Kinetics and Mechanisms of Hydrolytic Reactions of Methylated Cytidines under Acidic and Neutral Conditions

Jaroslaw Kusmierek,[a] Rainer Käppi,[b] Kari Neuvonen,[b] David Shugar[a,c] and Harri Lönnberg[b,*]

[a]Institute of Biochemistry and Biophysics, Polish Academy of Science, 02-532 Warszawa, Poland, [b]Department of Chemistry, University of Turku, SF-20500 Turku, Finland, and [c]Department of Biophysics, University of Warsaw, 02-089 Warszawa, Poland

Kusmierek, J., Käppi, R., Neuvonen, K., Shugar, D. and Lönnberg, H., 1989. Kinetics and Mechanisms of Hydrolytic Reactions of Methylated Cytidines under Acidic and Neutral Conditions. – Acta Chem. Scand. 43: 196–202.

First-order rate constants have been determined for acidic hydrolysis, and for acidic and acid buffer-catalysed, deamination of cytidine and a number of its methylated derivatives. Rate constants for deamination under neutral conditions (frequently referred to as spontaneous deamination) have also been determined. N^4-Methyl groups retard both deamination and hydrolysis, the former influence being much larger. A 6-methyl substituent retards deamination even more markedly, but accelerates hydrolysis. The effect of a 5-methyl group on the rates of both reactions is minor. The mechanisms of the deamination reactions under various conditions are discussed on the basis of structural effects, rates of hydrogen exchange at C5 and kinetic α-secondary isotope effects. Relevance of the data to enzyme-catalysed deamination and non-enzymatic deamination of cytosine residues in nucleic acids is briefly discussed.

The non-enzymatic deamination of cytosine residues in DNA is of considerable interest as a potential source of spontaneous mutagenesis.[1] Attention has more recently been extended to the behaviour of methylated cytosine residues, since the DNA of vertebrates, plants and bacteria contain 5-methylcytosine as a minor constituent,[2–5] and the DNA of some bacterial strains contain N^4-methylcytosine.[6,7] It has even been postulated that existence of the latter base in the DNA of thermophils may be related to enhanced resistance to thermally-induced deamination of N^4-methylcytidine (**3**) as compared with cytidine (**1**) and 5-methylcytidine (**2**).

Recent investigations by Ehrlich *et al.*[8] have demonstrated that the rate of spontaneous deamination of such bases in DNA decreases in the order 5-methylcytosine > cytosine > N^4-methylcytosine. This in part prompted us to undertake an extension of our previous[9,10] kinetic studies on the alkaline deamination of cytidine and its methyl and halogeno derivatives in neutral and acidic media, with a view to explaining, mechanistically, the influences of N^4-, 5- and 6-methyl groups. With this in mind, kinetic studies of hydrogen exchange at the ring C5 atom were also performed.

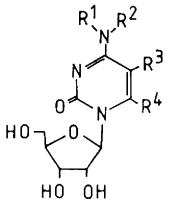
Results and discussion

Hydrolysis in aqueous acid. It has already been shown[11] that deamination of cytidine in aqueous acid is accompanied by hydrolysis to cytosine and D-ribose. The extent of the latter reaction, proceeding by a unimolecular cleavage of the protonated substrate to the free base and a resonance-stabilized carbonium ion,[12] increases appreciably with temperature, because its entropy of activation is slightly positive, while that of deamination is markedly negative.[11] Table 1 lists the first-order rate constants (monitored by HPLC) for competitive hydrolysis and deam-

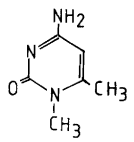
Table 1. First-order rate constants, $k_i/10^{-6}$ s^{-1}, for the hydrolysis and deamination of the monocations of cytidine and its derivatives at 363.2 K.[a]

	k(obs.)	k_h	k_d
Cytidine (**1**)	3.91(5)	0.86	3.05
5-Methylcytidine (**2**)	2.07(16)	0.37	1.70
N^4-Methylcytidine (**3**)	0.70(4)	0.43	0.27
N^4,5-Dimethylcytidine (**5**)	0.38(3)	0.22	0.16
N^4,N^4-Dimethylcytidine (**4**)	0.49	0.44	0.05
6-Methylcytidine (**6**)	58.1(9)	58.1(9)	–
1,6-Dimethylcytosine (**7**)	0.020(5)		0.02

[a]In aqueous hydrogen chloride (0.010 mol dm^{-3}) at an ionic strength of 0.10 mol dm^{-3} adjusted with sodium chloride.



1: $R^1 = R^2 = R^3 = R^4 = H$
2: $R^1 = R^2 = R^4 = H$, $R^3 = CH_3$
3: $R^1 = CH_3$, $R^2 = R^3 = R^4 = H$
4: $R^1 = R^2 = CH_3$, $R^3 = R^4 = H$
5: $R^1 = R^3 = CH_3$, $R^2 = R^4 = H$
6: $R^1 = R^2 = R^3 = H$, $R^4 = CH_3$



7

*To whom correspondence should be addressed.

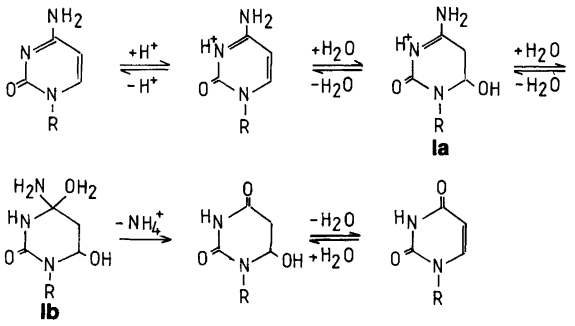
ination of cytidine and some of its methylated derivatives in aqueous hydrogen chloride (0.010 mol dm^{-3}). It will be seen that, for cytidine, hydrolysis accounts for 23 % of the reaction at 363.2 K. Methylation at C5 or N^4 affects only slightly the rate of hydrolysis; the small rate retardation observed may be accounted for by the electron-donating inductive effect of the methyl group, which enhances the electron density in the pyrimidine ring, so that it is a poorer leaving group. Despite this, hydrolysis constitutes the major reaction pathway with N^4-methylcytidines (**3**, **4**, **5**), because of their much lower rates of deamination relative to cytidine and 5-methylcytidine.

The behaviour of 6-methylcytidine (**6**) contrasts strikingly with that of the other methylcytidines, in that hydrolysis is seventy times more rapid than for cytidine, and is the only reaction detectable. For comparison, Notari *et al.*[13] have reported that 6-methylcytidine undergoes, in acetic acid buffers, hydrolysis to 6-methylcytosine, while cytidine is deaminated to uridine under the same conditions. A 6-methyl substituent also destabilizes the *N*-glycosidic bond in aqueous alkali to such an extent that the ionized 2′-hydroxy group partly displaces the 6-methylcytosine moiety, whereas no *N*-glycosidic bond cleavage is detectable with the parent cytidine.[9] These findings may be related to the known fact that the 6-methyl substituent constrains the nucleoside to the *syn* conformation.[14] It was proposed by Notari *et al.*[13] that the 5′-hydroxy group hydrogen-bonds to the oxygen atom at C2, thus decreasing the electron density of the pyrimidine ring, so that the pyrimidine is a better leaving group. Alternatively, one might assume that constraint to the *syn* conformation enhances non-bonded interactions between the carbonyl group and the sugar moiety; and, since these interactions are partially relieved on passage to the transition state, the *N*-glycosidic bond is destabilized.

Deamination in aqueous acid. As may be seen from Table 1, methylation at N^4 or C6 markedly retards deamination of cytidine in aqueous acid. The monocation of N^4-methylcytidine is deaminated at only 9 % of the rate for the cytidine cation, and this is further reduced to 1.5 % for the cation derived from the N^4,N^4-dimethyl derivative (**4**). Note also that N^4-methylation of the cation of 5-methylcytidine reduces the rate of deamination to 9 %, as for N^4-methylation of cytidine.

Deamination of 6-methylcytidine (**6**) is too slow, relative to hydrolysis, to be detected, but the data obtained with 1,6-dimethylcytosine (**7**) show that the 6-methyl substituent retards the rate of deamination by a factor of about 100, bearing in mind the fact that cytosine is deaminated four times less readily than cytidine.[15] The large rate-retarding effect of the 6-methyl group excludes the possibility that the deamination is initiated by nucleophilic attack by water at C4, as is the case in alkaline media.[9]

The foregoing effects of methyl substituents are also too large to be accounted for by their effects on the electron density of the pyrimidine ring, and hence its susceptibility



Scheme 1.

to nucleophilic attack. The pK_a values for the monocations derived from **1** and **4** (the most and least reactive species, respectively) differ by only 0.4 log units, the less reactive compound being the more acidic.[16] The large rate-retarding effects may, on the other hand, be accounted for by the mechanism for hydrolytic deamination proposed by Shapiro and Klein,[17,18] and subsequently refined by Wechter and Kelly.[19] As depicted in Scheme 1, the 5,6-double bond is initially saturated by nucleophilic attack of water at C6; the resulting 5,6-dihydro-6-hydroxycytidine (**Ia**) undergoes deamination to the corresponding uridine derivative, followed by dehydration to uridine. Accordingly, the first-order rate constant, k_d, for deamination may be expressed by eqn. (1), where the partial rate constants, k_1 and k_{-1}, refer to the reversible initial hydration of protonated cytidine, and k_2 is the rate constant for the deamination of the resulting intermediate **Ia**. The results of Johns *et al.*[20] on the competitive dehydration and deamination of the photochemically produced 3′-monophosphate of **Ia** suggest that the partial rate constants k_{-1} and k_2 are of the same order of magnitude in aqueous acid, so that neither of them may be neglected in the denominator of the right-hand side of eqn. (1). Methylation at C6 sterically hinders nucleophilic attack at C6. Hence the value of k_1, and consequently that of k_d, is markedly decreased. In turn, N^4-methyl groups may be expected to hinder, both sterically and inductively, displacement of the amino group by a molecule of water, leading to a diminution of k_2; hence, if k_{-1} is not originally negligible compared with k_2, the ratio $k_2/(k_{-1} + k_2)$ undergoes a parallel change, and k_d is decreased.

$$k_d = (k_1\, k_2)/(k_{-1} + k_2) \qquad (1)$$

The effects of methyl groups on the exchange rate of H5 furnish additional support for this reasoning. When the course of deamination of cytidine was followed by ^{1}H NMR spectroscopy in acidic deuterium oxide, replacement of H5 by deuterium was found to be considerably slower than deamination. The first-order rate constant for deuteriation, k_e, was 3×10^{-7} s^{-1} at 363.2 K, while that for deamination, k_d, was 1×10^{-6} s^{-1}. These data suggest that k_{-1} is smaller than k_2, and hence formation of the intermediate **Ia**, rather than its conversion into deamination products, is rate-limiting. By contrast, with N^4-methylcytidine the situation

is reversed; the rate of deuteriation was four times higher than the rate of deamination, the rate constants being 4×10^{-7} s^{-1} and 1×10^{-7} s^{-1}. Accordingly, the N^4-methyl group, which may be expected to retard hydrolytic deamination of **Ia**, but has only a small effect on the initial hydration, appears to change the rate-limiting step for deamination from the formation of **Ia** to the formation of **Ib** (or its breakdown). In other words, diminution of k_2 makes $k_{-1} > k_2$, decreasing simultaneously the value of k_d.

^{1}H NMR spectroscopy of aliquots withdrawn at appropriate time intervals from an equimolar solution of cytidine and its 6-deuterio derivative in aqueous hydrogen chloride (0.1 mol dm^{-3}) demonstrated that the latter disappeared slightly more rapidly, the kinetic α-secondary isotope effect, k(obs.,H)/k(obs.,D), being of the order of 0.8. While concurrent hydrolysis undoubtedly influences the observed value of the isotope effect, it seems reasonable to conclude that the principal reaction, i.e. deamination, exhibits an inverse isotope effect, consistent with the suggested sp^2 to sp^2 rehybridization of C6 in the rate-limiting step.[21] For comparison, acid-catalysed hydrolysis of uridine, via hydration of the 5,6-double bond, shows a small inverse α-secondary isotope effect: k(H6)/k(T6) = 0.87.[22]

A 5-methyl substituent also slightly retards deamination. This may be partly due to its electron-donating nature that decreases the electron density at C6, and may partly reflect the steric hindrance that a relatively bulky group adjacent to the reaction center forms to the nucleophilic attack.

In summary, the role of methyl groups on the rate of acidic deamination of cytidine may be accounted for by the mechanism depicted in Scheme 1, assuming that N^4-methylation changes the rate-limiting stage from the formation of the intermediate **Ia** to its deamination.

Buffer-catalysed deamination. Cytidine is known to undergo a buffer-catalysed deamination,[15,17,18,23] but the nature of catalysis remains controversial. Notari *et al.*[23] concluded that both acetic acid and acetate ion act as catalysts, while Garrett and Tsau[15] detected only acetic acid catalysis. Our own results are consistent with the latter proposal.

Table 2 lists the rate constants for deamination (k_d) and hydrolysis (k_h) of cytidine and some of its analogues in various acetic acid buffers as a function of buffer concentration. The catalytic constants, k(cat.), obtained for deamination from the slopes of the plots of eqn. (2), are listed in the same table.

$$k_d = k(\text{cat.})\{[\text{AcOH}] + [\text{AcO}^-]\} + \text{constant} \qquad (2)$$

Table 2. First-order rate constants, $k_i/10^{-6}$ s^{-1}, for the deamination and hydrolysis of cytidine and its derivatives in acetic acid buffers at 363.2 K,[a] and catalytic constants, k(cat.)/10^{-6} dm^3 mol^{-1} s^{-1}, for the buffer-catalysed deamination.

	[HOAc] / mol dm^{-3}	[NaOAc] / mol dm^{-3}	k(obs.)	k_h	k_d	k(cat.)
Cytidine (**1**)	1.0	0.20	3.67(8)	0.30	3.37	1.77(8)
	0.50	0.10	2.74(5)	0.36	2.38	
	0.10	0.020	1.73(4)	0.28	1.45	
	1.0	0.50	3.23(6)	0.18	3.05	1.42(2)
	0.50	0.25	2.13(5)	0.17	1.96	
	0.10	0.050	1.26(3)	0.13	1.13	
	1.0[b]	1.0	2.34(3)	0.10	2.24	0.83(3)
	0.50	0.50	1.54(2)	0.08	1.46	
	0.10	0.10	0.82(1)	0.07	0.75	
	0.50	1.0	1.61(2)	0.05	1.56	0.74(6)
	0.25	0.50	1.12(3)	0.05	1.07	
	0.05	0.10	0.59(2)	0.04	0.55	
	0.20	1.0	0.52(2)	0.02	0.50	0.20(2)
	0.10	0.50	0.41(2)	0.01	0.40	
	0.02	0.10	0.29(2)	0.01	0.28	
N^4-Methylcytidine (**3**)	1.0	1.0	0.72	0.06	0.66	0.27(1)
	0.50	0.50	0.48(3)	0.07	0.41	
	0.10	0.10	0.24(2)	0.06	0.18	
N^4,N^4-Dimethylcytidine (**4**)	1.0	1.0	0.09(2)	0.04	0.05	0.02(1)
	0.50	0.50	0.08(1)	0.04	0.04	
	0.10	0.10	0.03(1)	0.03	0.01	
1,6-Dimethylcytosine (**7**)	1.0	1.0	0.08(1)	0.0	0.07	0.01(1)
	0.50	0.50	0.07(1)	0.0	0.07	
	0.10	0.10	0.05(1)	0.0	0.05	

[a] Ionic strength adjusted to 1.0 mol dm^{-3} with sodium chloride. [b] $-\log([H^+]/\text{mol dm}^{-3}) = 4.66$.

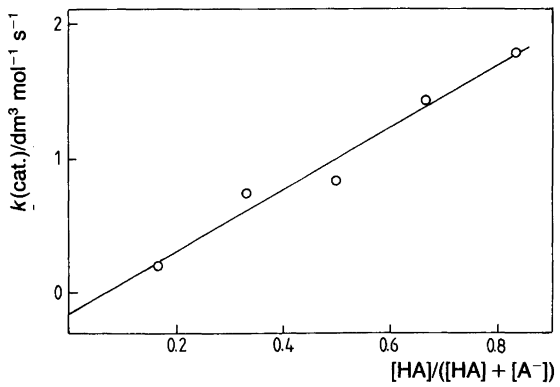


Fig. 1. Catalytic rate constants, k(cat.), for the deamination of cytidine in acetic acid buffers at 363.2 K plotted against the mole fraction of the non-dissociated acetic acid component of the buffers. Ionic strength adjusted to 1.0 mol dm^{-3} with sodium chloride.

As may be seen from Fig. 1, the values of k(cat.) are proportional to the mole fraction of the non-dissociated acetic acid component of the buffers, i.e. to the ratio of [AcOH]/{[AcOH] + [AcO$^-$]}. This is so only when catalysis by acetate ion is negligible compared with that of acetic acid. It is also predicated on the assumption that the 1-substituted cytosine with a saturated 5,6-double bond is fully protonated under the conditions of the reaction (Scheme 1); this is indeed the case, since it has been shown[24] long ago that saturation of the 5,6-bond of 1-substituted cytosines leads to an increase in the pK_a value of the protonated N3 of more than 2 units.

With 1,6-dimethylcytosine (**7**), deamination under the foregoing conditions is very slow (Table 2), excluding initial nucleophilic attack at C4 as a possible mechanism for the deamination of cytidine. In striking contrast with the situation in aqueous acid, hydrogen exchange at C5 of cytidine in acidic buffered media is considerably faster than deamination: e.g. in a buffer consisting of 0.50 mol dm^{-3} acetic acid and 0.10 mol dm^{-3} sodium acetate in deuterium oxide, the first-order rate constant for hydrogen exchange at C5 was 9.8×10^{-6} s^{-1} at 363.2 K (Table 3), while the rate constant for deamination was 1.2×10^{-6} s^{-1}. Hence buffer-catalysed deamination appears to proceed by rapid initial saturation of the 5,6-double bond, as proposed by Shapiro and Klein.[18] This suggestion is also consistent with the previous observation[20] that the 3′-monophosphate of **Ia** is dehydrated more rapidly than it is deaminated at pH 3–6.

Comparison of the data in Tables 2 and 3 reveals that hydrogen exchange at C5 is more susceptible to buffer catalysis than is the deamination of cytidine. This fact, together with the observation of Johns *et al.*,[20] according to which the deamination of the 3′-monophosphate of **Ia** is not catalysed by buffers, strongly suggests that hydration of the 5,6-double bond (and possibly its reverse reaction) must be the main target for the catalytic activity of acetic acid buffer in the deamination of cytidine. The observed linear dependence of k_d on the concentration of non-dissociated acetic acid may thus be accounted for by three

Table 3. First-order rate constants for the deuteriation of C5 of cytidine and its derivatives in acetic acid buffers at 363.2 K.[a]

	[HOAc] / mol dm^{-3}	[NaOAc] / mol dm^{-3}	$k_e/10^{-6}$ s^{-1}
Cytidine (**1**)	1.0	0.20	14.5(8)
	0.50	0.10	9.8(9)
	0.10	0.020	2.8(1)
	0.20	1.0	7.3(6)
	0.10	0.50	4.5(4)
	0.02	0.10	1.7(3)
N^4-Methylcytidine (**3**)	1.0	0.20	10.5(8)
	0.50	0.10	8.1(9)
	0.10	0.020	3.1(2)
N^4,N^4-Dimethylcytidine (**4**)	1.0	1.0	4.4(1)
	0.50	0.10	3.4(1)
	0.10	0.020	1.3(1)

[a] Ionic strength adjusted to 1.0 mol dm^{-3} with sodium chloride.

alternative mechanisms that are kinetically indistinguishable, *viz.* either saturation of the 5,6-double bond undergoes general acid-catalysis, or rapid initial protonation of the starting material is followed by general base catalysis or by nucleophilic attack by acetate ion.[25] The last two alternatives are more likely, since formation of **Ia** in buffer solutions is more rapid than in aqueous acid solutions containing only very weak bases or nucleophiles. The acetate ion either acts as a nucleophile in place of water, or it facilitates the attack of water as a general base.

Additional support for the above mechanism is provided by the effect of N^4-methyl substituents on the rates of deamination and C5-deuteriation. As shown in Table 2, the relative values for the catalytic constant, k(cat.), for deamination of **1**, **3** and **4** are 1, 0.3 and 0.02, respectively. By contrast, the rate of deuteriation was found to be rather insensitive to N^4-methylation (Table 3). This is consistent with the weak influence exerted by an N^4-methyl group on the basicity of the ring N3 and its inability to hinder sterically nucleophilic attack at C6. Consequently, the overall effect on the rate of formation of the saturated intermediate should remain small. The pronounced effect on the rate of deamination is most probably due to steric and inductive retardation of the displacement of the 4-amino group of **Ia** by a molecule of water, which constitutes the rate-limiting step of the buffered reaction.

Spontaneous deamination. Table 4 lists the rate constants for the deamination in triethanolamine buffer ([H$^+$] = 2.5 10^{-7}, calculated for 1:1 buffer by the acidity constant of the triethanolammonium ion at 363.2 K, I = 0.1 mol dm^{-3}).[26] Rates of deamination were virtually unaffected by buffer concentration or ratio of buffer components. The rate constants listed thus represent spontaneous deamination.

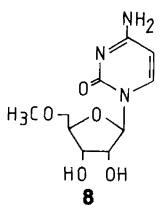
A 6-methyl substituent does not significantly retard

Table 4. First-order rate constants for the spontaneous deamination of cytidine and its derivatives at 363.2 K.[a]

	$k_d/10^{-6}$ s^{-1}
Cytidine (**1**)	0.24(1)[b]
5-Methylcytidine (**2**)	0.29(1)
N^4-Methylcytidine (**3**)	0.096(5)
N^4,5-Dimethylcytidine (**5**)	0.068(5)
N^4,N^4-Dimethylcytidine (**4**)	0.055(3)
5′-*O*-Methylcytidine (**8**)	0.15(1)
1,6-Dimethylcytosine (**7**)	0.070(3)

[a]Obtained in a triethanolamine/triethanolammonium chloride buffer (0.020/0.020 mol dm^{-3}), the ionic strength of which was adjusted to 0.10 mol dm^{-3} with sodium chloride. [b]8.8×10^{-8} s^{-1} at 358.2 K in Ref. 28.

deamination in triethanolamine buffer, in striking contrast with the situation in acidic media. The rate of deamination of 1,6-dimethylcytosine is only 3.4 times slower than for cytidine, which is approximately the difference in reactivity between cytosines and cytidines in general.[15] The rate-retarding effects of N^4-methyl groups are more marked, though smaller than in aqueous acid, the relative rate constants for **1**, **3** and **4** being 1, 0.40 and 0.23, respectively. 5-Methylcytidine is deaminated 1.2 times faster than cytidine, and N^4-methylation reduces the deamination rate to a quarter of the original value. Hence the structural effects differ from those observed in acidic solutions, and resemble more closely those in aqueous alkali:[9] 1, 1.9, 0.16 and 0.39 for **1**, **2**, **4** and **6**, respectively. Attack of hydroxide ion on the neutral molecule has been postulated to take place at C4.[9] Analogously, a molecule of water might attack this site without prior hydration of the 5,6-double bond. While the structural effects lend considerable support to this alternative, concurrent attack of water on C6 cannot be excluded, since cytidine undergoes deamination and C5-deuteriation at about the same rate. The first-order rate constants measured in a 1:1 buffer in deuterium oxide at 363.2 K were 0.94×10^{-7} s^{-1} and 1.5×10^{-7} s^{-1}, respectively. It is possible that routes involving an initial attack at C4 and C6 are approximately as favourable. This would explain why deuteriation and deamination occur almost equally as rapidly, and why the influences of N^4- and 6-methyl groups on the deamination rate are smaller than under acidic conditions. One might also envisage intramolecular nucleophilic attack of the 5′-hydroxy group at C6 competing with attack of water at C4. However, deamination of 5′-*O*-methylcytidine (**8**) is retarded by a factor of only 1.6 relative to cytidine. The difference in reactivity is too small to draw unequivocal conclusions; but it should be noted that the same structural modification is slightly rate-accelerating in aqueous alkali.[9]



8

Our results confirm the data of Erlich *et al.*[8] on the spontaneous deamination of methylated 2′-deoxycytidines, according to which N^4-methylation retards and 5-methylation accelerates the deamination by a factor of 2. It is noteworthy, however, that the rate-decelerating effect of N^4-methylation may be more marked if buffer-catalysis is involved in the heat-induced deamination of DNA.

Enzymic deamination. The foregoing results may have some relevance to the mechanism of enzymic deamination of cytosine nucleosides. Two alternative mechanisms have been proposed for this reaction, analogous to buffer-catalysed deamination. One involves attack of enzyme at C6 to form a saturated 5,6-adduct, followed by uncatalysed displacement of the 4-amino group by water and elimination of the enzyme to give uridine. The other is based on nucleophilic attack of the enzyme at C4, proceeding via a double-displacement mechanism to give uridine. The latter alternative is preferred, because 6-azacytidine and 5,6-dihydrocytidine are substrates of the enzymic reaction,[27] and cytidine in deuterium oxide solution buffered with phosphate, in the presence of a large excess of cytidine deaminase, showed no detectable deuteriation at C5 even with prolonged incubation.[28] An example of non-enzymatic deamination by nucleophilic attack at C4 is the reaction of the carcinogen 4-acetamido-*N*-acetoxystilbene with 1-methylcytosine in aqueous acetone, to give an N^3-adduct, followed by intramolecular nucleophilic attack at C4.[29] Support for nucleophilic attack at C4 is also provided by the transition-state inhibitors of Liu *et al.*[30] and of Ashley and Bartlett.[31]

Our results on spontaneous and alkaline deamination also suggest that nucleophilic attack on the unprotonated cytosine ring may take place at C4, although pre-equilibrium protonation of N3 appears to make C6 the preferred site of attack. Cytidine, 5-methylcytidine and N^4-methylcytidine all served as substrates for cytidine deaminase from *E. coli*, the relative rates being 1, 0.7 and 0.02, respectively. This finding is consistent with, but not conclusive evidence for, a direct displacement of the amino group. Enzymic deamination of 6-methylcytidine would provide a strong argument in favour of this mechanism. In contrast with Kreis *et al.*,[32] but in agreement with De Young *et al.*,[33] we have found that this compound is not a substrate for *E. coli* cytidine deaminase. However, it is also not an inhibitor, therefore is not bound by the enzyme, and no mechanistic conclusions can be drawn.

Experimental

Materials. Cytidine (**1**) and 5-methylcytidine (**2**) were products obtained from Sigma (St. Louis, MO., USA). Preparations of N^4,N^4-dimethylcytidine (**4**),[9] 6-methylcytidine

(**6**)[9] and 5′-*O*-methylcytidine (**8**)[34] have been previously described.

N⁴-*Methylcytidine* (**3**) was prepared from 2′,3′,5′-tri-*O*-benzoyluridine (obtained from Sigma) according to the procedure described by Divakar and Reese.[35] The product, isolated as the hydrochloride salt, was UV identical with that reported in the literature[16] and exhibited ^{1}H NMR signals (in D_2O, pD 4) at δ 8.09 (H6,d), 6.39 (H5,d), 6.18 (H1′,d), 4.60 (H2′,m), 4.48 (H3′,t), 4.41 (H4′,m), 4.18 (H5′,m), 4.09 (H5″,m) and 3.25 (N^4-CH_3,s).

N⁴,5-*Dimethylcytidine* (**5**) was synthesized analogously from commercial 2′,3′,5′-tri-*O*-benzoyl-5-methyluridine, prepared by condensation of trimethylsilylated thymine with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (Sigma).[36] The product, as the hydrochloride salt, was UV identical with that prepared by Fox *et al.*,[37] and exhibited ^{1}H NMR signals (D_2O, pD 2) at δ 8.02 (H6,s), 6.03 (H1′,d), 4.47 (H2′,m), 4.36 (H3′,t), 4.28 (H4′,m), 4.08 (H5′,m), 3.98 (H5″,m), 3.26 (N^4-CH_3,s) and 2.16 (5-CH_3,s).

1,6-Dimethylcytosine (**7**) was prepared as follows. 6-Methyluracil (1.5 g, Sigma) was treated with methyl iodide (6×0.15 cm³) in Me_2SO (5 cm³) in the presence of potassium carbonate (1.5 g). After 15 h, 75 % of the starting material had reacted, and the reaction mixture contained, in addition to unchanged 6-methyluracil (R_F 0.30 on silica gel with 2-propanol/chloroform 1/4, v/v), 1,6-dimethyluracil (R_F 0.45), 3,6-dimethyluracil (R_F 0.50) and 1,3,6-trimethyluracil (R_F 0.65). The methylated derivatives were identified on the basis of their UV spectra.[38] 1,6-Dimethyluracil was separated preparatively on TLC plates (silica gel, Merck) and crystallized from methanol. The product was converted into **7** by the method of Divakar and Reese.[35] However, prolonged treatments were needed both for the formation of the triazole derivative and its conversion into **7**. The product was crystallized as the hydrochloride from aqueous ethanol. Its UV spectrum was similar to that of **6** and it exhibited ^{1}H NMR signals (D_2O, pD 4) at δ 6.21 (H6,s), 3.60 (N^1-CH_3) and 2.53 (6-CH_3).

[5,6-$^2H_2$]*Cytidine* was prepared according to Rabi and Fox[39] with a modification of the desalting procedure. The reaction mixture, containing 1 g of cytidine, was loaded onto a strong cation-exchange resin (Dowex 50W X 8 100/200, NH_4^+-form, 2×27 cm) and eluted with distilled water. Fractions from 80 to 160 cm³ were concentrated and the cytidine was precipitated with acetone. ^{1}H NMR analysis showed that deuteriation was almost complete (>97 %). [5,6-$^2H_2$]Cytidine was converted into its [6-^{2}H] derivative during a 12-day incubation in an acetic acid buffer ([AcOH] = 0.2 mol dm^{-3}, [AcO^-] = 0.1 mol dm^{-3}) at 363.2 K. The mixture was loaded onto a strong cation-exchange resin (Dowex 50W X 8 100/200, H^+-form, 2×28 cm), washed first with aqueous 2-propanol (50 % v/v, 200 cm³), and then with aqueous ammonia (2 mol dm^{-3}) containing 50 % (v/v) of 2-propanol. Fractions from 180 to 200 cm³ of the latter eluent contained [6-^{2}H]cytidine. The evaporated residue was treated with charcoal in aqueous methanol, and the product crystallized from acetone containing 15 % (v/v) methanol. ^{1}H NMR spectroscopic measurements indicated that more than 95 % of the deuterium at C5 had been exchanged for protium.

Kinetic measurements by HPLC. The first-order rate constants for the hydrolysis and deamination of the compounds studied were determined by the HPLC technique described previously.[11]

Kinetic measurements by ^{1}H NMR spectroscopy. The first-order rate constants for the exchange of the C5 protium of the cytosine ring for deuterium were determined by recording the ^{1}H NMR spectra (Jeol GX 400) of aliquots withdrawn at suitable intervals. The integrals of the H5 and H6 signals were compared with that of the anomeric proton. No exchange of H6 could be detected under any of the conditions employed.

Kinetic α-secondary isotope effects for the deamination were determined by carrying out the reaction with an equimolar mixture of cytidine and its 6-deuterio derivative. Aliquots withdrawn at suitable intervals were neutralized, evaporated to dryness and dissolved in D_2O. With [6-^{2}H]cytidine the H5 signal appeared as a sharp singlet centered on the corresponding signal of cytidine [a doublet with J(H5,H6) of 7.8 Hz]. Careful integration of each of the three peaks allowed a reasonably accurate determination of the ratio of the concentration of cytidine and [6-^{2}H]cytidine as a function of time.

Acknowledgements. This investigation profited from the partial support of the Polish Academy of Sciences (CPBR 3.13.1.1.4 and 3.13.1.1.7). One of us (J.K.) is also indebted to the Academy of Finland, Council for the Natural Sciences, for a stipend during his stay at the University of Turku.

References

1. Singer, B. and Kusmierek, J. T. *Ann. Rev. Biochem. 52* (1982) 655.
2. Ehrlich, M. and Wang, R. Y.-H. *Science 212* (1981) 1350.
3. Vanyushin, B. F., Mazin, A. L., Vasilyev, V. K. and Belozersky, A. N. *Nature (London) 225* (1983) 948.
4. Hattman, S. *The Enzymes 14* (1983) 514.
5. Doerfler, W. *Ann. Rev. Biochem. 52* (1983) 93.
6. Janulaitis, A., Klimasauskas, S., Petrusyte, M. and Butkus, V. *FEBS Lett. 161* (1983) 131.
7. Ehrlich, M., Gama-Sosa, M. A., Carreira, L. H., Ljungdahl, L. G., Kuo, K. and Gehrke, C. W. *Nucleic Acids Res. 13* (1985) 1399.
8. Ehrlich, M., Norris, K. F., Wang, R. Y.-H., Kuo, K. C. and Gehrke, C. W. *Biosci. Rep. 6* (1986) 387.
9. Lönnberg, H., Suokas, P., Käppi, R. and Darzynkiewicz, E. *Acta Chem. Scand., Ser. B 40* (1986) 798.
10. Käppi, R. and Lönnberg, H. *Acta Chem. Scand., Ser. B 40* (1986) 768.

11. Lönnberg, H. and Käppi, R. *Nucleic Acids Res. 13* (1985) 2451.
12. Lönnberg, H. *Tetrahedron 38* (1982) 1517.
13. Notari, R. E., Witiak, D. T., De Young, J. L. and Lin, A. J. *J. Med. Chem. 15* (1972) 1207.
14. Schweizer, M. P., Banta, E. B., Witkowski, J. T. and Robins, R. K. *J. Am. Chem. Soc. 95* (1973) 3770.
15. Garrett, E. R. and Tsau, J. *J. Pharm. Sci. 61* (1972) 1052.
16. Szer, W. and Shugar, D. *Acta Biochim. Pol. 13* (1966) 184.
17. Shapiro, R. and Klein, R. S. *Biochemistry 5* (1966) 2358.
18. Shapiro, R. and Klein, R. S. *Biochemistry 6* (1967) 3576.
19. Wechter, W. J. and Kelly, R. C. *Collect. Czech. Chem. Commun. 35* (1970) 1991.
20. Johns, H. E., Le Blanc, J. C. and Freeman, K. B. *J. Mol. Biol. 13* (1965) 849.
21. Melander, L. and Saunders, W. H., Jr. *Reaction Rates of Isotopic Molecules,* Wiley, New York 1980, Chap. 6.
22. Prior, J. J. and Santi, D. V. *J. Biol. Chem. 259* (1984) 2429.
23. Notari, R. E., Chin, M. L. and Cardoni, A. *J. Pharm. Sci. 59* (1970) 28.
24. Janion, C. and Shugar, D. *Acta Biochim. Pol. 14* (1967) 293.
25. Bender, M. L., Bergeron, R. J. and Komiyama, M. *The Bioorganic Chemistry of Enzymatic Catalysis,* Wiley, New York 1984, Chap. 5.
26. Bates, R. G. and Allen, G. F. *J. Res. Natl. Bur. Stand., Sect. A 64* (1960) 343.
27. Evans, B. E., Mitchell, G. N. and Wolfenden, R. *Biochemistry 14* (1975) 621.
28. Frick, L., MacNeela, J. P. and Wolfenden, R. *Bioorg. Chem. 15* (1987) 100.
29. Scribner, J. D., Smith, D. L. and McCloskey, J. A. *J. Org. Chem. 43* (1978) 2085.
30. Liu, P. S., Marquez, V. E., Driscoll, J. S., Fuller, F. W. and McCormack, J. J. *J. Med. Chem. 24* (1981) 662.
31. Ashley, G. W. and Bartlett, P. A. *Biochem. Biophys. Res. Commun. 108* (1982) 1476.
32. Kreis, W., Watanabe, K. A. and Fox, J. J. *Chem.-Biol. Interact. 37* (1981) 41.
33. De Young, J. L., Notari, R. E. and Feller, D. R. *Res. Commun. Chem. Pathol. Pharmacol. 9* (1974) 163.
34. Kusmierek, J. T., Giziewicz, J. and Shugar, D. *Biochemistry 12* (1973) 194.
35. Divakar, K. J. and Reese, C. B. *J. Chem. Soc., Perkin Trans. 1* (1982) 1171.
36. Vorbruggen, H. and Bennua, B. *Chem. Ber. 114* (1981) 1279.
37. Fox, J. J., Van Praag, D., Wempen, I., Doerr, I. L., Cheong, L., Knoll, J. E., Eidinoff, M. L., Bendich, A. and Brown, G. B. *J. Am. Chem. Soc. 81* (1959) 178.
38. Shugar, D. and Fox, J. J. *Biochim. Biophys. Acta 9* (1952) 199.
39. Rabi, J. A. and Fox, J. J. *J. Am. Chem. Soc. 95* (1973) 1628.

Received November 7, 1988.