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The Two-Isozyme System of 3-Deoxy-D-*arabino*-Heptulosonate 7-Phosphate Synthase in *Nicotiana silvestris* and Other Higher Plants[1]

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ABSTRACT

Two isozymes of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase, denoted DS-Mn and DS-Co, were identified following DEAE-cellulose chromatography of crude extracts prepared from suspension-cultured cells of *Nicotiana silvestris*. The strikingly different properties of the isozymes allowed the development of assays for the selective detection of either isozyme in samples containing a mixture of the two. The DS-Mn isozyme required the sulfhydryl reductant, dithiothreitol, for activity and was stimulated by manganese. Activation by dithiothreitol was slow relative to catalysis, accounting for a hysteretic progress curve that was observed when reactions were started with inactive enzyme. The DS-Co isozyme was inhibited by dithiothreitol and required a divalent cation for activity. At optimal cation concentrations of 10 millimolar (magnesium), 0.5 millimolar (cobalt), and 0.5 millimolar (manganese), relative activities obtained were 100, 85, and 20, respectively. The substrate saturation curves with respect to erythrose 4-phosphate differed markedly when the two isozymes were compared. As little as 0.5 millimolar erythrose 4-phosphate saturated DS-Mn, whereas a 10-fold higher concentration was needed for saturation of DS-Co. The pH optimum of DS-Mn was 8.0, while that of the DS-Co isozyme was 8.6. Leaves of both *N. silvestris* and spinach also exhibited the DS-Mn/DS-Co isozyme arrangement, and the subcellular location of DS-Mn was shown to be the chloroplast compartment. By application of the differential assays for DAHP synthase isozymes, various monocotyledonous and dicotyledonous plants yielded data indicating the general presence of the DS-Mn/DS-Co isozyme pair in higher plants.

DAHP[2] synthase (EC 4.1.2.15) carries out the first catalytic step that is committed to formation of an initial precursor unique to the ultimate biosynthesis of the aromatic amino acids (L-phenylalanine, L-tyrosine, and L-tryptophan), a number of vitamin-like end products, and a host of other natural products in higher plants (10). The shikimate pathway interfaces with carbohydrate metabolism through the action of DAHP synthase which competes with other enzymes that utilize PEP or E4P.

A background of information about possible isozyme species corresponding to each catalytic step participating in aromatic biosynthesis and the separate subcellular location of such isozymes is just developing (16, 17). The best studied example is chorismate mutase, shown in *Nicotiana silvestris* to exist as two isozymes (11, 12): an allosterically controlled species (CM-1) being located in plastids and an unregulated species (CM-2) being located in the cytosol (7). Two isozymes of DAHP synthase were identified in *Vigna radiata* (23, 24), and were denoted DAHP synthase-Mn (stimulated by, but not requiring Mn) and DAHP synthase-Co (requiring Co, Mg, or Mn for activity). This pair of DAHP synthase isozymes was also isolated from leaves of *N. silvestris* (9). When assay conditions were optimized for either isozyme, the activity of the remaining isozyme was barely detectable.

The markedly distinct properties of DS-Mn and DS-Co from *N. silvestris* have been exploited to define assay conditions for exclusive assay of either one of the two isozymes in mixtures. A reliable methodology to discriminate DAHP synthase isozymes in crude extracts is particularly valuable for subcellular localization studies as well as assessment of the variation in isozyme levels in response to developmental and regulatory changes. Although DAHP synthase from a variety of plants has been studied (14, 15, 20–22, 26), particular assay conditions employed would have favored in most or all cases the detection of only DS-Mn (most often) or of only DS-Co. It appears that the isozyme pair of DS-Mn and DS-Co, spatially separated in different subcellular compartments, may be a conservative biochemical feature of higher plant cells—similar to the differentially compartmented isozyme pairs that make up duplicate pathways of carbohydrate metabolism (13).

MATERIALS AND METHODS

Cell Culture Procedures. A cell line designated ANS-1 (12), originally derived from a haploid plant of *N. silvestris*, was maintained in suspension culture. Cells were harvested at the indicated physiological stages of growth by vacuum filtration and then washed with 3% (w/v) mannitol. The resulting cell pack was quickly frozen in liquid N_2 and stored at −80°C.

Growth of *N. silvestris* Plants. Diploid *N. silvestris* plants were raised in a growth chamber under the following conditions: photoperiod, 12 h L: 12 h D; photosynthetic photon flux density, 175 $\mu E/m^2 \cdot s$ from fluorescent tubes; RH, 50 to 75%; temperature, 25°C during the L period and 20°C during the D period. *N. silvestris* plants grown on this regimen do not flower and grow rapidly to form a broad, nearly flat, rosette-shaped plant. Seeds were first germinated on 1% agar plates, then transferred in groups of 2 to 4 to the surface of hydrated peat-pellets in 6.5-cm deep trays sealed with plastic wrap, and covered with 2 layers of cheesecloth. After 3 to 4 weeks of growth, seedlings contained 2

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[2] Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; Epps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; PEP, phosphoenolpyruvate; E4P, D-erythrose 4-phosphate; L, light; D, dark.

to 3 sets of leaves and were thinned to 1 plant per peat-pellet. At this point, trays were again covered with plastic wrap, now punctured in several places to provide ventilation. These seedlings growing in peat-pellets served as stock for transplanting to 15 cm azalea pots filled with a premoistened 2:1 Jiffy Mix:perlite soilless potting mixture.

Immediately upon transplanting peat-pellet stock to the 15 cm container, plants were irrigated with approximately 400 ml of one-quarter strength Hoagland macro- and micronutrient medium (2). Feeding was continued at each watering, with a frequency determined by close inspection of soil moisture and leaf turgor. The interval between thorough waterings was 5 to 6 d for newly transplanted stock and decreased to 2 to 3-d for plants being conditioned for harvest. Plants were misted with distilled H_2O every 2 to 3 d. After 4 to 6 weeks, plants were still rapidly growing, had no senescent leaves, and were harvested 2 to 4 h after onset of the light period.

Cell Extracts. Frozen cell packs, immersed in liquid N_2, were ground to a fine powder in a Waring Blendor. Excess liquid N_2 was allowed to evaporate as the powdered cells were warmed to the point of thawing. Buffer (50 mM K-phosphate at pH 7.2) was quickly added to the powder at a ratio of 2 ml per 3g, and the slurry was mixed until completely thawed. Cell debris was removed by centrifugation at 38,000*g* for 15 min at 4°C. The supernatant was desalted on prepacked Sephadex G-25 columns (PD-10, Pharmacia, Piscataway, NJ) equilibrated with 10 mM K-phosphate (pH 7.2). Desalted cell extracts were stored at −20°C.

Leaf Extracts. Harvested leaves, with midribs removed, were frozen in liquid N_2 and ground to a fine powder in a Waring Blendor. Crude extracts were prepared as described for cell powders. Inclusion of 2-mercaptoethanol at 0.1% (v/v) was found to be necessary for recovery of DS-Mn activity from leaf extracts and was subsequently adopted as a component of extraction buffer for extracts prepared from suspension-cell cultures as well.

Ion-Exchange Chromatography. All procedures were carried out at 0 to 4°C. Powdered cells were extracted as described above. The extract was desalted by gel filtration on a 2.5 × 35 cm column of Sephadex G-25, equilibrated with 10 mM K-phosphate (pH 7.2). The resulting extract containing approximately 100 mg protein was applied to a 1.5 × 30 cm column of DEAE-cellulose (Whatman DE-52). The column was washed with three bed volumes of 10 mM K-phosphate equilibration buffer and then developed with 500 ml of a linear gradient of KCl (0–0.5 M) that was prepared in the same buffer. A flow rate of 0.5 ml per min was maintained, and fractions of 4 ml were collected. The conductivity of eluate fractions was monitored and related to KCl concentration using a standard curve. Fractions containing each DAHP synthase isozyme were pooled and concentrated to approximately 1 mg protein per ml by ultrafiltration through a PM-10 membrane (Amicon). The concentrated enzymes were desalted into 10 mM K-phosphate (pH 7.2) prior to immediate use or storage at −20°C.

Chloroplast Isolation and Preparation of Stromal Extracts. Chloroplasts were obtained from leaves of *N. silvestris* and spinach by aqueous homogenization in a Waring Blendor. Select, undamaged leaves from 2 to 3 plants were excised at the base, rinsed with cold (4°C) distilled H_2O, and blotted dry. Midribs were removed with a razor blade and the resulting leaf laminae were rolled into cylinders and sliced diametrically into segments to yield leaf slices approximately 3 mm wide by 20 to 30 mm long. Extraction medium, modified slightly from Mills and Joy (19) by the use of Hepes-KOH instead of Tricine-HCl, was prepared for use by stirring the solution in a 50% ethanol ice bath until a uniform slushy mixture was obtained. Fifty g of leaf slices were combined in a 1-L glass blender cup with 200 ml of extraction medium and homogenized 3 times with 2-s bursts at half speed. The brei was filtered through 2 layers of Miracloth sandwiched between 4 layers of cheesecloth to yield 200 ml of filtrate, of which 180 ml was distributed evenly between 50 ml centrifuge tubes (30 ml per tube). The filtered brei was underlayered with 14 ml of 40% (v/v) Percoll medium, modified from Mills and Joy (19) as for extraction medium. Chloroplasts were pelleted through the Percoll cushion by centrifugation for 3 min at 2500*g* in a Sorvall HB-4 rotor at 4°C. The supernatant, containing disrupted plastids and other organelles and their contents, was removed along with the 40% Percoll cushion by aspiration. For stromal extracts, each pellet was resuspended in 2 ml of 50 mM K-phosphate, centrifuged and the supernatant was desalted on PD-10 columns as described for crude extracts. Alternatively, the pellet in each tube was resuspended in 2 ml of extraction medium with the aid of a cotton swab and pooled to yield 13 ml of resuspended chloroplasts.

Purification of Chloroplasts on Discontinuous Sucrose Gradients. Resuspended chloroplasts (from the Percoll purification step) were further purified by sucrose density gradient centrifugation. Discontinuous gradients were prepared in polyallomer ultracentrifuge tubes by underlaying 9 ml each of 20% (w/v), 45% (w/v), and 60% (w/v) sucrose dissolved in fractionation buffer (7). Each gradient received 4 ml of resuspended chloroplasts, and was centrifuged at 4°C for 40 min at 50,000*g* in a Beckman SW25.1 rotor to separate intact from broken chloroplasts. Broken chloroplasts were retrieved in a volume of 1.45 ml from the 20 to 45% interface by puncturing the tube with an 18-gauge needle fitted to a 10-ml syringe. Intact chloroplasts were retrieved in a volume of 1.35 ml from the 45 to 65% interface in a similar manner.

Preparation of stromal extracts by hypotonic lysis was not practical due to the presence of high sucrose concentrations in the purified chloroplast suspensions. Chloroplast extracts were lysed by adding 5 μl of 10% (v/v) Triton X-100 to 1.0 ml of purified chloroplasts (final concentration = 0.05% v/v) followed by a 5-min incubation on ice to solubilize the chloroplast envelopes. Desalted chloroplast lysates (free of sucrose which interferes with the chemical assay for DAHP) were prepared by passing 0.9 ml of lysed chloroplasts through a PD-10 (G-25) column and collecting this in a final volume of 1.5 ml.

Comparative Survey. Seeds were sown in trays filled with perlite and irrigated daily with distilled H_2O. Following germination, whole seedlings were harvested into liquid N_2 and ground to a fine powder in a Waring Blendor. Fresh tissue from cauliflower florets and broccoli were homogenized in a Waring Blendor. Potato tuber tissue was ground in a mortar and pestle. Crude extracts were prepared as for *N. silvestris* leaves and assayed for DAHP synthase activity.

Analytical Assays. DAHP was assayed as described by Jensen and Nester (18) using the chemical method of Weissbach and Hurwitz (27) as adapted by Srinivasan and Sprinson (25). The *A* at 549 nm was measured in a thermostatically controlled autosampler cuvette set at 60°C. Chl and marker enzymes for plastid (nitrite reductase), cytosolic (nitrate reductase), microbody (catalase), and mitochondrial (fumarase) compartments were assayed as previously described (7). Protein concentrations were determined by the method of Bradford (4), with BSA (fraction V) as a standard. Sucrose concentrations were estimated with the use of a Bausch and Lomb hand-held refractometer.

Materials. Seeds of *N. silvestris* Speg. et Comes were originally obtained from Dr. L. G. Burk (United States Department of Agriculture Tobacco Research Laboratory, Oxford, NC). Spinach leaves were obtained from a local market. Biochemicals, Percoll, and Sephadex G-25 were obtained from Sigma Chemical Co. Protein assay reagent was from Bio-Rad. DEAE-cellulose (DE-52) ion-exchange resin was obtained from Whatman. Other

chemicals were obtained from commercial sources and were reagent grade or better.

RESULTS

Pattern of DAHP Synthase Activity in Crude Extracts. Crude extracts prepared from cultured cells of *N. silvestris* were tested for DAHP synthase activity under various conditions of catalysis as part of initial optimization experiments. Results given in Table I show that the presence of Mn or Co, as well as of DTT, produced effects that depended upon the combination of additions and upon the concentration of E4P used. Progressively higher activities were obtained in the presence of Co as E4P concentrations were raised; the additional presence of DTT was inhibitory. Activity with Mn varied similarly with increasing E4P. However, the additional presence of DTT in this combination stimulated activity. Furthermore, this effect was relatively greater (5-fold) at lower E4P concentrations.

Resolution of Two Isozymes. Chromatography of crude extract from *N. silvestris* on a column of DEAE-cellulose separated DAHP synthase into two peaks of activity. In light of results noted above in crude extracts the effects of E4P, divalent cation, and DTT were further examined on the separated isozymes. The activity profiles could be altered dramatically, depending upon the assay conditions used. The profiles obtained using three assay conditions are illustrated in Figure 1: one assay protocol that selectively measures the activity of DS-Mn; another that selectively measures the activity of DS-Co; and a third that measures the small residual activity of DS-Co under assay conditions lacking DTT, but otherwise optimized for DS-Mn. Hence, the DAHP synthase activity shown in Table I that was observed with Mn in the absence of DTT can be attributed to the DS-Co isozyme.

DAHP Synthase-Mn. The early eluting peak (at approximately 110 mM KCl) of DAHP synthase (Fig. 1) was not detectable unless DTT was present in the assay mixture, and this activity was further stimulated by manganese. The pH optimum for catalysis was 8.0. 2-Mercaptoethanol in the range of 1 to 10 mM could not substitute for DTT. Activation by DTT was slow relative to catalysis, thereby being the basis for the hysteretic (8) behavior of the enzyme. The enzyme could be activated prior to assay by incubation with DTT in the assay mixture lacking E4P (Fig. 2), thus eliminating the hysteresis. The pH optimum for activated enzyme preparations was between 7.0 and 7.5, 80% as much activity being found at pH 8.0. When reactions were started by addition of inactive enzyme, the pH optimum for catalysis was 8.0.

Table I. *Effects of Erythrose 4-Phosphate, DTT, and Divalent Cations upon DAHP Synthase Activity in Crude Extracts*

Crude extract from cultured cells of *N. silvestris* was prepared as described in "Materials and Methods." Reaction mixtures contained 50 mM K Epps buffer (pH 8.0), 2 mM PEP, 48 μg of crude-extract protein, and additions as indicated; divalent cations (chloride salts) were added at 0.5 mM. DTT was absent (−) or present (+) at a concentration of 0.5 mM.

Divalent Cation	DTT	Activity[a]	
		ΔA_{549}	%
	6.0 mM Erythrose 4-P		
Mn^{2+}	+	0.22	27
Mn^{2+}	−	0.19	23
Co^{2+}	+	0.62	76
Co^{2+}	−	0.82	100
	2.0 mM Erythrose 4-P		
Mn^{2+}	+	0.14	30
Mn^{2+}	−	0.08	17
Co^{2+}	+	0.32	70
Co^{2+}	−	0.46	100
	0.6 mM Erythrose 4-P		
Mn^{2+}	+	0.20	100
Mn^{2+}	−	0.04	20
Co^{2+}	+	0.11	55
Co^{2+}	−	0.19	95

[a] Reactions were run 20 min at 37°C. The highest activity obtained at each of the three E4P concentrations used was arbitrarily assigned a relative value of 100%.

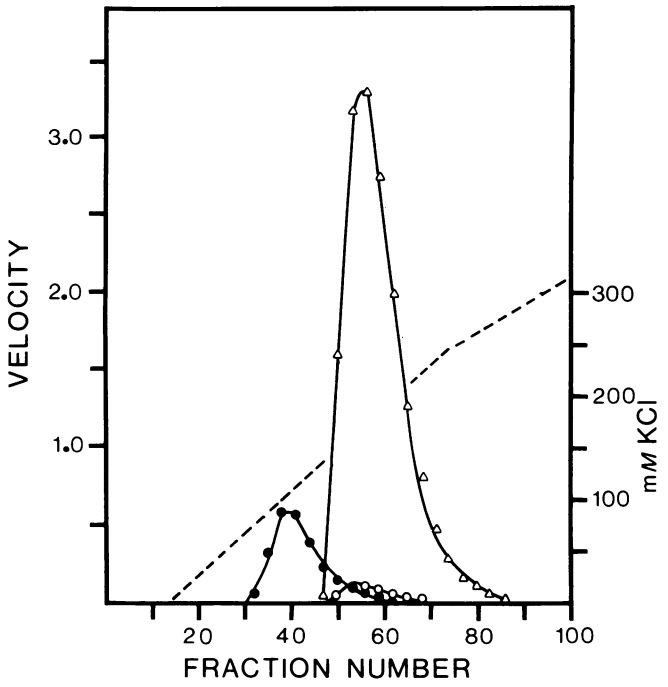


FIG. 1. DEAE-cellulose chromatography of crude extract from suspension-cultured cells of *N. silvestris*. The column was run as described in "Materials and Methods." The profiles illustrate the selective detection of each isozyme based on the assay conditions employed. ●——●, DS-Mn assay: 50 mM K Epps buffer (pH 8.0), 2 mM PEP, 0.5 mM E4P, 0.5 mM $MnCl_2$, and 0.5 mM DTT; ○——○, same as DS-Mn assay, but DTT omitted; Δ——Δ, DS-Co assay: 50 mM Epps buffer (pH 8.6), 2 mM PEP, 2 mM E4P, and 0.5 mM $CoCl_2$.

The effect of protein concentration on the degree of hysteresis displayed by DS-Mn in crude extracts was examined and is shown in Figure 3A. As the protein concentration in the assay mixture was increased, the lag-time before attainment of full activation decreased. Figure 3B shows the effect of hysteresis upon the reaction velocity *versus* protein concentration plot. Steady-state velocities obtained from the slopes of the linear portions of the progress curves yielded a plot that was linear and extrapolated to the origin as predicted by conventional enzyme kinetics. Velocities estimated from the standard 20-min assay yielded a plot that was also linear but that extrapolated to the abscissa. Hence, estimates of specific activity were made by plotting the slopes of linear portions of progress curves obtained at three or more protein concentrations.

The ability of sulfhydryl-blocking reagents to inactivate the DTT-activated DS-Mn was examined, and the effect of iodoacetamide addition is presented in Figure 4. Progress curves for two identical reactions are shown. Both reactions displayed a characteristic slow activation phase, leading to the attainment of a linear rate of product formation. During the linear phase, at t = 16 min, iodoacetamide was added to one reaction mixture and

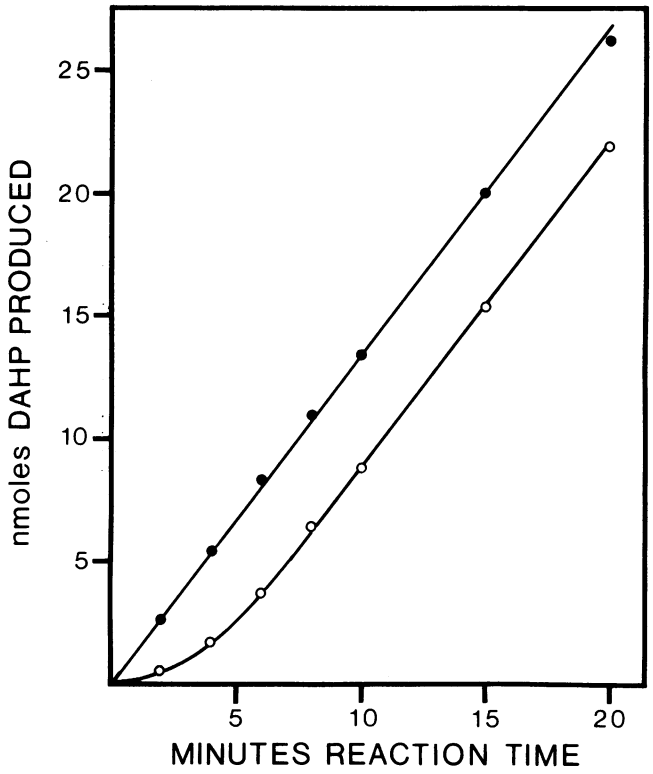


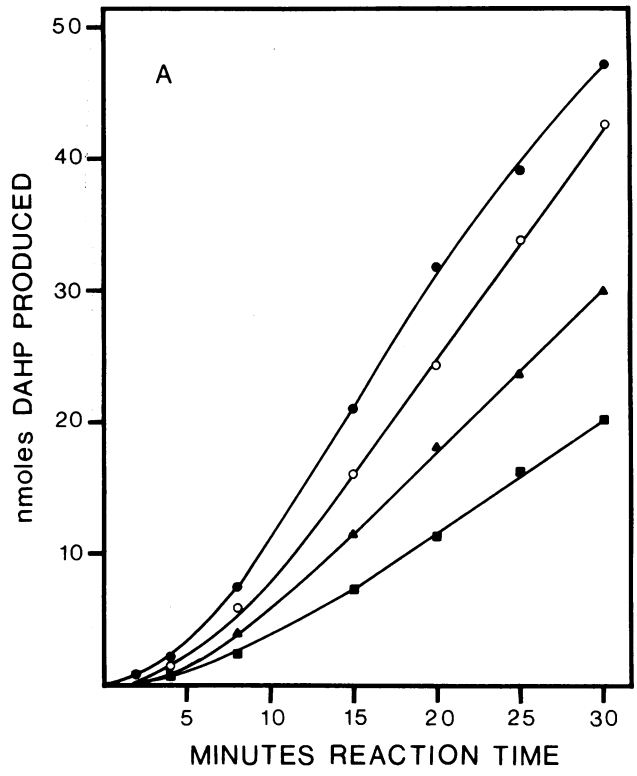
FIG. 2. Reaction progress curves for DS-Mn. Partially purified DS-Mn (1.1 mg of protein) was incubated for 15 min at 37°C in 0.5 ml of a solution containing 50 mM K Epps (pH 8.0), 3 mM PEP, 0.5 mM $MnCl_2$, and 0.5 mM DTT. To this mixture were added 1.5 ml of a solution containing 50 mM K Epps (pH 8.0), 3 mM PEP, 0.5 mM $MnCl_2$, 0.5 mM DTT, and 0.8 mM E4P to start the reaction. The control reaction, in a final volume of 2.0 ml, contained 50 mM K Epps (pH 8.0), 3 mM PEP, 0.6 mM E4P, 0.5 mM $MnCl_2$, 0.5 mM DTT, and 1.1 mg protein. Symbols: (●) activated prior to assay; (○) control reaction started with inactive enzyme.

distilled H_2O was added to the control. Within 2 min after addition of iodoacetamide, the reaction velocity decreased markedly and was nearly completely abolished after 15 min. The control reaction continued at a linear rate throughout the duration of the experiment. Under DS-Mn reaction conditions (pH 8.0), iodoacetamide is specific for sulfhydryl groups. This suggests that the DTT-activated DS-Mn can be inactivated by the covalent modification of one or more regulatory sulfhydryl groups that are essential for activity.

The substrate saturation curve of DS-Mn for E4P is shown in Figure 5. Optimal activities were obtained in the range of 0.5 to 1.0 mM, with substrate inhibition occurring at higher concentrations. The substrate saturation curve of DS-Mn for PEP was first-order, saturating at about 2.5 mM PEP (data not shown).

DAHP Synthase-Co. The second isozyme of DAHP synthase, eluting (Fig. 1) at approximately 170 mM KCl, required one of several divalent cations for activity. In contrast to DS-Mn, the progress curves obtained for DS-Co were linear. The enzyme displayed little activity below pH 7, its catalytic optimum being about pH 8.6.

Saturation curves with respect to the chloride salts of Mg, Co, and Mn are shown in Figure 6. The results illustrate a broad specificity for divalent-cation activators that has not been fully appreciated previously with respect to DS-Co (24). Although at low metal concentrations (0.5 mM) the order of activation strength was $Co^{2+} > Mn^{2+} > Mg^{2+}$, this order changed at approximately 0.75 mM to $Co^{2+} > Mg^{2+} > Mn^{2+}$ due to the inhibitory effects exerted by cobalt and manganese above their



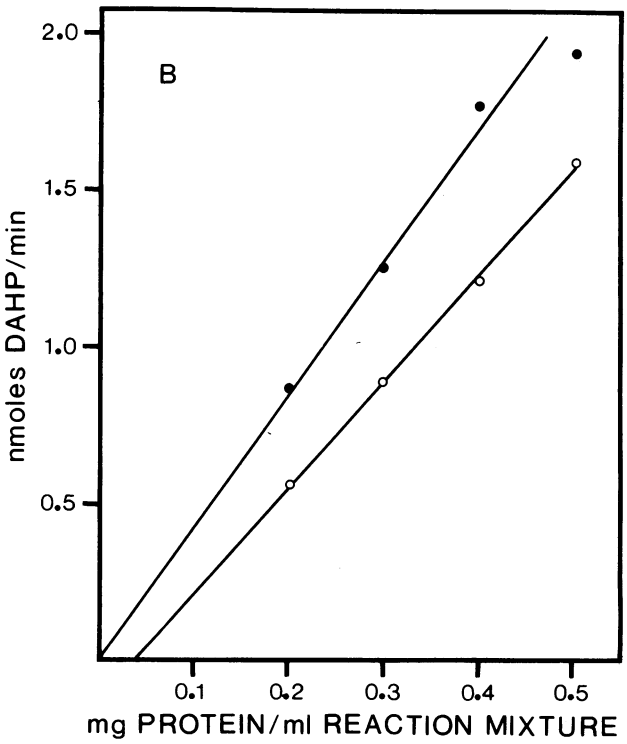


FIG. 3. Effects of protein concentration on degree of hysteresis displayed by DS-Mn in crude extract. Reaction conditions were as specified in the legend to Figure 2. A, Progress curves obtained at the following final protein concentrations (mg/ml): 0.2 (■), 0.3 (▲), 0.4 (○); 0.5 (●). B, Replot of nmol DAHP produced/min as a function of protein concentration, (●) determined from slope or (○) from 20-min end point.

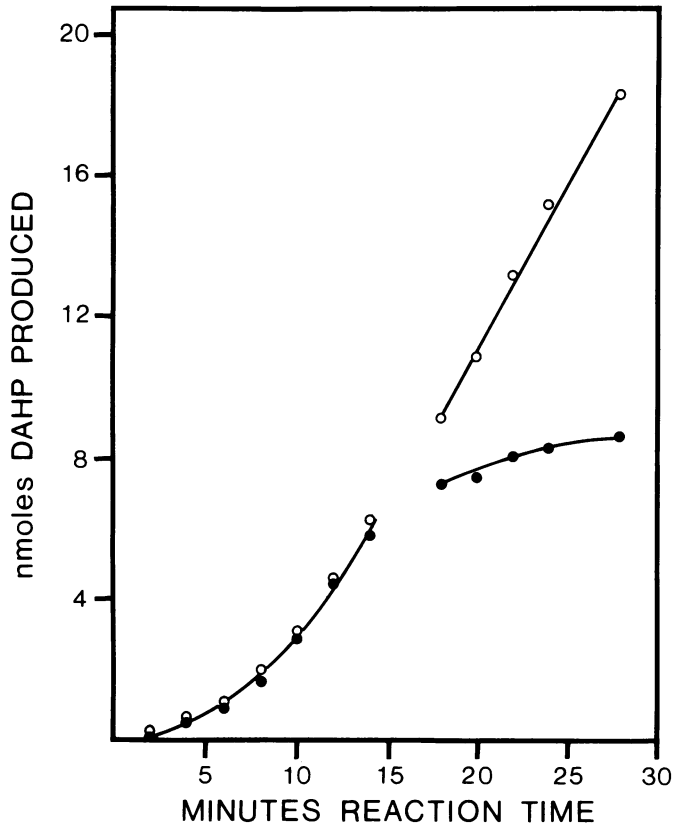


FIG. 4. Inactivation of partially purified DS-Mn by the sulfhydryl-blocking reagent, iodoacetamide. Two identical reactions were initiated and sampled periodically. At t = 16 min, iodoacetamide, at reaction pH, was added at a final concentration of 6.25 mM to one tube; distilled H_2O was added to the control. Symbols: (●) iodoacetamide treatment; (○) control.

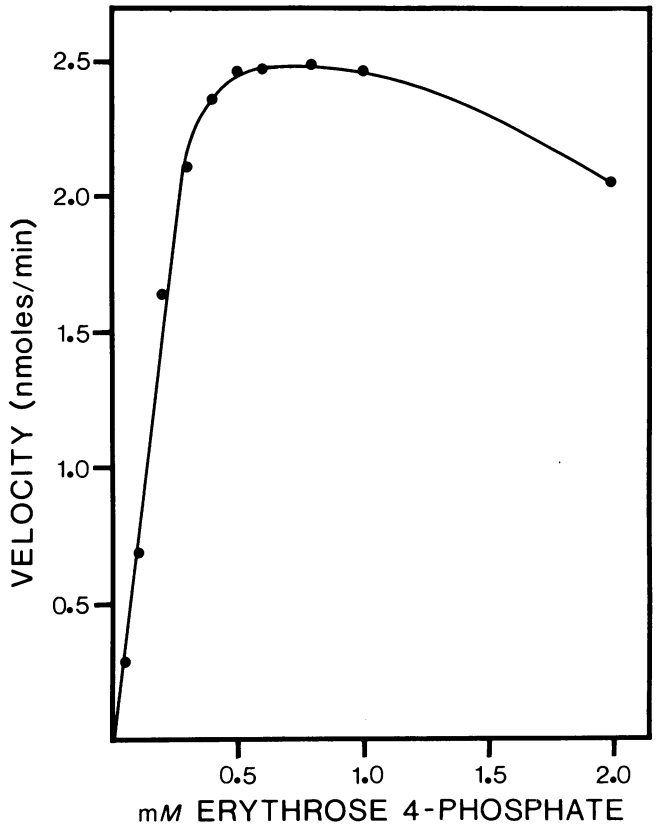


FIG. 5. Activity of DS-Mn as a function of erythrose 4-P concentration. Reaction mixtures (final volume, 200 μl) contained 50 mM K-Epps buffer (pH 8.0), 2 mM PEP, 0.5 mM $MnCl_2$, 0.5 mM DTT, and 50 μl of partially purified enzyme (66 μg protein). The enzyme was activated in this mixture at 37°C prior to the addition of E4P and the reaction was allowed to proceed for 20 min at 37°C.

optimal concentrations of 0.5 mM. $MgCl_2$ at 10 mM provided the greatest stimulation observed, and significant inhibition was not observed for Mg^{2+} up to 50 mM. The optimal concentration of $MgCl_2$ (10 mM) is distinctly more physiological than the optimal Co concentration (0.5 mM), and it is therefore likely that Mg is the functional activator *in vivo*. Ca and Zn at 0.5 mM were ineffective. The ability of Mn to substitute for the Co requirement of DS-Co presented a potential hindrance to the selective detection of DS-Mn in mixtures, particularly since DS-Co is often and perhaps always the major isozyme species in crude extracts.

An extremely useful variable in the selective isozyme assays proved to be the E4P concentration. DS-Co required E4P at concentrations that were an order of magnitude greater (Fig. 7) than required by DS-Mn (Fig. 5) for saturation. At E4P concentrations optimal for DS-Mn, the activity of DS-Co decreased to less than 20% of its maximum. The substrate saturation curve of DS-Co for PEP was first-order, saturating at about 1.5 mM PEP (data not shown).

Selective Detection of Isozymes. The finding that DS-Mn was inactive unless DTT was added to the reaction mixture provided a simple means for selective detection of DS-Co in isozyme mixtures. The selective detection of DS-Mn was not as easily achieved, but a combination of conditions that diminish the activity of DS-Co (lower pH, lower E4P concentration, use of Mn^{2+} as divalent cation, presence of DTT), while favoring the activity of DS-Mn, were successfully employed. Under such conditions of optimal DS-Mn activity, DS-Co was reduced to less than 2% of its potential activity. Selective detection of DS-Mn was accomplished with: 50 mM K Epps (pH 8.0), 3 mM PEP, 0.6 mM E4P, 0.5 mM $MnCl_2$, and 0.5 mM DTT. For the selective detection of DS-Co, an assay mixture of the following composition was used: 50 mM K Epps (pH 8.6), 2 mM PEP, 6 mM E4P, and either 10 mM $MgCl_2$ or 0.5 mM $CoCl_2$.

Subcellular Location of DS-Mn and DS-Co. Table II shows the relative proportions of the two DAHP synthase isozymes in whole leaf extracts from tobacco and spinach as revealed by the differential assay. The activity of DS-Co exceeded that of DS-Mn by a factor of 9 for *N. silvestris* and by a factor of 6 for spinach. In contrast, activity in chloroplast stromal extracts was exclusively that of DS-Mn. Activity of the cytosolic marker enzyme, nitrate reductase, indicated less than 2% contamination of the *N. silvestris* chloroplasts. These chloroplasts also contained a plastid isozyme of chorismate mutase (specific activity = 8.6 nmol/min·mg protein) that was determined to be virtually free of contamination by a second, cytosolic isozyme of chorismate mutase. The chorismate mutase isozymes were discriminated by means of an analysis of sensitivity to inhibition by L-tyrosine (7).

In a separate experiment, chloroplasts prepared in this manner were further purified by sucrose density gradient centrifugation which provided a clean separation of intact from broken chloroplasts (Fig. 8). A single gradient yielded 1.35 ml of purified chloroplasts with a Chl content of 0.445 mg/ml (40% of total) and 1.45 ml of broken chloroplasts with a Chl content of 0.632 mg/ml. Lysed chloroplasts yielded extracts with protein contents of 1.93 mg protein/mg of Chl from intact plastids and 0.55 mg protein/mg of Chl from the broken plastids. Approximately 11% of the total chloroplasts remained intact during purification, and these possessed less tha 0.4% of the cytosolic marker (nitrate reductase), only 0.12% of the microbody marker (catalase), and

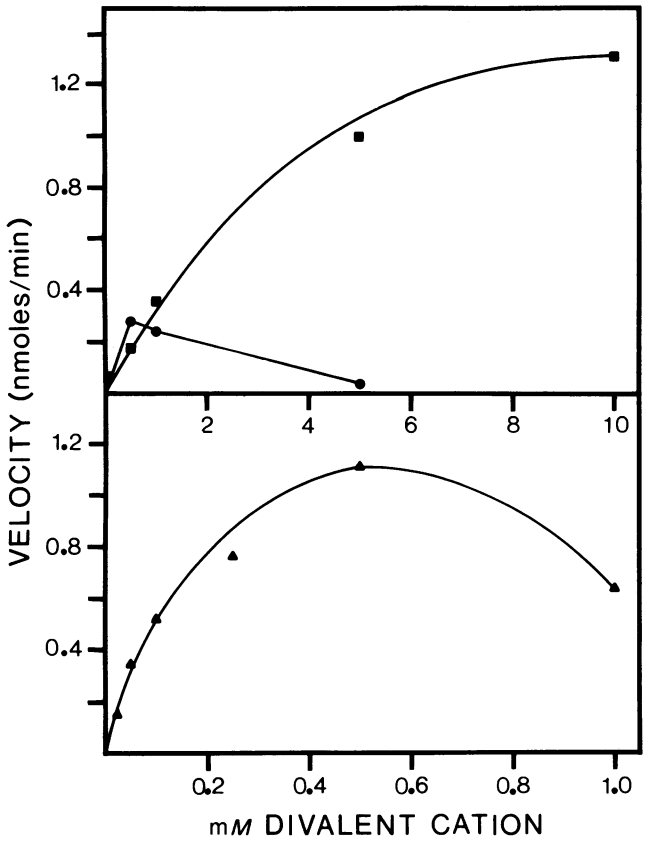


FIG. 6. Activity of DS-Co as a function of divalent cation concentration. Reaction mixtures (final volume, 200 μl) contained 50 mM K-Epps buffer (pH 8.6), 1 mM PEP, 6 mM E4P, 25 μl of partially purified enzyme (5 μg protein), and metal chloride as indicated. The reaction was started by the addition of enzyme and allowed to proceed for 20 min at 37°C. Symbols: (●) manganese; (▲) cobalt; (■) magnesium.

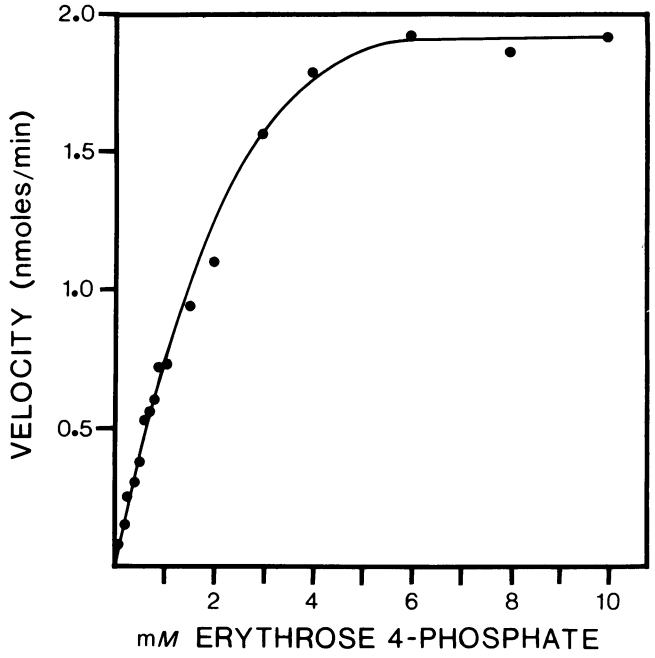


FIG. 7. Activity of DS-Co as a function of erythrose 4-P concentration. Reaction mixtures (final volume, 200 μl) contained 50 mM K Epps (pH 8.6), 2 mM PEP, 0.5 mM $CoCl_2$, 25 μl of partially purified enzyme (6 μg protein), and E4P as indicated. The reaction was started by the addition of enzyme and allowed to proceed for 20 min at 37°C.

Table II. *Subcellular Localization of DAHP Synthase Isozymes from Leaf Tissue*

Organism	Isozyme[a]	Specific Activity	
		Crude extract	Chloroplast stroma
		nmol DAHP/min·mg protein	
N. silvestris	DS-Mn	11.4	20.0
	DS-Co	100.0	3.0
Spinach	DS-Mn	9.8	7.5
	DS-Co	60.0	0

[a] DS-Mn Assay: 50 mM K Epps (pH 8.0), 3 mM PEP, 0.6 mM E4P, 0.5 mM $MnCl_2$, and 0.5 mM DTT. DS-Co Assay: 50 mM K Epps (pH 8.6), 2 mM PEP, 6 mM E4P, and 10 mM $MgCl_2$. The assay temperature was 37°C.

no detectable fumarase activity (mitochondrial marker).

Desalted extracts from broken and intact chloroplasts were assayed for DAHP synthase isozymes using the selective assay protocols. Activity of isozyme DS-Mn was found only in the extract from intact chloroplasts at a specific activity of 9.6 nmol/min·mg protein, equivalent to 30.8 nmol/min·mg Chl. No activity of isozyme DS-Co was detected in extracts from either intact or broken chloroplasts.

Comparative Survey of DAHP Synthase Isozymes. Results obtained by application of the differential assay protocols to crude extracts prepared from diverse plant species are shown in Table III. In all cases the highest activity was observed for isozyme DS-Co, ranging from specific activities of 6.8 nmol/min·mg protein for broccoli floret to 60.9 nmol/min·mg protein for rye seedlings. Since isozyme DS-Co is easiest to assay unambiguously in mixtures and since it inevitably yields higher specific activities than DS-Mn, it is striking that only DS-Mn has been recognized in a number of studies (15, 26).

The activity measured under assay-Mn conditions, but in the absence of DTT, undoubtedly is produced by isozyme DS-Co. Consistent with results obtained from partially purified DS-Co of *V. radiata* or *N. silvestris*, this residual activity of DS-Co (column 2, Table III) is only 0 to 3% of the full DS-Co activity (column 1, Table III). The highest residual activity of DS-Co was found in potato tuber (6%). It might seem appropriate to obtain DAHP synthase-Mn activities by subtracting assay-Mn (minus DTT) values from assay-Mn (plus DTT) values given in Table III. However, if DS-Co of other plants is inhibited by DTT as is the case in *N. silvestris*, then the values given under the (+DTT) column are the most accurate measurements of DS-Mn activities.

DISCUSSION

Isolated chloroplasts from spinach were shown to be capable of assimilating radiolabeled CO_2 or shikimate into aromatic amino acids (3, 6), thus indicating that an intact aromatic amino acid pathway must be located within the plastid compartment. Chloroplast-localized biosynthesis did not, however, account for total aromatic amino acid biosynthesis (6). Hence, a spatially separate pathway having its own complement of enzymes localized in another subcellular compartment might exist. Since the initial pathway precursors (PEP and E4P) are formed in the cytosol (1), this compartment is a likely subcellular location of a separate pathway. Indeed, chorismate mutase from *N. silvestris* was shown to exist as two isozymes (11), one located in the plastid and a second in the cytosol (7).

As was found for the two isozymes of chorismate mutase (11), dual isozymes of DAHP synthase were not restricted to suspension-cultured cells. Leaves from both *N. silvestris* and spinach

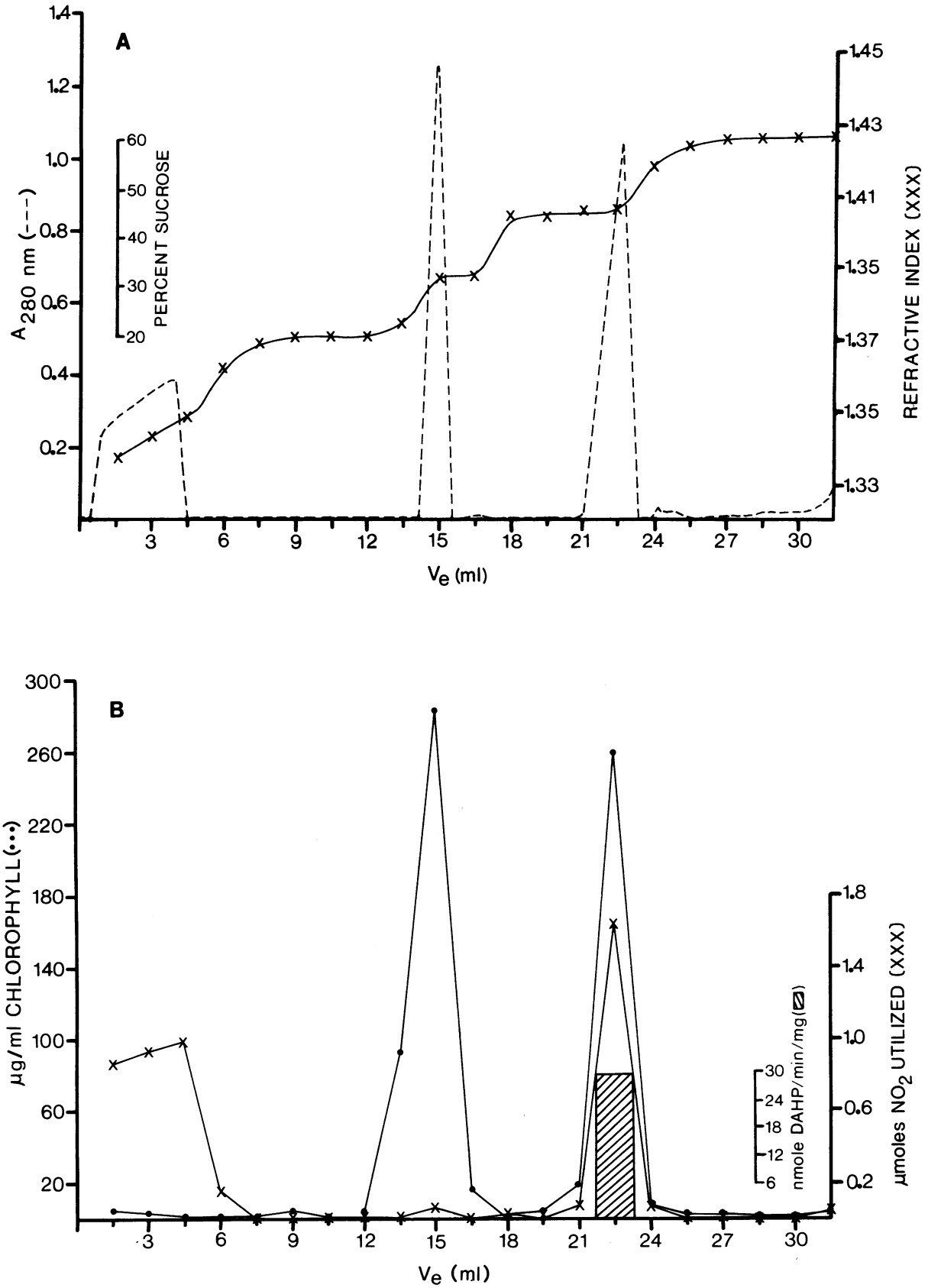


FIG. 8. Distribution of marker activities and DS-Mn following sucrose gradient centrifugation. Chloroplasts (6.9 mg Chl) that were purified through a cushion of Percoll (see "Materials and Methods") were applied to a discontinuous gradient of sucrose. Following centrifugation, the tube was punctured from the bottom and fractionated by upward displacement using an ISCO fractionator. A, The distribution of protein was monitored at 280 nm (left ordinate). Percent sucrose (inset) was calculated from the refractive index (right ordinate). B, Nitrite reductase (right ordinate), Chl (left ordinate). In an identical gradient, chloroplasts banding at the 20 to 45% sucrose interface and those sedimenting at the 45 to 60% sucrose interface were collected separately (see "Materials and Methods"), disrupted with a final concentration of 0.05% (v/v) Triton X-100, desalted on PD-10 (Sephadex G-25) columns to remove sucrose, and assayed for DS-Mn as described in "Materials and Methods." The histogram (inset) illustrates DS-Mn activity in the purified plastid fraction.

Table III. *Application of Differential Assay Conditions to Various Plant Sources*

Organism	Specific Activity		
	Assay-Co[a]	Assay-Mn[b]	
		−DTT	+DTT
	nmol DAHP/min·mg protein		
N. silvestris leaves	95.0	2.6	11.6
Cauliflower floret	41.6	ND[c]	10.1
Broccoli floret	6.8	ND	2.5
Soybean seedling	17.9	0.3	0.9
Alfalfa seedling	20.4	0.7	1.1
Squash seedling	11.6	0.2	1.6
Wheat seedling	49.7	0.7	2.2
Rye seedling	60.9	1.2	2.3
Potato tuber	41.6	2.7	12.3

[a] Reaction mixtures contained 50 mM K Epps (pH 8.6), 2 mM PEP, 6 mM E4P, and 10 mM $MgCl_2$. With cauliflower and broccoli 2 mM E4P was used and 0.5 mM $CoCl_2$ was substituted for $MgCl_2$. [b] Reaction mixtures contained 50 mM K Epps (pH 8.0), 3 mM PEP, 0.6 mM E4P, and 0.5 mM $MnCl_2$. DTT was absent (−DTT) or present (+DTT) at a final concentration of 0.5 mM. [c] ND, not detected. With cauliflower and broccoli 2 mM PEP and 0.5 mM E4P were used.

were shown to contain both DAHP synthase isozymes. Highly purified chloroplasts contained only the DTT-activated DS-Mn. Although attempts to demonstrate light activation of DS-Mn in leaves and in isolated chloroplasts have so far yielded erratic results, it seems reasonable to consider the possibility further that this isozyme may resemble redox-activated regulatory enzymes of the Calvin cycle (5) that are switched on during illumination with light. Thus, the activity of DS-Mn might be coordinated with the production of E4P by photosynthesis.

One striking property of DS-Mn was the hysteretic activation by DTT. The DTT-mediated conversion from inactive to active enzyme was found to be a slow process effected by protein concentration. That this phenomenon truly reflected hysteretic activation (*i.e.* slow transition to activated state in response to ligand addition) was confirmed by the ability to activate the enzyme prior to assay, thus eliminating the lag otherwise seen in the progress curve. The mode of action of DTT activation appeared to be the reduction of one or more disulfide bonds on the DS-Mn protein since the fully activated enzyme could be completely inactivated by the sulfhydryl-blocking reagent, iodoacetamide. Although the hysteretic effect of DTT upon mung bean DS-Mn was not fully appreciated by Rubin and Jensen (24), unpublished data (JL Rubin) indicate that DS-Mn of mung bean does indeed exhibit hysteretic behavior in response to DTT similar to the DS-Mn of *N. silvestris*.

The selective assays developed through characterization of partially purified isozymes prepared from mung bean (24) and from suspension-cultured cells of *N. silvestris* have proven useful for application to identification of the isozyme pair in various other plants. The remarkable similarity of the distinctive properties of the DAHP synthase isozymes from *N. silvestris* to those present in mung bean (24), spinach, and plants listed in Table III indicate the probable ubiquity of this isozyme pair in higher plants.

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LITERATURE CITED

1. AP REES T 1980 Integration of pathways of synthesis and degradation of hexose phosphates. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 3. Academic Press, New York, pp 1–42
2. BERRY WL 1978 Nutrition, containers, and media. *In* RW Langhans, ed, A Growth Chamber Manual: Environmental Control for Plants. Cornell University Press, Ithaca, pp 117–135
3. BICKEL H, L PALME, G SCHULTZ 1978 Incorporation of shikimate and other precursors into aromatic amino acids and prenylquinones of isolated spinach chloroplasts. Phytochemistry 17: 119–124
4. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72: 248–254
5. BUCHANAN BB 1980 Role of light in the regulation of chloroplast enzymes. Annu Rev Plant Physiol 31: 341–374
6. BUCKHOLTZ B, B REUPKE, H BICKEL, G SCHULTZ 1979 Reconstruction of amino acid synthesis by combining spinach chloroplasts with other leaf organelles. Phytochemistry 18: 1109–1111
7. D'AMATO TA, RJ GANSON, CG GAINES, RA JENSEN 1984 Subcellular localization of chorismate-mutase isoenzymes in protoplasts from mesophyll and suspension-cultured cells of *Nicotiana silvestris*. Planta 162: 104–108
8. FRIEDEN C 1970 Kinetic aspects of regulation of metabolic processes. The hysteretic enzyme concept. J Biol Chem 245: 5788–5799
9. GAINES CG, GS BYNG, RJ WHITAKER, RA JENSEN 1982 L-Tyrosine regulation and biosynthesis via arogenate dehydrogenase in suspension-cultured cells of *Nicotiana silvestris* Speg et Comes. Planta 156: 233–240
10. GILCHRIST DC, T KOSUGE 1980 Aromatic amino acid biosynthesis and its regulation. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 3. Academic Press, New York, pp 507–531
11. GOERS SK, RA JENSEN 1984 Separation and characterization of two chorismate-mutase isoenzymes from *Nicotiana silvestris*. Planta 162: 109–116
12. GOERS SK, RA JENSEN 1984 The differential allosteric regulation of two chorismate-mutase isoenzymes of *Nicotiana silvestris*. Planta 162: 117–124
13. GOTTLIEB LD 1982 Conservation and duplication of isozymes in higher plants. Science 216: 373–380
14. GRAZIANA A, AM BOUDET 1980 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase from *Zea mays*: general properties and regulation by tryptophan. Plant Cell Physiol 21: 793–802
15. HUISMAN OE, T KOSUGE 1974 Regulation of aromatic amino acid biosynthesis in higher plants. II. 3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase from cauliflower. J Biol Chem 249: 6842–6848
16. JENSEN RA 1985 Tyrosine and phenylalanine biosynthesis: relationship between alternative pathways, regulation and subcellular location. Recent Adv Phytochem 20: 57–82
17. JENSEN, RA 1986 The shikimate/arogenate pathway: link between carbohydrate metabolism and secondary metabolism. Physiol Plant 66: 164–168
18. JENSEN RA, E NESTER 1966 Regulatory enzymes of aromatic amino acid biosynthesis in *Bacillus subtilis*. I. Purification and properties of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase. J Biol Chem 241: 3365–3372
19. MILLS WR, KW JOY 1980 A rapid method for isolation of purified, physiologically active chloroplasts, used to study the intracellular distribution of amino acids in pea leaves. Planta 148: 75–83
20. MINAMIKAWA T 1967 A study on 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase in higher plants. Plant Cell Physiol 8: 695–707
21. MINAMIKAWA T, I URITANI 1967 3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase in sweet potato roots. J Biochem 61: 367–372
22. ROTHE GM, W MAURER, C MIELKE 1976 A study on 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase in higher plants. The existence of three isoenzymes in *Pisum sativum*. Ber Dtsch Bot Ges 89: 163–173
23. RUBIN JL, CG GAINES, RA JENSEN 1982 Enzymological basis for herbicidal action of glyphosate. Plant Physiol 70: 833–839
24. RUBIN JL, RA JENSEN 1985 Differentially regulated isozymes of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase from seedlings of *Vigna radiata* (L.) Wilczek. Plant Physiol 79: 711–718
25. SRINIVASAN PR, DB SPRINSON 1959 2-Keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate synthetase. J Biol Chem 234: 716–722
26. SUZICH JA, R RANJEVA, PM HASEGAWA, KM HERRMANN 1984 Regulation of the shikimate pathway of carrot cells in suspension culture. Plant Physiol 75: 369–371
27. WEISSBACH A, J HURWITZ 1959 The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. I. Identification. J Biol Chem 234: 705–709