Steric Course of the Reactions Catalyzed by 5-Enolpyruvylshikimate-3-phosphate Synthase, Chorismate Mutase, and Anthranilate Synthase†

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Abstract: To probe the steric course of several reactions in the shikimate pathway, (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol was synthesized and fed to cultures of *Klebsiella pneumoniae* mutant 62-1. The accumulated chorismic acid, labeled from endogenously formed (*E*)-[3-^{2}H,^{3}H]phosphoenolpyruvate, was isolated and degraded by hydrogenation of the side-chain double bond and cleavage of the ether linkage to D,L-lactic acid. Resolution of the latter and determination of the methyl group configuration in the two enantiomers demonstrated predominant *E* configuration of the [3′-^{2}H,^{3}H]chorismic acid. It follows that the addition and the elimination step in the 5-enolpyruvylshikimate-3-phosphate (ESP) synthase reaction proceed with opposite stereochemistry, i.e., anti/syn or syn/anti. Incubation of the above chorismic acid with anthranilate synthase produced [3-^{2}H,^{3}H]pyruvate of predominantly *S* configuration, indicating stereospecific protonation on the *re* face of the side chain. Conversion of the (*E*)-[3′-^{2}H,^{3}H]chorismic acid into phenylalanine and tyrosine with whole cells of *E. coli* and configurational analysis of these amino acids by conversion into pyruvate carrying a chiral methyl group with phenylalanine hydroxylase and/or tyrosine phenol-lyase indicated that the chorismate mutase reaction proceeds via a chair transition state.

The shikimate pathway is the major metabolic route leading to the formation of aromatic compounds in plants and microorganisms. The main features of the pathway are well understood,[2,3] but mechanistic details of many of the reactions remain to be elucidated. Stereochemical studies have dealt with a number of the pathway enzymes, but three stereochemical questions, revolving around the mode of attachment of the enolpyruvyl side chain of chorismate and its further transformations, have eluded an analysis. Elucidation of these questions requires the generation of 5-enolpyruvylshikimate-3-phosphate (ESP) (**1**) and chorismate (**2**) labeled asymmetrically in the side-chain methylene group and is complicated by the fact that the ESP synthase reaction involves an obligatory protonation/deprotonation at C-3 of phosphoenolpyruvate.[4,5] Specifically, the three stereochemical questions are (Figure 1) as follows: (a) In the ESP synthase reaction, does the (formal) addition of shikimate-3-phosphate to the enol double bond of phosphoenolpyruvate (PEP) proceed with the same stereochemistry as the subsequent (formal) elimination of phosphate and a proton, i.e., both syn or both anti, or do the two half reactions proceed with opposite stereochemistry, i.e., syn/anti or anti/syn? (b) In the chorismate mutase reaction, does C-1 of chorismate attack the side-chain methylene group on the *si* face, involving a chair transition state, or on the *re* face as predicted for a boat transition state? (c) In the anthranilate synthase reaction, does the protonation at the side-chain methylene group take place on the *si* or the *re* face?.

We report here results of studies which answer these three stereochemical questions. A preliminary account of part of this work has been published.[1]

Materials and Methods

Materials. Alcohol dehydrogenase (equine liver), L-lactate dehydrogenase (bovine heart), D-lactate dehydrogenase (*Lactobacillus leichmanii*), L-phenylalanine hydroxylase (rat liver), dihydrofolate reductase (chicken liver), dihydropteridine reductase (sheep liver), glucose dehydrogenase (calf liver), phenylalanine ammonia-lyase (*Rhodotorula glutinis*), and shikimate-3-phosphate were obtained from Sigma. Tyrosine phenol-lyase (*Citrobacter intermedius*) was a gift from Dr. T. Nagasawa and Prof. H. Yamada, Kyoto University, and anthranilate synthase was purified 50-fold (1.06 IU/mg) from *Klebsiella pneumoniae* mutant 62-1.[6] L-[U-^{14}C]Phenylalanine and sodium [^{3}H]borohydride were purchased from Amersham-Searle. 2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride, shikimic acid, tris(triphenylphosphine)rhodium(I) chloride, and pyridinium dichromate were obtained from Aldrich Chemical Co. All other chemicals and solvents were of reagent grade or of the highest grade commercially available and were used without further purification.

General Methods. Radioactive samples were counted in a Beckman LS 7500 liquid scintillation counter by using Aquasol-2 (Beckman) as a solvent and [^{14}C]- and [^{3}H]toluene as internal standards to determine counting efficiencies. Proton NMR spectra were recorded on a Brucker WP-200 superconducting NMR spectrometer and mass spectra on a Kratos MS-30 mass spectrometer. The configuration and chiral purity of chiral methyl groups was assayed on samples of sodium acetate by the method of Cornforth et al.[7] and Arigoni and co-workers,[8] using a procedure routinely employed in this laboratory.[9]

(1*R*,2*R*)-[1-^{2}H,^{3}H]Glycerol (Figure 4). A solution of 5.6 μL (ca. 100 μmol) of acetaldehyde in 2 mL of H_2O was reduced with sodium [^{3}H]-borohydride (ca. 25 μmol, 200 mCi) in a closed tube overnight at room temperature. One drop of acetone was then added to destroy any excess borohydride, and the solution was vacuum-distilled into a new flask. To the distillate was added 3 mL of 0.06 M Na-pyrophosphate-glycine buffer, pH 9, 4 mg of NAD^+, 4 mg of egg albumin, and 10 mg (12.6 IU) of horse liver alcohol dehydrogenase. The reaction was started by addition of 63.5 μL (500 μmol) of isopropylidene-(2*R*)-[1-$^2H_2$]glycerol[10] and left overnight at room temperature. Another 5 mg of alcohol dehydrogenase and 1 mg of NAD^+ were then added, and the reaction was continued for a further 5 h with stirring. Two milliliters of 0.5 N HCl was then added, and hydrolysis was allowed to proceed for 17 h at room temperature. The solution was taken to dryness on a rotary evaporator and the residue desalted by successive passage through columns of AG 1 × 8 (Cl^-) and AG 50 W × 8 (H^+) ion-exchange resin. The aqueous effluent containing the radioactivity was evaporated and the residue purified by preparative paper chromatography (Whatman No. 3, *n*-butyl alcohol/acetic acid/water 12:3:4, R_f (glycerol) = 0.51). The radioactive band was eluted with water, passed through a column of AG 50 W × 8, and rechromatographed on paper to give 11.25 mCi of material, which upon chromatographic purity analysis showed a single radioactive band coinciding with the R_f of authentic glycerol.[12]

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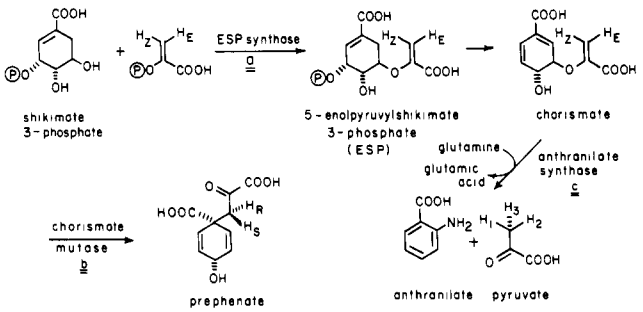

Figure 1. Three reactions in the shikimate pathway, which are the subject of stereochemical analysis.

Conversion of (1*R*,2*R*)-[1-^{2}H,^{3}H]Glycerol into Chorismate. Stereospecifically labeled chorismate was prepared by a modification of the method of Gibson.[13] The growth from a nutrient agar slope of *Klebsiella pneumoniae* 62-1 was inoculated into a 500-mL Erlenmeyer flask containing 100 mL of medium A. After 10 h of growth at 30 °C on a rotary shaker at 400 rpm, 10 mL of this seed culture was transferred to each of four 500-mL flasks containing 200 mL of medium A. After 6 h of growth at 30 °C and 400 rpm, the cultures were centrifuged and the pellets resuspended into four 500-mL flasks each containing 200 mL of medium B. The cultures were incubated for 16 h at 30 °C on a rotary shaker (400 rpm) and then immediately centrifuged (10 min, 6000 rpm). The supernatant was made alkaline by addition of 2 mL of 10 N NaOH and passed, with suction, through a column of Dowex 1-Cl (2.7 × 7 cm, flow rate 15–20 mL/min). The column was washed with water, and chorismate was then eluted with 1 M NH_4Cl (pH 8.5) at a flow rate of 1 mL/min. Fractions containing chorismate were pooled, acidified with 1 N HCl to pH 1.5, and extracted 5 times with 50-mL portions of ether. All these workup operations were carried out at 0–4 °C as rapidly as possible. The ether extracts were combined, washed with water, and dried over $MgSO_4$ at 4 °C overnight. The ether solution was then concentrated to a small volume and petroleum ether (bp 60–80 °C) was added until a faint cloudiness persisted. Cooling in a dry ice–acetone bath produced crude chorismate, which was recrystallized from ethyl acetate/petroleum ether to give 510 mg of colorless needles of >95% purity as judged by TLC. Radiochemical yields ranged from 0.5–2% based on glycerol.

Medium A

minimal salt soln	20 mL
5% (w/v) difco yeast extract	40 mL
5% (w/v) difco casamino acids	40 mL
D,L-tryptophan	51 mg
distilled water to 1 L, autoclave 15 min at 15 psi, and then add 16% (w/v) glucose (sterilized), 10 mL	

Minimal Salt Solution

$MgSO_4 \cdot 7H_2O$	15 g
citric acid·H_2O	100 g
K_2HPO_4	500 g
$NaNH_4HPO_4 \cdot H_2O$	175 g
distilled water	670 mL

Medium B

Na_2HPO_4	10.24 g
KH_2PO_4	1.08 g
glucose	10.8 g
shikimic acid	3.48 g
NH_4Cl	2.16 g
$MgCl_2$, 0.05 M	1.6 mL
L-tryptophan, 0.01 M	0.8 mL
distilled water to 800 mL, adjust pH to 7.5 with NaOH, and then add (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol, 0.5 mCi, 0.79 mmol	

Degradation of Chorismic Acid. Methyl 3-[(1-Carbomethoxyvinyl)oxy]-4-methoxybenzoate. Chorismate (175 mg, 0.77 mmol) biosynthesized from (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol and pyridinium dichromate (435 mg, 1.16 mmol) were dissolved in 5 mL of dry DMF and stirred for 4 h at 0 °C. Ten milliliters of water was added to the reaction mixture, the product was extracted into ethyl acetate (5 times), and the extract was washed with water and dried over $MgSO_4$. The product was esterified with diazomethane, followed by preparative-layer chromatography (silica gel, hexane/ethyl acetate 2:1) to give 112 mg (55% yield, 0.4 μCi) of a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.85 (dd, 1 H, *J* = 2, 8.6 Hz), 7.59 (d, 1 H, *J* = 2 Hz), 7.23 (d, 1 H, *J* = 8.6 Hz), 5.54 (d, 1 H, *J* = 2.4 Hz), 4.69 (d, 1 H, *J* = 2.4 Hz), 3.91 (s, 3 H), 3.83 (s, 3 H), 3.79 (s, 3 H); MS (EI), *m/z* M^+ = 266.0771, calcd for $C_{13}H_{14}O_6$ 266.0790.

Methyl 3-[(1-Carbomethoxyethyl)oxy]-4-methoxybenzoate. The previous compound (109 mg, 0.41 mmol) and 30 mg of tris(triphenylphosphine)rhodium chloride were placed in a two-neck flask, which was closed with rubber septums and purged 4 times with hydrogen gas. Dried and degassed benzene (4 mL) was then introduced via a needle and hydrogen gas was passed through the solution for 12 h with stirring at room temperature. The product was then purified by preparative-layer chromatography (silica gel, hexane/ethyl acetate 2:1) to give 95 mg (84%, 0.32 μCi) of the product as a colorless liquid: 1H NMR ($CDCl_3$) δ 7.70 (dd, 1 H, *J* = 2.0, 8.5 Hz), 7.49 (d, 1 H, *J* = 2.0 Hz), 6.90 (d, 1 H, *J* = 8.5 Hz), 4.82 (q, 1 H, *J* = 6.8 Hz), 3.89 (s, 3 H), 3.85 (s, 3 H), 3.74 (s, 3 H), 1.63 (d, 3 H, *J* = 6.8 Hz); MS (EI), *m/z* M^+ = 268.0985, calcd for $C_{13}H_{16}O_6$ 268.0947.

D,L-Lactate. The above compound (93 mg, 0.35 mmol) was saponified with 10% NaOH in methanol, sodium ions were removed with Amberlite IR 112 cation-exchange resin, and the solvent was evaporated under vacuum. The free acid (84 mg) was dissolved in 2 mL of anhydrous ethanol in a three-neck flask equipped with a dry ice condenser. Under an argon atmosphere, 30 mL of liquid ammonia was introduced into the reaction flask with cooling in a dry ice/acetone bath. Sodium metal (80 mg) was added in small pieces over a period of 1 h to the stirred solution, followed by stirring for an additional 1 h. Ammonium chloride (55 mg) was added, the ammonia was allowed to evaporate, and the residue was acidified to pH 2 with 1 N HCl and refluxed for 1 h. The solution was then lyophilized to dryness, the residue extracted with acetone, and the acetone extract purified twice by paper chromatography (Whatman No. 3, ethyl acetate/isopropyl alcohol/concentrated NH_4OH 45:35:20, descending, R_f(lactate) = 0.3). Extraction with methanol gave 7.2 mg of D,L-lacetate containing 0.045 μCi of tritium (6.5% radiochemical yield based on chorismate).

L-Lactate Dehydrogenase Reaction. One-third of the D,L-lactate sample (3.3 × 10^4 dpm) was incubated with 300 IU of L-lactate dehydrogenase and 200 mg of NAD^+ in the presence of 26.5 mg of FMN in 5 mL of phosphate buffer, pH 7.5, for 22 h at room temperature. Pyruvate produced in this enzymatic reaction was converted to acetate by addition of 1.5 mL of 30% hydrogen peroxide. After 20 min, excess hydrogen peroxide was decomposed by addition of platinum on charcoal, and the reaction mixture was steam-distilled. The distillate was brought to pH 9 with NaOH and evaporated to give 6400 dpm of sodium acetate for chirality analysis. A control experiment showed that hydrogen peroxide oxidation of D,L-lactate alone under the conditions used here did not produce radioactive acetate.

D-Lactate Dehydrogenase Reaction. Another third of the DL-lactate sample (3.3 × 10^4 dpm) was incubated with 227 IU of D-lactate dehydrogenase and 220 mg of NAD^+ in the presence of 66 mg of FMN in 5 mL of phosphate buffer, pH 7.5, for 15 h at 27–28 °C. Further treatment as in the case of the L-lactate dehydrogenase reaction gave 8800 dpm of sodium acetate for chirality analysis.

Reaction of Chorismate with Anthranilate Synthase. Tris·HCl buffer, pH 8.2 (100 μmol), chorismate (9.7 mg, 43 μmol, 1 μCi, pH adjusted to neutrality) biosynthesized from (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol, L-glutamine (50 μmol), $MgCl_2$ (50 μmol), NADH (50 μmol), L-lactate dehydrogenase (500 IU), and anthranilate synthase (5 IU) in a final volume of 1.65 mL were incubated for 2.5 h at 37 °C. The reaction mixture was acidified and immediately lyophilized and the residue extracted with methanol. Lactate was purified from the methanol extract by preparative-paper chromatography (Whatman No. 3, ethyl acetate/isopropyl alcohol/concentrated NH_4OH 45:35:20, descending). The L-lactate sample (0.26 μCi) was then oxidized to acetate by heating with 10 mL of oxidation mixture (153 mg of $K_2Cr_2O_7$, 24 mL of concentrated H_2SO_4, H_2O to 100 mL) on a steam bath for 20 min under N_2. Steam distillation, titration of the distillate with NaOH, and evaporation gave sodium acetate (0.058 μCi) for chirality analysis.

Conversion of Chorismate to Phenylalanine and Tyrosine. Chorismate (80.7 mg, 8.3 μCi) biosynthesized from (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol was fed to a 200-mL culture of *Escherichia coli* B (wild type) (medium: 2 g of glucose, 9.86 mg of $MgCl_2.7H_2O$, 1.52 mg of $FeSO_4 \cdot 7H_2O$, 1.98 g of $(NH_4)_2SO_4$, 63 mL of 1 M K-phosphate buffer, pH 7.0, distilled water to 1 L), which was incubated at 30 °C for 28 h on a rotary shaker (300 rpm). The cells were harvested by centrifugation, washed, and dried. The dried cells (114 mg, 1.5 μCi) were hydrolyzed by heating with 16 mL of freshly distilled 6 N HCl and 1 mL of ethanol at 115 °C for 3 days in a sealed, evacuated tube (2.4 × 10 cm). The hydrolysate was

(12) This material contains a large amount of (2*R*)-[1-^{2}H$_2$]- and some (1*S*,2*R*)-[1-^{2}H$_1$]glycerol, but the presence of these species is of no consequence in the subsequent experiments.

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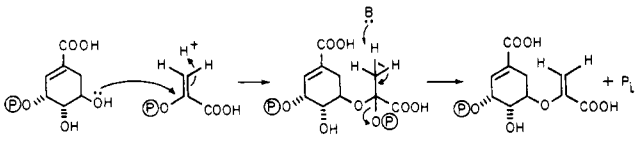

Figure 2. Reaction mechanism of ESP synthase proposed by Levin and Sprinson.[4]

filtered, the filtrate evaporated, and the residue dissolved in 10 mL of water (0.32 μCi). This solution was passed through a column of Dowex 1 × 2 (Cl^-) (1.7 × 4.5 cm), which was then eluted with 5% acetic acid. The active fractions were combined, concentrated, and then chromatographed on Dowex 50 W (OH^-) (1 × 120 cm column with a 2.8 × 110 cm jacket carrying 50 °C circulating water) as described by Stein and Moore.[14,15] The peaks of L-tyrosine (153–186 mL) and L-phenylalanine (174–210 mL) overlapped only slightly and about 70–80% of each amino acid could be recovered without contamination by the other. The yields were 1.3 mg of L-tyrosine (0.018 μCi) and 1.9 mg of L-phenylalanine (0.21 μCi).

Phenylalanine Ammonia-lyase Reaction.[16-18] The reaction mixture contained 6.1 μmol of L-phenylalanine (mixture of [U-^{14}C]-L-phenylalanine and aliquot of the above tritiated sample, $^3H/^{14}C$ 4.22), 0.13 IU of phenylalanine ammonia-lyase, and 100 μmol of Tris·HCl buffer, pH 8.5, in a volume of 4 mL. The reaction mixture was incubated at room temperature and monitored by measuring the UV absorption of *trans*-cinnamic acid ($\epsilon = 2.042 \times 10^4$ cm^{-1} M^{-1}). Aliquots were drawn at 15 min and 1.5, 5, 12, and 24 h and lyophilized to measure the amount of tritium released into the water. After 40 h the reaction mixture was acidified with HCl and extracted with ether. The ether extract was washed and dried, and 20 mg of unlabeled *trans*-cinnamic acid was added. The solution was then evaporated and the residue recrystallized from aqueous methanol. The product had a $^3H/^{14}C$ ratio of 3.59.

Phenylalanine Hydroxylase Reaction.[19,20] The reaction mixture contained 5.9 μmol of L-phenylalanine (0.106 μCi), 1.5 μmol of NADPH, 750 μmol of D-glucose, 3 μmol of 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine·HCl, 45 μmol of mercaptoethanol, 600 μmol of K-phosphate, pH 7.0, 104 IU of glucose dehydrogenase, 1.3 IU of phenylalanine hydroxylase, 22 IU of dihydropteridine reductase, and 3 IU dihydrofolate reductase (dialyzed prior to use against 0.01 M phosphate, pH 7.0, containing 5 mM mercaptoethanol for 1 h at 5 °C) in a total volume of 4.3 mL. The reaction mixture was incubated for 7 h at 25 °C with intermittent shaking. The reaction was then terminated by heating on a steam bath, the precipitated protein removed by centrifugation, and the combined supernatant and washings lyophilized to dryness. The residue was chromatographed on paper (four sheets, *n*-butyl alcohol/acetic acid/water 4:1:1, R_f(tyrosine) = 0.37) and the tyrosine band eluted with water. Radiochemical yield was 62%.

Degradation of Tyrosine with Tyrosine Phenol-lyase.[21] The reaction mixture contained 7.2 μmol of L-tyrosine (39 900 dpm), 300 μmol of K-phosphate buffer, pH 8.4, 1 μmol of pyridoxal phosphate, 80 μmol NADH, 1000 IU of L-lactate dehydrogenase, and 15 IU of tyrosine phenol-lyase in a volume of 3.6 mL. The two enzymes were dialyzed immediated prior to use against 0.01 M phosphate buffer, pH 7.0, containing 5 mM mercaptoethanol for 1 h at 5 °C. The reaction mixture was incubated for 4 h at 30 °C and then heated on a steam bath, acidified, and lyophilized to dryness. Lactic acid was extracted from the residue with methanol, purified by paper chromatography, and oxidized with dichromate as described above to give sodium acetate (3600 dpm).

Similarly, two samples of L-tyrosine (47 900 and 95 800 dpm), generated by hydroxylation of L-phenylalanine, were converted to sodium acetate (10 800 and 20 200 dpm).

Results

ESP Synthase. As part of a much earlier study,[22] we had attempted to investigate the steric course of the ESP synthase reaction by converting (*E*)- and (*Z*)-[3-^{3}H]phosphoenolpyruvate into chorismate with a cell-free extract of *Klebsiella pneumoniae* 62-1.[22] We observed[23] extensive washout of tritium (75–85%) and concluded that the remaining tritium would be evenly distributed between the two methylene positions of the side chain. This is in agreement with a mechanism proposed by Levin and Sprinson.[4] involving addition of the 5-OH group of shikimate-3-phosphate to the $\Delta^{2,3}$ double bond of PEP followed by loss of phosphate from the adduct (Figure 2), and with subsequent results indicating incorporation of solvent protons both into ESP and unreacted PEP[5,24,25] and equal loss of label from H_E and H_Z of PEP.[24,26] Obviously this approach is not suitable for the elucidation of the steric course of the ESP synthase reaction.

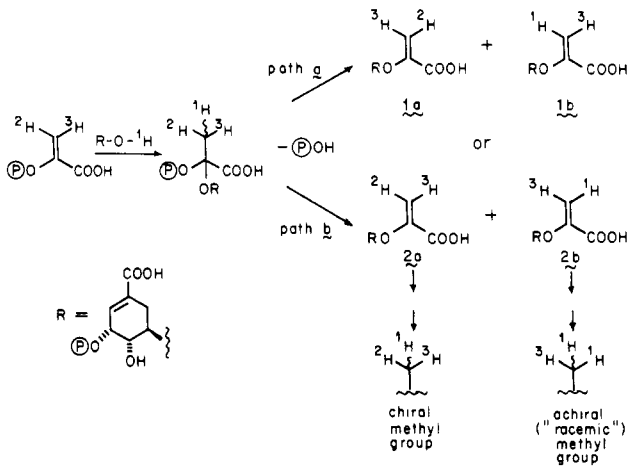

Figure 3. Stereochemical options in the ESP synthase reaction in H_2O with (*E*)-[3-^{2}H,^{3}H]PEP as substrate.

It occurred to us that the problem might be solved if conditions were chosen such that the methyl group formed transiently during the reaction is chiral. This would require as substrate a sample of PEP carrying deuterium in one methylene position, e.g., H_Z, and tritium in the other, e.g., H_E, in such a way that every tritiated molecule also contains deuterium. If the enzymatic synthesis of ESP is then carried out in normal water, the intermediate methyl group will now contain ^{1}H, ^{2}H, and ^{3}H in an asymmetric arrangement, assuming that the proton transfer is stereospecific. Subsequent abstraction of a hydrogen from this methyl group, very likely mediated by the same base group catalyzing the proton addition, will produce a mixture of two tritiated species of ESP, one resulting from loss of $^1H^+$ and the other from loss of $^2H^+$ (Figure 3). If the addition and the elimination step proceed with the same stereochemistry (anti/anti or syn/syn, path a, Figure 3), product set **1** will be obtained, whereas set **2** would result if the two steps proceed with opposite stereochemistry (anti/syn or syn/anti, path b, Figure 3). In the absence of an isotope effect in the deprotonation step, tritium in the product would be evenly distributed between the two methylene positions of the side chain. However, the two product sets would still be distinguishable, because in set **1** tritium in the H_Z position would have ^{2}H as a neighbor (species **1a**) whereas tritium in the H_E position would see ^{1}H in the other methylene position (species **1b**). In product set **2**, these relationships are reversed. If one converts these methylene groups into methyl groups by stereospecific addition of ^{1}H, species **1a** or **2a** will produce a chiral methyl group whereas **1b** or **2b** will give rise to an achiral methyl group. From the configuration of the resulting chiral methyl group and the steric course of the ^{1}H addition, the side-chain double bond configuration of the deuterated, tritiated ESP species can be deduced. If the ESP synthase reaction involves a primary kinetic isotope effect

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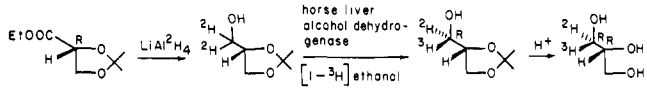

Figure 4. Synthesis of (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol.

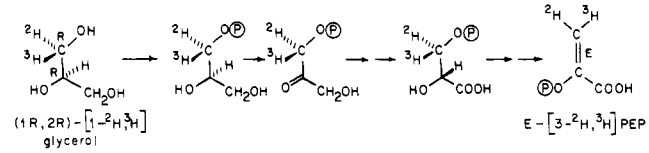

Figure 5. *Stereochemistry of PEP generated intracellularly from* (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol.

in the deprotonation step, as has since been demonstrated by Grimshaw et al.,[26] species **1a** or **2a** will be produced in excess over **1b** or **2b**, respectively, resulting in an uneven tritium distribution between the two methylene positions and allowing, potentially, other modes of analysis. A second requirement for the approach outlined above to be successful is that proton exchange with solvent, e.g., due to reversible binding to the enzyme and protonation/deprotonation of (substrate) PEP and (product) ESP must be minimized to retain significant chirality. In view of our experience with the cell-free extract and the known instability of chorismate synthase,[27] this is best accomplished, we reasoned, in an in vivo system of high metabolic flux which presumably provides tight coupling of the various enzymatic steps in the sequence. We therefore decided to carry out the enzymatic synthesis in whole cells of *Klebsiella pneumoniae* mutant 62-1, which accumulates large quantities of chorismate.[28] Since the cell membrane is not permeable to phosphate esters, like PEP, this required generating the stereospecifically labeled PEP within the cell from a suitable nonphosphorylated precursor. Glycerol was chosen as such a precursor.

To implement the approach outlined above we synthesized glycerol labeled stereospecifically with deuterium and tritium in the *pro-R*-hydroxymethyl group by the route shown in Figure 4. Equilibration of isopropylidene-(2*R*)-[1-^{2}H$_2$]glycerol[10] with [1-^{3}H]ethanol catalyzed by horse liver alcohol dehydrogenase followed by acid hydrolysis produced (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol as the only tritiated glycerol species.[12] Although the radiochemical yield is low, only 11.25 mCi of purified glycerol was obtained from ca. 200 mCi of [^{3}H]NaBH$_4$, this method has the advantage of great simplicity and convenience. Unfortunately it proved to be not practical to prepare the 1*S*,2*R* isomer by the same route from isopropylidene-(2*R*)-[1-^{3}H]glycerol and [1-^{2}H$_2$]ethanol. Unlike in the above situation, it is in this case essential that all the original label from the *pro-R* position of the isopropylideneglycerol be washed out; in our incubations we were not able to establish a clear end point for tritium exchange. Hence, we decided to carry out the investigation with only the 1*R*,2*R* isomer.

Samples of (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol, diluted with carrier material, were incubated with cells of *Klebsiella pneumoniae* mutant 62-1 in a medium supporting accumulation of chorismic acid. In an attempt to reduce the incorporation of label into the ring portion of chorismic acid, the cultures were supplemented with shikimic acid; however, as will be seen later, this was only moderately successful. Chorismic acid was obtained from these incubations in pure, crystalline form. On the basis of the known steric course of the glycerolkinase reaction[29] and on the work of Cohn et al.[30] on enolase, the (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol will generate, within the cells, PEP carrying deuterium in the H_Z and tritium in the H_E position (Figure 5). Hence, the resulting chorismate is formed from (*E*)-[3-^{2}H,^{3}H]PEP as a substrate.

To determine the side-chain configuration of the labeled chorismate formed, a sample was subjected to the degradation

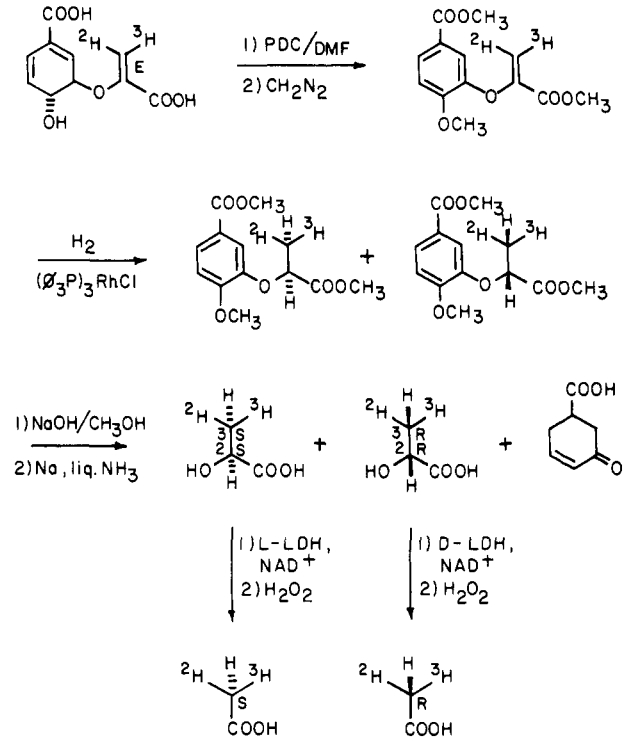

Figure 6. Degradation of chorismate to determine the side-chain configuration.

shown in Figure 6. Aromatization of the ring followed by hydrogenation of the side-chain double bond under homogeneous catalysis conditions and cleavage of the ether bond by Birch reduction produced D,L-lactate. By virtue of the strict cis addition of hydrogen in the hydrogenation reaction, in each enantiomer of the racemic lactate produced, the configurations at C-2 and C-3 will be correlated with each other in such a way that a deuterated, tritiated enolpyruvyl side chain of *E* configuration will produce (2*S*,3*S*)- + (2*R*,3*R*)-[3-^{2}H,^{3}H]lactate, whereas the corresponding side chain of *Z* configuration will result in (2*S*,3*R*)- + (2*R*,3*S*)-[3-^{2}H,^{3}H]lactate. In addition, in each case, the lactate will also contain tritiated methyl groups which are achiral because they arise from molecules containing tritium but not deuterium (species **1b** or **2b**, Figure 3). These will appear as racemic in the final assay, reducing the enantiomeric purity of the chiral methyl groups generated.

Separation of the two enantiomers of the D,L-lactate, accomplished by separate oxidation of aliquots to pyruvate with D- and L-lactate dehydrogenase, respectively, separates also the two enantiomeric chiral methyl groups generated in the hydrogenation reaction. Oxidation of the two pyruvate samples with H_2O_2[31] then gave two samples of acetic acid of, presumably, opposite configurations. These were subjected to chirality analysis by the method of Cornforth et al.[7] and Arigoni and co-workers.[8] This enzymatic procedure, which involves conversion of acetyl-CoA into malate with malate synthase followed by equilibration with fumarase, is based on a primary kinetic isotope effect in the malate synthase reaction. The configuration and enantiomeric purity of a chiral acetate sample is expressed as the *F* value,[9] a racemic sample giving *F* = 50 and enantiomerically pure (*R*)- and (*S*)-acetate showing *F* values of 79 and 21, respectively.[32] Duplicate analyses of the acetate from the oxidation with D-lactate dehydrogenase gave *F* = 55.1 and 56.4, indicating *R* configuration (17.6% and 22.0% ee, av 20% ee *R*). The materials from two oxidations with L-lacetate dehydrogenase, correspondingly, gave *F* = 45.5 and 44.5, indicating predominant *S* configuration (15.5% and 19.0% ee, av

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(32) The *F* value is the percentage of the tritium of malate remaining carbon bound upon equilibration with fumarase. *F* value and percent enantiomeric excess are related as: ee = {|*F* − 50|/29} × 100[%].

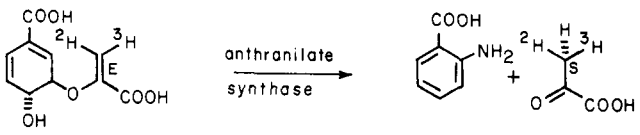

Figure 7. Steric course of the anthranilate synthase reaction.

17% ee *S*). Hence the stereochemical relationships between chorismate and the resulting acetate samples are as shown in Figure 6; i.e., the chorismate formed from (1*R*,2*R*)-[1-^{2}H,^{3}H]-glycerol via (*E*-[3-^{2}H,^{3}H]PEP has *E* configuration in the deuterated, tritiated side chain.

Anthranilate Synthase. Having at hand a sample of chorismate labeled stereospecifically in the side chain with known configuration, the stage was now set for experiments to elucidate the stereochemistry of the chorismate mutase and anthranilate synthase reactions. The steric course of the side-chain protonation in the latter reaction was determined by incubating an aliquot of the labeled chorismate with partially purified anthranilate synthase in the presence of excess lactate dehydrogenase and NADH. The lactate, resulting from the in situ reduction of the pyruvate generated from the chorismate side chain, was oxidized with dichromate to give acetate. Duplicate chirality analyses of this material gave *F* values of 44.0 and 45.1 (20.7% and 16.9% ee, av 19% ee *S*), indicating predominant *S* configuration of the pyruvate methyl group. It follows that the protonation of the enolpyruvyl side chain of chorismate in the anthranilate synthase reaction occurs on the *re* face (Figure 7).

Chorismate Mutase. To determine the steric course of the chorismate mutase reaction, another aliquot of the stereospecifically labeled chorismate was converted to phenylalanine and tyrosine by incubation with a wild-type culture of *E. coli*. The fermentation was carried out in a minimal medium, and the harvested cells were dried and subjected to acidic protein hydrolysis to liberate the amino acids. Tyrosine and phenylalanine were purified from the hydrolysate by preparative ion-exchange chromatography. Surprisingly, although the amounts of the two amino acids were comparable (1.3 mg vs. 1.9 mg from 114 mg of dried cells), the radiochemical yields based on chorismate differed substantially (tyrosine 0.22%, phenylalanine 2.5%).[33]

The configuration at the labeled methylene group of the tyrosine side chain was determined based on the same principle used for the configurational analysis of the chorismate, i.e., conversion of the methylene into a methyl group by stereospecific addition of an unlabeled hydrogen. The reaction used for this purpose is the α,β-elimination of phenol from tyrosine catalyzed by tyrosine phenol-lyase.[34] The amino acid side chain is obtained as pyruvate, following a protonation at C-β which has been shown[35] to occur stereospecifically on the same side from which the phenol substituent has departed, i.e., with retention of configuration at C-β. Incubation of the tyrosine from the protein hydrolysis with tyrosine phenol-lyase in the presence of excess lactate dehydrogenase and NADH produced lactate which was purified by paper chromatography and oxidized with dichromate to give sodium acetate in 9% radiochemical yield. Duplicate chirality analysis of this material gave *F* values of 55.1 and 57.0, corresponding to 19.3% and 24.1% ee, av 22% ee *R* isomer. The predominant *R* configuration of the methyl group indicates that the double-labeled methylene group in the tyrosine side chain has predominantly *R* configuration; i.e., C-1 of chorismate attacks the *si* face of the enolpyruvyl side chain methylene group (Figure 8).

The same stereochemistry was deduced for the chorismate mutase operating in phenylalanine biosynthesis. The labeled

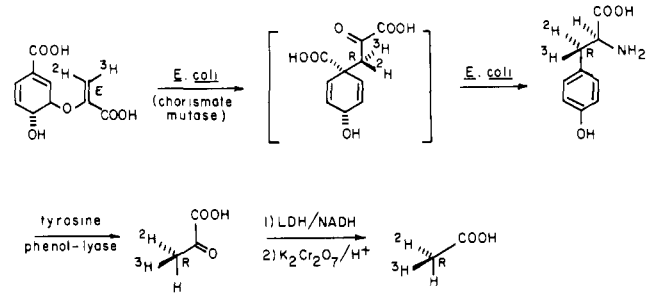

Figure 8. Steric course of the chorismate mutase reaction in *E. coli* and stereochemical analysis of the resulting tyrosine.

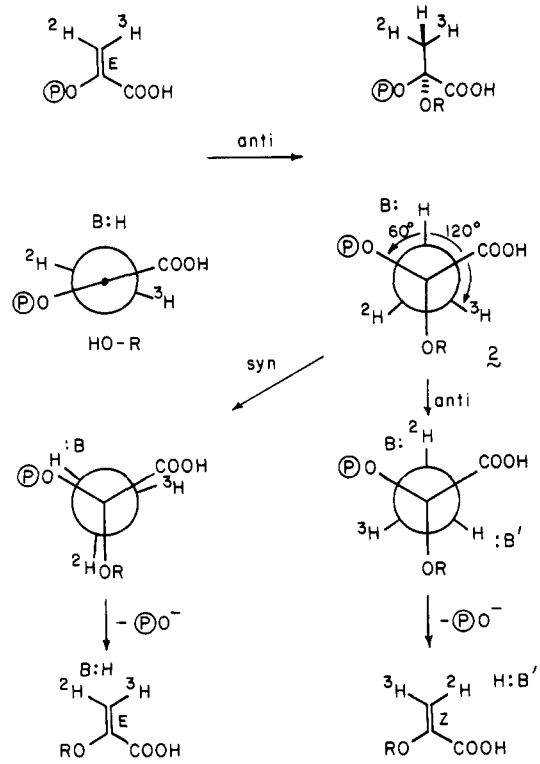

Figure 9. Stereochemistry of the ESP synthase reaction.

phenylalanine obtained from the *E. coli* protein hydrolysate was converted into tyrosine in 62% yield by oxidation with phenylalanine hydroxylase. Samples of the resulting tyrosine were then subjected to the enzymatic degradation with tyrosine phenol-lyase, and the lactate samples were oxidized to acetate. Chirality analysis of acetate samples from two separate experiments gave *F* values of 54.8 and 56.0, corresponding to 16.6% and 20.7% ee, av 19% *R* ee isomer. In view of the finding of Grimshaw et al.[26] that the deprotonation step in the ESP synthase reaction involves a deuterium isotope effect of nearly 2, an attempt was made to detect an unequal tritium distribution between the two methylene hydrogens of phenylalanine by incubation with phenylalanine ammonia-lyase. This enzyme eliminates the elements of ammonia from L-phenylalanine to give *trans*-cinnamic acid with stereospecific removal of the side-chain *pro*-3*S* hydrogen.[18,36] In a number of experiments, tritium losses from our labeled phenylalanine of 11–17% were seen. However, it was also found in the tyrosine phenol-lyase reaction that over 50% of the tritium of the amino acid resides in the aromatic ring. These figures and a consideration of the degrees of enantiomeric purity of the methyl groups observed made it clear that it would be difficult to confirm the chirality of the phenylalanine by this method, particularly when only one isotopic stereoisomer is available.

(33) The only difference in terms of labeling patterns is the absence of H-4 of chorismate in tyrosine. Since, based on known metabolic pathways, this position in the chorismate is not expected to carry much tritium, the reason for the difference in radiochemical yields must be sought in the differential kinetics of tyrosine and phenylalanine synthesis and utilization during the course of the experiment.

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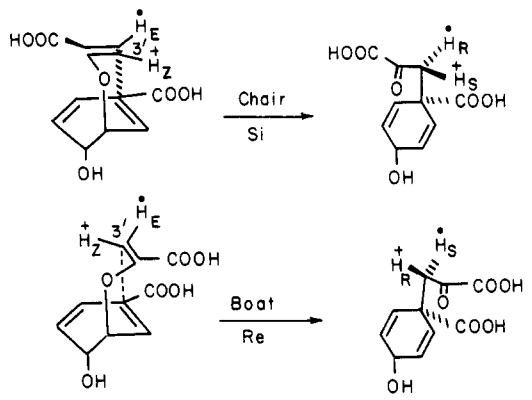

Figure 10. Two possible transition states in the chorismate mutase reaction.

Discussion

The work described here establishes the steric course of the reactions catalyzed by the enzymes ESP synthase, chorismate mutase, and anthranilate synthase. Our results on the first two enzymes agree with those reported recently in preliminary communications from the laboratories of Knowles[37,38] and Berchtold.[38,39] Their work differs from ours in that the ESP synthase and chorismate mutase reactions were carried out in vitro with isolated enzymes.

The finding that ESP synthase generates a deuterated, tritiated enolpyruvyl side chain of *E* configuration from (*E*)-[3-^{2}H,^{3}H]PEP, interpreted in terms of Levin and Sprinson's[4] addition/elimination mechanism (Figure 2), means that the two half reactions must occur with opposite stereochemistry, i.e., one syn and one anti. This is consistent with recent rsults from Abeles laboratory[25] indicating that the two half reactions are not concerted. They observed that ESP synthase in the presence, but not in the absence, of the substrate analogue 4,5-dideoxyshikimate-3-phosphate, which lacks the hydroxyl group involved in the addition reaction, catalyzes tritium exchange from solvent into PEP but not exchange between Pi and PEP nor "scrambling" of the bridge oxygen in PEP. They concluded that the formation of **2** (Figure 9) must proceed via a covalent enzyme–PEP intermediate, in which the methylene group of PEP is protonated. In the presence of the true substrate, shikimate-3-phosphate, **2**, could be formed from such an intermediate by replacement of an enzyme X^- group with ROH, or the process may involve a sequence of two addition/elimination cycles and three covalent intermediates, where C-3 would undergo protonation/deprotonation twice. The presence of an enzyme–PEP intermediate notwithstanding the stereochemical outcome of the ESP synthase reaction probably reflects a minimal motion pathway. As illustrated in Figure 9, the conversion of species **2** generated, for argument's sake, by an anti addition into product by a syn elimination requires only a 60° rotation around the C-2/C-3 axis. Although the rotation of the methyl group itself is of no concern, it has in fact been shown[26] to be fast, the proton addition and abstraction requires a base group on the enzyme which must change its orientation relative to the incoming and the leaving group at C-2. In an anti/syn or syn/anti addition/elimination sequence, a single base can mediate both the proton addition and abstraction at C-3 with only a modest amount of motion relative to the substituents at C-2. On the other hand, elimination of H^+ and PO_4^- from **2** with the same stereochemistry as the addition step, both anti or both syn, would necessitate a 120° rotation around the C-2/C-3 axis. This would either require a more extensive active site reorientation during the catalytic process or the reaction would have to involve two different base groups mediating proton addition and abstraction. The latter option obviously would require additional, diffusion-controlled proton-transfer steps which are likely to be slow. Thus, the stereochemistry observed for the ESP synthase reaction may be viewed as a consequence of an evolution toward catalytic efficiency.

The configurational purity of the chorismate side chain generated from PEP in the in vivo system is substantially below the theoretical maximum, as judged from the *F* values of the chiral acetate samples obtained in the degradation. If the starting (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol was enantiomerically pure, the observed[26] deuterium isotope effect in the ESP synthase reaction of about 2 predicts that ESP generated in a single protonation/deprotonation cycle should consist of 67% **2a** and 33% **2b** (Figure 3); the *F* values of the acetate samples from the degradation of this material would be 31 and 69. The observed *F* values indicate that C-3 of the enolpyruvyl moiety on the average undergoes protonation/deprotonation 3–4 times before its final conversion to chorismate. The true value will be lower since the glycerol may not be 100% enantiomerically pure and other metabolic processes in the in vivo system may contribute to racemization.

The stereochemical outcome of the anthranilate synthase reaction, formation of predominantly *S* methyl groups from the (*E*)-[3′-^{2}H,^{3}H]chorismate, indicates that the protonation has occurred predominantly on the *re* face of C-3 of the enolpyruvyl side chain of chorismate. This result is of no apparent mechanistic significance. Interestingly, however, it contrasts with the steeochemistry, *si* attack at C-3, observed for most of the enzymes catalyzing electrophilic attack at C-3 of PEP which have been studied so far.[40] A common evolutionary origin of these enzymes from the same ancestral protein has been suggested as the explanation for this stereochemical consistency.[40] If this is correct, anthranilate synthase is clearly not a member of this evolutionary family.

The chorismate mutase reaction, perhaps the only example of an enzyme-catalyzed pericyclic reaction, can proceed either through a chair transition state, involving *si* attack at C-3′, or through a boat transition state with *re* attack at C-3′. Calculations indicate a moderate preference for a chair transition state in the uncatalyzed reaction,[41] but in the enzyme-catalyzed process this could conceivably be superseded by other effects. The result of this study, showing that H_E of the chorismate side chain becomes H_R in the prephenate side chain methylene group, demonstrates that the enzymic process does indeed proceed through a chair transition state. Circumstantial evidence supporting this conclusion had already been provided earlier by Andrews et al.,[42] who studied several rigid analogues of chorismate and found that only a compound resembling the geometry of a chair transition state was an effective inhibitor of chorismate mutase. Of course, the demonstration that the reaction proceeds through a chair transition state does not prove that the process is also concerted. Addadi et al.[43] recently tried to probe for concertedness of the reaction by measuring secondary tritium isotope effects at C-3 and C-3′ of chorismate. While the results of the uncatalyzed reaction point to an early, unsymmetrical transition state, the absence of detectable secondary isotope effects in the enzymatic process indicates that here the rearrangement itself is not rate-limiting but that it is preceded by another, rate-limiting process. All the results are conveniently accommodated by a sequence of events involving binding to the enzyme of the most stable conformer of chorismic acid, in which the side chain at C-3 and the 4-OH are both equatorial,[44] a rate-limiting conformational change of the enzyme-substrate complex converting the chorismate to the diaxial conformer in which the side chain is properly oriented for a chair transition state, followed by rapid [3,3]-sigmatropic rearrangement to produce the enzyme-bound prephenic acid.

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Registry No. 1, 89771-75-5; **2**, 617-12-9; ESP synthase, 9068-73-9; chorismate mutase, 9068-30-8; anthranilate synthase, 9031-59-8; (1*R*,2*R*)-[1-^{2}H,^{3}H]glycerol, 90195-13-4; methyl 3-[(1-carbomethoxyvinyl)oxy]-4-methoxy benzoate, 81776-92-3; methyl 3-[(1-carbomethoxyethyl)oxy]-4-methoxybenzoate, 96454-31-8; tritiated D,L-lactic acid, 96553-57-0.

Complex Isomerization of Ketoses: A ^{13}C NMR Study of the Base-Catalyzed Ring-Opening and Ring-Closing Rates of D-Fructose Isomers in Aqueous Solution

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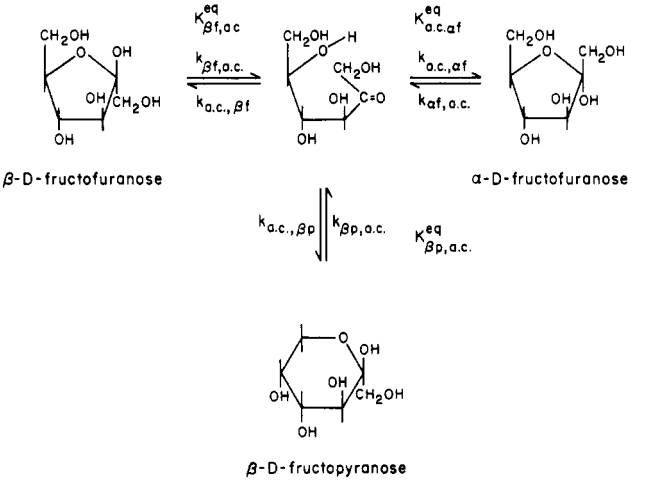
Abstract: In the present study, the isomerization of D-[2-^{13}C]fructose has been characterized under alkaline conditions with ^{13}C NMR spectroscopy. No detectible resonances arising from the open-chain hydrate or α-D-fructopyranose could be observed in ^{13}C NMR spectra acquired above pH 7 and between 17 and 50 °C. Resonances in spectra arising from the ^{13}C-enriched carbon of the α-furanose and β-furanose and the acyclic keto form of D-fructose were observed to broaden drastically with increasing pH or temperature while, in the same spectra, the line width of the anomeric carbon resonance of the β-pyranose form remained less than 2 Hz. Apparent first-order rate constants for ring opening of the α- and β-furanose forms of the sugar ($k_{\alpha f,a.c.}$, $k_{\beta f,a.c.}$) were determined by fitting measured line widths to a model for three-site exchange. At all pH values and temperatures studied, it was found that $k_{\beta f,a.c.}$ was nearly twice as large as $k_{\alpha f,a.c.}$ Second-order rate constants for furanose ring opening were determined from the pH dependence of the apparent first-order rate constants and were found to be (2.88 ± 0.5) × 10^6 and (3.6 ± 0.6) × 10^6 M^{-1} s^{-1} for ring opening of the α-furanose and β-furanose forms, respectively. Thermodynamic activation parameters, $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}_{298}$, and $\Delta S^{\ddagger}_{298}$, were determined from the temperature dependence of $k_{\alpha f,a.c.}$ and $k_{\beta f,a.c.}$ at pH 8.4. Under these conditions, $\Delta S^{\ddagger}_{298}$ was found to be positive for both furanose ring-opening reactions, in marked contrast to the large negative $\Delta S^{\ddagger}_{298}$ determined from furanose ring-opening rates of D-galactose, D-threose, D-erythrose, and 2-deoxy-D-ribose under acidic conditions. The positive $\Delta S^{\ddagger}_{298}$ values have been rationalized in terms of a model involving the organization of polar solvent around the stabilized D-fructofuranose anions. Values of $\Delta H°$, $\Delta G°_{298}$, and $\Delta S°_{298}$ characterizing interconversions between cyclic structures and between cyclic and acylic forms were determined from the temperature dependence of equilibrium constants at pH 8.4. In all cases, $\Delta G°_{298}$ is determined both in sign and magnitude by $\Delta H°$. Apparent first-order ring-closing rates to D-fructofuranoses were determined from ring-opening rate constants, $k_{\alpha f,a.c.}$ and $k_{\beta f,a.c.}$, and corresponding equilibrium constants, $K^{eq}_{\alpha f,a.c.}$ and $K^{eq}_{\alpha f,a.c.}$ At all temperatures studied, the ring-closing rate to β-fructofuranose was found to be roughly five times that of the corresponding rate to the α-furanose form. Inversion-transfer ^{13}C NMR experiments were carried out on D-[2-^{13}C]fructose at pH 8.4 and 27 °C in order to further characterize the relatively slow β-pyranose ring-opening and ring-closing rates ($k_{\beta p,a.c.}$ and $k_{a.c.,\beta p}$). From inversion-transfer data and equilbrium intensities, an upper limit of 0.1 s^{-1} was determined for $k_{\beta p,a.c.}$ and 20 s^{-1} for $k_{a.c.,\beta p}$. Under identical conditions, ring-closing rate constants to the α- and β-furanose forms were found to be about 80 and 500 s^{-1}.

Reducing sugars are known to exist in aqueous solution as complex equilibrium mixtures of isomeric forms, including pyranoses, furanose, and septanoses.[1-5] Interconversions between these forms is thought to proceed through a series of steps involving an initial base or acid-catalyzed ring opening to an acyclic keto or aldehyde intermediate in equilibrium with an open-chain hydrate (*gem*-diol), followed by a conformational rearrangement in acyclic structure and final ring-closing (Scheme I).[6,7] In the case of most simple pentoses and hexoses, ring-closing reaction rates are typically 10-1000 times faster than corresponding ring-opening rates,[8-12] making the amount of acyclic and open-chain forms present at equilbrium quite small (usually less than 1%). Many chemical and biochemical reactions involving monosaccharides involve only one of the isomeric forms present in

Scheme I



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