Proton Transfer from the C_5-*proR*/*proS* Positions of L-Dihydroorotate: General-Base Catalysis, Isotope Effects, and Internal Return[1]

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Received August 2, 1999

Abstract: Rate constants for C_5-*proR*/*proS*-hydron exchange from L-dihydroorotate were determined by ^{1}H NMR and detritiation in various oxygen-containing and amine buffers at 37 °C and ionic strength 1.0 M in aqueous solution. Thermodynamically unfavorable proton transfer from the C_5-*proR* and -*proS* positions ($pK_a = 20-21$) to oxygen-containing and amine bases shows general-base catalysis with a Brønsted β value of 0.84 ± 0.05 and 0.81 ± 0.08, respectively, which is consistent with a late, enolate-like transition state. General-base catalysis is detectable because there is a 160- or 85-fold negative deviation for the C_5-*proR* and -*proS* protons, respectively, from this correlation for deuterioxide ion. Deviations of $(k_H/k_T)_{obsd}$ and $(k_D/k_T)_{obsd}$ from the Swain−Schaad equation are consistent with internal return of the transferred hydron to a free C_5-carbanion/enolate intermediate from the conjugate general acid. This corresponds to an Eigen-type mechanism for hydron transfer, in which both proton transfer and diffusional separation of the C_5-carbanion/enolate• conjugate general-acid complex are partially rate-limiting, and a modest intrinsic barrier for C_5-hydron exchange. It is concluded that the C_5-carbanion/enolate can have a significant lifetime in aqueous solution and on dihydroorotate dehydrogenases (EC 1.3.1.14; EC 1.3.99.11).

Introduction

An important mechanistic question is how resonance delocalization of electron density contributes to the kinetic barrier for proton transfer from carbon and how biological catalysts handle this barrier.[2] The "normal acid"[3a] behavior[4] of carbon acids that give highly localized carbanions supports the conclusion that electron delocalization and the accompanying structural and solvation changes are responsible for the large intrinsic barriers[5] for proton transfer from most carbon acids: an increase in transition-state complexity is accompanied by a decreased reaction rate.[3b] Surprisingly, C(α)-proton transfer from thiazolium carbinols, ethyl acetate, and glycine methyl ester, which give resonance-stabilized products upon ionization and have late, product-like transition states, also exhibit normal behavior. This suggests that carbon acids can be as normal as any other acid with respect to rates of proton transfer if the mechanism for stabilization of the carbanion minimizes the changes in bond lengths and angles of heavy atoms, and associated unfavorable solvation changes, in the transition state for proton transfer. Retaining partial solvation of the reactant(s) in the transition state for proton transfer could minimize the contribution of solvent reorganization effects to the intrinsic barrier. The intrinsic barrier to enolization may also decrease when the relative stability of the resonance-stabilized enolate product is decreased by ground-state resonance stabilization of the keto tautomer by an adjacent heteroatom.[6a]

We report here an examination of proton transfer from the C_5-*proR*/*proS* position of L-dihydroorotate (**1**, Scheme 1) in aqueous solution. This model compound was selected for study because changes in bond lengths and angles upon ionization to form the enolate may be somewhat restricted due to the six-membered ring and resonance stabilization of the keto reactant by the nitrogen atom adjacent to the carbonyl group, which could contribute to normal acid behavior and a modest kinetic barrier for proton transfer. We are also interested in the magnitude of the kinetic barrier for C_5-*proR*/*proS* proton transfer that must be overcome for C_5-proton abstraction catalyzed by dihydroorotate dehydrogenases (EC 1.3.1.14; EC 1.3.99.11), and whether the C_5-carbanion/enolate (**2**) can exist as a discrete intermediate in aqueous solution or at an enzyme active site.[7]

The goal of the work reported here was to determine whether the rate-limiting step for thermodynamically unfavorable C_5-*proR*/*proS* proton transfer from **1** to buffer bases involves

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(1) This research was supported in part by a grant from the National Institutes of Health (GM 42878). NMR studies were performed in the Biochemistry NMR Facility at Johns Hopkins University, which was established by grants from the National Institutes of Health (GM 27512 and RR 06261).

(2) (a) Deslongchamps, P. In *Stereoelectronic Effects in Organic Chemistry*; Pergamon: New York, 1983; pp 340−359. (b) Stivers, J. T.; Washabaugh, M. W. *Bioorg. Chem.* **1991**, *19*, 369−383.

(3) (a) Proton transfers between the electronegative atoms (oxygen or nitrogen) of "normal" acids and bases are almost completely diffusion-controlled; only in a small region near $\Delta pK_a = 0$ is the proton-transfer step even partly rate determining (refs 22, 34a). (b) The reader is referred to refs 40, 41, and 46 for a discussion of the relative contributions from enthalpy and entropy to transition-state complexity.

(4) (a) Washabaugh, M. W.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 674−683. (b) Washabaugh, M. W.; Jencks, W. P. *Biochemistry* **1988**, *27*, 5044−5053.

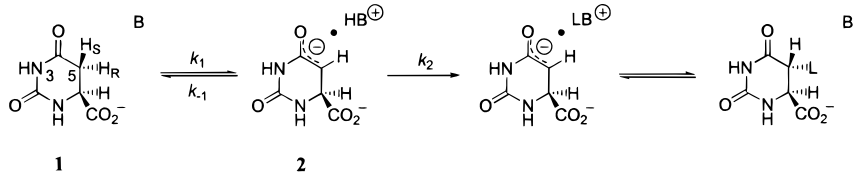
(5) The intrinsic barrier is the kinetic barrier when there is no thermodynamic driving force for a reaction.

(6) (a) Amyes, T. L.; Richard, J. P. *J. Am. Chem. Soc.* **1996**, *118*, 3129−3141. (b) Rios, A.; Richard, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 8375−8376.

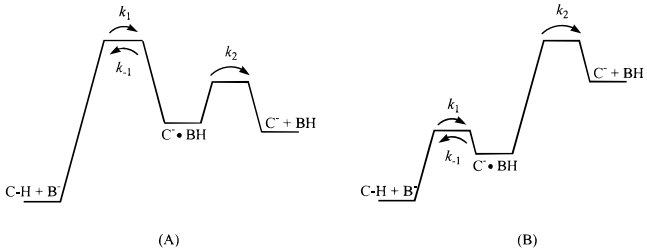
(7) (a) Pascal, R. A., Jr.; Walsh, C. T. *Biochemistry* **1984**, *23*, 2745−2752. (b) Hines, V.; Johnston, M. *Biochemistry* **1989**, *28*, 1227−1234.

10.1021/ja992753v CCC: $18.00 © 1999 American Chemical Society
Published on Web 12/10/1999

Scheme 1



1 **2**



(A) (B)

Figure 1. Hypothetical Gibbs free energy-reaction coordinate diagrams for thermodynamically unfavorable proton transfer to illustrate (panel A) rate-limiting proton transfer to form the carbanion·conjugate general-acid complex and (panel B) rate-limiting diffusional separation of the carbanion·conjugate general-acid complex.

diffusion-controlled separation of the products (k_2, Figure 1 and Scheme 1). If the C_5-*proR*/*proS* position behaves like a normal acid, and has a similar intrinsic barrier to proton transfer, both proton transfer (k_1) and diffusional separation of the products (k_2) would be partially rate-limiting. When both k_1 and k_2 are partially rate-limiting, k_{-1} competes with k_2, and the carbanion/enolate intermediate partitions between products and reactants. For base-catalyzed C−L exchange (where L = H, D, or T),[8] the extent of internal return (k_{-1}/k_2) can be measured from the breakdown of the Swain−Schaad equation (eq 1, with $y = 3.34$)[9] when both k_1 and k_2 are partially rate limiting. We have used

$$\log(k_H/k_T)_{obsd} = y \log(k_D/k_T)_{obsd} \quad (1)$$

this method to estimate the extent of internal return during proton exchange from C_5-*proR*/*proS* in aqueous solution.

In this paper we describe evidence that C_5-*proR*/*proS*-proton transfer from **1** proceeds through a free carbanion/enolate intermediate in aqueous solution, as expected for a normal acid. The magnitude of rate constants for C_5-*proR*/*proS*-proton transfer from **1** and isotope effects for C_5-*proR*/*proS*-proton transfer to buffer bases provide evidence for significant internal return of the abstracted proton to the C_5-carbanion/enolate (**2**) from a buffer acid, which is competitive with diffusional equilibration of the abstracted proton with solvent-derived protons, and a modest intrinsic barrier for proton transfer.

Experimental Section

Materials. All organic chemicals were reagent-grade and were purified by recrystallization. Reagent-grade inorganic chemicals were used as received. All water was prepared on a four-bowl Milli-Q water system including an Organex-Q cartridge (Millipore). All deuterated compounds were ≥99 atom % D and were purchased from Aldrich. [^{3}H]H_2O (1.0 Ci/mL) was purchased from New England Nuclear, and Ecolite was purchased from ICN. L-Dihydroorotic acid (**1**) was purchased from Aldrich and recrystallized from water: mp = 265−269 °C dec (uncorrected) (lit. 266 °C).[10] C_5-[^{3}H]-dihydroorotate was prepared from unlabeled **1** by exchange with [^{3}H]H_2O in L_2O (L = H or D). Partially neutralized buffer catalysts (0.2−0.4 M) and 0.1 M L-dihydroorotate in 1.5 mL of [^{3}H]L_2O (4 mCi/mL) were incubated at 37 °C for 10 h to 30 days. The exchange reaction was quenched by the addition of 3 M LCl to obtain pL < 3.5. The solvent, which contained [^{3}H]L_2O, was removed by evaporation under a water aspirator vacuum in a Savant Speed-Vac centrifugal concentrator; all operations were performed in a hood. Parallel experiments in D_2O and ^{1}H NMR examination of the exchanged substrate showed that isotopic labeling under these conditions occurs exclusively by exchange at C_5.

Methods. Solution pH was measured at 37 °C with an Orion Model SA 720 pH meter and a Radiometer GK2321C combination electrode standardized at pH 7.00 and 4.00 or 10.00. ^{1}H NMR spectra in D_2O were recorded on a Bruker AMX-300 NMR spectrometer using 3-(trimethylsilyl)propanesulfonate as an internal chemical shift standard. All reactions were carried out at 37 ± 0.1 °C (±1 °C in the NMR). Radioactivity was measured with a Beckman LS 7500 liquid scintillation counter.

Kinetics. Rate constants for L-dihydroorotate C_5-*proR*/*proS*-H → D and -T → D exchange in D_2O (likewise C_5-*proR*/*proS*-D → H and -T → H exchange in H_2O) were determined in the same reaction tube, rather than in parallel experiments.

Rate constants for L-dihydroorotate C_5-*proR*/*proS*-H → D exchange in D_2O were determined by ^{1}H NMR spectroscopy by measuring the integrated areas of the C_5-*proR* ($\delta \approx 3.0$ ppm, quartet) and C_5-*proS* ($\delta \approx 2.8$ ppm, quartet) peaks relative to the integrated area of the C_6−H peak (as the nonexchanging standard; $\delta \approx 4.1$ ppm, triplet) as a function of time.[11] The ionic strength was maintained at 1.0 M with KCl, and the concentration of **1** was 0.01 M, unless otherwise stated. The pseudo-first-order rate constants for proton exchange were obtained from the slopes of semilogarithmic plots of A_5/A_6 against time, where A is the integrated area of the C_5−H or C_6−H signal, respectively. These plots were linear for $>3t_{1/2}$ with ≥12 time points. Nonlinearity in such plots was observed only for proton exchange from the C_5-*proR* position with 1,4-diazabicyclo [2.2.2] octane as the buffer catalyst, where there was a decrease in the exchange rate over time that is attributed to a small secondary isotope effect on C_5-*proR* proton transfer as deuterium accumulates in the C_5-*proS* position. The pseudo-first-order rate constants in this case were determined for the first $t_{1/2}$ of proton exchange. When duplicate determinations of k_{obsd} were made, they agreed within ≤±8% of the average value. Rate constants for C_5-*proR*/*proS*-H → D in D_2O or -D → H exchange in H_2O were also determined using ^{1}H NMR spectroscopy as described below. For exchange reactions performed in the presence of 3-chloroquinuclidine, aliquots of quenched exchange-reaction solution (pL < 3.5; see below) were passed through a Sep-Pak CM column (Waters), which was previously equilibrated with L_2O and eluted with L_2O prior to analysis by ^{1}H NMR, to reduce the signals associated with buffer protons ($\delta \approx 3.2-3.5$ ppm).

Rate constants for C_5-*proR*/*proS*-T → D in D_2O or -T → H exchange in H_2O were determined by measuring nonvolatile, unexchanged tritium remaining in C_5-[^{3}H]-dihydroorotate. The ionic strength was maintained at 1.0 M with KCl. All tritium exchange reactions were performed in

(8) The term "hydron" refers to the hydrogen cation (L^+) without regard to nuclear mass. The specific names "proton" (^{1}H), "deuteron" (^{2}H), and "triton" (^{3}H) refer to the specific isotopes (Commission on Physical Organic Chemistry, IUPAC. *Pure Appl. Chem.* **1988**, *60*, 1115−1116) and are abbreviated here as: $^1H^+$, H; $^2H^+$, D; $^3H^+$, T.

(9) (a) Swain, C. G.; Stivers, E. C.; Reuwer, J. F., Jr.; Schaad, L. J. *J. Am. Chem. Soc.* **1958**, *80*, 5885−5893. Swain et al. used 3.26 for the exponent assuming the effective masses are in the ratio m_H:m_D:m_T of 1:2:3. (b) Streitwieser et al. used a value of 3.34 for the exponent based on the assumption that the effective masses are in the ratio of the reduced masses for ^{12}C−H, ^{12}C−D, and ^{12}C−T (Streitwieser, A., Jr.; Hollyhead, W. B.; Pudjaatmaka, A. H.; Owens, P. H.; Kruger, T. L.; Rubenstein, P. A.; MacQuarrie, R. A.; Brokaw, M. L.; Chu, W. K. C.; Niemeyer, H. M. *J. Am. Chem. Soc.* **1971**, *93*, 5088−5096).

(10) Miller, C. S.; Gordon, T.; Engelhardt, E. L. *J. Am. Chem. Soc.* **1953**, *75*, 6086−6087.

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a fume hood. The exchange reaction was initiated by dissolving 0.15 mmol of C_5-[^{3}H]-dihydroorotate (typically 50 mCi/mol), which contained 0.3−0.8 mmol buffer, in 60−230 μL of 3 M KOL and 0−375 μL of 3 M KCl to adjust the pL and ionic strength of the reaction solution, giving a final concentration of 100 mM substrate. The final concentration and fraction base of the buffers used in these tritium exchange experiments was as follows: 0.5 M potassium cacodylate (80% base); 0.3 M 1,4-diazabicyclo [2.2.2] octane hydrochloride (50% base); 0.2 M 3-chloroquinuclidine hydrochloride (50% base); 0.4 M tris(hydroxymethyl)aminomethane hydrochloride (80% base); and 0.2 M propargylamine hydrochloride (80% base). The reaction solution was incubated in a constant-temperature bath and was removed for about 10 s every 1 min to 24 h in order to obtain a 1-μL aliquot [using a P-10 Pipetman (Rainin)] that was immediately mixed with 10−20 μL of 1 M LCl in a liquid scintillation counting vial to quench the exchange reaction at pL < 2.0. The solvent, which contained exchanged tritium as [^{3}H]L_2O and aqueous LCl, was evaporated (see above) leaving any unexchanged C_5-[^{3}H]-dihydroorotate. The residue was dissolved in 1 mL of water, mixed with 10 mL of Ecolite, and the samples were counted for at least $10^{2.5}$ counts. A 1-μL aliquot of the reaction solution typically gave 6000 cpm for the initial time point (background = 30 ± 10 cpm). The end point was obtained after $>12t_{1/2}$ and was typically 200−500 cpm. The pseudo-first-order rate constants were obtained from the slopes of semilogarithmic plots of $(C_t - C_\infty)$ against time (C = cpm). These plots were linear for $>4t_{1/2}$ with >25 time points. When duplicate determinations of k_{obsd} were made, they agreed within <7% of the average value.

Measurements of pH were made at 37 ± 0.1 °C on the buffered solutions of **1** after exchange had occurred. The value of pD was obtained by adding 0.40 to the observed pH of the solutions in D_2O.[12] On the basis of measurements of pH at known concentrations of hydroxide ion at 37 °C and 1.0 M ionic strength, maintained with KCl, eq 2 was used to calculate the concentration of lyoxide ion at any pL. This equation includes the ion product of H_2O (pK_w^H = 13.63) or D_2O (pK_w^D = 14.55) at 37 °C.[13]

$$[OL^-] = 1.13 \times 10^{(pL - pK_w^L)} \quad (2)$$

Second-order rate constants for general-base-catalyzed C_5-*proR*/*proS*-H → D exchange, k_B, were obtained from the slope of plots of ≥4 values of k_{obsd} against buffer concentration, and were corrected for the fraction of free base of the buffer using standard graphical methods[14] and the apparent pK_a of the buffer (see below). Second-order rate constants for deuterioxide ion-catalyzed C_5-*proR*/*proS*-H → D exchange were obtained from the slope of a plot of observed pseudo-first-order rate constants, extrapolated to zero buffer concentration, against deuterioxide ion concentration in acetate-d_3-buffered solutions.

Determination of Catalyst pK_{BD} Values. Amine hydrochlorides, the potassium salts of carboxylic acids and phosphate, and cacodylic acid were dried in vacuo over P_2O_5 to constant weight before use. A 0.01 M solution of the catalyst at ionic strength 1.0 M (KCl) in freshly boiled H_2O was titrated potentiometrically under a nitrogen atmosphere with either 0.100 M KOH or 0.100 M HCl at 37 ± 0.1 °C according to the method of Albert and Sergeant.[15] Values of pK_{BH} for HPO_4^{2-} and $H_2NCH_2CH_2NH_3^+$ were determined spectrophotometrically using the indicator dye alizarine yellow.[15] A pK_{BH} value of 15 was estimated for the hydroxyl groups of tris(hydroxymethyl)aminomethane on the basis of pK_{BH} values for primary alcohols at low ionic strength in H_2O.[16] The catalyst pK_{BD} values in D_2O were calculated by adding ΔpK_a = 0.41 + 0.020 × pK_{BH} for the solvent deuterium isotope effect on the ionization of weak acids[17] to the values for pK_{BH} in H_2O.

Determination of the Extent of Internal Return. A steady-state treatment of Scheme 1 gives eq 3 for the observed rate constant for hydron exchange.

$$k_{obsd}{}^L = k_1{}^L k_2/(k_{-1}{}^L + k_2) \quad (3)$$

Defining the extent of internal return a^L as $k_{-1}{}^L/k_2$ and the equilibrium constant $K^L = k_1{}^L/k_{-1}{}^L$ where L = H, D, or T gives eqs 4−8, in which y = 3.34 and the ratio K^T/K^H is the equilibrium constant for CT + HOH ↔ CH + TOH as described previously.[9b,18]

$$A = (k_D/k_T)^y{}_{obsd}/(k_H/k_T)_{obsd} \quad (4)$$

$$B = (k_D/k_T)^y{}_{obsd}(K^T/K^H) \quad (5)$$

$$[1 - a^T(B^{1/y} - 1)]^y = A + a^T(A - B) \quad (6)$$

$$k_1{}^H/k_1{}^T = (k_H/k_T)_{obsd}[1 - a^T[(K^T/K^H)(k_H/k_T)_{obsd} - 1]]^{-1} \quad (7)$$

$$a^H = a^T(K^T/K^H)(k_1{}^H/k_1{}^T) \quad (8)$$

The equilibrium isotope effect, K^D/K^H, was measured for C_5-*proR*/*proS*-H → D exchange by determining the amount of deuterium incorporation in the C_5 position at equilibrium from D_2O in H_2O at 37 °C as described previously;[19] the pL was maintained by 0.2 M 1,4-diazabicyclo [2.2.2] octane hydrochloride (50% base). The equilibrium for deuterium incorporation at C_5 was established within 5 h. Values of K^D/K^H = 0.97 ± 0.01 and 1.0 ± 0.01 for the C_5-*proR*- and C_5-*proS*-H → D exchange were determined, respectively, and we assume a value of K^T/K^H = 1.0 ± 0.01 for the equilibrium isotope effect for C_5-*proR*/*proS*-T → D exchange. Equations for calculating the propagated errors in the calculated value of the Swain−Schaad exponent (y), the extent of internal return ($a^H = k_{-1}{}^H/k_2$), and the primary kinetic isotope effect for proton transfer ($k_1{}^H/k_1{}^T$) were derived previously from eqs 1, 7, and 8 with the standard error formula.[18] The propagated error in a^T was estimated by using the standard deviation of the values for a^T obtained by evaluating eq 6 with the minimum and maximum values of the variables.

Results

The kinetics of general-base-catalyzed C_5-*proR*/*proS*-hydron exchange from L-dihydroorotate at 37 °C and ionic strength 1.0 M, maintained with potassium chloride, in aqueous solution were followed by a combination of ^{1}H NMR and detritiation methods in the pL range 4.5−10.3 under pseudo-first-order conditions. Kinetic studies were limited to the pL range 4.5−10.3 because: (1) proton exchange is impracticably slow at pL < 4, (2) hydrolysis of **1** becomes significant at pL > 11,[20] and (3) deprotonation of the N_3 position (pK_a = 11.5)[20] is not significant in aqueous solution in this pL range. The pK_a value of 11.5 for the N_3 position in **1** demonstrates that in the pL range 4.5−10.3 the observed rate is for C_5-hydron exchange from the substrate which has N_3-H rather than N_3^-.[20]

The exchange reactions obey the rate law described by eq 9.

$$k_{obsd} = k'_{L_2O} + k_{LO^-}[LO^-] + k_{B_1}[Base_1] + k_{B_2}[Base_2] \quad (9)$$

The observed pseudo-first-order rate constant and second-order rate constants were determined as described in the Experimental Section. Typical data are shown in Figure 2 for C_5-*proR*-H → D exchange from **1** catalyzed by phosphate di- and trianions and cacodylate anion. The second-order rate constants, k_B, for

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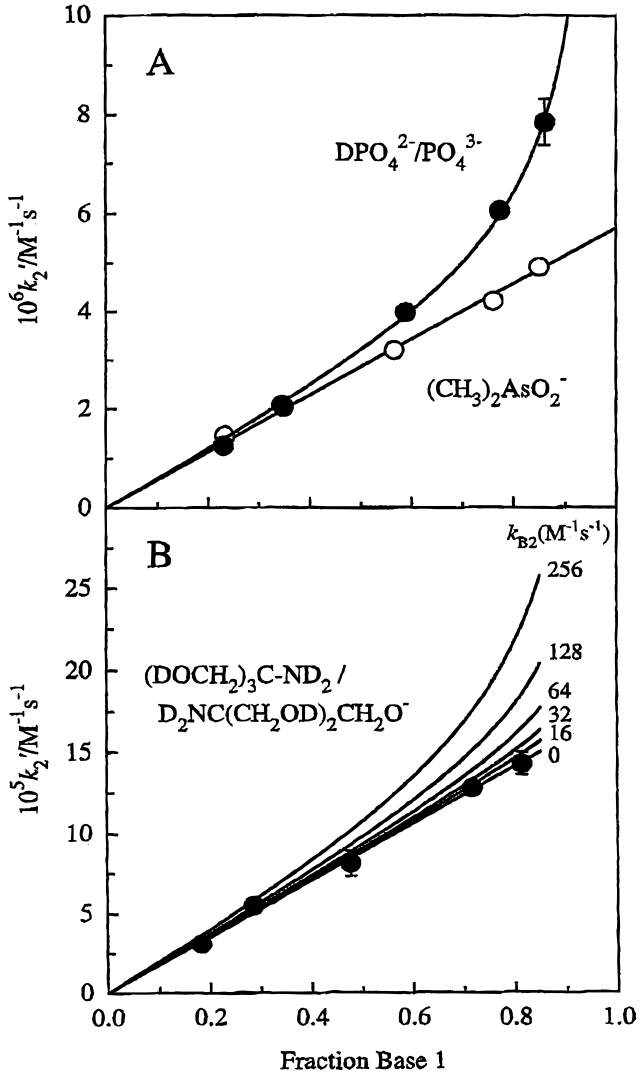

Figure 2. Dependence of the apparent catalytic constant, k_2' (= $(k_{obsd} - k_0)$/[buffer]$_{tot}$), on the composition of phosphate and cacodylate (panel A) and tris(hydroxymethyl)aminomethane (panel B) buffers for catalysis of C_5-*proR* proton transfer from L-dihydroorotate in D_2O at 37 °C, I = 1.0 M (KCl). The fraction of buffer base 1 refers to the fraction of phosphate dianion in the phosphate buffers and fraction of primary amine in the tris(hydroxymethyl)aminomethane buffers. The solid line drawn through the phosphate data is based on the rate law in eq 9 with $k_{B_1} = 5.37 \times 10^{-6}$ M^{-1} s^{-1}, $k_{B_2} = 4.39 \times 10^{-2}$ M^{-1} s^{-1}, pK_{BD} ($D_2PO_4^-$) = 6.85, and pK_{BD} (DPO_4^{2-}) = 11.77. The solid lines drawn through the tris(hydroxymethyl)aminomethane data are based on the rate law in eq 9 with $k_{B_1} = 1.77 \times 10^{-4}$ M^{-1} s^{-1}, k_{B_2} varied from 0 to 256 M^{-1} s^{-1}, pK_{BD} ($(DOCH_2)_3CND_3^+$) = 8.58, and pK_{BD} ($D_2NC(CH_2OD)_2CH_2OD$) = 15.71.

general-base catalysis of C_5-*proR*/*proS*-H → D exchange are summarized in Table 1. General-acid catalysis was detected with acetic-d_3 acid and protonated trifluoroethylamine but was not systematically evaluated. A second term for catalysis by buffer bases (k_{B_2}[Base$_2$]) is required in the rate law when dibasic buffer catalysts are used. The absence of upward curvature in Figure 2 for cacodylate, a monobasic catalyst with a pK_{BD} value that is similar to that of $D_2PO_4^-$ (Table 1), is consistent with this explanation. Values of the rate constants for catalysis of C_5-*proR*/*proS*-H → D exchange from **1** by deuterioxide ion and deuterium oxide are also reported in Table 1.

The dependence on pD of the pseudo-first-order rate constants, extrapolated to zero buffer concentration, for C_5-*proR*-H → D exchange from **1** is shown in Figure 3. The solid line in

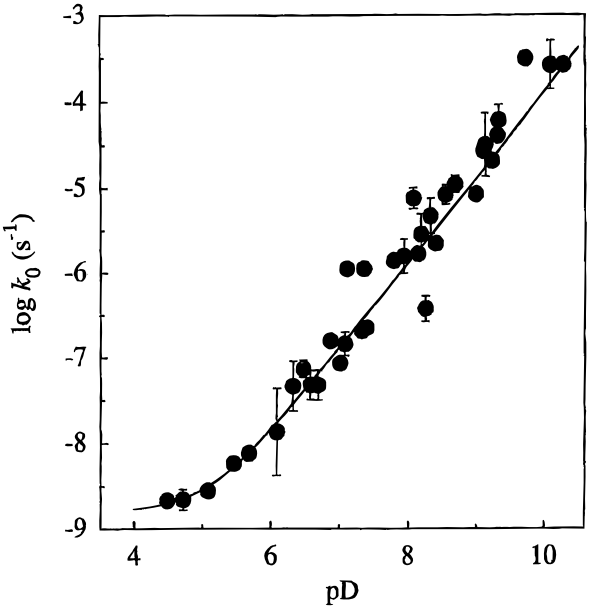

Figure 3. Dependence on pD of the observed pseudo-first-order rate constant, extrapolated to zero buffer concentration (k_0) for C_5-*proR* H → D exchange from L-dihydroorotate in D_2O at 37 °C, I = 1.0 M (KCl). The solid line drawn through the data is based on the rate law in eq 9 with $k'_{D_2O} = 1.59 \times 10^{-9}$ s^{-1} and $k_{DO^-} = 3.91$ M^{-1} s^{-1}.

Figure 3 was calculated using eq 9 with the rate constant for catalysis by DO^- and D_2O reported in Table 1: no specific-acid catalysis was detected. A similar dependence of the pseudo-first-order rate constants on pD was observed for C_5-*proS*-H → D exchange from **1** (data not shown).

Primary kinetic isotope effects for L-dihydroorotate C_5-hydron exchange, $(k_L/k_T)_{obsd}$, were determined by detritiation and ^{1}H NMR at 37 °C and 1.0 M ionic strength, maintained with potassium chloride, under pseudo-first-order conditions and are summarized in Table 2. Table 2 also contains several quantities that were calculated with the experimental values of $(k_L/k_T)_{obsd}$. These include the following: values of the Swain−Schaad exponent, y, to satisfy the Swain-Schaad equation (eq 1) in the range 1.5−2.6; extents of internal return, $a^L = k_{-1}^L/k_2$, for exchange of C_5-T and C_5-H from eq 6 and 8, respectively, in the range of 0.04−0.9 for the triton and 4 ± 3 for the proton; and a value for the primary kinetic isotope effect on the proton-transfer step itself, $k_1^H/k_1^T = 6.0$, from eq 7.

Biphasic kinetics were observed for C_5-T → L exchange in the presence of propargylamine (data not shown), which was attributed to rapid detritiation of the buffer catalyst. Parallel experiments in D_2O and ^{1}H NMR examination of the exchanged buffer showed that isotopic labeling under these conditions occurs by exchange at the acetylenic position.

Discussion

Nature of the Rate-Limiting Transition State. Figure 4 shows that the Brønsted plots for C_5-*proR* and -*proS* proton transfer from **1** in aqueous solution have slopes of β = 0.84 and 0.81, respectively, for catalysis by oxygen- and nitrogen-containing bases. General-base catalysis is readily detectable because of the approximately 160-fold (C_5-*proR*) and 85-fold (C_5-*proS*) negative deviations for catalysis by deuterioxide ion from the Brønsted plots. Omission of the rate constants for catalysis by tris(hydroxymethyl)aminomethane and propargylamine does not improve the fit of the data to the Brønsted plots

Table 1. Rate Constants for General-Base Catalysis of L-Dihydroorotate C_5-*proR*/*proS*-H → D Exchange[a]

catalyst	pK_{BD}[b]	*p*	*q*	$pK_{BD} + \log(p/q)$	C_5-*proR* k_B ($M^{-1}\,s^{-1}$)	C_5-*proR* $\log(k_B/q)$	C_5-*proS* k_B ($M^{-1}\,s^{-1}$)	C_5-*proS* $\log(k_B/q)$	k_B^R/k_B^S
D_2O	−1.74[c]	3	1	−1.26	$(2.88 \pm 0.48) \times 10^{-11}$	−10.54	$(4.19 \pm 0.32) \times 10^{-11}$	−10.38	0.7
DO^-	16.29[d]	2	1	16.59	3.91 ± 0.30	0.59	1.18 ± 0.20	0.07	3.3
D_3CCOO^-	5.11	1	2	4.81	$(9.26 \pm 0.76) \times 10^{-8}$	−7.33	$(3.74 \pm 0.09) \times 10^{-8}$	−7.73	2.5
$F_3CCH_2ND_2$	6.21	3	1	6.69	$(2.46 \pm 0.23) \times 10^{-6}$	−5.61	$(9.48 \pm 0.04) \times 10^{-7}$	−6.02	2.6
$(CH_3)_2AsO_2^-$	6.57	1	2	6.27	$(5.71 \pm 0.09) \times 10^{-6}$	−5.54	$(2.00 \pm 0.02) \times 10^{-6}$	−6.00	2.9
DPO_4^{2-}	6.85	2	3	6.67	$(5.37 \pm 0.22) \times 10^{-6}$	−5.75	$(9.02 \pm 0.10) \times 10^{-7}$	−6.52	6.0
$^+D_3N(CH_2)_2ND_2$	7.74	3	1	8.22	$(1.35 \pm 0.06) \times 10^{-4}$	−3.87	$(4.07 \pm 0.32) \times 10^{-5}$	−4.39	3.3
$(DOCH_2)_3CND_2$	8.58	3	1	9.06	$(1.77 \pm 0.02) \times 10^{-4}$	−3.75	$(3.72 \pm 0.09) \times 10^{-5}$	−4.43	4.8
$DCCCH_2ND_2$	8.66	3	1	9.14	$(3.41 \pm 0.20) \times 10^{-4}$	−3.47	$(7.52 \pm 0.36) \times 10^{-5}$	−4.12	4.5
$N(CH_2CH_2)_3N$	9.82	1	2	9.52	$(5.35 \pm 0.31) \times 10^{-3}$	−2.57	$(3.69 \pm 0.21) \times 10^{-3}$	−2.73	1.4
$D_2N(CH_2)_2ND_2$	10.88	3	2	11.06	$(1.64 \pm 0.18) \times 10^{-2}$	−2.09	$(6.11 \pm 1.04) \times 10^{-3}$	−2.52	2.7
PO_4^{3-}	11.77	1	4	11.17	$(4.39 \pm 0.35) \times 10^{-2}$	−1.96	$(4.63 \pm 0.16) \times 10^{-3}$	−2.94	9.5
$D_2NC(CH_2OD)_2CH_2O^-$	15.71	3	1	16.19	≤32	≤1.51	≤6.4	≤0.81	5.0

[a] At 37 °C and ionic strength 1.0 M (KCl) in D_2O. The rate constant k_B is defined in eq 9. Statistical corrections were made according to Bell and Evans.[14b] [b] Apparent pK_a of the conjugate acid at 37 °C and ionic strength 1.0 M (KCl) in D_2O (see text). [c] Calculated from the ion product of D_2O[13] based on a standard state of 55.0 M for pure D_2O at 37 °C, with $K_a^{D_2O} = K_w/[D_2O] = 10^{-14.55}/55.0$. [d] Rate constants for catalysis by OD^- are concentration based, $\gamma_{OD} = 0.89$.

Table 2. Catalysis of C_5-*proR*/*proS*-L Exchange from L-Dihydroorotate[a]

			proR					*proS*				
Catalyst	pK_a[b]	C_5-L/L_2O	$10^5 k_{obsd}$ (s^{-1})	$(k_L/k_T)_{obsd}$[c]	*y*[d]	k_{-1}^H/k_2[e,f] (k_{-1}^T/k_2)[e,h]	k_1^H/k_1^T[e,g]	$10^5 k_{obsd}$ (s^{-1})	$(k_L/k_T)_{obsd}$	*y*	k_{-1}^H/k_2 (k_{-1}^T/k_2)	k_1^H/k_1^T
$(CH_3)_2AsO_2^-$	6.57	H/D_2O	0.211 ± 0.008	3.4 ± 0.2	1.9 ± 0.2	—	—	0.077 ± 0.003	1.2 ± 0.1	—	—	—
		T/D_2O	0.063 ± 0.002			0.4 ± 0.1		0.063 ± 0.002			—	
		D/H_2O	0.140 ± 0.007	1.9 ± 0.1				0.058 ± 0.003	0.8 ± 0.1			
		T/H_2O	0.075 ± 0.002					0.075 ± 0.002				
$(LOCH_2)_3CNL_2$	8.58	H/D_2O	8.89 ± 0.22	23 ± 1	2.3 ± 0.1	—[i]	—	1.97 ± 0.21	5.0 ± 0.6	2.6 ± 0.4	—	—
		T/D_2O	0.39 ± 0.02			0.04 ± 0.02		0.394 ± 0.015			0.2 ± 0.1	
		D/H_2O	1.81 ± 0.12	3.9 ± 0.3				0.874 ± 0.060	1.9 ± 0.2			
		T/H_2O	0.47 ± 0.02					0.465 ± 0.023				
$LCCCH_2NL_2$	8.66	H/D_2O	6.33 ± 0.18	18 ± 2	1.8 ± 0.1	—	—	1.19 ± 0.03	3.4 ± 0.3	1.6 ± 0.2	—	—
		T/D_2O	0.35 ± 0.03			0.06 ± 0.01		0.350 ± 0.034			0.4 ± 0.1	
		D/H_2O	2.32 ± 0.16	5.0 ± 0.4				0.995 ± 0.054	2.1 ± 0.2			
		T/H_2O	0.46 ± 0.02					0.464 ± 0.023				
	9.59	H/D_2O	38.4 ± 2.0	3.3 ± 0.2	1.6 ± 0.2	—	—	23.9 ± 0.9	2.1 ± 0.1	1.5 ± 0.3	—	—
		T/D_2O	11.6 ± 0.6			0.4 ± 0.1		11.6 ± 0.6			0.9 ± 0.2	
		D/H_2O	22.5 ± 1.3	2.1 ± 0.2				16.8 ± 0.6	1.6 ± 0.1			
		T/H_2O	10.5 ± 0.5					10.5 ± 0.5				
	9.82	H/D_2O	97.4 ± 4.1	3.5 ± 0.2	2.0 ± 0.2	—	—	54.8 ± 0.8	2.0 ± 0.1	2.4 ± 0.3	4 ± 3	6 ± 4
		T/D_2O	27.6 ± 0.5			0.4 ± 0.1		27.6 ± 0.5			0.7 ± 0.2	
		D/H_2O	57.6 ± 2.4	1.9 ± 0.1				40.4 ± 1.0	1.3 ± 0.1			
		T/H_2O	30.4 ± 0.4					30.4 ± 0.4				

[a] At 37 °C and ionic strength 1.0 M (KCl) in L_2O. [b] Apparent pK_a of the conjugate acid at 37 °C and ionic strength 1.0 M (KCl) in L_2O (see text). [c] Values of $(k_H/k_T)_{obsd}$ and $(k_D/k_T)_{obsd}$ were calculated from the observed rate constants obtained in D_2O and H_2O, respectively (see text). [d] Exponent to satisfy the Swain−Schaad equation[9] defined by $\log(k_H/k_T)_{obsd} = y \log(k_D/k_T)_{obsd}$ (see text). [e] Defined in Scheme 1. [f] Calculated from eq 8. [g] Calculated from eq 7. [h] Calculated from eq 6 by using $(k_D/k_T)_{obsd}$ and $(k_H/k_T)_{obsd}$ with $K^T/K^H = 1.0 \pm 0.1$ (see text). [i] The calculated value is not meaningful due to imprecision in the measured rate constants (see text).

or change β significantly. There are no deviations in the reactions of amines that provide evidence for unfavorable steric effects by bulky catalysts or electrostatic stabilization of an ion pair intermediate involving the protonated amine and the C_5-carbanion/enolate (**2**, Scheme 1).

The Brønsted β values of >0.8 suggest a late, C_5-carbanion/enolate-like transition state, that proton transfer from C_5-*proR*/*proS* to buffer bases in aqueous solution is thermodynamically unfavorable, and that diffusional separation of the complex between **2** and the conjugate buffer acid is partially rate-limiting with proton transfer to form the complex. These conclusions are supported by the deviation of the values of $(k_H/k_T)_{obsd}$ and $(k_D/k_T)_{obsd}$ from the Swain−Schaad equation (Table 2). Large Brønsted β values have been reported for deprotonation of carbonyl-[6] and iminium ion-activated[2b,4] carbon acids, as well as carbon acids that require little electron delocalization and desolvation upon ionization,[21−23] when the proton-transfer step is strongly favorable in one direction.

The negative deviations from the Brønsted plots of the rate constants for catalysis by the strongly basic alcoholate anion of tris(hydroxymethyl)aminomethane (~10-fold) and deuteri-

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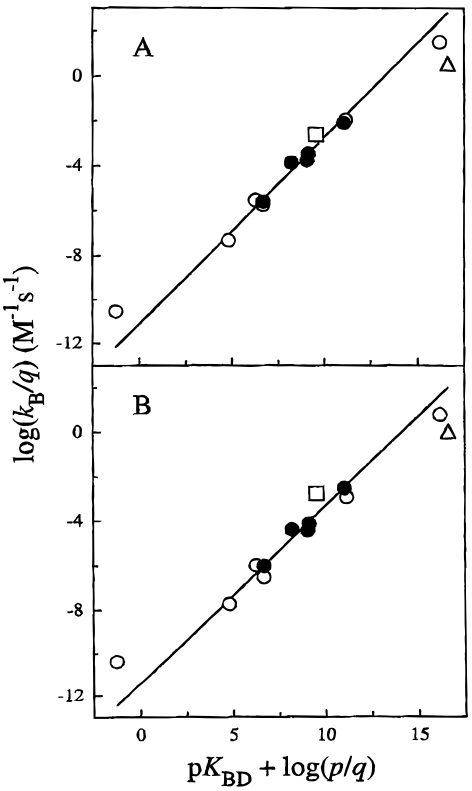

Figure 4. Statistically corrected Brønsted plots for general base catalysis of C_5-*proR* (panel A)- and C_5-*proS* (panel B)-H → -D exchange from L-dihydroorotate catalyzed by oxygen-containing buffers (○), primary amines (●), tertiary amine (□), and deuterioxide ion (△) in D_2O at 37 °C, I = 1.0 M (KCl). Statistical corrections were made according to Bell and Evans.[14b] The solid lines are drawn with slopes of $\beta = 0.84 \pm 0.05$ (C_5-*proR*; panel A) and $\beta = 0.81 \pm 0.08$ (C_5-*proS*; panel B).

oxide ion (~100-fold) are in the lower range of the negative deviations of between 10- and 1000-fold for catalysis by lyoxide ion that are usually observed for thermodynamically unfavorable proton transfers from carbon.[18] It is unlikely that these negative deviations represent a change in the rate-limiting step to rate-limiting diffusional encounter of the reactants because this would require a rate constant that is considerably smaller than the limiting value of 10^6 M^{-1} s^{-1} for diffusion-controlled encounter in aqueous solution.[24] The fact that no such negative deviation is observed for proton transfer between H_2O and H_3O^+ or H_2O and OH^- at $\Delta pK_a = 0$ may reflect proton transfer through solvent molecules,[25] which does not usually occur with carbon acids.[24,26,27] It is possible to explain the negative deviations for the reactions in which hydron transfer is largely rate-limiting by a requirement for partial desolvation of the lyoxide ion before reaction.[18] For reactions involving basic oxyanions as nucleophiles or catalysts, desolvation of the oxyanion appears to be ahead of bond formation to the oxyanion in the transition state.[26] No satisfactory explanation is available for deviations when diffusional separation of the products is largely rate-limiting.[18]

The observed primary kinetic isotope effects, $(k_D/k_T)_{obsd}$ and $(k_H/k_T)_{obsd}$, for catalysis of C_5-*proR* and -*proS*-hydron exchange from **1** in aqueous solution by oxygen-containing bases and amines are in the range 1.9−5.0 for $(k_D/k_T)_{obsd}$ and 3.3−23 for $(k_H/k_T)_{obsd}$ (Table 2). The relatively small values of $(k_H/k_T)_{obsd}$ could result from internal return or an asymmetrical transition state[28] or both. For example, $(k_H/k_T)_{obsd} = 3.3$ for catalysis of C_5-*proR*-hydron exchange from **1** by chloroquinuclidine may be compared with a value of ~19 that was calculated from the differences in zero-point vibrational stretching frequencies of C_5-H and C_5-D and the Swain−Schaad equation.[29] The isotope effects fit the Swain-Schaad equation (eq 1) with values of y in the range 1.6−2.3 (Table 2); these values differ significantly from the theoretical value of $y = 3.34$ for eq 1. These results provide evidence for internal return of the transferred hydron to **2** from the conjugate buffer acid, which contributes to the decreased observed primary kinetic isotope effect. The primary kinetic isotope effect is decreased because two transition states (hydron transfer and diffusional separation of the products) are partially rate limiting for base-catalyzed C_5-*proR* and -*proS*-hydron exchange from **1** in aqueous solution for these catalysts.[28] Significant internal return has been proposed to occur in several other carbanion-forming reactions according to a variety of experimental criteria.[2b,18,26b,30,31]

Streitwieser[31,32] showed that the amount of internal return, or the extent that the transferred hydron L is returned to the carbanion from the protonated buffer base before diffusional separation of the products, can be estimated from the y value of eq 1. Equation 1 describes the relationship between the primary kinetic isotope effects, $(k_D/k_T)_{obsd}$ and $(k_H/k_T)_{obsd}$. Internal return will contribute differently to the two isotope effects for reactions in which $k_2 \approx k_{-1}$ and $k_{-1}^H > k_{-1}^D > k_{-1}^T$ (Scheme 1).[28,31] For example, in reactions where k_{-1}^L/k_2 is small, k_{obsd} approaches k_1 (see eq 3), and eq 10 is obeyed. In reactions where k_{-1}^L/k_2 is large, the observed isotope effects approach the equilibrium isotope effects, and eq 11 is obeyed.[28]

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(29) A primary tritium kinetic isotope effect of $k_H/k_T \approx 19$ was calculated from the differences in zero-point vibrational stretching frequencies of C_5-H and C_5-D (ref 28, pp 130−131) by using $k_H/k_D = \exp[h(\nu_H - \nu_D)/2kT]$ and $k_H/k_T = (k_H/k_D)^{1.44}$ (ref 9); this treatment assumes that the differences in stretching vibrations upon deuteration dominate the measured isotope effect. Although stretching vibrations contribute substantially to the total zero-point energies, contributions from other vibrational modes certainly make significant contributions to the zero-point energies. The C_5-H stretching frequency of 3019 cm^{-1} for **1** was measured in a KBr pellet as described previously (ref 4b); a C_5-D stretching frequency of 2166 cm^{-1} for **1** was measured similarly.

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(32) Two simplifying assumptions are required to calculate the extent of internal return from the breakdown of eq 1 (ref 31): (1) There is no isotope effect on the rate constant for diffusional separation of the C_5-carbanion/enolate and the conjugate buffer acid (k_2). This is supported by the small isotope effect (≤3%) on the diffusion of HTO in H_2O and DTO in D_2O (Weingärtner, H. Z. *Phys. Chem., Neue Folge* **1982**, *132*, 129−149). (2) The solvent isotope effect on the primary kinetic isotope effect is negligible, which implies that k_{-1}^L/k_2 has no solvent isotope effect. The difference in ΔpK_a of 0.5 unit that arises from a change from D_2O to H_2O for the catalytic base is expected to result in a small increase in the extent of internal return. However, this solvent isotope effect is similar to the experimental error in the values (ref 18).

$$k_1^{H}/k_1^{T} = (k_1^{D}/k_1^{T})^{3.34} \quad (10)$$

$$K^{T}/K^{H} = (K^{T}/K^{D})^{3.34} \quad (11)$$

The values of the internal return ratio, k_{-1}^{L}/k_2, that were obtained from eq 1 are in the range 0.04−4 (Table 2) and indicate that internal return of the transferred hydron to **2** is significant. The extent of internal return should be interpreted conservatively because the errors are ±25% or more; these large errors arise because deviations from eq 10 as a consequence of internal return are typically small.[33] The results show that C_5-*proR*-hydron transfer catalyzed by tris(hydroxymethyl)aminomethane and propargylamine is subject to less internal return than C_5-*proS*-hydron transfer catalyzed by these catalysts, or the reactions catalyzed by an oxygen-containing catalyst or tertiary amines. A decrease in internal return is expected with decreasing basicity of the carbanion (see below).[18] The observed increase in the primary kinetic isotope effects for catalysis of C_5-*proR*-hydron transfer by tris(hydroxymethyl)aminomethane and propargylamine is consistent with predominantly rate-limiting hydron abstraction for these catalysts.

Correction of the value of $(k_H/k_T)_{obsd} = 2$ for C_5-*proS*-hydron abstraction catalyzed by 1,4-diazabicyclo [2.2.2] octane for the effects of internal return according to eq 7 gives $k_1^{H}/k_1^{T} \approx 6$ for the primary tritium kinetic isotope effect on the C_5-*proS* proton-transfer step itself. This shows that even moderate internal return markedly depresses isotope effects (Table 2).[28] This small isotope effect, compared to the calculated value of $k_1^{H}/k_1^{T} \approx 19$,[29] is also consistent with an asymmetrical transition state for the proton-transfer step.[26b]

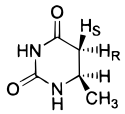
The magnitude of the Brønsted β values and isotope effects provide evidence that transfer of the C_5-*proR/proS* protons from 1 is similar, but not identical, to proton transfer from normal acids with electronegative atoms. Proton transfers between normal acids and bases are almost completely diffusion-controlled—only in a small region near $\Delta pK_a = 0$ is the proton transfer step even partly rate limiting, and for thermodynamically unfavorable ($\Delta pK_a < -3$) or favorable ($\Delta pK_a > 3$) proton transfers, diffusion-controlled separation of the products or encounter of the reactants becomes fully rate-limiting.[34,35] The Brønsted plots for these reactions follow "Eigen curves" with slopes of 0 and 1.0 in the favorable and unfavorable directions, respectively, and have a small transition region near $\Delta pK_a = 0$ of slope 0.5 where the proton-transfer step itself gives rise to a kinetic isotope effect. Brønsted β values of <1 and significant primary kinetic isotope effects are observed for **1** and related carbon acids because the proton transfer step is at least partly rate-limiting over a larger range of ΔpK_a.

Significant internal return of the abstracted hydron to **2** from the protonated buffer base establishes the Eigen mechanism for proton transfer from **1** in aqueous solution, which involves partitioning of this carbanion intermediate between products and reactants. This conclusion is relevant to the physiological role of **1** because it establishes that **2** can have a significant lifetime in aqueous solution and on dihydroorotate dehydrogenases.

Resonance, Solvation, and the Intrinsic Barrier. The "normal" acid behavior of C_5-proton transfer from **1**, thiazolium carbinols,[2b,4,18] ethyl acetate,[6a] and glycine methyl ester,[6b] suggests that carbonyl- and iminium ion-activated carbon acids can be as normal as any other acid with respect to rates of proton transfer if the mechanism for stabilization of the carbanion minimizes the changes in bond lengths and angles of heavy atoms, and associated unfavorable solvation changes, in the transition state for proton transfer.

A requirement for proper orbital alignment (stereoelectronic control) is widely accepted as an explanation of the stereoselective abstraction of protons α to carbonyl groups[36] and iminium ions,[37] although evidence supporting this explanation is limited and not without controversy.[38] The nonenzymatic C_5-proton exchange reactions of 6-substituted-5,6-dihydrouracils, including **1**, exhibit stereoselectivity that is similar to the stereopreferential abstraction of an axial α-proton for enolization of conformationally locked cyclohexanones;[36] stereopreferential abstraction of the C_5-*proR* proton was observed and explained as resulting from stabilization of the C_5-*proR* carbanion by orbital overlap of the p-orbital of the *quasi*-axial C_5-H_R bond and the p-orbitals of the C_4-carbonyl π-system.[11] This explanation is supported by the observation of preferential internal return in nonenzymatic C_5-*proS*-hydron exchange compared to the C_5-*proR* position (Table 2). The values of k_{-1}^{T}/k_2 for general-base catalysis of C_5-*proS*-hydron exchange are 2−7-fold greater than the values for C_5-*proR*-hydron exchange by the same catalyst. An increase in internal return is expected with increasing basicity (and decreasing stability) of a carbanion,[18] and the demonstration that internal return is more important for the C_5-*proS*-carbanion provides evidence that C_5-*proS*- is less stable than the C_5-*proR*-carbanion in aqueous solution.

The stereoselectivities of C_5-*proR*- to C_5-*proS*-proton transfer from **1** of 1−10:1 reported here (Table 1) are similar to previously reported values in the range 2-11:1 for **1** and other 6-substituted-5,6-dihydrouracils.[11] Differences in the stereoselectivity of C_5-*proR*- to C_5-*proS*-proton transfer from **1** of 11:1 compared to 2−3:1 for **3** and **4** were rationalized as being due



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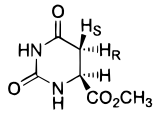

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to neighboring electrostatic effects that destabilize the conformer that would give rise to the C_5-*proS* carbanion. The progressive increase in stereoselectivity from 2.9:1 for cacodylate (one

(33) High precision for measured rate constants is required to obtain useful information about relative free energies of two transition states when both are partially rate-limiting (Albery, W. J.; Knowles, J. R. *J. Am. Chem. Soc.* **1977**, *99*, 637−638).

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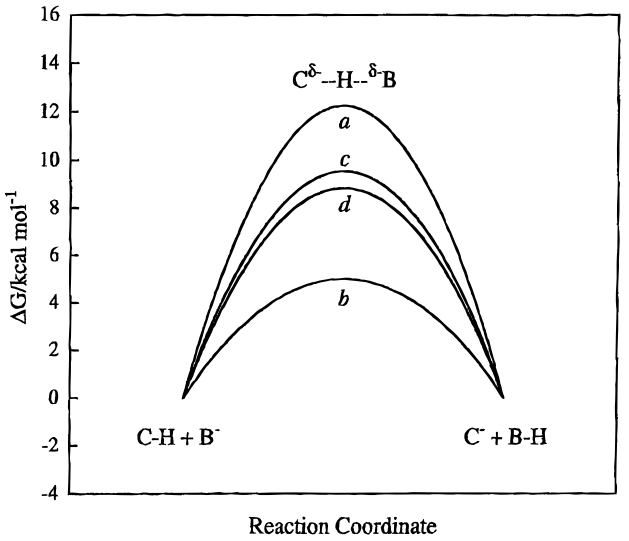


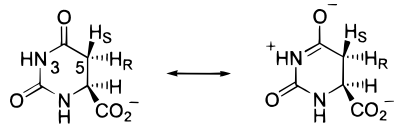
Figure 5. Gibbs free energy-reaction coordinate diagrams for thermoneutral proton transfer from carbons acids to an anionic base to illustrate the "intermediate" intrinsic kinetic barrier for the C_5 positions in L-dihydroorotate: (a) a carbonyl-activated carbon acid,[46a] (b) "normal" carbon acids (HCN[22] and thiamin[4a]), and (c) the C_5-*proS* and (d) C_5-*proR* positions of L-dihydroorotate.

negative charge) to 6.0:1 for phosphate dianion (two negative charges) and 9.5:1 for phosphate trianion (three negative charges), respectively, provides evidence that electrostatic repulsion resulting from the C_6-carboxyl group of **1** on approach of a negatively charged base contributes to the observed stereoselectivity.

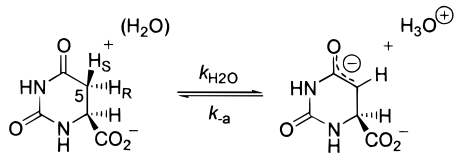
A principal reason for slow hydron exchange from carbon acids is the low acidity of most carbon acids, which arises from the low electronegativity of carbon.[39] Functional groups that decrease the pK_a of carbon acids also tend to make the transition state for the hydron transfer more complex and less stable, and this increase in transition-state complexity is accompanied by a decreased reaction rate.[40,41] The intrinsic kinetic barriers,[5,42] $\Delta G^{\ddagger}_{intr}$, for deprotonation of L-dihydroorotate of 8.8 kcal mol^{-1} at C_5-*proR* and 9.5 kcal mol^{-1} at C_5-*proS*, respectively, are larger than the ~5 kcal mol^{-1} barrier that is typical of "normal" acids,[43] but smaller than the intrinsic barrier of ≥12 kcal mol^{-1} barrier typical of carbonyl-activated carbon acids[44] (Figure 5). There are several possible reasons for the smaller-than-expected intrinsic barrier for proton transfer from the carbonyl-activated C_5-*proR/proS* position of **1**:

(1) The intrinsic barrier to proton transfer may decrease when the relative stability of the C_5-carbanion/enolate (**2**) is decreased by ground-state resonance stabilization of the keto tautomer as has been proposed for proton transfer from ethyl acetate.[6a] In the case of **1**, this can be accomplished by electron donation from N_3 to the carbonyl group (Scheme 2). Evidence consistent with such ground-state resonance stabilization comes from the

Scheme 2



Scheme 3



crystal structures of several 6-substituted-5,6-dihydrouracils, where the left half of the ring (N_1 to C_4) is almost completely planar.[45] In general, such an effect is possible when the atom adjacent to the carbonyl group is a heteroatom. The extent to which the relative resonance stabilization of the keto reactant and the enolate product is *expressed in the transition state* will dictate the magnitude of the decrease of the intrinsic barrier. No satisfactory calculation is available at this time for the relative contributions from enthalpy and entropy to transition-state complexity for proton transfer to and from **1** in aqueous solution.

(2) Large intrinsic barriers are typically associated with a lack of synchronization between concurrent reaction events such as bond formation/cleavage, solvation/desolvation, and development/loss of resonance.[46] The contribution of solvent reorganization effects to the intrinsic barrier could be minimized by retaining partial solvation of the reactant (**1**) in the transition state. The observation that oxygen anions are strongly solvated[47] suggests that, in the absence of ground-state resonance stabilization of **1**, retaining solvation in the keto reactant **1** and enolate product **2** would not be favorable. However, the negative charge on the carbonyl oxygen would be partly maintained in going from reactants to products when **1** is stabilized by resonance involving N_3 (Scheme 2), which could minimize solvent reorganization in the transition state for proton transfer. Since amines are less strongly solvated than oxygen anions,[47] the loss of the partial positive charge on N_3 in going from reactants to products is not expected to contribute significantly to solvent reorganization. A similar explanation has been proposed to partly account for the "normal" acid behavior of the C(α)-proton in an iminium-ion activated carbon acid.[2b]

p$K_a^{C_5H}$ Value for L**-Dihydroorotate.** Values of p$K_a^{C_5H}$ in the range 20−21 can be calculated for the C_5-*proR/proS* position in **1** in H_2O. This equilibrium constant in H_2O was obtained according to Scheme 3. The value of k_{H_2O} was calculated by multiplying the observed pseudo-first-order rate constant for the pD-independent, buffer-independent C_5-proton exchange reaction with D_2O by 2.6, to correct for the secondary solvent deuterium isotope effect on the rate constant of k_{H_2O}/k_{D_2O} = 2.6.[48] A value of 2×10^{10} M^{-1} s^{-1} was assumed for the diffusion-controlled reaction in the reverse direction. Values for k_{-a} in the range $(1-4) \times 10^{10}$ M^{-1} s^{-1} have been reported for protonation of amines[34a] and CN^-[22,26a] by H_3O^+. To our

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(42) Calculated using $\Delta G^{\ddagger}_{intr} = RT \ln(kT/hk_{intr})$ using $k_{intr} = 10^{6.3}$ M^{-1} s^{-1} (C_5-*proR*) and $k_{intr} = 10^{5.8}$ M^{-1} s^{-1} (C_5-*proS*), where the intrinsic rate constant, $k_{intr} = k_B$ at $\Delta pK_a = 0$, was obtained by extrapolation of the corresponding Brønsted plot (Figure 4). We assume that the Brønsted plots do not curve appreciably with increasing pK_{BD}.

(43) Calculated using $\Delta G^{\ddagger}_{intr} = RT \ln(kT/hk_{intr})$ using $k_{intr} = 10^{9}$ M^{-1} s^{-1} (Washabaugh, M. W.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 674−683; ref 22).

(44) Calculated using $\Delta G^{\ddagger}_{intr} = RT \ln(kT/hk_{intr})$ using $k_{intr} = 10^{3.8}$ M^{-1} s^{-1} (Bernasconi, C. F. *Adv. Phys. Org. Chem.* **1992**, *27*, 119−238).

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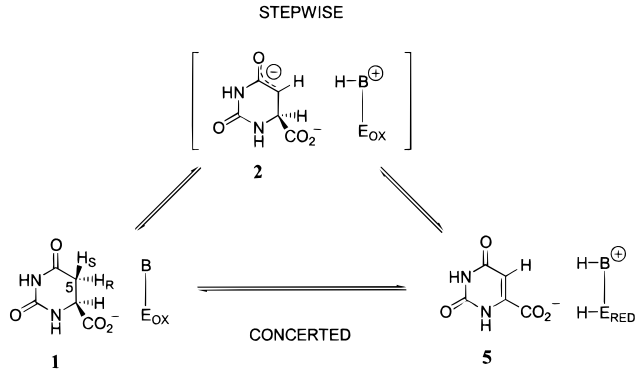
Scheme 4



knowledge, the smallest rate constant reported for diffusion-controlled reprotonation of a base by a buffer acid in aqueous solution is $k_{-a} = 4 \times 10^6$ M^{-1} s^{-1};[24] a p$K_a^{C_5H}$ value of 17 is obtained with this minimum value of k_{-a}.

The assumption of diffusion-controlled reprotonation of **2** by H_3O^+ is supported by the demonstration of an Eigen mechanism for proton transfer, in which both proton transfer and diffusional separation of the products are partially rate-limiting for relatively basic catalysts. In this simple three-step model for proton transfer, diffusion-controlled separation of the products becomes even more rate-limiting as the proton transfer becomes more thermodynamically unfavorable—the catalyst basicity decreases. For example, the large value of the internal return ratio, $k_{-1}^H/k_2 = 4$, for catalysis by 1,4-diazabicyclo [2.2.2] octane (pK_a = 9.82) (Table 2) is expected to become even larger for catalysis by the weak base H_2O (p$K_a = -1.74$). This assumption is also supported by the Brønsted β values >0.8 for catalysis of C_5-*proR/proS*-proton exchange by buffer bases, which indicate that the reverse protonation reaction has a Brønsted α value of <0.2 and that reprotonation is likely diffusion-controlled with a strong acid like H_3O^+. The values of p$K_a^{C_5H}$ in the range of 17−21 provide additional support for the conclusion that C_5-*proR/proS*-proton transfer from **1** is always thermodynamically unfavorable in aqueous solution.

Implications for Enzyme-Catalyzed Reactions. A fundamental problem in enzymology is how enzymes handle moderately unstable intermediates such as the C_5-carbanion/enolate. Dihydroorotate dehydrogenases catalyze the oxidation of **1** to orotate (**5**, Scheme 4). Scheme 4 shows that the enzyme could catalyze the reaction via either of two mechanisms: a stepwise mechanism (upper pathway); or a concerted mechanism (lower pathway). A p$K_a^{C_5H}$ value of ≤18 is required for enzyme-bound **1** if the C_5-carbanion exists as an intermediate in a stepwise mechanism;[49] however, the estimated value of p$K_a^{C_5H}$ = 20−21 for **1** shows that the C_5-carbanion is quite unstable in aqueous solution. Therefore, the enzyme either changes the relative thermodynamic stabilities of **1** and its C_5-carbanion, or it provides a one-step, concerted pathway that avoids the unstable carbanion intermediate.[50] There is precedent for stabilization of weakly acidic α-carbanions by enzymes; for example, pig kidney acyl-CoA dehydrogenase decreases the value of p$K_a^{\alpha-CH}$ for certain acyl-CoA analogues from 15 to 20 in aqueous solution to 7 at the active site.[51]

A stepwise mechanism for dehydrogenation with *anti* stereochemistry (upper pathway, Scheme 4) involving C_5-*proS*-hydron transfer from L-dihydroorotate (**1**), formation of the C_5-carbanion/enolate intermediate, followed by C_6-hydride or C_6-radical transfer to an enzyme-bound electron acceptor has been suggested for several dihydroorotate dehydrogenases; the enzymatic reaction shows stereoselectivity at C_5 opposite to that of nonenzymatic C_5-proton exchange.[7a,52] This stepwise mechanism is consistent with: (i) the aggregate isotope effects on V_{max} (^DV) and on $V_{max}/K_m[^D(V/K)]$ for the reaction with **1** catalyzed by the dihydroorotate dehydrogenase from *Crithidia fasciculata*,[7a] (ii) exchange at C_5 in several 5,6-dihydropyrimidines,[37b,53] (iii) the fact that **1** undergoes sodium ethoxide catalyzed exchange of the C_5 protons in refluxing ethanol-*d* without deuterium incorporation into the C_6 position,[52] and (iv) speculation that an enolate intermediate would be more susceptible to oxidation than **1** on the basis of nonenzymatic model reactions of enolates with riboflavin.[54] However, a concerted mechanism, as observed for acyl-CoA dehydrogenases,[55] has not been strictly ruled out.

Acknowledgment. We thank Cecile Pickart, Lawrence Grossman, and Paul Miller for helpful scientific discussions, advice, and encouragement.

JA992753V

(48) The solvent isotope effect of $k_{H_2O}/k_{D_2O} = 2.6$ was calculated as described previously (ref 4a) from a fractionation factor of $l^2 = (0.69)^2$ for HOD_2^+ and a solvent isotope effect of 1.22 for diffusional separation of HOD_2^+ from **2** (Millero, F. J.; Dexter, R., Hoff, E. *J. Chem. Eng. Data* **1971**, *16*, 85−87).

(49) The limiting p$K_a^{C_5H}$ value of ≤18 for enzyme-bound **1** was calculated as described previously (ref 4a) from $k_{cat} = 59$ s^{-1} at pH 6.0 for dihydroorotate dehydrogenase (ref 7a) and assuming pK_a = 7 for a catalytic base at the active site.

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