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Peptidoglycan Loss During Hen Egg White Lysozyme-Inorganic Salt Lysis of *Streptococcus mutans*

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Streptococcus mutans BHT was grown in Todd-Hewitt dialysate medium containing *N*-acetyl[^{14}C]glucosamine for 6 to 11 generations. After treatment with cold and hot trichloroacetic acid and trypsin, 52 to 65% of the radioactivity remained present in insoluble peptidoglycan-containing residues. Hen egg white lysozyme or mutanolysin treatment of the peptidoglycan residues resulted in the release of 80 and 97%, respectively, of the ^{14}C label to the supernatant fraction. Hydrochloric acid hydrolysates of such supernatants showed that essentially all of the radioactivity present in insoluble peptidoglycan fractions was present in compounds that comigrated on paper chromatography with glucosamine (~60%) or muramic acid (~30%). Treatment of whole cells with low and high concentrations of lysozyme alone resulted in losses of 45 and 70% of the insoluble peptidoglycan, respectively, yet release of deoxyribonucleic acid from cells was not detected. Sequential addition of appropriate concentrations of selected inorganic salts after lysozyme treatment did result in the liberation of deoxyribonucleic acid. Deoxyribonucleic acid release was correlated with a further release of peptidoglycan from the insoluble fraction. However, the total amount of peptidoglycan lost effected by the low concentration of lysozyme and NaSCN (lysis) was significantly less than the amount of peptidoglycan hydrolyzed by high concentrations of lysozyme alone (no lysis), suggesting that the overall amount of peptidoglycan lost did not correlate well with cellular lysis. The total amount of insoluble peptidoglycan lost at the highest salt concentrations tested was found to be greater than could be accounted for by lysozyme-sensitive linkages of the peptidoglycan, possibly implicating autolysins. The results obtained suggested that hydrolysis of peptidoglycan bonds in topologically localized, but strategically important, sites was a more significant factor in the sequence that results in loss of cellular integrity (lysis).

It has generally been accepted that lysozyme (EC 3.2.1.17) exerts its lytic effect upon susceptible bacteria, such as *Micrococcus luteus*, through hydrolysis of specific glycosidic linkages in its substrate, the peptidoglycan polymer of the bacterial cell wall (10, 26). Hydrolysis of a sufficient number of bonds in the peptidoglycan yields osmotically fragile cells which rupture at the plasma membrane, resulting in the release of high- and low-molecular-weight intracellular cytoplasmic contents (28). Although isolated cell walls of *M. luteus* are sensitive to lysozyme hydrolysis and are rapidly and completely dissolved by this enzyme (27), it is not clear how many or topologically which glycosidic bonds must be cleaved before lysis of whole cells ensues. It is also not known to what extent endogenous, autolytic peptidoglycan hydrolase enzymes present in the bacteria contribute to the hydrolytic process and cellular lysis. Evidence exists for participation of autolytic enzymes in lysozyme lytic systems (30).

Resistance to the lytic action of lysozyme is known to arise either because of the presence of other cell wall or cell membrane polymers which prevent access of lysozyme to its substrate (13) or through modification of peptidoglycan structure itself. It has been suggested that in some bacteria, e.g., *Streptococcus pyogenes* and *Lactobacillus casei*, the presence of cell wall polysaccharide interferes in part with lysozyme hydrolysis of the cell (8, 17, 18), whereas in other bacteria, e.g., *Lactobacillus fermentum*, lysis is not impeded (17). Examples of resistance due to peptidoglycan structure include changes in *O*-acyl or *N*-acetyl substituents of the peptidogly-

can. Holden et al. (12) attributed the increased lysozyme sensitivity of *Lactobacillus plantarum* grown with exogenous fatty acids in the absence of pantothenate to a reduction in cell wall *O*-acyl content. Logardt and Neujahr (20) observed that *L. fermentum* becomes less sensitive to lysozyme as the cells go into the stationary growth phase and correlated this resistance with an increase in the substitution of *O*-acetyl groups at the CH_2OH6-position of *N*-acetylmuramic acid residues. Other investigators (3, 4, 8, 33) have also noted that increased *O*-acyl wall content leads to the development of resistance to lysozyme. In addition, some bacteria, such as *Bacillus cereus*, have been shown to possess a deacetylase which hydrolyzes acetamido groups of *N*-acetylglucosamine residues (1). Because of the occurrence of glucosamine residues with free amino groups in the peptidoglycan, *B. cereus* is thought to be resistant to the lytic action of lysozyme (11). Resistance of streptococci to lysozyme has also been attributed, in part, to nonacetylated peptidoglycan-saccharide residues (6, 7, 16).

Although modifications in peptidoglycan structure probably contribute significantly to bacterial resistance to lysozyme, very little information exists in the literature concerning the presence and extent of lysozyme-sensitive bonds in the peptidoglycan of lysozyme-lysis-resistant bacteria (30). In studies with a strain of *B. cereus*, it has been shown recently that approximately 15% of the cell wall, presumed to be peptidoglycan, is sensitive to lysozyme (32). In experiments with *Micrococcus sodonensis* (*M. luteus*), Johnson and Campbell (15) obtained evidence which suggested that lysozyme-sensitive and lysozyme-resistant portions of the peptidoglycan do not exist as separate "islands," but rather as an interwoven peptidoglycan net. A determination of lysozyme-sensitive and lysozyme-resistant bonds should provide useful information to help clarify which and how many glycosidic bonds in the peptidoglycan must be hydrolyzed for cellular lysis to taken place. *Streptococcus mutans* BHT appears to be an excellent model system for this purpose. Lysozyme causes extensive damage to this bacterium as visualized by electron microscopy, although the cells apparently maintain their shape (2). However, the cells burst and undergo various degrees of lysis by further treatment with appropriate concentrations of inorganic salts (9, 23).

Our purpose therefore was, first, to develop reliable techniques for quantitating the sensitivity of peptidoglycan in whole cells of bacteria resistant to the lytic action of lysozyme alone, since such methods are not currently described in the literature, and, second, to attempt to correlate peptidoglycan damage with cellular lysis that occurs upon treatment with lysozyme and inorganic salts.

MATERIALS AND METHODS

Biochemicals. Hen egg white lysozyme (HEWL, 3× crystallized) and trypsin (bovine pancreas, type III) were obtained from Sigma Chemical Co., St. Louis, Mo. *N*-Acetyl-D-glucosamine (GlcNAc), D-glucosamine hydrochloride, and *N*-acetylmuramic acid were purchased from Pfansteihl Laboratories, Inc., Waukegan, Ill., and muramic acid was obtained from Calbiochem, San Diego, Calif. Sodium chloride, sodium thiocyanate, ammonium acetate, *n*-butanol, acetic acid, and trichloroacetic acid were purchased from Fisher Scientific Co., Pittsburgh, Pa. Sodium fluoride was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. [*methyl*-^{3}H]thymidine was a product of ICN Pharmaceuticals, Inc., Irvine Calif., and Filter-Solv was purchased from Beckman Corp., Somerset, N.J. *N*-Acetyl-D-[1-^{14}C]glucosamine ([^{14}C]GlcNAc), OCS, NCS, and ACS were purchased from Amersham Corp., Arlington Heights, Ill. Mutanolysin M-1 enzyme was kindly provided by Kanae Yokogawa, Dainippon Pharmaceutical Co., Osaka, Japan. It has been suggested that mutanolysin is a muramidase (34), although isolated enzyme preparations can be contaminated with protease activity (K. Yokogawa, personal communication). The enzyme was therefore further purified by gel filtration–ion-exchange chromatography to obtain a protease-free preparation.

Bacterial cultures. *S. mutans* BHT, a serotype *b* strain (18), was kindly provided by Harold Jordan, Forsyth Dental Center, Boston, Mass. Stock cultures grown to late exponential phase in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) were stored quick-frozen at −90°C in 1% skim milk. For routine use, frozen cultures were grown at 37°C as a 1:50 dilution in a dialysate of Todd-Hewitt broth (Difco) supplemented with a 0.5% (wt/vol) final concentration of D-glucose (Difco) and 0.3% (wt/vol) yeast extract (Difco) to late log phase (optical density of 0.80 at 675 nm, 1-cm light path). Freshly grown cells were then inoculated as a 1:1,600 dilution in a second transfer into dialysate medium, and at the late log phase of growth (optical density of 1.10 at 675 nm), bacteria were harvested and washed three times in ice-cold distilled water. Cells were suspended in 0.025 M ammonium acetate, pH 6.8, to an optical density of 0.40 at 700 nm (10^9 cells per ml, Petroff-Hausser counting chamber) immediately before experimental assays.

Radioisotopes were incorporated into *S. mutans* BHT by supplementing the second transfer into dialysate medium (10 ml) with either 10 μCi of [*methyl*-^{3}H]thymidine (specific activity, 1 mCi/ml, 72 Ci/mmol) per ml or 0.5 μCi of [^{14}C]GlcNAc (specific activity, 50 μCi/ml, 57.9 mCi/mmol) per ml.

Determination of percent incorporation of [^{14}C]GlcNAc label into peptidoglycan. Incorporation of [^{14}C]GlcNAc into the cell wall peptidoglycan fraction was determined, with modifications, by the methods outlined by Mychajlonka et al. (21) for

[^{3}H]lysine incorporation into peptidoglycan. Cells grown in the presence of [^{14}C]GlcNAc were suspended in 0.025 M ammonium acetate, and 2-ml samples were distributed into 10-ml plastic conical centrifuge tubes. To determine total [^{14}C]GlcNAc incorporation into whole cells, three of these 2-ml samples were directly applied to EHWP filters (0.5-μm pore size; Millipore Corp., Bedford, Mass.) on a 12-place sampling manifold (Millipore). Cells on the filters were washed three times with 3 ml of cold water and once with 3 ml of cold 95% ethanol with the aid of intermittent vacuum.

For determination of [^{14}C]GlcNAc label incorporation into cold and hot trichloroacetic acid precipitates and trypsin-resistant residues (peptidoglycan-containing fraction) (21), 2-ml samples were centrifuged at 2,100 × *g* for 15 min at 4°C. For each of five experiments, the cell pellets obtained from nine tubes were individually suspended in 2 ml of cold 10% trichloroacetic acid, and the tubes were placed in an ice bath for 1 h. Cold trichloroacetic acid precipitates of three tubes were trapped on EHWP filters and washed three times with 3 ml of cold 10% trichloroacetic acid and once with 3 ml of cold 95% ethanol. The remaining six tubes were removed from the ice bath and placed in a 95% water bath for 15 min and then returned to the ice bath to cool. Hot trichloroacetic acid precipitates were trapped on EHWP filters, which were washed as described above for cold trichloroacetic acid precipitates. Three of the six hot trichloroacetic acid precipitate filters were removed from the manifold, whereas the last three filters were left on the manifold and washed with 2 ml of 0.1 M sodium phosphate, pH 8.0. Vacuum was then released, and a slight positive pressure was applied to the manifold. Filters were then incubated with 2 ml of trypsin (1 mg/ml) in 0.1 M sodium phosphate buffer, pH 8.0, for 90 min at 37°C directly on the manifold. Suction was then applied, and trypsin-resistant residues (peptidoglycan fraction) were washed three times with 3 ml of cold water followed by a final wash with 3 ml of cold 95% ethanol.

All filters were placed in scintillation vials, allowed to dry overnight, and suspended in 1 ml of NCS followed by addition of 13 ml of OCS. Radioactivities in samples, as disintegrations per minute, were quantitated in a mark III liquid scintillation counter (Tracor Analytic, Austin, Tex.).

Determination of loss of insoluble peptidoglycan after treatment of isolated peptidoglycan with HEWL or mutanolysin. [^{14}C]GlcNAc-labeled peptidoglycan fractions from 2-ml samples were obtained as described above and washed with 3 ml of 0.025 M ammonium acetate, pH 6.8. Filters were then removed from the manifold and were placed separately in petri plates (10 by 35 mm) containing 2 ml of either HEWL or mutanolysin in 0.025 M ammonium acetate, pH 6.8, at final enzyme concentrations of 5, 50, and 500 μg/ml, in duplicate. After 18 h at 37°C, the filters were then placed back onto the manifold, washed three times with 3 ml of cold water and 3 ml of cold 95% ethanol, dried, and counted in the scintillation cocktail.

Paper chromatographic analyses of HEWL- and mutanolysin-solubilized peptidoglycan fragments. [^{14}C]GlcNAc-labeled peptidoglycan isolated on filters was incubated with either HEWL (100 μg per filter) or mutanolysin (10 μg per filter) in 0.025 M ammonium acetate, pH 6.8. After 18 h at 37°C, filters and reaction mixtures were applied under vacuum to the Millipore manifold system. Filters were washed three times with 3 ml of cold water, and each enzyme filtrate, which presumably contained solubilized peptidoglycan fragments, was lyophilized. The lyophilized material was hydrolyzed in 1 ml of 4 N HCl for 4 h at 105°C in vacuo. Hydrolysates were dried in vacuo over KOH pellets and were dissolved in 100 μl of water. Samples (5 μl) of each hydrolysate were spotted on a piece of chromatography paper (1 by 3.5 cm) for total radioactivity determination. The remainder of the hydrolysates were applied to 593-C paper (Schleicher & Schuell Co., Keene, N.H.), and descending paper chromatography was performed in *n*-butanol–acetic acid–water, 3:1:1, for 30 h, using GlcNAc, *N*-acetylmuramic acid, glucosamine hydrochloride, and muramic acid as standards (J. J. Pollock, Ph.D. thesis, Weizmann Institute of Science, Rehovot, Israel, 1969). Unlabeled *N*-acetylamino sugars were detected as fluorescent spots by the sodium hydroxide reagent (29), whereas amino sugars were revealed by 0.5% ninhydrin in acetone. Radioactivity on the chromatograms was quantitated by dividing the paper into 1-cm sections (3.5-cm strip width). The paper sections were then placed in scintillation vials, and 0.5 ml of water was then added (25). After the strips had been allowed to stand for 1 h at room temperature, 1 ml of Filter-Solv and 10 ml of ACS were added and the samples were counted.

Determination of loss of insoluble peptidoglycan after treatment of whole cells with HEWL and inorganic salts. Freshly harvested and washed [^{14}C]GlcNAc-labeled cells of *S. mutans* BHT in 0.025 M ammonium acetate were preincubated for 1 h with stirring at 37°C. Then, triplicate 2-ml samples were processed both for determination of total [^{14}C]GlcNAc in whole cells and for incorporation of the radiolabel into peptidoglycan (see above). HEWL was then added (final concentration of either 150 or 22.5 μg/ml), and incubation at 37°C was continued. Triplicate 2-ml samples were taken at 10, 40, 120, and 180 min for determination of label remaining in cells and in the peptidoglycan fraction. Radioactivity was also quantitated in controls incubated in the absence of lysozyme. After 3 h, NaSCN, NaF, or NaCl at a final concentration of 0.10 or 0.18 M was then added to HEWL-treated and untreated control cell suspensions, and triplicate 2-ml samples were removed at 1 min and after an additional 1 h of incubation and processed as described above. Loss of insoluble peptidoglycan was calculated by subtracting the peptidoglycan retained on filters after HEWL and inorganic salt treatment from the peptidoglycan values of the untreated controls.

Determination of release of [^{3}H]thymidine after treatment of whole cells with HEWL and inorganic salts. Cellular lysis was assayed by measurement of the liberation of DNA as macromolecular [^{3}H]thymidine from *S. mutans* BHT treated sequentially with HEWL and inorganic salts. The presence of macromolecular thymidine was determined by applying the released thymidine label to Sephadex G-25 columns to separate free thymidine from thymidine

incorporated into DNA (see companion paper [9]). A [^{3}H]thymidine-labeled cell suspension of *S. mutans* BHT in 0.025 M ammonium acetate, pH 6.8, was preincubated, with stirring, at 37°C. After 1 h, HEWL was added (final concentration of either 150 or 22.5 μg/ml). After an additional 3 h, 0.10 or 0.18 M NaSCN, NaCl, or NaF was added to the reaction mixtures. Incubation was continued for a further 5 h, and at designated times over the entire course of the incubation, 1-ml samples were removed, blended in a Vortex mixer, and processed for DNA release. Aliquots of 0.1 ml were taken from each tube for total tritium counts, and then the remaining sample was centrifuged at 2,100 × *g* for 20 min at 4°C (PR-6000 centrifuge; International Equipment Co., Needham Heights, Mass.). Resultant supernatants (0.1-ml samples) were similarly quantitated by using a cocktail consisting of 1 ml of Filter-Solv and 10 ml of ACS, and the percentages of [^{3}H]thymidine release were calculated.

RESULTS

Incorporation of [^{14}C]GlcNAc label into whole cell, cold trichloroacetic acid, hot trichloroacetic acid, and trypsin residues of *S. mutans* BHT. Table 1 shows the data of five different experiments in which *S. mutans* BHT was grown in supplemented Todd-Hewitt dialysate medium containing [^{14}C]GlcNAc. The resultant peptidoglycan-containing residues retained approximately 53 to 65% of the ^{14}C label after cold trichloroacetic acid, hot trichloroacetic acid, and trypsin treatment of whole cells. Although there was some variation in the incorporation of label in cold trichloroacetic acid, hot trichloroacetic acid, and trypsin residues between experiments, the results were very reproducible within each experiment (Table 1).

Susceptibility of peptidoglycan-containing residues to HEWL and mutanolysin hydrolysis and identification of radiolabel in enzyme-solubilized and acid-hydrolyzed fragments. Peptidoglycan susceptibility was assayed by measurement of the insoluble [^{14}C]-GlcNAc label retained on filters after treatment of isolated peptidoglycan (trypsin residues) with HEWL or mutanolysin. At 5 μg of mutanolysin per ml, virtually all (97%) of the insoluble peptidoglycan of *S. mutans* BHT was lost from filters, although addition of higher concentrations of the enzyme resulted in decreased hydrolysis (Table 2). In contrast, HEWL treatment did not appear to result in complete hydrolysis of the peptidoglycan residues, as a maximum of 80% of the insoluble peptidoglycan was lost during treatment with this enzyme. In comparison with mutanolysin, higher concentrations of HEWL did not result in decreased losses of peptidoglycan (Table 2). When the solubilized fragments from each enzyme-peptidoglycan digestion were acid hydrolyzed and chromatographed, incorporated radioactive label appeared in components having mobilities corresponding to glucosamine hydrochloride and muramic acid (Fig. 1). Radioactivity was only detected in areas of the chromatogram containing the two *N*-acetylamino sugars, indicating that [^{14}C]GlcNAc was not incorporated into other peptidoglycan components, such as amino acids. Of the total radioactivity present in hydrolysates, over 90% was recovered as the respective amino sugars on chromatograms, with twice as much radioactive label present in the glucosamine region as in the muramic acid region.

Kinetics of release of [^{14}C]GlcNAc label during incubation in the presence of high and low concentrations of HEWL followed by addition of NaSCN. Radioactivity as total GlcNAc and as insoluble peptidoglycan was determined over time in whole cells of *S. mutans* BHT by subtracting the radiolabel retained on filters after treatment with either 150 or 22.5 μg of HEWL per ml and a final concentration of 0.1 M NaSCN from that of untreated controls (Tables 3 and 4). In the experiment with 150 μg of HEWL per ml (Table 3), total peptidoglycan radioactivity (the amount lost plus that retained on filters) ranged from 54 to 62% (average of 59.9%) of the total [^{14}C]GlcNAc label during the incubation. In the experiment with the lower enzyme concentration (Table 4), values were from 53 to 58% (average of 56%), consistent with

TABLE 1. *Incorporation of [^{14}C]GlcNAc label into whole cell, cold trichloroacetic acid-precipitable, hot trichloroacetic acid-precipitable, and trypsin-treated residues of S. mutans BHT*

Prepn[a]	Incorporation of [^{14}C]GlcNAc label (%)[b]				
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
Cells	100	100	100	100	100
Cold trichloroacetic acid	73.9 ± 0.7	84.2 ± 1.3	86.7 ± 4.2	81.1 ± 0.8	74.3 ± 1.5
Hot trichloroacetic acid	58.7 ± 1.8	60.6 ± 0.5	74.1 ± 0.3	61.5 ± 1.7	61.6 ± 1.7
Trypsin residue (peptidoglycan)	55.8 ± 1.6	58.1 ± 1.0	65.5 ± 1.5	52.6 ± 3.6	57.8 ± 0.4

[a] Details of treatments are described in the text.

[b] The amounts ^{14}C present in samples of intact cells for experiments 1 through 5 were, respectively, 90,939, 77,856, 87,072, 70,455, and 91,610 dpm/ml of sample containing 2 × 10^9 cells. Each whole cell radioactivity value was set to 100%. Each value represents the mean of triplicate assays ± the standard error.

the range of percent incorporation noted in Table 1. In comparison with the lower concentration of HEWL, 150 μg of HEWL per ml released more total [^{14}C]GlcNAc label (52.2% as compared with 35.5%) from the cells (Tables 3 and 4). Addition of 0.1 M NaSCN to reaction mixtures led to a further small, but consistently

TABLE 2. *Loss of insoluble peptidoglycan of S. mutans BHT after treatment with HEWL or mutanolysin*

Prepn	Peptidoglycan[a]	
	Remaining in fraction (dpm)	Lost (%)[b]
Peptidoglycan fraction	54,035 ± 1,460	0
Peptidoglycan plus:		
5 μg of HEWL per ml	16,156 ± 1,063	70.1
50 μg of HEWL per ml	10,650 ± 596	80.3
500 μg of HEWL per ml	10,671 ± 147	80.3
5 μg of mutanolysin per ml	1,568 ± 160	97.1
50 μg of mutanolysin per ml	4,744 ± 266	91.2
500 μg of mutanolysin per ml	6,665 ± 426	87.7

[a] Peptidoglycan is defined as the insoluble fraction obtained after sequential treatment of cells with cold trichloroacetic acid, hot trichloroacetic acid, and trypsin (see text for details). Each value represents the mean of duplicate assays ± the standard error.

[b] Percentage of peptidoglycan lost = [(disintegrations per minute of peptidoglycan before enzyme treatment − disintegrations per minute of peptidoglycan after treatment)/disintegrations per minute of peptidoglycan before treatment] × 100.

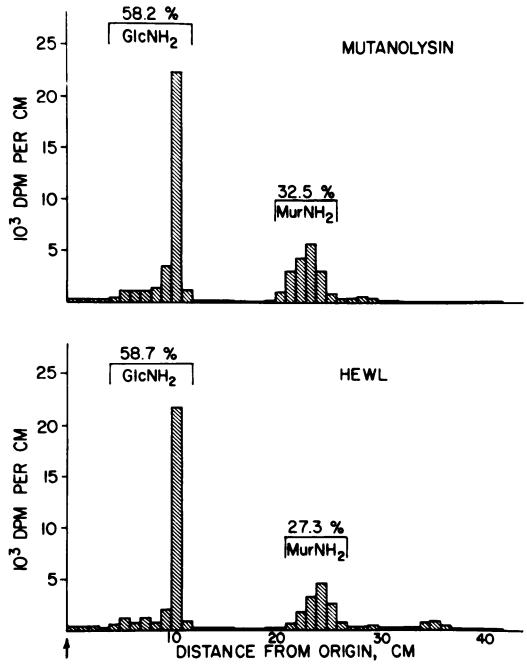

FIG. 1. *Paper chromatographic analyses of acid hydrolysates of HEWL- and mutanolysin-solubilized peptidoglycan fragments for distribution of [^{14}C]-GlcNAc label. See the text for details. Arrow indicates the origin on the descending paper chromatograms. Bracketed enclosures indicate the mobilities of glucosamine hydrochloride ($GlcNH_2$) and muramic acid ($MurNH_2$) standards. Values represent distributions of radioactive label as percentages of the total radioactivities present in acid hydrolysates before chromatography.*

TABLE 3. *Kinetics of release of [^{14}C]GlcNAc label from S. mutans BHT by a high concentration of HEWL alone and in the presence of NaSCN*

Incubation	[^{14}C]GlcNAc label					
	Total			As peptidoglycan		
	Retained on filter (dpm)	Released from filter (dpm)[a]	Lost (% of control)	Retained on filter (dpm)	Released from filter (dpm)[a]	Lost (% of control)
Untreated control[b]	98,732 ± 438			59,120 ± 1,897		
With HEWL[c]						
10 min	81,370 ± 2,085	17,362	17.6	43,878 ± 898	15,472	26.2
40 min	62,989 ± 124	35,743	36.2	31,013 ± 673	28,107	47.5
120 min	49,140 ± 899	49,592	50.2	19,188 ± 490	39,932	67.5
180 min	47,157 ± 2,455	51,575	52.2	17,410 ± 790	41,710	70.6
181 min[d]	45,739 ± 131	52,993	53.7	12,281 ± 1,074	46,839	79.2
240 min[d]	45,440 ± 1,139	53,292	54.0	12,085 ± 785	47,035	79.6

[a] Calculated at each time point by subtraction of radioactivity on filters after HEWL treatment from average radioactivity of untreated control.

[b] Average disintegrations per minute of all means of untreated controls for entire incubation period. Each mean was determined from triplicate assays at each time point.

[c] Final concentration of 150 μg/ml (see text for details of procedure).

[d] HEWL-treated cells contained 0.1 M NaSCN at these times. NaSCN was added after 3 h of incubation with HEWL (see text).

TABLE 4. *Kinetics of release of [^{14}C]GlcNAc label from S. mutans BHT by a low concentration of HEWL alone and in the presence of NaSCN*

Incubation	[^{14}C]GlcNAc label[a]					
	Total			As peptidoglycan		
	Retained on filter (dpm)	Released from filter (dpm)	Lost (% of control)	Retained on filter (dpm)	Released from filter (dpm)	Lost (% of control)
Untreated control	88,671 ± 1,061			49,671 ± 1,067		
With HEWL[b]						
10 min	81,834 ± 330	6,837	7.7	40,050 ± 932	9,621	19.4
40 min	71,889 ± 678	16,782	23.3	36,407 ± 439	13,264	26.7
120 min	60,320 ± 519	28,351	32.0	26,519 ± 1,839	23,152	46.6
180 min	57,211 ± 304	31,460	35.5	25,979 ± 1,295	23,692	47.7
181 min[c]	56,405 ± 363	32,266	36.4	22,879 ± 919	26,792	53.9
240 min[c]	56,394 ± 171	32,277	36.4	23,845 ± 600	25,826	52.0

[a] See footnotes to Table 3 for explanation of values.
[b] Final concentration of 22.5 μg/ml.
[c] HEWL-treated cells contained 0.1 M NaSCN at these times.

reproducible, loss of label. The additional loss of ^{14}C label after NaSCN addition became more obvious particularly at 150 μg of HEWL per ml when cells were treated with cold trichloroacetic acid, hot trichloroacetic acid, and trypsin to isolate the label retained in only the peptidoglycan fraction of the cell (Table 3). An examination of the data in Tables 3 and 4 also indicated that the majority (>80%) of the [^{14}C]GlcNAc label released by the treatment procedures was in fact peptidoglycan.

Correlation of peptidoglycan release with DNA release. In an effort to correlate peptidoglycan damage with the lytic process, the liberation of DNA as [^{3}H]thymidine was monitored after treatment with 150 μg of HEWL per ml and the sodium salts of fluoride, chloride, and thiocyanate at 0.1 and 0.18 M final concentrations (Fig. 2). Although HEWL alone effected the release of 17% of the [^{3}H]thymidine after 3 h of incubation, the released thymidine was not high-molecular-weight DNA, as the radioactive label appeared in the eluted position of free thymidine when supernatants of HEWL-treated cells of *S. mutans* BHT were applied to Sephadex G-25 columns. In contrast, when supernatants of HEWL–0.18 M NaSCN- or HEWL–NaCl-treated cells were applied to the columns, about 80% of the radioactivity was found in the void volume of the column, suggesting that high-molecular-weight DNA was liberated upon salt treatment. Moreover, electron microscopic observations have revealed that cell lysis takes place when appropriate concentrations of inorganic salts are added to the HEWL-treated cells (9; M. Cho et al., unpublished data). Identification of the [^{3}H]thymidine as DNA has been confirmed by precipitation of supernatants with

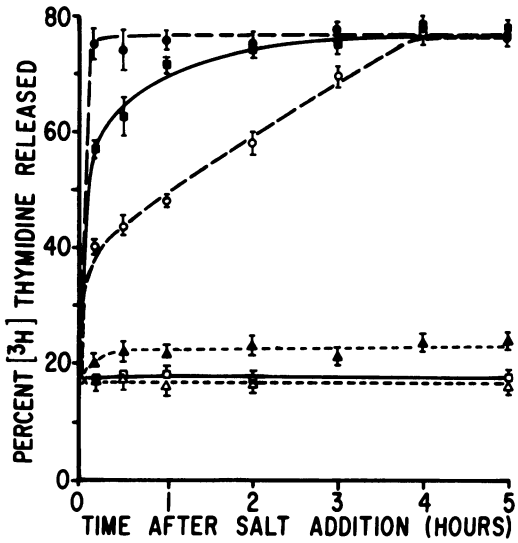

FIG. 2. *Kinetics of release of [^{3}H]thymidine from HEWL-treated S. mutans BHT by inorganic salts. At zero time before the addition of salts, cell suspensions were incubated with HEWL for a period of 3 h (see text for details). Symbols: ○, 0.1 M NaSCN; ●, 0.18 M NaSCN; □, 0.1 M NaCl; ■, 0.18 M NaCl; △, 0.1 M NaF; ▲, 0.18 M NaF; vertical bars, mean values of triplicate samples ± the standard errors.*

trichloroacetic acid and by the diphenylamine colorimetric assay for DNA (5). At 150 μg of HEWL per ml and a concentration of 0.1 M salt, only sodium thiocyanate caused the liberation of DNA (Fig. 2). Under these conditions, it was also observed that thiocyanate, but not chloride or fluoride, effected further release (8 to 12%) of peptidoglycan from HEWL-treated cells (Table 5). At the higher 0.18 M salt concentration,

sodium fluoride addition resulted in a small release of DNA (Fig. 2), and the loss of peptidoglycan was comparable to the loss with addition of 0.1 M sodium thiocyanate (Table 5). In contrast to 0.1 M chloride, with which DNA could not be detected in supernatants, a higher concentration of chloride, 0.18 M, resulted in a rapid release of DNA which plateaued at a release of approximately 80% of the total [^{3}H]-thymidine in the cells (Fig. 2). This plateau of ^{3}H release was also attained at both thiocyanate concentrations used and required 4 h of incubation with 0.1 M NaSCN but maximized within the first 5 min at 0.18 M NaSCN (Fig. 2). The rate of DNA release was faster with 0.18 M thiocyanate as compared with 0.18 M chloride (Fig. 2) or 0.1 M thiocyanate, although the additional peptidoglycan releases at the 0.18 M concentrations were about the same within the average of several experiments (Table 5). At 0.18 M chloride or thiocyanate, additional loss of peptidoglycan rose to approximately 20%, or about two to three times that observed with 0.18 M NaF or 0.1 M NaSCN (Table 5). In all experiments, the peptidoglycan released within the first 5 min of salt addition represented the maximum amount, as no further increase in peptidoglycan lost was observed during the incubation period.

Release of DNA was also noted at 22.5 μg of HEWL per ml in the presence of 0.1 M NaSCN, although the amount of [^{3}H]thymidine released (plateau levels of 45%, data not shown) was less than that obtained with 150 μg of HEWL per ml (Fig. 2). The smaller extent of lysis appeared to correlate with the smaller loss of insoluble peptidoglycan effected by the addition of NaSCN to cells treated with the lower concentration of HEWL (Table 4).

DISCUSSION

The studies described above indicate that [^{14}C]GlcNAc is efficiently incorporated into cells of *S. mutans* BHT. The majority of the [^{14}C]-GlcNAc (53 to 65%) was found to be present in peptidoglycan-containing residues after hot trichloroacetic acid and trypsin treatment (Table 1). The ^{14}C present in hot trichloroacetic acid- and trypsin-resistant residues was found to be GlcNAc and *N*-acetylmuramic acid (Fig. 1) in mutanolysin-hydrolyzable peptidoglycan (Table 2). Thus, it was concluded that over 50% of the [^{14}C]GlcNAc was incorporated into the glycan chains of the peptidoglycan of this strain. Also, the method used provides a reproducible, reliable, and sensitive assay for quantitating relative amounts of trichloroacetic acid-insoluble peptidoglycan retained by cells and, by difference, amounts of peptidoglycan lost to the supernatant medium. The advantages and limitations of the method used are of particular importance to the interpretation of the data obtained. Only ^{14}C-labeled peptidoglycan fragments completely released from, and not coprecipitated with, cell pellets and the peptidoglycan fractions of such pellets were measured. Thus, measurements did not include damaged peptidoglycan that remained insoluble during subsequent treatments.

Strain BHT of *S. mutans* loses viability relatively rapidly after exposure to HEWL (14). It seemed possible that hydrolysis of bonds in the wall peptidoglycan could be responsible for lethality. Indeed, isolated peptidoglycan-containing residues were partially solubilized by HEWL (Table 2), and 48 and 70% of [^{14}C]GlcNAc-labeled insoluble peptidoglycan was lost from intact cells upon exposure to low and high concentrations of HEWL, respectively. However, even after loss of 70% of their peptidoglycan (Table 3), the cells remained as turbid suspensions and failed to dissolve. The apparent greater susceptibility to HEWL of insoluble peptidoglycan residues (Table 2) than of peptidoglycan present in intact cells (Table 3) of *S. mutans* BHT might be attributed to the presence in intact cells of other cell wall polymers either covalently linked to or noncovalently associated with the pepti-

TABLE 5. *Peptidoglycan release from HEWL[a]-treated cells 5 min after addition of inorganic salts*

Inorganic sodium salt	Salt Concn (M)	Peptidoglycan lost (%)[b]			
		Expt. 1	Expt. 2	Expt. 3	Expt. 4
Fluoride	0.1	72.3 ± 0.3		66.9 ± 1.7	
	0.18		78.0 ± 1.2		76.2 ± 1.6
Chloride	0.1	69.8 ± 0.8		69.6 ± 1.1	
	0.18		89.8 ± 1.1	91.6 ± 0.3	92.5 ± 1.6
Thiocyanate	0.1	79.3 ± 1.4		82.2 ± 0.8	
	0.18		88.5 ± 1.0		90.3 ± 0.6

[a] Final concentration of 150 μg/ml.

[b] In the absence of inorganic salts, HEWL alone caused a maximum loss of 70.7 ± 0.9% of the insoluble peptidoglycan (see text and footnotes to Table 3 for details), which represented the average of the means obtained in the four experiments. Each value represents the mean of triplicate assays ± the standard error.

doglycan (14, 19, 31), to alterations in the three-dimensional organization of the peptidoglycan, or to chemical modification of the peptidoglycan itself during peptidoglycan isolation.

Although release of ^{14}C from isolated peptidoglycan-containing residues obtained after hot trichloroacetic acid extraction can be attributed solely to the action of HEWL (Table 2), it remains possible that release of ^{14}C from the peptidoglycan fraction of intact cells (Tables 3 and 4) could result from the combined action of HEWL and endogenous peptidoglycan hydrolase activities (autolysins). For example, glycosidic bonds present in the fraction of peptidoglycan resistant to HEWL but susceptible to mutanolysin might be susceptible to an endogenous peptidoglycan hydrolase. Logardt and Neujahr (20) have previously observed that in *L. fermentum*, the endogenous autolytic enzyme(s) preferentially degrades lysozyme-insensitive linkages.

The lack of release of high-molecular-weight DNA upon treatment of cells with HEWL (Fig. 2) is consistent with the absence of cellular dissolution (see companion paper [9]). Although some [^3H]thymidine was released from cells upon treatment with HEWL (Fig. 2), this was all low-molecular-weight, presumably "pool," thymidine. Cellular dissolution with the release of [^3H]thymidine as high-molecular-weight material (DNA) did occur (Fig. 2) when HEWL treatment was followed by the addition of NaSCN (0.1 or 0.18 M). A lower concentration of NaCl (0.1 M) or addition of NaF (0.18 M) failed to cause release of DNA (Fig. 2), in agreement with previous data (9, 23). Very rapid loss of cellular integrity, as judged by release of DNA (Fig. 2), and very rapid loss of additional cellular peptidoglycan (Table 5) upon exposure of cells to appropriate concentrations of inorganic salts may occur because the salts cause some sort of physical change in the cells that results in the further release of already damaged peptidoglycan. However, the salts may also activate autolytic enzymes, since the addition of 0.18 M NaCl or NaSCN to HEWL-damaged bacteria resulted in large increases in peptidoglycan damage (Table 5), which brought the total loss of insoluble peptidoglycan to values in the 90% region. This might suggest that HEWL-insensitive peptidoglycan bonds were being hydrolyzed, presumably through the action of an autolysin, as a maximum of only 80% of the bonds of the isolated peptidoglycan was sensitive to HEWL (Table 2). Our earlier studies would tend to support the presence and activation of an autolysin. A muramidase-inactive lysozyme derivative, an enzyme derivative incapable of hydrolyzing cell wall peptidoglycan, was still somehow able to elicit lysis of *S. mutans* BHT in the presence of salts, although to an apparent lesser extent than native lysozyme (24).

Additional loss of insoluble peptidoglycan does not seem to be the only factor involved in cellular lysis and loss of cellular DNA. Treatment with 0.18 M NaF and 0.1 M NaSCN resulted in very similar losses of insoluble peptidoglycan (6.4 and 8.6%, respectively [Table 5]), despite the fact that the former treatment failed to cause a significant release of DNA, whereas the latter treatment resulted in the release of 80% of the cellular DNA (Fig. 2). In addition, although the losses of peptidoglycan were virtually the same in the presence of 150 μg of HEWL per ml and either 0.18 M NaCl or 0.18 M NaSCN (Table 5), the rate of release of DNA was faster with NaSCN (Fig. 2). Conceivably, the inorganic salts may function in the destabilization of membrane structure when HEWL is bound to the cell (see companion paper [9]).

Although the data obtained indicate that HEWL treatment of cells of *S. mutans* BHT results in damage to and release of cell wall peptidoglycan, a correlation between the amount of overall peptidoglycan lost and either susceptibility to salt-induced lysis or extent of cellular lysis after salt treatment was not obtained. For example, in reaction mixtures containing 22.5 μg of HEWL per ml, 0.1 M NaSCN addition resulted in a small increase in loss of insoluble peptidoglycan (Table 4). Under these experimental conditions, the release of DNA and cell lysis do occur with an approximate 50% loss of total insoluble peptidoglycan (Table 4). Yet lysis did not take place with 150 μg of HEWL per ml alone, in the absence of salts, where 70% of the peptidoglycan was liberated (Table 3). Thus, it appears that the number of glycosidic bonds in the peptidoglycan that are hydrolyzed (and result in loss of peptidoglycan from the cells) may not be the important factor. It seems more likely that the topographic location of bonds in the peptidoglycan hydrolyzed may be important. For example, a loss of 50% of the peptidoglycan that is relatively evenly distributed over the surface of a *Streptococcus* cell with a relatively thick wall may leave enough wall to adequately cover and protect the cell. In contrast, hydrolysis of a much smaller number of bonds, concentrated at one location, could result in a hole in the protective surface large enough to result in explosion of the cell.

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