Biosynthesis of Diaminopimelic Acid and Lysine in *Escherichia coli*

1. THE INCORPORATION OF ^{14}C FROM VARIOUS ORGANIC PRECURSORS INTO THE DIAMINOPIMELIC ACID OF A LYSINE-REQUIRING MUTANT

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Diaminopimelic acid, which is widely distributed in bacteria, is converted into lysine by a specific decarboxylase (Work & Dewey, 1953; Dewey, Hoare & Work, 1954; Antia, Hoare & Work, 1957), and it has been suggested as an immediate precursor of lysine in some bacteria. A mutant of *Escherichia coli* deficient in the decarboxylase requires lysine for growth and accumulates diaminopimelic acid in the growth medium when lysine is restricted (Dewey & Work, 1952; Davis, 1952). As little is known of the biosynthesis of diaminopimelic acid, the incorporation of various precursors into this extracellular diaminopimelic acid is of interest.

Abelson and his colleagues, using the isotopic-dilution technique with *E. coli* B, suggested that some of the carbon atoms of intracellular diaminopimelic acid and lysine may be derived from aspartic acid (Abelson, 1954; Abelson, Bolton, Britten, Cowie & Roberts, 1953). Direct proof of the incorporation of aspartic acid into diaminopimelic acid was obtained by Gilvarg (1958) with cell-free extracts of *E. coli* mutant 26-26, which synthesized diaminopimelic acid from aspartic acid and pyruvate or glutamate in the presence of magnesium, adenosine triphosphate, diphosphopyridine nucleotide and triphosphopyridine nucleotide. *N*-Succinyl-L-diaminopimelic acid was identified as a precursor of diaminopimelic acid in another mutant, and the portion of the diaminopimelic acid molecule derived from aspartate was located (Gilvarg, 1957; Gilvarg, Edelman & Kindler, 1958).

We have now obtained evidence that most, but not all, of the cellular lysine of *E. coli* 9637 is derived directly from diaminopimelic acid. This evidence was obtained by the study, described in the next paper (Meadow & Work, 1959), of the incorporation of [^{14}C]diaminopimelic acid or [^{14}C]lysine into mutants of *E. coli* which required either or both of these amino acids for growth. The labelled diaminopimelic acid was extracted as described in this paper from the culture filtrate of a

lysine-requiring mutant of *E. coli* grown with suboptimum amounts of lysine. This method, which is that normally used for the production of diaminopimelic acid both in the laboratory (Work & Denman, 1953; Wright & Cresson, 1953) and on an industrial scale (Casida, 1956), gives a mixture of the *meso*- and *LL*-isomers (Hoare & Work, 1955). Perry & Foster (1955) used this method to prepare [^{14}C]diaminopimelic acid with a hydrolysate of a radioactive alga, *Scenedesmus obliquus*, as source of radioactivity. In our experiments the labelling was provided by [^{14}C]glucose. The incorporation of radioactivity both into this extracellular diaminopimelic acid and into that of the cells was compared when the mutant was also grown with trace amounts of other ^{14}C -labelled metabolites to determine whether the diaminopimelic acid accumulating in the growth medium under conditions of lysine-deficiency was derived from the same precursors as the intracellular diaminopimelic acid.

METHODS

Organism. The lysine-requiring mutant *E. coli* 26-26, derived from the parent strain *E. coli* A.C.T.C. 9637, was originally obtained from Dr B. Davis. Details of media and maintenance have been described by Meadow, Hoare & Work (1957). This mutant is particularly unstable and reversion was kept to a minimum by daily subculture in minimal medium containing lysine.

Materials. The radioactive materials (except acetate) were uniformly labelled with ^{14}C . D-[^{14}C]Glucose (specific activity 73.1 $\mu\text{C}/\text{mg.}$) was supplied by The Radiochemical Centre, Amersham, Bucks. The specific activities of sodium [^{14}C]aspartate and sodium [2- ^{14}C]acetate were 52 $\mu\text{C}/\text{mg.}$ and 92 $\mu\text{C}/\text{mg.}$ respectively. Sodium [^{14}C]pyruvate was prepared freshly from [^{14}C]pyruvamide (specific activity 43 $\mu\text{C}/\text{mg.}$) by hydrolysis with *N*-HCl for 90 min. at 100°.

Incorporation experiments. *E. coli* 26-26 was grown for 44 hr. with shaking at 37° in 250 ml. conical flasks each containing 100 ml. of minimal medium (Meadow *et al.* 1957) with 50 μM -L-lysine and 0.5% of glucose. Materials labelled with ^{14}C were sterilized by filtration through Dalton P5 Chamberland thimbles and added to the medium after autoclaving (10 lb./in.² for 10 min.) so that the solid from 1 ml. gave at least 5000 counts/min. The cells

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were harvested by centrifuging and washed with 0.9% NaCl soln. until 1 ml. of the washings contained no detectable radioactivity. The cells were acetone-dried, hydrolysed in sealed tubes overnight with 6*N*-HCl and freed from HCl by evaporation over KOH. The hydrolysed cells and the culture filtrates were fractionated by paper chromatography and their radioactivity was detected as described below.

Detection and estimation of diaminopimelic acid. The culture filtrate (0.1 ml.) was investigated directly by single-dimensional chromatography on Whatman no. 1 paper with methanol-water-10*N*-HCl-pyridine (80:17.5:2.5:10, by vol.) as solvent (Hoare & Work, 1955). After solvent-development and drying, the chromatograms were first scanned for radioactivity, then dipped in ninhydrin in acetone (0.1%, w/v) and heated at 100° for 2 min. The solvent system used separates the *meso*- and *LL*-isomers of diaminopimelic acid and the amount present in the spots was estimated by a modification of the reaction of lysine with acid ninhydrin (Chinard, 1952), proposed by Work (1957) for the estimation of diaminopimelic acid in solution. The piece of filter paper covered by both the diaminopimelic acid isomers was removed, cut into small pieces and immersed in a mixture of 0.5 ml. of water and 1 ml. of ninhydrin reagent (AnalaR acetic acid, 8 ml.; 0.6*M*-phosphoric acid, 2 ml.; AnalaR ninhydrin, 125 mg.). This mixture was heated at 100° for 5 min., diluted to 5 ml. with acetic acid and, after removing the filter paper by centrifuging, its absorption at 440 m μ was read in a Unicam spectrophotometer type SP. 500 in 1 cm. diameter tubes against the absorption of a blank prepared from adjacent amino acid-free areas of filter paper. A standard curve was constructed from known amounts (5–150 μ g.) of diaminopimelic acid subjected to the same chromatographic procedure.

The hydrolysed cells (approx. 5 mg. dry wt.) were investigated by two-dimensional chromatography on Whatman no. 4 paper with phenol-water-aq. NH₃ soln. (sp.gr. 0.880) (80:20:0.5, by vol.) and butan-1-ol-acetic acid-water (67:10:23, by vol.) as solvents. After they had been subjected to radioautography on Kodirex code 7 X-ray films for 6 weeks to detect the radioactive areas, the chromatograms were developed with ninhydrin.

Detection of radioactivity. Solutions were evaporated and the residues counted at infinite thinness, and solids were counted at infinite thickness, on 1 cm.² planchets with a thin mica end-window Geiger-Müller tube. The radioactivity of one-dimensional chromatograms was measured

by Dr G. Popják with an automatic recording and scanning device in which the paper strips were driven between two thin mica end-window Geiger-Müller counters (Hele, Popják & Lauryssens, 1957). Tracings were thus produced which could be related to the ninhydrin-treated chromatograms.

Extraction of diaminopimelic acid from the culture filtrate. The method used was essentially that of Work & Denman (1953), except for the use of an ion-exchange resin with a lower degree of cross-linking from which the diaminopimelic acid was relatively easily removed. The culture filtrate (200 ml.) was adjusted to pH 3 with HCl and boiled for 5 min. The resulting precipitate, previously found to be a lipoprotein-polysaccharide complex which accumulates in the growth medium at the same time as diaminopimelic acid (Meadow, 1958), was removed by filtration through Oxoid membrane filters (Oxo, Ltd., London). The filtrate was concentrated to dryness *in vacuo*, dissolved in water (20 ml.) and treated with an excess of charcoal (Farnell LS grade; approx. 5 g.) to remove some of the carbohydrates. It was filtered, redried and dissolved in 1.25*N*-HCl (10 ml.). This solution was run on to a column (36 cm. $\times$ 4.8 cm.) of Zeo-Karb 225 nominally 4½% cross-linked (100–150 mesh), which had been equilibrated with 1.25*N*-HCl after 'cycling' with 2*N*-NaOH, 4*N*-HCl and 2*N*-HCl. The amino acids were eluted from the column with 1.25*N*-HCl and examined in 0.3 ml. samples of the eluate fractions by paper chromatography with methanol-water-HCl-pyridine as solvent. In addition, diaminopimelic acid was estimated quantitatively in 0.1 ml. samples by reaction at 100° with ninhydrin in acid solution (Work, 1957). The fractions containing diaminopimelic acid were combined, concentrated *in vacuo* and decolorized with charcoal. The pH was adjusted to pH 6.2 with lithium hydroxide and diaminopimelic acid was precipitated by addition of 2 vol. of ethanol (Work, Birnbaum, Winitz & Greenstein, 1955).

RESULTS

Incorporation of ¹⁴C from the medium into the extracellular diaminopimelic acid produced by Escherichia coli 26-26

When *E. coli* 26-26 was grown under optimum conditions for the accumulation of diaminopimelic acid, with unlabelled glucose as energy source and trace amounts of [¹⁴C]acetate or [¹⁴C]aspartate, the

Table 1. Uptake of ¹⁴C-labelled materials from the growth medium by *Escherichia coli* 26-26

Cultures were grown for 44 hr. aerobically in minimal medium (see Methods section) containing 50 μ M-L-lysine with glucose (0.5%) as main energy source. Counts on solids from 1 ml. of medium are corrected for background and were made at infinite thinness with a thin mica end-window Geiger-Müller counter (counting efficiency approx. 2%).

	Amount of labelled material added to medium before growth (μ g./ml.)	Radioactivity		
		Before growth	After growth	Uptake (%)
		(counts/min./ml.)		
Aspartate	2.2	5 100	160	97
Acetate	1.9	7 800	1 400	82
Pyruvate	73.0	141 000	44 000	69
Glucose	7.0	10 380	727	93

greater part of the radioactivity had disappeared from the culture filtrate at the end of the incubation period of 44 hr. (Table 1). When 20 times as much ^{14}C was added, as pyruvate, 70 % of the radioactivity was lost from the medium during growth and the amount left in the culture filtrate was still considerably higher than that added as acetate or aspartate. Ninhydrin-developed chromatograms from all the culture filtrates showed two green spots corresponding to the *meso*- and *LL*-isomers of diaminopimelic acid (Hoare & Work, 1955); the total amount of diaminopimelic acid in each chromatogram was estimated to be about 20 μg . The radioactive areas on the chromatograms were detected by the automatic-recording device described in the Methods section. There was no detectable radioactivity in the areas occupied by diaminopimelic acid in the culture filtrates from media labelled with ^{14}C pyruvate and ^{14}C aspartate. The only peaks of radioactivity were in the same positions as those shown on chromatograms of the media before bacterial growth and were therefore due to unused radioactive metabolite. The culture filtrate from the acetate-labelled medium showed radioactivity in both the diaminopimelic acid spots. When the source of ^{14}C was glucose (diluted with unlabelled glucose as a source of energy) radioactive diaminopimelic acid was again produced. Since labelled glucose was more readily obtainable than acetate and since the cells would not easily become adapted to use acetate as sole source of carbon, it was decided to use ^{14}C glucose for the production of diaminopimelic acid.

Incorporation of ^{14}C into intracellular amino acids of Escherichia coli 26-26

The washed cells grown with the four sources of radioactivity were hydrolysed and the incorporation of ^{14}C into their intracellular amino acids was

Table 2. *Incorporation of ^{14}C aspartate into the intracellular amino acids of Escherichia coli 26-26 as shown by radioautography*

The culture was grown as described in Table 1, with added ^{14}C aspartate. Harvested cells were hydrolysed and the amino acids separated by paper chromatography and detected by ninhydrin-treatment and radioautography as described in the Methods section.

Amino acid	Intensity of radioautogram spot
Aspartic acid	++ ++
Glutamic acid	++ ++
Diaminopimelic acid	++ ++
Lysine	++ ++
Threonine	++
Leucine	++
Isoleucine	++
Other amino acids	+

studied by radioautography. The results obtained with pyruvate-grown and aspartate-grown cells were similar. In both, the diaminopimelic acid and the lysine of the cells were radioactive, although the diaminopimelic acid accumulating in the culture filtrate had been unlabelled. The radioautograph showed the preferential labelling of the aspartate 'family' of amino acids (Abelson, 1954); the highest radioactivities were in the aspartic acid, glutamic acid, threonine, isoleucine and leucine spots, as well as in diaminopimelic acid and lysine, although there was some labelling in the other amino acids (Table 2). The diaminopimelic acid present on the chromatograms of hydrolysed aspartate-grown cells and of the culture filtrate was eluted and analysed. Diaminopimelic acid (40 μg .) from the hydrolysed cells gave 50 counts/min. after correcting for background, whereas 40 μg . from the culture filtrate gave no counts above background.

When the cells were grown on unlabelled glucose with added ^{14}C glucose or ^{14}C acetate, all the intracellular amino acids including both diaminopimelic acid and lysine were radioactive. Here the extracellular diaminopimelic acid was also labelled.

Preparation of ^{14}C diaminopimelic acid from the culture filtrate of Escherichia coli 26-26 with ^{14}C glucose as source of radioactivity

Minimal medium (200 ml.) containing lysine (50 μM) and glucose (0.5 %; 100 μC) was used for the production of ^{14}C diaminopimelic acid. The culture filtrate was concentrated and separated on a column of Zeo-Karb 225 as described in the Methods section. After small amounts of aspartic acid, glycine, alanine, glutamic acid and valine had been eluted in the first 220 ml. of eluate from the column, diaminopimelic acid was detected in the 220-260 ml. fractions. The elution curve was almost symmetrical, showing that the *meso*- and *LL*-isomers were not being differentially eluted (see Hoare & Work, 1955). This was confirmed by paper chromatography. The final yield of diaminopimelic acid was 35 mg., of specific activity 0.10 $\mu\text{C}/\text{mg}$. Paper chromatograms of this ^{14}C diaminopimelic acid were made in the following solvents: methanol-water-10N-HCl-pyridine (80:17.5:2.5:10, by vol.); phenol-water-aq. NH_3 soln. (sp.gr. 0.880) (80:20:0.5, by vol.); butan-1-ol-acetic acid-water (67:10:23, by vol.). The radioactive and ninhydrin-positive spots were always superimposable, indicating that diaminopimelic acid was the only radioactive amino acid present.

DISCUSSION

This study has resulted in the unexpected finding that the diaminopimelic acid excreted into the medium by the lysine-requiring mutant of *E. coli*

under conditions of lysine-restriction was not always derived from the same precursors as the intracellular diaminopimelic acid. When the mutant was grown in media containing trace amounts of [^{14}C]aspartate or [^{14}C]pyruvate with unlabelled glucose as main energy source, the extracellular diaminopimelic acid contained at least 50 times less radioactivity than the intracellular diaminopimelic acid. The selective distribution of radioactivity in the intracellular amino acids suggested that both pyruvate and aspartate had been taken up unchanged, since they contributed relatively more radioactivity to aspartic acid, glutamic acid, threonine, leucine, lysine and diaminopimelic acid than to the other amino acids. These are the amino acids shown by isotopic-dilution techniques (Abelson, 1954) to be derived from any exogenous aspartic acid present in preference to the products of glucose metabolism.

The difference in radioactivity between the extracellular and intracellular diaminopimelic acid could be related to the differences in the constituents of the medium at the time of their synthesis. Intracellular diaminopimelic acid is formed during growth, that is, while the concentration of lysine in the medium is sufficient to allow normal protein and cell-wall synthesis, whereas the extracellular diaminopimelic acid is produced only after all the lysine has been exhausted from the medium in conditions of maximum aeration (Work, 1955). Roberts, Abelson, Cowie, Bolton & Britten (1955) have some evidence that exogenous lysine may promote new synthetic pathways in *E. coli* B; and it is possible that in the lysine-requiring mutant the exhaustion of exogenous lysine could result in an alternative metabolic sequence leading to the production of diaminopimelic acid by a route different from that normally used when the lysine concentration is adequate. An alternative hypothesis, that synthesis of extracellular diaminopimelic acid occurs only after the ^{14}C -labelled metabolite has been depleted from the medium, leaving unlabelled glucose as sole carbon source, is unlikely in view of the results with [^{14}C]acetate and [^{14}C]pyruvate as radioactive tracers. Growth with [^{14}C]acetate gave rise to ^{14}C -labelled extracellular diaminopimelic acid, whereas [^{14}C]pyruvate was not incorporated in this way and yet more pyruvate than acetate was left in the medium at the end of the experiment. There is, however, the possibility that pyruvate does not enter the cell during the later stages of growth when the extracellular diaminopimelic acid is produced, since if it could enter the cell it would be expected to give rise to labelled acetate and thence to labelled extracellular diaminopimelic acid.

These experiments suggest that intracellular diaminopimelic acid may be synthesized through a

route preferentially utilizing the carbon chain of aspartate or pyruvate, but that these compounds are not incorporated into the abnormal extracellular diaminopimelic acid. It cannot be too strongly emphasized that this extracellular accumulation of diaminopimelic acid occurs only under lysine-restriction and in fully aerated cultures at the end of the growth phase, when the available glucose-degradation products may be different from those produced by growth under conditions of less vigorous aeration. The mechanism of diaminopimelic acid accumulation under these circumstances is unknown, but it is possible that it involves some kind of overcompensation mechanism related to the lysine-deficiency. In the next paper evidence is presented which suggests that diaminopimelic acid is one, but not the only, precursor of lysine. In this case it is permissible to wonder why this mutant cannot use the alternative route when deprived of lysine. Perhaps the accumulation of diaminopimelic acid or its precursors may inhibit a step in the alternative pathway.

SUMMARY

1. *Escherichia coli* 26-26 was grown on a sub-optimum concentration of lysine with various ^{14}C -labelled compounds to determine which materials could act as carbon-chain precursors for diaminopimelic acid.

2. [^{14}C]Pyruvate and [^{14}C]aspartate were incorporated into the intracellular diaminopimelic acid but not into the extracellular diaminopimelic acid which accumulated in the culture filtrate.

3. [^{14}C]Glucose and [$2\text{-}^{14}\text{C}$]acetate were incorporated into the diaminopimelic acid both in the culture filtrate and in the cells.

4. A mixture of the *meso*- and *LL*-isomers of [^{14}C]diaminopimelic acid was isolated from the culture filtrate of the lysine-requiring mutant *E. coli* 26-26 grown with [^{14}C]glucose as main carbon source.

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Biosynthesis of Diaminopimelic Acid and Lysine in *Escherichia coli*

2. INCORPORATION OF [¹⁴C]DIAMINOPIMELIC ACID, LYSINE AND GLUCOSE

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Lysine is one of the few amino acids for which the biosynthetic pathway in bacteria is different from that found in other types of organisms (Work, 1955; Meadow, Hoare & Work, 1957). Much indirect evidence has accumulated which suggests that in *Escherichia coli* one such pathway involves the decarboxylation to lysine of diaminopimelic acid, an amino acid confined to bacteria (Work & Dewey, 1953; Hoare & Work, 1957). This evidence includes the absence of diaminopimelic acid decarboxylase from certain lysine-requiring mutants of *E. coli* (Dewey & Work, 1952), the accumulation of diaminopimelic acid by these mutants in lysine-deficient media (Davis, 1952) and the isolation of double mutants requiring both diaminopimelic acid and lysine for growth (Davis, 1952). In addition, isotopic-dilution experiments suggested that both lysine and diaminopimelic acid may be derived from the same precursor, aspartate, although not necessarily by the same route (Roberts, Abelson, Cowie, Bolton & Britten, 1955).

Even these few studies have produced various anomalies which cannot be explained by any single biosynthetic route to lysine. For instance, mutant 173-25 has not only an absolute requirement for diaminopimelic acid but, even when grown on diaminopimelic acid, it is stimulated by lysine (Davis, 1952), although its diaminopimelic acid

decarboxylase content is normal. This mutant can, however, be trained to dispense with lysine (Meadow *et al.* 1957) without any change in its diaminopimelic acid requirement or in its diaminopimelic acid decarboxylase activity, which suggests that a route to lysine other than that through decarboxylation of diaminopimelic acid may have been deficient or blocked in mutant 173-25 and then redeveloped by training. On the other hand, the only mutants known to lack diaminopimelic acid decarboxylase have an absolute requirement for lysine, which should not be the case were they able to produce lysine by a route different from that through diaminopimelic acid.

This paper describes direct evidence that diaminopimelic acid is taken up during growth of *E. coli* 9637 and certain of its mutants, that it is a precursor of lysine in these organisms, but that it does not provide the only biosynthetic route to lysine.

Some of these results have been presented in a preliminary report (Meadow & Work, 1959*a*).

METHODS

Materials. The radioactive materials used were uniformly labelled with ¹⁴C. [¹⁴C]Lysine (specific activity 100 µC/mg.) was supplied by The Radiochemical Centre, Amersham, Bucks. [¹⁴C]Diaminopimelic acid (specific activity 0.10 µC/mg.) was prepared as described in the preceding paper (Meadow & Work, 1959*b*); it was a mixture

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