# NUTRITIONAL REQUIREMENTS FOR THE FORMATION OF ARGININE DECARBOXYLASE IN *ESCHERICHIA COLI*[1]

GEORGE MELNYKOVYCH[2] AND ESMOND E. SNELL

*Department of Biochemistry, University of California, Berkeley, California*

Received for publication May 26, 1958

The nutritional requirements for growth and for optimum production of specific enzymes by a given organism may differ. Gale (1940), for example, observed considerable variations in the nutritional requirements for formation of three of the amino acid decarboxylases by the same strain of *Escherichia coli*. Glutamic acid decarboxylase was produced in a salts-glucose medium, and thus behaved like a constitutive enzyme. Lysine decarboxylase was adaptive, i. e., it was synthesized by the organism only in the presence of added substrate. The requirements for synthesis of arginine decarboxylase were more complex, for there was no appreciable enzyme formation by the cells grown in an arginine-supplemented chemically defined medium, unless it was fortified with casein hydrolyzate.

The present investigation arose in an effort to determine the nature of these additional requirements for arginine decarboxylase production. Particular attention was paid to the possible role of trace metals, since $Zn^{++}$ has been shown to be required for production of several pyridoxal phosphate enzymes in Neurospora (tryptophan synthetase (Nason, 1950); D-serine dehydrase and L-threonine dehydrase (Hoare and Snell, 1958)) and $Fe^{+++}$ activate the histidine decarboxylase of a lactobacillus (Guirard and Snell, 1954) and the aminobenzoic acid decarboxylase of *E. coli* (McCullough *et al.*, 1957).

## EXPERIMENTAL METHODS

*Escherichia coli* strain B was maintained in a basal medium composed of glucose, 2.0 per cent; $K_2HPO_4$, 0.1 per cent; NaCl, 0.1 per cent; sodium citrate, 0.05 per cent; $(NH_4)_2SO_4$, 0.4 per cent; and 0.5 per cent of 85 per cent lactic acid. The pH was adjusted to 6.8 with NaOH. The ingredients for 1 L of medium were dissolved in 200 ml of deionized water and extracted with 8-hydroxyquinoline (Waring and Werkman, 1943) to remove traces of ionic iron. The concentrated medium was stored under refrigeration in polyethylene bottles for not more than 10 days. Before use it was diluted to volume with deionized water and supplemented with recrystallized $MgSO_4$ (0.07 per cent). The flasks were autoclaved at 121 C for 5 min at which time the pH of the medium had fallen to 6.6.

Unless specified otherwise, the organism was grown from a 5 per cent inoculum grown in medium of low iron content for 19 to 20 hr at room temperature (approximately 24 C). For enzymatic studies the cells were harvested by centrifugation, washed once with deionized water, and suspended as required for individual experiments. Cell-free enzyme preparations were prepared by the method of Taylor and Gale (1945).

Arginine decarboxylase was determined by following carbon dioxide evolution from the amino acid at pH 4.25 (intact cells) or at pH 5.25 (cell-free preparations). The main compartment of each Warburg cup contained the cells or cell-free enzyme preparations, various additions as indicated with the individual experiments, and 0.05 M phthalate buffer in a volume of 3.0 ml. Into the side arm was placed 0.2 ml of a 0.0125 M solution of arginine hydrochloride. After equilibration in an atmosphere of nitrogen at 37 C, the substrate was added from the side arm and the carbon dioxide evolution measured. The enzyme activity is expressed as $Q_{CO_2}$, the $\mu$L of $CO_2$ evolved per mg of dry cells per hr.

## RESULTS

*Arginine decarboxylase production in stationary culture.* Partial anaerobiosis has been reported to favor formation of amino acid decarboxylases (Gale, 1940; Sher and Mallette, 1954). Our first experiments were therefore performed with cells grown in stationary culture in 100 ml of medium

[1] Supported in part by a grant (E-1575) from the United States Public Health Service.

[2] Postdoctoral Fellow of the National Institutes of Health, U. S. Public Health Service. Present address: Elgin State Hospital, Elgin, Illinois.

TABLE 1

*Effect of casein hydrolyzate and yeast extract (Difco) on growth and on synthesis of arginine decarboxylase in stationary cultures of Escherichia coli*

| Yeast Extract | Casein Hydrolyzate | Growth* | $Q_{CO_2}$ |
|---|---|---|---|
| % | % | *mg dry cells/ml* | |
| 0.5 | | 0.092 | 1240 |
| 0.25 | | 0.081 | 1240 |
| 0.025 | | 0.063 | 1140 |
| 0.01 | | 0.048 | 450 |
| | 0.5 | 0.087 | 1620 |
| | 0.25 | 0.073 | 1170 |
| | 0.025 | 0.056 | 1080 |
| | 0.01 | 0.029 | 1020 |
| | | 0.040 | 50 |

Incubation time, 20 hr.

* Cell yields given in this and subsequent tables were determined photometrically by use of a photoelectric colorimeter (540 mμ) and a separately constructed calibration curve relating optical densities to weight of cells in the suspension.

contained in 125 ml Erlenmeyer flasks. Cell growth and decarboxylase production under these conditions were relatively poor (table 1) even though the medium was supplemented with as much as 0.5 per cent of arginine. Addition of "vitamin-free," tryptophan-supplemented acid hydrolyzate of casein or of yeast extract (Difco) increased enzyme synthesis up to 30-fold, while the stimulation of growth was only about 2-fold. Mixtures of the B-vitamins, nucleic acid derivatives, trace elements, and amino acids were therefore tested separately for possible stimulatory action upon enzyme synthesis. Only the amino acid mixtures proved active. By appropriate deletion and substitution experiments it was found that a mixture of arginine, tyrosine, methionine, and asparagine substantially duplicated the effects of the mixture of 19 amino acids used as a control (table 2). This supplement increased growth about 40 per cent while arginine decarboxylase synthesis was increased about 20 times.

Addition of iron to the stationary cultures also stimulated enzyme synthesis (table 3). The stimulation was most pronounced when arginine, but not the other three amino acids (tyrosine, methionine, and asparagine), was omitted from the medium. Under these conditions, rather high decarboxylase production occurs in the absence of added substrate, again emphasizing the artificiality of classification of enzymes into "constitutive" and "inducible" types. Since arginine was used in rather high concentration and was not purified, a portion of

TABLE 2

*Effect of supplementary amino acids on growth and arginine decarboxylase production in stationary culture of Escherichia coli*

| Supplement Added per 100 ml of Basal Medium | Growth | Decarboxylase Activity ($Q_{CO_2}$) |
|---|---|---|
| | *mg dry cells/ml* | |
| Arginine·HCl, 500 mg | 0.050 | 60 |
| Arginine, 500 mg, + | | |
| (a) Tyrosine, 2.5 mg | 0.047 | 240 |
| (b) Methionine, 2.5 mg | 0.047 | 120 |
| (c) Asparagine, 40 mg | 0.044 | 90 |
| (d) Asparagine, 60 mg | 0.042 | 90 |
| (e) (a) + (b) | 0.047 | 540 |
| (f) (a) + (b) + (c)* | 0.054 | 800 |
| 19 Amino acids† | 0.059 | 950 |

Incubation time, 20 hr.

* Asparagine, 10 mg.

† The mixture of Sauberlich and Baumann (1948) was added at a level of 144.5 mg together with 490 mg of arginine hydrochloride.

TABLE 3

*Comparative effects of iron and amino acids on growth and formation of arginine decarboxylase in stationary cultures of Escherichia coli*

| Supplement Added per 100 ml Basal Medium | Growth | Final pH of Culture | $Q_{CO_2}$ |
|---|---|---|---|
| | *mg dry cells/ml* | | |
| None | 0.058 | 5.5 | 37 |
| $Fe^{+++}$, 22 μg | 0.068 | 5.2 | 108 |
| Arginine, 500 mg | 0.065 | 5.4 | 87 |
| Arginine plus $Fe^{+++}$, 22 μg | 0.068 | 5.3 | 115 |
| Amino acids* | 0.065 | 5.4 | 285 |
| Amino acids* + $Fe^{+++}$, 22 μg | 0.071 | 5.3 | 690 |
| Amino acids* + arginine, 500 mg | 0.073 | 5.2 | 770 |
| Amino acids* + arginine, 500 mg, + $Fe^{+++}$, 22 μg | 0.075 | 5.2 | 830 |

Incubation time, 19 hr.

* Tyrosine (2.5 mg), methionine (2.5 mg) and asparagine (10 mg); see table 2.

its effect may be due to contamination with traces of iron.

*Arginine decarboxylase production in aerated cultures.* Because of the high cell yields obtainable, the production of arginine decarboxylase in aerated cultures was investigated despite reports that aerobiosis influences enzyme production adversely. Cultures were grown in 100 ml of medium contained in 500 ml flasks which were aerated by shaking on a Brunswick rotary shaker for 18 or more hr. Under these conditions, growth was limited by the iron content of the medium (table 4). With iron supplementation alone, no decarboxylase was produced. However, in the presence of sufficient iron, decarboxylase production equal or superior to that in stationary cultures was obtained upon supplementation with a hydrolyzed casein preparation or a mixture of 19 amino acids. By empirical procedures similar to those used with stationary cultures, the specific amino acids required for near-maximum enzyme production were determined. This minimal supplement, listed in table 4, differs from that effective in stationary cultures chiefly in the presence of glutamic acid, which is necessary for full effectiveness, and in the amounts of individual amino acids supplied. Essentially no decarboxylase was formed in the absence of added $Fe^{+++}$. The traces formed in the cultures supplemented with the complete mixture of amino acids may have resulted from traces of iron in the amino acids.

TABLE 4

*Requirement for supplementary iron and amino acids for formation of arginine decarboxylase in shake cultures of Escherichia coli*

| Additions per 100 ml Basal Medium | | Growth | $Q_{CO_2}$ |
|---|---|---|---|
| Amino acids | $Fe^{+++}$ | | |
| | *μg* | *mg dry cells/ml* | |
| None | None | 1.4 | 0 |
| | 22.4 | 3.0 | 0 |
| | 112 | 3.1 | 0 |
| 19 Amino acids,* 867 mg | None | 3.3 | 18 |
| | 22.4 | 3.4 | 1500 |
| | 112 | 3.0 | 820 |
| Simplified amino acid supplement,† 330 mg | None | 1.85 | 0 |
| | 22.4 | 3.8 | 830 |
| | 112 | 3.4 | 1350 |

Incubation time, 18 hr.

* The mixture of Sauberlich and Baumann (1948) was used.

† L-Arginine·HCl, 60 mg; DL-methionine, 30 mg; L-tyrosine, 30 mg; L-asparagine, 120 mg; L-glutamic acid, 90 mg.

It seems likely that the substantial though limited growth in the aerated, Fe-deficient basal medium is made possible by the presence of traces of iron in the medium and in cells of the inoculum. This would indicate that the Fe-requirement for growth is less than that for production of arginine decarboxylase. This is demonstrated convincingly by the data of figure 1. The iron requirement for maximum enzyme

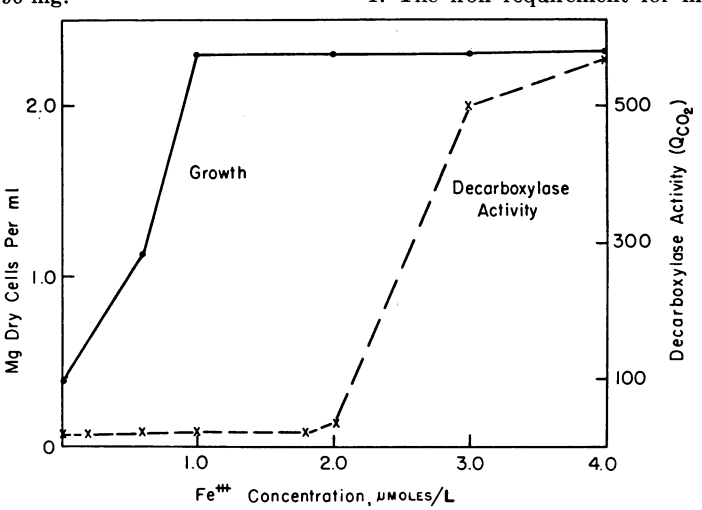


*Figure 1.* The comparative iron requirements for growth and synthesis of arginine decarboxylase in *Escherichia coli* strain B. Shake cultures in amino acid-supplemented basal medium. Incubated 24 hr at room temperature.

production is over three times that for maximum growth, and little or no enzyme production occurs at levels of $Fe^{+++}$ less than twice that required for maximum growth.

*Adaptive formation of arginine decarboxylase by resting cells.* To dissociate, at least in part, enzyme formation from increase in cell mass, requirements for adaptive formation of the arginine decarboxylase by resting cell suspensions of the organism were examined. Cells were grown under stationary conditions in the unsupplemented basal medium, resuspended in 0.0125 M phthalate buffer (pH 5.2), and incubated for 90 min at 30 C in the presence of 0.0125 M arginine, 1 per cent glucose, and the supplement to be tested. There was no net growth during this time, as judged from measurements of optical density, in any of the experiments reported. Following this induction period, cells were centrifuged, washed, and their arginine decarboxylase activity determined in the usual manner.

Supplementation during the induction period with those amino acids (tyrosine, methionine, and asparagine) that stimulated enzyme formation in growing cells was without effect on enzyme formation. In the presence of these amino acids, enzyme formation was stimulated by yeast extract, however. The effectiveness of the yeast extract was eliminated by ethylenediaminetetraacetic acid (Versene), and the stimulatory factor survived ashing. Although $Fe^{+++}$ alone was only slightly effective, it was found eventually that $Fe^{+++}$ plus phosphate ions largely duplicated the effect of the yeast ash. The magnitude of these effects are shown in table 5. The requirement for $Fe^{+++}$ was specific; $Cu^{++}$, $Zn^{++}$, $Co^{++}$, $Mn^{++}$, $Ni^{++}$, $Al^{+++}$, and $Mg^{++}$ at various levels failed to substitute for it. In some experiments, addition of $Mg^{++}$ together with $Fe^{+++}$ and $PO_4^{\equiv}$ gave slightly increased enzyme levels; the effect was, however, inconsistent. $Fe^{+++}$ and $PO_4^{\equiv}$ ions had relatively little effect in the absence of the amino acid supplement.

Antibiotics of the tetracycline group are known to inhibit the formation of certain enzymes, including arginine decarboxylase (Melnykovych, 1956). Table 6 demonstrates that relatively high concentrations of iron reverse this inhibitory effect of oxytetracycline.

*Experiments with cell-free arginine decarboxylase.* The cell-free histidine decarboxylase of Lactobacillus strain 30a is activated under some conditions by added $Fe^{+++}$ (Guirard and Snell, 1954), and this metal ion is an obligatory cofactor for an aminobenzoic acid decarboxylase of *E. coli* (McCullough *et al.*, 1957). These findings, together with the obligatory requirement for $Fe^{+++}$ for production of arginine decarboxylase demonstrated here, suggest, as one possibility, that $Fe^{+++}$ may be a functional portion of the enzyme. To test this possibility, cell-free preparations of the enzyme were prepared from acetone-dried cells of low iron content and fractionated with ammonium sulfate as described by Taylor and Gale (1945). Such preparations after dialysis were partially resolved with respect to pyridoxal phosphate, but were not activated by addition of ferric iron. Their activity was not reduced by incubation with $10^{-3}$ M Versene, 8-hydroxyquino-

TABLE 5

*Phosphate and iron requirements for induced formation of arginine decarboxylase by resting cell suspensions of Escherichia coli*

| Conc. | | $Q_{CO_2}$ |
|---|---|---|
| $Fe^{+++}$ | $PO_4^{\equiv}$ | |
| *μmoles/L* | *μmoles/L* | |
| — | — | 30 |
| 2.0 | — | 75 |
| — | 2500 | 115 |
| 0.2 | 2500 | 180 |
| 2.0 | 2500 | 240 |
| 2.0 | 10 | 80 |
| 2.0 | 100 | 270 |
| 2.0 | 1000 | 300 |

The induction period was 90 min; the induction medium also contained the simplified amino acid mixture given in table 3.

TABLE 6

*Reversal by $Fe^{+++}$ of oxytetracycline-induced inhibition of arginine decarboxylase synthesis in resting cells of Escherichia coli*

| $KH_2PO_4$ | $Fe^{+++}$ | Oxytetracycline HCl | $Q_{CO_2}$ |
|---|---|---|---|
| *mmoles/L* | *μmoles/L* | *ug/ml* | |
| — | — | — | 55 |
| 1.0 | — | — | 110 |
| 1.0 | 2.0 | — | 280 |
| 1.0 | 2.0 | 0.1 | 62 |
| 1.0 | 20.0 | 0.1 | 128 |
| 1.0 | 50.0 | 0.1 | 198 |
| 1.0 | 100.0 | 0.1 | 248 |

Conditions as in table 5.

line, *o*-phenanthroline, or sodium salicylate. These negative results do not exclude $Fe^{+++}$ as a component of the enzyme, but do show that if present it must be bound firmly to the enzyme protein. It is also possible that the iron functions only indirectly in synthesis of the enzyme, and and is not a component of the protein.[3] Purification and analysis of the enzyme may be required to decide between these two possibilities.

## DISCUSSION

Although *E. coli* synthesizes each of the amino acids at a rate that permits near-optimal growth, supplementary amino acids are required to permit optimal synthesis of arginine decarboxylase in either stationary or shaken cultures of this same organism. Billen and Lichstein (1950) earlier observed a similar relationship between growth and hydrogenase synthesis in *E. coli*. Of the seven supplementary amino acids necessary for optimal hydrogenase synthesis, four are needed for optimal formation of arginine decarboxylase. Requirements for synthesis of the latter enzyme are more exacting in aerated cultures, where growth is relatively rapid and heavy, than in stationary culture, and include a high requirement for iron. Supplementary iron and amino acids are also required for the induced formation of the decarboxylase in resting cell suspensions. These observations in *E. coli* recall those of Bellamy and Gunsalus (1945), Rodwell (1953), and Guirard and Snell (1954), who showed that various lactic acid bacteria require much more vitamin $B_6$ for synthesis of various amino acid decarboxylases than is required for growth, the exact level required varying, even in the same culture, with the decarboxylase examined. The observations emphasize anew the importance of the nutritional environment in defining the biochemical capacities of the cells obtained. By defining conditions under which high decarboxylase activity can be obtained with high cell yields, they also increase the convenience with which arginine decarboxylase can be obtained for use as a specific analytical or preparative reagent.

[3] Addition of pyridoxal or pyridoxal phosphate to iron-deficient cultures did not result in enzyme formation. The role of iron probably is not, therefore, in production of this coenzyme.

## SUMMARY

Although *Escherichia coli* strain B grows well in chemically defined media without added amino acids, it does not synthesize arginine decarboxylase. The substances present in casein digests that permit such synthesis were identified.

In nonaerated cultures, arginine, methionine, tyrosine, and asparagine largely duplicate the effects of casein digest; iron is also stimulatory. In aerated cultures where growth is much heavier, cells with high decarboxylase activity are produced if glutamic acid and iron are supplied in addition to these amino acids. Under these conditions, no decarboxylase is formed in the absence of added iron. The iron requirement for enzyme formation is much higher than that for growth. In the presence of optimal iron, substantial enzyme formation occurs in the absence of added arginine.

Adaptive formation of arginine decarboxylase by resting cell suspensions required addition of these same supplemental amino acids, and also of iron and phosphate ions. Oxytetracycline inhibited enzyme formation under these conditions; these inhibitory effects were alleviated by additional iron.

Partially purified cell-free preparations of arginine decarboxylase were prepared from cultures of *E. coli* grown with minimal amounts of iron for enzyme production. Although partially resolved with respect to pyridoxal phosphate, their activity was not enhanced by additions of iron, and was not decreased by several chelating agents. It thus remains to be determined whether iron is an essential component of the decarboxylase, or whether it functions indirectly in formation of the enzyme.

## REFERENCES

BELLAMY, W. D. AND GUNSALUS, I. C. 1945 Tyrosine decarboxylase. II. Pyridoxin deficient medium for apoenzyme production. J. Bacteriol., **50**, 95–103.

BILLEN, D. AND LICHSTEIN, H. C. 1950 Nutritional requirements for hydrogenase production by *Escherichia coli*. J. Bacteriol., **60**, 311–314.

GALE, E. F. 1940 The production of amines by bacteria. 1. The decarboxylation of amino acids by strain of *Bacterium coli*. Biochem. J. (London), **34**, 392–413.

GUIRARD, B. M. AND SNELL, E. E. 1954 Pyridoxal phosphate and metal ions as cofactors for histidine decarboxylase. J. Am. Chem. Soc., **76,** 4745–4746.

HOARE, D. AND SNELL, E. E. 1958 Some effects of metal ions in enzymatic reactions involving vitamin $B_6$. Proc. Symposium on Enzyme Chemistry, Tokyo and Kyoto, Japan, October, 1957, *in press.*

MCCULLOUGH, W. G., PILIGIAN, J. T., AND DANIEL, I. J. 1957 Enzymatic decarboxylation of the aminobenzoates. J. Am. Chem. Soc., **79,** 628–630.

MELNYKOVYCH, G. 1956 A study of the effects of antibiotics on the formation of amines by intestinal bacteria and their importance in the nutrition of the young rat. Ph.D. Thesis, University of Minnesota, Minneapolis.

NASON, A. 1950 Effect of zinc deficiency on the synthesis of tryptophan by *Neurospora* extracts. Science, **112,** 111–112.

RODWELL, A. W. 1953 The histidine decarboxylase of a species of *Lactobacillus*; apparent dispensability of pyridoxal phosphate as coenzyme. J. Gen. Microbiol., **8,** 233–237.

SAUBERLICH, H. E. AND BAUMANN, C. A. 1948 A factor required for the growth of *Leuconostoc citrovorum.* J. Biol. Chem., **176,** 165–173.

SHER, I. H. AND MALLETTE, M. F. 1954 Purification and study of L-lysine decarboxylase from *Escherichia coli* B. Arch. Biochem. Biophys., **53,** 354–369.

TAYLOR, S. E. AND GALE, E. F. 1945 Studies on bacterial amino acid decarboxylases. 6. Codecarboxylase content and action of inhibitors. Biochem. J. (London), **39,** 52–58.

WARING, W. S. AND WERKMAN, C. H. 1943 Iron requirements of heterotrophic bacteria. Arch. Biochem., **1,** 425–433.