Studies on the Bacteriolytic Activity of *Streptomyces albus*

Culture Filtrates. I. The Effect of Variations in Cultivation Conditions and the Screening of Various Enzyme Specificities

L. AKSNES and A. GROV

The University of Bergen, School of Medicine, The Gade Institute,
Department of Microbiology, N-5000 Bergen, Norway

Optimal conditions for production of bacteriolytic enzymes of *Streptomyces albus* G have been studied, and found to depend on stimulation and selection of strain, composition of medium, inoculum, and incubation. The culture filtrates lysed whole cells and mucopeptide/cell wall of *Staphylococcus aureus*, *Planococcus*, *Micrococcus luteus*, *M. conglomeratus*, *M. lyso-deikticus*, *Sarcina lutea*, and *Bacillus megaterium*. Results are presented to show that the lytic activity of the cultures is composed of an endo-*N*-acetylmuramidase, an *N*-acetylmuramyl-L-alanine amidase, several endopeptidases with specific activity on mucopeptide linkages, and caseinolytic activity. The specificities likely to be present are discussed in relation to apparently broken mucopeptide bonds.

Bacteriolytic enzymes have been isolated from a variety of sources including plants, animal tissues, and various microorganisms and, specially in the latter source, a great spectrum of specific lytic enzymes has been demonstrated.¹ Such enzymes, which are capable of solubilizing mucopeptide networks, the basic structure of all bacterial cell walls, belong to three classes: (1) Glycosidases or hexosaminidases which hydrolyse the polysaccharide (glycan) chains, (2) endo-peptidases, splitting the bonds within the peptides and their cross-links, and (3) acetylmuramyl-L-alanine amidases which cleave the junction between polysaccharides and peptides.¹⁻³ Proteolytic activity of soil microorganisms was discovered a long time ago,^{4,5} but the first systematic study was carried out by Welsch⁶ working with

Streptomyces albus strain G. Subsequent works,⁷⁻⁹ using fractional precipitation, ion exchange resin, and various test bacteria revealed that *Streptomyces* strains produce a complex system of lytic enzymes. This was confirmed by Ghuysen and collaborators (cf. Ref. 1) in their extensive work on the enzymes from *Streptomyces albus* G.

Bacteriolytic enzymes are of considerable interest in various fields; as potentially useful chemotherapeutics, in biochemical and genetical studies, and in the study of mucopeptide chemistry.¹

Streptomyces strains are reported to vary on cultivation in both growth and pigmentation¹⁰ as well as in the ability to produce lytic enzymes.^{7,11} Therefore, the aim of the present study was to examine the growth conditions and to select strains with high capability for synthesis of lytic enzymes.

MATERIALS AND METHODS

Chemicals. Bacto beef extract (B 126), bacto-peptone (B 118), special agar Noble, and trypsin were purchased from Difco Laboratories, Detroit, Mich., U.S.A.; hen egg-white lysozyme (3× crystalline) from Calbiochem. Inc., Los Angeles, Calif., U.S.A.; proteose peptone (code L46) and peptone bacteriological (L37) from Oxoid Ltd., London, England, and trypticase-soy from Baltimore Biological Laboratories, Baltimore, Md., U.S.A. Standard amino acids were obtained from Eastman Organic Chemicals Department, Rochester, N.Y., U.S.A.; dinitrophenyl(DNP)-amino acids from Nutritional Biochemicals Co., Cleveland,

Ohio, U.S.A.; casein, muramic acid, and glucosamine from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England; penta-glycine and ethylene-oxide from Fluka, CH-9470 Buchs, Switzerland, and Amberlite IRC-50 ion-exchange resin from the British Drug Houses Ltd., Poole, Dorset, England. Dialyzer tubing was purchased from A. H. Thomas Co., Philadelphia, Pa., U.S.A. Thin-layer plates (polygram Cel 300 and polygram sil N-HR) were from Machery, Nagel & Co., 516 Düren, BRD; Tabsorb (ethylene glycol adipate coated on chromosorb W), used as column material on gas chromatography, from Regis Chemical Co., Chicago, Ill, U.S.A., and trifluoroacetic acid anhydride from Pierce Chemical Co., Morton Grove, Ill, U.S.A. Trichloroacetic acid, 1-fluoro-2,4-dinitrobenzene (FDNB), diethylether, *p*-dimethylaminobenzaldehyde, acetic acid, chloroform, benzylalcohol, butanol, pyridine, ninhydrin, and all inorganic chemicals (reagent grade) were obtained from E. Merck, Darmstadt, BRD.

Organisms. *Streptomyces albus* G, *Staphylococcus aureus* Copenhagen (Sa), *Streptococcus pyogenes* A (Sp), *Planococcus* 2389 (Pc), *Micrococcus luteus* 144 (M-144), *Micrococcus conglomeratus* 84 (M-84), *Micrococcus lysodeikticus* NCTC 2665 (Ml), and *Sarcina lutea* (Sl) were from the laboratory stock of organisms at the Institute. *Bacillus megaterium* KM (Bm) was kindly provided by Dr. J.-M. Ghuyssen, Service de Bactériologie, Université de Liège, Belgium.

Media. The following media were made up to 1 l in deionized water: nutrient broth, pH 7.2, containing 3 g bacto-beef extract, 10 g bacto-peptone and 5 g NaCl. For cultivation of the halophilic *Planococcus* 60 g NaCl were added. Mucopeptide medium contained 2.5 g mucopeptide from Sa, 1 g K_2HPO_4 and 1 g $MgSO_4 \cdot 7H_2O$. Agar-mucopeptide medium contained in addition 10 g special agar Noble. The peptone media contained 10 g peptone, 1 g K_2HPO_4 , 1 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, and 2 g $NaNO_3$.

Growth and harvesting of the test-bacteria. The test-bacteria were grown aerobically on nutrient broth; 4 h inoculum (100 ml) was transferred to 900 ml fresh medium and incubated on a shaking machine at 37°C. The growth was followed spectrophotometrically at 600 nm on a Unicam SP 800 and the bacteria were harvested at the end of the exponential growth-phase, using a Sorvall RC-2 centrifuge (10 000 *g*). After washing in buffer of 0.15 M NaCl, 0.01 M phosphate, pH 7.2, the bacteria were stored at -25°C.

Heat-killed bacteria. Portions of test-bacteria were suspended in distilled water and placed in a boiling water bath for 15 min to denature autolytic enzymes,¹² then washed three times in water and freeze-dried.

Preparation of mucopeptide. Mucopeptides were prepared and analyzed as described earlier.¹³ To remove possible *O*-acetyl groups at the 6-position of muramic acid, the muco-

peptide preparations were further extracted for 20 min with 9 N NH_4OH at 20°C.¹⁴ After thorough washing with distilled water the mucopeptides were freeze-dried and examined by thin-layer and gas chromatography.

Preparation of cell wall. Isolation of mucopeptide from Bm was difficult since the bacteria clotted on suspension in trichloroacetic acid. Therefore, cell wall material was prepared from Bm according to the method used previously.¹⁵ As for mucopeptides, the cell wall was further trypsinated and extracted with 9 N NH_4OH , to remove *O*-acetyl groups¹⁴ and ester-linked *D*-alanine from teichoic acid,¹⁶ then washed thoroughly, freeze-dried and examined chromatographically.¹⁵

Ethylene-oxide treatment of mucopeptides/cell wall. To mask free amino groups of mucopeptide/cell wall preparations, a hydroxy-ethylation procedure described by Ghuyssen *et al.*³ was employed.

Analytical procedures

Thin-layer chromatography in one and two dimensions was performed on cellulose and silica plates in the following solvent systems: (A) Chloroform:benzyl alcohol:HAc (70:30:3, by vol.),¹⁷ (B) benzyl alcohol:chloroform:methanol: $H_2O:NH_3$ (25 %) (30:30:30:6:2, by vol.),³ (C) butanol:HAc: H_2O (4:1:1, by vol.),³ and (D) pyridine: H_2O (4:1, v/v).³ Ninhydrin¹⁸ and silver nitrate¹⁸ were used as colour reagents.

Gas chromatography of trifluoroacetylated amino acid butyl esters¹⁹ was carried out on a Perkin-Elmer 900 chromatograph fitted with flame ionization detector and a disc integrator. Tabsorb (80-100 mesh), in columns of 200 cm length and 2 mm inner diameter, was used as supporting material. The temperature was increased linearly (4°C/min) from 100°C to 200°C and was then held for 10 min at 210°C, the carrier gas (N_2) having a flow rate of 33 ml/min.

Tests for free groups. Estimation of the increase in free amino groups of enzymatically degraded mucopeptides/cell wall and the identification of *N*-terminal amino acids were carried out by the use of 1-fluoro-2,4-dinitrobenzene (FDNB) according to the description of Ghuyssen *et al.*² Soluble *N*-acetyl amino sugars were detected by the Morgan-Elson reaction² and the increase in reducing groups was measured by the method of Park and Johnson with modifications.^{3,20}

Reducing end-group determination. Samples of digested mucopeptide/cell wall, isolated oligosaccharides from preparative thin-layer chromatography and standard amino sugars (glucosamine and muramic acid) were reduced with $NaBH_4$.²¹ Reduced and hydrolyzed (3 N HCl for 4 h at 95°C in sealed tubes) preparations were desalted by extraction with pyridine and analyzed by thin-layer chromatography.

Selection of lytic strain and its cultivation. *Streptomyces albus* G was plated on agar-

mucopeptide medium and incubated at 28°C, separate colonies being then inspected. The diameter of the clearing zone around the colony, read after 4 days, was taken as a measure of the lytic activity. Colonies showing high lytic activity were replated on agar-mucopeptide medium every fourth day and incubated at 28°C. For production of enzymes the selected strain (a colony with high lytic activity) was replated once more on agar-mucopeptide medium, grown for 2 days and then transferred to 20 ml of the mucopeptide medium. After 30 h at 28°C on a shaker (Microid Flask Shaker, Griffin & Tatlock Ltd., London, England) operating at a rate of 50–60 rotations/min the inoculum was transferred to 1 l of proteose peptone medium and further incubated with shaking for 50–60 h.

Concentration and purification of enzymes by the use of Amberlite.^{14,22} After removal of mycelia by filtering through glass wool, the culture filtrate was mixed with one half its volume of distilled water and then 0.5 % (by weight) of Amberlite IRC-50 (H⁺) was added. After 1 h with stirring at 4°C, the pH of the suspension was adjusted to 5.0 with concentrated acetic acid and the stirring continued for 10 h. The ion-exchange resin was then isolated by centrifugation, washed in distilled water, resuspended in 10 % K₂HPO₄ (approx. 10 ml/g of dry resin) and, while stirring, 25 % NH₄OH was added until the pH was stable at 8.0. The suspension was further stirred for 5 h, centrifuged to remove the resin, and finally dialyzed against 0.01 M Tris-HCl pH 8.0. The dialyzed supernatant is further referred to as crude enzyme preparation (CEP).

Quantitative determination of mycelia. Duplicates of culture (20 ml each) were centrifuged at 3000 g. The mycelia were thoroughly washed in distilled water, dried at 90°C and weighed.

Test for bacteriolytic activity. Bacteriolytic activity was expressed as reduction in turbidity

of suspensions of bacteria or mucopeptide/cell wall compared to equal suspensions without enzyme. Heat-killed bacteria were washed twice in 0.01 M Tris-HCl buffer pH 8.0, and suspensions (0.5 ml) of bacteria and mucopeptide/cell wall in this buffer were adjusted to give a turbidity of 1.0 at 600 nm with 1.0 cm cell thickness after the addition of enzyme (0.25 ml). This corresponds to a cell concentration of about 5×10^8 cells/ml or a concentration of mucopeptides/cell wall of approximately 1 mg/ml. The suspensions were incubated at 37°C, the lytic activity, followed on a Unicam SP 800, being expressed as reduction in turbidity after 60 min (ΔT_{60}) when $\Delta T_{60} = (\Delta T_t/t) \times 60$. All measurements of activity were carried out before 50 % reduction in turbidity was reached.

Definition of bacteriolytic units. One lytic unit is defined as the activity, at the conditions described above, that gives a $\Delta T_{60} = 1.0$ in a suspension of Sa. Lytic units/ml is then referred to as $\Delta T_{60} \times 4$.

Test for caseinolytic activity. This was performed according to the method described by Petit *et al.*¹⁴ and expressed as reduction in turbidity after 60 min (ΔT_{60}) as for bacteriolytic activity.

EXPERIMENTS AND RESULTS

Substrates for lytic enzymes. The results of gas chromatographic examination of mucopeptides/cell wall with respect to amino acids are given in Table 1, showing a dominance of the amino acids characteristic of mucopeptides. The molar ratio of these amino acids (Table 2) and the absence of teichoic acids in the mucopeptide preparations, as found serologically by double diffusion in agar and chromatographically by

Table 1. Amino acid composition (mμmol/mg) of the mucopeptides/cell wall.

Amino acids	Mucopeptide of M-84	M-144	MI	Sa	Pc	Cell wall of Bm
Ala	1810	1070	1860	1080	720	1000
Gly	180	640	160	2430	210	60
Glu A	450	590	500	520	740	480
Lys	400	700	520	520	470	0
DAP	0	0	0	0	0	480
Val	20	40	60	60	240	0
Ile	70	30	50	50	210	0
Leu	50	100	120	80	370	30
Pro	100	20	30	30	160	0
Thr	60	40	40	50	160	0
Ser	90	20	20	100	100	0
Phe	0	40	50	80	150	0
Asp A	50	90	0	0	150	0
Tyr	0	0	0	30	0	0

Table 2. Molar ratios between the main amino acids of the mucopeptides/cell wall (Glu A = 1.0).

Amino acids	Mucopeptide of M-84	M-144	MI	Sa	Pc	Cell wall of Bm
Glu A	1.0	1.0	1.0	1.0	1.0	1.0
Ala	4.0	1.8	3.7	2.1	1.0	2.1
Gly	0.4	1.1	0.3	4.7	0.3	0.0
Lys	0.9	1.2	1.0	1.0	0.6	0.0
DAP	0.0	0.0	0.0	0.0	0.0	1.0

search for glycerol and ribitol,^{13,15} indicate that the preparations are satisfactorily pure.

Selection of lytic populations of Streptomyces albus G. Colonies with various lytic zones (0–0.5 cm diameter) appeared on agar-mucopeptide medium, each colony giving rise to new ones with variable lytic ability. However, upon further selection the extension of the highest lytic zones reached after 4 months a diameter of approximately 2.5 cm. There was no observable change in the maximal lytic activity of these colonies after 4 months, when the necessary replating and selections had been performed.

Influence of type of peptone on growth and enzyme production. Two litre flasks containing 0.5 ml of peptone medium made from various types of peptone were inoculated with 20 ml of *Streptomyces albus G* cultures and incubated with shaking at 28°C for 60 h. Five cultivations, a total of 5 × 4 flasks, were prepared for each peptone type, and the amount of mycelia and maximum lytic activity, measured on heat-killed cells of Sa and Bm, was determined. Considerable variations in lytic activity were observed within each peptone type and even in the flasks of the same cultivation. The variation in the amount of mycelia was small. Culture filtrates with low lytic activity appeared yellow-brownish in colour, whereas those with high activity were dark brown. The mean values of mycelia and lytic activity are listed in Table 3, indicating that the type of peptone apparently is of importance as far as lytic activity is concerned, proteose peptone being superior in this respect.

Effect of variation in the volume of inoculum. Three series of proteose peptone medium, each consisting of 0.25, 0.50, 0.75 and 1.0 l, were prepared in 2 l flasks, then inoculated with 20, 40 and 60 ml of culture and cultivated at 28°C

Table 3. The amount of mycelia and bacteriolytic activity in cultures of *Streptomyces albus G* using various types of peptone.

Type of peptone	Mycelia g/l	ΔT_{60} on Sa	ΔT_{60} on Bm
Bacto peptone	3.0	0.2	0.3
Proteose peptone	2.9	1.2	1.6
Peptone bacteriological	2.7	0.4	0.5
Trypticase Soy	2.6	0.2	0.3

Table 4. Bacteriolytic activity in cultures of *Streptomyces albus G* after direct inoculation and after preculture.

Inoculum	ΔT_{60} on Sa	ΔT_{60} on Bm
150 ml preculture	0.2	0.3
250 ml preculture	0.2	0.3
20 ml direct inoculum	1.2	1.6

with shaking for 60 h. The lytic activity in the different series, measured on heat-killed cells of Sa and Bm, showed rather small variations.

Effect of precultures on peptone medium. Precultures were prepared by transferring 20 ml inoculum to 150 and 250 ml of proteose peptone medium. After 15 h of incubation the precultures were used to inoculate 1 l portions (2 l flasks) of proteose peptone medium. The resultant lytic activities, taken as the mean values of two cultivations each of 4 flasks, are listed in Table 4, showing a remarkably lower activity after use of precultures than after a direct inoculum.

Growth and lytic spectrum. In accordance with the results obtained in the varied culture conditions, all further cultures were prepared using

Fig. 1. The amount of mycelia (---), bacteriolytic activity tested on Bm(1), Sa (3), Ml and Sl (4), M-144 and M-84 (5) and Sp (6), and caseinolytic activity (2) of a culture of *Streptomyces albus* G at various time intervals. Samples for testing were taken every fifth hour.

20 ml 30 h inoculum to 1 l proteose peptone medium in 2 l flasks, which were then incubated with shaking (50–60 rotations/min) at 28°C for 55 h.

Fig. 1 illustrates the growth (expressed as the amount of mycelia) and lytic activity (tested on all test-bacteria as well as casein) of a representative culture. Samples for testing were taken every fifth hour. Extracellular lytic activity was detected in the culture at the end of the exponential growth-phase and reached a maximum in the stationary phase of growth. Lytic activity was demonstrated by all test-bacteria, and the bacteriolytic as well as caseinolytic activities reached maximum at the same time interval. Although the total lytic activity varied from one culture to another, the ratios between the degree of lysis of different test-bacteria were found to be nearly constant. Solubilization of mucopeptides/cell wall with culture filtrates (taken at the time of maximum lytic activity) revealed smaller differences in the lytic spectrum compared to that tested on whole cells (Table 5). Mucopeptides were, however, rather variable as substrate for testing of lytic activities, probably due to heterogeneity with regard to molecular size. Incubation of

Table 5. Bacteriolytic activity of culture filtrate (at maximum) as measured on heat-killed bacteria and on mucopeptides/cell wall.

Bacteria	ΔT_{60} on heat-killed cells	ΔT_{60} on mucopeptide	ΔT_{60} on cell wall
Sa	1.8	1.2	—
M-144	0.5	0.7	—
M-84	0.5	0.9	—
Ml	0.9	0.6	—
Bm	3.0	—	1.7

ethylene-oxide-treated mucopeptides of Sa, M-144, M-84, Ml, and Pc and cell wall of Bm with the culture filtrate increased the amount of free amino groups and reducing groups in all samples, indicating the presence of both peptidases and hexosaminidases.

Lytic activities adsorbed to and eluted from Amberlite. Dialyzed eluate from the resin (CEP) lysed heat-killed bacteria and casein similar to the culture filtrate, indicating that all activities observed were adsorbed to and eluted from the resin. However, only about 50 % of the total bacteriolytic activity and 25 % of the caseinolytic activity were recovered. The remaining activity of the culture filtrate was not adsorbed by the addition of new resin. In spite of low recovery, a rapid concentration and marked purification of the enzymes were obtained. Thus, the absorbance at 280 nm of the culture filtrate (10× diluted) and CEP was 0.5 and 0.31, respectively. The ratios between degree of lysis on the various test-bacteria seemed similar for culture filtrate, for culture filtrate absorbed with Amberlite, and for CEP.

Samples of ethylene-oxide treated mucopeptides/cell wall (2 parts of 2 mg/ml) were digested with CEP (1 part) and egg-white lysozyme (1 part of 50 µg/ml), the free amino groups were traced with DNP and the mixtures then hydrolyzed. Chromatography of ether extracts in solvent system A and of water-saturated butanol extracts in system B revealed the amino acid derivatives listed in Table 6, which indicates that several types of linkages have been split on digestion with CEP.

Determination of hexosaminidase specificity. Bm cell wall (2 mg/ml in 0.01 M Tris-HCl, pH 8.0) was incubated with CEP at 37°C for 12 h.

Table 6. Dinitrophenyl-amino acids detected chromatographically after treatment of mucopeptides/cell wall with crude enzyme preparation (CEP) and egg-white lysozyme (E.L.).

	Mucopeptide ^a												Cell wall ^a	
	M-84 CEP	E.L.	M-144 CEP	E.L.	MI CEP	E.L.	Sa CEP	E.L.	Pc CEP	E.L.	Bm CEP	E.L.		
DNP-Ala	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DNP-Gly	—	—	—	—	—	—	+	+	+	—	—	—	—	—
DNP-Glu A	—	—	—	—	—	—	—	—	+	+	+	—	—	—
DNP- ϵ -Lys	—	—	+	+	+	+	—	—	—	—	—	—	—	—
DNP-DAP	—	—	—	—	—	—	—	—	—	—	—	+	+	+

^a + to +++: weak to strong colour reaction.

Samples of digested cell wall were reduced with NaBH₄, and both reduced and non-reduced samples were hydrolyzed and chromatographed in two dimensions using solvent systems C and D. A spot in the chromatogram of non-reduced samples corresponding to muramic acid was strongly reduced after treatment with NaBH₄, while a spot corresponding to muraminitol appeared on the chromatograms. The intensity of the spot corresponding to glucosamine remained unchanged and glucosaminitol was not detected in the reduced samples. This indicates that the only hexosaminidase present is an *N*-acetyl-muramidase.

Test on amidase activity. Mucopeptide of Sa (4 mg) was suspended in and digested with 2 ml (2 lytic units) of *N*-acetylglucosaminidase isolated from lysostaphin,²³ for 12 h at 37°C, pH 8.0. Undissolved material was removed by centrifugation and the supernatant further digested with CEP. Thin-layer chromatography in system C gave a ninhydrin and silver nitrate positive spot with an R_F of 0.25. Preparative chromatography in system C, elution of the material at R_F = 0.25, and chromatography of a 3 N HCl hydrolysate (4 h at 95°C) showed two spots corresponding to standard glucosamine and muramic acid. Neither in thin-layer nor gas chromatography could any amino acid be observed in 6 N HCl hydrolysate (18 h at 105°C) of a corresponding eluate. The presence of an oligosaccharide, free of amino acids, thus suggests that the CEP contains amidase activity in accordance with the observed free *N*-terminal alanine in digests of all mucopeptides/cell wall (Table 6).

Test on glycyl-glycine splitting. Treatment of penta-glycine (0.15 mg/ml) with CEP (enzyme/

substrate = 1/1 by vol., pH 8.0, 37°C for 12 h) was without effect as judged by free amino group determination and chromatography in system C.¹⁴

DISCUSSION

The selection of *Streptomyces albus* G gave colonies with increasing ability to produce bacteriolytic enzymes. However, the lytic activity of separate colonies varied after a number of successive selections. Without a continuous selection, the lytic activity of organisms from a colony with high activity diminished with time. This is in accordance with earlier observations on *Streptomyces albus* strains grown on nutrient broth.^{7,11} Consequently, continuous selection was found necessary to keep a stable lytic population, although this includes a risk of variation in the strain during the study. The experiment with precultures on peptone medium showed that a selected population rapidly loses its bacteriolytic activity on peptone medium. A direct inoculation from mucopeptide medium therefore seems necessary to obtain maximal lytic activity of the culture. The production of lytic enzymes apparently varied with type of peptone, this phenomenon not being explicable on the basis of the peptone composition.

The strain selections were performed on medium with mucopeptide from *S. aureus* Copenhagen, but the resultant CEP (Crude enzyme preparation) also lysed all the other test-bacteria. The lytic activity, however, varied considerably from one type of bacteria to another. This may be due to differences in mucopeptide structures, chemistry and/or thickness, and secondary effects of other com-

ponents present.^{3,24} Variations in the lytic spectrum of various cultures were not observed. Although the total activity varied, the ratios between activities on the different bacteria seemed nearly constant. This indicates that the production of lytic enzymes directed against different bacteria is intimately correlated. Of the characterized *Streptomyces* strains, only *Streptomyces albus* G^{6,11} is found to lyse all the test bacteria used in this study. In contrast to earlier observations on *Streptomyces albus* G²⁵ and *Streptomyces* S-35,²⁶ that lytic activity of the culture supernatant paralleled the amount of mycelia, the lytic activity in the present experiments appeared at the end of the exponential growth-phase. This may indicate that the lytic activity is due to endoenzymes released by autolysis.

The mucopeptide exhibits the mechanical strength of cell wall structures, and enzymatic hydrolysis of either the glycan chain or the peptide subunits (*cf.* Ref. 1) will usually cause lysis of the cell. The prepared mucopeptides/cell wall seemed pure and apparently suitable as substrates for enzymes as far as component composition and molar proportions are concerned (Tables 1 and 2). Except for a high content of alanine and small amounts of glycine in the *M. lysodeikticus* mucopeptide, the results of analyses of the other preparations agree fairly well with earlier reports. However, insoluble materials like these must be heterogeneous with respect to aggregation, content of natural free groups and groups set free during preparation and treatment.² Solubilization by enzymes¹² may reveal free groups not susceptible for reaction with reagents before digestion. The complex and macromolecular structure makes a complete masking of groups impossible.¹⁴ In spite of existent uncertainty, however, some suggestions as to the enzyme specificities of *Streptomyces albus* G culture may be made.

N-Acetylhexosaminidase, amidase, and endopeptidase activities have clearly been demonstrated, the latter activity most probably being composed of several enzymes. Since only muramic acid was found to be reduced by NaBH₄, the presence of only one hexosaminidase is likely, *i.e.* *N*-acetyl-muramidase. *M. conglomeratus* 84 has a high content of alanine¹³ and is comparable with *M. roseus* Thr—^{27,14}

having an alanine tripeptide bridge between the terminal D-alanine of one tetrapeptide chain and ϵ -lysine of an adjacent tetrapeptide. According to the results of digestion with CEP, followed by DNP-tracing, two linkages, D-alanyl-L-alanine and L-alanyl-L-alanine, may have been hydrolyzed. This corresponds to the activities of the earlier observed SA and MR endopeptidases.^{14,22} Also *Streptococcus pyogenes*, found to have an alanine dipeptide bridge,²⁸ is lysed by the same endo-peptidases as *M. roseus*.¹

The lack of activity of CEP on a pentaglycine preparation and the absence of detectable ϵ -DNP-lysine on examination of *S. aureus* mucopeptide digest indicate that, in the lysis of *S. aureus*, peptidase activity is mostly located to D-alanyl-glycine linkages.^{14,24} The tetrapeptides of *B. megaterium* KM mucopeptide contain diaminopimelic acid (DAP) instead of lysine and a direct linkage between D-alanine in one peptide to DAP in another is proposed.^{29,30} This linkage is apparently broken on digestion with CEP (Table 6), indicating the presence of the KM-endopeptidase activity,^{22,31} which has also been demonstrated in the culture filtrate of *Streptomyces* L₃.³²

In *Planococcus* 2389 mucopeptide the linkage between tetrapeptides is shown to be effected through D-glutamic acid (D-alanyl- γ -D-glutamyl- ϵ -lysine).³³ Results of DNP-tracing indicate that the D-alanyl- γ -D-glutamyl linkage is broken by digestion with CEP. This linkage has been found to be split by the KM-endopeptidase of *Streptomyces albus* G.³¹

Mucopeptides of both *M. lysodeikticus* NCTC 2665^{14,34} and *Sarcina lutea*³⁵ are found to contain D-alanyl- ϵ -lysine linkages. Increase of DNP- ϵ -lysine after treatment with CEP therefore, indicates splitting of the D-alanyl- ϵ -lysine bridge and an activity corresponding to the ML endopeptidase.^{14,22} The structure of mucopeptide of *M. luteus* 144 has not been revealed, but analyses indicate a structure similar to that of *M. lysodeikticus*. However, mucopeptide from *M. luteus* ATCC 398 has been found to contain the amino acids alanine, glutamic acid, glycine, and lysine at a ratio of 2:2:1:1 and a cross-bridge of γ -L-glutamyl-glycine³⁶ is proposed. Accordingly the result of DNP-tracing may indicate that the CEP splits the glycyl- ϵ -lysine bond of this

bridge, an activity which to our knowledge has not previously been observed in *Streptomyces* cultures.

An attempt to separate the observed endopeptidase, *N*-acetylmuramidase, *N*-acetylmuramyl-L-alanine amidase, and caseinolytic activities will be reported in a subsequent paper.

REFERENCES

1. Ghuysen, J.-M. *Bacteriol. Rev.* 32 (1968) 425.
2. Ghuysen, J.-M., Tipper, D. J. and Strominger, J. L. *Methods Enzymol.* 8 (1966) 685.
3. Strominger, J. L. and Ghuysen, J.-M. *Science* 156 (1967) 213.
4. Waksman, S. A. *J. Bacteriol.* 3 (1918) 509.
5. Lieske, R. *Morphologie und Biologie der Strahlenpilze*, Gebrüder Bornträger, Leipzig 1921.
6. Welsch, M. *Rev. Belge Pathol. Med. Exp.* 28, Suppl. 2 (1947) 1.
7. McCarty, M. *J. Exp. Med.* 96 (1952) 555.
8. Mory, Y., Kato, K., Matsubara, T. and Kotani, S. *Biken J.* 3 (1960) 129.
9. Mory, Y. and Kotani, S. *Biken J.* 5 (1962) 55.
10. Waksman, S. A. *Principles of Soil Microbiology*, Williams and Wilkins, Baltimore 1927, p. 304.
11. Ghuysen, J.-M. *Arch. Int. Physiol.* 65 (1957) 173.
12. Salton, M. R. J. *The Bacterial Cell Wall*, Elsevier, Amsterdam 1964.
13. Helgeland, S. and Grov, A. *Acta Pathol. Microbiol. Scand. Sect. B* 79 (1971) 819.
14. Petit, J. F., Munoz, E. and Ghuysen, J.-M. *Biochemistry* 5 (1966) 2764.
15. Grov, A. and Helgeland, S. *Acta Pathol. Microbiol. Scand. Sect. B* 79 (1971) 812.
16. Baddiley, J. *Proc. Roy. Soc. B* 170 (1968) 331.
17. Brenner, M., Niederwieser, A. and Pataki, G. In Stahl, E., Ed., *Thin-Layer Chromatography*, Springer, Berlin 1965, p. 4231.
18. Merck, E. *Chromatographie*, E. Merck AG, Darmstadt 1970.
19. Roach, D. and Gehrke, C. W. *J. Chromatogr.* 43 (1969) 303.
20. Thompson, J. S. and Shockman, G. D. *Anal. Biochem.* 22 (1968) 260.
21. Tipper, D. J. and Strominger, J. L. *Biochem. Biophys. Res. Commun.* 22 (1966) 48.
22. Ghuysen, J.-M., Dierickx, L., Coyette, J., Leyh-Bouille, M., Guinand, M. and Campbell, J. N. *Biochemistry* 8 (1969) 213.
23. Iversen, O.-J. and Grov, A. *Eur. J. Biochem.* 38 (1973) 293.
24. Ward, J.-B. and Perkins, H. R. *Biochem. J.* 106 (1968) 69.
25. Ghuysen, J.-M. *Biochim. Biophys. Acta* 40 (1960) 473.
26. Yoshimoto, T., Nakamishi, T., Fukumoto, J. and Tsuru, D. *Agr. Biol. Chem.* 35 (1971) 1775.
27. Salton, M. R. J. and Pavlik, J. G. *Biochim. Biophys. Acta* 39 (1960) 398.
28. Munoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J. F., Heymann, H., Bricas, E. and Lerancier, P. *Biochemistry* 5 (1966) 3748.
29. Bricas, E., Ghuysen, J.-M. and Dezélee, P. *Biochemistry* 6 (1967) 2598.
30. van Heijenoort, J., Elbaz, L., Dezélee, P., Petit, J. F., Bricas, E. and Ghuysen, J.-M. *Biochemistry* 8 (1969) 207.
31. Ghuysen, J.-M., Leyh-Bouille, M., Bonaly, R., Nieto, M., Perkins, H. R., Schleifer, K. H. and Kandler, O. *Biochemistry* 9 (1970) 2955.
32. Matsuda, T., Kotani, S. and Kato, K. *Biken J.* 11 (1968) 111.
33. Schleifer, K. H. and Kandler, O. *J. Bacteriol.* 103 (1970) 387.
34. Katz, W. and Strominger, J. L. *Biochemistry* 6 (1967) 930.
35. Campbell, J. N., Leyh-Bouille, M. and Ghuysen, J.-M. *Biochemistry* 8 (1969) 193.
36. Niebler, E., Schleifer, K. H. and Kandler, O. *Biochem. Biophys. Res. Commun.* 34 (1969) 560.

Received October 13, 1973.