The Formation of Phosphate End Groups in the Radiolysis of Polynucleotides in Aqueous Solution

Cherla P. Murthy, David J. Deeble, and Clemens von Sonntag

Max-Planck-Institut für Strahlenchemie, Stiftstraße 34–36,
D-4330 Mülheim a.d. Ruhr, Bundesrepublik Deutschland

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The polynucleotides poly(U), poly(C), poly(A) and poly(G) have been γ-irradiated in N_2O- and N_2O/O_2 (4:1)-saturated aqueous solutions. Hydroxyl radicals from the radiolysis of water react with the polynucleotides thereby producing among other lesions strand breaks. Strand breakage is connected with the formation of phosphomonoester end groups. Such end groups have been determined by measuring inorganic phosphate after a three hour incubation at 37 °C with acid or alkaline phosphatase.

In the absence of oxygen G(phosphomonoester end groups) (in units of μmol J^{-1}) are 0.47 (poly(U)), 0.17 (poly(C)) and ≤ 0.04 (poly(A) and poly(G)). In the case of poly(U) and poly(C) on heating the sample for one hour at 95 °C prior to incubation with phosphatases the above values increased by 0.14 and 0.07 μmol J^{-1}, resp., whereas such treatment of the purine polynucleotides still did not produce a measurable yield of phosphomonoester end groups. Comparing these values with G values for strand breakage taken from the literature, about two phosphomonoester end groups are formed per strand break in poly(U) while for poly(C) this ratio is about unity. The purine polynucleotides show very low yields of strand breakage in agreement with the negligible phosphomonoester yields.

In the presence of oxygen G(phosphomonoester end groups) are 0.46 (poly(U)), 0.21 (poly(C)), and ≤ 0.04 (poly(A) and poly(G)). On heating, these values increase, most markedly for poly(U) and poly(C). This is possibly linked to the decomposition of unstable hydroperoxides which are formed in high yields in poly(U) and poly(C) ($G = 0.7$ and 0.19 μmol J^{-1}, resp.).

It is known that at least in the case of poly(U), base radicals attack a sugar moiety and are the main precursors of these lesions. G(phosphomonoester end groups) are considerably lower in the case of the purine polynucleotides. Whether this is due to an inability of the base radicals to attack a sugar moiety or has other reasons must remain an open question.

Introduction

Although many aspects of free-radical-induced strand breaks in nucleic acids appear to be reasonably well understood (for reviews see ref. [1–3]), there still remain unresolved questions. The majority of work has been carried out on γ-irradiated aqueous solutions containing nitrous oxide, conditions under which the OH radical is the main attacking agent (*cf.* reactions (1)–(3), see below). In the studies on the formation of strand breaks in poly(U) [4, 5] and the concomitant release of unaltered uracil [6–8] it became apparent that base radicals must play a dominant role in inducing these lesions. In N_2O-saturated solutions G(strand breaks) = 0.24 μmol J^{-1} has been found for poly(U), whereas for DNA G(strand breaks) is only 0.084 μmol J^{-1} [1]. Thus these two kinds of polynucleotides appear to differ considerably in their mode of strand break formation. Three possible reasons for this difference come immediately to mind. The first of these may lie in the fact that in poly(U) the sugar moiety is ribose and in DNA 2-deoxyribose. A second explanation is that DNA is made up of more than one base and the OH- and H-adduct radicals of thymine, cytosine, adenine and guanine might behave differently from each other. Since radical transfer reactions within the polynucleotide can take place, a third reason could be that certain conformational restrictions might determine whether or not base radicals can induce strand breakage. In the present study we keep one of these "variables", the sugar moiety, constant, and have measured the formation of phosphomonoester end groups. These must be associated with strand breakage. Although the data cannot be used directly to explain the difference between poly(U) and DNA, it will form an integral part of the information required to enable the formulation of a detailed mechanism of nucleic acid strand breakage.

Reprint requests to Prof. Dr. C. v. Sonntag.

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Experimental

Poly(U), poly(A) and poly(C) were purchased from Boehringer/Mannheim, poly(G) was a P. L. Biochemical sample. All other chemicals used were of analytical grade. Details of sample preparation, irradiation conditions (dose rate 0.15 Gy s^{-1}) and post-irradiation treatment with borohydride and heating were identical to those already published [7, 8].

Phosphomonoester groups were converted into inorganic phosphate either at pH 8 using alkaline phosphatase (calf intestine, EC 3.1.3.1, purity grade 1, Boehringer/Mannheim) or at pH 5 using acid phosphatase (potato, EC 3.1.3.2, extra fine grade, Boehringer/Mannheim) at a final concentration of ca. 3 U cm^{-3}. In both cases incubation was at 37 °C for 3 h. The borohydride treatment did not lead to products inducing enzyme inactivation as tested by spiking selected samples with thymidine-5′-phosphate.

Inorganic phosphate was estimated spectrophotometrically using the vanadomolybdate reagent (14842 Kit) supplied by Merck (Darmstadt). The colour developed was stable for at least an hour, and the method gave reproducible results. After addition of the strongly acid reagent a turbidity developed which was removed by filtration through a moist Millex (0.2 μm) filter. The turbidity is due to precipitation of polynucleotides at the pH of ~ 0.9 attained. No phosphate loss was incurred on filtration. Although polynucleotides hydrolyse at low pH to produce phosphomonoester end groups, this does not yield inorganic phosphate, since the phosphatases are inactive at this pH.

In an attempt to determine products of sugar damage from irradiated poly(U) standard gas chromatographic techniques were employed [9]. An aliquot of an irradiated sample was ultrafiltered (Amicon, Microultrafiltration System 8 MC, Filter Diaflow YC 05). Then the filtrate along with an unfiltered sample were reduced with $NaBH_4$. These samples were trimethylsilylated and analyzed for products.

Results and Discussion

Aqueous solutions of polynucleotides (10^{-3} mol dm^{-3} in nucleotide units) were saturated either with N_2O or a 4:1 (v:v) mixture of N_2O and O_2 and subjected to ^{60}Co-γ-radiation. Under such conditions the solvated electrons from the radiolysis of water (reaction (1)) are converted into OH radicals (reaction (2)). In the absence of oxygen both the OH radicals and the H atoms react with the polynucleotides (reaction (3)) while in its presence the H atoms are largely scavenged by oxygen (reaction (4)).

$$H_2O \xrightarrow[\text{radiation}]{\text{ionizing}} {}^{\cdot}OH, {}^{\cdot}H, e^-_{aq}, H^+, H_2O_2, H_2 \quad (1)$$

$$e^-_{aq} + N_2O \longrightarrow {}^{\cdot}OH + N_2 + OH^- \quad (2)$$

$$\text{poly(N)} + {}^{\cdot}OH\,(H^{\cdot}) \longrightarrow \text{poly(N)}^{\cdot} \quad (3)$$

$$H^{\cdot} + O_2 \longrightarrow HO_2^{\cdot} \quad (4)$$

In the course of the subsequent free-radical reactions of the polynucleotide radicals strand breaks can occur. In the presence of oxygen, radicals derived from the pyrimidines and the sugar moiety are converted into peroxyl radicals. In contrast a considerable fraction of the purine-derived radicals appear not to react with oxygen [10, 11]. In N_2O/O_2-saturated pyrimidine polynucleotide solutions pyrimidine and sugar-derived peroxyl radicals are therefore intermediates in the strand-breaking process. When purines are present the situation is more complex, and any role of purine peroxyl radicals has not yet been identified.

In the case of DNA the nature of the products derived from the alteration of the sugar moiety [1–3] indicates that there must be two classes of strand breaks. In the first only one phosphomonoester end group is generated and the damaged sugar remains bound to the other phosphate (the base is released), while in the second class a whole damaged nucleoside is eliminated, *i.e.* two phosphomonoester end groups are formed per one strand break. In the absence of oxygen the radical at C(4′) is the precursor of both classes.

Phosphomonoester end groups have now been determined by measuring the inorganic phosphate after treatment of the irradiated polynucleotides with either alkaline or acid phosphatase. Initially both treatments were performed on the same irradiated samples and identical results were obtained. Therefore it was felt that only one kind of treatment is necessary and later only alkaline phosphatase was used. The yield of inorganic phosphate thus determined was linear with radiation dose. From the slopes of the yield *vs.* dose plots G values were calculated and are compiled in Table I. In some of these experiments the irradiated polynucleotides have been treated with sodium borohydride in order to destroy some potentially reactive groups such as carbonyl and hydroperoxyl functions. For comparative purposes reported values for G(strand breaks) are included in Table I.

Oxygen-free solutions

It can be seen that in N_2O-saturated solutions there is a one-to-one relationship between G(phosphomonoester end groups) and G(strand breaks) for poly(C), but for poly(U) this ratio is about two. If for poly(A) this ratio were also two we should have detected the formation of phosphomonoester end groups. Upon heating the irradiated poly(U) and poly(C) samples, G(phosphomonoester end groups) increased. Whether this increase is accompanied by an equivalent increase in strand breakage is not known. It must be noted that the determination of strand phosphomonoester end groups requires heating to 37 °C for 3 h while in the determination of single-strand breaks no such thermal treatment is involved. The large variation of G(phosphomonoester end groups) and G(strand breaks) among the polynucleotides and the differences in the ratio of G(phosphomonoester end groups)/G(strand breaks), strongly suggests that there are considerable differences in the mechanism of strand break formation.

Radical attack on poly(U) has been investigated in considerable detail and it is now clear that some 93% of the OH radicals add to the base, while the remaining 7% abstract H atoms from the sugar moiety [8]. Within an experimental error of about 5%, H atoms add exclusively to the base [6]. The base radicals can attack a sugar moiety and some of the resulting sugar radicals lead to strand breakage. As mentioned above, in DNA the radical at C(4′) can successively cleave both phosphodiester links, a reaction which would lead to two phosphomonoester end groups per strand break. With DNA it was possible to identify both the altered sugars released from the macromolecule [12, 13] and those that remained bound [14–17]. The same technique has now been applied to poly(U), and although those sugars expected from the C(4′)-mechanism have been identified in the radiolysis of ribose-5-phosphate [18–20] and ribose [21] (*i.e.* their determination is not an analytical problem), no trace of *free altered sugars* (*i.e.* sugars not bound to poly(U)) could be found when the standard technique was employed (see Experimental). Large quantities of unaltered uracil are found by HPLC, but there is no indication that stable altered nucleosides are released. It is therefore not possible to explain the production of two phosphomonoester end groups per strand break in poly(U) in terms of

Table I. G values ($\mu mol\ J^{-1}$) of phosphomonoester end groups (p.m.e.g.) produced upon γ-radiolysis of polynucleotides in aqueous solution (10^{-3} mol dm^{-3}) following the treatments shown. In the assay, samples are incubated for 3 h at 37 °C. Errors ± 15%. G values for single-strand breaks (s.s.b.) taken from the literature [24] are included for comparison. Note that G(p.m.e.g.) has been determined by incubating the samples for 3 h at 37 °C. When G(s.s.b.) was determined samples did not suffer such a thermal treatment.

Polynucleotide	Additional treatment	N_2O		N_2O/O_2	
		p.m.e.g.	s.s.b.	p.m.e.g.	s.s.b.
poly(U)	none	0.47	0.23	0.46	0.27
	heated	0.61		0.75	
	borohydride	0.44		0.29	
	borohydride/heated	0.59		0.60	
poly(C)	none	0.17	0.18	0.21	0.12
	heated	0.24		0.51	
	borohydride	0.15		0.11	
	borohydride/heated	0.23		0.28	
poly(A)	none	n.d.	0.049	n.d.	0.1
	heated	n.d.		0.05	
poly(G)	none	n.d.	0.0	≤ 0.04	0.0
	heated	n.d.		0.05	

n.d. = not detectable. Detection limit = 0.04 $\mu mol\ J^{-1}$.

the C(4′)-mechanism. Recently increasing evidence has been accumulated by Bothe and Schulte-Frohlinde (unpublished results, *cf.* [3]) showing that the C(2′) radical may be an important precursor of strand breakage. The 2′-radical will rapidly eliminate the phosphate at C(3′) to give a carbonyl function at C(2′), one phosphomonoester end group and the C(3′) radical. There is some experimental evidence from studies on ribose-5-phosphate [20] and on 5′-deoxycytidylic acid [22] that a C(3′) radical is capable of eliminating the C(5′) phosphate, possibly by forming an allylic radical. Such a mechanism would also give two phosphomonoester end groups per strand break. However, as with the 4′-mechanism an altered nucleoside radical would be released from the poly(U) chain leading to the same interpretative problems as mentioned above.

A ratio of two for G(phosphomonoester end groups)/G(strand breaks) could be accounted for if the initial number of breaks (forming only one phosphomonoester end group) were twice as high as observed but that the resulting sugar radicals dimerized. In fact, for many radicals the combination/disproportionation ratio is often > 5. In a pulse conductivity experiment (*cf.* ref. [23]) such a process would first lead to an increase in conductivity as breakage occurred, followed by a partial conductivity loss on dimerization. This, however, was not observed (*cf.* ref. [24]).

In poly(C) the high G(strand breaks) strongly suggests that, as in poly(U), base radicals play a dominant role in strand breakage. However, the ratio of G(phosphomonoester end groups)/G(strand breaks) is only unity in poly(C) while it is two in poly(U). This indicates that subsequent reactions must differ in these two systems.

While it is established in the case of poly(U), and also seems very likely for poly(C), that base radicals are the major precursors of strand breakage, this no longer appears to hold for the purine polynucleotides, poly(A) and poly(G). Their G values of phosphomonoester end group and strand break formation are both less than 0.1 μmol J^{-1}. Such low G values could well be due to direct OH attack at the sugar moiety. However, recent experiments with poly(A) at pH 5.8 indicate that G(adenine) is 0.15 immediately after irradiation and increases still further to 0.23 μmol J^{-1} upon post-irradiation heating (Deeble, Murthy, and von Sonntag, to be published). This high value might indicate that in poly(A) some radical transfer from the base to a sugar moiety may occur without, however, leading to efficient strand breakage. This is in contrast to poly(G) where even after post-irradiation heating G(guanine) ≤ 0.04 μmol J^{-1}. As well as damaged sugar moieties being responsible for release of unaltered base, the hydrolysis of labile base hydrates could also give free unaltered bases. Although this possibility has been excluded in the case of poly(U) [8], the appropriate experiments have not yet been made for poly(A).

The effect of oxygen

While the pyrimidine OH adduct radicals react with oxygen, it appears that some of the purine-derived radicals do not [10, 11]. In poly(U) base peroxyl radicals induce strand breakage by abstracting H atoms from a sugar moiety [25]. This results in the formation of hydroperoxides (G(RO_2H) = 0.7 μmol J^{-1} [7]). Some more hydroperoxides might be formed by the reaction of polynucleotide peroxyl radicals with $HO_2^{\cdot}/O_2^{\cdot-}$. It has been shown that $HO_2^{\cdot}/O_2^{\cdot-}$ is released in considerable quantities during the decay of poly(U) peroxyl radicals, and it has been suggested that this process may determine the lifetime of the macromolecular peroxyl radicals [26]. In poly(U) these hydroperoxides decay upon heating [7]. The observed increase of G(phosphomonoester end groups) from 0.46 to about 0.75 μmol J^{-1} could well be associated with this process. In fact destruction of the hydroperoxides with borohydride reduced G(phosphomonoester end groups) to about 0.3 μmol J^{-1}. However, not all the products formed upon $NaBH_4$ reduction are stable, and heating of such samples led to a pronounced increase in G(phosphomonoester end groups), although the yield was always lower than from the corresponding sample not borohydride-treated (Table I). It must be re-emphasized that the determination of phosphomonoester end groups involves a 3 h incubation at 37 °C during which time unstable products could decompose. In this respect borohydride not only destroys hydroperoxides but also reduces carbonyl functions both of which could be responsible for the effects observed on heating.

With poly(C) post-irradiation heating again increases G(phosphomonoester end groups) although initial and final yields are lower than for poly(U) (see Table I). As with poly(U) the decomposition of organic hydroperoxides (G(RO_2H) = 0.19; G(H_2O_2) =

0.17 μmol J^{-1}; determination as in ref. [7]) may participate in this effect. As with poly(U), borohydride treatment reduces G(phosphomonoester end groups) and again after hydroperoxide destruction, labile lesions are still present which on heating produce phosphomonoester end groups (Table I). Heating poly(C) in the presence of 10^{-4} mol dm^{-3} H_2O_2, a concentration twice that formed during irradiation leads to a negligible formation of phosphomonoester end groups.

In poly(A) without heating, G(phosphomonoester end groups) was about the detection limit of 0.04 μmol J^{-1}, on heating this value rises to 0.05 μmol J^{-1}. It is noted that our values are lower than those reported in the literature when N_2- and air-saturated solutions were studied [27]. We have no explanation for these discrepancies.

In the poly(G) system, both in the absence and presence of oxygen the yield of phosphomonoester end groups was below the detection limit of the assay.

Final Remarks

The present study has shown that the reactions that lead to strand breakage in polynucleotides and DNA are much more complex than envisaged before. Only when the sugar products have been identified will it be possible to present a more detailed mechanism. Very important observations have been presented by Adinarayana, Bothe and Schulte-Frohlinde [24] who show that strand breakage is very efficient for poly(U), but inefficient for poly(dU). In contrast, poly(A) shows a low efficiency for strand breakage while it is high in poly(dA). This raises anew the question of the conformational requirements for radical transfer reactions in polynucleotides.

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