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

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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-

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tive cell walls which, however, differ in the nature of their cross-links (Muñoz *et al.*, 1966). It is probable that this tetrapeptide, or variants of it of the form A-γ-D-glutamyl-B-D-alanine (in which A and B are both L-α-amino acids), is common to all bacterial cell wall peptidoglycans (*cf.* Ghuysen, 1968). Since its component amino acids (together with D-aspartate) are present in the cell wall of *B. sphaericus* (see below), this tetrapeptide sequence was expected to occur in the peptidoglycan of these cell walls, probably with D-aspartyl cross-links. Analysis of the data presented depends upon the assumption (to which no well-defined exceptions are known) that all of the peptide subunits will have a common tetrapeptide sequence.

Materials and Methods

Organism, Growth, and Preparation of Cell Walls. Spores of *B. sphaericus* 9602 were obtained from Dr. Alan Warth, and originated from one of the strains used by Powell and Strange (1957). This strain was found to have complex growth requirements, growing best on a medium consisting of "antibiotic medium no. 3" (Difco, 7.5 g), peptone (Difco, 2 g/l.), and beef extract (Difco, 3 g/l.). Each liter of cold, sterile medium was mixed with 10 ml of separately sterilized "spore salts" consisting of $CaCl_2 \cdot 2H_2O$ (13 g), $MgCl_2 \cdot 6H_2O$ (10 g), $MnSO_4$ (2.8 g), $ZnSO_4$ (0.5 g), $FeSO_4$ (0.1 g), and $CuSO_4$ (0.1 g) per l. of 0.1 N HCl. This was necessary for efficient spore maturation (but not for vegetative growth) both on agar gels and in liquid culture. Conical flasks (1000 ml) containing 500 ml of sterile medium were inoculated with about 10^8 spores, previously heat shocked for 30 min at 80°. Flasks were shaken vigorously at 32°, efficient aeration being necessary for rapid vegetative growth, and essential for sporulation. A lag phase of 3–4 hr was followed by exponential growth for 4 hr and linear growth for 2 hr, followed by a period of 1–2 hr during which cells shortened and growth rate declined into stationary phase. Cells were harvested after 10 hr when they began to shorten and were immediately broken. Cells (50 g wet weight) in 0.01 M KPO_4 (pH 7.5, 250 ml) were cooled in ice–water and sonicated in a Rosett cell immersed in an alcohol bath at −15° using a Branson J17V sonicator with a 0.75-in. step horn (Heat Systems, Inc., Melville, N. Y.). Breakage was usually complete in 30 min in the absence of beads, and the temperature could be maintained below 7°. After centrifugation, phase-contrast microscopy showed only cell fragments in the pellet, which, however, contained considerable pigment and large amounts of protein. Repeated sonication or breakage with glass beads in a Nossal disintegrator removed much pigment, but only small and diminishing amounts of protein. No further protein was removed by washing with 0.1 M KPO_4 (pH 8) or 1 M NaCl. Residual protein is, by operational definition, a component of the cell walls, though subsequent investigation showed it to be removable by 8 M urea or 0.01 M NaOH at room temperature.

Enzymes. Chalaropsis B enzyme (Hash, 1963) was a gift from Dr. J. Hash, Department of Microbiology, Vanderbilt University, Nashville, Tenn. The enzyme is an endo-*N*-acetylmuramidase which completely degrades the glycan of *S. aureus* cell wall peptidoglycan to disaccharides (Tipper *et al.*, 1964) and also hydrolyses *L. casei* cell wall peptidoglycan (Hungerer *et al.*, 1969). *Myxobacter* AL-1 enzyme (Ensign and Wolfe, 1964, 1965) was a gift from Dr. J. C. Ensign, Department of Bacteriology, University of Wisconsin, Madison, Wis. This enzyme is a bacteriolytic endopeptidase and protease (Ensign and Wolfe, 1964, 1965). Lysostaphin was a gift from Mead John Research Center, Evansville, Ind. *Streptomyces albus* amidase was a gift from Dr. J. M. Ghuysen, Service de Bacteriologie, Universite de Liege, Belgium. This enzyme hydrolyzes *N*-acetylmuramyl-L-alanine linkages between disaccharide and peptide in the products from *N*-acetylmuramidase hydrolysis of cell wall peptidoglycans (Ghuysen *et al.*, 1962).

Analyses. All techniques have been previously described (Hungerer *et al.*, 1969). Treatment for 40 hr at 115° in 6 N HCl was required for complete hydrolysis of N^{ϵ}-aspartyllysine linkages, and recovery of aspartic acid under these conditions was 93%.

Results

Cell Wall Composition. Analysis of extensively washed cell walls are given in Table I. The molecular weight of the peptidoglycan subunit (see below) is 1065, so that pure peptidoglycan would contain 0.94 μmole of muramic acid/mg. These walls contain 0.20 μmole of muramic acid/mg (*cf.* Tipper, 1968) and so contained 23% peptidoglycan. The walls contained 80 nmoles of phosphate/mg, and so contain little if any teichoic acid. Walls autolyzed readily in 0.02 M phosphate or Tris buffers (pH 7.5–9.0) but were totally resistant to autolysis after autoclaving for 20 min in water. The supernatant from autoclaving contained only traces of peptides. Treatment of autoclaved cell walls (1.4 g) with trypsin (0.1 mg/ml) in 0.1 M KPO_4–0.005 M $CaCl_2$ (pH 8.0, 150 ml) at 37° resulted in the slow release of soluble peptides, and analyses of the pellet (680 mg) after 3 hr are also given in Table I. Further treatment of the product with pronase (0.1 mg/ml) in 50 ml of the same buffer at 30° led to further solubilization of peptides, leaving peptidoglycan almost free of protein except for residual traces of glycine and serine (Table I). None of the supernatants contained hexosamines, and the final residue contained 0.45 μmole of muramic acid and 0.6 μmole of glucosamine per mg. Considerable ash, due to contaminating inorganic material, was left after combustion of these walls, which probably accounts for some of the nonpeptidoglycan material still present. The excess of glucosamine over muramic acid and glutamic acid suggests the presence of a second glucosamine-containing polymer (see below), which accounts for more of this nonpeptidoglycan material. The pronase-treated walls contain only 1.5 moles of alanine and 1.3 moles of ammonia per mole of glutamic acid. Since pronase has been reported to have weak hydrolytic activity against cell wall peptidoglycans (R. Plapp, private communication), trypsin-treated walls were used for structural investigations. Various preparations retained variable amounts of cell wall protein.

The cell wall protein, represented by the analyses of combined supernatants from trypsin and pronase hydrolysis, was rich in aspartate, glutamate, alanine, lysine, threonine, glycine, and valine, and was apparently free of histidine (Table I).

The cell walls of *S. faecium* (Ghuysen *et al.*, 1967; Kandler *et al.*, 1968) and *L. casei* (Hungerer *et al.*, 1969) contain N^{ϵ}-(D-isoasparaginyl)-L-lysine linkages, and hydrolysis of these walls with HCl leads to the production of the acid-stable compound N^{ϵ}-(aminosuccinoyl)-L-lysine (Swallow and Abraham, 1958). Differential hydrolysis of N^{ϵ}-(aminosuccinoyl)-L-lysine and α-peptide bonds is best achieved by concentrated HCl at

TABLE I: Analyses of *B. sphaericus* Cell Walls and Enzymically Derived Fractions.[a]

	Cell Walls	Trypsin Treated	Pronase Treated	Super, Trypsin, and Pronase	*Chalaropsis* B Hydrolysis			AL-1 Fractions		
					Super	Residue	Monomer	Pellet	Super	Dialysate
MurN	19	19	22	0	25	0	93	0.6	43	77
GlcN	31	31	30	0	35	2.1	100	4	154	83
Asp	88	29	19	60	32	28.8	168	49	15.1	150
Glu	77	28	22	48	31	20.0	163	38.2	13.7	149
Ala	90	43	33	50	49	26.4	179	42	18.0	213
Lys	62	31	22	38	29	18.1	151	34	17.5	153
NH_3	83	64	28	45						
His	0	0	0	0	0	0	0	0	0	0
Arg	8	tr	tr	6	1	3.9	8	6	1.7	4.3
Tre	54	6	0	49	6	23.8	67	42	3.6	33
Ser	25	4	1	22	3	11.2	31	19.3	2.4	19.0
Pro	14	3	0	15	3	4.0	25	13.7	2.1	9.5
Gly	37	6	1	32	5	16.1	59	29	5.0	34
Val	47	5	0	51	6	27.9	62	54	7.3	47
Ile	19	2	0	19	2	11.1	21	18.7	2.9	20
Leu	21	4	0	20	3	13.2	32	27	2.7	21
Tyr	13	32	0	9	2	4.9	9	9.4	0.9	8.2
Phe	15	3	0	12	2	7.9	18	16	1.6	9.5

[a] Data for the first six columns are presented as μmoles per 100 mg of untreated cell walls, for the *Chalaropsis* B monomer, as moles per 100 moles of glucosamine and for the AL-1 fractions as μmoles per total fraction. These fractions are nondialyzable pellet and supernatant (super), and dialysate (see text). Cysteine, tryptophan, and methionine were not determined. Histidine was not detected. Data for hexosamines are corrected for hydrolytic losses. The trypsin and pronase-treated cell walls contained 0.3 and 0.45 mole of glutamic acid per mg, respectively. MurN = muramic acid; GlcN = glucosamine; tr = trace.

80°, and under these conditions, *B. sphaericus* cell walls, the almost pure peptidoglycan resulting from protease hydrolysis of these cell walls, and enzymatically solubilized fragments of this peptidoglycan (see below) all give rise to N^{ϵ}-(aminosuccinoyl)-L-lysine on hydrolysis. In fact, most of the aspartic acid and lysine content of the peptidoglycan can be recovered as N^{ϵ}-(aminosuccinoyl)-L-lysine after this hydrolysis. The trypsin-solubilized peptides, however, do not give rise to N^{ϵ}-(aminosuccinoyl)-L-lysine, showing that the peptidoglycan, but not the cell wall protein, contains N^{ϵ}-(α- or β-aspartyl)-lysine linkages. N^{ϵ}-(Aminosuccinoyl)-L-lysine was recognized by its unique mobility (0.85 and 0.74 relative to lysine, on paper electrophoresis at pH 1.9 and 3.9, respectively) and its grey-purple color on heating at 70° with 0.2% ninhydrin containing 2% collidine. N^{ϵ}-(Aminosuccinoyl)-L-lysine was characterized by elution of the spot (located by ninhydrin-stained marker strips) and hydrolysis of the eluate with 6 N HCl for 24 hr at 115°, when almost complete hydrolysis to aspartic acid and lysine occurs. A synthetic sample (Hungerer *et al.*, 1969) was used for comparison and for quantitation by thin-layer chromatography on its bisdinitrophenyl derivative.

Configuration of Cell Wall Aspartic and Glutamic Acids. Commercial L-glutamate decarboxylase from *Clostridium welchii* (Worthington) has weak L-aspartate decarboxylase activity, but is inactive against the D isomers.

Conversion of L-[U-^{14}C]aspartate (100 nmoles, 10^4 cpm) into L-alanine was assayed by electrophoresis following incubation with enzyme (30 μg) and pyridoxal phosphate (10 nmole) for 18 hr at 37° in 0.05 M NaOAc (pH 5.2, 60 μl). Decarboxylation was 95% complete, but the prior admixture of a hydrolysate of *B. sphaericus* cell walls (180 nmoles of glutamate) almost completely inhibited the decarboxylation, probably due to the L-alanine present in the hydrolysate. Decarboxylation of L-glutamate (100 nmoles) was quantitative under these conditions, and was not inhibited by the cell wall hydrolysate. It was therefore necessary to fractionate hydrolysates before treatment with enzyme. Cell wall peptidoglycan (the residue from trypsin and pronase treatment of cell walls, 2 mg), cell wall protein (the supernatant from trypsin digestion), and N^{ϵ}-(aminosuccinoyl)-L-lysine (isolated by preparative paper electrophoresis at pH 3.9 of a concentrated HCl hydrolysate of cell wall, 5 mg) were separately hydrolyzed, and the products were fractionated by paper electrophoresis at pH 3.9. The eluted glutamate and aspartate spots were incubated with glutamate decarboxylase, and the products were dinitrophenylated and quantitated by thin-layer chromatography (*cf.* Ghuysen *et al.*, 1966). The results, given in Table II, show that peptidoglycan glutamate and aspartate had the D configuration, while the corresponding cell wall protein amino acids had the L configuration.

Solubilization of Trypsinized Cell Walls with Chalaropsis B Enzyme. Autoclaved cell walls (100 mg) were slowly solubilized by *Chalaropsis* B enzyme (2 mg) in 5 ml of 0.03 M NaOAc (pH 4.5) but the resultant mixture of protein and gly-

TABLE II: Configuration of Cell Wall Aspartic And Glutamic Acids.[a]

Material Hydrolyzed	Product of Decarboxylase Treatment			
	Asp	Ala	Glu	GABA
L-Aspartate	7	93		
D-Aspartate	90	10		
L-Glutamate			2	98
D-Glutamate			95	5
Peptidoglycan, aspartate band	85	15		
Peptidoglycan, glutamate band			88	12
Cell wall protein, aspartate band	13	87		
Cell wall protein, glutamate band			7	93
Cell wall ASL, aspartate band	82	18		

[a] Data are presented as per cent of the total product. All materials were hydrolyzed for 38 hr in 6 N HCl at 115°. Eluates of glutamate and aspartate from preparative electrophoresis of the products were incubated in 0.05 M NaOAc (pH 5.2, 95 μl) containing pyridoxal phosphate (2 mM), and L-glutamate decarboxylase (70 μg) for 18 hr at 37°. After centrifugation, aliquots (40 μl) of the supernatants were dinitrophenylated and quantitated by thin-layer chromatography. GABA = γ-aminobutyric acid; ASL = N^{ϵ}-(amino-succinoyl)-L-lysine.

copeptide was very viscous and difficult to fractionate satisfactorily, so all structural studies were performed on hydrolysates of trypsin-treated cell walls similar to those described in Table I. A sample of these walls (18.3 mg) was hydrolyzed at 37° in 0.01 M NaOAc (pH 4.5, 1 ml) with *Chalaropsis* B enzyme (0.1 mg). Maximum clarification occurred in about 24 hr, with release of 1.4 moles of reducing power/mole of glutamate (close to the value of 1.5 expected for release of one β-1,4-linked *N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide per peptide) and the solution was centrifuged after 48 hr. Amino acid analysis showed the supernatant to contain almost all of the glucosamine and all of the muramic acid. An aliquot (100 μl) of the supernatant was dried and redissolved in 0.1 M $NaBH_4$ (100 μl). After 18 hr at room temperature, the reduced product was again analyzed. It still contained 1.3 glucosamine residues per glutamic acid, but had no residual muramic acid, showing that *N*-acetylmuramidase action by the *Chalaropsis* B enzyme had been complete.

Fractionation and Analysis of the Products. Trypsinized cell walls (900 mg) were incubated at 37° in 0.01 M NaOAc (pH 4.5, 50 ml) containing *Chalaropsis* B enzyme (1.5 mg). Additional enzyme (0.5 mg) was added after 6 and 24 hr, and the suspension, which retained about 20% of its original turbidity, was centrifuged after 54 hr. The pellet (350 mg) on hydrolysis gave rise to all of the amino acids found in the trypsin supernatant of cell walls (Table I) and in approximately the same ratios (Table I). It contained no muramic acid, but about 6% of the initial glucosamine. The supernatant (500 mg) contained all of the muramic acid and equivalent amounts of peptidoglycan amino acids, plus additional amounts of these and other amino acids in proportions corresponding to about

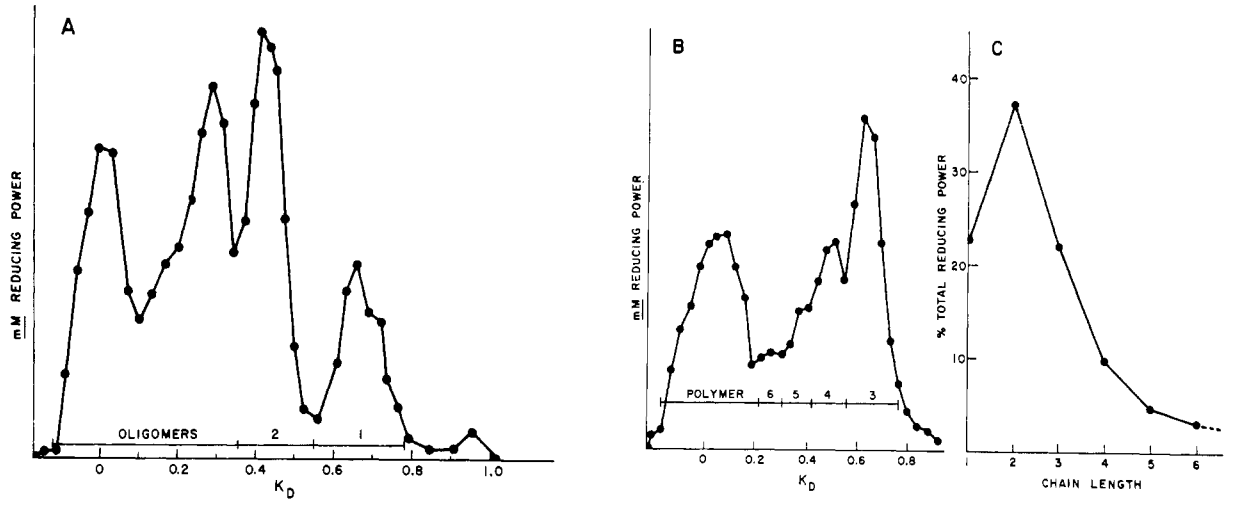

FIGURE 1: Sephadex G-50–G-25 fractionation of the supernatant from *Chalaropsis* B hydrolysis of trypsinized *B. sphaericus* cell walls. (A) Initial fractionation on Sephadex G-50 (100 × 1.5 cm) joined by a polyethylene capillary to Sephadex G-25 (100 × 1.5 cm), eluted with 0.1 M LiCl at room temperature. Fractions of 5 ml were collected each 20 min and aliquots (3 μl) were analyzed for reducing power. Since muramidase action had been complete and every peptide subunit carried a disaccharide with reducing *N*-acetylmuramic acid (see text), reducing power is a measure of the total number of peptide-disaccharide subunits present, irrespective of their degree of polymerization by peptide cross-links. V_e = elution volume, $V_0 = V_e$ for Blue Dextran, $V_0 + V_i = V_e$ for NaCl, and $K_D = (V_e - V_0)/V_i$. K_D is a measure of the fraction of V_i available to the solute. Indicated K_D values are for the combined columns. Fractions pooled were (1) K_D = 0.80 to 0.55 (monomer), (2) K_D = 0.55 to 0.35 (dimer), and K_D = 0.35 to −0.1 (higher oligomers), as indicated. (B) Refractionation of the higher oligomers from A on Sephadex G-50 (100 × 1.5 cm) joined to Sephadex G-50 (120 × 1 cm), eluted with 0.1 M LiCl. Fractions pooled were K_D = 0.2 to 0.18 (polymer), 0.18 to 0.30 ("hexamer"), 0.30 to 0.42 ("pentamer"), 0.42 to 0.55 (tetramer), and 0.55 to 0.75 (trimer), as indicated. (C) Relative proportions of reducing power in the fractions indicated by the bars marked 1 to 6 in Figure 1A,B. The numbers are approximations of the average chain length of each fraction (see text).

TABLE III: Analysis of Glycopeptide Oligomers from *Chalaropsis* B Enzyme Hydrolysis.[a]

Fraction	% of Total Glutamate	GlcN	MurN	MurN-6-P	Disaccharide	NH_3	Lys	Asp	Alanine			N Terminal		C Terminal		Chain Length
									Total	D	L	Asp	Lys	Ala	Lys	
Monomer	14.5	99	92	0	108	86	101	98	107	8	96	86	13	9	104	0.95
Dimer	31.7	100	100	0	96	97	112	107	146	59	102	45	6	3	50	1.9
Trimer	20.7	92	105	0	102	94	108	99	171	75	92	28	1	2	37	3.0
Oligomers	12.8	140	79	9.5	106	111	120	96	180	80	93	23	1	2	30	3.7
Polymer	20.4	812	56	36	95	217	103	92	170	80	97	20	2	1	22	4.4

[a] Fractions are named as indicated in Figure 1. Data are presented as moles per 100 moles of total glutamate in each fraction. Disaccharide is measured by both reducing power and 30-min Morgan–Elson procedures. For all fractions, as for 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid, the ratio (with *N*-acetylglucosamine as standard in each) of these determinations was 3.7. Chain length = 200/total N and C terminals. All fractions but monomer contained only traces of other amino acids. Monomer contained significant amounts of nonpeptidoglycan amino acids, approximately in the proportions found in the trypsin and pronase supernatant of cell walls (Table I). The corresponding amounts of peptidoglycan amino acids were subtracted from the data presented here, including 38% of the total glutamate of this fraction. GlcN = glucosamine; MurN = muramic acid; MurN-6-P = muramic acid 6-phosphate.

20% of the protein originally present in the cell walls (Table I). This supernatant thus contains most, if not all, of the peptidoglycan, and about 80% of the excess glucosamine of the cell walls, while the pellet consists almost entirely of residual cell wall protein.

The supernatant was fractionated on columns of Sephadex G-50 and G-25 with the results shown in Figure 1A. Aliquots of individual fractions were analyzed qualitatively for amino acid content by hydrolysis and electrophoresis. Almost all of the nonpeptidoglycan amino acids eluted after monomer, which contained the rest. The fractions up to and including trimer were pooled, desalted on Bio-Gel P2 (60 × 1.5 cm), eluted with water, and refractionated on two linked columns of Sephadex G-50, as shown in Figure 1B. The relative reducing power contents of the fractions indicated by the bars in Figure 1A,B are presented in Figure 1C. If their assigned numbers represent their chain lengths (in disaccharide-peptide subunits, see below), then an average peptide chain length of 1.9 subunits is obtained. Fractions 4–6 were pooled (oligomers) and analyses of all fractions are given in Table III. The nonpeptidoglycan amino acids in the monomer fraction are present in about the proportions found in the cell wall protein (Table I), and their amounts indicate that 38% of the glutamate of this fraction is derived from cell wall protein (Table I). The data for monomer in Table III have been corrected for the appropriate amounts of peptidoglycan components in this amount of cell wall protein impurity. On this basis, all fractions contain approximately equimolar glutamate, aspartate, ammonia, L-alanine, and lysine. The C-terminal amino acid is almost exclusively lysine, and the amounts of D- and total alanine also indicate that C-terminal peptide subunits lack D-alanine, since total alanine and D-alanine per glutamate are approximately $(2 - (1/n))$ and $(1 - (1/n))$, respectively, where n = glycopeptide chain length. The N-terminal amino acid is almost exclusively aspartate, though 10% of the total N-terminal residues are ε-lysine. These data are consistent with the total cell wall peptidoglycan contents of aspartate (0.9/glutamate) and alanine (1.4/glutamate) (Table I), and with the structures and proportions of peptides isolated after AL-1 enzyme hydrolysis (see below).

Each fraction contained about 1 mole of muramic acid/glutamate, and this totally disappeared on reduction overnight, at room temperature, with 0.1 M $NaBH_4$. Glucosamine remained unreduced. Combined with the hexosamine contents and reducing power data, this indicates that each glycopeptide subunit is accompanied by an *N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide. Little color was produced from these fractions in the 7-min Morgan–Elson procedure, and slow release of Morgan–Elson chromogen on heating in alkali gave the ratio of reducing power to 30-min Morgan–Elson colors, characteristic of the β-1,4-linked disaccharide (*cf.* Table III and Tipper, 1968).

The calculated chain lengths of the glycopeptide fractions agree with the predicted values based on elution volumes (Figure 1C). The polymer fraction contains all of the solubilized excess glucosamine, in an unidentified polymer excluded from the gel, and probably bound to a portion of the glycopeptide. This glycopeptide and the glycopeptide oligomers eluted with the complex contain 20% of the total solubilized glutamic acid, and have an average chain length of 4.4. The over-all average chain length of the glycopeptide is thus 2.2. Thus total N- and C-terminal amino acids should total 0.45 per glutamate (see

below), and if all C-terminal residues lack D-alanine, total cell wall alanine should be 1.55 per glutamate. The actual ratio for trypsin and pronase-digested cell walls is 1.5 (Table I).

Amidase Hydrolysis and Edman Degradation of Glycopeptides. An aliquot (0.2 μmole) of the glycopeptide dimer fraction was submitted to one cycle of Edman degradation. The product was devoid of N-terminal amino acids, indicating that all the N-terminal aspartyl linkages were β.

A sample (0.6 μmole) of the monomer fraction was incubated at 37° in 0.03 M NaOAc (pH 5.2, 100 μl) containing *S. albus* amidase. After 6 hr, 0.5 μmole of N-terminal alanine had been released, and an aliquot (0.2 μmole) of the product was submitted to Edman degradation. The product had lost all N-terminal alanine and most of its L-alanine (it retained its small amount of D-alanine) and had gained 0.8 mole of N-terminal glutamic acid. After a second cycle of degradation, N-terminal glutamate disappeared without release of N-terminal lysine, showing the glutamyl linkage to be γ. This monomer fraction thus consists mostly of $N^{\alpha \text{ or } \epsilon}$-(disaccharide-L-alanyl-D-isoglutamyl)-$N^{\alpha \text{ or } \epsilon}$-(β-D-aspartyl)-L-lysine, with a mole of ammonia on one of its three free carboxyl groups.

Hydrolysis of Cell Walls with AL-1 Enzyme. A. DETERMINATION OF LIBERATED END GROUPS. The *Myxobacter* AL-1 enzyme isolated by Ensign and Wolfe (1964, 1965) hydrolyzes cell walls of *S. aureus*, *S. epidermidis*, *Arthrobacter crystallopoietes*, and *L. casei*. In each case, hydrolysis of peptide bridges between D-alanine and the first amino acid (respectively, glycine, glycine, or L-serine, L-alanine, and D-isoasparagine) occurs first, followed by slower hydrolysis of *N*-acetylmuramyl-L-alanine linkages (amidase action) (Tipper *et al.*, 1967a; Tipper, 1969a,b; Hungerer *et al.*, 1969).

B. sphaericus cell walls, like *L. casei* cell walls, were solubilized very slowly by concentrations of AL-1 enzyme which rapidly hydrolyzed cell walls of *Staphylococci*. Fractionation of the resultant viscous solution proved very difficult, so trypsin-treated cell walls were used in the experiments described below. An extensively treated preparation (500 mg) was hydrolyzed with AL-1 enzyme, and samples were analyzed for N- and C-terminal amino acids with the results shown in Figure 2. All samples were first solubilized by hydrolysis with the *Chalaropsis* B enzyme, since recovery of terminal amino acids from insoluble peptides tends to be variable. The cell walls initially contained (per mole of glutamate) 0.34 mole of N-terminal aspartate, 0.04 mole of N^{ϵ}-terminal lysine, and traces of N-terminal alanine (total 9.39). They also contained small amounts of C-terminal alanine and 0.41 mole of C-terminal lysine. N- and C-terminal lysine did not change during hydrolysis by the AL-1 enzyme, but there was a parallel increase in N-terminal aspartate (maximally 0.89) and C-terminal alanine (maximally 0.50, sum with C-terminal lysine, 0.91). Release of N-terminal alanine also occurred (maximally 0.93), half appearing after release of C-terminal amino acids was complete. This release therefore results, at least in part, from amidase action. This pattern is similar to that previously observed on AL-1 hydrolysis of *L. casei* cell walls (Hungerer *et al.*, 1969) and other cell walls (*cf.* Tipper *et al.*, 1967a) where amidase action also follows hydrolysis of cross-bridge peptide linkages.

B. FRACTIONATION OF THE PRODUCTS. The analyses of the final products indicate about 90% hydrolysis of both D-alanyl-D-aspartyl cross-bridges and of *N*-acetylmuramyl-L-alanine linkages. These products should therefore consist, as in AL-1

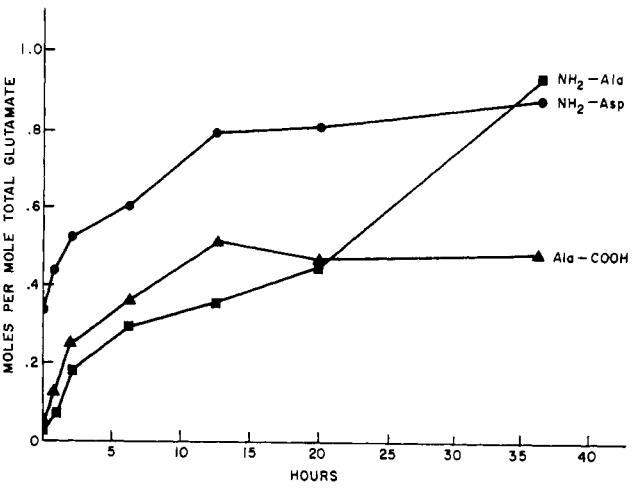

FIGURE 2: Kinetics of N- and C-terminal amino acid release on hydrolysis of trypsinized *B. sphaericus* cell walls with AL-1 enzyme. Trypsinized cell walls (500 mg, 210 μmoles of glutamic acid, 130 μmoles of muramic acid) in 30 ml of 0.03 M sodium barbital buffer (pH 9.0) were hydrolyzed with AL-1 enzyme (1 mg) at 37° in the presence of toluene. After 12 hr, an additional 1 mg of enzyme was added and incubation was continued to 40 hr. Aliquots (0.62 ml) were removed at the indicated intervals, dried, and solubilized by hydrolysis with *Chalaropsis* B enzyme (0.1 mg) in 1 ml of 0.05 M NaOAc (pH 4.5) for 48 hr at 37°. Aliquots (25 μl) of the solubilized samples were analyzed for N- and C-terminal amino acids.

hydrolysates of other cell walls (*cf.* Tipper *et al.*, 1967a), of subunits of the peptide and relatively peptide-free glycan from the peptidoglycan, together with other cell wall polymers, possibly covalently linked to a portion of the glycan. Attempts to fractionate this mixture by size using Sephadex or Bio-Gel, or according to charge using Dowex 50, resulted in clogging of the columns so that in order to fractionate the peptide subunits it was necessary to separate them from polymeric components by dialysis. Distribution of cell wall components in the resultant fractions is given in Table IV. After dialysis of the hydrolysis products against several changes of distilled water for 36 hr at 2°, the bag contents were centrifuged giving a pellet (76 mg). This pellet contained 38.2 μmoles of glutamate, or 19.1% of the total recovered (200 μmoles, Table IV). The nondialyzable supernatant was lyophilized giving a residue (141 mg) which contained 13.7 μmoles of glutamate, or 6.8% of the recovered glutamate. The dialysate contains the remaining 74% (149 μmoles) of glutamate. Analyses of the three fractions from dialysis are given in Table I. The recovery of glutamate in all fractions was 95%.

The pellet contained no detectable muramic acid and only 4 μmoles of glucosamine. Its amino acids had approximately the ratios found in cell wall protein, and this fraction therefore consists of residual insoluble cell wall protein. The soluble fractions contained a total of 120 μmoles of muramic acid, a recovery of 93% of that originally present in the cell walls. The dialysate contained equivalent amounts of glucosamine and muramic acid, presumably due to small glycan fragments (65% of the total) and apparently contained none of the secondary glucosamine polymer, which was found mostly in the nondialyzable supernatant, together with the residual 35% of the glycan.

Amino acid analyses (Table I) indicate that about 4 μmoles

TABLE IV: Distribution of Cell Wall Components after Dialysis of AL-1 Hydrolysate.[a]

	Glutamic Acid				Glucosamine		
	Total	Peptido-glycan	Protein	Muramic Acid	Total	Glycan	Polysac-charide
Pellet	38.2	0	38.2	0	4	0	4
Supernatant	13.7	9.5	4.2	43	154	47	97
Dialysate	149	116	33	77	83	83	0
Total	200	125.5	75.4	120	241	130	111
Original cell walls	211	130	81	130	188	130	58

[a] Data are presented as μmoles in the total fraction. The original cell walls (500 mg) had been stripped of much protein by trypsin hydrolysis, and contained 0.4 μmole of glutamate/mg. Data are corrected for the 14.5% in aliquots removed for analyses (see legend to Figure 2). The amount of total glutamic acid in protein is estimated from the amounts of nonpeptidoglycan amino acids (see Table I). The glucosamine in glycan is estimated from the muramic acid content, reflecting the normal 92% recovery of muramic acid. The discrepancy between total recovered polysaccharide glucosamine and that originally present is not explained.

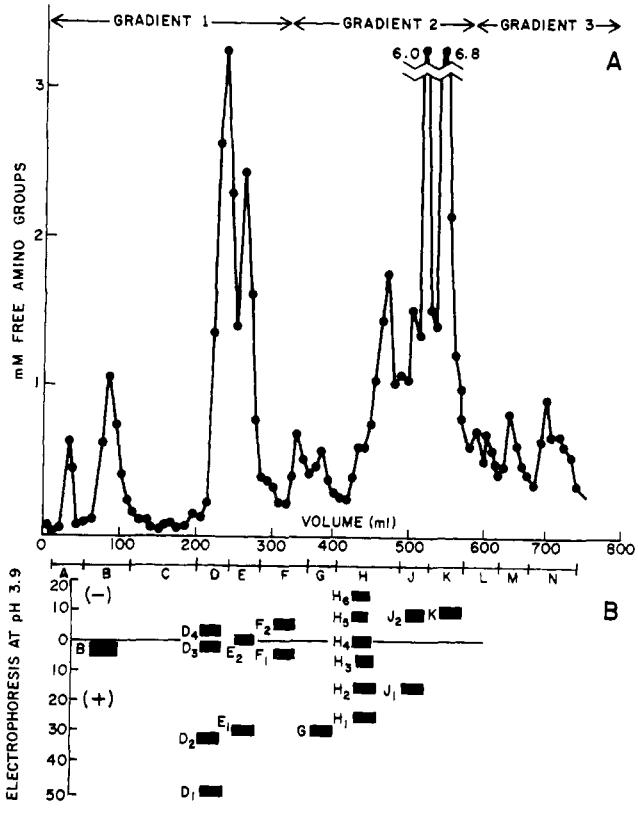

FIGURE 3: Fractionation of dialyzable peptides from AL-1 enzyme hydrolysis of *B. sphaericus* cell walls on Dowex 50. The concentrated dialysate was applied to a column of Dowex 50 (Beckman A4 resin, 1.5 × 15 cm) that had previously been equilibrated with pyridine–formic acid–acetic acid, buffer A (Hungerer *et al.*, 1969). It was eluted at 50° with the same three gradients of pyridine–acetate buffers previously described (Hungerer *et al.*, 1969), the changes in gradient system being indicated by the bars 1, 2, and 3. Aliquots (51 μl) of the fractions (3 ml) were analyzed for total N-terminal groups by dinitrophenylation with the results shown here (A). Fractions A to N were pooled as indicated, concentrated *in vacuo*, and redissolved in water (1 ml). Aliquots (10 μl) of these fractions were subjected to paper electrophoresis at pH 3.9. The mobilities of the major bands detected by ninhydrin are indicated by the lower diagram (B).

of the glutamate in the supernatant and about 33 μmoles of that in the dialysate are derived from cell wall protein. These two fractions thus contain about 125 μmoles of peptidoglycan glutamate, a recovery of 96%, 88% (actually about 98 μmoles after analyses) being in the dialysate.

The dialysate was concentrated, applied to a column of Dowex 50, and eluted with three gradients of increasing pH and ionic strength with the results shown in Figure 3A. Analysis of pooled fractions A to N showed that A contained large amounts of hexosamines, other fractions being free of hexosamines. Aliquots (10 μl) of fractions A to N were subjected to electrophoresis at pH 3.9 (Figure 3B). Ninhydrin detected only several very weak bands in A, C, L, and M, but all other fractions contained one or more major bands, that in fraction N having the mobility of free lysine. Aliquots (10 μl) of all fractions were hydrolyzed overnight in 6 N HCl at 105° and subjected to electrophoresis at pH 1.9. Fraction A contained large amounts of glucosamine and muramic acid, but relatively small amounts of amino acids, and this fraction therefore consists mainly of peptide-free glycan fractions. Fraction C, L, and M contained small amounts of all amino acids and so contained minor fragments of cell wall protein. They were not further investigated. Fraction N contained only lysine, and this fraction therefore consists of free lysine. Fractions G, J, and K contained peptidoglycan amino acids together with small amounts of other amino acids. All other fractions contained all amino acids, and fractions B, D, E, F, G, H, J, and K were further fractionated by preparative paper electrophoresis at pH 3.9 (2 hr, 40 V/cm on No. 3MM paper). Each solution was applied as a band 20 cm long, the center 18-cm region was cut out, and the end strips were developed with ninhydrin to detect bands, which were eluted from the center strip with 1 M acetic acid. Eluates were dried and redissolved in water (1 ml). Aliquots (10 μl) were dried and hydrolyzed for 8 hr at 80° in concentrated HCl. After electrophoresis of the products at pH 3.9, ninhydrin showed the presence of N^{ϵ}-(aminosuccinoyl)-L-lysine in bands E_1, G, H_2, J_1, J_2, and K only. Similar electrophoresis of 10-μl aliquots hydrolyzed for 48 hr at 115° showed that these same peptides contained the four peptidoglycan amino acids almost exclusive of others, while

TABLE V: Analyses of Dialyzable Peptides from AL-1 Hydrolysate of *B. sphaericus* Cell Walls.[a]

Peptides	Glu					Ala			N Terminal			C Terminal	
	Total μmoles	% of Total	NH_3	Lys	Asp	Total	D	L	Ala	Asp	Lys	Ala	Lys
E_1	8.5	12.3	0.10	1.00	0.90	2.09	1.20	1.13	0.74	0.88	0	0.81	0
G	4.5	6.5	0.13	1.05	0.86	1.06	0.04	1.16	0.82	0.86	0	0	0.80
H_2	2.5	3.6	0.07	0.97	0.80	2.02	0.91	1.00	0.90	1.03	0	0.82	0
J_1	1.2	1.7	0.13	1.09	0.79	1.10	0.12	0.92	0.87	0.79	0	0	0.78
J_2	30.5	44.2	0.96	1.13	0.93	1.98	0.88	0.93	0.85	0.80		1.13	0
K	22.0	31.7	1.28	1.04	0.82	0.19	0.10	0.98	1.07	0.86	0	0	0.86

[a] Data are expressed as moles per mole of total glutamate.

all other peptides contained a mixture of amino acids representative of the cell wall protein. Peptides of E_1, G, H_2, J_1, J_2, and K were purified by filtration on columns (2 × 60 cm) of Bio-Gel P2 (200 mesh), eluted with water, and their analyses are given in Table V. These peptides comprise all of the peptides derived from the cell wall peptidoglycan in the dialysate of the AL-1 hydrolysate. Their glutamate contents are given in Table V, the total being 69 μmoles. Since 8% of the fractions was used in analyses and about 10% in marker strips on electrophoresis, this corresponds to 84 μmoles, a recovery of about 86%, 76% of the peptidoglycan content of the original cell walls. Unless preferential loss of some fragment occurred, the ratios of these peptides should thus be similar to those in the original hydrolysate.

All peptides contained (per mole of glutamate) approximately 1 mole of L-alanine and 1 mole of N-terminal alanine. Peptides G, J_1, and K are almost devoid of D-alanine and contain only 1 mole of total alanine which must therefore be N-terminal L-alanine. These peptides also have C-terminal lysine. Peptides E_1, H_2, and J_2 contain an additional mole of total alanine and D-alanine, and have C-terminal alanine rather than lysine, so that if their N-terminal alanine also has the L configuration, their C-terminal alanine has the D configuration. This was demonstrated by analysis of the products of one cycle of Edman degradation of these three peptides, they retained about 1 mole of D-alanine, but lost all L-alanine. Thus, all peptides have about 1 mole of N-terminal L-alanine, like the amidase-hydrolyzed glycopeptide monomer, confirming that the AL-1 enzyme hydrolyzed the *B. sphaericus* peptidoglycan at most of its *N*-acetylmuramyl-L-alanine linkages.

Each peptide also contained 1 mole of glutamate, lysine, and N-terminal aspartate, as in the glycopeptide monomer which was shown to contain the sequence L-alanyl-D-glutamate (above). The two ammonia-free peptides H_2 and J_1 had identical mobilities at pH 3.9 (Figure 3) and gave identical patterns of N-terminal amino acids on Edman degradation. After one cycle, N-terminal alanine and aspartate disappeared, releasing N-terminal glutamate and N^{ϵ}-terminal lysine. As-

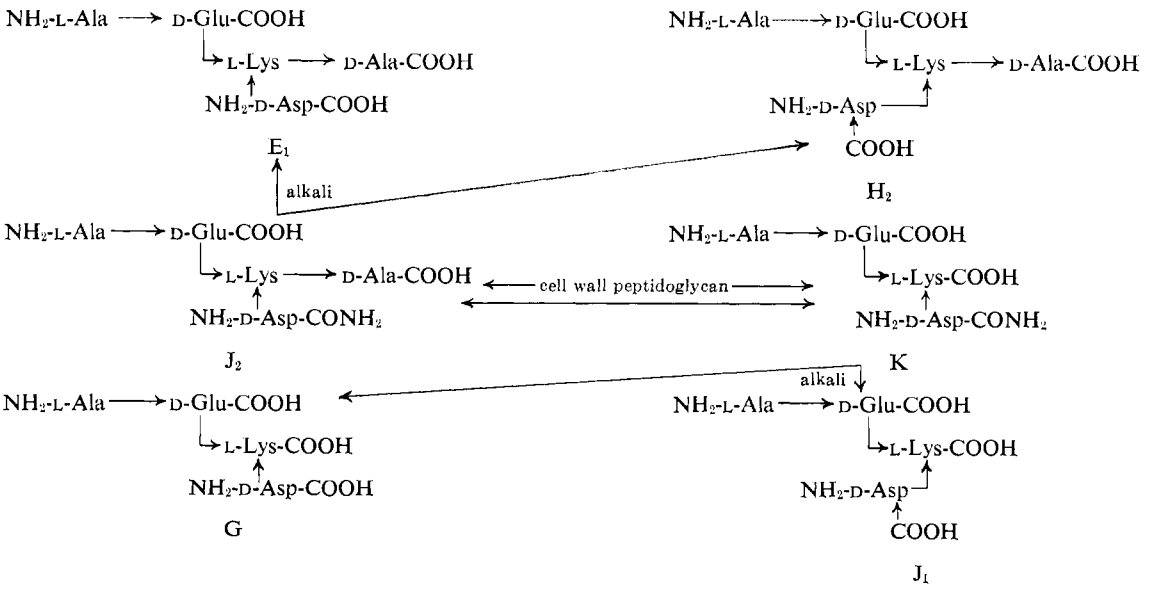

FIGURE 4: Structures of the peptides derived from peptidoglycan after hydrolysis of *B. sphaericus* cell walls with AL-1 enzyme. According to the current convention, vertical lines indicate linkage from or to groups not on the α-carbon atom of the amino acid.

suming that these peptides also have L-alanyl-D-glutamate, then they contain N^{ϵ}-(α-D-aspartyl)-lysine and N^{α}-(L-alanyl-D-isoglutamyl)-lysine. As expected, after a second cycle of Edman degradation, no N-terminal amino acids remained, confirming γ-glutamyl linkage. Thus J_1 is N^{α}-(L-alanyl-D-isoglutamyl)-N^{ϵ}-(α-D-aspartyl)-L-lysine, and H_2 is the same peptide with additional C-terminal D-alanine, as shown in Figure 4. The other two ammonia-free peptides, E_1 and G, also have the same mobility at pH 3.9, and while they have the same amino acid and N- and C-terminal analyses as, respectively, H_2 and J_1, they have a greater negative charge at pH 3.9. They are differentiated from H_2 and J_1 by Edman degradation, since after one cycle, N-terminal alanine and aspartate disappear, but only glutamate appears. This residual N terminus is lost in a second cycle of degradation without release of N-terminal lysine. These peptides are thus the β-aspartyl analogs of H_2 and J_1, as shown in Figure 4. Their greater negative charge is predictable since their aspartyl α-carboxylate group with its adjacent free amino group should have a lower pK than the aspartyl β-carboxylate group in peptides H_2 and J_1.

Peptides J_2 and K have the same total, N- and C-terminal analyses as E_1 or H_2 and G or J_1, respectively, but also contain 1 mole of ammonia. Their positive charge on electrophoresis at pH 3.9 is consistent with the loss of one negative charge. On Edman degradation, they give the same result as E_1 and G, showing them to have N^{ϵ}-(β-aspartyl)-lysine linkage. Their mole of ammonia is therefore on the α-carboxyl group of either their glutamate or their aspartate residues. These possibilities can be readily distinguished, since it has been demonstrated that the N-terminal D-isoasparaginyl but not the D-isoglutamyl residues in similar peptide fragments from the cell walls of *S. faecium* (Ghuysen *et al.*, 1967) and *L. casei* (Hungerer *et al.*, 1969) are highly labile to alkali. The D-isoasparaginyl residues in the latter, in particular, are slowly converted into a mixture of N^{ϵ}-(α- and β-D-aspartyl)-L-lysine derivatives during incubation at 37°, pH 9, the conditions of AL-1 hydrolysis. The ratio of α- to β-aspartyl peptides in the products from AL-1 hydrolysate of *L. casei* was about 1.4, as expected if N^{ϵ}-aminosuccinoyl-L-lysine were an intermediate in their production (Swallow and Abraham, 1958).

Action of Alkali on Peptides J_2 and K. Aliquots (100 μmoles) of all six peptides were incubated for 1 hr at 37° in 0.5 N NaOH, neutralized, and subjected to electrophoresis at pH 3.9 and 1.9. As shown in Figure 3, pH 3.9 distinguishes three pairs of peptides: K and J_2, H_2 and J_1, and E_1 and G. pH 1.9 separates all the peptides; mobilities relative to alanine (=100) and glutamate (=0) are 100, 86, and 48 for K, J_1, and G, respectively (all of which have C-terminal lysine), and 84, 70, and 12 for J_2, H_2, and E_1 (all of which have C-terminal alanine). Thus the C-terminal alanyl residues on peptides J_2, H_2, and E_1 have a lower pK than the C-terminal lysyl residues on the former peptides. All peptides are distinguished at this pH except J_1 and J_2, which are well separated at pH 3.9.

Peptides E_1, G_1, H_2, and J_1 had unchanged mobilities at pH 1.9 and 3.9 as expected from their initial lack of ammonia. Peptide J_2 (basic at pH 3.9) was converted into a mixture of E_1 and H_2 (acidic at pH 3.9), and peptide K (basic at pH 3.9) was converted into a mixture of G and J_2 (acidic at pH 3.9). These are exactly the results expected if J_2 and K have N-terminal iso-D-asparaginyl residues, but would not have occurred had these peptides had iso-D-glutaminyl residues. Aliquots (0.2 μmole) of peptides J_1 and K were treated with alkali as above and fractionated at pH 3.9. The peptide products were detected on marker strips with ninhydrin and eluted with 1 M acetic acid. They were then quantitated by total amino acid analysis. As expected, the ratio of α- to β-aspartyl peptide products (respectively, H_2 to E_1 and J_1 to G) in both cases was approximately 1:4.

The ratio of C-terminal lysine peptides (K + G + J_1) to C-terminal alanine peptides (J_2 + E_1 + H_2) in the AL-1 hydrolysate was 6:4, as expected, since 44% of the peptide subunits in the cell wall are uncross-linked and carry C-terminal lysine residues (see above), the other 56% being involved in D-alanyl-D-isoasparagine cross-links which are hydrolyzed by the AL-1 enzyme with release of C-terminal alanine; 26% of the C-terminal alanine peptides were deamidated (E_1 + H_2), the ratio of β to α peptide (E_1 to H_2) being 3.4:1. Similarly, 22% of the C-terminal lysine peptides were deamidated (G + J_1), the ratio of β to α peptide (G to J_1) being 3.7:1. Thus ammonia-free α- and β-aspartyl peptides occur in relative amounts consistent with their source being deamidation of D-isoasparaginyl peptides. Thus all cell wall peptides initially carry D-isoasparaginyl groups, as shown by analyses of the *Chalaropsis* B glycopeptides.

Glycan. Analysis of the products of hydrolysis of cell walls with the *Chalaropsis* B enzyme showed that the glycan had alternate *N*-acetylglucosamine and *N*-acetylmuramic acid residues with 1,4 linkages. Hydrolysis of the *N*-acetylmuramyl linkages by the *Chalaropsis* enzyme indicates that they are probably β-1,4, like the susceptible linkages in *S. aureus*. This was confirmed by isolation of *N*-acetylmuramyl-*N*-acetylglucosamine disaccharides from the cell walls.

A sample (9 μmoles of total hexosamine) of the nondialyzable supernatant from AL-1 hydrolysis was incubated overnight at 37° in 0.03 M KPO_4 (pH 7.5, 150 μl) containing lysostaphin (0.1 mg). Lysostaphin, a staphylolytic enzyme mixture produced by *S. epidermidis* strain W13 (Browder *et al.*, 1965), contains an endo-β-glucosaminidase whose preferred substrate is cell wall glycan stripped of its peptide side chains (Tipper *et al.*, 1967a). The products were fractionated on Bio-Gel P2 eluted with water. Fractions were analyzed for reducing power and contained a major peak of disaccharide (2.1 μmoles of total hexosamine) and a minor peak of higher oligosaccharide well separated from the excluded peak of glucosamine polymer. The disaccharide contained equimolar muramic acid and glucosamine, lost all of its glucosamine (but negligible muramic acid) on reduction, and had extinction coefficients in reducing power and 7-min Morgan–Elson determinations, respectively, 75 and 5% those for free *N*-acetylglucosamine, characteristic of 4-*O*-*N*-acetylglucosamine (*cf.* Ghuysen *et al.*, 1966).

Discussion

The peptidoglycan accounts for 23% of the weight of *B. sphaericus* vegetative cell walls and has the structure shown in Figure 5. Glycan chains of alternating 1,4-linked residues of *N*-acetylmuramic acid and *N*-acetylglucosamine are probably β linked, and if so, are identical in structure with those of *S. aureus* (Tipper *et al.*, 1967a). They are linked, in part, to a second glucosamine-containing polymer of unknown structure. Linkage to the third (protein) component of the cell walls has not been demonstrated. Every muramic acid resi-

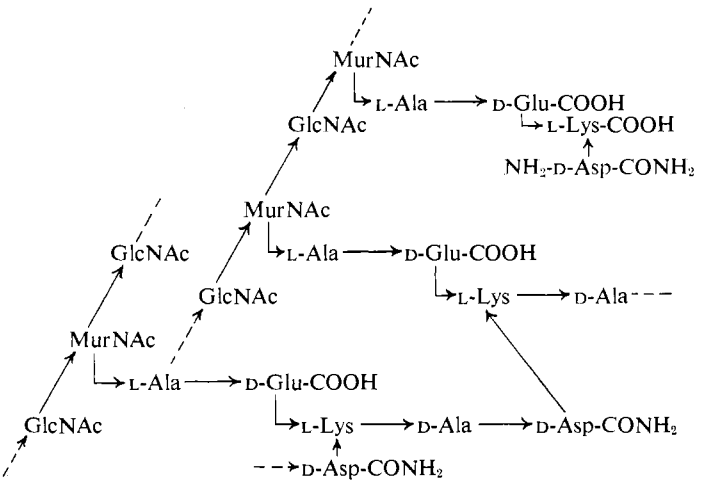

FIGURE 5: Structure of the peptidoglycan of *B. sphaericus* strain 9602. Two glycan chains are illustrated, connected by two cross-linked peptide subunits in the lower half of the figure. An uncross-linked monomer is shown at the upper right, with C-terminal lysine and N-terminal isoasparaginyl residues. These are also the termini of the cross-linked peptide sequences.

due is substituted by the peptide N^{α}-(L-alanyl-D-isoglutamyl)-N^{ϵ}-(D-isoasparaginyl)-L-lysine; 45% of these peptides is C terminal, the remainder being cross-linked between lysine and isoasparagine by D-alanyl residues to give oligomers of the peptide subunit of random chain length. This peptidoglycan thus resembles that of *L. casei* very closely, but differs in lacking ammonia on its glutamic α-carboxyl groups, and in lacking C-terminal D-alanine residues. It has been shown (Staudenbauer, 1968) that a 30,000*g* supernatant fraction from disrupted cells of *L. casei* catalyses the addition of D-aspartate, activated as D-aspartyl-β-phosphate, to the ϵ-amino group of lysine in UDP-*N*-acetylmuramyl-N^{α}-(L-alanyl-D-isoglutamyl-L-lysyl-D-alanyl-D-alanine. This nucleotide is almost certainly a precursor of the vegetative cell wall peptidoglycan of *B. sphaericus*, which therefore must have carboxypeptidases able to remove both C-terminal D-alanyl residues from the C-terminal peptide units of the peptidoglycan. Such activities have been found in *E. coli* (Izaki and Strominger, 1968) and in *B. subtilis* (Egan, 1969). The cross-links are presumably formed by a transpeptidation reaction involving loss of the terminal D-alanine residue, as demonstrated in *S. aureus* (Wise and Park, 1965; Tipper and Strominger, 1965, 1968) and *E. coli* (Izaki *et al.*, 1966, 1968; Araki *et al.*, 1966). *S. faecium* is known to contain the same peptide repeating unit as *L. casei* (Ghuysen *et al.*, 1967), so that these three families may have considerable genetic homology in their cell wall specific cistrons.

The peptidoglycan in the spores of *B. sphaericus* has been characterized, and consists of long glycan chains of alternating 1,4-linked residues of *N*-acetylglucosamine and either muramic lactam (54%), *N*-acetylmuramyl-L-alanine (18%), or *N*-acetylmuramyl-L-alanyl-D-isoglutamyl-*meso*-diaminopimelyl-D-alanine (28%) (Tipper, 1969b). The biosynthesis of these two structures during sporulation is under investigation.

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Specific Dissociation of Bacteriophage f_2 Protein to an 11S Component*

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ABSTRACT: The ribonucleic acid bacteriophage f_2 was partially disaggregated by guanidine hydrochloride. Approximately one-half of the protein was obtained as an apparently homogeneous 11S component. The molecular weight of this stable degradative intermediate indicates the presence of 19 ± 1 polypeptide chains. The morphology of the particle was investigated by electron microscopic examination of negatively stained specimens.

Electron microscopy of the small RNA-containing bacteriophages often fails to provide detail sufficiently convincing to permit classification of these viruses into one of the icosahedral classes. Bacteriophage R_{17}, for example, has been classified as a 32 capsomer virus by Vasquez *et al.* (1966) while Klug and coworkers (1966) feel a 60 capsomer model is the correct one. Evaluation of the latter data must await publication of the details of their work. This difficulty in direct interpretation of electron micrographs may result from the partial collapse of the virus particle during electron microscopic preparation, the possible existence of additional protein subunits in an internal protein core, or the possibility that the virus is not, in fact, icosahedral. Even if these problems are not encountered, capsomer definition in electron micrographs diminishes rapidly as the size of the morphological subunit decreases. The visualization of extended apparent local symmetry resulting from superposition of capsomers negatively contrasted on the top and bottom of the particle then becomes a rare event and the likelihood of predominantly bottom stained particles probably diminishes as the size of the virus decreases.

Any of the above difficulties may be responsible for ambiguities in interpreting the structure of the spherical RNA bacteriophages and we have therefore used an alternate approach in which bacteriophage f_2 was dissociated by solvent perturbation to yield a product from which an apparently homogeneous preparation of virus subunits was obtained accounting for a majority of the original intact viral protein.

Experimental Section

Materials. Crystalline ribonuclease was obtained from Worthington Biochemical Corp., and G-HCl from Eastman Organic Chemicals. Ultraviolet-absorbing impurities were removed from the latter by twice applying the following procedure. Norit A (1.7 g) was added to 50 ml of the guanidine-coning buffer, shaken, and allowed to stand for 15 min. The Norit A was removed by vacuum filtration using Whatman No. 42 filter paper. Bacteriophage f_2 was obtained from an infected Hfr strain of *Escherichia coli* (AB312) and purified according to the method of Cooper and Zinder (1963).

Gel Electrophoresis. Polyacrylamide gel electrophoresis was conducted in Lucite tubes using a continuous buffer system (0.04 M Tris adjusted to pH 9.0 or 7.75 with HCl or 0.03 M sodium acetate adjusted to pH 5.0 with acetic acid). The gels were stained with Amido Black and destained by diffusion into 10% acetic acid.

Electron Microscopy. The negative staining procedures and electron microscopic methodology used in our laboratory has been previously described (Haschemeyer, 1968). Approximate particle dimensions were obtained from photographs containing admixed crystalline catalase according to the procedure described by Luftig (1967, 1968).

Zone Centrifugation. Sucrose gradient experiments were performed in a Spinco Model L2-65-B preparative ultracentrifuge using the SW27 Ti swinging-bucket rotor. A 7-ml layer of 20% sucrose in TKC[1] buffer was initially placed at the bottom of each cellulose nitrate tube, followed by a 5–20% linear sucrose gradient throughout the rest of the tube. All gradients were allowed to stand 4–8 hr before overlayering

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[1] Abbreviation used is: TCK, 0.04 M Tris buffer–0.10 M KCl–0.005 M $CaCl_2$, titrated to pH 7.0 at 4° with HCl.