Assay and Properties of Diaminopimelate Epimerase from *Bacillus megaterium*

By P. J. WHITE*

Department of Microbiology, University of Sheffield, S10 2TN, and Twyford Laboratories, Twyford Abbey Road, London N.W.10

B. LEJEUNE† AND ELIZABETH WORK‡

Twyford Laboratories, Twyford Abbey Road, London N.W.10

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1. Diaminopimelate epimerase from a soluble extract of *Bacillus megaterium* N.C.I.B. 7581 was purified about 25-fold by fractionation with ammonium sulphate and chromatography on calcium phosphate gel-cellulose. The product was impure but was unstable on further purification. 2. Quantitative assay methods for the enzyme were devised in which *meso*- or LL-diaminopimelic acid may be the substrate. 3. Between 25° and 45° at pH 7.0 enzyme action leads to an equilibrium mixture containing 65% *meso*-isomer and 35% LL-isomer. 4. The initial rate of epimerization was 2–3 times as fast with LL-diaminopimelic acid as substrate as with the *meso*-isomer; a number of other amino acids were not racemized by the enzyme. The Michaelis constants at 37° were 6.7 mM (LL-isomer) and 100 mM (*meso*-isomer); with both substrates enzyme activity was maximal at pH 7–8. The relative rates of epimerization of LL-diaminopimelic acid at 25°, 37° and 45° were 0.77:1.00:1.15. 5. A thiol compound (of which 2,3-dimercaptopropan-1-ol was the most effective) was needed as an activator of the purified enzyme. 6. Carbonyl-binding reagents and several other compounds did not inhibit diaminopimelate epimerase. 7. Pyridoxal phosphate did not stimulate enzymic activity even in preparations that had been almost completely freed of derivatives of vitamin B₆ (as shown by microbiological assay).

Diaminopimelate epimerase (2,6-LL-diaminopimelate 2-epimerase, EC 5.1.1.7) is a bacterial enzyme that catalyses the stereochemical inversion of the groups attached to only one of the two asymmetric carbon atoms of diaminopimelic acid. For the enzyme to function, one of these atoms must be in the L-configuration, the other (where rearrangement occurs) may be in the L- or D-configuration. Thus either LL- or *meso*-diaminopimelic acid is a substrate; enzyme action leads to an equilibrium mixture of these isomers. The DD-isomer is not attacked. The enzyme was first described by Antia, Hoare & Work (1957), by whom it was called diaminopimelic acid racemase.

The formation of a mixture of LL- and *meso*-diaminopimelic acid by enzyme preparations

incubated with either pure isomer can be detected chromatographically on paper (Antia *et al.* 1957). Semiquantitative comparisons of the activities of different enzyme preparations can be made by comparing the times needed by the enzymes to produce a mixture of isomers, but the amounts of each isomer in the mixtures cannot be determined accurately by chromatography. Although the rate of epimerization might be measured quantitatively by polarimetry (Work, Birnbaum, Winitz & Greenstein, 1955) relatively large amounts of diaminopimelic acid would be needed to make solutions that caused a measurable optical rotation.

Quantitative assays on a small scale became feasible when diaminopimelate decarboxylase (*meso*-2,6-diaminopimelate carboxy-lyase, EC 4.1.1.20) was purified (White & Kelly, 1965). This enzyme converts *meso*-diaminopimelic acid quantitatively into L-lysine, and does not attack LL- or DD-diaminopimelate. Hence the rate at which the LL-isomer is converted into *meso*-isomer by diaminopimelate epimerase may be measured by the rate

* Present address: Department of Microbiology, University of Sheffield, S10 2TN.

† Present address: Laboratoire de Physiologie Végétale, Faculté des Sciences, 91-Orsay, France.

‡ Present address: Department of Biochemistry, Imperial College of Science and Technology, London S.W.7.

at which carbon dioxide is evolved when the epimerization is carried out in the presence of an excess of purified diaminopimelate decarboxylase. Alternatively the epimerization of either LL- or meso-diaminopimelic acid may be performed without added diaminopimelate decarboxylase. Samples are taken at intervals and epimerization is rapidly halted. Diaminopimelate decarboxylase is then added with cofactor and activator, and is allowed to convert all the meso-isomer in the sample into lysine. Finally the amount of lysine or of LL-diaminopimelic acid in the sample is assayed.

In the earlier work on diaminopimelate epimerase (reviewed by Work, 1962), the enzyme was obtained (after drying organisms with acetone) from a lysine-requiring mutant of *Escherichia coli*, which was used because it lacked diaminopimelate decarboxylase, so that the problem of separation of this enzyme from the epimerase was avoided. Diaminopimelate epimerase in crude extracts was active over a range of pH values from about 6.5 to 8.5, with optimum activity at pH 7.7–8.3. A partial loss of enzyme activity on storage overnight at -10° could be reversed by thiols. The enzyme was not activated by pyridoxal phosphate, although carbonyl-binding reagents caused an inhibition, which, however, was prevented or reversed by thiols but not by pyridoxal phosphate. No purification of the enzyme was achieved.

In the present work *Bacillus megaterium* was used as a source of the enzyme because extracts of these organisms had considerably greater diaminopimelate epimerase activity than had extracts of *E. coli*. Diaminopimelate decarboxylase was not entirely absent from *B. megaterium* (as it had been reported to be by Antia *et al.* 1957), but the activity of this enzyme was very low in the extracts. Quantitative assay procedures for diaminopimelate epimerase have been devised, and the enzyme has been partly purified and its properties have been studied in greater detail than before. A preliminary report of some of this work has been given (Lejeune, White & Work, 1965).

METHODS

Organisms. *B. megaterium* (National Collection of Industrial Bacteria no. 7581) was maintained on slopes of nutrient agar (Oxoid) that were incubated at 37° for 12–16 hr. and then stored at 2° . These organisms showed a very marked loss of viability when slope cultures were incubated for longer periods. Even when kept at 2° slope cultures did not remain viable for longer than 5–6 weeks.

E. coli (American Type Culture Collection no. 9637) was similarly maintained.

Saccharomyces carlsbergensis strain 4228c (National Collection of Yeast Cultures no. 534) was maintained on slopes of the medium M of Morris, Hughes & Mulder (1959) modified by using Oxoid malt extract (22.5 g./l.) in place of

John Bull brand malt extract. Cultures were incubated overnight at 30° , then kept at 2° .

Media. All media were made in glass-distilled water.

The medium for *B. megaterium* contained: NH_4Cl , 4 g.; K_2HPO_4 , 2.8 g.; KH_2PO_4 , 1.2 g.; $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$, 0.4 g.; DL-glutamic acid, 2 g.; glycerol, 10 ml.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 8 mg.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg.; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 16 mg.; water to 1 l. No adjustment of pH was made. A solution of FeSO_4 and one of MgSO_4 plus MnCl_2 were autoclaved (at 121° for 15 min.) separately and added to the rest of the medium after cooling. This medium is modified from that of Alfoldi (1958).

E. coli was grown in the medium of Davis & Mingioli (1950) as described by White & Kelly (1965).

S. carlsbergensis was grown (at 37°), when used for assays of pyridoxine, in the medium A of Morris *et al.* (1959) modified by using Difco 'vitamin-free' casein hydrolysate (Casamino acids) (2 g./l.) instead of a hydrolysate of casein prepared in the laboratory.

Growth and harvesting of *B. megaterium*. Organisms were grown in 2 l. conical flasks, each containing 500 ml. of medium. Each flask was inoculated with 1.0 ml. of a suspension in sterile water of organisms (about 2 mg. dry wt./ml.) from a slope culture that had been incubated for 8 hr. at 37° . The flasks were incubated at 37° while shaken mechanically at about 200 gyrations/min. on a model VS gyro-rotatory shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). Organisms were harvested after 40 hr., when the yield was about 1.5 mg. dry wt. of organisms/ml., by centrifuging at 20 000 g for 5 min. at 2° .

Harvested organisms were washed twice in 0.02 M-sodium phosphate buffer, pH 6.8, with a volume of buffer about one-quarter of that of the culture being used for each wash. The washed organisms were resuspended in 0.1 M-phosphate buffer, pH 6.8, containing BAL* (0.1 mM); this and all other phosphate buffers were made by mixing 0.2 M- Na_2HPO_4 and 0.2 M- NaH_2PO_4 in suitable proportions, and diluting appropriately with glass-distilled water. About 20 ml. of buffer was used to resuspend the organisms from 1 l. of culture, which gave about 50 mg. dry wt. of organisms/ml.

Purification of diaminopimelate epimerase. Except where stated otherwise, all steps were done at 2° with BAL (0.1 mM) present in buffers, which were prepared from glass-distilled water.

(1) Preparation of extract. Washed organisms, suspended in buffer as above, were broken by passage at 20 000 lb./in.² through a press of the type described by Milner, Lawrence & French (1950). The debris was removed by centrifugation at 20 000 g for 40 min. The supernatant liquid (extract) was carefully decanted from the pellet, which was rather loose when *B. megaterium* had been broken. Protein was measured in the extract and 0.1 M-phosphate buffer, pH 6.8, was then added to the extract to give a final concentration of 10 mg. of protein/ml. Subsequent steps were carried out only with extracts of *B. megaterium*.

(2) Fractionation with $(\text{NH}_4)_2\text{SO}_4$. Solid $(\text{NH}_4)_2\text{SO}_4$ (312 mg./ml. of extract) was added to give 50% saturation. The mixture was swirled until all the $(\text{NH}_4)_2\text{SO}_4$ had dissolved, then the precipitate was left to settle for 1 hr. The precipitate was removed by centrifuging at 20 000 g for 1 hr. The viscosity of the extract made this precipitate difficult to remove. More solid $(\text{NH}_4)_2\text{SO}_4$ (135 mg./ml. of

* Abbreviation: BAL, 2,3-dimercaptopropan-1-ol.

supernatant liquid) was added to give 70% saturation. After the mixture had been swirled and the precipitate left to stand for 1 hr. and centrifuged (for 30 min.) as before, the supernatant liquid was discarded and the precipitate was dissolved in 0.02M-borate buffer, pH 8.0, to a final volume about one-fifth of that of the original extract. Protein was measured in this solution and then borate buffer was added to dilute the solution to 10mg. of protein/ml. The borate buffer was prepared by mixing 0.2M- H_3BO_3 (70 ml.) and 0.05M- $\text{Na}_2\text{B}_4\text{O}_7$ (30 ml.) and making up to 1 l. with water.

Solid $(\text{NH}_4)_2\text{SO}_4$ (278 mg./ml. of solution) was added to give 45% saturation [supposing that no $(\text{NH}_4)_2\text{SO}_4$ was present before this addition]. The precipitate was removed after 1 hr. as before, and more solid $(\text{NH}_4)_2\text{SO}_4$ (64 mg./ml. of supernatant liquid) was added to give 55% saturation. The precipitate was centrifuged as before after 1 hr., and was redissolved in 0.02M-phosphate buffer, pH 6.8, to a final volume of about one-fifth of that of the original extract.

This solution was dialysed overnight against 0.02M-phosphate buffer, pH 6.8 (about 20 times the volume of the solution), to remove $(\text{NH}_4)_2\text{SO}_4$ and borate. The tubing for dialysis (Visking) was washed before use in several changes of water containing BAL (0.1 mM) and EDTA (1.0 mM) over a period of a week or two, to remove inhibitors.

(3) Chromatography on calcium phosphate gel-cellulose. Columns of calcium phosphate gel-cellulose were prepared by the method of Deeb & Hager (1964) as follows. Calcium phosphate gel, prepared by the procedure of Swingle & Tiselius (1951), was kept at 2° as a suspension in water, and its dry weight was determined. Whatman CF11 cellulose powder was washed successively with 0.5M-NaOH, water, 0.5M-HCl and water. The washed cellulose was kept at 2° as a suspension (about 10%, w/v) in water with a few drops of chloroform as preservative. The suspensions of gel and cellulose were stirred together in proportions of 1:5 (by dry wt.) and the mixture was washed several times by decantation with phosphate buffer, pH 6.8 (containing 0.1 mM-BAL), of the desired concentration, usually 0.02 M. The suspension was stirred magnetically at room temperature in a stoppered Buchner flask that was evacuated with a water pump to remove air from the suspension.

A column (2 cm. $\times$ 10 cm. after settling) was poured at room temperature from the de-aerated gel-cellulose slurry, and was washed with 0.02M-phosphate buffer, pH 6.8, containing 0.1 mM-BAL. To the column at 2° was applied 3–5 ml. of the non-diffusible residue from dialysis. The column was eluted with phosphate buffer, pH 6.8, at concentrations that were increased stepwise: 0.02 M, 0.05 M and 0.1 M. The rate of flow was about 10 ml./hr. and about 100 ml. of each buffer was used. The column effluent was collected in 5 ml. fractions, the E_{280} of each fraction measured and protein measured in selected samples. Fractions from the regions of relatively high E_{750} were tested manometrically for diaminopimelate epimerase activity. The enzymically active fractions (usually eluted by 0.05M-buffer) were combined.

Removal of derivatives of vitamin B₆ from diaminopimelate epimerase. The technique of Smith & Waygood (1961) was used with an extract of *B. megaterium*. The extract (10 mg. of protein/ml.) in 0.02M-phosphate buffer, pH 6.8 (20 ml. containing 0.1 mM-BAL), was mixed at 2° with 60 ml. of a solution of ammonium sulphate (390 g./l.) in a 1:10 mixture (v/v) of aq. ammonia solution (sp.gr. 0.88) and water. The

pH was 10. The mixture was left at 2° for 20 min., then was stirred rapidly at 2° while 1.0M-HCl (70–80 ml.) was added until the pH was 3 as judged with indicator paper. As quickly as possible the precipitate was collected by centrifuging at 2° at 15000 g for 2 min. and then rapidly suspended in an aqueous solution of ammonium sulphate (312 g./l.) that had previously been adjusted to pH 8.5 by addition of 2M-NaOH. The suspension was made up to 10 ml. and was stirred for 5 min. at 2°. The precipitate was again collected by centrifuging as before. The washing of the precipitate with ammonium sulphate was repeated twice, then the precipitate was redissolved in 0.02M-phosphate buffer, pH 6.8, containing 0.1 mM-BAL, and the volume was made up to 20 ml. This solution was again put through the entire process of precipitation from alkaline ammonium sulphate solution with acid followed by washing with ammonium sulphate solution. The solution from this second treatment was put through the entire process once more.

Measurement of diaminopimelate epimerase activity. Enzymic activity was roughly assayed by the method involving paper chromatography described by Work (1962). Quantitative assays were devised, involving manometric and colorimetric techniques.

(1) Manometric method. Warburg flasks were prepared, containing in the main compartment: diaminopimelate epimerase solution (0.1–0.5 ml., containing not more than 0.1 unit); BAL (2.5 μ moles); pyridoxal phosphate (10 μ g.); purified diaminopimelate decarboxylase (0.5 ml., containing not less than 0.3 unit); and 0.1M-phosphate buffer, pH 6.8, to adjust the total volume in the main compartment to 2.1 ml. The flasks were gassed with nitrogen (unnecessary except with crude extracts) at 37°. After equilibration, the substrate (LL-diaminopimelic acid, 26 μ moles in 0.4 ml. of 0.1M-buffer) was tipped from the side arm. The rate of evolution of CO_2 was measured for 1 hr. without a correction being made for retention of CO_2 by the buffer. No CO_2 was evolved in the absence of either diaminopimelate epimerase or the substrate.

(2) Colorimetric methods. (a) LL-Diaminopimelic acid as substrate.

Step 1. In a test tube (4 in. $\times$ 1/2 in.) were mixed: LL-diaminopimelic acid (20 μ moles); BAL (2 μ moles); and 0.1M-phosphate buffer, pH 7.0, to a total volume of 1.5–1.9 ml. The mixture was equilibrated at 37° and then at zero time diaminopimelate epimerase, also warmed to 37°, was added (0.1–0.5 ml., containing about 0.2 unit) to make the total volume in the tube 2 ml. The contents of the tube were quickly mixed, and a sample (0.4 ml.) was at once transferred to a small test tube (75 mm. $\times$ 8 mm.) that already contained 0.2 ml. of 0.2M-phosphate buffer, pH 6.8. This tube was capped with aluminium foil and was quickly put into boiling water where it was heated for 5 min. and then allowed to cool. The remainder (1.6 ml.) of the mixture was incubated at 37° and at intervals of 10 min. three further samples were removed and treated as above. Controls, each containing BAL (1.0 mM final concentration), were also set up: LL-diaminopimelic acid (10 μ moles) in 1.0 ml. of buffer; LL-diaminopimelic acid (5 μ moles) plus *meso*-diaminopimelic acid (5 μ moles) in 1.0 ml. of buffer; and diaminopimelate epimerase (same volume as in tubes with substrate) in buffer (to 2.0 ml. final volume). Samples (0.4 ml.) were taken from each control, and treated as above, at zero time and after incubation for 30 min. at 37°.

Step 2. After all samples had been boiled and cooled,

additions were made to each small tube as follows: pyridoxal phosphate (0.05 ml.; 0.1 mg./ml.); BAL (1 μ mole); and purified diaminopimelate decarboxylase (0.1 ml., containing not less than 0.05 unit). The tubes were again covered with foil and were incubated at 37° for 3 hr. with occasional mixing. Acetic acid was then added to each tube to make the total volume 1 ml. Usually the tubes were left overnight at this stage and the acetic acid prevented growth of contaminating organisms.

Step 3. A sample (0.4 ml.) from each small tube was transferred to a fresh 4 in. $\times$ $\frac{1}{2}$ in. tube and the amount of lysine in the sample was measured by the method of Shimura & Vogel (1966). Standards were also set up each in 0.4 ml. of 0.1 M-phosphate buffer, pH 6.8: LL-diaminopimelic acid (1.6 μ moles); LL-diaminopimelic acid (0.8 μ mole) plus DL-lysine hydrochloride (0.8 μ mole); and DL-lysine hydrochloride (1.6 μ moles). A blank (0.4 ml. of buffer) was also prepared. To each tube was added 0.1 ml. of 6 M-hydrochloric acid and 0.5 ml. of 15% (w/v) ninhydrin in 2-methoxyethanol. The tubes were covered with foil and heated for 1 hr. in boiling water. After cooling, 4 ml. of orthophosphoric acid (90%, w/v) was added and was thoroughly mixed with a glass rod. The E_{515} of the mixture was read (in 1 cm. glass cells) against the blank, and the amount of lysine (x μ moles) corresponding to the observed E_{515} was read from a standard curve. The amount of *meso*-diaminopimelic acid (μ moles/2 ml.) formed in the mixture incubated at step 1 (given by 12.5 x) was plotted against the time of sampling, and the rate of epimerization was determined from the slope of the line so obtained.

The standard curve for determination of lysine was established as follows: a series of 4 in. $\times$ $\frac{1}{2}$ in. tubes was prepared containing DL-lysine hydrochloride (y μ moles, where y was 0 to 1.6 μ moles) plus LL-diaminopimelic acid (1.6 minus y μ moles) in 0.4 ml. of 0.1 M-phosphate buffer, pH 6.8. Hydrochloric acid and ninhydrin were added as above and the tubes were treated as before. The E_{515} values (read against a blank as above) were plotted against the amounts of lysine present in the tubes.

(b) *meso*-Diaminopimelic acid as substrate.

Step 1. In a test tube (4 in. $\times$ $\frac{1}{2}$ in.) were mixed: *meso*-diaminopimelic acid (10 μ moles); BAL (1 μ mole); and 0.1 M-phosphate buffer, pH 7.0, to a total volume of 0.9 ml. The mixture was equilibrated at 37° then at zero time diaminopimelate epimerase (0.1 ml., containing about 0.2 unit), also warmed to 37°, was added. The contents of the tube were quickly mixed and a sample (0.1 ml.) was at once withdrawn and put into a small test tube (75 mm. $\times$ 8 mm.) already containing 0.4 ml. of 0.1 M-phosphate buffer, pH 6.8. Subsequent procedure was as in step 1 of method (a) above. Controls, each containing BAL (1.0 mM final concentration), were also set up: *meso*-diaminopimelic acid (10 μ moles) in 1.0 ml. of buffer; *meso*-diaminopimelic acid (5 μ moles) plus LL-diaminopimelic acid (5 μ moles) in 1.0 ml. of buffer; and diaminopimelate epimerase (0.1 ml.) made up to 1.0 ml. with buffer. Samples (0.1 ml.) were taken from each control and treated as above at zero time and after incubation for 30 min. at 37°.

Step 2. This was as in step 2 of method (a) above.

Step 3. A sample (0.5 ml.) from each small tube was transferred to a test tube (6 in. $\times$ $\frac{1}{2}$ in.) containing acetic acid (4 ml.) and the amount of diaminopimelic acid in the sample was measured by the method of Work (1957), in which lysine (up to 250 μ g./tube) does not interfere. Standards

were also set up each in 0.5 ml. of 0.1 M-phosphate buffer, pH 6.8: DL-lysine hydrochloride (0.5 μ mole); and DL-lysine hydrochloride (0.25 μ mole) plus LL-diaminopimelic acid (0.25 μ mole). A blank (0.5 ml. of buffer) was also prepared. Acetic acid (4 ml.) was also added to the standards and blank, then to every tube 0.5 ml. of ninhydrin reagent (250 mg. of ninhydrin dissolved in 4 ml. of 0.6 M-phosphoric acid and 6 ml. of acetic acid) was added, and the mixtures were heated at 37° for 90 min. The E_{440} was then read (in 1 cm. glass cells) against the blank, and the amount of diaminopimelic acid (z μ moles) corresponding to the observed E_{440} was read from a standard curve. The amount of LL-diaminopimelic acid (μ moles/ml.) formed in the mixture incubated at step 1 (given by 20 z) was plotted against the time of sampling, and the rate of epimerization was determined from the slope of the line so obtained.

The standard curve for measurement of diaminopimelic acid was drawn from the E_{440} values (read against a blank) of 0.1–0.5 ml. of a solution of LL-diaminopimelic acid (1 μ mole/ml.) in 0.1 M-phosphate buffer, pH 6.8, made up to 0.5 ml. with buffer and mixed with acetic acid (4 ml.) and ninhydrin reagent (0.5 ml.), then heated at 37° for 90 min.

The conditions described for the two colorimetric methods were the standard procedures. A number of variations of each method have been used, and these are described in the Results section.

The unit of enzymic activity was defined as recommended by the *Report of the Commission on Enzymes* (1961, p. 8), namely as the amount of enzyme that causes the formation of 1 μ mole of *meso*-diaminopimelic acid/min. (at 37°) from LL-diaminopimelic acid (colorimetric assay) or that causes the evolution of 1 μ mole of CO₂/min. (at 37°) from LL-diaminopimelic acid in the presence of excess diaminopimelate decarboxylase (manometric assay). Owing to retention of CO₂ the unit as determined manometrically is larger by about one-third than the unit determined colorimetrically.

Purified diaminopimelate decarboxylase. This was obtained from *E. coli* grown in 2 l. flasks as described by White & Kelly (1965). Purification was taken as far as precipitation with ammonium sulphate, and the redissolved enzyme was diluted so that the rate of evolution of CO₂ at 37° from *meso*-diaminopimelic acid at pH 6.8 was about 700 μ l./hr./ml. of solution (about 0.6 unit/ml.). This enzyme had no action on LL-diaminopimelic acid. Stock solutions of the enzyme were kept at –15°.

Other enzymes. Glutamate decarboxylase, lysine decarboxylase, D-amino acid oxidase and catalase were all purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks.

Determinations of L-glutamic acid and L-lysine. These were done with glutamate decarboxylase and lysine decarboxylase respectively, as described by Gale (1965).

Determinations of D-alanine, D-methionine and D-proline. These were carried out manometrically with D-amino acid oxidase and catalase, as described by Boulanger & Osteux (1965).

Microbiological assay of pyridoxine. The methods of extraction of vitamin B₆ (from diaminopimelate epimerase) and of its assay were those described by Morris *et al.* (1959).

Other determinations. The dry weight of washed suspensions of organisms was found by heating samples (1.0 ml.) to constant weight at 105° and deducting the dry weight of 1.0 ml. of the suspending fluid.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) as described by White & Kelly (1965). The E_{230}/E_{260} ratio of solutions was also used for rough estimation of protein and of nucleic acid (Layne, 1957).

Chemicals. *meso*-Diaminopimelic acid was isolated from a mixture of *meso*- and *LL*-isomers by fractional crystallization from aqueous ethanol, as described by White, Kelly, Suffling & Work (1964). *LL*-Diaminopimelic acid was isolated from the mother liquor by crystallization (from 1.0 M-HCl) of its salt with naphthalene-2-sulphonic acid, as described by Gilvarg (1959). The naphthalene-2-sulphonic acid (from British Drug Houses Ltd., Poole, Dorset) was recrystallized from dilute HCl before use. As was reported by Jušić, Roy, Schocher & Watson (1963) the derivative of *LL*-diaminopimelic acid forms the first crop of crystals only if this isomer predominates in a mixture of *LL*- and *meso*-isomers.

Other chemicals were commercial materials of A.R. grade where available.

RESULTS

Establishment of conditions for quantitative enzyme assays. Manometric assays can be carried out only at pH values at which diaminopimelate decarboxylase is active (i.e. pH 6.5–7.0; White & Kelly, 1965) and at which retention of carbon dioxide in solution is not great. Retention of carbon dioxide increases rapidly above pH 6.8 (which is optimum for the decarboxylase) and the activity of diaminopimelate epimerase at pH 6.8 is only slightly lower than it is at pH 7.0, which is the optimum for this enzyme (see below). Consequently pH 6.8 was adopted for manometric assays.

Since purified diaminopimelate decarboxylase functions only in the presence of pyridoxal phosphate and an activator (1.0 mM-BAL is most effective), both of these compounds must be present during the manometric assay, so that the cofactor and activator requirements of the epimerase cannot be examined by this method. Inhibitors of diaminopimelate decarboxylase interfere with the manometric assay, whether or not the epimerase is inhibited. Despite these restrictions on its use, the manometric assay is much more convenient to perform than is the colorimetric assay, which involves many more operations and takes considerably longer. The manometric assay is applicable to crude extracts (only extracts of *B. megaterium* and *E. coli* have been tested) since oxidation of diaminopimelate can easily be eliminated by gassing with nitrogen. The presence of diaminopimelate decarboxylase in an epimerase preparation does not matter in the manometric assay because it is essential that the decarboxylase is present in excess, and hence the equilibrium that would be produced by the epimerase alone (see below) is always disturbed in this assay.

An amount of diaminopimelate epimerase is used

that will form from *LL*-diaminopimelic acid about 5 μ moles of *meso*-isomer (about 110 μ l. of carbon dioxide)/hr. at 37°. In order for this rate of epimerization to determine the rate of evolution of carbon dioxide, rather more than a twofold excess of diaminopimelate decarboxylase must be present (Fig. 1). For the standard manometric assay conditions 10.5 μ moles of *LL*-isomer/ml. was in twofold excess of that needed to give the maximal rate of evolution of carbon dioxide. The rate of reaction was proportional to the amount of epimerase over about a tenfold range (Fig. 2).

Colorimetric assays have a wider range of application. The first incubation (of substrate with diaminopimelate epimerase) may be done at any desired pH value, since samples can be adjusted to pH 6.8 before the decarboxylase is added. Enzyme activity was maximal at pH 7.0–8.0 (see below) and pH 7.0 was generally adopted for colorimetric assays because this pH value is close to that used in the manometric assay. The first incubation may also be done in the absence of any added cofactor or activator as these compounds may be added later with the decarboxylase. However, an activator (1.0 mM-BAL was usually employed) of diaminopimelate epimerase was found to be necessary although the enzymic activity was unaltered when pyridoxal phosphate was omitted. The concentration of substrate (10 μ moles/ml.) was similar to that used in the manometric assay (10.5 μ moles/ml.).

Fig. 1. Amount of diaminopimelate decarboxylase needed (in the manometric assay) to demonstrate maximal activity of diaminopimelate epimerase. Warburg flasks contained partly purified epimerase (○, 0.1 ml.; △, 0.2 ml.; □, 0.3 ml.) with various amounts of decarboxylase and with other necessary additions (see the Methods section). The observed activity of the epimerase was maximal (0.33 unit/ml.) only when at least 2.1 units of decarboxylase were added/unit of epimerase.

Fig. 2. Relation between amount of partly purified diaminopimelate epimerase and rate of epimerization of LL-diaminopimelic acid. $\circ$, Colorimetric assay; Δ , manometric assay. Different solutions of enzyme were used in the two assays.

Samples were heated immediately after removal, to halt epimerization. The addition of an enzyme inhibitor or protein precipitant interferes with the subsequent action of diaminopimelate decarboxylase. This boiling destroys most of the BAL in the sample, and consequently BAL, as well as pyridoxal phosphate, was added to each sample after boiling so that both compounds would be present in excess during the incubation with the decarboxylase. During this incubation it is essential that all the *meso*-diaminopimelic acid in the sample is converted into lysine and incubation was therefore continued for at least 3 hr., within which time the decarboxylase was able to convert $4\mu\text{moles}$ of *meso*-isomer completely into lysine. Such an amount of *meso*-diaminopimelate would only be present in a sample if the LL-isomer were entirely converted into the *meso*-form by the epimerase, and this is not found to happen. As a control for the effectiveness of the decarboxylation, a known mixture of *meso*- and LL-isomers was included in every series of assays. Frequent chromatographic examinations were made to check that the epimerization step had produced a mixture of isomers and that the decarboxylation step had destroyed all the *meso*-isomer and had formed lysine.

In the final assay for lysine, the largest amount that could be present was $1.6\mu\text{moles}$ (in which case there would be no diaminopimelic acid). Since diaminopimelic acid interferes to some extent with the assay of lysine, the standard curve for lysine estimation (0 to $1.6\mu\text{moles}$) was established in the presence of correspondingly diminishing amounts of

LL-diaminopimelic acid (1.6 to $0\mu\text{moles}$) as described by Shimura (1963), so that the total amount of the two amino acids was always $1.6\mu\text{moles}$.

Colorimetric assays have been used for most of the investigations of the properties of purified diaminopimelate epimerase described below. When LL-diaminopimelate was substrate in this assay, the rate of epimerization was proportional to the amount of enzyme over about a tenfold range (Fig. 2).

Purification of diaminopimelate epimerase. Preliminary attempts were made to measure manometrically the diaminopimelate epimerase activity of washed organisms (only *E. coli* was tried) pretreated with cetyltrimethylammonium bromide and then incubated with LL-diaminopimelic acid under the conditions used for assay of diaminopimelate decarboxylase by White *et al.* (1964). The rate of evolution of carbon dioxide from the organisms (plus substrate) was considerably lower than that obtained with an equivalent amount of a soluble extract, but was unaltered when purified diaminopimelate decarboxylase was added to the organisms.

Extracts of *B. megaterium* tested manometrically showed greater diaminopimelate epimerase activity (about 0.1 unit/mg. of protein) than did extracts of *E. coli* (about 0.02 unit/mg. of protein). The activity in extracts of *B. megaterium* did not seem to be much affected by the stage of growth of the organisms from which extracts were made. The activity of diaminopimelate decarboxylase was always very low in the extracts of *B. megaterium*, never higher than 0.01 unit/mg. of protein, but this enzyme was clearly present.

Extracts of *B. megaterium* made with the press had higher diaminopimelate epimerase activity than had extracts made by ultrasonic disintegration, and were much more active than extracts of organisms dried with acetone. When organisms were put through the press only about two-thirds were broken, and the survivors were not broken on further passage through the press. The reason for this resistance has not been investigated, but it does not seem to be related to sporulation, no sign of which has been seen at any stage of growth in the medium used in these experiments. The yield of extract was only about 25ml. (containing about 15mg. of protein/ml.) from one load of the press (40ml. , containing about 50mg. dry wt. of organisms/ml.).

The extract was stored at -15° and the activity of diaminopimelate epimerase decreased only slightly over a month or two. The enzyme was stable overnight at 2° , but extracts became mouldy if kept long at this temperature. Steps in the purification of diaminopimelate epimerase from an extract are shown in Table 1. The enzyme was

Table 1. *Steps in the purification of diaminopimelate epimerase from B. megaterium*

Details of each step are given in the Methods section. Enzymic activity was measured manometrically. The values for 'total volume' and 'total activity' were corrected to allow for material removed for testing, and to adjust for the fact that not all the material obtained at one step was usually carried on to the next.

	Total vol. (ml.)	Total activity (units)	Activity (units/ml.)	Protein (mg./ml.)	Sp. activity (units/mg. of protein)	Recovery (%)	Purification factor
1. Extract	44	52	1.19	10	0.12		
2. 50–70%-satd.- (NH ₄) ₂ SO ₄ precipitate	10	33	3.34	6.9	0.48	64	4
3. 45–55%-satd.- (NH ₄) ₂ SO ₄ precipitate	10	16	1.6	2.2	0.73	31	6
4. Calcium phosphate gel-cellulose eluate (0.05 M-buffer)	23	2.8	0.12	0.042	2.8	5	23

stable on storage at -15° at stages 1–3, but the most pure material (stage 4) remained active for only about a week.

Antia *et al.* (1957) reported that diaminopimelate epimerase (from an extract of *E. coli*) was co-precipitated with nucleic acids by addition of streptomycin, and could be redissolved from the precipitate by stirring it with buffer. When streptomycin was added (as a 25%, w/v, solution) to extracts of *B. megaterium*, nucleic acids were precipitated and diaminopimelate epimerase activity disappeared from the supernatant liquid. However, it was not possible to extract the enzyme from the precipitate with 0.1 M-buffers in the range pH 6.5–8.5. Nucleic acids and diaminopimelate epimerase were also co-precipitated by protamine sulphate or by Mn^{2+} ions, but in neither case could the enzyme be redissolved from the precipitate.

The recovery of enzyme activity at each fractionation with ammonium sulphate was low (Table 1) although very little of the enzyme was present in fractions outside the limits used for fractionation. The recovery was not improved when ammonium sulphate was recrystallized from 1.0 mM-EDTA before use, or when ammonium sulphate 'specially low in heavy metals for enzyme work' (British Drug Houses Ltd.) was employed. The activity of the enzyme was unaffected by dialysis overnight.

Further purification was achieved by column chromatography on calcium phosphate gel-cellulose (Fig. 3). Generally the enzyme was eluted by 0.05 M-phosphate buffer, pH 6.8, but sometimes lower (0.02 M) or higher concentrations (0.10 M) removed the enzyme from the column. The recovery of enzyme activity was seldom better than 30% and no improvement was obtained when BAL was replaced in the eluting buffers by dithiothreitol (0.1 mM). A slightly lower degree of purification was obtained when DEAE-cellulose columns were used, and the recovery of enzymic activity was lower. A

Fig. 3. Elution of diaminopimelate epimerase from calcium phosphate gel-cellulose. Partly purified epimerase was applied to a column and eluted with phosphate buffer, pH 6.8, as described in the Methods section. Samples (0.5 ml.) from most fractions were treated with the reagents for determination of protein, and E_{750} values of the coloured reaction product were measured. ○, E_{750} ; ●, diaminopimelate epimerase activity.

still lower degree of purification was obtained when the dialysis residue was eluted through a column of Sephadex G-200 (Fig. 4), although the recovery from this column was high. The rather marked retardation of the enzyme by Sephadex G-200 suggests that the molecular weight of diaminopimelate epimerase is not very high, but no attempt was made to calibrate the column with proteins of known molecular weight. The enzymic activity relative to the protein was also retarded by passage through columns of Sephadex G-100 or G-75 (where retardation was very slight).

Fractions containing diaminopimelate epimerase from columns of calcium phosphate gel-cellulose (or Sephadex G-200) were applied to columns of

Fig. 4. Elution of diaminopimelate epimerase through Sephadex G-200. Partly purified enzyme (5 ml.) was applied to a column (3.3 cm. $\times$ 31 cm.) of Sephadex G-200, which was eluted at 2° with 0.01 M-phosphate buffer, pH 6.8, containing 0.1 mM-BAL. Fractions (10 ml.) were collected and samples (0.1 ml.) were treated with the reagents for measurement of protein and E_{750} values of the coloured reaction product were measured. ○, E_{750} ; ●, diaminopimelate epimerase activity. The void volume of the column was 80 ml. (measured with Blue Dextran) and the total volume of the wet Sephadex was 265 ml.

DEAE-cellulose, in 0.01 M-phosphate buffer, pH 6.8, containing 0.1 mM-BAL. The enzyme was retained by the cellulose, and could be eluted by 0.15 M-sodium chloride (in 0.01 M-buffer) but not by 0.1 M-sodium chloride in buffer. The recovery of enzymic activity was low (about 20%), but nevertheless the diaminopimelate epimerase so obtained had initially the highest specific activity (about 4 units/mg. of protein) that has been obtained. However, this enzyme rapidly lost activity, even when kept overnight at -15° with 0.1 mM-BAL, and no satisfactory method of stabilizing this material has been found.

Throughout the purification, diaminopimelate epimerase activity was closely associated with nucleic acids. After the second fractionation with ammonium sulphate the E_{280}/E_{260} ratio was 0.61 and after elution from calcium phosphate gel-cellulose the ratio was only 0.89. Most of the studies described below have been made with enzyme purified only as far as the second ammonium sulphate fractionation. This material has a specific activity of about 1 colorimetric unit/mg. of protein and is described as 'partly purified enzyme'. At this stage the solution had no other action on diamino-

Fig. 5. Activity of partly purified diaminopimelate epimerase at various pH values. Enzymic activity was assayed colorimetrically with the incubation at step 1 (see the Methods section) in various buffers (all 0.1 M): pH 5.5, sodium acetate buffer; pH 6.0–8.0, phosphate buffer; pH 8.0–8.5, borate buffer. Since the same solution of enzyme was not used throughout, the enzymic activity of a solution at one pH value was compared with the activity of the same solution at pH 7.0. ○, LL-Diaminopimelic acid as substrate; Δ, meso-diaminopimelic acid as substrate.

pimelic acid (in air) than to catalyse the inter-conversion of meso- and LL-isomers.

Properties of the purified enzyme. The rate of epimerization of LL-diaminopimelic acid was two to three times (varying with the batch of partly purified enzyme) the rate with meso-diaminopimelic acid. Activity was measured colorimetrically at various pH values with either LL- or meso-diaminopimelic acid as substrate (Fig. 5). When the LL-isomer was substrate, samples (0.4 ml.) in step 1 of the assay were added to tubes that already contained a volume of 0.2 M-sodium dihydrogen phosphate or 0.2 M-disodium hydrogen phosphate that would bring the pH value of the sample to 6.8, plus 0.2 M-phosphate buffer, pH 6.8, to bring the total volume of sample and buffer to 0.6 ml. When the meso-isomer was substrate and the sample volume was only 0.1 ml., the assay procedure was followed as described in the Methods section. The enzyme was active over a similar rather wide range of pH values with both isomers, and showed no sharply defined peak of activity with either. In crude extracts of *B. megaterium* and *E. coli* activity remained high at pH 8.5.

For each pH value at which the enzyme was tested, a freshly prepared solution of BAL (1.0 mM) in appropriate 0.1 M-buffer was kept at 37° throughout the period of sampling at step 1 of the colorimetric assay. At the end of this time the thiol groups of the BAL were titrated with 5,5'-dithiobis-

(2-nitrobenzoic acid) as described by Ellman (1959). The result was compared with the initial content of thiol groups (2/molecule); at pH 5.5 there was a 12% decrease of thiol groups and at pH 8.5 an 18% decrease during the incubation. Within these limits of pH the thiol groups were more stable.

The enzyme lost a little activity (about 20%) on keeping at 2° overnight at pH 5.5 (with 0.1mM-BAL), but lost 70% of its activity at pH 8.5 under the same conditions.

The initial rate of epimerization was measured colorimetrically with various concentrations of LL- or *meso*-diaminopimelic acid. Results plotted by the method of Lineweaver & Burk (1934) are shown in Fig. 6, and the Michaelis constants of diaminopimelate epimerase were found to be 6.7mM (for LL-isomer as substrate) and 100mM (for *meso*-isomer as substrate). For each concentration of LL-diaminopimelic acid that was used (a μ moles/ml.), assay results were read from a separate standard curve for lysine (0 to 0.16a μ mole) in the presence of LL-diaminopimelic acid (0.16a to 0 μ mole).

The rate of epimerization of LL-diaminopimelic acid was measured colorimetrically at various temperatures between 25° and 45°. The relative rates of reaction at 25°, 37° and 45° were 0.77:1.00:1.15. The logarithm of the initial rate of reaction was plotted against the reciprocal of the absolute temperature (Fig. 7). From the slope of this line the energy of activation at 37° was calculated to be 4.1 kcal./mole (Dixon & Webb, 1964a).

The stoichiometry of the epimerization reaction was investigated (Fig. 8). When LL-diaminopimelic acid was substrate the reaction led to a mixture containing 65% *meso*-isomer and 35% LL-isomer. Thus, at 37° and pH 7.0, $\Delta G^{0'}$ was -410 cal./mole. The same final equilibrium was reached when the *meso*-isomer was substrate. The total amount of

Fig. 6. Effect of substrate concentration on rate of enzymic epimerization of diaminopimelic acid. The initial rate of epimerization (v μ moles of product formed/hr./ml. of enzyme) was determined by colorimetric methods at various concentrations of substrate (s μ moles/ml.) at pH 7.0 and at 37°. (a) LL-Diaminopimelic acid as substrate. ○, Partly purified diaminopimelate epimerase; △, crude extract of *B. megaterium*. (b) *meso*-Diaminopimelic acid as substrate. The partly purified enzyme was not the same solution as that used in (a).

Fig. 7. Activity of partly purified diaminopimelate epimerase at various temperatures. The rate of epimerization of LL-diaminopimelic acid was measured by the colorimetric method, with the incubation in step 1 (see the Methods section) at various temperatures between 25° and 45°. The solution of enzyme contained 4mg. of protein/ml.

diaminopimelic acid was unchanged throughout, and no lysine was formed unless diaminopimelate decarboxylase was added. The composition of the mixture of isomers at the equilibrium position was the same at various temperatures between 25° and 45°. Consequently, between these temperatures, ΔH^0 for the epimerization of LL-diaminopimelic

acid is zero, so that $\Delta G^0 = -T\Delta S^0$, from which ΔS^0 at 37° is 1.3 entropy units/mole.

Diaminopimelate epimerase that had been purified by two fractionations with ammonium sulphate and then dialysed was incubated at pH 7.0 in the presence of BAL (1.0mm) and pyridoxal phosphate (10 μ g./ml.) at 37° with various possible substrates: D-glutamic acid, D-lysine hydrochloride, L-alanine, L-methionine and L-proline (all 1%, w/v). Samples (0.4 ml.) were taken at the start of incubation and after 70 min., and then heated for 5 min. in boiling water. Samples were then assayed for the optical isomer of the substrate compound they had contained (L-glutamic acid, L-lysine, D-alanine etc.). With none of these amino acids was any racemization detected.

Activators. In the colorimetric assay, partly purified diaminopimelate epimerase had only slight activity when BAL was not added during the first incubation. This slight activity when BAL was omitted was probably due to the presence in the enzyme solution of BAL (0.1 μ mole/ml.). Maximal activation was given by 1.0mm-BAL (Table 2). A number of other compounds were tested as activators, but none was more effective than BAL. Crude extracts of *B. megaterium* were fully active in the absence of an activator.

Attempts to demonstrate a requirement for pyridoxal phosphate as a cofactor. Some amino acid racemases have been found to require pyridoxal phosphate as cofactor, whereas others have not been shown to need this compound (Dixon & Webb, 1964b). All attempts to demonstrate such a requirement with diaminopimelate epimerase have been unsuccessful, although preliminary experiments had suggested that enzyme activity was stimulated by pyridoxal phosphate (Lejeune *et al.* 1965). The enzyme

Fig. 8. Composition of the mixture of isomers formed by the action of diaminopimelate epimerase. Substrate (○, LL-diaminopimelic acid, 10 μ moles/ml.; Δ, meso-diaminopimelic acid, 10 μ moles/ml.) was incubated at 37° with partly purified enzyme at pH 7.0 and the amount of meso-isomer present was determined at intervals. The total diaminopimelic acid was also determined (by direct assay of samples without incubation with diaminopimelate decarboxylase) and this remained constant at 10 μ moles/ml. throughout.

Table 2. Activation of diaminopimelate epimerase by various compounds

Diaminopimelate epimerase activity after $(\text{NH}_4)_2\text{SO}_4$ fractionation was measured colorimetrically (LL-isomer as substrate) with various compounds as possible activators and with pyridoxal phosphate (5 μ g./ml.) present throughout.

Compound	Concn. (mm)	Enzymic activity (% of rate with 1.0mm-BAL)
BAL	2.0	102
	1.0	100
	0.25	57
	0.05	34
No addition	—	0–10
BAL + EDTA	1.0 (both compounds)	100
2-Mercaptoethanol	1.0	0–10
2-Mercaptoethanol + EDTA	1.0 (both compounds)	20
Cysteine	1.0	44
Cysteine + EDTA	1.0 (both compounds)	100
Glutathione	1.0	0–10
Sodium sulphite + EDTA	1.0 (both compounds)	0–10
Dithiothreitol	1.0	100

Table 3. Activity of diaminopimelate epimerase in the presence and absence of pyridoxal phosphate after various treatments of the enzyme

Enzyme solutions that had been treated in various ways (see the text) were assayed microbiologically for pyridoxine and tested colorimetrically for diaminopimelate epimerase activity (LL-isomer as substrate) in the presence and absence of pyridoxal phosphate.

Enzyme solution	Pyridoxine (pmoles/mg. of protein)	Diaminopimelate epimerase activity (units/mg. of protein)	
		+ Pyridoxal phosphate (5 μ g./ml.)	– Pyridoxal phosphate
Crude extract	224	0.14	0.14
First acid precipitate	—	0.063	0.054
Second acid precipitate	9	0.029	0.034
Third acid precipitate	5	0.029	0.034
Crude extract after dialysis	—	0.12	0.12
Dialysed extract after Dowex treatment	—	0.22	—
Dialysed extract after charcoal treatment	—	0.22	—
Dialysed extract after Dowex and charcoal treatments	—	0.16	0.17
Eluate from calcium phosphate gel-cellulose column	390	1.8	2.2

purified from *B. megaterium* by ammonium sulphate fractionation and column chromatography was equally active in the absence or presence of added pyridoxal phosphate (Table 3). This purified enzyme was assayed for vitamin B₆ (*S. carlsbergensis* 4228c responds equally well to equimolar pyridoxine, pyridoxal or pyridoxamine) after release of 'bound forms' by autoclaving with acid. The hydrolysed enzyme supported only very slight growth of the assay organism, and only slightly stimulated the response of the organisms to sub-optimum amounts of pyridoxine. Because of this low response the content of vitamin B₆ in the enzyme could not be assayed accurately, but it was not higher than 0.4 nmole/mg. of protein (Table 3). This is equivalent to only 1 molecule of pyridoxine/25 molecules of protein of mol.wt. 100 000.

During the usual purification of the enzyme no special steps were taken to remove vitamin B₆. Special techniques to do this have been used, but in no case has this led to an enzyme preparation the activity of which could be stimulated by added pyridoxal phosphate. Extracts of *E. coli* show no diaminopimelate decarboxylase activity in the absence of added pyridoxal phosphate (White & Kelly, 1965). However, the activity of diaminopimelate epimerase was the same in these extracts (0.027 unit/mg. of protein) whether or not pyridoxal phosphate was added.

An extract of *B. megaterium* was dialysed overnight at 2° against 0.02 M-phosphate buffer, pH 6.8, containing 0.1 mM-BAL. Part (2 ml.) of the dialysis residue was eluted at 2° with the same buffer through a column (0.9 cm. $\times$ 7 cm.) of Dowex 1 (8% cross-linking; phosphate form; 50–100 mesh). The

protein-containing effluent was collected. Another part (2 ml.) of the dialysis residue was stirred at 2° with about 50 mg. of washed 'activated charcoal powder' (British Drug Houses Ltd.) for 10 min. and then the charcoal was removed by centrifuging. Some of the Dowex-column effluent (1 ml.) was similarly treated with charcoal. The various solutions of enzyme obtained in these ways were tested for diaminopimelate epimerase activity (Table 3). Even after treatment with both Dowex and charcoal the activity was the same whether or not pyridoxal phosphate was added.

Lawson & Quinn (1967) reported the partial resolution of apoenzymes of glutamate decarboxylase and histidine decarboxylase by use of the technique of Smith & Waygood (1961). Application of this method to an extract of *B. megaterium* led to the removal of most of the vitamin B₆ (Table 3). Only 5 pmoles of pyridoxine/mg. of protein remained, i.e. 1 molecule of pyridoxine/2000 molecules of protein of mol.wt. 100 000. However, the diaminopimelate epimerase activity of this solution was not increased by addition of pyridoxal phosphate.

Effects of possible inhibitors. Any compound that inhibits diaminopimelate decarboxylase will interfere with quantitative assays of diaminopimelate epimerase unless the inhibitor is removed before diaminopimelate decarboxylase is added. To devise separation methods for removal of inhibitors before assay of isomers of diaminopimelate would be very laborious. Instead, the activity of diaminopimelate epimerase was assessed by paper chromatography (see the Methods section). Samples (0.2 ml.) were taken 0, 30 and 90 min. after addition at 37° of

partly purified enzyme (0.3 mg.) to LL-diaminopimelic acid (10 μ moles) in 0.1 M-phosphate buffer, pH 7.0 (1.0 ml. total vol.), together with BAL (1.0 mM) and the possible inhibitor. The samples were treated as in step 1 of the colorimetric assay (LL-isomer as substrate), and then part (10 μ l.) of each was spotted on to paper. The following compounds were tested as inhibitors: cycloserine, hydroxylamine, iodoacetic acid, isoniazid, DL-penicillamine, potassium cyanide and semicarbazide (all 1.0 mM). 4-Deoxypyridoxine hydrochloride (50 μ g./ml.) was also tested. In the presence of BAL none of these compounds caused any inhibition of the enzyme; the same mixture of isomers of diaminopimelate was found as in the control at each time of sampling. It was thought possible that BAL might be preventing inhibition by some of these compounds, as was found by Antia *et al.* (1957). An extract was therefore prepared in the absence of BAL, and was used in similar tests of all the above compounds, but with BAL omitted. Again no inhibition was found by any of these compounds. A number of the compounds are effective inhibitors of diaminopimelate decarboxylase (White & Kelly, 1965); for instance hydroxylamine and semicarbazide (both 0.4 mM) each caused complete inhibition of this enzyme (assayed manometrically in the presence of BAL).

DISCUSSION

Diaminopimelate epimerase participates in the biosynthesis of lysine in *E. coli*, and is the only amino acid isomerase that is known to have a synthetic role. Early steps in the pathway to lysine lead to LL-diaminopimelic acid (Gilvarg, 1957; Peterkofsky & Gilvarg, 1961) and then diaminopimelate epimerase partly converts the LL-isomer into the *meso*-form, which alone is a substrate for diaminopimelate decarboxylase, by the action of which L-lysine is formed.

In the present work, relatively high and constant diaminopimelate epimerase activity was found in soluble extracts of *B. megaterium* prepared from organisms at various stages of growth. The amount of diaminopimelate decarboxylase in the same extracts was always lower, being only just enough to form lysine sufficiently rapidly to maintain synthesis of protein (assuming this to contain 5% of lysine) at the rate observed during exponential growth of the organisms. Possibly a relatively large amount of diaminopimelate epimerase is needed to produce *meso*-diaminopimelic acid at a concentration that allows the decarboxylase to function at its maximal rate. The epimerase must also provide *meso*-diaminopimelic acid for incorporation into the cell wall, which in *B. megaterium* contains both *meso*- and LL-isomers but no lysine (Hoare & Work,

1957; P. J. White, unpublished work with the strain used in the present study).

Extracts of *B. megaterium* and of *E. coli* were prepared by breaking organisms in a press, and diaminopimelate epimerase in the extracts was stable on storage at -15° (in the presence of BAL) for several months. Previously, organisms (*E. coli*) were dried with acetone before treatment in the press (Antia *et al.* 1957), and the enzyme had to be stabilized as a mercury derivative before it could be kept. Possibly this instability was caused by the acetone, since in the present work attempts to fractionate extracts with acetone led to a complete loss of activity. In contrast with the findings of Antia *et al.* (1957), diaminopimelate epimerase (from *B. megaterium*) was not completely inactivated by ammonium sulphate, although there were considerable losses of enzymic activity on fractionation with this salt. As was the case with diaminopimelate decarboxylase (White & Kelly, 1965), the greatest losses of diaminopimelate epimerase during purification occurred when the enzyme was eluted from solids on which it had been adsorbed. The stability of the enzyme diminished also on purification, and this factor, together with the low recovery, limited the degree of purity that was achieved. At the stage where purification was stopped the enzyme still contained nucleic acids.

After this partial purification, diaminopimelate epimerase, like the decarboxylase, was active only in the presence of a thiol compound. As was found by Antia *et al.* (1957), when such an activator is present the enzyme is insensitive to a variety of compounds (including carbonyl-binding reagents) that are frequently used as inhibitors of enzymes. Even in the absence of thiols diaminopimelate epimerase in a crude extract was not inhibited by carbonyl-binding reagents, which (in the presence of BAL) do inhibit diaminopimelate decarboxylase, for which pyridoxal phosphate is a cofactor. Also, the activity of the most highly purified enzyme or of an enzyme specially treated to remove derivatives of vitamin B₆ was not at all stimulated by addition of pyridoxal phosphate. Thus it is unlikely that pyridoxal phosphate is needed as a cofactor by diaminopimelate epimerase. However, no preparation of the enzyme was obtained that was completely free of vitamin B₆ derivatives; enzyme eluted from calcium phosphate gel-cellulose contained about 1 molecule of pyridoxine/25 molecules of protein. If this preparation were only 4% pure, there is a possibility that a pyridoxal phosphate cofactor is very firmly bound to the enzyme and is inaccessible to inhibitors.

Crude diaminopimelate epimerase (from *E. coli*) was stated by Work (1962) to show high activity between pH 6.8 and 8.3 with optimum activity at pH 7.7–8.3. In the present work partly purified

enzyme had maximal activity at pH 7.0-8.0, but there was a marked fall of activity between pH 8.0 and 8.5 that was not shown by crude extracts. The difference between these findings might be the result of the need by the purified enzyme for an activator, which (in the case of BAL) is less stable in alkaline solutions, and which might function less effectively as an activator at the higher pH values.

The colorimetric assay of diaminopimelate epimerase described in the present work differs from that described by Lejeune *et al.* (1965). The earlier method was devised before a procedure for assay of lysine in the presence of diaminopimelic acid (Shimura, 1963) had come to our notice. The value for the Michaelis constant of crude diaminopimelate epimerase (1.1 mM) determined at pH 8.0 by this earlier method (Lejeune *et al.* 1965) was wrong.

There was no evidence to suggest that diaminopimelate epimerase is really two enzymes, one acting on the LL-isomer and one on the *meso*-isomer, though the rate of epimerization of LL-diaminopimelate is the higher, as might be expected since this isomer is probably the natural substrate for the enzyme. The most highly purified enzyme used either isomer as substrate, and the same mixture of isomers was formed at equilibrium whichever isomer was the starting material. The range of pH values over which the enzyme was active was the same with both isomers.

Diaminopimelic acid is available commercially as a mixture of *meso*- and LL-isomers. Such mixtures are obtained from culture filtrates of mutants of *E. coli* lacking diaminopimelate decarboxylase (Work, 1963). The proportions of the two isomers in different commercial preparations of diaminopimelic acid are variable, but usually more than 65% of the *meso*-form is present, whereas the equilibrium mixture formed by purified diaminopimelate epimerase contains only 65% of this isomer. Probably the discrepancy is caused by unequal recoveries of the two isomers during purification and by the low rate of uptake of diaminopimelate by *E. coli* (Leive & Davis, 1965), which may prevent the equilibration of excreted and intracellular diaminopimelate.

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