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Release of Soluble Peptidoglycan from Growing Gonococci: Demonstration of Anhydro-Muramyl-Containing Fragments

RABINDRA K. SINHA AND RAOUL S. ROSENTHAL*

Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana 46223

Previous analysis of soluble peptidoglycan (PG) fragments released by exponentially growing gonococci implicated the combined action of both hexosaminidase and amidase activities in PG turnover. Current studies further characterized PG fragments which were labeled in the glycan with D-glucosamine and in the peptide moiety with *meso*-diaminopimelic acid or L- and D-alanine. Labeled PG fragments were isolated by gel filtration and characterized on the basis of (i) K_D values, (ii) free amino group analysis using fluorodinitrobenzene, (iii) borohydride reduction, (iv) alkali-catalyzed β-elimination, (v) paper chromatography in various solvents, (vi) electrophoretic mobility at various pH values, (vii) digestibility by *Charonia lampas* glycosidases, and (viii) content of labeled D- and L-alanine. A set of well-characterized PG fragments was used as standards. The monomer fraction (the major extracellular product) was found to contain two components. Most (about 80%) appeared to be *N*-acetylglucosaminyl-β-1 $\rightarrow$ 4-1,6-anhydro-*N*-acetylmuramyl-L-ala-D-glu-*meso*-diaminopimelic acid; the remainder was the corresponding disaccharide tetrapeptide containing a C-terminal D-alanine. An unusual feature of these products was the presence of the anhydro-muramyl (nonreducing) ends, reflecting the activity of a gonococcal transglycosylase, and the near absence of products containing detectable reducing ends. Otherwise, the structures of the monomer fragments were typical of those expected for a gram-negative bacterium (chemotype I). The corresponding peptide-cross-linked dimer and the free disaccharide also contained nonreducing ends, exclusively. Free peptides (products of amidase activity) consisted of both tripeptide and tetrapeptide. In summary, all gonococci examined appear to possess an unusual transglycosylase activity which contributes to the release of soluble PG fragments containing nonreducing, anhydro-muramyl ends. The release of these fragments in vivo might be a unique aspect of gonococci-host interactions.

Gonococci appear to be unusual among gram-negative bacteria in that they exhibit extensive turnover of their peptidoglycan (PG) layer during exponential growth (6-8, 13, 22) and shed the soluble PG fragments into the culture medium (13). Preliminary analysis of the soluble PG detected in gonococcal supernatants has revealed four major forms of PG fragments which were separated by gel filtration (13). These have been tentatively identified as forms of (i) cross-linked, bisdisaccharide peptide dimer (termed PGI in reference 13), (ii) disaccharide peptide monomer (PGII), (iii) free peptide (PGIIIa), and (iv) free disaccharide (PGIIIb). The presence of these forms suggested that turnover and release of PG by growing gonococci was mediated by both glycan-splitting (hexosaminidase) and peptide-splitting (*N*-acetylmuramyl-L-alanine amidase) activities (13).

The chemical structure of the soluble PG fragments released via turnover and of intact native gonococcal PG has not been completely defined. However, the composition of gonococcal PG appears to be chemotype I (16), typical of most gram-negative bacteria. It contains approximately equimolar amounts of muramic acid, glucosamine ($glcNH_2$), L-alanine, glutamic acid, diaminopimelic acid (DAP), and D-alanine (8, 9, 15, 24). The DAP residues in gonococcal PG are present in the *meso* form, exclusively (R. Roeske, unpublished data).

This laboratory is examining the hypothesis that the interaction between gonococcal PG and components of the host immune system and of the inflammatory response influences the consequences of gonococcal infections. As one test of the hypothesis, we are examining the biological activities of various forms of PG which, based on in vitro studies, may have the opportunity to interact in vivo with host cells and molecules. Among such forms of PG are those which are liberated by growing gonococci as a result of PG turnover. The purpose of this study was to further characterize the structure of these

PG fragments released by turnover. The fragments were unusual in that they possessed nonreducing (anhydro-muramyl) ends, exclusively. The glycan-splitting PG hydrolase (PG:PG-6-muramyl transferase; 3, 10) responsible for their release seems to represent a deviation from the more traditionally encountered bacterial hexosaminidases.

MATERIALS AND METHODS

Bacteria. *Neisseria gonorrhoeae* RD_5 (kindly supplied by F. E. Young, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.) was the principal organism used. Other strains and their suppliers were 2686 (D. S. Kellogg, Jr., Center for Disease Control, Atlanta, Ga.), CS7 (S. A. Morse, University of Oregon Health Sciences Center, Portland, Oreg.), and 7122 (T. M. Buchanan, University of Washington School of Medicine, Seattle, Wash.). These strains were originally isolated from local infections, with the exception of 7122, which has the properties of isolates from disseminated gonococcal infections.

In early studies, bacteria were maintained by serial passage on GCBS medium minus V-C-N inhibitor as described (13, 14, 20), and colonies were typed according to the criteria of Kellogg et al. (11). After publication of a scheme (18) for typing gonococci according to color and opacity characteristics of colonies and degree of piliation, bacteria were also passed on clear solid medium (18) and classified by the criteria of Swanson (18). Experiments involving strain 2686 were performed before initiation of Swanson's typing method; the "transparent" character of this strain was determined retrospectively by typing original lots of frozen isolates used for experiments. For strains RD_5 and CS7, only nonpiliated (p^-) bacteria were examined; for 2686 and 7122, both piliated (p^+) and p^- gonococci were used.

Growth and radiolabeling of bacteria. Gonococci were harvested from agar plates after 18 h and were grown in SGM broth containing 0.4% (wt/vol) pyruvic acid (or glucose) and DL-[*G*-^{3}H]DAP (Amersham/Searle Corp., Arlington Heights, Ill.) and D-[1-^{14}C]$glcNH_2$ as described (13). Gonococci were labeled continuously over approximately three generations during exponential growth. For "pulse-chase" experiments, bacteria were washed three times by centrifugation (10,000 × *g*, 1 min) in warm (37°C) broth containing 0.1 mM "cold" carrier (DAP, $glcNH_2$, or both). This resulted in at least a 10^4-fold dilution of free unincorporated radioactivity in the culture medium. Washed bacteria were then inoculated into broth containing 0.1 mM "cold" carrier. Samples taken immediately after inoculation indicated that virtually all of the ^{3}H and ^{14}C in cells was present as trichloroacetic acid-precipitable (macromolecular) materials. In some experiments liquid medium ($LGCB^+$; 15) containing pyruvate as the chief carbon source replaced SGM broth because $LGCB^+$ broth allowed a greater yield of exponential-phase gonococci. There was no detectable difference between cells grown in SGM or $LGCB^+$ broth in (i) the rate of PG turnover, (ii) the gel filtration pattern of soluble PG fragments released into the medium, and (iii) the composition of isolated fractions with respect to labeled components. The pH of liquid media was 7.2 ± 0.1.

Preparation of intact PG and soluble PG fragments. Purified intact PG was prepared by the trichloroacetic acid-sodium dodecyl sulfate extraction procedure as previously described (8, 13). Radiolabeled, purified disaccharide peptide monomer (approximately 1,000 daltons) and various peptide-cross-linked oligomers were isolated by gel filtration after hen egg white lysozyme or *Chalaropsis* B muramidase (Miles Inc., Elkhart, Ind.) digestion of intact PG (15).

Soluble PG fragments (PGI, PGII, PGIIIa, PGIIIb), released from exponentially growing gonococci that had been pulsed with DL-[^{3}H]DAP and D-[^{14}C]$glcNH_2$, were isolated from culture supernatants as described (13). DAP is incorporated specifically into the peptide side-chain of isolated PG; $glcNH_2$ is incorporated into both sugars of the glycan, exclusively (13). Briefly, supernatants from chase cultures were harvested by centrifugation (12,000 × *g*, 5 min, 25°C), heated (65°C, 20 min), and concentrated by flash evaporation or lyophilization. The concentrate was filtered on connected columns of Sephadex G-50 and G-25, and fractions were eluted with 0.1 M LiCl. This procedure resolved three major peaks of soluble PG which were tentatively identified as forms of dimer (PGI), monomer (PGII), and a mixture (PGIII) of free peptide and free disaccharide. PGIII was subsequently fractionated by gel filtration on Sephadex G-15 to isolate the free peptide (PGIIIa) and free disaccharide (PGIIIb).

Reference PG fragments, C8 (detected on chromatograms using ninhydrin) and [^{3}H]DAP-labeled X′ and X, were kindly supplied by J. van Heijenoort, Orsay, France, and U. Schwarz, Tübingen, Germany, respectively. C8 and X′ (see Fig. 5) have been reported to be identical and to be a disaccharide tetrapeptide derivative of chemotype I PG containing a nonreducing 1,6-anhydro-*N*-acetylmuramic acid end (10, 19); X is the analogous disaccharide tripeptide lacking the D-alanine residue (see Fig. 5). Compounds X′ and X were derived from *Escherichia coli* PG by using a partially purified transglycosylase obtained from *E. coli* W7 (10). Analogous PG fragments were obtained from gonococci by digesting purified gonococcal PG with the *E. coli* transglycosylase preparation. The enzymatic digestion was performed in the laboratory of U. Schwarz, Tübingen, using the following procedure. Gonococcal, [^{14}C]$glcNH_2$-labeled PG (1.7 mg; 4×10^6 dpm/mg) was incubated overnight with the partially purified enzyme in a total of 1.5 ml [10 mM tris(hydroxymethyl)aminomethane-maleate, 10 mM $MgSO_4$, 0.2% Triton X-100, pH 6.0] at 37°C. The mixture was then boiled for 4 min and lyophilized. After transport, the digest was rehydrated, and the monomer fraction was isolated by gel filtration on the Sephadex G-50 and G-25 columns and desalted over Sephadex G-15.

Analytical methods. The degree of peptide substitution of the amino groups of radiolabeled DAP residues was determined using the dinitrophenylation procedure of Ghuysen et al. (5). The basis for this reaction is that un-cross-linked DAP residues (containing a free epsilon amino group) are converted to mono-dinitrophenyl DAP (monoDNP-DAP); cross-linked residues are unavailable for derivatization. Typically, 50 μl of desalted PG sample, containing [^{3}H]-

DAP-labeled peptide, was reacted with 50 μl of 2% 1-fluoro-2,4-dinitrobenzene (FDNB) in alcohol and 25 μl of 10% sodium bicarbonate. After incubation for 60 min at 60°C, the reaction was stopped by the addition of 50 μl of 2 N HCl. The sample was then evaporated to dryness and hydrolyzed in 6 N HCl for 16 h at 110°C. Washed hydrolysate was applied to 3MM paper and subjected to descending chromatography in solvent I or II (see below) to separate [^{3}H]DAP and DNP[^{3}H]DAP. To insure that the standard reaction contained excess FDNB when samples containing an unknown amount of free amino groups were assayed, samples were also processed using a threefold greater concentration of FDNB. The additional FDNB did not change significantly the ratio of free [^{3}H]DAP to DNP-[^{3}H]DAP. Free [^{3}H]DAP and DNP-[^{3}H]DAP were quantitated by cutting paper strips (1 to 2 cm) and counting radioactivity (see below). Peptide-cross-linked, bisdisaccharide peptide dimer and un-cross-linked monomer were isolated from muramidase digests of purified DAP-labeled gonococcal PG. These fractions were treated as above and served as positive controls. Greater than 90% of the labeled DAP in control monomer preparations was converted to monoDNP-[^{3}H]DAP, whereas 50% of the DAP in dimer standards was recovered as monDNP-[^{3}H]DAP and 50% was recovered as free [^{3}H]DAP. These results are expected for these standards since virtually all of the DAP residues in un-cross-linked monomer and half of the DAP residues in cross-linked dimer should have an amino group available for reaction with FDNB. Free DAP (detected with ninhydrin), monoDNP-DAP, and diDNP-DAP were run as standards for paper chromatography.

Reducing sugar analysis was performed by incubating soluble PG fragments in 0.5 M $NaBH_4$ (in 0.05 N NaOH) for 3 h in the dark at room temperature. The mixture was then adjusted to pH 3.5 with 1 M acetic acid. The product was dried, hydrolyzed (4 N HCl, 100°C, 4 h), washed in water, and subjected to paper chromatography in solvent III (below) to separate glucosamine, muramic acid, and their respective alcohols. Paper strips were cut and counted for radioactivity. Standard amino sugars and their alcohols (prepared by the above procedure) were detected by ninhydrin or by radioactivity determination (for labeled standards). Internal positive controls, i.e., $glcNH_2$- and muramic acid-labeled monomer prepared using *Chalaropsis* muramidase and having a reducing muramic acid end, were also run to insure that borohydride reduction to muramicitol could occur in the experimental sample.

Samples of PG monomers were treated with 0.05 N NaOH at 37°C under conditions known to cause the β-elimination of the lactyl peptide moiety from disaccharide peptides which contain a reducing muramic acid residue (21). The residual glycan resulting from the β-elimination of disaccharide peptide monomer is thus a lactyl-less disaccharide. An internal standard, the PG monomer prepared using *Chalaropsis* B muramidase, was also treated in a similar fashion. Products of these reactions were analyzed by paper chromatography in solvent III.

PG monomers (labeled in both amino sugars of the glycan) were incubated at 37°C for 20 h in 100 μl (final volume) of 0.05 M citrate buffer (pH 4.6) containing 20 μg of a mixed glycosidase preparation from *Charonia lampas* (Miles Inc., Elkhart, Ind.). The major component of this preparation is a β-*N*-acetylglucosaminidase activity. This activity causes the release of a free reducing *N*-acetylglucosamine from the end of β-1 → 4-linked disaccharides that bears a free 4-hydroxy residue. Samples of the digests were subjected to borohydride treatment (or held as untreated controls) and analyzed for their content of $glcNH_2$, muramic acid, and their respective alcohols (see above).

Descending paper chromatography on Whatman 3MM paper was performed using: solvent I, *n*-butanol–acetic acid–water (5:1:2, vol/vol); solvent II (4:1:5, upper phase, vol/vol); and solvent III (3:1:1, vol/vol).

Electrophoresis was carried out on Whatmann 3MM paper using a flat-plate high-voltage electrophoresis unit (Savant Instruments, Hickeville, N.Y.). Solvents were formic acid-water (8:300, vol/vol), pH 2.1; acetic acid-pyridine-water (23:6:976, vol/vol), pH 4.0; acetic acid-pyridine-water (2:4:1,000, vol/vol), pH 5.1; and acetic acid-pyridine-water (8:200:2,800, vol/vol), pH 6.4. Samples were run typically at 40 V/cm for 120 min.

Determination of D- and L-alanine. The relative amounts of D- and L-alanine in purified intact PG and in isolated PG fragments released into the medium during growth was determined using D-amino acid oxidase. To obtain PG that contained ^{3}H in both D- and L-alanine, gonococci were grown in LGCB$^+$ containing 20 μCi of L-[2,3-^{3}H]alanine (ICN) per ml. Samples of [^{3}H]alanine-labeled PG were hydrolyzed (4 N HCl, 4 h, 110°C) and washed in distilled water by flash evaporation, and the pH was adjusted to 8.3 with KOH. Assay mixtures contained 50 μl of 2 M tris(hydroxymethyl)aminomethane buffer (pH 8.3), 25 μl of D-amino acid oxidase (2 U; Boehringer Mannheim), 20 μl of catalase (4,000 U; Sigma), and the experimental sample (in water). The volume was adjusted to 200 μl with water. The reaction mixture was incubated for 60 min at 37°C and subjected to electrophoresis (40 V/cm, 75 min) on Whatmann 3MM paper at pH 2.1 (formic acid). This procedure separates alanine from its neutral degradation product (pyruvic acid). As an internal control to show that D-amino acid oxidase was active and specific, D-[^{14}C]alanine and L-[^{14}C]alanine were added separately to additional samples of the experimental samples and treated as described above. Under these conditions D-[^{14}C]alanine, but not L-[^{14}C]alanine, should be converted to a product (pyruvate) which remains at the origin after high-voltage electrophoresis.

In purified intact PG, the ratio of D-[^{3}H]alanine disintegrations per minute to L-[^{3}H]alanine disintegrations per minute was approximately 0.85 (Table 1); obviously the bacteria racemized some of the labeled L-alanine to the D-stereoisomer. The above ratio of D-alanine to L-alanine is consistent with previous amino acid analysis of intact PG (15). Based on these studies (15) and assuming 1 mol of L-alanine per monomer unit, the ratio of D-alanine to L-alanine was approximately 0.8.

Determination of radioactivity. Radioactivity measurements of samples (dried on paper) by using a toluene cocktail containing NCS (Amersham/Searle

TABLE 1. *D-[^{3}H]- and L-[^{3}H]alanine in PG fragments and in the intact, parental PG*[a]

PG (or control)	D-Alanine dpm (% of total alanine dpm)	L-Alanine dpm (% of total alanine dpm)
D-[^{14}C]alanine (internal control)	95	5
L-[^{14}C]alanine (internal control)	0	100
Intact	46	54
PGII (monomer)	15	85
PGIIIa (free peptide)	33	67
Peak at $K_D = 1$ (free alanine)	100	0

[a] Gonococci were grown in the presence of L-[2,3-^{3}H]alanine. Samples were acid hydrolyzed, and the amounts of labeled D- and L-alanine were determined using D-amino acid oxidase.

Corp.), and of water-soluble materials by using Scintisol (Isolab, Inc., Akron, Ohio), were performed as described (13). Data were corrected for overlap and quench using calibration curves determined by external standardization, and radioactivity was reported as disintegrations per minute.

RESULTS

Release of PG fragments in various strains. Previous results from this laboratory (13) indicated that exponentially growing gonococci (strain RD_5, p^-, transparent [tr]) released at least four major forms of low-molecular-weight PG fragments as a result of PG turnover. These were tentatively identified as forms of bisdisaccharide peptide dimer (PGI), disaccharide peptide monomer (PGII), free peptide (PGIIIa), and free disaccharide (PGIIIb). Small amounts of soluble PG fragments with apparent molecular weights corresponding to disaccharide peptide trimers and tetramers were also detected. Of these products, the disaccharide peptide monomer, PGII, was consistently present in the highest amount.

To determine whether this pattern of PG release was characteristic of gonococci, several different strains were employed in the standard pulse-chase experiment, and the supernatants were analyzed by gel filtration for radiolabeled PG fragments. The release of [^{14}C]glcNH_2-labeled PG fragments (Fig. 1) by strain 2686 T4 (p^-, tr) is typical of other strains and colony types tested. These included RD_5 (p^-, opaque [op]), 2686 (p^+, tr), CS7 (p^-, tr), 7122 (p^+, tr), and 7122 (p^-, tr). In no case did the types of soluble PG fragments generated by these strains appear to differ qualitatively from those released by the prototype strain, RD_5 (p^-, tr) (13). RD_5 (p^-, op), the only opaque strain tested, appeared to release more of the larger-sized fragments, e.g., PGI, relative to monomer (PGII), than the transparent strains. Whether this is a general feature of PG turnover in opaque bacteria is currently under consideration. Otherwise, the distribution of the various fragments was similar for each of the strains tested. However, the method of analysis would not necessarily detect differences in the absolute amounts of soluble PG generated, because it could not be assumed that the specific activity of the labeled compounds in PG of various strains was identical.

The data for strain 2686 (Fig. 1) also include an additional PG fragment, intermediate in size between PGII and PGI, that was not noted previously (13). This peak was also present in the supernatants of RD_5 and other gonococci, but was sometimes not easily resolved from PGI. In experiments employing [^{3}H]DAP- and [^{14}C]-glcNH_2-labeled gonococci, this new peak was found to be enriched for the ^{14}C-labeled glycan compared to PGI and PGII. Further work is needed to identify this additional PG product. At present, the data are consistent with the possibility that it represents the glycan-linked tetrasaccharide, *N*-acetylglucosaminyl-*N*-acetylmuramyl-*N*-acetylglucosaminyl-*N*-acetylmuramic acid, which contains a peptide-side chain on only one of the two disaccharide units.

Radiochemical analysis of the soluble PG fragments. Because the turnover and release of soluble PG fragments seemed to be a general characteristic of gonococci, the next objective was to identify the chemical structure of the various fragments that were released into the medium. Because it seemed unrealistic to purify these fragments to chemical homogeneity in the presence of the many organic components, e.g., peptides, with similar properties in the rich undefined medium, strictly chemical means of identification were not attempted. A variety of standard radiochemical methods have traditionally been useful in characterizing PG fragments. Some of these methods, controlled with reference PG compounds, were used to identify the gonococcal PG fragments. Soluble PG products released by strain RD_5 were used throughout these experiments.

Free amino group analysis of [^{3}H]DAP residues in the various PG fragments isolated from culture supernatants was consistent with the hypothesis that PGII and PGIIIa were un-cross-linked disaccharide peptide monomer(s) and free peptide(s), respectively. As expected for un-cross-linked products, it was found (Fig. 2) that the majority (83 and 93%) of labeled DAP residues in PGII and PGIIIa, respectively, were free and available to react with FDNB and thus be derivatized to monoDNP-[^{3}H]DAP. By comparison, a reference PG monomer (un-cross-linked monomer obtained from gonococcal PG and containing a mixture of disaccharide tri- and tetra-

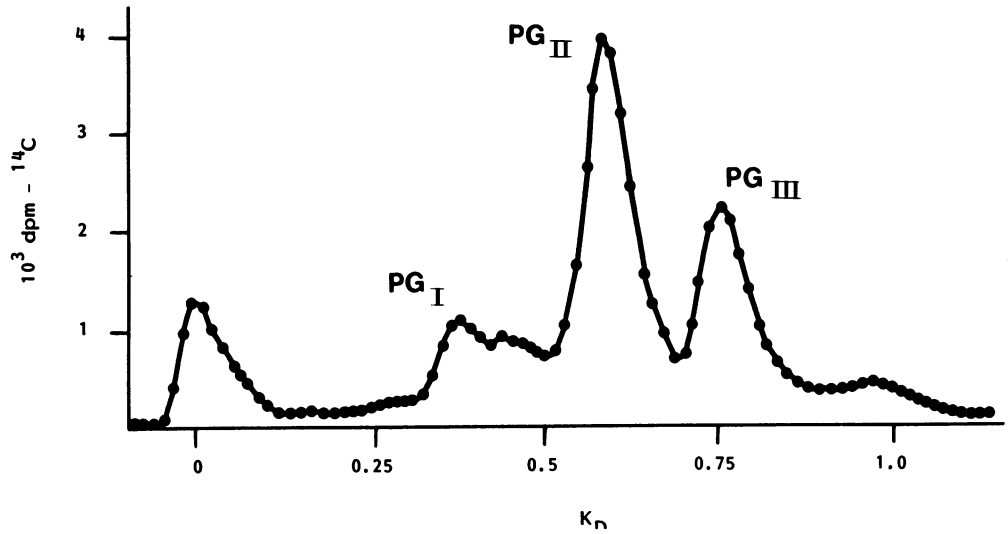

FIG. 1. *Gel filtration on connected columns of Sephadex G-50-25 of the supernatant of exponential strain 2686 (p^-, tr) grown in the presence of D-[1-^{14}C]glcNH$_2$.*

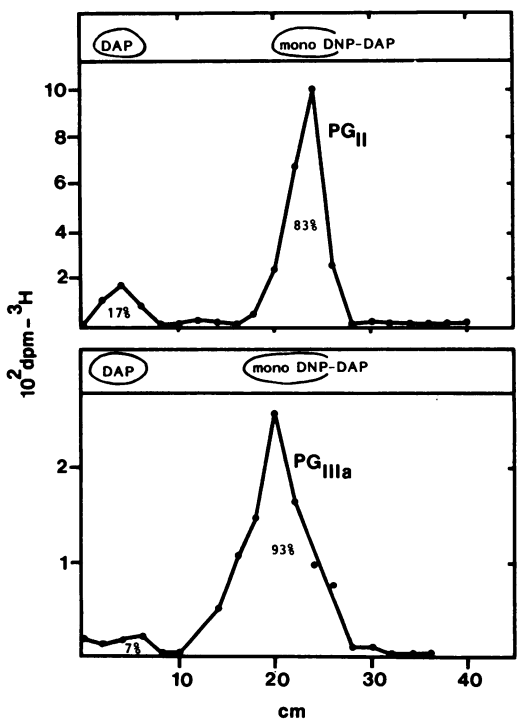

FIG. 2. *Free amino group analysis, using FDNB, of [^{3}H]DAP residues in soluble PG fragments. Desalted samples were treated with FDNB, acid hydrolyzed, and chromatographed on paper in solvent I to separate monoDNP-[^{3}H]DAP and free [^{3}H]DAP. The migrations of reference compounds are indicated. The amount of each product is expressed as percent of total ^{3}H recovered.*

peptides; 15) yielded approximately 90% monoDNP-[^{3}H]DAP in control experiments.

FDNB analysis of the presumed peptide-cross-linked dimer (PGI) consistently yielded approximately 65% monoDNP-[^{3}H]DAP (data not shown). This is significantly different from the 1:1 ratio of free DAP to DNP-DAP obtained for authentic peptide-cross-linked dimer. This difference was thought to be accounted for by the presence of small amounts of glycan-linked dimer and possibly some contaminating material from the newly recognized peak between PGI and PGII. These substances would have molecular weights identical, or similar, to peptide-cross-linked dimer and could contribute a greater than expected level of [^{3}H]DAP residues available for conversion to DNP-[^{3}H]DAP.

Further studies concentrated on radiochemical characterization and identification of the predominant, monomer fraction (PGII). For these experiments a "mixed-monomers" sample, which contained its own internal control, was employed. Mixed monomers were prepared by mixing a predetermined amount of radioactivity from (i) chase culture supernatants obtained from gonococci grown in the presence of [^{3}H]-glcNH$_2$, and (ii) *Chalaropsis* muramidase digest of purified PG isolated from gonococci grown in [^{14}C]glcNH$_2$. The mixture was filtered on the Sephadex G-50-25 columns. Mixed monomers were isolated by pooling the fractions as indicated in Fig. 3; this sample thus contained [^{3}H] glcNH$_2$-labeled monomer released from growing bacteria and [^{14}C]glcNH$_2$-labeled *Chalaropsis* monomer. As indicated previously, label added

as $glcNH_2$ was present as both $glcNH_2$ and muramic acid in both of the PG monomers that constitute mixed monomers.

The mixed-monomers sample was subjected to the borohydride reaction to assess which (if either) of the amino sugars in the experimental monomer was present as a reducing end. After acid hydrolysis, the ^{14}C-labeled control monomer (obtained by *Chalaropsis* digestion) yielded almost exclusively $glcNH_2$ and the reduced form (muramicitol) of muramic acid (Fig. 4). This is expected for a digestion product of a known muramidase and serves as a positive internal control for the reaction, i.e., reduction to the alcohol occurred in the sample. Unexpectedly, the borohydride treatment did not result in reduction to an alcohol in the ^3H-labeled chase monomer (PGII); essentially only [^3H]$glcNH_2$ and [^3H]muramic acid were found (Fig. 4), identical to controls (data not shown) which were hydrolyzed without prior borohydride treatment. This suggested that the experimental ^3H-labeled monomer contained neither a reducing $glcNH_2$ nor a reducing muramic acid. It should be pointed out that the minor peak at about 6 cm (Fig. 4) is not a product of borohydride reduction. This product, containing label derived from both the ^{14}C- and ^3H-monomers, was also present in control hydrolysates not treated with borohydride. It probably represents a breakdown product of one or both of the amino sugars, or incompletely degraded disaccharide. Although unlikely, it could not be completely ruled out at this time that this minor peak represented an additional amino sugar in the glycan moiety of gonococcal PG.

That the ^3H-monomer released from gonococci lacked a reducing end was confirmed by subjecting mixed monomers to treatment with mild alkali, i.e., the β-elimination reaction (21). This reaction causes the degradation of disaccharide peptide monomers into lactyl-less disaccharides and lactyl-peptides only when the muramic acid residue is at a reducing end (21). As expected, the *Chalaropsis* monomer was completely degraded by NaOH; the experimental

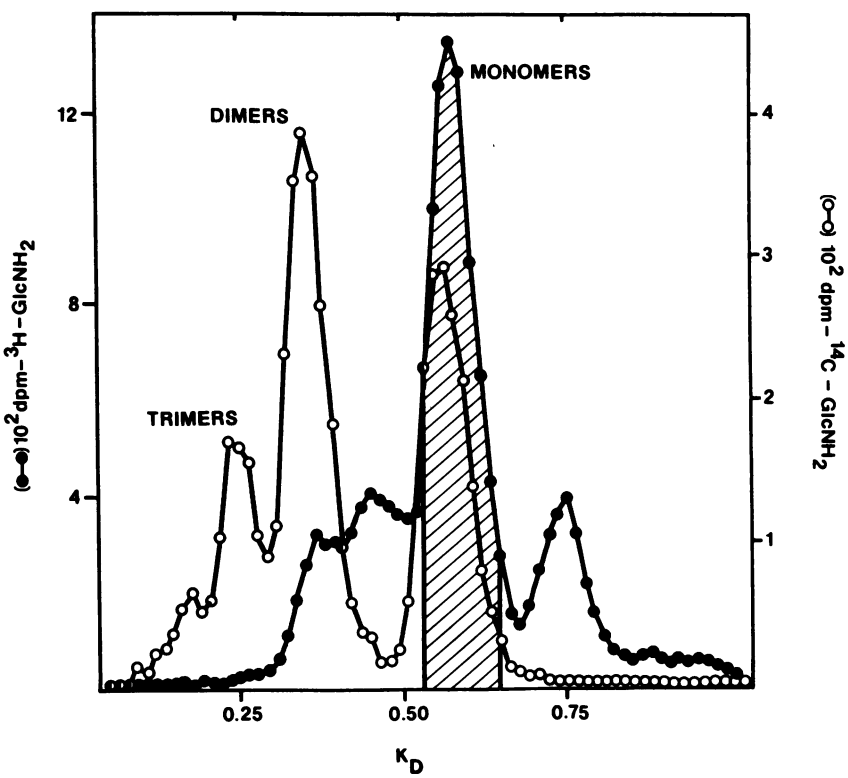

FIG. 3. *Gel filtration on Sephadex G-50–25 of a mixture of (i) the supernatant of exponential RD_5 grown in the presence of D-[^3H]$glcNH_2$ (●); and (ii) Chalaropsis B muramidase digest of D-[^{14}C]$glcNH_2$-labeled PG purified from strain RD_5 (○). The sample, mixed monomers, was formed by pooling fractions as indicated by the shading. The mixed-monomers sample thus contains ^3H-labeled monomer (PGII), released by growing gonococci, and ^{14}C-labeled Chalaropsis monomer. The latter contains a reducing muramyl residue and serves as an internal control for subsequent experiments.*

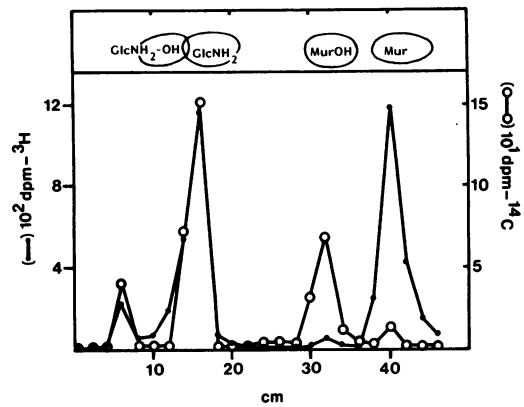

FIG. 4. *Paper chromatography in solvent III of the products after borohydride treatment of the mixed-monomers sample, i.e., [^{3}H]glcNH$_2$-labeled monomer (PGII) released by growing gonococci (●) plus [^{14}C]glcNH$_2$-labeled Chalaropsis monomer* (○). *Samples were treated with NaBH$_4$ and hydrolyzed before paper chromatography. Reference compounds, i.e., amino sugars (glcNH$_2$ and muramic acid) and their alcohols (glcNH$_2$-OH and MurOH), were detected using ninhydrin. The peak containing both labels at about 6 cm is not a product of borohydride reduction since it was also present in control samples that were hydrolyzed and chromatographed without prior borohydride treatment.*

monomer in the same tube, however, was unaffected.

In borohydride experiments, the free disaccharide (PGIIIb), similar to monomer (PGII), yielded almost exclusively glcNH$_2$ and muramic acid; the corresponding alcohols were not detected. The free disaccharide also yielded the relatively slow-moving, minor peak present in PGII (Fig. 4).

To our knowledge, the only precedent for a glycan-splitting PG hydrolase that releases nonreducing PG fragments with characteristics similar to those detected in gonococci is an *E. coli* murein transglycosylase. This activity was suggested (12) and subsequently investigated independently by two laboratories (10, 19). The novel feature of the products released from intact PG by this enzyme is the presence of a 1,6-anhydro-*N*-acetylmuramic residue at what would normally be the reducing end of the molecule (Fig. 5). Other than this feature, the structures of the various PG fragments liberated by the transglycosylase are identical to those liberated by "lysozyme-like" bacterial *N*-acetylmuramidases. The major product liberated by the action of *E. coli* transglycosylase on *E. coli* PG is a monomer fraction containing a mixture of disaccharide tri- and tetrapeptides (10, 19). These are nonreducing analogs of compounds C5 and C6, respectively (12, 23). Originally several terms were used for the transglycosylase-derived monomers. It is now known that C8, CA, and X′ are identical disaccharide tetrapeptides, and C7 and X are identical disaccharide tripeptides (10, 19). Their basic structure is compared to that of corresponding monomers obtained by *Charlaropsis* muramidase treatment (Fig. 5).

The ^{3}H-labeled monomer (PGII) released by gonococci was compared to reference PG compounds on the basis of K_D values, migration on paper in various solvents, and electrophoretic mobility. Reference compounds were (i) [^{14}C]-glcNH$_2$-labeled *Chalaropsis* monomer obtained from purified gonococcal PG, (ii) C8, (iii) [^{3}H]-DAP-labeled X and X′, and (iv) [^{14}C]glcNH$_2$-labeled "transglycosylase monomer" obtained from gonococcal PG by using the *E. coli* transglycosylase.

Paper chromatography of the PG fragments in solvent II (Fig. 6) indicated that the ^{3}H-labeled chase monomer (PGII) contained two components. The migration of the major (80 to 85% of the total disintegrations per minute) component was identical to that of compound X and to that of the minor component of the transglycosylase monomer. The minor, faster-moving peak corresponded to C8, X′, and the major component of the transglycosylase monomer.

Chalaropsis monomer was found in this experiment (Fig. 6) to consist mainly of three peaks. The major component (the one with the greatest mobility of the three) had an $R_{X'}$ identical to that reported for C6, the reducing disaccharide tetrapeptide obtained from *E. coli* PG (10, 23). The slowest-moving peak had an $R_{X'}$ equal to that reported for C5, the corresponding disaccharide tripeptide (10, 23). The nature of the other *Chalaropsis* monomer component is unknown. It was not resolved from the major component when chromatograms were run for shorter periods of time. A very small amount of ^{14}C in *Chalaropsis* monomer ran well ahead of the other components, with a migration identical to that of compound X.

The chase monomer (PGII) and all of the reference monomers had virtually identical electrophoretic mobilities at pH 2.1 and 5.1 (Fig. 7) and at pH 4.0. These data suggested the absence of significant peptide amidation in PGII. Under the conditions used, high-voltage electrophoresis (Fig. 7) did not resolve multiple monomer components in the manner achieved by paper chromatography (Fig. 6).

The above studies demonstrating (i) comigration of PGII components with certain reference PG compounds upon paper chromatography, high-voltage electrophoresis, and gel filtration, (ii) the lack of reducing sugars, and (iii) the presence of un-cross-linked DAP residues al-

most exclusively, suggested that monomer (PGII) is a mixture of *N*-acetylglucosaminyl-β-1 $\rightarrow$ 4-1,6-anhydro-*N*-acetylmuramyl peptides, the major component of which is the disaccharide tripeptide. To confirm the position of the amino sugars in the disaccharide, a mixed-monomers sample was treated with the *C. lampas* glycosidase. This was followed by borohydride treatment, acid hydrolysis, and paper chromatography to quantitate the amino sugars and their alcohols. It was found that ^{14}C-*Chalaropsis* monomer yielded both glucosaminitol and muramicitol (Fig. 8). ^{3}H-PGII yielded mainly glucosaminitol and muramic acid; relatively little muramicitol was detected (Fig. 8). This confirms that the glcNH$_2$ residue in the disaccharide of PGII contributes the number 1 carbon to the glycosidic linkage.

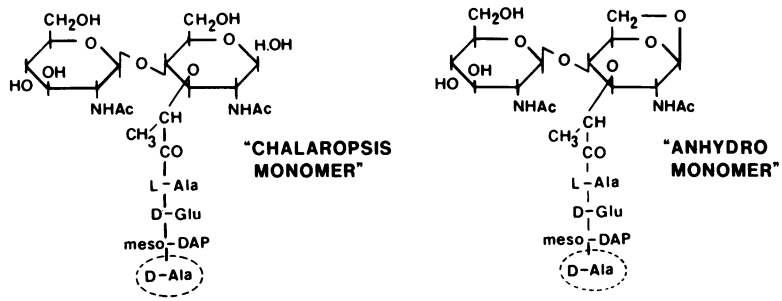

FIG. 5. *Comparison of the basic structures of disaccharide tetrapeptide monomers containing a reducing N-acetylmuramic acid end (Chalaropsis monomer) and a 1,6-anhydro-N-acetylmuramic acid, nonreducing end (anhydro monomer). Chalaropsis monomer, termed C6 elsewhere (10, 12, 19), is an expected product of the digestion of gonococcal PG with a muramidase. Anhydro monomer, termed C8, CA, and X' elsewhere (10, 19), is an expected product of the digestion of gonococcal PG with the E. coli transglycosylase (10). Digestion of gonococcal PG with either enzyme also gives rise to the corresponding disaccharide tripeptide monomers, lacking the* D*-alanine residues (circled). The anhydro-muramyl-containing disaccharide tripeptide has also been termed C7 and X (10, 19); the reducing disaccharide tripeptide has been referred to as C5 (10, 12, 19).*

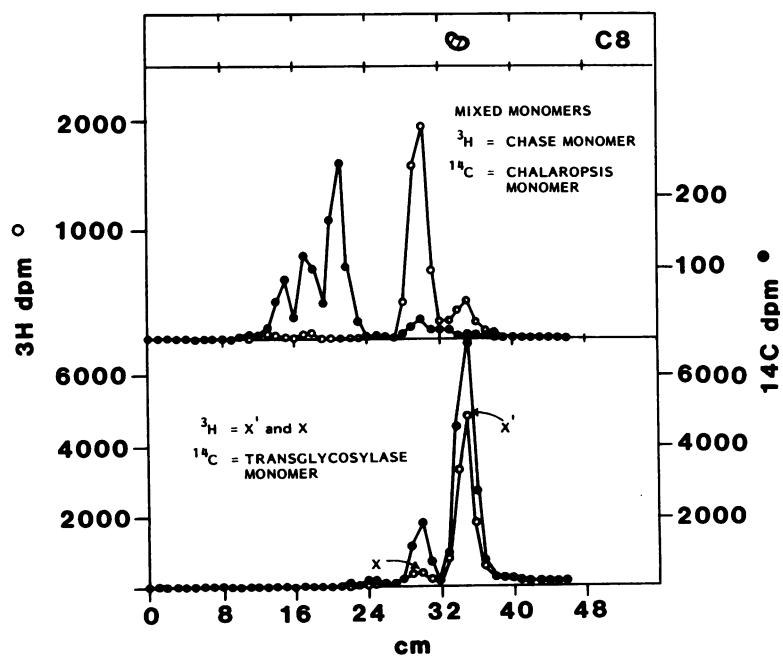

FIG. 6. *Paper chromatography in solvent II (upper phase) of the mixed-monomers sample, i.e., ^{3}H-chase monomer (PGII) released by growing gonococci plus ^{14}C-Chalaropsis monomer obtained by digestion of gonococcal PG with Chalaropsis muramidase, and several reference PG fragments. Compound C8 was detected by using ninhydrin.*

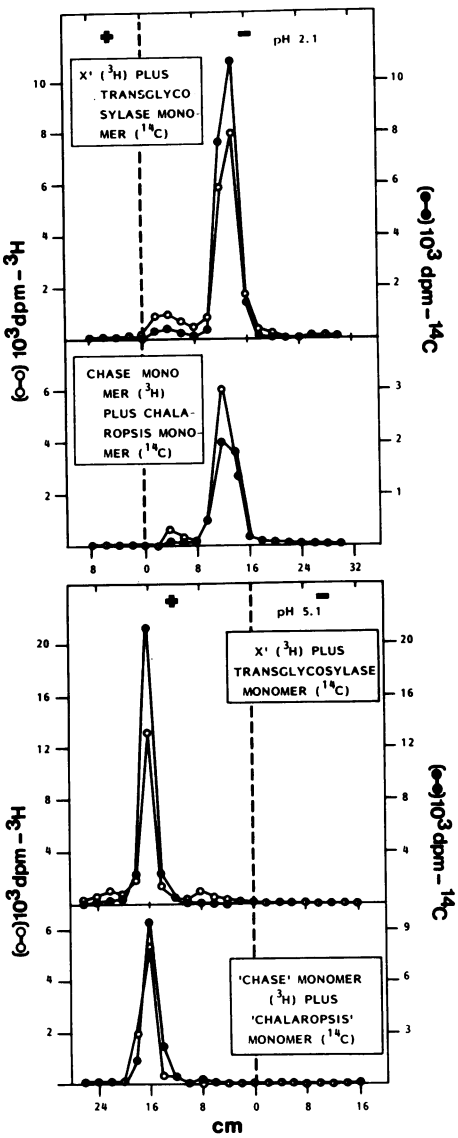

FIG. 7. *High-voltage electrophoresis at pH 2.1 and pH 5.1 of ^{3}H-chase monomer (PGII), released by growing gonococci, and of several reference PG fragments.*

As an independent determination of the nature of peptide chains, PG fragments released by growing bacteria and the homologous intact PG were analyzed for their content of labeled D- and L-alanine. In these experiments, gonococci were grown in the presence of L-[2,3-^{3}H]-alanine. PG fragments released into the supernatant were isolated by gel filtration, and intact PG was purified as before. The rationale for this experiment was that if some of the released fragments were relatively enriched for disaccharide tripeptide (as suggested by Fig. 6) compared to the parental PG from which they were derived, then the former should contain relatively smaller amounts of D-alanine. It was crucial that ^{3}H added as L-alanine be racemized to the D-stereoisomer and incorporated into PG as D-[^{3}H]alanine. This was the case, since 46% of the [^{3}H]alanine was present in intact PG as D-alanine (Table 1). Furthermore, the specific activity of ^{3}H in D- and L-alanine appeared to be approximately the same because, based on amino acid analysis of intact PG (15) and assuming 1 molecule of L-alanine per monomer unit, it is estimated that 44% of the alanine residues are in the D-form. From these amounts of D- and L-alanine (and assuming no significant levels of disaccharide pentapeptides) it was calculated that approximately 80% of the monomer units in intact PG are disaccharide tetrapeptides. The level of D-[^{3}H]alanine in chase monomer (PGII) was considerably less than in the parental PG (Table 1). This confirmed the paper chromatography experiments (Fig. 6) which showed that the released monomer consisted mainly of disaccharide tripeptides. The level (15%) of D-alanine in monomer (PGII) is equivalent to 20% disaccharide tetrapeptide molecules and 80% disaccharide tripeptide.

Free peptide (PGIIIa), however, contained relatively more D-alanine (and hence relatively more tetrapeptide) than monomer (PGII). This level (33%) of D-alanine in PGIIIa is equivalent to approximately equal molar amounts of free tetrapeptide and free tripeptide. Interestingly, the previously described large peak of free [^{3}H]alanine eluting at $K_D = 1.0$ on the Sephadex G-50-25 columns (13) was found to be D-alanine, exclusively. The release of free D-alanine from growing gonococci probably reflects the combined action of DD- and DL-carboxypeptidase activities which remove the terminal and subterminal D-alanine, respectively, from disaccharide pentapeptide fragments in the macromolecular PG (4). This is consistent with the finding of PG fragments containing tri- and tetrapeptides. A gonococcal DD-carboxypeptidase activity has been described previously (2).

In summary, the structure of the major PG fragment released from growing gonococci appears to be *N*-acetylglucosaminyl-β-1 $\rightarrow$ 4-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP. Additional components appear to be the analogous disaccharide tetrapeptide, free disaccharide, free tetra- and tripeptide, and cross-linked disaccharide peptide dimers.

DISCUSSION

Growing gonococci release soluble PG fragments into the growth medium concurrent with the extensive turnover of macromolecular PG

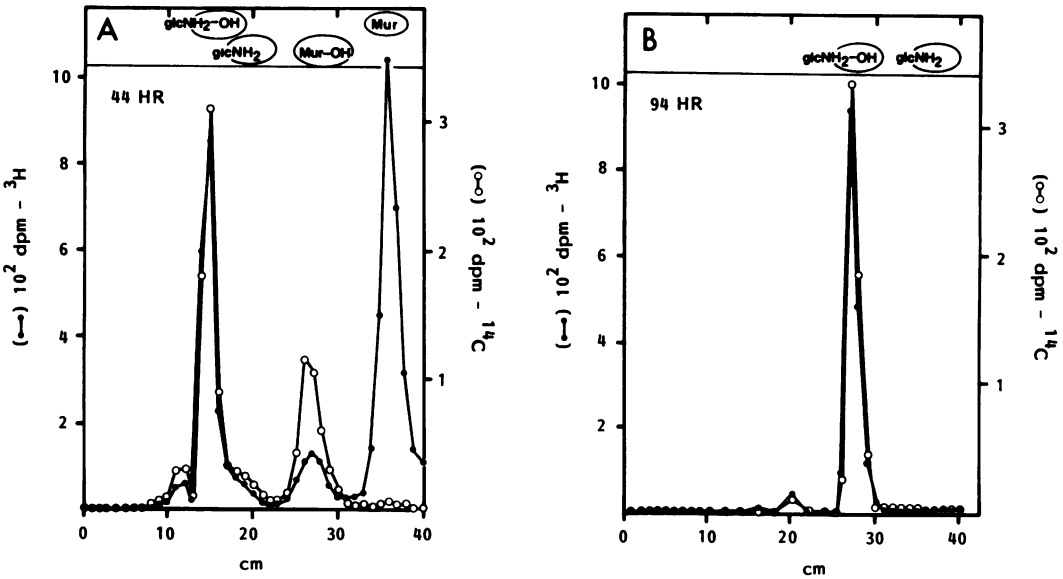

FIG. 8. *Paper chromatography of C. lampas glycosidase-treated mixed-monomers sample run for 44 h (A) and 94 h (B) in solvent III. The products obtained after glycosidase treatment were subjected to borohydride reduction and were acid hydrolyzed before paper chromatography. Reference compounds were as described in Fig. 4.*

(13). Initial characterization (13) of radiolabeled soluble fragments suggested that these were forms of (i) peptide-cross-linked bisdisaccharide peptide dimers, (ii) un-cross-linked disaccharide peptide monomers (the major component), (iii) free peptide, and (iv) free disaccharide. Current studies further characterized these PG products.

A rather surprising observation was that all of the glycan-containing fragments found in gonococcal supernatants appeared to possess nonreducing (1,6-anhydro) muramyl ends. This reflects the action of an unusual hexosaminidase (transglycosylase) activity, apparently identical to PG:PG-6-muramyl transferase previously detected in *E. coli* (3, 10). In *E. coli* the transglycosylase accounts for only a small amount of the total glycan-splitting, PG hydrolase activity. In contrast, our data indicate that growing gonococci release PG fragments with nonreducing ends, exclusively. It is possible, therefore, that these bacteria could contain the transglycosylase as their principal (if not only) glycan-splitting autolysin. Alternatively, gonococci could possess additional glycan-splitting activity which opens up reducing ends in the intact wall but which is not responsible for PG turnover and for the accompanying release of PG fragments into the medium. Such an activity would not be detected in our experiments because we examined supernatants only for evidence of PG hydrolase activity. In reference to this point, Hebeler and Young (9) characterized PG hydrolases in a Triton X-100 and NaCl extract of whole gonococci. Their study, which employed purified PG as substrate for the crude enzyme preparation, clearly demonstrated the presence of an L-alanine amidase. However, their enzyme preparation released very little reducing power from PG, which was interpreted as indicating the absence of significant glucosaminidase and muramidase activities in the autolysin-containing extract (9). It seems possible that a gonococcal transglycosylase could have been present in their extract, because the methods used (9) would not have detected this unusual type of PG hydrolase that releases PG fragments lacking a reducing end. In addition, Hebeler and Young (8) also determined the glycan chain length of gonococcal PG by using a well-established method based on reducing group analysis, and they reported that the average chain length was between 80 and 110 disaccharide units. In light of our findings suggesting that glycan chains in native PG could terminate in nonreducing ends, this parameter may be in need of reevaluation using techniques that evaluate chain length on different criteria.

The physiological role of PG turnover in gonococci and of the gonococcal transglycosylase responsible for release of nonreducing PG derivatives is unknown. Although there is only little precedent for either of these activities among gram-negative bacteria, most studies have employed gram-negative bacilli. Indeed, the control of cell shape, surface growth, and cell division in

gram-negative cocci has been virtually neglected as compared to gram-negative rods and to gram-positive bacteria. Thus it may be interesting to survey other gram-negative cocci for PG turnover and for transglycosylase activity to determine whether these properties are a general feature of gram-negative coccal growth. It also seems important to know whether gonococci (and other gram-negative cocci) also possess the more frequently encountered glucosaminidase and muramidase activities.

The major peak of soluble PG isolated by gel filtration was found to be the anhydro-muramyl-containing disaccharide peptide monomer. This peak typically accounted for greater than 50% of the total PG released by growing gonococci. Subsequent analysis of isolated monomer (Fig. 6 and Table 1) indicated that this fraction consisted of two components. Approximately 80% of the monomer fraction was identified as the disaccharide tripeptide, *N*-acetylglucosaminyl-β-1 $\rightarrow$ 4-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP; the remainder was the analogous disaccharide tetrapeptide containing a C-terminal D-alanine (see Fig. 5).

It is curious that whereas 80% of the released monomer (PGII) was found to be the disaccharide tripeptide, only a minor proportion (20%) of the un-cross-linked monomers in the intact parental PG was the disaccharide tripeptide. This apparent enrichment of disaccharide tripeptide in released monomer over the level present in the intact envelope might reflect (i) a relatively high affinity of the gonococcal hexosaminidase for tripeptide-substituted residues in intact PG, (ii) localization of the hexosaminidase in domains already enriched for tripeptide-containing glycan by the action of DL-carboxypeptidase, or (iii) release of DL-carboxypeptidase activity from cells and the subsequent conversion of disaccharide tetra- to tripeptide in the culture medium. In regard to the latter possibility, it should be noted that we have examined gonococcal supernatants for several kinds of enzymatic activities and, to date, have detected no PG hydrolase activity. Based on current (Fig. 6 and Table 1) and previous data (13, 15), it seems clear that gonococci possess a DL-carboxypeptidase activity (whether this activity is released from growing cells or not), in addition to DD-carboxypeptidase activity (2).

We have previously studied the extent of peptide cross-linking in native gonococcal PG and reported (15) that approximately 40% of the available DAP residues are substituted by peptide-cross-linking bonds. In contrast, the extent of cross-linking among soluble PG fragments (the majority of which were un-cross-linked monomers and free peptides) released from growing gonococci was only about 10%. It is not clear whether this large net decrease in cross-linking actually reflects the selective release of un-cross-linked fragments by native gonococcal PG hydrolases, or simply relates to the probability of breaking the required number of glycosidic linkages needed to completely excise the various PG fragments from the native PG matrix.

The principal rationale for identifying the soluble PG fragments released by growing gonococci was that these extracellular products might influence the outcome of host-gonococcus interactions (8, 13). Given the opportunity to interact with host cells and molecules, there is growing evidence that various PG derivatives possess an overall propensity to augment the activity of cells associated with the immune response and with inflammatory reactions (1, 17). It is perhaps significant that growing gonococci not only release large amounts of soluble PG fragments (an unusual characteristic among gram-negative bacteria studied to date), but also that the products appear to be rather unusual compared to those typically derived from bacteria. The novelty of these naturally occurring PG products relates to the presence of the nonreducing (anhydro-muramyl) ends; analogous products derived from PG using commercial lysozymes or bacterial hexosaminidases typically possess muramic acid or $glcNH_2$ as a reducing end. We speculate that the presence of the anhydro-muramyl ends could be biologically relevant since this type of modification, resulting in a less hydrophilic site in the glycan, might potentiate the interaction with host cell membranes. It is known that rather subtle changes (including those involving the sugar moieties) in the structure of PG derivatives can drastically alter biological activity (1). To our knowledge, anhydro-muramyl analogs have not yet been exploited. This class of naturally occurring derivatives, which might represent a unique aspect of gonococci-host interactions, is an interesting candidate for future biological testing.

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