Mechanism of RNA Cleavage by Imidazole. Catalysis *vs* Medium Effects

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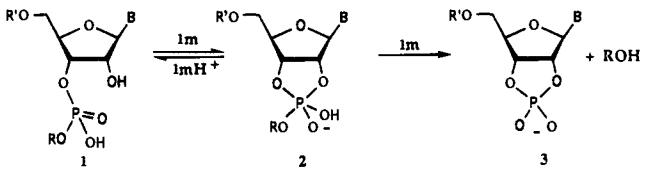
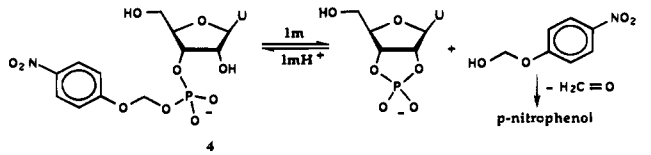
In a series of papers which have attracted widespread attention, Breslow and his co-workers presented evidence that the imidazole-catalyzed hydrolysis of RNA and various derivatives **1** (poly(U), UpU, ApA) is characterized by a "bell-shaped pH–rate profile.[1]" This evidence was interpreted in terms of a sequential bifunctional mechanism, in which the formation and breakdown of a pentacovalent phosphorane intermediate are catalyzed by different mechanisms.[2] This interpretation, which has potentially important implications for the mechanism of action of ribonucleases, has been sharply criticized,[3] and equally strongly defended, in particular by further experiments which extend the original data.[4] We report new results with a model substrate which parallel the original observations of the Breslow group but show that they represent not changes in rate-determining step but medium effects.



Central to the Breslow interpretation is the mechanistically unsymmetrical partitioning of the hydroxyphosphorane intermediate **2**. We found this surprising and have carried out a more detailed study of the basic reaction, using a wider range of general acid-base catalysts than was practical for the very slow[5] reactions studied in the original work.

Our chosen substrate (**4**) is an acetal ester of uridine 3′-phosphate. The leaving group (p-$NO_2C_6H_4OCH_2OH$) is designed to have a relatively high pK_a, not too far from that of the 5′-OH of a ribose derivative, and to break down rapidly when released to generate p-nitrophenol.[6] This permits continuous monitoring of the reaction, and we have collected good quality data for catalysis by many general bases.



Our results are consistent with the observations of Breslow and co-workers for the reactions of nucleotides.[1,2] Thus Figure 1, which shows our data for imidazole catalysis of the hydrolysis[7] of **4**, reproduces the "bell shape" they describe. However, we could not fit our data accurately to the kinetic equations generated by the Breslow mechanism: rather than describing a bell-shaped curve, the data fall on a good straight line up to 60–70% free base imidazole, before falling off at higher pH.[8]

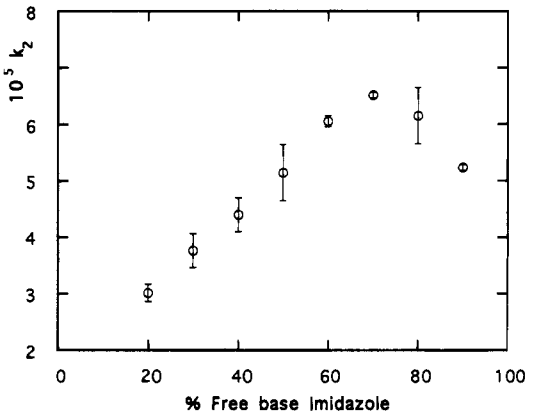


Figure 1. Catalysis of the hydrolysis of **4** by imidazole, as a function of the buffer ratio. Each point represents the second-order rate constant (in L mol^{-1} s^{-1}) for catalysis by imidazole at the buffer ratio indicated: error bars are calculated from the second-order plots, and they reflect different numbers of data points measured at different buffer ratios. (Data at 70 °C and ionic strength 1 M, in water.)

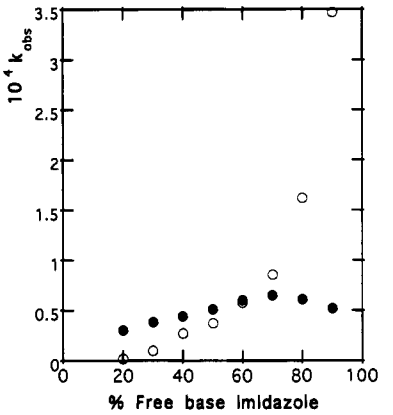


Figure 2. Second-order rate constants (filled circles, L mol^{-1} s^{-1}) for catalysis of the hydrolysis of **4** by imidazole, at varying buffer ratios, compared with the first-order rate constants (k_0, open circles, s^{-1}) for the background hydrolysis reaction at the same pH. (Data at 70 °C and ionic strength 1 M, in water.)

The interpretation of buffer catalysis data is always subject to a degree of uncertainty, because changing the concentration of the catalyst necessarily changes the medium. This uncertainty can be minimized by carefully designed experimentation[9] but is greatest where the increase in rate is proportionately small and high concentrations of catalyst are used. Figure 2 shows how the rate constants for catalysis of the hydrolysis of **4** by 1 M imidazole buffer, taken from Figure 1, compare with k_0, the first-order rate constant for the accompanying uncatalyzed reaction (obtained by extrapolation of the second-order plot

(1) Breslow, R.; Anslyn, E.; Huang, D.-L. *Tetrahedron*, **1991**, *47*, 2365.
(2) (a) Breslow, R.; Labelle, M. *J. Am. Chem. Soc.* **1986**, *108*, 2655. (b) Ansly, E.; Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 4473. (c) Breslow, R.; Huang, D.-L. *J. Am. Chem. Soc.* **1990**, *112*, 9621. (d) Breslow, R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1201.
(3) Menger, F. M. *J. Org. Chem.* **1991**, *56*, 6251. Haim, A. *J. Am. Chem. Soc.* **1992**, *114*, 8384.
(4) Breslow, R.; Xu, R. *J. Am. Chem. Soc.* **1993**, *115*, 10705.
(5) Which typically have half-lives of days at 80 °C and have to be followed by HPLC.
(6) The hydrolysis of 4 is several hundred times faster than that of UpU at the same pH (data of Lönnberg (Lönnberg, H. *J. Org. Chem.* **1991**, *56*, 5396): comparison at 70 °C). From this and other extrapolations we estimate a pK_a in the region of 11 for p-$NO_2C_6H_4OCH_2OH$.
(7) Each point in Figure 1 represents the second-order rate constant for catalysis by imidazole buffer and is derived from a good second-order plot, showing that the reaction is first order in the buffer. The data refer specifically to hydrolysis, rather than phosphoryl migration, which is not expected to be significant above pH 6 with our substrate.
(8) The data of Anslyn and Breslow[2b] can be viewed in a similar way. The maximum in the observed second-order rate constant (Figure 1) occurs at slightly higher pH (~70%, compared with ~50% free base imidazole for UpU) for our, more reactive, substrate.
(9) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969; p 585 ff.

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concerned to zero buffer concentration). Catalysis by imidazole at 80% and 90% free base takes place against a rapidly increasing background hydroxide-catalyzed reaction: at 90% free base the catalyzed reaction accounts for less than 12% of the observed rate of hydrolysis in the presence of 1 M (total) imidazole.[10]

The simplest explanation of the fall off in catalysis at high free base illustrated in Figure 1 would be a medium effect on this background reaction. This we tested by measuring the effects of added imidazole free base on the rate of hydrolysis of **4** at pH 9.5, where only the hydroxide-catalyzed reaction is observed. The rate of this reaction is indeed significantly depressed by added imidazole. The effect is proportional to the concentration added, and it amounts to 18% for 1 M imidazole:[11] rate depressions of similar magnitudes are caused by added pyrrole or dioxane.

These data allow us to correct the results shown in Figure 1 for the negative effect of increasing concentrations of imidazole on the background, hydroxide-catalyzed hydrolysis. The result is shown in Figure 3.[12] In the corrected plot the bell shape disappears, and with it the evidence for any but uncomplicated general base catalysis by imidazole of the hydrolysis of at least this RNA model.[12] A hydroxyphosphorane (**2**, or more likely the dianion) remains a likely intermediate in the reaction, but it is not required by the observed kinetics.

(10) Buffer catalysis disappears completely above about pH 9, where the background, hydroxide-catalyzed reaction becomes overwhelmingly predominant.

(11) The slope of the plot of k_0 *vs* imidazole concentration (four points, from 0 to 1.0 M, corrected to pH 9.50 to allow for minor pH changes) is $(-5.33 \pm 0.50) \times 10^{-3}$ M^{-1} s^{-1}. The depression in the observed rate as a function of imidazole concentration is thus $(-5.33 \times 10^{-3})k_{OH}[OH][Im]$. At pH 9.50 (0.3 M carbonate buffer, 70 °C, and ionic strength 1.0 M) in the absence of added imidazole, $k_{OH}[OH] = 2.94 \times 10^{-2}$ s^{-1}.

(12) The corrected plot (Figure 3, straight line, correlation coefficient $r = 0.996$ for eight data points) was obtained by adding to each data point (filled circle) the calculated negative medium effect on the hydroxide reaction $(5.33 \times 10^{-3})k_{OH}[OH][Im]$, obtained as described above:[11] 50% free base imidazole buffer gives a pH of 6.37 at 70 °C and ionic strength 1.0 M). The corrected plot has a small positive intercept at zero free base, corresponding to a small contribution from a general acid catalyzed reaction, or the kinetically equivalent general base catalyzed hydrolysis of the conjugate acid of **4**. The apparent rate constant (1.34×10^{-5}, compared with 8.16×10^{-5} M^{-1} s^{-1} for the general base catalyzed reaction) is of the order of magnitude expected for the latter reaction.[13]

(13) Chandler, A. J.; Hollfelder, F.; Kirby, A. J.; O'Carroll, F.; Strömberg, R. *J. Chem. Soc., Perkin Trans. 2* **1994**, 327.

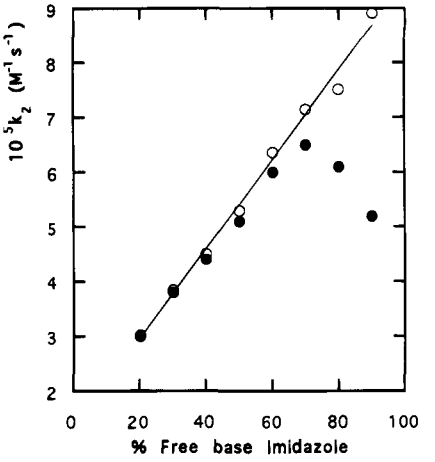

Figure 3. Catalysis of the hydrolysis of **4** by imidazole, as a function of the buffer ratio. The closed circles represent the data displayed in Figure 1, and the open circles represent the same data corrected for the medium effect of imidazole on the hydroxide-catalyzed reaction, as described in the text.[12] (Data at 70 °C and ionic strength 1 M, in water.)

The behavior of Breslow's substrates is closely similar to that of **4**, with maxima near 60% free base imidazole in the bell-shaped plots corresponding to Figure 1. For UpU the proportion of hydroxide- *vs* imidazole-catalyzed reaction appears to be comparable to that for **4**, though only two data points are available.[2b] For poly(U),[2a] the rate of the imidazole-catalyzed reaction is similar to that for UpU, and recent results from Lönnberg's laboratory[14] show that "under alkaline conditions the hydrolysis rates of poly(U) and 3′,5′-UpU are equal." We conclude that a common explanation of the bell shape is likely.[15]

Acknowledgment. R.E.M. thanks the Engineering and Physical Sciences Research Council of Great Britain for a studentship.

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(14) Kuusela, S.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1994**, 2109.

(15) The bell shape of Figure 1 also disappears in experiments where we correct for the medium effect by adding sufficient organic cosolvent (pyrrole) to keep constant the concentration of imidazole free base plus added cosolvent. We are currently extending these experiments to the imidazole-catalyzed hydrolysis of UpU.