The Autolytic Enzyme System of *Streptococcus faecalis*. II. Partial Characterization of the Autolysin and Its Substrate*

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ABSTRACT: A soluble autolysin has been isolated from cell walls of exponential (log) phase cultures of *Streptococcus faecalis* ATCC 9790 by means of gel filtration of trypsin-activated wall lysates. Trypsin and other proteolytic enzymes speed wall autolysis apparently by "activating" the autolysin. The autolysin seems to be closely associated with the wall as virtually complete wall dissolution is a prerequisite for obtaining the enzyme in solution. While the soluble autolysin can act on walls of *S. faecalis* and *Micrococcus lysodeikticus*, it is virtually inactive on walls from several other bacterial species. Borohydride reduction indicates cleavage of the glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in the polysaccharide peptidoglycan "backbone" and is thus an *N*-acetylmuramidase. Autolysin action released an isolatable highly cross-linked peptidoglycan derivative with an apparent molecular weight by gel filtration between 10,000 and 25,000 rather than dialyzable products.

Quantitation of released products suggests that the autolysin cleaves over 90% of the glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine in *S. faecalis* walls.

Previous studies (Shockman, 1963b, 1965; Shockman *et al.*, 1958, 1961; Toennies and Shockman, 1958; Conover *et al.*, 1966) have demonstrated that cells from exponential phase (log) cultures of *Streptococcus faecalis* 9790 will autolyze rapidly when placed under environmental conditions which prevent the continued synthesis of cell wall peptidoglycan (mucopeptide, glycopeptide, or murein). Inhibition of peptidoglycan synthesis (resulting in autolysis) can be caused either by the addition of a specific inhibitor, such as penicillin or cycloserine, or by deprivation of a nutritionally essential precursor of wall peptidoglycan, such as L-lysine, D-alanine, aspartic and glutamic acids, or glucose. The potential for cell autolysis reached a maximum toward the end of the exponential growth phase and thereafter the cells became resistant to autolysis (Shockman, 1965). Stationary-phase, valine-deprived (Val) or threonine-deprived (Thr) cultures resist autolysis (Shockman, 1965; Shockman *et al.*, 1961).

Two observations implicated an enzymatic attack on the rigid and protective cell wall as the initial step in cell autolysis. First, when an osmotically protective agent, such as 0.5 M sucrose, was provided, wall-free spherical, osmotically fragile protoplasts could be obtained from log cells, but not from stationary-phase Thr cells in the absence of an exogenous lytic enzyme, such as lysozyme (Shockman *et al.*, 1961). Second, isolated and purified walls from log, but not stationary-phase, Thr cultures slowly autolyzed when incubated in phosphate buffer (Shockman *et al.*, 1961).

We have now succeeded in releasing and partially purifying a soluble enzyme from walls of log cultures of *S. faecalis* which is capable of dissolving cell walls. Some of the properties of this enzyme have been examined.

Materials and Methods

Materials. Lysozyme (EC 3.2.1.17) and chymotrypsin (EC 3.4.4.5) were obtained from Worthington Biochemical Corp.; muramic acid from Cyclo Chemical Corp.; trypsin (EC 3.4.4.4), carboxypeptidase A (EC 3.4.2.1), pepsin (EC 3.4.4.1), and papain (EC 3.4.4.10) from General Biochemicals, Inc.; subtilisin BPN′ (EC 3.4.4.16) (Nagarse) from Enzyme Development Corp.; ficin (EC 3.4.4.12) from Mann Research Laboratories, Inc.; and pronase from Calbiochem. The plastic beads (styrene–divinylbenzene copolymer, 18–50 mesh) were a gift of the Dow Chemical Co. SDS[1] was a gift of the Du Pont Chemical Co. (Duponol 100). The 30% solution supplied was recrystallized twice from ethanol. Suramin was a gift of Imperial Chemical Industries. Bio-Gels P-60, P-30, and P-2 were purchased from Bio-Rad Laboratories. ε-Aminosuccinoyl-L-lysine was a gift of Dr. M. Ikawa. Pure disaccharide peptide from *Staphylo-*

* From the Department of Microbiology, Temple University School of Medicine, Philadelphia, Pennsylvania. *Received November 4, 1966.* This investigation was supported by U. S. Public Health Service Research Career Program Award 1-K3-AI-4792 from the National Institute of Allergy and Infectious Diseases to G. D. S. and by Research Grant GB 4466 from the National Science Foundation.

[1] SDS, sodium decyl sulfate; suramin, hexasodium *sym*-bis(*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamino-4,6,8-trisulfonate) carbamide; FDNB, 2,4-dinitro-1-fluorobenzene; TCA, trichloroacetic acid; SLS, sodium lauryl (dodecyl) sulfate.

coccus aureus Copenhagen and glucosamine 6-phosphate *N*-acetylase were kindly provided by Dr. J.-M. Ghuysen.

Cell wall preparations from other bacteria were kindly provided as generous gifts as follows: *S. aureus* Copenhagen by Drs. D. J. Tipper and J. L. Strominger, *Bacillus subtilis* 168 by Dr. F. Young, group A *Streptococci* strains TIM and Blackmore A 11 and group N *Streptococci* strains B207 and C559 by Dr. H. D. Slade, and *Chromobacterium violacium* and *Bacillus cereus* strain 5 by Dr. R. Wheat.

Cell Growth and Cell Wall Isolation. Large crops of *S. faecalis* ATCC 9790 (20–80 l.) were grown in the previously described (Shockman, 1963a) highly buffered synthetic medium in a New Brunswick fermentor. The pH of the cultures was maintained at 6.60, using the New Brunswick pH control unit. The arginine content of the medium was increased to 400 μg/ml (F. Feng and G. Toennies, personal communication), which resulted in exponential growth to cell turbidities in excess of 1.5 g of cellular dry wt/l. Log cells were grown in continuous cultures, maintaining cell densities in the growth vessel (10–12 l.) of about 1.0 g/l. dry wt. Thr and Val stationary-phase cells were grown in batch cultures (12–24 l.) in the same medium containing a limited supply of threonine (9.5 mg/l.) or valine (10.0 mg/l.) for 40–42 and 17–20 hr at 37° and harvested at densities of about 0.8 and 1.0 g cellular dry wt/l., respectively. Cells were harvested, washed four times, lyophilized, and stored in a desiccator at 4°. *Micrococcus lysodeikticus* was grown in the fermentor with aeration in the medium of Prasad and Litwack (1965) for about 48 hr.

Cells were disrupted by shaking with beads as previously described (Shockman *et al.*, 1957; Shockman, 1962), except that styrene–divinylbenzene copolymer beads (18–50 mesh) replaced glass beads (Ross, 1963). These beads generate less heat than glass beads and do not liberate alkali. The time of disruption was increased by about 5 min to ensure complete breakage. The walls were washed at least four times with water and twice with 0.1 M phosphate buffer, pH 7.4, on the centrifuge, and debris and undisrupted cells, if any, were removed by low-speed centrifugation. Walls were lyophilized and stored in a desiccator at 4°.

Chemical and Analytical Methods. Total hexosamines were determined after hydrolysis in 3 N HCl for 6 hr at 95° in sealed tubes and acetylation by the Morgan–Elson reaction in borate buffer (Ghuysen *et al.*, 1966), using the 30-min reaction time. Rhamnose was determined by the method of Dische and Shettles (1948); inorganic phosphate by the method of Lowry and López (1946); total phosphorus by the method of Boltz and Mellon (1947); free (terminal) amino groups by reaction with FDNB (Ghuysen *et al.*, 1966). Acetylation of walls was carried out according to the method of Park and Griffith (1964). De-*O*-acylation was carried out at pH 10.0 in carbonate–bicarbonate buffer (0.2 M) for 1 hr at 37° (Perkins, 1965). For reducing groups a modification of the Park and Johnson (1949) method was used (J. S. Thompson, 1967, in preparation). Protein was determined by the method of Lowry *et al.* (1951). TCA extraction was carried out by the method of Davison and Baddiley (1963). Amino acid analyses were performed on the Phoenix amino acid analyzer using Phoenix spheric resins and an accelerated system at 55°.

Paper Chromatography. Chromatography was carried out on Schleicher and Schuell paper no. 507-C in the following solvent systems: (A) pyridine–water (4:1) ascending (Salton and Ghuysen, 1960), (B) 1-butanol–acetic acid–water (3:1:1) descending (Salton and Ghuysen, 1960), and (C) 1-butanol–pyridine–water–acetic acid (60:40:30:3) descending (Primosigh *et al.*, 1961). Chromatograms were dried overnight *in vacuo* over fresh concentrated sulfuric acid and solid sodium hydroxide. Spots were visualized by dipping in 0.5% ninhydrin in acetone and heating at 70° for 20 min (Primosigh *et al.*, 1961; Toennies and Kolb, 1951).

Gel Filtration. This was carried out using Bio-Gel P-60 and a Pharmacia, Inc., column (1.5 × 70 cm) operated at 4° at a rate of 10 ml/hr. Fractions of 1.5–2.2 ml were collected.

Inactivation of Log Walls. Treatment with 2% SLS at 0° inactivated autolysis of log walls virtually completely. SDS was as effective as the dodecyl salt in inactivating autolysis and was much more soluble at 0°. Therefore, in nearly all experiments described in this paper, walls were inactivated as follows. About 100 mg of walls was suspended in 25 ml of 2% SDS and incubated in an ice bath for 1 hr with constant stirring. Walls were sedimented at 25,000*g* and the process was repeated. The walls were then washed four times with 40 ml of distilled water, suspended in 0.15 M maleate buffer (pH 7.0), and stored in the freezer. Storage of walls in maleate buffer resulted in more uniform and stable wall suspensions. Before use, the walls were removed from maleate and suspended in the assay buffer.

Lytic Assay. In an ice bath, 2.1 mg of SDS-treated log walls in 0.5 ml of 0.06 M sodium phosphate buffer (pH 6.7) was added to each tube. Enzyme and/or water was added to a total volume of 3.0 ml in 12-mm diameter round cuvets. The tubes were placed in a 37° water bath and the optical density at 450 mμ was determined at frequent intervals with a Bausch and Lomb Spectronic 20, equipped with an A. H. Thomas Co. absorbance digital readout. Absorbance readings were corrected to agree with Beer's law (Toennies and Gallant, 1949). One unit of enzyme was defined as the amount giving a decrease of 0.001 OD/hr. Some of the early assays described in this paper were carried out in a final concentration of 0.02 M phosphate buffer, pH 7, rather than in 0.01 M phosphate, pH 6.7.

Results

Lysis of Walls. Log walls autolyze in 0.01 M phosphate buffer. Addition of relatively large amounts (2–8 mg of protein) of the 105,000*g* supernatant fraction from mechanically disrupted log cells significantly increased the rate of lysis. Walls from stationary-

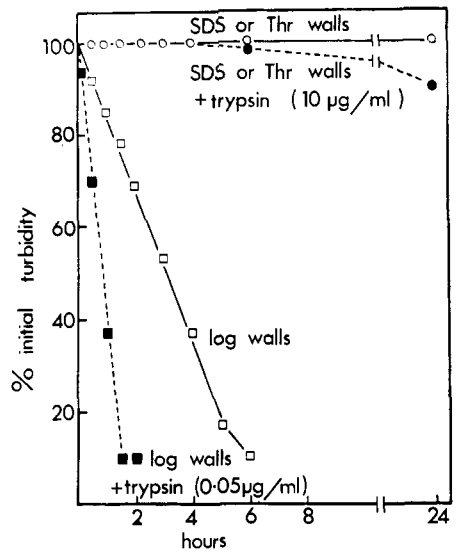

FIGURE 1: The effect of trypsin on autolysis of exponential phase (log), SDS-treated log (SDS), and walls from threonine-deprived cells (Thr).

phase Thr or Val cultures did not autolyze and as much as 8 mg of protein of the same supernatant fraction was virtually without effect on Thr walls. Autolysis of log walls could be inactivated, partially by heat (56° for 15 min), or completely by treatment with SDS at 0°.

Using SDS-inactivated log walls as substrate, the specific activity of the 105,000*g* supernatant fraction of log cells was low (5 units/mg of protein), but easily measurable. Procedures such as precipitation of nucleic acid with protamine sulfate, $MnCl_2$, or streptomycin and fractionation by means of ammonium sulfate precipitation or DEAE-cellulose batch absorption and elution (Allen *et al.*, 1964) failed to increase consistently the specific activity of the cytoplasmic enzyme.

The Effect of Proteolytic Enzymes on Wall Lysis. Montague (1964) reported that while trypsin had little action on isolated cell walls of *S. faecalis* NCTC 6782, it did cause a further release of rhamnose and phosphorus from autolyzed and partially lysozyme-degraded walls. When these walls were heated at 100° for 10 min and then partially degraded with lysozyme, trypsin had little or no effect. Also, Bleiweis and Krause (1965) found that treatment with trypsin activated autolysis of cell walls of a group D, strain D76 *Streptococcus*. Because of these reports, the effect of trypsin on autolysis of cell walls of *S. faecalis* 9790 was examined. The results of such an experiment are shown in Figure 1. While trypsin greatly increased the rate of autolysis of log walls, the enyzme had essentially no effect on the lysis of SDS-inactivated log walls or on walls from stationary-phase Thr or Val cultures. Table I shows that extremely low concentrations of trypsin were effective. Except for carboxypeptidase, all of the proteolytic enzymes tested increased the rate of autolysis

TABLE I: Effect of Trypsin Concentration on the Rate of Lysis of Log Walls.

Trypsin (μg/ml)	Lysis Rate (units)[a]
0.0	59
0.02	137
0.1	185
0.4	262
2.0	259
10.0	277
50.0	280

[a] One lysis unit equals a decrease of 0.001 OD/hr.

(Table II). Chymotrypsin was nearly as effective as trypsin.

Release of Enzyme. Since trypsin had no effect on detergent-inactivated walls it seemed unlikely that proteolytic digestion was a factor in itself, but that *via* proteolytic or esterase activity an endogenous wall lytic enzyme was being released or activated *in situ*. As shown in Figure 2, activity which dissolved SDS-inactivated walls was released to the supernatant fraction during trypsin-catalyzed wall autolysis or during wall autolysis in the absence of trypsin. This activity was not sedimented at 105,000*g* for 2 hr. Release of enzyme seemed to take place after wall dissolution, indicating that the enzyme may be an integral part of the wall structure. More enzyme was released by the trypsin-catalyzed wall autolysis (95 units/ml) than by simple autolysis (25 units/ml). Treatment of autolysates with trypsin after minimum turbidity had been reached caused no further enzyme release. When autolysates were allowed to remain at 37° after the minimum turbidity had been reached, a decrease in activity was observed. The lability of the enzyme, especially in the presence of proteolytic enzymes, is discussed below and probably accounts for the smaller yields of autolysin by means of simple autolysis. As shown in Table II, accelerated wall lysis caused by proteolytic enzymes other than trypsin sometimes resulted in release of autolysin. In all cases the amounts released were smaller than with trypsin, chymotrypsin being nearly as effective as trypsin. The decreased yield, in most cases, correlated with the ability of the proteolytic enzyme to inactivate the autolysin. Treatment of the 25,000*g* supernatant fraction of mechanically disrupted log cells (cytoplasmic fraction) with trypsin (12 μg/ml) for 1–2 hr did not increase the low levels of autolysin present.

Removal of Trypsin by Gel Filtration. The 25,000*g* supernatant fraction from trypsin-catalyzed log wall autolysis was placed on a column of polyacrylamide gel (Bio-Gel P-60). Log walls (100 mg) were suspended in 70 ml of 0.01 M phosphate, pH 6.7, containing 0.05 μg/ml of trypsin, and incubated at 37°. When the minimum turbidity was reached (6% of the initial

TABLE II: Effect of Proteolytic Enzymes on Wall Lysis and Release of Lytic Enzyme.

Expt	Enzyme	Amt (μg/ml)	Lysis Rate		Act. of Lysate on SDS Walls (units)	Inactivation of Lytic Enzyme by 30 μg/ml of Proteolytic Enzyme[b]
			Units[a]	—Control Units		
1	None	—	76	—	0	
1	Trypsin	0.1	452	376	100	1+
1	Pronase	10	422	346	0	4+
1	Papain	10	117	41	17	2+
1	Ficin	10	191	115	10	2+
1	Pepsin[c]	10	33	—	0	4+
1	Carboxypeptidase[d]	10	17	—	0	—
2	None	—	105	—	36	
2	Trypsin	0.05	390	285	86	
2	Subtilisin BPN′	10	363	258	46	—
2	Chymotrypsin	0.05	115	10	49	1+
2	Chymotrypsin	0.5	195	90	64	
2	Chymotrypsin	5.0	382	277	76	

[a] One lysis unit equals a decrease of 0.001 OD/hr (pH 6.7, 0.02 M phosphate). [b] 4+, complete inactivation in 1 hr at 37°; 2+, about 50% inactivation in 1 hr at 37°; 1+, 15–25% inactivation in 1 hr at 37°; —, not tested. [c] The rate of lysis shown is for incubation at pH 2 for 3.5 hr. Walls were then sedimented and incubated at pH 6.7 in 0.02 M phosphate. This resulted in a lysis rate twice that of a comparably incubated control. [d] The rate of lysis shown is for incubation at pH 7.5, 0.2 M phosphate, in the presence of LiCl for 3.5 hr. Walls were then sedimented and incubated at pH 6.7 in 0.02 M phosphate. This resulted in a lysis rate comparable to similarly incubated controls.

turbidity, in 70 min), 2 ml of lysate was removed for assay and the remainder was lyophilized. In the cold, the lyophilized digest was resuspended in water and centrifuged at 25,000*g* to remove insoluble material (fraction I). A portion of this soluble material, representing about 83 mg of the original wall, was applied to a Bio-Gel P-60 column, the column was eluted with 0.02 M phosphate buffer, pH 7.0, at 4°, and 1.5-ml fractions were collected. The elution pattern of this column is shown in Figure 3. The autolysin was excluded from this gel and could be recovered in the void volume along with most of the wall rhamnose and organic phosphorus (not shown) and about 10–20% of the Morgan–Elson positive *N*-acetylamino sugar groups. Trypsin was eluted as two peaks (Ganrot, 1966) approximately 1.8 and 2.4 times the void volume. The fractions containing enzyme were combined and lyophilized (fraction II). Preparations such as these have been designated "trypsin-free autolysin." Removal of trypsin was indicated not only by gel filtration profiles but also by an increase in stability of the autolysin (see below). The fractions containing the smaller molecular weight *N*-acetylamino sugar peaks were combined, lyophilized, desalted on a Bio-Gel P-2 column, and lyophilized again (fraction III).

The same Bio-Gel P-60 column was equilibrated and eluted with water. The elution pattern of the

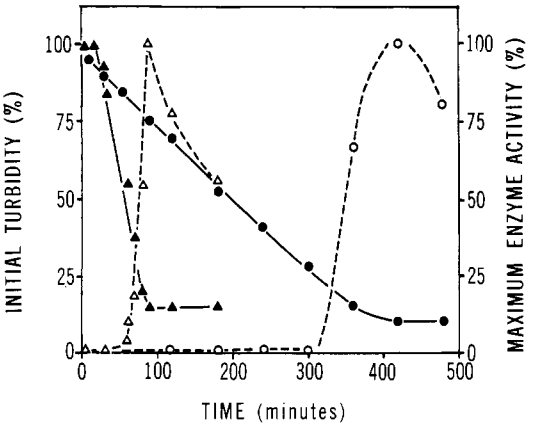

FIGURE 2: Release of soluble autolysin during wall lysis. Walls (0.7 mg/ml) were suspended in 0.02 M phosphate buffer, pH 7.0, in the absence (○) and presence (△) of 0.04 μg/ml of trypsin. The turbidity of the suspensions was followed (solid lines), samples were removed at various time intervals and centrifuged at 25,000*g* for 30 min, and the supernatant fraction was tested on SDS-treated log walls (dashed lines). Enzyme activity is expressed as per cent of maximum activity released in that experiment. More autolysin (95 units/ml) was released in the presence of trypsin than in its absence (25 units/ml).

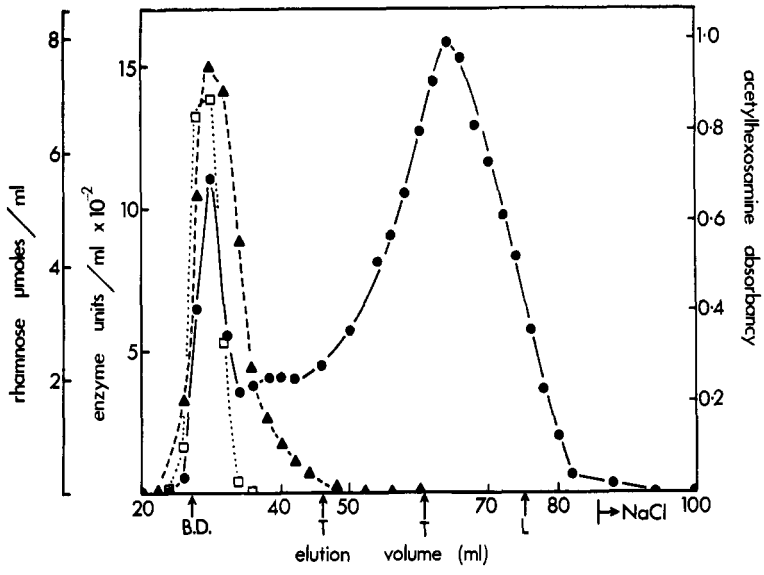

FIGURE 3: Separation of the products of trypsin-catalyzed log wall autolysis on Bio-Gel P-60 eluted with 0.02 M phosphate buffer, pH 7.0. The elution volumes for Blue Dextran (BD), trypsin (T), egg white lysozyme (L), and the volume at which NaCl begins to be eluted are indicated along the base of the figure. Two peaks were consistently obtained for trypsin. □, autolysin activity, units per milliliter; ▲, rhamnose, micromoles per milliliter; ●, *N*-acetylhexosamine (30 min), absorbancy.

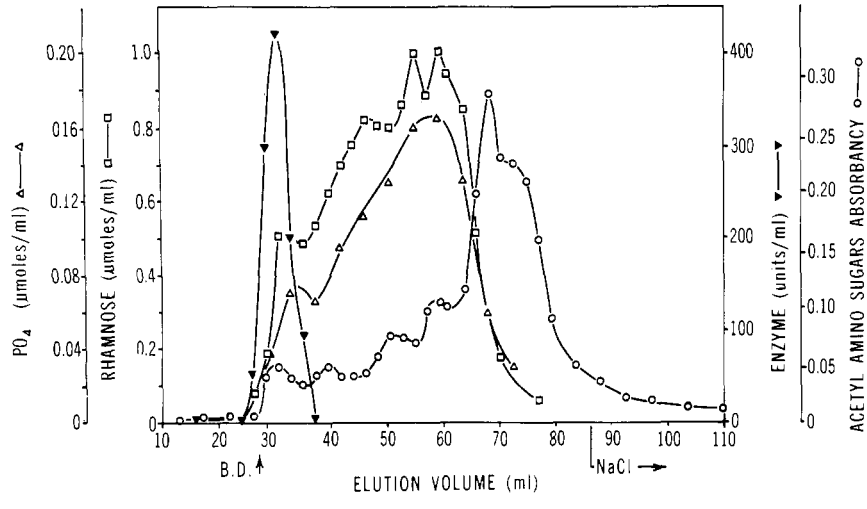

FIGURE 4: Separation on Bio-Gel P-60 of autolyzed log cell walls on a column equilibrated and eluted with water. Elution volumes for Blue Dextran (BD) and the volume at which NaCl begins to be eluted are indicated. ▼, autolysin activity, units per milliliter; □, rhamnose, micromoles per milliliter; Δ, phosphate, micromoles per milliliter; and ○, *N*-acetylamino sugar (30 min) absorbancy. Lysate from about one-half the amount of log walls used in the experiment illustrated in Figure 3 was applied to this column.

25,000*g* supernatant fraction of a similar trypsin-catalyzed log wall lysate is shown in Figure 4. Under these conditions, the autolysin was excluded from the column, but less *N*-acetylamino sugar and rhamnose was eluted with the enzyme. It is of interest to note that, even when not totally excluded from the gel, the rhamnose polysaccharide and the phosphorus-containing material appear to be eluted together. This fractionation indicates that the autolysin can be at least partially separated from *N*-acetylamino sugar, rhamnose and phosphate. At present we cannot distinguish between coincidental elution of other wall components with the autolysin and attachment of polysaccharide to the autolysin.

Action of Wall Autolysin. Walls from log and stationary-phase Thr and Val cells contained 31–39 mμmoles/

mg of reducing groups (glucose equivalents). Thr or Val walls did not significantly autolyze or release further reducing groups. Figure 5 shows that for all three conditions of log wall lysis (autolysis, trypsin-catalyzed autolysis, and autolysin action on SDS-treated walls) the increase in reducing groups corresponded closely to the observed reduction in turbidity. Figure 6A–C shows for the same three conditions that rhamnose and amino sugars were also released into solution as the turbidity decreased, while an increase in FDNB-reactive terminal amino groups was not observed. These results indicate that the amino sugar backbone of the peptidoglycan was cleaved rather than the peptide portion of that macromolecule. Rhamnose polysaccharide and the phosphate-containing teichoic acid polymer were released into solution as the peptidoglycan was dissolved.

The Nature of the Products Released by Trypsin-Catalyzed Wall Autolysis. The amounts of desalted fractions I and II material available were very small so that analytical results for these fractions are only approximations. Fraction I contained only 1.1% hexosamine, 0.5% rhamnose, 0.3% phosphorus, and 0.03 μmole/mg of amino-terminal groups. The amount of this small, insoluble residue varied from one batch of walls to another and probably represents mainly contaminating nonwall material not removed during wall preparation. Fraction II contained about 3% phosphorus, 14% hexosamine, 39% rhamnose, and 0.1 μmole/mg of amino-terminal groups. Although the Morgan–Elson reaction (Figure 3) indicates that some peptidoglycan is probably present, a large portion of fraction II can be accounted for as teichoic acid(s) and rhamnose polysaccharide(s). Armstrong *et al.* (1959) have obtained evidence that walls of this strain of *S. faecalis* contain a ribitol teichoic acid. The structure of this polymer in the walls of this organism has not yet been established.

Several preparations of fraction III have been examined. Phosphorus was either undetectable or barely detectable (0.01%) and rhamnose was extremely low (0.6–2.6%). The composition of fraction III was examined in greater detail using material pooled from several P-60 column runs, desalted, and repurified on a water eluted Bio-Gel P-30 column. This material contained (in micromoles per milligram) total hexosamine, 1.56 (after hydrolysis with 3 N HCl for 3 hr at 100°); glucosamine, 0.89 (by glucosamine 6-phosphate-*N*-acetylase after the same acid hydrolysis) (Ghuysen *et al.*, 1966); and the following amino acids: glutamic acid, 0.83; lysine, 0.84; aspartic acid, 0.74; and alanine, 1.4 (after hydrolysis with 6 N HCl for 20 hr at 120° and determined with the amino acid analyzer). Except for traces of a few other amino acids (*e.g.*, glycine, 0.02, and serine, 0.01) the only other ninhydrin-positive material present could be attributed to small amounts of glucosamine and muramic acid which survived hydrolysis (*ca.* 0.1 μmole) or the formation of the cyclic dipeptide ε-(aminosuccinoyl)lysine during acid hydrolysis (Ikawa, 1964). The formation of this cyclic dipeptide could result in reduced recoveries of lysine and aspartic acid. Assuming that the remaining amino sugar is muramic acid and that both muramic acid and glucosamine are *N*-acetylated, these amino sugars and amino acids accounted for over 90% of the dry weight of fraction III. Only 0.32 μmole/mg of terminal amino groups were present.

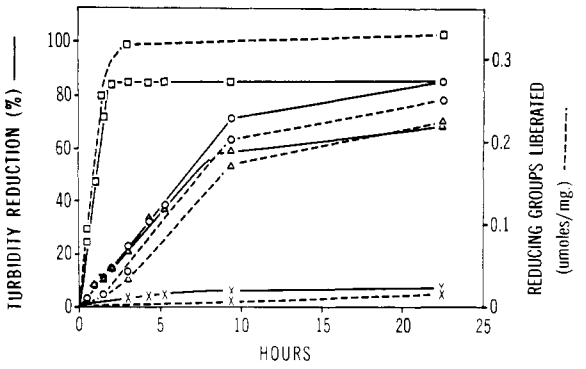

FIGURE 5: Release of reducing groups during wall lysis. Solid lines, turbidity; dashed lines, reducing groups liberated. O, log wall autolysis; □, trypsin (50 μg/ml) catalyzed wall autolysis; Δ, SDS-treated log walls plus autolysin; X, walls from Thr cells with and without trypsin.

The chemical analysis and apparent molecular size from gel filtration of fraction III (10,000–25,000 mol wt) suggested that it is a highly cross-linked peptidoglycan derivative that had been solubilized by enzymatic cleavage of the amino sugar "backbone." In order to determine the position of autolytic cleavage, a portion of fraction III was reduced with a tenfold molar excess of $NaBH_4$ (Tipper *et al.*, 1965) and hydrolyzed with 3 N HCl for 3 hr at 95°. HCl was removed by repeated evaporation *in vacuo* over NaOH and boric acid was removed by extracting seven times with methanol (Ghuysen and Strominger, 1963). Samples containing the equivalent of 34 and 85 μg of fraction III were subjected to one-dimensional paper chromatography in solvents A–C along with acid-hydrolyzed but unreduced fraction III and samples of unreduced and reduced glucosamine and muramic acid. Color development with ninhydrin showed that borohydride reduction resulted in a disappearance of the spot corresponding to muramic acid and the appearance of a reddish spot corresponding to muramitol. No change in the size or intensity of the glucosamine spot was observed, nor was a spot corresponding to glucosaminitol seen. Examination of the products of borohydride reduction by means of two differential amino sugar reactions (Ghuysen and Strominger, 1963; Strominger *et al.*, 1959) served only to confirm qualitatively the chromatographic data. These results indicate that the lysozyme-sensitive (β-1,4?) glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine is the sole cleavage point of the autolysin. Fraction III contained 0.70 μmole/mg of the disaccharide *N*-acetylglucosaminyl-*N*-acetylmuramic acid by the Morgan–

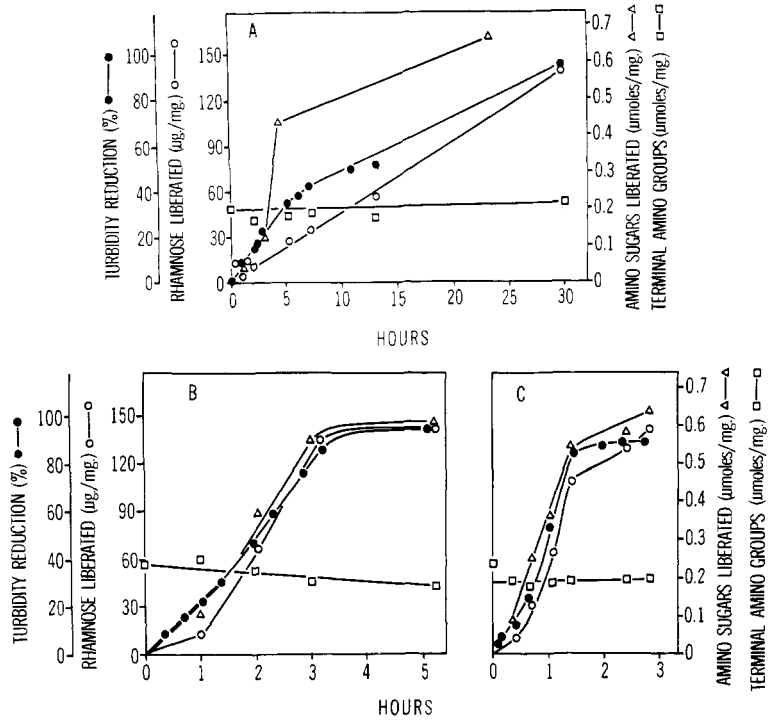

FIGURE 6: Correlation of release of amino sugars (Δ) and rhamnose (○) to the soluble fraction and amino-terminal groups (□) with turbidity reduction (●) during wall lysis. (A) Autolysis of log walls. (B) SDS-treated log walls plus autolysin. (C) Trypsin (4 μg/ml) catalyzed log wall autolysis.

Elson reaction after heating for 30 min in 1% borate using pure disaccharide from *S. aureus* as standard (Ghuysen *et al.*, 1966). Since peptide substitution decreases the molar extinction coefficient of the disaccharide from 9500 to 7500 (Muñoz *et al.*, 1966a,b), 0.70 μmole of disaccharide is equivalent to 0.88 μmole of disaccharide peptide, the quantity expected from the analytical data for hexosamines and amino acids.

The release of wall constituents to the soluble fraction after wall autolysis (85–94% drop in turbidity) indicates that over 90% of the glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine in the wall were broken. Per lysine or glutamic residue present in log walls (0.37 μmole/mg) (Toennies *et al.*, 1959) about 1.8 hexosamines (0.65 μmole/mg) and 0.8 disaccharide unit (0.29 μmole/mg) were released during wall autolysis. The decreased molar extinction coefficient in the Morgan-Elson reaction caused by peptide substitution of the disaccharide (Muñoz *et al.*, 1966a,b) indicates that the yield of disaccharide peptide/lysine residue in the wall is virtually 1.0.

Properties of the Trypsin-Free Enzyme. The trypsin-free autolysin acted on SDS-treated log walls to decrease the turbidity in a linear fashion until nearly the ultimate minimum turbidity was reached, in a manner essentially identical with that seen with the crude wall lysate (Conover *et al.*, 1966). The rate of decrease in turbidity was proportional to the amount of enzyme added. The reason for the zero-order kinetics is difficult to interpret in a system involving a soluble enzyme and an insoluble substrate. The same type of zero-order kinetics was also observed with trypsin-speeded autolysis of log walls. Autolysis of log walls in the absence of trypsin is not always linear.

The pH optima for the three conditions of wall autolysis are shown in Figure 7. All three conditions have a rather broad pH optimum, with significant rates of lysis at pH 5.6–8.4. The pH optimum for the trypsin-free autolysin is about the same as that for log wall autolysis (pH 6.7), while that for the trypsin-activated reaction on log walls appears to be a bit higher (about pH 7). It should be noted that the optimum for peptidase action of trypsin is about pH 8.

The optimum buffer concentrations for log wall autolysis, trypsin-catalyzed wall autolysis, and autolysin on SDS-treated log walls are all between 0.005 and 0.01 M in phosphate buffer, pH 6.9–7.0. The rate of autolysin action on SDS walls in 0.3 M phosphate buffer is about one-half that in 0.01 M. This contrasts to autolysis of whole log cells, which is nearly as rapid at 0.3 M as it is at 0.01 M phosphate and more rapid than autolysis in 0.05–0.1 M phosphate (Shockman *et al.*, 1961). Egg white lysozyme is as active on *S. faecalis* walls in 0.05 M as it is in 0.02 M phosphate. The protein concentration present in solutions of autolysin, containing as much as 4000 units/ml, was below the level detectable by absorption at 280 mμ.

Specificity of the Enzyme. The weakly active autolysin, from the cytoplasmic fraction, was inactive on walls from stationary-phase Val or Thr cultures of

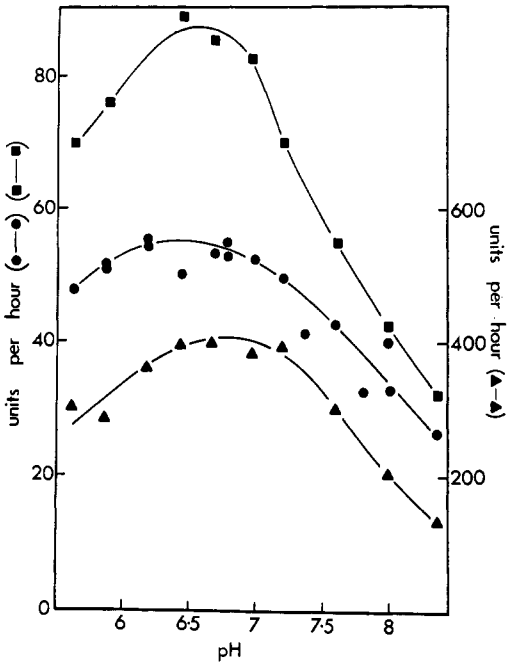

FIGURE 7: Influence of pH on wall lysis. ■, autolysis of log walls; ▲, trypsin (0.05 μg/ml) catalyzed autolysis of log walls; and ●, SDS-treated log walls plus trypsin-free autolysin. Phosphate buffer (0.02 M) was used throughout.

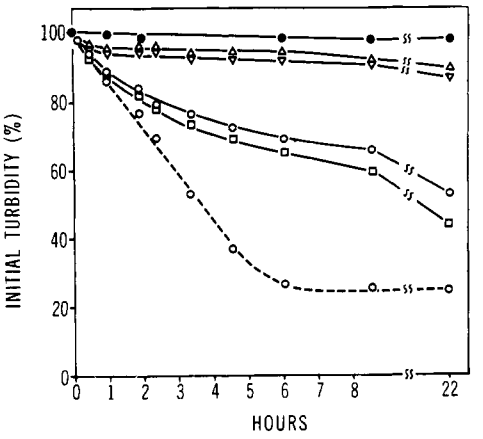

FIGURE 8: Effect of acetylation and de-*O*-acylation of *M. lysodeikticus* walls on autolysin activity. ●, control, no enzyme; ○, untreated walls; □, walls de-*O*-acylated at pH 10 for 1 hr at 37°; Δ, walls acetylated with acetic anhydride; and ∇, walls acetylated with acetic anhydride and then de-*O*-acylated. Only one control without added enzyme is shown. The others were essentially the same. The effect of the same amount of autolysin (65 units) on SDS-inactivated *S. faecalis* log walls is shown by the dashed line; phosphate 0.01 M, pH 7.0.

S. faecalis or on walls from stationary-phase *M. lysodeikticus*. The much more active trypsin-free autolysin did act on these substrates, but at a reduced rate compared to SDS-treated log walls (Table III and Figure 8). With *S. faecalis* wall substrates, in all cases the linearity of the reaction continued to near completion (*e.g.*, dashed line in Figure 8). On walls of *M lysodeikticus* (Figure 8), activity was not only lower but also did not follow zero-order kinetics. Brumfitt *et al.* (1958, 1959) and others (Prasad and Litwack, 1965; Abrams, 1958; Perkins, 1960, 1965) have presented evidence that resistance of *M. lysodeikticus* walls to lysozyme increases with the degree of *O*-acylation. We have confirmed this with *M. lysodeikticus* walls, obtaining results essentially the same as those of Perkins (1965). The extent of *O*- and *N*-acylation also affected the sensitivity of *S. faecalis* walls to lysozyme, but in a slightly different manner. De-*O*-acylation had virtually no effect, while acetylation significantly increased rather than decreased the rate of lysozyme digestion. For both *M. lysodeikticus* and *S. faecalis* walls, acetylated and then de-*O*-acylated walls were most sensitive to lysozyme. With the autolysin, a different situation was encountered. As shown in Table III, acetylation increased the rate of lysis of SDS-treated log walls, but had the opposite effect, if any, on walls from the other cell types. With *M. lysodeikticus* walls (Figure 8) the effect of *N*- and *O*-acylation on autolysin sensitivity was much different than that for lysozyme. De-*O*-acylation had little effect on the sensitivity to autolysin. Acetylation or acetylation followed by de-*O*-acylation drastically decreased the sensitivity of *M. lysodeikticus* walls to autolysin. Another comparison with lysozyme was made using TCA-extracted log walls as substrate (Figure 9). Lysozyme acted on the TCA residue of SDS-treated log walls more rapidly than it did on unextracted SDS-treated log walls. However, the same TCA residue was a poorer substrate than log walls for the autolysin.

TABLE III: Sensitivity to Autolytic Enzyme of Acetylated and Deacylated Walls.

Treatment	SDS-Treated Wall Substrates from		
	Log	Val	Thr
None	86[a]	44[a]	37[a]
pH 10, 1 hr, 37°	82	50	40
Acetylation	104	34	33
Acetylation then pH 10, 1 hr, 37°	107	54	42

[a] In lysis units. One lysis unit equals a decrease of 0.001 OD/hr.

Activity of trypsin-free autolysin was tested on walls of a few other bacteria. These walls were treated with SDS as stated above for *S. faecalis* walls in order to inactivate any wall lytic enzymes that might be present. The autolysin (115 units/ml) failed to decrease

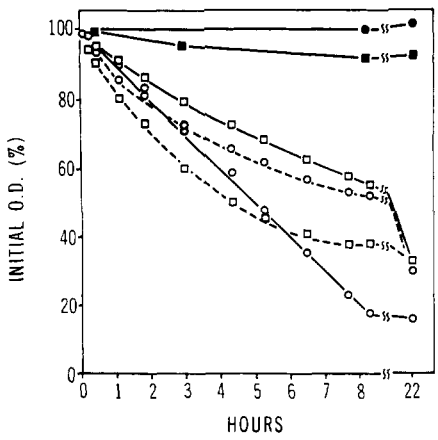

FIGURE 9: Effect of extraction of walls with TCA on lysozyme and autolysin sensitivity. Solid lines, autolysin (55 units), 0.01 M phosphate, pH 6.7; dashed lines, lysozyme (1 μg/ml), 0.05 M phosphate, pH 6.8. ●, unextracted walls, no enzyme; ■, TCA-extracted walls, no enzyme; O, unextracted walls plus enzyme; □, TCA-extracted walls plus enzyme. Only two controls are shown; the others were essentially the same regardless of buffer concentration.

significantly the turbidity of or to release *N*-acetylhexosamines from walls of *S. aureus* Copenhagen, *Chromobacterium violacium*, group A *Streptococci* strains TIM and Blackmore A 11, or group N *Streptococci* strains B207 and C559. Activity was barely detectable against walls of *Bacillus cereus* 5 (5 units/ml and a decrease of 19% in turbidity after 24 hr). Weak activity against walls of the transformable *B. subtilis* 168 was observed (16 units/ml and a decrease of 50% in turbidity after 24 hr). However, in this last case liberation of *N*-acetylhexosamines after 24 hr was only 13% of that obtained with an *S. faecalis* control that had lost 90% of its initial turbidity. In all cases controls, without added autolysin, did not significantly decrease in turbidity or liberate *N*-acetylhexosamines.

Effect of Inhibitors and Stimulators on Autolysin Activity. The autolysin does not seem to be a sulfhydryl enzyme. Activity on SDS-treated log walls was unaffected by the addition of mercaptoethanol (10^{-2} M), *p*-hydroxymercuribenzoate (10^{-4} M), *N*-ethylmaleimide (10^{-3} M), iodoacetate (10^{-4} M), EDTA (10^{-4} M), or magnesium sulfate (10^{-2} M). Autolysin (107 units/ml) was incubated with suramin for 1 hr at 37°. An extremely high concentration (12 mg/ml) completely inactivated the autolysin while lower, but still high, concentrations of suramin resulted in only partial inhibition (40% inhibition with 1.2 mg/ml and 12% inhibition with 0.12 mg/ml). Lysozyme (0.1 μg/ml) action on *M. lysodeikticus* cells has been reported to be 50% inhibited by an equal weight of suramin (Lominski and Gray, 1961).

Stability and Destruction of the Autolysin. The stability of the trypsin-free enyzme has been somewhat variable. Several preparations were stable for over 2 months at −10°. Another preparation lost all activity and a second 80% of its initial activity upon similar storage for 2 weeks. No significant decrease in activity was noted upon incubation at 37° in 0.002 M maleate (pH 4.8–6.0), phosphate (pH 6.5–7.0), or Tris·HCl (pH 7.2–8.6) for 17 hr. In 0.002 M phosphate (pH 7.0) bringing the autolysin to 56° (in approximately 4 min) was sufficient to cause 80% inactivation. Two minutes at 56° inactivated over 90% of the activity. The enzyme was inactivated by trypsin and other proteolytic enzymes (Table II). Incubation at 37° for 1 hr in the presence of 30 μg/ml of pronase completely inactivated 84 units/ml of the autolysin. After incubation of autolysin at 37° for 24 hr in the presence of 30 μg/ml of trypsin, 35–50% of the original activity was still present.

A number of other buffers can substitute for phosphate. Autolysis and autolysin assays can be carried out as effectively in Tris, acetate, or maleate buffers of suitable pH and ionic strength.

Discussion

Cellular autolysis has been observed in a wide variety of Gram-positive and Gram-negative bacteria (see reviews in Shockman, 1965; Salton, 1964; Stolp and Starr, 1965). In a few cases damage to the external rigid cell wall has been implicated as the primary event occurring, with a subsequent bursting of the contents of the cell through the damaged protective cell wall (Salton, 1964; Mohan *et al.*, 1965; Young and Spizizin, 1963; Weidel *et al.*, 1963). In still fewer instances enzymatic action on isolated walls has been demonstrated (Shockman, 1965; Young and Spizizin, 1963; Weidel *et al.*, 1963; Mitchell and Moyle, 1957). At the other extreme, a number of enzymes capable of hydrolyzing different bonds within wall peptidoglycan fragments have been isolated from *E. coli* and their properties determined (Pelzer, 1963a,b; Maas *et al.*, 1964; Weidel and Pelzer, 1964). However, information concerning the ability of these enzymes to dissolve either whole walls or undegraded peptidoglycan of *Escherichia coli* does not seem to be available. With the exception of the autolytic system of a transformable strain of *B. subtilis* (Young *et al.*, 1963, 1964, 1966), all information is either vague and descriptive of autolysis on a cellular level or complex in that a series of enzymes may be involved. Autolysis of *B. subtilis* appears to be due solely to an enzymatic hydrolysis of the amide linkage between muramic acid and L-alanine (Young *et al.*, 1964, 1966).

The autolytic system of *S. faecalis* seems to be of particular interest since activity is maximal during the exponential growth phase or, more specifically, toward the end of the exponential growth phase (Shockman, 1965). Thus, a somewhat inconsistent situation was found. When cells are synthesizing cell substance and dividing at their most rapid rate, they are most prone to autolysis. This, and several other observations, led to the hypothesis that the capacity of cells to autolyze was related to and perhaps required for cell (wall)

growth and division. In fact, we have previously suggested that "lysis is caused by a wall-lytic enzyme, which in normal growth may function in the opening of bonds in the hollow mucopolysaccharide peptide structure of the cell wall, in order to permit enlargement of the structure. While normal growth would involve a harmonious balance between the action of wall-lytic and wall-synthesizing enzymes, the lytic action would become dominant when wall synthesis is prevented . . ." (Toennies and Shockman, 1958). For such a function the autolysin would have to cleave the proper bond in the wall structure. Evidence has been obtained in two different laboratories, with cell-free systems from *S. aureus* and *M. lysodeikticus*, that one of the later stages in peptidoglycan synthesis is the formation of a membrane-bound *N*-acetylglucosamine-*N*-acetylmuramic acid pentapeptide derivative (Chatterjee and Park, 1964; Park, 1965; Anderson *et al.*, 1965). In order to insert this disaccharide peptide, perhaps into the middle of a linear polysaccharide chain, the glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine would need to be cleaved. In other words, the autolysin from *S. faecalis* seems to be splitting the correct bond for an involvement in wall biosynthesis. Under balanced growth conditions one would assume that the enzyme is closely controlled, perhaps by structural location as well as by activity and amounts synthesized, and closely linked to the biosynthetic system.

The increased rate of autolysis of log walls in the presence of proteolytic enzymes (Figure 1 and Table II) raises several interesting questions. The increased rate appears to be due to activation or release of the endogenous wall-bound lysin. The slow rate of log wall autolysis could be due to the location of the autolysin only at certain selected sites, such as the bands of new wall growth seen by the immunofluorescence technique (Cole and Hahn, 1962; Cole, 1965; Chung *et al.*, 1964). Release of soluble enzyme by trypsin could result in a less organized but more rapid dissolution process. Preliminary results with electron microscopy (in collaboration with Dr. Martha Rancourt) indicate that log wall autolysis occurs by a process consonant with the bands of new wall synthesis. During wall autolysis not only were hemispheres of walls seen (Mitchell and Moyle, 1957), but these progressively decreased in width, leaving toward the end of autolysis only end caps and slices of wall. These observations need to be confirmed and extended. The activation of autolysin by a proteolytic enzyme(s) certainly emphasizes the necessity of care in isolating the cell wall structure from whole cells (Weidel *et al.*, 1963), especially when proteolysis is used to remove cytoplasmic contaminants. The absence of lysis of walls from Val or Thr cells or of SDS-treated log walls in the presence of trypsin, plus, in the last case, the lack of an increase in amino-terminal groups (Figure 6) indicate that trypsin is not acting directly upon the peptide portion of the wall peptidoglycan, but that its action is indirect. It seems likely that the results obtained by Montague (1964) and Bleiweis and Krause (1965) were probably due to the activation or release of an autolysin.

The necessity to obtain dissolution of walls to obtain a soluble autolysin has been taken to indicate a close association of the autolysin with the cell wall. The relatively large amounts of autolysin obtained from cell walls as compared with the weakly lytic activity of the cytoplasm supports the wall location of the enzyme. Over 85% of the autolytic activity of log cells was found to be associated with the wall. Until recently walls of Gram-positive bacteria were thought to be devoid of enzymatic activities (Salton, 1964). However, unless walls have been treated with proteolytic enzymes relatively small amounts of "non-wall" amino acids are usually found (Salton, 1964; Toennies *et al.*, 1959). In walls of *S. faecalis*, amino acids other than lysine, alanine, glutamic and aspartic acids account for about 3% of the dry weight (Toennies *et al.*, 1959). Struve *et al.* (1966) obtained evidence for the presence of a 5′-nucleotidase in walls of *S. aureus* Copenhagen. The nucleotidase was not removed by washing walls with 4×10^{-3} M Triton X-100. Washing of *S. faecalis* walls with Triton X-100 does not affect their subsequent autolysis and as much as 0.01 M did not inhibit the action of the trypsin-free autolysin on SDS-treated log walls.

Although the autolysin cleaves the lysozyme-sensitive bond in the wall peptidoglycan substrate, it differs from egg white lysozyme in a number of respects. (1) While the autolysin is more active on walls of log phase *S. faecalis* than it is on walls of *M. lysodeikticus*, lysozyme is more active on walls of *M. lysodeikticus*. (2) Their action on the "mucopeptide residue" remaining after TCA extraction of both *S. faecalis* and *M. lysodeikticus* walls differs in that lysozyme dissolved these more rapidly while autolysin acted more slowly (Figure 9). Although TCA may not completely remove wall teichoic acid and rhamnose polysaccharide, these observations suggest that the presence of either or both of these polymers inhibits lysozyme, but favorably affects the activity of the autolysin. (3) Autolysin and lysozyme differ in ionic optima. (4) The zero-order kinetics seen with the autolysin on *S. faecalis* walls (Figure 8) differs from egg white lysozyme digestion of walls of either *S. faecalis* or *M. lysodeikticus*. The kinetics also differ from the second-order kinetics reported by Prasad and Litwack (1963) for the actions of egg white, rat kidney, and *Bacillus subtilis* lysozymes on *M. lysodeikticus* cells. The autolysin of a transformable strain of *B. subtilis* has been shown by Young (1966) to follow first-order kinetics. The *B. subtilis* autolysin also differs in that it is an amidase (Young, 1966). (5) The presence of *O*- and *N*-acyl groups affected lysozyme and autolysin action somewhat differently (Table III and Figure 8). The most striking of these effects was the greatly decreased sensitivity of *M. lysodeikticus* walls to autolysin, but an increased sensitivity to lysozyme, after acetylation followed by de-*O*-acylation (Figure 8). Similar treatment resulted in only a relatively small change in the sensitivity of *S. faecalis* walls to either autolysin

or lysozyme. Acylation with acetic anhydride also blocks free amino groups (Perkins, 1965) and free ε-amino groups of lysine are relatively frequent in walls of *M. lysodeikticus* (Salton, 1961). It is, therefore, possible that the nature and degree of cross-linking of the peptide portion of the peptidoglycan could affect autolysin sensitivity. (6) Although we have not made a direct comparison with the products of lysozyme digestion, the products of autolysis of log walls of *S. faecalis* are not dialyzable. Dialysis before application of lysates to Bio-Gel P-60 columns resulted in the same profile of acetylamino sugars, using the phosphate elution system (Figure 3). Fraction III was excluded from Bio-Gel P-10, but was resolved into two non-excluded components on Bio-Gel P-30, suggesting a molecular weight of between 10,000 and 25,000.

The resistance of walls from several bacterial species and the relative resistance of walls from stationary-phase Val or Thr *S. faecalis* or of *M. lysodeikticus* to autolysin remains to be explained. This degree of resistance is not nearly sufficient to explain the failure of Val or Thr cells or walls to autolyze. The 25,000*g* supernatant fraction from disrupted Thr cells did contain low levels of wall lytic activity. What appears to be a relatively rapid increase in resistance to cellular autolysis after the termination of exponential growth (Shockman, 1965) also remains to be explained.

Acknowledgments

We thank Dr. M. Prince Brigham and Mr. Earl Pearson of this institution for performing the amino acid analyses, Mr. Paul Lovett of this laboratory for the *M. lysodeikticus* walls, Dr. F. Neuhaus of Northwestern University for suggesting the plastic beads for cell disruption, Dr. M. Leyh-Bouille for the specific determination of *N*-acetylglucosamine, and Dr. J.-M. Ghuysen for kindly supplying standards of *N*-acetylmuramic acid and the *N*-acetylglucosamine-*N*-acetylmuramic acid disaccharide and for his hospitality to the senior author during the final stages of this work.

References

Abrams, A. (1958), *J. Biol. Chem. 230*, 949.

Allen, S. H. G., Kellermeyer, R. W., Stjernholm, R. L., and Wood, H. G. (1964), *J. Bacteriol. 87*, 171.

Anderson, J. S., Matsuhashi, M., Haskin, M. A., and Strominger, J. L. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 881.

Armstrong, J. J., Baddiley, J., Buchanan, J. G., Davison, A. L., Keleman, M. V., and Neuhaus, F. C. (1959), *Nature 184*, 247.

Bleiweis, A. S., and Krause, R. M. (1965), *J. Exptl. Med. 122*, 237.

Boltz, D. F., and Mellon, M. G. (1947), *Anal. Chem. 19*, 873.

Brumfitt, W. (1959), *Brit. J. Exptl. Pathol. 40*, 441.

Brumfitt, W., Wardlaw, A. C., and Park, J. T. (1958), *Nature 181*, 1783.

Chatterjee, A. N., and Park, J. T. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 9.

Chung, K. L., Hawirko, R. Z., and Isaac, P. K. (1964), *Can. J. Microbiol. 10*, 473.

Cole, R. M. (1965), *Bacteriol. Rev. 29*, 326.

Cole, R. M., and Hahn, J. J. (1962), *Science 135*, 722.

Conover, M. J., Thompson, J. S., and Shockman, G. D. (1966), *Biochem. Biophys. Res. Commum. 23*, 713.

Davison, A. L., and Baddiley, J. (1963), *J. Gen. Microbiol. 32*, 271.

Dische, Z., and Shettles, L. B. (1948), *J. Biol. Chem. 175*, 595.

Ganrot, P. O. (1966), *Acta Chem. Scand. 20*, 175.

Ghuysen, J.-M., and Strominger, J. L. (1963), *Biochemistry 2*, 1119.

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

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

Lominski, I., and Gray, S. (1961), *Nature 192*, 683.

Lowry, O. H., and López, J. A. (1946), *J. Biol. Chem. 162*, 421.

Lowry, O. H., Rosebrough, N. J., Farr, A. J., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Maas, D., Pelzer, H., and Weidel, W. (1964), *Z. Naturforsch. 19b*, 413.

Mitchell, P., and Moyle, J. (1957), *J. Gen. Microbiol. 16*, 184.

Mohan, R. R., Kronish, D. P., Pianotti, R. S., Epstein, R. L., and Schwartz, B. S. (1965), *J. Bacteriol. 90*, 1355.

Montague, M. D. (1964), *Biochim. Biophys. Acta 86*, 588.

Muñoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J. F., Heymann, H., Bricas, E., and Lefrancier, P. (1966a), *Biochemistry 5*, 3748.

Muñoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J. F., and Tinelli, R. (1966b), *Biochemistry 5*, 3091.

Park, J. T. (1965), Abstracts, 149th National Meeting of the American Chemical Society, Detroit, Mich., April, p 22C.

Park, J. T., and Griffith, M. (1964), *Proc. 3rd Intern. Symp. Flemings Lysozyme.*

Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 181*, 149.

Pelzer, H. (1963a), *Z. Naturforsch. 18b*, 950.

Pelzer, H. (1963b), *Z. Naturforsch. 18b*, 956.

Perkins, H. R. (1960), *Biochem. J. 74*, 186.

Perkins, H. R. (1965), *Biochem. J. 95*, 876.

Prasad, A. L. N., and Litwack, G. (1963), *Anal. Biochem. 6*, 328.

Prasad, A. L. N., and Litwack, G. (1965), *Biochemistry 4*, 496.

Primosigh, J., Pelzer, H., Maas, D., and Weidel, W. (1961), *Biochim. Biophys. Acta 46*, 68.

Ross, J. W. (1963), *Appl. Microbiol. 11*, 33.

Salton, M. R. J. (1961), *Biochim. Biophys. Acta 52*, 329.

Salton, M. R. J. (1964), The Bacterial Cell Wall, New York, N. Y., Elsevier.

Salton, M. R. J., and Ghuysen, J.-M. (1960), *Biochim. Biophys. Acta 45*, 355.

Shockman, G. D. (1962), *Biochim. Biophys. Acta 59*, 234.
Shockman, G. D. (1963a), *in* Analytical Microbiology, Kavanagh, F., Ed., New York, N. Y., Academic, p 567.
Shockman, G. D. (1963b), *Trans. N. Y. Acad. Sci. 26*, 182.
Shockman, G. D. (1965), *Bacteriol. Rev. 29*, 345.
Shockman, G. D., Conover, M. J., Kolb, J. J., Phillips, P. P., Riley, L. S., and Toennies, G. (161), *J. Bacteriol. 81*, 36.
Shockman, G. D., Kolb, J. J., and Toennies, G. (1957), *Biochim. Biophys. Acta 24*, 203.
Shockman, G. D., Kolb, J. J., and Toennies, G. (1958), *J. Biol. Chem. 230*, 961.
Stolp, H., and Starr, M. P. (1965), *Ann. Rev. Microbiol. 19*, 79.
Strominger, J. L., Park, J. T., and Thompson, R. E. (1959), *J. Biol. Chem. 234*, 3263.
Struve, W. G., Sinha, R. K., and Neuhaus, F. C. (1966), *Biochemistry 5*, 82.
Tipper, D. J., Ghuysen, J.-M., and Strominger, J. L. (1965), *Biochemistry 4*, 468.
Toennies, G., Bakay, B., and Shockman, G. D. (1959), *J. Biol. Chem. 234*, 3269.
Toennies, G., and Gallant, D. L. (1949), *Growth 13*, 7.
Toennies, G., and Kolb, J. J. (1951), *Anal. Chem. 23*, 823.
Toennies, G., and Shockman, G. D. (1958), *Proc. 4th Intern. Congr. Biochem. 13*, 365.
Weidel, W., Frank, H., and Leutgeb, W. (1963), *J. Gen. Microbiol. 30*, 127.
Weidel, W., and Pelzer, H. (1964), *Advan. Enzymol. 26*, 193.
Young, F. E. (1966), *J. Biol. Chem. 241*, 3462.
Young, F. E., and Spizizin, J. (1963), *J. Biol. Chem. 238*, 3126.
Young, F. E., Tipper, D. J., and Strominger, J. L. (1964), *J. Biol. Chem. 239*, PC3600.

The Relative Rates of Formation and Hydrolysis of the Thiosemicarbazones of Some Steroid Δ^4-3-Ketones*

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ABSTRACT: The rates of formation and hydrolysis of the thiosemicarbazones of a number of Δ^4-3-keto steroids and one saturated 3-keto steroid have been measured. They are found to depend on structural features of the steroids at points remote from the site of the reaction (long-range effects).

The rate-determining step in the formation and hydrolysis of the derivative is considered to be that in which carbon atom 3 changes from trigonal to tetrahedral.

There have been many studies of the variations in the rates of enzymic reactions with a number of structurally related steroid substrates (Talalay and Marcus, 1956; Langer *et al.*, 1959; Joshi *et al.*, 1963). However, only recently has attention been directed to the possible differences in intrinsic reactivity, other than isotope effects, of the functional groups undergoing the reaction (Joshi *et al.*, 1963; Ringold *et al.*, 1964a; Ringold *et al.*, 1966).

The present study was designed to discover if the reactivity of the Δ^4-3-ketone grouping toward thiosemicarbazide is affected by variation in the structure of the steroid molecule. Such differences in reaction rates may serve for comparison with kinetic studies of substrate specificity of enzymic reactions at the 3-position.

Experimental Section

Materials and Methods. Ethanol (95%) was refluxed with *m*-phenylenediamine for 2 hr and distilled, collecting the fraction distilling at 78 ± 0.5°. To the distillate was added a small amount of thiosemicarbazide and *p*-toluenesulfonic acid, the solution was redistilled, and the fraction boiling at 78 ± 0.5° was collected

* From the John Collins Warren Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02114. *Received December 14, 1966.* This is publication No. 1289 of the Cancer Commission of Harvard University. This investigation was supported by Public Health Service Grant No. CA02421-12 and CA01393-16 from the National Cancer Institute and Grant No. P95H from the American Cancer Society, Inc. A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists, Chicago, Ill., April 1967.

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