Proc. Natl. Acad. Sci. U. S. 56, 1661.
Koshland, D. E., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Ogilvie, J. W., and Sightler, J. H. (1968), *Biophys. J. 8*, A-90.
Patte, J. C., LeBras, G., Loviny, T., and Cohen, G. N. (1963), *Biochim. Biophys. Acta 67*, 16.
Patte, J. C., Truffa-Bachi, P., and Cohen, G. N. (1966), *Biochim Biophys. Acta 128*, 426.
Peterson, E. A., and Sober, H. A. (1956), *J. Am. Chem. Soc. 78*, 751.
Rabin, B. R. (1967), *Biochem. J. 102*, 22C.
Sanwal, B. D., Stachow, C. S., and Cook, R. A. (1965), *Biochemistry 4*, 410.
Sweeny, J. R., and Fisher, J. R. (1968), *Biochemistry 7*, 561.
Truffa-Bachi, P., van Rapenbusch, R., Janin, J., Gros, C., and Cohen, G. N. (1968), *European J. Biochem. 5*, 73.
Walter, C. (1965), Steady-State Applications in Enzyme Kinetics, New York, N. Y., Ronald, pp 175–179.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.
Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.
Zamenhof, S. (1957), *Methods Enzymol. 3*, 702.

Structure of the Cell Wall Peptidoglycan of *Lactobacillus casei* RO94*

K. D. Hungerer,† J. Fleck,‡ and D. J. Tipper§

ABSTRACT: The cell wall peptidoglycan of *Lactobacillus casei* RO94 contains almost equimolar proportions of D-glutamic acid, D-alanine, L-alanine, L-lysine, D-aspartic acid, *N*-acetylmuramic acid, *N*-acetylglucosamine, and two molar proportions of ammonia. Glycan chains of alternating, β-1,4-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid have an average chain length of about ten disaccharides and are substituted on every muramic acid residue by the pentapeptide N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanine, randomly cross-linked between D-alanine and D-isoasparagine to the extent of 55%. The *Myxobacter* AL-1 enzyme hydrolyses all of the D-alanyl-D-isoasparaginyl linkages and most of the *N*-acetylmuramyl-L-alanine linkages in the peptidoglycan resulting in the production of hexosamine-free peptide monomers and of an almost peptide-free complex of glycan and group C-specific polysaccharide. The group C-specific polysaccharide is linked to the glycan by highly acid-labile phosphodiester linkages and hydrolysis of the complex gives rise to muramic acid 6-phosphate.

The cell walls of *Lactobacillus casei*, like those of many gram-positive bacteria, contain D-glutamic acid, L-lysine, L-alanine, and D-alanine (Ikawa and Snell, 1960). These amino acids form the peptide component of UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-lysyl-D-alanyl-D-alanine which, together with variants in which L-alanine and lysine are replaced by other amino acids, is probably a biosynthetic precursor of all bacterial cell wall peptidoglycans (*cf.* Ghuysen *et al.*, 1968). The peptide moiety, lacking its C-terminal D-alanine residue, is found intact in the cell walls of several bacteria (Muñoz *et al.*, 1966; Jarvis and Strominger, 1967) and, as will be shown, not unexpectedly occurs also in the cell walls of *L. casei*.

Peptidoglycans containing this tetrapeptide are all cross-linked between D-alanine and the ε-amino group of lysine in a neighboring peptide, but differ in the nature of this link, *e.g.*, a direct linkage occurs in *Micrococcus lysodeikticus* (Petit *et al.*, 1966), pentaglycine with a variable extent of replacement by L-serine occurs in staphylococci (Tipper and Berman, 1969) and peptides of L-alanine and L-threonine occur in *Micrococcoccus roseus* (Petit *et al.*, 1966). *L. casei* cell walls contain aspartic acid (Cummins and Harris, 1956) and acid hydrolysis of these walls yields N^{ϵ}-(aminosuccinoyl)-L-lysine, a compound which is very resistant to acid hydrolysis and which is derived during hydrolysis from N^{ϵ}-(α- or β-aspartyl)-L-lysine (Swallow and Abraham, 1958). Thus drastic and prolonged hydrolysis is required if a reasonable yield of aspartic acid is to be obtained from these cell walls, and this leads to considerable racemization (Ikawa, 1964) and some destruction (see below) of the aspartic acid. Nevertheless, it is probable that at least 90% of the aspartic acid of the peptidoglycan has the D configuration (Ikawa, 1964). N^{ϵ}-(α- or β-D-aspartyl)-L-lysine therefore

* From the Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706. *Received April 7, 1969.* This investigation was supported by a U. S. Public Health Service research grant (HD 02972), a Career Research Development award (GM 38652), and a National Science Foundation research grant (GB-6416). A preliminary report of these results has been published (Hungerer, 1968).

† Present address: Max-Planck Institut für Immunobiologie, Freiburg, Germany.
‡ Present address: Institut de Bacteriologie, Strasbourg, France.
§ To whom inquiries should be addressed for reprints.

must occur in *L. casei* peptidoglycan, and by analogy with other cell wall peptidoglycans, is probably part of a cross-link between D-alanyl and L-lysyl residues in adjacent peptide subunits. The object of this paper is to test these predictions and in particular to determine the stereochemistry of the linkage of the aspartic acid residues. Information is also presented on the pattern of cross-linking of the peptide, on the structure and variation in chain length of the glycan of the peptidoglycan, and on the specificity of the *Myxobacter* AL-1 endopeptidase.

L. casei RO94 cell walls also contain a neutral polysaccharide containing glucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine (Hall and Knox, 1965) which carries the group C serological specificity (Knox and Hall, 1965a). The walls contain only 0.2% phosphorous, and at least 40% of this is involved in phosphodiester linkage of the reducing end of the neutral polysaccharide to the glycan of the peptidoglycan (Knox and Hall, 1965b). Hydrolysis of whole cells of *L. casei* 7461 walls yields muramic acid 6-phosphate (Verdier and Agren, 1959). We now find that hydrolysis of cell walls of *L. casei* RO94 with the *Myxobacter* AL-1 peptidase yields a complex of acidic glycan and neutral polysaccharide whose hydrolysis also yields muramic acid 6-phosphate. No teichoic acid has been detected in the cell walls of this organism.

Materials and Methods

Organism and Isolation of Cell Walls. Cultures of *L. casei* RO94 and preparations of cell walls used in preliminary experiments were kindly provided by Dr. K. W. Knox, Institute of Dental Research, Sydney, Australia. Cultures (30 l.) were grown at 35° in Tryptone, beef extract, glucose, yeast extract, and salts (MRS medium) as described by Rogosa and Sharpe (1960). Under these conditions, growth ceased after 25 hr, and cells were routinely harvested after 22 hr and immediately broken by shaking with glass beads (Superbrite, M & M Co., 0.18 mm) in a Nossal disintegrator for 3 min. After a second breakage, walls suspended in water were added slowly to boiling water and boiled for 15 min to destroy autolytic activities. The product was digested with ribonuclease and trypsin for 24 hr at 37° in 0.05 M KPO_4 at pH 8.0, washed twice with water, and lyophilized giving a yield of 5.3 g.

Enzymes. The *Chalaropsis* B enzyme (Hash, 1963) was a gift from Dr. J. Hash (Vanderbilt University, Nashville, Tenn.) and the *Myxobacter* AL-1 (Ensign and Wolfe, 1964, 1965) enzyme was a gift from Dr. J. C. Ensign (University of Wisconsin, Madison, Wis.). The π enzyme, a fraction of lysostaphin enriched in endo-*N*-acetylglucosaminidase activity (Browder *et al.*, 1965), was a gift from Dr. Browder (Mead Johnson & Co., Evansville, Ind.). Crystalline egg-white lysozyme was a commercial preparation from Armour & Co. *Streptomyces albus* *N*-acetylmuramyl-L-alanine amidase (Ghuysen *et al.*, 1962) was a gift from Dr. J.-M. Ghuysen (Université de Liege, Belgium). D-Lactate was determined with specific D-lactate dehydrogenase as previously described (Tipper, 1968); the enzyme was a gift from Dr. D. Dennis (Department of Biochemistry, Brandeis University, Waltham, Mass.).

N^{ϵ}-(α- and β-D-aspartyl)-L-lysine and N^{ϵ}-(Aminosuccinoyl)-L-lysine. The α-linked dipeptide was synthesized by coupling *N*-carbobenzyloxy-D-aspartic acid anhydride and L-lysine–copper complex as described by Ikawa (1964) followed by purification using paper electrophoresis at pH 1.9. The aminosuccinoyl derivative was prepared from a sample of the dipeptide hydrolyzed for 22 hr at 80° in concentrated HCl. The product was lyophilized and fractionated by preparative paper electrophoresis at pH 1.9. A sample (50 mg) of N^{ϵ}-(aminosuccinoyl)-L-lysine was hydrolyzed for 1 hr at 25° in 0.5 N NaOH (1 ml) and the products were fractionated by preparative paper electrophoresis at pH 1.9. The predominant band of N^{ϵ}-(β-D-aspartyl)-L-lysine was eluted with 0.5 M AcOH. The peptides were chromatographically and electrophoretically pure, and were identified by total and N-terminal amino acid determinations.

Analyses. Determinations of C- and N-terminal amino acids, total hexosamines, reducing power, total phosphate, and total D- and L-alanine were performed as previously described (Ghuysen *et al.*, 1966). Hydrolyses for N-terminal amino acids were 48 hr at 105° in 6 N HCl, with standards of dinitrophenylated and hydrolyzed free amino acids, N^{ϵ}-(β-aspartyl)-lysine and N^{α}-(glycyl)-lysine. Hexosamines were determined after hydrolysis for 8 hr at 100° in 3 N HCl using a Beckman-Spinco amino acid analyzer, and total amino acids were determined on the analyzer after hydrolysis for 40 hr at 115° in 6 N HCl (for lysine and aspartic acid) and for 18 hr at 105° in 6 N HCl for other amino acids. Under the latter conditions of hydrolysis, unhydrolyzed N^{ϵ}-(aminosuccinoyl)-L-lysine gave rise to a shoulder on the trailing edge of the lysine peaks. This shoulder was absent under the former conditions. Controls for recovery of lysine and aspartic acid were hydrolyzed N^{ϵ}-(β-aspartyl)-lysine and aspartic acid hydrolyzed under varying conditions with and without samples of peptidoglycan components from *Staphylococcus aureus* cell walls. Under standard conditions the recovery of aspartic acid was 93%. Chain lengths of glycan fractions were determined by the yield of formaldehyde on periodate oxidation following reduction with $NaBH_4$, as previously described (Tipper *et al.*, 1967a). Edman degradations were performed essentially as previously described (Tipper *et al.*, 1967b). Total hexoses were determined by a micro modification of an anthrone procedure (Shields and Burnett, 1960). Peptides in the eluates from Dowex 50 chromatography were quantitated by the ninhydrin procedure of Moore and Stein (1948), using alanine as standard.

Chromatography and Electrophoresis. Gel filtration on columns of Sephadex G-25 and G-50 (fine grade, Pharmacia, Uppsala, Sweden, 2 × 100 cm) in series was performed at room temperature using 0.1 M LiCl as eluent, as previously described (Tipper and Strominger, 1968). Similar columns and columns of Bio-Gel P2 (Bio-Rad, Richmond, Calif.; 1.5 × 75 cm) were also eluted with water at room temperature where indicated. For each column V_0 = the elution volume, V_e, of Blue Dextran (Pharmacia), $V_0 + V_i$ = the V_e for NaCl, and $K_D = (V_e - V_0)/V_i$. Columns of DEAE-Sephadex A-25 (1.5 × 50 cm) were prewashed with 1.0 M LiCl and water before use. After elution of unretarded material with several bed volumes of water, bound materials were eluted with a linear gradient of increasing LiCl concentrations up to 1.0 M. Peptides were fractionated on columns of Dowex 50 (Beckman resin A4, 2 × 20 cm) packed and operated at 50° using a modification of the elution system described by Jones (1964), which uses a gradient from pyridine–acetate buffers, pH 3.0 (buffer C) to pH 5.0 (buffer D). The column was washed with buffer D and then equilibrated with buffer B, a mixture of three volumes of buffer C and two volumes of buffer A (20 ml of

TABLE I: Composition of Cell Walls of *L. casei* RO94: Amino Acids and Amino Sugars.[a]

Lysine	98
Aspartic acid	78
Alanine	220
Ammonia	213
Glucosamine	97
Muramic acid	87
Galactosamine	220

[a] Data are expressed as moles per 100 moles of total glutamic acid. Cell walls (1 mg) were hydrolyzed with *Chalaropsis* B enzyme (0.05 mg) in 0.02 M NaOAc (pH 4.5, 100 μl) for 4 hr at 37°, sufficient time to give almost complete solubilization. Aliquots (15 μl) of the product were hydrolyzed in 3 and 6 N HCl as described in Methods. Ammonia determinations were derived from the 3 N HCl hydrolysate, with an unhydrolyzed aliquot as control for nonamide ammonia. Other amino acids present in amounts of 3–6 moles/100 moles of glutamic acid were glycine, threonine, serine, valine, and leucine. All other amino acids were present in smaller amounts. N-Terminal data (see below) indicate that about 10% of the peptide subunits is devoid of aspartic acid, so that the predicted content of aspartic acid is about 0.9 mole/mole of glutamic acid. However lower amounts were consistently found, possibly due to destruction during vigorous hydrolysis.

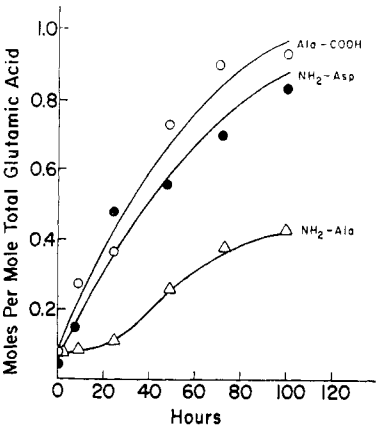

FIGURE 1: Kinetics of hydrolysis of *L. casei* cell walls with the *Myxobacter* AL-1 enzyme. Cell walls (11.3 mg) were incubated with AL-1 enzyme (1 mg/ml, 50 μl) in 0.03 M sodium barbital (pH 9.0, 1 ml). Aliquots of the supernatant (25 μl) were removed at intervals for the determination of C- and N-terminal amino acids. Samples of cell walls were also hydrolyzed with the *Chalaropsis* B enzyme (see below) for determination of initial C- and N-terminal amino acids. These included 0.1 mole of N^{ϵ}-terminal lysine and 0.07 mole of C-terminal lysine. These did not increase during AL-1 digestion, and no other N- or C-terminal amino acids other than those indicated were detected.

90% formic acid/l.). The first gradient was from buffer B (250 ml) to buffer C (250 ml), the second from buffer C (210 ml) to a mixture of buffer C (140 ml) plus buffer D (70 ml), and the third gradient was from this same mixture (200 ml) to buffer D (200 ml).

Quantitative and preparative paper electrophoresis was carried out on Whatman No. 3MM paper using buffer A (pH 1.9; pyridine–acetic acid–water, 15:50:2000, pH 3.9), buffer E (pH 4.6, pyridine–acetic acid–water, 1:1:80), and 0.05 M $Na_2B_4O_7$ (pH 9.2) at 30 V/cm using a Gilson Model D solvent-cooled high-voltage apparatus (Gilson Medical Electronics, Wis.). Peptide bands were eluted with 0.2 M acetic acid and subsequently purified by fractionation on Bio-Gel P2 to remove contaminants derived from the paper. Peptides, amino acids, and hexosamines were detected by dipping in ninhydrin reagent (0.2% ninhydrin, 0.5% pyridine, and 1% 2,4,6-trimethylpyridine in 95% ethanol) and heating at 70°. Oligosaccharides were detected by fluorescence after heating with NaOH as described by Sharon (1964). Peptides were also fractionated on thin-layer plates of silica gel G by elution with isobutyric acid–1 N triethylamine (3:2, v/v) and detected by spraying with the above ninhydrin reagent and heating at 80°.

Results

Analyses of Cell Walls. The cell walls contained 0.35 μmole of glutamic acid per mg, and their analyses are given in Table I. The group C-specific polysaccharide (C-antigen), isolated free of peptidoglycan components (see below), contained galactosamine, but no significant amounts of glucosamine. Thus the cell wall peptidoglycan contained 1 mole of glucosamine/mole of glutamic acid. However, Knox and Hall (1965a) isolated oligosaccharides containing glucosamine from the C-antigen of strain RO94. A culture of the organism used in the present studies was very kindly compared with the original strain by Dr. Knox, and found to be indistinguishable on the basis of fermentation tests with 18 sugars. Extracts of the two strains also reacted indistinguishably with specific antisera. At present we have no explanation of this discrepancy.

Kinetics of Hydrolysis of L. casei Cell Walls by Myxobacter AL-1 Enzyme. The results of hydrolysis of *L. casei* cell walls with the AL-1 enzyme are shown in Figure 1. Relatively rapid and parallel release of N-terminal aspartic acid and C-terminal alanine was followed, after a distinct lag, by the slower release of N-terminal alanine without further release of C-terminal amino acid. Since the peptide products have C-terminal D-alanine and N-terminal L-alanine and aspartic acid (see below), the rapid reaction corresponds to the hydrolysis of D-alanyl-D-aspartic acid peptide cross-links and the slower reaction to hydrolysis of *N*-acetylmuramyl-L-alanine linkages, resulting, as will be shown, in the production of hexosamine-free peptide monomers.

Fractionation of the Peptide Products of Hydrolysis of L. casei Cell Walls with AL-1 Enzyme. Cell walls (1.03 g, 360 μmoles of total glutamic acid) were hydrolyzed with AL-1 enzyme (20 mg) in 0.05 M sodium barbital buffer (pH 9.0, 50 ml) at 37°. Determination of N-terminal amino acids at intervals (*cf.* Figure 1) showed slow release of N-terminal aspartic acid and alanine. After 90 hr the product was centrifuged (20 min at 10,000*g*). The pellet (315 mg) contained amino acids of the peptidoglycan in normal ratios (*cf.* Table I) but contained only 30 μmoles of glutamic acid. Much of the pellet remained insoluble after HCl hydrolysis and consisted of inorganic material derived from the medium. Thus

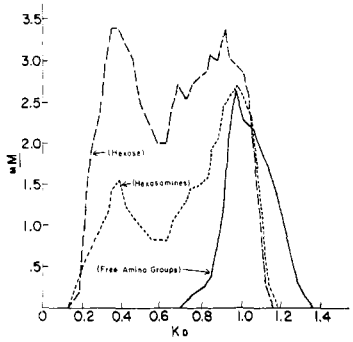

FIGURE 2: Fractionation on Sephadex G-50 of *L. casei* cell walls solubilized by hydrolysis with AL-1 enzyme. The supernatant, concentrated to 4 ml, was applied to a column of Sephadex G-50 (V_0 = 120 ml, V_i = 190 ml) and eluted with water. Aliquots (50 μl) were analyzed for free amino groups (———), for total hexosamines (·····), and for hexoses by the anthrone procedure (– – – –).

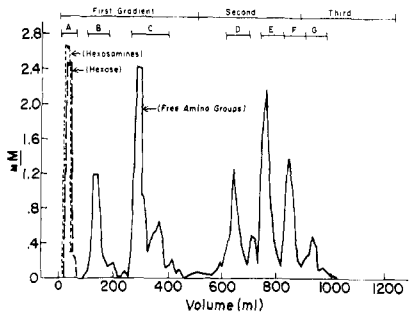

FIGURE 3: Fractionation on Dowex 50 of fraction II from Sephadex G-50 (Figure 2). The fraction was concentrated to 5 ml, adjusted to pH 2 with formic acid, and applied to a column of Dowex 50 and eluted as described in Methods. The volumes of the first, second, and third gradients were as indicated. Aliquots of fractions were analyzed for free amino groups (ninhydrin), hexoses (anthrone), and hexosamines (symbols as in Figure 2). The fractions indicated by bars A and G were pooled.

the solubilized material contained 326 μmoles of glutamic acid, 92% of the total peptidoglycan. The supernatant was fractionated on a column of Sephadex G-50 with the results shown in Figure 2. Two peaks of anthrone-positive material (C-antigen) and hexosamines (C-antigen and glycan) were found, the second overlapping a single peak containing all of the free amino groups and centered about K_D = 1 (totally included material). Fractions in the K_D range from 0 to 0.8 were pooled and concentrated (fraction 1). Fraction 1 contained, per mole of muramic acid, 1.0 mole of glucosamine, 2.9 moles of galactosamine, and 0.2 mole of glutamic acid. An aliquot (0.5 ml) of the solution (10 ml) of fraction 1 was fractionated on a column of DEAE-Sephadex A-25. Water (120 ml) eluted only small amounts of hexosamine, and a single broad peak of hexose (anthrone) and hexosamines was eluted between 0.1 and 0.4 M LiCl. Since the C-antigen is neutral and is unretarded by the column (see below), this fraction is thus a covalently linked complex of C-polysaccharide and glycan. It contained 40% of the muramic acid of the original cell walls and only 3% of their original peptide complement, indicating efficient *N*-acetylmuramyl-L-alanine amidase action by the AL-1 enzyme.

The second peak (fraction II, K_D = 0.8–1.3) is apparently a mixture of lower molecular weight fractions of a similar complex mixed with the peptides released by amidase action. This fraction was pooled, concentrated, and fractionated on Dowex 50 with the results shown in Figure 3. The glycan–C-antigen complex was eluted immediately in a fraction (A) virtually free of peptide. Peptide fractions B to G were analyzed qualitatively by paper electrophoresis at pH 1.9 and 3.9. Fraction B contained inorganic salt and was purified by refractionation on the same Dowex 50 column using the first gradient system. A single sharp peak (B_1) reacting with ninhydrin was separated from minor fractions and salts. Fractions B_1, C, D, E, F, and G were individually fractionated on Bio-Gel P2 columns (V_0 = 85 ml, V_i = 90 ml) eluted with buffer E. Each gave a single peak (mean K_D values 0.25–0.45) reacting with ninhydrin, except for C which gave two peaks, C_1 and C_2. C_2 was eluted at K_D = 1 and contained no amino acids detectable by paper electrophoresis after hydrolysis. It apparently consisted of ammonium salts and was discarded. The peptides in the pooled peaks were analyzed by paper electrophoresis at pH 1.9, 3.9, and 4.6, and by thin-layer and paper chromatography. Fraction B_1 contained a mixture of minor peptides acidic at pH 4.6, and one neutral band. C_1 and D carried a slight positive charge at pH 3.9, and peptides E, F, and G were positively charged at pH 3.9 and 4.6. Peptides C_1, D, and E gave single bands under all conditions while F and G each gave two bands, most clearly separated at pH 4.6. Peptides B_1, F, and G were fractionated by preparative paper electrophoresis at pH 4.6. B_1 gave three distinct bands of decreasing negative charge (B_α, B_β, and B_γ) and one neutral band (B_δ); F gave bands F_1 and F_2, mobility relative to lysine (at pH 4.6) 0.52 and 0.59, respectively, and G gave rise to bands G_1 and G_2, mobilities 0.70 and 1.0, respectively. The mobility of peptide E at pH 4.6 was 0.48. Amino acid analysis showed G_2 to be free lysine and it was discarded. Peptides F_1, F_2, and G_1 were fractionated on Bio-Gel P2 eluted with buffer E as before. Again each gave a single peak detectable with ninhydrin. Each now gave a single band on electrophoresis at pH 1.9, 3.9, and 4.6 and on thin-layer chromatography and is apparently homogeneous.

Analyses and Edman Degradation of the Peptides from AL-1 Digestion of L. casei Cell Walls. Proportions and analyses of the six major peptide fractions are presented in Table II. The total recovery of glutamic acid, allowing for analytical losses, was about 250 μmoles, 77% of the amount solubilized by the AL-1 enzyme.

All peptides contained, per mole of glutamic acid, 1 mole of L-alanine and about 1 mole of N-terminal alanine. Each also contained about 1 mole of N-terminal aspartic acid or N^ϵ-terminal lysine, and so all are uncross-linked monomers. After one cycle of Edman degradation, all peptides lost their N-terminal alanine which was replaced by 1 mole of N-terminal glutamic acid. At the same time, all of their L-alanine but none of their D-alanine was lost, indicating that all peptides contained the sequence (NH_2)-L-alanyl-D-glutamic acid. All peptides also contained 1 mole of lysine, which in peptide G_1 had its ε-amino group and carboxyl groups free, indicating N^α-(D-glutamyl)-L-lysine. The single mole of ammonia in this peptide must be on glutamic acid. In the second round of

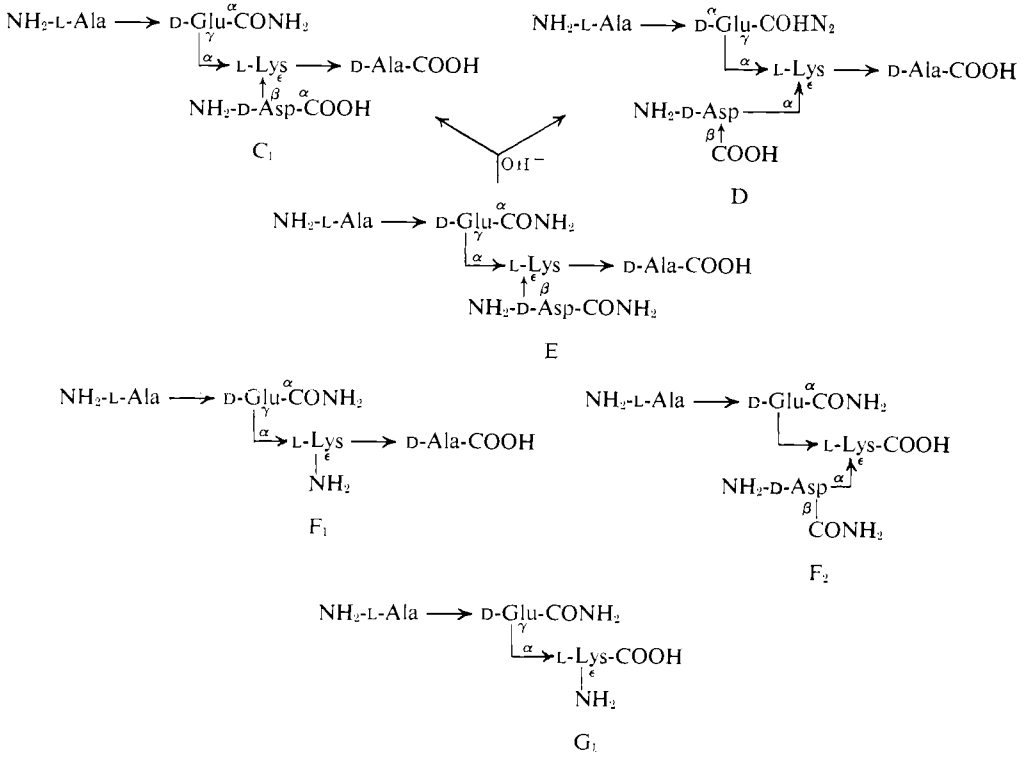

FIGURE 4: Structures of the neutral and basic peptides produced by hydrolysis of *L. casei* cell walls with AL-1 enzyme.

TABLE II: Analyses of Peptides from AL-1 Hydrolysate of *L. casei* Cell Walls.[a]

	% of Total Glu	Total Lys	Total Asp	Total Ala	D-Ala	L-Ala	NH_3	NH_2 Ala	NH_2 Asp	ϵ-NH_2 Lys	CO-OH Ala	CO-OH Lys	Color with Ninhydrin
C_1	46.5	73	84	200	88	112	95	96	83	0	64	9	Brown-blue
D	12	75	87	210	94	108	103	103	82	0	53	2	Deep blue
E	33	77	91	210	96	110	208	106	87	0	69	5	Blue-green-grey
F_1	2.5	75	8	200	82	102	114	92	5	75	64	4	Blue
F_2	4.5	91	100	140	0	124	132	103	69	0	0	37	Blue-green-grey
G_1	1.5	80	2	100	0	108	87	95	0	88	0	67	Blue

[a] All data are presented as moles per 100 moles of total glutamic acid. All peptides were free of hexosamines. Samples of unhydrolyzed peptide served as controls for ammonia content. The low molar proportions of lysine and aspartic acid are probably due to incomplete hydrolysis of N^{ϵ}-(aspartyl)-lysine linkages. The low yield of free lysine from hydrazinolysis of peptide F_2 is probably due to the acylation of both of its amino groups, since recovery of singly acylated amino acids was only about 65% (as in peptide G_1).

of Edman degradation, all peptides lost their N-terminal glutamic acid without release of α-*N*-terminal lysine, indicating γ-D-glutamyl-L-lysine linkage. Peptide G_1 is thus N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysine (Figure 4), and should thus have a net positive charge at pH 4.6, consistent with its electrophoretic mobility at this pH.

Peptide F_1 was identical with peptide G_1, except that, like peptides C_1, D, and E_1, it contained an additional mole of C-terminal D-alanine, as shown by the results of Edman degradation (above) and the C-terminal and total amino acid analyses (Table II). Peptide F_1 is thus N^{α}-(L-alanyl-D-isoglutamyl)-L-lysyl-D-alanine (Figure 4) which again is positively charged at pH 4.6.

The remaining four peptides, C_1, D, E, and F_2, each contained 1 mole of N-terminal aspartic acid instead of lysine, and each therefore contained N^{ϵ}-(aspartyl)-lysine. Peptides E and F_2 contain 2 moles of ammonia and therefore contain α- or β-aspartylamide. After one cycle of Edman degradation, all N-terminal aspartic acid (and alanine) disappeared in both E and F_2, but only glutamic acid appeared. Controls of N^{ϵ}-(α- and β-D-aspartyl)-L-lysine behaved as expected on Edman degradation, only the α analog giving rise to free lysine.

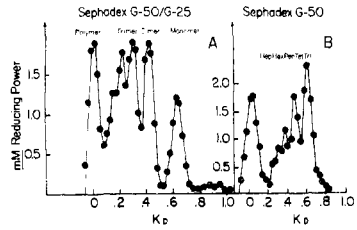

FIGURE 5: Gel filtration of glycopeptides produced by hydrolysis of *L. casei* cell walls with *Chalaropsis* B enzyme. The whole supernatant from hydrolysis was concentrated to 2 ml and applied in 0.1 M LiCl to a column of Sephadex G-50 (2.5 × 100 cm) connected by a polyethylene capillary to a column of Sephadex G-25 (2.5 × 100 cm). The combined values for V_0 and V_i were 380 and 480 ml, respectively, and figures given for K_D are for the combined columns. Fractions (4 ml) were collected each 15 min, and aliquots (3 μl) were analyzed for reducing power (A). Since each peptide subunit carries a disaccharide, reducing power measures the concentration of peptide subunits and the curve represents the distribution of oligomers by weight. Aliquots were also analyzed for hexose (not shown), all of which occurred in the excluded peak at $K_D = 0$ (C-antigen-glycopeptide complex). Fractions (4 ml) were combined as follows: K_D = 0.5–0.75 (monomer), 0.35–0.5 (dimer), 0.1–0.35, higher oligomers, and −0.1–0.1, C-antigen–glycopeptide complex. Fractions were concentrated to 2 ml and applied to a column (1.5 × 75 cm) of Bio-Gel P2 eluted with water. All reducing power eluted in salt-free peaks, K_D = 0–0.4. All fractions but monomer and dimer were combined, concentrated, and refractionated in 0.1 M LiCl on a column of Sephadex G-50 (2.5 × 100 cm) connected in series to a second column of Sephadex G-50 (1.5 × 120 cm). Aliquots (5 μl) of the fractions were analyzed for reducing power (B). Again figures quoted for K_D are for the combined columns. Fractions pooled were K_D = 0.7–0.54 (trimer), 0.2–0.54 (higher oligomers), and −0.1–0.2, (glycopeptide–C-antigen complex).

Thus both peptides E and F_2 contain N^{ϵ}-(D-isoasparaginyl)-L-lysine. Since peptide E has C-terminal D-alanine and peptide F_2 has C-terminal lysine, these peptides are, respectively, N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanine and the same peptide without the C-terminal D-alanine residue (Figure 4). Again both are positively charged at pH 4.6.

Peptides C_1 and D contain only 1 mole of ammonia and are neutral at pH 4.6. This mole of ammonia was retained after one cycle of Edman degradation and is therefore on glutamic acid. At the same time, peptide D lost its N-terminal L-alanine and D-aspartic acid and gained both N-terminal glutamic acid and N^{ϵ}-terminal lysine, the product being positively charged at pH 4.6. Peptide D is thus N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}-(α-D-aspartyl)-L-lysyl-D-alanine (Figure 4). Peptide C_1 had identical analyses except that no N^{ϵ}-terminal lysine was released on Edman degradation. It is thus the β-D-aspartyl analog of peptide D (Figure 4).

Action of Alkali on Peptide E. N^{ϵ}-(β-D-Isoasparaginyl)-lysyl peptides are very sensitive to alkali and are rapidly converted into a 1 to 4 mixture of N^{ϵ}-(α- and β-D-aspartyl)-lysyl peptides, presumably *via* the alkali-labile N^{ϵ}-(aminosuccinoyl)-lysyl peptide intermediate (Ghuysen *et al.*, 1967; Swallow and Abraham, 1958). In confirmation of this fact, treatment of peptide E (1 μmole) for 1 hr at 37° in 0.05 N NaOH (100 μl and 5 μmoles) resulted in complete conversion into a mixture of peptides C_1 and D. The products were fractionated by paper electrophoresis at pH 3.9, eluted with 0.1 M acetic acid, and analyzed for total amino acids after hydrolysis. Each contained 1 mole each of aspartic acid, glutamic acid, lysine, and ammonia, and 2 moles of alanine. The ratio of C_1 to D was 4.5 to 1, similar to their ratio in the products of AL-1 hydrolysis (Table II). Incubation of peptide E (0.25 μmole) for 24 hr under the conditions of AL-1 hydrolysis (37° in 0.05 M sodium barbital buffer, pH 9.0, 100 μl) also resulted in partial conversion into a mixture of peptides C_1 and D in about the same ratio, as shown by paper electrophoresis. Thus peptides C_1 and D are probably artifacts of base-catalyzed degradation of peptide E during isolation (see below). Peptide F_2 was converted into a mixture of two peptides, neutral at pH 4.6, by incubation for 1 hr at 37° in 0.05 N NaOH. The failure to isolate these products from the AL-1 digestion probably derives from the low total amount of peptide F_2 (Table II).

TABLE III: Analyses of the Acidic Peptides from AL-1 Hydrolysis of *L. casei* Cell Walls.[a]

Peptide	Total μmoles of Glutamate	Total Lys	Total Asp	Total Ala	Total D-Ala	Total L-Ala	NH_2 Ala	NH_2 Asp	D-Lactate
B_α	0.5	87	90	110	10	90	5	55	—
B_β	1.8	90	66	180	100	96	11	130	76
B_γ	1.0	80	87	200	98	110	7	95	100
B_δ	2.2	82	75	190	92	110	0	110	60

[a] Data are presented as moles per 100 moles of total glutamic acid. — indicates not determined.

Analyses of the Minor Acidic Peptides. The peptides in fraction B were all present in small amounts (Table III), and their analyses probably reflect partial purity (Table III). In contrast to the neutral and basic peptides, none contained much N-terminal alanine and three at least contained D-lactate. They are presumably D-lactyl peptide β-elimination products from peptide subunits attached to reducing end groups of *N*-acetylmuramic acid in the cell wall peptidoglycan, and so are artifacts of the prolonged incubation at pH 9.0 (*cf.* Ghuysen *et al.*, 1967; Tipper, 1968). B_α had C-terminal lysine, and the other peptides C-terminal alanine. Their analyses and electrophoretic mobilities indicate that B_δ, B_α, and B_γ are probably D-lactyl derivatives of E, C_1, and D, respectively, while B_α lacks C-terminal D-alanine.

Hydrolysis of L. casei Cell Walls with the Chalaropsis B N-Acetylmuramidase and Fractionaton of the Products. The *Chalaropsis* B enzyme (Hash, 1963) hydrolyzes all of the *N*-acetylmuramyl linkages in cell walls of *S. aureus* (Tipper *et al.*, 1964), *Staphylococcus epidermidis* (Tipper and Berman, 1969), and *Arthrobacter crystallopietes* (Krulwich *et al.*, 1967). This resulted in each case in the production of a soluble glycopeptide consisting of *N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides linked to the L-alanine N termini of the peptide subunits of the peptidoglycan, which retained their peptide cross-links intact. A portion of this glycopeptide in each case was covalently linked to teichoic acid and the rest consisted of a series of oligomers of the disaccharide-peptide subunit which could be fractionated on col-

TABLE IV: Analyses of *L. casei* Glycopeptide Oligomers.[a]

Fraction	Lys	Asp	NH_3	Ala	NH_2 Asp	NH_2 Lys	CO-OH Ala	CO-OH Lys	Mur	GlcN	GalN	Di-saccharide	Chain Length	% of Total Glutamic Acid
Monomer	78	68	167	143	63	30	62	40	86	92	0	99	1.05	15
Dimer	98	96	168	189	35	12	36	8	86	83	0	92	2.1	18
Higher oligomers	91	81	183	191	18	5	*b*	*b*	109	104	4	90	3–7	47
C-Antigen–glycopeptide complex	102	98	*b*	177	26	5	*b*	*b*	94	96	372	94	3.5	20

[a] Data are expressed as moles per 100 moles of total glutamic acid. Disaccharide was measured by both reducing power and 30-min Morgan–Elson procedures, using disaccharide-peptide from *S. aureus* as standard. For all four fractions, as for the *S. aureus* glycopeptide, the ratio of reducing power to 30-min Morgan–Elson color (with *N*-acetylglucosamine as standard for both) was 2.7–3.0, characteristic of 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid. This demonstrates the absence of larger oligomers of this disaccharide, which give very little Morgan–Elson color (*cf.* Ghuysen *et al.*, 1966). The chain length is the number of linked pentapeptide subunits, as deduced from the total N-terminal data. Apart from traces of glycine and serine, other amino acids were essentially absent. The total N terminal is 0.4 per glutamic acid indicating an approximate average chain length of 2.5 peptide subunits. Total C- and N-terminal lysine is 0.07 and 0.11 per glutamic, respectively. Total N-terminal aspartic acid is 0.29 per glutamic. [b] Not determined.

umns of Sephadex G-50 and G-25 connected in series (*cf.* Tipper and Strominger, 1968) showing the pattern of cross-linking within the original cell walls. This procedure was followed with *L. casei* cell walls with the additional aim of solubilizing the peptidoglycan at a low pH at which rearrangement of the isoasparaginyl residues should not occur, so that their true proportion in the cell walls could be determined.

Cell walls (818 mg) were incubated at 37° in 0.03 M sodium acetate (pH 4.5, 45 ml) with *Chalaropsis* B enzyme (0.6 mg). Reducing power of aliquots of (1 μl) was determined at intervals, and was 75% of maximum after 2 hr. After 18 hr release was 94% complete and the solution was opalescent. Additional enzyme (0.5 mg) was added and release of reducing power was complete at 30 hr by which time remaining insoluble material had precipitated. At 40 hr the solution was centrifuged. The pellet (47 mg) consisted of inorganic material and protein and was free of hexosamines. The supernatant was concentrated to 2 ml and fractionated on Sephadex G-50 and G-25 in 0.1 M LiCl with the results shown in Figure 5A. Fractions were pooled as indicated and desalted on Bio-Gel P2 columns. The complex with C-antigen was recombined with the oligomers up to trimer (K_D = 0.35) and refractionated on Sephadex G-50 with the results shown in Figure 5B. Again all of the hexose (and galactosamine, see below) occurred in the initial peak of excluded material (C-antigen–glycopeptide complex). Peaks were named as indicated and the total reducing power in each fraction was integrated. The complex with C-antigen contained 20% of the total reducing power (including monomer and dimer), the remainder being distributed among the glycopeptide oligomers as indicated in Figure 6. The number-average chain length, calculated from this distribution, is 2.2 peptide-disaccharide subunits. Analyses of the pooled fractions from Figure 5A are given in Table IV. All fractions contained one *N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide (and no N-terminal alanine) per glutamic acid residue, and lost all muramic acid on reduction, demonstrating that *N*-acetylmuramidase action had been complete. The absence of significant amounts of free disaccharide or of tetrasaccharide peptides indicates that substitution of muramic acid by peptide in the original peptidoglycan was also complete. In contrast to the glycopeptide monomer produced from *S. aureus* (Tipper and Strominger, 1965), that from *L. casei* does not contain an extra mole of D-alanine, and in fact has 40% C-terminal lysine. All of its aspartic acid is N terminal, and while aspartic acid is the predominant N-terminal amino acid, about one-third of the monomers and 11% of the total peptide subunits have N^{ϵ}-terminal lysine. These data are consistent with the structures and proportions of peptides produced by AL-1 digestion (Table II, Figure 4) and initial analyses of cell wall N-terminal groups. The total C- and N-terminal groups show the chain lengths of the fractions to be 1 and 2 for the monomer and dimer fractions, and higher for the others, the over-all average chain length being 2.5, close to that deduced from the

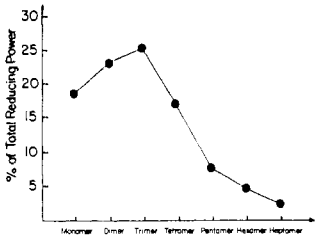

FIGURE 6: Distribution of total reducing power among oligomers of *L. casei* glycopeptide. Total reducing power in monomer to heptamer (Figure 5A,B) is called 100%. Because resolution of pentamer and higher oligomers is incomplete, the proportions for these oligomers are inexact although their combined contribution is accurate.

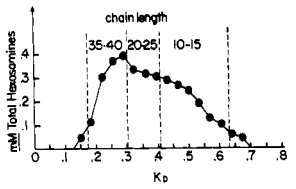

FIGURE 7: Fractionation on Sephadex G-50 of the glycan from *L. casei* cell walls. The glycan, isolated from the dilute H_2SO_4 hydrolysate of the complex with C-antigen, was applied to a column (1.5 × 100 cm) of Sephadex G-50 and eluted with water. Aliquots (100 μl) of the fractions (6 ml) were analyzed for total hexosamines, and fractions K_D = 0.17–0.3, 0.3–0.4, and 0.4–0.65 were pooled.

TABLE V: Analyses of Glycan Fractions from Sephadex G-50 (Figure 7).[a]

K_D	Mur	Glu	Ala	Asp	Mur-6-P	Total P
0.17–0.3	102	9	13	7	5	16
0.3–0.4	101	10	15	8	5	15
0.4–0.05	99	25	35	13	15	18

[a] Data are expressed as moles per 100 moles of glucosamine. Lysine was not determined. Muramic acid 6-phosphate (Mur-6-P) was assumed to give the same ninhydrin color yield as muramic acid for calculation of the amino acid analyzer data.

fractionation pattern in Figure 5. The total ammonia content is approximately equal to the total glutamic acid plus aspartic acid contents, indicating complete amidation of both in all fractions. This is consistent with the neutrality of all glycopeptide fractions on electrophoresis at pH 3.9 and 4.6.

Hydrolysis of the fraction containing higher glycopeptide oligomers with AL-1 enzyme at pH 9.0 led to the parallel release of C-terminal alanine and N-terminal aspartic acid, as for the whole cell walls. Release of N-terminal alanine was much slower. As expected, incubation of the monomer fraction with AL-1 enzyme resulted only in the slow release of N-terminal alanine without release of C-terminal amino acid.

Fractionation of the Glycopeptide Monomer and Identification of the Constituent Peptides. Peptides E and F_2, which have N-terminal isoasparagine, initially gave grey-green spots with the ninhydrin reagent described in Methods, while peptides F_1 and G_1, which have N^{ϵ}-terminal lysine, gave blue spots. These N-terminal groups should have pK's of about 8.9 and 9.5–10.5, respectively, and so the mixture of glycopeptide monomers was subjected to electrophoresis in borate buffer (pH 9.0). A negatively charged yellow band was separated from a neutral blue band. After elution, as expected, the blue band was found to have exclusively N^{ϵ}-terminal lysine and the yellow band to have exclusively N-terminal aspartic acid, the latter containing 60% of the total reducing power. (Lactyl peptide B_β also gave a yellow color with the ninhydrin reagent.) The eluted peptides each gave two identically colored bands on thin-layer chromatography in isobutyric acid–1 N triethylamine (3:2, v/v) followed by detection with the ninhydrin reagent. These presumably correspond, in each case, to peptides with C-terminal alanine and lysine. Aliquots (1 μmole) of the eluates from borate electrophoresis were incubated for 6 hr at 37° in 0.01 M sodium acetate (pH 5.2, 80 μl) containing sufficient *S. albus* amidase to hydrolyze them completely. Thin-layer chromatography and electrophoresis of the products and identification of their components by mobility and color with ninhydrin showed that the blue band gave rise to F_1 and G_1, while the yellow band gave rise to E and F_2, as expected. Thus the glycopeptide monomer contains disaccharide-L-alanyl-D-isoglutamyl-L-lysine with N-terminal lysine or isoasparagine and C-terminal lysine or alanine in the four possible combinations. It does not, however, contain any α- or β-aspartyl residues since no peptide C or D was produced. After one cycle of Edman degradation of the three glycopeptide fractions, all N-terminal aspartic acid and lysine disappeared without release of new N-terminal lysine, indicating that all aspartyl linkages are β. It can thus be concluded that all of the aspartic acid in *L. casei* peptidoglycan exists as isoasparagine residues.

Fractionation of Glycan from C-Antigen and Determination of Chain Length. Hydrolysis of the *L. casei* glycan–C-antigen complex (6 N HCl, 1 hr, 120°) and fractionation on Dowex 50 as described by Liu and Gotschlich (1967) led to the isolation of muramic acid 6-phosphate (about 12% of total muramic acid) identified by elution time on the amino acid analyzer and hydrolysis to muramic acid by *E. coli* alkaline phosphatase. The phosphodiester linkages between C-antigen and peptidoglycan in *L. casei* RO94 are readily hydrolyzed by dilute H_2SO_4 (Knox and Hall, 1965b), and glycan was isolated as follows: 0.66 N H_2SO_4 (1 ml) was added to an aliquot (5 ml) of the solution (10 ml) of the complex. After hydrolysis for 22 hr at 60°, neutralization with $Ba(OH)_2$ and centrifugation, the supernatant was concentrated and fractionated on DEAE-Sephadex A-25. All of the hexose (anthrone) and part of the hexosamine was eluted with water. This material contained galactosamine, but no glucosamine or muramic acid, and so consists entirely of C-antigen. The rest of the hexosamine was eluted in a broad peak between 0.3 and 0.7 M LiCl. These fractions were concentrated to dryness and extracted with 100% EtOH. The residue was washed twice with 100% EtOH and redissolved in water (2 ml). The EtOH supernatants contained virtually no hexosamine. The residue therefore contained most of the glycan of the sample hydrolyzed. It contained muramic acid and glucosamine in the ratio of 1:0.99, no galactosamine, and 0.14 mole of total phosphate/mole of muramic acid, 80% of which was released as inorganic phosphate on incubation with *E. coli* alkaline phosphatase. This glycan fraction was fractionated on Sephadex G-50 with the results shown in Figure 7. Three fractions were pooled as indicated, and in order of elution, accounted for 24, 32, and 44% of the total hexosamines, respectively. Their analyses are given in Table V. They each contained equimolar glucosamine and muramic acid, and, respectively, 0.1, 0.1, and 0.25 mole of peptide per mole of muramic acid, representing incomplete amidase action. An aliquot (0.6 μmole of hexosamine) of the fraction eluted at K_D = 0.17–0.3 was reincubated for 16 hr at 60° in 0.1 N H_2SO_4, neutralized, and refractionated on the G-50 column. It eluted at K_D = 0.16–0.27, and so fragmentation of the glycan during isolation was negligible.

Each of the three glycan fractions contained about 0.15

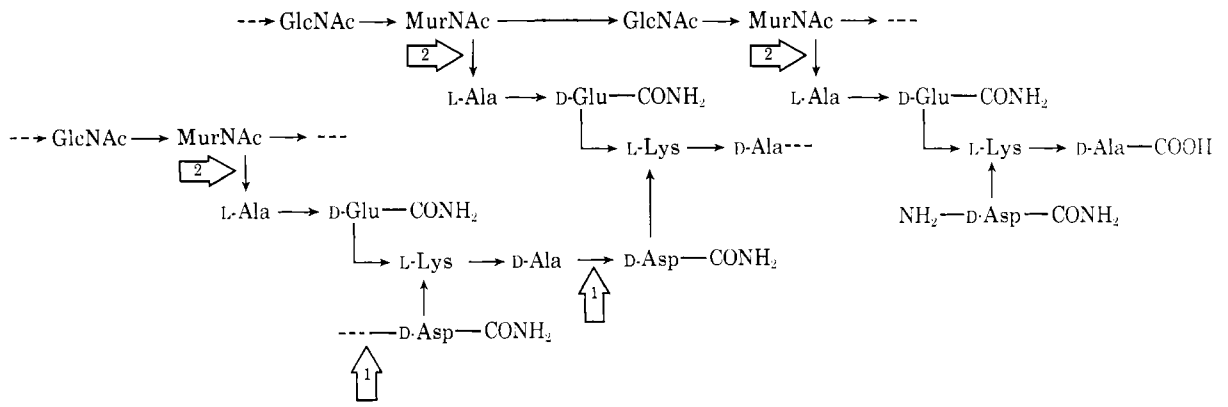

FIGURE 8: Structure of the repeating unit of the peptidoglycan of *L. casei*. One pair of peptide-cross-linked subunits, linking two adjacent glycan chains, and one of the four types of uncross-linked monomers is shown. Arrows indicate the points of hydrolysis by the *Myxobacter* AL-1 enzyme; (1) primary site of attack, (2) secondary site of attack.

mole of total phosphate per mole of muramic acid, but only one-third of this was accounted for after hydrolysis as muramic acid 6-phosphate in the fractions with higher molecular weight. Chain lengths were determined by reduction and periodate oxidation and are given in Figure 7. The over-all average chain length was 18–24 hexosamines or about 10 disaccharides.

Isolation of Disaccharides from the Glycan and Their Characterization. The glycan of *S. aureus*, stripped of its peptides, is the preferred substrate for the lysostaphin endo-β-*N*-acetylglucosaminidase (Tipper and Strominger, 1966). A sample of *L. casei* glycan (1.6 μmoles of total muramic acid) was hydrolyzed at 37° in 0.01 M KPO_4 (pH 7.5) with 21 μg of π enzyme (see Materials). Reducing power release was complete in 80 min and corresponded to 1.2 μmoles of 4-*O*-β-*N*-acetylmuramyl-*N*-acetylglucosamine (MG).[1] The product was desalted on Sephadex G-25 eluted with water, and the disaccharide peak (detected by reducing power) was pooled and concentrated.

The *S. aureus* glycan is a relatively poor substrate for lysozyme (Tipper *et al.*, 1967a), which hydrolyzes it to a mixture of di- and tetrasaccharides. A sample of *L. casei* glycan (3 μmoles of muramic acid) was hydrolyzed at 37° in 0.03 M KPO_4 (pH 6.5) with 100 μg of lysozyme. Reducing power release was complete after 3 hr and corresponded to 1.3 μmoles of 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid (GM). Disaccharide was fractionated from salt and tetrasaccharide on Sephadex G-25 eluted with water. The two products (detected with NaOH) had mobility on paper chromatography in butanol–acetic acid–water (3:1:1, v/v) and on electrophoresis at pH 3.9 identical with the authentic β-1,4-linked disaccharides from *S. aureus* cell walls (Tipper *et al.*, 1965; Tipper and Strominger, 1966). Mobilities at pH 3.9, relative to muramic acid, were 0.62 for MG and 0.73 for GM. Both disaccharides contained equimolar muramic acid and glucosamine. After reduction and hydrolysis, the product from π hydrolysis gave rise to muramic acid, glucosaminitol, and only traces of glucosamine and is, therefore, *N*-acetylmuramyl-*N*-acetylglucosamine. Conversely, the product from lysozyme digestion gave rise only to glucosamine after hydrolysis (muramicitol does not give a peak on the amino acid analyzer) and is, therefore, *N*-acetylglucosaminyl-*N*-acetylmuramic acid. Both disaccharides, after reduction, were rapidly oxidized in 10^{-3} M $NaIO_4$ at pH 4.5 to yield 1 mole of formaldehyde, indicating unsubstituted C-6 primary hydroxyl groups in the reducing moieties of both disaccharides. Finally, the molar color yields of the two disaccharides in the reducing power and 7- and 30-min Morgan–Elson procedures were identical with those of the corresponding authentic β-1,4-linked disaccharides, indicating 1,4 linkage in both since only this linkage inhibits the Morgan–Elson reaction. It can therefore be concluded that the majority, at least, of the glycoside linkages in the glycan of *L. casei* are 1,4. The susceptibility of these linkages to lysozyme, the *Chalaropsis* B enzyme, and the endo-*N*-acetylglucosaminidase of lysostaphin makes it almost certain that they have the β configuration. This is supported by the chromatographic identity of the disaccharides with the β-1,4-linked disaccharides from *S. aureus*.

Discussion

The structure of the repeating unit of the peptidoglycan of *L. casei* RO94, deduced from the present data, is shown in Figure 8. The isolation of disaccharides demonstrates the alternation of the sugar residues in the glycan. This has been done in several organisms, but only in *S. aureus* have the glycan linkages both been fully characterized as β-1,4 (*cf.* Tipper and Strominger, 1966; Tipper *et al.*, 1967a; M. Tomoeda and D. J. Tipper, unpublished results). The glycan of *Micrococcus lysodeikticus* is probably identical, though the anomericity of its *N*-acetylglucosaminidic linkages has not been conclusively demonstrated (Leyh-Bouille *et al.*, 1966). The glycan of *L. casei* is now shown to consist of alternating 1,4-linked residues of *N*-acetylglucosamine and *N*-acetylmuramic acid, probably β linked, and linked to a peptide through virtually every muramic acid residue. The cross-linked repeating unit of the peptide consists of the tetrapeptide N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine, common also to *S. aureus* and several other gram-positive organisms (Muñoz *et al.*, 1966), carrying D-isoasparaginyl residues on the ϵ-amino group of

[1] Abbreviations used, in addition to those listed in Biochemistry *5*, 1445 (1966), are: GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid; FDNB, fluorodinitrobenzene; MG, 4-*O*-β-*N*-acetylmuramyl-*N*-acetylglucosamine; GM, 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid.

its lysine residue and cross-linked between D-alanine and D-isoasparaginyl residues in adjacent subunits. The average extent of cross-linking is only 55%, resulting in a random pattern of oligomers of the peptide, with chain lengths between 1 and 4 predominating (Figure 6). *In vitro* studies with a cell-free system from *L. casei* have recently shown (Staudenbauer, 1968) that UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-lysyl-D-alanyl-D-alanine is a biosynthetic precursor of the peptidoglycan in this organism, as in *S. aureus*. D-Aspartic acid is activated as its β-phosphate in this organism and is added to the above nucleotide, forming N^{ϵ}-(β-D-aspartyl)-L-lysine linkages, in accord with the structural studies described herein. In the cell wall, the N-terminal subunits of the peptides carry predominantly N-terminal D-isoasparagine residues, but about 30% carry an N^{ϵ}-terminal lysine residue, either a result of autolysis, or of "oversight" by the enzyme which adds β-D-aspartyl residues. The C-terminal subunits carry a single residue of D-alanine or no residues on their lysine carboxyls. The C-terminal peptide subunits have therefore lost either one or two D-alanine residues, indicating the presence in *L. casei* of the appropriate carboxypeptidase activities. Such activities have previously been reported in *E. coli* (Izaki *et al.*, 1966) and in several other organisms (Izaki and Strominger, 1968). It has been suggested that these activities may control the extent of peptide cross-linking in these organisms. While in *L. casei*, no determined pattern of cross-linking is evident, only monomers and dimers occur in *E. coli*, where control of the pattern of cross-linking must also occur.

When this work was initiated, no D-aspartic acid containing peptidoglycan had been characterized. Recently published data (Ghuysen *et al.*, 1967) indicate that exactly the same peptide subunit accounts for at least 35% of the cell wall peptidoglycan of *Streptococcus faecalis*. More recently (Hungerer and Tipper, 1969), the identical subunit with no ammonia on the α-carboxyl group of its D-isoglutamyl residues has been shown to make up the repeating subunits of the cell wall peptidoglycan of *Bacillus sphaericus*.

The glycan fraction whose chain-length distribution is shown in Figure 7 was derived from the higher molecular weight fraction of the complex with C-antigen (Figure 2) and so may be deficient in smaller fragments. Nevertheless, the absence of chain lengths over 40 hexosamines is unique. Thus *S. aureus* glycan has an average chain length of 24 hexosamines (Tipper *et al.*, 1967a) but is polydisperse with fractions of both very short (average 6 hexosamines) and very long (at least 90 hexosamines) chain length. The same is true for *S. epidermidis* glycan (Tipper, 1969) and for the glycan from spherical cells of *Arthrobacter crystallopoietes* (Tipper *et al.*, 1967a). The glycan from the rod forms of this organism (Krulwich *et al.*, 1967) and from *Spirillum serpens* (Kolenbrander and Ensign, 1968) both had a much longer average chain length (about 100 hexosamines). However, the data reported here disprove a correlation between rod shape and long glycan chains. *B. subtilis* glycan also has a relatively short average chain length (25 hexosamines; Warth, 1968). The acid-labile phosphodiester linkage of the C-antigen to the peptidoglycan (Knox and Hall, 1965a,b) has been located, as expected, on the glycan, and presumably involves muramic acid since muramic acid 6-phosphate is a product of acid hydrolysis of both the walls (Verdier and Agren, 1959) and of the glycan–C-antigen complex. This confirms the recently published results of Knox and Holmwood (1968).

The bonds in *L. casei* peptidoglycan hydrolyzed by the AL-1 enzyme are indicated in Figure 8. Only one other enzyme, the *S. albus* SA endopeptidase, is known to hydrolyze D-alanyl-D-isoasparagine linkages (Ghuysen *et al.*, 1967). The AL-1 enzyme rapidly hydrolyzes D-alanylglycine, D-alanyl-L-serine, glycylglycine, and glycyl-L-serine linkages in the cell wall peptidoglycans of *S. aureus* and *S. epidermidis* (Tipper *et al.*, 1967a; Tipper and Berman, 1969). It also rapidly hydrolyzes D-alanyl-L-alanine linkages in the cell wall peptidoglycan of *A. crystallopoietes* (Krulwich *et al.*, 1967) and is now shown to hydrolyze D-alanyl-D-isoasparagine linkages, but more slowly. It hydrolyzed D-lactyl-L-alanine linkages slowest of all, and only when the D-lactyl group is part of *N*-acetylmuramic acid (Tipper, 1968), preferably in an intact glycan. The AL-1 enzyme is also proteolytic, and hydrolyzes L-prolyl-L-leucine, L-alanyl-L-leucine, L-valylcysteinsulfonic acid, and glycyl-L-phenylalanine linkages in a synthetic peptide and the oxidized β chain of insulin (Jackson and Wolfe, 1968). It thus has a unique specificity for either DD, DL, or LL linkages, or those containing glycine.

References

Browder, H. P., Zygmunt, W. A., Young, J. R., and Tavormina, P. A. (1965), *Biochem. Biophys. Res. Commun. 19*, 383.

Cummins, C. S., and Harris, H. (1956), *J. Gen. Microbiol. 14*, 583.

Ensign, J. C., and Wolfe, R. S. (1964), *J. Bacteriol. 87*, 924.

Ensign, J. C., and Wolfe, R. S. (1965), *J. Bacteriol. 90*, 395.

Ghuysen, J.-M., Bricas, E., Leyh-Bouille, M., Lache, M., and Shockman, G. D. (1967), *Biochemistry 6*, 2607.

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

Ghuysen, J.-M., Tipper, D. J., and Strominger, J. L. (1966), *Methods Enzymol. 8*, 685.

Ghuysen, J.-M., Tipper, D. J., and Strominger, J. L. (1968), *Compr. Biochem. 26A*, 53.

Hall, E. A., and Knox, K. W. (1965), *Biochem. J. 96*, 310.

Hash, J. H. (1963), *Arch. Biochem. Biophys. 102*, 379.

Hungerer, K. D. (1968), *Fed. Proc. 27*, 294.

Hungerer, K. D., and Tipper, D. J. (1969), *Biochemistry 8*, 3577.

Ikawa, M. (1964), *Biochemistry 3*, 594.

Ikawa, M., and Snell, E. E. (1960), *J. Biol. Chem. 235*, 1376.

Izaki, K., Matsuhashi, M., and Strominger, J. L. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 656.

Izaki, K., and Strominger, J. L. (1968), *J. Biol. Chem. 243*, 3193.

Jackson, R. W., and Wolfe, R. S. (1968), *J. Biol. Chem. 243*, 879.

Jarvis, D., and Strominger, J. L. (1967), *Biochemistry 6*, 2591.

Jones, R. T. (1964), *Cold Spring Harbor Symp. Quant. Biol. 29*, 297.

Knox, K. W., and Hall, E. A. (1965a), *Biochem. J. 94*, 525.

Knox, K. W., and Hall, E. A. (1965b), *Biochem. J. 96*, 302.

Knox, K. W., and Holmwood, K. J. (1968), *Biochem. J. 108*, 363.

Kolenbrander, P. E., and Ensign, J. C. (1968), *J. Bacteriol. 95*, 201.

Krulwich, T. A., Ensign, J. C., Tipper, D. J., and Strominger, J. L. (1967), *J. Bacteriol. 94*, 734, 741.

Leyh-Bouille, M., Ghuysen, J.-M., Tipper, D. J., and Strominger, J. L. (1966), *Biochemistry 5*, 3079.

Liu, T.-Y., and Gotschlich, E. C. (1967), *J. Biol. Chem. 242*, 471.
Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.
Muñoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J.-F., and Tinelli, R. (1966), *Biochemistry 5*, 3091.
Petit, J.-F., Muñoz, E., and Ghuysen, J.-M. (1966), *Biochemistry 5*, 2764.
Rogosa, M., and Sharpe, M. E. (1960), *J. Appl. Bacteriol. 23*, 130.
Sharon, N. (1964), *Proc. 3rd Symp. Fleming's Lysozyme, Milan* 44/RT.
Shields, R., and Burnett, W. (1960), *Anal. Chem. 32*, 885.
Staudenbauer, W. (1968), *Fed. Proc. 27*, 294.
Swallow, D. L., and Abraham, E. P. (1958), *Biochem. J. 70*, 364.
Tipper, D. J. (1968), *Biochemistry 7*, 1441.
Tipper, D. J. (1969), *Biochemistry 8*, 2192.
Tipper, D. J., and Berman, M. F. (1969), *Biochemistry 8*, 2183.
Tipper, D. J., Ghuysen, J.-M., and Strominger, J. L. (1965), *Biochemistry 4*, 468.
Tipper, D. J., Katz, W., Strominger, J. L., and Ghuysen, J.-M. (1967b), *Biochemistry 6*, 921.
Tipper, D. J., and Strominger, J. L. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1133.
Tipper, D. J., and Strominger, J. L. (1966), *Biochem. Biophys. Res. Commun. 22*, 48.
Tipper, D. J., and Strominger, J. L. (1968), *J. Biol. Chem. 243*, 3169.
Tipper, D. J., Strominger, J. L., and Ensign, J. C. (1967a), *Biochemistry 6*, 906.
Tipper, D. J., Strominger, J. L., and Ghuysen, J.-M. (1964), *Science 146*, 781.
Verdier, C.-H., and Agren, G. (1959), *Acta Chem. Scand. 13*, 1425.
Warth, A. D. (1968), Ph.D. Thesis, University of Wisconsin, Madison, Wis.

Cell Wall Polymers of *Bacillus sphaericus* 9602. I. Structure of the Vegetative Cell Wall Peptidoglycan*

K. D. Hungerer† and D. J. Tipper‡

ABSTRACT: Peptidoglycan constitutes 23% of the cell walls of *Bacillus sphaericus* strain 9602. Its glycan consists of alternating residues of *N*-acetylmuramic acid and *N*-acetylglucosamine, probably linked β-1,4 as in all known bacterial cell wall peptidoglycans. Every muramic acid residue of this glycan is substituted by N^{α}-(L-alanyl-D-isoglutamyl)-N^{ϵ}-(β-D-isoasparaginyl)-L-lysine tetrapeptides, 55% of which are cross-linked between L-lysine and D-isoasparagine by D-alanine residues to give oligomers of this peptide of random chain length. The subunits of this peptidoglycan thus differ from those of *Streptococcus faecium* and *Lactobacillus casei* RO94 only in lacking C-terminal D-alanine residues and amidation of their glutamic α-carboxyl groups. Their pattern of peptide cross-linking is very similar to that of *L. casei*. Much of the rest of the cell wall consists of protein which is trypsin and pronase sensitive. The walls also contain at least one other polymer, which contains glucosamine, and which is covalently bound to the peptidoglycan.

The bacterium *Bacillus sphaericus* 9602 is a representative member of its species which belongs to group 3 of the morphological classification of Smith *et al.* (1946), producing a near-terminal, spherical endospore of diameter considerably greater than that of the sporangium. It is also typical in that, unlike members of other bacillus species, its vegetative cell walls contain lysine and aspartic acid and little if any diaminopimelic acid (Powell and Strange, 1957). Its spores, however, do contain diaminopimelic acid, and this organism is therefore well suited to independent studies of spore and vegetative-type peptidoglycan biosynthesis. Such studies require detailed knowledge of vegetative and spore peptidoglycan structures, and this paper describes the results of work on the vegetative cell walls. Their peptidoglycan was found to have a structure very similar to that of *Lactobacillus casei* cell walls (Hungerer *et al.*, 1969) and it was possible to use many of the same techniques in the present studies, and to make direct comparisons of enzymically derived peptide fragments.

Extensive studies on the cell wall peptidoglycan of *Staphylococcus aureus* (*cf.* Muñoz *et al.*, 1966; Tipper *et al.*, 1967a,b; Jarvis and Strominger, 1967; Tipper, 1969a) have demonstrated that each of its subunits contains the amino acid sequence found in the nucleotide precursor that accumulates in this organism in the presence of penicillin (Strominger, 1959): *N*-acetylmuramyl-N^{α}-(L-alanyl-D-isoglutamyl)-L-lysyl-D-alanine. These tetrapeptides are cross-linked by pentapeptides of glycine and serine (Tipper, 1969a; Tipper and Berman, 1969), and the same tetrapeptide occurs in several other gram-posi-

* From the Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706. *Received May 16, 1969.* Supported by research grants from the U. S. Public Health Service (HD-02972) and the National Science Foundation (GB-6416) and a U. S. Public Health Research Career Program award (GM-38652).

† Present address: Max-Planck Institut für Immunbiologie, Freiburg, Germany.

‡ To whom reprint requests should be addressed.