NUTRITIONAL FACTORS STIMULATING THE FORMATION OF LYSINE DECARBOXYLASE IN *ESCHERICHIA COLI*

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ABSTRACT

MARETZKI, ANDREW (Pennsylvania State University, University Park) AND M. F. MALLETTE. Nutritional factors stimulating the formation of lysine decarboxylase in *Escherichia coli*. J. Bacteriol. **83**:720–726. 1962 — Inclusion of complex nitrogen sources in the induction medium was shown to be necessary for the synthesis of appreciable amounts of L-lysine decarboxylase by *Escherichia coli* B. Hy-case, a commercial acid hydrolyzate of casein, was especially effective in enzyme production, which was assayed manometrically after lysis of the bacteria from without by bacteriophage. Partial fractionation of the Hy-case, identification of the free amino acids, and addition of these amino acids to test media revealed stimulatory effects by methionine, threonine, proline, leucine, and tyrosine. A full complement of amino acids did not match the enzyme levels reached in the presence of Hy-case. Certain peptide fractions obtained from this mixture supplemented the effects of the amino acids in such a way as to suggest direct incorporation of peptide rather than transport or protective roles. Added purines, pyrimidines, iron, and water-soluble vitamins were without effect. Neither carbohydrates nor phosphorylated materials could be detected in the stimulatory fractions.

Early work (Gale and Epps, 1944; Sher and Mallette, 1954) has shown that the formation of L-lysine decarboxylase in appreciable amounts occurs only in media containing relatively complex nitrogen sources. Hydrolyzates of casein appear to be especially effective in stimulating this biosynthesis in *Escherichia coli*, suggesting that particular amino acids or peptides may play specific roles. Although such added materials were not necessary for growth of the strains studied, they may have replaced the normal biosynthetic materials, as shown by Warner (1956) with lysine in cultures of a coliform and of *Leuconostoc mesenteroides*.

Stimulation of the growth of microorganisms by added peptides has been reported repeatedly (for example, by Sandine, Speck, and Aurand, 1956). Stimulation of the biosynthesis of specific proteins in streptococci has been observed by Fox (1961), and Hartman and Zimmerman (1960) among others. In most cases of stimulation of growth or biosynthesis of proteins by peptides, it is assumed that the peptides are preferentially assimilated or serve to prevent destruction of the component amino acids. Inside the cells these peptides are then thought to be hydrolyzed and to function solely as effective sources of amino acids (Hartman and Zimmerman, 1960; Kihara, Ikawa, and Snell, 1961).

In other cases, however, peptides may play other roles, including that of serving as intermediates in protein biosynthesis. Inconclusive evidence for this possibility exists in the work of Turba, Leismann, and Kleinhenz (1957), who showed that peptides were labeled in a short time and that this label afterward appeared in proteins. Moreover, Raake (1957) was able to show a disappearance of peptides as protein accumulated in tissues of the pea. Investigations based on several other approaches suggest the direct participation of peptides in protein formation (Bishop, Leahy, and Schweet, 1960; Koningsberger, van der Grinten, and Overbeek, 1957; Tsuboi and Fujino, 1960; Brown and Brown, 1960; Fox, 1961).

The present report examines the stimulatory effect of a peptide-containing system in an attempt to elucidate the roles of nutritional factors in the biosynthesis of the specific protein, L-lysine decarboxylase.

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MATERIALS AND METHODS

E. coli B was grown at 37 C in liquid culture with lateral shaking at 200 cycles per min through an amplitude of 2 cm. Turbidity readings at 650 mμ (calibrated by plate and microscopic counts) were used to estimate cell populations. The cells were harvested by centrifugation for 15 min at 800 × *g* (2 to 4 C) and were washed once with 2.5 ml of 0.9% sodium chloride solution.

For use in the enzyme inductions, cells were grown in a standard glucose-synthetic medium described earlier (Weinbaum and Mallette, 1959) to a population of 6×10^8 cells/ml. After harvesting and washing, cells equivalent to 1.0 ml of this culture were incubated in the various experimental media.

Lysine decarboxylase synthesis was stimulated by inclusion of 1% L-lysine·HCl in the glucose-synthetic medium used as the negative control. This reference medium, containing lysine, was then used to assay the stimulatory effects of various added materials. Other media used in certain experiments included nutrient broth, containing 2.4% Difco nutrient broth, 0.5% NaCl, and 1% L-lysine·HCl; Hy-case, containing 5% Hy-case (a commercial acid hydrolyzate of casein, Sheffield Chemical, Norwich, N.Y.), and 1% L-lysine·HCl. A positive control medium consisting of 2% Hy-case and 1% L-lysine·HCl was included to demonstrate inducibility of each lot of cells.

Growth of the cells was quenched in ice water after the population reached 7×10^8 cells (about 4.5 generations). The cells were collected by centrifuging, washed, and suspended in 0.9% NaCl at a total volume of 1.4 ml. Unless otherwise stated, cells in such suspensions were lysed (Sher and Mallette, 1953) with bacteriophage of type $T_{2r}+$, using 100 phage particles per cell and shaking for 20 min at 37 C.

Lysine decarboxylase concentrations were determined by measuring CO_2 evolution manometrically at 37 C in a Warburg apparatus. The main cup of the Warburg flask contained 1.5 ml of 0.05 M phosphate buffer (pH 6.0), 0.5 ml of 0.004 M sodium pyridoxal phosphate, and 0.5 ml of cell suspension or lysate. The substrate, 0.5 ml of 0.067 M L-lysine·HCl, was tipped into the flask from the sidearm at zero time. Readings were recorded at 3-min intervals for a total of 15 min, and activities were calculated as the average μliters of CO_2/hr for each 10^8 cells.

Although activities relative to the controls were always consistent in different experiments or in repetitions of the same experiment, absolute activity values varied somewhat. Apparently these variations arose from the interaction of environmental fluctuations, as in ionic strength, and from physiological differences in the inocula and completeness of the cell lysis. For comparison of results from different experiments, it became necessary to employ a relative activity. Except for the absolute values in the preliminary experiment, the method used here reports activity as the ratio (in percentages) of the CO_2 evolved from cells of the experimental system to that of the positive control, each reduced by the CO_2 evolution in the negative control. Both positive and negative controls were included in every experiment. In this way, differences between presumably identical systems were limited to about ±10% of the activity. All experiments were repeated at least twice.

Electrophoresis was carried out using a Research Specialties Co. model E-800-2 apparatus with a pH 6.8 pyridine buffer (Turba, Pelzer, and Schuster, 1954), at 450 v (0.04 to 0.06 amp) for 7 hr on Whatman no. 17 filter paper previously washed with 0.1 N HCl and water. Sections of interest were eluted with water from the dried paper.

Of the active material obtained by electrophoresis, 250 to 500 mg were fractionated on columns of powdered Whatman no. 1 cellulose. Columns with diameters of 1.5 to 6 cm were packed dry to a height of 100 cm, and rates of flow were adjusted to 3 ml/hr to obtain maximal resolution, using *t*-butanol–*n*-propanol–water (2:2:1, v/v) as the solvent system.

For the qualitative examination of Hy-case, two-dimensional chromatography was carried out on Whatman no. 1 paper, using 80% redistilled phenol–water (w/v) in the long dimension and *n*-butanol–propionic acid–water (151:75:100, v/v) in the short dimension, or *t*-butanol–*n*-propanol–water (2:2:1, v/v) in the long dimension and 80% redistilled phenol–water (w/v) in the short dimension.

RESULTS

The preliminary experiment in Table 1 shows that the biosynthesis of lysine decarboxylase is markedly stimulated by the presence of relatively complex nitrogen sources. Although there are

TABLE 1. *Effect of various media on the formation of lysine decarboxylase in Escherichia coli*

Medium components*						Enzyme activity†	
Nutrient broth (2.4%)	Hy-case (5%)	Hy-case (1%)	Glucose plus salts	Glucose (2%)	Amino acids of casein (3.5%)	Cells intact	Cells lysed
+						7	30
+	+					30	89
	+			+		20	71
		+	+			—	15
			+			—	3
			+		+		9

* Each of the final media for growth and enzyme induction contained 1% lysine·HCl.

† Expressed as μliters of CO_2/hr (per 10^8 cells).

some effects from added amino acids, as will be shown later, not all of the amino acids of casein are required. Moreover, both nutrient broth and Hy-case are individually stimulatory, and in combination produce an even greater level of enzyme activity. Additions of water-soluble vitamins and purines and pyrimidines to simple media showed no significant enhancement of enzyme activity. This report is restricted to the study of the stimulatory factors in Hy-case.

When fractionation of this material was undertaken, only components behaving as electrically neutral substances in a pH 6.8 buffer system during electrophoresis significantly stimulated induction of lysine decarboxylase formation. This fraction also contained 58% of the total nitrogen. Subsequent cellulose-column chromatography permitted separation of this electrophoretically neutral material into nine relatively distinct ninhydrin-positive fractions. The enzyme-stimulating capacity of each fraction was tested individually, and none appreciably exceeded the negative control.

To evaluate the effects of mixtures of the free amino acids in these column fractions, the compositions of the fractions were studied by two-dimensional chromatography and by chromatography of the dinitrophenyl derivatives, as described by Biserte and Osteux (1951). The following amino acids, listed in the order of elution of the fractions from the column, were identified. Fraction *a* contained no free amino acids; fraction *b*, leucine-isoleucine; fraction *c*, phenylalanine; fraction *d*, valine and methionine; fraction *e*, tyrosine and α-amino-butyric acid; fraction *f*, proline; fraction *g*, alanine and threonine; fraction *h*, threonine; fraction *i*, glycine and serine. The identities of these substances were confirmed by elution and co-chromatography with authentic samples.

Solutions of commercial samples of the L-amino acids were prepared in concentrations corresponding to the total nitrogen content of the fractions in which they occurred, as determined by a micro-Kjeldahl procedure (Beet, 1955). For the sake of simplicity and to insure sufficiently high levels of the amino acids, the peptide content in each fraction was disregarded in computing the concentrations of the amino acids added. The amino acids from the neutral electrophoretic fraction account for 60 to 65% of the total Hy-case activity (Table 2). Assays after omission of amino acids from the mixture indicated their relative degrees of importance in the enzyme-forming system. Methionine, threonine, proline, leucine, and tyrosine appeared to be more stimulatory than did the other amino acids involved. However, omission of several amino acids at a time intensified the effects on enzyme formation. Free glutamic acid and aspartic acid were not a part of the neutral fraction, and their inclusion in the system produced no added stimulation. The stimulation by added Fe^{+++} in the arginine decarboxylase system (Melnykovych and Snell, 1958) was not observed in the formation of lysine decarboxylase.

Inability in these experiments to achieve enzyme formation comparable to that with Hy-case itself led to a search for stimulatory peptides. A sample of the original neutral fraction from electrophoresis was chromatographed in two dimensions. Among the ninhydrin-positive materials, two peptides were isolated, hydrolyzed, and chromatographed, demonstrating the component amino acids. One of these peptides was identical with a peptide in fraction *a* from the column. The other eight fractions from the column also contained peptides, though probably not all were different. These peptides appeared to decrease in size, from at least nine different amino residues in the peptide of fraction *a* to four kinds of residues in the peptide of fraction *i*.

Table 3 shows the effects of fractions from the column taken singly, in combination with one another, and in combination with the full synthetic amino acid complement. It was not

TABLE 2. *Stimulation of lysine decarboxylase (LD) formation by the amino acids of the neutral electrophoretic fraction*

Combination	LD activity (% of Hy-case control)	Combination	LD activity (% of Hy-case control)
Aa*	58	Aa-tyr	28
Aa, double concn	40	Aa-phe	55
Aa-ala†	50	Aa-leu, ser	25
Aa-ser	68	Aa-pro, tyr, meth	0
Aa-thr	30	Aa-phe, leu, meth, tyr, pro	0
Aa-gly	67	Aa-phe, gly, ala, ser	40
Aa-gly, ala	50	Pro, meth, thr	20
Aa-ser, thr	30	Aa + val	54
Aa-gly, ala, ser,	30	Aa + glu	50
Aa-gly, ala, ser, thr	0	Aa + asp	50
Aa-pro	23	Aa + Fe^{+++}	60
Aa-leu	37	Negative control	0
Aa-meth	9	Positive control	100

* Aa = amino acids of an electrophoresis fraction but without valine. Other amino acids (or Fe^{+++}) omitted or added as shown.

† Aa-ala represents the mixture of amino acids but with alanine omitted; ala = alanine, asp = aspartic acid, glu = glutamic acid, gly = glycine, leu = leucine, meth = methionine, phe = phenylalanine, pro = proline, ser = serine, thr = threonine, tyr = tyrosine, val = valine.

possible to determine the single effect on the enzyme system of the peptide in fraction *a*, since the cells autolyzed during the adaptive growth period when exposed to this fraction alone. However, from other mixtures which included *a*, it was evident that this fraction in the presence of the full amino acid complement did exert an additional stimulatory effect. All of the other individual fractions raised the level of lysine decarboxylase above that in the negative controls. But only fractions *a* and, especially, *f* improved enzyme formation above that obtained with the synthetic mixture of amino acids. Thus, only the peptides in these two fractions possessed any stimulatory powers beyond those of free amino acids.

Fraction *f* was subjected to further study since its presence in the medium stimulated lysine decarboxylase activity comparable to that in the original electrophoretic fraction and in Hy-case itself. Two-dimensional chromatograms of this fraction were examined with aniline-trichloroacetic acid spray (McCready and McComb, 1954) and with periodate-SO_2-rosaniline (Aronoff, 1956). Both tests were negative for carbohydrates. A report (Hipp, Groves, and McMeekin, 1957) that phosphopeptides from α-casein survive prolonged acid hydrolysis led to application of a spray reagent for phosphates (Aronoff, 1956) to two-dimensional chromatograms of *f*, with negative results. Furthermore, neutron activation of chromatograms (Benson et al., 1958) showed no subsequent zone due to P^{32}.

TABLE 3. *Stimulation of enzyme synthesis by fractions from column chromatography of the electrophoretic material*

Fractions added to the growth medium*	Lysine decarboxylase activity (% of Hy-case control)
a, b, c, d, e, f	71
a, b, c,	10
g, h, i	44
h, i	33
g, d	10
b	31
c	23
d	8
e	38
f	31
Aa†	58
Aa + *a*	77
Aa + *b, c*	68
Aa + *d*	65
Aa + *e*	43
Aa + *f*	125
Aa + *g*	67
Aa + *h*	41
Leu, phe, meth, pro, *a, g, h, i*	77

* When more than one fraction was used, equal volumes of each were evaporated to dryness and redissolved so as to make the mixture equivalent to 0.5 ml of 3.5% Hy-case.

† Amino acids of the electrophoresis fraction. The combination in this experiment did not include valine, which was shown to be an inactive component of the fraction at a later time.

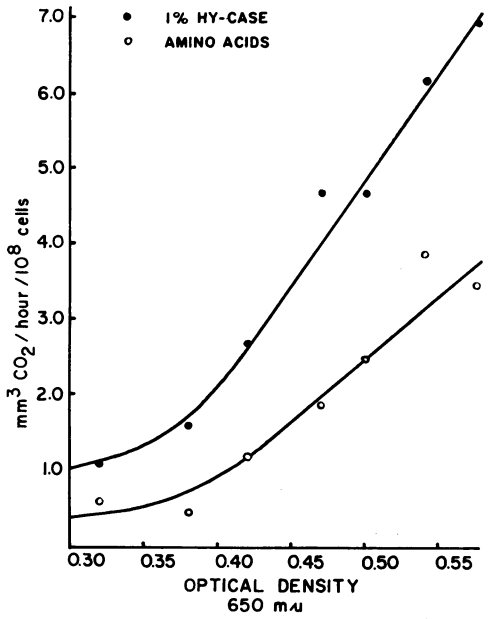

FIG. 1. *Stimulation of lysine decarboxylase formation by Hy-case compared with that due to free amino acids. The ordinate indicates the increase in enzyme activity in a given number of cells. The abscissa indicates increase in population (since optical density is numerically equal to turbidity, which in turn is directly proportional to population). In this case, the proportionality factor is 1.2 × 10⁹ cells of Escherichia coli/ml.*

Chromatography of fraction *f* in one dimension with isopropanol-formic acid-water (75:13:12, v/v), followed by ninhydrin treatment, revealed three areas in addition to proline, which was shown previously to be a free amino acid component of this fraction. These three areas differed chromatographically from the free amino acids of Hy-case, and hydrolysis followed by chromatography indicated that they were due to three or more peptides. Since doubling the proline concentration (the free amino acid in fraction *f*) in the standard free amino acid complement showed no enhancement of the enzyme activity, one or more of these peptides must be stimulatory.

Dinitrophenylation and chromatographic separation of the products, using the system of Biserte and Osteux (1951), revealed six dinitrophenyl derivatives of peptides in this active fraction *f*, but the distribution of the stimulating effect is not known. Attempts to obtain the several components in quantities large enough for study were unsuccessful.

In a further approach to the problem of demonstrating stimulatory action by materials other than free amino acids, the rate of enzyme formation as a function of the growth of *E. coli* B was investigated. The consistently higher enzyme activities stimulated by Hy-case at various stages in the growth of the cells are compared in Fig. 1 with activities obtained during growth in the mixture of free amino acids. Since the slopes are 1.43 for Hy-case medium and 0.70 for the simple amino acid medium, it is clear that enzyme formation in the cells exposed to Hy-case medium occurs at double the rate shown in cells grown in the mixture of amino acids. Hence, Hy-case must contain stimulatory factors in addition to the usual free amino acids.

DISCUSSION

Two significant factors in the synthesis of lysine decarboxylase by *E. coli* emerge as a result of this study. Certain preformed amino acids added to the medium are utilized by the organism to greater advantage than are others. Secondly, a distinct, synergistic role is played by stimulatory peptides in the presence of free amino acids.

The first conclusion supports the findings of Warner (1956) that the organism, in preference to synthesis, can utilize some preformed amino acids directly. The requirements for methionine, threonine, proline, leucine, and tyrosine in an inducing medium may be a reflection of their relative importance to the enzyme-synthesizing system, of their relative abundance in the protein molecule, or of the limited capacity of growing cells to synthesize these particular amino acids.

There was no indication that stimulatory substances other than amino acids and peptides are present in Hy-case. However, some peptides occurring in Hy-case must be stimulatory, as shown by both the fractionation and kinetic studies. The difference between the rates of enzyme formation in cells grown in the mixture of simple amino acids when compared to Hy-case suggests either direct incorporation of peptides or an increased availability of the component amino acids. However, doubling the amino acid concentration did not cause an enzyme increase, as expected from the hypothesis of amino acid or nitrogen availability. Likewise a particularly rapid diffusion of peptides to the site of synthesis seems unlikely, since such an advantage for peptides might be overcome at least in part by the improved availability of free amino acids

from an increased concentration. A requirement for a specific amino acid is also doubtful, since extra additions of the amino acids making up the peptide, plus those already present in the free amino acid complement, did not replace the stimulatory peptides.

The evidence suggests that certain peptide fragments may be utilized directly in forming the enzyme molecule. This suggestion is based on the assumption that the stimulatory peptides from Hy-case have at least a partial sequence corresponding to a similar alignment in the lysine decarboxylase molecule. An activation process resembling that for amino acids would be anticipated, and is suggested in the work of Koningsberger et al. (1957) and Tsuboi and Fujino (1960). Real proof of the incorporation of intact peptides is still not available in spite of the numerous suggestions that it must occur.

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