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3-Deoxy-D-*arabino*-Heptulosonate 7-Phosphate Synthase from Carrot Root (*Daucus carota*) Is a Hysteretic Enzyme[1]

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ABSTRACT

Roots of carrots (*Daucus carota*) contain three activities of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase, the enzyme that catalyzes the first step of the shikimate pathway. The three activities, enzymes I, II, and III, are separated by chromatography on phosphocellulose. Enzyme III, purified to electrophoretic homogeneity, has a native molecular weight of 103,000 and consists of two identical subunits of 53,000 daltons each. Double reciprocal plots of reaction velocity *versus* substrate concentration yield K_m values of 0.03 and 0.07 millimolar for P-enolpyruvate and erythrose-4-P, respectively. Both products, DAHP and orthophosphate, inhibit the enzyme. Enzyme III is a hysteretic enzyme that is activated by physiological concentrations of L-tryptophan and Mn^{2+}, both of which also partially eliminate the hysteretic lag. Feedback activation of carrot DAHP synthase by tryptophan is interpreted to be an early regulatory signal for polyphenol biosynthesis. The three carrot DAHP synthase isoenzymes share antigenic determinants.

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP[3]) synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate lyase [pyruvate phosphorylating], EC 4.1.2.15) catalyzes the first step in the shikimate pathway that leads to the synthesis of the aromatic amino acids in microorganisms and plants (28). This enzyme has been purified to homogeneity from *Neurospora crassa* (10, 19), *Bacillus subtilis* (13), *Escherichia coli* (17, 25), *Salmonella typhimurium* (11), *Schizosaccharomyces pombe* (2), and *Brevibacterium flavum* (29). The enzyme from cauliflower is the only plant DAHP synthase that has been substantially purified (14).

Microorganisms employ a variety of mechanisms to control carbon flow through the shikimate pathway (8). While shikimate kinase appears to be the key allosteric control point of this metabolic route in *B. subtilis* (12), DAHP synthase is the principal point of control in *E. coli* (20). In higher plants, up to 25% of the total carbon flow proceeds via the shikimate pathway. Apparently, some plants also regulate carbon flow along the shikimate pathway at the first step, because DAHP synthase isoenzymes (22, 23) and inhibitable forms of the enzyme (6, 21) have been reported for various plant tissues. The DAHP synthase activity of carrot cells grown in suspension cultures varies synchronously with the cell cycle (31).

This paper shows that carrot roots contain three forms of DAHP synthase, enzymes I, II, and III; enzyme III is purified to electrophoretic homogeneity. This paper also presents a kinetic analysis that identifies enzyme III as hysteretic; hysteresis is affected by tryptophan, a feedback activator of the enzyme.

MATERIALS AND METHODS

Reagents. PEP (4), erythrose-4-P (27), and DAHP (9) were synthesized and assayed as described previously (9, 25). All other reagents were obtained commercially at the highest purity available and were used without further purification.

Enzyme Assays. DAHP synthase was assayed by either of two methods (25). In method A, the reaction mixture contained 0.75 μmol of PEP, 0.3 μmol of erythrose-4-P, 0.07 μmol of L-tryptophan, 0.3 μmol of $MnCl_2$, and 100 μg of ovalbumin in a total volume of 0.1 ml of 50 mM bis-tris propane (pH 7.0). The reaction was initiated by adding 0.05 ml of appropriately diluted enzyme. The mixture was incubated for 20 min at 25°C. The reaction was stopped with TCA. DAHP was determined after degradation with $NaIO_4$ as the thiobarbiturate adduct. This endpoint assay was mainly used during the purification of the enzyme. In method B, 0.9 ml of appropriately diluted enzyme in 50 mM bis-tris propane (pH 7.0), containing 1 mM DTT and 150 μM PEP were prewarmed to 25°C. The reaction was started by addition of 100 μl of 2.5 mM erythrose-4-P dissolved in the same buffer. The disappearance of PEP was continuously monitored by reading A_{232}. This kinetic assay measures initial velocities and was used for most of the studies with the pure enzyme. One unit of DAHP synthase is defined as the amount of protein catalyzing the appearance of 1 μmol of DAHP (method A) or the disappearance of 1 μmol of PEP (method B) per min. Chorismate mutase was assayed as described (24).

Protein Determination. Protein was determined by the method of Lowry *et al.* (15). Since DTT, bis-tris propane, and tryptophan interfere with this assay, protein was precipitated with 20% TCA and resuspended in 0.1 N NaOH, containing 0.2 M Na_2CO_3, prior to determination. During enzyme purification, protein in tryptophan containing buffers was determined by the method of Bradford (3) with lysozyme as the standard.

Purification of DAHP Synthase III from Carrot Root (*Daucus carota*). All manipulations were carried out at 4°C.

Step I. Fresh carrot roots were washed extensively, rinsed with distilled H_2O, cooled to 4°C, and shredded in a food processor. About 1 kg of shredded carrots were added to 800 ml of ice-cold 50 mM bis-tris propane (pH 7.0), containing 50 mM 2-mercaptoethanol, 5 mM sodium meta-bisulfite, 2 mM $MnCl_2$, 30 μM $MgCl_2$, 1 mM tryptophan, 0.1 mM phenylmethylsulfonylfluoride (buffer A), and 75 g of PVP. The carrots were macerated with a Brinkmann homogenizer and then pressed through six layers of

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[3] Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; PEP, phosphoenolpyruvate.

Table I. *Purification of DAHP Synthase III*

The starting material was 5 kg of carrot root.

	Fraction	Total Vol	Total Protein	Total Enzyme Units	Specific Enzyme Activity	Enrichment	Yield
		ml	*mg*			*fold*	%
Step I	Cell extract	7,000	18,550				
Step II	0–50% $(NH_4)_2SO_4$	380	3,177	65.4[a]	0.021		
Step III	Phosphocellulose	63	30.9	16.5[b]	0.53	25	25
Step IV	1st Matrix Gel Orange A	36	5.3	14.3[b]	2.7	128	22
Step V	2nd Matrix Gel Orange A	24	0.84	5.4[b]	6.4	305	8
Step VI	Hydroxylapatite	4	0.71	5[b]	7.1	338	8

[a] These values represent total carrot DAHP synthase activity. [b] These values represent DAHP synthase III only.

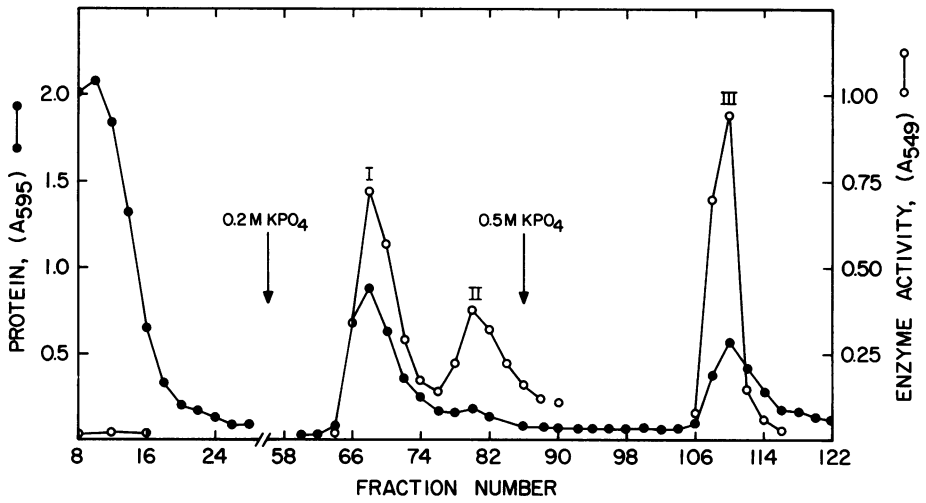

FIG. 1. Separation of carrot DAHP synthase isoenzymes by phosphocellulose column chromatography. Elution was with a step gradient as indicated by the arrows; (●), total protein; (○), DAHP synthase enzyme activity.

cheesecloth. The resulting juice was slowly mixed with one-twentieth volume of 2% protamine sulfate in buffer A and centrifuged for 30 min at 14,000*g*.

Step II. Finely ground $(NH_4)_2SO_4$ was added to the supernatant of step I to give 50% saturation. The precipitate was collected by centrifugation (90 min at 14,000*g*), resuspended in and dialyzed against buffer A. After 12 h the dialysis buffer was changed to 50 mM K-phosphate (pH 7.0), containing 20 mM 2-mercaptoethanol, 5 mM sodium meta-bisulfite, 0.2 mM $MnCl_2$, 3 μM $MgCl_2$, 1 mM tryptophan, and 0.1 mM phenylmethylsulfonyl fluoride (buffer B).

Step III. The protein solution obtained from 5 kg of carrots purified through Step II was clarified by centrifugation for 30 min at 40,000*g* and applied to a phosphocellulose column (Whatman P11; 1.5 × 110 cm) equilibrated with buffer B. DAHP synthase I/II and III were eluted by increasing the K-phosphate concentration to 0.2 and 0.5 M, respectively (Fig. 1). Fractions of 4.5 ml were collected at a flow rate of 30 ml/h.

Step IV. Fractions containing DAHP synthase III were pooled, and finely ground $(NH_4)_2SO_4$ was added to give 70% saturation. The precipitate was collected by centrifugation, resuspended in and dialyzed against buffer A followed by dialysis against 50 mM K-phosphate (pH 7.0), containing 1 mM tryptophan, 1 mM DTT, 0.2 mM $MnCl_2$, 3 μM $MgCl_2$, and 0.1 mM phenylmethylsulfonyl fluoride (buffer C). The protein solution was cleared by centrifugation and applied to a Matrix Gel Orange A column (Amicon; 2 × 15 cm) equilibrated with buffer C (pH 7.4). Fractions of 1.5 ml were collected at a flow rate of 15 ml/h.

Step V. Fractions containing DAHP synthase were pooled and dialyzed against 40 mM K-phosphate (pH 6.8), containing 1 mM DTT, 0.2 mM $MnCl_2$, and 3 μM $MgCl_2$. The dialysate was reapplied to the Matrix Gel Orange A column equilibrated with the dialysis buffer. DAHP synthase was eluted with 80 mM K-phosphate (pH 7.6), containing 1 mM DTT, 1 mM tryptophan, 0.2 mM $MnCl_2$, and 3 μM $MgCl_2$. Fractions of 1 ml were collected at a flow rate of 20 ml/h.

Step VI. Fractions containing DAHP synthase were pooled, dialyzed against 0.1 M K-phosphate (pH 7.0), containing 1 mM DTT, 1 mM tryptophan, 0.2 mM $MnCl_2$, and 3 μM $MgCl_2$, and applied to a hydroxylapatite column (BioGel HTP; 1.5 × 7 cm), equilibrated with the same buffer. DAHP synthase was eluted with a linear gradient obtained from 35 ml of equilibration buffer and 35 ml of 0.5 M K-phosphate (pH 7.0), containing 1 mM DTT, 1 mM tryptophan, 0.2 mM $MnCl_2$, and 3 μM $MgCl_2$. Fractions of 1 ml were collected at a flow rate of 10 ml/h.

SDS-PAGE. Protein samples were analyzed by SDS-PAGE (32), modified as described previously (17).

Preparation of Antibodies. DAHP synthase III purified through step VI (0.8–1.0 mg) and dialyzed against 10 mM K-phosphate (pH 7.4), containing 0.15 M NaCl, was mixed with an equal volume of Freund's complete adjuvant. The emulsion was injected intradermally into the back of a New Zealand White rabbit; intradermal booster injections of the same protein (100–200 μg) in Freund's incomplete adjuvant followed 8 and 10 weeks later. Sera from blood collected 7 d after the booster injections were assayed for anti-DAHP synthase III antibodies. The immunoglobulins of the sera were partially purified by three consecutive precipitations with solid $(NH_4)_2SO_4$ to 33% saturation.

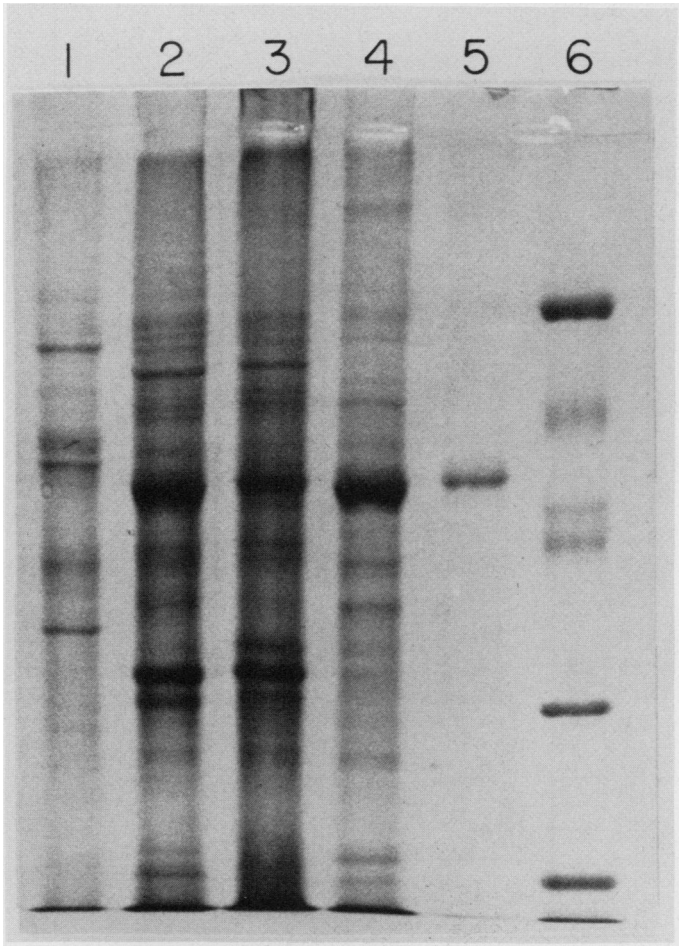

FIG. 2. SDS-PAGE. DAHP synthase activity containing fractions were pooled, and the proteins contained in the indicated sample volumes applied to the gel. Lane 1, 10 μl of the $(NH_4)_2SO_4$ pool; lane 2, 250 μl of the phosphocellulose pool; lane 3, 100 μl of the phosphocellulose pool after concentration with $(NH_4)_2SO_4$; lane 4, 1 ml of the first Orange A pool; lane 5, 1 ml of the second Orange A pool; lane 6, 1 μl of BioRad premixed mol wt standards.

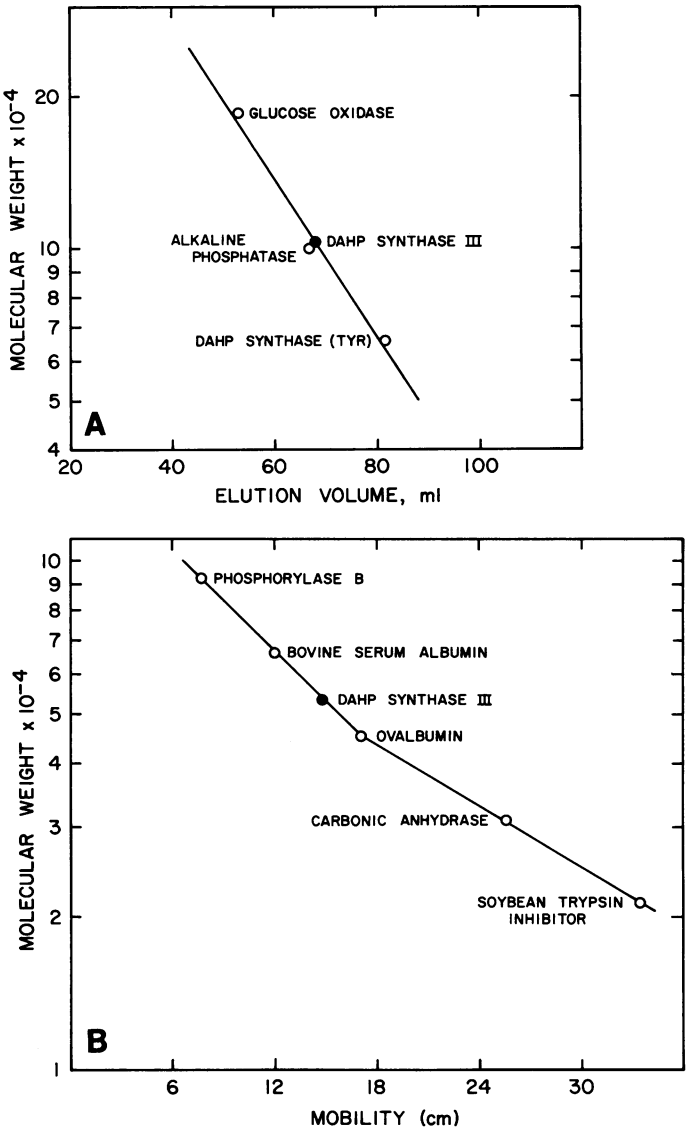

FIG. 3. Mol wt determinations. A, samples of purified native DAHP synthase III and the indicated standards were applied to a Sephadex G-150 column equilibrated with 0.25 M K-phosphate (pH 7.0), containing 1 mM tryptophan and 1 mM DTT. B, DAHP synthase and the indicated standards were denatured and subjected to SDS-PAGE.

RESULTS

Purification of DAHP Synthase from Carrot Root. DAHP synthase activity cannot be measured in crude cell extracts of carrot roots, due to the presence of compounds which interfere with the enzyme assay. However, the bulk of extractable carrot DAHP synthase activity is found in the protein precipitated from cell extracts with $(NH_4)_2SO_4$ at a final concentration of 50%. The specific enzyme activity in this fraction varies greatly with different batches of carrots; values range from 0.003 to 0.03 enzyme units/mg protein and depend at least in part on the freshness of the carrots. When carrots are stored at 4°C, 50% of the enzyme activity is lost in about 12 d; storage at −20°C does not preserve the activity either.

A summary of a typical purification is given in Table I. The enzyme purified through step III can be stored at 4°C for several months with little loss in activity. However, metals are necessary to maintain enzyme stability in such preparations. Thus, Mn^{2+} and Mg^{2+} have always been included in buffers. When enzyme purified through step II is dialyzed against K-phosphate containing no additional metals, 20% of the activity is lost; the remaining 80% is stable. However, if the protein precipitate of the $(NH_4)_2SO_4$ fractionation is directly resuspended in K-phosphate, 90% of the DAHP synthase activity is lost within 24 h at 4°C. This reproducible observation cannot be readily explained.

Upon phosphocellulose chromatography, DAHP synthase from carrot root is separated into three activities (Fig. 1). Two activities, eluted by 0.2 M K-phosphate, are designated DAHP synthase I and II; a third activity, DAHP synthase III, elutes at 0.5 M K-phosphate. DAHP synthase III accounts for approximately 50% of the total recovered enzyme activity. The relative amounts of DAHP synthases I and II vary with the different carrot preparations much more than the activity of DAHP synthase III.

DAHP synthase III binds L-tryptophan (see below). Upon binding of L-tryptophan, enzyme III apparently undergoes a substantial conformational change which is exploited in the purification scheme for this enzyme. At pH values above 7.0 in 50 mM K-phosphate, containing 1 mM tryptophan, the enzyme does not bind to Matrix Gel Orange A. However, when tryptophan is left out of the buffer and the pH is shifted to 6.8, the enzyme binds tightly to the matrix, and can be eluted from the matrix with 80 mM K-phosphate (pH 7.6), containing 1 mM

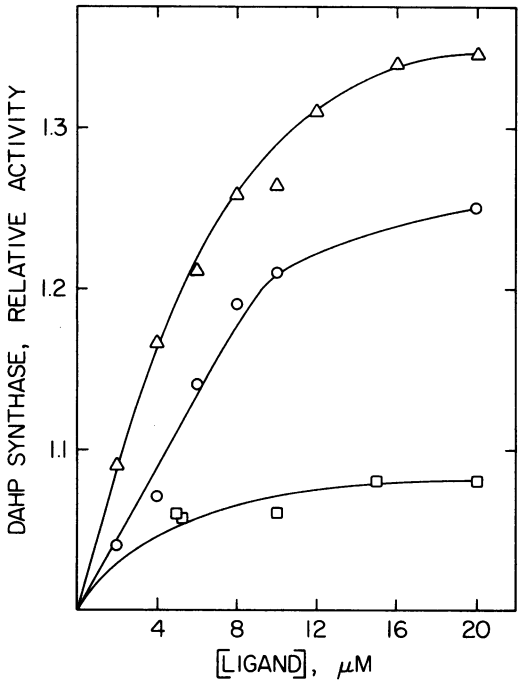

FIG. 4. Activation of DAHP synthase III by tryptophan (△), Mn^{2+} (○), and Mg^{2+} (□). The relative enzyme activity assayed by method B is plotted against the concentrations of the ligands.

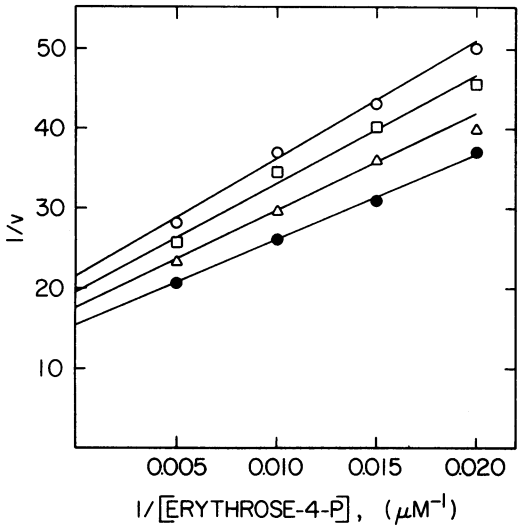

FIG. 5. Double reciprocal plots of reaction velocities as a function of erythrose-4-P concentrations. Enzyme (8 μg/assay) initial velocities were determined as ΔA_{232}/min within the first 90 s after addition of erythrose-4-P. PEP concentrations were 50 μM (○), 67 μM (□), 100 μM (△), and 150 μM (●).

tryptophan. Neither the pH shift nor the shift in the concentration of phosphate or tryptophan alone, but only the indicated combination of the three parameters can accomplish binding and elution.

By the six steps summarized in Table I, DAHP synthase III is purified to electrophoretic homogeneity (Fig. 2). The overall purification scheme for this enzyme (Table I) accomplishes a 338-fold enrichment with a yield of 8%. The actual purification is much greater, because the enzyme activity cannot be measured in the cell extract. Five kg of carrots yielded 0.74 mg of purified DAHP synthase III. If one assumes that 2% of the carrot root is protein, the six-step scheme of Table I represents a more than

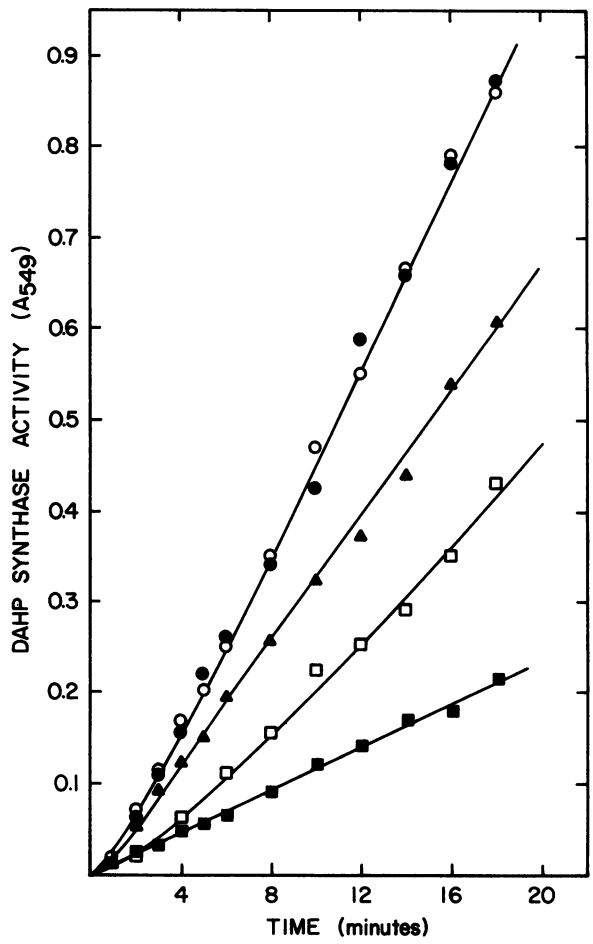

FIG. 6. Hysteresis of DAHP synthase III. The enzyme was assayed by method A. Pure DAHP synthase III, dialyzed against 50 mM bis-tris propane (pH 7.0), containing 1 mM DTT, was preincubated for 5 min at 25°C with PEP and ovalbumin (□); plus 0.8 mM tryptophan (▲); plus 0.5 mM Mn^{2+} (●); plus 0.8 mM tryptophan and 0.5 mM Mn^{2+} (○); plus 0.6 M 1,3-propanediol (■). The reaction was started with erythrose-4-P. Enzyme activity is plotted as the function of time after addition of erythrose-4-P.

10,000-fold purification for DAHP synthase III.

Mol Wt and Subunit Structure of Enzyme III. The mol wt of native DAHP synthase III from carrot root is 103,000 as estimated by gel filtration on Sephadex G-150 (Fig. 3A). The subunit mol wt of the enzyme is 53,000 as determined by SDS-PAGE (Fig. 3B). Thus, carrot DAHP synthase III appears to be a dimer.

Effects of Tryptophan and Metal Ions on Enzyme III. Carrot DAHP synthase III is activated by L-tryptophan (Fig. 4) and to a lesser degree by L-tyrosine (data not shown); the D-amino acids have no effect. None of the other 18 amino acids stimulates or inhibits this enzyme. The tryptophan activation is seen at all stages of purity.

While Mn^{2+} is necessary to stabilize the enzyme activity during the early stages of purification, μM concentrations of this metal (and to a lesser extent of Mg^{2+}) also stimulate the purified DAHP synthase III (Fig. 4). The enzyme is inhibited by metal chelators.

Enzyme Kinetics. Figure 5 shows double reciprocal plots of reaction velocities *versus* substrate concentrations. Linear regression analysis of the data yields K_m values of 0.03 and 0.07 mM for PEP and erythrose-4-P, respectively. Carrot DAHP synthase III is inhibited by both of its products, DAHP and Pi. With both substrate concentrations at four times K_m, 50% inhibition of the enzyme is obtained with 0.1 mM DAHP or with 35 mM Pi. Thus,

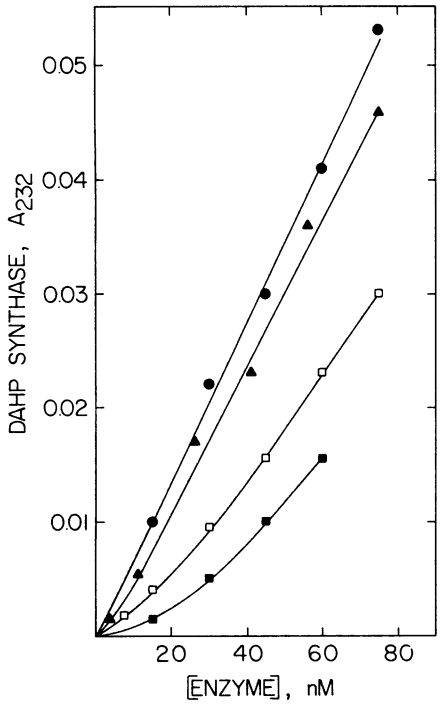

FIG. 7. Hysteresis of DAHP synthase III. The enzyme was assayed by method B. Initial velocities as a function of enzyme concentrations were determined as described in the legend to Figure 5; no additions (□); plus 0.1 mM tryptophan (▲); plus 20 μM Mn^{2+} (●); plus 0.6 M 1,3-propanediol (■).

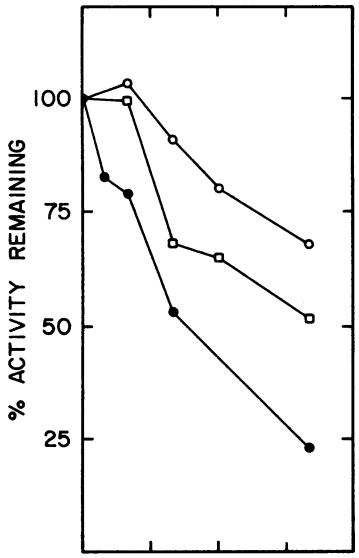

FIG. 8. Immunoinactivation of DAHP synthase isoenzymes by anti-DAHP synthase III antibodies. Increasing amounts of partially purified anti-DAHP synthase III antibodies are mixed with 0.05 units of DAHP synthase I (○), II (□), and III (●) eluted from a phosphocellulose column. The mixtures are incubated at 25°C for 20 min and then at 0°C for 2 h. Antigen-antibody complexes are removed by centrifugation and the supernatants assayed for DAHP synthase enzyme activity. The per cent activity remaining is plotted *versus* anti-DAHP synthase III antibodies; 100% activity is defined as the activity of supernatants from samples incubated with control serum.

the carrot DAHP synthase is not activated by its products.

Plots of enzyme activities as a function of time (Fig. 6) or enzyme concentration (Fig. 7) are nonlinear. The lag times before steady-state velocities are reached are typical for hysteretic enzymes (5, 18). Preincubation of the enzyme with either tryptophan or Mn^{2+} decreased the lag, but does not eliminate it entirely (Figs. 6 and 7). Also, 1,3-propanediol, which stabilizes the bacterial enzyme (25), inhibits the carrot enzyme (Figs. 6 and 7), and accentuates the effect of dilution upon reaction velocity (Fig. 7). The kinetic data identify the carrot DAHP synthase III as a hysteretic enzyme. Tryptophan, Mn^{2+}, and propanediol are ligands that affect the hysteresis of this protein.

Comparison of Carrot DAHP Synthases. Chromatography of carrot root extracts on phosphocellulose resolves three DAHP synthase activities (Fig. 1) which share some molecular properties. Antibodies directed against both native and SDS denatured carrot DAHP synthase III recognize and inhibit all three carrot DAHP synthases (Fig. 8) but none of the three *E. coli* isoenzymes.

DISCUSSION

Lignin, the polyphenolic glue that converts cellulose into wood is the second most abundant compound of higher plant cells. Since one precursor for lignin is phenylalanine, the shikimate pathway has to be very active during rapid growth of plants. To investigate the carbon flow regulation through this important plant pathway, we purified the first enzyme of this pathway, the DAHP synthase, from carrot root.

Carrot roots contain three DAHP synthase isoenzymes that can be separated by phosphocellulose chromatography. The predominant isoenzyme, DAHP synthase III, was purified to electrophoretic homogeneity. The carrot enzyme is the first plant DAHP synthase that has been obtained in pure form.

Carrot DAHP synthase III is a dimer with subunits of 53,000 D. While DAHP synthase from *B. subtilis* and *E. coli* are also oligomeric enzymes, but with subunit mol wt of approximately 40,000 (13, 17, 25), the subunit size of the enzyme from *B. flavum* (29) appears to resemble the carrot DAHP synthase III. However, in *B. flavum*, DAHP synthase and chorismate mutase form a bifunctional enzyme (30), whereas the purified carrot DAHP synthase III has no chorismate mutase activity. The carrot DAHP synthase and chorismate mutase are separated by chromatography on phosphocellulose from which chorismate mutase elutes in the flow through volume under the conditions given in Figure 1. Therefore, not only is the carrot enzyme the first plant DAHP synthase for which a native and a subunit mol wt is reported, the carrot enzyme is also the first monofunctional DAHP synthase with the larger mol wt subunit. This indicates functional differences between the bacterial and the plant enzymes.

The carrot enzyme III is also the first plant DAHP synthase for which monospecific antibodies have been prepared. The three carrot isoenzymes apparently share a broad range of antigenic determinants, in direct contrast to the three isoenzymes from *E. coli* (16; J. A. Suzich, K. M. Herrmann, unpublished data).

In microorganisms, feedback inhibition of DAHP synthase by aromatic amino acids is well established as a regulatory mechanism (8, 20). In higher plants, the enzyme from cauliflower is not affected by any aromatic amino acid (14), the corn seedling enzyme is inhibited by tryptophan (6), and the enzyme from pea leaves is inhibited by tyrosine with this inhibition reversed by tryptophan (21). In carrot root, DAHP synthase is not inhibited by any amino acid but *activated* by tryptophan (and to a lesser degree by tyrosine). Half maximal activation is obtained with less than 10 μM tryptophan, a concentration well below 111 μM, that has been estimated to be the free tryptophan concentration in carrot cells (34). Thus, the tryptophan activation may be of physiological significance. Tryptophan activation has also been

observed for the enzyme from carrot cells grown in suspension culture (31), and for the enzyme from potato tuber (J. Pinto, K. M. Herrmann, unpublished data).

Since DAHP synthases from different organisms catalyze identical reactions, the conformation of the polypeptide chain in the active sites should be very much alike. Therefore, it may be difficult to envision one allosteric ligand to inhibit the enzyme in one organism and to activate it in another. However, the tyrosine-sensitive DAHP synthase from *E. coli* is *inhibited* by tyrosine in buffers containing physiological substrate concentrations, and the enzyme is *activated* by tyrosine, when the PEP concentration falls below 10 μM (25). Inhibition and activation seem to be merely the consequences of a continuously changing sterical arrangement that depends upon the microenvironment. Analogously, one may not necessarily expect the active sites of the tryptophan-*inhibited E. coli* enzyme and the tryptophan-*activated* carrot enzyme to be structurally very different.

Tryptophan activation has previously been reported for another enzyme of aromatic amino acid biosynthesis; tryptophan activates the chorismate mutase of *B. flavum* through stabilization of the enzyme's quaternary structure (26). A similar mechanism is consistent with our kinetic analysis of the carrot DAHP synthase. Like many regulatory enzymes, carrot DAHP synthase III is hysteretic, and like other hysteretic enzymes, DAHP synthase III may undergo a conformational change or a change in the aggregation of subunits that proceeds slowly in relation to catalysis. Dissociation into less active subunits upon dilution would readily explain the nonlinear plot of enzyme activity as a function of enzyme concentration (Fig. 7). Tryptophan and Mn^{2+} would facilitate subunit aggregation, while 1,3-propanediol would prevent the aggregation into more active forms of the enzyme. A slow response to changes in the levels of substrates or ligands may be a time dependent buffer (5). In carrot, the slow response of DAHP synthase could delay an increase in PEP or erythrose-4-P levels from being immediately translated into increased carbon flow through the shikimate pathway. The reversal of the hysteretic behavior and the concomitant activation of the enzyme by physiological concentrations of tryptophan might be a mechanism to ensure an adequate supply of chorismate for phenylalanine and tyrosine biosynthesis. Phenylalanine and tyrosine, but not tryptophan, are substrates for phenylalanine ammonia lyase, one key enzyme of polyphenol biosynthesis (7). In carrot, the terminal pathway to tryptophan is controlled by tryptophan-mediated feedback inhibition of anthranilate synthase (33). Since tryptophan biosynthesis is energetically the most expensive of all amino acid biosynthetic pathways (1), tryptophan could also act as an early energy monitor for polyphenol biosynthesis through *feedback activation* of DAHP synthase. When the cell has sufficient energy to produce tryptophan at levels exceeding protein biosynthetic requirements, DAHP synthesis is enhanced and thereby more phenylalanine and tyrosine is available for the synthesis of secondary metabolites.

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