Further Studies on the Buffer-Catalyzed Cleavage and Isomerization of Uridyluridine. Medium and Ionic Strength Effects on Catalysis by Morpholine, Imidazole, and Acetate Buffers Help Clarify the Mechanisms Involved and Their Relationship to the Mechanism Used by the Enzyme Ribonuclease and by a Ribonuclease Mimic

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Abstract: The cleavage and isomerization of 3′,5′-uridyluridine catalyzed by morpholine buffers and by imidazole buffers has been reinvestigated. The key evidence for a previously proposed partitioning mechanism—in which the buffer acid BH^+ converts the substrate to a phosphorane monoanion intermediate which then partitions either to the 2′,5′ isomer of the substrate or (with buffer base catalysis) to the cleavage product—is confirmed. The negative catalytic effect of the buffer base on the isomerization reaction is not due to a medium effect. Indeed the medium effect on this reaction is in the opposite direction, strengthening the catalytic evidence. However, this branching mechanism with sequential bifunctional catalysis of the cleavage reaction is accompanied by an additional cleavage path using the buffer base only. This additional path, for which several alternative mechanisms are possible, is required by the results of improved studies on the imidazole catalysis. These show that the previously reported decrease in rate at a low BH^+/B ratio is due to ionic strength effects. The relative importance of these two pathways—one with kinetic dependence on the buffer acid and one without such dependence—depends on the buffer basicity/acidity. With the acidic buffer acetate/acetic acid, a buffer-acid-catalyzed mechanism for the cleavage and the isomerization is dominant. A bifunctional mechanism, in which one step involves simultaneous acid−base catalysis, seems most likely. The medium effects of added dioxane on all these reactions are sensible in terms of the detailed mechanisms proposed. The relationship of these results to the mechanisms of catalysis by ribonuclease and by an enzyme mimic is discussed.

Introduction

We have been studying the cyclization/cleavage of ribonucleic acids catalyzed by buffers. We wanted to determine the preferred mechanism used by normal nucleotides, not those more reactive analogs with such good leaving groups as *p*-nitrophenoxide ion. Such reactive leaving groups make the kinetic analyses easier, but they can certainly change the mechanism used. With the less reactive normal nucleotides, rather concentrated buffers are needed to achieve reasonable rates.

Our earliest work involved imidazole buffers, imitating the imidazole groups that are catalytic in the enzyme ribonuclease A. In this work we examined polyuridylic acid,[1,2] 3′,5′-uridyluridine (3′,5′-UpU, **1**),[3−8] and adenosyladenosine (ApA).[9] We found that imidazole buffers catalyzed the cyclization/cleavage of all these substrates (Figure 1). We also saw that these buffers catalyzed the isomerization of 3′,5′-UpU to 2′,5′-UpU (**2**), and vice versa, and similarly in the ApA series.

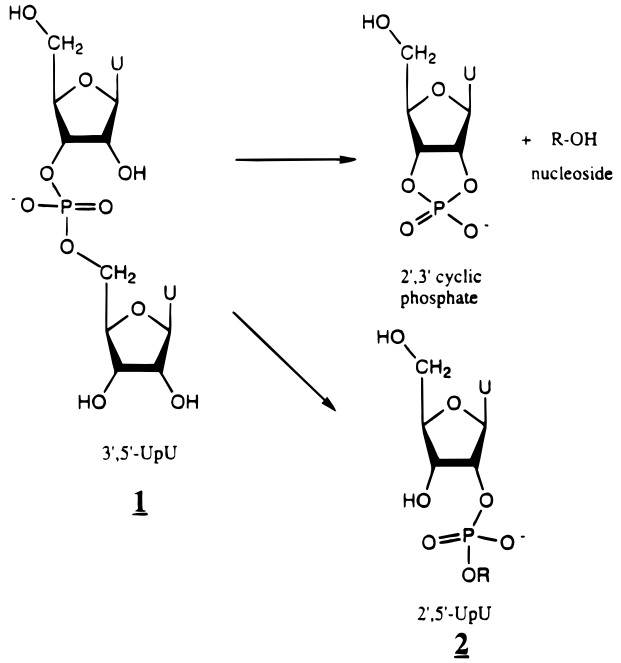

Figure 1. Cyclization/cleavage of a dinucleotide accompanied by isomerization.

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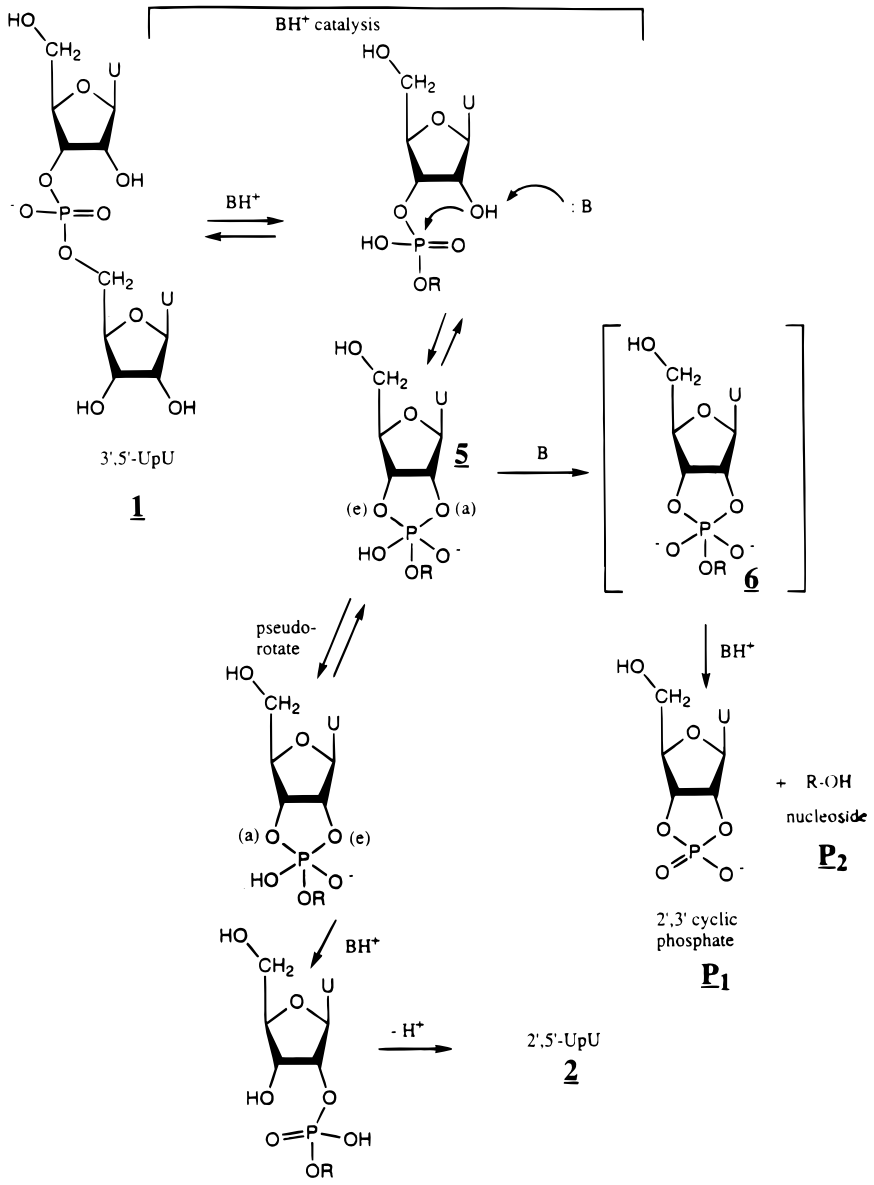


Figure 2. Our proposed mechanism for the sequential acid−base catalyzed cleavage of **1** by imidazole or morpholine buffers, with branching from the phosphorane intermediate **5** that leads to isomerization. This simplified scheme omits contributions that are made by nonbuffer species such as H^+ and OH^-, but they are included in Figure 3 and in previous treatments.[11,12] Also, as discussed previously, the pseudorotation of **5** may well involve its prior protonation to a neutral phosphorane. Note that the reversible protonation, followed by B catalysis, is just the specific acid/general base version of BH^+ catalysis. Its overall rate depends on $[BH^+]$, but not on $[H^+]$.

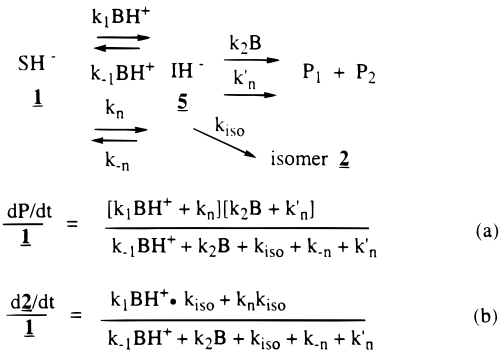
Our early work was performed without control of ionic strength. We saw apparent bell-shaped pH vs rate profiles for the cleavage process, indicating that both imidazole (Im) and imidazolium ion (ImH^+) were involved in the catalysis, and in a sequence with a phosphorane intermediate. Such a pH vs rate profile does not indicate which is the first catalyst and which the second in such a sequence. Evidence on this was obtained from a study of the isomerization reactions. They showed catalysis by ImH^+, and not by Im. In fact, with a constant concentration of ImH^+ the rate of isomerization actually *decreased* as Im was added.[7,9]

Such slowing of the rate by a catalyst, which we referred to as *negative catalysis*,[10] is consistent with a sequential process in which an intermediate branches: one branch leads to the isomer, while the other leads to cleavage with Im catalysis (Figure 2). Thus increasing [Im] increases the fraction of the intermediate that partitions along the cleavage path, leading to a decrease in the observed rate of isomerization.

In such a study the pH changes as buffer ratios change, and we corrected for this by subtracting the rates at lower buffer concentrations with the same pH's.[2,4,9] However, there were ambiguities in this study as well. (1) Ionic strength was not controlled, and it could be important. (2) The correction for the changing pH may not have been successfully made simply by the subtraction we did. In addition, (3) we saw that the isomerization reaction slowed on addition of Im; because of concern about medium effects, described further below, it was important to show that this slowing was due to *catalysis* of one leg of a partitioning mechanism, as in Figure 2, not simply because added Im made the medium less polar.

To address the first two points, we studied the cleavage and isomerization of 3′,5′-UpU by morpholine buffers.[11,12] Morpholine is more basic than is imidazole, so the negative catalytic

(10) Much ado has been made about our use of the phrase "negative catalysis". This term was first used by L. P. Hammett (*Physical Organic Chemistry*, 1st ed.; McGraw-Hill Book Company: New York, 1940; p 398). It simply means that a species that is functioning as a catalyst (i.e., it is not changed at the end of the reaction, and it is not simply an inhibitor of another catalyst) is observed to slow a reaction. Of course it does it by speeding another reaction in a branching process. I do not retract the use of this perfectly apt phrase by us or by Hammett.



$$\frac{dP/dt}{\underline{1}} = \frac{[k_1BH^+ + k_n][k_2B + k'_n]}{k_{-1}BH^+ + k_2B + k_{iso} + k_{-n} + k'_n} \quad (a)$$

$$\frac{d\underline{2}/dt}{\underline{1}} = \frac{k_1BH^+ \cdot k_{iso} + k_nk_{iso}}{k_{-1}BH^+ + k_2B + k_{iso} + k_{-n} + k'_n} \quad (b)$$

Figure 3. A condensed and simplified version of Figure 2 by which the substrate **1** of Figure 2 is reversibly converted to the phosphorane monoanion **5** with catalysis by the buffer acid BH^+ or nonbuffer species (H^+, OH^-, H_2O), and **5** is then converted to isomer **2** or, with catalysis by B or nonbuffer species, to cleavage products P_1 and P_2. The steady-state (in **5**) equations for cleavage (a) and for isomerization (b) are shown. The constants k_n, k_{-n}, and k'_n include concentrations of such nonbuffer species as OH^- and H^+, so they are functions of pH. The isomerization reaction shows *negative* catalysis (cf. ref 10) by B, which appears only in the denominator of eq b.

effect of the buffer base should be accentuated. Furthermore, we kept the buffer ratio constant and controlled ionic strength, while simply increasing the concentration of a given buffer. We saw that the pH was constant under these conditions. The cleavage rate simply increased with increasing buffer concentration at various ratios, but the isomerization rate showed *negative catalysis* again. That is, increasing the concentration of morpholine buffer led to a decrease in the isomerization rate with a 9/1 B/BH^+ buffer at constant pH and ionic strength, and also at 95/5 B/BH^+, but the decrease was smaller with an 8/2 buffer. This showed that it was the B component of the buffer that slowed the reaction (or a kinetically equivalent OH^-/BH^+-combination), as our partitioning mechanism would predict. The essence of the argument is presented in Figure 3. The detailed curves (e.g., Figure 4) could be fitted to the equations that such a mechanism requires,[11,12] but some questions remained.

First of all, the equations predicted that the initial decrease in rate of isomerization with increasing concentration of morpholine buffer would be followed by a plateau, as was observed (Figure 4). However, at higher buffer concentration we saw an increase in rate. This increase requires either an additional mechanism at higher buffer concentration or a medium effect[13] as high buffer concentration makes the solvent less polar.[12] The other question was whether a medium effect could explain even the initial decrease in rate, point 3 above.

Recently Kirby and Marriott have reported a study of medium effects on the cyclization/cleavage of compound **3**.[14] As they indicated, **3** is hundreds of times more reactive than is UpU, because of the better leaving group, and such increased reactivity could well change the mechanism. We[15] and others[16] have studied the imidazole buffer catalyzed cyclization/cleavage of the even more reactive compound **4** and see that only the Im plays a catalytic role. Thus whatever medium effects there are for substrate **3** might not be relevant to processes involving

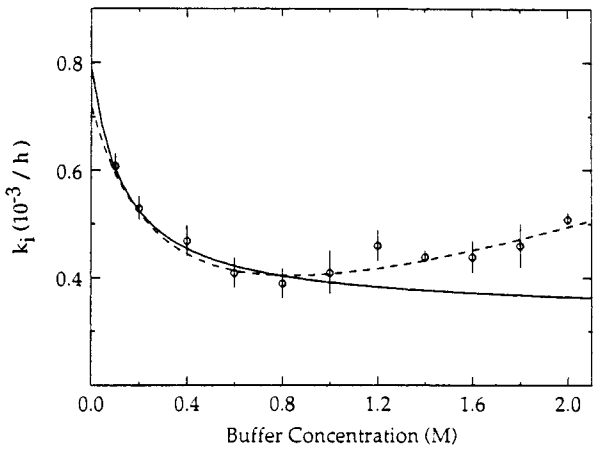
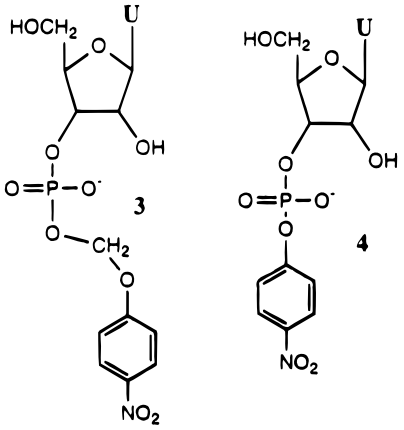


Figure 4. Pseudo-first-order rate constants as a function of buffer concentration for the isomerization of 3′,5′-UpU (**1**) to 2′,5′-UpU (**2**), from ref 12. All runs were at 80 °C, performed at least in duplicate with 9/1 morpholine/morpholine hydrochloride, with the buffer concentration being varied and the ionic strength being maintained constant at 0.2 M with NaCl. As described previously (ref 12), the measured pH is constant under these conditions. The solid line is that predicted (ref 12) from the kinetic treatment of the (expanded) mechanism of Figure 2. The dashed line shows an increase at high buffer concentrations that we ascribe to a medium effect (see Results and Discussion).

normal nucleotides such as UpU. However, the point certainly needed to be addressed.



With improved instrumentation the otherwise tedious kinetic studies with normal nucleotides such as UpU are more readily done. We have now performed new studies of ionic strength and medium effects on the imidazole and morpholine catalyses of cleavage and isomerization of 3′,5′-UpU. We have also examined the cleavage and isomerization of 3′,5′-UpU by acetate buffer. From these studies have emerged two conclusions: (1) the originally proposed BH^+/B sequence for cleavage of UpU by imidazole buffers (Figure 2) seems to play a role in these reactions. However, (2) this mechanism is accompanied by a cleavage process that has kinetic dependence only on B.

Some of the original evidence for our earlier mechanistic conclusions does not stand up to a careful re-examination with attention to the medium, but other evidence is actually strengthened when all the factors are fully controlled. The bifunctional mechanism is consistent with our findings with a synthetic ribonuclease artificial enzyme,[7,17−19] and with our proposals for the enzyme itself.[7] The two parallel processes are also related to theoretical treatments done by others.

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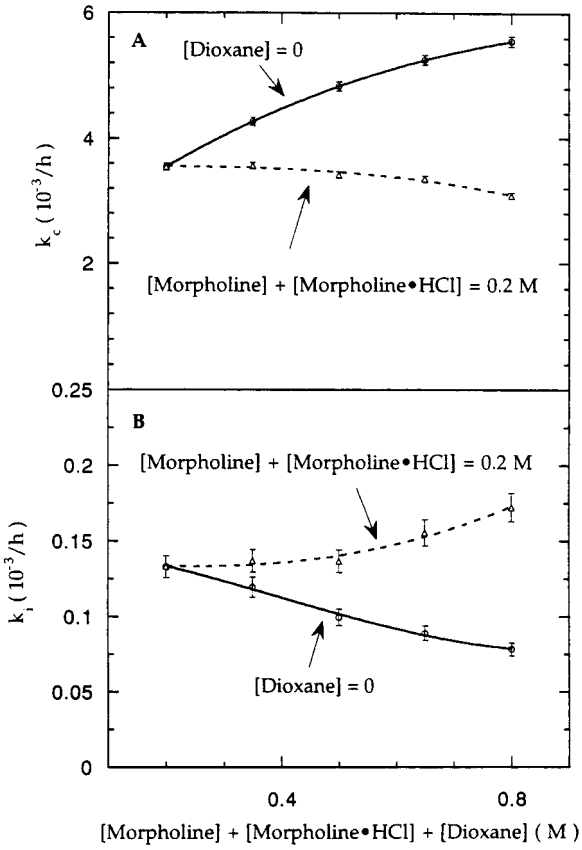


Figure 5. Pseudo-first-order rate constant for (A) the cleavage of 3′,5′-UpU and (B) the isomerization of 3′,5′-UpU to 2′,5′-UpU, both vs the buffer concentration at 60 °C: (○—) 9/1 morpholine/morpholine hydrochloride; (△- - -) 0.2 M morpholine buffer plus an amount of dioxane that brings the total concentration to the amount shown. The ionic strength was maintained at 0.2 M for all runs. The medium effect decreases the rate of the cleavage reaction, but it *increases* the rate of isomerization, opposite to the effect of the buffer.

Results and Discussion

Medium Effects on Morpholine Catalysis. With morpholine buffer we had already operated at constant ionic strength and constant pH.[11,12] Here the major question was the effect of the change in *medium* as buffer concentration increases. The increase in $[BH^+]$ is compensated by adjusting the ionic strength with NaCl, but the increase in [B] makes the medium less polar. With our new instrumentation that permits HPLC kinetic studies with automatic sampling, we have repeated the study of the cleavage and the isomerization of 3′,5′-UpU catalyzed by 9/1 morpholine/morpholinium ion buffer at constant ionic strength, as a function of total buffer concentration. For instrumental reasons the study was performed at 60 °C, not the 80 °C of the previous work. We saw that as before the cleavage rate increased as the buffer concentration increased (Figure 5A), and again we saw that the isomerization rate *decreased* with increasing buffer concentration (Figure 5B).

Kirby and Marriott added dioxane or pyrrole as mimics of imidazole that change the medium, but not the catalyst concentration.[14] Thus we examined the effect of added dioxane on the rate of cyclization/cleavage and the rate of isomerization of 3′,5′-UpU. The rate constants are listed in the supporting information and plotted in the various figures. The addition of dioxane to a morpholine buffer system slightly decreases the rate of the cleavage reaction (Figure 5A). However, Figure 5B reconfirms the negative rate effect of a basic morpholine buffer on isomerization and shows that the addition of dioxane leads to an *increase* in the rate of isomerization.

This shows that the negative rate effect of morpholine on the isomerization reaction is a true catalytic effect, and indeed our raw data underestimated the effect. If we correct for the medium effect, as indicated by the dioxane results, then the negative *catalytic* effect of morpholine is even larger. This is support for our proposal that the isomerization and the cleavage reaction branch from a common intermediate, with the cleavage branch being catalyzed by B. It does not rule out a kinetically equivalent alternative pathway for the cleavage reaction, as will be discussed below.

In our earlier work we had seen that the rate of isomerization showed a complex behavior as a function of buffer concentration.[12] As Figure 4 shows, the rate first decreases, then levels, then rises again at higher buffer concentration. The decrease and then leveling are as expected from our mechanism, from the equations that describe our mechanism (cf. Figure 3). However, the rise in rate at buffer concentrations higher than 1.0 M required an additional explanation. We pointed out[12] that this could be either a medium effect or the addition of further catalytic terms at high buffer concentrations, but with our new evidence we prefer the explanation[14] in terms of a medium effect.

As we show in Figure 5B, the addition of dioxane as a mimic of the medium effect of morpholine leads to a significant rate increase. After the rate drops to a plateau at 1.0 M in Figure 4, in accord with the kinetic equations, the medium effect keeps building with more buffer. This then must lead to an increase in rate after the plateau, as we observe.

These medium effects are sensible. In the isomerization reaction a substrate monoanion and a catalyst monocation first neutralize each other by reversible proton transfer, and then the phosphorane monoanion is formed in a step that restores charge separation. However, if the transition state occurs during that second step, the proton transfer will not yet be complete, so the net result is a decrease in charge on going from the original reactants to the transition state. This should lead to an *increase* in the rate when the medium is made less polar, as we observe. If isomerization involves protonation of **5** to form a neutral phosphorane, this will also lead to an increased rate with a less polar medium. Thus the medium effect supports both our evidence for this aspect of the mechanism and our particular interpretation of that evidence.

The medium effect on the cleavage reaction requires further comment. Even if the only pathway for cleavage were that shown in Figure 2, one would still expect the observed *decrease* in cleavage rate as the medium is made less polar. In the cleavage branch we invoke the conversion of a B of the buffer to a BH^+, and the phosphorane intermediate to a *dianion*. This increase in polarity could explain the slowing with a less polar medium.

However, evidence discussed later indicates that there is an additional pathway for the cleavage reaction, one that has kinetic dependence only on the buffer base B. With a strongly basic buffer like morpholine, this is the major cleavage path (with acetate buffer, described later, the buffer *acid* dominates the catalysis). As we will describe, this B-catalyzed cleavage process (one possible mechanism is shown in Figure 6; others are discussed later) also has a transition state more polar than the starting materials, and it should also be slowed by the addition of dioxane. Since this second path is the major one with basic buffers, the medium effect on cleavage reflects rate changes in this second path.

Re-Examining Imidazole Catalysis. In the studies with imidazole buffer catalysis, the original work did not involve control of ionic strength, nor did it examine the possible medium

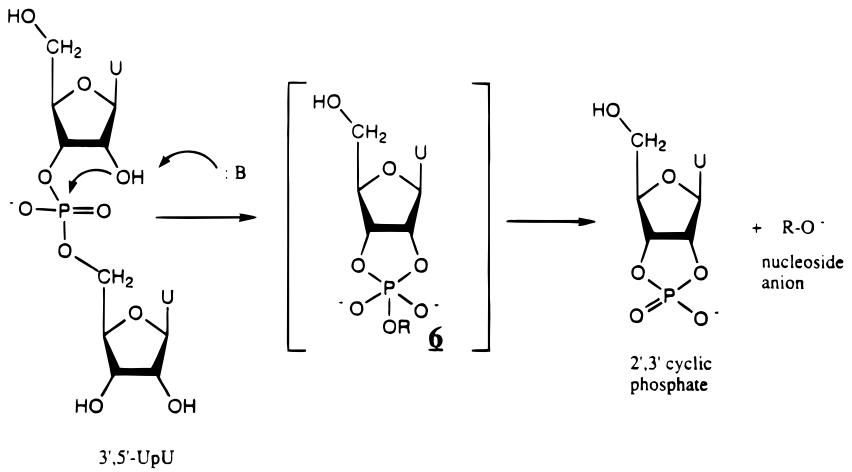


Figure 6. A cleavage mechanism with simple catalysis by the buffer base B. The intermediate dianion **6** is not a stable species, and it may not be fully formed before departure of the leaving group.

effects of nonpolar solvents that mimic imidazole. We have now addressed these deficiencies. We find that some of the original evidence for our sequential mechanism does not stand up to this more careful examination, but that other evidence for our mechanism is actually strengthened when these effects are taken into account. The sequential mechanism of Figure 2 still seems to be correct. However, there is also a pathway catalyzed by Im alone.

First of all, our new studies confirm that the cleavage process is catalyzed both by Im and by ImH^+. The rate constants are listed in the supporting information and plotted in the figures. Figure 7A shows the cleavage rate when $[ImH^+]$ is held at 0.5 M and [Im] is varied. We also show the data in Figure 7A with buffer concentration at 10% of the above values. In all cases ionic strength was held constant at 1.0 M with NaCl, so the pH's are the same for the two buffers at any given buffer ratio. The major catalyst is the buffer Im, not for example OH^-. Assuming that both lines correspond to the simplistic equation 1, the nonbuffer catalysis of the upper line contributes 6% at the low [Im], increasing to 7% at the high [Im] where $[OH^-]$ is higher.

$$k_c = k_{Im}[Im] + k_{nonbuffer} \quad (1)$$

As Figure 8A shows, holding [Im] constant at 0.5 M while increasing $[ImH^+]$ increases the cleavage rate. We do not have the data for this case at 10% of the buffer concentration, but from the low buffer data of Figure 7A the situation is clear. At a 1/1 ratio of [Im] to $[ImH^+]$, the rate at 10% of the high buffer concentration, the same concentration as in Figure 8A, was only 15% of the high buffer rate. Furthermore, the nonbuffer contribution in Figure 7A was only a few percent of the high buffer values and *increased* as the pH was increased. Thus in Figure 8A the increasing rate as $[ImH^+]$ is increased is the result of catalysis by ImH^+. A pH effect would be negligible and would go in the opposite direction.

From these data, it is clear that cleavage is catalyzed both by Im and by ImH^+. From the slopes of the lines in Figures 7A and 8A, Im catalysis is ca. 1.5 times as effective as is that of ImH^+ at equal concentrations, a relationship that will be seen again in the discussion of Figure 9. (Morpholine buffer has a stronger base, and a weaker acid, so the preference for base catalysis is even larger.)

We also examined the result of increasing the concentration of nonpolar solvent by adding dioxane to the medium. In contrast to the results with morpholine catalysis, the cleavage rate showed at most a *slight* decrease when dioxane was added. This medium effect may well explain the slight downward curvature seen in Figure 7A as Im is added to the medium. Its

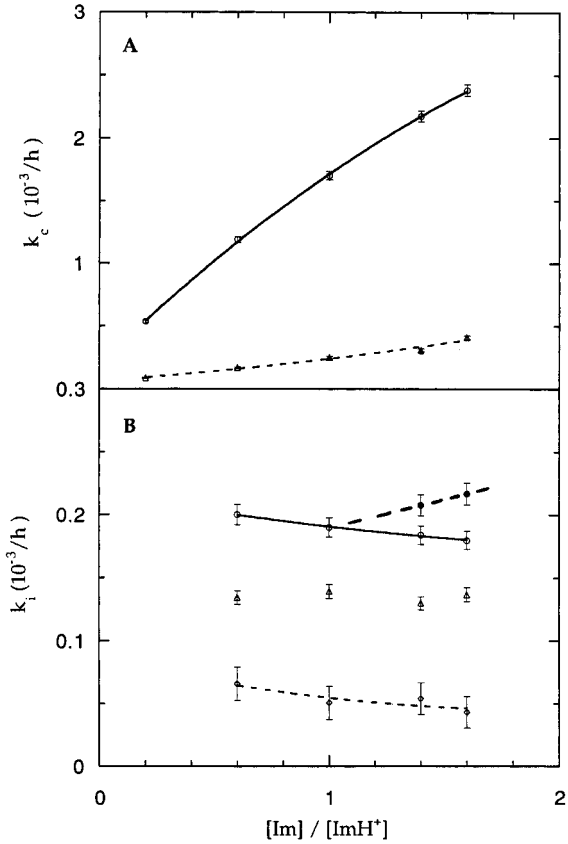


Figure 7. Observed pseudo-first-order rate constant for (A) the cleavage of **1**, followed by observing the formation of uridine, and (B) the isomerization of **1**, followed by observing the formation of **2**, at 60 °C and ionic strength 1.0 M (adjusted with NaCl) with $[ImH^+]$ held constant and [Im] varying. The pH varies (see Results and Discussion) as the buffer ratio changes: (○—) $[ImH^+]$ = 0.5 M; (△) $[ImH^+]$ = 0.05 M; (◇- - -) (in panel B) the difference between the 0.5 M and the 0.05 M data. Also shown in panel B (●— —) are two values when [Im] and $[ImH^+]$ were held at 0.5 M, and 0.2 and 0.3 M dioxane was added. This shows that the medium effect on this reaction goes in the opposite direction to the effect of [Im]. The lines are best fits, not theoretical.

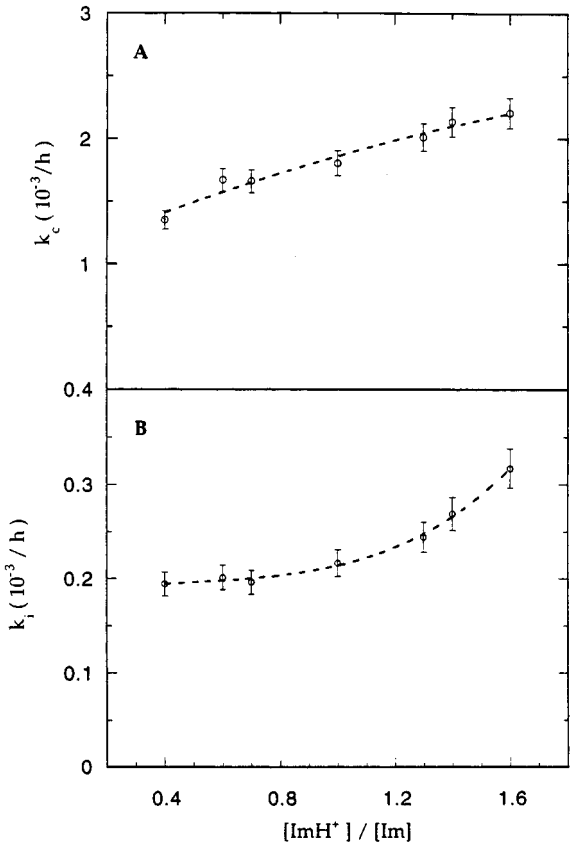


Figure 8. Observed pseudo-first-order rate constant for (A) the cleavage of **1**, followed by observing the formation of uridine, and (B) the isomerization of **1**, followed by observing the formation of **2**, at 60 °C and ionic strength 1.0 M (adjusted with NaCl) with [Im] = 0.5 M, as a function of [ImH$^+$]. The pH varies (see Results and Discussion) as the buffer ratio changes. The lines are best fits, not theoretical.

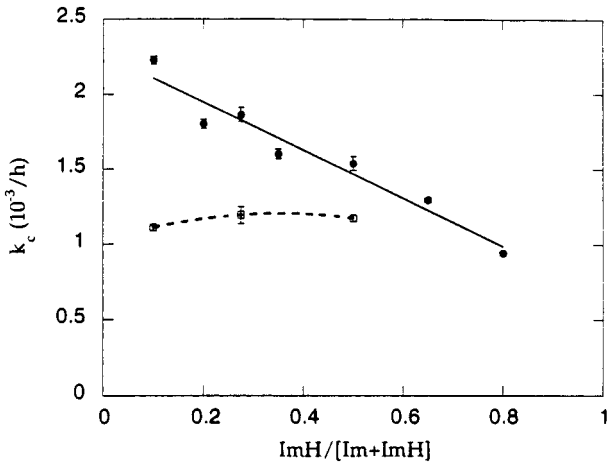


Figure 9. Observed pseudo-first-order rate constants for the cleavage of 3′,5′-UpU vs the state of protonation (BH^+/[BH^+ + B]) of 0.8 M imidazole buffer at 60 °C. The pH varies (see Results and Discussion) as the buffer ratio changes. The reaction was examined under conditions of both (□- - -) uncontrolled ionic strength and (●—) ionic strength controlled at 1.0 M with NaCl. The lines drawn are to emphasize the trends, not to fit a particular kinetic model.

catalytic effect is diminished at high concentrations by the medium effect.

Figures 7B and 8B show the same studies on the isomerization rate. These new data with control of ionic strength confirm our earlier conclusion that the isomerization reaction is catalyzed by ImH$^+$ (Figure 8B). The k_i shows a modest 50% increase over the range from 0.2 to 0.8 M [ImH$^+$] at constant ionic strength, but only a modest effect is expected. In the scheme of Figure 2 one expects *no* observable catalysis by ImH$^+$ if the first step is rapid and reversible compared with the subsequent step that leads to isomerization. That is, both the forward and reverse steps from **1** to **5** are catalyzed by BH^+, so one can see BH^+ in the kinetics only if that first step is partially rate determining. Since with imidazole buffers the cleavage is ca. 10 times as fast as the isomerization, the catalysis of the first step by ImH$^+$ makes a larger contribution to the overall *cleavage* rate than to the isomerization rate.

The data on isomerization, in Figure 7B, show a small (10%) *decrease* in rate as [Im] is increased at the high buffer concentration, and essentially no rate change at a buffer concentration 10-fold lower. This is what we first referred to as a negative catalytic effect, the slowing of isomerization by added Im.[9,10] We now see that this slowing is not a medium effect but is indeed evidence for a sequential mechanism with partitioning of an intermediate, as in Figures 2 and 3.

The critical data are shown in Figure 7B. Addition of dioxane causes an *increase* in the isomerization rate. As we discussed above with respect to the same findings in the morpholine case, this is quite reasonable for a reaction whose transition state is *less* polar than are the starting materials. As in the morpholine case, this shows that the *decrease* in rate actually caused by the basic component of the buffer is not a medium effect, but is the result of a partitioning mechanism in which the cleavage leg is catalyzed by B. As with the morpholine case, it is clear that the negative catalytic effect is larger than the raw data of Figure 7B show, compensated to some extent by a medium effect that works in the opposite direction. Thus if we corrected for the medium effect, the modest downward slope of the rate data in Figure 7B with increasing [Im] would be a considerably larger downward slope resulting from the catalytic partitioning effect.

Even when the medium effect is taken into account, the decrease in rate is small. It seems fairest to say that this new data does not refute the previously reported negative catalytic effect in the imidazole case—a negative effect that was much more pronounced in the morpholine case—but it is not strong support.

Our early studies leaned heavily on the observation of bell-shaped curves for the cleavage rate as a function of the imidazole buffer ratio. That is, we saw a rate maximum when both Im and ImH$^+$ were present, but the rate of cleavage decreased when the Im/ImH$^+$ ratio was high or low; the rate maximum occurred near a 1/1 ratio. However, these early studies were done without control of the ionic strength, and this could be a problem. Increasing Im while decreasing ImH$^+$ leads to a decrease in ionic strength, and this could have rate effects. Indeed, Perrin suggested that a medium effect or an ionic strength effect could account for our observed rate maximum.[20] We now find that the latter is indeed the case.

As Figure 9 shows, we can confirm the original finding[4] that the cleavage rate essentially levels off after a maximum when the Im/ImH$^+$ ratio is increased (when we subtracted the non-buffer-catalyzed rate, which is faster at the higher pH with a more basic buffer, this had led to a bell-shaped rate vs buffer composition curve[4]), but only if we do not adjust the ionic strength. When NaCl is used to maintain constant ionic strength, no such leveling is seen and the rate simply increases as the concentration of imidazole base increases. Thus the original evidence in favor of a sequential bifunctional mechanism for imidazole buffer catalysis of the cleavage reaction was flawed. However, extrapolation of the correct curve in Figure 9 to pure

(20) Perrin, C. L. *J. Org. Chem.* **1995**, *60*, 1239−1243.

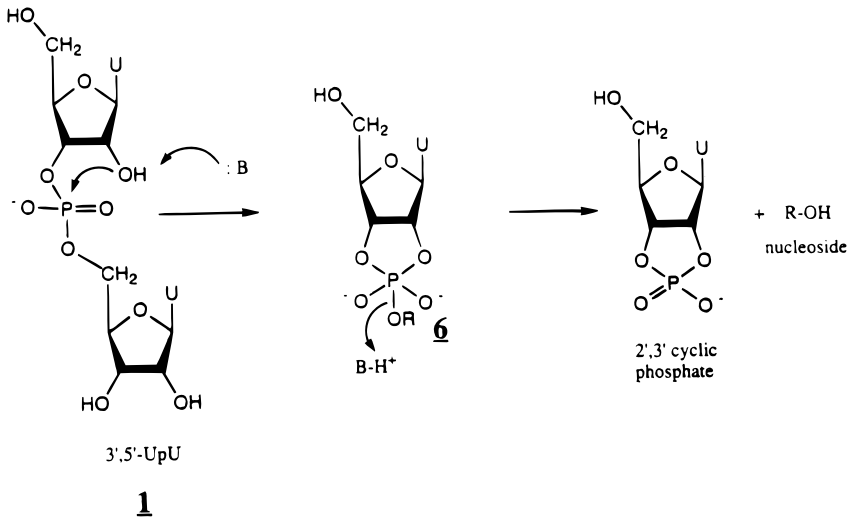


Figure 10. A possible pathway for the cleavage reaction that has kinetic order only in B. As discussed, the BH^+ catalysis would be kinetically invisible.

Im or pure ImH^+ indicates that there is an appreciable rate catalyzed by ImH^+, and a larger one catalyzed by Im. This is the relationship we saw in Figures 7A and 8A.

The ionic strength effect on the cleavage rate is reasonable. If ionic strength is not maintained with NaCl, decreasing the ImH^+ Cl^- concentration decreases the polarity of the medium, just as adding dioxane does. Thus the same slowing of the cleavage rate with decreasing polarity of the medium is seen.

Why is a bell-shaped curve not seen when the experiments are done correctly? The branching mechanism of Figure 2 *should* generate a bell-shaped curve. This is clear from Figure 3, where eq a has the product of B and BH^+ in the numerator. We propose that the bell-shaped curve is not seen in our case because it is hidden by an additional process: cleavage can also occur catalyzed by B alone. This extra process—which is the major one and even more important with a more basic buffer, as the buffer has more B and less BH^+—prevents the drop in rate when $[BH^+]$ becomes small at one end of the pH vs rate profile. A drop should occur if the sequential mechanism alone operated.

According to this proposal, cyclization to form a phosphorane can occur either with or without prior protonation. The reaction of the protonated phosphate will of course be intrinsically faster, but the operating pH is so far above the phosphate pK_a that only a small fraction of the phosphate is protonated. Thus the overall rate of cleavage catalyzed by base alone is greater than that for the bifunctional sequential process; it hides the rate decrease expected from the latter mechanism alone as $[BH^+]$ decreases while [B] increases.

Mechanism of the Reaction Catalyzed by B Alone. One possible mechanism is shown in Figure 10, with B converting substrate to an intermediate phosphorane dianion **6** and BH^+ carrying it forward to the cleavage products. As we have described elsewhere,[21] such a mechanism will show catalysis only by B, not by the BH^+ that is used in the later step. This mechanism cannot by itself explain the negative catalytic effects that we observe with buffer bases in the isomerization process, effects that go opposite to the medium effect and that require that some of the cleavage process go by a *base-catalyzed* branch from a common intermediate also involved in the isomerization process.

A related mechanism was shown in Figure 6. There the first-formed phosphorane dianion **6** fragments to products without further catalysis.

Calculations have indicated that such a phosphorane dianion will likely not be a stable intermediate,[22,23] and its fragmentation to a product alkoxide ion and a phosphate anion should be exothermic. (The monoanion **5** is predicted to be stable.) This version of cyclization without prior protonation can be thought of as a base-catalyzed displacement reaction, possibly with some addition character to it.

Perrin has suggested an alternative base-catalyzed mechanism to accommodate our published data (Figure 11).[20] In his scheme, the buffer base operates first to convert the substrate to the phosphorane dianion **6**, and this then rapidly protonates to form the phosphorane monoanion **5**. Both this scheme and that of Figure 2 convert the substrate to the same monanion **5**, and using a proton and a buffer base B, but in a different sequence. We had considered schemes related to that of Figure 11, but rejected them since they did not accommodate the apparent bell-shaped rate vs buffer composition curve. Since we have now shown that the bell-shaped curve was an artifact caused by variations in ionic strength, the scheme of Figure 11 must be reconsidered.

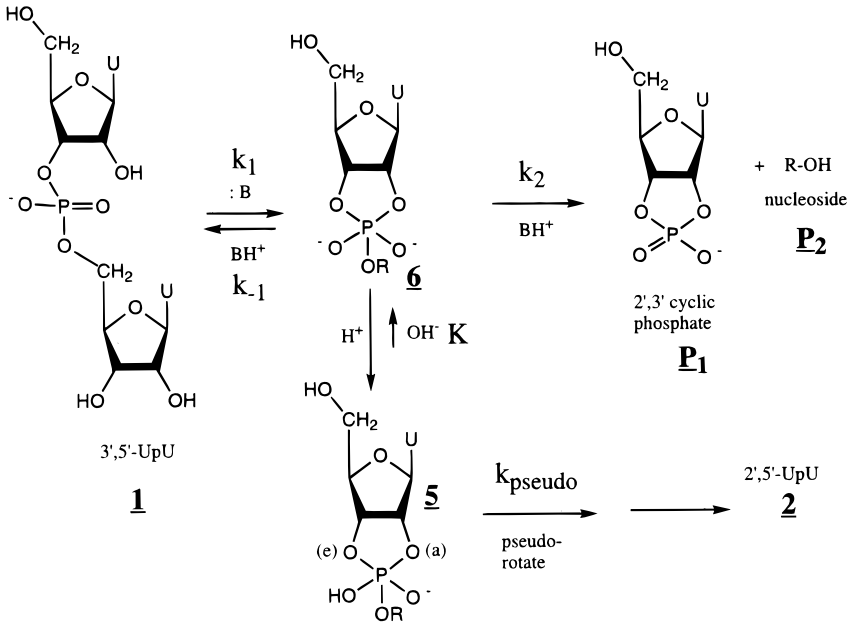
If the scheme as shown involved only buffer species, it alone could not explain our finding of negative catalysis of the isomerization by buffer base. That is, the rate of isomerization would follow a kinetic equation (eq c of Figure 11) in which the buffer base appears in the numerator as well as in some terms of the denominator. If those terms were completely dominant in the denominator, that could lead to a rate *independent* of [B], but never to a rate that *decreases* with increasing [B] as is observed. However, Perrin points out the possibility that our negative catalysis could be observed if the first step is catalyzed not only by B but also by OH^-. (He also suggests that the cleavage step in his scheme can be catalyzed both by BH^+ and by solvent species.) With the inclusion of the additional catalyst terms, the equation governing the isomerization rate is now eq d of Figure 11.

As eq d shows, one can account for the negative rate effect of [B] on the isomerization reaction *if* the second term in the numerator of the equation is significant; that is, if the catalysis by OH^- is comparable to the catalysis by B. From the data

(21) Breslow, R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1208−1211.

(22) (a) Haydock, K.; Lim, C.; Brünger, A. T.; Karplus, M. *J. Am. Chem. Soc.* **1990**, *112*, 3826−3831. (b) Lim, C.; Karplus, M. *J. Am. Chem. Soc.* **1990**, *112*, 5872−5873. (c) Dejaegere, A.; Lim, C.; Karplus, M. *J. Am. Chem. Soc.* **1991**, *113*, 4353−4355. (d) Dejaegere, A.; Karplus, M. *J. Am. Chem. Soc.* **1993**, *115*, 5316−5317.

(23) Wladkowski, B.; Krauss, M.; Stevens, W. *J. Am. Chem. Soc.* **1995**, *117*, 10537−10545.



$$k_{isom} = \frac{k_1B \bullet k_{pseudo}}{(k_{-1} + k_2)BH^+ \bullet K \bullet OH^- + k_{pseudo}} = \frac{k_1B \bullet k_{pseudo}}{k'B + k_{pseudo}} \quad (c)$$

$$k_{isom} = \frac{k_{pseudo}[k_1B + k_{OH}OH^-]}{k'B + k_{pseudo} + k''} \quad (d)$$

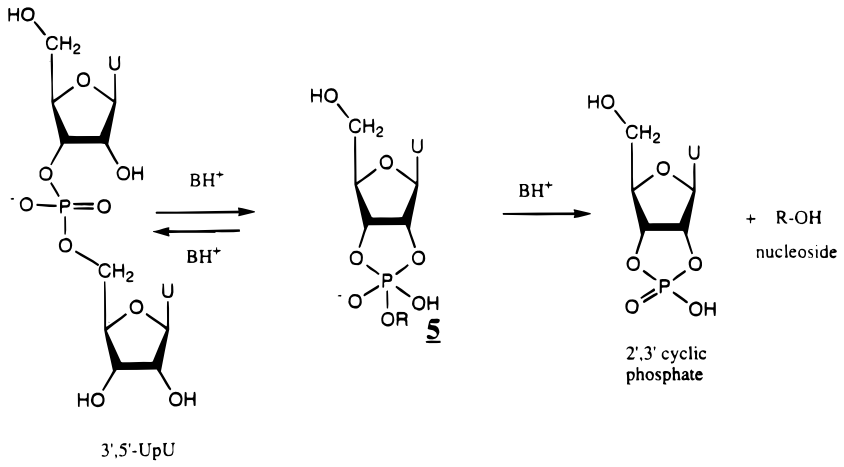
Figure 11. Scheme suggested by Perrin (ref 18) for the cleavage and isomerization of UpU. In this scheme the substrate is converted to the phosphorane monoanion **5** by a first step with B catalysis followed by a fast favorable protonation. The phosphorane anion **5** then goes on to the isomer **2** as in our scheme of Figure 2, or **5** is converted to the cleavage product by a sequential deprotonation by OH^-, then protonation by BH^+. This cleavage sequence is equivalent to B catalysis, so the overall process shows no kinetic order in BH^+, only in B. Equation c is the steady-state rate equation for isomerization of **1** to **2** if only buffer species play catalytic roles, and if the pH is such that **6** is rapidly and essentially completely protonated to form **5**. In this scheme the rate of formation of **5** is simply the rate of formation of **6**, which is rapidly protonated to **5**. Partitioning of **5**, which contributes the denominator of the equation, involves the isomerization reaction and also reversible conversion of **5** to **6**. It is shown as an equilibrium using H^+ and OH^-, but the situation does not change if BH^+ and B are used instead. k' is just a composite of the rate constants k_{-1} and k_2 with K. The cleavage leg with rate constant k_2 can also have a contribution from nonbuffer catalysts, but this does not affect the conclusions. This scheme in its simplest form (eq c) cannot account for the observed negative catalysis of isomerization by B, as discussed in the text, but it can account for it if the B in the first step is OH^- as well as buffer catalyst, as Perrin suggested, if that OH^- plays a significant catalytic role (eq d), and if **6** is stable enough to be a true intermediate (see Results and Discussion). In this equation, k_{OH} is the constant for such OH^- catalysis of the conversion of **1** to **6**, and k'' reflects the reversal of the first step by nonbuffer species. This is one of several schemes that account for the part of the cleavage catalyzed simply by B, but it does not account for the observed BH^+ process or the evidence that isomerization is catalyzed by BH^+ and H^+, not B and OH^-.

plotted in Figure 7A, the cleavage rate with imidazole buffer has a dependence on [Im] that is ca. 7 times as large for the upper curve—with [ImH^+] at 0.5 M and varying [Im]—as it is for the lower curve where all the buffer concentrations are 10% of those for the points above them. The pH's vary, but the pH at each buffer ratio is the same for the high and the low buffer concentrations. Thus for the cleavage reaction, the kinetic contribution of [OH^-] is about 40% that of [Im] at the low buffer concentration, but only 4% of that for [Im] at the high buffer concentration. From the data in Figure 7B it is clear that both the buffer and the nonbuffer species make a significant contribution to the rate of isomerization, but the catalyst is BH^+ or H^+, not B or OH^- as the Perrin scheme requires.

Our scheme of Figure 2 and the Perrin scheme of Figure 11 both postulate that the same intermediate phosphorane monoanion **5** is being formed in the isomerization and also in the cleavage reaction. The data of our Figures 7 and 8—and the data we have published previously—are consistent with the alternative of Figure 11 if a nonbuffer species makes a significant contribution to the first step, forming the intermediate, and if the dominant role of the basic buffer species B on the cleavage rate reflects mainly its role in the conversion of the intermediate to cleavage product, as our schemes both show.

Of course the Perrin scheme seems to use BH^+ for the cleavage branch, not B as in our scheme, but this is illusory. In our scheme the B is used to deprotonate the intermediate species **5**, and then the resulting BH^+ is used to protonate a leaving group. In the Perrin scheme the intermediate **5** is deprotonated by OH^-, and then BH^+ is used to protonate the leaving group. The two are kinetically equivalent. The rate of a process depending on the product [OH^-][BH^+] depends on [B], so either version could be used in either scheme equally well. In his scheme the dianion **6** could be protonated to the monanion **5** by BH^+, and the process then reversed by B. This would make the cleavage branch simply B catalyzed. Similarly, our scheme in Figure 2 could be modified to include reversible deprotonation of **5** to the dianion **6** by OH^-, then protonation of the leaving group by BH^+, without changing the kinetics.

The Perrin scheme of Figure 11 postulates that the phosphorane dianion **6** is a true intermediate with enough lifetime to protonate before fragmentation. Calculations suggest that this is unlikely,[22,23] that such a dianion cannot be a true intermediae



1 **5**

Figure 12. A cleavage mechanism that would show only BH^+ catalysis. As discussed in the text, it is excluded for the morpholine and imidazole buffers by the observed negative rate effect of B on the isomerization process.

but will fragment during its formation. The isomerization reaction requires a true intermediate that can protonate so that isomerization is possible. Our observed negative rate effects of buffer bases on isomerization require that such a protonated intermediate also be on the path to cleavage. This favors our mechanism of Figure 2 over the Perrin mechanism of Figure 11, but only if it is true that dianion **6** cannot be a true intermediate. At this point it seems best to consider both mechanisms as possible.

Subject to the above concerns, the Perrin scheme may be a viable alternative for the reaction catalyzed by B alone, but it does not deal with the reactions catalyzed by BH^+. Our data show that the cleavage reaction and, even more so, the isomerization reaction are catalyzed by BH^+. In those reactions the first step must be *protonation* of the substrate, in contrast to the Perrin scheme in which such protonation occurs after phosphorane formation and is kinetically invisible (since it is fast and essentially complete).

Are There Alternative Mechanisms for the BH^+-Catalyzed Reactions? We must also consider possible alternatives for the BH^+-catalyzed process. One of them is our scheme of Figure 2. It was originally based on the observation of a bell-shaped curve that now is seen to have an alternative explanation, so there is no longer direct evidence for catalysis of the cleavage leg of the partitioning scheme by B. The negative catalysis by B might involve only the scheme of Figure 11. Thus we must also consider paths in which BH^+ alone plays a role, without participation of B in a later step.

Such a path is shown in Figure 12. Here the BH^+ is used both to form the intermediate **5**, as in our Figure 2, and also to catalyze its decomposition to a cyclic *neutral* phosphate without deprotonation. However, in the same medium, that intermediate **5** is mainly being sent forward to cleavage products with catalysis by B, not BH^+ (as in our scheme, or in the equivalent and indistinguishable cleavage branch of the Perrin scheme). Otherwise one would not see negative effects of B on the rate of isomerization. One cannot postulate that the same intermediate **5** undergoes mainly a base-catalyzed decomposition mechanism if it is formed by one path, but uses a different acid-catalyzed decomposition mechanism in the same medium if it is formed by a different path. The path with BH^+ in its first step must be using B on the cleavage leg, not BH^+.

Thus we conclude that the scheme that best explains our observed BH^+ catalysis of cleavage and isomerization by imidazole buffers is Figure 2, as we originally proposed. It is accompanied[24,25] by a path using base catalysis alone—the schemes of Figures 6, 10, and 11 are all possible—that is of course unrelated to a bifunctional mechanism such as that seen with our mimic of ribonuclease, or with ribonuclease itself. However, when the leaving group is much more stable, in substrate **4**, base catalysis is all that occurs.[15,16] With such a good leaving group the more or less direct displacement reaction of Figure 6 will be favored.

Acetate Buffer. The balance between the path of Figure 2 and that of Figures 6, 10, and 11 will depend on the relative basicity and acidity of the buffer components, of course. We have examined the situation with sodium acetate and acetic acid as buffer catalysts. The pK_a of morpholine is 8.33, that of imidazole is 7.0, and that of acetic acid is 4.75. Thus a buffer-acid-catalyzed path should be favored with acetate buffer. This is what we observe.

In Figure 13 we plot the rate of cleavage and of isomerization of 3′,5′-UpU by various ratios of acetic acid to sodium acetate, both at a high buffer concentration and at a low one. (With this acidic buffer the isomerization rate is somewhat faster than the cleavage rate, in contrast to the situation with imidazole or morpholine buffers where the isomerization is considerably slower than the cleavage.) As the plots show, both the cleavage rate (Figure 13A) and the isomerization rate (Figure 13B) increase with increasing concentration of HOAc. In contrast to the data with morpholine or imidazole, there is no significant catalysis contributed by the buffer base, which is of course much weaker. That is, the rates extrapolated to [HOAc] = 0, with all the buffer as [AcO^-], are not significantly above zero. Thus mechanisms in which the first step is protonation of the substrate, as in the scheme of Figure 2, are the dominant ones here.

The data in Figure 13A,B show upward curvature and roughly fit a theoretical curve for *second-order* catalysis by HOAc. However, we must also consider medium effects. We have

(24) Haim (Haim, A. *J. Am. Chem. Soc.* **1992**, *114*, 8384−8388) argued that the isomerization and cleavage reactions did not proceed through a common intermediate. Our conclusion is that this could be partly correct: the cleavage reaction has an additional path that may bypass the isomerization intermediate **5**—unless it involves the scheme of Figure 11—as well as the one that passes through it.

(25) Kosonen and Lönnberg (Kosonen, M.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1995**, 1203−1209) describe relevant studies on the cleavage and isomerization of a uridine phosphate triester. Since their intermediate phosphorane anion does not carry a proton, it cannot show the base-catalyzed decomposition we invoke for our cases.

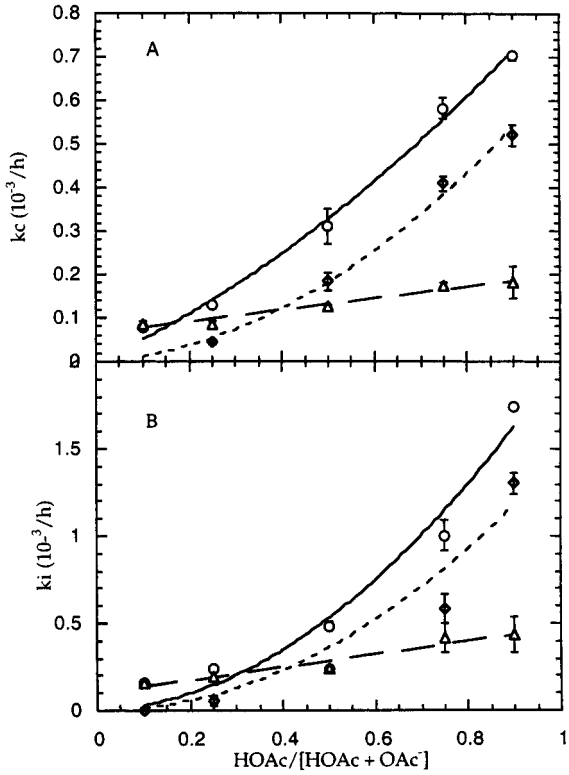


Figure 13. Observed pseudo-first-order rate constants for (A) the cleavage of 3′,5′-UpU and (B) the isomerization of 3′,5′-UpU, both vs the fraction of HOAc in acetate buffer at 60 °C. The pH of course varies as the buffer ratio changes: (○—) [buffer] = 1.0 M; (△– –) [buffer] = 0.1 M; (◇- - -) the difference between the 1.0 M and the 0.1 M data. Ionic strength was maintained at 1.0 M with NaCl. The curved lines shown are plots vs $[HOAc]^2$.

examined the effect of adding dioxane to a 0.3 M buffer consisting of 9/1 HOAc/AcO^-. When 0.6 M dioxane was added, the k_c went from 0.15×10^{-3} h^{-1} to 0.22×10^{-3} h^{-1}. With the same buffer and addition, k_i went from 0.60×10^{-3} h^{-1} to 0.68×10^{-3} h^{-1}. In this case *both* the isomerization and the cleavage reaction are faster when dioxane is added, although the effect is larger in the cleavage case. This contrasts with the *slowing* of cleavage by dioxane when the catalyst was morpholine or imidazole.

The difference reflects the difference in charge type for the buffer components. In the acetate buffer the acid form is neutral, so any process in which it protonates a substrate anion will not lead to net decreased charge. Similarly, since the acetate anion is negative, any process in which it removes a proton from the OH group of an intermediate will not lead to net increased charge, as such deprotonations did when a neutral base was involved. The medium effect of acetic acid should be even smaller than that of dioxane, so the upward curvatures in Figure 13 are probably not due simply to the medium effect.

We tentatively propose a mechanism (Figure 14) that accounts for the apparent second-order kinetics in HOAc. In the first step we write the reversible protonation of the substrate by buffer acid, just as in the mechanism of Figure 2. However, to account for the second molecule of HOAc that must appear in the transition state for cleavage, we propose that the cyclization to form a phosphorane—again catalyzed by the buffer base—is in this case also catalyzed by an HOAc molecule hydrogen-bonded to the neutral substrate. The cyclization would then lead directly to the fully protonated neutral phosphorane.

This is proposed mainly to handle the apparent second-order kinetics, but it is not unreasonable. In contrast to the cyclization process in the mechanism of Figure 2, this cyclization is driven by a much weaker base, so it might need the help that a hydrogen-bonded acid would provide.

It should also be noted that in the cyclization step an anionic base is removing a proton, so the transition state has a dispersed negative charge. Such a change from localized to dispersed negative charge should be *favored* by less polar solvents, in line with the observed medium effect. A similar change from localized to dispersed negative charge occurs in the fragmentation step that leads to cleavage products, again consistent with the observed medium effect here.

The kinetic expressions for this mechanism—derived by using the steady-state treatment—show that both cleavage and isomerization should be simply second-order in HOAc, as is apparently observed (acetate ion does not appear in the kinetic expression, since[21] it is formed from HOAc in a preceding step). Thus this mechanism seems a likely analog of the sequential acid−base bifunctional mechanism found for the other buffers, but it cannot be considered fully established until more work is done with the acetate buffer and other related catalysts. If it is correct, it has a step with *simultaneous* acid−base catalysis by the two buffer components, in direct analogy to the simultaneous bifunctional catalysis in the enzyme ribonuclease[7] and in our ribonuclease mimic.[7,17−19,26]

Relevance of These Studies to the Mechanism of Action of an Artificial Enzyme. We have described the hydrolysis of the cyclic phosphate **7** by a set of β-cyclodextrins carrying two imidazole groups attached to the primary carbons of the cyclodextrins.[7,17−19,26] Among the three isomers with imidazoles on neighboring glucoses (6A, 6B), on glucoses separated by one (6A, 6C), or on glucoses separated by two other glucose units (6A, 6D), the best catalyst was the 6A, 6B isomer.[17] Furthermore, the pH vs rate profile of this catalyst showed that it was operating with one basic imidazole ring and one acidic imidazolium ring. Finally, a proton inventory using deuterium isotope effects showed that the two catalytic groups were operating at the same time, in a *simultaneous* bifunctional process.[18] This differs from the sequential process seen with simple buffer catalysts (although the seeming second-order process with acetic acid buffers does require one simultaneous bifunctional step, as in the scheme of Figure 14).

The base group in such a mechanism is surely delivering the water nucleophile, but there are two possible roles for the ImH^+ in the hydrolysis of **7**. One could be to protonate the leaving oxygen, as in the scheme of Figure 15. The other is to protonate a phosphate anionic oxygen, so as to produce a phosphorane monoanion intermediate **8**, as in Figure 16. The intervention of such a phosphorane monoanion is of course the main thrust of our mechanistic conclusions in the buffer-catalyzed cleavage and isomerizations of UpU. The preferred geometry in the bifunctional catalyzed hydrolysis of compound **7** indicates that a phosphorane monoanion is also involved here.

Since both the nucleophile and the leaving group must depart in-line in a simultaneous displacement at phosphorus, a direct reaction such as that of Figure 15 would be best catalyzed by the A,D isomer of our catalyst set. Models show that the A,B isomer cannot simultaneously deliver a water to one face of the phosphorus while protonating a leaving group on the opposite face; yet the A,B isomer was the preferred catalyst. This indicates that the scheme of Figure 16 is preferred, the proton is being delivered to an anionic phosphate group, which is within reach, not to the leaving group. Then the resulting phosphorane monoanion **8** decomposes to product.

(26) Breslow, R.; Schmuck, C. *J. Am. Chem. Soc.* **1996**, *118*, 6601.

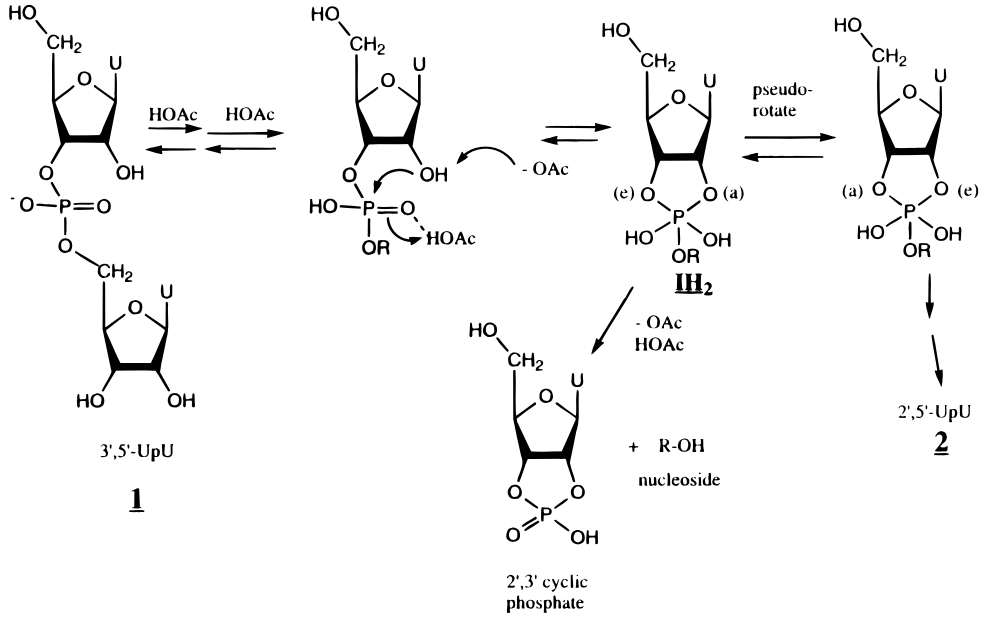


Figure 14. Likely mechanism for the cleavage and isomerization of 3′,5′-UpU catalyzed by acetic acid, to explain the apparent second-order dependence on [HOAc]. The scheme as shown is somewhat compressed: in the first step there is protonation of the substrate followed by hydrogen bonding of the second HOAc, and in the cleavage of the phosphorane IH_2 to the cyclic phosphate, the acetate probably deprotonates the phosphorane while the HOAc protonates the leaving group, as also occurs in the reversal of the formation of IH_2. The scheme differs from that of Figure 2 in that the cyclization step forms a neutral phosphorane in a simultaneous bifunctional process catalyzed by the buffer acid and the buffer base. This change reflects the greater acidity of HOAc than of the morpholinium or imidazolium cations, and the weaker basicity of acetate ion than of morpholine or imidazole. A steady-state treatment of this mechanism indicates that both k_c and k_i are simply proportional to $[HOAc]^2$, with no dependence on AcO^-. This is because the acetate ions used as catalysts in two of the steps are formed from HOAc in the preceding step and are thus kinetically invisible (ref 19).

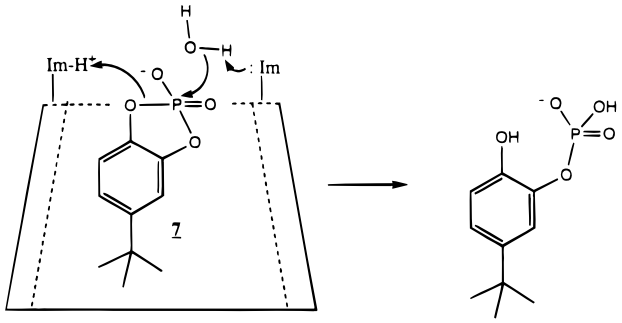


Figure 15. An *a priori* possible mechanism for the hydrolysis of substrate **7** by a cyclodextrin bis-imadazole. This excluded mechanism predicts that the most effective catalyst would have the two catalytic groups as far apart as possible, but that is not what is observed.

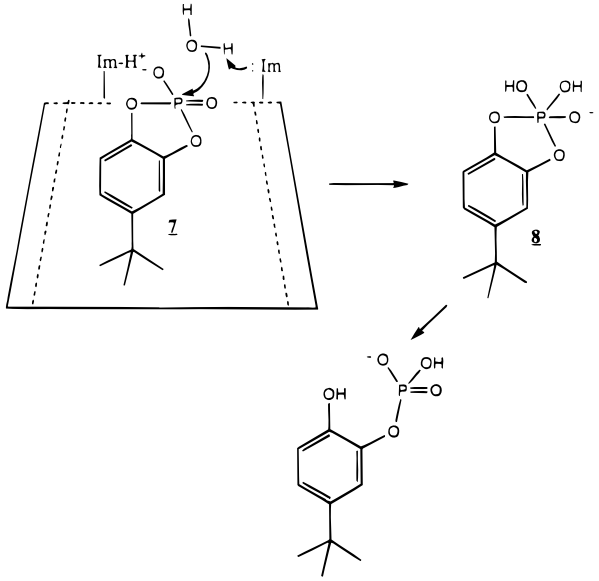


Figure 16. Correct mechanism for hydrolysis of substrate **7** by cyclodextrin bis-imidazole, which protonates the anionic oxygen and forms a phosphorane monoanion **8**. This explains the finding that the most effective cyclodextrin bis-imidazole isomer has the two catalytic groups on neighboring glucose units, mounted only 51° apart. This is a simultaneous analog of the mechanism in Figure 2.

As we have pointed out,[7] in such a simultaneous process there is no longer the question of whether the acid or the base operates first, as in the simple buffer schemes. Thus both our scheme of Figure 2 and the Perrin scheme of Figure 11 become identical when extrapolated to a *simultaneous* acid−base process. Of course in the Perrin scheme some proton-transfer steps are shown using H^+ or OH^-, but with any significant concentration of buffer catalysts, in solution or within a catalyst−substrate complex, all these transfers will be done by the catalyst species as in our scheme. Substitution of BH^+ and B for the H^+ and OH^- in the equilibration (Figure 11) between dianion **6** and monoanion **5** does not affect the validity of his suggestion.

Mechanism of the Enzyme Ribonuclease A.[27] On the basis of our conclusions from the previous buffer studies, and more seriously from the findings with the artificial enzyme that uses the principal catalytic group of the enzyme, we have suggested that the enzyme may well follow a related path (Figure 17), using simultaneous[28] acid−base catalysis. In most textbooks the enzyme mechanism is written as in Figure 18, with the basic imidazole group delivering the neighboring oxygen to the phosphate while the acidic imidazolium group protonates the

(27) For reviews, see: (a) Richards, F. M.; Wyckoff, H. W. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. 4. (b) Blackburn, P.; Moore, S. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1982; Vol. 15. (c) Campbell, C. L.; Petsko, G. A. *Biochemistry* **1987**, *26*, 8579−8584 and references cited therein.

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

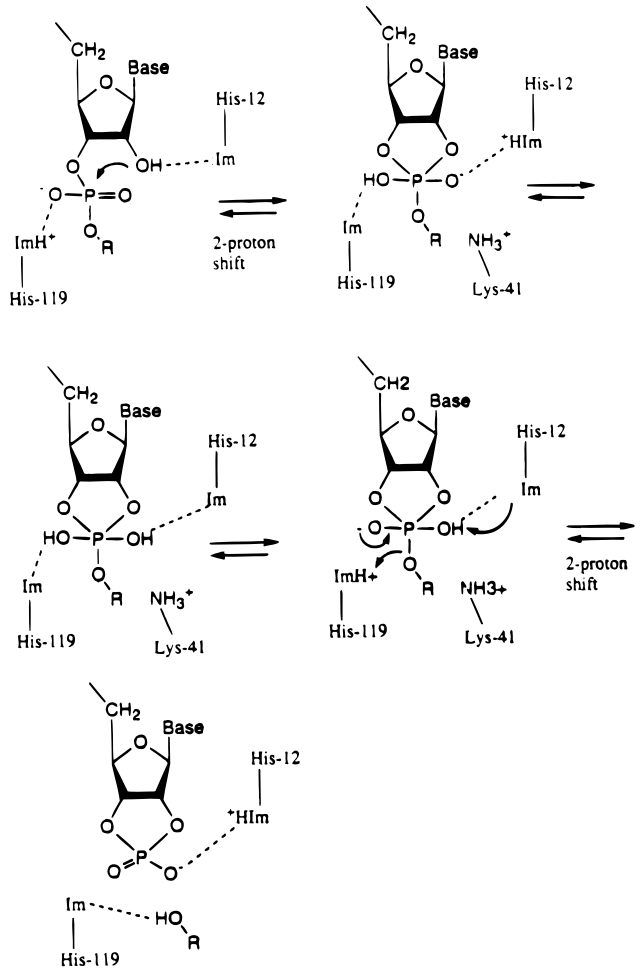


Figure 17. Mechanism we have proposed for the action of ribonuclease A, in which the first function of the imidazolium ion of His-119 is to protonate the phosphate anionic oxygen, leading to a phosphorane monoanion intermediate.

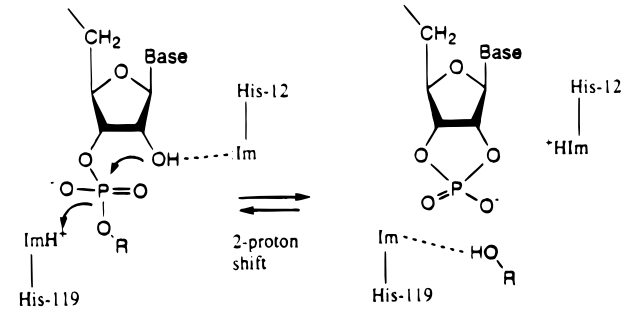


Figure 18. Classical mechanism for cyclization−cleavage of RNA by ribonuclease A, still favored by some workers.

leaving group. The resulting cyclic phosphate ester is then hydrolyzed in a second related process in which the scheme is reversed, but with water substituting for the nucleoside product. In at least one text our alternative is now shown as the likely path.[29]

In addressing the matter of an enzyme mechanism, analogy is useful but hardly definitive. However, we pointed out[7] that the determined structures of ribonuclease A with some bound anionic substrates or inhibitors showed that the imidazolium group is indeed hydrogen-bonded to an anionic phosphate oxygen, not to the neutral leaving group oxygen. Thus the proton transfer we postulate should occur readily. Furthermore, some calculations on the enzyme mechanism by the Karplus group[22] showed that our proposed process, with protonation to form a phosphorane intermediate, is energetically reasonable. Recent calculations by Wladkowski[23] come to the stronger conclusion that our phosphorane process is the preferred path for the enzyme, although in those calculations the phosphorane monoanion then goes on—with further protonation—to a neutral phosphorane. Inclusion of such an extra equlibration of the phosphorane monoanion with a neutral phosphorane would not affect the conclusions related to the schemes of either Figure 2 or Figure 11.

Contrary arguments about the enzyme mechanism have recently been advanced[30,31] and will be addressed elsewhere.

Summary of the Mechanistic Arguments

1. The cleavage of 3′,5′-UpU to form the 2′,3′-cyclic phosphate and its isomerization to 2′,5′-UpU are both catalyzed by morpholine buffer, imidazole buffer, and acetate buffer.

2. Isomerization with imidazole buffers involves a pathway in which the buffer acid converts substrate to a phosphorane intermediate. The intermediate can then branch: pseudorotation sends it to the isomer, while buffer base sends it to the cleavage products. This branching mechanism is indicated by the *negative* effect of buffer base on the isomerization rate. Studies of medium effects strengthen this evidence.

3. In addition to this cleavage mechanism—with buffer acid catalyzing the formation of an intermediate and buffer base sending the intermediate to cleavage products—there is an additional cleavage process in which only buffer base plays a role. This is the dominant mechanism with morpholine or imidazole buffers, hiding some of the kinetic expectations for the [BH^+, then B] mechanism.

4. With acetate buffer, acid catalysis dominates. Now both cleavage and isomerization involve conversion of 3′,5′-UpU to a phosphorane intermediate by the buffer acid. A proposed bifunctional process explains the detailed kinetic behavior.

5. The responses of all these reactions to medium effects are consistent with the proposed mechanisms.

6. The earliest evidence for sequential bifunctional catalysis by imidazole buffers—a bell-shaped rate of cleavage vs buffer ratio—was incorrect because of the neglect of ionic strength effects. Curiously, this early omission was the impetus for further experiments that actually established a role for this mechanism.

7. In an enzyme mimic, a simultaneous bifunctional mechanism operates that is related to the sequential bifunctional mechanism of buffer catalysis. Both processes involve conversion of a phosphate anion substrate to a phosphorane monoanion intermediate.

8. A mechanism has been proposed for the enzyme ribonuclease A that is essentially the same as that for the artificial enzyme.

Experimental Section

Materials. 3′,5′-Uridyluridine, uridine, imidazole, imidazole hydrochloride, acetic acid, and sodium acetate were purchased (Sigma) in the highest purity available. Morpholine (Aldrich) was redistilled, and morpholine hydrochloride was prepared from it. The 2.0 M stock solutions of the buffer components were prepared in advance and used in all the kinetic studies.

Kinetic Studies on UpU. The kinetic assay used in the current studies was similar to that described previously,[12] except that a fully automated HP1090 HPLC instrument was used in the current studies.

(29) Abeles, R. H.; Frey, P. A.; Jencks, W. P. *Biochemistry*; Jones and Bartlett: Boston, 1992.

(30) Thompson, J. E.; Raines, R. T. *J. Am. Chem. Soc.* **1994**, *116*, 5467−5468.

(31) Herschlag, D. *J. Am. Chem. Soc.* **1994**, *116*, 11631−11635.

The differences from the previous procedures are as follows: (1) Borosilicate glass 0.1 mL microvials were used as reaction vessels. (2) The reaction vials were incubated in the thermostated automatic sampler compartment of the HPLC instrument at 60.0 °C. (3) A 15 μL sample of the reaction solution was withdrawn periodically and automatically from the reaction vial and injected directly onto the column for analysis. The HPLC eluent was 4.5 mM pH 7 phosphate buffer with 4.0% MeOH, as before.[12] Since the sampling needle was part of the solvent delivery system in the chromatographic procedure, it was completely flushed with solvent between injections. As before,[12] an internal standard of potassium *p*-nitrobenzenesulfonate was used in the reaction mixture to calibrate the HPLC signals.

Typical Kinetic Run. To a reaction vial were added stock solutions of 15 μL of the potassium *p*-nitrobenzenesulfonate internal standard (200 μM) with 3′,5′-UpU (30 mM), 37.5 μL of imidazole (2.0 M), 37.5 μL of imidazole hydrochloride (2.0 M), 37.5 μL of NaCl solution (2.0 M), and 22.5 μL of deionized water. After stirring on a Vortex mixer the vial was incubated in the automatic sampler compartment of the HPLC instrument at 60 °C, and the instrument was programmed to withdraw 15 μL of the reaction solution at intervals over 13−50 h (the longer time for the isomerization study, which was slower with the basic buffers) and to inject it directly onto a C-18 reverse phase column. The integrated data furnished by the instrument were then calibrated with respect to the internal standard and analyzed with a standard computer program using the initial rate treatment. Only a few percent of the substrate was consumed.

Kinetic data for the runs are plotted in the figures of this paper and tabulated (155 rate constants) in the supporting information.

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Supporting Information Available: Tabulated rate constants and pH's that are plotted in the figures of the current paper (155 rate constants, 7 pH's) (6 pages). See any current masthead page for ordering and Internet access instructions.

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