Table 4. *Comparison of the oxidation of glucose, fructose, galactose and pyruvate by yeast cells grown anaerobically on galactose or fructose*

Cells were grown on 5.4% of the sugar for 12 hr. for fructose and 21 hr. for galactose. Both types of cell were thus at the same physiological age. F, Yeast grown on fructose; G, yeast grown on galactose. The Q_{O_2} without substrate was less than 2. Respiration was measured at 25°.

Substrate	Q_{O_2} at 1 hr.		Q_{O_2} at 2 hr.		Q_{O_2} at 3 hr.		Q_{O_2} at 4 hr.		Maximum Q_{O_2}		Time of maximum Q_{O_2} (hr.)	
	F	G	F	G	F	G	F	G	F	G	F	G
Glucose	<2	20	14	28	28	80	37	43	60	95	2.5	2.75
Fructose	<2	17	8	30	47	88	34	52	47	88	3.0	3.0
Galactose	<2	23	<2	38	<2	106	<2	75	<2	106	—	3.0
Pyruvate	<2	21	<2	23	<2	36	<2	50	<2	50	—	4.0

SUMMARY

1. The weight of cells obtained when yeast is grown anaerobically is the same when any of the three sugars, glucose, fructose and galactose, is used as substrate.

2. Yeast grown anaerobically on galactose retains its ability to respire. The activity of cytochrome *c* oxidase in these cells is about one-third of that of the aerobic inoculum.

3. In non-growing cells, casein hydrolysate added to the medium caused the development of a more rapid respiration with time when galactose or glucose was the substrate but not when lactate, pyruvate or ethanol was the substrate.

4. Casein hydrolysate stimulated the consumption of galactose and the production of carbon dioxide by yeast grown anaerobically on galactose.

5. Yeast grown anaerobically on galactose could respire immediately on glucose, fructose, galactose or pyruvate, but yeast grown anaerobically on fructose oxidized none of these substrates immediately but could adapt to oxidize glucose or fructose.

6. The results are discussed in the light of the repressive action of glucose on the synthesis of oxidative enzymes.

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Variation of Activity of Bacterial Diaminopimelate Decarboxylase under Different Conditions of Growth

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The amino acid α -diaminopimelic acid is mostly confined in Nature to the mucopeptide component of the cell wall of certain bacteria and blue-green algae, where it is usually present as the *meso*- or the *LL*-isomer. In some species, diaminopimelic acid is replaced in the mucopeptide by lysine. However,

even organisms that lack diaminopimelate may contain enzymes for which it is a substrate. One such enzyme is diaminopimelate decarboxylase (EC 4.1.1.20; *meso*-2,6-diaminopimelate carboxylase), which converts *meso*-diaminopimelic acid into lysine (Dewey & Work, 1952). The enzyme is

widely distributed in bacteria, and there is considerable evidence that it participates in the biosynthesis of lysine (see review by Work, 1960).

Dewey, Hoare & Work (1954) studied diaminopimelate decarboxylase in single strains of *Escherichia coli* and *Aerobacter aerogenes*. Similar, relatively-low enzymic activities were obtained when organisms were grown in crude or defined media. The decarboxylase activity of *E. coli* varied considerably with the age of cultures, declining rapidly after maximal growth was reached; that of *A. aerogenes* showed little variation with age. Addition of diaminopimelate or pyridoxine to the defined growth medium did not change the enzymic activity of *A. aerogenes*, but pyridoxal phosphate increased activity when added during assay. Denman, Hoare & Work (1955) briefly noted that a pyridoxine-requiring mutant of *E. coli* grown in the presence of lysine or excess of pyridoxine had lower diaminopimelate-decarboxylase activity than when grown in a minimal concentration of pyridoxine.

Inhibition of the formation of bacterial enzymes by compounds in the surrounding medium is now well known and has been called 'repression' (see review by Moyed & Umbarger, 1962). The compound causing repression is usually the product of the enzymic reaction, or the end-product of a sequence of reactions in which the enzyme participates. Repression is believed to be important in the control of biosynthetic pathways, because it affords a means of reducing the formation of enzymes when their end-products begin to accumulate.

The present investigation describes the effect of age of culture and composition of growth medium on the diaminopimelate-decarboxylase activities of certain bacteria. A preliminary report of part of this work has already been given (White, 1962). Repression of diaminopimelate decarboxylase by lysine in *E. coli* has also been described by Patte, Loviny & Cohen (1962).

METHODS

Organisms. All the organisms studied (except for one mutant of *E. coli*) could be grown in defined media without lysine or pyridoxine, and showed no lysine-decarboxylase activity when grown in such media.

The strains of *E. coli* and *A. aerogenes* used by Dewey *et al.* (1954) are no longer available, and other strains of these organisms were chosen. *A. aerogenes* 12409 was obtained from the American Type Culture Collection (A.T.C.C.). *E. coli* A.T.C.C. 11396 was obtained from Chas. Pfizer and Co. Inc., Groton, Conn., U.S.A. It is the parent of a mutant (not used in this study) that lacks diaminopimelate decarboxylase; this mutant and *A. aerogenes* 12409 are used in the commercial production of lysine (Casida, 1956). *E. coli* A.T.C.C. 9637 is the parent of the

mutants M81-29 and M122-64, which were obtained from Professor B. D. Davis. Mutant M81-29 was stated to require lysine or diaminopimelic acid for growth in a minimal medium, and mutant M122-64 to require lysine or diaminopimelic acid plus aspartic acid (Davis, 1952). A low concentration of L-lysine hydrochloride (0.5 mg./l.) has now been found sufficient to give full growth of mutant M81-29 (0.1 mg./l. gave half-maximum growth). Mutant M122-64 grew rather poorly in minimal medium plus DL-aspartic acid (200 mg./l.) and there was no marked improvement of growth on addition of lysine or diaminopimelic acid, whether or not aspartate was present.

Staphylococcus aureus was investigated because this species has no diaminopimelate in the cell wall, although it has an active decarboxylase (Antia, Hoare & Work, 1957). *S. aureus* H, JHM and R122 were obtained from Professor D. D. Woods, and have been previously studied (White, 1958). All the strains were coagulase-positive, and strain R122 was highly resistant to sulphonamide drugs. A penicillin-resistant substrain (JHMPR) was obtained after 24 transfers of strain JHM in liquid medium with increasing concentrations of benzylpenicillin. This substrain could grow after 24 hr. in concentrations of benzylpenicillin up to 3 µg./ml. (100 times as resistant as the parent). *S. aureus* strain Copenhagen was obtained from Dr J. L. Strominger. Lysine was thought to be required for growth of this strain, since washed organisms were reported to need lysine for biosynthesis of cell walls (Strominger & Threnn, 1959). However, the organisms were found to grow in the defined medium without lysine.

An asporogenous, indole-negative mutant (168-68 UV) of *Bacillus subtilis* strain Marburg was obtained from Dr P. Schaeffer. It was stored in the freeze-dried state and a fresh ampoule was used as a starting inoculum into broth at 37° for each experiment. The effect of age of culture could be investigated on vegetative cells without spores.

Bacillus megaterium strain Mox was obtained from Mlle H. Ionesco. This non-lysogenic substrain was derived from the lysogenic parent strain, 899 (1), by several transfers in medium containing oxalate.

All organisms except *B. subtilis* were maintained on slopes of nutrient agar (Oxoid). *A. aerogenes* was grown at 30°, other organisms at 37°. Stock cultures were transferred monthly and stored at 2°.

Media. All media and solutions were prepared from glass-distilled water. Batches (250 ml.) of medium were autoclaved at 115° for 7 min. Broth contained: Tryptone (Oxoid), 10 g.; glucose, 10 g.; yeast extract (Oxoid), 1.6 g.; K₂HPO₄, 5 g.; MgSO₄·7H₂O, 200 mg.; MnSO₄·4H₂O, 10 mg.; NaCl, 10 mg.; FeSO₄·7H₂O, 10 mg.; water to 1 l. (final pH adjusted to 6.8).

The basal defined medium for *E. coli* 9637 and *A. aerogenes* was the minimal medium of Davis & Mingioli (1950); glucose (0.2%) was usually added to the medium before autoclaving. For growth of mutants M122-64 and M81-29, supplements of DL-aspartic acid (200 mg./l.) or L-lysine hydrochloride (0.1 mg./l.) respectively were added. *E. coli* 11396 was grown in the same basal medium except that glucose was replaced by glycerol (1%, w/v).

The basal medium for staphylococci was derived from that of Gladstone (1937) and contained: N-NaOH 26 ml.; KH₂PO₄, 4.5 g.; FeSO₄·(NH₄)₂SO₄·6H₂O, 6 mg.; MgSO₄·7H₂O, 100 mg.; glucose (autoclaved separately), 7 g.; DL-alanine, 100 mg.; L-arginine monohydrochloride,

120 mg.; DL-aspartic acid, 100 mg.; L-cystine, 50 mg.; DL-glutamic acid, 240 mg.; glycine, 50 mg.; DL-histidine, 65 mg.; DL-isoleucine, 125 mg.; DL-leucine, 125 mg.; DL-methionine, 50 mg.; DL-phenylalanine, 50 mg.; DL-proline, 100 mg.; DL-serine, 25 mg.; DL-threonine, 100 mg.; DL-tryptophan, 20 mg.; DL-tyrosine, 100 mg.; DL-valine, 125 mg.; nicotinic acid, 1 mg.; thiamine, 200 µg.; biotin, 1 µg.; water to 1 l. (final pH adjusted to 7.4). *S. aureus* JHM, JHMPR and R122 grew poorly unless the medium was supplemented with (quantities/l.): L-asparagine, 400 mg.; L-cysteine hydrochloride, 50 mg.; adenine hydrochloride, 10 mg.; guanine, 10 mg.

The basal medium for *B. subtilis* contained: trisodium citrate dihydrate, 3.7 g.; K_2HPO_4 , 150 mg.; DL-asparagine, 8 g.; DL-tryptophan, 60 mg.; glycerol, 20 ml.; $MgSO_4 \cdot 7H_2O$, 50 mg.; $FeSO_4 \cdot 7H_2O$, 5 mg.; $CaCl_2 \cdot 6H_2O$, 50 mg.; $MnCl_2 \cdot 4H_2O$, 5 mg.; water to 1 l. Final pH was adjusted to 7.0 before addition of salts and tryptophan, which were autoclaved separately.

The basal medium for *B. megaterium* was derived from that of Alföldi (1958) and contained: NH_4Cl , 2 g.; K_2HPO_4 , 1.4 g.; KH_2PO_4 , 600 mg.; $Na_2SO_4 \cdot 10H_2O$, 200 mg.; DL-glutamic acid, 1 g.; glycerol, 10 ml.; $FeSO_4 \cdot 7H_2O$, 8 mg.; $MgSO_4 \cdot 7H_2O$, 10 mg.; $MnCl_2 \cdot 4H_2O$, 8 mg. (the salts were autoclaved separately); water to 1 l. (final pH adjusted to 7.2).

Supplements to basal media were added (before autoclaving) usually at the following concentrations: L-lysine hydrochloride, 45 mg./l.; diaminopimelic acid (mixture of *meso*- and *LL*-isomers), 90 mg./l.; pyridoxine, 10 mg./l.

Growth and harvesting of organisms. Organisms were grown in 2 l. conical flasks, containing 250 ml. of medium. The flasks were shaken mechanically at 200 gyrations/min. (Model VS Gyro-rotatory shaker, New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). Incubation was at 37°, except for *A. aerogenes*, which was grown at 30°.

Except for *B. subtilis* and *B. megaterium*, inocula were organisms from slope cultures on nutrient agar grown overnight, then suspended in water to about 0.5 mg. dry wt. of organisms/ml.; 0.5 ml. of this suspension was used to inoculate 250 ml. of medium. *B. subtilis* from a freeze-dried culture was grown overnight in broth (5 ml.); the organisms were centrifuged, washed with 0.9% NaCl (5 ml.) and added to 50 ml. of defined medium, which was incubated on the shaker. This culture, when in the logarithmic phase of growth, was used as inoculum (20 ml. for 250 ml. of medium). *B. megaterium* from a slope culture on nutrient agar was grown overnight in the defined medium (50 ml.) on the shaker. This culture was used as inoculum (1.0 ml. for 250 ml. of medium). Growth of organisms was assessed by measurement of the extinction in an EEL portable colorimeter (Evans Electroelenium Co. Ltd., Halstead, Essex), with $\frac{1}{4}$ in. diameter tubes and neutral density filter (no. 1.0). The relation between colorimeter reading and dry wt. of each type of organism was determined.

Organisms were harvested by centrifuging, washed twice in 0.02M-phosphate buffer, pH 6.8 (250 ml.), and then resuspended in 0.1M-phosphate buffer, pH 6.8, to a density of 10–30 mg. dry wt./ml. (assessed from the extinction). The 0.1M-buffer was made by mixing equal volumes of 0.1M- $NaH_2PO_4 \cdot 2H_2O$ and 0.1M- $Na_2HPO_4 \cdot 12H_2O$. The dry wt. of suspensions of organisms in 0.1M-buffer was subsequently determined by heating samples (1.0 ml.) to con-

stant wt. at 105° and deducting the dry wt. of the buffer (16 mg./ml.).

Measurement of enzymic activity. Activity was measured from the initial rate of decarboxylation of *meso*-diaminopimelic acid in the Warburg apparatus at 37° in an atmosphere of nitrogen. All reagents were dissolved in 0.1M-phosphate buffer, pH 6.8. The main compartment of the Warburg flasks contained: washed organisms (10–40 mg. dry wt.) suspended in buffer, 0.1 ml. of pyridoxal phosphate (1 mg./ml.), and, unless otherwise stated, cetyltrimethylammonium bromide (3 mg./ml.) to give 50–100 µg./mg. dry wt. of organisms (estimated from the extinction). The total liquid volume in the flasks before addition of substrate was adjusted to 2.1 ml. with buffer. After gassing with N_2 and equilibration, 0.4 ml. of a solution of *meso*-diaminopimelic acid (5 mg./ml.) was tipped from the side arm. For each organism at least two, and usually three, different volumes of organisms were tested, and the results were averaged. Controls without substrate were always included, and each organism was also tested for lysine decarboxylase by using 2 mg. of L-lysine hydrochloride as substrate in place of diaminopimelic acid. In some experiments, the reaction products from diaminopimelate were examined by paper chromatography as described by Work (1962).

During the early part of this work, organisms were made permeable to diaminopimelate by drying with acetone by the method of Gale & Epps (1944). Later it was found that treatment with cetyltrimethylammonium bromide produced higher enzymic activities. In certain experiments, suspensions of washed organisms were disrupted by passage at 4000 lb./in.² through the press described by Milner, Lawrence & French (1950), and then used for measurement of diaminopimelate decarboxylase. Cetyltrimethylammonium bromide was not added to the Warburg flasks when broken or acetone-dried organisms were used. As the yield of acetone-dried powder was only 79 and 62% of the dry weight of *E. coli* 9637 or *S. aureus* H respectively, these differences have been allowed for here in calculating Q_{CO_2} for acetone-dried organisms, so that the results may be directly compared with those obtained by other methods.

In organisms that contained lysine decarboxylase, diaminopimelate decarboxylase was measured by determining colorimetrically the initial rate of disappearance of diaminopimelate, and re-expressed as a rate of evolution of CO_2 (assuming no retention by the solution). In a 25 ml. conical flask shaken at 37° were mixed: washed organisms (about 100 mg. dry wt.), cetyltrimethylammonium bromide (6 mg.) and pyridoxal phosphate (0.4 mg.), all in 0.1M-phosphate buffer, pH 6.8, to a total volume of 8.4 ml. *meso*-Diaminopimelic acid (8 mg. in 1.6 ml. of buffer), also at 37°, was added. Immediately after this addition, and at 5 min. intervals, samples (1.0 ml.) were removed and added to ethanol (1.0 ml.) to inactivate the enzyme. After standing 1 hr. the mixture was centrifuged. Diaminopimelic acid was estimated in the supernatant liquid after dilution into an equal volume of water, by the method described by Work (1957) for determining diaminopimelic acid in the presence of lysine. The sample for test (0.5 ml.) was mixed with acetic acid (4 ml.) and ninhydrin reagent (0.5 ml.), in that order (ninhydrin reagent was prepared by dissolving 250 mg. of ninhydrin in 6 ml. of acetic acid and 4 ml. of 0.6M-phosphoric acid). The mixture was heated at

37° for 90 min. and then the extinction was measured at 440 m μ . Lysine (up to 250 μ g.) did not interfere with the estimation of 50 μ g. of diaminopimelate.

Results were expressed as Q_{CO_2} values (μ l. of CO_2 evolved/hr./mg. dry wt. of organisms), without correction of manometric results for retention of CO_2 by the buffer. An accurate correction cannot be made at pH 6.8 (Umbreit, Burris & Stauffer, 1959). One unit of diaminopimelate decarboxylase was defined as the amount of enzyme that causes the evolution of 22.4 μ l. of CO_2 /min. or 1344 μ l. of CO_2 /hr. at 37° from *meso*-diaminopimelate. Hence, units of enzyme/mg. dry wt. of organisms = $Q_{CO_2}/1344$, or units of enzyme/ml. of culture = $(Q_{CO_2}/1344) \times$ dry wt. of culture (mg./ml.). Because of the low activity of diaminopimelate decarboxylase, enzymic activities are expressed as milli-units (one-thousandth of a unit). Owing to retention of CO_2 at pH 6.8 the unit employed is larger by about one-third than that recommended by the *Report of the Commission on Enzymes* (1961, p. 8), namely the amount of enzyme that catalyses the transformation of 1 μ mole of substrate/min.

Chemicals. *meso*-Diaminopimelic acid was separated by fractional crystallization (Work, 1963) from an aqueous solution (25 mg./ml.) of a mixture of *meso*- and *LL*-isomers (generously given by Chas. Pfizer and Co. Inc.). Ethanol was added until there was a slight permanent turbidity at room temperature; after standing the precipitate was collected, dissolved in water and reprecipitated with ethanol. Two more precipitations gave *meso*-isomer containing no *LL*-isomer as determined chromatographically (Hoare & Work, 1957). *S*-(2-Aminoethyl)cysteine dihydrochloride was prepared from *L*-cysteine by the method of Cavallini, de Marco, Mondovì & Azzone (1955). *D*-Lysine was a gift from the late Dr J. P. Greenstein. Cetyltrimethylammonium bromide was obtained from Consolidated Midland Corp., Katonah, N.Y., U.S.A. Other chemicals were of analytical-reagent grade where available.

RESULTS

Establishment of conditions for enzyme assay. Suspensions of washed organisms do not decarboxylate diaminopimelic acid, presumably because of slow penetration of the compound; to overcome this difficulty, acetone-dried organisms were used previously (Dewey *et al.* 1954; Hoare & Work, 1955). Higher decarboxylase activities were often attained by treatment of organisms in the Warburg flasks with the surface-active compound cetyltrimethylammonium bromide at concentrations below about 200 μ g./mg. dry wt. of organisms; above this concentration there was inhibition of decarboxylase. With *B. subtilis*, there was a very narrow range of concentration over which the compound could be used successfully, and the organisms were broken by the press before assay. Use of the press to disrupt *E. coli* generally resulted in enzymic activities similar to those found with cetyltrimethylammonium bromide. Staphylococci could not be broken by the press.

Measurement of the enzymic activity of a suspension of *E. coli* 9637 by manometry and by the

colorimetric method gave Q_{CO_2} 22 and 27 respectively. Since no correction for retention of CO_2 has been made in the manometric method, this would be expected to give a Q_{CO_2} only about 75 % of that of the colorimetric method.

Addition of pyridoxal phosphate to the Warburg flasks increased the activity of diaminopimelate decarboxylase in all the organisms tested (Table 1). In *E. coli* 9637 and 11396, but no other organisms, the enzyme showed an absolute requirement for pyridoxal phosphate even after the organisms had been grown in the presence of pyridoxine. No cofactor activity was shown by pyridoxine. The amount of pyridoxal phosphate needed to achieve full decarboxylase activity in *E. coli* 9637 and *S. aureus* H is shown in Fig. 1. In *E. coli* 9637 there was a relatively high requirement (0.16 mM); this was the case in cells treated either by acetone-drying or with cetyltrimethylammonium bromide. This high concentration of the cofactor was used in enzyme assays on all organisms.

Effect of variations in composition of growth media on diaminopimelate-decarboxylase activity. Organisms grown in broth showed consistently low diaminopimelate-decarboxylase activities (Q_{CO_2} 3–6) (Table 1), in agreement with previous results (Dewey *et al.* 1954; Antia *et al.* 1957); no lysine-decarboxylase activity was detected. Organisms grown in basal media had activities 2–6 times as high as those of broth cultures, and there was considerable variation of activity between different species.

The effects of various amino acids or of pyridoxine in the growth medium on the cellular activity of diaminopimelate decarboxylase were investigated by adding these compounds to appropriate basal media. The results (Table 1) are given for organisms harvested at about the end of the logarithmic phase of growth; in younger cultures the enzymic activities of any one strain, though higher, bear the same general relation to each other for each medium. Growth in the presence of diaminopimelic acid (*meso*- + *LL*-isomers) had only a small effect on decarboxylase activity. A slight increase (not more than 20 %) was noted in some organisms, but it had no marked inducing effect.

L-Lysine caused a decrease (repression) of diaminopimelate-decarboxylase activity in certain organisms. In two strains of *S. aureus*, H and Copenhagen, activity was decreased to about 30 % of that obtained in lysine-free medium. In contrast, there was no effect in strains JHM and R122 or in the penicillin-resistant substrain JHMPR. Lysine lowered activity in all strains of *E. coli* that were examined, with the most marked effect in mutant M122–64, where activity was reduced by a factor of 6. Only the *L*-isomer of lysine caused repression; *D*-lysine had no effect on *S. aureus*, and in *E. coli*

Table 1. *Effect of composition of growth medium on diaminopimelate-decarboxylase activities of different organisms*

Organisms were grown on appropriate media, harvested near the end of logarithmic growth, washed and tested for diaminopimelate decarboxylase as described in Methods in the presence of pyridoxal phosphate and cetyltrimethylammonium bromide. Values in parentheses are for the same cells tested in the absence of pyridoxal phosphate.

Organism	Broth	Diaminopimelate-decarboxylase activity (Q_{CO_2}) after growth in						
		Basal medium with						Diamino-pimelate + pyridoxine
		No addition	meso- and LL-Diamino-pimelate (90 mg./l.)	L-Lysine (45 mg./l.)	D-Lysine (45 mg./l.)	Pyridoxine (10 mg./l.)	L-Lysine + pyridoxine	
<i>E. coli</i> 9637	4.8	30 (0)	36	14	50	16 (0)	6.1 (0)	23 (0)
<i>E. coli</i> M81-29	4.0	7.0*	7.9	3.0	—	9.8*	—	—
<i>E. coli</i> M122-64	5.8	10	9.1	1.5	—	6.4	—	—
<i>E. coli</i> 11396	3.0	18 (0)†	14†	4.4†	—	16 (0)†	—	—
<i>A. aerogenes</i> 12409	4.1	23 (4.5)	22	7.7	—	22 (7.6)	3.6	—
<i>S. aureus</i> H	4.2	23 (15)	23	8.1	19	22 (17)	11 (9)	27 (16)
<i>S. aureus</i> Copenhagen	—	10‡	—	3.7‡	—	—	—	—
<i>S. aureus</i> JHM	4.8	14 (4.7)	14	14	—	19 (3.9)	—	12 (5.8)
<i>S. aureus</i> JHMPR	—	15	—	13	—	—	—	—
<i>S. aureus</i> R122	5.6	23	28	27	—	27	—	—
<i>B. subtilis</i> Marburg	5.4	9.0	9.8	9.5	—	11	—	—
168-68 UV	—	—	—	—	—	—	—	—
<i>B. megaterium</i> Mox	—	7	—	6	—	7	—	—

* L-Lysine hydrochloride (0.1 mg./l.) added for this lysine-requiring mutant.

† Harvested during mid-logarithmic growth.

‡ Acetone-dried organisms.

Fig. 1. Requirement of diaminopimelate decarboxylase (in cell suspensions) for pyridoxal phosphate as a cofactor. Organisms were grown for about 18 hr. in basal media, washed and assayed manometrically for diaminopimelate decarboxylase in the presence of pyridoxine or various concentrations of pyridoxal phosphate. ●, *E. coli* 9637 with pyridoxine; ○, *E. coli* 9637 with pyridoxal phosphate; △, *S. aureus* H with pyridoxal phosphate.

9637 it caused an increase in enzymic activity. Raising the concentrations of L-lysine in basal media of *E. coli* 9637 and *S. aureus* H caused a progressive fall in diaminopimelate-decarboxylase activity of both organisms to about 40 % of the unrepressed values (Fig. 2). In *E. coli* 9637, lysine-decarboxylase activity appeared after growth in concentrations of lysine at and above 100 mg./l. Supplements to basal media had no effect on *B. megaterium* and *B. subtilis*.

Pyridoxine in the basal media had little effect on the diaminopimelate-decarboxylase activity of most species, with the exception of *E. coli* 9637, where it decreased enzymic activity to 55 % of the value in basal medium. The magnitude of repression depended on concentration up to 0.1 mg. of pyridoxine/l.; below 0.01 mg./l. there was no effect (Fig. 3). In one of the mutants (M122-64) of *E. coli* 9637, pyridoxine caused repression, whereas in the other mutant (M81-29) it increased the enzymic activity slightly. When lysine was already present in the growth medium, supplementation with pyridoxine caused additional repression both in *E. coli* 9637 and also in *A. aerogenes* (where pyridoxine alone had no effect).

The effects of amino acids other than lysine could be studied only in organisms, such as *E. coli*, which have no nutritional requirements for amino

acids. Staphylococci could not be used, as they need several amino acids for growth, and the strains studied differed in their requirements (White, 1958). Growth of *E. coli* 9637 in basal medium supplemented with a mixture of 18 amino

acids, other than lysine, caused some repression of diaminopimelate decarboxylase, though less than that caused by lysine alone (Table 2). By omission of various amino acids, singly or in groups, those responsible for repression in *E. coli* were found to be phenylalanine, tryptophan or tyrosine. The three compounds together were no more effective in decreasing activity than any one alone. The rate of growth in medium supplemented with phenylalanine was the same as in unsupplemented medium. In the presence of either lysine or pyridoxine, phenylalanine caused slight additional repression above that resulting from lysine or pyridoxine alone. The combination of lysine, pyridoxine and phenylalanine decreased diaminopimelate-decarboxylase activity to a level lower than that found in organisms grown in broth. The enzyme in *A. aerogenes* was not affected by growth in media supplemented with the mixture of 18 amino acids. In this organism, repression to a level below that found in broth culture was achieved by the combination of lysine and pyridoxine. No inhibition of the enzyme was found when lysine, pyridoxine and phenylalanine were added, together or separately, to Warburg flasks during enzyme assay of *E. coli* 9637.

Fig. 2. Effect of growth in the presence of lysine on the activity of diaminopimelate decarboxylase. Organisms were grown for about 18 hr. in basal media containing various concentrations of lysine, washed and assayed for diaminopimelate decarboxylase. Δ , *S. aureus* H; $\circ$, *E. coli* 9637. With *E. coli* the Q_{CO_2} at the two highest concentrations of lysine was measured by the colorimetric method and should be decreased by about 25% for comparison with the other values obtained by manometry, where no correction was made for retention of CO_2 .

Fig. 3. Effect of growth in the presence of pyridoxine on activity of diaminopimelate decarboxylase of *E. coli* 9637. Organisms were grown for about 18 hr. in basal medium with various concentrations of pyridoxine, washed and assayed manometrically for diaminopimelate decarboxylase.

Table 2. Effects of amino acids and pyridoxine in the growth medium on the activity of diaminopimelate decarboxylase of *E. coli* 9637

Organisms were grown for about 18 hr. at 37°, harvested, washed and tested for diaminopimelate decarboxylase. Details of the additions to the growth medium are as follows: (a) 18 amino acids (quantities/l.): DL-alanine, 100 mg.; L-arginine, 120 mg.; DL-aspartic acid, 100 mg.; L-asparagine, 400 mg.; L-cystine, 50 mg.; DL-glutamic acid, 240 mg.; glycine, 50 mg.; DL-histidine, 65 mg.; DL-isoleucine, 120 mg.; DL-leucine, 120 mg.; DL-methionine, 50 mg.; DL-phenylalanine, 50 mg.; DL-proline, 100 mg.; DL-serine, 25 mg.; DL-threonine, 100 mg.; DL-tryptophan, 20 mg.; DL-tyrosine, 100 mg.; DL-valine, 120 mg. (b) Phenylalanine, tryptophan and tyrosine: as in the 18 amino acids mixture. (c) 15 amino acids: the 18 amino acids without phenylalanine, tryptophan and tyrosine. (d) L-Lysine hydrochloride, 45 mg./l. (e) Pyridoxine, 10 mg./l.

Growth medium	Q _{CO₂}
Broth	4.8
Minimal medium	30
+ 18 amino acids	19
+ phenylalanine + tryptophan + tyrosine	22
+ phenylalanine	22
+ tryptophan	22
+ tyrosine	22
+ 15 amino acids	29
+ lysine	14
+ lysine + 18 amino acids	12
+ lysine + phenylalanine	12
+ pyridoxine	16
+ pyridoxine + phenylalanine	12
+ pyridoxine + lysine	7.4
+ pyridoxine + lysine + phenylalanine	3.7

Some changes of the carbohydrates in basal media were made (Table 3). There was little alteration in growth rate or diaminopimelate-decarboxylase activity when 0.2% glucose was replaced by 0.2% glycerol. When the carbohydrate concentrations were raised to 2% (w/v) the growth rates were increased, and there were various effects on decarboxylase activities. In *S. aureus* H and *E. coli* 9637, activity was decreased by 33 and 50% respectively after growth on 2% glycerol. In *E. coli* 11396 activity was decreased by 65% after growth on 2% glucose. Evolution of gas was high from control flasks of *A. aerogenes* incubated without substrate after growth in 2% glucose, and the diaminopimelate decarboxylase of these organisms had to be measured by the colorimetric method.

Effect of medium and age of culture on synthesis of enzyme. The age of the culture at the time of harvesting had a marked effect on the diaminopimelate-decarboxylase activity of most of the organisms studied. Young cultures always showed higher enzymic activities than those harvested at the end of logarithmic growth or later. Because of this, when the composition of the medium was varied, activities were usually measured at more than one stage of growth. The effect of age of culture was examined in detail with *E. coli* 9637 and *S. aureus* H (Figs. 4a and 4b). To compare the manner in which lysine and pyridoxine decreased the formation of enzyme in growing cultures, a 'differential plot' was used (Monod, Pappenheimer & Cohen-Bazire, 1952), in which the amount of enzyme/ml. of growing culture was plotted against the dry wt. of organisms/ml. of culture (Figs. 5a and 5b). In the absence of supplements, the total enzyme in the culture increased initially at a constant rate. In *E. coli* 9637 a maximum was reached near the end of logarithmic growth, after which total enzyme decreased rapidly, then approached a steady level. This decrease in enzyme after growth had ceased is shown in Fig. 5 (a), where the

amount of enzyme/ml. of culture is plotted against time of harvesting. Comparison of the differential rates of enzyme formation showed that growth in the presence of lysine decreased the initial rate of formation of decarboxylase by a factor of 4 in *E. coli* 9637. In contrast, pyridoxine did not affect the initial rate of decarboxylase formation, but stopped synthesis of the enzyme at an early stage of growth. When both lysine and pyridoxine were present together in the growth medium, enzyme formation was initially very slow, but later continued at the same rate as in medium supplemented only with lysine. There was little loss of enzyme from post-logarithmic phase cells grown in the presence of lysine or pyridoxine. Diaminopimelate in the basal medium had no effect either on the rate of formation or on the post-logarithmic loss of diaminopimelate decarboxylase, the curves for organisms harvested from basal or supplemented medium being identical. In *S. aureus* H, the amount of enzyme/ml. of culture increased linearly till the end of growth and then stayed approximately constant (Fig. 5b). In this organism, the effect of lysine was less marked than in *E. coli*, as it only halved the differential rate of enzyme formation.

Less-detailed work on *B. subtilis* grown in minimal medium showed that it behaved similarly to *E. coli*, with rises both in Q_{CO_2} and in total enzymic activity during early logarithmic growth, followed by a subsequent marked loss of enzyme from the culture.

The effect of an analogue of lysine, *S*-(2-aminoethyl)cysteine,

was examined in the hope that it would de-repress enzyme synthesis through competition with lysine, of which it is an antimetabolite (Shiota, Folk & Tietze, 1958; Shiota, Mauron & Folk, 1961). Aminoethylcysteine (1 mg./l. of basal medium) inhibited growth of *E. coli* 9637 for 24 hr. The

Table 3. *Effects of different concentrations of glucose or glycerol in the growth medium on the activity of diaminopimelate decarboxylase of various organisms*

Organisms were harvested near the end of logarithmic growth, washed and tested for diaminopimelate decarboxylase. Defined media (as in Methods) were used, with glucose or glycerol as carbohydrate source. Concentrations of glucose and glycerol are percentages (w/v). Glucose and glycerol were added after autoclaving to the medium for *S. aureus* H. With the other organisms, only the higher concentrations of glucose or glycerol were added after autoclaving.

Organism	Diaminopimelate-decarboxylase activity (Q_{CO_2}) after growth in medium containing			
	Glucose (0.2%)	Glycerol (0.2%)	Glucose (2%)	Glycerol (2%)
<i>S. aureus</i> H	21	19	18	12
<i>E. coli</i> 9637	32	35	34	17
<i>E. coli</i> 11396	8.6	8.5	3.3	9.2
<i>A. aerogenes</i> 12409	16	21	18*	20

* Measured by the colorimetric method. The value should be reduced by about 25% for comparison with the other results obtained by manometry, where no correction was made for retention of CO_2 .

inhibition was prevented by DL-lysine at one-eighth the molecular concentration of aminoethylcysteine. Diaminopimelate decarboxylase itself was not inhibited by aminoethylcysteine, and the compound was not a substrate for the enzyme from *E. coli* 9637 or *S. aureus* H. *E. coli* 9637 grown for 16 hr. in the absence of lysine with just sub-inhibitory concentrations of aminoethylcysteine

Fig. 4. Diaminopimelate-decarboxylase activity of organisms at various stages of growth. Organisms were grown for various times in basal media, washed and assayed manometrically for diaminopimelate decarboxylase. (a) *E. coli* 9637; (b) *S. aureus* H. $\circ$, Dry wt. of organisms in culture (found by measurement of extinction). Δ , Q_{CO_2} .

Fig. 5. Rates of formation of diaminopimelate decarboxylase during growth of organisms with various additions to the basal media. For calculation of the amount of enzyme in a culture see Methods. (a) *E. coli* 9637. The amount of enzyme/ml. is plotted during growth against dry wt. of culture and during the stationary phase against time of harvesting. $\circ$, Basal medium without addition; $\square$, L-lysine hydrochloride (45 mg./l.); $\blacksquare$, S-(aminoethyl)cysteine dihydrochloride (0.5 mg./l.); Δ , diaminopimelic acid (meso- + LL-isomers, 90 mg./l.); $\blacktriangle$, pyridoxine (10 mg./l.); $\bullet$, lysine and pyridoxine. (b) *S. aureus* H. The amount of enzyme/ml. is plotted against dry wt. of culture until growth stopped (straight lines calculated by the method of least squares). $\circ$, Basal medium without addition; $\square$, L-lysine hydrochloride (45 mg./l.).

(0.5 mg./l.) had a diaminopimelate-decarboxylase activity almost twice that of organisms grown for the same time in the absence of inhibitor. At first sight, this suggested that the analogue was preventing the repressive action of lysine; however, the initial rate of enzyme formation was very little affected by aminoethylcysteine (Fig. 5a). The cause of the apparent increase in enzymic activity was a retardation of growth, as a result of which the enzyme content was a maximum in organisms harvested at 16 hr., at which time control organisms had already lost 25 % of their enzyme.

DISCUSSION

In the present work, the diaminopimelate-decarboxylase activities of bacteria grown in broth were generally similar to those found previously, but organisms grown in defined media had activities (Q_{Co} , 20–50) considerably higher than those observed before (Dewey *et al.* 1954; Denman *et al.* 1955; Antia *et al.* 1957; Patte *et al.* 1962). Only with *Bacillus sphaericus* grown in complex media had similar activities been reported (Powell & Strange, 1957; Meadow & Work, 1958). Several factors may be responsible for these differences. One may be the use of different strains of organisms, as suggested by the marked variations in enzymic activity observed between strains of the same species (see also Dewey, 1954). Optimum conditions of growth, harvesting and enzyme assay are also important factors in producing maximum enzyme activity. For example, the diaminopimelate decarboxylase of *B. sphaericus* (Meadow & Work, 1958), *E. coli* 9637 and 11396 has no activity *in vitro* in the absence of added pyridoxal phosphate, and needs a much higher concentration of this cofactor for full activity than that previously recommended for assay. No explanation can be offered for the behaviour of the enzyme in these organisms, which may not be very exceptional in this respect. The absolute requirement for pyridoxal phosphate in *E. coli* 9637 was not abolished by growth in the presence of pyridoxine, but there is no doubt that some pyridoxine penetrated the organisms since it repressed the decarboxylase.

The lowering of diaminopimelate-decarboxylase activity (50–75 %) by growth in the presence of lysine is of the same order as that found by other workers in mutants of *E. coli* examined at only one stage of growth (Denman *et al.* 1955; Patte *et al.* 1962). Although lysine represses diaminopimelate decarboxylase in several organisms, the fact that organisms grown in broth usually have lower enzymic activity than those grown in defined medium containing lysine suggests that there are other nutritional factors regulating activity. The

failure of D-lysine to repress was surprising because this isomer can replace L-lysine for growth of lysine-requiring mutants of *E. coli* (Meadow, Hoare & Work, 1957). D-Lysine actually increased activity in *E. coli* 9637; owing to the small quantity of D-lysine available, this effect has not been further investigated.

In *E. coli* 9637 either pyridoxine or aromatic amino acids in the growth medium also repressed diaminopimelate decarboxylase. Denman *et al.* (1955) found that supplementation of the medium with pyridoxine halved decarboxylase activity in a mutant of *E. coli* 9637; a similar reduction has now been shown in the parent strain. The effect of pyridoxine seems to be distinct from that of lysine because repression by the two compounds together is greater than that of either singly. Moreover, lysine and pyridoxine have different effects on the course of the formation of the enzyme during growth. Lysine repressed enzyme formation from the start, whereas pyridoxine allowed enzyme to be formed initially at the unrepressed rate, and then caused a halt of enzyme synthesis. In other cases where the cofactor of an enzyme apparently caused repression, the effect has been attributed to stimulation by the cofactor of formation of the true repressor, which is the end-product of the enzymic reaction (Rowbury & Woods, 1961). Though it seems unlikely that stimulation of lysine synthesis is the basis of the effect of pyridoxine, the time-course of the reaction suggests that the presence of pyridoxine results in the formation of some other regulatory compound. Pyridoxine alone had no repressive effect in organisms other than *E. coli* 9637 or derived strains, but in *A. aerogenes* it reinforced repression by lysine.

The repressive effect of the aromatic amino acids is less marked, activity being reduced by only about 25 %. The effect seems to be distinct from those of pyridoxine and lysine, since there was a further repression by phenylalanine when lysine and pyridoxine were present together.

No explanation can be made of the way in which lysine, pyridoxine and aromatic amino acids affect the synthesis of the same enzyme. The chemical structures of the compounds show few similarities, and, as far as is known, the pathways of metabolism of these compounds are different, with no intermediate products in common. In these respects, and also in the fact that lysine, pyridoxine and aromatic amino acids can each separately repress, their effect differs from the 'multivalent repression' described by Freundlich, Burns & Umbarger (1963). These workers found in mutants of *E. coli* and *Salmonella typhimurium* that the enzymes concerned in the biosynthesis of valine, leucine and isoleucine were repressed only when all three of these compounds were present in the

medium. These three amino acids have very similar structures and the same enzymes catalyse some of the steps in their biosynthesis.

The presence of glucose in the growth medium is known to prevent the formation of many enzymes of carbohydrate metabolism. The mechanism is not understood, but glucose itself does not appear to be the inhibitory material (Magasanik, 1961). Angulo *et al.* (1960) showed that the type of carbohydrate used in the growth medium of a mutant of *E. coli* which lacked diaminopimelate decarboxylase affected the amount of diaminopimelate excreted into the medium; this suggests some regulatory effect by carbohydrates earlier in the biosynthetic pathway. In the present work, effects on diaminopimelate decarboxylase of replacing glucose by glycerol were very variable in different organisms.

Diaminopimelate decarboxylase of *S. aureus* JHM and R122, *B. subtilis* and *B. megaterium* was not repressed by any of the compounds tested, yet the broth-grown organisms had considerably lower activity than those grown in defined media. Presumably unidentified repressors are present in broth. Even in organisms that have been grown in broth, diaminopimelate decarboxylase was never completely repressed. The biosynthesis of lysine in *E. coli* is controlled not only by repression of this one enzyme, but also by feedback control of at least two other enzymes. One of the aspartate kinases and also the enzyme that condenses pyruvate and aspartate semialdehyde are inhibited by L-lysine (Stadtman, Cohen, Le Bras & de Robichon-Szulmajster, 1961; Yugari & Gilvarg, 1962); the former enzyme is also repressed by lysine. Exogenous lysine does not completely inhibit endogenous production of lysine by *E. coli* (Siddiqi, Kosloff, Putnam & Evans, 1952; Meadow & Work, 1959), and it thus appears that a residual biosynthesis of lysine must escape repression and feedback control.

In *S. aureus* H, *B. subtilis* and *E. coli* 9637 grown in basal media, the activity of diaminopimelate decarboxylase/mg. dry wt. of organisms (Q_{co_2}) was highest in the early stages of logarithmic growth, and subsequently decreased sharply. Similar results have been reported with ornithine carbamoyltransferase of *E. coli* grown in batch cultures (Gorini & Maas, 1958). In spite of the decrease in Q_{co_2} of diaminopimelate decarboxylase, the total enzyme in the culture (milliunits/ml.) rose at a constant rate during the logarithmic phase of growth. It seems that in very early stages of growth the enzyme is formed at a relatively higher rate, since the average amount of enzyme/organism (Q_{co_2}) is initially so high. The subsequent steady rate of formation of enzyme which is maintained during logarithmic growth may be controlled by regulatory amounts of lysine produced by the

enzyme first synthesized. The organisms growing during this period contain less of the enzyme than those that grew earliest, and the average amount of enzyme/unit weight of organisms (Q_{co_2}) falls.

At the end of logarithmic growth of *E. coli* 9637 the total enzyme in the culture (milliunits/ml.) declined rapidly, whereas in *S. aureus* H there was no loss. The cause of the decline of enzyme in *E. coli* is not known; the organisms do not lyse and the pH of the medium falls only from 6.8 to 6.6 during growth. Possibly the diaminopimelate decarboxylase breaks down to help provide an amino acid pool that may be used to form other enzymes or proteins that are more essential at this stage (Mandelstam, 1963). It is at this point that protein synthesis ceases in *Streptococcus faecalis*, though cell-wall synthesis continues (Shockman, Kolb & Toennies, 1958).

Even without lysine in the growth medium, the formation of diaminopimelate decarboxylase may be regulated by endogenous lysine synthesized by the organisms. Release from endogenous repression has, with other enzymes, been achieved in a number of ways (Moyed & Umbarger, 1962), some of which were tried in the present study. Limitation of growth of a lysine-requiring mutant by use of a suboptimum concentration of lysine was tried in two mutants of *E. coli* not blocked at diaminopimelate decarboxylase, but this did not result in higher enzymic activities. However, the extremely low lysine requirement of mutant M81-29 (less than 0.5 mg./l.) suggests that the synthesis of lysine may not have been eliminated. Another method of obtaining de-repression might be to make the synthesis of lysine rate-limiting for growth by supplementing the basal medium with amino acids and other growth factors. When the minimal medium was supplemented with amino acids other than those that repress diaminopimelate decarboxylase, there was no increase in the enzymic activity of *E. coli* 9637 and *A. aerogenes*. Growth factors other than pyridoxine have not been investigated. The lysine analogue, aminoethylcysteine, did not produce de-repression, although it acted as a growth inhibitor antagonizing lysine.

Antibiotics of the penicillin group are believed to be inhibitors of synthesis of bacterial cell walls (see review by Perkins, 1963). There have been suggestions that penicillin-resistant staphylococci containing lysine in their cell walls may sometimes change into Gram-negative rods with diaminopimelic acid in their walls (Briggs, Crawford, Abraham & Gladstone, 1957; Abraham, 1957). Although this observation has not been repeatable (Briggs, Crawford, Abraham & Gladstone, 1959), it suggested that in penicillin-resistant staphylococci there might be changes in the balance of enzymes (such as diaminopimelate decarboxylase) concerned in the metabolism of cell-wall components.

To investigate this possibility a penicillin-resistant substrain was developed; it did not, however, form any Gram-negative rods, and the activity of its diaminopimelate decarboxylase was the same as that of the parent strain.

SUMMARY

1. The activity of diaminopimelate decarboxylase was measured in various species of bacteria after growth in broth or defined media. Minimal defined media produced organisms with the highest activities; supplementation with certain amino acids or pyridoxine caused a reduction (repression) of activity of some species to the level found after growth in broth.

2. L-Lysine caused repression in *Escherichia coli* and *Aerobacter aerogenes*, and in certain, but not all strains of *Staphylococcus aureus*. The enzyme in *Bacillus subtilis* and *Bacillus megaterium* was not repressed. D-Lysine had no effect in *S. aureus* but caused a small increase of activity in *E. coli*.

3. In *E. coli*, repression was also caused by pyridoxine and by any one of the aromatic amino acids, phenylalanine, tryptophan or tyrosine. These effects were additive with that of L-lysine. Pyridoxine and L-lysine had different effects on the time course of enzyme synthesis.

4. Changes in the nature or amount of carbohydrate in the media had various effects on the amounts of enzyme in different organisms.

5. Highest activities were found in young cultures though enzyme continued to be formed until the end of logarithmic growth. Subsequently enzyme disappeared rapidly from *E. coli* and *B. subtilis* but not from *S. aureus*.

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