Reactions of Pyrimidine Nucleosides with Aqueous Alkalies: Kinetics and Mechanisms*

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Kinetics for the reactions of various cytosine and uracil nucleosides and their alkyl derivatives with aqueous sodium hydroxide have been studied by liquid chromatography. Blocking of the glycosyl hydroxyl groups with alkyl groups and changes in the glycon moiety configuration have been observed to exert only moderate effects on the rate of deamination of cytosine nucleosides. Methylation of the 4-amino group retards deamination considerably, while a methyl substituent at C5 is rate accelerating and at C6 only moderately rate retarding. These findings have been accounted for by a mechanism involving a rate limiting bimolecular displacement of the 4-amino group by a hydroxide ion. Analogous comparisons with uracil nucleosides suggest that the decomposition of uridine is initiated by an intermolecular attack of hydroxide ion on the C5 atom of the base moiety. In contrast, β-D-arabino- and β-D-lyxo-furanosyl derivatives appear to be cleaved via an intramolecular nucleophilic attack of the ionized 2′-hydroxyl group.

Detailed understanding of the alkaline solvolysis of nucleosides is helpful in attempting to elucidate the degradation of oligonucleotides and to develope methods for the removal of protecting groups during oligonucleotide synthesis. Among purine nucleosides, inosine has been shown to react via opening of the pyrimidine ring,[1-3] while adenosine,[4] 7-methylguanosine,[5-7] 9-(β-D-ribofuranosyl)purine,[8-10] and its 6-chloro derivative[11] are decomposed by cleavage of the imidazole ring. The kinetics for these multistep reactions have been examined to a limited extent.[4,10,12]

Among pyrimidine nucleosides, cytosine derivatives are largely deaminated to the corresponding uracil nucleosides,[13-15] but degradation to small fragments exhibiting no UV absorption also takes place.[14,16] For the deamination, two alternative mechanisms have been proposed.[14] Either a hydroxide ion directly displaces the amino group at C4, or the deamination step is preceded by a nucleophilic attack of hydroxide ion on C6 of the cytosine moiety. One of the aims of the present paper is to distinguish between these two pathways. Furthermore, the involvement of the glycosyl hydroxyl groups in the deamination of ribo and arabino nucleosides has been examined.

Uridines, with the exception of 5-halo substituted derivatives, are cleaved by complete fragmentation of the pyrimidine ring;[13,14,17] 5-halouridines and their β-D-arabinofuranosyl analogues yield, besides nonchromophoric products, numerous UV absorbing compounds, including uracil,[18] uridine,[18,19] 5-hydroxyuridine,[17-19] and imidazoline and barbituric acid nucleosides.[17,19,20] The initial step is either an inter or intramolecular nucleophilic attack on C5 or C6 of the base moiety. In the present paper, we try to elucidate which one of these alternatives operates in the hydrolysis of unsubstituted uridine and its β-D-arabino- and β-D-lyxo-furanosyl analogues.

Results and discussion

LC analyses of the aliquots withdrawn at different intervals from alkaline solutions of cytidine

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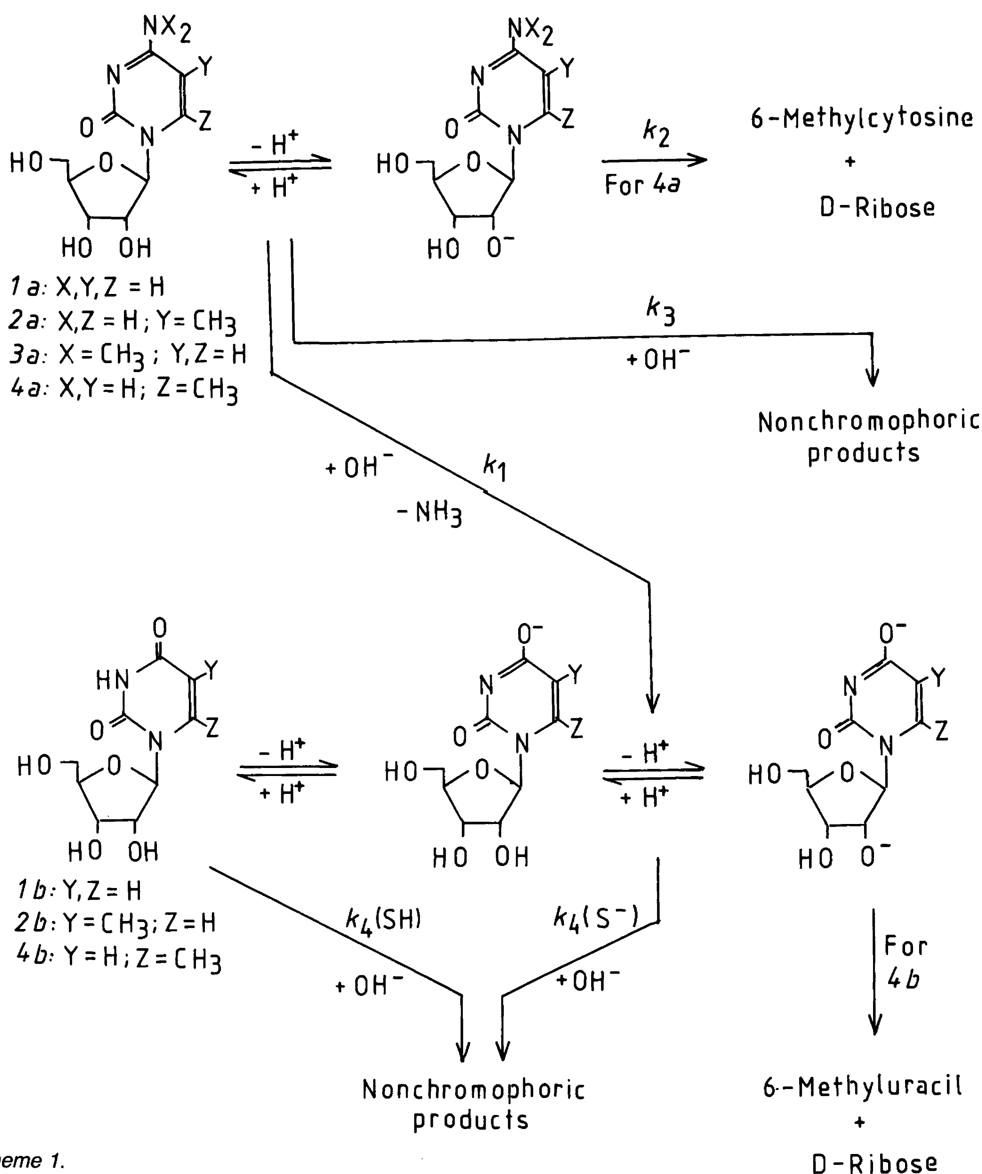


Scheme 1.

(*1a*) verified the previous suggestion[14,16] according to which cytidine is largely, but not quantitatively, deaminated to uridine (*1b*). Analogously, 5-methylcytidine (*2a*) and N^4,N^4-dimethylcytidine (*3a*) were observed to give 5-methyluridine (*2b*) and uridine, respectively, 6-methylcytidine (*4a*) was partly converted to 6-methyluridine (*4b*) and partly hydrolyzed to 6-methylcytosine and D-ribose; 6-methylcytosine was further deaminated to 6-methyluracil. Uridine and its methyl derivatives were slowly decomposed to products exhibiting no UV absorption. Accordingly, the alkaline cleavage of cytidines may proceed by the three parallel routes depicted in Scheme 1.

The values calculated from the LC data for the rate constants of the partial reactions are collected in Table 1. The rate constants obtained for the disappearance of *1a* and *1b* agree satisfac-

torily with those reported earlier.[14] However, the deamination of *1a* to *1b* occurs, according to our results, less quantitatively than suggested[14] on the basis of UV spectrophotometric measurements. The reason for this discrepancy is unknown. For the other compounds listed in Table 1, no kinetic data exist in the literature.

The pseudo first-order rate constants, k_1, for the deamination of *1a–4a* are approximately proportional to the concentration of hydroxide ion over the whole basicity range studied. Obviously, the ionization of the ribosyl hydroxyl groups does not play any important role in this partial reaction. The common electrolyte effects appear negligible. For example, the rate constant for the deamination of *1a* in 0.050 mol dm^{-3} aqueous sodium hydroxide remained unchanged within the limits of experimental errors, when the ionic strength was increased to 0.30 mol dm^{-3} with sodium chloride.

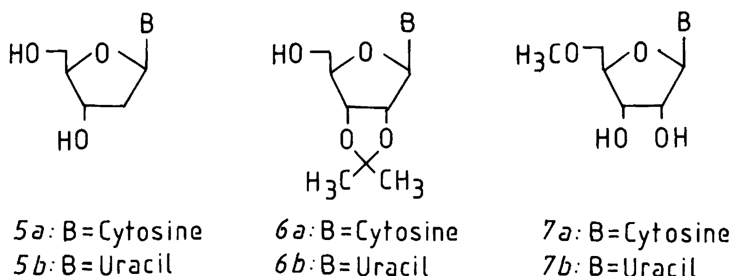
It is known that a methyl group bonded directly to the reaction center significantly retards nucleophilic attack of hydroxide ion. For example, hydroxide ion attacks on the carbonyl carbon of alkyl formates take place several hundred times more readily than on the carbonyl carbon of alkyl acetates.[21] The situation is similar with *p*-nitroformanilide and *p*-nitroacetanilide, both reacting by a rate limiting attack of hydroxide ion on the carbonyl carbon.[22] We have shown previously[23] that the half-life for the attack of hydroxide ion on C8 of 9-methylpurine is about 16 min in 0.50 mol dm^{-3} sodium hydroxide at 343.2 K. In contrast, 8,9-dimethylpuring remains almost unchanged for several h under the same conditions. Accordingly, C5 and C6 methyl groups can be expected to retard nucleophilic attacks on the respective sites of cytosine ring by a factor of 10^2–10^3. The fact that a 5-methyl group slightly accelerates and a 6-methyl group only moedrately retards the deamination of cytidine argues against rate limiting attack of hydroxide ion on C5 and C6, respectively. A direct displacement of the 4-amino group by hydroxide ion appears a more attractive mechanistic alternative. The large rate retardation that methylation of the 4-amino group results in is also in better agreement with the latter mechanism.

As mentioned above, 6-methylcytidine (*4a*) is the only cytosine nucleoside studied that yields

Table 1. Pseudo first-order rate constants for the disappearance of cytidine and its methyl derivatives in aqueous sodium hydroxide at 363.2 K, and the rate constants for the partial reactions involved.[a]

Compound	$[OH^-]$/mol dm^{-3}	$k_d/10^{-5}\ s^{-1}$	$k_1/10^{-5}\ s^{-1}$	$k_2/10^{-5}\ s^{-1}$	$k_3/10^{-5}\ s^{-1}$	$k_4/10^{-5}\ s^{-1}$
Cytidine (*1a*)	0.30	11.0(3)	6.9(3)	[b]	4.1(6)	1.60(2)
	0.25	9.1(3)	6.1(3)		3.0(6)	1.47(3)
	0.20	7.4(1)	4.4(2)		3.0(3)	1.21(3)
	0.15	5.5(1)	3.7(2)		1.8(3)	1.07(5)
	0.10	4.1(1)	2.6(1)		1.5(2)	0.86(5)
	0.050	2.2(1)	1.4(1)		0.8(2)	0.55(3)
5-Methylcytidine (*2a*)	0.25	10.3(1)	9.5(3)	[b]	0.8(4)	[c]
	0.20	9.4(2)	8.5(3)		0.9(5)	
	0.15	7.6(1)	6.9(3)		0.7(4)	
	0.10	5.1(1)	4.7(1)		0.4(2)	
	0.050	2.4(1)	2.2(1)		0.2(2)	
6-Methylcytidine (*4a*)	0.30	11.6(6)	2.7(3)	2.0(3)	6.9(11)	2.1(1)
	0.20	9.0(5)	1.7(2)	1.6(2)	5.7(9)	1.7(1)
	0.10	5.7(1)	0.9(1)	1.4(2)	3.3(4)	1.4(1)
N^4,N^4-Dimethylcytidine (*3a*)	0.30	1.27(8)	0.98(9)	[b]	0.3(2)	[d]
	0.20	0.94(3)	0.70(6)		0.2(1)	
	0.10	0.40(2)	0.30(3)		0.1(1)	

[a]For k_1, k_2 and k_3 see Scheme 1. $k_d = k_1 + k_2 + k_3$. k_4 is the first-order rate constant for the disappearance of the uridine formed as the deamination product of the corresponding cytidine. [b]Free base not detected. [c]*2b* was not markedly hydrolyzed within 120 h. [d]See the data given for *1a*.

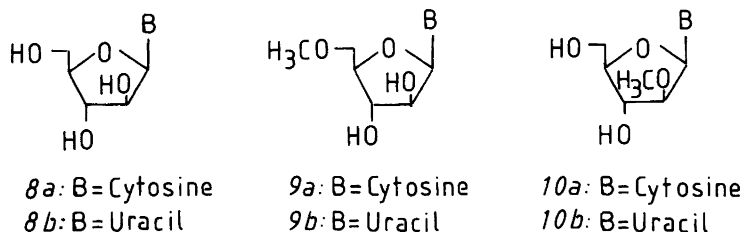


an intact pyrimidine base in aqueous alkali. Probably the steric strain caused by the methyl group adjacent to the ribofuranosyl group destabilizes the *N*-glycosidic bond. The pseudo first-order rate constants, k_2, for this partial reaction level off to a constant value at high alkalinities. Pos-

Table 2. Pseudo first-order rate constants for the disappearance of various cytosine nucleosides and their O′-alkyl derivatives in aqueous sodium hydroxide at 363.2 K, and the rate constants for the partial reactions involved.[a]

Compound	$[OH^-]$/mol dm^{-3}	$k_d/10^{-5}$ s^{-1}	$k_1/10^{-5}$ s^{-1}	$k_3/10^{-5}$ s^{-1}	$k_4/10^{-5}$ s^{-1}
2′-Deoxycytidine (*5a*)	0.30	10.4(2)	7.1(2)	3.3(4)	0.93(1)
	0.20	6.7(1)	4.8(1)	1.9(2)	0.67(2)
	0.10	2.9(1)	2.3(1)	0.6(2)	0.40(1)
2′,3′-*O*-Isopropylidenecytidine (*6a*)	0.20	11.5(2)	10.3(4)	1.2(6)	1.67(2)
	0.15	9.2(1)	8.0(2)	1.2(3)	1.44(2)
	0.10	6.0(1)	5.5(2)	0.5(3)	1.12(1)
	0.050	2.9(1)	2.6(1)	0.3(2)	0.88(1)
5′-*O*-Methylcytidine (*7a*)	0.30	10.3(2)	8.5(5)	1.8(7)	1.60(10)
	0.25	8.8(2)	7.4(3)	1.4(5)	1.40(11)
	0.20	6.8(1)	5.5(5)	1.3(6)	1.15(11)
	0.15	5.1(1)	4.2(2)	0.9(3)	0.95(7)
	0.10	3.6(1)	2.8(1)	0.8(2)	0.62(2)
	0.050	2.2(1)	1.6(1)	0.6(2)	0.46(1)
1-(β-D-Arabinofuranosyl)cytosine (*8a*)	0.30	22.9(3)	14.7(3)	8.2(6)	50.9(7)
	0.20	17.8(2)	10.6(3)	7.2(5)	50.9(6)
	0.10	10.9(1)	6.8(3)	4.1(4)	52.6(6)
1-(2′-*O*-Methyl-β-D-arabinofuranosyl)cytosine (*10a*)	0.30	7.11(10)	6.2(2)	0.9(3)	0.34(2)
	0.25	5.84(7)	5.0(1)	0.8(2)	0.30(2)
	0.20	4.69(5)	3.9(2)	0.7(3)	0.24(1)
	0.15	3.46(6)	2.9(2)	0.6(3)	0.21(1)
	0.10	2.32(4)	2.0(1)	0.3(1)	0.15(1)
	0.050	1.11(3)	1.0(1)	0.1(1)	0.10(1)
1-(5′-*O*-Methyl-β-D-arabinofuranosyl)cytosine (*9a*)	0.30	38.0(4)	30.0(5)	8.0(9)	83.4(14)
	0.20	27.8(3)	19.8(6)	8.0(9)	72.6(7)
	0.10	14.8(1)	10.4(5)	4.4(6)	65.5(12)
1-(β-D-Lyxofuranosyl)uracil (*11*)	0.30				36.2(9)
	0.20				34.6(9)
	0.10				37.7(9)

[a]See footnote ([a]) in Table 1. k_2 negligible for all the compounds listed.



sibly a mechanism similar to that of the alkaline hydrolysis of aryl aldofuranosides having a *trans*-1,2 configuration is utilized.[24] In other words, the 2′-hydroxyl group deprotonated in a rapid initial stage performs an intramolecular nucleophilic attack on the anomeric carbon atom with concomitant departure of the pyrimidine base.

Table 2 summarizes the kinetic data for the reactions of various cytosine nucleosides modified at the glycon moiety. With all these compounds, the corresponding uracil derivatives are the only UV absorbing products detected. The rate of deamination is proportional to the concentration of hydroxide ion over the whole basicity range, and the deamination takes place slightly more quantitatively than with cytidine. The compounds 2′-deoxycytidine (*5a*), 2′,3′-*O*-isopropylidenecytidine (*6a*) and 5′-*O*-methylcytidine (*7a*) are all deaminated to the corresponding uracil nucleosides (*5b–7b*) about as readily as cytidine. These findings corroborate the conclusion that the ribosyl hydroxyl groups do not participate in the deamination of cytidine.

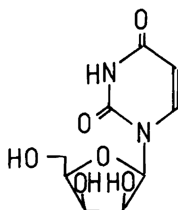
Compound *8a*, 1-(β-D-arabinofuranosyl)cytosine, reacts with alkalies more rapidly than cytidine.[16] Moreover, the disappearance of the starting material has been reported to proceed without appearance of the UV spectrum of 1-(β-D-arabinofuranosyl)uracil (*8b*).[16] On these bases, the decomposition of *8a* has been suggested[16] to involve an intramolecular nucleophilic attack of the 2′-hydroxyl group on the base moiety and a rapid subsequent opening of the pyrimidine ring. However, our results do not corroborate this argument. LC analyses clearly show the intermediary appearance of *8b*, but since it is decomposed more rapidly than *8a*, its mol fraction never exceeds 0.15. The rate constant, k_1, for the deamination is approximately double compared to that obtained with cytidine. Methylation of the 5′-hydroxyl group (*9a*) further accelerates the deamination by a factor of 2, while methylation of the 2′-hydroxyl group (*10a*) results in a similar retardation in rate. With all these compounds (*8a–10a*), k_1 is proportional to the concentration of hydroxide ion over the whole basicity range. This finding, together with the comparable reactivities of *1a*, *8a*, *9a* and *10a*, argues against the intramolecular participation of the arabinosyl hydroxyl groups in the deamination. Probably the mechanism suggested for the deamination of cytidine is utilized.

As seen from Table 1, the pseudo first-order rate constant, k_4, for the disappearance of uri-

Table 3. Parameters $k_4(S^-)$ and $k_4(SH)/K$ [eqn. (5)] for the alkaline decomposition of uridine and its alkyl derivatives at 363.2 k.

Compound	$k_4(S^-)/10^{-5}$ dm³ mol⁻¹ s⁻¹	$k_4(SH)K^{-1}/10^{-5}$ s⁻¹
Uridine (*1b*)	4.1(4)	0.41(5)
5-Methyluridine (*2b*)	[a]	
6-Methyluridine (*4b*)	3.6(3)	1.02(6)
2′-Deoxyuridine (*5b*)	2.7(1)	0.14(1)
2′,3′-*O*-Isopropylideneuridine (*6b*)	5.4(2)	0.61(3)
5′-*O*-Methyluridine (*7b*)	4.7(2)	0.21(4)

[a]No marked hydrolysis occurred within 120 h in 0.50 mol dm⁻³ sodium hydroxide.



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dine in aqueous alkali is not proportional to the concentration of hydrixide ion. Obviously, this results from the deprotonation of the base moiety in alkaline solutions. Ionization of the ribosyl hydroxyl groups hardly affects the shape of the curve of k_4 vs. $[OH^-]$, since the O′-alkylated uridines, *6b* and *7b*, exhibit rate profiles similar to that of uridine. Accordingly, the rate law obeyed may be expressed by eqn. (1), where $k_4(SH)$ and $k_4(S^-)$ denote the second-order rate constants for the disappearance of the neutral and anionic forms of the substrate, respectively. When [SH] and $[S^-]$ are expressed in terms of K and [S (total)], defined by eqns. (2) and (3), eqn. (4) is obtained for the observed first-order rate constant, k_4.

$$-\frac{d\,[S(total)]}{dt} = k_4(SH)[SH][OH^-] + k_4(S^-)[S^-][OH^-] \quad (1)$$

$$K = \frac{[S^-]}{[SH][OH^-]} \quad (2)$$

$$[S(total)] = [SH] + [S^-] \quad (3)$$

$$k_4 = \frac{k_4(SH)[OH^-] + k_4(S^-)K[OH^-]^2}{K[OH^-] + 1} \quad (4)$$

$$k_4 \simeq k_4(SH)/K + k_4(S^-)[OH^-] \quad (5)$$

The pK_a value for the deprotonation of the base moiety has been reported to be about 9.5 at 298.5 K.[25] Consequently, the substrate is almost completely deprotonated under the conditions employed in the kinetic measurements. Hence $K[OH^-]$ is much larger than unity, which leads to eqn. (5) for k_4.

The values of $k_4(S^-)$ and $k_4(SH)/K$ obtained for uridine and its alkylated derivatives are listed in Table 3. It should be noted that 6-methyluridine yields about 17% 6-methyluracil in addition to nonchromophoric products. The proportion of this reaction has been subtracted from the values of k_4 before the application of eqn. (5). Mechanistically, the release of 6-methyluracil may be explained as the release of 6-methylcytosine during the alkaline cleavage of *4a*.

The data in Table 3 indicate that alkylation of the ribosyl hydroxyl groups has only a small effect on $k_4(S^-)$. The influence that introduction of a methyl group at C6 of uridine results in is a moderate one, too. In contrast, 5-methyluridine (*2b*) is much more stable than uridine in aqueous alkali. During 120 h in 0.50 mol dm^{-3} sodium hydroxide at 363.2 K practically no decomposition occurred. These findings strongly suggest that the fragmentation of uridine is initiated by a nucleophilic attack of hydroxide ion on the C5 atom of the base moiety.

Compound *8b*, 1-(β-D-arabinofuranosyl)uracil, and 1-(β-D-lyxofuranosyl)uracil (*11*), both with *cis*-1,2 configurations, reacts with aqueous alkali much more rapidly than uridine. Moreover, the pseudo first-order rate constant, k_4, is independent of the concentration of hydroxide ion at $[OH^-] > 0.10$ mol dm^{-2}. Methylation of the 5′-hydroxyl group of *8b* slightly accelerates the alkaline decomposition, whereas blocking of the 2′-hydroxyl group retards the reaction by a factor of 100–300 and makes k_4 dependent on $[OH^-]$. Most probably the ionized 2′-hydroxyl group acts as an intramolecular nucleophile during the fragmentation of uracil nucleosides having a *cis*-1,2 arrangement. The pK_a value of the 2′-hydroxyl group of arabinofuranosyl residues has been reported to be 12.2 at the ionic strength of 1.0 mol dm^{-3} at 295.2 K.[26] Under the conditions employed in the kinetic measurements, the group is almost completely deprotonated, and hence the intramolecular attack on the base moiety, presumably on C6, is pH-independent. A similar mechanism has been suggested[19] for the reaction of the corresponding 5-halo derivatives with alkalies. In summary, the 2′-hydroxyl group appears to participate intramolecularly in the decomposition of 1-(β-D-arabinofuranosyl)uracil, but not in the deamination of its cytosine counterpart, *8a*. With cytidine and uridine, intermolecular nucleophilic attacks on the base moiety prevails; with cytidine, on C4 and with uridine, on C5.

Experimental

Materials. Cytidine (*1a*), 5-methylcytidine (*2a*), 2′-deoxycytidine (*5a*), 2′,3′-*O*-isopropylidenecytidine (*6a*), 1-(β-D-arabinofuranosyl)cytosine (*8a*) and the corresponding derivatives of uracil (*1b, 2b, 5b, 6b, 8b*) were commercial products of Sigma Chemical Company. Their purity was checked by LC, and they were employed as received. The preparation of 1-(2′-*O*-methyl-β-D-arabinofuranosyl)cytosine (*10a*) and -uracil (*10b*), 1-(5′-*O*-methyl-β-D-arabinofuranosyl)cytosine (*9a*) and -uracil (*9b*), and 1-(β-D-lyxofuranosyl)uracil (*11*) has been described earlier;[27] 5′-*O*-methylcytidine (*7a*) and -uridine (*7b*) were gifts of Dr. J. Kuśmierek. Their preparation has been reported elsewhere.[28] N^4,N^4-Dimethylcytidine (*3a*) was synthesized from cytidine by a transamination method of Shapiro and Weisgras.[29] The product exhibited a ^{13}C NMR spectrum identical with that reported by Chang *et al.*[30] 6-Methyluridine (*4b*) was prepared according to Vorbruggen *et al.*,[31] and converted to 6-methylcytidine (*4a*) via a 1,2,4-triazole derivative.[32] The ^{1}H and ^{13}C NMR spectra of *4a*,[33,34] and the ^{1}H NMR spectrum of *4b*[33] were identical with those reported. The latter compound was further characterized by comparing its LC behavior to that of an authentic sample received from Prof. D. Shugar.

Kinetic measurements. The LC technique described previously[10] was applied to follow the progress of the reactions of pyrimidine nucleosides with alkalies. However, the separations were carried out on a commercial μ-Porasil column (Waters Associates, 3.9×30 cm) using isocratic elution with mixtures of acetonitrile and acetic acid buffer (pH 4.3). The intermediates and products were identified by comparing the retention times and UV spectra to those of authentic samples. The peak heights were transformed to concentrations with the aid of calibration solutions of known concentrations.

Calculation of the rate constants. The pseudo first-order rate constants for the disappearance of cytosine and uracil nucleosides k_d and k_4, respectively, were calculated via the integrated first-order rate law. The first-order rate constants, k_1, for the deamination of cytosine nucleosides to uracil nucleosides were obtained from eqn. (6), where $[\mathrm{Urd}]_t$ is the concentration of the uracil derivative formed at time t, and $[\mathrm{Cyd}]_0$ is the initial concentration of the starting material.

$$\frac{[\mathrm{Urd}]_t}{[\mathrm{Cyd}]_0} = \frac{k_1}{k_4 - k_d}\,\mathrm{e}^{-k_d t} + \frac{k_1}{k_d - k_4}\,\mathrm{e}^{-k_4 t} \qquad (6)$$

The first-order rate constants for the release of the pyrimidine base (k_2) and the formation of nonchromophoric products (k_3) were obtained from eqns. (7) and (8), where x is the mol fraction of the pyrimidine base released.

$$k_2 = x k_d \qquad (7)$$
$$k_3 = k_d - (k_1 + k_2) \qquad (8)$$

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