INFECTION AND IMMUNITY, June 1980, p. 867-875
0019-9567/80/06-0867/09$02.00/0

Vol. 28, No. 3

Extent of Peptide Cross-Linking in the Peptidoglycan of *Neisseria gonorrhoeae*

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The extent of peptide cross-linking in peptidoglycan (PG) isolated from various strains of *Neisseria gonorrhoeae* was examined. Purified PG, specifically labeled in the peptide moiety with [^{3}H]diaminopimelic acid (DAP) and labeled in the glycan with [^{14}C]glucosamine and [^{14}C]muramic acid, was digested completely with Chalaropsis B muramidase. Gel filtration of the digest on connected columns of Sephadex G-50 and G-25 revealed four well-defined peaks corresponding to soluble PG fragments and containing a constant ratio of ^{3}H to ^{14}C. On the basis of (i) K_D values, (ii) amino acid composition, (iii) free amino group analysis of [^{3}H]DAP residues, (iv) borohydride reduction, (v) the β-elimination reaction, (vi) high-voltage electrophoresis, and (vii) paper chromatography in various solvents, the PG fragments were identified as un-cross-linked disaccharide peptide monomer, typical of chemotype I PG, and the corresponding peptide cross-linked dimers, trimers, and tetramers. The percent cross-linking of PG basically reflects the percentage of DAP residues that are involved in peptide cross-linking bonds. This value was estimated from the distribution of labeled fragments that resulted from the enzymatic digestion of PG and was confirmed by the analysis of free amino groups in [^{3}H]DAP of intact PG. Although there were subtle, strain- and medium-dependent differences in percent cross-linking, these values varied only over a relatively narrow range (36 to 44%). The percent cross-linking of PG in the prototype strain, RD_5, grown in a standard gonococcal medium ($LGCB^+$) was 41.0 ± 2.0%. This is a relatively high degree of peptide cross-linking for a gram-negative bacterium. We also confirmed previous observations that the extent of PG cross-linking among isogenic gonococci was higher in strains, e.g., FA140 and FA136, carrying loci that govern increased resistance to multiple drugs.

During the past few years the importance of peptidoglycan (PG) as a biological effector molecule has become increasingly apparent. In particular, intact PG or soluble derivatives of PG seem to have a propensity to augment the activities of cells and molecules associated with the immune response and with inflammation (35). Confirmed activities include adjuvanticity (1, 3), mitogenicity (9, 37), consumption of complement (15, 44), and arthritogenicity (5, 27).

This laboratory is examining the hypothesis that the interaction between the PG of *Neisseria gonorrhoeae* and the components of the inflammatory response might influence the consequences of gonococcal infection. Gonococci are unusual among gram-negative bacteria in that they exhibit extensive turnover of PG during exponential growth (14, 19, 42) and release the soluble products (principally a disaccharide peptide monomer) into the medium (30). Gonococci also possess a great tendency to undergo autolysis in buffer or in medium depleted of certain nutrients (10, 18).

The composition of native, gonococcal PG appears to be chemotype I (34), typical of most gram-negative bacteria. Gonococcal PG contains approximately equimolar amounts of muramic acid, glucosamine ($GlcNH_2$), L-alanine, glutamic acid, diaminopimelic acid (DAP), and D-alanine (19, 46); presumably the amino sugars are *N*-acetylated. The DAP is in the *meso*-configuration exclusively (R. Roeske, personal communication). The peptide cross-linking of chemotype I PG involves a direct bond between the free epsilon amino group of a DAP residue on one glycan chain and the carboxyl group of the subterminal D-alanine on a different chain (34). The presence of amidated amino acids and *O*-acetylated amino sugars and the possible significance of trace levels of glycine and aspartate in purified preparations of gonococcal PG (19, 30, 46) have not been established.

The percentage of peptide cross-linking in gonococcal PG has not been previously reported. However, Guymon et al. (17) determined the relative degrees of cross-linking among an isogenic set of *N. gonorrhoeae* strains and made the interesting observation that nonspecific re-

sistance to several antibiotics correlated with a relatively high extent of peptide cross-linking. However, their data could not be interpreted in terms of the absolute extent of cross-linking. The extent of PG cross-linking in the commensal *N. perflava* has been reported and is relatively high for gram-negative bacteria; approximately 50% of the DAP moieties are involved in cross-linking bonds (28).

As part of our efforts to understand the structural basis for the interaction between PG and host, an initial objective is to examine, in detail, the molecular architecture of gonococcal PG. The current study assessed the extent of peptide cross-linking. Gonococcal PG was found to be more extensively cross-linked than most gram-negative bacteria with ca. 40% of the epsilon amino groups on DAP residues substituted by cross-linking bonds. In addition, we confirmed previous observations (17) that multiple antibiotic resistance in gonococci was correlated with a small increase in the extent of peptide cross-linking.

MATERIALS AND METHODS

Bacteria. *N. gonorrhoeae* strains (and their sources) are: RD_5 (F. E. Young, Rochester, N.Y.), JW31 (S. A. Morse, Portland, Ore.), 2686 (D. C. Kellogg, Atlanta, Ga.), and FA19, FA136, FA140, and BR87 (P. F. Sparling, Chapel Hill, N.C.). The latter four strains are part of an isogenic set of gonococcal strains that differ in susceptibilities to several antibiotics (17). FA19 is the wild type; both FA136 (*penA2* and *mtr-2*) and FA140 (*penA2, mtr-2, penB2,* and *ompA*) are relatively resistant to various drugs, detergents, and dyes; BR87 is similar to FA140 but also contains the *str-7* and *env-2* genes. In BR87, the *env* marker mediates drug hypersensitivity even in the presence of the resistance genes.

Gonococci were routinely maintained on solid medium (GCBS; 31, 39). Colonial type 4 bacteria, selected according to the criterion of Kellogg et al. (26), were used throughout. Bacteria used in experiments were also typed according to the scheme of Swanson (36). All strains regularly grew as "transparent" colonies.

Growth and radiolabeling of bacteria. Gonococci were cultured in liquid medium as previously described with SGM broth (30) as modified from that of Hebeler and Young (20) or $LGCB^+$ broth, which is $LGCBS^+$ (31) without Kellogg's supplement. Pyruvate (0.4%, wt/vol) was substituted for glucose as the carbon source to increase the specific activity of D-[^{14}C]glucosamine in PG (30, 41). Specific labeling of the peptide and glycan moieties of isolated PG with [*G*-^{3}H]DAP (Amersham, Arlington Heights, Ill.) and D-[1-^{14}C]glucosamine (ICN, Irvine, Calif.), respectively, and preparation of purified PG from late-exponential-phase bacteria were performed as described (30). ^{14}C added to cultures as $GlcNH_2$ was present in the glycan of PG as both $GlcNH_2$ and muramic acid. The time of cell harvest among different cultures was rigidly standardized; this is potentially important because both growth phase and growth rate influence the extent of PG cross-linking in other bacteria (33). The doubling time for all strains was between 60 and 75 min.

Fractionation of enzymatically digested PG. Purified, radiolabeled PG (typically 1 mg/ml) was incubated at 37°C in 0.05 M sodium acetate (pH 4.7) in the presence of *Chalaropsis* sp. muramidase (30 U/mg of PG; Miles Laboratories, Elkhart, Ind.). Typically, incubation was for 6 h. To insure that the digestion was complete, we also analyzed the reaction products of some digestions after additional periods of incubation. For the latter experiments, the reaction mixtures were heated at 80°C for 15 min to inactivate the enzyme. The Chalaropsis digests of PG were filtered on connected columns of Sephadex G-50 and G-25 (eluted with 0.1 M LiCl), and fractions were counted for radioactivity as indicated below. This simple gel filtration method is well-established and has been applied previously to the fractionation of soluble fragments of gonococcal PG (30). The method readily separates the major, expected products of muramidase digestion of gonococcal PG, i.e., un-cross-linked disaccharide peptide monomers (ca. 1,000 molecular weight), peptide cross-linked bisdisaccharide peptide dimers (ca. 2,000 molecular weight), and trisdisaccharide peptide trimers (ca. 3,000 molecular weight), and provides some resolution of the higher oligomers. The expected products of a hypothetical segment of muramidase-digested PG are shown in Fig. 1.

Analytical methods. Free amino group analysis of [^{3}H]DAP residues in isolated PG fragments was performed by using the 2:4-dinitrofluorobenzene (FDNB) procedure as described (13). After reaction with FDNB, the samples were acid hydrolyzed, applied to Whatman 3MM paper, and chromatographed in butanol-acetic acid-water (BAW; 4:1:5 [vol/vol, upper phase]) to separate free [^{3}H]diaminopimelic acid (DAP) from mono-2,4-dinitrophenyl (DNP) [^{3}H]DAP. Free DAP, mono-DNP-DAP, and di-DNP-DAP were used as standards. By this procedure, the epsilon amino groups of all [^{3}H]DAP residues in un-cross-linked monomer should be available to react with FDNB and thereby yield only mono-DNP-[^{3}H]DAP. By similar reasoning, the ratio of mono-DNP[^{3}H]-DAP-free [^{3}H]DAP in peptide cross-linked dimers and trimers theoretically should be 1:1 and 1:2, respectively.

Borohydride reduction of isolated PG fragments containing a terminal, reducing *N*-acetylhexosamine was performed as described (13) by using $NaBH_4$. After reaction with $NaBH_4$, samples were acid hydrolyzed and chromatographed on paper in BAW (3:1:1, vol/vol) to separate muramic acid, $GlcNH_2$, and their respective alcohols. Standard amino sugars and their alcohols (prepared by the above procedure) were detected by ninhydrin or by radioactivity determination. Treatment of isolated PG fragments with 0.05 M NaOH under conditions known to cause the β-elimination of lactyl peptides from disaccharide peptide monomers or oligomers containing a reducing muramic acid was performed as described previously (32, 40). Products of this reaction were subjected to paper chromatography in BAW (3:1:1, vol/vol) to separate

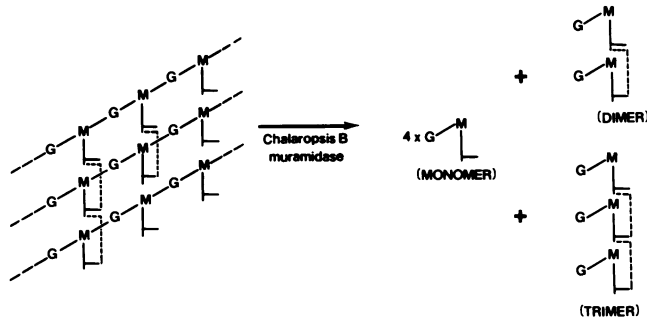

FIG. 1. *Expected products of the complete digestion of a hypothetical segment of PG with Chalaropsis B muramidase. G = N-acetylglucosamine, M = N-acetylmuramic acid, ⊢ = tetrapeptide, ⌐ = direct peptide linkage between subunits. The enzyme cleaves the glycan backbone to release glycan fragments having a reducing muramic acid end.*

undegraded PG fragments, e.g., disaccharide peptide labeled in both amino sugars of the glycan backbone, from the free glycan, e.g., disaccharide. Electrophoresis (typically at 40 V/cm for 120 min) was performed on Whatman 3MM paper by using a flat-plate high-voltage electrophoresis unit (Savant Instruments, Hickeville, N.Y.). Solvents were formic acid-water (8:300, vol/vol, pH 2.0) and acetic acid-pyridine-water (2:4:1,000, vol/vol, pH 5.1). To determine the distribution of radioactivity on all chromatograms, we cut paper strips and analyzed them for radioactivity (see below). Amino acid analysis was performed with a Beckman model 119CL amino acid analyzer.

Determination of percent cross-linking. The extent of cross-linking was estimated from the distribution of the various radiolabeled PG products obtained by Chalaropsis muramidase digestion and isolated by gel filtration. The relative amounts of monomer, dimer, trimer, and tetramer were obtained by determining the ^3H (or ^{14}C) disintegrations per minute under each peak and dividing by the total disintegrations per minute eluted from the column. This method was spot checked by planimetry, which gave almost identical results. The equation used to determine the percent cross-linking was modified slightly from that of Dezélee and Shockman (7). Percent cross-linking was defined as 0.5 × percent dimer + 0.667 × percent trimer + 0.75 × percent tetramer. We used 0.75 (rather than 0.80; reference 7) as the constant in the last (tetramer) term since it appeared that there were few or no oligomers higher than tetramer in the muramidase digests of gonococcal PG. As a confirmation, the extent of cross-linking in intact PG of strain RD_5 was determined directly by the FDNB method described above. To insure accessibility of the reactive amino groups in PG to FDNB, the turbid, aggregated, intact PG suspension was disaggregated with a Branson model 350 Sonifier. This procedure clarified and solubilized the labeled PG (PG remained in the supernatant after centrifugation at 36,000 × *g* for 35 min). By either method, i.e., distribution of muramidase-soluble products or terminal amino group analysis of sonicated PG, the parameter "percent cross-linking" basically reflects the percentage of the total number of DAP residues that are engaged in cross-linking peptide bonds.

Radioactivity determination. To prepare samples for scintillation counting, aqueous samples were dissolved in Scintisol, and samples, dried on paper, were dissolved in NCS-toluene as previously described (30). Radioactivity was measured in a Packard model 3255 scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). All data were corrected for quenching and reported as disintegrations per minute. Quench calibration curves for ^3H and ^{14}C efficiencies and for overlap of ^{14}C into ^3H channels were obtained by external standardization.

RESULTS

Muramidase digestion of gonococcal PG. [^{3}H]DAP- and [^{14}C]$GlcNH_2$-labeled intact PG, isolated from exponential-phase strain RD_5, were treated for 6 h with Chalaropsis muramidase and filtered on the Sephadex G-50-25 series (Fig. 2). Four well-separated peaks containing both [^{3}H]DAP-labeled peptide and [^{14}C]-$GlcNH_2$-labeled glycan were recovered. The ratio of ^3H to ^{14}C was constant essentially throughout. Based on their K_D values, these peaks corresponded to PG fragments with sizes expected for the monomer (ca. 1,000 molecular weight) and the various cross-linked oligomers, i.e., dimer, trimer, and tetramer. Virtually all of the disintegrations per minute present in the intact PG were recovered in the soluble fragments. Because the calculation of percent cross-linking ultimately depends, in part, on the distribution of these fragments, it was important to ensure that the enzymatic digestion had gone to completion. Therefore, PG was incubated with Chalaropsis enzyme under standard conditions for 3, 6, and 12 h and then subjected to gel filtration. The distribution of the four peaks was the same at 12 h as at 6 h (Table 1), indicating that digestion was complete by 6 h.

Identification of the soluble PG fragments. To confirm the presumptive identification of the gel filtration fractions, we isolated

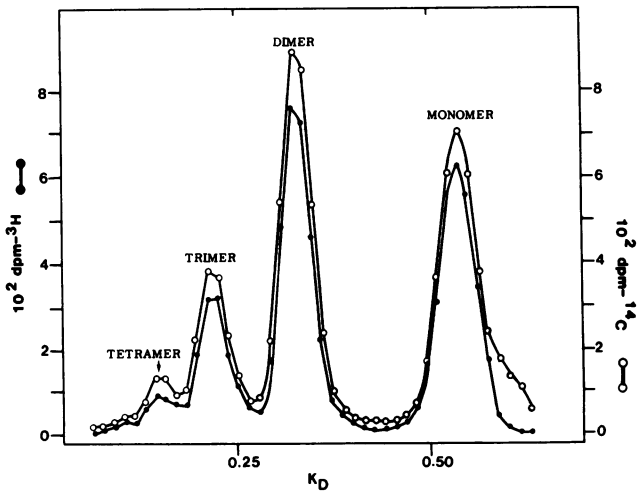

FIG. 2. *Gel filtration on connected columns of Sephadex G-50 and G-25 of the Chalaropsis muramidase digest of isolated PG obtained from strain RD_5 grown in SGM. ^3H was added to the culture as DAP; ^{14}C was added as $GlcNH_2$. These specifically label the peptide and glycan moieties, respectively, of gonococcal isolated PG. Label added as [^{14}C]$GlcNH_2$ was present in isolated PG as both $GlcNH_2$ and muramic acid.*

peak materials, desalted them on a Sephadex G-15 column, and subjected them to a battery of radiochemical analyses. Test samples of isolated fragments were treated with FDNB, hydrolyzed, and paper chromatographed to quantitate free [^{3}H]DAP and mono-DNP-[^{3}H]DAP. This analysis for free amino groups (Fig. 3) confirmed the presumptive identification of un-cross-linked monomers, and peptide-cross-linked dimers and trimers. Integration of the areas under the peaks for FDNB-treated monomer (Fig. 3), for example, indicated that 91% of the DAP residues were free to react with FDNB and, thereby, be derivatized to mono-DNP-DAP. The theoretical value for un-cross-linked monomer is 100%. Analogous values of percent mono-DNP-DAP for the presumed peptide-cross-linked dimers and trimers were 52% and 35%, respectively (Fig. 3). These correspond closely with the theoretical values for dimer and trimer of 50 and 33%.

The data (Fig. 3) also indicate that after treatment with FDNB and acid hydrolysis, the ^{14}C present in each of the PG fragments remained as $GlcNH_2$ and muramic acid only (identical to acid hydrolysates of control PG samples that were not treated with FDNB). The corresponding DNP derivatives of the hexosamines, which are readily separated from the free hexosamines, were not detected in significant amounts, suggesting that amino groups of both $GlcNH_2$ and muramic acid are blocked and presumably are *N*-acetylated. Amino acid analysis of PG fragments (Table 2) was also compatible with the tentative identification of monomer and dimer. The composition of these fragments, like intact PG, was that expected for chemotype I, which contains direct crossbridges involving DAP and D-ala residues. These data (Table 2), which were obtained for isolated fragments and the same batch of intact PG from which they were derived, show relatively low amounts of $GlcNH_2$ in monomer and dimer as compared to other experiments in which $GlcNH_2$ was present in amounts approximately equimolar to glutamic acid. The possible significance of small amounts of glycine in fragments and in intact PG is currently unknown. The composition of PG or PG fragments was essentially the same, regardless of the type of growth medium used.

TABLE 1. *Distribution of soluble fragments obtained by digestion of intact PG with Chalaropsis muramidase*

Time of incubation (h)	Distribution of PG fragments (% of total)			
	Monomer	Dimer	Trimer	Tetramer
3	38	37	18	8
6	38	40	17	5
12	38	40	17	5

Borohydride treatment of monomer and dimer, containing label in both $GlcNH_2$ and muramic acid, followed by acid hydrolysis and paper chromatography, yielded only $GlcNH_2$ and muramicitol as the final products, indicating that muramic acid was at the reducing end. This was confirmed by the results of the β-elimination reaction, i.e., mild alkali removed the free disaccharide from ^{14}C-labeled monomer. These data are to be expected for PG fragments obtained as

a result of Chalaropsis muramidase digestion.

The monomer fractions were also subjected directly to paper chromatography in BAW (4:1:5, vol/vol, upper phase) and run for 7 days. Under these conditions the monomer was resolved, essentially, into two peaks. Based on their R_f values, the major peak (ca. 70% of the ^{14}C-labeled monomer) appeared to be the disaccharide tetrapeptide, identical to compound C_6 (the disaccharide tetrapeptide monomer obtained from *E. coli* PG by muramidase action; 22). The R_f of the minor peak (30% of the monomer) was consistent with that of C5, the corresponding disaccharide tripeptide obtained from *E. coli* PG (22). The presence of the disaccharide tripeptide suggests the activity of a gonococcal LD-carboxypeptidase. This observation that monomer consisted of both disaccharide tetra- and tripeptides is in agreement with the ratio of alanine to glutamic acid of <2.0 to 1 that consistently was obtained for acid hydrolysates of gonococcal PG (Table 2). On the basis of high voltage electrophoresis at pH 2.0 and 5.1, isolated monomer comigrated with a reference disaccharide peptide monomer (X', 22) isolated from *E. coli* PG (chemotype I) and provided by U. Schwarz, Tubingen, W. Germany. Both were cationic at pH 2.0 and anionic at pH 5.1. Identical electrophoretic mobilities of isolated monomer and of reference monomer X' suggests the absence of significant peptide amidation in the gonococcal monomer.

Together, the above data suggest that the soluble PG fragments obtained by Chalaropsis digestion and isolated by gel filtration do in fact represent un-cross-linked monomers and peptide cross-linked dimers, trimers, and tetramers as indicated (Fig. 2).

Determination of percent cross-linking. Based on the distribution of the Chalaropsis-

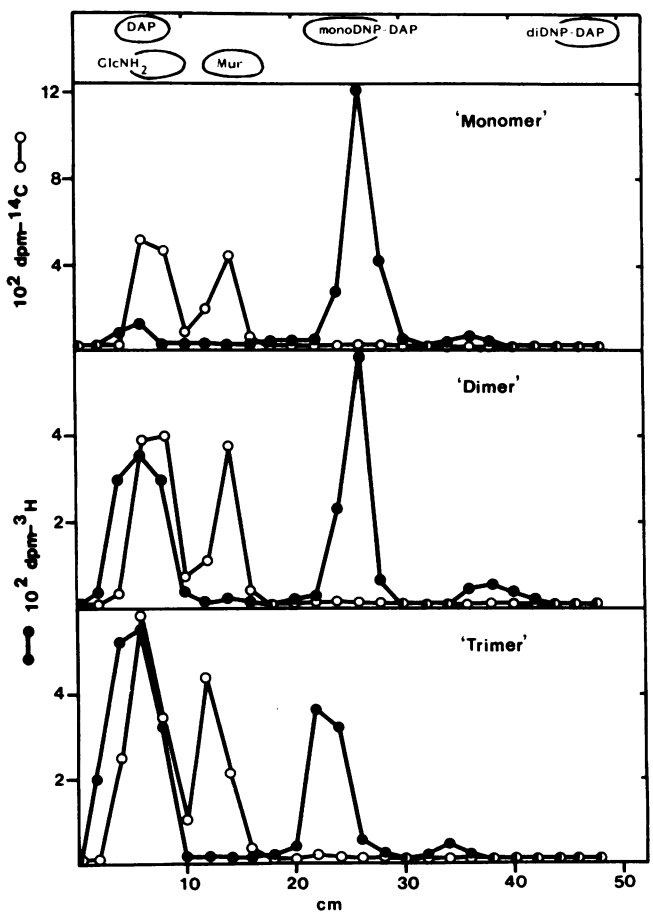

FIG. 3. *Free amino group analyses of isolated PG fragments labeled in the peptide with [^{3}H]DAP and in the glycan with [^{14}C]GlcNH$_2$ and [^{14}C]muramic acid. Samples were treated with FDNB, acid hydrolyzed, and subjected to paper chromatography in BAW (4:1:5, vol/vol, upper phase) to separate the free amino acid or amino sugars from their respective DNP derivatives.*

soluble fragments, the PG of strain RD_5 grown in $LGCB^+$ was estimated to be ca. 42% peptide cross-linked (Table 3). The data (Table 3) also indicate that PG obtained from the same strain grown in SGM was slightly, but significantly, less cross-linked. The extent of PG cross-linking in strain RD_5 was confirmed, independently, by measurement of free amino groups of DAP residues in sonicated, native PG by using FDNB (Fig. 4).

The extent of PG cross-linking in other gonococcal strains did not appear to differ remarkably from RD_5; all of the values fell into a relatively narrow range of 36 to 44% (Table 3). Within this range, the data (Table 3) reveal subtle differences among the isogenic strains which differ in drug susceptibility, i.e., the PG of the relatively resistant FA140 and FA136 was slightly more extensively cross-linked than that of the wild type, FA19. This tends to confirm the earlier observations of Guymon et al. (17). It should be pointed out that in one experiment involving analysis of the PG of FA19, BR87, and FA140, the basic experimental design was modified slightly in an attempt to enhance the ability to detect rather small differences in percent PG cross-linking. In this experiment, a single batch of [^{3}H]$GlcNH_2$-PG (400 μg) from FA19 was mixed with 1 mg of either [^{14}C]$GlcNH_2$-PG from FA140 (relatively drug resistant) or [^{14}C]-$GlcNH_2$-PG from BR87 (hypersusceptible). The mixtures were treated with Chalaropsis muramidase under the standard conditions and analyzed on the Sephadex G-50-25 columns. It was felt that the concomitant treatment and analysis of FA19-[^{3}H]PG (the internal reference) plus one or the other of the [^{14}C]PGs might provide a more direct means of comparison to FA19. For the FA19 versus BR87 pair, the values for percent cross-linking were 38.7 and 39.4%, respectively. For the FA19 versus FA140 set, values were 38.5 and 43.4%, respectively.

TABLE 2. *Amino acid analysis of isolated monomer and dimer obtained by Chalaropsis digestion and of the parental intact PG from which they were derived*

Amino acid[a]	Molar ratio[b]		
	Monomer	Dimer	Intact PG
Alanine	1.66	1.78	1.80
Glutamic acid	1.00	1.00	1.00
DAP	0.80	0.94	0.91
Muramic acid	1.01	0.93	1.04
$GlcNH_2$	0.63	0.64	0.98
Glycine	0.20	0.11	0.08
Aspartic acid	ND[c]	ND	0.02

[a] No other amino acids were detected; the sensitivity of the analyses was such that amino acids present at a molar ratio to glutamic acid of approximately 0.01 or greater would have been detected. Hydrolysis was with 4 N HCl for 5 h at 110°C.

[b] In respect to glutamic acid ≡ 1.00.

[c] Not detected.

DISCUSSION

The physiological consequences related to the extent of PG cross-linking are poorly understood, but the "tightness" of the PG matrix undoubtedly contributes to many of the traditional functions assigned to the PG "corset" of diverse bacteria, e.g., shape determination and maintenance of physical integrity. In general, the PG obtained from gram-positive bacteria is more extensively cross-linked than that of gram-negative bacteria. Typically, the values for percent cross-linking (in terms of percent diamino

TABLE 3. *Extent of peptide cross-linking in gonococcal PG*

Strain	Medium	No. of independent experiments	Distribution of PG fragments[a] (% of total ± SD)				Cross-linking[b] (% ± SD)
			Monomer	Dimer	Trimer	Tetramer	
RD_5	SGM	5	37.6 ± 0.9	38.6 ± 1.9	17.0 ± 0.7	7.0 ± 2.5	36.0 ± 1.4
RD_5	$LGCB^+$	7	28.6 ± 2.1	39.6 ± 2.2	22.8 ± 0.8	9.2 ± 3.8	41.9 ± 2.0
2686	SGM	1	33.3	37.4	18.9	10.4	39.1
JW31	$LGCB^+$	1	30.9	40.1	23.5	5.5	39.8
FA19[c]	$LGCB^+$	2[d]	34.4	35.5	21.1	9.0	38.6
FA140[c]	$LGCB^+$	1	27.8	35.0	24.9	12.4	43.4
BR87[c]	$LGCB^+$	2	31.7	35.8	21.2	11.2	40.5
FA136	$LGCB^+$	1	25.0	41.3	21.8	11.9	44.1

[a] From gel filtration patterns of PG fragments obtained by digestion of intact PG with Chalaropsis muramidase. SD, Standard deviation.

[b] Percent peptide cross-linking = 0.5 × percent dimer + 0.667 × percent trimer + 0.75 × percent tetramer.

[c] One experiment employing PG from FA19, FA140, and BR87 was performed slightly differently than the others to enhance the ability to detect small differences in percent cross-linking. For description, see text.

[d] Average of two determinations with a single batch of PG. See text.

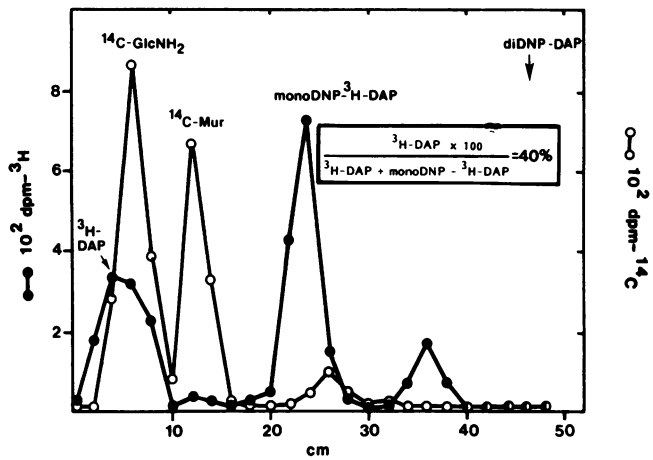

FIG. 4. *Free amino group analysis of purified, sonicated PG obtained from strain RD_5 grown in SGM. PG was labeled in the peptide with [^{3}H]DAP and in the glycan with [^{14}C]GlcNH$_2$ and [^{14}C]muramic acid (Mur). Paper chromatography of acid hydrolysates of FDNB-treated samples was as in Fig. 3. The amount of [^{3}H]DAP and mono-DNP-[^{3}H]DAP was determined by totalling the disintegrations per minute under the respective peaks. The small peak at 36 cm was thought to be a breakdown product of mono-DNP-DAP due to acid hydrolysis and was therefore counted as mono-DNP-[^{3}H]DAP disintegrations per minute. The percentage of total ^{3}H disintegrations per minute that are present as free [^{3}H]DAP is an index of percent PG cross-linking.*

acid residues involved in cross-linking bonds) in gram-positive bacteria is greater than 50% and may reach as high as 90% (4, 7, 12). Gram-negative PG is generally less than 50% cross-linked, e.g., *E. coli*, 25 to 30% (38, 43); *Vibrio*, 30% (24, 45); *Proteus* spp., 33 to 37% (25, 28); *Moraxella* sp., 37% (28); and *Pseudomonas*, 25 to 45% (11, 21, 28). The range of values for percent PG cross-linking (36 to 44%) obtained here indicates that gonococci are slightly more extensively cross-linked than most other gram-negative bacteria. Interestingly, the PG of the commensal *Neisseria perflava* is particularly highly cross-linked (50%) for a gram-negative bacterium (28).

The relatively high extent of PG cross-linking among gonococci, including the highly autolytic strain RD_5 (18), is in contrast to the overall great fragility and autolytic tendency associated with these bacteria (10, 18). If percent cross-linking, per se, were the critical determinant and inversely proportional to the degree of osmotic fragility, one might expect gonococci to be relatively resistant to autolysis. Obviously, other factors, e.g., deregulation of potentially suicidal autolytic enzymes (some of which may exert their activity by reducing PG cross-linkage below some critical level) and membrane stability (41) are more fundamental determinants of the autolytic behavior of gonococci, as they are in other bacteria (6).

Very subtle medium- and strain-dependent differences in percent PG cross-linking in gonococci were observed (Table 3). Previous demonstrations of medium-dependent changes in PG cross-linking have been made in staphylococci (33). In the latter studies (33), the effects of medium composition probably related to the external concentration of certain amino acids, e.g., glycine and serine, which are used directly as (or can be interconverted to) components critical to the formation of interpeptide bridges. Because gonococcal PG appears to contain direct crossbridges between DAP and D-alanine residues, such a mechanism probably does not apply. The data (Table 3) confirm some key observations of Guymon et al. (17) that the extent of PG cross-linking among isogenic gonococci was higher in those strains carrying loci, e.g., *mtr-2* and *penB2*, which govern increased resistance to multiple drugs. In terms of absolute percent cross-linking, however, our observed differences, e.g., FA19 versus FA140 or FA136, were very small.

The physiological significance, if any, of the medium- and strain-related differences in percent cross-linking of gonococcal PG is unknown. In some bacteria, alterations in extent of PG cross-linking have been associated with obvious physiological and morphological changes, e.g., morphogenesis in *Myxococcus* sp. (23) and *Bacillus* (29). In other cases, however, absolute differences in extent of PG cross-linking that were substantially greater than the differences observed here had no detectable effect on cell morphology or function (33). In considering pos-

sible effects of altered cross-linking it would, then, seem important to know not only that a net change in percent cross-linking occurs, but also how such changes are distributed throughout the PG layer. For example, if the events leading to a small net change in cross-linking occurred randomly and, hence, were diluted throughout the entire PG matrix, then local perturbations in any given domain (and the observable physiological consequences) likely would be minimal. However, a net change in percent cross-linking of the same magnitude could be accounted for, entirely, by rather large perturbations in a small number of discrete sites. The physiological importance of such alterations could be considerable. For example, substantial changes in PG cross-linking at or near sites of attachment of PG-associated, outer membrane proteins that serve as water-filled channels for the passage of numerous solutes (8) might alter the number, the spatial distribution, and the conformation of functional pore proteins. Any combination of these effects could conceivably influence the overall permeability characteristics of the intact bacterium. Such a mechanism might apply to the original observations (17), confirmed here, that increased resistance to multiple drugs is associated with increased levels of PG cross-linking. It should be noted that an intact PG layer has been reported, previously, to be critical to the maintenance of the outer permeability barrier in gram-negative bacteria (2).

As indicated previously, the primary objective of these studies was to characterize more completely the structure of gonococcal PG as part of our ongoing examination of biological activities mediated by gonococcal PG. In general, little is known about the structural basis for the biological activities of PG. The manifestations of some activities, e.g., attraction of leukocytes, arthritogenicity, and complement consumption, are apparently dependent upon size; these activities are eliminated or substantially reduced when PG fragments below some critical size threshold are tested (15, 16, 27). Arthritogenicity and complement consumption, specifically, have been reported to require glycosidically linked polymers of the repeating disaccharide subunit (15, 27). In collaboration with others, we have found that various forms of gonococcal PG consume complement (B. H. Petersen, manuscript in preparation) and are directly toxic for fallopian tube organ cultures (M. A. Melly, Z. A. McGee, and R. S. Rosenthal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B90, p. 32). With knowledge of the extent of PG cross-linking in gonococci and the ability to isolate variously cross-linked fragments, we hope to determine the role of peptide cross-linking (among other structural variables of PG) in these and other biological activities.

ACKNOWLEDGMENTS

The technical assistance of Martin J. Scheidt and Steven Underwood, the supervision of amino acid analysis by Roger W. Roeske, and critical review of the manuscript by W. S. Wegener are gratefully acknowledged.

This investigation was supported by Public Health Service grant R01 AI 14826 from the National Institute of Allergy and Infectious Diseases.

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