

# Kinetics of Reactions of Pyrimidine Nucleoside 2'- and 3'-Monophosphates under Acidic and Neutral Conditions: Concurrent Phosphate Migration, Dephosphorylation and Deamination

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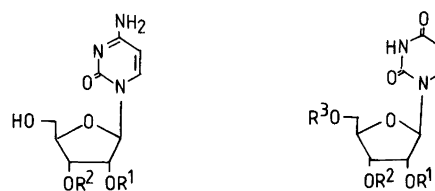
First-order rate constants for the following reactions of cytidine and uridine monophosphates have been determined over the acidity range from pH 7 to  $H_0 - 0.7$ : (i) interconversion of the 2'- and 3'-monophosphates of cytidine and uridine, (ii) dephosphorylation of the 2', 3'- and 5'-monophosphates, and (iii) deamination of cytosine nucleotides to uracil nucleotides. Competition between these reactions under various conditions is discussed, and the data on phosphate migration and phosphoester hydrolysis are compared with those reported earlier for adenosine monophosphates.

Migration of a phosphate group between the 2'- and 3'-positions of ribofuranose residues is a reaction frequently encountered, when oligoribonucleotides or their constituents are treated with aqueous acids or bases, or incubated at elevated temperatures. Monomeric nucleoside 3'-monophosphates, for example, undergo isomerization to 2'-phosphates in aqueous acid,<sup>1-3</sup> or, at high temperatures, even under neutral conditions.<sup>3</sup> Similarly, imidazole buffers have been shown to catalyze the conversion of dinucleoside 3',5'-monophosphates into the corresponding 2',5'-isomers.<sup>4</sup> The alkaline hydrolysis of these compounds, in turn, yields a mixture of 2'- and 3'-monophosphates, probably *via* intermediary formation of a cyclic 2',3'-phosphodiester.<sup>5-7</sup> Finally, 2-chlorophenyl esters of dinucleoside 3',5'-monophosphates are converted under mildly acidic conditions into a mixture of 2',5'- and 3',5'-phosphodiesters and 2'- and 3'-phosphomonoesters.<sup>8,9</sup> This kind of isomerization of the 3',5'-phosphodiester bond is perhaps the most harmful side reaction of the chemical synthesis of oligoribonucleotides. Accordingly, detailed understanding of the factors that affect the rate of phosphate migration is highly desirable. We have previously described,<sup>3</sup> as the first part of the studies aimed to explain phosphate migration mechanically, the kinetics and mechanism for the interconversion of adenosine 2'- and 3'- monophosphates (2'- and 3'-AMP) under acidic and neutral conditions. In the present paper these studies are extended to the corresponding pyrimidine nucleotides, 2'- and 3'-monophosphates of cytidine (2'- and 3'-CMP) and uridine (2'- and 3'-UMP). The investigation was undertaken to find out whether the base

moiety structure has any influence on the course of phosphate migration, as it has on the hydrolysis of dinucleoside 3',5'-monophosphates,<sup>5,6</sup> and to provide quantitative data on the competition between these processes and hydrolytic deamination of cytosine nucleotides.

## Results and discussion

**Product distributions.** HPLC analyses of aliquots withdrawn at appropriate intervals from acidic solutions of pyrimidine nucleoside 2'- and 3'-monophosphates revealed that the interconversions of both 2'- and 3'-CMP (**1a**, **1b**) and 2'- and 3'-UMP (**2a**, **2b**) proceed at high hydronium ion concentrations considerably faster than other potential reactions such as dephosphorylation, deamination and depyrimidination. In fact, the equilibrium composition consisting of 40 % 2'-NMP and 60 % 3'-NMP was attained at pH < 1 before any other products appeared. However, in less acidic solutions several parallel reactions take place. Figs. 1 and 2 show the time-dependent product distribu-



**1a**  $R^1 = \text{PO}(\text{OH})_2$ ,  $R^2 = \text{H}$   
**1b**  $R^1 = \text{H}$ ,  $R^2 = \text{PO}(\text{OH})_2$   
**1c**  $R^1 = R^2 = \text{H}$

**2a**  $R^1 = \text{PO}(\text{OH})_2$ ,  $R^2 = R^3 = \text{H}$   
**2b**  $R^1 = R^3 = \text{H}$ ,  $R^2 = \text{PO}(\text{OH})_2$   
**2c**  $R^1 = R^2 = R^3 = \text{H}$   
**2d**  $R^1 = R^2 = \text{H}$ ,  $R^3 = \text{PO}(\text{OH})_2$

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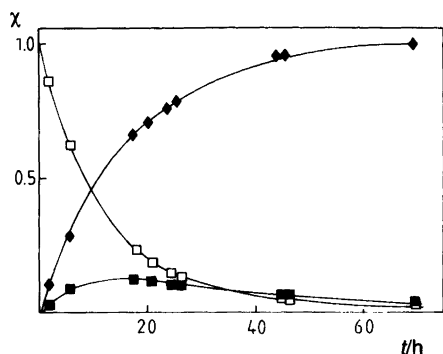


Fig. 1. Time-dependent product distribution for reactions of 2'-UMP (**2a**) in a formic acid buffer ( $[\text{HCOOH}]/[\text{HCOONa}] = 0.010/0.010 \text{ mol dm}^{-3}$ ,  $I = 0.10 \text{ mol dm}^{-3}$  with NaCl) at 363.2 K:  $\square$ , 2'-UMP (**2a**);  $\blacksquare$ , 3'-UMP (**2b**);  $\blacklozenge$ , uridine (**2c**).

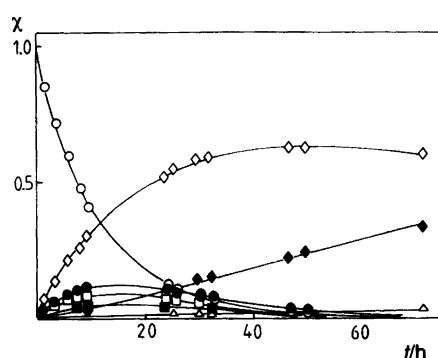
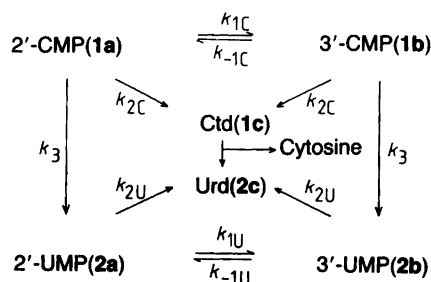
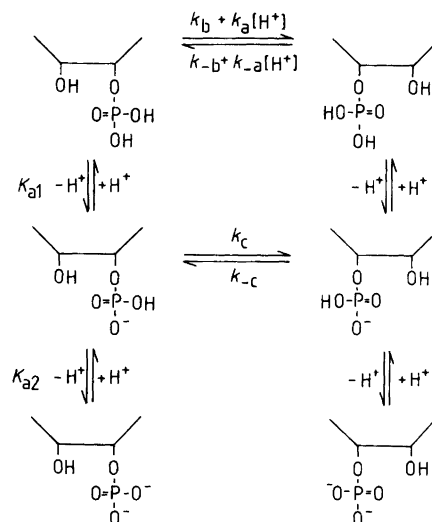


Fig. 2. Time-dependent product distribution for reactions of 2'-CMP (**1a**) in a formic acid buffer ( $[\text{HCOOH}]/[\text{HCOONa}] = 0.010/0.010 \text{ mol dm}^{-3}$ ,  $I = 0.10 \text{ mol dm}^{-3}$  with NaCl) at 363.2 K:  $\circ$ , 2'-CMP (**1a**);  $\bullet$ , 3'-CMP (**1b**);  $\diamond$ , cytidine (**1c**);  $\square$ , 2'-UMP (**2a**);  $\blacksquare$ , 3'-UMP (**2b**);  $\blacklozenge$ , uridine (**2c**);  $\triangle$ , cytosine.

tions observed for the reactions of 2'-UMP and 2'-CMP in a 1:1 formic acid buffer. In both cases dephosphorylation to the corresponding nucleosides (**1c**, **2c**) competes with phosphate migration. Appearance of 2'- and 3'-UMP among the reaction products of 2'-CMP further indicates that cytosine nucleotides are also partly deaminated to uracil derivatives. Depyrimidination is of minor importance, since the mole fraction of free cytosine remained below 0.01 during the first two half-lives of the disappearance of the cytosine nucleotides. At later stages some cytosine is accumulated



Scheme 1.



Scheme 2.

due to competitive hydrolysis and deamination of cytidine.<sup>10,11</sup> Cytosine was not markedly deaminated nor was uridine hydrolyzed to uracil during the time that the course of the reactions was followed. Accordingly, the reactions that pyrimidine nucleoside 2'- and 3'-monophosphates undergo under slightly acidic conditions may be summarized by Scheme 1.

**Phosphate migration.** We have shown previously<sup>3</sup> that the interconversion of 2'- and 3'-AMP proceeds by intramolecular attack of the 2'- or 3'-hydroxy function on the phosphorus atom of the neighboring phosphate group and subsequent breakdown of the resulting pentacoordinated intermediate either back to the starting material, or, after pseudorotation, to its positional isomer. In other words, the phosphate migration takes place predominantly by an 'adjacent association' mechanism without the intermediary appearance of cyclic 2',3'-monophosphate. The reaction is first order with respect to hydronium ion at  $\text{pH} < 1$ , approaches a second-order dependence at  $1 < \text{pH} < 2$ , and becomes pH-independent at  $2 < \text{pH} < 6$ . Scheme 2 describes the minimal reaction pattern needed to explain this kind of pH-rate profile. The observed first-order rate constant,  $k_1$ , may thus be expressed by eqn. (1), assuming that the acidity constants of 2'- and 3'-phosphate groups are equal.

$$k_1 = \frac{\frac{k_a}{K_{a1}} [\text{H}^+]^2 + \frac{k_b}{K_{a1}} [\text{H}^+] + k_c}{\frac{K_{a2}}{[\text{H}^+]} + 1 + \frac{[\text{H}^+]}{K_{a1}}} \quad (1)$$

An analogous eqn. may be written for  $k_{-1}$ . The rate constants observed for the interconversions of 2'- and 3'-CMP (Fig. 3) and 2'- and 3'-UMP (Fig. 4) obey these

Table 1. Partial rate constants for the interconversion of nucleoside 2'- and 3'-monophosphates at 363.2 K.<sup>a</sup>

Comp.	$k_a$	$k_{-a}$	$k_c$	$k_{-c}$
	$10^{-3} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$	$10^{-3} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$	$10^{-6} \text{ s}^{-1}$	$10^{-6} \text{ s}^{-1}$
2'/3'-AMP <sup>b</sup>	6.4	3.0	7.8	3.4
2'/3'-CMP	7.3	5.0	7.1	3.4
2'/3'-UMP	8.4	7.4	5.1	3.1

<sup>a</sup>The rate constants indicated in Scheme 2.  $\text{p}K_{a1} = 1.3$  and  $\text{p}K_{a2} = 6.2$ . The rate constants  $k_b$  and  $k_{-b}$  are of the order of  $10^{-5} \text{ s}^{-1}$ , but their values were not obtained with reasonable accuracy. <sup>b</sup>From Ref. 3.

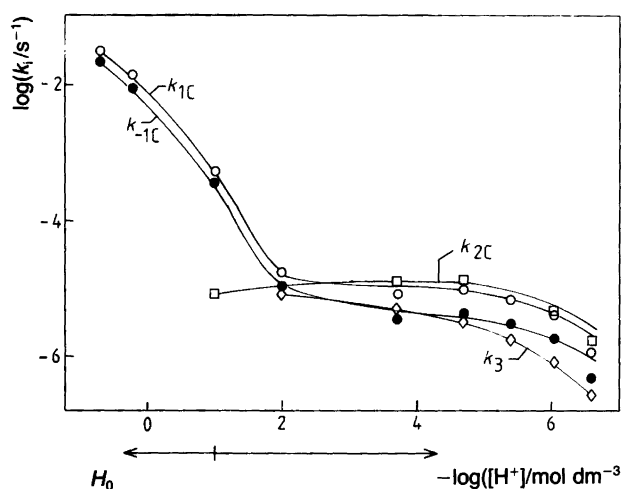


Fig. 3. pH-rate profiles for reactions of 2'- and 3'-CMP (1a, 1b) at 363.2 K ( $I = 0.10 \text{ mol dm}^{-3}$  with NaCl). The rate constants indicated are defined in Scheme 1.

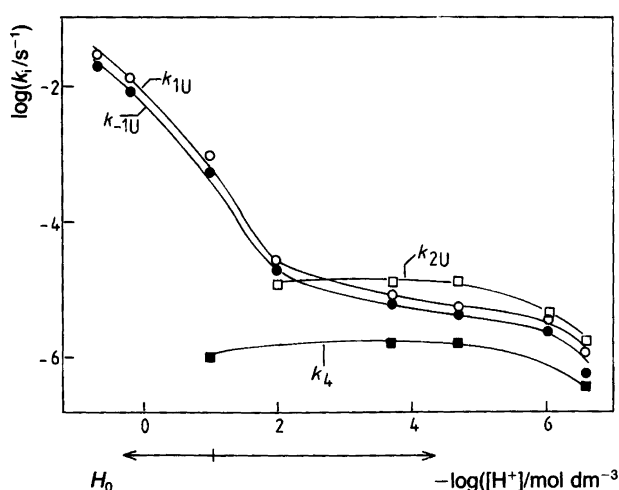


Fig. 4. pH-rate profiles for reactions of 2'-, 3'- and 5'-UMP (2a, 2b, 2d) at 363.2 K ( $I = 0.10 \text{ mol dm}^{-3}$  with NaCl). Rate constants,  $k_{1U}$ ,  $k_{-1U}$  and  $k_{2U}$ , are defined in Scheme 1.  $k_4$  is the first-order rate constant for the dephosphorylation of 5'-UMP. Hydrolysis to uracil competes with dephosphorylation of 5'-UMP at pH 1.

equations, the values obtained by least-squares fitting<sup>13</sup> for the partial rate constants ( $k_a$ ,  $k_{-a}$ ,  $k_c$  and  $k_{-c}$ ) being rather similar to those reported<sup>3</sup> for the mutual isomerization of 2'- and 3'-AMP (Table 1). Uncatalyzed migration of the dihydrogenphosphate group does not predominate under any conditions, and hence its rate constants ( $k_b$  and  $k_{-b}$ ) were not obtained with a reasonable accuracy. Accordingly, the chemical nature of the base moiety has practically no influence on the rate of phosphate migration.

It has been reported<sup>5</sup> that dinucleoside 3',5'-monophosphates derived from 3'-UMP or 3'-CMP are hydrolyzed in aqueous acid two to three times as fast as their purine counterparts. The rate acceleration was attributed to intramolecular hydrogen bonding between the 2'-hydroxy group and the O<sup>2</sup> atom of the pyrimidine base, which would enhance the nucleophilicity of the 2'-oxygen atom.<sup>5,12</sup> The results of the present study do not, however, support this conclusion. Migration of the phosphate group from 3'- to 2'-position is also initiated by a nucleophilic attack of the 2'-hydroxy function, but is not enhanced by pyrimidine bases. The reactivity differences between various dinucleoside monophosphates could alternatively be accounted for by the effects of intramolecular stacking interactions on the conformation and thus reactivity of the phosphodiester grouping.

*Side reactions.* The structure of the base moiety has practically no effect on the rate of dephosphorylation, either. As with 2'- and 3'-AMP, dephosphorylation of 2'/3'-CMP and 2'/3'-UMP competes with phosphate migration at low hydronium ion concentrations, the rates of these two concurrent processes being almost equal at pH > 2 (Figs. 3 and 4). The 2'- and 3'-phosphomonoesters are hydrolyzed approximately as readily, since the appearance of uridine was observed to obey strictly first-order kinetics under conditions where phosphate migration took place concurrently with dephosphorylation. The reactive species is the phosphate monoanion, as may be deduced from the shape of the pH-rate profile [eqn. (2)]. The rate constants,  $k_d$ , obtained for the hydrolysis of this species are practically equal with all the nucleotides studied:  $1.6 \times 10^{-5} \text{ s}^{-1}$  with AMP and

$$k_2 = k_d / ([H^+]K_{a1}^{-1} + 1 + K_{a2}[H^+]) \quad (2)$$

$1.4 \times 10^{-5} \text{ s}^{-1}$  with UMP and CMP. Dephosphorylation of 5'-UMP exhibits a similar dependence of rate on acidity (Fig. 4), the value of  $k_d$  being somewhat smaller than that of 2'-/3'-monophosphates,  $3 \times 10^{-6} \text{ s}^{-1}$  (Fig. 2). The reactivity difference of this magnitude is too small to be taken as a serious indication of participation of the neighboring hydroxy function in the hydrolysis of 2'- and 3'-phosphomonoesters. Moreover, in those cases where the hydrolysis of phosphomonoesters has been shown to be enhanced by intramolecular general acid catalysis of a hydroxy group, the most reactive species is the phosphate dianion and not the monoanion.<sup>14</sup> Most probably all the nucleoside monophosphates studied are dephosphorylated by the mechanism presented for simple alkyl monophosphates.<sup>15</sup> In other words, a proton transfer from the phosphate hydroxy ligand to the esterified oxygen atom results in unimolecular rupture of the P-O bond with concomitant formation of a metaphosphate ion preassociated with a molecule of water.<sup>16</sup>

Deamination of 2'- and 3'-CMP to the corresponding derivatives of uracil also competes with their interconversion at low hydronium ion concentrations (Fig. 3). The proportion of this reaction is greatest at pH 2-3, i.e. under conditions where the acid-catalyzed phosphate migration no longer plays a significant role, but the base moiety still remains protonated ( $pK_a$  4). It has been shown previously<sup>11</sup> that cytidine is deaminated in aqueous acid by the rate-limiting addition of a molecule of water on the 5,6-double bond and subsequent deamination and dehydration of the resulting saturated intermediate to uridine. The rate of this reaction is thus proportional to the extent of protonation of the base moiety. In buffer solutions where the base moiety is unprotonated, the deamination reaction exhibits general acid catalysis of the buffer acid.<sup>11</sup> In other words, the deamination rate is a linear function of buffer concentration. The pH-rate profile depicted in Fig. 3 thus refers mainly to hydronium-ion catalyzed reaction at  $\text{pH} < 3$ , and to general acid catalyzed reaction at  $\text{pH} > 4$ . In the latter region the values of rate constants depend on the buffer concentration employed ( $0.010 \text{ mol dm}^{-3}$  in Fig. 3). Comparison of the data in Fig. 3 with those reported<sup>11</sup> for cytidine reveals that 2'- and 3'-CMP are deaminated from 2 to 3 times as readily as cytidine over the whole acidity range studied. The reason for this small rate acceleration remains unknown.

## Experimental

The cytosine and uracil nucleotides were products of Sigma, and they were used as received, after checking of purity by HPLC.

Kinetic measurements were carried out by the HPLC method described previously.<sup>3</sup> The hydronium ion concentrations, adjusted with hydrogen chloride, and formate, acetate, maleate and triethanolamine buffers, were calcu-

$$[\text{UMP}]_t = [\text{CMP}]_0 \frac{k_3}{k_{2U} - k_t} [\exp(-k_t t) - \exp(-k_{2U} t)] \quad (3)$$

$$k_t = k_{2C} + k_3 \quad (4)$$

lated from the  $pK_a$  values of the buffer acids under the experimental conditions.<sup>17-20</sup>

First-order rate constants,  $k_1$  and  $k_{-1}$ , for the interconversion of the nucleoside 2'- and 3'-monophosphates were calculated as described previously.<sup>3</sup> Rate constants,  $k_{2U}$ , for the dephosphorylation of 2'- and 3'-UMP were obtained as first-order rate constants for the formation of uridine, which did not react further under the experimental conditions. The mixture of 2'- and 3'-CMP underwent parallel dephosphorylation and deamination, the rate constants of which ( $k_{2C}$  and  $k_3$ , respectively) were calculated from eqns. (3) and (4), where  $[\text{UMP}]_t$  refers to the sum concentration of 2'- and 3'-UMP at moment  $t$  and  $[\text{CMP}]_0$  is the initial sum concentration of 2'- and 3'-CMP. The values of  $k_t$  were determined as the first-order rate constant for the disappearance of 2'- and 3'-CMP.

## References

- Brown, D. M. and Todd, A. R. *J. Chem. Soc.* (1952) 52.
- Cohn, W. E. *J. Am. Chem. Soc.* 72 (1950) 2811.
- Oivanen, M. and Lönnberg, H. *J. Org. Chem.* 54 (1989) 2556.
- Anslyn, E. and Breslow, R. *J. Am. Chem. Soc.* 111 (1989) 4473.
- Witzel, H. *Justus Liebigs Ann. Chem.* 635 (1960) 182.
- Koike, T. and Inoue, Y. *Chem. Lett.* (1972) 569.
- Brown, D. M. *Basic Princ. Nucleic Acid Chem.* 2 (1974) 1.
- Reese, C. B. and Skone, P. A. *Nucleic Acids Res.* 13 (1985) 5215.
- Pathak, T. and Chattopadhyaya, J. *Acta Chem. Scand., Ser. B* 39 (1985) 799.
- Lönnberg, H. and Käppi, R. *Nucleic Acids Res.* 13 (1985) 2451.
- Kusmierek, J., Käppi, R., Neuvonen, K., Shugar, D. and Lönnberg, H. *Acta Chem. Scand.* 43 (1989) 196.
- Witzel, H. and Barnard, E. A. *Biochem. Biophys. Res. Commun.* 7 (1962) 289.
- Ruckdeschel, F. R. *Basic Scientific Subroutines*, Byte/McGraw-Hill; Petersborough NH, 1981, Vol. 2, p. 75.
- Bunton, C. A. and Chaimovich, H. J. *J. Am. Chem. Soc.* 88 (1966) 4082.
- Westheimer, F. H. *Chem. Rev.* 81 (1981) 313.
- For a discussion on the role of metaphosphate intermediate see: Burgess, J., Blundell, N., Cullis, P. M., Hubbard, C. D. and Misra, R. *J. Am. Chem. Soc.* 110 (1988) 7900 and references therein.
- Harned, H. S. and Embree, N. D. *J. Am. Chem. Soc.* 56 (1934) 1042.
- Harned, H. S. and Ehlers, R. W. *J. Am. Chem. Soc.* 54 (1932) 1350.
- Adell, B. Z. *Phys. Chem., Abt. A* (1940) 161.
- Bates, R. G. and Allen, G. F. *J. Res. Natl. Bur. Stand., Sect. A* 64 (1960) 343.

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