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 $^{18}$O 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 D$_2$O reaction medium showed incorporation of approximately 1.3 atoms of deuterium, and consisted of 39% d$_2$, 53% d$_3$, and 8% d$_4$. Enolpyruvate phosphate isolated from the same reaction mixture had 0.72 atom of deuterium with a similar d$_2$:d$_3$ 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 $^3$H 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 5-hydroxyl 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 $^{18}$O 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.

ES-3-P synthetase represents a rare type of reaction in which the enolpyruvyl moiety of enolpyruvyl 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 P$_1$ then yields ES-3-P. This mechanism is supported by studies of the reaction in D$_2$O and in H$_2$O, and with [18O]enolpyruvate-P, which are described in the present report.

EXPERIMENTAL PROCEDURE

$[^{18}$O]Enolpyruvate-P—A solution of 3.0 g of freshly recrystallized bromopyruvic acid (2) in 5 g of H$_2$O$_2$O (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 $^{18}$O into two additional 3.0-g lots of bromopyruvic acid. The combined products were reconstituted and dried with P$_2$O$_5$ to yield 1.4 g of white crystals. Behavior on melting: first transition 52–55°; m.p. of glass, 73.5–76° (2). $^{18}$O concentrations in the carboxyl group was determined by deacylating the bromopyruvic acid in diphenylamine-diphenylmethane (4, 5). Determinative yields of CO$_2$ were obtained at reflux temperature (270°) in 5 min, but considerable reaction occurred also at 100°. The following $^{18}$O values were obtained in CO$_2$ evolved under the indicated conditions: (a) at 100° for 25 min, 8.79 atom % excess; (b) discarding the CO$_2$ 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 $^{18}$O in the carboxyl group.

The carboxyl oxygen of the bromopyruvic acid can be calculated by the following formula: 1

The abbreviations used are: ES 3 P, 5-enolpyruvylshikimate 3-phosphate; NMR, nuclear magnetic resonance.

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2 The numbering of the ring carbon atoms follows Chemical Abstracts usage.
yield) by the method of Clark and Kirby (6, 7). The $^{18}O$ concentration of this product was $5.25 \pm 0.09$ atom % excess (average of three analyses), or the same atoms of $^{18}O$ excess per mole, within experimental error, as in the bromopyruvic acid ($6 \times 5.25/100 = 0.315; 3 \times 10.3/100 = 0.309$). Although decarboxylation of enolpyruvate-P in refluxing diphenylamine-diphenylmethane gave essentially quantitative yields of CO$_2$, the $^{18}O$ 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. $^{18}O_2$ obtained by refluxing in quinoline showed somewhat higher results (7.87 atom % excess). The $^{18}O$ in a solution (1 ml) of 0.5 pmole of shikimate-3-P, 500 pmoles of Na$_2$H$_2$PO$_4$, 1.1 mmoles of KF, 5.5 mmoles of Tris maleate buffer (pH 6.1), and 0.4 to 0.6 mmoles of K$_2$CO$_3$ was measured as previously described (13). Saline extracts on DEAE-cellulose were free of phosphatase and could be studied by P$_1$ release (14).

Isolation of P$_1$ from ES-3-P Synthetase Reaction with $[^{18}O]$Enolpyruvate-P—A solution (110 ml) containing 110 pmoles of the $[^{18}O]$enolpyruvate-P, 110 pmoles of shikimate-3-P, 1.1 mmoles of K$_2$SO$_4$, 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°C 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 pmoles) was converted to K$_2$HPO$_4$ for $^{18}O$ analysis (3). Found was $3.18$ atom % excess.

Formation of [HI]ES-3-P in Trivalent 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°C 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 57% 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 [3H]BaES-3-P was 6.62 × 10^6 dpm per mole.

A sample of the original incubation mixture (1.5 ml) was freeze-dried, the condensed water was diluted 100-fold (w/w), and 0.0500 g was assayed by scintillation counting under standard conditions. A total activity of 399 ± 8 mCi (average of four independent analyses) was obtained for the [3H] present in the reaction mixture, or 3.20 × 10^6 dpm per mg.

[3H]BaES-3-P (40 mg) was converted to the acid with Dowex 50-H⁺, and hydrolyzed in 0.1 N HCl (1). The solution was evaporated to dryness in a vacuum, and the residue was treated several times with water and evaporated to dryness. The barium salt of shikimate-3-P was isolated. It had no radioactivity.

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° 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° for 8 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° for 6 hours, harvested by centrifugation in the cold, and washed once with cold 0.05 M Tris succinate buffer, pH 0.8 (this buffer, pH 0.8, was used throughout, and all operations were at 2° to 4°). 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 subjected by 50-ml portions to oscillation in a M.S.E. 100-watt ultrasonic integrator for three periods of 2½ min each, alternating with 30-s rest periods, and were centrifuged at 2° 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 hr). They were either stored at 15° 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 500 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 280:260. 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 69, 60, 64, and 66 (Fraction II), and tubes 65 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°. Activity remained unchanged for over 1 year.

**Isolation of [3H]ES-3-P from Reactions Conducted in D₂O**—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 mmoles of Tris chloride (pH 7.1) and 5 mmole of KH₂PO₄, were brought to dryness by rotary evaporation in a vacuum, and exchanged twice with D₂O (99.8 mole %) by dissolving the residues in this solvent and removing it by evaporation. The above concentrates were dissolved in D₂O, mixed, made up to 450 to 490 ml, and warmed to 37°.

In one experiment the enzyme preparation was a fraction obtained by chromatography of a Salmonella extract as described above, and was dialyzed against saturated (NH₄)₂SO₄. The precipitate was separated by centrifugation, and dissolved in 50 ml of 0.01 M Tris buffer in D₂O (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, approx. 500 units) was diluted to 20 ml with 0.05 M Tris succinate buffer in D₂O, 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 D₂O buffer.

The enzyme solution was added to give a final volume of 500 ml, and the course of the reaction at 37° 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 ml of KOH to pH 8.0 and heating in a boiling water bath for 5 min. D₂O was removed by evaporation in a vacuum, and the residue was dissolved in ml of water. Shikimate-3-P, enolpyruvate-P, and ES-3 separated by chromatography on Dowex 1 × 8 (Cl⁻) and isolated as barium salts (1).

**Conversion of Barium Salts to Methoxyl 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 AgNO₃ solution. Ethanol (2 volumes) was added, the mixture was stored at 4° 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 AgO₂, 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 enriching 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 D₂O. 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 an internal standard of sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Spectra of the methyl derivatizes were obtained in CDCl₃ with tetramethylsilane as internal standard. The extent of D₂O 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 [¹⁸O]enolpyruvate-P was labeling of the carboxyl oxygen of bromopyruvate by exchange with H₂¹⁸O. Analysis of the CO₂ obtained by thermal decarboxylation of the bromopyruvate in diphenylamine-diphenylmethane showed considerable incorporation of ¹⁸O into the carboxyl group (8.84 atom % excess). The ¹⁸O in the carboxyl oxygen, calculated by difference between the total ¹⁸O and that of the carboxyl group, was 13.2 atom % excess.

The atoms of ¹⁸O excess per mole in both bromopyruvate and enolpyruvate-P were identical, i.e. 0.309 and 0.315, respectively. Although the ¹⁸O of the CO₂ from bromopyruvate decarboxylation was constant (see "Experimental Procedure"), the isotope concentration in CO₂ from enolpyruvate-P was variable and lower than expected, presumably owing to exchange with phosphate oxygen atoms. (A similar labeling of CO₂ occurred when alanine was decarboxylated in the presence of KH₂¹⁸PO₄). The ¹⁸O of the CO₂ oxygen was, therefore, assumed to be the same as that of the carboxyl oxygen of bromopyruvate, i.e. 13.2 atom % excess.

The [¹⁸O]enolpyruvate-P was incubated with shikimate-3-P and a bacterial extract containing ES-3-P synthetase. The Pi formed in the reaction contained 3.18 atom % excess ¹⁸O, corresponding to 4 × 3.18 = 12.7 for ¹⁸O in the C—O—P oxygen. This value was very close to the 13.2 atom % excess calculated for the ester linkage of the enolpyruvate-P. The synthesis of ES-3-P, therefore, took place with C—O cleavage of the enolpyruvate-P.

A similar incubation of shikimate-3-P and enolpyruvate-P was conducted in the presence of H₂¹⁸O. The ES-3-P was isolated, purified as previously described (1), and reprecipitated to constant activity. The product had an activity of 6.62 × 10⁶ dpm per mole, corresponding to 21% of the specific activity of the medium (i.e. 21% of 2 atoms of H₂O, or 0.42 atom). Acid hydrolysis of the [¹³C]ES-3-P gave shikimate-3-P devoid of radioactivity. In a similar experiment ¹⁸O was not incorporated into enolpyruvate-P when incubated with enzyme in the absence of shikimate-3-P.

A further investigation was undertaken of the ES-3-P synthetase reaction in D₂O. A much more purified enzyme preparation was used in this work, and reactants as well as ES-3-P were isolated and studied by NMR and mass spectrometry. The results of the second experiment described under "Experimental Procedure" are shown in Table I. Essentially identical results were obtained in the first experiment.

The 60 MHz NMR spectrum of ES-3-P, Fig. 1, showed 2 vinyl methylene protons as doublets at δ 4.71 (H₃, and 5.21 (H₄), J₂₋₃ = 2.5 Hz. Assignment of these resonances, H₃ trans to the carboxyl and H₄ cis to the carboxyl, was based on the observation that a β proton cis to the carboxyl group of an α,β-unsaturated carboxylic acid absorbed downfield relative to the corresponding trans β 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 summarized in a straightforward manner (Table II), and closely resembled those in the spectrum of shikimic acid (24), except that H-3 in ES-3-P was also coupled with phosphorus, J₃₋₅ = 8.5 Hz.

The spectrum of enolpyruvate-P showed the vinyl methylene protons at δ 5.18 (H₃, and 5.35 (H₄), each as two overlapping doublets owing to geminal coupling (J₁₂₋₃₋₄) and coupling with phosphorus. This assignment has recently been unequivocally established by Cohn et al., in a NMR study of 1-[¹³C]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 δ 5.18 and 5.35, it was determined that J₁₂₋₃₋₄ = 2.4 Hz and J₁₂₋₃₋₄ = 2.8 Hz, respectively. Similarly, the vinyl protons of trimethyl enolpyruvate-P appeared at δ 5.63 (H₃, and 5.97 (H₄), each as two overlapping doublets, and it was determined that J₁₂₋₃₋₄ = 2.4 Hz and J₁₂₋₃₋₄ = 2.8 Hz, respectively. It may be seen that the relative magnitudes of J₁₂₋₃₋₄ and J₁₂₋₃₋₄ of enolpyruvate-P have been reversed in the trimethyl ester (cf. Reference 25). The protons of the carboxylic and phosphonic ester methyl groups appeared, respectively, as a singlet at δ 3.88, and a doublet at δ 3.92 (J₁₂₋₃₋₄ = 11.5 Hz).

Labeled species of ES-3-P and trimethyl enolpyruvate-P showed diminished intensities only in resonances assigned to vinyl methylene protons. Deuterium was equally distributed between H₃ and H₄ in both compounds. In 60 MHz spectra H₃ of [¹³C]ES-3-P was quite distinct, but H₄ was obscured by other resonances. However, in a 100 MHz spectrum at δ 0° H₃ and H₄ were well resolved singlets of equal intensity. Both had small shoulders on the downfield side probably owing to the

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**Table I**

| Compound isolated | NMR | Mass spectrometry |
|-------------------|-----|---------------------|
|                   | Atoms of deuterium | Atoms of deuterium | m/² | Abundance | d₁ | d₂ | d₃ |
| ES-3-P            | 1.1 | 1.3a                | 394 | 8         | 53 | 30 |
| Enolpyruvate-P    | 0.75| 0.72a               | 210 | 50        | 28 | 22 |
| S-3-P             | 0.0 | 2.5b                | 324 | 100       | 0  | 0  |

a Pentamethyl derivative.

b Trimethyl ester.
Fig. 1. Nuclear magnetic resonance spectrum of potassium 5-enolpyruvylshikimate 3-phosphate, pD 7.9, in D₂O at 60 MHz.

TABLE II

| Chemical shifts (δ values) | H-2 | H-3 | H-4 | H-5 | H-6e | H-6a | HA | HB |
|---------------------------|-----|-----|-----|-----|------|------|-----|-----|
| H-2                       | 6.52| 1.88| 4.1 | 4.5 | 2.08 | 2.20 | 4.71| 5.21|

| Coupling constants (Hz)  | J₁₂,₂ | J₁₂,₄ | J₁₂,₆ | J₁₄,₆ | J₁₆,₆ | J₁₆,₆₆ | J₄₆,₆₆ | J₆₆,₆₆ |
|--------------------------|-------|-------|-------|--------|--------|----------|----------|----------|
| J₁₂,₂                    | 4     | 2     | 4     | 8.5    | 8      | 5        | 6        | 18.5     |
| J₁₂,₄                    |       |       |       |        |        |          |          |          |
| J₁₂,₆                    |       |       |       |        |        |          |          |          |
| J₁₄,₆                    |       |       |       |        |        |          |          |          |
| J₁₆,₆                    |       |       |       |        |        |          |          |          |
| J₁₆,₆₆                   |       |       |       |        |        |          |          |          |
| J₄₆,₆₆                   |       |       |       |        |        |          |          |          |
| J₆₆,₆₆                   |       |       |       |        |        |          |          |          |

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 HA = HB, the value obtained for HB was multiplied by 2.

DISCUSSION

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 P₁ 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 P₁ released in the reaction contained all of the ¹⁸O present in the ester oxygen of the enolpyruvate-P. When the reaction was carried out in ³H₂O, 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 [²H]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 D₂O. As shown in Table I, more than 1
atom of deuterium was introduced into ES-3-P from a nearly proton-free D₂O medium. Mass spectrometry of peptamethyl 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

\[ \text{RO} \text{COH} \]

and

\[ \text{RO} \text{COH} \]

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 isotope equilibrium was not reached between the vinyl methylhydrogens of ES-3-P and those of enolpyruvate-P under the experimental conditions described above. In the absence of initial rate data on deuterium incorporation into product and starting material it is not possible to calculate isotope effects for the forward and reverse steps of the equilibrium. However, the difference in extent of labeling between ES-3-P and enolpyruvate-P suggests that the postulated intermediate breaks down faster to ES-3-P than to enolpyruvate-P. Isotope effects in protonation of vinyl carbon atoms as well as elimination reactions may account for the 3-fold greater incorporation of deuterium than of tritium into ES-3-P under similar experimental conditions.

Addition-elimination mechanisms are prevalent in reactions of enol ethers. The acid-catalyzed synthesis of enol ethers from enolic compounds and CH₃OH was found to occur with complete transfer of ¹⁸O 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 hydrolysis.

(27)

The only other reaction known in which the enolpyruvyl moiety of enolpyruvyl-P is transferred apparently unchanged, occurs in the formation of UDP N-acetyleneopyruvylglucosamine, an intermediate in the synthesis of UDP-N-acetylmuramic acid (28, 29). The mechanism of the latter reaction is probably similar to that of ES-3-P synthetase.

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