Homoserine and Diaminobutyric Acid in the Mucopeptide-Precursor-Nucleotides and Cell Walls of some Plant-Pathogenic Corynebacteria

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The nucleotide precursors of cell-wall mucopeptide were prepared by vancomycin inhibition of *Corynebacterium insidiosum* and *Corynebacterium poinsettiae*. The amino acid in the third position in the former peptide was shown by optical rotatory dispersion of the bisdinitrophenyl derivative to be L-diaminobutyric acid. Homoserine in the nucleotide from *C. poinsettiae* was catalytically oxidized to aspartic acid, which was then shown to be the L-isomer by optical rotatory dispersion of the trinitrophenyl compound. The sequence for *C. insidiosum* was UDP-N-acetylmuramyl-glycyl-isoglutamyl-(γ -acetyl)-diaminobutyryl-alanyl-alanine. The cell walls of *C. insidiosum* and *Corynebacterium sepe-donicum* contained diaminobutyric acid that was optically inactive. It is proposed, therefore, that the primary peptide chain contains the L-isomer with its γ -amino group blocked by acetylation and that cross-linking is achieved by means of the D-isomer, analogous to D-ornithine in *C. poinsettiae*.

The precursor nucleotides for the synthesis of the mucopeptide component of bacterial cell walls have the general composition UDP-N-acetylmuramyl-X-D-isoglutamyl-Y-D-alanyl-D-alanine (Rogers & Perkins, 1968; Perkins, 1969a), where X is most commonly L-alanine, but can be L-serine as in *Butyribacterium rettgeri* (Guinand, Ghuysen, Schleifer & Kandler, 1969) or glycine as in *Corynebacterium poinsettiae* (Perkins, 1967) and *Microbacterium lacticum* (Schleifer, Plapp & Kandler, 1968). The residue Y, on the other hand, is commonly meso-diaminopimelic acid or L-lysine, but can be L-ornithine as in *Micrococcus radiodurans* (Work, 1965) and *B. rettgeri* (Miller, Plapp & Kandler, 1966) or LL-diaminopimelic acid as in propionibacteria (Allsop & Work, 1963), *Streptomyces* species and *Clostridium perfringens* (Leyh-Bouille, Bonaly, Ghuysen, Tinelli & Tipper, 1970a). The mucopeptides of some bacteria contain L-2,4-diaminobutyric acid (e.g. *Corynebacterium tritici*) or D-ornithine (e.g. *C. poinsettiae*) (Perkins & Cummins, 1964) and D-ornithine is accompanied by homoserine (Perkins, 1965). When *C. poinsettiae* was inhibited with vancomycin, precursor nucleotide accumulated containing glycine, glutamic acid, homoserine and alanine (mol.prop. 1:1:1:2) but no ornithine (Chatterjee & Perkins, 1966a), and the structure UDP-N-acetylmuramyl-glycyl-D-isoglutamyl-homoseryl-D-alanyl-D-alanine was later

assigned (Perkins, 1967). The homoserine was of unknown configuration, although it seemed probable that it might belong to the L-series, in accordance with the known occurrence at the same position in the sequence of L-lysine, LL-diaminopimelic acid, the L-centre of meso-diaminopimelic acid (Diringer & Jušić, 1966; Bricas, Ghuysen & Dezélee, 1967) and L-ornithine (Miller *et al.* 1966). The present work establishes the configuration of the homoserine occurring in the mucopeptide precursor of *C. poinsettiae* and at the same time discusses the configuration of 2,4-diaminobutyric acid in some related Corynebacteria. A preliminary account of some of this work has appeared (Perkins, 1968).

METHODS

Growth of organisms. *C. poinsettiae* (N.C.P.P. 177) was grown at 30°C, *Corynebacterium insidiosum* (N.C.P.P. 1110) at 25°C and *Corynebacterium sepe-donicum* (N.C.P.P. 378) at 22°C in shaken-flask cultures in Hedley-Wright broth containing 1% (w/v) of glucose.

Preparation of cell walls. Cell walls were prepared by breakage with glass beads, differential centrifuging, washing with M-NaCl and trypsin digestion as described by Perkins & Nieto (1970).

Isolation of nucleotides. (a) *C. insidiosum.* A 3-day culture was diluted in 10 vol. of fresh broth and shaken until growth recommenced. The cells were then collected by centrifuging and resuspended in 30 mM-sodium phosphate buffer, pH 7, at 15 mg dry wt./ml. This suspension

was used to inoculate four conical flasks (2 litres) each containing broth-glucose (500ml) to a cell density of 1 mg dry wt./ml. After shaking for 2.5 h chloramphenicol was added (final concentration 40 μ g/ml), followed 15 min later by vancomycin hydrochloride (20 μ g/ml). Shaking was continued for 2 h and the cells were then collected by centrifuging. The cells were extracted with 25% (w/v) trichloroacetic acid (18 ml) at 0°C for 15 min, centrifuged and re-extracted with a further 12 ml. Trichloroacetic acid was removed from the extracts by shaking with ether and NaOH was added to neutralize the solution. At this stage the extract contained 35 μ mol of bound *N*-acetylhexosamine (Reissig, Strominger & Leloir, 1955). The volume was decreased under vacuum to 5 ml and the polysaccharide also present (probably the same as the extracellular product described by Gorin & Spencer, 1961, 1964) was precipitated by the addition of 5 ml of ethanol. The supernatant was dried under vacuum, redissolved in water, applied to a column (100 mm $\times$ 22 mm) of Sephadex G-25 and eluted with water on a fraction collector (5 ml fractions). Samples (0.3 ml) of each fraction with high E_{260} were taken and their bound *N*-acetylhexosamine content was measured. Two peaks were obtained, a major one in fractions 20–25 and a minor one in fractions 26–31, the fractions being numbered from the addition of the sample. The latter contained UDP-*N*-acetylglucosamine. Fractions 20–25 were pooled, concentrated and applied as a streak to washed paper for chromatography overnight in ethanol-1 M-ammonium acetate (5:2, v/v). Two main u.v.-absorbing bands were observed, one at 1–11 cm (band A) and one at 17–24 cm (band B). Each was eluted in water and its bound *N*-acetylhexosamine content measured. The slow band contained 13 μ mol and the fast band 6.5 μ mol. These products were further purified by paper chromatography for 24 h in isobutyric acid-aq. 0.5 M-ammonia (5:3, v/v). Once again the u.v.-absorbing bands were eluted. Purified band A contained 10.5 μ mol and purified band B 5.2 μ mol of bound *N*-acetylhexosamine. Samples were hydrolysed (6 M-HCl at 105°C for 18 h) and amino acids were separated by electrophoresis in 0.25 M-formic acid, in parallel with quantitative marker mixture. Colour was developed by dipping in 0.45% ninhydrin in acetone followed by incubation at 50°C for 1 h (Chatterjee & Perkins, 1966b) and elution in acetone-water (7:3, v/v), and the E_{370} was measured.

Band B contained UDP, bound *N*-acetylhexosamine, glutamic acid, glycine, 2,4-diaminobutyric acid and alanine in the following molar proportions: 1.01, 1.06, 1.00, 1.02, 1.00, 1.93. Band A contained the same components in the same proportions, but in addition contained aspartic acid and *N*-methyl-leucine attributable to bound vancomycin, as indicated by Chatterjee & Perkins (1966b). The *N*-methyl-leucine/diaminobutyric acid molar ratio was 1.11:1.

(b) *C. poinsettiae*. The same procedure was used, except that the vancomycin concentration was 25 μ g/ml and the ethanol precipitation was unnecessary, since the trichloroacetic acid extract did not contain much polysaccharide. As before nucleotides with and without bound vancomycin were isolated (Chatterjee & Perkins, 1966b). The yield of the former was about 10 μ mol/g dry wt. of cells and of the latter about 20 μ mol. The nucleotide without bound vancomycin contained UDP, bound *N*-acetylhexo-

amine, glutamic acid, glycine, homoserine and alanine in the following molar proportions: 1.0, 1.0, 1.0, 0.9, 1.0, 2.1.

(c) *Staphylococcus aureus* H. The same procedure was followed. The isolated nucleotide (yield 35 μ mol/g dry wt. of cells) contained UDP, bound *N*-acetylhexosamine, glutamic acid, lysine and alanine in the following molar proportions: 1.0, 1.0, 1.0, 1.0, 3.0.

Oxidation of homoserine. Platinum oxide catalyst (Johnson, Matthey and Co. Ltd., London E.C.1, U.K.) was suspended in water, reduced by H₂ at room temperature, filtered and dried. This catalyst (6 mg) was added to a mixture of 10 mM-homoserine (1 ml) and saturated NaHCO₃ (20 μ l) and stirred magnetically at 54°C under O₂ for 2 h. T.l.c. in ethanol-water (2:1, v/v) and paper electrophoresis in collidine-acetate buffer, pH 7 (Newton & Abraham, 1954), showed complete conversion of homoserine into aspartic acid.

The nucleotide-pentapeptide from *C. poinsettiae* (1.07 μ mol in 1 ml) was treated with saturated NaHCO₃ (20 μ l), the pH value then being 8.3. Reduced catalyst (2 mg) was added and O₂ was passed through the solution. The flask was then stoppered and magnetic stirring was continued at 60°C for 1 h. Further catalyst (4 mg) was then added and stirring was continued for a further 11 h. A sample (50 μ l) was then hydrolysed (6 M-HCl at 105°C overnight) and subjected to paper electrophoresis in collidine-acetate buffer, pH 7. The oxidized sample, unlike the untreated material, showed the presence of aspartic acid, although a little homoserine lactone, characterized by its cationic yellow spot, was still visible. The remainder of the oxidized nucleotide was hydrolysed and the amino acids were separated by electrophoresis on washed paper in 0.25 M-formic acid (3 h, 9 V/cm). The aspartic acid was eluted with water.

Preparation of trinitrophenyl derivatives. The method of Okuyama & Satake (1960) was employed. 2,4,6-Trinitrobenzenesulphonic acid was a crystalline sample prepared by A. J. Garrett. A portion (20 mg) was dissolved in water (0.7 ml) together with L-aspartic acid (13.3 mg) and NaHCO₃ (23 mg). The mixture was left at room temperature in the dark for 2 h. It was then acidified with HCl to pH 1 and kept at 2°C in the dark. Trinitrophenyl-L-aspartic acid separated as fine needles, which were filtered and dried *in vacuo* over NaOH and P₂O₅ [yield 5 mg, m.p. 146°C (Kofler), ϵ_{350} 1.64 $\times$ 10⁴ in solution in 1% NaHCO₃; Okuyama & Satake (1960) give for the crystalline monohydrate prepared in the same way m.p. 151–152°C, ϵ_{350} 1.34 $\times$ 10⁴ in solution in 4% NaHCO₃]. A sample subjected to t.l.c. on Kieselgel G (activated at 100°C for 30 min and then left in air for some hours) in chloroform-methanol-acetic acid (95:5:1, by vol.) gave R_F 0.19 (DNP-glutamic acid has R_F 0.40). Trinitrophenyl-D-aspartic acid monohydrate was prepared in the same way.

The aspartic acid derived from the oxidized nucleotide-pentapeptide was dissolved in 1% NaHCO₃ (0.1 ml) and ethanolic 0.5% trinitrobenzenesulphonic acid (0.2 ml) was added. The mixture was left in the dark at room temperature for 2 h and the ethanol was removed *in vacuo*. Water was added and ether extraction was performed twice. Then 1 drop of 2 M-HCl was added and the product was extracted into ether (two 0.5 ml portions). The extract was chromatographed by t.l.c. as above, most of the material corresponding to authentic trinitrophenyl-

aspartic acid. The silica was scraped off and eluted with acetone-water (3:1, v/v).

Optical rotatory dispersion. The apparatus was a Polaromatic 62 (Bellingham and Stanley, London N.15, U.K.). Samples of trinitrophenylaspartic acid (approx. 0.1 mm) or bis-DNP-diaminobutyric acid (approx. 50 μ M) in 0.125 M-NaHCO₃ were placed in a cell of 10 mm light-path and the instrument was set for a full-scale deflexion of 50 millidegrees. The concentration of the experimental sample was determined by measurement of E_{350} .

Partial hydrolysis of nucleotide-pentapeptide and separation of resultant peptides. The precursor nucleotide from *C. insidiosum* (4.5 μ mol) was dissolved in water (0.9 ml), M-HCl (50 μ l) was added and the mixture was heated at 100°C for 5 min. After cooling, 0.5 M-Na₂CO₃ was added dropwise to pH 9.5 and heating at 100°C was repeated for 4 min. The cooled mixture was acidified with 1 drop of acetic acid and dried *in vacuo*. Then 0.5 ml each of acetic acid and conc. HCl were added, and the tube was sealed and incubated at 35°C for 4 days. The acids were removed under vacuum and the product was transferred to a column (50 mm $\times$ 7 mm) of Zeo-Karb 225 (H⁺ form) resin and eluted first with water and then with 2 M-NH₃. Each eluate was transferred to Whatman no. 3 paper (previously thoroughly washed by irrigation with 1 M-ammonium acetate followed by water) and chromatographed in butan-1-ol-acetic acid-water (63:10:27, by vol., upper phase), the aqueous eluate for 16 h and the ammoniacal one for 24 h.

The aqueous eluate gave a fast-running band, detectable by the method of Rydon & Smith (1952), R_{Leu} 1.15. The remainder of the band was eluted and hydrolysed in 6 M-HCl at 105°C overnight. It was found to contain equimolecular proportions of glycine and glutamic acid. Like the corresponding material from *C. poinsettiae* (Perkins, 1967) the peptide also contained lactic acid, as shown by a colour reaction with H₂SO₄ and 4-hydroxybiphenyl.

The ammoniacal eluate gave nine ninhydrin-positive bands, the first being at the origin and the seventh in the alanine position. Each band was eluted and its components were further separated by electrophoresis on washed paper in 0.25 M-formic acid (3 h, 9 V/cm), ninhydrin-positive regions again being eluted with water. These peptides were hydrolysed before and after dinitrophenylation to determine their sequences.

Bis-DNP-diaminobutyric acid. Samples of cell-wall or precursor nucleotide were hydrolysed in 6 M-HCl at 105°C for 18 h. The diaminobutyric acid was purified by electrophoresis on washed paper in 0.25 M-formic acid and the basic band was eluted. This product was converted into bis-DNP derivative by reaction with ethanolic 0.5% (v/v) 1-fluoro-2,4-dinitrobenzene-0.125 M-NaHCO₃ (2:1, v/v) and extraction of the product from the acidified reaction mixture into ether. The extract was chromatographed by t.l.c. on silica gel G as described for trinitrophenylaspartic acid. Authentic bis-DNP-L-diaminobutyric acid was prepared in the same way. The concentration of solutions in 0.125 M-NaHCO₃ was determined from E_{350} (ϵ 30300).

Determination of acetyl. Samples of the precursor nucleotides from *C. poinsettiae*, *C. insidiosum* and *S. aureus* H were freed of small molecules by filtration on Sephadex G-25, since the nucleotides are almost excluded by the gel (Rosenthal & Sharon, 1964). Portions (0.2 μ mol) were dissolved in water (10 μ l) and transferred to a 21 cm

capillary tube with 1.7 mm bore, followed by washings of water (10 μ l and 5 μ l). Each addition was brought to the bottom of the tube by centrifuging. Conc. HCl (5 μ l) was then added, followed by water (5 μ l). The tube was sealed, heated at 105°C for 2 h and then cooled in ice. It was then opened near the top and ether (about 0.2 ml) was added. Fatty acids were then extracted by vigorous shaking with inversion, care being taken to ensure that the aqueous layer moved right up and down the tube each time. Finally the aqueous layer was shaken down and the tube was covered with an ether-washed rubber collar and a piece of detached tube. Samples (1 μ l) were applied with a Hamilton syringe to a g.l.c. column.

Standards were provided by treating a solution of *N*-acetylglucosamine in the same way, and solvent blanks and an unhydrolysed control were also examined. The volume of ether for each sample was determined by measuring the length of capillary it occupied (usually 7–8 cm) and the peak areas from the g.l.c. were corrected accordingly. The instrument was a Pye Argon and conditions were as follows: gas, argon 50 cm³/min; support, Gas-Chrom G (100–200 mesh); phase, 20% Carbowax terephthalic acid; temperature: injection 160°C, column 150°C; ionization detector, 1500 V, sensitivity $\times$ 1; chart speed, 24 in/h. Peak areas were measured with a planimeter.

RESULTS

Configuration of homoserine. Precursor nucleotide from *C. poinsettiae* contains UDP-*N*-acetylmuramic acid joined to a peptide chain with the sequence glycyl - isoglutamyl - homoseryl - alanyl - alanine (Perkins, 1967). The configuration of the homoserine was not known. During acid hydrolysis and subsequent drying homoserine is in equilibrium with its lactone and it may therefore be expected to racemize very rapidly. This difficulty was overcome by oxidation of the hydroxymethyl group of homoserine to a carboxyl group, thus yielding aspartic acid, which is much less liable to racemize during subsequent acid hydrolysis. The oxidation was performed catalytically with gaseous oxygen, much of the homoserine of the nucleotide being converted into aspartic acid. The aspartic acid in turn was converted into its trinitrophenyl derivative, since the DNP derivative was found to have rather a small specific rotation relative to its u.v. absorption. The optical rotatory dispersion of the trinitrophenylaspartic acid derived from the homoserine of the precursor nucleotide was compared with that of authentic D- and L-compounds (Fig. 1). It was evident that the experimental sample was of the L-configuration, the specific rotation being equivalent to that derived from a mixture of 85% L- and 15% D-aspartic acid. Such a degree of racemization may well have occurred during acid hydrolysis and it seems safe to assume that L-homoserine was present in the nucleotide-peptide from *C. poinsettiae*.

The alanine and glutamic acid from the same

hydrolysate were also converted into their trinitrophenyl derivatives and their optical-rotatory-dispersion curves were compared with those of authentic compounds of known configuration. As expected, both were of the D-configuration that has always been found for the glutamic acid and terminal alanine dipeptide of mucopeptide precursors.

Peptide sequence of the precursor from C. insidiosum. The nucleotide precursor accumulated by *C. insidiosum* in the presence of vancomycin was

Fig. 1. Optical rotatory dispersion of trinitrophenyl-aspartic acid. —, Authentic compounds: D-, 0.103 M; L-, 0.102 M. ----, Sample obtained by oxidation of the homoserine in the precursor nucleotide of *C. poinsettiae*, 0.111 M. The light-path was 10 mm and the solvent was 0.125 M-NaHCO₃.

found to contain glycine, glutamic acid, diamino-butyric acid and alanine in the molar proportions 1:1:1:2. Nucleotide complexed with vancomycin was also present in the trichloroacetic acid extracts (Chatterjee & Perkins, 1966b), but this was not used for structural investigation. The purified precursor was first briefly hydrolysed to remove the UDP, then heated in alkaline solution to remove by β -elimination all of the muramic acid but the lactyl side chain (Perkins, 1967; Ghuysen, Bricas, Leyh-Bouille, Lache & Shockman, 1967; Tipper, 1968) and finally partially hydrolysed in a mixture of hydrochloric acid and acetic acid at 35°C for 4 days. The resulting peptides were separated by paper chromatography followed by electrophoresis, their composition and proposed sequences being given in Table 1.

The peptides I–V were consistent with the sequence muramyl-glycyl-glutamyl-diaminobutyric acid-alanyl-alanine. This bore a close resemblance to the sequence established for the precursor nucleotide from *C. poinsettiae* (Perkins, 1967) the only difference being the substitution of diamino-butyric acid for homoserine. The glutamic acid was presumably γ -linked, as had always been observed for these compounds (Ito & Strominger, 1964; Muñoz *et al.* 1966; Perkins, 1967). However, the electrophoretic behaviour of the peptides II, III and IV was incompatible with their amino acid composition, since at pH 7 peptides II and IV were acidic instead of neutral and peptide III was neutral instead of basic. Further, dinitrophenylation yielded DNP-glutamic acid as the only DNP derivative from peptides II and IV, free diamino-butyric acid being recovered, whereas peptide II gave only mono-DNP-diaminobutyric acid. This derivative was compared with authentic γ -DNP-diaminobutyric acid by paper chromatography in phosphate buffer (Levy, 1954); authentic com-

Table 1. *Peptides isolated from a partial hydrolysate of the precursor nucleotide of C. insidiosum*

The nucleotide was degraded as described in the Methods section. Abbreviation: Dab, 2,4-diaminobutyric acid.

Peptide	Chromatography in butanol-acetic acid-water	Electrophoresis		Components of peptide after dinitrophenylation reaction	Proposed sequence
		In 0.25 M-formic acid	At pH 7		
I	$R_{\text{Leu}} = 1.15$	—	—	Lactic acid, Gly, Glu	Lactyl-Gly-Glu
II	$R_{\text{Gly}} = 1$	Slower than Asp	Acidic	DNP-Glu, Dab	Glu- γ -Ac-Dab
III	$R_{\text{Ala}} = 1.25$	Just behind Ala	Neutral	α -DNP-Dab, (Ala) ₂	γ -Ac-Dab-Ala-Ala
IV	Between Gly and Glu	Slower than Asp	Acidic	DNP-Glu, Dab, (Ala) ₂	Glu- γ -Ac-Dab-Ala-Ala
V	$R = 1.25$	Just ahead of Gly	Neutral	DNP-Ala, Ala	Ala-Ala

Table 2. Composition of cell walls and nucleotide precursor

Samples were hydrolysed in 6M-HCl at 105°C for 18 h. The cell-wall hydrolysates were analysed on an automatic amino acid analyser. Amino acids in the nucleotide hydrolysate were determined by quantitative paper electrophoresis (see the Methods section). N.D., Not determined.

	<i>C. insidiosum</i>		<i>C. sepedonicum</i>
	Nucleotide	Cell wall	Cell wall
UDP	1.01	—	—
<i>N</i> -Acetylhexosamine	1.06	N.D.	N.D.
Glycine	1.02	1.1	1.1
Glutamic acid	1.00	1.0	1.0
Diaminobutyric acid	1.00	1.7	2.0
Alanine	1.93	1.0	1.0

pound R_F 0.44, compound from peptide II R_F 0.50. Hence only the α -amino groups of diaminobutyric acid can have been free in the parent peptide.

These results all suggested that the γ -amino group of diaminobutyric acid was blocked in the nucleotide precursor itself. Treatment of the nucleotide precursor with fluorodinitrobenzene under the usual conditions produced no DNP derivative, whereas the corresponding compound from *S. aureus* H yielded a DNP-substituted product, from which ϵ -DNP-lysine was isolated. Similarly, the isolated cell walls of *C. insidiosum* and *C. sepedonicum* yielded hardly any DNP derivative when treated with fluorodinitrobenzene. Evidently there was indeed some substituent on the amino group of diaminobutyric acid not involved in peptide linkage. To determine the nature of the substituent, the nucleotide precursors from *C. insidiosum*, *C. poinsettiae* and *S. aureus* H were hydrolysed and their acetyl contents were determined by g.l.c. Whereas the latter two compounds contained only 1 molar proportion of acetyl (that present on the muramic acid, as shown by the fact that they gave the reaction for *N*-acetylhexosamine after removal of UDP by brief acid hydrolysis) the compound from *C. insidiosum* contained 2 molar proportions of acetyl. It therefore appeared that the γ -amino group of diaminobutyric acid was substituted by an acetyl group.

The ether extracts of the hydrolysates of all three nucleotides showed in addition to acetic acid another unidentified small peak (retention time relative to acetic acid 1.24), which was not present in hydrolysates of *N*-acetylglucosamine. The area of this peak was not more than 13% of that of the acetic acid peak.

Configuration of diaminobutyric acid. Analysis of the cell walls of *C. insidiosum* and *C. sepedonicum* showed that they contained an excess of diaminobutyric acid over glutamic acid (Table 2). Since the nucleotide precursor of *C. insidiosum* contained diaminobutyric acid with an acetylated γ -amino

Fig. 2. Optical rotatory dispersion of bis-DNP-diaminobutyric acid. —, Authentic L-compound. ----, Sample from the precursor nucleotide of *C. insidiosum*. - · - ·, Samples from the isolated cell walls of *C. insidiosum* or *C. sepedonicum*. The concentration was 49.5 μ M. The light-path was 10mm and the solvent was 0.125M NaHCO_3 .

group, it seemed possible that the cross-linking was effected by a second molecule of diaminobutyric acid, analogous to the D-ornithine cross-links found in *C. poinsettiae* (Perkins, 1967). If so, then the completed mucopeptide would be expected to have approximately equimolar proportions of D- and L-diaminobutyric acid, since that in the primary peptide chain should have the L-configuration like the homoserine in *C. poinsettiae* and the corresponding amino acids in other bacteria (Perkins, 1969a).

Diaminobutyric acid samples from hydrolysates of the cell walls of *C. insidiosum* and *C. sepedonicum* and the precursor nucleotide of *C. insidiosum* were converted into their bis-DNP derivatives and optical-rotatory-dispersion curves were prepared

(Fig. 2). From the rotation at 392nm the diaminobutyric acid from the hydrolysed precursor nucleotide was calculated to contain 88% of the L-isomer, whereas that from the cell-wall samples was almost optically inactive.

DISCUSSION

The relationship between cell-wall composition and antigenic structure of corynebacteria was studied by Cummins (1962), but as pointed out by Perkins & Cummins (1964) some of the plant-pathogenic species contained diamino acids other than lysine or diaminopimelic acid. It is now clear that *C. insidiosum* and *C. sepedonicum* also fall into this category, and that in *C. poinsettiae* (and presumably also in *C. betae* and *C. flaccumfaciens*; Perkins, 1965) the third amino acid of the peptide chain in the mucopeptide precursor is homoserine, cross-linking being mediated by D-ornithine. All these species therefore have mucopeptides belonging to Type IV of Ghuysen (1968). It is of note that *C. poinsettiae*, *C. insidiosum*, *C. tritici* and *C. sepedonicum* all fell within one group of greater than 80% similarity, according to a taxonomic survey by da Silva & Holt (1965), who did not include cell-wall components among the characters assessed.

The sequence of amino acid configuration in the primary peptide chain attached to the carboxyl group of muramic acid, wherever it has been determined, has been L- (or glycine), D-, L- [or the L-centre of diaminopimelic acid (Diringer & Jušić, 1966; Bricas *et al.* 1967) or β -hydroxydiaminopimelic acid (Perkins, 1969b)], D-, D-, L-Homoserine and L-diaminobutyric acid can now be added to the list of L-amino acids known to occur in the third position. Cross-linking in mucopeptides takes place by a transpeptidation reaction, in which the terminal D-alanine residue of the precursor pentapeptide is lost and the sub-terminal D-alanine residue becomes transferred to the appropriate accepting amino group (Ghuysen, 1968). The specificity of this reaction for the acceptor group is considerable, and it has been proposed that in structures such as that found in *C. insidiosum* acetylation of the L-diaminobutyric acid in the primary chain may be necessary to permit the transpeptidation to the D-diaminobutyric acid employed in cross-linking (Leyh-Bouille *et al.* 1970b). Such masking could be permanent, as in *C. insidiosum* where very few free amino groups are found in the isolated cell walls, or the acetyl groups could be removed at a later stage to yield free amino groups in the final structure.

The identification of one molar proportion of acetyl in the nucleotides of *C. poinsettiae* and *S. aureus* H conforms with the usual substituent for the amino group of muramic acid. A small peak of

other ether-extractable material was observed on g.l.c. but not identified with any simple volatile acid. It is noteworthy that in the cell walls of mycobacteria, and also in a partial nucleotide precursor accumulated by *Mycobacterium phlei* in the presence of cycloserine, N-acetylmuramic acid was replaced by N-glycolylmuramic acid (Petit, Adam, Wietzerbin-Falszpan, Lederer & Ghuysen, 1969; Petit, Adam & Wietzerbin-Falszpan, 1970), but evidently the plant-pathogenic corynebacteria are not exceptional in this respect.

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REFERENCES

- Allsop, J. & Work, E. (1963). *Biochem. J.* **87**, 512.
- Bricas, E., Ghuysen, J.-M. & Dezelée, P. (1967). *Biochemistry, Easton*, **6**, 2598.
- Chatterjee, A. N. & Perkins, H. R. (1966a). *Biochem. J.* **100**, 32 p.
- Chatterjee, A. N. & Perkins, H. R. (1966b). *Biochem. biophys. Res. Commun.* **24**, 489.
- Cummins, C. S. (1962). *J. gen. Microbiol.* **28**, 35.
- da Silva, G. A. N. & Holt, J. G. (1965). *J. Bact.* **90**, 921.
- Diringer, H. & Jušić, D. (1966). *Z. Naturf.* **21b**, 603.
- Ghuysen, J.-M. (1968). *Bact. Rev.* **32**, 425.
- Ghuysen, J.-M., Bricas, E., Leyh-Bouille, M., Lache, M. & Shockman, G. D. (1967). *Biochemistry, Easton*, **6**, 2607.
- Gorin, P. A. J. & Spencer, J. F. T. (1961). *Can. J. Chem.* **39**, 2274.
- Gorin, P. A. J. & Spencer, J. F. T. (1964). *Can. J. Chem.* **42**, 1230.
- Guinand, M., Ghuysen, J.-M., Schleifer, K. H. & Kandler, O. (1969). *Biochemistry, Easton*, **8**, 200.
- Ito, E. & Strominger, J. L. (1964). *J. biol. Chem.* **239**, 210.
- Levy, A. L. (1964). *Nature, Lond.*, **174**, 126.
- Leyh-Bouille, M., Bonaly, R., Ghuysen, J.-M., Tinelli, R. & Tipper, D. J. (1970a). *Biochemistry, Easton*, **9**, 2944.
- Leyh-Bouille, M., Ghuysen, J.-M., Bonaly, R., Nieto, M., Perkins, H. R., Schleifer, K. H. & Kandler, O. (1970b). *Biochemistry, Easton*, **9**, 2961.
- Miller, I., Plapp, R. & Kandler, O. (1966). *Biochem. biophys. Res. Commun.* **25**, 415.
- Muñoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J.-F., Heymann, H., Bricas, E. & Lefrancier, P. (1966). *Biochemistry, Easton*, **5**, 3748.
- Newton, G. G. F., & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
- Okuyama, T. & Satake, K. (1960). *J. Biochem., Tokyo*, **47**, 454.
- Perkins, H. R. (1965). *Biochem. J.* **97**, 3 c.
- Perkins, H. R. (1967). *Biochem. J.* **102**, 29 c.
- Perkins, H. R. (1968). *Biochem. J.* **110**, 49 p.
- Perkins, H. R. (1969a). *Adv. Pharmac. Chemother.* **7**, 293.
- Perkins, H. R. (1969b). *Biochem. J.* **115**, 797.
- Perkins, H. R. & Cummins, C. S. (1964). *Nature, Lond.*, **201**, 1105.
- Perkins, H. R. & Nieto, M. (1970). *Biochem. J.* **116**, 83.
- Petit, J.-F., Adam, A. & Wietzerbin-Falszpan, J. (1970). *FEBS Lett.* **6**, 55.

- Petit, J.-F., Adam, A., Wietzerbin-Falszpan, J., Lederer, E. & Ghuysen, J.-M. (1969). *Biochem. biophys. Res. Commun.* **35**, 478.
- Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
- Rogers, H. J. & Perkins, H. R. (1968). *Cell Walls and Membranes*, pp. 285-311. London: E. and F. N. Spon Ltd.
- Rosenthal, S. & Sharon, N. (1964). *Biachim. biophys. Acta*, **83**, 378.
- Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, **169**, 922.
- Schleifer, K. H., Plapp, R. & Kandler, O. (1968). *Biachim. biophys. Acta*, **154**, 573.
- Tipper, D. J. (1968). *Biochemistry, Easton*, **7**, 1441.
- Work, E. (1965). *Nature, Lond.*, **201**, 1107.