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Alternative Allosteric Effects Exerted by End Products upon a Two-Substrate Enzyme in *Rhodomicrobium vannielii**

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ROY A. JENSEN AND WILLIAM C. TRENTINI‡
From the Department of Microbiology, Baylor College of Medicine, Houston, Texas 77025

SUMMARY

The regulatory enzyme, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase of *Rhodomicrobium vannielii* was studied. Both phenylalanine and tyrosine are competitive inhibitors with respect to the substrate, erythrose 4-phosphate. Tryptophan does not influence enzyme activity *per se*, but potentiates the effect of phenylalanine or tyrosine. Kinetic experiments led to the conclusion that alternative kinetic pathways to the ternary complex existed, and that the one forming (phosphoenolpyruvate-enzyme) as the initial binary complex was kinetically preferred. Accordingly, the ratio of the two substrates had a marked influence upon reaction velocity. The observed ability of phenylalanine and tyrosine to either activate or inhibit enzyme activity, depending upon the ratio of the two substrates was consistent with the postulated mechanism. An extremely wide range of enzyme activity exists, on the one hand, because of the stimulation of activity by one or two amino acid end products, and on the other hand, because of the potent concerted inhibition in the simultaneous presence of all three amino acids. The physiological consequences of this pattern of control for 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase are discussed.

Allosteric regulation in branched metabolic pathways is relatively complex. Multiple end product derivatives of such pathways control the activity of the enzyme catalyzing the initial reaction of the pathway in a number of alternative ways. The biochemical route of synthesis for aromatic amino acids illustrates such a branching pathway. Various allosteric patterns of control for 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase were previously described with data collected from a large number of bacterial genera (1). One of the infrequent patterns, found in *Rhodomicrobium vannielii*, was one in which aromatic end products acted together synergistically to produce concerted feedback inhibition.

Concerted feedback inhibition was originally described by Datta and Gest (2) and by Paulus and Gray (3) for other pathways of different microorganisms. It describes an allosteric pattern in which multiple end products act in synergistic combination to produce feedback inhibition. Individual end products characteristically produce little or no feedback inhibition. The concerted influence of the combination of multiple end products to produce inhibition is even more striking for DAHP[1] synthetase in *R. vannielii* because when one or more of the end products are absent, individual end products potentially can stimulate enzyme activity.

MATERIALS AND METHODS

Cultivation of Organism—R. vannielii (strain C-2859) was grown in the minimal medium (YE^-) of Trentini and Starr (4) and Trentini (5), modified to contain 0.1% sodium lactate and 0.1% tris(hydroxymethyl)aminomethane. Supplemented media were obtained by additions to YE^- to give the following final concentrations: 0.1% Difco yeast extract (YE^+); phenylalanine, tyrosine, or tryptophan, 50 μg per ml; and a mixture containing 50 μg per ml each of phenylalanine, tyrosine, and tryptophan, plus 0.5 μg per ml each of *p*-aminobenzoic acid and *p*-hydroxybenzoic acid. Stock cultures were kept at 4° following growth in anaerobic roll tubes in either YE^- or YE^+. Experimental cultures were grown in either large test tubes or liter bottles immersed in the center of a constant temperature water bath held at 34° ± 0.1°. The rectangular bath was illuminated through the short axis by a Sylvania R-32 movie lamp placed on opposite sides of the bath in order to give a total incident light reading of 850 foot candles at the bath's center. The voltage was kept constant at 65 volts of alternating current. The entire growth area was contained in a ventilated box, free from extraneous light. Anaerobiosis, agitation, and CO_2 supply were accomplished by sparging the cultures with 95% N_2-5% CO_2. The media was initially sparged for 1 to 2 hours prior to inoculation to allow for pH and gas equilibrations. Cultures were allowed to reach maximum growth rates for the appropriate growth condition by diluting them before shading densities were reached. In this manner at least 10 doublings and two transfers occurred before experimental vessels were inoculated. Cells used in these experiments were permitted to grow beyond shading densities and were harvested in mid or late exponential phase of growth.

Preparation of Extracts—Whole cell pellets obtained by centrifugation were stored for durations up to 8 months at −80° without loss of DAHP-synthetase activity. Preliminary experi-

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‡ Present address, Department of Biology, Mount Allison University, Sackville, New Brunswick, Canada.

[1] The abbreviation used is: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

ments revealed a decrement of enzyme activity in cell-free extracts compared to the activity measured in whole cell preparations which were treated with toluene to make the cells freely permeable to small molecules. However, the addition of 10^{-3} M glutathione to a 0.04 M potassium phosphate buffer, pH 6.8, containing 0.1 M KCl, yielded stable enzyme preparations which were comparable in specific activity to that of whole cell preparations. Cells were disrupted by three 1-min bursts of sonic oscillation with the use of a Müller apparatus. Small molecules were removed by passing the extract through Sephadex G-25 at 4°. Cell-free extracts were stable at −80° for at least several months in the presence of glutathione.

Analytical Procedures—DAHP synthetase activity was assayed by a colorimetric determination of DAHP produced by the enzymatic condensation of erythrose-4-P and P-enolpyruvate (6). The reaction mixture contained 8 μmoles of phosphate buffer, pH 6.8, 0.2 μmole of glutathione, and 20 μmoles of KCl plus the indicated amounts of erythrose-4-P and P-enolpyruvate in a final volume of 0.2 ml. Reaction times were 20 min unless otherwise indicated. A molar extinction coefficient of 4.5×10^4 at 549 nm was used for DAHP. Other procedural details were carried out as previously described (7). Erythrose-4-P is unstable and its decay was monitored in order to permit concentration corrections by use of reference standards as previously described (7).

Protein concentrations were estimated by the method of Lowry *et al.* (8) with bovine serum albumin as a reference standard. With whole cell preparations this procedure was preceded by hydrolysis at 100° for 10 min after the sample was made 0.2 N in NaOH.

Whole Cell Enzyme Assays—Culture samples, usually 100 ml in volume, were centrifuged and washed twice with 10 ml of 0.04 M potassium phosphate in 0.10 M KCl, pH 6.8. Cell suspensions were concentrated by resuspending the cell pellet obtained from the final centrifugation in 0.5 ml of buffer. The cells were shaken at 37° with 0.1 ml of toluene for 10 min and then assayed in the usual manner for DAHP synthetase activity.

Chemicals—All biochemicals were purchased from Sigma except for amino acids and erythrose-4-P which were obtained from Calbiochem. All chemicals were of reagent quality.

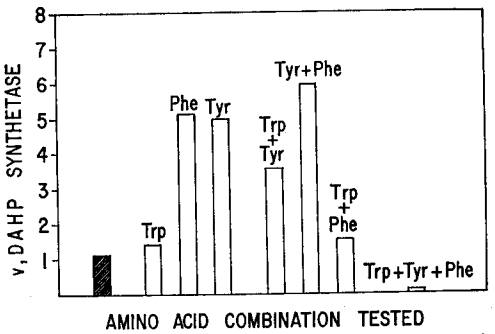

FIG. 1. The effect of aromatic amino acids upon reaction velocity of DAHP synthetase in *R. vannielii*. Each reaction mixture contained P-enolpyruvate and erythrose-4-P at final concentrations of 2.0 mM (conditions of substrate inhibition by erythrose-4-P). Reaction velocity, *v*, is expressed as nanomoles of DAHP formed per min per ml of reaction mixture. The *hatched bar* indicates *v* in the control reaction lacking amino acids. Amino acids (tryptophan (*Trp*), tyrosine (*Tyr*), and phenylalanine (*Phe*)) were added to a final concentration of 0.2 mM. A crude extract from a culture grown in a minimal medium was prepared by sonic disruption. Each reaction mixture contained 125 μg of protein.

RESULTS

Preliminary Enzymology—The assay for DAHP synthetase activity was done under conditions of proportionality with respect to reaction time and protein concentration. The pH optimum was determined to be 6.8 from a pH curve which showed a broad peak between 6.6 and 7.4. Activity was stable at 37° for at least 30 min. Inorganic phosphate inhibited activity 50% at a concentration of 0.5 M. However, there was no significant inhibition by the phosphate concentration used in the buffer (0.04 M). In addition, the inorganic phosphate generated as a product of the enzyme reaction was at least an order of magnitude lower than inhibitory concentrations. Chorismate and prephenate (2 mM) were not feedback inhibitors of DAHP synthetase (9) in *R. vannielii*. Enzyme activity also was not influenced by *p*-hydroxybenzoate, *p*-aminobenzoate, 2,3-dihydroxybenzoate, or 3,4-dihydroxybenzaldehyde.

Concerted Feedback Inhibition—Fig. 1 illustrates in histogram form the effect of aromatic amino acids upon the activity of DAHP synthetase in *R. vannielii*. Both phenylalanine and tyrosine stimulated enzyme activity about 5-fold at concentrations of 0.2 mM. Tryptophan at 0.2 mM also had a slight stimulative effect. A mixture of tyrosine and phenylalanine were somewhat more stimulative than either alone. Tryptophan, in combination with either phenylalanine or tyrosine, decreased the activating influence of the latter two amino acids. The combination of all three aromatic amino acids acted in a concerted fashion to produce a potent inhibition of enzyme activity (about 85% inhibition at 0.2 mM concentrations of each amino acid).

Kinetic Analysis—The kinetic behavior of DAHP synthetase in *R. vannielii* was examined as a basis for understanding the unusual pattern of amino acid inhibition shown in Fig. 1. The enzyme is strongly inhibited by high concentrations of erythrose-4-P. This is shown in Fig. 2 in which reaction velocity is followed as a function of erythrose-4-P as the variable substrate at several fixed concentrations of P-enolpyruvate. The concentration of erythrose-4-P required to produce substrate inhibition was increased as the fixed concentration of P-enolpyruvate was increased. It appears that an optimum ratio of P-enolpyruvate to erythrose-4-P exists with a value of about 2.

The same effect of substrate ratios was observed from the plot of reaction velocity as a function of P-enolpyruvate as the variable substrate (Fig. 3). Erythrose-4-P fixed at 2 mM was inhibitory at the highest concentration of P-enolpyruvate used

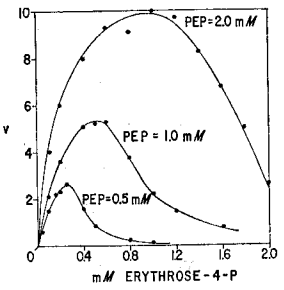

FIG. 2. Substrate saturation curve for DAHP synthetase of *R. vannielii*. Reaction velocity, *v*, is expressed as nanomoles of DAHP formed per min per ml of reaction mixture. The concentration of the variable substrate, erythrose-4-P, is given on the *abscissa*. P-enolpyruvate (*PEP*) was used as the fixed substrate at the three concentrations shown. A crude extract from a culture grown in YE^+ medium was prepared by sonic disruption. The reaction mixture contained 105 μg of protein.

(2 mM) and became increasingly inhibitory at lower concentrations of P-enolpyruvate. In Fig. 3 one can find a particular concentration of P-enolpyruvate for which each of the fixed erythrose-4-P concentrations (1.0, 0.4, or 0.2 mM) is the most active. The latter reflects the crucial influence of substrate ratios upon enzyme activity. When P-enolpyruvate was the variable substrate, the substrate saturation curves were sigmoid in shape. As the fixed concentration of erythrose-4-P was decreased, the distance along the abscissa between the ordinate and the point of inflection of the rate curve was decreased. The inhibition at high concentrations of erythrose-4-P in Fig. 2 and the decreased activity at low concentrations of P-enolpyruvate in Fig. 3 (*i.e.* in the early sigmoid portion of the curve) both document the necessity for a favorable ratio of P-enolpyruvate to erythrose-4-P to permit maximum catalytic activity.

The data of Fig. 2 were replotted in double reciprocal form as shown in Fig. 4. Points on the portions of curves showing substrate inhibition were omitted from Fig. 4. The *inset* shows a secondary plot of the extrapolated ordinate values (v'^{-1}) as a function of the reciprocal of the various fixed concentrations of P-enolpyruvate used (PEP^{-1}).

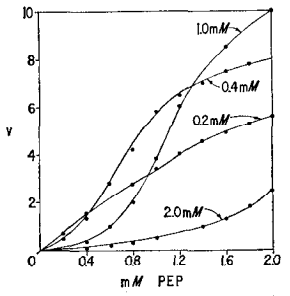

FIG. 3. Substrate saturation curve for DAHP synthetase of *R. vannielii*. Reaction velocity, *v*, is expressed as nanomoles DAHP formed per min per ml of reaction mixture. The concentration of the variable substrate, P-enolpyruvate (*PEP*), is shown on the *abscissa*. The concentrations of erythrose-4-P (fixed variable) used are identified on the curves. Aliquots of the same extract described in Fig. 2 were used.

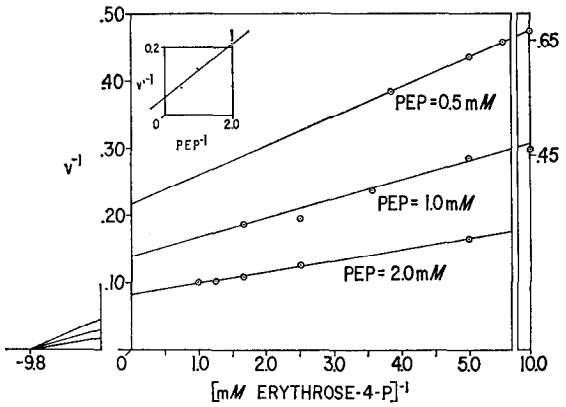

FIG. 4. Double reciprocal plot of reaction velocity as a function of erythrose-4-P. P-enolpyruvate (*PEP*) was maintained constant at three different concentrations as indicated on the curves. The data shown in Fig. 2 were replotted with points from the first order portions of the curves. Reciprocals of the initial reaction velocities (v^{-1}) are given on the same scale along both *left* and *right ordinates*. (However, note that the *right ordinate* has been adjusted to account for the foreshortening of the *abscissa*.) The *inset* is a secondary plot of the reciprocal of the apparent V_{max} values against the reciprocal of P-enolpyruvate concentration.

A V_{max} value of 21.7 nmoles of DAHP formed per min per mg of protein was determined by extrapolation to the *ordinate* of the *inset*. Since the substrate saturation curves shown in Fig. 3 deviate from Michaelis-Menten kinetics, replots of these data analogous to those shown in Fig. 4 are not shown.

Kinetic Influence of Phenylalanine and Tyrosine—Phenylalanine is a competitive inhibitor of DAHP synthetase with respect to erythrose-4-P, as is clearly shown in Fig. 5. Substrate saturation curves done in the presence of several concentrations of phenylalanine show the alteration of the apparent K_m for erythrose-4-P. The double reciprocal plot produced lines which passed through the same velocity extrapolation on the *ordinate*. The influence of phenylalanine upon the normal substrate saturation curves shown in Figs. 2 and 3 are illustrated in Figs. 6 and 7. When erythrose-4-P was the variable substrate, phenylalanine displaced the curve to the right. Since phenylalanine decreases the apparent concentration of erythrose-4-P, a higher concentration of erythrose-4-P was required to produce substrate inhibition. Therefore, at high erythrose-4-P concentrations, phenylalanine had an activating effect. At low concentrations of erythrose-4-P, phenylalanine was inhibitory. Similarly, Fig. 7, phenylalanine activates enzyme activity at low P-enolpyruvate concentrations and inhibits activity at high P-enolpyruvate concentrations. Hence, phenylalanine potentially can produce activating or inhibitory consequences, depending upon the ratio of substrates that are present. Data obtained with tyrosine were nearly identical with those shown for phenylalanine. Substrate saturation curves done in the presence of tryptophan nearly superimposed with those determined in the absence of tryptophan. Inhibition found in the presence of the combination of tryptophan, tyrosine, and phenylalanine was also competitive with respect to erythrose-4-P.

Phenylalanine Inhibition Curves—Phenylalanine inhibition (or activation) curves determined in the presence of inhibitory concentrations of erythrose-4-P may show peculiar shapes which change in direction. This is illustrated by Table I which shows reaction velocities in the presence of phenylalanine taken from

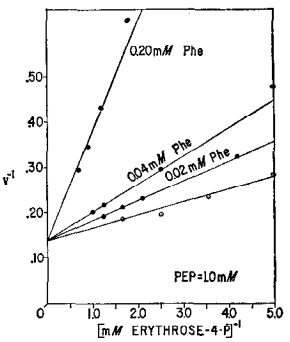

FIG. 5. Competitive inhibition of DAHP synthetase in *R. vannielii*. Double reciprocal plot of reaction velocity as a function of erythrose-4-P concentration in the presence of phenylalanine (*Phe*) and in the absence of phenylalanine (⊙). The final concentration of phenylalanine in the reaction mixture is identified on each curve. P-enolpyruvate (*PEP*) was constant at 1.0 mM. The same extract and protein concentration specified in Fig. 2 were used. Note that only points from the first order portion of the substrate saturation curve (Fig. 6) are included. At high concentrations of erythrose-4-P, phenylalanine stimulates activity so that curves expressing velocity in the presence of phenylalanine cross the *control curve* which swings sharply upward as the result of substrate inhibition.

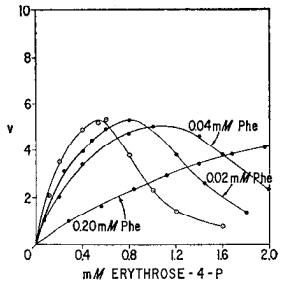

FIG. 6. Influence of phenylalanine on the substrate saturation curve with erythrose-4-P as the variable substrate. The concentration of P-enolpyruvate (*PEP*) was maintained constant at 1.0 mM. The rate curves obtained in the absence of phenylalanine (○) and in the presence of several phenylalanine concentrations (●) are plotted. The same extract and protein concentration specified in Fig. 2 were used.

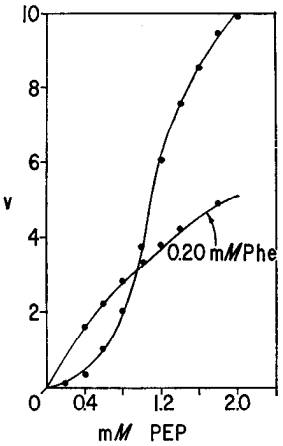

FIG. 7. Influence of phenylalanine (*Phe*) on the substrate saturation curve with P-enolpyruvate (*PEP*) as the variable substrate. The concentration of erythrose-4-P was maintained constant at 1.0 mM. The same extract and protein concentration specified in Fig. 2 were used.

the data of Fig. 6 at four concentrations of erythrose-4-P. For example, at an erythrose-4-P concentration of 1.2 mM with P-enolpyruvate constant at 1.0 mM, phenylalanine stimulates activity more at 0.04 mM than at either 0.02 or 0.20 mM. Likewise, a concentration of erythrose-4-P can be found for which each of the other two phenylalanine concentrations is the most stimulative. In general, curves which express reaction velocity as a function of phenylalanine (or tyrosine) concentration at an inhibitory concentration of erythrose-4-P will pass through a velocity maximum. The concentration of phenylalanine required to achieve the velocity maximum will increase as the ratio of erythrose-4-P to P-enolpyruvate is increased, as indicated in Table I.

Repression of Synthesis of DAHP Synthetase—The specific activity of DAHP synthetase was nearly identical regardless of whether extracts were prepared from cells grown in a minimal medium or from cells grown in a yeast extract medium (Table II). The addition of aromatic amino acids, individually, or in combination to minimal medium did not decrease the specific activity of DAHP synthetase. Therefore, the enzyme is either constitutive or is already maximally repressed upon conditions of growth in minimal medium.

TABLE I

Biphasic influence of phenylalanine concentration on reaction velocity

These data were calculated from the results plotted in Fig. 6.

Concentration of erythrose-4-P	Relative reaction velocity[a] at phenylalanine concentration of:		
	0.02 mM	0.04 mM	0.20 mM
mM			
0.4	0.80	0.69	0.27
0.8	1.39	1.29	0.39
1.2	2.71	3.71	2.21
1.6	2.50	4.75	4.75

[a] Reaction velocities are expressed as relative numbers by comparison with the velocity of the corresponding control point in Fig. 6.

TABLE II

Repression of DAHP synthethse in R. vanniclii

Cells were grown as described under "Materials and Methods," harvested, and assayed for DAHP synthetase activity in cell-free extracts. Specific activities, calculated as units per min per mg of protein were arbitrarily normalized to the specific activity obtained after growth in a minimal medium. Final concentrations of protein in reaction mixtures ranged from 0.15 to 0.25 mg/0.2 ml.

Growth condition	Relative specific activity
Minimal	1.0
Minimal + phenylalanine	1.1
Minimal + tyrosine	0.9
Minimal + tryptophan	1.2
Minimal + Aro[a]	1.2
Yeast extract	1.1

[a] Mixture of aromatic metabolites (see "Materials and Methods").

DISCUSSION

Sigmoid substrate saturation curves have often been taken to implicate cooperative interactions between enzyme subunits. Such non-Michaelian rate curves seem to be a common characteristic of regulatory enzymes. Allosteric effectors may accentuate the cooperativity of the substrate saturation curve, while activators produce rate curves which approach the form of a rectangular hyperbola. Such a situation was observed for the DAHP synthetase of *R. vannielii*. Fig. 7 illustrates a sigmoid substrate saturation curve with P-enolpyruvate as the variable substrate. Phenylalanine (or tyrosine) converts the sigmoid-shaped rate curve to one of near hyperbolic shape. However, it is possible to explain all of the kinetic data on the basis of the mechanism for DAHP synthetase, a two-substrate enzyme.

If one considers a mechanism in which there are two permissible kinetic pathways to a ternary complex, then the following sequences of interactions would occur.

$$\text{(Ery-4-P)} + (E) \rightarrow \text{(Ery-4-P—}E\text{)} \xrightarrow{\text{(PEP)}} \text{(Ery-4-P—}E\text{—PEP)} \rightarrow \text{products} \quad (1)$$

$$(E) + \text{(PEP)} \rightarrow (E\text{-PEP}) \xrightarrow{\text{(Ery-4-P)}} \text{(Ery-4-P—}E\text{—PEP)} \rightarrow \text{products} \quad (2)$$

where *E* is DAHP synthetase, PEP is phosphoenolpyruvate, and Ery-4-P is erythrose 4-phosphate. If one further assumes that Equation 2 expresses a sequence that is preferred kinetically to that expressed by Equation 1, then one expects substrate saturation curves which are consistent with those of Figs. 2 and 3. A high ratio of erythrose-4-P to P-enolpyruvate will favor Sequence 1 above since the probability of forming the binary complex, erythrose-4-P-*E*, will be high. Similarly, a high ratio of P-enolpyruvate to erythrose-4-P will favor the formation of binary complex *E*-P-enolpyruvate which initiates the kinetically preferred pathway of Sequence 2 above. Ferdinand (10) has suggested that the existence of alternate kinetic pathways could account for the substrate saturation curves found for phosphofructokinase, a two-substrate enzyme which has a striking similarity to DAHP synthetase in this respect.

Hence, erythrose-4-P potentially is inhibitory to the over-all rate of the reaction if its concentration sufficiently exceeds that of P-enolpyruvate. When erythrose-4-P is the variable substrate in a rate curve determination, this effect manifests itself as substrate inhibition. Inhibition occurs when the variable concentration of erythrose-4-P is elevated sufficiently beyond the fixed concentration of P-enolpyruvate. On the other hand, when P-enolpyruvate is the variable substrate, inhibition is observed at low P-enolpyruvate concentrations, producing a sigmoid curve. The point of inflection marks the shift from the nonpreferred pathway at low P-enolpyruvate concentrations to the kinetically preferred pathway at high P-enolpyruvate concentrations. The point of inflection of a series of rate curves is, of course, shifted to the left as lower fixed concentrations of erythrose-4-P are used.

Phenylalanine and tyrosine are competitive inhibitors of the DAHP synthetase reaction with respect to erythrose-4-P. Tryptophan potentiates these specificities of tyrosine and phenylalanine strongly, although it has little effect alone. Because the reaction velocity is so sensitive to substrate ratios, phenylalanine or tyrosine potentially can either increase or decrease the reaction velocity. When P-enolpyruvate is present in excess, phenylalanine or tyrosine will increase the rate by decreasing the apparent concentration of erythrose-4-P. Our observation of biphasic inhibition curves (see Table I) is consistent with this mechanism. In either case the presence of all three amino acids affects the binding of erythrose-4-P so severely that the consequence is a potent inhibition. These studies reinforce the point made by Atkinson and Walton (11) that the complexities inherent in two-substrate enzyme reactions can account for "allosteric" phenomena without necessarily involving protein-protein interactions.

Extrapolation of these results to the situation *in vivo* depends upon the intracellular concentrations of erythrose-4-P and P-enolpyruvate, a matter of speculation. If P-enolpyruvate were always in excess in the cell, then the regulatory situation would be a modified concerted feedback inhibition (2, 3) in which two end products inhibit enzyme activity individually, and these effects are strongly potentiated in the presence of all three end products. On the other hand, if erythrose-4-P has a relatively high intracellular concentration, then DAHP synthetase activity possesses a wide range of activity, depending upon the combinations of aromatic amino acids in contact with the enzyme. The potential ambivalent effects mediated by aromatic amino acids depending upon the existing substrate ratios seems to be consistent with economy at the physiological level. Under conditions where aromatic amino acid synthesis is limiting to growth, it is probable that one end product will be the restrictive metabolite. By definition, the other two will be in relative excess. This would therefore stimulate DAHP synthetase activity, and would accelerate the formation of the limiting amino acid. Eventual excess of all end products would feedback inhibit DAHP synthetase strongly, and economically curb the further entry of metabolites into the pathway. The increased range of DAHP synthetase activity owing to the ability of end products to act as both positive and negative effectors may be significant in view of the possibility that the enzyme may not be subject to repression control.

Another important possibility is that the ratios of P-enolpyruvate and erythrose-4-P may reflect an influence of intracellular energy levels upon the over-all activity in the aromatic amino acid pathway. P-enolpyruvate, of course, is an important high energy compound. Low P-enolpyruvate concentrations would reflect deficient cellular energy resources, and would depress the activity level of DAHP synthetase. Hence, the system would appear to be equipped to respond to both general metabolic conditions and to specific end product conditions through the same mechanism.

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