Secondary Tritium Isotope Effects as Probes of the Enzymic and Nonenzymic Conversion of Chorismate to Prephenate†

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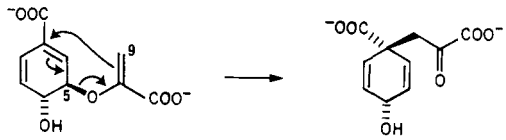
ABSTRACT: To obtain information about the degree of concert of both the nonenzymic and the enzyme-catalyzed rearrangement of chorismate to prephenate, we have determined the secondary tritium isotope effects at the bond-making position (C-9) and the bond-breaking position (C-5) of chorismate. The isotope effects were determined by the competitive method, using either [5-^{3}H,7-^{14}C]chorismate or [9-^{3}H,7-^{14}C]chorismate as the substrate. In the nonenzymic reaction (pH 7.5, 60 °C), k_H/k_T is 1.149 ± 0.012 for bond breaking (C-9) and 0.992 ± 0.012 for bond making (C-5). This indicates an asymmetric transition state in which the new bond is hardly, if at all, formed, while the bond between C-5 and oxygen is substantially broken. In the enzymic reaction (pH 7.5, 30 °C), the values of k_H/k_T in both positions are unity within experimental error. It is most likely that the isotope effects are suppressed in the enzymic process and that the rate-limiting transition state occurs *before* the rearrangement itself. The kinetically significant transition state presumably involves *either* the binding step of the small equilibrium proportion of the axial conformer of the substrate *or* an isomerization of enzyme-bound chorismate from the more stable conformer in which the carboxyvinyloxy group is equatorial to that in which this group is axial. Rearrangement would then proceed relatively rapidly from the higher energy axial conformer.

The enzyme chorismate mutase catalyzes the conversion of chorismate (I) to prephenate (II) at the branch point of the metabolic pathway for the biosynthesis of the aromatic amino



I II

acids tyrosine, phenylalanine, and tryptophan. The reaction, which is formally a Claisen rearrangement, is particularly interesting, being one of a very small group of possible pericyclic processes in intermediary metabolism. The 3,3-sigmatropic rearrangement also proceeds thermally, and it has been calculated (Andrews et al., 1973) that the uncatalyzed reaction is accelerated by a factor of more than 10^6 both by the chorismate mutase from *Streptomyces aureofaciens* (Görisch, 1978) and by the bifunctional enzyme chorismate mutase–prephenate dehydrogenase from *Aerobacter aerogenes* (Andrews et al., 1973). From the known activation parameters for the uncatalyzed reaction, it is clear that the enzyme is more than just an entropy trap (Westheimer, 1962) since the value of $\Delta S^{\ddagger}$ for the uncatalyzed process is equivalent to a factor of only 10^3 in the reaction rate (Andrews et al., 1973). It has been proposed, however, that the rate enhancement may derive from (i) enzymic stabilization of the less stable ring conformation of chorismate in which the carboxyvinyloxy group is axial (Edwards & Jackman, 1965) [this has been calculated to be 7 kcal/mol less stable than the equatorial conformer (Andrews et al., 1973)] and (ii) the removal of the remaining rotational degrees of freedom of the axial conformer in the enzyme–substrate complex (Andrews et al., 1973). Other factors, such as are known to affect the rates of pericyclic reactions (Evans & Golob, 1975; Gajewski, 1980; Burrows & Carpenter, 1982), cannot, however, be excluded. Some effort has been made to define the geometry of the transition state for the enzyme-catalyzed transformation by using substrate analogues designed to mimic the transition state (Andrews et al., 1977; Görisch, 1978), but no direct evidence is yet available either on the stereochemistry of the rearrangement or on the symmetry of the transition state in terms of bond making and bond breaking.

As part of an investigation of the mechanism of the chorismate mutase reaction, we have asked the question of the extent to which the reaction is concerted. For an uncatalyzed pericyclic reaction, there are three approaches to the problem of whether the process is concerted or stepwise. First, the stereochemical course of the reaction may be inconsistent with the predictions of the rules of orbital symmetry for concerted processes (Woodward & Hoffmann, 1970). This criterion cannot be used profitably for chorismate mutase: enzymic reactions are universally stereospecific, and both the possible stereospecific paths of the rearrangement of chorismate to prephenate (via a chair or a boat transition state) are "allowed" transformations. Second, the observed activation energy may be compared with that calculated for a stepwise reaction via a biradical intermediate, and a low experimental value may suggest some degree of concert. This approach is obviously unhelpful for a catalyzed reaction. Third, the degree of bond formation and cleavage at the transition state may be probed for the reaction centers undergoing a change in hybridization by studying the secondary hydrogen isotope effects at these centers. This is, in principle at least, applicable to an enzyme-catalyzed process. In the present case, C-9 of chorismate has two attached hydrogens and in the course of the rearrangement changes its hybridization from sp^2 to sp^3. Deuterium or tritium substitution at C-9 should therefore accelerate the rearrangement if the new bond is partially formed at the transition state. Conversely, C-5 has one attached hydrogen and in the course of the rearrangement changes its hybridization from sp^3 to sp^2. 5-Deuterio- or 5-tritio-labeled chorismate should therefore rearrange more slowly than the unlabeled molecule if the bond between C-5 and oxygen is

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partially broken at the transition state. (We shall defer, for the moment, consideration of the question of allylic delocalization.) Both bond-forming and bond-cleaving processes can therefore in principle be probed, the magnitude of the effect on the reaction rate providing a measure of the extent to which the carbon center has changed its hybridization at the transition state.

For enzyme-catalyzed reactions, however, attempts to observe kinetic isotope effects may be vitiated because (for primary effects) the transition state(s) involving the isotope may be kinetically insignificant, or (for secondary effects) the rate-limiting transition state may occur before the step(s) in which atom rehybridization takes place (Northrop, 1975). In such cases, one learns about the relative kinetic significance of different steps in the overall catalyzed reaction. A comparison of the magnitudes of the isotope effects in the enzymic and nonenzymic reactions therefore provides information about the mechanism of catalysis.

We report here the secondary tritium isotope effects determined by the competitive method with ^{14}C-labeled material, for the rearrangement both of [5-^{3}H]chorismate and of [9-^{3}H]chorismate, in the uncatalyzed thermal reaction and in the rearrangement catalyzed by chorismate mutase–prephenate dehydrogenase from *Escherichia coli*.

Experimental Procedures

Materials

$NaH^{14}CO_3$ (52 mCi/mmol), D-[3-^{3}H]glucose (12.3 Ci/mmol), and ^3H_2O (5 Ci/mL) were purchased from Amersham (Chicago, IL). DEAE-cellulose (DE-52) was obtained from Whatman (Clifton, NJ), AG1-8X (Cl^- form, 200–400 mesh) and Chelex 100 (Na^+ form, 200–400 mesh) were from Bio-Rad (Richmond, CA), silica gel preparative thin-layer chromatography plates (0.5 mm, 20 cm × 20 cm) were from E. Merck (Darmstadt, Germany), cellulose plates (0.16 mm, 20 cm × 20 cm) were from Eastman Kodak (Rochester, NY), and ribulose 1,5-bisphosphate, shikimic acid, phenyllactic acid, and phosphoenolpyruvate were from Sigma (St. Louis, MO).

Shikimate 3-phosphate was prepared by Dr. C. E. Grimshaw from fermentation of *Aerobacter aerogenes* A-170-40 (Knowles & Sprinson, 1970), a mutant deficient in 5-enolpyruvoylshikimate-3-phosphate synthetase. Unlabeled 5-enolpyruvoylshikimate 3-phosphate was prepared by the method reported earlier (Grimshaw et al., 1982). Unlabeled chorismic acid was isolated from the fermentation of *Klebsiella pneumoniae* 62-1 (formerly *Aerobacter aerogenes* 62-1; Gibson & Gibson, 1964) by modification of the method of Gibson (1968). Following ether extraction of the pooled column eluates, the chorismic acid solution was treated with animal charcoal and then filtered, after partial evaporation of the solvent under reduced pressure. Two recrystallizations (see below) from ether–methylene chloride–hexane (1:1:2, by volume) gave crystalline chorismic acid (700 mg from 2 L of culture). The material was characterized by ^{1}H NMR (Edwards & Jackman, 1965), by thin-layer chromatography [silica gel GHLF plates from Analtech (Newark, DE) eluted with toluene–acetic acid (12:7 v/v); R_f 0.3], and by enzymic assay using chorismate mutase (see below). Chorismic acid was stored as a dry solid at −20 °C and was subjected to charcoal treatment and recrystallization before every isotope effect experiment.

Phosphoglycerate kinase (rabbit muscle), glyceraldehyde-phosphate dehydrogenase (rabbit muscle), glycerophosphate dehydrogenase (rabbit muscle), enolase (yeast), phosphoglycerate mutase (rabbit muscle), hexokinase (yeast), transaldolase (yeast), pyruvate kinase (rabbit muscle), lysozyme (hen egg white), and glucose-6-phosphate dehydrogenase were purchased from Sigma. Ribulose-1,5-bisphosphate carboxylase from *Rhodospirillum rubrum* was purified to homogeneity by Dr. J. Sue following the method of Kornberg & Lascelles (1960). Crystalline triosephosphate isomerase (chicken muscle) was prepared by Dr. J. Belasco according to Putman et al. (1972) and McVittie et al. (1972). 5-Enolpyruvoylshikimate-3-phosphate synthetase was partially purified from *K. pneumoniae* 62-1 by Dr. C. E. Grimshaw and S. G. Sogo and had a specific activity of 1.5 units/mg. Cell extract from *E. coli* 83-24 (Davis 1951) was prepared following the method of Floss et al. (1972).

For the conversion of 5-enolpyruvoylshikimate 3-phosphate to chorismate, a cell extract from *K. pneumoniae* 62-1 was prepared as follows. Cells were grown as for the production of unlabeled chorismic acid and washed with 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8.0, containing ethylenediaminetetraacetic acid (EDTA) (1 mM) and dithioerythritol (1 mM). The washed cells were resuspended in the same buffer (1 g wet weight/4 mL of buffer) and broken in French press at 20000 psi. Cell debris was removed by centrifugation (30 min, 37000*g*) and the supernatant was dialyzed overnight against 2 L of the above buffer. The protein concentration was 20 mg/mL. The extract was flash frozen in liquid N_2 and stored at −70 °C, under which conditions the activity was maintained for 3–4 months.

For the conversion of shikimate to chorismate, *K. pneumoniae* 62-1 (5 g, grown and washed as described above) was suspended in 0.5 M sucrose solution (15 mL), containing 50 mM Tris-HCl buffer, pH 8.0, dithioerythritol (1 mM), and *p*-toluenesulfonyl fluoride (0.1 mM). A solution of lysozyme (17 mg) in 0.1 M EDTA (1.7 mL), pH 8.0, was added, and the suspension was gently agitated at 0 °C for 30 min. The resulting spheroplasts were collected by centrifugation (20 min, 2000*g*), washed with 25 mM Tris-HCl buffer, pH 8.0, containing EDTA (1 mM) and dithioerythritol (1 mM), and then broken with vigorous stirring (5 min) in the same buffer (20 mL). The lysate was centrifuged (40 min, 37000*g*), and the supernatant was stored at −70 °C.

Chorismate mutase–prephenate dehydrogenase was isolated from *E. coli* JFM 30. This strain contains a plasmid that encodes the enzyme, and at least 8% of the total extractable protein is the mutase–dehydrogenase. The enzyme was partially purified as follows (Sampathkumar & Morrison, 1982a). Nucleic acid was precipitated from the cell lysate by treatment with $MnCl_2$, and manganese ion was removed from the supernatant by treatment with Chelex 100. The material was fractionated by ammonium sulfate precipitation (40–53% saturated) and then subjected to ion-exchange chromatography on DEAE-cellulose in 100 mM *N*-ethylmorpholine hydrochloride buffer, pH 7.0, containing EDTA (1 mM), dithioerythritol (1 mM), sodium citrate (1 mM), and glycerol (10% v/v), eluting with a linear gradient of KCl (0–0.5 M). The specific activity of the enzyme sample was 16 units/mg. The enzyme was stored in 0.1 M *N*-ethylmorpholine hydrochloride buffer, pH 7.0, containing EDTA (1 mM), dithioerythritol (1 mM), sodium citrate (1 mM), and glycerol (10% v/v) at −70 °C. The enzyme was stable under these conditions for several months without any loss of activity.

Enzyme Assays. Chorismate mutase was assayed at 30 °C in reaction mixtures containing 50 mM *N*-ethylmorpholine–4-morpholineethanesulfonate buffer, pH 7.5, containing EDTA (1 mM), dithioerythritol, (1 mM), bovine serum albumin (0.1 mg/mL), and chorismate (0.4 mM) in a total volume of 3 mL. Chorismate consumption was followed spectrophotometrically

at its λ_{max} (275 nm, ϵ = 2630 M^{-1} cm^{-1}). Divided cuvettes were used to balance the protein absorbance at 275 nm. One unit of enzyme activity is defined as the amount of enzyme that will convert 1 μmol of chorismate to prephenate in 1 min. The same conditions were used to assay the chorismate content of solutions, using 0.3 unit of enzyme in a total volume of 3 mL.

Ribulosebisphosphate carboxylase activity was determined by the method of Racker (1962). One unit of enzyme is defined as the amount that will convert 1 μmol of ribulose 1,5-bisphosphate to 2 μmol of 3-phosphoglycerate in 1 min at 30 °C.

5-Enolpyruvoylshikimate-3-phosphate synthetase activity and 5-enolpyruvoylshikimate 3-phosphate were assayed as described by Grimshaw et al. (1982).

Glucose 6-phosphate was determined by spectrometric measurement of NADPH using glucose-6-phosphate dehydrogenase in solutions of 0.1 M triethanolamine bicarbonate buffer, pH 7.6, containing $MgCl_2$ (5 mM) and $NADP^+$ (0.1 mg/mL).

Erythrose 4-phosphate was determined by the transaldolase assay of Racker (1974).

Synthesis of Labeled Substrates. (*A*) [7-^{14}C]*Chorismic Acid.* Ribulose-1,5-bisphosphate carboxylase (1 mL, 3.5 units) was dialyzed overnight against 2 L of carbonate-free 100 mM triethanolamine hydrochloride buffer, pH 7.8, containing $MgCl_2$ (20 mM), EDTA (4 mM), and dithioerythritol (1 mM). The recovered activity was 1.5 units/mL. To the enzyme (1 unit) in the above buffer (3 mL) were added fresh ribulose 1,5-bisphosphate (30 μmol) and $NaH^{14}CO_3$ (1 mCi, 19 μmol). This solution was kept for 3 h at 30 °C under argon and then acidified with 2 N HCl (0.5 mL). The residual $^{14}CO_2$ was collected in an N_2 stream in 1 N NaOH. After the pH of the reaction solution was adjusted to 7.5, the product, 3-phospho[1-^{14}C]glycerate, was purified by chromatography on a column (7 mL) of AG1-8X equilibrated with 0.16 mM HCl and eluted with a gradient (70 mL + 70 mL) of 0.16–100 mM HCl. The phosphoglycerate eluted at 60 mM HCl and was isolated in 96% yield with a specific radioactivity of 24.6 mCi/mmol.

3-Phospho-D-[1-^{14}C]glycerate was converted in situ to phosphoenolpyruvate and coupled to shikimate phosphate in an incubation comprising 20 mM triethanolamine hydrochloride buffer, pH 7.7 (4 mL), containing $MgSO_4$ (6 mM), EDTA (1 mM), shikimate 3-phosphate (5 mM), 2,3-bisphosphoglycerate (10 μM), 3-phospho-D-[1-^{14}C]glycerate (1.25 mM, 24.6 mCi/mmol), enolase (0.5 unit), phosphoglycerate mutase (5 units), and 5-enolpyruvoylshikimate-3-phosphate synthetase (0.6 unit). The production of 5-enolpyruvoylshikimate 3-phosphate was monitored at 240 nm and leveled off after 75 min. At this point, the reaction was stopped by boiling the solution for 2 min, and the cooled solution was applied to a column (7 mL) of DEAE-cellulose equilibrated with 20 mM NH_4HCO_3 and eluted with a linear gradient (50 mL + 50 mL) of 20–400 mM NH_4HCO_3. Phosphoenolpyruvate eluted at 215 mM, shikimate 3-phosphate at 225 mM, and the product, 5-[7-^{14}C]enolpyruvoylshikimate 3-phosphate (80% yield, 24.2 mCi/mol), at 315 mM NH_4HCO_3.

5-[7-^{14}C]Enolpyruvoylshikimate 3-phosphate was converted to [7-^{14}C]chorismate by adaption of the method of Morell et al. (1967). The reaction mixture comprised 100 mM Tris-HCl buffer, pH 8.0 (5 mL), containing EDTA (0.4 mM), dithioerythritol (10 mM), KF (10 mM), $MgSO_4$ (10 mM), NADH (10 μmol), FAD^+ (5 μmol), 5-[7-^{14}C]enolpyruvoylshikimate 3-phosphate (5 μmol, appropriately diluted with unlabeled material), and a cell-free extract or spheroplast lysate (1 mL) from *K. pneumoniae* 62-1. The mixture was kept at 37 °C in the absence of O_2 for 75 min, the optimal incubation time being determined for each batch of cell extract from the time at which the yield of chorismate was maximal. The reaction was stopped by acidification, the precipitated protein was removed after 15 min by centrifugation at 0 °C, and the resulting supernatant was extracted into ether (5 × 6 mL) at 4 °C. More than 70% of the ^{14}C was extracted into ether. The pooled etheral solution was evaporated to about 3 mL, and unlabeled chorismic acid (30–70 mg) was added. The solution was filtered through Celite and animal charcoal, and the solution was further reduced by evaporation to 1 mL. The addition of cold methylene chloride (~1 mL) caused the chorismic acid to crystallize. When necessary, a small quantity (<0.1 mg) of seed crystals of unlabeled chorismic acid was added. After nucleation, cold hexane (~2 mL) was added slowly, and the solution was then left at −20 °C. The yield of crystalline chorismic acid was 50–60% based on 5-enolpyruvoylshikimate 3-phosphate. This recrystallization procedure was used in all experiments involving chorismic acid.

(*B*) [9-^{3}*H*]*Chorismic acid* was synthesized as follows. The reaction mixture (1 mL) comprised 100 mM potassium succinate buffer, pH 6.25, containing KF (50 mM), K_2HPO_4 (1 mM), bovine serum albumin (0.1 mg/mL), phosphoenolpyruvate (5 μmol), shikimate 3-phosphate (5 μmol), ^3H_2O (1 Ci), and 5-enolpyruvoylshikimate-3-phosphate synthetase (1 unit). The solution was incubated at 30 °C for 6 h, and the reaction was terminated by heating to 70 °C for 10 min. The tritiated water was removed by repeated bulb to bulb distillation, and the product, 5-[9-^{3}H]enolpyruvoylshikimate 3-phosphate (80% yield, 13.2 μCi/μmol), was purified as described above. This material was converted into [9-^{3}H]-chorismate as described above for the 7-^{14}C-labeled material.

(*C*) [5-^{3}*H*]*Chorismic Acid.* D-[3-^{3}H]Glucose 6-phosphate was prepared by incubating D-[3-^{3}H]glucose (0.64 mCi, 20 μmol) in 100 mM triethylammonium bicarbonate buffer, pH 7.6 (4 mL), containing KCl (0.1 M), $MgSO_4$ (10 mM), ATP (2 μmol), phosphoenolpyruvate (20 μmol), pyruvate kinase (2 units), and hexokinase (1 unit) for 2 h at 30 °C. The product was purified on a column (20 mL) of DEAE-cellulose equilibrated with 5 mM triethylammonium bicarbonate, pH 7.6, and eluted with a linear gradient (200 mL + 200 mL) of 5–200 mM triethylammonium bicarbonate. [3-^{3}H]Glucose 6-phosphate eluted at 85 mM buffer (90% yield, 30 mCi/mmol), and 10 μmol was subjected to lead tetraacetate oxidation to [1-^{3}H]erythrose 4-phosphate according to the procedure of Sieben et al. (1966). The product mixture, after evaporation of the acetic acid, was dissolved in 100 mM triethylammonium bicarbonate, pH 7.6 (3 mL), containing $MgSO_4$ (5 mM) and $NADP^+$ (4 μmol), and any residual glucose 6-phosphate was oxidized by the addition of glucose-6-phosphate dehydrogenase (0.5 unit) and incubation at 30 °C until the A_{340nm} was constant. The mixture was then applied to a column (20 mL) of DEAE-cellulose equilibrated with 5 mM triethylammonium bicarbonate, pH 7.6, and eluted with a linear gradient (200 mL + 200 mL; 5–250 mM) of the same buffer. D-[1-^{3}H]Erythrose 4-phosphate eluted at 110 mM buffer and was isolated in 66% yield. It was stored at room temperature to minimize dimerization (Blackmore et al., 1976).

D-[1-^{3}H]Erythrose 4-phosphate (6.5 μmol) was incubated in 40 mM potassium phosphate buffer, pH 7.6 (7 mL), containing $MgSO_4$ (4 mM), $CoCl_2$ (20 μM), NAD^+ (6 μmol), phosphoenolpyruvate (20 μmol), and aged cell extract (1 mL,

5 mg of protein) from *E. coli* 83-24. After 1 h at 37 °C, NADPH (15 μmol) and a further portion of cell extract (0.5 mL) was added, and the incubation was monitored at 340 nm for NADPH consumption (Floss et al., 1972). The product, [5-^{3}H]shikimate, was purified on cellulose preparative thin-layer plates, eluting with 7:3 v/v 2-propanol:30% NH_4OH. Shikimate has an R_f of 0.35 in this system. The product was extracted from the plate into 50 mM triethylammonium bicarbonate buffer in 20% yield. Repeated cocrystallization of a sample with unlabeled shikimic acid showed no loss of radioactivity and confirmed the identity of the product.

[5-^{3}H]Shikimate (~5 μmol) was incubated in 100 mM Tris-HCl buffer, pH 8.0 (5 mL), containing EDTA (0.4 mM), dithioerythritol (10 mM), $MgSO_4$ (10 mM), KCl (50 mM), NADH (10 μmol), FAD^+ (5 μmol), ATP (2.5 μmol), phosphoenolpyruvate (17.5 μmol), pyruvate kinase (2 units), and spheroplast lysate (1 mL) from *K. pneumoniae* 62-1 at 37 °C in the absence of O_2 for 50–75 min (the optimum time being determined as described above). The product, [5-^{3}H]chorismate, was isolated and purified as described above for [7-^{14}C]chorismate.

To check that the ^{3}H label was in the 5-position in this chorismate sample, a portion of [7-^{14}C, 5-^{3}H]chorismate (20 mg; ~1 μCi/mmol of ^{3}H; ^{3}H:^{14}C ratio of 10.4) was dissolved in dry pyridine (200 μL), and acetic anhydride (200 μL) was added (Ife et al., 1976). After 20 min at room temperature in the absence of O_2, the acetic acid was removed under vacuum with added toluene, water (500 μL) was added, and the product was extracted into ether. The product, 3-[(1′-carboxyvinyl)oxy]benzoic acid, was purified by preparative thin-layer chromatography on silica gel [eluting with 1:6 (v/v) methanol:methylene chloride, R_f 0.4] and had a ^{3}H:^{14}C ratio of 0.7. At least 93% of the ^{3}H label in the [5-^{3}H]chorismate was therefore in the 5-position.

Methods

Ultraviolet measurements were made on a Perkin-Elmer 554 spectrophotometer with an adjustable temperature control. Scintillation counting was performed on a Beckman LS 233 instrument. Scintillation cocktails were made up with scintillation-grade toluene (Fisher, Rochester, NY), Biosolve (Beckman, Fullerton, CA), and Liquifluor (New England Nuclear, Boston, MA) (85:10:5, by volume). Counting efficiencies were determined as a function of the "external standard ratio": calibration curves were periodically checked by using standard [^{3}H]- and [^{14}C]hexadecane samples quenched with varying amounts of acetone. Typical counting efficiencies were 0.39 (channel 1) and <0.001 (channel 2) for ^{3}H, and 0.2 (channel 1) and 0.62 (channel 2) for ^{14}C. Counting efficiencies were constant from sample to sample. Samples, typically containing (1–3) × 10^4 dpm of ^{3}H, were dissolved in water (200 μL) and scintillation cocktail (12 mL) and were counted for 3 × 10 min each; dpm values were calculated for ^{3}H and for ^{14}C from the average of the counts for the three counting cycles. Three separate samples were taken from each crystallization, and the mean of the ^{3}H:^{14}C ratio was calculated. A weighted mean of the ^{3}H:^{14}C ratios was then calculated for the different recrystallizations, provided that the average value for a given recrystallization fell within the standard error of the mean value for all recrystallizations.

Kinetic Isotope Effect Measurements. For each experiment, samples of doubly labeled [5-^{3}H, 7-^{14}C]- or [9-^{3}H, 7-^{14}C]-chorismate were prepared. The latter was produced by mixing the singly labeled 5-enolpyruvoylshikimate 3-phosphate precursors and converting these together, by using the spheroplast lysate from *K. pneumoniae* 62-1, into chorismate. The former was made by mixing [5-^{3}H]shikimate with 5-[7-^{14}C]enolpyruvoylshikimate 3-phosphate and converting these together (with the same enzyme extract) into chorismate. The ratio of ^{3}H:^{14}C was routinely between 5 and 7. The doubly labeled chorismic acid was diluted with unlabeled material (~70 mg) and recrystallized as described above, 3 × ~0.2 mg of crystals (containing ~10^4 cpm of ^{3}H) being removed (after filtration and air-drying) for scintillation counting. The bulk was immediately recrystallized. Four recrystallizations (each in 75–80% recovered yield) were normally performed, leaving ~30 mg for the rearrangement reaction.

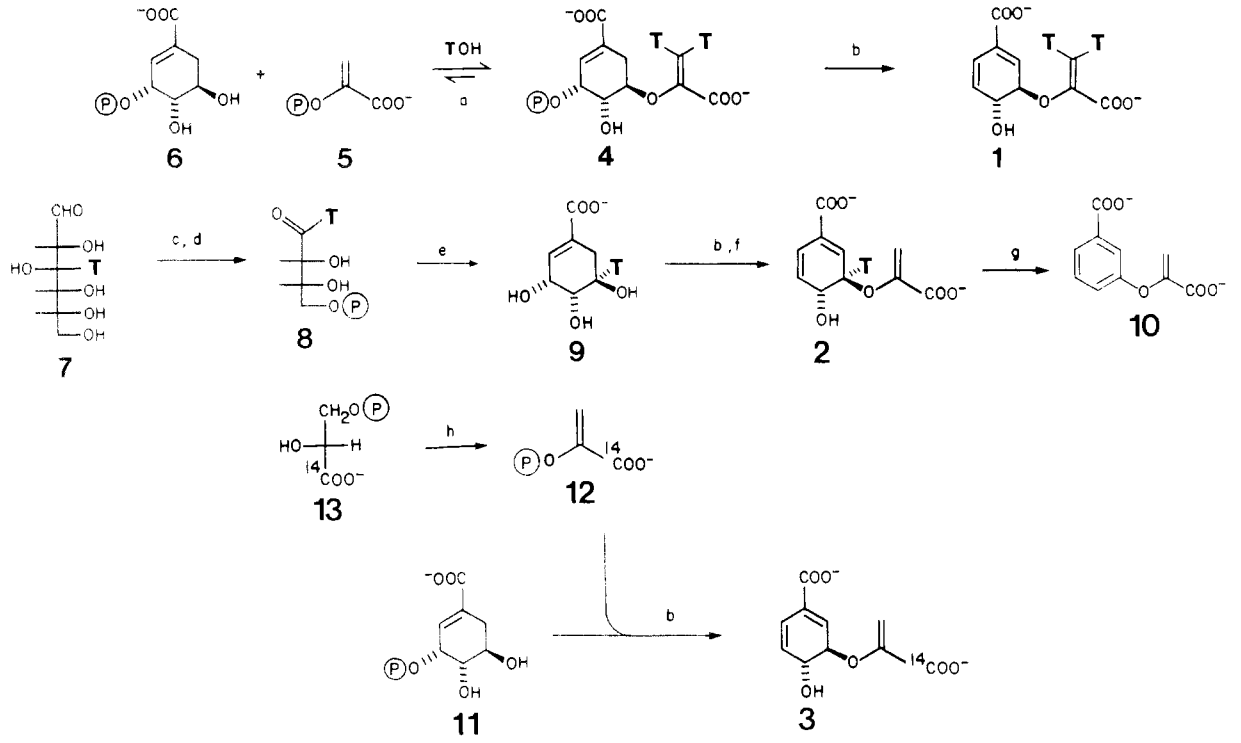
For the *chorismate mutase catalyzed reaction*, the crystalline chorismic acid was dissolved in 50 mM *N*-ethylmorpholine–morpholineethansulfonate buffer, pH 7.5, containing EDTA (1 mM), dithioerythritol (1 mM), and bovine serum albumin (0.1 mg/mL) at 30 °C to make a 4 mM chorismate solution. An appropriate amount of enzyme (~3 units) was then added to effect ~50% conversion in 15 min. A portion of the incubation was monitored spectrophotometrically at 275 nm and frequently exchanged with the bulk reaction mixture. The measured $\Delta\epsilon$ is 2340 M^{-1} cm^{-1}. At the appropriate extent of reaction, the mixture was cooled rapidly to 3 °C, and $NaBH_4$ (10 mg in 1 mL of water) was added with stirring to reduce the prephenate. After 15 min, the solution was acidified to pH 1 with 2 N HCl and left for 20 min at 30 °C to convert the reduced prephenate to phenyllactic acid. Chorismic acid and phenyllactic acid were then extracted into ether, and 10 mg of each of the unlabeled compounds was added. The two acids were separated and purified on preparative thin-layer plates of silica gel, eluting with toluene–acetic acid (12:7 v/v; chorismic acid R_f 0.30, phenyllactic acid R_f 0.50). Small amounts of *p*-hydroxybenzoic acid (R_f 0.61) could also be detected. The silica samples containing chorismic acid and phenyllactic acid were each washed with hexane, and the compounds were then extracted from the silica into ether, and 30 mg of unlabeled material was added. Chorismic acid was recrystallized as described above, and phenyllactic acid was recrystallized from ether–hexane at −20 °C. Each material was recrystallized at least 3 times, the ^{3}H:^{14}C ratio being measured after each crystallization. The variation of values from different runs of the same experiment is probably due to spontaneous decomposition of chorismate to fluorescent impurities which, even in tiny amounts, can influence the accuracy of the counting. Indeed, the scatter among samples from a given experiment consistently increased as the time between sample preparation and counting increased. For this reason, the time between sample isolation and counting was kept as short as possible. The extent of the reaction was obtained from the value of A_{275nm} at the time of quench and was confirmed by enzymic assay of the remaining chorismate after the borohydride reduction.

For the *nonenzymic reaction*, crystalline chorismic acid was incubated at 60 °C in the same buffer as that used in the enzyme-catalyzed reaction. The progress of the reaction was followed at 275 nm. Under these conditions, the $t_{1/2}$ of chorismate is ~35 min. The extent of reaction was determined both by spectroscopic measurements (apparent $\Delta\epsilon$ 1860 M^{-1} cm^{-1}: side reactions interfere in this case) and by using the rate constant determined by Andrews et al. (1973) of 26.5 × 10^{-5} s^{-1}. The workup procedure was as described above. In the thermal reaction, the ratio of prephenate to *p*-hydroxybenzoate is ~4.

Results

Isotopically labeled samples of chorismate [9-^{3}H (**1**), 5-^{3}H (**2**), and 7-^{14}C (**3**)] were synthesized enzymatically by using

Scheme I: Synthetic Routes to [9-^{3}H]-, [5-^{3}H]-, and [7-^{14}C]Chorismate



[a] 5-Enolpyruvoylshikimate-3-phosphate synthetase. [b] Spheroplast lysate from *K. pneumoniae* 62-1. [c] Hexokinase–ATP. [d] Lead tetraacetate. [e] Extract from *E. coli* 83-24. [f] Phosphoenolpyruvate. [g] Acetic anhydride–pyridine. [h] Phosphoglycerate mutase and enolase.

the enzymes of the shikimic acid pathway. The routes followed are shown in Scheme I. The tritium label was introduced into the 9-position of 5-enolpyruvoylshikimate 3-phosphate (**4**) by allowing it to equilibrate with shikimate 3-phosphate (**5**) and phosphoenolpyruvate (**6**) in the presence of P_i, 5-enolpyruvoylshikimate-3-phosphate synthetase, and tritiated water. It is known that incorporation of tritium occurs under these conditions at both vinylic positions in equal amounts (Ife et al., 1976; Levin & Sprinson, 1964; Grimshaw et al., 1982). For the synthesis of [5-^{3}H]chorismate (**2**), [3-^{3}H]glucose (**7**) was converted to [1-^{3}H]erythrose 4-phosphate (**8**), which was then transformed by an extract of *E. coli* 83-24 into [5-^{3}H]-shikimate (**9**). This labeled shikimate was then converted into [5-^{3}H]chorismate by a spheroplast lysate from *K. pneumoniae* 62-1. The position of the label was confirmed by chemical conversion of [5-^{3}H, 7-^{14}C]chorismate into 3-[(1′-[^{14}C]-carboxyvinyl)oxy]benzoic acid (**10**) by the selective removal of the C-5 hydrogen in the aromatization of chorismate mediated by acetic anhydride–pyridine (Ife et al., 1976). The ^{3}H:^{14}C ratio fell from 10.4 in the chorismate to 0.7 in the benzoate derivative, showing that at least 93% of the ^{3}H label was attached to C-5 in chorismate. Finally, [7-^{14}C]chorismate was made from phospho[1-^{14}C]enolpyruvate (**12**) [obtained in situ from 3-phospho-D-[1-^{14}C]glycerate (**13**)] and shikimate 3-phosphate (**11**) in the presence of phosphoglycerate mutase, enolase, and 5-enolpyruvoylshikimate-3-phosphate synthetase. The product, 5-[7-^{14}C]enolpyruvoylshikimate 3-phosphate, was then converted to [7-^{14}C]chorismate (**3**) by using the lysate from *K. pneumoniae* 62-1.

Samples of doubly labeled chorismate were synthesized from the appropriate precursors on the day of the experiment and were crystallized to a constant ^{3}H:^{14}C ratio. When the procedure described under Methods was used, the formation of oils and of amorphous precipitates (a frequent occurrence if published methods are employed) was eliminated. Four recrystallizations, each in 75–80% yield, were performed in 1 day. The ^{3}H:^{14}C ratio was stable after the second recrystallization. Purified, doubly labeled chorismate was then partially converted to prephenate, either at 30 °C in the presence of chorismate mutase or at 60 °C by a thermal reaction. The enzymic reaction was stopped (at about 50% conversion) after ~15 min, during which time the spontaneous breakdown of chorismate is less than 1.5%. After immediate reduction of the prephenate product with borohydride, the reduced prephenate was converted to phenyllactate in acid at 30 °C. Under the same conditions, less than 0.5% of the chorismic acid decomposed. With this procedure, no detectable exchange of the hydrogens α to the carbonyl group of prephenate occurs during the enzymic reaction or during workup. In the course of the thermal reaction (~30 min, 60 °C), however, some 15% loss of ^{3}H from the product of the rearrangement of [9-^{3}H]chorismate was observed, due to the partial aromatization of prephenate to phenylpyruvate and the rapid enolization of the latter. The kinetic isotope effect for the uncatalyzed rearrangement of [9-^{3}H]chorismate at 60 °C was therefore evaluated *only* from the ^{3}H:^{14}C ratio of the remaining chorismate.

The remaining chorismic acid and the product phenyllactic acid were extracted into ether from acidic solution, and, after chromatographic separation, each was diluted with unlabeled material and subjected to repeated recrystallization to a constant ^{3}H:^{14}C ratio. Results from a representative reaction are presented in Table I. The secondary kinetic isotope effects were evaluated from the following relationships (Ropp, 1960). For the recovered starting material

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

and for product

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

where k_H/k_T is the isotope effect, f is the fractional extent of reaction, r = (^{3}H:^{14}C in product)/(^{3}H:^{14}C in substrate at start),

Table I: Values of the ^3H:^{14}C Ratio in the Uncatalyzed Thermal Rearrangement of [5-^3H,7-^{14}C]Chorismate[a]

compound	crystallization	^3H:^{14}C	weighted mean
chorismic acid before reaction	1	5.681 ± 0.077	5.749 ± 0.010
	2	5.755 ± 0.018	
	3	5.750 ± 0.014	
	4	5.737 ± 0.021	
chorismic acid remaining after reaction[b]	1	6.119 ± 0.025	6.164 ± 0.013
	2	6.166 ± 0.014	
	3	6.150 ± 0.037	
phenyllactic acid product[b]	1	5.183 ± 0.028	5.205 ± 0.017
	2	5.219 ± 0.022	
	3	5.172 ± 0.051	

[a] pH 7.5, 60 °C. [b] Extent of reaction, 42.8%.

and r' = (^3H:^{14}C in recovered substrate)/(^3H:^{14}C in substrate at start).

The isotope effects were measured by comparing the specific radioactivity ratio in the starting material both with that of the recovered substrate and with that of the reaction product (however, see Table II, footnote *b*). These two independent determinations were always in good agreement.

In Table II, we report the secondary tritium kinetic isotope effects for the enzymic and nonenzymic rearrangement of [9-^3H]- and [5-^3H]chorismate. The errors given for r and r' are statistical errors which reflect the reproducibility of the measurements.

Discussion

Nonenzymic Thermal Rearrangement. Secondary deuterium isotope effects have been determined for a number of Claisen and Cope rearrangements, and the values obtained have been used to define both the earliness (or lateness) of the transition state and the degree of concert of the process (Gajewski, 1980; Humski et al., 1970; McMichael & Korver, 1979; Gajewski & Conrad, 1979). For both the archetypal aliphatic and aromatic Claisen rearrangements (involving allyl vinyl ether and allyl phenyl ether, respectively), the decelerating effect of deuteriums at the C–O bond-breaking position was larger than the accelerating effect of deuteriums at the C–C bond-making position. Thus, in the rearrangement of 3-oxa-1,5-hexadiene (allyl vinyl ether) at 160 °C, Gajewski & Conrad (1979) found $k_H/k_{4\text{-}D} = 1.092$ and $k_{6\text{-}D}/k_H = 1.025$. Comparison with the estimated equilibrium isotope effects of 1.27 and 1.16, respectively, indicates an early transition state (Gajewski & Conrad, 1979) and suggests (if we assume a linear relationship between isotope effect and bond order) that the C–O bond is about one-third broken while the new bond is about one-sixth formed.

The results in Table II show that in the uncatalyzed rearrangement of chorismate at 60 °C, k_H/k_T is 1.149 ± 0.012 for the bond-breaking site and 0.992 ± 0.012 for the bond-making site. Estimation of the equilibrium isotope effects at 60 °C [from the deuterium fractionation factors at 25 °C (Hartshorn & Shiner, 1972) and by using the Swain–Schaad relationship (Swain et al., 1958)] gives $K_H/K_T = 1.36$ and 0.813 for the bond-breaking and bond-making sites, respectively. These results suggest that C–O bond is about 40% broken at the transition state, while the new C–C bond is not detectably formed. The extent of bond cleavage is consistent with a somewhat later transition state for the reaction of chorismate compared with that of allyl vinyl ether: the chorismate reaction is expected to be less exergonic due to the loss of conjugation of the ring double bonds in chorismate (Andrews & Haddon, 1979). Further, we should expect greater delocalization from the ring π electrons of any putative intermediate deriving from heterolytic or homolytic cleavage of the chorismate C–O bond. Our results do not signal any detectable formation of the new C–C bond, though it is not possible to state that the rearrangement of chorismate is a stepwise process. What is clear is that at the transition state, the incipient C–C bond is hardly, if at all, formed, while the C–O bond is substantially broken.

Chorismate Mutase Catalyzed Rearrangement. For the enzymic reaction, we find (Table II) that the secondary tritium isotope effects are both unity within experimental uncertainty. These results can be interpreted in two ways. First, we might conclude that the isotope effects *are* expressed but are undetectably small. A secondary tritium isotope effect determined by the competitive method necessarily evaluates the effect of V_{max}/K_m (Simon & Palm, 1966) and therefore senses any change in the nature of the carbon–hydrogen bond that has occurred between the starting material (free chorismate) and the transition state of the rate-limiting step. The equilibrium isotope effects at 30 °C for the two positions in chorismate are estimated to be 1.43 and 0.775 for the bond-breaking and bond-making sites, respectively. [These values are derived from the data reported by Hartshorn & Shiner (1972) and are gratifyingly close to estimates of 1.48 and 0.78 based upon the experimental results collected by Cleland (1980).] Since an isotope effect of 1.15 is observed for the bond-breaking position in the nonenzymic reaction, the transition state would have to be improbably "early" to account for the observed *enzymic* values of 1.0. That is, if the isotope effects are fully expressed, we should have to postulate a transition state for the rearrangement so early that virtually no bond making or breaking had yet occurred. This is unlikely.

The second explanation is that the isotope effects are suppressed because the rate-limiting transition state *precedes* the isotopically sensitive step. As discussed below, this would not only account for the observed isotope effects of 1.0 but would

Table II: Tritium Kinetic Isotope Effects on the Nonenzymic and Enzymic Rearrangement of [5-^3H,7-^{14}C]- and [9-^3H,7-^{14}C]Chorismate[a]

rearrangement	position of ^3H label	r'	r	extent of reaction (%)	k_H/k_T
nonenzymic	5	1.106 ± 0.004	0.909 ± 0.002	50.1	1.149 ± 0.012
	5	1.072 ± 0.003	0.905 ± 0.003	42.8	
	9	1.003 ± 0.003	*b*	48.3	0.992 ± 0.012
	9	0.989 ± 0.004	*b*	43.7	
enzymic	5	0.987 ± 0.003	0.984 ± 0.004	39	1.003 ± 0.020
	5	1.010 ± 0.004	0.983 ± 0.005	25	
	5	0.984 ± 0.002	1.014 ± 0.003	68.9	
	9	1.005 ± 0.005	0.988 ± 0.003	46.5	1.012 ± 0.004

[a] r' and r are as defined under Results. [b] Under the conditions of the thermal reaction, the tritium label is not stably bound in the product due to enolization. In this case, therefore, the isotope effect is obtained only from the specific radioactivity of the recovered substrate.

also be consistent with a mechanistic pathway in which chorismate binds to the enzyme, and the enzyme–chorismate complex then suffers a slow (isotopically insensitive) conformational change that brings the carboxyvinyl group *axial*, this step being followed by the sigmatropic rearrangement in a relatively rapid step. This explanation is more probable, and we explore several ways of confirming it.

As has been successfully practiced by Cleland (Cook & Cleland, 1981), it is often possible to slow down the "catalytic" step of an enzyme-catalyzed reaction (i.e., that step in which substrate covalency changes occur) by running the reaction at a nonoptimal pH. This technique can cause the transition state of the catalytic step to become rate limiting, allowing the full expression of both primary and secondary kinetic isotope effects. The obvious requirement is that V_{max} should fall as one moves away from the pH optimum for the reaction. Unfortunately, this approach is not helpful for the chorismate mutase reaction, since the enzyme is too unstable at the low pH extreme (below pH 6), and at high pH, the observed decrease in V_{max}/K_m is due to a rise in K_m rather than to a fall in V_{max} (P. Sampathkumar, private communication). In a second approach, we explored the possibility that coupling the chorismate mutase and the prephenate dehydrogenase activity of the enzyme by the inclusion of NAD^+ (a known activator of the mutase; Sampathkumar & Morrison, 1982b) would accelerate the putative isotopically insensitive step and allow expression of the isotope effects. However, the mutase reaction is only speeded up about 5-fold by the addition of NAD^+, which would not be enough fully to unmask a kinetically insignificant transition state. Indeed, it has been pointed out (Northrop, 1975) that a rate difference of at least 100-fold is needed in order to see full expression of a suppressed isotope effect.

In an attempt to confirm the observed effects of isotopic substitution, we considered the possibility of running the reaction uphill from prephenate to chorismate: the rate-limiting transition step would then *follow* the isotopically sensitive one, and the full secondary isotope effect (equal to the equilibrium effect, which would thereby be determined experimentally) would be measured. In principle, the mutase reaction could be run in the direction prephenate to chorismate by coupling with anthranilate synthetase (the enzyme that converts chorismate to anthranilate). This approach would be feasible if the chorismate $\rightleftharpoons$ prephenate equilibrium does not lie too far toward prephenate. The overall equilibrium constant for the mutase reaction has never been determined, and some preliminary experiments were performed to examine this. After long incubations of [7-^{14}C]chorismate with the mutase, chorismate and prephenate were separated by repeated ion-exchange chromatography, and a prephenate:chorismate ratio of 600 was obtained. (Until the equilibrium has been approached from the prephenate side, however, this must be taken as a lower limit for the equilibrium constant.) An equilibrium as unfavorable as this makes any investigation of the back-reaction a difficult problem in the absence of extremely efficient coupling enzyme systems, and this approach needs further evaluation.

In summary, it appears that competitive experiments of the kind reported here cannot yield direct information about the nature of the transition state in the mutase-catalyzed reaction. What we can conclude, however, on the basis of the known kinetic parameters of the enzyme and of the absence of measurable secondary isotope effects, is that the catalysis may proceed in one of two ways. First, the enzyme may have a rigid binding site that selectively binds the conformer of chorismate that has the carboxyvinyloxy group *axial*, which is required for the reaction to occur by elementary application of the minimal motion postulate. On the basis that both subunits of the enzyme dimer of 88 000 daltons are independently active, k_{cat} is approximately 25 s^{-1}, the apparent K_m is 60 μM (in the presence of bovine serum albumin), and k_{cat}/K_m is about 4×10^5 M^{-1} s^{-1} (Sampathkumar, 1978). The real K_m (for the axial conformer) would be much less than 60 μM because of the low concentration of free axial conformer. If we assume that the "on" rate is limited only by diffusion, and has a rate constant of 10^9 M^{-1} s^{-1} (Hammes & Schimmel, 1970), then we can estimate that the equilibrium population of axial chorismate is 1 in 2×10^3, which corresponds to a $\delta\Delta G$ of 4.5 kcal/mol. This value compares with that of 7 kcal/mol calculated by Andrews et al. (1973) for the free-energy difference between axial and equatorial conformers of chorismate. The measured acceleration of 10^6-fold with respect to the uncatalyzed reaction would then be achieved by the enzyme through the selective binding of the axial conformer and the partial freezing of the rotation in the carboxyvinyloxy group.

The second possible mechanistic pathway for the enzyme involves the binding of chorismate in its preferred equatorial conformation with a rate constant of 10^5 M^{-1} s^{-1}, followed by a conformational isomerization of the enzyme–substrate complex that puts the carboxyvinyloxy group axial, this complex then rearranging to enzyme–prephenate. The observed k_{cat} of 25 s^{-1} would then represent either the conformational change or the rearrangement. It must be remembered that the rearrangement could be rate limiting for V_{max}: the isotopic effect measurements we have made by the competitive method relate to V_{max}/K_m. It would, of course, be interesting to determine the secondary isotope effects on V_{max} by using specifically deuterated substrates in noncompetitive experiments. The problems of precision and accuracy for such experiments may, however, prove overwhelming.

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Registry No. **1**, 86456-74-8; **2**, 86456-75-9; **3**, 86456-76-0; **4**, 86456-77-1; **5**, 138-08-9; **6**, 63959-45-5; **7**, 2535-38-8; **8**, 86456-78-2; **9**, 86456-79-3; **10**, 16929-37-6; **13**, 63877-11-2; **I**, 617-12-9; chorismate mutase, 9068-30-8; ribulose 1,5-bisphosphate, 2002-28-0; 5-[7-^{14}C]enolpyruvoylshikimate 3-phosphate, 86456-80-6; D-[3-^{3}H]glucose 6-phosphate, 86456-81-7

References

Andrews, P. R., & Haddon, R. C. (1979) *Aust. J. Chem. 32*, 1921–1929.

Andrews, P. R., Smith, G. D., & Young, I. G. (1973) *Biochemistry 12*, 3492–3498.

Andrews, P. R., Cain, E. N., Rizzardo, E., & Smith, G. D. (1977) *Biochemistry 16*, 4848–4852.

Blackmore, P. F., Williams, J. F., & MacLeod, J. K. (1976) *FEBS Lett. 64*, 222–226.

Burrows, C. J., & Carpenter, B. K. (1981) *J. Am. Chem. Soc. 103*, 6984–6986.

Cleland, W. W. (1980) *Methods Enzymol. 64*, 104–125.

Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 20*, 1797–1805.

Davis, B. D. (1951) *J. Biol. Chem. 191*, 315–325.

Edwards, J. M., & Jackman, L. M. (1965) *Aust. J. Chem. 18*, 1227–1239.

Evans, D. A., & Golob, A. M. (1975) *J. Am. Chem. Soc. 97*, 4765–4766.

Floss, H. G., Onderka, D. K., & Carroll, M. (1972) *J. Biol. Chem. 247*, 736–744.
Gajewski, J. J. (1980) *Acc. Chem. Res. 13*, 142–148.
Gajewski, J. J., & Conrad, N. D. (1979) *J. Am. Chem. Soc. 101*, 2747–2748.
Gibson, F. (1968) *Biochem. Prep. 12*, 94–97.
Gibson, M. I., & Gibson, F. (1964) *Biochem. J. 90*, 248.
Görisch, H. (1978) *Biochemistry 17*, 3700–3705.
Grimshaw, C. E., Sogo, S. G., & Knowles, J. R. (1982) *J. Biol. Chem. 257*, 596–598.
Hammes, G. G., & Schimmel, P. R. (1970) *Enzymes, 3rd Ed. 2*, 67–114.
Hartshorn, S. R., & Shiner, V. J. (1972) *J. Am. Chem. Soc. 94*, 9002–9012.
Humski, K., Malojcič, R., Borcič, S., & Sunko, D. E. (1970) *J. Am. Chem. Soc. 92*, 6534–6538.
Ife, R. J., Ball, L. F., Lowe, P., & Haslam, E. (1976) *J. Chem. Soc., Perkin Trans. 1*, 1776–1783.
Knowles, P. F., & Sprinson, D. B. (1970) *Methods Enzymol. 17A*, 351–352.
Kornberg, H. L., & Lascelles, J. (1960) *J. Gen. Microbiol. 23*, 511–516.
Levin, J. G., & Sprinson, D. B. (1964) *J. Biol. Chem. 239*, 1142–1150.
McMichael, K. D., & Korver, G. L. (1979) *J. Am. Chem. Soc. 101*, 2746–2747.
McVittie, J. D., Esnouf, M. P., & Peacocke, A. R. (1972) *Eur. J. Biochem. 29*, 67–73.
Morell, H., Clark, M. J., Knowles, P. F., & Sprinson, D. B. (1967) *J. Biol. Chem. 242*, 82–90.
Northrop, D. B. (1975) *Biochemistry 14*, 2644–2651.
Onderka, D. K., & Floss, H. G. (1969) *J. Am. Chem. Soc. 91*, 5894–5896.
Putman, S. J., Coulson, A. F. W., Farley, I. R. T., Riddleston, B., & Knowles, J. R. (1972) *Biochem. J. 129*, 301–310.
Racker, E. (1962) *Methods Enzymol. 5*, 266–270.
Racker, E. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) 2nd ed., Vol. 3, pp 1391–1393, Academic Press, New York.
Ropp, G. A. (1960) *J. Am. Chem. Soc. 82*, 842–852.
Sampathkumar, P. (1978) Ph.D. Thesis, Australian National University.
Sampathkumar, P., & Morrison, J. F. (1982a) *Biochim. Biophys. Acta 702*, 204–211.
Sampathkumar, P., & Morrison, J. F. (1982b) *Biochim. Biophys. Acta 702*, 212–219.
Sieben, A. S., Perlin, A. S., & Simpson, F. J. (1966) *Can. J. Biochem. 44*, 663–669.
Simon, H., & Palm, D. (1966) *Angew. Chem., Int. Ed. Engl. 5*, 920–933.
Swain, C. G., Stivers, E. C., Reuwer, J. F., & Schaad, L. J. (1958) *J. Am. Chem. Soc. 80*, 5885–5893.
Westheimer, F. H. (1962) *Adv. Enzymol. Relat. Areas Mol. Biol. 24*, 441–482.
Woodward, R. B., & Hoffmann, R. (1970) *The Conservation of Orbital Symmetry*, Verlag Chemie, Weinheim, West Germany.

Enzymic Detection of Uracil in a Cloned and Sequenced Deoxyribonucleic Acid Segment†

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ABSTRACT: Uracil can occur in DNA either as a result of utilization of dUTP by DNA polymerases or from in situ deamination of cytosine. The latter results in transition mutations following the next round of replication. We describe a technique for the detection of uracil in DNA by a modification of the Maxam–Gilbert sequencing procedure. Reaction of end-labeled DNA with uracil-DNA glycosylase followed by 1 M piperidine results in scission products corresponding to locations of uracils. These are detected by autoradiography following electrophoresis through a sequencing gel. Comparison of these scission products with the DNA sequences elucidates the mechanism of origin of the DNA uracils. The technique was tested with a cloned human DNA sequence grown in a dut⁻,ung⁻ strain of *Escherichia coli*, which incorporates uracil in place of thymine in its DNA, and by chemical deamination of cytosines in that sequence. The technique was expanded by use of alkaline and enzymic probes to investigate possible accumulation of uracil, base losses, and other modifications in human liver and brain DNA. No damaged DNA moieties were detected. This method is applicable to the study of any recoverable reiterated sequence by any enzyme preparation that can recognize modifications in DNA.

Uracil, while not a normal component of DNA, may arise either as a result of utilization of dUTP in place of dTTP by DNA polymerases or from in situ deamination of cytosine (Lindahl, 1979). Replicative polymerases have the same K_m for dUTP as for dTTP and will utilize dUTP if it is available (Schlomai & Kornberg, 1978; Dube et al., 1979). Deamination of cytosine in DNA may occur either spontaneously (Lindahl & Nyberg, 1974) or by reaction with environmental agents such as sodium bisulfite or nitrous acid (Lindahl, 1979). If uracil derived from cytosine is not removed, transition mutations (G-C → A-T) will be observed following the next round of replication (Drake & Baltz, 1976).

Two mechanisms normally exclude uracil from DNA. Deoxyuridine-5′-triphosphate nucleotidohydrolase (dUTPase) hydrolyzes dUTP to dUMP, making dUTP unavailable as a

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