Observation of a Secondary Tritium Isotope Effect in the Chorismate Synthase Reaction†

Shankar Balasubramanian,‡ John R. Coggins,§ and Chris Abell*,‡

University Chemical Laboratory, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, England, and Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland

Received July 12, 1994; Revised Manuscript Received September 8, 1994®

ABSTRACT: Chorismate synthase, the seventh enzyme on the shikimate pathway, catalyzes the formation of chorismate from 5-enolpyruvylshikimate 3-phosphate (EPSP). This reaction involves the loss of phosphate from C(3) and hydrogen from the C(6) *pro-R* position of EPSP. In order to probe the mechanism of this reaction, [3-^{3}H,^{14}C]EPSP has been synthesized and a secondary *V/K* tritium kinetic isotope measured for the reaction catalyzed by *Neurospora crassa* chorismate synthase. A small but significant value of $k_H/k_T = 1.047 \pm 0.012$ was observed. The reaction is also shown to be effectively irreversible. Previous experiments have measured a primary deuterium isotope effect on *V/K* at C(6) [Balasubramanian, S., Abell, C., & Coggins, J. R. (1990) *J. Am. Chem. Soc. 112*, 8581−8583], and there is additionally evidence in support of a flavin intermediate in the mechanism [Ramjee, M. N., Coggins, J. R., Hawkes, T. R., Lowe, D. J., & Thorneley, R. N. F. (1991) *J. Am. Chem. Soc. 113*, 8566−8567]. In the light of these observations the reaction mechanism probably involves cleavage of the C(6)−H and C(3)−O bonds in *distinct* but partially rate determining steps.

The enzyme chorismate synthase (EC 4.6.1.4) catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate (**1**, EPSP)[1] to chorismate (**2**) in the seventh step of the shikimate pathway (Ganem, 1978). This enzymatic transformation is formally an *anti*-1,4-elimination of the C(3) phosphate and the C(6) *pro-R* hydrogen (Hill & Newkome, 1969; Onderka & Floss, 1969; Onderka *et al.*, 1972). The mechanism for this reaction is unknown. There is no well-studied enzyme reaction which can serve as a precedent for this transformation, and surprisingly little is known about the non-enzymic elimination of allylic phosphates.

The overall *anti* stereochemistry of the chosimate synthase reaction was the only piece of mechanistic information available for many years. Model studies of 1,4-eliminations have demonstrated a preference for *syn* stereochemistry for concerted mechanisms (Hill & Bock, 1978; Toromanoff, 1980; Moss & Rickbourne, 1986; Olwegard & Ahlberg, 1990), and furthermore, theoretical arguments based on frontier orbital symmetry predict that a concerted E2′ elimination should also proceed with *syn* stereochemistry (Fukui, 1965; Anh, 1968). These contrasting stereochemical results have prompted a series of mechanistic proposals for the chorismate synthase reaction, some of which are summarized in Scheme 1 (Ganem, 1978; Onderka *et al.*, 1972, Hawkes *et al.*, 1990). Recently, several experiments have been carried out in an attempt to distinguish between these various proposals. IsoEPSP (**3**) was synthesized and shown to be a competitive inhibitor, rather than a substrate, for chorismate synthase (Bartlett *et al.*, 1986). (6*R*)-6-FluoroEPSP was shown to be a good competitive inhibitor of the enzyme, but did not result in the accumulation of either isoEPSP (**3**) or an enzyme-bound species such as **4** (Balasubramanian *et al.*, 1991a). Finally, in pre-steady-state experiments using the *Escherichia coli* enzyme, no significant burst or lag in phosphate release was detected (Hawkes *et al.*, 1990).

The mechanistic proposals in Scheme 1 ignore the interesting fact that chorismate synthase has a requirement for a reduced flavin cofactor (Welch *et al.*, 1974; Hasan & Nester, 1978; White 1988). However, recent experiments have demonstrated that there is a spectral change associated with the flavin during the catalytic reaction (Ramjee *et al.*, 1991) and, furthermore, that an enzyme-bound flavin semiquinone radical is formed stoichiometrically in the presence of the substrate analogue (6*R*)-6-fluoro EPSP (Ramjee *et al.*, 1992). In the light of these results a mechanistically functional role for the flavin must now be seriously considered, suggesting that the reaction mechanism is more complex than was previously considered.

A feature of the transformation of **1** to **2** is the change in hybridization at C(3) from sp^3 to sp^2. The degree of bond breakage can be probed in the transition state by studying the secondary hydrogen isotope effect at this center. Specifically [3-^{3}H]EPSP should undergo elimination more slowly than [^{14}C]EPSP in a competitive isotope experiment, providing that bond cleavage at C(3) is isotopically sensitive and precedes the irreversible step. Previously we reported a primary deuterium isotope effect on *V* and *V/K* at C(6) for *Neurospora crassa* chorismate synthase (Balasubramanian *et al.*, 1990). The observation of an isotope effect on *V/K* at C(6) was encouraging, as it demonstrated that isotope effects associated with bond breaking are not totally sup-

† This work was funded by the SERC and ICI Agrochemicals through a CASE Award to S.B.

* Author to whom correspondence should be sent: University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K. Tel: +44-223-336405. FAX: +44-223-336362. E-mail: CA26@CUS.CAM.AC.UK.

‡ University of Cambridge.

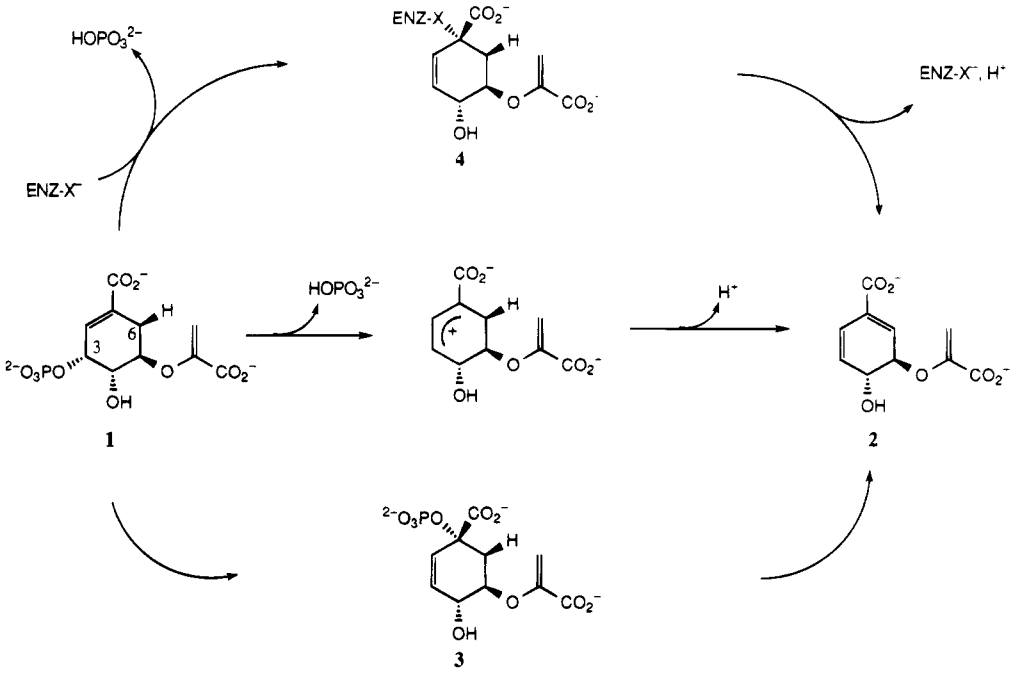
§ University of Glasgow.

® Abstract published in *Advance ACS Abstracts,* November 15, 1994.

[1] Abbreviations: EPSP, 5-enolpyruvylshikimate 3-phosphate; FMN, flavin mononucleotide; PEP, phosphoenolpyruvate.

0006-2960/95/0434-341$09.00/0

Scheme 1: Some Suggested Mechanisms for the Enzymic Conversion of EPSP to Chorismate



pressed. This would have been the case if substrate binding had been the rate-limiting step, as was observed for chorismate mutase, the next enzyme on the shikimate pathway (Addadi *et al.*, 1983).

In this paper we report the synthesis of [3-^{3}H,^{14}C]EPSP. This was used as a substrate for the *N. crassa* chorismate synthase, and a small, but significant, secondary tritium isotope effect on *V/K* at C(3) was observed.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]$NaBH_4$ (9.09 Ci/mmol) and [^{14}C]shikimic acid (21.9 mCi/mmol) were purchased from New England Nuclear/Dupont (Stevenage, England). Amberlite (Cl^- form, CG400, 100−200 mesh) and Dowex (H^+ form, W-50) were obtained from BDH Resins (Lutterworth, England), and DEAE-Sephacel (Cl^- form) was from Pharmacia (Uppsala, Sweden). [Bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris), quinic acid, shikimic acid, ATP, phosphoenolpyruvate, FMN, and NADPH were purchased from Sigma (Poole, England). Amberlite CG-400 was converted to the acetate form by overnight elution with 2 N sodium acetate, and DEAE-Sephacel was converted to the bicarbonate form by elution with 1 M triethylammonium bicarbonate (TEAB, pH 7.8, 20 vol). Dowex W-50 (H^+) was subjected to precycling with water (deionized, 10 vol), sodium hydroxide (5%, 10 vol), water (deionized, 10 vol), HCl (10%, 20 vol), and finally water (deionized, 20 vol), before use. The Na^+ form of Dowex W-50 was prepared by elution with aqueous sodium chloride (5%, 20 vol), followed by washing with water (deionized, 20 vol). Dichloromethane, hexane, methanol, and triethylamine were all freshly distilled from calcium hydride. A stock solution of TEAB (1 M, pH 7.8) was freshly prepared by passing carbon dioxide gas into triethylamine (1 M) at room temperature and used within 24 h.

Unlabeled chorismic acid was prepared from an overexpressing strain of *Aerobacter aerogenes* by the method of Gibson (1968) and was stored desiccated at −20 °C. Prior to each experiment chorismic acid was treated with activated charcoal and recrystallized from ether/dichloromethane/hexane (Guilford *et al.*, 1987). Unlabeled EPSP was prepared as the barium salt from shikimic acid using shikimate kinase and EPSP synthase (Balasubramanian & Abell, 1991b).

Apyrase (potato, grade VII) was purchased from Sigma (Poole, England), and alkaline phosphatase was from Boehringer Mannheim (Lewes, England). Dehydroquinase (Duncan *et al.*, 1986), shikimate dehydrogenase (Anton & Coggins, 1988), shikimate kinase (Millar *et al.*, 1986), and EPSP synthase (Duncan *et al.*, 1984), all from *E. coli*, were purified from recombinant *E. coli* strains by the indicated published procedures. Chorismate synthase was purified from wild-type *N. crassa* by the method of White *et al.* (1988) and had a specific activity of 11.5 units/mg.

Enzyme Assays. Chorismate synthase was assayed at 25 °C in a reaction mixture containing 50 mM Bis-Tris-HCl buffer, pH 7.0, KCl (50 mM), NADPH (20 μM), FMN (10 μM), and EPSP (50 μM) in a total volume of 1 mL. Assays were subjected to a 5-min preincubation of all components except EPSP, at 25 °C, and were started by the addition of EPSP. The reaction rate was monitored by the appearance of the diene chromophore of chorismic acid at 275 nm ($\Delta\epsilon_{275}$ = 2630 M^{-1} cm^{-1}; Gibson, 1970).

Synthesis

(A) [3-^{3}H]Shikimic Acid. Quinic acid (**5**) was oxidized to 3-dehydroquinic acid (**6**) by aqueous nitric acid following the published procedure of Grewe and Haworth (1968). 3-Dehydroquinic acid (**6**) was stored desiccated at −20 °C as the ammonium salt.

The ammonium salt of 3-dehydroquinate (**6**, 850 mg, 4.07 mmol) was dissolved in phosphate buffer (100 mL, 20 mM), and *E. coli* dehydroquinase (100 units) was added. After overnight incubation at 30 °C the completion of the reaction

was confirmed by HPLC (retention time (**6**) = 15.5 min). The reaction was stopped by acidification with Dowex-H^+ (~5 g), and after removal of the resin, the reaction mixture was loaded directly onto a prepared column of Amberlite CG-400 (100−200 mesh, acetate form) of dimensions 20 × 2 cm, at a flow rate of 1 mL min^{-1}. 3-Dehydroshikimic acid (**7**) binds to form a sharp light-colored band at the top of the column. The column was then eluted with a linear gradient of 0.5 to 6.0 M acetic acid (200 mL + 200 mL) at a flow rate of 1 mL min^{-1} and thereafter with 6.0 M acetic acid, and 10-mL fractions were collected. The chromophore of **7** (λ_{max} = 234 nm) was difficult to detect by UV against a background of acetic acid. However, the fractions could be analyzed by spotting a drop onto a TLC plate and viewing under a UV source after air drying. All UV-active fractions (40−49) were pooled and concentrated to dryness *in vacuo*, with a bath temperature no higher than 40 °C, to give a clear oil (649 mg, 3.77 mmol, 93% crude yield), which crystallized when left under high vacuum. Recrystallization from hot ethyl acetate gave the acid **7** (381 mg, 2.22 mmol, 55% recrystallized yield) as white crystals with physical properties identical to those published (Haslam *et al.*, 1963).

3-Dehydroshikimic acid (**7**, 89 mg, 0.52 mmol) was dissolved in water (5 mL), passed through a small column (10 × 1 cm) of Dowex W-50 (Na^+ form), and lyophilized to give the sodium salt of **7** (71 mg, 0.37 mmol, 71%). The sodium salt was then dissolved in dry methanol (5 mL) and gently stirred in a flask equipped with a drying tube, at room temperature. Unlabeled sodium borohydride (8.5 mg, 0.22 mmol) was added, and HPLC analysis of an aliquot of the reaction mixture (containing ~0.1% of the mixture) indicated that less than 20% reaction had occurred (retention times: shikimic acid = 13.0 min; 3-epishikimic acid = 13.8 min; 3-dehydroshikimic acid = 15.5 min). [^{3}H]$NaBH_4$ (100 mCi, 9.09 Ci/mmol) was added to the reaction mixture, which was stirred overnight at room temperature. The reduction was completed by the addition of more unlabeled sodium borohydride (35 mg, 0.92 mmol), followed by stirring for 15 min. A small sample of the reaction mixture (containing ~0.1% of the mixture) was injected onto an HPLC column, and the eluant containing both shikimic acid (retention time = 13.0 min) and 3-epishikimic acid (retention time = 13.8 min) was analyzed by scintillation counting. Most of the radioactivity present (> 95%) was eluted in the combination of both peaks, which appeared in a ratio of 1:4 (shikimic acid:3-epishikimic acid). The reaction mixture was concentrated to dryness *in vacuo* and then redissolved in H_2O (15 mL) and acidified with Dowex W-50 (H^+) to quench any unreacted borohydride. The solution was brought to ~pH 7.0 by the addition of 10% NaOH (~2−4 drops) prior to loading onto a column of DEAE-Sephacel (bicarbonate form, 12 × 2 cm), which had been preequilibrated with 5 mM TEAB (100 mL). The column was washed with 5 mM TEAB (25 mL) and then eluted with a linear gradient of 5 to 500 mM TEAB (100 mL + 100 mL). Fractions (5 mL) were collected and assayed for radioactivity. The mixture of epimers (**8** and **9**) eluted at 175 mM TEAB (fractions 12−16). These fractions were pooled and concentrated to dryness to give the triethylammonium salt of the mixture of C(3) epimers **8** and **9** as a white solid (80 mg, 0.30 mmol, 80% combined chemical yield) which had a specific radioactivity (^{3}H) of 90.9 mCi/mmol (27% combined radiochemical yield). The mixture was stored in methanol (15 mL) at −20 °C.

[3-^{3}H]Shikimic acid was purified from the mixture in two steps. About one-third of the epimeric mixture (**8** and **9**, 0.096 mmol, 8.7 mCi ^{3}H) was concentrated to dryness, diluted with unlabeled shikimic acid (17.4 mg, 0.1 mmol) and dissolved in 50 mM Bis-Tris buffer (pH 7.0, 20 mL) in the presence of 7.5 mM ATP, 2.5 mM $MgCl_2$, and 50 mM KCl. The final concentration of [3-^{3}H]shikimic acid was 6.0 mM. *E. coli* shikimate kinase (10 units) was added, and the reaction was incubated overnight at 30 °C. When a small amount of reaction mixture was analyzed by HPLC, all of the radioactivity eluted in two peaks at 6.6 min (shikimate 3-phosphate) and at 13.8 min (3-epishikimic acid) in the ratio 1:4, suggesting that [3-^{3}H]shikimic acid had been selectively and quantitatively phosphorylated. The mixture was purified on a column of DEAE-Sephacel (bicarbonate form, 15 × 2 cm) which had been preequilibrated with 5 mM TEAB at a flow rate of 1 mL min^{-1}. A linear gradient of 5 to 400 mM TEAB (150 mL + 150 mL) was applied, and 5-mL fractions were collected. The residual [3-^{3}H]epishikimic acid (**9**) eluted at 100 mM TEAB (fractions 12−18) and [3-^{3}H]-shikimate 3-phosphate eluted at 220 mM TEAB (fractions 31−36). The radioactivity in the eluant fell to background levels between these products.

The identity of [3-^{3}H]shikimate 3-phosphate was confirmed by its co-elution with a sample of unlabeled shikimate 3-phosphate by HPLC (retention time = 6.5 min). Concentration of the pooled fractions gave [3-^{3}H]shikimate 3-phosphate as the tris[triethylammonium] salt (50 mg, 0.090 mmol), which had a specific radioactivity of 14.48 mCi/mmol (19% radiochemical yield from a mixture of **8** and **9**). The salt was stored in methanol (5 mL) at −20 °C. [3-^{3}H]-Shikimate 3-phosphate (0.090 mmol, 14.48 mCi/mmol) was concentrated to dryness and then redissolved in 20 mM Tris-HCl buffer (pH 9.0, 4.5 mL) which contained $MgCl_2$ (2 mM). Alkaline phosphatase (Boehringer Mannheim, 2 units) was added, and the reaction was incubated overnight at 30 °C. Completion of dephosphorylation was verified by co-injection of a small sample of the reaction mixture (~0.1 mCi) with an unlabeled mixture of shikimic acid and shikimate 3-phosphate (<0.1 mg of each) onto an HPLC column. Most of the radioactivity (>95%) co-eluted with shikimic acid (retention time = 13.0 min). The reaction mixture was diluted to 10 mL with H_2O and loaded directly onto a column of DEAE-Sephacel (bicarbonate form, 10 × 2 cm), preequilibrated with 5 mM TEAB. After the column was washed with 5 mM TEAB (25 mL), a linear gradient of 5 to 500 mM TEAB (100 mL + 100 mL) was applied, and 5-mL fractions were collected. [3-^{3}H]Shikimic acid eluted at 150 mM TEAB (fractions 11−13). These fractions were pooled and concentrated to dryness *in vacuo* to give the triethylammonium salt of (**8**) (21 mg, 0.078 mmol, 87% chemical yield from shikimate 3-phosphate), which had a specific radioactivity of 14.23 mCi/mmol (85% radiochemical yield).

To confirm that the ^{3}H was in the 3-position of this shikimic acid sample, a small portion (~0.2 μCi) of **8** was first mixed with [^{14}C]shikimic acid to give a dual-labeled sample (**10**) with a ^{3}H:^{14}C ratio of 10.4 ± 0.1. Some of the dual-labeled shikimic acid (**10**, ^{3}H:^{14}C = 10.4 ± 0.1, 4 mM) was selectively oxidized to 3-dehydroshikimic acid by incubation with *E. coli* shikimate dehydrogenase (~3 units)

in the presence of $NADP^+$ (10 mM) and phosphate buffer (100 mM, pH 7.0). The resulting shikimic acid and 3-dehydroshikimic acid were separated by HPLC, and the ^3H:^{14}C ratios were determined. For the recovered shikimic acid the ^3H:^{14}C ratio was unchanged at ^3H:^{14}C = 10.5 ± 0.1, whereas the ratio in the recovered 3-dehydroshikimic acid had fallen to below 0.1 (^3H:^{14}C = 0.04 ± 0.03), due to specific removal of tritium by the enzyme. This confirmed that at least 99% of the tritium was in the required 3-position of shikimic acid. In addition all of the ^{14}C radioactivity for a given HPLC injection was recovered quantitatively in the two peaks collected, confirming that no detectable ^{14}C contaminants were present.

(B) 5-Enolpyruvyl[3-^3H,^{14}C]shikimate 3-Phosphate (**12**). [^{14}C]Shikimic acid (23.9 mCi, specific radioactivity [^{14}C] = 21.9 mCi/mmol, in ethanol/toluene [2:1] solution) was added to [3-^3H]shikimic acid (**8**, 133 mCi, specific radioactivity (^3H) = 14.23 mCi/mmol, in methanol) and gently vortexed. The solvents were removed to give a dual-labeled sample (**10**) with an approximate ^3H:^{14}C ratio of 6. A small sample containing ~2.5 mCi ^3H was removed and diluted with 40 mg of unlabeled shikimic acid. After four recrystallizations from hot methanol/dichloromethane, each in about 70% yield, the ^3H:^{14}C ratio was accurately determined to be 6.080 ± 0.008. The dual-labeled shikimic acid (**10**, 128 mCi, ^3H) was diluted with 11 mg (0.063 mmol) of unlabeled shikimic acid to give a dual-labelled sample with a calculated specific radioactivity of 1.78 mCi/mmol (^3H). The diluted sample was made up in Bis-Tris-HCl buffer (50 mM, pH 7.0) at 45 mM concentration (16 mL volume), in the presence of PEP (50 mM), ATP (50 mM), and $MgCl_2$ (2.5 mM). *E. coli* shikimate kinase (10 units) and EPSP synthase (2 units) were added, and the reaction was incubated for 3 h at 30 °C. Apyrase (3 units, Sigma grade VII) was added, and the reaction was left overnight at 30 °C. The reaction was stopped by heating at 100 °C for 5 min, and the precipitated protein was removed by centrifugation.

The reaction mixture was loaded onto a prepared column of DEAE-Sephacel (bicarbonate form, 10 × 2 cm), which had been preequilibrated with 4 mM TEAB at a flow rate of 1 mL min^{-1}. The column was washed with 4 mM TEAB (25 mL) and eluted with a linear gradient of 4 to 450 mM TEAB (100 mL + 100 mL), and 5-mL fractions were collected. Residual [3-^3H,^{14}C]shikimate (**10**) eluted in fractions 9−13 (125 mM TEAB), and [3-^3H,^{14}C]shikimate 3-phosphate (**11**), in fractions 19−23 (240 mM). AMP elutes before shikimate 3-phosphate at 220 mM TEAB and was detectable by its absorbance at 254 nm. [3-^3H,^{14}C]EPSP (**12**) eluted in fractions 30−35 (360 mM TEAB). These fractions were pooled and concentrated under vacuum to give the tetrakis[triethylammonium] salt of [3-^3H,^{14}C]EPSP (**12**, 35.2 mg, 48.3 mmol, 67% chemical yield). The specific radioactivity of the [3-^3H,^{14}C]EPSP was 1.86 mCi/mmol (^3H) (based on the mass). The specific radioactivity based on enzyme assay of a small sample of **12** was measured at 1.87 mCi/mmol (^3H). [3-^3H,^{14}C]EPSP was stored as the tetrakis-[triethylammonium] salt in dry methanol (at 10 mCi/mL, ^3H) at −20 °C and was stable for at least a month under these conditions.

To verify that the ^3H:^{14}C ratio was unchanged from that of the starting material, [3-^3H,^{14}C]shikimic acid (**10**), a small sample of **12** (~2 μCi ^3H) was degraded back to shikimic acid in two steps. The initial step was dephosphorylation by alkaline phosphatase (2 units) in Tris buffer (50 mM, pH 9.0), containing $MgCl_2$ (5 mM), at 30 °C, which was followed by acidification to ~pH 1 with concentrated hydrochloric acid and hydrolysis of the enolpyruvyl side chain at 100 °C for 10 min. The resulting labeled shikimic acid was directly HPLC purified and diluted with 40 mg of unlabeled shikimic acid, and the ^3H:^{14}C ratio was determined over four recrystallizations.

Methods

Ultraviolet measurements were made on a Perkin-Elmer Lambda 2 UV/visible spectrophotometer at 25 °C using 1-mL quartz cells. All HPLC described was carried out on a Bio-Rad Aminex HPX 87H organic acids semipreparative column (300 × 7.8 mm). The eluant used for HPLC was always 50 mM aqueous formic acid at a flow rate of 0.6 mL min^{-1}. HPLC was carried out using an LKB HPLC system. Radioactivity was assayed by scintillation counting in Pharmacia Hi-Safe II Optiphase scintillation fluid using a Canberra Packard 2000 CA Tri-Carb liquid scintillation analyzer. At the start of these ^3H/^{14}C dual-labeling studies fresh ^3H and ^{14}C quench calibration curves were prepared using a series of [^3H]- and [^{14}C]toluene standards (purchased from Canberra Packard) quenched with different amounts of nitromethane. It was verified by the manufacturers that these standards were compatible with the scintillation fluid used when counting samples. To verify the validity of quench curves, samples containing either ^3H or ^{14}C were counted in dual mode. The "spill-down" or "spill-up" of dpm values of ^3H or ^{14}C was typically ~0.2% or less. During the experiment the stability of the counter was periodically checked by counting a quenched standard or reevaluating the ^3H:^{14}C ratio of an "old" sample of [3-^3H,^{14}C]shikimic acid, which was always well within ±1% of the original ratio. The Auto Efficiency Control (AEC; see manufacturer's handbook for details) was used for all dual-label counting to ensure that counting efficiency was kept constant from sample to sample. Counting efficiencies were typically 0.42 (channel A) and 0.055 (channel B) for ^3H and 0.11 (channel A) and 0.84 (channel B) for ^{14}C. Recrystallized samples (shikimic acid or chorismic acid) were first air dried, and then 2 aliquots of each, typically containing ~0.5 mg of crystals (2−6 × 10^3 dpm ^3H), were removed and dissolved in 200 μL of H_2O and 10 mL of scintillation fluid. This was carried out for each of four recrystallizations. Each of the eight samples was counted for 3 cycles of 10 min. The analysis of the ^3H:^{14}C counting data was based on that used by Addadi *et al.* (1983) and by Guilford *et al.* (1987). For each crystallization, six ^3H:^{14}C values were obtained and the mean value ± standard error was calculated. A weighted mean was calculated provided that the mean value for a given crystallization fell within the standard deviation of the mean value for all counts over all four recrystallizations (i.e., all 24 values). Weighted mean values have been expressed with limits of ± standard deviation.

Measurement of Secondary Tritium Kinetic Isotope Effect. [3-^3H,^{14}C]EPSP (**12**, 7.5 mCi ^3H, specific activity ^3H = 1.86 mCi/mmol, ^3H:^{14}C = 6.084 ± 0.005) was converted to the sodium salt by passing through a small column of Dowex W-50 (5 × 1 cm, Na^+ form) directly into a buffered receiver containing 100 mM Bis-Tris-HCl (25 mL, pH 7.0). The solution was diluted to 50 mL with H_2O to give an approximately 80 μM solution of **12** in 50 mM Bis-Tris-

HCl (pH 7.0). This concentration and specific radioactivity of **12** in the solution was standardized by transformation of a small sample by chorismate synthase and counting for radioactivity. The specific radioactivity of **12** was in the range 1.865 ± 0.015 mCi/mmol (^{3}H) for all experimental runs, indicating that the sample of [3-^{3}H,^{14}C]EPSP (**12**) used was stable (to within ±1% error) over the course of experiments (within 4 weeks). The solution was made up to 20 μM with respect to NADPH and 10 μM with respect to FMN and kept in a foil-covered brown glass reaction vessel throughout. This precaution was due to indications of EPSP instability in the presence of light, NADPH, and FMN, from independent studies (not described).

The turnover of [3-^{3}H,^{14}C]EPSP (**12**) to [3-^{3}H,^{14}C]-chorismic acid was initiated by the addition of *N. crassa* chorismate synthase (~0.2 unit). Immediately after the reaction mixture was swirled, 2 × 1 mL aliquots were removed and monitored by UV spectrophotometry at 275 nm using a two-cell time-drive program. The two portions were frequently exchanged with the bulk reaction mixture during the reaction, which was carried out at 25 °C (room temperature). At the appropriate extent of conversion (usually 40−60%, which occurred after ~10 min) the bulk reaction mixture was quenched by pouring onto a bed of Dowex W-50 (10 g, H^+ form) on an ice bath. The fractional extent of conversion (*f*) was determined from the increase in absorbance at 275 nm at the time of the quench and verified by the complete conversion of portions monitored by UV. Both methods of evaluation were always within experimental error (±2%), and the average value of *f* was used in calculations. Unlabeled chorismic acid (5 mg) was added to the reaction mixture, and the [3-^{3}H,^{14}C]chorismic acid produced was extracted into ether (3 × 50 mL) with usually ~85−90% efficiency (by radioactivity assay). After drying over sodium sulfate, the extract was concentrated to dryness (bath temperature 20 °C). Unlabeled chorismic acid (35 mg) was added, the sample was recrystallized four times by the method described above, and the ^{3}H:^{14}C ratio was determined. On the second day the residual aqueous reaction mixture (which had been kept frozen at −20 °C overnight) containing [3-^{3}H,^{14}C]EPSP (**13**) was stirred with activated charcoal (~200 mg) for 5 min (to remove FMN and NADPH) and then filtered through a column of celite (3 × 1 cm) under reduced pressure; the column was finally washed with H_2O (25 mL). The filtrate was concentrated to ~10 mL under high vacuum (bath temperature 50 °C) and then heated at 100 °C for 10 min to hydrolyze the enolpyruvyl side chain. After cooling to room temperature, the solution was neutralized by the addition of 1 M TEAB (~1 mL, pH 7.8), and the pH was adjusted to ~pH 9 by the addition of 0.1 M NaOH (~1.5 mL). Alkaline phosphatase (2 units) was added, and the reaction mixture was incubated at 30 °C for 15 min. Completion of degradation was verified by HPLC (co-elution of radioactivity with unlabeled shikimic acid; retention time = 13.0 min). The solution was concentrated to dryness under high vacuum, and the residue was redissolved in 50 mM HCO_2H (1 mL). Unlabeled shikimic acid (5 mg) was added, and the [3-^{3}H,^{14}C]shikimic acid was purified by multiple injections onto an HPLC column (retention time = 13.0 min). The purified [3-^{3}H,^{14}C]-shikimic acid was concentrated to dryness, diluted with unlabeled shikimic acid (35 mg), and recrystallized four times from hot methanol/dichloromethane, and the ^{3}H:^{14}C ratio was determined.

Estimation of the Equilibrium Constant. To run the reaction to equilibrium, the reaction mixture was set up exactly as described above, but after addition of chorismate synthase (~0.2 unit) the reaction was left for an hour to proceed to completion, as determined by UV spectrophotometry. More chorismate synthase (~0.2 unit) was then added, and the reaction mixture was left for 3 h to ensure that isotopic equilibrium had been reached. After the usual workup procedure (Dowex-H^+) a sample of the reaction mixture (containing ~0.1 μCi ^{3}H) was injected onto an HPLC column together with unlabeled EPSP and chorismic acid (~0.1 mg of each). The chorismic acid was then extracted from the bulk mixture into twice the usual amount of ether (6 × 50 mL). The ^{3}H:^{14}C ratio of the chorismic acid was determined as described above.

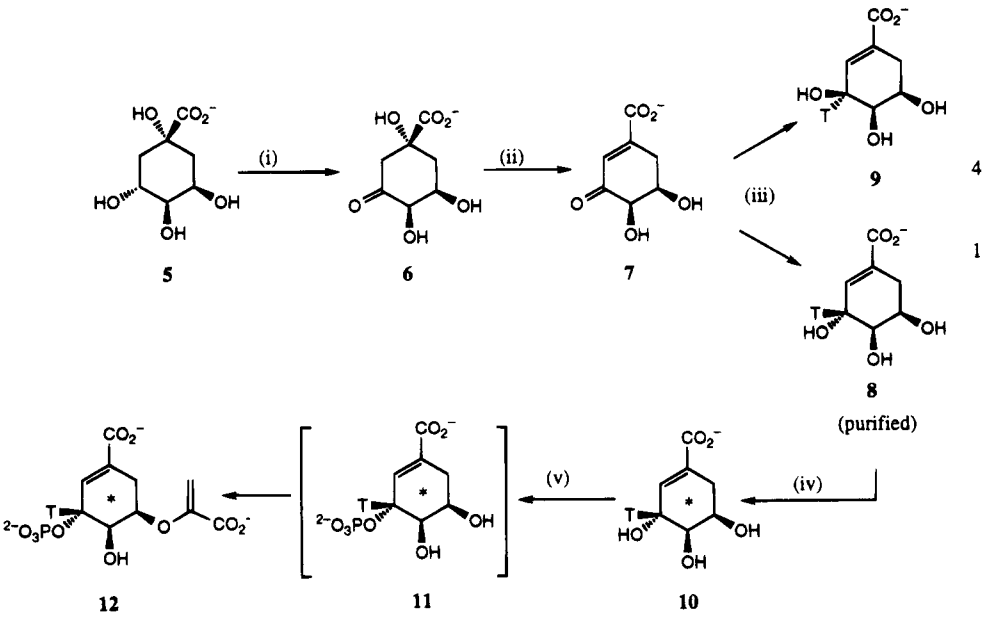
RESULTS

The dual-labeled substrate [3-^{3}H,^{14}C]EPSP (**12**) was synthesized using a combination of non-enzymatic steps together with reactions mediated by various enzymes of the shikimate pathway (Scheme 2). 3-Dehydroquinic acid (**6**) was prepared from quinic acid (**5**) by selective oxidation of the C(3) axial hydroxyl group using nitric acid (Grewe & Haworth, 1968). 3-Dehydroquinic acid (**6**) was then dehydrated to 3-dehydroshikimic acid (**7**) enzymically using dehydroquinase (3-dehydroquinate dehydratase, EC 4.2.1.10). The tritium label was introduced into the 3-position by sodium borotritiide reduction of 3-dehydroshikimic acid (**7**), which gave a 1:4 mixture of [3-^{3}H]shikimic acid (**8**) and 3-epi[3-^{3}H]shikimic acid (**9**) that was not easily separable. The contaminating (but major) epimer (**9**) was removed by selectively phosphorylating the [3-^{3}H]shikimic acid (**8**) using shikimate kinase to give [3-^{3}H]shikimate 3-phosphate. This was then easily separated from the 3-epi[3-^{3}H]shikimic acid (**9**) by ion-exchange chromatography. Finally, pure [3-^{3}H]-shikimate 3-phosphate was dephosphorylated by alkaline phosphatase to give uncontaminated **8**.

The position of the ^{3}H in **8** was confirmed by selective oxidation of a sample of dual-labeled shikimic acid (**10**). This was prepared by mixing together [3-^{3}H]shikimic acid (**8**) and [U-^{14}C]shikimic acid to give a ^{3}H:^{14}C ratio of 10.4 ± 0.1 (for this experiment only). The [3-^{3}H,U-^{14}C]shikimate (**10**) was used as a substrate for shikimate dehydrogenase. This enzyme selectively removes the C(3) hydrogen in an $NADP^+$-dependent oxidation to form dehydroshikimate (**7**). The ^{3}H:^{14}C ratio in the 3-dehydroshikimic acid formed was less than 0.1 (^{3}H:^{14}C = 0.04 ± 0.03), whereas the ^{3}H:^{14}C ratio in the residual shikimic acid was unchanged at 10.5 ± 0.1. This confirmed that more than 99% of the tritium was in the required 3-position of shikimic acid.

Dual-labeled shikimic acid (**10**) for the isotope experiments was prepared by mixing together [3-^{3}H]shikimic acid (**8**) and [U-^{14}C]shikimic acid to give a ^{3}H:^{14}C ratio of 6.080 ± 0.008, which was determined after dilution with unlabeled material and subsequent recrystallization to a constant ^{3}H:^{14}C ratio. The dual-labeled shikimic acid (**10**) was transformed to dual-labeled EPSP (**12**) by incubation with shikimate kinase and EPSP synthase in the presence of a slight excess of co-substrates ATP and PEP. The residual ATP and ADP were degraded to AMP and inorganic phosphate, with

Scheme 2[a]



[a] (i) 50% aqueous HNO_3, 0 °C, 2 days, 30%. (ii) Dehydroquinase (100 units), 30 °C. The transformation was carried out on 3-dehydroquinic acid (40 mM) in 100 mL of H_2O, pH 7.0 (potassium phosphate, 100 mM), and was followed at 234 nm. (iii) [^{3}H]$NaBH_4$, MeOH. [3-^{3}H]Shikimic acid was separated by ion-exchange chromotography on DEAE-Sephacel (eluant, 5 to 500 mM gradient of TEAB). (iv) Mixed with [^{14}C]shikimic acid. (v) Shikimate kinase (10 units) and EPSP synthase (2 units), 3 h, 30 °C. Transformations were carried out on [3-^{3}H,^{14}C]shikimic acid (45 mM) in 10 mL of H_2O, pH 7.0 (Bis-Tris-HCl, 50 mM), which contained ATP (50 mM), PEP (50 mM), and $MgCl_2$ (2.5 mM). *Denotes uniform ^{14}C labeling.

Table 1: Values of the ^{3}H:^{14}C Ratio in the Chorismate Synthase Catalyzed Conversion of [3-^{3}H,^{14}C]EPSP[a]

compound	crystallization	^{3}H:^{14}C	weighted mean
EPSP before reaction[b]	1	6.000 ± 0.017	6.084 ± 0.005
	2	6.036 ± 0.021	
	3	6.099 ± 0.012	
	4	6.084 ± 0.006	
EPSP remaining after reaction[b]	1	6.197 ± 0.010	6.288 ± 0.010
	2	6.261 ± 0.016	
	3	6.313 ± 0.015	
	4	6.283 ± 0.021	
chorismic acid product	1	5.863 ± 0.022	5.920 ± 0.009
	2	5.915 ± 0.027	
	3	5.941 ± 0.012	
	4	5.900 ± 0.025	

[a] These data relate to experiment 3 in Table 2. [b] Ratio determined by degradation to shikimic acid prior to recrystallization.

apyrase, to facilitate the purification of **12** by ion-exchange chromatography. A small portion of [3-^{3}H,^{14}C]EPSP (**12**) was degraded back to [3-^{3}H, ^{14}C]shikimic acid (**10**) by alkaline phosphatase followed by acid hydrolysis, and the ^{3}H:^{14}C ratio was found to be unchanged (^{3}H:^{14}C = 6.084 ± 0.005). The same batch of substrate [3-^{3}H,^{14}C]EPSP (**12**) was used for each experiment. The specific radioactivity, which was checked before each experiment, was consistently 1.865 ± 0.015 μCi (^{3}H)/μmol, confirming that the bulk stock of [3-^{3}H,^{14}C]EPSP was stable.

[3-^{3}H, ^{14}C]EPSP (**12**) was partially transformed to [3-^{3}H,^{14}C]-chorismate by *N. crassa* chorismate synthase. The extent of reaction was accurately monitored by UV spectrophotometry at 275 nm, and the reaction was stopped by rapid acidification (to ~pH 2 at 0 °C) at 40, 45, and 62% conversion, respectively, in three separate experiments. The chorismic acid product was directly extracted from the acidic mixture into ether, diluted with unlabeled material, and recrystallized to a constant ^{3}H:^{14}C ratio.

Analysis of the residual EPSP was less straightforward. The aromatic material (FMN, NADPH, and aromatized product) was first removed by charcoal treatment followed by filtration through celite. The dual-labelled EPSP was then degraded to shikimic acid. The recovered dual-labelled shikimic acid was HPLC purified, then diluted with unlabelled material and recrystallised to a constant ^{3}H:^{14}C ratio. Table 1 shows the data obtained for a representative experimental run in which the reaction was quenched at 45% conversion. The secondary kinetic isotope effects were determined from the relations (1) and (2) (Ropp, 1960):[2]

For product

$$k_H/k_T = \ln(1 - f)/\ln(1 - rf) \quad (1)$$

and for recovered starting material

$$k_H/k_T = \ln(1 - f)/[\ln[r'(1 - f)] \quad (2)$$

where k_H/k_T is the isotope effect, f is the fractional extent of reaction, r = (^{3}H:^{14}C ratio in recovered product)/(^{3}H:^{14}C ratio in substrate at start), and r' = (^{3}H:^{14}C ratio in recovered substrate)/(^{3}H:^{14}C ratio in substrate at start). The ratios r and r' were evaluated for three experiments in which the extent of reaction was varied from 40% to 62%, and the resulting isotope effects are presented in Table 2. The overall

[2] Since the distribution of ^{14}C is general throughout the shikimate skeleton, two of the labeled carbons [C(3) and C(6)] undergo a change in hybridization (sp^3 to sp^2) during the chorismate synthase reaction which may result in fractionation of ^{14}C. The ratio of any ^{14}C isotope effect to the ^{3}H isotope effect is likely to be less than 5%, and therefore fractionation of ^{14}C has been assumed to be negligible.

Table 2: Secondary Tritium *V/K* Isotope Effects on the Chorismate Synthase Catalyzed Transformation of [3-^{3}H,^{14}C]EPSP

	r'	r	extent of reaction (%)	k_H/k_T[a]
1	1.025 ± 0.002	0.970 ± 0.002	62 ± 2	1.039 ± 0.013
2	1.031 ± 0.002	0.966 ± 0.002	40 ± 2	1.055 ± 0.009
3	1.034 ± 0.002	0.973 ± 0.002	45 ± 2	1.048 ± 0.010
		overall k_H/k_T = 1.047 ± 0.012[b]		

[a] The values in this column are averages of those obtained from r and from r' for a given experiment. [b] This value is the mean overall experiments, and the standard deviation is quoted as the error margin.

value of k_H/k_T quoted (1.047) is the mean, and the overall error limit (±0.012) is the standard deviation of the six values obtained from the three experiments.

In a parallel control experiment the transformation of dual-labeled EPSP by chorismate synthase was allowed to run to equilibrium. Greater than 98% of the bulk radioactivity was extracted, suggesting that at least 98% chorismic acid was formed at equilibrium. This was confirmed by HPLC co-injection of some of the reaction mixture (before extraction) with unlabeled EPSP and chorismic acid. More than 99% of the radioactivity co-eluted with the chorismic acid peak. The apparent equilibrium constant for *N. crassa* chorismate synthase was on this basis estimated to have the *lower limit* $K_{eq} = 2.4 \times 10^{-3}$ M (from K_{eq} = {[chorismate][Pi]}/[EPSP]) under these conditions. The extracted chorismic acid was diluted with unlabeled material and recrystallized to a constant ^{3}H :^{14}C ratio, which was found to be unchanged (6.085 ± 0.006) from that of the substrate EPSP (^{3}H :^{14}C = 6.084 ± 0.005).

DISCUSSION

Using [3-^{3}H,^{14}C]EPSP as a substrate, a secondary kinetic isotope effect associated with rehybridization at C(3) has been detected in the chorismate synthase reaction. Three experiments were performed, and in each the magnitude of the kinetic isotope effect was calculated both from the ^{3}H:^{14}C ratio of the product, chorismic acid, and also from that of the unreacted starting material, EPSP. The six values give a k_H/k_T = 1.047 ± 0.012, which, although small, is statistically significant.

The small size of the *V/K* isotope effect measured in this experiment was not unexpected. The k_{cat}/K_m value of 1.5×10^7 M^{-1} s^{-1} for *N. crassa* chorismate synthase[3] is close to the diffusion-controlled association limit (Knowles & Albery, 1977), suggesting that formation of the E·S complex is partially rate limiting in *V/K*. This is also evident in the primary C(6) deuterium isotope effects, where the smaller $(V/K)^D$ = 1.6 ± 0.1, compared with V^D = 2.7 ± 0.2 (Balasubramanian *et al.*, 1990), is consistent with an enzyme reaction with a high commitment to catalysis (Northrop, 1975). The measured value of the primary C(6) deuterium isotope effect on V (V^D = 2.7 ± 0.2) would be consistent with a slow product release step if there were a high value for the intrinsic isotope effect at this center. It is interesting to note that chorismate mutase, the next enzyme on the shikimate pathway, also has a high commitment to catalysis and likewise catalyzes an irreversible reaction. However, for chorismate mutase it appears that substrate binding is sufficiently rate limiting in *V/K* to completely suppress the expression of *V/K* isotope effects (Addadi *et al.*, 1983).

For mechanistic steps involving an isotopically sensitive bond rehybridization, the relative magnitudes of the intrinsic kinetic isotope effect and the equilibrium isotope effect can be taken as a measure of the extent of rehybridization in the transition state. In principle intrinsic isotope effects may be determined through measurement of both deuterium and tritium isotope effects on *V/K* at the same center (Northrop, 1975). However, the small size of the measured tritium isotope effect at C(3) precludes an accurate experimental determination of the corresponding deuterium isotope effect. Our measured value of k_H/k_T = 1.047 ± 0.012 may be used as a lower limit for the value of the intrinsic kinetic isotope effect at C(3). An estimation of the equilibrium isotope effect at 25 °C, using tabulated values of deuterium fractionating factors (Hartshorn & Shiner, 1972) and the Swain−Schaad relationship (Swain *et al.*, 1958), gives a value of k_H/k_T = 1.44 at the C(3) center. The extent of C(3) bond rehybridization in the transition state therefore has a lower limit of 10% (assuming a linear relationship between isotope effect and bond order).

Despite the difficulty in assessing the significance of the size of the isotope effect at C(3) in mechanistic terms, the actual observation of an isotope effect is itself significant. The combination of a *V/K* isotope effect at C(6) and the absence of a secondary tritium isotope effect at C(3) would not be expected for a synchronous concerted mechanism. Since this is not the case, a concerted mechanism involving cleavage of the C(6)−H and C(3)−O bonds in the same transition state must remain a possibility. A stepwise mechanism is also possible, in which the bond-breaking steps occur in distinct but partially rate determining steps. In principle these two cases can be resolved by use of double isotope fractionation experiments;[4] however, this would be experimentally challenging for such a small effect at C(3). On the basis of our estimation of the apparent equilibrium constant in this paper, the overall enzyme-catalyzed transformation of EPSP to chorismate is effectively irreversible,[5] as is the transformation catalyzed by chorismate mutase (Addadi *et al.*, 1983). This has implications for the case of a stepwise mechanism, which are as follows: If a covalent catalytic step is assumed to be the irreversible step in the mechanism, the C(3) and C(6) *V/K* isotope effects require that it be the second of the two bond-cleavage steps, since a *V/K* isotope effect will not be expressed for events which follow an irreversible step. Following on from this, the first bond-breaking step must therefore be reversible.

We were unable to resolve the possibility of a stepwise mechanism from one that is strictly concerted on the basis of isotope effects. However, there is other good experimental

[3] Based on the values k_{cat} = 33 s^{-1} (White *et al.*,1988) and K_m(EPSP) = 2.2 μM (Balasubramanian *et al.*, 1990).

[4] For example, see Belasco *et al.* (1983).

[5] A result recently confirmed by additional experiments (M. Brown, unpublished results).

[6] These experiments were carried out on *E. coli* chorismate synthase; however, the formation of enzyme-bound flavin semiquinone radical has now been observed for *N. crassa* chorismate synthase under similar conditions (M. Brown, unpublished results). We have therefore assumed the role of the flavin to be general for both enzymes.

[7] A free radical mechanism was suggested by Bartlett and co-workers prior to the observation of a flavin semiquinone: see Bartlett *et al.* (1989).

evidence available to help resolve the two cases. While it has been known for some time that chorismate synthase requires a reduced flavin cofactor for catalytic activity (Welch *et al.*, 1974; Hasan & Nester, 1978; White, 1988), the role of the flavin in the chorismate synthase reaction had remained cryptic until recently. We now believe that flavin is actively involved in the reaction mechanism. This view is supported by the observation of a spectral change (λ_{max} = 400 nm) associated with the flavin during the catalytic reaction (Ramjee *et al.*, 1991), and furthermore, by the stoichiometric formation of an enzyme-bound flavin semiquinone radical, in the presence of the substrate analogue (6*R*)-6-fluoroEPSP (Ramjee *et al.*, 1992).[6] These experiments have led to proposals for the involvement of a covalent flavin adduct (Ramjee *et al.*, 1991) or radical intermediates (Ramjee *et al.*, 1992).[7] The interpretation of these observations necessitates that the chorismate synthase mechanism be stepwise rather than concerted.

In summary, we have shown the presence of a small but significant tritium *V/K* isotope effect at C(3) for the chorismate synthase reaction. This, taken together with the deuterium isotope effects measured at C(6), in addition to the spectral evidence for the formation of some flavin intermediate during the mechanism, has provided several new leads. The mechanism is likely to involve cleavage of the C(6)−H and C(3)−O bonds in *distinct* but partially rate determining steps. Owing to the overall reaction being irreversible, the former bond-cleavage step must be reversible, and therefore it is the latter bond-cleavage step that is presumed to be irreversible. The determination of intrinsic isotope effects at C(3) and C(6) would facilitate a more quantitative analysis of the observed isotope effects. However, it would be mechanistically more informative to next determine whether loss of the hydrogen at C(6) or of phosphate at C(3) is reversible, since this would reveal the order of bond breaking. Such information may be accessed through isotope-exchange experiments.

ACKNOWLEDGMENT

We thank John Greene for assistance in the purification of proteins and Dr. Dave Griffin for his interest in the project.

REFERENCES

Addadi, L., Jaffe, E. K., & Knowles, J. R. (1983) *Biochemistry 22*, 4494−4501.

Anh, N. T. (1968) *J. Chem. Soc., Chem. Commun.*, 1089−1090.

Anton, I. A., & Coggins, J. R. (1988) *Biochem. J. 249*, 319−326.

Balasubramanian, S., Abell, C., & Coggins, J. R. (1990) *J. Am. Chem. Soc. 112*, 8581−8583.

Balasubramanian, S., Davies, G. M., Coggins, J. R., & Abell, C. (1991a) *J. Am. Chem. Soc. 113*, 8945−8946.

Balasubramanian, S., & Abell, C. (1991b) *Tetrahedron Lett. 32*, 963−966.

Bartlett, P. A., Maitra, U., & Chouinard, P. M. (1986) *J. Am. Chem. Soc. 108*, 8068−8071.

Bartlett, P. A., McLaren, K. L., Aldberg, D. G., Fassler, A., Nyfeler, R., Lauhon, C. T., & Grissom, C. B. (1989) *Proceedings for the Society for Chemical Industry Pesticides Group Meeting* ; BCPC Monograph 42, pp 155−170, Lavenham Press, Lavenham, Suffolk, England.

Belasco, J. G., Albery, W. J., & Knowles, J. R. (1983) *J. Am. Chem. Soc. 105*, 2475−2477.

Duncan, K., Lewendon, A., & Coggins, J. R. (1984) *FEBS Lett. 170*, 59−63.

Duncan, K., Chaudhuri, S., Campbell, M. S., & Coggins, J. R. (1986) *Biochem. J. 238*, 475−483.

Fukui, K. (1965) *Tetrahedron Lett. 28*, 2427−2432.

Ganem, B. (1978) *Tetrahedron 34*, 3353−3383.

Gibson, F. (1968) *Biochem. Prep. 12*, 94−97.

Gibson, F. (1970) *Methods Enzymol. 17*, 362−364.

Grewe, R., & Haendler, H. (1968) *Biochem. Prep. 11*, 21−26.

Guilford, W. J., Copley, S. D., & Knowles, J. R. (1987) *J. Am. Chem. Soc. 109*, 5013−5019.

Hartshorn, S. R., & Shiner, V. J. (1972) *J. Am. Chem. Soc. 94*, 9002−9012.

Hasan, N., & Nester, E. W. (1978) *J. Biol. Chem. 253*, 4999−5004.

Haslam, E., Haworth, R. D., & Knowles, P. F. (1963) *Methods Enzymol. 6*, 498−501.

Hawkes, T. R., Lewis, T., Coggins, J. R., Mousdale, D. M., Lowe, D. L., & Thorneley, R. N. F. (1990) *Biochem. J. 265*, 899−902.

Hill, R. K., & Newkome, G. R. (1969) *J. Am. Chem. Soc. 91*, 5893−5894.

Hill, R. K., & Bock, M. G. (1978) *J. Am. Chem. Soc. 100*, 637−639.

Knowles, J. R., & Albery, W. J. (1977) *Acc. Chem Res. 10*, 105−111.

Millar, G., Lewendon, A., Hunter, M. G., & Coggins, J. R. (1986) *Biochem. J. 237*, 427−437.

Moss, B. J., & Rickbourne, B. (1986) *J. Org. Chem. 51*, 1992−1996.

Northrop, D. B. (1975) *Biochemistry 14*, 2644−2651.

Olwegard, M., & Ahlberg, P. (1990) *Acta Chem. Scand. 44*, 642−644.

Onderka, D. K., & Floss, H. G. (1969) *J. Am. Chem. Soc. 91*, 5894−5896.

Onderka, D. K., Floss, H. G., & Carroll, M. (1972) *J. Biol. Chem. 247*, 736−744.

Ramjee, M. N., Coggins, J. R., Hawkes, T. R., Lowe, D. J., & Thorneley, R. N. F. (1991) *J. Am. Chem. Soc. 113*, 8566−8567.

Ramjee, M. N., Balasubramanian, S., Abell, C., Coggins, J. R., Davies, G. M., Hawkes, T. R., Lowe, D. J., & Thorneley, R. N. F. (1992) *J. Am. Chem. Soc. 114*, 3151−3153.

Ropp, G. A. (1960) *J. Am. Chem. Soc. 82*, 842−852.

Swain, C. G., Stivers, E. C., Reuwer, J. F., & Schaad, L. J. (1958) *J. Am. Chem. Soc. 80*, 5885−5893.

Toromanoff, E. C. R. (1980) *Sceances Acad. Sci. Ser. C. 290*, 81−84.

Welch, G. R., Cole, K. W., & Gaertner, F. H. (1974) *Arch. Biochem. Biophys. 282*, 437−442.

White, P. J., Millar, G., & Coggins, J. R. (1988) *Biochem. J. 251*, 313−322.

White, P. J. (1988) Ph.D. Thesis, University of Glasgow, U.K.

Wibbenmeyer, J., Brundage, L., Padgette, S. R., Likose, J. J., & Kishore, G. M. (1988) *Biochem. Biophys. Res. Commun. 153*, 1760−1766.