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Biological Reactions of Peroxynitrite: Evidence for an Alternative Pathway of Salicylate Hydroxylation

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Salicylate hydroxylation has often been used as an assay of hydroxyl radical production *in vivo*. We have examined here if hydroxylation of salicylate might also occur by its reaction with peroxynitrite. To test this hypothesis, we exposed salicylate to various concentrations of peroxynitrite, *in vitro*. We observed the hydroxylation of salicylate at 37°C by peroxynitrite at pH 6, 7 and 7.5, where the primary products had similar retention times on HPLC to 2,3- and 2,5-dihydroxybenzoic acid. The product yields were pH dependent with maximal amounts formed at pH 6. Furthermore, the relative concentration of 2,3- to 2,5-dihydroxybenzoic acid increased with decreasing pH. Nitration of salicylate was also observed and both nitration and hydroxylation reaction products were confirmed independently by mass spectrometry. The spin trap N-*t*-butyl-α-phenylnitrone (PBN), with or without dimethyl sulfoxide (DMSO), was incapable of trapping the peroxynitrite decomposition intermediates. Moreover, free radical adducts of the type PBN/$^{\bullet}CH_3$ and PBN/$^{\bullet}OH$ were susceptible to destruction by peroxynitrite (pH 7, 0.1 M phosphate buffer). These results suggest direct peroxynitrite hydroxylation of salicylate and that the presence of hydroxyl radicals is not a prerequisite for hydroxylation reactions.

Keywords: 2,5-dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, N-*t*-butyl-α-phenylnitrone, dimethyl sulfoxide, electron spin resonance

Abbreviations: 2,5-DHB, 2,5-dihydroxybenzoic acid; 2,3-DHB, 2,3-dihydroxybenzoic acid; PBN, N-*t*-butyl-α-phenylnitrone; DMSO, dimethylsulfoxide; ESR, electron spin resonance

INTRODUCTION

Salicylate hydroxylation has been used as an assay for hydroxyl radical generation in many biological systems.[1,2,3] For example, recent evidence from our laboratory has demonstrated salicylate hydroxylation in normal muscle during contraction with intense stimulation.[4] This has usually been taken to imply Fenton-type chemistry wherein $^{\bullet}OH$ is generated from superoxide and hydrogen peroxide in systems that were subjected to various insults. However, it would seem unlikely that in relatively normal tissue, sufficient Fe^{2+} would be available to catalyze the Fenton reaction. Recently, there has been evidence that normal muscle can generate $O_2^{\bullet-}$ and $NO^{\bullet}$ during muscle contraction[5,6] which can combine to form peroxynitrite. Peroxynitrite, seems to possess hydroxyl radical-like reactivity

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during its decomposition to nitrate.[7] It has been shown to hydroxylate or nitrate phenylalanine,[8] participate in the formation of dityrosine from phenylalanine,[8] as well as modify cysteine and methionine residues.[9] While the peroxynitrite anion is relatively unreactive, it is the short-lived conjugate acid (peroxynitrous acid) that is the reactive species.[10] We hypothesized that peroxynitrite might be responsible for *in vivo* salicylate hydroxylation. Our results *in vitro* support the possibility that this reaction pathway may be responsible for some salicylate hydroxylation products measured *in vivo*.

MATERIALS AND METHODS

Materials: Sodium salicylate, 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). 5-Nitrosalicylate was from Aldrich Chem. Co. (Milwaukee, WI).

Methods: Peroxynitrite was synthesized as previously described.[9] Briefly, a solution of 0.6 M $NaNO_2$ (in double distilled water) was mixed with a solution of 0.6 M H_2O_2/0.6 M HCl at a rate of 26 mL/min through two plastic syringes connected to form a T-junction. The products were then allowed to immediately drain into a solution of 1.5 M NaOH. All solutions were ice cold prior to mixing. Excess H_2O_2 was destroyed by aspirating the peroxynitrite solution through a filtration apparatus layered with MnO_2 which was repeated until the bubbling stopped. Peroxynitrite concentration was determined spectrophotometrically ($\varepsilon_{302} = 1670\ M^{-1} \cdot cm^{-1}$).[9]

Reaction of salicylate with peroxynitrite: Salicylate stock solutions were prepared at various pH values in 0.1 M potassium phosphate buffer. Peroxynitrite was carefully added down the side of an Eppendorf tube containing salicylate solution and vortexed. In control experiments peroxynitrite was allowed to decompose at pH 7, followed by salicylate addition to the reaction mixture after 3 minutes. In additional controls $NaNO_2$ or H_2O_2 was omitted from the incubation with salicylate to eliminate peroxynitrite formation and test for residual hydroxyl radical activity if any.

Reaction of salicylate with hydrogen peroxide: Varying amounts of H_2O_2 (8.8 M stock in double distilled water) were mixed with a salicylate stock solution (pH 7, 0.1 M potassium phosphate buffer) followed by addition of trace ferrous ammonium sulfate.

Detection and analysis of products by HPLC: The products were analyzed by reversed phase HPLC (C-18 Ultrasphere analytical column Beckman model—UM5, 4.6 × 250 mm) which was preceded by a UM5, 4.6 × 45 mm precolumn, with 5% acetic acid/2 g/L ammonium acetate buffer at pH 3 as the eluant. The flow rate was 0.7 mL/min with UV detection at 243 nm. For references, authentic samples of 2,3-DHB, 2,5-DHB (Sigma Chem. Co.) and 5-nitrosalicylate (Aldrich Chemical Co.) were measured.

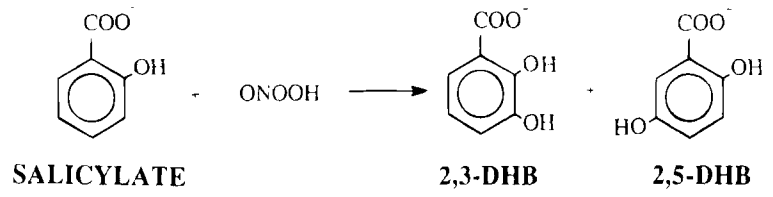
Mass spectrometry analysis: The reaction products were analyzed by electron ionization mass spectrometry on a VG 70-250S high resolution mass spectrometer at 70 eV.

ESR: PBN (50–90 mM) solution with and without DMSO (4 M) at pH 7 were mixed vigorously and then measured immediately on a Varian E-9 X-band (9.5 GHz) spectrometer. Typical instrument settings were: microwave power, 20 mW; modulation frequency, 100 kHz; field set, 3380 G; scan range, 100 G; modulation amplitude, 1 G; scan time, 8 minutes; and time constant, 0.3 s.

RESULTS

Hydroxylation of Salicylate by Peroxynitrite

Addition of peroxynitrite to salicylate (pH 7, 0.1 M phosphate buffer, 37°C) resulted in the

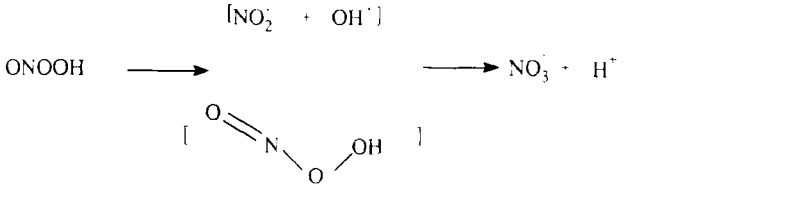


SCHEME I

formation of 2,5-DHB and 2,3-DHB (Scheme I) as shown by HPLC (Fig. 1A). These products co-eluted with 2,3- and 2,5-DHB standards (Fig. 1C). Note that additional peaks were observed from the salicylate reaction with peroxynitrite (Fig. 1A) *vs* ·OH from the Fenton reaction (Fig. 1B). Referring to Fig.2A, the product yields of 2,5-DHB were greater than 2,3-DHB, as expected.[11] Across the ratios of peroxynitrite to salicylate, the yields of each product were ststistically indistinguishable. As the ratio of (initial) peroxynitrite to salicylate increased, the relative yield of 2,3-DHB with respect to 2,5-DHB decreased as shown in Figure 2B, approaching statistical significance ($p = 0.056$). The formation of 2,5- and 2,3-DHB's, from the reaction between peroxy-nitrite and salicylate, as a function of pH is shown in Figure 3A. As the pH was increased, the product yields decreased ($p < 0.05$), consistent with the formation of peroxynitrous acid at lower pH, which is more reactive than peroxy-nitrite anion ($pK_a = 6.8$[12]). The relative yield of 2,3-DHB with respect to 2,5-DHB decreased as a function of increasing pH as shown in Figure 3B ($p < 0.05$). A comparison of the direct reaction of salicylate with hydroxyl radicals *vs* peroxynitrite (Figure 2A) indicates that the latter species is a less effective hydroxylating agent (Table I). The absolute yield of hydroxylated products was less than 2% of the initial peroxynitrite added. Electron ionization mass spectroscopy showed the presence of 2,3-DHB, 2,5-DHB and nitrosalicylate as products from the reaction between peroxynitrite and salicylate (data not shown). It is pertinent to note that nitrosalicylate formation from Fe^{3+}-EDTA was reported by Beckman *et al.*[13] who obtained an 8.8% yield of nitrosalicylate with respect to added peroxynitrite.

The Hydroxylating Species

In order to determine whether hydroxyl radicals were produced during the decomposition of peroxynitrite (Scheme II), we incubated solutions of 5–90 mM PBN with and without 4 M DMSO with varying concentrations of peroxynitrite (pH 7, 0.1 M phosphate). If free hydroxyl radicals were released during peroxynitrite decomposition one would expect the PBN trapped species



VIBRATIONALLY EXCITED STATE

SCHEME II

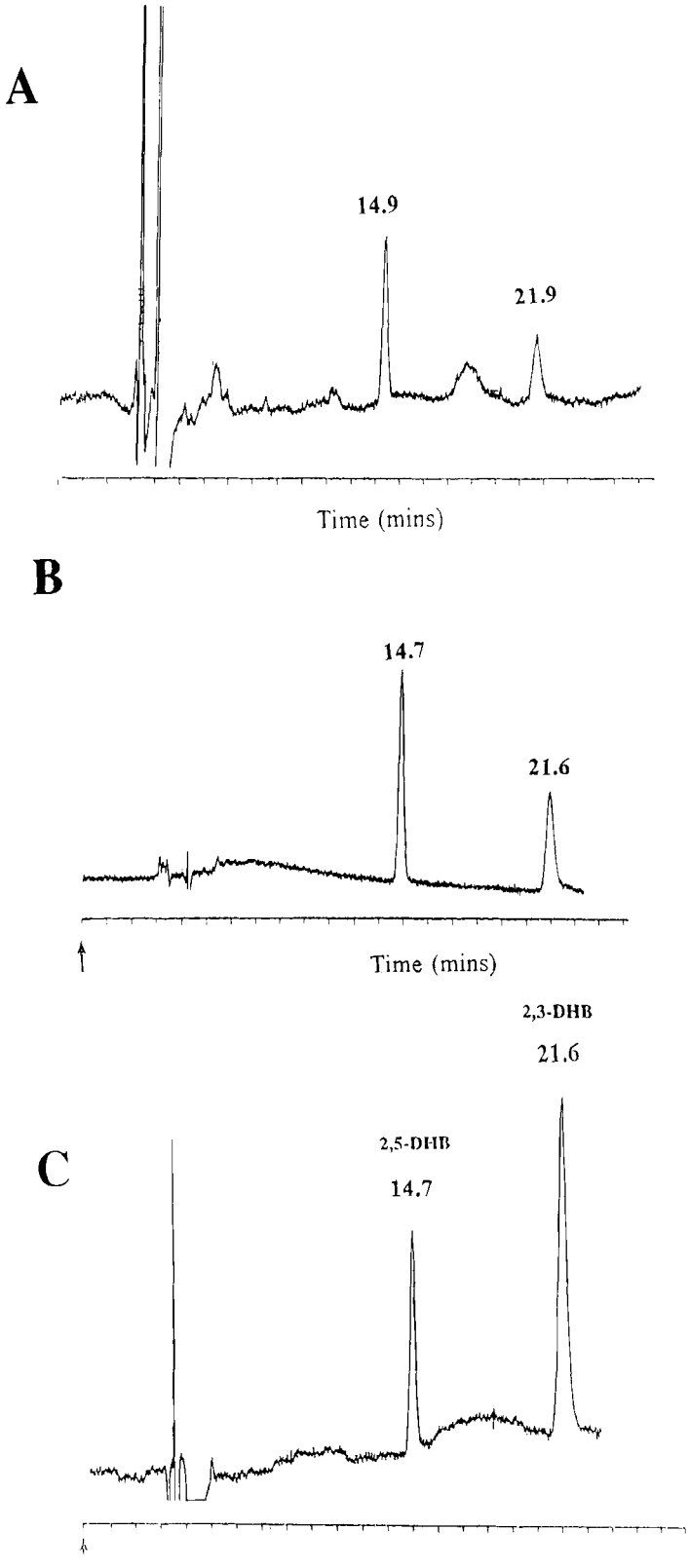


FIGURE 1 Hydroxylation of salicylate by peroxynitrite. HPLC chromatograms of: (**A**) salicylate (1 mM) after incubation with peroxynitrite (1 mM). (**B**) salicylate (1 mM) incubated with $^{\bullet}OH$ generated from the Fenton reaction using 0.3 mM H_2O_2 and trace Fe^{2+}. (**C**) 2,5-DHB and 2,3-DHB standards. The salicylate peak eluted at 73 mins (not shown). All reactions were carried out at pH 7 (0.1 M phosphate buffer) and at 37°C.

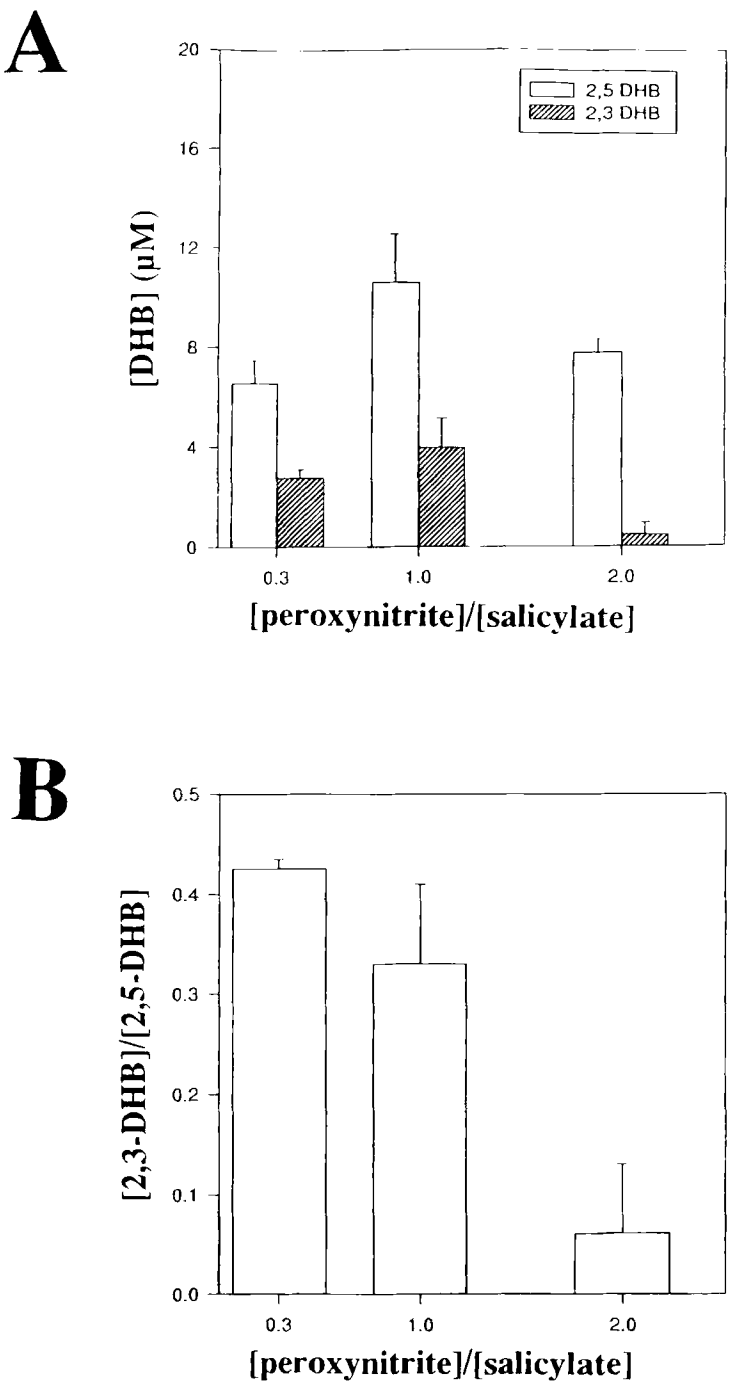


FIGURE 2 Yield of hydroxylated products. (**A**) 2,5-DHB and 2,3-DHB produced as a function of peroxynitrite to salicylate (error bars represent standard deviations obtained from 3 runs). (**B**) Ratio of 2,3-DHB to 2,5-DHB as a function of peroxynitrite to salicylate. [salicylate] = 1mM. All reactions were carried out at pH 7 (0.1 M phosphate) and at 37°C.

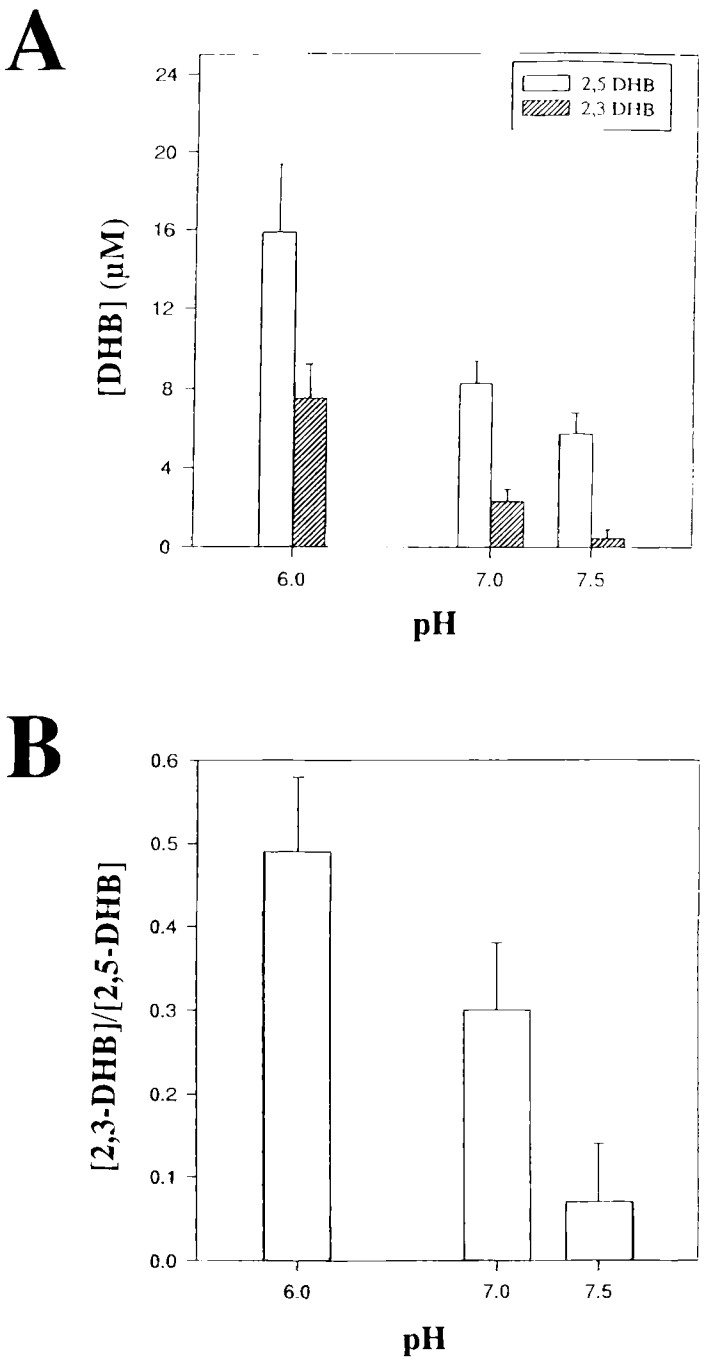


FIGURE 3 Yield of hydroxylated products as a function of pH. (**A**) 2,5-DHB and 2,3-DHB (error bars represent standard deviations obtained from 3 runs). (**B**) Ratio of 2,3-DHB to 2,5-DHB as a function of pH. [salicylate] = 1mM, [peroxynitrite] = 1mM. All reactions were carried out in 0.1 M phosphate and at 37°C.

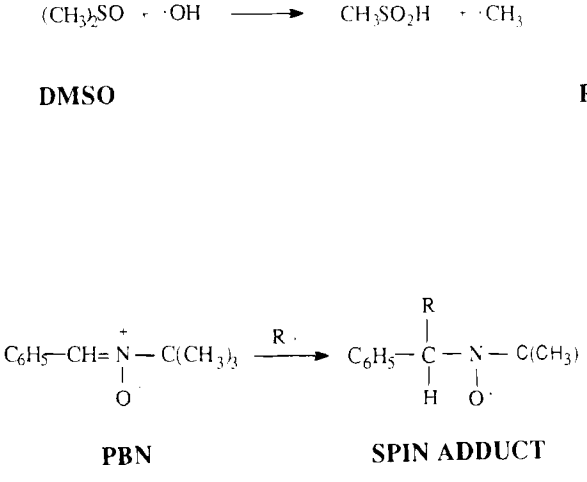
TABLE I Product formation from the reaction of salicylate with •OH from the Fenton reaction. The salicylate concentration was 1 mM (pH 7, 0.1 M phosphate).

H_2O_2/Salicylate	2,5-DHB (μM)	2,3-DHB (μM)
1:1	187 ± 10	74 ± 10
0.3:1	47 ± 10	29 ± 9

shown in Scheme III. However, PBN alone was unable to trap the peroxynitrite decomposition intermediates. In the presence of DMSO only a trace signal was observed, which was independent of the peroxynitrite concentration (data not shown). We exposed solutions of previously prepared spin adducts i.e. PBN/•CH_3 (Fig. 4A) and PBN/•OH (pH 7, 0.1 M phosphate) first to hydroxyl radicals from the Fenton reaction and then to peroxynitrite. Upon the addition of •OH, the signal corresponding to the PBN/•CH_3 adduct grows (Fig. 4B), which is due to the excess PBN and DMSO present in the solution that are capable of trapping additional hydroxyl radicals (Scheme III). When peroxynitrite was added to the solution in Fig. 4B, most of the signal disappeared (Fig. 4C). When peroxynitrite that had *decomposed* was added to the spin adduct, there was no effect on the spectral parameters (data not shown). If hydroxyl radicals were released during the decomposition of peroxynitrite, it is likely that they would be trapped by the excess PBN/DMSO leading to a **growth** in the signal intensity (as observed upon addition of hydrogen peroxide to the solution containing the PBN/•CH_3 adduct as in Figure 4B). The **loss** in intensity of a stable adduct implies that some **other mechanism** occurs in the decomposition process.

DISCUSSION

We have shown that peroxynitrite is capable of hydroxylating salicylate. The products, albeit in low yield, are physiologically relevant since 2,3-DHB formation from salicylate has been observed in tissues under various forms of physiological oxidant stress.[4] The low product yield in the reaction between peroxynitrite and various substrates is well documented, with the maximum theoretical yield for generation of hydroxylated and nitrated products being 20–25% of added peroxynitrite.[8,14,15,16,17] There does not appear to be any obvious trend in the amounts of 2,3-DHB and 2,5-DHB formed when the initial peroxy-nitrite concentration is increased from 0.5 mM to 2 mM. This could be due to the fact that



SCHEME III

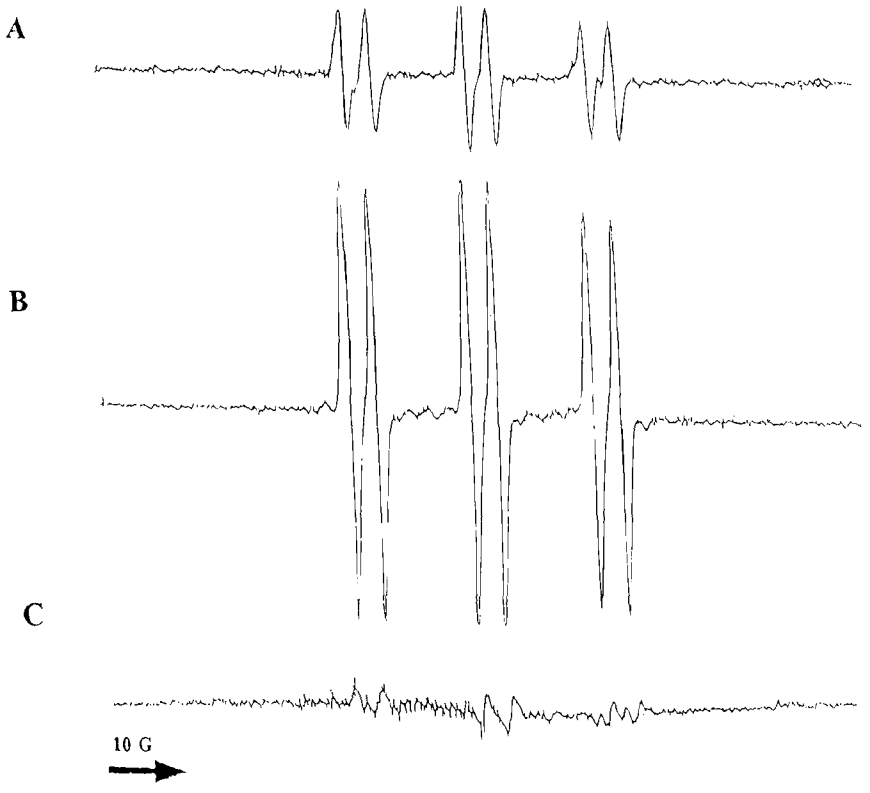


FIGURE 4 ESR spectra of PBN/$^{\bullet}CH_3$ adducts. (**A**) 50 mM PBN and 4 M DMSO incubated with 2 mM H_2O_2 and trace Fe^{2+}. (**B**) 9 mM H_2O_2 added to the above mixture. (**C**) 2.72 mM peroxynitrite was added to the above mixture (in figure 2B). All reactions were carried out at pH 7 (0.1 M phosphate buffer, 37°C).

although the reaction of peroxynitrite with substrates is first order in peroxynitrite, it may occur only at low concentrations of peroxynitrite and excess substrate[8,10] and that the peroxynitrite concentrations chosen in our experiments (i.e. 0.5 mM up to 2 mM) occur near where this type of behavior falls off. The relative product yields (with respect to added peroxynitrite) in the reaction between peroxynitrite and various substrates have been found to decrease at higher peroxynitrite[8,14] concentrations and our results are consistent with this observation. The pH dependence of product yields in the reaction between peroxynitrite and salicylate is consistent with the involvement of peroxynitrite in this reaction in the form of peroxynitrous acid (pK_a = 6.8) that is capable of both hydroxylation and nitration reactions.[12] The hydroxylation of salicylate by peroxynitrite appears to be specific in that the 2,3-DHB/2,5-DHB ratio appears to decrease as a function of an increase in the pH of the medium. This observation would then appear to suggest that the salicylate assay *in vivo* might also be influenced by the local pH of the surrounding medium, when peroxynitrite acts as the hydroxylating agent. Therefore, the predominance of 2,3-DHB vs. 2,5-DHB under physiological conditions[4] may be, in part, due to the combined influences of low pH and low peroxynitrite concentration in vivo. Unlike peroxynitrite based hydroxylation, 2,3-DHB and 2,5-DHB yields from the reaction between salicylate and hydroxyl radicals appears to be proportional to the amount of H_2O_2 in the Fenton reaction, indicating that 1 mM salicylate used seems to be an efficient trap.

It has been suggested that the hydroxylating ability of peroxynitrite is linked to the vibrationally excited *trans-isomer* of peroxynitrous acid.[12] Alternatively, there have been reports that small amounts of hydroxyl radicals are generated during the decomposition of peroxynitrous acid.[8] However, our results do not suggest the presence of free hydroxyl radicals in this system especially since we were unable to consistently trap any hydroxyl radicals in our ESR experiments using traps that are specific for $^{\bullet}OH$.[18,19,20,21] Moreover, a major and critical difference between $^{\bullet}OH$ and peroxynitrite lies in the ability of the latter species to destroy stable spin adducts.

In conclusion, these results suggest that peroxynitrite competes with hydroxyl radicals in aromatic hydroxylation within the biological pH range. Considering the highly efficient mechanisms for sequestering free metal ions (i.e. to inhibit Fenton chemistry), and the high levels of $O_2^{\bullet-}$ and $NO^{\bullet}$ in many cellular systems, it seems likely that peroxynitrite may be the sole or major hydroxylating species in many cases. Therefore the salicylate assay should be done with the cognizance that hydroxylated products can be formed via peroxynitrite. Furthermore, these results demonstrate that assays of hydroxyl radicals such as PBN/DMSO using ESR,[18,19,20,21] may be ineffective in systems where peroxynitrite is also formed, since it destroys the ESR signal arising from methyl or hydroxyl adducts. Although the yields of hydroxylated products of salicylate are small, they are very *significant* because these are the yields obtained in vivo in biological systems.[4] Furthermore we have observed that nitric oxide and peroxynitrite mediate the hydroxylation of salicylate in the fatigued diaphragm (unpublished results) indicating that the salicylate assay is not an exclusive indicator of hydroxyl radicals produced from the Fenton reaction but also from peroxynitrite. Consequently, distinguishing between peroxynitrite and hydroxyl radical in tissue preparations remains difficult using currently available assays and may require simultaneous analysis of nitrated compounds formed from peroxynitrite or $NO^{\bullet}$-derived species.

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