Purification and Kinetics of Tyrosine-sensitive 3-Deoxy-d-arabino-heptulosonic Acid 7-Phosphate Synthetase from Salmonella*

(Received for publication, October 17, 1972)

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SUMMARY

Tyrosine-sensitive 3-deoxy-d-arabino-heptulosonic acid 7-phosphate synthetase was prepared by a relatively simple procedure from an operator constitutive (tyrO) strain of Salmonella typhimurium in highly purified, nearly homogeneous form. It had a molecular weight of approximately 100,000 and was independent of Co2+ for activity. In the reaction of d-erythrose 4-phosphate with enolpyruvate phosphate labeled with 32P in the C—O—P linkage, 32P was recovered almost entirely in orthophosphate released, indicating a C—O cleavage mechanism. The kinetic properties of the enzyme were studied by measuring release of 32P from [32P]enolpyruvate phosphate. With sufficiently low levels of substrates a sequential mechanism was indicated, rather than a ping-pong mechanism as previously reported from other laboratories. Rate equations were derived for bireac tant mechanisms in which conversion of the ternary complex of enzyme and substrates to that of enzyme and products is irreversible. Expected inhibition patterns were deduced and used to analyze inhibition data. Product inhibition patterns by orthophosphate and 3-deoxy-d-arabino-heptulosonic acid 7-phosphate indicated an ordered reaction in which enolpyruvate phosphate was the first substrate to bind to enzyme and orthophosphate the first product to be released. A mechanism for the reaction is suggested which is in accord with these kinetic results, as well as orthophosphate release with C—O cleavage and stereospecific addition of erythrose 4-phosphate to enolpyruvate phosphate. The kinetics of inhibition of enzyme by d-erythritol 4-phosphate indicated that it was an apparent analog of 3-deoxy-d-arabino-heptulosonate 7-phosphate rather than of erythrose 4-phosphate.

* This work was supported by grants from the American Cancer Society, the American Heart Association, the United States Public Health Service, and the National Science Foundation.
‡ Present address, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. Career Investigator Fellow of the American Heart Association, 1971-72. Supported in part by National Institutes of Health Training Grant GM235 and Predoctoral Fellowship FOI GM38232. Part of this report is from a dissertation submitted in February 1970 in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Sciences, Columbia University.
§ Career Investigator of the American Heart Association.

3-Deoxy-d-arabino-heptulosonate 7-phosphate synthetase (EC 4.1.2.15) catalyzes the first committed reaction of aromatic amino acid biosynthesis (1).

Enolpyruvate-P + d-erythrose-4-P + H2O → P1 + DAHP

Most bacterial species, molds, and yeast have three isoenzymes for this activity under control of tyrosine, phenylalanine, and tryptophan, respectively (2, 3). As part of a study of these isoenzymes in Salmonella typhimurium we have prepared highly purified tyrosine-sensitive DAHP synthetase and have investigated some of its properties. A convenient source of the enzyme was an operator constitutive strain which had derepressed levels of tyrosine operon enzymes (4).

The kinetic properties of phenylalanine- (5) and tyrosine sensitive (6) DAHP synthetases of Escherichia coli have been investigated previously. Parallel lines were obtained in double reciprocal plots of initial velocity against substrate concentration, and a ping-pong mechanism (7) was suggested (5, 6) which comprised two partial reactions.

Enzyme + enolpyruvate-P ⇌ pyruvyl enzyme + P1 (1)
Erythrose-4-P + pyruvyl enzyme ⇌ DAHP + enzyme (2)

Our initial studies of the reaction showed that P1 release occurred with C—O cleavage of enolpyruvate-P (8). A ping-pong mechanism could therefore be rationalized by suggesting that an enolpyruvyl enzyme was formed by nucleophilic attack of enzyme at C-2 of enolpyruvate-P, protonation at C-3, and elimination of P1. An addition-elimination mechanism for enolpyruvyl enzyme formation would predict incorporation of a proton from the medium at C-3 of DAHP. Floss et al. (9) have recently confirmed the small incorporation of H1 and H2 into DAHP, and have shown that the reaction is stereospecific. Hence, formation of a methyl group intermediate, by analogy with the mechanism suggested for 3-enolpyruvylshikimate-3-P synthetase (10), is unlikely. Since a two-step mechanism is otherwise difficult to reconcile with
C—O cleavage of enolpyruvate-P, we reinvestigated the kinetics of the DAHP synthetase reaction. The results reported here indicate that the mechanism is ordered sequential rather than ping-pong. A portion of this work was published in preliminary form (8).

**EXPERIMENTAL PROCEDURE**

**Materials**

Monoecyclohexammonium enolpyruvate-P was prepared according to Clark and Kirby (11). This compound labeled with C—O in C—O—P oxygen and barium 5-enolpyruvylshikimate-3-P were prepared according to published procedures from this laboratory (10, 12). Bis-cyclohexammonium β-erythritol-4-P (13) was a generous gift of Professor C. E. Ballou. BarDAHP was prepared according to an unpublished procedure. Cyclohexammonium salts were used directly, and barium salts were converted to potassium salts. Erythrose-4-P for routine assays was prepared by the method of Ballou and MacDonald (14). The solution obtained in this preparation was brought to pH 4.0 to 5.0 with KOH, assayed for erythrose-4-P with DAHP synthetase (in the presence of excess enolpyruvate-P), adjusted to 0.01 M, and stored at 4°C. Aliquots were neutralized before use in the assay. Erythrose-4-P for kinetic measurements was prepared before use by converting the barium salt hydrate (15) to the free acid (15) and adjusting to pH 5.0 with KOH. An advantage of this method is that the product is essentially free of dimers. Concentration of erythrose-4-P was assayd with transaldolase (16), which was a generous gift of Professor B. L. Horecker. [U-14C]-Erythrose-4-P (2 μCi per μmole) was prepared from [U-14C]glucose-6-P (15), and fractions from Dowex 1 formate containing erythrose-4-P were pooled. The solution was passed through Amberlite IR-120 (H+), extracted continuously with ether for 48 hours to remove formic acid, concentrated in a vacuum, and adjusted to pH 5 with KOH.

[32P]Enolpyruvate-P was prepared from pyruvate and [β,γ-32P]ATP with enolpyruvate-P synthetase (17, 18), which was a generous gift of Dr. Mildred Cohn. [β,γ-32P]ATP (19), purified on Dowex 1 formate (19), was isolated by absorption on Norite, elution with ethyl-15 M NH₄OH (40:1:60), and lyophilization of the resulting solution (18). Equimolar amounts of ATP, pyruvate, and MgCl₂ were incubated with enolpyruvate-P synthetase and 2 mg of bovine serum albumin in 2.5 ml of 0.1 M M, Hepes buffer, pH 7.4, was substituted for Tris maleate. M, Hepes buffer, resuspended in the same, and adjusted to pH 7.4. Enzyme preparations containing glycerol required dilution to give a final concentration in the reaction mixture of 0.08% glycerol or less, since higher concentrations interfere with the periodate-thiobarbituric assay. Specific activity is defined as micromoles of DAHP per hour per mg of protein. Protein was determined by the method of Lowry and Lopez (23). Protein was determined by the method of Lowry et al. (24).

**Purification of DAHP Synthetase**

Unless otherwise stated all operations were at 2 to 4°C. Volumes and activities of fractions are in Table I. “Buffer” refers to potassium phosphate buffer, pH 7.0. Strain SG-12, previously described as jpr-2 (4), was grown in 15 liters of minimal medium (21) with vigorous shaking at 37°C to optical density of 0.87 at 660 nm. Cells were harvested by centrifugation (4,000 × g), and stored at −18°C until used. The thawed cells (30 g) were washed with 350 ml of 0.03 M barium buffer, resuspended in the same buffer (final volume 120 ml), and disrupted by subjecting 60 ml portions to oscillation in a 100-watt M.S.E. ultrasonic disintegrator at 4°C for three 15-min periods with 5 min rest periods. The suspension was centrifuged at 48,000 × g for 1 hour, and the extract was washed at 60 ml per hour under pressure of a peristaltic pump on a column (2.5 × 50 cm) of DE-52 which was equilibrated with 0.01 M buffer. The column was washed with 1 column volume of 0.03 M buffer and was eluted at 60 ml per hour with a linear gradient prepared from 2 liters of 0.01 M buffer and 2 liters of 0.15 M buffer. Fractions of 20 ml were collected. The major DAHP synthetase activity was in Fractions 54 to 66, which were pooled and diluted with an equal volume of 0.001 M buffer (final buffer concentration 0.035 M).

The solution was absorbed on a DE-52 column (1.6 × 10 cm) at 30 ml per hour and the column was washed as described previously. It was eluted at 30 ml per hour with a linear gradient of 0.001 M buffer (final buffer concentration 0.035 M).

**Rad Laboratories, and DEAE-cellulose was Whatman DE-52.**

Other materials were obtained from commercial sources.

**Methods**

DAHP synthetase activity was determined at 37°C in a 10-min incubation as described previously (21), except that 0.30 ml of 0.2 M Hepes buffer, pH 7.4, was substituted for Tris maleate. Bovine serum albumin (100 μg) was added to all purified fractions. Enzyme preparations containing glycerol required dilution to give a final concentration in the reaction mixture of 0.08% glycerol or less, since higher concentrations interfere with the periodate-thiobarbiturate assay. Specific activity is defined as micromoles of DAHP per hour per mg of protein. Protein was determined by the method of Lowry et al. (24).

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incubation mixtures.

of isolated Pi were corrected for dilution by Pi present in the reaction mixture, which was incubated at 37°C for 1 hour, and Pi was isolated. In a control experiment denatured enzyme and 100 mmoles of 18O-enolpyruvate-P and erythrose-4-P as indicated, 0.1 M ammonium molybdate in 1 M sodium acetate buffer, pH 4.0, containing 0.1 M mercuric acetate (to hydrolyze enolpyruvate-P). 14P present in labeled enolpyruvate-P was determined at the same time with 0.1 ml of solution and 0.9 ml of acetate buffer. 14P was assayed in 10 ml of a phosphor solution prepared from 2 parts of a solution of 0.5% 2,5-diphenyloxazole (PPO) and 0.03% 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in toluene, and 1 part of ethanol (v/v).

Inhibition Studies

Inhibition by indicated levels of added DAHP or n-erythritol-4-P was measured by rate of 14P release as described above.

### Isolation of Pi Released in DAHP Synthetase Reaction from 18O-Enolpyruvate-P

A solution (110 ml) containing 110 μmoles of 18O-enolpyruvate-P (13.2 atoms % excess 18O in C—O—P oxygen), 6.6 mmoles of Tris maleate buffer, pH 6.8, 10 mg of bovine serum albumin, and DAHP synthetase (1.5 mg of protein, specific activity 250 μmoles per hour per mg), was incubated at 37°C for 1 hour. The reaction mixture was brought to pH 8.0 with 15 μM NH4O2 and DAHP was determined. The solution was treated with 3.0 ml (0.1 ml per 4 μmoles of Pi) of magnesium mixture (26) and adjusted to pH 9.0 with 15 μM NH4O2. MgNH4PO4 was removed by centrifugation in the cold, washed with 4 μM NH4O2, dried, and dissolved in a slurry of Dowex 50W-X8 (H+) which was transferred to a 1-ml column of the resin. The column was washed four times with 0.3 ml of water, combined eluate and washings were evaporated to dryness, and KH2PO4 was isolated (26) and analyzed for 18O (27). 14P, isolated as described above from a similar reaction mixture containing H34O and unlabeled substrates, as well as water evaporated from it, were also analyzed for 18O. In a control experiment denatured enzyme and 100 μmoles of KH2H18O4 were added to the reaction mixture, which was incubated at 37°C for 1 hour, and Pi was isolated. 18O values of isolated Pi were corrected for dilution by Pi present in the incubation mixtures.

### Formation of DAHP in 3H2O or 2H2O

A solution (100 ml) containing 300 μmoles of enolpyruvate-P, 300 μmoles of erythrose-4-P, 5.5 μmoles of potassium phosphate buffer, pH 6.8, 10 mg of bovine serum albumin, DAHP synthetase (6 mg of protein, specific activity 250 μmoles per hour per mg), and 250 mCi of 3H2O was incubated at 37°C for 1 hour. The reaction was stopped by chilling in ice, and the amount of DAHP was determined. Dowex 1-X8 (Cl−, 200 to 400 mesh) was allowed to stand overnight in 3 x HCl, washed with water until free of Cl−, and freed of fine particles. The reaction mixture was absorbed at 12 ml per hour on a column (1.8 x 20 cm) of the resin, and eluted with a linear gradient prepared from 250 ml of water and 250 ml of 0.6 M NH4Br adjusted to pH 7.0 with NH4OH. Fractions of DAHP contaminated with Pi (in approximately 0.24 M NH4Br) were pooled, and the solution was diluted with an equal amount of water and rechromatographed as described above. DAHP fractions were pooled and concentrated to 5 ml, and the solution was treated with a 3-fold excess of barium bromide and 2 volumes of absolute ethanol. BaDAHP (186 mg) was collected by centrifugation, washed, dissolved in 6 ml of water, and precipitated by adding 12 ml of absolute ethanol. The product (in 0.1 ml, 5.295 mg per ml) and an aliquot of the incubation mixture diluted with 10% were assayed by scintillation counting in 10 ml of Bray’s solution (28). Activity of 3H2O present in the reaction mixture was 236 mCi or 9.5 x 108 dpm per ml, and that of BaDAHP was 3.4 x 109 dpm per ml. DAHP was also incubated with enzyme in 3H2O under the above conditions, isolated, and assayed for 3H. Similar incubations were carried out in a medium of 3H2O, and DAHP samples were isolated and analyzed (29).

### Determination of Initial Velocities

Incubation times were chosen within range of linear rates, which were determined in preliminary rate experiments at lowest and highest substrate concentrations. Rate measurements in duplicate or triplicate were made in 1.0 ml containing enolpyruvate-P and erythrose-4-P as indicated, 0.1 M Hepes buffer, pH 7.4 (ionic strength 0.05), 5% glycerol, and [14]P-enolpyruvate-P (2 to 5 x 104 cpm). Solutions were incubated at 25°C for 10 min immediately after adding [14]P-enolpyruvate-P, and reaction was initiated by adding 3 to 5 μl of appropriately diluted enzyme. Reaction was terminated by adding 1.0 ml of a solution of 5% ammonium molybdate in 1 M H2SO4, and carrier Pi (1 μmole) was added immediately. The solution was extracted (30) within 20 s with 4.00 ml of water-saturated benzene-isobutanol (1:1) and 3 ml of the organic phase taken for liquid scintillation counting. 14P present in [14]P-enolpyruvate-P was determined in exactly the same manner, with enzyme omitted, for each concentration of substrates. 14P present as enolpyruvate-P was determined similarly for each curve by treating 0.1 ml of enzyme-free reaction mixture with 0.9 ml of 0.1 M sodium acetate buffer, pH 4.0, containing 0.1 M mercu-}

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The procedure was modified for measuring inhibition by added Pi, since marked quenching of counts occurred when Pi was higher than 2 mM, and phosphomolybdic acid was precipitated when Pi was higher than 4 mM. A solution (1.0 ml) of indicated concentrations of substrates and Pi, [32P]enolpyruvate-P (5 × 10^4 cpm), 5% glycerol, and enough Hepes buffer, pH 7.4, to bring final ionic strength to 0.1, was incubated at 25°C for 10 min. Reaction was started by adding enzyme, and stopped after 10 min by adding 0.05 ml of 5.4 M H2SO4 (final pH, 2.5). Aliquots of 0.1 ml were removed and added to 0.9 ml of water, and the resulting solution, treated with carrier Pi to 2 μM, was assayed as described above.

Substrate Saturation Curves

Initial velocities were determined by the modified procedure described for determination of Pi inhibition, except that 1 μmole of carrier Pi was added.

Exchange Reactions

1. Exchange of 32P with Enolpyruvate-P—A solution (1.0 ml) containing 10 μmoles of Hepes buffer, pH 7.4, 1 mg of bovine serum albumin, 0.2 mg of DAHP synthetase (specific activity 1200), 0.5 mCi of [32P]Pi, 1.0 μmole of enolpyruvate-P, and 10 μmoles of Pi, was incubated at 25°C for 10 hours. A similar solution was prepared and incubated which had only 1.0 μmole of Pi. Analysis of aliquots indicated that enzyme activity was still present. The solutions were diluted to 10 ml and chromatographed on columns (1 × 10 cm) of Dowex 1-X8 (Cl−) as described previously for isolation of enolpyruvate-P.

2. Exchange of 32P with Enolpyruvate-P—A solution (1.0 ml) containing 0.032 μmole of [32P]enolpyruvate-P, 0.44 μmole of DAHP, 100 mg of glycerol, 100 μmoles of Hepes buffer, pH 7.4, and 0.4 mg of DAHP synthetase (specific activity 1200), was incubated at 25°C. A similar solution was prepared and incubated which had only 1.0 μmole of Pi. Analysis of aliquots indicated that enzyme activity was still present in the reaction mixtures after 24 hours. Enzyme-4-P was eluted with 0.02 M HCl and DAHP with 0.05 M HCl. Aliquots of column fractions were assayed for 32P.

3. Exchange of 32P, with Enolpyruvate-P under Conditions of Enzyme Inhibition by Pi—A solution (1 ml) containing 5 mCi of [32P]Pi, 0.1 μmole of enolpyruvate-P, 0.036 μmole of erythrose-4-P, 20 μmoles of potassium phosphate buffer, pH 7.4, 5% glycerol, and 6 μg of DAHP synthetase (specific activity 450) was incubated at 25°C for 25 min. Carrier enolpyruvate-P (1 μmole) was added, and the diluted solution was chromatographed on a column (0.7 × 6.0 cm) of Dowex 1-X8 (Cl−) as described above. Enzyme activity with and without added Pi was determined under the same conditions except that 1 mg per ml of bovine serum albumin was present instead of 5% glycerol.

Analysis of Results

We adopted the nomenclature and system of kinetic constants proposed by Cleland (7). Kinetic constants were calculated from initial velocity and product inhibition data by a line-intercept computer program (method of least squares), and all lines were drawn from slope and intercept values so obtained.

RESULTS AND DISCUSSION

Purification and Properties of DAHP Synthetase (tp)—The phenylalanine-sensitive isoenzyme was the major DAHP synthetase in our strain of S. typhimurium and constituted approximately 75% of total activity (Fig. 1). However, in a 4-fluorophenylalanine-resistant operator mutation, SG-12, tyrosine-controlled enzymes were constitutively derepressed 10- to 20-fold, and DAHP synthetase (tp) was 75 to 80% of the total activity. The concentration of tryptophan-regulated enzyme was low, and phenylalanine-regulated enzyme was both widely separated from tyrosine enzyme and unstable during purification in absence of enolpyruvate-P (Fig. 1). Hence, extracts of SG-12 were an excellent source of tyrosine-inhibitable enzyme. As seen from Table I only 3 column chromatographic steps afforded good yields of highly purified and stable enzyme which was 90% inhibitable by L-tyrosine. The presence of phosphate and maintenance of high protein concentrations were important in the procedure. A purification of 150- to 200-fold was achieved, and the activity was more than 2000-fold that of tyrosine-inhibitable enzyme in wild type cells (specific activity 0.5). However, our preparation is not homogeneous. Gel electrophoresis (31) of 13,000 μg samples of enzyme at 4°C and pH 7.5 showed one major and one very small protein band, and at pH 9, one major and two faint bands. The major band in both gels coincided with DAHP synthetase activity. Gel chromatography on Sephadex G-100 was carried out as described under 'Experimental Procedure,' and a molecular weight of approximately 100,000 was derived by conventional methods (32).

A buffer which did not affect enzyme activity was sought for initial rate measurements. Phosphate was inhibitory as would be expected from a reaction product. Furthermore, the enzyme showed considerable sensitivity to ionic strength greater than 0.1, and it is likely that this accounts for severe losses of activity in our attempts to chromatograph ammonium sulfate fractions on DEAE-cellulose. pH profiles of DAHP synthetase at 0.10
Effect of Co²⁺ and EDTA on activity of DAHP synthetase ( tyr)

Activities were determined in Hepes buffer assay mixture as described under “Experimental Procedure” with 1.7 µg of a hydroxylapatite fraction, specific activity 370.

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<tr>
<th>Addition</th>
<th>DAHP synthetase activity</th>
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<tr>
<td></td>
<td>µM</td>
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<tr>
<td>Co²⁺</td>
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<tr>
<td>0.066</td>
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<tr>
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<td>0.669</td>
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<tr>
<td>0.030</td>
<td>106</td>
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<tr>
<td>0.069</td>
<td>106</td>
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Co²⁺ and unaffected by EDTA. With a highly purified preparation of phenylalanine-inhibitable DAHP synthetase, Co²⁺ showed only slight stimulation of enzyme protected by enolpyruvate-P; it was more effective with partially inactivated enzyme (34a). Our preparation was stabilized by tyrosine and glycerol.

**Substrate Saturation**—The effect of varying concentrations of enolpyruvate-P on the rate of DAHP synthetase indicated that the rate was independent of substrate concentration above 0.5 mM enolpyruvate-P at a fixed concentration of 0.1 mM erythrose-4-P. There appeared to be substantial substrate inhibition by erythrose-4-P above 0.4 mM. Saturation of DAHP synthetase without inhibition occurred between 0.2 mM and 0.3 mM erythrose-4-P, at a fixed concentration of 0.1 mM enolpyruvate-P. Inactivation by erythrose-4-P has recently been reported for the tyrosine-inhibitable enzyme from Salmonella (33) and the tryptophan inhabitable enzyme from Streptomyces (34).

**Initial Velocity**—A double reciprocal plot of initial velocity against erythrose-4-P concentration at fixed concentrations of enolpyruvate-P is shown in Fig. 2. As found by previous authors (5, 6, 33, 34), a parallel initial velocity pattern was obtained which is described by the equation:

\[
\frac{1}{v} = \frac{V_{\text{max}}}{K_A A} + \frac{V_{\text{max}}}{K_B B} + \frac{1}{V_{\text{max}}}
\]

where A and B are concentrations of substrates, and Kₐ and Kₜ are Michaelis constants. Secondary plots obtained from Fig. 2 gave Kₐ values of 1.5 \times 10⁻⁵ M for enolpyruvate-P and 1.3 \times 10⁻⁴ M for erythrose-4-P. These values are approximately 100 times lower than those reported previously for either E. coli (6) or Salmonella (33) enzymes.

A parallel initial velocity pattern is consistent with a ping-pong mechanism in which release of Pᵢ occurred between the binding of enolpyruvate-P and that of erythrose-4-P to the enzyme. However, as pointed out previously (7, 35), other kinetic reaction mechanisms can under certain conditions give double reciprocal plots of initial velocity which are a series of parallel lines (cf. 6). In a sequential reaction A + B → P + Q,
the rate equation is given by:

\[
\frac{v}{V_{\text{max}}} = \frac{K_A K_B}{K_A K_B + K_A + K_B + 1}
\]

where \(K_{IA}\) is the dissociation constant of enzyme and \(A\). Plots of reciprocal initial velocity versus reciprocal substrate concentration for either substrate will yield a series of lines which intersect at a point dependent on the ratio of \(K_{IA}/K_A\). Equation 4 in double reciprocal form, with \(A\) as the variable substrate and \(B\) as the changing fixed substrate, is given by:

\[
\frac{1}{v} = \frac{K_A}{V_{\text{max}}} \left( \frac{1 + K_A K_B}{K_A B} \right) + \frac{1}{V_{\text{max}}} \left( \frac{1 + K_B}{B} \right)
\]

When the ratio \(K_{IA}/K_A\) is small, Equation 5 approximates Equation 6 for a ping-pong mechanism and the lines appear nearly parallel.

\[
\frac{1}{v} = \frac{K_A}{V_{\text{max}}} \left( \frac{1}{A} \right) + \frac{1}{V_{\text{max}}} \left( \frac{1 + K_B}{B} \right)
\]

The vertical coordinate of the intersection point for a sequential reaction, derived from Equation 5, is \(1/V_{\text{max}} (1 - K_A/K_{IA})\). As \(K_{IA}/K_A\) decreases, the intersection point becomes more negative, with minus infinity corresponding to parallel lines. The possibility was therefore considered that the mechanism of DAHP synthetase was sequential and the observed parallel lines of reciprocal initial velocity plots were due to a small value for \(K_{IA}/K_A\).

**Isotope Exchange Studies**—Exchange by DAHP synthetase of labeled \(P_i\) with enolpyruvate-P is predicted by a ping-pong mechanism. Our attempts to show exchange (see "Experimental Procedure") of \(^{32}\text{P}\) into enolpyruvate-P were negative, as was also reported recently by Nagano and Zalkin (33). Exchange of \([U-^{14}\text{C}]\) erythrose-4-P with DAHP in the absence of \(P_i\) is predicted by a ping-pong mechanism and in the presence of \(P_i\) by a sequential mechanism. Exchange of \([U-^{14}\text{C}]\) erythrose-4-P into DAHP did not occur either in the presence or absence of \(P_i\). Reversal of the reaction could not be demonstrated when enolpyruvate-P and erythrose-4-P were incubated with DAHP synthetase in the presence of 5 nCi of \(^{32}\text{P}\) and sufficient \(P_i\) to inhibit the rate of the forward reaction by 45%. Reversal of only \(1 \times 10^{-4} \) to \(1 \times 10^{-4}\) \(\%\) could have been easily detected by synthesis of \(^{32}\text{P}\) enolpyruvate-P. Absence of exchange of \(P_i\) into enolpyruvate-P or erythrose-4-P into DAHP is not surprising in view of the known irreversibility of the reaction, which was estimated to have a free energy change of approximately -16 to -17 Cal (1).

In a ping pong mechanism Reaction 1 would be expected to occur in the forward direction, even if the reverse reaction did not take place (as shown by lack of exchange of \(P_i\) into enolpyruvate-P). \(^{32}\text{P}\) Enolpyruvate-P was incubated with a 10-fold excess of enzyme, and \(^{32}\text{P}\) was determined with and without an excess of erythrose-4-P. The results, shown in Table III, indicate that the release of \(^{32}\text{P}\) from \(^{32}\text{P}\)-enolpyruvate-P did not occur even under the most favorable conditions, unless erythrose-4-P was also present. The absence of this reaction tends to rule against a ping-pong mechanism.

**Initial Velocities at Low Substrate Concentrations**—With high activity \(^{32}\text{P}\)-enolpyruvate-P, it was possible to determine initial velocities at substrate concentrations close to \(K_m\), i.e., approximately 10-fold lower than in Fig. 2. The results, shown in Fig. 3, indicate that under these conditions the lines in a double reciprocal plot intersect and that the reaction is sequential. The parallel lines obtained in Fig. 2 must therefore be the result of a small \(K_{IA}/K_A\) ratio. If enolpyruvate-P is assumed to be the first substrate to bind to enzyme, the slope to intercept ratio from a secondary plot of slopes of Fig. 2 against reciprocal of velocities at substrate concentrations close to \(K_m\) is predicted by a sequential mechanism. The value of \(K_{IA} = 4.7 \times 10^{-4}\) \(\text{m}\) for enolpyruvate-P is approximately three times lower than its \(K_m\). Because of experimental difficulty in determining accurate initial velocities at low substrate concentrations, this value probably represents the upper limit of \(K_{IA}\) and the actual value is probably smaller.

**Product Inhibition**—Product inhibition studies of the DAHP synthetase reaction were carried out to distinguish between an ordered and random sequential mechanism. "Steady state" kinetics was assumed in deriving rate equations for ordered reactions in the forward direction (Mechanisms 1 and 2, Table IV) according to the methods of King and Altman (36) and Cleland (87). It was also assumed that the conversion of ternary complex \(EAB \rightarrow EPQ\) was irreversible. The rate equation for Mechanism 1 (Ordered Bi Bi) is:

\[
\frac{v}{V_{\text{max}}} = \frac{K_{IA} K_B}{K_A + K_B + 1} + \frac{K_A Q}{K_{IQ} A} + \frac{K_{IA} K_B}{K_{IQ} A} + \frac{K_A K_B}{K_P} + \frac{P}{K_{PI}} + \frac{K_A P Q}{K_{IQ} A}
\]

It differs from the equation for the reversible mechanism (7) only by absence of \(P\) and \(AP\) terms in the denominator. The rate equation for Mechanism 2 (Ordered Bi Bi with dead-end

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<th>Additions</th>
<th>cpm \times 10^{-4}</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>3.50</td>
</tr>
<tr>
<td>DAHP synthetase</td>
<td>2.81</td>
</tr>
<tr>
<td>DAHP synthetase + erythrose-4-P</td>
<td>32.5</td>
</tr>
<tr>
<td>Mercuric acetate</td>
<td>32.4</td>
</tr>
</tbody>
</table>

Table III

**Attempt to show partial reaction of DAHP synthetase with \(^{32}\text{P}\)-enolpyruvate-P**

Reaction mixtures (1 ml) contained 50 mM Hepes buffer, pH 7.4, 0.10 \(\mu\)M \(^{32}\text{P}\)-enolpyruvate-P, 5% glycerol, and where indicated, -0.1 \(\mu\)M (0.1 mg) DAHP synthetase, 0.1 mM erythrose-4-P, and 80 mM mercuric acetate in sodium acetate, pH 4.0. \(P_i\) (1 \(\mu\)M) was included with enzyme additions to facilitate exchange of \(^{32}\text{P}\) possibly bound to protein. (\(P_i\) could not effectively hinder binding of enolpyruvate-P (A) to enzyme, since from \(K_{IA}\) and \(K_{EPQ}\) (Table V), \(E/A/E/P_1 \approx 60\) for the above concentrations of \(A\) and \(P_i\).) Incubation was for 10 min at 25°, and reaction was stopped and \(^{32}\text{P}\) determined as described under "Experimental Procedure."
Inhibition by several levels of DAHP with nonsaturating concentrations of substrates is shown in Fig. 6 for variable erythrose-4-P and in Fig. 7 for variable enolpyruvate-P. DAHP was a noncompetitive inhibitor against erythrose-4-P and a competitive inhibitor against enolpyruvate-P. This result is in accord with Mechanism 2 (Table IV). The reaction catalyzed by DHAP synthetase is therefore ordered sequential, with enolpyruvate-P the first substrate (A) to bind to the enzyme, and P1 the first product (P) to be released.6 DAHP inhibition with variable erythrose-4-P at higher (0.1 mM) enolpyruvate-P was found to be somewhat less inhibitory, i.e., more nearly noncompetitive, than at lower (0.04 mM) enolpyruvate-P (Fig. 6).

δ-Erythritol-4-P Inhibition—δ-Erythritol-4-P was tested as an inhibitor for the DAHP synthetase reaction in the expectation that it would be an analog of the substrate erythrose-4-P and provide additional evidence by means of dead-end inhibition patterns for assignment of Mechanism 2. Inhibition by several levels of δ-erythritol-4-P, with nonsaturating concentrations of substrates is shown in Fig. 8 for variable erythrose-4-P, and Fig. 9 for variable enolpyruvate-P. δ-Erythritol-4-P was more nearly an uncompetitive inhibitor against erythrose-4-P at high (0.1 mM) but not saturating levels of enolpyruvate-P, and a competitive inhibitor against enolpyruvate-P. This is the same inhibition pattern as that observed for DAHP. δ-Erythritol-4-P appears to act as an analog of DAHP and competes with enolpyruvate-P for free enzyme. Thus, similarities in structure between DAHP and δ-erythritol-4-P predominate over similarities between δ-erythrose-4-P and its corresponding alcohol. Apparently an aldehyde function at C-1 is essential to bind an analog at the erythrose-4-P binding site, since not only erythrose-4-P, but also erythrose, ribose, and ribose-5-P inactivate the enzyme (34).

**Kinetic and Inhibition Constants—Secondary plots of slopes or intercepts (where applicable) were obtained from lines in figures of either initial velocity or product inhibition.** Kinetic constants and inhibition constants were obtained from horizontal intercepts of these secondary plots (which were all linear). Values of kinetic constants for Mechanism 2 are shown in Table V.

**Mechanism of Reaction of Enolpyruvate-P with Erythrose-4-P—**

Results of studies of the DAHP synthetase reaction with enolpyruvate-P labeled with \(^{32}P\) in the C—O—P oxygen are shown in Table VI. It may be seen that nearly all of the label was present in P1 released. In spite of care to avoid impurities in isolation of KH\(_2\)PO\(_4\) there was occasionally a small contamination of labeled phosphate, as shown also with a control of KH\(_2\)PO\(_4\). As expected, \(^{32}P\) from the medium was not incorporated into P1.

An enzyme-erythrose-4-P-DAHP dead-end complex (Mechanism 4) would show the same inhibition pattern as Mechanism 2. However, formation of such a complex is very unlikely.

---

**Table IV**

*Product inhibition patterns for irreversible bireactant sequential mechanisms*

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Inhibitory product</th>
<th>Variable A (unsaturated)</th>
<th>Variable B (unsaturated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ordered Bi Bi</td>
<td>P</td>
<td>UC</td>
<td>UC</td>
</tr>
<tr>
<td>2. Ordered Bi Bi with dead-end</td>
<td>P</td>
<td>Q</td>
<td>C</td>
</tr>
<tr>
<td>E-P complex (+ or — dead-end E-A-P complex)</td>
<td>Q</td>
<td>C</td>
<td>NC</td>
</tr>
<tr>
<td>3. Random Bi Bi with dead-end</td>
<td>P</td>
<td>Q</td>
<td>C</td>
</tr>
<tr>
<td>E-B-P complex</td>
<td>Q</td>
<td>C</td>
<td>NC</td>
</tr>
<tr>
<td>4. Random Bi Bi with dead-end</td>
<td>P</td>
<td>Q</td>
<td>C</td>
</tr>
<tr>
<td>E-A-P and E-B-P complexes</td>
<td>Q</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

a E, enzyme; A and B, reactants; P and Q, products; C, competitive; NC, noncompetitive; UC, uncompetitive.
b A is first substrate to bind to enzyme and P first product to be released.

where \(K_i\) is dissociation constant of E-P dead-end complex and \(K'_i\) is dissociation constant of E-A-P dead-end complex.

**Rate equations for random mechanisms in the forward direction (Mechanisms 3 and 4, Table IV)** were derived by methods of "rapid equilibrium" kinetics, and it was assumed that conversion of ternary complex \(EAB \rightarrow EPQ\) was rate-limiting and irreversible. Theoretically most random mechanisms resemble "rapid equilibrium" random mechanisms in initial velocity and product inhibition studies (37). The rate equation for Mechanism 3 (Random Bi Bi with dead-end E-B-P complex) is given by Equation 9, while that for Mechanism 4 (Random Bi Bi with dead-end E-A-P and E-B-P complexes) is given by Equation 10:

\[
v = \frac{K_{iA}K_B}{AB} \left( \frac{1 + P}{K_i} \right) + \frac{K_A}{A} \left( \frac{1 + P}{K_i} \right) + \frac{K_B}{B} \left( \frac{1 + P}{K_i} \right) + 1 + \frac{K_A}{K_i} + \frac{K_A}{Q} + \frac{K_A}{P} + \frac{K_B}{P} + \frac{K_B}{Q} + \frac{K_B}{P} + \frac{K_A}{P}
\]

(8)

where \(K_{AB}\) is the dissociation constant of E-B and P from dead-end E-B-P complex, and \(K_{BP}\) is the dissociation constant of E-A and P from dead-end E-A-P complex.

Product inhibition equations were derived from rate equations, and expected inhibition patterns for various mechanisms are summarized in Table IV.

Inhibition by several levels of P1 with nonsaturating concentrations of substrates is shown in Fig. 4 for variable erythrose-4-P and in Fig. 5 for variable enolpyruvate-P. P1 was a noncompetitive inhibitor against both substrates. This inhibition pattern (Table IV) may be given by either an ordered mechanism, or random mechanism with formation of dead end complexes (Mechanism 2 or 4, respectively). Hence, P1 inhibition cannot be used to distinguish between an ordered and random mechanism.
Formation of $P_i$ must therefore have occurred by cleavage of the $C-O$ bond, i.e. with elimination of orthophosphate, rather than displacement of phosphoryl by a nucleophile and $P-O$ cleavage (1). The latter type of reaction was found in pyruvate kinase (38) and implicated in enolpyruvate-P carboxylase (39) and enolpyruvate-P carboxykinase (40). Release of $P_i$ by $C-O$ cleavage of enolpyruvate-$P$ was first observed in this laboratory in 1963 in the 5-enolpyruvylshikimate-3-$P$ synthetase reaction, which occurred with incorporation of 1.3 atoms of $^2$H from the medium at $C-3$ of the enolpyruvyl side chain (10). This reaction was therefore formally interpreted as occurring by protonation of $C-3$, attack at $C-2$ by hydroxyl of shikimate-$5-P$, and elimination of $P_i$.

A pyruvyl enzyme intermediate (Equation 1), postulated in

\[ \text{Enolpyruvate} + \text{Erythrose-4-P} \rightarrow \text{Product} + P_i \]

...was found to be a good substrate for DAHP synthetase. The kinetics of the reaction were studied in detail, and the results are presented in Table V.

**Table V**

<table>
<thead>
<tr>
<th>Kinetic constant</th>
<th>Experimental values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (µmoles/min/mg of protein)</td>
<td>0.242 ± 0.008</td>
</tr>
<tr>
<td>$K_A$ (µM)</td>
<td>14.9 ± 2.9</td>
</tr>
<tr>
<td>$K_B$ (µM)</td>
<td>13.2 ± 1.4</td>
</tr>
<tr>
<td>$K_C$ (µM)</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td>$K_D$ (mM)</td>
<td>56.8</td>
</tr>
<tr>
<td>$K_{F,DAHP}$ (mM)</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>$K_{F,DAHP}$ (mM)</td>
<td>27.5 ± 3.0</td>
</tr>
<tr>
<td>$K_i_{Erythritol-4-P}$ (mM)</td>
<td>4.0 ± 2.0</td>
</tr>
</tbody>
</table>
C-3, and HOH would displace the enzyme to yield the postulated
formation of Pi, as shown in Scheme 1. Alternatively, a nucleophilic
attack of enolpyruvate-1', addition of erythrose-4-P at C-3, and elimina-
tion of Pi would displace the enzyme to yield the postulated
intermediate. This mechanism is in accord with release of Pi by C-O
cleavage, stereospecific addition of erythro-4-P to enolpyruvate-P, and
kinetics of the reaction. A similar mechanism may occur in other enzyme reactions involving addition
of an aldehyde to C-3 of enolpyruvate P, e.g. synthesis of 3-deoxy
D-manno-octulosonic acid 8-phosphate (41) and salic acid 9-phosphate (42). In spite of similarities with 5-enolpyruvylshikimate-
3-P synthesis (C-O cleavage), and carboxylation reactions of enolpyruvate-P (addition by an electrophile at the si face (43)),
the DAHP synthetase reaction has unique features of its own. As pointed out previously, it does not involve a pro	onation of C-3, as in the former reaction, or phosphoryl displacement with
P-O cleavage, as in the latter reactions.

**Acknowledgments**—We thank Dr. W. B. Latta for help with a large scale purification of enzyme and with the gel electrophoresis experiments; Dr. L. Ponticorvo for help with 18O and 2H analyses; Dr. Mildred Cohn for generous supplies of purified enolpyruvate-P synthetase, Dr. C. E. Ballou for his last sample of D
erithritol-4-P, and Dr. A. M. Gold and Dr. W. W. Cleland for many helpful and critical discussions. We acknowledge the excellent technical assistance of Miss Ann-Maj Norman.

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**Table VI**

<table>
<thead>
<tr>
<th>Labeled compound added</th>
<th>Isolated compound</th>
<th>Found</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>14O-Enolpyruvate-P</td>
<td>KH₂PO₄</td>
<td>3.14₄</td>
<td>3.30₄</td>
</tr>
<tr>
<td>14O-Enolpyruvate-P</td>
<td>KH₂PO₄</td>
<td>2.98</td>
<td>3.30</td>
</tr>
<tr>
<td>14O-Enolpyruvate-P</td>
<td>KH₂PO₄</td>
<td>3.28</td>
<td>3.30</td>
</tr>
<tr>
<td>KH₂P(O)</td>
<td>KH₂PO₄</td>
<td>2.55</td>
<td>2.80</td>
</tr>
<tr>
<td>H₂O</td>
<td>KO₂H</td>
<td>0.008</td>
<td>0.345</td>
</tr>
<tr>
<td>H₂O + DAHP (no substrates)</td>
<td>DAHP</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>DAHP</td>
<td>0.012</td>
<td></td>
</tr>
<tr>
<td>H₂O + DAHP (no substrates)</td>
<td>DAHP</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>H₂O + DAHP (no substrates)</td>
<td>DAHP</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

* Corrected for about 10% dilution of labeled P, by that found present in reagents plus enzyme.

Atoms % excess 18O in C-O-P/atoms of oxygen in P₁ = 14.3/4, or 3.60 atoms % excess 18O expected in P₁, for C-O cleavage.

* Calculated 0.07% of excess 18O or 3H, neglecting isotope, effects, for irreversible formation of DAHP. Similar incorporation
  of 14O and 3H from the medium was reported with a crude enzyme preparation (9).

**Scheme 1.** Hypothetical mechanism for DAHP synthetase reaction.
Radiat. Isotopes 1, 208
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Purification and Kinetics of Tyrosine-sensitive 3-Deoxy-d-arabino-heptulosonic Acid 7-Phosphate Synthetase from Salmonella
Albert B. DeLeo, Jean Dayan and D. B. Sprinson


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