1 H, cyclopropyl), 2.41 (d, J = 6 Hz, 1 H cyclopropyl), 5.84 (d, J = 2 Hz, 1 H, Ar), 6.53 (br d, 1 H, Ar), 7.1-7.8 (m, 9 H, Ar). MS (70 eV), m/e (relative abundance) 265 (100), 84 (78), 350 (69). Molecular ion calcd for $C_{22}H_{16}Cl_2$, m/e 350.0675; found 350.0670. 2,7-Dibromocyclopropane: 79% yield; ^{1}H NMR ($CDCl_3$) δ 1.78 (s, 3 H), 2.09 (d, J = 6 Hz, 1 H), 2.40 (d, J = 6 Hz, 1 H), 5.84 (d, J = 2 Hz, 1 H), 6.52 (br d, 1 H), 7.2-7.7 (m, 9 H). MS (70 eV), m/e (relative abundance) 265 (100), 440 (66), 280 (31). Molecular ion calcd for $C_{22}H_{16}Br_2$, m/e 437.9628; found 437.9623. 2,7-Diiodocyclopropane: 76% yield; ^{1}H NMR ($CDCl_3$) δ 1.7 (s, 3 H), 2.08 (d, J = 6 Hz, 1 H), 2.39 (d, J = 6 Hz, 1 H), 6.03 (d, J = 1 Hz, 1 H), 6.50 (br d, 1 H), 7.2-7.7 (m, 9 H). MS (10 eV), m/e (relative abundance) 265 (96), 534 (100). Molecular ion calcd for $C_{22}H_{16}I_2$, m/e 533.9340; found 533.9339. 2,2',7,7'-Tetraiodo dimeric azine: 5% yield; mp >250 °C. Anal. Calcd for $C_{26}H_{12}N_2I_4$: C, 36.31; H, 1.41; N, 3.26. Found: C, 36.36; H, 1.61; N, 3.56. MS (70 eV), m/e (relative abundance) 860 (69), 733 (18), 324 (24). Molecular ion calcd for $C_{26}H_{12}N_2I_4$, m/e 859.7185; found 859.7191.

Triplet-Sensitized Irradiation of XDAF (X = H, Cl, Br, I) in the Presence of Methyl Alcohol. Four nitrogen-purged 5.0 M MeOH/CH_3CN solutions (25.0 mL) containing one of the XDAF (1.5×10^{-3} M for X = H, Cl, Br; 1.0×10^{-3} M for X = I) and 2,3-benzofluorenone (4.5×10^{-3} M) were prepared. The samples were individually irradiated (>398 nm) to completion (50 min) through a Corning 3-74 filter with a 200-W high-pressure mercury arc lamp. Under these conditions, nearly all the light is absorbed by the 2,3-benzofluorenone. The solvent was removed under vacuum. The ^{1}H NMR spectra were recorded with p-dioxane as internal standard. Three products were observed in each case. X = H: ether 84%, fluorene 7%, 9-fluorenyl dimer 4%. X = Cl: ether 26%, dichlorofluorene 9%, tetrachloro-9-fluorenyl dimer 22%. X = Br: ether 29%, dibromofluorene 6%, tetrabromo-9-fluorenyl dimer 20%. X = I: ether 28%, diiodofluorene 6%, tetraiodo-9-fluorenyl dimer 14%.

Triplet-Sensitized Irradiation of XDAF with α-Methylstyrene. Four N_2-purged solutions of α-methylstyrene (0.1 M) in CH_3CN (25 mL) and benzofluorenone were prepared and irradiated according to the procedure described above. The expected cyclopropanes are the major products (X = H, 95%; X = Cl, 94%; X = Br, >95%; X = I, 92%).

Irradiation of XDAF with (E)-α-Methyl-β-deuteriostyrene.[28a,b] Three N_2-purged solutions of the deuteriated styrene in CH_3CN (A, 0.1 M; B, 2.15 M; C, neat) containing CDAF (1×10^{-2} M) were prepared and irradiated (350 nm) until ca. 80% of the diazo compound was consumed (UV). The solvent was removed and the unreacted α-methylstyrene was collected by bulb-to-bulb distillation. The ^{1}H NMR spectra of the cyclopropanes is the same as for the undeuteriated sample except that the cyclopropyl protons appear as singlets. Integration of these spectra gives the stereochemical results reported above. The NMR spectrum of the recovered α-methylstyrene showed that no significant isomerization had occurred. A control experiment showed that cyclopropane formation required light. Similar experiments were performed for BDAF and for IDAF.

Triplet-Sensitized Photolysis of XDAF with (E)-α-Methyl-β-deuteriostyrene. Two N_2-purged solutions of the deuteriated styrene in CH_3CN (A, 0.1 M; B, 1.0 M) containing CDAF (1×10^{-2} M) and benzofluorenone were irradiated through a Corning 3-74 filter (>398 nm). After irradiation the solvent was removed and ^{1}H NMR spectra of the product and unreacted deuteriated methylstyrene were recorded. The unreacted styrene was not isomerized. An additional control experiment showed that the cyclopropanes are not isomerized by the reaction conditions. The stereochemistry of the cyclopropane product was determined from integration of the NMR spectrum. Retention of configuration was 0% for A and <5% for B. Similar experiments were carried out (0.1 M α-methylstyrene) for BDAF, IDAF, and DAF. For these three compounds, cyclopropane was formed with 0%, 0%, and 16% retention, respectively.

Triplet-Sensitized Competition between Methyl Alcohol and α-Methylstyrene. Four samples were prepared containing CDAF (1.5×10^{-3} M), benzofluorenone (4.5×10^{-3} M), methyl alcohol (5.0 M), and α-methylstyrene (0.03-0.3 M) in CH_3CN (25 mL). The samples were purged with N_2 and irradiated (>398 nm) for 50 min when >95% of the diazo compound was consumed (UV). The yields of the previously identified ether and cyclopropane products were determined by NMR spectroscopy and plotted as shown on Figure 4.

BDAF was similarly examined (α-methylstyrene 0.03-0.2 M) as was IDAF (9.0×10^{-4} M). For DAF the concentration of methyl alcohol was lowered to 1.0 M because at the higher concentration an insignificant yield of cyclopropane resulted. The results of these experiments are also shown on Figure 4.

Finally, a related competition experiment with the α-methylstyrene concentration held constant (0.1 M, X = Cl, Br; 1.0 M, X = H) and the methyl alcohol concentration varied (1.0-4.0 M, X = Cl, Br; 0.05-0.4 M, X = H) gives exactly analogous results.

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The Conformational Equilibrium of Chorismate in Solution: Implications for the Mechanism of the Non-Enzymic and the Enzyme-Catalyzed Rearrangement of Chorismate to Prephenate

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Abstract: The temperature variation of the ^{1}H NMR coupling constants of chorismic acid and of the bis(tetra-n-butylammonium) salts of chorismate and of 4-O-methylchorismate in water and in methanol has been studied. The results show that 10-40% of each of these species is present in the pseudo-diaxial form in aqueous solution at 25 °C. In methanol solution, chorismate exists as the pseudo-diequatorial conformer. The rate of the Claisen rearrangement of chorismate is 100 times slower in methanol than in water, while the rearrangements of chorismic acid and of 4-O-methylchorismate are slowed by 11-fold and 7-fold, respectively. These results together suggest that the non-enzymic rearrangement of chorismate involves a dipolar transition state having some of the character of a tight ion pair between the enol pyruvate anion and the cyclohexadienyl cation. The relatively small difference in the free energies of the two conformers of chorismate in aqueous solution further suggests that the enzyme chorismate mutase can directly select the pseudo-diaxial conformer (from which the Claisen rearrangement occurs) from solution.

Chorismate (**1**) occupies a central position in the biosynthesis of the three aromatic amino acids and of a number of other metabolites in microorganisms and plants.[1] In the path to phenylalanine and tyrosine, chorismate undergoes what is formally

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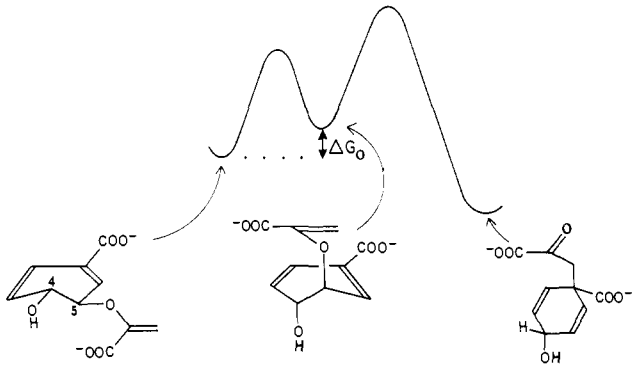
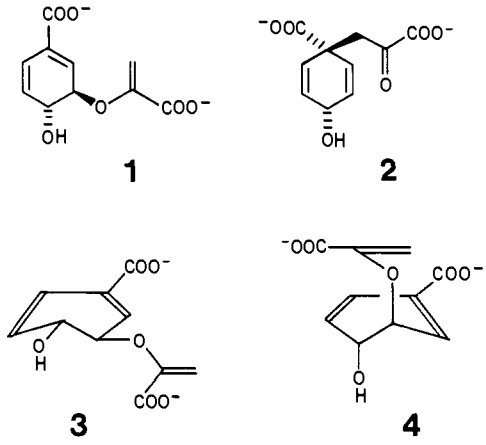


Figure 1. Free energy profile for the conformational equilibrium of chorismate and the rearrangement of the pseudo-diaxial conformer to prephenate.

a Claisen rearrangement to prephenate (**2**). While this transformation occurs reasonably easily in the absence of catalysts (the half-life of chorismate in neutral buffer at 50 °C is about 90 min), the enzyme chorismate mutase accelerates the rearrangement by more than 10^6-fold.[2] The mutase, as the only characterized enzyme catalyzing what appears to be a pericyclic reaction, has attracted considerable attention,[3-7] but the mechanism of the enzymic process remains mysterious. In this and the following paper, we report the results of experiments designed to define the conformation of chorismate in solution and to illuminate the nature of both the non-enzymic and the enzyme-catalyzed transformations.



The non-enzymic rearrangement of chorismate is an intramolecular reaction, and we have earlier shown that it proceeds via a transition state of chair-like geometry.[8] The conformer of chorismate from which rearrangement occurs has the hydroxyl group and the enol pyruvoyl group axial (**4**), yet the more stable conformer that predominates in solution is the pseudo-diequatorial one (**3**).[9] In order to understand what the enzyme must achieve to accelerate the rearrangement, we must define the position of the conformational equilibrium. Early extended Hückel calculations[2] suggested that the potential energy difference between the pseudo-diaxial and pseudo-diequatorial conformers of chorismate was as high as 7 kcal/mol (equivalent to a ratio of 10^5 at 25 °C). Later, however, using MINDO/3, it was concluded[10] that the chorismate ring is close to planarity and that the diequatorial conformation is "only marginally" favored. As will be evident from what follows, it is important to know whether the proportion of the pseudo-diaxial conformer is a significant or only a vanishingly small fraction of the total chorismate in solution. While there was a hint in the literature,[11] from the solvent dependence of the $J_{H,H}$ coupling constants, that changes in the conformational equilibrium of chorismate in solution could be observed, no previous work allowed an estimate of the position of this equilibrium. In the terms of Figure 1, we need to have some idea of the value of ΔG_0.

In an attempt to define the nature of the transition state for the rearrangement of chorismate to prephenate, we earlier measured[7] secondary tritium isotope effects to probe the extent of bond breaking (using [5-^{3}H]chorismate) and of bond making (using [9-^{3}H]chorismate) at the transition state of both the non-enzymic and the mutase-catalyzed reaction. The non-enzymic reaction showed a substantial effect ($k_H/k_T = 1.15$) at the bond-breaking position and no detectable effect at the bond-making position, indicating 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 contrast, the values for k_H/k_T for the mutase-catalyzed reaction were unity in both positions. It is possible that these isotope effects are suppressed in the enzymic reaction because the rate-limiting transition state occurs *before* the rearrangement itself. This led us[7] to suggest that the enzymic process follows one of two paths. In the first possibility, the enzyme would have a rigid binding site that selectively binds the pseudo-diaxial conformer, and the reaction of this minor conformer would be diffusion controlled. The second possibility is that the enzyme binds chorismate in its preferred pseudo-diequatorial conformation and that binding is followed by a conformational isomerization of the enzyme–substrate complex that converts the substrate into the diaxial form appropriate for the covalent rearrangement. While there are other possibilities for the identity of the isotopically silent rate-limiting transition state (a number of which are examined in the succeeding paper[12]), it is clear that progress in our understanding of the mechanism of the enzymic process requires some definition of the nature of the substrate in solution. This paper addresses that issue.

Experimental Section

Materials. Chorismic acid was isolated from the fermentation of *Klebsiella pneumoniae* 62-1 by the method of Gibson[13] as modified by Addadi et al.[7]

Prephenate was prepared by equilibration of chorismate with chorismate mutase in 50 mM *N*-ethylmorpholine-2-(*N*-morpholino)ethanesulfonic acid buffer, pH 7.5, containing EDTA (1 mM), dithioerythritol (1 mM), and bovine serum albumin (0.1 mg/mL). The product prephenate was purified by chromatography on a column (20 mL) of DEAE-cellulose equilibrated with 40 mM triethylammonium bicarbonate buffer, pH 7.5, and eluted with a linear gradient (150 mL + 150 mL, 40–150 mM) of triethylammonium bicarbonate buffer, pH 7.5. The fractions containing prephenate were concentrated by repeated evaporation of added 2-propanol, using NaOH to maintain the pH above 7.0.

Phenylpyruvate was prepared by treatment of prephenate with 0.001 N HCl for several hours.

p-Hydroxybenzoic acid was purchased from Aldrich. D_2O (99.8%) and methanol-d_4 were obtained from Merck and Co. DCl was obtained from Stohler/Kor. 4-*O*-Methylchorismate was a generous gift from Dr. R. Padykula.

Methods. ^{1}H NMR spectra of chorismic acid in 0.1 N DCl in D_2O and in methanol-d_4, of bis(tetrabutylammonium)chorismate in D_2O, and of bis(tetrabutylammonium)-4-*O*-methylchorismate in D_2O and in methanol-d_4 were taken on a Bruker AM500 spectrometer. Spectra of

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bis(tetrabutylammonium)chorismate in methanol-d_4 were taken on a Bruker AM300 spectrometer. Data resolution was optimized by applying negative line broadening and Gaussian multiplication prior to Fourier transformation.

Reactions of bis(tetrabutylammonium)chorismate in H_2O and in methanol were carried out by heating solutions (0.8 mM) at 50 °C and analyzing portions at intervals by high-performance liquid chromatography on a Pharmacia Polyanion SI HR5/5 column. The column was washed with 750 mM sodium phosphate buffer, pH 7.0, and then with H_2O, prior to sample injection. After injection, the column was washed with H_2O and eluted with 113 mM sodium phosphate buffer, pH 7.0. The amounts of the components of the reaction mixture were estimated by cutting and weighing. Calibration curves were prepared for chorismate, prephenate, phenylpyruvate, and *p*-hydroxybenzoate. Concentrations of standard solutions were determined by assay with chorismate mutase for chorismate,[7] by assay with prephenate dehydrogenase for prephenate,[14] by A_{320nm} in the presence of NaOH (0.8 N) for phenylpyruvate,[15] and by weight for *p*-hydroxybenzoate. Reactions of chorismic acid in 0.1 N HCl and in methanol were carried out by heating solutions (1 mM) at 50 °C and analyzing portions at intervals for the remaining starting material with chorismate mutase. The distribution of products was determined by NMR.

Reactions of bis(tetrabutylammonium)-4-*O*-methylchorismate in H_2O and in methanol were carried out by heating solutions (1 mM) at 50 °C and analyzing portions at intervals for the remaining starting material by the HPLC procedure described above. In this case, only the area under the 4-*O*-methylchorismate peak was determined. The distribution of products was determined by NMR.

In all cases, the rate of disappearance of starting material was fitted to the equation $A = A_0e^{-kt}$. The rate constants for appearance of individual products were determined from the average of the measured product ratios. Under some conditions, prephenate and 4-*O*-methylprephenate rapidly decompose to phenylpyruvate. In these cases, the rate constant for the Claisen rearrangement was determined from the sum of the amounts of prephenate and phenylpyruvate formed.

Results and Discussion

The Conformation of Chorismate in Solution. NMR provides the best means for determining conformations of molecules in solution. The well-known Karplus relationship and its various modifications provide a basis for relating the coupling constant between two protons on adjacent carbon atoms to the dihedral angle between them.[16-19] For a molecule that is interconverting rapidly between two conformational states, the observed coupling constant is the weighted average of the coupling constants for the individual conformers X and Y, J^X and J^Y

$$J^{obsd} = fJ^X + (1 - f)J^Y \quad (1)$$

where f is the fraction of conformer X at equilibrium. Therefore, if J^X and J^Y can be determined, the distribution of the molecule between the conformers can be determined. In ideal cases, J^X and J^Y can be determined by obtaining spectra at temperatures low enough that the interconversion between conformers is slow and separate resonances for the two conformers can be observed. For many molecules, however, rapid equilibration between conformers persists at low temperatures so that it is not possible to obtain spectra for either, never mind both, of the individual conformers. In such cases, values for J^X and J^Y may be estimated by using another method that involves measuring the temperature dependences of the observed coupling constants. A change in an observed coupling constant with temperature can be interpreted as being due to a change in f (i.e., a shift in the conformational equilibrium) as long as the intrinsic coupling constants for each of the two conformers, J^X and J^Y, are themselves independent of temperature. This independence is important, since the goal of the analysis is to determine f from the value of J^{obsd} at a particular temperature. Vicinal coupling constants that are independent of temperature for conformationally rigid systems have been reported by several authors.[20-22]

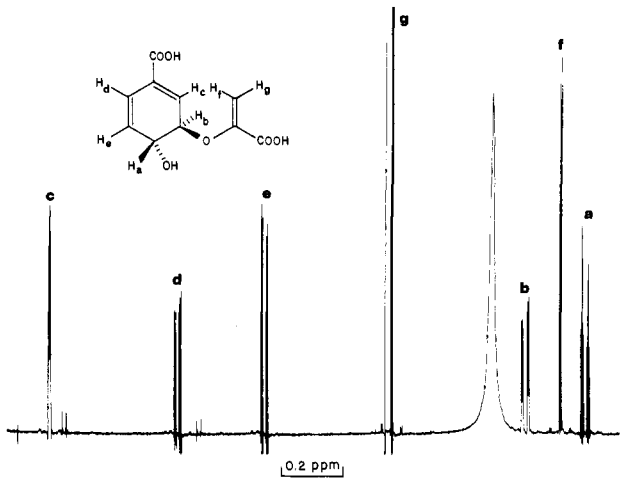

Figure 2. ^{1}H NMR spectrum of chorismic acid in methanol-d_4. The chemical shifts (relative to CH_3OH at 3.30) are as follows: H_a 4.56; H_b 4.80; H_c 6.72; H_d 6.20; H_e 5.85; H_f 4.66; and H_g 5.34.

Estimates for the values of J^X and J^Y may be obtained by the method of Bowmaker et al.[22] Any two observed coupling constants J_u and J_v that vary with temperature may be related by elimination of f from the pair of equations of the form of eq 1, to give

$$J_u^{obsd} = \frac{J_v^{obsd}(J_u^X - J_u^Y)}{(J_v^X - J_v^Y)} + \frac{(J_v^X J_u^Y - J_v^Y J_u^X)}{(J_v^X - J_v^Y)} \quad (2)$$

This equation expresses the fact that a shift in the conformational equilibrium occurring over a range of temperature should result in *related* changes in the observed values of the coupling constants. A plot of J_u^{obsd} vs. J_v^{obsd} gives values for the slope and intercept. These values are functions of the coupling constants for the individual conformers, which can be solved to give estimates for J_u^X, J_u^Y, J_v^X, and J_v^Y, the intrinsic coupling constants for conformers X and Y. Provided that these intrinsic coupling constants are independent of temperature, then eq 1 may be used to estimate f (and therefore ΔG_0) from the observed coupling constants at a particular temperature.

The conformations of chorismic acid and of bis(tetra-*n*-butylammonium)chorismate in solution have been investigated by studying the temperature dependences of several coupling constants. A typical spectrum of chorismic acid in methanol is shown in Figure 2. Peak assignments are taken from Young et al.[23] Four coupling constants—$J_{a,b}$, $J_{a,e}$, $J_{b,c}$, and $J_{d,e}$—are first order and easily determined from the spectral data. These four coupling constants contain information about the conformation of the molecule and can be analyzed as described above on the basis of an equilibrium between two limiting conformations. For example, the dihedral angle between protons a and b is about 170° in the pseudo-diequatorial conformer, but only 70° in the pseudo-diaxial conformer. The Karplus relationship predicts that $J_{a,b}$ should be larger for the diequatorial conformer than for the diaxial conformer. Similar analysis for $J_{a,e}$ and $J_{b,c}$ shows that, in contrast to $J_{a,b}$, these coupling constants should be smaller in the diequatorial conformer than in the diaxial conformer. Finally, the dihedral angle between protons d and e does not change between the two conformers, and $J_{d,e}$ should be the same for both. $J_{d,e}$ can therefore serve as a control for the temperature independence of the intrinsic constants. Only data for which $J_{d,e}$ is independent of temperature are used in this analysis.

The variations of the observed coupling constants with temperature between 245 and 325 K for chorismic acid in methanol are shown in Figure 3. The changes in the coupling constants are consistent with a shift toward the diequatorial conformer as

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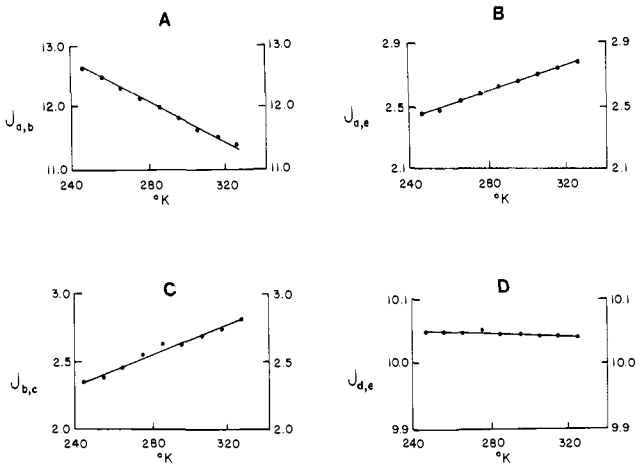

Figure 3. Observed H–H coupling constants (in Hz) for chorismic acid in methanol-d_4 as a function of temperature. The coupling protons are identified by subscript letters (see Figure 2).

the temperature is lowered. That is, $J_{a,b}$ increases and $J_{a,e}$ and $J_{b,c}$ decrease. $J_{d,e}$ is essentially independent of temperature, as required.

Relating observed coupling constants to each other as described by Bowmaker et al.[22] gives

$$J_{a,b}^{obsd} = (-3.793)(J_{a,e}^{obsd}) + 21.90 \qquad r = 0.999 \qquad (3)$$

$$J_{a,b}^{obsd} = (-2.627)(J_{b,c}^{obsd}) + 18.75 \qquad r = 0.992 \qquad (4)$$

$$J_{a,e}^{obsd} = (0.6945)(J_{b,c}^{obsd}) + 0.8273 \qquad r = 0.996 \qquad (5)$$

The expressions for the slopes and intercepts of eq 3–5 provide a series of six equations in the six intrinsic coupling constants. Unfortunately, these six equations are not independent, and sets of solutions for the values of the intrinsic coupling exist. None of the coupling constants can be determined experimentally, since the coupling constants do not become temperature independent at the low end of the experimental temperature range due to the presence of only one conformer. Any acceptable set of solutions must, however, fall within acceptable limits for each of the six intrinsic coupling constants. These limits are discussed below.

First, $J_{a,b}$ for the pseudo-diequatorial conformer must be greater than 12.6 Hz, the largest observed value, and analogously, $J_{b,c}$ and $J_{a,e}$ for this conformer must be less than 2.3 and 2.5 Hz, respectively. Narrower limits on these coupling constants for the diequatorial conformer and limits on the coupling constants for the diaxial conformer can be obtained from model compounds. The vicinal coupling constants for a variety of conformationally rigid cyclohexenones[24] and cyclohexanes[20] have been determined, and reasonable estimates for the coupling constants for chorismate can be deduced from these data. Thus for rigid cyclohex-2-en-1-ones, the values for $J_{3,4axial}$ are around 1.9 Hz, and those for $J_{3,4equatorial}$ are around 6 Hz.[24] Conservatively assuming an uncertainty of 1 Hz leads to estimates for $J_{a,e}$ and $J_{b,c}$ for the pseudo-diequatorial conformer of chorismate of 1.4–2.4 Hz and for $J_{a,e}$ and $J_{b,c}$ for the pseudo-diaxial conformer of chorismate of 5.5–6.5 Hz. On the basis of the J values for chorismate in methanol, which appears to reside exclusively in the diequatorial form (see below), the range for $J_{a,e}$ for the diequatorial form becomes 0.75–2.4 Hz. From the published data on conformationally restricted cyclohexanes,[17] analogous arguments lead to estimates for $J_{a,b}$ of chorismate of 2.8–3.8 Hz for the diaxial conformer and 12.5–13.5 Hz for the diequatorial conformer. The range of $J_{a,b}$ values for the diequatorial conformer is constrained a little more by the observed value, since $J_{a,b}^{obsd}$ is 12.6 Hz at 245 K. The boundary conditions for all the relevant J values are listed in Table I. Now, using eq 3–5 and values for $J_{a,b}^{eq}$ and $J_{a,b}^{ax}$ over their expected ranges, we may calculate the other four J values

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Table I. Predicted Ranges (Hz) for the J Values of Chorismic Acid in Methanol

	diequatorial conformer	diaxial conformer
$J_{a,b}$	12.6–13.5	2.8–3.8
$J_{a,e}$	1.4–2.4[a]	5.5–6.5
$J_{b,c}$	1.4–2.4	5.5–6.5

[a] This becomes 0.75–2.4 Hz, if the value for chorismate in methanol is included (see text).

Table II. Coupling Constants for Chorismic Acid in Methanol[a]

	$J_{a,b}^{eq}$	$J_{a,b}^{ax}$	$J_{a,e}^{eq}$	$J_{a,e}^{ax}$	$J_{b,c}^{eq}$	$J_{b,c}^{ax}$	f^b
1	12.6	2.8	2.45	5.03	2.34	6.07	0.91
2	12.6	3.8	2.45	4.77	2.34	5.69	0.90
3	13.0	2.8	2.35	5.04	2.19	6.07	0.88
4	13.0	3.8	2.35	4.78	2.19	5.69	0.87
5	13.5	2.8	2.22	5.04	2.00	6.07	0.84
6	13.5	3.8	2.22	4.78	2.00	5.69	0.82

[a] Based on assumed values for $J_{a,b}^{eq}$ and $J_{a,b}^{ax}$, and eq 3–5. [b] At 25 °C.

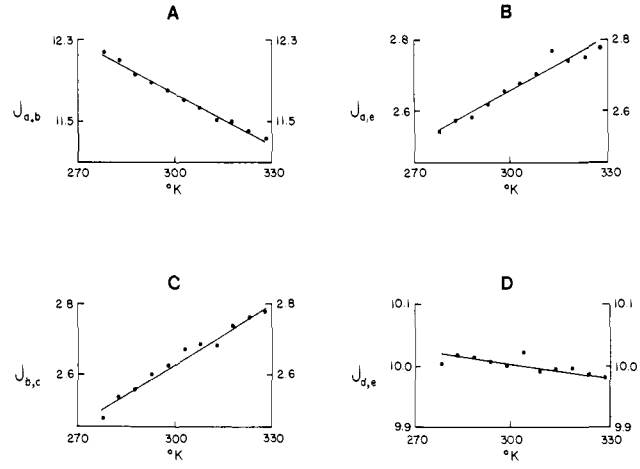

Figure 4. Observed H–H coupling constants (in Hz) for bis(tetra-*n*-butylammonium)chorismate in D_2O as a function of temperature. The coupling protons are identified by subscript letters (see Figure 2).

and f, the fraction of chorismate in the pseudo-diequatorial conformer at 298 K. These data are shown in Table II, from which two conclusions are evident. First, all the J values fall within, or very close to, the ranges predicted in Table I. Second, and more importantly, the value of f is relatively insensitive to the actual choice of limiting coupling constants. The fraction of the pseudo-diequatorial conformer only varies between 0.82 and 0.91. It seems clear that, in methanol at 25 °C, *both* conformers of chorismic acid are present in reasonable amounts and that the ratio of diequatorial to diaxial conformers is between 5 and 10. While the data do not permit a more precise estimate of the equilibrium position, the important conclusion is that significant amounts of pseudo-diaxial conformer are present at normal temperatures. The value of ΔG_0 (Figure 1) is about 0.9–1.4 kcal/mol.

The conformational equilibria of the bis(tetrabutylammonium) salts of chorismate and of 4-*O*-methylchorismate in D_2O and in methanol and of chorismic acid in 0.1 N DCl in D_2O have also been investigated. For the aqueous solutions, the temperature range is restricted by the freezing point of water at the lower end and by the rapid rate of the Claisen rearrangement at the upper end. These constraints are not serious, however, and the temperature dependences of the coupling constants for the bis(tetrabutylammonium) salt of chorismate in D_2O over the temperature range 278 to 328 K are shown in Figure 4. Expressing the coupling constants as functions of each other, as before, gives

$$J_{a,b}^{obsd} = (-3.257)(J_{a,e}^{obsd}) + 20.42 \qquad r = 0.985 \qquad (6)$$

$$J_{a,b}^{obsd} = (-2.841)(J_{b,c}^{obsd}) + 19.23 \qquad r = 0.988 \qquad (7)$$

$$J_{a,e}^{obsd} = (0.8300)(J_{b,c}^{obsd}) + 0.4750 \qquad r = 0.962 \qquad (8)$$

Table III. Conformational Preferences and Relative Rearrangement Rates of Chorismic Acid, Chorismate, and 4-*O*-Methylchorismate

compound	solvent[a]	proportion of pseudo-diequatorial conformer[b]	k_{H_2O}/k_{MeOH}[c]
chorismate[d]	water	~0.88	100
chorismate	methanol	>0.98	
chorismic acid	water	~0.83	11
chorismic acid	methanol	~0.87	
4-*O*-methylchlorismate[d]	water	~0.60	7
4-*O*-methylchorismate	methanol	~0.65	

[a] Perdeuterio solvents were used for NMR measurements of conformational equilibria. Unlabeled solvents were used for rearrangement rate measurements. [b] 25 °C. [c] 50 °C. [d] Bis(tetra-*n*-butylammonium) salt.

Solving for the intrinsic coupling constants yields values of f (the equilibrium proportion of diequatorial conformer) between 0.83 and 0.92 at 25 °C. These and all other values for f are collected in Table III, from which it is clear that chorismate, 4-*O*-methylchorismate, and chorismic acid all exist in water in both conformations, predominantly as the pseudo-diequatorial form.

In contrast to the above results, the spectra of bis(tetrabutylammonium)chorismate *in methanol* show a different pattern. From 254 to 316 K, these spectra show the following characteristics. (i) H_a and H_b resonate at the same frequency. [This appears to be a fortuitous result of the conformation and solvent dependence of the chemical shifts, since in aqueous methanol-d_4 (50%, v/v) δ_a and δ_b are closer together than in D_2O but are still distinct.] (ii) $J_{a,e}$ is only 0.75 Hz. (iii) The spectrum is *independent* of temperature over the temperature range investigated. This suggests that there is no shift in the conformational equilibrium and that the molecule is present exclusively (>98%) as the diequatorial conformer at all temperatures investigated. The conformation is not dictated by the bulkiness of the tetrabutylammonium counterions, since a spectrum of disodium chorismate in methanol is very similar to that of bis(tetrabutylammonium)chorismate in methanol. The conformational preference of the chorismate dianion in methanol could in principle be a consequence either of a stronger charge–charge repulsion in the less polar solvent or of stronger intramolecular hydrogen bonding (in methanol) between the C-4 hydroxyl group and the side-chain carboxylate. In either case, the pseudo-diequatorial form of the substrate would be favored. Since, however, both conformers of 4-*O*-methylchorismate are present at equilibrium in water *and* in methanol (see Table III), we can conclude that intramolecular hydrogen bonding between the C-4 hydroxyl group and the side-chain carboxylate dominates the conformational preference of chorismate itself.

Implications for the Mechanism of the Non-enzymic Process. The effect of solvent on the equilibrium between chorismate conformers is a necessary part of any interpretation of the solvent effect on the rate of the non-enzymic rearrangement of chorismate to prephenate. Study of the uncatalyzed reaction is complicated by the fact that changing the solvent also leads to the appearance of products other than prephenate. While in water, chorismate rearranges smoothly to prephenate and only about 15% goes to *p*-hydroxybenzoate, considerably more of this elimination product appears when the solvent is methanol. In aprotic solvents such as acetonitrile or dimethyl sulfoxide, the predominant product is *O*-(1-carboxyvinyl)-3-hydroxybenzoate deriving from the loss of water from chorismate. These problems notwithstanding, analysis of the amount of remaining starting material (chorismate) and of the amounts of the products (prephenate, phenylpyruvate, and *p*-hydroxybenzoate) during the incubation of bis(tetra-*n*-butylammonium)chorismate at 50 °C in water or methanol gives rate constants for the Claisen rearrangement of 0.44 h^{-1} in water and 0.0043 h^{-1} in methanol. The rearrangement is evidently 100 times slower in methanol than in water. A change in rate of this magnitude is rather large for a simple pericyclic process. Thus (even though such solvent sensitivities are not readily predictable[25]) the rate of the ortho-Claisen rearrangement of allyl aryl ethers is only reduced by 20-fold or so when the solvent is changed from aqueous ethanol to tetradecane,[26] and the rate of the rearrangement of *trans,trans*-3-oxa-4-methyl-1,5,7-nonatriene is reduced by only 6-fold on going from methanol-water (2:1, v/v) to ethanol.[25]

There are two factors that could contribute to the solvent effect on the rearrangement of chorismate to prephenate. First, the ground state of the reaction could be preferentially stabilized in methanol by the selective population of the pseudo-diequatorial conformer from which rearrangement cannot occur. Such stabilization could arise from an intramolecular hydrogen bond between the side-chain carboxylate and the hydroxyl group at C-4 (Figure 1), the importance of which is emphasized by the finding that *both* conformational isomers of 4-*O*-methylchorismate exist in methanol, whereas only the pseudo-diequatorial conformer of chorismate itself is observed in that solvent. Undoubtedly some of the slowing effect of methanol on the chorismate rearrangement can be attributed to this source. Not *all* the rate reduction need be thus assigned, however, since our experimental results only show that the percentage of chorismate in the pseudo-diaxial form falls from 10–20% in water to (say) <2% in methanol. The solvent effect on the conformational equilibrium therefore accounts for at least 10-fold of the observed 100-fold reduction in the rate of the rearrangement of chorismate in methanol. A second contribution to the observed solvent effect could derive from a destabilization of the transition state for the rearrangement in the less polar medium. Thus, not only are the two carboxylate groups necessarily closer together in the transition state than in either of the ground state conformations, but the transition state may be intrinsically dipolar with some of the character of a tight ion pair between the enolpyruvate anion and the cyclohexadienyl cation. This possibility is consistent with the rates of rearrangement of a range of chorismate analogues in a variety of solvents[25,27] and has considerable attractiveness for the enzyme-catalyzed reaction in that it would provide a mechanistic handle that the enzyme could manipulate for catalytic purposes.

In an effort to assess the relative importance of the effects of solvent on the conformational equilibrium and on the transition state, we have determined the conformational equilibria and the rearrangement rates of bis(tetrabutylammonium)-4-*O*-methylchorismate and of chorismic acid in water and in methanol. The conformational equilibria of these molecules are only slightly affected by the change from water to methanol (see Table III), and any solvent effect on the rates of rearrangement is better interpreted in terms of transition-state polarity. The Claisen rearrangement of 4-*O*-methylchorismate is slowed by a factor of 7 when the solvent is changed from water to methanol (Table III). While this effect can be attributed to transition-state destabilization in methanol, we cannot be sure how much is due to the greater difficulty of bringing the two carboxylate groups closer together in the transition state and how much is due to charge separation at the transition state. In an attempt to minimize the electrostatic contribution (of the approximation of the carboxylates in the transition state), we have determined the solvent effect on the rearrangement of chorismic *acid* in water and in methanol. The rate of this Claisen rearrangement is 11-fold slower in methanol relative to water (Table III). Since the effect of solvent change on the conformational equilibrium is small for chorismic acid, and since there will be little electrostatic consequence, it seems clear that the transition state for the rearrangement reaction is more polar than the ground state. This conclusion is in agreement with recent results from other groups.[25,27] The postulate of a dipolar transition state for the non-enzymic rearrangement presented here is complemented by similar arguments for the enzyme-catalyzed reaction, which are advanced in the following paper.[12]

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Implications for the Mechanism of the Enzyme-Catalyzed Reaction. The relatively small difference in the free energies of the two conformers of chorismate in neutral aqueous solution has implications for the mechanism of the enzyme-catalyzed reaction. As discussed in the introduction, the rearrangement requires a pseudo-diaxial orientation of ring substituents, and if the equilibrium proportion of diaxial conformer had been vanishingly small, we should have had to conclude that the enzyme would bind the available diequatorial conformer and then isomerize it to the diaxial form. Thus the observed value[28] of k_{cat}/K_m is 1.2×10^6 M^{-1} s^{-1} (based upon the total chorismate concentration), and if the equilibrium proportion of the pseudo-diaxial conformer had been less than about 0.1%, the k_{cat}/K_m for this conformer as substrate would have exceeded the acceptable ceiling for an encounter-controlled enzymic process of around 10^9 M^{-1} s^{-1}. This would then have required that the enzyme first recognize and bind the more abundant pseudo-diequatorial form. However, with the knowledge that the diaxial conformer exists at reasonable levels (10–20%) in solution and that conformer interconversion is fast, there is no need to postulate an enzyme-catalyzed conformational change: the enzyme can select the diaxial conformer directly. While these arguments are tinged with teleology, mechanistic economy strongly suggests that the rate-limiting transition state of the chorismate mutase reaction does *not* involve a conformer interconversion. These arguments are developed in the following paper.[12]

(28) We have found k_{cat} = 51 s^{-1} and K_m = 41 μM, with highly purified enzyme, in 50 mM *N*-ethylmorpholine-2-(*N*-ethylmorpholino)ethanesulfonic acid buffer, pH 7.6, containing EDTA (1 mM), dithioerythritol (1 mM), and bovine serum albumin (0.1 mg/mL), at 30 °C. These values can be compared with k_{cat} = 25 s^{-1} and K_m = 60 μm reported by Sampathkumar, P. Ph.D. Thesis, Australian National University, 1978.

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On the Mechanism of the Chorismate Mutase Reaction

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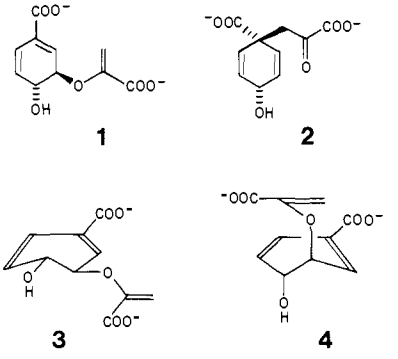
Abstract: To probe the mechanistic pathway of the reaction catalyzed by chorismate mutase, the secondary tritium isotope effect at C-4 of chorismate, k_H/k_T, has been determined. The small *inverse* effect of 0.96 rules out pathways that involve general acid catalysis at the C-4 hydroxyl group. This result, the existence of a significant (2.2-fold) solvent deuterium isotope effect (in the enzymic but *not* in the non-enzymic reaction), and all other results that bear upon this reaction are consistent with a pathway in which the rate-limiting heterolytic cleavage of the chorismate ether bond is assisted by attack of an enzymic nucleophile at C-5, to give an intermediate that collapses in an S_N2' process to yield prephenate.

The intramolecular rearrangement of chorismate (**1**) to prephenate (**2**) is catalyzed by the enzyme chorismate mutase and is a key step in the formation of tyrosine and phenylalanine via the shikimate pathway in bacteria, fungi, and higher plants.[1] This Claisen rearrangement appears to be the only example of a formal pericyclic reaction in primary metabolism. In contrast to most enzyme-catalyzed reactions, the non-enzymic rearrangement proceeds smoothly and can, therefore, be directly compared with the catalyzed reaction. Stereochemical studies have shown that the enzymic[2] and the non-enzymic[3] reaction each proceeds through a transition state of chair-like geometry. The reaction mechanism has been probed further through the use of secondary isotope effects. The non-enzymic rearrangement shows a secondary tritium isotope effect at C-5, the site of bond breaking, but none at C-9, the site of bond formation,[4] which suggests either a stepwise reaction or a very unsymmetrical transition state. However, no isotope effect at either site was observed for the enzyme-catalyzed rearrangement.[4] This result does not preclude similar pathways for the catalyzed and uncatalyzed processes, since the intrinsic isotope effects on the enzymic rearrangement could be masked by a rate-limiting transition state that precedes an isotopically sensitive rearrangement step.[4]

The activation parameters for the enzymic reaction have been determined and have been compared with those for the spontaneous thermal rearrangement. It was suggested that the enzymic rate acceleration of more than 10^6 could be the result of a decrease in the entropy of activation to near zero and a reduction in the enthalpy of activation by about 5 kcal/mol.[5] While deductions

Chart I



based on the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for reactions in structured solvents such as water are risky, it does appear that chorismate mutase is acting as more than just an entropy trap.[6]

There are (at least!) four ways in which chorismate mutase could conceivably effect the rate enhancement that is observed

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