Bacteriolytic Enzymes from *Staphylococcus aureus*

SPECIFICITY OF ACTION OF ENDO- β -N-ACETYLGLUCOSAMINIDASE

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The bacteriolytic enzyme with an isoelectric point of 9.5 that is produced by all strains of *Staphylococcus aureus* investigated was purified from strain M18 (Wadström & Hisatsune, 1970). This enzyme released reducing groups from cell walls of *Micrococcus lysodeikticus* and was thus shown to be a bacteriolytic hexosaminidase. Although dinitrophenylation and acid hydrolysis of cell walls hydrolysed by a partially purified enzyme gave DNP-alanine and DNP-glycine from staphylococcal peptidoglycan, which indicated the presence of a peptidase and probably also an *N*-acetylmuramyl-L-alanine amidase, hydrolysis of cell walls by the extensively purified enzyme did not give any DNP-amino acids. The enzyme digest was purified by Amberlite CG-120 and Sephadex G-10 chromatography. Reduction by sodium borohydride of the disaccharide obtained was followed by acid hydrolysis and paper chromatography. Glucosamine completely disappeared after this treatment and a new spot identical with glucosaminitol appeared. The muramic acid spot remained unchanged. The purified enzyme was found to be devoid of exo- β -N-acetylglucosaminidase activity. These results are compatible with the action of a bacteriolytic endo- β -N-acetylglucosaminidase. It is also proposed that this enzyme is probably identical with the staphylococcal lysozyme. The mode of action of this has not previously been investigated.

In the preceding paper (Wadström & Hisatsune, 1970) the purification of an extracellular hexosaminidase from *Staphylococcus aureus* strain M18, was described. Crude supernatants from several strains, cultivated for purification and characterization of a variety of extracellular proteins produced by pathogenic staphylococci (*S. aureus*), showed that all strains produced bacteriolytic activity. Preliminary work (Wadström & Hisatsune, 1968) showed that an alkaline enzyme with an isoelectric point of 9.5 ± 0.1 degraded cell walls from *Micrococcus lysodeikticus* and *S. aureus* strain 3528, as well as peptidoglycan of the same staphylococcal strain. The degradation products reacted as *N*-acetyl amino sugars and possessed free reducing groups. These results seemed to be compatible with the action of mucopeptide *N*-acetylmuramyl-hydrolase, i.e. endo- β -N-acetylmuramidase or lysozyme (EC 3.2.1.17), or with the action of a bacteriolytic glucosaminidase. Such an enzyme is known to be produced by strains of *Staphylococcus epidermidis* (Schindler & Schuhardt, 1964). It is also a constituent of the preparation of lysostaphin from strain K-6W1 (Browder, Zygmunt, Young &

Tavormina, 1965). Tipper (1969) and Korman, Lis & Sharon (1970) reported the existence of an endo- β -N-acetylglucosaminidase in the autolytic enzyme system of *S. aureus* strain Copenhagen and strain HS 968. In both cases, however, very low activity was found in the extracellular fluid. The extracellular lysozyme, produced by *S. aureus*, has not been extensively purified, and thus not characterized physicochemically or according to its mode of action (for references see Wadström & Hisatsune, 1970). The aim of this investigation was to analyse the products of cell wall degradation after hydrolysis with the purified enzyme to reveal its mode of action, substrate specificity and bacteriolytic spectrum. Experiments were usually also performed with hen's-egg-white lysozyme, which is the most thoroughly investigated bacteriolytic hexosaminidase (Chipman & Sharon, 1969). The comparatively high molecular weight, instability on purification and great sensitivity to EDTA and heavy metal ions seemed not to be compatible with a lysozyme (Jollés, 1964, 1967; Chipman & Sharon, 1969; Howard & Glazer, 1969).

MATERIALS AND METHODS

Materials. A detailed description was given by Wadström & Hisatsune (1970). Lysozyme, trypsin (EC 3.4.4.4), *p*-nitrophenyl β -N-acetylglucosaminide,

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DNP-amino acids, muramic acid, *N*-acetylglucosamine and *M. lysodeikticus* whole cells (freeze-dried) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. and Amberlite CG-120, type II, lot 4280, was from Rohm and Haas, Philadelphia, Pa., U.S.A. Bovine serum albumin was a gift from KABI, Stockholm, Sweden. T.l.c. was carried out on Eastman chromatogram sheets, type K 301R, silica gel, from Eastman Distillation Products Industries, Rochester, N.Y., U.S.A. Sephadex and Dextran Blue 2000 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Cell-wall digests were dialysed in 23/32 tubing (Visking; Union Carbide, Chicago, Ill., U.S.A.).

Specimens of bacterial strains were kindly provided as follows: *Bacillus subtilis* strain 168 from Dr J. Jonasson; *Bacteroides necrophorus* from Dr A. Wahren; *S. epidermidis*, *Clostridium butyricum*, *Bacillus megaterium*, *M. lysodeikticus* N.C.T.C. 2665, *Bacillus cereus*, *Escherichia coli* B, 0111:B4, *Pseudomonas aeruginosa* and *Streptococcus pyogenes* were all kindly supplied by N.C.T.C., Colindale, London N.W.9, U.K. Whole cells or cell walls of *Lactobacillus acidophilus*, *Micrococcus roseus* R-27 (IP), *Sarcina lutea* R 262 (IP), *Micrococcus mucoruber*, *Micrococcus varians* and *Micrococcus flavus* were generously provided by Dr J.-M. Ghuyssen. Cell walls of *Mycobacterium tuberculosis* BCG (lot no. 06, Lederle) and cell walls of *Listeria monocytogenes* were kindly provided by Dr E. Ribí and cell walls of *Bordetella pertussis* by Dr P. Novotny. D-Glucose derivatives for inhibition experiments were kindly supplied by Dr A. Neuberger and Dr B. M. Wilson.

If not otherwise stated all chemicals were of analytical grade.

Cell growth. A high cell yield of all the bacterial species mentioned above was obtained in the modified CCY medium, CCY₁ (Arvidsson, Holme & Wadström, 1970) on shaking-tables overnight. The *Bacillus* species were grown in a glucose-broth medium, heavily inoculated, and harvested after 4 h. All cells were washed twice in 0.01 M-sodium phosphate buffer, pH 7.0, and then freeze-dried.

Preparation of cell walls. Cells of *M. lysodeikticus* and *S. aureus* strain 3528 were grown in a Biotec FL 103 fermentor (Biotec, Bromma, Sweden) in the CCY₁ medium to cell densities of 4–8 g dry wt./l. This medium was found to be superior to media previously described for cultivation of *M. lysodeikticus* (Smolelis & Hartsell, 1949). Active aeration and pH control were found necessary for a high yield. The harvested cells were washed twice with cold 0.01 M-sodium phosphate buffer, pH 7.2. Procedures for disintegration of cells by using a Braun homogenizer, model MSK (B. Braun Apparatebau, Melsungen, Germany), essentially followed the method of Bleiweis, Karakawa & Krause (1964) with a slight modification. The washed cells were diluted 1:3 (v/v) with 0.01 M-sodium phosphate buffer, pH 7.2, and 30 ml of the bacterial suspension was homogenized with 33 g of glass beads (diameter 0.1–0.12 mm) and 0.2 ml of tri-*n*-butyl phosphate in 75 ml stoppered shaking flasks. The temperature of the flasks was kept below 4°C by the continuous flow of an appropriate amount of CO₂ into the chamber. Disintegration for 2 min at 4000 oscillations/min was repeated twice for *M. lysodeikticus* and three times for *S. aureus* cells. The suspensions of disintegrated cells were separated from the glass beads by filtration through a glass filter. The filters

were rinsed with buffer, and the wash was combined with filtrates and diluted with buffer to a final volume of 300 ml. After homogenization with a Sorvall Omni-Mixer the suspensions were centrifuged at 4000 rev./min (Sorvall SS-34 rotor) for 10 min. The pellet was discarded and the supernatant was centrifuged at 10500 rev./min for 30 min to recover the crude cell-wall material in the pellet. The cell walls were successively subjected to the following treatments: washing with cold 1 M-NaCl, two digestions with trypsin (crystalline, Sigma grade III, 60 mg in 300 ml of 0.068 M-sodium phosphate buffer, pH 7.7, at 37°C for 1 h), washing with 0.068 M-phosphate buffer, pH 7.7, heating to 100°C for 10 min, washing with water twice, and the preparation was finally freeze-dried. The yield of cell walls from 6 g (dry wt.) of cells was approx. 450 mg.

Preparation of staphylococcal peptidoglycan. Cell walls of *S. aureus* strain 3528 (760 mg dry wt.) were suspended and extracted with 40 ml of 10% (w/v) trichloroacetic acid for 24 h at 4°C as described by Armstrong, Baddiley, Buchanan, Carss & Greenberg (1958). This volume was then centrifuged (13000 rev./min for 20 min; Sorvall SS-34 rotor), the pellet suspended in the initial volume of trichloroacetic acid and the whole procedure was repeated for another two periods of 24 h. The supernatants containing the cell-wall teichoic acids were collected and precipitated with acetone (Kato *et al.* 1962) and then freeze-dried. The pellet, i.e. the peptidoglycan fraction, was washed three times with water after the third and last extraction and freeze-dried. The yield was 170 mg of teichoic acid and 560 mg of peptidoglycan. The peptidoglycan preparation was essentially free from hot acid-extractable D-alanine.

Enzymic digestion of the cell walls and peptidoglycan. Hen's-egg-white lysozyme (0.9 mg) in 1 ml of 0.02 M-sodium phosphate buffer, pH 6.5, was added to the suspension of 150 mg of cell walls of *M. lysodeikticus* in 29 ml of the same buffer preincubated at 37°C for 30 min. The reaction mixture was shaken at 37°C and appropriate samples were taken at time-intervals for analyses. Cell walls of *M. lysodeikticus* and peptidoglycan of *S. aureus* strain 3528 were digested with 450 units of purified glucosaminidase (isoelectric point 9.5; 15 units/ml) under the same conditions.

Digests of cell walls of *M. lysodeikticus* and staphylococcal peptidoglycan were dialysed for 24 h against water. The diffusate, i.e. the diffusible fraction (DF) and the dialysis residue, i.e. the nondiffusible fraction (NDF), were collected and freeze-dried separately. The diffusible fractions were then subjected to chromatography on Amberlite CG-120 (DF CG-120) and Sephadex G-10 (DF CG-120, G-10).

Amberlite chromatography. Amberlite CG-120, type II, was washed and equilibrated as described by Hirs, Moore & Stein (1953). Columns (2 cm × 20 cm) were packed and the cell-wall saccharides were eluted with water. This material was freeze-dried, dissolved and chromatographed on Sephadex G-10.

Sephadex G-10 chromatography. Sephadex G-10 (fine grade; exclusion limit mol.wt. 700) was packed in columns (1.0 cm × 57.5 cm) and operated by downward flow at 4°C with water (0.8 ml/h). Fractions (2.5 ml) were collected and assayed for *N*-acetyl amino sugars (Morgan-Elson reaction; 30 min heating) and free amino groups. The void volume was 15 ml as determined by chromato-

graphy of Dextran Blue. Fractions positive for the Morgan-Elson reaction were freeze-dried.

Paper chromatography. The diffusible fraction treated as described above was hydrolysed in 4M-HCl (final concentration) for 3 h at 105°C and then chromatographed on Whatman 3MM paper (Shockman, Thompson & Conover, 1967). The following three solvent systems were used: (A) pyridine-water (4:1, v/v, ascending), (B) butan-1-ol-acetic acid-water (3:1:1, by vol., descending) and (C) butan-1-ol-pyridine-water-acetic acid (60:40:30:3, by vol., descending). Spots were made visible by dipping the dried chromatograms in 0.5% ninhydrin in acetone and heating at 70°C for 20 min.

N-Terminal group determination. A considerable release of free amino group was observed at the same time as the appearance of reducing power during the lytic action of the partially purified enzyme (isoelectric point 9.5) on the cell walls of *M. lysodeikticus*. Freeze-dried digests (6 mg) of the cell walls (5 mg of cell walls and 15 units of enzyme/ml, 0.02M-sodium phosphate buffer, pH 6.5, 10 h at 37°C) and the diffusible fraction (DF no. 1A and 1B, 6 mg) were subjected to dinitrophenylation by using 1-fluoro-2,4-dinitrobenzene and trimethylamine (Fraenkel-Conrat, Harris & Levy, 1955; Sanger & Thompson, 1953). The ether-extractable DNP-amino acid fractions were subjected to t.l.c. with the following solvent systems: (D) toluene-pyridine-ethyl chlorohydrin-0.8M-NH₃ (50:15:33:30, by vol.), (E) chloroform-methanol-acetic acid (95:5:1, by vol.), (F) chloroform-2-methyl butan-2-ol-acetic acid (70:30:3, by vol.) and (G) propan-1-ol-33% NH₃ (sp.gr. 0.85) (1:3, v/v).

Enzyme assays. In preliminary work a modification of the turbidimetric method described by Jollés (Jollés, 1962; Parry, Chandan & Shahani, 1965) was used as described by Wadström & Hisatsune (1970).

The glucosaminidase activity was assayed in three different ways: (1) by the linear increase of transmittance ($\Delta T_{640\text{nm}}^{0.1\text{cm}}/\text{min}$); (2) by the decrease in extinction ($\Delta E_{640\text{nm}}^{1.0\text{cm}}/\text{min}$); (3) by the determination of μmol of reducing groups released per unit time (Momose, Inaba, Mukai & Watanabe, 1960a,b). These methods were used in the same incubation mixture; whole cells, cell walls, or peptidoglycan suspended in a 3% solution of NaCl, were diluted (1:2, v/v) with 0.15M-tris-HCl buffer, pH 7.0. A 0.1 ml portion of the test specimen was mixed with 2 ml of this suspension ($E_{640}^{0.1\text{cm}}$ 0.8). If not otherwise stated, this incubation mixture was used for measurement of the linear decrease in extinction. One unit of enzyme activity is defined as the amount of enzyme in the test system required to give an initial linear decrease of 0.1 extinction unit/min at 37°C. This corresponds to the lytic activity obtained by 2 μg of hen's-egg-white lysozyme in 0.05M-tris-HCl buffer, pH 6.5. The relative bacteriolytic activity on different bacterial whole cells and cell walls is given in proportion to the reference system, a suspension of whole cells of *M. lysodeikticus*. All assays were carried out on a Beckman DB-G spectrophotometer.

If not otherwise stated, purified glucosaminidase (100 units/ml, sp. activity 1200 units/mg) was dialysed against 0.05M-sodium phosphate buffer, pH 6.5, and used for the characterization, as described in the Results section.

Inhibition by α - and β -2-acetamido-2-deoxy-D-glucoses. The turbidimetric method of Prasad & Litwack (1963) modified by Neuberger & Wilson (1967) was used. A

1.6 ml portion of a cell suspension containing 0.02M-tris-HCl buffer, pH 7.0, final concentration was pipetted into the cuvette, 1.0 ml of the inhibitor was added, and finally 0.1 ml of enzyme solution (50 units/ml).

The rate of lysis was recorded as $\Delta E_{450}/\text{min}$, and the percentage of inhibition was calculated from the linear part of the curve in the presence and absence of inhibitor. Assay controls were run without the addition of inhibitor. Hen's-egg-white lysozyme (final concentration 1.0 $\mu\text{g}/\text{ml}$) was inhibited to 50% by the three compounds in corresponding concentrations (Neuberger & Wilson, 1967).

Exo- β -N-acetylglucosaminidase. The enzyme was purified from pig epididymis (Findlay & Levvy, 1960) and diluted in 0.1% (w/v) bovine serum albumin for assay. Determination of the enzyme activity was performed as described by Levvy & Conchie (1966) by using *p*-nitrophenyl β -N-acetylglucosaminide as a substrate and measuring the released *p*-nitrophenol. Crude cell extract, crude culture supernatant and purified glucosaminidase (200 units/ml) were assayed at pH 4.4 and 6.5 in the same assay system.

Chemical and analytical methods. Reducing-sugar activity in the soluble fraction after enzymic hydrolysis of cell walls was determined by the method of Momose *et al.* (1960a,b) and free amino groups and *N*-acetyl amino sugar (Morgan-Elson reaction, 30 min heating) as described by Ghuyssen, Tipper & Strominger (1966). Amino sugar was determined as described by Belcher, Nutten & Sambrook (1954) after hydrolysis (2M-HCl at 100°C for 2 h) and was followed by evaporation of acid *in vacuo* in the presence of KOH and P₂O₅. Glucose was used as a standard for reducing-power determination, *N*-acetylglucosamine for *N*-acetyl amino sugar determination, glycine for free amino group determination, and glucosamine hydrochloride for the amino sugar reaction. The procedures of reduction of cell-wall digest with NaBH₄ were based on the method described by Ghuyssen *et al.* (1966). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) or by E_{280} measurements in a Zeiss PMQ spectrophotometer with bovine serum albumin as a standard.

RESULTS

Enzymic activity: lysis of *M. lysodeikticus* cells and cell walls. In primary work on isolation and characterization of staphylococcal glucosaminidase in crude culture supernatants and after isoelectric focusing, a modification of the method described by Jollés was used (Wadström & Hisatsune, 1968). After an initial lag phase, which varied for different samples of crude or partially purified enzyme, the rate of lysis was shown to be proportional to the enzyme concentration in about a fivefold dilution range. Comparisons between the initial increase in transmittance (T_{640}), the initial decrease in extinction (E_{640}), and the release of reducing groups on incubation of crude, partially purified (steps 1-3 and step 5; Wadström & Hisatsune, 1970) and purified glucosaminidase were made. Heat-stable inhibitors in crude material, probably derived from autolysed cell walls, were found to inhibit the

Fig. 1. (a) Time-course relationship of digestion of purified cell walls of *M. lysodeikticus* at 37°C with purified staphylococcal glucosaminidase (steps 1, 2, 3, 5 and 6; Wadström & Hisatsune, 1970) in 0.067M-sodium phosphate buffer, pH 6.5, at 37°C. At intervals, samples were removed and assayed for free amino and reducing groups and for *N*-acetylhexosamine. ○, Reducing power; ●, free amino groups; △, *N*-acetylhexosamine. (b) Digestion under the same conditions, but with an extensively purified glucosaminidase (steps 1-6; Wadström & Hisatsune, 1970); symbols as in (a). (c) Digestion under the same conditions, but with hen's-egg-white lysozyme (30 µg/ml); symbols as in (a).

activity of the purified enzyme. Thus the determination of activity in a crude supernatant gives an uncertain value due to the influence of inhibitor(s). No absolute linear relationship could be shown for crude material either. However, for purified glucosaminidase the determination of $\Delta E_{640}/\text{min}$ and the release of reducing groups gave a linear relationship with an enzyme diluted in an eightfold range with an accuracy of about $\pm 10\%$ and $\pm 3\%$ respectively. If not otherwise stated, the measurement of $\Delta E_{640}/\text{min}$ was used in all tests below. The initial decrease in extinction from an E_{640} of 0.8 was automatically recorded.

Mode of action. A specimen of cell walls of *M. lysodeikticus* (5mg/ml) was suspended in 0.067M-sodium phosphate buffer, pH 6.5, and incubated with 15 units of purified enzyme/ml in a total volume of 30ml. At intervals during incubation at 37°C samples were removed to measure the increase in transmittance and the release of free amino and reducing groups. As shown in Fig. 1, reducing groups and free amino groups were, in parallel with the increase in transmittance, rapidly and steadily liberated for 1h and then more

gradually for 3h. Reducing sugar ($3.5 \mu\text{mol/ml}$) and free amino groups ($5 \mu\text{mol/ml}$) were liberated under these experimental conditions, whereas the increase in released *N*-acetylhexosamine was very low.

Isolation of disaccharide from lytic products of the cell walls of *M. lysodeikticus*. The diffusible fraction of lysozyme digests of the cell walls of *M. lysodeikticus* is known to consist not only of disaccharide but also of tetrasaccharide and other large oligosaccharides (Salton & Ghuyssen, 1960; Perkins, 1960a,b; Mirelman & Sharon, 1967). An attempt to isolate pure disaccharides from the digests was made by using Amberlite CG-120 ion-exchange resin and Sephadex G-10 (Scheme 1). Cell walls (1.5g) of *M. lysodeikticus* were digested at 37°C with 5500 units of partially and extensively purified glucosaminidase (isoelectric point 9.5) respectively in 65ml of 0.05M-ammonium acetate buffer, pH 6.5, containing bovine serum albumin (0.5mg/ml) as a stabilizer. After incubation for 20h with shaking, the digests were dialysed against 200ml of water at 2°C for 24h and the dialysis residue was freeze-dried. The dialysis was repeated three times and the diffusible fractions were freeze-

dried and pooled. Ammonium acetate remaining was removed by repeated evaporation *in vacuo* in the presence of fresh concentrated sulphuric acid and phosphorus pentoxide. The yield of the diffusible fraction (DF no. 1) was 560mg after digestion with the partially purified enzyme (DF no. 1A) and 280mg after digestion with the extensively purified enzyme (DF no. 1B).

Scheme 1. Purification of disaccharide from cell walls of *M. lysodeikticus* digested with purified staphylococcal glucosaminidase.

To separate the disaccharide components from other free amino group components, 330mg of DF no. 1A and 280mg of DF no. 1B were subjected to treatment with Amberlite CG-120. Elution with 450ml of water (DF, CG-120) yielded 150mg of material for DF no. 1A and 60mg for DF no. 1B. The total amount of DF no. 1A CG-120 and DF no. 1B CG-120 was dissolved in 2ml of water and applied to a Sephadex G-10 column. The Morgan–Elson-positive material (30min heating) was entirely eluted in the included volume (Figs. 2a and 2b). Free amino groups were also detected in the same fractions. No positive amino sugar reaction was observed for the hydrolysates (2M-hydrochloric acid final concentration, 3h at 105°C) of each fraction from the void volume, whereas a strong reaction was observed in those from the included volume. This indicates that oligosaccharides that are not sensitive to the Morgan–Elson reaction, and consequently might not be detected with this reaction, were not present in the fractions from the void volume. The amino sugar components of DF no. 1A and 1B should be almost entirely disaccharide, since the molecular weight of the disaccharide, i.e. *N*-acetylmuramyl-*N*-acetylglucosamine, is 497. Fractions positive for the Morgan–Elson reaction from the included volume were freeze-dried (DF CG-120 and G-10). The yield was 128mg dry wt. for DF no. 1A and 55mg dry wt. for DF no. 1B.

Characterization. Paper chromatography of acid hydrolysates of DF no. 1A CG-120 and G-10 showed only two amino sugar components, glucosamine and muramic acid, and only traces of amino acids were

Fig. 2. (a) Sephadex G-10 gel filtration of a digest of cell walls of *M. lysodeikticus* with a partially purified glucosaminidase (see Fig. 1). The sample (83mg in 2ml of water) was applied on the column (1cm × 57.5cm) and eluted with water at the flow rate of 4.8ml/h at 4°C. Samples (1.4ml) of the eluent were collected. ●, *N*-acetylaminosugar; ○, free amino groups. (b) Sephadex G-10 gel filtration after digestion with an extensively purified glucosaminidase (see Fig. 1); symbols as in (a).

observed. These acid hydrolysates were also found to be strongly positive both for the reducing sugar and for the Morgan-Elson reaction without being hydrolysed. Quantitative sugar analysis showed that this fraction consisted entirely of glucosamine and muramic acid in 1:1 ratio. These results indicate that this fraction (DF, G-10) is the disaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid.

The reducing end of the DF no. 1B, CG-120, G-10 and the sugar component of the DF no. 1 were determined by reduction with sodium borohydride and then paper chromatographic examination of the acid hydrolysates. Samples DF no. 1B, CG-120, G-10 (2mg) and DF no. 1 (4mg) were reduced with 0.6ml of an unbuffered 0.1M-sodium borohydride solution. *N*-Acetylglucosamine, glucosamine and muramic acid (Sigma) were treated in the same way and used as controls. After 3h reaction at room temperature, 0.3ml of concentrated hydrochloric acid was added and the material was hydrolysed for 3h at 100°C. It was then evaporated to dryness in the presence of sodium hydroxide and phosphorus pentoxide. Unchanged samples and controls were treated in the same way. After paper chromatography, with three solvent systems (Shockman *et al.* 1967), unchanged samples showed strong spots of glucosamine and muramic acid by reaction with ninhydrin reagent. However, after reduction the glucosamine spot disappeared entirely and a new pinkish spot identical with the spot of glucosaminitol, the reduction product of glucosamine, appeared. This spot was easily identified by the slow development of its pinkish colour with ninhydrin reagent on the paper chromatogram. In contrast, the muramic acid spot remained unchanged, and no spot corresponding to muramitol was observed. On the chromatogram of DF no. 1A and 1B spots for amino acids were observed that were probably not of cell-wall peptide origin. However, only traces of amino acids were observed on the chromatogram of DF no. 1B, CG-120, G-10. These results show that glucosamine was at the reducing end of the sugar components of the DF no. 1. Thus, the hexosaminidase (isoelectric point 9.5) was characterized as an *N*-acetylglucosaminidase. The ratio between the Morgan-Elson colour after 30min of reaction and the liberated reducing power is tenfold higher after hydrolysis of the glucosidic bonds liberating muramic acid at the reducing end than *N*-acetylglucosamine (D. J. Tipper, personal communication). Thus, the results shown in Table 1 should also be compatible with the action of an endo- β -*N*-acetylglucosaminidase.

The cell-wall mucopeptide of *S. aureus* strain 3528 was also digested under the same conditions as used for the *M. lysodeikticus* cell walls. From 600mg of the staphylococcal mucopeptide as much as

Table 1. *Ratio calculated for the colour developed after Morgan-Elson reaction (30min heating) to reducing power*

The ratios of colour developed for *N*-acetylglucosamine-*N*-acetylmuramic acid and *N*-acetylmuramic acid-*N*-acetylglucosamine were kindly given by Dr D. J. Tipper.

Staphylococcal glucosaminidase:	
DF CG-120, G-10	0.01
Hen's-egg-white lysozyme:	
NDF staphylococcal peptidoglycan	0.1
<i>N</i> -Acetylglucosamine- <i>N</i> -acetylmuramic acid	0.25
<i>N</i> -Acetylmuramic acid- <i>N</i> -acetylglucosamine	0.02

460mg of the diffusible fraction was obtained after digestion with the partially purified enzyme. Subsequent Amberlite CG-120 treatment yielded 135mg of the material and after Sephadex G-10 gel filtration 115mg of the disaccharide fraction was obtained for the partially purified enzyme. This was also entirely eluted in the included volume. Considerable adsorption of material on Amberlite CG-120 resin was observed in this case. After digestion of 600mg of staphylococcal mucopeptide with the extensively purified enzyme, only a few mg of disaccharide was obtained after Amberlite and Sephadex chromatography.

N-Terminal group determination. T.l.c. was performed on the diffusible fraction after hydrolysis of *M. lysodeikticus* whole cells and staphylococcal mucopeptide with a partially purified (steps 1-3 and step 5) and extensively purified (steps 1-6) glucosaminidase (Table 1 in Wadström & Hisatsune, 1970). A lysozyme digest was used as a control. Only traces of DNP-alanine were observed in digests of lysozyme and the extensively purified glucosaminidase, although the partially purified preparation released *N*-terminal alanine in the digest.

Although the L- or D-configuration of the *N*-terminal alanine has not yet been established, this result suggests that the partially purified enzyme preparation may contain *N*-acetylmuramyl-L-alanine amidase activity and/or cell-wall peptidase activity which cleave the linkage between a lysine and a D-alanine residue of the cell-wall peptide. Release of *N*-terminal alanine and glycine was observed in the digestion of staphylococcal peptidoglycan with a partially purified enzyme, whereas only a trace of it was demonstrated after digestion by lysozyme or an extensively purified enzyme.

Inhibition experiments. Neuberger & Wilson (1967) have investigated the conditions for inhibition of hen's-egg-white lysozyme with some α - and β -derivatives of 2-acetamido-2-deoxy-D-glucose. The specificity of the inhibition by β -linked compounds was high. This is in accordance with the

β -(1 $\rightarrow$ 4)-linkage of the *N*-acetylglucosaminyl-*N*-acetylmuramic acid polymer in the cell-wall glycan. Staphylococcal glucosaminidase was also inhibited selectively about ten times more effectively by methyl 2-acetamido-2-deoxy- β -D-glucoside than by the corresponding α -derivative. This clearly shows that the enzyme should be classified as a β -*N*-acetylglucosaminidase.

Exo- β -N-acetylglucosaminidase. A crude cell extract and culture supernatant of strain M18 and purified glucosaminidase were all devoid of *exo- β -N-acetylglucosaminidase* activity in assays performed at pH 4.4 and 6.5. Control experiments were performed with *exo- β -N-acetylglucosaminidase* from pig epididymis under the same assay conditions.

Effect on whole cells and cell walls. Whole cells of

S. aureus strain 3528 and *M. lysodeikticus* were disintegrated to obtain cell walls (see the Materials and Methods section). The sensitivity and the accuracy of the test were investigated by using whole cells and cell walls (Table 2). The accuracy of the test in double experiments and at different dilutions of enzyme was not significantly increased ($\pm 10\%$) when cell walls were used.

Decrease in turbidity of suspensions of bacterial cells and cell walls was measured as described by Hash (1967). Staphylococcal cell walls, treated with cold 10% (w/v) trichloroacetic acid to remove teichoic acid, were found to aggregate spontaneously. Ultrasonic treatment (MSE Sonifier; amplitude 4–8 μ m; 20 s) was necessary to permit measurement of the decrease in extinction of a stable suspension.

Table 2. Survey of the lytic activity of purified glucosaminidase against various micro-organisms

If not otherwise stated, whole cells were used and controls without addition of enzyme were run. In no case was an autolytic activity so significant that heat-treated cells had to be used.

	Glucosaminidase (units/ml)*	Hen's-egg-white lysozyme (units/ml)†
<i>M. lysodeikticus</i> N.C.T.C. 2665‡	100	100
Cell walls	140	205
<i>B. subtilis</i> 168	300	105
<i>B. megaterium</i> KM	270	90
<i>Sarcina lutea</i> R 262	160	100
<i>M. mucoruber</i>	145	80
<i>S. epidermidis</i>	125	10
<i>S. aureus</i> strain Wood 46	80	5
<i>S. aureus</i> strain V8	20	0
<i>S. aureus</i> strain M18	10	0
Whole cells, strain 3528	40	0
Cell walls, strain 3528	80	5
Mucopeptide, strain 3528	35	0
<i>M. flavus</i>	15	10
<i>Lactobacillus acidophilus</i> cell walls	30	15
<i>B. cereus</i>	20	10
<i>Pseudomonas aeruginosa</i> N.C.T.C. 1999	20	5
<i>M. varians</i> N.C.T.C. 7281	15	0
<i>M. roseus</i> R-27	0	0
Cell walls	5	0
<i>Bordetella pertussis</i> cell walls	10	0
<i>Mycobacterium tuberculosis</i> BCG		
Cell walls	2	0
Cell walls, lipid free	5	0
<i>Streptococcus pyogenes</i> group A type 18	0	0
<i>E. coli</i> B	0	0
Cell walls	20	5
<i>Bacteroides necrophorus</i>	0	0
<i>Listeria monocytogenes</i> cell walls	0	0

* Expressed in assay units (E_{640} units/ml) according to the standard procedure of the turbidimetric method.

† The same conditions were used as footnote* except that 0.15 M-tris-HCl buffer, pH 6.5, was used.

‡ Cells suspended in 3% (w/v) NaCl were also heated in a boiling-water bath and afterwards used in the standard assay system were found to be a less susceptible substrate (75 units/ml for glucosaminidase).

In these experiments *M. lysodeikticus* was found to be more susceptible to lysis than *S. aureus* strain 3528 when experiments with whole cells and cell walls were compared. A complete clarification of *S. aureus* whole cells and cell walls was not obtained as for *M. lysodeikticus*, and the decrease in extinction did not exceed 70% of maximum lysis after 12 h of incubation.

The susceptibility of whole cells of several strains of *S. aureus* was also investigated. Strain Wood 46 was most sensitive, although whole cells of strain M18, the source of the purified glucosaminidase, were quite resistant.

Endo- β -*N*-acetylglucosaminidase was assayed on several Gram-positive and Gram-negative bacteria (Table 2). A similar amount of glucosaminidase and hen's-egg-white lysozyme (100 units/ml), calculated from lysis of *M. lysodeikticus* whole cells under standard assay conditions, was used in all these experiments to give an estimation of the specificity for different species and the relative specific activities. Ultrasonic treatment, as described above, was necessary to obtain stable suspensions of whole cells and cell walls of several of the species studied. Some *Bacillus* and micrococcal species were found to be most susceptible, whereas *M. roseus*, *Strep. pyogenes* and *E. coli* were quite resistant. The susceptibility of whole cells of *S. epidermidis* was greater than that of cells of *S. aureus*, and both species were quite resistant to the action of lysozyme. Thus it is apparent that there is a great difference between the specificity of this enzyme and lysozymes from hen's-egg white as well as bovine and human milk (Vakil, Chandan, Parry & Shahani, 1969) and the F1 endo- β -*N*-acetylmuramidase from *Streptomyces* (Munoz, Ghuysen, Leyh-Bouille, Petit & Tinelli, 1966).

Effect on peptidoglycan. After removal of teichoic acid and other polyelectrolytes by trichloroacetic acid extraction of cell walls of *S. aureus*, the susceptibility to the glucosaminidase decreased, whereas the resistance to hen's-egg-white lysozyme persisted unchanged as shown in Table 2.

DISCUSSION

A bacteriolytic *N*-acetylglucosaminidase that lyses intact cells of *M. lysodeikticus* was purified from a culture supernatant of *S. aureus* strain M18 (Wadström & Hisatsune, 1970). Two different glycosidic hydrolases (EC 3.2.1.-), i.e. muramidase and glucosaminidase, are able to dissolve bacterial cell walls by degradation of the glycan moiety of the cell-wall peptidoglycan found in nearly all bacterial cell walls hitherto investigated (Ghuysen, 1968). Lysozyme or muramidase, i.e. mucopeptide *N*-acetylmuramyl hydrolase (EC 3.2.1.17), shows *N*-acetylglucosaminidase activity toward chitin saccharides and certain synthetic substrates, but it

acts on the cell-wall glycan exclusively as an endo-*N*-acetylmuramidase, cleaving the *N*-acetylglucosaminyl-*N*-acetylmuramic acid polymer only at the glycosidic bond of the *N*-acetylmuramic acid residues (Chipman & Sharon, 1969). After enzymic digestion of *M. lysodeikticus* cell walls and reduction of the oligosaccharides formed by sodium borohydride, the disappearance of *N*-acetylglucosamine and the corresponding appearance of the reduction product glucosaminitol could be demonstrated. The staphylococcal hexosaminidase (isoelectric point 9.5) strain M18 was therefore characterized as an *N*-acetylglucosaminidase.

The only endo- β -*N*-acetylglucosaminidases hitherto reported are the streptococcal muralysin, a phage-induced lytic enzyme (Barkulis, Smith, Boltralik & Heymann, 1964), and a hexosaminidase found in a potent staphylolytic agent, lysostaphin (Browder *et al.* 1965), isolated from a strain of *S. epidermidis*, and also a cell-bound autolytic enzyme of *S. aureus* (Tipper, 1969; Korman *et al.* 1970). It is considered that the present investigation is the first on an extracellular endo- β -*N*-acetylglucosaminidase from *S. aureus*. Martin & Klemper also reported on an endo-*N*-acetylglucosaminidase from *Clostridium perfringens* lytic for cell-wall peptidoglycan of Gram-negative bacteria. This enzyme does not, in fact, attack the cell walls of *M. lysodeikticus* (Martin & Klemper, 1970) like the enzyme of *S. aureus* strain M18.

Endo- β -*N*-acetylglucosaminidases seem to be rarely found in nature and have not yet been listed with a systematic name and EC number (Florkin & Stotz, 1965). On the other hand, the exo- β -*N*-acetylglucosaminidase (β -2-acetoamido-2-deoxy-D-glycoside acetoamidodeoxyglycohydrolase or chitobiase, EC 3.2.1.29) is found in mammalian tissues, e.g. calf brain (Frohwein & Gatt, 1967), moulds, e.g. in *Dictyostelium discoideum* (Loomis, 1969), *Aspergillus niger* (Bahl & Agrawal, 1969), and many bacterial cells (Woollen, Heyworth & Walker, 1961; Woollen, Walker & Heyworth, 1961; Berkeley, Drozd & Broughall, 1969), but hitherto not in *S. aureus*. Neither the crude material nor the purified enzyme liberated a significant amount of *p*-nitrophenol from *p*-nitrophenyl β -*N*-acetylglucosaminide at pH 4.4 and 6.5 in the assay system for exo- β -*N*-acetylglucosaminidase (Levy & Conchie, 1966). Inhibition experiments in which structural analogues of *N*-acetylglucosamine were used (Neuberger & Wilson, 1967) showed that methyl 2-acetoamido-2-deoxy- β -D-glucoside has a more than five times stronger inhibitory effect on this enzyme than did methyl 2-acetoamido-2-deoxy- α -D-glucoside. These results taken together indicate that the enzyme is an endo- β -*N*-acetylglucosaminidase.

A strong correlation between extracellular lysozyme production and pathogenicity of staphylo-

cocci has been reported (Kashiba, Niizu, Tanaka, Nozu & Amano, 1959; Jay, 1966; Hawiger, 1968b). Lysozyme production was also interpreted as an aid for the identification of potentially pathogenic strains of staphylococci (Grossgebauer, Schmidt & Langmaack, 1968). The results reported raise the question of whether the extracellular lytic enzyme of pathogenic staphylococci that has been reported as lysozyme is a true muramidase or a glucosaminidase. Release of reducing power and material reacting as *N*-acetyl amino sugar from *M. lysodeikticus* whole cells (Richmond, 1959) and cell walls, and peptidoglycan from *S. aureus* strain Zak (Hawiger, 1968a) were the only evidences that a crude extracellular product (Richmond, 1959), or a partially purified extracellular enzyme, might be a lysozyme similar to hen's-egg-white lysozyme. This will consequently make re-examination of the enzymic properties of these lysozymes from different strains necessary, with special reference to their substrate specificity.

The isolated saccharides from the hydrolysed cell walls of *M. lysodeikticus* were purified by chromatography on Amberlite CG-120 and Sephadex G-10 (DF CG-120, G-10) and were considered to be entirely *N*-acetylmuramyl-*N*-acetylglucosamine disaccharide according to the following criteria: (1) glucosamine or the reducing end was entirely consumed by reduction with sodium borohydride leaving an unchanged muramic acid residue; (2) sugar determination showed that the fraction consists almost entirely of amino sugar, and almost no amino acids were demonstrated in hydrolysates by paper chromatography; (3) the fraction was eluted in the included volume of Sephadex G-10 chromatography. The exclusion limit is a molecular weight of 700, and the theoretical molecular weight of *N*-acetylmuramyl-*N*-acetylglucosamine is 497. Thus, chromatography on Amberlite CG-120 and Sephadex G-10 were considered to be suitable methods for the purification of disaccharides from the cell-wall digests. Hen's-egg-white lysozyme degrades the cell-wall glycan of *M. lysodeikticus* to di-, tetra- and octa-saccharides that are separable by ion-exchange chromatography (Sharon, 1967). They probably cause product-inhibition of the enzyme and retard further degradation of the cell-wall glycan and the oligosaccharides. Staphylococcal glucosaminidase was found to degrade the polyglycan into disaccharides alone. This has also been observed for the glucosaminidase in lysostaphin (Leyh-Bouille, Ghuysen, Tipper & Strominger, 1966). The *N*-acetylmuramyl-*N*-acetylglucosamine obtained by this procedure will be useful for future experiments with lysozymes and glucosaminidases to correlate the binding of various saccharides with the function of these enzymes (Chipman & Sharon, 1969).

Tipper (1969) has reported the existence of endo- β -*N*-acetylglucosaminidase in autolysed cell walls from *S. aureus* strain Copenhagen and no greater quantities were found extracellularly. The autolytic enzyme system of *S. aureus* strain Copenhagen (Tipper, 1969) and strain Oeding (Huff & Silverman, 1968; Huff, Silverman & Adams, 1969) contains acetylmuramyl-L-alanine amidase and an endopeptidase hydrolysing the pentaglycine bridge in the peptide moiety of the cell wall. The contaminating amidase and/or peptidase present in partially purified glucosaminidase were also found to be basic proteins perhaps identical with the enzymes of the strains Copenhagen and Oeding and two of the components in lysostaphin (Wadström & Vesterberg, 1970).

The sensitivity of different bacterial species to the purified enzyme was investigated. Table 2 shows that the purified glucosaminidase from strain M18 acts on many Gram-positive bacteria. *B. megaterium* and *M. lysodeikticus* were among the most susceptible organisms, although *B. cereus* and several Gram-negative rods were markedly insensitive both to the staphylococcal glucosaminidase and to hen's-egg-white lysozyme. *S. aureus*, *S. epidermidis* and *Strep. pyogenes* whole cells were degraded more slowly than *M. lysodeikticus*. These characteristics are similar to those of hen's-egg-white lysozyme and mammalian muramidase. The muramidase produced by the fungus *Chalaropsis* is more active on staphylococcal cell walls (Hash & Rothlauf, 1967).

This investigation has shown that the purified hexosaminidase from strain M18 has a mode of action different from an endo- β -*N*-acetylmuramidase, i.e. a lysozyme. Since the structure, mode of action and enzymic properties for several lysozymes from different sources have been extensively investigated, further characterization of this staphylococcal endo- β -*N*-acetylglucosaminidase is in progress to make further comparisons possible between a bacteriolytic muramidase and a glucosaminidase with similar specificities. Some of the enzymic properties of the staphylococcal enzyme, such as optimum pH for the stability and action, heat stability and influence of monovalent and divalent ions on the enzymic activity, have already been investigated and compared with those of hen's-egg-white lysozyme (Wadström, 1970).

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