Turnover of the Cell Wall Peptidoglycan during Growth of Neisseria gonorrhoeae and Escherichia coli. Relative Stability of Newly Synthesized Material

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The peptidoglycan of a number of strains of Neisseria gonorrhoeae and Escherichia coli turned over during exponential growth as monitored by the loss of radioactivity (supplied as [14C]glucosamine) from SDS-insoluble material. However, no turnover of the peptide side chains of E. coli peptidoglycan was observed (monitored by diaminopimelic acid) even though turnover of glycan material was occurring. Turnover rates of 9 to 15% per generation were recorded for all of the N. gonorrhoeae strains studied except for the autolytic variant RD5 which showed a higher rate of turnover (20 to 26% per generation). In contrast to previous interpretations, these rates of turnover were not affected by benzylpenicillin, unless sufficient antibiotic was present to affect culture turbidity, when lysis occurred. Examination of the fragments (monomer, dimer and their O-acetylated counterparts, and oligomers) produced by Chalaropsis B muramidase treatment of prelabelled peptidoglycan revealed that no fraction of the peptidoglycan was immune from turnover. However, peptidoglycan pulse-labelled for only 10 min did not show immediate turnover. The lapse of time before turnover commenced was strain dependent, with a maximum value of 1.5 generations. This work confirms that the peptidoglycan of N. gonorrhoeae undergoes a period of maturation and suggests that only mature peptidoglycan turns over.

INTRODUCTION

Turnover of cell wall peptidoglycan has been observed in a number of Gram-positive bacteria. However, not all bacterial species exhibit peptidoglycan turnover, suggesting that turnover is not an essential feature of peptidoglycan metabolism (for review see Daneo-Moore & Shockman, 1977).

Turnover of the peptidoglycan of the Gram-negative coccus Neisseria gonorrhoeae, during exponential growth of the bacterium, has been reported by many groups of workers (Hebeler & Young, 1976a; Goodell et al., 1978; Rosenthal, 1979), and turnover rates of up to 50% per generation have been recorded. During our studies of the peptidoglycan metabolism of N. gonorrhoeae and the effects of β-lactam antibiotics (Brown & Perkins, 1979; Blundell et al., 1980; Blundell & Perkins, 1981; Lear & Perkins, 1983), it became apparent that the peptidoglycan of N. gonorrhoeae 1L260 did not turn over so rapidly as that of strains previously reported (Hebeler & Young, 1976a; Goodell et al., 1978; Rosenthal, 1979). The present investigation of the peptidoglycan turnover in a number of strains of N. gonorrhoeae has indicated that (i) the peptidoglycan of N. gonorrhoeae turns over more slowly than formerly reported: (ii) newly synthesized peptidoglycan appears to be immune from such turnover until it has undergone a period of maturation which may take up to 1.5 generations of growth; (iii) benzylpenicillin does

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Abbreviation: Apm, diaminopimelic acid.
not seem to increase the rate of peptidoglycan turnover unless it is affecting growth (inhibition or lysis), even though extensive biochemical changes are known to be occurring.

METHODS

Organisms and growth conditions. N. gonorrhoeae strain FA19 is a penicillin-sensitive laboratory strain obtained from P. F. Sparling (Sparling et al., 1975). Strain 1L260 is also a penicillin-sensitive isolate described earlier (Brown & Perkins, 1979). Strain RD4, a highly autolytic isolate extensively used in laboratory investigations, was obtained from R. S. Rosenthal (Rosenthal, 1979). Strain P9-2 is a piliated type 1 cell strain obtained from J. E. Heckels and stored as described by Diaz & Heckels (1982).

For experiments involving Escherichia coli, three strains were used: (i) C600 (F thi-1 thr-1 leuB6 lacY1 tonA21 supE44 Δ) is a K12 derivative; (ii) HB101 (F hsdS20 (rK- mB- recA1 ara-14 proA2 lacY1 galK2 leuE2 proS2 L20 (Sm') xyl-5 mtl-l supE44 Δ-) is a K12-Δ hybrid; (iii) W7 (dap lys) is a K12 derivative obtained from Dr T. E. Park. Strain W7 is auxotrophic for both diaminopimelic acid (A2pm) and lysine, so these amino acids were added to a final concentration of 2 and 200 μg ml⁻¹ respectively.

Cultures of N. gonorrhoeae were grown on chocolate agar made from 8% (v/v) horse blood in Columbia agar base (Lab M, London, UK) and stored at −70 °C in nutrient broth (Oxoid no. 2) containing 20% (w/v) glycerol. For each series of experiments, chocolate agar plates were inoculated from the stored cultures and grown overnight at 37 °C in an atmosphere of 5% (v/v) CO₂ in air. Liquid medium was proteose peptone no. 3 (Difco) (Wegener et al., 1977) (pH 7.2) with the addition of the growth supplement of Mayer et al. (1974) except that the glucose was replaced with pyruvate (Rosenthal, 1979). This liquid medium was similarly equilibrated with 5% (v/v) CO₂ overnight at 37 °C and then inoculated with organisms to an OD₆₅₀ of 0.1 as previously described (Brown & Perkins, 1979). The bacteria were grown for a generation or two into exponential phase.

Cultures of E. coli were grown overnight at 37 °C in proteose-peptone medium with pyruvate as carbon source as for N. gonorrhoeae. The cultures were diluted into fresh pre-warmed medium to an OD₆₅₀ of about 0.1 and grown into exponential phase. These cultures were then labelled as described below for N. gonorrhoeae.

Radiolabelling of peptidoglycan for turnover measurement. Peptidoglycan was labelled in two ways: (i) exponentially, so that the bulk of the labelled peptidoglycan was biosynthetically old material, and (ii) pulse-labelled, so that the labelled peptidoglycan was biosynthetically new material, as follows.

(i) Exponentially growing cultures of N. gonorrhoeae or E. coli were diluted to an OD₆₅₀ of 0 and D-[1,14C]glucosamine hydrochloride (54 mCi mmol⁻¹; 20 MBq μmol⁻¹) was added to a final concentration of 0.05 μCi ml⁻¹. The culture was then grown for 2 to 2.5 generations in the presence of this label at 37 °C. For the double labelling of the peptidoglycan of E. coli, strain W7 was used. This strain was labelled with D-[1,14C]glucosamine hydrochloride as described above and with (meso + DL)-[G-3H]A2pm (0·25 to 1 Ci mmol⁻¹; 9·25 to 37 GBq mmol⁻¹) at a final radioactive concentration of 1 μCi ml⁻¹, with an A2pm concentration of 2 μg ml⁻¹. A portion of this culture was then centrifuged at room temperature (2750 g, 5 min), and the radioactive medium was carefully removed from the cell pellet, which was freed of medium as completely as possible. The pellet was resuspended in the original volume of medium [pre-warmed and pre-equilibrated with 5% (v/v) CO₂ in air] containing unlabelled D-glucosamine hydrochloride at a final concentration of 10 μM. If A2pm had been used to label peptidoglycan, then in the subsequent turnover part of the experiment, this amino acid was added to a final concentration of 100 μg ml⁻¹. This culture was used to inoculate flasks containing similarly pre-warmed and pre-equilibrated medium, which also contained 10 μM-glucosamine hydrochloride, to an OD₆₅₀ of about 0.1. Growth of these cultures was monitored (as OD₆₅₀) at intervals and samples were taken for turnover determination.

(ii) Cultures of N. gonorrhoeae, growing exponentially at an OD₆₅₀ of 0·35 to 0·45, were pulse-labelled with [6,3H]glucosamine hydrochloride (34·6 Ci mmol⁻¹; 1·28 TBq mmol⁻¹) added to a final concentration of 2 μCi ml⁻¹, for 10 min (one-sixth to one-ninth of a generation depending upon the strain). The culture was then harvested and resuspended in fresh, pre-warmed and pre-equilibrated medium containing 10 μM unlabelled glucosamine hydrochloride. This culture was used to inoculate flasks containing pre-warmed pre-equilibrated medium (plus 10 μM-glucosamine hydrochloride) to an OD₆₅₀ of about 0·1. Samples for growth measurement (OD₆₅₀) and turnover determination were then taken at intervals. Cultures resumed exponential growth after dilution and centrifugation without lag, with mean generation times of 60 to 90 min depending upon the strain. Concentrations of glucosamine greater than about 30 μM inhibited growth, so glucosamine was only added to 10 μM after radiolabelling.

Determination of turnover. Labelled cultures, resuspended in unlabelled medium, were grown for a further 2 to 2.5 generations, with the OD₆₅₀ being measured at intervals. Samples (0·5 ml) were taken at various times, added to an equal volume of 10% (w/v) SDS, pH 7·2, in water and heated for 20 min in a boiling water bath. After cooling, the samples were filtered through glass fibre discs (Whatman GF/C; 2·4 cm diam.), which were washed twice with water (10 ml) and dried. Radioactivity was determined by liquid scintillation counting. Turnover was defined as the diminution of the radioactivity in these samples with time, the radioactivity of the later samples being expressed as a percentage of the zero time value.
Peptidoglycan turnover in *N. gonorrhoeae*

For experiments involving simultaneous measurement of the turnover of peptidoglycan and protein of *N. gonorrhoeae*, cultures were extensively labelled with D-[1-14C]glucosamine hydrochloride (final concentration 0.05 μCi ml⁻¹) and phenyl[2,3-3H]alanine (final concentration 0.5 μCi ml⁻¹) to label peptidoglycan and protein respectively according to the procedure (i) described above. In the subsequent turnover experiment, D-glucosamine hydrochloride and phenylalanine were added to the growth medium to 10 μM and 1 mM final concentrations respectively. Samples (0.5 ml) were taken into an equal volume of ice-cold 10% (w/v) TCA, kept for 30 min in the cold, filtered through glass fibre filters (GF/C) and then washed with 4 × 5 ml ice-cold 5% (w/v) TCA followed by 4 × 5 ml ethanol. The filters were then dried and radioactivity was measured, correction being made for spill-over of 14C-radioactivity into the 3H-channel of the liquid scintillation counter (Intertechnique). Preliminary experiments on peptidoglycan turnover in strain 1L260 showed that similar turnover rates were obtained by monitoring loss of radioactivity from either SDS-insoluble or TCA-precipitable material, although approximately twice the amount of radioactivity was found in TCA-precipitable material as in SDS-insoluble material.

In both of these preparations the radioactivity was totally converted by *Chalaropsis* B muramidase into recognizable fragments of peptidoglycan (J. K. Blundell & H. R. Perkins, unpublished results). Measurement of the turnover of the peptidoglycan of *E. coli* labelled with D-[1-14C]glucosamine for 2 to 2.5 generations was exactly as for *N. gonorrhoeae*.

For experiments where the effects of benzylpenicillin on peptidoglycan turnover were measured, this antibiotic was added at zero time in the turnover period.

All experiments were done at least three times. The results shown are of representative experiments. The radioactivity determinations at each time point were the mean of at least duplicate samples, which in any case differed little.

*Digestion of labelled peptidoglycan and analysis by TLC.* During a turnover experiment on an extensively labelled culture of *N. gonorrhoeae* 1L260, 10 ml samples were taken at each time point and added to an equal volume of 10% (w/v) SDS and boiled for 20 min. A peptidoglycan fraction was then isolated, digested with muramidase and analysed by TLC, and the radioactivity in each fraction was determined according to the method of Blundell & Perkins (1981). TLC resolved the solubilized fragments into the following fractions, starting from the fastest-moving fraction to the slowest and origin material as described by Blundell & Perkins (1981): O-acetylated monomer; monomer; di-O-acetylated dimer; mono-O-acetylated dimer; dimer; trimers and higher oligomers which were unresolved; and origin material.

*Chemicals.* All chemicals used were the best grade commercially available. Radiochemicals were obtained from Amersham and benzylpenicillin from Sigma. *Chalaropsis* B muramidase was kindly provided by Dr J. B. Ward.

**RESULTS**

*Turnover of extensively labelled peptidoglycan of* *N. gonorrhoeae*

The turnover of biosynthetically old peptidoglycan was studied in extensively labelled cultures. The peptidoglycan of *N. gonorrhoeae* strains 1L260, RD₃, P9-2 and FA19 was extensively labelled with D-[1-14C]glucosamine for 2 to 2.5 generations and, after removal of the label, turnover was followed in exponentially growing cultures. Turnover occurred without lag and according to first-order kinetics (Fig. 1). The rates of turnover were less than those reported by other workers (Hebeler & Young, 1976a; Goodell et al., 1978; Rosenthal, 1979). Typical rates, expressed as the percentage of radioactivity lost from the peptidoglycan fraction per generation of growth (as determined by OD₆₅₀), for the various strains were as follows: 1L260, 9–13%; RD₃, 20–26%; P9-2, 10–12%; FA19, 13–15%. To check whether the observed turnover was due to lysis, perhaps aggravated by centrifugation and resuspension, cultures of *N. gonorrhoeae* 1L260 and RD₃ were dual-labelled with D-[1-14C]glucosamine and phenyl[2,3-3H]alanine for 2 to 2.5 generations to label peptidoglycan and protein respectively. In both strains, turnover of peptidoglycan was accompanied by only a relatively small turnover of protein (Fig. 2). Hence the observed turnover of peptidoglycan could not be attributed to widespread damage and lysis of the cells.

Goodell et al. (1978) reported that the presence of penicillin increased the rate of peptidoglycan turnover of *N. gonorrhoeae*. Furthermore, Blundell & Perkins (1981) found that a major effect of penicillin on *N. gonorrhoeae* was to lower the degree of O-acetylation of its peptidoglycan, at penicillin concentrations ranging from those insufficient to affect culture turbidity to those causing morphological changes and death of a large proportion of the cells. Since such observations are of prime importance for understanding both the mechanism of
Fig. 1. Turnover of peptidoglycan of *N. gonorrhoeae*. The peptidoglycan of four strains of *N. gonorrhoeae* was labelled with D-[1-14C]glucosamine for 2 to 2.5 generations of exponential growth. The bacteria were harvested, resuspended in fresh medium containing unlabelled glucosamine and re-incubated (see Methods). During this period OD_{675} was measured (open symbols) and samples (0.5 ml) were taken to prepare hot SDS-insoluble material collected on GF/C filters, the radioactivity of which was measured (see Methods). The radioactivity was recorded as a percentage of that present in the samples at zero time (filled symbols). One hundred percent corresponds to the following amount of radioactivity per sample for each of the strains of *N. gonorrhoeae* used: (a) 1L260 (O, ●), 3955 c.p.m.; RD5 (□, ■), 2982 c.p.m.; (b) P9-2 (O, ●), 1377 c.p.m.; FA19 (□, ■), 5505 c.p.m.

Fig. 2. Turnover of protein and peptidoglycan of *N. gonorrhoeae*. Exponentially growing cultures of *N. gonorrhoeae* 1L260 (a) and RD5 (b) were labelled for 2 to 2.5 generations with phenyl[2,3-3H]alanine and D-[1-14C]glucosamine. The bacteria were then harvested, resuspended in fresh medium containing 10 μM-glucosamine and 1 mM-phenylalanine and re-incubated (see Methods). Growth (OD_{675}, □) was then followed and samples (0.5 ml) were added to an equal volume of ice-cold 10% (w/v) TCA for turnover determination. The precipitates collected on GF/C filters were dried and their radioactivity was measured. Corrections were made for spill-over of 14C-radioactivity into the 3H-channel. Turnover was recorded as described in Fig. 1. For 1L260 (a), 100% refers to 2039 c.p.m. for [14C]peptidoglycan (●) and 1850 c.p.m. for phenyl[3H]alanine (O). For RD5 (b), 100% refers to 3159 c.p.m. for [14C]peptidoglycan (●) and 1766 c.p.m. for phenyl[3H]alanine (O).
Peptidoglycan turnover in *N. gonorrhoeae* was examined to study the effect of penicillin upon the biosynthesis and metabolism of its peptidoglycan. Cultures of *N. gonorrhoeae* strains 1L260, RD₅, and P9–2 were labelled as before and benzylpenicillin was added at zero time in the turnover part of the experiment. Benzylpenicillin increased turnover of peptidoglycan only when there were observable effects of the antibiotic upon culture turbidity (i.e. growth inhibition and/or cell lysis) and not at lower concentrations, at which O-acetylation of peptidoglycan would be markedly decreased (Blundell & Perkins, 1981) (Fig. 3). Only strain P9–2 showed slightly increased turnover at 0·1 μg benzylpenicillin ml⁻¹, when culture turbidity was unaffected (Fig. 3c).

**Turnover of pulse-labelled peptidoglycan**

Various groups of workers, with different species of Gram-positive bacteria, have reported that pulse-labelled peptidoglycan, i.e. newly synthesized material, only undergoes turnover after action of penicillin upon *N. gonorrhoeae* and the biosynthesis and metabolism of its peptidoglycan, we examined the effect of penicillin upon extensively labelled peptidoglycan. Cultures of *N. gonorrhoeae* strains 1L260, RD₅, and P9–2 were labelled as before and benzylpenicillin was added at zero time in the turnover part of the experiment. Benzylpenicillin increased turnover of peptidoglycan only when there were observable effects of the antibiotic upon culture turbidity (i.e. growth inhibition and/or cell lysis) and not at lower concentrations, at which O-acetylation of peptidoglycan would be markedly decreased (Blundell & Perkins, 1981) (Fig. 3). Only strain P9–2 showed slightly increased turnover at 0·1 μg benzylpenicillin ml⁻¹, when culture turbidity was unaffected (Fig. 3c).
Exponentially growing cultures of *N. gonorrhoeae* were pulse-labelled with D-[6-³H]glucosamine for 10 min (approximately one-sixth to one-ninth of a generation depending upon the strain used) and turnover of this labelled material (newly synthesized peptidoglycan) was monitored. With each strain there was a lag before turnover became apparent (Fig. 4). This lag was strain dependent, being about 60 to 90 min with strains 1L260 and FA19, and about 30 to 60 min for strain RD₅. When turnover of this pulse-labelled material finally occurred, it did so at rates almost identical to those found for extensively labelled bacteria (see Fig. 1). In a few experiments with all three strains, a rapid loss of up to 10% of the radioactivity occurred during the first 15 min, but thereafter the pattern exactly resembled that shown in Fig. 4.

**Is there a specific peptidoglycan fraction(s) undergoing turnover?**

The peptidoglycan of *N. gonorrhoeae* is known to be partially O-acetylated (Blundell *et al.*, 1980; Blundell & Perkins, 1981; Dougherty, 1983a, b; Rosenthal *et al.*, 1982; Swim *et al.*, 1983).
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Fig. 5. Turnover of E. coli peptidoglycan. Exponentially growing cultures of E. coli labelled for 2 to 2.5 generations with D-[1-14C]glucosamine were harvested by centrifugation and resuspended in fresh medium containing 10μM-glucosamine to OD675 = 0.1. Growth (OD675, □) was then followed and samples (0.5 ml) were taken into equal volumes of either 10% (w/v) SDS or 10% (w/v) ice-cold TCA (see Methods). The insoluble material was collected on GF/C filters and radioactivity was measured. (a) Strain C600 [100% = 8434 c.p.m. in TCA-precipitable material (○) and 4589 c.p.m. in SDS-insoluble material (●)]; (b) strain HB101 [100% = 10536 c.p.m. in TCA-precipitable material (○) and 4703 c.p.m. in SDS-insoluble material (●)].

Units of the peptidoglycan exist in both the O-acetylated and non-O-acetylated form, representing fractions that could theoretically undergo turnover at different rates. To determine if a specific fraction (or fractions) of the peptidoglycan was undergoing turnover, a culture of strain IL260 was extensively labelled with D-[1-14C]glucosamine and turnover was monitored as described. At each time interval, an extra sample of culture was taken and the peptidoglycan of this sample was isolated. This peptidoglycan was digested with Chalaropsis B muramidase and the solubilized products were analysed by TLC (see Methods). Measurement of radioactivity showed that all the fractions appeared to be equally susceptible to turnover.

Turnover of the peptidoglycan of E. coli

To see whether in our hands turnover of the peptidoglycan of N. gonorrhoeae was different from that of other Gram-negative bacteria we studied E. coli. Although turnover of peptidoglycan has been reported not to occur in E. coli (van Tubergen & Setlow, 1961; Mauck et al., 1971), Chaloupka & Strnadová (1972) observed some turnover in an A2pm-dependent mutant of E. coli, when [14C]A2pm had been used to label the peptidoglycan. In our experiments, we labelled exponentially growing cultures of two different E. coli strains (HB101 and C600) for 2 to 2.5 generations with D-[1-14C]glucosamine and then centrifuged and resuspended the bacteria in fresh medium containing 10μM-glucosamine. SDS-insoluble (sacculus peptidoglycan) and TCA-precipitable (total macromolecular) fractions were prepared and peptidoglycan turnover was measured. The results suggested that there was indeed peptidoglycan turnover in both E. coli strains, equivalent to a loss of radioactivity from TCA-precipitable material of about 8% per generation (Fig. 5). This turnover followed first-order kinetics as found in N. gonorrhoeae (Hebeler & Young, 1976a; Goodell et al., 1978; Rosenthal, 1979; this paper). The loss of radioactivity from SDS-insoluble material indicated an even higher rate of turnover but the kinetics were more complex. To ensure that D-[1-14C]-glucosamine was labelling peptidoglycan specifically, labelled SDS-insoluble material was
isolated, freed from unincorporated label, digested with *Chalaropsis* B muramidase and analysed by TLC. More than 96% of the SDS-insoluble material was solubilized by this procedure into products indicative of peptidoglycan, i.e. mainly monomers (disaccharide-peptide) and dimers (bis-disaccharide-cross-linked peptide) with a small amount of oligomeric material (chiefly trimers).

TCA-precipitable material was similarly treated with *Chalaropsis* B muramidase and the solubilized products were analysed by TLC. Unlike the SDS-insoluble material, only about 50% of the TCA-precipitable material was solubilized by the muramidase. However, the solubilized products of digestion of TCA-precipitable material were indicative of peptidoglycan, as mentioned above for the SDS-insoluble fraction. Thus, the TCA-precipitable fraction probably contained some lipopolysaccharide which has glucosamine components and which is resistant to muramidase treatment.

Previous work, indicating that turnover of *E. coli* peptidoglycan occurred, utilized cells pre-labelled with A2pm and differed markedly from our results in which a glucosamine label was used. We therefore double-labelled *E. coli* W7 in its peptidoglycan with D-[1-14C]glucosamine and [G-3H]A2pm and monitored turnover of peptidoglycan. In agreement with both our findings and those of other workers, such double-labelled cultures showed that there was indeed loss of material labelled with glucosamine but no loss of material labelled with A2pm (Fig. 6). Thus, it appeared that the glycan part of peptidoglycan was undergoing turnover, but that the peptide side chains were either not undergoing turnover or were somehow being retained within the macromolecule.

**DISCUSSION**

Two regimes were used to label the peptidoglycan of *N. gonorrhoeae*. In the first the bulk of the peptidoglycan was biosynthetically 'old' since labelling was continued for 2 to 2.5 generations. In the second the radioactive peptidoglycan was biosynthetically 'new' since labelling was for only
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10 min. In our hands biosynthetically old peptidoglycan turned over, but at lower rates than those recorded by others (Hebeler & Young, 1976a; Goodell et al., 1978; Rosenthal, 1979). This turnover proceeded without lag, according to first-order kinetics, with rates between 9 and 15% per generation for three different strains of *N. gonorrhoeae* (IL260, FA19, and the piliated variant P9–2). Only the autolytic strain RD3 showed a higher rate of turnover of between 20 and 26% per generation, far lower than the rates of up to 50% per generation reported by Hebeler & Young (1976a). An explanation for these discrepancies might lie in the different radioactive precursors used to label peptidoglycan, namely glucosamine in our work and A2pm in many previous studies. Goodell et al. (1978) using *N. gonorrhoeae* strain Reeves reported a turnover rate of glucosamine-labelled peptidoglycan of 13% per generation, which agrees well with the values reported in this work, but if they labelled with A2pm turnover rates of about 26% per generation were obtained. However, it is noticeable that relatively enormous quantities of radioactive A2pm had to be used to obtain only a few counts, so that the labelled product could conceivably have been unrepresentative of the whole. On the other hand, Rosenthal (1979) using *N. gonorrhoeae* RD3 obtained a turnover rate of 31 to 40% per generation regardless of whether glucosamine or A2pm had been the source of label, compared with 50% per generation quoted by Hebeler & Young (1976a), who used the same strain and labelled with A2pm. Since A2pm labels the peptide side chains, and as *N. gonorrhoeae* has an active N-acetylmuramyl-L-alanine amidase which cleaves the amide link between N-acetylmuramic acid and l-alanine (Hebeler & Young, 1976b), different values for the turnover of the peptidoglycan, depending upon which precursor was used, could perhaps be expected. However, loss of such chains should lead to the appearance of unsubstituted disaccharide units in the Chalaraopsis muramidase digests, and we found no evidence for these.

Problems associated with growing *N. gonorrhoeae* in liquid media and the risks of autolysis are well documented (Kellogg et al., 1963; Morse & Bartenstein, 1974; Hebeler & Young, 1976b) and it could be argued that most, if not all, of the observed turnover of peptidoglycan might be a consequence of cell lysis. However, our results show that in cultures of *N. gonorrhoeae* simultaneously labelled in the peptidoglycan and in the protein, the rate of turnover of protein was much less than that of peptidoglycan. Thus even the relatively low turnover of peptidoglycan observed by us was not apparently due to cell lysis. A similar conclusion was reached by Hebeler & Young (1976a) in regard to the much higher level of peptidoglycan turnover that they observed.

Penicillin did not affect the rate of peptidoglycan turnover unless culture turbidity was also affected. Goodell et al. (1978) and Sinha & Rosenthal (1981) reported that penicillin increased the rate of peptidoglycan turnover, but the drug concentrations that they used were sufficient to initiate lysis. As the peptidoglycan of *N. gonorrhoeae* is also partially O-acetylated (Blundell et al., 1980; Blundell & Perkins, 1981) specific fractions of the peptidoglycan (O-acetylated vs non-O-acetylated) might undergo turnover, while others do not. It seems that no specific fraction of peptidoglycan (monomers, dimers, oligomers or their O-acetylated derivatives) was immune from turnover. A similar observation was made by Mauck et al. (1971) with *Bacillus subtilis* in regard to substitution of the peptidoglycan with teichoic acids.

We did not examine the products of peptidoglycan turnover in *N. gonorrhoeae* but presumably they were the same as those reported earlier, namely (a) disaccharide peptide monomer (80% anhydromonomer tripeptide and 20% monomer tetrapeptide); (b) cross-linked, bis-disaccharide peptide dimer; (c) free peptide and (d) free disaccharide (Rosenthal, 1979; Sinha & Rosenthal, 1980; Sinha & Rosenthal, 1981).

Our results with three different strains of *E. coli* indicated that peptidoglycan turnover was occurring at about 8% per generation in experiments where TCA-precipitation was used to collect the peptidoglycan and considerably more when the SDS-insoluble fraction was used. However, this extensive turnover was only observed if the peptidoglycan was labelled with glucosamine rather than with A2pm. Such findings suggest that even though the glycan part of peptidoglycan is being turned over, the peptide moiety is not, or it is being retained within the macromolecule. Thus, these findings might explain why some workers have not observed peptidoglycan turnover in *E. coli* (van Tubergen & Setlow, 1961; Mauck et al., 1971) while other
workers have observed turnover (Chaloupka & Strnadová, 1972; this study). In general, turnover has not been observed with A$_2$pm-labelled peptidoglycan [except by Chaloupka & Strnadová (1972), who observed turnover with an A$_2$pm auxotroph of E. coli under A$_2$pm starvation].

The initial immunity to turnover of pulse-labelled (newly synthesized) peptidoglycan in N. gonorrhoeae was particularly interesting. The lag before turnover began appeared to be strain dependent, being shorter (30 to 60 min) in the autolytic isolate RD$_5$ compared with 60 to 90 min for IL260 and FA19. The immunity of peptidoglycan to turnover has been observed with Gram-positive bacteria, e.g. Lactobacillus acidophilus (Boothby et al., 1973) and B. subtilis (Mauck et al., 1971; Pooley, 1976), but has not been reported in N. gonorrhoeae. Wegener et al. (1977) reported that 'old' and 'newly synthesized' peptidoglycan underwent degradation at approximately the same rates in resting cell suspensions of N. gonorrhoeae, but the relevance of such findings to turnover in growing bacteria is unclear. The lag before newly synthesized peptidoglycan was turned over suggested that peptidoglycan must undergo a period of maturation before turnover could occur. The time scale of this maturation process (60 to 90 min with IL260) agreed closely with the results of Lear & Perkins (1983), who found that cross-linking and O-acetylation of newly synthesized peptidoglycan was maximal 60 to 90 min after the pulse. Similar evidence for maturation of N. gonorrhoeae peptidoglycan was found by Dougherty (1983b). Thus N. gonorrhoeae peptidoglycan appears to undergo maturation processes after incorporation into the sacculus, as has been shown in E. coli (de Pedro & Schwarz, 1981).

Pooley (1976) reported that in B. subtilis newly synthesized peptidoglycan was immune from turnover until it had matured. He suggested that peptidoglycan was laid down as layers starting from the cytoplasmic membrane and that only peptidoglycan that had reached the outer layers underwent turnover, thus accounting for the lag or period of immunity to turnover. Such a model is probably not applicable to N. gonorrhoeae, since Gram-negative organisms have only a very thin cell wall, consisting of a few layers of peptidoglycan at most compared with the multilayered, thick cell wall of Gram-positive bacteria. In N. gonorrhoeae the lag before turnover might represent the time required for progression of peptidoglycan from its site of synthesis to its site of turnover, perhaps in a radial or linear fashion, during which time maturation occurs and thus permits turnover.

How such maturation occurs is as yet unknown, but it might possibly be by chemical modification, i.e. O-acetylation, a proportion of which takes place after incorporation of monomer units into saccular peptidoglycan in N. gonorrhoeae (Lear & Perkins, 1983) and also in Proteus mirabilis (Gmeiner & Kroll, 1981). In E. coli, newly synthesized peptidoglycan contains pentapeptide subunits, is less cross-linked and carries less lipoprotein than mature material (de Pedro & Schwarz, 1981).

In the relatively autolytic gonococcal isolate RD$_5$, the peptidoglycan has a much reduced level of O-acetylation (18% vs 45–53% observed with other strains; Rosenthal et al., 1982) and a shorter lag before newly synthesized peptidoglycan begins to turnover. Perhaps here the lower degree of O-acetylation correlates with faster maturation and earlier susceptibility to turnover.

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REFERENCES


Peptidoglycan turnover in N. gonorrhoeae


