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Vanadyl-induced Fenton-like reaction in RNA

An ESR and spin trapping study

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Vanadyl (VO^{2+}) complexed to RNA reacts with hydrogen peroxide in a Fenton-like manner producing hydroxyl radicals ('OH). The hydroxyl radicals can be spin trapped with 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) forming the DMPO-OH spin adduct. In addition, in the presence of ethanol the formation of the hydroxyethyl radical adduct of DMPO (DMPO-ETOH) confirms the production of hydroxyl radicals by the RNA/VO^{2+} complex. When the reaction between the RNA/VO^{2+} complex and H_2O_2 is carried out in the presence of the spin trap 2-methyl-2-nitrosopropane (MNP), radicals produced in the reaction of 'OH with RNA are trapped. Base hydrolysis of the MNP-RNA adducts (pH 12) followed by a reduction in the pH to pH 7 after hydrolysis is complete, yields an MNP adduct with a well-resolved ESR spectrum identical to the ESR spectrum obtained from analogous experiments with poly U. The ESR spectrum consists of a triplet of sextets ($a_N = 1.48$ mT, $a_N^{\beta} = 0.25$ mT and $a_H^{\beta} = 0.14$ mT), indicating that the unpaired nitroxide electron interacts with the nuclei of a β-nitrogen and β-hydrogen. The results suggest that the 'OH generated in the RNA/VO^{2+} reaction with H_2O_2 add to the C(5) carbon of uracil forming a C(6) carbon centered radical. This radical is subsequently spin trapped by MNP.

Vanadium; Vanadyl; Hydrogen peroxide; Hydroxyl radical; Electron spin resonance; Spin trapping

1. INTRODUCTION

The purpose of this work is to study, using ESR and spin trapping, vanadyl-induced Fenton-like reactions in RNA. Spin trapping is a technique by which a short-lived free radical is reacted with a spin trap, usually a nitrone or nitroso compound, to give a longer-lived nitroxide spin adduct which can be identified by ESR [1].

The oxycation of vanadium(IV), vanadyl (VO^{2+}), has proven to be an effective spin probe in biological systems [2–4]. Vanadyl has a single unpaired electron in its lowest nondegenerate vanadium d_{xy} orbital [5]. In a magnetic field, this electron interacts with the ^{51}V nucleus (99.7% abundant) which has a nuclear spin, I = 7/2, generating an isotropic ESR spectrum at room temperature consisting of 8 sharp lines [6]. However, when VO^{2+} is bound to a membrane component or to a slowly tumbling large molecule (protein, nucleic acid) in solution, it yields an anisotropic ESR spectrum. This spectrum resembles the ESR spectrum of immobilized vanadyl in a polycrystalline state or frozen solution [7]. The versatility of vanadyl as spin probe and its capacity to yield important information with regard to metal properties in biological systems, is due mainly to this susceptibility of the vanadyl ESR spectrum to the motion of the cation in solution.

Other aspects of the VO^{2+} chemistry are also of interest in biology. Similar to Fe(II) and Cu(I), VO^{2+} participates in Fenton-like reactions reacting with H_2O_2 to produce hydroxyl radicals ('OH) [8–11]. Using vanadyl ESR, Brooks et al. [8] studied the kinetics of this reaction. Recently, Keller et al. [9,10] using spin trapping have studied the effects of vanadyl on lipid peroxidation and oxidation of NADH. In both studies vanadyl-induced 'OH were implicated. Also, using spin trapping in aqueous solutions of VO^{2+} and Fe^{2+} salts, Carmichael [11] has shown that VO^{2+} generates, in a Fenton-like reaction, approximately half the 'OH as compared to FE^{2+}. This does not necessarily have to be the case when the reactions occur in biological systems. In biological systems these metals are complexed and may contain a large variety of ligands that could possibly alter their chemical properties.

An increasing interest has developed with regard to the role of metal ions in nucleic acid damage [12]. However, most of the emphasis has been placed on Fenton-driven iron and copper reactions. Vanadyl forms complexes with nucleic acids (RNA, DNA) and with ribonucleosides [13–16]. The effects of vanadyl-ribonucleoside complexes on enzymes important to recombinant DNA technology are well documented [14–16]. Therefore, since it is possible that vanadyl may be associated in some manner (still unclear) with nucleic acids in the cell, knowledge of the chemistry of these

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vanadyl-nucleic acid complexes is important. For this reason, in the present study VO^{2+}/RNA complexes were reacted with H_2O_2 in order to determine their capacity to generate ·OH in a Fenton-like manner. In addition, it is of interest to determine the fate of the ·OH following their formation.

2. MATERIALS AND METHODS

Vanadyl sulfate was obtained from Fisher Scientific Co. (Fair Lawn, NJ). The concentration of vanadyl was determined spectrophotometrically (λ = 750 nm, ϵ = 18 $M^{-1}\cdot cm^{-1}$) [17]. Hydrogen peroxide was obtained from Sigma (St. Louis, MO) and its concentration was determined by titration with potassium permanganate [18]. The spin traps 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and 2-methyl-2-nitrosopropane (MNP) were purchased from Aldrich (Milwaukee, WI). DMPO was purified following the method of Buettner and Oberley [19]. In this method, DMPO is successively treated with activated charcoal until all free radical impurities are eliminated as verified by ESR. The concentration of DMPO was measured spectrophotometrically (λ = 227 nm, ϵ = 8 × 10^3 $M^{-1}\cdot cm^{-1}$) [20]. MNP (3–5 mg/ml) was dissolved in water by stirring in the dark at 45°C as described by Makino et al. [21]. The spin trap 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS) was purchased from Sigma and was used without further purification by dissolving directly in water at the desired concentration. Metal-free water was used in all solutions. This water was prepared by further treating, in a separating funnel, water obtained from a Sybron/Barnstead NANO pure system with a 0.001% dithizone (Sigma) solution in carbon tetrachloride. The water was heated to boiling in a water bath in order to eliminate all residual organic material. All glassware was kept permanently soaking in a 1:1 mixture of sulfuric and nitric acids to eliminate trace metals. Immediately prior to use, the glassware was rinsed with metal-free water and dried under a stream of nitrogen.

Calf liver RNA, poly A, poly C, poly G and poly U were purchased from Sigma. The polynucleotides were used as standards and model systems for RNA. To eliminate any metals bound to these nucleic acids, solutions of RNA and polynucleotides were dialyzed, first against several changes (volume ratio 1:250) of 0.01 M EDTA and then, against several changes of metal-free water to eliminate the EDTA. The nucleic acid solutions were then lyophilized and stored until required.

For experiments requiring DMPO, this spin trap was added (0.1 M final) to a 1:1 mixture of VO^{2+}/nucleic acid (1 × 10^{-3} M) followed by addition of H_2O_2 (1 × 10^{-3} M). To avoid chelation of VO^{2+} by MNP in the experiments requiring this spin trap, the MNP was mixed with the H_2O_2 and added to the VO^{2+}/nucleic acid solution. Except for MNP which had a final concentration of 2.5 × 10^{-2} M, the concentration of all other species in the MNP experiments was the same as in the experiments requiring DMPO. The solutions containing the MNP-nucleic acid spin adducts were immediately extracted with petroleum ether to eliminate any ditertiary butyl nitroxide (d-tBN) impurity which may be formed and is frequently generated in experiments with MNP [22]. The solutions were then purged with nitrogen to eliminate any residual petroleum ether. In addition, the petroleum ether extraction and the nitrogen bubbling also eliminate all the excess or unreacted MNP due to the higher solubility of this spin trap in nonpolar solvents and its volatility.

The spin-trapped nucleic acids were subjected to base hydrolysis (pH 12) with dilute NaOH. Once the hydrolysis was complete as judged by the resolution of the ESR spectra, the pH of the solutions were lowered to pH 7 and the final ESR spectra were recorded. ESR spectra were also recorded immediately after addition of the H_2O_2. The ESR spectra were recorded on a Varian E-109 spectrometer at 100 kHz magnetic field modulation. The instrument was set at a magnetic field: 3700 G (for VO^{2+}), 3350 G (for spin adducts); microwave frequency: 9.510 GHz; microwave power: 10 mW; modulation amplitude: 10 G (for VO^{2+}), 0.5 G (for spin adducts); scan range: 2000 G (for VO^{2+}), 100 G (for spin adducts); and unless otherwise specified, the time constant and scan times were 0.25 s and 4 min, respectively. Spectral parameters were obtained by computer simulation, generating theoretical ESR spectra that best match the experimental ESR spectra.

3. RESULTS AND DISCUSSION

When vanadyl sulfate (1 × 10^{-3} M) is mixed with H_2O_2 (1 × 10^{-3} M) in the presence of DMPO (0.1 M), the ESR spectrum shown in fig.1A is obtained. This spectrum consists of the typical isotropic 8-line VO^{2+} spectrum superimposed by another ESR spectrum consisting of 4 sharp lines. Reducing the instrument scan range from 2000 G to 100 G and the modulation amplitude from 10 G to 0.5 G, these additional 4 lines become well resolved into the 1:2:2:1 pattern shown in fig.1B. This ESR spectrum with hyperfine coupling constants, $a_N = a_H^\beta$ = 1.49 mT, corresponds to the DMPO-OH spin adduct [23]. Since DMPO-OH may be formed by pathways other than the direct addition of ·OH to DMPO, an experiment was done reacting vanadyl sulfate (1 × 10^{-3} M) with H_2O_2 (1 × 10^{-3} M) in the presence of D,L-histidine (1 × 10^{-2} M) and the spin trap MNP (1 × 10^{-2} M) in order to verify the generation of ·OH. The spin adduct produced in this reaction yields an ESR spectrum (fig.1C) in which each line in the primary nitroxide triplet is split into 12 lines in a 1:1:2:2:3:3:3:3:2:2:1:1 pattern. This pattern is obtained when the unpaired nitroxide electron interacts with the nuclei of a nitrogen and 3 hydrogens from the histidine molecule. Fig.1D shows the computer simulation that matches the experimental ESR spectrum (fig.1C). This computer simulation was generated using hyperfine coupling values of 1.52 mT, 0.19 mT, 0.38 mT, 0.19 mT and 0.073 mT for the primary nitroxide nitrogen, the histidine nitrogen and the 3 histidine protons, respectively. These hyperfine coupling constants are consistent with the known spectral parameters for the histidyl radical adduct of MNP formed following ·OH reaction with histidine [24]. In addition, when higher concentrations (0.1 M) of D,L-histidine are used and the reaction is carried out at pH 7, the spin adduct obtained is predominantly a triplet of doublets with hyperfine coupling constants a_N = 1.57 mT and a_H^β = 0.34 mT. These values are also consistent with previous data obtained for the spin trapping of histidyl radicals following the reaction of ·OH with histidine [24]. The results shown in fig.1 confirm that ·OH are formed at the concentrations of VO^{2+} and H_2O_2 used.

Although solutions of vanadyl salts react with H_2O_2 generating ·OH, the question still remains whether this cation bound to a large biological molecule, such as a nucleic acid, will react in the same manner. Therefore, VO^{2+} (1 × 10^{-3} M) chelated to RNA (1 × 10^{-3} M) was mixed with H_2O_2 (1 × 10^{-3} M) in the presence of the

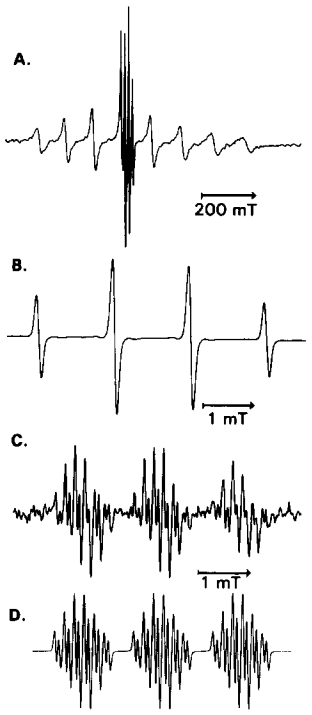

Fig.1. (A) ESR spectrum of $VOSO_4$ (1×10^{-3} M) following its reaction with H_2O_2 ($\times 10^{-3}$ M) in the presence of DMPO (0.1 M). (B) DMPO-OH ESR spectrum observed in the center region of (A) after reducing the instrument microwave power, modulation amplitude and scan range. (C) ESR spectrum of the MNP-histidyl adduct from the reaction of $VOSO_4$ with H_2O_2 (same concentrations as in (A)) in the presence of histidine (1×10^{-3} M) and MNP (2.5×10^{-2} M). (D) Computer simulation of the ESR spectrum in (C). Instrument receiver gain was set at: 1.25×10^4, 2×10^3 and 1.6×10^5 for (A), (B) and (C), respectively. The time constant and scan time for (C) were 2 s and 30 min, respectively.

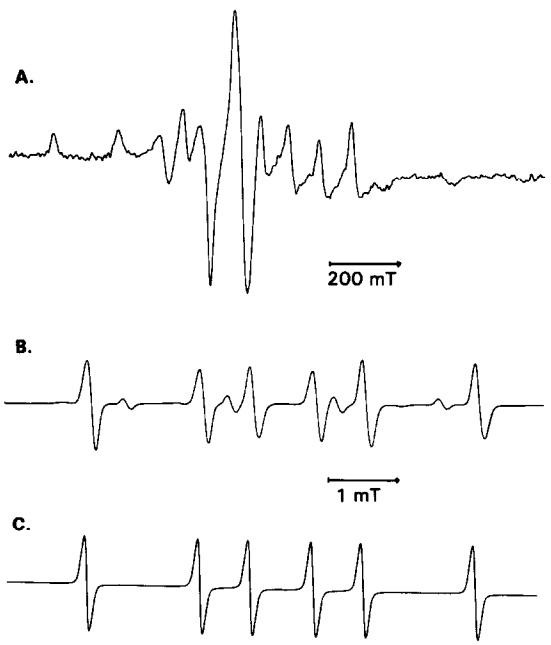

Fig.2. (A) ESR spectrum of VO^{2+} (1×10^{-3} M) bound to RNA (1×10^{-3} M) in the presence of DMPO (0.1 M). (B) ESR spectrum of DMPO-hydroxyethyl adduct following reaction of mixture in (A) with H_2O_2 (1×10^{-3} M) in the presence of ethanol (1.7 M). (C) Computer simulation of ESR spectrum in (B). Instrument receiver gain was set at: 2×10^4 and 2.5×10^3 for (A) and (B), respectively.

spin trap DMPO (0.1 M). Fig.2 shows the results of this experiment. Fig.2A is the typical anisotropic ESR spectrum obtained from immobilized VO^{2+} when bound to a large slowly tumbling molecule in solution [25]. This spectrum was obtained in the presence of DMPO and therefore, shows that all VO^{2+} remains bound to the nucleic acid in the presence of this spin trap. In order to verify that the VO^{2+}/RNA complex does generate ·OH when mixed with H_2O_2, the reaction was carried out in the presence of ethanol (1.7 M). The predominant spin adduct formed (fig.2B) consists of a triplet of doublets and can be computer-simulated using hyperfine coupling constants, $a_N = 1.58$ mT, $a_H^\beta = 2.28$ mT (fig.2C). These spectral parameters are consistent with those previously reported for DMPO-hydroxyethyl radical formed following the reaction of ·OH with ethanol [23]. Although at the concentration of ethanol used all ·OH formed should react with this reagent, the additional ESR lines observed in fig.2B show that a small fraction of DMPO-OH is also formed. The formation of this additional DMPO-OH has previously been observed in Fenton-like reactions involving VO^{2+} and possibly originates from the oxidation of DMPO by a reactive vanadium intermediate [11].

It is of interest to determine whether the ·OH generated by the VO^{2+}/RNA complex react with RNA itself. Therefore, solutions of the VO^{2+}/RNA complex, prepared in a similar fashion to the previous experiments, were mixed with H_2O_2 (1×10^{-3} M) in the presence of the spin trap MNP (2.5×10^{-2} M). However, in this experiment the H_2O_2 and MNP were added simultaneously as a mixture to the VO^{2+}/RNA solution. This was done in order for the reaction to occur instantaneously at the RNA molecule and to avoid chelation of VO^{2+} by MNP which occurs when MNP is mixed with VO^{2+}/RNA prior to the addition of H_2O_2. Fig.3A shows the ESR spectrum of the VO^{2+}/RNA complex. Addition of the H_2O_2 and MNP mixture to the solution containing the VO^{2+}/RNA complex, causes the disappearance of the VO^{2+}/RNA ESR spectrum and yields the sharp 3-line ESR spectrum shown in fig.3B. Reducing the instrument scan range and the modulation amplitude in a similar fashion as shown in fig.1, the ESR spectrum in fig.3B becomes better resolved showing 3 broad lines (fig.3C) typical of a nitroxide radical partially immobilized by a large molecule in solution. This result indicates that ·OH produced in the reaction between VO^{2+}/RNA and H_2O_2 react with RNA generating nucleic acid radicals which are spin trapped by MNP.

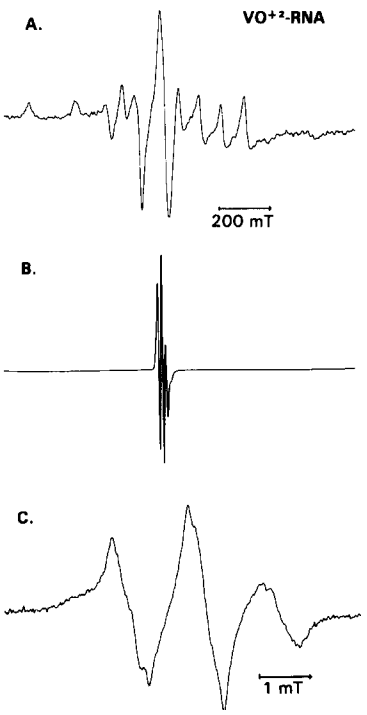

Fig.3. ESR spectra of: (A) VO^{2+}/RNA complex; (B) following reaction of VO^{2+}/RNA with H_2O_2 (1×10^{-3} M) in the presence of MNP (2.5×10^{-2} M); (C) spin-trapped RNA (same as in (B)) after reducing the instrument modulation amplitude and scan range. Instrument receiver gain was set at: 2×10^4 for (A) and (C); 2×10^3 for (B).

To determine the location of the trapped radicals in the RNA molecule, the spin-trapped RNA was hydrolyzed (pH 12) with dilute sodium hydroxide. Because the ribose sugars in RNA contain a 2′-hydroxyl group this nucleic acid is susceptible to base hydrolysis [26]. This method is effective for hydrolyzing spin-trapped RNA because it is strictly chemical and it avoids any specificity complications that could occur, due to the bound spin trap, during enzymatic hydrolysis with RNase. It must be noted that base hydrolysis is not completely free of complications in spin trapping experiments of the nature described in this work using MNP. Control experiments using polynucleotides show that at pH 12 and only in the presence of MNP, polynucleotides yield identical spin adducts as those obtained following their reaction with ·OH. However, this problem can be circumvented by extracting the solutions with petroleum ether and bubbling with nitrogen immediately prior to raising the pH to pH 12. MNP is volatile and is more soluble in non-polar solvents than in water. Therefore, petroleum ether extraction not only eliminates any ditertiary butyl nitroxide (d-tBN), an impurity frequently formed in experiments with MNP [22], it also eliminates the excess unreacted MNP. Nitrogen bubbling through the samples eliminates the residual MNP remaining in the solution and also the residual petroleum ether. Following these precautions, the hydrolysis products observed by ESR originate only from the spin trapped nucleic acids formed subsequently to their reaction with ·OH. Once the hydrolysis at pH 12 of the spin-trapped RNA is complete, the resolution of the broad ESR spectrum (fig.3C) increases permitting the identification of the spin adducts. Fig.4A shows the ESR spectrum of the hydrolyzed spin-trapped RNA after reducing the pH to pH 7. This spectrum consists of a triplet of sextets indicating that the unpaired nitroxide electron is interacting with the nuclei of a secondary nitrogen and a secondary hydrogen. The computer-generated theoretical spectrum that best matches the experimental spectrum (fig.4A) is shown in fig.4B). This simulation was obtained using the hyperfine coupling constants, a_N = 1.48 mT, a_N^β = 0.25 mT and a_H^β = 0.14 mT. Since all bases in RNA contain at least one position where addition of MNP would permit the interaction of the unpaired nitroxide electron with the nuclei of a β-nitrogen and β-hydrogen, similar experiments to the VO^{2+}/RNA reaction with H_2O_2 were carried out using poly A, poly C, poly G and poly U as model systems and standards for RNA. Fig.4C shows the ESR spectrum (pH 7) of the spin adduct obtained from the hydrolysis of the spin-trapped poly U. This spectrum is identical to the spectrum obtained from the RNA experiment (fig.4A) and is matched by the computer simulation (fig.4B). The only position in uracil that addition of MNP would produce an ESR spectrum consisting of a triplet of sextets is the C(6) carbon. Therefore, the results show that following the reaction of VO^{2+}/RNA complex with H_2O_2 only radicals at the C(6) uracil carbon were spin trapped. This result is consistent with the results obtained following γ-radiolysis of RNA solutions containing MNP (28). Although hydrolysis of the spin trapped poly C also yields a spin adduct (pH 7) with ans ESR spectrum consisting of a triplet of sextets with hyperfine coupling constants, a_N = 1.49 mT, a_N^β = 0.27 mT and a_H^β = 0.16 mT, this spectrum is different to the ESR spectra of the spin adducts originating from RNA and poly U. In experiments with poly A, the ESR spectrum was lost when reducing the pH to pH 7. However, at pH 12 the ESR spectrum for hydrolyzed spin-trapped poly A is different from the ESR spectrum for the hydrolyzed spin-trapped RNA. This suggests that in RNA no adenine radicals were spin trapped. In experiments using poly G no radicals were spin trapped. Therefore, it is possible that in the RNA experiments guanine radicals may have been formed but were not observed by spin trapping.

There are 4 immediate reasons that could possibly explain the spin trapping of RNA radicals only at the uracil C(6) position: (1) vanadyl binds to RNA regions rich in uracil; (2) the stability of spin adducts for different bases is not equal; (3) ·OH reacts with all bases followed by spin density migration to uracil; and (4) the MNP spin trapping efficiency is not equal for radicals produced in different bases. It must be noted that ·OH will react at approximately equal rates with all RNA

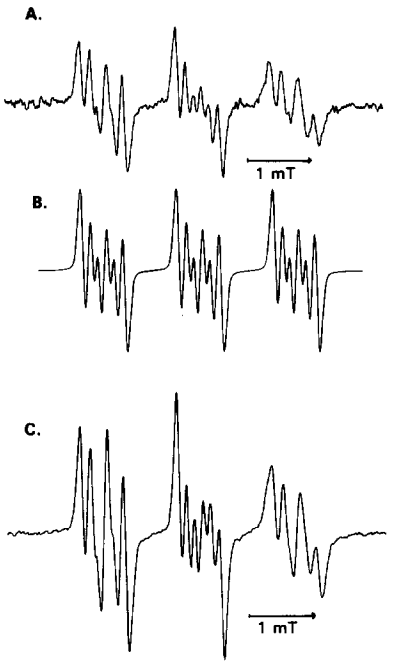

Fig.4. ESR spectra at pH 7 obtained after base hydrolysis (pH 12) of solutions containing MNP-RNA spin adduct (A) and MNP-poly U spin adduct (C). Computer simulation of these spectra is shown in (B). Instrument receiver gain was set at: 5×10^4 and 2×10^4 for (A) and (C), respectively. Both spectra were recorded with 8 min scan time and 0.5 s time constant.

bases [27].

It is unlikely that VO^{2+} binding to RNA regions rich in uracil can be the cause of the observed results. Identical results are obtained in spin trapping experiments with RNA using γ-radiolysis as the source of $^{\cdot}OH$ [28]. Furthermore, in this case experiments were also done using different sources of RNA (calf liver and bakers yeast) to insure base composition variability. The stability of the spin adducts is also an unlikely reason for observing only a uracil adduct in the RNA experiments. Although the results from the poly A experiments may suggest that spin adduct stability plays a role in the observed RNA results, it is improbable because the stability of the sping adducts originating from poly C and poly U is equal, yet no cytosine spin adducts are observed in the RNA experiments.

It is conceivable that an intramolecular mechanism may exist by which following $^{\cdot}OH$ reaction with any of the RNA bases, the radical spin density may migrate to its most stable location in the nucleic acid prior to its reaction with MNP. In this case, the results suggest the C(6) carbon of uracil. However, this cannot be definitely concluded without prior knowledge of the MNP spin trapping efficiency for radicals formed on all RNA bases. As estimated from the intensity of the spin adduct ESR spectra, the MNP spin trapping of radicals formed in poly U is approximately twice and 5 times more efficient than for poly C and poly A, respectively. If this observation can be extrapolated to the RNA case, the results may suggest that in RNA spin trapping of base radicals by MNP occurs preferentially at uracil followed by cytosine, adenine and guanine. No observed spin trapping results in the experiments using poly G suggest low spin trapping efficiency by MNP for poly G radicals. In an attempt to determine whether other base radicals could be spin trapped in RNA, similar experiments were done using another nitroso spin trap, DBNBS, instead of MNP. However, in these experiments the intensity of the ESR spectrum of the spin-trapped RNA was much lower compared to the ESR spectrum of RNA spin-trapped by MNP. Furthermore, the DBNBS spin-trapped RNA was not stable and decomposed during hydrolysis at pH 12.

In conclusion, although the results appear to favor spin trapping efficiency as the major reason in RNA for observing only spin-trapped uracil at the C(6) carbon, it must be noted that certain experimental facts favor an intramolecular spin density migration mechanism. For instance, the concentration of MNP (2.5×10^{-2} M) in the experiments was sufficiently high to allow trapping of any base radical formed. The total amount of base radicals that can be formed cannot surpass the concentration of $^{\cdot}OH$ generated. In turn, the concentration of $^{\cdot}OH$ formed is limited by the initial VO^{2+} and H_2O_2 concentrations (1×10^{-3} M). It should be expected that a 25-fold excess of MNP would offset any of the differences in MNP spin trapping efficiencies observed for polynucleotide radicals.

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