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An Improved Technique for the Preparation of *Streptomyces* Peptidases and *N*-Acetylmuramyl-L-alanine Amidase Active on Bacterial Wall Peptidoglycans*

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ABSTRACT: Separation and purification of *N*-acetylmuramyl-L-alanine amidase, of aminopeptidase, and of the four bacteriolytic MR, SA, ML, and KM endopeptidases are described. The activities of the enzymes are discussed with respect to the nature of the sensitive linkages and to the rates of hydrolysis of these linkages when the enzymes act upon isolated walls or upon soluble compounds. In some cases, a high degree of polymerization of the substrate enhances the enzyme activity upon the sensitive linkages.

A species of *Streptomyces* has been shown to secrete into the culture media at least six enzymes capable of hydrolyzing specific CO–NH linkages within the bacterial wall peptidoglycans. These enzymes, which have proven to be valuable tools in the study of the wall structure, are the following: (1) the lytic SA endopeptidase (Muñoz *et al.*, 1966b) (typical substrate, walls of *Staphylococcus aureus*), (2) the lytic ML endopeptidase (Ghuysen *et al.*, 1968) (typical substrate, walls of *Micrococcus lysodeikticus*), (3) the lytic MR endopeptidase (Petit *et al.*, 1966) (typical substrate, walls of *Micrococcus roseus*), (4) the lytic KM endopeptidase (van Heijenoort *et al.*, 1969; Guinand *et al.*, 1969) (typical substrates, walls of *Bacillus megaterium* KM and walls of *Butyribacterium rettgeri*), (5) the nonlytic *N*-acetylmuramyl-L-alanine amidase (Munoz *et al.*, 1966b) (typical substrate, the disaccharide peptide N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine isolated from walls of *S. aureus*), and (6) a nonlytic aminopeptidase (Muñoz *et al.*, 1966b; Petit *et al.*, 1966) (typical substrate, glycylglycyl-L-alanine). Among these enzymes the MR endopeptidase is unique in that it functions also as a protease, in that it is active on a wide range of proteins, such as casein.

The goal of the present paper is to briefly describe the production, isolation, and purification of these six enzymes and to report some of their properties.

Material and Methods

Walls. Walls of *S. aureus* Copenhagen, *M. lysodeikticus*, *M. roseus* R27, *B. megaterium* KM, and *B. rettgeri* (from heated cells; Guinand *et al.*, 1969) were prepared by mechanical disruption using glass beads. The purification sequence included treatment with trypsin. Walls from cells of *Lactobacillus acidophilus* 63 AM Gasser (Pasteur Institute, Paris) harvested in the logarithmic phase were prepared in a similar manner, with the exception that the treatment with trypsin was omitted. Extreme care was taken in order to prevent autolysis. The endogenous wall autolysin was finally inactivated by heating the purified walls for 20 min at 100°, in water.

Soluble Compounds from Degraded Walls. (1) The *S. aureus* disaccharide tetrapeptide monomer N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-γ-D-isoglutaminyl)-L-lysyl-D-alanine (Muñoz *et al.*, 1966b),

* From the Service de Bactériologie, Université de Liège, Liège, Belgium. *Received August 1, 1968.* This research has been supported in part by U. S. Department of Agriculture Grant UR-E4-10-2 under Public Law 480 and by the Fonds de la Recherche Fondamentale Collective, Brussels, Belgium (Contract No. 515).

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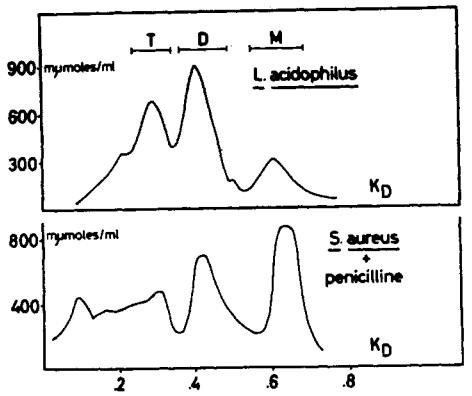

FIGURE 1: Filtration on the Sephadex G-50 and G-25 column systems of autolyzed walls of *L. acidophilus* and of endo-*N*-acetylmuramidase treated walls of *S. aureus* (the extent of peptide cross-linking has been decreased by growing the cells in the presence of penicillin). Prior to filtration on the above system, enriched preparations of walls peptidoglycan debris were obtained by filtration on a Sephadex G-75 column (*L. acidophilus*) or by treatment with ECTEOLA cellulose (*S. aureus*). The elution profiles show the distribution of the reducing groups, relative to β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides. Starting materials, 200 mg of walls. Fractions volume, 8 ml. M = disaccharide peptide monomer; D = bisdisaccharide peptide dimer; and T = trisdisaccharide peptide trimer.

(2) the *M. lysodeikticus* and *Sarcina lutea* peptide dimers *i.e.*, two peptide monomers N^{α}-[L-alanyl-γ-(α-D-glutamylglycine)]-L-lysyl-D-alanine linked through a N^{ϵ}-(D-alanyl)-L-lysine linkage (Ghuysen *et al.*, 1968; Campbell *et al.*, 1969), (3) the *Escherichia coli* bisdisaccharide peptide dimer, in which two tetrapeptides L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine are linked through a D-alanyl-(D)-*meso*-diaminopimelic acid linkage (van Heijenoort *et al.*, 1969), (4) the *B. rettgeri* peptide dimer in which two tetrapeptides N^{α}-(L-seryl-γ-D-glutamyl)-L-ornithyl-D-alanine are joined together by a D-lysine or by a D-ornithine residue as represented in Figure 8 in Guinand *et al.* (1969) were prepared as previously described. (5) The *S. aureus* bisdisaccharide-peptide dimer, *i.e.*, a dimer in which the former *S. aureus* disaccharide tetrapeptide monomer and a disaccharide pentapeptide monomer with two D-Ala residues at the C terminal are joined together through a pentaglycine bridge extending from the ε-amino group of the lysine residue of the pentapeptide to the C-terminal D-alanine residue of the tetrapeptide, was prepared essentially as previously described (Muñoz *et al.*, 1966b). Walls were prepared from cells grown in the presence of penicillin (in order to decrease the extent of peptide cross-linking in the wall peptidoglycan); the walls were solubilized by F_1 endo-*N*-acetylmuramidase; the teichoic acid–glycopeptide complexes were retained on a column of ECTEOLA-cellulose (OH^- form, in water) while the teichoic acid free peptidoglycan debris emerged in the water eluate (for conditions of cell growth in the presence of penicillin and of wall degradation and fractionation, see Muñoz *et al.*, 1966b). The isolated peptidoglycan debris (about 70% of the peptidoglycan units present in the original walls) were further fractionated (Figure 1, lower part) into disaccharide peptide monomer (M), bisdisaccharide peptide dimer (D), and a higher oligomer fraction by filtration in 0.1 M LiCl on the linked Sephadex G-50 and G-25 columns system described in Ghuysen *et al.* (1968). The monomer and the dimer were filtered again on the above Sephadex columns system, and next in water on a Sephadex G-25 column. Analytical data showed that the isolated bisdisaccharide peptide dimer contained, besides the internal pentaglycine bridge, an uncross-linked pentaglycine chain at its N terminus. These opened pentaglycine chains were completely degraded by the *Streptomyces* aminopeptidase as described in Petit *et al.* (1966) and Muñoz *et al.* (1966b). The free glycine residues were removed from the dimer by further filtration in water on Sephadex G-25. The aforementioned K_D values of the dimer (Figure 1), together with the analytical data (disaccharide, 1; Lys, 1; Glu, 0.96; Gly, 2.88; Ala, 2.40; N^{ϵ}- Lys, 0.47; *N*-Gly, 0.0; and *N*-Ala, 0.0), were in agreement with the above proposed structure. (6) The *L. acidophilus* bisdisaccharide peptide dimer, *i.e.*, a dimer in which one disaccharide pentapeptide N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanine and one disaccharide hexapeptide with two D-Ala residues at the C terminal, are joined by means of a direct bond involving the terminal D-alanine residue of the pentapeptide and the N-terminal D-isoasparagine residue of the hexapeptide; and (7) the *L. acidophilus* trisdisaccharide peptide trimer in which one disaccharide hexapeptide and two disaccharide pentapeptides are linked through the aforementioned D-alanyl-D-isoasparagine linkages were isolated from walls prepared from log-phase cells allowed to autolyze at 37°, for 6 hr, in 0.05 M citrate buffer (pH 5). During this process, similar to that previously described in the case of *Streptococcus faecalis* ATCC 9790 (Shockman *et al.*, 1967), an endo-*N*-acetylmuramidase autolysin hydrolyzes the glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine (actual data, 300 mμmoles of disaccharide units was detected in the autolysate, per mg of walls or per 340 mμequiv of peptidoglycan units). The autolysate was filtered through a Sephadex G-75 column (130 × 1.7 cm) in 0.05 M citrate buffer (pH 5). An excluded material, containing the active autolysin, was separated from a fraction of K_D = 0.75. This latter fraction contained 85% of the peptidoglycan units present in the original walls. It was further fractionated by filtration in 0.1 M LiCl on the linked Sephadex G-50 and G-25 column system (Figure 1, upper part). The disaccharide peptide monomer, the bisdisaccharide peptide dimer, and the trisdisaccharide peptide trimer were again and separately filtered on the above Sephadex column system. Finally they were desalted by filtration in water on Sephadex G-25. The disaccharide peptide monomer (disaccharide, 1; Lys, 1; Ala, 3.32; Glu, 1.10; Asp, 0.97; and *C*-Ala, 0.94) was found identical with the *S. faecalis* monomer N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-Ala-D-isoGln)-N^{ϵ}-(D-isoAsn)-L-Lys-D-Ala previously studied (Ghuysen *et al.*, 1967), with the exception that the *L. acidophilus* monomer terminates by a D-Ala-D-Ala dipeptide. The chemical data for the *L. acidophilus* dimer (disaccharide, 1; Lys, 1; Ala, 2.60; Glu, 1; Asp, 1.02;

and *C*-Ala, 0.54) and for the trimer (disaccharide, 1; Lys, 1; Ala, 2.38; Glu, 1.05; Asp, 1.05; and *C*-Ala, 0.34), together with their K_D values on the Sephadex column system, were in agreement with the above-proposed structures. A complete characterization of the dimer and of the trimer will be given elsewhere. In terms of the peptidoglycan units present in the original walls, the actual recoveries of the monomer, dimer, and trimer were 10, 37, and 30%, respectively.

Analytical Methods. N- and C-terminal groups were measured by the fluorodinitrobenzene technique and the hydrazinolysis technique, respectively (Ghuysen *et al.*, 1966, 1968). Proteins were measured by the Folin–Ciocalteu method as modified by Lowry *et al.* (1951). All pH values were determined at 20–25°.

Routine Estimation of Enzymatic Activities. (1) The bacteriolytic activities were measured by following the decrease in turbidity (absorption at 550 mμ) of a standard wall suspension (1 mg/ml) made up in the appropriate buffer. (2) Caseinolytic activity was measured by incubating an aliquot (10–50 μl) of the enzyme preparations, at 37° for 10–20 min, with 500 μl of a casein solution (0.2% in 0.05 M phosphate buffer, pH 8). The hydrolysis was stopped by the addition of 4.5 ml of 1% trichloroacetic acid. Turbidity was measured with a Pulfrich nephelometer. (3) Aminopeptidase activity was measured by determining by the fluorodinitrobenzene technique the amount of free glycine liberated from the tripeptide Gly-Gly-L-Ala, after incubation at 37° with an aliquot of the enzyme preparation. (4) *N*-Acetylmuramyl-L-alanine amidase activity was measured by estimating by the fluorodinitrobenzene technique the amount of free peptide liberated from the *S. aureus* disaccharide tetrapeptide monomer after incubation at 37° with an aliquot of the enzyme preparation. The free cationic peptide was also separated from the neutral disaccharide peptide by electrophoresis on Whatman No. 3MM paper, for 2 hr, in pyridine–acetic acid–water (2:9:1000, v/v) buffer (pH 3.7), using an Electrorheophor Pleuger apparatus. The extent of the hydrolysis was roughly estimated after location of the compounds with ninhydrin.

Combined Gel Filtration–Gel Electrophoresis on Sephadex G-50. This apparatus (Figure 2) is a modification of the one described in Dierickx and Ghuysen (1962), used for sucrose gradient electrophoresis. The U-tube was filled with 0.003 M (μ = 0.01) sodium phosphate buffer (pH 6.45) (at 20°). Glass beads were added, as shown, to provide a bed for the resin. Sephadex G-50 (fine particle size 20–80 μ), previously equilibrated against 0.003 M phosphate buffer (pH 6.45), was added to one side and the column (volume 460 ml) was packed in the usual way. For the first fractionation step (gel filtration) the sample was applied to the top of the column and elution was commenced (descending) with the same buffer. Elution was continued until 110 ml, which represents two-thirds of the void volume of this system, had been collected *via* the capillary at the bottom of the column. For the second step (electrophoresis) elution was stopped and a current of 20 mA, 7 V/cm was applied to the electrodes. Migration was toward the anode (*i.e.*, ascending the column) and rapidly migrating contaminating materials (*i.e.*, pigments) were washed clear by

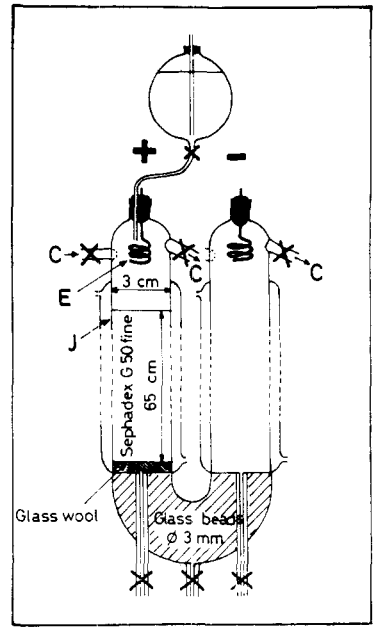

FIGURE 2: Schema of the apparatus used for combined gel filtration–gel electrophoresis on Sephadex G-50. (J) Jacket with circulating cold water (4°); (E) platin electrodes; (C) circulating phosphate buffer (when gel electrophoresis is in operation). For more details, see Dierickx and Ghuysen (1962).

the continuous (for this step only) lateral flow of buffer across the upper surface of the column. After 40-hr electrophoresis with the enzymes still on the column, the current was switched off and the third step, again gel filtration in the descending direction, was begun. Eluent fractions were collected *via* the capillary at the bottom of the column and assayed for activity.

CM-cellulose columns were prepared by equilibrating the resin, batchwise, against 0.2 M Tris buffer (pH 8.6). The columns were packed and washed with water or with a selected Tris buffer, pH 8.6. Adsorption and elution conditions (fractions volume 8 ml) are given in the text. Concentration of the various effluent fractions was achieved by dialysis against dry Carbowax 4000 (B. D. H.).

Specific Activities of Purified Enzymes on Soluble Substrates. Substrates containing 10–40 mμequiv of the sensitive linkages were incubated for various times, at 37°, with 0.5–10 μg of purified enzymes in final volumes of 30 μl of either 0.03 M acetate buffer (pH 5.5) (for the *N*-acetylmuramyl-L-alanine amidase) or 0.005–0.01 M Veronal buffer (pH 9) (for the MR, SA, ML, and KM endopeptidases). The hydrolytic actions of the *N*-acetylmuramyl-L-alanine amidase (the liberation of free peptide from disaccharide peptide monomer) and of the endopeptidases (the liberation of peptide monomers or disaccharide peptide monomers from peptide dimers or from bisdisaccharide peptide dimers and trisdisaccharide peptide trimers) result in the exposure of N-terminal groups. The extent of the hydrolyses was estimated by N-terminal group measurements and controlled by appropriate chromatographic or electrophoretic analyses under conditions which provided the separation of the de-

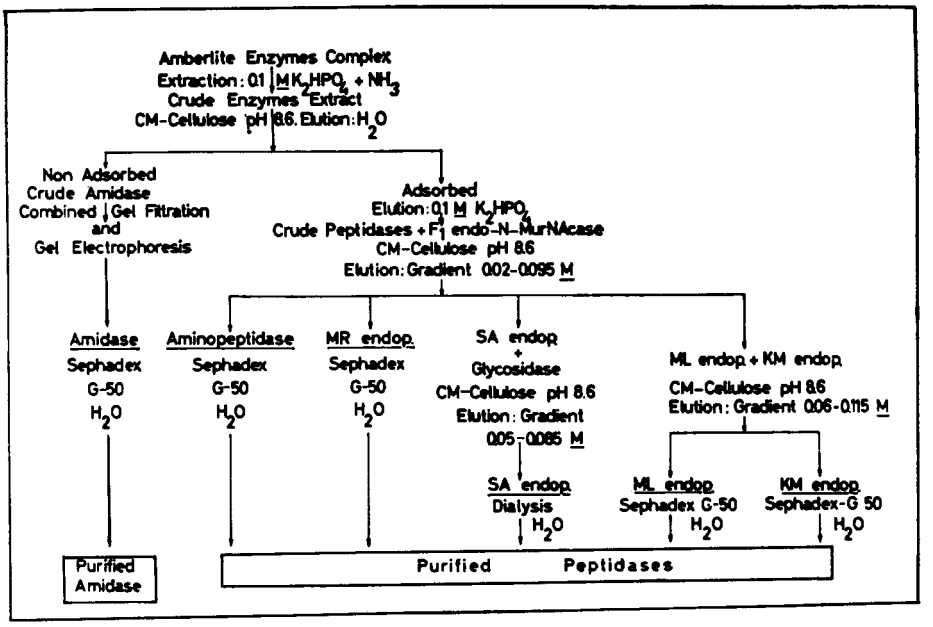

FIGURE 3: Flowsheet: summary of the steps involved in the purification of the *N*-acetylmuramyl-L-alanine amidase, of the aminopeptidase, and of the bacteriolytic MR, SA, ML, and KM endopeptidases.

graded products from the original substrates. The techniques used were: (1) for the amidase activity upon the *S. aureus* disaccharide peptide monomer, by paper electrophoresis at pH 3.7; (2) for the SA endopeptidase activity upon the *S. aureus* bisdisaccharide peptide dimer, by descending paper chromatography in isobutyric acid–0.5 M NH_4OH (5:3, v/v); (3) for the SA endopeptidase activity upon the *L. acidophilus* bisdisaccharide peptide dimer and trisdisaccharide peptide trimer, by paper electrophoresis in 0.1 M formic acid; (4) for the ML endopeptidase upon *S. lutea* peptide dimer and (5) for the KM endopeptidase upon the *B. rettgeri* peptide dimer, chromatography on thin-layer plates of Stahl's silica gel G (Merck) in isobutyric acid–0.5 M NH_4OH (5:3 v/v); and (6) for the KM endopeptidase upon the *E. coli* bisdisaccharide peptide dimer, by paper electrophoresis at pH 6.4 in pyridine–acetic acid–water (10:1:1000, v/v).

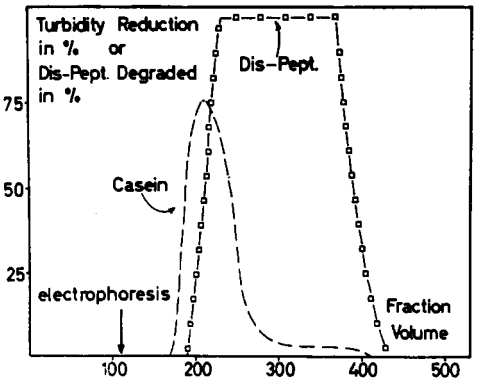

FIGURE 4: Purification of the *N*-acetylmuramyl-L-alanine amidase by combined gel filtration–gel electrophoresis on Sephadex G-50. The curves show activity of the eluent fractions on the designated substrates. For description of the apparatus, and conditions, see text. Aliquots of the fractions were analyzed for caseinolytic activity (caseinase A), and for hydrolysis of the *S. aureus* disaccharide peptide monomer. The casein solution was incubated 15 min, at 37°, with 100 μl of each fraction. The disaccharide peptide (10 mμmoles) was incubated for 3 hr at 37° with 5 μl of each fraction, supplemented with 5 μl of 0.2 M acetate buffer (pH 5.4), in a final volume of 30 μl.

Experimental Section

Selection and Cultivation of Streptomyces Strains. Selection was carried out as previously described (Ghuysen *et al.*, 1965). Agar plates containing 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and supplemented with 1% (w/v) cell walls of either *S. aureus*, *M. lysodeikticus*, or *B. megaterium* were incubated with various strains of *Streptomyces*. Those strains which showed a 1-cm zone of lysis on each side of the mycelium after 4 days at 28° were selected. One of the selected strains was grown for 20 hr at 28° with shaking, in a medium containing 0.8% peptone oxoid, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4, 0.2% $NaNO_3$, 0.03% KCl, 0.003% $Co(NO_3)_2 \cdot 6H_2O$, plus 0.1% (w/v) autoclaved, dried whole cells of *S. aureus*. After two successive subcultures of increasing size, 75 l. of culture in logarithmic phase was used to inoculate a 500-l. tank of the same staphylococcal cell enriched medium. This culture was grown at 28° with mechanical stirring, and an air flow rate of 100 l./min at an air pressure of 0.6 kg/cm². Bacteriolytic and caseinolytic activities were maximal after about 50 hr.

Isolation of the Crude Enzyme Complex. Mycelia were removed from the culture by filtration, the filtrate was cooled to 0°, and the crude enzyme complex was adsorbed on Amberlite CG50 or XE64 H^+ (5 kg of resin/500 l. of filtrate) by adjusting the pH to 5 with acetic acid. The enzyme–resin complex was then collected by filtration, lyophilized, and stored at 0°. This enzyme–resin complex can be held for months under these conditions without detectable loss of activity.

Initial Separation of the N-Acetylmuramyl-L-alanine Amidase and the Peptidases. All of the manipulations hereinafter described were carried out at 4° (see flow

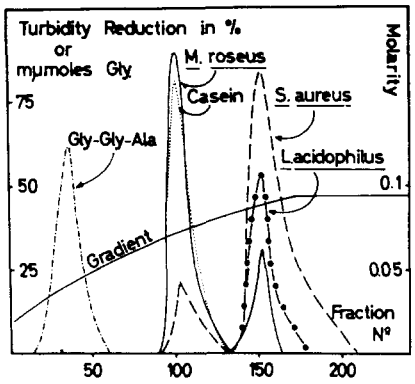

FIGURE 5: Separation of aminopeptidase (substrate, Gly-Gly-L-Ala), of MR endopeptidase (substrate, *M. roseus* walls and casein), and of SA endopeptidase (substrate, *S. aureus* and *L. acidophilus* walls), on CM-cellulose. The curves show the activity of the eluent fractions on the designated substrates. For conditions of chromatography, see text. Aliquots of the fractions (8 ml) were analyzed for aminopeptidase activity, for caseinolytic activity, and for lytic activity upon bacterial walls of *M. roseus*, *S. aureus*, and of *L. acidophilus* (heat inactivated). Walls (500 μg) were incubated for 1 hr (*M. roseus*), for 4 hr (*S. aureus*), and for 22 hr (*L. acidophilus*) at 37° with 50 μl of each fraction in a final volume of 500 μl. The tripeptide Gly-Gly-L-Ala (250 mμmoles) was incubated for 4 hr with 25 μl of each fraction and 8 μl of 0.2 M K_2HPO_4 in a final volume of 60 μl. The glycine was measured as its dinitrophenyl derivative. Casein solution (500 μl) was incubated 15 min, at 37°, with 25 μl of each fraction.

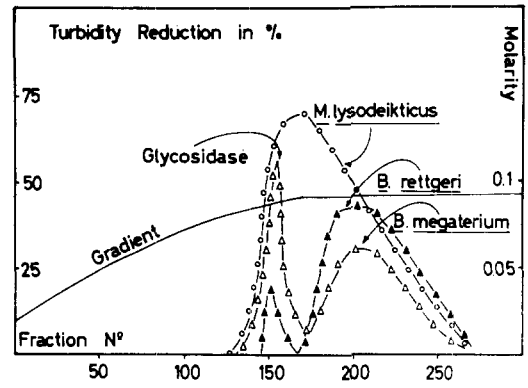

FIGURE 6: Separation of ML endopeptidase (substrate, *M. lysodeikticus* walls) and of KM endopeptidase (substrate, *B. megaterium* and *B. rettgeri* walls) on CM-cellulose. The curves show the activity of the effluent fractions on walls of the designated species. Walls (500 μg) were incubated for 1 hr (*M. lysodeikticus*), for 5 hr (*B. megaterium*), and for 22 hr (*B. rettgeri*) at 37° with 50 μl of each fraction (8 ml) in a final volume of 500 μl. Fractions 140–170 contain a contaminating glycosidase (see text).

sheet in Figure 3). The Amberlite-adsorbed enzyme complex (200 g) (*i.e.*, the equivalent of about 15 l. of the original culture) was suspended in about 2 l. of 0.1 M K_2HPO_4 and the pH of the suspension was brought to 7.5 by dropwise addition of concentrated ammonia with vigorous stirring. The resin was removed by filtration and the filtrate was clarified by centrifugation. The volume of the supernatant was reduced to 50 ml by dialysis against dry Carbowax. The concentrated extract was divided into two 25-ml aliquots and each of them was filtered in water on a 75 × 4 cm column of Sephadex G-50 (fine). The initial fractions, collected at the void volume of the gel, were turbid. These were clarified by centrifugation and added to those fractions subsequently collected which were found to be free of phosphate. The final solution, about 900 ml, was adsorbed on a 300-ml column of CM-cellulose (pH 8.6) which had been washed with water. Nonadsorbed material was collected by eluting the column with water and following the appearance of material absorbing at 278 mμ. These fractions were pooled and concentrated to a volume of 50 ml. The resultant dark brown solution represented the crude *N*-acetylmuramyl-L-alanine amidase preparation. The material adsorbed to the CM-cellulose column was then eluted with 0.1 M K_2HPO_4. All of the fractions which were found lytic for walls of *S. aureus* were pooled and concentrated to a volume of 25 ml. The resultant yellowish solution represented the crude peptidase preparation.

Further Purification of the N-Acetylmuramyl-L-alanine Amidase. A 10-ml aliquot of the crude *N*-acetylmuramyl-L-alanine amidase preparation was layered on the top of the Sephadex G-50 column in the combined gel filtration–gel electrophoresis apparatus, and the filtration–electrophoresis–filtration sequence described in Material and Methods was carried out. The final eluent fractions were analyzed for their caseinolytic activity and for their capability of hydrolyzing the *S. aureus* disaccharide peptide monomer (*i.e.*, amidase activity) (see Material and Methods). Figure 4 shows the elution profiles of the caseinase and of the amidase. The fractions collected between 270 and 380 ml were pooled and concentrated to 5 ml.

Further Purification of the Crude Peptidases. The crude peptidase preparation (25 ml) was dialyzed for 6 hr against 0.01 M Tris buffer (pH 8.6) and was adsorbed on a 150-ml column (2 × 50 cm) of CM-cellulose (pH 8.6) washed with a 0.02 M Tris buffer (pH 8.6). After adsorption of the crude peptidases, the column was washed with 200 ml of the 0.02 M Tris buffer (pH 8.6) and then eluted with an increasing gradient of Tris buffer (pH 8.6) (mixing chamber at constant volume, 2 l. of 0.02 M Tris buffer; adding solution, 0.2 M Tris buffer). Figure 5 shows the elution profile of the aminopeptidase (active on the tripeptide Gly-Gly-L-Ala), of the MR endopeptidase (active on casein, on walls of *M. roseus*, and, at a much lower rate, on walls of *S. aureus*), and of the SA endopeptidase (active on walls of *S. aureus*, *L. acidophilus*, and *M. roseus*). Figure 6 shows the elution profile of the ML endopeptidase (active on walls of *M. lysodeikticus*) and of the KM endopeptidase (active on walls of *B. rettgeri* and *B. megaterium* KM). As is also shown in Figure 6 two fractions were obtained which exhibited lytic activity on walls of *B. megaterium*. The peak eluted at fraction 150 was shown to contain glycosidase activity as evidenced by the release of reducing groups from the degraded *B. megaterium* walls. The peaks represented in Figures 5 and 6 were collected and designated as follows: aminopeptidase, samples 20–50; MR endopeptidase, samples 90–110; SA endopeptidase plus glycosidase, samples 140–160; and ML endopep-

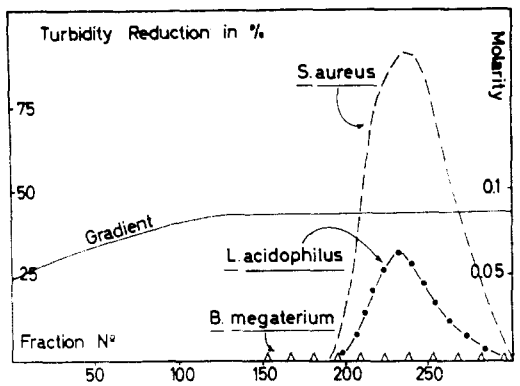

FIGURE 7: Purification of SA endopeptidase on CM-cellulose. The curves show the lytic activity of effluent fractions on walls of the designated species. For conditions, see text. Starting material, fractions 140–160 of Figures 5 and 6. Walls (500 μg) were incubated for 10 hr (*S. aureus*) and for 24 hr (*L. acidophilus* and *B. megaterium*), at 37° with 100 μl of each fraction in a final volume of 500 μl.

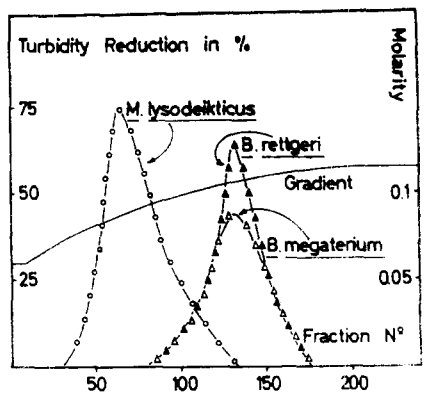

FIGURE 8: Purification of ML endopeptidase (substrate, walls of *M. lysodeikticus*) and KM endopeptidase (substrates, walls of *B. rettgeri* and *B. megaterium*) on CM-cellulose. The curves show the lytic activity of the effluent fractions on walls of the designed species. For conditions, see text. Starting material, fractions 160–250 of Figure 6. Walls (500 μg) were incubated for 30 min (*M. lysodeikticus*) and for 15 hr (*B. rettgeri* and *B. megaterium*) at 37° with 150 μl of each fraction in a final volume of 500 μl.

tidase plus KM endopeptidase, samples 160–250. Each of the fractions was pooled and concentrated to a volume of 5 ml.

Separation of the SA Endopeptidase from the Glycosidase. The concentrated fractions 140–160 (Figures 5 and 6) were dialyzed for 6 hr against 0.05 M Tris buffer (pH 8.6). The solution was adsorbed on a 200-ml column of CM-cellulose (pH 8.6) washed with 0.05 M Tris buffer (pH 8.6). The elution was carried out with an increasing gradient of Tris buffer (pH 8.6) (mixing flask, at constant volume, 2 l. of 0.05 M Tris buffer; adding solution, 0.15 M Tris buffer). Figure 7 shows the elution profile of the SA endopeptidase. All of the fractions were found completely inactive upon walls of *B. megaterium* and of *M. lysodeikticus* and contained no detectable glycosidase activity. It is possible that the contaminating glycosidase detected in Figure 6 was some F_1 endo-*N*-acetylmuramidase (Muñoz *et al.*, 1966a). This enzyme, however, is known to be firmly fixed on CM-cellulose and cannot normally be eluted by the buffer concentration used. The SA endopeptidase present in fractions 205–280 (Figure 7) was concentrated to a volume of 5 ml.

Separation of the ML and the KM Endopeptidases. The concentrated fractions 160–250 (Figure 6) were dialyzed for 6 hr against 0.06 M Tris buffer (pH 8.6). The enzymes were adsorbed on a 180-ml column of CM-cellulose (pH 8.6) washed with 0.06 M Tris buffer (pH 8.6). The elution was carried out with an increasing gradient of Tris buffer (pH 8.6) (mixing flask, at constant volume, 2 l. of 0.06 M Tris buffer; adding solution, 0.2 M Tris buffer). As shown in Figure 8, the ML endopeptidase and the KM endopeptidase were sequentially eluted in that order. Fractions 50–95 (ML endopeptidase) and fractions 115–160 (KM endopeptidase) were pooled separately and concentrated to a volume of 5 ml.

Final Purification of the Streptomyces Enzymes. The isolated and concentrated (5 ml) *N*-acetylmuramyl-L-alanine amidase, aminopeptidase, and MR, ML, and KM endopeptidases were filtered in water on a 400-ml (100 × 2.25 cm) column of Sephadex G-50. None of the enzymes was excluded from the gel and all of them were eluted before the salts. Figure 9 illustrates the gel filtration of the KM endopeptidase. For each enzyme, the fractions exhibiting the highest activity were pooled (fractions 35–39 in Figure 9, for example). These solutions constituted the final purified enzymatic preparations. The SA endopeptidase was found, at this stage of purity, to be retained on the Sephadex column. As a result, for this enzyme only, the final purification step consisted of dialysis against water for 15 hr. It was noted that even after this dialysis, a small amount of small molecular weight carbowax compounds which had penetrated the dialysis tubing during the concentration steps persisted. The yields of each of the six enzymes purified were from 15 to 25 mg of protein per 15 l. of original culture filtrate. Protein was estimated by the technique of Lowry *et al.* (1951) with the exception of the SA endopeptidase which precipitated under the assay conditions. In this latter case, protein was estimated by measuring absorbance at 278 mμ. The purified amidase, aminopeptidase, and endopeptidases are partially denaturated by freeze drying. In aqueous solutions, they are completely destroyed by heating, 2 min, at 100°. They can be stored in frozen state, at −20°, for weeks without detectable loss of activity.

Properties of the Purified Enzymes. Figure 10 shows the kinetics of wall solubilization by the four endopeptidases. A first-order reaction was not observed in all cases. The specific activities of the enzymes upon bacterial walls were calculated from the incubation times, under the conditions indicated in the legend of Figure 10, which were required to bring about a reduction of turbidity of 50% in a standard wall suspension. In these calculations, it was assumed that a $\log \Delta_0/\Delta_t = 0.3$ corresponded to the solubilization of 50% of the wall materials. The results (Table I) are expressed in micrograms of walls dissolved per micrograms of enzyme per hour. Table I also presents the specific activities of the enzymes upon

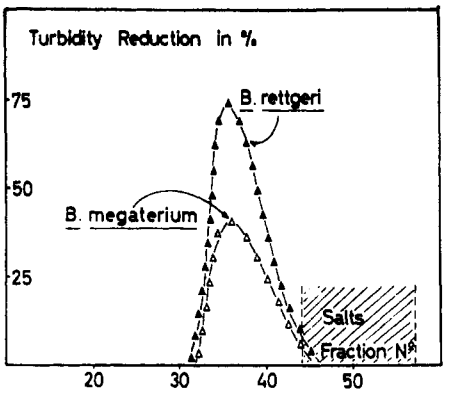

FIGURE 9: Purification of KM endopeptidase on Sephadex G-50 (in water). The curves show the lytic activity of the effluent fractions on walls of the designated species. Starting material, fractions 115–160 of Figure 8. Walls (500 μg) were incubated for 2 hr (*B. megaterium*) and for 8 hr (*B. rettgeri*) with 100 μl of each fraction, supplemented with 50 μl of 0.1 M Veronal buffer (pH 9) in a final volume of 500 μl.

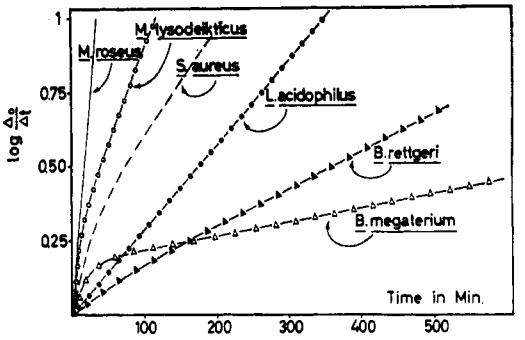

FIGURE 10: Kinetics of wall solubilization by the purified endopeptidases. The lines or curves are designated according to the species of walls used as substrate. Walls (500 μg) were incubated at 37° in a final volume of 500 μl: (A) in water in the case of *B. rettgeri* and in 0.03 M Veronal buffer (pH 9) in the case of *B. megaterium*, in the presence of 40 μg (in proteins) of KM endopeptidase; (B) in 0.01 M Veronal buffer (pH 9) in the cases of *S. aureus* and *L. acidophilus*, in the presence of 40 μg (in proteins) of SA endopeptidase; (C) in 0.01 *M* Veronal buffer (pH 9) in the case of *M. lysodeikticus*, in the presence of 10 μg (in proteins) of ML endopeptidase; (D) in 0.01 M Veronal buffer (pH 9) in the case of *M. roseus*, in the presence of 80 μg (in proteins) of MR endopeptidase. Results are expressed as $\log \Delta_0/\Delta_t$ with Δ_0 = optical density at 550 mμ of the the original wall suspension and Δ_t = optical density after various times of incubation.

defined soluble substrates (see Material and Methods), expressed in millimicroequivalents of hydrolyzed linkages per micrograms of enzyme per hour.

Discussion

The peptide moiety of the bacterial peptidoglycans is composed of subunits having the general sequence (L-Ala- or L-Ser- or Gly-)-γ-D-Glu-(L-Lys- or *meso*-diaminopinelic acid or L-Orn-)-D-Ala. The peptide subunits substitute, at their N termini, the carboxyl groups of the *N*-acetylmuramic acid residues of the glycan chain. The γ-carboxyl group of glutamic acid is joined to the α-amino group of L-lysine or to the amino group located on the L-carbon of *meso*-diaminopimelic acid. The cross-linkings between peptide subunits vary according to the species but always involve the C-terminal D-alanine residues of the peptide subunits. Cross-linking between peptide subunits may occur directly through such linkages as D-Ala-(D)-*meso*-DAP in the cases of *B. megaterium* and *E. coli* (van Heijenoort *et al.*, 1969) and N^{ϵ}-(D-Ala)-L-Lys in the case of *M. lysodeikticus* and some other *Micrococcaceae* (Ghuysen *et al.*, 1968; Campbell *et al.*, 1969), or through additional intervening peptides. For example, in some *Micrococcaceae*, the bridge consists of several glycine and/or L-amino acids (Petit *et al.*, 1966; Muñoz *et al.*, 1966b) extending from the ε-amino group of L-lysine to the C-terminal D-alanine, while in *Streptococcus faecalis* (Ghuysen *et al.*, 1967) a D-isoasparaginine residue, at an identical location, serves as the bridge between peptide subunits. In *B. rettgeri* (Guinand *et al.*, 1969) and in some *Corynebacteria* (Perkins, 1967), one D-lysine or one D-ornithine extends from the α-carboxyl group of glutamic acid to the C-terminal D-alanine, forming N^{α}-(D-Ala)-D-Lys or N^{α}-(D-Ala)-D-Orn linkages (for a review, see Ghuysen, 1968). All of the six *Streptomyces* enzymes studied in the present report hydrolyze CO-NH linkages in bacterial peptidoglycans but none of them acts on linkages located within the peptide subunits. They can be grouped into four types (Table I). (1) The *N*-acetylmuramyl-L-alanine amidase hydrolyzes linkages involving the N termini of the peptide subunits. (2) The SA, ML, and KM endopeptidases hydrolyze linkages involving the C-terminal D-alanine of the peptide subunits. Their specificity depends upon the type of bridging between peptide subunits. (3) The aminopeptidase hydrolyzes those peptide bridges composed of glycine and/or L-amino acid residues when they have been opened at their N termini by the SA endopeptidase. (4) The MR endopeptidase, the only true protease active on casein, hydrolyzes linkages located within those peptide bridges composed of several L-amino acids and, at a much slower rate, of several glycine residues.

Streptomyces sp thus appear to be a remarkable source of highly specific enzymes. *N*-Acetylmuramyl-L-alanine amidase, a very anionic protein at pH 8.6, can be easily separated from the aminopeptidase and the four endopeptidases which are cationic and are thus adsorbed on CM-cellulose at this pH and under conditions of low ionic strength. Further purification of the amidase was carried out by combined gel diffusion–gel electrophoresis on Sephadex G-50. The isolation and purification of the aminopeptidase and of the four endopeptidases are difficult tasks. They have been performed making use of the influence of small variations in ionic strength of the buffer on their adsorption–elution properties on CM-cellulose at alkaline pH. It has not been established that the final preparations are in fact homogeneous with respect to their protein content. They are, however, sufficiently devoid of mutual contamination to be used as analytical tools. The specificity of these enzymes has been essential in elucidating the structure of the walls of a large number of bacterial species. It should be kept

TABLE I: Properties of *Streptomyces* Enzymes Acting upon CO–NH Linkages in Bacterial Peptidoglycans.

Enzymes	Linkages Hydrolyzed	Species	Sensitive Substrates and Specific Activity[a]	
			Walls (μg)	Soluble Compounds (mμequiv)
Amidase	D-Lactyl-L-Ala	*S. aureus*[b]	0	Disaccharide peptide monomer (30) Bisdisaccharide peptide dimer (15)
	D-Lactyl-L-Ser	*B. rettgeri*[c]	0	Disaccharide peptide (monomer and oligomer) +
SA endopeptidase	D-Ala-L-Ala	*M. roseus*[b]	+	
	D-Ala-Gly	*S. aureus*[b]	12.5	Bisdisaccharide peptide dimer (2.8)
	D-Ala-D-isoAsn	*S. faecalis*[d]	+	
		L. acidophilus[e]	3.6	Bisdisaccharide peptide dimer; incomplete hydrolysis (see text) Trisdisaccharide peptide trimer (0.05)
ML endopeptidase	N^{ϵ}-(D-Ala)-L-Lys	*M. lysodeikticus*[f]	100	
		S. lutea[f]	+	Peptide dimer (4.4)
KM endopeptidase	D-Ala-(D)-*meso*-diaminopimelic acid	*B. megaterium*[g]	1.1	
		E. coli[g]		Bisdisaccharide peptide dimer (20)
		Envelope	0	
		Rigid layer	+	
	N^{α}-(D-Ala)-D-Lys and N^{α}-(D-Ala)-D-Orn	*B. rettgeri*[c]	1.9	Bisdisaccharide peptide dimer (0.25)
MR endopeptidase	L-Ala-L-Thr	*M. roseus* R27[h]	19	
	L-Ala-L-Ala	*M. roseus* Thr^{-}[h]	+	
	Gly-Gly	*S. aureus*[h]	+	
Aminopeptidase[b,h]	Nonlytic exopeptidase which hydrolyzes (1) L-Ala-L-Ala, L-Ala-L-Thr, and Gly-Gly; (2) N^{ϵ}-(L-Ala- or L-Thr- or Gly-)-L-Lys and (3) L-Ala-D-isoGln linkages			

[a] Expressed in micrograms of walls solubilized or in millimicroequivalents of linkages hydrolyzed, per micrograms of enzyme, per hour. For conditions, see text and legend of Figure 10. For a description of the soluble compounds, see Material and Methods. (+) The substrate is sensitive to the enzyme but the specific activity has not been measured. [b] Munoz *et al.* (1966b). [c] Guinand *et al.* (1969). [d] Ghuysen *et al.* (1967). [e] Materials and Methods. [f] Ghuysen *et al.* (1968); Campbell *et al.* (1969). [g] van Heijenoort *et al.* (1969). [h] Petit *et al.* (1966).

in mind, however, that the fact that intact walls or envelopes of a given species are not degraded by a particular enzyme does not necessarily mean that the susceptible linkage is not present in the walls. To be functional *N*-acetylmuramyl-L-alanine amidase requires that the glycan first be split and has, thus, no bacteriolytic action. Another dramatic example can be found in the envelopes of *E. coli* which are not lysed by KM endopeptidase, albeit the bisdisaccharide peptide dimer isolated from these envelopes is hydrolyzed at a rate of 20 mμequiv/μg of protein per hr, by the same enzyme (Table I). It is clear that the outer lipoprotein and lipopolysaccharide layers of the envelope prevent the enzyme from reaching the D-Ala-*meso*-(D)-diaminopimelic acid linkages within the underlying peptidoglycan. As is expected, the *E. coli* rigid layer (Weidel and Pelzer, 1964), *i.e.*, the peptidoglycan sacculus with protein globules attached to it, obtained after stripping the lipoprotein and lipopolysaccharide polymers from the envelopes, was found to be solubilized by the KM endopeptidase (we are indebted to Dr. J.-F. Petit for a generous gift of isolated *E. coli* rigid layer). Differences in susceptibility to lysis by a given peptidase of walls of various species can be, of course, a direct consequence of variations in the enzymatic susceptibility of the sensitive peptide linkages. The rate of hydrolysis of D-Ala-D-Lys and D-Ala-D-Orn linkages in *B. rettgeri* peptide dimer, by the KM endopeptidase, is 80 times smaller than the rate of hydrolysis, by the same enzyme, of the D-Ala-(D)-*meso*-diaminopimelic acid linkage in the *E. coli* bisdisaccharide peptide dimer. It may be that the KM endopeptidase works best as a carboxypeptidase since the link hydrolyzed in the *E. coli* peptide dimer (see Figure 3 in van Heijenoort *et al.*, 1969) and the link hydrolyzed in *B. rettgeri* peptide dimer (see Figure 8 in Guinand *et al.*, 1969) are in positions α to a free carboxyl group. It would be important that specific activity of the KM endopeptidase be also determined with the peptide dimer from *Bacillus subtilis* in which case, as briefly reported by Warth and Strominger (1968), the carboxyl group located on the D-carbon of *meso*-diaminopimelic acid is amidated.

A very incomplete hydrolysis, approximately equal to 50%, of the *L. acidophilus* bisdisaccharide peptide dimer into disaccharide peptide monomers (Table I) was brought about by incubating 15 mμmoles of the dimer with 18 μg of SA endopeptidase for 24 hr at 37° (final volume, 30 μl of 0.01 M Veronal buffer, pH 9). Under the same conditions of incubation, 10 mμmoles of tris-disaccharide peptide trimer (Table I), *i.e.*, containing 20 mμequiv of peptide bridges, was completely hydrolyzed into disaccharide peptide monomers. Paper electrophoresis study of the kinetics of the enzymatic degradation of the trimer obviously showed that the direct degradation of the trimer into monomers by concomitant hydrolysis of the two bridges is a much faster reaction than the sequential degradation of the trimer *via* the transitory formation of dimers. The trimer evidently appears to fit much better the substrate requirements of the peptidase than the dimer does. A high degree of polymerization, or perhaps a tridimensional netlike structure of the substrate, is thus likely to enhance the activity of the enzyme on the sensitive linkage. In agreement with this hypothesis, one can estimate, since about 250 mμequiv of peptide bridges is hydrolyzed for each solubilized mg of *L. acidophilus* inactivated walls and since 1 μg of enzyme dissolves, per hr, 3.6 μg of walls (Table I), that 1 μg of this enzyme hydrolyzes, per hr, 0.9 mμequiv of peptide bridges when it acts on walls, a value which is about 20 times higher than that observed if the trimer is used as substrate (Table I). With *S. aureus*, 1 μg of SA endopeptidase dissolves, per hr, 12.5 μg of walls, that is to say it hydrolyzes, per hr, 6 mμequiv of D-Ala-Gly linkages, a value which is only twice that observed if the bisdisaccharide peptide dimer is used as substrate. With *M. lysodeikticus*, similar estimations show that the rate of hydrolysis of N^{ϵ}-(D-Ala)-L-Lys linkage (50 mμequiv/mg of walls) does not depend on the substrate used, 5 mμequiv of linkages in the walls and 4.4 mμequiv in the peptide dimer being hydrolyzed, per hr, per μg of enzyme.

Further investigations must be done before an unfailing and reproducible procedure can be proposed for the preparation and the purification of the *Streptomyces* enzymes. Complications arise from the following observations. (1) Besides the enzymes which are described in this paper, the *Streptomyces* sp. secrete at least two endo-*N*-acetylmuramidases, *i.e.*, the F_1 (Muñoz *et al.*, 1966a) and the "32" (Ghuysen *et al.*, 1962) enzymes, both of which are powerful lytic agents toward the walls used as substrates for the endopeptidases. (2) The walls from a given bacterial species can be degraded by different endopeptidases, as, for example, those of *M. roseus* which are sensitive both to the SA and the MR endopeptidases. (3) The resulting effect of two enzyme preparations working in conjunction upon a sensitive wall substrate is sometimes synergistic (Ghuysen and Salton, 1957) or inhibiting (see the above discussion on the influence of the degree of polymerization of the substrate on the enzymatic activity). (4) *Streptomyces* sp. are susceptible to variations. From the foregoing it follows that the result of a lytic test performed with any type of bacterial wall in order to control the selection and the growth of a *Streptomyces* strain is only indicative of the secretion in the external medium of a "lytic complex." This lytic complex must be analyzed by fractionation before one can estimate the actual yield of the individual enzymes. Using this technique it has been repeatedly observed that batches of crude enzyme complexes, although prepared from the same selected strain grown under the same conditions, contained variable amounts of lytic endo-*N*-acetylmuramidases and endopeptidases. From the foregoing, it also follows that the procedure proposed for the enzyme preparation and purification must be refined by using well-defined soluble substrates specifically sensitive to each of the *Streptomyces* enzymes. Moreover, these substrates are needed to meaningfully determine the activity recovery and the improvement in specific activity (in terms of protein content) after each step of purification. This improvement has now been made possible since, as a result of the present work and of others recently presented (Campbell *et al.*, 1969; Guinand *et al.*, 1969; van Heijenoort *et al.*, 1969), such substrates have been isolated and are well characterized.

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A General Method for Fractionation of Nucleic Acids on the Basis of Sequence Homology*

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ABSTRACT: Techniques used for detection of homologous nucleic acid duplexes on nitrocellulose disks were adapted for use in preparative fractionation of genetically specific nucleic acids. The technique used consists of preparation of a deoxyribonucleic acid–nitrocellulose complex, drying the complex at high temperature, preincubation in a polymer solution, batch incubation of the deoxyribonucleic acid–nitrocellulose with the preparation to be purified, and subsequent elution of the nucleic acids of interest. Mixtures of labeled deoxyribonucleic acid from bacteria and bacteriophage were purified about 50-fold; recovery was about 70%. The same technique was applied to the preparation of messenger ribonucleic acid specific for T2 bacteriophage, yielding milligram quantities of T2 messenger ribonucleic acid. These techniques are applicable to a variety of experimental situations in which a relatively large amount of genetically specific nucleic acid is desired.

Methods of separation of nucleic acids on solid-phase nitrocellulose fall into two classes. The first depends only upon the interactions of the nucleic acids with the nitrocellulose; the second depends, in addition, upon interactions between complementary[1] sequences in the nucleic acids. Although both classes of methods may, in special circumstances, be used for preparative fractionation of genetically specific nucleic acids, the second class is applicable to a greater variety of problems in biochemistry and genetics.

Nygaard and Hall (1963) discovered that nitrocellulose membrane filter disks bind DNA–RNA hybrids

* From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. *Received June 4, 1968.* ORNL is operated by the Union Carbide Corp., Nuclear Division, for the U. S. Atomic Energy Commission. This research is supported, in part, by U. S. Public Health Service Grant GM-10,692 from the National Institute of General Medical Sciences.

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[1] The vocabulary of this paper was chosen to conform mainly with the usage suggested by Britten (1968). I have, however, retained the terms "homologous" and "heterologous" on the grounds that Britten's suggested term "identical" is almost certain to be understood in its ordinary sense, and is more likely to cause confusion in certain types of experiments (*e.g.*, strand separation) than is "homologous."