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Stimulation of Lysine Decarboxylase Production in *Escherichia coli* by Amino Acids and Peptides[1]

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A commercial hydrolysate of casein stimulated production of lysine decarboxylase (EC 4.1.1.18) by *Escherichia coli* B. Cellulose and gel chromatography of this hydrolysate yielded peptides which were variably effective in this stimulation. Replacement of individual, stimulatory peptides by equivalent amino acids duplicated the enzyme levels attained with those peptides. There was no indication of specific stimulation by any peptide. The peptides were probably taken up by the oligopeptide transport system of *E. coli* and hydrolyzed intracellularly by peptidases to their constituent amino acids for use in enzyme synthesis. Single omission of amino acids from mixtures was used to screen them for their relative lysine decarboxylase stimulating abilities. Over 100 different mixtures were evaluated in establishing the total amino acid requirements for maximal synthesis of lysine decarboxylase by *E. coli* B. A mixture containing all of the common amino acids except glutamic acid, aspartic acid, and alanine increased lysine decarboxylase threefold over an equivalent weight of casein hydrolysate. The nine most stimulatory amino acids were methionine, arginine, cystine, leucine, isoleucine, glutamine, threonine, tyrosine, and asparagine. Methionine and arginine quantitatively were the most important. A mixture of these nine was 87% as effective as the complete mixture. Several amino acids were inhibitory at moderate concentrations, and alanine (2.53 mM) was the most effective. Added pyridoxine increased lysine decarboxylase activity 30%, whereas other B vitamins and cyclic adenosine 5'-monophosphate had no effect.

The role of casein hydrolysates in satisfying nutritional requirements for biosynthesis of specific enzymes in *Escherichia coli* has received widespread attention. Melnykovych and Snell (15) reported that a tryptophan-supplemented, acid hydrolysate of casein increased production of arginine decarboxylase. Formic hydrogenlyase and hydrogenase were produced by *E. coli* grown on a basal medium containing glucose and salts and supplemented with an acid hydrolysate of casein, but were not present when the organism was grown in basal medium (2, 3). The amount of inducible nitrate reductase system of *E. coli* was considerably higher in casein hydrolysate medium than it was in glucose-salts medium (27). Similar effects of casein hydrolysates on enzyme formation in *Streptococcus faecalis* (4), *Pseudomonas saccharophila* (14), *Aspergillus niger* (23), *Bacillus subtilis* (20), *Myxococcus virescens* (8), and *Clostridium welchii* (16) have been noted.

Lysine decarboxylase in *Bacterium cadaveris* and *E. coli* strain 86 was first reported by Gale and Epps (6). It was shown to be an inducible enzyme by Sher and Mallette (21). The presence of lysine in the medium was sufficient for induction of some enzyme. However, formation of lysine decarboxylase was much enhanced in media containing additional organic nitrogen. In one study (22) using *E. coli* B, Hy-Case (a commercial hydrolysate of casein) was more stimulatory than other sources of nitrogen. This hydrolysate was later shown (13) to contain stimulatory amino acids and peptides. Although detailed analysis was prevented by limited sensitivity of the manometric assay method employed (13), no mixture of amino acids was found which duplicated a combination of amino acids and peptides. Therefore, it was suggested that certain peptides might play specific roles in enzyme formation. In the present study, the stimulatory factors in casein hydrolysate that increased lysine decarboxylase production are identified, and the nutritional environment is defined for maximal enzyme biosynthesis in vivo.

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MATERIALS AND METHODS

Microorganism. *Escherichia coli* B was used throughout this investigation. Stock cultures were carried on nutrient agar slants, transferred weekly, and subcultured for experimental use by inoculation of 10 ml of glucose-salts medium (28) with cells from a slant. This suspension was incubated at 37 C with shaking until the turbidity at 650 nm indicated 5×10^8 cells/ml.

Enzyme induction. Medium for induction of enzyme biosynthesis contained the following components listed in grams per liter of distilled water: $Na_2HPO_4 \cdot 12H_2O$, 8.0; KH_2PO_4, 10.0; NH_4Cl, 1.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.41; glucose, 20.0; and L-lysine monohydrochloride, 10.0. Casein hydrolysate (Hy-Case, Sheffield Chemical), amino acids, or peptides were added to 10-ml portions of this medium and sterilized by filtration through a type GS membrane filter, 0.22 μm pore size (Millipore Corp.). A 0.1-ml sample of stock cell suspension in glucose-salts medium (5×10^8 cells/ml) was added to 10.0 ml of induction medium, and the system was shaken for 12 h at 37 C.

One reference mixture, termed the Lu mixture (composition formulated by W. W.-W. Lu in M.S. thesis, Pennsylvania State Univ., 1969), was taken as the starting point for evaluating the effects of amino acids. It contained the following amino acids in μmol/25 mg of total mixture: L-alanine, 16.1; glycine, 13.3; L-leucine, 32.4; L-proline, 49.2; L-methionine, 8.7; D,L-serine, 50.2; D,L-threonine, 34.9; and L-tyrosine, 14.0. Another mixture corresponded in composition to Hy-Case in which the amino acids determined with a Technicon amino acid analyzer were (in μmol/25 mg): alanine, 10.2; arginine, 5.7; aspartic acid, 13.6; glutamic acid, 39.1; glycine, 8.3; histidine, 4.6; isoleucine, 10.0; leucine, 19.6; methionine, 4.7; phenylalanine, 5.7; proline, 25.1; serine, 14.5; threonine, 9.6; tyrosine, 7.0; and valine, 15.7.

Preparation of enzyme. Induced cultures were centrifuged at 5 C and $3000 \times g$ for 10 min. Cells were washed by suspension in 10.0 ml of cold 0.9% NaCl solution and recentrifuged. Washed cells were suspended in 10.0 ml of cold 0.05 M phosphate buffer (pH 7.0) in a cellulose nitrate tube, chilled in ice, and treated for five 30-s periods with a flat-tipped, 13-mm stephorn of a Branson Sonifier. Mixtures were cooled for 1 min between each sonication period. The resulting suspensions were centrifuged at 5 C for 20 min at $4,000 \times g$, and supernatants were taken as lysine decarboxylase extracts. Protein in these extracts ranged from 0.1 to 0.25 mg/ml and was determined by the method of Lowry et al. (11) at 500 nm with the use of bovine serum albumin as the reference protein.

Assay of enzyme. The lysine decarboxylase assay was modified from that of Lu and Mallette (12) based on reaction of lysine with ninhydrin. This assay was checked by comparison with a manometric method which measured carbon dioxide liberated on decarboxylation of lysine to cadaverine. Enzyme extract (1.0 ml), 1.5 ml of 0.02 M phosphate buffer (pH 7.0), and 0.2 ml of 4×10^{-4} M pyridoxal phosphate solution were combined and allowed to stand 10 min. Lysine (0.50 ml of 0.0250 M, 12.5 μmol) was added, and the mixture incubated at 37 C for 45 min. Residual lysine was determined by adding 0.6 ml of 6 N hydrochloric acid solution and 1.0 ml of 2.5% ninhydrin in methyl cellosolve (wt/vol), capping the tubes with aluminum foil, and heating at 100 C for 70 min. After cooling to 30 C, 4.0 ml of concentrated phosphoric acid was added and thoroughly mixed. Absorbance was read at 515 nm in 1-cm cuvettes with a Beckman DU spectrophotometer. A set of lysine standards was run in each experiment. Results were reported on a specific activity basis as percentages of the lysine decarboxylase activities of controls containing mixtures of amino acids appropriate to individual experiments. Reproducibility was ±7%.

Isolation of stimulatory peptides. Peptides 1 to 7 were separated by extraction for 25 h at 25 C with stirring of 60 g of Hy-Case in 500 ml of ethanol:chloroform (1:1 vol/vol) and chromatography of the extract on a column of Whatman no. 1 cellulose (2.5 by 74 cm). The eluant was 1-butanol:4 N NH_3:ethanol (11:8:4 vol/vol/vol) at a flow rate of 7.8 ml/h, and 3-ml fractions were collected. Peptide A was obtained by extraction for 24 h at 25 C of 20 g of Hy-Case with 200 ml of 1-butanol:acetic acid:water (60:15:25 vol/vol/vol) and subsequent chromatography of the extract on a Sephadex G-15 column (1.5 by 75 cm). The eluant was 1-butanol:acetic acid:water (60:15:25 vol/vol/vol), and the flow rate was 10 ml/h. Three-ml fractions were collected. All fractions were checked by two-dimensional paper chromatography as below. Those having the same composition were combined and evaporated under reduced pressure at 40 C. Residues were taken up in 50 ml of water and dried in vacuo.

Composition of stimulatory peptides. Peptides were hydrolyzed in 6 N HCl at 105 C for 24 h in sealed Pyrex tubes. The resulting amino acids were identified by two-dimensional paper chromatography. Chromatograms were developed in the long dimension with phenol:water (80:20 wt/vol) for 24 h and in the short dimension with 1-butanol:propionic acid:water (200:100:141 vol/vol/vol) for 14 h. Amino compounds were located by spraying with 0.5% ninhydrin in acetone (wt/vol) and developed at 100 C for 3 min. Relative ratios of amino acids in peptides were estimated both by comparison with known amounts of standard amino acids producing comparable color intensities when chromatographed as above and by gas-liquid chromatographic analysis according to Roach and Gehrke (19) as *N*-trifluoroacetyl *n*-butyl esters.

RESULTS

Peptides stimulating lysine decarboxylase. A 2-mg portion of one peptide fraction and 10 mg of the amino acid mixture of Lu were placed in individual induction media. Induced cells were assayed for lysine decarboxylase activity, reported in Table 1 as percentages of a control having 10 mg of the Lu mixture only. Addition of larger or smaller amounts of these peptides to

TABLE 1. *Stimulation of lysine decarboxylase by peptides*

Peptide[a]	Relative lysine decarboxylase activity[b]
1	118
2	113
3	101
4	85
5	79
6	185
7	98
A	135

[a] Isolated from casein hydrolysate as described in Materials and Methods.

[b] Expressed as percentage of specific activity of a control containing the amino acid mixture of Lu for which the lysine decarboxylase activity was 0.226 μmol per min per mg of protein.

the Lu amino acid mixture did not improve enzyme levels. Furthermore, only when peptides were added with a complex mixture of amino acids was significant enzyme activity detected. Stimulatory peptides 1, 2, 6, and A were separately hydrolyzed, and their amino acid compositions were determined. They were replaced in induction media by their equivalent amino acids as measured by paper and gas chromatography after hydrolysis of the individual peptides. Weights were corrected for the difference between molecular weights of free amino acids and their residues in peptides, so that replacement was mole per mole. Lysine decarboxylase activity was measured as before. The amino acid compositions of the four active peptides and the enzyme levels reached by replacement with amino acids are shown in Table 2. Comparison of Tables 1 and 2 reveals that stimulation by peptides 1, 2, 6, and A is due solely to the amino acids present in them.

The observation that *E. coli* can utilize peptides as a source of amino acids has been discussed by Payne, Gilvarg, Simmonds, and their colleagues (17, 18, 24, 25). Peptides are transported into *E. coli* via one or more systems for dipeptides and one system for oligopeptides of three or more residues. Transport is followed by cleavage to amino acids by intracellular peptidases for subsequent use in protein synthesis. The general specificities defined for oligopeptide transport (18) would certainly allow the stimulatory oligopeptides isolated from Hy-Case to penetrate *E. coli* cells. Furthermore, at least three classes of peptidases have been found in *E. coli*, dipeptidases, aminopeptidases, and endopeptidases, all of which are intracellular and constitutive (24). Aminopeptidases and endopeptidases have specificities capable of cleaving the Hy-Case peptides isolated in this study to amino acids and dipeptides that could be attacked in turn by dipeptidases. The above systems are presumably used by *E. coli* to acquire amino acids important for lysine decarboxylase biosynthesis from exogenous Hy-Case peptides.

Amino acid requirements for biosynthesis of lysine decarboxylase. The demonstration that stimulatory peptides could be replaced by free amino acids led to a search for requirements for maximal lysine decarboxylase activity. Hy-Case was hydrolyzed, and its amino acid composition was determined. A corresponding mixture of amino acids was prepared and used as a positive control in lysine decarboxylase induction. To determine the best possible mixture, the concentration of each common L-amino acid was varied individually from zero to twice its concentration in Hy-Case. Concentrations of other amino acids were kept constant at the level occurring in Hy-Case. Amino acids not present in the original hydrolysate were also tested for potential activity. Over 100 different amino acid mixtures were screened. The effects of methionine, aspartic acid, and alanine are displayed graphically in Fig. 1 to 3 as representative of the three types of behavior observed for individual amino acids.

The most striking effect was that of methionine. Lysine decarboxylase activity was very low without added methionine, although relatively small amounts permitted full expression of lysine decarboxylase synthesis. Arginine also was important because its optimal addition approximately doubled the capacity of the cell to produce lysine decarboxylase when compared to a medium without arginine. Nevertheless, a mixture of methionine and arginine together at their optimal levels gave only 45% of the lysine

TABLE 2. *Replacement of stimulatory peptides by amino acids*

No.	Peptide composition[a]	Relative lysine decarboxylase activity[b]
1	Pro,Ile,Leu,Val,Phe	114
2	Ile,Leu,Phe	110
6	Arg,Ser,Gly,Lys	202
A	Pro,Ile,Met,Gln	129

[a] Amino acids were present as single residues in these peptides.

[b] Percentage of control containing amino acid mixture of Lu for which the lysine decarboxylase activity was 0.226 μmol per min per mg of protein.

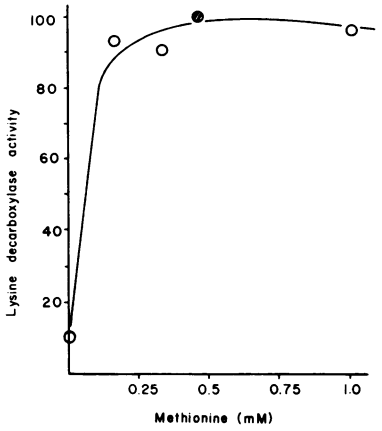

FIG. 1. *Effect of methionine on lysine decarboxylase production. The mixture of amino acids corresponding to Hy-Case was taken as the control, and relative lysine decarboxylase activities are expressed as percentages of that control. The shaded circle indicates concentration in the control for which the lysine decarboxylase activity was 0.509 μmol per min per mg of protein.*

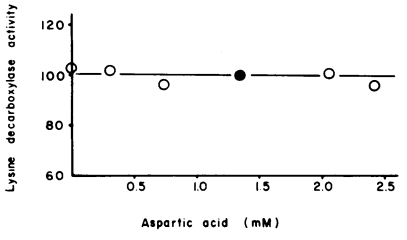

FIG. 2. *Effect of aspartic acid on lysine decarboxylase production. The data are reported on the basis described in the legend of Fig. 1.*

decarboxylase of a control containing a full complement of amino acids. Inhibitory effects were observed for moderate concentrations of alanine (41% at 2.53 mM, see Fig. 3) and to a lesser extent for glycine, arginine, phenylalanine, and tryptophan. Variations in concentrations of several amino acids caused substantial changes in lysine decarboxylase concentration. The relative importances of amino acids to lysine decarboxylase formation in such experiments are summarized in Table 3.

A "complete" amino acid mixture for maximum lysine decarboxylase production contained optimal concentrations of the amino acids of Table 3. A second amino acid mixture, termed "abridged" mixture, contained the nine most stimulatory amino acids of the table. Compositions of these mixtures are listed in Table 4 and differed from Hy-Case by addition of tryptophan, glutamine, asparagine, and cystine; omission of alanine, aspartate, and glutamate; increase in arginine and methionine; and other minor changes. Only L-amino acids were used.

Relative effectiveness of Hy-Case and amino acid mixtures. Taking the response from 0.65 mg/ml of the complete mixture as 100%, relative lysine decarboxylase-stimulating abilities of equivalent concentrations of Hy-Case and the abridged mixture were 44 and 87%, respectively. Three times as much Hy-Case (1.90 mg/ml) matched the enzyme production of the complete amino acid mixture. Further increases of Hy-Case in the induction medium up to 10 mg/ml did not improve lysine decarboxylase production. Although a mixture of the nine most important amino acids accounted for a substantial amount of lysine decarboxylase activity, the mixture containing 16 amino acids was necessary for maximum lysine decarboxylase production.

Other nutritional factors and lysine decarboxylase production. Effects on enzyme levels by thiamine, nicotinamide, riboflavin, calcium pantothenate, biotin, folic acid, vitamin B_{12}, cyclic adenosine 5'-monophosphate, and pyridoxine were tested by addition of 0.1 to 10 μg/ml to the complete amino acid mixture of Table 4. Only pyridoxine increased lysine decarboxylase. At a concentration of 6.6 μg/ml with the complete amino acid mixture present, an increase of 30% in lysine decarboxylase was obtained.

With *p*-chloromercuribenzoate, Sher and Mallette (22) showed that sulfhydryl groups were important for lysine decarboxylase activity. These sulfhydryl groups might be oxidized during handling of the enzyme thus leading to low activity. Moreover, even though pyridoxal phosphate was added during assay for lysine decarboxylase, loss of coenzyme during cell rupture and handling of extracts might lead to denaturation and decreased activity. To check these possibilities, 0.01% mercaptoethanol and 1×10^{-4} M pyridoxal phosphate were added to buffer for preparation of enzyme extracts. They

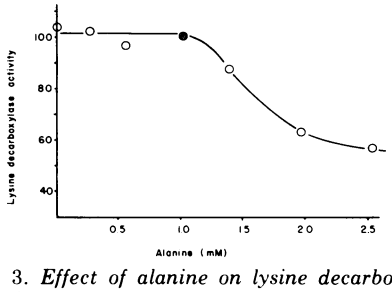

FIG. 3. *Effect of alanine on lysine decarboxylase production. The data are reported on the basis described in the legend of Fig. 1.*

TABLE 3. *Relative importance of amino acids in lysine decarboxylase production*

Amino acid[a]	Lysine decarboxylase activity		
	Control[b] without listed amino acid	Maximum[c] in supplemented mixture	Increase[d] (%)
Met	0.051	0.509	900
Arg	0.269	0.550	104
CySSCy	0.509	0.728	43
Leu	0.428	0.595	39
Ile	0.423	0.564	33
Gln	0.509	0.651	28
Thr	0.464	0.580	25
Tyr	0.415	0.519	25
Asn	0.509	0.621	22
Gly,Ser,Trp,Val, His,Phe,Pro	0.494–0.478	0.520–0.545	>5 but <15

[a] Listed in decreasing order of stimulation of synthesis of lysine decarboxylase.

[b] Lysine decarboxylase activity (in μmol per min per mg of protein) produced in a mixture of amino acids corresponding to Hy-Case except that amino acids listed in the first column were omitted individually. Composition of Hy-Case is given in Materials and Methods.

[c] Lysine decarboxylase activity (in μmol per min per mg of protein) as the maximum produced by variable addition as in Fig. 1 to 3 of amino acids listed in the first column to a mixture otherwise corresponding in composition to Hy-Case.

[d] Expressed as percentage of increase of lysine decarboxylase activity in the 3rd column over that in the 2nd column (difference between values of the 3rd and 2nd columns divided by that of the 2nd column ×100).

had no effect on lysine decarboxylase activity. Because most assays were conducted within 2 h of enzyme isolation, denaturation of this rather stable enzyme (22) was not detectable.

DISCUSSION

A medium containing amino acids (methionine, arginine, cystine, leucine, isoleucine, glutamine, threonine, tyrosine, and asparagine) extensively stimulated the formation of lysine decarboxylase. Therefore, preformed amino acids were utilized directly, although *E. coli* B can synthesize these amino acids for normal growth. The importance of a particular amino acid to a given protein product probably results from the extent to which this amino acid facilitates formation of the protein molecule and may be related to its proportion in the specific protein. For example, 9.7% tyrosine was found in purified lysine decarboxylase by Sher and Mallette (22), and tyrosine stimulates (Table 3) formation of lysine decarboxylase. In the same study, sulfhydryl groups were shown to be important for enzyme activity, and cystine in the medium improved enzyme production. In the opposite sense, unimportance of certain amino acids in enzyme production might suggest either a limited requirement in the enzyme molecule or abundant production by the cell. The situation may be more complex than this simple correlation suggests. For instance, conditions that produce lysine decarboxylase also produce considerable amounts of tyrosine and arginine decarboxylases. A strain of *Streptococcus*, nutritionally exacting toward arginine, was found (5) to produce high levels of the degradative enzyme, arginine dihydrolase. Selectively degradative action of tyrosine decarboxylase on the free amino acid caused a tyrosine requirement by *S. faecalis* (9). Thus, high levels of such enzymes may underlie the need for corresponding amino acids under special conditions of enzyme biosynthesis.

In an earlier study (13), it was suggested that peptides might play some specific role not entirely filled by free amino acids. The present results make this possibility quite unlikely. This revised opinion is due in part to the improved assay procedure, but much more so to the inability of the original work to evaluate inhibitory effects by certain amino acids at concentrations beyond their optima. Phenylalanine, tryptophan, glycine, and arginine are stimulatory at characteristic concentrations, but all are inhibitory at higher concentrations. In addition, alanine, never stimulatory, becomes inhibitory (see Fig. 3). Therefore, Maretzki and Mallette (13) did not match the stimulation by peptides with increased concentrations of amino acids because of inhibition by one or more of these amino acids.

Kihara and Snell (10) also concluded from extensive studies on peptides and growth of

TABLE 4. *Composition of complete and abridged amino acid mixtures*

Amino acid	Complete mixture (μmol/25 mg)	Abridged mixture (μmol/25 mg)
Arg	9.0	13.8
Asn	14.2	21.9
CySSCy	5.2	8.0
Gln	17.1	26.4
Gly	8.3	
His	6.0	
Ile	11.9	18.3
Leu	23.8	36.6
Met	12.6	19.4
Phe	3.8	
Pro	16.3	
Ser	11.9	
Thr	7.9	12.2
Trp	6.1	
Tyr	8.6	13.3
Val	18.7	

lactic acid bacteria and other microorganisms that direct incorporation of peptides into protein without prior hydrolysis did not occur. The presence of systems for peptide transport and intracellular hydrolysis in *E. coli* explains why peptides containing lysine decarboxylase-stimulating amino acids would themselves be stimulatory. The peptide uptake and peptide hydrolysis systems of *E. coli* have been investigated in detail, and several reviews have appeared (17, 18, 24, 25).

The presence of 1% lysine in the medium as inducer adds a regulatory aspect to this particular system. Lysine represses and feedback inhibits aspartokinase III (26). Thus, a system, which synthesizes three stimulatory amino acids, methionine, isoleucine, and threonine from aspartic acid, is at least partially shut off. Of the inducible enzymes in *E. coli* surveyed in the introduction, it is interesting to note that all require methionine and tyrosine for maximum activity, and two require arginine and cystine. These amino acids must be available in the medium for maximal synthesis of certain inducible enzymes.

As pointed out above, some amino acids inhibit lysine decarboxylase production. This effect may be due to properties of the participating amino acid-uptake systems. For example, alanine may inhibit by preventing uptake of glycine and serine by the serine-alanine and glycine-alanine transport systems. Inhibitory effects of phenylalanine and tryptophan at higher concentrations, although less potent than alanine, may be due to competition with stimulatory tyrosine for sites on the phenylalanine-tyrosine-tryptophan permease. These relationships emphasize that measurement of enzyme levels before and after additions to the medium is not enough to evaluate the effect of an amino acid on enzyme production. A range of concentrations must be considered in establishing optimal levels.

Pyridoxine also aided lysine decarboxylase production, presumably because *E. coli* converts it to pyridoxal phosphate, the coenzyme functioning with lysine decarboxylase. This pyridoxine effect is similar to those observed by Bellamy and Gunsalus (1) for tyrosine decarboxylase and by Guirard and Snell (7) for histidine decarboxylase, who showed that certain lactic acid bacteria require much more pyridoxine for synthesis of amino acid decarboxylases than for growth. The coenzyme may stabilize lysine decarboxylase within the cell, or it may act at a different level by aiding in interconversions of intracellular amino acids. Such observations reflect the importance of the nutritional environment in defining biochemical capacities of cells. Furthermore, isolation of pure lysine decarboxylase will be facilitated by these conditions which combine high enzyme activities with high cell yields.

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