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STUDIES ON PEPTIDES, GLYCOPEPTIDES AND ANTIGENIC POLYSACCHARIDE-GLYCOPEPTIDE COMPLEXES ISOLATED FROM AN L-11 ENZYME LYSATE OF THE CELL WALLS OF *MYCOBACTERIUM TUBERCULOSIS* STRAIN H37Rv

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SUMMARY 1. Digestion of delipidated cell walls of *Mycobacterium tuberculosis* strain H37Rv by *Flavobacterium* L-11 enzyme caused cleavage of about 50% of the linkages between glycan and peptide moieties of the peptidoglycan and solubilization of 20% of the cell wall materials.

2. Solubilized cell wall materials were fractionated into portions containing peptides and amino acids (PA and PB), soluble glycopeptides consisting of amino sugars and animo acids (HM-3), and complexes of glycopeptide with polysaccharides of arabinose, galactose, and mannose (HM-1 and HM-2).

3. Electrophoretically homogeneous peptides were isolated from the PA and PB portions by ion exchange column chromatography. Analyses of constituent, N-terminal and C-terminal amino acids and Edman degradation revealed that these peptides were a tetrapeptide (L-Ala-D-Glu-DAP-D-Ala), a tripeptide (L-Ala-D-Glu-DAP), octapeptides consisting of two molecules of the tetrapeptides, and a heptapeptide made up of one tetrapeptide and one tripeptide. Determination of terminal amino acids liberated by treatment of the octapeptides and the heptapeptide with D-alanyl-DAP endopeptidase of *Streptomyces* L-3 enzyme proved that these peptides consisted of dimeric forms of peptide subunits and had a cross link between the carboxyl group of C-terminal D-alanine of one tetrapeptide subunit and the α- or α'-amino group of α,α'-diaminopimelic acid of another tetra- or tripeptide subunit. Besides the peptides described above, evidence for a new tetrapeptide subunit (D-Ala-D-Glu[^{2}Gly]^{L}DAP) was obtained. It was also shown that the carboxyl groups of glutamic acid and α,α'-diaminopimelic acid residues of the peptides isolated were substituted to various extents by ammonia, and these substituted carboxyl groups were deamidated by the action of the L-3 enzyme.

4. The antigenicities of five representative subfractions obtained by re-gelfiltration of HM-1 and HM-2 were studied by the agar gel precipitin test with anti-whole cell rabbit serum. Three of them, which were complexes of glycopeptide and poly-

saccharides with different contents of hexose and pentose, gave a single precipitin line fusing with that of arabinogalactomannan isolated from cold tricholoroacetic acid extracts of the cell walls. The others, an arabinose polysaccharide with little glycopeptide and an arabinose-rich polysaccharide-glycopeptide complex, had no precipitating activities, but effectively inhibited precipitation of antiserum by the antigens.

5. A peptide moiety of glycopeptide subfractions obtained by re-gelfiltration of HM-3 contained glycine and aspartic acid besides alanine, glutamic acid and α,α'-diaminopimelic acid. Analyses of two reduced glycopeptide subfractions by periodate oxidation showed that their glycan moieties had average chain lengths of about nine and six amino sugar residues and that they had N-acylmuramic acid and N-acetylglucosamine respectively, at their non-reducing ends.

6. Evidence was obtained suggesting a possible role of phosphorus in linkage between glycopeptide and antigenic polysaccharide.

INTRODUCTION

A previous paper from this laboratory reported that BCG cell walls partially lysed by treatment with egg white lysozyme had a definitive sensitivity to the lytic action of *Flavobacterium* L-11 enzyme, but that native BCG cell walls were scarcely solubilized by this enzyme (Kotani et al., 1962, 1963). The enzyme preparations used at that time were obtained from the culture supernatants of *Flavobacterium sp.* (L-11 bacterium) grown without aeration in 0.1% casamino acid medium. They were active on the cell walls of *Staphylococcus aureus* and *Micrococcus lysodeikticus*, but did not solubilize the walls of group A *Streptococcus pyogenes* and *Corynebacterium diphtheriae*. But the lytic spectrum of the enzyme was broadened by improving the conditions of culture of the L-11 bacterium. Thus the enzyme(s) produced by bacterium grown with aeration in 2% glucose-broth effectively solubilized the cell walls of group A *S. pyogenes* (Hamada et al., 1968), *C. diphtheriae* (Matsuda et al., 1967), *Lactobacillus plantarum* (Matsuda et al., 1968) and other bacteria, which had previously been refractory to the L-11 enzyme.

The susceptibility of mycobacterial cell walls to the L-11 enzyme therefore was reinvestigated. Some enzyme preparations were found to have a definite lytic action on the native cell walls of *Mycobacterium tuberculosis* strain H37Rv. The present work was to determine the chemical and immunological properties of some structural units of the cell walls of this organism, solubilized by digestion with the L-11 enzyme.

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MATERIALS AND METHODS

1. *Preparation of cell walls*

M. tuberculosis strain H37Rv was grown as a surface pellicle at 37 C for three weeks on Sauton's synthetic medium dispensed in Roux's bottles. The culture was mixed with formalin at 0.2% final concentration and stood overnight at 4 C with occasional shaking. Cells were collected on a glass filter, and washed with deionized water and acetone. Acetone-dried cells (70 g) were disrupted with a Braun mechnical cell homogeniser (Model MSK, B. Braun Apparatebau Melsungen, West Germany). The cells (5 g) were placed in a 75 ml shaking flask with 30 g of glass beads (0.10 to 0.11 mm diameter, B. Braun cat No. 54140) and 35 ml of 0.01 M potassium phosphate buffer, pH 7.0 containing 0.1% Tween 80 and 1 M sodium chloride. The flask was

shaken for 20 min at 2,000 oscillation/min, and was cooled continuously with a stream of liquid carbon doxide passing around it through a flexible steel capillary tube. The contents of the flask were checked for unbroken cells by acid-fast stain (the Ziehl-Neelsen method), and disruption of cells seemed to be satisfactory. The disrupted cell suspension derived from 70 g of starting material, was separated from the glass beads, and centrifuged at 13,000×g for 20 min. The precipitate was reexamined with acid-fast stain and was found to be heavily contaminated with unbroken cells. The "disrupted" cells, therefore, were retreated in the Braun homogeniser. 3 g (wet weight) portions of the "disrupted" cells were shaken in 30 ml of deionized water containing 0.25 ml of tri-*n*-butylphosphate as an antifoaming agent with 30 g of glass beads (0.17 to 0.19 mm diameter, cat. No. 54150) at 2,000 oscillations/min for 15 min, as described above. The redisrupted cell suspension was separated from the glass beads and centrifuged at 1,000×g for 20 min. The supernatant fluid containing practically no unbroken cells was reserved, and the sediment was again disrupted in the homogeniser in the same way. The contents of the flask were separated from the glass beads. They were essentially free from acid-fast cells, and were combined with the supernatant fluid obtained previously. This pooled suspension of disrupted cells was centrifuged at 10,000×g for 20 min. The sediment was suspended in 210 ml of 0.1 M Tris-HCl buffer, pH 7.2, containing 40 mg pronase P (Kaken Chemicals Co., Tokyo; 4,500 P. U. K./g) and 5% (v/v) ethanol, and was incubated at 45 to 50 C for 18 hr. The digested cell walls were centrifuged, and redigested with pronase P as described above. Fully digested cell walls were washed thoroughly with deionized water, and lyophilized. The yield was 3.681 g.

A portion (3.5 g) of the purified cell walls was extracted with 70 ml of chloroform-methanol (2: 1, v/v) at room temperature for one day. The extracted walls were collected on a sintered glass filter, and reextracted. The extraction procedures were repeated until the extracts left no significant amount of residue on evaporation (ten times in all). Delipidated cell walls obtained in this way (2.71 g dry weight) were used as the strating material for the L-11 enzyme digestion in this study.

2. *Cell wall lytic enzyme preparations*

L-11 enzyme: The crude enzyme preparation used here (lot D24) was obtained by ammonium sulfate precipitation from the culture supernatant of the L-11 bacterium grown at 28 C with vigorous aeration in 2% glucose-broth in a 200 liter tank. The culture was grown at the Fuji pilot plant (Mishima, Shizuoka) of the Kyowa Fermentation Industry, Tokyo. A portion (2 g) of the crude enzyme was dissolved in 20 ml of 0.01 M potassium phosphate buffer, pH 8.0, and applied to a Sephadex G-75 column (3.5×75 cm). Fractions (10 ml each) eluted with the above buffer at a flow rate of 60 ml/hr were assayed for lytic activity on *S. aureus* (strain Copenhagen) cell walls. The assay was performed by incubating 0.1 ml aliquots of fractions with 0.1 ml portions of a cell wall suspension (2 mg/ml) in 0.01 M potassium phosphate buffer, pH 8.0, at 37 C. The extent of lysis of the cell walls was determined by visual inspection after one and three hr incubation. Fractions showing strong lytic activity were pooled. This partially purified enzyme preparation had a potency of 5.6 units/ml. Potency was determined as previously reported (Suguinaka et al., 1967).

L-3 enzyme: A partially purified specimen prepared by Dr. T. Katayama in this laboratory was used. This enzyme specimen was obtained from a crude enzyme preparation by negative adsorption using a DEAE-cellulose column and then fractionation by isoelectric focusing. The crude preparation was obtained by precipitation with ammonium sulfate from the culture filtrate of *Streptomyces sp.* (L-3 bacterium) grown at 30 C for seven to ten days in a modification of McCarty's medium (Mori et al., 1960, Mori and Kotani, 1962).

3. *Analytical procedures*

Free and total amino groups, and total amino sugars: These were determined by the method of Ghuysen, Tipper and Strominger (1966). For determination of total amino groups and amino sugars, test specimens were hydrolyzed in 4 N HCl for 8 hr and in 3 N HCl for 4 hr at 100 C, respectively.

Amino acids and amino sugars: The amino acid and amino sugar composition of the delipidated cell walls were determined on a hydrolyzate (with 6 N HCl at 100 C, for 14 hr) using a Hitachi KLA-3 Amino Acid Analyzer (Hitachi Ltd., Tokyo). Determination of the constituent amino acids in peptides and of amino acids isolated from cell wall digests, was made on hydrolyzed specimens (4 N HCl at

100 C, for 8 hr unless otherwise stated) by thin layer and paper chromatographies by the methods of Ghuysen, Tipper and Strominger (1966) and of Primosigh et al. (1961), respectively.

N-terminal and C-terminal amino acids: The method used was essentially that of Ghuysen, Tipper and Strominger (1966). Hydrazinolysis was performed by treatment of dried specimen with redistilled, anhydrous hydrazine at 100 C for 7 hr.

Edman degradation analysis: A modification of the method of Tipper et al. (1967) was employed (Kato et al., 1968).

Determination of isomers of alanine and glutamic acid: The isomers of total alanine were determined as follows. An aliquot (containing about 50 mμ moles of total alanine) of the hydrolyzed specimen was incubated with 20 μl of a mixture consisting of 30 μl of D-amino acid oxidase solution, 200 μl of a catalase solution (2 mg/ml, crystalline preparation from bovine liver, potency 19,000 Sigma units/mg, Sigma Chemicals Co., St. Louis, Mo., U.S.A.), and 1,500 μl of 0.1 M pyrophosphate buffer, pH 8.3. After 3 hr incubation at 37 C, the reaction mixture was heated at 100 C for 3 min to stop the enzyme action. The oxidase was omitted from the control mixture. The alanine contents of the test and control mixtures were measured by thin layer chromatography. The amounts of D-alanine reduced and L-alanine remaining after D-amino acid oxidase treatment were calculated. For detrmination of the optical configuration of N-terminal and C-terminal alanine residues, a test specimen was subjected to dinitrophenylation, and then hydrolyzed. The hydrolyzate was dried *in vacuo* and dissolved in deionized water. The solution was shaken with *n*-butanol to remove DNP-alanine derived from the N-terminal residue. Alanine isomers of non-N-terminal alanine in the aqueous phase were assayed as described above. Then, the configuration of the N-terminal and C-terminal alanine residues were calculated.

L-Glutamic acid decarboxylase (from *Escherichia coli*, Seikagaku Fine Biochemicals, Tokyo) was used in determination of the isomers of total glutamic acid. An aliquot (containing 100 mμmoles of total glutamic acid) of hydrolyzed specimen was incubated with 125 μg of L-glutamic acid decarboxylase in 100 μl of 0.025 M acetic acid buffer, pH 4.4. After 3 hr incubation at 37 C, the reaction mixture was heated at 100 C for 3 min, and centrifuged at 2,000 $\times$ g for 10 min. The precipitate was washed. The supernatant and washing fluid were combined and dried *in vacuo*. The amount of isomers of glutamic acid residues was calculated from the difference in the glutamic acid contents of the test and decarboxylase-free control.

Ammonia: The ammonia content in hydrolyzate of test peptides was measured by the method of Okuda and Fujii (1966, a modification of the method of Fawcett and Scott, 1960). This method, however, was not applicable to Edman degradation products, since colour development was inhibited by the reagents. The microdiffusion method described by Conway (Ballentine, 1957) was used for determination of ammonia in Edman degradation products; ammonia was trapped in 0.01 M H_2SO_4 in the center well in the diffusion chamber and estimated by the method of Okuda and Fujii.

Neutral sugars: Qualitative analyses were done by paper chromatography. Test specimens were hydrolyzed in 2 N H_2SO_4 at 100 C for 4 hr, and neutralized with barium hydroxide. An aliquot of the supernatant was applied to Toyo Roshi, No. 51A, paper, and developed by the descending technique. *n*-Butanol-acetic acid-water (4: 1: 5, v/v/v) or ethylacetate-pyridine-water (8: 2: 1, v/v/v) was used as solvent. Spots of sugar were detected by the method of Trevelyan, Procter and Harrison (1950). Quantitative determinations of pentose and hexose were performed by the orcinol and anthrone methods as described by Ashwell (1957), respectively. Galactose was determined by the Galactostat system of Worthington Biochemical Corp. (Freehold, N. J., U.S.A.). Free reducing sugars were measured with unhydrolyzed specimens by the method of Park and Johnson (1949).

Phosphorus: This was measured by the method of Lowry et al. (1954).

Determination of average chain length and the non-reducing end of glycan by periodate oxidation: The procedure used was a modification of the method of Tipper, Strominger and Ensign (1967). Aliquots (240 μl, containing 150 to 200 mμmoles of amino sugars) of specimens were reduced with 24 μl of fresh, unbuffered 1.0 M sodium borohydride solution at room temperature for 5 hr. The reduced specimens were neutralized with 24 μl of 1.0 M acetic acid and supplemented with 360 μl of deionized water. For determination of formaldehyde liberated, the reduced specimen (324 μl) was mixed with 40 μl of 0.1 M sodium acetate buffer, pH 4.5 and 20 μl of 0.01 M sodium periodate and oxidized at room tem-

perature in the dark. At intervals, aliquots (60 μl) were withdrawn and immediately mixed with 20 μl of 1.0 M sodium arsenite to stop the oxidation, and frozen at −20 C. Formaldehyde liberated was measured by the method of Suzuki and Strominger (1960). Oxidation of N-acetylglucosamine, under the same conditions, served as a standard for formaldehyde production. Periodate consumption was determined as follows. An aliquot (50 μl) of the reduced and neutralized specimen was oxidized by addition of 50 μl of 0.02 M sodium periodate in 0.02 M sodium acetate buffer, pH 4.5, at room temperature in the dark. Aliquots of 5 μl were removed at intervals, and immediately diluted with 400 μl of deionized water. Periodate consumption was measured by the decrease in optical density at 225 mμ. An appropriate amount of ethyleneglycol was oxidized under the same conditions for 24 hr, as a reference to calculate the amount of periodate consumed from the optical density reduction.

4. *Immunological methods*

Preparation of antiserum: Acetone-dried cells (80 mg) of strain H37Rv were suspended in 2 ml of physiological saline containing 100 mg dihydrostreptomycin and 20,000 units of penicillin G. The suspension was vigorously mixed with 4 ml of Drakeol (Pennsylvania Oil Co., Pa., U.S.A.) and 2 ml of Arlacel A (Atlas Chemical Industry, U.S.A.) to make a water in oil emulsion. The antigen was injected into the foot pads (0.25 ml each, 1.0 ml in total) of normal adult rabbits weighing about 3 kg. Three similar injections were given at weekly intervals. One week after the fourth injection, the rabbits received one intraveneous injection of 2 ml of the cell suspension in physiological saline (2 mg/ml). They were bled one week later. These antiserum specimens were mixed with sodium azide (0.1%, final concentration), and stored at 4 C.

Gel diffusion method: A solution of 1% agar (Difco special noble agar, Difco Laboratories, Mich., U.S.A.) in 0.01 M veronal buffer, pH 8.0, was allowed to solidify on a glass plate (4.5×4.5 cm) to give a layer about 5 mm thick. A central well (for antiserum) and six outer wells (for antigens) were cut out as shown in Figs. 13 and 14. The diameter of wells was about 5 mm. Agar plates were kept in a cold room for 5 days and their precipitin lines were examined (Kabat and Mayer, 1961).

Precipitin inhibition test: An aliquot (70 μl) of antiserum was incubated with 10 μl of the test specimen or physiological saline at 37 C for 2 hr. The test and control serum specimens were allowed to react with appropriate antigens as described in the preceding paragraph.

RESULTS

1. *Composition of the delipidated cell walls*

The delipidated cell walls used in this study had the composition summarized in Table 1. They contained approximately one mole each of muramic acid, glucosamine, L-alanine, D-glutamic acid, α, α'-diaminopimelic acid (DAP), and 0.6 mole of D-alanine and 2.6 mole of bound ammonia as major components of the basal peptidoglycan. In addition 0.4 mole of L-glutamic acid was found together with small,

TABLE 1. *Composition of the delipidated cell walls of M. tuberculosis strain H37Rv*

Constituent	mμmoles/mg	Molar ratio	μg/mg	
Muramic acid	114	0.80	26.2	
Glucosamine	162	1.14	29.1	
L-Alanine	116	0.82	10.3	
D-Alanine	81	0.57	7.2	
L-Glutamic acid	60	0.42	8.8	
D-Glutamic acid	133	0.94	19.6	
α, α'-Diaminopimelic acid	142	1.00	27.1	136.7
Threonine	8.4	0.059	1.0	
Glycine	6.4	0.045	0.5	
Serine	2.3	0.016	0.2	
Aspartic acid	2.1	0.015	0.3	
Ammonia[a]	374	2.63	6.4	
Hexose (as galactose)	728	5.13	132	
Pentose (as arabinose)	880	6.20	132	268.4
Phosphorus	143	1.01	4.4	
(Total)			405.1	

Other amino acids detected in trace amounts were lysine, leucine, isoleucine and valine.

Corrections for the destruction of constituents, especially of muramic acid, during the hydrolysis of a test specimen were not done.

[a] Estimated by the method of Okuda and Fujii.

but significant amounts of threonine (0.059 mole), glycine (0.045 mole), serine (0.016 mole), and aspartic acid (0.015 mole). Trace amounts of lysine, leucine, isoleucine, and valine were also detected. The total amount of these major and minor amino acids and ammonia was about 14% of the delipidated cell walls on a weight basis. Polysaccharides of arabinose, galactose, and mannose, on the other hand, constituted one quarter of the delipidated walls. Phosphorous (one mole/mole of DAP) was also an essential component. More than half the delipidated cell walls by weight was not accounted for by the above-mentioned constituents. The bulk of this unidentified material seemed to consist of mycolic acids, since high contents of these characteristic fatty acids are reported to be present in mycobacterial cell walls (Kotani et al., 1963; Kanetsuna, 1968; and others). But their content was not determined in this study.

Analyses of N-terminal and C-terminal amino acids indicated that the DAP residues with one amino group free (these are referred to hereafter as mono-NH_2-DAP) was less than 10% of the total DAP residues, and there was a negligible amount of amino acid residues with free carboxyl groups (alanine and glycine were detected as C-terminals, but their amounts were too small to estimate accurately). The peptidoglycan of the strain H37Rv cell walls seemed to have extensive cross-linkages between basal peptide subunits through one of the amino groups of DAP residues.

2. *Digestion of delipidated cell walls with the L-11 enzyme*

A portion (2,500 mg) of the delipidated cell walls was digested by incubation with 1,500 units of the L-11 enzyme at 37 C for 144 hr. The enzyme was added in three portions; first 750 units and then 375 units each after 48 and 96 hr incubation. The total volume of digestion mixture increased from 250 ml initially to 500 ml at the end of the incubation. But the final concentrations of potassium phosphate buffer, pH 8.0, and sodium azide (as preservative) were kept constant at 0.01 M and 0.1% respectively.

Determination of the optical density and free amino and reducing groups on aliquots withdrawn from the incubation mixture at intervals and heated at 100 C for 5 min to stop the reaction, showed that the delipidated cell walls were solubilized by the L-11 enzyme, liberating a maximum of 340 mμmoles of free amino groups/mg cell walls (Fig. 1). No detectable increase in free reducing sugars occurred during the incubation. Solubilization of the cell walls was incomplete, and the decrease in optical density of the incubation mixure did not exceed 41%. For analysis of amino acid residues with amino groups liberated by the enzyme action, aliquots of the incubation mixture were subjected to dinitrophenylation and hydrolyzed, and the N-terminal amino acids were measured by thin layer chromato-

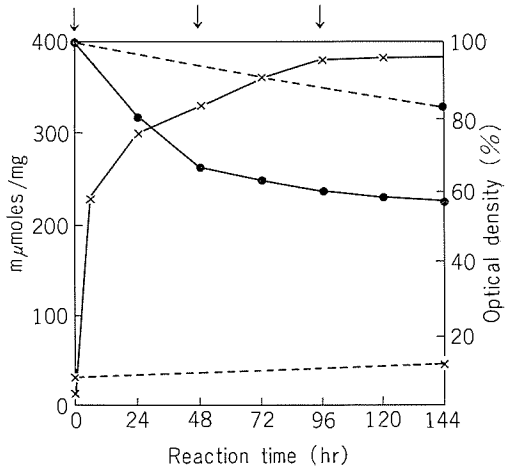


FIGURE 1. *Solubilization of the delipidated cell walls with liberation of free amino groups by the L-11 enzyme.*

↓ *Time of addition of enzyme*
●——● *Optical density (test)*
●------● *Optical density (control)*
×——× *Free amino groups (test)*
×------× *Free amino groups (control)*

There was no significant liberation of reducing groups under the action of the L-11 enzyme.

graphy. As shown in Fig. 2, N-terminal alanine (L-isomer) and mono-NH_2-DAP increased during the incubation (60 and 16 mμmoles/mg cell walls, and about 0.5 and 0.1 mole/mole of total DAP residues, respectively). Possible release of C-terminal amino acids was studied by the hydrazinolysis method. Small amounts (about 10 mμmoles/mg) of alanine, glycine, and glutamic acid were recovered from the hydrazinolyzate of the digested cell walls. The significance of these small amounts is questionable in view of the inherent errors of the analysis. The finding that 0.5 mole/mole of DAP of N-termianl L-alanine was liberated without concomitant liberation of C-terminal amino acids indicates that about half the N-acylmuramic acid→L-alanine linkages between the glycan and peptide moieties in the peptidoglycan were hydrolyzed by the L-11 enzyme. There is no direct evidence for release of the carboxyl groups of muramic acid residues, but the above result is supported by the observation that more than half the total amino groups of the delipidated cell walls were recovered in the lower molecular weight portions which contained neither amino sugars nor neutral sugars (*see* Table 2). The significance of the liberation of mono-NH_2-DAP is questionable, since the amount was small and the concomitant liberation of C-terminal amino acid was not confirmed as described above. The increase of mono-NH_2-DAP during digestion of the cell walls may be explained by supposing that measurement of free amino groups of DAP residues in peptidoglycan becomes more efficient as the peptidoglycan becomes smaller as the result of cleavage of linkages between peptide and glycan moieties. Matsuda et al. (1968) in a study on the digestion of *L. plantarum* cell walls by the L-11 enzyme noted an increase in the number of mono-NH_2-DAP residues, presumably by the same mechanism. However, it is possible that the increase in mono-NH_2-DAP is due to hydrolysis of linkages involving the amino groups of DAP residues. It should be added in this connection that, unlike the enzyme used in a previous study, one preparation of the L-11 enzyme was found which effectively hydrolyzed the D-alanine→DAP linkages in *L. plantarum* cell wall peptidoglycan (unpublished observation).

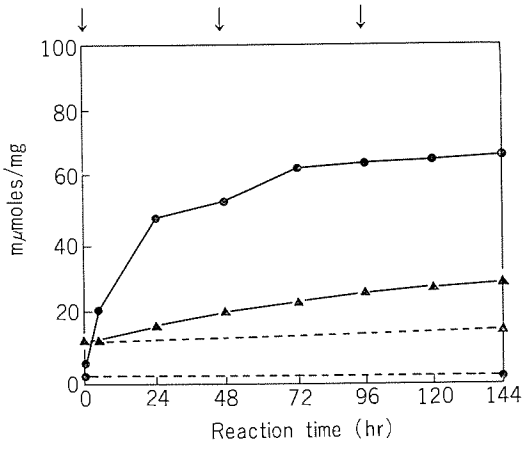


FIGURE 2. *Liberation of N-terminal amino acids by hydrolysis of the delipidated cell walls with the L-11 enzyme.*

↓ *Time of addition of enzyme*
●——● *N-terminal alanine (test)*
●------● *N-terminal alanine (control)*
▲——▲ *mono-NH_2-DAP (test)*
▲------▲ *mono-NH_2-DAP (control)*

3. *Fractionation of an L-11 enzyme digest of the delipidated cell walls*

A digest from 2,500 mg of delipidated cell walls incubated with the L-11 enzyme for 144 hr was centrifuged at 10,000×g for one hr. The insoluble residue (1,975 mg in a lyophilized state, about 79% of the delipidated cell walls) was washed twice with 20 ml portions of deionized water. The supernatant and two washing fluids were combined and concentrated to about 2 ml under reduced pressure at room temperature using a rotary evaporator. The concentrated solution (solubilized cell wall matrials) was submitted to gel filtration on three successive Sephadex columns (1.5 to 2.0 cm diameter×98 cm length) of G-50 fine, G-50 coarse, and G-25 coarse gels (all beads; Pharmacia, Uppsala, Sweden), connected in series by capillary polyethylene tubes. The

external (Vo) and internal (Vi) volumes were determined by filtration of blue dextran 2,000 (Pharmacia) and sodium chloride, respectively. The material was applied to the top of the G-50 fine column. Elution was carried out with deionized water at a flow rate of 30 ml/hr. Aliquots of each fractions of eluate (10 ml) were assayed for free and total amino groups, amino sugars, hexoses and pentoses. The results in Fig. 3 and Table 2 show that the solubilized cell wall materials were roughly separated into five portions. The higher moelcuar weight fractions, HM-1 and HM-2, contained virtually all of neutral sugars and some of the amino sugars and total amino groups. These two portions differed from each other in their relative contents of hexose and pentose. The fraction of medium molecular weight, HM-3, consisted of amino sugars and amino acids, and contained only little neutral sugars. The lower molecular weight fractions (PA and PB), on the other hand, consisted entirely of peptides or amino acids. Sodium azide, added to the incubation mixture as a preservative, was eluted in the fractions following the PB portion. The fractions containing sodium azide developed an unusual colour in assays of free and total amino groups. No amino acids were detected by paper or thin layer chromatography in hydrolyzates of these fractions.

As shown in Table 2, a little more than 50% of the total amino groups in the delipidated walls, was recovered in the PA and PB portions, while about 30% remained in the insoluble residue. Nearly half the amino sugars solubilized were eluted in the HM-3 portion with a little hexose and pentose. The bulk of solubilized neutral sugars (less than 30% of the total neutral sugars present in the delipidated cell walls), on the other hand, was obtained in the HM-1 and HM-2 portions with amino sugars and total amino groups. Thus, the digest of the delipidated cell walls was roughly separated into neutral polysaccharide-glycopeptide complexes (HM-1 and HM-2), soluble glycopeptide (HM-3), and peptides and amino acids (PA and PB). This was confirmed by the following analyses.

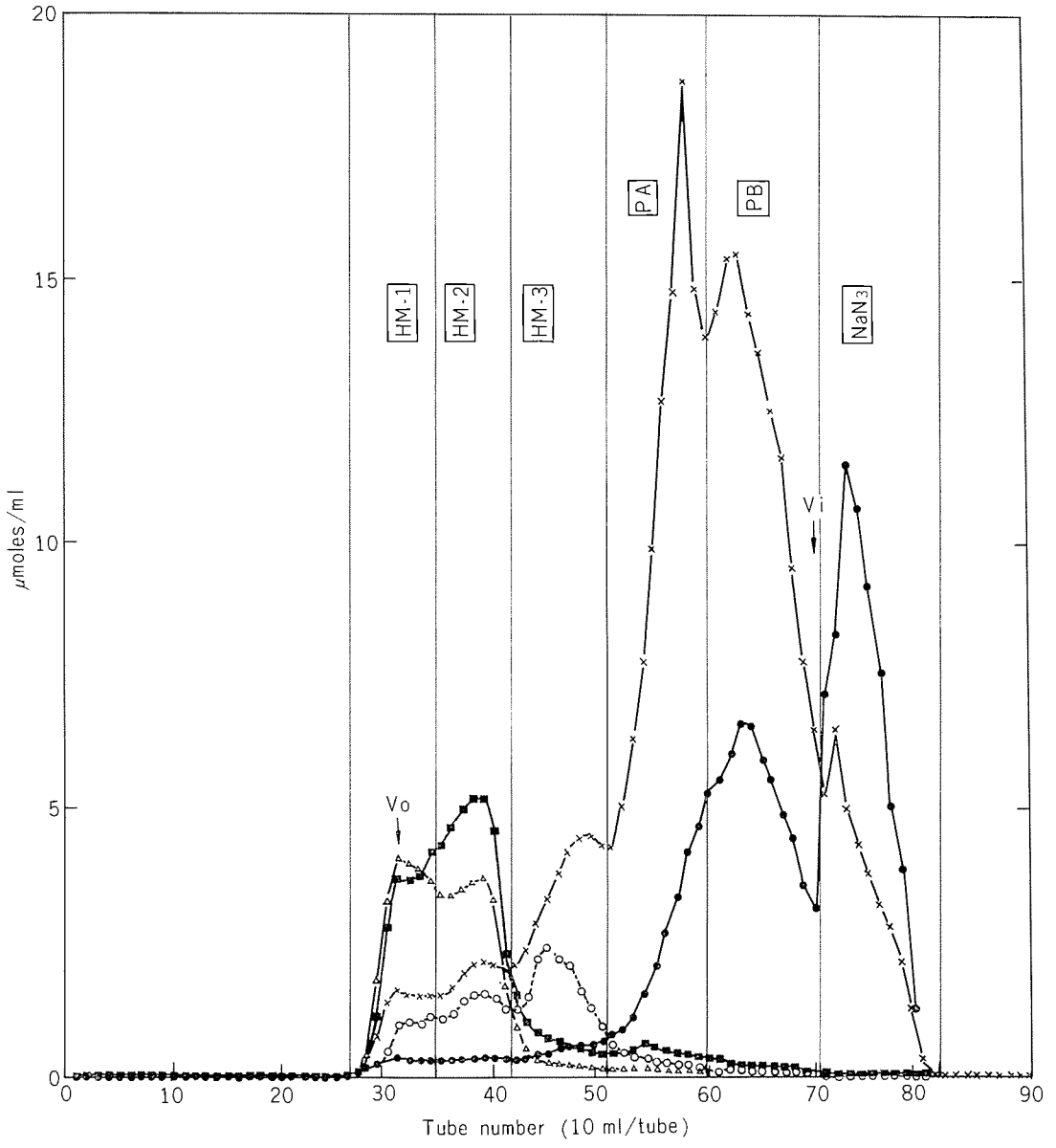
4. *Isolation of monomeric and dimeric forms of basal peptide subunits and amino acids from the PA and PB portions*

The PA and PB portions were each concentrated to about 2 ml using a rotary evaporator. These concentrated solutions were each acidified with formic acid (2% in a final concentration), and applied to a column (0.9 cm × 70 cm) of Amberlite CG-120 (type 2; Rohm and Haas, Philadelphia, Pa., U.S.A.) and chromatographed as described in previous papers (Kato et al., 1968a). Elution was performed first with 500 ml of 0.2 M pyridine-acetate buffer, pH 3.1, and then by linear gradient elution with 500 ml of the above buffer in the mixing chamber and 500 ml of 2.0 M pyridine-acetate buffer, pH 5.0 in the reservoir. The temperature of the column was maintained at 50 C during elution. Fractions of 10 ml were collected and aliquots of each were assayed for

TABLE 2. *Distribution of the cell wall constituents in five soluble fractions and an insoluble residue*

Constituent	Insoluble residue	Soluble fractions						Recovery
		HM-1	HM-2	HM-3	PA	PB	Total	
Total amino groups	33.5	3.7	4.6	14.2	31.0	32.2	85.7	119.2
Amino sugars	45.2	7.7	11.4	22.3	0	0	41.4	86.6
Hexoses	63.8	13.1	14.9	3.6	0	0	31.6	95.4
Pentoses	70.2	11.2	8.8	1.1	0	0	21.1	91.3

Values are expressed as percentage of the contents of the respective constituents in the delipidated cell walls.



×——× *Total amino groups*
●——● *Free amino groups*
○------○ *Amino sugars*
△——△ *Pentoses*
■——■ *Hexoses*

FIGURE 3. *Gel filtration on successive columns of Sephadex G–50 fine, G–50 coarse and G–25 coarse of the cell wall materials solubilized by digestion with the L–11 enzyme.*

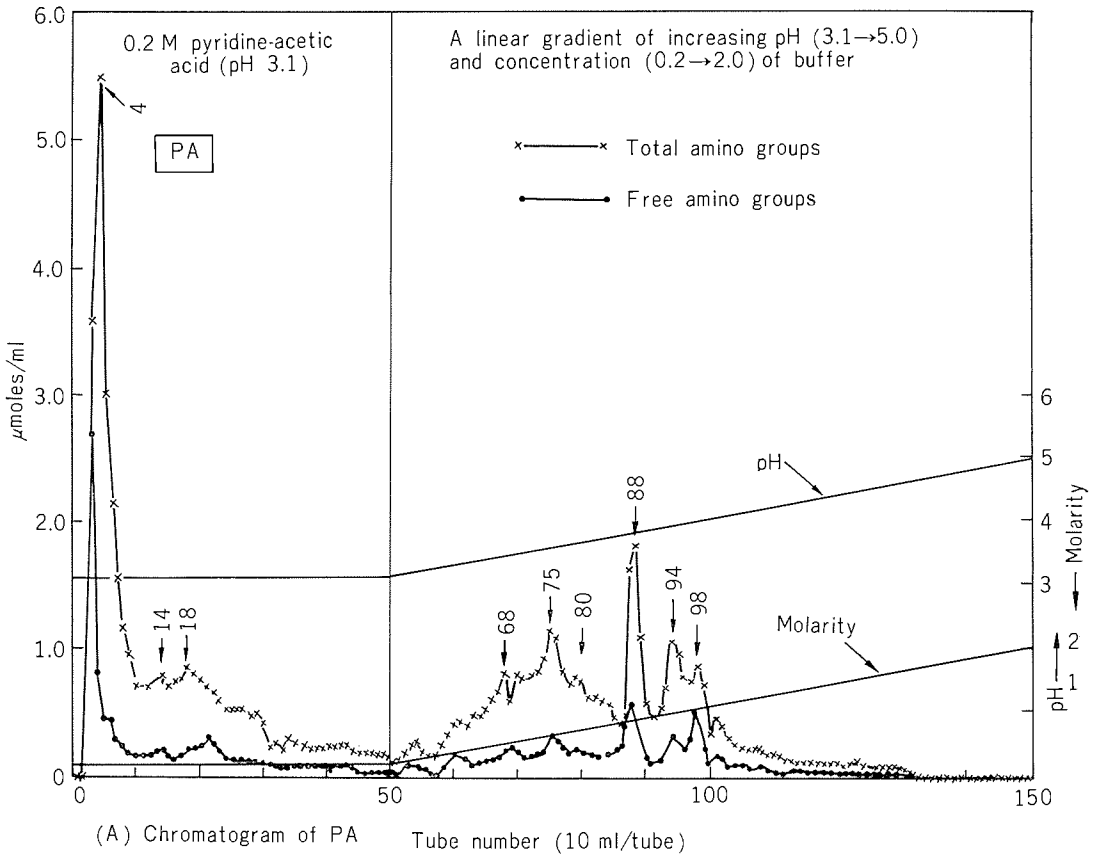


FIGURE. 4 *Fractionation of PA and PB by chromatography on an Amberlite CG-120 column.*

free and total amino groups. On chromatography of PB, linear gradient elution was not achieved for technical reasons. Therefore the fractions in tubes Nos. 51 to 150 were pooled, and evaporated to dryness to remove the pyridine-acetate buffer. The residue was dissolved in one ml of deionized water, and acidified with formic acid. The solution was applied to a column of Amberlite, and eluted with 200 ml of 0.2 M buffer, pH 3.1, and with a linear gradient of increasing pH and concentration of buffer.

The chromatograms obtained for PA and PB are shown in Fig. 4, (A) and (B). The peak fractions containing more than 0.75 μmole/ml* of total amino groups were subjected to paper electrophoresis at pH 1.9 and 5.0 to test their homogeneities. Among the peak fractions examined, PA-68 (this means the fraction eluted in tube No. 68; similar abbreviations are used hereafter), PA-75, PA-80, PA-88, PA-94, PA-98, PB-87, PB-93, and PB-96 gave a single or essentially single ninhydrin-positive spot on electrophoresis at both pH values, and so were sufficiently pure to use for chemical

* Further studies were abandoned about peak fractions which contained less than 0.75 μmoles/ml of total amino groups, since it was known in a previous work on the L-11 enzyme digests of BCG cell walls that shortage of the materials made it very difficult to perform sufficient analyses on such fractions.

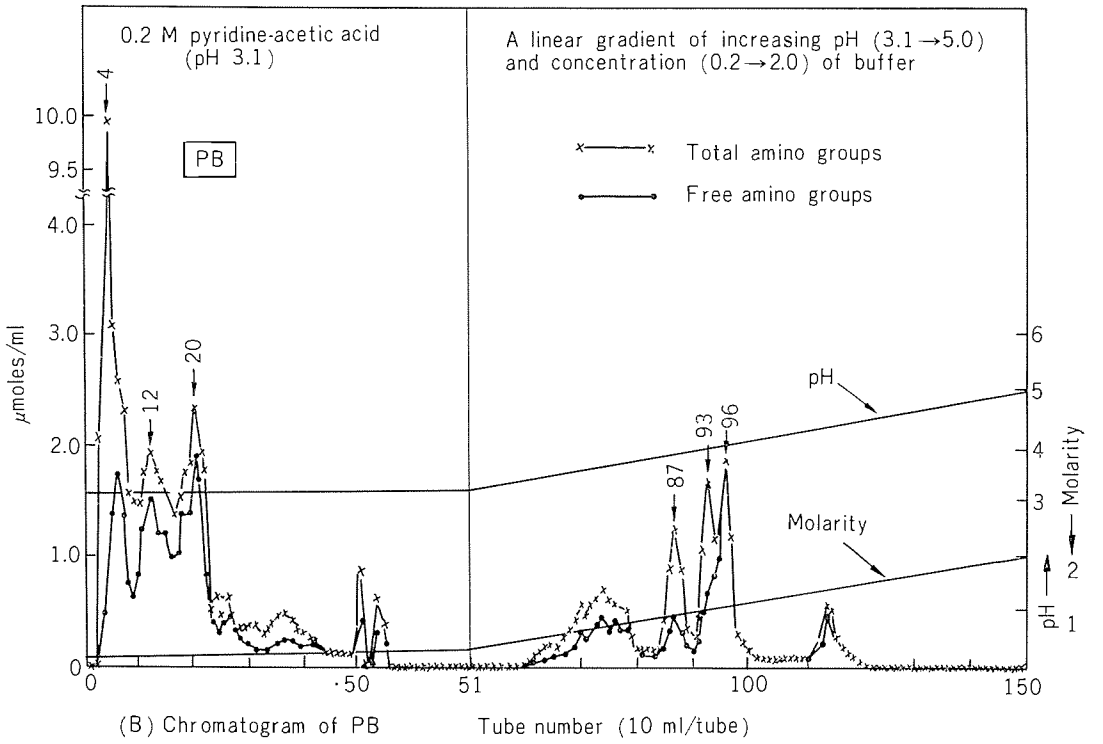


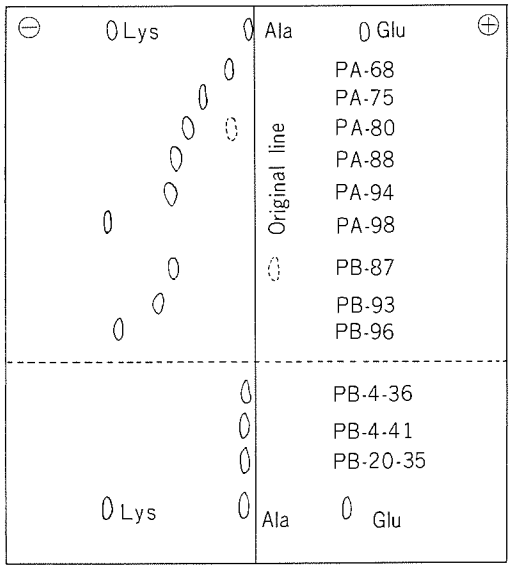
FIGURE 4. *Fractionation of PA and PB by chromatography on an Amberlite CG-120 column (continued).*

analyses without further purification (Fig. 5). PA-4, PA-14, PA-18, PB-4, PB-12 and PB-20 were not homogeneous, giving two or more ninhydrin-positive spots on electrophoresis at one or both pH values. Therefore, the contents of tubes around each of the latter fractions, except PA-14 and PA-18, were pooled separately (For example, the contents of tubes Nos. 16 to 23 were combined in the case of PB-20). PA-14 and PA-18 were not purified further because their contents of total amino groups were low. The pooled fractions were each concentrated to about 2 ml in a rotary evaporator. The concentrated solutions were adjusted to pH 9.0 with 2 N NaOH, and applied to a Dowex 1 column (0.9 cm × 70 cm) equilibrated with collidine-pyridine-acetic acid buffer, pH 8.0 (Schroeder et al., 1962). Stepwise elution was carried out as illustrated in Fig. 6. The peak fractions, PB-4-36 (a fraction eluted in tube No. 36 by Dowex 1 column chromatography of pooled fraction PB-4), PB-4-41 and PB-20-35 were shown to be electrophoretically homogeneous (*see* Fig. 5). PB-4-4 was ninhydrin-negative, and seemed to consist of substances other than peptides or amino acids, possibly derived from the resin.

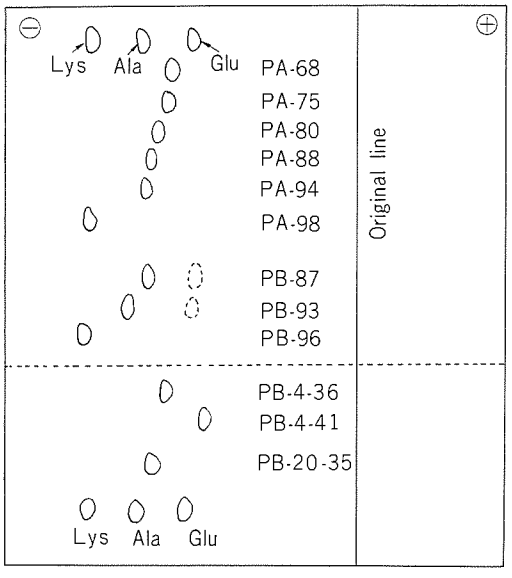
5. *Identification of peptides and amino acids isolated from the L-11 enzyme digest of the delipidated cell walls*

1) Monomeric peptide subunits

The contents of total, N-terminal and C-terminal amino acids and ammonia of PB-87, PB-93, and PB-4-41 are shown in Table 3. The data are expressed as molar ratios to the total DAP residues. Fig. 7, (A) and (B), illustrate the chemical structures of PB-87 and PB-93 respectively, which are compatible



(A) 0.1 M pyridine-acetic acid, pH 5.0



(B) Formic acid-acetic acid-water
(5 : 15 : 80, v/v/v), pH 1.9

FIGURE 5. *Patterns of electrophoresis of peptides and amino acids isolated from PA and PB by ion exchange chromatography.*
Voltage: 25 volt/cm Time: 150 min

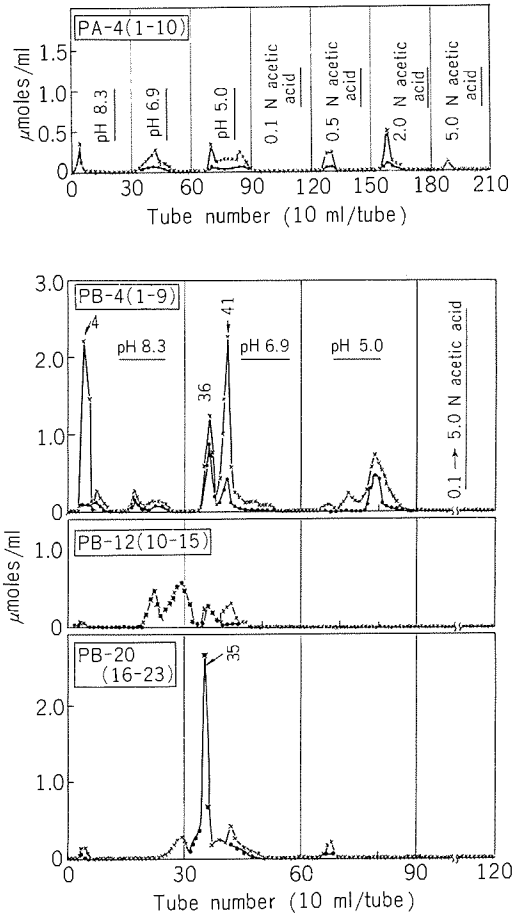


FIGURE 6. *Fractionation of fractions from Amberlite by chromatography on Dowex 1 column.*

×——× *Total amino groups*
●——● *Free amino groups*

with the analytical results. The sequence of amino acids in the tetrapeptide, PB-87, was determined by Edman degradation analysis. In the first cycle of degradation, N-terminal alanine and mono-NH_2-DAP disappeared and these were replaced by N-terminal glutamic acid. The N-terminal glutamic acid disappeared during the second cycle of degradation, but no new N-terminal amino acids appeared. From the proposed structure, ammonia should be released at this stage, but this could not be

TABLE 3. *Analyses of total, N-terminal and C-terminal amino acids, and ammonia in monomeric peptide subunits*

Constituent	PB-87		PB-93		PB-4-41	
	A	B	A	B	A	B
L-Ala	1.2	1.0	0.9	1.0	1.7	1.7
D-Ala	0.9	1.0	0	0		
Glu	0.9	1.0	1.0 (D)	1.0 (D)	1.1	1.0
DAP	1.0	1.0	1.0	1.0	1.0	1.0
Gly					0.3	0.3
NH_3	2.3	2.0	2.3	2.0	0.7	1.0
NH_2-Ala	1.0 (L)	1.0 (L)	1.0 (L)	1.0 (L)	0.9	1.0
mono-NH_2-DAP	1.0	1.0	1.1	1.0	0.9	1.0
Ala-COOH	1.1 (D)	1.0 (D)	tr	0	0.8	0.7
DAP-$(COOH)_2$			0	0	0	0
Gly-COOH					0.3	0.3

Values are expressed as moles per mole of total DAP.
A: Experimental values
B: Values calculated on the basis of the proposed structure (see Fig. 7).
L or D in parenthesis denotes the L-isomer or D-isomer, respectively.

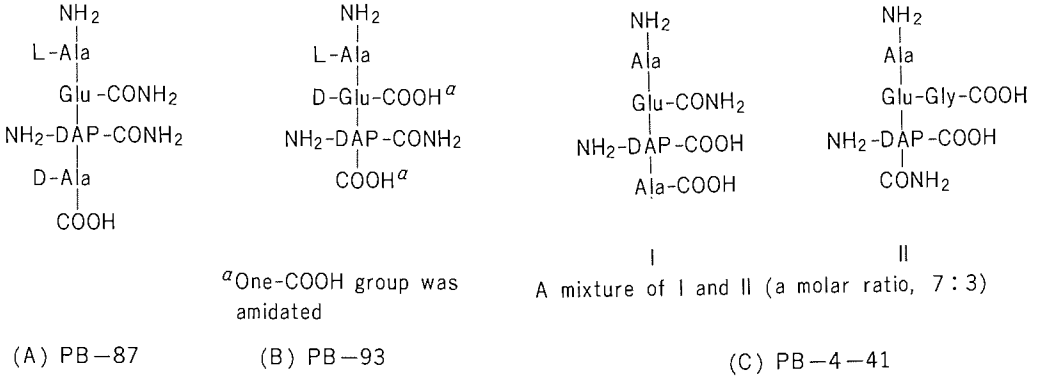


FIGURE 7. *Possible structures of the peptide monomer subunits.*

shown by the method of Okuda and Fujii used in this experiment since Edman reagents interfered with the reaction.

PB-4-41 was rather complicated. There was two kinds of C-terminal amino acid; 0.8 mole of C-terminal alanine and 0.3 mole of C-terminal glycine/mole of total DAP residue. The molar ratios of glycine (0.3 mole) and ammonia (0.7 mole) to total DAP residues were far from unity. These facts suggest that PB-4-41 may be a mixture of two peptides, not separated by electrophoresis; that is (L-)Ala-(D-)Glu$^{\alpha}$(Gly)$^{\varepsilon}$DAP and (L-)Ala-(D-)Glu($^{\alpha}CONH_2$)$^{\varepsilon}$DAP-(D-)Ala in a molar ratio of 3 : 7, as illustrated in Fig. 7, (C). This possibility was supported by the findings that during the second cycle of the Edman degradation, about 0.3 mole of glycine and about 0.7 mole of ammonia appeared in place of the one mole of N-terminal glutamic acid which had replaced about one mole each of N-terminal alanine and mono-NH_2-DAP in the first cycle

TABLE 4. *Analyses of total, N-terminal and C-terminal amino acids and ammonia in dimers of peptide subunits*

Constituent	PA-68	PA-75	PA-80	PA-88	Octa-peptide[a]	PA-94	Hepta-peptide[a]
L-Ala	2.0	2.0	2.0	1.1	1.0	0.9	1.0
D-Ala				1.0	1.0	0.5	0.5
Glu	1.1	1.1	1.1	1.1 (D)	1.0 (D)	0.9	1.0
DAP	1.0	1.0	1.0	1.0	1.0	1.0	1.0
NH_3	0.6	1.0	1.5	2.5	0.5~2.0	1.8	2.0
NH_2-Ala	0.9	1.1	1.1	1.1 (L)	1.0 (L)	0.8 (L)	1.0 (L)
mono-NH_2-DAP	0.4	0.5	0.5	0.6	0.5	0.4	0.5
Ala-COOH	0.4	0.4	0.6	0.5 (D)	0.5 (D)	0.2	0
DAP-$(COOH)_2$						0	0

Values are expressed as moles per mole of total DAP.

[a] Values calculated on the basis of the proposed structures (see Fig. 8).

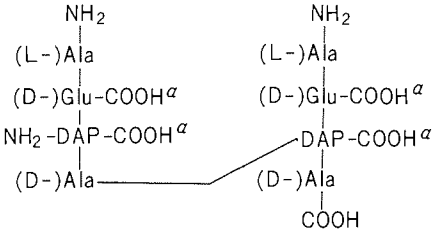
L or D in parenthesis denotes the L-isomer or D-isomer, respectively.

of degradation. In this experiment, the microdiffusion method of Conway which was not affected by Edman reagents was used to determine liberated ammonia.

2) Dimeric forms of peptide subunits

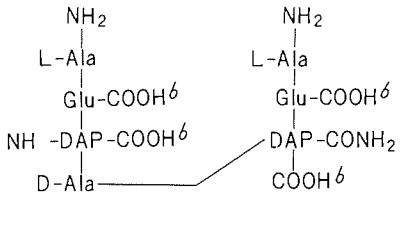
As summarized in Table 4, the peak fractions PA-68, PA-75, PA-80 and PA-88 had essentially the same total, N-terminal and C-terminal amino acid compositions. However, they differed in their ammonia contents. They all contained 2 moles of alanine (1 mole of L-isomer and 1 mole of D-isomer so far as determined), one mole of glutamic acid and one mole of DAP. Their N-terminal amino acids were alanine (about 1 mole/mole of total DAP residue) and mono-NH_2-DAP (about 0.5 mole), and their C-terminal amino acid was alanine (about 0.5 mole). Analyses of PA-88 showed that the glutamic acid residues were D-isomers, and the N-terminal and C-terminal alanine residues were the L- and D-isomers, respectively.

The above analytical results indicate that PA-68, PA-75, PA-80 and PA-88 are peptide dimers composed of two tetrapeptide monomers



[a] One, 2, 3 or 4 of the carboxyl groups of glutamic acid and DAP residues were substituted by ammonia (amidated) in PA—68, PA—75, PA—80 and PA—88, respectively.

(A) PA—68, PA—75, PA—80 and PA—88



[b] Three -COOH groups were amidated.

(B) Pa—94

FIGURE 8. *Possible structures of the dimeric forms of peptide subunits.*

TABLE 5. *Changes in N-terminal and C-terminal amino acids and ammonia caused by treatment of peptide monomers and dimers with the L-3 enzyme*

Constituent	PA-88		PA-94		PB-87		PB-93	
	A	B	A	B	A	B	A	B
NH_2-Ala	1.1	1.3(1.0)	0.8	1.0(1.0)	1.0	0.9(1.0)	1.0	1.1(1.0)
mono-NH_2-DAP	0.6	0.9(1.0)	0.4	0.7(1.0)	1.0	1.1(1.0)	1.1	1.0(1.0)
Ala-COOH	0.5	0.9(1.0)	0.2	0.4(0.5)	1.1	1.1(1.0)	tr	tr (0)
DAP-$(COOH)_2$	0	tr (0)	0	0.4(0.5)	0	0 (0)	0	0.5(1.0)
NH_3	2.5	0.6(0^a)	1.8	0.2(0^a)	2.3	0.7(0^a)	2.3	0.4(0^a)

Values are expressed as moles per mole of total DAP.

A: Values before treatment with the L-3 enzyme.

B: Values after hydrolysis by incubation with the L-3 enzyme.

Values in parenthesis are calculated assuming complete hydrolysis of the susceptible linkages by the L-3 enzyme.

[a] It was assumed that that the $CONH_2$ groups of (*iso*-)glutamine as well as DAP residues were deamidated.

(L-Ala-D-Glu-DAP-D-Ala) connected by a linkage between the carboxyl group of C-terminal alanine in one peptide subnit and α- or α'-amino group of the DAP residue in another. The carboxyl groups of glutamic acid and DAP residues were substituted to various degrees with ammonia (Fig. 8). PA-94 differed from the above octapeptides, in its total content of D-alanine (0.5 mole/mole of total DAP) and in the absence of C-terminal amino acids. PA-94 seems to be a heptapeptide composed of one tetrapeptide monomer and one tripeptide monomer (L-Ala-(D-)Glu-DAP) connected by a D-Ala→DAP linkage.

Edman degradation analysis was carried out on PA-88. In the first cycle of degradation N-terminal L-alanine (about 1 mole/mole of total DAP) and mono-NH_2-DAP (about 0.5 mole) disappeared and about 1 mole of glutamic acid became the new N-terminal. No terminal amino acids appeared during the second cycle. The DAP residues participitating in the linkage between two peptide monomers are not susceptible to the first cycle of degradation since their amino groups are not free. Therefore, these DAP residues should be detected as mono-NH_2-DAP during the second cycle of degradation if they are linked with α-carboxyl groups of the glutamic acid residues on the N-terminal side. This was not the case. Edman degradation is known to proceed along the α-peptide bonds, so the above finding suggests that the linkages between the glutamic acid and DAP residues in the basal peptide subunits are of the γ-type.

3) Treatment of peptide monomers and dimers with *Streptomyces* L-3 enzyme

Aliquots (300 mμmoles) of PA-88, PA-94, PB-87 and PB-93 were dried *in vacuo*. The residues were each redissolved in 300 μl of 0.02 M potassium phosphate buffer, pH 7.8 containing 2.4 units (against *C. diphtheriae* walls) of *Streptomyces* L-3 enzyme. The solutions were incubated at 37 C for 24 hr, to study the liberation of N-terminal and C-terminal amino acids, decrease in bound ammonia, and changes in electrical charge during digestion with the L-3 enzyme. The results are shown in Table 5.

Concomitant increases in mono-NH_2-DAP and C-terminal alanine occurred in PA-88 and PA-94. This suggests that the cross linkages in these peptide dimers were hydrolyzed by the D-alanine→DAP endopeptidase of the L-3 enzyme. The increase in C-terminal DAP in PA-94, on the other hand, is probably due to the action of the DAP-amide amidase of the L-3 enzyme. The liberation of am-

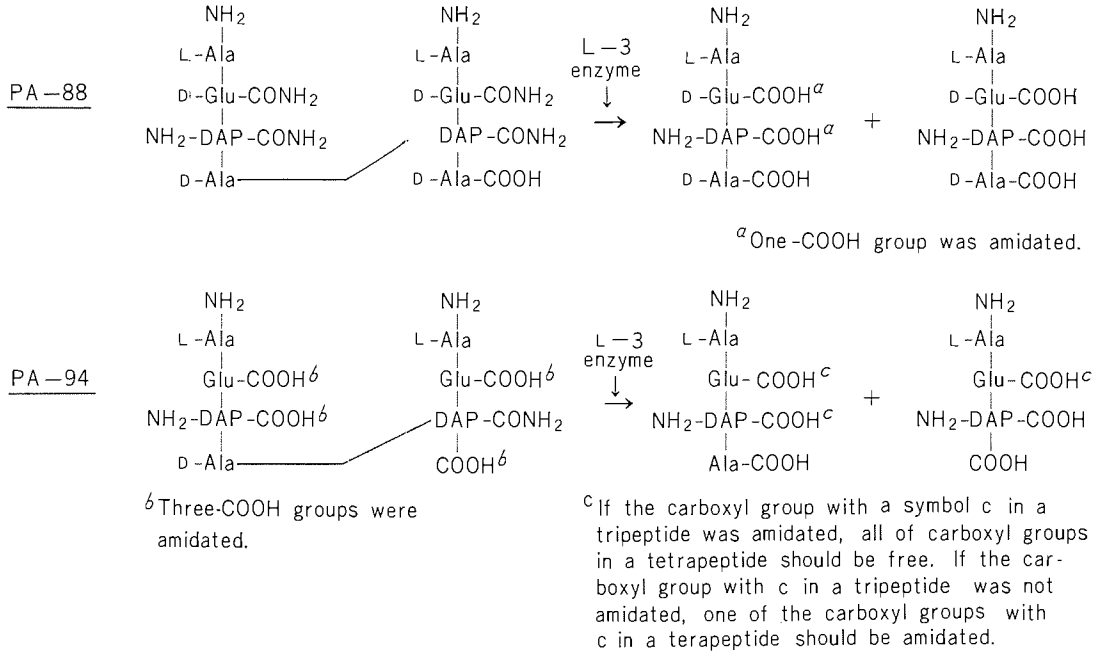


FIGURE 9. *Possible mechanism of hydrolysis of the dimeric forms of peptide subunits by the L–3 enzyme.*

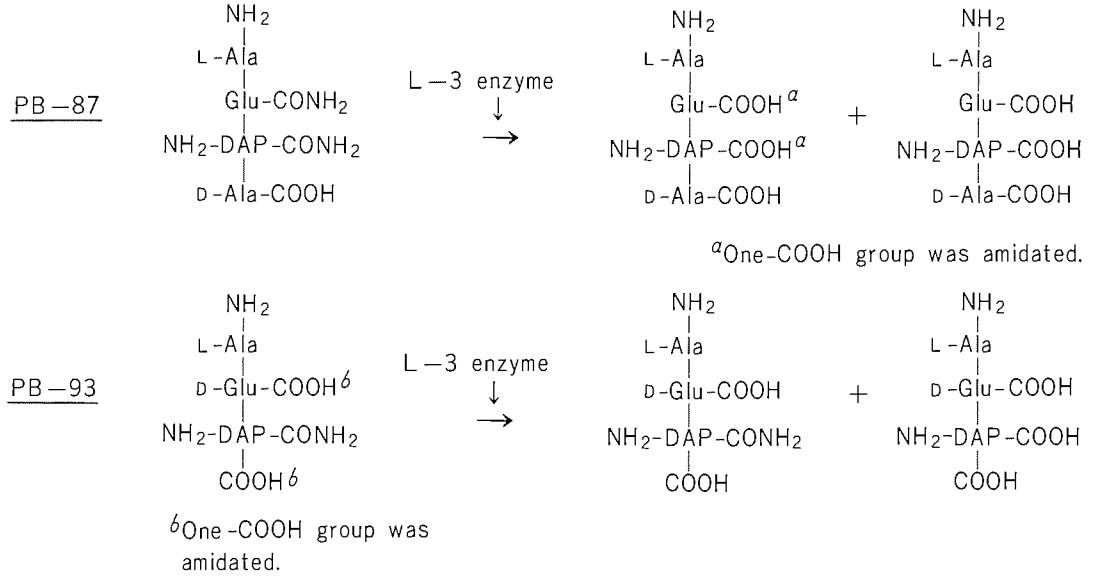


FIGURE 10. *Possible mechanism of hydrolysis of the peptide monomer subunits by the L–3 enzyme.*

monia in this and other peptides, however, cannot be fully explained as due to deamidation of DAP-amides only. A possible explanation is that it represents deamidation of (*iso*-) glutamine as well as DAP residues. This is also compatible with the findings that on paper electrophoresis at pH 5.0 the basic test peptides, PA-88 and PA-94, gave an acidic digestion product as well as neutral one. A possible mechanism for the hydrolysis of the dimeric forms of peptide subunits by the L-3 enzyme is presented in Fig. 9.

Treatment of the monomeric peptide subunits, PB-87 and PB-93, with the L-3 enzyme resulted in partial deamidation of the DAP-amides and (*iso*-)glutamine residues, and in formation of both acidic and neutral hydrolysis products from the originally single basic peptide. An increase in C-terminal DAP due to deamidation of the DAP-amides was also noted in tripeptide, PB-93. Fig. 10 shows the hydrolysis proposed for PB-87 and PB-93 schematically.

4) Other peak fractions

PB-96, PB-4-36 and PB-20-35 appeared to be single amino acids or a mixture of amino acids, since there were no significant differences between their contents of free and total amino groups. They were identified by analyses of unhydrolyzed and hydrolzyed specimens by paper chromatography and paper electrophoresis, and thin-layer chromatography of the DNP-derivatives. It was found that PB-96 was lysine, PB-4-36 a mixture of threonine and serine (in a molar ratio of 1.0: 1.7), and PB-20-35 a mixture of valine, isoleucine and leucine (1.0: 3.2: 1.4). Similar analyses showed that PA-98 was a mixture of 1 mole lysine and 0.2 mole of a peptide composed of alanine, glutamic acid and DAP in molar ratios of 1.5: 1.0: 1.0. Further analysis of this peptide was not possible due to shortage of material.

6. *Chemical and immunological properties of HM-1, HM-2 and HM-3*

1) Refiltration on Sephadex columns

Fractions 27 to 35 (HM-1), 36 to 41 (HM-2), and 42 to 51 (HM-3) respectively, were pooled and concentrated to about 2 ml with a rotary evaporator. The each was submitted to refiltration on Sephadex columns; HM-1 on two columns of G-75 coarse (1.2 cm×95 cm and 1.0 cm×95 cm) connected in series, HM-2 on a G-75 fine, column (1.2 cm×95 cm) and HM-3 on G-15 column (1.2 cm×95 cm) or G-25 fine, column (1.2 cm×95 cm). Fractions of 4 or 10 ml were collected. Aliquots of fractions were assayed for hexoses, pentoses, amino sugars and total amino groups. Neutral sugars were not determined in HM-3 since it contained virutally none. The results are shown in Fig. 11.

HM-1 was separated into two major portions; a wide peak in tubes Nos. 23 to 45, containing more hexose than pentose and a small amount of glycopeptide components, and the other containing almost exclusively of arabinose with a trace of glycopeptide.

HM-2 was roughly separated into fractions around tube No. 22 and those about tube No. 36. The former contained nearly equal amounts of hexose and pentose, and the latter was rich in pentose. It also seemed to contain fractions rich in hexose and total amino groups (around tube No. 27).

HM-3, which was eluted as a single peak near the Vo volume from a G-15 column, gave two peaks of total amino groups on filtration through G-25 column.

2) Chemical properties of the subfractions of HM-1, HM-2 and HM-3

Representative subfractions separated by refiltration of HM-1, HM-2 and HM-3 on Sephadex columns were analyzed (Table 6). The peptide portion of all the subfractions contained a significant amount of glycine and aspartic acid in addition to alanine, glutamic acid and DAP. The low molar ratio of DAP to muramic acid or glucosamine indicates that the greater part of the muramic acid residues in the glycan portion of these subfractions was not substituted by peptides. The terminal amino acids of HM-3-42 (a subfraction in tube No. 42 from HM-3) and HM-3-46 were

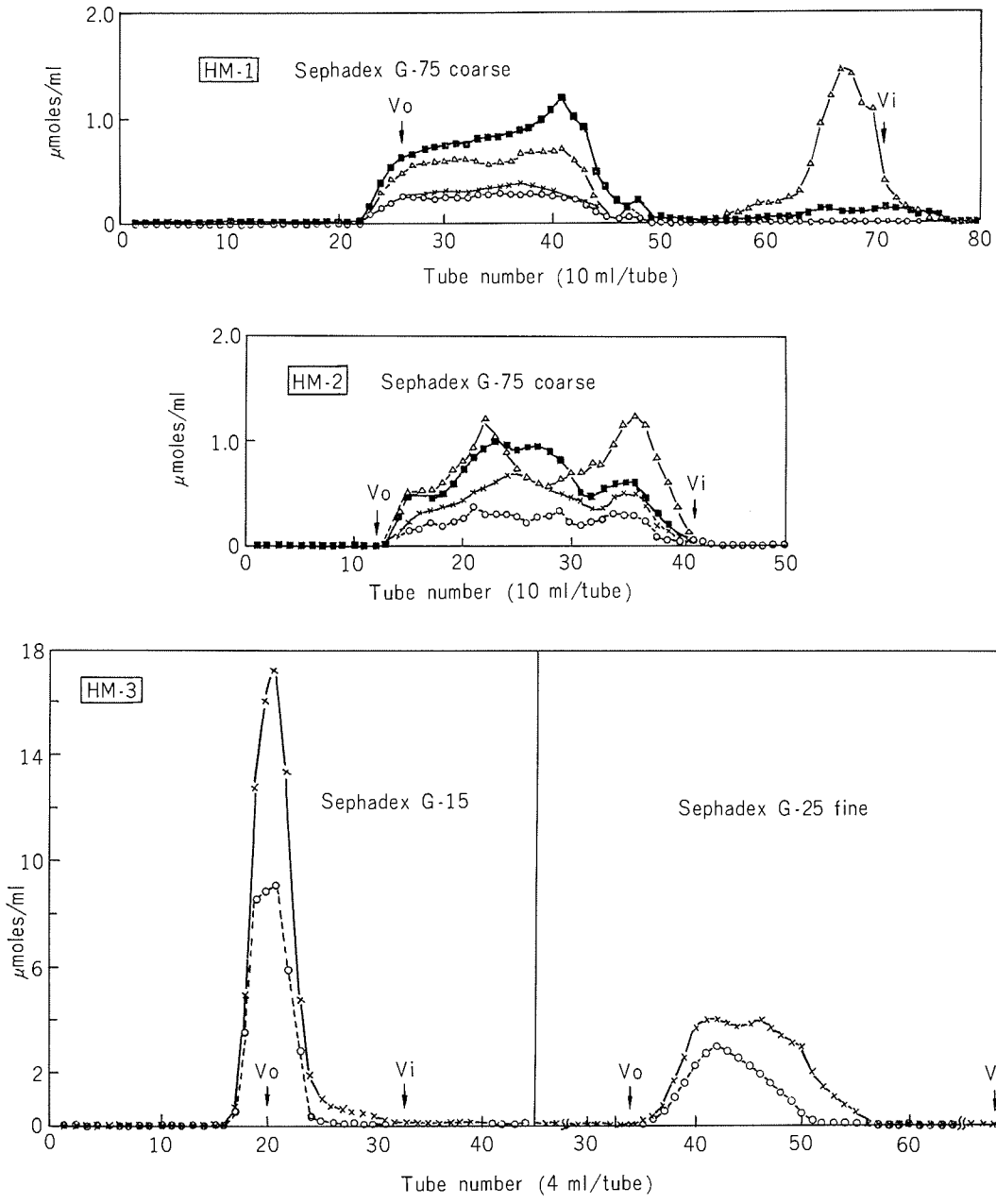


FIGURE 11. *Gel filtration patterns of HM–1, HM–2 and HM–3.*

■——■ *Hexoses*
△——△ *Pentoses*
×——× *Total amino groups*
○------○ *Amino sugars*

TABLE 6. *Composition of subfractions of HM-1, HM-2 and HM-3*

Sufractions[a]	Neutral sugars			Amino acids					NH_3	Amino sugars		Phos-phorus
	Ara	Gal[b]	Man[c]	Ala	Glu	DAP	Gly	Asp		GlcN	Mur	
HM-1-27	27.6	24.3	9.2	1.8	1.2	1.0	3.1	0.8	ND[d]	5.2	3.1	0.75
HM-1-41	32.9	43.9	11.8	2.0	1.2	1.0	1.5	0.9	ND	5.0	3.2	0.98
HM-2-22	41.8	39.1	11.5	1.9	1.1	1.0	1.7	0.5	ND	3.5	2.3	1.57
HM-2-36	71.8	26.2	8.0	2.0	1.2	1.0	2.4	0.8	ND	5.5	4.2	2.65
HM-3-42	1.2	2.4		0.9	1.2	1.0	0.5	0.4	1.2	3.0	2.5	0.05
HM-3-46	0.4	0.7		1.8	1.3	1.0	0.8	0.4	0.4	1.8	1.5	0

Values are expressed as moles of per mole total DAP.

Ara: arabinose, Gal: galactose, Man: mannose, GlcN: glucosamine, Mur: muramic acid

[a] For example, HM-1-27 indicates the subfraction of HM-1 in tube No. 27.

HM-1-67 (pooled) had the following composition (mμmoles/100 μl): arabinose 3,434, amino sugars 108, total amino groups 465, phosphorus 10, hexose <25. Alanine, glutamic acid, DAP, glycine and aspartic acid were detectable.

[b] Determined with a Galactostat.

[c] Calculated by substracting the value for galactose from that for total hexose.

[d] Not determined.

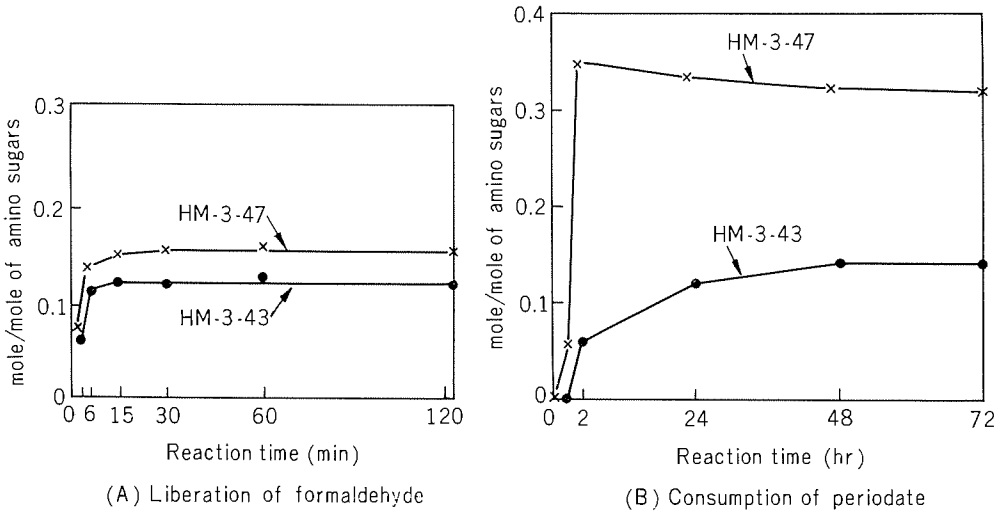


FIGURE 12. *Oxidation of reduced specimens of HM-3-43 and HM-3-47 by periodate.*

analyzed; 0.6 and 0.8 mole N-terminal alanine, 0.1 and 0.1 mole mono-NH_2-DAP, and 0.2 and 0.3 mole C-terminal alanine were found, respectively. The results suggest that there are extensive corss-linkages in a peptide portion of the test glycopeptides through the α- or α'-amino groups of DAP residues, and that the bulk of peptide subunits are linked with a glycan portion indirectly, *viz.* through the peptide subunit connected with a glycan portion by a muramyl→L-alanine bond. It may be added here that treatment of HM-3 (a concentrated specimen before refractionation containing 5.4 μmoles of total amino groups and 2.3 μmoles of amino sugars) with the L-11 enzyme (10 units) or L-3 enzyme (9 units) in 0.01 M potas-

sium phosphate buffer, pH 7.8, at 37 C for 24 hr did not result in any significant liberation of free amino groups.

To obtain information on the average chain length and nature of the non-reducing amino sugar of the HM-3 subfractions, specimens of HM-3-43 and HM-3-47* were reduced with sodium borohydride and oxidized with sodium periodate. The maximum release of formaldehyde during the oxidations of HM-3-43 and HM-3-47 amounted to 0.11 and 0.15 mole/mole of total amino sugars, respectively (Fig. 12). Thus these subfractions had average chain lengths of 9 and 6 amino sugar residues, respectively. For this calculation it was assumed that the small amount of polysaccharide of neutral sugars present in the specimens did not contribute significantly to the production of formaldehyde. Periodate consumptions during the oxidation, on the other hand, were shown to be 0.15 mole (HM-3-43) and 0.32 mole (HM-3-47)/mole of total amino sugars. It was recently demonstrated that a glycan portion of mycobacterial cell wall peptidoglycan had alternating β-1,4-linked residues of N-acetylglycosamine and N-glycolylmuramic acid (Petit et al., 1969; Adam et al., 1969; Azuma et al., 1970). During periodate oxidation, 1 mole of a glycan with the repeating unit cited above should liberate 1 mole of formaldehyde and consume 2 moles of periodate (1 mole each at both reducing and non-reducing ends) if the glycan has a non-reducing glucosamine in the pyranose ring form. However, one mole of the glycan should produce 1 mole of formaldehyde and consume 1 mole of periodate at the reducing end only if it has N-substituted muramic acid in the pyranose ring form as the non-reducing end (Leyh-Bouille et al., 1966). Thus the above data suggest that the non-reducing end was mainly muramic acid in HM-3-43 and exclusively glucosamine in HM-3-47. It may be added here that when a concentrated specimen of HM-3 (containing 5.4 μmole total amino groups and 2.3 μmole amino sugars) was incubated with 20 μg of crystalline egg white lysozyme in 0.025 M potassium phosphate buffer, pH 7.0 at 37 C for 24 hr, a significant amount of reducing groups was liberated and the molar ratio of total amino sugars to reducing groups decreased from 8 to 4.

Phosphorus was present in subfractions of HM-1 and HM-2 containing polysaccharides of neutral sugars, but was virtually absent in those from HM-3 containing little neutral sugars. Furthermore the phosphorus content was roughly proportional to the content of neutral sugars, especially pentose (the molar ratios of phosphorus to pentose in all subfractions of HM-1 and HM-2 were 0.027 to 0.037, except in HM-1-67 [pooled] where the ratio was as low as 0.003).

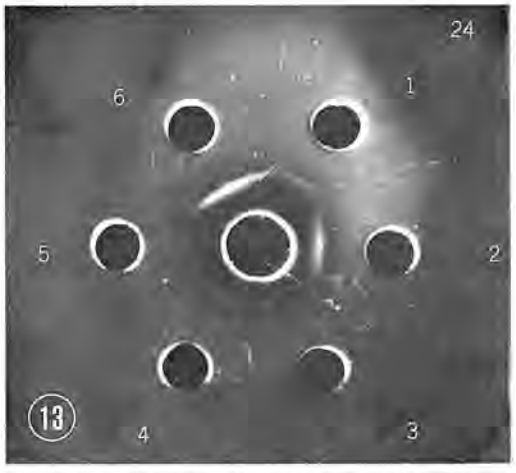


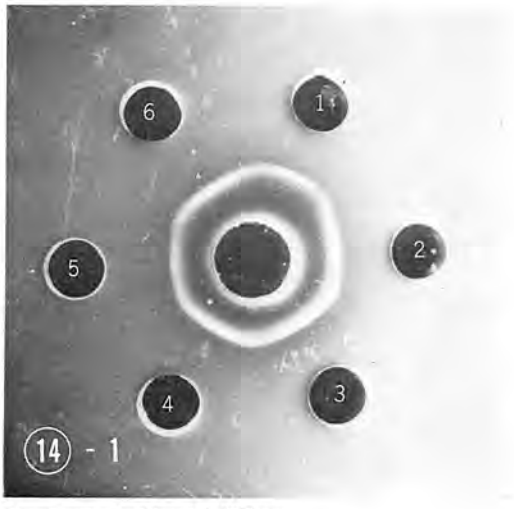
FIGURE 13. *Reaction of subfractions of HM-1 and HM-2 with anti-H37Rv whole cell rabbit serum (immunodiffusion in agar)*

The center well contained rabbit antiserum (80 μl) prepared against strain H37Rv whole cells. The outer wells contained the following antigen solutions (5 mμmoles of arabinose equivalents/40μl). 1: HM-1-27, 2: HM-1-41, 3: HM-1-67, 4: HM-2-22, 5: HM-2-36, 6: arabinogalactomannan extracted from H37Rv cell walls by cold trichloroacetic acid.

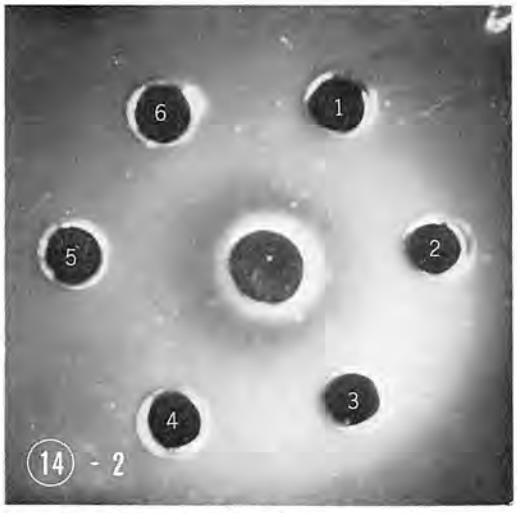
* These fractions were submitted to periodate oxidation in place of HM-3-42 and HM-3-46, since the latter fractions had been exhausted in other analyses.

3) Immunological properties of subfractions of HM-1 and HM-2

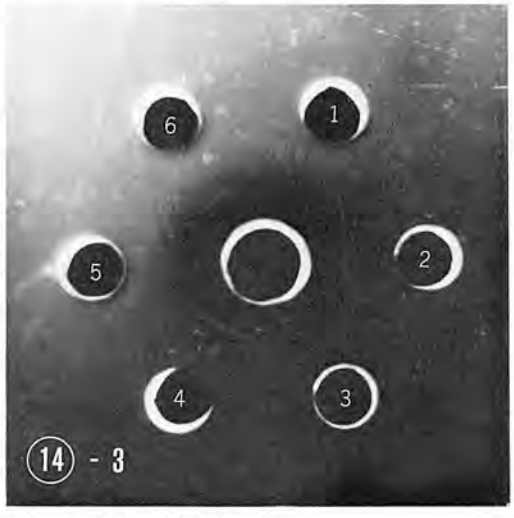
The reaction of representative subfractions of HM-1 and HM-2 with anti-H37Rv whole cell rabbit serum were studied by the Ouchterlony method. HM-1-27, HM-1-41 and HM-2-22 each gave a single precipitin line common to all of them and to arabinogalactomannan. The latter was obtained by ethanol (60 to 80%, v/v) precipitation of the extracts from H37Rv cell walls with 5% trichloroacetic acid in the cold. The minimum concentrations of these subfractions giving a positive reaction were 1.2, 1.6 and 6 mμmoles (as arabinose)/40 μl, respectively. Unlike these fractions, HM-1-67 (pooled) and HM-2-36 did not precipitate the antisera even at concentrations of 1,400 and 800 mμmoles (as arabinose)/40 μl, respectively (Figs. 13 and 14). Their possible inhibitory effects of the latter two fractions against precipitation of the antiserum by HM-1-27, HM-1-41, HM-2-22 and arabinogalactomannan were tested. It was shown that 40 mμmoles (as arabinose) of HM-1-67 completely inhibited the precipitating activity of the antiserum, and a similar amount of HM-2-36 caused marked inhibition (Fig. 14).



A. Control without inhibitors



B. Inhibition with HM–1–67



C. Inhibition with HM–2–36

FIGURE 14. *Precipitin inhibiting activities of HM–1–67 and HM–2–36 (immunodiffusion in agar).*

The center well contained the antiserum (70 μl) which had been incubated with the specimen (40 mμmoles of arabinose equivalents/10 μl) or physiological saline at 37 C for two hr. The outer wells contained the following antigen solutions (20 mμmoles of arabinose equivalents/40 μl). 1: HM–1–27, 3: HM–1–45, 5: HM–2–22, 2, 4 and 6: arabinogalactomannan.

DISCUSSION

There have been many studies on the cell walls of *Mycobacteria* during the past 12 years. Mycobacterial cell walls have been shown to be composed of a basal, rigid peptidoglycan layer containing muramic acid, glucosamine, alanine (D- and L-isomers), D-glutamic acid and *meso*-α,α′-diaminopimelic acid, and special structures such as antigenic arabinogalactan or arabinogalactomannan and mycolic acids (Cummins and Harris, 1958; Kotani et al., 1959; Belknap, Camien and Dunn, 1961; Takeya, Hisatsune and Nakashima, 1961; Cummins, 1962; Kotani et al., 1963; Takeya and Hisatsune, 1963; Takeya, Hisatsune and Inoue, 1963; DeWijs and Jollès, 1964; Misaki et al., 1966; Misaki and Yukawa, 1966; Cummins et al., 1967; Kanetsuna, 1968; Azuma, Yamamura and Fukushi 1968; Kotani et al., 1968a, 1968b; Kanetsuna, Imaeda and San Blas, 1968; Migliore and Jollès, 1968; Kanetsuna, Imaeda and Cunto, 1969; Cunto, Kanetsuna and Imaeda, 1969; Petit et al., 1969; Adam et al., 1969; Azuma et al., 1969; Azuma et al., 1970; Wietzerbin-Falszpan et al, 1970; Kanetsuna and San Blas, 1970; Acharya and Goldman, 1970). Other compounds have been found in the cell wall fraction isolated from disrupted mycobacterial cells, though these may not be cell wall components in a strict sense. These include socalled free lipids easily extractable from isolated cell walls with neutral organic solvents without hydrolysis (Kotani et al., 1959) and the tuberculin-active peptides reported by Azuma et al. (1969).

The peptide moiety of the peptidoglycan was found to be composed of a tripeptide (Ala-Glu-DAP) and a tetrapeptide (Ala-Glu-DAP-Ala) as building blocks (Kotani et al., 1968a on BCG; Migliore and Jollès, 1968 on *M. tuberculosis var. hominis*; Petit et al., 1969 on *Mycobacterium smegmatis*; Wietzerbin-Falszpan et al., 1970 on *M. smegmatis*, *Mycobacterium phlei* and BCG). The cross-linkages between the basal peptide subunits were demonstrated by isolation of possible dimeric or oligomeric forms of peptide subunits in the studies of Kotani et al. (1968a) and of Petit et al. (1969). The former authors studied the peptides isolated from L-11 enzyme digests of BCG cell walls which were extracted with neutral organic solvents and 5% trichloroacetic acid without heating. Petit et al. studied the peptidoglycan obtained from delipidated cell walls of *M. smegmatis* by alkaline treatment and mild acid hydrolysis. This was solubilized by *Myxobacter* AL_1 enzyme, and the resulting peptides were analyzed. However, both groups gave insufficient evidence for the existence of dimeric or oligomeric forms of peptide subunits.

In the present study, we established the existence of dimeric forms of two tetrapeptide or of one tetrapeptide and one tripeptide, by isolating them in a sufficiently pure state for the chemical and enzymatic analyses. The cell walls used here were not exposed to any treatment which could cause cleavage of linkages between the building blocks. Neither a trimer of peptide subunits, such as that suggested in a previous study on BCG cell walls, nor oligomers $(Ala\text{-}Glu\text{-}DAP\text{-}Ala)_n$, n having an average value of 4, as reported by Petit et al. was obtained. The cell wall peptidoglycan of *M. tuberculosis* strain H37Rv definitely has a network structure composed of tetrapeptide and tripeptide subunits cross-linked by D-alanine→DAP bonds as in the cell walls of *Escherichia coli* (Weidel and Pelzer, 1964), *C. diphtheriae* (Kato, Strominger and Kotani, 1968) and *L. plantarum* (Matsuda, Kotani and Kato, 1968a, 1968b). The presence of extensive cross-linkages between peptide subunits through α- or α′-amino groups of DAP residues was indicated by terminal amino acid analyses of delipidated cell walls themselves.

The overall structure of the peptide moiety of mycobacterial cell wall peptidoglycan, however, seems to be more complicated. This is indicated by the facts that evidence was found for the existence of a new tetrapeptide, $Ala\text{-}Glu(^{\alpha}Gly)^{\varepsilon}DAP$, and that a peptide

moiety of the soluble glycopeptides isolated from the L-11 enzyme digest contained glycine and aspartic acid besides the usual three amino acids as the essential constituents. It should be pointed out that no tetrapeptide of the type cited above has so far been reported in any of the bacterial cell walls, though it has been demonstrated that a tetrapeptide, L-Ala-D-Glu($^{\alpha}$Gly)^{L}L-Lys-D-Ala, is a basal peptide subunits of *Micrococcus lysodeikticus* cell walls (Tipper et al., 1967; Ghuysen et al., 1968; Kato et al., 1968b).

Another noteworthy finding was that peptides were isolated, in which the carboxyl groups of glutamic acid and DAP residues were substituted by ammonia to various extents. This does not necessarily mean that the peptide subunits in native peptidoglycan are amidated to various extents, since deamidation may occur during isolation of the peptides. Moreover, both (*iso*-)glutamine and DAP-amides were shown to be susceptible to deamidation by the L-3 enzyme. Previous studies (Kato, Strominger and Kotani, 1968; Matsuda, Kotani and Kato, 1968a, 1968b; Matsuda et al., 1968) showed that the L-3 enzyme exerted DAP-amide amidase activity on the cell walls of *C. diphtheriae* and *L. plantarum*. In these studies (*iso*-)glutamine residues were not found to be deamidated by the L-3 enzyme. It has recently been shown that a basic pentapeptide, N$^{\alpha}$-(L-alanyl-D-*iso*glutaminyl)-N$^{\varepsilon}$-glycyl-L-lysyl-D-alanine, isolated from the L-11 enzyme digest of *S. aureus* (strain Copenhagen) cell walls changed to a neutral peptide on treatment with the L-3 enzyme, probably by deamidation of the *iso*glutamine residues. Further work is necessary on the action of the L-3 enzyme on *iso*glutamine residues in bacterial cell walls.

Recent studies by Petit and others (Petit et al., 1969; Adam et al., 1969; Azuma et al., 1970) showed that the amino groups of muramic acid residues in mycobacterial cell wall peptidoglycan were substituted by glycolyl groups, not by acetyl groups as in the cell walls of other bacterial species (except *Nocardia*). Thus a glycan portion of mycobacterial cell walls consists of the repeating disaccharide unit, N-acetylglucosamine-β-1,4-N-glycolylmuramic acid. Analyses by periodate oxidation of soluble glycopeptides obtained in this study showed that their glycans had an average chain length of 6 to 9 amino sugar residues. These values are considerably lower than those reported for the cell walls of other bacterial species. For example there are 19 to 25 amino sugar residues in the glycan of *S. aureus* (strain Copenhagen) cell walls (Tipper, Strominger and Ensign, 1967). Analyses by periodate oxidation also indicated that the non-reducing ends in soluble glycopeptides were partly muramic acid and partly glucosamine. The reducing ends in soluble glycopeptides have been studied by identification of amino sugar alcohol formed by reduction of soluble glycopeptide with sodium borohydride, but no definite conclusions have been obtained.

Studies on subfractions of HM-1 and HM-2 showed that these polysaccharide-glycopeptide complexes were not homogeneous with regard to their relative contents of hexose and pentose, and their antigenic reactivities. This finding is noteworthy since native cell walls were used as starting material in this study, and no detectable release of reducing groups was recognized during solubilization of the cell walls by the L-11 enzyme. It was found in this laboratory that an inducible enzyme (M-2 enzyme), formed by a soil bacterium isolated by the enrichment culture method, depolymerized the antigenic polysaccharides from the cell walls of Mycobacterial species and *C. diphtheriae*, and deprived them of their reactivities with antisera (Kotani, Matsubara and Sakagoshi, 1966; Kotani et al., 1968b, Kato, 1969). Polysaccharide fragments rich in arabinose were isolated from enzymatic hydrolysates of BCG arabinogalactan and of H37Rv arabinogalactomannan. These were shown to have potent inhibitory activities against the precipitation of antiserum by antigenic polysaccharides (Kato, 1969). These findings seem compatible with the present observation that HM-1-67, almost exclusively consisting of arabinose and HM-2-

36 rich in arabinose, combined with the corresponding antibodies, but did not precipitate them.

The linkage between peptidoglycan and polysaccharides of hexose and pentose was first suggested by Misaki and Yukawa (1966) from studies on BCG cell walls. It was confirmed by Kanetsuna (1968) in the cell walls of several species of *Mycobacteria*. The latter author demonstrated that a polysaccharide of neutral sugars, arabinose and galactose, behaved as an ion both on ion-exchange chromatography and electrophoresis, always moving with the components of glycopeptide. Additional evidence for this was provided in this study by isolation of polysaccharide-glycopeptide complexes from the L-11 enzyme digest of native cell walls of strain H37Rv. In connection with the role of phosphate in the linkage between polysaccharide and glycopeptide, it should be noted that muramic acid-6-phosphate was found by Liu and Gotschlich (1967) in *Mycobacterium butyricum* cell walls, by Kanetsuna (1968) in BCG cell walls, and by Cunto, Kanetsuna and Imaeda (1969) in *M. smegmatis* cell walls. Amino acid analysis of an acid hydrolyzate of delipidated cell walls and a residue remained insoluble after the L-11 enzyme treatment indicated the possible presence of this compound in *M. tuberculosis* strain H37Rv (unpublished observation). Consequently, the polysaccharide seems to be linked through phosphodiester linkages to muramic acid residues in the peptidoglycan. Evidence for this obtained in the present work was that, whereas little or no phosphorus was present in glycopeptide subfractions which were virtually free from neutral sugars, phosphorus seemed to be an essential component of polysaccharide-glycopeptide complexes, and the molar ratios of phosphorus to arabinose in these complexes were fairly constant although these complexes were not homogeneous. It should be pointed out, however, that the findings mentioned above did not necessarily exclude the existence of other types of linkage between neutral polysaccharide and glycopeptide. In fact, Kanetsuna and San Blas (1970) in their study on cell walls of BCG and *M. smegmatis* presented evidence indicating that there were two types of linkage between arabinogalactan and glycopeptide, and some of arabinogalactan might be linked glycosidically to an amino sugar (possibly glucosamine) of peptidoglycan.

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