pH- and buffer-independent cleavage and mutual isomerization of uridine 2′- and 3′-alkyl phosphodiesters: implications for the buffer catalyzed cleavage of RNA

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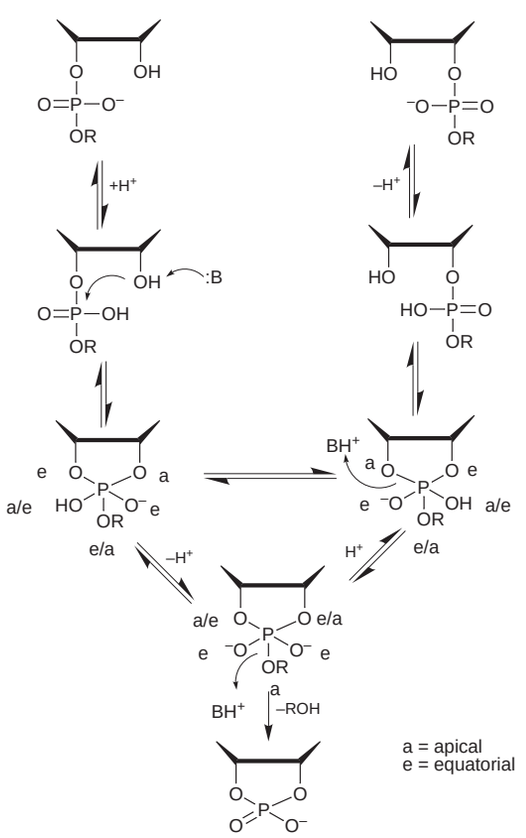
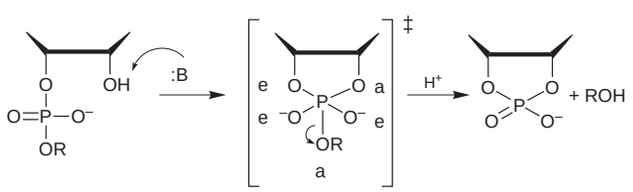
Concurrent isomerization of the ethyl, 2-ethoxyethyl, 2,2-dichloroethyl and 2,2,2-trichloroethyl esters of uridine 3′-phosphate to their 2′-counterparts and cleavage to uridine 2′,3′-cyclic phosphate have been studied over a wide pH-range ($H_0 = -0.2$ to pH 9) at 363.2 K. The buffer-independent pH-rate profiles obtained show involvement of four distinct kinetic terms in the cleavage reaction, *viz.* dependence of rate on $[H^+][SH]$, $[SH]$, $[S^-]$ and $[S^-][H^+]^{-1}$ (S^- denotes the diester monoanion). The β_{lg} values (lg = leaving group) for the partial reactions are -0.04 ± 0.04, -0.19 ± 0.12, -0.59 ± 0.12 and -1.10 ± 0.05, respectively. The isomerization, in turn, shows dependence of rate on $[H^+][SH]$, $[SH]$ and $[S^-]$, the β_{rg} values (rg = remnant group) being -0.23 ± 0.04, -0.23 ± 0.11 and -0.03 ± 0.01. The mechanisms of various partial reactions are evaluated by comparing these values with those of specific and buffer catalyzed reactions of the corresponding 3′-phosphotriesters, regarded as mimetics of the neutral ionic form of diesters. Furthermore, the mechanistic significance of the dissimilar competition between the cleavage and isomerization with diesters and triesters is discussed.

Introduction

The kinetics and mechanism of the cleavage of the phosphodiester bonds of RNA by general acids and bases have received considerable interest,[1–5] to a great extent as a model for the action of RNase A. Scheme 1 shows the mechanism suggested originally by Anslyn and Breslow[6] and refined later by Breslow *et al.*[2] Accordingly, the phosphodiester monoanion is assumed to undergo a rapid initial protonation, and the neutral phosphate is then attacked by the 2′-hydroxy function assisted by the buffer base. The monoanionic phosphorane intermediate obtained is decomposed in two alternative manners: uncatalyzed rate-limiting pseudorotation and subsequent cleavage of the P-O3′ bond give the 2′,5′-isomer (isomerization), while deprotonation to the phosphorane dianion followed by general acid catalyzed departure of the 5′-linked nucleoside yields a 2′,3′-cyclic phosphate (cleavage). Concurrent with this sequential general acid/base catalyzed cleavage reaction, interpreted as specific acid/general base catalysis for the formation and specific base/general acid catalysis for the breakdown of the intermediate, a simple general base catalyzed cleavage has been suggested to take place: general base catalyzed attack of the 2′-hydroxy function on monoanionic phosphate gives a dianionic phosphorane, which is decomposed solely to cleavage products without any kinetically visible catalysis (Scheme 2).[2] Breslow's



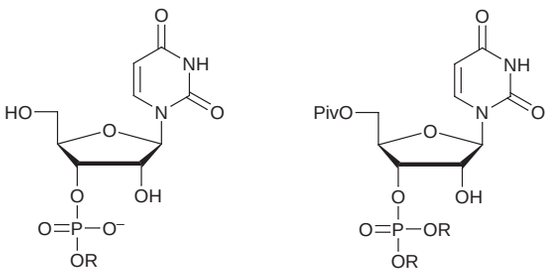
Scheme 1



Scheme 2

mechanism has been criticized, and alternative interpretations for his observations have been suggested,[4,7–11] but the refined mechanism has also been defended.[1,2] One curious feature of the mechanism depicted in Scheme 1 is catalytically asymmetric breakdown of the monoanionic phosphorane intermediate: the endocyclic fissions are assumed to be general acid catalyzed, while a similar exocyclic fission takes place only after deprotonation to a dianionic intermediate. We have tried to evaluate the feasibility of this mechanistic model by comparative kinetic

measurements of the cleavage and isomerization of uridine 3′-phosphodiesters (**1a–d**) and 3′-phosphotriesters (**2a–d**) under neutral conditions. The underlying idea is that a monoanionic phosphorane derived from a 3′-triester (**3b**) constitutes a reasonably good model for a monoanionic phosphorane derived from a 3′-diester (**3a**). The diester intermediate (**3a**) may, however, be deprotonated to a dianionic phosphorane, while this is not possible for the triester derived phosphorane (**3b**). Accordingly, if a dianionic phosphorane is on the path-



1 **a**: R = Et
b: R = CH_2CH_2OEt
c: R = CH_2CHCl_2
d: R = CH_2CCl_3
e: R = CH_2CH_2Cl

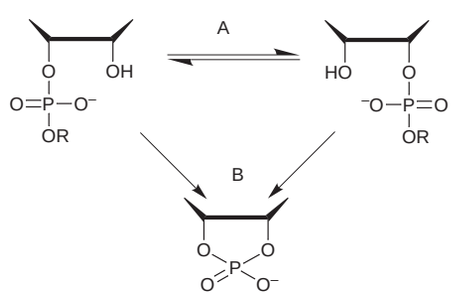
2 **a**: R = Pr^i
b: R = Et
c: R = Me
d: R = CH_2CH_2OMe

3a **3b**

way of the cleavage of diesters, as assumed by Breslow, competition between isomerization and cleavage of diesters is expected to differ considerably from that of triesters. The transition state structures of the pH-independent reactions are further elucidated by comparing their β_{lg} values to those observed for the hydronium and hydroxide ion catalyzed reactions, proceeding by departure of alcohol or alkoxide ion, respectively, and to the β_{lg} values reported previously[12] for the triester reactions. Additionally, the mechanisms of the reactions *via* a neutral phosphorane are briefly discussed.

Results

The concurrent intramolecular transesterifications of the alkyl esters of uridine 3′-phosphate[13] (**1a–d**) to uridine 2′,3′-cyclic phosphate (Reaction **B** in Scheme 3) and uridine 2′-alkylphosphates (Reaction **A** in Scheme 3) were followed at



Scheme 3

various pH values at 363.2 K by determining the composition of the aliquots withdrawn at appropriate intervals by reversed-phase (RP) HPLC. The pH–rate profiles obtained for the buffer-independent isomerization (**A**) and cleavage (**B**) are presented in Figs. 1 and 2, respectively. These profiles show, consistent with previous studies with uridylyl(3′,5′)uridine,[14] that the isomerization rate depends on three distinct kinetic terms ([SH][H^+], [SH] and [S^-]) and the cleavage rate on four

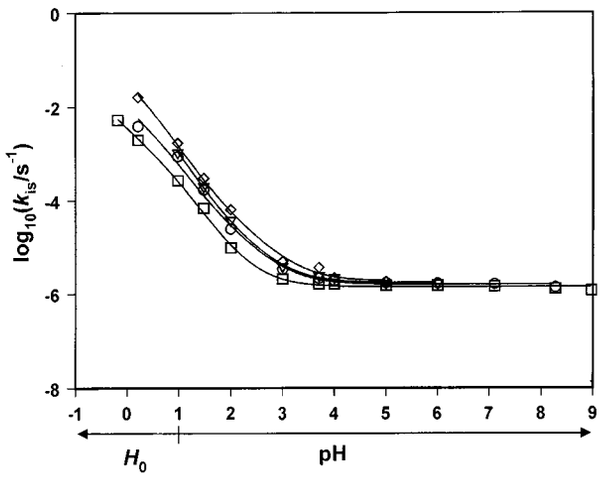


Fig. 1 pH–rate profiles for the mutual buffer-independent isomerization of uridine 3′- and 2′-alkylphosphates at 363.2 K. The ionic strength was adjusted to 0.1 mol dm^{-3} with NaCl, except at $H_0 < 1$. k_{is} denotes the sum of the rate constants of the opposite reactions. Notation: **1a** (□), **1b** (○), **1c** (▽), **1d** (◇).

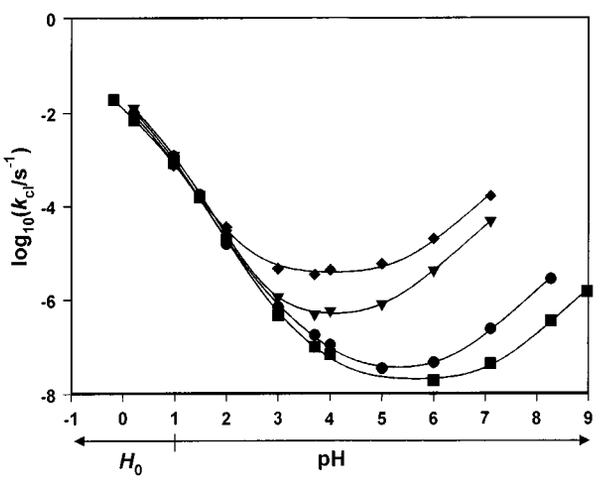


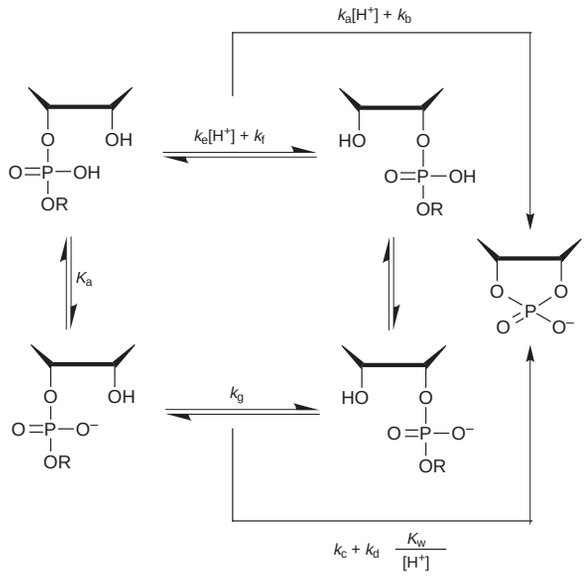
Fig. 2 pH–rate profiles for the buffer-independent cleavage of uridine 3′-alkylphosphates at 363.2 K. The ionic strength was adjusted to 0.1 mol dm^{-3} with NaCl, except at $H_0 < 1$. Notation: **1a** (■), **1b** (●), **1c** (▼), **1d** (◆).

terms ([SH][H^+], [SH], [S^-] and [S^-][H^+]$^{-1}$). Accordingly, the pseudo-first-order rate constants, k_{cl}, for the cleavage reaction (Reaction **B**) were fitted to eqn. (1) to obtain the partial rate and equilibrium constants ($k_a - k_d$ and K_a) indicated in Scheme 4.

$$k_{cl} = \frac{\frac{k_a}{K_a}[H^+]^2 + \frac{k_b}{K_a}[H^+] + k_c + \frac{k_a K_w}{[H^+]}}{\frac{[H^+]}{K_a} + 1} \quad (1)$$

Similarly, the partial rate constants for the isomerization ($k_e - k_g$), indicated in the same scheme, were obtained by fitting the pseudo-first-order rate constants, k_{is}, to eqn. (2). All the rate constants referring to isomerization (k_{is}, $k_e - k_g$) are sums of the rate constants for the forward and reverse reactions. At equilibrium, both isomers were observed to be present at equal concentration, indicating that the isomerization is as fast in both directions. Table 1 lists the values obtained.

$$k_{is} = \frac{\frac{k_e}{K_a}[H^+]^2 + \frac{k_f}{K_a}[H^+] + k_g}{\frac{[H^+]}{K_a} + 1} \quad (2)$$



Scheme 4

Table 1 Partial rate constants for the cleavage and isomerization of uridine 3′-alkylphosphates at 363.2 K ($I = 0.1$ mol dm^{-3} with NaCl)[a]

	1a	1b	1c	1d
K_a/mol dm^{-3}	0.073	0.14	0.11	0.16
$k_a/10^{-2}$ dm^3 mol^{-1} s^{-1}	1.32	2.2	2.3	1.8
$k_b/10^{-4}$ s^{-1}	0.27	0.9	0.55	3
$k_c/10^{-7}$ s^{-1}	0.19	0.30	4.2	35
$k_d/10^{-2}$ dm^3 mol^{-1} s^{-1}	0.45	4.1	960	3790
$k_e/10^{-2}$ dm^3 mol^{-1} s^{-1}	0.38	1.1	1.8	3.4
$k_f/10^{-4}$ s^{-1}	0.46	3.2	2.4	6.3
$k_g/10^{-6}$ s^{-1}	1.40	1.58	1.74	1.92

[a] For the rate and equilibrium constants see Scheme 4.

In addition to diesters **1a**–**d**, the reactions of the 2-chloroethyl ester (**1e**) were also studied. While this compound reacted at pH < 2 and at pH > 8 analogously to **1a**–**d**, it somewhat unexpectedly gave at pH 2 to 8, in addition to the 2′-isomer, two unidentified products in approximately a 1 : 1 ratio. Both of these compounds exhibited in their electrospray MS spectra signals at m/z −367 (100%), suggesting that the 2-chloro substituent of **1e** and its 2′-isomer had been displaced by a hydroxy group. The formation of these products was pH-independent and almost two orders of magnitude faster than what could be expected for the cleavage to the 2′,3′-cyclic phosphate.

Discussion

Partial reactions

Figs. 3 and 4 show as illustrative examples the contributions of various partial reactions to the observed rate constant of isomerization (k_{is}) and cleavage (k_{cl}) of two diesters, **1a** and **1c**. The effect of the leaving group on the rate of the partial reactions predominating under strongly acidic and alkaline conditions, *i.e.* the effect on k_a, k_d and k_e in Scheme 4, has been discussed previously.[13] The β_{lg} values obtained in the present study at elevated temperature (363.2 K) are almost equal to those observed earlier[13] at 298.2 K: β_{lg} for k_a −0.04 ± 0.04 (−0.12 ± 0.05), for k_d −1.10 ± 0.05 (−1.28 ± 0.05), and β_{rg} (rg = remnant group) for k_e −0.23 ± 0.04 (−0.18 ± 0.02) (the previously reported values are given in parentheses). Accordingly, attention is paid in the following only to those partial reactions predominating under neutral conditions, *i.e.* to k_b, k_c, k_f and k_g in Scheme 4.

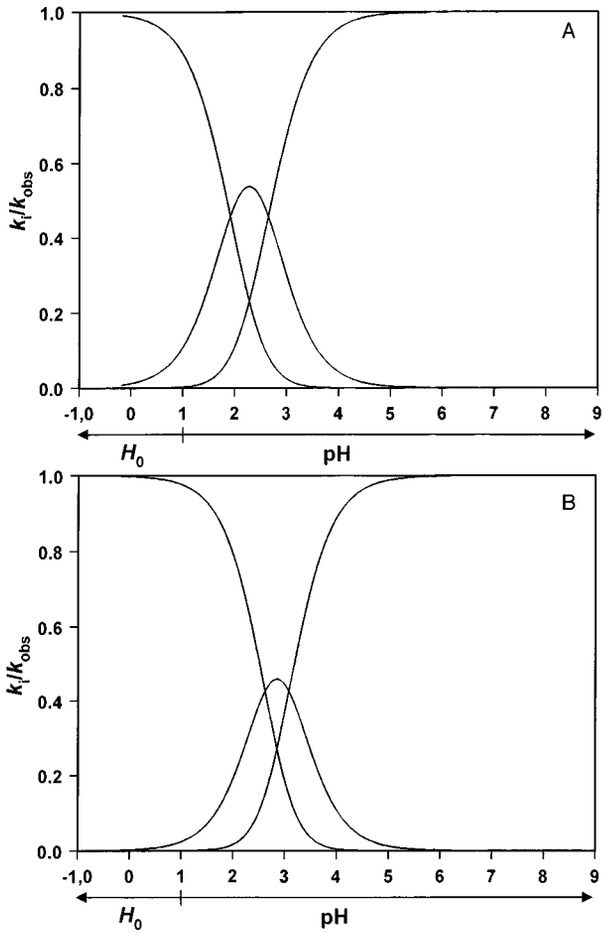


Fig. 3 Contributions of various kinetic terms to the rate constant constant of buffer-independent isomerization of uridine 3′-alkylphosphates. The curves from left to right refer to partial reactions exhibiting the dependence of rate on [H$^+$][SH], [SH] and [S$^-$], respectively (S stands for the phosphodiester monoanion). Field A shows the data for the ethyl ester **1a** and field B for the 2,2-dichloroethyl ester **1c**.

pH-independent cleavage of phosphodiester monoanions

As seen from Figs. 3 and 4, the pH-independent isomerization of the phosphodiester monoanion (k_g) predominates at pH > 4 and the pH-independent cleavage, depending on the acidity of the leaving group, at pH 4 to 7. The isomerization is markedly insensitive to the acidity of the esterified alcohol, whereas the β_{lg} value for the cleavage is moderately negative, −0.59 ± 0.12 (Fig. 5). The isomerization rate of **1a**–**d** is 70- to 0.8-fold greater than the cleavage rate of the same compound, the difference in rate gradually decreasing with the increasing acidity of the esterified alcohol. Assuming that both reactions take place *via* a common phosphorane intermediate, as previously suggested,[5] the most plausible mechanisms may be depicted by Scheme 5: a monoanionic phosphorane obtained by a pH-independent reaction of a minor tautomer of the starting material either undergoes pseudorotation and subsequent breakdown to the 2′-isomer, or is decomposed to 2′,3′-cyclic phosphate by Reactions **C** or **D**. Since the isomerization is usually faster than cleavage, the breakdown of the monoanionic intermediate to the 2′,3′-cyclic phosphate must be (at least partially) the rate-limiting step of the cleavage reaction. If the cleavage takes place *via* Reaction **D**, the situation may well be compared to the hydroxide ion catalyzed reactions of triesters **2a**–**d**, which also proceed *via* a common monoanionic phosphorane[12,15] (Scheme 6). With triesters **2a**–**d**, the partition of the monoanionic phosphorane to the isomerization and cleavage products is very unsymmetrical. The hydroxide ion catalyzed isomerization is 10^5 times faster than the cleavage.[12] This suggests that the

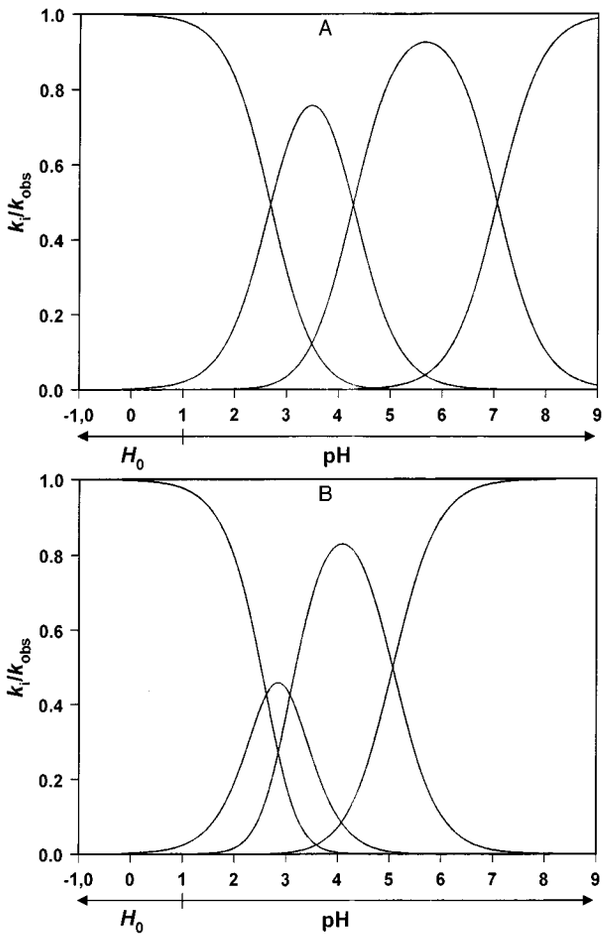
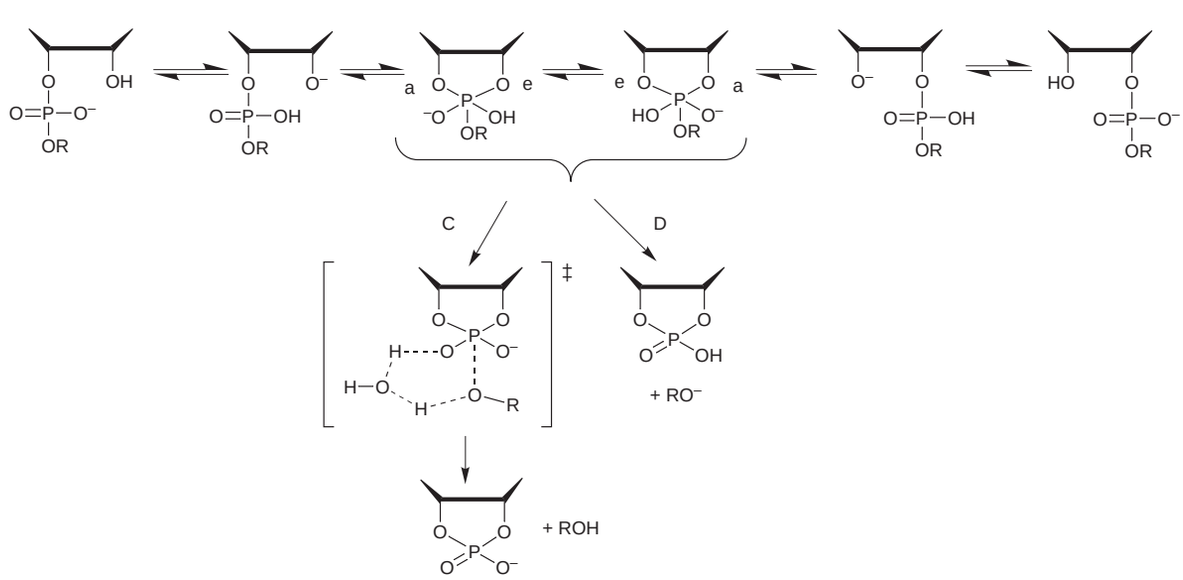


Fig. 4 Contributions of various kinetic terms to the rate constant constant of buffer-independent cleavage of uridine 3′-alkylphosphates. The curves from left to right refer to partial reactions exhibiting the dependence of rate on $[H^+][SH]$, $[SH]$, $[S^-]$ and $[S^-][H^+]^{-1}$, respectively (S^- stands for the phosphodiester monoanion). Field A shows the data for the ethyl ester **1a** and field B for the 2,2-dichloroethyl ester **1c**.

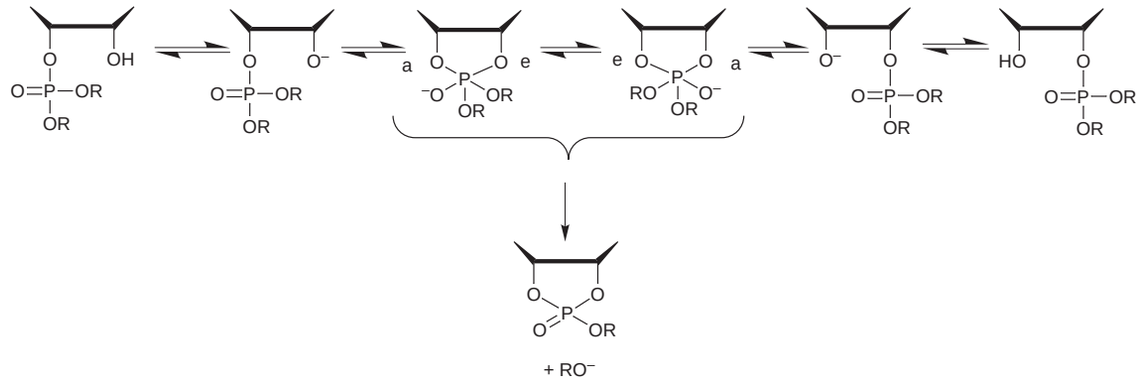
breakdown *via* the monoanionic phosphorane derived from diesters would also be several orders of magnitude slower than the isomerization, if Reaction **D** in Scheme 1 is utilized. This is not the case. As mentioned above, with diesters the formation of isomerization products is favoured only by a factor of 70 or less. This marked difference in the behaviour of diesters and triesters may be taken as an indication of dissimilar mechanisms. Evidently the presence of a dissociable proton in the monoanionic phosphorane intermediate of the diester reactions (Scheme 5) really plays a role. While the triesters have to utilize Reaction **D**, deprotonation of the monoanionic phosphorane offers for diesters a more favourable reaction pathway. Since the reaction must, however, remain pH-independent and deprotonation of the monoanionic phosphorane is not thermodynamically favoured under neutral conditions ($pK_a \gg 7$),[1] this deprotonation must be followed by a kinetically visible protonation. Consistent with the mechanism suggested by Breslow for the buffer catalyzed cleavage, one may assume that the departure of an alkoxy group from the dianionic phosphorane is accompanied by partially rate-limiting protonation of the leaving oxygen. For comparison, we have previously presented evidence that over a relatively wide pH-range from 3 to 7 a related reaction, *viz.* a general acid catalyzed departure of the alkoxy group from the monoanionic phosphorane, is the predominant mechanism for the cleavage of triesters **2a**–**d**.[12] Uncatalyzed cleavage of the alkoxide ion overcomes this reaction only at pH > 7.

The β_{lg} value of -0.59 ± 0.12 observed for the pH-independent cleavage of diesters **1a**–**d** appears to be consistent with Reaction **C** in Scheme 5. The corresponding value for the hydroxide ion catalyzed cleavage, which proceeds by displacement of the alkoxide ion with 2′-oxyanion *via* a dianionic pentacoordinated transition state or marginally stable phosphorane intermediate (Scheme 7*a*),[13] is considerably more negative, -1.10 ± 0.05 (-1.28 ± 0.05 at 298.2 K[13]). For the cleavage *via* cationic phosphorane (Scheme 7*b*), where the leaving alkoxy group undoubtedly departs as a neutral alcohol, and the bond rupture is at least partially rate-limiting,[5,13] the β_{lg} value is -0.04 ± 0.04. Hence, the β_{lg} value of -0.59 might well refer to partial protonation of the leaving group in the transition state. For comparison, the β_{lg} values reported[12] for triesters **2a**–**d** are: -1.26 ± 0.07 for the cleavage *via* departure of an alkoxide ion from the monoanionic phosphorane (Scheme 8*a*), -0.94 ± 0.13 for the cleavage *via* general acid catalyzed departure of the alkoxy group from the monoanionic phosphosphorane (hydronium ion is assumed to act as a general acid, Scheme 8*b*; with carboxylic acid buffers $\beta_{lg} = -1.0$), and -0.51 ± 0.13 for the cleavage *via* departure of alcohol from monocationic phosphorane (Scheme 8*c*). Though the β_{lg} values are somewhat more negative with triesters than with diesters, a similar trend is evident.

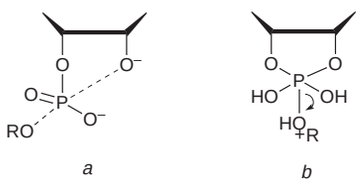
In summary, the preceding discussion suggests that the



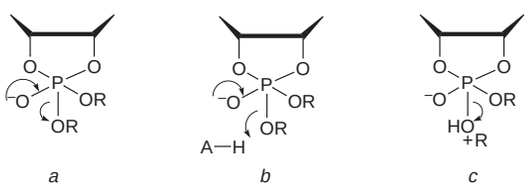
Scheme 5



Scheme 6



Scheme 7



Scheme 8

pH-independent cleavage of **1a**–**d** proceeds by Reaction **C** rather than Reaction **D** in Scheme 5. Accordingly, the present results do not argue against the sequential specific base/general acid catalyzed mechanism suggested by Breslow for the breakdown of the monoanionic phosphorane intermediate derived from uridylyl(3′,5′)uridine, although they cannot be taken as conclusive evidence for Breslow's mechanism, either.

pH-independent isomerization

The pH-independent isomerization of **1a**–**d** (Scheme 5) is quite insensitive to the acidity of the esterified alcohol, $\beta_{rg} = -0.03 \pm 0.01$ (Fig. 5), in marked contrast to the corresponding partial reaction of phosphotriesters (**2a**–**d**), *viz.* the hydroxide ion catalyzed isomerization (Scheme 6). The β_{rg} value of the latter reaction has been reported[12] to be -1.10 ± 0.16. With both diesters and triesters the increasing acidity of the esterified alcohol(s) facilitates the formation of the phosphorane intermediate by lowering the electron density at phosphorus, and this effect is undoubtedly greater with triesters than with diesters, since triesters bear two electron-withdrawing alkyl groups instead of one of diesters. Even if this fact is taken into account, the marked insensitivity of the diester isomerization to the electronegativity of the alkyl group appears to need an explanation. The most plausible mechanism for the reaction is the attack of the 2′-oxyanion on neutral phosphate, followed by pseudorotation, which may at least partially be rate-limiting (Scheme 5).[5,15,16] According to the rules of Westheimer,[17] a strongly electron-withdrawing alkyl group may be expected to adopt an apical position upon formation of the phosphorane intermediate. Since pseudorotation places the same alkyl group in equatorial position, the pseudorotation barrier is increased with the increasing electronegativity of the alkyl group. Accordingly, the polar effects on the formation of the phosphorane intermediate and its pseudorotation are opposite. With triesters, having two identical alkyl groups, the height of the pseudorotation barrier does not depend on the electronegativity of the alkyl groups, since one of them is always apical and the other equatorial.

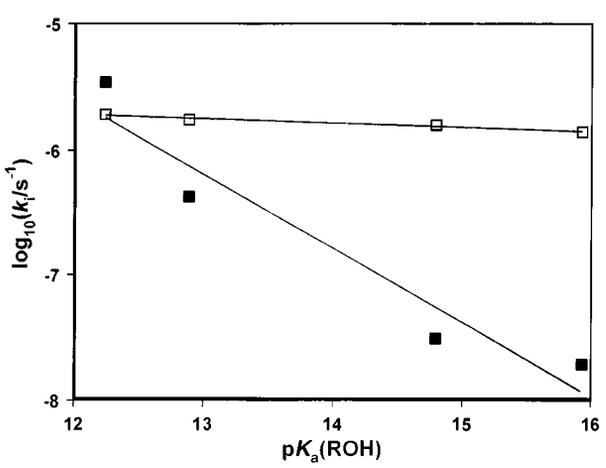
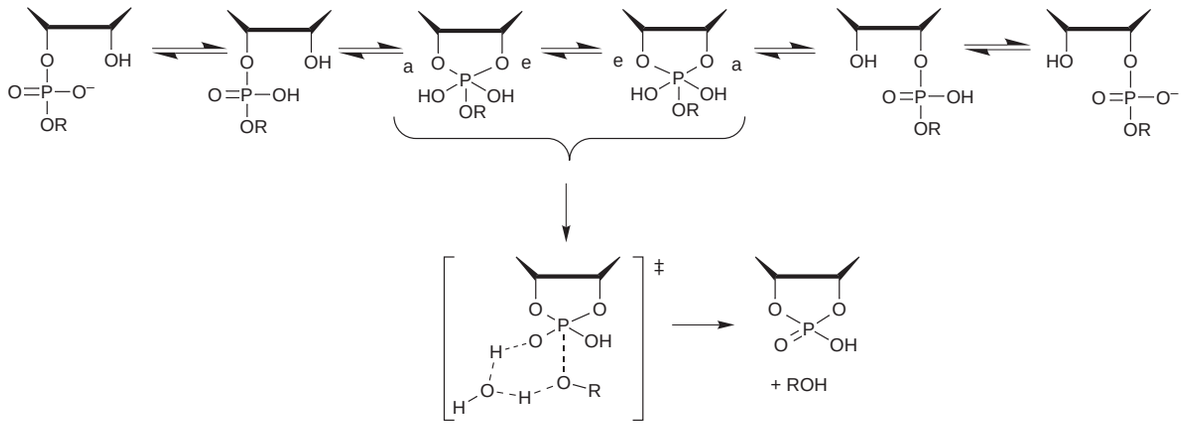


Fig. 5 Logarithmic first-order rate constants for the pH-independent cleavage (k_c, ■) and isomerization (k_g, □) of uridine 3′- and 2′-alkylphosphate monoanions plotted against the pK_a value of the esterified alcohol.[20] The data refer to 363.2 K and $I = 0.1$ mol dm^{-3}. $\beta_{lg} = -0.59 \pm 0.12$ for the cleavage and $\beta_{rg} = -0.03 \pm 0.01$ for the isomerization.

Uncatalyzed reactions of neutral phosphodiesters

As seen from Figs. 3 and 4, the kinetic term referring to the dependence of rate on [SH] predominates both with the isomerization and cleavage of **1a**–**d** over a narrow acidity range around pH 3. The most straightforward mechanistic description for this partial reaction is the attack of the 2′-hydroxy function on neutral phosphate followed by (or concerted with) the water mediated proton transfer from the attacking nucleophile to the phosphoryl oxygen (Scheme 9).[5] Pseudorotation of the neutral phosphorane intermediate obtained, and subsequent cleavage of the P-O3′ bond after (or concerted with) the water mediated proton transfer from the phosphoryl oxygen to the leaving oxygen lead to isomerization. Protonation of the alkoxy oxygen, in turn, results in the cleavage reaction. *Ab initio* calculations on neutral phosphorane derived from methyl ethylene phosphate suggest that the pseudorotation barrier is lower than that of endocyclic or exocyclic PO bond cleavage.[18] Accordingly, pseudorotation hardly limits the rate of isomerization. The β values of these partial reactions, $\beta_{rg} = -0.19 \pm 0.12$ for the isomerization and $\beta_{lg} = -0.23 \pm 0.11$ for the cleavage (Fig. 6), are consistent with the essential features of the proposed mechanism, *viz.* partially rate-limiting attack and departure of neutral alcohol. For comparison, the β values for the isomerization and cleavage *via* a monocationic phosphorane, both preceeding by the attack and departure of alcoholic oxygen, are $\beta_{rg} = -0.23 \pm 0.04$ and β_{lg} -0.04 ± 0.04, respectively.[12]



Scheme 9

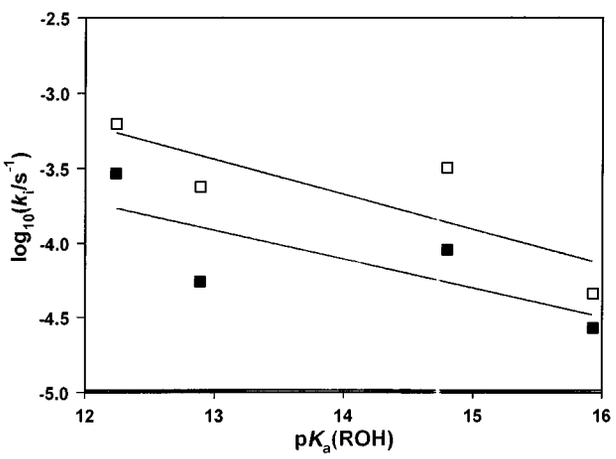


Fig. 6 Logarithmic first-order rate constants for the cleavage (k_b, ■) and isomerization (k_f, □) of neutral uridine 3′- and 2′-alkylphosphates plotted against the pK_a value of the esterified alcohol.[20] The data refer to 363.2 K and $I = 0.1$ mol dm^{-3}. $\beta_{lg} = -0.19 \pm 0.12$ for the cleavage and $\beta_{rg} = -0.23 \pm 0.11$ for the isomerization.

Table 2 Retention times of uridine 3′-alkylphosphates, their 2′-isomers and hydrolysis products on a Hypersil ODS column[a]

	t_R/min		
Compound	3′-Isomer	2′-Isomer	Eluent[b]
1a	7.4	5.1	3.5
1b	10.6	6.4	5.0
1c	10.4	5.5	7.0
1d	9.1	4.9	12.0
1e	7.4	4.6	5.0
2′,3′-cUMP	5.0		0.0
UMP	5.8	7.0	0.0

[a] Column 4 × 250 mm, particle size 5 μm, flow rate ml min^{-1}. The absorptions were measured at $\lambda = 260$ nm. [b] Acetonitrile content (%, v/v) in an acetic acid–sodium acetate buffer (0.045–0.015 mol dm^{-3}), containing 0.1 mol dm^{-3} ammonium chloride.

Experimental

Materials

The syntheses of the alkyl esters of uridine 3′-phosphate (**1a**–**e**) have been described previously.[13]

Kinetic measurements

The pH of the reaction solutions was adjusted with aqueous hydrogen chloride or with formic acid, acetic acid, HEPES and glycine buffers. The reactions were carried out as described earlier,[13] the initial concentration of the starting material being approximately 2×10^{-4} mol dm^{-3}. The progress of the reactions was followed by analyzing the composition of the aliquots withdrawn at appropriate intervals by RP HPLC. The retention times and chromatographic conditions are indicated in Table 2. The pseudo-first-order rate constants for the cleavage of **1a**–**d** to uridine 2′,3′-cyclic phosphate (k_{cl}) and their isomerization to the 2′-diesters (k_{is}) were calculated by the rate laws of irreversible and reversible first-order reactions, respectively.[19] The buffer-independent rate constants were obtained by extrapolating the rate constants observed at low buffer concentrations to the buffer concentration zero. As an illustrative example, the experimental rate constants obtained with the 2,2-dichloroethyl ester (**1c**) at two buffer concentrations are listed in Table 3.

Table 3 Observed pseudo-first-order rate constants for the cleavage ($k_{cl,\ obs}$) and isomerization ($k_{is,\ obs}$) of uridine 3′-(2,2-dichloroethyl)phosphate (**1c**) at 363.2 K ($I = 0.1$ mol dm^{-3} with NaCl)

Buffer acid	[HA]/mol dm^{-3}	[A$^-$]/mol dm^{-3}	$k_{cl,\ obs}/10^{-6}$ s^{-1}	$k_{is,\ obs}/10^{-6}$ s^{-1}
Formic acid	0.050	0.010	3.83 ± 0.05	9.4 ± 0.3
	0.025	0.005	2.48 ± 0.02	6.61 ± 0.09
	0.020	0.020	1.442 ± 0.015	3.85 ± 0.09
	0.010	0.010	0.967 ± 0.004	3.12 ± 0.04
	0.010	0.020	0.92 ± 0.03	2.89 ± 0.04
	0.005	0.010	0.74 ± 0.02	2.52 ± 0.04
Acetic acid	0.010	0.020	0.723 ± 0.016	1.89 ± 0.06
	0.005	0.010	0.76 ± 0.06	1.76 ± 0.05
HEPES[a]	0.040	0.010	4.69 ± 0.08	1.90 ± 0.03
	0.020	0.005	4.43 ± 0.11	1.78 ± 0.06
	0.010	0.030	47.5 ± 0.9	2.18 ± 0.17
	0.005	0.015	47.3 ± 1.2	*b*

[a] *N*′-(2-Hydroxyethyl)piperazine-*N*-ethanesulfonic acid. [b] Not detectable.

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