***O*-Acetylation of Peptidoglycan in *Neisseria gonorrhoeae*. Investigation of Lipid-linked Intermediates and Glycan Chains Newly Incorporated into the Cell Wall**

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(Received 1 April 1986; revised 26 May 1986)

Radioactive labelling of the amino sugars in gonococcal peptidoglycan was followed by treatment with *Chalaropsis* muramidase and TLC separation of the products. Even after very brief periods of labelling (0.5 min) the peptidoglycan was already cross-linked to some 80% of the final value and little change occurred within 2 min. The remaining cross-linking was achieved only over a period of about one generation time. Streptomycete endopeptidase was used to show the extent to which new chains were cross-linked to old. Even at the earliest times many cross-linked units contained new material in both moieties and by 3 min there was little distinction in relative labelling, indicating that in *Neisseria gonorrhoeae* most newly synthesized glycan chains are cross-linked to other new chains rather than to pre-existing peptidoglycan. A model is proposed in which newly polymerized monomer units are predestined either towards dimer formation with other new chains, which are then rapidly *O*-acetylated and not further cross-linked, or towards the formation of trimers and higher oligomers, the latter being a slower process. Although significant *O*-acetylation of peptidoglycan was detectable even at the earliest times, efforts to detect *O*-acetylated lipid intermediates were unsuccessful. The chief lipid intermediate found was apparently the disaccharide-peptide unit linked to undecaprenol.

INTRODUCTION

The peptidoglycan of *Neisseria gonorrhoeae* has been shown (Hebeler & Young, 1976) to have a chemical composition of muramic acid, glucosamine, alanine, glutamic acid, diaminopimelic acid in the ratio 1 : 1 : 2 : 1 : 1 and thus to be of type A₁ (Schleifer & Kandler, 1972). It has been established (Blundell *et al.*, 1980) that unlike in *Escherichia coli*, approximately 50% of the muramic acid residues are *O*-acetylated. The immediate response of gonococci to low concentrations of β -lactam antibiotics is a decrease in *O*-acetylation rather than in peptide cross-linking (Blundell & Perkins, 1981; Dougherty, 1985). Using short pulses of radioactive glucosamine added to the growth medium, we have been able to follow the progress of incorporation of precursor into *O*-acetylated and non-*O*-acetylated subunits in SDS-insoluble peptidoglycan (Lear & Perkins, 1983). In these experiments peptidoglycan was isolated from culture samples taken at intervals after addition of radioactivity and was then digested with *Chalaropsis* muramidase B (Hash & Rothlauf, 1967). The resultant fragments were separated by TLC (Martin & Gmeiner, 1979) into monomers (disaccharide peptide), dimers (bisdisaccharide cross-linked peptide) and oligomers [unresolved in this system, but consisting mainly of trimers (Rosenthal *et al.*, 1980)]. With labelling periods from 3 min to 90 min it was shown that newly synthesized peptidoglycan was both less *O*-acetylated and less cross-linked than mature peptidoglycan.

Cross-linking very rapidly (3 min) achieved 80% of its final value, but the remainder of the process was much slower, taking a further 60 min. *O*-Acetylation rose quickly over 10 min to about 65% of its maximum, the process being completed by 60 min. We concluded that newly

synthesized peptidoglycan underwent a maturation process involving both further peptide cross-linking and *O*-acetylation. Peptidoglycan maturation involving increases in cross-linking has been observed in *E. coli* (de Pedro & Schwarz, 1981) whilst increases in both cross-linking and *O*-acetylation have been reported in *Proteus mirabilis* (Gmeiner & Kroll, 1981) and *N. gonorrhoeae* (Lear & Perkins, 1983; Dougherty, 1983a). However, no study to date has resolved the question of whether all or only a proportion of the *O*-acetyl groups are added to newly synthesized peptidoglycan after its insertion into the cell wall. We have addressed this problem in two ways: first, by investigating the peptidoglycan labelled with very short pulses (0.5–2 min) of radioactive glucosamine, and second, by isolating the lipid (C_{55} -undecaprenol)-linked precursor of peptidoglycan (Higashi *et al.*, 1967, 1970). Very early *O*-acetylation could conceivably take place at the lipid-intermediate stage of peptidoglycan biosynthesis, as for instance has been shown for amidation (Siewert & Strominger, 1968).

METHODS

Organisms and growth conditions. *Neisseria gonorrhoeae* strain 1L260 (Brown & Perkins, 1979), grown overnight on chocolate agar plates, was used to inoculate proteose peptone no. 3 (Difco) liquid medium, with pyruvate as carbon source (Blundell & Perkins, 1981). Cultures were incubated with shaking at 37 °C.

Radioactive labelling: isolation and analysis of peptidoglycan. Cultures (75 ml) were grown to OD_{675} 0.2. A 10 ml sample was transferred to a separate flask for radioactive labelling, and incubation of this and the parent culture continued. Then either 25 μ Ci (0.925 MBq) of D -[6- 3 H]glucosamine hydrochloride [40 Ci mmol $^{-1}$, 1.48 TBq mmol $^{-1}$, Amersham] ml $^{-1}$ (for short-term experiments, series 1) or 1.25 μ Ci (46 kBq) of D -[1- 14 C]glucosamine hydrochloride [54 mCi (2 GBq) mmol $^{-1}$, Amersham] ml $^{-1}$ (for longer-term experiments, series 2) was added to the smaller culture at OD_{675} 0.3, and the culture was swirled rapidly to ensure thorough mixing. Samples (2 ml) were added to an equal volume of 10% (w/v) SDS and heated at 100 °C for 20 min (Blundell *et al.*, 1980). Samples (10 ml) of the parent culture were similarly treated. Incorporation of radioactivity was measured by filtering 100 μ l volumes of labelled samples on glass microfibre discs (GF/C, Whatman), which were then washed and used for measurement of radioactivity (Blundell & Perkins, 1981). SDS-insoluble peptidoglycan was isolated from the labelled culture samples after each had been mixed with unlabelled material from samples of the parent culture. Insoluble material collected by centrifugation (Blundell & Perkins, 1981) was digested with *Chalaropsis* muramidase B and the fragments were separated by TLC in isobutyric acid/1 M-ammonia (5:3, v/v) (Martin & Gmeiner, 1979). After drying, TLC plates were sprayed with En 3 Hance (New England Nuclear) and spots were detected by fluorography. Spots identified by comparison with previously identified markers were cut out and their radioactivity was measured in a scintillation counter.

Endopeptidase treatment of mono-*O*-acetylated dimers. After scintillation counting, strips of TLC corresponding to spots identified as mono-*O*-acetylated dimer were washed three times with toluene to remove scintillant and then were eluted with water. After drying over NaOH under reduced pressure, samples were resuspended in 75 μ l 0.5 M-Tris/HCl pH 7.5 containing 5 mM-MgCl $_2$ and 15 μ g DD-carboxypeptidase from *Streptomyces albus* G (Ghuysen *et al.*, 1970), and incubated at 37 °C overnight. This enzyme has been shown to have endopeptidase activity (Leyh-Bouille *et al.*, 1970) and will cleave both *O*-acetylated and non-*O*-acetylated dimers derived from *N. gonorrhoeae* peptidoglycan into their constituent subunits (unpublished results). The products of endopeptidase treatment were separated by TLC, detected and quantified as above.

Isolation of the C_{55} -undecaprenol-linked precursors of peptidoglycan. Cultures (2 l) were grown to OD_{675} 0.4. D -[1- 14 C]Glucosamine hydrochloride [56.8 μ Ci mmol $^{-1}$, 2.10 GBq mmol $^{-1}$; Amersham] was added (final concentration 0.08 μ Ci ml $^{-1}$) and incubation continued for 10 min. Cultures were then cooled rapidly by pouring onto 1:2:1 of crushed frozen liquid medium, and cells were harvested by centrifugation at 5200 *g* for 20 min at 2 °C. The pellets were quickly resuspended in 190 ml boiling water and heated at 100 °C for 10 min. After cooling, cellular material was collected by centrifugation (115000 *g*, 90 min), resuspended in 50 ml 50 mM-sodium phosphate buffer pH 7.2 and extracted with an equal volume of butan-1-ol/6 M-pyridinium acetate pH 4.2 (2:1, v/v) to remove lipid intermediate (Anderson *et al.*, 1967). The aqueous phase was re-extracted twice with butan-1-ol. The combined butan-1-ol extracts were washed three times with water and dried by evaporation under reduced pressure. The residue was resuspended in chloroform/methanol (1:1, v/v), applied to the origin of a paper chromatogram (3 MM, Whatman) and fractionated by descending chromatography in isobutyric acid/1 M-ammonia (5:3, v/v) for 18 h (Anderson *et al.*, 1967). Sample tracks were cut into 0.5 cm strips and radioactivity was detected by scintillation counting. Of the two major peaks, peak I ran just behind the solvent front (R_F 0.9) as expected for lipid-linked intermediates (Anderson *et al.*, 1967). Peak II (R_F 0.7) was unidentified, but on hydrolysis (4 M-HCl, 4 h, 100 °C) was found to contain no muramic acid (results not shown) and was therefore deemed to be neither peptidoglycan nor one of its precursors. Chromatogram strips corresponding to peak I were washed with toluene to remove scintillant, then immersed in 0.1 M-HCl and heated to 100 °C for 10 min to hydrolyse the

pyrophosphoryl link between the lipid and glycan portions of lipid intermediate (Siewert & Strominger, 1968). Solubilized material was eluted with water and fractionated by descending paper chromatography (3 MM, Whatman) in butan-1-ol/acetic acid/water (4:1:5, by vol., upper phase) for 72 h. Radioactive spots were detected as described before, eluted with water, hydrolysed with 4 M-HCl at 100 °C for 4 h in sealed tubes, and the liberated sugars separated by descending paper chromatography (3 MM, Whatman) in butan-1-ol/acetic acid/water for 34 h.

Source of marker compounds. Monomer and *O*-acetylated monomer were obtained essentially as by Chapman & Perkins (1983). Other gonococcal peptidoglycan fragments were purified as described by Blundell & Perkins (1981). *N*-Acetylmuramyl pentapeptide, a gift of N. McKendrick, was made from *Bacillus licheniformis* UDP-*N*-acetylmuramyl pentapeptide (Ward, 1974) by hydrolysis with 0.1 M-HCl at 100 °C for 10 min. Muramic acid was purchased from Sigma. Unlabelled marker compounds were detected on chromatograms by spraying with 0.4% (w/v) ninhydrin (Sigma) in acetone, or silver nitrate/sodium hydroxide (Trevelyan *et al.*, 1950).

Enzymes. *Chalaropsis* muramidase B was given by Dr J. B. Ward and DD-carboxypeptidase from *S. albus* G by Professor J.-M. Ghuyens.

RESULTS

Cultures incubated with radioactive glucosamine incorporated label very rapidly into SDS-insoluble material. After as little as 0.5 min significant amounts of radioactivity appeared in the cell wall. The composition of newly synthesized peptidoglycan as deduced from the proportions of radioactivity found in the various *Chalaropsis* muramidase fragments separated by TLC is shown in Table 1. The degrees of *O*-acetylation and peptide cross-linking were calculated as before (Blundell & Perkins, 1981). The TLC method does not distinguish between *O*-acetylated monomers and monomers in which the anhydro form of muramic acid is present (Swim *et al.*, 1983) but there is no reason to suppose that this would involve more than a small proportion of the units at any time point (Blundell & Perkins, 1985*b*). Calculations of cross-linking would in any case be unaffected. Because of the possibility of asymmetric labelling of the mono-*O*-acetyldimer fraction in newly synthesized peptidoglycan (Gmeiner & Kroll, 1981), mono-*O*-acetyldimer spots were eluted from TLC plates and further digested by *S. albus* G DD-carboxypeptidase. Mono-*O*-acetylated dimer, isolated from cultures of *N. gonorrhoeae* labelled with radioactive glucosamine for about two generation times, when treated with this enzyme, yields monomer and *O*-acetylated monomer with approximately equal amounts of radioactivity (the minor difference was assumed to be due to chemical loss of *O*-acetyl during the digestion: cf. Table 1). In cultures pulsed for 0.5–1.5 min this ratio was about 2:1, decreasing to 5:3 for pulses of 2 min and approaching 1:1 within 15 min (Table 1). The proportion of newly synthesized subunits in the dimer fraction that were *O*-acetylated was also calculated from these results (Table 1).

After 0.5 min monomer *O*-acetylation was only 22% of its final, mature value whilst that of newly synthesized dimers was 42% of maximum. The latter proportion also increased somewhat within the next 2–3 min. The degree of peptide cross-linking was calculated after allowance had been made for the lower specific activity of the *O*-acetylmonomer moiety of the mono-*O*-acetyldimer observed at short labelling times (see Table 1). At 0.5 min the degree of cross-linking was 28.6, 80% of the final value of 35.6 observed after long-term incorporation (90 min).

The presence of *O*-acetyl groups in newly synthesized SDS-insoluble peptidoglycan within 0.5 min of the bacteria being pulsed with glucosamine prompted us to look for *O*-acetylation of the lipid-linked intermediates of peptidoglycan synthesis. Lipid-linked precursor was isolated as fast-migrating glucosamine-labelled material (R_f 0.9) on paper chromatography of a butan-1-ol/pyridinium acetate extract of *N. gonorrhoeae*. To investigate the glycan portion of the molecule, the pyrophosphoryl link between lipid and glycan was broken by brief hydrolysis with 0.1 M-HCl. Parallel experiments showed that these conditions produced no significant hydrolytic removal of *O*-acetyl groups from peptidoglycan fragments (data not shown) and Ward & Perkins (1973) have shown that they do not result in hydrolysis of glycan chains. The fragments released were characterized by further paper chromatography in butan-1-ol/acetic acid/water (Fig. 1). Over 80% of the radioactivity was recovered in a single peak with a chromatographic mobility similar to that of disaccharide peptides (non-*O*-acetylated

Table 1. *O*-Acetylation and cross-linking of radioactively labelled peptidoglycan

For experimental details see Methods. The label was supplied as D-[6-³H]glucosamine in series 1 and as D-[1-¹⁴C]glucosamine in series 2. Results are the mean of two separate experiments, each with duplicate samples.

Expt series no.	Time after addition of radioactive GlcN (min)	Total incorporation into peptidoglycan (MBq)	Percentage of radioactivity found as						Percentage of radioactivity in mono- <i>O</i> -acetyl-dimer recovered after endopeptidase digestion as		Degree of cross-linking†	Percentage of newly synthesized peptidoglycan <i>O</i> -acetylated‡	
			Percentage of radioactivity found as						Percentage of radioactivity in mono- <i>O</i> -acetyl-dimer recovered after endopeptidase digestion as			Fraction:	
			Monomer	<i>O</i> -Acetyl-monomer	Dimer	Mono- <i>O</i> -acetyl-dimer	Di- <i>O</i> -acetyl-dimer	Oligomer	Monomer*	<i>O</i> -Acetyl monomer*		Monomer	Dimer
1	0.5	0.14	46.5	6.2	21.3	10.9	3.7	10.4	67.0	33.0	28.6	11.8	20.3
	1	1.15	41.5	7.8	22.4	11.7	3.9	10.6	67.0	33.0	30.1	15.8	20.4
	1.5	3.33	38.4	8.8	23.9	10.7	4.6	12.3	66.7	33.3	31.5	18.6	20.8
	2	5.79	41.3	8.8	21.2	10.9	4.3	12.7	61.3	38.7	29.2	17.6	23.4
2	3	1.24	33.0	6.9	26.2	12.3	5.5	12.4	56.0	44.0	32.8	17.3	24.8
	15	25.61	26.6	11.6	24.8	13.5	6.5	12.8	52.3	47.7	33.0	30.4	28.9
	45	67.27	21.1	14.9	18.6	15.8	8.7	16.0	52.0	48.0	34.3	41.4	37.8
	90	97.15	16.6	18.1	13.1	15.0	11.8	21.1	50.0	50.0	35.6	52.2	48.3

* After correction for slight loss of *O*-acetyl groups by chemical hydrolysis during the endopeptidase digestion. Uncorrected values for the 90 min sample were 51.3% monomer and 48.7% *O*-acetylmonomer.

† Degree of cross-linking, as defined by Blundell & Perkins (1981), has been calculated after allowing for the fact that the dimer (and presumably trimer/oligomer) fractions are unequally labelled in their two (or more) disaccharide moieties. This has been done by enhancing the proportion of each dimer (or trimer/oligomer) in line with the higher specific radioactivities found in the monomer portion of the mono-*O*-acetyl-dimer, thus compensating for unlabelled moieties utilized in forming cross-linked fragments. For example, for the sample labelled for 0.5 min the *O*-acetylmonomer portion is deficient in radioactivity by 34% (67–33). Hence the total mono-*O*-acetyl-dimer fraction if labelled to the same specific radioactivity as the monomer would have 1.34 times as much label, i.e. 10.9×1.34 units = 14.6. If one makes the reasonable assumption that the dimer and di-*O*-acetyl-dimer fractions are relatively unlabelled to the same extent, then their corrected values become $21.3 \times 1.34 = 28.5$ and $3.7 \times 1.34 = 5.0$ respectively and the modified total for all dimers becomes 48.1. By similar reasoning, and assuming that only the terminal unit of trimer (oligomer) has specific radioactivity lowered to the same extent as in the dimers, trimer radioactivity may be corrected to $10.4 \times 1.20 = 12.5$. The total corrected radioactivity then equals label in monomers, 52.7 units + label in dimers, 48.1 units + label in oligomer, 12.5 units = 113.3 units. Of this total the corrected dimers = 42.4% and the corrected oligomer = 11.0%. Then degree of cross-linking (Blundell & Perkins, 1981) = $42.4 \times 0.5 + 11.0 \times 0.67 = 28.6$.

‡ These values are calculated from columns 4–8, with the values for *O*-acetylation of dimer corrected as in columns 10 and 11. Thus for the 0.5 min sample, the percentage *O*-acetylation of monomers is $100 \times 6.2/(6.2 + 46.5) = 11.8\%$ and of dimers is $100 \times [3.7 + (0.33 \times 10.9)]/(21.3 + 10.9 + 3.7) = 20.3\%$.

Fig. 1. Descending paper chromatography of radioactivity solubilized by acid hydrolysis (0.1 M-HCl, 10 min, 100 °C) from lipid intermediate labelled in the glycan portion of the molecule by D-[1-¹⁴C]glucosamine. Solvent: butan-1-ol/acetic acid/water (4:1:5, by vol., upper phase). Chromatogram tracks were cut into 0.5 cm strips, for measurement of radioactivity in a scintillation counter. Sample (lower trace) and standard monomers (upper trace) were compared. Unlabelled *N*-acetylmuramylpentapeptide (—) was detected by spraying with ninhydrin. In this system monomers (disaccharide peptides) from *N. gonorrhoeae* ran as four spots, in order from the origin: disaccharide tripeptide (M3) disaccharide tetrapeptide (M4), followed by their respective *O*-acetylated derivatives (OM3, OM4).

monomers) but considerably slower than that of *O*-acetylated disaccharide peptides (*O*-acetylated monomers). A minor faster-running peak had a relative chromatographic mobility (R_{C6}) of 2.67, similar to that of *N*-acetylglucosamine (R_{C6} 2.6) (Chapman & Perkins, 1983). No peak was detected with a chromatographic mobility corresponding to that of *O*-acetylated monomers.

Hydrolysis of the major peak with 4 M-HCl yielded two radioactive spots on descending paper chromatography in butan-1-ol/acetic acid/water, corresponding to glucosamine and muramic acid. The muramic acid spot contained only 37% of the activity found in the glucosamine spot. However, no carrier was added and it is known that very small amounts of muramic acid are relatively labile under the conditions used for hydrolysis and appreciable loss would be expected. Rosenthal (1979) observed similar losses in some experiments. Parallel hydrolysis of the faster-running peak (R_{C6} 2.67) showed that it contained no labelled material with the mobility of muramic acid.

DISCUSSION

Previous work showing that peptidoglycan newly synthesized by *N. gonorrhoeae* is less cross-linked and less *O*-acetylated than mature peptidoglycan (Lear & Perkins, 1983) has been extended to very short labelling times. Pulses of radioactive glucosamine as short as 0.5 min labelled SDS-insoluble peptidoglycan, which was shown to be both cross-linked and *O*-acetylated to a significant degree. Transpeptidation was especially rapid, with the degree of cross-linking being 80% of that of mature peptidoglycan after a 0.5 min pulse. There was little increase in cross-linking over the 2 min time course, suggesting that the slower secondary transpeptidation noted previously in periods greater than 3 min (Lear & Perkins, 1983) did not commence appreciably before that time. Whether there was a delay in the onset of secondary cross-linking extending as long as 10 min, as observed in *E. coli* by de Pedro & Schwarz (1981), cannot be deduced from our experiments.

During periods up to 2 min, *O*-acetylation was much less near to completion than cross-linking, but did not approach zero even at the shortest labelling time. *O*-Acetylation of newly synthesized cross-linked peptidoglycan was considerably greater than that of the uncross-linked material (Table 1). These differences diminished with time but were still perceptible even in the 3–10 min period (Lear & Perkins, 1983). The fact that, throughout the early stage of synthesis, the cross-linked peptidoglycan was consistently more highly *O*-acetylated than the uncross-linked polymer of necessity implied that pre-formed dimers were preferentially *O*-acetylated. However, the pattern of *O*-acetylation does not suggest that all *O*-acetylated monomers arise by cleavage of *O*-acetylated dimers, even though a suitable endopeptidase is known to exist (Chapman & Perkins, 1983; Blundell & Perkins, 1985*a*). The only way for such a mechanism to operate would be if primary synthesis, cross-linking, *O*-acetylation and endopeptidase action all took place within about 1 min, whereafter the endopeptidase ceased for some reason to act, since otherwise one would expect more evidence of continuing peptide splitting, which did not appear to occur. Our experiments, in which pre-formed mono-*O*-acetylated dimer was split into its constituent monomer and *O*-acetylated monomer units by the use of exogenous endopeptidase, clearly showed that some of the latter subunits were derived from unlabelled and therefore biosynthetically 'old' glycan chains. Unlike in *P. mirabilis*, where most of the mono-*O*-acetylated dimer fraction carried radioactivity exclusively in the non-*O*-acetylated subunit (Gmeiner & Kroll, 1981), we found that only a relatively small proportion of the *O*-acetylated subunits were derived from 'old' chains. Asymmetric labelling of the mono-*O*-acetylated dimer fraction was greatly diminished after 3 min and had virtually disappeared by 15 min (Table 1). We therefore conclude that in *N. gonorrhoeae* a large proportion of the newly synthesized glycan chains are cross-linked to other newly synthesized chains rather than to pre-existing older unlabelled chains with their higher degree of *O*-acetylation. This would mean that, once begun, a glycan chain would become inaccessible to further dimer formation soon after 3 min, a conclusion in accord with the very high proportion (92%) of the final degree of cross-linking achieved within the same period.

The secondary cross-linking during the maturation period was evidently involved mainly with the formation of oligomers, while the proportion of dimers remained unchanged (Table 1 and Lear & Perkins, 1983). A possible explanation for this would be that newly polymerized monomer units are predestined, either by local factors in the gonococcal wall or by their topography within the growing cell, e.g. septal rather than peripheral, to follow one of two pathways. The first, and major, route would involve rapid dimer formation mainly by cross-linkage to similar chains being simultaneously synthesized. These dimer units would undergo relatively rapid *O*-acetylation but would not become further cross-linked to form trimers or higher oligomers. The second, minor route would be the main channel by which the trimer/oligomer component was formed. Here monomer units would be programmed *ab initio* to form multiple cross-links and would not draw on the corpus of dimer units to make trimers/oligomers. This second route to cross-linking would be relatively slow and would account for the fact that pulse-labelled dimer units do not contribute to trimer/oligomer formation during a chase period, the radioactive label arising in the latter components solely at the expense of monomers (Lear & Perkins, 1983).

The presence of a significant degree of *O*-acetylation of SDS-insoluble peptidoglycan even after the shortest labelling periods led us to investigate the possibility that *O*-acetylation occurred at the lipid-intermediate stage. Extraction of the lipid intermediates by the procedure of Anderson *et al.* (1967) yielded material with the characteristics of undecaprenol-linked peptidoglycan precursor. This major lipid intermediate of peptidoglycan synthesis contained the disaccharide-peptide (presumably pentapeptide) but there was no evidence for a corresponding *O*-acetylated intermediate. The absence of any detectable *O*-acetylation of the lipid-linked intermediates would suggest that the initial *O*-acetylation occurs in conjunction with the primary transpeptidation whereby newly synthesized peptidoglycan is cross-linked into the cell wall. The rapidity of these processes implies that they may all occur at the surface of a single enzyme or enzyme-complex. The suggestion that in *E. coli* two of these activities (transglycosylation and transpeptidation) may be dual functions of single enzymes (penicillin-

binding proteins PBP1a, 1b or 3) is already well known (Ishino *et al.*, 1980, 1981; Suzuki *et al.*, 1980). So far as *N. gonorrhoeae* is concerned, Dougherty (1983*b*, 1985) has suggested a close correlation between the function of PBP2 and the process of *O*-acetylation. A *penA* strain, in which PBP2 had much less affinity for penicillin than the wild-type, also showed less decrease in *O*-acetylation in response to low concentrations of penicillin (Dougherty, 1985). Like ourselves, he suggests a close association between *O*-acetylation and peptidoglycan synthesis as a possible explanation for his results. However, in our hands, a *penA2* strain (FA102) behaved similarly to the wild-type (FA19) in showing a 40% decrease in *O*-acetylation of the monomer fraction in response to concentrations of benzylpenicillin approximately equal to the respective MIC values (J. K. Blundell & H. R. Perkins, unpublished results). It may be, therefore, that *penA* resistant strains differ in their response, either via PBP2 or by some other mechanism.

Our experiments on the relative effects of penicillin on early and late *O*-acetylation and cross-linking will form the substance of a later communication.

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