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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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of the *meso*- and *LL*-isomers, known to have equal biological activities for the mutants (Meadow *et al.* 1957).

Organisms. All organisms originated from the parent strain *E. coli* A.C.T.C. 9637. *E. coli* 26-26 required lysine for growth, *E. coli* D was diaminopimelic acid-dependent and *E. coli* 173-25 was also diaminopimelic acid-dependent and was stimulated by lysine. Their source and growth characteristics have been described by Meadow *et al.* (1957).

Growth for incorporation experiments. All organisms were grown at 37° for 18 hr. in minimal medium (Meadow *et al.* 1957) containing glucose (0.5%) and amino acid supplements. After growth the mutants were subcultured into minimal medium with and without amino acids to check that no reversion had occurred. For growth of *E. coli* 26-26 the medium contained 0.2 mM-L-lysine, previously shown to be the concentration necessary for optimum growth (Meadow *et al.* 1957). *E. coli* 9637, *E. coli* D and *E. coli* 173-25 were grown in the presence of diaminopimelic acid (*meso*- and *LL*-) (0.1 mM) and L-lysine (0.1 mM); these were the conditions found to give optimum growth of *E. coli* 173-25 and were used for mutant D to provide identical growth conditions for these two mutants.

The amounts of [¹⁴C]lysine and [¹⁴C]diaminopimelic acid used in each experiment are shown in Table 1. The radioactivity removed during growth was determined by counting, at infinite thinness, the solid from 0.5 ml. of the original medium and of the culture filtrate after removing the bacteria by centrifuging at 10 000 *g* for 10 min.

In another experiment *E. coli* 173-25 and *E. coli* D were grown with unlabelled diaminopimelic acid, unlabelled lysine and [¹⁴C]glucose (1.0 μ C/ml. of medium) diluted to 0.5% with unlabelled glucose.

Preparation of cell fractions. (a) Trichloroacetic acid fractions: 25 ml. batches were grown for each experiment: the cells were harvested by centrifuging and washed with water until 0.5 ml. of the washings contained no detectable radioactivity. The washed cells were extracted with cold and then with hot trichloroacetic acid (5%, w/v) according to the method of Roberts *et al.* (1955). The extracted materials and residues were hydrolysed in sealed tubes with 6N-HCl at 100° for 18 hr.

(b) Cell walls: 600 ml. batches of media were used for each experiment. After washing as before, the harvested cells were disintegrated mechanically at 4° (Mickle, 1948) and treated with trypsin and pepsin to prepare 'purified' cell walls (Cummins & Harris, 1956). The walls were further extracted with aqueous phenol (90%, w/v) (Weidel & Primosigh, 1958) and the phenol was removed from each fraction by dialysis against tap water. The phenol-soluble material and the insoluble residues were then hydrolysed separately with 6N-HCl as before.

Chromatography. The hydrolysed cell fractions were evaporated to dryness over KOH, redissolved in water (2 ml.) and separated chromatographically on Whatman no. 4 paper with phenol-water-aq. NH₃ soln. (sp.gr. 0.880) (80:20:0.5, w/v/v) and butan-1-ol-acetic acid-water (67:10:23, by vol.) as solvents. The volumes applied were 0.25 ml. of the trichloroacetic acid fractions and 0.5 ml. of the cell-wall fractions. This chromatographic system separated diaminopimelic acid from the other amino acids but did not always separate lysine from arginine. The dried chromatograms were placed in contact with X-ray plates (Kodirex code 7) for 3-6 weeks to detect the radioactive areas. Those areas corresponding to lysine and diamino-

pimelic acid were then cut out and eluted with water. The eluate from the lysine-containing area was subjected to paper electrophoresis in 0.5M-Na₂CO₃ buffer, pH 11.5, to remove arginine and histidine (Harris & Warren, 1954). After drying the electrophoresis strips, the lysine was again detected by radioautography and eluted. All eluates were made up to a volume of 2 ml.

Measurement of radioactivity. Triplicate samples (0.2 ml.) of the eluates from the chromatograms and electrophoresis papers were dried on to 1 cm.² planchets and counted at infinite thinness with a windowless flow counter (S.C. 16 Tracerlab, Chicago, U.S.A.) with a conventional scaler (counting efficiency 10-5%). Each sample was counted twice to at least 10 000 counts and the results given are means of the activities of the three samples. Duplicate estimations agreed within 5%. Specific activities were calculated in terms of counts/min./ μ g. of carbon (calculated from the weight of diaminopimelic acid or lysine estimated).

Estimation of diaminopimelic acid and lysine. Diaminopimelic acid was estimated in eluates from chromatograms (Meadow & Work, 1959b). Lysine was similarly determined after electrophoresis except that the heating time at 100° was prolonged to 1 hr. and the standard curve ranged from 5 to 100 μ g. Estimations were carried out in duplicate on 0.5, 0.2 and 0.1 ml. samples of the eluates and the results given are the mean values.

RESULTS

Table 1 shows the amounts of [¹⁴C]lysine and [¹⁴C]diaminopimelic acid removed from the medium during growth of the three mutants of *E. coli* in various experiments. Chromatograms of the media after growth showed only trace amounts of several amino acids other than those added and there was only slight labelling detectable when the chromatograms were examined by radioautography, except in the diaminopimelic acid or lysine originally added.

Incorporation of [¹⁴C]lysine

Trichloroacetic acid fractions. The results obtained by extraction with trichloroacetic acid of the three mutants and the parent strain grown in the presence of [¹⁴C]lysine were similar. The soluble fractions contained little or no detectable radioactivity and only traces of ninhydrin-positive material and were therefore not investigated further. The insoluble residues were radioactive and were investigated by chromatography, electrophoresis and radioautography. Exogenous [¹⁴C]lysine had been incorporated only into lysine and to a small extent into two other positions, X and Y, which did not correspond with any ninhydrin-positive spot. There was no detectable radioactivity in diaminopimelic acid.

The diaminopimelic acid and lysine of these fractions were separated, estimated and counted as described in the Methods section and the results are given in Table 2. Most (80-90%) of the [¹⁴C]lysine removed from the medium was recovered from the

Table 1. *Uptake of [¹⁴C]lysine and [¹⁴C]diaminopimelic acid by mutants of Escherichia coli*

The mutants were grown at 37° for 18 hr. in minimal medium, containing [¹⁴C]lysine or [¹⁴C]diaminopimelic acid with glucose (0.5%) as main carbon source. Unlabelled lysine and diaminopimelic acid were added to give the following final concentrations: mutant 26-26, L-lysine (0.2 mM); mutants 173-25 and D, diaminopimelic acid (0.1 mM) and L-lysine (0.1 mM). The experiments are numbered for references with other tables.

Mutant	Expt. no.	[¹⁴ C]Amino acid	Radioactivity of medium (counts/min./ml.)	
			Before growth	After growth
26-26	1	Lysine	17 500	9 540
173-25	2		14 900	12 000
D	3		14 400	12 400
173-25	4		35 600	33 460
D	5		36 000	34 200
173-25	6	Diaminopimelic acid	440	140
D	7		430	110
173-25	8		450	270
D	9		440	243

Table 2. *Incorporation of [¹⁴C]lysine into the trichloroacetic acid-insoluble fractions of mutants of Escherichia coli*

The mutants were grown in minimal medium with unlabelled glucose and amino acid supplements and [¹⁴C]lysine as shown in Table 1. After growth they were harvested, washed and extracted with trichloroacetic acid as described in the Methods section. The insoluble residues were hydrolysed and the lysine and diaminopimelic acid separated on paper and estimated in eluates as described in the Methods section. Values shown were obtained with the eluates from one chromatogram to which one-eighth of the total hydrolysate had been applied. The radioactivity recovered is calculated as the percentage of that removed from the medium found in the lysine of the hydrolysed fraction.

Mutant	Expt. no.	Amino acids in eluate					Radio- activity recovered (%)
		Diaminopimelic acid		Lysine			
		Amount (μ g.)	Radioactivity (counts/min.)	Amount (μ g.)	Radioactivity (counts/min.)	Specific activity	
						(counts/min./ μ g. of carbon)	
26-26	1	5	0	37	21 800	1200	87
		6	0	40	23 400	1180	94
173-25	2	18	0	76	7 600	218	86
		20	0	86	8 200	193	92
D	3	10	0	47	4 800	207	79
		13	0	57	5 500	196	90

Table 3. *Incorporation of [¹⁴C]lysine into cell-wall fractions of mutants of Escherichia coli*

After growth, as shown in Table 1, cell walls were prepared from the harvested cells, fractionated with phenol (90% w/v), hydrolysed and the diaminopimelic acid and lysine separated on paper and estimated in eluates as described in the Methods section. Values shown were obtained with eluates from a single chromatogram to which one-quarter of the total hydrolysate had been applied. The recovery of radioactivity is calculated as the percentage of that removed from the medium found in the lysine and diaminopimelic acid of the hydrolysed wall fractions.

Mutant	Expt. no.	Wall fraction	Amino acids in eluate					Radio-activity recovered (%)
			Diaminopimelic acid		Lysine			
			Amount (μg.)	Radio-activity (counts/min.)	Amount (μg.)	Radioactivity (counts/min.)	Specific activity (counts/min./μg. of carbon)	
173-25	4	Phenol-insoluble	71	0	649	160 200	501	50
			82	0	722	140 900	499	44
		Phenol-soluble	18	0	120	28 840	488	9
			21	0	132	25 630	485	8
D	5	Phenol-insoluble	64	0	483	124 000	521	46
			60	0	466	113 000	494	42
		Phenol-soluble	17	0	78	18 900	492	7
			19	0	90	21 600	487	8

Table 4. Incorporation of [¹⁴C]diaminopimelic acid into the trichloroacetic acid-insoluble fractions of diaminopimelic acid-requiring mutants of *Escherichia coli*

After growth with [¹⁴C]diaminopimelic acid, the cells were investigated as in Table 2. DAP, Diaminopimelic acid.

Amino acids in eluates									
Diaminopimelic acid					Lysine				
Expt. no.	Amount (μg.)	Radio-activity (counts/min.)	Specific activity (counts/min./μg. of carbon)	Amount (μg.)	Radio-activity (counts/min.)	Specific activity (counts/min./μg. of carbon)	Specific activity (DAP) Specific activity (lysine)	Radio-activity recovered (%)	
Mutant 173-25	6	137	51.6	30.5	620	41.2	1.25	81	
	5	112	50.6	25	510	41.4	1.23	58	
D	7.5	180	54.2	48	740	31.3	1.73	91	
	7	166	51.6	50	755	30.6	1.68	89	

Table 5. Incorporation of [¹⁴C]diaminopimelic acid into cell-wall fractions of diaminopimelic acid-requiring mutants of *Escherichia coli*

After growth with [¹⁴C]diaminopimelic acid, the cells were harvested and cell walls prepared and treated as described in Table 3. DAP, Diaminopimelic acid.

Amino acids in eluates									
Diaminopimelic acid					Lysine				
Expt. no.	Amount (μg.)	Radio-activity (counts/min.)	Specific activity (counts/min./μg. of carbon)	Amount (μg.)	Radio-activity (counts/min.)	Specific activity (counts/min./μg. of carbon)	Specific activity (DAP) Specific activity (lysine)	Radio-activity recovered (%)	
Mutant 173-25									
	Wall fraction								
	Phenol-insoluble	62	1465	53.4	420	8800	1.25	38	
	Phenol-soluble	68	1587	52.7	435	9240	1.22	41	
D									
	Phenol-insoluble	15	340	51.2	62	1280	1.22	6	
	Phenol-soluble	19	452	51.8	82	1708	1.22	8	
D									
	Phenol-insoluble	70	1620	52.3	648	9960	1.68	40	
	Phenol-soluble	65.5	1500	51.8	603	8920	1.72	36	
		23.5	521	50.1	140	2084	1.66	9	
		18	405	50.9	117	1621	1.81	7	

lysine of the trichloroacetic acid-insoluble fractions of all three mutants. The diaminopimelic acid was not labelled. The specific activity of lysine in mutant 26-26 cannot be compared with that in the other two mutants since the media were different. The results with mutants 173-25 and D, which were obtained with identical media, show that exogenous lysine accounted for 10 % of the cellular lysine in both mutants.

Cell walls. Mutants D and 173-25 were grown in the presence of [^{14}C]lysine and the cell walls isolated and extracted with phenol (Table 3). The fraction soluble in phenol yielded on hydrolysis many amino acids, including some lysine and diaminopimelic acid, whereas the residues contained mainly glucosamine, muramic acid, glutamic acid, alanine, lysine and diaminopimelic acid, but with traces of several other amino acids. The diaminopimelic acid was again unlabelled, but only 60 % of the radioactivity removed from the medium was found in the lysine of the two wall fractions. The specific activities of the lysine in the two wall fractions were similar and there were no significant differences between the results for the two mutants, both of which derived about 10 % of their lysine from the growth medium.

Incorporation of [^{14}C]diaminopimelic acid

Trichloroacetic acid fractions. As with lysine, no radioactivity was detected in the trichloroacetic acid-soluble fractions of the two diaminopimelic acid-requiring mutants grown with labelled diaminopimelic acid. Radioautographs of chromatograms from the hydrolysed trichloroacetic acid-insoluble residues of the parent strain and of the two mutants showed that [^{14}C]diaminopimelic acid was incorporated by all three types of cells. ^{14}C was present only in the lysine and diaminopimelic acid spots and sometimes in the unidentified spots X and Y, previously found to be labelled on the chromatograms of cells grown with [^{14}C]-

lysine. There was no essential difference between the three radioautographs except that those from the two mutants showed more strongly radioactive spots.

Measurements of the radioactivity of the lysine and diaminopimelic acid in the trichloroacetic acid-insoluble fractions showed that about 80 % of the ^{14}C taken up from the medium had been incorporated into these two amino acids (Table 4). The specific activity of diaminopimelic acid was higher than that of lysine.

Cell walls. Table 5 shows the distribution of radioactivity in the diaminopimelic acid and lysine of isolated cell-wall fractions of the diaminopimelic acid-requiring mutants grown with [^{14}C]diaminopimelic acid. Both the diaminopimelic acid and the lysine of the two fractions were labelled, the total amount of radioactivity in the two fractions representing approximately 50 % of that removed from the medium. There were no significant differences between the degrees of labelling of the two fractions, but the specific activity of diaminopimelic acid was considerably higher than that of lysine, particularly in mutant D. In this mutant only 55 % of the lysine was derived from the exogenous diaminopimelic acid supplied, whereas in mutant 173-25 80 % of the lysine was derived from the [^{14}C]diaminopimelic acid.

Incorporation of [^{14}C]glucose

The previous experiments had suggested that part of the lysine was derived from a precursor other than diaminopimelic acid. This was confirmed by growing the two diaminopimelic acid-requiring mutants in media containing unlabelled lysine and diaminopimelic acid and [^{14}C]glucose. The resulting cell walls contained unlabelled diaminopimelic acid (Table 6), confirming that both mutants were unable to synthesize diaminopimelic acid. All the other amino acids, including lysine, were labelled. The specific activity of the

Table 6. *Incorporation of [^{14}C]glucose into cell-wall fractions of diaminopimelic acid-requiring mutants of Escherichia coli*

After growth with [^{14}C]glucose and unlabelled diaminopimelic acid and lysine, the cells were harvested and cell walls prepared and treated as described in Table 3.

Mutant	Wall fraction	Amino acids in eluates			
		Diaminopimelic acid		Lysine	
		Amount ($\mu\text{g.}$)	Radio- activity (counts/ min.)	Amount ($\mu\text{g.}$)	Radioactivity (counts/min.)
173-25	Phenol-insoluble	40	0	200	11 000
	Phenol-soluble	12	0	40	2 370
D	Phenol-insoluble	50	0	420	48'220
	Phenol-soluble	15	0	100	11 680
					Specific activity (counts/min./ $\mu\text{g. of carbon}$)
					112
					120
					233
					237

lysine from mutant D was double that from mutant 173-25, showing that about 20% of the lysine in walls of mutant D and 10% of that in mutant 173-25 was derived from glucose.

DISCUSSION

These experiments show the general lack of metabolic interchange between lysine or diaminopimelic acid and the other cellular constituents of *E. coli*. *meso*Diaminopimelic acid and lysine are substrates for several enzymes known to be present in the strain used (Meadow *et al.* 1957). Two specific decarboxylases convert either *meso*diaminopimelic acid into lysine or lysine into cadaverine; and diaminopimelic acid racemase produces an equilibrium mixture of the *meso*- and *LL*-isomers (Antia, Hoare & Work, 1957), a necessary reaction for the incorporation (as *meso*diaminopimelic acid and lysine) of the *meso*- and *LL*-[^{14}C]diaminopimelic acid supplied in our experiments. In addition, transaminases can catalyse the transfer of amino groups from all isomers of diaminopimelic acid and lysine to keto acids (Meadow & Work, 1958). However, very little redistribution of ^{14}C was found in cells grown with [^{14}C]lysine or [^{14}C]diaminopimelic acid. About 90% of the [^{14}C]lysine taken up from the medium was recovered in the lysine of the trichloroacetic acid-insoluble fractions only and traces of radioactivity were found in two other unidentified materials (X and Y) in these fractions. These labelled materials (X and Y) were not investigated further. [^{14}C]Diaminopimelic acid was incorporated both by the diaminopimelic acid-requiring mutants and by the parent strain; it was converted only into lysine and the unidentified materials X and Y. Thus transamination reactions apparently played little part in the normal metabolism of either diaminopimelic acid or lysine in *E. coli* and insignificant decarboxylation of lysine can have occurred.

This metabolic inertness of lysine had previously been shown with *E. coli* grown in the presence of carboxyl-labelled lysine (Siddiqi, Kozloff, Putnam & Evans, 1952), but some results of Roberts *et al.* (1955) suggested a redistribution of exogenous [^{14}C]lysine into threonine, isoleucine, arginine and proline. Roberts *et al.* were, however, unable to find any evidence for incorporation of diaminopimelic acid during growth of *E. coli* B for 2 hr. with [^{14}C]glucose, since there was no decrease of the labelling of intracellular diaminopimelic acid or lysine as a result of adding unlabelled diaminopimelic acid to the growth medium. The results were interpreted as indicating that diaminopimelic acid is not on the direct biosynthetic route to lysine, but this interpretation is not valid as diaminopimelic acid was apparently not incorpo-

ated by the bacteria in these experiments. It is not possible to say whether the short growth time or the different strain used by Roberts *et al.* could account for the divergence between their results and ours, or whether the low concentrations of diaminopimelic acid in the whole cells and hence the low radioactivities involved made changes in radioactivity difficult to detect.

One of the main difficulties in all experiments involving the study of diaminopimelic acid in Gram-negative bacteria is the relatively low concentration of this amino acid in whole cells (0.4% dry wt.). It was to avoid this difficulty that we used isolated cell walls, which in *E. coli* contain all the cellular diaminopimelic acid (Hoare & Work, 1957) but comprise only about 10% of the dry weight (Salton, 1956). We also concentrated the diaminopimelic acid-containing component of the walls by extraction with 90% phenol. This method of fractionation was used by Weidel & Primosigh (1958) to separate the walls of *E. coli* B into two components, a phenol-soluble lipoprotein and a phenol-insoluble lipopolysaccharide-peptide. The latter fraction, which represented only 20% of the wall weight, contained, in addition to the lipopolysaccharide, glutamic acid, alanine, glycine, lysine, diaminopimelic acid, glucosamine and muramic acid. This simple amino acid pattern, combined with the two hexosamines, is typical of the mucopeptide which comprises the main rigid structure of the walls of Gram-positive bacteria, except that in these bacteria lysine and diaminopimelic acid never occur together (see Work, 1957). Our results on the amino acid contents of the two wall fractions confirmed in general those of Weidel & Primosigh, except that diaminopimelic acid was also present to a small extent in the phenol-soluble fraction and trace amounts of several other amino acids as well as the seven main components were present in the phenol-insoluble residue. We have found that the divergences between these results are due to strain differences rather than to differences in techniques.

The mutants which were exacting for either diaminopimelic acid or lysine incorporated these amino acids very efficiently. Both the cell-wall fractions together contained less ^{14}C than the trichloroacetic acid-insoluble fractions, which is to be expected since the trichloroacetic acid-insoluble fraction contains both the intracellular protein and the cell walls. Similar results have been reported for lysine incorporation into cell walls of *Staphylococcus aureus* (Gale, Shepherd & Folkes, 1958).

The experiments with the diaminopimelic acid-requiring mutants, grown in identical media containing both diaminopimelic acid and lysine, suggest that these mutants obtain lysine by at least three different means: by direct incorporation of exogenous lysine; by decarboxylation of

exogenous diaminopimelic acid; by a route from glucose which does not involve diaminopimelic acid. The difference between the two mutants may depend on the proportionately different utilization of these routes rather than the development of new biosynthetic pathways. In spite of the different effects of lysine on their growth, both mutants obtained 10% of their wall lysine directly from the medium, showing that the independence of lysine of mutant D was not due to impermeability to this amino acid. However, the amount of wall lysine (50–60%) derived from diaminopimelic acid in mutant D was less than that in mutant 173–25 (80%), although the diaminopimelic acid decarboxylase activities of the mutants were the same (Meadow *et al.* 1957). The lysine requirement of mutant 173–25 cannot therefore have been due to lack of penetration of diaminopimelic acid as suggested by Davis (1955). On the contrary, it seems more probable that the lysine independence of mutant D was achieved by an increased utilization of the third source of this amino acid. This hypothesis was supported by the results of experiments with [^{14}C]glucose, in which only 10% of the wall lysine of mutant 173–25, as compared with 20% of that of mutant D, was derived from glucose. The source of lysine in mutant D grown without exogenous lysine was not studied, so it is not possible to decide whence this mutant would derive its lysine under this condition. There is some evidence that exogenous lysine may effect the endogenous metabolism of diaminopimelic acid and lysine. Thus the presence of lysine causes lysis of mutants D and 173–25 grown in limiting concentrations of diaminopimelic acid (Meadow *et al.* 1957; Rhuland, 1957); the mechanism of this effect has not been studied. Roberts *et al.* (1955) suggested that exogenous lysine may initiate a series of metabolic steps leading to the synthesis of arginine and proline from lysine; however, the lack of redistribution of ^{14}C from lysine and diaminopimelic acid in our experiments make such new metabolic steps unlikely in the diaminopimelic acid-requiring mutants.

Although these experiments provide an explanation for the difference between the amino acid requirements of mutants 173–25 and D, they do not explain the partial lysine requirement of mutant 173–25. The alternative route to lysine from glucose was shown to be operative in this mutant and yet, even when supplemented by the route through diaminopimelic acid, it was unable to meet the full lysine requirements of the growing cell. In mutant 26–26, which lacks diaminopimelic acid decarboxylase (Dewey & Work, 1952), the absolute requirement for lysine suggests that the alternative route to lysine through glucose must be absent or inoperative.

Lysine was not converted into diaminopimelic acid in our experiments, in confirmation of the results of Roberts *et al.* (1955). This result was to be expected in mutant 26–26, which has no diaminopimelic acid decarboxylase, but in mutant 173–25 and D, which contain normal levels of decarboxylase (Meadow *et al.* 1957), the failure to transfer ^{14}C from lysine to diaminopimelic acid can be taken as evidence for the virtual irreversibility of diaminopimelic acid decarboxylase. The identical requirements of the two mutants for diaminopimelic acid also support this view.

SUMMARY

1. Mutants of *Escherichia coli*, requiring either lysine or diaminopimelic acid or both of these amino acids for growth in a salts-glucose medium, were grown with [^{14}C]lysine, [^{14}C]diaminopimelic acid or [^{14}C]glucose and the radioactivities of the lysine and diaminopimelic acid in the trichloroacetic acid-insoluble fractions and fractionated cell walls were investigated.

2. [^{14}C]Lysine was taken up by all the mutants tested. Of the radioactivity incorporated, 90% was found in the lysine of the trichloroacetic acid-insoluble fractions, whereas the cell walls contained 50–60%. Diaminopimelic acid was not labelled; 10% of the cell-wall lysine of the diaminopimelic acid-requiring mutants was derived from that supplied exogenously.

3. [^{14}C]Diaminopimelic acid was incorporated into both diaminopimelic acid and lysine of the diaminopimelic acid-requiring mutants and the parent strain. Eighty per cent of the cell-wall lysine of mutant 173–25 (diaminopimelic acid-dependent, lysine-stimulated) and 50–60% of that from mutant D (diaminopimelic acid-dependent) was derived from the exogenous diaminopimelic acid.

4. Mutants 173–25 and D grown with [^{14}C]glucose contained [^{14}C]lysine but unlabelled diaminopimelic acid. Twenty per cent of the wall lysine in mutant D and 10% of that in mutant 173–25 was derived from glucose.

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The Estimation of Anserinase Activity by a Low-Temperature Ninhydrin Reaction

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The properties of an enzyme which cleaves anserine into 1-methylhistidine and β -alanine have been reported previously (Jones, 1955, 1956). In the latter communication, reference was made to an improved procedure for the assay of anserinase. The present paper describes the experimental basis of this procedure.

In the earlier study, enzyme activity was measured by either the chromatographic estimation of liberated β -alanine or the analysis of deproteinized reaction mixtures for increase in amino nitrogen by a ninhydrin colorimetric procedure (Moore & Stein, 1948), the degree of hydrolysis being calculated according to Fleisher (1953) and Schwartz & Engel (1950). The chromatographic procedure is lengthy, but can be used to estimate small amounts of anserine hydrolysis with considerable accuracy. The Schwartz & Engel procedure is relatively rapid but less sensitive. This low sensitivity, which is a particular disadvantage in the measurement of initial velocity constants, is due to the high colour yields given by unhydrolysed anserine relative to those given by small quantities of liberated amino acids at a reaction temperature of 100°.

During the course of an examination of the free amino compounds of fish muscle it was noticed that anserine gave a weak yellow-orange spot on ninhydrin-sprayed chromatograms developed at room temperature, whereas its constituent amino acids gave more typical purplish colorations. It seemed possible, therefore, that similar effects might occur in solution and allow the direct colorimetric

determination of anserinase by a method with low or zero anserine 'blanks' and consequent higher potential sensitivity at low levels of hydrolysis.

EXPERIMENTAL

Materials

Anserine. This was isolated from frozen pike (*Esox lucius*) by ion-exchange chromatography on sulphonated-polystyrene and Dowex 2 resins (Jones, 1955). After being recrystallized twice, its m.p. was 240°, decomp. (corr.). Linneweh & Linneweh (1930) record m.p. 238–239° (Found: N, 23.2. Calc. for $C_{10}H_{16}O_3N_4$: N, 23.3%).

DL-1-Methylhistidine. Anserine (3 g.) was hydrolysed in barium hydroxide (14%, w/v) for 8 hr. at 120°. The barium was removed as the sulphate and the hydrolysate was displaced from a column of Dowex 2 (OH) resin (15 cm. $\times$ 1.8 cm., 80–100 mesh) with 0.02N-HCl through successive columns (12 cm. $\times$ 1 cm., 100–200 mesh and 10 cm. $\times$ 0.5 cm., 200–400 mesh). The rate of flow from the last column was 0.2 ml./min. Uncontaminated DL-1-methylhistidine fractions were obtained. These were concentrated and, after recrystallizing from water, gave 1.4 g. of the amino acid, m.p. 251.5° decomp. (corr.). Linneweh & Linneweh (1930) reported m.p. 248–252° (Found: N, 22.6. Calc. for $C_7H_{11}O_3N_2H_2O$: N, 22.5%).

β -Alanine. This was obtained in uncontaminated fractions during the separation of the hydrolysate of anserine on Dowex 2. The fractions were concentrated and after being recrystallized from aqueous ethanol gave 800 mg. of the amino acid, m.p. 200.5° (corr.). Holm (1905) reported m.p. 200° (Found: N, 15.6. Calc. for $C_3H_7O_2N$: N, 15.7%).

Ninhydrin reagent. This was prepared according to Moore & Stein (1948).