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

Evangelos Bricas, Jean-Marie Ghuysen, and Philippe Dezélée

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.

Dinitrophenylation of amino acids often enhances their optical rotations (Alderton, 1953; Rao and Sober, 1954), thus providing a way of determining their configurations with only minute amounts of material. Owing to this property, Diringer and Jusic (1966) recently determined the configuration of the asymmetrical carbon of the *meso*-DAP which in the *E. coli* peptide subunit has a free amino group. The mono-DNP-*meso*-DAP obtained after dinitrophenylation and acid hydrolysis of this peptide had a molar optical rotation $[M]_D$ equal to $+250° \pm 10\%$. Since synthetic di-DNP-LL-DAP and synthetic mono-DNP-LL-DAP have $[M]_D$ respectively equal to -444 and $-231°$ (Jusic *et al.*, 1963b), the above value of $+250° \pm 10\%$ provided evidence that in the *E. coli* peptide subunit the free amino group of the *meso*-DAP residue is on the asymmetrical carbon having the D configuration.

The present paper deals with the isolation of both *meso*- and DD-DAP residues from the cell walls of *Bacillus megaterium* KM and with determination of which, D or L, carbon of *meso*-DAP has the amino group engaged in peptide linkage to glutamic acid.

Materials and Methods

B. megaterium cell walls were prepared and trypsin reated according to standard procedures (Petit *et al.*, 1966). This wall preparation contains (millimicromoles per milligram): Glu 440, DAP 490, Ala 750, total hexosamine 1080, glucosamine (as determined by a specific enzymatic procedure) 725, organic phosphorus 240, and glucose 330. Phosphorus, glucose, part of the glucosamine residues, and glycerol constitute a phosphomucopolysaccharide complex covalently linked to the peptidoglycan (Ghuysen, 1964). About 340 mμmoles (per mg of walls) of DAP residues have one of their amino groups free and are thus not engaged in peptide cross-linkages. The enzymes and analytical methods used throughout the present work have been described in previous papers dealing with the study of bacterial peptidoglycans containing L-lysine (Ghuysen *et al.*, 1966b; Petit *et al.*, 1966; Muñoz *et al.*, 1966a,b; Ghuysen *et al.*, 1967), with the exception of the endopeptidase which was prepared as follows. *Streptomyces albus* G was grown for 70 hr, at 28°, with shaking in 500 ml of the following medium: peptone 0.5%, K_2HPO_4 0.1%, $Co(NO_3)_2 \cdot 6H_2O$ 0.003%, $MgSO_4 \cdot 7H_2O$ 0.05%, and $NaNO_3$ 0.05% supplemented with dried, autoclaved cells of *B. megaterium* KM 0.1%. The culture filtrate was passed through a carboxymethylcellulose column previously equilibrated against 0.01 M Tris-HCl buffer (pH 8). After absorption, the column was washed with the same buffer and then treated with 0.2 M Tris buffer (pH 8). Fractions of the effluent were tested for lytic activity upon *B. megaterium* walls. As evidenced by the analysis of the degraded walls, some fractions contained both an endo-*N*-acetylmuramidase and an endopeptidase which respectively hydrolyzed the *N*-acetylmuramic glycosidic linkages in the polysaccharide moiety of the peptidoglycan and induced the appearance of free amino groups of the DAP residues in the peptide moiety. This endopeptidase is probably similar to the one from the *E. coli* autolytic system (Weidel and Pelzer, 1964) and to the endopeptidase L_3 secreted by another *Streptomyces* (Mori *et al.*, 1960; Ghuysen *et al.*, 1966a). The yield of the endopeptidase used in the present work was highly variable; its purification is still under study.

Chromatographic Solvents. The following solvents were used: (I) methanol–pyridine–10 N HCl–H_2O (80:10:2.5:17.5) (Rhuland *et al.*, 1955), (II) *t*-amyl alcohol saturated with 0.1 M phthalate buffer (pH 6) (Jusic *et al.*, 1963a), (III) benzyl alcohol–chloroform–methanol–water–15 N ammonia (30:30:30:6:2), (IV) chloroform–methanol–acetic acid (85:14:1) (at 0°), (V) 1-butanol–acetic acid–water (40:10:50, upper phase), and (VI) 1-butanol–pyridine–acetic acid–water (30:20:6:24).

Chromatographic Separation of DAP Isomers. On Whatman No. 1 paper, using solvent I, LL-DAP has a higher R_F than DD- or *meso*-DAP which migrate together (detection: ninhydrin). On Whatman No. 4 paper (previously dipped in 0.1 M phthalate buffer (pH 6) and dried), using solvent II, di-DNP-LL- and -DD-DAP migrate together and more rapidly than di-DNP-*meso*-DAP. On silica gel (Kieselgel G, according to Stahl, Merck) thin layer plates, using solvent III, DNP-Glu, di-DNP-*meso*-DAP, di-DNP-LL- or -DD-DAP and DNP-Ala present increasing R_F values. DNP-Ala and DNP-OH migrate together but were, however, separable (after the plates were dried in a cold air stream) by further development in the same direction as above with solvent IV. Sequential chromatography with solvents III and IV was used for routine analysis of amino acids, after 6 N HCl hydrolysis at 115° for 15 hr and after dinitrophenylation. Mono-DNP-DAP (also after 6 N HCl hydrolysis as above) was isolated on silica gel plates using solvent III or on Whatman No. 1 papers using solvents V or VI. *Optical rotations* were measured using a Perkin-Elmer Model 141 polarimeter, with tubes of 1 dm of about 1-ml content. *Optical rotatory dispersion* was recorded with the aid of a FICA spectropolarimeter. *Absorption spectrum* was recorded with a Cary Model 15 spectrophotometer.

Experimental Section

Isolation of Disaccharide Peptide Monomer and Disaccharide Peptide Oligomer from B. megaterium Cell Walls. Walls (1 g) were treated for 10 hr, at 37°, with 10 ml of the *Streptomyces* endopeptidase preparation (see Material and Methods) supplemented with 10 mg of *Streptomyces* F_1 endo-*N*-acetylmuramidase (Muñoz *et al.*, 1966a) in a final volume of 200 ml of 0.02 M Veronal buffer (pH 8.8). The soluble digest contained, per milligram of original walls, about 420 mμmoles of mono-N-terminal DAP and about 460 mμmoles of reducing groups equivalent to *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid disaccharide. Owing to the composition of the cell walls (see Materials

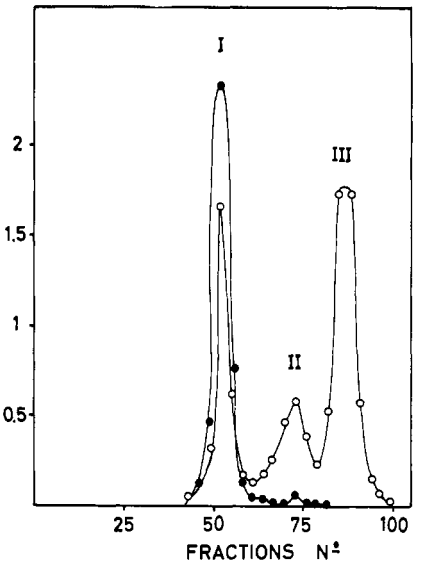

FIGURE 1: Fractionation of the degraded *B. megaterium* cell walls. The soluble products from 100 mg of walls were filtered in 0.05 N LiCl on two columns (each of 100 and 1.5 cm) of Sephadex G-50 and Sephadex G-25 connected in series (V_0 = 175 ml; $V_0 + V_i$ = 350 ml). Fractions of 3.5 ml were collected. Total hexosamine and glucose (this latter compound is indicative of the phosphomucopolysaccharide complex) were estimated on 50-μl aliquots. For total hexosamine estimation, hydrolysis was performed in 3 N HCl for 3 hr at 100°. For glucose estimation the samples were freeze dried and the residues were treated at 100° in sealed tubes for 2 hr with 20 μl of 2 N HCl. The hydrolysates were rapidly lyophilized in a high vacuum chamber, and D-glucosamine was enzymatically estimated (using the Glucostat reagents; Worthington Co.). Organic phosphorus determinations (not shown) paralleled the glucose ones. Terminal amino groups (not shown) were detected only in both disaccharide peptide monomer and disaccharide peptide oligomer fractions. I = phosphomucopolysaccharide complex; II = disaccharide peptide oligomer; and III = disaccharide peptide monomer. Units are micromoles per milliliter. (●) Glucose and (○) total hexosamine.

and Methods) this latter figure indicates that the polysaccharide moiety of the peptidoglycan has been virtually completely hydrolyzed. The above N-terminal group analysis indicates that *B. megaterium* walls are characterized by a low degree of peptide cross-linking and that not all of the DAP residues can be transformed into mono-N-terminal DAP even after exhaustive endopeptidase action (as in the conditions described above). Sephadex filtration (Figure 1) of the degraded cell walls yielded *seriatim* the phosphomucopolysaccharide complex (excluded from the gel) still associated with a minor amount of peptidoglycan debris, a disaccharide peptide oligomer associated with traces of the phosphomucopolysaccharide compound, and a disaccharide peptide monomer free of other cell wall constituents. Each of the three fractions was desalted by filtration in water on Sephadex G-25, bead form. The actual yields from 1 g of cell walls were 400 mg of the phosphomucopolysaccharide fraction, 80 mg of the disaccharide peptide oligomer fraction, and 250 mg of the disaccharide peptide monomer fraction. When expressed in terms of glutamic acid, these values represent actual yields of 5, 16, and 57% for the phosphopolysaccharide fraction, the disaccharide peptide oligomer fraction, and the disaccharide peptide monomer fraction, respectively.

Analyses of the Disaccharide Peptide Monomer and of the Disaccharide Peptide Oligomer Fractions. The results are shown in Table I and are expressed in moles per mole of glutamic acid. Total hexosamine determination on acid hydrolysates (3 N HCl, 3 hr, 100°) was carried out after chemical acetylation, using the

TABLE I: Analysis of the Disaccharide Peptide Monomer Fraction and of the Disaccharide Peptide Oligomer Fraction from Cell Walls of *B. megaterium*.[a]

	Disaccharide Peptide Monomer	Disaccharide Peptide Oligomer
A. Disaccharide moiety		
Total hexosamine	1.67	1.85
D-Glucosamine	0.86	0.90
B. Total amino acids		
Glutamic acid	1	1
meso-DAP	0.90	0.60
DD- or LL-DAP	0	0.40
Alanine	1.80	1.75
NH_3	0	0
C. Amino-terminal groups		
Mono-*N*-DAP	1.06	0.45
D. Carboxyl-terminal group[b]		
Glutamic acid	0.10	0.02
meso-DAP	0.30	0.16
DD- or LL-DAP	0	trace
Alanine	0.30	0.19

[a] Data expressed in moles per mole of glutamic acid.
[b] None of the values reported have been corrected. Yields of hydrazinolysis of tripeptides Ala-Gly-Gly and Gly-Gly-Ala were 68–76% of the theoretical values. *Remark:* Owing to the yields of production of both disaccharide peptide monomer and disaccharide peptide oligomer fractions and to the content of DD- or LL-DAP in this latter fraction, the content of *meso*-DAP in the intact walls is 85–90% of the total DAP. Traces of DAP residues other than *meso*-DAP are detected (but cannot be accurately determined) after dinitrophenylation and thin layer chromatography of cell wall acid hydrolysates.

Morgan–Elson reaction with the 7-min heating at 100° in 1% borate (Ghuysen *et al.*, 1966). Glucosamine in the same hydrolysates was estimated using the Morgan–Elson reaction as above but after enzymatic acetylation with the yeast D-glucosamine 6-phosphate *N*-acetylase (Brown, 1962). Both fractions had identical molar extinction coefficients in the Morgan–Elson reaction, after 30 min of heating at 100° in 1% borate, which were compatible with the presence of β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide units. Chromatography of acid hydrolysates (6 N HCl, 120°, 15 hr) in various systems (see Materials and Methods) showed that *meso*-DAP was the only isomer of DAP present in the disaccharide peptide monomer fraction while both *meso*-DAP and either LL- or DD-DAP or both occurred in the disaccharide peptide oligomer fraction. All of the DAP residues in the disaccharide peptide monomer fraction but only 50% of them in the disaccharide peptide oligomer fraction had one of their amino groups free, which is compatible with a monomeric and a dimeric structure, respectively. Neither of the two fractions contained amide ammonia, which explains their electrophoretic properties (Figure 2). On paper electrophoresis at pH 5 (pyridine–acetic acid–water, 4:2:1000) (2 hr, 20 v/cm), the disaccharide peptide oligomer had almost the same mobility toward the anode as glutamic acid. Under the same conditions, the disaccharide peptide monomer had a slightly lower mobility. In 0.1 N formic acid, both fractions were slightly positively charged. Hydrolysis of the disaccharide peptide monomer with the *Streptomyces* *N*-acetylmuramyl-L-alanine amidase produced about 0.8 mole of N-terminal L-alanine/mole of glutamic acid.[2] On paper electrophoresis (Figure 2) in 0.1 N formic acid, the neutral free disaccharide was well separated from the basic free peptide. The disaccharide was eluted from the paper and further purified by filtration in water on Sephadex G-25, bead form. It was indistinguishable by chromatography in various solvent systems from authentic *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid and had in the usual Morgan–Elson procedure (after 30-min heating at 100° in 1% borate; Ghuysen *et al.*, 1966a,b) the characteristic molar extinction coefficient of 9500. The free peptide was also eluted from the paper. On filtration in water on two columns of Sephadex G-25, bead form, and Sephadex G-15, bead form, connected in series, it was eluted after a volume of 200 ml which was equal to the $V_0 + V_i$ of the system. The isolated peptide could not be degraded by the *Streptomyces* aminopeptidase, an enzyme which is known to liberate the N-terminal L-alanine residue from the peptide N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine (Muñoz *et al.*, 1966b) thus exposing the amino group of the isoglutaminyl residue. The lack of activity of the aminopeptidase upon the *B. megaterium* peptide probably results from the vicinity of a free carboxyl group on the glutamic acid residue. The sequence Ala-Glu was, however, demonstrated by Edman degradation (Tipper *et al.*, 1967) of the peptide; after one cycle of the degradation, N-terminal alanine was quantitatively replaced by N-terminal glutamic acid (concomitantly most of the mono-*N*-terminal DAP groups disappeared). After the second cycle of the degradation, all N-terminal groups had disappeared. Hydrazinolysis of both the disaccharide peptide monomer and disaccharide peptide oligomer fractions (Table I) showed the presence of equivalent amounts of C-terminal alanine and C-terminal DAP, and that of a much smaller amount of C-terminal glutamic acid. In the disaccharide peptide monomer, the sum of all of the C-terminal groups amounted to 0.7/mole of total glutamic acid. This yield was similar to that obtained with the tripeptides used as controls and thus confirms the monomeric structure of the peptides present in this fraction. In the disaccharide peptide oligomer fraction, the C-terminal groups altogether amounted to only 0.37/mole of total glutamic acid, which confirms the dimeric (in average) characteristic of the components of this fraction.

Isolation from the Disaccharide Peptide Monomer Fraction of the meso-DAP Residues as Mono-DNP-meso-DAP. This fraction (10 mg) (*i.e.*, about 10 μmoles of DAP) was dissolved in 6 ml of 1% borate and mixed with 600 μl of a 0.01 M FDNB solution in ethanol. The solution was maintained at 60° for 30 min, acidified to 6 N HCl, and maintained (in sealed tube) at 115° for 15 hr. The hydrolysate was diluted with water to 2 N HCl and extracted three times with ether. Mono-DNP-DAP was then extracted from the aqueous phase with water-saturated 1-butanol (three times). The butanol solution was evaporated under vacuum and the residue, dissolved in water, was adsorbed on a small column (5 × 1 cm) of Ecteola-cellulose (the resin in OH^- form had been previously batchwise equilibrated against a 0.2 M LiCl solution and then washed with water until disappearance of Cl^- ions). The column was washed with 250 ml of water and then treated with a 0.1 M LiCl solution. Mono-DNP-DAP was readily eluted. It was finally desalted by filtration in water on a column of Sephadex G-10, bead form (130 × 1.5 cm). First LiCl and then mono-DNP-DAP were eluted. The elution curves, however, overlapped. A second filtration on the same column was carried out on those fractions which were already relatively salt free. This, coupled with the fact that about 35% of destruction of the mono-DNP-DAP occurred during hydrolyses of the dinitrophenylated monomer fraction, explains the low yield of the procedure which amounted to about 20% of the theoretical value (actual yield, 2 μmoles of mono-DNP-*meso*-DAP). Analysis of the original monomer fraction (see preceding section and Table I) had shown that *meso*-

[2] Disaccharide peptide monomer (50 mμmoles) was treated with 10 μg, in proteins, of amidase, for 5 hr at 37°, in a final volume of 50 μl of 0.02 M acetate buffer (pH 5.4). The *B. megaterium* disaccharide peptide monomer is not as good a substrate as the amidated disaccharide peptide monomer of *S. aureus*, *M. roseus*, *S. pyogenes*, and *S. faecalis* previously studied (Muñoz *et al.*, 1966b; Ghuysen *et al.*, 1967), which necessitated the use of a high amount of enzyme. This may be related to the acidic properties of the substrate presently studied.

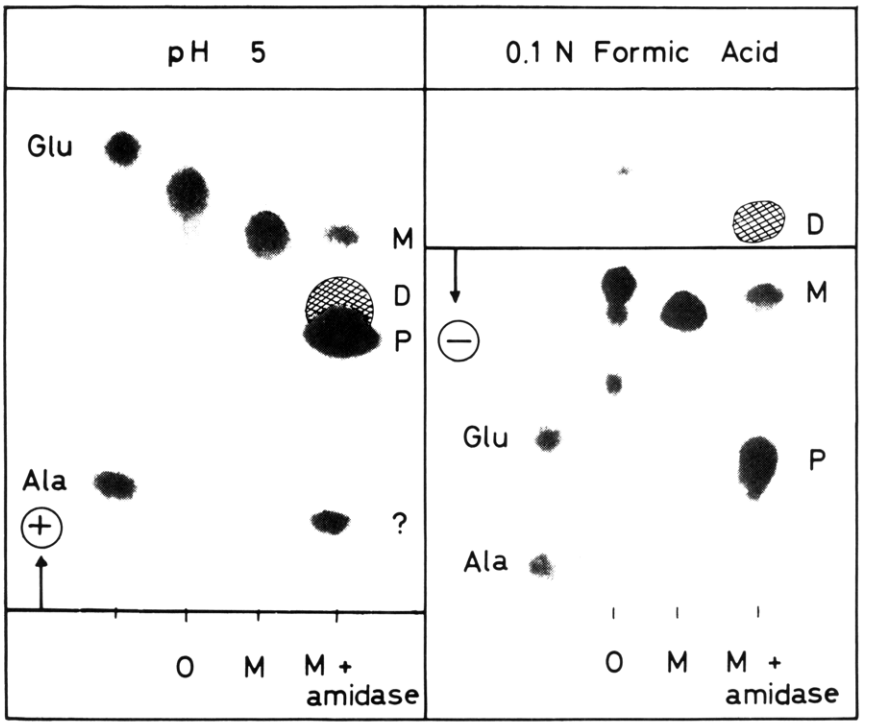

FIGURE 2: Paper electrophoresis of the disaccharide peptide oligomer fraction (0), the disaccharide peptide monomer fraction (M), and the amidase-treated disaccharide peptide monomer fraction (M + amidase). Electrophoreses were carried out at pH 5 (pyridine–acetic acid–water, 4:2:1000) and in 0.1 N formic acid, for 2 hr, at 15 v/cm. At pH 5, samples were spotted at 10 cm from the cathode. In 0.1 N formic acid, samples were spotted at the center of the paper sheet (*i.e.*, at 20 cm from the electrodes). Detection was made by ninhydrin for the amino groups (as shown in the figure) and by ultraviolet fluorescence for the disaccharide moieties. Before amidase treatment, the spots detected by ninhydrin and fluorescence coincided except for two minor ninhydrin-positive materials appearing only on electrophoresis in 0.1 N formic acid. After amidase treatment, a small amount of unhydrolyzed disaccharide peptide monomer (M) was separated from free peptide (P) and from free disaccharide (D) (represented by hatched areas). As shown in the figure, free peptide and free disaccharide are poorly separated at pH 5. Their preparative separation was therefore performed in 0.1 N formic acid. The unhydrolyzed disaccharide peptide monomer was also quantitatively recovered. The yield of the amidase hydrolysis was 78% of the theoretical value.

DAP was the only isomer present. The isolated mono-DNP-*meso*-DAP was, therefore, representative of the whole original fraction. This natural mono-DNP-*meso*-DAP was indistinguishable from synthetic racemic mono-DNP-*meso*-DAP by paper chromatography in solvent V (R_F 0.39), and by paper electrophoresis at pH 5 (same buffer as above) and in 0.1 M formic acid. After dinitrophenylation, the resulting di-DNP-*meso*-DAP was indistinguishable from synthetic di-DNP-*meso*-DAP on paper chromatography in solvent II and on silica gel thin layer chromatography in solvent III.

Isolation of the Mono-N-Terminal DAP Residues from the Disaccharide Peptide Oligomer Fraction and Their Characterizations as Mono-DNP-meso-DAP. The same procedure as above was applied but 20 mg of the fraction (*i.e.*, about 20 μmoles of DAP) was used. In this case, however, the mono-DNP-DAP obtained after the final Sephadex filtration was contaminated by an unidentified dinitrophenyl compound which was more acidic on paper electrophoresis at pH 5 and presented R_F 0.90 on paper chromatography in solvent V. The mono-DNP-DAP was purified by paper chromatography in this latter system. After extraction from the paper with ethanol and evaporation under vacuum, the residue was dissolved in 1 N HCl. The solution was extracted with ether and then with water-saturated 1-butanol. The butanol extract was evaporated under vacuum to yield about 1 μmole of mono-DNP-DAP which was characterized as mono-DNP-*meso*-DAP by paper and silica gel thin layer chromatography in the form of the di-DNP derivative, in the solvent systems II and III.

Isolation of the Non-N-terminal DAP residues, as Di-DNP-DAP, from the Disaccharide Peptide Oligomer Fraction. As indicated above, the fraction was dinitrophenylated and hydrolyzed in 6 N HCl. After butanol extraction of the free mono-DNP-*meso*-DAP, the aqueous phase contained most of the amino acids which in the original fraction had no free amino group. After freeze drying, the residue (containing boric acid

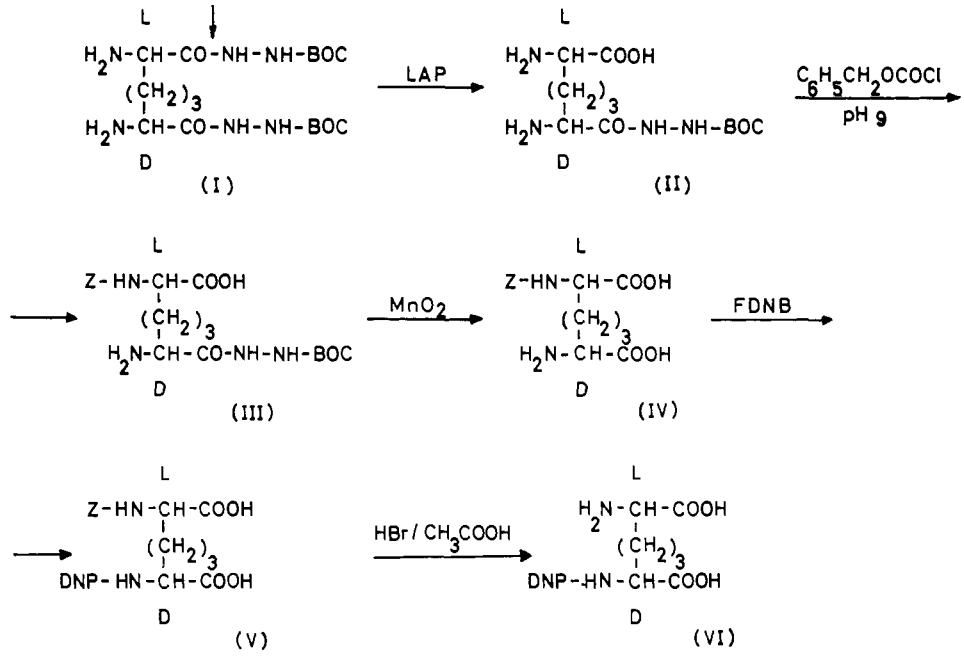

FIGURE 3: Scheme for the stereospecific synthesis of the mono-DNP-(D)-*meso*-DAP. Abbreviations: BOC = $(CH_3)_3$-COCO; Z = $C_6H_5CH_2OCO$; and LAP = leucine aminopeptidase.

from the dinitrophenylation reaction) was dissolved n water and the pH was adjusted to 9.2 with NaOH. The solution (18 ml) was mixed with 2 ml of a 5% potassium borate solution and with 2 ml of the FDNB reagent. After dinitrophenylation, the solution was acidified to 2 N HCl and the DNP-amino acids were extracted with ether. The residue of the ether extract was submitted to preparative paper chromatography in solvent II in presence of 0.1 M phthalate buffer (pH 6). In this system, DNP-Glu, di-DNP-*meso*-DAP, di-DNP-LL- or -DD-DAP, and DNP-Ala have R_F relative to DNP-OH, respectively, equal to 0.064, 0.220, 0.44, and 0.92. Traces of di-DNP-*meso*-DAP were detected, but this was not further studied. The di-DNP-LL- or -DD-DAP was eluted from the paper with 0.1 N HCl (in order to minimize the solubilization of phthalic acid). The solution was supplemented with concentrated HCl (final concentration, 2 N) and was then extracted with ether. After evaporation of ether, the di-DNP-LL- or -DD-DAP was dissolved in the minimum of glacial acetic acid, leaving a residue consisting of a few crystals of phthalic acid. The acetic acid solution was lyophilized, yielding about 0.8 μmole of di-DNP-LL- or -DD-DAP.

Synthesis of Mono-DNP-(D)*-meso-DAP.*[3] In order to further identify the natural compounds mono-DNP-*meso*-DAP obtained from both disaccharide peptide monomer and disaccharide peptide oligomer fractions, one of the two possible isomers, namely the mono-DNP-(D)-*meso*-DAP, was synthesized according to the procedure which is schematically represented in Figure 3. Stereospecific hydrolysis with leucine aminopeptidase from calf lens (VEB Arzneimittelwerk, Dresden) or leucine aminopeptidase from hog kidney (Worthington) of the symmetrical hydrazide derivative of *meso*-DAP (compound I) yielded the nonsymmetrical compound II (Bricas and Nicot, 1966; Dezélée and Bricas, 1967). There is an important difference of pK between the two amino groups of compound II owing to the fact that the amino group of the L asymmetric carbon of *meso*-DAP is on an α carbon bearing a free carboxyl group, whereas the amino group on the D asymmetric carbon of *meso*-DAP is on an α carbon bearing a protected hydrazide function. Thus if an acylation is carried out at pH 9, the amino group on the L carbon, being to a great extent unprotonated, should be more reactive than the amino group on the D carbon, which is still nearly completely protonated. Indeed, benzyloxycarbonylation assays carried out on compound II, at pH 9 under pH-Stat control, show that mainly compound III is obtained. This compound was desalted on Dowex 50-X8 (H^+ form).

The substituted hydrazide of this compound was oxidized by activated MnO_2 (Attenburrow *et al.*, 1952) according to Kelly (1963). The resulting compound IV was characterized in the form of its manganese chelate; $[\alpha]_D^{22}$ $-18 \pm 0.5°$ (*c* 0.6, 1 N HCl). *Anal.* Calcd for $C_{15}H_{10}N_2O_4 \cdot 0.5Mn \cdot 0.5H_2O$: C, 49.8; H, 5.85; N, 7.78. Found: C, 49.23; H, 5.7; N, 7.81.

[3] In order to specify on which one of the two asymmetrical carbons of *meso*-DAP are placed the substituted amino groups, we advocate the use of the notation (L) or (D) written immediately *before* the abbreviation *meso*-DAP. Similarly we propose to write (L) or (D) immediately *after meso*-DAP in order to distinguish between the carboxyl-substituted groups.

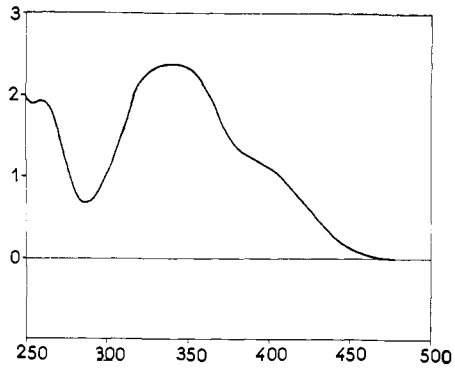

FIGURE 4: Ultraviolet spectrum of synthetic mono-DNP-(D)-*meso*-DAP in acetic acid. Optical density *vs.* wavelength in millimicrons.

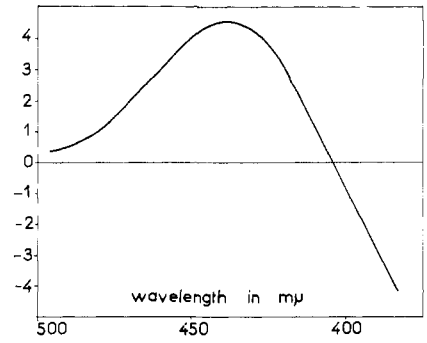

FIGURE 5: Anomalous optical rotatory dispersion curve of synthetic mono-DNP-(D)-*meso*-DAP, between 500 and 380 mμ, showing a positive Cotton effect. Cuvet: 0.2 dm; concentration = 0.03885%; solvent = acetic acid. Rotation in $^1/_{1000}$ degrees.

It was homogeneous on thin layer silica gel chromatography in solvent VI (R_F 0.59). Compound IV (58 mg) was then dinitrophenylated according to Ghuysen *et al.* (1966a,b). Compound V was treated for 30 min at 25° with a saturated hydrobromic acid solution in glacial acetic acid. Compound VI, mono-DNP-(D)-*meso*-DAP, was finally crystallized from water at pH 3.2; yield, 9.5 mg; $[\alpha]_D^{22}$ +66.8 ± 1.3° (*c* 0.07, glacial acetic acid). *Anal.* Calcd for $C_{13}H_{16}N_4O_8 \cdot 0.5\ H_2O$: C, 42.7; H, 4.7. Found: C, 42.68; H, 4.69. It was homogeneous on thin layer silica gel chromatography in solvent VI (R_F 0.55).

The configuration and the optical homogeneity of compound IV have also been indirectly corroborated by determining the optical properties of the mono-DNP-*meso*-DAP derived from it.[4] As will be developed in the Discussion the configuration of this compound can be checked by comparing, in the manner reported by Diringer and Jusic (1966), its optical properties with these of synthetic mono-DNP-LL-DAP and mono-DNP-*meso*-DAP obtained from the natural products.

Molar optical rotations, $[M]_D$, of the synthetic mono-DNP-(D)-*meso*-DAP and of both natural mono-DNP-*meso*-DAP compounds in glacial acetic acid were found to be identical within the limits of experimental error. $[M]_D$ values were, respectively, +238 ± 5° for the synthetic compound, +248 ± 6° for the natural compound from the disaccharide peptide monomer fraction, and +220 ± 20° for the natural compound from the disaccharide peptide oligomer fraction. The amounts used for these determinations ranged between 0.5 and 1.5 μmoles. Under the same conditions, the natural di-DNP-(LL- or -DD)-DAP from the disaccharide peptide oligomer fraction had an $[M]_D$ equal to +426 ± 20°. Since synthetic di-DNP-LL-DAP as prepared by Jusic *et al.* (1963b) had an $[M]_D$ equal to −444°, the configuration DD can be assigned to the natural compound.

Optical Rotatory Dispersions of Synthetic and Natural Mono-DNP-(D)-meso-DAP. The ultraviolet spectrum of mono-DNP-(D)-*meso*-DAP in acetic acid shows a maximum absorption at 340 mμ and a shoulder at 418 mμ (Figure 4). In the vicinity of this latter absorption band, mono-DNP-(D)-*meso*-DAP exhibits anomalous optical rotatory dispersions with a positive Cotton effect centered on λ_0 418 mμ (Figure 5). Between 460 and 600 mμ in a region sufficiently distant from the former active band, the optical rotatory dispersion (Figure 6) followed the simplified Drude equation $[M] = K/(\lambda^2 - \lambda_0^2)$. Plotting $I/[M]$ *vs.* λ^2 (Figure 7), the extrapolations of the resulting straight lines on the wavelength axis gave, for both synthetic and natural compounds, identical values of λ_0^2 corresponding to 418 mμ. Moreover, the K values were found to be 0.416 (±0.006) deg × μ^2 for the synthetic product and 0.424 (±0.009) deg × μ^2 for the natural one, which is further proof for the identity of the two compounds. Maximum errors were calculated on the basis of 1% precision in the estimation of sample concentrations and on the basis of 0.5-mdeg precision in the measurement of the rotary angles for the synthetic product and of 1 mdeg for the natural one. It is not known whether the compounds exhibit a positive Cotton effect at 340 mμ; the strong absorption at this wavelength precluded the determination of the optical rotations.

Discussion

The polysaccharide moieties of all bacterial wall peptidoglycans so studied are composed of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. In contrast to this, the peptide moieties

[4] In order to establish more directly the configuration of IV, we are undertaking a series of experiments. On the one hand, the action of L- and D-aminoacidoxidases on IV will be studied, and on the other hand, the nonsymmetrical dipeptide L-Ala-(D)-*meso*-DAP, which had already been obtained by the stereospecific action of leucine aminopeptidase on the synthetic tripeptide bis(L-Ala)-*meso*-DAP (Nicot and Bricas, 1963), will be prepared from IV.

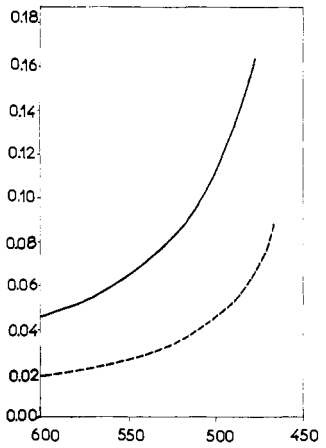

FIGURE 6: Optical rotatory dispersions, between 460 and 600 mμ, of mono-DNP-(D)-*meso*-DAP. Dispersion curve of synthetic and natural compound: concentration = 0.07185% for the synthetic compound and 0.02725% for the natural one. Solvent: acetic acid. Cuvet 1 dm. Rotation is in degrees. Wavelength is in millimicrons. (———) Synthetic product and (– – – –) natural product.

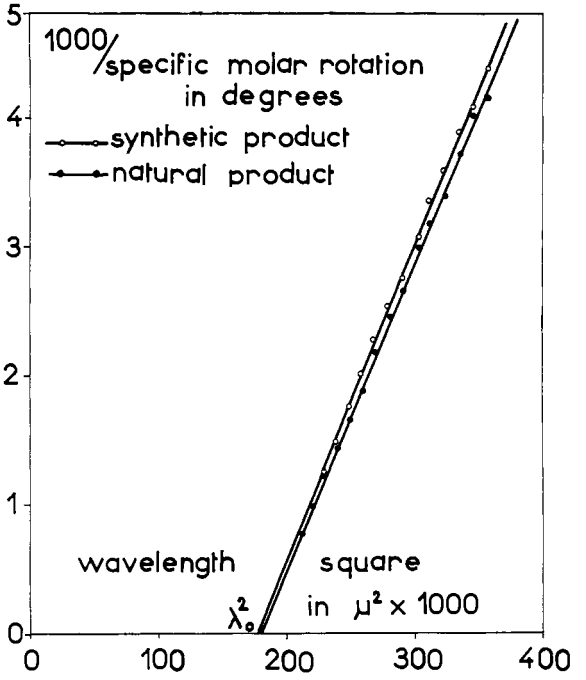

FIGURE 7: Optical rotatory dispersion between 460 and 600 mμ of mono-DNP-(D)-*meso*-DAP. Graphical representation of the simplified Drude equation for both synthetic and natural compounds. Determination of the center of positive Cotton effect (λ_0 418 mμ) and *K* coefficients.

of the bacterial peptidoglycans present numerous structural variations, both in the sequence of the amino acids of the peptide subunits and in the nature of the cross-links between these subunits. N^{α}-(L-Alanyl-D-isoglutaminyl)-L-lysyl-D-alanine is the peptide subunit for the wall peptidoglycan of *Staphylococcus aureus*, *Micrococcus roseus*, *Streptococcus pyogenes* (Muñoz, *et al.*, 1966b), and *Streptococcus faecalis* (Ghuysen *et al.*, 1967). A similar sequence occurs in *Micrococcus lysodeikticus* but the D-isoglutamine residue is replaced by the dipeptide α-D-glutamylglycine (Tipper and Strominger, 1965; Mirelman and Sharon, 1966; Tipper *et al.*, 1967). In the former L-lysine-containing peptidoglycans, the peptide cross-links extend from the N^{ϵ}-terminal lysine of one peptide to the C-terminal D-alanine of another, either by direct bonding (Petit *et al.*, 1966; Tipper *et al.*, 1967; Katz and Strominger, 1967; Mirelman and Sharon, 1966) or by peptide bridges. Glycine, L-alanine, L-threonine, and D-isoasparagine (Petit *et al.*, 1966; Ghuysen *et al.*, 1967; Tipper *et al.*, 1967) are known to be constituents of these bridges which may be composed of one or two different amino acids forming chain lengths of one to five residues. With many bacterial peptidoglycans, additional stereochemical problems arise from the fact tht L-lysine is replaced by *meso*-diaminopimelic acid. Important structural features of these *meso*-DAP-containing tetrapeptide subunits remained, however, to be unravelled. Recently, Diringer and Jusic (1966) showed that the optical rotation of the mono-DNP-*meso*-DAP isolated after dinitrophenylation and acid hydrolysis of the *E. coli* peptide subunit had that value expected for a mono-DNP-(D)-*meso*-DAP. Therefore, they concluded that the amino group engaged in peptide linkage to glutamic acid was located on the L carbon of *meso*-DAP.

The present paper is a first report describing some structural features of another DAP-containing peptidoglycan which is found in walls of the Gram-positive *B. megaterium* KM. Again, the polysaccharide moiety was found to be quantitatively cleaved into β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides, thus strengthening the hypothesis mentioned above according to which such an alternation of the two *N*-acetylhexosamine residues may well be a structure common to all bacterial walls. About 85% of the DAP residues in *B. megaterium* peptidoglycan are *meso* isomers. Most of these residues have one amino group free, which is indicative of a low degree of peptide cross-linking. A *Streptomyces* endopeptidase, different from the SA, ML, and MR endopeptidases previously described (Petit *et al.*, 1966), was prepared (its production by the *Streptomyces* is, however, still casual and no refined procedure of purification can yet be proposed). This enzyme is capable of liberating one amino group from the few *meso*-DAP residues which in the native walls have both amino groups unexposed. The paucity of the number of the linkages split during this process, the occurrence of many native free carboxyl groups, and the relative inaccuracy of the C-terminal group determination precluded the identification of the carboxyl groups which appeared as a consequence

of the endopeptidase treatment. After fractionation of the endo-*N*-acetylmuramidase- and endopeptidase-treated cell walls, the *meso*-DAP residues which all have one amino group free were recovered predominantly in a disaccharide peptide monomer fraction with only minor amounts appearing in a disaccharide peptide oligomer fraction. Both fractions were obtained separated from the nonpeptidoglycan phosphomucopolysaccharide complex previously studied (Ghuysen, 1964). Analysis of the disaccharide peptide monomer fraction and its degradation by enzymatic and chemical means showed that the disaccharide units were substituted by an assortment of peptide monomers, mainly L-Ala-D-Glu-*meso*-DAP and L-Ala-D-Glu-*meso*-DAP-D-Ala. Neither the disaccharide peptide monomer fraction nor the disaccharide peptide oligomer fraction had amide ammonia, in agreement with their electrophoretic properties. The *meso*-DAP residues were isolated from both fractions as monodinitrophenyl derivatives. They exhibited in acetic acid molar optical rotations ($[M]_D$ +220 and +248°) almost identical with that previously observed with the *E. coli* mono-DNP-*meso*-DAP (+250°) (Diringer and Jusic, 1966). The location of the dinitrophenyl substituent on the D carbon, as previously proposed by these authors in the cases of the mono-DNP-*meso*-DAP isolated from *E. coli*, has been thus confirmed also in the case of the mono-DNP-*meso*-DAP derivative isolated from one other species of bacteria. Moreover, the mono-DNP-*meso*-DAP derivative synthetized according to the procedure shown in Figure 3 has a molar rotation of +238 ± 5°, identical, within the limits of experimental error, with the values of the $[M]_D$ of the same derivative isolated from two different species of bacteria. On the other hand, both synthetic and natural mono-DNP-*meso*-DAP exhibited the same anomalous rotatory dispersion with Cotton effect centered on 418 mμ, and followed, between 450 and 600 mμ, the simplified Drude equation with identical *K* coefficients.

The agreement between the optical properties of the compounds isolated from two different bacteria, as well as with those of the synthetic compound, serves as evidence for their stereochemical homogeneity. This implies that all three derivatives are identical and therefore that the DNP group in all three cases is located on the D carbon. Consequently the peptide linkage Glu-*meso*-DAP existing in both *B. megaterium* and *E. coli* peptide subunits involves the amino group located on the L carbon of the *meso*-DAP.

About 15% of the total DAP residues of the *B. megaterium* peptidoglycan was recovered with the two amino groups unexposed, as constituents of the disaccharide peptide oligomer fraction. This small number of DAP residues which thus had escaped the endopeptidase action were isolated and characterized as DD isomers once again on the basis of the molar optical rotation of their bisdinitrophenyl derivatives (+426 ± 20%). From this, it can thus be concluded that DD-DAP for which the occurrence in a few bacteria had been previously suspected is indeed a possible constituent of the bacterial wall peptidoglycan.

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The Peptide N^{α}-(L-Alanyl-D-isoglutaminyl)-N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanine and the Disaccharide *N*-Acetylglucosaminyl-β-1,4-*N*-acetylmuramic Acid in Cell Wall Peptidoglycan of *Streptococcus faecalis* Strain ATCC 9790*

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ABSTRACT: A major portion of the cell wall peptidoglycan in *Streptococcus faecalis* is composed of the disaccharide tetrapeptide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine. The tetrapeptides are cross-linked through single D-isoasparaginyl residues extending from the C-terminal D-alanine of one tetrapeptide unit to the N^{ϵ}-terminal L-lysine of another. It is the first time that the occurrence of an isoasparaginyl residue in a natural product has been described. The *Streptomyces* SA endopeptidase cleaves D-alanyl-D-isoasparaginyl linkages and is thus the first enzyme known to hydrolyze D–D peptide bonds. Treatment of the disaccharide N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}-(D-isoasparaginyl)-L-lysyl-D-alanine with 10 equiv of NaOH at 37° for 1 hr results in deamidation of the isoasparaginyl residue together with migration of the aspartyl–lysine peptide bond giving rise to a mixture of N^{ϵ}-(β-aspartyl)- and N^{ϵ}-(α-aspartyl)lysyl peptides. Under the same alkaline treatment, the *N*-acetylmuramyl residue undergoes a lactyl elimination which results in the production of acyl peptides and a Morgan–Elson prochromogenic compound, without hydrolysis of the glycosidic linkage. This conversion, interpreted to be the result of a β elimination, also occurs in the other disaccharide peptide monomers previously isolated from *Staphylococcus aureus*, *Micrococcus roseus*, *and Streptococcus pyogenes*.

The structures of the peptide subunits and of the peptide cross-links in the cell wall peptidoglycan of several Gram-positive bacterial species have been recently determined after stepwise degradation with specific *Streptomyces* enzymes (Ghuysen *et al.*, 1965; Petit *et al.*, 1966; Muñoz *et al.*, 1966a). These studies have demonstrated that the basal peptide subunit of the wall peptidoglycan of three species (*Staphylococcus aureus* Copenhagen, *Micrococcus roseus* R27, and *Streptococcus pyogenes*, group A, type 14) has the structure N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine (Muñoz *et al.*, 1966a). On the other hand, five types of peptide cross-links between the ϵ-amino group of lysine of one peptide subunit and the terminal alanine carboxyl group of another were found (Petit *et al.*, 1966; Tipper *et al.*, 1967). These bridges are pentaglycine in *S. aureus* Copenhagen, tri-L-alanine in *M. roseus* thr⁻, tri-L-alanyl-L-threonine in *M. roseus* R27, di-L-alanine in *S. pyogenes* type 14, L-alanine in *Arthrobacter crystallopoietes*, and direct bonding with no additional amino acids between the C-terminal alanine residue of one peptide subunit and the ϵ-amino group of lysine of another in *Micrococcus lysodeikticus*. Until now, all of the peptide bridges examined contained either glycine or neutral L-amino acids. D-Aspartic acid is present in cell walls of *Streptococcus faecalis* and of numerous *Lactobacillus* spp in amounts nearly equivalent to that of L-lysine and D-glutamic acid (Cummins and Harris, 1956; Toennies *et al.*, 1959; Ikawa and Snell, 1960; Ikawa, 1964; Plapp and Kandler, 1966; Shockman *et al.*, 1967). When walls of *Lactobacillus brevis* were hydrolyzed in 11 N HCl at 80° for 43 hr a derivative of aminosuccinimide, ϵ-(aminosuccinyl)lysine, was isolated and on treatment with dilute sodium hydroxide was converted into a mixture

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