation, having both a complex set of membrane-bound peptidoglycan-synthesizing enzymes and preexisting murein, but was less complicated than viable cell populations with their various mechanisms of adaptation and resistance. For example, Ishiguro et al. (5) found that peptidoglycan biosynthesis is apparently under *relA* control in ether-treated bacteria (ETB), and Kraus et al. (7) showed that peptidoglycan synthesized in ETB more closely resembled peptidoglycan made in vivo than did that made by membrane preparations. Penicillin and other β-lactam antibiotics have been proposed to inhibit peptidoglycan transpeptidases by acting as substrate analogs of the terminal D-alanyl-D-alanine residues of the peptide cross-bridge precursors (24, 27), forming stable acyl-enzyme complexes. With the development of methods to study the penicillin-binding proteins (PBPs) of bacteria, it became possible to determine experimentally how stable these complexes are by measuring their deacylation rates. The acyl enzymes formed by penicillin G with the high-molecular-weight PBPs of *Escherichia coli*, which are thought to be the killing targets of β-lactams, were indeed found to be long-lived (half-lives, >60 min), while PBPs 5 and 6 deacylated relatively rapidly (18). Because radioactivity labeled β-lactams other than penicillin G are not readily available, deacylation rates for other β-lactams have not been measured directly and it has generally been assumed that they are similar to the rates for penicillin G. The present study demonstrates that (i) peptidoglycan synthesis in ether-permeabilized *E. coli* is dependent on high-molecular-weight PBPs; (ii) reactivation of peptidoglycan synthesis and, therefore, presumably deacylation of the acyl-enzyme complex proceed at different rates for different β-lactams; and (iii) this reactivation is relatively slow for penicillin G, but quite rapid for some other drugs. (Some of the results described herein were presented earlier in preliminary form [M. K. Talbot, F. Schaefer, and J. G. Christenson, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1116, 1984; M. K. Talbot, F. Schaefer, V. Brocks, and J. G. Christenson, 26th ICAAC, abstr. no. 123, 1986].)

MATERIALS AND METHODS

Uridine-5'-diphospho-N-acetyl-d-[U-14C]glucosamine (UDP-[14C]GlcNAc; ca. 300 mCi/mmol), 2,6-diamino[1,7,14C]pimelic acid (mixed D, L, and meso isomers; 281 mCi/mmol), L-[U-14C]leucine (348 mCi/mmol), and [3H]benzylpenicillin (10.6 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, Ill. Filtron X and En'Hance scintillation media are products of National Diagnostics and Dupont, NEN Research Products, Boston, Mass., respectively. Egg white lysozyme was purchased from Worthington Diagnostics, Freehold, N.J., and N-acetylmuramidase from Strepto-
myces globisporus was from Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill. Plates for thin-layer chromatography (TLC) were plastic-backed Polygram Sil G, 0.25-mm-thick silica gel (Brinkmann Instruments, Inc., Westbury, N.Y.). Nucleotides, sarcosyl, and sodium dodecyl sulfate (SDS) were from Sigma Chemical Co., St. Louis, Mo.

Cefamandole, penicillin G, cephaloridine, cepalexin, and cephalothin were from Eli Lilly & Co., Indianapolis, Ind.; mezlocillin and azlocillin, from Miles Pharmaceuticals; cloxacinil and methicillin, from Bristol Laboratories, Syracuse, N.Y.; cefradin, from Abbott Laboratories, North Chicago, Ill.; cefotaxime, from Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.; cefoxitin, from Merck Sharp & Dohme, West Point, Pa.; ampicillin, from Wyeth Laboratories; carbenicillin, from Roerig; amdinocillin and ceftriaxone, from Roche Laboratories; Hoffmann-La Roche Inc., Nutley, N.J.; and phosphonomycin and 1-aminoethyl phosphonic acid, from Sigma. Flavomycin and moenomycin A were the generous gifts of G. Seibert of Hoechst AG.

Bacterial strains. E. coli B ATCC 23226 and Bacillus cereus ATCC 11778 were obtained from the American Type Culture Collection, Rockville, Md. E. coli K-12 JE5707 (Thi" Dap" Lys"), originally from Y. Hirota, was the gift of S. Makoever.

β-Lactamases. B. cereus penicillinase was purchased from Calbiochem-Behring, La Jolla, Calif. The other enzymes were preparations partially purified in our laboratories, essentially following published procedures (1, 12, 17). Each enzyme was assayed with the specified drug by standard spectrophotometric methods.

The capability of each β-lactamase to completely hydrolyze the appropriate β-lactam was assessed by agar diffusion microbiological assay of reaction mixtures before and after incubation with the enzyme. β-Lactamases were inactivated after the 10-min incubation, before assay, by addition of an equal volume of acetonitrile.

Preparation of UDP-MurNAC-pentapeptide. UDP-N-acetylmuramyl-pentapeptide (UDP-MurNAC-pentapeptide) was prepared from B. cereus ATCC 11778 by the procedure of Moore et al. (14). The concentration was determined by an assay for muramyl peptide based on that of Strominger (19). The results agreed well with the UDP concentration based on ε₅₆₀ = 1.0 × 10⁴. In addition, several preparations were assayed by a high-pressure liquid chromatography procedure based on that of Flouret et al. (3) and subjected to amino acid analysis. The major high-pressure liquid chromatography peaks had the proper retention time for UDP-MurNAC-pentapeptide and was active in the pep tidoglycan synthesis assay; the minor peaks showed no activity in the assay. The ratio of glutamate/alanine/diaminopimelate was 1:2:7:0.85.

Determination of peptidoglycan synthesis. Ether-treated cells of E. coli B were prepared by the method of Mirelman et al. (13). Protein content was determined by the method of Lowry et al. (10) and adjusted to 10 mg/ml in “basic medium,” which is composed of 80 mM KC1, 40 mM Tris hydrochloride (pH 7.4), 7 mM MgCl₂, 2 mM [ethylene-bis(oxyethylenenitrilo)tetraacetic acid, 0.4 mM spermidine·3HCl, and 0.5 M sucrose (13). The cells were stored frozen at −25°C and remained active for several months. Assays were conducted by using a modification of the procedures of Mirelman et al. (13). Standard reaction mixtures contained the following final concentrations in a reaction volume of 200 μl: 50 mM Tris hydrochloride (pH 8.3), 25 mM MgCl₂, 0.5 mM 2-mercaptopethanol, 10 mM disodium ATP, 2.1 μM UDP-[¹⁴C]GlcNAc (1.85 kBq), 75 μM UDP-MurNAC-pentapeptide, and ETB (1 mg of protein per ml). Reaction mixtures were incubated with shaking at 30°C for 40 min. Reactions were terminated by addition of 1 ml of 4% SDS and boiling for 30 min. SDS-insoluble material was collected on 0.22-μm filters and washed with 2% SDS and water. Dried filters were counted in Filtron X.

Measurement of cross-linking in ETB. Inhibition of cross-linking was measured by TLC of the muramidase-digested products of the peptidoglycan synthesis assay. Assays were conducted by using a modification of the procedures of Mirelman et al. (13). Standard reaction mixtures contained the following in a final volume of 100 μl: 50 mM Tris hydrochloride (pH 8.3), 25 mM MgCl₂, 0.5 mM 2-mercaptopethanol, 10 mM disodium ATP, 4.1 μM UDP-[¹⁴C]GlcNAc (2.33 kBq), 100 μM UDP-MurNAC-pentapeptide, and ETB (5 mg of protein per ml). Reaction mixtures were incubated with shaking at 30°C for 40 min. Reactions were terminated by the addition of 0.5 ml of 20% trichloroacetic acid (TCA) and were placed on ice for at least 30 min. Precipitates were collected by centrifugation and washed three times with water. Pellets were suspended in 100 μl of a 100-μg/ml solution of N-acetylmuramidase (from S. globisporus) in 25 mM sodium phosphate buffer (pH 6.5) with 0.1 mM MgCl₂. Samples were incubated in a shaking water bath at 37°C overnight. After digestion, samples were evaporated and suspended in 20 μl of water. The entire volume of each sample was spotted onto plastic-backed silica gel TLC plates. Each plate was also spotted with [¹⁴C]-labeled monomer and dimer standards. Plates were developed twice in isobutyric acid–1 N ammonia (5:3) (11). Plates were air dried, sprayed with En'Hance, and placed in contact with Kodak X-Omat film at ~80°C for at least 3 days. Exposed film was developed, and areas corresponding to bands on the autoradiograph were cut from the TLC plates and counted in Filtron X. Results are reported as percentage of total radioactivity present as cross-linked species [radioactivity in dimer or in (dimer plus trimer) divided by total radioactivity].

Preparation of [¹⁴C]-labeled monomer and dimer fragments. Purified monomer and dimer fragments were prepared by the method of Gmeiner (4) from peptidoglycan isolated from E. coli K-12 JE5707 (Thi" Dap" Lys") grown in the presence of [¹⁴C]diaminopimelic acid. The labeled peptidoglycan was digested with egg white lysozyme (100 μg/ml in 0.1 M ammonium acetate, 0.01 M EDTA [pH 7.9]) for 18 h. Monomer and dimer fragments were separated on a Sephadex G-50 column connected in series with a Sephadex G-25 column and eluted with 0.1 M LiCl. A 5-μl amount of each fraction was counted, and fractions corresponding to the two radioactive peaks were pooled. A sample of each peak was spotted on silica gel TLC plates and developed twice in isobutyric acid–1 N ammonia (5:3) (11). Separation was visualized by autoradiography. The Rₐ of these monomer and dimer fragments (0.29 and 0.14) were in good agreement with those reported by Gmeiner (4). Separated fragments were stored at −25°C.

PBPs. An adaptation of the method of Spratt (18) was used to determine the affinity of β-lactams for the PBPs of ETB. Samples were prepared by incubating ETB (0.1 mg of protein) with the test compound for 10 min at 30°C. [³H]benzyl penicillin (10 μCi; 0.877 nmol) was added, and incubation was continued for 10 min. The reaction was terminated by the addition of 5 μl of 10% sarcosyl and incubation at room temperature for 5 min. Cold acetone, 400 μl, was added, and the samples were incubated at 0°C for 5 min. Precipitates...
TABLE 1. Effects of omissions and additions on [14C]GlcNAc incorporation by ETB

| Omission(s) | Addition(s) | [14C]GlcNAc incorporation (% of control) |
|-------------|-------------|------------------------------------------|
| MgCl₂       |             | 1.0                                      |
| UDP-MurNAc-pentapeptide | 3.4 |          |
| ETB         |             | 1.0                                      |
| Mercaptoethanol |         | 90                                       |
| NH₄Cl       |             | 124                                      |
| ATP         |             | 101                                      |
| ATP         | UTP         | 2.0                                      |
| ATP         | UDP         | 1.0                                      |
| ATP         | UTP         | 0.7                                      |
| UDP         |             | 1.0                                      |
| MurNAc      |             | 64                                       |

* Control incorporation was approximately 35 pmol of [14C]GlcNAc, 18,000 cpm.

were collected by centrifugation at 12,500 × g for 5 min; pellets were air dried and suspended in sample buffer. Samples were boiled for 3 min just prior to loading.

Samples were fractionated on polyacrylamide gels as described by Then and Kohl (23). Electrophoresis was performed at a constant current of 35 mA, using the discontinuous buffer system of Laemmli and Favre (9). Gels were fixed, stained with Coomassie blue, and destained by the usual procedures. Gels were prepared for autoradiography by using En3Hance following the manufacturer’s suggestions. Gels were dried between dialysis membrane sheets and exposed to two sheets of Kodak X-Omat film, which were developed after 7 to 14 days of exposure at −80°C. Results were quantitated by densitometry, using a Beckman DU-8B spectrophotometer equipped with a slab gel scanning system.

RESULTS

Assay conditions and kinetics. Peptidoglycan synthesis in ETB was completely dependent on the presence of Mg²⁺, UDP-MurNAc-pentapeptide, and ether-treated cells (Table 1). Omission of mercaptoethanol or ATP had little effect, but we continued to include both in our standard assay mixture for consistency with the literature and because of formation of a precipitate in the absence of ATP. Mirelman et al. (13) also included 50 mM NH₄Cl in their assay mixture, but we found that incorporation of [14C]GlcNAc was slightly greater in the absence of NH₄Cl and so omitted it from our standard mixture.

Incorporation of [14C]GlcNAc was not affected by substitution of GTP or CTP for ATP (data not shown), but UTP and UDP strongly inhibited the reaction in either the presence or the absence of ATP. This may be due to competition with UDP-linked peptidoglycan precursors for a binding site on one or more enzymes. Consistent with this, MurNAc was also an inhibitor of incorporation, though much weaker than the uridine nucleotides.

The dependence of the rate of incorporation of [14C]GlcNAc on the concentrations of UDP-MurNAc-pentapeptide and UDP-GlcNAc followed Michaelis-Menten kinetics reasonably well for both substrates. The kinetic constants determined from Lineweaver-Burk plots (Fig. 1) were as follows: UDP-MurNAc-pentapeptide – Kₘ = 14 μM, Vₘₕ = 0.0175 nmol/min per mg of protein; UDP-GlcNAc – Kₘ = 5.3 μM, Vₘₕ = 0.0275 nmol/min per mg of protein. Similar results were obtained when other plotting methods were used and when the outlying point in the plot for UDP-GlcNAc was omitted.

[14C]GlcNAc incorporation was strongly inhibited by β-lactam antibiotics (see below) and other inhibitors of the later steps of peptidoglycan synthesis (such as vancomycin, tunicamycin, flavomycin, moenomycin, and enduracidin), but not by inhibitors of the earlier steps (such as cycloserine, alafosfalin, and fosfomycin).

Correlation of PBP affinity and inhibition of peptidoglycan synthesis. When ether-treated cells of E. coli B were labeled with [3H]penicillin G, subjected to SDS-polyacrylamide gel electrophoresis, and fluorographed, a typical pattern of PBPs emerged (Fig. 2). The affinity of various β-lactam antibiotics for these PBPs was estimated by their ability to inhibit binding of [3H]penicillin G. The ability of the same drugs to inhibit peptidoglycan synthesis in ETB was also determined; the results, expressed as the concentrations required for 50% inhibition of [3H]penicillin G binding or of [14C]GlcNAc incorporation, are compared in Table 2. To test the correlation between activity in the peptidoglycan synthesis assay and affinity for the various PBPs, Spearman rank order correlation coefficients (rₛ) were calculated with the following results: PBP 1A, rₛ = 0.776, 0.002 < P < 0.005; PBP 1Bs, rₛ = 0.848, P < 0.001; PBP 2, rₛ = −0.030, P > 0.5 (not significant); PBP 3, rₛ = 0.474, 0.1 < P < 0.2 (not significant); PBP 4, rₛ = 0.153, P > 0.5 (not significant). Thus, a strong positive correlation was found between the
activity of the drugs as inhibitors of peptidoglycan synthesis and their affinity for PBPs 1A and 1Bs. This result is consistent with the results of others indicating that PBP 1Bs is the major peptidoglycan synthetase of *E. coli* and that PBP 1A can substitute for it under some conditions (20, 21).

There was no significant correlation for PBP 2, 3, or 4.

**Time course of[^14]C]GlcNAc incorporation and its inhibition by ampicillin.** Peptidoglycan synthesis, as measured by[^14]C]GlcNAc incorporation, proceeded with a slight downward curvature for at least 40 min (Fig. 3). In the presence of 10 μg of ampicillin per ml, peptidoglycan synthesis was inhibited progressively, ceasing completely after about 20 min.

**Peptidoglycan synthesis in ETB “preinhibited” by ampicillin.** Apparently, the peptidoglycan transpeptidases had been converted to inactive penicilloyl enzymes by interaction with ampicillin for 20 min. If this is the case, ETB preincubated with ampicillin before addition of substrate should not incorporate[^14]C]GlcNAc at all. Moreover, if the penicilloyl enzymes are sufficiently long-lived, it should be possible to isolate preinhibited ETB. Preinhibited ETB were prepared by incubating the standard ETB preparation with an excess of ampicillin (1,000 μg/ml) for 1 h. The drug was removed by centrifugation, and the ETB were washed and suspended in their original volume of basic medium. Preincubated control ETB were prepared by the same procedure without ampicillin. The preincubated ETB were compared with untreated (not preincubated) ETB in the standard assay. The preinhibited ETB had greater peptidoglycan-synthesizing activity than untreated ETB assayed in the presence of ampicillin (Fig. 4). It appeared that, although the murein-synthesizing activity of ETB could be completely inhibited by ampicillin, either some portion of the activity was not inhibited by ampicillin under the preincubation conditions (i.e., in the absence of substrates) or the inhibition was readily reversed upon removal of ampicillin. It was soon found, however, that when β-lactamase was used to remove the drug, more activity was recovered. ETB were preincubated with 1,000 μg of ampicillin per ml and then treated with β-lactamase or washed to remove the drug or both. The enzyme-treated ETB had greater peptidoglycan-synthesizing activity than the washed ETB (Fig. 5), suggesting that treatment of the ETB after preincubation governed the recovery of activity; i.e., fully inhibited peptidoglycan synthetases were reactivated after removal of the ampicillin.

**Reactivation of peptidoglycan synthesis after inhibition by various β-lactams.** Similar experiments were conducted with a variety of β-lactam antibiotics. ETB were preincubated with 1,000 μg of the drug per ml for 1 h. Sufficient β-lactamase was added to hydrolyze the remaining free drug.

![FIG. 2. PBP pattern in ether-treated E. coli B.](image-url)

**TABLE 2. Inhibition of peptidoglycan synthesis, PBPs, and bacterial growth by various β-lactams**

| Antibiotic     | Peptidoglycan synthesis | PBP 1A | PBP 1Bs | PBP 2 | PBP 3 | PBP 4 | PBP 5 | PBP 6 | MIC (μg/ml) |
|----------------|-------------------------|--------|---------|-------|-------|-------|-------|-------|------------|
| Cefotaxime     | 0.37                    | 0.09   | 1.3     | 3.2   | 0.01  | 1.6   | >10   | >10   | <0.008     |
| Ceftriaxone    | 2.0                     | 0.28   | 3.0     | 47    | 24    | >50   | >50   | >50   | >128       |
| Metronidazole  | 2.3                     | 0.26   | 1.2     | 0.20  | <0.01 | 3.5   | >10   | >10   | >0.125     |
| Cefadroxil     | 2.4                     | 0.045  | 3.2     | >10   | 1.0   | 1.7   | 0.28  | 0.70  | 2          |
| Penicillin G   | 2.6                     | 1.6    | 3.3     | 2.6   | 1.4   | 2.5   | 20    | 20    | 8          |
| Cefaclor      | 3.3                     | 0.09   | 1.7     | >5    | 1.8   | 2.8   | >5    | >5    | 4          |
| Ampicillin     | 3.7                     | 0.45   | 2.4     | 0.61  | 0.20  | 0.06  | >10   | 4.5   | 1          |
| Carbenicillin  | 6.1                     | 0.34   | 4.8     | 3.3   | 0.40  | 0.55  | >10   | >10   | 1          |
| Cloxacillin    | 6.6                     | 0.95   | 8.0     | 0.95  | 0.60  | 0.60  | 6.0   | >7.5  | >128       |
| Cephalexin     | >100                    | 6.5    | >50     | 22    | 14    | 1.5   | >50   | >100  | >0.4       |
| Methicillin    | >100                    | 8.5    | 95      | 14    | <2.5  | 6.5   | >250  | 160   | >128       |
| Amdinocillin   | >100                    | 160    | >250    | <2.5  | 65    | 150   | >250  | >250  | >0.063     |
and incubation was continued for another 10 min. At this point, substrates were added and \(^{14}\text{C}\)GlcNAc incorporation was measured at intervals for the next 40 min. The results (Fig. 6) indicated that ETB incorporated only 11\% as much GlcNAc as the control after inhibition by penicillin G and only 14\% after inhibition by azlocillin. However, inhibition by ampicillin, ceftriaxone, cefotaxime, and cefalothin was reversed to a much greater extent, yielding 30 to 50\% of control synthesis.

The effectiveness of drug removal by \(\beta\)-lactamases was tested by using a microbiological assay of samples of sham reaction mixtures. Ampicillin, cephalothin, penicillin G, and azlocillin were undetectable. Residual ceftriaxone and cefotaxime were detected at about 0.1 to 0.2 \(\mu\)g/ml after \(\beta\)-lactamase treatment. The reactivation experiments for these compounds were repeated, but the initial concentration was reduced to 100 \(\mu\)g/ml (a concentration which reduces peptidoglycan synthesis to <3\% of the control) and the reaction mixtures were treated with \(\beta\)-lactamase and washed before the extent of reactivation was measured. No antimicrobial activity was detected in these reaction mixtures, and the results of the reactivation experiments were essentially identical to the original results.

It should be noted that the curves shown in Fig. 6 are concave upward, indicating that the rate of peptidoglycan synthesis steadily increased during the course of the experiment. This increasing rate suggests that the reactivation of the peptidoglycan synthetases continued during the course of the experiment. The final degree of reactivation was

![FIG. 3. Time course of \(^{14}\text{C}\)GlcNAc incorporation (•) and inhibition of incorporation by ampicillin at 10 \(\mu\)g/ml (○).](image1)

![FIG. 4. Effect of 1,000 \(\mu\)g of ampicillin per ml during preincubation on \(^{14}\text{C}\)GlcNAc incorporation. ETB were preincubated with 1,000 \(\mu\)g of ampicillin per ml for 1 h. The drug was removed by centrifugation and washing. Control ETB were prepared by the same procedure without ampicillin. Incorporation of \(^{14}\text{C}\)GlcNAc was then measured as in the standard assay. Included for comparison are assay results measured in the presence and absence of ampicillin, using ETB which had not been preincubated.](image2)

![FIG. 5. Effectiveness of different methods of drug removal. ETB were preincubated with 1,000 \(\mu\)g of ampicillin per ml for 1 h. Drug was removed by washing (□) or \(\beta\)-lactamase treatment (△) (Calbiochem-Behring penicillinase) for 10 min. Both positive (preincubated and assayed without drug, ○) and negative (preincubated and assayed with drug, □) controls are included.](image3)

![FIG. 6. Resumption of peptidoglycan synthesis in ETB after preincubation with 1,000 \(\mu\)g of different \(\beta\)-lactams per ml and removal of excess antibiotic by \(\beta\)-lactamase. Sufficient \(\beta\)-lactamase was added to hydrolyze the remaining free drug, and incubation was continued for 10 min. ETB were then assayed for 40 min; samples were removed and processed at the indicated times. Controls were preincubated without drug or enzyme. Samples preincubated with drugs and then assayed for 40 min without \(\beta\)-lactamase treatment incorporated <2\% of the control. Results are expressed as percentage of 40-min control incorporation. Penicillins were hydrolyzed with the \(\beta\)-lactamase from E. coli TEM-1, cefotaxime and ceftriaxone were hydrolyzed with the \(\beta\)-lactamase from P. vulgaris 1028\(\beta\)-C, and cephalothin was hydrolyzed with the \(\beta\)-lactamase from Enterobacter cloacae P99.](image4)
estimated by calculating the slopes of the GlcNAc incorporation curves at 40 min. These results (Table 3) indicate that peptidoglycan synthetases inactivated by ampicillin were almost completely reactivated after removal of the drug, while only 15% of control activity was recovered after removal of penicillin G or azlocillin. Reactivation after treatment with the other drugs tested fell between these extremes.

Peptidoglycan synthesis involves both transglycosylation and transpeptidation, and the experiments described above depend on both types of reaction. Conceivably, the apparent reversal of inhibition could be explained by a stimulation of transglycosylation, rather than reactivation of transpeptidation. We therefore examined the extent of cross-linkage of TCA-insoluble peptidoglycan synthesized by ETB preinhibited by ampicillin, ceftriaxone, and penicillin G, with and without removal of excess drug. The results (Table 4) indicate that all three drugs inhibited cross-linking by 80 to 90% before β-lactamase treatment. This inhibition was reduced to 29% after β-lactamase treatment for ampicillin and ceftriaxone, but only to 62% for penicillin G. The cross-linking assay thus confirmed the results of the peptidoglycan synthesis assay in that there was a considerable degree of reactivation, which was less for penicillin G than for other β-lactams.

Reactivation of peptidoglycan synthesis in the absence of protein synthesis. Tuomanen (25) has reported that peptidoglycan synthesis occurring after the inactivation of all mature PBPs could be explained by a rapid de novo synthesis of PBPs. Our results could also be explained by de novo synthesis of peptidoglycan synthetases if ETB were capable of protein synthesis under the conditions used. ETB were indeed found to incorporate [14C]leucine into TCA-insoluble material in the standard reaction mixture, which contains 10 mM ATP. In the absence of ATP, [14C]leucine incorporation was reduced by 97%, but peptidoglycan synthesis reactivated at least as well in the absence of ATP as in its presence after inhibition by ampicillin (Table 3). Reactivation of peptidoglycan synthesis after inhibition by ampicillin is therefore independent of protein synthesis.

DISCUSSION

We have found that ETB provided with substrates and Mg2+ provide a sensitive and convenient system for the study of peptidoglycan synthesis and its inhibition by antibiotics. The results of this assay, in conjunction with MIC determinations and assays such as affinity for PBPs, may help to elucidate the various mechanisms of action of antibiotics and the mechanisms of bacterial resistance. Several observations readily illustrate how the information derived from the ETB assay may supplement MIC results. For example, both of the antistaphylococcal penicillins, cloxacillin and methicillin, were inactive against E. coli B at 128 μg/ml, but cloxacillin was a fairly effective inhibitor of peptidoglycan synthesis while methicillin was among the weakest compounds tested. As another example, MICs of both cephalaxin and cephaloridine were 4 μg/ml, but cephalaxin had very poor activity and cephaloridine had very good activity in the peptidoglycan synthesis assay. These observations are not surprising when it is remembered that MIC determinations, unlike the ETB assay, are strongly affected by permeability barriers and β-lactamases and that the ETB assay measures effects on only one of several PBPs that are lethal targets. Unlike the PBP assay, the ETB assay is also sensitive to inhibitors of several steps in peptidoglycan synthesis.

The response of ETB to non-β-lactam inhibitors of peptidoglycan synthesis was consistent with expectations. Since UDP-MurNAc-pentapeptide was provided, the assay was insensitive to inhibitors of the cytoplasmic steps. It was, however, sensitive to inhibitors acting at later stages, including tunicamycin, which is thought to inhibit phospho-N-acetylmuramyl-pentapeptide translocase, the enzyme immediately following formation of the nucleotide-linked pentapeptide (22, 26). It is interesting to note that peptidoglycan synthesis could be completely inhibited by inhibitors of either transpeptidation (i.e., β-lactams) or transglycosylation (e.g., moenomycin), indicating that both transpeptidation and transglycosylation are required for synthesis of SDS-insoluble peptidoglycan and that we are observing formation of the complex macromolecule, rather than extension of the preexisting murin by addition of monomers by either type of reaction alone.

Our results for the affinities of the various β-lactam antibiotics for the PBPs of ether-treated E. coli B are in good agreement with previously published data (see, for example, reference 2). The activity of various β-lactams as inhibitors of peptidoglycan synthesis correlates well with their affinity for PBPs 1A and 1B. This is consistent with the finding that PBP 1B is the major peptidoglycan synthetase of E. coli (15, 16, 20, 21). The role of PBP 1A is not clear, but it is known that it can substitute for defective PBP 1B. Recently, Kraus and Höltje (8) used specific mutants to show that PBP 1B is the major peptidoglycan-synthesizing activity in ETB and is poorly compensated for by PBP 1A. We found that the rank order of affinities of the β-lactams tested was almost the same for PBP 1A as for PBP 1B, but PBP 1A was always more sensitive. In view of the published results and the fact that the correlation of peptidoglycan synthesis-inhibition with PBP affinity is somewhat better for PBP 1B, the

| Drug                        | Rate of peptidoglycan synthesis (% of control) |
|-----------------------------|-----------------------------------------------|
| Penicillin G                | 15                                            |
| Azlocillin                  | 15                                            |
| Cefotaxime                  | 46                                            |
| Cephalexin                  | 65                                            |
| Ceftriaxone                 | 72                                            |
| Ampicillin                  | 74                                            |
| Ampicillin (no ATP)         | 112                                           |

|$^a$ Cross-linking was calculated as radioactivity present as dimer/total radioactivity. Percent inhibition was calculated as percent cross-linking in the treated samples/percent cross-linking in the untreated (control) samples.

|$^b$ β-Lactamases used were from Serratia marcescens U91 (class III; penicillin and ampicillin) and Proteus vulgaris 1028B-C (ceftriaxone).

|$^c$ ND, Not determined.

| Treatment of ETB | % Inhibition of cross-linking after preincubation with: |
|------------------|--------------------------------------------------------|
|                  | Ampicillin | Ceftriaxone | Penicillin G |
| β-Lactamase      | 29         | 29          | 62           |
| Washed           | 65         | ND          | 83           |
| Drug not removed | 81         | 80          | 90           |
present assay appears to measure primarily peptidoglycan synthesis catalyzed by PBP 1Bs.

It must be noted that a compound such as aminocillin, which is a poor inhibitor of peptidoglycan synthesis in this assay, may have very good antibacterial activity. This is a result of the critical importance of quantitatively minor cross-linking reactions to bacterial viability and suggests the value of such reactions as targets for antibiotic action.

It has been proposed and widely confirmed that β-lactam antibiotics are substrate analogs for the transpeptidases catalyzing the cross-linking of peptidoglycan and form stable acyl-enzyme complexes with membrane-bound penicillin-sensitive enzymes, including the major peptidoglycan synthetases. The formation of such long-lived complexes between these enzymes and radiolabeled penicillin G allowed the detection of the PBPs. Spratt (18) measured the deac- 
ylation rates of labeled benzylpenicilloyl-PBPs of E. coli and found that the complexes with the high-molecular-weight PBPs were quite stable, while PBPs 5 and 6 deacetylated with half-lives of 5 and 19 min, respectively. This method of measuring deacetylation rates depends on the availability of radiolabeled β-lactams. Except for penicillin G, such labeled compounds are not readily available; therefore, this approach has not been widely used for other β-lactams.

We used ETB to measure the lifetimes of a series of acyl-envelope complexes. This method measures the reactiva-
tion of peptidoglycan synthetase activity by measuring the amount of product formed after removal of the inhibitor. The applicability of this method depends mainly on the availability of a suitable β-lactam to inactivate free antibiotic. We found 0.1- to 0.2-μg/ml residual concentrations of the relatively β-lactamase-stable cephalosporins ceftriaxone and cefotaxime after β-lactamase treatment. It should be noted that these residual concentrations are well below the 50% inhibitory concentrations of these drugs for PBP 1Bs. The reactivation results for these compounds were confirmed by using a procedure that left no detectable drug.

ETB may more closely resemble the in vivo interactions between the membrane-bound peptidoglycan synthetases and β-lactam antibiotics than the membrane preparations frequently used to determine PBP affinity. ETB seem to be essentially intact but permeabilized cells and retain many characteristics of normal cells, including the close association of the membrane and preexisting peptidoglycan. In addition, ETB allow the measurement of enzyme activity and its inhibition by β-lactam antibiotics rather than simply measurement of the relative affinity of inhibitors for the enzymes.

The reactivation of peptidoglycan synthetases initially detected in the peptidoglycan synthesis assay was subsequently confirmed in an assay which specifically measures the cross-linking step of peptidoglycan synthesis. The ability of ampicillin, ceftriaxone, and penicillin G to inhibit cross-linking in ETB showed a pattern of reversal after β-lacta-

mase treatment similar to that seen in the peptidoglycan synthesis assay.

Two significant differences between the peptidoglycan synthesis and cross-linking assays should be noted. First, the peptidoglycan synthesis assay measures SDS-insoluble, “mature” peptidoglycan (13), while the cross-linking assay measures TCA-insoluble material that may include a consid-
erable amount of “immature” peptidoglycan. Second, the cross-linking assay indicates the ratio of cross-linked to uncross-linked units in material that is sufficiently polymer-
ized to be TCA precipitable; there may be much less such material in the presence of an antibiotic than in the control. Thus, 50% inhibitory concentrations measured in this assay are concentrations required for a 50% reduction in the dimer/monomer ratio, not the concentrations required for a 50% reduction in cross-link formation. That the former are much greater than the latter is suggested by the fact that 50% inhibitory concentrations for the cross-linking assay are typically about 10-fold greater than those for peptidoglycan synthesis. Tuomanen (25) has also reported the resumption of peptidoglycan synthesis after the inactivation of all ma-
ture PBPs, but concluded that this result could be explained by rapid de novo synthesis of PBPs. After removal of excess penicillin and resumption of protein synthesis, peptidogly-

can synthesis began immediately, although no PBPs could be detected for 10 min. This raised the possibility that our results could be explained by a similar rapid synthesis of new, active PBPs, rather than deacetylation of preexisting, inhibited PBPs. It is unlikely that ETB synthesize protein under the conditions used in our experiments, but ETB do incorporate [14C]leucine into TCA-insoluble material, possibly by charging tRNA. To eliminate this possibility, ETB were tested in the absence of ATP, and it was found that ETB can synthesize peptidoglycan but not incorporate [14C]leucine under these conditions. Reactivation of amin-
cillin-inhibited peptidoglycan synthesis was also readily observed in the absence of ATP. Since this recovery of activity could not have been due to de novo synthesis of PBPs, it is attributed to deacetylation of the inhibited PBPs.

As noted above, the activity measured probably corre-
sponds to that of PBP 1Bs. Rapid deacetylation of PBP 1Bs was not expected, in view of Spratt's (18) deacetylation times of >60 min for the higher-molecular-weight PBPs of E. coli inhibited by penicillin G. In fact, our results for penicillin G do not contradict the expected long half-life of PBP 1Bs since ETB preincubated with penicillin G recover only 11% of the control activity after 40 min. Our results do indicate that acyl enzymes formed by PBP 1Bs with other β-lactams are not equally long-lived. Tuomanen (25) concluded that a very small amount of newly synthesized PBPs, <10% of normal total amount, may allow the resumption of pepti-
doglycan synthesis in E. coli. A very slow deacetylation rate of penicillin may liberate a small amount of PBP 1Bs, sufficient to resume synthesis at the low observed rate. The rapid recovery of ETB pretreated with other β-lactams may reflect a previously undetected difference in the binding kinetics of this class of compounds.

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