the yolk. On the 14th day there is a pronounced surge in the liver which precedes a greater surge of activity in skeletal muscle on the 18th day. These surges of activity probably represent a pattern of functional differentiation of certain cells in the embryo, and in its liver and muscle.

SUMMARY

1. An increase of glutamic dehydrogenase (GDH) activity in yolk of the hen's egg has been shown to occur in the mid-incubation period.

2. Generally, the levels of GDH in embryonic tissues are less than those of tissues of the hen except for the blastoderm and 2-day-old embryo, which have GDH levels as high as those of some hen tissues.

3. GDH has been found to increase *pari passu* with total protein in the whole embryo and yolk sac from the fourth and sixth day of incubation respectively.

4. GDH activity in the chick embryo and its extra-embryonic tissues during development has been shown to have similarities with certain other enzyme activities.

5. In certain tissues of chick embryos during later developmental stages considerable surges of activity are found to occur which may be associated with functional differentiation.

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Interrelationships between Lysine and αε-Diaminopimelic Acid and their Derivatives and Analogues in Mutants of *Escherichia coli*

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The little that is known of the biosynthesis or metabolism of lysine in bacteria suggests that the biosynthetic pathway is different from that in other species (for review see Work, 1955; Strassman, Thomas & Weinhouse, 1956). *meso*-αε-Diaminopimelic acid, which is converted into L-lysine by enzymic decarboxylation (Dewey, Hoare & Work, 1954), has now been established as a lysine precursor in some bacteria. We have investigated further relationships between lysine and diaminopimelic acid by studying the growth of certain diaminopimelic acid- and lysine-requiring mutants derived from *Escherichia coli* 9637 (Davis, 1952)

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The lysine-requiring mutant 26-26 was blocked at the diaminopimelic acid decarboxylase step (Dewey & Work, 1952) and accumulated diaminopimelic acid during growth (Davis, 1952). The diaminopimelic acid-requiring mutant 173-25 had no such block, although it had a partial requirement for lysine (Davis, 1952). In this Laboratory, training of the latter mutant to grow in decreasing concentrations of lysine has produced a further mutant, D, which required diaminopimelic acid but not lysine.

The effects of lysine analogues and derivatives on the growth of the lysine-requiring mutants 26-26 and 173-25 were investigated. Most of the compounds tested were already known to affect lysine metabolism in other species. ω-*N*-Acetyl-lysine replaced lysine for the rat but the α-*N*-acetyl derivative was inactive (Neuberger & Sanger, 1944). ε-Hydroxy-α-aminocaproic acid prevented lysine utilization in rats (Gaudrey, 1954), and either inhibited growth or replaced lysine in neurospora mutants (Good, Heilbronner & Mitchell, 1950); but it had no effect on lactobacilli or the lysine-requiring *E. coli* mutants 173-25 and 26-26 (Ravel, Woods, Felsing & Shive, 1954; Davis, 1952). D-Lysine did not replace L-lysine in the rat, but spared the lysine requirement of certain neurospora mutants (Schweet, Holden & Lowy, 1954). Lysine polymers inhibited the growth of *Staphylococcus aureus*, *E. coli* and *Leuconostoc mesenteroides*, the most highly polymerized compounds being the most active (Katchalski, Bichowski-Slomnitzki & Volcani, 1953; McLaren & Knight, 1953), and were tested for their activity against the mutants.

Enzyme activities of the two diaminopimelic acid-requiring mutants were compared with a view to determining whether the additional lysine requirement of mutant 173-25 was the result of a change in the relative activities of the enzymes known to metabolize diaminopimelic acid and lysine (Antia, Hoare & Work, 1957).

The two diaminopimelic acid-requiring mutants were found to lyse during the logarithmic-growth phase when grown in the presence of lysine and low concentrations of diaminopimelic acid. The effects on this lysis of various derivatives and analogues of diaminopimelic acid and lysine were studied. Among the compounds tested was lanthionine, the monosulphur analogue of diaminopimelic acid and a constituent of the antibiotic nisin (Newton, Abraham & Berridge, 1953; Alderton & Fevold, 1951). Lanthionine had been reported to replace diaminopimelic acid for the growth of mutant 173-25 (Seibert, Soto-Figueroa, Miller & Seibert, 1954); it did not do so under our conditions of test but had other interesting effects.

It was hoped that conditions might be found whereby the diaminopimelic acid-requiring mutants could be used for microbiological assay. Davis (1952) had reported that the use of mutant 173-25 was complicated by the facts that excess of lysine was inhibitory and that the optimum concentrations of lysine varied with diaminopimelic acid concentration. We found the main complication to be lysis.

Some of these results have already been presented in a preliminary report (Meadow & Work, 1956).

METHODS

Chemicals

αε-Diaminopimelic acid. Isomers were obtained as described by Hoare & Work (1955). Unless otherwise stated, *meso*diaminopimelic acid was used.

Diaminodicarboxylic acids. These were unresolved materials prepared by Dr D. H. Simmonds (1954).

Polylysines. These were obtained from Dr B. E. Volcani.

N-Acetyl-lysines. ω-*N*-Acetyl-L-lysine was prepared by a modification of the method of Neuberger & Sanger (1943), the ions being removed by electrodialysis (Found: C, 50·8; H, 8·6; N, 14·7%. $C_7H_{16}O_3N_2$ requires C, 51·0; H, 8·5; N, 14·9%). α-*N*-Acetyl-L-lysine was prepared by Dr A. Neuberger & Dr F. Sanger (1943).

Pure D- and L-amino acids. These were obtained from Dr J. P. Greenstein. Unless otherwise stated, the lysine employed was commercial L-lysine hydrochloride.

Lanthionine. This was commercial material consisting of *meso-* and LL-isomers.

Casein hydrolysates. These were prepared according to the method of Askonas, Campbell & Work (1954).

Organisms

All organisms were grown at 37° and originated from the parent strain *E. coli* ACTC 9637. This organism grows well in the minimal medium described below. It was maintained on nutrient agar, stored at 4° and subcultured at 3-monthly intervals. It was subcultured into minimal medium 24 hr. before use.

E. coli 26-26. This was originally obtained from Dr B. Davis. It required the addition of lysine (200 μM) to minimal medium for optimum growth. It was maintained and stored in the same way as *E. coli* 9637. It was subcultured into minimal medium containing lysine (200 μM) 24 hr. before use.

E. coli 173-25. This was also obtained from Dr Davis. It required 100 μM-diaminopimelic acid (*meso-* or LL-) and 100 μM-lysine for optimum growth in minimal medium (Hoare & Work, 1955). It was originally stored at 4° on minimal-medium agar containing lysine (100 μM) and diaminopimelic acid (100 μM), but after some time it was lost and a fresh subculture obtained from Dr Davis. It was then stored as a freeze-dried culture. Three days before use this was subcultured into minimal medium containing 100 μM-diaminopimelic acid and 100 μM-lysine and thereafter subcultured daily in this medium.

E. coli D. This was produced from mutant 173-25 by subculture into a series of tubes containing minimal medium plus 100 μM-diaminopimelic acid and twofold dilutions of lysine from 100 μM downwards, and also into a control tube containing no lysine. By daily transfer for about 4 weeks from the lowest concentration of lysine in which good growth occurred into lower concentrations of

lysine, the mutant D was produced. It grew well in minimal medium containing 100 μM-*meso*- or -LL-diaminopimelic acid and was not stimulated by lysine. It was at first stored at 4° on minimal medium agar plus 100 μM-*meso*diaminopimelic acid and subcultured monthly. Under these conditions it was not stable and regained its lysine requirements. It was obtained afresh from mutant 173-25 as before and freeze-dried for storage. It was used in the same way as mutant 173-25, except that lysine was omitted from the medium.

Growth tests

The minimal medium was a modification of that used by Davis & Mingioli (1950) and contained: K_2HPO_4, 7 g.; KH_2PO_4, 3 g.; sodium citrate, 0·5 g.; $MgSO_4,7H_2O$, 0·1 g.; $(NH_4)_2SO_4$, 1 g.; water to 1 l.; pH was 7·0. Sterile glucose was added after autoclaving (10 lb./in.^{2} for 10 min.) to give a final concentration of 1% (w/v). For minimal-medium agar, agar (2%, w/v) was incorporated. Minimal medium was distributed in 6 in. × $\frac{3}{4}$ in. glass tubes to give 10 ml. final volumes. Polylysines, leucovorin and pyridoxal phosphate were sterilized by filtration through Dalton P5 Chamberland thimbles. Except where stated, all other additions were made before autoclaving. All tests were set up at least in duplicate and included three control tubes, intended to detect reversion, containing minimal medium alone, minimal medium plus diaminopimelic acid or minimal medium plus lysine. The inoculum was usually one drop (0·02 ml.) of an 18 hr. culture, washed three times with normal saline and resuspended in its original volume of saline. For the penicillin experiments, heavy suspensions of logarithmically growing cells were produced by aeration with a stream of air during incubation; the organisms were washed as before and 3 ml. was used as inoculum. The tubes were incubated in a water bath at 37°. Growth was measured in terms of opacity at 675 mμ in the Unicam absorptiometer (type SP. 350) by placing the tubes directly in the apparatus and using a control tube for obtaining a zero reading. Corrections were made to bring net turbidity into a linear relationship with bacterial concentrations (Toennies & Gallant, 1949*a*). In order to detect reversion, the organisms from each tube were subcultured at the end of every experiment into minimal medium alone and into minimal medium containing the amino acid(s) necessary for growth, and incubated for 24 hr.

Enzyme tests

E. coli 9637 was grown for 24 hr. at 37° with aeration by agitation in 4 l. of minimal medium containing Difco acid casein hydrolysate (0·25 g./l.). The mutants were grown similarly with the addition of lysine and diaminopimelic acid as required. Before harvesting they were tested for reversion as previously described. The cells were harvested by centrifuging and washed three times with normal saline before drying with acetone and storage at −10°. Diaminopimelic acid and lysine decarboxylases were measured in the Warburg apparatus at appropriate pH as described by Hoare & Work (1955) and Hoare (1956). Racemase activity was determined chromatographically (Antia *et al.* 1957).

Chromatography

Whatman no. 1 paper was used throughout. The presence of diaminopimelic acid in the culture filtrates was demonstrated by paper chromatography in methanol–water–10N-HCl–pyridine (80:17·5:2·5:10) as used by Hoare & Work (1955). The contents (10 ml.) of the tubes were filtered through Dalton P5 Chamberland thimbles to remove the bacteria, mixed with a knife point of activated charcoal (British Drug Houses Ltd.), evaporated to dryness on a water bath and resuspended in 0·1 ml. of water. Activated charcoal was removed by centrifuging and the whole solution examined chromatographically. Examination of minimal medium with diaminopimelic acid added before or after this treatment showed that diaminopimelic acid was not removed by the activated charcoal.

Bioautography (Winsten & Eigen, 1950) was used to detect growth-stimulating factors on chromatograms. Minimal-medium agar, containing sufficient (1 μM) synthetic diaminopimelic acid to give heavy growth only in the presence of a stimulating factor and 2:3:5-triphenyltetrazolium chloride (0·1%) as indicator, was seeded at 48° with a washed suspension of *E. coli* D (1·6 ml. of suspension/100 ml. of agar) prepared as for growth tests. After development overnight in solvents, dried chromatograms were applied to the agar for 20 min. Controls, consisting of the same substances applied to the chromatography paper but not irrigated with the solvent, were also placed on the plates. After incubation for 18 hr. at 37°, areas where heavy growth had occurred were red. Lysis was visible as a clearing in the agar medium, which was otherwise slightly cloudy owing to trace growth of the organisms. The distances moved by the amino acids in various solvents were determined by making duplicate chromatograms, one of which was applied to the agar plate while the other was dipped in ninhydrin in acetone (0·1%, w/v) and heated at 100° for 2 min.

RESULTS

Growth characteristics of E. coli 173-25 *and D*

The findings of Davis (1952) on the absolute requirement of mutant 173-25 for diaminopimelic acid and the relative requirement for lysine were confirmed (see Table 1), although the diaminopimelic

Table 1. *Effects of lysine, diaminopimelic acid (DAP) and lanthionine on growth of* E. coli 173-25 *and* E. coli *D for* 18 *hr. in minimal medium*

Growth conditions and technique are as described in Methods. +, added to the medium; −, not added to the medium.

Mutant	Lysine (100 μM)	DAP (100 μM)	Lanthionine (10 μM)	Turbidity at 675 mμ, corrected
173-25	−	−	−	0
	+	−	−	0
	−	+	−	102
	+	+	−	284
	−	−	+	0
	+	−	+	0
	−	+	+	0
	+	+	+	290
D	−	−	−	0
	+	−	−	0
	−	+	−	278
	+	+	−	278
	−	−	+	0
	+	−	+	0
	−	+	+	0
	+	+	+	270

acid concentration (100 μM) required for optimum growth was five times higher than that reported by Davis. Mutant D responded only to diaminopimelic acid (Table 1) and an approximately linear relationship existed between diaminopimelic acid concentration (0–50 μM) and the final optical density [Fig. 5, curve (*a*)]. On the other hand, with mutant 173-25 there was no such linear relationship; study of the growth curves in the presence of lysine revealed that, with low concentrations of diaminopimelic acid, decreases in turbidity occurred after varying periods of logarithmic growth. Fig. 1 represents a series of such growth curves obtained with mutant 173-25 grown in lysine (100 μM) and varying concentrations of diaminopimelic acid. Both the lag time (0–5 hr. after inoculation) and the growth rate in the logarithmic phase (5–10 hr. after inoculation) were independent of diaminopimelic acid concentration; but between 6 and 10 hr. after inoculation there was a decrease in turbidity in the tubes containing concentrations of diaminopimelic acid below 20 μM. This decrease continued at an approximately logarithmic rate and occurred first in media containing the lowest concentrations of diaminopimelic acid. After this phase of fall in turbidity (hereafter referred to as lysis), there was a slight rise in turbidity which was not due to reversion of the culture since, 24 hr. after inoculation, tests for reversion showed that the organisms still retained their original growth requirements. At diaminopimelic acid concentrations higher than 20 μM no lysis occurred.

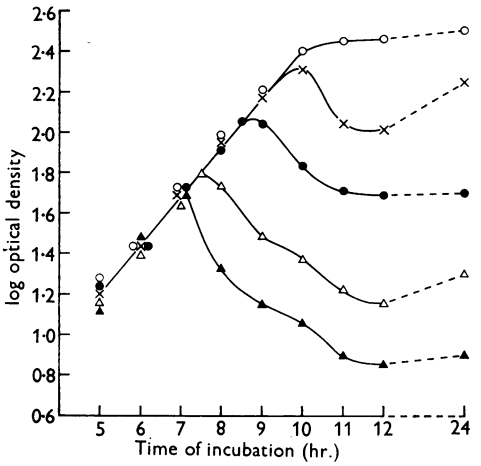

Fig. 1. Response of *E. coli* 173-25 to various concentrations of *meso*diaminopimelic acid in the presence of L-lysine (100 μM). Growth was at 37° in tubes containing 10 ml. of minimal medium plus amino acids; inoculum was 0·02 ml. of washed cells from an overnight culture. Optical density was measured at 673 mμ. Concentrations of *meso*diaminopimelic acid (μM): ○, 40; ×, 20; ●, 10; △, 5; ▲, 2·5.

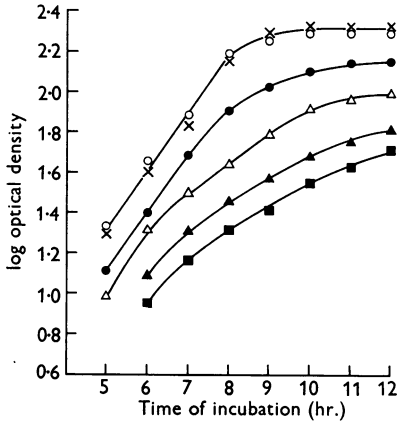

Fig. 2. Response of *E. coli* 173-25 to various concentrations of L-lysine in the presence of *meso*diaminopimelic acid (50 μM). Methods as in Fig. 1. Concentration of L-lysine (μM): ×, 100; ○, 50; ●, 10; △, 5; ▲, 1; ■, no lysine.

At various stages of the growth curve, the organisms in certain tubes were centrifuged out and the culture filtrates examined for diaminopimelic acid. Since very small amounts of diaminopimelic acid (1·9–38 μg./tube) were present originally in the media in which growth and lysis occurred, paper chromatography proved to be the only feasible method of examination. Even here it was necessary to examine the whole contents (10 ml.) of the tubes. Fortunately, the solvent usually employed for the detection of diaminopimelic acid on paper chromatograms (methanol–water–HCl–pyridine) can be used to separate substances mixed with high concentrations of salts. No preliminary desalting or treatment other than removal of carbohydrates by activated charcoal was found necessary before concentration of 10 ml. of culture media and application to a single spot. In all tubes where lysis had occurred, no free diaminopimelic acid was found (i.e. less than 0·5 μg. was present), whereas those in which logarithmic growth was taking place contained detectable amounts. In the control tubes containing more than 20 μM-diaminopimelic acid, where no lysis was observed, diaminopimelic acid was found in the medium in all stages of growth. All the tubes examined contained lysine.

When mutant D, which grew normally in the absence of lysine, was grown with lysine in the medium, the result was identical with that obtained with mutant 173-25 on the same medium; lysis occurred in the same concentration range of diaminopimelic acid (Fig. 3).

Effect of growth conditions on lysis. Changes in the age and size of the inoculum of both mutants were investigated. With inocula grown for 6, 9, 12, 15 and 24 hr. there was no change in the time of lysis or the

level of diaminopimelic acid below which lysis occurred. The lag phase could be shortened to 2 hr. and the time of lysis brought forward slightly by increasing the inoculum size, but lysis still occurred only in the logarithmic phase; the total growth achieved before lysis and the concentration of diaminopimelic acid below which lysis occurred were unchanged.

The concentration of glucose in the medium was varied between 1 and 0·05 %, below which level the amount of growth was too small to be measured. Within this range lysis was unaffected by glucose concentration and in all further experiments 1 % (w/v) glucose was used. Changes in phosphate concentration between 0·7 mM and 0·7 M had no effect on lysis.

The effect of lysine on the growth and lysis of these two mutants was investigated in some detail. In the presence of optimum concentrations of diaminopimelic acid (50 μM), the mass of growth of mutant 173-25 was raised by increasing concentrations of lysine and there was no lysis (Fig. 2). These results show the marked difference in this mutant between its absolute requirement for diaminopimelic acid and its relative requirement for lysine, since shortage of diaminopimelic acid in the presence of excess of lysine caused lysis, whereas shortage of lysine in the presence of excess of diaminopimelic acid did not. The growth of mutant D was not affected by the addition of lysine to the medium (Table 1). At lower concentrations of diaminopimelic acid (below 20 μM), where lysis occurred, variations in lysine concentration from 50 to 500 μM had no effect on the growth or lysis of either mutant; at concentrations of lysine below 50 μM and with these low concentrations of diaminopimelic acid, growth of mutant 173-25 was too poor to be followed photometrically. The growth of mutant D could be followed in various concentrations of diaminopimelic acid both with and without lysine. Here there was no lysis in the absence of lysine or in concentrations below 50 μM; above this level lysis occurred, although the initial growth rate was unchanged (Fig. 3). Thus the presence of lysine was proved to be an essential factor in lysis.

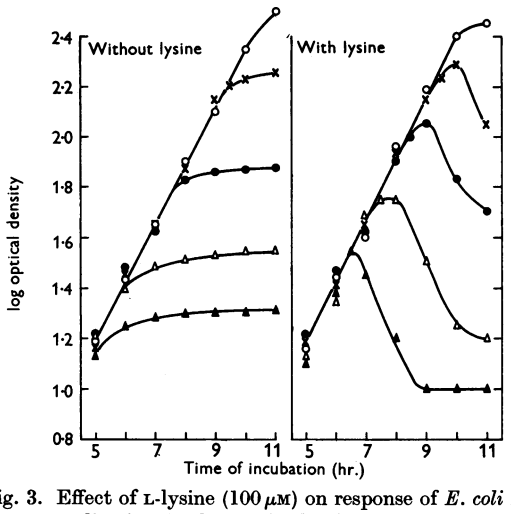

Fig. 3. Effect of L-lysine (100 μM) on response of *E. coli* D to *meso*diaminopimelic acid. Methods as in Fig. 1. *meso*Diaminopimelic acid concentration (μM): ○, 40; ×, 20; ●, 10; △, 5; ▲, 2·5.

Effect of diaminopimelic acid isomers and analogues on growth. The growth characteristics of the mutants were unchanged by replacing *meso*diaminopimelic acid by the LL-isomer. DD-Diaminopimelic acid did not support growth. Various samples of synthetic diaminopimelic acid produced the same effects (lysis or growth) as the *meso*-isomer except that concentrations 1·2–2 times higher were required. This variability of activity was probably due to differences between the samples in the relative proportions of the isomers, caused by their differential solubilities.

Lanthionine was incapable of replacing diaminopimelic acid for growth in the presence of lysine. In the absence of lysine, lanthionine inhibited growth of both mutants; in the presence of optimum concentrations of both lysine and diaminopimelic acid it had no effect (Table 1). In suboptimum concentrations of diaminopimelic acid and 100 μM-lysine (the conditions found to produce lysis), lanthionine 10 μM prevented lysis, though it had no effect on the lag period or growth rate of either mutant. Fig. 4*B* shows the effect of lanthionine on the growth and lysis of mutant 173-25. Similar results were obtained with mutant D. Nisin was also tested for its effect on growth; even at the highest concentration used (3000 units or 0·1 mg./ml.) it had no effect on growth or lysis in the presence or absence of lysine.

Cystine did not replace diaminopimelic acid. It inhibited the growth of mutant 173-25 on lysine and diaminopimelic acid and of mutant D on diaminopimelic acid with or without lysine. Its effects were investigated in more detail with mutant D, where good growth without lysis took place in the absence of lysine. The inhibition of growth by cystine was competitive, being overcome by increasing the diaminopimelic acid concentration (Fig. 5). Cystathionine, diaminosebacic acid, diaminoadipic acid, diaminosuccinic acid, diaminosuberic acid, 2:6-piperidinedicarboxylic acid, dipicolinic acid and $\alpha\epsilon$-diamino-$\alpha\epsilon$-dimethylpimelic acid (all mM) and 0·1 mM-$\alpha\epsilon$-diamino-β-hydroxypimelic acid (isomers A and B, Stewart & Woolley, 1956) were incapable of supporting the growth of either mutant in the absence of diaminopimelic acid, whether or not lysine was present. None of these substances prevented lysis or inhibited growth.

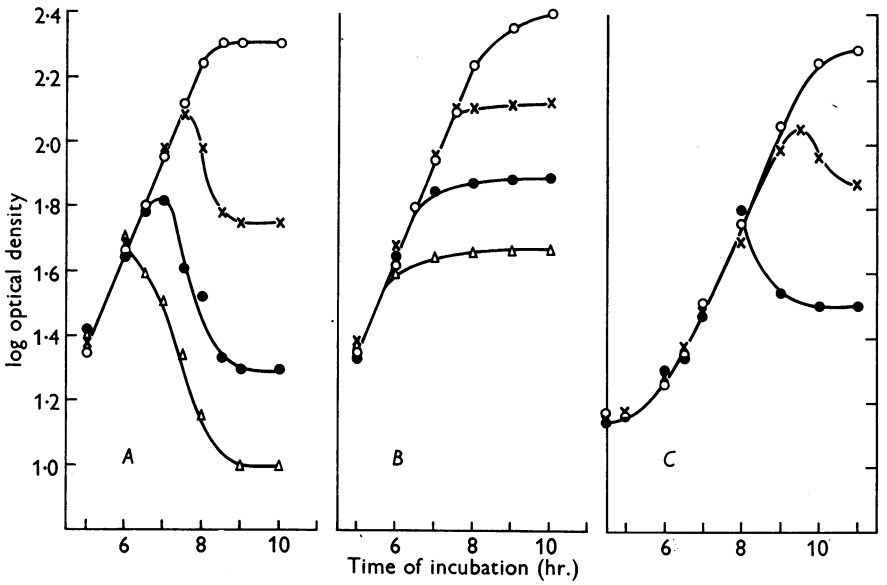

Fig. 4. Effect of lanthionine and tetralysine on response of *E. coli* 173-25 to *meso*diaminopimelic acid in the presence of L-lysine (100 μM). Methods as in Fig. 1. *meso*Diaminopimelic acid concentration (μM): ○, 40; ×, 20; ●, 10; △, 5. *A*, No further additions; *B*, plus lanthionine (10 μM); *C*, plus tetralysine (100 μM).

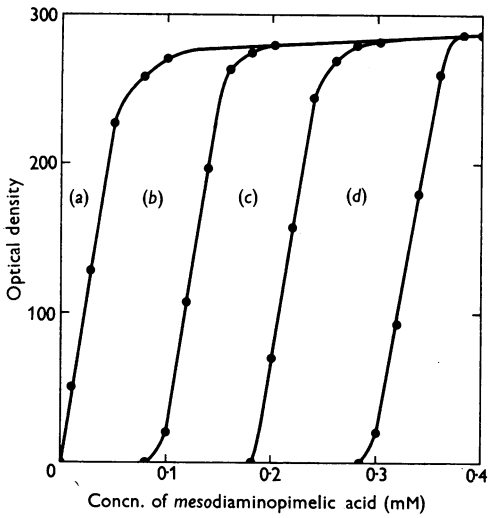

Fig. 5. Competitive inhibition by cystine of growth of *E. coli* D in the presence of various concentrations of *meso*diaminopimelic acid. Incubation for 18 hr.; other conditions were as in Fig. 1. Cystine concentration (μM): (*a*) 0; (*b*) 25; (*c*) 50; (*d*) 100.

Further growth requirements. In order to investigate further the suitability of mutants 173-25 and D for the microbiological assay of diaminopimelic acid, a large number of naturally occurring substances were tested for effects on growth in the presence of optimum (100 μM) or suboptimum (10 μM) concentrations of diaminopimelic acid. None of the following compounds had any effect in concentrations up to those shown: Vitamins: thiamine, riboflavin, pteroylglutamic acid, pyridoxal phosphate, pantothenate, nicotinamide, biotin and inositol (all 10 μM); leucovorin, riboflavin phosphate and vitamin B_{12} (μM); pyridoxine and *p*-aminobenzoic acid (100 μM). Purines and pyrimidines: xanthine, adenine, guanine, uracil, hypoxanthine, uridine, thymine and thymidine (100 μM); 6-methylaminopurine (10 mM). Other growth factors: orotic acid (100 μM); glutamine, cholesterol and shikimic acid (mM); *p*-hydroxybenzoic acid and glutathione (10 mM); biocytin (10 μg./ml.).

Commercial acid hydrolysate of casein (Difco vitamin-free) markedly increased the growth and decreased the lag phase to 2 hr. in both optimum and suboptimum concentrations of diaminopimelic acid (in the latter lysis did not occur). This growth stimulation was investigated in more detail for mutant D (Table 2), since the absence of lysis in low concentrations of diaminopimelic acid had suggested that the organism might prove more useful than mutant 173-25 for microbiological assay. The absolute requirement for diaminopimelic acid was not eliminated by casein hydrolysate (5 mg./ml.) although the minimum amount necessary for growth was lowered approximately tenfold. Substitution of casein hydrolysate by a mixture of pure L-amino acids (Block & Bolling, 1945) did not

produce growth stimulation. Other samples of casein were examined and showed varying degrees of growth stimulation; in every case acid hydrolysates were more active than the unhydrolysed caseins. No activity was given by hydrolysates of several samples of goat casein and also of a pure sample of cow α-casein. The possible effect of trace metals was eliminated by the finding that ashed Difco casein hydrolysate was inactive. The difference in activities between various caseins might have been due to the formation of amino acid–sugar complexes during the time between milking and preparation of the caseins. Accordingly, samples of casein were prepared from cow's and goat's milk which had either stood 24 hr. at room temperature or had been obtained freshly from the same animals. After hydrolysis, the caseins from the two samples of cow's milk were identical in their growth-promoting activities, whereas the goat samples were both inactive.

Table 2. *Effect of Difco casein acid hydrolysate (CH) on the growth of* E. coli *D for 18 hr. in minimal medium containing* meso*diaminopimelic acid (DAP)*

DAP (μM)	CH (μg./ml.)	Turbidity corrected at 675 mμ
100	5000	440
100	500	300
100	50	270
100	0	260
10	5000	150
10	500	80
10	50	20
10	0	25

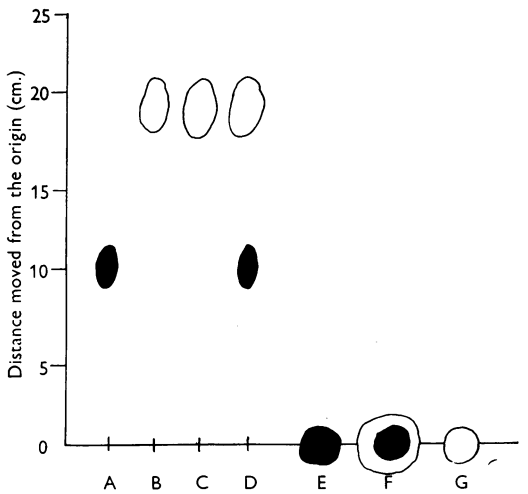

Fig. 6. Bioautography of *E. coli* D. Minimal-medium agar, containing synthetic diaminopimelic acid (μM) and 2:3:5-triphenyltetrazolium chloride (0·1%), was seeded with an 18 hr. washed culture of mutant D. Chromatogram (Whatman no. 1) was irrigated overnight with methanol–ammonia (sp.gr. 0·880)–water (200:10:20), dried for 6 hr. and laid on the agar plate for 20 min. The plate was incubated for 18 hr. at 37°. Materials were applied at the following positions: A and E, synthetic diaminopimelic acid (4 μg.); B and F, Difco casein hydrolysate (125 μg.); C and G, L-lysine (4 μg.); D, casein hydrolysate (125 μg.) + diaminopimelic acid (4 μg.) + L-lysine (4 μg.). Spots E, F and G were controls and were not irrigated in the solvent. Solid spots indicate areas of growth (red colour); open spots indicate areas of lysis (clearing).

These experiments were complicated by the very high reversion rate of mutant D to amino acid independence in the presence of casein hydrolysate. All tubes were set up in quadruplicate and even then there was occasionally reversion in all tubes. The factor causing growth stimulation was therefore investigated, using solid media (where reversion was easier to detect) and paper chromatography followed by bioautography on seeded-agar plates. Since Difco casein hydrolysate was the most active of the samples tested, this mixture was chosen for further investigation. The following chromatographic solvents were tested (figures in parentheses indicate parts by volume): *n*-butanol–water (90:10, 80:20, 60:40); *n*-butanol–ammonia (sp.gr. 0·880) (90:10); *n*-butanol–acetic acid (70:30); ethanol–ammonia (sp.gr. 0·880)–water (200:10:20); methanol–ammonia (sp.gr. 0·880)–water (200:10:20, 100:10:20, 100:40:20); methanol–water–10N-HCl–pyridine (80 : 17·5 : 2·5 : 10); *tert.*-butanol–water (70:30); 2:6-lutidine–water (5:3·5); propan-1-ol–water (40:10); methyl cellosolve (2-ethoxymethanol)–10N-HCl (100:1). Areas of growth (red colour) due to diaminopimelic acid were approximately the same size whether or not the chromatography paper had been irrigated, and lysine caused lysis both before and after irrigation. The effect of casein hydrolysate, however, was different in the control (unirrigated) and the irrigated strips from all solvents. The control spots showed an inner area of growth stimulation (red colour) as well as an outer ring of lysis (clearing), but after irrigation the growth-stimulating effect disappeared, leaving only a lysis zone in the position of lysine (Fig. 6). It seemed likely therefore that the growth stimulation was due to several factors which separated on paper chromatography and thereby lost their activity. Since hydrolysed pure α-casein was inactive, it is possible that the active complex originated from substances absorbed on the casein and subsequently removed in varying amounts according to the method of preparation and purification. No further attempt was made to identify the factors involved in this growth-promoting effect.

Effect of isomers and analogues of lysine on growth and lysis. D-Lysine showed approximately 20% of the activity of L-lysine in all the systems tested. Its

effect on the growth of mutant 173-25 in the presence of optimum concentrations of diaminopimelic acid and on growth of the lysine-requiring mutant 26-26 in minimal medium are shown in Table 3. With both isomers present their effects were additive. In the presence of D-lysine and suboptimum concentrations of diaminopimelic acid, both mutants 173-25 and D grew and lysed as in the presence of L-lysine, except that the minimum concentration of D-lysine (300 μM) causing lysis was approximately five times higher than that of L-lysine.

Fig. 7 compares the effect of L-lysine and its ω- and α-*N*-acetyl derivatives on the growth of the

Table 3. *Effect of* L- *and* D-*lysine on growth of* E. coli 26-26 *and* E. coli 173-25 *for* 18 *hr. in minimal medium*

– Indicates that no figures are available.

L-Lysine (μM)	D-Lysine (μM)	Turbidity corrected at 675 mμ 26-26	173-25*
0	0	0	95
10	0	0	100
50	0	30	120
100	0	75	240
200	0	150	244
400	0	180	244
0	50	0	110
0	250	25	180
0	500	70	236
0	1000	154	240
0	2000	175	240
50	50	–	160
50	250	–	230
50	500	–	240
100	250	95	–
100	500	150	–
100	1000	185	–

* *meso*Diaminopimelic acid (0·1 mM) was present in the medium.

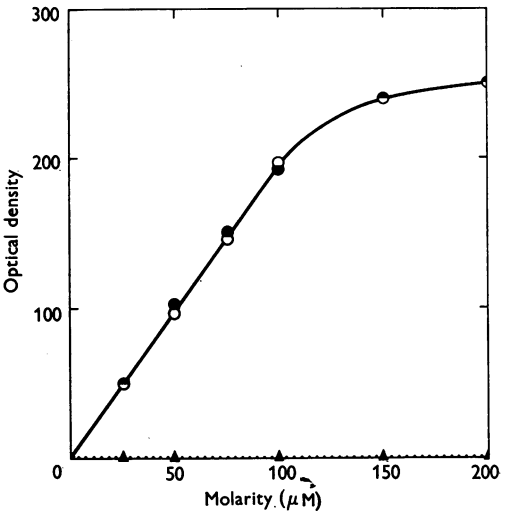

Fig. 7. Response of *E. coli* 26-26 to L-lysine and to *N*-acetyl-lysines. Incubation was for 18 hr. Other methods were as in Fig. 1. ●, L-Lysine; ○, α-*N*-acetyl-L-lysine; ▲...▲, ω-*N*-acetyl-L-lysine.

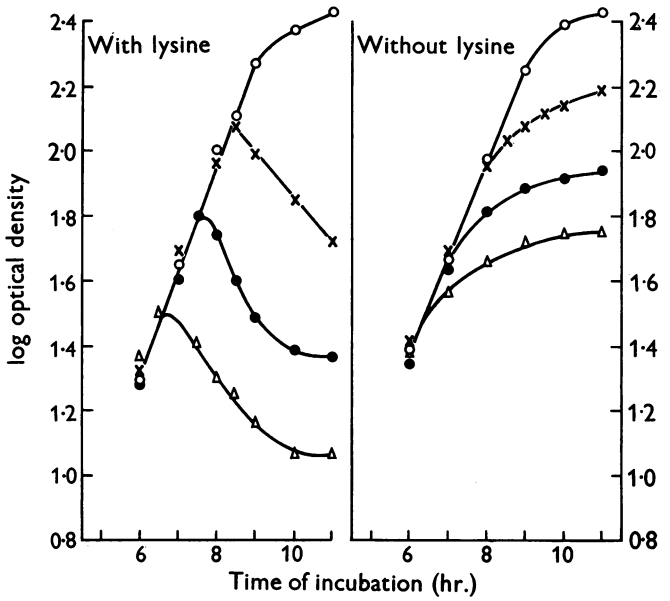

Fig. 8. Response of *E. coli* 173-25 to various concentrations of *meso*diaminopimelic acid in the presence of α-*N*-acetyl-L-lysine (100 μM) with and without L-lysine (100 μM). Methods were as in Fig.1. Concentration of *meso*diaminopimelic acid (μM): ○, 40; ×, 20; ●, 10; △, 5.

Table 4. *Concentrations of polylysines required to inhibit* 18 *hr. growth of* E. coli *in minimal medium containing appropriate supplements*

E. coli 9637 was tested in unsupplemented minimal medium. Amino acids (100 μM) present as supplements for the *E. coli* mutants examined were: mutant D, *meso*diaminopimelic acid; mutant 26-26, L-lysine; mutant 173-25, *meso*diaminopimelic acid and L-lysine. Figures represent lowest concentration of polylysines (μg./ml.) which inhibited growth. DP: Degree of polymerization, i.e. number of lysine molecules/molecule of polylysine.

	E. coli strain			
Polylysine	9637	D	26-26	173-25
Dilysine HCl	>500	>500	50	50
Trilysine HCl	>500	>500	35	40
Tetralysine HBr*	240	200	20	20
Polylysine HBr* (DP 32)	60	60	6	6
Polylysine HBr* (DP 200)	60	60	7	6

* Control tubes containing equivalent amounts of NaBr showed no inhibition.

lysine-requiring mutant 26-26. Lysine and α-*N*-acetyl-lysine showed equal activity, mole for mole, but ω-acetyl-lysine was inactive. This replacement of lysine by α-*N*-acetyl-lysine was not due to removal of the acetyl group during autoclaving since sterilization by filtration had no effect on growth-promoting activity. α-*N*-Acetyl-lysine also replaced lysine in enhancing the growth of mutant 173-25 in the presence of optimum concentrations of diaminopimelic acid, whereas ω-acetyl-lysine had no such activity. The effect of these substances on the growth of mutants 173-25 and D in suboptimum concentrations of diaminopimelic acid was also investigated. α-*N*-Acetyl-lysine did not prevent the lysis caused by lysine but, when it was substituted for lysine, there was no lysis (Fig. 8) even though, in mutant 173-25, growth was stimulated. ω-Acetyl-lysine (100 μM) had no effect either on growth or lysis.

Cadaverine, ornithine, ε-hydroxy-α-aminocaproic acid, 2-(4′-piperidyl)-1-aminopropionic acid (Harris & Work, 1950) and *S*-(β-aminoethyl)cysteine (Lindley, 1956), all 0·01–1 mM, had no effect, whether in replacing lysine for growth, in causing or preventing lysis, or in inhibiting growth for any of the mutants. Cystine and lanthionine (mM) did not affect growth of mutant 26-26.

Di-, tri-, tetra- and poly-lysines did not replace lysine for the growth of the lysine-requiring mutants and were inhibitory towards both the parent strain and all three mutants (Table 4). The inhibitory activity was related to the degree of polymerization, the most highly polymerized form was the most active. The lysine-requiring mutants were more sensitive than the parent strain or mutant D. The effect of subinhibitory concentrations of these polylysines on growth of 173-25 and D in the presence of suboptimum concentrations of diaminopimelic acid was tested. They caused an increased lag period and less total growth but they did not prevent lysis, as shown in Fig. 4*C*, where the organisms used were the more sensitive mutant 173-25.

Table 5. *Enzyme activities of* E. coli

Cells were grown as described in Methods and tested as acetone-dried powders. Decarboxylase tests were carried out in the Warburg apparatus: cells (40 mg.) were suspended in 2·5 ml. of 0·1 M-phosphate buffer [pH 5·5 for lysine, pH 6·8 for diaminopimelic acid (DAP)]. Substrates: L-lysine hydrochloride (6 mg.) or *meso*diaminopimelic acid (2 mg.); atmosphere, air; temperature, 37°. Q_{CO_2}, μl. of CO_2/mg./hr. Racemase test was by paper chromatography. Cells (3 mg.) were shaken at 37° with *meso*- or LL-DAP (0·3 mg. in 0·15 ml. of water) in 0·1 M-borate buffer, pH 8·5 (0·3 ml. containing 30 μg. of streptomycin). Samples (0·1 ml.) were removed at 30, 60 and 120 min., deproteinized with ethanol (0·2 ml.) and centrifuged: supernatants (0·1 ml.) were applied to Whatman no. 1 paper, which was irrigated overnight with methanol–water–10 N-HCl–pyridine (80:17·5:2·5:10). Spots developed with ninhydrin in acetone (0·1 %) at 100° for 2 min. Racemization was shown by equal concentrations of LL- and *meso*-DAP present in reaction mixture: ++, after 1 hr., +, after 2 hr.

	Decarboxylase activities (Q_{CO_2})		
E. coli	Lysine	DAP	DAP racemase
9637	4·4	4·2	+
173-25	4·3	5·2	++
D	4·3	5·4	++

Enzyme activities

The activities of diaminopimelic acid and lysine decarboxylases and of diaminopimelic acid racemase of acetone-dried cells of mutants 173-25, D and of the parent strain (9637) were determined (Table 5). The mutants had similar diaminopimelic acid decarboxylase and racemase activities, which were higher than those of the parent strain. The lysine decarboxylase activities of the three strains were identical.

Cell-wall composition

The composition of purified cell-wall preparations (Cummins & Harris, 1956*a*) from acetone-dried cells grown as for enzyme tests were kindly

determined for us by Dr C. S. Cummins and Dr H. Harris (London Hospital). The amino acid and sugar components of the cell walls of the two diaminopimelic acid-requiring mutants and of the parent strain were found to be indistinguishable by paper chromatography. The cell walls contained diaminopimelic acid, aspartic acid, glutamic acid, alanine, leucine and isoleucine in approximately equal amounts, together with slightly smaller amounts of lysine, threonine, serine, arginine, valine and tyrosine, and traces of proline and methionine. The sugars present in each were glucose, galactose, glucosamine and the 'unknown hexosamine' (Strange & Powell, 1954; Strange, 1956).

Further studies on lysis

In order to determine whether true lysis was responsible for the decrease in turbidity, the cultures were examined by electron and phase-contrast microscopy. Recently, Lederberg (1956) reported that addition of penicillin (1500 units/ml.) to heavy suspensions of *E. coli* B growing in nutrient media caused protoplast formation. The effects on the mutants of penicillin and of our lysogenic conditions were therefore compared.

Microscopy. Tubes originally containing 50 and 20 μM-diaminopimelic acid were removed 12 hr. after inoculation, and the bacterial debris was concentrated by centrifuging at 4°. Electron microphotographs of the debris showed that, in the tubes in which lysis had occurred, about 25 % of the cells appeared to have lost their protoplasmic contents and the cell wall was left as a shell. In samples from the control tube (50 μM-diaminopimelic acid) no abnormal cells were observed. Phase-contrast microscopy was used to watch the cultures during lysis, droplets of inoculated media being suspended in liquid paraffin at 34°. In the media containing 20 μM-diaminopimelic acid or less, the cells grew and divided normally for 2 or 3 hr. and then showed abnormal behaviour. Some apparently normal motile rods remained capable of dividing whereas others formed spherical bodies with diameters approximately twice those of the rods. Some of these sperical bodies, which resembled the protoplasts obtained from *E. coli* by Zinder & Arndt (1956), subsequently lysed, leaving an approximately circular 'ghost' of the same size. Particulate debris was also seen. In the control drops (50 μM-diaminopimelic acid) the rods grew and divided for 14 hr. and no spherical bodies or particulate debris appeared.

Penicillin effect. Sensitivity tests in minimal medium with the necessary amino acid additions for the mutant concerned showed that the parent strain and the mutants were all inhibited by the same concentration of penicillin (4 units/ml.). By the use of heavy inocula described in Methods growth with and without addition of penicillin (100 units/ml.) before inoculation was followed turbidimetrically for 6 hr. after inoculation. The three mutants, 173-25, D and 26-26, and the parent strain, *E. coli* 9637, behaved identically. Without penicillin turbidity increased logarithmically for about 4 hr. and then levelled off. In the presence of penicillin, turbidity increased for about 2 hr. and then decreased logarithmically. This lytic effect of penicillin was not altered by the presence of lysine and diaminopimelic acid (mM) during growth or by their addition during lysis.

DISCUSSION

The lysis of the two diaminopimelic acid-requiring *E. coli* mutants (173-25 and D) in limiting concentrations of diaminopimelic acid might be a similar type of phenomenon to that observed by Toennies & Gallant (1949*b*) in the lysine-requiring organism *Streptococcus faecalis*. This organism lysed in the logarithmic phase of growth after lysine had been exhausted from a medium of high phosphate content. The *E. coli* mutants lysed when the diaminopimelic acid had disappeared from a medium containing lysine; phosphate concentration was not critical. That lysine itself had a lytic effect was shown clearly in experiments with mutant D, which does not require lysine for growth; lysis was dependent on the presence of lysine but not on its concentration. In mutant 173-25, where lysine stimulated growth, α-*N*-acetyl-L-lysine replaced L-lysine as a growth factor but did not cause lysis.

These lytic phenomena should be considered in conjunction with the present state of knowledge of the bacterial cell wall. In the Gram-positive bacteria the amino acid composition of the cell walls is relatively simple (Cummins & Harris, 1956*a*, *b*). Diaminopimelic acid is a constituent, except in most cocci (including *S. faecalis*) and lactobacilli, where it is replaced by lysine. Other major amino acid constituents are invariably alanine and glutamic acid with sometimes aspartic acid and glycine. Walls of fewer Gram-negative organisms have been examined but those of *E. coli* contain a much more complicated amino acid mixture, including both diaminopimelic acid and lysine (Salton, 1953*a*; C. S. Cummins & H. Harris, private communication). In media of high osmotic pressure the wall can be removed without impairment of many synthetic functions of the organism (Weibull, 1953; Salton, 1953*b*; McQuillen, 1956; Lederberg, 1956; Zinder & Arndt, 1956; Mitchell & Moyle, 1956). The resultant spherical body is known as a 'protoplast'; on reduction of osmotic pressure it lyses, leaving an empty case or 'ghost' and small granules. In cultures growing in normal media, lysis, observed by a continued decrease in turbidity,

could be due to the successive or simultaneous combination of two events: first the formation of protoplasts, either through failure to form new cell wall or through dissolution of already existing wall; second, lysis of the protoplasts.

In the experiments in which lysis occurred in *E. coli* and *S. faecalis*, the limiting essential metabolite was a cell-wall constituent and was exhausted from the medium at the beginning of lysis. The possibility that *E. coli* lysed through protoplast formation is suggested by the following observations. Broken cell-wall envelopes were revealed by electron microscopy of the debris after lysis: the formation and subsequent disintegration of circular bodies during lysis was seen under the phase-contrast microscope. Decreases in turbidity were also caused by addition of penicillin to heavy suspensions of logarithmically growing cultures. Such treatment of *E. coli* B in the presence of sucrose (20 %, w/v) is known (Lederberg, 1956) to produce stable protoplasts.

It is therefore possible that, in *E. coli*, a shortage of diaminopimelic acid may have resulted in failure to lay down new cell wall at the point of division. This could result in either complete disintegration of the cell or escape of the protoplast from the fractured enclosing cell wall; the unsuitable osmotic conditions would then cause rupture of the protoplast and lysis would occur. If this were the cause of lysis, it is difficult to account for the absence of lysis during growth of the lysine-requiring *E. coli* 26-26. A deficiency of lysine might have been expected to prevent cell-wall formation in *E. coli*, as it may have done in *S. faecalis*. Under these conditions *E. coli* 26-26 accumulates and excretes large amounts of diaminopimelic acid (Davis, 1952), and it is possible that this excess of diaminopimelic acid prevented lysis; in the diaminopimelic acid-requiring mutants lysis was prevented by increasing diaminopimelic acid concentration. The contributive effect of lysine in the lysis of *E. coli* and the prevention of lysis by lanthionine have not been explained. Neither lysine nor lanthionine spared diaminopimelic acid as a growth factor and these are therefore unlikely to have been incorporated into the cell wall in its place. Examination of the cell-wall composition of cells grown under conditions giving rise to lysis has not been possible owing to poor growth. When grown in optimum concentrations of diaminopimelic acid, both the diaminopimelic acid-requiring mutants and the parent strain had similar cell walls. The complete inactivity of *S*-(β-aminoethyl)-cysteine [$H_2N \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$] for any of the mutants is surprising. As the mono-sulphur analogue of lysine it might have been expected to interfere in growth or lysis, especially as lanthionine interfered with both the growth-promoting effect of diaminopimelic acid and the lytic effect of lysine.

The lysis of mutant 173-25 during growth in the presence of lysine might explain some of the quantitative differences between our results and those of Davis (1952). The inactivity of D-lysine in replacing L-lysine for growth, reported by Davis, may have been due to his use of insufficient quantities of D-lysine. Both samples of D-lysine were obtained from the same source and were free from traces of L-isomer (Birnbaum, Levintow, Kingsley & Greenstein, 1952). Our results with lanthionine differ substantially from those of Seibert *et al.* (1954), who concluded that lanthionine could replace diaminopimelic acid for growth of mutant 173-25. These authors used bioautography with solvent systems which did not differentiate between diaminopimelic acid and lanthionine, so it is possible that their conclusions were based on prevention of lysis rather than stimulation of growth.

Cystine, like lanthionine, inhibited growth of both diaminopimelic acid-requiring mutants in the absence of lysine. With mutant D inhibition by cystine was strictly competitive and the molar ratios for complete inhibition were about two of cystine to one of diaminopimelic acid. As cystine had no effect on diaminopimelic acid decarboxylase (Dewey *et al.* 1954) the reaction inhibited is probably not that of decarboxylation to lysine.

Since the diaminopimelic acid requirements of mutants 173-25 and D were absolutely specific, the possibility of the use of these mutants for the microbiological assay of diaminopimelic acid was entertained. The only substance so far reported to replace diaminopimelic acid for mutant 173-25 is β-hydroxydiaminopimelic acid (Gilvarg, 1956). On solid media and under carefully controlled conditions C. Gilvarg (personal communication) has shown that isomer A (Stewart & Woolley, 1956) is active in this respect. We have confirmed these results under the same conditions but the inactivity of this β-hydroxy derivative in liquid media suggests that it does not entirely replace diaminopimelic acid. Mutant 173-25 was unsuitable for assay because of lysis at low concentrations of diaminopimelic acid. Even with mutant D, where there was a straight-line relationship between diaminopimelic acid concentration and turbidity, this relationship was altered by lysine, which caused lysis, and by casein hydrolysate which increased growth. Since casein hydrolysate also prevented lysis, it was thought that turbidimetric assay in its presence might prove possible, but the rate of reversion was too high to give reliable results. It must be concluded that turbidimetric estimation of the growth of neither mutant in liquid media can be used for assay of diaminopimelic acid in materials containing lysine and other amino acids. L. E. Rhuland (private

communication) reports that bioautography or plate-diffusion assays can be used; our work on bioautography also shows that it could be adapted for assay purposes.

It was hoped that comparison of the action of the *meso*- and LL-isomers of diaminopimelic acid on growth of the mutants might give some indication of the order in which the isomers are synthesized by *E. coli*. However, they were found to be identical in all respects, as was previously found on solid media (Hoare & Work, 1955). This equivalence is probably due to rapid interconversion of the isomers by the enzyme diaminopimelic acid racemase (Antia *et al.* 1957).

The experiments with lysine derivatives provide further evidence that the lysine metabolism of bacteria is different from that of other species. In contrast with the results in rats (Neuberger & Sanger, 1944), α-*N*-acetyl-L-lysine replaced L-lysine for growth, whereas ω-acetyl-L-lysine was inactive. The effect of the α-acetyl derivative on lysis of the diaminopimelic acid-requiring mutants suggests that it was not converted into lysine before use. When both lysine and α-*N*-acetyl-lysine were present, lysine was apparently used preferentially and then caused lysis, but when lysine was replaced by the α-acetyl derivative lysis did not occur, although growth was stimulated. Although ε-hydroxy-α-aminocaproic acid can either substitute for lysine or inhibit growth in certain neurospora mutants (Good *et al.* 1950), it did not interfere in the growth or lysis of *E. coli*. Davis (1952) showed that α-aminoadipic acid, known to be involved as a growth factor rather than as an inhibitor in lysine metabolism in other species (Work, 1955), had no effect on mutants 173-25 and 26-26. D-Lysine, although less active than L-lysine, completely replaced it for growth or lysis of *E. coli*. Certain neurospora mutants are reported to utilize D-lysine, but their lysine requirements were not completely satisfied by it (Schweet *et al.* 1954).

The antibacterial polylysines were most active against the lysine-requiring mutants. The increased susceptibility of mutant 173-25 over that of mutant D supports the view that the partial lysine requirement of mutant 173-25 is due to failure of a biosynthetic mechanism, rather than to a secondary effect due to partial inhibition by a metabolite accumulated before the genetic block, as suggested by Davis (1952). The lysine requirement of mutant 173-25 is difficult to explain if the only pathway of lysine formation is assumed to be through decarboxylation of diaminopimelic acid. Training the mutant to dispense with lysine did not alter either the requirement for diaminopimelic acid or the activity of its decarboxylase, and the resulting mutant D was identical in these respects with mutant 173-25. Evidently the training process did not increase the rate of lysine production from diaminopimelic acid, but stimulated an alternative route of lysine biosynthesis.

SUMMARY

1. The logarithmic growth rate of the diaminopimelic acid-requiring *Escherichia coli* mutants 173-25 and D in a salts–glucose medium containing lysine was independent of the concentration of added diaminopimelic acid. At concentrations below 20 μM lysis occurred during the logarithmic phase after exhaustion of diaminopimelic acid from the medium. Mutant D, in contrast with mutant 173-25, had no requirement for lysine; in the absence of lysine it did not lyse.

2. During the period of lysis, phase-contrast microscopy showed the appearance of spherical bodies which later lysed. Electron micrographs after lysis showed cell envelopes which had lost their protoplasmic contents.

3. Lysis was observed during the logarithmic growth phase of all strains of *E. coli* examined grown in the presence of penicillin. Lysine and diaminopimelic acid had no effect on this lysis.

4. DD-Diaminopimelic acid, cystine, lanthionine and several straight-chain diaminodicarboxylic acids did not act as growth factors for the diaminopimelic acid-requiring mutants. Lanthionine prevented lysis in the presence of lysine and inhibited growth in its absence. Nisin had no such effect. Cystine inhibited growth, the inhibition being overcome competitively by increasing concentrations of diaminopimelic acid.

5. The lysine requirements of mutants 173-25 and 26-26 were satisfied by α-*N*-acetyl-lysine but not by ω-acetyl-lysine. α-*N*-Acetyl-lysine did not produce lysis of mutants 173-25 and D when it replaced lysine. ε-Hydroxy-α-aminocaproic acid and *S*-(β-aminoethyl)cysteine had no effect on growth or lysis. D-Lysine produced all the effects of L-lysine when present in concentrations five times higher. Polylysines did not affect lysis but they inhibited growth, the lysine-requiring mutants 26-26 and 173-25 being more sensitive than the parent strain or mutant D.

6. Mutant D, which was derived from mutant 173-25 by training it to dispense with lysine, resembled mutant 173-25 in its diaminopimelic acid requirements and in its diaminopimelic acid decarboxylase activity. The cell walls of the two mutants were indistinguishable from each other and from the parent wild strain, all containing rather more diaminopimelic acid than lysine.

7. Hydrolysates of certain caseins stimulated the growth of the diaminopimelic acid-requiring mutants. The factors responsible could not be identified.

8. It is suggested that the lysis of the diaminopimelic acid-requiring mutants in the presence of lysine and suboptimum concentrations of diaminopimelic acid was due partly to the shortage of diaminopimelic acid, which is an essential cell-wall constituent. This would result in failure to synthesize cell wall, causing either protoplast formation or complete disintegration of the cell.

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