General base catalysis *vs.* medium effects in the hydrolysis of an RNA model †

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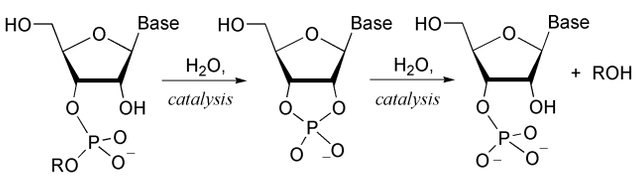
The interpretation of buffer catalysis data is always subject to a degree of uncertainty, because changing the concentration of the catalyst necessarily changes the medium. The history of the imidazole-catalysed reactions of ribonucleotide derivatives is a classic illustration of the potential problems, which are complicated further by significant sensitivity to ionic strength and specific salt effects. Results are reported for the hydrolysis of 4-nitrophenoxymethyl uridine 3′-phosphate, which releases the chromophoric *p*-nitrophenolate on cleavage of the P–OCH_2OAr bond. In the case of catalysis by imidazole, a bell-shaped dependence on the buffer ratio is explained in terms of a solvent effect on the background, hydroxide-catalysed reaction. Results for six other amine bases with $pK_a = 7 \pm 1$ show that, in this system, non-linear behaviour is the norm rather than the exception.

Phosphodiesterases are of special interest for the study of the chemical mechanisms of enzyme action, not least because their substrates are otherwise extraordinarily stable to hydrolysis.[1,2] The enzymes concerned thus include some of the most efficient catalysts known, but the enormous accelerations involved mean that direct comparisons of their catalytic mechanisms with those of the corresponding non-enzymic ("model") reactions are difficult, if not impossible.

This problem is significantly reduced in the case of the ribonucleases. In the P–O cleavage of ribonucleotides (Scheme 1) the

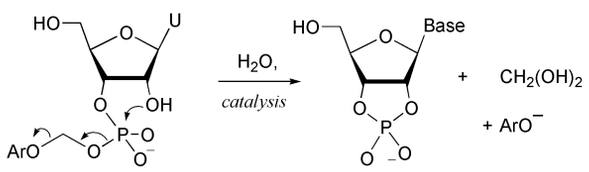


Scheme 1

common nucleophile is the 2′-hydroxy group of the substrate itself,[2] acting with an effective molarity (EM)[3] of some 10^7 M. The smaller accelerations involved mean that the non-enzymic reactions, though still very slow, can be followed near pH 7 at elevated temperatures even for unactivated substrates like UpU and RNA itself.[4–7] Such work depends on separation techniques such as HPLC or capillary electrophoresis (CE), because there is no significant UV change on hydrolysis.

We report a study of the general base catalysed hydrolysis of a new class (**4**) of ribonucleotide,[8] designed to allow P–O cleavage to be followed continuously for an alkoxide leaving group. The initial product from the cleavage of the P–OCH_2-OAr bond of **4** is the hemiacetal $ArOCH_2OH$ or its anion, expected to break down much faster than it is formed under the conditions of our reactions, to give formaldehyde and the phenol. Thus at pHs close to or above the pK_a, P–O cleavage can be measured directly by following the release of the (chromophoric) phenolate anion, and there is in principal no limit on the number of data points that can be collected.

† Electronic supplementary information (ESI) available: tables of primary kinetic data and derived rate constants. See http://www.rsc.org/suppdata/p2/b1/b109325h/

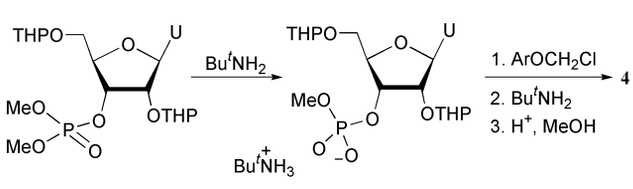


We have studied two acetal esters of uridine 3′-phosphate, **4a** and **4b**, with Ar = 4-nitrophenyl and 3,4-dichlorophenyl, respectively. The OH of the hemiacetal leaving group $ArOCH_2OH$ was designed to have a pK_a (estimated below as about 11) not too far below that of the 5′-OH of a ribose derivative. We have collected good quality data for catalysis by many general bases, and have measured pH–rate profiles for the hydrolysis of both substrates, derived from extended buffer studies (*vide infra*). We worked mostly with the 4-nitrophenol derivative **4a**, and results refer to this compound unless otherwise indicated. The hydrolysis of **4a** is at least one hundred times faster than that of UpU at the same pH [k_0 is 3×10^{-8} s^{-1} for the hydrolysis of UpU at 80 °C,[7] and of the order of 10^{-6} s^{-1} for the hydrolysis of **4b** at 70 °C (see below)].

Results and discussion

Synthesis of uridine phosphate acetal esters

The dimethyl phosphate triesters **5** (Scheme 2, mixture of



Scheme 2

diastereoisomers) were prepared by phosphorylation of the 2′,5′-*bis*(tetrahydropyranyl) protected uridine *via* standard phosphorus(III) chemistry.[9]

Demethylation of the triester mixture with *tert*-butylamine (Scheme 2) gave the *tert*-butylammonium salts of the

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monomethyl phosphate diesters **6**,[10] which were alkylated by the relevant α-chloroether in the presence of silver(I) oxide. The resulting aryloxymethyl methyl triesters were themselves demethylated with *tert*-butylamine. Finally the THP protecting groups were removed by the method of Williams and co-workers,[11] using methanol as the solvent. This last step proved the most difficult: methanol was necessary to dissolve the substrate, but it slowed THP cleavage and accelerated the target cyclisation reaction of the deprotected substrate. This cyclisation was followed by rapid methanolysis of the resulting 5-membered ring cyclic phosphodiester, to give substantial amounts (at least 60%) of a mixture of methyl uridine 2′- and 3′-phosphates.

The final product mixture contained some 40% of the desired aryloxymethyl uridine phosphate diester **4** and small amounts of its uridine 2′-phosphate isomer. HPLC separation of the product mixture allowed characterisation of the two isomers: ^{31}P NMR of the mixtures resulting from acid catalysed removal of the THP protecting groups showed that the 2′ isomer represented only ten percent of the total. The crude mixture could be used directly for kinetic studies: only the aryloxymethyl uridine phosphates react under the conditions used, and good quality kinetic data were obtained. The deprotected diesters, as the *tert*-butylammonium salts, were stored in methanol at −18 °C, and proved to be stable over several months.

Kinetic measurements

The hydrolyses of the arylacetal esters **4a** and **4b** of uridine 3′-phosphate were followed under pseudo-first order conditions for 3 to 4 half-lives over the pH range 1 to 10, in aqueous buffers at 70 °C, in the thermostatted cell compartment of a Gilford 2600 UV spectrophotometer: with special emphasis on buffer catalysis in the pH-range 6 to 8 most relevant to enzyme chemistry. The release of the phenol and/or phenolate anion was followed at 408 nm (pH > 5.6) for 4-nitrophenoxymethyl uridine phosphate **4a** and for 3,4-dichlorophenoxymethyl uridine phosphate **4b**, at 245 and 305 nm (below and above pH 8, respectively). Plots of absorbance against time were fitted as simple exponentials to give first order rate constants: correlation coefficients for pseudo-first order plots were typically 0.999 or better. Buffers were made up to a constant ionic strength of 1.0 M (KCl) except where specifically indicated, and three or more concentrations were used, up to a maximum of 1 M. First order rate constants (k_0) for the spontaneous or water-catalysed reaction and second order rate constants (k_2) for buffer catalysis were obtained as the intercepts and slopes of plots of observed first order rate constant against buffer concentration in the usual way. Where buffer catalysis was not significant the data points were averaged to give k_0.

The pH–rate profile (Fig. 1) for the hydrolysis of 3,4-dichlorophenoxymethyl uridine phosphate **4b** was fitted by the method of least squares to eqn. (1):

$$k_{obs} = k_H a_H + k_0 + k_{OH} K_w / a_H \qquad (1)$$

k_0 is the pH-independent rate constant for the substrate anion, k_H the rate constant for the acid catalysed reaction, and k_{OH} the rate constant for hydroxide-ion catalysis. Using a value for K_w of 15.8×10^{-14} mol^2 dm^{-6} at 70 °C[12] gave $k_H = 0.20 \pm 0.02$ mol^{-1} dm^3 s^{-1} and $k_{OH} = 29.5 \pm 2.8$ mol^{-1} dm^3 s^{-1}. The buffer independent reaction is very slow, in the region $10^{-(6-7)}$ (the least squares fit gives a value of 2 ± 16 s^{-1}). No attempt was made to allow for the first pK_a of the phosphate diester acid, which is expected to be around 0.7.[13] The hydrolysis of 4-nitrophenoxymethyl uridine phosphate **4a** was followed at and above pH 5.63: only the final term of eqn. (1) was significant, the fit giving $k_{OH} = 78 \pm 3$ dm^3 mol^{-1} s^{-1}. The data (Fig. 1, filled circles) fall off from a line of unit slope at pH > 9, consistent with conversion to a species reacting a few times more slowly by the ionization of a group of p$K_a \approx 9.5$. This is in the region of the pK_a of the 3-NH of the uracil group: ionisation would be expected to make the 2-OH group more weakly acidic.

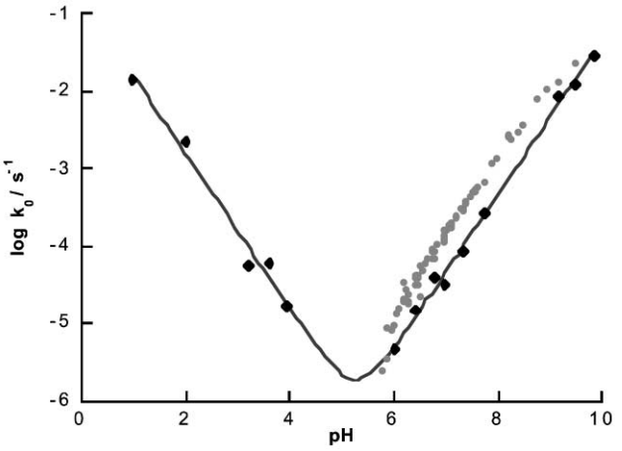


Fig. 1 pH–rate profile for the hydrolysis of **4a** (●) and **4b** (◆). The points are experimental and the curve for **4b** is calculated using the rate constants given in the text. (Data at 70 °C and ionic strength 1.0 M, in water.)

The pK_a of the hemiacetal leaving group

Our best estimate of the pK_a of the *p*NPCH$_2$OH leaving group of **4a** comes from our own data for the hydrolysis of its acetate ester at 25 °C,[14] and the good linear free energy relationship between k_{OH} and the leaving group measured by Bruice *et al.*[15] for the hydrolysis of acetate esters under the same conditions [eqn. (2)].

$$\log (k_{OH}/s^{-1} \text{ at } 25\ ^\circ C) = 3.12 \pm 0.16 - 0.25 \pm 0.01 pK_{LG} \qquad (2)$$

The acetate ester pNPCH$_2$OAc is hydrolysed (as an ester[14] rather than an acetal) in the pH region (0–14), with $k_{OH} = 2.48$ dm^3 mol^{-1} s^{-1} at 25 °C. This allows us to estimate a pK_a of 10.8 ± 0.7 for the hemiacetal pNPCH$_2$OH derived from 4-nitrophenol. From its slightly lower rate constant for alkaline hydrolysis, and the β_{LG} of 0.54 for aryl uridine 3′-phosphates measured by Williams[11] at 25 °C (corrected to 0.47 at 70 °C) we estimate pK_a = 11.2 for the corresponding hemiacetal derived from 3,4-dichlorophenol.

Buffer catalysis

Catalysis by imidazole buffers is summarised in Fig. 2. The curve is not of the form expected for simple general acid or general base catalysis, or a combination of the two, but shows a

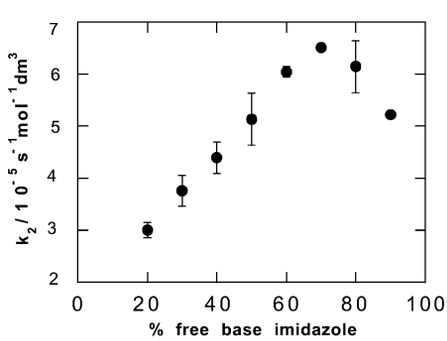


Fig. 2 Catalysis of the hydrolysis of **4a** by imidazole as a function of the buffer ratio. Each point represents the second-order rate constant (in dm^3 mol^{-1} s^{-1}) for catalysis by imidazole at the buffer ratio indicated: error bars are calculated from the second-order plots, and reflect the different numbers of data points measured at different buffer ratios. (Data at 70 °C and ionic strength 1.0 M, in water.)

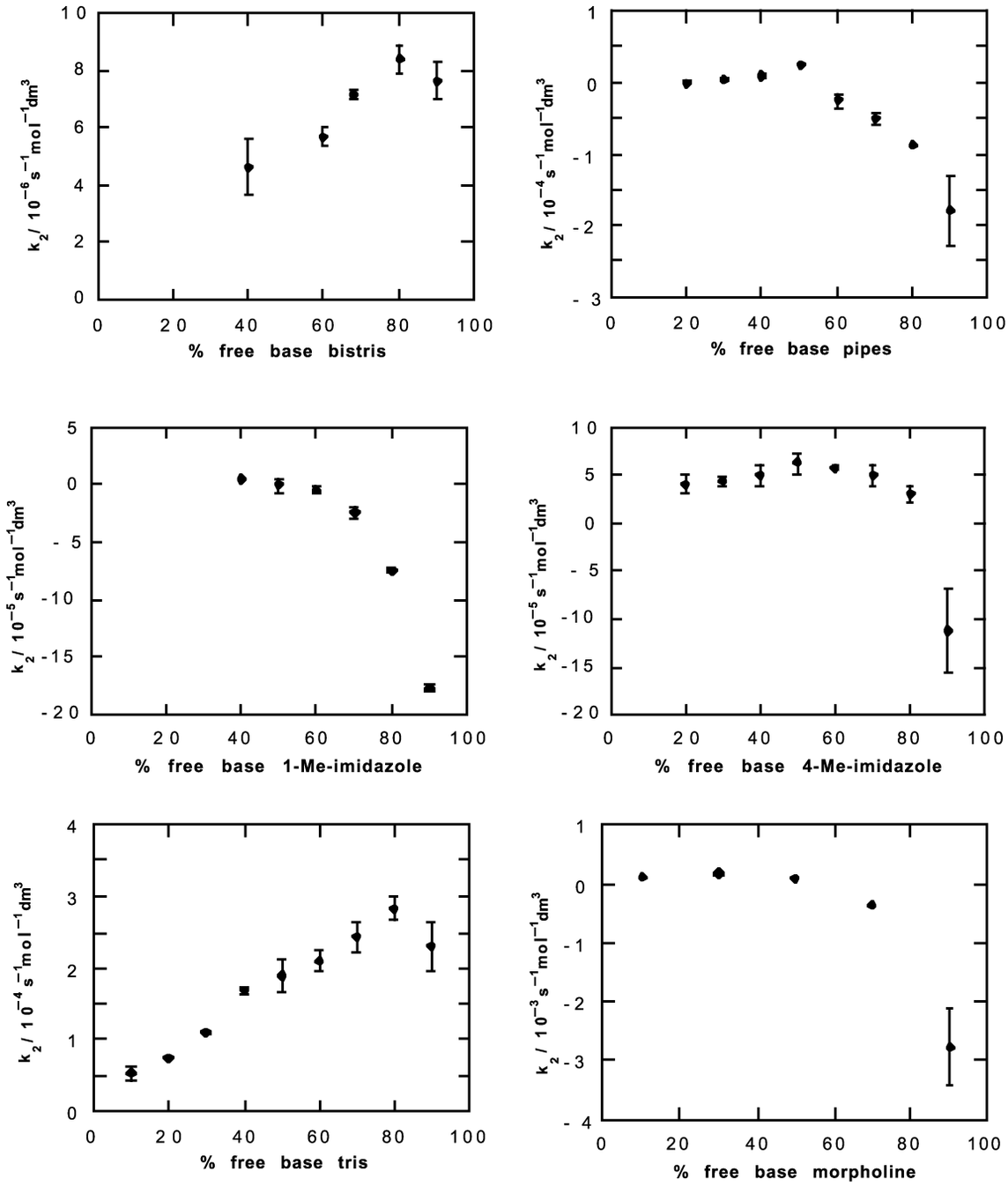


Fig. 3 Catalysis of the hydrolysis of **4a**, at 70 °C and ionic strength 1.0 M, by six amine buffers increasing in pK_a between 6–8. Second order rate constants are plotted against the percentage of free base, as for Fig. 2.

rate maximum near 70% free base, similar to the bell-shaped curve observed by Anslyn and Breslow for imidazole catalysis of the hydrolysis of UpU and poly-U. However, we were unable to fit the data to the kinetic mechanism suggested by Anslyn and Breslow:[5] 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.

To investigate buffer catalysis further, we extended our studies to simple derivatives of imidazole, and other potential amine catalysts with pK_as between 6 and 8. Fig. 3 shows results for catalysis by 2,2′,2″-nitrilotris[2,2-bis(hydroxymethyl)ethanol] (pK_a, under our reaction conditions, 6.12), PIPES [piperazine-1,4-bis(ethanesulfonic acid), pK_a 6.49], 1-methylimidazole (pK_a 6.54), 4-methylimidazole (pK_a 6.87), tris [tris(hydroxymethyl)-aminomethane, pK_a 7.29] and morpholine (pK_a 7.97). The data are reported as summary plots of k_2 against the percentage free base in the buffer, to allow direct comparison with the results obtained with imidazole buffers (Fig. 2)

Salt *vs.* solvent effects

The wide range of behaviour shown in the full set of buffer plots for the cleavage of the 4-nitrophenoxymethyl uridine 3′-phosphate **4a** (Figs. 2 and 3) shows that the observed effects are far from simple. The bell-shaped curve is not general: where one can be discerned the rate maximum does not change systematically with the pK_a of the buffer; and many of the buffer catalysis rate constants are actually negative—that is to say, reaction is slower at higher buffer concentrations. It seems clear that we are dealing here with medium effects. These could be of two kinds: specific salt effects, resulting from the gradual replacement of potassium ions of the KCl used to maintain ionic strength by the organic ammonium cations of the buffer; or solvent effects, resulting from the increasing amounts of organic cosolvent present at higher concentrations of amine buffer. (For example, a 1 M 90% free base morpholine buffer contains

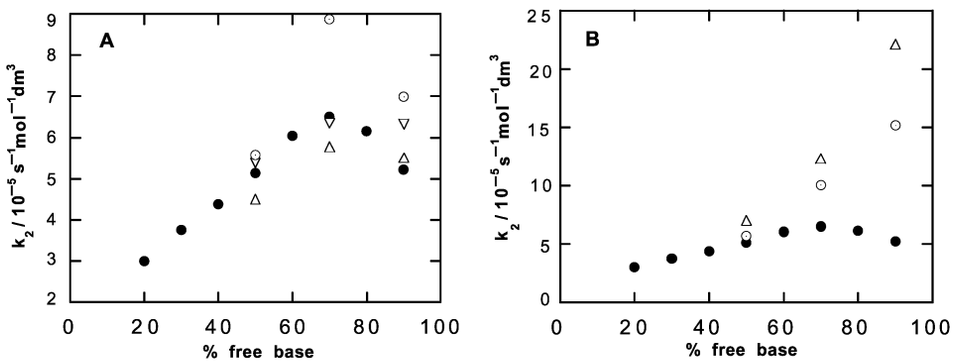


Fig. 4 Medium effects on the imidazole-catalysed P–O cleavage reaction of **4a** (see the text). The data from Fig. 1 (filled circles) are included in both plots for comparison. **A**: salt effects. Ionic strength *either*: maintained at 1.0 M with KCl (filled circles) or piperidine·HCl (triangles); *or*: varied, by diluting the highest concentration buffer [initial ionic strength 1.0 M KCl (circles) or piperidine·HCl (inverted triangles)] without adding salt. **B**: effects of added pyrrole [ionic strength maintained at 1.0 M with KCl (circles) or piperidine hydrochloride (triangles)].

8% v/v of morpholine, which acts as both catalyst and cosolvent.)

To try to distinguish between these possibilities we ran two series of experiments: (1) using piperidine hydrochloride (a strong base of pK_a 11.2, so present only as the conjugate acid below pH 8) rather than KCl to maintain ionic strength at 1.0 M, and (2) adding pyrrole (chosen as a close but inert analogue of imidazole) as cosolvent, to maintain a constant total (molar) concentration of organic solute [imidazole free base plus pyrrole]. The effects on the shape of the imidazole buffer plot are shown in Fig. 4.

The use of organic salts in imidazole buffers has only a small effect on the "bell-shape" (Fig. 4, plot **A**). Solvent effects, by contrast, are dramatic (plot **B**). In the presence of added pyrrole the "bell-shape" disappears completely. Evidently one or both of the reactions contributing to the total observed pseudo first order rate constant is very sensitive to the effects of relatively small amounts of organic cosolvent; and the effect increases with pH.

Fig. 5 shows a comparison of rate constants for the two

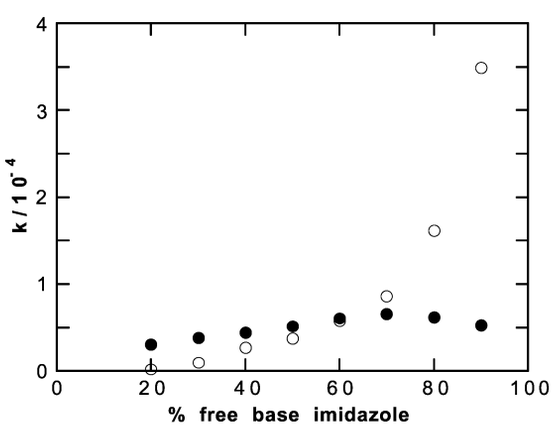


Fig. 5 Second-order rate constants (closed circles, $dm^3\ mol^{-1}\ s^{-1}$, data from Fig. 1) for catalysis of the hydrolysis of **4a** by imidazole at varying buffer ratios, compared with the first-order rate constants (k_0, open circles, s^{-1}) for the background hydrolysis reaction at the same pH. (Data at 70 °C and ionic strength 1 M, in water.)

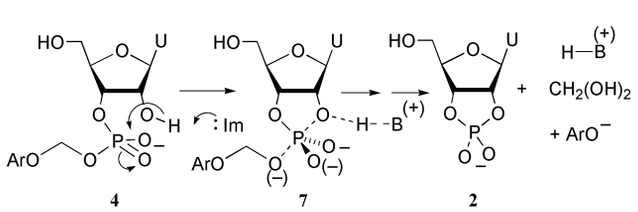
hydrolysis reactions of **4a**: k_2 for the reaction catalysed by 1 M imidazole buffer and k_0, the first-order rate constant for the accompanying buffer-independent reaction (obtained by extrapolation of the second-order plot to zero buffer concentration).

Catalysis by imidazole at 80 and 90% free base takes place against a rapidly increasing background hydroxide-catalyzed reaction: at 90% free base the reaction catalysed by 1 M imidazole buffer accounts for less than 12% of the observed rate of hydrolysis. We showed in our preliminary communication [8] that the rate of the hydroxide-catalyzed reaction is depressed by increasing concentrations of imidazole at higher pH, and that this factor can account quantitatively for the curvature (the bell-shape) in the plot of Fig. 2. The results for the six other general bases used (Fig. 3) can be treated in the same way to produce acceptably linear plots. The negative solvent effects on k_{OH} are quantitatively comparable to that used for imidazole, though morpholine and—perhaps surpisingly—PIPES have significantly larger effects.

Conclusions

The reactions of compounds **4** studied in this work are simplified versions of those of ribonucleotides like UpU. With the better hemiacetal leaving group, any intermediate phosphorane will have a shorter lifetime, with cleavage substantially faster relative to isomerisation: which is not therefore expected for **4a** in the region of pH under investigation. Thus our results for the hydrolysis of **4a** tell us specifically about the P–O cleavage reaction. The mechanism is clearly associative, with the formation of the new P–O bond running ahead of the cleavage: and if a phosphorane is an intermediate its formation rather than its breakdown must be rate determining because the hemiacetal is a better leaving group than the 2′-OH of ribose.

We have suggested that the main reaction flux for the hydrolysis and isomerisation of the chimeric nucleotide TTUTT near pH 7 is through the phosphorane monoanion.[7] The only evidence consistent with this pathway for the hydrolysis of **4a** is the minor contribution of catalysis by the imidazolium cation. Since the phosphorane dianion is not expected to have a significant lifetime with a leaving group as good as the hemiacetal $ArOCH_2O^-$ we see no reason to suggest a mechanism for the main reaction other than classical general base catalysis, with bond-making and bond-breaking more or less coupled in the transition state **7** (Scheme 3).[16]



Scheme 3

Using the model substrate **4a** we have reproduced Breslow's original bell-shaped buffer plot for imidazole catalysis of ribonucleotide hydrolysis using a model substrate, extended the number of buffers used and shown that the deviation from linearity is the result of a solvent effect on the background,

hydroxide-catalysed hydrolysis reaction. More recent data[17] show that for UpU the hydroxide-catalysed reaction is less important *vis á vis* the imidazole-catalyzed reaction than it is for **4a**, accounting for only some 30% of the observed reaction at 90% free base imidazole. The solvent effect we observe therefore appears not to be large enough to account for the bell-shaped curves observed with poly-U and UpU.[5] Nevertheless, solvent effects on the imidazole-catalysed reactions are clearly complex, making it difficult to define rate laws with confidence.[7,17,18] Buffer catalysis of the hydrolysis and isomerisation of the ribonucleotides is further complicated by a marked sensitivity to ionic strength.[7,17] It is this medium effect which was the source of the original bell-shaped curves observed with poly-U and UpU: the bell-shape was not observed when the reactions were run at constant ionic strength:[17] under these conditions the rate simply increased with the concentration of imidazole free base.

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 but is greatest where the increase in rate is proportionately small and high concentrations of catalyst are used. The history of the imidazole-catalysed reactions of ribonucleotide derivatives is a classic illustration of the potential problems.

Experimental

2′,5′-Di-*O*-tetrahydropyran-2-yl-3′-*O*-uridine dimethyl phosphate 5

N,*N*-Diisopropylethylamine (0.25 ml, 1.5 mmol) was added dropwise to a stirred solution of methyl phosphorodichloridite (0.07 ml, 0.7 mmol) in THF (7 ml) at −78 °C, under argon. After five minutes, 2′,5′-di-*O*-tetrahydropyran-2-yluridine[19] (0.24 g, 0.6 mmol in 2 ml of THF) was added dropwise. The mixture was stirred for 100 minutes at 0 °C and then methanol (0.08 ml, 1.8 mmol) was added at −78 °C. One hour later, iodine (0.37 g, 1.4 mmol in 5 ml THF and 2 ml H_2O) was added with pyridine (0.25 ml, 2.9 mmol) at room temperature. After five minutes the solvent was removed *in vacuo*, the residue taken up in dichloromethane (20 ml) and aqueous sodium hydrogen sulfite (8 ml of a 5% solution in water) added. The phases were separated, the organic layer was washed (10 ml water, 10 ml brine), dried (magnesium sulfate) and evaporated *in vacuo*. The triester was usually used without further purification but could be purified by chromatography (SiO_2, 5% MeOH–CH_2Cl_2) to yield the *triester* **5** as a gum (mixture of diastereoisomers); R_f (5% MeOH–CH_2Cl_2) 0.07; δ_H (250 MHz, $CDCl_3$) 9.10 (1 H, broad s, NH), 8.1–7.8 (1 H, m, NC*H*=CH), 6.3–6.1 (1 H, m, C^1H), 5.7–5.6 (1 H, m, NCH=C*H*), 5.4–3.4 (11 H, m, C^2H, C^3H, C^4H, C^5H_2 and 2 × OC(R)HO, 2 × CH_2O), 3.8–3.7 (6 H, m, 2 × $POCH_3$) and 1.9–1.4 (12 H, m, 6 × CH_2); δ_P ($CDCl_3$) −140.6 (4 signals, 2 coincident).

tert-Butylammonium salt of 2′,5′-di-*O*-tetrahydropyran-2-yl-3′-*O*-uridine methyl phosphate 6

tert-Butylamine (20 ml) was added to the crude triester **5** (200 mg, 0.4 mmol) and refluxed (46 °C) overnight. The solvent was removed *in vacuo* and the residue chromatographed (SiO_2, 5% MeOH + 0.5% *t*-$BuNH_2$–CH_2Cl_2) to yield the diester (200 mg, 52% from 2′,5′-di-*O*-tetrahydropyran-2-yluridine) as a glass (mixture of four diastereoisomers); R_f (10% MeOH–CH_2Cl_2) 0.02; δ_H (250 MHz, $CDCl_3$) 7.98, 7.92, 7.83 and 7.80 (1 H, d, *J* 8, NC*H*=CH), 6.6 (4 H, broad s, 4 × NH), 6.19 and 6.13 (½ H, d, *J* 7, C^1H), 6.12 and 6.01 (½ H, d, *J* 5, C^1H), 4.8–3.3 (11 H, m, C^2H, C^3H, C^4H, C^5H_2, 2 × OC(R)HO and 2 × CH_2O), 3.6–3.4 (3 H, m, $POCH_3$), 1.8–1.3 (12 H, m, 6 × CH_2) and 1.26 (9 H, s, $(CH_3)_3CN$); δ_P ($CDCl_3$) −141.4 (4 almost coincident signals); *m*/*z* 580 (100%, MH^+) and 505 (100, $(M\text{-}t\text{-}BuNH_3^+)^-$); (Found: M^+, 580.2676; $C_{24}H_{43}N_3O_{11}P$ requires *M*, 580.2635).

3,4-Dichlorophenoxymethyl methyl 2′,5′-di-*O*-tetrahydropyran-2-yl-3′-*O*-uridine phosphate

Silver(I) oxide (0.4 g, 1.7 mmol), the diester **6** (0.5 g, 0.9 mmol) and 3,4-dichlorophenoxymethylene chloride[20] (0.65 ml, 4.5 mmol) in acetonitrile (10 ml) were stirred under argon, in the dark at 50 °C for two days. The mixture was taken up in dichloromethane, filtered, evaporated *in vacuo*, taken up in more dichloromethane, washed (water, brine) and dried (magnesium sulfate). Chromatography (SiO_2, 2 to 5% MeOH–CH_2Cl_2, graded elution) yielded the *triester* (503 mg, 86%) as a gum (mixture of diastereoisomers); R_f (5% MeOH–CH_2Cl_2) 0.07; δ_H ($CDCl_3$) 8.4–7.7 (1 H, m, NC*H*=CH), 7.4–7.3 (1 H, m, ArH), 7.2 (1 H, m, ArH), 6.9 (1 H, m, ArH), 6.2–6.1 (1 H, m, C^1H), 5.9–5.6 (3 H, m, $POCH_2O$ and NCH=C*H*), 5.0–3.5 (11 H, m, C^2H, C^3H, C^4H, C^5H_2 and 2 × OC(R)*H*O, 2 × CH_2O), 3.8–3.7 (3 H, m, $POCH_3$) and 1.8–1.4 (12 H, m, 6 × CH_2); δ_P ($CDCl_3$) −143.1 (coincident signals).

4-Nitrophenoxymethyl methyl 2′,5′-di-*O*-tetrahydropyran-2-yl-3′-*O*-uridine phosphate

Silver(I) oxide (80 mg, 0.34 mmol), diester **6** (100 mg, 0.17 mmol) and 4-nitrophenoxymethylene chloride[21,22] (0.13 ml, 1.0 mmol) in acetonitrile (2 ml) was stirred under argon, in the dark at 50 °C for two days. The mixture was taken up in dichloromethane, filtered, evaporated *in vacuo*, taken up in more dichloromethane, washed (water, brine) and dried (magnesium sulfate). Chromatography (SiO_2, 2 to 5% MeOH–CH_2Cl_2, graded elution) yielded the *triester* (28 mg, 25%) as a gum (mixture of diastereoisomers); R_f (5% MeOH–CH_2Cl_2) 0.1; δ_H (200 MHz, $CDCl_3$) 9.05, 8.99, 8.96 and 8.91 (1 H, broad s, NH), 8.3–8.2 (2 H, m, 2 × ArH), 8.1–7.7 (1 H, m, NC*H*=CH), 7.2–7.1 (2 H, m, 2 × ArH), 6.3–6.1 (1 H, m, C^1H), 5.9–5.7 (3 H, m, $POCH_2O$ and NCH=C*H*), 5.2 (1 H, m, C^3H), 4.8–3.4 (10 H, m, C^2H, C^4H, C^5H_2, 2 × OC(R)HO and 2 × CH_2O), 3.9–3.7 (3 H, m, $POCH_3$) and 1.8–1.4 (12 H, m, 6 × CH_2); δ_P ($CDCl_3$) −143.2 (coincident signals).

tert-Butylammonium salt of 3,4-dichlorophenoxymethyl 2′,5′-di-*O*-tetrahydropyran-2-yl-3′-*O*-uridine phosphate

tert-Butylamine (40 ml) was added to the triester made above (502 mg, 0.74 mmol) and the solution refluxed (46 °C) overnight. The solvent was removed *in vacuo* and the residue chromatographed (SiO_2, 5 to 10% MeOH + 0.5% *t*-$BuNH_2$–CH_2Cl_2, graded elution) to yield the *diester* (445 mg, 81 %) (mixture of diastereoisomers) as plates, mp 129–131 °C (from ethanol); R_f (10% MeOH–CH_2Cl_2) 0.10; δ_H (400 MHz, $CDCl_3$) 8.5–7.7 (1 H, m, NC*H*=CH), 7.3 (1 H, m, ArH), 7.1 (1 H, m, ArH), 6.9 (1 H, m, ArH), 6.2–5.7 (2 H, m, C^1H and NCH=C*H*), 5.6–5.4 (2 H, m, $POCH_2O$), 5.0–3.3 (11 H, m, C^2H, C^3H, C^4H, C^5H_2, 2 × OC(R)HO and 2 × CH_2O), 1.8–1.3 (12 H, m, 6 × CH_2) and 1.27 (9 H, s, $(CH_3)_3CN$); δ_C ($CDCl_3$) 169.7 (4 signals, C=O), 155.8 (2 signals, aromatic C–O), 155.4 (4 signals, C=O), 144.5 (m), 132.6 (m), 130.6 (m), 125.2 (m), 118.0 (m), 115.7, 102–95 (m), 89–87 (m), 82 (m), 78–77 (m), 73 (m), 66 (m), 64–62 (m), 52–49 (m), 31–28 (m), 25.2 (2) and 20–18 (m); δ_P ($CDCl_3$) −145.3 (coincident signals); *m*/*z* 665 (100%, $(M\text{-}t\text{-}BuNH_2)^-$).

tert-Butylammonium salt of 4-nitrophenoxymethyl 2′,5′-di-*O*-tetrahydropyran-2-yl-3′-*O*-uridine phosphate

tert-Butylamine (10 ml) was added to the triester made above (180 mg, 0.27 mmol) and the solution refluxed (46 °C) overnight. The solvent was removed *in vacuo* and the residue chromatographed (SiO_2, 5 to 10% MeOH + 0.5% *t*-$BuNH_2$–CH_2Cl_2, graded elution) to yield the *diester* (109 mg, 56%) (mixture of diastereoisomers) as plates, mp 100–102 °C (from ethanol); R_f (10% MeOH–CH_2Cl_2) 0.09; δ_H (200 MHz, $CDCl_3$) 8.2–8.1 (2 H, m, 2 × ArH), 8.08, 7.95, 7.90 and 7.82 (1 H, d, *J* 8,

NC*H*=CH), 7.3–7.1 (6 H, broad s, 2 × ArH, NH and RNH_3^+), 6.2–6.0 (1 H, m, C^1H), 5.9–5.6 (3 H, m, $POCH_2O$ and NCH=C*H*), 4.8–3.3 (11 H, m, C^2H, C^3H, C^4H, C^5H_2, 2 × OC(R)*H*O and 2 × CH_2O), 1.8–1.4 (12 H, m, 6 × CH_2) and 1.35 (9 H, s, $(CH_3)_3CN$); δ_C ($CDCl_3$) 163.8, 161.6, 150.9, 142.5, 140.1 (m), 125.7, 115.9, 115.6, 101.9 (m), 99.2 (m), 97.6, 88.1, 86.5 (m), 82.6 (m), 73.5 (m), 66.4 (m), 63.8 (m), 62.0 (m), 53.4, 51.3, 30.0 (m), 28.0 (m), 25.3 (m) and 19.4 (m); δ_P ($CDCl_3$) −144.9 (4 signals, 2 coincident); *m*/*z* 717 (25%, M^+) and 642 (100, (M-*t*-BuNH_3)$^-$); (Found: M^+, 717.2785; $C_{30}H_{46}O_{14}N_4P$ requires *M*, 717.2748).

tert-Butylammonium salt of 3,4-dichlorophenoxymethyl 3′-*O*-uridine phosphate 4b

Amberlyst 15-H ion exchange resin (20 mg) was added to a solution of the protected diester (50 mg) in methanol (4 ml). After stirring at room temperature, for 150 minutes, the solution was filtered. A drop of *tert*-butylamine was added and the solvent removed under reduced pressure to yield the crude *diester* **4b** which was used without further purification; δ_H (200 MHz, CD_3OD) 8.06 (1 H, d, *J* 10, NC*H*=CH), 7.43 (1 H, d, *J* 6, ArH), 7.24 (1 H, d, *J* 2, ArH), 7.08 (1 H, dd, 6 and 2, ArH), 6.2–5.6 (2 H, m, NCH=C*H* and C^1H), 5.57 (2 H, d, *J* 12, $POCH_2OAr$), 5.0 (6 H, broad s, RNH_3^+, NH and 2 × ROH), 4.8–3.8 (5 H, m, C^2H, C^3H, C^4H and C^5H_2) and 1.28 (9 H, s, $(CH_3)_3CN$); δ_P (CD_3OD) −143.2. The product contained a little protected diester **8**, cyclised product and 3,4-dichlorophenol.

tert-Butylammonium salt of 4-nitrophenoxymethyl 3′-*O*-uridine phosphate 4a

Amberlyst 15-H ion exchange resin (20 mg) was added to a solution of the protected diester (25 mg) in methanol (4 ml). After stirring, at room temperature, for 150 minutes, the solution was filtered. A drop of *tert*-butylamine was added and the solvent removed under reduced pressure to yield the crude *diester* **4a** which was used without further purification; δ_H (250 MHz, CD_3OD) 8.20 (2 H, d, *J* 9, 2 × ArH), 7.95 (1 H, d, *J* 10, NC*H*=CH), 7.23 (2 H, d, *J* 9, 2 × ArH), 6.1–5.8 (2 H, m, C^1H and NCH=C*H*), 4.9 (6 H, broad s, RNH_3^+, NH and 2 × ROH), 4.7–3.7 (5 H, m, C^2H, C^3H, C^4H and C^5H_2) and 1.30 (9 H, s, $(CH_3)_3CN$). The product contained a little protected diester, cyclised product and 4-nitrophenol.

The crude mixture could be separated using reverse phase HPLC (Hichrom Spherisorb S50DS1–25217 semi-preparative column; 2 ml min^{-1} flow rate) and a solvent system containing 1 part acetonitrile to 4 parts 0.1 M triethylammonium acetate buffer (50% free acid, pH 4.5). From the spectra of the mixtures it was possible to estimate the isomer ratio.

After HPLC purification: major isomer (85–90%, 3′-*O*-phosphate, retention time 38 min); δ_H (250 MHz, CD_3OD) 8.21 (2 H, d, *J* 9, 2 × ArH), 8.03 (1 H, d, *J* 8, NC*H*=CH), 7.26 (2 H, d, *J* 9, 2 × ArH), 5.91 (1 H, d, *J* 5, C^1H), 5.71 (1 H, d, *J* 2, OCH_AH_BO), 5.67 (1 H, d, *J* 2, OCH_AH_BO), 5.63 (1 H, d, *J* 8, NCH=C*H*), 4.59 (2 H, m, C^5H_2), 4.24 (1 H, m, C^3H), 3.96 (1 H, m, C^2 or ^4H) and 3.55 (1 H, m, C^2 or ^4H); δ_P (CD_3OD) −143.3.

After HPLC purification: minor isomer (10–15%, 2′-*O*-phosphate, retention time 41 min); δ_H (250 MHz, CD_3OD) 8.22 (2 H, d, *J* 9, 2 × ArH), 7.91 (1 H, d, *J* 8, NC*H*=CH), 7.27 (2 H, d, *J* 9, 2 × ArH), 5.98 (1 H, d, *J* 6, C^1H), 5.73 (1 H, d, *J* 2, OCH_AH_BO), 5.68 (1 H, d, *J* 2, OCH_AH_BO), 5.65 (1 H, d, *J* 8, NCH=C*H*), 4.63 (2 H, m, C^5H_2), 4.29 (1 H, m, C^3H), 3.82 (1 H, m, C^2 or ^4H) and 3.56 (1 H, m, C^2 or ^4H); δ_P (CD_3OD) −143.5.

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