ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1979, p. 28-36
0066-4804/79/07-0028/09$02.00/0

Vol. 16, No. 1

In Vitro Synthesis of Peptidoglycan by β-Lactam-Sensitive and -Resistant Strains of *Neisseria gonorrhoeae*: Effects of β-Lactam and Other Antibiotics

CHRISTINE A. BROWN AND HAROLD R. PERKINS*

Department of Microbiology, Life Sciences Building, University of Liverpool, Liverpool L69 3BX, England

Received for publication 20 April 1979

The synthesis in vitro of peptidoglycan by *Neisseria gonorrhoeae* was studied in organisms made permeable to nucleotide precursors by treatment with ether. Optimum synthesis occurred at 30°C in tris(hydroxymethyl)aminomethane-maleate buffer (0.05 M; pH 6) in the presence of 20 mM Mg^{2+}. The incorporation from uridine 5'-diphosphate-*N*-acetyl-[^{14}C]glucosamine into peptidoglycan, measured after precipitation of the cells with trichloroacetic acid, was sensitive to the β-lactam antibiotics, bacitracin, diumycin, and tunicamycin and relatively resistant to spectinomycin and tetracycline. Differences in sensitivity between preparations from a β-lactamase producer and a laboratory segregant derived from it were not great. Synthesized peptidoglycan was also fractionated into sodium dodecyl sulfate-soluble and -insoluble portions. β-Lactam antibiotics at concentrations equivalent to the minimal inhibitory concentrations for growth of the organisms did not inhibit peptidoglycan synthesis, but rather caused a small enhancement. At higher concentrations, above about 0.5 μg/ml, incorporation into sodium dodecyl sulfate-insoluble material was progressively inhibited, whereas the amount of sodium dodecyl sulfate-soluble product increased greatly, more than compensating for the loss of the precipitable fraction. Similar observations were made with three strains, and also with the β-lactam clavulanic acid, normally considered as a β-lactamase inhibitor rather than as itself an effective antibiotic.

Until recently, gonococci had always been observed to be highly sensitive to penicillin, and even though some strains existed that were relatively more resistant this did not prevent penicillin from remaining the preferred treatment. In 1976, strains that had acquired high levels of resistance attributable to their production of a TEM-type β-lactamase (1, 18, 19, 26) were isolated in various parts of the world, and infections with these could no longer be treated by single massive doses of benzylpenicillin. Later evidence showed that the gene for the β-lactamase was carried on plasmids of 4.4×10^6 daltons in some strains and 3.2×10^6 daltons in others, but that its base sequence was at least 70% similar in each case (3, 21). It was, nevertheless, possible to treat infections with these strains by using β-lactams resistant to TEM-β-lactamase, for which the fundamental attack on gonococcal wall synthesis could be expected to be the same as for penicillin. β-Lactams are well known to interfere with the synthesis of bacterial cell walls by preventing the cross-linking of peptidoglycan (4), and there is good evidence that this mechanism is also involved in their action on gonococci (22). As in other organisms, their bactericidal effect is mediated by autolysis (22, 32) which is promoted by penicillin action. To gain further insight into the precise way in which β-lactams can kill gonococci, whether or not they bear β-lactamase genes on their plasmids, we have investigated directly the effect of these antibiotics on the in vitro biosynthesis of peptidoglycan from its nucleotide precursors. The basic composition of the gonococcal peptidoglycan is known to be the same as that of other gram-negative bacteria (6), and the work that follows describes the development of a system based on previous work with *Escherichia coli* (13), in which ether treatment was used to make the organisms permeable to nucleotide-linked precursors. Peptidoglycan synthesis was then studied in the presence and absence of antibiotics.

(This work forms part of a thesis by C. A. Brown for a fellowship of the Institute of Medical Laboratory Sciences, London England.)

MATERIALS AND METHODS

Organisms and growth conditions. Three strains of *Neisseria gonorrhoeae* were used. Strain

FA19 was a penicillin-sensitive laboratory strain originally obtained from P. F. Sparling. The other two strains were derived from an isolate obtained by A. Percival from a patient in Liverpool; one, 1L261, (Percival no. 771+) was resistant to penicillin by reason of plasmid-borne β-lactamase production, and the other, 1L260 (Percival no. 771−) was a penicillin-sensitive laboratory segregant that had lost the plasmid concerned (F. Flett, S. Rayter, J. R. Saunders, and G. O. Humphreys, personal communication). Cultures were grown on chocolate agar made from 8% horse blood in Columbia agar base (Oxoid Ltd., London) and stored at −70°C in nutrient broth (Oxoid no. 2) containing 20% glycerol. For each series of experiments, chocolate agar plates were inoculated from the stored cultures and grown overnight at 36°C in an atmosphere of 10% CO_2 in air. The colony morphology of all strains used was T_3 or T_4. After the organisms had been checked for the presence or absence of β-lactamase by using the chromogenic cephalosporin 87/312 (16), the plates were flooded with medium, the organisms were scraped from the surface of the agar with a wire loop, and the suspension was transferred to 500-ml batches of prewarmed medium in 1-liter conical flasks. Each flask received the collected growth from one plate. The medium was the broth medium of Mayer et al. (10), except that GC medium base (Difco Laboratories, Detroit, Mich.) was replaced by brain heart infusion (Becton, Dickinson, & Co., Cockeysville, Md.). In some later experiments, the basal medium was protease-peptone no. 3 (Difco) (31). For each basal medium, the supplement described by Mayer et al. (10) was added at 1% (vol/vol).

The flasks were placed in a CO_2 incubator and purged with 10% CO_2 before being covered and shaken at 120 rpm on a rotary shaker at 36°C. Growth was continued to early exponential phase, usually about 4 h, by which time the mean generation time was about 1 h.

Assay of peptidoglycan synthesis. The cells from 3 liters of culture were harvested at 16,300 × *g* for 10 min and suspended in 60 ml of the basic medium of Mirelman et al. (13). They were then harvested at 12,000 × *g* as rapidly as possible, suspended in 8 ml of the same basic medium, and gently mixed with 8 ml of ether for 1 min (28). The ether-treated cells were recovered exactly as described earlier (28). The final pellet was suspended in 200 μl of tris (hydroxymethyl)aminomethane (Tris)-maleate buffer (0.05 M; pH 6), containing 0.02 M Mg^{2+} and 1 mM 2-mercaptoethanol, and used immediately.

For assay of peptidoglycan synthesis, samples contained, in a final volume of 70 μl, 10 nmol of uridine 5′-diphosphate (UDP)-*N*-acetylglucosamine (UDPGlcNAc), 10 μl of a 5× concentration of Tris-maleate buffer as described above, 10 μl of 4.5 mM UDP-*N*-acetylmuramic acid L-alanyl-D-isoglutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine (UDP-MurNAc-pentapeptide), prepared as described by Ward (29), UDP-[^{14}C]GlcNAc (0.125 μCi), and ether-treated cells (20 μl) containing 0.5 to 1 mg of protein (8). The procedure was as follows. All components except the radioactive precursor and the UDP-MurNAc-pentapeptide were prepared on ice, and the cells were added. This mixture was incubated for 30 min at 30°C to allow any endogenous peptidoglycan precursor to be used up. The UDP-MurNAc-pentapeptide and UDP-[^{14}C]GlcNAc were then added together with antibiotic when appropriate, and incubation was continued for 30 min.

In some experiments, the reaction was terminated by the addition of 1 ml of 5% (wt/vol) trichloroacetic acid, and the resulting precipitate was filtered on glass microfiber filters (GF/C, Whatman, England) and washed four times with 5 ml of 5% trichloroacetic acid and four times with 5 ml of ethanol. After being dried in an oven, the filters were covered with scintillation fluid containing 2(4′-*t*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole (Butyl-PBD) in 1 liter of toluene, and their radioactivity was measured in a scintillation counter (Intertechnique, Dover, N.J.). The efficiency of counting was 80%.

In experiments in which incorporation into insoluble peptidoglycan was being measured, the samples after incubation were treated by a modification of the method previously described (33). The reaction was terminated by adding sodium dodecyl sulfate (SDS) to a final concentration of 5% (wt/vol) and heating at 100°C for 20 min. After cooling, the insoluble residue was sedimented in a microcentrifuge (Jobling 320) at 14,000 × *g* for 5 min. The supernatant was removed and retained. The residue was washed with 1% SDS, this supernatant being combined with the first, and the pellet was transferred in 1% SDS to a glass microfiber filter, which was washed twice with water (5 ml). After they were dried in an oven, the radioactivity retained on the filters was measured as before.

The bulk of the SDS was removed from the supernatants by precipitation at 4°C and by centrifuging, and the final supernatant was dried over P_2O_5 in vacuo before being resuspended in water (50 μl) and transferred as a 2-cm streak to the origin of a paper chromatogram (Whatman 3MM). Descending chromatography was in isobutyric acid–0.5 M ammonia (5:3, vol/vol) for 48 h. After drying, the origin of the chromatogram was removed and its radioactivity was taken as a measure of the SDS-soluble peptidoglycan synthesized. Since a small proportion of this material was inevitably carried down with the SDS that precipitated on cooling, this precipitate was redissolved and its radioactivity was measured in aqueous scintillant (butyl-PBD, 6 g; toluene, 1 liter; Triton X 100, 0.5 liter; water, 150 ml). In calculations of radioactivity incorporated into the SDS-soluble fraction, correction was routinely made for loss by this route. Confirmation that the SDS-soluble material was indeed peptidoglycan was obtained by degrading it with lysozyme, which converted the polymer entirely to low-molecular weight products.

In all experiments, the radioactivity incorporated into peptidoglycan was taken to be the excess uptake observed in a sample to which the UDP-MurNAc-pentapeptide had been added. The control without this component generally represented only 5 to 10% of the incorporation in the full assay mixture.

MIC. Where required, the minimal inhibitory concentration (MIC) of antibiotics was measured by a plate method for *N. gonorrhoeae* exactly as described by Shannon et al. (23).

Antibiotics. The antibiotics used were commercial

samples, except for the following gifts, which are gratefully acknowledged: clavulanic acid from Beecham Research Ltd., Brockham Park, Surrey, England; spectinomycin from Upjohn Ltd., Crawley, Sussex, England; cefuroxime and cephalexin from Glaxo Research Ltd., Greenford, Middlesex, England; cefoxitin from Merck Sharp & Dohme, West Point, Pa.; tunicamycin and diumycin from R. B. Sykes.

RESULTS

Establishment of conditions for *in vitro* synthesis of peptidoglycan. The method used for in vitro synthesis of peptidoglycan was adapted from that applied to *E. coli* (13), in which permeability to peptidoglycan precursors was achieved by treatment of cells from exponential cultures with diethyl ether. Gonococci of strain 1L260 treated with ether and incubated with peptidoglycan precursors at pH 8 showed incorporation of label from radioactive UDP-GlcNAc, dependent upon the presence of UDP-MurNAc-pentapeptide, into trichloroacetic acid precipitates of the etherized cells. However, in these early experiments the incorporation in the absence of added UDP-MurNAc-pentapeptide was rather high, probably because of the high level of endogenous precursor that was available for synthesis. To avoid this complication, subsequent preparations were first incubated with non-radioactive UDP-GlcNAc alone, so that the pool of UDP-MurNAc-pentapeptide could be depleted. Then the radioactivity and the exogenous peptidoglycan precursor were added together, and under these conditions incorporation into the control cells represented only a small proportion of that in the complete assay. Although some incorporation was observed at pH 8, incubation over a range of pH values showed that the optimum occurred at pH 6 (Fig. 1). Furthermore, in trials of a variety of buffers at this pH, Tris-maleate was found to be the best.

Other experiments were conducted to establish the optimum Mg^{2+} concentration. For this purpose, $MgCl_2$ was omitted from the basic medium used for washing the harvested cells (see Materials and Methods) and also from the buffer used to wash the cells after ether treatment. The assay was conducted in Tris-maleate buffer without added magnesium, which was then supplied over a range of final concentrations up to 100 mM. The results showed that the optimum concentration of Mg^{2+} lay between 10 and 25 mM, and a repeated experiment within this range gave an optimum value of 20 mM (Fig. 2). At this concentration, examination of a range of incubation temperatures showed that 30°C was the optimum (Fig. 3).

Before using the etherized cell preparation to study the effects of antibiotics on peptidoglycan synthesis, it was necessary to show that incorporation was linear and that the assay mixture contained sufficient peptidoglycan precursor. Under the standard conditions, as set out in Materials and Methods, incorporation was essentially linear up to 30 min and showed only a small decline from linearity after 60 min (Fig. 4).

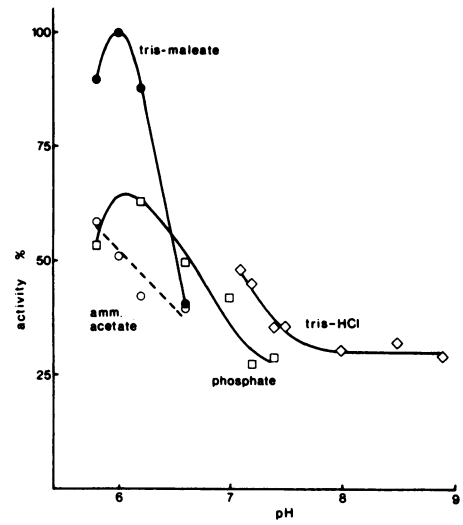

FIG. 1. *Peptidoglycan synthesis measured by incorporation into trichloroacetic acid precipitates: the effect of changes of buffer and pH. The incorporation under optimum conditions is shown as 100%.*

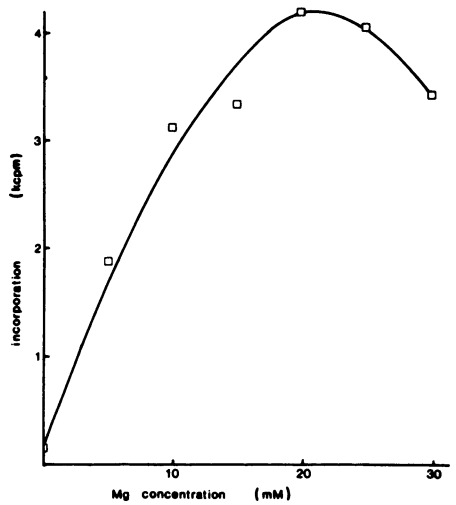

FIG. 2. *Peptidoglycan synthesis measured by incorporation into trichloroacetic acid precipitates: the effect of Mg^{2+} concentrations.*

In experiments in which the UDP-MurNAc-pentapeptide concentration was doubled or quadrupled, no difference in peptidoglycan synthesis was observed.

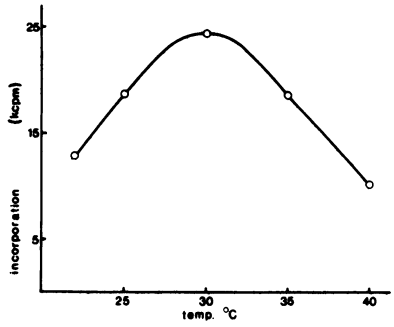

FIG. 3. *Peptidoglycan synthesis measured by incorporation into trichloroacetic acid precipitates: the effect of temperature.*

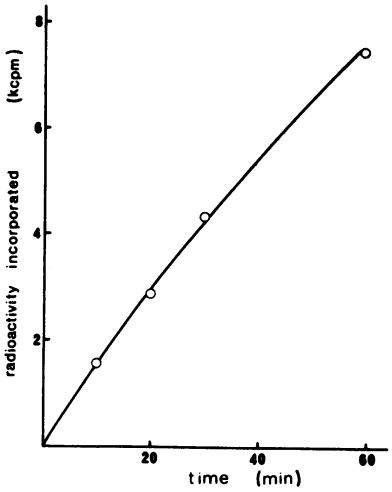

FIG. 4. *Time dependence of incorporation into trichloroacetic acid-precipitated peptidoglycan.*

Effect of antibiotics on peptidoglycan synthesis measured in trichloroacetic acid precipitates. Once conditions for the incorporation of peptidoglycan precursors into trichloroacetic acid precipitates had been standardized, it was possible to study the effects of various antibiotics. Two strains were examined in this series of experiments, one sensitive to penicillin (1L260) and one resistant by reason of plasmid-coded β-lactamase (1L261) (Table 1). Antibiotics known to affect various stages of peptidoglycan synthesis in other organisms were also effective here. The least active was tunicamycin at 1 μg/ml (cf. the relative insensitivity of cell-free synthesis of peptidoglycan in *Bacillus licheniformis* 94 [30]). Bacitracin, which prevents the recycling of undecaprenyl-pyrophosphate (24), caused 85 to 90% inhibition, as did diumycin, which interferes with the utilization of the GlcNAc-MurNAc-(pentapeptide)-P-P-undecaprenol intermediate (7, 9). The simultaneous addition of clavulanic acid, a known inhibitor of TEM β-lactamase (20), brought the penicillin-induced inhibition of peptidoglycan synthesis in the β-lactamase producer 1L261 from its initially low level to the same value as in the sensitive strain 1L260, although the same concentration of clavulanic acid alone had no inhibitory effect. As expected, added clavulanic acid produced little enhancement of penicillin inhibition when the sensitive strain was used. In fact, unlike most bacteria, *N. gonorrhoeae* are relatively sensitive to clavulanic acid (15), and later experiments showed that the peptidoglycan synthesis by ether-treated cells was affected in much the same way by this as by other β-lactam antibiotics.

Peptidoglycan synthesis measured in trichloroacetic acid precipitates compared with SDS-soluble and SDS-insoluble fractions. Although trichloroacetic acid precipitates

TABLE 1. *Inhibition by various antibiotics of the in vitro synthesis of peptidoglycan*

Antibiotic	Concn (μg/ml)	% Inhibition of peptidoglycan synthesis[a]	
		Strain 1L260 (β-lactamase negative)	Strain 1L261 (β-lactamase positive)
Benzylpenicillin	10	42.3	36.5
Benzylpenicillin + clavulanic acid	10; 2[b]	51.4	83.4
Bacitracin	10	88.8	85.5
Tetracycline	10	5.4	19.4
Spectinomycin	10	5.8	12.4
Cefuroxime	10	57.3	80.6
Cefoxitin	10	77.3	—
Diumycin	1	88.4	89.9
Tunicamycin	1	26.0	—

[a] Peptidoglycan synthesis was measured by the radioactivity incorporated into trichloroacetic acid precipitates as described in the text. —, Not examined.

[b] First value refers to benzylpenicillin; second value refers to clavulanic acid.

undoubtedly contained the peptidoglycan, it is known from work with other organisms that in the presence of β-lactam antibiotics soluble material excreted by the cells can also be co-precipitated with them (27). Therefore, in detailed experiments concerned with the action of the β-lactam antibiotics, the ether-treated cells, after the period of incorporation, were either collected as trichloroacetic acid precipitates as described above, or extracted with hot SDS as described in Materials and Methods, to yield SDS-insoluble and SDS-soluble fractions. In the latter, only the polymeric material was considered, being collected as the product that remained on the origin of a paper chromatogram.

The effect of different concentrations of cefuroxime on the incorporation of radioactivity from UDP-[^{14}C]GlcNAc into trichloroacetic acid precipitates is shown in Fig. 5. Two β-lactamase-negative (1L260, FA19) and one β-lactamase-positive (1L261) strains were used, the absolute amount of incorporation in the controls being dependent upon the individual preparation and therefore not for direct comparison. In all of these, inhibition of peptidoglycan synthesis by cefuroxime followed the same pattern. At concentrations up to about 0.05 μg/ml there was a slight increase in incorporation, and thereafter a rapid continuous decrease occurred up to 10 μg/ml, the highest concentration used in this experiment. The cefuroxime concentration that caused a 50% inhibition of the maximum incorporation achieved was about 1 μg/ml for strains 1L261 and FA19, and about 5 μg/ml for strain 1L260. These concentrations were greater by about two orders of magnitude than the MICs of cefuroxime for the same strains, which were 0.014, 0.012, and 0.010 μg/ml, respectively.

The effect of benzylpenicillin on the biosynthesis of the SDS-soluble and SDS-insoluble fractions of the peptidoglycan of the β-lactamase-negative strain 1L260 is shown in Fig. 6. Without antibiotic, approximately equal amounts of radioactivity were incorporated in the peptidoglycan of both fractions. With doses of penicillin from 0.01 to 1 μg/ml, the amount of SDS-insoluble product actually increased, the highest point of the curve representing an increment of 84% over the control. Higher doses of antibiotic decreased the synthesis of SDS-insoluble peptidoglycan almost to zero. The effects on the synthesis of SDS-soluble peptidoglycan polymer were quite different; at concentrations up to 0.1 μg of penicillin per ml, there was no great change (but certainly no decrease corresponding to the increase in insoluble material) and thereafter up to 50 μg/ml there was a vast increase in the amount of incorporation, far outweighing any concomitant decrease in the production of the SDS-insoluble fraction. Thus, at 1 μg of penicillin per ml there was already 2.4 times as much soluble polymer synthesized as in the control, although the insoluble product was still in excess of its control value. At 50 μg/ml, the synthesis of soluble polymer had increased 5.6-fold. The changes at low penicillin concentration thus correspond to those observed with trichloroacetic acid-precipitated material, where again a slight rise was consistently seen (Fig. 5).

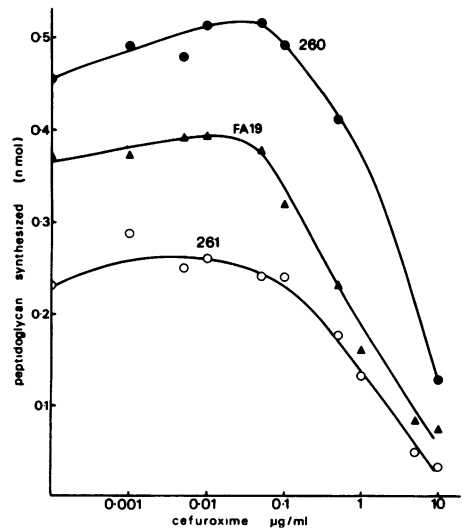

FIG. 5. *Effect of cefuroxime concentration on the synthesis of peptidoglycan measured in trichloroacetic acid precipitates. Preparations from strains 1L260, 1L261, and FA19 were used. For convenience, the control values are plotted on the ordinate axis.*

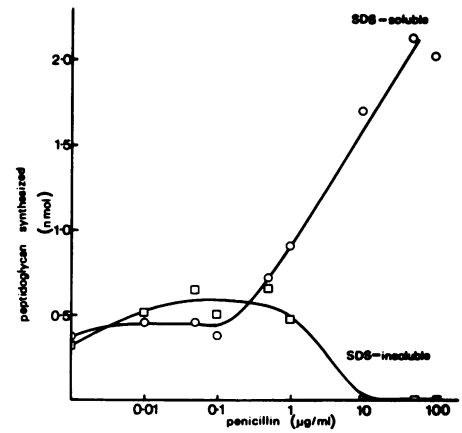

FIG. 6. *Effect of benzylpenicillin concentration on the synthesis of SDS-soluble and SDS-insoluble peptidoglycan in strain 1L260. Control values are plotted on the ordinate axis.*

However, the large amount of SDS-soluble polymer produced at higher drug concentrations was evidently not trichloroacetic acid-precipitable, since it did not appear in the latter fraction. Direct attempts to precipitate the material that had been dissolved in SDS with trichloroacetic acid were unsuccessful.

β-Lactamase production prevented the relative effects of penicillin upon peptidoglycan synthesis in strains 1L261 and 1L260 from being compared, but cefuroxime, which is resistant to TEM-β-lactamase (17), was used in this way (Fig. 7). Again the effects upon the synthesis of SDS-insoluble and SDS-soluble peptidoglycan were of the same general type just described for benzylpenicillin and 1L260, and once more the higher concentrations of cefuroxime produced full inhibition of the synthesis of insoluble material and a considerable enhancement in production of the soluble polymer, particularly noticeable with the β-lactamase producer for which at 100 μg/ml the increase was 6.8-fold.

That these responses to β-lactams were not confined to the strains isolated in Liverpool can be seen from comparable experiments with strain FA19, which has been widely used in genetic and biochemical studies elsewhere. With this strain, far less of the synthesized peptidoglycan appeared in the SDS-insoluble fraction, even in the absence of antibiotic, but increasing concentrations of benzylpenicillin again led to a small rise in incorporation into both fractions up to 0.01 μg/ml, and thereafter to an almost total inhibition of the synthesis of SDS-insoluble material and a disproportionate enhancement of the production of SDS-soluble polymer (Fig. 8).

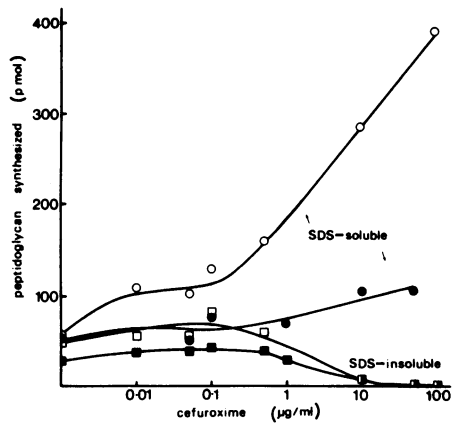

FIG. 7. *Effect of cefuroxime concentration on the synthesis of SDS-soluble and SDS-insoluble peptidoglycan. Symbols: ○, □, strain 1L261; ●, ■, strain 1L260. Control values are plotted on the ordinate axis.*

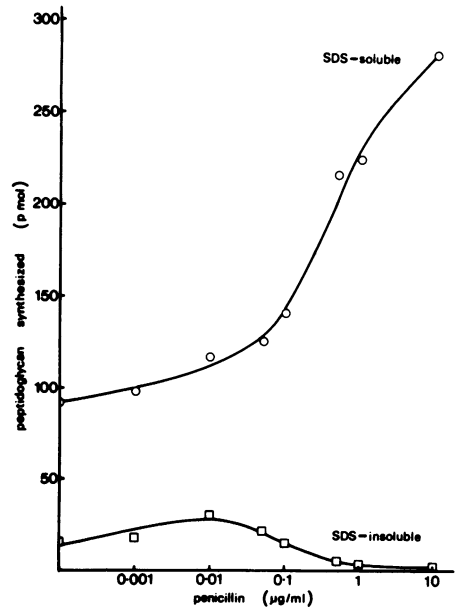

FIG. 8. *Effect of benzylpenicillin concentration on the synthesis of SDS-soluble and SDS-insoluble peptidoglycan in strain FA19. Control values are plotted on the ordinate axis.*

Thus, at 10 μg of antibiotic per ml the increase over the control for strain FA19 was about threefold, compared with strain 1L260 for which the corresponding increase was fourfold (Fig. 6). The MICs of benzylpenicillin against these strains were 0.010 and 0.21 μg/ml, respectively.

Clavulanic acid is fairly active against gonococci, MICs of 0.5 to 5 μg/ml (11) and 0.1 μg/ml (15) having been reported. Its effect on in vitro peptidoglycan synthesis by preparations from strain FA19 was also examined (Fig. 9). At concentrations below 1 μg/ml it had little effect on the synthesis of the SDS-insoluble material and produced a slight increase in the amount of trichloracetic acid-precipitable or of SDS-soluble material (examined separately). As with the other β-lactam antibiotics, higher concentrations inhibited the synthesis of the TCA-precipitable and SDS-insoluble fractions and caused an increase in the amount of SDS-soluble polymer. Thus its unusual effectiveness as an antibiotic against *N. gonorrhoeae* is paralleled by its effects on peptidoglycan synthesis in vitro.

Other β-lactam antibiotics were also examined. The amidino analog of penicillin mecillinam, which is known to bind specifically to the penicillin-binding proteins of *Escherichia coli* and to cause that organism to be converted to ovoid shapes (25), had only a slight effect upon peptidoglycan synthesis by strain 1L260, as mea-

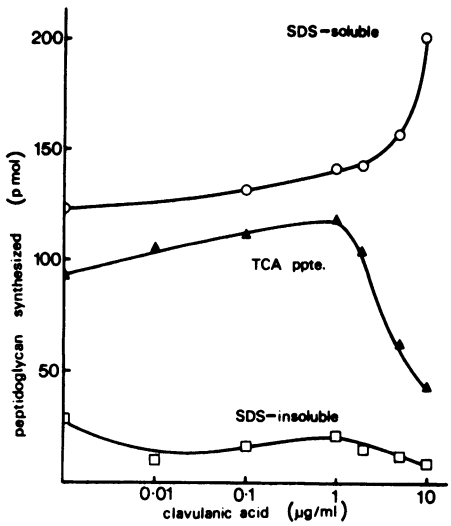

FIG. 9. *Effect of clavulanic acid concentration on the synthesis of peptidoglycan in trichloroacetic acid precipitates and also in the SDS-soluble and SDS-insoluble fractions. Strain FA19. Control values are plotted on the ordinate axis.*

sured by incorporation into trichloroacetic acid precipitates. Mecillinam at 0.1 μg/ml caused a 6% increase in synthesis, and higher concentrations caused a steady decrease, but even at 100 μg/ml synthesis was only 28% less than that in the control preparation.

Cephalexin has been reported to cause higher degrees of peptidoglycan cross-linking and inhibition of DD-carboxypeptidase activity in *E. coli* PAT84 (14), changes which may account for the appearance of filamentous cells. Its effect on peptidoglycan synthesis in etherized *N. gonorrhoeae* strain FA19 was, however, surprisingly small. Concentrations up to 100 μg/ml produced hardly any decrease in the synthesis of SDS-insoluble material, and only a 63% increase in the amount of the SDS-soluble fraction. The amount of incorporation into the trichloroacetic acid-precipitate was unaffected up to 10 μg/ml, and even at 100 μg/ml had decreased by only 24%. The MIC of cephalexin for strain FA19 was high (0.78 μg/ml) compared with the MICs of benzylpenicillin and cefuroxime (0.010 and 0.012 μg/ml, respectively), a fact which corresponds well with the relative insensitivity to cephalexin of the in vitro system.

DISCUSSION

Cell-free synthesis of peptidoglycan in *N. gonorrhoeae* was sensitive to the antibiotics that are known to inhibit this process in other organisms and was resistant to substances that act in other ways. The high level of susceptibility to bacitracin may indicate that the amount of undecaprenol phosphate normally present in these ether-treated cells is rather low, since bacitracin prevents the recycling of the undecaprenol pyrophosphate that is needed to allow further synthesis of peptidoglycan to proceed (24). It is evident, therefore, that the cell-free system has the general properties that we expected.

The optimum pH value for synthesis was pH 6, in contrast to observations with *E. coli*, for which pH 8.3 was employed (13) or with toluenized *Micrococcus luteus* (optimum pH 8) (S. J. Thorpe and H. R. Perkins, unpublished data). On the other hand, the optimum Mg^{2+} concentration was the same for each organism (20 mM). Experiments on the turnover of peptidoglycan in *N. gonorrhoeae* (6) showed that in growth medium 50% of peptidoglycan-specific label had disappeared from the cells within 1 h at 37°C. Wegener et al. (32) found that when 10 μg of penicillin per ml was added to cells kept at pH 6 rather than to exponential cultures, the rate of hydrolysis of peptidoglycan was halved, although the cells were protected from autolysis under these conditions. In partial contrast, Goodell et al. (5) found that at pH 6.4 penicillin had no effect on the loss of peptidoglycan labeled with [^{3}H]diaminopimelic acid, but caused increased loss when the label was [^{3}H]glucosamine. In any event, it seems unlikely that the optimum synthesis at pH 6 observed in our experiments was attributable solely to any inhibition of peptidoglycan lysis at that pH value, although that may well have represented an additional factor.

Consistently, low concentrations of β-lactam antibiotics produced a small increase in the in vitro synthesis of peptidoglycan measured either as a trichloroacetic acid precipitate or as SDS-insoluble or SDS-soluble material. A possible explanation would be that, since gonococci contain a DD-carboxypeptidase which is highly sensitive to β-lactam antibiotics (2), this enzyme was attacking the UDP-MurNAc-pentapeptide added to the etherized cells and rendering it unsuitable for peptidoglycan synthesis. Penicillin would then be seen to act by inhibiting the enzyme and sparing the substrate. This explanation is unlikely for the following reasons: (i) the enzyme has a pH optimum of 8 to 10 and is relatively inactive at pH6 (24) (ii) the incorporation was almost linear long after the 30-min period used in the tests and was therefore apparently insensitive to the disappearance of substrate (iii) increasing the UDP-MurNAc-pentapeptide concentration fourfold did not prevent the enhancement of synthesis observed at low penicillin concentrations.

An alternative explanation would be that low β-lactam concentrations were inhibiting the cross-linking of some relatively minor class of peptidoglycan, the function of which was to control the synthesis of a major type of cross-linked peptidoglycan. At higher concentrations of β-lactam, the cross-linking of this major type of peptidoglycan would also be inhibited and incorporation into the SDS-insoluble and trichloroacetic acid-precipitate fractions would fall, which is what was observed.

A different phenomenon was the observation that high concentrations of β-lactam antibiotics produced a stimulation of the synthesis of SDS-soluble peptidoglycan polymer far in excess of any concomitant disappearance of the SDS-insoluble material. It is evident that a simple transfer from one category to the other, as observed in *B. licheniformis* (11) or *M. luteus* (12, 33), could not account for our results with *N. gonorrhoeae*, which suggest that the synthesis of un-cross-linked polymer was normally limited by the presence of the cross-linked fraction. Thus, when β-lactams prevent the formation of cross-links, uncontrolled synthesis of the un-cross-linked polymer proceeds, giving the high yields observed in our experiments. Alternatively, β-lactams as possible analogs of acyl-D-alanyl-D-alanine might interact with an allosteric site on the synthetic enzyme, designed cooperatively to enhance peptidoglycan synthesis when plenty of UDP-MurNAc-pentapeptide substrate is available.

ACKNOWLEDGMENTS

This investigation was supported by the Medical Research Council, London, England (Programme grant no. G974/775).

We also thank J. R. Saunders (Medical Research Council grant no. G976/811) for assistance with the growth of gonococci.

LITERATURE CITED

1. **Ashford, W. A., R. G. Golash, and V. G. Hemming.** 1976. Penicillinase-producing *Neisseria gonorrhoeae*. Lancet ii:657–658.
2. **Davis, R. H., and M. R. J. Salton.** 1975. Some properties of a D-alanine carboxypeptidase in envelope fractions of *Neisseria gonorrhoeae*. Infect. Immun. **12**:1065–1069.
3. **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.
4. **Ghuysen, J.-M.** 1977. The concept of the penicillin target from 1965 until today. J. Gen. Microbiol. **101**:13–33.
5. **Goodell, E. W., M. Fazio, and A. Tomasz.** 1978. Effect of benzylpenicillin on the synthesis and structure of the cell envelope of *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **13**:514–526.
6. **Hebeler, B. H., and F. E. Young.** 1976. Chemical composition and turnover of peptidoglycan in *Neisseria gonorrhoeae*. J. Bacteriol. **126**:1180–1185.
7. **Linnett, P. E., and J. L. Strominger.** 1973. Additional antibiotic inhibitors of peptidoglycan synthesis. Antimicrob. Agents Chemother. **4**:231–236.
8. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
9. **Lugtenberg, E. J. J., J. A. Hellings, and G. J. van de Berg.** 1972. Inhibition of peptidoglycan synthesis by the antibiotic diumycin A. Antimicrob. Agents Chemother. **2**:485–491.
10. **Mayer, L. W., K. K. Holmes, and S. Falkow.** 1974. Characterization of plasmid deoxyribonucleic acid from *Neisseria gonorrhoeae*. Infect. Immun. **10**:712–717.
11. **Miller, J. M., C. N. Baker, and C. Thornsberry.** 1978. Inhibition of β-lactamase in *Neisseria gonorrhoeae* by sodium clavulanate. Antimicrob. Agents Chemother. **14**:794–796.
12. **Mirelman, D., R. Bracha, and N. Sharon.** 1974. Penicillin-induced secretion of a soluble, uncross-linked peptidoglycan by *Micrococcus luteus* cells. Biochemistry **13**:5045–5053.
13. **Mirelman, D., Y. Yashouv-Gan, and U. Schwarz.** 1976. Peptidoglycan biosynthesis in a thermosensitive division mutant of *Escherichia coli*. Biochemistry **15**: 1781–1790.
14. **Mirelman, D., Y. Yashouv-Gan, and U. Schwarz.** 1977. Regulation of murein biosynthesis and septum formation in filamentous cells of *Escherichia coli* PAT84. J. Bacteriol. **129**:1593–1600.
15. **Neu, H. C., and K. P. Fu.** 1978. Clavulanic acid, a novel inhibitor of β-lactamases. Antimicrob. Agents Chemother. **14**:650–655.
16. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**:283–288.
17. **O'Callaghan, C. H., R. B. Sykes, A. Griffiths, and J. E. Thornton.** 1976. Cefuroxime, a new cephalosporin antibiotic: activity in vitro. Antimicrob. Agents Chemother. **9**:511–519.
18. **Percival, A., J. Rowlands, J. E. Corkill, C. D. Alergant, O. P. Arya, E. Rees, and E. H. Annels.** 1976. Penicillinase-producing gonococci in Liverpool. Lancet ii:1379–1382.
19. **Phillips, I.** 1976. β-Lactamase-producing penicillin-resistant gonococcus. Lancet ii:656–657.
20. **Reading, C., and M. Cole.** 1977. Clavulanic acid: a beta-lactamase-inhibiting beta-lactam from *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. **11**:852–857.
21. **Roberts, M., L. P. Elwell, and S. Falkow.** 1977. Molecular characterization of two beta-lactamase-specifying plasmids isolated from *Neisseria gonorrhoeae*. J. Bacteriol. **131**:557–563.
22. **Salton, M. R. J.** 1977. Enzymatic basis for β-lactam antibiotic activity, p. 177–196. *In* R. B. Roberts (ed.), The gonococcus. John Wiley & Sons, Inc., New York.
23. **Shannon, R., A. J. Hedges, and R. J. Edwards.** 1975. Distribution of levels of penicillin resistance among freshly isolated strains of *N. gonorrhoeae*. Application of a novel sensitivity assay. Br. J. Vener. Dis. **51**:246–250.
24. **Siewert, G., and J. L. Strominger.** 1967. Bacitracin, an inhibitor of the dephosphorylation of lipid-pyrophosphate, an intermediate in biosynthesis of bacterial cell walls. Proc. Natl. Acad. Sci. U.S.A. **57**:767–773.
25. **Spratt, B. G.** 1975. Distinct penicillin binding proteins involved in the division, elongation and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S.A. **72**:2999–3003.
26. **Turner, G. C., J. G. Ratcliffe, and D. Anderson.** 1976. Penicillinase-producing *Neisseria gonorrhoeae*. Lancet ii:793.
27. **Tynecka, Z., and J. B. Ward** 1975. Peptidoglycan synthesis in *Bacillus licheniformis*. The inhibition of cross-

linking by benzylpenicillin and cephaloridine *in vivo* accompanied by the formation of soluble peptidoglycan. Biochem. J. **146**:253–267.

28. **Vosberg, H.-P., and H. Hoffman-Berling.** 1971. DNA-synthesis in nucleotide-permeable *Escherichia coli* cells. I. Preparation and properties of ether-treated cells. J. Mol. Biol. **58**:739–753.
29. **Ward, J. B.** 1974. The synthesis of peptidoglycan in an autolysin-deficient mutant of *Bacillus licheniformis* NCTC 6346 and the effect of β-lactam antibiotics, bacitracin and vancomycin. Biochem. J. **141**:227–241.
30. **Ward, J. B.** 1977. Tunicamycin inhibition of bacterial wall polymer synthesis. FEBS Lett. **78**:151–154.
31. **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.
32. **Wegener, W. S., B. H. Hebeler, and S. A. Morse.** 1977. Cell envelope of *Neisseria gonorrhoeae*: penicillin enhancement of peptidoglycan hydrolysis. Infect. Immun. **18**:717–725.
33. **Weston, A., J. B. Ward, and H. R. Perkins.** 1977. Biosynthesis of peptidoglycan in wall plus membrane preparations from *Micrococcus luteus:* direction of chain extension, length of chains and effect of penicillin on cross-linking. J. Gen. Microbiol. **99**:171–181.