On the Mechanism of 5-Enolpyruvylshikimate 3-Phosphate Synthetase*

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SUMMARY

The enzymic synthesis of 5-enolpyruvylshikimate 3-phosphate (ES-3-P) from enolpyruvate phosphate (labeled with 3O in the C—O—P oxygen) and shikimate 3-phosphate occurred with C—O cleavage of the pyruvate ester. ES-3-P formed in a D2O reaction medium showed incorporation of approximately 1.3 atoms of deuterium, and consisted of 39% d2, 53% d3, and 8% d4. Enolpyruvate phosphate isolated from the same reaction mixture had 0.72 atom of deuterium with a similar d2:d3 ratio. In both compounds, the nuclear magnetic resonances of the vinyl methylene protons were diminished equally in intensity. ES-3-P formed in a tritiated medium contained 0.42 atom of 3H in the vinyl methylene hydrogens.

A reversible addition-elimination mechanism is proposed in which protonation of carbon 3 of enolpyruvate phosphate is associated with a nucleophilic attack on carbon 2 by the 3-hydroxy group of shikimate 3-phosphate. A methyl group of unrestricted rotation is formed in the resulting postulated intermediate. Elimination of orthophosphate then yields ES-3-P, and elimination of shikimate 3-phosphate yields enolpyruvate phosphate.

A procedure was developed for preparing enolpyruvate phosphate with 3O in the C—O—P oxygen. The isolation of shikimate 3-phosphate was improved. ES-3-P synthetase from Salmonella was purified 80-fold by a simple procedure.

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ES-3-P synthetase represents a rare type of reaction in which the enolpyruvyl moiety of enolpyruvate phosphate is transferred apparently unchanged to a recipient molecule. The ES-3-P synthetase reaction was previously considered (1) a reversible addition-elimination reaction (Scheme 1). Protonation of carbon 3 of enolpyruvate-P, facilitated through electron donation by the ester oxygen, was assumed to be associated with a nucleophilic attack on carbon 2 of enolpyruvate P by the 3-hydroxyl group of shikimate-3-P. Elimination of P1 then yielded ES-3-P. This mechanism is supported by studies of the reaction in D2O and in 3H2O, and with [3O]enolpyruvate-P, which are described in the present report.

EXPERIMENTAL PROCEDURE

[3O]Enolpyruvate-P—A solution of 3.0 g of freshly recrystallized bromopyruvic acid (2) in 5 g of H3PO4 (20.1 atom % excess) was allowed to stand in the dark for 24 hours at room temperature. The water was removed by lyophilization and was reutilized in an identical manner to incorporate 3O into two additional 3.0-g lots of bromopyruvic acid. The combined products were recrystallized three times from purified, dry chloroform to yield 7.8 g of white crystals. Behavior on melting: first transition 52–55°; m.p. of glass, 73.5–76° (2). The concentration (3) 10.30 ± 0.02 atom % excess (average of three independent analyses).

The concentration in the carboxyl group was determined by decarboxylating the bromopyruvic acid in diphenylamine-diphenylmethane (4, 5). Quantitative yields of CO2 were obtained at reflux temperature (270°) in 5 min, but considerable reaction occurred also at 100°. The following % values were obtained in CO2 evolved under the indicated conditions: (a) at 100° for 25 min, 8.79 atom % excess; (b) discarding the CO2 after the above treatment, and refluxing for 5 min, 8.99 atom % excess; and (c) refluxing directly for 5 min, 8.74 atom % excess. The average of these three analyses was 8.84 atom % excess 3O in the carboxyl group.

The carbonyl oxygen of the bromopyruvate can be calculated by difference: (3 × 10.3) – (2 × 8.84) = 13.2 atom % excess 3O.

Labeled bromopyruvic acid (3.93 g) was converted to the monocylohexylamine salt of enolpyruvate-P (3.64 g, 58%)

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yield) by the method of Clark and Kirby (6, 7). The $^{18}O$ concentration of this product was 5.25 ± 0.09 atom % excess (average of three analyses), or the same atoms of $^{18}O$ excess per mole, within experimental error, as in the bromopyruvic acid (6 × 5.25/100 = 0.315; 3 × 10.3/100 = 0.309).

Although decarboxylation of enolpyruvate-P in refluxing diphenylamine-diphenylmethane gave essentially quantitative yields of CO$_2$, its $^{18}O$ concentration (6.4 to 6.9 atom % excess) was variable and lower than expected from the value found in the decarboxylation of the bromopyruvic acid. C$^{18}O_2$ obtained by refluxing in quinoline showed somewhat higher results (7.87 atom % excess).

The evolved CO$_2$ contained 7.18 atom % excess $^{18}O$.

Exchange with phosphate oxygens—probably as a result of internal symmetrical peak (1). A minor phosphorylated impurity was detected in paper electrophoresis for 1 hour at pH 5.5 and 900 volts (0.1 mmol acetate buffer; length of paper, 50 cm). This minor component may have resulted from isomerization of shikimate 3-phosphate to 4-phosphate (9). Hydrolysis of both barium salts with alkaline phosphatase gave rise to 1.0 mole of shikimate and 1.0 mole of P$_i$ per equivalent weight of 500 (9).

**Preparation of Shikimate-3-P**—Several minor changes were introduced into the procedure of Weiss and Mingioli (9). *Aerobacter aerogenes* A-170-40 was grown as previously described (9) with rapid shaking (900 ml in a 2-liter flask) for 90 hours at 37°, and cells were removed by centrifugation. The culture fluid (12 liters) was assayed for shikimate 3 P by determining shikimate (10) on appropriate samples before and after hydrolysis with potato acid phosphatase. Found were 1020 mg of shikimate-3-P and 50 mg of shikimate per liter. In a typical hydrolysis, a solution (1 ml) of 0.5 μmole of shikimate-3-P, 500 μmoles of acetate buffer, pH 5.0, and 0.4 μg of potato acid phosphatase, was incubated at 37° for 3 hours, heated at 100° for 3 min, and clarified by centrifugation. A 0.2-ml sample of the supernatant solution was analyzed for shikimate (10). The results were in good agreement with values obtained by bioassay (1).

Granulated charcoal (400 g of Nuchar C190, 30 mesh) was heated in boiling water for 30 min and allowed to settle overnight. Floating particles were removed by decantation, and the procedure was repeated. A chromatographic column (50 × 7.5 cm) fitted with a coarse sintered glass plate and a layer of glass wool was filled with water, a slurry of the charcoal was poured into the column while water was withdrawn at the bottom. The charcoal (43 × 7.5 cm) was covered immediately with a 2- to 3-cm layer of Berkshire sand.

The culture filtrate was brought to pH 3 with HCl, treated with 0.5 ml of toluene, and loaded on the column at a rate of 300 to 400 ml per hour. The column was then washed with 2 liters of the following solvents adjusted to pH 2.5 with HCl: water, 1.5, 5, and 10% ethanol, and finally with 500 ml of 5% unacidified ethanol. Shikimate-3-P was eluted with 2500 ml of 5% ethanol, 3 liters of 10% ethanol, and 2 liters of 25% ethanol. The fractions were combined and concentrated in a vacuum. The residual colorless syrup was diutred with water to 150 ml and contained 7 g of shikimate-3-P. The solution was adjusted to pH 7 with NaOH and treated with 90 ml of 0.5 M barium acetate. Absolute ethanol (950 ml) was added to give 80% (v/v) ethanol, and the solution was allowed to stand overnight at 0°. The precipitate was collected by centrifugation, washed twice with 200 ml of 75% ethanol and once with 200 ml of absolute ethanol, and dried over P$_2$O$_5$ in a vacuum. Yield was 12.8 g of a white powder. Continued elution of the column with 10 liters of 25% ethanol gave a slightly yellow syrup (assaying 2.0 g of shikimate-3-P) from which were isolated 2.9 g of slightly tan barium salt.

Both preparations of shikimate-3-P gave one spot in descending chromatography on paper with a butanol-acetic acid-water (100:6.2:2, v/v) (11) and with n-propyl alcohol-15% NH$_4$OH-water (6:3:1, v/v) (12), an acid molybdate spray (12) being used to detect phosphate esters. Chromatography on Dowex 1-Cr at pH 9 with a lithium chloride gradient gave one symmetrical peak (1). A minor phosphorylated impurity was detected in paper electrophoresis for 1 hour at pH 5.5 and 900 volts (0.1 mmol acetate buffer; length of paper, 50 cm). This minor component may have resulted from isomerization of shikimate 3-phosphate to 4-phosphate (9). Hydrolysis of both barium salts with alkaline phosphatase gave rise to 1.0 mole of shikimate and 1.0 mole of P$_i$ per equivalent weight of 500 (9).

**Assay of ES-3-P Synthetase Activity**—Disappearance of enolpyruvate-P was measured as previously described (13). Active fractions obtained by chromatography of Salmonella extracts on DEAE-cellulose were free of phosphatase and could be studied by P$_i$ release (14).

**Isolation of P$_i$ from ES-3-P Synthetase Reaction with [18O]Enolpyruvate-P**—A solution (110 ml) containing 110 μmoles of the [18O]enolpyruvate-P, 110 μmoles of shikimate-3-P, 1.1 mmoles ofKF, 5.5 mmoles of Tris maleate buffer (pH 6.1), and 0.4 to 0.6 saturated (NH$_4$)$_2$SO$_4$ fraction (70 mg of protein) of *Escherichia coli* K-12 mutant 58-278 (1) was incubated at 37° for 1 hour. Approximately 3 ml of 15% NH$_4$OH were added to pH 8.0, and MgNH$_4$PO$_4$ was precipitated by adding 2 ml of magnesium mixture and 11 ml of 15% NH$_4$OH (15). The MgNH$_4$PO$_4$ (60 μmoles) was converted to K$_2$H$_2$PO$_4$ for $^{18}O$ analysis (3). Found was 3.18 atom % excess.

**Formation of [H]ES-3 P in Tris-Buffered Reaction Mixture**—ES-3-P was synthesized, on the preparative scale previously described, with a 0.4 to 0.6 saturated (NH$_4$)$_2$SO$_4$ fraction of *E. coli* K-12 58-278 (1). The reaction mixture (500 ml) contained approximately 400 mCi of $^3$H$_2$O, and was incubated at 37° for 80 min. The yield of barium salt of ES-3-P was 144 mg, which was 90% pure by 2,4-dinitrophenylhydrazine assay and 87% pure by bioassay (1). A sample (46 mg) was dissolved in 17 ml of water, and BaES-3-P was reprecipitated with 34 ml of absolute ethanol to yield 34 mg of product. Bioassay indicated a purity of 95%; radioactivity, measured as described below, was unchanged. Samples of the barium salt were combusted in O$_2$ in sealed Vycor tubes, the resulting water was added to scintillation mixture,
and counted under standard conditions (16). Alternatively, samples were combusted in a Packard “Oxidizer” and the resulting water was counted similarly. The activity of $^{3}{\text{H}}\text{BaES-3-P}$ was 6.62 × 10^6 dpm per mole.

A similar reaction mixture was incubated as described above except that shikimate-3-P was omitted. Enolpyruvate-P was isolated by chromatography (1), precipitated as barium salt in the presence of a 6-fold excess of barium acetate, and converted to the crystalline tricyclohexylammonium salt. The latter was pure by enzymic assay and had no radioactivity (yield, 50% based on initial enolpyruvate-P).

Purification of ES-3-P Synthetase from Salmonella typhimurium—Wild type cells of Salmonella LT-2 were grown overnight at 37°C in 5 ml of enriched medium (0.2% casein hydrolysate and 0.2% yeast extract), and 1 ml was transferred into 50 ml of the same medium. These cultures were shaken at 37°C for 6 hours, and 10-ml aliquots were inoculated into 500 ml of minimal medium (17) in 2-liter flasks. The cells were grown with rapid shaking at 37°C for 6 hours, harvested by centrifugation in the cold, and washed once with cold 0.05 M Tris buffer, pH 8.0, and stored at 4°C. The yield of wet cells was 38 g from 7 liters of medium. Chilled suspensions of cells (1 g/4 ml of 0.01 M buffer) were disrupted by subjecting 50-ml portions to oscillation in a M.S.E. 100-watt ultrasonic disintegrator for three periods of 2 min each, alternating with 30-s rest periods, and were centrifuged at 2°C for 45 min at 45,000 × g. The resulting extracts contained approximately 25 mg of protein (18) per ml, and had a specific activity of 2.2 units (micromoles of ES-3-P per mg per hour). They were either stored at 15°C or chromatographed as described below.

Whatman DEAE-cellulose (DE-52, 150 g) was stirred vigorously three times in 1 liter-portions of 0.01 M buffer and fine particles were removed as completely as possible. It was then stirred overnight in 300 ml of 0.2 M buffer, fine particles were again removed, and the remainder was washed thoroughly with 0.01 M buffer and used to prepare a column, 2.5 × 45 cm. The crude extract (140 ml) was applied to the top of the column under slight pressure, and chromatographed with a linear gradient of 1000 ml of 0.01 M buffer in the mixing chamber and 1000 ml of 0.2 M buffer in the reservoir. Fractions of 10 ml were collected at a flow rate of 0.5 ml per min. Protein concentration was estimated from the ratio of absorption at A_{280}:A_{260}. A sharp peak of ES-3-P synthetase activity appeared in tubes 59 to 67 (approximately 2.0 mg of protein per tube) which was divided into three fractions comprising tubes 61 through 63 (Fraction I), tubes 59, 60, 64, and 65 (Fraction II), and tubes 66 and 67 (Fraction III). These fractions were concentrated immediately to approximately 7 ml by ultrafiltration on a Diaflo membrane, and had specific activities of 175, 85, and 60, respectively (recovery 35%). Fraction I was divided into 1-ml portions which were mixed with 1 ml of glycerol and stored at −15°C. Activity remained unchanged for over 1 year.

Isolation of $^{3}{\text{H}}\text{BaES-3-P}$ from Reactions Conducted in D_{2}O—Monocyclohexylammonium enolpyruvate-P (6, 7) was deionized, neutralized to pH 7.0 with KOH, and its purity checked with pyruvate kinase coupled to lactate dehydrogenase (19). Potassium shikimate-3-P was prepared similarly and analysed for esterified phosphate (14). Solutions containing, respectively, 0.5 mmole of enolpyruvate-P, 0.5 mmole of shikimate-3-P, 25 mmole of Tris chloride (pH 7.1) and 5 mmole of KF were brought to dryness by rotary evaporation in a vacuum, and exchanged twice with D_{2}O (99.8 mole %) by dissolving the residues in this solvent and removing it by evaporation. The above components were dissolved in D_{2}O, mixed, made up to 150 to 490 ml, and warmed to 37°C.

In one experiment the enzyme preparation was a fraction obtained by chromatography of a Salmonella extract as described above, and was dialyzed against saturated (NH_{4})_{2}SO_{4}. The precipitate was separated by centrifugation, and dissolved in 50 ml of 0.01 M Tris buffer in D_{2}O (pD 6.8). The protein concentration was 0.27 mg per ml with a specific activity of 44, thus affording approximately 600 units. In a second experiment, 4 ml of Fraction I (stored in 50% glycerol as described above, approximately 500 units) was diluted to 20 ml with 0.05 M Tris succinate buffer in D_{2}O, pH 7.0, and concentrated to 1 to 2 ml by ultrafiltration. This procedure was repeated three times and the concentrated solution was diluted with 8 ml of D_{2}O buffer.

The enzyme solution was added to give a final volume of 500 ml, and the course of the reaction at 37°C was followed by assaying enolpyruvate-P disappearance. Equilibrium was reached at 50% conversion in 105 min, and the reaction was stopped after 135 min by adding 1.0 N KOH to pH 8.0 and heating in a boiling water bath for 5 min. D_{2}O was removed by evaporation in a vacuum, and the residue was dissolved in water. Shikimate-3-P, enolpyruvate-P, and ES-3 separated by chromatography on Dowex 1 X8 (Cl−) and isolated as barium salts (1).

Conversion of Barium Salts to Methyl Esters and Ethers—The barium salts of shikimate-3-P, enolpyruvate-P, and ES-3-P were converted to the acids with Dowex 50-H, neutralized with KOH, and treated with twice the calculated amount of AgNO_{3} solution. Ethanol (2 volumes) was added, the mixture was stored at 4°C overnight, and the silver salt was separated by centrifugation, washed with 67% ethanol, and dried in a vacuum. A suspension of the powdered silver salt in methyl iodide was treated with an excess of AgNO_{3}, refluxed with stirring for 3 hours, cooled, diluted with ether, and filtered (20). The clear filtrate was evaporated to dryness, and the residue was dissolved in chloroform and applied to a 20-cm² preparative thin layer chromatography plate (silica gel, 2 mm thick) which was then developed with acetone-chloroform (1:4 v/v). Product bands were located by staining the edges with iodine vapor, scraped off the plate, and eluted with chloroform.

Mass Spectra—Mass spectra of the methyl derivatives were obtained on a CEC 21-110C spectrometer with perfluorokerosene...
as a reference. The concentration of deuterated species was determined by enlarging the molecular ion region, and calculating intensities of \( M^+ \), \( M^+ + 1 \), and \( M^+ + 2 \) from the average of several scans (21). Corrections were made for natural abundances from the spectra of standards.

Nuclear Magnetic Resonance Spectra—Solutions of the barium salts of enolpyruvate-P and ES-3-P were deionized, neutralized with KOH to pH 7.5, evaporated to dryness, and exchanged several times with \( D_2O \). NMR spectra were recorded at an approximate concentration of 0.25 m on a Varian A-60 spectrometer. Chemical shifts were determined in parts per million downfield from tetramethylsilane as internal standard. The extent of deuteration incorporation was determined by integrating NMR spectra of labeled and unlabeled salts of ES-3-P and trimethyl esters of enolpyruvate-P (in which the O-methyl proton resonances served as internal standards).

RESULTS

The first step in the synthesis of \( [\text{\textsuperscript{18}}O]\text{enolpyruvate-P} \) was labeling of the carbonyl oxygen of bromopyruvate by exchange with \( \text{H}_2\text{\textsuperscript{18}}O \). Analysis of the \( \text{CO}_2 \) obtained by thermal decarboxylation of the bromopyruvate in diphenylamine-diphenyl-sulfonate. Spectra of the methyl derivatives were obtained in \( \text{CDCl}_3 \) with tetramethylsilane as internal standard. The phosphate oxygen atoms. (A similar labeling of \( \text{CO}_2 \) occurred when methane showed considerable incorporation of \( \text{\textsuperscript{18}}O \) into the boxylation of the bromopyruvate in diphenylamine-diphenyl-sulfonate. Spectra of the methyl derivatives were obtained in \( \text{CDCl}_3 \) with tetramethylsilane as internal standard. The extent of \( \text{\textsuperscript{18}}O \) incorporation was determined by integrating NMR spectra of labeled and unlabeled salts of ES-3-P and trimethyl esters of enolpyruvate-P (in which the O-methyl proton resonances served as internal standards).

The spectrum of enolpyruvate-P showed the vinyl methylene protons at \( \delta 5.71 \) and \( 5.21 \) (\( H_B \)), \( J_{AB} = 2.5 \) Hz. Assignment of these resonances, \( H_A \) trans to the carboxyl and \( H_B \) cis to the carboxyl, was based on the observation that a \( \beta \) proton cis to the carboxyl group of an \( \alpha,\beta \)-unsaturated carboxylic acid absorbed downfield relative to the corresponding trans \( \beta \) proton. (22). The vinyl methylene protons of chorismic acid were assigned in a similar manner (23). The ring proton resonances, from 60 and 100 MHz spectra, were analyzed and assigned in a straightforward manner (Table II), and closely resembled those in the spectrum of shikimic acid (24), except that \( \text{H}_3 \) in ES-3-P was also coupled with phosphorus, \( J_{3-P} = 8.5 \) Hz.

The spectrum of enolpyruvate-P showed the vinyl methylene protons at \( \delta 5.18 \) (\( H_A \)) and 5.35 (\( H_B \)), each as two overlapping doublets owing to geminal coupling (\( J_{AB} \)) and coupling with phosphorus. This assignment has recently been established unequivocally by Cohn et al. in a NMR study of \( \text{I}^{-}\text{[\text{\textsuperscript{3}}P]}\text{enolpyruvate-P} \) (25). It was not possible to evaluate separately each coupling constant, but from the separation between the outer peaks of the apparent triplets at \( \delta 5.18 \) and 5.35, it was determined that \( J_{AB} = 2.4 \) Hz and \( J_{AP} = 2.8 \) Hz, respectively. Similarly, the vinyl protons of trimethyl enolpyruvate-P appeared at \( \delta 5.63 \) (\( H_A \)) and 5.97 (\( H_B \)), each as two overlapping doublets, and it was determined that \( J_{AB} = 4.85 \) Hz, and \( J_{AP} = 4.65 \) Hz, respectively. It may be seen that the relative magnitudes of \( J_{AB} \) and \( J_{AP} \) of enolpyruvate-P have been reversed in the trimethyl ester (cf. Reference 25). The protons of the carbonylic and phosphoric ester methyl groups appeared, respectively, as a singlet at \( \delta 3.88 \), and a doublet at \( \delta 3.92 \) (\( J_{3-P} = 11.5 \) Hz).

The doubly labeled species of ES-3-P and trimethyl enolpyruvate-P showed diminished intensities only in resonances assigned to vinyl methylene protons. Deuteration was equally distributed between \( H_A \) and \( H_B \) in both compounds. In 60 MHz spectra of \( \text{HE} \) ES-3-P was quite distinct, but \( H_A \) was obscured by other resonances. However, in a 100 MHz spectrum at 60\( ^\circ \) \( H_A \) and \( H_B \) were well resolved singlets of equal intensity. Both had small shoulders on the downfield side probably owing to the

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<th>Compound isolated</th>
<th>NMR</th>
<th>Mass spectrometry</th>
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<tr>
<td></td>
<td></td>
<td>Atoms of deutium</td>
</tr>
<tr>
<td>ES-3-P</td>
<td>1.1</td>
<td>1.3\textsuperscript{a}</td>
</tr>
<tr>
<td>Enolpyruvate-P</td>
<td>0.73</td>
<td>0.72\textsuperscript{a}</td>
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<tr>
<td>S-3-P</td>
<td>0.9</td>
<td>0.0\textsuperscript{a}</td>
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\( ^{a} \) Pentamethyl derivative.  
\( ^{b} \) Trimethyl ester.

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small amount of unlabeled molecules. Integration of the 60 MHz spectra showed that 1.1 atoms of deuterium was incorporated into ES-3-P, and 0.75 atom into trimethyl enolpyruvate-P.

The amount of deuterium labeling observed by NMR was corroborated by mass spectrometry (Table I). All the methyl derivatives gave intense molecular ions suitable for determination of deuterium concentration by comparison of spectra obtained with the labeled and unlabeled compounds. By this method ES-3-P had 1.3 atoms deuterium, and enolpyruvate-P had 0.72 atom deuterium. The proportion of labeled species was higher in ES-3-P than in enolpyruvate-P, but the ratio of singly labeled to doubly labeled molecules was essentially the same in both compounds. Shikimate-3-P was unlabeled, as would be expected.

Assuming $H_A = H_B$, the value obtained for $H_B$ was multiplied by 2.

The mechanism of ES-3-P formation has previously been considered (1) as a reversible addition-elimination reaction (Scheme 1). It was assumed that protonation of carbon 3 of enolpyruvate-P, resulting from electron donation by the ester oxygen, was associated with a nucleophilic attack by the 5-hydroxyl group of shikimate-3-P on carbon 2. Elimination of P$_i$ from the postulated intermediate could yield ES-3-P, and in reverse direction, elimination of shikimate-3-P would yield enolpyruvate-P. This mechanism predicts C—O cleavage of the ester bond and exchange of hydrogen from the medium with vinyl methylene protons of ES-3-P and enolpyruvate-P. Both predictions have been realized in the experiments described above.

The $P_i$ released in the reaction contained all of the $^{18}O$ present in the ester oxygen of the enolpyruvate-P. When the reaction was carried out in $^3$H$_2$O, 21% of 2 atoms of tritium (0.42 atom) was found in ES-3-P. It is reasonable to assume that the tritium was introduced on the enolpyruvyl side chain of ES-3-P, since there are no known enzymic reactions that labilize the carbon-bound hydrogen atoms of shikimate-3-P. Furthermore, acid hydrolysis of [3H]ES-3-P gave shikimate-3-P devoid of radioactivity. The tritium must have been introduced during ES-3-P formation, since enolpyruvate-P did not become labeled when incubated with enzyme extract in the absence of shikimate-3-P. Although low labeling in ES-3-P could have resulted from isotope effects, the relatively small incorporation of tritium from the medium made it difficult to interpret the result in relation to mechanism of the reaction.

ES-3 P and enolpyruvate P were therefore isolated from an enzymic synthesis in D$_2$O. As shown in Table I, more than 1

### Table II

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<thead>
<tr>
<th>Chemical shifts ($\delta$ values)</th>
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<tr>
<td>H-2</td>
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<td>6.52</td>
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<th>Coupling constants (Hz)</th>
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<tr>
<td>$J_{2.3}$</td>
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atom of deuterium was introduced into ES-3-P from a nearly proton-free D2O medium. Mass spectrometry of pentamethyl
ES-3-P showed 1.3 atoms of deuterium distributed in 8% d0
species, 53% d1, and 39% d2. The further observation that the
vinyl proton magnetic resonances were equally diminished in
intensity indicates that the two possible d1 species

and

were present in essentially equal proportions, with the remainder
being either unlabeled or doubly labeled. A similar distribution
of deuterium was found in enolpyruvate-P, although only 50% of
the molecules were labeled compared to 92% in ES-3-P. These results indicate that a methyl group with unrestricted
rotation was formed at carbon 3 of enolpyruvate-P (Scheme 1).

It is not clear why isotopic equilibrium was not reached be-

between the vinyl methylene hydrogens of ES-3-P and those of
enol ethers. The acid-catalyzed synthesis of enol ethers from
acetaldehyde (27).

Addition-elimination mechanisms are prevalent in reactions of
enol ethers. The acid-catalyzed synthesis of enol ethers from
enolic compounds and CH3180H was found to occur with com-
plete transfer of 180 to the products (26). The results were
interpreted as suggesting an addition-elimination mechanism for
acid-catalyzed synthesis and hydrolysis of enol ethers. A similar
conclusion was reached in a study of enol ether hy-

The only reaction known in which the enolpyruvyl moiety of enolpyruvyl-P is transferred apparently unchanged,
occurs in the formation of UDP N-acetylglucosamine, an intermediate in the synthesis of UDP-N-acetylmuramic
acid (28, 29). The mechanism of the latter reaction is probably

calculated isotope effects for the forward and reverse steps of

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