Proton Transfer from Heterocyclic Compounds. Part 10.[1] Adenine Mono- and Poly-nucleotides

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Detritiation rate constants from the C-8 position of adenosine 5'-monophosphate, adenosine 3'-monophosphate, adenosine 3',5'-cyclic phosphate, and poly(adenosine 5'-monophosphate) have been measured over a pH range at 85°. The rate–pH profiles are of the same form as for adenosine with the important difference that for the first two compounds an additional plateau region is obtained in the pH range where ionisation of the secondary phosphoric acid function occurs. The reactivity of the species formed ($AMPH^-$) is greater than it would otherwise be because it can also exist as a zwitterion (H^+AMP^{2-}). Exchange from the polymer is *ca.* 30 times slower than for the mononucleotides.

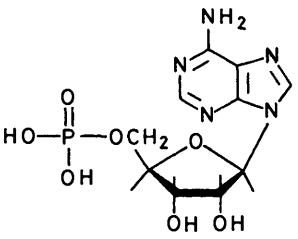
NUCLEOTIDES labelled with tritium at the C-8 position are widely used in biochemical research.[2] However very little mechanistic information relating to the possible loss of tritium is available in sharp contrast to the situation that exists with respect to the corresponding nucleosides [3-5] and bases.[6,7] Shelton and Clark [8] reported on the incorporation of tritium into various nucleotides and also the purine residues of DNA. Maslova and co-workers [9-11] have used hydrogen–tritium exchange in adenosine 5'-monophosphate and poly(adenosine 5'-monophosphate) in order to assess the effect of conformational changes on the rate and Schimmel *et al.*[12-14] have shown that the rate of exchange at the C-8 position of the purine residues of nucleic acids is sensitive to the local environment of the particular base. Thomas and Livramento [15] have developed a laser Raman spectroscopic method to study hydrogen–deuterium exchange reactions; in the C-8 exchange of poly(adenosine 5'-monophosphate) the Arrhenius plot showed regions of different activation energy (the discontinuity occurring at 60°), probably as a result of conformational changes.

In previous studies of isotopic hydrogen exchange from the C-8 position of purines we have shown [16] that the presence of a proton adjacent to this site can lead to large rate accelerations (*ca.* 10^8—10^9). Similarly metal ions [1] can also bring about large rate enhancements (10^4—10^6). In contrast the development of negative charge adjacent to the exchanging group makes the anion very unreactive although examples have recently been reported [16] where both mono- and di-anionic forms contribute to the overall rate.

These experiments led us to believe that a study of the effect of the phosphate group on rates of detritiation from the C-8 position would be worthwhile and for this reason we chose adenosine 5'-monophosphate (5'-AMP) (1), adenosine 3'-monophosphate (3'-AMP) (2), and adenosine 3',5'-cyclic phosphate (c-AMP) (3). At the same time a similar study on poly(adenosine 5'-monophosphate) [(poly (A) (4)] was undertaken in order to assess the magnitude of possible conformational effects.

EXPERIMENTAL

Materials.—All compounds were commercially available either as the sodium or potassium salts. The tritiated nucleotides were prepared by incubating a mixture of the appropriate salt (*ca.* 50 mg) and tritiated water (20 μl, 5 Ci ml^{-1}) at 85° for 18 h. The tritiated water was then removed



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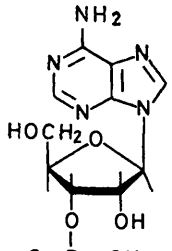

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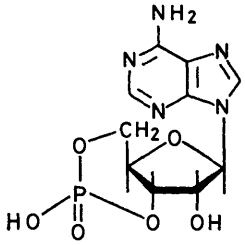

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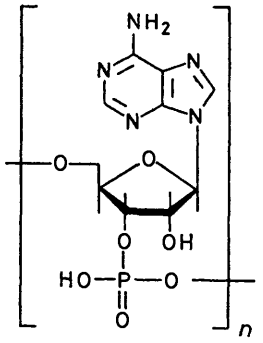

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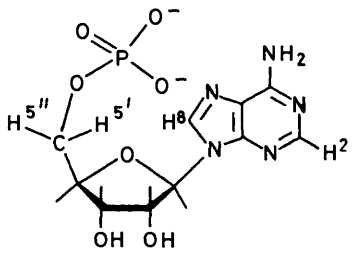


(5)

by lyophilisation. A small amount of water was then added to the solid to exchange labile tritium and the water removed again. With the exception of poly(adenosine

5′-monophosphate) good incorporation of tritium was achieved (specific activities were in the region of 0.1 Ci mmol^{-1}) and the specificity of labelling was checked by tritium n.m.r. spectroscopy; [17] no incorporation into the C-2 position (which in the case of adenine is known to be *ca.* 2 000 times less reactive [18] than the C-8 position) had occurred.

Kinetics.—The rates of detritiation were measured in the pH range 1.95—11.90 at 85° using the procedures described previously.[6] In view of the fact that nucleotides are known to undergo hydrolysis in both acidic and basic media it was customary to monitor the u.v. spectra of the reaction solutions over the duration of the detritiation experiments.

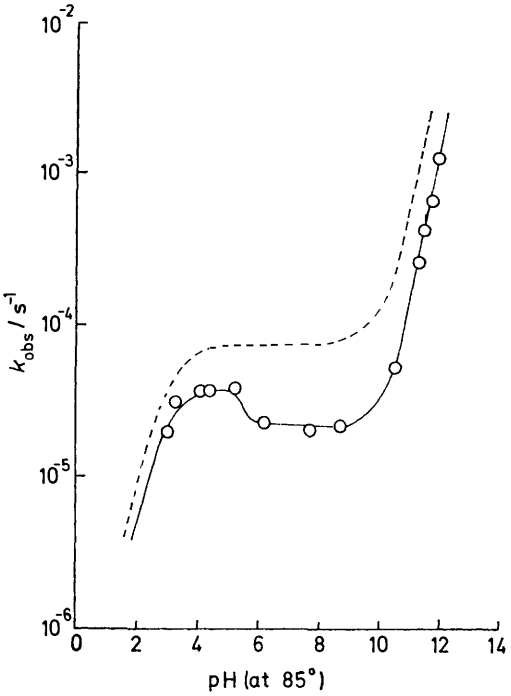


FIGURE 1 Rate–pH profile for the detritiation of [8-^{3}H]-5′-adenosine monophosphate in aqueous buffers at 85°; the curve is computed using equation (3) and values given in the text; the dotted curve gives the previously reported [4] data for adenosine

In acid solutions [19] the corresponding base and ribosyl phosphate are formed and at very high pH the corresponding nucleoside and inorganic phosphate are the products.[20] Only in the case of poly(adenosine 5′-monophosphate) at high pH (>9) did hydrolysis accompany the detritiation; under these conditions the detritiation of the hydrolysis products,[21] 2′- and 3′-adenosine monophosphate was measured. Good first-order plots were obtained, indicating, as would be expected, that both components detritiate at approximately the same rate.

RESULTS AND DISCUSSION

The detritiation rate constants ($k_{obs.}$) for the three [8-^{3}H]adenine nucleotides 5′-AMP, 3′-AMP, and c-AMP are given in Table 1 and plotted in the form of rate–pH profiles in Figures 1–3 respectively. Also shown in Figure 1 is the corresponding rate–pH profile for adenosine [4] which had previously been interpreted in terms of hydroxide ion attack on the neutral form (at high pH)

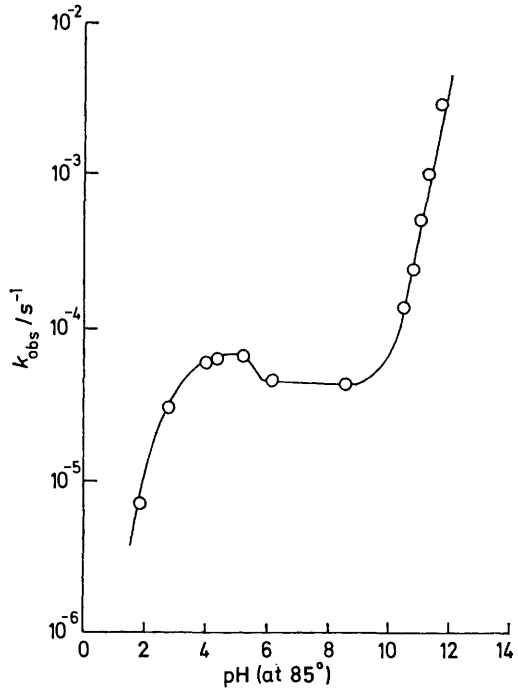


FIGURE 2 Rate–pH profile for the detritiation of [8-^{3}H]-3′-adenosine monophosphate at 85°. The curve is computed using equation (3) and values given in the text

and the protonated substrate (at low pH); c-AMP gives a similar plot and the results for this compound can be explained in the same manner. However in the case of both 5′- and 3′-AMP there is a significant rate decrease at $5 \leqslant pH \leqslant 6$, the range where secondary phosphate

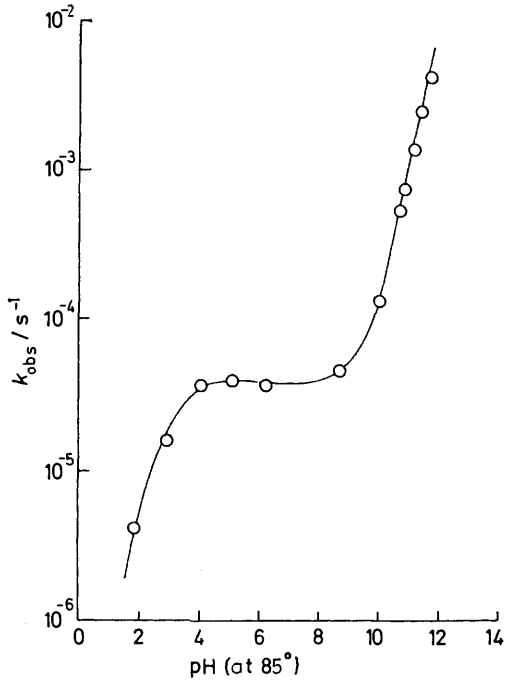


FIGURE 3 Rate–pH profile for the detritiation of [8-^{3}H]-adenosine 3′,5′-cyclic monophosphate in aqueous buffers at 85° The curve is computed using equation (7) and values given in the text

ionisation is known to occur.[22-24] If therefore the rate decrease is a consequence of the ionisation of the secondary phosphate group no such finding should be observed in the case of cyclic AMP and this is indeed the case (*cf.* Figures 1 and 3).

If we can represent the 5′-(or 3′-)AMP molecule by

TABLE 1

Detritiation rate constants ($k_{obs.}$) for [8-^{3}H]-5′-adenosine monophosphate (1), [8-^{3}H]-3′-adenosine monophosphate (2), and [8-^{3}H] adenosine 3′,5′-cyclic monophosphate (3) at 85°

	$10^5 k_{obs.}/s^{-1}$		
pH (at 85°)	(1)	(2)	(3)
1.95		0.69	0.43
2.95	1.91		
3.25	3.05		
4.10	3.51	5.80	3.78
4.41	3.62		
4.44		6.20	
5.19	3.78		
5.20		6.55	4.21
6.25	2.30	4.62	3.80
7.73	2.03		
8.70	2.16	4.50	4.83
10.07			13.8
10.51	5.25		
10.63		13.6	51
10.89		23.9	77.6
11.16		49.4	138
11.30	26		
11.44		100	247
11.46	41		
11.73	65		
11.80		280	412
11.90	123		

$AMPH_2$ the total nucleotide concentration in solution ($[AMP]_T$) is given by equation (1). The concentration of H^+AMPH_2 can be neglected as this species is only formed at very low pH,[25] outside the range of the present study. The prefixed H^+ in $^+HAMPH^-$ denotes base protonation and the suffixed H^- represents primary phosphate

$$[AMP]_T = [H^+AMPH^-] + [AMPH^-] + AMP^{2-}] \quad (1)$$

ionisation. The respective ionisation constants are given by $K_a' = [AMPH^-][H^+]/[H^+AMPH^-]$ and $K_a'' = [AMP^{2-}][H^+]/[AMPH^-]$. If the proposed mechanism involves rate-determining hydroxide ion attack on all three species present in solution we have equation (2).

$$\text{Rate} = k_{obs.}[AMP]_T = k[H^+AMPH^-][OH^-] + k'[AMPH^-][OH^-] + k''[AMP^{2-}][OH^-] \quad (2)$$

Combination of equations (1) and (2) together with the different ionisation constants yields equation (3). For

$$k_{obs.} = \frac{kK_w}{[H^+] + K_a' + \frac{K_a'K_a''}{[H^+]}} + \frac{k'K_w}{[H^+] + K_a'' + \frac{[H^+]^2}{K_a'}} + \frac{k''[OH^-]}{1 + \frac{[H^+]}{K_a''} + \frac{[H^+]^2}{K_a'K_a''}} \quad (3)$$

$K_a' \gg [H^+] \gg K_a''$ and $k \gg k'$ and k'' equation (3) simplifies to $k_{obs.} = kK_w/K_a'$, which corresponds to the first plateau for 5′- and 3′-AMP at pH *ca.* 4—5. Thus k can be evaluated from a knowledge of K_w (pK_w 12.50 at 85°) and K_a' (pK_a' was chosen by a trial and error procedure to give the best fit to the experimental data). At high pH, for $K_a'' \gg [H^+]$, equation (3) reduces to (4)

$$k_{obs.} = k'K_w/K_a'' + k''[OH^-] \quad (4)$$

so that a plot of $k_{obs.}$ against $[OH^-]$ should be linear, with a slope k'' and intercept $k'K_w/K_a''$.

For cyclic AMP, equations (5) and (6) apply,

$$[AMP]_T = [H^+AMP^-] + [AMP^-] \quad (5)$$

$$\text{Rate} = k_{obs.}[AMP]_T = k_T[H^+AMP^-] + k_T'[AMP^-] \quad (6)$$

leading to equation (7) where $K_a' = [AMP^-][H^+]/[H^+AMP^-]$. Equation (7) is similar to that previously

$$k_{obs.} = \frac{k_TK_w}{[H^+] + K_a'} + \frac{k_T'K_a'[OH^-]}{[H^+] + K_a'} \quad (7)$$

derived for adenosine [4] the only difference being that k_T and k_T' do not (because of the phosphate group) correspond exactly to the protonated and neutral forms of adenosine. At high pH equation (7) reduces to (8) so

$$k_{obs.} = k_TK_w/K_a' + k_T'[OH^-] \quad (8)$$

that all three adenine nucleotides should yield a linear $k_{obs.}$ against $[OH^-]$ plot and this is found to be the case

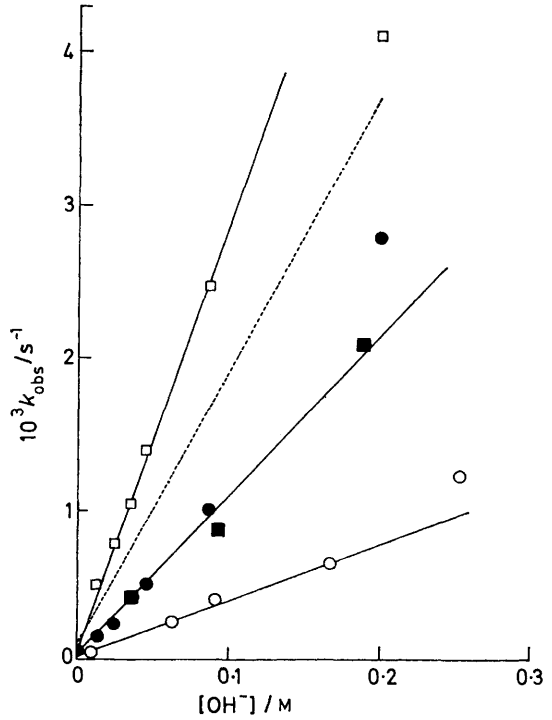


FIGURE 4 Plot of $k_{obs.}$ against hydroxide ion concentration for the detritiation of 5′-AMP (○); 3′-AMP (●); 3′,5′-c-AMP (□); poly A (■). Data for adenosine [4] (– – – –) are also included

(Figure 4). The derived second-order rate constants together with both experimental and literature equilibrium constants are summarised in Table 2. The relevant values for adenosine [4] at 85° are 2.15×10^5 and

1.9×10^{-2} l mol^{-1} s^{-1} for hydroxide ion attack on the protonated and neutral forms of the substrate respectively; the pK_a value is 2.9.

At low pH the hydroxide-catalysed rate constants (k, k_T) for the base-protonated species cover a narrow range (factor of two difference) and decrease in the order adenosine > 3′-AMP > 5′-AMP > 3′,5′-c-AMP. Not too much significance can be attached to these small differences bearing in mind that the respective pK_a values are not known to better than ± 0.1 pK units. Nevertheless it is clear that the phosphate group influences exchange at the C-8 position only marginally. Similar comments apply to the reactions at high pH where the rate constants (k'' and k_T') decrease slightly in the order 3′,5′-c-AMP > adenosine > 3′-AMP > 5′-AMP.

Where the phosphate group is influential however is in the intermediate pH region (4—6) where in the case of 3′- and 5′-AMP secondary ionisation allows the species AMPH$^-$ to undergo hydroxide catalysed exchange and leads to an additional plateau in the rate–pH profile. It is not immediately apparent why the rate constant (k') for this process should be *ca.* 10^4 times greater than the k'' and k_T' values. Other, non-kinetic investigations however provide relevant information. First, ^{1}H n.m.r. studies [21-23] concerned with the influence of the 5′-phosphate group in purine 5′-nucleotides on the acidity of 8-H provide evidence for an anti-conformation being predominant in these compounds. So also do solid-state *X*-ray crystallographic studies.[27] The anti-conformation of 5′-AMP (5) shows the phosphate group in juxtaposition to the C-8 position whereas in the 3′-nucleotides it cannot approach this position; consequently the n.m.r. parameters of the latter are little changed. However the detritiation rate data for both 3′- and 5′-AMP show similar effects upon secondary phosphate ionisation suggesting that the factors influencing the kinetic results are different from those affecting the n.m.r. measurements.

Secondly, the results of ultrasonic absorption studies [28] in solutions of 5′-AMP show that the absorption is at a maximum at a pH of 5.05. This is probably a consequence, at least in part, of direct proton exchange between different ionised forms of the substrate. We are therefore led to believe that the enhanced reactivity of the species AMPH$^-$ is probably due to the formation of the zwitterion ($\overset{+}{H}$AMP^{2-}) *via* the equilibrium (9). Isotopic hydrogen exchange studies on various xanthines [16] as well as 1-methyl-guanosine and -ionosine [29] have led us to suggest that zwitterionic species are involved there as well. In the present study we therefore take the view that the measured second-order rate constant k' for both 3′- and 5′-AMP is a composite function involving rate-determining hydroxide ion attack on the species AMPH$^-$ and H$^+$AMP^{2-} {equation (10) where $K_{zw} = [H^+AMP^{2-}]/[AMPH^-]$}. Although k_{AMPH^-} can be estimated to be

$$k' = k_{AMPH^-} + K_{zw}k_{H^+AMP^{2-}} \qquad (10)$$

ca. 10^4 smaller than k' no value can be given to $k_{H^+AMP^{2-}}$ as K_{zw} has not yet been determined.

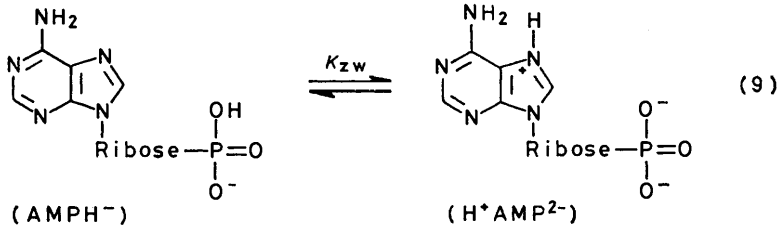
The fact that k' for 5′-AMP is higher (by a factor of 2.3) than for 3′-AMP may simply be a consequence of different K_{zw} values. It is however possible that the close proximity of the phosphate group in 5′-AMP to the N-7 position enables more facile proton migration, particularly with participation of solvent molecules (Scheme).

TABLE 2

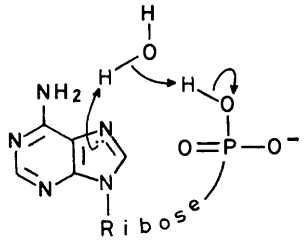
Derived kinetic and equilibrium data at 85° for [8-^{3}H]adenine mononucleotides

Compound	pK_a'	pK_a'' [c]	k	Rate constants (l mol^{-1} s^{-1}) k_T	k'	k_T'	k''
3′-AMP	3.1 (3.65) [a]	5.65	1.6×10^5		3.2×10^2		1×10^{-2}
5′-AMP	3.0 (3.80) [a]	5.65	1.2×10^5		7.3×10^2		0.4×10^{-2}
3′,5′-c-AMP	3.1 (3.60) [b]			1.0×10^5		2.8×10^{-2}	

[a] Lit.,[26] values at 25°. [b] Lit.,[22] value in D_2O at 32°. [c] Values extrapolated to 85° using literature data.[22]



(AMPH$^-$) (H$^+$AMP^{2-}) (9)



SCHEME

Although the detritiation rate data for poly(adenosine 5′-monophosphate) (Table 3) are not as extensive as for the other compounds it is clear that the rate–pH profile (Figure 5) is similar to that for 3′,5′-c-AMP and adeno-

sine, and indicative of only one ionisable proton in the pH range studied. At low pH equation (11) holds, where K_a' is the ionisation constant for N-1 protonation

$$k_{obs.} = kK_w/([H^+] + K_a') \quad (11)$$

and k is the second-order rate constant for detritiation of the protonated polymer. The best fit to the experimental data gave pK_a' 4.3 (compared to the literature

TABLE 3

Detritiation rate constants ($k_{obs.}$) for [8-^{3}H]poly(5'-adenosine phosphate) at 85°

pH (at 85°)	$10^5 k_{obs.}/s^{-1}$
4.10	0.34
4.30	0.85
4.63	2.18
5.02	2.23
7.41	2.27
8.70	3.03
11.08	41.5
11.48	87.5
11.78	209

value [30] at 22° of 5.87) and hence $k = 3.5 \times 10^3$ l mol^{-1} s^{-1}. As mentioned previously, at pH values in excess of 9 the polymer hydrolyses [24] to a mixture of 2'- and 3'-AMP and the detritiation rate data therefore refer to a mixture of these compounds. Both are of similar reactivity and the dashed line in Figure 4 is reproduced

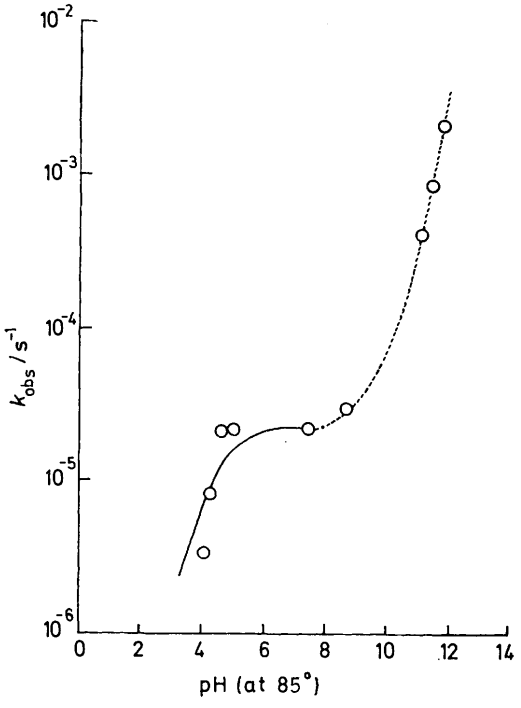


FIGURE 5 Rate–pH profile for the detritiation of [8-^{3}H]-5'-polyadenosine monophosphate at 85°

from the data shown in Figure 2 for 3'-AMP at high pH. In the plot of $k_{obs.}$ against hydroxide ion concentration (Figure 4) the coincidence of the points for poly(A) and 3'-AMP is clearly evident.

The pK_a' value for poly(A) is more than one unit higher than that of the monomer, 5'-AMP, and the second-order rate constant for the detritiation of the base-protonated species *ca.* 30 times less than the value for 5'-AMP. This behaviour is in accord with the findings of Maslova *et al.*[9-11] and Thomas and Livramento,[15] expressed in terms of the observed first-order rate constants. Poly(A) exists as hydrogen-bonded double-strand helices at low pH,[31] whilst single-strand helices predominate in neutral solution accompanied by extensive base-stacking.[32] Clearly all these factors could contribute to the rate reduction. The rate–pH profile however shows no evidence for a structural transition as a function of pH as observed by Steiner and Beers [33] at pH *ca.* 6. In view of the somewhat higher temperature used in the present study we are inclined to believe that the poly(A) exists as a single stranded structure over the pH range 4—9 with but little base stacking.

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