Enzymic Assays for Isomers of 2,6-Diaminopimelic Acid in Walls of *Bacillus cereus* and *Bacillus megaterium*

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An enzymic assay for individual isomers (*meso*-, LL- and DD-) of 2,6-diaminopimelate was developed. The enzyme 2,6-diaminopimelate decarboxylase specifically attacked *meso*-diaminopimelate and was used to measure this isomer manometrically. The *meso*- and LL-isomers were measured together manometrically in a coupled assay with diaminopimelate decarboxylase and diaminopimelate epimerase (which converts LL-diaminopimelate into *meso*-diaminopimelate). The DD-isomer was not attacked by either enzyme and was measured, as residual diaminopimelate after the coupled assay, by a colorimetric method, which was also used to measure total diaminopimelate before enzymic treatments. The coupled enzymes were also used to prepare pure DD-isomer from chemically synthesized diaminopimelate. A mixture of diaminopimelate isomers was present in walls of four strains of *Bacillus megaterium* [in each about 75% (w/w) *meso*-, 18% LL- and 7% DD-] and in walls of two strains of *Bacillus cereus* (about 85% *meso*-, 8% LL- and 7% DD-). One strain of *B. cereus* contained at least 95% *meso*-diaminopimelate, with only traces of LL- and DD-isomers. Peptidoglycan from *Escherichia coli* was assayed as containing at least 95% *meso*-isomer. The proportion of isomers in the wall of a strain of *B. megaterium* remained constant after growth in a variety of different media.

The amino acid 2,6-diaminopimelate is widespread among prokaryotes, being a precursor in the biosynthesis of lysine (Work, 1960) and also a unique component of many bacterial peptidoglycans (Schleifer & Kandler, 1972). Diaminopimelate contains two asymmetric centres and exists in three isomeric forms, the DD-, the LL- and the optically inactive *meso*-isomer. Early studies with qualitative methods which combined paper chromatography and enzymic attack showed that the *meso*- and LL-isomers were present in bacterial walls, and occasionally trace amounts of what was presumed to be DD-isomer were also detected (Hoare & Work, 1957). The presence of *meso*- or LL-diaminopimelate in bacterial walls is perhaps not surprising, as both isomers are intermediates in the biosynthesis of L-lysine (Work, 1960). However, the occurrence of DD-diaminopimelate has rarely been reported, and a biosynthetic route to this isomer is unknown.

Usually only a single isomer of diaminopimelate is present in walls of a given bacterial strain, and the *meso*-isomer is the most common. A mixture of isomers has occasionally been detected (Work, 1970). Walls of *Bacillus megaterium* are particularly interesting because the diaminopimelate-isomer com-

positions in different strains range from *meso*- plus LL- (Hoare & Work, 1957; White *et al.*, 1969), *meso*- plus DD- (Bricas *et al.*, 1967) and *meso*- alone (Pitel & Gilvarg, 1970). Wickus & Strominger (1972) showed that an enzyme system from *B. megaterium* QMB1551 could incorporate DD-diaminopimelate into a peptidoglycan precursor, probably as a specialized cross-bridge. Whether the DD-isomer was actually present in walls of this strain was not investigated. *Bacillus cereus*, which is similar to *B. megaterium* both morphologically and nutritionally (White, 1972), was reported to contain only the *meso*-isomer (Hoare & Work, 1957) and there may therefore be taxonomic significance in the distribution of diaminopimelate isomers. However, the proof of isomer configuration, from optical activity, has only been done with a few bacteria (Work, 1970), and this proof cannot be done on a mixture of isomers without preliminary resolution.

Most analyses of isomers of diaminopimelate have relied on chromatographic data alone, with the solvent system of Rhuland *et al.* (1955) used to separate the LL-isomer from *meso*- plus DD-, which run together. For the examination of a mixture of all isomers, the *meso*-/DD-isomers from the above system must be resolved further, e.g. as their di-Dnp* derivatives (Jušić *et al.*, 1963a). A two-stage

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* Abbreviation: Dnp-, 2,4-dinitrophenyl-.

chromatographic separation procedure to do this has been proposed (Jušić *et al.*, 1963a; Work, 1971), but seems to have been very rarely (if ever) used.

Such a system is time-consuming and not quantitative. Some disadvantages of chromatographic separation may be avoided by exploiting the stereospecificity of enzymes. Purified diaminopimelate decarboxylase (EC 4.1.1.20; *meso*-2,6-diaminopimelate carboxy-lyase) is specific for the *meso*-isomer (White & Kelly, 1965) and diaminopimelate epimerase (EC 5.1.1.7; 2,6-LL-diaminopimelate 2-epimerase) interconverts the LL- and *meso*-isomers (White *et al.*, 1969). Neither enzyme attacks the DD-isomer. The need for a quantitative assay of diaminopimelate isomers prompted investigation of the best conditions for using diaminopimelate decarboxylase and diaminopimelate epimerase. Isomers of diaminopimelate in walls of *B. megaterium* and *B. cereus* were examined in detail because of the taxonomic relatedness of these organisms and the conflicting results on the isomer composition of the former species. A preliminary report of this work has appeared (Day & White, 1973).

Methods

Organisms

Bacillus megaterium strains 7581, 8291, 9376 and KM, and *Bacillus cereus* strains 6349, 7464, 9373 and 9373AI and their origins were described by White (1972). *Escherichia coli* A.T.C.C. 9637 was the strain used by White & Kelly (1965) in the purification of diaminopimelate decarboxylase. All organisms were maintained on slopes of nutrient-agar (Oxoid, Basingstoke, Hants., U.K.) which were incubated at 37°C for 8–16h and then kept at 2°C, with monthly transfer.

Media

All media were made in glass-distilled water. Strains of *B. megaterium* and *B. cereus* were grown at 37°C in minimal media A1 or A2 (White, 1972), containing nutritional supplements for some strains (quantities are mg/l): for strain 7581, biotin (0.02); for strain 9376, L-threonine (60) and L-valine (60); for strains 9373 and 6349, glycine (50) and L-methionine (50); for strain 7464, DL-aspartic acid (100), DL-glutamic acid (240), DL-histidine (60), glycine (50) and L-methionine (50). *E. coli* was grown at 37°C in the medium described by Davis & Mingioli (1950), modified by autoclaving the glucose in the medium at 115°C for 7 min. For 10-litre batches of medium the glucose was autoclaved separately.

Growth conditions

Organisms were usually grown in 2-litre conical flasks on a rotary shaker at about 200 gyrations/min

(model VS Gyro-rotary shaker; New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.). For *E. coli*, each flask (250 ml of medium) was inoculated with 0.5 ml of a suspension in sterile water of organisms (about 0.5 mg dry wt/ml) from an overnight culture on nutrient-agar. *B. megaterium* and *B. cereus* were similarly grown in flasks containing 500 ml of medium, and each flask was inoculated with 1.0 ml of suspension in sterile water of organisms (about 2 mg dry wt/ml) from a nutrient-agar slope that had been incubated for about 8 h at 37°C. Occasionally larger quantities of organisms (10–20 litres of medium) were grown in a fermenter (Micro-ferm; New Brunswick Scientific Co.) under conditions of vigorous aeration (3 litres/min) and stirring (150 rev./min). Growth of cultures was assessed from turbidity measured in 6 mm-diameter tubes in an EEL photoelectric colorimeter (Evans Electroselenium Co., Halstead, Essex, U.K.) with neutral-density filter (no. 1.0) and water for zero setting.

Preparation of walls

Walls of *Bacillus* species were prepared from organisms harvested usually from defined medium, and always in late-exponential phase. In no case were any spores seen by phase-contrast microscopy. All manipulations were done at 2°C unless otherwise stated. The organisms were washed by centrifuging and were resuspended in 0.1 M-sodium phosphate buffer, pH 6.8, to give about 50 mg dry wt. of organisms/ml, and were then broken by passage at 1.4×10^8 Pa through a pressure cell of the type described by Milner *et al.* (1950). Debris was recovered by centrifuging at 20000 g for 30 min and the supernatant liquid was decanted. The upper layer of the pellet was loose and contained mainly broken organisms (crude walls), which were scraped off and resuspended in water. This suspension was heated at 60°C for 10 min to inactivate lytic enzymes. Unbroken organisms were removed from this suspension by centrifuging (three times) at low speed (2000 g, 5 min). The pellet was discarded each time to leave a suspension containing crude walls.

The wall material was recovered by centrifuging, and was resuspended in 100 mM-Tris/HCl buffer, pH 7.8, containing crystalline trypsin (100 µg/ml; from bovine pancreas; BDH Chemicals, Poole, Dorset, U.K.) to give about 10 mg dry wt. of walls/ml of buffer. The suspension was incubated at 37°C with slow shaking for 4 h. Wall material was isolated by centrifuging (10000 g; 10 min) and washed (volumes same as with Tris/HCl buffer above) with 1.0 M-NaCl (×2) and then water (×4). The walls were freeze-dried from water and finally dried under vacuum over CaCl₂.

Peptidoglycan from *E. coli* was prepared by a procedure modified from that of Schocher *et al.*

(1962). Organisms grown in minimal medium were harvested in late-exponential phase, washed by centrifuging (5000 *g* for 10 min), and broken in the pressure cell as described above. Debris was recovered by centrifuging at 20000 *g* for 30 min and the supernatant material discarded. The pellet was resuspended in water, heated at 60°C for 10 min, and whole organisms were removed by repeated centrifuging (three times) at 4000 *g* for 5 min. Crude walls in the supernatant liquid were obtained by centrifuging (12000 *g*, 10 min) and washed in 1.0M-NaCl ($\times 2$), then water ($\times 4$).

The wall material was resuspended in 100mM-Tris/HCl buffer, pH 7.8 (containing 1mM-MnCl₂), with deoxyribonuclease and ribonuclease (each 50 μ g/ml) and incubated at 37°C for 4 h. Deoxyribonuclease (bovine pancreas) was from BDH; ribonuclease (bovine pancreas) was from Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K. Trypsin (100 μ g/ml) was then added, with a few drops of toluene, and the incubation continued overnight. Walls were recovered by centrifuging and the sedimented material was resuspended in 10% (w/v) sodium dodecyl sulphate. The suspension was shaken overnight at 37°C and the wall material was isolated by centrifuging, washed six times with water and freeze-dried.

Preparation of enzymes

Diaminopimelate decarboxylase was purified from extracts of *E. coli* A.T.C.C. 9637 as described by White & Kelly (1965). Purification was usually taken only as far as precipitation with (NH₄)₂SO₄, followed by dialysis, and then enzyme was stored at -15°C until required. To achieve satisfactory co-precipitation of nucleic acids and diaminopimelate epimerase (which would interfere with enzymic assays of *meso*-diaminopimelate; see the Results section) it was necessary to prepare an extract with the pressure cell operated at not greater than 0.7×10^8 Pa. When a higher pressure was used, nucleic acids and diaminopimelate epimerase were more difficult to precipitate with protamine plus streptomycin.

Diaminopimelate epimerase was purified from extracts of *Bacillus megaterium* as described by White *et al.* (1969). Purification was normally taken as far as the second (NH₄)₂SO₄ fractionation, followed by dialysis and storage of the enzyme at -15°C until required. Enzymic activities were measured manometrically (and expressed in manometric units) as described for diaminopimelate decarboxylase by White and Kelly (1965) and for diaminopimelate epimerase by White *et al.* (1969).

Hydrolysis of walls

For the analysis of amino acids, walls were hydrolysed in 6M-HCl at 105°C for 18 h. For analysis of

amino sugars, wall was hydrolysed in 4M-HCl at 105°C for 4 h. After heating, the hydrolysates were filtered through Whatman no. 1 paper, which was then rinsed with water, and the filtrate dried under vacuum over CaCl₂ and NaOH pellets. Each hydrolysate was resuspended in water at least twice and the drying was repeated to remove HCl.

Chemical assays

Hexosamines were measured by the method of Levy & McAllan (1959). Protein was measured by the method of Lowry *et al.* (1951) in terms of a standard bovine albumin powder (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.).

Characterization of diaminopimelate

Diaminopimelate concentrations were measured by the colorimetric method of Work (1957), in which excess of lysine interferes very little. A calibration curve was prepared by using pure *meso*-diaminopimelate (see below).

The proportions of isomers of diaminopimelate were determined by enzymic methods. *Meso*-Diaminopimelate was assayed from the CO₂ evolved during incubation with purified diaminopimelate decarboxylase, which specifically converts *meso*-diaminopimelate into L-lysine plus CO₂. Residual diaminopimelate after this assay was either the LL-isomer or the DD-isomer or both. A coupled assay with diaminopimelate epimerase and diaminopimelate decarboxylase was used to measure LL- and *meso*-diaminopimelate together; both isomers were converted into L-lysine plus CO₂. Any residual diaminopimelate after this assay was the DD-isomer. The amount of LL-isomer was the difference between the measurement of *meso*-isomer alone and the measurement of *meso*- plus LL-isomer in the coupled assay.

These enzymic assays were done manometrically in the Warburg apparatus at 37°C. Reagents for the assays were dissolved in 0.1M-sodium phosphate buffer, pH 6.8 (made in glass-distilled water). The main compartment of the Warburg flask contained the enzyme preparation in buffer, 10 μ g of pyridoxal phosphate and 1mM-2,3-dimercaptopropan-1-ol. One side arm of the flask contained diaminopimelate as substrate. The total volume of reaction mixture was 2.5 ml. A second side arm contained 0.1 ml of 1M-citric acid (in water), which was tipped into the reaction mixture at the end of enzymic evolution of CO₂ to determine the total CO₂ evolved, since at the assay pH value there is considerable retention of CO₂ in solution. Any diaminopimelate remaining in the incubation mixture was measured by the colorimetric assay after prior removal of protein from the mixture (1 ml) by adding 1 ml of aqueous trichloroacetic acid (10%, w/v). After 5 min the

precipitate was removed by centrifuging (10000 g; 5 min). Trichloroacetic acid did not interfere in the subsequent assay for diaminopimelate in the supernatant liquid.

Diaminopimelate (1.0–2.5 mg per Warburg flask) was assayed for *meso*-isomer with purified diaminopimelate decarboxylase (0.08–0.12 manometric unit per flask; about 0.1 mg of protein). For assay of *meso*- plus LL-isomers, diaminopimelate epimerase was used (0.09–0.12 manometric unit per Warburg flask; about 0.15 mg of protein) together with at least 0.3 unit of diaminopimelate decarboxylase (about 0.3 mg of protein). Whenever possible, deproteinized incubation mixtures were examined by paper chromatography (in solvent I, below) as a qualitative check of the assays.

A mixture of pure isomers of diaminopimelate could be assayed directly by enzymic methods, but that from hydrolysed walls had first to be freed of unknown inhibitors of the enzymes by elution from a column of Dowex 50. The hydrolysate from about 60 mg dry wt. of wall was dissolved in water (5 ml) and adsorbed on the resin (Bio-Rad AG 50W, X8; H⁺ form; 6 ml bed volume). The column was then washed with water (40 ml) and eluted with 1 M-HCl (50 ml) followed by 1 M-NH₃. Alanine and glutamate were eluted with the HCl, and diaminopimelate appeared in the NH₃ eluate. Fractions containing diaminopimelate, detected by the colorimetric assay or by chromatography, were pooled and dried on a rotary evaporator. There was no separation on diaminopimelate isomers by the column.

Chromatography of diaminopimelate and its derivatives

Free diaminopimelate was separated by descending chromatography on paper (Whatman no. 1) with the solvent (I) described by Rhuland *et al.* (1955) (methanol/water/10 M-HCl/pyridine, 32:7:1:4, by vol.). In this system LL-diaminopimelate has a higher mobility than either the *meso*- or DD-isomers, which run almost together (DD-diaminopimelate runs very slightly behind the *meso*-isomer). Amino acids were detected by dipping chromatograms in ninhydrin (0.2%, w/v, in acetone) and heating at 95°C for 5 min. Solvent II, described by Perkins (1965) [methanol/water/pyridine/98% (w/v) formic acid, 80:19:10:1, by vol.], was used when diaminopimelate was subsequently to be eluted from chromatograms, so as to avoid eluting pyridine hydrochloride, which interfered in further manipulations.

Dinitrophenyl derivatives of diaminopimelate were separated on thin-layer plates of silica gel G (E. Merck Laboratory Chemicals, Darmstadt, Germany) in solvent III, described by Bricas *et al.* (1967) (benzyl alcohol/chloroform/methanol/water/15 M-NH₃, 15:15:15:3:1, by vol.). The di-Dnp

derivative of *meso*-diaminopimelate travels more slowly than the di-Dnp derivatives of LL- and DD-diaminopimelate, which migrate together in this system. Dinitrophenyl derivatives of authentic isomers of diaminopimelate were prepared by the methods of Jušić *et al.* (1963a,b).

Characterization of DD-diaminopimelate from walls

The source of DD-diaminopimelate from walls of *B. megaterium* 7581 was the diaminopimelate remaining after the conversion of the *meso*- and LL-isomers into L-lysine in the coupled assay.

For determination of its optical activity, a total of 7.5 μmol of wall DD-diaminopimelate was used as starting material. The incubation mixtures from several coupled assays were deproteinized and then eluted from a Dowex 50 column (H⁺ form, 5 ml bed volume) with pyridine/acetic acid buffer, pH 6.5 (0.617 M-pyridine in water containing 70 mM-acetic acid) and fractions containing diaminopimelate were pooled and dried on a rotary evaporator. Traces of contaminating lysine in the diaminopimelate were separated by chromatography on water-washed paper with solvent II, and diaminopimelate was eluted with water. The di-Dnp derivative was prepared, as described by Perkins (1969), and separated by t.l.c. (solvent III). The di-Dnp-DD-diaminopimelate was eluted (acetone/10 mM-NH₃, 1:1, v/v), dried and finally redissolved in 1 ml of Na₂CO₃ (10%, w/v), which was extracted with 2 × 0.5 ml of ether. The Na₂CO₃ solution was then acidified to pH 2 with 2 M-HCl and extracted with 2 × 0.5 ml of ether. Light petroleum (b.p. 40–60°C) was added dropwise to the ether extract until it became faintly turbid, and the solution was left at 2°C overnight. The precipitate was collected by centrifuging (10000 g; 5 min) and the optical activity measured in acetic acid (Jušić *et al.*, 1963b).

Preparation of diaminopimelate isomers

(1) *meso*- and LL-isomers. Diaminopimelate obtained from cultures of mutants of *E. coli* that require lysine for growth ('fermentation diaminopimelate') contains a mixture of *meso*- and LL-isomers only (Work, 1963). Such diaminopimelate was used as a convenient starting material to isolate pure samples of *meso*- and LL-diaminopimelate, by a fractional crystallization procedure (White *et al.*, 1969). The *meso*- and LL-isomers so isolated were at least 95% pure as judged by the enzymic analyses described above.

(2) DD-Isomer. Diaminopimelate was synthesized chemically as described by Saleh & White (1976). This material consists of 50% (w/w) *meso*-, 25% LL- and 25% DD-diaminopimelate isomers (assayed enzymically). *meso*-Diaminopimelate was first partly

removed from the isomer mixture by fractional crystallization, to leave a material rich in LL- and DD-isomers. Then LL-diaminopimelate (and any remaining *meso*-isomer) was converted into L-lysine with diaminopimelate epimerase and diaminopimelate decarboxylase, but the DD-isomer was unchanged. The mixture of L-lysine and DD-diaminopimelate was separated by ion-exchange chromatography on Amberlite IR-120 resin (H^+ form) eluted with pyridine/acetate buffer, pH 6.5, and the DD-isomer was isolated by crystallization.

The yield was 706 mg of DD-diaminopimelate ($\frac{1}{2}H_2O$), from 2.75 g of a mixture of LL- (45%, w/w), DD- (45%) and *meso*- (10%) isomers. The material had an $[\alpha]_D^{24}$ of -8.6° (in water). Work (1963) gave a $[\alpha]_D^{24}$ for DD-diaminopimelate of -8.45° (in water). The elementary analysis was determined. Found: C, 42.4 (42.2); H, 7.4 (7.6); N, 13.7 (14.1); values in parentheses are for DD-diaminopimelate, $\frac{1}{2}H_2O$. This diaminopimelate and its di-Dnp derivative had the expected R_f values (in solvents I and III respectively) of the DD-isomers.

Results

Conditions for quantitative assay of diaminopimelate isomers

The principle was to measure the total concentration of diaminopimelate in a sample by colorimetric assay, and then to assay for the different isomers enzymically. Diaminopimelate decarboxylase alone was used to measure *meso*-diaminopimelate; any diaminopimelate remaining after this assay should be LL- or DD-isomer, or both. A coupled assay using diaminopimelate epimerase plus diaminopimelate decarboxylase was used to measure *meso*- plus LL-diaminopimelate; any remaining should be the DD-isomer. The concentrations of substrate and enzyme at which the enzymic assays were fully quantitative were investigated.

The extent of conversion of a fixed amount of substrate with different concentrations of enzyme is shown in Fig. 1. The extent of reaction with a constant amount of enzyme but with different amounts of substrate is shown in Fig. 2. These results established the conditions used as a routine for the quantitative assays of *meso*- and LL-diaminopimelate. *Meso*-Diaminopimelate (1.0–2.5 mg) was assayed from total CO_2 evolved after incubation with diaminopimelate decarboxylase (0.08–0.12 unit). LL-Diaminopimelate (1.0–2.5 mg) was assayed from total CO_2 evolved in the coupled assay with diaminopimelate epimerase (0.09–0.12 unit). For the assay of the *meso*-isomer the s.e.m. was 1.2%, based on six replicate assays, and in the assay of the LL-isomer the s.e.m. was 1.9%, also based on six replicate assays.

The precision of the assay was investigated

Fig. 1. Extent of decarboxylation of *meso*- or LL-diaminopimelate with different amounts of diaminopimelate decarboxylase and diaminopimelate epimerase

Conversion of diaminopimelate into lysine was measured manometrically at $37^\circ C$ from the CO_2 released in enzymic assays (see the Methods section). Citric acid was tipped (to measure dissolved CO_2) when the enzymic reaction had stopped. O, Constant amount of *meso*-diaminopimelate (2.0 mg/flask) and a variable amount of diaminopimelate decarboxylase; ●, constant amount of LL-diaminopimelate (2.5 mg/flask) and a variable amount of diaminopimelate epimerase (together with a threefold excess of diaminopimelate decarboxylase).

Fig. 2. Extent of enzymic decarboxylation of *meso*- or LL-diaminopimelate at different concentrations of either substrate

Procedure was as in Fig. 1. O, Constant amount of diaminopimelate decarboxylase (0.1 unit/flask) and a variable concentration of *meso*-diaminopimelate; ●, constant amount of diaminopimelate epimerase (0.095 unit/flask) plus diaminopimelate decarboxylase (0.3 unit/flask) and a variable concentration of LL-diaminopimelate.

Table 1. *Enzymic and colorimetric assays of standard mixtures of diaminopimelate isomers*

Standard mixtures of authentic diaminopimelate isomers were assayed by using a combination of enzymic and colorimetric methods. *meso*-Diaminopimelate and *meso*- plus LL-diaminopimelate were assayed manometrically with diaminopimelate decarboxylase and with diaminopimelate epimerase plus diaminopimelate decarboxylase respectively. The residual diaminopimelate after enzymic incubations was measured colorimetrically with acidic ninhydrin. It was present as the LL- and/or DD-isomer after decarboxylase treatment and the DD-isomer after treatment with decarboxylase plus epimerase. All results are the means of at least two determinations. A₂pm, diaminopimelate.

Isomer and % (w/w) of total A ₂ pm in standard mixture	A ₂ pm isomers detected [as % (w/w) of total]					
	After decarboxylase treatment		After decarboxylase plus epimerase treatment			
	Manometric assay*	Colorimetric assay†	Manometric assay*	Colorimetric assay†		
<i>meso</i> - 100	98.5	}	}	}		
LL- 0	1.5				Not done	Not done
DD- 0						
<i>meso</i> - 95	95.5	}	}	}		
LL- 5	5.5				103	1.0
DD- 0						
<i>meso</i> - 80	81	}	}	}		
LL- 10	19.5				87	12
DD- 10						
<i>meso</i> - 90	88.5	}	}	}		
LL- 5	12				94	6.5
DD- 5						

* From total CO₂ evolved in the assay.

† The residual diaminopimelate after enzymic incubation.

with mixtures of the authentic isomers (Table 1). These mixtures were made up to contain a high proportion of the *meso*-isomer, because this was expected to be the dominant isomer in bacterial walls (Hoare & Work, 1957). Close agreement was observed between the measured and actual compositions of the mixtures, with both manometry and colorimetric assays of residual diaminopimelate after enzymic incubations. These two measurements usually agreed within 1–2% of the theoretical, and were a useful check on the assay. However, the colorimetric assay tended to record an apparent residual low concentration of diaminopimelate from assays in which the diaminopimelate had been entirely converted into lysine, as judged by manometry and chromatography. Lysine with acidic ninhydrin does produce a little colour in the assay (Work, 1957). Interference by lysine was only slight and corrections were not usually made, so that DD-diaminopimelate may be overestimated in walls. The apparent presence of very small amounts of residual diaminopimelate (less than 2% of the total) after enzymic incubations was always regarded with suspicion and was checked by chromatography.

Amino acids and diaminopimelate isomers in walls of B. megaterium and B. cereus

The amino acids and isomers of diaminopimelate found in walls of some strains of *B. megaterium* and

B. cereus are shown in Table 2. The amino acid analyser did not give estimates of glycine and serine, which had retention times on the ion-exchange column similar to those of glucosamine and muramic acid. However, significant quantities of these two amino acids were not detected by paper chromatography. The overall amino acid compositions of both species of *Bacillus* were similar, with the typical peptidoglycan amino acids alanine, glutamate and diaminopimelate predominating. In every case except *B. cereus* 7464, the molar ratio of diaminopimelate/glutamate was greater than 1. Walls of *B. megaterium* contained about 75% *meso*-, 18% LL- and 7% DD-diaminopimelate (w/w), whereas strains of *B. cereus* contained proportionally more *meso*-diaminopimelate and less LL-isomer. Three of the four strains of *B. cereus* contained about 85% *meso*-, 8% LL- and 7% (w/w) DD-diaminopimelate, but the other (strain 7464) contained at least 95% *meso*-isomer. The amounts of the LL- and DD-isomers in this wall preparation were too small to measure by the enzymic method, although both isomers were detected by paper chromatography after the *meso*-diaminopimelate assay. Strain *B. cereus* 7464 is more fastidious in its nutritional requirements than are the other strains examined (White, 1972).

The effect of a change in the composition of the growth medium on wall diaminopimelate isomers was investigated with *B. megaterium* 7581 (Table 3). The

Table 2. *Amino acids, hexosamine and diaminopimelate isomers in walls of some strains of B. megaterium and B. cereus*
 The amino acids and diaminopimelate isomers were measured in acid hydrolysates of wall material isolated from organisms grown in their respective minimal media (see the Methods section). Amino acids were determined with an automatic analyser. Diaminopimelate isomers were measured by using the enzymic and colorimetric methods (Table 1), supplemented with examination by paper chromatography (solvent I), before and after enzymic incubations. The colorimetric assay was also used to measure total diaminopimelate before enzymic incubations. n.d., Not detected. A₂pm, diaminopimelate.

	Amino acid concentration ($\mu\text{mol/g}$ dry wt. of wall) in:							
	Strains of <i>Bacillus megaterium</i>				Strains of <i>Bacillus cereus</i>			
	7581	KM	9376	8241	9373	9373AI	6349	7464
Alanine	974	831	771	1120	758	780	606	478
Glutamate	481	409	414	582	424	434	305	411
Diaminopimelate	574	522	500	805	507	515	400	373
Aspartate	27	23	19	13	44	48	38	40
Valine	n.d.	70	78	n.d.	98	116	22	55
Isoleucine	n.d.	25	19	n.d.	35	41	n.d.	34
Phenylalanine	n.d.	18	n.d.	n.d.	16	24	n.d.	n.d.
Threonine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12	25
Hexosamine (total)	987	973	1040	1280	1275	1330	1062	775
A ₂ pm isomers, as % (w/w) of total A ₂ pm								
<i>meso</i> -	75	76	74	77	85	85	84	95
LL-	18	17	19	17	8	8	8	5*
DD-	7	8	7	7	7	7.5	8	

* The amount of diaminopimelate that was not *meso*-isomer in strain 7464 was too small to establish the proportions of LL- and DD-isomers. Both of these isomers were detected by paper chromatography.

Table 3. *Diaminopimelate isomers in walls of Bacillus megaterium* 7581 after growth in different media

Walls were isolated from *B. megaterium* 7581 after growth in different media, and the diaminopimelate isomer content was examined enzymically as in Table 1. Medium A1 contained glycerol (1%, w/v) as carbon source and medium A2 contained glucose (1%, w/v). Biotin (20 $\mu\text{g/litre}$) was added to these minimal media for strain 7581.

Growth medium	Diaminopimelate isomer (% w/w)		
	<i>meso</i> -	LL-	DD-
A1	75	18	7
A2	76	17	7
A1+L-lysine (100mg/l)	75	18	7
Nutrient broth	75	17	8

proportion of isomers remained the same after growth in minimal medium with a change of carbon source or with a supplement of L-lysine, which might have been expected to exert a regulatory effect on the synthesis of diaminopimelate (Grandgenett & Stahly, 1971). Growth in a complex medium did not alter the proportion of wall diaminopimelate isomers, although this medium would presumably not contain diaminopimelate. The effect of a supplement of diaminopimelate was not examined, because at the time when these experiments were done it was thought that *B. megaterium* would be relatively

impermeable to it, as is *Escherichia coli* (Davis, 1952; Leive & Davis, 1965).

Evidence for the presence of DD-diaminopimelate

The presence of *meso*- and LL-diaminopimelate in bacterial wall preparations is not unusual (Schleifer & Kandler, 1972), and in the present work the evidence for identity was based on both enzymic and chromatographic data. The finding in walls of *B. megaterium* and *B. cereus* of DD-diaminopimelate (for which no biosynthetic route has been described) was more unexpected. Confirmation of identity of this isomer, other than its resistance to attack by diaminopimelate decarboxylase plus diaminopimelate epimerase, and chromatographic mobility in solvent I (where it co-chromatographs with the *meso*-isomer), was therefore sought. The properties of the di-Dnp derivative of wall DD-diaminopimelate were examined (Table 4). This derivative can be separated from the *meso*- derivative by t.l.c. (Bricas *et al.*, 1967). Derivative formation has the further advantage of enhancing optical rotation, so that measurements can be made on smaller amounts of sample (Rao & Sober, 1954). The di-Dnp derivative of residual diaminopimelate after the coupled enzymic assay had the chromatographic mobility of the DD-isomer and not the *meso*-isomer. The di-Dnp derivative of *meso*-diaminopimelate is internally compensated and has no optical activity.

Table 4. Evidence for the identification of DD-diaminopimelate from walls of *Bacillus megaterium* 7581

The DD-diaminopimelate from *B. megaterium* after enzymic attack in the coupled assay was characterized to check its authenticity. The diaminopimelate was first separated from L-lysine and then converted into its di-Dnp derivative with fluorodinitrobenzene reagent. The chromatographic properties of wall di-Dnp-DD-diaminopimelate were examined on thin-layer plates in solvent III. The optical rotation of wall di-Dnp-DD-diaminopimelate was measured at a concentration of about 0.2% (w/v) in acetic acid (Jušić *et al.*, 1963b). This concentration was determined from a graph of concentration of authentic di-Dnp-DD-diaminopimelate (in acetic acid) against A_{360} . A₂pm, diamino pimelate.

	Properties of di-Dnp derivatives of A ₂ pm	
	$R_{Dnp-A_{11a}}$	Specific optical rotation $[\alpha]_D^{22-24}$
Authentic di-Dnp-meso-A ₂ pm	0.73	0
Authentic di-Dnp-DD-A ₂ pm	0.78	+46.2
<i>B. megaterium</i> di-Dnp-DD-A ₂ pm	0.78	+44.1

Although there is no published value for the specific rotation of synthetic di-Dnp-DD-diaminopimelate, the value for di-Dnp-LL-diaminopimelate is $-85 \pm 5^\circ$ (Jušić *et al.*, 1963b) and therefore the DD-diaminopimelate derivative has the expected opposite sign of rotation.

Characterization of diaminopimelate in peptidoglycan from *E. coli*

Although LL- and DD-isomers were never present in major proportions in acid hydrolysates of walls, the possibility that they arose wholly from acid-induced racemization of meso-diaminopimelate seems unlikely. The hydrolysis conditions did not produce detectable racemization of a single isomer of free diaminopimelate, but racemization might occur during the hydrolysis of peptide bonds. Peptides containing diaminopimelate of a known configuration were not available to check this possibility; instead a compromise was made by examining the hydrolysis products of peptidoglycan of *E. coli*, which is reported to contain only the meso-isomer (Diringer & Jušić, 1966). By the enzymic methods, this material was assayed as at least 95% meso-diaminopimelate, although trace amounts of LL- and DD-isomers (not more than 5% of the total) were detected by paper chromatography. Such trace amounts of these isomers may have arisen from meso-diaminopimelate by racemization during hydrolysis, but this extent of racemization cannot account for the proportion of isomers found in strains of *B. megaterium*.

Discussion

The use of stereospecific enzymes has several advantages over the earlier methods for assigning a configuration to diaminopimelate isomers, particularly in the quantitative analysis of mixtures of isomers, and the fact that no preliminary resolution or derivative formation is required. The finding of all three isomers of diaminopimelate in hydrolysates of walls of *B. megaterium* 7581 was not altogether surprising. The LL-isomer has previously been detected in this strain by chromatography (Hoare & Work, 1957; White *et al.*, 1969) and the amount of the DD-isomer was possibly below the sensitivity limits of the qualitative enzymic method of Hoare & Work (1957). Lack of sensitivity may also account for reports of meso-isomer only in *B. cereus* (Schleifer & Kandler, 1972). One strain (7464) of *B. cereus* contained at least 95% meso-isomer, which is close to previous reports of meso- only, but the other strains of *B. cereus* contained only about 85% meso-isomer. This is the first report of a mixture of diaminopimelate isomers in the walls of *B. cereus*. The range of isomers reported in the present work is not altogether similar to other findings, particularly in *B. megaterium*, where two groups of workers have failed to detect the LL-isomer (Bricas *et al.*, 1967; Pitel & Gilvarg, 1970). This may be an indication of strain differences, although *B. megaterium* KM was used by both Bricas *et al.* (1967) and ourselves; also the four strains of *B. megaterium* examined in the present work, collected from different sources (White, 1972), had similar compositions. The isomer composition in one strain was constant after growth in four different media and so cultural conditions probably do not explain the range in reported values. This constancy of proportions also argues against there being a mixture of three different strains (each having a different isomer of diaminopimelate in its wall) present in one supposedly pure culture.

The presence of more than one isomer of any diamino acid in the same wall is not a common occurrence in bacteria (Work, 1970). However, certain *Corynebacterium* species have a mixture of L- and D-2,4-diaminobutyrate in their walls (Perkins, 1968; Fiedler & Kandler, 1973) and *Butyribacterium rettgeri* contains L- and D-ornithine (Guinand *et al.*, 1969). Walls of *Actinomyces* contain a mixture of diamino acids more frequently than do those of any other types of organism. Different amounts of the three diaminopimelate isomers have been detected, together with α -hydroxydiaminopimelate (Hoare & Work, 1957; Yamaguchi, 1965; Perkins, 1969). Although meso-diaminopimelate, and to a lesser extent the LL-isomer, may occur alone as the only diamino acid in walls, the DD-isomer only occurs as a small proportion, accompanied by another

isomer of diaminopimelate in larger proportion. Reports of the presence of DD-diaminopimelate are rare and so far have been confined to *Micromonospora* (Hoare & Work, 1957), *Actinomyces* (Hoare & Work, 1957; Perkins, 1969) and *B. megaterium* KM (Bricas *et al.*, 1967). It is notable that a minor amount of LL-diaminopimelate in addition to the *meso*-isomer has been recorded for *B. megaterium* KM (Salton, 1957). The present work shows that DD-diaminopimelate may commonly occur in hydrolysates of walls of *B. megaterium* and *B. cereus*. Certainly the absence of DD-isomer should not be assumed without rigid proofs. However, it is by no means clear whether DD-diaminopimelate arises wholly or in part from acid-induced racemization of other diaminopimelate isomers during hydrolysis. Examination of *E. coli* peptidoglycan, in which the diaminopimelate has been firmly identified as the *meso*-isomer by optical studies (Diringer & Jušić, 1966), showed the diaminopimelate to be at least 95% *meso*-isomer, but with a small amount of the other two isomers. Peptidoglycan of *E. coli* and *B. megaterium* is structurally similar (van Heijenoort *et al.*, 1969) and it appears therefore that racemization during hydrolysis alone cannot account for the proportion of isomers detected in some strains of *B. megaterium* and *B. cereus*.

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References

- Bricas, E., Ghuysen, J.-M. & Dezelée, P. (1967) *Biochemistry* **6**, 2598–2607
- Davis, B. D. (1952) *Nature (London)* **169**, 534–536
- Davis, B. D. & Mingioli, E. S. (1950) *J. Bacteriol.* **60**, 17–28
- Day, A. & White, P. J. (1973) *J. Gen. Microbiol.* **77**, xix
- Diringer, H. & Jušić, D. (1966) *Z. Naturforsch. Teil B* **21**, 603–604
- Fiedler, F. & Kandler, O. (1973) *Arch. Mikrobiol.* **89**, 51–66
- Grandgenett, D. P. & Stahly, D. P. (1971) *J. Bacteriol.* **105**, 1211–1212
- Guinand, M., Ghuysen, J.-M., Schleifer, K. H. & Kandler, O. (1969) *Biochemistry* **8**, 200–207
- Hoare, D. S. & Work, E. (1957) *Biochem. J.* **65**, 441–447
- Jušić, D., Roy, C., Schocher, A. J. & Watson, R. W. (1963a) *Can. J. Biochem. Physiol.* **41**, 817–820
- Jušić, D., Roy, C., Schocher, A. J., Watson, R. W. (1963b) *Can. J. Chem.* **41**, 2715–2718
- Leive, L. & Davis, B. D. (1965) *J. Biol. Chem.* **240**, 4362–4369
- Levy, G. A. & McAllan, A. (1959) *Biochem. J.* **73**, 127–132
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Milner, H. W., Lawrence, N. S. & French, C. S. (1950) *Science* **111**, 633–634
- Perkins, H. R. (1965) *Nature (London)* **208**, 872–873
- Perkins, H. R. (1968) *Biochem. J.* **110**, 47P–48P
- Perkins, H. R. (1969) *Biochem. J.* **115**, 797–805
- Pitel, D. W. & Gilvarg, C. (1970) *J. Biol. Chem.* **245**, 6711–6717
- Rao, K. R. & Sober, H. A. (1954) *J. Am. Chem. Soc.* **76**, 1328–1331
- Rhuland, L. E., Work, E., Denman, R. F. & Hoare, D. S. (1955) *J. Am. Chem. Soc.* **77**, 4844–4846
- Saleh, F. & White, P. J. (1976) *J. Gen. Microbiol.* **96**, 253–261
- Salton, M. R. J. (1957) *Nature (London)* **180**, 338–339
- Schleifer, K. H. & Kandler, O. (1972) *Bacteriol. Rev.* **36**, 407–477
- Schocher, A. J., Bayley, S. T. & Watson, R. W. (1962) *Can. J. Microbiol.* **8**, 89–98
- van Heijenoort, J., Elbaz, L., Dezelée, P., Petit, J.-F., Bricas, E. & Ghuysen, J.-M. (1969) *Biochemistry* **8**, 207–213
- White, P. J. (1972) *J. Gen. Microbiol.* **71**, 505–514
- White, P. J. & Kelly, B. (1965) *Biochem. J.* **96**, 75–84
- White, P. J., Lejeune, B. & Work, E. (1969) *Biochem. J.* **113**, 589–601
- Wickus, G. G. & Strominger, J. L. (1972) *J. Biol. Chem.* **247**, 5297–5306
- Work, E. (1957) *Biochem. J.* **67**, 416–423
- Work, E. (1960) *Colloq. Int. C. N. R. S.* **92**, 143–169
- Work, E. (1963) *Methods Enzymol.* **6**, 624–634
- Work, E. (1970) *Int. J. Syst. Bacteriol.* **30**, 425–433
- Work, E. (1971) *Methods Microbiol.* **5A**, 361–418
- Yamaguchi, T. (1965) *J. Bacteriol.* **89**, 444–453