The Stereoisomers of $\alpha\epsilon$ -Diaminopimelic Acid

2. THEIR DISTRIBUTION IN THE BACTERIAL ORDER ACTINOMYCETALES AND IN CERTAIN EUBACTERIALES*

By D. S. HOARE† AND ELIZABETH WORK

Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

(Received 7 June 1956)

The occurrence in various Eubacteriales of two of the three stereoisomers of $\alpha\epsilon$ -diaminopimelic acid (1:5-diaminopentane-1:5-dicarboxylic acid) has been reported in a preliminary survey (Hoare & Work, 1955*a, b*). The most common form was the *meso* isomer, but the LL isomer occurred in *Clostridium perfringens* (syn. *Cl. welchii*) and the two propionibacteria examined. In an earlier study of the Actinomycetales (carried out before reference isomers of diaminopimelic acid were available), the nocardia were found to resemble *Mycobacterium tuberculosis* in their diaminopimelic acid content; streptomycetes and an actinomycetes contained what appeared, at the time, to be another amino acid differing slightly from diaminopimelic acid in its behaviour towards chromatography and enzymes (Work, 1953*a, b*; Work & Dewey, 1953*a, b*). This amino acid (then referred to as 'A') was isolated and tentatively identified as a higher homologue of diaminopimelic acid, but has now been shown to be LL-diaminopimelic acid.

This paper describes the isolation and identification of LL-diaminopimelic acid from hydrolysed streptomycetes and also the distribution of the three stereoisomers among representative members of the order Actinomycetales. In view of the finding of the less common LL isomer in certain propionibacteria and clostridia, further examinations are reported of the diaminopimelic acid isomer contents of related organisms.

The following paper includes an examination of the distribution of two bacterial enzymes known to metabolize diaminopimelic acid, and compares the enzyme and diaminopimelic acid isomer contents of various organisms (Antia, Hoare & Work, 1957). A preliminary report has been given of these findings (Hoare & Work, 1956).

MATERIALS AND METHODS

Diaminopimelic acid. Reference isomers were prepared by enzymic resolution of synthetic diaminopimelic acid (Work, Birnbaum, Winitz & Greenstein, 1955). The *meso* and LL isomers were also prepared by fractional crystallization of

the isomer mixture obtained from the culture filtrate of the lysine auxotroph of *Escherichia coli* 26-26 (Hoare & Work 1955*b*). Crude LL-diaminopimelic acid (amino acid 'A') was prepared from hydrolysed *Streptomyces* MA by the method used for the isolation of *meso*diaminopimelic acid from *Corynebacterium diphtheriae* (Work, 1951).

Bacteria. Organisms were grown as described in the succeeding paper, unless otherwise stated (Antia *et al.* 1957), or were used as dried cell preparations kindly presented by various workers. Hydrolysates of bacteria were prepared containing the equivalent of 83 mg. of dried cell/ml.; ethanol extracts were made from dried bacteria (Work & Dewey, 1953*a*). Cytoplasmic fractions from *Esch. coli* were prepared from the lyophilized cytoplasmic contents (580 mg.) obtained after removal of cell walls (this was kindly provided by Dr M. R. J. Salton). The preparation was dissolved by overnight stirring at +2° in 0.1 M bicarbonate buffer, pH 8.2 (25 ml.), containing 0.6 M-KCl. Insoluble debris was removed by centrifuging at 600 *g* and the supernatant solution was diluted with water (100 ml.). Successive centrifugings at 1300, 2700 and 105 000 *g* produced sediments, none being produced at 6600 *g*. The sediments and final supernatant solution were analysed for protein, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) by the method of Schneider (1945). Each fraction was also hydrolysed and examined by paper chromatography for diaminopimelic acid.

Paper chromatography. Two-dimensional chromatograms were made on Whatman no. 4 paper, with phenol (NH₃ atmosphere) and butanol-acetic acid as solvents (Work & Dewey, 1953*a*). When crude hydrolysates were examined for diaminopimelic acid, overlap with cystine was prevented by pretreating the solution with H₂O₂ (Dent, 1948). One-dimensional chromatography was carried out on Whatman no. 1 paper, by overnight irrigation with methanol-water-10N-HCl-pyridine (80:17.5:2.5:10, by vol.), the spots being developed by dipping the sheets in ninhydrin in acetone (0.1%, w/v) and heating at 100° for 2 min. (Rhuland, Work, Denman & Hoare, 1955; Hoare & Work, 1955*b*). The characteristic green diaminopimelic acid spots, which faded to a permanent yellow colour, were easily distinguished from the faster-fading purple spots given by other amino acids, the majority of which had higher *R_F* values than diaminopimelic acid. Lysine and cadaverine spots were well separated from diaminopimelic acid.

The one-dimensional system was used to separate LL-diaminopimelic acid from the *meso* and DD isomers. Crude hydrolysates were not usually suitable for direct examination unless diaminopimelic acid was present in sufficiently high concentration to be apparent as strong spots after chromatography of 20 μ l. of hydrolysate. When larger amounts of hydrolysate had to be used, the diaminopimelic

* Part 1: Hoare & Work (1955*b*).

† Present address: Biochemistry Department, University of California, Berkeley, California, U.S.A.

acid spots were obscured or displaced by streaking of other amino acids; this could be partly eliminated by treating the hydrolysate with a small amount of H_2O_2 before running the chromatogram. The neutral amino acid fractions prepared by electrodialysis or paper electrophoresis of hydrolysates (Work & Dewey, 1953a; Hoare & Work, 1955b) were more generally used without H_2O_2 treatment, as the next slowest moving amino acid was well separated from diaminopimelic acid; the equivalent of 30–40 μ l. of hydrolysate could then be used if necessary. Neutral fractions could not be used in greater amounts without further purification, effected by HCl fractionation on a column of Zeo-Karb 225 (Work & Denman, 1953).

Enzymic tests for isomers. One-dimensional chromatography did not differentiate between *meso*- and *DD*-diaminopimelic acid; these isomers were identified enzymically by means of diaminopimelic acid decarboxylase and L-amino acid oxidase, both of which attack *meso*- but not *DD*-diaminopimelic acid (Hoare & Work, 1955b; Work, 1955). Neither enzyme attacked diaminopimelic acid in untreated bacterial hydrolysates, but both were satisfactory when used on neutral amino acid fractions. Crude diaminopimelic acid decarboxylase was provided by any acetone-dried cell preparations of *Aerobacter aerogenes* or *Esch. coli* which had been grown on glucose-salt medium and which had a Q_{CO_2} (μ l. of CO_2 /mg. dry wt./hr.) greater than 3.0. These preparations converted both *LL*- and *meso*-diaminopimelic acid into lysine. The cells (10 mg.) were suspended in 0.25M phosphate buffer, pH 7.0 (0.3 ml.), and shaken overnight at 37° with the neutral amino acid fraction under test (0.3 ml.). Samples (0.2 ml.) taken at the beginning and end of incubation were deproteinized in ethanol (0.4 ml.) and the supernatant solutions (0.1–0.2 ml.) were examined by one-dimensional paper chromatography. Suitable controls were included, of known isomers incubated with enzyme, either alone or plus test solution. The absence of *DD* isomer was proved when the strong '*meso*' diaminopimelic acid spot, present in the zero-time sample of the test solution, was not found in the incubated sample. If diaminopimelic acid persisted after incubation, the presence of some *DD* isomer was suggested. When both *DD* and *meso* isomers were present, incubation produced a decrease in concentration of diaminopimelic acid, accompanied by formation of lysine (shown by two-dimensional chromatography of another portion of incubate). In any case, when *DD*-diaminopimelic acid appeared to be present, the test was repeated with L-amino acid oxidase from *Neurospora*; 3 mg. of enzyme (activity Q_{O_2} (μ l. of O_2 /mg./hr.) = 10) in 0.1M borate buffer, pH 8.6 (0.5 ml.), was incubated for 6 hr. in O_2 atmosphere with the test solution (0.3 ml.).

Partially purified diaminopimelic acid decarboxylase from *Aero. aerogenes* (Hoare & Work, 1955b) was used in a few cases. It was relatively specific for *meso*-diaminopimelic acid and had little or no action on the *LL* isomer.

Optical rotations. These were measured on 5% (w/v) solutions in 5N-HCl in 20 cm. tubes at 24°.

Chinard reaction. This was carried out as described by Chinard (1952).

RESULTS

Isolation and identification of *LL*-diaminopimelic acid from *Streptomyces*

Streptomyces MA (300 g. dry wt.) was hydrolysed and processed as in the preparation of *meso*-

diaminopimelic acid. The neutral fraction (containing crude amino acid 'A') obtained by electrodialysis was contaminated with small amounts of other neutral amino acids. These were removed by twice precipitating with ethanol the barium salt of 'A' (Jones & Moeller, 1928), but considerable amounts of 'A' remained in solution. 'A' was later purified by ion exchange on Zeo-Karb 225 (Work & Denman, 1953). Repeated precipitation of 'A' as the hydrochloride from aqueous acetone gave 300 mg. of pure material. It had $[\alpha]_D + 36.2$. (Found: C, 37.3; H, 6.7; N, 12.4; Cl, 15.2. Diaminopimelic acid monohydrochloride, $C_7H_{15}O_4N_2Cl$, requires C, 37.1; H, 6.6; N, 12.3; Cl, 15.7. $[\alpha]_D$ for *LL* isomer = +38.5.)

Amino acid 'A' resembled authentic *LL*-diaminopimelic acid in all respects. Thus it behaved identically on paper chromatography in all solvent systems which separated the isomers (Rhuland *et al.* 1955), and gave the characteristic ninhydrin colour reaction on paper. On reaction with ninhydrin in acid solution (Chinard, 1952), the products from 'A' and synthetic diaminopimelic acid gave identical absorption spectra. The free amino acid forms of both 'A' and *LL*-diaminopimelic acid were very soluble in water, crystallized with one molecule of water of crystallization (10%, w/v) and formed gels, rather than crystals, on treatment of aqueous solutions with ethanol (Work *et al.* 1955). 'A' and *LL*-diaminopimelic acid were only slightly decarboxylated by partially purified preparations of diaminopimelic acid decarboxylase, the extent of decarboxylation depending on the particular enzyme preparations used (Hoare & Work, 1955b). Crude decarboxylase preparations from *Aero. aerogenes* completely decarboxylated both samples at identical rates.

Diaminopimelic acid isomers in the *Actinomycetales*

The results of this examination are shown in Table 1. Six species of *Mycobacterium* all contained *meso*-diaminopimelic acid in sufficient amounts to be easily identifiable when 10 μ l. of the whole hydrolysate was examined (see Rhuland *et al.* 1955, Fig. 3). The neutral fractions showed no trace of the *LL* isomer and after testing with crude or purified decarboxylase were free from residual diaminopimelic acid, proving the absence of the *DD* isomer. Seven different species of *Nocardia* all resembled the *mycobacteria* in their high content of *meso*-diaminopimelic acid, but many of them contained traces of the *LL* isomer (Fig. 1, spots A and B). When the decarboxylation of the neutral amino acid fractions of certain *nocardia* was carried out with partially purified decarboxylase, *LL*-diaminopimelic acid remained, but the use of crude enzyme resulted in complete decarboxylation of all the diaminopimelic acid. The *LL* isomer in some species of *Nocardia* was

Table 1. *Distribution of diaminopimelic acid (DAP) isomers among certain bacteria*

Bacterial hydrolysate (10 μ l. = 0.8 mg. dry wt.) and neutral fraction from hydrolysate were examined by paper chromatography on Whatman no. 1 paper in methanol-water-10N-HCl-pyridine (80:17.5:2.5:10, by vol.). Diaminopimelic acid was identified by position and by colour of spots (olive-green, fading to permanent yellow) on dipping in ninhydrin in acetone (0.1%, w/v) and heating for 2 min. at 100°. DD and *meso* isomers were identified as described in the text; LL isomer was identified by the position on the chromatogram. + + +, DAP spot strong, isomer easily identified in hydrolysate (10 μ l.); + +, DAP spot visible in hydrolysate (20 μ l.); +, isomer identifiable only in electrodialysate (20 μ l.); tr., isomer visible only in larger amounts of electrodialysate (30 μ l. or more) or in column eluate; + -, isomer stable to action of DAP decarboxylase and L-amino acid oxidase; —, isomer not detected.

Organism	Strain and source*	Grown by†	Diaminopimelic acid isomer		
			<i>meso</i>	LL	DD
Actinomycetales					
<i>Mycobacterium tuberculosis</i> var. <i>bovis</i> B.C.G.	‡	See Work & Dewey (1953a)	+++	—	—
<i>Myco. tuberculosis</i> var. <i>bovis</i>	‡	See Work & Dewey (1953a)	+++	—	—
<i>Myco. avium</i>	‡	See Work & Dewey (1953a)	+++	—	—
<i>Myco. phlei</i>	NCTC 8151	(1)	+++	—	—
<i>Myco. smegmatis</i>	NCTC 3821	(1)	+++	—	—
<i>Myco. stercois</i>	NCTC 8159	(1)	+++	—	—
<i>Nocardia asteroides</i>	W.C. 3308 and UCHMS	(2) and local	+++	—	—
<i>N. leishmanii</i>	A, 341 LSTM	Local	+++	tr.	—
<i>N. corallina</i>	W.C. 3406	(2)	+++	—	—
<i>N. rubra</i>	N.R.L. Ottawa 497	Local	+++	tr.	—
<i>N. coeliaca</i>	NCTC 2570	Local	+++	tr.	—
<i>N. madurae</i>	NCTC	Local	+++	tr.	—
<i>N. lutea</i>	NCTC 576	Local	+++	tr.	—
<i>Actinomyces</i> (aerobic species)	‡	Local (5)	—	+++	—
<i>Streptomyces griseus</i>	W.C. 3463	2	+	++	—
<i>Streptomyces fradiae</i>	W.C. 3556 B	2	tr.	++	—
<i>Streptomyces</i>	MA Clevedon‡	3	—	+·	—
<i>Streptomyces</i> sp.	Clevedon§	3	+	++	—
<i>Streptomyces</i>	XZ-6 Clevedon‡	3	tr.	++	—
<i>Streptomyces</i>	X-15 Clevedon‡	3	—	++	—
<i>Streptomyces</i>	'Cutler' Clevedon	3	—	++	—
<i>Streptomyces</i>	BCC-75 Clevedon‡	3	—	++	—
<i>Micromonospora</i>	W.C. 3450	2	++	++	+·
<i>Micromonospora</i>	W.C. 3452	2	++	++	+·
<i>Micromonospora</i>	LSTM‡	Local (5)	++	++	+·
Eubacteriales					
<i>Propionibacterium</i>	¶	Local	—	+++	—
<i>Bacillus cereus</i>	NRRL 569	Local (5)	++	—	—
<i>B. subtilis</i> **	Cambridge	Local (5)	++	—	—
<i>B. megatherium</i>	Lab. strain, Porton	Porton	++	+	—
<i>Clostridium butyricum</i>	NCTC 7423	Local (5)	++	—	††
<i>Cl. novyi</i> (syn. <i>Cl. oedematiens</i>)	Albiston LIPM	4	++	—	—
<i>Cl. acetobutyricum</i> **	UCL	UCL	++	—	—
<i>Cl. sporogenes</i>	NCTC 532	Local (5)	+	—	††
<i>Cl. bifermentans</i>	NCTC 2914	Local (5)	+	—	††
<i>Cl. perfringens</i> (syn. <i>Cl. welchii</i>) D**	See Work & Dewey (1953a)	—	—	++	—
<i>Cl. welchii</i> A	S, 107	4	—	++	—
<i>Cl. kluyveri</i>	Sheffield‡	UCL	++	—	—
<i>Cl. tetani</i>	S, 279	4	+	—	—

* The following abbreviations are used for sources: UCHMS, Department of Bacteriology, University College Hospital Medical School; LSTM, Department of Medical Mycology, London School of Hygiene and Tropical Medicine; Clevedon, Medical Research Council Antibiotics Research Station; Cambridge, Medical Research Council Unit for Chemical Microbiology; LIPM, Lister Institute of Preventive Medicine, Elstree; UCL, Department of Biochemistry, University College, London; Sheffield, Department of Microbiology, University of Sheffield; Porton, M.R.E., Ministry of Supply; NCTC, National Collection of Type Cultures; NRRL, Northern Region Research Laboratory.

† See list of acknowledgements.

‡ Tai & van Heyningen (1951).

§ These strains have not been further classified.

|| Also, unidentified amino acid giving same colour reaction, see text.

¶ See text and Table 5 (Antia *et al.* 1957), 6 types of organisms.

** Reported by Hoare & Work (1955b).

†† Decarboxylase resistant spot.

so low in concentration that it was observed only when the chromatograms were overloaded with *meso* isomer; overloading was not possible unless the diaminopimelic acid was further purified by ion-exchange chromatography. Spots X 1 and 2 show the type of chromatogram obtained with 5 μ g. of LL-diaminopimelic acid and 30 or 50 μ g. of *meso* isomer; in the absence of other amino acids it is not difficult to detect such low proportions of the LL isomer. However, the reverse process, detection of low amounts of *meso* isomer in the presence of the

Fig. 1. Techniques of paper chromatography and enzymic digestion as used in the detection of diaminopimelic acid isomers in hydrolysates of micro-organisms, illustrated on members of the Actinomycetales. X 1-5. Chromatograms of LL- and *meso*-diaminopimelic acid, one isomer being present in excess. 1, 5 μ g. of LL+30 μ g. of *meso*; 2, 5 μ g. of LL+50 μ g. of *meso*; 3, 30 μ g. of LL+5 μ g. of *meso*; 4, 50 μ g. of LL; 5, 50 μ g. of LL+5 μ g. of *meso*. A-F. Chromatograms of either neutral fractions or eluates from Zeo-Karb 215 columns from various Actinomycetales, and of results (denoted by suffix d) of treatment of these fractions with crude diaminopimelic acid decarboxylase as in Methods. A, *Nocardia asteroides* (eluate); B, *Nocardia rubra* (neutral fraction); C, *Streptomyces* MA (neutral fraction); D, *Streptomyces* (Tai & van Heyningen, 1951) (neutral fraction); E, *Micromonospora* W.C. 3452 (eluate); F, *Micromonospora* LSTM (neutral fraction). Chromatography on Whatman no. 1 paper was carried out with overnight irrigation by methanol-water-10N-HCl-pyridine (80:17.5:2.5:10, by vol.); colours were developed by ninhydrin in acetone (0.1%, w/v) and heating for 2 min. at 100°. Diaminopimelic acid spots (solid outlines containing numbers) and spot U (unknown) gave characteristic olive-green colour fading to permanent yellow; lysine (solid spots designated L) were brownish purple and faded to brown; other neutral amino acids (dotted outlines) were purple, fading rapidly. Numbers on spots indicate colour strength on an arbitrary scale ranging from 1 (just visible) to 6 (maximum intensity).

LL isomer, is not so easy, owing to a tendency of the LL isomer, when present in amounts higher than 30 μ g., to 'tail' and so overlap slightly the *meso* spot (X 4). Careful matching against controls will enable this 'tailing' to be distinguished from the true presence of *meso* isomer, as, in this case, the doublespot (X 5) presents a club-shaped appearance.

The only actinomycete examined (an aerobic species) contained a high concentration of LL isomer and no *meso* or DD. This was confirmed with both purified and crude decarboxylase preparations, only the latter producing significant decarboxylation. The streptomycetes all contained LL isomer, at lower concentrations than in the actinomycete, and in some strains lesser amounts of *meso* isomer were present; no DD isomer was found (spots C and D).

Two species of *Micromonospora* contained all three isomers; two well-defined diaminopimelic acid spots were observed on chromatograms and, after treatment with crude decarboxylase, a single spot remained in the *meso* position, considerably reduced in intensity from the original spot, while lysine was formed (E and Ed). Control experiments in which either *meso*- or DD-diaminopimelic acid was added to the original solution showed that the added *meso* isomer, but not the added DD compound, was decarboxylated. Similar results were obtained with L-amino acid oxidase, but the residual diaminopimelic acid spot was weaker than after decarboxylase treatment.

Another species of *Micromonospora* (LSTM) contained *meso*-, DD- and LL-diaminopimelic acids, and an unknown amino acid which gave the characteristic diaminopimelic acid colour reaction on methanol-water-HCl-pyridine chromatograms, but which travelled more slowly than diaminopimelic acid (spot F). Added isomers of diaminopimelic acid travelled to their expected positions. This unidentified substance, which is neutral and stable to decarboxylase and peroxide, had previously been observed to travel more slowly than diaminopimelic acid in the phenol direction of two-dimensional chromatograms (Work & Dewey, 1953b). No further work has been carried out on this amino acid; it has been observed in hydrolysates made from three different preparations of this organism, which was very slow-growing and required 2-3 months to produce sufficient cells for hydrolysis.

Diaminopimelic acid isomer distribution in certain Eubacteriales

The preliminary results on the LL-diaminopimelic acid content of two species of *Propionibacterium* have been confirmed for other species. The following organisms were examined (the strain numbers being given in Table 5, Antia *et al.* 1957), the number in parentheses indicating the number of strains:

Propionibacterium arabinosus (1), *Pr. jensenii* (3), *Pr. pentosaceum* (2), *Pr. petersonii* (1), *Pr. theonii* (2), *Pr. zeae* (1). All contained similar amounts of LL-diaminopimelic acid, the concentration being sufficiently high for a strong spot to be apparent on chromatography of 10 μ l. of hydrolysates. No *meso* isomer could be detected in the neutral fractions; and no diaminopimelic acid remained after treatment with crude decarboxylase. No diaminopimelic acid was found in microbacteria (two strains), *Lactobacillus casei* and *Lb. brevis* (two strains); *Lb. plantarum* (syn. *Lb. arabinosus*) had been previously found to contain mesodiaminopimelic acid (Hoare & Work, 1955b).

Among the Bacillaceae, the finding of LL-diaminopimelic acid in *Clostridium welchii* A (Hoare & Work, 1955b, fig. 4) was confirmed with different growth media. All other clostridia examined contained only the *meso* isomer; *Cl. tetani*, previously found to be lacking in diaminopimelic acid (Work & Dewey, 1953a), was re-examined in fresh culture and found to contain lower concentrations of diaminopimelic acid than other clostridia. In certain clostridia, indicated in Table 1, the mesodiaminopimelic acid was found to be partly resistant to decarboxylation; on examination of fresh hydrolysates of the same cultures, this observation could not be repeated, and the amino acid was always completely oxidized by L-amino acid oxidase. The reason for this effect has not been further investigated. *meso*Diaminopimelic acid was previously found in *Bacillus subtilis* and *B. megatherium* and is herewith described in *B. cereus*. However, Mr R. E. Strange, Microbiological Research Department, Porton, Wiltshire, observed small amounts of LL isomer in addition to the *meso* isomer in another culture of *B. megatherium*. At his request we checked this finding, and with his permission are including it among our results.

Diaminopimelic acid content of various intracellular fractions

Fractional centrifuging of the cytoplasmic contents of *Esch. coli* produced three sediments, all free from diaminopimelic acid. Those fractions sedimenting at the lower speeds contained mainly protein with small amounts of DNA; the pellet produced by the highest speed contained RNA, DNA and protein in the proportions 4.3:9.2:59.5. The supernatant liquid remaining after 4 hr. centrifuging at 105 000 g contained a low concentration of mesodiaminopimelic acid. Partially purified diaminopimelic acid decarboxylase from *Aero. aerogenes* did not contain diaminopimelic acid. Only the *meso* isomer of diaminopimelic acid was found in the hexosamine-containing peptide excreted by germinating spores of *B. megatherium* (Strange & Powell, 1954).

The ethanolic extracts of a few species were examined. In *Lb. arabinosus* and *Cl. welchii* (A), no diaminopimelic acid was found in the extracts, either before or after hydrolysis. Approximately equal amounts of *meso*- and LL-diaminopimelic acid were present together in the unhydrolysed ethanolic extracts from *Pr. arabinosus*, *Pr. pentosaceum*, *B. cereus* and *B. subtilis*; the culture of *B. megatherium* in which only the *meso* isomer was found in whole cells had only this isomer free in its ethanolic extract.

In certain organisms, diaminopimelic acid was demonstrable in the ethanolic extracts only after hydrolysis; these include *Pr. jensenii* (*meso* and LL isomers), *Streptomyces* MA (LL isomer), *Cl. cylindrosporum*, *Cl. oedaematiens*, *Myc. smegmatis*, *Myc. tuberculosis* var. *bovis* and *Micromonospora* WC 3452, the last five organisms having only the *meso* isomer. The amounts of diaminopimelic acid in ethanolic extracts varied from about 2 to 10% of that present in whole cells, the highest concentrations being found in bacilli.

DISCUSSION

The original mistaken identification of LL-diaminopimelic acid from streptomyces was due mainly to ignorance of the striking differences between many of the properties of the *meso* and LL isomers. When reference isomers were available, investigation of their physical and chromatographic properties and behaviour towards enzymes revealed the identity of the LL isomer with the unknown amino acid ('A'). Although it is now known that separation of isomers by paper chromatography is more reproducible on Whatman no. 1 paper than on the no. 4 originally used, the difference between the R_f values of 'A' and the mesodiaminopimelic acid from *C. diphtheriae* was sufficient to differentiate between the two substances. Also the stability of 'A' to partially purified diaminopimelic acid decarboxylase contrasted sharply with the behaviour of diaminopimelic acid from other microorganisms (Work & Dewey, 1953a; Dewey, Hoare & Work, 1954, Table 8). Had the less specific crude preparation of acetone-dried cells been used, this difference in properties would not have been noticed; indeed, when crude cells were later substituted for purified decarboxylase, or when the method of purification of decarboxylase had been altered (Hoare & Work, 1955b), the previous findings on the complete stability of 'A' towards decarboxylase could not be repeated. It is now known that the earlier preparations of diaminopimelic acid decarboxylase were the more specific towards the *meso* isomer, whereas crude cells attack both isomers.

The preliminary investigation on the distribution of diaminopimelic acid in the Actinomycetales

(Work, 1953*a*) was left uncompleted pending the identification of amino acid 'A'. The earlier findings have now been mainly confirmed by the improved paper-chromatography method; the only differences are that several isomers were often found together in the same organism. This was not the case among mycobacteria, but in the nocardias and streptomycetes minor amounts of a second isomer were sometimes found. The species of *Micromonospora* examined contained roughly similar proportions of LL- and *meso*-diaminopimelic acid. Smaller amounts of DD isomer may also have been present, but there is uncertainty about the identification of this enzyme-resistant form of diaminopimelic acid. It was always found in micromonosporae, but its amount was not consistent in different preparations of the same organism, or when two different enzymes were used to identify it. In one species of this organism, the presence of a third slow spot resembling diaminopimelic acid in its ninhydrin colour reaction is of interest.

In the Actinomycetales, LL-diaminopimelic acid is a common constituent, whereas in the Eubacteriales it is only rarely found. It is possible that phase of growth may influence the overall cellular content of diaminopimelic acid, or even the proportion of isomers. This has not yet been investigated, but it might explain the present finding of *meso*-diaminopimelic acid in *Cl. tetani*, whereas previously a culture of unspecified history was found to be free from the amino acid (Work & Dewey, 1953*a*). The Gram-positive cocci provide a group of Eubacteriales from which diaminopimelic acid is nearly always absent, only a few exceptions among the micrococci being known (Cummins & Harris, 1956*a*). The majority of lactobacilli, which are classified in the same family as the streptococci, also appear to be free from diaminopimelic acid. *Lb. arabinosus*, the only member of this genus which we originally examined (Work & Dewey, 1953*a*), is the only species known to contain diaminopimelic acid (Cummins & Harris, 1956*b*); our finding of the *meso* isomer in this organism has been confirmed by isolation (Ikawa & O'Barr, 1955).

One of the major sites for diaminopimelic acid in the bacterial cell is the wall, where it is invariably accompanied by glutamic acid, alanine and glucosamine (Salton, 1953; Cummins & Harris, 1956*a-c*). An insoluble, high-molecular-weight peptide, contained in spore coats of bacilli and liberated in soluble form on standing or on germination, is made up entirely from these same substances (Strange & Powell, 1954; Strange & Dark, 1956; Powell & Strange, 1956). The detailed investigations of Cummins & Harris on the constituents of the cell walls of Gram-positive Eubacteriales and of Actinomycetales have agreed with our results on

the distribution of diaminopimelic acid in whole bacterial cells. There appeared to be certain patterns of cell-wall constituents which were often consistent with bacterial taxonomy. This is particularly striking in regard to the distribution of the different isomers of diaminopimelic acid. Thus the cell walls of the mycobacteria, nocardia and corynebacteria were all distinguished by their contents both of large amounts of *meso*-diaminopimelic acid and of arabinose and galactose. Cell walls of streptomycetes and aerobic actinomycetes, on the other hand, contained LL-diaminopimelic acid and glycine, both of which were also constituents of propionibacteria, but which were not usually found in the cell walls of other genera. In organisms lacking in diaminopimelic acid, the cell walls always contained lysine, as in the majority of lactobacilli, where aspartic acid also occurred. The exception, *Lb. arabinosus*, had *meso*-diaminopimelic acid, but no lysine or aspartic acid, in its cell walls.

The soluble, non-protein cell contents often, but not invariably, contain diaminopimelic acid, either free (see also Work, 1949; Work & Dewey, 1953*a*; Blass, Lecomte & Macheboeuf, 1951), or bound so as to be apparent only after hydrolysis. The free form may represent the cellular metabolic pool, and its amount and isomer content may depend on the metabolic state of the cells at harvesting. Under certain cultural conditions, the *Esch. coli* mutant 26-26, lacking in diaminopimelic acid decarboxylase, accumulated both LL- and *meso*-diaminopimelic acid in the soluble fraction and excreted them into the culture filtrate (Hoare & Work, 1955*b*). Both isomers were also present in the soluble fractions of some propionibacteria and bacilli, although only one isomer was found in hydrolysates of whole cells of both types of organisms and walls of propionibacteria. We have already suggested that definite cellular fractions may be connected with different functions of diaminopimelic acid (Work & Dewey, 1953*a*); these findings emphasize the wisdom of studying such fractions. The nature of the bound soluble fraction is not known; some of it might be unpolymerized or degraded forms of the peptide units from the cell walls.

No homogeneous bacterial protein free from large amounts of carbohydrate has been found to contain diaminopimelic acid. In all cases where the presence of diaminopimelic acid was reported, either the protein was not shown to be pure, as with shigella endotoxin, where only the most rigorously purified sample was free from diaminopimelic acid (Work & Dewey, 1953*a*), or the 'protein' contained polysaccharide (Seibert, Figueroa & Dufour, 1955) and was probably derived from the cell wall. The total proteins from protoplasts of *B. megatherium* contained no diaminopimelic acid, although the

protoplasts were derived from cells containing it (McQuillen, 1956). The nucleic acid-containing fractions from *Esch. coli* were prepared from lyophilized cytoplasmic contents and cannot therefore be considered as homogeneous. Their freedom from diaminopimelic acid is, however, of interest and indicates absence of contamination from cell-wall fragments.

Diaminopimelic acid is evidently not confined exclusively to bacteria and blue-green algae, as suggested by Work & Dewey (1953*a*). It has been found in *Chlorella ellipsoidia* by Fujiwara & Akabori (1954); we have confirmed this result, but were unable to identify the isomer because of the very low concentration of diaminopimelic acid in the strain examined (Gerneck 211/1*a*, Cambridge Collection of Algae and Protozoa). Systematic examination and fractionation of other micro-organisms unrelated to bacteria may reveal other species containing diaminopimelic acid. The fact that this has not yet occurred suggests that diaminopimelic acid is probably not a common major cellular constituent outside the Schizomycetes.

SUMMARY

1. An unidentified amino acid, previously isolated from streptomycetes, was identified as LL-diaminopimelic acid.

2. Among the members of the bacterial order Actinomycetales, the LL isomer is present in hydrolysates of whole cells of streptomycetes and an aerobic actinomycete, with small amounts of *meso* isomer in some species. The *meso* isomer occurred in nocardias and mycobacteria, with traces of LL isomer in certain nocardias. In micromonosporae, both isomers were present in similar proportions, with possibly a small amount of DD-diaminopimelic acid.

3. Among selected Gram-positive Eubacteriales, cells of *Clostridium welchii* and propionibacteria contained LL-diaminopimelic acid only, whereas *meso* isomer was present in all other clostridia and bacilli.

4. No diaminopimelic acid was found in nucleic acid-containing fractions which could be sedimented from *Escherichia coli* extracts, but the material remaining in solution after high-speed centrifuging contained *meso* isomer.

5. Ethanolic extracts of cells often, but not invariably, contained diaminopimelic acid, either free or in a bound form liberated by hydrolysis. The isomers present were not always confined to those found in the whole cells from which the extracts were made.

We are grateful to Dr J. P. Greenstein for diaminopimelic acid and to Dr H. Newey for L-amino acid oxidase. Cells were grown for us by the following, to whom we are very

grateful (numbers are used for reference in Table 1): Dr R. Codner (3), Dr S. A. Waksman (2), Dr D. Dolby (4), Dr P. Meadow (1), Miss M. Antia (5), Dr G. E. Fogg. Spore peptide was kindly supplied by Mr R. E. Strange and cell extracts of *Clostridium cylindrosporum* by Dr B. Wright. Analyses were carried out by Drs Weiler and Strauss. Technical assistance was given by Mr R. Denman and Mr D. Irving. London University Research Fund provided grants for apparatus, and the Rockefeller Research Fund of University College Hospital Medical School provided a maintenance grant to D.S.H. E.W. is very grateful to the Belgian Federation of University Women for a travelling fellowship, and to Dr J. Brachet for hospitality and advice during fractionation of *Escherichia coli*.

REFERENCES

- Antia, M., Hoare, D. S. & Work, E. (1957). *Biochem. J.* **65**, 448.
 Blass, J., Lecomte, O. & Macheboeuf, M. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1552.
 Chinard, F. P. (1952). *J. biol. Chem.* **199**, 91.
 Cummins, C. S. & Harris, H. (1956*a*). *Int. Bull. bacterial Nomen. Taxon.* **6**, 111.
 Cummins, C. S. & Harris, H. (1956*b*). *J. gen. Microbiol.* **14**, 583.
 Cummins, C. S. & Harris, H. (1956*c*). *J. gen. Microbiol.* **15**, ix.
 Dent, C. E. (1948). *Biochem. J.* **43**, 169.
 Dewey, D. L., Hoare, D. S. & Work, E. (1954). *Biochem. J.* **58**, 523.
 Fujiwara, T. & Akabori, S. (1954). *J. chem. Soc. Japan*, **75**, 993.
 Hoare, D. S. & Work, E. (1955*a*). *Biochem. J.* **60**, ii.
 Hoare, D. S. & Work, E. (1955*b*). *Biochem. J.* **61**, 562.
 Hoare, D. S. & Work, E. (1956). *J. gen. Microbiol.* **15**, xiii.
 Ikawa, M. & O'Barr, J. S. (1955). *J. biol. Chem.* **213**, 877.
 Jones, D. B. & Moeller, O. (1928). *J. biol. Chem.* **79**, 429.
 McQuillen, K. (1956). *Bacterial Anatomy, Symp. Soc. gen. Microbiol.* p. 127.
 Powell, J. F. & Strange, R. E. (1956). *Biochem. J.* **63**, 661.
 Rhuland, L. E., Work, E., Denman, R. F. & Hoare, D. S. (1955). *J. Amer. chem. Soc.* **77**, 4844.
 Salton, M. R. J. (1953). *Biochim. biophys. Acta*, **10**, 512.
 Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
 Seibert, F. B., Figueroa, E. S. & Dufour, E. H. (1955). *Amer. Rev. Tuberc.* **5**, 704.
 Strange, R. E. & Dark, F. A. (1956). *Biochem. J.* **62**, 459.
 Strange, R. E. & Powell, F. J. (1954). *Biochem. J.* **58**, 80.
 Tai, T. Y. & van Heyningen, W. E. (1951). *J. gen. Microbiol.* **5**, 110.
 Work, E. (1949). *Biochim. biophys. Acta*, **3**, 400.
 Work, E. (1951). *Biochem. J.* **49**, 17.
 Work, E. (1953*a*). *J. gen. Microbiol.* **9**, ii.
 Work, E. (1953*b*). *Proc. 6th Congr. int. Microbiol., Rome*, **1**, 161.
 Work, E. (1955). *Biochim. biophys. Acta*, **17**, 410.
 Work, E., Birnbaum, S. M., Winitz, M. & Greenstein, J. P. (1955). *J. Amer. chem. Soc.* **77**, 1916.
 Work, E. & Denman, R. F. (1953). *Biochim. biophys. Acta*, **10**, 183.
 Work, E. & Dewey, D. L. (1953*a*). *J. gen. Microbiol.* **9**, 394.
 Work, E. & Dewey, D. L. (1953*b*). *Proc. 6th Congr. int. Microbiol., Rome*, **1**, 50.