The Acidity of Uracil and Uracil Analogs in the Gas Phase: Four Surprisingly Acidic Sites and Biological Implications

Mary Ann Kurinovich and Jeehiun K. Lee
Department of Chemistry, Rutgers University, The State University of New Jersey, Piscataway, New Jersey, USA

The gas phase acidities of a series of uracil derivatives (1-methyluracil, 3-methyluracil, 6-methyluracil, 5,6-dimethyluracil, and 1,3-dimethyluracil) have been bracketed to provide an understanding of the intrinsic reactivity of uracil. The experiments indicate that in the gas phase, uracil has four sites more acidic than water. Among the uracil analogs, the N1-H sites have ΔH_{acid} values of 331–333 kcal mol^{-1}; the acidity of the N3 sites fall between 347–352 kcal mol^{-1}. The vinylic C6 in 1-methyluracil and 3-methyluracil brackets to 363 kcal mol^{-1}, and 369 kcal mol^{-1} in 1,3-dimethyluracil; the C5 of 1,3-dimethyluracil brackets to 384 kcal mol^{-1}. Calculations conducted at B3LYP/6-31+G* are in agreement with the experimental values. The bracketing of several of these sites involved utilization of an FTMS protocol to measure the *less* acidic site in a molecule that has more than one acidic site, establishing the generality of this method. In molecules with multiple acidic sites, only the two most acidic sites were bracketable, which is attributable to a kinetic effect. The measured acidities are in direct contrast to in solution, where the two most acidic sites of uracil (N1 and N3) are indifferentiable. The vinylic C6 site is also particularly acidic, compared to acrolein and pyridine. The biological implications of these results, particularly with respect to enzymes for which uracil is a substrate, are discussed. (J Am Soc Mass Spectrom 2002, 13, 985–995) © 2002 American Society for Mass Spectrometry

Accurate measurements of the acidities and basicities of nucleic bases and nucleic base derivatives is essential for understanding issues of fundamental importance in biological systems. Hydrogen bonding modulates recognition of DNA and RNA bases, and the interaction energy between two bonded complementary nucleobases is dependent on the intrinsic basicity of the acceptor atoms as well as on the acidity of donor NH groups [1, 2]. In addition, understanding the intrinsic reactivity of nucleic bases can shed light on key biosynthetic mechanisms in which nucleobases are substrates [3–8].

The gas phase is a valuable environment in which to examine the properties and reactivity of biological molecules. Biological media, from intracellular environs to the interior of proteins, are seldom aqueous in nature. For example, it has been shown that the interior of proteins is often nonpolar, causing shifts in acidity and basicity and changes in reactivity compared to behavior in aqueous solution [9–11]. The gas phase is the "ultimate" nonpolar environment and therefore allows one to establish intrinsic reactivity in the absence of solvent, and extrapolate the effects of media. Establishing thermochemical properties of the nucleobases is of interest for purely chemical reasons, but is also valuable for biological reasons, providing a basis for understanding the role of media on reactivity [9, 12–14]. In essence, gas phase experiments can provide the link between calculations and condensed phase data.

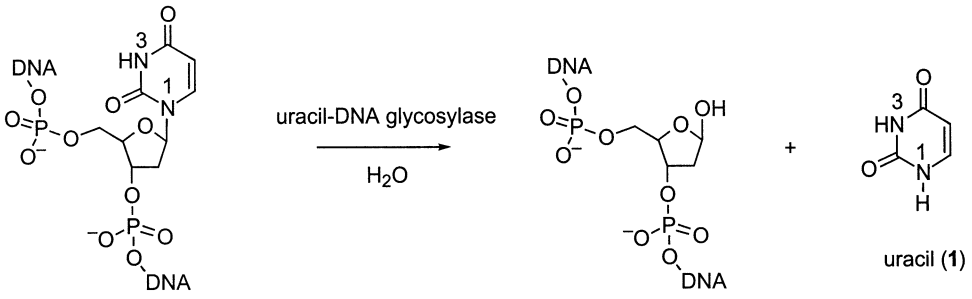
Recently, our studies of nucleobases have focused on the pyrimidine base uracil. While the proton affinities of the most basic sites of the major nucleobases, deoxyribonucleosides, and deoxyribonucleotides have been bracketed, gas phase acidities of the nucleobases are largely unknown [15–18]. Our studies have also been motivated by our interest in two pyrimidine-related enzymes, uracil-DNA glycosylase (UDGase) and orotidine 5'-monophosphate decarboxylase (ODCase). UDGase catalyzes a genome-protecting reaction that cleaves misincorporated uracil from DNA, through an unknown mechanism that presumably involves some form of N1-deprotonated uracil as a leaving group (Scheme **1**) [4, 5, 19–23]. ODCase lies along the pyrimidine de novo synthetic pathway, and catalyzes the decarboxylation of orotate ribose-5'-monophosphate to ultimately form uracil ribose-5'-monophosphate (Scheme **2**), via a mechanism that presumably involves some form of C6-deprotonated uracil [6, 24–27]. Both mechanisms are hotly debated, the central question being how the uracil anion—whether the $N1^-$ in

Published online June 27, 2002

Address reprint requests to Dr. J. K. Lee, Department of Chemistry, Rutgers University, 610 Taylor Road, Piscataway, NJ 08854, USA. E-mail: jklee@rutchem.rutgers.edu

1044-0305/02/$20.00
PII S1044-0305(02)00410-5

Received December 20, 2001
Revised April 3, 2002
Accepted April 3, 2002



Scheme 1 Uracil cleavage catalyzed by uracil-DNA glycosylase.

UDGase or the $C6^-$ in ODCase—is made an energetically favorable entity [4, 5, 7, 19–23, 28–60].

In earlier work, we examined the acidities of the N1 and N3 sites of uracil and discovered that the N1 proton is 14 kcal mol^{-1} more acidic than the N3 proton. This was particularly intriguing in light of the fact that the N1 and N3 solution-phase pK_a's are indistinguishable [61, 62]. Herein, we describe a more in-depth study of the acidity of pyrimidine nucleobases. We utilize our method for measuring multiple acidic sites on a molecule in an FTMS to establish the acidity of a series of uracil derivatives, and compare those results to calculated values to benchmark the computational methods. Computationally and experimentally, we establish that uracil has *four* sites more acidic than water in the gas phase. The biological implications of these results, particularly with regard to UDGase and ODCase, are also discussed.

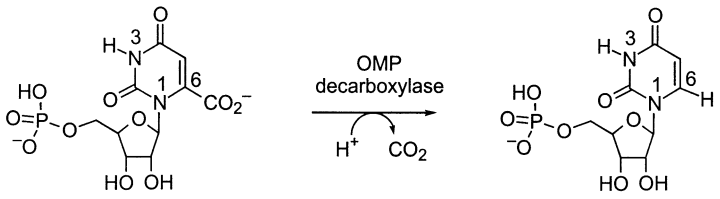
Experimental

All experiments were conducted on a dual-cell Finnigan 2001 Fourier transform mass spectrometer (Bremen, Germany). Each side of the 2 inch cubic dual cell is pumped down to a baseline pressure of less than 1×10^{-9} torr. The dual cell is positioned colinearly with the magnetic field produced by a 3.3 T superconducting magnet.

Neutral samples were introduced into the FT mass spectrometer using a Finnigan heated batch inlet system, a home-built heated batch inlet system, via a pulsed valve system, or by means of a heated solids probe. All chemicals were available commercially and were used as received. Most ions were produced by proton transfer to hydroxide. Hydroxide was generated by pulsing water into the cell and sending an electron beam (typically 6 eV, 8 μA, beam time 5 ms) through the center of the cell. A trapping potential of -2 V was applied to the cell walls perpendicular to the magnetic field at all times except when ions were transferred from one cell to another. Transfer is accomplished by temporarily grounding (50–150 μs) the conductance limit plate, the trapping plate separating the two cells. The ions then can pass through a 2 mm hole in the center of the conductance limit plate. Transferred ions were cooled with argon [63, 64].

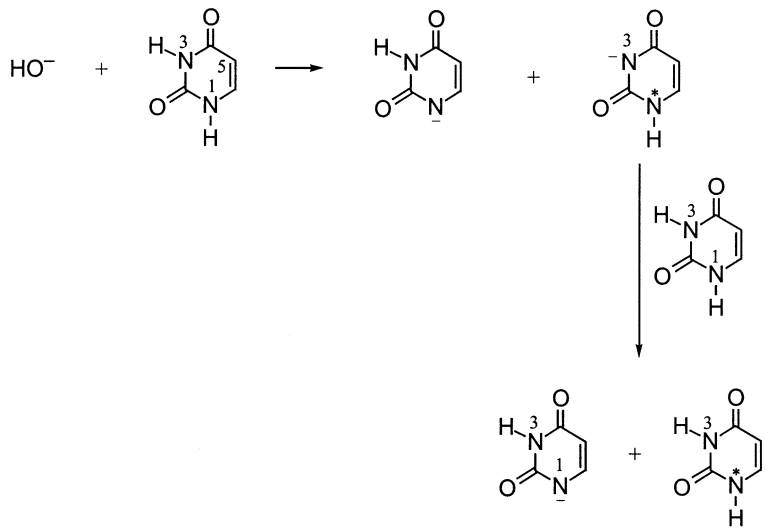
Acidity bracketing was utilized to measure the gas phase acidities. Species of known acidities are allowed to react with the substrate of unknown acidity. The ability of the anionic conjugate base of the substrate of unknown acidity to deprotonate relatively stronger acids, and the inability of the anion to deprotonate weaker acids (stronger bases) allow one to bracket the acidity of the unknown. Where possible, the reverse reaction is also explored. Rapid proton transfer (i.e., near the collision rate) was taken as evidence that the reaction was exothermic and is indicated by a + in the Tables.

We have recently developed a Fourier transform mass spectrometry (FTMS) method, building upon earlier work in the flowing afterglow, for the bracketing of less acidic sites in molecules that have multiple acidic sites; the experimental procedure has been described previously [14, 65–78]. Briefly, using uracil as an example, when hydroxide is used to deprotonate uracil, two ions are formed, the N1-deprotonated uracil and the N3-deprotonated uracil. When the ions are allowed to stay in an environment where there is a constant pressure of neutral uracil, the $N3^-$ ion isomerizes to $N1^-$ (Scheme **3**). We then transfer the $N1^-$ ion to the



orotate ribose 5'-monophosphate uracil ribose 5'-monophosphate

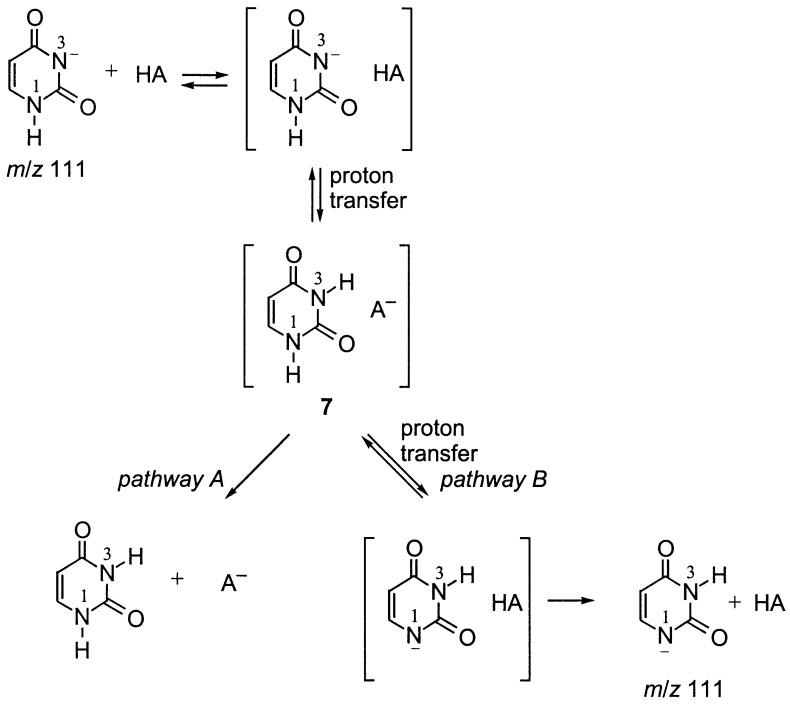
Scheme 2 Decarboxylation catalyzed by orotidine 5'-monophosphate decarboxylase.



Scheme 3 Uracil-catalyzed isomerization of anion formed from deprotonation of the less acidic N3 site of uracil.

second cell, where the reference acid is added at a constant pressure, and we monitor proton transfer. We refer to these conditions as "more acidic" conditions, because we allow for isomerization to the more acidic site before transfer. If, instead, the $(M - 1)^-$ of uracil (which is some mixture of $N1^-$ and $N3^-$) is transferred from the neutral uracil environment directly (within 200 ms) to the second cell, then the $N3^-$ will not isomerize and that ion can be bracketed. We will refer to this method as "less acidic" conditions. One experimental caveat, described further in the Discussion, is that when bracketing the less acidic site, Complex 7 in Scheme **4** can partition via either Pathway A or Pathway B. If Pathway B is followed, proton transfer is undetectable. In some cases, deuterated acids can be used to observe both pathways. For example, reaction of a mixture of N1 and N3 ions with a deuterated acid such as DCOOD results in signal for A^- and for *m/z* 112, described in an earlier paper [14]. Such an experiment also indicates that under these conditions, the less acidic $N3^-$ ion comprises about 5−10% of the total $(M - 1)^-$ signal.

Throughout the text, the term "gas phase acidity" is used to refer to the enthalpic (ΔH) change associated with deprotonation. Calculations were conducted at



Scheme 4 Possible pathways for bracketing of less acidic site of uracil.

Table 1. Calculated gas phase acidities of the different sites of uracil, 1-methyluracil, 3-methyluracil, 6-methyluracil, 5,6-dimethyluracil, and 1,3-dimethyluracil at B3LYP/6-31+G* in kcal mol^{-1}[a]

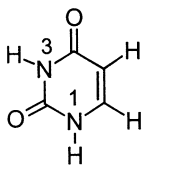
Structure	N1	N3	C5	C6
Uracil	329.0	342.6	376.1	361.5
1-Methyluracil	—	343.8	377.3	362.9
3-Methyluracil	331.3	—	378.4	363.5
6-Methyluracil	330.5	344.1	377.8	—
5,6-Dimethyluracil	331.7	344.6	—	—
1,3-Dimethyluracil	—	—	378.7	365.6

[a]At 298 K.

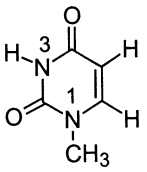
B3LYP/6-31+G* using Gaussian94 and Gaussian98 [79, 80]. Frequencies were conducted on all structures and no scaling factor was applied. All acidities reported are at 298 K.

Computational Results

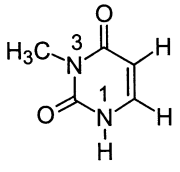
Calculated acidities for the Compounds 1–6 (uracil, 1-methyluracil, 3-methyluracil, 6-methyluracil, 5,6-dimethyluracil, and 1,3-dimethyluracil) are summarized in Table 1. Calculations were conducted at B3LYP/6-31+G*.



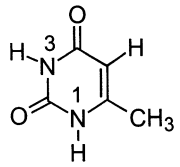
1



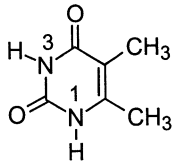
2



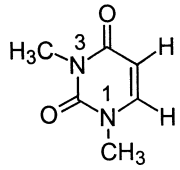
3



4



5



6

Uracil. The calculated values for the acidity of the N1 and N3 sites of uracil are 329.0 and 342.6 kcal mol^{-1}, respectively. Despite solution phase pK_a's that are indifferentiable, the gas phase N1 and N3 acidities are calculated to be 13.6 kcal mol^{-1} apart, with the N1 site being more acidic. Zeegers-Huyskens et al. have also previously calculated uracil deprotonation energies at B3LYP/6-31++G**; these values compare favorably to our numbers (332.5 and 345.8 kcal mol^{-1} for N1 and N3, respectively) [1].

When considering the acidity of uracil, the N1 and N3 protons are the most obvious sites. Interestingly, however, the C5 and C6 sites are calculated to be more acidic than might be expected. The predicted C5-H acidity is 376.1 kcal mol^{-1}; the C6-H calculated acidity is 361.5 [81].

1-Methyluracil. This compound is of interest to us because the N1 site is blocked, and experimentally, this will allow us to concentrate on the other remaining acidic sites. There are three potentially acidic sites on 1-methyluracil: N3, C5, and C6. These values are calculated to be quite close to the values of the parent uracil (343.8, 377.3, 362.9 kcal mol^{-1}, respectively).

3-Methyluracil. As with 1-methyluracil, 3-methyluracil is valuable experimentally, because the 3-position is blocked. 3-Methyluracil has three predicted acidic sites: N1, C5, and C6; these values are also comparable to those of uracil: 331.3, 378.4, 363.5 kcal mol^{-1}, respectively.

6-Methyluracil. 6-methyluracil has three acidic sites, N1, N3, and C5, which are calculated to be 330.5, 344.1, and 377.8 kcal mol^{-1}, comparable to the other uracil derivatives.

5,6-Dimethyluracil. Experimentally useful because only the N1 and N3 can be bracketed, 5,6-dimethyluracil has N1 and N3 calculated acidities that are in keeping with the other uracils (331.7 and 344.6 kcal mol^{-1}).

1,3-Dimethyluracil. Use of this compound allows us to block both the N1 and N3 sites and concentrate on the lower acidity sites C5 and C6. These sites have calculated acidities of 378.7 and 365.6 kcal mol^{-1}. Our results are also in agreement with calculations by Gronert et al., who calculated the C6 acidity of 1,3-dimethyluracil at MP2/6-31+G**//HF/6-31+G* (367.6 kcal mol^{-1}) and at B3LYP/6-31+G**//HF/6-31+G* (366.0 kcal mol^{-1}) [81].

Calculations predict that methylation changes acidity very little; therefore, these methylated analogs can be used experimentally to establish the validity of the earlier bracketing of the N1 and N3 sites in uracil.

Experimental Results

Uracil. The N1 and N3 sites of the parent uracil have been bracketed by us previously, to be 333 ± 4 and 347 ± 4 kcal mol^{-1}, respectively [14]. The acidity of uracil has also been measured by Marshall and coworkers, who bracketed the N1-H of uracil; their value is in agreement with ours (ΔG_{acid} = 328.9 ± 0.3 kcal mol^{-1}) [82].

1-Methyluracil. The results for the bracketing of 1-methyluracil can be found in Tables 2 and 3. When we run under conditions in which we should see only the most acidic site (Experimental), we find that the conjugate base of *para*-trifluoro-aniline (ΔH_{acid}[*p*-CF_3PhNH_2]

Table 2. Summary of results of proton transfer from reference acids and bases to 1-methyluracil N3

Reference compound	ΔH_{acid}[a]	Proton transfer[b] Reference acid	Proton transfer[b] Conjugate base
$HCCl_3$	357.6 ± 2.1	−	+
$CH_3CHCHCHO$	354.7 ± 2.1	−	+
p-CF_3PhNH_2	353.3 ± 2.1	−	+
CF_3COCH_3	349.2 ± 2.1	−	+
CH_3COOH	348.1 ± 2.2	+	+
HCOOH	345.3 ± 2.9	+	−

[a]Acidities are in kcal mol^{-1} and come from reference 87.
[b]+ Indicates the occurrence; − denotes the absence of proton transfer.

= 353.3 kcal mol^{-1}), the enolate of trifluoroacetone ($\Delta H_{acid}[CF_3COCH_3]$ = 349.2 kcal mol^{-1}), and acetate ($\Delta H_{acid}[CH_3COOH]$ = 348.1 kcal mol^{-1}) all deprotonate 1-methyluracil, while formate (ΔH_{acid}[HCOOH] = 345.3 kcal mol^{-1}) does not. The conjugate base of 1-methyluracil also deprotonates acetic acid, implying close-to-thermoneutral reactions, since the reactions proceed in both directions. The conjugate base of 1-methyluracil deprotonates formic acid (ΔH_{acid}[HCOOH] = 345.3 kcal mol^{-1}). Based on these results, we bracket the most acidic site of 1-methyluracil to be 348 ± 3 kcal mol^{-1}.

We can also run under conditions in which the less acidic site(s) is bracketable (Experimental). Under these conditions, we find that the conjugate base of 1-methyluracil can deprotonate 2-fluoroaniline (ΔH_{acid}[*m*-F-C_6H_6N] = 362.6 kcal mol^{-1}), but cannot deprotonate 4-fluoroaniline (ΔH_{acid}[*p*-F-C_6H_6N] = 364.3 kcal mol^{-1}). We therefore bracket only one site under these conditions, with a ΔH_{acid} of 363 ± 3 kcal mol^{-1} (Table 3).

3-Methyluracil. Acidity bracketing results for 3-methyluracil are summarized in Tables 4 and 5. The more acidic site of 3-methyluracil brackets to 333 ± 2 kcal mol^{-1} (Table 4). Reactions with pyruvic acid ($\Delta H_{acid}[CH_3COCOOH]$ = 333.5 kcal mol^{-1}) and hydrochloric acid (ΔH_{acid}[HCl] = 333.4 kcal mol^{-1}) proceed in both directions. The conjugate base of 3-methyluracil deprotonates difluoroacetic acid ($\Delta H_{acid}[C_2H_2F_2O_2]$ = 331.0 kcal mol^{-1}) but difluoroacetate does not deprotonate 3-methyluracil.

Table 3. Summary of results of proton transfer from reference acids and bases to 1-methyluracil C6

Reference compound	ΔH_{acid}[a]	Proton transfer[b] Reference acid
CH_3COCH_3	369.1 ± 2.1	−
CH_3CHO	365.8 ± 3.7	−
4-Fluoroaniline	364.3 ± 2.1	−
2-Fluoroaniline	362.6 ± 2.2	+
Pyrrole	358.6 ± 2.2	+
$CH_3CHCHCHO$	354.7 ± 2.1	+

[a]Acidities are in kcal mol^{-1} and come from reference 87.
[b]+ Indicates the occurrence; − denotes the absence of proton transfer.

Table 4. Summary or results of proton transfer from reference acids and bases to 3-methyluracil N1

Reference compound	ΔH_{acid}[a]	Proton transfer[b] Reference acid	Proton transfer[b] Conjugate base
CH_3COCH_3	369.1 ± 2.1	−	+
CF_3COCH_3	349.2 ± 2.1	−	+
CH_3COOH	348.1 ± 2.1	−	+
HCOOH	345.3 ± 2.9	−	+
$CH_3COCH_2COCH_3$	343.8 ± 2.1	−	+
m-CF_3PhOH	339.3 ± 2.1	−	+
$CH_3CHClCOOH$	337.0 ± 2.1	−	+
$CH_3COCOOH$	333.5 ± 2.9	+	+
HCl	333.4 ± 0.1	+	+
CHF_2COOH	331.0 ± 2.1	+	−
$CF_3COCH_2COCH_3$	328.3 ± 2.9	+	−

[a]Acidities are in kcal mol^{-1} and come from reference 87.
[b]+ Indicates the occurrence; − denotes the absence of proton transfer.

The less acidic site of 3-methyluracil was bracketed as shown in Table 5. Under "less acidic" conditions, the conjugate base of 3-methyluracil deprotonates 2-fluoroaniline (ΔH_{acid}[*m*-F-C_6H_6N] = 362.6 kcal mol^{-1}) but does not deprotonate 4-fluoroaniline (ΔH_{acid}[*p*-F-C_6H_6N] = 364.3 kcal mol^{-1}). We therefore bracket this site to be 363 ± 3 kcal mol^{-1}.

6-Methyluracil. The conjugate base of 6-methyluracil deprotonates difluoroacetic acid ($\Delta H_{acid}[C_2H_2F_2O_2]$ = 331.0 kcal mol^{-1}) and 1-trifluoro-2,4-pentadione ($\Delta H_{acid}[C_5H_5F_3O_2]$ = 328.3 kcal mol^{-1}), but does not deprotonate HCl (ΔH_{acid}[HCl] = 333.4 kcal mol^{-1}). Cl^- is also able to deprotonate 6-methyluracil. We therefore bracket the more acidic site of 6-methyluracil to be 331 ± 3 kcal mol^{-1} (Table 6).

The more basic conjugate base of 6-methyluracil does not deprotonate crotonaldehyde ($\Delta H_{acid}[C_4H_6O_2]$ = 354.7 kcal mol^{-1}, Table 7), but is able to deprotonate trifluoroacetone ($\Delta H_{acid}[C_3H_3F_3O]$ = 349.2 kcal mol^{-1}). We therefore bracket the less acidic site of 6-methyluracil to be 352 ± 5 kcal mol^{-1}.

Table 5. Summary of results of proton transfer from reference acids and bases to 3-methyluracil C6

Reference compound	ΔH_{acid}[a]	Proton transfer[b] reference acid
CH_3COCH_3	369.1 ± 2.1	−
CH_3CHO	365.8 ± 3.7	−
4-Fluoroaniline	364.3 ± 2.1	−
2-Fluoroaniline	362.6 ± 2.2	+
Pyrrole	358.6 ± 2.2	+
CH_3COOH	348.1 ± 2.1	+
HCOOH	345.3 ± 2.1	+

[a]Acidities are in kcal mol^{-1} and come from reference 87.
[b]+ Indicates the occurrence; − denotes the absence of proton transfer.

Table 6. Summary of results of proton transfer from reference acids and bases to 6-methyluracil N1

Reference compound	ΔH_{acid}[a]	Proton transfer[b] Reference acid	Proton transfer[b] Conjugate base
$CH_3COCH_2COCH_3$	343.8 ± 2.1	−	+
m-CF_3PhOH	339.3 ± 2.1	−	+
$CH_3COCOOH$	333.5 ± 2.9	−	+
HCl	333.4 ± 0.1	−	+
CHF_2COOH	331.0 ± 2.1	+	+
$CF_3COCH_2COCH_3$	328.3 ± 2.9	+	−

[a]Acidities are in kcal mol^{-1} and come from reference 87.
[b]+ Indicates the occurrence; − denotes the absence of proton transfer.

5,6-Dimethyluracil. The results for the bracketing studies of the more acidic site of 5,6-dimethyluracil are summarized in Table 8. Reactions with pyruvic acid ($\Delta H_{acid}[CH_3COCOOH]$ = 333.5 kcal mol^{-1}) and hydrochloric acid ($\Delta H_{acid}[HCl]$ = 333.4 kcal mol^{-1}) proceed in both directions. The conjugate base of 5,6-dimethyluracil deprotonates trifluoropentadione ($\Delta H_{acid}[CF_3COCH_2COCH_3]$ = 328.3 kcal mol^{-1}), but the enolate of the pentadione does not deprotonate 5,6-dimethyluracil. We therefore bracket the most acidic site of 5,6-dimethyluracil to be 333 ± 2 kcal mol^{-1}.

The less acidic site of 5,6-dimethyluracil brackets to 349 ± 3 kcal mol^{-1}. While the conjugate base of 5,6-dimethyluracil is unable to deprotonate trifluoroacetone ($\Delta H_{acid}[C_3H_3F_3O]$ = 349.2 kcal mol^{-1}), it does deprotonate acetic acid ($\Delta H_{acid}[C_2H_4O_2]$ = 348.1 kcal mol^{-1}, Table 9).

Table 7. Summary of results of proton transfer from reference acids and bases to 6-methyluracil N3

Reference compound	ΔH_{acid}[a]	Proton transfer[b] reference acid
Pyrrole	358.6 ± 2.2	−
$CH_3CD_2NO_2$	355.9 ± 2.2	−
$CH_3CHCHCHO$	354.7 ± 2.1	−
CF_3COCH_3	349.2 ± 2.1	+
CH_3COOH	348.1 ± 2.1	+
HCOOH	345.3 ± 2.1	+
$CH_3COCH_2COCH_3$	343.8 ± 2.1	+
m-CF_3PhOH	339.3 ± 2.1	+

[a]Acidities are in kcal mol^{-1} and come from reference 87.
[b]+ Indicates the occurrence; − denotes the absence of proton transfer.

Table 8. Summary of results of proton transfer from reference acids and bases to 5,6-dimethyluracil N1

Reference compound	ΔH_{acid}[a]	Proton transfer[b] Reference acid	Proton transfer[b] Conjugate base
CF_3COCH_3	349.2 ± 2.1	−	+
CH_3COOH	348.1 ± 2.1	−	+
HCOOH	345.3 ± 2.2	−	+
$CH_3COCH_2COCH_3$	343.8 ± 2.1	−	+
m-CF_3PhOH	339.3 ± 2.1	−	+
$CH_3COCOOH$	333.5 ± 2.9	+	+
HCl	333.4 ± 0.1	+	+
$CF_3COCH_2COCH_3$	328.3 ± 2.1	+	−

[a]Acidities are in kcal mol^{-1} and come from reference 87.
[b]+ Indicates the occurrence; − denotes the absence of proton transfer.

Table 9. Summary of results of proton transfer from reference acids and bases to 5,6-dimethyluracil N3

Reference compound	ΔH_{acid}[a]	Proton transfer[b] Reference acid
$CH_3CHCHCHO$	354.7 ± 2.1	−
CF_3COCH_3	349.2 ± 2.1	−
CH_3COOH	348.1 ± 2.2	+
HCOOH	345.3 ± 2.9	+
$CH_3COCH_2COCH_3$	343.8 ± 2.1	+
m-CF_3PhOH	339.3 ± 2.1	+

[a]Acidities are in kcal mol^{-1} and come from reference 87.
[b]+ Indicates the occurrence; − denotes the absence of proton transfer.

1,3-Dimethyluracil. The more acidic site of 1,3-dimethyluracil was bracketed as shown in Table 10. The conjugate bases of 2-fluoroethanol ($\Delta H_{acid}[C_3H_5FO]$ = 371.2 kcal mol^{-1}) and acetone ($\Delta H_{acid}[C_3H_6O]$ = 369.1 kcal mol^{-1}) deprotonate 1,3-dimethyluracil, but the enolate of 3-pentanone ($\Delta H_{acid}[C_5H_{10}O]$ = 368.6 kcal mol^{-1}) does not. The conjugate base of 1,3-dimethyluracil does not deprotonate acetone, but does deprotonate 3-pentanone. Based on these results, we bracket the more acidic site of 1,3-dimethyluracil to be 369 ± 2 kcal mol^{-1}.

The results for the bracketing of the less acidic site of 1,3-dimethyluracil are summarized in Table 11. While the conjugate base of 1,3-dimethyluracil deprotonates methanol ($\Delta H_{acid}[CH_3O]$ = 381.8 kcal mol^{-1}), it cannot deprotonate 2-methylfuran ($\Delta H_{acid}[C_5H_6O]$ = 383.9 kcal mol^{-1}). This less acidic site brackets to 384 ± 3 kcal mol^{-1}.

Table 10. Summary of results of proton transfer from reference acids and bases to 1,3-dimethyluracil C6

Reference compound	ΔH_{acid}[a]	Proton transfer[b] Reference acid	Proton transfer[b] Conjugate base
$C(CH_3)_3CHO$	387.4 ± 4.1	−	+
cyclohexene	386.5 ± 5.1	−	+
$CH_2CHC(CH_3)CH_2$	385.6 ± 5.1	−	+
CH_3OH	381.8 ± 1.0	−	+
CH_3CH_2OH	378.3 ± 1.0	−	+
$CH(CH_3)_2OH$	375.9 ± 1.2	−	+
CH_2CHCH_2OH	373.5 ± 2.9	−	+
CH_2FCH_2OH	371.2 ± 2.9	−	+
CH_3COCH_3	369.1 ± 2.1	−	+
$CH_3CH_2COCH_2CH_3$	368.6 ± 2.2	+	−
$CH_3COCH_2CH_3$	367.2 ± 2.4	+	−
CH_3CHO	365.8 ± 3.7	+	−
2-Fluoroaniline	362.6 ± 2.2	+	−
Pyrrole	358.6 ± 2.2	+	−

[a]Acidities are in kcal mol^{-1} and come from reference 87.
[b]+ Indicates the occurrence; − denotes the absence of proton transfer.

Table 11. Summary of results of proton transfer from reference acids and bases to 1,3-dimethyluracil C5

Reference compound	ΔH_{acid}[a]	Proton transfer[b] Reference acid
$CH_3C(CH_2)C(CH_2)CH_3$	388.1 ± 2.1	−
Cyclohexene	386.5 ± 5.1	−
$CH_2CHC(CH_3)CH_2$	385.6 ± 5.1	−
2-Methylfuran	383.9 ± 3.1	−
CH_3OH	381.8 ± 1.0	+
CH_3CH_2OH	378.3 ± 1.0	+
$CH(CH_3)_2OH$	375.9 ± 1.2	+

[a]Acidities are in kcal mol^{-1} and come from reference 87.
[b]+ Indicates the occurrence; − denotes the absence of proton transfer.

Discussion

A summary of the experimental results for the compounds in this study are in Table 12, with assignations of the site that is believed to have been measured. Corresponding calculated values, computed at B3LYP/6-31+G*, are in parentheses. The calculations and experimental data are in agreement, within experimental error. B3LYP/6-31+G* therefore appears to be a reasonable method and level at which to calculate gas-phase enthalpies of deprotonation of nucleobases [1, 2, 14, 81, 83–85].

N1 and N3 acidities. The acidity of uracil is of particular interest because it impacts directly on issues of biological relevance. In solution, the N1 and N3 acidities of uracil are indistinguishable [61, 62]. Measuring the acidity of uracil yields a pK_a of 9.5, a mixture of N1 and N3 deprotonation. 3-Methyluracil has a pK_a of 10.0, while 1-methyluracil has a pK_a of 9.8. The fact that the acidities are indistinguishable is of interest since glycosylation of a nucleobase to make a nucleic acid takes place specifically at N1; the sites should be similarly reactive, yet nature glycosylates specifically at N1. Also of interest is the deglycosylation of uracil, which is effected by UDGase (Scheme **1**). The mechanism appears to involve some form of N1-deprotonated uracil as a leaving group; however, because uracil has a relatively high pK_a, deprotonated uracil should be a mediocre leaving group and mechanistic hypotheses have focussed on ways in which the uracil could be activated to encourage its leaving group ability [4, 5, 19–23].

Intriguingly, although the N1 and N3 acidities of uracil are indifferentiable in solution, calculations predict they should be 13.6 kcal mol^{-1} apart in the gas phase [1, 14]. Measurement of the parent uracil under our "more acidic" and "less acidic" conditions are in agreement with these calculations (N1-H, 333 kcal mol^{-1}, N3-H, 347 kcal mol^{-1}, Table 12) [14]. However, measurement of a less acidic site is, by virtue of the experiment, somewhat limited. First, the reaction can only be conducted in one direction; with uracil, that is $N3^-$ plus reference acids (HA). Second, the formation of the [uracil $N3^-$ · HA] complex followed by proton transfer results in ion molecule Complex 7 (Scheme **4**). Complex 7 can partition in two ways: The complex can separate, thus producing a signal for A^- that indicates proton transfer; *or*, A^- can deprotonate the N1-H of uracil, which simply yields the $[M - H]^-$ of uracil, which is indistinguishable from the starting anion at *m/z* 111. Should the ion-molecule Complex 7 "choose" to take the latter pathway exclusively, it will appear that proton transfer does not take place, which is an experimental caveat. One motivation, therefore, for the study of the methylated analogs of uracil is to block more acidic sites to measure less acidic sites, and confirm the measurements of the parent uracil [67–69].

Calculations predict that methylation should affect acidities very little. For example, while uracil N1 has a predicted deprotonation enthalpy of 329 kcal mol^{-1}, 3-methyluracil N1-H is calculated to be 331 kcal mol^{-1}. Although differing by 2 kcal mol^{-1}, certainly N1 is still nowhere near the less acidic site, N3. The N3-H of the parent uracil has a calculated acidity of 343 kcal mol^{-1}, while the N3-H of 1-methyluracil (which conveniently blocks the more acidic N1 site) is calculated to be 344 kcal mol^{-1}.

Experimentally, the methylated analogs behave as predicted. The N1-H of 3-methyluracil brackets to 333 kcal mol^{-1}, while the N3-H of 1-methyluracil brackets to 348 kcal mol^{-1}, comparable to the N1 and N3 protons in the parent uracil (333 and 347 kcal mol^{-1}, respectively). Among all the analogs with an available N1 proton, the N1-H acidities bracket to between 331–333 kcal mol^{-1}, and are essentially the same within experimental error. The N3 sites bracket to 347–352 kcal mol^{-1}. Therefore, there is indeed a disparity between the N1

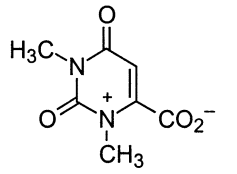
Table 12. Summary of experimental and calculated gas phase acidities of the different sites of uracil, 1-methyluracil, 3-methyluracil, 6-methyluracil, 5,6-dimethyluracil, and 1,3-dimethyluracil, in kcal mol^{-1}[a,b,c]

Structure	N1	N3	C5	C6
Uracil	333 ± 4 (329.0)	347 ± 4 (342.6)	NM (376.1)	NM (361.5)
1-Methyluracil	NA	348 ± 3 (343.8)	NM (377.3)	363 ± 3 (362.9)
3-Methyluracil	333 ± 2 (331.3)	NA	NM (378.4)	363 ± 3 (363.5)
6-Methyluracil	331 ± 3 (330.5)	352 ± 5 (344.1)	NM (377.8)	NA
5,6-Dimethyluracil	333 ± 2 (331.7)	349 ± 3 (344.6)	NA	NA
1,3-Dimethyluracil	NA	NA	384 ± 3 (378.7)	369 ± 2 (365.6)

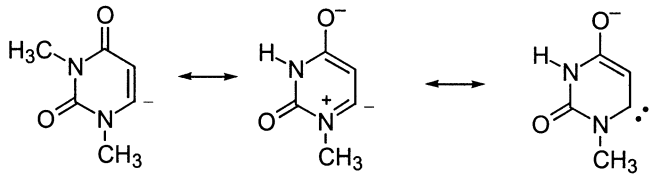
[a]Values in parentheses are calculated at 298 K at B3LYP/6−31+G*.
[b]NA is not applicable (site is alkylated).
[c]NM is not measured (see text).

and N3 acidities in the gas phase, and solvation causes these acidities to coalesce. Nature may take advantage of this differential acidity in a nonpolar environment such as an enzyme active site to glycosylate specifically at N1. Furthermore, our results show that N1-deprotonated uracil may not be a poor leaving group in the gas phase; its proton affinity is the same as that of chloride. Therefore, in a nonpolar cavity, N1-deprotonated uracil may be easier to cleave than the solution phase pK_as imply. In keeping with our predictions, recent experimental studies with UDGase indicate that the *anionic* base is most probably a leaving group, contrary to previous hypotheses that involved protonation of the uracil before cleavage [22, 31–33].

C5 and C6 acidities. Through these studies, we have also found that the N1 and N3 sites are not the only positions of relatively high acidity. Uracil has four potentially acidic sites, the N1, the N3, the C5, and the C6. Calculations predict that the C5 and C6 positions will have acidities of 376.1 and 361.5 kcal mol^{-1}, respectively. Our experimental results are in agreement with computational predictions (Table 12); the C6 in 1-methyluracil and 3-methyluracil brackets to 363 kcal mol^{-1}, and 369 kcal mol^{-1} in 1,3-dimethyluracil. The C5 of 1,3-dimethyluracil brackets to 384 kcal mol^{-1}. Our results are also consistent with earlier work by Gronert et. al, who bracketed the C6 site of 1,3-dimethyluracil (via decarboxylation of orotate in Structure 8) to be 369.9 ± 3.1 kcal mol^{-1} [81].



8



9

The vinylic protons of acrolein calculate to 382.6 (terminal) and 374.5 (adjacent to carbonyl) kcal mol^{-1}, and the acidity of pyridine is 391 kcal mol^{-1} [86, 87]. The C6 proton of uracil therefore does appear to be unusually acidic. This anion is of particular biological interest, because the final step in the de novo synthesis of pyrimidine nucleotides involves decarboxylation of orotate ribose 5'-monophosphate to ultimately yield uracil ribose 5'-monophosphate, presumably via some form of the C6 anion as an intermediate (Scheme **2**). The reaction is catalyzed by orotidine 5'-monophosphate decarboxylase, a key antitumor target, via a mechanism that is hotly debated. The nature of the C6 anionic intermediate resulting from decarboxylation of orotate is of course the focus of mechanistic studies—how stable is it, and how does the enzyme catalyze the reaction? We have established, through earlier computational studies, that the C6 anion may garner special stability because of its resonance Structure 9, a carbene-ylide [7, 49, 60]. The C5 anion, with a calculated proton affinity of 376 kcal mol^{-1}, is comparable in proton affinity to the conjugate base of acrolein. The present work establishes experimentally the stability of the C6 ion, as manifested by its acidity. Interestingly, the transformation of orotate to uracil is the only known biochemical decarboxylation where the resultant anion has no pi system into which to delocalize. Our results establish that, despite its lack of pi stabilization, the resultant C6 anion is not as unfavorable in a nonpolar environment as one might initially think, despite its lack of pi stabilization, which may be related to catalysis by ODCase.

Kinetic effects. We have found that for each uracil analog, we are able to bracket the acidities of the two most acidic positions. Therefore, although hydroxide (ΔH_{acid} = 390.7 kcal mol^{-1}) is sufficiently basic to deprotonate all four sites of uracil, we bracket only the N1 and the N3 sites, not the C5 and the C6. Likewise, with 1-methyluracil we bracket only the N3 and C6 sites, although the C5 site has a proton; with 3-methyluracil we bracket N1 and C6, not C5; the pattern continues with all the uracils.

We initially speculated that the issue might be one of ion mobility, which has been observed in the reaction of deuterated reagents with *p*-difluorophenyl anions [88]. For example, with uracil, perhaps the $N3^-$ reacts mostly via Pathway A in Scheme **4**, whereas the $C5^-$ and $C6^-$ ions react primarily via Pathway B and therefore cannot be bracketed. One possible reason for this difference in behavior is that after the $N3^-$ accepts a proton, the resultant conjugate base may not be mobile enough to move easily past the 2-carbonyl and around the ring and deprotonate the N1 site (that is, Pathway B could be somewhat suppressed). If this were true, however, presumably, the C6 in 3-methyluracil should not be bracketable, but it is. More likely, our bracketing only the two most acidic sites of each uracil is a kinetic effect. Hydroxide probably removes the proton from the third least acidic site most infrequently [65, 66]. Additionally, the anion formed from the least acidic site will be particularly prone to isomerization, because there are *two or more* sites with which it could react. For example, with 3-methyluracil, there are three potentially acidic sites, the N1, the C5, and the C6. Reaction with hydroxide should result in the least amount of $C5^-$. Because there is so little of $C5^-$, it will be a difficult position to bracket. In addition,

the $C5^-$ will also rearrange readily to the $N1^-$ and the $C6^-$ ions, which will further decrease odds of bracketing the C5 position. We believe that these two effects—minimal formation of the $C5^-$ and facile isomerization to $N1^-$ and $C6^-$—make bracketing the third and fourth least acidic sites in a molecule improbable.

Conclusions

We have discovered that uracil has four surprisingly acidic sites. Calculations and experiments indicate that methylation is an effective means of targeting specific sites for acidity bracketing. Among all the uracil analogs, the N1-H acidities bracket to between 331–333 kcal mol^{-1}. The N3 sites all bracket to 347–352 kcal mol^{-1}. The C6 in 1-methyluracil and 3-methyluracil brackets to 363 kcal mol^{-1}, and 369 kcal mol^{-1} in 1,3-dimethyluracil. The C5 of 1,3-dimethyluracil brackets to 384 kcal mol^{-1}. The C6 carbon vinylic site is particularly acidic with respect to acrolein and pyridine, which has implications with regard to ODCase. The relatively stability of the $C6^-$ means that decarboxylation of orotate to form uracil in a nonpolar environment is more facile than might be expected, which might constitute an enzymatic advantage. The measured gas phase N1 and N3 acidities are in direct contrast to those in solution, where the N1 and N3 are close enough in acidity to be unresolvable. Such a separation of acidities in the gas phase may be why N1 is the preferred site of glycosylation, and why UDGase cleavage of uracil from DNA is not unfavorable, since the uracil $N1^-$ is relatively stable in a nonpolar environment. Calculations conducted at B3LYP/6-31+G* are in agreement with the experimental values. The bracketing of several of these sites involved utilization of a novel protocol to measure the *less* acidic site in a molecule that has more than one acidic site, establishing the generality of this method. In molecules with more than two acidic sites, only the two most acidic sites were bracketable, which is attributable to a kinetic effect.

Acknowledgments

The authors thank the National Science Foundation (CHE-0092215), ACS-PRF (grant no. 32732-G4), the Rutgers Environmental Health Sciences Exploratory Research Grant Program, and the Rutgers Busch Grant Program for support, and the National Center for Supercomputing Applications, the High Performance Computing Center at the University of Kentucky, and the Boston University Scientific Computing Facilities for computational resources. They are also grateful to Professor Scott Gronert for valuable discussions.

References

1. Nguyen, M. T.; Chandra, A. K.; Zeegers-Huyskens, T. Protonation and Deprotonation Energies of Uracil. *J. Chem. Soc., Faraday Trans.* **1998,** *94,* 1277–1280.
2. Chandra, A. K.; Nguyen, M. T.; Uchimaru, T.; Zeegers-Huyskens, T. Protonation and Deprotonation Enthalpies of Guanine and Adenine and Implications for the Structure and Energy of Their Complexes with Water: Comparison with Uracil, Thymine, and Cytosine. *J. Phys. Chem. A* **1999,** *103,* 8853–8860.
3. Saenger, W. *Principles of Nucleic Acid Structure.* Springer-Verlag: New York, 1984.
4. Dianov, G.; Sedgwick, B.; Daly, G.; Olsson, M.; Lovett, S.; Lindahl, T. Release of 5'-Terminal Deoxyribose-Phosphate Residues from Incised Abasic Sites in DNA by the *Escherichia coli* RecJ Protein. *Nucleic Acids Res.* **1994,** *22,* 993–998.
5. Savva, R.; McAuley-Hecht, K.; Brown, T.; Pearl, L. The Structural Basis of Specific Base-Excision Repair by Uracil-DNA Glycosylase. *Nature* **1995,** *373,* 487–493.
6. Radzicka, A.; Wolfenden, R. A Proficient Enzyme. *Science* **1995,** *267,* 90–93.
7. Lee, J. K.; Houk, K. N. A Proficient Enzyme Revisited: The Predicted Mechanism for Orotidine Monophosphate Decarboxylase. *Science* **1997,** *276,* 942–945.
8. Lundegaard, C.; Jensen, K. F. Kinetic Mechanism of Uracil Phosphoribosyltransferase from *Escherichia coli* and Catalytic Importance of the Conserved Proline in the PRPP Binding Site. *Biochem.* **1999,** *38,* 3327–3334 , and references therein.
9. Jordan, F.; Li, H.; Brown, A. Remarkable Stabilization of Zwitterionic Intermediates May Account for a Billion-fold Rate Acceleration by Thiamin Diphosphate-Dependent Decarboxylases. *Biochem.* **1999,** *38,* 6369–6373.
10. Simonson, T.; Brooks, C. L., III. Charge Screening and the Dielectric Constant of Proteins: Insights from Molecular Dynamics. *J. Am. Chem. Soc.* **1996,** *118,* 8452–8458.
11. Gilson, M. K.; Honig, B. H. The Dielectric Constant of a Folded Protein. *Biopolymers* **1986,** *25,* 2097–2119.
12. Lowry, T. H.; Richardson, K. S. *Mechanism and Theory in Organic Chemistry; 3rd ed.* Harper and Row, Publishers: New York, 1987.
13. McEwen, W. K. A Further Study of Extremely Weak Acids. *J. Am. Chem. Soc.* **1936,** *58,* 1124–1129.
14. Kurinovich, M. A.; Lee, J. K. The Acidity of Uracil from the Gas Phase to Solution: The Coalescence of the N1 and N3 Sites and Implications for Biological Glycosylation. *J. Am. Chem. Soc.* **2000,** *122,* 6258–6262.
15. Greco, F.; Liguori, A.; Sindona, G.; Uccella, N. Gas-Phase Proton Affinity of Deoxyribonucleosides and Related Nucleobases by Fast Atom Bombardment Tandem Mass Spectrometry. *J. Am. Chem. Soc.* **1990,** *112,* 9092–9096.
16. Green-Church, K. B.; Limbach, P. A. Mononucleotide Gas-Phase Proton Affinities as Determined by the Kinetic Method. *J. Am. Soc. Mass Spectrom.* **2000,** *11,* 24–32.
17. Wilson, M. S.; McCloskey, J. A. Chemical Ionization Mass Spectrometry of Nucleosides. Mechanisms of Ion Formation and Estimations of Proton Affinity. *J. Am. Chem. Soc.* **1975,** *97,* 3436–3444.
18. Meot-Ner (Mautner), M. Ion Thermochemistry of Low-Volatility Compounds in the Gas Phase. 2. Intrinsic Basicities and Hydrogen-Bonded Dimers of Nitrogen Heterocyclics and Nucleic Bases. *J. Am. Chem. Soc.* **1979,** *101,* 2396–2403.
19. Shroyer, M. J. N.; Bennett, S. E.; Putnam, C. D.; Tainer, J. A.; Mosbaugh, D. W. Mutation of an Active Site Residue in *Escherichia coli* Uracil-DNA Glycosylase: Effect on DNA Binding, Uracil Inhibition and Catalysis. *Biochem.* **1999,** *38,* 4834–4845.
20. Drohat, A. C.; Xiao, G.; Tordova, M.; Jagadeesh, J.; Pankiewicz, K. W.; Watanabe, K. A.; Gilliland, G. L.; Stivers, J. T. Heteronuclear NMR and Crystallographic Studies of Wild-Type and H187Q *Escherichia coli* Uracil DNA Glycosylase: Electrophilic Catalysis of Uracil Expulsion by a Neutral Histidine 187. *Biochem.* **1999,** *38,* 11876–11886.
21. Drohat, A. C.; Jagadeesh, J.; Ferguson, E.; Stivers, J. T. Role of Electrophilic and General Base Catalysis in the Mechanism of

Escherichia coli Uracil DNA Glycosylase. *Biochem.* **1999,** *38,* 11866–11875.
22. Drohat, A. C.; Stivers, J. T. NMR Evidence for an Unusually Low N1 pKa for Uracil Bound to Uracil DNA Glycosylase: Implications for Catalysis. *J. Am. Chem. Soc.* **2000,** *122,* 1840–1841.
23. Luo, N.; Mehler, E.; Osman, R. Specificity and Catalysis of Uracil DNA Glycosylase. A Molecular Dynamics Study of Reactant and Product Complexes with DNA. *Biochem.* **1999,** *38,* 9209–9220.
24. McClard, R. W.; Black, M. J.; Livingstone, L. R.; Jones, M. E. Isolation and Initial Characterization of the Single Polypeptide that Synthesizes Uridine-5'-Monophosphate from Orotate in Ehrlich ascites carcinoma. Purification by Tandem Affinity Chromatography of Uridine-5'-Monophosphate Synthase. *Biochem.* **1980,** *19,* 4699–4706.
25. Bruice, T. C.; Benkovic, S. Bioorganic Mechanisms, Vol. II. W. A. Benjamin: New York, 1966.
26. Bender, M. L. *Mechanisms of Homogeneous Catalysis from Protons to Proteins.* Wiley-Interscience: New York, 1971.
27. Snider, M. J.; Wolfenden, R. The Rate of Spontaneous Decarboxylation of Amino Acids. *J. Am. Chem. Soc.* **2000,** *122,* 11507–11508.
28. Parikh, S. S.; Walcher, G.; Jones, G. D.; Slupphaug, G.; Krokan, H. E.; Blackburn, G. M.; Tainer, J. A. Uracil-DNA Glycosylase-DNA Substrate and Product Structures: Conformational Strain Promotes Catalytic Efficiency by Coupled Stereoelectronic Effects. *Proc. Natl. Acad. Sci. U.S.A.* **2000,** *97,* 5083–5088.
29. Xiao, G.; Tordova, M.; Jagadeesh, J.; Drohat, A.; Stivers, J. T.; Gilliland, G. L. Crystal Structure of *Escherichia coli* Uracil DNA Glycosylase and Its Complexes with Uracil and Glycerol: Structure and Glycosylase Mechanism Revisited. *Proteins Struct. Funct. Genet.* **1999,** *35,* 13–24.
30. Stivers, J. T.; Pankiewicz, K. W.; Watanabe, K. A. Kinetic Mechanism of Damage Site Recognition and Uracil Flipping by *Escherichia coli* Uracil DNA Glycosylase. *Biochem.* **1999,** *38,* 952–963.
31. Drohat, A. C.; Stivers, J. T. *Escherichia coli* Uracil DNA Glycosylase: NMR Characterization of the Short Hydrogen Bond from His187 to Uracil O2. *Biochem.* **2000,** *39,* 11865–11875.
32. Dong, J.; Drohat, A. C.; Stivers, J. T.; Pankiewicz, K. W.; Carey, P. R. Raman Spectroscopy of Uracil DNA Glycosylase-DNA Complexes: Insights into DNA Damage Recognition and Catalysis. *Biochem.* **2000,** *39,* 13241–13250.
33. Werner, R. M.; Stivers, J. T. Kinetic Isotope Effect Studies of the Reaction Catalyzed by Uracil DNA Glycosylase: Evidence for an Oxocarbenium Ion-Uracil Anion Intermediate. *Biochem.* **2000,** *39,* 14054–14064.
34. Jiang, Y. L.; Stivers, J. T. Reconstructing the Substrate for Uracil DNA Glycosylase: Tracking the Transmission of Binding Energy in Catalysis. *Biochem.* **2001,** *40,* 7710–7719.
35. Dinner, A. R.; Blackburn, G. M.; Karplus, M. Uracil-DNA Glycosylase Acts by Substrate Autocatalysis. *Nature* **2001,** *413,* 752–755.
36. Beak, P.; Siegel, B. Mechanism of Decarboxylation of 1,3-Dimethylorotic Acid. A Model for Orotidine 5'-Phosphate Decarboxylase. *J. Am. Chem. Soc.* **1976,** *98,* 3601–3606.
37. Acheson, S. A.; Bell, J. B.; Jones, M. E.; Wolfenden, R. Orotidine-5'-Monophosphate Decarboxylase Catalysis: Kinetic Isotope Effects and the State of Hybridization of a Bound Transition-State Analogue. *Biochem.* **1990,** *29,* 3198–3202.
38. Levine, H. L.; Brody, R. S.; Westheimer, F. H. Inhibition of Orotidine-5'-Phosphate Decarboxylase by 1-(5'-Phospho-β-D-Ribofuranosyl)barbituric Acid, 6-Azauridine 5'-Phosphate, and Uridine 5'-Phosphate. *Biochem.* **1980,** *19,* 4993–4999.
39. Smiley, J. A.; Paneth, P.; O'Leary, M. H.; Bell, J. B.; Jones, M. E. Investigation of the Enzymatic Mechanism of Yeast Orotidine-5'-Monophosphate Decarboxylase Using ^{13}C Kinetic Isotope Effects. *Biochem.* **1991,** *30,* 6216–6223.
40. Smiley, J. A.; Jones, M. E. A Unique Catalytic and Inhibitor-Binding Role for Lys93 of Yeast Orotidylate Decarboxylase. *Biochem.* **1992,** *31,* 12162–12168.
41. Smiley, J. A.; Benkovic, S. J. Selection of Catalytic Antibodies for a Biosynthetic Reaction from a Combinatorial cDNA Library by Complementation of an Auxotrophic *Escherichia coli*: Antibodies for Orotate Decarboxylation. *Proc. Natl. Acad. Sci. U.S.A.* **1994,** *91,* 8319–8323.
42. Smiley, J. A.; Benkovic, S. J. Expression of an Orotate Decarboxylating Catalytic Antibody Confers 5-Fluoroorotate Sensitivity to a Pyrimidine Auxotrophic *Escherichia coli*: An Example of Intracellular Prodrug Activation. *J. Am. Chem. Soc.* **1995,** *117,* 3877–3878.
43. Smiley, J. A.; Hay, K. M.; Levison, B. S. A Reexamination of the Substrate Utilization of 2-Thioorotidine-5'-Monophosphate by Yeast Orotidine-5'-Monophosphate Decarboxylase. *Bioorg. Chem.* **2001,** *29,* 96–106.
44. Silverman, R. B.; Groziak, M. P. Model Chemistry for a Covalent Mechanism of Action of Orotidine 5'-Phosphate Decarboxylase. *J. Am. Chem. Soc.* **1982,** *104,* 6434–6439.
45. Cui, W.; DeWitt, J. G.; Miller, S. M.; Wu, W. No Metal Cofactor in Orotidine 5'-Monophosphate Decarboxylase. *Biochem. Biophys. Res. Comm.* **1999,** *259,* 133–135.
46. Ehrlich, J. I.; Hwang, C.-C.; Cook, P. F.; Blanchard, J. S. Evidence for a Stepwise Mechanism of OMP Decarboxylase. *J. Am. Chem. Soc.* **1999,** *121,* 6966–6967.
47. Nakanishi, M. P.; Wu, W. Mechanism of Decarboxylation of 1,3-Dimethylorotic Acid Revisited: Trapping of the Reaction Intermediate. *Tetrahedron Lett.* **1998,** *39,* 6271–6272.
48. Wu, W.; Ley-han, A.; Wong, F. M.; Austin, T. J.; Miller, S. M. Decarboxylation of 1,3-Dimethylorotic Acid Revisited: Determining the Role of N-1. *Bioorg. Med. Chem. Lett.* **1997,** *7,* 2623–2628.
49. Singleton, D. A.; Merrigan, S. A.; Kim, B. J.; Beak, P.; Phillips, L. M.; Lee, J. K. ^{13}C Kinetic Isotope Effects and the Mechanism of the Uncatalyzed Decarboxylation of Orotic Acid. *J. Am. Chem. Soc.* **2000,** *122,* 3296–3300.
50. Appleby, T. C.; Kinsland, C.; Begley, T. P.; Ealick, S. E. The Crystal Structure and Mechanism of Orotidine 5'-Monophosphate Decarboxylase. *Proc. Natl. Acad. Sci. U.S.A.* **2000,** *97,* 2005–2010.
51. Harris, P.; Poulsen, J.-C. N.; Jensen, K. F.; Larsen, S. Structural Basis for the Catalytic Mechanism of a Proficient Enzyme: Orotidine 5'-Monophosphate Decarboxylase. *Biochem.* **2000,** *39,* 4217–4224.
52. Miller, B. G.; Snider, M. J.; Short, S. A.; Wolfenden, R. Contribution of Enzyme-Phosphoribosyl Contacts to Catalysis by Orotidine 5'-Phosphate Decarboxylase. *Biochem.* **2000,** *39,* 8113–8118.
53. Miller, B. G.; Hassell, A. M.; Wolfenden, R.; Milburn, M. V.; Short, S. A. Anatomy of a Proficient Enzyme: The Structure of Orotidine 5'-Monophosphate Decarboxylase in the Presence and Absence of a Potential Transition State Analog. *Proc. Natl. Acad. Sci. U.S.A.* **2000,** *97,* 2011–2016.
54. Wu, N.; Mo, Y.; Gao, J.; Pai, E. F. Electrostatic Stress in Catalysis: Structure and Mechanism of the Enzyme Orotidine Monophosphate Decarboxylase. *Proc. Natl. Acad. Sci. U.S.A.* **2000,** *97,* 2017–2022.
55. Warshel, A.; Strajbl, M.; Villa, J.; Florian, J. Remarkable Rate Enhancement of Orotidine 5'-Monophosphate Decarboxylase is Due to Transition-State Stabilization Rather than to Ground-State Destabilization. *Biochem.* **2000,** *39,* 14728–14738.
56. Warshel, A.; Florian, J.; Strajbl, M.; Villa, J. Circe Effect versus Enzyme Preorganization: What Can Be Learned from the

Structure of the Most Proficient Enzyme? *Chem. Bio. Chem.* **2001,** *2,* 109–111.
57. Begley, T. P.; Appleby, T. C.; Ealick, S. E. The Structural Basis for the Remarkable Catalytic Proficiency of Orotidine 5'-Monophosphate Decarboxylase. *Curr. Opin. Struct. Biol.* **2000,** *10,* 711–718.
58. Houk, K. N.; Lee, J. K.; Tantillo, D. J.; Bahmanyar, S.; Hietbrink, B. N. Crystal Structures of Orotidine 5'-Monophosphate Decarboxylase: Does the Structure Reveal the Mechanism of the Most Proficient Enzyme? *Chem. Bio. Chem.* **2001,** *2,* 113–118.
59. Rishavy, M. A.; Cleland, W. W. Determination of the Mechanism of Orotidine 5'-Monophosphate Decarboxylase by Isotope Effects. *Biochem.* **2000,** *39,* 4569–4574.
60. Phillips, L. M.; Lee, J. K. Theoretical Studies of Mechanisms and Kinetic Isotope Effects on the Decarboxylation of Orotic Acid and Derivatives. *J. Am. Chem. Soc.* **2001,** *123,* 12067–12073.
61. Nakanishi, K.; Suzuki, N.; Yamazaki, F. Ultraviolet Spectra of N-Heterocyclic Systems. I. The Anions of Uracils. *Bull. Chem. Soc. Jpn.* **1961,** *34,* 53–57.
62. Kimura, E.; Kitamura, H.; Koike, T.; Shiro, M. Facile and Selective Electrostatic Stabilization of Uracil N(1)-Anion by a Proximate Protonated Amine: A Chemical Implication for Why Uracil N(1) is Chosen for Glycosylation Site. *J. Am. Chem. Soc.* **1997,** *119,* 10909–10919.
63. Amster, I. J. Fourier Transform Mass Spectrometry. *J. Mass. Spec.* **1996,** *31,* 1325–1337 , and references therein.
64. Marshall, A. G.; Grosshans, P. B. Fourier Transform Ion Cyclotron Resonance Mass Spectrometry: The Teenage Years. *Anal. Chem.* **1991,** *63,* 215A–229A , and references therein.
65. O'Hair, R. A. J.; Gronert, S.; DePuy, C. H.; Bowie, J. H. Gas-Phase Ion Chemistry of the Acetic Acid Enolate Anion $[CH_2CO_2H]^-$. *J. Am. Chem. Soc.* **1989,** *111,* 3105–3106.
66. Grabowski, J. J.; Cheng, X. Gas-Phase Formation of the Enolate Monoanion of Acetic Acid by Proton Abstraction. *J. Am. Chem. Soc.* **1989,** *111,* 3106–3108.
67. Wenthold, P. G.; Squires, R. R. Biradical Thermochemistry from Collision-Induced Dissociation Threshold Energy Measurements. Absolute Heats of Formation of *ortho*-, *meta*-, and *para*-Benzyne. *J. Am. Chem. Soc.* **1994,** *116,* 6401–6412.
68. Wenthold, P. G.; Paulino, J. A.; Squires, R. R. The Absolute Heats of Formation of *o*-, *m*-, and *p*-Benzyne. *J. Am. Chem. Soc.* **1991,** *113,* 7414–7415.
69. Wenthold, P. G.; Squires, R. R. Determination of the Gas Phase Acidities of Halogen-Substituted Aromatic Compounds Using the Silane-Cleavage Method. *J. Mass. Spec.* **1995,** *30,* 17–24.
70. Hare, M. C.; Marimanikkuppam, S. S.; Kass, S. R. Acetamide Enolate: Formation, Reactivity, and Proton Affinity. *Int. J. Mass Spectrom.* **2001,** *210/211,* 153–163.
71. Kass, S. R.; Guo, H.; Dahlke, G. D. The Formation and Reactivity of the Thiomethyl Anion. *J. Am. Soc. Mass Spectrom.* **1990,** *1,* 366–371.
72. Anderson, K. K.; Kass, S. R. Vinyl Anion Synthesis in the Gas Phase. *Tetrahedron Lett.* **1989,** *30,* 3045–3048.
73. Chou, P. K.; Kass, S. R. (E) and (Z) Vinyl Anions. The Formation and Characterization of Regio- and Stereoisomers in the Gas Phase. *J. Am. Chem. Soc.* **1991,** *113,* 4357–4359.
74. Sachs, R. K.; Kass, S. R. 3-Carbomethoxycyclopropen-3-yl Anion. Formation and Characterization of an Antiaromatic Ion. *J. Am. Chem. Soc.* **1994,** *116,* 783–784.
75. Baschky, M. C.; Peterson, K. C.; Kass, S. R. Stereospecificity in the Gas Phase. Formation and Characterization of Configurationally Stable Cyclopropyl Anions. *J. Am. Chem. Soc.* **1994,** *116,* 7218–7224.
76. Merrill, G. N.; Dahlke, G. D.; Kass, S. R. β-Cyanoethyl Anion: Lusus naturae. *J. Am. Chem. Soc.* **1996,** *118,* 4462–4468.
77. Reed, D. R.; Kass, S. R. An Experimental Determination of the α and β C–H Bond Dissociation Energies in Naphthalene. *J. Mass. Spec.* **2000,** *35,* 534–539.
78. Ingemann, S.; Nibbering, N. M. M. On the Dipole Stabilized Carbanions Derived from Methyl Formate and *N, N*-Dimethylformamide. *J. Org. Chem.* **1985,** *50,* 682–689.
79. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Gill, P. M. W.; Johnson, B. G.; Robb, M. A.; Cheeseman, J. R.; Keith, T.; Petersson, G. A.; Montgomery, J. A.; Raghavachari, K.; Al-Laham, M. A.; Zakrzewski, V. G.; Ortiz, J. V.; Foresman, J. B.; Peng, C. Y.; Ayala, P. Y.; Chen, W.; Wong, M. W.; Andres, J. L.; Replogle, E. S.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Binkley, J. S.; Defrees, D. J.; Baker, J.; Stewart, J. P.; Head-Gordon, M.; Gonzalez, C.; Pople, J. A. *GAUSSIAN94, Rev. E.2.* Gaussian, Inc.: Pittsburgh, 1995.
80. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A. Jr.;; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Baboul, A. G.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Andres, J. L.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. *GAUSSIAN98.* Gaussian, Inc.: Pittsburgh, 1998.
81. Gronert, S.; Feng, W. Y.; Chew, F.; Wu, W. The Gas Phase Acid/Base Properties of 1,3-Dimethyluracil, 1-Methyl-2-pyridone, and 1-Methyl-4-pyridone: Relevance to the Mechanism of Orotidine 5'-Monophosphate Decarboxylase. *Int. J. Mass Spectrom.* **2000,** *196,* 251–258.
82. Li, W.; Santos, I.; Marshall, A. G. Determination of the Gas-Phase Acidities of Uracil and 5-Fluorouracil by FT-ICR Mass Spectrometry. *1996 ASMS Abstracts* Portland, Oregon, May, **1996**.
83. Chandra, A. K.; Nguyen, M. T.; Zeegers-Huyskens, T. Theoretical Study of the Interaction Between Thymine and Water. Protonation and Deprotonation Enthalpies and Comparison with Uracil. *J. Phys. Chem. A* **1998,** *102,* 6010–6016.
84. Graindourze, M.; Smets, J.; Zeegers-Huyskens, T.; Maes, G. FT-IR Spectroscopic Study of Uracil Derivatives and their Hydrogen-Bonded Complexes with Model Proton Donors. Part 6. Proton Transfer in Uracil·HCl Complexes Isolated in Concentrated or Strongly Annealed Argon Matrices. *J. Mol. Struct. (THEOCHEM)* **1994,** *318,* 55–64.
85. Smets, J.; Graindourze, M.; Zeegers-Huyskens, T.; Maes, G. FT-IR Spectroscopic Study of Uracil Derivatives and their Hydrogen-Bonded Complexes with Model Proton Donors. Part 5. Complexes of Uracils with Hydrogen Chloride in Argon Matrices. *J. Mol. Struct. (THEOCHEM)* **1994,** *318,* 37–53.
86. Lias, S. G.; Bartmess, J. E.; Liebman, J. F.; Holmes, J. L.; Levin, R. D.; Mallard, W. G.NIST Thermochemical Tables. *J. Phys. Chem. Ref. Data* **1988,** *17*, Suppl. 1.
87. *NIST Chemistry WebBook, NIST Standard Reference Database Number 69, November 1998*; Mallard, W. G.; Linstrom, P. J., Eds.; National Institute of Standards and Technology: Gaithersburg 1998; http://webbook.nist.gov.
88. Kato, S.; DePuy, C. H.; Gronert, S.; Bierbaum, V. M. Gas Phase Hydrogen/Deuterium Exchange Reactions of Fluorophenyl Anions. *J. Am. Soc. Mass Spectrom.* **2000,** *10,* 840–847.