Biochemistry

 Volume 5, Number 10 October 11, 1966

Structure of the Cell Wall of *Micrococcus lysodeikticus*. I. Study of the Structure of the Glycan*

Melina Leyh-Bouille, Jean-Marie Ghuysen, Donald J. Tipper, and Jack L. Strominger

ABSTRACT: The glycan portion of the cell wall of *Micrococcus lysodeikticus*, or at least the greatest part of it, consists of linear chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues, both in the pyranose ring form. Both glycosidic links are 1,4.

The links from *N*-acetylglucosamine to *N*-acetylmuramic acid and probably the links from *N*-acetylmuramic acid to *N*-acetylglucosamine are in the β configuration. At least 40% of the *N*-acetylmuramic residues are not substituted by peptide. This structure is based on the quantitative isolation of the disaccharides *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid and *N*-acetylmuramyl-(β?)-1,4-*N*-acetylglucosamine, of the trisaccharides *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramyl-(β?)-1,4-*N*-acetylglucosamine and *N*-acetylmuramyl-(β?)-1,4-*N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid, and of the tetrasaccharide *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramyl-(β?)-1,4-*N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid. An octasaccharide the structure of which has not been fully elucidated has also been isolated.

It is generally said that the glycan portion of bacterial cell wall peptidoglycans is made up of alternating units of *N*-acetylglucosamine[1] and *N*-acetylmuramic acid and that the carboxyl groups of the MurNAc residues are involved in amide linkages to terminal L-alanine residues of the peptide portion of the polymer. Such a structure, however, has only been proven in one case. The peptidoglycan of *Staphylococcus aureus* strain Copenhagen cell walls was quantitatively degraded into β-1,4-GlcNAc-MurNAc disaccharides by means of lysis with various *endo*-*N*-acetylmuramidases (Ghuysen and Strominger, 1963a,b; Tipper *et al.*, 1965) followed by hydrolysis of the amide linkage between the disaccharides and the peptide moiety by *N*-acetylmuramic acid-L-alanine amidase. The complementary β-1,4-MurNAc-GlcNAc disaccharides were obtained in quantitative yield from these same cell walls after lysis with lysostaphin which contains a lytic peptidase and an *endo*-*N*-acetylglucosaminidase (Browder *et al.*, 1965; Tipper and Strominger, 1966). After isolation and purification, their structures were established.

Earlier studies (Salton and Ghuysen, 1960; Perkins, 1960) carried out on walls of *Micrococcus lysodeikticus* provided data which suggested that the linear polysaccharide chains were formed of repeating β-1,6-GlcNAc-MurNAc disaccharide units linked to each other by β-1,4 linkages. Other observations, however, raised questions regarding this proposal. First, it was based on structural studies carried out on isolated di- and tetrasaccharides which altogether represented only a small part of the total hexosamine content of the peptidoglycan. Secondly, some properties of the natural, isolated GlcNAc-MurNAc disaccharide, for which a β-1,6 linkage had been proposed, were different

* From the Service de Bactériologie, Université de Liège, Liège, Belgium, and Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin. *Received June 6, 1966.* This research has been supported in part by a grant (UR-E4-10-2) made by the U. S. Department of Agriculture under P. L. 480 (to J. M. G.) and by grants from the U. S. Public Health Service (AI-06247 and AM-08672) and National Science Foundation (GB-4552) (to J. L. S.). This paper is from a dissertation submitted by M. L.-B. in partial fulfilment of the requirements for a Ph.D. degree, University of Liège, Belgium (1965).

[1] Abbreviations used: GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid.

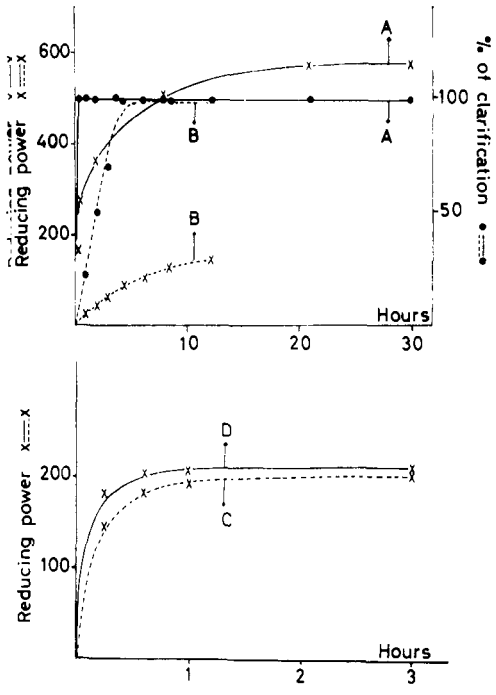

FIGURE 1: Kinetics of the digestion of the cell walls of *M. lysodeikticus* with *endo-N*-acetylmuramidase (lysozyme) and with *endo-N*-acetylglucosaminidase (from (lysostaphin). Aliquots were removed from the digests at intervals and centrifuged to remove insoluble material. The pellets were resuspended in water for turbidity determinations at 550 mμ, and reducing power was determined on aliquots of the supernatants. Results are expressed in per cent of clarification and in millimicromoles of reducing groups per milligram of walls relative to an *N*-acetylglucosamine standard. Conditions of incubations: (A) 4 mg of cell walls was treated with 40 μg of lysozyme in 300 μl of 0.01 M phosphate buffer, pH 6. (B) Same as in A, but 4 μg of lysozyme was used. (C) Walls (200 μg) were treated with 5.5 μg of *endo-N*-acetylglucosaminidase from lysostaphin, in 40 μl of 0.01 M phosphate buffer, pH 7.5. (D) Walls (200 μg) were treated for 2 hr with 3 μg of *Myxobacter* enzyme in 30 μl of 0.007 M Veronal buffer, pH 9. The soluble digest was heated 5 min at 100° and 1.33 μl of 0.1 N HCl, 4 μl of 0.1 M phosphate buffer, pH 7.5, and 5.5 μg of *endo-N*-acetylglucosaminidase were added (final volume, 40 μl)

from these of an authentic, synthetic β-1,6 disaccharide (Jeanloz *et al.*, 1963; Sharon *et al.*, 1966). In consequence, the structure of the polysaccharide moiety of the *M. lysodeikticus* peptidoglycan has been reinvestigated and the results obtained are presented in this paper. A preliminary account has appeared (Leyh-Bouille and Tipper, 1965).

Materials and Methods

Cell Walls and Enzymes. Cell walls of *M. Lysodeikticus* were prepared according to standard procedure and were trypsin treated, as previously described (Petit *et al.*, 1966). *endo-N*-Acetylmuramidase (egg white lysozyme, Armour) and lysostaphin (kindly supplied by Mead-Johnson Co., Evansville, Ind.) were used in order to dissolve the cell walls. The *Streptomyces N*-acetylmuramyl-L-alanine amidase was prepared as previously described (Ghuysen *et al.*, 1962) and a similar enzyme from *Myxobacter* (Ensign and Wolfe, 1966) was a gift of Dr. J. Ensign. β-*exo-N*-Acetylglucosaminidase from pig epididymis (*exo*-β-GlcNAcase) was prepared according to Sanderson *et al.* (1962). D-Glucosamine 6-phosphate *N*-acetylase was prepared from yeast according to Brown (1962) and glucosamine deaminase from *Escherichia coli B* according to Risse and Lüderitz (1964). These two latter enzymes were a generous gift from Dr. O. Lüderitz.

Analytical Procedures. The analytical procedures which were used throughout the present studies have been previously described (Ghuysen *et al.*, 1966). Acetylamino sugars were determined employing the Morgan–Elson reagent using either 7 or 30 min of heating in 1% borate. Amino sugars were determined employing the same procedure (7 min of heating) after chemical acetylation. Glucosamine was specifically acetylated with yeast D-glucosamine 6-phosphate *N*-acetylase. In some cases, glucosamine was deaminated with the *E. coli* deaminase. Reducing groups were determined by the Park–Johnson ferricyanide procedure. Free amino acids and free amino groups were measured with dinitrofluorobenzene. Formaldehyde was determined with the H_2SO_4–chromotropic acid reagent, measuring the absorption at 570 mμ (Suzuki and Strominger, 1960). Periodate in presence of iodate was determined on the basis of a differential molar extinction coefficient at 224 mμ ($\Delta\epsilon$) of 8730 for periodate and iodate (Dixon and Lipkin, 1954). Free D-glucose was estimated using the "glucostat" reagents from Worthington Biochemical Corp., Freehold, N. Y.

Column Chromatography. Sephadex G-75, medium grade, G-50, medium grade, G-25, medium grade and fine grade (bead form), were obtained from Pharmacia, Uppsala, Sweden, and carboxymethylcellulose (CMC) (Cellex CM), from Calbiochem, Los Angeles, Calif. CMC was used either in the H^+ form (the resin was treated with 1 N NH_4OH, washed with water, treated with 2 N acetic acid, and finally washed with water) or in the Na^+ form (the resin was treated with a 0.5 N HCl–1 M NaCl solution, washed with water, treated with 1 N NH_4OH, washed again with 0.5 M NaCl solution, and finally with water).

Paper chromatography was carried out by the descending technique on Whatman No. 1 paper using the following solvents: (A) 1-butanol–acetic acid–water (3:1:1), (B) isobutyric acid–0.5 N NH_4OH (5:3), (C) 1-butanol–acetic acid–water (25:6:25, upper phase), and by the two-dimensional technique using first the solvent A and then solvent D (pyridine–water, 8:2).

Paper electrophoresis was carried out on Whatman 3MM paper (40 × 40 cm) in an Electrorheophor apparatus (Pleuger, Antwerp, Belgium), using pyridine–acetic acid–water (2:10:1000) buffer, pH 3.9.

Detection on Paper. Oligosaccharides were detected with diphenylamine–trichloroacetic acid spray (Hough *et al.*, 1950) or by fluorescence after the paper had been dipped in a 0.5 N NaOH solution made up in ethanol–1-propanol (6:4) and heated for 10 min at 120° (Sharon, 1964). Amino acids and free amino groups were detected with ninhydrin spray (0.5% in isopropyl alcohol–water, 9:1). Free glucose was detected with "glucostat" spray (Worthington Biochem. Corp., Freehold, N. Y.).

Experimental Section

Enzymatic Digestion of Walls of M. lysodeikticus

Cell walls of *M. lysodeikticus* have a high intrinsic content of N-terminal groups, about 400 mμmoles/mg. Most of them are amino-terminal ε-lysine. No difference in the appearance of these groups in soluble products was observed when lysis of the cell walls was carried out by the two *endo-N*-acetylhexosaminidases.

A. Lysozyme Digestion (Figure 1A, B). Hydrolysis of only a few glycosidic bonds by lysozyme in 0.01 M phosphate buffer, pH 6, is sufficient to induce complete solubilization of the walls. The minimum number of reducing groups for each milligram of completely solubilized walls is equivalent to about 110 mμmoles of GlcNAc. At completion of the reaction, however, 570 mμmoles of reducing groups per milligram is found in the digest.

B. Digestion with Lysostaphin (Figure 1C). Working in 0.01 M phosphate, pH 7.5, this enzyme digests the cell walls of *M. lysodeikticus* and, at completion of the reaction, releases 220 mμmoles/mg of reducing groups.

C. Digestion with Myxobacter Enzyme. This enzyme preparation hydrolyses *N*-acetylmuramyl-L-alanine linkages and a number of peptide bonds inside the pentaglycine bridges in cell walls of *S. aureus* (D. J. Tipper, J. C. Ensign, and J. L. Strominger, unpublished data). In 0.007 M Veronal buffer, pH 9, this enzyme preparation also lyses cell walls of *M. lysodeikticus*. The kinetics of the digestion shows that the hydrolysis of a single type of bond is involved. For each milligram of digested walls, 200 mμmoles of amino-terminal alanine appears as a result of the hydrolysis of *N*-acetylmuramyl-L-alanine linkages. After solubilization with the *Myxobacter* enzyme, the *endo-N*-acetylglucosaminidase liberated the same number of reducing groups as from the intact cell walls (Figure 1D).

Preparation of the Disaccharide, MurNAc-GlcNAc, from Cell Walls of M. lysodeikticus

Digestion with endo-N-Acetylglucosaminidase. Cell walls (200 mg) were incubated for 18 hr at 37° with 250 μg of the *Myxobacter* enzyme preparation in 10.5 ml (final volume) of 0.02 M Veronal buffer, pH 9. As the clarification proceeded, the pH was maintained at 8.5–9 by addition of 2 N NaOH. When the incubation was over, the digest was neutralized and heated 5 min in boiling water, and 1.2 ml of 0.1 M phosphate buffer, pH 7.5, and 3 mg of lysostaphin were added. After 8 hr at 37°, the digestion was stopped. The digest contained, per milligram of treated walls, 200 mμmoles of amino-terminal alanine, 370 mμmoles of amino-terminal ε-lysine, and 220 mμmoles of reducing groups. It was filtered in water on a column of Sephadex G-25, bead form ($V_0 + V_i$ = 340 ml) (Figure 2). About 85% of material containing reducing groups and amino-terminal groups were eluted between 275 and 340 ml. This fraction was freeze dried and the residual material was passed over a column (2.5 × 22 cm) of CMC (Na^+) using water as eluent (Figure 3). First material containing reducing groups was eluted between 90 and 135 ml and next, material containing amino-terminal groups, between 110 and 155 ml. Fractions between 110 and 130 ml were pooled, freeze dried, and refractionated on the same column. The pooled reducing fractions from the two columns contained only GlcNAc and MurNAc; no amino acids were present. This fraction contained 100 μmoles of total hexosamines, *i.e.*, a yield of 54% of the total hexosamine from 200 mg of walls. It will be characterized below as the disaccharide, MurNAc-GlcNAc.

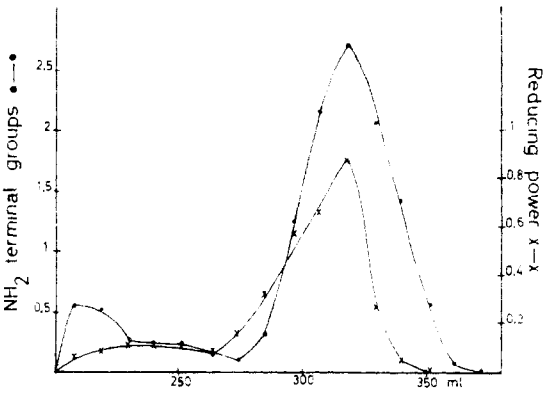

FIGURE 2: Preparation of the disaccharide MurNAc-GlcNAc from cell walls of *M. lysodeikticus*. Walls of *M. lysodeikticus* were digested with *Myxobacter N*-acetylmuramyl-L-alanine amidase, then treated with the *endo-N*-acetylglucosaminidase of lysostaphin, and finally filtered on Sephadex G-25, fine grade. Results are expressed in millimicromoles per microliter of reducing groups (relative to *N*-acetylglucosamine) and of amino-terminal groups (relative to alanine). Fractions eluted between 275 and 340 ml contain the disaccharide MurNAc-GlcNAc and small peptides.

Preparation of the Disaccharide GlcNAc-MurNAc, of the Tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc and of Higher Oligosaccharides from Cell Walls of M. lysodeikticus

Digestion with endo-N-Acetylmuramidase. The walls were digested with lysozyme and the incubation was stopped as soon as clarification had occurred in order

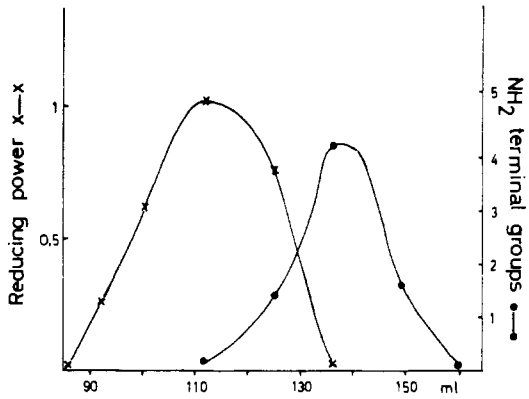

FIGURE 3: Preparation of the disaccharide MurNAc-GlcNAc from cell walls of *M. lysodeikticus*. The mixture of free disaccharide and free peptides obtained after filtration of the cell wall digest on Sephadex G-25 (Figure 2) was chromatographed on a column of CM-cellulose (Na^+) using water as the eluent. Results are expressed as in Figure 2. The disaccharide MurNAc-GlcNAc is eluted between 90 and 135 ml.

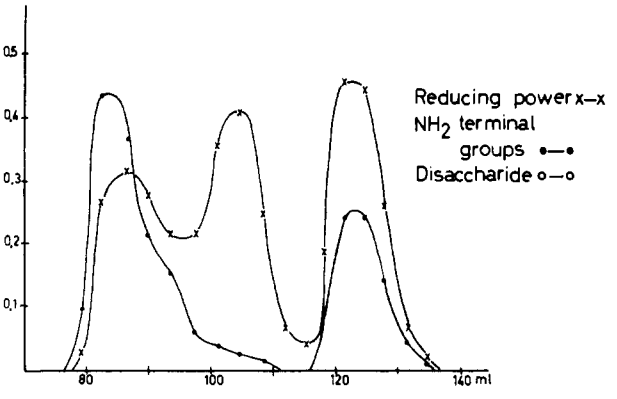

FIGURE 4: Preparation of the disaccharide GlcNAc-MurNAc and higher oligosaccharides from cell walls of *M. lysodeikticus*. The portion of the lysozyme digest of the cell walls, which was not retained on CM-cellulose (H^+), was filtered on a column of Sephadex G-25, medium grade. Results are expressed as in Figure 2. Disaccharide has been measured on the basis of an $\epsilon_{585m\mu}$ 9000, employing the Morgan–Elson reaction after 30 min of heating in borate. Higher oligosaccharides do not give this reaction. Fraction 1, 80–95 ml; fraction 2, 95–110 ml; fraction 3, 115–135 ml; only fraction 3 contains disaccharide.

to obtain good yields of higher oligosaccharides. Reducing groups equivalent to only 120 mμmoles of GlcNAc/mg of cell walls were present in the digest.

A. PROCEDURE 1. Walls (1 g) were treated with 1 mg of lysozyme for 5 hr at 37° in 0.01 M phosphate buffer, pH 6.0. The digest was passed over a 1200-ml column of CMC (H^+) using water as eluent. Most of the oligosaccharides substituted by peptide is absorbed on the column under these conditions. The material not adsorbed under these conditions (70% of the reducing groups present and 10% of the total amino-terminal groups) was freeze dried and then filtered on a column of Sephadex G-25, medium grade ($V_0 + V_i$ = 140 ml), using 0.1 M LiCl as eluent (Figure 4). Three fractions were obtained with peaks having the following elution volumes: fraction 1 (85 ml), fraction 2 (105 ml), and fraction 3 (125 ml). Fraction 3 was the only one which gave a positive Morgan–Elson reaction after 30 min of heating in borate. This fraction was desalted by filtration in water on Sephadex G-25, medium grade, yielding after lyophilization the disaccharide GlcNAc-MurNAc which will be characterized below (4.5% of the total hexosamine content of the walls). Fraction 2 was slightly contaminated by materials of fraction 1. It was repurified by repeated chromatography on the Sephadex G-25 column in 0.1 M LiCl and then desalted as indicated for fraction 3. It yielded the tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc (10% of the total hexosamine content of the walls). Fraction 1 was a mixture of free oligosaccharides and oligosaccharide–peptide complexes, which were not absorbed on the CMC column. It was adjusted to pH 5.5 by the addition of 1 M acetate buffer (final concentration 0.025 M), 2 ml of a solution of *N*-acetylmuramyl-L-alanine amidase (Ghuysen and Strominger, 1963a) was added, and the mixture was incubated for 1 hr at 37°. In these conditions, reaction was complete and 1 mμmole of new amino-terminal groups appeared for 4 mμmoles of total hexosamine. The digest was filtered on the same CMC (H^+) column as above. The material which was not adsorbed (90% of the reducing groups and 25% of the amino-terminal groups of the amidase-treated fraction 1) was freeze dried and then filtered on the Sephadex G-25 column, in 0.1 M LiCl. The reducing groups (75%) were found present in a high molecular weight fraction together with some free peptide material. This reducing material was further purified by filtration on a Sephadex G-50 column ($V_0 + V_i$ = 150 ml), also in 0.1 M LiCl. It then behaved as a low molecular weight compound (elution volume, 117–157 ml). After freeze drying and desalting on a Sephadex G-25 column, this material was chromatographed on paper using solvent B. Detection on marker strips showed a major component of R_F 0.18 and minor components with R_F 0.25 and 0.31. The component of R_F 0.18 was eluted from the paper and after freeze drying, it was repurified on Sephadex G-25 in water. It yielded an octasaccharide (2% of the total hexosamine of the walls).

B. PROCEDURE 2. Cell walls (120 mg) were digested with lysozyme as described in procedure 1 and the digest was submitted to paper electrophoresis at pH 3.9. The material was applied as a band near the cathode. After 5 hr of electrophoresis at 20 v/cm, the electrophorheogram was divided in three zones (Figure 5): zone A, from 30 to 40 cm toward the anode, was rich in glucose and represented the nonpeptidogly-

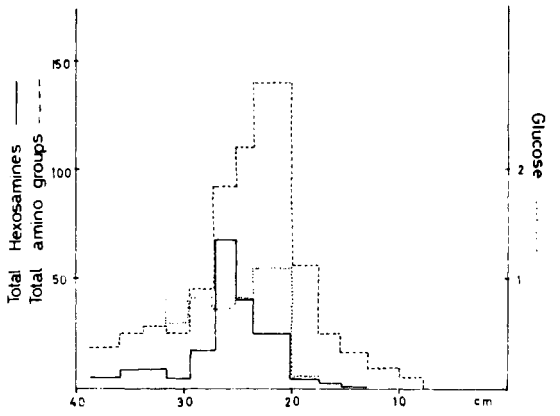

FIGURE 5: Preparation of disaccharide GlcNAc-MurNAc and higher oligosaccharides from cell walls of *M. lysodeikticus*. Paper electrophoresis at pH 3.9 of walls of *M. lysodeikticus* digested with lysozyme. The lysozyme digest was applied as a band near the cathode (at 0 cm). After electrophoresis, a marker strip was cut into segments of 1-cm length. Each segment was extracted with water and the lyophilized eluates were analyzed for total amino acids, total hexosamine, and glucose after appropriate HCl hydrolyses. The results are expressed as micromoles per milligram of walls (for amino acids and hexosamine) or as micrograms per milligrams of walls (for glucose) in each paper segment from the cathode (0 cm) to the anode (40 cm). Zone B corresponds to a migration of 25–30 cm.

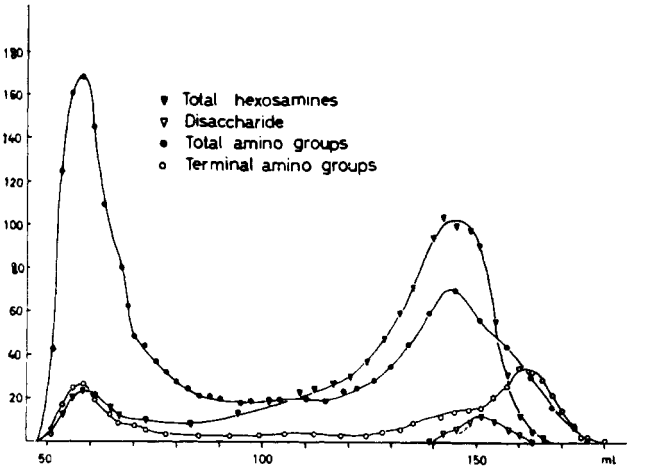

FIGURE 6: Preparation of disaccharide GlcNAc-MurNAc and higher oligosaccharides from cell walls of *M. lysodeikticus*. The material extracted from zone B (Figure 5) after paper electrophoresis of a lysozyme digest of cell walls was filtered on Sephadex G-25 medium grade. Results are expressed as mμmoles/50 μl.

can components of the cell wall; zone B, from 25 to 30 cm, contained 74.5 μmoles of hexosamines, *i.e.*, 67% of the total hexosamine of the digest; zone C, from 17 to 25 cm, was rich in peptide components. After extraction of zone B with water and freeze drying the extract, the residue was filtered on a Sephadex G-75 column ($V_0 + V_i$ = 170 ml) in water (Figure 6). The low molecular weight fraction (elution volume, 130–155 ml) contained 45% of the total hexosamine of the cell wall digest. It was freeze dried and filtered on Sephadex G-25, medium grade ($V_0 + V_i$ = 130 ml), in water. This procedure again yielded three fractions: fraction 1 (elution volume, 65–83 ml; 18% of the total hexosamines content of the digest), fraction 2 (83–95 ml; 13% of the hexosamines), fraction 3 (95–115 ml; 9% of the hexosamines), in higher yield than had been obtained previously. All three fractions contained only oligosaccharides which were devoid of peptide substituents. Only fraction 3 gave a positive Morgan–Elson reaction after 30 min of heating in borate. This fraction was further purified by preparative paper chromatography in solvent A (R_F 0.42) and finally, by filtration on Sephadex G-25 in water. It yielded the disaccharide GlcNAc-MurNAc. Fraction 2 was similarly further purified by paper chromatography in solvent A (R_F 0.28) and filtration on Sephadex G-25, yielding the tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc. Fraction 1 was not further purified. It has been designated as "mixed oligosaccharides." It contained, as shown by paper chromatography in solvent C, three oligosaccharides with R_{GlcNac} 0.07, 0.19, and 0.38. These data suggest that they could be, respectively, octasaccharide (50%), hexasaccharide (25%), and tetrasaccharide (25%). These estimates of the fractions of the total hexosamines of fraction 1 were determined by extracting each oligosaccharide from the relevant bands (after detection on marker strips) and measuring the total hexosamine content of each extract.

Preparation of the Trisaccharides MurNAc²-GlcNAc¹-MurNAc and GlcNAc³-MurNAc²-GlcNAc, of the Disaccharide MurNAc²-GlcNAc, and of a Mixture of the Disaccharides GlcNAc³-MurNAc and GlcNAc¹-MurNAc by Enzymatic Degradations of the Isolated Tetrasaccharide GlcNAc³-MurNAc²-GlcNAc¹-MurNAc. A. PREPARATION OF THE TRISACCHARIDE MURNAC²-GLCNAC¹-MURNAC. Pure tetrasaccharide (3.88 μmoles) GlcNAc³-MurNAc²-GlcNAc¹-MurNAc was treated at 37° with 500 μl of the pig epididymis *exo*-β-*N*-acetylglucosaminidase in a final volume of 840 μl of 0.025 M citrate buffer, pH 4.2, in the presence of NaCl (0.15 M) and of bovine serum albumin (0.015%). The extent of color development in the Morgan–Elson reaction after 7 min of heating in borate indicated that the reaction was complete after 3 hr. Filtration of the digest on Sephadex G-25, bead form ($V_0 + V_i$ = 56 ml), yielded free GlcNAc (elution volume, 50 ml) and trisaccharide (elution volume, 35 ml). The trisaccharide, after freeze drying of the fraction, was further purified by paper chromatography in solvent A (R_F 0.40). It was extracted from the paper with water and the lyophilized eluate was submitted to a final purification by filtration on the Sephadex G-25 bead form (yield, 1.68 μmoles).

TABLE I: Characterization of Free Oligosaccharides from Cell Walls of *M. lysodeikticus*.

Oligosaccharide[c]	Chemical Compn in % of Total Hexosamines: Before Reduction GlcN[a]	MurN[a]	After Reduction HexN[a]	GlcN[a]	Action of[b] *exo*-β-GlcNAcase	Solvents: A, R_F	B, R_F	C, R_{GlcNAc}
A (isolated from cell wall digests).								
X-Y	51.9	55.5	50	50	2.1	0.39	0.44	0.82
X^3-Y^2-X^1-Y	52	51.4	75	50.3	4.24	0.25	0.32	0.38
X-Y-X-Y-X-Y-X-Y	49.6			45.6	8.00		0.18	0.07
Y-X	46.5		46	0	—	0.38		
B (isolated after further degradation of the tetrasaccharide X^3-Y^2-X^1-Y)								
X^3-Y^2-X	64.3		63.3	33	2.83	0.26		
Y^2-X^1-Y	33		65	33	—	0.41		
X^3-Y and X^1-Y					2.00	0.39		
Y^2-X	40		50	0	—	0.40		

[a] GlcN = glucosamine; MurN = muramic acid; HexN = total hexosamine. [b] Moles of total hexosamine which give rise to 1 mole of free GlcNac on hydrolysis with the *exo*-β-GlcNAcase. In the case of samples indicated by a dash, no GlcNAc was released by this enzyme. [c] X = *N*-acetylglucosamine, Y = *N*-acetylmuramic acid.

B. PREPARATION OF THE TRISACCHARIDE GLCNAC[3]-MURNAC[2]-GLCNAC AND OF THE DISACCHARIDE MURNAC[2]-GLCNAC. The tetrasaccharide GlcNAc[3]-MurNAc[2]-GlcNAc[1]-MurNAc (8.2 μmoles) was treated with 300 μg of lysostaphin in a final volume of 2 ml of 0.01 M phosphate buffer, pH 7.5. After 20 hr of incubation at 37°, the color development of the digest in the Morgan–Elson reaction, after 7 min of heating in borate buffer, was equivalent to 1 mole of free *N*-acetylhexosamine/mole of original tetrasaccharide. The digest, however, was more complex than expected and its analysis showed that some *exo*-β-acetylglucosaminidase activity, liberating GlcNAc from the nonreducing end of the tetrasaccharide, was present with the large amount of enzyme and prolonged incubation employed. Filtration of the digest on a column of Sephadex G-25, fine grade ($V_0 + V_i$ = 50 ml), in water yielded two fractions. Fraction 1, with an elution volume of 46–54 ml, was a mixture of free MurNAc (91%) and free GlcNAc (9%). Fraction 2, with an elution volume of 37–46 ml, was, after freeze drying, submitted to a preparative paper chromatography in solvent A. Two components of R_F 0.25 and 0.41 were extracted from the paper with water. These two eluates were freeze dried and the material was then filtered in water on Sephadex G-25 fine grade. The component with R_F 0.25 yielded trisaccharide GlcNAc-MurNAc-GlcNAc (elution volume 25–35 ml, 3.6 μmoles). The component with R_F 0.41 yielded free *N*-acetylglucosamine (elution volume, 50–60 ml) and disaccharide MurNAc-GlcNAc (elution volume, 40–48 ml; 1.44 μmoles).

C. PREPARATION OF THE DISACCHARIDES GLCNAC[3]-MURNAC AND GLCNAC[1]-MURNAC. Preliminary experiments showed that tetrasaccharide GlcNAc[3]-MurNAc[2]-GlcNAc[1]-MurNAc is not completely hydrolyzed by lysozyme. Tetrasaccharide (30 mμmoles) was treated at 37°, for 16 hr, with 0.2, 2, and 20 μg of lysozyme in 20 μl of 0.01 M phosphate buffer, pH 6.2. On the basis of the color development of the digests when the Morgan–Elson reaction was applied after 30 min of heating in borate, the extents of the hydrolysis were found equal to 0, 35, and 60%, respectively. Tetrasaccharide (3.4 μmoles) was treated for 21 hr with 2.5 mg of lysozyme in 2.5 ml of the phosphate buffer. By filtration of the digest on a column of Sephadex G-25, bead form (1.5 × 125 cm), a mixture of the disaccharides GlcNAc[3]-MurNAc and GlcNAc[1]-MurNAc (elution volume, 103–124 ml; yield, 4.5 μmoles) was separated from the residual tetrasaccharide (elution volume, 80–90 ml). When reincubated with fresh lysozyme, this tetrasaccharide fraction was again partially hydrolysed into disaccharides, as was the original tetrasaccharide. It has also been observed that none of the fractions collected after filtration on Sephadex of the lysozyme-treated tetrasaccharide was lytic for *M. lysodeikticus*, although lysozyme which has not been incubated with tetrasaccharide does not lose its lytic activity when filtered under the same conditions. These observations could conceivably be explained by the binding of disaccharides to the active sites of the enzyme (Johnson and Phillips, 1965).

Characterization of the Oligosaccharides (Tables I–II)

Chromatography. Each oligosaccharide, about 100 mμmoles, gave one component on paper chromatography in solvents A–C, detectable with the diphenyl-

TABLE II: Characterization of Free Oligosaccharides from Cell Walls of *M. lysodeikticus*.

Compound[a]	Reducing Power (rel color yields)	Molar Extinction Coefficients (585 mμ) in the Morgan–Elson Reaction	
		b	*c*
X	1	20,000	14,000
X-Y	1.53	3,500	9,500
X-Y-X-Y	1.17	Very low	1,000
X-Y-X-Y-X-Y-X-Y	0.89	Very low	Very low
Y-X	0.71	Very low	1,600
Y		19,000	13,500
Y-X-Y		750	1,100
X-Y-X		Very low	1,500

[a] X = *N*-acetylglucosamine; Y = *N*-acetylmuramic acid. [b] This column heated in borate for 7 min. [c] This column heated in borate for 30 min.

amine–trichloracetic acid reagent or by fluorescence after alkali treatment.

Chemical Composition. Each oligosaccharide gave rise on acid hydrolysis to equal amounts of glucosamine and muramic acid (Table I). After hydrolysis with 3 N HCl for 3 hr at 95°, the two hexosamines were detected with ninhydrin spray after two-dimensional chromatography in solvents A and D. The total hexosamine contents of the hydrolysates were determined using the Morgan–Elson reaction, with 7 min of heating in borate, after chemical reacetylation. Glucosamine was measured as GlcNAc after its specific acetylation with the yeast D-glucosamine 6-phosphate *N*-acetylase. Muramic acid was estimated as the difference between total hexosamine and glucosamine. In some cases, the glucosamine was specifically deaminated with the *E. coli* glucosamine deaminase and then muramic acid was measured as MurNAc after chemical acetylation. None of these hydrolysates contained D-glucose. Each oligosaccharide was also hydrolyzed with 6 N HCl for 15 hr at 100° and the free amino acids were estimated by thin layer chromatography on silica gel after dinitrophenylation. The contaminations by amino acids varied from 0 to 4% relative to hexosamine on a molar basis.

$NaBH_4$ Reduction. Samples (30 μl) of each oligosaccharide (100–400 mμmoles of total hexosamines) were mixed with 3 μl of a fresh 1 M $NaBH_4$ solution. After 4 hr at room temperature, 6 μl of 1 N HAc was added. After freeze drying, 50 μl of absolute methanol was added and the solutions were evaporated. This treatment (to remove boric acid) was repeated three times. The residues were then hydrolyzed with 3 N HCl for 3 hr at 95°. Total hexosamines and glucosamine were measured as above. By comparison of these analyses with those of the nonreduced oligosaccharides, the size of the oligosaccharides and the nature of the reducing groups were determined (Table I).

Incubation with exo-β-N-Acetylglucosaminidase from Pig Epididymis (exo-β-GNAcase). About 50 mμmoles

TABLE III: Periodate Oxidation of the Reduced Oligosaccharides from the Cell Walls of *M. Lysodeikticus*.[a]

Oligosaccharide[b]	Periodate Consumption and Formaldehyde Production				Glucosamine Content (after Hydrolysis)		
	After 5–10 min of Oxidation		After 30 hr of Oxidation				
	IO_4 Uptake	HCHO Produced	Addnl IO_4 Uptake	Addnl HCHO Produced	Before Reduction	After Reduction	After Reduction and 30 hr of Oxidation
A (isolated from cell wall digests)							
X-Y	0.97	1.05	1.01	0	1.08	1	0.22
X^3-Y^2-X^1-Y	0.97	0.94	1	0	2.06	2.06	1.39
X-Y-X-Y-X-Y-X-Y	0.95	1.12	1.17	0	3.97	3.60	2.80
Y-X	0.94	0.96	0	0	0.93	0	0
B (isolated after further degradation of the tetrasaccharide X^3-Y^2-X^1-Y)							
X^3-Y^2-X	1.16	1.12	0.93	0	1.93	1.00	0.19
Y^2-X^1-Y	1.01	0.97	0	0	0.99	0.99	1.00
X^3-Y and X^1-Y	0.90	1.04	1.25	0	1.00	1.00	0.12
Y^2-X	1.06	0.99	0	0	0.80	0	0

[a] Results are expressed as moles of periodate, formaldehyde, and glucosamine per mole of oligosaccharide. [b] X = *N*-acetylglucosamine; Y = *N*-acetylmuramic acid.

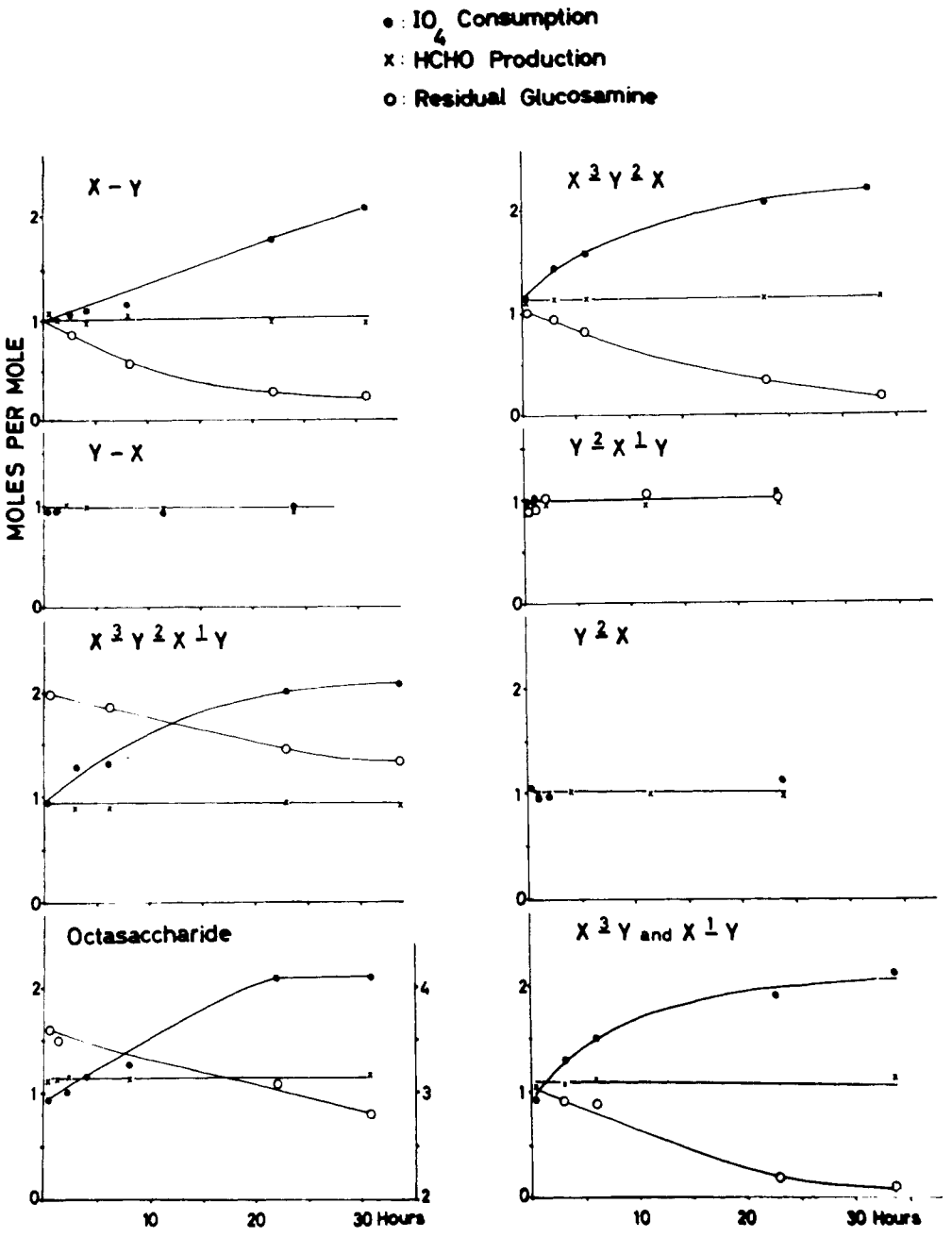

FIGURE 7: Periodate oxidation of reduced oligosaccharides from cells walls of *M. lysodeikticus*. At the indicated times, aliquots were taken for the determination of periodate consumption, formaldehyde production, and residual glucosamine (after hydrolysis). The right-hand ordinate for octasaccharide applies only to residual glucosamine.

of each oligosaccharide was treated with 17 μl of the enzyme preparation in a final volume of 30 μl of 0.04 M citrate buffer, pH 4.2. Liberation of free *N*-acetylglucosamine was complete after 3 hr of incubation at 37°. This was determined by applying to the digests the Morgan–Elson reaction with 7 min of heating in borate under which conditions the color yields from the nontreated oligosaccharides are small (Table II). This enzyme specifically removes the GlcNAc residue β-glycosidically linked at the non-reducing end of the oligosaccharides and therefore provides further evidence of the size of the oligosaccharide (Table I).

Morgan–Elson Reaction and Reducing Power. Molar extinction coefficients (ε) in the Morgan–Elson reaction with 7 and 30 min of heating in borate and reducing power, relative to GlcNAc, were also determined for

each oligosaccharide (Table II). Only the disaccharide GlcNAc-MurNAc gives a high color yield in the Morgan–Elson reaction and has a high reducing equivalent. This has been ascribed to an unusual lability of the glycosidic linkage under the alkaline conditions of these reactions (Tipper *et al.*, 1965). It is noteworthy that higher oligosaccharides with MurNAc as the reducing group (MurNAc-GlcNAc-MurNAc and GlcNAc-MurNAc-GlcNAc-MurNAc) do not behave in this manner.

Periodate Oxidation of the Oligosaccharides (Table III and Figure 7). About 150 mμmoles of each oligosaccharide was treated with 10 μl of 0.2 M $NaBH_4$ for 4 hr at room temperature. The pH was adjusted to 5 with 0.2 M acetic acid (about 10 μl). Sodium periodate (4.50 μl 0.1 M) was added and the volumes were adjusted to 50 μl with water. Oxidation was allowed to proceed at room temperature in the dark and aliquots were withdrawn at intervals for the determination of periodate consumption, formaldehyde production, and residual *N*-acetylglucosamine.

PERIODATE CONSUMPTION. An aliquot of each sample was diluted to 100 μl with water and the absorption of the solution was measured at 224 mμ. The results were calculated with reference to an oxidized ethylene glycol standard which gave the reported differential molar extinction coefficient for the oxidation of periodate to iodate. The above conditions of oxidation (150 mμmoles of reduced saccharide oxidized with 450 mμmoles of periodate) were used in order to ensure minimum errors in the determination of the consumed periodate. That complete oxidation occurred in these conditions was checked by oxidizing 90 mμmoles of reduced saccharide with 450 mμmoles of periodate for 6 and 24 hr.

FORMALDEHYDE PRODUCTION. An aliquot of each sample was mixed with 20 μl of 0.2 M sodium arsenite and, after 15 min at room temperature, with 100 μl of the chromotropic acid reagent. The solutions were heated for 30 min in a boiling water bath and cooled to room temperature, and the adsorptions were measured at 570 mμ. Oxidized ethylene glycol was used as a formaldehyde standard.

RESIDUAL *N*-ACETYLGLUCOSAMINE. Periodate present in the aliquot was destroyed by addition of 0.02 M ethylene glycol. After acid hydrolysis, glucosamine was acetylated enzymatically. GlcNAc was determined using the Morgan–Elson reaction.

All reduced saccharides consumed 1 mole of periodate within 5–10 min with formation of 1 mole of formaldehyde (Figure 7, Table III). No further uptake of periodate or formation of formaldehyde was observed with oligosaccharides in which the nonreducing end group was MurNAc (*e.g.*, MurNAc-GlcNAc and MurNAc-GlcNAc-MurNAc). A second mole of periodate was consumed slowly and without production of formaldehyde by oligosaccharides in which the nonreducing end was GlcNAc. With this latter group of oligosaccharides, one GlcNAc slowly disappeared with a rate similar to the rate of uptake of the second mole of periodate. This rate is itself identical with the rate of oxidation of *p*-nitrophenyl-β-*N*-acetylglucosaminide.

Discussion

Structure of the Isolated Oligosaccharides

Disaccharide X-Y.[2] *exo*-β-GlcNAcase completely hydrolyzes this disaccharide into its component *N*-acetylhexosamines. $NaBH_4$ destroys 50% of the total hexosamines but none of the glucosamine (Table I). The reduced disaccharide consumes 1 mole of periodate rapidly with concomitant formation of 1 mole of formaldehyde. A second mole of periodate is consumed slowly with parallel destruction of the glucosamine (Table III). These data are compatible only with a 1,4-linked disaccharide and with the GlcNAc as nonreducing group and in the pyranose ring form (Figure 8). If the GlcNAc residue were in the furanose ring form and the linkage were 1,6, oxidation would have given rise to the fast uptake of 1 mole of periodate with production of 1 mole of formaldehyde and with destruction of glucosamine, and then to the slow uptake of a second mole of periodate by destruction of the muramicitol residue. If the GlcNAc residue were in the furanose ring form and the linkage were 1,4, oxidation would have given rise to the fast uptake of 2 moles of periodate with production of 2 moles of formaldehyde. A 1,6 linkage with GlcNAc in the pyranose ring form is similarly excluded (Figure 8).

Disaccharide Y-X. exo-β-GlcNAcase has no action on this disaccharide. $NaBH_4$ destroys 50% of the total hexosamines and 100% of the glucosamine (Table I). The reduced disaccharide consumes rapidly 1 mole of periodate with production of 1 mole of formaldehyde and these values are not modified on prolonged oxidation (Table III). These data are compatible only with a 1,4-linked disaccharide with the MurNAc as the reducing group in the pyranose ring form (Figure 8). If MurNAc were in the furanose ring form, this latter residue would have consumed 1 mole of periodate and would have produced 1 mole of formaldehyde; therefore, the total consumption of periodate and the total production of formaldehyde would have been, respectively, 3 and 1 for a 1,6 link, 2 and 2 for a 1,4 link, and 3 and 2 for a 1,3 link.

Tetrasaccharide X^3-Y^2-X^1-*Y. exo*-β-GlcNAcase liberates one GlcNAc from four total hexosamine residues. $NaBH_4$ destroys 25% of the total hexosamines but none of the glucosamine (Table I). GlcNAc, therefore, is at the nonreducing end and MurNAc at the reducing end of a tetrasaccharide. The complete sequence and the nature of the links were demonstrated by periodate oxidation of the tetrasaccharide and by the isolation and characterization by periodate oxidation of the saccharides prepared by enzymatic degradation of the tetrasaccharide, *i.e.*, the "left" trisaccharide X^3-Y^2-X, the "right" trisaccharide Y^2-X^1-Y, the "internal" disaccharide Y^2-X, and the mixture of "left"

[2] X = GlcNAc; Y = MurNAc.

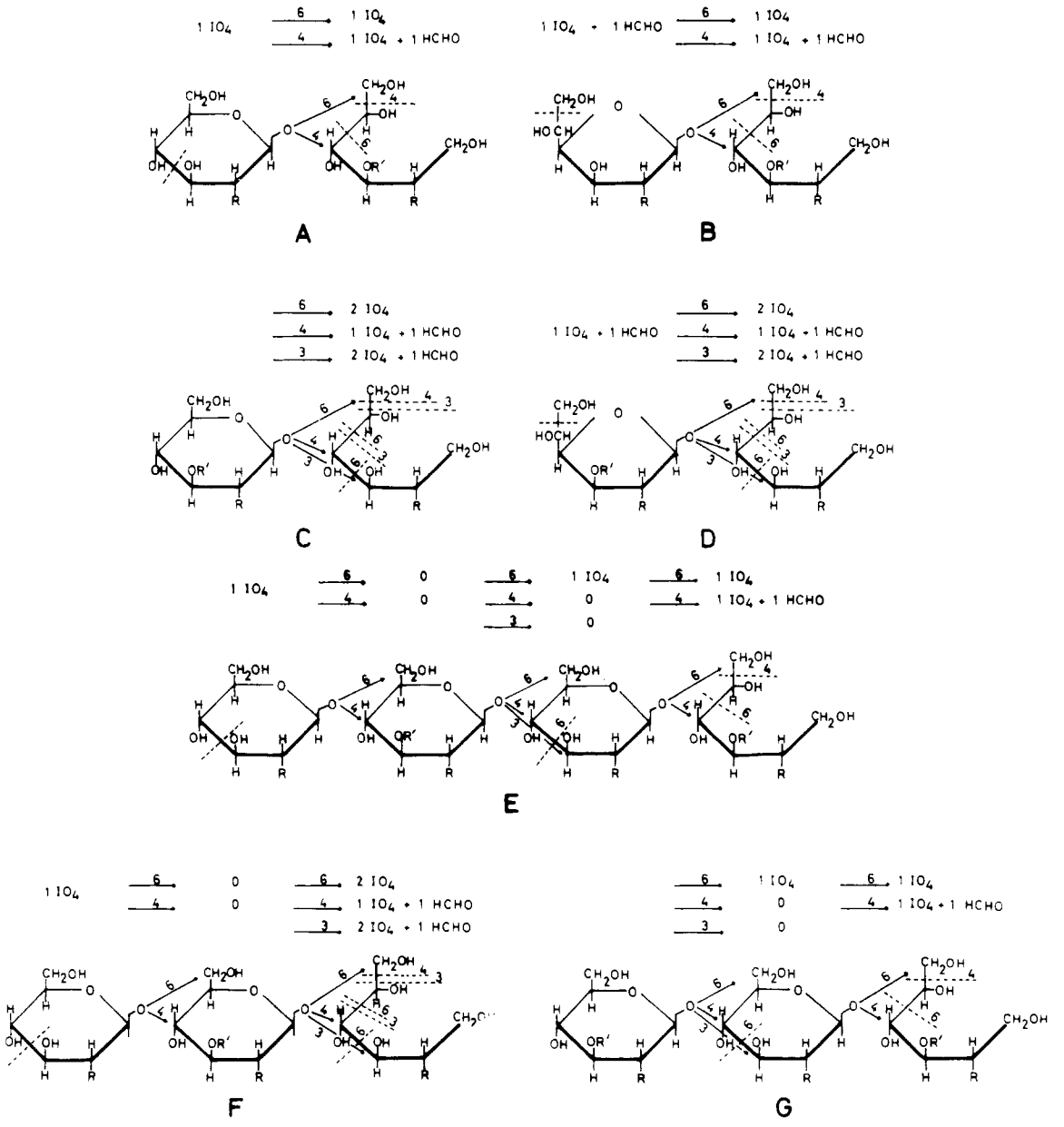

FIGURE 8: Theoretical periodate oxidation of reduced oligosaccharides composed of alternating GlcNAc and MurNAc residues. The theoretical contribution to the periodate uptake and to the formaldehyde production of each *N*-acetylhexosamine residue in the various oligosaccharides is shown for all possible types of glycosidic linkages. In the case of X-Y and Y-X, the structures have the nonreducing sugar in the pyranose ring form (Figure 8A, C) and in the furanose ring form (Figure 8B, D). The theoretical oxidation for each type of linkage (numbered arrows) can be calculated by following the relevant dotted lines. (A and B): disaccharide X-Y; (C and D): disaccharide Y-X; (E): tetrasaccharide X^3-Y^2-X^1-Y; (F): trisaccharide X^3-Y^2-X; (6); trisaccharide Y^2-X^1-Y. R = $NHCOCH_3$; R′ = $CH_3CHCOOH$.

and "right" disaccharides X^3-Y and X^1-Y. Disaccharides Y^2-X and X^3-Y + X^1-Y have the same characteristics as the equivalent disaccharides Y-X and X-Y directly isolated from the cell wall digest. *exo*-β-GlcNAcase liberates one GlcNAc from three total hexosamine residues in the case of trisaccharide X^3-Y^2-X and has no action on trisaccharide Y^2-X^1-Y (Table I). $NaBH_4$ destroys one hexosamine out of three in both trisaccharides; this hexosamine is GlcNAc in trisaccharide X^3-Y^2-X and is not GlcNAc in trisaccharide Y^2-X^1-Y (Table I). Periodate oxidations (Table III and Figure 8) of reduced tetrasaccharide X^3-Y^2-X^1-Y and of reduced trisaccharide Y^2-X^1-Y indicate that link 1 is 1,4 and link 2 is 1,3 or 1,4, but not 1,6; oxidation of reduced

disaccharide Y^2-X and reduced trisaccharide X^3-Y^2-X indicates that link 2 is 1,4. Finally, oxidation of the mixture of reduced disaccharides X^3-Y and X^1-Y indicates that both links 3 and 1 are 1,4. Therefore, the alternating sequence X-Y-X-Y in the tetrasaccharide with all of the sugars linked 1,4 is established.

Octasaccharide X-Y-X-Y-X-Y-X-Y. *exo*-β-GlcNAcase liberates one GlcNAc from eight total hexosamines residues. $NaBH_4$ does not affect the GlcNAc content of the compound (Table I). Reduced saccharide (1 mole) consumes 1 mole of periodate rapidly with production of 1 mole of formaldehyde and slowly a second mole of periodate with destruction of 25% of the glucosamine content. Therefore, MurNAc and GlcNAc are at the reducing and the nonreducing end, respectively, of an octasaccharide. The X-Y link at the reducing end is certainly 1,4; no formaldehyde would be produced if it were 1,6. None of the Y-X links can be 1,6, since this would result in consumption of additional periodate and destruction of additional GlcNAc.

Anomeric Configuration of the Glycosidic Linkages. Hydrolysis by the *exo*-β-GlcNAcase, which is devoid of activity on α-*N*-acetylglucosaminides, establishes that the GlcNAc is linked in the β configuration at the nonreducing end of disaccharide X-Y, tetrasaccharide X^3-Y^2-X^1-Y, and trisaccharide X^3-Y^2-X. Link 1 in the tetrasaccharide X^3-Y^2-X^1-Y is also in the β configuration since the mixture of disaccharides X^3-Y and X^1-Y is completely hydrolyzed into free *N*-acetylhexosamines by *exo*-β-GlcNAcase. Disaccharide Y-X, directly isolated from the walls, or disaccharide Y^2-X, isolated after further degradation of the tetrasaccharide X^3-Y^2-X^1-Y, are resistant to the *exo*-β-GlcNAcase. However, tetrasaccharide X^3-Y^2-X^1-Y is split into disaccharides by lysozyme which is known to hydrolyze the central β linkage in tetra-*N*-acetylchitotetraose. This evidence that lysozyme splits β linkages supports the hypothesis that in the oligosaccharides from cell walls of *M. lysodeikticus* the links from MurNAc to GlcNAc are also in the β configuration. It is hoped that this can be established by other means.

Structure of the Oligosaccharide Chains in Cell Walls of M. lysodeikticus

Cell walls (1 mg) of *M. lysodeikticus* contain 920 mμmoles of total hexosamines, *i.e.*, 460 mμmoles of disaccharide units. After successive treatment of the cell walls with the *Myxobacter* enzyme and with lysostaphin, the actual yield in purified disaccharide Y-X was 54%, in terms of total hexosamines. Before any fractionation was applied, the cell wall digest had reducing groups equivalent to 225 mμmoles of GlcNAc (Figure 1). Since 1 mole of disaccharide Y-X has a reducing power equivalent to 0.71 mole of GlcNAc (Table II), it can be concluded that free disaccharides Y-X amounted in the digest to about 340 mμmoles/mg of walls. Therefore, *at least* 74% of the *N*-acetylmuramyl-*N*-acetylglucosaminyl linkages in the cell wall polysaccharide are 1,4, probably in the β configuration.

After exhaustive digestion of 1 mg of cell walls with lysozyme, 570 mμmoles of reducing groups, relative to GlcNAc, was liberated (Figure 1). Since disaccharide X-Y is the end product of the lysozyme digestion and since it is equivalent in the reducing power determination to 1.5 GlcNAc (Table II), it can be concluded that in this case complete hydrolysis of the cell wall polysaccharide has induced the appearance of about 380 mμmoles of X-Y disaccharide units. Therefore, at least 83% of the *N*-acetylglucosaminyl-*N*-acetylmuramic acid linkages in the walls is 1,4 and in the β configuration. The isolated disaccharide X-Y was obtained with a much lower yield for two reasons. First, in order to isolate not only disaccharides but also larger oligosaccharides, the lysozyme incubation was stopped as soon as the clarification of the walls had occurred and long before the release of reducing groups was maximum (Figure 1). Second, in order to estimate the occurrence of nonpeptide-substituted segments in the cell wall polysaccharide, the lysozyme digest was not treated with *N*-acetylmuramyl-L-alanine amidase. In terms of total hexosamine residues, the actual yields in disaccharide X-Y and tetrasaccharide X-Y-X-Y were, respectively, 4.5 and 10%, employing the first procedure of fractionation, and 9 and 13%, employing the second procedure of fractionation. Using this latter procedure, a third preparation (designated "mixed oligosaccharides") containing free tetra-, hexa- and octasaccharides was also obtained which represented 18% of the total hexosamine content of the walls. These data indicate that at least 40% of the disaccharide units in the polysaccharide of *M. lysodeikticus* cell walls is not substituted with peptide. This finding has been confirmed by degrading the cell walls with the *Streptomyces* F_1 *endo*-*N*-acetylmuramidase (Muñoz *et al.*, 1966) and is in accord with the extent of conversion of the COOH groups of muramic acid residues into CH_2OH groups by direct esterification on the intact cell walls and then reduction with $LiBH_4$ (Salton, 1961). From the foregoing it can be concluded that the glycan portion of the cell walls of *M. lysodeikticus*, or at least the greatest part of it, consists of linear chains made up of 1,4-linked alternating GlcNAc and MurNAc residues. Both GlcNAc and MurNAc residues are in the pyranose ring form. The linkages GlcNAc-MurNAc and probably the linkages MurNAc-GlcNAc are in the β configuration. At least, 40% of the MurNAc residues have their carboxyl groups free.

Comparative Structure of Cell Walls of M. lysodeikticus and S. aureus

The glycan portions of the cell walls of *M. lysodeikticus* and of *S. aureus* present marked differences. In *S. aureus*, about 50% of the muramic acid residues have *O*-acetyl groups on C_6 and virtually all of them are substituted with peptides (Ghuysen and Strominger, 1963a,b). Both properties may contribute to the lysozyme resistance of *S. aureus*. Indeed although the *O*-acetylation of cell walls of *M. lysodeikticus* (Brumfitt, 1959) does make them resistant to lysozyme, the removal of these groups from the cell walls of *S. aureus* or from the insoluble peptidoglycan freed from

teichoic acid by trichloracetic treatment does not render them very lysozyme sensitive (this *O*-acetylpeptidoglycan can be hydrolyzed using large amounts of lysozyme; Mandelstam and Strominger, 1961). The distribution of disaccharide units without peptide substitution in the *M. lysodeikticus* polysaccharide chains is unknown. It is clear, however, that they can form large segments reaching the size of octasaccharides. This low order of cross-linking in cell walls of *M. lysodeikticus* occurs not only at the polysaccharide–peptide junctions but also in the peptide moiety itself, since less than 50% of the lysine residues have their ϵ-amino groups engaged in peptide cross-linking (Petit *et al.*, 1966). This also contrasts with the virtual absence of free amino groups in the cell wall peptidoglycan of *S. aureus* (Ghuysen *et al.*, 1965). The peptidoglycan of *M. lysodeikticus* is, therefore, a much looser network. This may explain why the solubilization of the cell walls with an *endo-N*-acetylhexosaminidase is brought about by the hydrolysis of a few glycosidic bonds in the case of *M. lysodeikticus* and requires the hydrolysis of all sensitive glycosidic bonds in the case of *S. aureus* (Ghuysen and Strominger, 1963a).

Finally, it must be recalled, as already pointed out (Salton, 1961), that in order to accommodate the molar ratios glucosamine:muramic acid:Ala:Glu:Lys:Gly found in cell walls of *M. lysodeikticus* roughly equal to 1:1:2:1:1:1 and to accommodate the nonsubstituted disaccharide units of the glycan moiety, some of the hypothetical peptide subunits, L-Ala-D-Glu-γ-L-Lys-D-Ala with Gly substituted on the α-COOH group of glutamic acid (D. J. Tipper, K. Kato, D. Jarvis, W. Katz, J. L. Strominger, and J. M. Ghuysen, unpublished data), must somehow form larger peptide units.

References

Browder, H. P., Zygmut, W. A., Young, J. R., and Tavormina, P. A. (1965), *Biochem. Biophys. Res. Commun. 3*, 383.

Brown, D. H. (1962) *in* Methoden der Enzymatischen Analyse, Bergmeyer, H. U., Ed., Weinheim, Verlag Chemie, p 151.

Brumfitt, W. (1959), *Brit. J. Exptl. Pathol. 40*, 441.

Dixon, J. S., and Lipkin, D. (1954), *Anal. Chem. 26*, 1092.

Ensign, J. C., and Wolfe, R. S. (1966), *J. Bacteriol. 91*, 524.

Ghuysen, J. M., Leyh-Bouille, M., and Dierickx, L. (1962), *Biochim. Biophys. Acta 63*, 286.

Ghuysen, J. M., and Strominger, J. L. (1963a), *Biochemistry 2*, 1110.

Ghuysen, J. M., and Strominger, J. L. (1963b), *Biochemistry 2*, 1119.

Ghuysen, J. M., Tipper, D. J., Birge, C. H., and Strominger, J. L. (1965), *Biochemistry 4*, 2245.

Ghuysen, J. M., Tipper, D. J., and Strominger, J. L. (1966), *Methods Enzymol. 8* (in press).

Hough, L., Jones, J. K. N., and Wadman, W. H. (1950), *J. Chem. Soc.*, 1702.

Jeanloz, R. W., Sharon, N., and Flowers, H. M. (1963), *Biochem. Biophys. Res. Commun. 13*, 20.

Johnson, L. N., and Phillips, D. C. (1965), *Nature 206*, 761.

Leyh-Bouille, M., and Tipper, D. J. (1965), *Bull. Soc. Chim. Biol. 47*, 1905.

Mandelstam, M. H., and Strominger, J. L. (1961), *Biochem. Biophys. Res. Commun. 5*, 466.

Muñoz, E., Ghuysen, J. M., Leyh-Bouille, M., Petit, J. F., and Tinelli, R., *Biochemistry 5*, 3091 (this issue; following paper).

Perkins, H. R. (1960), *Biochem. J. 74*, 182.

Petit, J. F., Muñoz, E., and Ghuysen, J. M. (1966), *Biochemistry 5*, 2764.

Risse, H. J., and Lüderitz, O. D. (1964), *Biochem. Z. 3*, 411.

Salton, M. R. J. (1961), Proceedings of the 2nd Symposium on Fleming's Lysozyme, Milan, April, Vol. II, p 1/Xii.

Salton, M. R. J., and Ghuysen, J. M. (1960), *Biochim. Biophys. Acta 45*, 355.

Sanderson, A. R., Strominger, J. L., and Nathenson, S. G. (1962), *J. Biol. Chem. 237*, 3603.

Sharon, N. (1964), Proceedings of the 3rd Symposium on Fleming's Lysozyme, Milan, April, 44/RT.

Sharon, N., Osawa, T., Flowers, H. M., and Jeanloz, R. W. (1966), *J. Biol. Chem. 241*, 223.

Suzuki, S., and Strominger, J. L. (1960), *J. Biol. Chem. 235*, 2768.

Tipper, D. J., Ghuysen, J. M., and Strominger, J. L. (1965), *Biochemistry 4*, 468.

Tipper, D. J., and Strominger, J. L. (1966), *Biochem. Biophys. Res. Commun. 22*, 48.