The Enzymatic Formation and Isolation of 3-Enolpyruvylshikimate 5-Phosphate*

J. G. Levin† and D. B. Sprinson‡

From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York 32, New York

(Received for publication, July 23, 1963)

Previous investigations (1-5) have established in some detail the path of aromatic biosynthesis in bacteria, starting with glucose and leading to the formation of shikimate 5-phosphate (Fig. 1). This was also true of the conversion of prephenate to phenylalanine (6) and tyrosine (7), and of anthranilate to tryptophan (8-10). On the other hand, it was not known how shikimate 5-phosphate is converted to prephenate and to anthranilate. The present study was undertaken to clarify this part of the pathway.

Several multiple aromatic auxotrophs blocked beyond shikimate were shown to accumulate this compound and shikimate-5-P (11, 12). Other strains, presumably blocked later, accumulated, in addition to shikimate and shikimate-5-P, an acid-labile compound which was readily hydrolyzed to shikimic acid (11). A barium salt of this compound was isolated, and on the basis of its hydrolysis to equimolar amounts of shikimic and pyruvic acids and its ability to react with periodate, it was tentatively assigned the structure of 3- or 5-enolpyruvylshikimate.¹ Later it was observed² that a product with similar chromatographic and chemical properties was formed in extracts of Escherichia coli from shikimate-5-P plus enolpyruvate-P.

Further study of this reaction, described below, demonstrated that the first product formed is 3-enolpyruvylshikimate-5-P, which is then dephosphorylated. 3-Enolpyruvylshikimate-5-P, unlike enolpyruvylshikimate, is converted to prephenate by extracts of E. coli.³ Preliminary reports of this investigation have appeared elsewhere (13, 14).

EXPERIMENTAL PROCEDURE

Materials—The barium salts of enolpyruvylshikimate (more than 90% pure) and shikimate-5-P were generous gifts of Professor B. D. Davis, who also provided the mutant strains used in this work.

The following materials were obtained from commercial sources: enolpyruvate-P (tricyclohexylammonium salt) and shikimic acid, California Corporation for Biochemical Research;

• This work was supported by grants from the American Cancer Society, the American Heart Association, and the National Institutes of Health of the United States Public Health Service.

† Present address, National Institutes of Health, Bethesda 14, Maryland. This report is from a dissertation submitted in April 1962 by Judith G. Levin in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

‡ Career Investigator of the American Heart Association.

¹ C. Gilvarg, unpublished results quoted by B. D. Davis (1).

² E. B. Kahan, unpublished results quoted in (12).

³ M. J. Clark, and D. B. Sprinson, unpublished observations (cf. (13)).
responds to 5-dehydroquinate and to 5-dehydroshikimate in addition to shikimate (19), but since the former substances were not present in any of the samples, appearance of turbidity was attributed solely to growth on shikimate. The range of the assay is between 0 and 10 μg of shikimate.

Light absorption measurements were carried out on a Beckman DU spectrophotometer or a Cary recording spectrophotometer, model 11S. Infrared spectra were recorded with a Perkin-Elmer infrared spectrophotometer, model 21.

Preparation of Enzyme—E. coli K12 mutant 58-278, a phenylalanine auxotroph (20) which accumulates prephenic acid (21, 22), was grown with aeration for 18 hours at 35°C on Medium A enriched with 0.2% yeast extract and 0.2% Casamino acids. The cells were harvested by centrifugation at 3°C, washed twice with 1/30 potassium phosphate buffer, pH 7.2, suspended in 0.01 M tris-chloride buffer, pH 8.2 (20 ml per 5 g of wet bacteria), and subjected to sonic oscillation at 9 kc, with cooling, for 30 minutes. Centrifugation for 40 minutes at 16,000 x g in a Lourdes centrifuge yielded a slightly turbid yellow-brown supernatant solution. All subsequent operations were carried out at 2°C.

The cell-free extract (116 ml) was treated with 17 ml of 2% protamine sulfate solution, and the resulting precipitate was removed by centrifugation for 35 minutes at 18,000 x g and discarded. A mixture of 29.2 g of (NH₄)₂SO₄ and 2.9 g of K₂CO₃ was finely ground in a mortar and added slowly with stirring to 120 ml of the clear supernatant solution. After stirring for another 20 minutes, the suspension was left standing for 15 minutes; the precipitate was then removed by centrifugation for 20 minutes at 15,000 x g and discarded. The procedure was repeated on the supernatant solution with a mixture of 16.9 g of (NH₄)₂SO₄ and 1.7 g of K₂CO₃. After additional stirring for 20 minutes, the suspension was left standing for 1 hour. The precipitate was removed by centrifugation at 18,000 x g, dissolved in 20 ml of 0.01 M tris-chloride buffer, pH 8.2, and dialyzed exhaustively against 0.01 M tris-chloride buffer, pH 7.4 (five changes of 4 liters each during a 22-hour period).

A summary of the partial purification of the enzyme is given in Table I. All fractions were stable for at least 9 months at –20°C. In the experiments described below, the ammonium sulfate fraction was used.

Assay for ES-5-P and for ES-5-P Synthetase—The activity of the enzyme was determined by measuring the amount of ES-5-P formed from enolpyruvate-P and shikimate-5-P. One unit of enzyme was defined as that amount of enzyme which will catalyze the formation of 1 μmole of ES-5-P in 1 minute under the following conditions. The incubation mixture contained 1 μmole of enolpyruvate-P, 1 μmole of shikimate-5-P, 50 μmoles of tris-maleate buffer, pH 6.1, and enzyme fraction in a total volume of 1.0 ml. When the crude extract or protamine supernatant solution was assayed, 10 μmoles of KF were added to prevent further conversion of ES-5-P to prephenate. This conversion did not occur in the ammonium sulfate fraction; with long incubation periods of the latter fraction at pH 8.2 (e.g. Tables II and III), fluoride also inhibited the hydrolysis of ES-5-P to enolpyruvylshikimate. The reaction was started by the addition of enzyme after preincubation of the other constituents at 37°C for 2 minutes. After 15 minutes at 37°C, the reaction was terminated by addition of 0.02 ml of 1 N KOH followed by heating in a boiling water bath for 2 minutes; proteins were removed by centrifugation.

---

**Fig. 1.** Position of shikimate 5-P in the path of biosynthesis of the aromatic amino acids.

**Fig. 2.** Typical bioassay curve for shikimic acid obtained with *A. aerogenes* A170-14351.
**TABLE I**

Partial purification of ES-5-P synthetase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>Total activity (umoles/mg x 100)</th>
<th>Specific activity (umoles/mg x 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protamine supernatant solution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate (0.4 to 0.6 saturated)</td>
<td>26.5</td>
<td>17</td>
<td>60</td>
<td>13.4</td>
</tr>
</tbody>
</table>

**TABLE II**

Effect of fluoride on formation of enolpyruvylshikimate and release of orthophosphate

The reaction mixture contained 1.0 m mole of enolpyruvate-P, 1 m mole of shikimate-5-P, 50 m moles of Tris-chloride buffer, pH 8.2, 10 m moles of KF (where added), and 0.05 ml of enzyme (1 mg of protein) in a final volume of 1.4 ml. After incubation at 37°C for 20 hours, proteins were precipitated by the addition of 0.8 ml of 25% trichloroacetic acid to 1.2 ml of incubation mixture, and were removed by centrifugation and filtration. A 0.2-ml aliquot of the filtrate was heated in a boiling water bath for 10 minutes and used for the assay of shikimate ("bioassay procedure"). A 0.8-ml aliquot was used for phosphate determination (10).

<table>
<thead>
<tr>
<th>F⁻ addition</th>
<th>Enolpyruvylshikimate formed</th>
<th>Orthophosphate released and calculated</th>
<th>ES-5-P formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Found)</td>
<td>Calculated from enolpyruvylshikimate formed</td>
<td>Calculated from ES-5-P formed</td>
</tr>
<tr>
<td></td>
<td>µmole</td>
<td>µmole</td>
<td>µmole</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>0.45</td>
<td>1.06</td>
<td>0.90</td>
</tr>
<tr>
<td>+</td>
<td>0.10</td>
<td>0.68</td>
<td>0.20</td>
</tr>
</tbody>
</table>

³ Sum of phosphate (0.20 µmole) resulting from formation of enolpyruvylshikimate and phosphate expected from shikimate released only by combined phosphatase and acid hydrolysis (0.92 - 0.38 = 0.54 µmoles) in a similar experiment (Table II) with the same extract.

To determine enolpyruvylshikimate, two aliquots were taken from the incubation mixture. One aliquot, the shikimate blank, was pasteurized at 65°C for 10 minutes and used directly for shikimate assay (A) (see "Analytical Procedures"). The other aliquot was acidified with 6 N HCl to a final concentration of 0.1 N, heated in a boiling water bath for 10 minutes, and assayed for additional shikimate produced (B). To estimate ES-5-P and unreacted shikimate-5-P, a third aliquot of the reaction mixture, containing between 0.1 and 0.3 µmole of shikimate in all its derivatives, was incubated for 1 hour at 37°C with 40 µmoles of Tris-chloride buffer, pH 8.2, 10 µmoles of NaCl, and 50 µg of alkaline phosphatase in a total volume of 1.0 ml. An aliquot of this solution was used directly for shikimate assay (C). A second aliquot was acidified with 6 N HCl to a final concentration of 0.1 N, heated in a boiling water bath for 10 minutes, and assayed for shikimate (D) released from enolpyruvylshikimate, shikimate-5-P, and ES-5-P. Calculations:

Enolpyruvylshikimate = B - A
Shikimate-5-P = C - A
ES-5-P = D - (A + B + C)

(In a typical assay, the values for the blank and for enolpyruvylshikimate were negligible.)

The above procedure for the determination of ES-5-P, shikimate-5-P, or enolpyruvylshikimate will be referred to henceforth as the "bioassay procedure."

Where indicated, ES-5-P synthetase activity was also estimated by enzymatic determination of unreacted enolpyruvate-P (17).

Isolation of ES-5-P—A solution (475 ml) containing 0.5 mmole of enolpyruvate-P, 0.5 mmole of shikimate-5-P, 5 mmoles of KF, and 25 mmoles of Tris-chloride buffer, pH 7.2, was incubated at 37°C for 30 minutes. The 0.4 to 0.6 saturated (NH₄)₂SO₄ fraction (25 ml, 20 mg of protein per ml) was added, and incubation was continued for 1 hour. After the addition of 1 N NaOH to pH 8, the solution was heated for 5 minutes in a boiling water bath and filtered. Assay of an aliquot (0.5 ml) for enolpyruvate-P (17) indicated a conversion to ES-5-P of 79%.

Dowex 1-X8 (chloride form, 200 to 400 mesh) was allowed to stand overnight in 3 N HCl, washed until the washings were free of chloride, and thoroughly freed of fine particles. A column 50 cm high and 3 cm in diameter was prepared, covered with a 0.5-cm layer of acid-washed (3 N HCl) Berkshire sand, and equilibrated with 0.01 M Tris-chloride buffer, pH 9.0 (by passage of 3 liters of buffer overnight). The filtered incubation mixture was loaded on the column at a rate of 1.5 ml per minute, and the filtrate was heated in a boiling water bath for 10 minutes and used for the assay of shikimate ("bioassay procedure"). A 0.8-ml aliquot was used for phosphate determination (18).

**TABLE III**

Evidence for formation of ES-5-P

The reaction mixture contained 1.0 µmole of enolpyruvate-P, 1.0 µmole of shikimate-5-P, 20 µmoles of Tris-chloride buffer, pH 8.2, 10 µmoles of KF, and 0.05 ml of enzyme (1 mg of protein) in a final volume of 1.0 ml. After incubation for 20 hours at 37°C, the reaction was terminated by heating at 65°C for 2 minutes.

<table>
<thead>
<tr>
<th>Compound obtained²</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid hydrolysis</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.60</td>
</tr>
<tr>
<td>Shikimate</td>
<td>0.11²</td>
</tr>
</tbody>
</table>

² To determine pyruvate released from enolpyruvylshikimate or from ES-5-P by acid hydrolysis, acid-labile enolpyruvate-P was first converted enzymically to lactate (17) with a 0.25 ml aliquot of the reaction mixture. To the resulting volume of 0.95 ml was added 0.05 ml of 6 N HCl, and the mixture was heated in a boiling water bath for 10 minutes. Precipitated proteins were removed by centrifugation, and 0.5 ml of the supernatant solution was used for the determination of pyruvate (18). Enolpyruvylshikimate, measured as shikimate released by brief acid hydrolysis, was determined on a 0.2-ml aliquot of the reaction mixture. Shikimate released by alkaline phosphatase, and shikimate present after both alkaline phosphatase and acid hydrolysis, were determined on a separate 0.4-ml aliquot of the reaction mixture (see "Assay for ES-5-P and for ES-5-P Synthetase").

³ Control experiments, with shikimate-5-P as substrate, showed that in the presence of the fluoride used in these incubations, the alkaline phosphatase was somewhat inhibited. The shikimate recovered in all its derivatives is, therefore, less than 1 µmole per ml.

² This value is designated "enolpyruvylshikimate" in Table II.
p-aminobenzoic acid, and 0.01 pg of p-hydroxybenzoic acid per 20 pg of L-phenylalanine, 5 pg of L-tryptophan, 0.01 pg of an organism under "Analytical Procedures." Medium A (100 ml), shown to accumulate enolpyruvylshikimate (11). Stock cultures strains used, gave a negative result.

pmoles) was converted to the potassium salt. Shikimate (2.0 pmoles) was set free. The precipitate was collected by centrifugation, washed four times with 10-ml portions of cold absolute ethanol. After standing overnight at 0°, the precipitate was removed by centrifugation, washed four times with 10 ml portions of 67% ethanol, and dried in a vacuum over P2O5. The yield was 810 mg, containing 320 pmoles of ES-5-P by "bioassay."

The crude product was stirred with 10 ml of water, and the suspension was cleared by centrifugation. After the supernatant solution was removed with a pipette, this process of extraction was repeated seven more times. The combined extract (approximately 80 ml) were treated with 160 ml of absolute ethanol. After standing overnight at 0°, the precipitate was collected by centrifugation, washed four times with 10 ml portions of cold 67% ethanol, and dried in a vacuum over P2O5. The yield was 196 mg of white solid, containing 290 pmoles of ES-5-P by "bioassay."" At 100° under reduced pressure, this salt showed an absorption spectrum (Fig. 3). Without fluoride (at pH 8.2 and 100°) and enolpyruvylshikimate, and ES-5-P by the "bioassay procedure."

In a typical elution pattern, shikimate-5-P was found in Fractions 90 to 100; enolpyruvylshikimate, in Fractions 103 to 110; enolpyruvate-P, in Fractions 114 to 125; and ES-5-P, in Fractions 162 to 188. In the case of the last three compounds, rapid identification was performed routinely by the reaction with 2,4-dinitrophenylhydrazine (18) after acid hydrolysis.

Enzymatic Formation of ES-5-P—It may be seen from Table II that incubation of the enzyme with equimolar amounts of shikimate-5-P and enolpyruvate-P resulted in the formation of a 45% yield of enolpyruvylshikimate. However, the inorganic phosphate released was always slightly, but significantly, higher than that calculated from the enolpyruvylshikimate formed (Equation 1).

\[
\text{Shikimate-5-P + enolpyruvate-P} \rightarrow \text{enolpyruvylshikimate} + 2 \text{HPO}_4'^- \] (1)

In the presence of fluoride, the formation of enolpyruvylshikimate was strongly inhibited, but the release of orthophosphate was much higher than that calculated from this equation.

Further analysis of the incubation mixtures containing fluoride, presented in Table III, showed that pyruvate liberated by hydrolysis with dilute acid was 6 times greater than would be expected from the amount of enolpyruvylshikimate formed. Shikimate released by alkaline phosphatase (from unreacted shikimate-5-P) was 0.97 µmole whereas 0.90 µmole should have remained unreacted if the 0.11 µmole of enolpyruvylshikimate were the only product formed. Furthermore, when these incubation mixtures were subjected to acid hydrolysis after treatment with alkaline phosphate, an additional 0.54 µmole of shikimate was set free.

It would appear from these results that shikimate-5-P and enolpyruvate-P are converted (in the presence of fluoride) to a compound which, like enolpyruvylshikimate, yields pyruvate on hydrolysis with dilute acid but, unlike enolpyruvylshikimate, does not give rise to shikimic acid on acid hydrolysis until after dephosphorylation by phosphatase. On the reasonable assumption that the phosphate in this compound is still esterified to the hydroxy group on C-5 and that dephosphorylation produces enolpyruvylshikimate, it is suggested that shikimate-5-P (Compound I) and enolpyruvate-P react to form ES-5-P (II) and orthophosphate (Fig. 3). Without fluoride (at pH 8.2 and after prolonged incubation), ES-5-P is dephosphorylated to enolpyruvylshikimate (III) whereas with fluoride, dephos-
phorylation is inhibited and the phosphorylated compound is accumulated.\(^8\)

The phosphate released (Table II) in excess of that calculated from the enolpyruvylshikimate produced may be explained by assuming that ES-5-P is formed in addition to enolpyruvylshikimate. In the presence of fluoride, 0.08 \(\mu\)mole of phosphate was liberated from 1 \(\mu\)mole of shikimate-5-P and 1 \(\mu\)mole of enolpyruvylshikimate. This corresponds fairly closely to the value of 0.2 \(\mu\)mole calculated from the formation of 0.1 \(\mu\)mole of enolpyruvylshikimate, plus the 0.54 \(\mu\)mole expected from formation of 0.54 \(\mu\)mole of ES-5-P (Table III). Without fluoride, ES-5-P was largely dephosphorylated to enolpyruvylshikimate, but, as pointed out earlier, the amount of orthophosphate released (0.06 \(\mu\)moles) was somewhat higher than that expected (0.90 \(\mu\)mole) from the yield of enolpyruvylshikimate alone. Results obtained by a more detailed analysis of incubation mixtures without fluoride were in accord with the view that they contained small amounts of ES-5-P.

Further examination of the ES-5-P synthetase reaction showed that in short incubation periods at lower pH (5.4 to 0.2), high yields of ES-5-P were obtained even in the absence of fluoride and only negligible amounts of enolpyruvylshikimate were produced. These are the conditions described for the assay of the enzyme activity. ES-5-P was also isolated on a large scale by chromatography on Dowex 1 (CI) and obtained as an essentially pure barium salt (see "Experimental Procedure").

Properties of ES-5-P—As shown in "Experimental Procedure," ES-5-P gave rise to the expected proportions of phosphate, pyruvate, and shikimate. Pyruvate was readily released by heating in dilute acid, but not by boiling at pH 8 to 9 (this was routine procedure for inactivating the enzyme in the assay method). These properties would be those expected from an enol ether structure.

Further information on the structure of ES-5-P was obtained by comparing the infrared spectra of the barium salts of ES-5-P, enolpyruvylshikimate, and shikimate-5-P (Fig. 4). All three compounds show two bands resulting from the absorption of the carboxylate ion at 6.35 and 7.13 \(\mu\). No absorption is observed in the carbonyl region, indicating the absence of a lactone or ester structure. Only ES-5-P and enolpyruvylshikimate show a band at 8.2 \(\mu\) characteristic of the C—O vibration of vinyl ethers. On the other hand, only ES-5-P and shikimate-5-P show bands at 8.9 to 0.3 \(\mu\) (ionic phosphate) and at 10.25 \(\mu\) (—Oalkyl). These observations are in accord with the structure of ES-5-P shown in Fig. 3.

Accumulation of ES-5-P in Culture Filtrates—It may be seen from Table IV that, in addition to the previously observed shikimate, shikimate-5-P, and enolpyruvylshikimate (11), \(\_\right\) aerogenes A170-44 and \(\_\left\) aerogenes A170-44 and E. coli M159-2 accumulated a compound with the properties of ES-5-P. The excretion of ES-5-P into the growth medium by mutants presumably blocked after ES-5-P is evidence for its formation in the intact cell, and lends support for the role of ES-5-P as a physiologically active intermediate.

Properties of ES-5-P Synthetase—The formation of ES-5-P was directly proportional to protein concentration at low concentrations of enzyme (Fig. 5A), and was linear with time up to 25 minutes (Fig. 5B). The effect of pH on the rate of formation of ES-5-P is shown in Fig. 6. In Tris-maleate buffer, the optimal pH was between 5.4 and 6.2. The decrease in activity with increasing pH was observed in Tris-chloride buffer as well as in Tris-maleate buffer. Exhaustive dialysis of the enzyme did not lead to decreased enzymatic activity (see "Preparation of Enzyme"). Premiscation of the enzyme with EDTA, and treat-

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Shikimate</th>
<th>Shikimate-5-P</th>
<th>Enolpyruvylshikimate</th>
<th>ES-5-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A170-44</td>
<td>46</td>
<td>13</td>
<td>34</td>
<td>12</td>
</tr>
<tr>
<td>M159-2</td>
<td>2</td>
<td>0.8</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>
ment with Dowex 1 (Cl), Dowex 50 (Na+), or charcoal had no effect on the formation of ES-5-P. It therefore appears that there are no metal or cofactor requirements for this enzymatic activity.

A study of the stoichiometry of the reaction (exemplified in Table V) showed that the equilibrium is 75 to 80% in the direction of ES-5-P formation. The effect on the reaction rate of varying concentrations of enolpyruvate-P (Fig. 7) and shikimate-5-P (Fig. 8) was determined, and the results were plotted (23) for the determination of the $K_m$ values. The $K_m$ for enolpyruvate-P was found to be $2.4 \times 10^{-4}$ M, and for shikimate-5-P, $3.4 \times 10^{-4}$ M.

**Reversibility of Reaction**—Incubation of equimolar amounts of enolpyruvate-P and shikimate-5-P with enzyme in the presence of a 10-fold higher concentration of orthophosphate indicated an inhibition of ES-5-P synthesis, suggesting that high concentrations of phosphate might cause a reversal of the ES-5-P synthetase reaction. This suggestion was confirmed by the results presented in Table VI. ES-5-P and either an equivalent amount or a 25-fold excess of phosphate were incubated with ES-5-P synthetase. The enzyme was inactivated by heating, and enolpyruvate-P was determined enzymatically (17). ES-5-P was also incubated with a 25-fold excess of phosphate in the presence of the pyruvate kinase system (ADP, MgCl₂, KCl, and pyruvate kinase) in order to convert enolpyruvate-P to pyruvate. This is necessary to inactivate NADH oxidase, which would interfere with the assay for enolpyruvate-P.

![Fig. 5](image1)

**Fig. 5.** Effect of protein concentration (A) and time (B) on ES-5-P formation. See "Assay for ES-5-P and for ES-5-P Synthetase" for details. The concentration of protein in B was 0.2 mg per ml.

![Fig. 6](image2)

**Fig. 6.** Variation of ES-5-P synthetase activity with pH. Each reaction mixture contained 3 μmoles of enolpyruvate-P, 3 μmoles of shikimate-5-P, 150 μmoles of Tris-maleate buffer, and enzyme (0.60 mg of protein) in a final volume of 3.0 ml. After incubation at 37° for 15 minutes, aliquots were withdrawn for determination of unreacted enolpyruvate-P (17). The pH was measured at the end of the reaction.

![Fig. 7](image3)

**Fig. 7.** Dependence of the rate of ES-5-P formation on concentration of enolpyruvate-P. In addition to the latter, the reaction mixtures contained, in a final volume of 1 ml, 1 μmole of shikimate-5-P (or 2 μmoles when the enolpyruvate-P concentration equaled or exceeded 1 μmole per ml), 50 μmoles of Tris-maleate buffer, pH 6.1, and enzyme (0.2 mg of protein). After incubation at 37° for 15 minutes, aliquots were withdrawn for the determination of ES-5-P by the "bioassay procedure."

![Fig. 8](image4)

**Fig. 8.** Dependence of the rate of ES-5-P formation on shikimate-5-P concentration. In addition to the latter, the reaction mixtures contained, in a final volume of 1 ml, 1 μmole of enolpyruvate-P (or 2 μmoles when the shikimate-5-P concentration equaled or exceeded 1 μmole per ml), 50 μmoles of Tris-maleate buffer, pH 6.1, and enzyme (0.2 mg of protein). The remaining procedure was as described in Fig. 7.

**Table V**

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Enolpyruvate-P (μmoles)</th>
<th>Shikimate-5-P (μmoles)</th>
<th>ES-5-P (μmoles)</th>
<th>Enolpyruvate-shikimate (μmoles)</th>
<th>Orthophosphate (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.90</td>
<td>5.15</td>
<td>0.00</td>
<td>0.00</td>
<td>0.30</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>1.20</td>
<td>3.80</td>
<td>0.10</td>
<td>4.00</td>
</tr>
<tr>
<td>Change</td>
<td>−3.90</td>
<td>−3.95</td>
<td>+3.80</td>
<td>+0.10</td>
<td>+3.70</td>
</tr>
</tbody>
</table>

The reaction mixture contained enolpyruvate-P, shikimate-5-P, 50 μmoles of KF, 220 μmoles of Tris-maleate buffer, pH 6.1, and 0.3 ml of 1:5 diluted enzyme (1 mg of protein) in a total volume of 5.0 ml. Before and after an incubation period of 2 hours at 37°, aliquots were withdrawn for the determination of enolpyruvate-P, shikimate-5-P, ES-5-P, enolpyruvate-shikimate, and orthophosphate. In separate incubation mixtures it was shown that the further addition of enzyme had no effect on the point of equilibrium.

This is necessary to inactivate NADH oxidase, which would interfere with the assay for enolpyruvate-P.
of orthophosphate, pyruvate, and shikimate per mole of ES-5-P, shikimate-5-P and enolpyruvylshikimate in addition to ES-5-P structure of enolpyruvate-P from the enol ether structure of ES-5-P and the reversal of the synthetase reaction to yield the enol ester structure of enolpyruvate-P. Being more negatively charged at pH 9 than any of the other compounds, ES-5-P was eluted last from Dowex 1 (Cl) columns. Although rigorous proof of structure is as yet unavailable, considerable evidence is at hand in support of Structure II (Fig. 3). This consists of the infrared spectra (Fig. 4) discussed earlier, the lability to the equilibrium constant of the ES-5-P synthetase reaction is 12 (Equation 2, concentrations are in micromoles per ml).

\[
K = \frac{[\text{ES-5-P}] [\text{orthophosphate}]}{[\text{shikimate-5-P}] [\text{enolpyruvate-P}]} = \frac{[0.76][0.74]}{[0.24][0.20]} = 12 \quad (2)
\]

The average value obtained from this and similar experiments was 15. It follows that this reaction (Equation 3) is accompanied by a negative free energy change of 2000 calories.

\[
\Delta F' = -2000 \text{ cal} \quad (3)
\]

The facile formation of enolpyruvate-P from ES-5-P demonstrates that enolpyruvyl ethers are high energy compounds. From the known free energy of hydrolysis of enolpyruvate-P (24) and the free energy change of the ES-5-P synthetase reaction, it is possible to calculate the free energy of hydrolysis of ES-5-P (Equations 4, 5, and 6).

\[
\text{Enolpyruvate-P}^- + \text{H}_2\text{O} \rightarrow \text{pyruvate}^- + \text{HPOT}^-, \quad \Delta F' = -13,300 \text{ cal} \quad (4)
\]

\[
\text{ES-5-P}'^- + \text{HPOT}^- \rightarrow \text{enolpyruvate-P}^-, \quad \Delta F' = +2,000 \text{ cal} \quad (5)
\]

Sum: ES-5-P"^- + H_2O \rightarrow shikimate-5-P"^- + pyruvate"^-, \quad \Delta F' = -11,300 \text{ cal} \quad (6)

This small difference in free energy of hydrolysis between enolpyruvate-P and ES-5-P' would be expected, since the enolpyruvate structure is common to both compounds. It is of interest that in all other known reactions of enolpyruvate-P the enol structure is not preserved in the product. These are the pyruvate kinase reaction and the various condensation reactions of C-3 of enolpyruvate-P with the carbonyl group of HCOO^- or of a sugar (e.g. D-erythrose-4-P, N-acetyl-D-mannosamine-6-P). The latter reactions, in contrast with the ES-5-P synthetase reaction, are associated with a large negative free energy change (3).

The ES-5-P synthetase reaction may be looked upon as taking place in two stages (Fig. 9). In the first stage, the electron-donating bridge oxygen of the phosphate group causes polarization of the double bond of enolpyruvate-P so as to favor protonation of the phosphate group in ES-5-P remains attached at C-5, and that the enolpyruvyl residue is attached to the same carbon atom as in enolpyruvylshikimate. There is independent evidence that in the latter compound the enolpyruvyl sidechain is on C-3. When enolpyruvylshikimate is treated with periodate, a compound is formed with an absorption maximum at 235 to 240 mp attributable to an α,β unsaturated aldehyde structure,
FIG. 9. Hypothetical mechanism for ES-5-P synthetase reaction

The enzymatic conversion of ES-5-P to prephenate may be rationalized as an elimination of phosphate, concerted with (2, 13), or independent of (14, 27), migration of enolpyruvate to C-1. In either case, the origin of the migrating group and its terminus are on the same side of the ring. The recent demonstration (28) that the pyruvate side chain of prephenate (Compound V, Scheme 1) is trans to the hydroxyl on C-4 is in accord with the evidence discussed earlier that the enolpyruvyl group in ES-5-P is attached at C-3, i.e. also trans to the hydroxyl on C-4.

It has been shown by Rivera and Srinivasan (29) that ES-5-P is an obligatory intermediate in the formation of anthranilic acid, the amide nitrogen of glutamine being the source of the amino group. The previous suggestion (2) that a branch point in aromatic biosynthesis may occur immediately after shikimate-5-P must therefore be revised. Furthermore, since mutant strains blocked immediately after ES-5-P (Table IV) require a complete aromatic supplement for growth (11), the branch point compound cannot be ES-5-P, but must be an intermediate produced at some later stage in the pathway. Such an intermediate (IV), formed from ES-5-P and serving as a precursor of prephenate (V), was suggested recently (14, 27), on the basis of a proposal by Woodward\(^{11}\) that enolpyruvylshikimate (assumed at that time to be the 5-enolpyruvyl derivative) is converted to prephenate by a Claisen rearrangement and dehydration. Support for the above scheme has been furnished by the isolation of Compound IV (chorismic acid) from culture filtrates of an organism blocked in the synthesis of both prephenate and anthranilate (30, 31). Although the path between ES-5-P and IV is still unclear, it is known that IV is converted enzymatically to prephenate and to anthranilate, and spontaneously at neutral pH to prephenate (30, 31). The accumulation of trans-2,3-dihydro-3-hydroxyanthranilic acid by a mutant strain of \textit{Streptomyces aureofaciens} (32) suggests the formation of an intermediate, VI (Scheme 2), from chorismic acid and glutamine. Compound VI would undergo elimination of pyruvate to yield anthranilate or hydrolysis to yield the accumulated hydroxyamino acid.

**SUMMARY**

In the presence of partially purified extracts of \textit{Escherichia coli}, shikimate 5-phosphate and enolpyruvate phosphate reacted to yield 3-enolpyruvylshikimate 5-phosphate and orthophosphate. The name 3-enolpyruvylshikimate 5-phosphate synthetase is tentatively proposed by R. B. Woodward, February 8, 1954, after a colloquium presented by B. D. Davis at Harvard University.
tatively suggested for this enzymatic activity. 3-Enolpyruvylshikimate 5-phosphate was isolated as an essentially pure barium salt by anion exchange chromatography on Dowex 1 (Cl). The chemical properties of 3-enolpyruvylshikimate 5-phosphate and its infrared spectrum were consistent with the proposed structure.

High yields of 3-enolpyruvylshikimate 5-phosphate and orthophosphate were obtained from equimolar concentrations of shikimate 5-phosphate and enolpyruvate phosphate. Starting with 3-enolpyruvylshikimate 5-phosphate and an excess of orthophosphate, the reaction was readily reversed, indicating that enol ethers are high energy compounds. Cofactor requirements for the reaction could not be demonstrated. An approximate free energy change of $-2000$ cal was calculated for the forward reaction.

The role of 3-enolpyruvylshikimate 5-phosphate as a precursor of prephenic acid and as a common intermediate in the biosynthesis of the aromatic amino acids was discussed.

Acknowledgments—We are grateful to Professor M. Sprecher for helpful discussions, and to Dr. P. F. Knowles for developing an improved procedure for the isolation and purification of 3-enolpyruvylshikimate 5-phosphate.

REFERENCES

15. WARBURG, O., AND CHRISTIAN, W., Biochem. Z., 310, 384 (1941-1942).
The Enzymatic Formation and Isolation of 3-Enolpyruvylshikimate 5-Phosphate
J. G. Levin and D. B. Sprinson


Access the most updated version of this article at http://www.jbc.org/content/239/4/1142.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/239/4/1142.citation.full.html#ref-list-1