On the Mechanism of Catalysis by Ribonuclease: Cleavage and Isomerization of the Dinucleotide UpU Catalyzed by Imidazole Buffers

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Abstract: Uridylyl(3′,5′)uridine [(3′,5′)-UpU] undergoes cleavage to uridine 2′,3′-cyclic phosphate and uridine, catalyzed by imidazole buffers. Kinetic studies indicate a sequential bifunctional mechanism, with one buffer component catalyzing the formation of a phosphorane monoanion intermediate and the other catalyzing its forward decomposition to products. From a study of the buffer-catalyzed isomerization of (3′,5′)-UpU to (2′,5′)-UpU, the first catalyst is identified as imidazolium ion and the second as imidazole. The resulting detailed mechanism is chemically reasonable. It is also consistent with what is known about bovine pancreatic ribonuclease. Thus, a related multistep mechanism is suggested for the enzymatic reactions as well.

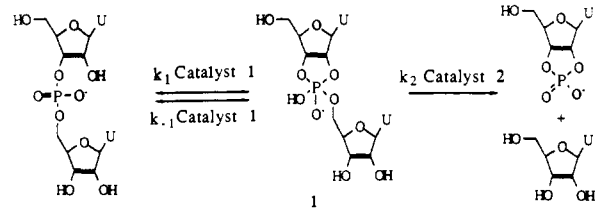
We have described a study of the cleavage of poly(ribouridylic acid) [poly(U)] catalyzed by imidazole buffers.[1] The assay that made the kinetic studies possible had been developed previously.[2] We found that imidazole buffer does indeed catalyze this cleavage and the reaction shows in part[1] a bell-shaped pH vs rate profile. This was of particular interest since the enzyme bovine pancreatic ribonuclease A also uses imidazole groups of two histidine residues as the principal catalytic groups in the cleavage of RNA, and the enzyme also shows a bell-shaped pH vs rate profile.[3]

Although a bell-shaped pH–rate profile could mean simultaneous catalysis of the reaction by a basic imidazole and an acidic imidazolium ion, and such simultaneous catalysis is commonly written for the enzyme mechanism, our kinetic studies showed that this was not occurring in the chemical model system.[1] The rate of the buffer-catalyzed reaction was proportional to the *first* power of the buffer concentration, but a simultaneous mechanism would require second-order dependence. Various control experiments established that we were not dealing with a third-order mechanism (second order in buffer) disguised by complexing of reagents.[1] Instead, the observed kinetics indicated that we were dealing with *sequential bifunctional catalysis* (Scheme I).

The reaction is bifunctional since an acid and a base catalyst are involved, but one of them (catalyst 1) catalyzes a first step while the other one (catalyst 2) catalyzes a second step. Each step has only one catalyst species in it, so the overall reaction is pseudo-first-order in imidazole buffer no matter which step is rate limiting, or even if the the two are of comparable rate. With an excess of catalyst 1 the second step is rate limiting; with an excess of catalyst 2 the first step is the slow one. There is a rate maximum when both catalysts are present and both steps are accelerated, so the pH vs rate profile is bell-shaped. This was the first *kinetic* evidence for an intermediate in phosphate substitution reactions. The intermediate was required to be an isomer of the poly(U), a monoanionic phosphorane (see Discussion).

We were able to fit our detailed kinetic data according to this scheme, including additional terms for imidazolium ion catalysis alone, for imidazole catalysis alone, and for catalysis by nonbuffer species.[1] However, there were some ambiguities with regard to the sequential bifunctional catalysis. The most prominent one was that the scheme worked equally well whether the basic imidazole was catalyst 1 or catalyst 2, with the acidic imidazolium ion playing the other role. We wanted to clear this up so we could establish a well-defined mechanism. Another problem was the unlikely possibility that the pH dependence reflected a pK of the *substrate*, not of the catalyst. Although the normal pK's do not

Scheme I. Alternative Sequential Bifunctional Mechanisms and Their Common Rate Expression



Mechanism 1:
Catalyst 1 = Imidazole
Catalyst 2 = Imidazolium

Mechanism 2:
Catalyst 1 = Imidazolium
Catalyst 2 = Imidazole

$$\text{Rate} = \frac{k_1k_2[\text{UpU}][\text{Cat. 1}][\text{Cat 2}]}{k_{-1}[\text{Cat. 1}] + k_2[\text{Cat. 2}]}$$

fit this explanation, there might be some unusual ionizable groups in a polymer. Thus, we have examined the imidazole-catalyzed cleavage of UpU, a simple dimer.

We had previously described an assay for the cleavage of (3′,5′)-UpU, forming uridine and uridine 2′,3′-cyclic phosphate.[2] We now find that the imidazole-catalyzed cleavage of UpU also shows in part a bell-shaped pH vs rate profile and pseudo-first-order kinetics in buffer, just as poly(U) did. Thus no unusual properties of a polymer need be invoked. However, the details of the catalyzed processes that UpU undergoes let us solve the kinetic ambiguity, establishing which is catalyst 1 and which is catalyst 2 (Scheme I).

Cleavage of UpU, or of poly(U), involves attack on the 3′,5′-phosphodiester group by the C-2′ hydroxyl. The product of cleavage is a 2′,3′-cyclic phosphate ester of uridine, with a new 5′-OH group on the uridine residue that departs (Scheme I). With UpU these are the actual products, while for internal reactions of poly(U) both the uridine 2′,3′-cyclic phosphate and the uridine leaving group are still attached to polymer chains. However, in both systems another reaction is also possible. Attack on the 3′,5′-phosphodiester group by the 2′-hydroxyl group can lead to migration instead of cleavage, forming a 2′,5′-phosphodiester isomer of the starting material. This migration is difficult to follow with poly(U), but with the dimer UpU the 3′,5′ and 2′,5′ isomers are easily distinguished. We had already shown that an HPLC assay could be used to follow this isomerization.[2] By observing the dependence of this migration on pH and buffer concentration, we are now able to clarify the entire mechanistic picture, and in a way that proves to be chemically satisfying.

After we reported our studies on the catalyzed cleavage of poly(U) and pointed out that our kinetics did not resolve the ambiguity between a [base, then acid] and an [acid, then base] sequence, Taira proposed a solution[4] based on theoretical con-

(1) Breslow, R.; LaBelle, M. *J. Am. Chem. Soc.* **1986**, *108*, 2655–2659.
(2) Corcoran, R.; LaBelle, M.; Czarnik, A. W.; Breslow, R. *Anal. Biochem.* **1985**, *144*, 563–568.
(3) (a) Herries, D. G.; Mathias, A. P.; Rabin, B. R. *Biochem. J.* **1962**, *85*, 127. (b) del Rosario, E. J.; Hammes, G. G. *Biochemistry* **1969**, *8*, 1884.

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siderations. He suggested that some features of our work could be understood in stereoelectronic terms if the sequence involved first base, then acid, not the alternative. Whatever the merits of stereoelectronic arguments may be in these systems, our kinetic studies have now established that the alternative sequence is actually the correct one: the first catalyst is the acidic imidazolium ion and the second one the basic imidazole. Thus the stereoelectronic argument of Taira is apparently not relevant here.

Experimental Methods

Materials. Uridylyl(3′,5′)uridine, uridylyl(2′,5′)uridine, and uridine were purchased from Sigma Chemical Co. and used as received. Imidazole was purchased from Aldrich and recrystallized from benzene. All stock solutions, buffers, and eluents were prepared from deionized water.

Kinetic Method. The assay to determine the rate of formation of uridine and of (2′,5′)-UpU from (3′,5′)-UpU has been described in detail previously.[2] The same precautions (to avoid ribonuclease impurities) and assay procedure were used except for the following points: (a) The capillary tubes used as kinetic vessels were not silylated before use. (b) The imidazole buffers were prepared by weighing and dissolving calculated amounts of imidazole and imidazole hydrochloride to form the desired molar ratio of protonated to unprotonated buffer. (c) The standard plots required by the assay were made from solutions that were 550 μM in potassium *p*-nitrobenzenesulfonate and 6, 12, 24, 48, 96, and 192 μM in both uridine and (2′,5′)-UpU. (d) The substrate stock solution was prepared as 64 mM (3′,5′)-UpU and 2.2 mM potassium *p*-nitrobenzenesulfonate. (e) The HPLC eluent was 4.5 mM pH 7 phosphate buffer containing 4.0% MeOH. A flow rate of 1.2 mL/min yielded base-line resolution of uridine (5.4 min), internal standard (6.5 min), (2′,5′)-UpU (7.9 min), and (3′,5′)-UpU (13.9 min).

The kinetic assay for the cleavage and isomerization of (2′,5′)-UpU was exactly the same as that for (3′,5′)-UpU except for one point. The eluent used was 4.5 mM pH 7 phosphate buffer containing 3.5% MeOH. A flow rate of 1.2 mL/min yielded base-line resolution of uridine (8.1 min), internal standard (9.7 min), (2′,5′)-UpU (11.8 min), and (3′,5′)-UpU (20.8 min).

The acetate buffers were prepared by syringing and weighing calculated amounts of acetic acid and sodium acetate, respectively, to form the desired molar ratio of protonated to unprotonated buffer.

Data Collection. The sealed capillary tubes used as reaction vessels were incubated in an oven at 80 ± 1 °C. A capillary tube was removed from the oven every 1–2 h, broken, and analyzed. To obtain each rate, either seven or eight points were collected. Every rate plot had a correlation coefficient of 0.985 or better and was repeated two to three times. The errors plotted in the figures are plus and minus two standard deviations of the repetitive runs.

Results

Cleavage of (3′,5′)-UpU or of (2′,5′)-UpU at the 5′ P–O ester bond produces uridine and uridine 2′,3′-cyclic monophosphate. Under some of the cleavage reaction conditions, the (3′,5′)-UpU is found to isomerize to (2′,5′)-UpU and vice versa. The compounds uridine, (2′,5′)-UpU, and (3′,5′)-UpU are quantitatively detected by high-pressure liquid chromatography, and thus the cleavage and isomerization of both UpU isomers can be followed. We have shown previously that the assay is reliable and leads to good reproducible kinetic results, when we examined the simple acid cleavage of (3′,5′)-UpU.[2] Furthermore, in all of this work we obtain a good linear response over the first 10% of reaction; for reaction over this range a logarithmic treatment is not required. The cleavage and isomerization of (2′,5′)-UpU can also be quantitatively followed by an assay differing only in HPLC conditions (see Experimental Methods).

Over the buffer concentration range used (0.2–2.0 M), the pseudo-first-order rate constants for the buffer-catalyzed cleavage reactions appear first order in total concentration of imidazole buffer. This is true for both (3′,5′)-UpU and (2′,5′)-UpU, as displayed in Figures 1 and 2, respectively. There is no sign of upward deviation from linearity in the dependence upon buffer concentration, even though Figure 2 possesses some scatter. The slopes of the lines are clearly dependent upon the degree of protonation of imidazole buffer. In Figures 1A and 2A the observed rate is simply plotted versus buffer concentration; the intercepts are the buffer-independent rates at each pH. The buffer-independent rate was then subtracted from each observed rate of cleavage for that line, to generate the lines of Figures 1B and 2B, representing only the buffer-*dependent* rates.

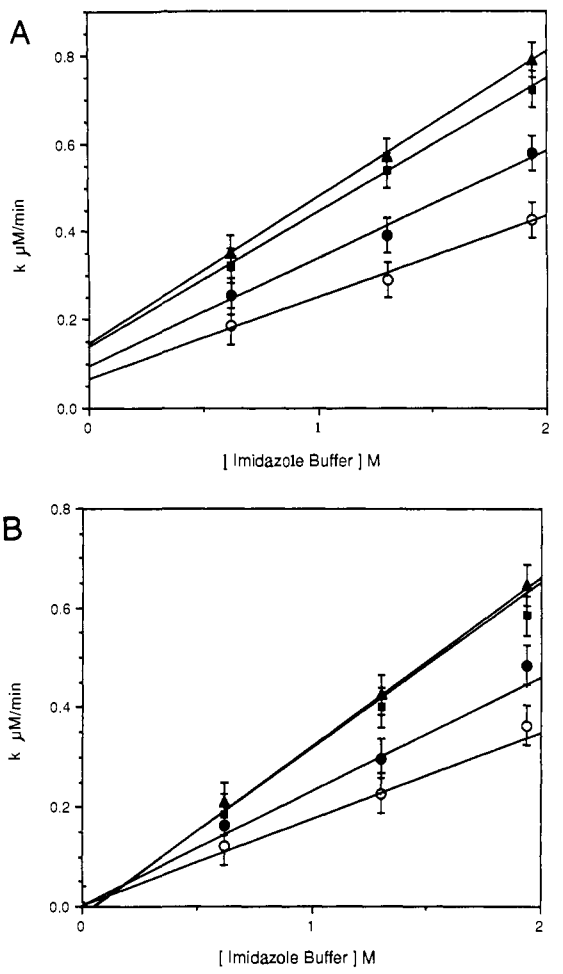


Figure 1. Plot of the observed pseudo-first-order rate constant for (3′,5′)-UpU cleavage as a function of imidazole buffer concentration at four different stages of protonation of the buffer: (●) $ImH^+/Im = 0.0$, (■) $ImH^+/Im = 0.4$, (▲) $ImH^+/Im = 0.6$, and (○) $ImH^+/Im = 1.0$. Part A displays the experimental data. Part B shows only the buffer-dependent data (see Results). The lines drawn in part A are computer-generated least-squares fits. The lines drawn in part B were calculated by our kinetic treatment.

The observation that the degree of protonation of the buffer determines the slope of the lines in Figures 1 and 2 is further illustrated in plots of the cleavage rate vs state of protonation of the imidazole buffer (Figures 3 and 4). Again the parts A are the raw data, while the B panels are corrected by substracting the buffer-independent rate for each point. The data in these parts B display a bell-shaped state-of-protonation (analogous to pH) vs rate profile. The bell curve of Figure 3B is quite symmetrical, with the buffer-catalyzed term on the basic side being slightly greater than the term on the acid side. The bell curve of Figure 4B, however, has its maximum considerably displaced toward the basic side.

The data of Figures 3 and 4 indicate catalysis of cleavage by both imidazole *and* imidazolium for both (3′,5′)-UpU and (2′,5′)-UpU substrates. These results confirm the previous findings for poly(U).[1] As discussed for poly(U), any interpretation of the cleavage data involving simultaneous catalysis by both imidazole and imidazolium is ruled out since Figures 1B and 2B show a clean first-order dependence of the rate constants on the buffer concentration. No square term in overall buffer concentration is detectable even at very high buffer concentrations, such as 2 M. Several control experiments, previously discussed,[1] also show that a third-order mechanism (second order in the buffer catalyst) has

(4) Taira, K. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 1903.

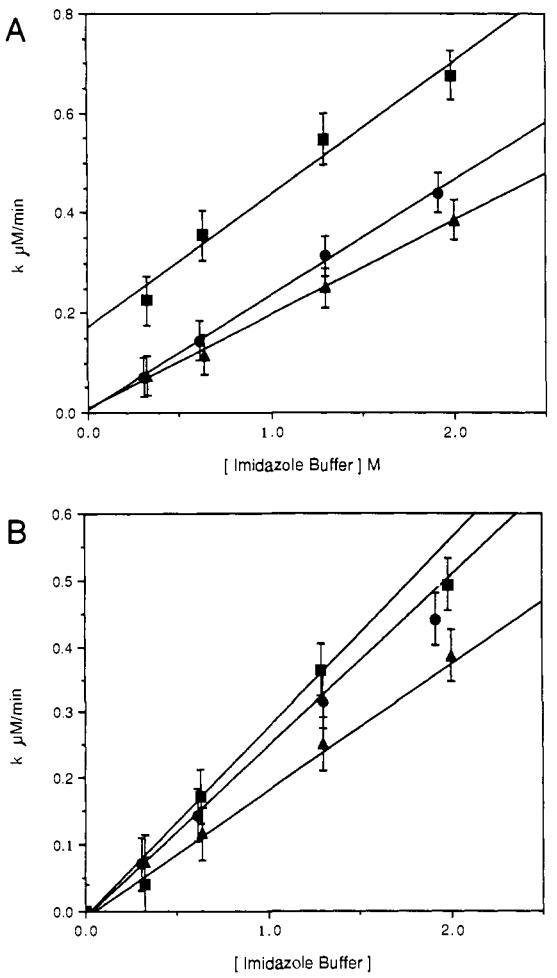


Figure 2. Plot of the observed pseudo-first-order rate constant for (2′,5′)-UpU cleavage as a function of imidazole buffer concentration at three different states of protonation of the buffer: (■) $ImH^+/Im = 0.0$, (●) $ImH^+/Im = 0.6$, and (▲) $ImH^+/Im = 0.87$. Part A displays the experimental data. Part B shows only the buffer-dependent data. The lines drawn in part A are computer-generated least-squares fits. The lines drawn in part B were calculated by our kinetic treatment.

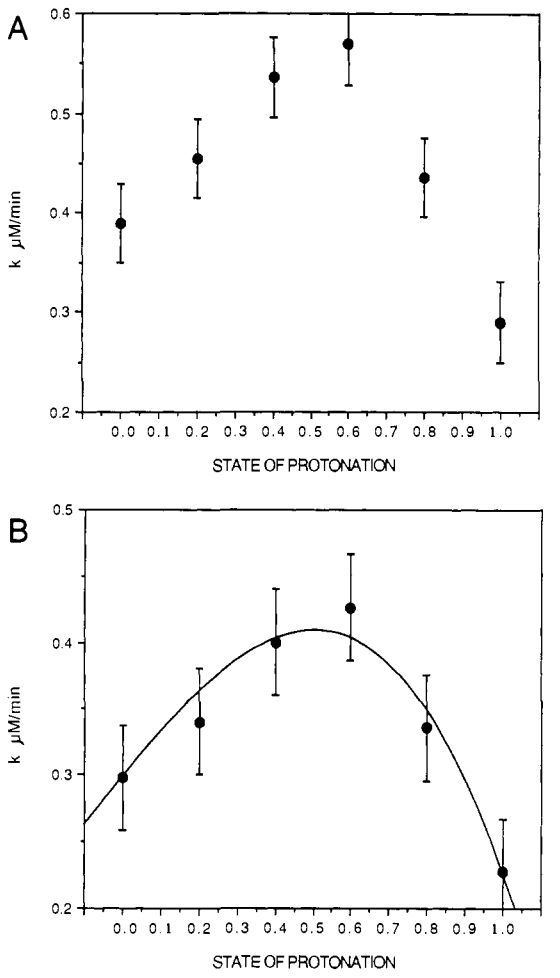


Figure 3. Buffer-catalyzed rate constant for hydrolysis of (3′,5′)-UpU at 1.3 M buffer vs the state of protonation of the buffer. Part A displays the experimental data. Part B shows only the buffer-dependent data. The curve in part B was calculated by our kinetic treatment.

not been disguised by complexing of reagents.

In addition to confirming that the results obtained for cleavage of poly(U) are also seen with both (3′,5′)-UpU and (2′,5′)-UpU, we extended the studies to the isomerizations of the phosphate linkage. Figures 5 and 6 display the dependence of the rate of isomerization upon total imidazole buffer concentration. The curves of Figures 5B and 6B show no deviation from linearity in buffer concentration, although Figure 6B has some scatter. Figures 5A and 6A show that, in contrast to the cleavage reactions, the buffer-independent rates of isomerization were quite significant with respect to the buffer-dependent rates. In these studies, isomerization was typically 5–10 times slower than cleavage.

As was observed for the cleavage reactions, the slopes of the lines are clearly dependent upon the state of protonation of the buffer. However, in contrast to the cleavage reactions, the slopes of the isomerization lines simply increase with increased protonation of the buffer. This point is clear in plots of the rate of isomerization vs state of protonation of the buffer (Figures 7B and 8B). A plot of the buffer-dependent pseudo-first-order rate constants for isomerization vs the state of protonation (for a 1.3 M total imidazole concentration) continues to increase in a near-linear fashion, with a maximum rate using only imidazole hydrochloride. Although Figures 7 and 8 possess significant scatter, it is clear that other alternatives such as imidazole-dependent isomerization or simultaneous or sequential bifunctional imidazole–imidazolium-dependent isomerization are not operative. The buffer-catalyzed isomerization reaction is catalyzed only by imidazolium cation.

In order to further substantiate that the isomerization is catalyzed by a buffer acid term and is not dependent upon some other variable that we were not controlling (such as ionic strength), we examined the acetic acid catalyzed isomerization of (2′,5′)-UpU. Figure 9 displays a plot of the rate of isomerization of (2′,5′)-UpU versus state of protonation of acetate. The plot again shows that the isomerization rate increases with increasing concentration of the acidic buffer component.

Discussion

We first review the conclusions from our study of poly(U).[1] A bell-shaped profile of pH vs rate can indicate simultaneous acid–base catalysis of a reaction, but it can also indicate a change in rate-determining step. A simultaneous cleavage mechanism would yield second-order kinetics with respect to the buffer concentration. Since the reaction remains first order with respect to buffer over the concentrations studied, a simultaneous cleavage mechanism is ruled out. The alternative mechanism involves a change in rate-determining step. This requires a two step sequential mechanism, so there must be a reaction intermediate between the two steps. The only logical intermediate between UpU and the uridine 2′,3′-cyclic monophosphate is a five-coordinate phosphorus species, a phosphorane, in which the nucleophile has attacked the phosphorus but the leaving group is still attached.[5]

The intermediate in our reactions must be an *isomer* of the starting material, with a single negative charge. As discussed previously,[1] a dianionic phosphorane intermediate would remove

(5) (a) Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1*, 70–78. (b) Hudson, R. F.; Brown, C. *Acc. Chem. Res.* **1972**, *5*, 204.

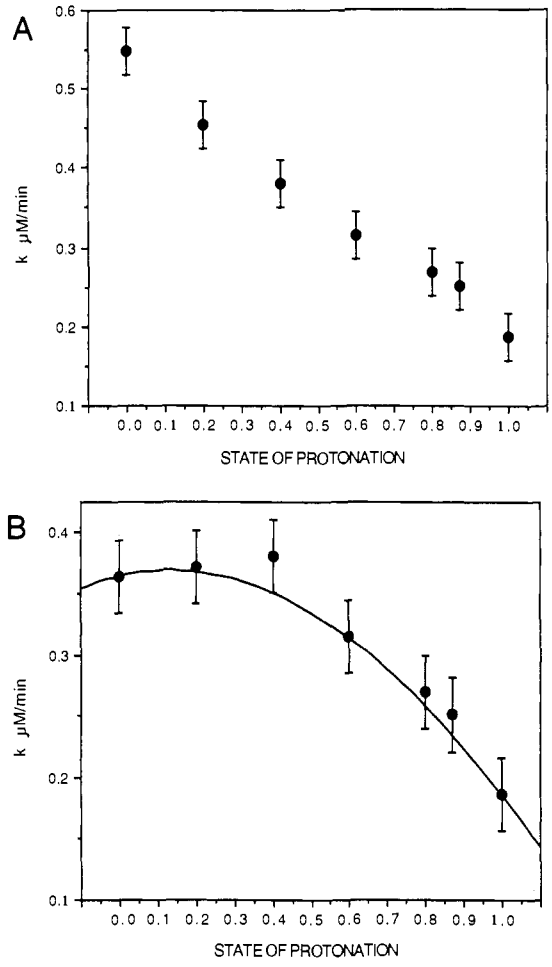
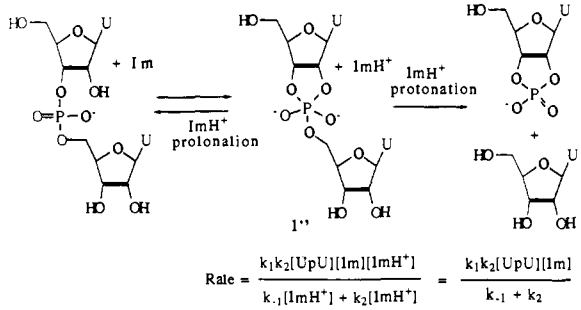


Figure 4. Buffer-catalyzed rate constant for hydrolysis of (2′,5′)-UpU at 1.3 M buffer vs the state of protonation of the buffer. Part A displays the experimental data. Part B shows only the buffer-dependent data. The curve in part B was calculated by our kinetic treatment.

Scheme II. A Mechanism That Does Not Fit the Kinetics because It Would Show Catalysis Only by Imidazole



observable catalysis by imidazolium cation, and a neutral intermediate would remove observable catalysis by imidazole. For instance, if the intermediate were a dianion formed by proton removal from imidazole catalysis (Scheme II), then the resulting imidazolium cation would have to catalyze both the second step *and* the reverse of the first step. Increasing the concentration of imidazolium ion would not increase the fraction of the intermediate that underwent cleavage rather than return to starting material, so imidazolium cation would not affect the rate of formation of the products. This is clear from the kinetic equation corresponding to Scheme II.

Scheme II is closely related to the mechanism used by chymotrypsin to cleave peptides, although the anionic intermediate in that case is a tetrahedral carbon species, not a phosphorane. In the first step for chymotrypsin an imidazole acts as a base, while in the second step the resulting imidazolium cation protonates the

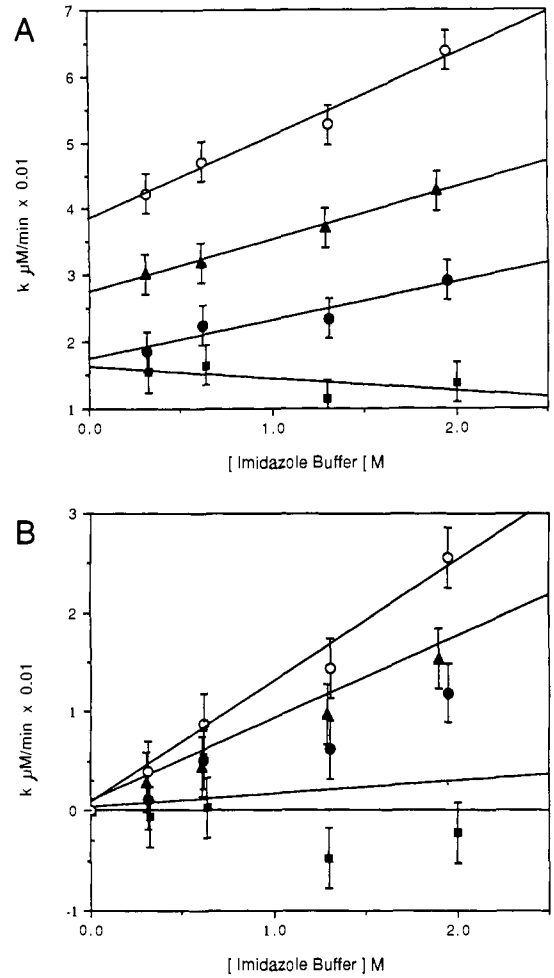


Figure 5. Plot of the observed pseudo-first-order rate constant for (3′,5′)-UpU isomerization as a function of imidazole buffer concentration at four different states of protonation of the buffer: (■) $ImH^+/Im = 0.0$, (●) $ImH^+/Im = 0.2$, (▲) $ImH^+/Im = 0.8$, and (○) $ImH^+/Im = 1.0$. Part A displays the experimental data. Part B shows only the buffer-dependent data. The lines drawn in part A are computer-generated least-squares fits. The lines drawn in part B were calculated by our kinetic treatment. The kinetic treatment for the 0.2 M imidazolium data is low compared to the experimental data.

leaving group. It is well known that the pH vs rate curve for chymotrypsin rises to a plateau at high pH and does not drop to form a bell-shaped curve. It is usually stated that this failure to see a rate drop at high pH, when the intermediate imidazolium ion should be titrated away, is because the imidazolium ion is inaccessible or the rate of proton transfer is too fast. As the equation of Scheme II makes clear, *this kind of mechanism cannot show a pH dependence reflecting imidazolium ion concentration.* Thus the chymotrypsin pH curve is exactly as expected for its mechanism, and no special explanations are required.

Scheme I displays two different mechanisms which differ solely by the order in which the acid and base catalyze a sequential cleavage reaction. Along with each mechanism is given the kinetic expression. Either of these mechanisms can explain the results of Figures 1–4 and of our previous studies on poly(U). For example, the first step in mechanism 1 (Scheme I) requires a base, and the second step requires an acid. If starting with pure base we increase the acid concentration, the second step speeds up and accelerates the reaction, but the first step slows. Eventually the first step becomes rate limiting. At this point additional acid depletes the base further to slow the first step, and the overall reaction rate slows down. In mechanism 2 the acid acts first, then the base.

The kinetic expressions describing mechanisms 1 and 2 are identical except for the interchange of imidazole and imidazolium. Thus, there is a kinetic ambiguity associated with whether the

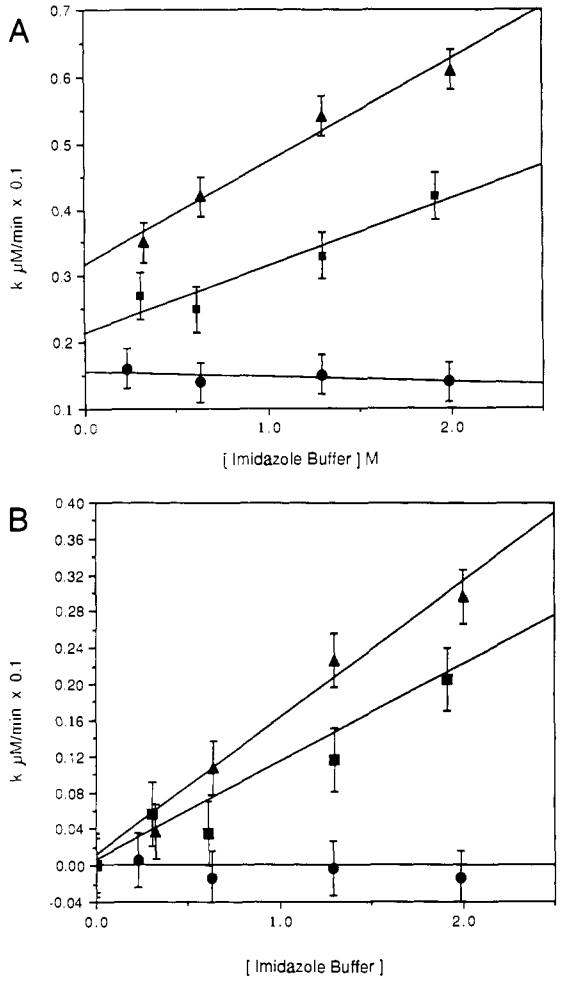


Figure 6. Plot of the observed pseudo-first-order rate constant for (2′,5′)-UpU isomerization as a function of imidazole buffer concentration at three different states of protonation of the buffer: (●) ImH^+/Im = 0.0, (■) ImH^+/Im = 0.6, and (▲) ImH^+/Im = 0.87. Part A displays the experimental data. Part B shows only the buffer-dependent data. The lines drawn in part A are computer-generated least-squares fits. The lines drawn in part B were calculated by our kinetic treatment.

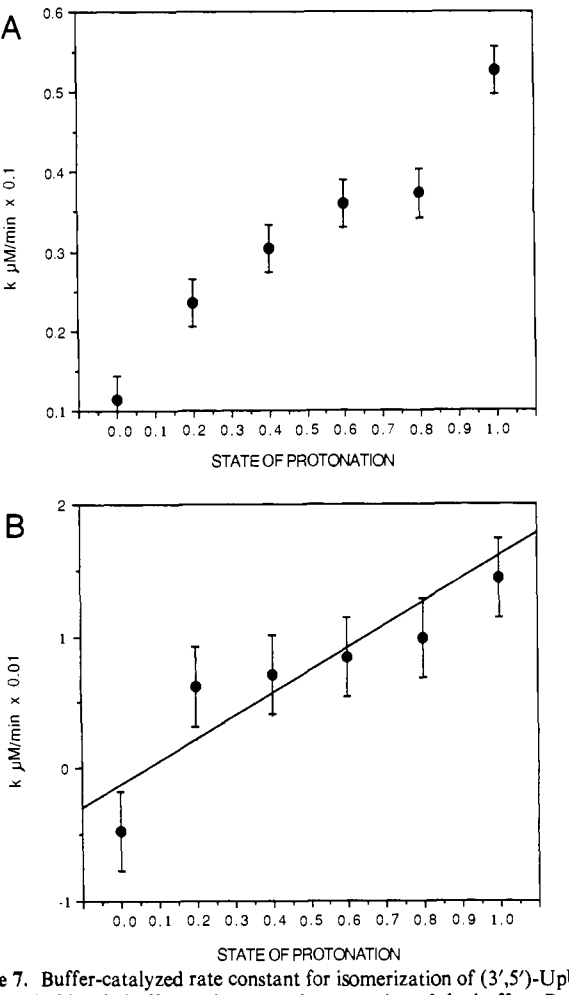


Figure 7. Buffer-catalyzed rate constant for isomerization of (3′,5′)-UpU at 1.3 M imidazole buffer vs the state of protonation of the buffer. Part A displays the experimental data. Part B shows only the buffer-dependent data. The line in part B was calculated by our kinetic treatment.

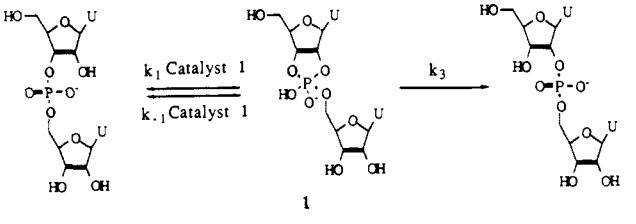
first or second steps are acid (or base) catalyzed, as in our previous work.[1]

It is commonly believed that phosphate substitution reactions proceed through five-coordinate phosphorus intermediates.[5] This study and our previous study provide kinetic evidence for this intermediate. The isomerization of (3′,5′)-UpU to (2′,5′)-UpU must also go through an intermediate. We assume that the isomerization reaction occurs at least initially through the *same* five-coordinate phosphorane monoanion intermediate as is involved in the cleavage reaction, with the same first step to form that intermediate. Two paths branch from the intermediate, one leading to cleavage and the other (with probable change in protonation state) to isomerization. The assumption that isomerization and cleavage proceed via the same intermediate is completely reasonable. In the same medium both reactions should follow the same lowest energy pathway from substrate to five-coordinate intermediate. The kinetics of the isomerization reaction thus allows us to solve the kinetic ambiguity in the cleavage mechanism.

Scheme III displays two different isomerization mechanisms and the corresponding kinetic expressions, which differ only in whether the first step is acid or base catalyzed. In mechanism 3, the base catalyzes the first step; this corresponds to cleavage mechanism 1. Conversely, in mechanism 4 the first step is acid catalyzed and thus corresponds to cleavage mechanism 2.

Intermediate **1** has the configuration shown in Scheme IV, with the 2′ O–P ester bond in an apical position and the 3′ O–P ester

Scheme III. Alternative Mechanisms for the Buffer-Catalyzed Isomerization of (3′,5′)-UpU to (2′,5′)-UpU

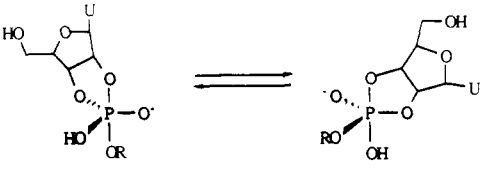


Mechanism 3
Catalyst 1= Imidazole

$$\text{Rate} = \frac{k_1 k_3 [\text{UpU}][\text{Cat. 1}]}{k_{-1}[\text{Cat. 1}] + k_3}$$

Mechanism 4
Catalyst 1= Imidazolium

Scheme IV. Pseudorotations Required for the Isomerization Reactions



1 2

R= Uridine

bond in an equatorial position.[5] In hydrolysis and substitution reactions of phosphate esters, the attacking group always occupies

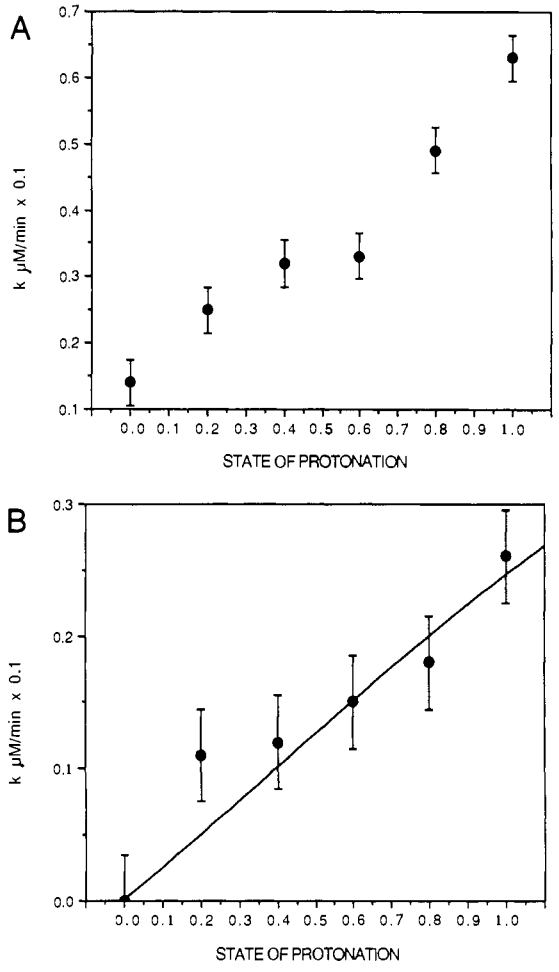


Figure 8. Buffer-catalyzed rate constant for isomerization of (2′,5′)-UpU at 1.3 M imidazole buffer vs the state of protonation of the buffer. Part A displays the experimental data. Part B shows only the buffer-dependent data. The line in part B was calculated by our kinetic treatment.

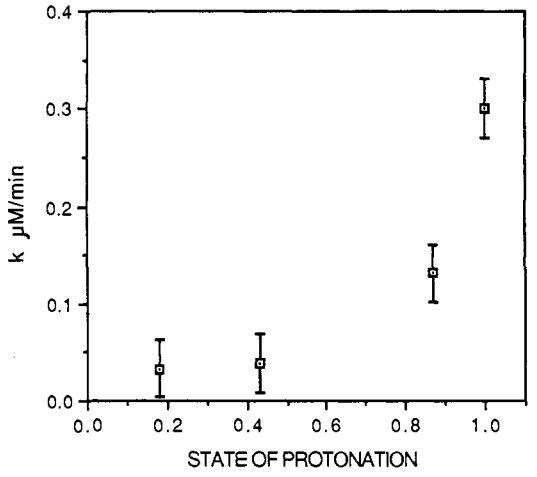


Figure 9. Buffer-catalyzed rate constant for isomerization of (2′,5′)-UpU at 1.3 M acetate buffer vs the state of protonation of the buffer.

an apical position.[5] The leaving group must also occupy an apical position. Therefore, in order to isomerize a 3′,5′-diester to a 2′,5′-diester via the initially formed cyclic phosphate **1**, a pseudorotation must occur to yield **2**, placing the 3′-oxygen in an apical position.[5,6]

The kinetic results show that with imidazole buffers the isomerization of (3′,5′)-UpU is always 5–10 times slower than cleavage. This is also true when (2′,5′)-UpU is the starting material. Qualitatively, in both cases cleavage is seen to be significantly faster than isomerization with imidazole catalysts (but not with acetic acid catalyst, vide infra), so it is clear that the second step of the cleavage sequence is faster than the pseudorotation with which it competes. Since the rate of the first step is comparable to that of the second cleavage step, faster or slower depending on which side of the bell curve is being examined, this first step is also fast compared with pseudorotation. This is supported by our detailed kinetic model (vide infra), which shows that with imidazole buffer catalysis the intermediate reverts to starting material, or cleaves to product, more rapidly than it pseudorotates.

Figures 5–8 demonstrate that isomerizations are catalyzed by imidazolium ion, not by imidazole, with no bell-shaped curve. As we increase the proportion of imidazolium, we find an increase in isomerization relative to cleavage (compare the rates in Figures 3 and 4 to those in Figures 7 and 8). *This proves that the second step of the cleavage reaction cannot use imidazolium ion as catalyst.* Cleavage must use imidazole as the catalyst for the second step, so that the proportion of isomerization vs cleavage can increase as the buffer goes from imidazole to imidazolium ion. The increase in rate of isomerization relative to cleavage would not occur if *both* the isomerization and the second step of cleavage were catalyzed by imidazolium ion. Instead, the proportion of cleavage and isomerization would remain constant. *Therefore, since isomerization increases relative to cleavage with increasing acid, the first step of the cleavage reaction uses imidazolium and the second one imidazole.* This is the reverse of the choice that had been suggested on the basis of stereoelectronic arguments.[4]

The interpretation of the catalysis of isomerization by imidazolium ion is not simple. At first one might think that this requires imidazolium ion to catalyze the "rate-determining" step; the slowest step is pseudorotation. However, in complex reaction sequences all kinetic effects do not necessarily involve a single rate-determining step. We believe that this is the situation here: the imidazolium ion catalyzes the first step, forming the intermediate, although the second pseudorotation step is slower (once pseudorotation has occurred, subsequent reactions are rapid, and their catalysis would not show up in the kinetics). This conclusion is supported by our detailed kinetic modeling (vide infra), showing that the experimental observations fit our interpretation.

In qualitative terms, the rate of isomerization depends on the rate constant for pseudorotation and on the steady-state concentration of intermediate **1**. This steady-state concentration is less than the equilibrium value because **1** is being bled off by cleavage, and to a lesser extent by isomerization. Increasing the concentration of imidazolium ion increases the rate of formation of **1**, while decreasing the concentration of imidazole decreases the rate of cleavage of **1**. In a more acidic buffer both effects increase the steady-state concentration of **1** and thus the rate of isomerization. Thus we expect that imidazolium ion will catalyze isomerization even if it plays a role only in the first, relatively rapid, step.

We also examined the possibility that imidazolium ion might catalyze the pseudorotation step. The transition state for pseudorotation, a square pyramid, would probably be more stable relative to the starting trigonal bipyramid if it were neutral rather than anionic.[7] Thus one might invoke imidazolium ion as a catalyst for the protonation of **1**, to yield **1′** and facilitate pseudorotation.

We believe that this can be excluded as an explanation of the kinetics. The estimated first and second pK_a's of **1′** are 9 and 13, respectively.[8] In all our media except the most basic, the intermediates will thus exist largely as **1′**. The neutral form **1′** is a reversible dead end in the cleavage reactions: without the negative charge it would have to cleave to a protonated phosphate

(6) Berry, R. S. *J. Chem. Phys.* **1960**, *32*, 933.

(7) The hybridization change on pseudorotating through a square-pyramidal transition state is such that a neutral five-coordinate phosphate should pseudorotate faster than an anionic phosphate. Support for this can be derived from Holmes, R. R. *J. Am. Chem. Soc.* **1978**, *100*, 433–446.

(8) Kluger, R.; Covitz, F.; Dennis, E.; Williams, D.; Westheimer, F. H. *J. Am. Chem. Soc.* **1969**, *91*, 6066–6072.

cation, a very high energy species. The conversion of **1** to **1′** will not cause the isomerization reaction to show observable general-acid catalysis. Proton transfers between heteroatoms are faster than processes involving heavy atoms, except for some rapid unimolecular reactions,[9] so **1** and **1′** will be in rapid equilibrium and buffer catalysis of their interconversion will not be kinetically detectable. However, as mentioned above we can account for our experimental observations without the need to invoke imidazolium ion catalysis of the pseudorotation process.

The acetate/acetic acid buffer catalysis can also be understood in these terms. Presumably intermediate **1** is formed in the same way, by protonation of the phosphate anion and then general-base-assisted attack by the 2′-OH group. At the low pH of this buffer the equilibrium will be greatly shifted toward **1′**, the neutral intermediate. The equilibrium between starting material and five-coordinate intermediates will be shifted to the right by this protonation, and cleavage will be greatly slowed since it requires proton loss from the intermediate. Now pseudorotation is actually the fastest process that the intermediates can undergo, so with this buffer at low pH the isomerization runs faster than the cleavage.

Modeling of the Kinetics. The kinetic expressions accompanying mechanisms 2 and 4 do not fully describe the behavior of the data in the figures. The cleavage expressions correctly accommodate the maximum rate when both imidazole and imidazolium are present but predict a rate of zero when one of them is missing. The termini in the bell curves of Figures 3 and 4 are quite significant in comparison to the maximum rates when both catalysts are present. In order to model the kinetic data we must include terms to account for the end points. The possibilities have been previously discussed:[1] there can be additional pathways catalyzed only by Im and only by ImH^+, most likely with the other catalytic functions performed by water, hydronium, or hydroxide.

The buffer-independent catalysis by water species is quite significant in both the cleavage and isomerization reactions, as comparison of the parts A and B of Figures 1 and 2 and Figures 5 and 6 shows. It is particularly significant for the isomerization reaction: the buffer-independent rate was comparable to the buffer-dependent rate, and under some conditions greater (Figures 5A and 6A).

The water catalysis adds more kinetic terms similar to eq 2 and allows for non-zero termini. In order to model the data for buffer catalysis, we have included $k'[Im]$ and $k''[ImH^+]$ terms. Even though we are considering only the buffer-catalyzed reactions, a water-catalyzed term k_w must be added to the denominator, expressing the fact that the decomposition of **1** back to starting material has a buffer-independent term in its rate. This finally leads to eq 1 for cleavage and eq 2 for isomerization. Optimization of the relative rate constants for the data presented lead to the model curves shown along with each figure. The rate constants are listed in Table I. It should be stressed that these rate constants were found to fit the data but are not the sole solutions. The fact that we were able to fit our proposed mechanisms to the data does, however, lend credence to our interpretation.

$$\text{rate of cleavage} = \frac{k_1k_2[\text{UpU}][\text{ImH}^+][\text{Im}]}{k_{-1}[\text{ImH}^+] + k_2[\text{Im}] + k_3 + k_w} + k'[\text{Im}] + k''[\text{ImH}^+] \quad (1)$$

$$\text{rate of isomerization} = \frac{k_1k_3[\text{UpU}][\text{ImH}^+]}{k_{-1}[\text{ImH}^+] + k_2[\text{Im}] + k_3 + k_w} \quad (2)$$

Implications of the Acid–Base Mechanism. In this study we suggest a mechanism (Scheme V) in which imidazolium reversibly converts UpU to **1** and imidazole catalyzes the cleavage of **1** to uridine and uridine 2′,3′-cyclic monophosphate. In the first step nucleophilic attack by the 2′-OH occurs with general-base assistance *after protonation of the phosphate.* The proton placed on the phosphate plays the role of an electrophilic catalyst, increasing the electrophilicity of the phosphorus and allowing for more facile attack by the 2′-OH. This step is thus catalyzed by a combination of proton and imidazole, which is of course kinetically equivalent to imidazolium cation. In the reversal of the formation of **1**, the same sequence runs backward with the imidazolium delivering a proton to the C-2 oxygen leaving group. As usual, a specific-acid/general-base mechanism in the forward direction implies the kinetically equivalent general-acid mechanism in the reverse. After ring opening the resulting phosphoric acid ester loses its proton to form the stable anion.

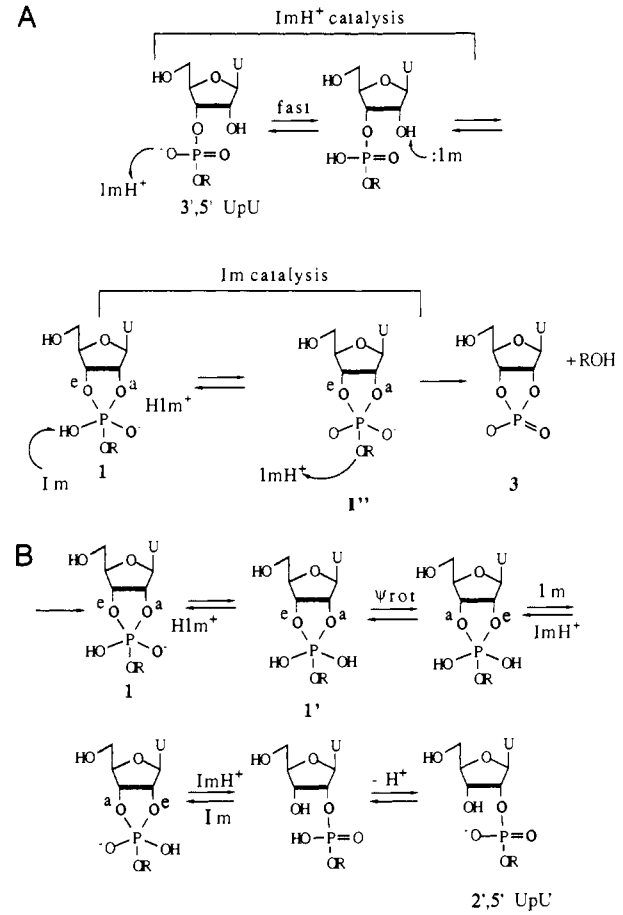
The second step in the cleavage is formally catalyzed by imidazole. Although this might mean simply that imidazole removes the proton from intermediate **1** while the 5′-oxygen is ejected as an anion, another kinetically equivalent mechanism seems more attractive. The departure of the 5′-oxygen in the forward cleavage of **1** can be assisted by imidazolium ion just as the departure of the 2′-oxygen is assisted in the reverse of the first reaction. However, for forward cleavage the intermediate **1** must be (reversibly) converted to the dianion **1″**, in an equilibrium whose position will depend on the concentration of OH^-. Thus the reaction will depend on the product of the concentrations

Table I. Calculated Relative Rate Constants for the Kinetic Model Presented in Equations 1 and 2

rate constant	(3′,5′)-UpU (μM/min)	(2′,5′)-UpU (μM/min)
k_1	1.30	1.54
k_2	4.80	0.65
k_3	0.39	0.40
k_{-1}[a]	1.00	1.00
k_w	15.0	6.0
k'	0.23	0.28
k''	0.18	0.14

[a] k_{-1} was arbitrarily set to 1.00.

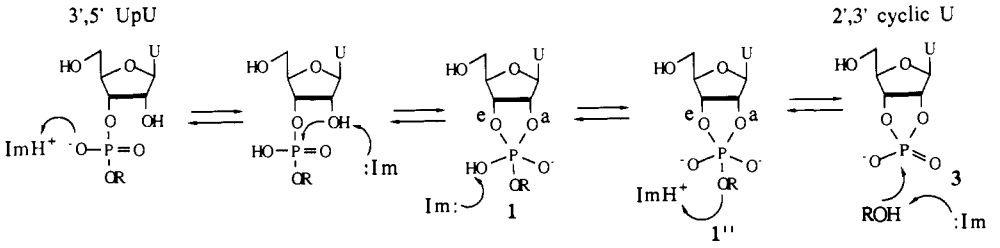
Scheme V. Our Preferred Mechanisms for the Imidazole-Catalyzed Reactions of UpU[a]



[a] Cleavage (A) goes through the pathway (3′,5′)-UpU to **1** to **1″** to **3**; isomerization (B) prefers (3′,5′)-UpU to **1** to **1′** to (2′,5′)-UpU.

(9) Cf.: Eigen, M. *Angew. Chem., Int. Ed. Engl.* **1964**, *3*, 1.

Scheme VI. Intermediate 1 Is Formed by Internal Attack on *Protonated* (3',5')-UpU but by External Attack on *Unprotonated* 3 Followed by Protonation



$[ImH^+][OH^-]$; this is of course kinetically equivalent to imidazole concentration. It must be emphasized that there is no experimental evidence that distinguishes between these two possible interpretations of "imidazole" catalysis.

In either case, we again face the question we raised[1] previously: Why is the cleavage of intermediate **1** catalyzed by imidazolium ion when the endocyclic 2'-oxygen is departing but by imidazole when the exocyclic 5'-oxygen is the leaving group? Taira[4] had suggested a stereoelectronic explanation for the reverse possibility, when it was not clear that the first catalyst was imidazolium ion, but his explanation is not readily adapted to the real situation. In fact, he used his explanation to argue for the [Im, then ImH^+] sequence that we have now excluded. We believe that the dilemma can be explained in energetic terms.

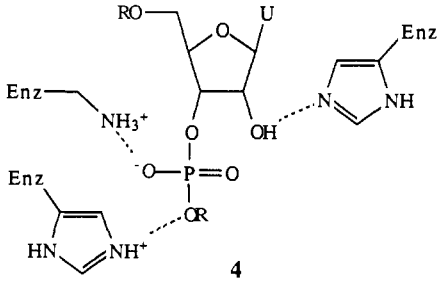
Conversion of **1** back to starting material can form the protonated phosphate diester, whose loss of a proton and formation of the more stable anion comes as a later step. However, cleavage in the forward direction forms the strained 2',3'-cyclic phosphate **3**. If the proton is removed first, the product can directly be the phosphate anion. The cyclic phosphate **3** and any transition state resembling it are strained,[5a] but more stable than they would be in this pH region if they had not yet lost the proton.

Another way to describe the situation is to consider the reverse of these two cleavages (Scheme VI), attack of the 2'-OH of UpU or of the 5'-OH of the product upon **3**, to form intermediate **1** again. The phosphate diester anion of UpU is so stable that attack by the 2'-OH group, assisted by imidazole general base, can occur only if the phosphate is activated by prior protonation. In contrast, the strained phosphate monoanion **3** is sufficiently reactive that it can be attacked by a 5'-OH/imidazole combination *without* requiring prior protonation, so the immediate product is dianion **1''** that picks up a proton later to form **1**.

We have also recently seen that the cyclization reaction of UpU can be catalyzed by imidazole with a Zn^{2+}.[10] The Zn^{2+} probably coordinates to the phosphate anion in a way similar to that we have invoked for the proton in our UpU and poly(U) series. Finally, we should point out that the reactions shown as "fast" in Scheme V involve reversible protonations. These are usually thought of as involving H^+ or OH^-, but with the buffer concentrations we are using there is no doubt that such proton transfers would be performed by imidazole or imidazolium cation. The actual catalysts used in fast reversible equilibria do not show up in the kinetics.

Relevance to the Enzymatic Process. As with our previous work, these results call attention to the need to consider a sequential mechanism for cleavage and hydrolysis of RNA by ribonuclease, not just the concerted mechanism often written. At first sight, however, we seem to have the reverse of the catalytic mechanism that might have been thought of for the enzyme. In a sequential mechanism, one might have expected the imidazole base to act in the first part of the mechanism, deprotonating the 2'-hydroxyl group as it adds to the phosphorus atom.[11] The resulting five-coordinate phosphorus intermediate would then lose the 5'-OH group of the departing nucleotide with general-acid assistance by imidazolium ion. Our kinetics indicate imidazolium ion in the first step and imidazole in the second for our model RNA cleavage reactions.

The difference is deceiving. As we suggest, an early step indeed involves an imidazole acting as a base on the 2'-OH group *after a proton is added to the phosphate anion.* A later step probably involves an imidazolium ion acting as a general acid to protonate the 5'-oxygen of the leaving group *after an imidazole removes the proton from the five-coordinate intermediate.* The enzyme might of course simply find a way to stabilize the anions without protonating them, possibly by hydrogen bonding to a lysine ammonium cation. If the proton were not added to the phosphate in the first step, it would not have to be removed later in the sequence. In place of protonating the phosphate in the first step, the enzyme might simply substitute structure **4**, with electrostatic and hydrogen-bonding stabilization of charge. However, we believe that the detailed mechanism we have discovered for the model system is also very likely for the enzyme ribonuclease itself (cf. Scheme VII).



Our key finding is that in the model system an imidazolium cation protonates the phosphate anion in the first step. Thus in Scheme VIIA we propose protonation of the substrate by the ImH^+ of His-119 in the enzyme. X-ray studies of bovine pancreatic ribonuclease and its complexes show that the ImH^+ of His-119 can be found in various locations (this flexibility is needed in our mechanism); in a complex of the enzyme with phosphate substrates it is located near the phosphate anion oxygen.[11,12] Titration studies also indicate that phosphate anion substrates bind to an imidazolium cation of the enzyme.[13] The lysine-41 cation is *not* ion paired with the phosphate, although it is found coordinated to a vanadate ion in a complex that resembles a phosphorane and is considered a transition-state analogue.[14] Thus we suggest that the original negative charge of the substrate is paired with His-119, and that Lys-41 swings in to stabilize the monoanionic phosphorane intermediate.

A proton transfer from His-119 ImH^+ to the substrate would generate **5**. When the Im of His-12 delivers the oxygen from C-2', forming the five-coordinate intermediate **6**, the anionic **6** could

(10) Breslow, R.; Huang, D.-L.; Anslyn, E. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1746–1750.

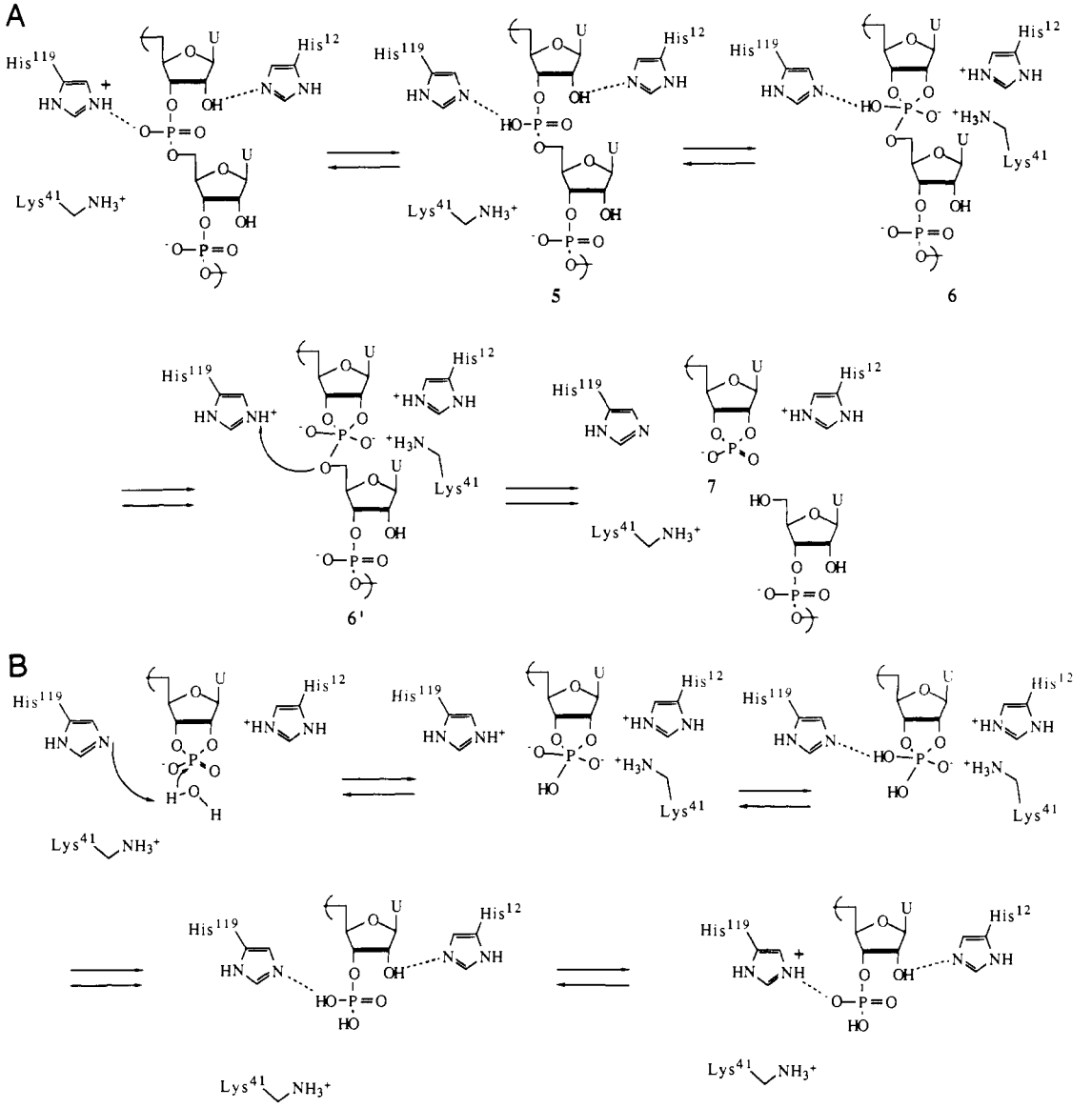
(11) (a) Blackburn, P.; Moore, S. *The Enzymes*; Academic Press, New York, 1982; Vol. 15, Chapter 12, pp 317–433. (b) Richards, F. M.; Wycoff, H. W. *The Enzymes*; Academic Press: New York, 1971; Vol. 4, Chapter 24, pp 647–806. (c) Deakyne, C. A., Allen, J. C. *J. Am. Chem. Soc.* **1979**, *101*, 3951.

(12) Campbell, C. L.; Petsko, G. A. *Biochemistry* **1987**, *26*, 8579–8584 and references cited therein.

(13) Reference 11b, pp 759–769.

(14) Alber, T.; Gilbert, W. A.; Ponzi, D. R.; Petsko, G. A. In *Mobility and Function in Proteins and Nucleic Acids*; R., Porter; M., O'Connor; J. Whelan, Eds.; Pitman: London, 1982; p 4. See also Wlodawer, A.; Miller, M.; Sjolin, L. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3628.

Scheme VII. A Mechanism for RNA Cleavage by Bovine Pancreatic Ribonuclease That Fits What Is Known about the Enzyme and Parallels the Mechanism of the Model System[a]



[a] His^{12}, His^{119}, and Lys^{41} are catalytic groups of the enzyme. Cyclization occurs by pathway A and hydrolysis of the cyclic phosphate by pathway B. As discussed, the first two steps of pathway A (and last two steps of pathway B) may proceed in concert.

be stabilized by Lys-41-NH_3^+ ion pairing. Removal of the proton again by His-119 would generate the dianion **6′**; this could fragment with protonation of the leaving group oxygen by the His-119 ImH^+ group. Adding a proton to the phosphate stabilizes the transition state for addition of the 2′-OH group, but the proton is lost again in order to stabilize the transition state for loss of the 5′-OH group. The flexibility of histidine on rotation about the CH_2–imidazole bond might well permit these last two proton transfers involving His-119 (from the phosphorane, and to the leaving group) to be performed by two different nitrogen atoms.[15]

Unfortunately, it is not possible to get the same kind of kinetic information on the enzymatic process as on the model systems we have examined. However, the enzyme is probably no more likely to attack the unstrained phosphate anion of the substrate, to produce a phosphorane dianion, than our model system was. Prior protonation of the phosphate anion would be catalytically useful. As in our model system, however, ring opening attack on the cyclic phosphate **7**, in hydrolysis or in alcoholysis that reverses its formation, will have less need for protonation of the phosphate anion because of the well established high reactivity of such strained phosphates.[5a]

The sequence we suggest for hydrolysis of the cyclic phosphate **7** (Scheme VIIB), based on the mechanism used by the model system, does indeed form the phosphorane dianion by His-119 imidazole assisted attack of a water molecule. This dianion is then protonated by the newly formed imidazolium ion before it extrudes the 2′-oxygen, an extrusion assisted by the imidazolium ion of His-12. Without such intermediate protonation the dianion **6′** will simply reverse the first step and revert to cyclic phosphate.

In the enzymatic reaction all the groups are held in place, so steps involving several catalytic groups might occur at once. For example, the proton transfer in the first step of Scheme VIIA that changes one hydrogen bond into another might occur in concert with the attack by the 2′-OH in the second step. If this merged [step 1/step 2] were rate determining, as it can be in our model system, it could show a two-proton inventory[16] in H_2O/D_2O mixtures. Matta and Vo have reported a two-proton inventory

(15) In the enzyme the equilibrium proton transfers can be directly ascribed to imidazole groups, while in the buffer-catalyzed reactions the protons can in principle be delivered and removed by other species. However, with the high concentrations of buffer used it seems likely that these transfers are performed by imidazole species even in the model system.

(16) Matta, M. S.; Vo, D. T. *J. Am. Chem. Soc.* **1986**, *108*, 5316.

in the hydrolysis of cytidine 2',3'-cyclic monophosphate catalyzed by ribonuclease A.[17] Although the proton inventory method[16] is ambiguous about the precise roles of the protons whose transfers are being detected, one interpretation is that the hydrolysis involves the mechanism of Scheme VIIB in which the last two steps are merged (corresponding to the first two steps of Scheme VIIA), so that two protons are moving.

It should be noted that by this mechanism, and indeed most others, *the enzyme oscillates between two states*. In its resting state His-12 is unprotonated, while His-119 is an imidazolium ion. After the steps of Scheme VIIA, which produce the cyclic phosphate product, the enzyme protonation states are reversed. The new base/acid pair is now ideally set up to perform the steps of Scheme VIIB, hydrolysis of the cyclic phosphate, that will return the enzyme to its original state. This may explain why the resting enzyme is not[18] an outstanding catalyst for the hydrolysis of added cyclic phosphate substrates: it has not been put into the correct state of protonation by the reactions of Scheme VIIA. Such oscillation in catalyst structure accompanying multistep reactions can be invoked for many other enzymes as well.

The mechanism of Scheme VII, possibly with steps 1 and 2 merged, is consistent with everything known about the enzyme so far. It also takes advantage of the catalytic effects we have seen in our model studies. Thus, at this time we consider Scheme VII to be the most likely enzyme mechanism; it directly parallels Scheme V for the model system. As with carboxypeptidase A,[19] a model system has given us additional insight into the probable details of the enzymatic process.

Acknowledgment. We thank the National Institutes of Health for support of this work and the National Science Foundation for a postdoctoral fellowship for E.A.

Registry No. (3',5')-UpU, 2415-43-2; (2',5')-UpU, 13493-13-5; uridine, 58-96-8; 2',3'-cyclic uridine monophosphate, 606-02-0; imidazole, 288-32-4; imidazole HCl, 1467-16-9; ribonuclease, 9001-99-4.

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Synthesis and Photochemical Deoligomerizations of a Series of Isomeric Disilyliron Complexes: $(\eta^5\text{-}C_5H_5)Fe(CO)_2Si_2Ph_{3-n}Me_{2+n}$[1]

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Abstract: A series of six disilyl complexes of the general type $(\eta^5\text{-}C_5H_5)Fe(CO)_2Si_2Ph_{3-n}Me_{2+n}$, representing three isomeric pairs, have been synthesized and characterized. Photolysis of each complex leads to the formation of a series of monosilyl complexes $(\eta^5\text{-}C_5H_5)Fe(CO)_2SiPh_{3-n}Me_n$ via a silylene expulsion process. The product distribution from photolysis of the isomeric pairs validates a mechanism involving intermediacy of dynamically equilibrating silyl(silylene) complexes.

The interactions of transition-metal complexes with silanes has long been an area of interest involving both stoichiometric and catalytic transformations of the silicon compounds. Many silyl transition-metal complexes are known including σ- and π-bonded organosilyl and silyl groups.[2-4] The particular interaction of oligosilanes with metal complexes has been longstanding,[5-10] with a particular recent resurgance of interest due to the potential of polysilanes as preceramic, photoresist, and photoconducting materials.[11-13]

Previous studies in our laboratories showed that oligosilane derivatives of the transition metals are readily synthesized from the transition-metal systems $[(\eta^5\text{-}C_5H_5)Fe(CO)_2]^-$ (Fp),[5,8,14] $[(\eta^5\text{-}C_5H_5)Ru(CO)_2]^-$ (Rp), and $[Re(CO)_5]^-$.[10] Malisch et al. have reported the related $[(\eta^5\text{-}C_5H_5)Mo(CO)_3]^-$ systems,[6] and Nicholson et al. have reported $[Mn(CO)_5]^-$ complexes.[7] The results of these studies indicate that the oligosilane complexes may be thermally and oxidatively stable (Fe, Ru, Re) or unstable (Mo).

Of particular interest are the results of photochemical treatment of the Fp complexes where facile deoligomerizations yield, inter alia, monosilyl complexes (eq 1, 2).[8]

$$FpSiMe_2SiMe_3 \xrightarrow{h\nu} FpSiMe_3 + \text{polymer} \quad (1)$$

$$FpSiMe_2SiPh_3 \xrightarrow{h\nu} \underset{85\%}{FpSiPh_2Me} + \underset{8\%}{FpSiMe_2Ph} + \underset{7\%}{FpSiPh_3} + \text{polymer} \quad (2)$$

These studies suggested a mechanism involving initial photochemical expulsion of CO to produce a 16e iron complex followed

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