Structure of the Cell Wall of *Corynebacterium diphtheriae*. I. Mechanism of Hydrolysis by the L-3 Enzyme and the Structure of the Peptide*

Keijiro Kato,† Jack L. Strominger‡ and Shozo Kotani

ABSTRACT: The lysis of cell walls of *Corynebacterium diphtheriae* by the L-3 enzyme from *Streptomyces* has been investigated. The primary lytic activity in the partially purified enzyme is a bridge-splitting enzyme which catalyzes the hydrolysis of D-alanyl-*meso*-diaminopimelic acid linkages. The preparation also contains acetylmuramyl-L-alanine amidase activity, an enzyme which catalyzes hydrolysis of an amide of diaminopimelic acid and possibly a D-alanine carboxypeptidase. Through the use of this enzyme preparation, as well as the peptidase from *Myxobacterium*, it has been shown that the peptide of the cell wall of this organism contains both tetrapeptide (L-Ala-D-Glu-*meso*-Dap-D-Ala) and tripeptide (L-Ala-D-Glu-*meso*-Dap) subunits. In most of these peptides both the carboxyl group of D-glutamic acid and that of *meso*-diaminopimelic acid are present as amides. The extent of cross-linking of these units to each other through D-alanyl-*meso*-diaminopimelic acid cross-bridges to form dimers, and possibly higher oligomers, is discussed. A small number of tetra- and tripeptide subunits containing a single amide residue on the D-glutamic acid, and a small number of pentapeptide diamide subunits (containing a second D-alanine residue at the carboxyl terminus) were also present.

The structures of the cell walls of gram-positive bacteria which contain lysine as the dibasic amino acid (*e.g.*, *Staphylococcus aureus* and *Micrococcus lysodeikticus*) have been studied by chemical analyses of solubilized products of enzymatic lysis (see, for example, Strominger and Ghuysen, 1967) and also through biosynthetic investigations (Strominger *et al.*, 1967). The structure of the cell wall of the gram-negative bacillus, *Escherichia coli*, which contains diaminopimelic acid rather than lysine has also been extensively studied (Weidel and Pelzer, 1964). In general, the rigid layer of these walls contains peptidoglycan strands whose peptide subunits are cross-linked through interpeptide bridges. In *M. lysodeikticus* the carboxyl group of the terminal D-alanine residue in one peptide subunit is linked directly to the ε-amino group of an L-lysine residue in another subunit; a similar linkage occurs in *E. coli* except that the ε-amino group is that of *meso*-diaminopimelic acid. In *S. aureus* a small peptide, pentaglycine, is interposed between the D-alanine and L-lysine residues.

Mori *et al.* (1960) isolated the L-3 enzyme from *Streptomyces*. This enzyme lyses cell walls of *Corynebacterium diphtheriae*. The availability of this enzyme enabled us to investigate the structure of the cell wall of this gram-positive organism, which contains diaminopimelic acid as the dibasic amino acid. In this paper kinetic studies of the mechanism of enzymatic lysis and the analyses of solubilized products of the cell wall of *C. diphtheriae* are described.

Materials and Methods

Cell Walls. C. diphtheriae, strain Park–Williams No. 8, a substrain of Toronto–Harvard, grown in the modified Taylors medium for toxin production, was used. Cell walls were prepared by differential centrifugation of sonically disrupted cells. The cell walls were purified by treatment at 37°, for 2 hr, with trypsin in 0.01 M phosphate buffer (pH 7.8), washed with the buffer and then water, and freeze dried.

Delipidated cell walls were prepared according to Anderson (1943). The cell walls were treated with 40 volumes of ethyl alcohol–ethyl ether mixture (1:1, v/v) for 2 days at room temperature. Treatment in this solvent was repeated three more times. Finally, the cell walls were extracted in chloroform at room temperature for 2 days. This procedure was also repeated three more times.

Enzymes. A. L-3 ENZYME was a concentrate of a culture filtrate of *Streptomyces* sp. and was purified by column chromatography on Duolite C-10. The procedure for purification of the enzyme was reported by Mori and Kotani (1962).

B. THE PEPTIDASE from *Myxobacterium* (Ensign and Wolfe, 1965, 1966; Tipper *et al.*, 1967), *N*-acetylmuramyl-L-alanine amidase (Ghuysen *et al.*, 1962), and endo-*N*-acetylmuramidase (*Chalaropsis* B enzyme; Hash, 1963) were generous gifts from Drs. J. C. Ensign

* From the Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706, and Department of Microbiology, Osaka University Dental School, Osaka, Japan. *Received April 1, 1968.* Supported by research grants from the U. S. Public Health Service (AI-06247) and National Science Foundation (GB-4552).

† Present address: Department of Microbiology, Osaka University Dental School, Osaka, Japan.

‡ Present address: Biological Laboratories, Harvard University, Cambridge, Mass. 02138. Please send reprint requests to this address.

(Department of Bacteriology, University of Wisconsin), J.-M. Ghuysen (Service de Bactériologie, Université de Liège), and J. C. Hash (Department of Microbiology, Vanderbilt University School of Medicine), respectively.

Analytical Procedures. Determination of total, NH_2-terminal, COOH-terminal, and free amino acids, total hexosamine, and reducing power were carried out according to Ghuysen *et al.* (1966). For determination of NH_2-terminal and total amino acids, materials were hydrolyzed at 100° in 4 N HCl for 8 and 12 hr, respectively. Hydrazinolysis for the determination of COOH-terminal amino acid was performed at 100° for 6 hr. The determinations of reducing power and pentose were performed by the methods of Park and Johnson (1949) and Ashwell (1957), respectively.

Thin-Layer Chromatography. Dinitrophenylated derivatives of NH_2- and COOH-terminal amino acid and amino acids in acid hydrolysate of peptides were determined by quantitative thin-layer chromatography as described by Ghuysen *et al.* (1966). The following solvents were used: solvent 1, 1-butanol–1% ammonium hydroxide (1:1, upper phase); 2, chloroform–methyl alcohol–acetic acid (84:15:1); 3, benzyl alcohol–methyl alcohol–chloroform–water–ammonium hydroxide (30:30:30:6:2); 4, *t*-amyl alcohol–chloroform–methyl alcohol–water–acetic acid (30:30:30:20:3) (organic phase); and 5, triethylamine–1% ammonium hydroxide (1:1, upper phase).

Thin-Layer Chromatography of Dinitrophenyl Peptides. A peptide fraction (10–30 mμmoles) was lyophilyzed, redissolved in 40 μl of 1% sodium borate, and 12 μl of 0.1 M dinitrofluorobenzene solution (in ethyl alcohol) was added. After 90-min incubation at 60°, the solution was acidified to 2 N HCl (10 μl of concentrated HCl) and then treated with 150 μl of ethyl ether. The extraction of free dinitrophenyl-amino acid by ether was repeated twice more, and the water phase which contained the DNP peptide was dried. The DNP peptide was then dissolved in 10–30 μl of 0.04 M ammonium hydroxide or in methyl alcohol and developed on silica gel G in solvent 4 or 5. In solvent 4 development was carried out for 2 hr at 2° and in solvent 5 development for 2 hr at 2° was repeated three times. The R_F values of DNP peptides were compared with DNP-tetraglycine. The latter moved 2.6–2.8 cm in solvent 4 at 2° after 2-hr development and after three developments (2 hr each) in solvent 5 at 2° it moved 2.8 cm from the origin.

Results

Composition of the Cell Walls of C. diphtheriae. The cell walls (2 mg) were hydrolyzed at 100° with 4 N HCl for 12 hr. An aliquot was analyzed on a Beckman–Spinco amino acid analyzer (Table I). The cell walls contained, as major components, glutamic acid, alanine, diaminopimelic acid, glucosamine, and muramic acid, in the ratio 1.0:1.69:0.94:0.95:1.09 and, as minor components, leucine, serine, threonine, aspartic acid, lysine, and galactosamine. The proportion of L- and D-alanine was 1.0–0.69 (determined enzymatically, see Ghuysen *et al.*, 1966). The content of glutamic acid was 430 mμmoles/mg of cell wall, and that of ammonia was 2 moles/mole of glutamic acid.

TABLE I: Composition of Cell Wall of *C. diphtheriae*.[a]

Components	μmoles/mg of Cell Walls	Ratio
Lysine	0.034	0.08
Ammonia	0.825	2.0
Aspartic acid	0.052	0.125
Threonine	0.034	0.08
Serine	0.052	0.125
Glutamic acid	0.43	1.0
Glycine	0.069	0.166
Alanine	0.70	1.69
L-Alanine	0.41	0.95
D-Alanine	0.29	0.65
Diaminopimelic acid	0.39	0.93
Isoleucine	0.017	0.041
Leucine	0.052	0.125
Glucosamine	0.41	0.95
Muramic acid	0.47	1.09
Galactosamine	0.035	0.08

[a] Data were obtained from the Beckman–Spinco amino acid analyzer. L- and D-alanine were measured enzymatically (Ghuysen *et al.*, 1966). A correction was made for hydrolytic loss of muramic acid.

Hydrolysis of C. diphtheriae Cell Walls by the L-3 Enzyme. Determination of free amino groups and measurement of turbidity indicated that free amino groups were released in parallel with turbidity reduction. After a 3-hr incubation under the conditions employed turbidity reached about half of the initial value, and release of amino groups reached 1.1 moles/mole of glutamic acid (Figure 1). Finally, after 48-hr incubation, turbidity was reduced by 80% and 2.5 moles of amino groups/glutamic acid was released. Reducing groups, however, showed no change during lysis indicating that no cleavage of the cell wall polysaccharide had occurred. These results indicated that the L-3 enzyme preparation contained peptidase(s) or amidase(s), but no glycosidase(s).

Nature of NH_2- and COOH-Terminal Amino Acids Released by the L-3 Enzyme. Analysis of terminal amino acids released by the L-3 enzyme acting on either cell wall or on the glycopeptide solubilized by treatment of the cell wall with B enzyme of *Chalaropsis* (Hash, 1963) were carried out.

A. DIGESTION OF THE CELL WALL BY THE B ENZYME OF *Chalaropsis.* A preliminary treatment with this acetylmuramidase was carried out in order to obtain a soluble preparation which could be used for analysis of NH_2- and COOH-terminal groups present in the isolated cell walls. The cell wall suspension (20 μl) (30 mg/ml, made into a homogeneous suspension by brief treatment in a sonic oscillator) was incubated

TABLE II: Release from *C. diphtheriae* Cell Walls of Total Free NH_2 Groups and of NH_2- and COOH-Terminal Amino Acids.[a]

Tube[b]	Total Free NH_2 Groups 0 hr	44 hr	Δ	NH_2-Terminal Amino Acids	0 hr	44 hr	Δ	COOH-Terminal Amino Acids	0 hr	44 hr	Δ
A. B enzyme, then L-3 enzyme	0.64	1.68	1.04	Ala	0	0.08	0.08	Ala	0.29	0.81	0.52
				Dap	0.31	0.85	0.54	Dap	0.08	0.34	0.26
						Total	0.62			Total	0.78
B. L-3 enzyme	0.29	1.67	1.38	Ala		0.32	0.32[c]	Ala	0.27	0.78	0.50
				Dap		0.74	0.43	Dap	0.07	0.36	0.29
				Unknown[d]		0.15	0.15			Total	0.79
						Total	0.90				

[a] Data are expressed as moles per mole of glutamic acid. See text for details. [b] Part A is the hydrolysis of *solubilized glycopeptide* (*i.e.*, cell walls previously treated with the B enzyme) by the L-3 enzyme. Part B is the hydrolysis of intact cell walls by the L-3 enzyme. The kinetics of the latter is shown in Figure 2. [c] These values were obtained by subtracting the values above for the 0-hr sample of the solubilized glycopeptide. [d] The unknown substance detected on thin-layer chromatogram as a DNP derivative may be an ether-soluble peptide. The mobility of this material on silica gel G in solvents 1 and 2 was slower than DNP-glutamic acid.

at 37° with 2 μl of the *Chalaropsis* B enzyme (1 mg/ml) in 45 μl of 0.01 M acetate buffer (pH 4.7). A cell wall suspension in the same buffer but without B enzyme was incubated as a control.

Reducing power increased and reached a maximum after 2 hr. Further increase after prolonged incubation was slight. The net increase of reducing power after 22-hr incubation was 0.8 mole/mole of glutamic acid. The turbidity of the reaction mixture was also rapidly reduced. In a large-scale digestion of the cell wall with the B enzyme, 60% of the wall material was solubilized as measured by weight. The composition of the material not solubilized has not yet been investigated. It could, for example, be lipids. The fraction solubilized by the B enzyme contained almost 0.6 mole of NH_2 groups/mole of glutamic acid. Half of this was a free amino group of diaminopimelic acid (Table IIA, see 0-hr sample); the remainder consisted of small amounts of a variety of materials.

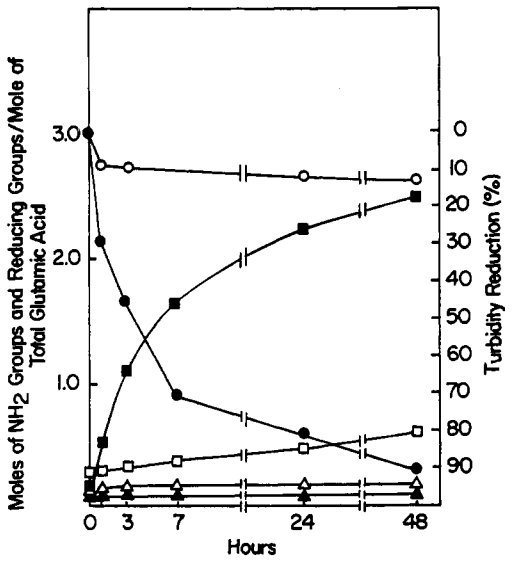

FIGURE 1: Release of free amino groups and reducing power and reduction of turbidity during lysis of *C. diphtheriae* cells walls with L-3 enzyme. For control and enzyme, respectively, ● and ○ = turbidity; △ and ▲ = reducing power, □ and ■ = free NH_2 groups.

B. DIGESTION BY THE L-3 ENZYME OF THE INTACT CELL WALL AND OF THE GYLCOPEPTIDE SOLUBILIZED BY THE *Chalaropsis* B ENZYME. Digestion of the cell wall and of the soluble glycopeptide by L-3 enzyme was carried out by the addition of the enzyme solution directly into the incubation mixtures described above. The reaction mixture was divided in half (45 μl) and each half was adjusted to pH 8.0 by the addition of 4 μl of 0.1 M K_2HPO_4 solution. L-3 enzyme (20 μl) (6 units/ml) or water (20 μl) (as control) was added and the final volume of the mixture was brought to 90 μl with water.

The reaction mixtures were incubated at 37° and aliquots (2–10 μl) were removed for determination of NH_2- and COOH-terminal amino acids and for total free NH_2 groups (Table II, Figure 2). When the solubilized glycopeptide was used as substrate, equivalent amounts of NH_2-terminal diaminopimelic acid and COOH-terminal alanine were released. Little or no NH_2-terminal alanine was formed. A small amount of COOH-terminal diaminopimelic acid was also released. These results can be interpreted as due to the hydrolysis of a direct linkage between the COOH-terminal D-alanine residue of one tetrapeptide subunit (L-Ala-D-Glu-*meso*-Dap-D-Ala) and the free amino group of diaminopimelic acid in another subunit. The release of COOH-terminal diaminopimelic acid

TABLE III: Configuration of NH_2- and COOH-Terminal Alanine Released by the L-3 Enzyme.[a]

	Measurement of NH_2-Terminal Alanine				Isolated COOH-Terminal Alanine	
	Control		After Dinitrophenylation			
	L-Ala	D-Ala	L-Ala	D-Ala	L-Ala	D-Ala
Proportion of L- and D-alanine (%)	67	33	58	42	9	91
Ratio of alanine to total glutamic acid	1.11	0.55	0.76	0.55		

[a] *C. diphtheriae* cell walls (4.3 mg) were incubated with 160 μl of L-3 enzyme (6 units/ml) in 600 μl of 0.01 M phosphate buffer (pH 8.0), containing 0.1% NaN_3 (as a bactericidal agent) at 37° for 72 hr. Cell walls incubated in the same buffer system but without L-3 enzyme were employed as the control. See text for further details.

is due to further hydrolysis catalyzed by other enzymes in the L-3 preparation (see below). When the intact cell wall was the substrate, similar results were obtained except that an additional slow release of 0.3 residue of NH_2-terminal alanine was observed with no measurable change in the COOH-terminal groups released. This additional activity is due to hydrolysis of the *N*-acetylmuramyl-L-alanine linkage (see below).

Configuration of NH_2- and COOH-Terminal Alanine Released by L-3 Enzyme. NH_2-TERMINAL ALANINE. Both cell wall and cell wall lysates prepared as described in Table III were suspended in 300 μl of 2% sodium borate and mixed with 30 μl of 0.1 M DNFB.[1] After 30-min incubation at 60°, 150 μl of concentrated HCl was added and the samples were hydrolyzed at 100° for 7 hr. Ether-soluble DNP-amino acids were removed. The residual acid solution containing ether-insoluble DNP-amino acids and non-NH_2-terminal amino acids was diluted with water to give a concentration of HCl of 1.5 N. The diluted solution was applied to a small column (5 ml) of Dowex 50-H (Bio-Rad, Los Angeles, Calif., 200–400 mesh, previously washed and equilibrated with 1.5 N HCl).

Alanine was eluted between 8 and 12 ml of 1.5 N HCl. It was located by thin-layer chromatography of its DNP derivative. The alanine-containing fractions were lyophilyzed. Total alanine and the proportion of L and D isomers were measured (Table III).

The amount of D-alanine in the cell wall lysate was unchanged by dinitrophenylation. However, the decrease in the amount of L-alanine (0.35 mole/mole of glutamic acid, Table III) was equivalent to the amount of NH_2-terminal alanine released by the L-3 enzyme (Table II). Thus the alanine released as NH_2-terminal alanine during lysis of *C. diphtheriae* cell wall by L-3 enzyme is the L isomer.

COOH-TERMINAL ALANINE. Hydrazinolysis was carried out by heating the cell wall lysate in 300 μl of hydrazine at 100° for 6 hr. Hydrazine was evaporated over H_2SO_4 in a vacuum desiccator. The sample was resuspended in 400 μl of water, then mixed with 80 μl of benzaldehyde, and shaken vigorously for 1 hr at room temperature. Treatment with benzaldehyde was repeated once more. The water phase that separated after centrifugation was extracted with ethyl ether, and the residual ether in water phase was evaporated at 37°. The water phase (300 μl) was then acidified by addition of 100 μl of 6 N HCl and alanine was separated by Dowex 50 column chromatography as described above. Enzymatic measurement of the configuration of this COOH-terminal alanine indicated that 91% of it was the D isomer (Table III).

Large-Scale Digestion of C. diphtheriae Cell Wall by L-3 Enzyme. C. diphtheriae cell wall (150 mg) was incubated at 37° with 5 ml of L-3 enzyme (6 units/ml) in 11.1 ml of phosphate buffer (pH 7.8) (0.01 M final concentration) containing 0.1% NaN_3. Aliquots were removed for the determination of free amino groups, NH_2-terminal amino acid, and turbidity reduction (3, 10, and 3 μl, respectively).

At zero time the incubation mixture contained 0.1 mole of total free amino groups/mole of glutamic acid and 0.44 mole of NH_2-terminal diaminopimelic acid. After 48-hr digestion by the L-3 enzyme, the lysate contained 1.92 moles of free amino groups, 0.89 mole of NH_2-terminal Dap, 0.52 mole of NH_2-terminal alanine, and 0.1 mole of free alanine. These data are similar to those recorded in Table II. The lysate was

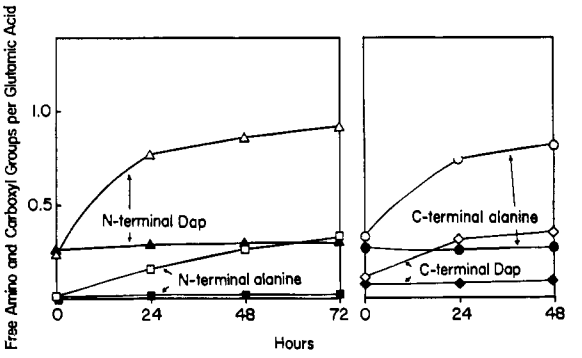

FIGURE 2: Free amino and carboxyl groups released during lysis of cell walls of *C. diphtheriae* by L-3 enzyme. Open symbols represent the digest, closed symbols the control.

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: DNFB, dinitrofluorobenzene.

TABLE IV: Molar Proportions of Amino Acids and Amino Sugars in the Fractions Obtained after Lysis with the L-3 Enzyme by Filtration on a Sephadex G-25 Column.[a]

	Polysaccharide Fraction 30	Peptide Fractions			
		45	49	53	59
Lysine	0	0	0	0.34	0.24
Ammonia	5.50	1.84	1.78	1.68	0.46
Aspartic acid	0	0	0	0	0.34
Threonine	0	0	0	0	0.10
Serine	0	0	0	0	0.02
Glutamic acid	1.00	1.00	1.00	1.00	1.00
Glycine	0	0	0	Trace	0.25
Alanine	2.00	1.85	1.92	1.64	1.52
Isoleucine	0	0	0	0	0.07
Leucine	0	0	0	0	1.17
Diaminopimelic acid	0	0.55	1.14	1.08	1.00
Glucosamine	25.00	0	0	0	0
Muramic acid	22.50	0	0	0	0

[a] Data are expressed as moles per mole of glutamic acid. Each fraction was hydrolyzed at 100° for 12 hr in 4 N HCl prior to analysis.

centrifuged at 34,000*g* for 60 min and the clear supernatant was lyophilized.

Separation of Polysaccharide and Peptide Components by Filtration of the L-3 Enzyme Lysate on a Column of Sephadex G-25. The sample described above was dissolved in 2 ml of water and applied to a Sephadex G-25 column (2 × 90 cm) (Figure 3). The sample was followed by water. Analyses of free amino groups, reducing groups, pentose, and hexosamine revealed that high molecular weight fractions (fractions 28–42) contained the hexosamine and pentose of the cell wall and the low molecular weight fractions (fractions 45–71) contained almost all of the free amino groups of the lysate, but no hexosamine. Reducing groups were extremely small in amount.

The amino acid and amino sugar components of various fractions were determined with the amino acid analyzer (Table IV). The high molecular weight fraction (fraction 30) contained all the glucosamine and muramic acid of the wall (in addition to the pentose, one of the components of the antigenic polysaccharide of this organism) and about 0.04 mole of peptide/mole of glucosamine. The polysaccharide fraction is therefore virtually free of peptide. Fraction 30 also contained 0.015 mole of total organic phosphate. About 0.01 mole of hexosamine was present as hexosamine phosphate (eluted from the long column of the amino acid analyzer just prior to aspartic acid). Further characterization of the polysaccharide fraction will be reported subsequently.

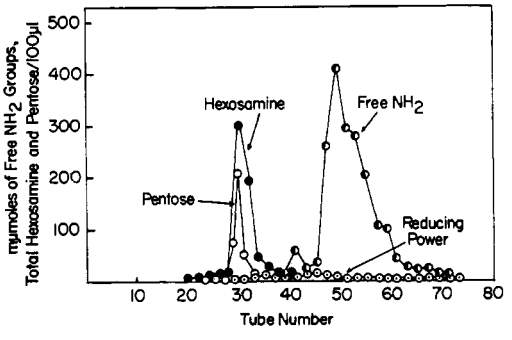

FIGURE 3: Filtration of the L-3 enzyme lysate of *C. diphtheriae* cell wall on a column of Sephadex G-25. A column of Sephadex G-25 (Pharmacia, fine grade, 2 × 90 cm, V_0 = 135 ml) was used. The sample was followed by water. The flow rate was 0.5 ml/min and 8-ml fractions were collected.

Analyses of three of the low molecular weight fractions (fractions 45, 49, and 53) showed that they were peptides composed of alanine (2), glutamic acid (1), and diaminopimelic acid (1–0.5) (Table IV). These fractions also contained about 1.8 moles of bound ammonia. The later fractions of the column effluent (*e.g.*, fraction 59) contained both the major and the minor amino acids of the cell wall.

Thin-layer chromatography of DNP derivatives of aliquots of various fractions indicated that the late fractions contained mainly free amino acids while the earlier fractions contained a number of peptides. A column of Bio-Gel P_2 was employed to separate the low molecular weight amino acids from the high molecular weight peptides. The capability of the Bio-Gel P_2 column used (Bio-Rad, 100–200 mesh, 2 × 35 cm) for separating amino acids from small peptides was demonstrated by separation of glycine and tetraglycine (6 mg of each) in a preliminary experiment.

The low molecular weight fractions from the Sephadex G-25 column (fractions 45–56) were lyophilized, redissolved in 1 ml of water, and applied to the Bio-Gel P_2 column. The sample was followed by water and 1.4 ml was collected in each tube at a flow rate

TABLE V: Amino Acid Composition, NH_2- and COOH-Terminal Amino Acid, and Configuration of the Alanine in Peptides 1 and 2 Isolated from *C. diphtheriae* Cell Walls after Lysis by the L-3 Enzyme.

	Molar Ratio of Amino Acids			NH_2-Terminal Amino Acids		COOH-Terminal Amino Acids		Per Cent of D- and L-Ala	
	Glu	Ala	Dap	Ala	Dap	Ala	Dap	D-Ala	L-Ala
Peptide 1	1.0	1.8	0.9	0.8	0.7	1.0		43.5	56.5
Peptide 2	1.0	0.9	0.7	0.9	0.9		0.6	0	100

of 8.4 ml/hr. Aliquots (50 μl) of each fractions were allowed to react with DNFB. The samples were acidified and extracted with ether to differentiate the ether-insoluble DNP peptides and the ether-soluble DNP-amino acids. Thin-layer chromatography of these DNP derivatives revealed that the peptides were eluted early (effluent volume, 42–56 ml) while the amino acids were eluted later (effluent volume, 57–72 ml).

Separation of the Peptides on a Column of Dowex 50. The peptide-containing fractions of the Bio-Gel P_2 column eluate were combined, lyophilized, and applied to a column of Dowex 50X-8. The details of the column, column operation, and development were the same as described by Jones (1964). Two major peptide peaks were eluted between pH 3.5 and 3.8 (Figure 4). Several minor peptide peaks were also present. The homogeneity of the two major peptides (peptides 1 and 2) was examined by thin-layer chromatography of their DNP derivatives on silica gel G using solvents 4 and 5. In each case one major component (*R* (DNP-tetraglycine) 1.07 and 1.0 in solvent 4 and 1.07 and 1.0 in solvent 5) was found. Several minor components of lower mobility were sometimes detected but these are known to be due to incomplete dinitrophenylation of the major peptides.

Rechromatography of the Two Peptide Fractions on Dowex 1. A column of Dowex 1-Cl-X2 (Bio-Rad, Richmond, Calif., 200–400 mesh, 0.9 × 100 cm) was converted into the acetate form and equilibrated with the starting buffer (*N*-ethylmorpholine-containing pyridine–acetic acid buffer (pH 9.0) (Schroeder *et al.*, 1962)). The fractions of peptide 1 (fractions 16–19, Figure 4) were lyophilized and redissolved in 1 ml of the starting buffer. This solution was applied to the Dowex column. This was followed by a gradient started with 500 ml of this buffer at pH 9.0 in the mixing flask. Then 500 ml of buffer containing increasing concentrations of acetic acid (0.2, 0.5, 1.0, and 2.0 N) were added to the reservoir. The pH of the effluent dropped rapidly at the beginning of the addition to pH 8.0 and then more gradually. An aliquot (20 μl) of each fraction (10 ml) was lyophilized and free amino groups were determined by the DNP method. One major (peptide 1) and one minor (peptide 1′) peak were eluted successively in the pH range 7.5–6.5 (at about 760 and 808 ml).

Peptide 2 was also chromatographed on the same column. However, collidine buffer (1% collidine, 1% pyridine, and 0.075% acetic acid) (pH 8.1) (modification of Schroeder *et al.*, 1962) was used as the starting buffer, and buffer containing increasing concentrations (0.01, 0.02, 0.05, and 0.1 N) of acetic acid was added to the reservoir. Peptide 2 was eluted as a single peak at pH 6.2 (360 ml).

Analyses of Peptides 1 and 2. AMINO ACID COMPOSITION and NH_2- AND COOH-TERMINAL AMINO ACIDS. Peptide 1 contained glutamic acid, diaminopimelic acid, and alanine in the ratio 1:1:2 (Table V). After treatment with DNFB, all of the diaminopimelic acid was recovered as mono-DNP-diaminopimelic acid. One of the alanine residues was NH_2 terminal and one was COOH terminal. One of the alanine residues was L-alanine and the other was D-alanine. The NH_2-terminal alanine can be assumed to be L-alanine and COOH-terminal residue D-alanine.

Peptide 2 contained glutamic acid, diaminopimelic acid, and alanine in the ratio 1:1:1 (Table V). After treatment with DNFB, 1 mole each of NH_2-terminal DNP-alanine and mono-DNP-diaminopimelic acid per mole of glutamic acid was obtained. The COOH-terminal amino acid was diaminopimelic acid. The alanine residue had the L configuration. The glutamic acid of both peptides was insensitive to the L-glutamic

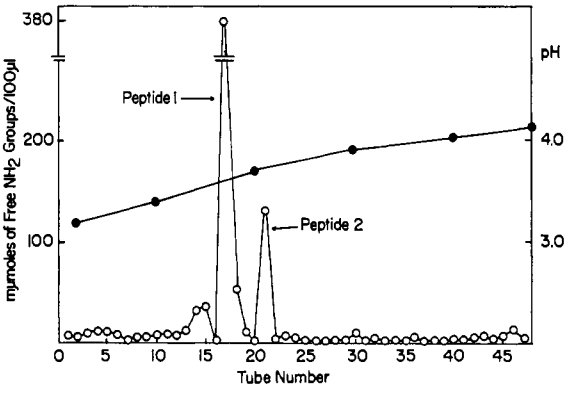

FIGURE 4: Separation of cell wall peptides on Dowex 50. The peptide fraction from the Bio-Gel P_2 column was lyophilized, redissolved in 2 ml of 2% formic acid, and applied to a Dowex 50-X8 column (Spinco type 15A resin, 0.9 × 17 cm). The column was equilibrated with 0.2 M pyridine buffer (pH 3.1) (Schroeder *et al.*, 1962). Development was carried out with 500 ml of a linear gradient of pyridine–acetic acid beginning with 0.2 M pyridine at pH 3.1 and ending with 2.0 M pyridine at pH 5.0. The column was kept at 50° and fractions were collected at room temperature. The flow rate was 36 ml/hr. Aliquots (20 μl) were removed for the determination of amino group with DNFB.

TABLE VI: Edman Degradation of Peptides 1 and 2 Obtained after Lysis with the L-3 Enzyme.[a]

	Degradation Step	NH_3		NH_2-Terminal Groups	
		Free	Total	Ala	Glu
Peptide 1	0	0	24.5	62	0
	1	0	26.2	0	42
	2	7.4	8.3	0	0
Peptide 2	0	0	58.0	51	0
	1	0	24.8	0	34
	2	9.3	13.0	0	0
L-Isoglutamine	0	0	45.0	0	42
	1	23.5	25.8	0	0

[a] Data are expressed as millimicromoles in the sample analyzed.

acid decarboxylase of *E. coli;* thus, it is the D isomer. The diaminopimelic acid was the *meso* isomer, easily distinguished from the LL or DD isomers by thin-layer chromatography of the diDNP derivatives on silica gel G in solvent 1.

These analyses thus showed that these materials were peptide monomers, peptide 1 being a tetrapeptide and peptide 2 a tripeptide.

PAPER ELECTROPHORESIS OF THE PEPTIDES. Examination of peptides by paper electrophoresis was carried out at pH 2.0 in 2% formic acid and 5.0 in 0.1 M pyridine acetate buffer at 2°. At pH 2.0 both peptides moved toward the anode, showing slightly different mobilities. Peptide 1 moved 12.8 cm and peptide 2 12.4 cm at a potential gradient of 14 V/cm for 2.5 hr. At pH 5.0 both peptides stayed near the origin, indicating that both were uncharged at this pH. However, the peptides, Ala-Glu-Dap-Ala and Ala-Glu-Dap, would each contain two positively charged amino groups and three negatively charged carboxyl groups at pH 5.0. These data suggested that one of the carboxyl groups might be an amide. Presumably, in the tripeptide at least, this could not be a Dap-carboxyl group. Such a group would not be measured as a COOH-terminal amino acid since it would yield a hydrazide in the procedure used.

EDMAN DEGRADATION OF THE PEPTIDES. Peptide 1 (150 mμmoles) and peptide 2 (100 mμmoles) were subjected to two cycles of degradation with phenyl isothiocyanate by an method modified from that of Konigsberg (Tipper *et al.*, 1967) (Table VI).

In both peptides NH_2-terminal alanine disappeared and NH_2-terminal glutamic acid appeared after the first cycle. The recovery of NH_2-terminal glutamic acid was 61–68% when calculated on the basis of the yield of glycine from Ala-Gly-Gly. At the first cycle the NH_2-terminal group of diaminopimelic acid also disappeared since it became substituted by the phenylthiocarbamyl group.

At the second cycle NH_2-terminal glutamic acid disappeared and free ammonia was liberated. The yield of free ammonia was low, but a similarly low yield was obtained from the standard, isoglutamine. These results indicate that the second amino acid of both peptides 1 and 2 was glutamic acid and that the α-carboxyl group of glutamic acid was substituted by ammonia.

The analyses of amino acid composition, NH_2-, and COOH-terminal amino acid showed that peptide 1 is a tetrapeptide containing one carboxamide group: its sequence must be L-Ala-D-iso-Glu(NH_2)-*meso*-Dap-D-Ala. Peptide 2 is a tripeptide containing one carboxamide group; its sequence must be L-Ala-D-iso-Glu(NH_2)-*meso*-Dap.

Hydrolysis of C. diphtheriae Cell Wall by Myxobacterium Enzyme. The *Myxobacterium* enzyme (Ensign and Wolfe, 1964–1966) is a cell wall lytic enzyme active especially on *Arthrobacter* and *S. aureus*. Kinetic studies of lysis of cell walls showed that the enzyme-catalyzed hydrolysis of both acetylmuramyl-L-alanine linkages and of the polyglycine cross-bridges in the wall of *S. aureus* (Tipper *et al.*, 1967). Its action on the wall of *C. diphtheriae* was examined in the hope that it might hydrolyze only the former type of linkages in these walls, and thus provide a means of obtaining the cross-linked polypeptide subunits of the cell wall.

C. diphtheriae cell walls (900 μg) were suspended in 100 μl of 0.01 M Veronal buffer (pH 8.9) containing 19 μl of *Myxobacterium* enzyme solution and incubated at 37°. Free amino groups were released to a value of 1.2 moles/mole of glutamic acid after 18 hr and 1.53 moles after 72 hr. Examination of the nature of the NH_2-terminal groups released indicated that after 72-hr incubation 0.69 mole of alanine/mole of glutamic acid was released (Figure 5). There was no increase of NH_2-terminal Dap indicating that no cross-bridges had been opened. The NH_2-terminal alanine released had the L configuration (Table VII) indicating that the acetylmuramyl-L-alanine linkage had been split.

Large-Scale Lysis of C. diphtheriae Cell Wall by Myxobacterium Enzyme and Fractionation of the Lysate on Columns of Sephadex G-50 and G-25. Cell walls of *C. diphtheriae* (300 mg) were incubated with 2 ml of

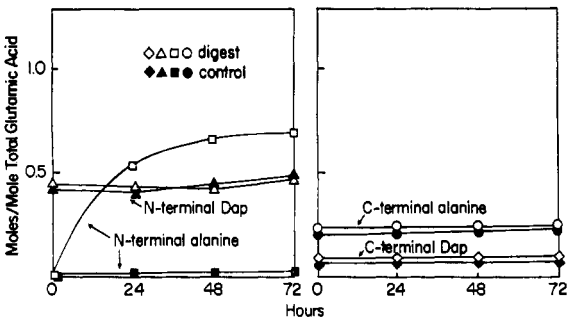

FIGURE 5: Kinetics of the release of NH_2- and COOH-terminal amino acids during the lysis of *C. diphtheriae* cell wall by *Myxobacterium* enzyme. Open symbols show the incubation mixture of cell walls with enzyme and closed symbols are the control mixture of cell walls without enzyme.

Myxobacterium enzyme in 20 ml of 0.01 M sodium barbital buffer (pH 8.9) at 37°. After 72-hr incubation, the lysate was centrifuged at 34,000*g* for 15 min in the Servall centrifuge. The residue was washed twice with water. The clear supernatant solution and washings were combined and concentrated to 3 ml. This solution was subjected to get filtration on columns of Sephadex G-50 and Sephadex G-25 run in tandem (Tipper and Strominger, 1968). The tandem column was developed with water.

Various measurements indicated that there were two high molecular weight peaks which contained hexosamine and pentose, and two low molecular weight fractions which contained free amino groups (Figure 6). Thin-layer chromatography of the DNP derivatives of the material in the peaks with free amino group showed that the first peak at tubes 65–71 contained two peptides and the second peak at tubes 73–79 contained several additional peptides and free amino acids.

Separation of Peptide Peak 1 on a Column of Dowex 50. Lyophilized material recovered from peak 1 (tubes 65–71, Figure 6) was redissolved in 2 ml of 2% formic acid and applied to the top of the column. The column was developed first with 400 ml of 0.2 M pyridine–acetic acid buffer (pH 3.1). No material with free amino groups was eluted with buffer. Then a linear gradient

TABLE VII: Configuration of the NH_2-Terminal Alanine Released after Lysis of the Cell Wall of *C. diphtheriae* by the *Myxobacterium* Enzyme.[a]

	D-Alanine	L-Alanine
Control	0.41	0.91
After dinitrophenylation	0.55	0.49
Δ	0	−0.47

[a] The method employed depends upon the disappearance of one or both of the isomers of NH_2-terminal alanine after dinitrophenylation and is described earlier in the text. Data are expressed as moles of D- or L-alanine per mole of glutamic acid.

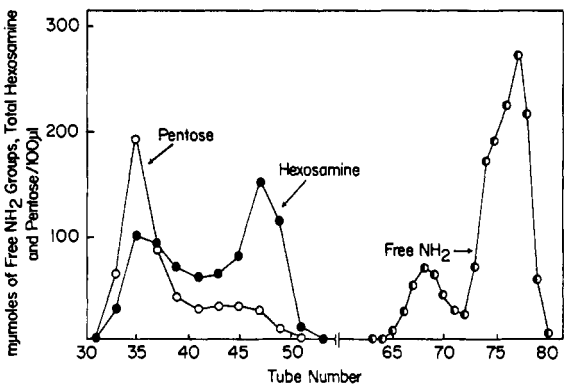

FIGURE 6: Filtration on columns of Sephadex G-50 and G-25 of *C. diphtheriae* cell walls by the *Myxobacterium* enzyme. Columns of Sephadex G-50 (1.4 × 114 cm) and G-25 (2.5 × 100 cm) were operated in tandem. Water was the eluent at a flow rate of 30 ml/hr. Fractions of 10 ml were collected. Analyses of free amino groups, hexosamines (after hydrolysis at 95° for 4 hr), and pentose were carried out.

was applied with 400 ml of 0.2 M pyridine–acetic acid buffer (pH 3.1) in the mixing vessel and 400 ml of 2.0 M pyridine–acetic acid buffer (pH 5.0) in the reservoir.

Two materials with free amino groups were eluted successively in the pH range 3.3–3.5 (Figure 8A). Analyses of these two fractions are presented below.

Separation of Peptides and Amino Acids of Peak 2 on Columns of Sephadex G-10 and Dowex 50. Since peak 2 (tubes 73–79, Figure 6) was presumed to contain free amino acids as well as peptides, a preliminary separation was carried out on a column of Sephadex G-10 (2.5 × 48 cm). The material of peak 2 was lyophilized, redissolved in 2.5 ml of water, and applied to the top of the column. The column was developed with water at a rate of 50 ml/hr (Figure 7).

Determination of free amino groups and thin-layer chromatography of the DNP derivatives of materials in each tube was carried out, and the effluent was divided into three fractions (Figure 7) with reference to the materials found. These three fractions were

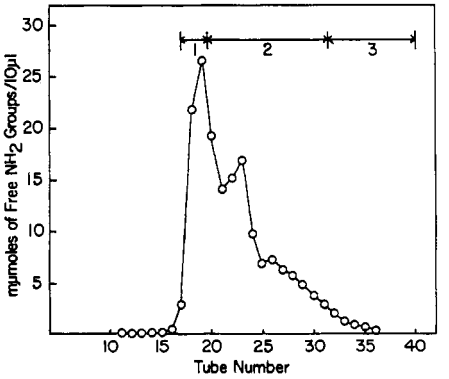

FIGURE 7: Filtration on a column of Sephadex G-10 of peak 2 (Figure 5) of Sephadex G-50 and G-25 column. Details of the separation are given in the text. Aliquots (10 μl) of the column eluate were taken for the determination of free NH_2 groups.

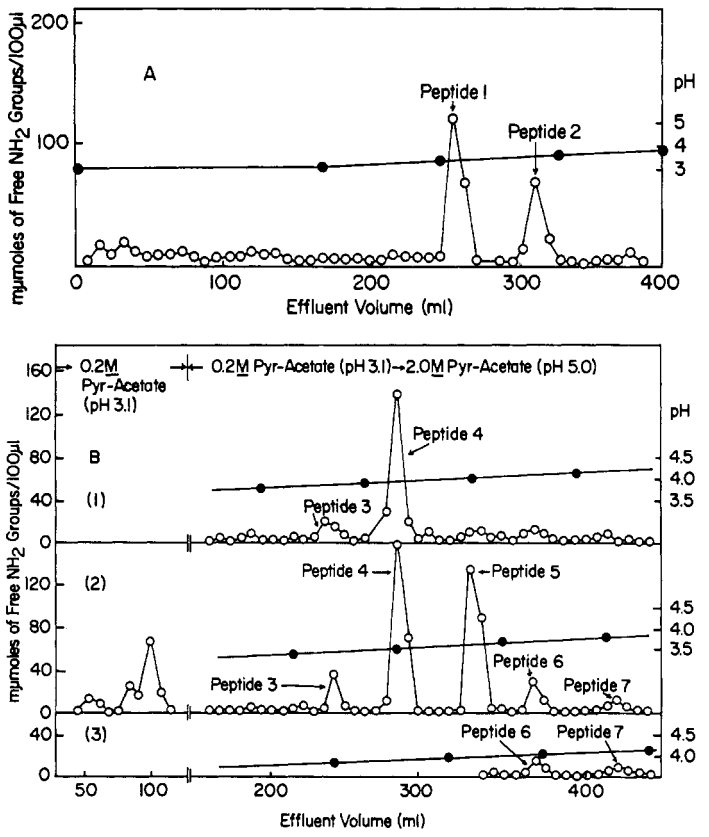

FIGURE 8: Separation on a column of Dowex 50 of the peptides obtained from cell walls after lysis with the *Myxobacterium* enzyme. The Dowex 50-X8 column (Spinco type 15A resin, 0.9 × 17 cm) was equilibrated with 0.2 M pyridine–acetic buffer (pH 3.1) (Schroeder *et al.*, 1962). First, 0.2 M pyridine buffer (pH 3.1) was employed as the eluent and then a linear gradient was applied beginning with this buffer in the mixing vessel and 2.0 M pyridine–acetate buffer (pH 5.0) in the reservoir. The column was kept at 50° and fractions were collected at room temperature at a flow rate of 50 ml/hr. Free amino groups were measured with DNFB. (A) Material recovered from peak 1 (tubes 65–71, Figure 6) was lyophilized, redissolved in 2 ml of 2% formic acid, and applied to the column. (B) The three fractions from the Sephadex G-10 column (Figure 7) were lyophilized, redissolved in 2 ml of 2% formic acid, and applied separately to the column. (1) Fraction 1, tubes 17–19; (2) fraction 2, tubes 20–31; (3) fraction 3, tubes 32–40.

lyophilized separately and each was subjected to chromatography on a Dowex 50 column (Figure 8B).

Two peptide fractions were found in the first fraction, eluted in the pH range of 3.8–4.0. The second fraction was more complicated. Some free amino acids were eluted early in the column at pH 3.1. Five peptide fractions were eluted later, two of which were present in the first fraction. The third fraction yielded two peptides, both of which were also present in the second fraction. Analyses (see below) indicated that peptides eluted at similar position were in fact identical. The five peptides obtained from this column will be referred to as peptides 3–7.

Analyses of the Seven Peptides Isolated from the Myxobacterium Enzyme Lysate of C. diphtheriae Cell Walls. AMINO ACID COMPOSITION, NH_2- AND COOH-TERMINAL AMINO ACIDS, AND CONFIGURATION OF THE

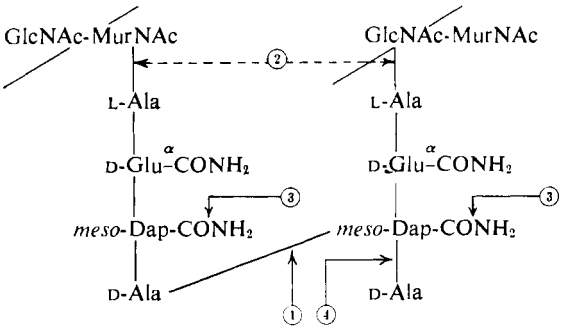

FIGURE 9: Points of cleavage of the octapeptide dimer and of the peptidoglycan of the cell wall of *C. diphtheriae* by the L-3 enzyme. This representation is of two subunits of the peptidoglycan. The octapeptide dimer results from hydrolysis at 2 by an acetylmuramyl-L-alanine amidase, such as that found in the *Myxobacterium* enzyme. Hydrolysis at 1 is catalyzed by the D-Ala-*meso*-Dap bridge-splitting enzyme in the L-3 preparation and that at 3 by the diaminopimelic acid amidase. The data obtained suggest the presence of a D-alanine carboxypeptidase (4) (see text), but the existence of this enzyme has not been proven. When the L-3 preparation acts on cell walls, hydrolysis at 2 is also catalyzed.

ALANINE (TABLE VIII). All the peptides isolated are composed of alanine, glutamic acid, and diaminopimelic acid. The ratio of NH_2-terminal alanine and NH_2-terminal Dap to total amino acid indicated that peptides 1 and 2 were monomers. They are identical with the tetrapeptide monoamide and tripeptide monoamide isolated from the L-3 enzyme lysate.

Peptide 3 had three alanine residues, one of which was L and two of which were the D isomer. This peptide was a pentapeptide which contained two amide residues.

Peptides 4 and 5 were also a tetra- and tripeptide monomer, respectively. They differed from peptides 1 and 2 in that each contained two amide residues. Peptide 5 did not give any COOH-terminal amino acid after hydrazynolysis, suggesting that the carboxyl group of the COOH-terminal diaminopimelic acid of this tripeptide might be substituted by ammonia. Treatment of peptides 4 and 5 with the Edman reagent yielded ammonia at the first cycle indicating that in each case the diaminopimelic acid was an amide and that the carboxamide was adjacent to an α-amino group.

Analyses of peptides 6 and 7 indicated again that almost all of the L-alanine was NH_2 terminal, but the NH_2-terminal diaminopimelic acid was only half of the total amount of diaminopimelic acid. These data indicated that these two peptides were dimers. The analyses were compatible with the structure of an octapeptide for peptide 6 (two tetrapeptide diamide units linked *via* a bond between the terminal alanine carboxyl group in one and an amino group of diaminopimelic acid in the other) and a heptapeptide for peptide 7 (one tetrapeptide diamide unit and one tripeptide diamide unit, similarly linked).

PAPER ELECTROPHORESIS was carried out at pH 5.0 as described above. Peptides 1 and 2 were neutral and stayed near the origin. Peptides 3–7 migrated toward the cathode between alanine (neutral, 0.8 cm) and lysine (charge +1, 7.5 cm). The migrations were:

TABLE VIII: Amino Acid Composition, NH_2- and COOH-Terminal Amino Acids, and Configuration of the Alanine in Each Peptide Isolated from *C. diphtheriae* Cell Wall after Lysis by the *Myxobacterium* Enzyme.

Peptide	Molar Ratio of Amino Acids and Ammonia				NH_2-Terminal Amino Acids		COOH-Terminal Amino Acids		Per Cent of D- and L-Ala	
	Glu	Ala	Dap	NH_3	Ala	Dap	Ala	Dap	D-Ala	L-Ala
1	1.00	2.04	0.98	1.19	0.77	0.82	0.67	(0.33)	49.5	50.5
2	1.00	1.01	1.07	1.19	0.98	0.93		0.89	3.5	96.5
3	1.00	2.90	0.98	1.72	1.06	1.04	1.00		66.5	33.5
4	1.00	2.05	1.40	1.85	1.03	0.92	0.50	0.11	50.7	49.3
5	1.00	1.00	1.01	1.89	0.86	0.90	0.20	0.13	6.0	94.0
6	1.00	2.10	1.03	1.87	1.05	0.53	0.35		48.5	51.5
7	1.00	1.49	0.82	1.77	1.10	0.50	0.10	0.11	36.0	64.0

TABLE IX: NH_2- and COOH-Terminal Amino Acids Produced by the Action of L-3 Enzyme on Peptides 6 and 7.[a]

	Incubn Time (hr)	NH_2-Terminal Amino Acids		COOH-Terminal Amino Acids	
		Ala	Dap	Ala	Dap
Peptide 6	0	0.97	0.48	0.36	0.08
	24	1.02	1.17	0.91	0.51[b]
	Δ	0.05	0.69	0.55	0.43
Peptide 7	0	0.93	0.41	0.13	0
	24	0.93	1.27	0.51	1.25
	Δ	0.00	0.86	0.43	1.25

[a] Data are expressed as moles per mole of total glutamic acid in the substrates. [b] The origin of the COOH-terminal Dap from peptide 6 is not clear. This octapeptide should be hydrolyzed to two tetrapeptide subunits, each with a COOH-terminal D-alanine residue. In the large-scale experiment with the L-3 enzyme some free alanine was found. It is possible therefore that this preparation also contains a D-alanine carboxypeptidase which could account for the appearance of COOH-terminal Dap in peptide 6.

peptide 1, 0.8 cm; peptide 2, 0.8 cm; peptide 3, 3.6 cm; peptide 4, 4.1 cm; peptide 5, 4.4 cm; peptide 6, 4.8 cm; and peptide 7, 5.1 cm. These data were compatible with the fact that peptides 3–7 had one negative charge less than peptides 1 and 2, due to the presence of a second amide residue.

Hydrolysis of Peptide Dimers (Peptides 6 and 7) by the L-3 Enzyme. Comparison of the results obtained with the L-3 enzyme lysate and those obtained with the *Myxobacterium* enzyme lysate suggested that the L-3 enzyme contained an amidase which removed the amide groups of diaminopimelic acid (but not those of glutamic acid) as well as an enzyme which catalyzed hydrolysis of the D-Ala-*meso*-Dap cross-link. This question was examined using the peptide dimers as substrates. The proposed structures of peptides 6 and 7 and points of cleavage by the L-3 enzyme are shown in Figure 9.

Peptides 6 and 7 (about 120 mμmoles of each) were lyophilized and redissolved in 80 μl of water. L-3 enzyme solution (100 μl, 6 units/ml) and 20 μl of 0.1 M phosphate buffer (pH 8.0) containing 1.1% NaN_3 were added. The reaction mixtures were incubated at 37° for 48 hr. Analyses of the released NH_2- and COOH-terminal groups were carried out. The materials were also examined by paper electrophoresis at pH 5.0.

RELEASE OF NH_2-TERMINAL AMINO ACIDS. The amount of NH_2-terminal diaminopimelic acid increased from about 0.5 to about 1 residue per glutamic acid residue in each of the peptides, indicative of cleavage of all of the cross-links (Table IX).

RELEASE OF COOH-TERMINAL GROUPS. COOH-terminal D-alanine (about 0.5 mole) was released also from both peptides by the action of L-3 enzyme (Table IX). The appearance of a mole of COOH-terminal diaminopimelic acid in peptide 7 is compatible with the hydrolysis of an amide on the terminal diaminopimelic acid of the heptapeptide. Some COOH-terminal diaminopimelic acid also appeared in peptide 6. Its origin is not clear, but its appearance as the result of the action of a D-alanine carboxypeptidase is one possibility (see footnote *b* in Table IX).

PAPER ELECTROPHORESIS. Examination of peptides by paper electrophoresis revealed that, while peptides 6 and 7 were positively charged (mobilities: peptide 6

was 4.8 cm and peptide 7 was 5.1 cm in 0.1 M pyridine acetate buffer (pH 5.0)), the products of L-3 enzyme digestion (peptide monomers) were neutral. This result suggested that an amide residue had been removed from either diaminopimelic acid or glutamic acid. It has been shown above that it is the diaminopimelic acid residue which lacks an amide group after lysis of cell walls by the L-3 enzyme and it is therefore presumed that this amide is also the one hydrolyzed in the isolated peptide dimers.

Discussion

The lysis of cell walls of *C. diphtheriae* by the L-3 enzyme preparation is, therefore, due to the action of a D-alanine-*meso*-diaminopimelic acid endopeptidase which catalyzes the hydrolysis of interpeptide bridges connecting the peptide subunits. Although other activities are present, the bridge-splitting enzyme is more active in the preparation than the other activities which have been measured and the decrease of turbidity of the cell suspension parallels the increase of amino groups due to the opening of the interpeptide bridges. Solubilization of cell walls as a consequence of bridge-splitting enzymes of this type has been found in several different bacteriolytic enzyme preparations using a variety of organisms (Strominger and Ghuysen, 1967). A specific D-Ala-*meso*-Dap bridge-splitting enzyme has also been found as one of the components of the *E. coli* autolytic complex (Pelzer, 1963).

A second activity in the preparation, an acetylmuramyl-L-alanine amidase, is a much weaker activity. The other activities, an enzyme which deamidates an amide of diaminopimelic acid and possibly a D-alanine carboxypeptidase, catalyze hydrolyses which would not result in solubilization of cell walls. Both the acetylmuramyl-L-alanine amidase and the D-alanine carboxypeptidase have been found in a variety of other microbial sources (see Strominger and Ghuysen, 1967) but the diaminopimelic acid deamidase has not been described previously.

Some picture of the structure of the peptide portion of the peptidoglycan of the cell wall of *C. diphtheriae* has emerged from this study. The main peptide units substituted on the acetylmuramic acid residues of the glycan are tetrapeptides (L-Ala-D-Glu-*meso*-Dap-D-Ala) and tripeptides (L-Ala-D-Glu-*meso*-Dap) in which the carboxyl groups of both the D-glutamic acid and the *meso*-diaminopimelic acid residues are present as amides. Some units also occur in which only the D-glutamic acid residues bear the amide. It is not clear whether these latter units were introduced into the wall during growth of the cells or whether they were artificially produced in some manner (*e.g.*, through the action of an autolytic enzyme during preparation of the cell walls).

A portion of the tetrapeptide and tripeptide subunits are cross-linked to each other through D-Ala-*meso*-Dap bridges. The extent to which such cross-linking occurs in the wall of *C. diphtheriae* is not absolutely clear from the present study. On the one hand, the isolation of the peptide subunits after lysis of cell walls with the *Myxobacterium* enzyme (presumably acting as an acetylmuramyl-L-alanine amidase) suggest that the total of cross-linked units may not exceed 20% (see Figure 8). On the other hand, the analysis of the NH_2-terminal groups of diaminopimelic acid found after solubilization of cell walls suggest that only 30–40% of such amino groups are free (Table II, Figure 5). A value of 50% would be obtained if all of the peptide subunits were present as dimers. Lower values, such as that found, suggest that some higher oligomers must also be present in the native wall. The data, therefore, suggest that some cleavage of interpeptide bridges may have occurred on treatment of the walls with the *Myxobacterium* enzyme even though no evidence of such cleavage was perceived in the experiment of Figure 5. It is sometimes difficult to measure an increase of new NH_2-terminal groups over a high initial blank. Therefore, it would be desirable to obtain the peptide subunits by an alternative route. Such a method would be solubilization of the wall with an endoacetylmuramidase (B enzyme from *Chalaropsis*) followed by hydrolysis with the *N*-acetylmuramyl-L-alanine amidase from *Streptomyces* (Ghuysen *et al.*, 1962). Preliminary experiments carried out with this method of obtaining the peptide subunits indicated that after release of 0.8–0.9 residue of NH_2-terminal alanine and subsequent chromatography on columns of CM-cellulose and Dowex 50 the per cent of peptide dimers present in the mixture was larger than that recorded in Figure 8, and evidence for small amounts of slightly larger oligomers (perhaps trimers) was present. Thus, a further fractionation and analysis of peptides obtained by this means will be necessary in order to reconstruct a picture of the nature of the peptide subunits actually present in the cell wall of *C. diphtheriae*.

References

Anderson, R. T. (1943), *Yale J. Biol. Med. 15*, 311.

Ashwell, G. (1957), *Methods Enzymol. 3*, 73.

Ensign, J. C., and Wolfe, R. S. (1964), *J. Bacteriol. 87*, 924.

Ensign, J. C., and Wolfe, R. S. (1965), *J. Bacteriol. 90*, 395.

Ensign, J. C., and Wolfe, R. S. (1966), *J. Bacteriol. 91*, 524.

Ghuysen, J.-M., Leyh-Bouille, M., and Dierickx, L. (1962), *Biochim. Biophys. Acta 63*, 286.

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

Hash, J. H. (1963), *Arch. Biochem. Biophys. 102*, 379.

Jones, R. J. (1964), *Cold Spring Harbor Symp. Quant. Biol. 29*, 297.

Mori, Y., Kato, K., Matsubara, T., and Kotani, S. (1960), *Biken's J. 3*, 139.

Mori, Y., and Kotani, S. (1962), *Biken's J. 5*, 55.

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

Pelzer, H. (1963), *Z. Naturforsch. 18b*, 950, 956.

Schroeder, W. A., Jones, R. T., Cormick, J., and McCalla, K. (1962), *Anal. Chem. 34*, 1570.
Strominger, J. L., and Ghuysen, J.-M. (1967), *Science 156*, 213.
Strominger, J. L., Izaki, K., Matsuhashi, M., and Tipper, D. J. (1967), *Federation Proc. 26*, 9.
Tipper, D. J., and Strominger, J. L. (1968), *J. Biol. Chem. 243*, 3169.
Tipper, D. J., Strominger, J. L., Ensign, J. C. (1967), *Biochemistry 6*, 906.
Weidel, W., and Pelzer, H. (1964), *Advan. Enzymol. 26*, 193.

Heterogeneity in Protein Subunits of Human Serum High-Density Lipoproteins*

B. Shore and V. Shore

ABSTRACT: The protein moieties of high-density lipoproteins of human serum contain comparable quantities of two polypeptide subunits of different amino acid sequence. These peptides in urea solutions were separated by polyacrylamide gel electrophoresis and by chromatography on DEAE-cellulose. The carboxyl-terminal sequences -Thr-Gln and probably -Lys-Tyr-Lys-Asn-Leu-Thr were elucidated by the actions of carboxypeptidases A and B on the lipid-free protein moieties of two fractions (1.083–1.124 and 1.126–1.195 g/cc) of lipoproteins and on the peptides fractionated on DEAE-cellulose. Glutamic acid γ-hydrazide, indicative of C-terminal glutamine, in addition to threonine, was found among the products of hydrazinolysis of the protein.

The protein contains approximately 2 moles of C-terminal glutamine plus threonine per 30,000 g of protein.

The protein moiety of the entire human serum high-density lipoprotein fraction (1.065–1.195 g/cc) is commonly thought to consist of one protein, the α protein, which occurs as identical subunits whose number varies with the density and molecular weight of the parent lipoprotein (Shore, 1957; Scanu, 1966; Levy and Fredrickson, 1965; Gustafson *et al.*, 1966). There are, however, some preliminary reports which do suggest heterogeneity in the proteins of high-density lipoproteins (Shore and Shore, 1966; Alaupovic *et al.*, 1967).

In the present study, the presence in high-density lipoproteins of two nonidentical peptides in comparable amounts is indicated (1) by the action of carboxypeptidases A and B on the protein moiety, (2) by the finding of glutamic acid γ-hydrazide, indicative of C-terminal glutamine, and threonine as products of hydrazinolysis of the protein, (3) by polyacrylamide gel disc electrophoresis of the protein in 8 M urea solution at pH 8.8, and (4) by separation of the peptide with C-terminal glutamine from that with C-terminal threonine by DEAE-cellulose column chromatography.

Materials and Methods

Lipoprotein fractions of densities 1.083–1.124 and 1.126–1.195 g/cc (HDL_2 and HDL_3, respectively) were isolated from human serum of individual donors. HDL_3 was prepared as described previously (Shore and Shore, 1967); HDL_2, the lipoproteins which floated between solvent densities 1.083 and 1.124 g/cc, were separated from less dense lipoproteins by two centrifugations at a solvent density of 1.083 g/cc (20°) for 36 hr at 39,000 rpm in a 40.3 rotor at 13–14° in a Spinco Model L centrifuge. The HDL_2 was then concentrated by two flotations at a solvent density of 1.124 g/cc for 48 hr at 39,000 rpm at 13–14°. The protein moieties of these lipoprotein fractions were obtained in lipid-free, water-soluble form with essentially complete recovery as described previously (Shore and Shore, 1967).

Carboxypeptidase A (COA-DFP 6131), a three-times-crystallized, diisopropylfluorophosphate-treated enzyme in water suspension, was obtained from Worthington Biochemical Corp. (Freehold, N. J.). Aliquots of the enzyme suspension were dissolved in 2 M LiCl at pH 8 before use. Carboxypeptidase B (COB-DFP 7GA) was obtained as a frozen solution from the same source. The presence of a small amount of carboxypeptidase A in the carboxypeptidase B preparation or *vice versa* was not excluded. The proteins in water solution (2 mg/ml) were digested with carboxypeptidase A, or

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