Mechanisms of catalysis by imidazole buffers of the hydrolysis and isomerisation of RNA models

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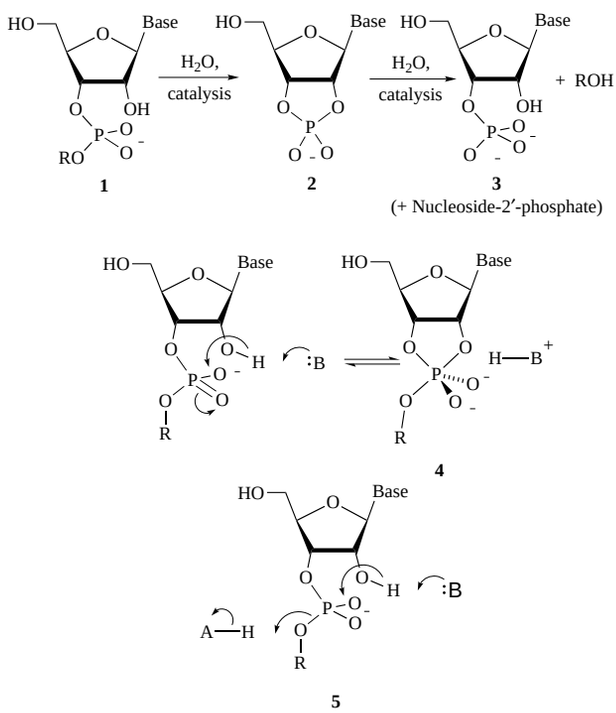
We report second-order rate constants for catalysis by 0.1–0.7 M imidazole–imidazolium ($Im–ImH^+$) buffers of the hydrolysis and isomerisation of the dinucleoside monophosphate uridyl(3′→5″)uridine, a comparative study of the same reactions of the chimeric oligonucleotide TTUTT, and systematic measurements of medium and ionic strength effects on the reactions. At the concentrations used in this work: (*i*) the hydrolysis of 3′,5″-UpU is catalysed both by imidazole and, less effectively, by its conjugate acid, in reactions which are of the first order in the general acid or base. (*ii*) Isomerisation to 2′,5″-UpU is catalysed only by ImH^+, as is the isomerisation of TTUTT: there is no evidence for 'negative catalysis'. (*iii*) The hydrolysis of TTUTT is also catalysed by both imidazole and by its conjugate acid, but in this case catalysis by ImH^+ is the more effective. At constant ionic strength we see no sign of the bell-shaped dependence on the degree of protonation of the buffer seen previously with poly-U. (*iv*) The emergence of the 'bell-shape' in the reaction of UpU is shown to depend on the ionic strength of the medium.

We conclude that the hydrolysis of the internucleoside bond in these phosphodiesters involves two, parallel, reaction pathways: (*a*) a more or less concerted general base catalysed reaction; and (*b*) a two-step process, involving the rate determining general acid catalysed breakdown of the phosphorane monoanion intermediate 10. Near pH 7, in the presence of Im and ImH^+, the two pathways run at comparable, but very slow rates. Ribonucleases logically combine the catalytic advantages of both pathways.

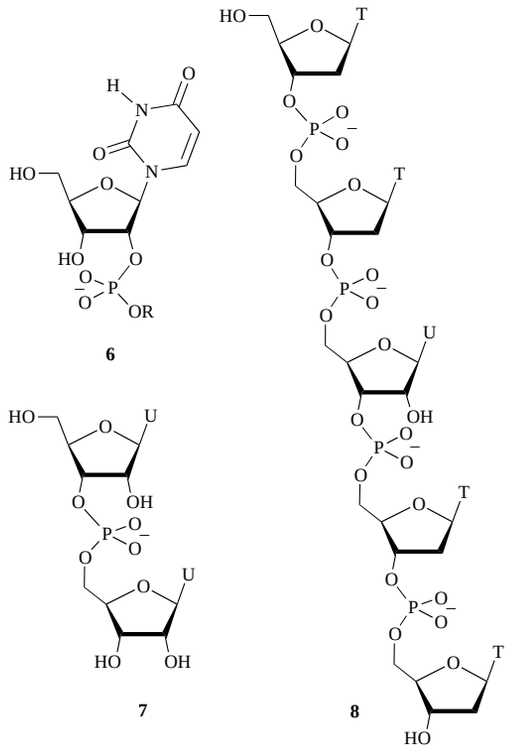
The ribonucleases (RNAses) are of special interest in the study of the chemical mechanisms of enzyme action. The phosphodiester group forms part of the backbone of the nucleic acids; it is extraordinarily stable to hydrolysis, so that enzymes catalysing the reaction are among the most efficient known; and the nucleophile—the ribose 2′-hydroxy group—is part of the substrate, attached adjacent to the 3-phosphodiester group to a cyclic structure of limited conformational flexibility. So the geometry of nucleophilic attack by oxygen on phosphodiester phosphorus, which is one key to high efficiency,[1] is well-defined, and this part of the process can be conveniently studied as an intramolecular reaction in simple systems **1**. Recent work in the area (critically reviewed by Perreault and Anslyn[2]) has concentrated on mechanisms for catalysis of the primary reaction **1**→**2** when the leaving group RO(H) is poor—typically a nucleoside 5′-OH group. The mechanisms concerned, and the available data, have been the subject of some controversy and a certain amount of confusion, which the present work is intended to help resolve.

The requirements for catalysis are in principle simple. The nucleophilic OH must lose a proton, which near pH 7 requires a general base; and the leaving group must gain one, from a general acid stronger than water. In the best understood enzyme, RNAse A, the imidazole groups of two active-site histidines are involved, one of them as the conjugate acid. So the most directly relevant model work is that using imidazole buffers, mixtures in different ratios of the free base (Im) and the imidazolium cation (ImH^+). A detailed mechanism for catalysis by this buffer system has been presented (and extrapolated to the enzyme mechanism) by Breslow and his co-workers.[3] A number of authors have criticised aspects of this work,[2] which nevertheless remains the most substantial experimental contribution to the problem.

Important unanswered questions include the status of possible pentacovalent (phosphorane) intermediates (*e.g.* **4**) and the degree of concertedness of bond-making and -breaking at phosphorus (**5**).[2] It ought to be possible to answer these questions by systematic experiments on simple systems. In practice



these present considerable technical difficulties. No change of chromophore is involved, so reactions have to be followed by separation-analysis of aliquots, usually by HPLC. The reactions in imidazole buffers (near pH 7) are extremely slow, with typical half-lives for hydrolysis even at 80 °C of several weeks; and catalysis is modest. For this reason concentrated buffer solutions have been used in the great majority of published experiments; but the problem then arises of distinguishing weak catalysis from the medium effects of molar quantities of what amounts to an added organic cosolvent. Finally, up to three competing reactions may be involved in the disappearance of a



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simple system **1**: hydrolysis to **2**, the reversible isomerisation to the 2′-phosphate (*e.g.* the uridylyl derivative **6**), and the subsequent hydrolysis of **6**: all of which may occur both spontaneously and with buffer catalysis; and all of which are subject to the same technical difficulties. So a major problem has been the availability of accurate, self-consistent kinetic data, obtained under controlled conditions, on which to base an analysis and a mechanistic interpretation.

We report an extensive set of careful, systematic measurements of medium and ionic strength effects on the rates of hydrolysis and isomerisation of the dinucleoside monophosphate uridyl(3′→5″)uridine (3′,5″-UpU, **7**) in imidazole–imidazolium buffers: and a comparative study of the chimeric oligonucleotide TTUTT **8**, which undergoes the same reactions at the unique in-chain uridylyl residue, in a situation which better models the environment in RNA.

Results and discussion

Kinetic results and analysis

The disappearance of the starting material **7** and the formation of its 2′,5″-isomer **6** were followed (at 80 °C) for 1–2 half-lives. To avoid ambiguities resulting from processing, the data obtained were fitted to a full rate equation for parallel first-order processes (for details see the Experimental section). This procedure gives, in principle, first-order rate constants for the hydrolysis and isomerisation of both isomers, with the advantage that no simplifying assumptions have to be made. We made measurements at four different buffer concentrations, at four different buffer ratios, and report derived second-order rate constants for catalysis by imidazole and its conjugate acid. We have deliberately kept to relatively low concentrations of imidazole buffers (up to 0.7 M total buffer), to minimise medium and specific salt effects of buffer constituents. But we have also addressed the question of ionic strength and medium effects directly, paying particular attention to the effects on the background, buffer-independent reactions. Published measurements report mostly first-order rate constants, measured at high buffer concentrations, without control of ionic strength.

Hydrolysis. The data appear in Figs. 1–6, and Tables 1–5. Fig. 1 shows typical second-order plots for the hydrolysis of 3′,5″-UpU at a constant ionic strength of 1.0 M. The plots give

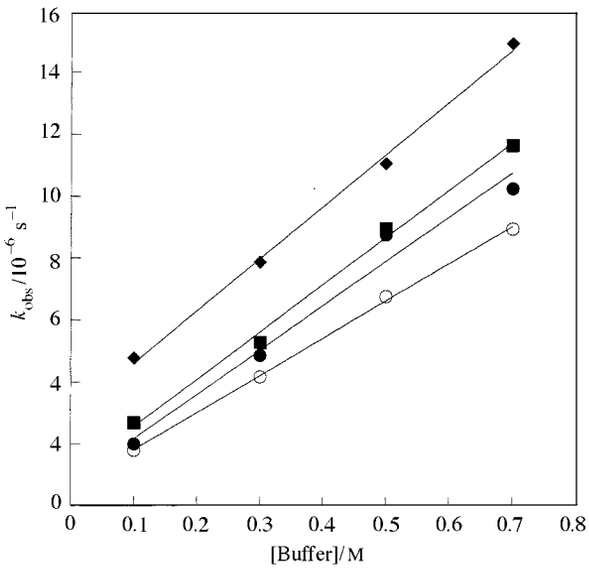


Fig. 1 Second-order plots for catalysis by imidazole buffers of the hydrolysis of 3′,5″-UpU, at 80 °C and ionic strength 1.0 M (KCl). Data are for buffers containing 30, 40, 60 and 80% free base.

acceptable straight lines, consistent with previous conclusions that catalysis involves only first order terms in [Im] and/or [ImH$^+$]. The slopes and intercepts of these lines give, respectively, second order rate constants k_2 for buffer catalysis, and first-order rate constants k_0 for the uncatalysed reaction at the pH of the buffer concerned. Full data sets as shown in Fig. 1 were collected for both hydrolysis and isomerisation, at two different ionic strengths, 0.5 and 1.0 M (KCl). These results are summarised in Table 1.

As reported earlier,[4] catalysis by imidazole buffers is modest: compared to the uncatalysed reaction, 0.7 M buffers enhance the hydrolysis by factors of 5–20, depending on the conditions. Thus even in the most concentrated buffer solutions used, the proportion of the uncatalysed reaction varies from 5% to as high as 20%, increasing with pH. [This is still far less than for the hydrolysis of model compound **1** (R = $CH_2OC_6H_4NO_2$-*p*), with a moderately good leaving group, for which the buffer-independent hydrolysis is dominant at higher pH.[5]] Catalysis of the isomerisation of 3′,5″-UpU is weaker still. The largest rate enhancement is observed in 30% free base solutions, where 0.7 M buffer enhances the rate of isomerisation by a factor of 1.8. For isomerisation the uncatalysed reaction is generally at least as important as the buffer-catalysed reaction. Similarly, and in contrast to earlier reports on imidazole catalysis, observed first-order rate constants for isomerisation and hydrolysis are comparable. In 0.1 M 30% free base imidazole solution isomerisation is twice as fast as hydrolysis; but as the buffer concentration or the base content increases, the rate of the hydrolysis increases more rapidly, until in 0.7 M 80% free base solution hydrolysis is four times faster than isomerisation.

First-order rate constants for buffer-independent hydrolysis and isomerisation are shown as a function of pH in Fig. 2. Consistent with an earlier report[6] the rate of hydrolysis is first-order in hydroxide ion concentration above pH 6, but the rate of isomerisation is independent of pH in this region. Below about pH 7 buffer-independent isomerisation is faster than hydrolysis. Comparison of the results for hydrolysis at ionic strength 0.5 and 1.0 M shows that higher ionic strength favours hydrolysis, while isomerisation appears to be not much affected. This is consistent with the increased charge developed in the transition state dianion[6] for the hydroxide-catalysed hydrolysis. The isomerisation reaction is pH-independent: the transition state carries the same charge as the reactant, and the rate is not significantly affected by the change in ionic strength.

Table 1 Rate constants for the spontaneous (k_0) and buffer-catalysed (k_2) hydrolysis and isomerization of 3′,5″-UpU, at 80 °C and constant ionic strength (I)

		Ionic strength = 0.5 M			
		Hydrolysis		Isomerisation	
Buffer (%) free base	pH	k_0/s^{-1}	k_2/dm^3 mol^{-1} s^{-1}	k_0/s^{-1}	k_2/dm^3 mol^{-1} s^{-1}
30	5.85	$3.0 \pm 5.9 \times 10^{-8}$	$1.21 \pm 0.13 \times 10^{-6}$	$2.40 \pm 0.07 \times 10^{-7}$	$2.94 \pm 0.15 \times 10^{-7}$
40	6.09	$-0.4 \pm 6.2 \times 10^{-8}$	$1.51 \pm 0.13 \times 10^{-6}$	$2.53 \pm 0.01 \times 10^{-7}$	$2.26 \pm 0.03 \times 10^{-7}$
60	6.45	$6.9 \pm 3.3 \times 10^{-8}$	$1.60 \pm 0.07 \times 10^{-6}$	$2.53 \pm 0.03 \times 10^{-7}$	$1.23 \pm 0.07 \times 10^{-7}$
80	6.88	$1.87 \pm 0.15 \times 10^{-7}$	$1.52 \pm 0.03 \times 10^{-6}$	$2.27 \pm 0.03 \times 10^{-7}$	$1.12 \pm 0.01 \times 10^{-7}$
		Ionic strength = 1.0 M			
		Hydrolysis		Isomerisation	
Buffer (%) free base	pH	k_0/s^{-1}	k_2/dm^3 mol^{-1} s^{-1}	k_0/s^{-1}	k_2/dm^3 mol^{-1} s^{-1}
30	5.97	$6.10 \pm 1.21 \times 10^{-8}$	$1.21 \pm 0.03 \times 10^{-6}$	$2.88 \pm 0.01 \times 10^{-7}$	$2.04 \pm 0.01 \times 10^{-7}$
40	6.11	$7.4 \pm 7.5 \times 10^{-8}$	$1.44 \pm 0.16 \times 10^{-6}$	$2.84 \pm 0.03 \times 10^{-7}$	$2.26 \pm 0.07 \times 10^{-7}$
60	6.45	$1.04 \pm 0.34 \times 10^{-7}$	$1.53 \pm 0.07 \times 10^{-6}$	—	—
80	6.94	$2.94 \pm 0.31 \times 10^{-7}$	$1.69 \pm 0.07 \times 10^{-6}$	$2.30 \pm 0.01 \times 10^{-7}$	$6.15 \pm 2.2 \times 10^{-8}$

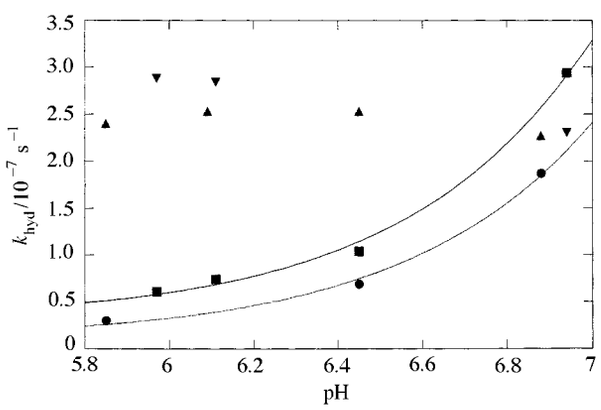


Fig. 2 pH-dependence of the spontaneous (buffer-independent) hydrolysis and isomerisation (triangles) of 3′,5″-UpU, at 80 °C and constant ionic strength (KCl). Data (from Table 1) are for 0.5 M (▼,●) and 1.0 M (▲ and ■). The curves are calculated from the equation $k_0 = k_0' + k_{OH}[OH]$, with rate constants k_0' of 9.5×10^{-9} and 3×10^{-8} s^{-1} and k_{OH} of 9.43×10^{-2} and 0.122 dm^3 mol^{-1} s^{-1}. K_w at 80 °C and ionic strength 1.0 M is 12.61.

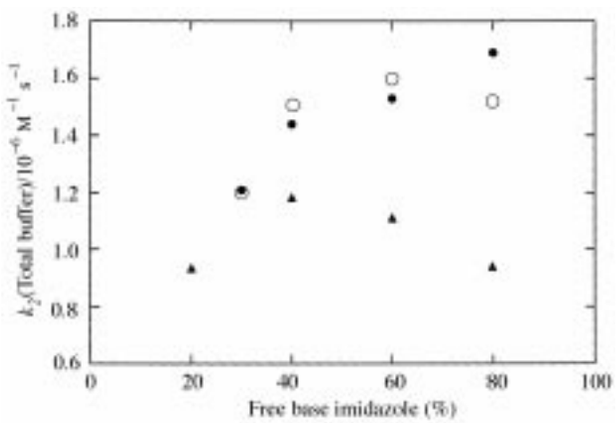


Fig. 3 Effects of ionic strength on second order rate constants for catalysis by imidazole buffers of the hydrolysis of 3′,5″-UpU, at 80 °C. Data for ionic strength 1.0 (●) and 0.5 M (○) are taken from this work (Table 1). Individual first-order rate constants were satisfactorily reproducible, and two independent sets of data measured at ionic strength 0.5 M gave the same second-order rate constants, within experimental error. The third set of data points (▲) is derived from the work of Anslyn and Breslow,[4] where the ionic strength was not held constant, but varied (as the concentration of ImH$^+$). We calculated the latter set of rate constants starting from the published rates at 0.5 M ImH$^+$,[4] finally dividing by a factor of 2.2 to match our own number at 0.5 M KCl. (There are known problems involved in reproducing the quantitative aspects of this work, but relative rates are considered to be reliable.[24])

The most striking observation in previous solution studies was the apparent bell-shaped pH-dependence of rate constants for the hydrolysis of UpU and poly-U catalysed by imidazole–imidazolium buffers.[4,7] This kinetic feature was the basis for the detailed mechanisms proposed by Breslow and co-workers for the model reaction, and its extrapolation to the enzyme situation. It turns out that the shape of this curve is very sensitive to the ionic strength of the medium. Fig. 3 shows second-order rate constants for imidazole-catalysed hydrolysis, taken from Table 1, as a function of the percentage of free base form of the buffer: and compares them with the original data of Anslyn and Breslow (adapted, somewhat empirically, to the same scale, as described in the caption to the Figure). At ionic strength 1.0 M the plot is close to linear (correlation coefficient 0.955 for four datapoints), with rate constants increasing as the percentage of imidazole free base increases: at 0.5 M the rate constants level out at high percentage free base; and where no salt is added (data of Anslyn and Breslow) they fall further still at high % free base, where the ionic strength is lowest: it is under these conditions that the bell-shape appears. Breslow *et al*. have found a similar, approximately linear, pH-dependence of the observed first-order rate constants obtained at a constant buffer ([Im] + [ImH$^+$]) concentration of 0.8 M when the ionic strength is kept constant at 1.0 M.[3] With TTUTT (see below) we observe a linear dependence even at ionic strength 0.5 M.

The usual methods of analysing catalysis in terms of separate contributions from Im and ImH$^+$, plus any higher order terms, cannot be applied with complete confidence to these data, but there is certainly a significant catalytic term in imidazole free base, and almost certainly one also in ImH$^+$. Simple extrapolation of the least squares line through the data at ionic strength 1.0 M gives second order rate constants of $1.9 \pm 0.2 \times 10^{-6}$ and $1.0 \pm 0.2 \times 10^{-6}$ dm^3 mol^{-1} s^{-1} for catalysis by Im and ImH$^+$, respectively. Only if the medium and ionic strength effects can be explained and quantified convincingly would a more detailed analysis be justified for this system.

The behaviour at high percentages of free base could be an effect of ionic strength on the imidazole buffer catalysed reaction, an effect on the buffer-independent reaction; or of course on both. The second-order rate constants are obtained as slopes

Table 2 The effect of ionic strength on observed rate constants for hydrolysis and isomerisation of 3′,5″-UpU at 80 °C

Free base (%)	[Buffer]/M	*I*/M	$k_{hyd}/10^{-6}$ s^{-1}	$k_{isom}/10^{-7}$ s^{-1}
50	0.7	0.35	12.6 ± 0.2	4.3 ± 0.1
50	0.7	0.5	12.7 ± 0.6	4.1 ± 0.1
50	0.7	1.0	13.3 ± 0.2	4.1 ± 0.1
80	0.3	0.2	5.2 ± 0.1	2.8 ± 0.1
80	0.3	0.5	6.1 ± 0.1	2.8 ± 0.1
80	0.3	1.0	7.7 ± 0.1	2.7 ± 0.1
80	0.7	0.2	12.9 ± 0.3	3.1 ± 0.1
80	0.7	0.5	13.1 ± 0.3	3.1 ± 0.1
80	0.7	1.0	15.4 ± 0.3	3.0 ± 0.1

of plots (*e.g.* Fig. 1) of k_{obs} *vs*. concentration: k_{obs} values include contributions from both buffer-independent and buffer-catalysed reactions, and the procedure assumes that the rate of the buffer-independent reaction remains constant as the buffer concentration increases. This is not necessarily the case. Fig. 2 shows that the buffer-independent hydrolysis of UpU is faster in a more polar (higher ionic strength) medium. If the background hydrolysis rate is reduced in media of lower polarity the slopes of the second-order plots will underestimate the second-order rate constants. This sort of effect has been observed in the hydrolysis of **1** (R = $CH_2OC_6H_4NO_2$-*p*), where the buffer-independent hydrolysis is the predominant reaction, and explains the apparent bell-shaped pH-dependence for catalysis by imidazole buffers in that case.[5] The results shown in Table 2 indicate that the ionic strength effect on UpU hydrolysis also operates primarily on the buffer-independent reaction: the rate increase observed when the ionic strength is increased from 0.2 to 1.0 M is independent of buffer concentration at 80% free base buffer; and is smaller at lower pH (50% free base buffer), where the hydroxide catalysed reaction makes a smaller contribution to the buffer-independent hydrolysis. This is consistent with an effect of ionic strength that is primarily on the hydroxide-catalysed, buffer-independent hydrolysis reaction:† there is no significant effect on isomerisation. Thus the curvature apparent in the plots shown in Fig. 3 is most likely not an effect on the kinetics of the buffer-catalysed reaction.

The polarity of the medium is also affected by increasing concentrations of imidazole. In experiments designed to evaluate any such medium effect the concentration of neutral organic solute (and hence, at least approximately, the polarity of the medium) was held constant by adding appropriate concentrations of an organic cosolvent. If the polarity of the medium is constant the slope of the plots of k_{obs} *vs.* buffer concentration should refer exclusively to the catalytic effect. Fig. 4 compares results (one example, taken from a number of such experiments) obtained in the presence and absence of a balancing concentration of dimethylformamide (DMF). In this case the correction, for whatever reason, introduces curvature. One formal possibility is that the hydrolysis is second order in buffer concentration, and the effect is normally concealed by a medium effect acting in the opposite direction. [A weak apparent second-order dependence on imidazole concentration (up to 2 M) has been reported for the hydrolysis of uridine and cytidine-2′,3′-cyclic phosphates.[8]] The interpretation of these results is further complicated by the effect of the added co-solvent on the pH of the imidazole buffers: the points in Fig. 4 have been corrected for the pH change, on the assumption that only the buffer-independent reaction is affected, and that its rate is not suppressed by a medium effect. The corrected data do not fit the second-order model convincingly: the results of many experiments of this sort, both with UpU and with our more reactive model substrate **1** (R = $CH_2OC_6H_4NO_2$-*p*)[9] are complex (see Breslow *et al*.[3] for examples).

† Note that since the rate constants from this work, plotted as circles in Fig. 3, are second order, measured at (almost) constant pH and constant ionic strength, the buffer-independent reaction has been factored out as far as possible. If the (small) difference between the two curves (open *vs.* closed circles) is an effect on the buffer-independent reaction, as the results in Table 2 suggest, then it must be a specific salt effect, of imidazolium *vs.* potassium, on hydroxide or transition state activity coefficients.

Table 3 First-order rate constants of hydrolysis and isomerisation of 3′,5″-UpU in 0.1 M 50% free imidazole base solutions of increasing DMF concentration

[DMF]/M	pH	$k_{hyd}/10^{-7}$ s^{-1}	$k_{isom}/10^{-7}$ s^{-1}
0.1	6.33	1.8 ± 0.1	2.53 ± 0.05
0.3	6.28	1.7 ± 0.2	2.50 ± 0.05
0.5	6.25	1.3 ± 0.1	2.26 ± 0.04
0.7	6.21	1.5 ± 0.1	2.11 ± 0.04

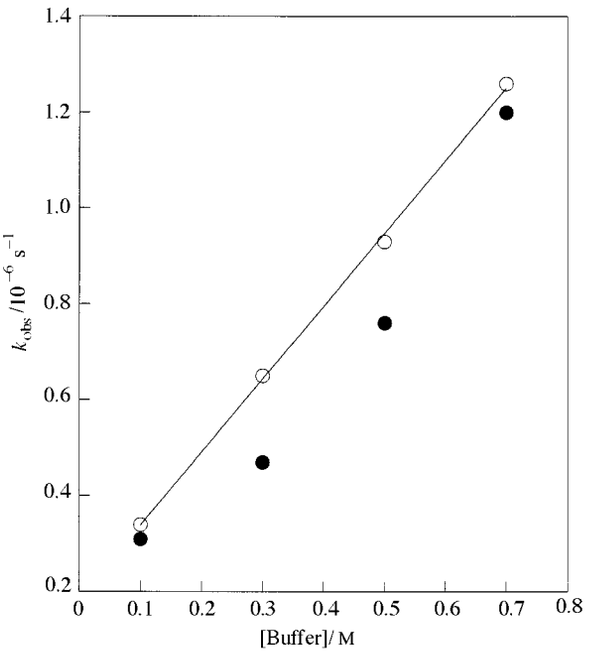


Fig. 4 The effect of added organic co-solvent on the hydrolysis of 3′,5″-UpU catalysed by 30% free base imidazole buffers, at 80 °C, pH 5.89 and ionic strength 0.5 M. ○ No added co-solvent, ● [Imidazole] + [DMF] kept constant at 0.7 M.

Isomerisation. Second-order rate constants for the imidazole buffer catalysed isomerisation of 3′,5″-UpU to its 2′,5″-isomer appear in Table 1. At ionic strength 0.5 M the rate constants clearly increase with decreasing pH, consistent with general acid catalysis of isomerisation.[3,4] The three rate constants referring to ionic strength 1.0 M appear to exhibit more complex behaviour, but since the reaction is very slow, and catalysis modest, too much weight should not be attached to small differences. The values obtained at ionic strength 0.5 M are not consistently higher or lower than those at ionic strength 1.0 M, and more extensive data would be needed to establish reliable patterns of behaviour. We conclude simply that the effects of changing ionic strength on isomerisation are small. Similarly, changing the polarity of the medium by adding small amounts of organic cosolvent does not seem to affect the rate of isomerisation: second-order rate constants in 30% free base imidazole solutions, at ionic strength 0.5 M, with and without a balancing concentration of added dioxane, were $3.1 \pm 0.2 \times 10^{-7}$ and $2.9 \pm 0.2 \times 10^{-7}$ dm^3 mol^{-1} s^{-1}, respectively. Similarly, in 80% free base solutions with balancing concentrations of DMF added, the observed rate constants in the presence and in the absence of cosolvent are similar.

Table 3 shows the results of another kind of experiment, in which increasing concentrations (up to 0.7 M) of DMF were added while the buffer concentration was held constant: the observed rates of both hydrolysis and isomerisation decreased

Table 4 Rate constants for spontaneous (k_0) and buffer-catalysed (k_2) hydrolysis and isomerization of TTUTT, at 80 °C and ionic strength 0.5 M (KCl)

Buffer (%) free base	pH	k_0/s^{-1}	$k_2/dm^3\ mol^{-1}\ s^{-1}$	k_0/s^{-1}	$k_2/dm^3\ mol^{-1}\ s^{-1}$
30	5.85	$1.2 \pm 13 \times 10^{-8}$	$2.42 \pm 0.30 \times 10^{-7}$	$3.28 \pm 0.30 \times 10^{-7}$	$5.80 \pm 0.66 \times 10^{-7}$
40	6.07	$-1.7 \pm 1.3 \times 10^{-7}$	$2.32 \pm 0.28 \times 10^{-6}$	$2.18 \pm 0.76 \times 10^{-7}$	$6.50 \pm 1.40 \times 10^{-7}$
60	6.48	$2.81 \pm 0.50 \times 10^{-7}$	$1.76 \pm 0.11 \times 10^{-6}$	$2.70 \pm 0.70 \times 10^{-7}$	$2.75 \pm 1.30 \times 10^{-7}$
80	6.88	$2.18 \pm 0.57 \times 10^{-7}$	$1.63 \pm 0.12 \times 10^{-6}$	$3.80 \pm 0.40 \times 10^{-7}$	$0.90 \pm 0.80 \times 10^{-7}$

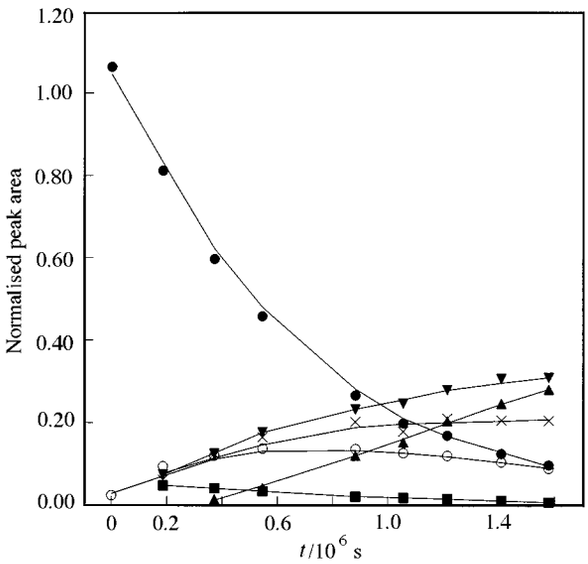


Fig. 5 Typical product distribution during the hydrolysis of TTUTT (**8**), followed by capillary electrophoresis for 2–3 weeks at 80 °C. Notation: ● 3′,5″-TTUTT, ▼ Product **a** (3′,5″-TpT, **4**), × Product **d** (TTU-2 and/or 3 phosphate), ○ Product **c** (2′,5″-TTUTT, **6**), ▲ Product **e** (TTU, **8**), ■ Product **b** (TTU-2′,3′-cyclic phosphate).

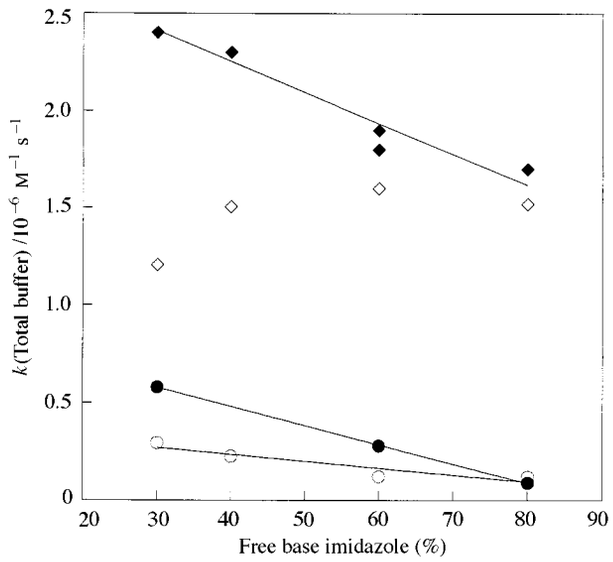


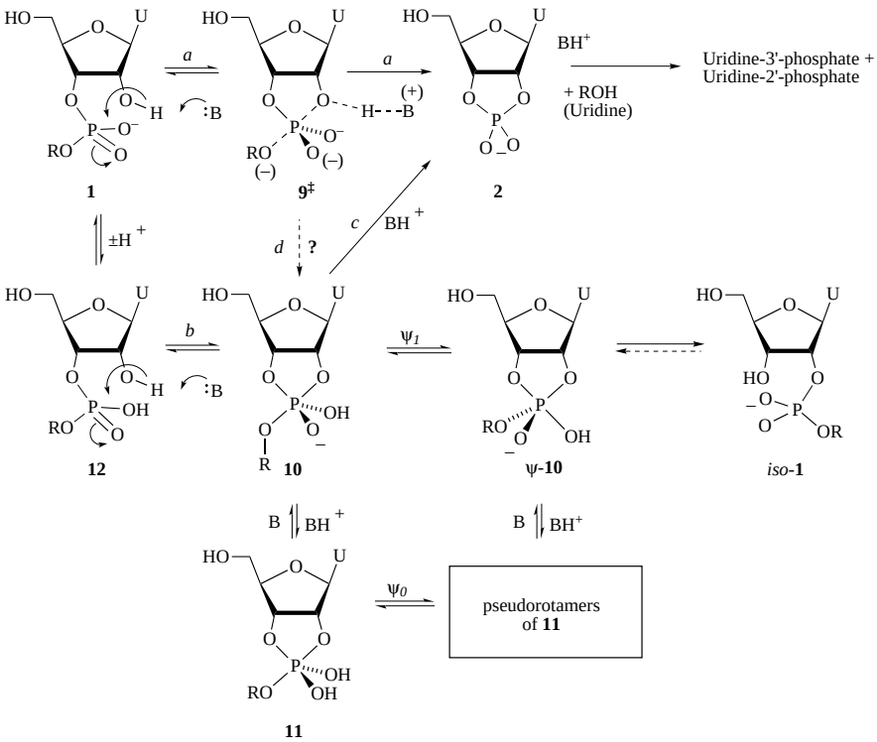
Fig. 6 Second-order rate constants for catalysis by imidazole buffers of the hydrolysis (◆) and isomerisation (●) reactions of TTUTT as a function of buffer ratio, at ionic strength 0.5 M and 80 °C. Two independent sets of data at 40 and 60% free base gave second-order rate constants identical within experimental error. Data for the hydrolysis (◇) and isomerization (○) of 3′,5″-UpU, measured under the same conditions (open symbols) are included for comparison. Extrapolations of the three linear plots give rate constants for catalysis by ImH^+ of the hydrolysis and isomerisation of TTUTT of $2.89 \pm 0.12 \times 10^{-6}$ and $8.73 \pm 0.08 \times 10^{-7}\ dm^3\ mol^{-1}\ s^{-1}$, respectively, and for the isomerisation of UpU of $3.76 \pm 0.57 \times 10^{-7}\ dm^3\ mol^{-1}\ s^{-1}$. Also a rate constant of $1.31 \pm 0.33 \times 10^{-6}\ dm^3\ mol^{-1}\ s^{-1}$ for the hydrolysis of TTUTT catalysed by imidazole free base.

by some 20%. The decrease in pH can account for the fall in k_{hyd}, but isomerisation is independent of pH. This modest decrease in the rate of isomerisation in the presence of 0.7 M DMF contrasts with the small rate increase observed by Breslow *et al.*,[3] when adding 0.3 M dioxane at 1.0 M imidazole buffer, 50% free base (60 °C). Medium effects are evidently complex—and not obviously informative.

Reactions of TTUTT. Studies on imidazole catalysis of the hydrolysis of polyuridylic acid were the first to show a bell-shaped pH–rate profile.[7] Recent measurements of the rates of buffer independent hydrolysis and isomerisation of poly-U have shown that the polymeric substrate can be up to ten times more reactive than 3′,5″-UpU under acidic conditions.[10] The metal ion promoted hydrolysis of different phosphodiester substrates also shows differences in kinetic behaviour between dinucleoside monophosphates and oligonucleotides.[21] So studies with 3′,5″-UpU as a model do not necessarily reveal all the essential features of buffer dependent RNA hydrolysis.

TTUTT **8**, though otherwise a typical oligonucleotide, expected to behave kinetically more like RNA than UpU, contains only a single reactive ribosyl phosphodiester bond. This greatly simplifies the product—and hence the kinetic—analysis. We followed the disappearance of TTUTT by capillary electrophoresis, as described in the Experimental section. The disappearance of TTUTT is over twice as fast as that of UpU in the more acidic buffers, so that the initial cyclic monophosphate product (*cf.* **2**) can be observed under some conditions. Thus all the expected products of reaction at the reactive UpT site appeared. They were identified, in the absence of authentic samples, on the basis of their kinetic and electrophoretic behaviour. The course of a typical experiment, run over 2–3 weeks, is illustrated in Fig. 5. Rate constants for the hydrolysis and isomerisation of TTUTT, derived from such experiments at four different buffer concentrations at each of four different buffer ratios (as for UpU, *cf.* Fig. 1, above), at ionic strength 0.5 M, are given in Table 4. (Some plots show slight curvature. This is most marked in 30% free base solutions: but the data are not accurate enough to establish that the effect is real. Second-order rate constants were determined by a least-squares fit of the data to straight lines.)

Rate constants for buffer-independent hydrolysis and isomerisation are similar to those for the reactions of 3′,5″-UpU under the same conditions, and show the same dependence on pH (*cf.* Fig. 2). The buffer-catalysed isomerisations are also closely similar: again isomerisation is catalysed exclusively by ImH^+, though with a rate constant about twice as large for TTUTT as for UpU. (Intercepts at 100% free base are zero, within experimental error, in both cases.) But at a constant ionic strength of 0.5 M there is a clear difference in behaviour for buffer-catalysed hydrolysis. Fig. 6 compares second-order rate constants for the buffer-catalysed hydrolysis and isomerisation of TTUTT with the data for the same reactions, under the same conditions, of 3′,5″-UpU. Whereas the rate constant for hydrolysis of UpU catalysed by ImH^+ is ill-defined, though clearly lower than that for catalysis by imidazole free base, buffer-catalysed hydrolysis of TTUTT is well-behaved, and can be extrapolated to give good second-order rate constants for both Im and ImH^+-catalysed reactions, of $1.31 \pm 0.33 \times 10^{-6}$ and $2.89 \pm 0.12 \times 10^{-6}\ dm^3\ mol^{-1}\ s^{-1}$, respectively.



Scheme 1

Mechanisms of catalysis by imidazole buffers

The sensitivity to ionic strength of catalysis by imidazole buffers apparent in Fig. 3 makes it difficult to define rate laws, and thus the composition of rate determining transition states, with complete conviction for the UpU system. However, some conclusions (1–4 below) are firm, especially as the kinetic complications largely disappear at constant high ionic strength, and are not a serious problem for the reactions of TTUTT. We base our discussion on Scheme 1, which outlines parts of the mechanism consistent with the accumulated evidence, and involves the predominant ionic forms of species long enough lived to attain proton transfer equilibrium, plus the key transition state **9**‡.

(1) The general base catalysed reaction of the diester anion **1** (B = imidazole) (like the specific base catalysed reaction) can lead directly to hydrolysis, by way of a dianionic transition state (**9**‡, path *a*). If an intermediate phosphorane dianion **4** (above) is involved it does not live long enough to reach prototropic equilibrium: so it does not (quite) appear in Scheme 1. This part of the mechanism is generally accepted.[2]

(2) Parallel to the reaction catalysed by imidazole free base are two reactions catalysed by ImH^+ (Scheme 1, second row). Isomerisation to the 2′-phosphate (iso-**1**) is catalysed exclusively by ImH^+, and is presumed to involve pseudorotation (ψ_1) of the phosphorane monoanion (**10**⇌ψ-**10**) and subsequent loss of the 3′-oxygen. The observed isomerisation is the strongest evidence for the presence of an intermediate on the reaction pathway, since it is generally accepted that isomerisation requires a phosphorane monoanion. Isomerisation is the slowest of the observed buffer-catalysed reactions, so pseudorotation is likely to be the slowest (observed) reaction of **10** (see below). This is strong evidence that it lives long enough to attain prototropic equilibrium.

(3) If the phosphorane monoanion **10** is an intermediate it can break down in two other ways: with loss of either the 2′-oxygen, to regenerate starting material (reverse of step *b*); or of the exocyclic leaving group (OR), accounting for the observed ImH^+-catalysis of hydrolysis (step *c*). This ImH^+-catalysed reaction is slower for UpU but for TTUTT it is over twice as fast as the reaction catalysed by free base imidazole.

(4) Simple proton transfers involving high energy phosphorane intermediates **10** and **11** will be diffusion-controlled reactions catalysed in the thermodynamically favourable direction by the buffer. Rate constants for diffusion-controlled proton transfers involving lyonium or lyate ions may be up to 30 times faster,[12] but the concentrations of Im and ImH^+ near pH 7 are up to 10^5 times greater than those of H_3O^+ or HO^-.

The main uncertainty, not resolved in Scheme 1, lies in the details of the formation and cleavage of the phosphorane monoanion **10**, and its status *vis à vis* its conjugate acid. Best estimates of the pK_a values of hydroxyphosphoranes (pK_1 ranging from 6.5 to 11)[2] suggest that the neutral, dihydroxy form **11** could be dominant near pH 7. So reactions we discuss in terms of ImH^+-catalysed reactions of the phosphorane monoanion **10** may in fact represent the kinetically equivalent reactions of **11** catalysed by imidazole acting as a general base. This kinetic ambiguity cannot be finally resolved, but mechanisms involving **11** as an intermediate on the reaction pathway seem unlikely.‡ If the monoanion **10** does live long enough to achieve prototropic equilibrium then its formation by deprotonation of (the possibly more stable) **11** will be a rapid equilibrium process, kinetically independent of buffer concentration. [If not, proton transfer from **11** to Im leads to the same encounter complex (**10** in Scheme 1) as is formed by diffusion together of **10** and ImH^+.] We observe pH-independent and ImH^+-catalysed isomerisation reactions of the (substrate) anion. Consistent with this conclusion, the phosphorane monoanion, rather than the neutral form, also appears to be the key intermediate in the isomerisation reactions of corresponding dimethyl triesters, where the substrate is neutral.[13]

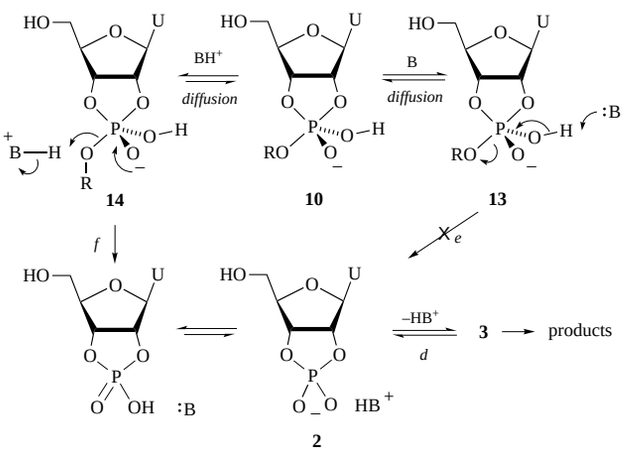
(5) The phosphorane monoanion **10** may be formed directly, *via* general base catalysed attack of the 2′-OH on the protonated phosphodiester group **12** (Scheme 1, path *b*), as suggested by Anslyn and Breslow;[4] or from the anion (step *d*). The reactions *via* paths *a*, *b* and *c* can be expected to go at comparable rates: though the conjugate acid **12** is present in 10^{5-6} times lower concentration near pH 7 it is predicted to be 10^{5-6} times

‡ With the possible exception of a 'by-pass' route to ψ-**10** *via* the (presumably faster) pseudorotation of **11**. This would not affect the discussion of pseudorotation rates that follows.

more reactive than the diester anion **1**.[14] And though the phosphorane dianion **4** may be too unstable to exist, this can be only by a narrow margin:[2] it would be generated (or almost generated) *via* transition state **9**‡ as its encounter complex with ImH^+, in which the thermodynamically favourable proton transfer to form **10** + Im might be expected to be at least as easy as P–O bond cleavage.

(6) The mechanism of formation of **10** is probably not accessible to direct kinetic investigation because its breakdown is the likely rate determining step in the non-enzymic reaction. In the pH 7 region, under conditions similar to our experiments, loss of the exocyclic OMe group is a minor pathway in the hydrolysis of methyl ethylene phosphate (which may be presumed to go *via* the phosphorane monoanion);[15] and some 10^5 times slower than ring-opening in the cleavage of a dimethyl uridine phosphate.[13]

We conclude that the phosphorane anion **10** carries the main reaction flux for the hydrolysis, as well as the isomerisation of TTUTT, and a significant proportion also for both reactions of UpU. **10** could be partitioned in three ways, by up to four different mechanisms in each case: for simplicity we concentrate on the reactions involving buffer catalysis. Its rate determining breakdown could be both general acid and general base catalysed (Scheme 2), thus accounting—since it is isomeric with the



Scheme 2

starting anion **1**—for the observed catalysis by ImH^+ and a proportion of the catalysis by Im (in parallel with the direct path *a*). [This general base catalysis would follow the classical mechanism: complete removal of the OH proton of **10** by Im would generate the unstable dianion **4**, so incipient proton removal must trigger a concerted P–O bond cleavage (**13**), to generate the cyclic phosphate directly.] However, the absence of general base catalysis of isomerisation (see below) suggests that this pathway is not significant for hydrolysis (*via* path *e*, Scheme 2) either. General acid catalysis (**14**, step *f*) produces the conjugate acid of the initial cyclic phosphate product **2** in its encounter complex with Im, which will be rapidly converted to **2** by the thermodynamically favourable proton transfer followed by the diffusion away of ImH^+.§ In either case the fastest reaction of **10** is expected to be reversion to starting material.

Finally, the slowest observed reaction of **10** is pseudorotation, leading by way of ψ-**10** to isomerisation (Scheme 1). ψ-**10** can break down by all the mechanisms available to **10**, but only two are actually observed, the spontaneous and the general acid catalysed isomerisation. Since pseudorotation itself is not subject to buffer catalysis (assuming that **10** lives long enough to reach prototropic equilibrium), it cannot be exclusively rate determining for isomerisation (otherwise general acid catalysis would not be observed).¶ It is, however, slow enough to make the general acid catalysed cleavage and isomerisation of **10** go at comparable rates. So we can infer relative rates for the partitioning of **10** (see Scheme 1): $v_\psi < v_c < v_b$, with differences of no more than an order of magnitude in each case: and thus $k_\psi < k_c < k_b$.

Implications for RNAse mechanisms. The ribonucleases catalyse the same core hydrolysis reaction studied in this work, though the proton transfer catalysis involved is clearly far more efficient. The basic requirements—for a general base to activate the attacking nucleophile and a general acid to assist the departure of the leaving group—are well-defined; and recent work shows how precise positioning of catalytic groups can make this sort of general acid–base catalysis highly efficient.[16] The main area of uncertainty, as discussed above for the model reactions, lies in the exact status of the pentacoordinate species involved in the phosphate transfer process. In terms of catalytic efficiency an intermediate on the reaction pathway is an advantage only if it is stable enough to lower the energy of transition states for both P–O formation and cleavage without being so stable that it introduces a significant energy sink. Our current understanding of the chemistry of these processes suggests that in an efficient enzyme system P–O formation and cleavage will be strongly coupled, in a process which takes advantage of the availability of stable pentacovalent structures, without necessarily involving a full intermediate. The structure and charge distribution of such a rate determining transition state should be close to those of a phosphorane, providing a basis for understanding the dynamic binding[17] involved.

The simplest concerted mechanism in model systems involves general base catalysis and a dianionic transition state (**9**‡). This is the main pathway with very good leaving groups. Poor leaving groups need an additional proton from a general acid, eventually to assist the departure of the leaving group but also to stabilise the phosphorane intermediate as the monoanion. We have suggested (Scheme 1, above) how this proton could be the one removed from the substrate OH group, but in no mechanism of catalysis where the same proton does both jobs can it be optimally efficient in both roles. Model studies of catalysis by diamines are consistent with this conclusion: the monocations are particularly effective as general bases in the cleavage of ApA at pH 8,[18] and in a system specifically designed to study the departure of the (MeO···H) leaving group the dications are particularly effective general acids.[19] In both cases 1,2-diamines, where the geometry is optimal for proton transfer to both nucleophile/leaving group and the PO_2^- group oxygens, are the best catalysts.

Thus the intrinsic chemistry points rather clearly to the need for a separate source of stabilisation for the developing second negative charge at the PO_2^{2-} centre. In route *b*,*c* this is provided in advance by a full proton transfer, to neutralise the monoanion, but this leaves the proton in a less than optimal position for transfer to the leaving group oxygen. As has been pointed out by a number of authors,[2] the lysine NH_3^+ group present in the active site of RNAse A is a weak enough general acid to transfer a proton, *via* a strong hydrogen-bond, to the developing PO_2^{2-} of a phosphorane dianion. Even if the geometry were unchanged from the Michaelis complex, hydrogen bonding to the weakly basic PO_2^- groups of the substrate and the product would not be much stronger than to solvent water.

§ We note that the general acid catalysed reaction (reverse of path *b* in Scheme 1) produces the protonated diester **12**: this is further evidence consistent with the likely importance of the pathway *via* **12** in the forward reaction *via* **10**.

¶ We agree with a referee that the absence of general base catalysis of the breakdown of **10** is surprising. But the evidence that isomerisation, and thus the breakdown of ψ-**10**, is not general base catalysed, is unambiguous. The two intermediates differ in that the axial OH of ψ-**10** will be a weaker acid: but the faster ring-opening of ψ-**10** (compared with exocyclic cleavage of **10**) should more than compensate.

Summary

Our results confirm some of the main conclusions of previous workers, and resolve some kinetic complications.[2] The hydrolysis of 3′,5″-UpU is catalysed both by imidazole and, less effectively, by its conjugate acid, in reactions which are of the first order in the general acid or base. Its isomerisation to 2′,5″-UpU is catalysed only by ImH^+. Observed first order rate constants for hydrolysis show marked sensitivity to ionic strength. This is identified primarily as an effect on the specific base catalysed reaction. At the buffer concentrations used in this work there is no sign of the apparent 'negative catalysis' observed by Breslow and Huang[20] on increasing [Im] at constant [ImH^+].‖

The hydrolysis of TTUTT is also catalysed by both imidazole and by its conjugate acid, but in this case catalysis by ImH^+ is the more effective. At constant ionic strength we see no sign of the bell-shaped dependence on the degree of protonation of the buffer seen by Breslow and Labelle with poly-U.[7] The isomerisation reaction, of TTUTT, like the corresponding reaction of UpU, is catalysed exclusively by ImH^+. The intrinsic chemistry indicates that a separate source of stabilisation for the developing second negative charge at the PO_2^{2-} centre is needed for optimal catalytic efficiency.

Conclusions

We conclude that the hydrolysis of the internucleoside bond in UpU and TTUTT involves two, parallel, reaction pathways: (*a*) a more or less concerted general base catalysed reaction; and (*b*) a two-step process, involving the rate determining general acid catalysed breakdown of the phosphorane monoanion intermediate **10**. Near pH 7, in the presence of Im and ImH^+, the two pathways run at comparable, but very slow rates. Ribonucleases can combine the catalytic advantages of both pathways, with the general base and the general acid acting in concert on coupled P–O bond making and breaking processes; but for maximum efficiency a third, Brønsted acid, group is required.

Experimental

Materials

The solid phase synthesis of TTUTT (**8**), by the phosphoramidite method on a 10 μM scale, and the solution-phase synthesis of 3′,5″-UpU have been described previously.[11,21] Imidazole was recrystallised from acetone. Dioxane and DMF were freshly distilled. Water was triply distilled and degassed by purging with argon.

Reaction solutions

Buffer solutions were made by mixing imidazole and HCl solutions. The pH was measured at 80 °C. The solutions were then diluted to appropriate concentrations, the ionic strength adjusted with KCl and the pH checked again. The pH varied by a maximum of 0.08 pH units within a dilution series.

Kinetic measurements

Reactions were carried out in sealed glass tubes. The volume of reaction solution in each tube was 100 μl for 3′,5″-UpU and 25 μl for TTUTT. The substrate concentration was 0.1 mM for 3′,5″-UpU and 1 mM for TTUTT. Potassium *p*-nitrobenzenesulfonate was used as an internal standard: its concentration was adjusted to give a peak of similar size to that of the substrate at the beginning of the reaction. The temperature was maintained at 80.0 ± 0.5 °C with a water bath. The glass tubes were removed at appropriate intervals over at least one half-life of the hydrolysis reaction. Samples were stored in the freezer until analysed. For analysis, UpU samples were diluted to 400 μl: TTUTT samples were not diluted.

‖ At the highest concentration (0.14 M) of [ImH^+] common to our measurements at all four buffer ratios, the rate of isomerisation is constant within experimental error ($3.0 \pm 0.1 \times 10^{-7}$ s^{-1}): as expected, since the rate of isomerisation is independent of [Im] or pH.

Table 5 Identification of products of hydrolysis of TTUTT by CE

Compound (see Fig. 5)	Migration time/min	Identified as
	28	3′,5′-TTUTT (**8**)
a	16	3′,5′-TpT
b	35	TTU-2′,3′-cyclic phosphate
c	27.5	2′,5′-TTUTT
d	41	TTU-2′ plus -3′phosphate
e	29	TTU

HPLC analysis of UpU runs

UpU reactions were analysed by RP-HPLC using a Hypersil RP-18 column (250 × 4 mm, 5 μm particle size). The eluent was acetic acid–acetate buffer (0.1 M, pH 4.3) containing 0.1 M NH_4Cl, and UV-detection at 260 nm was employed. The retention times of 2′,5″- and 3′,5″-UpU and the internal standard were 5, 8 and 11 min, respectively. For the calculation of the rate constants, the integrals of the UpU isomers were divided by that of the internal standard.

Analysis of TTUTT samples by CE

TTUTT samples were analysed by capillary electrophoresis. Fused silica capillaries of total length 87 cm were used. The run buffer was 0.5 M boric acid, pH 9.3, the applied voltage was 30 kV, and UV-detection at 254 nm was employed. Under these conditions, the migration time of the starting material was approximately 28 min and that of the internal standard 42 min, though migration times varied somewhat within a given series of runs. Migration times were longer with fresh run buffer, decreasing as more samples were analysed. The buffer was changed after every five samples to ensure optimal separation.

As the reaction proceeded, several products appeared. These were identified on the basis of their kinetic and electrophoretic behaviour, since authentic standards were not available. Normalised integrals of different signals as a function of time are shown for a typical run in Fig. 5. The assignment of the signals and their approximate migration times are shown in Table 5. Compounds **a** and **b** appear to be the initial hydrolysis products, 3′,5″-TpT and TTU trimer bearing a 2′,3′-cyclic phosphate function at its 3′-terminus. The product containing the cyclic phosphate function does not accumulate to a large extent, but rapidly reacts further, consistent with the higher reactivity of 2′,3′-cyclic phosphate functions compared with internucleosidic phosphodiester bonds. In contrast, the integral of **a** increases steadily, indicating that this product does not react further; as would be expected for a compound consisting only of deoxyribonucleoside linkages. Compound **c** is taken to be the 2′,5″-isomer of the starting material. It is formed as an initial product, and then reacts further. As the reaction proceeds, **c** and the starting material form a 1 : 1 equilibrium mixture, as observed previously for 2′,5″- and 3′,5″-isomers of dinucleoside monophosphates.[6] CE analysis of UpU isomers is also consistent with this assignment: the migration times of 2′,5″- and 3′,5″-UpU are very similar, with that of the 2′,5″-isomer slightly shorter. This appears also to be the case with the corresponding TTUTT isomers. In Fig. 5 and Table 5, **d** refers to a pair of signals which were only partially separated. This pair of compounds appear as secondary products, and it is logical to suppose that they are the two TTUp trimers, with either a 2′- or a 3′-monophosphate group. Consistent with this assignment, they are formed in a 1 : 2 ratio, as reported for 2′- and 3′-nucleoside monophosphates produced from other nucleotides. Finally, product **e** is formed after longer reaction times: it is most likely the dephosphorylated TTU trimer and was not involved in the kinetic measurements.

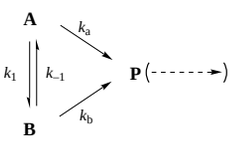
Consistent with these assignments, the sum of the normalised integrals of products on the 5″-side of the scissile phosphodiester bond corresponds to that of the products on the 3′-side. For every time-point the sum of the integrals of peaks **b**, **d** and **e** is 1.5 times larger than that of **a**, as expected for products consisting of two (TpT) and three (TTU) chromophoric units.

The electrophoretic behaviour of the compounds is also consistent with the assignments. 3′-Phosphorylated trimers can be expected to have the longest migration time because of their high negative charge. The corresponding cyclic phosphodiester is of the same size, but less charged than an acyclic monoester. It is therefore less attracted to the anode, and so migrates faster, as observed. The charge on the dephosphorylated product **c** is smaller still, and it migrates faster than the products bearing a terminal monophosphate group. The charge-to-size ratio of TTU and the starting material are nearly the same, but since the TTUTT molecule is larger, it is more easily carried by the electro-osmotic flow, and hence appears to migrate faster.

Peak areas were normalised for the calculation of the rate constants. The integrals were first divided by the migration time because in CE the integrals are dependent on the migration time. Finally the values obtained for the TTUTT-isomers were divided by that of the internal standard. The values thus obtained are proportional to the concentration of the compound, as shown by various calibration experiments. The data obtained by CE are slightly less accurate than those obtained by HPLC: the maximum standard deviation of the data fits observed was some 2% higher than for the HPLC experiments.

Appendix: Kinetic Analysis

The isomerisations of the 2′,5″- and 3′,5″-isomers of UpU (**A** and **B**) and their hydrolysis, initially to the cyclic 2′,3′-uridine phosphate (**P**) can be represented by the scheme:



Under pseudo-first-order conditions, the rate of change of the concentrations of **A** and **B** is given by

$$\mathrm{d}[\mathbf{A}]/dt = p[\mathbf{A}] + q[\mathbf{B}] \text{ and } d[\mathbf{B}]/dt = r[\mathbf{A}] + s[\mathbf{B}], \text{ where}$$

$$p = -(k_1 + k_a),\ q = k_{-1},\ r = k_1,\ s = -(k_{-1} + k_b).$$

This is a homogeneous system of linear first-order differential equations with constant coefficients, which can be solved analytically[22] to give the integrated rate laws:

$$[\mathbf{A}] = \frac{1}{z_1 - z_2}\{y_2 e^{z_1 t} - y_1 e^{z_2 t}\}$$

$$[\mathbf{B}] = \frac{1}{q(z_1 - z_2)}\{(z_1 - p)y_2 e^{z_1 t} - (z_2 - p)y_1 e^{z_2 t}\} \quad (1)$$

where

$$z_{1,2} = \frac{p + s}{2} \pm \sqrt{\tfrac{1}{4}(p - s)^2 + qr}$$

$$y_i = [\mathbf{B}]_0 q - [\mathbf{A}]_0(z_i - p)$$

Rate constants were determined by least-squares fitting of measured concentrations $[\mathbf{A}]_i$ and $[\mathbf{B}]_i$ at various times to eqn. (1),[22] *i.e.* by minimising

$$\chi^2 = \sum_{i=1}^{N}\left(\frac{[\mathbf{A}]_i - [\mathbf{A}]_{calc}}{\sigma_{A,i}}\right)^2 + \sum_{i=1}^{N}\left(\frac{[\mathbf{B}]_i - [\mathbf{B}]_{calc}}{\sigma_{B,i}}\right)^2$$

The initial concentrations $[\mathbf{A}]_0$ (and $[\mathbf{B}]_0$ if not exactly zero), were also treated as fit parameters. The σ values were estimated, conservatively, on the basis of a limited reproducibility analysis. The computations were carried out using a custom-written Turbo Pascal implementation of the Broyden–Fletcher–Goldfarb–Shanno variant of the Davidon–Fletcher–Powell minimisation algorithm.[23] Errors (more accurately, confidence limits) for fitted parameters were calculated as the square roots of the diagonal elements of the inverse of the Hessian matrix (the matrix of second-order partial derivatives) of χ^2 at its minimum.

In the great majority of experiments only the 2′,5″-isomer **A** was initially present ($[\mathbf{B}]_0 = 0$), and the concentration of isomer (**B**) remained relatively small. Thus meaningful values could be obtained only for k_1 and k_a, and these are the values reported. (Confidence limits for k_{-1} and k_b were of the same order of magnitude as their derived values.)

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