Stereochemistry of the 3-Deoxy-D-arabino-heptulosonate 7-Phosphate Synthetase Reaction and the Chorismate Synthetase Reaction*

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SUMMARY

(2)- and (E)-3-[3-3H]P-enolpyruvate were prepared from [1-1H]glucose and [1-1H]mannose with the glycolytic enzymes and were converted into shikimates titrated at C-6 with non-labeled erythrose-4-P and cell-free extract of Escherichia coli mutant 83-24. Degradation to malate and analysis by the fumarase reaction showed that (2)-[3-3H]P-enolpyruvate had produced [6-3H]shikimate of predominantly 6R configuration and the E isomer had given predominantly (6S)-[6-3H]shikimate. The DAHP synthetase reaction therefore is stereospecific with respect of C-3 of P-enolpyruvate, involving si attack at this center. As a consequence, the transient formation of a methyl group at C-3 of P-enolpyruvate as part of the reaction, as proposed by DeLeo and Sprinson (1968) Biochem. Biophys. Res. Commun., 32, 873), is unlikely.

Further conversion of (6R)- and (6S)-6-[3-3H]shikimate into chorismate showed that the pro-6R hydrogen is eliminated in this process. Thus, the 1,4-conjugate elimination of phosphoric acid in the chorismate synthetase reaction is anti, rendering a concerted (E2') mechanism unlikely.

The shikimic acid pathway is the main biosynthetic route leading to the formation of aromatic compounds in higher plants and microorganisms. It is involved in the biosynthesis of such essential compounds as the aromatic amino acids phenylalanine, tyrosine, and tryptophan, a number of quinones, e.g. ubiquinone, plastoquinone, vitamin K, and the tocopherols, and the vitamin folic acid as well as in the formation of a vast number of so-called secondary metabolites, like many alkaloids, plant phenols, and a number of antibiotics. The individual steps of the pathway have been identified by mutant and tracer techniques and have been shown in cell-free systems, and most of the enzymes have been at least partially purified (1-4). Current emphasis is on the study of regulatory and genetic aspects of the pathway (4-6). Surprisingly, the detailed chemistry of many reactions of the pathway is not very well known. Plausible mechanisms have been proposed for most of the reactions, but there is frequently not much experimental evidence to support these or to distinguish them from possible alternatives. In order to contribute to the understanding of the biochemical reaction mechanisms operative in this biosynthetic pathway we decided to investigate the stereochemistry of some of the reactions. In this paper we report on the stereo course of two reactions of the shikimic acid pathway, the formation of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) from P-enolpyruvate and erythrose-4-P and the formation of chorismic acid from its precursor, 5-enolpyruvylshikimic acid 3-P (ESP). Preliminary accounts of this work have been published (8, 9).

EXPERIMENTAL PROCEDURES

Materials—The chemicals used were of reagent grade or of the highest purity commercially available. They were used without further purification. Erythrose-4-P was liberated from its dimethylacetal as described by Ballou et al. (10) and was kept as a frozen aqueous solution. Reference DHQ was prepared by oxidation of quinic acid as described by Haslam et al. (11).

3H:14C ratio was usually ascertained by conversion into malate and furmarate to be at least 96% stereospecifically labeled (13), was provided by Dr. I. A. Rose, Philadelphia. Doubly labeled compounds were obtained by mixing the singly labeled species, and constancy of their 3H:14C ratio was usually ascertained by rechromatography of an aliquot in at least one system.

1 The abbreviations used are: DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; ESB, 5-enolpyruvylshikimic 3-phosphate; DHQ, 3-dehydroquinate.

2 Throughout this paper we use the systematic numbering of shikimic acid (7) as in Fig. 1 rather than the traditional one.

3 We are indebted to Dr. H. F. Guenther and Miss G. Schneider for the first of these 3-P-[1-14C]glycerate preparations.
The glycolytic enzymes and fumarase were purchased from Calbiochem, Los Angeles, and phosphomannose isomerase (40 i. u. per mg of protein) was a gift from Boehringer and Sons, Mannheim. These enzymes were used without dialysis. Anthranilate synthetase, native enzyme (14), and Component I (15), purified from Salmonella typhimurium were a gift from Dr. H. Zalkin, Lafayette. The phenylalanine- and tyrosine-sensitive isoenzymes of DAHP synthetase from Escherichia coli were partially purified as described by Smith et al. (16). The tyrosine-sensitive enzyme was inhibited 66% by tyrosine and over 85% by tyrosine + phenylalanine and the phenylalanine-sensitive enzyme was inhibited over 90% by phenylalanine. DHQ synthetase was partially purified by Smith et al. (16). The tyrosine-sensitive enzyme was inhibited 66% by tyrosine and over 85% by tyrosine + phenylalanine. The phenylalanine- and tyrosine-sensitive isoenzymes of DSHP 4,Salmonella typhimurium were a gift from Dr. H. Zalkin, Lafayette. Acetosyringone, RF 0.2. Thin layer chromatography was done with 0.25 mm thick pre-coated silica gel plates (Merek) and the following systems: System H: disoonylpropyl ether-water-formic acid (50:1:3); malate, RF 0; fumarate, RF 0.7. System H: toluene-ethyl formate-formic acid (5:4:1), run three times; shikimic acid, RF 0.2 to 0.3.

The following spray reagents were used to visualize compounds on chromatograms: Reagent A, potassium permanganate; 0.25 g of KMnO4 and 0.5 g of NaHCO3 in 100 ml of water; Reagent B, ammonium molybdate; 0.5 g of ammonium molybdate is dissolved in 5 ml of water; 1.5 ml of 25% HCl and 2.5 ml of 70% HCO3 are added and, after cooling to room temperature, the solution is made up to 50 ml with acetone. The chromatograms are sprayed and then exposed to short wave ultraviolet light; Reagent C, bromphenol blue: 25 mg of bromphenol blue and 100 mg of citric acid in 50 ml of water; Reagent D, periodate-aniline; (Solution a) 320 mg of NaNO3 in 34 ml of 1 N acetic acid and 40 ml of 1 N sodium acetate, (Solution b) 3% aniline in ethanol. Chromatograms are sprayed with Solution a, dried for 15 to 20 min at room temperature, and then sprayed with Solution b.

**Assay Procedures—DAHP was determined colorimetrically with the thioarbitrurate assay of Srinivasan and Spirnson (22). In some cases the yields of enzyme reactions were estimated by chromatographing an aliquot of the reaction mixture, scanning the chromatogram for 14C, and integrating the area under the entire curve and the peak area of the desired compound. Protein was determined by the method of Warburg and Christian (23).**

**Radioactivity Determinations—**Radioactivity of substances in solution was determined in a Beckman LS 100 liquid scintillation counter, with 2,5-diphenyloxazole (PPO) and 1,4-bis(5-phenyloxazolyl)benzene (dimethylPOPPO) in toluene or toluene-ether-water-formic acid (30:1:3) toluene-ether-water-formic acid (30:1:3) or toluene (1:1:1:1); tritium standard in the form of labeled toluene, or both. Radioactive substances on chromatograms were located by scanning in a Packard model 7201 chromatogram scanner.

(E)- and (Z)-(3-14C)P-enolpyruvate—The reaction mixture for the conversion of glucose or mannose into 3-P-glycerate contained in a volume of 3 ml: [1-14C]glucose ([1-3H]mannose), 1 μmole or less; Tris buffer, pH 8.2, 150 μmoles; ATP, 0.5 μmole; NAD+, 5 μmoles; Na2HAsO4, 9 μmoles; MglCl, 12 μmoles; pyruvate kinase, 20 i. u.; glucose-P isomerase (mannose-1' isomerase), 10 i. u.; phosphofructokinase, 20 i. u.; aldolase, 10 i. u.; triose-P isomerase, 10 i. u.; and glyceraldehyde-P dehydrogenase, 10 i. u. The reaction was initiated with 10 i. u. of hexokinase. In order to keep its concentration low, ATP was regenerated with pyruvate kinase and 5 μmoles of P-enolpyruvate, which were added in portions of 0.5 μmole over a period of 3 hours. The reaction was terminated when no more change in absorption at 340 nm occurred for a period of 15 min. The protein was precipitated by the addition of 0.2 ml of 10% trichloroacetic acid, removed by centrifugation, and washed twice with 0.5 ml of water. The combined supernatants were lyophilized and the 3-P-glycerate isolated by successive chromatography in Systems A and C. Reference 3-P-glycerate spotted alongside the radioactive material was detected by spraying with the ammonium molybdate reagent. The yields were somewhat variable, but in two typical runs, 100 μCi of [1-14C]glucose and 100 μCi of [1-3H]mannose gave 65 μCi of (3S)-3-P-[3-3H]glycerate and 40 μCi of (3R)-3-P-[3-3H]glycerate, respectively.

The 3-P-glycerate samples were further converted into P-enol-
pyruvate in a reaction mixture which contained in a volume of 1 ml: 3-P-glycerate, 1 to 2 μmoles; triehanolamine buffer, pH 7.6, 50 μmoles; MgCl₂, 5 μmoles; EDTA, 0.2 μmole; dithiothreitol, 10 μmoles; NAD⁺, 1 μmole; ATP, 1 μmole; FAD, 0.5 μmole; P-enolpyruvate, 0.5 μmole; fructose-6-P, 1 μmole; shikimic acid, 0.5 μmole; and cell-free extract corresponding to another 2.5 mg of protein. The reaction was carried out at 37°C for 14 hours. After this time, isocitrate dehydrogenase, 1 i.u.; D,L-isocitrate, 50 μmoles; NAD⁺, 1 μmole; and cell-free extract corresponding to another 2.5 μg of protein were added and the incubation was continued for 30 min. The protein was precipitated with 2 N HCl and removed by centrifugation. The shikimic acid was isolated and purified by successive chromatography in System B and D and its radiochemical purity was established in System I. Reference shikimic acid was located by spraying the chromatograms with permanganate reagent. The yields varied considerably from incubation to incubation. Usually, between 30 and 45% of pure material were isolated, but in a number of runs yields as high as 80 to 90% have been obtained. In some experiments, the P-enolpyruvate was generated from 2-P-glycerate directly in the reaction mixture. Since the cell-free extract contains enough of the two enzymes necessary, this required only the substitution of 3-P-glycerate for P-enolpyruvate. 3-[14C]P-enolpyruvate was prepared in the same way from 2-[14C]glycerate.

Conversion of 3-[14C]P-enolpyruvate to 3-[14C]P-enolpyruvate — The reaction mixture for the conversion of 3-[14C]P-enolpyruvate into shikimic acid contained in a volume of 1 ml: potassium phosphate buffer, pH 7.4, 50 μmoles; MgCl₂, 5 μmoles; erythrose-4-P, 5 μmoles; (S)- or (R)-3-[14C]P-enolpyruvate, 1.5 μmoles; and aged cell-free extract of E. coli 83-24 corresponding to 8 mg of protein. The mixture was incubated at 37°C for 14 hours. After this time, isocitrate dehydrogenase, 1 i.u.; D,L-isocitrate, 50 μmoles; NAD⁺, 1 μmole; and cell-free extract corresponding to another 2.5 μg of protein were added and the incubation was continued for 30 min. The protein was precipitated with 2 N HCl and removed by centrifugation. The shikimic acid was isolated and purified by successive chromatography in System A. The further conversion followed closely the procedure of Hanson and Ross (27) for the degradation of quinate to citrate. The residues were dissolved in 0.1 ml of water. 0.1 ml of 1 N periodic acid was added, and the reaction mixtures were kept for 1 hour at 25°C in the dark. They were then applied to columns of 2 ml of Dowex 1-X8 formate (50 to 100 mesh), which were eluted with 10 ml of 3 N HCOOH. Fractions of 1 ml were collected and assayed for radioactivity. The radioactive fractions were combined, evaporated to dryness, and kept at 10°C. The radioactive malate samples were eluted with water and aliquots were counted. At this point, 15 and 9%, respectively, of the 14C of the (6R)- and (6S)-3-[14C]-shikimate samples were recovered as malate, not accounting for the various aliquots taken for chromatography and counting, which consumed roughly half of the material.

Degradation of Shikimate—(6R)- and (6S)-[6-2H]shikimate (2.5 μCi) were each mixed with about 0.9 μCi of tritium [1, 6-3H]shikimate and the two samples, each about 0.75 μCi, were esterified by refluxing for 4 hours with 2 ml of 5% methanolic HCl. Completeness of the esterification was shown by chromatography in System A. The solvent was evaporated and the residue dried for 10 min at 10°C to remove HCl gas. The residues were dissolved in 1 ml of absolute methanol and oxidized for 1 min at 0°C. The oxonides were immediately reduced by addition of 40 mg of solid NaBH₄ to the cooled solutions and after 30 min excess NaBH₄ was destroyed with 0.5 ml of acetone. The solutions were diluted with 2 ml of water and passed through columns of 1 ml of Dowex 50-H⁺ (50 to 100 mesh), which were washed with 15 ml of water. Eluate and washings were combined, evaporated to dryness in a rotary evaporator, and dissolved three times in a few milliliters of methanol followed by evaporation to remove boron. Completeness of the ozonolysis was confirmed by chromatography in System A. The further conversion followed closely the procedure of Hanson and Ross (27) for the degradation of quinate to citrate. The residues were dissolved in 0.1 ml of water. 0.1 ml of 1 N periodic acid was added, and the reaction mixtures were kept in the dark at 25°C for 30 min. Sodium ions were then removed by passage through columns of 1 ml of Dowex 50-H⁺ (50 to 100 mesh), which were washed with 10 ml of 3 N HCOOH. Fractions of 1 ml were collected and assayed for radioactivity. The radioactive fractions were combined, evaporated to dryness, and kept at 10°C. The radioactive malate samples were eluted with water and aliquots were counted. At this point, 15 and 9%, respectively, of the 14C of the (6R)- and (6S)-[1, 6-2H]-shikimate samples were recovered as malate, not accounting for the various aliquots taken for chromatography and counting, which consumed roughly half of the material.

Conversion of DAP into DHQ—The incubation mixture containing in a volume of 0.5 ml: potassium phosphate buffer, pH 7.4, 20 μmoles; fumarase, 20 pg of protein; carrier (S)-malate, 0.2 μmole; and radioactive (R,S)-malate. The reaction was carried out at 25°C and followed spectrophotometrically at 240 nm. Fifteen minutes after equilibration had been reached, the reaction mixtures were evaporated to dryness and aliquots of the residues were counted to determine by difference the amount of tritium released into water. The remainder was chromatographed in System H and the fumarate bands were eluted with a mixture of methanol-water-concentrated ammonia (5:3:2) to give 7.3 x 10⁶ and 9.3 x 10⁶ dpm of 14C, respectively.
and DAHP synthetase, 0.2 mg of protein. The reaction mixture was previously incubated for 10 min at 37° before addition of the DAHP. The conversion was complete after 30 min. The mixture was chromatographed quantitatively in System F.

RESULTS

In the DAHP synthetase reaction, the carbonyl carbon atom of erythrose-4-P is linked to the methylene carbon atom of P-enolpyruvate. The reaction could involve either \( \text{re} \) or \( \text{si} \) attack (28) at the latter position. Chorismate synthetase catalyzes the 1,4-conjugate elimination of the elements of phosphoric acid from C-3 and C-6 of ESP to give chorismic acid. In this reaction, the phosphate and the proton could be eliminated on the same side of the ring or on opposite sides, resulting in loss of either the pro-\( S \) or the pro-\( R \) hydrogen from C-6 of ESP. To examine these questions, we prepared the two isomers of P-enolpyruvate tritiated stereospecifically at C-3 from [\( \text{3}^\text{H} \)]glucose and [1-\( \text{3}^\text{H} \)]mannose by the route outlined in Fig. 1. The two hexoses were each phosphorylated with hexokinase, and stereospecific labeling at C-1 was achieved by conversion into fructose-6-P with phosphoglucomutase and phosphomannose isomerase, respectively. The stereochemistry of these two isomerase reactions is known (20, 30) to be such that upon further conversion of the intermediate fructose-6-P samples with the glycolytic enzymes in the presence of arsenate to make the reaction sequence irreversible [1-\( \text{3}^\text{H} \)]glucose gives rise to (3S)-3-P-\([3^\text{H}]\]glycerate and [1-\( \text{3}^\text{H} \)]mannose produces (6R)-3-P-\([3^\text{H}]\]glycerate. These two samples were further converted into P-enolpyruvate by the action of phosphoglyceromutase and enolase. The latter enzyme has recently been shown (31) to catalyze anti elimination of the elements of water from 2-P-glycerate. Consequently, the P-enolpyruvate derived from [1-\( \text{3}^\text{H} \)]glucose has \( Z \) configuration and the one from [1-\( \text{3}^\text{H} \)]mannose has \( E \) configuration. Using unlabeled erythrose-4-P and a cell-free extract of \( E. \) coli mutant S3-24, which accumulates shikimic acid, these two samples of [\( \text{3}^\text{H} \)]P-enolpyruvate were then converted into shikimic acid. Alternatively, the [3-\( \text{3}^\text{H} \)]P-enolpyruvate could be generated from 3-P-[\( \text{3}^\text{H} \)]glycerate directly in the reaction mixture without further addition because the cell-free extract did contain sufficient enolase and phosphoglyceromutase activity.

The resulting two specimens of [6-\( \text{3}^\text{H} \)]shikimate were degraded according to Fig. 1 in order to determine their configuration at the labeled carbon atom 6. They were mixed with racemic [1,6-\( \text{14}^\text{C} \)]shikimate, esterified with methanolic HCl, and subjected to ozonolysis and NaBH\(_4\) reduction of the ozonide, followed by periodate cleavage, bromine oxidation, and hydrolysis of the ester. This degradation produced two samples of malate, each presumably racemic at C-2, which contained as their C-3 the original carbon atom 6 of shikimate. These were analyzed by incubation with fumarase, which catalyzes the reversible anti elimination of the hydroxyl group and the pro-\( 3R \) hydrogen from (2S)-malate to

\[\text{malate} \rightarrow \text{fumarate} \]

*For nomenclature see Reference 32.*
give fumarate, but does not react with (2S)-malate (33, 34).

Thus, at equilibrium the enzyme will have released all the tritium from (2S,3R)-[3-3H]malate into water, whereas that of (2R,3S)-[3-3H]malate remains in the fumarate and malate. The results of this degradation are summarized in Table I. It can be seen that the shikimate obtained from (Z)-[3-3H]P-enolpyruvate gave rise to a malate sample which in the fumarase reaction lost most of its tritium. Consequently, this specimen is predominantly (6S)-[6-3H]shikimate. The shikimate from (E)-[3-3H]P-enolpyruvate produced malate which retained most of its tritium in the fumarase reaction and therefore must have predominantly 6S configuration. The tritium in the two malate samples is scrambled considerably between the two heterotopic positions at C-3. As shown below, 12 to 18% of this scrambling is already inherent in the starting shikimate samples. However, some additional scrambling appears to occur in the course of the degradation, presumably by chemical enolization during workup at the stage of the maleic acid semialdehyde. This assumption is supported by the observation of a slight decrease of the 3H:14C ratios in the conversion of shikimate to (2R,S)-malate.

A aliquots of the (6S)- and (6S)-[6-3H]shikimate were mixed with [7-14C]-shikimate obtained enzymatically from [1-14C]P-enolpyruvate and the reactions were carried out with this double labeled sample.

### Table I

<table>
<thead>
<tr>
<th>Material obtained from</th>
<th>Shikimate</th>
<th>Fumarate</th>
<th>Tritium of (2S)-malate released into water</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)-[3-3H]P-enolpyruvate</td>
<td>2.56</td>
<td>2.93</td>
<td>88%</td>
</tr>
<tr>
<td>(E)-[3-3H]P-enolpyruvate</td>
<td>2.26</td>
<td>2.54</td>
<td>90%</td>
</tr>
<tr>
<td>(E)-[3-3H]P-enolpyruvate</td>
<td>0.64</td>
<td>2.10</td>
<td>72%</td>
</tr>
</tbody>
</table>

* Relative to malate.

### Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate</th>
<th>P-enolpyruvate</th>
<th>Shikimate</th>
<th>Chorismate</th>
<th>Anthranilate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(Z)-[3-3H]P-enolpyruvate</td>
<td>14.0</td>
<td>2.5</td>
<td>18</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>(E)-[3-3H]P-enolpyruvate</td>
<td>14.3</td>
<td>11.7</td>
<td>82</td>
<td>0.06</td>
</tr>
<tr>
<td>3</td>
<td>(Z)-[3-3H]P-enolpyruvate</td>
<td>19.6</td>
<td>9.3</td>
<td>18</td>
<td>0.77</td>
</tr>
<tr>
<td>4</td>
<td>(E)-[3-3H]P-enolpyruvate</td>
<td>5.0</td>
<td>5.2</td>
<td>88</td>
<td>0.02</td>
</tr>
<tr>
<td>5</td>
<td>(E)-[1-14C,3-3H]P-enolpyruvate</td>
<td>2.78</td>
<td>2.30</td>
<td>86</td>
<td>2.11</td>
</tr>
</tbody>
</table>

* Relative to shikimate.

b Analyzed and found to be at least 96% stereospecifically labeled (13).
TABLE III  
Exchange of tritium from [3-3H]P-enolpyruvate during DAHP synthetase reaction

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme</th>
<th>Incubation time (min)</th>
<th>Yield of DAHP</th>
<th>Tritium found in water (% of DAHP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phe-sensitive</td>
<td>45</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Phe-sensitive</td>
<td>15</td>
<td>12</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Phe-sensitive</td>
<td>40</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Phe-sensitive</td>
<td>40</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Tyr-sensitive</td>
<td>45</td>
<td>6.05</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>Tyr-sensitive</td>
<td>40</td>
<td>5.0</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

 * Complete reaction mixture.
 b Reaction mixture previously incubated in the absence of erythrose-4-P, followed by incubation with erythrose-4-P for same length of time.
 c P-enolpyruvate incubated with heat-denatured enzyme.
 d n.d., not determined.

TABLE IV  
Incorporation of tritium from ¹H₂O and ¹⁷H₂O into DAHP during DAHP synthetase reaction

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme</th>
<th>Incubation time (min)</th>
<th>Yield of DAHP</th>
<th>Tritium found in water (% of DAHP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phe-sensitive</td>
<td>45</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Phe-sensitive</td>
<td>15</td>
<td>12</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Phe-sensitive</td>
<td>40</td>
<td>13</td>
<td>13</td>
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<td></td>
<td>Phe-sensitive</td>
<td>40</td>
<td>13</td>
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</tr>
<tr>
<td></td>
<td>Tyr-sensitive</td>
<td>45</td>
<td>6.05</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>Tyr-sensitive</td>
<td>40</td>
<td>5.0</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

 a Complete reaction mixture.
 b Reaction mixture previously incubated in the absence of erythrose-4-P, followed by incubation with erythrose-4-P for same length of time.
 c P-enolpyruvate incubated with heat-denatured enzyme.
 d n.d., not determined.

DISCUSSION

In this paper, improved procedures for the preparation of isotopically labeled shikimic acid and chorismic acid are described. So far, [¹³C]shikimic acid has usually been prepared by photosynthesis using leaves of Ginkgo biloba and ¹⁴CO₂ (e.g., Reference 39) or, in one case, by a five-step chemical synthesis starting from methyl [2,3,4,13C₄]acrylate (40). Labeled chorismic acid has been obtained by fermentation of [U-¹³C]glucose with A. aerogenes mutant 62-1 (41). Compared to these methods, the enzymatic syntheses described here offer one or several of the following advantages: (a) The yields are better, (b) the procedures are simple, (c) they produce only the biologically active stereoisomer, and (d) they can be used to introduce specific and even stereospecific labels in a variety of positions. In our laboratory, the enzymatic methods have been used to prepare [7-¹³C]shikimic acid and [1,6,7-¹³C]shikimic acid and the 6R and 6S isomers of [6-¹³C]-shikimic acid, which were used in this project as well as in the synthesis of the sterechemistry of napthoquinone biosynthesis (42) and of the dehydroshikimate dehydratase reaction (43), and to prepare various ring and side chain-labeled chorismic acids, which were used in a study of the anthranilate synthetase reaction (26). A similar enzymatic synthesis of labeled shikimic acid has been developed by Scharf and Zenk (44).

The results of this study allow some conclusions regarding the DAHP synthetase reaction. Kinetic data from Staub and Dénès (45) and Moldovanyi and Dénès (46), and Nagano and Zalkin (47) suggest that this reaction proceeds by a "ping-pong" mechanism (48), although the latter authors feel that they cannot exclude a sequential mechanism. Because of its ability to protect the enzyme against denaturation (47, 45, 46), P-enolpyruvate is assumed to be the substrate which is bound first by the enzyme. DeLeo and Sprinson (37) as well as Nagano and Zalkin (47) carried out ¹⁴O experiments which showed that the reaction involves C-O rather than P-O bond cleavage. On the basis of these results, the mechanism shown in Fig. 2 was proposed (37). In support of this mechanism, DeLeo and Sprinson reported "that DAHP
formed in a medium containing \( ^{3}H\text{H}_{2}O \) was extensively labeled" as would be expected from their suggested mechanism. Our data, on the other hand, do not support the assumption of the intermediate formation of a methyl group at C-3 of P-enolpyruvate. It is evident from Table II that the reaction at this carbon atom is at least predominantly stereospecific, and therefore at least in the majority of molecules the 2 methylene protons of P-enolpyruvate cannot have become part of a freely rotating methyl group with the concomitant loss of their identity. Our experiments do, however, confirm DeLeo and Sprinson’s observation (37) of tritium incorporation into DAHP when the reaction is carried out in tritiated water. Although lack of sufficient material prevented us from determining the position of the tritium, it seems reasonable to assume that it is located at C-3, since some tritium is also lost from this position when [3-\( ^{3}H\)P]P-enolpyruvate is used as the substrate.

The amount of tritium incorporated is much less than the expected value for obligatory protonation of the methylene group (0.67 \( \mu \)mole of hydrogen per \( \mu \)mole in the absence of isotope effects) and the experiment with \( ^{3}H\text{HOH} \) indicates that the low incorporation is not due to a product isotope effect (38). The result of the former experiment also suggests that the protonation step which leads to the incorporation of tritium is reversible (49). However, the enzyme apparently does not catalyze exchange of tritium at C-3 of P-enolpyruvate in the absence of erythrose-4-P as might be expected for a ping-pong mechanism as suggested by DeLeo and Sprinson (37), and this finding could be taken as evidence in support of a sequential mechanism. An incorporation of tritium from \( ^{3}H\text{HOH} \) at C-3 of DAHP and the observed partial scrambling of the stereospecific tritium label could be explained in at least two ways. One possibility would be that a methyl group is formed by protonation of C-3 of P-enolpyruvate as an obligatory step in the over-all reaction, but that its lifetime is extremely short and in most cases the same proton is abstracted again before the methyl group can undergo rotation. The rate constant for the over-all reaction catalyzed by DAHP synthetase can be estimated from data given by Srinivasan and Sprinson (22) to be in the order of \( 10^{9} \) sec\(^{-1} \). Proton transfers, for example from the imidazolium ion, have rate constants in the order of \( 10^{5} \) sec\(^{-1} \) (50) while rate constants for the unrestricted rotation of methyl groups are \( >10^{8} \) sec\(^{-1} \).

Thus, this possibility is extremely unlikely, unless one wants to make the assumption that the enzyme dramatically restricts the rotation of the methyl group. As an alternative which is more in line with the available evidence, the tritium exchange and the scrambling of the label could be due to formation of an intermediate carbocation at C-3 of the enolpyruvate residue, which normally attacks C-1 of erythrose-4-P but can undergo reversible protonation as a side reaction.

The results of this study define the steric course of the DAHP synthetase reaction. The side of attack at the carbonyl group of erythrose-4-P was already evident from the known configuration of DAHP at C-4 (36). Since (Z)-[\( ^{3}H\text{H}_{2}\text{O} \)]P-enolpyruvate gives rise to (6R)-[\( ^{3}H\text{H}_{2}\text{O} \)]shikimate, the intermediate [3-\( ^{3}H\)DAHP] must have 2S configuration. Consequently, the stereochemistry of the DAHP synthetase reaction is as shown in Fig. 3, i.e. it involves \( \beta \) attack at C-3 of P-enolpyruvate and \( \alpha \) attack at C-1 of erythrose-4-P. Although without mechanistic significance, the finding of \( \beta \) attack at C-3 of P-enolpyruvate is interesting in view of recent studies by Rose et al. (13) on the stereochemistry of a number of phosphopyruvate carboxylases. The four enzymes examined by these authors all catalyze the addition of CO\(_{2}\) at the \( \beta \) face of P-enolpyruvate. Pyruvate kinase, which has also been studied recently (53-55), catalyzes the \( \alpha \) addition of a proton at C-3 of P-enolpyruvate.

Chorismate synthetase has been investigated by Morelli et al. (25) who noted the analogy of the reaction to the 1,4-conjugate (E2') eliminations in the 9,10-dihydroanthracene series studied by Cristol et al. (56). If the reaction proceeds by a concerted mechanism, theoretical considerations (57, 58) in agreement with the results of Cristol et al. (56) predict that the elimination of phosphate and the proton should occur at the same side of the plane of the ring or \( \beta \) as visualized in Fig. 4. \( \beta \) pyramidal elimination would require the loss of the \( \phi\)-6\( \phi \) hydrogen of ESP. The experimental finding that the \( \phi\)-6\( \phi \) hydrogen is lost indicates that the reaction involves an \( \alpha \) elimination. The same conclusion was reached independently by Hill and Newkome (59) with a somewhat different approach. These authors synthesized (6R)- and (6S)-[6-\( ^{2}H\)H]shikimate from methyl acrylate deuterated in the cis- or trans-hydrogen of the methylene group, respectively, via a Diels-Alder reaction with \( \text{trans,trans-1,4-diisocyanate} \) and determined the incorporation of deuterium from these two samples into phenylalanine and tyrosine by a shikimate-dependent mutant of \( E. \) coli. The assignment of the configuration at C-6 of their two deuterated shikimic acids depended critically on the correct assignment of the configuration at C-1 of the Diels-Alder adduct, which had been a matter of controversy (60, 61) and which they (62) as well as others (63, 64) recently re-examined by spectroscopic methods. Obviously, the agreement of their results and ours on the stereochemistry of the chorismate synthetase reaction constitutes an independent confirmation for the correctness of this spectroscopic assignment of the configuration of the Diels-Alder adduct. The finding that chorismate synthetase catalyzes an \( \alpha \) elimination of the elements of phosphoric acid from ESP renders a concerted (E2') mechanism less likely. As a possible alternative, one could envisage a two-stage X-group mechanism (65) as shown in Fig. 5. In the first step an anionic group of the enzyme displaces the phosphate in an \( S_{N}2\) reaction which would

4 Calculated from the activation energy for rotation (51) according to the Eyring equation (32).
be 

the pro-R hydrogen from C-6. Another explanation has been proposed by Rose (67), who pointed out the possible importance of special separation of the catalytically active sites on the enzyme. The reaction obviously requires catalytic groups at least one acid and one base, which could easily neutralize each other if located closely together on the same side of the ring system of the bound substrate. Thus, the advantage of maximal separation of the two catalytic groups when located on opposite sides of the ring could be the reason for the observed stereoechemistry of the chorismate synthetase reaction.

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REFERENCES

Stereochemistry of the 3-Deoxy-D-arabino-heptulosonate 7-Phosphate Synthetase
Reaction and the Chorismate Synthetase Reaction
H. G. Floss, D. K. Onderka and M. Carroll


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