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INDUCTION OF L-ASPARAGINASE SYNTHESIS IN *ESCHERICHIA COLI*

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SUMMARY

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) synthesis in *Escherichia coli* ATCC 9637 was studied. The EC-2 isoenzyme was synthesized if the culture was grown under anaerobic conditions or under conditions of moderate aeration after oxygen has been used up from the medium as detected by an oxygen electrode. Sodium lactate added into the medium in a concentration of 25–100 μmoles/ml stimulated L-asparaginase EC-2 synthesis in moderately-aerated cultures and rendered it possible to some extent even in the presence of oxygen. The substrate, L-asparagine, induced an additional synthesis of EC-2 isoenzyme but only under anaerobic conditions or if lactate was present in the growth medium. No other amino acids tested exhibited a similar effect. Two other strains ML 30 and B behaved in a similar way. The L-asparaginase EC-1 isoenzyme was synthesized in the cells constitutively and its synthesis was not markedly affected by any of the conditions tested. However, we observed direct dependence between EC-1 and EC-2 synthesis: if the synthesis of EC-2 was stimulated, the synthesis of the former decreased or stopped altogether depending on the degree of stimulation of L-asparaginase EC-2 synthesis.

INTRODUCTION

L-Asparaginase (L-asparagine amidohydrolase EC 3.5.1.1), which was originally isolated from guinea pig serum[1], shows marked antineoplastic activity. Mashburn and Wriston[2] and Schwartz *et al.*[3] isolated an asparaginase with similar properties from *Escherichia coli* cells. The *E. coli* asparaginase is formed by two isoenzymes EC-1 and EC-2, of which only EC-2 inhibits the growth of tumours.

Data describing cultivation conditions leading to an effective synthesis of L-asparaginase in *E. coli* cultures are incomplete and often contradictory. Hernádi *et al.*[5] reported that the EC-1 isoenzyme formation starts in the early logarithmic phase and reaches a maximum in the late logarithmic phase of growth whereas the

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beginning of EC-2 isoenzyme synthesis is in the late logarithmic phase and reaches its peak in the stationary phase of growth. EC-1 isoenzyme is synthesized constitutively according to Broome[6].

Some authors isolated L-asparaginase EC-2 from cells grown in highly aerated cultures[7,8]. On the other hand Cedar and Schwartz[9] and others[10] observed that the synthesis of EC-2 isoenzyme is inversely dependent on aeration. In agreement with their view Bilimora[11] and Stejskalová *et al.*[12] recommended growing the culture under conditions of moderate aeration. Boeck *et al.*[13] and Svobodová[14] used an oxygen electrode and found that synthesis of EC-2 L-asparaginase in *E. coli* cells starts after depletion of oxygen from the cultivation medium. Also our present paper presents some observations which confirm their findings.

In *Serratia marcescens* L-asparaginase synthesis is induced by its substrate L-asparagine[15], in *Pseudomonas* both L-asparagine and L-aspartic acid serve as inducers[16,17]. The induction takes place in both organisms under aerobic conditions.

The mechanism of regulation of L-asparaginase synthesis in *E. coli* by external agents is not clear as yet. A number of authors attempted to elucidate whether L-asparagine or related compounds can induce its synthesis, however, their results are contradictory.

Robison and Berk[18] described a stimulation of EC-2 L-asparaginase synthesis by L-aspartic acid in some *E. coli* strains but they did not find any effect of L-asparagine or glutamine. Netrval and Smékal[7] observed that high concentrations of L-asparagine had some small stimulatory effect on the enzyme production. Others who studied this problem did not find any stimulation of L-asparaginase synthesis by L-asparagine[9,11].

Results presented in this paper demonstrate that the EC-2 L-asparaginase of *E. coli* is an inducible enzyme. Its formation is induced if oxygen is depleted from the medium or if lactate is present. Under anaerobic conditions or in the presence of lactate L-asparagine is an inducer of enzyme synthesis.

Part of these results come from the diploma work of Svobodová[14].

MATERIALS AND METHODS

Bacterial strains and cultivation

E. coli ATCC 9637, B and ML 30 were used. The cells were grown in minimal medium M 56[19] with 0.4% glycerol as the sole source of carbon. Aerobic cultivations were performed on a laboratory reciprocal shaker at 37 °C. The aeration of the culture was regulated by the number of strokes: vigorous aeration, 120 strokes/min; moderate aeration, 84 strokes/min. Sodium lactate and L-asparagine were added before the inoculation of the culture both in a concentration of 80 μmoles/ml unless otherwise indicated.

3 ml of an overnight liquid culture were inoculated into 60 ml of medium in 250-ml erlenmeyer flasks. The growth of the culture was followed by turbidity measurements on the Klett–Summerson photocolorimeter with a red filter. 100 Klett units corresponded to 0.4 mg dry wt of *E. coli* cells/ml of the culture. At time intervals given samples were taken for absorbance measurements, enzyme estimation and in some cases oxygen dissolved in the medium was estimated in parallel flasks. Anaerobic cultivations were performed in 25-ml erlenmeyer flasks filled up almost

completely with minimal medium with glycerol supplemented with sodium fumarate at a concentration of 300 μmoles/ml (under anaerobic conditions sodium fumarate served as an acceptor of electrons). The medium was bubbled with nitrogen for 10 min and then inoculated with 4 ml of culture growing in a stationary culture for two days at 37 °C. Immediately after the flasks were closed with rubber stoppers and placed into a thermostat at 37 °C. For each estimation a new flask was used.

L-Asparaginase estimation

5-ml samples of culture were mixed with chloramphenicol (final concn 100 μg/ml) and centrifuged at 0 °C in a Janetzki KG centrifuge. After washing with 0.1 M NaCl the cells were resuspended in 1 ml 0.1 M NaCl. The suspension was mixed with 1 drop toluene and shaken in closed test tubes for 5 min in a water-bath shaker at 37 °C. EC-1 and EC-2 isoenzymes were estimated in a toluenized suspension by the method of Campbell *et al.*[4] with some modifications: (a) The samples were deproteinized, by the method of Archibald[20], after incubation with L-asparagine. (b) The samples were mixed with oxidizing reagent[21] before the addition of Nessler solution[22].

Oxygen dissolved in the medium

This was measured by Clark's method[23] with a polarographic electrode. Partial pressure of oxygen was expressed as a percentage of the value corresponding to the medium completely saturated with oxygen.

Chemicals

Chemicals used were of analytical grade.

RESULTS

L-Asparaginase synthesis under different conditions of aeration

Fig. 1 (left part) shows that under conditions of moderate aeration the concentration of oxygen dissolved in the medium fell to zero after about 1 h of cultivation. The synthesis of L-asparaginase EC-2 in the cells started 30 min after the oxygen had been used up (see also Fig. 3). If the culture was vigorously aerated (Fig. 1, right part) the concentration of oxygen did not become zero during the whole course of cultivation and in consequence no enzyme was synthesized. It follows further from Fig. 1 that the synthesis of L-asparaginase EC-1 was independent of oxygen concentration and its course did not differ under conditions of low and high aeration, respectively.

The effect of sodium lactate on L-asparaginase synthesis

Sodium lactate added into the medium stimulated the synthesis of L-asparaginase EC-2 both under conditions of low and high aeration while EC-1 isoenzyme synthesis was not markedly influenced (Fig. 1). However, for deeper analysis see the last paragraph of the Results. It follows from Figs 1 and 4 that in the presence of lactate L-asparaginase EC-2 formation was stimulated even if oxygen could be detected in the growth medium. In such a case, however, the amount of enzyme synthesized was considerably lower than under anaerobic conditions.

The effect of lactate increased with its increasing concentration up to the

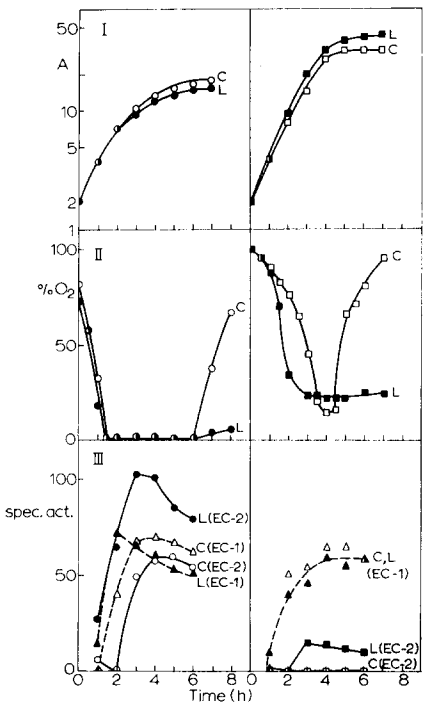

Fig. 1. L-Asparaginase synthesis in conditions of moderate and vigorous aeration. Cells of *E. coli* ATCC 9637 were cultivated under conditions of moderate (left part) and vigorous (right part) aeration. Part I, growth curves; Part II, percentage of oxygen dissolved in the medium; Part III, specific activity ($\times 10^3$) of L-asparaginases EC-1 and EC-2. L, sodium lactate; C, control (no addition into the medium).

amount of 100 μmoles/ml of medium; at higher concentrations its effects became considerably lower (Fig. 2).

A more comprehensive experiment shown in Fig. 3 demonstrated again (see Fig. 1), that L-asparaginase EC-2 synthesis started 30 min after the depletion of oxygen from the medium. Addition of lactate immediately after oxygen has been used up shortened this time interval by about 10 min. If lactate was added 30 min after dissolved oxygen reached zero, enzyme production started immediately but at a higher rate than in the control cells.

The main metabolic pathway of lactate utilization leads *via* pyruvate and the citric acid cycle. For this reason we tested the effect of some citric acid cycle intermediates on L-asparaginase EC-2 synthesis. We found that none of the compounds tested (pyruvate, oxaloacetate, fumarate) showed any stimulatory effect comparable with that of lactate (results not given).

Effect of L-*asparagine on* L-*asparaginase synthesis*

It follows from Fig. 4 that under conditions of low aeration and anaerobiosis in the presence of L-asparagine the cells synthesized approximately a double amount of L-asparaginase EC-2 than the control ones without L-asparagine. If the culture was vigorously aerated we found a very low, nevertheless reproducible, stimulatory effect of L-asparagine.

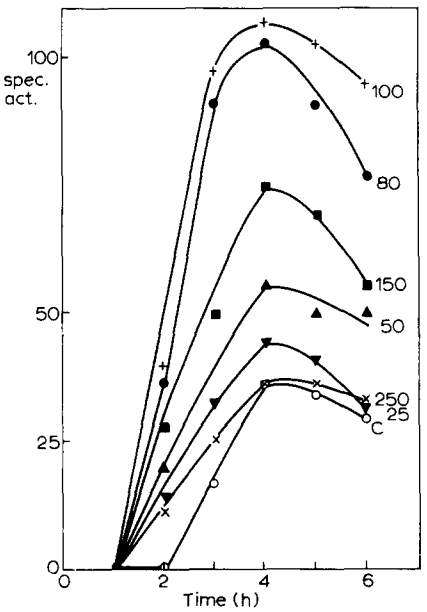

Fig. 2. Effect of different concentrations of lactate on L-asparaginase EC-2 synthesis. *E. coli* ATCC 9637 cells were cultivated under conditions of moderate aeration. Sodium lactate in concentrations (μmoles/ml) indicated in the graph was added into the medium before the inoculation of the culture. C, control without lactate. Spec. act. values $\times 10^3$.

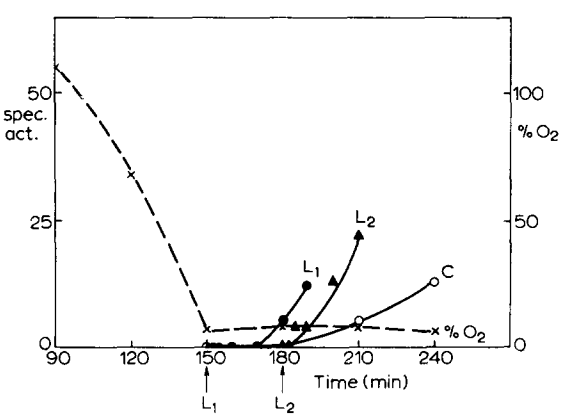

Fig. 3. Effect of lactate added after depletion of oxygen on L-asparaginase EC-2 synthesis. *E. coli* ATCC 9637 cells were cultivated under conditions of moderate aeration. γ-Asparaginase EC-2 activity was estimated in the course of the experiment in the control culture (C). Immediately after depletion of oxygen from the medium, lactate in a concentration of 80 μmoles/ml was added into an aliquot of the control culture as indicated by the arrow (L_1). From this aliquot in very short time intervals samples were taken in which the activity of L-asparaginase EC-2 was estimated. 30 min after the depletion of oxygen into another aliquot of the control culture lactate was added (L_2) in a concentration of 80 μmoles/ml. From this part of the culture as well samples were taken in very short time intervals for enzyme estimation. Spec. act. values $\times 10^3$.

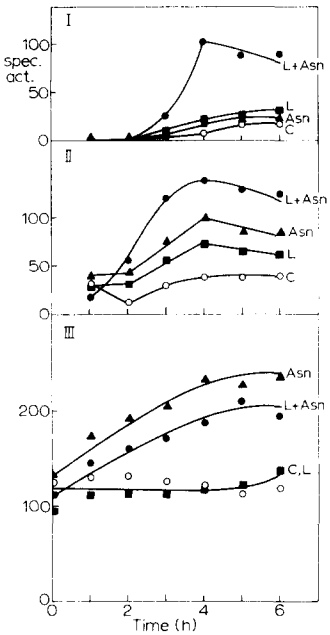

Fig. 4. Induction of L-asparaginase EC-2 synthesis under different conditions of aeration. Cells of *E. coli* ATCC 9637 were cultivated under conditions of vigorous (I) and moderate aeration (II) and under anaerobic conditions (III). When cells were cultivated anaerobically for each enzyme estimation a new flask was used up. C, control; L, lactate; Asn, L-asparagine. Spec. act. values $\times 10^3$.

In the aerated cultures the effect of L-asparagine was enhanced by a simultaneous addition of sodium lactate into the medium. In vigorously aerated cultures we observed a 10-fold stimulation (compared with control cells without lactate and L-asparagine) of L-asparaginase EC-2 synthesis if L-asparagine and lactate were added into the growth medium.

Fig. 5 shows that chloramphenicol inhibited the lactate and/or L-asparagine-

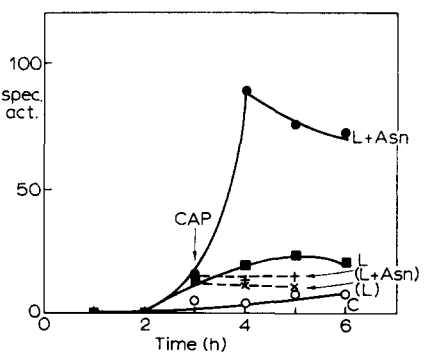

Fig. 5. Effect of chloramphenicol on L-asparaginase EC-2 synthesis. ATCC 9637 cells were grown under conditions of vigorous aration. 3 h after inoculation protein synthesis was stopped by thes addition of chloramphenicole (100 μg/ml), as indicated by the arrow into aliquots of the culture tested. Cultures with chloramphenicol were further incubated under the same conditions. C, control; L, lactate; Asn, L-asparagine, CAP, chloramphenicol. Spec. act. values $\times 10^3$.

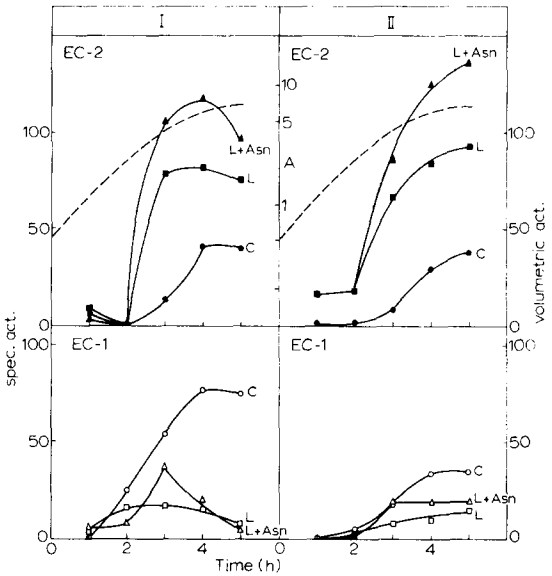

Fig. 6. Dependence of EC-1 L-asparaginase activity on L-asparaginase EC-2 synthesis. L-Asparaginase EC-1 and EC-2 activity was followed in *E. coli* ATCC 9637 control cells and in cells cultivated respectively with sodium lactate and sodium lactate with L-asparagine. The cultivation was performed under conditions of moderate aeration. The results were expressed both in terms of specific activity (I) and volumetric activity (II). – – – –, growth curve; C, control; L, lactate; Asn, L-asparagine. Both ordinate values $\times 10^3$.

stimulated L-asparaginase EC-2 synthesis. We can thus conclude that both compounds evoked a *de novo* synthesis of the enzyme.

We observed again a decrease of EC-1 activity if the synthesis of L-asparaginase EC-2 was stimulated. These results are given in Fig. 6.

Effect of amino acids

The effect of some amino acids on L-asparaginase EC-2 synthesis was tested under conditions in which the effect of L-asparagine was most pronounced, *i.e.* when the culture was vigorously aerated and lactate was present in the medium. Table I

TABLE I

EFFECT OF SOME AMINO ACIDS ON L-ASPARAGINASE EC-2 SYNTHESIS

The cells of ATCC 9637 were cultivated under conditions of vigorous aeration. The control culture contained sodium lactate. The cultures tested contained sodium lactate and the given amino acid (80 μmoles/ml). Both lactate and the amino acid were added into the medium before inoculation of the culture. pH was adjusted to 7.0 in all these cases.

Amino acid tested	*Spec. act. ($\times 10^3$)*
Control	68.0
L-Leucine	15.2
L-Glutamine	79.5
L-Arginine	79.0
Glycine	86.5
L-Alanine	0
L-Aspartic acid	37.2
L-Asparagine	164.0

shows that only L-asparagine stimulated L-asparaginase EC-2 synthesis from all the amino acids tested. L-leucine, L-alanine and L-aspartic acid inhibited the synthesis of this enzyme.

Testing of other strains

The previous experiments were all performed with *E. coli* ATCC 9637. As it follows from Table II lactate and/or L-asparagine stimulated L-asparaginase EC-2 synthesis also in other *E. coli* strains, namely ML 30 and B.

TABLE II

EFFECT OF SODIUM LACTATE AND L-ASPARAGINE ON L-ASPARAGINASE EC-2 SYNTHESIS IN SOME *E. coli* STRAINS

The cultures were grown under conditions of vigorous aeration. The table contains the maximum values of enzyme activity reached in the course of a 5-h cultivation.

Additions to medium	*Spec. act. ($\times 10^3$)*		
	ATCC 9637	*ML 30*	*B*
None	7	0	1
Lactate	23	19	14
L-Asparagine	18	3	1
Lactate + L-asparagine	103	27	24

Interdependence of L-asparaginases EC-1 and EC-2 synthesis

As it can be seen from Fig. 6 (Part I) simultaneously with an increase of L-asparaginase EC-2 synthesis a decrease of EC-1 activity was observed (see also Fig. 1). This decrease could not be observed in the control culture and on the other hand it was a maximum where the stimulation of EC-2 synthesis had been most pronounced, *i.e.* where lactate and L-asparagine were added simultaneously into the medium.

When the enzymatic activities were plotted as units produced per ml of culture (Part II) it became clear that the constitutive synthesis of L-asparaginase EC-1 proceeds in the control cells regularly during the whole course of the logarithmic phase. However, if the synthesis of L-asparaginase EC-2 was strongly induced (lactate with L-asparagine present in the medium), the synthesis of L-asparaginase EC-1 stopped even during the logarithmic phase of growth.

DISCUSSION

Cultivation conditions which would allow optimum synthesis of L-asparaginase in *E. coli* cells were studied by a number of investigators; nevertheless, their conclusions, which were not based on a knowledge of regulation mechanisms, were mostly empirical. The aim of our experiments was to find the essential parameters which would influence L-asparaginase formation.

We have found, in accordance with Broome[6], that L-asparaginase EC-1 synthesis was not markedly influenced by experimental conditions and we share his opinion that it is synthesized constitutively in *E. coli*. Our results confirmed the finding of Hernádi *et al.*[5] that the formation of L-asparaginase EC-1 coincides with the loga-

rithmic phase of growth of the culture. We assume that there could exist some correlation between the synthesis of EC-1 and EC-2 isoenzymes. In most cases, shown in this paper, both enzymes are synthesized simultaneously. However, abnormal stimulation of EC-2 synthesis leads to an earlier cessation of EC-1 formation. There could be several reasons for this effect, *e.g.* competition for precursors or for sites of synthesis *etc.*, but we have not enough evidence to be able to discuss this effect more deeply.

The mechanism of EC-2 isoenzyme synthesis is in the focus of interest of many authors because of its antineoplastic activity. Several papers reported recently that the presence of oxygen in the medium represses L-asparaginase EC-2 synthesis[9,13,14,24], however, most authors did not define precisely the degree of aeration. We showed that the onset of enzyme synthesis takes place about 30 min after the depletion of oxygen by the growing culture. According to Fowler[25] and Rosolová and Kaprálek[26] this is the time which the culture needs for establishing the anaerobic metabolism, if there is a shift from aerobic into anaerobic conditions.

It has been reported that oxygen represses the formation of some enzymes in microorganisms, *e.g.* tetrathionate reductase in *Citrobacter*[27] and some enzymes taking part in the citric acid cycle in *E. coli*[28,29], nitrate reductase in *Bacillus stearothermophilus*[30] and others. This effect is partly understandable in enzymes taking part in redox metabolism, however, it is difficult to interprete the inhibitory effect of oxygen on L-asparaginase EC-2 synthesis. We could imagine that L-asparaginase could take part in some redox system, namely in the fumarate–succinate system where it could play a role as a precursor of fumarate. This possibility was tested by an unpublished experiment in which we tried to replace fumarate by L-asparagine in the medium for anaerobic cultivation. In this case L-asparagine was not able to replace fumarate, the culture did not grow and thus the assumption that L-asparagine could be a precursor of fumarate could not be verified.

In the presence of lactate the culture synthesized some amount of L-asparaginase EC-2 even under conditions of vigorous aeration. In the presence of lactate the moderately aerated culture needed less time after the depletion of oxygen to start *de novo* L-asparaginase EC-2 synthesis and the amount of enzyme synthesized was higher. This could mean that the presence of lactate in the medium brought about conditions similar to anaerobic ones. This idea was suggested to us also by the fact that under anaerobic conditions lactate had no effect on L-asparaginase EC-2 synthesis. However, we could not find in the literature any evidence supporting this speculation.

None of the citric acid cycle intermediates tested could replace lactate in the medium, but it is not excluded that these compounds are not transported effectively into the cells.

In our experiments L-asparaginase EC-2 synthesis was induced specifically from all amino acids tested only by its substrate L-asparagine, however, for this effect the absence of oxygen or the presence of lactate are a prerequisite. This effect can be found also in some other systems: tetrathionate reductase of *Citrobacter*[27] and nitrate reductase of *B. stearothermophilus*[30] which are induced by their respective substrates in the absence of oxygen. However, these enzymes cannot be synthesized in the absence of externally added substrate as it is in the case of L-asparaginase EC-2.

We assume that we have observed at least two different ways of stimulation of L-asparaginase synthesis which differ by their mechanisms. The first includes the

effect of oxygen depletion and presence of lactate. The second might be a true induction of enzyme synthesis by its substrate, L-asparagine.

In two other *E. coli* strains (ML 30 and B), L-asparaginase EC-2 was inducible under the conditions described in this paper although it is possible that other strains could react differently[13].

We conclude that, at least in some strains of *E. coli*, L-asparaginase, EC-2 is an inducible enzyme. Its synthesis is regulated by the amount of oxygen dissolved in the growth medium and by some substances as sodium lactate and/or by the substrate of the enzyme L-asparagine. The EC-1 isoenzyme is synthesized constitutively.

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