JOURNAL OF BACTERIOLOGY, Feb. 1974, p. 796-804

Vol. 117, No. 2
Printed in U.S.A.

Specificity of the Autolysin of *Streptococcus (Diplococcus) pneumoniae*

LAWRENCE V. HOWARD[1] AND HARRY GOODER

Department of Bacteriology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

Received for publication 10 September 1973

A *Streptococcus (Diplococcus) pneumoniae* autolysin, partially purified from cellular autolysates, was optimally active at pH 7.0 and was stimulated by monovalent cations. Addition of autolysin to walls resulted in the appearance of only N-terminal L-alanine, whereas no glycosidase activity was observed. Walls which had been solubilized by autolysin were separated by gel filtration into a low-molecular-weight peptide containing amino acids in the same ratios found in intact walls and a high molecular fraction containing the amino acid-deficient peptidoglycan backbone. Thus, the major activity is an *N*-acetylmuramyl-L-alanine amidase. In addition, walls undergoing spontaneous lysis revealed no glycosidase activity but showed an increase in only N-terminal alanine. Autolysin, which was bound to walls in saline, was almost completely removed when walls were washed in distilled water, and all of the activity was recovered in the water wash fluid.

Research on bacterial autolysis has been stimulated by a desire to determine the functions of autolysins and the mechanisms by which these potentially lethal enzymes are controlled. Some postulated functions depend on enzymes of a given specificity. For example, a muramidase would provide the proper receptor for the insertion of new disaccharide-peptide subunits into a growing peptidoglycan polymer (18), whereas a muramyl-L-alanine amidase would be necessary for the formation of the "head-to-tail" type of cross-linking bridge (5).

Autolysins have been demonstrated to be important factors in the killing of bacteria by antibiotics which inhibit cell wall biosynthesis. These antibiotics are less lethal for mutants of *Streptococcus pneumoniae* (23) and *Bacillus licheniformis* (15) which have decreased ability to autolyze compared to the wild-type cells. In the presence of penicillin, D-cycloserine, or phosphonomycin, pneumococcal cells which do not autolyze due to the incorporation of ethanolamine into their walls were killed at a slower rate than cells which were capable of autolysis (22).

Pneumococcal cell walls consist of peptidoglycan (12) and a ribitol teichoic acid which is the C-polysaccharide antigen and contains D-glucose, galactosamine phosphate, choline, and a diaminotrideoxyhexose (3, 12, 21). Tomasz (22) reported that pneumococcal cells were no longer competent for transformation, formed long chains, were not bile soluble, and did not autolyze when ethanolamine replaced choline in the wall. Mosser and Tomasz (12) reported that walls which had been solubilized by the autolytic enzyme yielded a low-molecular-weight peptide fraction which contained primarily alanine, glutamic acid, and lysine but no amino sugars and a high-molecular-weight fraction which consisted of the teichoic acid polymer and the amino acid-deficient polysaccharide backbone of the peptidoglycan. Thus, the major lytic activity in their enzyme preparations appeared to be an amidase which removed the peptide chain from the polysaccharide of the peptidoglycan.

In the present studies we have studied the cellular distribution and specificity of the autolytic system of a highly autolytic type 19 pneumococcus. An autolysin preparation partially purified from cellular autolysates was used to solubilize walls, and the specificity of the system was determined by end-group analysis and examination of the products of lysed walls. The specificity of the autolysin was also examined by using walls undergoing spontaneous lysis.

MATERIALS AND METHODS

Organism. An unencapsulated strain of *Streptococcus (Diplococcus) pneumoniae* was isolated from

[1] Present address: Department of Microbiology, Harvard University School of Public Health, Boston, Mass. 02115.

the blood of a patient at North Carolina Memorial Hospital, Chapel Hill, N.C. The strain was identified as a type 19 pneumococcus by the Center for Disease Control, Chamblee, Ga.

Growth media and cultural conditions. Cells which were used as a source of autolysin or heated prior to wall isolation were grown in Todd-Hewitt broth (BBL) supplemented with an additional 0.5% glucose. Native cell walls, which were used for studies on spontaneous wall autolysis, were isolated from cells which had been grown in infusion broth (BBL) containing 0.1% glucose. Incubation was at 37 C; a sterile pH electrode was placed in all cultures, and a constant pH of 7.0 was maintained by means of a Beckman model K automatic titrator which added 0.5 N NaOH to 1.5-liter culture volumes or 1.0 N NaOH to 12-liter culture volumes.

Preparation of walls as substrate for autolysin. Todd-Hewitt broth in 12-liter quantities was given a 2% inoculum of an overnight culture and incubated at 37 C. At the end of the exponential phase of growth, the cells were passed through a copper coil, 7 m in length and 4 mm in diameter, which was immersed in a 65 C water bath, and the cells were maintained at this temperature for an additional 10 min. This treatment was reported to inhibit autolysis of a pneumococcal strain (21) and is sufficient to inactivate the autolytic enzyme of the strain used in the present studies. After the culture cooled, the cells were harvested at 1,500 × *g* for 30 min and washed once in saline and once in distilled water. The cells were suspended in water at a concentration of 40 mg (dry weight) per ml and were disrupted for 5 min at 0 to 5 C in a Braun homogenizer by the procedure of Huff et al. (7). Samples, which were Gram stained and examined microscopically, contained less than 5% whole cells.

The suspension of disrupted cells was centrifuged at 755 × *g* for 10 min to sediment whole cells and debris, which were discarded. After one saline wash and one distilled water wash at 27,000 × *g* for 30 min, walls were incubated with 1% sodium dodecyl sulfate with continuous stirring for 1 h at 37 C. Walls were washed four times in distilled water, suspended in 0.01 M sodium phosphate at pH 8.0, and incubated with 0.10 mg of trypsin per ml for 12 h at 37 C in the presence of a few drops of chloroform. Walls were washed once in the phosphate buffer and twice in distilled water and then were lyophilized.

Isolation of native cell walls. Walls which were obtained from unheated cells were capable of undergoing spontaneous autolysis and were termed native walls. These walls were prepared and washed at 4 C. The cells from 1.5 liters of a 9-h culture were washed twice in saline and suspended in 24 ml of saline. A 6-ml volume of the cell suspension and an equal amount of no. 13 Ballotini beads per cup were shaken on the Mickle tissue disintegrator for 6 min. One drop of octanol was added to prevent frothing. The disrupted cells were centrifuged at 755 × *g* for 10 min to sediment whole cells and debris, and the resulting supernatant fluid was removed and centrifuged at 12,000 × *g* for 30 min to deposit the walls.

Preparation of autolysin. Cells from 1.5 liters of a 10-h culture were washed twice in saline at 4 C and suspended in 50 ml of 0.2 M sodium phosphate, pH 6.0. Autolysis was allowed to proceed for 1.5 h at 37 C, and the soluble fraction of the autolysate was obtained by centrifugation at 29,000 × *g* for 30 min. The sediment was suspended in 50 ml of the phosphate buffer and centrifuged, and the supernatant fluid was pooled with the first soluble fraction. The remaining steps were done at 4 C, and centrifugations were at 12,000 × *g* for 20 min. To the 100 ml of crude extract, 4.0 ml of a solution of 20% streptomycin sulfate was added with constant stirring. The enzyme preparation was stirred for 30 min, and after centrifugation the insoluble material was discarded. Solid ammonium sulfate was added to a final concentration of 40% of saturation, and after 30 min the precipitate was collected by centrifugation. The pellet was dissolved in 10 ml of 0.01 M sodium phosphate, pH 6.0, and 5.0 ml of 1-butanol was added with stirring (11). Stirring was continued for 15 min, and, after centrifugation to sediment denatured proteins, the aqueous phase was dialyzed against 1.0 liter of 0.01 M sodium phosphate, pH 6.0, overnight. Enzyme activity was stable over a period of several weeks when stored at 4 C. Recovery of activity after ammonium sulfate precipitation was 80 to 90% and resulted in approximately a fivefold increase in specific activity. The butanol step resulted in 55 to 75% recovery and about a threefold purification.

Assay of autolysin. The assay of autolytic enzyme activity was based on the rate of turbidity decrease in a pneumococcal wall suspension. Lyophilized walls were suspended in the appropriate buffer and sonicated for 5 s by using a Blackstone model BP-2 ultrasonic probe (supplied by Fisher Scientific Co.), to disperse any clumped material. The assay was routinely carried out in a total volume of 3.0 ml of 0.1 M sodium phosphate, pH 7.0, by using a wall suspension which had an optical density (OD) of 0.60 to 0.65 at 500 nm at the start of the assay. After the addition of autolysin, the OD, at 500 nm, was continuously recorded in a Beckman DB spectrophotometer where the mixture was maintained at 37 C by means of a circulating water bath. One enzyme unit is the amount of enzyme which causes a decrease in 0.10 OD units during a 10-min interval in the linear portion of the curve. The rate of lysis was proportional to enzyme dilution when the turbidity decrease was between 0.025 and 0.250 OD units per 10 min. Specific activity was defined as enzyme units per milligram of protein. The spontaneous lysis of walls was measured in a Klett-Summerson colorimeter with a no. 54 filter.

Conditions for wall lysis during examination of the specificity of the autolysin. Lyophilized walls were suspended in 9.0 ml of 0.01 M sodium phosphate, pH 7.0, containing 0.1 M NaCl, and lysis was commenced by the addition of 1.0 ml of an enzyme preparation containing approximately 0.5 mg of protein. Controls consisted of walls alone or walls with enzyme which had been inactivated by heating at 100 C for 10 min. The turbidity of the wall suspensions was measured with the Klett-Summerson colorimeter with a no. 54 filter. Autolysin at a concentration equal to that in the mixture was incubated alone

where indicated. All incubations were at 37 C. Samples from walls undergoing lysis and controls were removed at various time intervals and heated in a boiling water bath for 3 min to inhibit lysis.

Hydrolysis and ion exchange chromatography. Walls or wall fractions were hydrolyzed in 0.5 ml of 6 N HCl for 18 h at 105 C in evacuated glass vials. Amino acids and amino sugars in hydrolysates were determined by ion exchange chromatography by using an automatic amino acid analyzer marketed by Beckman Instruments, Inc. Elution positions and color yields of the individual substances were determined by chromatography of two dilutions of amino acids and amino sugars found in the pneumococcal wall. Hydrolysates were diluted 1/10 and 0.1 ml of this dilution was analyzed directly. No attempt was made to compensate for losses due to destruction of amino sugars as our interest was primarily in the nature of the components rather than the exact quantitative composition.

Analytical procedures. Reducing power (20) and ninhydrin-positive material (16) were determined according to published procedures. N-terminal amino acids were identified and quantitated by dinitrophenylation by the procedure of Ghuysen et al. (6). Hydrolysis of dinitrophenylated (DNP) peptides was at 105 C for 8 h. Alanine, glutamic acid, lysine, and serine, individually and as mixtures, were used as standards.

When the amount of an N-terminal amino acid in walls was measured, four concentrations in duplicate of an amino acid or authentic ϵ-DNP lysine were subjected to the entire procedure. Release of N-terminal D- and L-alanine was examined by the decrease in free D- or L-alanine after dinitrophenylation and hydrolysis of wall samples. The alanine was purified by chromatography on Dowex 50, and after recovery the total amount of D- and L-alanine was determined by using D-amino acid oxidase and L-alanine α-ketoglutarate transaminase (6).

Protein determinations. Protein was determined by the method of Lowry et al. (9) as modified by Neidhardt and Boyd (13) using crystalline bovine serum albumin as a standard.

Gel filtration. Columns of Sephadex were packed according to the instructions of the manufacturer, and the void volumes were determined with blue dextran. Wall fractions were desalted on a Sephadex G-10 column, 0.8 cm in diameter and 27 cm in length, which was run at a flow rate of 6 ml/h.

Formamide extraction of walls. Cell walls (60 mg) were suspended in formamide (50 ml) and heated with constant stirring at 150 C for 20 min. After cooling the mixture for 2 h at 4 C, it was centrifuged at 37,000 $\times$ g for 30 min. The sediment was washed three times with deionized water at 4 C and lyophilized.

RESULTS

Properties of autolysin. Walls were suspended in 0.01 M sodium phosphate containing 0.1 M NaCl at pH values from 5.5 to 8.0. The autolysin was diluted with the buffer at the appropriate pH and assayed. The OD decrease in a 10-min interval at each pH value is recorded in Fig. 1. The greatest rate of turbidity decrease was at pH 7.0, with one-half of this rate occurring at 8.0 and between 5.5 and 6.0.

Lytic activity was assayed in 0.01 M phosphate containing varying amounts of NaCl. The rate of lysis in the presence of higher concentrations of NaCl was approximately 50% greater than when lysis occurred in 0.01 M phosphate alone (Table 1); stimulation of activity by KCl also was observed between 0.01 and 0.025 M and was identical to NaCl stimulation up to 0.1 M, which was the highest concentration tested.

Lytic activity was not affected by 0.002 M $MgCl_2$, 0.01% or 0.1% sodium deoxycholate, or 0.01 or 0.1 M ethanolamine in the assay mixture. Each of these substances caused no detectable lysis when incubated with walls alone. Choline at concentrations between 0.02 to 0.05 M brought about a 50% decrease in the rate of lysis.

Distribution of autolytic activity in cellular fractions. The amount of lytic activity in

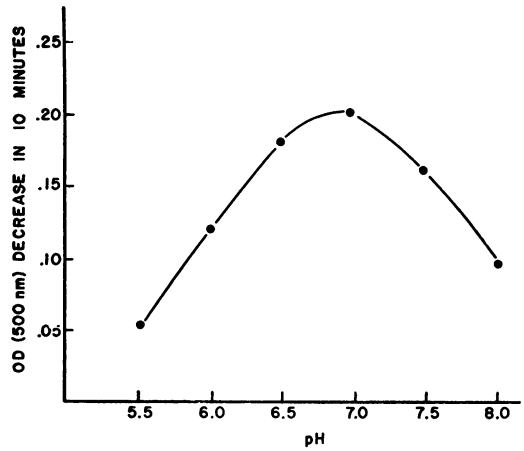

FIG. 1. *Effect of pH on autolysin activity in 0.01 M sodium phosphate containing 0.1 M sodium chloride.*

TABLE 1. *Effect of NaCl on activity of autolysin*[a]

Molarity of NaCl	OD (500 nm) decrease in 10 min
0.40	0.28
0.20	0.28
0.10	0.27
0.05	0.26
0.025	0.26
0.013	0.21
None	0.19

[a] All assays were done in the presence of 0.01 M sodium phosphate, pH 7.0.

the cytoplasmic fraction, saline and water wash of walls and autolyzed walls was measured. Native walls were isolated and the cytoplasmic fraction of the disrupted cell suspension was saved for assay after the removal of walls by centrifugation. The sedimented walls were suspended in saline and divided equally into two samples. Each sample was sedimented and the supernatant fluid was saved (first wash of walls). One sample of the walls was suspended and washed twice in saline and the wash liquid was saved (saline wash 1 and 2). The other sample of the walls was suspended and washed twice in deionized water and the wash liquid was also saved (water wash 1 and 2). Both washed wall preparations were finally suspended in 0.1 M sodium phosphate, pH 7.0, at 37 C. Lysis of saline-washed walls was complete in 20 min, whereas the water-washed walls had lysed only after 3 h of incubation.

The number of enzyme units in each fraction derived from one-half of the disrupted cell suspension is recorded in Table 2. The cytoplasmic fraction contained 20.5 enzyme units, and the first wash of the wall suspension had 2.0 units. The wash fluid from the two subsequent saline washes of half of the wall suspension contained little activity, whereas the two wash fluids from water-washed walls had a total of 12.6 units. The lysate of saline-washed walls contained 6.9 enzyme units, but less than 1.2 units were found in lysate of water-washed walls. Thus, the autolytic activity was removed from walls during washing in distilled water, as evidenced by the recovery of enzyme activity in the wash fluid and by the reduced amount of enzyme in lysates of water-washed walls. About 50% of the total activity was present in the cytoplasmic fraction.

In separate experiments when autolysates of saline-washed walls were centrifuged at 12,000 × *g* for 30 min, approximately one-half of the enzyme activity in the wall autolysate was sedimented.

TABLE 2. *Distribution of lytic activity in cytoplasm, saline, and water washes of walls, and lysates of walls*

Fraction tested	Total enzyme units derived from one-half of culture
Cytoplasm	20.5
First wash of cell walls	2.0
Saline wash 1	0.7
Saline wash 2	<0.4
Water wash 1	4.2
Water wash 2	8.4
Lysate of saline-washed cell walls	6.9
Lysate of water-washed cell walls	<1.2

Amino acid and amino sugar analysis of pneumococcal walls. Walls were hydrolyzed in 6 N HCl for 18 h at 105 C and in 4 N HCl for 8 h at 100 C, and the ninhydrin-positive components were examined on the Beckman amino acid analyzer. Two unidentified peaks, containing material which eluted from the long column in early positions, were found when walls were hydrolyzed in 4 N HCl and were present in lesser amounts when hydrolysis was in 6 N HCl. The two peaks were in approximately the same positions as the muramic acid phosphate and galactosamine 6-phosphate which Liu and Gotschlich (8) found in hydrolysates of the pneumococcal C-polysaccharide. The remaining substances were readily identifiable, and all were present in greater amounts when hydrolysis was in 6 N HCl. The quantity of each component identified in the 6 N HCl hydrolysate is expressed in micromoles per milligram of wall in the first column of Table 3. The molar ratios of lysine, glutamic acid, alanine, muramic acid, and glucosamine were 1.1:1.0:2.3:0.8, and :0.9, respectively. Pneumococcal walls also contained approximately 0.5 and 1.1 residues of serine and galactosamine per mole of glutamic acid, respectively. Aspartic acid and glycine which were present in very low concentrations were the only other amino acids detected.

Reducing power of walls during lysis. Pneumococcal walls at a concentration of 1.5

TABLE 3. *Amino acid and amino sugar analysis of cell wall fractions*[a]

Major components	Cell walls (μmol/mg)	Pellet (μmol/mg)	Fraction A (μmol/mg)	Fraction B (μmol/mg)
Lysine	0.43	0.56	0.09	1.77
Glutamic acid	0.39	0.45	0.08	1.70
Alanine	0.89	0.87	0.17	3.98
Serine	0.21	0.22	0.04	0.88
Muramic acid	0.33	0.39	0.27	—
Glucosamine	0.34	0.32	0.24	—
Galactosamine	0.44	0.33	0.46	—
Minor components				
Aspartic acid	0.01	0.03	0.01	0.07
Glycine	Trace	0.04	0.01	0.09
Isoleucine	—	0.01	Trace	—
Leucine	—	0.01	Trace	—

[a] Samples were hydrolyzed in 6 N HCl at 105 C for 18 h. —, Not detected.

mg/ml were incubated with autolysin for 90 min, and at periodic intervals the turbidity and reducing power of the mixture were determined. Incubation resulted in approximately a 60% decrease in turbidity, but no change in the reducing power of the walls was detected (Fig. 2). No change in reducing power was found when walls or autolysin were incubated alone at concentrations comparable to those in the mixture. The reducing power present in the autolysin preparation accounts for the difference in values between the wall plus autolysin mixture and wall control.

In similar experiments a known glycosidase, lysozyme, was tested with the wall material remaining after formamide extraction (peptidoglycan). Pneumococcal peptidoglycan, but not intact wall, was found to be sensitive to attack by lysozyme. The insoluble residue after formamide extraction of pneumococcal walls was incubated at a concentration of 1.0 mg/ml with 10 μg of egg-white lysozyme per ml for 200 min at 37 C. There was a 25% decrease in turbidity and a concomitant increase in reducing power, which increased about threefold during the incubation period (Fig. 3). No change occurred

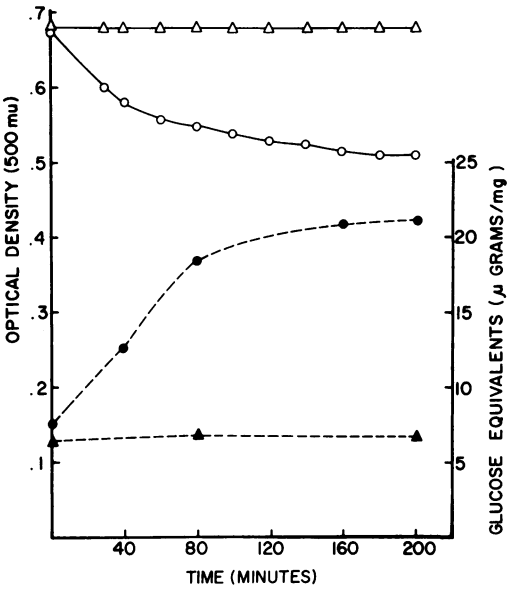

FIG. 3. *Lysis of pneumococcal peptidoglycan by lysozyme. Solid lines, OD; dashed lines, glucose equivalents. Symbols:* O, ●, *peptidoglycan plus lysozyme;* △, ▲, *peptidoglycan alone.*

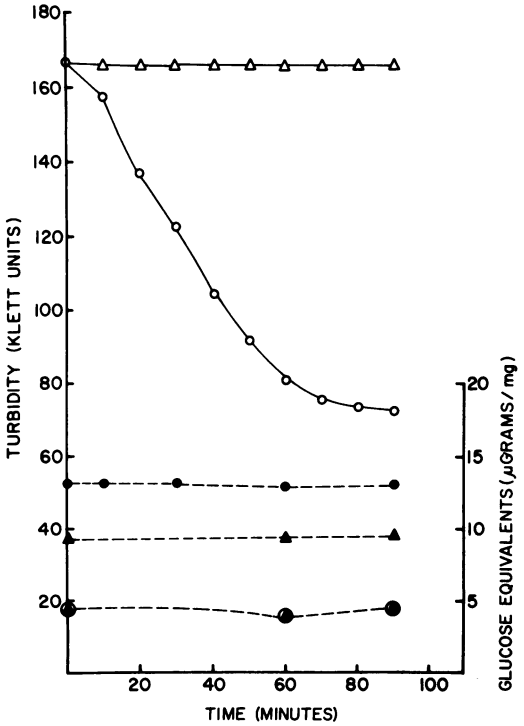

FIG. 2. *Reducing power of walls during lysis. Solid lines, turbidity; dashed lines, glucose equivalents. Symbols:* O, ●, *walls plus 53 units enzyme;* △, ▲, *walls alone;* ⊘, *enzyme alone.*

in the turbidity or reducing power in the mixture containing only peptidoglycan. Lysozyme incubated alone had an insignificant amount of reducing power.

Appearance of free amino groups during lysis. In preliminary experiments lysis of walls by autolysin resulted in an increase in ninhydrin-positive material of 0.24 μmol/mg of wall material. Walls incubated alone showed no change in turbidity or number of groups reacting with ninhydrin. The autolysin preparation had no detectable amino groups before or after incubation.

The nature of amino groups liberated during lysis was investigated. Walls at a concentration of 1.8 mg/ml were incubated with autolysin for 110 min, and samples were removed for determination of N-terminal amino acids by the dinitrophenylation procedure. Enzymatic lysis of the walls resulted in approximately a 60% reduction in turbidity, whereas no change occurred when walls were incubated alone (Fig. 4). Chromatography of the ether-extractable DNP amino acids after dinitrophenylation and hydrolysis revealed only DNP alanine in samples from intact and lysed walls. No DNP amino acids were observed in samples from enzyme incubated alone. There was 0.15 μmol of N-terminal alanine in intact walls as shown by the control wall preparation and also by the 0-min sample from lysing walls (Fig. 4). When walls

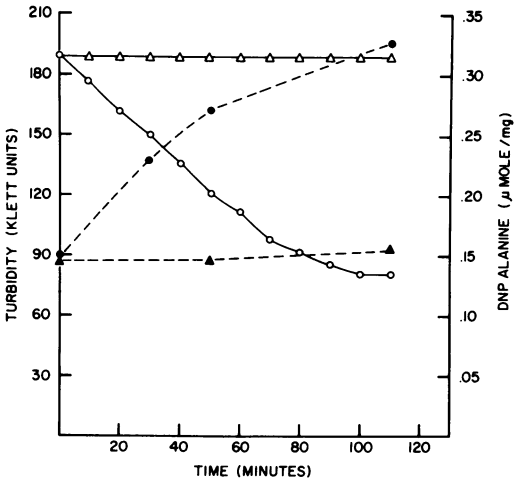

FIG. 4. *Release of N-terminal alanine during lysis of walls. Solid lines, turbidity; dashed lines, DNP alanine. Symbols:* O, ●, *walls plus 53 units enzyme;* Δ, ▲, *walls alone.*

were incubated with enzyme, an additional 0.18 μmol of N-terminal alanine appeared concomitantly with turbidity decrease, whereas there was no change in the walls which were incubated without enzyme.

The N^{ϵ}-DNP-lysine remaining in the aqueous phase after ether extraction was purified by chromatography. During lysis of the walls or incubation of walls alone, the number of free N^{ϵ}-lysine groups remained constant at 0.08 μmol/mg of wall material.

Configuration of N-terminal alanine released during lysis. Pneumococcal walls at a concentration of 1.8 mg/ml were incubated with autolysin and, as a control, with autolysin which had been heated at 100 C for 10 min. The turbidity of the walls which were incubated with active autolysin decreased from a Klett reading of 224 to a final value of 90 in 140 min. No change in turbidity occurred when walls were incubated with heated enzyme. At 0 and 160 min of incubation, samples were removed, heated with fluorodinitrobenzene (FDNB), hydrolyzed, and extracted with ether.

The amount of D- and L-alanine in wall samples after the removal of N-terminal alanine is recorded in Table 4. The quantity of L-alanine in the 0-min samples and in the 160-min sample from the control was approximately 0.60 μmol/mg, whereas 0.41 μmol/mg was found in samples from lysed walls. The amount of non-N-terminal D-alanine did not change during lysis and was the same in lysed walls and the control. The 0.16 μmol/mg difference in L-alanine between samples from lysed and unlysed walls is in good agreement with data from the previous experiment in which 0.18 μmol of N-terminal alanine per mg of walls appeared during lysis. Walls contained a total of 0.28 μmol of D-alanine per mg by enzymatic assay of hydrolysates.

Fractionation of lysed walls. Autolysin (1.1 mg [dry weight]) was added to 100 mg of walls in 50 ml of 0.01 M sodium phosphate, pH 7.0, containing 0.02 M NaCl. After 195 min of incubation at 37 C, the turbidity had dropped from 208 Klett units to a final reading of 85. After an additional 30-min incubation time, the lysate was lyophilized, suspended in 3.5 ml of water, and stirred to disperse clumped material. The lysate was centrifuged at 17,300 × *g* for 20 min at 4 C. The pellet was washed two times in cold distilled water and, after lyophilization, weighed 29 mg.

The supernatant fluid was chromatographed on a Sephadex G-50 column. The ninhydrin-positive material was present in a peak eluting at the void volume and in a second peak containing material of lower molecular weight (Fig. 5). Fractions 24 through 32 were pooled and lyophilized (fraction A), and fractions 45 through 65 were treated similarly (fraction B). Fraction A was suspended in 1.5 ml of distilled water, desalted on a Sephadex G-10 column, and lyophilized in preweighed hydrolysis tubes. Fraction B was treated in a similar manner.

Samples of the pellet, fraction A, and fraction B were hydrolyzed, and the hydrolysates were analyzed on the Beckman amino acid analyzer. The composition of each fraction and of intact walls is presented in Table 3. The pellet resembled the intact walls in the relative proportions of constituents except for a slight decrease in the amount of galactosamine. Fraction A contained muramic acid and glucosamine in approximately equimolar proportions and was deficient in lysine, glutamic acid, alanine, and serine. The molar ratios of the amino acids were essentially unchanged when compared to the

TABLE 4. *Amount of L- and D-alanine in cell wall samples after the removal of N-terminal alanine*

Samples	Amount of alanine in:	
	Walls plus active enzyme (μmol/mg)	Walls plus heated enzyme (μmol/mg)
L-Alanine		
0 Min	0.57	0.58
160 Min	0.41	0.62
D-Alanine		
0 Min	0.15	0.16
160 Min	0.15	0.15

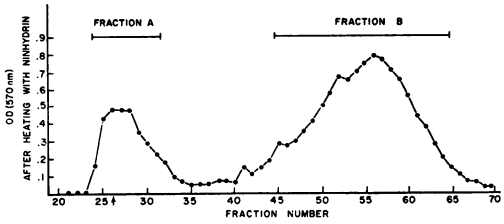

FIG. 5. *Gel filtration of soluble fraction of wall lysate on Sephadex G-50. The void volume is indicated by the arrow. The column was 2.5 by 40 cm and had been equilibrated with 0.01 M sodium phosphate, pH 7.0, containing 0.02 M NaCl. Flow rate was 30 ml per h, and fractions of approximately 3.0 ml were collected, of which 0.5 ml was tested for ninhydrin-positive material.*

intact cell walls. There was approximately a threefold decrease in the amount of amino acids compared to glucosamine and muramic acid. This fraction was enriched in galactosamine. Fraction B contained only lysine, glutamic acid, alanine, serine, and small amounts of aspartic acid and glycine. None of the amino sugars was detected in this fraction. The molar ratios of the amino acids were essentially the same as in intact walls, and the amino acids recovered account for 95% of the total weight in this hydrolysate.

The analysis of wall fragments produced by lysis and the observation that N-terminal L-alanine appeared during lysis indicates that the autolysin has *N*-acetylmuramyl-L-alanine amidase activity.

Spontaneous lysis of native walls. Although native walls were isolated and washed at 4 C, they frequently lysed during preparation or within several minutes after suspension in buffer at 37 C. Thus, only small volumes of culture could be handled rapidly enough to minimize lysis. These small volumes precluded analysis of the end products of autolysis but did allow a limited investigation of the appearance of free amino groups and possible changes in reducing power.

Native walls were isolated and washed once in saline. One-half of the material was washed twice in cold saline and, for a control, the other half was washed in cold distilled water. The saline- and water-washed walls were suspended in 0.025 M sodium phosphate, pH 7.0, and incubated at 37 C. The turbidity of the saline-washed walls decreased about 45% during incubation, whereas the water-washed walls showed only a slight decrease in turbidity (Fig. 6). Lysis of saline-washed walls was accompanied by a 50% increase in ninhydrin-reactive material from 0.25 μmol/ml to 0.37 μmol/ml, whereas walls washed in water gave only a 10% increase to 0.27 μmol/ml.

There was no change in the reducing power of walls undergoing spontaneous lysis. Comparison of dinitrophenylated samples of native walls before and after autolysis revealed that an increase in only N-terminal alanine occurred during lysis.

DISCUSSION

In the present studies the action of the autolysin of the pneumococcus was studied in more detail than previously reported. Our data indicates that the autolytic enzyme of the pneumococcus is an *N*-acetylmuramyl-L-alanine amidase. Lysis of walls by a preparation of the autolytic enzyme was accompanied by an increase in only N-terminal L-alanine and resulted in the release of the low-molecular-weight peptide moiety from the peptidoglycan backbone. Glycosidase activity was not detected when the autolytic enzyme was added to pneumococcal walls or when native walls underwent spontaneous autolysis.

Mosser and Tomasz (12) found that pneumococcal walls, after solubilization by an autolytic enzyme preparation, were fragmented into a fraction of low molecular weight constituting the peptide portion of the peptidoglycan and a second fraction of high molecular weight containing the amino acid-deficient glycan chain and the teichoic acid. The authors concluded

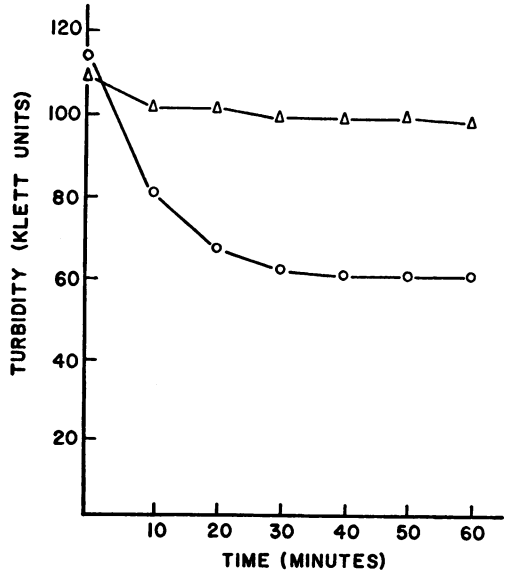

FIG. 6. *Changes in turbidity and amount of ninhydrin-positive material released during lysis of native walls. Symbols:* O, *saline-washed walls;* Δ, *water-washed walls.*

that a major activity in their autolytic enzyme preparation appeared to be amidase which cleaved the amide bond between alanine and muramic acid.

The possibility of autolysins in pneumococcal cells in addition to the amidase cannot be excluded. The major autolytic activity of *Staphylococcus aureus* H is an amidase which was present predominantly in the soluble fraction of disrupted cells; however, a glucosidase not demonstrable in this fraction was present in a small quantity in cell wall preparations (19). Wild-type *B. subtilis* 168, which contains predominantly amidase activity (24), was demonstrated to have a glycosidase in a sporulation mutant (2). In the present studies, by using partially purified enzyme, other autolysins could have been lost or inactivated during the purification steps necessary to reduce the large amount of contamination present in cellular autolysates. In addition, other autolysins might not be active under the conditions of incubation used or may have been inactivated by proteolysis (2). Although walls autolyzing spontaneously revealed no glycosidase activity, an enzyme of this specificity may not attach to walls of disrupted cells. Nevertheless, the amidase has now been demonstrated to be the major autolytic activity in two pneumococcal strains and is probably the enzyme responsible for the propensity of pneumococci to autolyze and for the bile solubility of this species (12, 22).

The typical components of peptidoglycan, lysine, glutamic acid, alanine, muramic acid, and glucosamine were found in pneumococcal walls in approximately the same quantity as previously reported (12). Serine, the only other amino acid present to a large extent in our wall hydrolysates, had a ratio to glutamic acid of 1 to 2. Serine appears to be a component of the peptide of the peptidoglycan, since it is present in the low-molecular-weight fraction of lysed cell walls in an unchanged ratio to the other amino acids. This ratio of serine to other amino acids is maintained also in the insoluble and high-molecular-weight fractions of lysed walls. Mosser and Tomasz (12) reported the presence of a number of amino acids not typical of peptidoglycan in their wall preparations and speculated that the walls used in their studies might have a protein component such as an autolytic enzyme. In contrast, we found only lysine, glutamic acid, alanine, and serine, and very small quantities of aspartic acid and glycine in our walls. Analysis of the free amino groups of walls indicated that 20 to 30% of the ϵ-amino groups of lysine are free, in agreement with the observation that the peptide units of pneumococcal peptidoglycan are primarily in the form of dimers and trimers (12). Our finding, that approximately 30% of the total alanine in walls in the D-isomer, is within the value obtained by Manning (10) for pneumococcal C-polysaccharide, which contained 25 to 33% of its total alanine in the D-configuration.

The amounts of non-N-terminal L- and D-alanine presented in Table 4 may not represent the exact quantities present in cell walls due to an unknown amount of alanine in the added enzyme preparation. In addition the conditions of hydrolysis were mild in the FDNB procedure and may not give optimal yields of non-N-terminal amino acids. However, this experiment clearly demonstrates that it is the L-isomer of alanine which becomes N-terminal during lysis.

The purification of autolysins has proven to be difficult due in part to problems in separating these enzymes from cell wall material (1, 17). High concentrations of salt have been used in separating autolysins from walls of *Streptococcus faecalis* (14) and *Bacillus subtilis* (4). In the present studies, the removal of pneumococcal autolysin from walls in distilled water was demonstrated by the much reduced quantity of enzyme in lysates of water-washed walls and by the recovery of the lytic activity in the water wash fluid. In addition, water-washed walls completely lysed only over a period of several hours, whereas saline-washed walls lysed in 30 min or less. Removal of autolysin from walls caused no apparent loss in lytic activity. Adsorption of pneumococcal autolysin to walls in the presence of NaCl and subsequent elution in distilled water might yield soluble enzyme of high purity free of contaminating wall material.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-04577 from the National Institute of Allergy and Infectious Diseases. L.V.H. was supported by Public Health Service grant GM-01138 from the National Institute of General Medical Services.

LITERATURE CITED

1. Brown, W. C., D. K. Fraser, and F. E. Young. 1970. Problems in purification of a *Bacillus subtilis* autolytic enzyme caused by association with teichoic acid. Biochim. Biophys. Acta **198**:308-315.
2. Brown, W. C., and F. E. Young. 1970. Dynamic interaction between cell wall polymers, extra-cellular proteases and autolytic enzymes. Biochem. Biophys. Res. Commun. **38**:564-568.
3. Brundish, D. E., and J. Baddiley. 1968. Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. Biochem. J. **110**:573.
4. Fan, D. P. 1970. Cell wall binding properties of the *Bacillus subtilis* autolysin(s). J. Bacteriol. **103**:488-493.
5. Ghuysen, J. M. 1968. Use of bacteriolytic enzymes in the

determination of wall structure and their role in cell metabolism. Bacteriol. Rev. **32**:424-464.
6. Ghuysen, J. M., D. J. Tipper, and J. L. Strominger. 1966. Enzymes that degrade bacterial cell walls, p. 685-699. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
7. Huff, E., H. Oxley, and C. S. Silverman. 1964. Density-gradient patterns of *Staphylococcus aureus* cells and cell walls during growth and mechanical disruption. J. Bacteriol. **88**:1155-1162.
8. Liu, T. Y., and E. C. Gotschlich. 1963. The chemical composition of pneumococcal C-polysaccharide. J. Biol. Chem. **238**:1928-1934.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265-275.
10. Manning, J. M. 1971. Chromatographic determination of the D- and L-amino acid residues in pneumococcal C-polysaccharide. J. Biol. Chem. **246**:2926-2929.
11. Morton, R. K. 1955. Methods of extraction of enzymes from animal tissues, p. 25-51. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 1. Academic Press Inc., New York.
12. Mosser, J. L., and A. Tomasz. 1970. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J. Biol. Chem. **245**:287-298.
13. Neidhardt, F. C., and R. F. Boyd. 1965. Cell biology. Burgess Publishing Co., Minneapolis, Minn.
14. Pooley, H. M., and J. M. Porres-Juan, and G. D. Shockman. 1970. Dissociation of an autolytic enzyme-cell wall complex by treatment with unusually high concentrations of salt. Biochem. Biophys. Res. Commun. **38**:1134-1140.
15. Rogers, H. J., and C. W. Forsberg. 1971. Role of autolysins in the killing of bacteria by some bacteriocidal antibiotics. J. Bacteriol. **108**:1235-1243.
16. Rosen, H. 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys. **67**:10-15.
17. Shockman, G. D., and M. C. Cheney. 1969. Autolytic enzyme system of *Streptococcus faecalis*. V. Nature of the autolysin-cell wall complex and its relationship to properties of the autolytic enzyme of *Streptococcus faecalis*. J. Bacteriol. **98**:1199-1207.
18. Shockman, G. D., J. S. Thompson, and M. J. Conover. 1967. The autolytic enzyme system of *Streptococcus faecalis*. II. Partial characterization of the autolysin and its substrate. Biochemistry **6**:1054-1065.
19. Singer, H. J., E. M. Wise, Jr., and J. T. Park. 1972. Properties and purification of *N*-acetylmuramyl-L-alanine amidase from *Staphylococcus aureus* H. J. Bacteriol. **112**:932-939.
20. Thompson, J. S., and G. D. Shockman. 1968. A modification of the Park and Johnson reducing sugar determination suitable for the assay of insoluble materials: its application to bacterial cell walls. Anal. Biochem. **22**:260-268.
21. Tomasz, A. 1967. Choline in the cell wall of a bacterium: novel type of polymer-linked choline in pneumococcus. Science **157**:694-697.
22. Tomasz, A. 1968. Biological consequences of the replacement of choline by ethanolamine in the cell wall of pneumococcus: chain formation, loss of transformability, and loss of autolysis. Proc. Nat. Acad. Sci. U.S.A. **59**:87-93.
23. Tomasz, A., A. Albino, and E. Zananti. 1970. Multiple antibiotic resistance in a bacterium with suppressed autolytic system. Nature (London) **227**:138-140.
24. Young, F. E. 1966. Autolytic enzyme associated with cell walls of *Bacillus subtilis*. J. Biol. Chem. **241**:3462-3467.