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Effects of β-Lactam Antibiotics on Peptidoglycan Synthesis in Growing *Neisseria gonorrhoeae*, Including Changes in the Degree of *O*-Acetylation

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Low concentrations of β-lactam antibiotics caused an increased uptake of radioactive glucosamine into the sodium dodecyl sulfate-insoluble peptidoglycan of growing *Neisseria gonorrhoeae*. There was no appreciable change in the (small) amount of sodium dodecyl sulfate-soluble polymer present in the cultures. The sodium dodecyl sulfate-insoluble product in control cells was only partially dissolved by egg-white lysozyme (about 40%), but could all be released by the *Chalaropsis* B muramidase. In cells exposed to β-lactams the proportion of labeled peptidoglycan susceptible to lysozyme increased to 60%. Examination of the *Chalaropsis* B digests by thin-layer chromatography showed that they contained disaccharide-peptide monomers with and without *O*-acetylation and bis-disaccharide-peptide dimers with one or two *O*-acetyl groups, or with none. β-Lactam antibiotics caused a decrease in the degree of *O*-acetylation but did not greatly affect the amount of peptidoglycan cross-linking. They also had the effect of enlarging the bacteria and conserving and thickening the septa that could be observed in thin sections under the electron microscope. The relationship between these results and the effects of β-lactams on in vitro synthesis of peptidoglycan by ether-treated *N. gonorrhoeae* is discussed.

We have previously shown that peptidoglycan synthesis occurring in vitro when ether-treated *Neisseria gonorrhoeae* cells are given nucleotide precursors is affected by β-lactam antibiotics (3). At or below the minimum inhibitory concentration for growth the antibiotics produced a small overall increase in peptidoglycan synthesis, whereby amounts of both the sodium dodecyl sulfate (SDS)-insoluble fraction and the SDS-soluble fraction were enhanced. Higher antibiotic concentrations caused an inhibition of synthesis of cross-linked material (the SDS-insoluble product) and a much more than compensatory increase in the amount of SDS-soluble polymeric product (3). To determine whether these responses to β-lactams were confined to the ether-treated cells, we have examined peptidoglycan synthesis during growth in the presence or absence of antibiotics by measuring the uptake of radioactively labeled glucosamine as previously described (14, 20). We characterized as peptidoglycan the radioactivity incorporated into the SDS-insoluble fraction of the cells, first by showing that almost all the label was equally distributed between glucosamine and muramic acid. However, we found that, contrary to previous reports (14, 20), the material was not all solubilized by egg-white lysozyme, but required the muramidase B from *Chalaropsis* (6) to obtain complete solution. Further examination of labeled and unlabeled preparations showed that the peptidoglycan of *N. gonorrhoeae* contains *O*-acetyl groups equivalent to about 50% of the muramic acid residues (2). The *Chalaropsis* enzyme digests have now been fractionated by thin-layer chromatography (TLC) as described for *Proteus mirabilis* (10), which has partially *O*-acetylated peptidoglycan (10, 11). The same *O*-acetylated and unsubstituted peptidoglycan fragments have been demonstrated in both organisms. Changes in the degree of *O*-acetylation in response to low concentrations of β-lactam antibiotics are described, reminiscent of those formerly observed in unstable spheroplast L-forms of *P. mirabilis* grown in the presence of benzylpenicillin (10). Earlier reports on the morphological effects of sub-inhibitory concentrations of penicillin on bacteria described gross alterations in the septal region of *N. gonorrhoeae* (9) and increases in cell size (1, 22). Even at the very low levels of β-lactams used in our experiments, similar changes were observed and measured.

(This work forms part of the presentation by J. K. Blundell for the degree of Ph.D. from the University of Liverpool.)

MATERIALS AND METHODS

Organisms, growth conditions, and radioactive labeling. *N. gonorrhoeae* strain FA19, a penicillin-sensitive laboratory strain, and strain FA102, a *penA2* penicillin-resistant mutant produced by a transformation cross between FA19 and the relatively highly resistant non-β-lactamase producer FA48, were obtained from P. F. Sparling (17). Strains 1L260 and 1L261 were, respectively, penicillin-sensitive and penicillin-resistant isolates described earlier (3). *P. mirabilis*, a stock laboratory strain, was grown in nutrient broth and labeled with *N*-acetyl-[^{14}C]glucosamine essentially as described (10).

Cultures of *N. gonorrhoeae* were grown in chocolate agar made from 8% (vol/vol) horse blood in Columbia agar base (Lab M, London) and stored at −70°C in nutrient broth (Oxoid no. 2) containing 20% (vol/vol) 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% CO_2 in air. Liquid medium was proteose peptone no. 3 (Difco) (20) (pH 7.2) with the addition of the growth supplement of Mayer et al. (12), except that the glucose was replaced with pyruvate (14). The medium was equilibrated with 5% CO_2 overnight at 37°C and then inoculated with organisms to an absorbancy at 675 nm (A_{675}) of 0.04 as previously described (3). Radioactive glucosamine (Radiochemical Centre, Amersham, U.K.) was then added either as D-[1-^{3}H]-glucosamine hydrochloride (30 Ci/mmol) to a concentration of 1 μCi/ml of culture or as D-[1-^{14}C]-glucosamine hydrochloride (58 mCi/mmol) to a final concentration of 0.25 μCi/ml. Incubation with shaking at 37°C was continued until a culture density of A_{675} = 0.1 was achieved; at this time equal samples (50 ml) were taken and shaken in 250-ml flasks. This point was taken as time 0 in all experiments. Absorbancy (A_{675}) was monitored at intervals, and after 30 min antibiotic was added as required.

Measurement of radioactivity incorporated into peptidoglycan. Samples (0.5 ml) were taken at intervals, added to an equal volume of 10% (wt/vol) SDS in water, and heated in a boiling-water bath for 20 min. After cooling they were filtered on glass-fiber disks (GF/C Whatman), which were then washed twice with water (10 ml), dried at 80°C for 1 h, and transferred to counting vials. Radioactivity was measured as described before (3). It was shown in preliminary experiments that by the time A_{675}-0.8 had been reached, about 30% of the added radioactivity had been incorporated into trichloroacetic acid-insoluble material and that about 90% of this was also SDS insoluble.

Preparation of labeled peptidoglycan and subsequent enzyme digestion. From labeling experiments using [^{14}C]glucosamine as just described, bulk samples of 10 ml were taken after 2 h of incubation, added to an equal volume of 10% (wt/vol) SDS, and heated at 100°C as before. The pH value was checked after boiling, and the SDS-insoluble material was collected by centrifuging at 130,000 × *g* and washing in water as previously described (2).

Samples were digested by muramidases as follows. (i) Lysozyme: the SDS-insoluble product was suspended in 0.1 M ammonium acetate buffer (pH 6.5) (150 μl) and digested with egg-white lysozyme (Sigma) (200 μg/ml) at 37°C for 24 h. After addition of water, the insoluble portion was sedimented and washed as before, and the supernatant fluid was dried by rotary evaporation and suspended in 200 μl of water. Samples (5 μl) were counted in aqueous scintillation fluid. (ii) *Chalaropsis* B enzyme: insoluble pellets, either directly as the initial SDS-insoluble fraction or after lysozyme digestion as described, were suspended in 0.03 M triethylamine acetate buffer (pH 4.7) (150 μl) and incubated at 37°C with 50 μg *Chalaropsis* B enzyme (kindly donated by J. H. Hash or, in later experiments, J. B. Ward) per ml for 24 h. Supernatant fluids and pellets were recovered as before. Pellets after *Chalaropsis* enzyme treatment contained no more than 4% of the radioactivity initially present in the SDS-insoluble fraction and usually much less.

TLC of enzyme-digested peptidoglycan. Samples of solubilized material (ca. 100,000 dpm) were transferred to the origin of a 0.25-mm silica gel plastic TLC plate (Polygram SilG/UV_{254}; Macherey-Nagel, Germany) and run twice in *iso*-butyric acid–1 M ammonia (5:3, vol/vol) as described (10). After drying, radioactive spots were detected by autoradiography using X-ray film (Kodak X-Omat H) exposed for 2 to 3 days.

Spots thus identified were cut from the TLC plate, and the radioactivity was measured in nonaqueous scintillation fluid. If the radioactive samples were to be recovered, they were eluted in water after scintillant had been removed by washing in toluene.

Mild alkali treatment of peptidoglycan fragments. The eluted samples were adjusted to pH 10 with ammonia and incubated at 37°C for various periods. After incubation the samples were acidified with acetic acid, rotary evaporated to dryness, and reexamined by TLC.

Electron microscopy. Samples (1 ml) were taken during the experiments in which growing bacteria were treated with β-lactam antibiotics, harvested by centrifuging for 3 min on a high-speed bench centrifuge, and suspended in 1 ml of 0.1 M potassium phosphate buffer (pH 7.2) containing 2.5% (wt/vol) glutaraldehyde. After again being centrifuged, the bacteria were covered with 1 ml of the same glutaraldehyde buffer and left overnight at 4°C. Samples were stained with 1% (wt/vol) OsO_4 and block stained with 70% (vol/vol) ethanol saturated with uranyl acetate. After dehydration through an alcohol series, the samples were embedded in Taab 812 medium mix and heated at 60°C for 24 h. Sections were cut, stained with lead citrate, mounted on copper grids to gold thickness, and examined in a Cora 500 electron microscope.

Measurement of cell volume. The size distribution of the bacteria was measured using a Z_BI Coulter electronic particle counter fitted with a 30-μm probe and linked to a Coulter Channelyzer (Coulter Electronics Ltd., Hertfordshire, U.K.). At regular time intervals, samples of cultures with and without thienamycin (0.01 and 0.1 μg/ml) were diluted in Isoton II (Coulter Electronics Ltd.) and measured immediately. The instrument was calibrated by using polystyrene beads of known volume to calculate the cell volume.

Antibiotics. The benzylpenicillin was from Sigma;

N-formimidoyl thienamycin (briefly referred to hereafter as thienamycin) (MK0787) was a gift from Merck, Sharp and Dohme, Rahway, N.J., by courtesy of B. G. Christensen; and cefuroxime was obtained from Glaxo Research Ltd., Greenford, Middlesex, England.

RESULTS

Labeling of peptidoglycan during growth and effect of β-lactam antibiotics. Growing cultures of *N. gonorrhoeae*, with or without added antibiotic, were supplemented with radioactive glucosamine, and uptake into SDS-insoluble material was followed in parallel with measurements of absorbancy. The radioactivity was identified as representing peptidoglycan by hydrolyzing samples of the labeled product in 4 M HCl for 4 h at 100°C, removing HCl in a vacuum, and separating the liberated glucosamine and muramic acid by paper chromatography (Whatman 3MM; butan-1-ol–acetic acid–water, 4:1:5, vol/vol, upper phase). Spots were located with ninhydrin, and their radioactivity was measured. The only labeled products corresponded to glucosamine and muramic acid, and the latter compound contained about 80% as much radioactivity as the glucosamine. Since muramic acid is known to be labile under the conditions used for hydrolysis, it is reasonable to assume that the two components of the peptidoglycan were essentially equally labeled and that almost all the label was in peptidoglycan, as previously reported (5).

Regardless of the strain, low concentrations of β-lactam antibiotics (in the region of the minimum inhibitory concentration) caused an increase in the incorporation of glucosamine into the SDS-insoluble peptidoglycan. Thus with strain FA19, benzylpenicillin at 0.001 μg/ml had hardly any effect on the absorbancy of the growing culture, but nevertheless caused a large rise in glucosamine incorporation (Fig. 1). The minimum inhibitory concentration of benzylpenicillin for this strain, determined by a plate method (16), was 0.01 μg/ml (3). When this concentration of antibiotic was added to the growing cells, the effect upon increase of absorbancy was slight until 180 min, but nevertheless the incorporation of radioactivity was depressed at all times after the addition of penicillin (Fig. 1). For the last 60 min during which absorbancy was followed, the amount of incorporated glucosamine declined, probably corresponding to a release of peptidoglycan as observed by others (5, 21).

Evidence that the enhanced uptake of radioactivity in the presence of 0.001 μg of penicillin per ml represented an actual increase in peptidoglycan labeling was obtained by hydrolyzing the product to glucosamine and muramic acid as

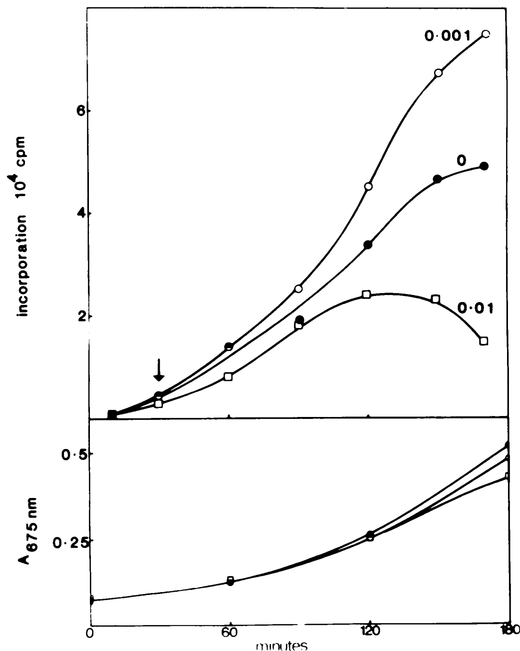

FIG. 1. *Incorporation of [^{3}H]glucosamine into the SDS-insoluble peptidoglycan of growing N. gonorrhoeae FA19. Benzylpenicillin was added at 30 min (arrow).* ●, *Control;* ○, *0.001 μg/ml;* □, *0.01 μg/ml. The lower portion of the graph shows turbidity (A_{675}) in the same experiment.*

described above. The individual amino sugars also showed about the same degree of increased radioactivity as the parent peptidoglycan.

Enhanced uptake of glucosamine was not confined to one β-lactam antibiotic or to one strain, since similar results were observed with strain 1L260 and benzylpenicillin or *N*-formimidoyl thienamycin, a β-lactam with minimum inhibitory concentrations in the range of 0.031 to 0.25 μg/ml for gonococci (8). As shown in Fig. 2, 0.01 μg of thienamycin per ml had no apparent effect on A_{675} of the culture, but at 0.1 μg/ml both the culture density and glucosamine uptake ceased to increase during the 3rd hour. At both concentrations, viability had decreased to only about 10% of the control value 80 min after addition of thienamycin. Similar increases in peptidoglycan synthesis were also obtained when strain 1L261 (a β-lactamase producer) was treated with 0.05 or 0.1 μg of the β-lactamase-resistant antibiotic cefuroxime per ml.

Evidence that the enhancement of peptidoglycan labeling was peculiar to β-lactam antibiotics was obtained by performing experiments in which tetracycline, at concentrations that were found to be just inadequate to inhibit growth (A_{675} increase) under our conditions (0.1 and 0.2

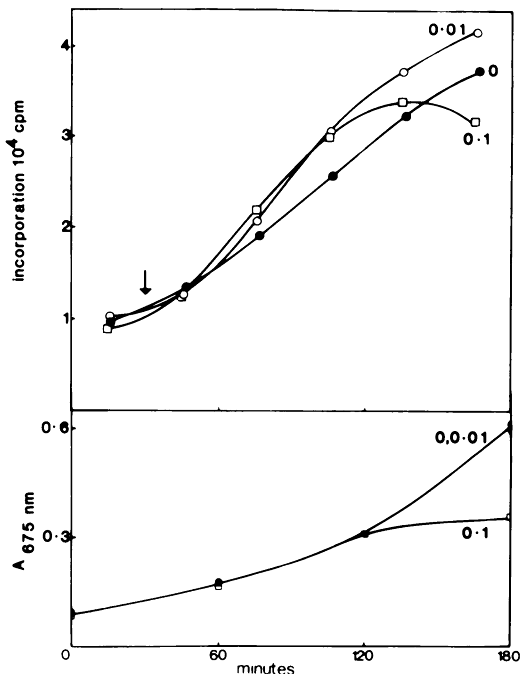

FIG. 2. *Incorporation of [^{3}H]glucosamine into the peptidoglycan of strain 1L260. Thienamycin was added at 30 min (arrow).* ●, *Control;* ○, *0.01 μg/ml;* □, *0.1 μg/ml. The lower portion of the graph shows A_{675} in the same experiment.*

μg/ml), was added during incorporation of [^{3}H]glucosamine. No enhancement of peptidoglycan labeling was observed; indeed, there was even a small diminution of uptake.

Presence of SDS-soluble peptidoglycan and its response to penicillin. Since peptidoglycan synthesized by ether-treated *N. gonorrhoeae* contained a large proportion of SDS-soluble polymer (3), it was necessary to establish whether the growing bacteria also synthesized peptidoglycan of this type. Experiments similar to those already described were conducted with strain 1L260, except that after removal of the SDS-insoluble fraction by centrifugation, the remaining supernatant was treated to remove excess SDS (3) and chromatographed on Whatman 3MM paper as formerly described (3). The polymeric material remaining on the origin was counted in nonaqueous scintillation fluid. The total radioactivity recovered as SDS-soluble polymer in any sample represented not more than 10% of the amount found in the SDS-insoluble peptidoglycan. Penicillin at 0.1 μg/ml had little effect on incorporation into the SDS-soluble fraction; if anything, it produced a diminution of radioactivity rather than the enhancement observed either with the SDS-insoluble fraction in the same experiment, or with in vitro synthesis of both SDS-insoluble and -soluble fractions (3).

Susceptibility of the peptidoglycan to muramidases. Samples of peptidoglycan taken towards the end of experiments similar to those shown in Fig. 1 and 2 were first treated with lysozyme and then, after recovery of the insoluble pellet by centrifuging, with *Chalaropsis* B muramidase, as described before (2). Preliminary experiments showed that the original lysozyme treatment was essentially complete since repeated digestion did not yield any significant increase in solubilization. The proportions of the radioactive peptidoglycan rendered soluble by the enzymes are shown in Table 1.

Whereas in control cultures of strain 1L260 approximately half of the peptidoglycan was lysozyme resistant, low concentration of penicillin or thienamycin caused a considerable increase in lysozyme susceptibility. The same pattern was observed with strain FA102, and in each case increases in β-lactam concentration led to even more lysozyme-sensitive peptidoglycan, so that as much as 88% of the whole became susceptible to the enzyme.

The presence of *O*-acetyl groups in the peptidoglycan. The results just described led us to seek the reason for the lysozyme resistance of a proportion of the peptidoglycan of *N. gonorrhoeae*, and as already reported (2), clear evi-

TABLE 1. *Solubilization of labeled peptidoglycan by successive treatments with lysozyme and Chalaropsis B muramidase*

Strain	Antibiotic	Concn (μg/ml)	% of total radioactivity[a]: Released by: Lysozyme	Released by: *Chalaropsis* B muramidase	Remaining in pellet
1L260	Benzylpenicillin	0	48.9	50.9	0.2
		0.1	59.0	39.0	2.0
1L260	*N*-Formimidoyl thienamycin	0	47.7	51.7	0.6
		0.01	71.9	27.5	0.6
		0.1	87.9	11.7	0.4
FA102	Benzylpenicillin	0	51.0	48.0	1.0
		0.05	63.0	36.0	1.0
		0.1[b]	79.0	20.0	1.0

[a] Radioactivity from [^{3}H]glucosamine was incorporated during growth, and at 1.5 to 2.5 h after the time of antibiotic addition, when growth as measured by A_{675} was essentially unaffected, samples were taken simultaneously from control and treated flasks. The SDS-insoluble peptidoglycan was prepared by centrifuging, and muramidase treatments, first with lysozyme and then with *Chalaropsis* enzyme, were performed as described in the text.

[b] In this flask growth had ceased in response to the antibiotic.

dence for O-acetylation was obtained. To study the degree of O-acetylation we followed closely the procedure employed with *P. mirabilis* (10). Direct comparison of the two organisms showed that *Chalaropsis* B digests of the *N. gonorrhoeae* peptidoglycan produced the same fragments, O-acetylated and non-O-acetylated, as *P. mirabilis*. An autoradiograph after TLC of the digest from *N. gonorrhoeae* is shown in Fig. 3. Comparison of lane a (control) and lane b (with penicillin, 0.1 μg/ml) shows clearly that the antibiotic has caused the monomer fraction of the peptidoglycan to become less O-acetylated. The relationship between the separated fragments and the relative sensitivity to lysozyme and *Chalaropsis* B enzyme is shown in lanes c and d. Lysozyme solubilization yielded much material that was sufficiently polymeric to remain at the origin of the chromatogram; the recognizable smaller fragments were mainly non-O-acetylated and mono-O-acetylated dimer, with only a trace of the di-O-acetylated fragment, and a considerable amount of non-O-acetylated monomer with very little of its O-acetylated analog. Subsequent *Chalaropsis* B digestion of the insoluble residue gave the reverse picture (Fig. 3d), in which essentially no origin material remained and the major fragments belonged to the O-acetylated category.

The observation of solubilized but chromatographically immobile material in lysozyme digests matches our previous results with paper chromatography in the same solvent (2). Further treatment of eluted origin material with *Chalaropsis* B enzyme yielded the same small-molecular-weight fragments that direct application of the latter enzyme produced. Traces of labeled material running even faster than O-acetylated monomer were always observed, even in the digest from *P. mirabilis*, but were not identified.

Further evidence for the identification of the relatively fast-running components as O-acetyl derivatives was provided by mild alkali treatment of the eluted spots. Treatment of the supposed O-acetylated monomer from *N. gonorrhoeae* or *P. mirabilis* with ammonia at pH 10 for 2 to 6 h resulted in 60 to 80% conversion to the unsubstituted monomer, the remainder of the radioactivity being found as apparently unchanged material or as a very fast-moving component, thought to represent β-elimination products (19) (Fig. 4). The O-acetylated dimers were also converted to their less O-acetylated counterparts. Under the same conditions initially non-O-acetylated monomer and dimer were converted partially to the same β-elimination products, with the majority remaining unchanged.

Effects of β-lactam antibiotics on cross-linking and the degree of O-acetylation. The precise composition of the labeled peptidoglycan in relation to cross-linking and O-acetylation was determined by direct measurement of the radioactivity in the fragments produced by *Chalaropsis* B digestion (Table 2). The results show that, in strain 1L260, benzylpenicillin at 0.1 μg/ml caused a shift from O-acetylated monomer to the unsubstituted fragment and a comparable but smaller effect on dimer. Similar

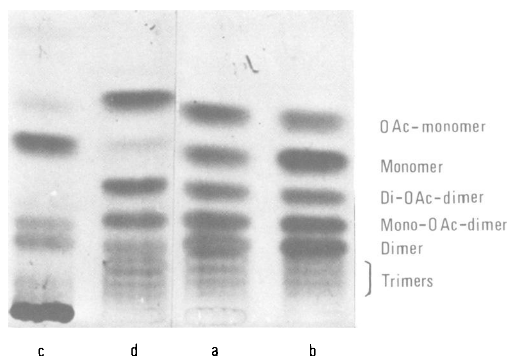

FIG. 3. *Autoradiogram of TLC of lysozyme and Chalaropsis muramidase digests of peptidoglycan from strain 1L260, labeled during growth with [¹⁴C]glucosamine. Samples were taken 130 min after transfer. TLC plate 1: (a) Control culture, Chalaropsis digest; (b) culture with 0.1 μg of benzylpenicillin per ml added 30 min after transfer, Chalaropsis digest. TLC plate 2: (c) Like (a), but lysozyme digest supernatant; (d) Chalaropsis digest of the sedimentable residue from (c).*

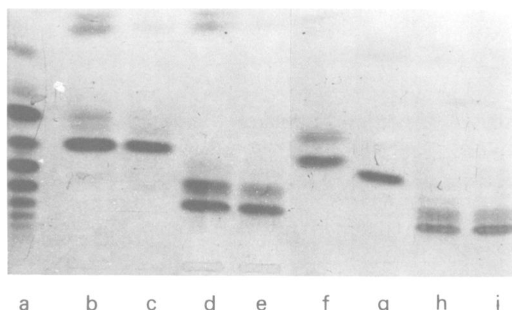

FIG. 4. *Autoradiogram of TLC of peptidoglycan fragments after treatment with ammonia, pH 10, 6 h. Lanes a to e, Experiment 1, P. mirabilis; lanes f to i, experiment 2, N. gonorrhoeae 1L260. Lane a, Untreated whole Chalaropsis digest for comparison; for designation of spots see Fig. 3. In lanes b to i, the original fragments were eluted from similar TLC plates, incubated with ammonia to remove O-acetyl groups as described in the text, and rerun. Original fragments were as follows: (b, f) O-acetylated monomer; (c, g) non-O-acetylated monomer; (d, h) di-O-acetylated dimer; (e, i) mono-O-acetylated dimer.*

TABLE 2. *Composition of SDS-insoluble peptidoglycan deduced from Chalaropsis B digestion and TLC*

Organism	Benzylpenicillin concn (μg/ml)	Total radioactivity[a] (%) recovered as:						% of peptide cross-linking[b]
		Multimer[c]	Dimer	Mono-*O*-acetylated dimer	Di-*O*-acetylated dimer	Monomer	*O*-acetylated monomer	
N. gonorrhoeae 1L260	0	13	14	16	14	16	20	31
	0.1[d]	14	16	15	12	22	13	31
	0.5[e]	12	13	14	11	31	14	27
N. gonorrhoeae FA19	0	32	11	14	12	12	15	40
	0.01[d]	38	13	11	8	15	9	41
P. mirabilis	0	7	7	14	25	12	31	27

[a] Cultures were grown and labeled with [^{14}C]glucosamine in experiments similar to that of Fig. 1 as described in the text, and samples were taken at 130 min. The total of approximately 100,000 dpm obtained from the TLC of each experiment included the small amount of radioactivity present in the very fast-moving spots (see Fig. 3 and the text) but not reported in this Table.

[b] Percentage of peptide cross-linking was calculated (15) as 0.5 × percent total dimer + 0.667 × percent multimer, on the assumption that the multimer was largely trimer.

[c] Multimer represented material running as trimer (10), but slow-running and origin material was also included. The latter represented only a very small proportion with strain 1L260 or *P. mirabilis*, but was considerably greater, up to 50% of the multimer fraction, in strain FA19.

[d] In these experiments the uptake curve closely resembled that shown for 0.1 μg of thienamycin per ml in Fig. 2.

[e] In this experiment the uptake curve closely resembled that shown for 0.01 μg of penicillin per ml and strain FA19 in Fig. 1.

changes were observed with strain FA19 and 0.01 μg of penicillin per ml. The degree of cross-linking observed for strain FA19 control cultures (40%) was very close to the value of 38.6% previously reported (15). These low concentrations of penicillin, which caused an enhancement of peptidoglycan labeling (e.g., Fig. 1), did not affect the overall degree of cross-linking in either strain. However, it was noticeable that penicillin at 0.5 μg/ml, which prevented an increase in culture density in strain 1L260, did cause a perceptible decrease in cross-linking, mainly attributable to the larger proportion of the total peptidoglycan that appeared in the non-*O*-acetylated monomer fraction.

The results for *P. mirabilis*, included for comparison, show that with our strain the percentage of peptide cross-linking (27%) is very close to the value of 25% calculated from the results of Martin and Gmeiner (10).

The degree of *O*-acetylation of the cross-linked and un-cross-linked fragments of peptidoglycan is calculated in Table 3. The values for both strains of *N. gonorrhoeae* grown in the absence of penicillin are closely comparable to the proportion of measurable *O*-acetyl groups relative to total diaminopimelic acid calculated from direct chemical estimations (2). Under conditions in which enhanced uptake of radioactivity to peptidoglycan occurred while the turbidity of the culture remained unaffected, there was a substantial decrease in the degree of *O*-acetylation, which was more noticeable in the monomer than the dimer fraction. With strain 1L260 more penicillin caused an additional decrease in the *O*-acetylation of monomer, but no further change in that of the dimer.

TABLE 3. *Degree of O-acetylation in monomer and dimer fractions of peptidoglycan*

Organism	Benzylpenicillin concn (μg/ml)	% *O*-acetylation[a]		
		Monomer	Dimer	Monomer + dimer
N. gonorrhoeae 1L260	0	55	52	53
	0.1	37	46	41
	0.5	30	47	38
N. gonorrhoeae FA19	0	56	51	53
	0.01	38	42	40
P. mirabilis	0	72	70	71

[a] The experiment is the same as that used for Table 2. Percentage of *O*-acetylation represents the molar proportion of *O*-acetyl groups divided by the total disaccharide (*N*-acetylglucosamine-*N*-acetylmuramyl) units present in the fraction, ×100.

Concomitant morphological effects. Since it is known that β-lactam antibiotics affect the morphology and size of *N. gonorrhoeae* (1, 5, 9, 22), we attempted to correlate these changes with the biochemical effects already described. We examined cross-sections of bacteria under conditions where overall peptidoglycan synthesis had been enhanced by thienamycin (Fig. 5)

or by benzylpenicillin (not shown). Both β-lactams caused observable septa in a far greater proportion of the cells, with frequent thickening of the septal region and apparent extrusion of blebs (5, 9, 22). At the same time the average size of the cells increased (1, 22) to an extent that was related to the drug concentration (Table 4). The progress of the enlargment was followed by direct measurement in the Coulter Counter during an experiment similar to that shown in Fig. 2. Increases in cell size in the presence of thienamycin were rapid and continuous (Fig. 6), and 80 min after addition of the drug at 0.01 μg/ml the cell volume (1.5 μm^3) was closely similar to that deduced from the examination of thin sections (1.6 μm^3; Table 4).

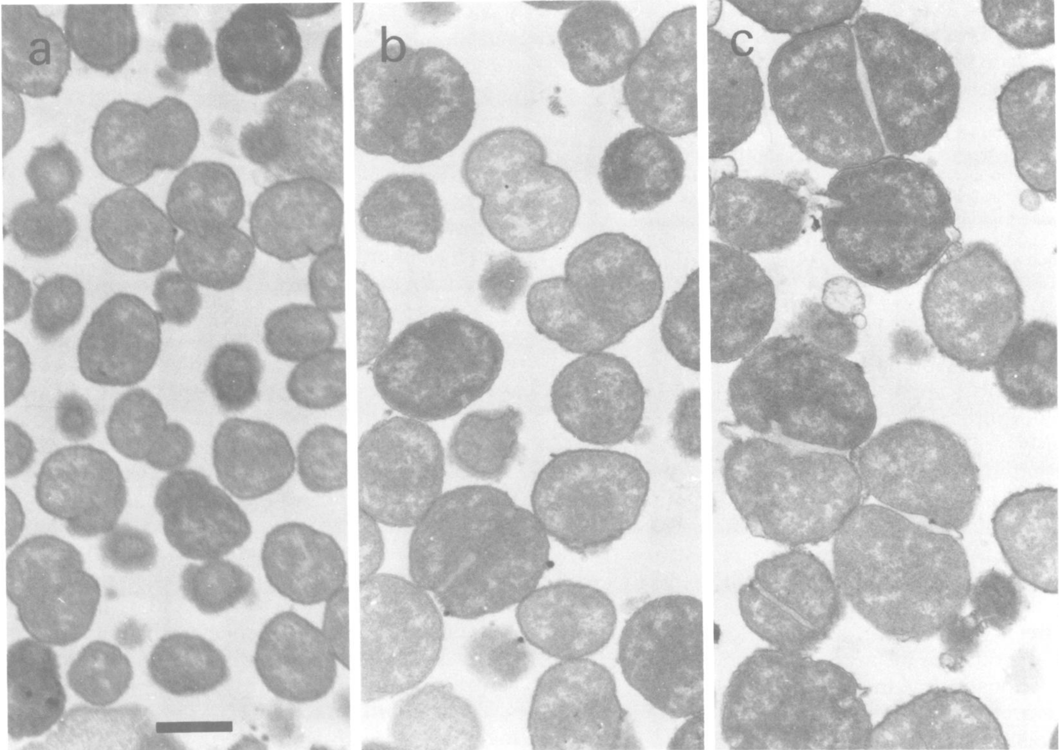

FIG. 5. *Electron micrographs of thin sections of N. gonorrhoeae 1L260. Cells were fixed 80 min after addition of thienamycin: (a) control; (b) 0.01 μg/ml; (c) 0.1 μg/ml. Bar, 1 μm.*

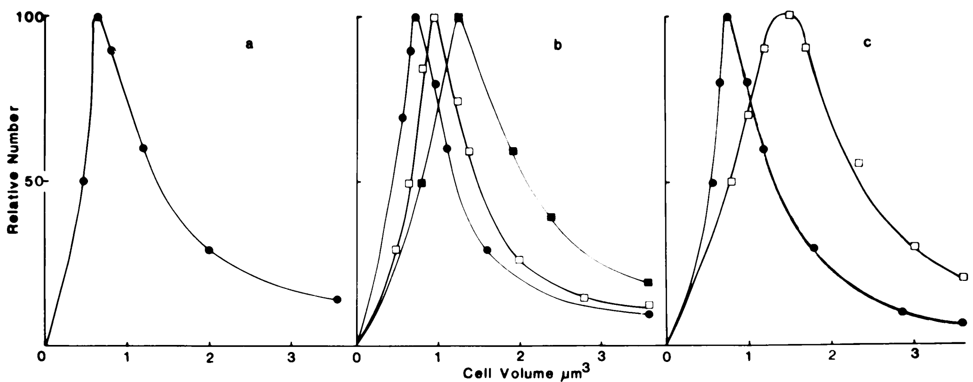

FIG. 6. *Size distribution of growing N. gonorrhoeae 1L260, as measured by the Coulter Counter. (a) Before addition of antibiotic. (b) At 35 min after and (c) 78 min after addition of thienamycin: ●, control; □, 0.01 μg/ml; ■, 0.1 μg/ml. After 78 min with 0.1 μg of thienamycin per ml, damage to the cells was such that an accurate Coulter measurement could not be obtained.*

TABLE 4. *Size of N. gonorrhoeae as measured by electron microscopy*

Culture[a]	Dimensions of bacteria[b] (μm, mean ± SD)		Vol[c] (μm³)
	Major axis	Minor axis	
Control	1.34 ± 0.13 (34)	1.12 ± 0.11 (34)	0.9
Thienamycin (0.01 μg/ml)	1.57 ± 0.24 (45)	1.32 ± 0.14 (45)	1.6
Thienamycin (0.1 μg/ml)	2.02 ± 0.34 (36)	1.61 ± 0.25 (36)	3.0

[a] Samples were taken from parallel cultures 110 min after time 0 and 80 min after addition of antibiotics in the experiment shown in Fig. 2.

[b] Figures in parentheses indicate the number of complete bacteria measured within an arbitrarily placed square. Since the cross-sections were not circular, the major and minor axes were measured in all cases. SD, Standard deviation.

[c] Calculated from the mean dimensions on the assumption that each cell was a short cylinder capped by two hemispheres.

DISCUSSION

Our previous work (3) showing that β-lactam antibiotics enhanced the synthesis of peptidoglycan (both SDS insoluble, and therefore presumably cross-linked, and SDS soluble) in ether-treated cells has now been confirmed with growing organisms, in which the bulk of the incorporation of radioactive glucosamine was into SDS-insoluble peptidoglycan. Contrary to the widely held view that an inhibition of the cross-linking of peptidoglycan is one of the primary effects of β-lactam antibiotics, which leads by some route or other to the death of bacteria (13), it appears that in *N. gonorrhoeae* gross inhibition of cross-linking does not occur at an early stage. This lack of effect on cross-linking, suggested by measurement of overall synthesis (Fig. 1 and 2), has been substantiated by detailed examination of the degree of cross-linking deduced from the composition of *Chalaropsis* B muramidase digests (Table 2). When bacteria were exposed to penicillin in concentrations sufficient to cause gross changes in morphology and the death of a large proportion of the cells, peptidoglycan cross-linking was unaffected. This observation did not, of course, preclude some specialized subpopulation of peptidoglycan from having suffered decreased cross-linking at a time when the overall effect was in the opposite sense.

While peptidoglycan cross-linking remained unaffected, the degree of *O*-acetylation of the peptidoglycan was greatly diminished (Tables 2 and 3). The precise relationship of this parameter to the inhibitory effect of penicillin on bacteria is not yet understood, particularly as the phenomenon has only been previously reported in the unstable L-forms of *P. mirabilis* (10). There, too, the effects on the degree of *O*-acetylation were far more dramatic than the rather small decreases in cross-linking. It could be argued from the results with *N. gonorrhoeae* that in the presence of β-lactam antibiotics a type of peptidoglycan somewhat low in *O*-acetylation is synthesized at the expense of a more highly *O*-acetylated subpopulation, or that the over-production of peptidoglycan (Fig. 1 and 2) is not accompanied by a concomitant increase in *O*-acetylation.

Enhanced peptidoglycan synthesis and diminished *O*-acetylation were accompanied by morphological changes. Enlargement of the cells, already apparent after only one-third of a generation time, proceeded until cell volume had increased two- to threefold and thickened septa had developed. The higher the antibiotic concentration the greater was the degree of enlargement. Evidently the processes that control peptidoglycan deposition and cell shape and division in *N. gonorrhoeae* are an early target for penicillin action, which must be mediated through a particularly susceptible enzyme. Dougherty et al. (4) have demonstrated the presence of three major penicillin-binding proteins (PBPs) in many strains of *N. gonorrhoeae*, and it might be supposed that one of these must have the key role implied by our results. Examination of wall-defective mutants (16) should prove valuable here. So far we have only examined overall peptidoglycan synthesis in one resistant mutant of the *penA2* type (strain FA102), and here enhancement was elicited as with the parent strain FA19, although at higher penicillin concentrations.

Very recently Barbour (1) examined the binding of specific β-lactams to the PBPs of *N. gonorrhoeae*. He distinguished between cephaloridine and penicillin, which bound to all three PBPs and produced spheroplasts at minimum inhibitory concentrations, and mecillinam, which bound only to PBP2 and produced enlarged, apparently intact cells. In our experiments thienamycin, known to bind to the PBP2 of *Escherichia coli* (18), produced morphological and biochemical effects similar to those of penicillin, with some evidence for extrusion of cellular material that might presage spheroplast formation. The different recorded affinities for gonococcal PBPs (1) suggest that that approach, coupled with the biochemical studies described here and in earlier work (3, 5, 14, 15, 21), should lead towards a fuller understanding of how β-lactams kill gonococci.

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