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Investigation of Spin-Trapping Artifacts Formed by the Forrester-Hepburn Mechanism

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Abstract

Free radical detection with ESR spin trapping relies on the specific addition of the radical to nitron/nitroso compounds. It has been proposed that spin traps can react also in biological systems to give false-positive results. For nitron spin traps, the reaction with nucleophiles, first described by Forrester and Hepburn, has been discussed as the most critical source of artifacts.

For artifact identification, the ESR preincubation method may be used, which employs isotopically marked spin traps. Here we investigated the influence of fast sulfite-hydroxylamine equilibrium chemistry on the validity of this assay. Using the (faster) aspiration technique, we found that the Forrester-Hepburn mechanism also contributes to DMPO/ $\bullet\text{SO}_3^-$ adduct formation during ferricyanide-mediated sulfite oxidation, but no evidence for artifactual DMPO/ $\bullet\text{SO}_3^-$ formation was found if the more potent horseradish peroxidase was used. This is ESR evidence that the Forrester-Hepburn mechanism can occur under mild conditions, depending on the experimental details.

This technique can also be used to test for other artifact mechanisms. We investigated the known *ene* reaction of DBNBS and tryptophan in more detail. We found that a strong artifact signal is induced by light; however, with atypically long incubations, we found that the artifact is also formed thermally.

Keywords

Forrester-Hepburn mechanism; spin trapping; false-positive; artifact; nucleophile

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Introduction

ESR spin trapping is a method commonly used for the detection of free radicals [1]. In the process of spin trapping, ESR-detectable nitroxyl radicals are formed by the reaction of primary radicals with nitrones or nitroso compounds [2]. This is necessary for detection, especially in biological systems, since primary radicals usually exhibit a short half-life and attain only very low concentrations, often in the subnanomolar range. However, the spin-trapping technique also introduces the possibility of secondary reactions causing false positive signals. Among those artifact mechanisms, the Forrester-Hepburn mechanism [3] is considered the most serious under biologically relevant conditions [4]. In that mechanism, the nucleophilic reaction of the substrate with the spin trap yields the respective hydroxylamine, which may then be subsequently oxidized to the same product as derived from genuine spin trapping. To occur in a biological system, the artifact mechanism requires a good nucleophile in water. Thus, sulfite as well as thiols and pseudohalogenides have been considered. In biological systems, the highly abundant glutathione may be of importance. In order to investigate whether a signal is generated by the Forrester-Hepburn mechanism, Timmins *et al.* described a procedure using isotopically labeled spin traps (Scheme 1) [5, 6]. Briefly, the spin trap and the substrate are preincubated, and then an isotopically labeled derivative of the same spin trap is added concomitantly with the oxidizing agent. The signal ratio of the two spin-trap isotopes indicates whether it originated by the spin trapping of the radical (equal ratio of isotope signals) or in an artifactual reaction during preincubation (higher proportion of the first isotope).

Although it has been shown that this technique is suitable for detecting artifacts originating in the Forrester-Hepburn mechanism [5-7], limitations of this procedure have to be considered. If artifact formation does occur, the hydroxylamine of the first spin trap is formed during preincubation. However, if this reaction is highly reversible, the second hydroxylamine may reach a concentration equal to the first hydroxylamine before the ESR experiment is finished (See scheme 2). In scheme 3, the timeline of a preincubation experiment in a system with DMPO+R⁻ hydroxylamine equilibrium is outlined. The DMPOH/R hydroxylamine would form during the preincubation phase. The same happens with the D₃-DMPOH/R hydroxylamine once it has been added. Fast re-equilibration (Scheme 2) could result in a 50/50 isotope ratio; since the assay relies on an increased hydroxylamine concentration derived from the preincubation phase, this would cause false-negative results if the hydroxylamine re-equilibration is fast compared to the time necessary for oxidant addition and ESR detection.

Hydroxylamine equilibrium chemistry has been reported for sulfite and DMPO [8]. Also, the existence of the Forrester-Hepburn mechanism was proposed because, in a sulfite oxidation experiment, ferricyanide was reduced faster in the presence of DMPO [8]. We have now investigated whether this faster reduction may be due to oxidation of the DMPO/•SO₃⁻ adduct as an alternative explanation and have reinvestigated our preincubation experiments with respect to the equilibrium chemistry. Using the aspiration sample loading technique [9], we shortened the delay time between the addition of the second spin trap and the end of data acquisition. In experiments with thiols, we used ferricyanide as a mild oxidant and, to increase the rate of nucleophilic reaction, also did experiments in alkaline pH. Although, to the best of our knowledge, no spin-trapping experiments have been reported in such an alkaline environment, such a pH should further increase the chance of artifact detection. For example, the respective pK_a values of the investigated thiols (GSH 9.65 [10], Cys 8.18 [11]) mean that at a pH of 9, the fraction of deprotonated thiol (which actually carries out the nucleophilic attack) would be 7 times larger with cysteine and more than 30 times larger with glutathione than at a neutral pH.

The requirement of isotopically labeled spin traps is a fundamental drawback of the Timmins method of testing for the Forrester-Hepburn artifact. Here we also describe several variations of the Timmins isotope experiment: Instead of the labeled spin trap, which is expensive and labor-intensive, we used isotopically labeled substrates (which may be commercially available) or merely compared the signals with and without preincubation. The same method was also used to investigate factors causing the known artifact formation by the *ene* reaction of tryptophan with the nitroso spin trap DBNBS [12]. DBNBS forms artifact signals with certain amino acids, which makes it less favorable for experiments with enzymes. On the other hand, the radical adduct of this water-soluble nitroso spin trap may provide structural information on the radical species not achievable from a nitron spin trap. We also investigated whether the artifact with tryptophan, the most profound of those artifacts, is light- and/or temperature-dependent.

Materials and methods

Chemicals

Sodium sulfite (Na_2SO_3), potassium cyanide (KCN), potassium ferricyanide $\text{K}_3[\text{Fe}(\text{CN})_6]$, reduced L-glutathione (GSH), cysteine, hydrogen peroxide (30%), horseradish peroxidase (type VI), diethylenetriaminepentaacetic acid (DTPA), and potassium phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Isotopically labelled KCN (^{13}C , 99 %), Tryptophan (Indole-3- ^{13}C , 99 %) and azide (1- ^{15}N , 98 %+) was obtained from Cambridge Isotope Laboratories (Andover, MA). Chelex-100 resin was purchased from Biorad (Hercules, CA). DMPO was purchased from Dojindo (Rockville, MD). D₃-DMPO was synthesized [13] and purified by charcoal treatment [14] and subsequent double distillation. DBNBS was synthesized [15] and purified as previously described [16]. 4-POBN was purchased from Axxora (Farmingdale, NY) and purified by recrystallization from hexane/acetone. See Scheme 1 for the chemical structures of the spin traps used.

ESR spectroscopy

ESR spectra were recorded with a Bruker ElexSys E-500 spectrometer equipped with an ER 4122 SHQ cavity. The instrument operates at 9.75 GHz (X-Band). We used 20 mW microwave power, a modulation frequency of 100 kHz and modulation amplitude of 1.0 G. For standard preincubation and control experiments, the time constant and conversion time were 40.96 ms and receiver gain was 2.5×10^3 . The field scan range was 80–120 G as shown in the plot axis with a resolution of 1024 points. For the concentration dependence, 3 scans were averaged. For preincubation experiments, the first spin trap (final concentration 50 mM) was mixed with the substrate (10 mM). After 15 min of incubation at room temperature, the second spin trap (50 mM) was added, and radical generation was initiated immediately with HRP (1 mg/ml) and 350 μM hydrogen peroxide or 1 mM ferricyanide. The solution (total volume 50 μl) was mixed, transferred into capillaries, tuned and measured. For the aspiration preincubation experiments, the concentrations were the same as with the conventional preincubation experiment, but the sample volume was 1.5 ml. The first DMPO isotope (50 mM) was preincubated for 20 min, and then the second DMPO isotope and the oxidant were added at the same time. The sample was aspirated into the flat cell, and the measurement was started 30 s after addition of the second isotope; parameters were the same as for the standard preincubation experiment, but the scan width was 25 G, the number of points 512, the receiver gain 5×10^3 , and the time constant and the conversion time 5.12 ms. After 3 min, the same sample was scanned again.

In some of the variations of the preincubation assay, isotopically labeled substrates were used instead of isotopically labeled spin traps. For preincubation, the spin trap (100 mM) and the first isotope-labeled substrate (10 mM) were mixed and incubated for 15 min, then

the second isotope-labeled substrate (10 mM) was added together with 1 mM ferricyanide and the sample measured immediately (flat cell, volume 500 μ l). In the isotope-free experiment, the intensity of a preincubation experiment was compared to the intensity of an experiment without preincubation [flat cell, 400 μ l solution, in carbonate buffer (200 mM) with 25 μ M DTPA]. Parameters were the same as for the standard preincubation experiment except for receiver gain (1×10^4 with ^{13}CN and DBNBS); eight scans were accumulated.

Artifact formation from the *ene* reaction was investigated in a system of 10 mM DBNBS and 20 mM tryptophan in the absence of oxidation agents, with incubation times of 30 min at room temperature and 3 h at room temperature and 4 °C. As described in the results, samples were handled in the dark or exposed to room light or projector light (Kodak Carousel 4600; distance from the front lens 50 mm). For all ESR experiments, we used phosphate buffer (100 mM) with 25 μ M DTPA at pH 7.4 or 9.0, unless noted otherwise. Each set of measurements was carried out 3 times; representative experiments are shown. The simulation software WinSim [17] was employed to determine the coupling constants.

For fast scans with the aspiration technique, sulfite was incubated with one spin-trap isotope for 20 min. Then the second spin-trap isotope with ferricyanide or HRP/H₂O₂ was added in quick sequence, the sample was mixed, aspirated into the flat cell and measured 30 s and 3 min after the addition of the second spin trap.

For the stopped-flow experiment, a Harvard apparatus PHD 2000 infuse-withdraw pump (equipped with 2 syringes) was connected with a Y connector to a Bruker Aqua-X capillary in a Bruker E-500 EPR spectrometer to inject the solutions into the measuring cell from two syringes (Syringe 1: D3-DMPO and SO_3^{2-} , preincubated for 20 min; syringe 2: ^{14}N -DMPO and $[\text{Fe}(\text{CN})_6]^{3-}$). Upon injection, the samples were mixed in the Y connector to a final concentration of 100 mM DMPO, 10 mM SO_3^{2-} and 1 mM $[\text{Fe}(\text{CN})_6]^{3-}$. Data acquisition (1 scan ca 750 ms; 5000 scans) was triggered after the sample injection pump finished. The custom-made trigger device used an inverse Schmitt trigger with an output pulse of 5 V triggered by the falling slope of the pump operation signal. A total sample volume 1.5 ml was injected. In the figure, the intensities of the respective low-field peaks normalized to their maximal intensities are shown.

NMR spectroscopy

10 mM glutathione or cysteine was mixed with 100 mM ^{14}N -DMPO in deuterated phosphate buffer, incubated for 15 min at room temperature and the ^1H -NMR spectrum measured. Also, a reference spectrum of DMPO incubated for 15 min in the deuterated buffer was recorded. The deuterated phosphate buffer contained 100 mM Na_3PO_4 and 100 μ M DTPA in D_2O , and the pD was adjusted to 9.0 with NaOD. ^1H NMR spectra were acquired at 25°C on a Varian INOVA 600 spectrometer operating at a ^1H frequency of 599.763 MHz, with a Varian triple resonance, actively shielded Z-gradient probe. All 1D ^1H NMR spectra were acquired using a 1 s presaturation delay, 1 s acquisition time, and 30° excitation pulse to allow adequate recovery of the proton resonances for quantitative integration.

For the cyano hydroxylamine equilibrium experiment, 20 ml of potassium cyanide and 100 mM DMPO in deuterated phosphate buffer, pH 7.4, were mixed, and ^1H spectra were recorded with parameters as described in the experimental section every 15 min for 10 h. Then, 100 mM benzaldehyde was added to the sample and recording (every 5 min for the first hour, then every 15 min) was continued for another 11 h. To estimate the fraction of hydroxylamine, the intensities of the two methyl peaks of the DMPOH/CN (1.06 ppm and 0.89 ppm) were added and divided by the sum of the methyl DMPO peak (1.24 ppm) and DMPOH/CN peaks.

UV/Vis spectroscopy

10 mM substrate was mixed with 1 mM ferricyanide – either in the presence or absence of 100 mM DMPO – and the time course (15 min) was measured on a Cary 300 UV/Vis spectrophotometer ($\lambda = 420$ nm).

Results

Fast scans - the validity of negative results

As a consequence of the potentially fast hydroxylamine equilibrium with DMPO and the nucleophiles (Scheme 2, see discussion), we modified the preincubation experiment to measure the ESR spectrum as quickly as possible after addition of the second spin-trap isotope. Since the experimental procedure required a larger sample volume, we used the easily obtainable trideutero- D_3 -DMPO as the second isotope. Due to the nuclear spin of 1 (deuterium) instead of $\frac{1}{2}$ (proton), the D_3 -DMPO/ $\bullet SO_3^-$ signal consists of nine lines ($a^N = 14.5$ G and $a^D = 2.43$ G) (Figure 1). According to the spectral simulation, the fraction of residual DMPO signal ($a^N = 14.48$ G and $a^H = 16.21$ G, Lit. [18]: $a^N = 14.7$ G and $a^H = 15.9$ G) resulting from incomplete H/D exchange is $< 4\%$. The control experiment (f) (spin trap plus ferricyanide) shows that hydroxylamine impurities from the synthesis using isotope exchange in strong alkaline medium have been successfully removed from the D_3 -DMPO; otherwise, a signal would have been present in (f) since ferricyanide would oxidize contaminating hydroxylamines to nitroxides [19].

For the preincubation experiment (same concentrations, but 50 mM DMPO or D_3 -DMPO), we used the aspiration technique and scanned only a part of the spectrum (25 G), which allowed us to complete the measurement < 35 s after addition of the second spin trap. This is considerably faster than the ~ 3 min for the conventional experiment. Using those conditions, we saw artifact contribution with potassium ferricyanide (Figure 2A). The difference between the preincubation experiment (a) and the same experiment with the order of spin traps reversed (b) was calculated. It consists of the signal of the isotope present during preincubation in experiment (a) superimposed upon the negative inverted signal of the isotope present in experiment (b). However, the analysis of the same samples scanned 3 min after addition of the second spin trap resulted in virtually the same signal in (a) and (b), which is the same result as for a genuine signal (Figure 2B), meaning the sensitivity for the artifact has been lost. However, when horseradish peroxidase/ H_2O_2 was used instead of ferricyanide, no measurable artifact contribution was found after either 30 s or 3 min (Figure 3 A and B).

The concentration dependence of the signal intensity allows insight into the decomposition pathway of the free radicals [20, 21]. With ferricyanide, the adduct signal was proportional to the square root of ferricyanide concentration (Figure 4), indicating a second-order decay mechanism, presumably (ferricyanide-independent) disproportionation.

Fast scan ESR preincubation experiments were also carried out with cysteine and glutathione; however, with neither ferricyanide nor horseradish peroxidase/hydrogen peroxide did we find a detectable artifact contribution in the ESR experiment (data not shown). With a pH of 9.0, the same result was obtained (data not shown). Hydroxylamine formation with the thiols was investigated by NMR spectroscopy, but, in accordance with previous work [7], it was not detectable even if investigated at pH 9 (not shown). Additionally, the ferricyanide reduction rate was checked in the presence and absence of DMPO by means of UV/Vis spectroscopy. With GSH, ferricyanide reduction in the presence of sulfite barely increased after addition of the spin trap (Figure 5A). With cysteine (Figure 5B), the ferricyanide reduction was fast even at concentrations of substrate as low as 1 mM (half life, $\tau_{1/2} \approx 3.3$ min). Here (Figure 5B) a faster ferricyanide decay (half life, $\tau_{1/2}$

≈ 2.2 min) was observed in the presence of DMPO. This would be due to the oxidation of hydroxylamine, oxidation of the radical adduct or some unknown reason. Taken together, these results provide no evidence that glutathione-DMPO nitroxide adduct was formed from artifact chemistry when ferricyanide was used for glutathione oxidation.

The DMPO-cyanide system was investigated with respect to hydroxylamine formation (20 mM CN⁻, 100 mM DMPO) in an overnight experiment, as has been done similarly with sulfite and DEPMPO [22]. When 100 mM benzaldehyde was added, the estimated percentage of hydroxylamine dropped from approximately 7% to approximately 1%. This is interpreted as re-equilibration chemistry of the hydroxylamine (Supplemental data 2).

Variations of the Timmins et al. method

First, the experiment with cyanide was repeated with isotopically labeled cyanide instead of isotopically labeled spin trap. KCN (¹²C or ¹³C) was incubated with 1 mg/ml horseradish peroxidase and 350 μM hydrogen peroxide. A six-line signal was detected with ¹²CN⁻ (data not shown; DMPO/¹²CN[•], coupling constants: $a^N = 15.44$ G and $a^H = 18.90$ G; Lit.[23]: $a^N = 15.5$ G and $a^H = 18.9$ G) and a 12-line signal with ¹³CN⁻ (data not shown, DMPO/¹³CN[•], $a^N = 15.44$ G, $a^H = 18.90$ G and $a^{13C} = 12.95$ G; Lit.[23]: $a^N = 15.5$ G, $a^H = 18.9$ and $a^{13C} = 13.0$ G). Then, the preincubation experiment was carried out. DMPO was preincubated with 10 mM KCN (¹²C) for 30 min and then 10 mM KCN (¹³C) was added concomitantly with 1mg/ml horseradish peroxidase and 350 μM hydrogen peroxide. The resulting spectrum consisted mainly of the DMPO/¹²CN[•] six-line signal (Figure 6a), whereas the same experiment with the order of substrate isotopes reversed (¹³CN⁻ during preincubation and ¹²CN⁻ together with enzyme and hydrogen peroxide) showed the twelve-line signal of DMPO/¹³CN[•] (Figure 6b). The difference of 6a and 6b is a superposition of the ¹²C adduct and the inverted ¹³C adduct, indicating that the signal originates almost entirely from the Forrester-Hepburn mechanism, which is in accordance with the findings we reported before [7]. The experiment was also repeated with 4-POBN; there was no detectable signal at all (data not shown).

We also investigated the sodium azide signal. ESR preincubation experiments with 10 mM ¹⁵N¹⁴N¹⁴N⁻/¹⁴N⁻₃ and HRP/H₂O₂ as the oxidant were carried out, as well as ferricyanide consumption measurements (UV/Vis) and NMR experiments at pD 9 (data not shown). The preincubation experiment gave a negative result for nucleophilic addition, and virtually no FeCN was consumed in the presence or absence of DMPO; our experiments gave no indication of artifact contribution to the reported azide signal [24, 25]. In addition, no hydroxylamine formation was detected with NMR when 100 mM DMPO was added to 10 mM azide.

Artifact identification may also be accomplished by comparing signal intensities with and without preincubation, eliminating the need for isotopically labeled substances. In this variation, 10 mM cyanide, 100 mM DMPO, 1 mg/ml HRP and 350 μM H₂O₂ in phosphate buffer (pH = 9.0) were used for signal generation. Indeed, it was found that after 15 min preincubation, the signal was much stronger than without preincubation (Figure 7); the DMPO/CN[•] adduct was again identified as artifactual.

The ene reaction artifact

First, we investigated whether the known artifact signal with tryptophan could be produced in a light-dependent reaction. Indeed, a strong artifact signal was induced when 10 mM DBNBS and 20 mM tryptophan were exposed to light from a slide projector in the presence of 0.25 mg/ml HRP and 100 μM H₂O₂ (Figure 8A). When the same experiment was carried out in the dark, almost no artifact contribution was detected (Figure 8B), demonstrating that

the tryptophyl radical is formed enzymatically [26]. Next, we asked which factors were necessary for occurrence of artifact signals. We found that the presence of oxidants was not required since tryptophan and DBNBS were sufficient for signal occurrence (Figure 9A). In the dark, almost no signal was detectable (Figure 9b). However, in long incubations (3h), the artifact signal was not decreased by a change from normal room light to dark room conditions, but was decreased by a lower temperature. This suggests the occurrence of more than one pathway of artifact formation, including one which is very slow and occurs in the dark at room temperature.

Discussion

We did not detect an artifact contribution for the sulfite radical adduct in our first study [7], but it was proposed that hydroxylamine formation was a reversible process which could render a potentially occurring artifact contribution undetectable [27]. However, the method we applied here can detect the artifact in a system with reversible hydroxylamine equilibration depending on the rate of the various reactions. The DMPOH/CN hydroxylamine is formed in a reversible equilibrium (Supplemental Figure 2), but the signal in the corresponding ESR experiment was clearly identified as artifact [7]. For sulfite, Potapenko showed indirect kinetic evidence for the Forrester-Hepburn artifact in experiments with DMPO [8]. In that publication, the ferricyanide consumption rates of the sulfite oxidation experiment were investigated by means of UV-Vis spectroscopy in the presence and absence of DMPO. It was found that in the presence of the spin trap, the ferricyanide reduction was much faster; this increased rate was interpreted as evidence of the Forrester-Hepburn mechanism [8].

To exclude the possibility that the elevated ferricyanide decay was due to oxidation of the DMPO-sulfite radical adduct by FeCN, we investigated the decomposition mechanism of $\text{DMPO}^{\bullet}\text{SO}_3^-$ in the $[\text{Fe}(\text{CN})_6]^{3-}$ system. The concentration dependence of the steady-state signal intensity gives insight into the decomposition pathway of radicals [20, 21]. We found that the signal intensity was proportional to the square root of the initial concentration (Figure 4), which indicates a disproportionation mechanism for radical decay. Ferricyanide-mediated adduct oxidation would have caused the signal to be independent of ferricyanide concentration over a wide concentration range since ferricyanide would both generate and degrade the radical adduct. So, in the sulfite/ferricyanide system, artifact chemistry seemed to be the explanation for the increased consumption of the oxidant. Thus, we repeated our preincubation experiments with sulfite and carried out the experiment with significantly faster sample handling. The mixed sample was loaded within seconds into the ESR spectrometer using an aspiration technique. As did Potapenko *et al.* [8], we used ferricyanide as a mild oxidant to make genuine radical formation less favorable (see below). With these experimental conditions, we indeed found proof for the Forrester-Hepburn artifact in the ESR preincubation assay: The proportion of the isotope present during preincubation was elevated significantly 30 s after mixing (Figure 2a). The artifact contribution seen in the experiment (~35%) has to be considered a lower limit for actual artifact occurrence. When the same samples were rescanned 3 minutes after addition of the second isotope (Figure 2b), the signals were virtually identical, which would normally be interpreted as a genuine signal. This finding shows that the negative result of a conventional preincubation experiment (~3 min after addition of the second spin trap) with sulfite was misleading.

These results are in accordance with a fast sulfite/hydroxylamine equilibrium (scheme 2). At first, the hydroxylamine formed with the first isotope during preincubation is present in excess, but the second hydroxylamine equilibrates fast enough to result in an ESR signal with isotope ratios close to 50:50 after 3 min (see scheme 3). However, our data show that

the method is sensitive to the Forrester-Hepburn artifact with sulfite if scanned quickly. For illustration, see supplementary results: Normalized data of a stopped-flow experiment and the integration times for preincubation experiments. Only within the first minute is the radical formation of the preincubated isotope (D_3 -DMPO) faster, which is detectable within the timeframe of the aspiration experiment. If horseradish peroxidase and hydrogen peroxide were used instead of ferricyanide (Figure 3), no evidence for artifact formation was found in the fast aspiration experiment.

As discussed earlier, negative results of any preincubation experiment may be unreliable due to fast equilibrium chemistry. However, we were able to detect artifact formation with ferricyanide. The change to HRP as oxidant accelerated the adduct formation rates due to its higher one-electron reduction potential, but should not have a major influence on the sulfite/hydroxylamine equilibrium because radical generation is a nano-to-micromolar process, whereas the hydroxylamine formation is millimolar. Considering the one-electron reduction potentials, dependence of artifact occurrence on the oxidant is plausible. Potassium ferricyanide may yield a higher artifact proportion since its one-electron reduction potential is somewhat lower than that of sulfite (0.46 V [28] and 0.63 V [29]). In contrast, the one-electron reduction potential of horseradish peroxidase Compound I/Compound II (1.0 V) is higher [30]; thus, radical formation is a favorable process with HRP. Occurrence of sulfite radicals in enzyme-mediated sulfite oxidation has been proven by direct ESR, which is free of spin-trapping artifacts [31]. In comparison to the radical addition process, the Forrester-Hepburn mechanism is not favorable, as determined by density functional theory studies [32]. Also, immuno-spin-trapping experiments with sulfite [33] are not affected by the substance-mediated Forrester-Hepburn mechanism as the sulfite hydroxylamine of DMPO is washed out and the immuno-spin-trapping signal originates from protein radicals, which is also indirect evidence for genuine sulfite radical adduct formation. These findings corroborate spin-trap adduct formation in the HRP system due to a radical process.

We also reinvestigated thiols (glutathione and cysteine) with the sample aspiration preincubation experiment. With horseradish peroxidase, artifact contribution was not found at either physiological pH or at pH 9 (data not shown). The high pH experiments were carried out because this would promote artifact formation. According to the Henderson-Hasselbalch equation, at pH 9 the deprotonated anion (which carries out the nucleophilic attack) would be increased sevenfold for cysteine and more than 30-fold for glutathione. However, at this pH the signal decay was found to be faster, so with cysteine the sample had to be scanned after 2 min. Additionally, NMR experiments to detect hydroxylamine formation were carried out at pH 9.0 with negative results (not shown). Furthermore, UV-Vis experiments at pH 7.4 (GSH: Fig. 5) resulted in no evidence of the Forrester-Hepburn mechanism being the major source of the signal. Considering the one-electron reduction potentials of cysteine (−220 mV) [34] and glutathione (−240 mV) [35], we would expect genuine radical formation to be a strongly favorable process. That is reflected in the ferricyanide reduction data (UV/Vis experiments); both processes are fast in the absence of spin trap. Taken together, we see these results as evidence that radical formation and subsequent spin trapping does outcompete the artifact mechanism with thiols.

The contribution of the artifact mechanism with DMPO and sulfite (with ferricyanide as the oxidant) and possibly with cysteine is in agreement with the previous observation that the equilibrium constant for the nucleophilic addition to another nitron trap, DEPMPO, increases in the order glutathione < cysteine < < sulfite [36]. In the case of cyanide, we have previously observed that the much higher positive charge on the β -carbon of DEPMPO relative to DMPO [37] makes nucleophilic attack more likely [7]. The fact that the formation of hydroxylamine from DMPO and cysteine is undetectable argues that some other factor may be responsible for the increase in ferricyanide reduction in the DMPO/cysteine system.

A disadvantage of the Timmins method is the requirement for isotopically labeled spin traps. With the known Forrester-Hepburn artifact in the presence of cyanide, we tested variations of this method. Certainly, an isotopically labeled substrate can be used instead of the labeled spin trap. This approach is especially useful if radical generation is investigated with a variety of spin traps. Alternatively, the cyanide artifact could be detected merely by comparison of intensities with and without preincubation, as shown with DMPO (Figure 6). However, the Timmins approach (Figure 7) should be considered more reliable with respect to experimental error. If the Forrester-Hepburn mechanism does occur, it would be reflected by a change in the isotope ratio in the ESR signal. We calculate the difference between the respective experiments with the order of spin traps reversed to easily indicate such a change. If the difference does not have the characteristic shape (the second isotope's signal superimposed on the inverted signal of the first isotope), it will be identified as experimental imprecision. But with the intensity comparison experiment, one does rely on quantitative EPR. As discussed earlier, both methods may be affected by false-negative results due to fast hydroxylamine equilibrium, but a positive result is still direct evidence of the Forrester-Hepburn mechanism. The isotope-free approach may be worth considering when the acquisition of isotopically labeled substances is labor-intensive (synthesis of spin traps) and/or expensive (^{33}S labeling for sulfite, GSH and cysteine).

Another source of false-positive signals in spin trapping is the *ene* reaction followed by one-electron oxidation, which is known for nitroso spin traps [12]. Many mechanisms have been discussed for the nitroso *ene* reaction, of which the Diels-Alder-type reaction is considered the most likely; however, diradical or zwitterionic processes are also possible (See Scheme 4) [38]. Although not originating in the Forrester-Hepburn mechanism, the preincubation assay also reveals this kind of artifact since the artifact is formed from an intermediate produced by the reaction between the substrate and spin trap. We found that light does increase this type of artifact generation (Figure 8). For the light-dependent signal, the presence of a chemical oxidant was not required (Figure 9a and b), so that type of artifact is apparent in the controls. We didn't investigate if the role of light is to increase the rate of hydroxylamine formation or to photo-oxidize the hydroxylamine. Depending on the reactants, radical formation with aromatic nitroso compounds and occurrence of the *ene* reaction may or may not be light dependent [39]. After a long incubation period of 3 hours, a strong signal was obtained even under daylight conditions (Figure 9c). Since a signal of comparable intensity was obtained under dark room conditions, we conclude that the artifact can also be generated by a light-independent mechanism. Indeed, if the sample temperature was kept at 4 °C, the signal was significantly decreased. The pericyclic reaction is a thermally allowed, suprafacial reaction, but also slow autoxidation seems possible. As a result, while light exposure of DNBBS samples should be avoided, working in the dark cannot eliminate artifact formation entirely. However, it should be noted that the concentrations and incubation times were chosen to favor development of artifact formation and are much higher than those of typical spin-trapping experiments. Thus, each particular experiment needs to be investigated individually to determine whether it is affected by the *ene* reaction artifact.

Conclusions

Here we have investigated the occurrence of the Forrester-Hepburn mechanism in ESR spin trapping with the Timmins preincubation method, using high substrate and spin-trap concentrations that would give the best prospects for artifact occurrence. With refined experimental conditions, we were able to prove that the Forrester-Hepburn mechanism does contribute to the signal with sulfite if the relatively weak oxidant ferricyanide is used. With horseradish peroxidase, no significant artifact contribution was found. Regarding limitations of the method, we learned that the underlying artifact chemistry can render negative results

unreliable. Thus, negative results should be interpreted as “absence of evidence” rather than “evidence of absence”.

The systems in which we detected occurrence of the Forrester-Hepburn mechanism had two properties: strong hydroxylamine formation as measurable by NMR, and an oxidant that was too weak to thermodynamically favor generation of the respective free radical species. Our findings show that the Forrester-Hepburn mechanism should be heeded when spin-trapping experiments with nucleophilic substrates are interpreted, since artifactual radical adduct formation can occur with DMPO under mild conditions in aqueous buffers. However, demonstrated examples of misinterpretation due to the Forrester-Hepburn mechanism are rare.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Cys	L-cysteine
DBNBS	3,5-dibromo-4-nitrosobenzenesulfonate
DMPO	5,5-dimethyl-1-pyrroline- <i>N</i> -oxide
DMPOH/CN	1-hydroxy,2-cyano,5,5-dimethylpyrrolidine
GSH	L-glutathione reduced
4-POBN	<i>N</i> -tert-butyl- α -(4-pyridyl)nitron- <i>N'</i> -oxide

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Highlights

- Investigation of false positives in ESR spin-trapping experiments with nucleophiles
- The Forrester-Hepburn mechanism can cause artifacts under mild conditions
- Forrester-Hepburn mechanism may dominates if free radical reaction is unfavorable

Figure 1.

Spin trapping of the $\text{SO}_3^{\bullet-}$ radical with DMPO and D₃-DMPO. 100 mM D₃-DMPO, 10 mM sulfite and 1 mM ferricyanide results in a 9-line signal of intensity similar to DMPO (a). The fraction of residual undeuterated DMPO signal in (c) was estimated to be < 4% by means of spectral simulation. With both spin traps, the signal virtually vanished if either ferricyanide (b and e) or substrate (c and f) was omitted.

Figure 2.

Preincubation measurement with sulfite and ferricyanide. 10 mM sulfite was preincubated for 20 min with 50 mM spin trap (a: DMPO, b: D₃-DMPO), then the other spin trap (50 mM) as well as 10 mM ferricyanide was added. A) The sample was measured 30 s after addition of the second spin trap. The fraction of the isotope present during preincubation increased (x: DMPO, o: D₃-DMPO). The spectral shape of the difference (c) indicates an artifact. B) The same samples rescanned 3 minutes after addition of the second spin trap. The spectral shapes of (a) and (b) are virtually the same, and the difference consists mainly of noise (c). Since the intensity increases during incubation, the scale in figure A) is increased by a factor of 2.

Figure 3.

Preincubation measurements with sulfite and horseradish peroxidase. 10 mM sulfite was preincubated for 20 min with 50 mM spin trap (a: DMPO, b: D₃-DMPO), then the other spin trap (50 mM) as well as 1 mg/ml HRP and 350 μ M H₂O₂ was added. Evidence for the Forrester-Hepburn artifact was not detected either after 30 s (A) or 3 min (B).

Figure 4.

Dependence of the signal intensity on the ferricyanide concentration. The signal is proportional to the square root of $[\text{Fe}(\text{CN})_6]^{3-}$ concentration. This indicates that $\text{DMPO}/\cdot\text{SO}_3^-$ decomposes by disproportionation rather than ferricyanide-mediated oxidation to the nitrone. Concentrations: 100 mM DMPO, 10 mM SO_3^{2-} and 1mM $[\text{Fe}(\text{CN})_6]^{3-}$ as indicated.

Figure 5.

Ferricyanide consumption during thiol oxidation in the presence or absence of ferricyanide. 1 mM ferricyanide and 10 mM glutathione (A) or 1 mM cysteine (B) were monitored by UV/Vis spectroscopy (420 nm) for 15 min in the absence (b) and presence (c) of DMPO; a): Ferricyanide in the presence of DMPO alone.

Figure 6.

Preincubation experiment with DMPO and isotopically labeled cyanide. 100 mM DMPO was incubated with 10 mM $^{12}\text{CN}^-$ (a) or $^{13}\text{CN}^-$ (b) for 15 min, then the other isotope of the substrate (10 mM) was added concomitantly with horseradish peroxidase (1mg/ml) and hydrogen peroxide (350 μM). The signal consists mainly of the isotope present during preincubation, and the signal shape of the difference (c) indicates that the signal is originating in the Forrester-Hepburn mechanism.

Figure 7.

DMPO spin-trapping and isotope-free preincubation experiment with the cyanide radical. A 20 min preincubation of 10 mM substrate and 100 mM DMPO (b) before addition of 1 mg/ml horseradish peroxidase and 350 μM H_2O_2 results in a significantly higher signal than without preincubation (a), indicating a Forrester-Hepburn artifact. This experiment was carried out at pH 9 using chelexed carbonate buffer.

Figure 8.

Preincubation experiment with tryptophan and DBNBS. If exposed to bright light during 30 min preincubation (A), a significant artifact contribution is detectable. In the dark, no artifact contribution is visible (B). Conditions: 20 mM $^{12/13}\text{C}$ tryptophan, 10 mM DBNBS, 0.25 mg/ml HRP and 100 μM H_2O_2 .

Figure 9.

DBNBS spin trapping of the tryptophan radical in the absence of oxidants. A strong artifact signal was formed with 20 min bright light exposure (a and b), but also upon 3 h incubation at room temperature (c). Here, a change from room light to darkness (d) did not reduce the signal, but lowering the temperature during incubation (from room temperature to 4 °C) decreased the artifact signal (e), indicating that the adduct may be formed in a thermal reaction in addition to a light-controlled one.

Scheme 1.
Chemical structures of the spin traps

Scheme 2.

Hydroxylamine formation with DMPO and nucleophiles

Scheme 3.

The preincubation experiment and hydroxylamine formation

Scheme 4.
Potential mechanisms for the *ene* reaction (from[38], modified)