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HYDROXYL RADICAL-INDUCED REACTIONS IN POLYADENYLIC ACID AS STUDIED BY PULSE RADIOLYSIS†
Part I. Transformation Reactions of Two Isomeric OH-Adducts

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The absorption spectra of polyadenylic acid (polyA) radicals in N_2O saturated aqueous solution have been measured as a function of time (up to 15 s) following an 0.4 μs electron pulse. The spectra and their changes were analysed by comparison with those from monomeric adenine derivatives (nucleosides and nucleotides) which had been studied by Steenken.[1]

The reaction of $OH^{\bullet}$ radicals with the adenine moiety in polyA results in the formation of two hydroxyl adducts at the positions C-4 [polyA4$OH^{\bullet}$] and C-8 [polyA8$OH^{\bullet}$]. Each OH-adduct undergoes a unimolecular transformation reaction before any bimolecular or other unimolecular decay occurs. These reactions are characterized by different rate constants and pH dependencies. The polyA4$OH^{\bullet}$ adduct undergoes a dehydration reaction to yield a neutral N^6 centered radical (rate constant $k_{deh} = 1.4 \times 10^4 s^{-1}$ at pH 7.3). This reaction is strongly inhibited by H^+. In comparison with the analogous reactions in adenosine phosphates, the kinetic pK value for its inhibition is two pH units higher. This shift is the result of the counter ion condensation or double-strand formation. The polyA8$OH^{\bullet}$ adduct undergoes an imidazole ring opening reaction to yield an enol type of formamidopyrimidine radical with the resulting base damage ($k_{r.o.} = 3.5 \times 10^4 s^{-1}$ at pH 7.3). This reaction in contrast is strongly catalysed by H^+ and OH^-, similar as for adenosine but different compared to the nucleotides.

KEY WORDS: Polynucleotides, polyadenylic acid, purines, pulse radiolysis, strand breaks, hydroxyl radicals.

INTRODUCTION

The mechanism of hydroxyl radical-induced strand break formation of DNA is difficult to elucidate. The reason is that the hydroxyl radicals react with the bases in single-stranded DNA to ~95% (from a comparison with polyU[2]) and in double-stranded DNA to ~80%[3]. Since there are four different bases in the DNA and every base has more than one position suitable for a reaction with the hydroxyl radicals, many (at least ten[4]) different types of base radicals are formed initially in the reaction of DNA with hydroxyl radicals. With the present methods a mixture of such a number of radicals is difficult to analyse. One way out of this difficulty is the study of

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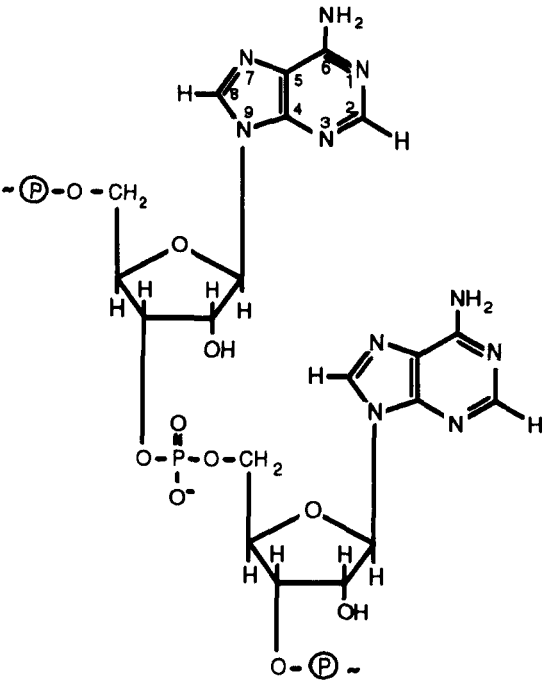
polynucleotides carrying only one base. Polynucleotides with uracil as the only base (polyU) have been extensively investigated in the past.[5-10] In the absence of oxygen the main feature of the pathway to strand break formation is the addition of the hydroxyl radical to the base followed by H abstraction from a sugar. The sugar radicals then lead to strand break formation. Similar studies of polynucleotides with purines as base were hampered by the fact that they show much lower yields of strand break formation[11] and that the reaction of hydroxyl radicals with the purines and their derivatives were not known in detail. The situation with respect to the latter point has improved recently since the groups of O'Neill,[12-16] Steenken,[1,17-22] Cadet,[23] and van Hemmen[24] have carefully studied purine radical chemistry. Especially the reactions of hydroxyl radicals with adenine derivatives and their fast transformations have been elucidated.[21] We have undertaken a pulse radiolytic study of polyadenylic acid (polyA) in deaerated aqueous solutions and have followed the spectral changes of the radicals of the adenine moiety. PolyA is a model for ribonucleic (RNA) since it has a hydroxyl group at C-2′ in the sugar whereas DNA has no hydroxyl group at C-2′. A section of polyA is presented below.



It is hoped that the reaction of the base radicals nevertheless allow to draw conclusions relevant for the DNA. The present work describes the initial changes in the absorption spectrum of polyA and the comparison with changes observed in adenosine phosphates. The result is that these changes are very similar. The initial reactions of the adenine-OH adducts in polyA are not strongly influenced by the polymeric structure of polyA in the pH range $7 \leqslant \text{pH} \leqslant 10$, but at pH $\sim$ 6 drastic changes have been observed which are due to the known[25] fact that at pH $\sim$ 6 the structure of polyA changes from a single-stranded to a double-stranded form.

MATERIALS AND METHODS

The potassium salt of polyadenylic acid, polyA, was obtained from Boehringer, Mannheim, and used without further purification. PolyA was dissolved in water by continuous stirring for 12 h. The water was purified with a millipore ion exchange unit (Milli-Q system). The concentration of polyA (typically 0.2 mM) was adjusted spectrophotometrically using a molar extinction coefficient $\varepsilon = 10\,150\,M^{-1}cm^{-1}$ at 257 nm (*in mononucleotide units*).[11]

Measurements were made at room temperature (~20°C) and at naturally occurring pH of ~7.5. Other pH values were adjusted with $HClO_4$ or KOH solutions. The aqueous solutions of polyA were saturated with N_2O in order to convert e^-_{aq} into OH ($e^-_{aq} + N_2O + H_2O \rightarrow OH + OH^- + N_2$).

The pulse experiments were carried out with a 2.8 MeV van der Graaff accelerator with 0.4 μs pulses of 3–6 Gy in typical experiments. The computer-controlled pulse radiolysis apparatus is described elsewhere.[26] Dosimetry was carried out with N_2O saturated 0.01 M KSCN solutions taking $\varepsilon(SCN_2^-) = 7600\,M^{-1}cm^{-1}$ at 480 nm and G(OH) = 6.0.[27]

RESULTS AND DISCUSSION

Production of OH Adducts

The rate constant for OH addition to polyA at neutral pH is known to be $(9 \pm 1) \times 10^8\,M^{-1}s^{-1}$,[28] $(9.1 \pm 0.5) \times 10^8\,M^{-1}s^{-1}$ or $(9.7 \pm 0.5) \times 10^8$ (depending on the method)[11] and $(5.5 \pm 0.2) \times 10^8\,M^{-1}s^{-1}$ at pH 5.[11] At a concentration of polyA of 0.2 mM and doses of (3–6) Gy about (2–4) μM of OH radicals are formed and these are scavenged within ~20 μs in a pseudo first-order reaction. In Figure 1 the absorption spectra of transients formed in pulse-irradiated N_2O-saturated aqueous solution of polyA and their evolution with time are presented. The initial spectrum (curve 1) is recorded after completion of the reaction with OH radicals (after 20 μs). This spectrum is transformed within 150–300 μs into another spectrum (curve 2) in different ways at different spectral regions, i.e. an increase of optical density (OD↑) in the region 320–350 nm and for $\lambda \geqslant 540$ nm and a decrease (OD↓) in the region 350–540 nm was observed (see insets to Figure 1). These spectra (curves 1 and 2) intersect at 3 isobestic points. It was suspected earlier that more than one site of the base moiety in polyA is attacked by OH.[28] Other spectra (curves 3–5 in Figure 1) were recorded after 3 ms–15 s and demonstrate the subsequent reactions of the adenine radicals.

Transformation Reactions of OH-Adducts

Rate constants. The changes of the absorption spectrum of the OH adduct to polyA as described above and shown in the insets to Figure 1 are of first-order with respect to time. The corresponding first-order constants are listed in Table 1 and are compared with those[21] of some naturally occurring monomeric purine systems of the adenine family. Their rate constants decrease from the free base to the ribose-substituted adenine OH adduct and decrease further on introduction of a phosphate group at C-3′. The introduction of a phosphate group at C-5′ has a smaller influence than that at C-3′, most probably because of a greater distance between the base radical and C-5′ as compared to C-3′.[21] The rate constants for all phosphates have

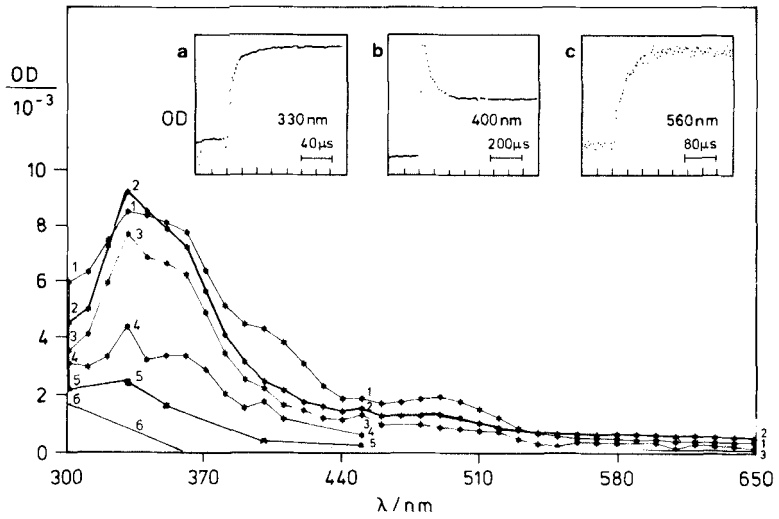


FIGURE 1 Absorption spectra recorded after electron pulses of 6 Gy/pulse to N_2O saturated aqueous solutions of 0.2 mM polyadenylic acid at pH = 7.3 and 20°C. (1) 20 μs after pulse (after completion of the reaction with $OH^{\cdot}$); (2) 300 μs after pulse (after completion of transformation reactions); (3) 3 ms after pulse; (4) 0.75 s after pulse; (5) 7.5 s after pulse; (6) 15 s after pulse. The insets show the ring opening (a) at 300 nm, (c) at 560 nm, and the dehydration reaction (b) at 400 nm.

TABLE I

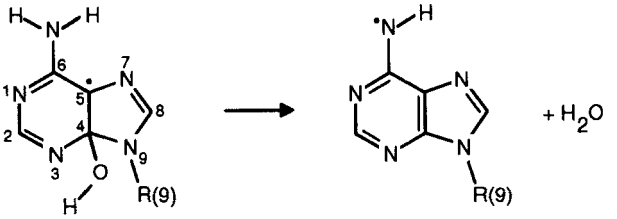
Rate constants[a] for the transformation reactions of the OH-adducts of polyA[b] as compared with those for 9-substituted adenine derivatives[c]

Compound	pH	k [s^{-1}] OD build up (nm)	OD decay (nm)
Adenine	7.0	1.3×10^5 (300) 1.2×10^5 (550)	1.3×10^5 (400)
Adenosine	7.3	2.6×10^4 (330) 2.2×10^4 (550)	1.7×10^4 (400)
Adenosine 3-monophosphate	5	1.3×10^4 (330) 1.4×10^4 (600)	1.1×10^4 (400)
Adenosine 5-monophosphate	5	2.1×10^4 (330) 2.6×10^4 (580)	1.9×10^4 (400)
Adenosine 3,5-diphosphate	5	1.2×10^4 (320) 1.9×10^4 (580)	1.3×10^4 (400)
Polyadenylic acid (polyA)	7.3	3.5×10^4 (330) 2.6×10^4 (600)	1.4×10^4 (400)
	5.0[d]	1.1×10^5 (330)	1.3×10^3 (400)

[a] Measured in aqueous solutions at 20°C.
[b] This work.
[c] From Ref. 21.
[d] See also Figures 2 and 3.

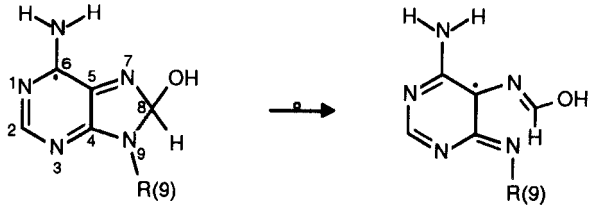
been measured at pH 5 where the phosphates exist as monoanions as does the ribose phosphate moiety in polyA in neutral solution. The rate constants for the adenosine 3′,5′-diphosphate lie between those for both monophosphates. For polyA at pH 7.3 the decay ($OD_{\downarrow}^{400\,nm}$) was found to be practically the same as for adenosine 3,5-diphosphate but the build up ($OD_{\uparrow}^{330\,nm}$) is nearly 3 times faster. The similarity of the processes observed in polyA (Figure 1, Table I) and in N-9 substitutedpurines from the adenine family indicates that they are most probably analogous. Steenken and coworkers[1,21] have identified two processes in adenine derivatives following reaction with OH radicals.

(1) Dehydration of the OH adduct on C-4, symbolized as A4OH$^{\bullet}$. This reaction is characterized by an optical density decrease (OD↓) in the 350–520 nm region (reaction 1).



A4OH$^{\bullet}$ A(-H)$^{\bullet}$ (1)

(2) Ring opening of the OH adduct at C-8, A8OH, a reaction which is characterized by an optical density build up (OD↑) in the spectral region ~330 nm and ~580 nm (reaction 2).



A8OH$^{\bullet}$ A$^{\bullet}$r.o. (2)

From the fact that the spectral changes observed in polyA occur in the same spectral region and in the same direction as observed for the OH adducts to monomeric adenine derivatives and from the result that the rate constants are very similar we conclude that both reactions, the dehydration reaction (1) and the ring-opening reaction (2), take place also in polyA.

These two predominant types of reactions (reactions 1 and 2) have been further characterized in the monomeric adenine derivatives by substituent effects, different activation parameters, different pH dependencies and different redox reactivities with respect to scavengers.[17,21] From these parameters the substituent effects could not be used for further identification of type 1 and type 2 reactions in polyA for obvious

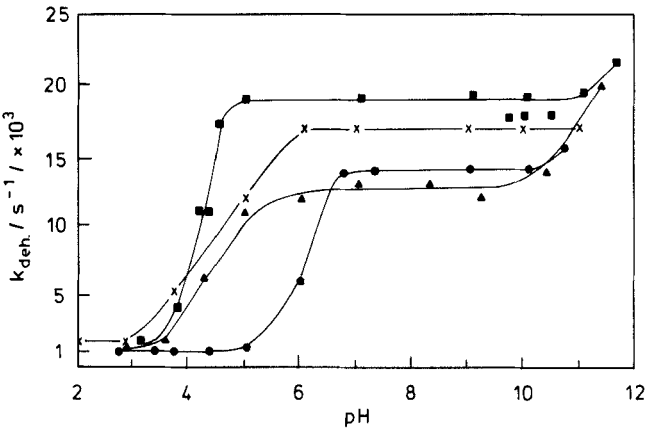


FIGURE 2 Dependence on pH of the rate constants at 20°C for transformation of A4OH (dehydration reaction 1) in: circles, polyA, 0.2 mM; squares, adenosine 5′-monophosphate, 0.4 mM; triangles,[32] adenosine 3′-monophosphate, 0.4 mM; crosses, adenosine, 0.4 mM (taken from Ref. 21 and recalculated for 20°C).

reasons. Temperature dependence could not be used, since for the OH adduct of adenosine 3′,5′-diphosphate, the best model for polyA (see Table I), the activation parameters differ only very slightly, practically within the limit of experimental error.[21] We chose, therefore, the pH dependence for further characterization of type 1 and 2 reactions in polyA.

b) *pH Dependence of rate constants.* In Figures 2 and 3 the pH dependencies of the transformation reactions of the polyA OH adducts are presented. They are quite different for OD↓ (400 nm) (Figure 2) and OD↑ (330 nm) (Figure 3), which changes were assigned to dehydration and ring opening reactions, respectively. For the dehydration reaction (Figure 2) of the adenosine phosphate 4OH adducts there is a

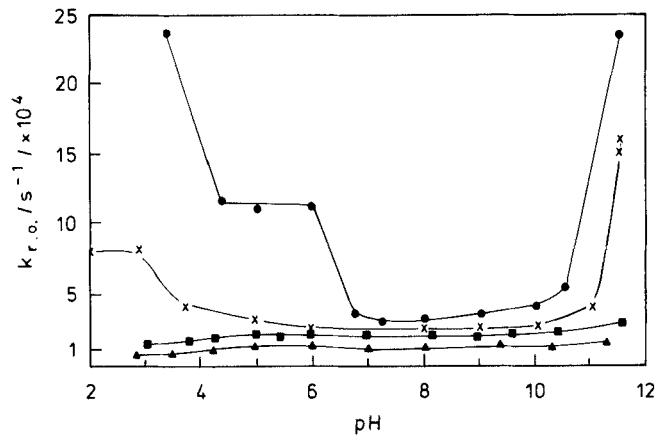
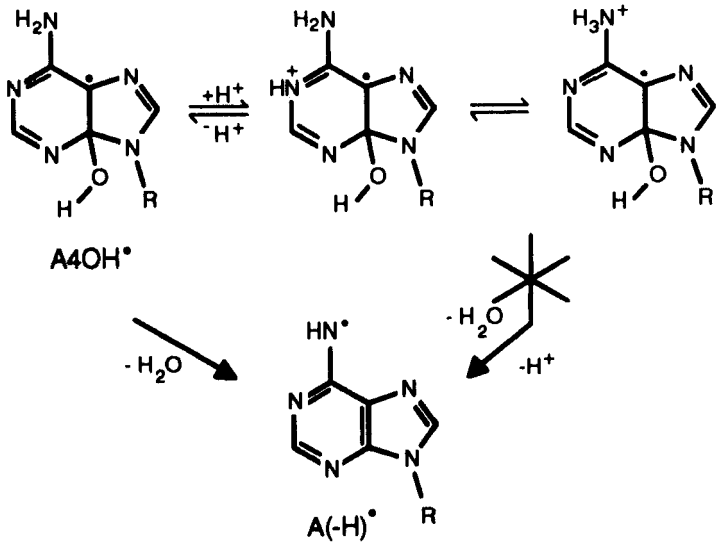


FIGURE 3 Dependence on pH of the rate constant at 20°C for transformation of A8OH (ring opening reaction 2) in: circles, polyA, 0.2 mM; squares, adenosine 5′-monophosphate, 0.4 mM; triangles,[32] adenosine 3′-monophosphate, 0.4 mM; crosses, adenosine, 0.4 mM (taken from Ref. 21 and recalculated for 20°C).

broad range ($5 \leqslant pH \leqslant 10$) where the rate constants (k_{deh}) practically do not change. For polyA4OH such a plateau is observed too but it lies between $7 \leqslant pH \leqslant 10$. In going to the acidic region the rate constants decrease and a new (lower) plateau value ($k_{obs}^{deh} \sim 1 \times 10^3 s^{-1}$) is reached with an apparent kinetic pK of ~ 6.3 for the dehydration rate constant (Figure 2). A similar decrease is observed (Figure 2) for the 4OH adducts in nucleotides and adenosine but with an apparent kinetic pK $\sim$ 4.5 for the dehydration rate constant.

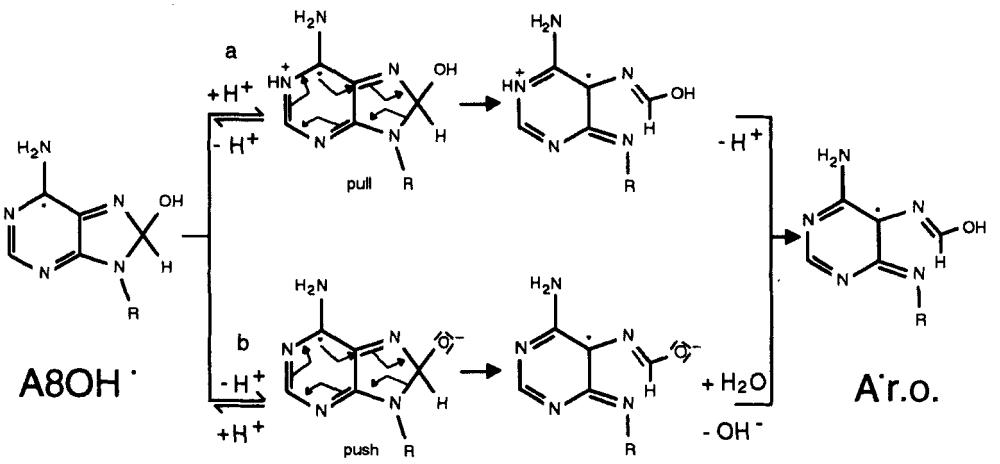
The difference in the apparent kinetic pK values is ~ 2 units between polyA and adenosine phosphates. Such an effect is often observed when comparing rates or equilibrium constants involving ions of polyelectrolytes and their monomeric analogues.[1,25,29] The phenomenon reflects the higher proton (or cation) concentration near the surface of a polyanion due to counter ion condensation. The comparison of both kinetic pK values shows that the polynucleotide is a stronger apparent base than the mononucleotides. Therefore, the H^+ inhibition of the dehydration reaction (1) in polyA at pH $\sim$ 6.3 may be explained by the same reaction scheme (I) which was postulated for the adenosine[21] and should be valid also for nucleotides.



Scheme 1.

Another, however related explanation is that around pH 6 the formation of a double-stranded form of polyA sets in.[25,30,31] At pH values of 6–7 (depending on ionic strength and temperature)[31] addition of acid to polyA solution does not result in an decrease of the pH in the bulk of the solution.[25] The added protons are used up to protonate polyA, most possibly at N1 of the adenine moiety.[31] After addition of 0.2–0.5 protons per adenine moiety, polyA exhibits a double-stranded structure. The double-strands are predicted to contain two hydrogen bonds per adenine pair, both involving hydrogens from amino groups[25] and the N7 nitrogen from the imidazole ring on one case and one of the oxygens of the phosphate group in the second case, but other possibilities are also discussed.[31] The added protons are necessary to

stabilise the double helix. The formation of double-stranded polyA upon H^+ addition has a similar effect as protonation of adenine moiety depicted in the reaction scheme I, i.e., it retards the formation of the N^6 centered A(–H)$^{\cdot}$ radical. The ring opening reaction, on the other hand, is strongly accelerated by H^+ below pH 7 and by OH^- above pH 10 in polyA (Figure 3). The increase of the rate constant ($k_{r.o.}$) below pH 7 occurs in two steps. One step is again at pH ~ 6.3, the pH value at which the formation of double-stranded polyA sets in. The second step is below pH 4.3. At pH 3.4 the rate constant for the increase at 330 nm approaches the value of the pseudo first order OH addition to polyA (at pH 7.3) and the latter reaction begins to be rate determining. The acceleration of the ring opening reaction was observed earlier in the case of adenosine[21] (but not for the corresponding nucleotides) and was explained by H^+ and OH^- catalysis (Scheme II).



Scheme 2.

The increase of $k_{r.o.}$ in adenosine[21] (see also Figure 3) occurs at pH values around 3.7. We assume that this increase corresponds to the increase observed with polyA around pH 6.3 (first step). The reason is the high local proton concentration at the surface of polyA due to ioncondensation and the presence of double-strands. The difference in these two (for adenosine and polyA) kinetic pK values is greater than the difference in the kinetic pK values observed for dehydration. Both pK values are shifted to ~6.3 in polyA. This could mean that not the basicity of the polyA8OH$^{\cdot}$ radical alone is responsible for the kinetic pK value but the onset of double strand formation. The acceleration at pH ~ 4 (second step) could be due to the fact (know from polyA titration curve[33]) that further addition of acid to solutions containing double-stranded polyA leads to the protonation of each adenine moiety.

The increase of $k_{r.o.}$ in polyA at pH ~ 11 is similar to that recently reported for adenosine[21] and may be explained by OH^- catalysis of the 8OH adducts (Scheme II).[21] Such catalysis was not observed in the case of nucleotides (see Figure 3). The reason for this finding is not known. At pH 11.5 the rate constant for the catalyzed ring opening reaches up to the value of the rate constant of the pseudo first-order OH addition to polyA (at pH 7.3) and the latter reaction is rate determining. In the basic

region the structure of polyA does not change, i.e. polyA remains single-stranded as it is at pH 7.3.

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