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

3. PROPERTIES AND DISTRIBUTION OF DIAMINOPIMELIC ACID RACEMASE, AN ENZYME CAUSING INTERCONVERSION OF THE LL AND *meso* ISOMERS

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A partially purified preparation of diaminopimelic acid decarboxylase from *Aerobacter aerogenes* was relatively specific for the *meso* isomer of diaminopimelic acid, but the crude enzyme in acetone-dried cells decarboxylated both *meso* and LL isomers, (Hoare & Work, 1955*a*). The initial step in the decarboxylation of LL-diaminopimelic acid was shown, in a preliminary communication, to be conversion into the *meso* isomer (Hoare, 1955). This paper describes experiments leading to recognition of this step and of the enzyme system responsible for it. The enzyme converts either the LL or the *meso* isomer into a mixture of the two forms and is called diaminopimelic acid racemase. Some properties of the enzyme in cell-free systems are described and compared with those of diaminopimelic acid decarboxylase. In order to assess the significance of diaminopimelic acid racemase in bacterial metabolism, its distribution in selected bacteria is examined and compared with that of diaminopimelic acid decarboxylase. An attempt is made to correlate these distributions with the known occurrence of the isomers of diaminopimelic acid in these bacteria (Work & Dewey, 1953; Hoare & Work, 1955*a*, 1957).

Preliminary reports have already presented some of these results (Hoare & Work, 1955*b*, 1956; Work, 1955).

METHODS

The three stereoisomers of diaminopimelic acid were prepared as described (Hoare & Work, 1955*a*, 1957).

Bacteria. Aero. aerogenes was grown as described by Hoare & Work (1955*a*). *Escherichia coli* ACTC 9637 was grown with aeration for 12 hr. at 37° on a similar medium; *Esch. coli* lysine auxotroph 26–26 (Davis, 1952) was grown as described by Work & Denman (1953), except that the L-lysine content of the medium was 30 mg./l. unless otherwise stated. Other bacterial cells were grown on suitable media. All cells were harvested by centrifuging, washed twice with water or saline and immediately dried with acetone at $-10°$. They were stored at $-10°$ over a desiccant.

Enzyme preparations and tests. All solutions of amino acids and buffers were made in glass-distilled water. Partially purified diaminopimelic acid decarboxylase was prepared from *Aero. aerogenes*, as described by Hoare & Work (1955*a*), and partially purified lysine decarboxylase was obtained from *Esch. coli* B (Hoare, 1956). Decarboxylase assays were carried out at 37° in N_2 in the Warburg apparatus; lysine decarboxylase was estimated at pH 5·5 with L-lysine (13 mM) as substrate; diaminopimelic acid decarboxylase was assayed at pH 6·8 with *meso*diaminopimelic acid (4 mM) as substrate (Dewey, Hoare & Work, 1954). For the estimation of diaminopimelic acid decarboxylase in bacterial cells, acetone-dried cells (40 mg.) suspended in 0·1 M phosphate buffer and pyridoxal phosphate (10^{-5} M) (final vol. 2·5 ml.) were used for each test, and blanks without substrate were included. When the gas output was slight, or the blank value was high, the deproteinized overnight reaction products (0·1–0·3 ml.) were examined for the decarboxylation products, lysine and cadaverine, by paper chromatography (Hoare & Work, 1957) and compared with the blank solutions similarly treated. If no lysine or cadaverine was found in the reaction products, diaminopimelic acid decarboxylase was assumed to be absent. When insufficient cells were available for quantitative tests, the reaction products were examined qualitatively by paper chromatography after incubation of cells with *meso*-diaminopimelic acid at pH 6·8, as described for diaminopimelic acid racemase tests.

Qualitative tests for diaminopimelic acid racemase were carried out on bacteria as follows: acetone-dried cells (3 mg.), suspended in 0·1 M borate buffer, pH 8·5 (0·3 ml.), were incubated with shaking at 37° with either *meso*- or LL-diaminopimelic acid (0·3 mg. in 0·15 ml. of water). Streptomycin was added to a final concentration of 30 μg./ml. to prevent bacterial contamination during overnight incubation. Samples (0·1 ml.) were transferred to ethanol (0·2 ml.) at times 0, 1 and 18 hr. (overnight) and the supernatants examined for diaminopimelic acid isomers by one-dimensional paper chromatography (Hoare & Work, 1957). If, after 1 hr., equal amounts of both LL- and *meso*-diaminopimelic acid were present in the reaction mixture, high racemase activity was indicated. When no isomer interconversion was found after incubation overnight, racemase was considered to be absent. The test was most conveniently carried out with *meso*diaminopimelic acid as substrate, because of the difficulty in detecting slight conversion of the LL into the *meso* isomer (Hoare & Work, 1957). When the cells contained very active diaminopimelic acid decarboxylase, it was necessary to use the LL isomer as substrate, because, in this case, even at pH 8·5, the *meso* isomer was completely decarboxylated to lysine (or cadaverine when lysine decarboxylase was also present) rather than partially racemized to the LL isomer. Under these conditions, racemase activity was indicated by conversion of the LL isomer

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Table 1. *Decarboxylation of* meso- *and* LL-*diaminopimelic acid (DAP) at* pH 6·8 *by acetone-dried bacteria*

Dried cells (40 mg.) were suspended in 2·5 ml. of 0·1 M phosphate buffer containing pyridoxal phosphate (10 μg.) and diaminopimelic acid (2 mg.); atmosphere, N_2: temp., 37°. $Q_{CO_2} = \mu$l. of CO_2/mg./hr. Organisms 1–4 were examined at the beginning of this work, and the others were studied later.

Organism and source*	Q_{CO_2} with *meso*DAP	Q_{CO_2} with LL-DAP	Lag with LL-DAP (min.)	Q_{CO_2} ratio: LL/*meso*
(1) *Aero. aerogenes*, Str. 1; Cambridge	7·8†	2·8	20	0·36
(2) *Esch. coli* B; Carnegie Inst., Washington	12·0†	4·5	10	0·38
(3) *Micrococcus lysodeikticus*; Cambridge	1·2	0·45	40	0·38
(4) *Sarcina lutea*; Cambridge	3·6	2·4	10	0·67
(5) *Staphylococcus citreus*, 36; London	3·3	0·8	0	0·24
(6) *Sporosarcina ureae*; Delft	9·3	0	—	—
(7) *Propionibacterium pentosaceum*; NCTC 8070	0·77	0·51	30	0·66
(8) *Bacillus cereus*; NRRL 569	0	0	—	—

* For definition of contractions see Table 5 and Hoare & Work (1957) Table 1.
† Lysine decarboxylase present.

into lysine or cadaverine; in the absence of racemase no decarboxylation products were detected.

Cell-free extracts containing diaminopimelic acid racemase were prepared at 2° from suspensions of *Esch. coli* 26–26 (1 g.) in 0·01 M phosphate buffer, pH 6·8 (40 ml.). Cells were disintegrated either by hand-grinding with Celite (Dewey *et al.* 1954) or by passage at 12 000 lb./in.^{2} through an apparatus described by Milner, Lawrence & French (1950). Cell residues were removed by centrifuging at 15 000 *g*, and the cell-free extract was either used immediately or stabilized by addition of $HgCl_2$ (0·14 mM) and stored at −10°. Tests of racemase activity were carried out in 0·1 M borate buffer (pH 8·5) with *meso*diaminopimelic acid (0·4 mg./ml.) as substrate. If $HgCl_2$ had previously been added, the enzyme was reactivated by addition of sodium sulphide (final concentration 14 mM) 15 min. before the test was carried out. Solutions were incubated at 37°, and after any three convenient times (e.g. 5, 10 and 20 min.) according to the activity of the enzyme preparation, samples (0·1 ml.) were transferred into ethanol (0·2 ml.) and the supernatants (0·1 ml.) were examined for diaminopimelic acid isomers by paper chromatography.

RESULTS

Recognition of an enzyme system converting LL-*diaminopimelic acid into the* meso *isomer*

Acetone-dried preparations of various bacteria were examined manometrically for decarboxylase activity towards the three stereoisomers of diaminopimelic acid. Organisms known to contain an active decarboxylase attacked both the LL and *meso* isomers (Table 1, nos. 1–4). The DD isomer was not attacked. The LL isomer was always decarboxylated at a lower rate, and there was often a preliminary lag period. The same decarboxylation products, lysine and cadaverine, were formed from both isomers, cadaverine being formed only when lysine decarboxylase was also present (organisms 1 and 2). The results with *Aero. aerogenes* have already been presented in detail in a previous paper, where the relative specificity for the *meso* isomer of the partially purified diaminopimelic acid decarboxylase was also illustrated (Hoare & Work, 1955*a*, Fig. 3 and Table 2). This difference in specificities of the crude and purified enzymes suggested that acetone-dried cells contained an enzyme system which resulted in the ultimate decarboxylation of the LL isomer, and which was almost absent from the partially purified decarboxylase. The presence of two enzymes acting on either the *meso* or the LL isomer was suggested by the variation in the relative rates of decarboxylation of the *meso* and LL isomers shown by different bacteria.

The possibility that decarboxylation of LL-diaminopimelic acid was brought about by L-lysine decarboxylase was discounted by the following facts. Both *meso*- and LL-diaminopimelic acid were decarboxylated by *Sarcina lutea* and *Micrococcus lysodeikticus*, although no lysine decarboxylase was found in either organism. Also, LL-diaminopimelic acid was not decarboxylated by preparations of lysine decarboxylase free from diaminopimelic acid decarboxylase. Such preparations were acetone-dried *Bacillus cadaveris* (Gale & Epps, 1944) or partially purified lysine decarboxylase from *Esch. coli* B (Table 2).

In an attempt to separate the two enzymes acting on diaminopimelic acid in *Aero. aerogenes*, the cell-free extract was subjected to the purification procedure used for *meso*diaminopimelic acid decarboxylase (Hoare & Work, 1955*a*), all fractions being assayed manometrically for decarboxylation of the LL or *meso* isomer. The rate of decarboxylation of the LL isomer was considerably decreased, and the lag period increased, by prolonged dialysis against 10^{-4} M dimercaptopropanol (BAL). Protamine treatment resulted in loss of activity towards the LL isomer, both from the precipitate and the supernatant. This attempt at separation was abandoned and an alternative source of the enzyme attacking only the LL isomer was sought.

Table 2. *Specificity of lysine decarboxylase*

A standard reaction was carried out in Warburg apparatus at pH 5·5, with 6 mg. of lysine hydrochloride or 2 mg. of diaminopimelic acid (DAP). Atmosphere, N_2; temp., 37°.

Enzyme				
Source	Amount (mg.)	Substrate	Time (min.)	Gas output (µl.)
*B. cadaveris** (cells)	20	L-Lysine	5	135
	40	LL-DAP	40	0
	40	*meso*DAP	40	0
Esch. coli B (purified)	1	L-Lysine	10	85
	1	L-Lysine + LL-DAP	10	92
	1	LL-DAP	30	2
	1	*meso*DAP	30	0

* Provided by Dr A. Meister.

For this purpose an examination was made of the lysine auxotroph of *Esch. coli* 26–26 (grown on medium containing 10 mg. of lysine/l.), which lacks diaminopimelic acid decarboxylase (Dewey & Work, 1952). In experiment 1 (Fig. 1), LL-diaminopimelic acid (2 mg.) was incubated for 5·5 hr. at 37° in a Warburg flask containing acetone-dried *Esch. coli* 26–26 (40 mg.) suspended in buffer at pH 6·8 (2·5 ml.) as in a standard decarboxylase assay. A control flask contained boiled cell suspension. No gas evolution occurred in either flask. The reaction mixtures were deproteinized by heating at 100° for 5 min. and centrifuging; similar concentrations of diaminopimelic acid were found in each supernatant solution on examination of portions (0·1 ml.) by paper chromatography in phenol–ammonia. (The system for separation of isomers was not yet developed.) The remainder of each solution was evaporated to dryness at 50°, dissolved in 0·05M phosphate buffer, pH 6·8 (0·5 ml.), and placed in the side bulbs of Warburg flasks, the main compartments of which contained buffered solutions of partially purified diaminopimelic acid decarboxylase. A control flask contained untreated LL-diaminopimelic acid. The LL-diaminopimelic acid which had been previously incubated with unboiled mutant cells was decarboxylated to lysine, while the untreated sample, or that incubated with boiled cells, was not significantly decarboxylated. In Expt. 2, LL-diaminopimelic acid was added to flasks containing *Esch. coli* 26–26, or purified diaminopimelic acid decarboxylase, or a mixture of the two preparations. Significant decarboxylation occurred only when both preparations were present, but in this case there was a preliminary lag compared with the control flask where *meso* isomer was decarboxylated. Previous experiments had demonstrated the complete absence of any decarboxylase activity against *meso* isomer in *Esch. coli* 26–26 when it was

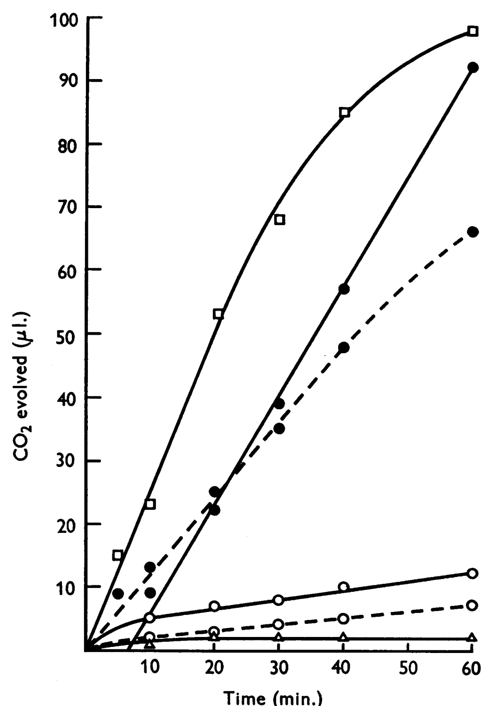


Fig. 1. Manometric demonstration of conversion of LL-diaminopimelic acid into the *meso* isomer by *Esch. coli* 26–26. Incubations were carried out in Warburg flasks at 37°, with enzyme in 0·1M phosphate buffer, pH 6·8 (2 ml.), and diaminopimelic acid (2 mg. in 0·4 ml.) was tipped in from the side arm. Expt. 1 (interrupted lines). ○, LL Isomer tested with partially purified diaminopimelic acid decarboxylase (5 mg.); ●, LL isomer, preincubated with *Esch. coli* 26–26 (40 mg.) for 5·5 hr., boiled, concentrated and tested with decarboxylase. Expt. 2 (solid lines). ○, LL Isomer + decarboxylase (5 mg.); △, LL isomer + *Esch. coli* 26–26 (40 mg.); ●, LL isomer + decarboxylase + *Esch. coli*; □, *meso* isomer + decarboxylase.

examined, either as a suspension of acetone-dried cells or as a cell-free extract; neither did these cell preparations inhibit or enhance activity of crude or purified diaminopimelic acid decarboxylase preparations.

Properties of diaminopimelic acid racemase in Esch. coli 26–26

These experiments suggested that the apparent decarboxylation of LL-diaminopimelic acid was due to two enzymes. One enzyme, referred to below as diaminopimelic acid racemase, converted the LL isomer into the *meso* form; the other enzyme, diaminopimelic acid decarboxylase, acted on the *meso* isomer. It was also shown that *Esch. coli* 26–26 was a source of racemase free from decarboxylase. However, before further investigations on diaminopimelic acid racemase could be made, a method of assay had to be found. Quantitative decarboxylation of the *meso* isomer formed from the LL isomer could not be used, because a preparation of decarboxylase specific to the *meso* isomer could not be conveniently made (Hoare & Work, 1955*a*) and all bacteria examined at that time decarboxylated both isomers or neither one.

Assay. When it was found that the chromatographic solvent methanol–water–pyridine separated LL- and *meso*-diaminopimelic acids (Rhuland, Work, Denman & Hoare, 1955), it was possible that the action of diaminopimelic acid racemase might be demonstrated by paper chromatography. An overlap between lysine and diaminopimelic acid prevented application of the reaction to any systems which contained diaminopimelic acid decarboxylase in addition to the racemase. Addition of hydrochloric acid to the solvent system produced separation of both diaminopimelic acid isomers from lysine and cadaverine, and provided a system for the qualitative assay of racemase, applicable even in the presence of decarboxylase. When an attempt was made to examine, by paper chromatography, the action of the racemase in *Esch. coli* 26–26, it was found that the 'enzyme blank', with no added substrate, gave strong spots of both *meso*- and LL-diaminopimelic acid. This was due to the high intracellular concentration of these diaminopimelic acid isomers which had been built up by *Esch. coli* 26–26 during growth on the medium containing only 10 mg. of lysine/l. (Hoare & Work, 1955*a*). Since no intracellular diaminopimelic acid accumulated when the organism was grown on lysine in concentrations of 30 mg./l. or over, such cells were tested as a source of racemase. They proved satisfactory, being free from decarboxylase and intracellular diaminopimelic acid.

The pH optimum of the racemase in these acetone-dried cells of *Esch. coli* 26–26 was between 8 and 8·5 in both phosphate and borate buffers. The enzyme in acetone-dried cells and cell-free extracts acted on either LL- or *meso*-diaminopimelic acid, producing identical final reaction mixtures containing approximately equal amounts of each isomer, as shown by comparison with known amounts of LL- and *meso*-diaminopimelic acids (Fig. 2). Also, no LL-diaminopimelic acid was formed from the DD isomer, indicating that the DD form was not racemized to the *meso* isomer, since further racemization would have then converted the *meso* into the LL form. No inhibition by the DD isomer was observed, even in concentration five times that of the substrate. When fresh cell-free extracts of these cells were incubated at pH 8·5 with *meso*diaminopimelic acid under the conditions described in methods for the standard racemase test, activity was detectable after 3 min.; after 10 min. the reaction was complete, as judged by the relative intensities of the LL- and *meso*-diaminopimelic acid spots in the reaction mixture.

Activation and inhibition. Freshly prepared extracts were not activated by thiols such as BAL, 2-mercaptoethanol, glutathione (all at 10^{-4} M) or by sodium sulphide (10^{-2} M). Storage at $-10°$ for 24 hr. resulted in a partial loss of activity which was completely reversed by thiols or sodium sulphide;

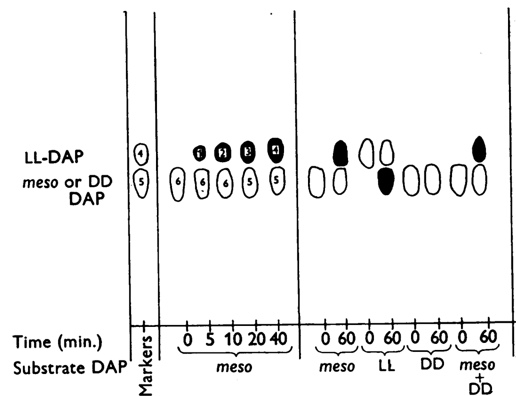


Fig. 2. Racemization of diaminopimelic acid by cell-free extracts of *Esch. coli* 26–26. The extract (0·5 ml.) was prepared as described in Methods; it was incubated in 0·1 M borate buffer, pH 8·5 (0·5 ml.), with diaminopimelic acid isomer (0·5 mg.). Samples (0·1 ml.) were removed at specified times and deproteinized with ethanol (0·2 ml.); the supernatant (0·1 ml.) was examined by chromatography on Whatman no. 1 paper in methanol–water–10N-HCl–pyridine (80:17·5:2·5:10, by vol.). The spots were developed with ninhydrin in acetone (0·1%, w/v). Black spots indicate reaction products, unshaded spots substrate; numbers on spots indicate colour strength on arbitrary scale varying from 1 (just visible) to 6 (maximum intensity). Markers of *meso*- and LL-diaminopimelic acid (10 μg.).

longer storage resulted in gradually increasing inactivation, which eventually became irreversible after about 4 days. Dialysis produced loss of activity unless carried out against comparatively high concentrations (10^{-3}M) of BAL, when some activity was retained overnight. Additions of pyridoxal phosphate, α-oxoglutarate, boiled extracts of rat liver or *Aero. aerogenes* were all without effect on the activities of fresh, stored or dialysed racemase preparations.

The effects of inhibitors on freshly prepared cell-free extracts of diaminopimelic acid racemase are shown in Table 3. The enzyme is a 'sulphydryl' enzyme, being inhibited by low concentrations of –SH-binding reagents (such as *p*-chloromercuribenzoates or heavy metals) and afterwards reactivated by thiols. This probably accounts for the instability towards storage. Inhibition by carbonyl-binding reagents, such as hydroxylamine, hydrazine and semicarbazide, suggests that a carbonyl group is essential for enzyme activity. The inhibitory action of all these reagents, as well as of *iso*nicotinic acid hydrazide, was prevented or reversed by the presence of thiols (10^{-4}M), but not by pyridoxal phosphate. There was no inhibition by potassium cyanide at concentrations as high as 10^{-2}M, even after pre-incubation of the enzyme with it at pH 6·0, 6·8 or 8·5. 5′-Deoxypyridoxal phosphate was without effect, nor did the chelating agents 8-hydroxyquinoline or ethylenediaminetetraacetic acid inhibit racemization. D- or L-Lysine did not cause inhibition.

Attempts at purification. The instability of diaminopimelic acid racemase in cell-free extracts prevented any fractionation of the free enzyme; for example, even in the presence of 10^{-3}M BAL, activity was lost on attempted fractionation with magnesium or ammonium sulphates or with acetone. At the suggestion of Dr Emil Smith, the enzyme was stabilized as a mercury derivative; the mercury salt (Hg racemase) was easily dissociated by 10^{-2}M sodium sulphide or by 10^{-4}M thiols, and was stable to storage at −10° for 4 months. All fractionation operations were carried out on the Hg racemase, with reactivation by sodium sulphide before testing. The Hg racemase was stable to dialysis against 10^{-2}M phosphate buffer, pH 5·8–6·8 at +2° for 24 hr., but lost considerable activity on 3 days' dialysis and was not then reactivated by pyridoxal phosphate.

Purification by acetone fractionation of protamine-treated extracts, the method used for diaminopimelic acid decarboxylase, could not be used on fresh racemase or Hg racemase. Precipitation of nucleic acid from extracts containing Hg racemase by excess of protamine sulphate (2 mg./ml.) resulted in loss of activity from the solution, and the precipitate could not be redissolved. By halving the amount of protamine used, about half the nucleic acid and protein were precipitated, as indicated by change in optical densities at 280 and 260 mμ, and the solution retained all its activity. Acetone fractionation of this solution did not produce clear-cut separations, probably owing to the presence of nucleic acids. All activity was lost by precipitation with ammonium sulphate. The use of streptomycin (10 mg./ml. of extract) as a nucleic acid precipitant also removed all racemase from solution, but in this case the precipitate could be redissolved in borate buffer (pH 8·5) and all activity recovered. Acidification of Hg racemase solutions to pH 4·8 produced a heavy precipitate, which, after washing with water, could be redissolved by raising the pH to 6·0 or above, and contained full racemase activity. Neither acid nor streptomycin precipitation removed any nucleic acid from the racemase, as shown by the constancy of the ratios of optical densities at 280 and

Table 3. *Effect of inhibitors on diaminopimelic acid racemase in fresh cell-free extract of* Esch. coli 26–26

The test was carried out as described in Methods. In the absence of inhibitor, racemization was complete in 20 min.; complete inhibition was shown by the presence of only one isomer of diaminopimelic acid in the reaction mixture after the time shown.

Inhibitor	Concn. (M)	Reaction time (min.)	Inhibition
Mercuric chloride	10^{-4}	60	Complete
Cupric sulphate	10^{-5}	60	Complete
p-Chloromercuribenzoate	10^{-3}	60	Complete
EDTA	10^{-3}	120	None
Oxine	10^{-4}	40	None
Cyanide	10^{-2}	40	None
Hydroxylamine	10^{-3}	120	Complete
Hydrazine	10^{-2}	120	Partial
Semicarbazide	10^{-2}	120	Partial
*iso*Nicotinic acid hydrazide*	10^{-3}	120	Partial
	10^{-2}	120	Complete
Deoxypyridoxal phosphate	10^{-4}	120	None

* Pre-incubated with enzyme for 30 min.

260 mμ. These facts suggest that in *Esch. coli* 26–26 diaminopimelic acid racemase may exist in close association with nucleic acid.

The pH optimum of the racemase after storage as the Hg salt was very similar to that of the enzyme tested in acetone-dried cell suspensions, being between about 7·7 and 8·3. The alkalinity of sodium sulphide made it unsuitable for reactivation of the Hg racemase for the pH investigations, so BAL (10^{-4} M) was used. This proved to be slightly acid, but the pH of the final reaction mixture was always checked. Slight racemase activity was observed at pH of 9·1 or 6·0, and at pH 6·8 the activity was only slightly lower than at pH 8·3. Unfortunately the method of estimation does not allow a true pH–activity relationship to be established.

Diaminopimelic acid racemase, purified by streptomycin precipitation, was examined for alanine racemase activity as follows. The streptomycin-precipitated Hg racemase (10 mg.) dissolved in 0·1 M aminotrishydroxymethylmethane (tris) buffer, pH 7·3 (1 ml.), was mixed with 0·1 M sodium sulphide (0·7 ml.). A portion (0·4 ml.) was tested over 40 and 100 min. with *meso*diaminopimelic acid (0·2 mg.) for diaminopimelic acid racemase. To the remainder, L-alanine (0·175 mg.) and pyridoxal phosphate (10 μg.) were added; a portion of the reaction mixture was removed immediately as a control and immersed for 5 min. in boiling water. The remaining preparation was incubated at 37° for 40 or 100 min. and then boiled. Protein was removed by centrifuging from the control and incubates, and each supernatant (0·4 ml.) was treated with a dialysed solution (0·15 ml.) of L-amino acid oxidase from *Crotalus adamanteus* (rattlesnake) venom (20 mg. of dried venom per ml. of 0·1 M tris buffer, pH 7·3). Both mixtures were gassed with oxygen, corked and shaken at 37° for 3 hr., after which samples (0·2 ml.) were deproteinized with ethanol and examined by one-dimensional paper chromatography. The results showed that after 40 min. incubation with racemase preparation a small amount of L-alanine had been converted into the D isomer, since there was a faint alanine spot left after treatment with L-amino acid oxidase, whereas in the boiled control sample all the alanine had disappeared after incubation with oxidase. Considerably more D-alanine was apparent when the incubation period was extended to 100 min. but racemization was not yet complete. Diaminopimelic acid was completely racemized in 40 min. by the racemase preparation. This showed that, although alanine racemase was present, it was less active than diaminopimelic acid racemase.

Comparison of inhibition of diaminopimelic acid racemase and decarboxylase in normal bacterial cells

Diaminopimelic acid racemase resembled diaminopimelic decarboxylase in being inhibited by most reagents which bind either thiol or carbonyl groups; the racemase differed from decarboxylase in its stability to potassium cyanide and in its

Table 4. *Comparative effects of inhibitors on diaminopimelic acid decarboxylase and racemase acting simultaneously*

Expt. *A*, *Esch. coli* ACTC 9637, 10 mg. as cell suspension. Expt. *B*, *Aero. aerogenes*, 5 mg. as cell-free extract. Incubations were carried out at 37° in 0·1 M buffer (0·6 ml.) at the pH shown, with 0·2 mg. of either *meso*- or LL-diaminopimelic acid (DAP). Samples (0·2 ml.) were transferred at times shown to 0·4 ml. of ethanol. A portion (0·1 ml.) of supernatant liquid was examined by paper chromatography with methanol–water–pyridine–HCl solvent. Reaction products are indicated by strength, judged by eye, of ninhydrin spots, varying from + + + (maximum strength) to + − (just visible); − indicates not visible.

Expt.	Inhibitor (M)	pH	Substrate DAP	Time (min.)	Reaction products *meso*DAP	LL-DAP	Lysine
A	.	8·5	LL	15	+	+	+ +
	.	8·5	LL	60	−	−	+ + +
	.	6·8	*meso*	15	+ −	+ −	+ +
	.	6·8	*meso*	60	−	−	+ + +
	KCN, 10^{-2}	8·5	LL	15	+	+	+ −
	KCN, 10^{-2}	8·5	LL	60	+	+	+
	KCN, 10^{-2}	6·8	*meso*	15	+	+	+ −
	KCN, 10^{-2}	6·8	*meso*	60	+ −	+ −	+ +
	$CuSO_4$, 10^{-5}	8·5	LL	15	−	+ +	−
	$CuSO_4$, 10^{-5}	8·5	LL	60	+ −	+ +	−
	$CuSO_4$, 10^{-5}	6·8	*meso*	15	+ +	−	+ −
	$CuSO_4$, 10^{-5}	6·8	*meso*	60	+ +	−	+ −
B	.	8·5	LL	120	+	+	+ −
	.	6·8	*meso*	120	+ −	+ −	+ +
	NH_2OH, 10^{-3}	8·5	LL	120	−	+ + +	−
	NH_2OH, 10^{-3}	6·8	*meso*	120	+ + +	−	−
	NH_2OH + BAL, 10^{-3}	8·5	LL	120	+	+	−
	NH_2OH + BAL, 10^{-3}	6·8	*meso*	120	+	+	−

insensitivity to carbonyl-binding reagents acting in the presence of added thiols. It was possible that these differences might have been due to the different sources of the two enzymes, since the racemase was obtained from cells of a mutant *Esch. coli*, the abnormal constituents of which might have interfered with added inhibitors. Accordingly, the two enzymes were investigated simultaneously in preparations of normal bacterial cells, either *meso*-diaminopimelic acid at pH 6·8 or LL-diaminopimelic acid at pH 8·5 being used as substrate (Table 4). In the absence of inhibitors, the net result was conversion of either isomer of diaminopimelic acid into lysine; this was due to the overlapping pH ranges of the two enzymes and to the fact that the equilibrium mixture produced by the racemase was continually changed by decarboxylation of the *meso* isomer. However, by examination of reaction products at specified times, it was possible to investigate inhibition of either enzyme. *Esch. coli* 9637, the parent strain of mutant *Esch. coli* 26–26, at pH 8·5 completely converted LL-diaminopimelic acid into lysine in 1 hr., whereas after 15 min. the reaction products included both *meso*diaminopimelic acid and some unchanged LL isomer. At pH 6·8, *meso*-diaminopimelic acid was mostly decarboxylated to lysine, but a small amount had been racemized to the LL isomer after 15 min. In the presence of 10^{-2} M potassium cyanide racemase was not inhibited, as shown by the interconversion of the diaminopimelic acid isomers at either pH, but the decarboxylase was partially inhibited, since lysine formation was reduced, especially after 15 min. In contrast, cupric sulphate (10^{-5} M) caused almost complete inhibition of racemase and a considerable inhibition of decarboxylase. In cell-free extracts of *Aero. aerogenes*, both racemase and decarboxylase were completely inhibited by 10^{-3} M hydroxylamine; with BAL (10^{-3} M) also present, racemization, but not decarboxylation, occurred.

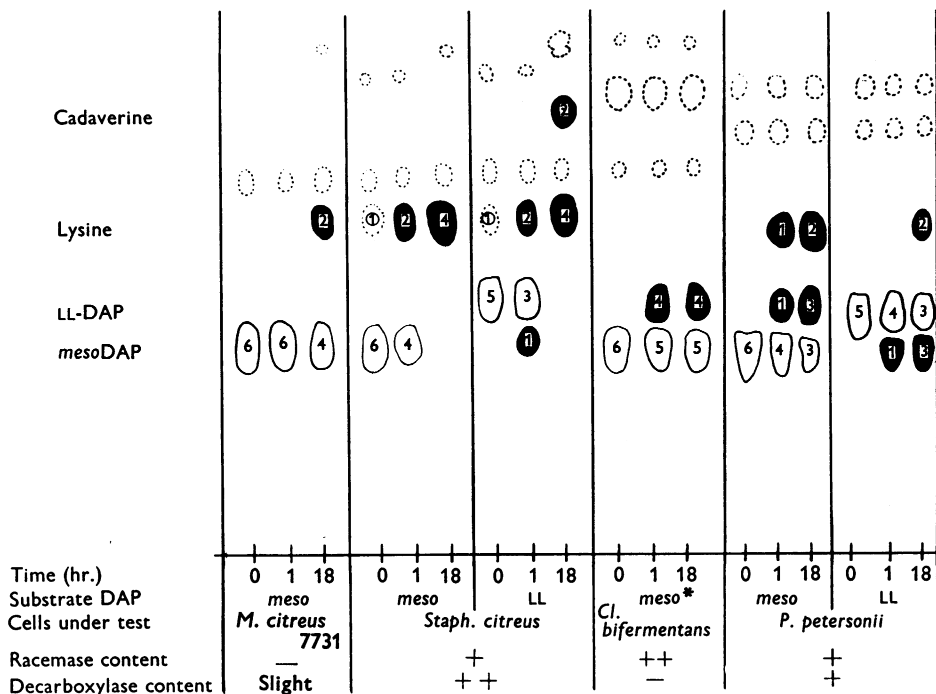


Fig. 3. Some typical tests for diaminopimelic acid racemase on various species of bacteria. Cells (3 mg.) were incubated at 37° with one isomer (LL or *meso*) of diaminopimelic acid (DAP) as substrate (3 mg. in 0·15 ml. of water) in 0·1 M borate buffer, pH 8·5 (0·3 ml., containing 30 μg. of streptomycin). Samples (0·1 ml.) were taken at the times specified and deproteinized with ethanol (0·2 ml.); the supernatant (0·1 ml.) was examined by chromatography in methanol–water–10 N-HCl–pyridine (80:17·5:2·5:10, by vol.) on Whatman no. 1 paper. The spots were developed with ninhydrin in acetone (0·1 %, w/v). Dotted spots indicate intracellular amino acids (unrelated to test). Unshaded spots indicate substrate, and black spots indicate reaction products; numbers on spots indicate colour strength of spot on an arbitrary scale, varying from 1 (just visible) to 6 (maximum intensity). The units of racemase activity, as in Table 5, are: + +, complete racemization in 1 hr.; +, partial racemization in 1 hr.

* With LL-DAP reaction products also identical at 1 and 18 hr.

Table 5. *Diaminopimelic acid decarboxylase and racemase contents of various bacteria in relation to diaminopimelic acid (DAP) isomer distributions*

Enzyme tests were as described in Methods. The contents of decarboxylase are indicated as Q_{CO_2} ($=\mu$l./mg./hr.) or, when detected by chromatography, as + or sl. Racemase contents, indicated as degree of racemization: + +, complete in 1 hr.; +, partial in 1 hr.; sl., only detectable after 18 hr.

Organism	Strain and source*	Growth conditions: Medium†	Temp.	Time (days)	Decarboxylase (Q_{CO_2})	Racemase	DAP
Micrococcaceae							
Staphylococcus aureus	Oxford	A	37°	3	0·8	.	0
	Oxford	D	37	2	2·9	+ +	0
Staph. citreus	36 (London)	C	37	0·75	3·2	+	0
Micrococcus citreus	NCTC 7731	C	37	0·75	sl.	sl.	*meso*
	NCTC 7731	C	37	1	0	0	.
M. varians	NCTC 7281	C	37	1·3	0·8	+ +	*meso*
M. lysodeikticus	Cambridge	A	25	3	3·0	+ +	0
	Cambridge	B	25	3	3·8	.	0
Sarcina lutea	Cambridge	A	25	3	2·7	+ +	0
	Cambridge	B	25	3	5·8	+ +	0
Sporosarcina urea	Delft	E	Room	2	6·9‡	0	0
	Edinburgh	E	Room	2	3·1	0	0
Lactobacillaceae							
Strep. faecalis	R Cambridge	G2	37	4	0	0	0
	ATCC 9790	G1	37	3	0	0	0
	ATCC 9790	H	37	3	0	0	0
Strep. lactis	NCIB 8586	J	37	1	0	0	0
Leuconostoc mesenteroides	P 60 Cambridge	G2	37	1–5	0	0	0
	NCIB 6990	K	25	1	0	0	0
Ln. citrovorum	NCIB 7837	K	25	1	0	0	0
Microbacterium lacticum	NCTC 8541	G1	30	4	1·1	+	0
Lactobacillus plantarum (syn. *Lb. arabinosus*)	ATCC 8014	G2	37	1–5	0	+	*meso*
Lb. casei	ATCC 7469	G2	37	2	0·5	sl.	0
Lb. brevis	L3 Birmingham	L	30	2	0	sl.	0
	L4 Birmingham	L	30	1	0	0	0
Propionibacterium arabinosum	NCTC 5958	M	30	3	1·4	+	LL
Pr. jensenii	NCTC 8069	N	30	3	+	+ +	LL
	NCTC 8071	M	30	3	sl.	sl.	LL
Pr. pentosaceum	NCTC 8070	N	30	3	1·5	+ +	LL
	van Niel	M	30	3	2·7	+ +	LL
Pr. petersonii	NCTC 5962	M	30	4	+	+	LL
Pr. thoenii	NCTC 5966	M	30	4	sl.	0	LL
	NCTC 8072	M	30	3	sl.	0	LL
Pr. zeae	NCTC 5967	N	30	3	3·3	+ +	LL
Bacillaceae							
Bacillus subtilis	Cambridge	F	37	.	0	+	*meso*
B. cereus	NRRL 569	F	37	1	0	+ +	*meso*
Clostridium butyricum	NCTC 7423	P	37	2	0	0	*meso*
Cl. novyi (syn. *Cl. oedematiens*)	Albiston LIPM	Q	35	1	0	0	*meso*
	Albiston LIPM	Q	35	3	0	0	*meso*
Cl. acetobutyricum	UCL	P	.	.	0	.	*meso*
Cl. sporogenes	NCTC 532	P	37	3	0	0	*meso*
Cl. bifermentans	NCTC 2914	P	37	1	0	+ +	*meso*
Cl. perfringens (syn. *Cl. welchii*) A	S107 LIPM	R	35	0·35	0	+	LL
	S107 LIPM	P	35	0·7	0	+	LL
Cl. tetani	S279 LIPM	P	35	3	2·6	sl.	*meso*
Actinomycetales							
Mycobacterium phlei	NCTC 8151	S	37	7	0·2	+	*meso*
Myco. stercoris	NCTC 8159	S	37	7	0·5	+	*meso*
Myco. smegmatis	NCTC 3821	S	37	7	0·2	+	*meso*
Actinomyces	Aerobic sp.	F	30	5	0	0	LL
Nocardia asteroides	UCHMS	G2	37	2	0·7	+	*meso*

* For most abbreviations see Hoare & Work, 1957, table 1. Others are: London, Dept. of Bacteriology, London Hospital. Edinburgh, Edinburgh and East of Scotland College of Agriculture. Birmingham, Dept. of Applied Biochemistry, The University. Delft, Microbiological Laboratory, Technical High School. ATCC, American Type Culture Collection.

† Growth media were as follows: (Figures in parentheses indicate %, w/v, except for R and S). A, Nutrient agar; B, glucose (1) nutrient agar; C, serum (5), glucose (1), nutrient agar; D, CCY agar (Gladstone & Fildes, 1940); E, urea (0·5), peptone (1), Lab Lemco (1); F, nutrient broth; G1, glucose (1), nutrient broth; G2, glucose (2), nutrient broth; H, blood (1), glucose (1), nutrient broth; J, glucose (0·5), tryptone (1), yeast extract (1), K_2HPO_4 (0·5); K, glucose (0·5), tryptone (1), yeast extract (1), K_2HPO_4 (0·5), tomato juice (20); L, wort broth; M, yeast extract (0·2), sodium lactate (2); N, yeast extract (0·2), sodium lactate (2), salts; P, cooked meat broth; Q, sodium sulphate meat broth; R, van Heyningen (1948); S, Dubos & Davis (1946).

‡ Increased by storage.

Distribution of diaminopimelic acid racemase and decarboxylase in certain bacteria

The activities of diaminopimelic acid racemase and decarboxylase were determined in various Micrococcaceae, Lactobacteriaceae, Bacillaceae and a few Actinomycetales. Some examples of typical racemase tests on acetone-dried organisms are shown in Fig. 3. The results are summarized in Table 5, and presented with the diaminopimelic acid isomer content of the organisms, reported in the preceding paper (Hoare & Work, 1957). Since no systematic investigation of the effect of growth conditions on enzyme activity was made, the conditions of growth have been included in the table. Both enzymes were present in many, but not all, organisms examined. There was no relation between racemase content and isomer distribution; thus *Clostridium welchii* had a racemase activity similar to that of other clostridia, although it differed in containing only LL-diaminopimelic acid. Various degrees of racemase activities were found in other clostridia, all of which contained *meso*diaminopimelic acid. With the exception of *Cl. tetani*, no clostridia or bacilli examined contained measurable amounts of diaminopimelic acid decarboxylase, there being no detectable decarboxylation of either the LL or *meso* isomer (Table 1, no. 8).

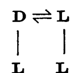
Many of the Micrococcaceae, which contained no diaminopimelic acid, had very active diaminopimelic acid racemase and decarboxylase. Two strains of *Micrococcus*, examined because they were found by Cummins & Harris (1956) to have diaminopimelic acid in their cell walls, contained the *meso* isomer, but had less racemase and decarboxylase than the staphylococci. Considerable variations in the decarboxylase contents of Micrococcaceae resulted from changes of culture medium, the richer the medium in nutrients and the higher the cell yield, the higher were the resultant decarboxylase levels. Since low yields were obtained of the two micrococci which contained diaminopimelic acid, it is possible that the low enzyme activities were a consequence of poor growth. *Sporosarcina ureae* was without racemase activity, although high decarboxylase levels were found in both strains; the activity rose on storage of cells at $-10°$, probably owing to loss of an enzyme competing for the substrate. No detectable decarboxylation of the LL isomer occurred (Table 1, no. 6).

The Lactobacteriaceae fell into two main groups, the coccal organisms—streptococci and leuconostocs—which contained no detectable racemase, decarboxylase or diaminopimelic acid; and the rod-shaped bacteria, nearly all of which contained one or both of these enzymes and, in certain cases, also diaminopimelic acid. Thus the propionibacteria, all having high levels of LL-diaminopimelic acid, contained levels of racemase varying from very high to undetectable. All propionibacteria showed decarboxylase activities when tested chromatographically; quantitative estimation of activity was often difficult to carry out, because of very high endogenous gas production; this did not occur with other organisms. Among the closely related lactobacilli and microbacteria, only *Lb. arabinosus* contained diaminopimelic acid (the *meso* isomer), yet most of them contained racemase or decarboxylase. Among the Actinomycetales, the three mycobacteria examined contained low racemase and decarboxylase activities, as did *Nocardia asteroides*. An aerobic actinomycete, containing LL-diaminopimelic acid, had no detectable racemase or decarboxylase.

DISCUSSION

Several bacterial amino acid racemases are known; they all act on both optical isomers and produce racemic mixtures. Alanine racemase is widely distributed among bacteria, but has not been found in other micro-organisms or in animal tissues; its pH optimum is about 8·5; it requires pyridoxal phosphate for activation, even in acetone-dried cell preparations (Wood & Gunsalus, 1951; Marr & Wilson, 1954). Glutamic acid racemase in *Lb. arabinosus* also has pH optimum of 8, but there are conflicting reports as to its requirement for pyridoxal phosphate (Ayengar & Roberts, 1952; Narrod & Wood, 1952). Threonine racemase in *Esch. coli* requires ATP or AMP as a cofactor, and there is no mention of a pyridoxal phosphate requirement (Amos, 1954). Racemization *in vitro* of amino acids was produced by heating at pH 10 with pyridoxal and metal salts; at pH 5, transamination, but no direct racemization, occurred (Olivard, Metzler & Snell, 1952).

Any consideration of racemization of diaminopimelic acid is complicated by the fact that the amino acid has two asymmetric carbon atoms; it has three isomeric forms, since the *meso* form is symmetrical about the γ-carbon atom. The enzyme which catalyses the interconversion of *meso* and LL-diaminopimelic acids has been called diaminopimelic acid racemase. The reaction catalysed may be written diagrammatically as



Thus the intramolecular rearrangement produced by the enzyme involves the groups attached to only one of the asymmetric carbon atoms; this carbon may be in the D or the L configuration. The other asymmetric carbon atom must be in the L configuration, since the DD isomer is not attacked. Although

no accurate measurements were made on the composition of the equilibrium mixture, paper chromatography showed that it consists of an approximately equal mixture of the LL and *meso* isomers. Since the reaction product is not a racemic mixture of optical antipodes, it might be argued that the enzyme is not a racemase in the usual sense of the word; true racemization does, apparently, occur at one of the two asymmetric centres. The properties of diaminopimelic acid racemase, particularly the alkaline pH optimum, are similar to those of the other bacterial amino acid racemases. The possibility that it might be the same enzyme as one of the other racemases with a broad specificity has not been entirely eliminated, even for alanine racemase; however, alanine racemase occurs in streptococci (Wood & Gunsalus, 1951) from which diaminopimelic acid racemase is missing, so it is probable that the two enzymes are different.

*meso*Diaminopimelic acid is a substrate for the two enzymes, diaminopimelic acid racemase and decarboxylase. Until this wide survey of distribution of these two enzymes was undertaken, we were unable to determine whether diaminopimelic acid decarboxylase had an absolute specificity for the *meso* isomer or whether it also acted very slowly on the LL isomer. Fractionated decarboxylase preparations had slight activity towards the LL isomer (Hoare & Work, 1955*a*), and no bacteria had been then found which possessed an active decarboxylase but no racemase. However, the *Sporosarcina* species, on which we report in this paper, contain no diaminopimelic acid racemase and have strong decarboxylase activity towards only *meso*diaminopimelic acid (Table 1). The absolute specificity of the decarboxylase is thus proved in this organism. It is interesting to note that the point of attack of both racemase and decarboxylase on *meso*diaminopimelic acid is at the asymmetric centre in the D configuration, the products being LL-diaminopimelic acid and L-lysine respectively. The L configuration for the other asymmetric centre appears to be necessary for enzyme activity. It is therefore not surprising that D-lysine and DD-diaminopimelic acid do not inhibit either enzyme. As far as specificity to homologues is concerned, diaminopimelic acid decarboxylase has no activity against other diaminodicarboxylic acids (Dewey *et al.* 1954); the specificity of the racemase is not known, because the method of testing activity cannot be applied to other amino acids as their isomers cannot be resolved by paper chromatography. Both diaminopimelic acid racemase and decarboxylase are sulphydryl enzymes; the racemase is the more sensitive to oxidation but can be stabilized as its mercury salt. Direct proof is not available as to whether pyridoxal phosphate is involved in racemase function. The inhibition of racemase by aldehyde-binding reagents resembles that of diaminopimelic acid decarboxylase, which is activated by pyridoxal phosphate (Hoare, 1956). The stability of racemase to potassium cyanide cannot be explained, since this reagent is generally an efficient inhibitor of enzymes activated by pyridoxal phosphate. The reversal by thiols of the inhibition of racemase by *iso*nicotinic acid hydrazide and carbonyl-binding reagents is also unexplained; it does not occur with diaminopimelic acid decarboxylase. A similar reversal of *iso*nicotinic acid hydrazide inhibition of cysteine sulphinic acid decarboxylase was noted by Davison (1956); this enzyme is a 'sulphydryl' enzyme requiring pyridoxal phosphate.

In the study of the distribution of these two enzymes known to metabolize diaminopimelic acid, only a few families of Gram-positive Eubacteriales were examined in detail. These families were selected because the commonly occurring *meso*-diaminopimelic acid was not invariably present; thus in the Micrococceae and the majority of Lactobacteriaceae (propionibacteria excepted) no diaminopimelic acid occurs, while among the Bacillaceae an exception was *Cl. welchii*, with its content of LL-diaminopimelic acid. It was hoped that some clue might be obtained as to the reason for this distribution, but this was not found, except in the Streptococcaceae. The enzyme contents of organisms containing *meso*diaminopimelic acid did not differ in any one respect from those containing the LL isomer.

No full investigation of the effect of phase of growth on enzyme activities has been made; it is known that different species differ markedly in this respect, as with diaminopimelic acid decarboxylase contents of *Esch. coli* and *Aero. aerogenes* (Dewey *et al.* 1954). In the staphylococci, improvement in growth medium raised the activity of diaminopimelic acid decarboxylase. Since it is possible that some losses of enzyme activities might have occurred through the use of unsuitable growth conditions, it might be wiser to place more reliance on the positive results of enzyme activity rather than on the odd negative anomaly. However, when nearly all members of a family or genus lack a given enzyme, it is felt that more importance can be attributed to the results. Thus, among the Bacillaceae, diaminopimelic acid decarboxylase is absent from all except *Cl. tetani* (*B. cadaveris* was not included in Table 5; it had a very active lysine decarboxylase but no diaminopimelic acid decarboxylase, see Table 2). The Streptococcaceae also provide a consistent group, containing neither diaminopimelic acid nor its racemase or decarboxylase; possibly in these nutritionally exacting cocci, diaminopimelic acid plays no part in cellular metabolism. These Streptococcaceae are sharply differentiated from the other Eubacteriales, even

from the related lactobacilli and propionibacteria, in all of which diaminopimelic acid appears to be a metabolite, judged by its presence in the cell or by the activity of enzymes attacking it. The majority of the other Gram-positive cocci in the family Micrococcaceae also lack diaminopimelic acid; the presence of active racemase and decarboxylase suggests that these organisms may utilize diaminopimelic acid as a transitory metabolite but do not usually incorporate it into their cell walls. The fact that two exceptional micrococci contain diaminopimelic acid (Cummins & Harris, 1956) supports the hypothesis that diaminopimelic acid is a metabolite in Micrococcaceae.

One known metabolic function of diaminopimelic acid in *Esch. coli* is to act as a precursor of lysine through the action of diaminopimelic acid decarboxylase (Work, 1955). The racemase is known also to be functional in the metabolism of this organism, since the lysine auxotroph, *Esch. coli* 26–26, blocked at the decarboxylase step, accumulates a mixture of equal amounts of LL- and *meso*-diaminopimelic acids. Also, the diaminopimelic acid auxotroph, *Esch. coli* 173–25, responds equally well to LL- or *meso*-diaminopimelic acids (Hoare & Work, 1955*a*).

This study of the distribution of diaminopimelic acid racemase has not answered the questions of the function of the racemase in metabolism, or as to which isomer of diaminopimelic acid is synthesized first. The racemase is even more widely distributed among bacteria than is the decarboxylase. It is possible that LL-diaminopimelic acid might be synthesized first from precursors in the LL configuration; the *meso* isomer, found in most bacterial cells, may be subsequently formed by racemization. The finding of only the LL isomer of diaminopimelic acid in whole cells of *Cl. welchii* and propionibacteria is not due to lack of racemase. Since most propionibacteria also contain decarboxylase, it is possible that the racemase in these organisms is acting simply in the enzyme sequence necessary for lysine formation. The intracellular soluble amino acids of propionibacteria resemble a racemase equilibrium mixture in their content of both *meso*- and LL-diaminopimelic acid (Hoare & Work, 1957). In the clostridia, with no diaminopimelic acid decarboxylase, lysine is probably not normally formed endogenously; if it is, it may be derived from some source other than diaminopimelic acid.

SUMMARY

1. The enzyme diaminopimelic acid racemase converts either LL- or *meso*-diaminopimelic acid into a mixture of the two isomers. It has no action on DD-diaminopimelic acid.

2. This enzyme, acting in sequence with diaminopimelic acid decarboxylase (which is specific for *meso*diaminopimelic acid), is responsible for the apparent decarboxylation of LL-diaminopimelic acid by many acetone-dried bacteria.

3. The properties of diaminopimelic acid racemase have been examined in cell-free extracts of *Esch. coli* 26–26, which are free from decarboxylase. The pH optimum is about 7·7–8·3. The enzyme has a sensitive sulphydryl group, but can be stabilized as an inactive mercury complex and subsequently reactivated. It is not activated by pyridoxal phosphate, but is inhibited by carbonyl-binding reagents; this inhibition is reversed by thiols.

4. Diaminopimelic acid racemase is even more widely distributed among bacteria than is the decarboxylase. The distribution of these enzymes bore no relation to the occurrence of the isomers of diaminopimelic acid. Only among the Streptococcaceae were both these enzymes and diaminopimelic acid absent. Decarboxylase was absent from most Bacillaceae, but racemase was usually present.

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Note added in proof. Since this paper went to press diaminopimelic acid decarboxylase has been found in *B. sphaericus* (cf. p. 457).

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Connective Tissue Growth Stimulated by Carrageenin

2. THE METABOLISM OF SULPHATED POLYSACCHARIDES*

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Robertson & Schwartz (1953) have shown that the subcutaneous injection of carrageenin in guinea pigs causes the formation of large amounts of easily separated connective tissue. In the course of preliminary experiments, which have been briefly reported by Jackson (1956), this method has been found to provide a very convenient experimental model for biochemical studies on connective-tissue formation and removal. The new tissue so formed is readily separated from surrounding skin, fascia and muscle and amounts of the order of 10–20 g. of granuloma tissue/guinea pig are regularly obtained in the early phase of the response to a single injection of 50 mg. of carrageenin. In the earlier paper of this series some observations on the formation and removal of collagen in the carrageenin-induced granuloma are reported (Jackson, 1957). The present paper is concerned with the metabolic behaviour of sulphated polysaccharides from the same granulation tissue, and from skin and rib cartilage of normal animals.

It has been shown by Layton (1950) that there is an increased uptake of sulphate *in vitro* by granulation tissue formed after muscle injury. Kodicek & Loewi (1955) studied the uptake of [^{35}S]sulphate by mucopolysaccharides of forming connective tissue after tendotomy in guinea pigs. They found that the uptake of ^{35}S reached a maximum from the 7th to 15th days and returned to low values on the 23rd day after operation. The radioactivities measured by them relate to total organic [^{35}S]sulphate in the granulation tissue after preliminary extraction with Ringer solution, and therefore loss of unknown amounts of soluble organic [^{35}S]sulphate in this fraction. Some 67 % of the [^{35}S]sulphate in the Ringer-washed granulation tissue was shown to be present in an alkali-extractable polysaccharide having a mobility on paper ionophoresis similar to chondroitin sulphuric acid and which was metachromatic to toluidine blue. There remained some 25 % of [^{35}S]sulphate activity unaccounted for, of which apparently only a small part is contained in residue material. But paucity of extracted material made further characterization impossible. In the present experiments the uptake of [^{35}S]sulphate into three fractions of the granuloma and other tissues of guinea pigs has been studied. The first fraction is that extracted along with neutral salt-soluble collagen, i.e. by neutral 0·2 M sodium chloride. The second fraction is brought into water or dilute salt solution by digestion with activated papain, which destroys the protein linkages. Finally, there is an organic sulphate fraction in residual material which resists dissolution even by papain digestion and which will be referred to as residue sulphate.

* Part 1: Jackson (1957).

METHODS

Treatment of animals

Guinea pigs were treated as described in the preceding paper, Jackson (1957). In the main series of carrageenin experiments thirty-six guinea pigs were used, each was injected with 50 mg. of carrageenin subcutaneously, and killed in batches of three at each interval of time. A further twenty-one guinea pigs were used for the determination of specific activities in normal rib cartilage and skin, again three guinea pigs for each interval of time. The 625 μc of ^{35}S injected was chosen to give a dosage of 1 μc/g. body wt. Any corrections necessary in individual groups of animals to bring the dosage to this level were small, but have been made throughout where required.