Progress of $O$-Acetylation and Cross-linking of Peptidoglycan in Neisseria gonorrhoeae Grown in the Presence of Penicillin

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The synthesis, cross-linking and $O$-acetylation of gonococcal peptidoglycan in growing cells were studied by following incorporation of radioactive glucosamine and separation of the SDS-insoluble peptidoglycan into uncross-linked (monomer) and cross-linked (dimer and oligomer) fragments. Cultures to which penicillin or piperacillin at concentrations near the minimum growth inhibitory concentration (MIC) had been added 20 min before the radioactive label were compared with controls. The $\beta$-lactams affected the early stage of cross-linking (up to 3 min) but had no effect thereafter. The deficit of cross-linking, however, did not recover. The $O$-acetylation, particularly of the monomer fraction, was decreased by $\beta$-lactams even at concentrations that had no effect on culture optical density, viable counts or overall peptidoglycan synthesis. These effects on $O$-acetylation occurred mainly after the first 3 min of incorporation, rather than before. $O$-Acetylation of the oligomer fraction was also followed. Here penicillin led to increased levels of $O$-acetylation during the early stages of incorporation but the final value was never exceeded; indeed at higher drug concentrations the later stages of $O$-acetylation of oligomers were inhibited (e.g. almost completely at $2.5 \times$ MIC).

INTRODUCTION

The peptidoglycan of Neisseria gonorrhoeae is partially $O$-acyetylated (Blundell et al., 1980) on the C6 position of the muramic acid residues (Perkins et al., 1983). We have previously shown that newly synthesized peptidoglycan differs in chemical composition from mature peptidoglycan (Lear & Perkins, 1983, 1986). New peptidoglycan was both less highly cross-linked and much less $O$-acyetylated than mature peptidoglycan. After a maturation process lasting for 0.5–1 generation time and involving further transpeptidation and $O$-acyetylation, the final degrees of peptide cross-linking and $O$-acyetylation were achieved. Dougherty (1983a) has also inferred such a maturation process in N. gonorrhoeae, by observing differences between biosynthetically 'new' and 'old' peptidoglycan. Similarly, in Proteus mirabilis, Gmeiner & Kroll (1981) concluded that $O$-acetylation succeeds the incorporation of non-$O$-acyetylated murein subunits into the murein sacculus, although this clear temporal distinction was not observed in N. gonorrhoeae (Lear & Perkins, 1986). In Escherichia coli a maturation process involving further transpeptidation of newly inserted peptidoglycan has also been observed (de Pedro & Schwarz, 1981).

High concentrations of benzylpenicillin cause inhibition of cell wall biosynthesis, rapid loss of viability, induction of wall hydrolase activity, and cell lysis in N. gonorrhoeae (Hebeler & Young, 1975; Wegener et al., 1977; Goodell et al., 1978; Sinha & Rosenthal, 1981). Low concentrations of $\beta$-lactams (at or below the minimum inhibitory concentration for growth – MIC), when added to growing cultures, cause cellular enlargement and appreciable septal thickening (Lorian & Atkinson, 1976; Westling-Håggström et al., 1977; Blundell & Perkins, 1981). They also cause a marked decrease in the $O$-acytylation of peptidoglycan, especially of the uncross-linked disaccharide-peptide (monomer) components (Blundell & Perkins, 1981). Little or no effect was
seen on the overall degree of peptide cross-linking. Dougherty (1983b) similarly found that the primary effect of penicillin, at a concentration equal to the MIC, on the sensitive strain FA19, was a slight change in peptide cross-linking and a sharp decline in the degree of O-acetylation.

The present work examines the action of penicillin upon the post-synthetic modifications of newly incorporated peptidoglycan, including for the first time the degree of O-acetylation of the oligomer fraction. The action of piperacillin, which in E. coli specifically affects the maturation of peptidoglycan (de Pedro & Schwarz, 1981), was also examined.

METHODS

Growth conditions and radioactive labelling. N. gonorrhoeae strain IL260, described previously (Brown & Perkins, 1979), grown on chocolate agar plates overnight, was used to inoculate pyruvate-supplemented proteose peptone no. 3 (Blundell & Perkins, 1981) to an OD<sub>675</sub> of 0.04. Cultures were incubated in a shaking water bath at 37°C until a culture density of OD<sub>675</sub> = 0.1 was achieved, and were then divided equally into control (to which no antibiotic was to be added) and antibiotic cultures. Incubation was continued to OD<sub>675</sub> = 0.25, when benzylpenicillin was added to the antibiotic cultures at various final concentrations. After 20 min [sufficient time to ensure that binding of antibiotic to the penicillin-binding proteins had reached completion (Dougherty et al., 1980)] [D-l-<sup>14</sup>C]glucosamine hydrochloride (54 μCi μmol<sup>-1</sup>, 2.0 MBq μmol<sup>-1</sup>; Amersham) was added to a final concentration of 1 μCi ml<sup>-1</sup> (37 kBq ml<sup>-1</sup>). Incubation was continued for a further 90 min (1.25 generations).

Analysis of peptidoglycan – incorporation of radioactivity, purification, composition. At various time intervals after the addition of radioactivity, 2 ml culture samples were removed into an equal volume of 10% (w/v) SDS and heated at 100 °C for 20 min (Blundell et al., 1980). Incorporation of radioactivity into peptidoglycan was measured by filtering duplicate 50 μl volumes on glass microfibre discs (GF/C, Whatman). The filters were washed with water, dried and their radioactivity was measured in a scintillation counter (Blundell & Perkins, 1981).

Peptidoglycan was prepared from the remainder of each sample essentially as described by Blundell & Perkins (1981). Briefly, SDS-insoluble material was collected by centrifugation at 130000 g for 45 min, washed, digested with Chalaropsis muramidase B (Hash & Rothlauf, 1967) (given by Dr. J. B. Ward) and the resulting solubilized material was prepared for TLC. Samples were spotted on to the origin of 0.25 mm silica gel plastic TLC plates (Polygram SilG/UV254; Macherey-Nagel, Germany) and the chromatogram was developed twice with iso-butyric acid/l M-ammonia (5:3, v/v) (Martin & Gmeiner, 1979). Radioactive spots were detected by autoradiography on X-ray film (Kodak X-Omat H), and identified by comparison with previously characterized markers as monomer (disaccharide peptide), O-acetyl monomers, dimer (bis-disaccharide cross-linked peptide), mono-O-acetyl dimer, di-O-acetyl dimer and oligomers (the latter were not further divided into sub-fractions). Each spot was cut out from the chromatogram and radioactivity was measured in a non-aqueous scintillant (Scintran, Cocktail ‘O’, BDH).

Endopeptidase treatment of oligomers. This was done by the method previously used for dimers (Lear & Perkins, 1986). After separation of peptidoglycan fragments by TLC, strips corresponding to the oligomer region were washed to remove scintillant and eluted with water. The dried samples were digested overnight with D-d-carboxypeptidase from Streptomyces albus G in buffer at pH 7.5. The products were separated by TLC into O-acetylated and non-O-acetylated monomers, detected and quantified as before.

Determination of viable count. A method to optimize the determination of culture viability was devised because of the tendency of gonococci to form large clumps of cells in liquid culture. A brief period of sonication, sufficient to break up any cell clumps without damaging the organisms themselves, was used. Sonication at an amplitude of 4-5 μm for 14 s maximized viable counts [measured as colony forming units (c.f.u.) ml<sup>-1</sup>]. The period of sonication was critical, with viable counts peaking sharply at 14 s. Routinely, 3 ml culture samples were removed and placed in a sterile glass tube (75 x 13 mm), covered with a Subaseal through which was passed the tapered probe, terminal diameter 3 mm. Sonication (MSE sonicator) for 14 s followed, and the sample was then serially diluted so that viable counts could be determined by the method of Miles & Misra (1938), on Gonococcal Agar plates. Colonies were counted after plates had been incubated at 37 °C for 48 h.

RESULTS

To establish the conditions in which the effects of benzylpenicillin on gonococcal peptidoglycan could best be studied and interpreted, concentrations of the antibiotic above and below the MIC (0.21 μg benzylpenicillin ml<sup>-1</sup> for N. gonorrhoeae IL260; Brown & Perkins, 1979) were used. Their effects on culture OD<sub>675</sub>, viable counts and incorporation of [<sup>14</sup>C]glucosamine into SDS-insoluble material (96% of it peptidoglycan: Blundell & Perkins, 1981) are shown in Fig. 1. Concentrations of penicillin at or below 0.1 μg ml<sup>-1</sup> had no effect on OD<sub>675</sub>; even 0.5 μg ml<sup>-1</sup> produced no decline until more than 90 min after administration. Viable counts were
unaffected by 0.01 μg penicillin ml⁻¹ (not shown) and only declined to a limited extent about 80 min after addition of 0.1 μg ml⁻¹. Despite the relatively small effect of 0.5 μg penicillin ml⁻¹ on culture OD₆₇₅, this concentration brought a rapid and progressive decrease in viable counts. The corresponding changes in peptidoglycan synthesis can also be seen in Fig. 1. The two lower penicillin concentrations produced an appreciable enhancement, as formerly observed when penicillin was added to growing cultures undergoing long-term labelling (Blundell & Perkins, 1981), whereas 0.5 μg penicillin ml⁻¹ occasioned a slight decrease in uptake during the first 45 min of labelling, followed by a period of decline when radioactivity was being lost. It is against these contrasting effects on culture OD₆₇₅, viable counts and peptidoglycan synthesis that the following detailed observations on peptidoglycan composition have to be considered.

Cross-linking

The effects of benzylpenicillin on the incorporation of glucosamine into the various fractions (monomers, dimers, oligomers) found in Chalaropsis muramidase digests of peptidoglycan are shown in Fig. 2. As shown earlier (Lear & Perkins, 1983, 1986) much of the newly synthesized peptidoglycan became cross-linked within 3 min, the remaining changes taking about one generation time to complete. The effects of penicillin may be considered in relation to these two phases of incorporation. For the material incorporated within 3 min, penicillin at 0.1 μg ml⁻¹ produced an approximately 25% increase in the proportion of uncross-linked material (monomers) with corresponding decreases in both dimers and oligomers (Fig. 2a, b). The higher penicillin concentration of 0.5 μg ml⁻¹ resulted in a further increase of some 20% in the proportion of monomer fraction, most of this change coming at the expense of the dimers while

**Fig. 1.** Effects of penicillin on growth (OD₆₇₅), viable counts and uptake of glucosamine into peptidoglycan. (a) Viable counts; (b) incorporation of [¹⁴C]-glucosamine into SDS-insoluble peptidoglycan; (c) OD₆₇₅. Penicillin was added, followed 20 min later by [¹⁴C]glucosamine (see Methods for details) as shown by arrows. Concentration (μg ml⁻¹) of benzylpenicillin: ○—○, none; ▲—▲, 0.01; ●—●, 0.1; ■—■, 0.5. Where symbols for lower penicillin concentrations are not shown, this indicates that no difference from the control was detected. Results represent replicate samples from a typical experiment.
Fig. 2. (a, b, c) Effects of penicillin on incorporation of glucosamine into peptidoglycan monomers (○), dimers (△) and oligomers (□). The percentage of total radioactivity in these fractions separated by TLC of SDS-insoluble peptidoglycan first digested by Chalaropsis muramidase is shown. (a) Without penicillin; (b) 0-1 μg penicillin ml⁻¹; (c) 0-5 μg penicillin ml⁻¹. (d) The degree of cross-linkage as defined by Blundell & Perkins (1981). Concentration (μg ml⁻¹) of benzylpenicillin: ○····· ○, none; △····· △, 0-01; ···· ···· ····, 0-1; □—□, 0-5. Each experiment consisted of a control without penicillin and two or more parallel cultures with added drug. The values represent the means of duplicate results for two (0-01 μg penicillin ml⁻¹) or three (0-1 and 0-5 μg penicillin ml⁻¹) separate experiments and the mean of all experiments for the controls. Individual values for proportion of total radioactivity did not differ by more than about 10%.

the oligomers remained relatively unaffected (Fig. 2b, c). In the control culture, there was a gradual decrease with time of the proportion of monomers with little change in dimers and a corresponding increase in the proportion of oligomers. This 'maturation' process (Lear & Perkins, 1983), during which some further cross-linking occurred, was clearly not inhibited by penicillin; at a concentration of 0-1 μg ml⁻¹ some shift to increased oligomer still took place and the initial shortfall of dimers was to some extent restored with a more rapid loss of monomers (Fig. 2a, b). Similar changes were observed at 0-5 μg penicillin ml⁻¹, except that the changes that took place after the first 3 min were speeded up, with the major conversion of monomers to dimers and oligomers occurring in the following 12 min. Alternatively, this period could be considered as a delay to the 15th min of some of the cross-linking that in controls occurred within the first 3 min.

The changes in the proportions of monomers, dimers and oligomers just described may also be summarized in the form of the degree of cross-linking (Fig. 2d). Here it is seen that the values for 0-01 μg penicillin ml⁻¹ (omitted from Fig. 2a–c for the sake of clarity) were little changed from...
Penicillin and N. gonorrhoeae peptidoglycan

Fig. 3. Proportion of peptidoglycan subfractions (monomers, dimers, oligomers) O-acetylated.
Concentration (µg ml⁻¹) of benzylpenicillin; ○—○, none; ▲—▲, 0·01; ■—■, 0·1; □—□, 0·5. For other details see Methods and Fig. 2.

the control without antibiotic, either during the initial 3 min or subsequently. The decreases in cross-linking brought about by higher concentrations of penicillin mostly affected the initial 3 min period, the gains during the remainder of the 90 min being very similar whether penicillin was present or not. Perhaps the only noticeable difference here was a slight tendency for cross-linking to decline in the last 60 min when 0·5 µg penicillin ml⁻¹ was present. Here, however, the bacteria were rapidly losing viability and even overall labelling was declining, i.e. peptidoglycan was being lost from the cells, although little decline in culture OD₆₇₅ had ensued at that stage (Fig. 1).

Degree of O-acetylation

The separation of Chalaropsis muramidase digests by TLC was also used to determine the degree of O-acetylation of the monomer and dimer fractions (Blundell & Perkins, 1981). The degree of O-acetylation of the oligomer fraction (mainly trimers) was determined for the first time by using an endopeptidase to convert oligomers into O-acetylated and non-O-acetylated monomers, the relative proportions of which could then be measured by further application of TLC and measurement of radioactivity. The TLC method does not distinguish between O-acetylated monomers and monomers in which the anhydro form of muramic acid is present but there is no reason to suppose that this would involve more than a small proportion of the units at any time point. Further, decreases in O-acetylation brought about by penicillin are not accompanied by any change in the proportion of anhydro units found in the monomer fraction, so that changes in the degree of O-acetylation were in reality even more pronounced than they appeared (Blundell & Perkins, 1985). The effects of penicillin on the O-acetylation of the monomers, dimers and trimers in the original digest are shown in Fig. 3. Once more, the effects can be considered in relation to (i) the first 3 min and (ii) the development of O-acetylation thereafter. For the first 3 min, penicillin had relatively little effect on O-acetylation of the monomer fraction, which remained between 21 and 26%; the higher penicillin concentrations (0·1 and 0·5 µg ml⁻¹) if anything increased the O-acetylation of the dimer fraction, while penicillin at all concentrations used greatly enhanced O-acetylation of the oligomers. Between 3 and 90 min the doubling of the O-acetylation of monomers that occurred in the controls was greatly diminished by all concentrations of penicillin, the samples at 90 min with 0·1 and 0·5 µg penicillin ml⁻¹ having little more O-acetylation than they had at 3 min. O-Acetylation of the
Table 1. Effects of piperacillin on newly synthesized and mature peptidoglycan

A mid-exponential-phase culture of *N. gonorrhoeae* IL260 was divided into two; one culture was incubated with piperacillin for 20 min before addition of \( [^{14}C] \)glucosamine to both cultures. Samples were taken for analysis (see Methods) after a further 5 min ('new') and 90 min ('mature'). Results for benzylpenicillin (100 ng ml\(^{-1}\); 3 and 90 min) are given for comparison. Values for piperacillin represent the results of two separate experiments each done in duplicate, and for penicillin three experiments.

<table>
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<th>Antibiotic</th>
<th>Conc (ng ml(^{-1}))</th>
<th>Monomers</th>
<th>Dimers</th>
<th>Oligomers</th>
<th>Monomers</th>
<th>Dimers</th>
<th>Oligomers</th>
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<td>16.5</td>
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<tr>
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<td>27.9</td>
<td>35.3</td>
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Piperacillin both delays the onset, and reduces the rate, of secondary transpeptidation of newly synthesized peptidoglycan in *E. coli* (de Pedro & Schwarz, 1981). In this organism new material loosely cross-linked with the pre-existing sacculus undergoes a maturation process by which the final degree of cross-linking is achieved. Since gonococcal peptidoglycan undergoes a similar maturation, the possibility that piperacillin might uncouple secondary transpeptidation from the process which incorporates newly synthesized peptidoglycan into the cell wall was investigated.

Experiments similar to those described above for benzylpenicillin were done with piperacillin at concentrations of 10 and 50 ng ml\(^{-1}\), chosen because although near the MIC, which lay somewhere between these two values, they had no effect on culture OD\(_{675}\) within the 210 min of the whole experiment. The values for the proportions of monomers, dimers and oligomers in muramidase digests and for the degrees of \( O \)-acylation of the monomers and dimers for 'new' and 'mature' peptidoglycan are given in Table 1. The corresponding values for benzylpenicillin (0.1 \( \mu g \) ml\(^{-1}\)) are included for comparison.

The major effects of piperacillin on cross-linking were on the initial stage (up to 5 min in this experiment), where 50 ng ml\(^{-1}\) produced a decrease from 31.5% in the control to 25.5%. The further cross-linking achieved during the 5 to 90 min period was unaffected by the drug. Correspondingly, the effects of piperacillin on the proportions of monomers, dimers and oligomers in the main resembled those of penicillin, i.e. an increase in the proportion of monomers, an initial but not a final effect on the proportion of dimers and a decrease at both stages in the proportion of oligomers. The decreases in \( O \)-acylation of monomers observed with penicillin also paralleled those brought about by piperacillin. The greatest difference between the two drugs was in the \( O \)-acylation of dimers, where piperacillin, particularly at 50 ng ml\(^{-1}\), inhibited the second-stage \( O \)-acylation to an even greater extent than penicillin.
DISCUSSION

The present series of experiments shows that concentrations of penicillin too low to affect culture OD675, viable counts or overall peptidoglycan synthesis and cross-linking in *N. gonorrhoeae* IL260 nevertheless produce detectable effects on peptidoglycan composition. Thus at 0.01 µg ml⁻¹, a concentration representing only some 5% of the nominal MIC value (Brown & Perkins, 1979), benzylpenicillin led to a substantial decrease in the second-stage O-acetylation of peptidoglycan monomer that occurred between 3 and 90 min after the addition of radioactive label. The other main change was that the oligomer synthesized during the first 3 min was much more O-acetylated in the presence of the drug. Only those peptidoglycan units labelled by incorporation of radioactive glucosamine into their amino sugars were being examined, and in the presence of penicillin the proportion of those units O-acetylated in the oligomer fraction (38%) was higher than in the monomer (21%) or the dimer (28.5%) fractions. It follows, therefore, either that O-acetylated units were incorporated into oligomers in preference to non-O-acetylated units or that O-acetylation of disaccharide units newly added to make oligomers was enhanced in the presence of penicillin. Higher concentrations of penicillin produced, in this respect only, essentially similar effects. We have proposed (Lear & Perkins, 1986) that oligomers might arise from a special group of monomer units as it were designated for that purpose and separate from those destined to become dimers. If this were true, then changes brought about by penicillin specifically in the O-acetylation of oligomers would be easily accounted for. To some extent, although not at the lowest concentration, penicillin also caused the short-term O-acetylation of the dimer fraction to rise above control values.

The progress of O-acetylation of dimers during the later stages of incorporation was largely unaffected by penicillin. In the oligomer fraction, the early increase in O-acetylation was compensated by a decrease in rate during the later stages so that the final value was never exceeded (Fig. 3); at higher penicillin concentrations (0.1 and 0.5 µg ml⁻¹) the later phases of O-acetylation were respectively diminished and completely eliminated. These results could to some extent reflect the incorporation of new monomer units with a decreased proportion O-acetylated (Fig. 3) but the final level of O-acetylation in oligomers remained higher than in the monomers, so that a further explanation must be sought.

It may be, therefore, that the O-acetylation of dimers and higher oligomers occurs by a process distinct from that involved with monomers, and therefore subject to different effects in response to penicillin. The ultimate resolution of the relationship between penicillin-binding proteins, peptidoglycan cross-linking and O-acetylation in *N. gonorrhoeae* (see discussion in Lear & Perkins, 1986) would need to take account of possible different routes of synthesis and modification, and the extreme sensitivity of the O-acetylation process.

The effects of penicillin on cross-linking could best be summarized by saying that any long-term decreases seen after 90 min (e.g. from 35.6% down to 30.5% and 24.8% at 0.1 and 0.5 µg penicillin ml⁻¹, respectively) were already manifest at 3 min (from 31.4% down to 25.8% and 21%, respectively). Thus the second-stage slower development of the final degree of cross-linking was essentially unaffected by penicillin. The main difference observed at the highest concentration (0.5 µg ml⁻¹) was an apparent speeding up of the second stage of cross-linking, followed by a period where a decrease, or at least no further increase, occurred. It must be borne in mind that these bacteria were undergoing overall loss of peptidoglycan (Fig. 1) and the picture is further complicated by the fact that under those circumstances new synthesis continues even while old material is being lost (Perkins et al., 1983).

Piperacillin, implicated in affecting secondary transpeptidation in *E. coli* (de Pedro & Schwarz, 1981) had no similar effect in *N. gonorrhoeae*. The only different effect of piperacillin relative to penicillin was an even greater decrease in second-stage O-acetylation of the dimer fraction, at concentrations chosen so that each drug had about the same effect on cross-linking. The influence of piperacillin on the O-acetylation of oligomers was not examined. The extensive turnover in growing *E. coli* of SDS-insoluble peptidoglycan observed by Greenway & Perkins (1985) is now widely accepted, and this must surely influence the apparent maturation of newly synthesized peptidoglycan in this bacterium. However, in *N. gonorrhoeae* turnover was not more than about 10% per generation and was in any case subject to a delay of about one generation
time before it affected newly incorporated radioactive glucosamine (Greenway & Perkins, 1985) so that its effect in the present experiments must have been negligible.

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REFERENCES


