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Comparative in Vivo Nitrogen-15 Nuclear Magnetic Resonance Study of the Cell Wall Components of Five Gram-Positive Bacteria†

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ABSTRACT: The proton-decoupled 9.12-MHz ^{15}N NMR spectra of ^{15}N-labeled *Bacillus subtilis*, *Bacillus licheniformis*, *Staphylococcus aureus*, *Streptococcus faecalis*, and *Micrococcus lysodeikticus* intact cells, isolated cells walls, and cell wall digests have been examined. The general characteristics of Gram-positive bacteria ^{15}N NMR spectra are described and spectral assignments are provided, which allow in vivo ^{15}N NMR to be applied to a wide range of problems in bacterial cell wall research. The qualitative similarity of the intact cell and cell wall spectra found in each bacteria allowed the ^{15}N resonances observed in the proton broad-band noise-decoupled ^{15}N NMR spectra of intact cells to be assigned to cell wall components. Each of the five Gram-positive bacteria displayed a unique set of cell wall ^{15}N resonances, which reflected variations in the primary structure of peptidoglycans and the amounts of teichoic acid and teichuronic acid in the cell wall, as well as the dynamic properties of the cell wall polymers. Spectral assignments of cell wall ^{15}N resonances assigned to teichoic D-Ala residues, teichuronic acid and acetamido groups, and peptidoglycan acetamido, amide, peptide, and free amino groups have been made on the basis of specific isotopic labeling and dilution experiments, comparison of chemical shifts to literature values, determination of pH titration shifts, cell wall fractionation experiments, and comparative analysis of the cell wall lysozyme digest spectra in terms of the known primary sequences of peptide chains. All the peptidoglycan ^{15}N peptide resonances observed in the intact cells and isolated cell walls could be accounted for by residues in the bridge or crossbar regions of the peptide chains, which indicated that only the cross-linking groups had a high degree of motional freedom. Thermal- and pH-induced conformational changes around the cross-linking D-Ala residues were detected in the *B. licheniformis* cell wall lysozyme digest products. Comparison of the proton broad-band noise-decoupled and gated decoupled intact cell and cell wall ^{15}N spectra indicated that broad-band proton decoupling resulted in nulling of cytoplasmic resonances and enhancement of the cell wall resonances by the $^{15}N\{^1H\}$ nuclear Overhauser effect.

The chemical compositions of the Gram-positive bacterial cell walls and the primary structures of cell wall peptidoglycans, teichoic acids, and teichuronic acids have been thoroughly characterized (Ghuysen & Shockman, 1973; Tipper, 1970; Hughes, 1968). Much less is known about the physical and structural properties of bacterial cell wall polymers (Rogers, 1974) due to difficulties in making conformational and dynamic measurements on peptidoglycans. This has prevented the adequate testing of various three-dimensional structural models proposed for bacterial cell wall peptidoglycan (Tipper, 1970; Keleman & Rogers, 1971; Braun et al., 1973; Oldmixon et al., 1974; Formanek et al., 1974).

Nuclear magnetic resonance spectroscopy is a powerful tool for probing the conformations, noncovalent bonding interactions, and molecular motion of polymeric molecules.

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However, the complexity of the NMR[1] spectra of intact cells and isolated cellular organelles has usually prevented the technique from being applied to analysis of cellular structural components under physiologically relevant conditions. Recent observations (Lapidot & Irving, 1977a) of relatively simple, well-resolved proton-decoupled 9.12-MHz ^{15}N NMR spectra from packed cell pellets of ^{15}N-labeled *Escherichia coli*, *Bacillus licheniformis*, and baker's yeast and ^{15}N-Gly-labeled *Staphyllococcus aureus* pointed to the possibility of using ^{15}N NMR to probe the structural and dynamic properties of native cellular components. The technique has proved particularly suitable for studying cell wall polymers in intact bacterial cells under physiologically relevant conditions. ^{15}N nuclear magnetic resonances of cell wall components have been used to study the effect of organization on the dynamic properties of cell envelope components in *E. coli* (Irving & Lapidot, 1977), the contributions of noncovalent bonding interactions to molecular motions of peptidoglycan in *B. licheniformis* (Lapidot & Irving, 1977b, 1978a,b; Irving & Lapidot, 1978a), the physical interactions between vancomycin and the cell wall in *B. licheniformis* (Irving & Lapidot, 1978b), and the nature of the molecular motions of the peptidoglycan pentaglycine bridge in *S. aureus* cell walls (Lapidot & Irving, 1978c). The scope of in vivo ^{15}N NMR measurements might be extended to additional problems in bacterial cell wall research, such as (1) the relation between the packing arrangements of cell wall polymers and the gross morphological and mechanical features of the cell wall, (2) changes in the conformations and packing of cell wall polymers that occur during the turnover and translocation of cell wall material, (3) the mechanism by which new peptidoglycan is inserted into the cell wall without disturbing the mechanical properties of the existing wall, and (4) the physicochemical basis of the bactericidal action of antibiotics. In order to stimulate research in this direction and also to provide a firmer basis for the past applications of ^{15}N NMR to topics in cell wall research, we here report a survey of the ^{15}N NMR spectra of the intact cells, isolated cell walls, and cell wall digests of five uniformly ^{15}N-enriched Gram-positive bacteria.

The objectives of this paper are to provide definitive spectral assignments for peptidoglycan, teichoic acid, and teichuronic acid nitrogen groups and to determine the biochemical and dynamic basis for both the differences found in the ^{15}N NMR spectra of the five Gram-positive bacteria and changes observed in the cell wall resonances on going from the intact cell to the isolated cell wall to the cell wall lysozyme digest.

Materials and Methods

^{15}N-Enriched Reagents. [^{15}N]Ammonium chloride enriched to 90–95% ^{15}N was prepared by standard methods from [^{15}N]nitric acid (obtained from the Isotope Separation Plant of the Weizmann Institute). Standard procedures were used to prepare [^{15}N]glycine (Schoenheimer & Ratner, 1938), L-[$^{15}N_{\omega}$]lysine (Fink et al., 1944), and DL-[^{15}N]alanine (Shemin, 1946). The ^{15}N-labeled amino acid mixture was obtained from the protein hydrolysate of baker's yeast grown at 37 °C with aeration on ^{15}N minimum salts media supplemented with glucose (5 g/L) and Difco yeast carbon base (10 g/L). The amino acid mixture was purified by ion-exchange chromatography on Dowex H-50. The composition of the ^{15}N-labeled amino acid mixture, as determined on a Beckman amino acid analyzer, was 14.4 Lys, 3.5 His, 5.0 Arg, 0.2 Asp, 0.6 Thr, 0.3 Ser, 2.2 Glu, 3.8 Pro, 100 Gly, 130 Ala, 41 Cys, 10.7 Val, 2.2 Met, 14.0 Leu, 2.6 Tyr, 8.1 Phe (mg/g). *N*-Acetylglucosamine was received as a gift from Professor David Mirelman.

Growth Conditions. ^{15}N-Labeled *B. licheniformis* (ATCC 9945) and ^{15}N-labeled *B. subtilis* (ATCC 6633) were grown at 37 °C with aeration and gyrorotatory shaking to mid-log growth phase [OD_{680} 0.15–0.20] on Spizizen media (Spizizen, 1958) in which $(NH_4)_2SO_4$ (2 g/L) was replaced with $^{15}NH_4Cl$ (1 g/L). ^{15}N-Labeled *S. aureus* H and *M. lysodeikticus* (NTCC 2665) were grown at 30 and 37 °C, respectively, with aeration and gyrorotatory shaking to mid-log growth phase [OD_{680} 0.1–0.5] on McVeigh–Hobdy (McVeigh & Hobdy, 1952) media in which $(NH_4)_2SO_4$ (1 g/L) was replaced by $^{15}NH_4Cl$ (0.5 g/L) and synthetic amino acid mixture was replaced by ^{15}N-labeled amino acid mixture (1 g/L). ^{15}N-Gly-labeled *S. aureus*, ^{15}N-Ala-labeled *S. aureus*, and $^{15}N_{\omega}$-Lys-labeled *S. aureus* were grown on McVeigh–Hobdy media by individually replacing glycine, alanine, and lysine in the synthetic amino acid mixture with [^{15}N]glycine (150 g/L), DL-[^{15}N]alanine (150 mg/mL), and L-[$^{15}N_{\omega}$]lysine (100 mg/L), respectively. ^{15}N-Labeled *S. faecalis* (ATCC 9790) was grown at 37 °C with aeration and gyrorotatory shaking to mid-log growth phase [OD_{680} 0.1–0.15] on Shockman media (Shockman, 1963) in which the synthetic amino acid mixture was replaced with ^{15}N-labeled amino acid mixture (1 g/L).

Preparation of Cell and Cell Wall Samples. Upon completion of growth, 4% $NaDodSO_4$ was added to the culture media to inactivate autolysins (Rogers & Forsberg, 1971), shaking was continued for 10 min at 37 °C, and the bacterial cells were rapidly harvested by centrifugation (Sorval RC2-B rotor, 10000 rpm, 10 min, 37 °C). The cell pellet was washed three times with distilled water at 37 °C to remove traces of $NaDodSO_4$. Cell walls were prepared by mechanical disruption of cells in a Braun tissue homogenizer by shaking at maximum speed with 0.10–0.11-mm diameter ballatoni glass beads (B. Braun Melsunsen, West Germany, No. 541540) for 3 min at 4 °C. The supernatant was decanted from the glass beads and centrifuged (10000*g*, 10 min, 4 °C) to remove unbroken cells. Cell walls were obtained from the supernatant by centrifugation (15000*g*, 15 min, 4 °C), and the pH was adjusted to pH 7.0 by addition of concentrated HCl, when needed. Intact cells and cell walls were packed into 10-mm NMR tubes (Wilmad No. 513-7PP) to heights of 2–2.5 cm by centrifugation at 3000 rpm (IEC HN-S centrifuge) for 0.5 and 2 h, respectively.

Cell wall lysozyme digests were prepared by suspending 1–2 cm^3 of packed cell walls in 10 mL of distilled water containing 2–3 mg of salt-free lysozyme (Worthington Biochemical Co.) and incubating at pH 7.0 for 5 h at 37 °C with gentle shaking. The reaction mixture was boiled for 5 min to precipitate proteins and centrifuged (15000*g*, 15 min). The supernatant was concentrated to 1 cm^3 by rotatory evaporation, after which the pH was adjusted by addition of concentrated HCl or NaOH.

Partially autolyzed ^{15}N-Ala-labeled *S. aureus* cell walls were prepared by incubating for 5 h at 37 °C, pH 9.0, cell walls that were obtained from cells that had been harvested without prior inactivation of autolysins by $NaDodSO_4$ treatment. Before packing into an NMR tube, the cell walls were washed three times with distilled water and adjusted to pH 7.0.

[1] Abbreviations used: GlcNAc, *N*-acetylglucosamine; GlcUA, glucuronic acid; Gdn·HCl, guanidine hydrochloride; *meso*-DAP, *meso*-diaminopimelic acid; MurNAc, *N*-acetylmuramic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; $NaDodSO_4$, sodium dodecyl sulfate.

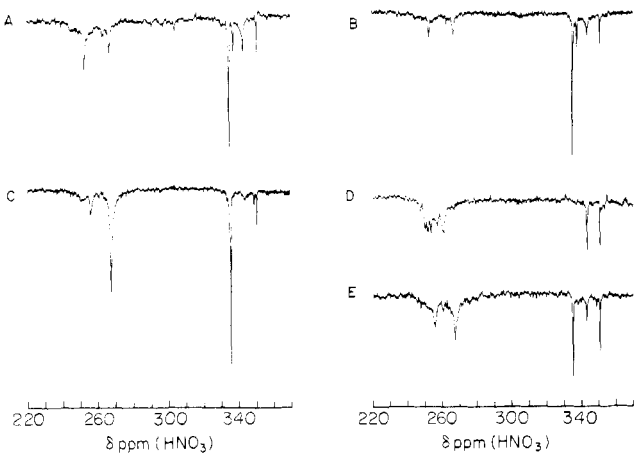


FIGURE 1: The proton-decoupled 9.12-MHz ^{15}N NMR spectra at 27 °C of five ^{15}N-enriched intact Gram-positive bacterial cells. Assignments of resonances to regions 1–10 of the peptidoglycan peptide chain shown in Figure 3, teichoic acid (TA), and teichuronic acid (TUA) are as follows: 350.89 (4 M $^{15}NH_4Cl$ in 2 M HCl external reference); *B. licheniformis* 252.8 (TUA), 266.8 (3 or 6), 335.6 (TA), 337.9 (10), 343.6 (protein Lys-N_ω); *B. subtilis* 253.4 (TUA), 266.8 (3 or 6), 335.5 (TA), 337.8 (10), 343.6 (proton Lys-N_ω); *S. faecalis* 255.7 (7), 260.3 (8), 267.3 (3), 268.4 ($CONH_2$ in 8), 335.3 (TA), 343.3 (10 or protein Lys-N_ω); *M. lysodeikticus* 250.2 (1), 251.5 (9), 253.6 (glycan), 256.2 (7 or 2), 260.3 ($NHCH_2CO^-$ in 3), 343.6 (10); *S. aureus* 250.2–251.7 (9), 255.7 (7), 267.3 (8), 343.9 (10), 349.2 (N-terminal glycyl residue in non-cross-linked 8). (A) *B. licheniformis*. (B) *B. subtilis*. (C) *S. aureus*.

Teichoic acid and teichuronic acid were extracted from *B. licheniformis* cell walls (2 cm^3) with 10 mL of 5% (w/v) trichloroacetic acid at 35 °C (Hughes, 1968). Cell walls were washed several times with distilled water and their pHs adjusted to pH 6 by addition of concentrated NaOH. Teichoic acid was extracted from *B. licheniformis* cell walls by incubating 2 cm^3 of packed cell walls suspended in 100 mL of 0.1 N NaOH at 35 °C under nitrogen for 24 h (Hughes & Tanner, 1968). The cell walls were washed extensively with distilled water and adjusted to pH 7.0 by addition of dilute HCl before packing into an NMR tube.

Nuclear Magnetic Resonance Measurements. ^{15}N NMR measurements were performed on a Brucker WH-90 pulse Fourier transform spectrometer operating at 9.12 MHz. Proton broad-band noise-decoupled spectra were obtained with the following spectrometer conditions: 90° pulse angle of 28-μs duration, 2-KHz spectral width, 1-s recycle time, 10000 accumulations, 2K Fourier transform of data, 2-Hz exponential filter, digital resolution of ca. 1 Hz. Spectral accumulations were repeated using 3-s delays to verify that partial saturation had not occurred. Gated decoupled spectra (proton irradiation on only during accumulation of free induction decays) were obtained using delays (greater than ten times the estimated T_1 values) of 4 s for cells and cell walls and 10 s for lysozyme digests. Field stabilization was accomplished by locking on the deuterium resonance of D_2O in a 5-mm concentric tube inserted into the 10-mm sample tube. $^{15}NH_4Cl$ (4 M) in 2 M HCl in a 2-mm tube inserted into the 5-mm tube of D_2O provided an external reference signal at 350.89 ppm upfield from $H^{15}NO_3$. Chemical shifts are estimated to be accurate to ±0.05 ppm.

Line widths and relative amplitudes of the lysozyme digest spectra were determined by matching the observed bandshapes, with computer simulated spectra consisting of superimposed Lorentzian-shaped lines of varying intensities, line widths, and chemical shifts, using programs written in SPEAKEZ and interactive graphics on a Tektronics 4014A visual display unit.

Results and Discussion

The proton-decoupled 9.12-MHz ^{15}N NMR spectra of pellets of intact cells and cell walls of five ^{15}N-enriched Gram-positive bacteria are shown in Figures 1 and 2. The five Gram-positive bacteria were chosen for the variations in the primary structures of their peptidoglycan peptide chains and the cell wall acidic polysaccharides, which are summarized in Figure 3 and Table I. The remarkable qualitative similarities between the ^{15}N NMR spectra of the intact cell and the isolated cell walls found in each of the five Gram-positive bacteria indicate that the resonances observed in the intact cell spectra originate primarily from cell wall components.

The relatively simple, intense, and well-resolved ^{15}N NMR spectra obtained for Gram-positive bacteria can be accounted for by the relatively large mass of the Gram-positive cell wall (15–20% dry weight of the cell), the high percentage of nitrogen in the cell wall (5–10%), the relatively small number of different types of nitrogen functional groups (five–ten types of peptide bonds, two types of free amino groups, and two–three types of *N*-acetylhexosamineacetamide groups) (Salton,

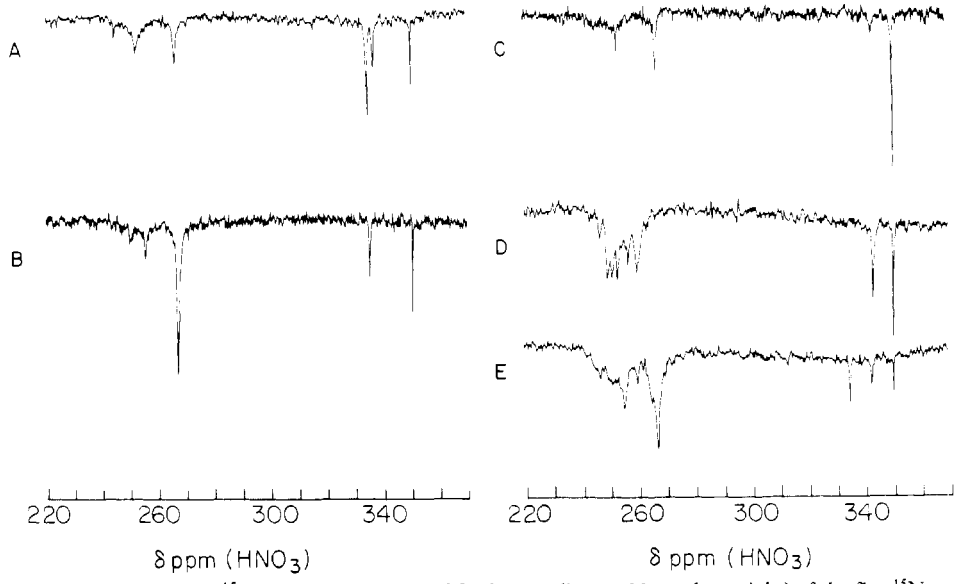


FIGURE 2: The proton-decoupled 9.12-MHz ^{15}N NMR spectra at 27 °C of cell walls (~100 mg dry weight) of the five ^{15}N-enriched Gram-positive bacteria shown in Figure 1. (A) *B. licheniformis*. (B) *S. aureus*. (C) *B. subtilis*. (D) *M. lysodeikticus*.

Table I: Composition of the Cell Walls of Five Gram-Positive Bacteria

bacteria	R[a]	R'[a]	bridge	accessory polymer
B. licheniformis	OH, NH_2	COO^-, $CONH_2$	direct	teichoic acid, teichuronic acid
B. subtilis	OH, NH_2	COO^-, $CONH_2$	direct	teichoic acid
S. faecalis	NH_2	H	$-NHCHCH_2CO-$ (CONH$_2$)	polysaccharides
M. lysodeikticus	$NHCH_2COO^-$	H	(L-Ala-γ-D-Glu-L-Lys)$_{1-6}$ (Gly; D-Ala)	trace amounts of *N*-acetyl polysaccharide amines
S. aureus	NH_2	H	$(-NHCH_2CO-)_5$	teichoic acid

[a] Refer to the R and R' groups in Figure 3.

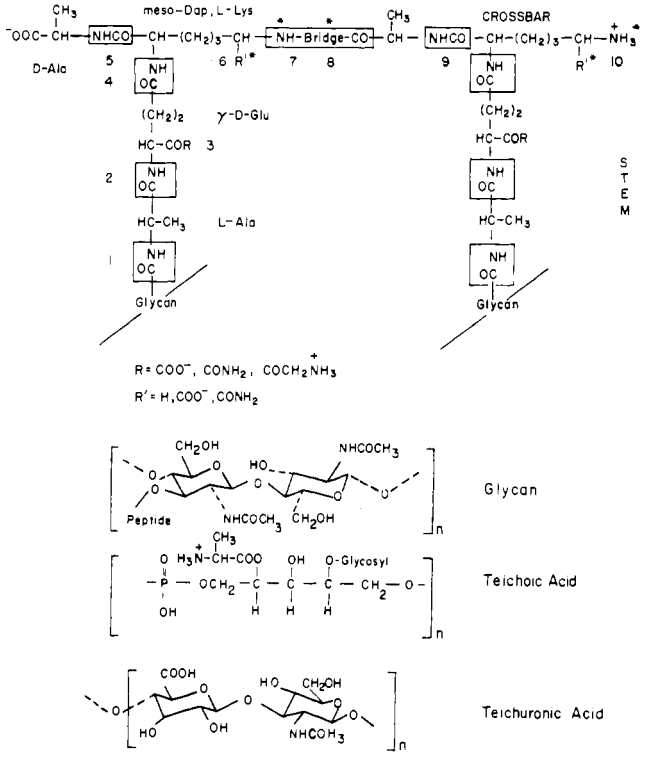


FIGURE 3: The primary structure of cell wall polymers according to Ghuysen (1968). The different types of peptide nitrogens are marked and numbered for reference in the text.

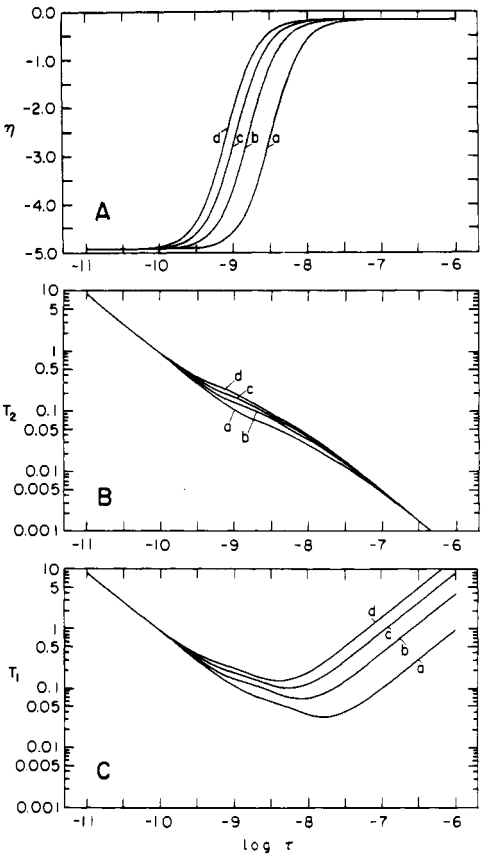


FIGURE 4: The dependence of the $^{15}N\{^1H\}$ nuclear Overhauser effect (A), the ^{15}N spin–spin relaxation time (B), and the ^{15}N spin–lattice relaxation time (C) on correlation time (τ) for relaxation occurring via dipolar interactions of ^{15}N with a single proton (r = 0.9 A) in molecules undergoing isotropic reorientation. Curves are given for 9.12 (a), 18.24 (b), 27.36 (c), and 36.48 (d) MHz.

1964), and the high degree of mobility at several sites along the peptidoglycan and acidic polysaccharide chains. A typical 2000 Hz wide, 2K Fourier transformed ^{15}N NMR spectra of 1.0–1.5 cm^3 of packed cells or cell walls (also the approximately 300 and 100 mg dry weight, respectively) containing 1.30-μm equivalents of each type of nitrogen group required the accumulation of 10000 free induction decays over the course of about 4 h. Several cell wall and gated decoupled spectra were on the limits of a Brucker WH-90 spectrometer sensitivity. Variations in the packing of cell wall samples can lead to dramatic changes in the absolute spectral intensities. Contamination with paramagnetic ions can lead to variations in the intensities of nuclear Overhauser enhanced free amino ^{15}N resonances (Irving & Lapidot, 1975).

As seen in Figures 1 and 2, each Gram-positive bacteria has a unique ^{15}N NMR spectrum, which probably reflects differences in the cell wall composition and dynamic structure. The basic strategy which has been used to account for these differences has been to assign the peptidoglycan ^{15}N NMR resonance obtained for cell wall lysozyme digests in terms of the primary structures given in Figure 3 and Table I and then to determine which peptidoglycan resonances observed in the lysozyme digest spectra are nulled in the whole cells and cell wall spectra. Cell wall lysozyme digests are particularly useful in making assignments of peptidoglycan resonances since cleavage of the MurNAc–GlcNAc β(1-4) glycosidic linkages in glycan strands introduces a great deal of motional freedom into peptide chains without changing their primary structures. Thus, deviations in relative intensities found on going from the lysozyme digests to the intact, isolated, and native cell walls can only result from a change in the mobility along the peptide chains. As the correlation time of a ^{15}NH group, whose relaxation is dominated by ^{15}N–^1H dipolar interactions, increases, the nuclear Overhauser enhancement factor approaches zero and T_2 relaxation times that determine line widths (lw = $1/\pi T_2$) decrease (Figure 4), leading to a reduction in absolute signal intensity and line broadening. Both of these effects can lead to the disappearance of a ^{15}N resonance. Gated decoupled measurements are required to distinguish between these possibilities. The change in the ^{15}N chemical shift of a resonance on going from the lysozyme digest can result only from a conformational change which

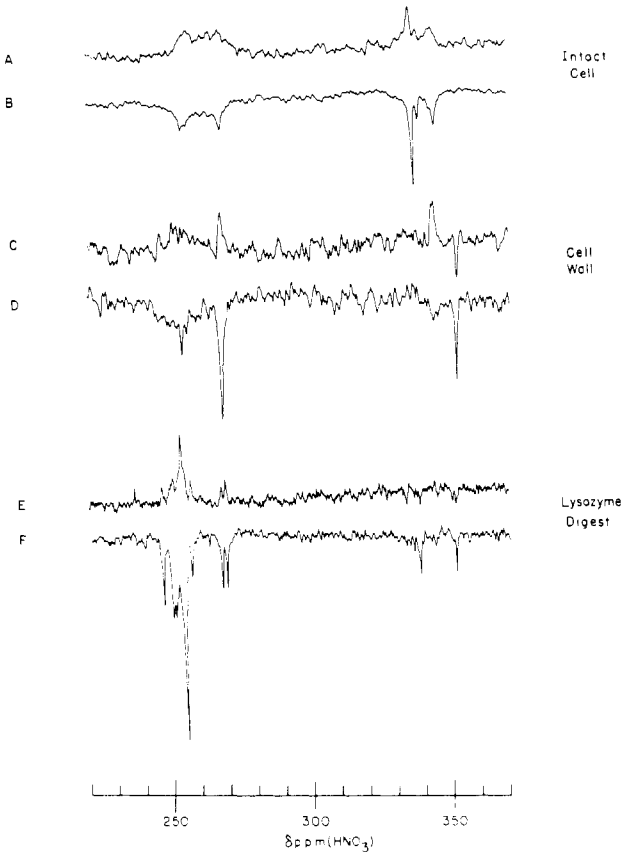


FIGURE 5: The gated decoupled (A, C, E) and broad-band noise-decoupled (B, D, F) ^{15}N NMR spectra of *B. subtilis* cell, cell walls, and cell wall lysozyme digest.

alters either the time-averaged electronic structure or the mode of solvation of the peptide group.

^{15}N resonances observed in the lysozyme digest spectra have been assigned on the basis of specific isotope labeling and dilution experiments, the reported ^{15}N chemical shifts of oligopeptides and 2-deoxy-2-aminohexose derivatives, cell wall fraction studies, and a taxonomic comparison of lysozyme digest spectra. In the following sections, spectral assignments are made for *B. subtilis*, *B. licheniformis*, *S. aureus*, *S. faecalis*, and *M. lysodeikticus* cell wall resonances. Variations in resonance intensities are accounted for in terms of the dynamic structure of the peptidoglycan network.

B. subtilis. The proton broad-band decoupled and gated decoupled 9.12-MHz ^{15}N NMR spectra of ^{15}N-labeled *B. subtilis* intact cells, cell walls, and cell wall lysozyme digest at pH 7.0 and 27 °C are shown in Figure 5. The proton gated decoupled ^{15}N NMR spectra of the intact cells displays a broad envelope of resonances in the amide region (250–270 ppm). Proton broad-band noise decoupling leads to the nuclear Overhauser effects' nulling of many components of the amide envelope, leaving two well-resolved resonances at 253.4 and 266.8 ppm. These two amide resonances apparently originate from the cell wall since they occur in the proton-decoupled ^{15}N spectrum of isolated cell walls. The change in their relative intensities observed on going from the intact cell to the isolated cell wall proabably results from a change in mobility, which alters the NOE factor.

The 253.4-ppm resonance has been assigned to a derivative of GlcNAc since the resonance is abolished by specific isotope dilution of GlcNAc derivatives, achieved by growing ^{15}N,^{14}N-GlcNAc-labeled *B. subtilis* on ^{15}N minimum salts media supplemented with nonlabeled GlcNAc. The 266.8-ppm resonance is assigned to the amide NH group of amidated

Table II: ^{15}N Spectral Parameters of the *B. subtilis* Cell Wall Lysozyme Digest at pH 7.0, 30 °C

resonance	rel area[a]	line width (Hz)	NOE
246.1	0.33	8	−3.8
249.4	0.32	8	−3.8
250.3	0.37	8	−2.8
251.1	0.07	8	−8.7
252.3	0.46	10	−1.8
253.3	1.00	7	−3.0
254.3	0.43	8	−1.6
256.4	0.30	6	−1.6
267.0	0.23	6	−3.1
268.8	0.32	6	−2.2

[a] Of the gated decoupled spectrum.

Table III: pH Titration Shifts of ^{15}N Resonances of the *B. subtilis* Cell Wall Lysozyme Digest at 29 °C

pH 1.5	pH 3.5	pH 5.5	pH 11.0
250.1	248.2	245.9	245.5
250.1	249.7	249.2	249.7
250.1	250.2	250.3	250.6
253.3	251.9		
254.0	252.9	251.0	254.3
254.4	253.85	252.2	252.0
257.3	254.7	252.7	252.8
256.6	256.4	256.2	256.5
266.9	267.0	267.0	267.2
269.2	268.9	268.7	268.9

D-Glu and *meso*-DAP residues of cell wall peptidoglycan since this resonance is not found in the cell wall ^{15}N spectra of *E. coli* (Irving & Lapidot, 1977) whose peptidoglycan is identical with that of *B. subtilis*, except for absence of amidated groups (van Heijenoort et al., 1969).

Lysozyme digestion of the *B. subtilis* cell wall leads to the appearance of additional resonances in the amide region, whose chemical shifts, line widths, NOE factors, and relative intensities (Table II) were determined by computer simulation of the band shapes obtained by the superposition of Lorentzian shaped lines. The 253.3- and 254.3-ppm resonances found in the ^{15}N-labeled *B. subtilis* lysozyme digest spectrum of ^{15}N-labeled *B. subtilis* cell wall can be assigned to ClcNAc derivatives since they are absent in the ^{15}N NMR spectrum of lysozyme digest spectra of ^{15}N,^{14}N-GlcNAc-labeled *B. subtilis*. The chemical shift of the 253.4-ppm resonance is in good agreement with the reported chemical shifts of α and β anomeric forms of GlcNAc and GlnNAc (Coxon, 1977; Butto & Roberts, 1977). We can tentatively assign the 253.4- and 254.3-ppm resonances to the acetamido groups of Mur-NAc-β(1-4)-GlcNAc disaccharide units of peptidoglycan, derived from GlcNAc (Pooley, 1976a,b). It was not possible to assign the remaining resonances in the lysozyme digest spectrum to specific peptide groups on the basis of reported chemical shifts of peptide groups since the effects of amidation and the presence of iso(amino acids) in peptide backbone have not yet been evaluated. However, the resonances of peptide groups adjacent to free carboxylic acid groups can be identified on the basis of their pH titration shifts.

The shifts of peptidoglycan peptide resonances can be conveniently followed as a function of pH in the lysozyme digest spectra of ^{15}N,^{14}N-GlcNac-labeled *B. subtilis* cell walls, where peptide resonances are not marked by the intense acetamido resonances. The lysozyme digest spectra of GlcNAc diluted cell walls at pH 11.0, 7.0, 3.5, and 1.5 are shown in Figure 6 and the titration shifts are summarized in Table III. On going to low pH, the 246.1-, 249.4-, and 250.3-ppm resonances coalesce into a single resonance at 250 ppm. The

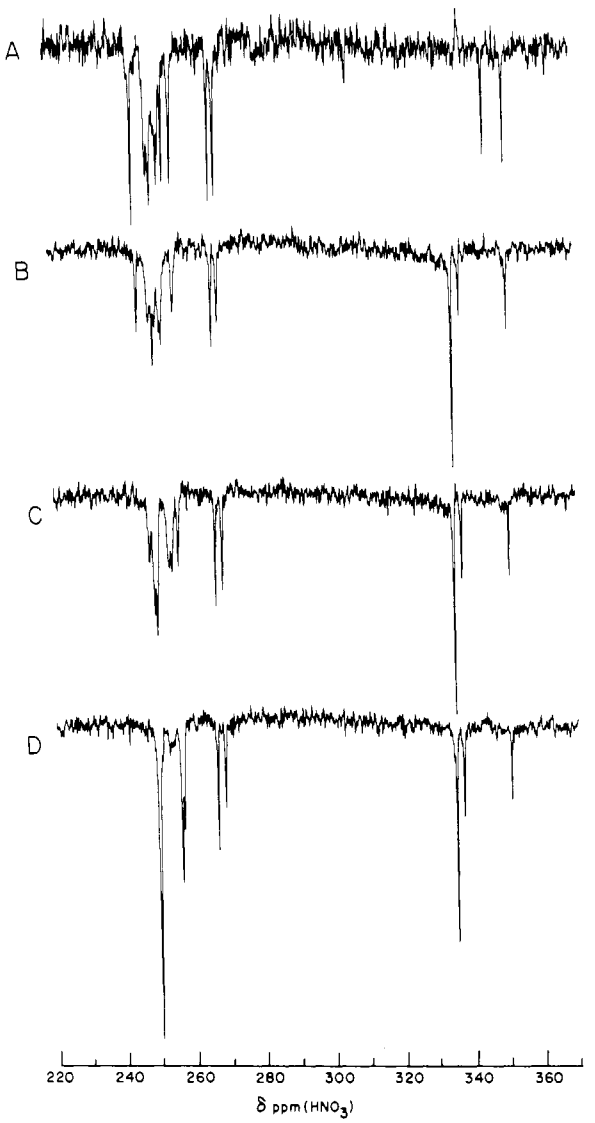


FIGURE 6: The proton broad-band noise-decoupled ^{15}N NMR spectra of the ^{15}N,^{14}N-GalNAc-labeled *B. subtilis* cell wall lysozyme digest at pH (A) 11.0, (B) 7.0, (C) 3.5, (D) 1.5.

4.1-ppm shift of the 246.1-ppm resonance is in reasonable agreement with shifts reported for C-terminal peptide groups upon protonation of carboxylate groups (Irving & Lapidot, 1976; Markowski et al., 1977). The shift of only 0.9 ppm found for the 249.4-ppm resonance probably results from a conformation effect associated with elimination of salt bridging rather than from electronic effect associated with protonation on adjacent carboxylate groups. The coalescence of the three resonances into a single resonance, with a chemical shift characteristic of alanine, suggests that 246.1-, 249.4-, and 250.3-ppm resonances originate from the three types of alanyl peptide bonds shown at positions 1, 5, and 7 in Figure 3. The 246.1-ppm resonance can be readily assigned to the C-terminal D-Ala at position 5. We tentatively assign the 249.4-ppm resonance to the D-Ala at position 7 in the crossbar region and the 250.1-ppm resonance to the L-Ala at position 1 in the stem region of the peptidoglycan peptide chain. On going to low pH, the 251.1- and 252.3-ppm resonances shift to 255 ppm and therefore originate from γ-D-Glu and *meso*-DAP groups at positions 3 and 7 (R = R′ = OH), respectively. The resonance at 256.4 ppm, which does not shift at low pH, can be assigned to *meso*-DAP residue at position 4 by elimination. This resonance is likely to be degenerate with amidated γ-

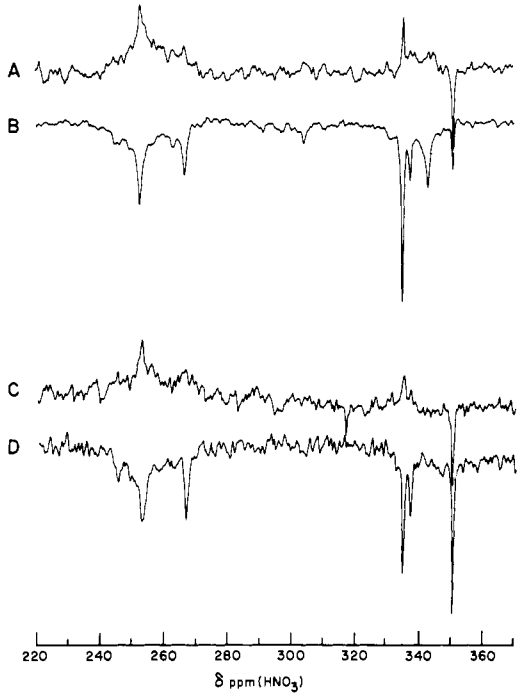


FIGURE 7: The gated decoupled (A, C) and broad-band noise-decoupled (B, D) ^{15}N NMR spectra of ^{15}N-labeled *B. licheniformis* cells (A, B) and cell walls (C, D).

D-Glu and *meso*-DAP groups at positions 3 and 7 (R = R′ = NH_2), respectively. The amide groups of amidated γ-D-Glu and *meso*-DAP occur at 267.0 and 268.8 ppm. It is interesting to note the broadened line widths are observed at intermediate pH values, which indicate that protonation of carboxylate groups involving cleavage of salt bridges might occur at an intermediate exchange rate on the NMR time scale. The shift of the 251.1-ppm resonance to 254 on going from pH 7.0 to 11.0 is puzzling since the only ammonium group in the peptide chain, the *meso*-DAP amino group at position 10, is far removed from any peptide bond. Apparently the shift results from a conformational change associated with breakage of a salt bridge. The characterization of the conformational change must await the definitive assignment of the 251.1-ppm resonance.

The amino region of the broad-band noise-decoupled ^{15}N NMR spectrum of ^{15}N-labeled *B. subtilis* cells displays three resonances at 335.6, 337.9, and 343.6 ppm. The 343.6-ppm resonance can be assigned to protein Lys-N_ω free amino groups on the basis of its chemical shift (Lapidot & Irving, 1977; Gust et al., 1975). Although the 335.6- and 337.9-ppm resonances are not seen in the cell wall and lysozyme spectra shown in Figure 5, they can be readily observed in the lysozyme digest spectra shown in Figure 6 and must originate from cell wall components. As demonstrated in a later section, the 335.6-ppm resonance can be assigned to the free amino group of D-Ala residues of teichoic acids, while the 337.9-ppm resonance is assigned to the free amino group of *meso*-DAP residues of peptidoglycan. The NOE factors of the amino groups are notoriously sensitive to the presence of trace amounts of paramagnetic metal ions (Irving & Lapidot, 1975). The sporadic absence of the free amino resonances in cell wall and lysozyme digest spectra probably results from coordination of free amino groups with trace amounts of metal ions, which results in nulling of the ^{15}N resonance due to a partial ^{15}N{^{1}H} NOE enhancements.

B. licheniformis. The proton broad-band noise-decoupled and gated decoupled 9.12-MHz ^{15}N NMR spectra of ^{15}N-

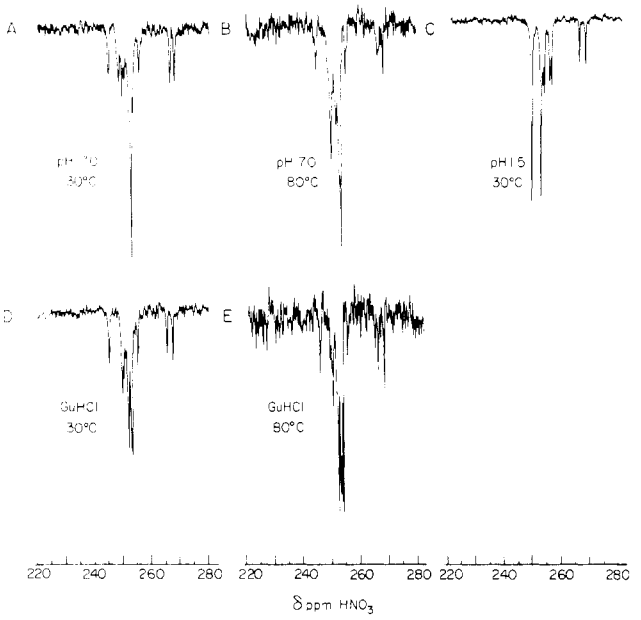


FIGURE 8: The proton broad-band noise-decoupled ^{15}N NMR spectra of *B. licheniformis* cell wall lysozyme digest at (A) 30 °C, pH 7.0, (B) 80 °C, pH 7.0, (C) 30 °C, pH 1.5, (D) 30 °C, suspended in 6 M guanidine hydrochloride, (E) 80 °C, suspended in 6 M guanidine hydrochloride.

Table IV: ^{15}N Spectral Parameters of the *B. licheniformis* Cell Wall Lysozyme Digest at pH 7.0, 30 °C

resonance	rel area[a]	line width
245.6	0.19	4
246.0	0.12	4
248.7	0.02	3
249.3	0.31	7
250.4	0.29	7
251.1	0.21	7
252.0	0.18	6
252.8	0.14	5
253.5	1.00	5
254.2	0.14	3
256.6	0.18	5
267.3	0.25	5
268.9	0.13	3

[a] Of broad-band noise-decoupled spectrum.

labeled *B. licheniformis* intact cells and isolated cell walls are shown in Figure 7. The proton broad-band noise-decoupled spectrum of cell wall lysozyme digest is shown in Figure 8, and the chemical shifts, line widths, and relative amplitudes are summarized in Table IV. The *B. licheniformis* intact cell spectra are qualitatively similar to the spectra of *B. subtilis*, except that the weak resonance at 253.4 ppm in *B. subtilis* is replaced by a much more intense resonance at 252.8 ppm. The 252.8-ppm resonance is present in the lysozyme digest of *B. licheniformis* but not in *B. subtilis*. Peptidoglycans of *B. licheniformis* and *B. subtilis* have identical primary structures; however, *B. licheniformis* cell walls contain, in addition to teichoic acid, teichuronic acid, a polymer of GlnNAc and GlcUA (Hughes, 1968). The 252.8-ppm resonance is significantly attenuated in the intact cell, cell wall, and lysozyme digest spectra of *B. licheniformis* cells grown on media containing nonlabeled GlcNAc. Since GlnNAc is a derivative of GlcNAc, we can assign the 252.8-ppm resonance to acetamido groups of GlnNAc residues of cell wall teichuronic acid. The observation of teichuronic acid ^{15}N NMR resonances in the intact cell indicates that this acidic polysaccharide has a high degree of mobility. It is interesting to note the chondroiton 4-sulfate residues, which are analogues of teichurnoic acid groups, have a similarly high degree of mobility in nasal cartilage proteoglycan (Brewer & Keiser, 1975; Torchia et al., 1977). The assignments of the 252.8- and 335.6-ppm resonances to teichoic and teichuronic acids, respectively, are supported by the absence of these resonances in cell walls, whose teichoic and teichuronic acids have been removed by extraction with trichloroacetic acid (Figure 9A). Other changes are noted in the spectrum of teichoic and teichuronic acid free cell walls, which are probably associated with changes in cell wall structure due to a disturbance in the charge balance. Selective removal of teichoic acid by mild alkaline hydrolysis leads to the disappearance of the 335.6-ppm resonance (Figure 9B).

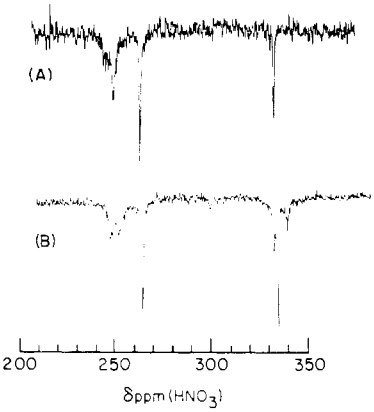


FIGURE 9: The proton broad-band noise-decoupled 9.12-MHz ^{15}N NMR spectra at 27 °C of *B. licheniformis* cell walls without teichoic acid (A) and without teichoic and teichuronic acids (B).

Table V: Effects of Temperature, pH, and Guanidine Hydrochloride on the ^{15}N Chemical Shifts of *B. licheniformis* Cell Wall Lysozyme Digest

pH 7.0, 30 °C	pH 1.5, 30 °C	pH 7.0, 80 °C	6 M Gdn·HCl, 30 °C	6 M Gdn·HCl, 80 °C
245.6	250.3	245.7	245.6	245.9
245.8	250.7	245.7	245.6	245.9
249.3	250.3	250.9	250.3	249.9
250.3	250.3	251.6	250.7	250.5
251.3	256.8	259.8	251.3	251.6
252.7	257.4	252.6	252.4	252.2
257.4	253.5	253.4	253.3, 253.5	252.8, 253.3
254.1	254.3	254.5	254.7	253.8
256.2	256.6	256.1	255.7	255.6
267.1	267.1	267.3	265.8	266.2
268.8	269.2	269.2	267.9	268.5

The observation of the 245.6-ppm C-terminal D-Ala resonance in the *B. licheniformis* intact cell and cell wall spectra might represent either a decrease in the degree of subunit cross-linking or a change in conformation of peptidoglycan peptide chains. It is interesting to note that C-terminal alanine residues are usually absent in cell wall lysozyme digest preparation of *B. licheniformis* and *B. subtilis* (Mirelman & Sharon, 1968; Hughes, 1970). This has been ascribed to the action of a cell wall D-Ala carboxypeptidase during the preparation of the cell walls. In the present study, cell wall enzymes have been inactivated by $NaDodSO_4$ extraction before harvesting and this probably accounts for the observation of relatively intense C-terminal D-Ala ^{15}N resonances in the lysozyme digest spectra reported here.

The proton broad-band noise-decoupled ^{15}N spectra of *B. licheniformis* lysozyme digest have been examined in aqueous solution at 30 and 80 °C at pH 7.0 and at pH 1.5 at 30 °C and in 6 M guanidine hydrochloride at 30 and 80 °C. The

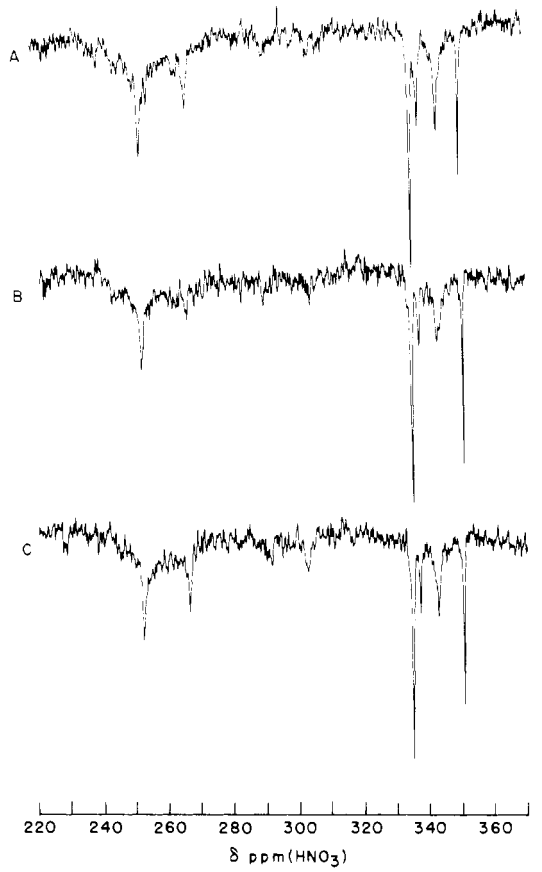


FIGURE 10: The proton broad-band noise-decoupled 9.12-MHz ^{15}N NMR spectra of (A) normal ^{15}N-labeled *B. licheniformis* cells, (B) ^{15}N-labeled *B. licheniformis* cells after one generation of growth on labeled media, (C) ^{15}N-labeled *B. licheniformis* cells after two generations of growth on nonlabeled media.

changes in the ^{15}N chemical shifts are summarized in Table V. The shifts observed on going to low pH parallel those found in *B. subtilis* lysozyme digest. At elevated temperatures only the 249.4-ppm resonance, which was assiged to D-Ala at region 5 in the crossbar region, shifts significantly in the upfield direction. The smaller upfield shifts observed for other resonances are typical of peptide nitrogens exposed to solvent (Llinas & Wuthrich, 1978). The 249.4-ppm resonance also shifts upfield in 6 M Gdn·HCl, while other resonances show downfield shifts associated with interaction of peptide carbonyl groups with protic Gdn·HCl NH groups. These resonances shift upfield in Gdn·HCl at elevated temperatures, which confirm their exposure to solvent molecules.

The observation of teichoic acid, teichuronic acid, and peptidoglycan free amino and amide resonances suggests that these groups have a high degree of mobility in the native cell wall. The possibility exists that a high degree of mobility does not occur uniformly throughout the cell wall but is limited to either the inner layer, containing nascent peptidoglycan strands, or to the outer layer, where turnover due to autolysis occurs. Since new cell wall material migrates to the outer surface during the course of two cell generations (Pooley, 1976a,b), chase dilution of the cell wall with nonlabeled peptidoglycan for one and two generations would be expected to reveal differences in mobility throughout the cell wall. As seen in Figure 10, no significant qualitative differences are observed in the spectra of completely labeled cells harvested one and two generations after growth on nonlabeled media. This indicates that mobile peptidoglycan subunits are not localized in the inner cell wall layer and cannot be accounted for by nascent peptidoglycan chains.

S. aureus. We now turn to coccal bacteria, whose peptidoglycan differs from that of the baccilli in two important respects. L-Lys replaces *meso*-DAP. Cross-linking of peptidoglycan subunit does not occur through the formation of a direct peptide bond between N-terminal L-Lys-N_ϵ and C-terminal D-Ala, but rather via a bridge consisting of an amino acid or peptide chain (see Figure 3) (Ghuysen & Shockman, 1973).

The proton broad-band noise-decoupled 9.12-MHz ^{15}N NMR spectra of intact cells and isolated cell walls of ^{15}N-labeled *S. aureus* display resonances at 250.4, 255.7, 267.3, 335.6, 343.9, and 349.2 ppm (Figures 1c and 2c). The ^{15}N NMR spectrum of specifically labeled ^{15}N-Gly-labeled *S. aureus* (Figure 11A) displays a free amino resonance at 349.2 ppm and an intense peptide resonance at 267.3 ppm, the position of nonterminal glycyl groups with water-solvated, random conformations. The 267.3- and 349.2-ppm resonances observed in ^{15}N-labeled *S. aureus* cells can be conclusively assigned to the nonterminal and N-terminal glycyl residues of the pentaglycine bridge in cell wall peptidoglycan. The ^{15}N NMR spectra of $^{15}N_\omega$-Lys-labeled *S. aureus* cells and cell walls (Figure 11B) display a peptide resonance at 255.1 ppm, in addition to the Lys-N_ω free amino group resonance at 343.6 ppm. The 255.1-ppm resonance can be assigned to L-Lys-N_ω peptide bond at position 7 of Figure 3. ^{15}N-Ala-labeled *S. aureus* cells (Figure 11C) display an intense resonance at 335.6

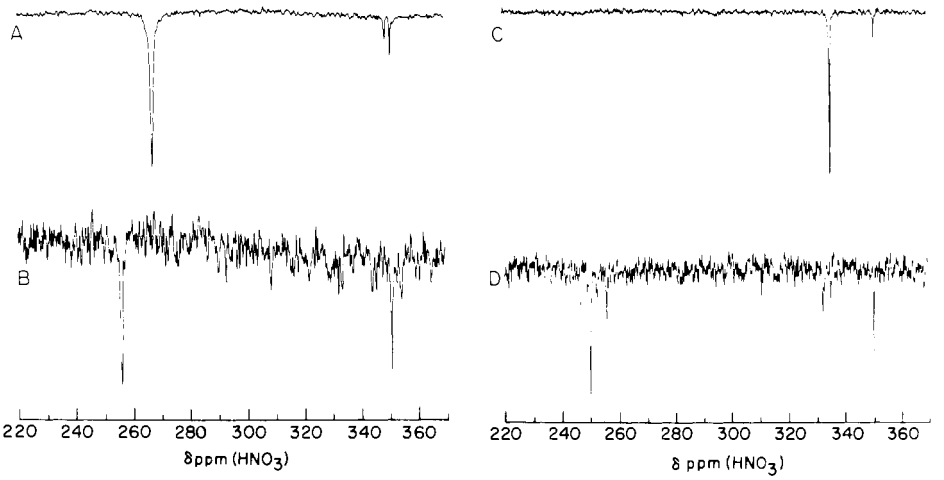


FIGURE 11: The proton broad-band noise-decoupled 9.12-MHz ^{15}N NMR spectra of (A) specifically labeled ^{15}N-Gly-labeled *S. aureus* cells, (B) specifically labeled $^{15}N_\omega$-Lys-labeled *S. aureus* cell walls, (C) specifically labeled ^{15}N-Ala-labeled *S. aureus* cells, (D) specifically labeled ^{15}N-Ala-labeled *S. aureus* cell wall autolysate.

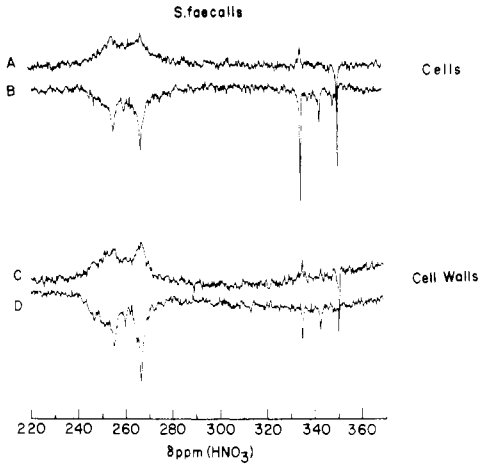


FIGURE 12: The gated decoupled (A, C) and broad-band noise-decoupled (B, D) ^{15}N NMR spectra of ^{15}N-labeled *S. faecalis* cells (A, B) and cell walls (C, D).

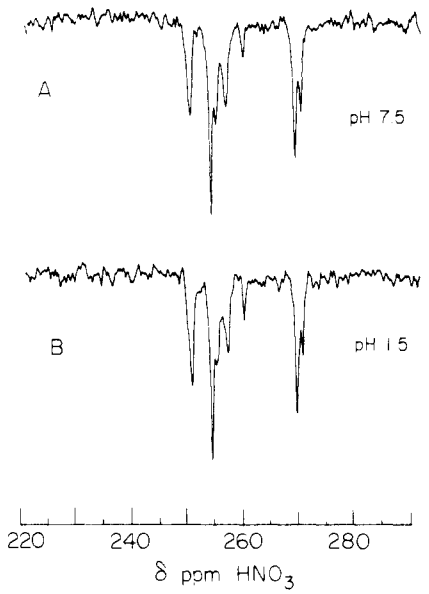
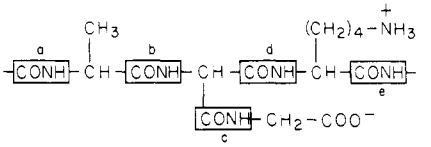


FIGURE 13: The proton broad-band noise-decoupled spectrum of ^{15}N-labeled *S. faecalis* cell wall lysozyme digest at (A) pH 7.5 and (B) pH 1.5.

ppm, which is in agreement with assignment of this resonance to teichoic acid in *B. licheniformis*. *S. aureus* cell walls are resistant to lysozyme; however, they can be autolyzed by incubation at 37 °C at pH 9.0 when $NaDodSO_4$ inactivation of cell wall enzymes is omitted before harvesting. The ^{15}N-Ala-labeled *S. aureus* cell wall autolysate displays a resonance at 250.2 ppm, which has been assigned in *B. subtilis* to an alanine group (Figure 11D) at either position 1 or 9. Although the 250.2-ppm resonance corresponds to the 250.4-ppm resonance found in ^{15}N-labeled *S. aureus* cells, the resonance was not observed in ^{15}N-Ala-labeled *S. aureus* intact cells (Figure 11C). The use of the autolysate in place of lysozyme digest to obtain the chemical shifts of mobile peptidoglycan subunits has the disadvantage that peptide resonance L-Ala at position 1 might be attenuated due to the autolytic cleavage of MurNAc L-Ala peptide bond (Ghuysen, 1968).

S. faecalis. The proton broad-band noise-decoupled and gated decoupled 9.12-MHz ^{15}N NMR spectra of ^{15}N-labeled *S. faecalis* intact cells and cell walls are shown in Figure 12. The proton broad-band noise-decoupled spectrum of ^{15}N-labeled *S. faecalis* cell wall lysozyme digest at pH 7.0 and 27 °C is shown in Figure 13. *S. faecalis* intact cells display resonances at 255.7, 260.3, 267.3, 268.4, 335.3, and 343.3 ppm. Although the resonances at 260.3 and 267.3 ppm have chemical shifts practically identical with C-terminal and nonterminal glycyl residues, respectively, the 260.3-ppm resonance does not shift on going to low pH and *S. faecalis* cell wall peptidoglycan does not contain glycyl residues (Ghuysen, 1968). *S. faecalis* peptidoglycan contains amide groups at both γ-D-Glu (position 3) and iso-D-Asn (bridge) (Ghuysen et al., 1967). The 267.3- and 268.4-ppm resonances can be assigned to amide NH_2 nitrogens by analogy with *B. licheniformis* and *B. subtilis* spectra. Similarly the 335.3- and 343.3-ppm amino resonances are assigned to free amino groups teichoic acid and Lys-N_ω, respectively.

Chart I



Unlike the *B. subtilis* lysozyme digest spectrum, the resonances in the spectra of *S. faecalis* cell wall lysozyme digest do show shift on going to low pH and closely resemble the *B. subtilis* lysozyme digest spectra at pH 1.5. In *S. faecalis* peptidoglycan, all the carboxylate groups are amidated and to the first approximation amidation and protonation of free carboxylate groups produce equivalent shifts of adjacent peptide nitrogens. The 250.1-ppm resonance can be assigned to alanine groups at positions 1 and 9, the 253.7-ppm resonance to MurNAc and GlcNAc acetamido groups, the 254.6-ppm resonance to amidate γ-D-Glu, and the 256.4-ppm resonance to Lys-N_α at position 4 and Lys-N_ω at position 7. By elimination the new resonance at 259.6 ppm is assigned to D-isoasparagine peptide bond in the bridge at position 8. The *S. faecalis* lysozyme digest spectrum displays two types of amide NH_2 resonances, one at 269.7 ppm and another at 268.7 ppm. The latter resonance is also found in *B. licheniformis* and *B. subtilis* lysozyme digest spectra and can be assigned to amide NH_2 of γ-D-Glu (position 3), which *B. subtilis*, *B. licheniformis*, and *S. faecalis* have in common. By elimination, the 269.7-ppm resonance of *S. faecalis* is assigned to D-iso-Asn and the 267.1-ppm resonance of *B. licheniformis* and *B. subtilis* is assigned to amidated *meso*-DAP (position 6). The amide NH_2 resonances are sensitive to solvation effects (Table IV), which probably explain the downfield shifts observed on going from lysozyme digests to the intact cell walls. Due to the solvation shifts of amide resonances, it is not possible to make a definitive assignment of the 266.8-ppm resonance in *B. licheniformis* and *B. subtilis* and 267.3-ppm resonance in *S. faecalis*.

The absence of C-terminal Ala resonance at 246 ppm in the lysozyme digest spectra may be indicative of either a high degree of cross-linking or the attachment of a D-iso-Asn residue to C-terminal alanine groups. The free γ-carboxylate groups of D-iso-Asn are too far removed from the peptide nitrogen to perturb the chemical shifts of the D-iso-Asn peptide group.

M. lysodeikticus. The peptidoglycan network of *M. lysodeikticus* differs from those of the other two bacteria studied in two important respects. Not all the glycan MurNAc residues carry peptide chains. The bridge consists of long peptide chains (L-Ala-γ-D-Glu(Gly)-L-Lys-D-Ala)$_{1-6}$ (Chart I) (Ghuysen et al., 1967). *M. lysodeikticus* peptidoglycan has been termed a "loose" network due to the long bridges and low degree of covalent cross-linking (Ghuysen, 1972). The intact cell spectra of *S. aureus* and *S. faecalis* have dem-

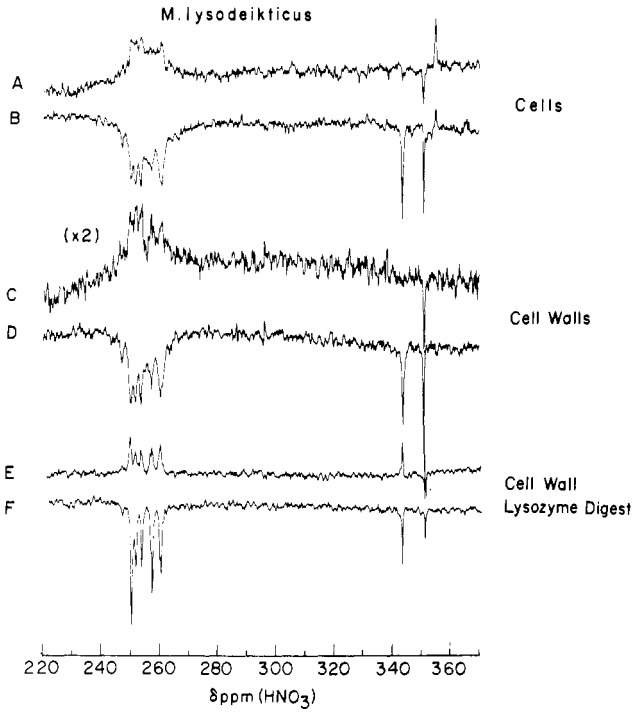


FIGURE 14: The gated decoupled (A, C, E) and broad-band noise-decoupled (B, D, F) ^{15}N NMR spectra of ^{15}N-labeled *M. lysodeikticus* cells (A, B), cell walls (C, D), and cell wall lysozyme digest (E, F).

Table VI: ^{15}N Spectral Parameters of the *M. lysodeikticus* Cell Wall Lysozyme Digest at pH 7.0, 30 °C

resonance	rel area[a]	line width (Hz)	NOE
247	0.31	4	−1.7
249.9	1.64	4	−3.1
251.6	0.85	4	−3.1
253.5	1.00	4	−2.7
256.6	0.70	4	−2.1
257.1	1.00	4	−3.4
2.598	0.73	4	−2.3
260.2	1.09	4	−2.6

[a] Of the gated decoupled spectrum.

onstrated that a high degree of mobility exists in the bridge region of peptidoglycan in the native cell wall; accordingly, a complete set of (L-Ala-γ-D-Glu(Gly)-L-Lys-D-Ala)$_n$ ^{15}N cross-bridge resonances would be expected to occur in the *M. lysodeikticus* intact cell and cell wall spectra. This is the case, as seen in the proton broad-band noise-decoupled and gated decoupled 9.12-MHz ^{15}N NMR spectra of ^{15}N-labeled *M. lysodeikticus* intact cells, isolated cell walls, and cell lysozyme digests shown in Figure 14. The chemical shifts, line widths, NOE factors, and relative intensities of the lysozyme digest spectra were obtained by computer simulation and are given in Table VI. The 6.2-ppm upfield shifts observed for the 247-, 259.3-, and 260.2-ppm resonances in the lysozyme digest spectra on going from pH 1.5 (Figure 15) allow these resonances to be assigned to C-terminal Ala group and to two kinds of C-terminal glycyl groups, respectively. The two types of C-terminal glycyl resonances might be accounted for by the glycyl residues in the stem (position 3 in Figure 3) and bridge (position c in Chart I) regions. The 256.5-ppm resonance can be readily assigned to the Lys-N_α peptide bond (position e in Chart I) by analogy with the peptidoglycan of *S. faecalis*. The 249.9-ppm resonance probably corresponds to the D-Ala peptide (at position e in Chart I). The L-Ala peptide bond (position d in Chart I) appears shifted upfield to 251.6 ppm

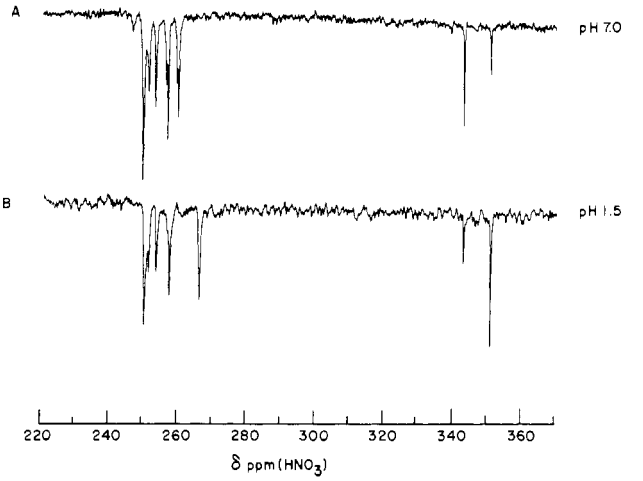


FIGURE 15: The proton broad-band noise-decoupled 9.12-MHz ^{15}N NMR spectra at 27 °C of ^{15}N-labeled *M. lysodeikticus* cell wall lysozyme digest (A) at pH 7.0 and (B) at pH 1.5.

as a result of an N-terminal nearest-neighbor effect associated with the substitution of D-Ala residue in place of MurNAc. The substitution of γ-D-Glu (position b in Chart I) with a charged C-terminal glycyl group in place of an amide NH_2 can lead to changes in the solvation of γ-D-Glu peptide bond, which might explain the downfield shift of the resonance from 254.4 to 253.6 ppm. These assignments are tentative and require specific isotope labeling for their verification.

Comparison of Intact Cell Spectra. The differences in the five Gram-positive bacteria intact cell spectra (Figure 1) are readily explained by variations in cell wall composition, as summarized in Figure 3. Substitution of DAP in place of Lys in *B. licheniformis* and *B. subtilis* leads to the appearance of a new amino free amino resonance at 337.8 ppm and the replacement of the Lys-N_ω peptide resonance at 255.9 ppm with the resonance of the DAP peptide at 253 ppm. The stronger intensity of the 253-ppm resonance in *B. licheniformis* results from the acetamido resonance of teichuronic acid. The substitution of γ-D-Glu carboxylic acid group in *M. lysodeikticus* with a glycyl residue instead of amide NH_2 leads to the appearance of a new resonance at 260.7 ppm and the absence of amide resonances in the vicinity of 267 ppm. *M. lysodeikticus* cell walls do not contain teichoic acid and that explains the absence of the 335.6-ppm resonance, which dominates the amino region of the other four intact cell spectra.

Mobility along the Peptidoglycan Peptide Chain. The observation of a ^{15}N NMR resonance in the intact cell or cell wall depends on the resonance having a relatively narrow (<70 Hz) line width and a large negative nuclear Overhauser enhancement. Normally we assume that when a proton-decoupled resonance is not observed, it has been broadened beyond detection by the large dipole interaction that results from a low degree of motional freedom. However, in the case of proton-decoupled ^{15}N NMR, normally narrow resonances can be lost as a result of partial quenching of the nuclear Overhauser enhancement by factors other than mobility (e.g., paramagnetic ions) (Irving & Lapidot, 1975). In this case, relatively narrow resonances would be observed in gated decoupled spectra (proton decoupling only during spectral accumulation) but not in broad-band noise-decoupled spectra. Examination of gated decoupled and broad-band noise-decoupled spectra of *B. licheniformis*, *B. subtilis*, *S. faecalis*, and *M. lysodeikticus* cells and cell walls (Figures 5, 7, 12, and 14) shows that the peptidoglycan stem resonances (250, 251, and 256 ppm) have not been nulled by proton decoupling. They probably occur in the low-intensity broad envelope, which

has a nuclear Overhauser enhancement factor of about −2.

Some insights into the mobility gradient in peptidoglycan can be obtained by examining from which positions along the peptide chain (Figure 3) the resonances of the intact cell originate. The DAP and Lys-N_ω peptide resonances, the pentaglycyl resonance, and the DAP and iso-Asn amide NH_2 resonances of *B. licheniformis*, *B. subtilis*, *S. aureus*, and *S. faecalis* all originate from either the crossbar or bridge regions of the peptide. No resonance was observed, which could be shown to originate from the stem region of the peptide. Neither was any resonance observed which could be uniquely assigned to the glycan strands. We can conclude *that in a tight peptidoglycan matrix, the glycan strands and the peptide stems are rigid, while considerable mobility exists in the peptide crossbar and bridge regions.* It is reasonable that peptidoglycan stem residues, whose resonances were not observed, have a low degree of motional freedom because they engage in interactions that contribute to the function of the cell wall.

M. lysodeikticus has a "loose" peptidoglycan matrix, in which not all the *N*-acetylmuramic acid residues are substituted with peptide. Furthermore, the bridges consist of replicas of the peptide itself (L-Ala-γ-D-Glu(Gly)-L-Lys(D-Ala)$_{1-6}$). The low degree of cross-linking is believed to introduce considerable "looseness" into *M. lysodeikticus* peptidoglycan (Ghyusen, 1972). This is borne out by the intact cell spectrum of *M. lysodeikticus*, which is the only intact cell spectrum that displays all the resonances observed in the lysozyme digest. *In a "loose" peptidoglycan there is an apparently high degree of mobility throughout the entire peptidoglycan peptide chain.*

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