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ALLOSTERIC INHIBITION OF 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHETASE BY TYROSINE, TRYPTOPHAN AND PHENYLPYRUVATE IN *PSEUDOMONAS AERUGINOSA*

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SUMMARY

1. In *Pseudomonas aerginosa* the allosteric control of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase, EC 4.1.2.15, by tyrosine has been known for some time. However, a more detailed analysis of the enzyme in crude extracts revealed sensitivity to inhibition by tryptophan and phenylpyruvate in addition to tyrosine. It is concluded that a single enzyme exists bearing three inhibitor specificities. Testing of various inhibitor combinations revealed no synergistic or antagonistic effects of aromatic metabolites. Combinations of the three inhibitors produced cumulative or less-than-cumulative effects.

2. Tyrosine and phenylpyruvate were both capable of producing more than 90% inhibition at saturating concentrations of substrates, but tyrosine was about 18 times more potent than phenylpyruvate on a molar basis. Although inhibition by tryptophan was limited to a maximum of 25–30%, relatively low concentrations were effective. It is concluded that inhibition by either tyrosine or tryptophan is competitive with respect to phosphoenolpyruvate and noncompetitive with respect to erythrose-4-*P*. On the other hand, phenylpyruvate inhibition appears to be competitive with respect to erythrose-4-*P* and noncompetitive with respect to phosphoenolpyruvate.

3. Hence, one metabolite of each terminal branch of the aromatic amino acid pathway exhibits allosteric influence upon the activity of DAHP synthetase, an observation in accord with expectations for balanced participation of multiple end products in the regulation of a branched pathway. The quantitative effects actually realized *in vivo* would depend in complex fashion upon the intracellular concentrations of tyrosine, tryptophan and phenylpyruvate and upon the intracellular concentrations of erythrose-4-*P* and phosphoenolpyruvate.

4. A variety of physiological experiments with wild type and mutant cells led

Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DAH, 3-deoxy-D-*arabino*-heptulosonate.

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to the conclusion that the synthesis of DAHP synthetase is not controlled by repression in *P. aeruginosa*.

INTRODUCTION

The enzyme, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase, EC 4.1.2.15, provides the first common precursor that is specific for the biosynthesis of the three aromatic amino acids. Efficient regulation of this enzyme, situated at a metabolic branch point, would be expected to be of great metabolic importance. It has, in fact, been found that DAHP synthetase is usually subject to feedback inhibition in microorganisms[1,2]. Many of the various control patterns for DAHP synthetase which exist among microorganisms can be reconciled with a scheme of regulation which seems efficient at the physiological level. Some of the best known patterns include isoenzymic feedback inhibition, sequential feedback inhibition, concerted feedback inhibition, and cumulative feedback inhibition (see ref. 2). All of these control patterns involve the balanced participation of all three end products, either directly or indirectly, in the mechanism of regulation.

In contrast, an apparent uni-effector control by a single aromatic amino acid characterizes a surprisingly large number of microbial DAHP synthetases[1,2]. *A priori* one might predict that a single DAHP synthetase subject to feedback inhibition by tyrosine, for example, would result in growth inhibition by exogenous tyrosine, *i.e.* near-complete inhibition of the activity of DAHP synthetase by tyrosine would cause starvation for precursors needed for phenylalanine and tryptophan biosynthesis. Indeed, this outcome is realized in several microorganisms, *e.g. Thiobacillus neapolitanus*[3] and the blue-green bacterium, *Agmenellum quadruplicatum* (L. O. Ingram and R. A. Jensen, unpublished results). Both of the latter organisms possess a single phenylalanine-inhibitable DAHP synthetase and consequently are growth-inhibited by exogenous phenylalanine in a defined medium. A comparable outcome is achieved artificially in the laboratory with *Escherichia coli* mutants which have lost one or more isoenzymes of DAHP synthetase[4].

However, apparent uni-effector DAHP synthetases occur in other microorganisms which are not growth inhibited by the corresponding aromatic amino acid. For example, *Serratia marcescens* and various Pseudomonad species appear to possess only tyrosine-inhibitable DAHP synthetases and *Streptomyces coelicolor* possesses only a tryptophan-inhibitable DAHP synthetase[1,2,5]; yet neither tyrosine nor tryptophan inhibits the growth of these bacteria.

Since *P. aeruginosa* is amenable to genetic analysis[6] and is currently the subject of ongoing studies of gene–enzyme relationships, it was judged to be a suitable system for a detailed experimental analysis of the regulatory features of DAHP synthetase.

MATERIALS AND METHODS

Organism

Strain 1 of *P. aeruginosa* was originally acquired from B. W. Holloway[6]. MC 36 was derived from strain 1 by nitrosoguanidine mutagenesis as previously described[7]. Regulatory mutants 4FT-1 and TA-2 were selected as mutants appearing spontane-

ously on solid medium containing 4-fluorotryptophan and β-2-thienylalanine, respectively[8].

Cultivation of P. aeruginosa

The composition of a minimal salts medium containing glucose at a final concentration of 0.5% has been described[9]. Growth rates at 37 °C were estimated by following culture turbidities using a Klett–Summerson colorimeter. It was important to use a No. 54 green filter in order to avoid inaccuracies due to the spectral characteristics of the pyocyanine pigment produced by *P. aeruginosa*. Continuous cultivation of mutant MC 36 in minimal salts–glucose medium at 37 °C was accomplished in a New Brunswick chemostat (Bioflow Model C30). The doubling time was decreased to 95–100 min by the appropriate adjustment of the influx of the growth limiting amino acid. Following steady state growth for 8–10 h the entire working volume (350 ml) was harvested prior to the preparation of extracts.

Preparation of extracts

Extracts were prepared from cells grown at 37 °C with vigorous shaking, usually 200 ml of minimal salts–glucose medium supplemented as indicated. Harvest of cells in the late exponential phase of growth, unless otherwise indicated, was accomplished by centrifugation, washing with 200 ml of 0.04 M potassium phosphate buffer (pH 7.0) containing 0.1 M KCl, and resuspension in 4–5 ml of the buffer. Cells were broken by sonication, using two 30-s pulses from a sonic probe (Biosonik, Bronwill Instruments), maintaining the preparation as close to ice temperature as possible. Cell debris was separated by centrifugation at 27 000 × *g* for 20 min. Nucleic acid was removed by precipitation after adding a sufficient volume of a freshly prepared 2% stock of protamine sulfate to give a final concentration of 0.2%. After centrifugation, the extract was dialyzed overnight at 4 °C against 2 changes of 1000 vol. of 0.04 M potassium phosphate buffer (pH 7.0) containing 0.1 M KCl.

Mutant MC 36 was starved in the absence of aromatic amino acids in an attempt to demonstrate derepression of DAHP synthetase as indicated in Table II. In this experiment 400 ml of cells were grown in minimal salts–glucose medium in the presence of 100 μg each of tyrosine, phenylalanine and tryptophan per ml at 37 °C with vigorous shaking. *p*-Aminobenzoate, *p*-hydroxybenzoate, 2,3-dihydroxybenzoate and 3,4-dihydroxybenzaldehyde were also present at a final concentration of 1 μg/ml. One-half of the culture was harvested in the late exponential phase of growth. The other half was centrifuged and resuspended in minimum salts–glucose medium lacking aromatic supplements. Starvation was continued during further incubation at 37 °C with shaking for 5 h. Extracts were prepared as previously described. The first extract should contain a maximally repressed level of DAHP synthetase whereas the second should contain a maximally derepressed level of DAHP synthetase.

Analytical procedures

The assay of DAHP synthetase was carried out according to the procedure of Srinivasan and Sprinson[10] as modified[11]. In crude extracts conditions of proportionality with respect to both time and protein concentration were used in the assay of DAHP synthetase activity. The assay gives a linear relationship of activity to protein in the range of 15–125 μg of crude extract protein in the reaction mixture. Reaction mixtures

contained 8 μmoles of potassium phosphate buffer (pH 6.8) 20 μmoles of KCl *plus* the indicated amounts of erythrose-4-*P* and phosphoenolpyruvate in a final reaction volume of 0.2 ml. Reaction times were 20 min unless otherwise indicated. DAHP concentration was estimated by using a molar extinction coefficient of $4.5 \cdot 10^4$ at 549 nm. The stated concentrations of erythrose-4-*P* may be inaccurate by as much as 30% due to the decay of erythrose-4-*P* during storage. It was not thought necessary to correct concentrations by the use of reference standards as previously described[11]. However, relative values used to determine factor differences within any given experiment can be considered to be correct. High substrate concentrations of erythrose-4-*P* (above 1 mM) may produce some absorbance at 549 nm. Hence, blanks were run for erythrose-4-*P* in order to correct for this effect. Specific activities of DAHP synthetase are expressed as nmoles DAHP formed per minute per mg of protein at 37 °C.

Protein concentrations were estimated by the procedure of Lowry *et al.*[12] using bovine serum albumin as a standard.

Preparation of DAH

Aromatic amino acid-requiring auxotrophs of *Bacillus subtilis* lacking 5-dehydroquinate synthetase activity accumulate DAHP which can be purified relatively easily[13]. Usually about half of the accumulated material is recovered in the dephosphorylated form (DAH) due to phosphatase activity present in whole cells and/or crude extracts. DAHP and DAH separate cleanly when eluted from P2 gel columns (Calbiochem).

Inhibition of the thiobarbituric assay system

Tryptophan is reactive with periodate and may at sufficiently high concentrations inhibit the chemical assay system for DAHP. Appropriate controls were included in inhibition experiments to detect any possible non-enzymatic effects. DAH was used as a standard in experiments in which compounds were tested as inhibitors of the chemical assay system.

Substrates for DAHP synthetase

Trisodium phosphoenolpyruvate was obtained from Sigma. D-Erythrose-4-*P* was obtained from Sigma as the sodium salt (84% pure) or from Calbiochem as the dicyclohexylammonium derivative. The latter product was hydrolyzed as specified by Calbiochem and stored in a 9 mM stock solution. The Sigma product was made up in 40 mM potassium phosphate buffer and stored as a 20 mM stock solution. Small batches of these substrate preparations were maintained at a temperature of 4 °C since freezing apparently results in dimer formation of erythrose-4-*P* (D. B. Sprinson, personal communication).

The erythrose-4-*P* obtained from Sigma produced a strong apparent substrate inhibition beginning at about 2 mM (*e.g.*, about 80% inhibition at 6 mM erythrose-4-*P* and 2 mM phosphoenolpyruvate); the Calbiochem product did not exhibit substrate inhibition. Hence, it would appear that the effect can be attributed to some impurity in the Sigma product. The substrate efficiencies of the two substrate preparations were about equal up to 2 mM concentrations.

Chemicals

Barium salts of chorismate (86% pure) and prephenate (96% pure) were prepared by the method of Gibson[14] and used as the potassium salts following the addition of excess potassium sulfate. All amino acids, anthranilic acid, and protamine sulfate (salmon) were obtained from Calbiochem. β-2-Thienylalanine and D,L-4-fluorotryptophan, bovine serum albumin, shikimic acid, sodium phenylpyruvate and 2-thiobarbituric acid were from Sigma Chemical Company. 4-Hydroxyphenylpyruvic acid was purchased from Mann Research. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine and 3,4-dihydroxybenzaldehyde were obtained from Aldrich Chemicals. Phenylpyruvate was examined by thin-layer chromatography and found to be free from contamination with tyrosine, tryptophan or phenylalanine.

RESULTS

Identification of allosteric effectors

A variety of aromatic compounds were tested as inhibitors of DAHP synthetase (Table I) in *P. aeruginosa*. Both substrates were present at 0.5 mM (the saturating concentration of each substrate is about 2 mM). The values shown in Table I represent the average of three or more determinations, and appropriate experiments were performed to ensure that the values represent initial velocities. As previously noted in species of *Pseudomonas*[1,2,5,15], tyrosine is an effective inhibitor of DAHP synthetase. In previous comparative analyses of DAHP synthetase[1] only qualitatively obvious inhibitor effects obtained at relatively low inhibitor concentrations were noted. Table I shows data suggesting that the *P. aeruginosa* enzyme may also possess allosteric specificity for tryptophan and phenylpyruvate. The common intermediates of the aromatic pathway (shikimate, chorismate and prephenate) did not inhibit DAHP synthetase significantly. Likewise, the tyrosine intermediate, *p*-hydroxyphenylpyruvate, and the tryptophan intermediate, anthranilate, were ineffective as inhibitors. Higher concentrations of test inhibitors up to 1 mM were also used with negative results. At high inhibitor concentrations corrections for inhibition of the chemical assay system were often necessary (see Materials and Methods). Vitamin derivatives of the aromatic pathway such as *p*-aminobenzoate, *p*-hydroxybenzoate, 2,3-dihydroxybenzoate and 3,4-dihydroxybenzaldehyde did not exert simple or synergistic inhibitory effects on DAHP synthetase (S. L. Stenmark, unpublished results).

Further data, in the form of inhibition curves, are presented in Fig. 1. At substrate concentrations of 2 mM each of erythrose-4-*P* and phosphoenolpyruvate, tyrosine was by far the best inhibitor, complete inhibition being approached at relatively low concentrations. Tryptophan was also effective on a molar basis, but inhibition was incomplete with maximal inhibition occurring at about 30%. Phenylpyruvate was a poor inhibitor on a molar basis, but near-complete inhibition could be approached at high concentrations. Ionic strength or pH effects were eliminated as possible trivial explanations for inhibition of DAHP synthetase by phenylpyruvate. Under the assay conditions used (Fig. 1), 50% inhibition of DAHP synthetase activity by phenylpyruvate and tyrosine was achieved at 1.5 mM and 0.085 mM, respectively. Half-maximal inhibition by tryptophan was achieved at a concentration of about 0.080 mM.

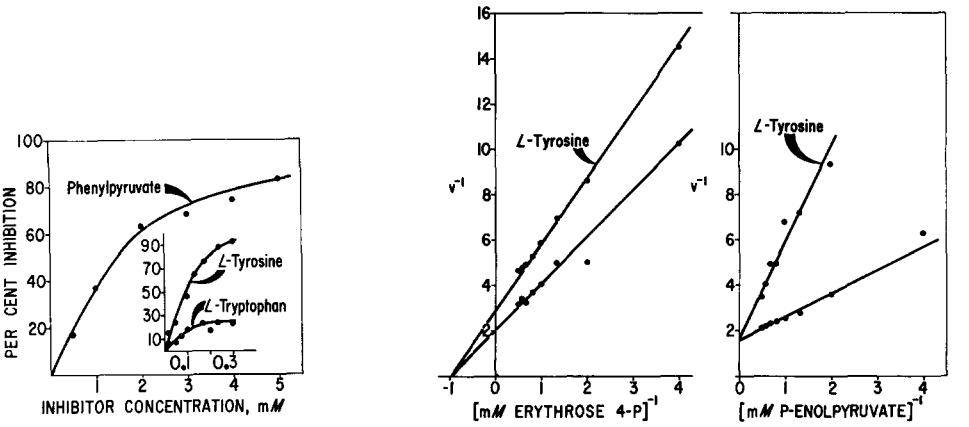

Fig. 1. Inhibition curves showing inhibition of DAHP synthetase activity as a function of tyrosine, tryptophan or phenylpyruvate concentration. The concentrations of erythrose-4-*P* and phosphoenolpyruvate were each 2 mM. Extracts were prepared from exponential phase cultures of strain 1 of *P. aeruginosa* and assayed for activity at 37 °C as described in Materials and Methods. The specific activities of extracts used in the determinations of the curves (from left to right) were 41.8, 53.1 and 50.0 nmoles DAHP per min/mg protein. Per cent inhibition was calculated by relating activity measured in the presence of inhibitor to the average of two reaction velocities measured in the absence of inhibitor. The abscissa and ordinate of the inset express the same parameters shown on the othre abscissa and ordinate scales.

Fig. 2. Double-reciprocal plots of initial velocity of DAHP synthetase as a function of substrate concentration. An extract of strain 1 was obtained from cells grown in minimal salts–glucose medium and harvested in the exponential phase of growth. The specific activity was 48.2, and reaction mixtures contained 115 μg of protein. L-Tyrosine, when present, was used at a final concentration of 0.1 mM. The concentrations of the variable substrate, either erythrose-4-*P* (left) or phosphoenolpyruvate (right) are indicated along the abscissa scale. The fixed substrate, either phosphoenolpyruvate (left) or erythrose-4-*P* (right) was present at a final concentration of 2 mM. Velocity of DAHP synthetase, indicated on the ordinate scales as v^{-1}, is expressed as $\Delta A_{549\ nm}$ per 10 min at 37 °C.

Combinations of aromatic metabolites were tested in order to screen for regulatory relationships in which inhibition might be reversed by another compound, or in which inhibition might be potentiated, *i.e.* synergistic allosteric combinations. None of these were found. Various possible combinations of tyrosine, tryptophan and phenylpyruvate resulted in cumulative or less-than-cumulative levels of inhibition.

Kinetic analysis of inhibitions

Kinetic results obtained from crude extracts assayed with tyrosine are shown in Fig. 2. Tyrosine is a competitive inhibitor with respect to phosphoenolpyruvate and inhibits noncompetitively with respect to erythrose-4-*P*. Data obtained with tryptophan and shown in Fig. 3 were qualitatively similar. As with tyrosine, tryptophan inhibited in strictly competitive fashion with respect to phosphoenolpyruvate and non-competitively with respect to erythrose-4-*P*.

The kinetic analysis of phenylpyruvate inhibition produced results which were symmetrically opposite to those obtained with tyrosine or tryptophan. Phenylpyruvate, as shown in Fig. 4, was a competitive inhibitor with respect to erythrose-4-*P* and a noncompetitive inhibitor with respect to phosphoenolpyruvate. In contrast to results obtained with crude extracts, phenylpyruvate does not inhibit the activity

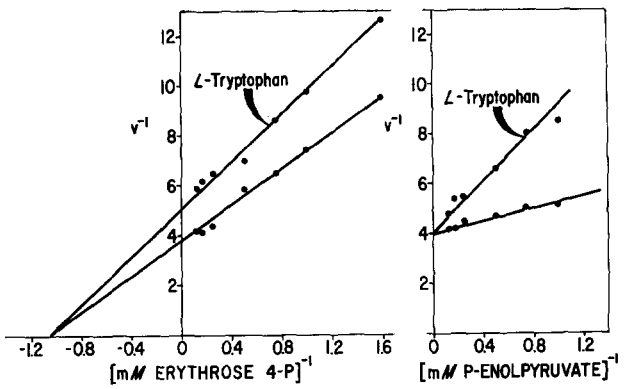

Fig. 3. Double-reciprocal plots of initial velocity of DAHP synthetase as a function of substrate concentration. An extract having a specific activity of 48.2 was prepared from exponentially grown cells of strain 1. The reaction mixtures contained 60 μg of extract protein. L-Tryptophan, when present, was used at a final concentration of 0.17 mM. The concentrations of the variable substrate, either erythrose-4-*P* (left) or phosphoenolpyruvate (right) are indicated along the abscissa scale. The fixed substrate, either phosphoenolpyruvate (left) or erythrose-4-*P* (right) was present at a final concentration of 2 mM. Velocity of DAHP synthetase, indicated on the ordinates as v^{-1}, is expressed as $\Delta A_{549\ nm}$ per 10 min at 37 °C.

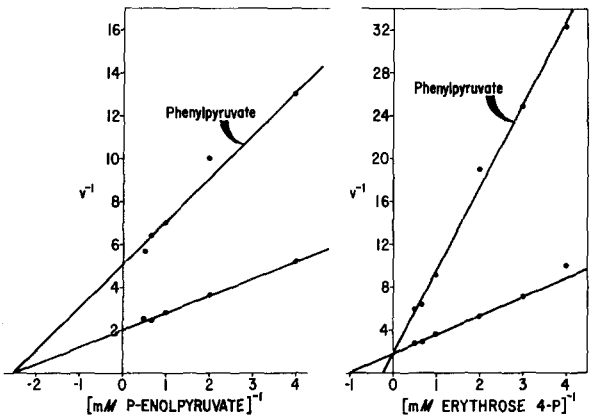

Fig. 4. Double-reciprocal plots of initial activity of DAHP synthetase as a function of substrate concentration. An extract of strain 1 was prepared from cells grown in minimal salts–glucose medium and harvested in the exponential phase of growth. The specific activity was 48.2, and reaction mixtures contained 115 μg of protein. Phenylpyruvate, when present, was used at a final concentration of 1.5 mM. The concentrations of the variable substrate, either phosphoenolpyruvate (left) or erythrose-4-*P* (right) are indicated along the abscissa scale. The fixed substrate, either erythrose-4-*P* (left) or phosphoenolpyruvate (right) was present ar a final concentration of 2 mM Velocity of DAHP synthetase, indicated on the ordinate scales as v^{-1}, is expressed as $\Delta A_{549\ nm}$ per 10 min at 37 °C.

of partially purified DAHP synthetase eluted from a Sephadex G-100 column. The elution profile obtained and the method used are described in ref. 15.

Experiments similar to those given in Figs 2, 3 and 4 using different inhibitor concentrations consistently produced plots indicating simple competitive or non-competitive mechanisms of inhibition.

TABLE I

INHIBITION OF DAHP SYNTHETASE ACTIVITY BY AROMATIC METABOLITES

Reaction vessels contained 140 μg of crude extract protein from strain 1 possessing a specific activity for DAHP synthetase of 60.9. Inhibition values were calculated by relating velocity determinations obtained in the presence of 0.17 mM inhibitor to the velocity measured in the absence of inhibitor. The cells used as the source of extract were grown in minimal salts–glucose medium at 37 °C and harvested in exponential phase. Extract was added to a mixture of substrates (each at 0.5 mM concentration) and inhibitor in the assay procedure for DAHP synthetase.

Inhibitor	*% Inhibition*
L-Tyrosine	90
p-Hydroxyphenylpyruvate	0
L-Phenylalanine	0
Phenylpyruvate	24
L-Tryptophan	29
Anthranilate	0
Shikimate	1
Chorismate	0
Prephenate	4

TABLE II

ABSENCE OF REPRESSION OF DAHP SYNTHETASE IN *P. aeruginosa*

*Strain designation**	*Description*	*Growth supplement***	*Specific activity*
1	Wild type prototroph	Fructose	40.7
1	Wild type prototroph	Glucose	53.1
1	Wild type prototroph	L-Tyrosine	45.8
1	Wild type prototroph	Aromatic amino acids	64.5
1	Wild type prototroph	L-Phenylalanine	51.4
1	Wild type prototroph	Shikimate	55.9
1	Wild type prototroph	L-Tryptophan	42.0
MC 36	Aromatic auxotroph	Aromatic amino acids	47.2
MC 36	Aromatic auxotroph	Glucose***	39.9
1	Wild type prototroph	Glucose	43.3
4 FT-1	Tryptophan excretor	Glucose	28.8
TA-2	Phenylalanine excretor	Glucose	34.9

* MC 36 is blocked before shikimate and requires either shikimate or all three aromatic amino acids for growth. 4FT-1 and TA-2 are prototrophic derivatives of the parental strain 1, and are regulatory mutants of tryptophan and phenylalanine synthesis, respectively.

** Cells were grown in a basal salts minimal medium. Aromatic metabolite supplements were added to a final concentration of 100 μg/ml. The source of carbon during growth was either 0.5% glucose or 0.5% fructose. Aromatic amino acids denote the combination of tyrosine, phenylalanine and tryptophan.

*** Cells of MC 36 in the exponential phase of growth were starved in minimal salts–glucose medium at 37 °C for 5 h (see Materials and Methods for other details).

Repression of DAHP synthetase?

The results summarized in Table II document fairly rigorously the conclusion that the synthesis of DAHP synthetase is subject to little or no control by repression. As shown in the top portion of Table II, various aromatic supplements fail to influence the specific activity of DAHP synthetase relative to the activity found under conditions of growth in a minimal salts–glucose medium. However, these data *per se* do not exclude the possibility that endogenous aromatic synthesis in a minimal salts–glucose medium is set at a sufficiently high level to bring about maximal levels of repression.

Three additional approaches were used which have been successful in other microorganisms for demonstrating derepression of repressible enzyme systems. (i) An auxotroph blocked in the common portion of the aromatic pathway was grown to exponential phase and starved for aromatic end-products for 5 h. Although this procedure results in a near-complete derepression of enzymes in the tryptophan pathway of *P. aeruginosa* (D. L. Pierson, unpublished data), the specific activity of DAHP synthetase was not elevated. In addition the mutant MC 36 (Table II) was cultured in a New Brunswick chemostat at 37 °C under conditions of growth limitation for each of the three aromatic amino acids. Specific activities for DAHP synthetase of 50.1, 53.6 and 53.8 nmoles DAHP per min/mg protein were measured in extracts prepared from tryptophan-, tyrosine- and phenylalanine-limited cultures, respectively. Hence, no derepression could be demonstrated under steady-state conditions of end-product limitation, the most rigorous methodology available. (ii) Regulatory mutants which overproduce one of the aromatic end-products in many microorganisms often are derepressed in the synthesis of DAHP synthetase, a physiological consequence of the greater total production of end-products[5]. However, a regulatory mutant which overproduces tryptophan due to constitutive synthesis of tryptophan pathway enzymes has levels of DAHP synthetase which are comparable to those found in wild type extracts; likewise, a mutant which excretes phenylalanine is unaffected in level of DAHP synthetase. (iii) The biosynthesis of aromatic end-products in *P. aeruginosa*

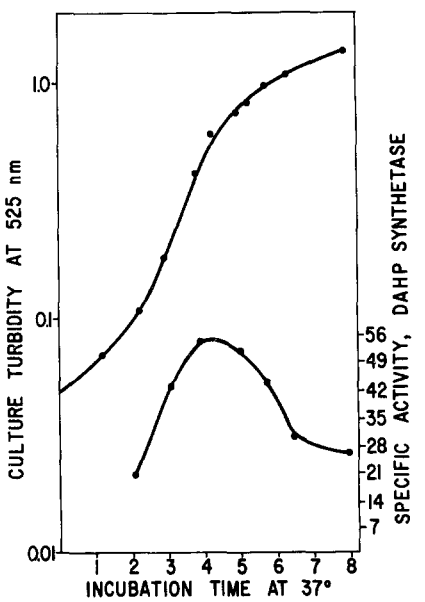

Fig. 5. Variation of DAHP synthetase activity during the growth cycle. A 200 ml volume of minimal glucose medium at 37 °C was inoculated from a broth culture of strain 1 and grown overnight to a turbidity at 525 nm of 1.81. The cells were centrifuged and resuspended as a homogenous suspension in a 10 ml volume of medium. A 3 ml portion of this was used as an inoculum for 2 l of medium in a 4 l Fernbach flask. The culture was incubated with shaking at 37 °C and growth was followed (left ordinate) by turbidity measurements taken at 525 nm in a Gilford spectrophotometer. At intervals, as indicated, 200-ml samples were removed and the cells were harvested by centrifugation. Specific activities of DAHP synthetase (right ordinate, lower curve) in these samples were determined as described under Materials and Methods.

has been shown to vary quantitatively in response to the particular source of carbon present in the medium[16]. Hence, fructose, for example, appears to be a relatively poor source of initial substrate(s) entering the general aromatic pathway. Under such metabolic conditions DAHP synthetase might be expected to derepress in order to compete more effectively for al lower level of such multifunctional metabolites as phosphoenolpyruvate and/or erythrose-4-*P*. However, no derepression of DAHP synthetase occurred when fructose was used in place of glucose as the carbon source.

A repressible DAHP synthetase was initially anticipated because specific activities measured in crude extracts prepared from various strains cultured under a variety of growth conditions varied about two-fold. However, in light of the finding (Fig. 5) that specific activities of samples harvested from various phases of the growth cycle varied about two-fold, it appears that these variations in batch culture extracts can be accounted for as differences in specific activity which occur during the growth cycle.

DISCUSSION

A multi-branched biochemical pathway poses certain complexities with respect to the regulation of the initial enzyme activity, a catalytic function which must somehow respond appropriately to the combined overall level of the end-products produced. This is the case with DAHP synthetase, the first enzyme specifically involved in the eventual synthesis of the three aromatic amino acids from phosphoenolpyruvate and erythrose-4-*P*. The various uni-effector DAHP synthetases[1] contrast with various multi-effector patterns[2] that have received the most attention in branched pathways. *P. aeruginosa* was examined as a representative example of such a paradoxical system. No nutritional conditions were found to support the possible existence of an inadequate regulatory system. Thus, no physiological circumstances could be found which led either to growth inhibition (a consequence of hyper-regulation) or to excretion of either tyrosine or other aromatic compounds (a consequence of hypo-regulation). Hence, the pattern of control in *P. aeruginosa* appears to be efficient at the cellular level, and various novel possibilities were considered for insight into the nature of the allosteric control pattern.

Tyrosine could provide an adequate and dominating control function if it were the "last" end-product formed endogenously and if exogenous tyrosine were impermeable to the cell membrane. However, exogenous tyrosine is known to be actively transported into cells of *P. aeruginosa*[17], a conclusion that is further supported by the existence of nutritional mutants which require tyrosine for growth. Since exogenous tyrosine does not inhibit growth, the excellent inhibition of DAHP synthetase achieved *in vitro* by tyrosine must only be partially realized *in vivo*. However, many leaky aromatic auxotrophs already under nutritional stress due to a partial block in the common pathway of aromatic synthesis are growth-inhibited by tyrosine, but not by phenylalanine or tryptophan. In wild type cell populations the fractions of uninhibited DAHP synthetase activity appears to be sufficient to guarantee enough precursor for phenylalanine and tryptophan synthesis. A sufficiently high internal level of phosphoenolpyruvate would favor a continuing state of incomplete inhibition by tyrosine. The substantial ability of *P. aeruginosa* to degrade tyrosine[8] may be another factor which might act to maintain internal tyrosine levels below some threshold value.

The finding that phenylalanine indirectly regulates the activity of DAHP synthetase via its transamination product, phenylpyruvate, provides evidence for a balanced system in which each of several allosteric metabolites exerts a fractional portion of the overall control function. Hence, in the final analysis the regulation resembles that of many other systems in which both phenylalanine and tyrosine (and to a lesser extent, tryptophan) participate in allosteric control of DAHP synthetase. Perhaps phenylpyruvate is a more stable and constant representative metabolite of its branch of the pathway than phenylalanine because of the degradation of phenylalanine (pseudomonads are unusual among bacteria in their possession of phenylalanine hydroxylase activity[18]). Alternatively, exogenous phenylalanine may be converted rapidly to phenylpyruvate by transamination. It is of further interest in connection with the completeness of allosteric control of DAHP synthetase that even in the presence of excess tyrosine and phenylalanine (or phenylpyruvate), normal amounts of the phenazine pigment[19] and aromatic vitamins (which are all known to be derived from chorismate) are formed.

Although end products such as amino acids, purines or pyrimidines ordinarily are expected to exercise control functions because they are concentrated in intracellular pools, examples of control by intermediary metabolites are not unusual. The control of DAHP synthetase in *Bacillus* species[20] by sequential feedback inhibition[21] is a striking example. The molecular mechanism probably is intimately related to a protein complex of DAHP synthetase and chorismate mutase[22]. Such an organizational arrangement presumably permits allosteric control by the particularly low concentrations of intermediary metabolite(s) associated with a component of the complex as substrate or product. In this context DAHP synthetase in *P. aeruginosa* does not appear to be associated with prephenate dehydratase[15].

The possible existence of undetected labile regulatory isoenzymes of DAHP synthetase seems unlikely in the light of the following findings: (i) The enzyme activity studied is feedback inhibited by three metabolites, a circumstance sufficient to accommodate the overall control of a single enzyme. (ii) In most systems of regulatory isoenzymes, each isoenzyme species is specifically repressible by a different end-product, and this provides a molecular basis for fractional control by each end-product of total activity. A variety of experiments show that in *P. aeruginosa* repression of DAHP synthetase is not produced by end-product supplementation nor is derepression produced by end-product starvation. A non-repressible DAHP synthetase is wholly consistent with the general lack of repression control of biosynthetic enzymes in pseudomonads. (iii) The ability of either tyrosine or phenylpyruvate to almost completely inhibit activity departs from the strict additivity of inhibitor effects noted to characterize various regulatory isoenzyme systems. (iv) DAHP synthetase activity present in crude extracts elutes with good recovery (>95%) from gel filtration columns as a single symmetrical peak[15]. When DAHP synthetase is eluted from Sephadex G-100, sensitivity to inhibition by phenylpyruvate (but not to tyrosine nor to tryptophan) is lost. This desensitization could reflect the separation of an associated regulatory protein or selective denaturation of the allosteric site. Less likely, it could possibly reflect the loss of a protein converting phenylpyruvate to some true inhibitor. Such a conversion would need to be very rapid since no progressive increase of inhibition is found as a function of incubation time during enzyme assay. However, such a situation would not alter the physiological interpretation since inhibition of DAHP

synthetase would still be equated with high levels of intracellular phenylpyruvate.

It now seems probable that a detailed examination of other apparent uni-effector DAHP synthetase systems will reveal complexities in control relationships that, in fact, involve several or all of the multiple end-products of the pathway. Hence, "uni-effector" control may often only be the fortuitous observation of the most obvious allosteric effect, perhaps prejudiced by the particular conditions of *in vitro* assay used. An example of the degree of complexity possible is the DAHP synthetase of *Rhodomicrobium vannielii* described by Jensen and Trentini[16]. In this case conditions of assay can be chosen which give apparent uni-effector control patterns. Phenylalanine and tyrosine are competitive inhibitors with respect to erythrose 4-*P*. Tryptophan, ineffective alone, potentiates the effects of phenylalanine and tyrosine. The ratio of the two substrates has a marked influence upon the reaction velocity. Depending on substrate ratios, tyrosine or phenylalanine can either activate or inhibit enzyme activity. Accordingly, it is possible to choose conditions of substrate and allosteric effector concentration arbitrarily to yield any result between activation and inhibition.

The results of kinetic analyses indicate that tyrosine would be the most effective inhibitor metabolite when the intracellular concentration of phosphoenolpyruvate is low and the concentration of erythrose-4-*P* is high. The converse would hold for phenylpyruvate when the intracellular concentrations of phosphoenolpyruvate and erythrose-4-*P* are high and low, respectively. Since tyrosine is a potent inhibitor which is competitive with phosphoenolpyruvate, it would appear that the most drastic change that could influence the activity of DAHP synthetase would be a decreased intracellular level of phosphoenolpyruvate. Phosphoenolpyruvate is an important high energy compound, and low cellular supplies of phosphoenolpyruvate would reflect deficient energy resources. The allosteric DAHP synthetase of *P. aeruginosa* appears to possess a degree of complexity that may reflect the capability of a common mechanism to respond to both specific end product conditions and to general metabolic conditions.

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