

Orotic Acid Decarboxylation in Water and Nonpolar Solvents: A Potential Role for Desolvation in the Action of OMP Decarboxylase[†]

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ABSTRACT: OMP decarboxylase (ODCase) generates a very large rate enhancement without the assistance of metals or other cofactors. The uncatalyzed decarboxylation of 1-methylorotate in water is shown to involve the monoanion, although uncharged 1-methylorotic acid is decarboxylated at a similar rate. To measure the extent to which the rate of the nonenzymatic decarboxylation of orotate derivatives might be enhanced by their removal from solvent water, the 1-phosphoribosyl moiety of OMP was replaced with 1-substituents that would allow it to enter less polar solvents. When the tetrabutylammonium salt of 1-cyclohexylorotate was transferred from water to a series of dipolar aprotic solvents, its rate of decarboxylation increased markedly, varying with the relative ability of each solvent to release the substrate in the ground state from stabilization by solvent water acting as a proton donor. These findings are consistent with the view that separation of the substrate from solvent water may contribute, at least to a limited extent, to the rate enhancement produced by ODCase. This enzyme's active site, like that of another cofactorless enzyme recently shown to produce a rate enhancement similar in magnitude (uroporphyrinogen decarboxylase), is equipped with an ammonium group positioned in such a way as to balance the electrostatic charge of the carboxylate group of the substrate and later supply a proton to the incipient carbanion in a relatively waterless environment.

Compared with the sluggish pace of the uncatalyzed reaction, orotidine 5'-phosphate decarboxylase (ODCase,¹ EC 4.1.1.23) generates one of the largest rate enhancements known to be produced by any enzyme, and it achieves this feat as a pure protein catalyst, without the assistance of metal ions or other cofactors (1). Of the eight invariant amino acid residues that are conserved in ODCase from all species that have been examined, seven make direct contact with the substrate, and mutation of any one of those residues to alanine leads to a drastic loss of activity (2). But only one of those eight residues (Lys-93 in the yeast sequence) approaches the pyrimidine ring at a position near the site of CO₂ elimination, as indicated by the structure of the enzyme complex with the transition state analogue 6-hydroxyuridine 5'-phosphate (3). The other seven residues appear to play a less direct role in the action of ODCase, bracing the substrate in position for effective catalysis and assisting the folding of loops that enclose the substrate during the central chemical events in catalysis (3, 4).

An enzyme's tendency to bind the substrate while the enzyme is in an open configuration, and then enclose the altered substrate in the transition state (S[‡]), has been observed in transition state analogue complexes formed by triosephosphate isomerase (5, 6),

adenosine deaminase (7), cytidine deaminase (8), and ODCase (9). There appear to be several possible reasons why that tendency might promote effective catalysis. First, closure of an initially open active site would be expected to allow maximization of the solid angle of contact between the enzyme (a large molecule) and the altered substrate in the transition state (usually a small molecule), and hence maximization of their forces of mutual attraction (10). Second, closure of an initially open active site tends to involve removal of the substrate from solvent water, which would, in itself, be expected to enhance reactivity, at least in some cases. Earlier work has shown, for example, that the nonenzymatic elimination of CO₂ from 2-keto acids in the presence of thiamine (11) and the uncatalyzed decarboxylation of 3-carboxy-6-benzisoxazole (CBI) (12) are greatly accelerated when these reactions are conducted in nonpolar solvents.

In this work, we set out to determine the extent to which removal of orotate derivatives from solvent water, by transfer into nonpolar solvents, might enhance their spontaneous rate of decarboxylation.

Earlier, the uncatalyzed decarboxylation of orotate derivatives was found to proceed so slowly in water that it was necessary to conduct experiments at elevated temperatures if rate constants were to be determined within a reasonable period of time. Because the 1-glycosidic bonds of pyrimidine nucleosides undergo hydrolysis much more rapidly than the carboxylate group is eliminated from orotate, those experiments on nonenzymatic decarboxylation were conducted using the relatively stable 1-methyl derivative of orotic acid and related molecules (13–22). In our experiments, we sought to replace the phosphoribosyl moiety of OMP with nonpolar substituents that would allow orotate to enter relatively nonpolar solvents in the presence of alkylammonium counterions. The 1-cyclohexyl substituent proved to be suitable for that purpose.

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¹Abbreviations: OMP, orotidine 5'-monophosphate; ODCase, orotidine 5'-monophosphate decarboxylase; 1-MeU, 1-methyluracil; 1-ChxU, 1-cyclohexyluracil; U6AA, uracil-6-acetic acid; 1-MeO, 1-methylorotic acid; 1-ChxO, 1-cyclohexylorotic acid; 3-MeO, 3-methylorotic acid; 1,3-Me₂O, 1,3-dimethylorotic acid; TBA, tetrabutylammonium; 1-ChxO-TBA, 1-cyclohexylorotate tetrabutylammonium salt; 3-MeU, 3-methyluracil; 1,3-Me₂U, 1,3-dimethyluracil; 6-MeU, 6-methyluracil; DMAc, *N,N*-dimethylacetamide; CBI, 3-carboxy-6-benzisoxazole; FNB, 4-fluoro-nitrobenzene; UroD, uroporphyrinogen decarboxylase.

Table 1: ^1H NMR Chemical Shifts of Derivatives of Orotic Acid and Uracil in D_2O

	C5H	1-Me	3-Me	C1'H ^a	CH ₂ ^b		C6H	C5H	1-Me	3-Me	C1'H ^a	CH ₃ ^b
orotate	6.14					U	7.48	5.75				
1-MeO	5.74	3.29				1-MeU	7.55	5.74	3.32			
3-MeO	6.20		3.23			3-MeU	7.44	5.81		3.22		
1,3-Me ₂ O	5.78	3.34	3.25			1,3-Me ₂ U	7.53	5.83	3.36	3.26		
1-ChxO	5.58			3.72		1-ChxU	7.69	5.79				4.27
U6AA	5.63				3.36	6-MeU		5.59				2.14

^aMultiplet arising from the cyclohexyl C1'H group linked to N1 of the pyrimidine ring. ^bThe CH₂ singlet of U6AA and the CH₃ singlet of 6-MeU were also used in the calculations.

MATERIALS AND METHODS

Orotic acid, 1-methyluracil (1-MeU), 1-cyclohexyluracil (1-ChxU), and uracil-6-acetic acid (U6AA) were purchased from Sigma-Aldrich Corp. 1-Methylorotic acid (1-MeO) and 1-cyclohexylorotic acid (1-ChxO) were prepared from the corresponding uracil derivatives as described by Landesman (23). In both cases, the 1-alkyluracil in a dry DMF solution was treated with bromine (in CCl_4) to generate the 5-bromo derivative, followed by reaction with potassium cyanide in the presence of 18-crown-6 to produce the 6-cyano derivative, and finally, the 6-cyano derivative was heated in 1 N NaOH to yield the corresponding 1-alkylorotic acid. 3-Methylorotic acid (3-MeO) and 1,3-dimethylorotic acid (1,3-Me₂O) were prepared as described by Curran and Angier (24). The identity of each product was confirmed by ^1H NMR and UV spectrometry in acidic, neutral, and basic aqueous solutions (25), and their purities, as measured by ^1H NMR, were judged to be >95%.

To measure the effect of the changing pH on the rate of decarboxylation, reaction mixtures containing 1-MeO (0.01 M) were prepared in HCl, and in buffers (sodium acetate, potassium phosphate, sodium borate, and sodium carbonate, at 0.1 M), and KOH distributed over the H_0 –pH range between ca. –1 and 11 at 25 °C. The heats of ionization of these anionic buffers are relatively small (26), as would also be expected to be true of the –COOH groups of orotic acid derivatives. Thus, the state of ionization of these orotate derivatives in buffered solution is not expected to change much with an increase in temperature in the acid range [$\text{p}K_{\text{a}1}$ for 1-MeO ($\text{p}K_{\text{a}1} \sim 0.6$ at 25 °C)] (25). Because the heats of ionization of the N₃ proton of uracil derivatives [$\text{p}K_{\text{a}2}$ for 1-MeO (~ 9.8 at 25 °C)] (26) do not appear to have been reported, we used a pH meter to determine the temperature dependence of $\text{p}K_{\text{a}2}$ for 1-MeO as described in Results.

^1H NMR spectra were recorded with a Varian Unity 500 MHz spectrometer, equipped with a high-sensitivity cryoprobe operated using Solaris 9 software. To obtain a signal-to-noise ratio suitable for accurate measurement of the integrated intensities of the resonances arising from both the starting material and product in the kinetic experiments described below, we employed 4–16 transients with a 60 s pulse delay.

For rate measurements in aqueous solution, samples (0.1 mL) of orotate derivatives (0.025 M) in potassium phosphate buffer (0.1 M, pH 7.0) were sealed under vacuum in quartz tubes and heated in Thermolyne 47900 ovens for times sufficient to achieve 15–85% reaction. For analysis by proton NMR, samples (0.1 mL) were then diluted with D_2O [0.5 mL, containing 0.01 M pyrazine (4H, δ 8.60) as an integration standard]. For rate measurements in nonpolar solvents, the tetrabutylammonium salt of 1-cyclohexylorotic acid (1-ChxO-TBA) was prepared by titration of 1-ChxO with tetrabutylammonium hydroxide to pH 7, lyophilized

to remove water, dried over magnesium perchlorate, and dissolved in the nonpolar solvent. To minimize residual ^1H signals arising from the solvent during analysis by ^1H NMR, reaction mixtures were prepared in $\text{DMSO-}d_6$, acetone- d_6 , dioxane- d_8 , chloroform- d_1 , dimethylformamide- d_7 , dimethylacetamide- d_8 , or tetrahydrofuran- d_6 purchased from Sigma-Aldrich Corp.; in formamide- d_3 purchased from CDN Isotopes, Inc.; in methylformamide- d_4 purchased from Medical Isotopes, Inc.; or in acetonitrile- d_3 purchased from Cambridge Isotope Laboratories. Samples dissolved in nonpolar solvents were frozen by immersion in a dry ice/acetone bath and sealed in quartz tubes under vacuum. After being heated, samples were prepared for analysis by ^1H NMR as described above, except that $\text{DMSO-}d_6$ was used as the solvent instead of D_2O in some cases. D_2O and $\text{DMSO-}d_6$ were found to be devoid of NMR signals in the region used for observing signals arising from the pyrimidine protons and from the C1' proton of the 1-cyclohexyl substituent. ^1H NMR spectra were recorded as described above.

To follow the progress of reactions conducted in aqueous solution, the integrated intensities of the 1-alkyl protons and the CH protons of the pyrimidine ring were used to determine the concentrations of the reactant and product (Table 1). For reactions conducted in nonpolar solvents, the integrated intensities of the 1-ChxO resonances at ~ 5.58 ppm (C5H, singlet) and ~ 3.72 ppm (C1'H, multiplet) and the 1-ChxU resonances at ~ 7.69 ppm (C6H, doublet), ~ 5.79 ppm (C5H, doublet), and ~ 4.27 ppm (C1'H, multiplet) were used instead. Under all conditions examined, decarboxylation followed simple first-order kinetics to completion, and the resulting rate constants yielded linear Arrhenius plots when they were plotted as a logarithmic function of $1000/T$ (kelvin).

RESULTS

Effects of pH on the Rate of Decarboxylation of 1-MeO in Water. At 160 °C, the rate of decarboxylation of 1-MeO remained essentially constant in the acidic and neutral range (Figure 1) but decreased at higher pH values, presumably in response to the ionization of the N₃ proton. Since hydroxide ion displaces silicate from quartz in alkaline solution at elevated temperatures (27), experiments at pH > 8 were also conducted in Teflon-lined bombs. The heat of ionization of the N₃ proton of 1-MeO was also determined by monitoring the pH of a solution of millimolar 1-MeO (0.07 M, pH 9.8 at 22 °C) half-titrated with KOH, as a function of increasing temperature. A van't Hoff plot of those data (not shown) yielded a heat of ionization of 6.1 kcal/mol.

The $\text{p}K_{\text{a}}$ value of the –COOH group of 1-MeO is 0.7 (25). Because carboxylic acids tend to undergo decarboxylation in their anionic (–COO[–]) forms (28), the absence of any significant effect of changing pH on the rate of decarboxylation of 1-MeO

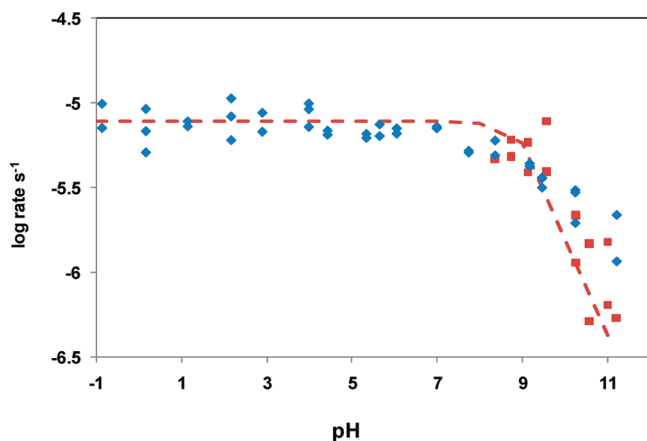


FIGURE 1: Effects of pH on $\log k$ (s^{-1}) for decarboxylation of 1-MeO at 160 °C in various buffers (see the text). Data were obtained using quartz tubes (blue diamonds) or Teflon cups in steel bombs (red squares). The solid line is calculated for the equation $\log k = 5.1[1\text{-MeO}^-] - 6.3[1\text{-MeO}^{2-}]$, where 1-MeO^- is the monoanion and 1-MeO^{2-} is the dianion of 1-methylorotate and the pK_a value of 1-MeO is 9.8.

between $H_o - 1$ and pH 3 is somewhat surprising. As in certain other cases in which formally uncharged carboxylic acids have been found to be unexpectedly reactive (29), the actual reactant in the range between $H_o - 1$ and pH 3 seems likely to be a rare zwitterionic form of the reactant (1-MeO^\pm) in which the carboxylate group remains negatively charged and the reactant is protonated at another position (N1, O2, or O4).² Such a zwitterion would be expected to be much more rapidly decarboxylated than 1-MeO^- . As a result, the reactivity of the major species (1-MeO^-) might (by coincidence) appear to remain unchanged by protonation.

Effects of 1-Substituents on the Rate of Decarboxylation of Orotate Derivatives in Water. In potassium phosphate buffer (0.1 M, pH 7), the decarboxylation of orotate derivatives followed simple first-order kinetics, yielding linear Arrhenius plots with similar slopes (Figure 2) that were extrapolated to estimate the rates of decarboxylation of these molecules at ordinary temperatures.

Of the reactants examined here, the monoanions of orotate and 3-MeO underwent decarboxylation most reluctantly, with extrapolated rate constants of 1.3×10^{-17} and $3.9 \times 10^{-17} s^{-1}$, respectively, at 25 °C. These very slow decarboxylations were accompanied by a concurrent reaction that led to opening of the pyrimidine ring and ultimately to the release of acetaldehyde (see below). 1,3-Me₂O and 1-MeO reacted considerably more rapidly, with rate constants of 2.4×10^{-15} and $3.4 \times 10^{-15} s^{-1}$, respectively, at 25 °C, and no ring opening accompanied decarboxylation. 1-Cyclohexylation of orotate resulted in a further increase in the rate constant for decarboxylation, to $4.9 \times 10^{-14} s^{-1}$ at 25 °C, with no concurrent ring opening. Heats and

²However, the C5 proton of 1-MeO showed an increase in chemical shift at pH < 2, confirming protonation of 1-MeO, to yield an acid with a pK_a value of ~ 0.7 (25) (data not shown).

³We also tested the effect of inserting a methylene group between C6 of orotic acid and its carboxylate group, by examining the behavior of U6AA as a reactant. The insertion of that methylene group enhanced the rate constant for decarboxylation by almost 7 orders of magnitude, to $1.7 \times 10^{-10} s^{-1}$ at 25 °C. In contrast, the acetate ion has been shown to undergo no detectable decarboxylation after 14 days at 360 °C (29). The reasons for this unusual reactivity remain to be established, but the decarboxylation of U6AA presumably generates a 6-methyl anion in which a negative charge might be delocalized to either of the oxygen atoms at C2 or C4.

entropies of activation for each of these reactions, obtained from Arrhenius plots, are listed in Table 2.³

For the decarboxylation of 1-MeO at 25 °C ($3.4 \times 10^{-15} s^{-1}$), the rate constant obtained in our experiments was somewhat larger than a value ($2.8 \times 10^{-16} s^{-1}$) reported earlier by one of us (1), based on UV spectrophotometric analysis of the progress of decarboxylation. We attribute that difference to the superiority of the ¹H NMR method described here, which allowed independent identification and more precise quantitation of the reactant and product at each stage of the reaction.

Ring Opening of Orotate and 3-Methylorotate in Water. During the very slow decarboxylation of aqueous solutions of orotate and 3-MeO at temperatures between 180 and 250 °C, other products were formed in significant amounts. Two of those products were identified as acetaldehyde and the corresponding gem-diol, by the characteristic aldehyde proton (~ 9.62 ppm, quartet, $J = 3$ Hz) and the methine proton of the covalent hydrate (~ 5.2 ppm, quartet, $J = 5.2$ Hz), and the doublets for the respective methyl groups (~ 2.19 ppm aldehyde, 1.28 ppm hydrate). Those products would be expected to arise from the opening of the pyrimidine ring between N3 and C4 to generate the corresponding derivative of ureidosuccinaldehyde, followed by elimination of acetaldehyde. Additional minor peaks appeared in the aliphatic region of the ¹H NMR spectra, presumably representing other products of ring opening. Decomposition by this alternative route proceeded at $\sim 57\%$ of the rate of decarboxylation of orotate, and at $\sim 32\%$ of the rate of decarboxylation of 3-MeO. No significant ring opening was observed at any temperature for 1-MeO, 1,3-Me₂O, 1-ChxO, or U6AA, whose rate of decarboxylation was considerably more rapid than that of orotate or 3-MeO. Rate constants for the decarboxylation of orotate and 3-MeO were obtained by applying the observed product ratio [as reflected in the appearance of acetaldehyde and its hydrate compared with the product of decarboxylation (U or 3-MeU)] to the first-order rate constant observed for the disappearance of the substrate. Because that correction was substantial, particularly for orotate, the rate constants for orotate and 3-MeO in Table 2 should be considered approximate.

Decarboxylation of 1-Cyclohexylorotate in Nonpolar Solvents. To permit the ionized form of 1-ChxO to enter organic solvents at concentrations sufficient for monitoring its decomposition, we converted the acid to its tetrabutylammonium salt (1-ChxO-TBA). TBA was selected to both enhance solubility of 1-ChxO in organic solvents and to provide a counterion that did not contain a proton, which could hydrogen bond to the orotate carboxylate and potentially reduce its reactivity toward decarboxylation. In a series of solvents ranging from formamide to acetone, we observed quantitative conversion of 1-ChxO to 1-ChxU. The logarithm of the first-order rate constants of these reactions, plotted as a function of absolute temperature, yielded linear Arrhenius plots as shown in Figure 3.

Extrapolation of these Arrhenius plots to 25 °C showed that decarboxylation in formamide, *N*-methylformamide (NMF), and dimethylformamide (DMF) proceeded ~ 10 -, ~ 100 -, and ~ 1000 -fold more rapidly, respectively, than decarboxylation in water. Acetonitrile and dimethyl sulfoxide produced rates of decarboxylation that were intermediate between those observed in NMF and DMF. Decarboxylation proceeded most rapidly ($\sim 3 \times 10^{-11} s^{-1}$) in dimethylacetamide, tetrahydrofuran, dioxane, and acetone. The thermodynamic activation parameters observed in these solvents are listed in Table 3.

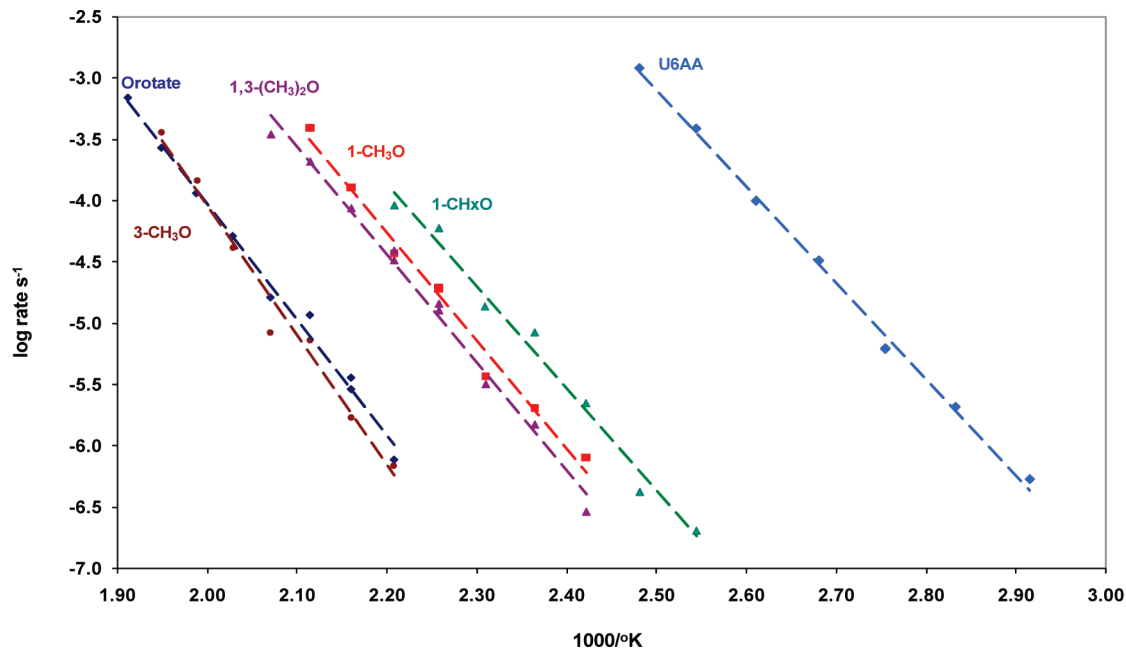


FIGURE 2: Rate constants ($\log k, \text{s}^{-1}$) for the decarboxylation of orotic acid derivatives in potassium phosphate buffer (0.1 M, pH 7.0), plotted as a function of the reciprocal of absolute temperature.

Table 2: Thermodynamics of Activation for Decarboxylation of Orotic Acid Derivatives in Potassium Phosphate Buffer (0.1 M, pH 7.0)						
	temp range (°C)	$k_{25^\circ\text{C}} (\text{s}^{-1})$	$\log k_{25^\circ\text{C}}$	$\Delta H^\ddagger (\text{kcal/mol})$	$\Delta G^\ddagger (\text{kcal/mol})$	$T\Delta S^\ddagger (\text{kcal/mol})$
orotate	180–250	1.3×10^{-17}	−16.89	42.8	40.4	2.5
3-MeO	180–250	3.9×10^{-17}	−16.41	47.8	42.4	5.4
1,3-Me ₂ O	140–210	2.4×10^{-15}	−14.62	39.7	37.3	2.4
1-MeO	140–200	3.4×10^{-15}	−14.47	39.8	37.1	2.8
1-chxO	120–180	4.9×10^{-14}	−13.31	37.3	35.5	1.8
U6AA	70–130	1.7×10^{-10}	−9.77	35.1	30.7	4.4

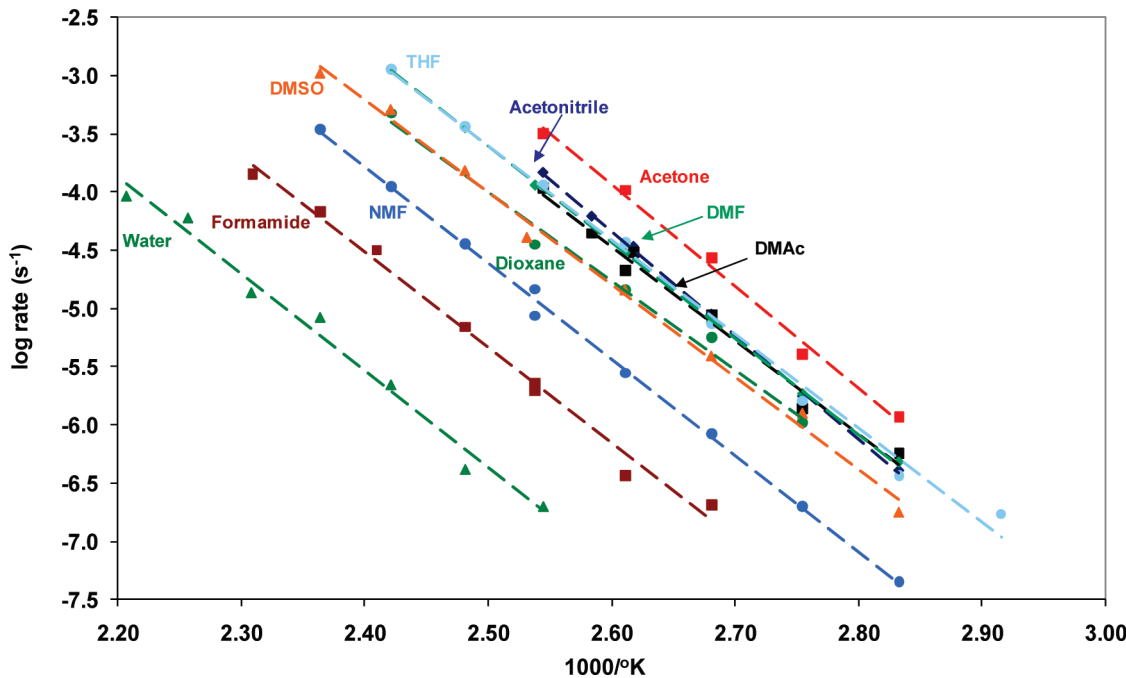


FIGURE 3: Rate constants ($\log k, \text{s}^{-1}$) for the decarboxylation of 1-ChxO-TBA salt in various solvents, compared with 1-ChxO in potassium phosphate buffer (0.1 M, pH 7), plotted as a function of the reciprocal of absolute temperature.

6-Deuteration during Decarboxylation in Nonpolar Solvents. In our experiments, deuterated solvents were used to

reduce interference by proton signals arising from the solvent. To generate uracil derivatives in these solvents, it would be necessary

Table 3: Thermodynamics of Activation for Decarboxylation of the TBA Salt of 1-ChxO in Nonpolar Solvents

	temp range (°C)	$k_{25^\circ\text{C}}$ (s^{-1})	$\log k_{25^\circ\text{C}}$	ΔH^\ddagger (kcal/mol)	ΔG^\ddagger (kcal/mol)	$T\Delta S^\ddagger$ (kcal/mol)
water	120–180	4.9×10^{-14}	–13.31	37.3	35.5	1.8
formamide	110–160	4.3×10^{-13}	–12.37	37.0	34.2	2.8
NMF	80–150	2.1×10^{-12}	–11.68	37.2	33.3	3.9
acetonitrile	80–120	8.3×10^{-12}	–11.08	40.3	32.5	7.8
DMSO	80–150	1.6×10^{-11}	–10.80	35.7	32.1	3.6
DMF	80–140	2.2×10^{-11}	–10.66	37.1	31.9	5.1
Me ₂ -acetamide	80–120	2.9×10^{-11}	–10.54	36.2	31.7	4.5
tetrahydrofuran	70–140	3.0×10^{-11}	–10.52	36.5	31.7	4.7
dioxane	90–140	3.0×10^{-11}	–10.52	34.3	31.7	2.6
acetone	80–120	3.0×10^{-11}	–10.52	39.3	31.7	7.6

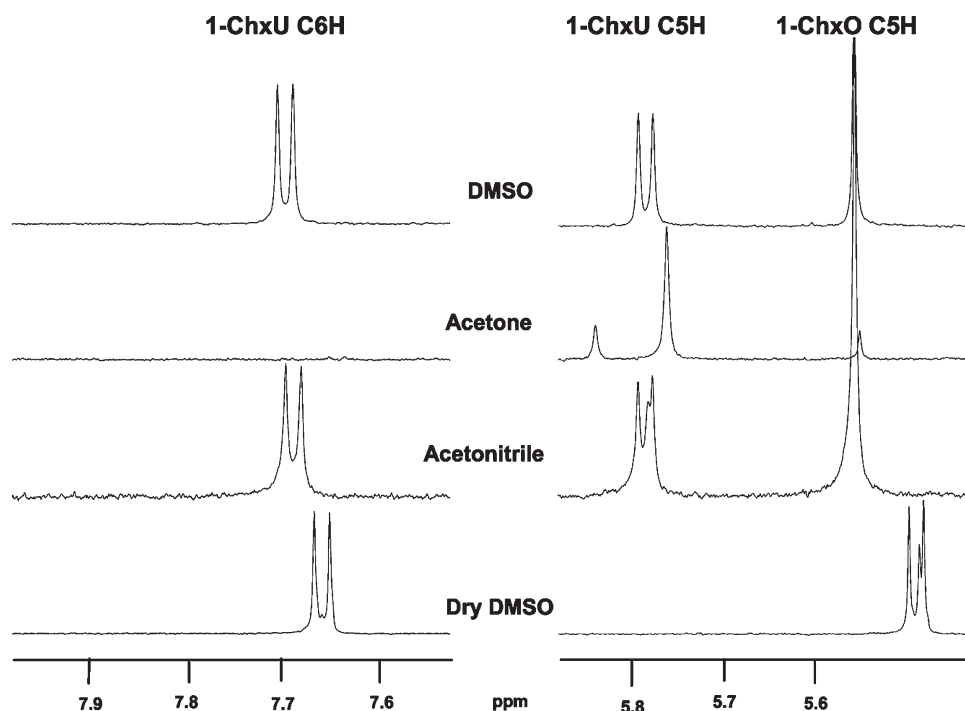


FIGURE 4: ^1H NMR spectra of the C6 and C5 protons of 1-ChxU arising from the decarboxylation of 1-ChxO conducted in three solvents. The top three samples were diluted in D_2O , while the fourth was diluted in $\text{DMSO}-d_6$. When traces of H_2O were present in these reaction mixtures, two doublets ($J = 7.9$ Hz) were produced when the C6 carbanion extracted a proton from water. In acetone- d_6 , the C6 carbanion extracted a deuteron from the solvent, leaving only the C5 proton resonance as a singlet. Acetonitrile- d_3 and “dry” DMSO showed evidence of both reactions. In $\text{DMSO}-d_6$, the resonances were shifted upfield from their positions in D_2O .

to abstract a proton or deuteron from the solvent unless traces of water were present in the solvent. In some cases, deuteration of C6 was in fact observed in the ^1H NMR spectra, with a singlet for the 5-proton of 1-ChxU resulting from the loss of the 6-proton coupling due to deuteration (Figure 4).

The extent of 6-deuteration varied in different solvents. Thus, the product of 1-ChxO decarboxylation in $\text{DMSO}-d_6$ showed two doublets arising from 6-protonation by traces of H_2O (top spectrum in Figure 4). However, in acetone- d_6 , only the C5 proton resonance of the product appeared as a singlet, implying deuteration at C6. In solvents in which limited amounts of water were present, a mixture of protonated and deuterated products was observed, as illustrated by the two bottom spectra of Figure 4. Similarly, Beak and Siegel (14) reported deuteration at C6 when 1,3-Me₂O was heated in sulfolane- d_4 at 206 °C. In these cases, the observed deuteration at C6 implies an ability of the carbanion to extract deuterium even from solvents of very weak acidity. That property is not unexpected, in view of the estimated $\text{p}K_a$ values of acetone (20), acetonitrile (25), Me₂-acetamide (25), and DMSO (31), compared with an estimated $\text{p}K_a$ value of ~ 34 for the 6CH group of UMP (30).

DISCUSSION

Table 2 shows that the rate of decarboxylation of orotate derivatives in water is markedly affected by the nature of the substituent at N1. In earlier experimental work on the non-enzymatic reactivity of orotic acid derivatives in water and other solvents, 1-methylated derivatives were used to circumvent complications arising from the instability of the 1-phosphoribofuranosyl substituent at the elevated temperatures that were needed to observe decarboxylation (1, 13, 14). In this work, the slowest rates of decarboxylation were observed for unsubstituted orotate and 3-MeO, which also underwent slow but significant ring opening with release of acetaldehyde at elevated temperatures. However, 1-MeO and 1,3-Me₂O undergo decarboxylation ~ 100 -fold more rapidly than does orotate, and a further 10-fold increase in rate was produced by the presence of a 1-cyclohexyl substituent, introduced for the purpose of enhancing solubility in nonpolar solvents. Model building discloses a steric conflict between the 1-substituent and the 6-carboxylate group if the latter is oriented in the same plane as the pyrimidine ring. That

Table 4: Effects of Solvent on Rate Constants ($\log k$, s^{-1}) Reported for 1-ChxO Decarboxylation (this work), CBI Decarboxylation (12), and Attack of Azide on 1-Fluoro-4-nitrobenzene (31)

	ChxO	CBI ^a	azide and FNB ^b	acidity ^c	basity ^c	acidity and basity
water	-13.3	-5.13	0.0	1.00	1.00	2.00
formamide	-12.4	-3.13	0.8	0.66	0.99	1.65
NMF	-11.7	-2.09	—	—	—	—
acetonitrile	-11.1	0.60	3.9	0.37	0.86	1.22
DMSO	-10.8	1.00	3.9	0.34	1.08	1.41
DMF	-10.7	1.56	4.5	0.30	0.93	1.23
acetone	-10.5	1.38	4.9	0.25	0.81	1.06
dioxane	-10.5	-1.39	—	0.19	0.67	0.86
DMAc	-10.5	2.20	5.0	—	—	—
linear regression ^d						
<i>n</i>		9	7	7	7	7
<i>R</i> ²		0.81	0.98	0.98	0.25	0.87
slope		0.36	0.53	(-3.8)	(-3.9)	(-2.7)

^a $\log k$ (s^{-1}) for decarboxylation of CBI (12). ^b $\log k$ (s^{-1}) for attack of azide on FNB (31). ^cSwain's scales of acidity (anion solvating tendency) and basity (cation solvating tendency), based on a survey of solvent effects on the rates of 77 reactions (34). ^dThe number of data points (*n*), squared regression coefficient (*R*²), and slope are shown for linear regression of $\log k$ (s^{-1}) observed for decarboxylation of ChxO in this work (column 2), vs the logarithms of rate constants recorded for CBI decarboxylation (column 3) and for attack of azide on FNB (column 4), and against Swain's scales of acidity (anion solvating tendency, column 5), basity (cation solvating tendency, column 6) (34), and overall polarity (acidity and basity, column 7).

steric conflict, present in 1-substituted forms of orotate but not in orotate or in 3-MeO, is presumably relieved in carbanionic intermediates approaching the transition state for decarboxylation, accounting for their differing rates of reaction.

Substantial increases in the rate of decarboxylation of 1-ChxO were observed in nonaqueous solvents. Table 4 shows the logarithms of the rate constants for decarboxylation of 1-ChxO (obtained by extrapolation to 25 °C) in various solvents, and also some values recorded in the literature for two well-characterized, quite different reactions that involve delocalization of negative charge as the substrates proceed from the ground state to the transition state: the reaction of azide with 4-fluoronitrobenzene (FNB) at 100 °C (31) and the decomposition of 3-carboxybenzisoxazole (CBI) at 30 °C (32–34).⁴ Despite their differences in mechanism, (see Figure 5) all three of these reactions can be seen to be very closely related to each other in the relative ordering of their kinetic responses to the various solvents (as indicated by the *R*² values for linear regression at the foot of each column of Table 4) the span of relative reactivities is greater for azide attack on FNB and for decomposition of CBI than for decarboxylation of 1-ChxO (as indicated by the slopes in Table 4).

Such reactions, in the words of Parker, “are faster in dipolar aprotic solvents because the reactant anion is much more solvated by protic than by dipolar (aprotic) solvents and this outweighs any effects due to transition state anion ... solvation” (35). Also shown are Swain's scales of “acidity” (anion solvating tendency) and “basity” (cation solvating tendency), based on a survey of the rates of 77 reactions in various solvents (36). In keeping with Parker's analysis, the rate of

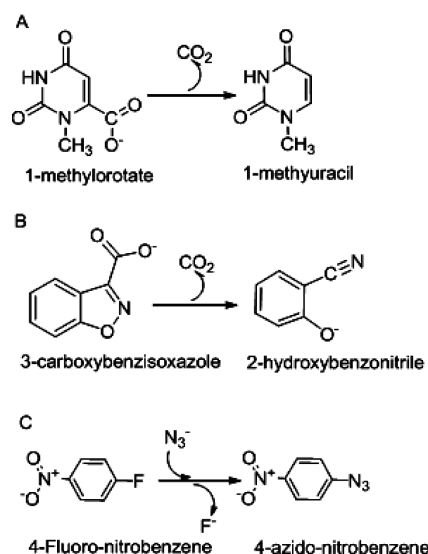


FIGURE 5: Decarboxylation of 1-methylorotate, decarboxylation of 3-carboxybenzisoxazole, and reaction of NaN_3 with 4-fluoronitrobenzene. These reactions are characterized by delocalization of charge in the transition state.

1-ChxO decarboxylation shows a strong negative correlation (slope = -3.8) with Swain's acidity scale and no significant correlation with his basity scale. One is led to infer that orotate derivatives are markedly stabilized in their ground states (relative to their transition states) by H-bonding to solvent water and that this effect tends to disappear in solvents with increasingly aprotic character.

These findings are consistent with the possibility that extraction of the substrate from solvent water enhances the value of k_{cat} and contributes to the rate enhancement (k_{cat}/k_{non}) produced by OMP decarboxylase. It should be recognized that simple desolvation of a hydrophilic substrate would not, by itself, be expected to have much effect on the value of k_{cat}/K_m , because desolvation is expected to increase K_m to the same extent that it enhances k_{cat} (37, 38). Nevertheless, if a substrate is equipped with substituents distant from the site of bond making and breaking, whose interaction with the enzyme can be used to draw the scissile portion of the substrate into a nonaqueous environment that

⁴Although the decomposition of CBI involves a decarboxylation, that decarboxylation occurs in concert with the opening of the isoxazole ring and does not involve formation of a discrete carbanion intermediate as indicated by the lack of incorporation of solvent tritium into the product (32). Benzisoxazole (BI), which lacks a 3-carboxylate group, decomposes by an E2 elimination reaction in the presence of acetate as a catalyst and shows kinetic solvent effects (exemplified by a 7 order of magnitude rate increase from water to hexamethylphosphoramide) closely comparable in magnitude with those observed for CBI, but when BI reacts in the presence of triethylamine, the kinetic solvent effect was reduced to less than a single order of magnitude. Hilvert et al. (34) suggested that large solvent effects might be associated with the presence of a carboxylate functionality in the reactant or the catalyst, rather than solvent interactions with the polarizable benzisoxazole.

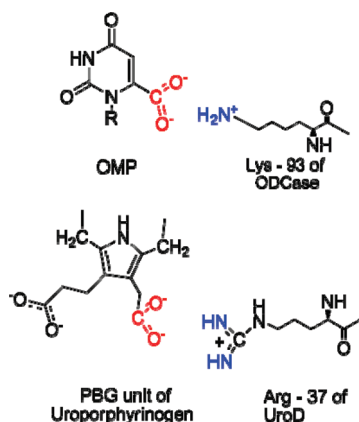


FIGURE 6: Proposed similarity of interaction of the ammonium groups of Lys-93 in ODCase and Arg-37 of urogen III decarboxylase with the carboxylate groups of their respective OMP and urogen III substrates.

enhances the reaction rate, then those substituents can be considered to contribute indirectly to catalysis by paying the cost of desolvating the scissile part of the substrate. In the case of ODCase, the phosphoribosyl group (39), and more specifically the phosphoryl group (40), of substrate OMP make very large contributions (11–12 kcal/mol in free energy) to stabilizing the altered substrate in the transition state by interacting with basic groups at the active site, without themselves playing any direct role in bond breaking (37). In a somewhat similar way, quaternary ammonium and phosphonium ions form ion pairs with the thiophenoxide ion, enabling it to enter the organic layer of a two-phase system where it reacts rapidly with 1-bromooctane, in the first well-characterized example of phase transfer catalysis (41).

The effects of solvent on the decarboxylation of 1-ChxO presented here are less pronounced than those observed for the reaction of azide with FNB or the decomposition of CBI (see slopes in Table 4), suggesting that simple desolvation of the substrate is unlikely to account for more than a limited part of the catalytic power of ODCase. Moreover, the effects of structural modification make it clear that much of this enzyme's catalytic effect depends on specific interactions between the substrate and polar residues of this enzyme's active site (2, 42). In these model experiments, a positively charged ammonium group was necessary to assist in the removal of the carboxylate group from water. In ODCase, Lys-93 may play a comparable role in assisting the extraction of the substrate from solvent water during formation of the enzyme–substrate complex. Thus, the affinity of ODCase for OMP is substantially reduced when Lys-93 is converted to alanine or when OMP is converted to UMP (3). Kinetic isotope effects indicate that, after substrate binding, the enzyme reaction proceeds in stepwise fashion, with carbanion formation preceding incorporation of the C6 proton into product UMP (42–47). The ammonium group of Lys-93 presumably stabilizes the carbanion generated by CO₂ elimination and furnishes the proton that takes its place (11).

The likelihood of that scenario receives unexpected support from the recent finding that another enzyme reaction, involving a very different substrate (uroporphyrinogen), proceeds with a $t_{1/2}$ of 2.3×10^9 years in water in the absence of the enzyme (48). Like ODCase, uroporphyrinogen decarboxylase (UroD, EC 4.1.1.37) catalyzes a difficult decarboxylation reaction without the assistance of metals or other cofactors. The only structural feature shared by these enzymes is the presence at the active site of a

single basic side chain (Lys-93 in the case of yeast ODCase and Arg-37 in the case of UroD) that is indispensable for catalysis (Figure 6). During the evolution of their active sites, these enzymes appear to have converged on a common strategy in which cationic side chains are in position to assist in the extraction of the substrate from solvent water into a relatively waterless nonpolar cavity, stabilize carbanions that approach the transition state in structure, and furnish a proton to generate the decarboxylated product. Other active site side chains appear to play important, but indirect, roles in maintaining the structural surroundings in which these events occur.

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