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# Differentially Regulated Isozymes of 3-Deoxy-D-*arabino*-Heptulosonate-7-Phosphate Synthase from Seedlings of *Vigna radiata* [L.] Wilczek[1]

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JUDITH L. RUBIN AND ROY A. JENSEN*
*Center for Somatic-cell Genetics and Biochemistry, State University of New York at Binghamton, Binghamton, New York 13901*

## ABSTRACT

**Two isozymes of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (EC 4.1.2.15) designated DS-Mn and DS-Co were separated from seedlings of *Vigna radiata* [L.] Wilczek by DEAE-cellulose column chromatography. DS-Mn was activated 2.6-fold by 0.4 millimolar manganese, had an activity optimum of 7.0, and was substrate inhibited by erythrose-4-phosphate (E4P) concentrations in excess of 0.5 millimolar. In contrast, DS-Co had an activity optimum at pH 8.8, required E4P concentrations of at least 4 millimolar to approach saturation, and exhibited an absolute requirement for divalent cation (cobalt being the best). Regulatory properties of the two isozymes differed dramatically. The activity of DS-Mn was activated by chorismate (noncompetitively against E4P and competitively against phosphoenolpyruvate), and was inhibited by prephenate and L-arogenate (competitively against E4P and noncompetitively against phosphoenolpyruvate in both cases). Under standard assay conditions, L-arogenate inhibited the activity of DS-Mn 50% at a concentration of 155 micromolar and was at least 3 times more potent than prephenate on a molar basis. Weak inhibition of DS-Mn by L-tryptophan was also observed, the magnitude of inhibition increasing with decreasing pH. The pattern of allosteric control found for DS-Mn is consistent with the operation of a control mechanism of sequential feedback inhibition governing overall pathway flux. DS-Co was not subject to allosteric control by any of the molecules affecting DS-Mn. However, DS-Co was inhibited by caffeate (3,4-dihydroxycinnamate), noncompetitively with respect to either substrate. The striking parallels between the isozyme pairs of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase and chorismate mutase are noted—one isozyme in each case being tightly regulated, the other being essentially unregulated.**

The first enzyme of the shikimate pathway, DAHP[2] synthase [EC 4.1.2.15], catalyzes the condensation of PEP and E4P to form DAHP. L-Phenylalanine, L-tyrosine, and L-tryptophan are major aromatic endproducts of this multibranched biosynthetic pathway in bacteria, fungi, and plants. In higher plants, these amino acids serve as precursors for a vast array of secondary metabolites such as alkaloids, flavonoids, lignin precursors, coumarins, indole derivatives, and other phenolic compounds (12).

In mung bean we showed (25) that total DAHP synthase activity was distributed between two separable and quite distinctive isozymes. One isozyme (DS-Co) had an absolute requirement for divalent cation (cobalt being preferred). The second isozyme (DS-Mn) was stimulated by, but did not require, manganese. Although *Nicotiana silvestris* is not closely related to *V. radiata*, a pair of isozymes similar to the *V. radiata* isozymes were noted in *N. silvestris* (7, 8). This latter uniformity of isozyme makeup is not reflected, however, by descriptions in the literature of DAHP synthase from corn (14), cauliflower (15), pea (20, 22, 23), tea (26), and carrot (28). Since we have found that assay conditions that favor the optimal detection of one isozyme are inadequate for detection of the second isozyme and vice versa, it is quite possible that this two-isozyme system may have escaped recognition in some other studies. For example, although a single enzyme that appears similar to DS-Mn was found in cauliflower (15) and in carrot (28), the assay conditions used would have masked the possible presence of a coexisting DS-Co.

A general perception appears to have developed that DAHP synthase in higher plants is not subject to allosteric control (12). This has followed from the general finding that aromatic amino acids fail to inhibit DAHP synthase activity and because the midpathway intermediate, shikimic acid, may accumulate to high concentrations in some plant tissues (31). In a few cases where some evidence for allosteric regulation has been found *e.g.* inhibition by L-tryptophan (14), inhibition by L-tyrosine (22) or activation by either L-tryptophan or L-tyrosine (28), effects were rather weak and not easily reconciled with any pattern that equates with efficient overall pathway control. Against this background of little evidence for allosteric regulation at the level of DAHP synthase, it is nevertheless almost heretical to envision early-pathway feedback control to be absent. As discussed previously (16), lack of early-pathway control would be especially unusual where late-pathway control circuits are known to exist. In prokaryotes, DAHP synthase is in fact an enzyme that provides premier examples where allosteric regulation was initially thought to be absent until new control patterns such as sequential feedback inhibition and concerted feedback inhibition (see 18 for review) were recognized.

Since the distinctly different isozymes of DAHP synthase in *V. radiata* suggested to us a vehicle for some form of differential regulation, we have carried out a detailed evaluation of possible allosteric sensitivities of DS-Mn and DS-Co to a variety of possible effector molecules.

## MATERIALS AND METHODS

**Plant Material.** Seeds of *Vigna radiata* [L.] Wilczek were germinated as previously described (24). Four-d-old seedlings

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[2] Abbreviations: DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; PEP, phosphoenolpyruvate; E4P, erythrose-4-phosphate.

were frozen intact in liquid $N_2$ and ground to a fine powder in a Waring Blendor. The powder was stored at −80°C prior to the preparation of crude extract.

**Column Chromatography.** Crude extract was prepared and desalted as previously described (25). Chromatography of crude extract (331 mg protein) on DEAE-cellulose, and further purification of DS-Co via hydroxylapatite column chromatography was conducted as previously described (25) except that a salt gradient of 450 ml was applied to a 2.5 × 10-cm column of hydroxylapatite, and fractions of 3.4 ml were collected.

**Enzyme Assays.** Phosphatase activity was assayed by measuring formation of *p*-nitrophenol from *p*-nitrophenylphosphate, as described previously (25). DAHP synthase was assayed by the method of Srinivasan and Sprinson (27). Reaction mixtures (200 μl) for DS-Co contained 2 mM PEP, 4 mM E4P, 1 mM $CoCl_2$, 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid buffer (pH 8.8), and enzyme. For the assay of DS-Mn, reaction mixtures contained 2 mM PEP, 0.5 mM E4P, 0.4 mM $MnCl_2$, 50 mM Pipes buffer (pH 7.0), 0.2 M NaF, and enzyme, unless otherwise noted.

All effectors that were found to inhibit or activate the DAHP synthase isozymes were examined for possible interference with the chemical assay for DAHP. Authentic DAHP for use as a standard was prepared enzymically using DAHP synthase-Tyr from *Pseudomonas aeruginosa*, partially purified as described previously (29), and enzyme was removed by ultrafiltration (PM-10 membrane, Amicon). Prephenate, L-arogenate, and chorismate (0.025–2.5 mM) did not reduce chromagen formation in the assay when tested in the presence of 50 nmol of DAHP. Although caffeate did diminish chromagen formation slightly (5% decrease at 0.2 mM), it was concluded that inhibition of DS-Co by caffeate was not artifactual since an equivalent concentration of caffeate (0.2 mM) caused enzyme inhibition that was well over an order of magnitude greater than what could be accounted for by inhibition of the chemical assay for DAHP. The known ability of L-tryptophan to inhibit the chemical assay for DAHP was offset by use of controls where appropriate concentrations of L-tryptophan were added to reaction mixtures following the termination of the enzymic reaction but prior to the chemical determination of enzymically produced DAHP.

**Protein Determination.** Protein concentration was determined by the method of Bradford (2) using BSA as standard.

**Materials.** Chorismate (87% pure) was prepared from culture supernatants of *Klebsiella pneumoniae* 62-1 according to the method of Gibson (9). Barium prephenate (91% pure) was prepared from culture supernatants of an L-tyrosine auxotroph of *Salmonella typhimurium* (6) and was converted to the potassium salt with excess $K_2SO_4$ prior to use. L-Arogenate was prepared from culture supernatants of a triple auxotroph of *Neurospora crassa* ATCC 36373 (19) and was prepared according to the method of Zamir *et al.* (32).

Caffeic acid, periodic acid, and thiobarbituric acid were from Aldrich Chemical Co. DEAE-cellulose (DE52) and hydroxylapatite (Bio-Gel HTP), were obtained from Whatman Ltd. and Bio-Rad Laboratories, respectively. Sephadex G-25, XAD-4, and all biochemicals not specified above were obtained from Sigma Chemical Co.

## RESULTS

**Chromatographic Separation of DAHP Synthase Isozymes.** DEAE-cellulose chromatography of *V. radiata* crude extract yielded two stable peaks of DAHP synthase activity, designated DS-Mn and DS-Co (Fig. 1). We previously showed (25) that DS-Co coeluted with one isozyme of chorismate mutase, and Figure 1 also shows considerable contamination of DS-Co with phosphatase activity. Further purification of DS-Co via hydroxylapatite column chromatography yielded an enzyme preparation that was completely resolved from all phosphatase and chorismate mutase activity (Fig. 2). This served as the source of DS-Co for all experiments; specific activity was 206 nmol/min·mg at 37°C.

DS-Mn from DEAE-cellulose overlapped shikimate dehydrogenase activity as previously noted (25) and was also contaminated with considerable phosphatase activity (Fig. 1). Attempts to resolve DS-Mn from phosphatase activity via a variety of additional chromatographic techniques were unsuccessful due to apparent enzyme instability. Therefore, the DS-Mn preparation recovered from DEAE-cellulose was the source of this isozyme for all experiments. To minimize interference by contaminating phosphatase activities present in this enzyme preparation, 0.2 M NaF was included in all assays unless specified otherwise. The presence of this inhibitor decreased phosphatase activity by 90%. It is of interest that in crude extract preparations DS-Mn activity was stimulated 2.3-fold in the presence of 0.2 M NaF, while DS-Co activity was stimulated only slightly under these conditions. Since E4P levels are 8-fold lower in DS-Mn assay protocols, the NaF stimulation may reflect vulnerability of E4P to phosphatase-catalyzed cleavage.

The partial purification of the two isozymes is summarized in Table I. The apparent activity of DS-Co in crude extracts is probably underestimated, perhaps because of interference by phosphatase activity. Thus, more than the apparent starting activity was recovered following both chromatography steps, and it may be that the actual purification was several-fold less than shown. On the other hand, the apparent activity of DS-Mn in crude extracts is probably overestimated, primarily because of the ability of the DS-Co isozyme (see later section) to function to a limited extent in the presence of manganese. Thus, the actual recovery and purification of DS-Mn was probably significantly greater than indicated by a comparison of the values shown in Table I.

**Differential Characteristics of DAHP Synthase Isozymes.** DS-Mn was relatively unstable in crude extracts, in contrast to DS-Co. After storage of crude extract at 0°C for 24 h, DS-Mn and DS-Co lost 79 and 11% of their original activities, respectively.

Characterization of partially purified DS-Mn and DS-Co revealed additional differential properties of these two isozymes. DS-Mn displayed activity in the absence of added divalent cation, but was activated 2.6-fold under standard assay conditions in the presence of 0.4 mM $Mn^{2+}$ (Fig. 3). In contrast, DAHP synthase-Co was inactive in the absence of divalent cations, and concentrations of $Co^{2+}$ in the range of 0.5 to 1.0 mM yielded maximal activity. Both isozymes showed steeply declining activities as cation concentrations were increased above the optimal level (Fig. 3). The requirement of DS-Co for $Co^{2+}$ could be partially replaced by $Mg^{2+}$ and $Mn^{2+}$ (chloride salts). In the presence of 5 mM $Mg^{2+}$ or 1 mM $Mn^{2+}$, relative activities of 71 or 17%, were obtained, respectively. No inhibition of enzyme activity was noted with $Mg^{2+}$ concentrations up to 12 mM.

The two isozymes differed significantly with respect to pH optima for activity at 37°C. The pH optimum for DS-Mn was 7.0, while the pH optimum for DS-Co was 8.8 (Fig. 4). Each isozyme exhibited no more than 25 to 30% of maximal activity when assayed at the pH optimum of the other isozyme.

An additional differential characteristic of these two isozymes was the substrate saturation curve with respect to E4P (Fig. 5). With DS-Mn, concentrations of E4P in excess of about 0.5 mM caused substrate inhibition. In contrast, significantly higher concentrations of E4P were required for saturation of DS-Co, and substrate inhibition was not observed even at high E4P concentrations. Thus, at 0.5 mM E4P (optimal for DS-Mn), DS-Co only achieved about 40% of its potential activity. At 6.0 mM E4P (saturating for DS-Co), DS-Mn activity was diminished to about 38% of its maximum due to substrate inhibition. Substrate

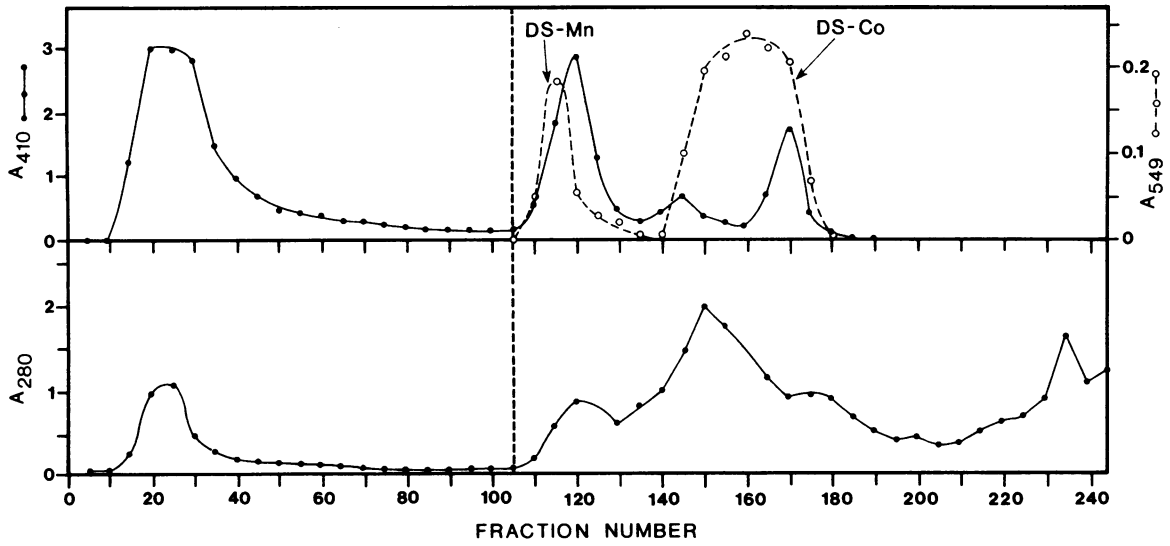


FIG. 1. DEAE-cellulose column chromatography resolved two peaks of DAHP synthase (- - - -, top). DS-Mn and DS-Co eluted at 12 and 145 mM KCl, respectively. A major peak of phosphatase activity eluted in wash fractions (——, top), while three additional peaks eluted in gradient fractions. The bottom panel depicts the protein profile ($A_{280}$). Phosphatase activity is expressed as $A_{410}$ = 1.0 = 308 nmol/min·4.2-ml fraction. DAHP synthase activity is expressed as $A_{549}$ = 1.0 = 186.5 nmol/min·4.2-ml fraction.

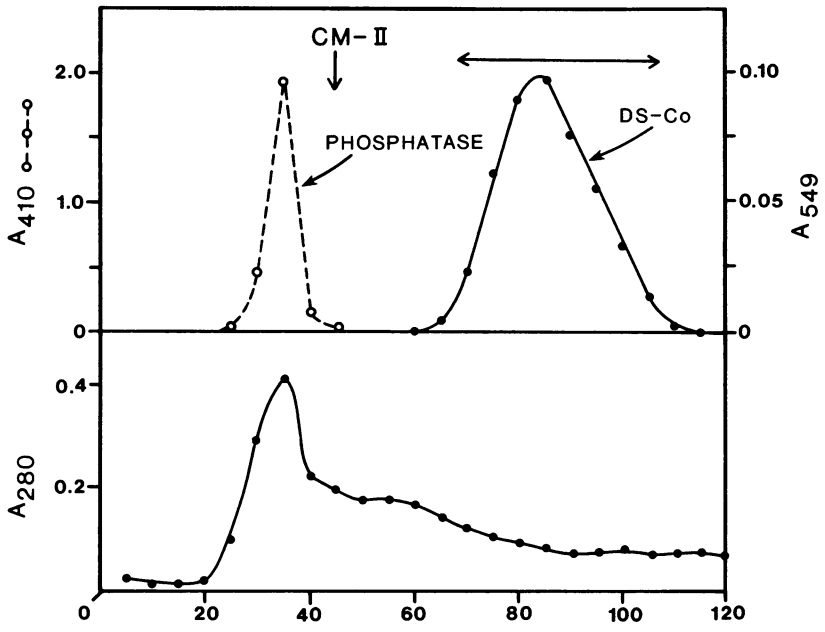


FIG. 2. The peak position of chorismate mutase-II is shown by the arrow. Fractions containing DS-Co were pooled as indicated by the horizontal double-arrowed line. Phosphatase activity is expressed as $A_{410}$ = 1.0 = 374 nmol/min·3.4-ml fraction. DS-Co activity is expressed as $A_{549}$ = 1.0 = 151 nmol/min·3.4-ml fraction. The protein eluate profile ($A_{280}$) is shown in the bottom panel.

Table I. *Partial Purification of DS-Co and DS-Mn Isozymes from Mung Bean Seedlings*

| Enzyme Preparation | Vol | Total Protein | Total Activity | Specific Activity | Recovery | Purification |
|---|---|---|---|---|---|---|
| | *ml* | *mg* | *nmol/min* | *nmol/min·mg* | % | *-fold* |
| Partial purification of DS-Co | | | | | | |
| Crude extract | 45.8 | 331.4 | 1160 | 3.5 | 100 | 1.0 |
| DEAE-cellulose | 53.0 | 227.9 | 2347 | 10.3 | 202 | 2.9 |
| Hydroxylapatite | 16.2 | 8.2 | 1588 | 205.9 | 137 | 58.1 |
| Partial purification of DS-Mn | | | | | | |
| Crude extract | 45.8 | 331.4 | 1627 | 4.91 | 100 | 1.0 |
| DEAE-cellulose | 15.0 | 20.3 | 162 | 7.98 | 10 | 1.63 |

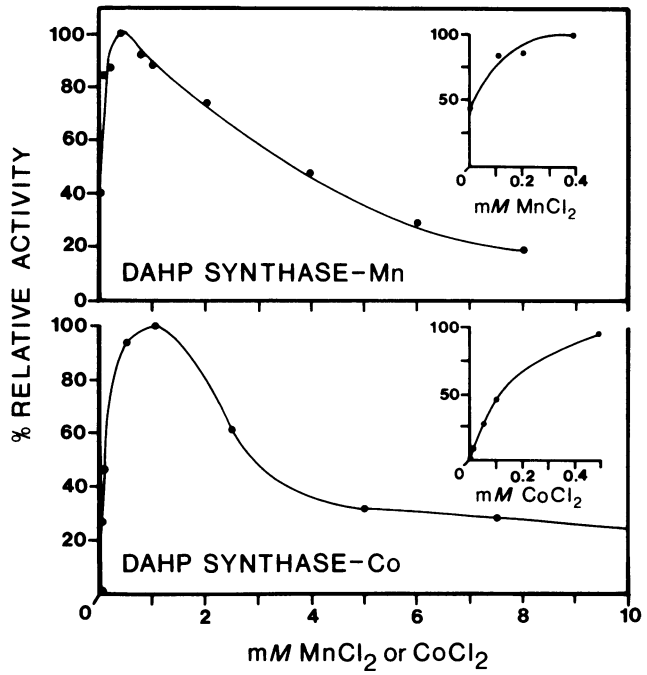


FIG. 3. Effect of divalent cations on the activation of DAHP synthase isozymes. $Mn^{2+}$ activates DS-Mn (top) while DS-Co is strictly dependent upon $Co^{2+}$ for activity (bottom). The insets expand the scale in the range of 0 to 0.5 mM $Mn^{2+}$ or $Co^{2+}$. A relative activity of 100% corresponds to an activity of 0.66 nmol/min (4.9 nmol/min·mg) for DS-Mn and to an activity of 1.31 nmol/min (104.5 nmol/min·mg) for DS-Co. The chloride salts of $Mn^{2+}$ and $Co^{2+}$ were utilized, and substrate concentrations were as follows: 1.6 mM PEP and 3.2 mM E4P for DS-Mn, and 4 mM PEP for DS-Co. NaF was not present in the assay of DS-Mn.

saturation curves with respect to PEP leveled off at about 2 mM for both DS-Mn (E4P fixed at 0.5 mM) and DS-Co (E4P fixed at 4 mM). When the standard assay conditions for the two DAHP synthase isozymes (see "Materials and Methods") were used, neither partially purified isozyme displayed any activity when assayed under the alternative conditions.

**Sensitivity of DAHP Synthase-Co to Allosteric Control.** The three aromatic amino acids, shikimate, and the three cyclohexadienyl intermediates (chorismate, prephenate, and L-arogenate) all failed to inhibit DS-Co, but caffeate (3,4-dihydroxycinnamate) was inhibitory (Fig. 6). Fifty percent inhibition was obtained in the presence of 180 μM caffeate. Double-reciprocal plots revealed that inhibition was noncompetitive with respect to both E4P and PEP (Fig. 7).

**Sensitivity of DAHP Synthase-Mn to Allosteric Control.** L-Tryptophan was a weak inhibitor of DAHP synthase-Mn at pH 7.0. Using the standard assay conditions, concentrations of L-tryptophan in the range of 0.05 to 1 mM yielded no greater than 20% inhibition of enzyme activity (data not shown). However, sensitivity of DS-Mn to inhibition by L-tryptophan increased with decreasing pH (Fig. 8). At pH 5.0, 44% inhibition of activity was obtained in the presence of 0.4 mM L-tryptophan. However, at pH 5, DS-Mn exhibits very little activity (as shown in Fig. 4).

DS-Mn activity was responsive to allosteric effects of three shikimate-pathway intermediates: chorismate, prephenate, and L-arogenate (Fig. 9). Chorismate activated DS-Mn, with 0.5 mM chorismate producing a 40% increase in activity. Both prephenate and L-arogenate inhibited DS-Mn activity. Prephenate concentrations of about 460 μM were required for 50% inhibition of enzyme activity. L-Arogenate was a more potent inhibitor of DS-Mn than prephenate, 50% inhibition of enzyme activity occurring at 155 μM L-arogenate. Greater than 75% inhibition was obtained with 625 μM L-arogenate.

The effects of chorismate, prephenate, and L-arogenate on the kinetic parameters of DS-Mn were determined graphically utilizing Hanes-Woolf plots, and the results are summarized in Table II. Both L-arogenate and prephenate displayed noncompetitive inhibition with respect to PEP and competitive inhibition with respect to E4P. In contrast, the activator, chorismate, decreased the apparent $K_m$ of PEP and increased the apparent $V_{max}$ with respect to E4P.

We considered the possibility that L-arogenate preparations might contain a contaminating compound that might be the true inhibitor of DS-Mn. This possibility was eliminated by results given in Table III where L-arogenate was quantitatively converted to L-phenylalanine enzymically. A partially purified arogenate dehydratase preparation from *Pseudomonas aeruginosa* (3) was used for specific enzymic conversion. The enzymically treated preparation of L-arogenate failed to inhibit DS-Mn as expected if L-arogenate is the true inhibitory metabolite. Analytical evaluation of chorismate and prephenate preparations are routinely carried out to assure the absence of significant amounts of organic impurities, and especially the absence of detectable amounts of aromatic-pathway intermediates.

## DISCUSSION

**DAHP Synthase-Co.** The catalytic properties of DS-Co were strikingly different from those of DS-Mn. DS-Co possessed an absolute requirement for a divalent cation, cobalt being the most effective. At the pH optimum of 8.8, using high concentrations of E4P and 1.0 mM cobalt, the DS-Co enzyme was very active. Under the same assay conditions, activity of DS-Mn was not detectable.

DS-Co was unaffected by aromatic amino acids or by pathway intermediates that might conceivably function as allosteric agents. Hence, this isozyme appears to lack feedback control. However, the secondary metabolite, caffeate, was an effective inhibitor. This regulation could have physiological significance. For example, if unregulated biosynthesis of L-phenylalanine overflows into secondary metabolite formation at appropriate developmental times, caffeate may act as a feedback sensor of what can be regarded as endproducts of an extended pathway, acting to slow early-pathway catalysis at DS-Co. It seems suggestive that an otherwise unregulated isozyme of chorismate mutase (chorismate mutase-2) from *Nicotiana silvestris* is a second example in higher plants of an aromatic-pathway isozyme located at a metabolic branchpoint that is subject to inhibition by caffeate (13). Although chorismate mutase-2 of *V. radiata*, synonymous with CM-II (25), appears to be exceptional among chorismate mutase-2 isozymes in its lack of sensitivity to caffeate inhibition (11), it is possible that the isozyme became desensitized during the purification regimen employed.

**DAHP Synthase-Mn.** Optimal conditions for assay for DS-Mn with respect to pH optimum, divalent cation ($Mn^{2+}$) employed for activation, and E4P concentration are incompatible with the detection of DS-Co activity. In *N. silvestris*, it has recently been found that DTT activates DS-Mn, producing a marked hysteretic response (R. J. Ganson and R. A. Jensen, unpublished data). DTT was present in the buffer used for crude extracts and for DEAE-cellulose chromatography of DS-Mn in this study as described before (25), and preliminary experiments indicate that *V. radiata* DS-Mn is also activated by DTT.

The multimetabolite control of DS-Mn is complicated, but amenable to a physiologically attractive interpretation. Figure 10 illustrates DS-Mn in relationship to its four allosteric agents and to the aromatic amino acids. If a metabolic state of aromatic amino acid sufficiency is considered, L-tryptophan would partially inhibit DS-Mn and would activate chorismate mutase-1 (10). The latter effect would diminish intracellular chorismate

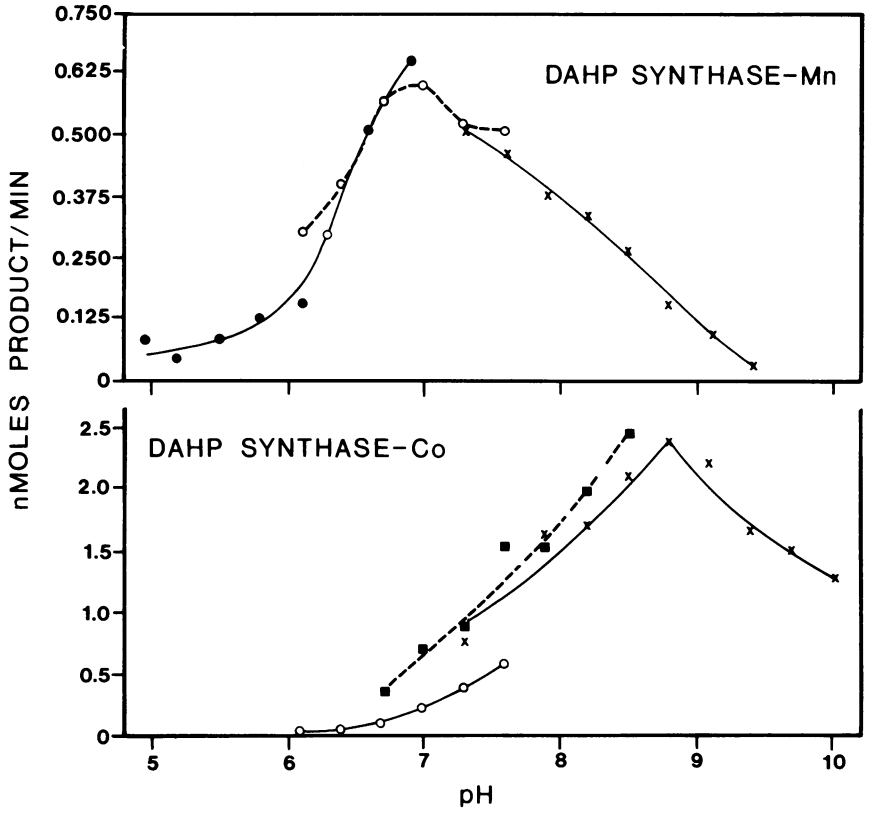


FIG. 4. pH optima for activity of DAHP synthase isozymes. Partially purified DAHP synthase isozymes were assayed in the presence of 50 mM (top) or 100 mM (bottom) buffers at the indicated pH values. Buffers utilized were as follows: (●), Mes; (○), Pipes; (×), Epps; and (■), Hepes. Substrate concentrations were the same as for Figure 3, and divalent cation concentrations were as follows: 2 mM $Mn^{2+}$ for DS-Mn and 0.5 mM $Co^{2+}$ for DS-Co. NaF was not present in assay for DS-Mn.

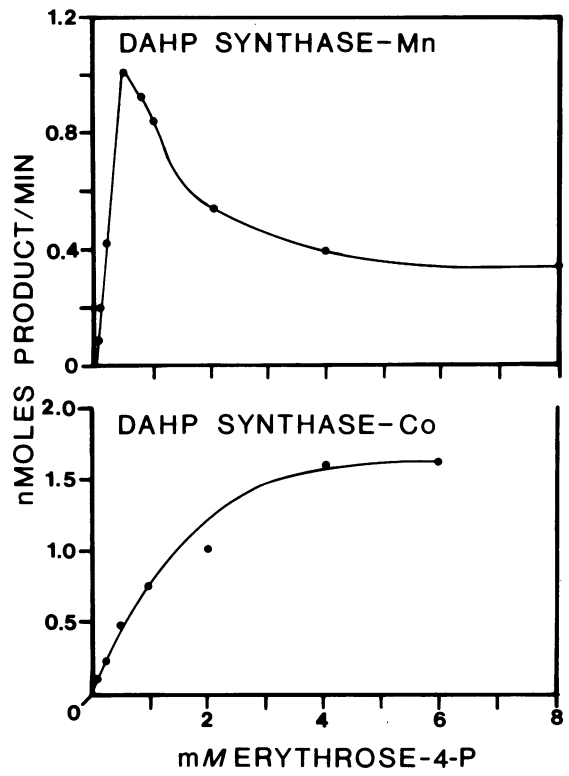


FIG. 5. Substrate saturation curves with respect to E4P at fixed concentrations of PEP. Partially purified DAHP synthase preparations were assayed with varied concentrations of E4P, utilizing 1.6 and 2 mM PEP for DS-Mn and DS-Co, respectively. NaF was not present in the assay for DS-Mn.

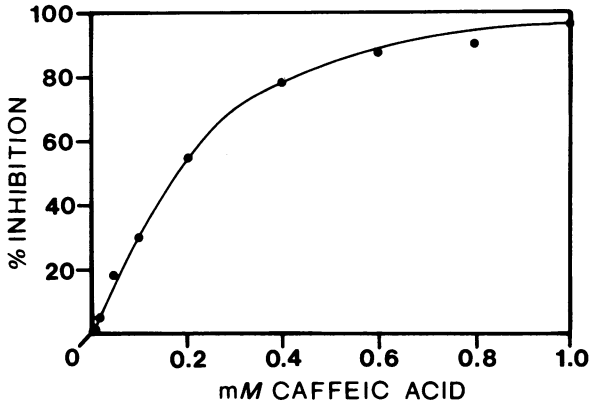


FIG. 6. Caffeic acid inhibition of DS-Co. Enzyme was assayed under the standard conditions at the indicated concentrations of caffeic acid, which was freshly prepared. Fifty percent inhibition was obtained with 0.18 mM caffeic acid.

levels and increase prephenate levels, thereby jointly decreasing an activation effect and increasing an inhibition effect exerted upon DS-Mn. Increased prephenate would favor increased L-arogenate levels via prephenate aminotransferase, an exceedingly active enzyme in higher plants (1, 24). If L-phenylalanine and/or L-tyrosine exert significant product inhibition effects upon arogenate dehydratase and/or arogenate dehydrogenase *in vivo*, then overall pathway control could be quite tight since L-arogenate is the most effective inhibitor molecule of DS-Mn. Such an overall pattern would be a third variation of sequential feedback inhibition—a control pattern first described in *Bacillus subtilis* (17, 21), with a second variation described recenty in xanthomonad bacteria (30).

At the other extreme, if one envisions the consequences of aromatic amino acid limitation, L-tryptophan levels would now

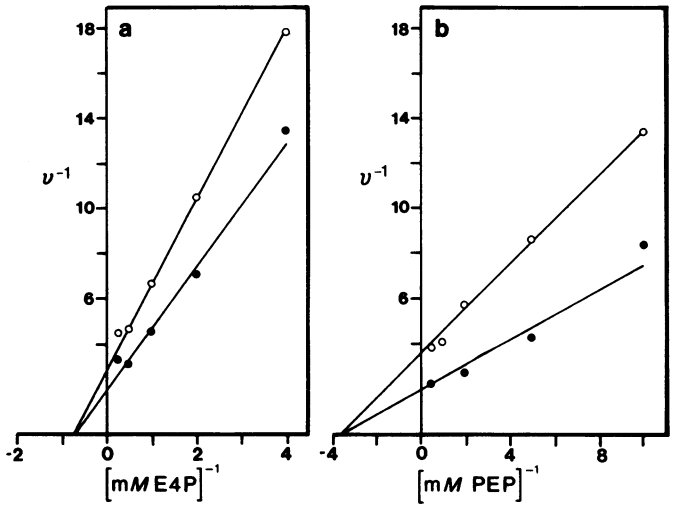


FIG. 7. Noncompetitive inhibition of DS-Co by caffeic acid. Enzyme was assayed in the presence (O) or absence (●) of 0.2 mM caffeic acid, which was freshly prepared. A velocity ($v$) of 1 equals 4.44 nmol DAHP formed/min.

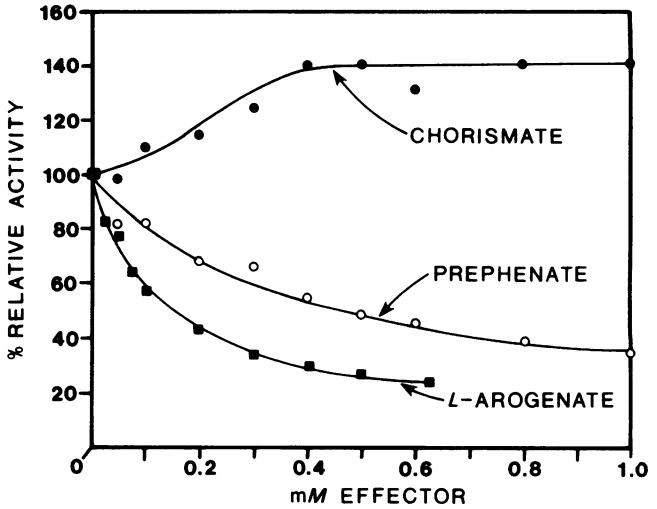


FIG. 9. Effect of chorismate, prephenate, and L-arogenate on DS-Mn activity. Partially purified enzyme was assayed under the standard conditions at the indicated concentrations of effectors. A relative activity of 100% represents the activity obtained in the absence of added effectors.

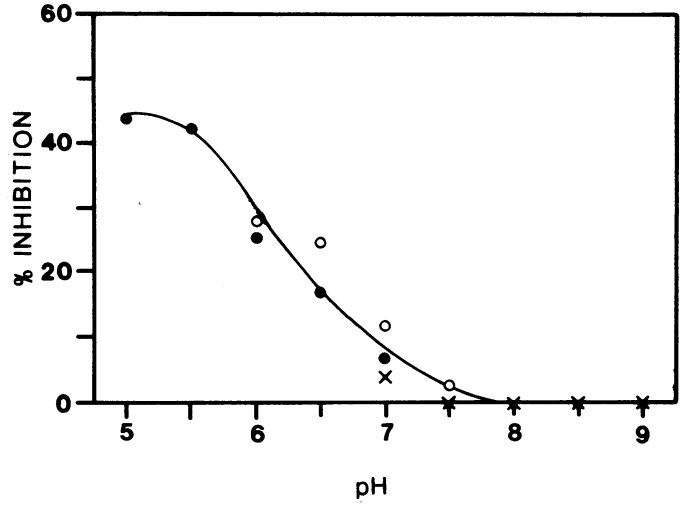


FIG. 8. pH-dependence of inhibition of DS-Mn by L-tryptophan. Enzyme was assayed in the presence of or absence of 0.4 mM L-tryptophan with the following buffers present at 50 mM: (●), Mes; (O), Pipes; or (×) Epps.

be inadequate to inhibit DS-Mn or to activate chorismate mutase-1. The latter effect might tend to increase chorismate concentrations enough to activate DS-Mn. Since conditions of L-phenylalanine and L-tyrosine limitation are supposed, prephenate and L-arogenate molecules will be pulled toward endproduct formation. The latter effect, coupled with the decreased conversion of chorismate to prephenate would tend to deplete the inhibitory prephenate and L-arogenate molecules from the system. The sum of these molecular events would promote the maximal activity state of DS-Mn.

Legitimate reservations about the above *in vivo* interpretations follow from the likelihood that *in vivo* concentrations of at least chorismate and prephenate are orders of magnitude lower than those used *in vitro* to demonstrate allosteric effects. (Since L-arogenate is an amino acid, *in vivo* levels conceivably could occupy an expandable pool.) However, '*in vivo*' concentration determinations are in fact *in vitro* measurements of average intracellular concentrations that do not take in account the variation that may occur in the microenvironment of the cell (or in the plastid in the case of DS-Mn). Additionally, in cases where activation or inhibition is competitive, the *in vivo* effects would be much more dramatic than indicated by standard assays carried out at near-saturating substrate. For example, in standard assays,

Table II. *Effects of Chorismate, Prephenate, and L-Arogenate on Apparent Kinetic Parameters of DS-Mn*

Partially purified enzyme was assayed under the standard conditions: (a) at constant PEP (2 mM) with E4P varied between 0.2 and 0.5 mM; or (b) at constant E4P (0.5 mM) with PEP varied between 0.4 and 2 mM. Determinations were made in the absence or presence of 0.1 mM L-arogenate, 0.5 mM prephenate, or 0.5 mM chorismate. Kinetic effects were determined graphically via Hanes-Woolf plots.

| Effector | Effect | Kinetic Effect with Respect to: | |
|---|---|---|---|
| | | PEP | E4P |
| L-Arogenate | Inhibition | Noncompetitive | Competitive |
| Prephenate | Inhibition | Noncompetitive | Competitive |
| Chorismate | Activation | Competitive | Noncompetitive |

Table III. *Verification that L-Arogenate is an Inhibitor Molecule for DS-Mn*

Partially purified DS-Mn and DS-Co preparations were assayed in the presence or absence of 0.13 mM L-arogenate. To confirm that DS-Mn was specifically inhibited by L-arogenate rather than by some unknown contaminant, a sample of L-arogenate was quantitatively converted to L-phenylalanine utilizing partially purified arogenate dehydratase from *Pseudomonas aeruginosa*. Data are expressed as per cent activity relative to that obtained in the absence of effector. The specific activities of DS-Mn and DS-Co were 8.0 and 206 nmol/min·mg protein at 37°C, respectively.

| Effector | % Relative Activity | |
|---|---|---|
| | DS-Mn | DS-Co |
| None | 100 | 100 |
| Untreated L-arogenate | 64 | 95 |
| Enzyme-treated L-arogenate[a] | 98 | 98 |

[a] Arogenate dehydratase partially purified as in reference 3 was added to give a final specific activity of 0.4 nmol/min·mg protein, and the preparation was incubated 24 h at 37°C. Extract protein was removed by ultrafiltration (PM-10 membrane, Amicon).

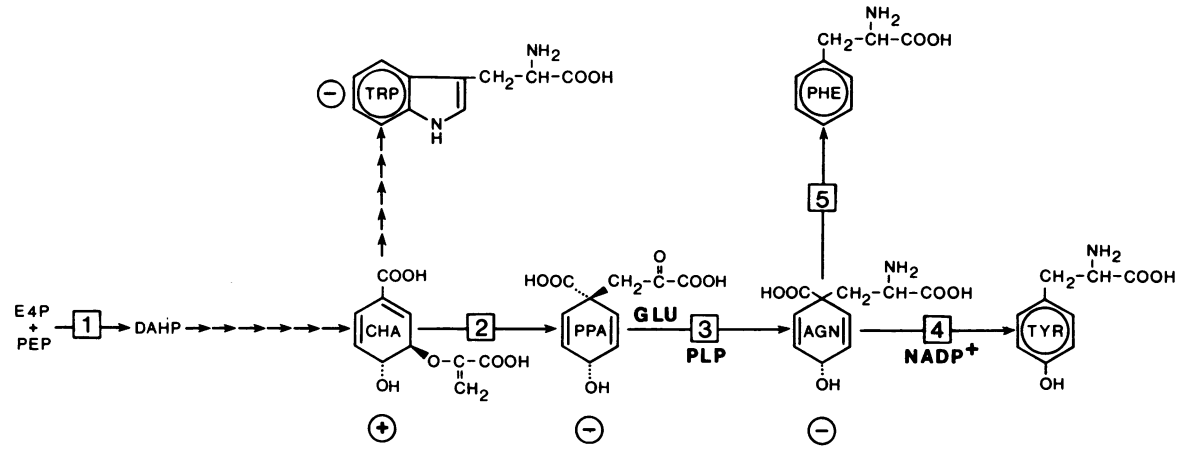


FIG. 10. Relationship of effector molecules to DS-Mn and to aromatic amino acid endproducts in *V. radiata*. Enzymes specific for phenylalanine biosynthesis have not yet been described in higher plants, but arogenate dehydratase has been detected recently in both *N. silvestris* and in spinach chloroplasts (33). Arogenate dehydrogenase requiring $NADP^+$, has been described in mung bean (24). Although prephenate dehydrogenase has not been detected in a number of higher plants examined, this activity (not shown) was detected in mung bean seedlings (24). The possibility that the latter activity may be present only during the developmental time of seedling germination is being examined. The transamination of prephenate is carried out by [AT-C], an aminotransferase previously denoted PPA-AT in mung bean (24), and similar to the recently characterized aminotransferase activity in *N. silvestris* (1). CM-1 is an isozyme of chorismate mutase that is activated by L-tryptophan and feedback inhibited by L-tyrosine and L-phenylalanine in mung bean (10). DS-Mn is responsive to allosteric activation by chorismate (+) and to feedback inhibition by prephenate, L-arogenate and L-tryptophan (−). Enzymes are designated as follows: (1), DS-Mn; (2), chorismate mutase-1; (3), AT-C; (4), arogenate dehydrogenase; (5), arogenate dehydratase. CHA, chorismate; PPA, prephenate; AGN, L-arogenate; TYR, L-tyrosine; PHE, L-phenylalanine; TRP, L-tryptophan; PLP, pyridoxal 5′-phosphate.

the CM-1 isozyme of chorismate mutase in *N. silvestris* exhibits a $K_a$ of 1.5 μM for L-tryptophan, even though mM levels of L-tryptophan produce less than 2-fold activation at saturating levels of substrate (13).

**Perspective on Significance of Isozyme Pairs.** Both chorismate mutase and DAHP synthase exist in *V. radiata* as isozyme pairs (10, 11, 25, this paper), with one isozyme of each pair subject to allosteric control by aromatic-pathway metabolites, indicating a more or less conventional operation of feedback control. The second isozyme of each pair is not feedback regulated by any biosynthetic-pathway metabolites, although the control of these isozymes by caffeate in some higher plants (13, this paper) may be significant as signals of feedback control within the boundaries of a larger biochemical unit. There is some basis for projection that other higher plants may possess similar isozyme pairs of chorismate mutase and DAHP synthase. Another branchpoint enzyme, anthranilate synthase, may exist as a mixture of feedback-sensitive and feedback-resistant isozymes (4). In *N. silvestris*, the regulated chorismate mutase-1 has been shown to occupy the plastid compartment while the unregulated chorismate mutase-2 is located in the cytosol (5). It will be interesting to see whether the subcellular locations of other aromatic-pathway isozyme pairs will parallel those of chorismate mutase. Indeed, our results in *N. silvestris* with DS-Co and DS-Mn isozymes fit this expectation (8), and evidence from other laboratories is also suggestive along these lines (20).

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