Quantitative Evidence for the Mechanism of RNA Cleavage by Enzyme Mimics. Cleavage and Isomerization of UpU by Morpholine Buffers

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Abstract: The cleavage of 3′,5″-UpU to form the 2′,3′-cyclic phosphate and uridine and the simultaneous isomerization of the substrate to 2′,5″-UpU have been studied in morpholine buffer. The total rate of cleavage with morpholine buffer at 90/10 base to acid ratio and constant ionic strength shows an increase with increasing buffer concentration up to 1 M, but the isomerization rate shows a *decrease*, followed by a constant rate below that of the uncatalyzed process. A similar increase in rate of cleavage—but decrease in rate of isomerization—is also seen with a 95/5 morpholine/morpholinium buffer and with data that can fit the theoretical equations using those parameters that are common for the 90/10 buffer. With 80/20 morpholine/morpholinium the negative effect of buffer on the rate of isomerization is smaller and not clearly seen. These observations are consistent with the predictions from a kinetic treatment of a previously proposed mechanism: the substrate is converted to a phosphorane intermediate by buffer acid in the common first steps of both processes, but the paths then branch from that intermediate. The cleavage path shows buffer base catalysis, but the isomerization path does not. At higher buffer concentrations an additional catalytic process seems to have been detected. The dependences of the buffer-catalyzed reactions on buffer ratios are also consistent with those reported earlier for imidazole catalysis. The early imidazole data fit the theoretical predictions of our mechanistic treatment. They are also consistent with our mechanism according to a criterion suggested by a critic. The results confirm previous findings. They also furnish detailed evidence for some of the steps that were previously only suggested.

Introduction

Hydrolysis of ribonucleic acid (RNA) by the enzyme ribonuclease A involves ester interchange with the 2′ hydroxyl group to form a cyclic phosphate, with cleavage of the chain. The resulting 2′,3′-cyclic phosphate ester (cf. **3**) is then hydrolyzed by the enzyme. Catalysis of both the cyclization and the hydrolysis uses the imidazole rings of histidine-12 and histidine-119, although lysine-41 also plays a role.

Some years ago we initiated an investigation of the cleavage of RNA by imidazole buffer.[1] As we have described, a number of other research groups have studied similar questions, although generally with phenyl esters rather than with the normal leaving group of RNA itself.[2-8] We examined the imidazole-catalyzed cleavage of polyuridylic acid (polyU),[9,10] of 3′,5′-uridyluridine (UpU),[11] of the isomeric 2′,5″-UpU, and of 3′,5″-adenosyladenosine (ApA).[12] The kinetic studies indicated sequential bifunctional catalysis—one buffer component catalyzed the conversion of the substrate to a phosphorane intermediate (cf. **5**), and then the other buffer component catalyzed the conversion of this phosphorane intermediate to the cleavage product. However, we were initially unable to specify whether basic imidazole (Im) or acidic imidazolium ion (ImH^+) was the first buffer catalyst. Further studies clarified the picture.

We saw that the cleavage of 3′,5″-UpU (**1**, base = uridine) was accompanied by its isomerization to 2′,5″-UpU (**2**, base = uridine) and *vice versa*.[9,11] Furthermore, the isomerizations were catalyzed only by the acidic buffer component ImH^+.[11] It is

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(1) For reviews, see: (a) Breslow, R.; Anslyn, E.; Huang, D.-L. *Tetrahedron* **1991**, *47*, 2365–2376. (b) Breslow, R. *Acc. Chem. Res.* **1991**, *24*, 317–324.

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(11) Anslyn, E.; Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 4473–4482. Some errors in this paper can cause confusion. Thus the figure legends refer to the plots as involving rate *constants*, but the plots are actually of *rates* that would be observed, as the units listed indicate, for the reaction run with substrate at 1.6 mM (they were run at 16 mM, but the analytical method introduced a constant factor of ten because of an overlooked dilution). Also, in a few cases where the theoretical line was calculated to be a gentle curve, but the error bars exceeded the curvature, a simple straight line was drawn instead. There are also errors in Table I, listing the constants that generate the theoretical lines. The values of k_3 should be 0.10 (shown 0.39) and 0.06 (shown 0.40), apparently a transcription error. The other numerical values are correct, but k_1, k_2, and k_{-1} have units of (10^{-4} M^{-1} min^{-1}), k_3 and k_w have units of (10^{-4} min^{-1}), while k' and k'' have units of (10^{-6} min^{-1}). These corrections affect any attempts to reproduce the quantitative aspects of the paper but have no effect on the overall conclusions. These errata and those in ref 12 were submitted to *J. Am. Chem. Soc.* as Corrections on August 20, 1993, but the editor decided—in part because of the time elapsed—that correction here was sufficient.

(12) Breslow, R.; Huang, D.-L. *J. Am. Chem. Soc.* **1990**, *112*, 9621–9623. Equations 1 and 2 are incorrect and should read as follows (cf. eq 1 and 2 of the current paper):

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

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

The equations were corrected in the following: Breslow, R. *Acc. Chem. Res.* **1991**, *24*, 317–324. Also, the values listed in Table I are not pseudo-first-order rate constants; they are initial rates of the reactions using 3 mM substrate, with units of mM h^{-1}. Pseudo-first-order rate constants—including those with negative values because of the subtraction of the rates observed at zero [buffer]—can be obtained simply by dividing the values of Table I by 3 mM. The data with and without this subtraction are listed in the supplementary material to the current paper. These corrections do not affect the conclusions of this paper—in particular, the slowing of the isomerization reaction by one buffer component that we describe as "negative catalysis".

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known[13] that phosphate migrations of this type must go through a phosphorane intermediate that undergoes pseudorotation, interconverting some apical and equatorial groups. If the cleavage and the isomerization went through the *same* phosphorane intermediate, they would have a common first step catalyzed by ImH^+, and Im would be the second catalyst in the cleavage sequence. We proposed a mechanism (related to that of Figure 1) corresponding to this concept.[11]

Further evidence of a different sort came from studies on imidazole-catalyzed cleavage and isomerization of 3′,5″- and 2′,5″-ApA.[12] We saw again that isomerization was catalyzed by ImH^+, not by Im, and we also saw that Im contributed in a *negative* way to the isomerization.[14] With buffers rich in the basic Im component we saw a *decrease* in the rate of isomerization as the buffer concentration increased. When we held the ImH^+ concentration constant, we saw that increasing the concentration of Im decreased the isomerization rate (correcting for the rate effects of simple pH changes on the nonbuffer catalyzed part of the rate by extrapolating to zero buffer concentration).

As we have described,[1] such a negative effect was expected for the proposed mechanism. A steady-state treatment of the originally proposed mechanism,[1b,12] using the partitioning method of Cleland,[17-19] led to the first term of eq 1 and to eq 2. Note that [Im] appears only in the denominator of eq 2 for isomerization. Thus increasing [Im] should slow this reaction (physically, it decreases the concentration of **5** by increasing its partitioning along the cleavage path). It speeds the cleavage, since [Im] is also in the numerator of eq 1.

The extra terms in eq 1 were added simply to accommodate the fact that the observed reaction does not slow to zero when one or the other buffer component is missing. We indicated that this probably indicated that solvent species could act in sequential combination with the buffer components, as shown in Figure 1. However, we did not have enough data to justify the implied more elaborate form of these extra terms. As we will show later, our new evidence now allows us to change the form of these terms to reflect the details of Figure 1.

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

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

We have discussed elsewhere[11] the detailed mechanism we deduced from these studies. A two-step sequence for conversion of the starting material to the intermediate **5** was just the specific acid/general base version of ImH^+ catalysis. The mechanistic preference in the model system led us[1,11,20] to reconsider the enzyme ribonuclease A itself.

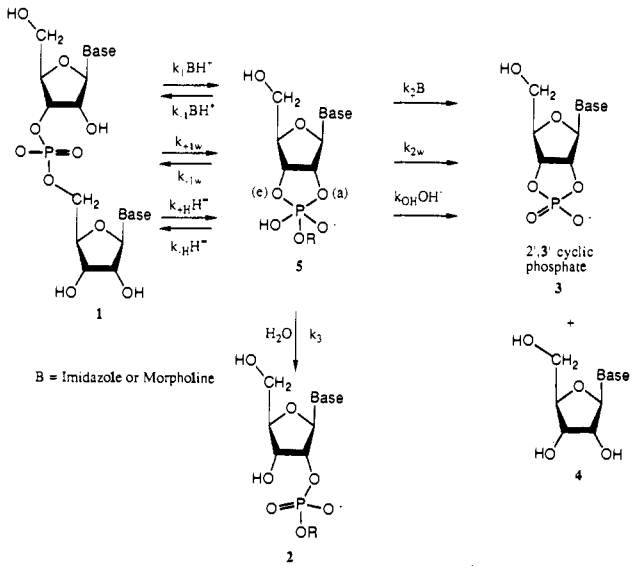

Figure 1. The detailed mechanism for buffer-catalyzed cleavage and isomerization of dinucleotides, whose derived eqs 3 and 4 fit the observed data.

We proposed a new enzyme mechanism based on our findings. In contrast to the situation in simple buffer solution, we noted that the ImH^+ and Im could operate *simultaneously* in the enzyme, not sequentially. This is consistent with proton inventory evidence for the enzyme.[21] Structural evidence for the enzyme fits our mechanism better than it does the classical one, and pathway calculations by the Karplus group also support the simultaneous version of our new proposals.[22] Of course once the acid and base operate simultaneously there is no longer the distinction between the base, then acid and the acid, then base sequence that is of concern in the simple buffer system. What carries over to the enzyme is the proposal that the first function of the acid catalyst is to protonate the phosphate anion oxygen, not the leaving group.

We were stimulated by the geometric implications of our new mechanism to redesign a mimic of ribonuclease, which produced a considerably better catalyst.[23,24] Furthermore, others have designed novel and effective ribonuclease mimics based on our mechanism.[25] However, we felt it desirable to obtain additional detailed kinetic data, with careful fitting to the theoretical curves, to confirm our conclusions in this important and unusual mechanistic case. While the kinetic data on polyU[10] were well fit by theory, those on UpU[11] and ApA[12] were more limited. Thus we have examined the cleavage and isomerization of 3′,5″-UpU by morpholine buffers. The data obtained can indeed fit the theoretical equations for our mechanism, and they also add more information about the chemistry.

Results

The observed pseudo-first-order rate constants for cleavage (k_c) and for isomerization (k_i) of 3′,5″-UpU by various buffers are listed in Tables I–III and plotted in Figures 3–5.

Discussion

The Data Obtained with Morpholine Buffers. The striking kinetic effects with imidazole buffer were contributed by its basic Im component, that promoted the cleavage branch of the

(13) Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1*, 70.

(14) We described the coefficient of the catalyst term in the *observed* rate as an *experimental* catalytic rate constant. This would be normal usage if the rate had increased with catalyst concentration. It corresponds to the type of equation that is used[15] to describe catalytic terms that add to an uncatalyzed rate: $k_{\text{observed}} = k_{\text{uncatalyzed}} + k_{\text{cat}}[\text{catalyst}]$. As used in this way, k_{cat} is not a theoretical rate constant, just an observed coefficient. Some chemists object to this usage in our case, since the observed coefficient has a negative value. We chose to distinguish between the *experimental* observation of a downward slope in rate as the catalyst concentration is increased—as in Figures 3A and 4A—and the *theoretical* explanation that is formulated as in eq 3. In eq 3 all theoretical rate constants are of course positive, but that for [base] appears in the denominator so it is the exponent—not the coefficient—that is negative. The observation of a rate decrease as [catalyst] increases is related to the common ion effect and other partitioning effects in carbonium ion chemistry. However, there are not many other cases in which a *catalyst* has this effect. Hammett[16] refers to "negative catalysis", but with a different meaning from ours.

(15) Cf.: Bruice, T. C.; Benkovic, S. J. *Bioorganic Mechanisms*; Benjamin: New York, 1975; Vol. 1, pp 4–16.

(16) Hammett, L. P. *Physical Organic Chemistry*; McGraw-Hill: New York, 1940; p 398.

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(19) Cf.: also Breslow, R. *J. Chem. Ed.* **1990**, *67*, 228–229, for an application of these ideas to chymotrypsin kinetics.

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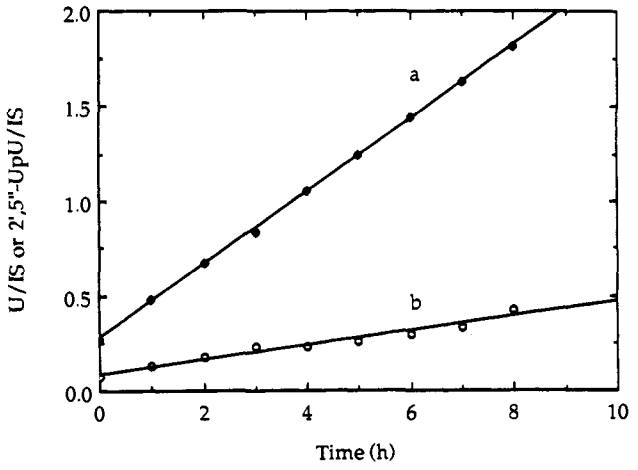

Figure 2. A typical kinetic run, with [morpholine buffer]$_{total}$ = 0.2 M, and B/BH$^+$ = 8/2; curve a, uridine (U); curve b, 2',5''-UpU; IS, internal standard.

Table I. Pseudo-First-Order Rate Constants for the Cleavage and Isomerization of 3',5''-UpU (4 mM) Catalyzed by Various Concentrations of Morpholine Buffer with a 90/10 Base to Acid Ratio, at 80 °C

concn of buffer (M)	k_i (10^{-3} h^{-1})	k_c (10^{-3} h^{-1})
0.10	0.61 ± 0.02	8.1 ± 0.3
0.20	0.53 ± 0.02	9.6 ± 0.3
0.40	0.47 ± 0.03	12.8 ± 0.3
0.60	0.41 ± 0.03	15.1 ± 0.3
0.80	0.39 ± 0.03	17.0 ± 0.4
1.00	0.41 ± 0.04	21.1 ± 1.1
1.20	0.46 ± 0.03	23.1 ± 0.4
1.40	0.44 ± 0.01	23.8 ± 1.5
1.60	0.44 ± 0.03	27.6 ± 0.1
1.80	0.46 ± 0.04	29.7 ± 2.1
2.00	0.51 ± 0.01	32.6 ± 0.3

Table II. Pseudo-First-Order Rate Constants for the Cleavage and Isomerization of 3',5''-UpU(4mM) Catalyzed by Various Concentrations of Morpholine Buffer with an 80/20 Base to Acid Ratio, at 80 °C

concn of buffer (M)	k_i (10^{-3} h^{-1})	k_c (10^{-3} h^{-1})
0.10	0.46 ± 0.03	3.53 ± 0.02
0.20	0.43 ± 0.03	4.87 ± 0.07
0.40	0.47 ± 0.03	7.05 ± 0.10
0.60	0.50 ± 0.03	9.36 ± 0.10
0.80	0.50 ± 0.03	11.94 ± 0.08
1.00	0.52 ± 0.03	14.20 ± 0.40

Table III. Pseudo-First-Order Rate Constants for the Cleavage and Isomerization of 3',5''-UpU(4mM) Catalyzed by Various Concentrations of Morpholine Buffer with a 95/5 Base to Acid Ratio, at 80 °C

concn of buffer (M)	k_i (10^{-3} h^{-1})	k_c (10^{-3} h^{-1})
0.10	0.55 ± 0.03	16.8 ± 0.4
0.20	0.50 ± 0.03	18.9 ± 0.6
0.40	0.48 ± 0.03	23.9 ± 0.3
0.60	0.46 ± 0.04	27.5 ± 0.5
0.70	0.42 ± 0.03	28.2 ± 0.3
0.90	0.44 ± 0.03	29.9 ± 1.0

mechanism. As predicted from this, we had found[11] that the less basic acetate buffer (pK_a 4.75; all pK's are reported for 25 °C, but the reactions were performed at 80 °C) promoted isomerization of UpU more than cleavage, in contrast to imidazole buffer (pK_a 7.0). Thus we went to morpholine buffer (pK_a 8.33) and a high 9/1 B/BH$^+$ ratio, with which we expected even more striking negative catalytic effects. To avoid any uncertainty about correcting for pH changes we varied the buffer concentration but kept a constant buffer ratio and maintained ionic strength with added NaCl. The pH was constant (at 25 °C) at 9.3.

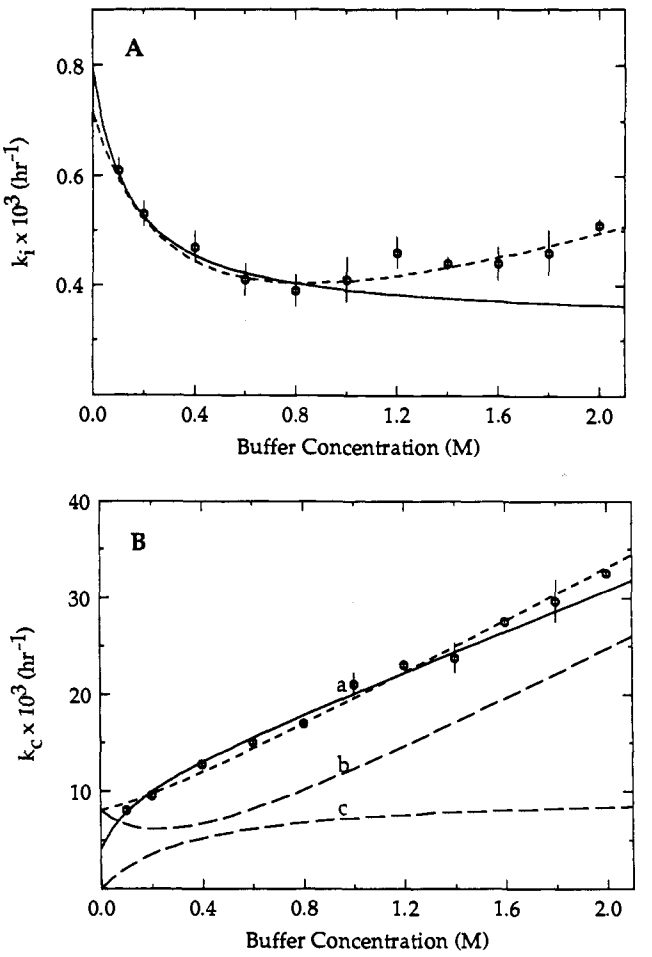

Figure 3. Pseudo-first-order rate constants as a function of buffer concentration for (A) isomerization of 3',5''-UpU to 2',5''-UpU and (B) simultaneous cleavage of 3',5''-UpU to form uridine 2',3'-cyclic phosphate and uridine. All runs were at 80 °C, performed at least in duplicate and analyzed as described previously[11,12] but with 9/1 morpholine/morpholine·HCl, varying the buffer concentration and maintaining constant ionic strength at 0.2 M with NaCl. The solid curve in (A) was obtained by fitting eq 3 to the observed points up to 1.0 M buffer. The intercept for [buffer] = zero is from this curve fitting; it is thus uncertain, but clearly above the observed points. The solid curve a in (B) was obtained by fitting eq 4 to the observed points up to 1.0 M buffer, using the same denominator as in (A). The dashed curves b and c are for the first and second terms of eq 4, respectively; they add up to curve a. The procedures for curve fitting are described in the Experimental Section; the parameters derived from this curve fitting are listed in Table IV. We also show the observed pseudo-first-order rate constants over the full experimental buffer concentration range (up to 2.0 M) with 9/1 morpholine/morpholine·HCl and constant (0.2 M) ionic strength. The dashed curve in A was obtained by fitting eq 5 to the observed points. The top dashed curve in B was obtained by fitting eq 6 to the observed points using the same denominator as in A. The procedures for curve fitting are described in the Experimental Section; the parameters derived from this curve fitting are listed in Table V.

The data are shown in Figure 3. The isomerization shows the kinds of negative effects mentioned earlier. With all buffer concentrations the rate of isomerization is slower than the (extrapolated) uncatalyzed rate, when [buffer] = 0. Further, in the early part of the curve there is an observed downward slope of the rate as buffer concentration is increased, corresponding to a negative observed coefficient for [buffer]. The data with buffer concentrations up to 1.0 M can be fit to the curve calculated from eq 3, which is that expected for the detailed mechanism of Figure 1. The derived curve-fitting parameters—which are composites of the various rate constants in eqs 3 and 4—are defined in the Experimental Section under "data treatment" and listed in Table IV. Standard deviations are listed for these fitting parameters, but the value of "*d*" is set as errorless and the same for the two

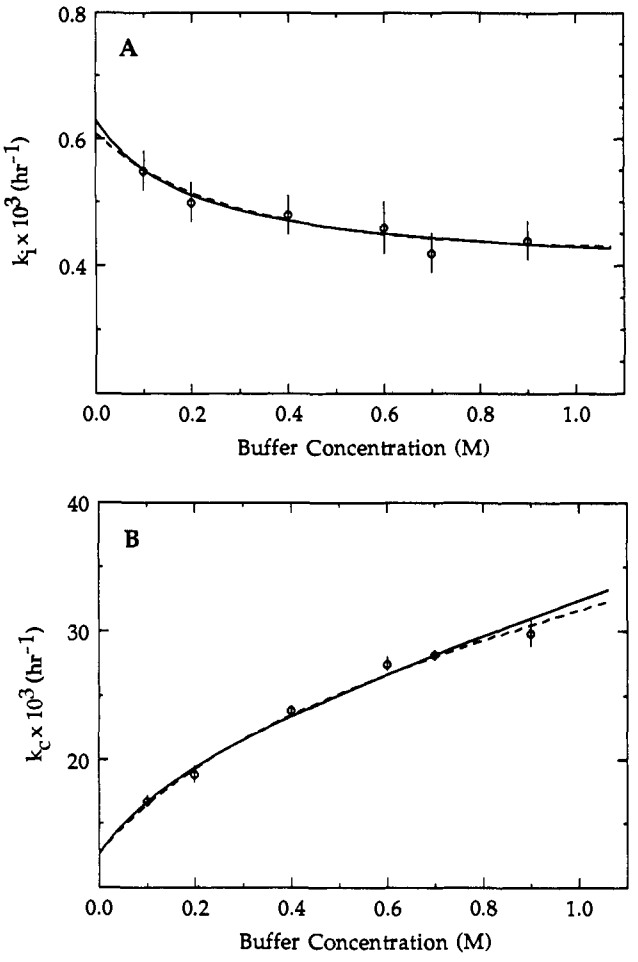

Figure 4. Pseudo-first-order rate constants as a function of buffer concentration for (A) isomerization of 3′,5″-UpU and (B) the simultaneous cleavage with 9.5/0.5 morpholine/morpholine·HCl. The solid lines are derived by fitting eqs 3 and 4, while the dashed lines are from fitting eqs 5 and 6. The values of the parameters from this curve fitting are listed in Tables IV and V.

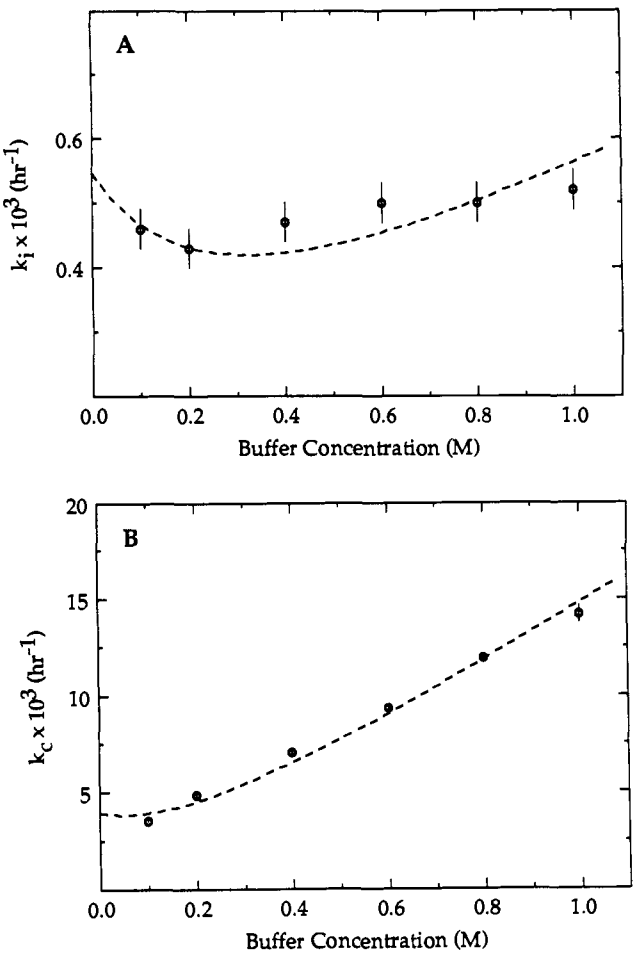

Figure 5. Pseudo-first-order rate constants as a function of buffer concentration for (A) isomerization of 3′,5″-UpU and (B) the simultaneous cleavage with 8/2 morpholine/morpholine·HCl. The dashed curves in both parts were derived by fitting the points to eqs 5 and 6 using the same method as in Figure 3, but carrying over from Figure 3 all values that are unaffected by the buffer ratio and pH. The values of the parameters from this curve fitting are listed in Table V.

buffer ratios, since it (k_2/k_3) does not depend on pH or on buffer concentrations. As the error ranges show, the fit is not very sensitive to the precise value of some of the parameters.

Relatively few data are being fitted to the many parameters required by our mechanism. For this reason, the precise values of the fitting parameters should not be taken too seriously. For instance, the standard deviation for the parameter "c" with the 95/5 buffer is larger than the value itself, so it encompasses meaningless negative numbers.

$$k_{\text{isomerization of UpU}} = \frac{k_1 k_3[\mathrm{BH^+}] + k''_{\mathrm{w}} k_3}{k_{-1}[\mathrm{BH^+}] + k_2[\mathrm{B}] + k_3 + k_{\mathrm{w}}} \quad (3)$$

B = imidazole or morpholine

$$k_{\text{cleavage of UpU}} = \frac{k_1 k_2[\mathrm{BH^+}][\mathrm{B}] + k'_{\mathrm{w}}}{k_{-1}[\mathrm{BH^+}] + k_2[\mathrm{B}] + k_3 + k_{\mathrm{w}}} + \frac{k'[\mathrm{B}] + k''[\mathrm{BH^+}]}{k_{-1}[\mathrm{BH^+}] + k_2[\mathrm{B}] + k_3 + k_{\mathrm{w}}} \quad (4)$$

In terms of the scheme of Figure 1, the constants[26] are defined as follows: k_{w} (units of h^{-1}) = $k_{-1\mathrm{w}} + k_{2\mathrm{w}} + k_{\mathrm{OH}}[\mathrm{OH^-}] + k_{-\mathrm{H}}[\mathrm{H^+}]$; k'_{w} (units of h^{-2}) = $(k_{+1\mathrm{w}} + k_{+\mathrm{H}}[\mathrm{H^+}])(k_{2\mathrm{w}} + k_{\mathrm{OH}}[\mathrm{OH^-}])$; k' (units of h^{-2}) = $k_{+1\mathrm{w}}k_2 + k_1 k_{\mathrm{OH}}[\mathrm{OH^-}][\mathrm{BH^+}]/[\mathrm{B}]$; k'' (units of h^{-2}) = $k_1 k_{2\mathrm{w}} + k_{+\mathrm{H}} k_2[\mathrm{H^+}][\mathrm{B}]/[\mathrm{BH^+}]$; k''_{w} (as used in this equation, units of h^{-1}) = $k_{+1\mathrm{w}} + k_{+\mathrm{H}}[\mathrm{H^+}]$. Of course expressions such as $[\mathrm{OH^-}][\mathrm{BH^+}]/[\mathrm{B}]$ and $[\mathrm{H^+}][\mathrm{B}]/[\mathrm{BH^+}]$ are just equilibrium constants for the buffer; they are written out here so their derivation will be clear.

(26) Note that some of the constants are actually *products* of rate constants, so they have dimensions of (time)$^{-2}$. One cannot add terms with different dimensionality.

Table IV. Fitting Parameters for Figures 3 and 4 Based on Eqs 3 and 4

parameters[a] (units)	B:BH$^+$ (95:5)	B:BH$^+$ (90:10)
a (M)	0.017 ± 0.02	0.034 ± 0.02
b (h)	129 ± 17	300 ± 32
c (Mh)	27 ± 40	43 ± 36
d (M^{-1})[b]	34	34
e	3.1 ± 0.1	3.6 ± 0.2
f (M)	0.34 ± 0.03	0.18 ± 0.06

[a] These parameters involve combinations of the constants in the equations. They are defined in the Experimental Section under data treatment. The errors shown are the calculated standard deviations. [b] Set the same for the two buffer ratios and assumed errorless in the standard deviation calculation.

The curvature observed in the isomerization line of Figure 4A reflects the changed contributions with increasing [buffer] of the mathematical components in the numerator and denominator of eq 3. When the buffer catalyzed terms eventually dominate the uncatalyzed terms, $k_{\text{isomerization}}$ becomes independent of [buffer] and a plateau should result. Before that, the denominator predicts a decrease in rate from added amounts of the basic buffer component. Since this is a strong base and makes up 90% of the buffer, it dominates the increase expected from the acidic buffer component in the numerator.

Table V. Fitting Parameters for Figures 3–5 Based on Eqs 5 and 6

parameters (units)[a]	B:BH$^+$ (95:5)	B:BH$^+$ (90:10)	B:BH$^+$ (80:20)
a (M)	0.018 ± 0.29	0.0087 ± 0.011	0.014 ± 0.07
b (M^2)	0.014 ± 0.11	0.016 ± 0.005	0.018 ± 0.02
c (Mh)	57 ± 750	63 ± 23.2	95 ± 134
d (M^{2}h)	23 ± 188	22.3 ± 8.7	33 ± 37
e[b]	9.7	9.7	9.7
f(M)	1.78 ± 0.04	0.62 ± 0.03	0.21 ± 0.05
g (M^2)	0.29 ± 0.02	0.18 ± 0.02	0.13 ± 0.02

[a] These parameters involve combinations of the constants in the equations. They are defined in the Experimental Section under data treatment. The errors shown are the calculated standard deviations. [b] Set the same for the three buffer ratios and assumed errorless in the standard deviation calculation.

However, curvature in the early [buffer] region is not seen in the cleavage data of Figure 4B, even though eq 4 for that process has the same terms in the common denominator (which is simply made up of terms for all the ways in which the intermediate can partition) that led to curvature in Figure 4A in the low [buffer] region. This noncurvature in Figure 4B can be explained only if we add the new details shown in Figure 1, in which we explicitly include catalysis by species such as water, OH^-, and H^+ in sequential combination with the buffer components.

We had originally[1,10–12] written kinetic terms (cf. eq 1) such as k'[Im] and k''[ImH$^+$] to take account of our experimental observations that catalysis did not fall to zero when only one of the buffer components was present. We pointed out that this probably reflected alternative paths in which k'[Im] corresponded to a first step with water catalysis and a second catalyzed by Im (or the kinetically equivalent ImH$^+$ followed by OH^-), but we did not have sufficient data to justify a more complex kinetic expression. Now, however, our data require the addition of these details to the mechanism in Figure 1, and the explicit inclusion of kinetic terms corresponding to them in eq 4.

To make it clear what is happening, we have separated the first term in eq 4 from the second term. The first term is like the first term in eq 1, combining the expression for uncatalyzed reaction along with that for sequential catalysis in which the first step uses BH$^+$ and the second step uses B. The second term corresponds to paths in which nonbuffer components also play a role along with the buffers; it replaces the simple additive terms in eq 1. As can be seen, the essentially straight line of Figure 4B shows no curvature because the opposite curvatures contributed by the two terms *compensate* in the [buffer] region in which uncatalyzed reactions are still competitive. If the extra two terms are not given the common partitioning denominator, they do not straighten the line. We are able to fit these data using the same parameters as were derived for Figure 4A.

When the 9/1 morpholine buffer is increased up to 2.0 M, the cleavage data (Figure 3B) still fall on the same line, but now some upward curvature is seen (Figure 3A) for isomerization at high [buffer]. This effect seems to be real, and if it does not reflect a medium effect it can be accommodated by assuming that the isomerization path can show some minor catalysis by BH$^+$, only evident at high [buffer]. In eqs 5 and 6 we add the corresponding terms to the numerator of eq 3 and to all the denominators. With this addition we fit the isomerization data with the corresponding curve in Figure 3A, and the cleavage data with the corresponding curve in Figure 3B (that is not distinguishable from the curve using eq 4). The fitting parameters—which are composites of the rate constants in eqs 5 and 6—are listed in Table V. Again, they are defined in the Experimental Section under "data fitting." Here the parameter "e" (k_2/k'_3) was set errorless and the same for all three buffer ratios. Again, the standard deviations for this fitting show that the precise values of several of the constants are not critical to the fit.

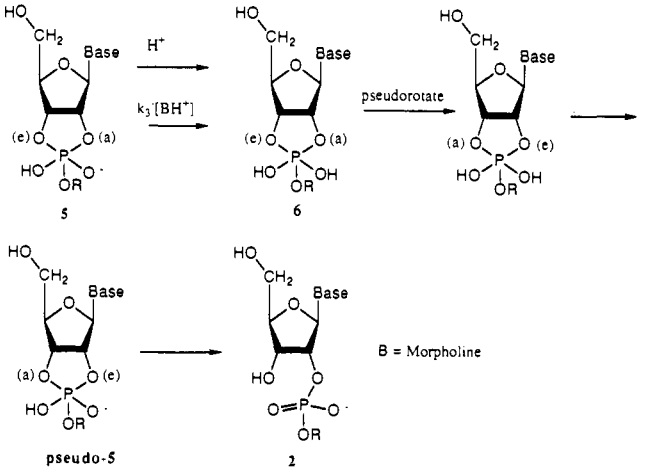

Figure 6. The modification of the scheme of Figure 1 that will explain the upward curvature seen in Figure 3 for the isomerization reaction, but not the cleavage reaction, with very high [buffer].

$$k_{\text{isomerization of UpU}} = \frac{(k''_w + k_1[BH^+])(k_3 + k_3'[BH^+])}{(k_{-1} + k_3')[BH^+] + k_2[B] + k_3 + k_w} \quad \text{B = imidazole or morpholine} \tag{5}$$

$$k_{\text{cleavage of UpU}} = \frac{k_1k_2[BH^+][B] + k'_w}{(k_{-1} + k_3')[BH^+] + k_2[B] + k_3 + k_w} + \frac{k'[B] + k''[BH^+]}{(k_{-1} + k_3')[BH^+] + k_2[B] + k_3 + k_w} \tag{6}$$

We have argued[11] that pseudorotation of the phosphorane is probably faster with a fully protonated phosphorane (**6**) than with the initially formed monoanion (**5**). This is consistent with studies on the H^+ and OH^- catalyzed reactions of dinucleotides.[27] Thus it is likely that the BH$^+$ catalysis of isomerization seen at high [buffer] reflects protonation of the phosphorane monoanion. Buffer catalyzed proton transfers involving tetrahedral intermediates in *carbonyl* reactions are well precedented;[28] they can be seen kinetically, even though normally proton transfers between heteroatoms are essentially diffusion controlled,[29] because protonation competes with rapid unimolecular decomposition of such intermediates. This minor addition (Figure 6) to the overall mechanism permits us to fit all the data over the entire concentration range.

We follow the cleavage kinetics by monitoring the formation of uridine (**4**, base = U) using HPLC, but we also see the other product, uridine-2′,3′-cyclic phosphate (**3**, base = U). At high morpholine buffer concentrations less of this cyclic phosphate is seen, and a new product appears in the HPLC. We identify this as the cleavage product from nucleophilic attack by morpholine on the cyclic phosphate, also formed when authentic samples react under these conditions. As expected, no such nucleophilic cleavage product was seen with *N*-methylmorpholine. Since the morpholine nucleophilic reaction occurs after the cleavage step and does not affect the concentration of the uridine whose appearance we use to follow the kinetics, this morpholine side reaction does not compromise our studies. In related work it was seen that after OH^- catalyzed cyclization there could be hydrolysis of the 2′,3′-cyclic phosphate product.[27]

As a further confirmation of the observed decrease in the rate of isomerization as the buffer concentration is increased, we have

(27) Järvinen, P.; Oivaenen, M.; Lönnberg, H. *J. Org. Chem.* **1991**, *56*, 5396–5401.
(28) cf. Cunningham, B. A.; Schmir, G. L. *J. Am. Chem. Soc.* **1966**, *88*, 551.
(29) Eigen, M. *Angew. Chem., Int. Ed. End.* **1964**, *3*, 1.

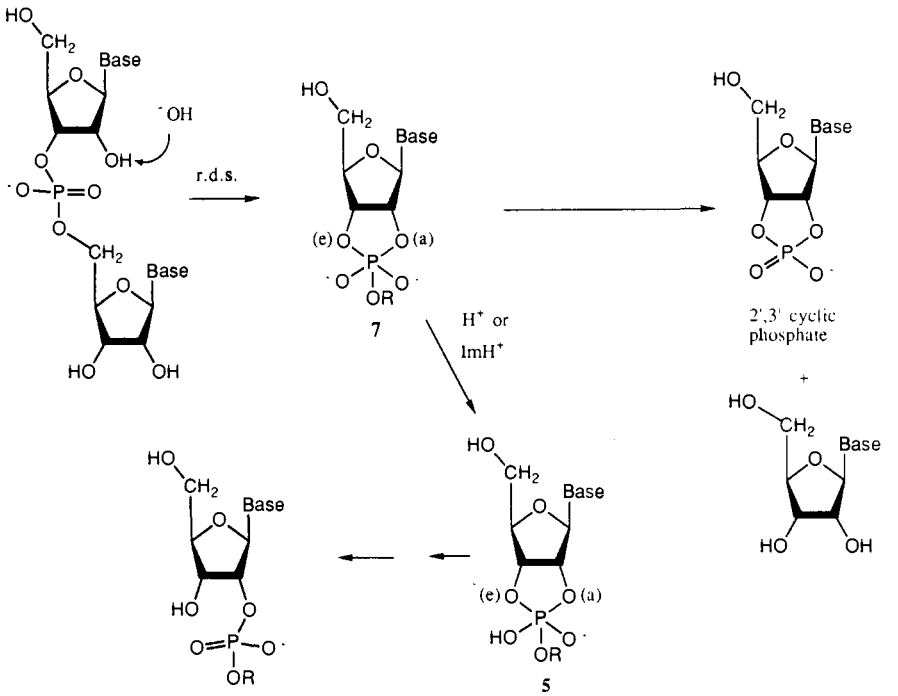

Figure 7. A new mechanism for cleavage and isomerization at high pH, where [OH^-] is high enough, and [H^+] low enough, that cyclization occurs without prior protonation, to form the phosphorane dianion **6**.[27] Trapping of **6** by BH^+ or H^+ converts it to **5** and permits isomerization.

also looked at a 9.5/0.5 morpholine/morpholinium ion buffer (pH 9.6). Figure 4A shows that here too we see a decrease in the rate of isomerization with increasing buffer at the beginning of the curve, down to a plateau near 1.0 M. These data also fit eqs 3 and 4, as Figures 4 (parts A and B) show, and with identical parameters for those of Figures 3 in all cases where they should be identical. The parameters are listed in Table IV. Their variation from the parameters for the 9/1 buffer are reasonable in terms of the predicted effects of differing pH's and buffer ratios.

We have also looked at the cleavage and isomerization of UpU by morpholine buffer at a B/BH^+ ratio of 8/2 (pH 8.9). The data are shown in Figure 5 (parts A and B). As expected, with the less basic buffer the initial negative catalytic effect on isomerization seen with 9/1 buffer is diminished and no longer clearly seen, since the basic buffer effect in the denominator does not so greatly dominate the acidic buffer effect in the numerator. However, again we can fit the data with curves for eqs 5 and 6, corresponding to the mechanism of Figure 1 with the modification of Figure 6. As indicated in Table V, these fits can be achieved with the same value for the parameter that is common to the 9/1, 9.5/0.5, and 8/2 studies and with sensible trends among the other parameters.

It is interesting that both the cleavage rates *and* the isomerization rates, extrapolated to zero [buffer], are smaller for the 8/2 buffer than for the 9/1. At the higher pH one might have expected the isomerization to be slower, since it is acid catalyzed. Still–as expected–at all buffer concentrations a larger *proportion* of the product comes from isomerization with the 8/2 buffer than with the 9/1. The observation that at these high pH's the nonbuffer catalyzed rate of isomerization actually *increases* as the pH is raised further may indicate an additional new mechanism at high pH and low [buffer].

It is well-known that RNA is cleaved by strong base,[27] and with the basic morpholine buffer we have moved into the pH region at which first-order dependence on [OH^-] was observed.[27] At high [OH^-] there is a direct hydroxide-catalyzed mechanism (Figure 7) in which a phosphorane *di*anion **7** is formed in the rate determining step (the change to a more anionic mechanism at high pH is well precedented in *carbonyl* hydrolysis chemistry). Then this dianion can cleave directly, but if it becomes protonated it can undergo the pseudorotation that leads to isomerization.

Protonation is apparently required for isomerization, since we confirm the report[27] that reaction of UpU with 0.1 N NaOH alone leads to cleavage but no detectable isomerization. The concentration of H^+ is negligible at this high pH but is essentially 10^4 higher at the pH 9.3 of the 9/1 buffer or pH 8.9 of the 8/2 buffer. Protonation could involve the H^+ of the medium or perhaps the morpholinium ion BH^+. In either case such protonation should be more effective with the 8/2 than with the 9/1 buffer, consistent with our observation that a higher fraction of the product is the isomer with the 8/2 buffer. Since protonation comes after the rate-determining closure step, and only affects the partitioning of the intermediate, the proton source does not show up in the kinetics.

The extrapolated rate of isomerization at [buffer] = zero is also lower with the 9.5/0.5 morpholine/morpholinium buffer (pH 9.6) than with the 9/1 buffer (pH 9.3). Since in the mechanism of Figure 7 the dianion **7** is formed in a process first-order in [OH^-]—but *two* protons are needed to convert this to the fully protonated phosphorane **6** that is most likely the species that can pseudorotate rapidly—a pH optimum for the overall process is not unreasonable.

The mechanism of Figure 7 may operate at low [buffer] and high pH, where OH^- contributes appreciably to the rate. However, when the buffer catalysis dominates catalysis by OH^-, apparently we still see the (BH^+, then B) sequence with morpholine buffer that we saw with imidazole. This is the only reasonable explanation of the downward slope in the early parts of Figures 3A–5A.

This study has focussed on examining the rates of cleavage and isomerization at varying buffer concentrations with constant buffer ratios; these rates can be fit exactly to appropriate theoretical equations and, with the pH held constant, no corrections need be made for pH effects. Still, we briefly examined the cleavage and isomerization of UpU with *varying* morpholine buffer ratios. As in our earliest study,[10] this was done with high (1.0 M) buffer concentrations, to maximize the buffer contribution to the observed rates. Even so, in this case the effects of changing pH's are greater, since the morpholine buffers are more basic and OH^- catalysis is significant.

Varying the concentration indicated that at the high pH of the basic morpholine buffer the correction for *nonbuffer* catalyzed cleavage reaction was very large. (By contrast, in our studies[10,20]

on the cleavage of polyU with imidazole buffers the correction for nonbuffer catalysis was at most 8%.) As mentioned above, others have studied the very effective OH^- dependent cleavage reaction.[27] Since the correction is so large, and—as described below—the theoretical justification for a simple subtraction of the uncatalyzed rate is not sound, we do not trust and did not attempt a serious quantitative treatment of this data.

How Is This Work Related to the Imidazole Studies? The finding that the reaction rate for cleavage reached a maximum with both Im and ImH^+ present, but that the rate was first-order in [buffer], established a two-step sequence: one buffer component reversibly converts the substrate to a phosphorane anion, whose cleavage is catalyzed by the second buffer component. This was originally seen in the studies with polyU.[10] We have discussed this work more recently, and replotted the data.[20] In that work we plotted both the raw rate data obtained over nine well-defined buffer ratios and pH's and also the data after subtracting the rate at the same pH in the absence of buffer catalysts. As we will mention, there is a question about how to do such a correction, but in this case the largest correction was only 8%, so its details do not matter. Both the corrected *and* the uncorrected data showed a rate maximum when the two buffer components were present, and they showed a linear dependence on buffer concentration.

We were initially unable to decide whether the first catalyst was Im or ImH^+[10] and only later could resolve the matter. The first evidence was the finding[11] that isomerization of UpU was catalyzed only by ImH^+, not by Im. In this work, as in our earlier studies on polyU, we examined both the raw rate data and also the rates with [buffer] extrapolated to zero, to obtain an estimate of the amount of the observed rate that was contributed by solvent species alone. We also fit the data—corrected by subtracting the [buffer] = zero values—to equations obtained from eqs 1 and 2 by subtracting terms 7 and 8. This has evoked the valid criticism[30,31] that such correction terms are buffer independent only when [buffer] = zero.

Even so, we were able to fit the experimental data to these imperfect equations. Details of this fitting are being deposited as supplementary material; as we show in the supplementary material, this fitting was successful because the buffer terms were small compared with the nonbuffer terms in the denominator of terms 7 and 8. In later work, including the present study, we have written equations only for the uncorrected rates in order to avoid this problem.

$$\frac{k'_w}{k_{-1}[ImH^+] + k_2[Im] + k_3 + k_w} \quad (7)$$

$$\frac{k''_w}{k_{-1}[ImH^+] + k_2[Im] + k_3 + k_w} \quad (8)$$

The current work shows that with morpholine buffer there is also a two-step mechanism for cleavage, in which BH^+ acts to generate an intermediate and B is the catalyst that sends it along the cleavage pathway. The negative catalysis of isomerization seen in Figures 3A and 5A is possible only if a buffer component acts in the second step of cleavage to speed the cleavage pathway, changing the partitioning and leading to a lower observed rate of isomerization. Rigorously, such negative catalysis shows only that isomerization goes through an intermediate and that there is a second pathway from that intermediate—catalyzed by buffer—that leads to a different product. However, there can be no doubt that this second path is the one leading to the observed cleavage reaction. The fact that this negative catalytic effect is diminished in Figure 4A, in which the buffer is less basic, shows that the second catalyst in cleavage is the *basic* buffer component, as we had concluded for imidazole catalysis.

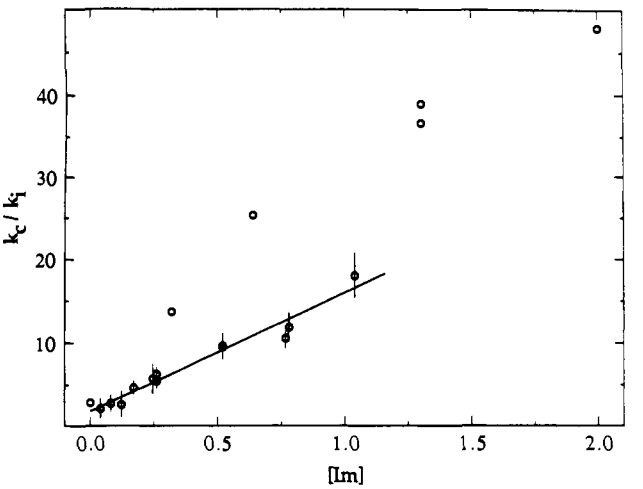

Figure 8. A plot of all the data for cleavage and isomerization of 2′,5″-UpU (from ref 11), using eq 9. The upper points shown without error bars are for data obtained in unbuffered runs using only one buffer component. Only the reliable data obtained with defined buffer ratios are shown with error bars and used to fit eq 9.

Is There a Common Intermediate for Isomerization and Cleavage? Recently it has been claimed ("The Proposed Mechanism Is Incompatible with the Kinetic Measurements")[31] that such a common intermediate is excluded by a kinetic analysis of our published data on imidazole catalyzed UpU cleavage and isomerization, although an alternative mechanism was not proposed nor were new data contributed. A common intermediate is extremely likely on the face of it, since both processes must, by independent evidence, proceed through a phosphorane intermediate—cleavage because of the bell shaped curve and linear [buffer] dependence and isomerization because of the requirement for pseudorotation. However, we have reexamined our earlier data in light of this critique.

Haim has pointed out that if our mechanism is correct, and cleavage and isomerization proceed from a common intermediate—the cleavage path showing catalysis by Im and by nonbuffer species but the isomerization path showing only nonbuffer catalysis—then eq 9 should be followed. In his Figures 4, 5, and 6 Haim plotted some of our published data[11] on cleavage and isomerization of 2′,5″-UpU using such an equation.

$$k_c/k_i = k_a[Im] + k_b \quad (9)$$

We have aggregated all our published data on 2′,5″-UpU cleavage and isomerization, obtained with defined buffer ratios, in Figure 8. Of course k_b is not independent of pH, but with the imidazole concentrations used k_b is small compared with k_a[Im], so aggregation of the data generated with different buffer ratios may not introduce large errors.

As the reader can see in Figure 8, the data obtained with defined buffering fit a straight line. (Even the less reliable data from essentially unbuffered runs using only one buffer component follow the same general trend.) Thus such a plot does not exclude a common intermediate for cleavage and isomerization, contrary to Haim's assertions,[31] it actually furnishes additional support for our proposed mechanism.

As mentioned above, we and Anslyn are depositing as supplementary material to this paper the details of the previous[11] fit of the *corrected* data (corrected by subtracting the observed rate at the same pH but with [buffer] extrapolated to zero) for UpU cleavage and isomerization by imidazole buffers to the

(30) Menger, F. *J. Org. Chem.* **1991**, *56*, 6251–6252. The claim that microscopic reversibility was violated in ref 11 is incorrect, as a reading of that paper will confirm. The author writes out a detailed mechanism that he ascribes to us, but this mechanism appears nowhere—either explicitly or implicitly—in our publications. We did not propose that water catalyzed a reaction in only one direction, we simply (p 4479) wrote equations for the reactions catalyzed only by buffer in which we had subtracted terms (7) and (8) from eqs 1 and 2. With such a subtraction the terms k'_w and k''_w disappear from the numerators, but of course the term k_w does not disappear from the common denominator.

(31) Haim, A. *J. Am. Chem. Soc.* **1992**, *114*, 8384–8388.

(roughly) corrected equations in which we had subtracted terms (7) and (8). The data indeed fit the equations, as we originally had claimed,[11] and surprisingly well considering the approximation in the correction and the roughness of some of the data. However, there are errors in Table I of ref 11, that lists the values of the constants needed to generate the lines. The data can indeed be fit (as shown in the supplementary material) with lines generated from eqs 1 and 2 of that paper—and with no change in the numerical values listed in Table I except for the values of k_3. These should be 0.10 and 0.06 in columns 2 and 3; they were apparently mistranscribed. However, k_1, k_2, and k_{-1} have units of 10^{-4} M^{-1} min^{-1}, k_3 and k_w have units of 10^{-4} min^{-1}, while k' and k'' have units of 10^{-6} min^{-1}. The concentration of the substrate UpU was taken as 16 mM. These ambiguities and errors may explain why Haim had trouble reproducing our lines from the published values in Table I.

We have also examined fitting of the *uncorrected* data from ref 11 to eqs 3 and 4 of this paper. These early somewhat qualitative data had significant published error bars, some with limited buffering and all without control of ionic strength, *as we ourselves first pointed out.*[11] (In preliminary unpublished work, we saw that ionic strength effects were small.) Even so, as figures being deposited in supplementary material show, all the published data that have defined buffer ratios can be fitted by the theoretical lines. With few points and many parameters, we do not take such a fit seriously *except* to refute the claim[31] that a fit is impossible.

The current study clearly shows that there is a common intermediate for the morpholine catalyzed reaction, since only a branching scheme from a common intermediate can explain the partitioning effects that we describe as negative, the *decrease* in isomerization rate as [buffer] is increased. Since we had found related effects previously for imidazole catalysis with ApA, it seems clear that there is a common intermediate there as well.

Others have concluded that H^+ catalysis of dinucleotide cleavage and isomerization also proceeds through a common intermediate.[27] Furthermore, as indicated above, we have no difficulty fitting our published data to the predictions of our mechanism, in contrast to claims elsewhere.[31] The current study, in which careful control of pH and ionic strength was included, produced data that *require* a common intermediate to fit the observed kinetics, and they require a pathway in which the cleavage leg of this partitioning scheme is catalyzed by the buffer base.

Conclusions

These studies certainly confirm our previous conclusion that buffer catalyzed cleavage and isomerization of UpU (and of ApA) proceeds through a common intermediate, whose formation is catalyzed by the acidic buffer component and whose cleavage is catalyzed by the buffer base. By extension, the same mechanism must apply for polyU. Even with the earlier somewhat qualitative arguments for the mechanism, it seemed highly likely. However, the quantitative curve fitting possible in the present study not only confirms the correctness of the general mechanism previously porposed, it also furnishes evidence for the details of some individual steps that could not be deduced from the earlier studies.

As described, the mechanistic conclusions have furnished insight into the mechanism of the enzyme ribonuclease A. They have also guided the synthesis of improved enzyme mimics.

Experimental Methods

Materials and Buffers. Nucleotides and uridine were obtained from Sigma. Morpholine (Aldrich) was redistilled before use. Buffers were prepared by mixing solutions of morpholine and morpholine hydrochloride in the stated ratios and then slightly adjusted by addition of a little more of one buffer solution (to compensate for any concentration deviations) to obtain the theoretical pH value. Aliquots of the same stock buffer solution were used to prepare all the concentrations used. On dilution and adjustment to ionic strength 0.2 M with NaCl, they still had the theoretical pH.

Kinetic Experiments. The kinetic studies were done as previously described,[11] in capillary sample tubes incubated at 80 °C. Ionic strength was kept constant at 0.2 M with added NaCl. HPLC analysis with an internal standard was used to follow the appearance of uridine (cleavage) and of 2′,5″-UpU (isomerization). Differences from previous procedures are emphasized here: (1) The capillary tubes used as kinetics vessels were not silylated before use. (2) The standard calibration plots required by the assay were made from solutions that were 12 μM in internal standard (potassium *p*-nitrobenzenesulfonate) and 2, 6, 12, 25, 40, 60, 80, 100, and 200 μM in 2′,5″-UpU and 2, 6, 12, 25, 75, 100, and 200 μM in uridine. (3) The substrate stock solution was prepared as 16 mM 3′,5″-UpU and 480 μM standard in deionized water. (4) The morpholine buffer stock solution was prepared with use of 3.2 M morpholine solution and 3.2 M morpholine·HCl as described above. (5) The HPLC eluent was 4.5 mM pH 7 phosphate buffer containing 4.0% of MeOH. A flow rate of 1.0 mL/min yielded baseline resolution of uridine (2.1 min), internal standard (2.9 min), 2′,5″-UpU (4.3 min), and 3′,5″-UpU (8.7 min). (6) The HPLC solvent delivery system was Waters Model 510. The UV detector was Waters 440 at a sensitivity of 0.01, and the chromatograms were integrated using a Spectra-physics Data-jet integrator. A 10 μM sample loop in a Rheodyne 7125 injector and Rainin instrument company's Microsorb column (catalog no.: 80-205-c3) were used.

A Typical Procedure for the Measurement of the Rate Constant of Cleavage and Isomerization of 3′,5″-UpU (4 mM) Catalyzed by 1.0 M Morpholine Buffer (B:BH^+ = 90:10) at 80 °C. To a 250-μL centrifuge tube were added stock solutions of 50 μL of internal standard and 3′,5″-UpU, 62.5 μL of morpholine (B:BH^+ = 9:1), and 20 μL of 1 M NaCl solution, and 67.5 μL of deionized water via micropipet and the mixture was stirred on a vortex mixer. The solution was divided among 9–11 capillary tubes with a syringe. The capillary tubes were flame sealed, incubated in an 80 °C oven controlled by an I^2R L6-1000SS Therm-O-Watch, and then periodically removed from the oven during the experiment. After a defined time, 10 μL of the reaction mixture in a capillary tube was transferred to a 250-μL centrifuge tube with a syringe, diluted with 90 μL of deionized water, stirred on a vortex mixer, and frozen in dry ice or injected to HPLC for analysis. The concentration of the substrate 3′,5″-UpU was 4 mM and ionic strength was 0.2 M in the reaction. The pseudo-first-order rate constant was obtained by initial rate treatments.

Data Treatment. Reactions were run to only a few percent conversion of substrate, so a logarithmic treatment was unnecessary. Results of a typical run are shown in Figure 2.

All points in the plots are the results of at least duplicate runs. Based on the proposed kinetic model, computer fitting of theoretical curves to the observed points was done by nonlinear least squares numerical analysis programs (Igor and Passage) for the Macintosh computer and a program (stepit) written for the Vax computer for more complex cases. Different fitting programs gave consistent results with regard to the fitting itself and fitting parameters. In order to do the actual fitting, eqs 3 and 4 were divided by k_1k_3 for both denominator and numerator to form the following fitting function:

$$k_i = \frac{\delta x + a}{bx + c}$$

$$k_c = \frac{\delta(1-\delta)dx^2 + ex + f}{bx + c}$$

where $a(M) = k''_w/k_1$; $b(h) = (k_{-1}\delta + k_2(1-\delta))/k_1k_3$; $c(Mh) = (k_3 + k_w)/k_1k_3$; $d(M^{-1}) = k_2/k_3$; $e = (k'(1-\delta) + k''\delta)/k_1k_3$; $f(M) = k'_w/k_1k_3$; $\delta = [BH^+]/([BH^+] + [B])$; $x = [BH^+] + [B]$. Equations 5 and 6 were divided by $k_1k'_3$ for both denominator and numerator to form a different set of fitting equations

$$k_i = \frac{\delta x^2 + ax + b}{cx + d}$$

$$k_c = \frac{\delta(1 - \delta)ex^2 + fx + g}{cx + d}$$

where a (M) $= (k_1k_3 + k'_3k''_w)\delta/k_1k'_3$; b (M^2) $= k_3k''_w/k_1k'_3$; c (Mh) $= ((k_{-1} + k'_3)\delta + k_2(1 - \delta))/k_1k'_3$; d (M^{2}h) $= (k_3 + k_w)/k_1k'_3$; $e = k_2/k'_3$; f (M) $= (k'(1 - \delta) + k''d)/k_1k'_3$; g (M^2) $= k'_w/k_1k'_3$; $\delta = [BH^+]/([BH^+] + [B])$; $x = [BH^+] + [B]$. These equations are used in the fitting, and the derived parameters are listed in Tables IV and V.

With a given buffer ratio the parameters of the common denominators of the fitting equations—or of eqs 3 and 4—were set the same when fitting the cleavage or the isomerization data as in Figures 3–5. However, almost all the parameters change with different buffer ratios and the resulting different pH's, and there are no simple relationships between them. Thus, fitting was done independently for different buffer ratios, setting only parameter d of Table IV or parameter c of Table V constant since those parameters do not involve terms that vary with buffer ratio or with pH dependent "water catalysis". With such a procedure standard deviations—which would be available only within a given plot—do not reflect the overall fitting accuracy since they do not include a consideration of the errors in the data points themselves. Nonetheless, the standard deviations of the fitting parameters are listed in Tables IV and V. The accuracy of fit of the theoretical treatment to the experimental results can best be judged visually in Figures 3–5.

Acknowledgment. This work was supported by the NIH and the ONR. We acknowledge helpful suggestions from referees and editors.

Supplementary Material Available: Individual rate constants from which the average values in Tables I–III were calculated, original data for refs 11 and 12, and details of the plots of the data in ref 11 (37 pages). Ordering information is given on any current masthead page.