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The Configuration of 2,6-Diamino-3-hydroxypimelic Acid in Microbial Cell Walls

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β-Hydroxydiaminopimelic acid, together with some diaminopimelic acid, occurs in the cell-wall mucopeptide of certain Actinomycetales. These components were converted into their di-DNP derivatives and separated by chromatography. Hence the relative proportions present in the cell walls of a number of species were measured. The problem of acid-induced inversion of configuration was studied. Of the diaminohydroxypimelic acids isomer B (see Scheme 2; amino groups *meso*, hydroxy group *threo* to its neighbouring amino group) always predominated but a small proportion of isomer D (amino groups L, hydroxy group *erythro*) also occurred. The configuration of the diaminohydroxypimelic acids was determined by periodate oxidation to glutamic γ-semialdehyde, which underwent spontaneous ring-closure. Reduction with sodium borohydride produced optically active proline, the configuration of which was determined by direct measurement of the optical rotation of DNP-proline. Un-cross-linked diaminohydroxypimelic acid in the cell wall was oxidized with periodate in the presence of ammonia. Since the remaining amino group was bound in peptide linkage, ring-closure was prevented and borohydride reduction of the aldehyde–ammonia presumed to be present resulted in the formation of ornithine. The quantity of ornithine was used as a measure of the degree of cross-linking.

The chromatographically slow-moving component recognized in hydrolysates of some Actinomycetales (Hoare & Work, 1957; Becker, Lechevalier & Lechevalier, 1965; Yamaguchi, 1965) was isolated from *Ampullariella regularis* and shown to be 2,6-diamino-3-hydroxypimelic acid (Perkins, 1965). This aminoacid can exist as four pairs of optical enantiomorphs and these racemic pairs were synthesized by Stewart (1961) and identified on the basis of relative configurations of the three asymmetric centres as A, B, C and D (see Scheme 2). Comparison of the properties of the compound from *Amp. regularis* with the synthetic isomers showed that it was isomer B (Perkins, 1965). At the same time, synthetic isomer B was found to separate on paper chromatography in methanol–water–pyridine–hydrochloric acid, the solvent used by Rhuland, Work, Denman & Hoare (1955), into two parts, and the supposition was made that this represented separation of the two optical enantiomorphs present, a phenomenon already well established for diaminopimelic acid (Rhuland *et al.* 1955). The isomer from *Amp. regularis* was the same as that in the slower of the two isomer B spots and was therefore presumably one optical enantiomer. The present work defines the configuration of this enantiomer and also those of the chromatographically separable forms of isomers C and D. The proportion of the various isomers of diaminodicarboxylic acids in hydrolysates of the cell walls of various Actinomycetales is also presented.

METHODS

Cell walls. *Amp. regularis* was grown and cell walls were prepared as described by Yamaguchi (1965). Samples of the cell walls of *Ampullariella digitata*, *Actinoplanes utahensis*, *Actinoplanes philippinensis*, *Actinoplanes* sp. E_{3-15}, *Amorphosporangium auranticolor* and *Streptosporangium album* were kindly given by Dr Yamaguchi.

Isolation of diaminohydroxypimelic acids. Cell walls were hydrolysed (6M-HCl for 16hr. at 105°). The hydrolysates were streaked on Whatman no. 3 paper (previously washed with M-ammonium acetate and water) and chromatographed overnight in solvent *A* [methanol–water–pyridine–98% (w/v) formic acid (80:19:10:1, by vol.)]. The slow-moving bands were detected by development of control strips with ninhydrin and the remainder of the substances in the slow-moving bands were eluted with water. The racemic pairs of synthetic isomers B, C and D were resolved chromatographically by a similar procedure. Isomer A gave only one spot (Perkins, 1965).

Crystals of the naturally occurring isomer B were obtained as follows. A sample of material from the slowest band from

Amp. regularis (about 6 μmoles) was dried in a desiccator over NaOH and H_2SO_4, dissolved in water, decolorized with charcoal, dried and dissolved in 0·3 ml. of water. Ethanol was added until slight turbidity persisted (0·5 ml.) and crystals formed in the cold, m.p. (Kofler) 234° (decomp.); Stewart (1961) quotes m.p. 239° for isomer B.

Separation and determination of di-DNP derivatives. Samples of the diaminohydroxypimelic acids were converted into their di-DNP derivatives by reaction with 1-fluoro-2,4-dinitrobenzene in ethanol–0·125 M-$NaHCO_3$ (2:1, v/v) and extraction of the product from the acidified reaction mixture into ether. The di-DNP derivatives of the racemic pairs A, B, C and D gave four clearly distinct spots when chromatographed on Whatman no. 1 paper overnight in solvent *B* [butan-1-ol–water–aq. NH_3 (sp.gr. 0·880) (20:19:1, by vol.)] (Stewart, 1961).

For determination, coloured areas of the paper were eluted with acetone–water (1:1, v/v) and the eluate was dried and dissolved in a measured volume of acetone–water (1:1, v/v). A suitable sample was added to 0·125 M-$NaHCO_3$ and the extinction read at 350 nm. In calculating the relative proportion of the isomer present, the assumption was made that all the di-DNP derivatives had the same molar extinction.

Prolonged hydrolysis of synthetic isomers. Diaminohydroxypimelic acid, isomer B (0·4 mg.) prepared by Stewart (1961) was dissolved in 6 M-HCl (0·6 ml.). Three samples (0·2 ml.) were sealed in ampoules and heated at 105° for periods of 8–56 hr. Each was then freed of HCl by evaporation *in vacuo*, dissolved in 0·2 ml. of 0·125 M-$NaHCO_3$ and treated with 0·4 ml. of ethanolic 0·5% (v/v) fluorodinitrobenzene. The resulting di-DNP derivatives of isomers B and C were extracted, chromatographed and determined as described above. In another experiment isomer C and isomer D were each hydrolysed for 16 hr. and the interconversion of isomers was measured in the same way.

Mono-DNP derivatives of diaminohydroxypimelic acid, isomer B. A sample of isomer B was treated with fluorodinitrobenzene (1 mol.prop.) under the usual conditions. After ether extraction from both alkaline and acid solution to remove unchanged reagent and any di-DNP derivative formed, the aqueous layer was chromatographed on paper in phosphate buffer (Levy, 1954). It gave two coloured spots, R_F 0·56 and 0·65 (dinitrophenol has R_F 0·21). The spots were identified as follows. A sample of the mixture of mono-DNP derivatives was treated with $NaIO_4$ and left at room temperature in the dark for 30 min. Excess of ethane-1,2-diol was added to destroy residual periodate and the sample was again chromatographed. Only the slower spot had disappeared and it was therefore presumably 2-amino-6-dinitrophenylamino-3-hydroxypimelic acid, the fast periodate-stable spot being 6-amino-2-dinitrophenylamino-3-hydroxypimelic acid.

A sample of the cell walls of *Amp. regularis* (27 mg.) was converted into the DNP derivative and hydrolysed in 4 M-HCl for 4 hr. After ether extraction the aqueous layer was dark brown, but obviously contained some yellow substance. It was filtered through a column (8 mm. × 250 mm.) of Sephadex G-25, which retained the dark material. Elution with water produced three yellow bands, the central one being by far the most intense. This material was concentrated and a sample was treated with 0·1 M-$NaIO_4$ (10 μl for.) 10 min. and then with 0·1 M-ethane-1,2-diol (10 μl.). Oxidized and unoxidized samples were chromatographed in phosphate buffer (Levy, 1954) and compared with the mono-DNP derivatives of *meso*-diaminopimelic acid and of diaminohydroxypimelic acid, isomer B. The sample was periodate-resistant and matched 6-amino-2-dinitrophenylamino-3-hydroxypimelic acid. The weak bands from the Sephadex column did not correspond to any of these marker substances. One corresponded to a marker of ϵ-DNP-lysine but they were not further characterized.

Conversion of diaminohydroxypimelic acid into proline. A typical experiment was as follows. Diaminohydroxypimelic acid samples, either isomer B or D isolated from cell walls or the enantioners of the synthetic compounds separated as slow or fast spots from chromatograms in solvent *A*, about 1 μmole in 20 μl., were treated with 2 M-NH_3 (40 μl.) and 0·2 M-$NaIO_4$ (10 μl.) and water (70 μl.) Each mixture was left in the dark at room temperature (10 min.) and then a small amount of $NaBH_4$ was added (an excess) and after mixing, the tube was stored at 2° overnight. Excess of NH_3 was removed with a rotary evaporator, excess of $NaBH_4$ was destroyed by addition of a drop of acetic acid and the sample was dried and used for making DNP-proline. Water (0·15 ml.) was added, followed by saturated $NaHCO_3$ (0·05 ml.) and ethanolic 0·5% (v/v) fluorodinitrobenzene (0·4 ml.), and the sample was left in the dark at room temperature. L-Proline (1 μmole) was treated in the same way. After 2 hr. the ethanol was removed *in vacuo*, unchanged reagent was extracted into ether, a drop of 2 M-HCl was added and the DNP-proline was extracted into ether. The ether extract was applied as a band (5 cm.) to a thin layer of Kieselgel G (20 cm. × 20 cm.) (activated at 100° for 30 min. and left to cool for at least 60 min.) and the chromatogram was developed in solvent *C* (chloroform–methanol–acetic acid, 95:5:1, by vol.). The solvent ran 18 cm., dinitrophenol 14·2 cm., DNP-proline 10·2 cm. and the reaction product showed a strong band equivalent to DNP-proline and a weaker band at 4·7 cm. This last band was DNP-glycine (see Scheme 3). The bands of DNP-proline were scraped from the glass plate with a spatula and transferred to a pointed centrifuge tube. The DNP-proline was removed by two extractions with acetone–water (3:1, v/v), followed by centrifuging. Samples of the extracts were dried *in vacuo* and dissolved in 0·125 M-$NaHCO_3$ for polarimetry. A 0·1 mM solution was convenient for measurement of rotation. For authentic DNP-L-proline (Sigma) $\epsilon_{max.} = \epsilon_{390} = 19500$. The optical rotatory dispersion was measured on a Polarmatic 62 apparatus (Bellingham and Stanley, London, N.15) with a cell of 5 mm. light-path and full-scale deflexion of 50 millidegrees. The cell holds about 0·25 ml., hence 25 nmoles of DNP-proline was sufficient.

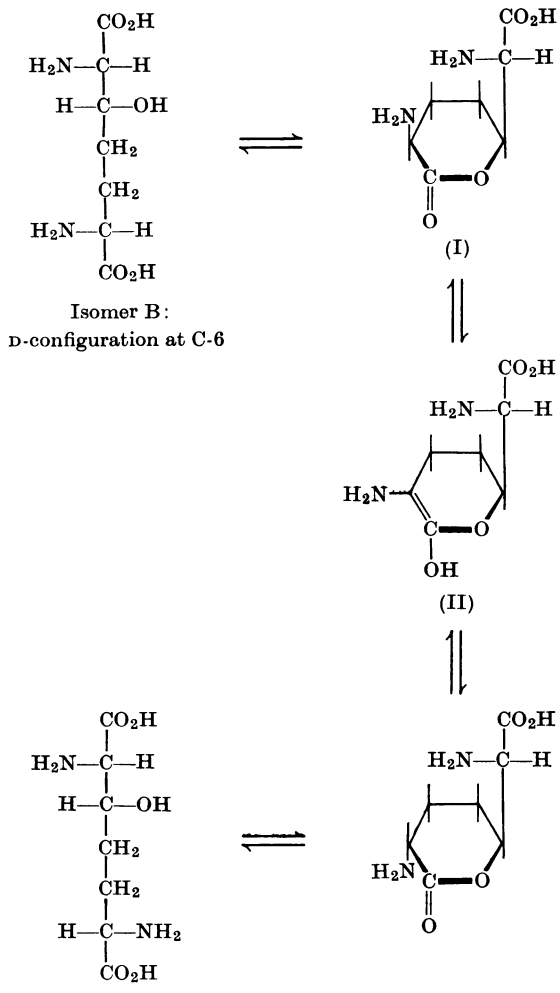
Conversion of un-cross-linked diaminohydroxypimelic acid into ornithine. A sample of the freeze-dried cell wall of *Amp. regularis* (2·5–3·0 mg.) in a pointed centrifuge tube was treated with 2 M-NH_3 (0·15 ml.) and then with 0·2 M-$NaIO_4$ (0·15 ml.), the whole being kept in the dark and thoroughly mixed with a glass rod at intervals over 1·5 hr. Preliminary experiments with various time-intervals showed that 1–1·5 hr. was the best. Then excess of solid $NaBH_4$ was added and after thorough mixing the sample was stored at 2° overnight. It was then diluted to 10 ml. and centrifuged, the supernatant was carefully removed and the cell-wall pad was resuspended in 10 ml. of water and again centrifuged. The final residue was resuspended in 6 M-HCl (1 ml.) and hydrolysed at 105° overnight. The HCl was removed *in vacuo*, water was added and again removed *in vacuo*. The

hydrolysate was then dissolved in water (15mg. of original cell walls/ml.) and 0·05ml. was used for the ninhydrin reaction at pH0·9 (Work, 1957), heating period 5min., scaled down to give 1ml. final volume. The extinctions of the ninhydrin-treated samples were read in a spectrophotometer (Unicam SP.500) with 1cm.-light-path micro cells and an absorption curve was plotted. Ornithine concentration was calculated from the increase in E_{510} of the sample relative to an untreated control.

Recovery of ornithine and determination of optical configuration. A further sample of the hydrolysate just described was applied to washed Whatman no. 3 paper and subjected to electrophoresis in 0·25M-formic acid (10v/cm. for 3hr.). The basic band corresponding to marker ornithine and detected by ninhydrin was eluted and used for two purposes. First, a sample was treated with acid ninhydrin (Work, 1957) and shown to give an extinction curve exactly like that for authentic ornithine. Secondly, a sample was converted into its di-DNP derivative, which was then purified by t.l.c. in solvent *C*. (Di-DNP-ornithine and major spot in unknown, R_F 0·36; di-DNP-lysine and minor spot in unknown, R_F 0·53). The lysine presumably came from a small amount of protein contaminant in the cell-wall preparation, as it was also present, together with small amounts of non-mucopeptide amino acids, in hydrolysates of untreated material. The di-DNP-ornithine band was removed and eluted as described for DNP-proline and then used for measurement of optical rotatory dispersion. Authentic di-DNP-L-ornithine (Sigma), dissolved in aq. 1% (w/v) $NaHCO_3$, had $\epsilon_{max.} = \epsilon_{350} = 30300$. A 0·05mM solution was convenient for measurement of rotation in a cell of 5mm. light-path.

RESULTS

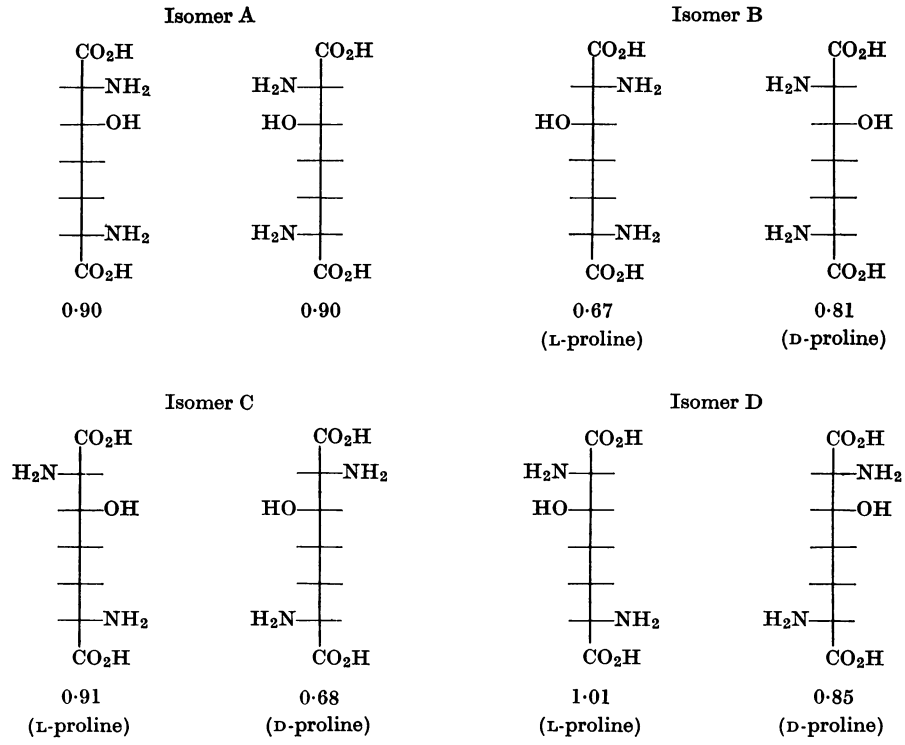
Interconversion of diaminohydroxypimelic acid isomers during hydrolysis. Since diaminohydroxypimelic acid can only be identified in bacterial cell walls after acid hydrolysis, the stability of the isomers under hydrolytic conditions is important. Hamilton & Anderson (1955) reported racemization at the α-carbon atom of the analogous hydroxylysine, during reflux in 6M-hydrochloric acid. It may be envisaged that formation of the δ-lactone (I) will lead by way of the enol (II) to racemization of configuration at C-6 (Scheme 1). Thus isomers B and C would be expected to interconvert, likewise isomers A and D (Scheme 2).



Scheme 1.

The result of prolonged acid hydrolysis of synthetic isomer B is shown in Fig. 1. Evidently interconversion takes place fairly rapidly during acid hydrolysis. In a parallel experiment, after hydrolysis for 16hr. synthetic isomer C was recovered as 80% unchanged and 20% isomer B, whereas synthetic isomer D was recovered as 80% unchanged and 20% isomer A. At the time of these experiments (1967) the sample of isomer B prepared by Dr J. M. Stewart was found to contain a small proportion of isomer C. This had not been noticed in 1965 and it seems possible that some interconversion had occurred during storage.

Diaminodicarboxylic acid isomers in cell-wall hydrolysates. Samples of cell walls were hydrolysed in 6M-hydrochloric acid for 16hr. at 105°. The diaminodicarboxylic acids were separated in solvent *A* and converted into their di-DNP derivatives, which were in turn separated in solvent *B*, eluted and determined. This method distinguishes between LL-diaminopimelic acid and DD-diaminopimelic acid, since the latter runs much more slowly than the former in solvent *A* but overlaps *meso*-diaminopimelic acid (Perkins, 1965; Rhuland *et al.* 1955). The di-DNP derivatives of *meso*- and DD-diaminopimelic acid, however, are easily separated in solvent *B*. The proportions of the various diaminodicarboxylic acids found are given in



Scheme 2. Configuration of diaminohydroxypimelic acid isomers. The numbers represent R_F with respect to *meso*-diaminopimelic acid in methanol–water–pyridine–conc.HCl (32:7:4:1, by vol.). In parentheses are the optical isomers of proline obtained from the non-hydroxylated end of the molecule by the procedure given in Scheme 3.

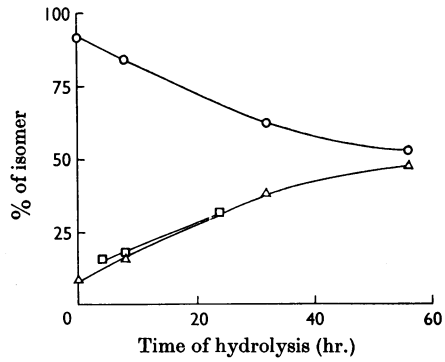


Fig. 1. Interconversion of diaminohydroxypimelic acid isomers during acid hydrolysis. A sample of synthetic isomer B (racemate) was hydrolysed in 6 M-HCl in a sealed tube at 105°. The isomers were separated as di-DNP derivatives as described in the text: ○, isomer B; △, isomer C. The curve also shows the increase in the proportion of isomer C (□) observed in hydrolysates of the cell walls of *Amp. regularis*. Only the slowest band containing isomers B and C was eluted from the chromatogram in this experiment, the small proportion of isomer D being ignored.

Table 1. The ratio of diaminohydroxypimelic acids to diaminopimelic acids differed considerably in the samples of cell walls from various species. Wherever diaminohydroxypimelic acids occurred, isomer B predominated. As indicated above, isomer C must have arisen at least partly by conversion from isomer B. The relative proportions of these two isomers recovered from the cell walls of *Amp. regularis* hydrolysed for different times are shown in Fig. 1. It is evidently impossible to say whether a small part of the isomer C may have been present before hydrolysis. Cell walls of *Amp. regularis*, *Amp. digitata*, *Amor. auranticolor* and *Act. utahensis* also contained an appreciable proportion of isomer D. This was unlikely to have arisen during hydrolysis, since prolonged hydrolysis of synthetic isomers B or C did not lead to any detectable amount of isomers A or D.

Configuration of the isomers of diaminohydroxypimelic acid. The isomer B isolated from the cell walls of *Amp. regularis* was one of the two enantiomers found in the synthetic compound (Perkins, 1965). The procedure used to establish the configura-

Table 1. *Diaminodicarboxylic acids in cell-wall hydrolysates.*

Cell-wall samples were hydrolysed in 6M-HCl at 105° for 16hr. The amino acids were measured as di-DNP derivatives (see the Methods section) and the values in the table are the percentage of each isomer of the total diaminodicarboxylic acids found. n.d. Not detected; *, small amount not measured.

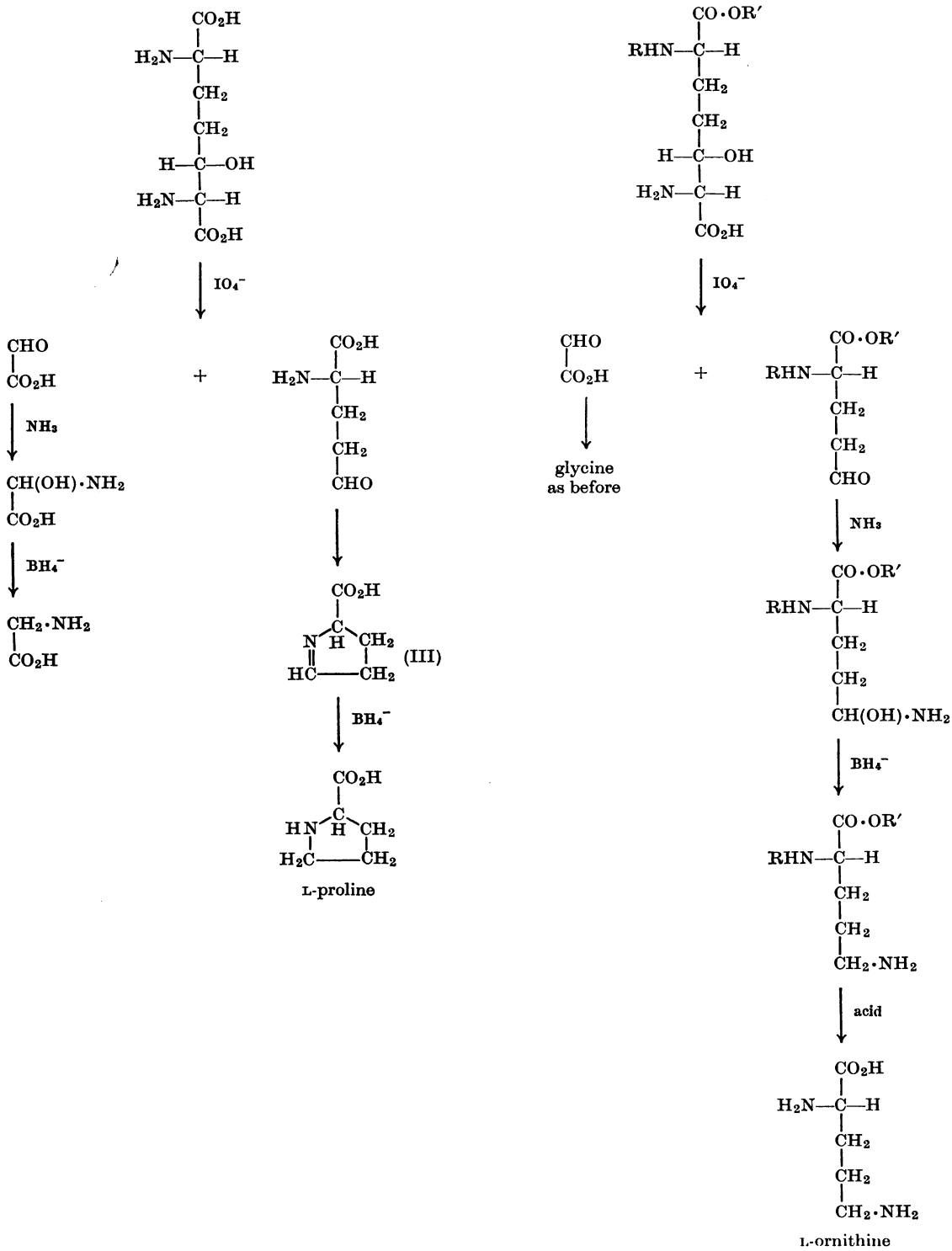
Organism	Isomer of 2,6-diamino-3-hydroxypimelic acid				Isomer of 2,6-diaminopimelic acid		
	A	B	C	D	*meso-*	LL-	DD-
Amp. regularis	*	67·5	20·5	6·5	4·5	0·8	n.d.
Amp. digitata	2·0	56·2	15·4	3·7	15·9	6·9	n.d.
Amor. auranticolor	*	39·2	10·8	4·8	40·0	5·3	n.d.
Act. utahensis	1·6	15·1	6·0	3·5	57·7	16·2	n.d.
Act. philippinensis	n.d.	2·9	1·1	n.d.	73·3	18·6	4·2†
Act. sp. E_{3-15}	n.d.	12·5	5·4	n.d.	64·0	14·0	4·1†
Str. album	0	0	0	0	68·6	27·1	4·4

† In this experiment DD-diaminopimelic acid could possibly have been confused with diaminohydroxypimelic acid, isomer D.

tion of the asymmetric centre at C-6 is outlined in Scheme 3. The bond between the vicinal hydroxyl and amino groups of hydroxylysine was split by periodate under alkaline conditions (Van Slyke, Hiller & MacFadyen, 1941) and diaminohydroxypimelic acid behaved in the same way. The two products were glyoxylic acid and glutamic γ-semialdehyde, the latter compound undergoing spontaneous ring-closure to form 1-pyrroline-5-carboxylic acid (III) (Vogel & Davis, 1952). Alkaline conditions were achieved by addition of ammonia, and glyoxylic acid was converted to some extent into the corresponding aldehyde–ammonia. After 10min. an excess of sodium borohydride was added to the mixture, since preliminary experiments had shown that rapid reduction of periodate was complete at that time. The borohydride reduced 1-pyrroline-5-carboxylic acid to proline and the presumed aldehyde–ammonia of glyoxylic acid to glycine. In a trial experiment with tartaric acid under similar reaction conditions 1μmole gave 0·64μmole of glycine, a 32% yield. Glyoxylic acid, however, underwent rapid over-oxidation even at room temperature. Some glycollic acid may also have been produced on reduction, but it was not looked for. Proline was identified chromatographically and by the characteristic blue colour it yielded with isatin. The proline and glycine were converted into their DNP derivatives and separated by t.l.c. In a typical experiment 1μmole of diaminohydroxypimelic acid, isomer A, gave 0·67μmole of DNP-proline and 0·19μmole of DNP-glycine. In this particular instance the DNP-proline was optically inactive because the isomer A was a racemic mixture, not separable on paper chromatography in solvent *A* (Perkins, 1965).

The optical rotatory dispersions of the DNP-proline samples prepared in this way were examined. DNP-proline derived from a crystalline sample of the diaminohydroxypimelic acid, isomer B, from *Amp. regularis* gave the curve shown in Fig. 2, which also includes authentic DNP-L-proline. The molecular rotations at the peak at 292nm. were as follows: DNP-L-proline, $[M]^{25}_{292}$ +40600°; product from naturally occuring isomer B, $[M]^{25}_{292}$ +38900° ([*M*] = mol.wt. × [α]/100). As indicated in Table 1, the cell walls of *Amp. regularis* contained some isomer D. This was isolated from chromatograms and converted into DNP-proline, which proved to be of the L-configuration (Fig. 3). Samples of synthetic racemic diaminohydroxypimelic acids, isomers B, C and D (Stewart, 1961), were resolved by chromatography in solvent *A*. The purified enantiomers were then converted into DNP-proline, and the optical configuration was determined. An example of DNP-D-proline obtained in this way is shown in Fig. 3. The optical isomers of proline found are included in Scheme 2.

Position of the hydroxyl group of diaminohydroxypimelic acid bound in cell walls. The diamino acids of bacterial cell walls are commonly involved in cross-linking and where *meso*-diaminopimelic acid occurs the amino group of the L-centre is bound to the γ-carboxyl group of the D-glutamic acid in the primary peptide chain (Ghuysen, 1968). In *Amp. regularis* the cross-linking was extensive, as judged by the difficulty of dinitrophenylation of any free amino groups, but nevertheless it might be expected that in any un-cross-linked molecules the bound amino group would belong to the primary chain. The hydroxyl group might therefore be next to a bound amino group or next to a free one. Only in the latter case would the molecule be attacked by periodate. The problem was approached in two ways. The cell walls of *Amp. regularis* were dinitrophenylated and hydrolysed in 4M-hydrochloric acid



Scheme 3. Periodate oxidation and borohydride reduction of free and bound diaminohydroxypimelic acid. R represents the γ-glutamyl residue in the cell wall and R′ is D-alanine or H.

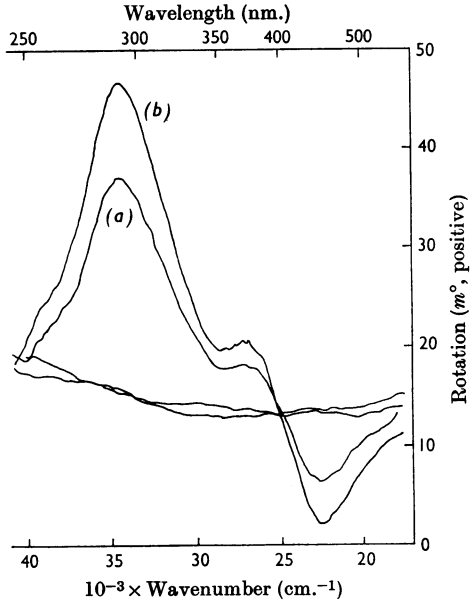


Fig. 2. Optical rotatory dispersion of DNP-L-proline. Curve *a*, authentic compound (0·104mM); curve *b*, sample derived from crystalline diaminohydroxypimelic acid isomer B, isolated from the cell walls of *Amp. regularis* (0·160mM). The other two curves are solvent blanks. The light-path was 5mm. The solvent was 0·125M-$NaHCO_3$. Arbitrary zero on ordinate scale.

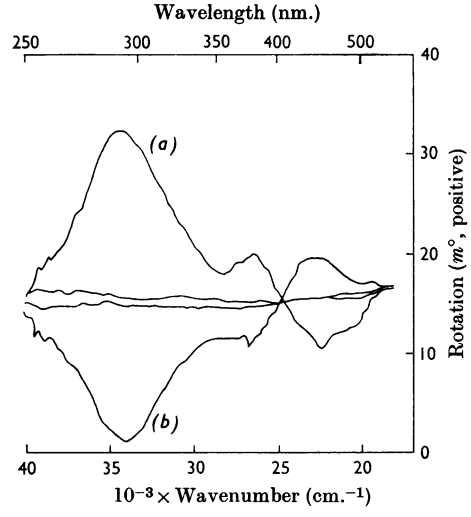


Fig. 3. Optical rotatory dispersion of DNP-proline. Curve *a*, sample derived from diaminohydroxypimelic acid, isomer D, isolated from the cell wall of *Amp. regularis*; curve *b*, sample derived from synthetic diaminohydroxypimelic acid, isomer D, enantiomer running more slowly during chromatography in solvent *A*. The other two curves are solvent blanks. The light-path was 5mm. The solvent was 0·125M-$NaHCO_3$. Arbitrary zero on ordinate scale.

for 4hr. The mono-DNP derivative present in the ether-extracted aqueous layer was purified by column chromatography on Sephadex G-25 and then chromatographed on paper before and after periodate oxidation. The single spot observed was resistant to periodate, separated from mono-DNP-*meso*-diaminopimelic acid and ε-DNP-lysine and matched one of the mono-DNP derivatives of synthetic isomer B that was also periodate-resistant. Hence the DNP group was attached next to the hydroxyl group, and the amino group bound in the cell walls must have been at the other end of the molecule. It was not possible to obtain a quantitative result from this experiment, but the amount of mono-DNP derivative was very small.

In the second method the cell walls were treated with periodate in the presence of ammonia and then reduced with sodium borohydride. Under these conditions any diaminohydroxypimelic acid with a free hydroxyl group next to a free amino group would be converted into ornithine (Scheme 3), since preliminary experiments had shown that under similar conditions *n*-butyraldehyde was reduced to *n*-butylamine. The cell walls were washed and hydrolysed and a sample of the hydrolysate was heated with ninhydrin in acid conditions (Work, 1957). The characteristic peak of ornithine was observed in the treated sample, whereas almost none occurred in the control (Fig. 4). Ornithine was additionally characterized by separation of the basic amino acids on paper electrophoresis, followed by chromatography in methanol–water–pyridine–conc. hydrochloric acid (32:7:4:1, by vol.) and development with ninhydrin, when it also gives a distinctive colour (Perkins & Cummins, 1964). In the sample of cell walls of *Amp. regularis* examined, the content of diaminohydroxypimelic acid determined by the acid ninhydrin reaction, by using the maximum at 420nm., was 3·6μmoles/10mg. The oxidized and reduced sample contained 0·38μmole/10mg. of ornithine, suggesting that about 10% of the molecules were un-cross-linked. This value was much higher than would have been indicated by the dinitrophenylation reaction, but it seems possible that the latter reaction was prevented by inaccessibility of the sites.

The ornithine produced as described above was also used to confirm that the amino group of the L-centre of diaminohydroxypimelic acid was bound in the primary chain. It was converted into its di-DNP derivative, which was purified and used for examination of optical rotatory dispersion. The curve for authentic di-DNP-L-ornithine is given in Fig. 5. The material derived from cell walls gave a curve of exactly the same shape, with a Cotton effect centred on 370nm. The authentic compound had $[M]^{25}_{389} + 88800°$, and that for the cell wall product was $[M]^{25}_{389} + 56100°$. This would corres-

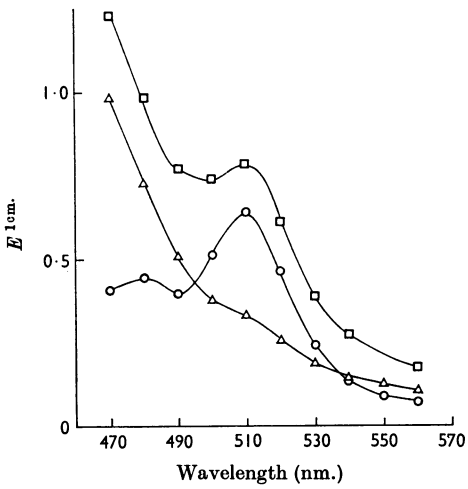


Fig. 4. Extinction curves in acid ninhydrin reaction (pH 0·9). A sample of the cell walls of *Amp. regularis* was treated with $NaIO_4$, then with $NaBH_4$, washed and hydrolysed as described in the Methods section. The acid ninhydrin reaction (Work, 1957) was in a final volume of 1 ml. ○, Ornithine (40 nmoles); △, control cell walls, no periodate; □ periodate-treated cell walls. The high extinction at low wavelengths in the cell wall samples is due to the presence of diaminohydroxypimelic acid isomers. These show no peak at 510 nm.

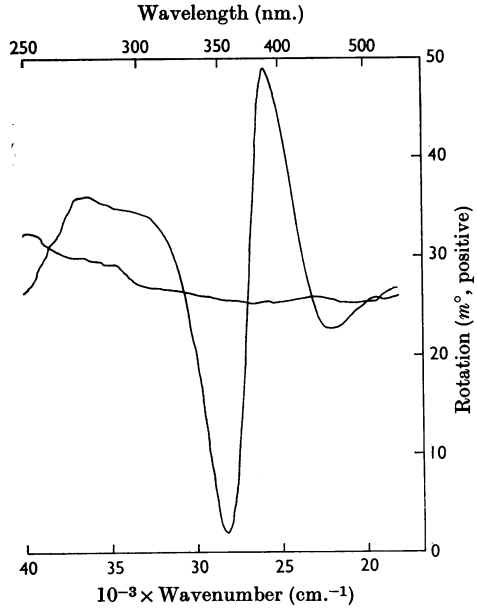


Fig. 5. Optical rotatory dispersion of di-DNP-L-ornithine (concentration 52·8 μM). The light-path was 5 mm. The solvent was 0·125 M-$NaHCO_3$. The relatively horizontal curve is a solvent blank. Arbitrary zero on ordinate scale.

pond to a mixture of D- and L-isomers with 82% in the L-form. Such a degree of racemization may possibly have occurred during acid hydrolysis. Thus this result confirms the idea that the L-centre of diaminohydroxypimelic acid is the one linked into the primary amino acid chain of the mucopeptide in *Amp. regularis*.

DISCUSSION

The occurrence of diaminohydroxypimelic acid in microbial cell walls seems to be confined to certain members of the Actinomycetales, in particular the *Actinoplanes* (Yamaguchi, 1965; Szaniszlo & Gooder, 1967). In all these studies the hydroxy acid was detected as a slow-moving component on paper chromatograms. This method would have detected some isomers of 2,6-diamino-3-hydroxypimelic acid, but others could well have been mistaken for *meso*-diaminopimelic acid (Perkins, 1965). Table 1 shows that isomer B was the isomer occurring in highest proportion in six species, although large amounts of diaminopimelic acid were sometimes also present. Isomer C was detected, probably because of inversion during hydrolysis, but a small amount of isomer D also occurred, and this was unlikely to have resulted from inversion. It seems possible that these hydroxylated acids arise by oxidation of diaminopimelic acid that has already become incorporated either in cell-wall mucopeptide precursors or in the cell wall itself. Such a biosynthetic sequence has been suggested for the *threo*-3-hydroxyglutamic acid found in the cell walls of *Microbacterium lacticum* (Schleifer, Plapp & Kandler, 1968). In that organism the cell walls of bacteria grown in almost anaerobic conditions contained only traces of the hydroxylated compound, its place being taken by glutamic acid. In *Actinoplanes meso*-diaminopimelic acid, bound by its L-centre and undergoing oxidation at the distal end to give a hydroxyl group *threo* to the amino group at the D-centre, would then give rise to diaminohydroxypimelic acid, isomer B, slow-moving enantiomer. LL-Diaminopimelic acid, which was also always present in smaller amount (see also Szaniszlo & Gooder, 1967), would, on oxidation at the distal end to give a hydroxyl group *erythro* to the vicinal amino group (in this case at an L-centre), yield isomer D, fast-moving enantiomer (Scheme 2). These were the isomers observed in *Amp. regularis*. Conceivably one enzyme could catalyse oxidation next to an L-amino acid centre to yield an *erythro*-hydroxyl group or next to a D-amino acid centre to give a *threo*-hydroxyl group. The latter case has been observed for the D-glutamic acid of *M. lacticum* (Schleifer *et al.* 1968). The fact that the isomer B from the other species also corresponded to the slowest-moving chromatographic spot and isomer D to the *meso*-diaminopimelic acid position suggested

that in these species too the same absolute configurations of the isomers occurred. The presence of the L-centre in the primary amino acid chain of mucopeptide corresponds to observations in bacteria having *meso*-diaminopimelic acid in their cell walls (Diringer & Jušić, 1966; Bricas, Ghuysen & Dezélée, 1967).

It remains possible, however, that the diaminopimelic acid is hydroxylated before incorporation into mucopeptide precursors. Sundharadas & Gilvarg (1966) observed that an auxotroph of *Escherichia coli* requiring diaminopimelic acid for growth (strain 173-25) would grow in the presence of added diaminohydroxypimelic acid, isomer D (but not A, B, or C), so long as lysine was also present. Further, the cell walls of these organisms now contained diaminohydroxypimelic acid instead of *meso*-diaminopimelic acid, but it was not known which isomer was present in the cell wall. Hence at least in *E. coli* diaminohydroxypimelic acid could replace diaminopimelic acid during the complete process of mucopeptide synthesis.

The borohydride reduction of an aldehyde in the presence of ammonia to the corresponding amine proved a convenient method for detecting un-cross-linked diaminohydroxypimelic acid, which was converted into ornithine. A similar procedure might be applied to the hydroxylysine in collagen, after periodate oxidation, which would only occur if both the ε-amino and δ-hydroxyl groups were free. Similarly the corresponding aldehyde observed in tropocollagen (Paz *et al.* 1969) could be reduced to ornithine without prior periodate treatment.

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REFERENCES

Becker, B., Lechevalier, M. D. & Lechevalier, H. A. (1965). *Appl. Microbiol.* **13**, 236.
Bricas, E., Ghuysen, J. M. & Dezélée, P. (1967). *Biochemistry*, **6**, 2598.
Diringer, H. & Jušić, D. (1966). *Z. Naturf.* **21b**, 603.
Ghuysen, J. M. (1968). *Bact. Rev.* **32**, 425.
Hamilton, P. B. & Anderson, R. A. (1955). *J. biol. Chem.* **213**, 249.
Hoare, D. S. & Work, E. (1957). *Biochem. J.* **65**, 441
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
Paz, M. A., Lent, R. W., Faris, B., Franzblau, C., Blumenfeld, O. O. & Gallop, P. M. (1969). *Biochem. biophys. Res. Commun.* **34**, 221.
Perkins, H. R. (1965). *Nature, Lond.*, **208**, 872.
Perkins, H. R. & Cummins, C. S (1964). *Nature, Lond.*, **201**, 1115.
Rhuland, L. E., Work, E., Denman, R. F. & Hoare, D. S. (1955). *J. Amer. chem. Soc.* **77**, 4844.
Schleifer, K. H., Plapp, R. & Kandler, O. (1968). *Biochim. biophys. Acta*, **154**, 573.
Stewart, J. M. (1961). *J. Amer. chem. Soc.* **83**, 435.
Sundharadas, G. & Gilvarg, C. (1966). *J. biol. Chem.* **241**, 3276.
Szaniszlo, P. J. & Gooder, H. (1967). *J. Bact.* **94**, 2037.
Van Slyke, D. D., Hiller, A. & MacFadyen, D. A. (1941). *J. biol. Chem.* **141**, 681.
Vogel, H. J. & Davis, B. D. (1952). *J. Amer. chem. Soc.* **74**, 109.
Work, E. (1957). *Biochem. J.* **67**, 416.
Yamaguchi, T. (1965). *J. Bact.* **89**, 444.