Intramolecular Carboxyl Group Participation in the Hydrolysis of an Acyl Imidazole

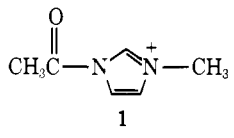
Jerry H. Smith

Contribution from the Department of Chemistry, Marquette University, Milwaukee, Wisconsin 53233. *Received October 17, 1975*

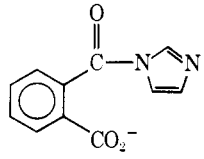
Abstract: Hydrolysis of the sodium salt of *N*-*o*-carboxybenzoylimidazole (**2**), *N*-benzoylimidazole (**3**), and *N*-benzoyl-*N'*-methylimidazolium fluorosulfonate has been examined over a wide pH range. The hydrolysis of **2** is catalyzed by the neighboring carboxyl group, which shows an effective concentration of 80 000 M when compared to intermolecular acetic acid catalyzed hydrolysis of **3**. The mechanism of intramolecular carboxyl group participation is thought to involve attack of the carboxylate anion on the amide carbonyl with the imidazole in a protonated state. The carboxylate anion is not effective in displacing the unprotonated imidazole group. The relevance of these results to the mechanism of carboxypeptidase catalysis is discussed.

The participation of an ionized carboxyl group has been implicated in the mechanisms of action of certain enzymes which catalyze the hydrolysis of peptide bonds.[1] For example, carboxypeptidase catalyzed hydrolysis of peptides shows a pH dependency consistent with the involvement of an ionized glutamic acid side chain.[2] This is somewhat surprising in view of the paucity of examples of nonenzymatic carboxylate ion catalyzed amide hydrolysis. Catalysis of amide hydrolysis by the protonated carboxyl group is well documented.[3]

Only one unambiguous example of carboxylate ion catalyzed amide hydrolysis, the acetate catalyzed hydrolysis of *N*-acetyl-*N'*-methylimidazolium ion (**1**), has been reported.[4] The



1

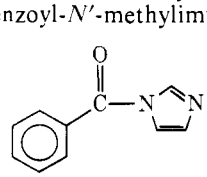


2

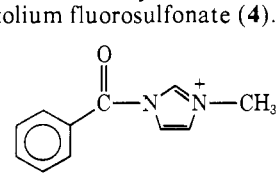
leaving group of **1** has a pK_a of 7.0, which is only 2.2 units above that of the acetate group. With amides such as *N*-acetylimidazole, which have a poorer leaving group (p$K_a \approx$ 14.2), catalysis by acetic acid and not by acetate is observed.[5]

Intramolecular catalysis by the carboxylate anion has not been observed in the hydrolysis of amides, although there are many examples of intramolecular catalysis by a protonated carboxyl group.[3] It might be expected that intramolecular participation by carboxylate would be important in the hydrolysis of amides with leaving group pK_a's considerably higher than 7. For example, it is known that the hydrolysis of esters is catalyzed by a neighboring carboxylate group when the pK_a of the alcohol leaving group is as high as 13.5.[6]

To determine the importance of intramolecular carboxylate catalysis in the hydrolysis of amides, we have prepared the sodium salt of *N*-*o*-carboxybenzoylimidazole (**2**) and report here its hydrolytic properties. For comparative purposes, we have also studied the hydrolysis of *N*-benzoylimidazole (**3**) and *N*-benzoyl-*N'*-methylimidazolium fluorosulfonate (**4**).



3



4

Results

Three experimental methods were used to obtain the observed pseudo-first-order rate constants k_{obsd} reported in this work. A spectrophotometric method using unbuffer solutions was employed in the pH ranges below 2 and above 10.8 where the concentrations of hydrogen ion and hydroxide ion were much greater than the substrate concentration. In the pH range 3-6, a spectrophotometric method using buffered solutions was employed, and k_{obsd} was obtained by extrapolation to zero buffer concentration. A pH-stat method was used in the pH range 6-12. In several cases where two methods were used in the same pH range, the k_{obsd} values obtained were in close agreement. All kinetic runs were carried out at 25 ± 0.1 °C at an ionic strength of 0.1 maintained with either NaCl or KCl.

Hydrolysis of *N*-*o*-Carboxybenzoylimidazole Sodium Salt (2). The hydrolysis of **2** was studied over the pH range 2-12.5 and the results obtained are shown in Figure 1. The points are experimental and the solid line between pH 10 and 12.5 is the computer fit of the data to the equation

$$k_{obsd} = k_H^S(H^+) + k_{OH}^S K_W/(H^+) \quad (1)$$

where k_H^S is the rate constant for the hydrogen ion catalyzed reaction of **2** (unprotonated), k_{OH}^S refers to hydroxide catalyzed hydrolysis of **2**, and K_W is the ion product of water. The values of these parameters are listed in Table I. No water-catalyzed reaction of **2** corresponding to k_0^S was observed.

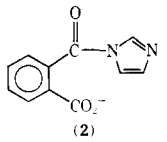
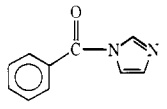
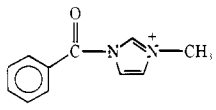
The solid line between pH 2 and 9 in Figure 1 represents hydrolysis of phthalic anhydride (**5**). The line was drawn through eight data points which have been omitted for the sake of clarity. The limiting rate constant in acid solution which corresponds to water catalyzed hydrolysis of **5** was calculated to be 1.12×10^{-2} s^{-1}. Bruice[6] obtained a value of 1.23×10^{-2} s^{-1} for this term at ionic strength equal to 1.0. The profile for hydrolysis of **5** was extended to high pH by extrapolation (dashed line) assuming a first-order dependency in hydroxide concentration. Several kinetic runs were made in the high pH region, but each was too fast to measure.

Hydrolysis of *N*-Benzoylimidazole (3). The hydrolysis of **3** was studied over the pH range 1-10.5. The results obtained are shown in Figure 2, where the points (solid circles) are experimental and the solid line is the computer fit of the data to eq 2. The values of the parameters of eq 2 are listed in Table I,

$$k_{obsd} = \frac{K_a^{SH}}{(H^+) + K_a^{SH}}\left[\frac{k_0^{SH}(H^+)}{K_a^{SH}} + k_0^S + \frac{k_{OH}^S K_W}{(H^+)}\right] \quad (2)$$

where K_a^{SH} is the acid dissociation constant of the conjugate acid (SH) of **3**, k_0^{SH} is the water-catalyzed reaction of SH, k_0^S is the water-catalyzed reaction of S, and k_{OH}^S is the hydroxide-catalyzed reaction of S. The results obtained for **3** are qualitatively similar to those obtained by Jencks[5] for the hydrolysis of *N*-acetylimidazole and to those obtained by Thornton[7] for the hydrolysis of **3** at 15 °C.

Table I. Hydrolytic Data for Acyl Imidazoles at 25 °C, μ = 0.1[a]

Substrate (S)	k_H^S, M^{-1} s^{-1}	k_0^S, s^{-1}	k_{OH}^S, M^{-1} s^{-1}	k_0^{SH}, s^{-1}	pK_a^{SH}	k_{HOAc}^S, M^{-1} s^{-1}	k_{OAc}^S, M^{-1} s^{-1}
 (2)	(2.42 ± 0.74) × 10^8	0[b]	2.12 ± 0.19	2.42 × 10^5[c]			
 (3)		(9.12 ± 1.54) × 10^{-5}	(2.92 ± 0.22) × 10^2	(3.93 ± 0.36) × 10^{-2}	3.34 ± 0.07	5.3 × 10^{-2}	0[b]
 (4)		(3.88 ± 0.14) × 10^{-2}	(1.19 ± 0.03) × 10^5			0[b]	8.6 × 10^{-1}

[a] Rate constants are given with standard deviations. [b] Too small to measure. [c] See text for calculations of this parameter.

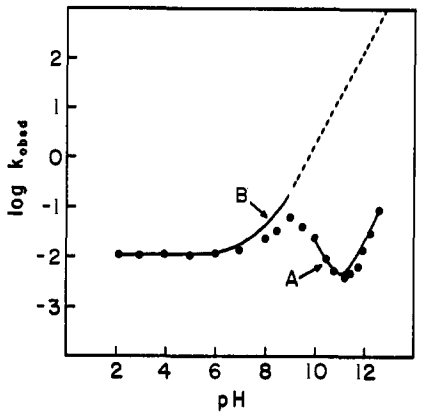


Figure 1. (A) Hydrolysis of *N*-*o*-carboxybenzoylimidazole sodium salt (**2**). (B) Hydrolysis of phthalic anhydride (**5**).

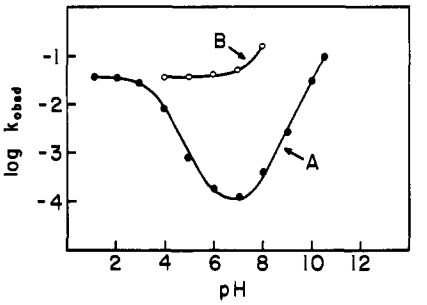


Figure 2. (A) Hydrolysis of *N*-benzoylimidazole (**3**). (B) Hydrolysis of *N*-benzoyl-*N'*-methylimidazolium fluorosulfonate (**4**).

Acetic Acid Catalyzed Hydrolysis of 3. The hydrolysis of **3** was followed spectrophotometrically in acetic acid buffers at pH 4.0, 5.0, and 6.0. At each pH, the results fit the expression, $k_{obsd} = k_0 + k_{HOAc}^S(HOAc)$, where the total buffer concentration was varied in the range 0.01-0.10 M. The average value of k_{HOAc}^S is given in Table I.

Hydrolysis of *N*-Benzoyl-*N'*-methylimidazolium Fluorosulfonate (4). The hydrolysis of **4** was studied over the pH range 4-8 and the results are given in Figure 2. The points (open circles) are experimental and the solid line is the computer fit to the equation

$$k_{obsd} = k_0^S + \frac{k_{OH}^S K_W}{(H^+)} \quad (3)$$

where k_0^S and k_{OH}^S refer to the water-catalyzed and hydroxide-catalyzed reactions of **4.** The values obtained for these parameters are listed in Table I. These results are qualitatively similar to those obtained by Jencks[4] for the hydrolysis of **1** and by Thornton[7] for the hydrolysis of **4** at 15 °C.

Acetate-Catalyzed Hydrolysis of 4. The hydrolysis of **4** was followed spectrophotometrically in acetic acid buffers at pH 4.0, 5.0, and 6.0. At each pH, the results fit the expression, $k_{obsd} = k_0 + k_{OAc}^S$ (OAc^-), where the total buffer concentration was varied in the range 0.01-0.10 M. The average value determined for k_{OAc}^S is listed in Table I.

Discussion

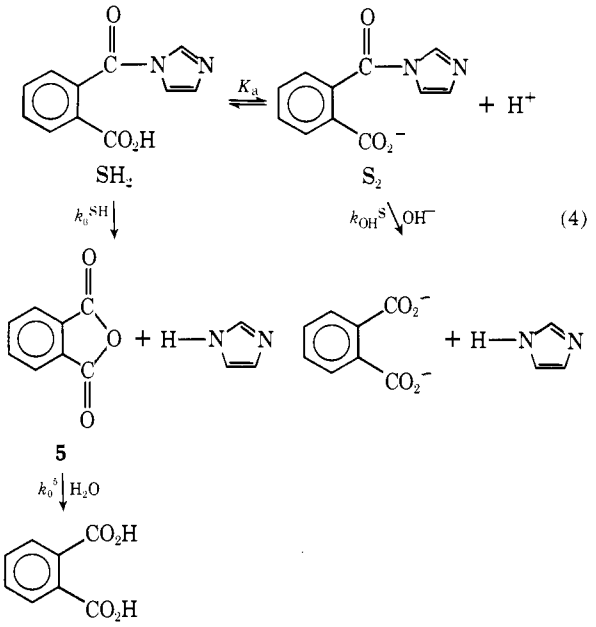
The pH profile for the hydrolysis of **2** is shown in Figure 1. It can be seen that, in the pH range 2-7, the profile is identical with that found for the hydrolysis of phthalic anhydride (**5**) under the same reaction conditions. At pH values between 7 and 9, k_{obsd} for **2** rises with pH in a manner similar to but not identical with that for **5.** Above pH 9, the pH dependency for **2** deviates dramatically from that for **5.**

These observations can be interpreted in the following manner. In the pH range 11-12.5, the reaction of **2** with hydroxide is observed with a second-order rate constant of 2.12 M^{-1} s^{-1} (see Table I). This is slower by a factor of 140 than hydroxide attack on **3,** an effect which can be attributed to electrostatic inhibition of hydroxide attack by the negatively charged carboxylate group.

In the pH range 10-11, the observed rate constant increases with decreasing pH and shows a first-order dependency on hydrogen ion concentration. The second-order rate constant k_H^S (eq 1) was found to be 2.42 × 10^8 M^{-1} s^{-1}. Below pH 10, k_{obsd} shows less than a first-order dependency on hydrogen ion concentration and reaches a relative maximum at pH 9 before falling to a limiting value of 1.07 × 10^{-2} s^{-1} below pH 7. This value corresponds well to k_{obsd} for hydrolysis of phthalic anhydride (**5**) in this region.

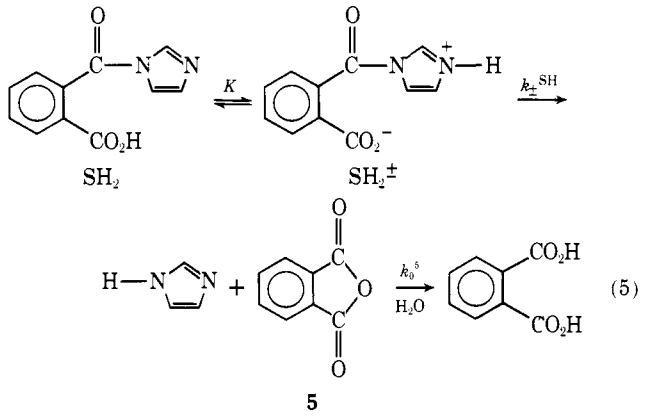
In eq 4 is shown a pathway which is consistent with these findings. Above pH 11, the predominant reaction is hydroxide attack on the unprotonated form of **2** (S_2). Below pH 11, the predominant reaction is hydrogen ion catalyzed hydroysis of S_2, which will be discussed below. In the pH range 8-10, there is a change from rate-determining formation of **5** above pH 10 to rate-determining hydrolysis of **5** below pH 8. Thus, below neutrality, **2** is converted rapidly to **5** and the observed rate constant that is measured is k_0[5].

We return now to the hydrogen ion catalyzed reaction of S_2, which represents the water catalyzed reaction of SH_2 as shown



in eq 4. The second-order rate constant $k_H{}^S$ is related to $k_0{}^{SH}$ by the expression, $k_0{}^{SH} = k_H{}^S K_a{}^{SH}$. Since the reaction of **2** is too fast to be observed below neutral pH, $K_a{}^{SH}$ cannot be measured. The value of $pK_a{}^{SH}$ is estimated to be approximately 3.0.[8] Using this value, the first-order rate constant $k_0{}^{SH}$ for intramolecular carboxyl group catalyzed hydrolysis of **2** is estimated to be 2.4×10^5 s^{-1}. This appears to be the largest rate constant reported for carboxyl catalyzed amide hydrolysis.

The mechanism of hydrolysis of SH_2 shown in eq 4 is kinetically indistinguishable from the mechanism given in eq 5,



where the proton in the reactive form of SH_2 is on the imidazole nitrogen. The rate parameters are related by the expression, $k_\pm{}^{SH} = k_0{}^{SH}K$, where K is the ratio of the acid dissociation constant of the imidazole proton of $SH_2{}^\pm$ to that of the carboxyl proton of SH_2. The value of pK_a for the $SH_2{}^\pm$ imidazole proton should be similar to the value of 3.34 determined for the imidazole proton of **3** (see Table I). Using this value and a value of 3.0 for the pK_a of SH_2, K is calculated to be about 0.46. The value of $k_\pm{}^{SH}$ is thus estimated to be 1×10^5 s^{-1}, which represents the rate constant for attack of the neighboring carboxylate anion on the imidazole-protonated form of the substrate.

In order to choose between the mechanisms of eq 4 and 5, we have investigated the acetic acid catalyzed hydrolysis of *N*-benzoylimidazole (**3**) and *N*-benzoyl-*N'*-methylimidazolium ion (**4**). This approach was taken by Jencks to determine the mechanism of reactions of **1** with nucleophilic reagents of the form HY.[4] He was able to show that the preferred mechanism was reaction of Y^- with the protonated substrate. We have reached a similar conclusion for the acetic acid catalyzed reaction of **3**.

The rate constants for hydrolysis of **3** and **4** are shown as a function of pH in Figure 2. The rate constant for water-catalyzed hydrolysis of **4** is essentially identical with the limiting rate constant for **3** in the acidic pH range, indicating that hydrolysis of **3** at low pH results from attack of water on the protonated substrate.

The hydrolysis of **3** is catalyzed by acetic acid with a second-order rate constant $k_{HOAc}{}^S$ equal to 5.3×10^{-2} M^{-1} s^{-1}. The rate constant $k_{OAc}{}^{SH}$ for the kinetically equivalent pathway involving reaction of acetate with protonated **3** can be calculated from the expression, $k_{OAc}{}^{SH} = k_{HOAc}{}^S K$, where K is the ratio of the acid dissociation constant of **3** to that of acetic acid. The value of $k_{OAc}{}^{SH}$ obtained in this manner is 1.36 M^{-1} s^{-1}, which compares well with the value of 0.86 M^{-1} s^{-1} found for the reaction of acetate with **4** (Table I). These findings substantiate a mechanism analogous to that in eq 5 for the reaction of **3** with acetic acid and support the contention that hydrolysis of **2** proceeds via the mechanism of eq 5 as well.

From the aforementioned arguments, it can be concluded that a neighboring carboxylate anion is capable of displacing the protonated imidazole group with $pK_a = 7.0$.[10] The effective concentration of the carboxylate anion in **2**, calculated as the ratio of $k_\pm{}^{SH}$ for **2** to $k_{OAc}{}^{SH}$ for **3**, is about 80 000 M. However, the neighboring carboxylate anion of **2** is not able to displace the unprotonated imidazole group with $pK_a = 14.2$. This is evidenced by the fact that the observed rate constants for hydrolysis of **2** between pH 10 and 12.5 are adequately described by the two-parameter relationship of eq 1; the $k_0{}^S$ term is too small to detect. This establishes an upper limit of about $pK_a = 14$ for the leaving group in order to observe intramolecular catalysis by an ionized carboxylate group.

In contrast, the enzyme carboxypeptidase is capable of using the ionized carboxyl group of Glu-270 to catalyze the hydrolysis of amides with very poor leaving groups ($pK_a \approx 30$). It has been proposed that another group at the active site, Tyr-248, assists by donating a proton to the leaving group in a general acid catalyzed process.[11] This would seem to be a very important mode of catalysis for carboxypeptidase. It enables the enzyme to function with substrates having leaving group pK_a values at least 16 units more unfavorable than the upper limit for intramolecular carboxylate participation established in this work.

Experimental Section

General. NMR spectra were recorded on a Varian A-60A instrument and ir spectra on a Perkin Elmer Model 137 instrument. Melting points were taken on a Thomas–Hoover apparatus and are uncorrected. pH measurements were made with either a Radiometer pH meter equipped with a combination electrode or with a Corning Model 12 pH meter fitted with a glass electrode and a calomel reference electrode.

***N*-*o*-Carboxybenzoylimidazole, Sodium Salt (2).** To 0.54 g of NaH (50% oil dispersion) (11.3 mmol) suspended in 5 ml of DMF was added with stirring, under nitrogen at 10 °C, 0.76 g of imidazole (11.2 mmol) dissolved in 5 ml of DMF. To the resulting, almost homogeneous solution was added 1.67 g of phthalic anhydride (11.3 mmol) in 5 ml of DMF over a period of 0.5 h. The resulting solution was allowed to warm to room temperature and was stirred for an additional hour. The white precipitate that formed was collected by filtration and washed repeatedly with dry ether. The material was dried under vacuum over P_2O_5 at 70 °C for several hours to give a white solid which decomposed without melting when heated to about 260 °C. The ir spectrum showed carbonyl stretching at 5.83 μ and carboxylate anion stretching at 6.3 and 7.2 μ. The NMR spectrum (Me_2SO-d_6) gave multiplets in the region 8.1–6.9 relative to DSS. This material was used in the kinetic studies without further purification.

***N*-Benzoyl-*N'*-methylimidazolium Fluorosulfonate (4).** To 536 mg of *N*-benzoylimidazole (**3**)[12] (3.1 mmol) in 5 ml of dry ether was added

with stirring, under nitrogen at 5 °C, 0.25 ml of methyl fluorosulfonate (3.1 mmol) over a period of 15 min. The oily precipitate was stirred at 4 °C overnight to give a white solid, which was washed repeatedly with dry ether and dried under vacuum over P_2O_5. The slightly hydroscopic material decomposed in the range 60–80 °C. The NMR spectrum in CD_3CN gave the following peaks relative to Me_4Si: δ 9.1 (1 H, s), 7.9–7.5 (7 H, m), and 4.0 (3 H, s). This material was used in the kinetic studies without further purification.

Kinetic Measurements. Pseudo-first-order rate constants were determined either spectrophotometrically at 260 nm using a Cary 14 spectrophotometer or by a pH stat method using a Radiometer apparatus which included a TTT60 titrator, PHM62 pH meter, ABU12T buret, and a REC61 servograph. Stock solutions of **3** were prepared in acetonitrile and were stable for weeks when stored at −15 °C. Stock solutions of **4** were prepared in acetonitrile and used the same day. Stock solutions of **2** were prepared in Me_2SO and were used within 2 h. No solvent was found for 2 which afforded solutions stable for more than 2 h. Typically, 10–50 μl of stock solution was used to initiate the reaction. In many pH-stat runs, **2** was added as a solid directly to the reaction vessel. Results using this technique compared well with results using fresh stock Me_2SO solutions. The concentration of substrates was approximately 3×10^{-2} M for the pH-stat runs and about 2.5×10^{-4} M for the spectrophotometric runs. The pseudo-first-order rate constants were fit to the appropriate rate expression using a nonlinear least-squares computer program.

Acknowledgment. Support from the Research Corporation is gratefully acknowledged.

References and Notes

(1) "The Enzymes", Vol. III, P. D. Boyer, Ed., Academic Press, New York, N. Y., 1971.
(2) E. T. Kaiser and B. L. Kaiser, *Acc. Chem. Res.*, **5**, 219 (1972).
(3) A. J. Kirby and A. R. Fersht, *Prog. Bioorg. Chem.*, **1**, 1 (1971).
(4) R. W. Wolfenden and W. P. Jencks, *J. Am. Chem. Soc.*, **83**, 4390 (1961).
(5) W. P. Jencks and J. Carriuolo, *J. Biol. Chem.*, **234**, 1272 (1959).
(6) J. W. Thanassi and T. C. Bruice, *J. Am. Chem. Soc.*, **88**, 747 (1966).
(7) M. Choi and E. R. Thornton, *J. Am. Chem. Soc.*, **96**, 1428 (1974).
(8) The $pK_a^{SH^+}$ for **2** should be about the same as the first pK_a for phthalic acid, 2.98.[9]
(9) W. R. Maxwell and J. R. Partington, *Trans. Faraday Soc.*, **33**, 670 (1937).
(10) T. C. Bruice and G. L. Schmir, *J. Am. Chem. Soc.*, **80**, 148 (1958).
(11) W. N. Lipscomb et al., *Brookhaven Symp. Biol.*, **21**, 24 (1968).
(12) M. Caplow and W. P. Jencks, *Biochemistry*, **1**, 883 (1962).

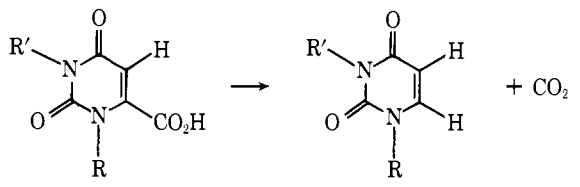
Mechanism of Decarboxylation of 1,3-Dimethylorotic Acid. A Model for Orotidine 5′-Phosphate Decarboxylase

Peter Beak* and Brock Siegel

Contribution from the Roger Adams Laboratory, University of Illinois, Urbana, Illinois 61801. *Received June 2, 1975*

Abstract: The decarboxylation of 1,3-dimethylorotic acid (**1**) is shown to proceed by separate pH-determined pathways in sulfolane at 180–220 °C. Although a process involving ionization of **1** is the major pathway in the presence of excess base, decarboxylation is initiated by zwitterion formation in the neutral solvent. Measurements of the rate of loss of carbon dioxide from 6-carboxy-2,4-dimethoxypyrimidine (**5**) and 1-methyl-2,4-dimethoxypyrimidinium-6-carboxylate betaine (**7**) are used to estimate the equilibrium and rate constants for the zwitterionic pathway. Comparison of the rate constant for decarboxylation of **7** with k_{cat} for orotidine 5′-phosphate decarboxylase shows that the biological catalysis can be satisfactorily accounted for if the enzyme provides a site which displaces the equilibrium in favor of the zwitterionic form of orotidylic acid. It is also noted that the inhibitor 6-azauridine monophosphate, which has a greater affinity for the enzyme than does the substrate, provides a partial model for the intermediate formed on loss of carbon dioxide from the zwitterion.

The temperatures in excess of 180° which are required to promote decarboxylation of 1,3-dimethylorotic acid (**1**) to 1,3-dimethyluracil (**2**) are in sharp contrast to the ambient



1, R = R′ = CH_3 **2**, R = R′ = CH_3
3, R = *R*-5-P; R′ = H **4**, R = *R*-5-P; R′ = H

temperatures at which orotidine 5′-phosphate decarboxylase catalyzes the conversion of orotidylic acid (**3**) to uridylic acid (**4**). The enzymatic conversion of **3** to **4** is an essential step in nucleic acid biosynthesis and also appears to be biomechanistically novel. In virtually all known biochemical decarboxylations an intermediate is involved in which the electron pair remaining after breakage of the carbon–carbon bond can be stabilized by delocalization into a π orbital.[1] For decarboxylation of **3**, however, no such stabilization is apparent, and mechanisms involving intermediates with electron pairs in an sp^2 orbital orthogonal to the π system have been suggested.[2]

This report details our determination of the pathways of decarboxylation of **1**.[3] The understanding gained of that process provides a mechanism which can account for the catalytic action of the enzyme in the conversion of **3** to **4**.

Results

At 206° in sulfolane, 1,3-dimethylorotic acid (**1**) evolves carbon dioxide quantitatively in 3 h, and 1,3-dimethyluracil (**2**), the only product observable by NMR, can be isolated in 79% yield. The rate of the reaction can be followed by observation of the C-6 proton by NMR, by titration of the residual acid, or by continuous monitoring of the carbon dioxide evolved. A rate constant of 7.6 (±0.2) $\times 10^{-4}$ s^{-1} is obtained by the three methods and the reaction is first order to more than 90% completion. In the presence of *N,N*-diethylaniline, the observed first-order rate constant rises to a plateau of 3.1 $\times 10^{-3}$ s^{-1} as the concentration of base increases, as shown in Figure 1a.[4] If the latter value is taken as the limiting rate for decarboxylation of the carboxylate anion of **1**, reaction in the absence of added base via the same intermediate would require that **1** be ca. 25% ionized in neat sulfolane. However, conductivity studies set an upper limit of 3% on the degree of ionization of **1** in sulfolane at 206°.[6] Figure 1b shows that a