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Structure of the Cell Wall of *Staphylococcus aureus*. VIII. Structure and Chemical Synthesis of the Basic Peptides Released by the *Myxobacterium* Enzyme*

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ABSTRACT: The basic peptide fraction obtained after hydrolysis of the cell wall of *Staphylococcus aureus* by the *Myxobacterium* enzyme has been separated into two major components, a pentapeptide and a hexapeptide. Various data indicate that the pentapeptide has the structure L-alanyl-D-isoglutaminyl-(N^{ϵ}-glycyl)-L-lysyl-D-alanine and the hexapeptide has the structure L-alanyl-D-isoglutaminyl-(N^{ϵ}-glycylglycyl)-L-lysyl-D-alanine.

The pentapeptide, and in addition the tetrapeptide lacking any glycine, have been synthesized chemically and compared to the isolated material.

A bacteriolytic enzyme from a species of *Myxobacterium* has been purified and characterized (Ensign and Wolfe, 1965, 1966). Investigations of the mode of action of this enzyme have revealed that it brings about bacteriolysis as a consequence of the hydrolysis of three linkages within the peptidoglycan of the cell wall (Tipper *et al.*, 1967a). The pentaglycine cross bridge is hydrolyzed at two positions with the liberation of both tri- and tetraglycine. Both COOH-terminal D-alanine and COOH-terminal glycine are liberated during this hydrolysis. The acetylmuramyl-L-alanine linkage in the peptidoglycan is also hydrolyzed at a slower rate. As a consequence of the hydrolysis of these three bonds a teichoic acid–polysaccharide complex, an intact polysaccharide, and a low molecular weight peptide fraction are formed. The polysaccharide fraction has been characterized and the peptide has been separated into a neutral fraction, composed of tri- and tetraglycine, and a basic fraction. The purpose of the present paper is to report the resolution of the two major components of the basic peptide fraction and the analyses of these materials. As a further proof of the structure of the isolated materials, chemical synthesis of one of these peptides as well as of the tetrapeptide common to many peptidoglycans has been carried out. The tetrapeptide has also been synthesized in parallel work carried out by Muñoz *et al.* (1966),[1] who, in addition, isolated it following enzymatic degradation of the cell walls of several different bacteria. The acetylmuramyl tetrapeptide has been obtained in small amounts during earlier studies of the cell wall of *Staphylococcus aureus* (Ghuysen *et al.*, 1965).

Materials and Methods

Analyses of total amino acids and NH_2-terminal and COOH-terminal amino acids were carried out as described by Ghuysen *et al.* (1966). Total amino acid analyses were also carried out with the Beckman-Spinco amino acid analyzer. Stepwise degradation of peptides with the Edman reagents was carried out as described by Sjöquist (1957, 1959). This technique has also been employed recently in this laboratory for the study of other peptides derived from the cell wall (Tipper *et al.*, 1967b). The dehydration and reduction of amides was carried out as described by Ressler and Kashelikar (1966). We wish to thank Dr. Ressler for the use of the facilities of her laboratory and for assistance with these analyses.

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[1] In earlier synthetic work, the protected pentapeptide with the sequence L-Ala-D-Glu-L-Lys-D-Ala-D-Ala was synthesized (Garg *et al.*, 1962; Tesser and Nivard, 1964). At about the same time both the unprotected pentapeptide and the acetylmuramyl pentapeptide were synthesized (Lanzilotti *et al.*, 1964) and shown to be identical with the material derived from the uridine nucleotide, uridine diphosphate acetylmuramyl pentapeptide. These materials differ from the compounds synthesized here and by Munoz *et al.* (1966) in lacking an amide on the α-COOH of glutamic acid and in carrying a second D-alanine residue at the COOH terminus.

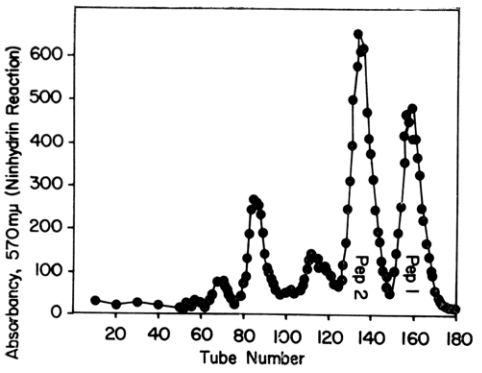

FIGURE 1: Separation of the basic peptides on Dowex 50-H^+. A column of Dowex 50 W-X8 (8.5 × 2.4 cm) was employed in a water-jacketed column at 50°. The column was developed as described by Jones (1964). It was equilibrated with a buffer at pH 3.1 containing, in 4 l., 64.5 ml of pyridine and 1115 ml of acetic acid. This buffer (500 ml) was placed in the mixing flask and 500 ml of buffer at pH 5.0 containing, in 4 l., 645 ml of pyridine and 573 ml of acetic acid was placed in the reservoir. The basic peptide fraction (109 mg) was applied to the column in 10 ml of 2% formic acid. Fractions of about 4 ml were collected every 4 min. An aliquot of each fraction was analyzed with ninhydrin.

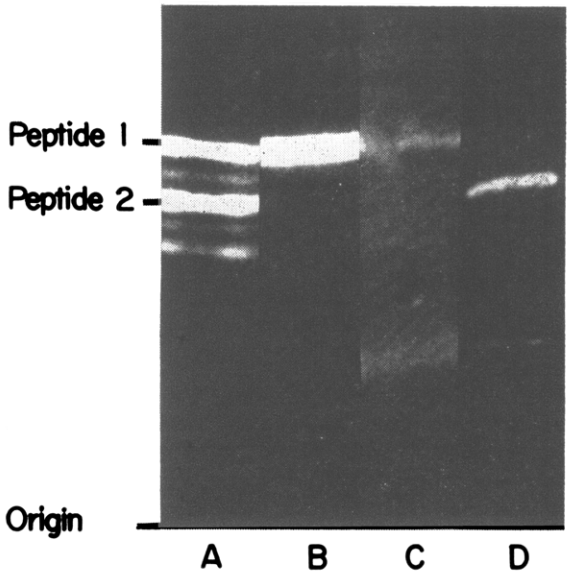

FIGURE 2: Thin layer chromatography of the bis-DNP derivative of the peptides. Aliquots of each peptide (approximately 0.2 μmole in 50 μl of water) were employed. Triethylamine (10% in ethanol, 15 μl) was added followed by 50 μl of 0.1 M 2,4-dinitrofluorobenzene in ethanol. After being heated for 30 min at 60°, the solutions were evaporated to dryness and applied with glacial acetic acid to a thin layer plate of silica gel G. The plate was then developed at 4°, once in ethyl acetate–methanol–toluene–isopropyl ether–acetic acid–water (30:20:10:40:2:6) and then four times in dioxane–methanol–toluene–isopropyl ether–acetic acid–water (25:12:15:60:2:2). Each development required about 1 hr. The compounds shown are (A) mixed peptide fraction, (B) peptide 1 after chromatography on Dowex 50 and Dowex 1, (C) the trailing edge of the main peptide peak from Dowex 1 showing the impurities in peptide 1, and (D) peptide 2 after Dowex 50 chromatography. The impurities seen in peptide 2 could not be removed by chromatography on Dowex 1. They are small in amount and do not reproduce well in this illustration.

Elementary analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Optical rotations were measured on a Zeiss polarimeter.

Results and Discussion

Separation of the Basic Peptide Fraction. Preliminary results indicated that the basic peptide fraction obtained from the *Myxobacterium* digest of cell walls of *Staphylococcus aureus* (Tipper *et al.*, 1967a) could be separated by high-voltage paper electrophoresis at pH 1.9 or 4.6 into two main components. Thin layer chromatography of the bisdinitrophenyl (DNP) derivative of this peptide fraction (see below) also indicated the presence of more than one peptide. Amino acid analyses revealed that the molar ratios of the amino acids in the mixed peptide fraction was Glu:Lys:Ala:Gly 1:1:2:1.6.

Fractionation on a column of Dowex 50-X8 separated the mixed peptide fractions into two major and three or more minor components (Figure 1). Thin layer chromatography of the DNP derivatives of the two major components revealed that each was still contaminated by small amounts of other peptides (Figure 2). Rechromatography of each of these two components on Dowex 50 did not separate contaminants from either of these major peptide fractions. They were each, therefore, rechromatographed on a column of Dowex 1-acetate which resulted in the separation from one of the major peptides of all detectable peptide contaminants (Figure 3). The second peptide fraction could not be separated from the minor components (see Figure 2) by chromatography on Dowex 1-acetate. The amount of the contaminating materials judged from the amount of DNP derivatives formed was not more than 10% of the total and the analyses reported below were carried out on this material.

In a preparative scale run, 109 mg of the mixed peptide fraction was separated. The yield of the two major peptide fractions was 31 mg of peptide 1 (28%) and 42 mg of peptide 2 (38%).

Analysis of the Two Peptide Fractions. Peptide 1 contained glutamic acid, alanine, lysine, glycine, and ammonia in the ratio 1:2:1:1:1 (Table I). Peptide 2 had the same molar ratios except that 2 glycine

TABLE I: Analyses of Isolated Peptides.[a]

	Glutamic Acid	Glycine	Alanine	Lysine	NH_3	γ-Amino-butyric Acid[b]
Synthetic tetrapeptide						
Total	0.78	0	1.58	0.81	0.76	
Synthetic pentapeptide						
Total	0.64	0.62	1.26	0.62	0.63	0.17
Peptide 1 (pentapeptide)						
Total	0.55	0.54	1.08	0.53	0.56	0.14
Amino terminal		0.53	0.53			
Carboxyl terminal			0.47			
Peptide 2 (hexapeptide)						
Total	0.79	1.42	1.51	0.75	0.76	0.19
Amino terminal		0.78	0.79			
Carboxyl terminal			0.73			

[a] Data are expressed as micromoles per milliliter of a solution of each peptide. [b] After dehydration and reduction. The procedure of Ressler and Kashelikar (1966) does not result in quantitative conversion.

residues were present. Measurements of NH_2-terminal groups indicated that each of these peptides contained one NH_2-terminal alanine and one NH_2-terminal glycine residue. Measurements of COOH-terminal groups indicated that each peptide contained COOH-terminal alanine. No other COOH-terminal groups were detected (Table I). Peptide 1 is, therefore, a pentapeptide and peptide 2 a hexapeptide.

Chemical Synthesis of L-*Alanyl*-D-*isoglutaminyl*-L-*lysyl*-D-*alanine and of* L-*Alanyl*-D-*isoglutaminyl*-(*N*$^{\epsilon}$-*glycyl*)-L-*lysyl*-D-*alanine.* In order to obtain definitive proof of the structure of these compounds, the chemical synthesis of the simpler of the two compounds isolated, the pentapeptide, was undertaken. The cell wall tetrapeptide which differs from the pentapeptide in lacking a single glycine residue has also been synthesized. Fully protected derivatives of both peptides were first synthesized and then all of the protecting groups were removed simultaneously by treatment with hydrogen in the presence of a palladium catalyst. The route to the syntheses of both of the protected peptides was the same and involved, as a final step, coupling through the γ-carboxyl group of glutamic acid where no possibility for racemization exists. Thus, carbobenzoxy-L-alanyl-D-isoglutamine was coupled, by means of a mixed anhydride, with ε-carbobenzoxy-L-lysyl-D-alanine-*p*-nitrobenzyl ester and ε-(carbobenzoxyglycyl)-L-lysyl-D-alanine nitrobenzyl ester to give the required protected tetrapeptide and pentapeptide, respectively.

The following compounds were prepared as previously described: carbobenzoxy-L-alanine (Bergmann and Zervas, 1932), carbobenzoxy-D-alanine (Hunt and du Vigneaud, 1938), ε-carbobenzoxy-L-lysine (Bergmann *et al.*, 1935), carbobenzoxyglycine *p*-nitrophenyl ester (du Vigneaud *et al.*, 1960), carbobenzoxy-L-alanine *p*-nitrophenyl ester (du Vigneaud *et al.*, 1964), and α-*o*-nitrophenylsulfenyl-(ε-carbobenzoxy)-L-lysine dicyclohexylammonium salt (Zervas *et al.*, 1963). These compounds gave satisfactory analytical values and their melting points and optical rotations were in agreement with the published values.

D-*Alanine p-Nitrobenzyl Ester Hydrobromide.* Carbobenzoxy-D-alanine (11.2 g, 50 mmoles) was converted to the corresponding *p*-nitrobenzyl ester by the method of Schwarz and Arakawa (1959). The product was

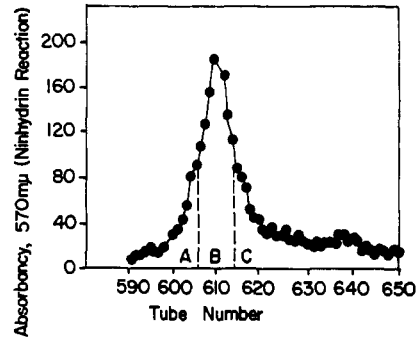

FIGURE 3. Further chromatography of peptide I. A column of Dowex 1-acetate X2 (200–400 mesh, 125 × 1.2 cm) was employed. One-fourth of peptide 1 from the experiment of Figure 1 was applied to the column. Initially the mixing vessel contained 1 l. of buffer containing 15 ml of pyridine, 25 ml of *N*-ethylmorpholine, and 0.3 ml of glacial acetic acid at pH 8.9. The reservoir contained 1 l. of 0.1 N acetic acid. After 900 ml of the reservoir had been employed it was replaced by 1 l. of 0.5 N acetic acid. The column was operated at 37°. Fractions of about 2 ml were collected each 6 min (see Schroeder *et al.*, 1962).

crystallized from ethyl acetate (40 ml) and petroleum ether (bp 38–52°) (25 ml) to give 15.2 g, mp 100–101°, $[\alpha]_D^{30}$ +14.3° (*c* 1.8, ethanol). Garg *et al.* (1962) reported mp 99–100°, $[\alpha]_D^{30}$ +21.9° (pyridine). Tesser and Nivard (1964) reported mp 102°, $[\alpha]_D^{20}$ +1.2° (*c* 1.5, $CHCl_3$).

Anal. Calcd for $C_{18}H_{18}N_2O_6$ (358.3): C, 60.4; H, 5.06; N, 7.82. Found: C, 60.4; H, 5.19; N, 7.93.

Treatment of carbobenzoxy-D-alanine *p*-nitrobenzyl ester (7.2 g, 20 mmoles) with a solution of hydrogen bromide in acetic acid and crystallization of the product from methanol (25 ml) and ether (30 ml) gave D-alanine *p*-nitrobenzyl ester hydrobromide, 5.8 g, mp 182–183°, $[\alpha]_D^{25}$ +2.9° (*c* 3.1, dimethylformamide). Tesser and Nivard (1964) reported mp 177°, $[\alpha]_D^{22}$ +4.5° (*c* 2, dimethylformamide).

Anal. Calcd for $C_{10}H_{13}N_2O_4Br$ (305.1): C, 39.4; H, 4.29; N, 9.18. Found: C, 39.2; H, 4.25; N, 9.38.

ε-Carbobenzoxyglycyl-L-lysine. L-Lysine hydrochloride (8.0 g, 44 mmoles) was dissolved in boiling water (70 ml) and basic copper carbonate (6.0 g) was added to the solution in small portions. The hot mixture was filtered and the filter was rinsed with hot water (25 ml). The filtrate was reduced to approximately 40 ml *in vacuo* and dimethylformamide (20 ml) was added, followed by triethylamine (6.0 ml). The solution was cooled in ice water and a solution of carbobenzoxyglycine *p*-nitrophenyl ester (13.2 g, 40 mmoles) in dimethylformamide (40 ml) was added dropwise during 5 hr with vigorous stirring. When the addition had been completed, stirring was continued for a further 20 hr. The mixture was reduced to a small volume and diluted with water (100 ml). The precipitate was filtered off, suspended in a mixture of ethanol (100 ml) and ether (100 ml), filtered again, and washed on the filter with ether (200 ml).

The dipeptide complex was suspended in 1 N HCl (200 ml) and the mixture was stirred. A stream of hydrogen sulfide was bubbled through the suspension for 4 hr, followed by a stream of air for 4 hr, and the mixture was filtered through a pad of cellulose powder. The filtrate was neutralized to pH 6 with ammonium hydroxide solution and the precipitate which formed was filtered, washed with ethanol, and dried. The crude product was crystallized from a mixture of acetic acid (75 ml) and water (100 ml) to give 8.4 g, mp 244–246° dec, $[\alpha]_D^{26}$ +10.0° (*c* 1.2, glacial acetic acid). A sample was recrystallized from the same solvent mixture and dried for analysis for 10 hr at 40° over P_2O_5 *in vacuo;* the melting point was unchanged.

Anal. Calcd for $C_{16}H_{23}N_3O_5$ (337.4): C, 57.0; H, 6.87; N, 12.5. Found: C, 56.9; H, 7.03; N, 12.3.

Material possessing similar properties was obtained in lower yield by the mixed anhydride procedure, as described by Theodoropoulos (1958); the latter author reported mp 250–252° dec, $[\alpha]_D^{16}$ +8.7° (*c* 1.03, glacial acetic acid).

α-o-Nitrophenylsulfenyl-(ε-carbobenzoxyglycyl-)-L-lysine Dicyclohexylammonium Salt. *o*-Nitrophenylsulfenyl chloride was prepared from bis-*o*-nitrophenyl disulfide by the method of Hubacher (1943). ε-Carbobenzoxyglycyl-L-lysine (6.75 g, 20 mmoles) was dissolved in a mixture of water (10 ml), dioxane (25 ml), and 2 N sodium hydroxide (10 ml), and the solution was cooled in ice water and stirred vigorously. During a period of 20 min the additions of a solution of *o*-nitrophenylsulfenyl chloride (4.7 g, 25 mmoles) in dioxane (20 ml) in 2-ml portions and 2 N sodium hydroxide in 1-ml portions were made. The mixture was diluted with water (250 ml) and filtered. The filtrate was acidified with 2 N sulfuric acid (20 ml) and extracted with ethyl acetate (three 75-ml portions). The combined extracts were washed with water (three 150-ml portions) and saturated sodium chloride solution (100 ml) and dried over anhydrous sodium sulfate for 2 hr. Removal of the solvent *in vacuo* left a yellow oil which was taken up in ethyl acetate (50 ml). Dicyclohexylamine (4.0 ml) was added to the solution; a crystalline precipitate soon began to form and the mixture was left to stand for 1 day at 6°. The product was filtered, washed with ether, and dried *in vacuo* yielding 11.6 g. Recrystallization from methanol (100 ml) and water (75 ml) gave 10.1 g, mp 169–170°, $[\alpha]_D^{28}$ −31.3° (*c* 0.53, dimethylformamide). A further recrystallization from the same solvent mixture left the melting point unchanged.

Anal. Calcd for $C_{34}H_{49}N_5O_7S$ (671.8): C, 60.8; H, 7.35; N, 10.4. Found: C, 60.6; H, 7.52; N, 10.3.

α-o-Nitrophenylsulfenyl-(ε-carbobenzoxyglycyl)-L-lysyl-D-alanine p-Nitrobenzyl Ester. A solution of D-alanine *p*-nitrobenzyl ester hydrobromide (1.7 g, 5.5 mmoles) in dimethylformamide (10 ml) was added to a suspension of α-*o*-nitrophenylsulfenyl-(ε-carbobenzoxyglycyl)-L-lysine dicyclohexylammonium salt (3.4 g, 5 mmoles) in dimethylformamide (50 ml). The suspension was stirred vigorously for 5 hr before a solution of *N,N'*-dicyclohexylcarbodiimide (1.1 g, 5 mmoles) in dimethylformamide (10 ml) was added. The mixture was stirred for approximately 24 hr, the precipitate was filtered, and the filtrate was evaporated under vacuum to an oily residue. This was treated with warm ethyl acetate (50 ml), the suspension was filtered, and, after being cooled, the filtrate was washed with water (25 ml), 1 N sulfuric acid (25 ml), water (20 ml), 1 N potassium bicarbonate solution (25 ml), and saturated sodium chloride solution (25 ml), and dried over anhydrous sodium sulfate. Removal of ethyl acetate under vacuum gave a tacky solid which crystallized on being triturated with ethyl acetate (approximately 5 ml). The crystals were filtered and recrystallized from tetrahydrofuran (10 ml) and petroleum ether (10 ml) to give 1.5 g, mp 125–126°, $[\alpha]_D^{20}$ −9.4° (*c* 1.17, dimethylformamide). Thin layer chromatographic analysis in the solvent system isopropyl ether–ethanol–pyridine–water (150:40:25:25) revealed only one yellow spot.

Anal. Calcd for $C_{32}H_{36}N_6O_{10}S$ (696.7): C, 55.2; H, 5.21; N, 12.1. Found: C, 55.0; H, 5.30; N, 11.9.

α-o-Nitrophenylsulfenyl-(ε-carbobenzoxy)-L-lysyl-D-alanine p-Nitrobenzyl Ester. In a similar manner, α-*o*-nitrophenylsulfenyl-(ε-carbobenzoxy)-L-lysine dicyclohexylammonium salt (4.1 g, 6.6 mmoles) and D-alanine

p-nitrobenzyl ester hydrobromide (2.1 g, 6.6 mmoles) were coupled to give, after recrystallization from tetrahydrofuran (20 ml) and petroleum ether (20 ml), α-*o*-nitrophenylsulfenyl-(ε-carbobenzoxy)-L-lysyl-D-alanine *p*-nitrobenzyl ester, 2.7 g, mp 156–157°, $[\alpha]_D^{31}$ −10.8° (*c* 1.6, dimethylformamide). A sample of the product was analyzed by means of thin layer chromatography in the solvent system isopropyl ether–ethanol–pyridine–water (150:40:25:25) and found to be homogeneous.

Anal. Calcd for $C_{30}H_{33}N_5O_9S$ (639.7): C, 56.3; H, 5.20; N, 11.0. Found: C, 56.1; H, 5.05; N, 10.8.

ε-Carbobenzoxyglycyl-L-lysyl-D-alanine p-Nitrobenzyl Ester Hydrochloride. A warm solution of α-*o*-nitrophenylsulfenyl-(ε-carbobenzoxyglycyl)-L-lysyl-D-alanine *p*-nitrobenzyl ester (1.2 g, 1.7 mmoles) in nitromethane (20 ml) was diluted with benzene (50 ml) and cooled in ice water. Nitromethane (5 ml), saturated with hydrogen chloride gas, was added and after 10 min the solution was evaporated *in vacuo* to an oil. Trituration with a 1:1 mixture of ethyl acetate and ether (two 20-ml portions), with decantation, led to solidification. The crude product was crystallized from ethanol (10 ml) and ether (20 ml) to give 0.8 g, mp 149–150°, $[\alpha]_D^{31}$ +20.8° (*c* 1.1, ethanol).

Descending paper chromatographic analysis in the solvent system *t*-amyl alcohol–isopropyl ether–acetic acid–water (4:3:1:5) showed the material to be homogeneous, R_F 0.63. A sample was recrystallized from nitromethane–ether and dried for analysis for 5 hr over P_2O_5 at 40° *in vacuo;* the melting point was unchanged.

Anal. Calcd for $C_{26}H_{34}ClN_5O_8$ (580.0): C, 53.8; H, 5.91; N, 12.1. Found: C, 53.6; H, 5.90; N, 11.9.

α-*o*-Nitrophenylsulfenyl-(ε-carbobenzoxy)-L-lysyl-D-alanine *p*-nitrobenzyl ester (1.6 g, 2.5 mmoles) was converted to ε-carbobenzoxy-L-lysyl-D-alanine *p*-nitrobenzyl ester hydrochloride under closely similar conditions. The material was crystallized from ethanol (15 ml) and ether (25 ml) to give 1.1 g, mp 145–146°, $[\alpha]_D^{30}$ +26.0° (*c* 1.2, ethanol). Descending paper chromatographic analysis in the solvent system *t*-amyl alcohol–isopropyl ether–acetic acid–water (4:3:1:5) revealed a single, ninhydrin-positive spot, R_F 0.75.

Anal. Calcd for $C_{24}H_{31}ClN_4O_7$ (523.0): C, 55.1; H, 5.98; N, 10.7. Found: C, 55.0; H, 5.95; N, 10.6.

Carbobenzoxy-L-alanyl-D-isoglutamine. Carbobenzoxy-D-glutamic acid was prepared from D-glutamic acid (13.5 g, 90 mmoles) according to the method of Bergmann and Zervas (1932). Recrystallization of the product from ethyl acetate (20 ml) and petroleum ether (50 ml) gave 23.5 g, mp 120–121°, $[\alpha]_D^{25}$ +7.2° (*c* 3.0, glacial acetic acid).

Anal. Calcd for $C_{13}H_{15}NO_6$ (281.3): C, 55.5; H, 5.38; N, 4.98. Found: C, 55.6; H, 5.33; N, 4.99.

Carbobenzoxy-D-isoglutamine was obtained from carbobenzoxy-D-glutamic acid (8.8 g, 30 mmoles) by application of the mixed anhydride procedure in the manner described by Ressler (1960) for the preparation of carbobenzoxy-L-isoglutamine. Two crystallizations of the product from ethanol and petroleum ether gave 3.9 g, mp 175–176°, $[\alpha]_D^{27}$ +6.1° (*c* 0.8, methanol).

Anal. Calcd for $C_{13}H_{16}N_2O_5$ (280.3): C, 55.7; H, 5.75; N, 10.0. Found: C, 55.8; H, 5.96; N, 9.92.

The purity of all preparations of the product was assayed routinely by subjecting small samples to hydrogenolysis followed by paper electrophoresis in 0.2 M pyridine–acetic acid buffer (pH 4.0) for 2.5 hr at 20 v/cm. Under these conditions reference samples of L-glutamine and L-isoglutamine had moved 1.0 and 6.7 cm toward the cathode, respectively, whereas D-glutamic acid moved 2.3 cm toward the anode.

Carbobenzoxy-D-isoglutamine (2.8 g, 10 mmoles) was dissolved in a mixture of ethanol (60 ml), acetic acid (4 ml), and water (30 ml) and the solution was treated with hydrogen in the presence of 10% palladium–charcoal catalyst (0.3 g) for 1.5 hr. The mixture was filtered and the filtrate was evaporated *in vacuo* to give an oil which soon began to crystallize. The crude product was recrystallized from water (10 ml) and ethanol (25 ml) to give 1.2 g, $[\alpha]_D^{24}$ −21.0° (*c* 2.1, water).

This crystalline solid was dissolved in water (20 ml) and triethylamine (1.6 ml) was added, followed by a solution of carbobenzoxy-L-alanine *p*-nitrophenyl ester (3.4 g, 10 mmoles) in dimethylformamide (20 ml). The yellow solution was left to stand at room temperature for 2 days before trifluoroacetic acid (5 ml) was added and the solvent was removed *in vacuo*. The residue was triturated with hot ethyl acetate (two 20-ml portions), filtered, dried, triturated with ice-cold water (25 ml), and dried *in vacuo* over P_2O_5 to give 3.0 g. Two successive crystallizations from ethanol–ether gave 2.5 g, mp 128–129°, $[\alpha]_D^{30}$ +8.7° (*c* 2.3, dimethylformamide).

Anal. Calcd for $C_{16}H_{21}N_3O_6$ (351.4): C, 54.7; H, 6.02; N, 12.0. Found: C, 54.6; H, 6.31; N, 11.9.

Carbobenzoxy-L-alanyl-D-isoglutaminyl-(ε-carbobenzoxyglycyl-)-L-lysyl-D-alanine p-Nitrobenzyl Ester. A solution of carbobenzoxy-L-alanyl-D-isoglutamine (0.35 g, 1 mmole) and triethylamine (0.14 ml, 1 mmole) in dimethylformamide (25 ml) was cooled to −25°. The three-necked reaction flask was fitted with a thermometer, soda lime tube, and mechanical stirrer. Ethyl chloroformate (0.1 ml, 1 mmole) was added and the mixture was stirred for 15 min during which time the temperature was maintained between −15 and −25°. A solution of (ε-carbobenzoxyglycyl)-L-lysyl-D-alanine *p*-nitrobenzyl ester hydrochloride (0.8 g, 1 mmole) and triethylamine (0.14 ml, 1 mmole) in dimethylformamide (15 ml) was cooled to −10° and added to the solution of mixed anhydride. After 2 hr the mixture was evaporated *in vacuo* to a solid residue which was triturated with hot ethanol (25 ml) and filtered. The crude product was reprecipitated from pyridine (10 ml) and water (30 ml) to give 580 mg. Two successive precipitations from aqueous dimethylformamide gave 410 mg, mp 233–234°, $[\alpha]_D^{27}$ 0° (*c* 0.55, dimethylformamide).

Anal. Calcd for $C_{42}H_{52}N_8O_{13}$ (876.9): C, 57.5; H, 5.98; N, 12.8. Found: C, 57.4; H, 6.09; N, 12.7.

L-Alanyl-D-isoglutaminyl-(ε-glycyl)-L-lysyl-D-alanine. Carbobenzoxy-L-alanyl-D-isoglutaminyl-(ε-carbobenz-

TABLE II: Chromatographic and Electrophoretic Mobilities of Synthetic Pentapeptide and Isolated Peptides.

	Chromatography Solvents[a]				Electrophoresis[b]	
	A	B	C	D	pH 1.9	pH 4.6
Synthetic pentapeptide	0.66	0.56	0.84	0.49	25.9	28.7
Peptide 1 (pentapeptide)	0.66	0.56	0.84	0.49	25.9	28.7
Peptide 2 (hexapeptide)	0.58	0.45	0.84	0.49	24.5	27.4

[a] Data are recorded as R_F (A) of the bis-DNP derivatives on silica gel G after one 1-hr development in ethyl acetate–methanol–toluene–isopropyl ether–acetic acid–water (30:20:10:40:2:6) followed by four 1-hr developments in dioxane–methanol–toluene–isopropyl ether–acetic acid–water (25:12:15:60:2:2) at 4°; (B) of the bis-DNP derivatives on silica gel G after two 1-hr developments in ethyl acetate–*n*-propyl alcohol–methyl ethyl ketone–4% acetic acid (3:1:1:3); (C) of the free peptide on Whatman No. 1 filter paper in isobutyric acid–1 N NH_4OH (5:3); and (D) of the free peptide on Whatman No. 1 filter paper in pyridine–ethanol–water (8:7:5). [b] Data are recorded as migration (in centimeters) toward the cathode on electrophoresis at pH 1.9 (0.6 N formic acid–1.5 N acetic acid) for 1.5 hr, and at pH 4.6 (0.5 M pyridinium acetate) for 2.5 hr on Whatman No. 3MM filter paper at a potential gradient of 37.5 v/cm.

oxyglycyl)-L-lysyl-D-alanine *p*-nitrobenzyl ester (20 mg, approximately 25 μmoles) was dissolved in glacial acetic acid (20 ml). Palladium oxide (20 mg) was added and the mixture was hydrogenolyzed for 3 hr at room temperature. The mixture was diluted with water (25 ml) and filtered, and the filtrate was evaporated *in vacuo* to a residue which was dissolved in water (5 ml). A small aliquot was applied as a band to Whatman No. 3MM filter paper and electrophoresis was carried out for 2.5 hr in 0.5 M pyridinium acetate (pH 4.6) with a potential gradient of 37.5 v/cm. Staining a longitudinal section of the paper with ninhydrin revealed one major band and one minor band which had moved 28.7 and 4.6 cm toward the cathode, respectively. The faster moving band was eluted with a formic acid–water mixture. When the eluted material was subjected to electrophoresis, a very small amount of the more slowly moving material was found to have been generated.

Another aliquot (1.0 ml) of the solution of free peptide was electrophoresed as a band under the same conditions. The material represented by the faster moving band was eluted and when assayed by reaction with 2,4-dinitrofluorobenzene was found to give 3.9 μmoles of peptide. A sample of this peptide solution was hydrolyzed in 6 N hydrochloric acid at 100° for 8 hr. Amino acid analyses indicated the presence of glutamic acid, lysine, alanine, glycine, and ammonia in the molar ratio 1:1:2:1:1 (Table I).

Carbobenzoxy-L-alanyl-D-isoglutaminyl-(ε-carbobenzoxy)-L-lysyl-D-alanine p-nitrobenzyl Ester. The protected tetrapeptide was prepared on the same scale, and by a procedure essentially the same as that described for the protected pentapeptide. Purification by successive precipitations from aqueous pyridine and aqueous dimethylformamide gave 350 mg, mp 223–224° dec, $[\alpha]_D^{22}$ 0° (*c* 1.5, dimethylformamide).

Anal. Calcd for $C_{40}H_{49}N_7O_{12}$ (819.8): C, 58.6; H, 6.02; N, 12.0. Found: C, 58.8; H, 6.12; N, 11.9.

The protecting groups were removed in the same manner as described for the protected pentapeptide. A sample of the free peptide was hydrolyzed in 6 N HCl for 10 hr at 110°. Amino acid analyses indicated the presence of glutamic acid, lysine, alanine, and ammonia in the molar ratio 1:1:2:1 (Table I).

Comparison of the Isolated and Synthetic Peptides. In addition to the analyses reported above, these materials have been compared by their chromatographic and electrophoretic mobilities. By these techniques the synthetic and isolated pentapeptide were identical (Table II).

Dehydration and Reduction of the Isolated and Synthetic Peptides. The presence of an amide on the

TABLE III: NH_2-Terminal Amino Acids in Peptides during Edman Degradation.[a]

Compound	Cycles of Degradation	NH_2-Terminal Groups			
		Glu	Gly	Ala	ε-Lys
Synthetic pentapeptide	0		6.23	5.89	
	1	4.21			4.40
	2				
Peptide 1 pentapeptide	0		0.36	0.34	
	1	0.23			0.28
	2				0.03
Peptide 2 hexapeptide	0		1.31	1.37	
	1	0.96	1.14		
	2				0.90

[a] Data are recorded as micromoles per milliliter of a solution of each peptide. Where no value is recorded, none of this compound could be detected.

α-carboxyl group of glutamic acid in the peptidoglycan has previously been demonstrated by analyses of other materials and by stepwise Edman degradation (Tipper and Strominger, 1965; Tipper *et al.*, 1967b). As proof of the occurrence of a primary amide in these peptides the isolated penta- and hexapeptides and the synthetic pentapeptide have been dehydrated with ethylene-chlorophosphite in triethyl phosphite and then reduced with sodium in liquid ammonia containing methanol to yield an amino acid, as employed by Ressler and Kashelikar (1966). In each case, γ-aminobutyric acid was formed (Table I, Figure 4). This degradation provided unambiguous evidence that the α-carboxyl group of glutamic acid was present as a primary amide in the peptide, and also indicated indirectly that glutamic acid was linked to lysine through its γ-carboxyl group.

Edman Degradation of the Isolated and Synthetic Peptides. Both the isolated and the synthetic pentapeptide had NH_2-terminal glycine and alanine residues. After the first cycle of reaction with the Edman reagents, both of the NH_2-terminal groups disappeared and NH_2-terminal glutamic acid and an ϵ-amino group of lysine appeared. All NH_2-terminal groups disappeared during the second reaction cycle (Table III).

The isolated hexapeptide initially also had NH_2-terminal alanine and glycine residues. After the first reaction cycle, it had NH_2-terminal glutamic acid and glycine residues, and after the second reaction cycle a free ϵ-amino group of lysine. This degradation thus clearly shows that glycylglycine is substituted on the ϵ-amino group of lysine in the hexapeptide.

The isolation and chemical synthesis of the pentapeptide provide a definitive proof of the nature of the repeating subunit of the peptidoglycan. These studies also provide strong support for the mode of hydrolysis of the peptidoglycan of *S. aureus* proposed for the *Myxobacterium* enzyme (Tipper *et al.*, 1967a).

The tetrapeptide lacking a glycine residue has also been synthesized in the present work and by Muñoz *et al.* (1966). The latter authors found the synthetic tetrapeptide to be identical to the material isolated from a number of bacterial species. Thus, the chemical nature of the repeating peptide unit of the peptidoglycan of bacterial cell walls has been firmly established. The ϵ-amino group of lysine (free in the tetrapeptide and substituted by a glycine residue in the pentapeptide) and the free carboxyl group of its terminal D-alanine residue are the functional groups involved in its polymerization through interpeptide bridges.

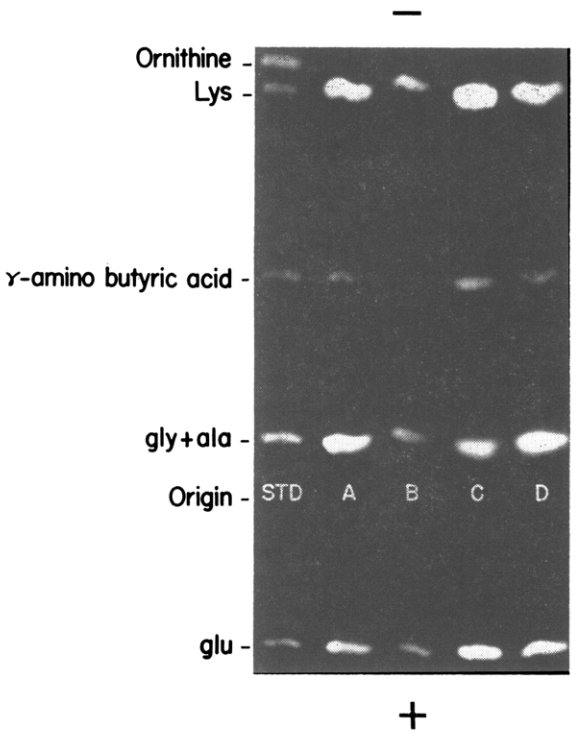

FIGURE 4: Formation of γ-aminobutyric acid after dehydration and reduction of the isolated and synthetic peptides. The synthetic pentapeptide (A), peptide 1 (C), and peptide 2 (D) were subjected to dehydration with ethylenechlorophosphite, reduced with sodium in liquid ammonia containing methanol (Ressler and Kashelikar, 1966), and then hydrolyzed in 6 N HCl at 110° for 15 hr. An aliquot of the resulting amino acid mixture was subjected to paper electrophoresis at pH 4.6. Also shown is a sample of the synthetic pentapeptide (B) which was hydrolyzed with acid *without* prior dehydration and reduction.

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The Cell Wall Peptidoglycan of *Bacillus megaterium* KM. I. Studies on the Stereochemistry of α,α'-Diaminopimelic Acid*

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ABSTRACT: α,α'-Diaminopimelic acid (DAP) occurs in the wall peptidoglycan of *Bacillus megaterium* KM predominantly in the form of its *meso* isomer (about 85% of the total residues) and, in minor amounts, in the form of its DD isomer. The amino groups on the L carbon of the *meso*-DAP residues are involved in peptide linkages to the glutamic acid residues. Most of the amino groups on the D carbon of the *meso*-DAP residues are free; some of them are substituted, thus probably serving to cross-link peptide subunits. These amino groups can be liberated by a *Streptomyces* endopeptidase. None of the DD-DAP residues have amino groups free. Moreover, these groups are not liberated by endopeptidase treatment. The peptidoglycan upon enzymatic degradation yields mainly two fractions. A major fraction is composed of disaccharide peptide monomer subunits containing only the *meso* isomer of DAP. A second minor fraction is composed of disaccharide peptide oligomers containing both *meso* and DD isomers of DAP. The *meso*-DAP residues isolated as monodinitrophenyl derivatives from both fractions have optical rotations and optical rotatory dispersions identical with that of synthetic monodinitrophenyl-*meso*-DAP obtained by dinitrophenylation of the amino group on the D carbon. The assignment of the DD configuration to the DAP residues which are not *meso* rests upon the optical rotatory properties of their bisdinitrophenyl derivatives.

Diaminopimelic acid (α,α') (DAP)[1] is a constituent of the peptide subunit of the peptidoglycan of many bacterial cell walls (see review by Salton, 1964). In *Escherichia coli*, the peptide subunits have the sequence L-Ala-D-Glu-*meso*-DAP-D-Ala. Some of these subunits are interlinked through peptide bonds extending from the amino group of the *meso*-DAP residue of one peptide to what is probably the carboxyl group of the C-terminal D-alanine residue of another peptide (Weidel and Pelzer, 1964). LL-DAP, either alone or together with *meso*-DAP, has also been encountered in acid hydrolysates of a limited number of bacteria (Hoare and Work, 1955, 1957; Allsop and Work 1963; Tinelli, 1966). The characterization of LL- and *meso*-DAP residues rests upon their chromatographic separation according to Rhuland *et al.* (1955), the decarboxylation of the *meso* isomer using a specific *meso*-DAP decarboxylase, and the specific enzymatic epimerization of LL-DAP into *meso*-DAP. With three species of *Micromonospora*, the *meso*-DAP spot obtained after paper chromatography in the Rhuland *et al.* (1955) solvent system was found only partially sensitive to *meso*-DAP decarboxylase (Hoare and Work, 1957). Since DD-DAP has the same property as *meso*-DAP in the above chromatographic system, it was suggested that in the walls of these three microorganisms, part of the DAP might occur in the DD form.

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[1] Abbreviations used: DAP, α,α'-diaminopimelic acid; FDNB, fluorodinitrobenzene.