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Metabolic Regulation by Homoserine in *Escherichia coli* B/r

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A mathematical analysis of branched pathway regulation has led to the prediction of a novel homoserine control in *Escherichia coli* B. Experimental support for such control is presented in this paper. Homoserine, the precursor of both threonine and methionine, inhibits nicotinamide adenine dinucleotide phosphate ($NADP^+$)-specific glutamate dehydrogenase (EC 1.4.1.4), the enzyme catalyzing the first reaction in ammonia assimilation. Physiological and biochemical evidence for this effect are offered. Homoserine depresses the growth rate of the organism, and glutamate, the product of the inhibited reaction, reverses this effect. The $NADP^+$-specific glutamate dehydrogenase activity in cell-free extracts is inhibited by homoserine, and this inhibition parallels the restriction of growth rate. These effects are found in other enteric bacteria which share a similar overall pattern of control for the amino acids derived from aspartate. On the other hand, a sampling of more distantly related species which have different pathways and/or regulatory patterns provides no evidence for homoserine inhibition of the glutamate dehydrogenase reaction.

The quantitative relationships among the component parts of a complex biochemical system must be delicately coordinated in order for such a system to function normally. As an example, let us consider a branched biosynthetic pathway regulated by the "nested pattern" of feedback inhibition. In this pattern of feedback inhibition, each of the end products inhibits the first reaction in the branch specific to its own synthesis as well as the first reaction in the common sequence. Thus, each end product regulates both the synthesis and the degradation of the branch-point metabolite. From mathematical analysis one can predict that these two regulations by a given end product must be balanced in a specific way for the proper functioning of the system (24). This prediction provides the following explanation for the formation of multienzyme complexes among the regulatory enzymes of many branched pathways: these complexes are formed to maintain the two inhibitions by a common end product at their proper relative levels. Dysfunctional behavior is predicted when this control pattern breaks down.

One type of dysfunctional behavior occurs when an end product in a branched pathway inhibits the synthesis of the branch-point metabolite much more strongly than the degradation of that metabolite. The addition of such an end product to the cellular environment causes the endogenous level of the other end product to decrease, thus starving the organism for the second end product and resulting in a reduced growth rate. The predictions and experimental evidence for this type of disorder have been discussed elsewhere (24).

Another type of dysfunctional response is predicted when an end product inhibits the degradation of the branch-point metabolite much more strongly than the synthesis of that metabolite, which is the converse of the first situation described. This condition leads to an unstable system that might be manifested by a continual increase in the level of the branch-point metabolite. In contrast to the first type of disorder, for which there is abundant experimental evidence, we know of no examples of this second type.

Escherichia coli B, however, presents a paradoxical case. In this organism, homoserine is the branch-point metabolite leading to the synthesis of both threonine and methionine. Methionine strongly inhibits the first step in its own biosynthesis from homoserine but has no inhibitory effect on the synthesis of homoserine (19). Although this fits the pattern for the second type of dysfunction, there is no evidence for

homoserine secretion in cross-feeding experiments or other evidence of dysfunction.

Of course, repression or derepression of enzyme synthesis could be involved in long term compensation. Nevertheless, in view of the rapidly varying conditions which the cell often experiences, an inefficient control at the metabolic level would seem to have negative selective value.

To resolve this apparent paradox, we have postulated that the homoserine level in this organism is subject to control by a hitherto unrecognized mechanism and in this paper present evidence from studies with *E. coli* B which tends to support this prediction. Homoserine is found to inhibit the nicotinamide adenine dinucleotide phosphate ($NADP^+$)-specific glutamate dehydrogenase (EC 1.4.1.4), an enzyme responsible for primary amination in this organism (12) and, hence, a first step in the flow of nitrogen to the amino acids derived from aspartate.

MATERIALS AND METHODS

Organisms. The principal organism used in this study was *E. coli* B/r. *E. coli* B184, obtained from G. N. Cohen, is a mutant of *E. coli* B that lacks homoserine dehydrogenase (EC 1.1.1.3) activity and, therefore, is unable to synthesize homoserine from aspartate semialdehyde. This mutant was used previously in our laboratory (26) to demonstrate control by repression in its threonine synthetase system (homoserine kinase, [EC 2.7.1.39] and threonine synthase [EC 4.2.99.2]). This strain, however, was observed to undergo spontaneous, high-frequency transitions in colony morphology (rough ⇆ smooth), similar in appearance to those described in other organisms (2, 29). We were concerned that this heterogeneity in colony morphology might reflect a corresponding heterogeneity in membrane and/or cell wall properties and possibly in transport of the required nutrient homoserine. If this were true, the utility of the strain for physiological studies at limiting homoserine concentrations would be severely compromised by the introduction of a gross population effect. For this reason, the homoserine dehydrogenase lesion in this organism was transferred to the more morphologically homogeneous B/r strain by low-multiplicity transduction with P1 phage (9). The desired auxotroph was enriched in minimal medium plus homoserine and subjected to penicillin selection in minimal medium according to the method of Davis (7). The resulting mutant strain, designated H36, is presumably isogenic with the wild-type B/r in all other respects.

Other organisms studied to a limited extent were *E. coli* HfrH and *Salmonella typhimurium* LT2 obtained from S. Cooper, *Rhodopseudomonas spheroides* 02 (a derivative of van Neil's 2.4.1 strain) and *Rhodospirillum rubrum* 06 (ATCC 25903) obtained from D. I. Friedman, *Pseudomonas fluorescens* PF014 and *Bacillus subtilis* L obtained from R. H. Olsen, and *B. licheniformis* (a derivative of Halvorson's *B. terminalis* strain) obtained from E. Juni.

Media. Three types of media were used: nutrient broth (Difco), a minimal medium (8), and a complex synthetic medium (18). The minimal and complex synthetic media were supplemented with amino acids as indicated. In addition, minimal medium was routinely supplemented with isoleucine (1 mM) and methionine (1 mM), although omission of these does not significantly affect the results reported in this paper. All amino acids were of the L isomer, except in the case of homoserine, where both the L isomer and the DL racemic mixture were used. In the latter case, homoserine concentrations are given in terms of the L isomer. The D isomer of homoserine had no effect on growth rate or the activity of glutamate dehydrogenase.

Growth conditions and measurements. Cells were grown overnight in a glucose-limiting minimal medium at 37 C, except for *R. spheroides*, *R. rubrum*, and *P. fluorescens*, which were grown in nutrient broth in the dark at room temperature. The overnight cultures, except for *P. fluorescens*, were centrifuged in the cold (0 to 4 C) at 12,000 × *g* for 10 min and resuspended in a small volume of minimal medium without glucose. A small portion of this suspension (usually 0.1 ml) then was used to inoculate 10 ml of fresh medium of the desired composition. In the case of *P. fluorescens*, a 1-ml portion of the overnight culture was diluted in 10 ml of minimal medium.

Cultures were grown in sterile, capped, standard test tubes (18 by 150 mm) held at an angle and mechanically shaken in a water bath (New Brunswick model G76) at 37 C. (*R. spheroides* and *R. rubrum* were incubated at 30 C, and *P. fluorescens* at 30 C in the dark.) Variations over a twofold range in the frequency of oscillation or the volume of culture had no effect on the growth rate of *E. coli* B/r in minimal medium, and from this we conclude that growth rate is not restricted by surface-limited diffusion of oxygen under these conditions.

Growth was monitored turbidimetrically at 525 nm in a Fisher Electrophotometer (model II). Optical density (OD) readings were taken by removing the culture tube from the water bath, wiping it dry, and placing it directly in an electrophotometer. This procedure took less than 30 s and was done at intervals of 10 min or more. Identical growth rates in minimal medium were obtained from triplicate cultures of *E. coli* B monitored at 5-, 10-, and 20-min intervals, indicating that this method of measurement introduced negligible error.

Preparation of extracts. An 80-ml amount of a mid-log culture (0.1 OD or 10^8 cells/ml) was harvested by centrifugation in the cold (0 to 4 C) at 12,000 × *g* for 10 min. The pellet was resuspended in 10 ml of double-distilled water and subjected to sonic disruption (Heat Systems-Ultrasonics Inc., model W140) at 0 C for three 1-min intervals, each followed by a 1-min cooling period. "Cell-free" extracts were obtained by centrifugation of the sonically treated suspension in the cold at 12,000 × *g* for 10 min to remove whole cells and debris. The extracts were kept on ice during use and stored at 4 C thereafter.

The protein content of these extracts was measured by the colorimetric method of Lowry et al. (16), with bovine serum albumin as standard.

Enzyme assays. Glutamate dehydrogenase activity was assayed either by the oxidative deamination of glutamate as measured by an increase in OD at 340 nm caused by the reduction of $NADP^+$, according to the method of Halpern and Umbarger (12) or by the reductive amination of α-ketoglutarate as measured by the decrease in OD at 340 nm due to the formation of $NADP^+$, according to a modification of the method of von Tigerstrom and Campbell (31). In the latter case, the assay system was composed of the following, in a total volume of 3 ml: reduced NADP (NADPH), 0.22 μmol; sodium α-ketoglutarate, 29 μmol; NH_4Cl, 115 μmol; potassium phosphate buffer. pH 8, 120 μmol; and 0.05 ml of extract containing approximately 50 to 100 μg of protein.

The initial rate of the reaction was calculated from absorbance readings at 340 nm taken at 60-s intervals in a Zeiss spectrophotometer (model PMQ II) for a period of 6 to 8 min (points were conveniently linear in this range) at room temperature. The instrument was zeroed on a water blank at the start of each interval, and a control cuvette, lacking glutamate or ammonia but otherwise identical in composition to the experimental cuvette, was run in all cases.

Aspartate-glutamate transaminase (EC 2.6.1.1) activity in crude extracts was determined by the spectrophotometric measurement of oxalacetate in a Zeiss spectrophotometer according to the procedure of Jenkins, Yphantis, and Sizer (13).

All enzyme assays were done in duplicate.

RESULTS

The theoretical prediction that the branch-point metabolite homoserine should be subject to control in *E. coli* B prompted a search for some physiological manifestation of this putative regulatory mechanism. The first successful clue was provided by an earlier study involving the threonine synthetase system in *E. coli* B 184 (26). The growth rate of this homoserine auxotroph increases with increasing homoserine concentration in the medium (Fig. 1). Although a slight reduction in growth rate sometimes was observed at high concentrations, this effect was dismissed as nonspecific at the time because of the high concentrations involved and the weakness of the inhibition observed. This effect subsequently was reexamined in H36 mutant of *E. coli* B/r.

Physiological behavior: restriction of growth rate by homoserine. Figure 2 shows the growth rate of *E. coli* H36 as a function of homoserine concentration. Addition of excess homoserine (final concentration, 13 mM) to a culture of *E. coli* H36, growing in minimal medium with an optimal homoserine concentration, resulted in an immediate reduction of the growth rate. This abrupt change in behavior

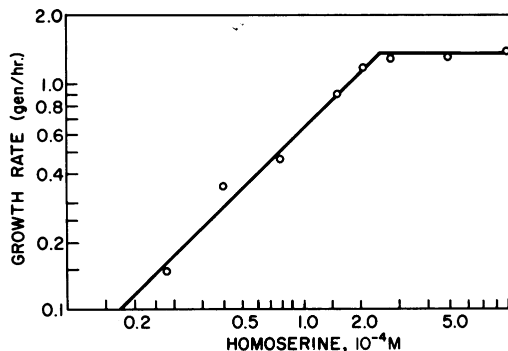

FIG. 1. *Exponential growth rate of the homoserine auxotroph E. coli B184 as a function of the added homoserine concentration. Cells were grown in minimal medium with the indicated concentrations of* L*-homoserine, as described in Materials and Methods.*

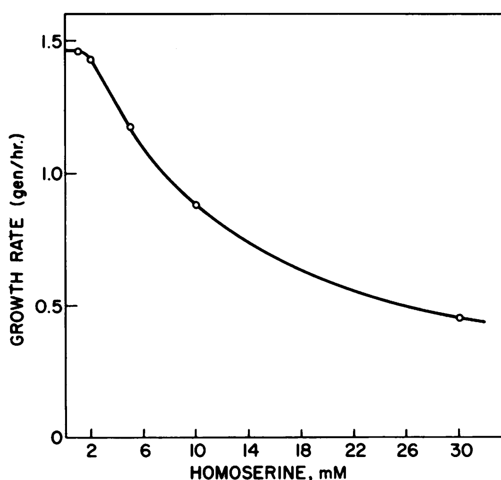

FIG. 2. *Restriction of growth rate in E. coli H36 by elevated concentrations of homoserine. Cells were grown in minimal medium with the indicated concentrations of* L*-homoserine, as described in Materials and Methods.*

suggests an inhibition of enzymatic activity as the primary effect rather than a repression of enzyme synthesis. Furthermore, growth at the reduced rate was followed for more than four generations in the high homoserine medium and no change in rate was observed. Derepression as a secondary adaptation to the initial homoserine effect seems unlikely in view of the persistent reduction in growth rate.

A logical point for homoserine to act is the aspartokinase (EC 2.7.2.4) reaction, the step usually thought of as the first important reaction in the flow of carbon to the amino acids related to aspartate (Fig. 3). There is evidence

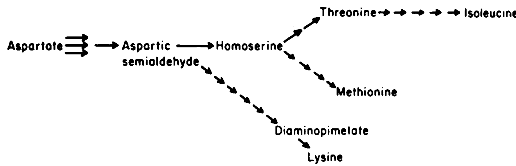

FIG. 3. *Biosynthetic pathways for the aspartate family of amino acids in E. coli B. Only the end products and key intermediates are shown. Parallel arrows indicate isozymes catalyzing the same reaction, and consecutive arrows denote the successive reactions in a pathway.*

for such inhibition in *E. coli* K-12 (20). This resembles product inhibition since aspartokinase and homoserine dehydrogenase activities in this organism are found within a single isozyme (20). In *E. coli* B, however, since the pattern of control is different (19), it is possible that there may be no homoserine inhibition of the aspartokinase reaction. In any event, according to this mechanism, the restriction in growth rate must be due to a starvation of the organism for one or more of the aspartate-related end products, excluding those subsequently derived from homoserine. However, the addition of lysine and diaminopimelate to the medium in millimolar concentrations did not relieve the depressed growth rate of H36 caused by homoserine.

Neither was the depression of growth rate eliminated by any of the end products derived from homoserine, i.e., threonine, methionine, or isoleucine. Thus, the homoserine effect is not simply a substrate inhibition of homoserine transport or of any subsequent reaction that would deprive the organism of these metabolites.

From the foregoing results, we concluded that the point where homoserine must act is somewhere outside the aspartate family of biosynthetic pathways. Hence, a more extensive search for this location was begun. The restriction of growth rate by homoserine was removed when cells were grown in a nutrient broth medium. The effect also was reversed when the minimal medium with a high (15 mM) concentration of homoserine was supplemented with Casamino Acids (2 mg/ml). The components of this medium were systematically examined to find the amino acid (or acids) responsible for the reversal of the homoserine effect.

Physiological behavior: relief of homoserine-restricted growth by amino acids. Since homoserine occurs in a rather complex set of branched metabolic pathways, one must entertain the possibility that any regulatory enzyme affected by it might also involve the *concerted* action of several other metabolites. Since it is impractical to examine all combinations, and to ensure that relief by such a combination of amino acids would not be missed, we eliminated them sequentially from the medium. After a preliminary selection of eight amino acids that produced complete relief of the depression in growth rate by homoserine, we further reduced this number as follows. All combinations of the eight amino acids taken seven at a time were tested for their ability to relieve the restriction of growth rate. The combination that produced the maximal relief was selected for examination in a similar fashion; i.e., all combinations of the seven amino acids were then tested six at a time and so on. Figure 4 shows a typical test and illustrates the calculation of "percent-relief."

The maximal relief can be plotted as a function of the number of amino acids remaining, and several types of relationships can be anticipated. Three possibilities are shown in Fig. 5. Case A would be expected if each of the amino acids contributed independently and equally to the total relief. Case B depicts the synergistic relief by a combination of three amino acids, none of which has any effect without the accompanying presence of the other two. The other five amino acids in this case do not contribute to the relief. In case C there are four amino acids that do not contribute to the relief and four that contribute equally well.

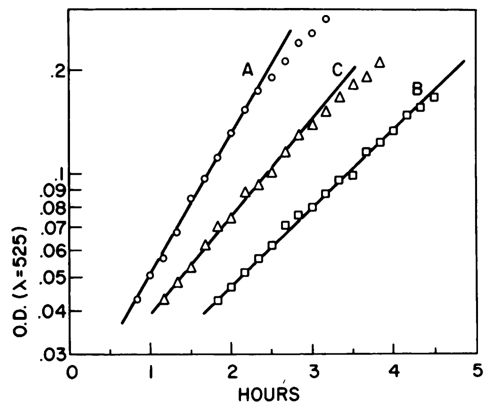

FIG. 4. *Test for relief of homoserine-restricted growth. E. coli H36 was grown in minimal medium supplemented with: (A) 0.5 mM homoserine to produce the optimal growth rate (○); (B) 15 mM homoserine to produce the restricted growth rate (□); (C) 15 mM homoserine plus the experimental combination of amino acids in millimolar concentrations (Δ). The percent relief is given by the following ratio: $100 (D_b - D_c)/(D_b - D_a)$ where D_a, D_b, and D_c are the doubling times for the three cases above. In this example, glutamate is the amino acid being tested for its ability to relieve the growth restriction.*

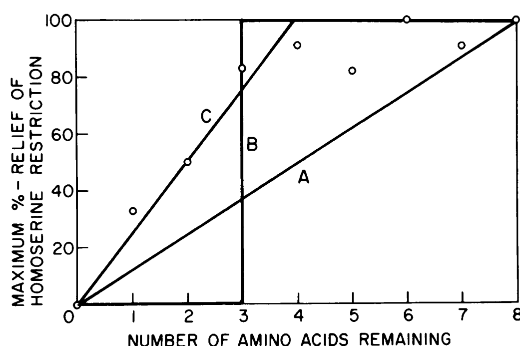

FIG. 5. *Sequential elimination of the amino acids contributing the least to relief of homoserine-restricted growth of E. coli H36. Three ideal cases are indicated: (A) equal and independent contributions by all amino acids: (B) synergistic relief by three amino acids and no contribution by the others, and (C) four amino acids contributing equally and the others providing no contribution. The experimental data plotted in this figure agree closely with case (c). See text for additional discussion.*

The actual data, which also are plotted in Fig. 5, agree most closely with the hypothetical case C. Table 1 shows the amino acids involved and their order of elimination. The last combination to produce essentially complete reversal of the homoserine restriction is glutamate, histidine, arginine, and glutamine. Having established that no strictly concerted action among the amino acids was involved, we examined all of the amino acids individually (Table 2). Several amino acids alone were able to produce partial relief of homoserine-restricted growth. Since glutamate is required either as substrate or amino donor for the synthesis of all other amino acids, it seems likely that homoserine is interfering with glutamate production, possibly the glutamate dehydrogenase reaction. The partial relief of homoserine-restricted growth by the addition of an amino acid could then be understood in terms of the glutamate sparing that would result from known patterns of feedback inhibition (27). The inability of glutamate itself to produce complete relief is probably due to poor uptake of glutamate. Support for this derives from the fact that the growth rate of *E. coli* B/r grown on glutamate as the sole source of nitrogen is three to four times less than that observed for these cells grown on ammonia (experimental results not shown).

Molecular mechanism: inhibition of NADP$^+$-specific glutamate dehydrogenase by homoserine. Since physiological evidence indicated glutamate dehydrogenase as a likely point of homoserine control, we measured the activity of this enzyme in crude extracts of *E. coli* B/r. As expected, this activity was inhibited by homoserine, a maximal inhibition of 30% being achieved with a 30 mM concentration (Fig. 6).

Glutamate is a key branch point in the nitrogen metabolism of *E. coli* (27). Because of

TABLE 1. *Relief of homoserine-restricted growth by amino acid additions*[a]

Amino acid additions[b]	Percent relief[c]
Glu, His, Arg, Gln, Gly, Hyp, Asp, Cys	100
Glu, His, Arg, Gln, Gly, Hyp, Asp	91
Glu, His, Arg, Gln, Gly, Hyp	100
Glu, His, Arg, Gln, Gly	82
Glu, His, Arg, Gln	91
Glu, His, Arg	83
Glu, His	50
Glu	33
—	0

[a] All combinations of the eight amino acids taken seven at a time were tested for their ability to relieve the restriction of growth rate. The combination that produced maximal relief was selected for examination in a similar fashion, i.e., all combinations of the remaining seven amino acids were then tested six at a time, and so on.

[b] Amino acids were added to minimal medium supplemented with homoserine (final concentration, 15 mM) as described in Materials and Methods. All amino acids had a final concentration of 1.0 mM.

[c] For the method of calculation, see Figure 4.

TABLE 2. *Relief of homoserine-restricted growth by single amino acid additions*

Amino acid addition[a]	Percent relief[b]	Amino acid addition[a]	Percent relief[b]
Gly	39	Ser	18
Gln	36	Cys-Cys	16
Glu	33	Thr	11
Tyr	32	Trp	10
Ala	31	Phe	9
Leu	30	Hyp	6
Val	28	Asn	—[c]
Asp	26	Lys	—[c]
Arg	19	Cys	—[c]
His	19		

[a] Amino acids were added to minimal medium supplemented with homoserine (final concentration, 15 mM) as described in Materials and Methods. All amino acids had a final concentration of 1.0 mM except Tyr (1.75 mM) and Cys-Cys (saturated solution).

[b] For the method of calculation, see Fig. 4.

[c] Produced further restriction of growth.

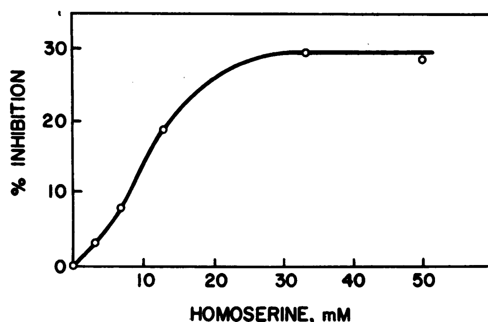

FIG. 6. *Inhibition by homoserine of glutamate dehydrogenase activity from E. coli B/r. Extracts were prepared and assays were performed in the reverse direction with the indicated homoserine additions, as described in Materials and Methods.*

its central position, we should expect sophisticated controls to influence its fate. The inhibition of glutamate dehydrogenase by homoserine represents part of a "nested pattern" of control (24). In a normally functioning system characterized by the nested pattern of control, homoserine also would be expected to inhibit the first reaction specific to its synthesis from glutamate, i.e., the aspartate-glutamate transaminase reaction. However, this control system does not respond to the addition of homoserine in a normal, functional manner; in fact, the growth rate of the organism is decreased. According to the mathematical analysis previously presented (24), the resulting dysfunctional behavior is exactly what is expected if the inhibition of glutamate utilization via aspartate transaminase is much weaker than the corresponding inhibition of glutamate dehydrogenase.

Molecular mechanism: effect of homoserine on aspartate-glutamate transaminase activity. The aspartate transaminase activity was examined in extracts of *E. coli* B/r. Homoserine has no inhibitory effect on this activity at concentrations up to 13 mM, in agreement with the predictions in the previous paragraph. The inhibition of the aspartate aminotransferase by hydroxylamine, an analogue of homoserine and a known inhibitor of transaminase activity, was examined as a control to demonstrate that we could detect inhibition by this type of compound in our assay system. Hydroxylamine was indeed a strong inhibitor at 0.1 mM concentrations in our assay. Homoserine's failure to inhibit aspartate aminotransferase activity is in agreement with the findings of Polyanovskii and Torchinskii (23). These authors examined the inhibition of pig heart transaminase activity by a series of eight cyclic derivatives of hydroxylamine. Of these, the lactone of homoserine (α-aminobutyrolactone) was the only one that did not produce any inhibition. They also studied the aliphatic analogues of several of these compounds, homoserine not included, and found them to be consistently more potent inhibitors.

Control pattern correlations. Homoserine inhibits glutamate dehydrogenase activity in *E. coli* B/r. How general is the occurrence of this mechanism? Is it unique to *E. coli* B/r, to all organisms which utilize this incomplete form of the nested pattern of control, or to the nested pattern itself; or will it be found wherever the pathways of the aspartate family exist, even in the absence of a nested pattern of control? In an attempt to answer this, we have made a cursory examination of the effect of homoserine on growth rate and glutamate dehydrogenase activity in several different species selected to represent a diversity of regulatory patterns.

Two additional enteric bacteria were examined: *E. coli* HfrH, a K-12 strain, and *S. typhimurium* LT2. Both of these strains exhibit the nested pattern of control involving isozymes of aspartokinase and homoserine dehydrogenase (3, 19) although they differ somewhat from *E. coli* B in the details of the overall pattern (19). The effect of homoserine on the growth behavior and glutamate dehydrogenase activity in these organisms was similar to that obtained with *E. coli* B/r.

In other distantly related organisms that we examined, the control patterns in the aspartate family of amino acids have been less thoroughly characterized, but at least portions of their overall patterns have been established. *R. rubrum* has a novel control mechanism involving the specific reversal by one end product of the inhibitory effect of another end product (5). The growth rate of this species and the activity of $NADP^+$-specific glutamate dehydrogenase in its crude extracts were unaffected by high (13 mM) concentrations of homoserine.

B. subtilis utilizes a mechanism involving the concerted action of threonine and lysine to inhibit the activity of a single aspartokinase in what appears to be a nested pattern (21). The steady-state growth rate of this organism is unaffected by high (15 mM) concentrations of homoserine, and no $NADP^+$-specific glutamate dehydrogenase activity could be detected in extracts of *B. subtilis*. This is in agreement with recent findings that the *Bacillus* group may utilize an entirely different route for primary amination and is not believed to possess an $NADP^+$-specific glutamate dehydrogenase (10).

P. fluorescens has a control mechanism very

similar to that of *B. subtilis*, as Cohen, Stanier, and LeBras (4) have shown in their comparative study of regulation in the aspartate family of amino acids. Crude extracts of *P. fluorescens* exhibited a low $NADP^+$-specific glutamate dehydrogenase activity that was not inhibited by homoserine. However, unlike *B. subtilis* and more like the remaining organisms to be discussed, the growth rate of *P. fluorescens* was depressed strongly by high (15 mM) concentrations of homoserine.

A mechanism of control resembling the sequential pattern is known to exist for the pathways of the aspartate-related amino acids in *R. spheroides* (6) and *B. licheniformis* (15, 28). We did not detect $NADP^+$-specific glutamate dehydrogenase activity with our methods in extracts from either of these species, although, using the techniques of Phibbs and Bernlohr (22), we have measured this activity in *B. licheniformis* and found little inhibition by homoserine. In both organisms, the rate of growth was sharply curtailed by the addition of 15 mM homoserine.

The commercially available glutamate dehydrogenase from bovine liver (Sigma Chemical Co.), with which so much work has been done (11), was also examined. The enzyme's activity was tested with NAD^+ and $NADP^+$ cofactors, in the forward (reductive amination of α-ketoglutarate) as well as reverse (oxidative deamination of glutamate) direction, in both phosphate and tris(hydroxymethyl)aminomethane buffers. No inhibition by homoserine was observed; this is not surprising in view of the fact that homoserine is not an intermediate normally found in higher animals.

From this brief survey (Table 3) it would appear that homoserine inhibition of glutamate dehydrogenase is associated in some way with the nested pattern of control involving aspartokinase isozymes. Of course, many cases would have to be examined in much greater detail to establish inductively such a generality.

TABLE 3. *Summary of homoserine effects on growth rate and glutamate dehydrogenase activity for different organisms*

Organism	Control pattern of the aspartate family	Restriction of growth rate[a] (%)	Inhibition of glutamate dehydrogenase activity[b] (%)
E. coli B	Nested, isozyme	49	24
E. coli K-12[c]	Nested, isozyme	81	13
S. typhimurium	Nested, isozyme	49	23
R. rubrum	Specific reversal	0	0
B. subtilis	Nested, concerted	0	ND[d]
P. fluorescens	Nested, concerted	100	0
B. licheniformis	Sequential	100	5
R. spheroides	Sequential	100	ND[d]
Bovine liver	NA[d]	NA[d]	0

[a] Cells grown in minimal medium with and without homoserine (final concentration, 15 mM). See Materials and Methods for additional detail.

[b] Activity in cell-free extracts determined with and without homoserine (final concentration, 13 mM) as described in Materials and Methods.

[c] This organism was grown as described in Materials and Methods except for the addition of excess thiamine, a growth requirement for this organism.

[d] NA, not applicable; ND, no glutamate dehydrogenase activity detected.

DISCUSSION

The branched pathway for the synthesis of threonine and methionine from aspartate in *E. coli* B exhibits a "nested pattern" of inhibitory control (24). In this case, the pattern is incomplete because methionine does not inhibit homoserine dehydrogenase or aspartokinase, which would reduce the synthesis of the branchpoint metabolite homoserine (19). Although mathematical analysis indicates that this unbalanced control pattern can result in dysfunctional behavior, this organism appears to function normally. We have predicted an additional homoserine control to obviate this difficulty.

An incidental observation from a previous study in this laboratory led to the demonstration that homoserine in high concentrations decreases the growth rate of *E. coli* B. Subsequently, it was shown that homoserine inhibits glutamate dehydrogenase activity in cell-free extracts of *E. coli* B. Two questions arise from these observations: is this mechanism of glutamate dehydrogenase inhibition responsible for the physiological effect, and does it normally provide a regulatory function in vivo?

The following evidence supports an affirmative answer to the first question. Homoserine slows the growth of *E. coli* B, but the restriction is relieved by the addition of glutamate, the product of the inhibited reaction. The restriction also is relieved by a number of other amino acids that could spare glutamate by feedback-inhibiting their synthesis at an early stage. Growth cannot be inhibited completely nor can the corresponding activity of glutamate dehydrogenase. However, this answer is not conclusive in view of the following genetic evidence.

Homoserine-resistant mutants have been obtained by treatment with nitrosoguanidine (1). Most are mutants for homoserine transport (Templeton and Savageau, in press). One type of mutation, however, originally thought to be

within the structural gene for glutamate dehydrogenase (25), could not be transduced back into the wild-type parent and is now considered to be multiply mutant in functions other than glutamate dehydrogenase. This evidence indicates that either another site of homoserine action might be responsible for the growth-retarding effect or that a mutation at another site can compensate for the homoserine inhibition of glutamate dehydrogenase.

The second question is more difficult to answer. There have been other reports of metabolites that modify enzymes in distant or unrelated pathways (14, 17), but the relevance of these effects for the regulation of metabolic networks in vivo is difficult to assess. They may indeed be subtle phenomena involved in the intergration of the larger metabolic networks, as Jensen (14) has speculated. However, these cross-pathway phenomena usually occur only at relatively high concentrations or in mutant strains, which suggests the possibility of an artifact.

The fact that homoserine inhibition occurs at relatively high concentrations (about 10 mM for half-maximal inhibition) is also the prime argument against the in vivo role we have ascribed to this inhibition. However, it could well be that homoserine can normally achieve high concentrations in the cell. We know that it takes high concentrations of homoserine to support a normal growth rate for the homoserine auxotroph H36, and at higher concentrations the inhibition of growth rate parallels the in vitro inhibition of glutamate dehydrogenase activity, indicating that endogenous and exogenous concentrations of homoserine may be similar. Nevertheless, there are additional arguments in favor of a regulatory role for homoserine.

A rationale for homoserine inhibition does exist. According to this rationale, such a control would provide a necessary supplement to the pattern of regulation previously known to exist in the branched pathway synthesizing threonine and methionine. Figure 7 is a graphical representation of the relevant interactions. (This system represents the portion of the biosynthetic pathways for the aspartate family of amino acids that results when the organism is grown in a medium with excess lysine and isoleucine. The lysine and isoleucine pathways are eliminated from consideration in view of their repression and inhibition by these amino acids.) This system is a hybrid of the prototype patterns of feedback regulation previously discussed (24). The pattern is "nested" as far as threonine is concerned and "sequential" with regard to methionine, the two patterns being mixed and overlapping.

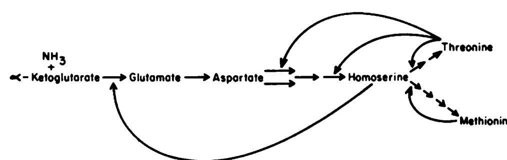

FIG. 7. *Mixed and overlapping patterns of control involving sequential and nested interactions. This is a simplified version of a branched pathway segment from the overall system in Fig. 3. These pathways may be thought of as those remaining when the others have been repressed and inhibited by growth in the presence of excess lysine and isoleucine.*

The analysis of this system leads to further implications that have been experimentally verified. For example, growth retardation upon the addition of homoserine implies an imbalance in the control of glutamate synthesis and degradation. According to the previous analysis (24), this behavior would result from an insufficient homoserine inhibition of the aspartate-glutamate transaminase activity. The transaminase activity in *E. coli* B was tested and found to be unaffected by high concentrations of homoserine. As another example, glutamate dehydrogenase is the first enzyme common to many pathways involved in nitrogen flow. As such, no single end product should be able to inhibit its activity completely. Otherwise, the organism would be starved for the other nitrogenous end products. Accordingly, homoserine is able to inhibit only about 30% of this key activity. A rise in homoserine concentration might be viewed as a signal that all of the end products of the aspartate family are available in excess. It is not an unreasonable estimate that the products of aspartate might account for 30% of the cell's nitrogen.

One also might expect that, if the homoserine control was an integral part of the overall pattern of regulation, it might not be found in other types of organisms that utilize a different pattern of regulation. The data in Table 3, although incomplete, do tend to support this expectation. The fact that glutamate dehydrogenase from several sources is not affected by homoserine shows that there is not simply a fortuitous relationship between homoserine and some structural feature of this enzyme necessary for its activity. The fact that the growth of several organisms is strongly inhibited by homoserine, even though the glutamate dehydrogenase reaction is either absent or unaffected by homoserine, suggests that homoserine might

have additional sites of action. However, other explanations involving secondary effectors (e.g., threonine) would also have to be considered.

Additional evidence in support of the regulatory role we have described is provided by the mathematical analysis of the model in Fig. 7. The analysis shows that the homoserine control can indeed stabilize an otherwise unstable system (in preparation).

Although repression control of glutamate dehydrogenase in *E. coli* has been well established (12, 32), we are aware of no previous report of control by inhibition. In fact, the enzyme from *E. coli* is reported to lack the interesting allosteric and polymerizing effects associated with the animal enzyme (11). Thus, homoserine inhibition of glutamate dehydrogenase appears to be the first instance of an allosteric regulation for this key enzyme in nitrogen metabolism. Since this reaction is the first in a set of branching pathways, other effectors undoubtedly will be found to modulate the activity of this enzyme. In an examination of other nitrogenous products for their allosteric effect on glutamate dehydrogenase, we observed a glutamine: α-ketoglutarate amidotransferase (oxidoreductase, $NADP^+$) activity in extracts of *E. coli* B/r. This novel activity was first described in *Aerobacter aerogenes* by Tempest, Meers, and Brown (30). In *E. coli* B/r, it appears to interact with glutamate dehydrogenase, unlike the case in *Aerobacter*. A description of these and other preliminary findings have recently been published (25). This amidotransferase activity in *E. coli* B/r is also partially inhibited by homoserine. However, preliminary experiments indicate that the other key enzyme of ammonia assimilation, glutamine synthetase, is not affected by homoserine.

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