Structure of the Cell Wall of *Micrococcus lysodeikticus*. II. Study of the Structure of the Peptides Produced after Lysis with the *Myxobacterium* Enzyme*

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ABSTRACT: The bacteriolytic enzyme obtained from a species of *Myxobacterium* hydrolyzes cell walls of *Micrococcus lysodeikticus* by catalyzing hydrolysis of acetylmuramyl-L-alanine linkages. After this hydrolysis the intact glycan and the intact peptide subunits have been separated. The latter have been fractionated into two major and one minor peptide component. One of the major peptide components is the dimer of two pentapeptides (L-Ala-D-γ-Glu(α-Gly)-L-Lys-D-Ala) connected by a D-alanyl-N^{ϵ}-lysine linkage. The glycine residues on the α-carboxyl group of glutamic acid have a free carboxyl group. The other major peptide fraction contains either three or four pentapeptide units connected in a similar manner. The structure of the cell wall of *M. lysodeikticus* is discussed in the light of these findings and earlier investigations.

In the peptidoglycan of bacterial cell walls, the glycan consists of alternating residues of acetylglucosamine and acetylmuramic acid. All of the acetylmuramic acid residues are substituted by a peptide containing L-alanine, D-alanine, D-glutamic acid, and a dibasic amino acid, most commonly L-lysine or *meso*-diaminopimelic acid. These peptidoglycan strands are then cross-linked through peptide bridges involving the dibasic amino acid, which thus serves as the branch point in the formation of the cross-linked branched peptide. In the past 5 years the structure of the peptidoglycan of *Staphylococcus aureus* has been extensively investigated and the main features of both the glycan and peptide structures are known (Mandelstam and Strominger, 1961; Ghuysen *et al.*, 1965; Tipper *et al.*, 1967; Muñoz *et al.*, 1966b). More recently, the peptidoglycans of two other micrococci have been studied, namely *Micrococcus roseus* strain R27 (Petit *et al.*, 1966) and *Micrococcus lysodeikticus* (Leyh-Bouille *et al.*, 1966; Muñoz *et al.*, 1966a).

In the preceding paper in this series the structure of the glycan in *M. lysodeikticus* was studied (Leyh-Bouille *et al.*, 1966). In the present work the structure of the peptide has been investigated. This peptide was obtained after hydrolysis of cell walls with a bacteriolytic enzyme obtained from a species of *Myxobacterium* (Ensign and Wolfe, 1965, 1966; Tipper *et al.*, 1967). This enzyme is a proteinase which also hydrolyzes some of the peptide linkages which are found in the cell wall. As will be shown, its action on *M. lysodeikticus* cell walls is limited to hydrolysis of the acetylmuramyl-L-alanine linkages. After hydrolysis of these linkages, the glycan and the peptide have been separated. The composition of the peptide fraction and studies of its structure are reported in the present paper.

Materials and Methods

General Procedures. Cell walls were prepared from commercially available cells of *M. lysodeikticus* (Miles Laboratories, Elkhart, Ind.) according to standard procedures, including disintegrating of cells in a Sorvall Omni-Mixer high-speed homogenizer and differential centrifugation. We are grateful to Dr. J. F. Petit for the cell wall preparation employed, to Dr. J. Ensign for a gift of the *Myxobacterium* enzyme, and to Dr. J.-M. Ghuysen for *N*-acetylmuramyl-L-alanine amidase. Total hexosamines were measured by the Morgan–Elson reaction, following essentially the method of Reissig *et al.* (1955), as modified for a microdetermination (see Ghuysen *et al.*, 1966). Sephadex G-25 (fine grade) (Pharmacia, Uppsala, Sweden) and Dowex 50-X8 (50–100 mesh) were used for gel filtration and ion-exchange chromatography, respectively, of the cell wall peptides.

Determination of Total, NH_2-Terminal, and COOH-Terminal Amino Acids. For total amino acids, samples containing 20–50 mμmoles of peptide were hydrolyzed in a total volume of 70–100 μl of 4 N HCl for 6 hr at 100°. The hydrolysate was dried over NaOH and treated with dinitrofluorobenzene[1] in 50 μl of a 1% $K_2B_4O_7$ solution at 60° for 30 min.

For NH_2-terminal amino acid determination, the

* From the Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin. *Received September 2, 1966.* Supported by research grants from the U. S. Public Health Service (AI-06247) and National Science Foundation (GB-4552) and by a travel grant (to W. K.) from the Fulbright Commission.

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[1] Abbreviations used: DNFB, dinitrofluorobenzene; UDP, uridine diphosphate.

peptide sample was dried *in vacuo*, redissolved in 50 μl of 1% $K_2B_4O_7$, and treated with DNFB under the above conditions. The reaction mixture was acidified with concentrated HCl to give a final HCl concentration of 4 N and hydrolyzed for 6 hr at 100°.

Ether-soluble dinitrophenylamino acids were extracted from the above solutions with 200 μl of ether three times. The ether was then evaporated by brief warming. ε-Mono-DNP-lysine, remaining in the aqueous phase, was extracted twice with the same volume of butanol–1% ammonium hydroxide (1:1) (organic phase). The solvent was drawn off *in vacuo*. COOH-terminal amino acid determination was carried out by hydrazinolysis and subsequent dinitrophenylation of the COOH-terminal amino acid.

DNP-amino acids obtained in these three procedures were measured by thin layer chromatography on silica gel G. The dry residues of both ether-soluble DNP-amino acids and of ε-mono-DNP-lysine were redissolved in 20.5 μl of ammoniacal butanol. Usually 3 μl of this solution were chromatographed at room temperature in butanol–1% ammonium hydroxide (1:1) (upper phase). Chromatography was stopped after the solvent covered slightly more than one-half of the plate. After drying thoroughly in a cool air stream, the chromatogram was further developed at 4° in chloroform–methanol–acetic acid (84:15:1).

Individual DNP-amino acid spots were transferred into 1-ml tubes and extracted from the silica gel by buzzing vigorously several times in 200–275 μl of 0.05 M NH_4OH. The absorbance at 362 mμ of these solutions was measured. Details of all of these methods have recently been published (Ghuysen *et al.*, 1966).

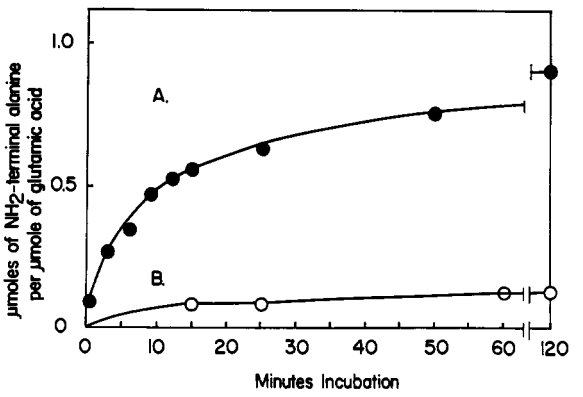


FIGURE 1: Time course of liberation of NH_2-terminal alanine by the *Myxobacterium* enzyme. (A) The incubation mixture contained 100 μl of a cell wall suspension (10.2 mg/ml), 12 μl of 0.1 M barbital buffer (pH 9.0), and 9 μl of *Myxobacterium* (1 mg of protein/ml). Aliquots of 12 μl were removed after various times of incubation at 37° and inactivated by heating in a boiling-water bath. The NH_2-terminal alanine in these samples was then measured. No other NH_2-terminal amino acids were found and no NH_2-terminal alanine was released in a control incubation from which the *Myxobacterium* enzyme was omitted. (B) The incubation mixture contained 100 μl of cell wall suspension (10.2 mg/ml), 9 ml of lysozyme (1 mg/ml), and 12 ml of 0.2 M ammonium acetate buffer (pH 5.2). After 2-hr incubation at 37°, by which time the suspension had completely cleared, the sample was dried *in vacuo*, reconstituted by addition of 100 μl of water, and then treated identically with A.

Results

Composition of the Cell Walls of M. lysodeikticus. The cell walls employed contained muramic acid, glucosamine, glutamic acid, alanine, lysine, and glycine in the molar ratio 1:1:1:2:1:1 (Table I). Traces of several other amino acids were present in an amount not exceeding 2% of any of the major constituents above. The amount of glutamic acid present (taken as representative of the amount of the repeating unit of the cell wall) was 0.45 μmole/mg of cell wall.

TABLE I: Analyses of Cell Wall of *M. lysodeikticus*.[a]

	μmoles/mg of Cell Walls	Ratio
Glutamic acid	0.45	(1.00)
Glycine	0.50	1.10
Lysine	0.38	0.85
Alanine	0.84	1.87
L-Alanine	0.43	0.96
D-Alanine	0.41	0.91
Muramic acid	0.42	0.94
Glucosamine	0.54	1.19

[a] Data were obtained from the Beckman-Spinco amino acid analyzer except for D- and L-alanine which were measured enzymatically (see Ghuysen *et al.*, 1966).

Kinetics of Hydrolysis of Cell Walls by the Myxobacterium Enzyme. During lysis of the cell walls by the *Myxobacterium* enzyme, no measurable release of reducing groups or of COOH-terminal groups occurred. NH_2-terminal alanine was released and reached a maximum level equivalent to 0.90 μmole/μmole of glutamic acid in the cell wall (Figure 1). The treatment with *Myxobacterium* enzyme was also followed by treatment with the endoacetylglucosaminidase from lysostaphin which fragments the cell wall polysaccharide into disaccharide units, as has been done previously (Leyh-Bouille *et al.*, 1966). The measured release of NH_2-terminal alanine was the same as occurred on treatment with the *Myxobacterium* enzyme alone. If the walls were first solubilized by fragmentation of the polysaccharide with egg-white lysozyme and then treated with the *Myxobacterium* enzyme, no liberation of N-terminal L-alanine occurred (Figure 1).

By contrast the acetylmuramyl-L-alanine amidase from *Streptomyces albus* G did not cleave any linkages in the cell wall of this organism and consequently

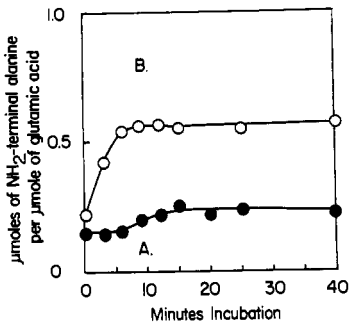


FIGURE 2: Liberation of NH_2-terminal L-alanine by *N*-acetylmuramyl-L-alanine amidase from *Streptomyces albus* G. (A) Cell wall suspension (100 ml, 10.2 mg/ml), 60 μl of water, 11 μl of 1% bovine serum albumin, 5 μl of 1 M ammonium acetate buffer (pH 5.2), and 9 μl of the amidase preparation (2 mg/ml) were incubated at 37° for various intervals. Aliquots of 20 μl were removed for a measurement of NH_2-terminal amino acids. The only NH_2-terminal amino acid liberated was alanine. (B) The cell wall suspension was solubilized by lysozyme, as is described in Figure 1B. After incubation for 2 hr at 37°, the sample was lyophilized, reconstituted by addition of 160 μl of water, and then treated identically with the sample described in A.

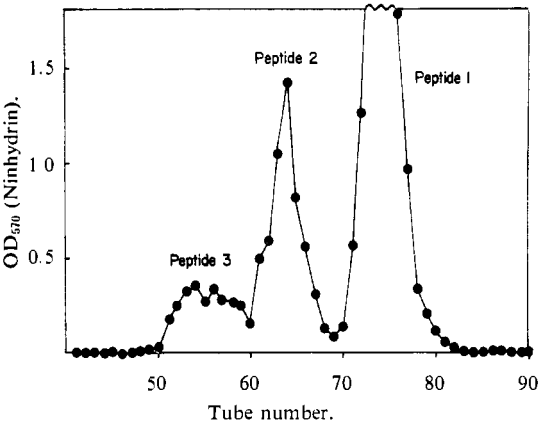


FIGURE 4: Separation of the peptide fraction by filtration on a column of Sephadex G-25. Sephadex G-25 (fine grade, bead form) was employed in a column 99 × 0.9 cm. It was equilibrated with 0.05 M pyridinium acetate buffer (pH 4.5). The sample was applied in a volume of 1 ml, and the column was then developed with the same buffer. Fractions of 0.65 ml were collected every 3 min. Aliquots (20 μl) were analyzed for amino groups by the ninhydrin reaction, measured at 570 mμ.

did not solubilize the cell wall. If, however, the cell walls were first solubilized with egg-white lysozyme and then treated with the acetylmuramyl-L-alanine amidase, NH_2-terminal alanine appeared and reached a maximum equivalent to only 0.6 μmole/μmole of glutamic acid (Figure 2).

Release of NH_2-terminal alanine by both of these enzymes without concomitant release of COOH-terminal groups as measured by the technique employed is indicative of cleavage of the acetylmuramyl-L-alanine linkage since the COOH groups of acetylmuramic acid released by this cleavage are not measured by the technique employed.

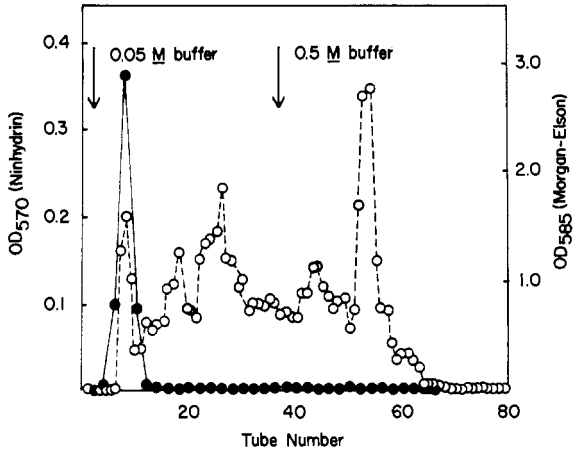


FIGURE 3: Separation of polysaccharide and peptides by chromatography on a column of Dowex 50. For details, see text. Fractions of 12 ml were collected every 4.5 min. Aliquots of 20 μl were analyzed for amino groups with ninhydrin (measured at 570 mμ) and for total hexosamine by the Morgan–Elson reaction (measured at 585 mμ).

Fractionation of a Large-Scale Cell Wall Lysate Prepared with the Myxobacterium Enzyme. Cell walls (111 mg) were suspended in 4 ml of 0.02 M barbital buffer (pH 9.0). The *Myxobacterium* enzyme (0.25 ml, 1 mg/ml) was then added and the mixture was incubated for 18 hr at 37°. The pH was checked at intervals of about 2 hr and adjusted with 2 N sodium hydroxide, a total of 10 μl being required during the incubation to keep the pH constant. The lysate prepared in this way should contain peptide fraction free of hexosamine and a polysaccharide fraction, perhaps still substituted by some peptides. An effort was made to separate these on a column of CM-cellulose as had been previously accomplished for a mixture of the *disaccharide* and peptide obtained from the same cell walls (Leyh-Bouille *et al.*, 1966). In this case, however, the peptide fraction *was not* absorbed on CM-cellulose but instead came through the column with the polysaccharide, perhaps due to a strong ionic interaction of the peptide and the polysaccharide. The entire material was, therefore, pooled, lyophilized, and applied to a column of Dowex 50-H^+ form (25 × 2.7 cm), equilibrated with 0.05 M pyridinium acetate (pH 4.3). All of the hexosamine together with a small amount of peptide came straight through the column. The remainder of the peptides were eluted slowly by the 0.05 M buffer and finally were swept off the column

TABLE II: Analyses of Fraction A (polysaccharide) and Fraction B (peptide) from *M. lysodeitkicus* after Lysis with the *Myxobacterium* Enzyme.[a]

	Amino Acids (mμmoles/μl)				Total Hexosamines (mμmoles/μl)
	Glutamic Acid	Glycine	Lysine	Alanine	
Fraction A (tubes 1–15)					
Total amino acids	2.2	2.1	1.5	3.ff	22.5
Fraction B (tubes 16–70)					
Total amino acids	7.65	8.30	7.85	15.95	
NH_2-terminal amino acids	—	—	3.73	7.34	0.0
COOH-terminal amino acids	—	7.02	—	3.78	

[a] These materials were separated on a column of Dowex 50 (Figure 3).

with 0.5 M pyridinium acetate (pH 4.3). Examination of the material present in the various peaks between tubes 16 and 70 (Figure 3) indicated that it was composed of a relatively small number of peptides smeared through the elution profile. The materials present in tubes 16–70 were, therefore, pooled, lyophilized, dissolved in a small volume of water, and applied to a column of Sephadex G-25 (Figure 4). This procedure separated the peptides into two major components termed peptides 1 and 2 in peaks 1 (tubes 70–80) and 2 (tubes 60–68) and one or more minor components termed peptide 3 in peak 3 (tubes 51–59). No hexosamine was present in any of these fractions.

Analyses of the Fractions Obtained by Fractionation on Dowex 50. Amino acid and hexosamine analyses of the two pooled peaks obtained from the Dowex 50 column (Table II) revealed that peak A contained all of the hexosamine and about 15% of the amino acids present in the original cell wall. The amino acid analyses, although not accurate because of the small amounts present, indicate that the four major cell wall acids were present in this fraction in approximately the same molar proportions as in the original cell wall. The NH_2-terminal alanine of this fraction was very low, and it is believed that the amino acids present represent peptides which remain linked to polysaccharide. For some unknown reason, a small portion of the acetylmuramyl-L-alanine linkages was not cleaved by the *Myxobacterium* enzyme despite long incubation.

The material in peak B contained about 85% of the cell wall amino acids in the same molar proportions as are found in the original cell wall (Table II). About one-half of the alanine was NH_2 terminal and one-fourth of it was COOH terminal. As in the original cell wall virtually all of the glycine was COOH terminal and about one-half of the lysine had a free ε-amino group.

Edman degradation of this mixed peptide fraction was carried out (Tipper *et al.*, 1967). After the first cycle of reaction with the Edman reagents both the NH_2-terminal alanine and the free ε-amino group of lysine disappeared. The only new amino-terminal group which appeared was glutamic acid. After the second cycle of reaction with the Edman reagents this amino-terminal glutamic acid disappeared and NH_2-terminal glycine appeared. Moreover, the NH_2-terminal glycine which appeared was free glycine, as was demonstrated by the fact that the dinitrophenyl (DNP) derivative was extractable by ether (without prior acid hydrolysis) and chromatographed as DNP-glycine. Thin layer chromatography of the DNP derivative of this mixed peptide fraction indicated that it contained two major and one minor component (Figure 5).

Analyses of the Fractions Obtained by Separation of the Mixed Peptide on Sephadex G-25. Total amino

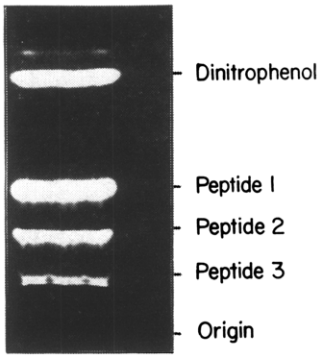


FIGURE 5: Thin layer chromatography of the mixed peptide fraction. A mixture containing 15 μl of the peptide solution, 60 μl of water, 125 μl of 1.5% $K_2B_4O_7$, and 15 μl of 0.1 M DNFB in ethanol were heated at 60° for 30 min. The solution was lyophilized and redissolved in 40 μl of 0.04 N NH_4OH. This solution was used for thin layer chromatography on silica gel H. The plate was developed for 2.5 hr at room temperature in the dark in *t*-amyl alcohol–chloroform–methyl alcohol–water–glacial acetic acid (30:30:30:20:3), dried, and then developed again.

acid analyses revealed that each of the three fractions contained four amino acid components in the same molar ratio as was found in the mixed peptide fraction; *i.e.*, each contained glutamic acid, glycine, lysine, and alanine in the ratio 1:1:1:2 (Table III). Striking differences were found in the NH_2-terminal and COOH-terminal groups. In each of these fractions one-half of the two alanine residues was NH_2 terminal and all of the glycine was COOH terminal. In peak 1, one-fourth of the two alanine residues was COOH terminal and one-half of the lysine had a free ϵ-amino group. In peak 2, 15% of the two alanine residues was COOH terminal and 25% of the lysine had a free ϵ-amino group. In peak 3, the COOH-terminal alanine and free ϵ-amino groups of lysine were still lower but could not be determined accurately because of the small amounts of material available.

Thin layer chromatography revealed that each of the three materials migrated as a single band, corresponding to the bands observed in the mixed peptide fraction. The relative amounts of the three peptides were: peak 1, 62%; peak 2, 32%; and peak 3, 6%.

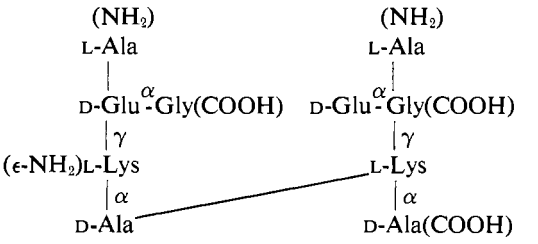
TABLE III: Analyses of the Peptides Obtained by Filtration on Sephadex G-25.[a]

	Amino Acids			
	Glutamic Acid	Glycine	Lysine	Alanine
		Peptide 1		
Total amino acids	5.28	5.60	5.12	10.7
NH_2-terminal amino acids	—	—	2.81	4.97
COOH-terminal amino acids	—	5.48	—	2.75
		Peptide 2		
Total amino acids	2.72	2.99	2.45	5.53
NH_2-terminal amino acids	—	—	0.61	2.05
COOH-terminal amino acids	—	2.26	—	0.81
		Peptide 3		
Total amino acids	0.58	0.56	0.55	1.28
NH_2-terminal amino acids	—	—	0.09	0.66
COOH-terminal amino acids	—	0.61	—	0.23 (?)

[a] Data are expressed as micromoles per milliliter of the solution of each peptide.

Discussion

The data obtained are compatible with the following structure for peptide 1 (free amino and carboxyl groups shown in parentheses).



The NH_2-terminal amino acid is alanine and it is attached to a glutamic acid residue as revealed by the Edman degradation. This degradation (Tipper *et al.*, 1967) also established that glycine is attached to the α-carboxyl group of glutamic acid. Moreover, all of the glycine has a COOH-terminal group as was shown by hydrazinolysis. The lysine must, therefore, be attached to the γ-carboxyl group of glutamic acid. This mode of attachment has been demonstrated directly only in the case of the peptide in the uridine nucleotide precursor of the peptidoglycan isolated from *S. aureus* (Ito and Strominger, 1964; Lanzillotti *et al.*, 1964). More recently, the γ-glutamyl-lysine linkage in peptides isolated from cell walls has been demonstrated by comparison of the isolated materials with chemically synthesized peptides containing either an α- or γ-glutamyl-lysine linkage (Muñoz *et al.*, 1966b).

The presence of a direct bridge between D-alanine and the ϵ-amino group of lysine is implied by the lack of any additional amino acids to form an interpeptide bridge (as occurs in *S. aureus*) and by the reduction of the number of COOH-terminal alanine residues and free ϵ-amino groups of lysine. The isolation of an enzyme which specifically cleaves this bridge has permitted the demonstration directly of the fact that the bridge consists of a D-alanyl-N^{ϵ}-lysine linkage (Petit *et al.*, 1966). The number of pentapeptide units which are linked together in this way in peptide 1 is calculated by the reciprocal of the number of free ϵ-amino groups of lysine or of COOH-terminal alanine groups per repeating unit. For peptide 1 each of these terminal groups is one-half residue per glutamic acid residue and thus the number of pentapeptide units linked together is 2, *i.e.*, peptide 1 is a dimer of two pentapeptide units.[2]

Peptide 2 gave similar analyses to peptide 1, except

[2] One other structure is compatible with the analytical data obtained, that of a tetrapeptide (L-Ala·D-γ-Glu(α-Gly)-L-Lys) linked to a hexapeptide (L-Ala·D-γ-Gly(α-Gly)·L-Lys·D-Ala·D-Ala) through an L-lysyl-N^{ϵ}-lysine linkage. This structure is not compatible with the hydrolysis catalyzed by the ML-endopeptidase (Petit *et al.*, 1966), nor is it compatible with the mechanism of biosynthesis of the interpeptide cross-links. The latter has been demonstrated in *E. coli* and a similar mechanism is believed to occur in *M. lysodietkicus*.

for the percentage of free ε-amino groups of lysine and COOH-terminal groups of alanine. The calculation of the number of pentapeptides linked to each other is made from these two data. In this case the proportion of free ε-amino groups of lysine (0.25) gives a value of four pentapeptide units while the proportion of free COOH-terminal groups of alanine (0.3/repeating unit, *i.e.*, 15% of two residues) gives a value of three pentapeptide units. Both of these analyses are subject to some inaccuracies and the difference, 5%, in the percentage of terminal groups measured by the two methods is not significant. The peptide is either a trimer or a tetramer composed of three or four pentapeptide units. The methods employed are at the present time not sufficiently accurate to distinguish between these possibilities. Peptide 3, the minor component, may be a higher oligomer of pentapeptide units or possibly a mixture of higher oligomers but the amount of material obtained has not been sufficient for adequate characterization.

These results may be compared to data previously obtained in studies of the products of lysis of cell walls of *M. lysodeikticus* by egg-white lysozyme. In those studies it was shown that about 50% of the wall was converted to a dialyzable form by the enzyme (Ghuysen and Salton, 1960; Ghuysen, 1961). The dialyzable fraction was composed of the disaccharide, acetylglucosaminyl-acetylmuramic acid, of a tetrasaccharide composed of two disaccharide units and of two disaccharide-peptide fractions. One of the latter was a disaccharide-pentapeptide (containing glutamine:glycine:lysine:alanine, 1:1:1:2) and the second appeared to be a dimer of this compound in which the two units were linked through a peptide linkage involving the ε-amino group of one of the pentapeptides. These two materials together accounted for about 8% of the total cell wall.[3] The remainder of the peptide present in the cell wall of this organism was in the nondialyzable fraction which has not been characterized further. The occurrence of the disaccharide pentapeptide is noteworthy in view of the failure to find any trace of the pentapeptide monomer in the present studies. The total amount found in earlier studies was small, perhaps 4% of the total, but this amount should have been detected if it had been present in the material examined in the present study. It is possible that the difference is due to a difference in the starting material employed in these two studies, but in any case an uncross-linked pentapeptide cannot account for a major portion of the peptide structure of the cell wall of *M. lysodeikticus*.

The NH_2-terminal and COOH-terminal groups in cell walls of *M. lysodeikticus* have also been examined previously (Salton, 1961). The amount of COOH-terminal glycine found (500 μmoles/g) is approximately the same as the total glycine content of the wall and is compatible with the fact that in the present study it has been found that all of the glycine present in the cell wall has a COOH-terminal group. The high content of free ε-amino groups of lysine (400 μmoles/g) and low amount of COOH-terminal alanine (76 μmoles/g) are, however, not in accord with data obtained in the present study. In analyses of the peptide fractions reported here the COOH-terminal alanine was equivalent to the free ε-amino groups of lysine[4] and the value obtained was intermediate between the two values reported previously. We have no explanation for these discrepancies.

In considering the structure of the cell wall of this organism a major problem presents itself. After lysis of cell walls of *M. lysodeikticus* with egg-white lysozyme or with the F_1 acetylmuramidase, 40–50% of the glycan is released as disaccharide and oligosaccharide fractions unsubstituted by peptide (Leyh-Bouille *et al.*, 1966; Muñoz *et al.*, 1966a). Nevertheless, the original analyses reveal that the number of pentapeptide subunits is approximately equivalent to the number of disaccharide units. Although these analyses are not extremely accurate, any possible excess of disaccharide units could not exceed 10% of the number of pentapeptide subunits. Thus, the mode of linkage of the pentapeptide subunits which are not attached to disaccharide units is obscure. Analysis of the amino-terminal groups in lysozyme digest of cell walls revealed that the number of free amino groups of alanine was not greater than 3% of the total,[4] thus confirming data obtained earlier (Salton, 1961). These data exclude the possibility that dimers or trimers of pentapeptide units are linked through only one of their NH_2-terminal alanine residues to a single disaccharide. In that case considerable amounts of NH_2-terminal L-alanine would be present in the cell walls. It remains possible that such a mode of linkage occurs but that the NH_2-terminal L-alanine residues not linked to disaccharide are substituted by some other substance, *e.g.*, by an acetyl residue. This hypothesis would require however that in addition to splitting the acetylmuramyl-L-alanine linkage, the *Myxobacterium* enzyme would also split the unknown substituent on L-alanine. All of these data need to be reconciled before a complete structure of the cell wall of this organism can emerge.

The structure of the peptides in *M. lysodeikticus* can be compared with that in two other organisms which have been extensively investigated, *S. aureus* and *M. roseus* (Ghuysen *et al.*, 1965; Petit *et al.*, 1966). In both of these latter organisms tetrapeptide subunits are connected by a small peptide bridge, a pentaglycine bridge in the case of *S. aureus* and a tri-L-alanyl-L-threonine bridge in the case of *M. roseus*. Moreover, in both of

[3] Recently, the disaccharide pentapeptide has again been isolated from *M. lysodeikticus* and further characterized. Evidence that it contains glycine linked to the α-COOH group and lysine linked to the γ-COOH group of glutamic acid has been presented (Mirelman and Sharon, 1966).

[4] Analyses of NH_2-terminal groups have been carried out on cell walls solubilized by egg-white lysozyme in order to insure accessibility of all NH_2 groups to DNFB. The values found were: free ε-amino groups of lysine, 0.142 μmole/mg; and NH_2-terminal alanine, approximately 0.014 μmole/mg, compared to 0.45 μmole of peptide subunit/mg. Thus only 32% of the lysine has a free ε-amino group. This value for ε-DNP-lysine in the cell wall is compatible with the composition of the peptides obtained.

these latter organisms the size of the peptide linked oligomers which are formed in this way is much larger than that which is found in *M. lysodeikticus*. Although small oligomers exist in *S. aureus* and *M. roseus*, the major part of the structures are composed of oligomers of ten units or larger in the former and of four units in the latter. This type of structure is referred to as a tight peptidoglycan network in contrast to the looser mesh which appears to exist in the cell wall of *M. lysodeikticus*. A similar situation exists in both *Escherichia coli* (Weidel and Pelzer, 1964) and *Corynebacterium diphtheriae* (K. Kato and J. L. Strominger, unpublished data, 1966) where tetrapeptide units are directly connected to each other by D-alanyl-*meso*-diaminopimelic acid linkage. Also, in both of these organisms the predominant peptide-linked oligomer is a dimer and higher oligomers do not occur in these organisms.

A further point of comparison among these organisms is the nature of the substituents linked on the α-carboxyl group of glutamic acid. In both *S. aureus* and *M. roseus* the α-carboxyl group is an amide, while in *M. lysodeikticus* a single glycine residue is substituted in this position (Tipper and Strominger, 1965; Tipper *et al.*, 1967; Muñoz *et al.*, 1966b). The net electrical charge in the peptidoglycan in *M. lysodeikticus* is, therefore, considerably different than that in the other two organisms. Finally, it may be mentioned that about 10% of the peptide units in the peptidoglycan of *S. aureus* consists of decapeptides in which a pentapeptide (glutamic acid:lysine:alanine, 1:1:3) bearing an extra D-alanine at its terminus is substituted on the ϵ-amino group of lysine by a pentaglycine chain. These units represent the growing points of the wall and are referred to as nascent peptidoglycan units (Tipper and Strominger, 1965; Tipper, 1966). Such units have not been encountered in the peptidoglycan of *M. lysodeikticus* during the present study. Their absence is presumably owing to the fact that the cell walls employed were obtained from stationary phase cultures while those employed in the study of *S. aureus* were obtained from mid-log-phase cultures.

The biosynthesis of the peptidoglycan of *M. lysodeikticus* has been extensively studied (Anderson *et al.*, 1965, 1966; Matsuhashi *et al.*, 1965; Katz *et al.*, 1967). From the point of view of the present structural study several points should be emphasized. No evidence was obtained during these studies of biosynthesis for the incorporation of acetylmuramic acid residues unsubstituted by peptide, *i.e.*, UDP-acetylmuramic acid itself did not appear to be a precursor. As in the case of *S. aureus* the only active nucleotide precursors were UDP-acetylmuramyl-L-Ala·D-Glu·L-Lys·D-Ala·D-Ala and UDP-acetylglucosamine. Thus, the unusual structural feature of the occurrence of unsubstituted acetylmuramic acid residues in the peptidoglycan may require some novel biosynthetic steps following the assembly of the peptidoglycan chains. Secondly, in the biosynthetic pathway the terminal D-alanine residue of UDP-acetylmuramyl-L-Ala·D-Glu·L-Lys·D-Ala·D-Ala is eventually removed in a cross-linking reaction. In the peptide-linked dimer or trimer in the cell wall of *M. lysodeikticus* the terminal pentapeptide unit (L-Ala·D-γ-Glu(α-Gly)·L-Lys·D-Ala) which is not involved in the cross linking has also lost its terminal D-alanine residue. This amino acid must be removed by a mechanism different from that which is involved in cross-linking, perhaps by a D-alanine carboxypeptidase, as is believed to occur in a similar situation in the peptidoglycan of *E. coli* (Izaki *et al.*, 1966). Finally, it should be mentioned that the glycine residue substituted on the α-carboxyl group of glutamic acid is added at the stage of lipid intermediates (Katz *et al.*, 1967) by a mechanism similar to that in which this α-carboxyl group becomes amidated in *S. aureus*.[5] The acceptor for this reaction is disaccharide (-pentapeptide)-P-P-phospholipid.

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The Binding of Divers Detergent Anions to Bovine Serum Albumin*

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ABSTRACT: The binding of long-chain alkyl sulfates and sulfonates (C_8 to C_{14} sulfates and C_8 to C_{12} sulfonates) to bovine serum albumin (BSA) has been studied at pH 5.6 and two temperatures, 2 and 23°. Although all ligand–protein complexes are shown to undergo a small conformation change at molal ratios greater than the saturation value for the high-energy binding sites, only dodecyl and myristyl sulfate–protein complexes exhibit massive disorganization at high molal binding ratios. The total number of available binding sites on the native macromolecule is shown to be approximately ten for all ligands investigated. However, these ten sites are not all of the same energy. The number of sites in the highest energy set for the sulfates increases with hydrocarbon tail length, but the interaction energy varies only slightly. The sulfonates, on the other hand, bind not only to different numbers of sites but also with different energies when the length of the hydrocarbon tail is altered. It is apparent that the binding affinities are strongly dependent on the character of the polar portion of the ligand and are greatly influenced by the difference between the CS bond in sulfonates and COS bonds in sulfates. A mathematical model is described which predicts protein stabilization as well as disorganization in the presence of bound species, and some experimental data presented herein are analyzed by means of this theoretical treatment.

The intramolecular forces responsible for maintaining secondary and tertiary structure of proteins can be altered by environmental changes such as increasing the temperature of the system, binding small molecules, or increasing the residual electronic charge on the macromolecule. Disruption of these forces results in a conformation change of the protein. For example, when small organic molecules are bound to proteins, a ligand–protein interaction may be substituted for a structure stabilizing segment–segment or side-chain–side-chain interaction, and conformational changes may occur. It is the aim of the present series of investigations to provide a systematic study of protein binding and its effects on the stability of secondary and tertiary structure. Thus, through an understanding of the binding forces and the manner in which they alter the protein conformation, significant information and insight into the nature of protein structure can be obtained.

The first paper of this series (Ray *et al.*, 1966) presented an historical background and the results of binding measurements on a series of related ligands (octane, octanol, octyl sulfate, octyl sulfonate, dodecanol, and dodecyl sulfate) to a single protein in the native state, bovine serum albumin (BSA).[1] The results of this preliminary investigation can be summarized as follows. (1) Different numbers of sites on the native BSA molecule are available to the above ligands. (2) The binding energy and the number of sites in the highest affinity set appear to increase

* From the Department of Chemistry, Georgetown University, Washington, D. C. 20007. *Received October 19, 1966.* This work was supported by Grant GM 12085-03 from the U. S. Public Health Service to one of us (J. S.).

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[1] Abbreviation used: BSA, bovine serum albumin.