JOURNAL OF BACTERIOLOGY, Dec. 1974, p. 1124-1132

Vol. 120, No. 3
Printed in U.S.A.

Mis-Regulation of 3-Deoxy-D-Arabino-Heptulosonate 7-Phosphate Synthetase Does Not Account for Growth Inhibition by Phenylalanine in *Agmenellum quadruplicatum*

ROY A. JENSEN, S. STENMARK-COX, AND LONNIE O. INGRAM

Department of Microbiology, Baylor College of Medicine, Houston, Texas 77025, and Department of Microbiology, University of Florida, Gainesville, Florida 32601

Received for publication 13 September 1974

The growth of the blue-green bacterium, *Agmenellum quadruplicatum*, is inhibited in the presence of L-phenylalanine. This species has a single, constitutively synthesized 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase. L-Phenylalanine inhibits DAHP synthetase non-competitively with respect to both substrate reactants. Other aromatic amino acids do not inhibit the activity of DAHP synthetase. A common expectation for branch-point enzymes such as DAHP synthetase is a balanced pattern of feedback control by all of the ultimate end products. It seemed likely that growth inhibition might equate with defective regulation within the branched aromatic pathway. Accordingly, the possibility was examined that mis-regulation of DAHP synthetase by L-phenylalanine in wild-type cells causes starvation for precursors of the other aromatic end products. However, the molecular basis for growth inhibition cannot be attributed to L-phenylalanine inhibition of DAHP synthetase for the following reasons: (i) DAHP synthetase enzymes from L-phenylalanine-resistant mutants are more, rather than less, sensitive to feedback inhibition by L-phenylalanine. (ii) Shikimate not only fails to antagonize inhibition, but is itself inhibitory. (iii) Neither the sensitivity nor the completeness of L-phenylalanine inhibition of the wild-type enzyme in vitro appears sufficient to account for the potent inhibition of growth in vivo by L-phenylalanine. The dominating effect of L-phenylalanine in the control of DAHP synthetase appears to reflect a mechanism that prevents rather than causes growth inhibition by L-phenylalanine. The alteration of the control of DAHP synthetase in mutants selected for resistance to growth inhibition by L-phenylalanine did indicate that the cause for this metabolite vulnerability can be localized within the aromatic amino acid pathway. Apparently, an aromatic intermediate (between shikimate and the end products) accumulates in the presence of L-phenylalanine, causing toxicity by some unknown mechanism. It is concluded that phenylpyruvate, potentially formed by transamination of L-phenylalanine, is an unlikely cause of growth inhibition. Although several significant questions remain unanswered, our results suggest that single-effector control of DAHP synthetase, the first regulatory enzyme activity of a branched pathway, may be more appropriate than it would seem a priori.

The inhibition of microbial growth by various organic metabolites is a common phenomenon, though diverse in its detail of expression for individual organisms. The frequency of occurrence of such toxicities in heterotrophic microorganisms is perhaps not fully appreciated (8). The susceptibility of autotrophic bacteria to such metabolite toxicities, on the other hand, is particularly well known (19, 23). We recently described the growth inhibition of *Agmenellum quadruplicatum*, a photoautotrophic species, by L-phenylalanine (8). The growth inhibition, once established in the presence of L-phenylalanine, was immediately reversed by L-tyrosine. Three alternatives were considered (8) that might account for the inhibitory effect of L-phenylalanine upon growth: (i) L-phenylalanine might prevent formation of precursors required for L-tyrosine biosynthesis through hyperinhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase. (ii) Accumulated phenylpyruvate, a keto acid produced by

transamination of L-phenylalanine, might somehow cause inhibition of L-tyrosine biosynthesis. (iii) Accumulated phenylpyruvate might inhibit one or more reactions of a seemingly unrelated biochemical pathway. In case (iii) reversal of inhibition of L-tyrosine could be explained in terms of its competition with L-phenylalanine for transport into the cell.

The data reported in this communication permit the elimination of what initially appears to be the most feasible explanation, i.e., possibility (i) above. The data indicate that alternatives (ii) and (iii) are also unlikely. These results may have general applicability to a better appreciation of the nature of the single-effector type of DAHP synthetase, known to be commonly distributed in nature (12, 14).

MATERIALS AND METHODS

Organisms and cultivation. The origin and cultivation of the following strains were described previously (9, 24): *A. quadruplicatum* strain BG1, *Anacystis nidulans* (Kratz-Myers strain), and *Coccochloris elabens* strain 17A. *Oscillatoria williamsii* strain Mev (filamentous, marine) and *Schizothrix calcicola* strain Man (filamentous, marine) were originally described by Van Baalen (26). Growth media (liquid and solid), methods of cultivation, and procedures for measurement of growth were given previously for strain BG1 of *A. quadruplicatum* (9); other species of blue-green bacteria were handled in the same manner unless otherwise specified.

Isolation of mutants in A. quadruplicatum. Approximately 10^8 cells taken from a liquid culture of *A. quadruplicatum* growing exponentially in mineral salts medium at 39 C were spread on solid ASP_2 mineral salts medium supplemented with 0.2 mM L-phenylalanine. A photograph illustrating the physical appearance of spontaneous mutants arising after 5 or more days of incubation was published previously (8). None of the resistant clones was ever observed to excrete metabolites that either reversed or accentuated the inhibition of surrounding cells. Similar results were obtained when 0.2 mM β-2-thienylalanine or 4-fluorophenylalanine were used in place of L-phenylalanine. Resistant mutants selected in the presence of L-phenylalanine, β-2-thienylalanine, or 4-fluorophenylalanine were cloned and purified under the same conditions used for the initial selection (8). The mutants, when first selected, exhibited apparent differences in colony size and/or differences in relative resistance to L-phenylalanine and analogues of L-phenylalanine. Over 100 mutants representing six tentative classes were purified, and the 27 mutants listed in Table 3 were chosen for enzymological analysis. The tentative classifications of mutants were discarded when it was found that no obvious correlations of particular enzymological changes could be made with variations of resistance phenotype.

Extract preparation. Cell pellets obtained by centrifugation of cultures in the exponential phase of growth were stored at −20 C (up to 6 months) prior to extract preparation. The whole cells were resuspended in 4 ml of 0.04 M potassium phosphate buffer, pH 6.8, at 4 C. Cell clumps were dispersed with a 30-s pulse of ultrasound energy at ice temperature using a Bronwill sonifier. Cells were treated with lysozyme (5 mg/ml) for 30 min at 37 C. The preparation was chilled over ice to 4 C and sonically treated for an additional minute in order to disrupt lysozyme-weakened cells and to decrease deoxyribonucleate viscosity. The volume was increased to 6 ml with buffer, and the preparation was centrifuged for 2 h at 35,000 rpm in a type 65 fixed-angle rotor of a Beckman preparative ultracentrifuge. About 4 ml of clear extract was decanted. The extract was dialyzed overnight at 4 C against 1,000 volumes of buffer.

Biochemicals. All L-amino acids, keto acids, β-2-thienylalanine, 4-fluorophenylalanine, the potassium salt of erythrose 4-phosphate, trisodium phosphoenolpyruvate, and Sephadex G-200 were obtained from Sigma Chemical Co. Lysozyme (3× crystallized) was obtained from Worthington Biochemical Corp. Molecular weight standards for Sephadex G-200 gel chromatography were from Mann Research Laboratories. Other chemicals were of analytical grade.

Analytical procedures. Protein concentrations were estimated by the method of Lowry et al. (21). The procedure for the assay of DAHP synthetase activity has been described previously (13). Unless indicated otherwise, both substrates were used at 2 mM concentrations, and the reaction time was 20 min at 37 C.

Prephenate/phenylpyruvate transaminase (24) was assayed for 20 min at 45 C utilizing L-leucine as the amino donor and phenylpyruvate as the keto acceptor. A decrease in absorbance of phenylpyruvate at 320 nm was measured. The reaction mixture contained 0.05 ml of 10 mM phenylpyruvate and 0.05 ml of 40 mM L-leucine (prepared in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 8.8). The addition of pyridoxal 5'-phosphate was unnecessary. Presumably this cofactor is firmly bound to the enzyme. The reaction was initiated by the addition of 0.1 ml of enzyme preparation and terminated by the addition of 3 ml of 2.67 N NaOH.

The assay for prephenate dehydratase (24) was carried out for 30 min at 37 C. The formation of the phenylpyruvate product was measured as increasing absorbance at 320 nm. The reaction mixture contained 0.10 ml of 10 mM potassium prephenate and 0.05 ml of 0.05 M tris(hydroxymethyl)aminomethane buffer at pH 8.8. The reaction was initiated by addition of 0.15 ml of enzyme preparation and terminated with 0.9 ml of 2.67 N NaOH.

The assay for chorismate mutase (24) was carried out for 20 min at 37 C. The prephenate product, converted to phenylpyruvate by acid treatment, was measured by reading absorbance at 320 nm. The reaction mixture contained 0.10 ml of 4 mM chorismate and 0.20 ml of 0.05 M tris(hydroxymethyl)-aminomethane buffer, pH 7.8. The reaction was initiated by addition of 0.10 ml of enzyme preparation and terminated with 0.10 ml of 20% trichloroacetic acid. Incubation was continued for 10 min at 37 C,

and the mixture was diluted fourfold in 2.67 N NaOH prior to spectrophotometric measurement.

RESULTS

Single-effector pattern of regulation in blue-green bacteria. Comparative enzymology (12, 14) has shown that the single-effector type of DAHP synthetase is of surprisingly frequent occurrence in microorganisms. (Occasionally, an apparent single-effector pattern may prove to involve multiple effectors [10].) The single-effector pattern may be common in the blue-green group of bacteria (Table 1; 27). The DAHP synthetase activities of *A. quadruplicatum*, *C. elabens*, and *O. williamsii* (Table 1) were inhibited by L-phenylalanine but not by L-tyrosine. On the other hand, the enzyme of *A. nidulans* was inhibited by L-tyrosine but only little, if any, by L-phenylalanine. The enzyme of *S. calcicola* was inhibited by either L-tyrosine or L-phenylalanine, a circumstance that could reflect either multiple-effector binding sites on a single protein or the presence of regulatory isoenzymes. A priori, the control of a common-pathway enzyme, such as DAHP synthetase, by a single end product of the branched pathway seemingly violates ordinary rules of regulation, creating the potentiality for growth inhibition. Hence, one obvious hypothesis to account for growth inhibition in *A. quadruplicatum* by L-phenylalanine (8) would be the apparent regulatory deficiency of a DAHP synthetase under the sole control of L-phenylalanine (Table 1).

L-Phenylalanine-sensitive DAHP synthetase of A. quadruplicatum. The enzymological characteristics of DAHP synthetase in *A. quadruplicatum* were studied further. Figure 1 shows the inhibition profile obtained for the enzyme; the inhibition curve exhibits uncomplicated kinetics (also see Fig. 3 and 4). A relatively high concentration of L-phenylalanine (0.2 mM) was required to obtain 50% inhibition of activity, and maximal inhibition was about 70%. It is not unusual in DAHP synthetase systems of isoenzymes that one isoenzyme is largely derepressed while another is maximally repressed (11). Experiments of the type that have successfully provided evidence in other systems for the presence of such a "masked" isoenzyme were negative. Thus, growth in the presence of various aromatic supplements that might act as regulatory signals for repression or derepression did not significantly influence

TABLE 1. *End-product inhibition of DAHP synthetase in species of blue-green bacteria*

Organism	Sp act[a]	% Inhibition[b]		
		Phe	Tyr	Aro
A. quadruplicatum	54.9	57	1	57
A. nidulans	23.7	6	53	49
O. williamsii	59.0	73	0	71
C. elabens	69.6	77	0	81
S. calcicola	38.4	24	52	69

[a] Specific activity is expressed as nanomoles of DAHP per minute per milligram of extract protein in a reaction mixture lacking inhibitor molecules.

[b] Percent inhibition was calculated by equating the specific activity of the control reaction to 100% activity (i.e., 0% inhibition). L-Phenylalanine (Phe), L-tyrosine (Tyr), and a mixture of the three aromatic amino acids (Aro) were used at final concentrations of 0.5 mM. L-Tryptophan was also tested as an inhibitor and was found (data not shown) not to inhibit any of the DAHP synthetases assayed.

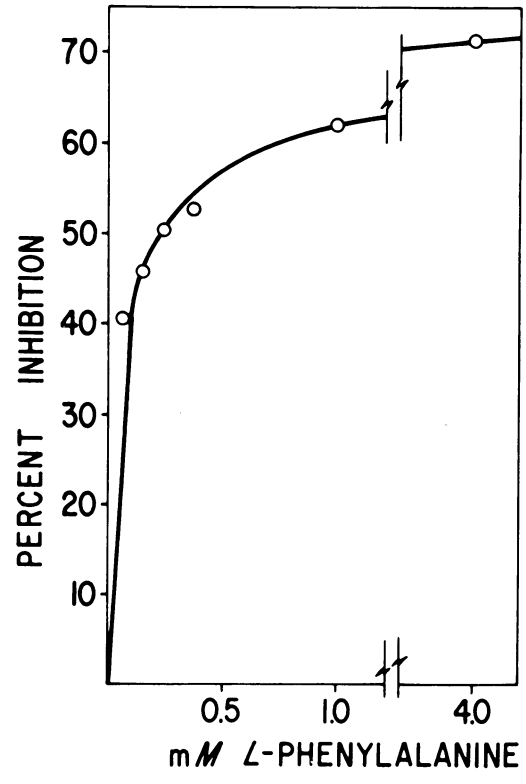

FIG. 1. *Effect of L-phenylalanine upon activity of DAHP synthetase from A. quadruplicatum. A crude extract was prepared and assayed in the presence of 2 mM phosphoenolpyruvate, 2 mM erythrose 4-phosphate plus the indicated concentration of L-phenylalanine. The control reaction, lacking inhibitor, exhibited a specific activity of 47.5 nmol of DAHP per min per mg of extract protein (37 C).*

either the specific activity of DAHP synthetase or its sensitivity to inhibition by L-phenylalanine (Table 2).

Partial purification leading to the recovery of a single species of DAHP synthetase, under conditions of no appreciable loss of the original activity present in crude extracts, also supports the presence of a single L-phenylalanine-sensitive DAHP synthetase in *A. quadruplicatum*. Figure 2 illustrates the elution of enzyme activity as a single, sharply defined band after gel filtration. DAHP synthetase was not co-eluted with other key enzymes of aromatic biosynthesis (Fig. 2). Additionally, it is known that pretyrosine dehydrogenase ($\bar{M}$ 64,000), a newly found enzyme of L-tyrosine biosynthesis (15), is not co-eluted with DAHP synthetase.

Enzymological analyses of inhibition-resistant mutants. Mutants were selected for a phenotype of resistance to L-phenylalanine or to L-phenylalanine analogues during growth (see Materials and Methods). If inhibition of DAHP synthetase by L-phenylalanine were, in fact, the molecular basis for growth inhibition, then at least some mutants would be expected to have a feedback-resistant (or at least a less-sensitive) DAHP synthetase. DAHP synthetase was indeed altered in many of the mutants. However, in direct opposition to our expectations (Table 3), the altered DAHP synthetase found in most of the mutants tested was hypersensitive to L-phenylalanine. None of the mutants was found to differ from wild type in enzymological properties of chorismate mutase, prephenate dehydratase, or prephenate/phenylpyruvate transaminase (see ref. 15 and 24 for descriptions of these enzymes). Mutants such as strains P3, P9, and P12 (Table 3), which exhibit no enzymological differences that we could detect compared with wild type, very likely are transport mutants. The increase in sensitivity to inhibition by L-phenylalanine found in many mutants was usually accompanied by an increased sensitivity to L-tyrosine and occasionally to L-tryptophan. The inhibition of activity in the simultaneous presence of all three aromatic amino acids was not significantly greater than inhibition in the presence of L-phenylalanine alone. The limited inhibition by L-tyrosine and L-tryptophan probably occurs through weak binding at the L-phenylalanine binding site of DAHP synthetase.

The kinetic mechanism of inhibition was examined in crude extracts from wild type, and these data were compared with similar analyses of the enzyme from mutant P11. The kinetic results are shown in graphical form as double reciprocal plots (Fig. 3 and 4). In both wild-type BG1 and mutant P11, inhibition by L-phenylalanine is noncompetitive with respect to both the phosphoenolpyruvate and erythrose 4-phosphate substrates. The molecular basis for

TABLE 2. *Effect of aromatic amino acids present during growth upon synthesis of DAHP synthetase in A. quadruplicatum*

Amino acid supplement[a]	Sp act[b]	% Inhibition (0.1 mM Phe)
None	51.9	42
L-Phenylalanine	57.0	46
L-Tyrosine	47.2	43
L-Tryptophan	43.9	51
Aro	50.4	47

[a] The indicated amino acids were present at an initial concentration of 0.2 mM. In the presence of L-phenylalanine (Phe), cells cultured in mineral salts medium were suspended to a turbidity of 0.10 in L-phenylalanine-containing medium for 16 h. Aro refers to a mixture of the three aromatic amino acids, each present at 0.2 mM concentration.

[b] Specific activity is expressed as nanomoles of DAHP per minute per milligram of extract protein in a reaction mixture lacking inhibitor molecules.

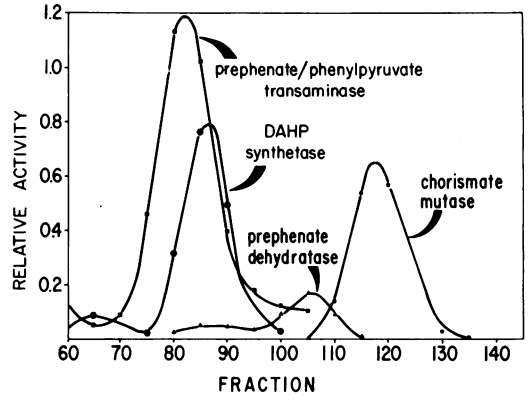

FIG. 2. *Elution of DAHP synthetase from a gel-filtration column. A 3-ml volume of extract (70 mg) was applied to a Sephadex G-200 column (2.5 by 100 cm) that was equilibrated at 4 C with 0.04 M potassium phosphate buffer, pH 6.8. The flow rate was about 15 ml/h, and eluate fractions of 2.5-ml volume were collected at 4 C. The column was calibrated by the method of Andrews (1) using chymotrypsinogen, ovalbumin, bovine serum albumin, and gamma globulin as molecular weight standards. Molecular weight estimates for the enzymes as denoted from left to right are 130,000, 120,000, 60,000, and 33,000. A relative activity of 1.0 on the ordinate scale corresponds to absorbancy increments of 0.050, 0.033, 0.033, and 0.050 per min at wavelengths of 320, 549, 320, and 320 nm for the enzymes, as shown from left to right, respectively.*

TABLE 3. *Alteration of DAHP synthetase in mutants of A. quadruplicatum resistant to L-phenylalanine inhibition*

Organism[a]	% Inhibition[b]			
	Phe	Tyr	Trp	Aro
Wild type	48	2	0	47
R 1	85	9	0	86
R 2	86	12	0	86
R 4	87	10	5	83
R 5	85	7	0	83
R 6	85	17	4	87
R 7	85	12	8	88
R 8	84	14	8	88
R 9	84	14	7	86
R 10	85	18	2	87
R 11	86	13	9	84
R 12	88	11	14	90
R 13	84	13	4	87
R 14	86	17	0	91
R 15	82	24	0	87
P 1	77	8	1	72
P 3	56	4	0	50
P 4	91	26	2	92
P 5	69	5	0	63
P 6	62	4	0	59
P 7	90	20	2	89
P 8	93	23	8	92
P 9	57	0	1	49
P 10	92	14	0	90
P 11	84	12	2	86
P 12	51	3	0	51
P 13	86	9	0	87
P 14	69	5	2	66

[a] All mutants were derived from strain BG1 (wild type) by their phenotypes of resistance to L-phenylalanine (series P) or to analogues of L-phenylalanine (series R).

[b] Percent inhibition was calculated by relating activity found in the presence of L-phenylalanine (Phe), L-tyrosine (Tyr), L-tryptophan (Trp), or all three (Aro) to that of a control reaction mixture lacking inhibitors. All test inhibitors were present at a final concentration of 0.2 mM.

the hypersensitivity of the mutant enzyme is that the apparent value of the V_{max} is decreased more with the mutant enzyme than with wild type in the presence of a given concentration of L-phenylalanine. The K_m for either substrate remains unaltered in mutant P11.

Inhibitor-antagonist relationships. Although shikimate bypasses the DAHP synthetase reaction, it was ineffective as an antagonist of growth inhibition by L-phenylalanine. In fact, concentrations in excess of 0.4 mM were inhibitory to growth (8), a result that is consistent with shikimate being a precursor of some intracellular inhibitor. Other key intermediates, such as chorismate or prephenate, are unstable and appear to be poorly transported into many microbial cells. (To our knowledge, chorismate and prephenate have not been successfully used in nutritional studies with any experimental system.) We have not yet obtained pretyrosine [β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)alanine], a newly found precursor of L-tyrosine in species of blue-green bacteria (15, 24), in sufficient amounts for use in growth experiments.

The most effective antagonists of the growth inhibition produced by L-phenylalanine were found to be L-tyrosine, L-alanine, L-leucine, L-methionine, L-tryptophan, and L-isoleucine (8). The possibility that these amino acids might antagonize growth inhibition through reversal of the L-phenylalanine-mediated inhibition of DAHP synthetase was tested. High concentrations of amino acids (L-tyrosine, L-leucine, L-methionine, L-alanine, L-isoleucine, L-

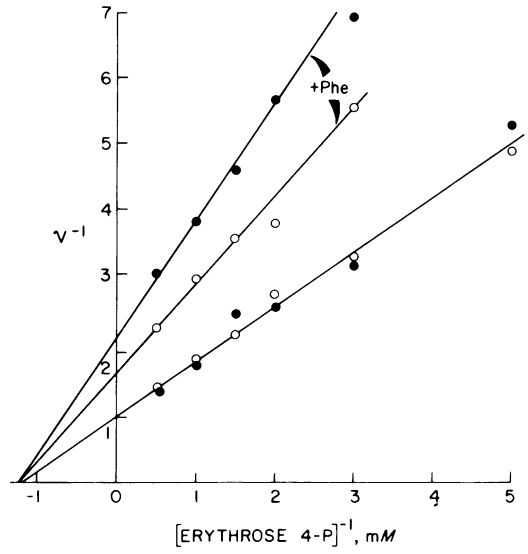

FIG. 3. *Kinetic analysis (double reciprocal plot) of L-phenylalanine inhibition of DAHP synthetase in wild-type A. quadruplicatum strain BG1 (○) and in mutant isolate P11 (●). L-Phenylalanine (Phe), when present, was used at a final concentration of 0.1 mM. The reciprocal of initial velocity (v) is plotted against the reciprocal of the concentration of erythrose 4-phosphate, the variable substrate. Phosphoenolpyruvate was present at a fixed concentration of 2 mM. A value of 1 on the ordinate scale corresponds to a specific activity of 70 nmol of DAHP formed per minute per mg of protein.*

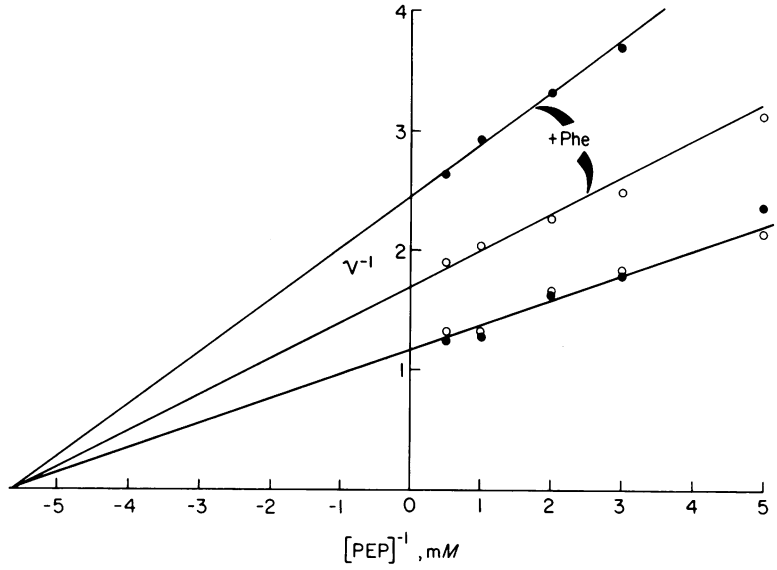

FIG. 4. *Kinetic analysis (double reciprocal plot) of* L-*phenylalanine inhibition of DAHP synthetase in wild-type A. quadruplicatum strain BG1* (○) *and in mutant isolate P11* (●). L-*Phenylalanine (Phe), when present, was used at a final concentration of 0.1 mM. The reciprocal of initial velocity (v) is plotted as the reciprocal of concentration of phosphoenolpyruvate (PEP), the variable substrate. Erythrose 4-phosphate was present at a fixed concentration of 2 mM. A value of 1 on the ordinate scale corresponds to a specific activity of 70 nmol of DAHP formed per min per mg of protein.*

glutamate, or L-histidine at 0.5 mM; L-tryptophan at 0.1 mM) were without effect upon enzyme activity, whether tested in the presence (at 0.2 mM) or absence of L-phenylalanine.

Phenylpyruvate effects. Phenylpyruvate, though less potent than L-phenylalanine, also caused growth inhibition in *A. quadruplicatum* (8). This very likely is the result of the intracellular transamination of phenylpyruvate to L-phenylalanine. However, the possibility that phenylpyruvate might be the actual intracellular inhibitor of growth by virtue of interference with the metabolism of some other keto acid was considered. Such interference could lead to partial starvation for a given keto acid or to excess levels of the keto acid, in which case the presence of the keto acid would reverse L-phenylalanine inhibition of growth or sensitize cells to inhibition, respectively. Various keto acids were examined for ability to either antagonize or sensitize cells to L-phenylalanine inhibition on solid medium. One-milligram amounts of crystalline α-ketoglutarate, oxalacetate, pyruvate, and glyoxylate were placed in the center of agar plates containing L-phenylalanine and seeded with an initial population of about 10^8 cells of *A. quadruplicatum*. After incubation for 5 days at 39 C, most of these compounds were found to be inhibitory per se at high concentrations (as verified on media lacking L-phenylalanine), but none of the keto acids altered the degree of L-phenylalanine inhibition throughout the range of keto acid concentration provided by the diffusion gradient. Various other organic compounds also did not influence the degree of growth inhibition caused by L-phenylalanine; these included glucose, citrate, and glycerol.

DISCUSSION

Hypothesis of the mis-regulated DAHP synthetase can be eliminated. The most obvious explanation to account for growth inhibition of *A. quadruplicatum* in the presence of L-phenylalanine is that hyperinhibition of DAHP synthetase by L-phenylalanine leads to L-tyrosine and/or L-tryptophan starvation. The ability of L-tyrosine, when present in the growth medium, to antagonize L-phenylalanine-mediated inhibition of growth (8) is consistent with this hypothesis. However, the finding that L-phenylalanine-resistant mutants exhibit increased sensitivity of DAHP synthetase to L-phenylalanine inhibition implies that the control of DAHP synthetase by L-phenylalanine not only does not explain sensitivity of growth to L-phenylalanine, but actually seems to provide

a regulatory basis for resistance to growth inhibition.

Numerous other metabolite sensitivities are known (4, 19, 28, 29) that are analogous in that a branch-point enzyme is regulated by a metabolite which also causes inhibition of growth. In view of our results with *A. quadruplicatum*, it is possible that the interpretation of mis-regulation of a branch-point enzyme, advanced in these other systems as an explanation for growth inhibition, is incorrect.

A toxic aromatic intermediate. Since increased inhibition of DAHP synthetase leads to resistance to growth inhibition caused by L-phenylalanine, it seems probable that toxicity is caused by some aromatic intermediate subsequent to the DAHP synthetase reaction. Previously puzzling observations that were inconsistent with the mis-regulation hypothesis are compatible with the toxic intermediate interpretation, namely, that: (i) sensitivity of the wild-type DAHP synthetase to inhibition by L-phenylalanine is poor, and inhibition is incomplete (i.e., inhibition saturates at about 70%). (ii) No excretor-type mutants were found when selecting for L-phenylalanine-resistant mutants; (note that a feedback-resistant DAHP synthetase leading to increased production of a toxic intermediary metabolite[s] might cause lethality or slow growth). (iii) Shikimate does not reverse L-phenylalanine inhibition of growth (an expectation of the DAHP synthetase mis-regulation hypothesis); shikimate is itself inhibitory (suggestive of some post-shikimate molecule[s] being the intracellular inhibitor).

Phenylpyruvate is not the inhibitor metabolite. The possibility that phenylpyruvate is the toxic intermediate was considered because: (i) an active L-phenylalanine/phenylpyruvate transaminase activity exists in *A. quadruplicatum* (24), (ii) exogenous phenylpyruvate inhibits growth (8), and (iii) a generalized keto acid toxicity has often been claimed to be a physiological characteristic of autotrophic microorganisms (2).

High concentrations of L-phenylalanine (or a derivative metabolite such as phenylpyruvate) could inhibit some essential reaction in an apparently unrelated biochemical pathway. Precedent examples of such phenomena are known (e.g., 28, 29). In one instance, pyruvate kinase of skeletal muscle is specifically inhibited by L-phenylalanine (3). (By remarkable coincidence with respect to antagonistic relationships [8], a number of nonaromatic amino acids reverse the inhibition of pyruvate kinase by L-phenylalanine.) However, the possibility that the site of growth inhibition by L-phenylalanine or phenylpyruvate is external to the aromatic amino acid pathway is unlikely based on the evidence provided by L-phenylalanine-resistant mutants. The alterations of DAHP synthetase found in such mutants would not be expected to affect the concentration of phenylpyruvate formed from L-phenylalanine or the susceptibility of some putative enzymatic target in another biochemical pathway. It is likewise difficult to support a mechanism of growth inhibition based upon the inhibition of some reaction within the aromatic pathway by phenylpyruvate. For example, one could hypothesize that phenylpyruvate (formed from L-phenylalanine) might saturate the common transaminase that is used to convert prephenate to pretyrosine (24), thereby causing starvation for L-tyrosine. Again, this mechanism of inhibition is difficult to reconcile with an increased sensitivity of DAHP synthetase to feedback inhibition in mutants leading to a resistant phenotype. Neither L-phenylalanine nor phenylpyruvate inhibits pretyrosine dehydrogenase activity (24), eliminating this enzyme of L-tyrosine synthesis as the site of action.

The inhibitor is probably an aromatic intermediate. Since a decrease in DAHP synthetase catalysis (i.e., increased feedback inhibition in mutants) leads to resistance, some accumulated aromatic derivative of DAHP synthetase action must be toxic. Because prephenate dehydratase in *A. quadruplicatum* is more sensitive to feedback inhibition by L-phenylalanine (24) than is DAHP synthetase, the presence of exogenous phenylalanine in wild type may trap a toxic intermediate somewhere between the two control points under regulation by L-phenylalanine. The decreased flow of early pathway precursors in resistant mutants would lead to decreased levels of compounds such as prephenate (a keto acid) or pretyrosine (an amino acid). These compounds might accumulate when L-phenylalanine is supplied to wild type, owing to the blocking of prephenate flow into the L-phenylalanine branch of the pathway (i.e., feedback inhibition of prephenate dehydratase by L-phenylalanine).

Relationship of metabolite toxicity and its regulation of a common-pathway enzyme. The inhibition of growth by amino acid metabolites is of frequent occurrence. Qualitatively, the phenomenon of toxicity of L-phenylalanine for species of *Thiobacillus* (16–18, 22, 23) is similar to our observations made in *A. quadruplicatum*. An entirely analogous circumstance of single-effector regulation of a common

enzyme within a branched pathway and threonine inhibition of growth was described in *Methylococcus capsulatus* (5, 7). If one assumes that hyperregulation of a common enzyme step within a branched pathway accounts for growth inhibition, then one class of resistant mutants ought to possess feedback-resistant enzymes. It may be significant that such mutants were not obtained in *T. neapolitanus* and *M. capsulatus*, reminiscent of our findings with *A. quadruplicatum*. In *T. neapolitanus* the mechanism of resistance proposed for one mutant having an altered DAHP synthetase (but not feedback resistant) requires difficult assumptions about endogenous pools (18). Only permease mutants were found in *M. capsulatus* (6).

Even where a set of regulatory isoenzymes exists, a "dominant isoenzyme" (11) frequently makes up the major fraction of enzyme formed in wild type. A general possibility is that the dominant role of an amino acid end product as an effector molecule for a common enzyme of a branched pathway may reflect a control that in nature prevents the endogenously formed amino acid from inhibiting growth (by whatever mechanism of inhibition is involved). According to this interpretation, the single-effector type of DAHP synthetase is not the defective regulatory device that initially seems to be the case. Since the wild-type cells are sensitive, rather than resistant, to inhibition by L-phenylalanine, it seems likely that conditions favoring the selection of resistant mutants have not occurred in nature. Thus, the nutritional environment may seldom, if ever, possess sufficient L-phenylalanine to inhibit growth; or perhaps inhibitory concentrations of L-phenylalanine usually occur in the presence of antagonistic amino acids. The same considerations can be made for the well-known valine sensitivity of *Escherichia coli* K-12, a wild-type strain (20, 25).

ACKNOWLEDGMENTS

These studies were supported by Public Health Service research grant AM-13105 (R.A.J.) from the National Institute of Arthritis, Metabolism, and Digestive Diseases and grant VC 127 (L.O.I.) from the American Cancer Society. R. A. J. is the recipient of Public Health Service Career Development Award GM-22778 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Andrews, P. 1965. The gel-filtration behavior of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595-606.
2. Borichewski, R. M. 1967. Keto acids as growth-limiting factors in autotrophic growth of *Thiobacillus thiooxidans*. J. Bacteriol. **93**:597-599.
3. Carminatti, H., L. Jiménez de Asúa, B. Leiderman, and E. Rozengurt. 1971. Allosteric properties of skeletal muscle pyruvate kinase. J. Biol. Chem. **246**:7284-7288.
4. Carr, N. G. 1973. Metabolic control and autotrophic physiology, p. 31-65. *In* N. Carr and B. Whitton (ed.), The biology of blue-green algae. Blackwell Scientific Publications, Oxford.
5. Eccleston, M., and D. P. Kelly. 1972. Assimilation and toxicity of exogenous amino acids in the methane-oxidizing bacterium *Methylococcus capsulatus*. J. Gen. Microbiol. **71**:541-554.
6. Eccleston, M., and D. P. Kelly. 1972. Competition among amino acids for incorporation into *Methylococcus capsulatus*. J. Gen. Microbiol. **73**:303-314.
7. Eccleston, M., and D. P. Kelly. 1973. Inhibition by L-threonine of aspartokinase as a cause of threonine toxicity to *Methylococcus capsulatus*. J. Gen. Microbiol. **75**:223-226.
8. Ingram, L. O., and R. A. Jensen. 1973. Growth inhibition by L-phenylalanine in *Agmenellum quadruplicatum*: a clue to some amino acid relationships. Arch. Mikrobiol. **91**:221-233.
9. Ingram, L. O., D. Pierson, J. F. Kane, C. Van Baalen, and R. A. Jensen. 1972. Documentation of auxotrophic mutation in blue-green bacteria: characterization of a tryptophan auxotroph in *Agmenellum quadruplicatum*. J. Bacteriol. **111**:112-118.
10. Jensen, R. A., D. H. Calhoun, and S. L. Stenmark. 1973. Allosteric inhibition of 3-deoxy-*D-arabino*-heptulosonate 7-phosphate synthetase by tyrosine, tryptophan and phenylpyruvate in *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **293**:256-268.
11. Jensen, R. A., and D. S. Nasser. 1968. Comparative regulation of isoenzymic 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetases in microorganisms. J. Bacteriol. **95**:188-196.
12. Jensen, R. A., D. S. Nasser, and E. W. Nester. 1967. The comparative control of a branch-point enzyme in microorganisms. J. Bacteriol. **94**:1582-1593.
13. Jensen, R. A., and E. W. Nester. 1966. Regulatory enzymes of aromatic amino acid biosynthesis in *Bacillus subtilis*. I. Purification and properties of 3-deoxy-*D-arabino*-heptulosonate 7-phosphate synthetase. J. Biol. Chem. **241**:3365-3372.
14. Jensen, R. A., and J. Rebello. 1970. Comparative allostery of microbial enzymes at metabolic branch-points: evolutionary implications, p. 105-121. *In* C. J. Corum (ed.), Development of industrial microbiology, vol. 11, Plenum Press, New York.
15. Jensen, R. A., and S. L. Stenmark. 1975. The ancient origin of a second microbial pathway for L-tyrosine biosynthesis in prokaryotes. J. Mol. Evol., vol. 4-3.
16. Johnson, C. L., and W. Vishniac. 1970. Growth inhibition in *Thiobacillus neapolitanus* by histidine, methionine, phenylalanine, and threonine. J. Bacteriol. **104**:1145-1150.
17. Kelly, D. P. 1969. Regulation of chemoautotrophic metabolism. I. Toxicity of phenylalanine to Thiobacilli. Arch. Mikrobiol. **69**:330-342.
18. Kelly, D. P. 1969. Regulation of chemoautotrophic metabolism. III. DAHP synthetase in *Thiobacillus neapolitanus*. Arch. Mikrobiol. **69**:360-369.
19. Kelly, D. P. 1971. Autotrophy: concepts of lithotrophic bacteria and their organic metabolism. Annu. Rev. Microbiol. **25**:177-210.
20. Leavitt, R. J., and H. E. Umbarger. 1962. Isoleucine and valine metabolism in *Escherichia coli*. XI. Valine inhibition of *Escherichia coli* strain K-12. J. Bacteriol. **83**:624-630.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin

phenol reagent. J. Biol. Chem. **192**:265-275.
22. Lu, M. C., A. Matin, and S. C. Rittenberg. 1971. Inhibition of growth of obligately chemolithotrophic Thiobacilli by amino acids. Arch. Mikrobiol. **79**:354-366.
23. Rittenberg, S. C. 1969. The roles of exogenous organic matter in the physiology of chemolithotrophic bacteria. Advan. Microbiol. Physiol. **3**:159-196.
24. Stenmark, S. L., D. L. Pierson, G. I. Glover, and R. A. Jensen. 1974. Blue-green bacteria synthesize *L*-tyrosine by the pretyrosine pathway. Nature (London) **247**:290-292.
25. Tatum, E. L. 1946. Biochemical mutations in bacteria. Cold Spring Harbor Symp. Quant. Biol. **11**:278-283.
26. Van Baalen, C. 1962. Studies on marine blue-green algae. Bot. Mar. **4**:129-139.
27. Weber, H. L., and A. Bock. 1968. Comparative studies on the regulation of DAHP synthetase activity in blue-green and green algae. Arch. Mikrobiol. **61**:159-168.
28. Weber, G., R. Glazer, and R. A. Ross. 1969. Regulation of human and rat brain metabolism: inhibitory action of phenylalanine and phenylpyruvate on glycolysis, protein, lipid, DNA and RNA metabolism. Advan. Enzyme Regul. **8**:13-36.
29. Wolfson, P. J., and T. A. Krulwich. 1972. Inhibition of isocitrate lyase: the basis for inhibition of growth of two *Arthrobacter* species by pyruvate. J. Bacteriol. **112**:356-364.