Direct EPR Detection of the Carbonate Radical Anion Produced from Peroxynitrite and Carbon Dioxide*

(Received for publication, December 14, 1998, and in revised form, February 4, 1999)

Marcelo G. Bonini, Rafael Radi, Gerardo Ferrer-Sueta, Ana M. Da C. Ferreira, and Ohara Augusto

From the *Departamento de Bioquímica and |Departamento de Química, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil (05599-970) and |Departamento de Bioquímica, Facultad de Medicina and **Unidad Asociada Enzimología, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay

The biological effects of peroxynitrite have been recently considered to be largely dependent on its reaction with carbon dioxide, which is present in high concentrations in intra- and extracellular compartments. Peroxynitrite anion (ONOO−) reacts rapidly with carbon dioxide, forming an adduct, nitrosoperoxocarboxylate (ONOOCO2), whose decomposition has been proposed to produce reactive intermediates such as the carbonate radical (CO2•−). Here, by the use of rapid mixing continuous flow electron paramagnetic resonance (EPR), we directly detected the carbonate radical in flow mixtures of peroxynitrite with bicarbonate-carbon dioxide over the pH range of 6–9. The radical was unambiguously identified by its EPR parameters (g = 2.0013; line width = 5.5 G) and by experiments with bicarbonate labeled with 13C. In this case, the singlet EPR signal obtained with 12C bicarbonate splits into the expected doublet because of 13C (a(13C) = 11.7 G). The singlet spectrum of the unlabeled radical was invariant between pH 6 and 9, confirming that in this pH range the detected radical is the carbonate radical anion (CO2•−). Importantly, in addition to contributing to the understanding of nitrosoperoxocarboxylate decomposition pathways, this is the first report unambiguously demonstrating the formation of the carbonate radical anion at physiological pHs by direct EPR spectroscopy.

Peroxynitrite is formed from the very fast reaction between nitric oxide and superoxide anion (k = (6.7 – 19) × 109 M−1 s−1) (see Reaction 1) (1, 2). The compound is a potent oxidant that has been receiving increasing attention as a potential pathogenic mediator in human diseases and as a cellular toxin in host defense mechanisms against invading microorganisms (3–6). At present, a significant part of the biological reactivity of peroxynitrite is ascribed to the adduct produced by its reaction with carbon dioxide (7–13). The peroxynitrite anion (ONOO−), which is the predominant form at physiological pHs (pK6 = 6.8) (see reaction 2, Table II) (2, 3), reacts fast with carbon dioxide (pH-independent k = 5.8 × 106 M−1 s−1 at 37 °C) (11), producing an adduct whose structure is proposed to be [(ONOOCO2)−, nitrosoperoxocarboxylate] (see reaction 3, Table II) (7). Taking into account the concentrations of carbon dioxide in equilibrium with bicarbonate present in physiological fluids, model calculations have suggested that most of the peroxynitrite that might be formed in these fluids will produce the carbon dioxide adduct before reacting with other biological targets (5, 13).

Carbon dioxide modulates the reactivity of peroxynitrite by altering reaction rates, product yields, and product distribution (7–13). In these reactions, formation of the adduct nitrosoperoxocarboxylate is rate-limiting, as first proposed by Lymar and Hurst (7). This suggestion was confirmed by other authors (8–13), and the current proposal is that in the absence of substrates, the carbon dioxide adduct decomposes to nitrate and carbon dioxide, but in their presence, a fraction of the adduct (~35%) can engage in one-electron oxidations and aromatic nitrations, probably through homolysis to nitrogen dioxide and carbonate radical anion (Scheme 1A). The participation of the carbonate radical in the oxidation of substrates was inferred from the reactivity of peroxynitrite with inorganic ions in the presence of carbon dioxide (11,12).

The pathway shown in Scheme 1A is similar to the one initially proposed to explain the oxidations mediated by peroxynitrite in the absence of carbon dioxide (Scheme 1B). It has been proposed that peroxynitrous acid (ONOOCO2), the adduct formed between peroxynitrite and carbon dioxide, is the primary oxidant in peroxynitrite-mediated oxidations, probably as an unstable intermediate in the oxidation of substrates before reducing to hydroxyl radical (19,20). The controversy was difficult to resolve because spin trapping of the hydroxyl radical with DMPO, the best unambiguous method for detecting the hydroxyl radical by EPR (21), was compromised by the reactivity of peroxynitrite with spin traps

* This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP) (to O. A.), and Swedish Agency for Research on Development Cooperation, Consejo Nacional de Investigaciones Científicas y Técnicas e Projetos (FINEP) (to O. A.), and Swedish Agency for Research on Development Cooperation, Consejo Nacional de Investigaciones Científicas y Técnicas e Projetos (FINEP) (to O. A.).

** To whom correspondence should be addressed: Instituto de Química, Universidade de São Paulo, Caixa Postal 26077, 05599-970, São Paulo, SP, Brazil. Tel.: 55-11-818-3873; Fax: 55-11-8182186; E-mail: oaugusto@quin.iqup.br.

‡ From theDepartment of Biochemistry and *Department of Chemistry, Institute of Chemistry, University of São Paulo, São Paulo, Brazil (05599-970) and *Department of Chemistry, Faculty of Medicine and †Unidad Asociada Enzimología, Faculty of Sciences, University of the Republic, Montevideo, Uruguay.

1 Unless otherwise specified, the terms peroxynitrite and bicarbonate are used to refer to the sum of peroxynitrite anion (ONOO−) and peroxynitrous acid (ONOOH) and to the sum of CO2, H2CO3, HCO3−, and CO32−, respectively. IUPAC-recommended names for peroxynitrite anion, peroxynitrous acid, nitrosoperoxocarboxylate (ONOOCO2), and nitrite oxide are oxoperoxynitrat(I), hydrogen oxoperoxynitrate, 1-carboxylato-2-nitrosodioxiane, and nitrogen monoxide, respectively.

2 The abbreviation used is: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide.

This paper is available on line at http://www.jbc.org
and spin adducts (15, 19). A direct demonstration of the participation of the carbonate radical in oxidations mediated by peroxynitrite in the presence of carbon dioxide (Scheme 1A) could be anticipated to be similarly difficult. There is only one report of spin trapping of the carbonate radical with nitromethane in irradiated aqueous solutions at pH 12 (22). At physiological pHs, the DMPO-carbon dioxide radical anion produced during irradiation of bicarbonate and DMPO solutions was incorrectly attributed to the carbonate radical adduct (23). We have tried to trap the carbonate radical in systems containing peroxynitrite and bicarbonate under different experimental conditions but did not succeed. Consequently, we considered it worth trying to detect the carbonate radical directly by continuous fast flow EPR of peroxynitrite and bicarbonate solutions. Our results provided unambiguous evidence for the formation of the carbonate radical in these mixtures.

EXPERIMENTAL PROCEDURES

Materials—All reagents were purchased from Merck, Aldrich, or Sigma and were analytical grade or better. Sodium bicarbonate labeled with $^{13}$C (98%) was obtained from Isotec (Miamisburg, OH). Peroxynitrite was synthesized from sodium nitrite (0.6 M) and hydrogen peroxide (0.65 M) in a quenched-flow reactor (3, 9); excess hydrogen peroxide was used to minimize nitrite contamination (24). To eliminate excess hydrogen peroxide, peroxynitrite was treated with manganese dioxide. Synthesized peroxynitrite contained low levels of contaminating hydrogen peroxide (< 1%) and nitrate (10–30%) that were determined as described previously (2) by the titanyl method and by absorbance measurements at 354 nm ($\varepsilon = 24.8 M^{-1} cm^{-1}$), respectively. The concentration of peroxynitrite stock solutions was determined spectrophotometrically at 392 nm using an extinction coefficient of 1670 $M^{-1} cm^{-1}$. All solutions were prepared with distilled water treated in a Millipore Milli-Q system.

EPR Experiments—The EPR fast-flow spectra were recorded at room temperature ($25 \pm 2^\circ C$) on a Bruker EMX spectrometer operating at 9.68 GHz and 100 KHz field modulation equipped with a Bruker ER 4117 D-MTV dielectric mixing resonator with a 9-mm distance between the mixing cell and the resonator center. Sodium bicarbonate solutions were prepared in 0.1–1 M phosphate buffer, pH 6.9–7.1, and mixed with water, pH 12. The spectra were recorded 15 ms after mixing under a continuous flow of 7 ml/min as described under “Experimental Procedures” except for the spectrum (B) that was scanned immediately after stopping the flow. The final concentrations of peroxynitrite, bicarbonate, and phosphate buffer were 10, 50, and 500 mM; the final pH was 6.9. Instrumental conditions: microwave power, 2 mW; modulation amplitude, 5G; time constant 0.16 s; scan rate 0.84 G/s. Each spectrum is the accumulation of two scans.

RESULTS

Fast flow mixing of concentrated solutions of peroxynitrite (10 mM final concentration) and bicarbonate (50 mM final concentration) in 0.5 M phosphate buffer at pH 6.9 led to the detection of a one-line EPR spectrum (Fig. 1A) that was not detectable after stopping the flow (Fig. 1B). Also, no signal was detected in fast flow mixtures of the buffer with either bicarbonate (Fig. 1C) or peroxynitrite alone (Fig. 1D), indicating that the radical is produced from the reaction between peroxynitrite and carbon dioxide. Accordingly, the EPR signal varied significantly with the pH of the mixtures, and the intensities were maximum around pH 6.4 (Fig. 2). This value is similar to those obtained for maximum decomposition rates of peroxynitrite in the presence of carbon dioxide, which was in the pH range of 6.2–6.5 (7, 9). These values are consistent with a rate-limiting reaction between the peroxynitrite anion ($pK_a = 10803$)

FIG. 1. EPR continuous flow spectra of the carbonate radical produced from mixing peroxynitrite and bicarbonate solutions at room temperature. A, peroxynitrite (20 mM) and bicarbonate (100 mM) solutions in 1 M phosphate buffer, pH 6.9; B, same as A but with the spectrum scanned after stopping the flow; C, peroxynitrite (20 mM) mixed with 1 M phosphate buffer, pH 7.1; D, bicarbonate (100 mM) mixed with water, pH 12. The spectra were recorded 15 ms after mixing (flow of 7 ml/min) as described under “Experimental Procedures” except for the spectrum (B) that was scanned immediately after stopping the flow. The final concentrations of peroxynitrite, bicarbonate, and phosphate buffer were 10, 50, and 500 mM; the final pH was 6.9. Instrumental conditions: microwave power, 2 mW; modulation amplitude, 5G; time constant 0.16 s; scan rate 0.84 G/s. Each spectrum is the accumulation of two scans.

FIG. 2. EPR one-line spectra of peroxynitrite and bicarbonate solutions at room temperature. A, peroxynitrite (20 mM) and bicarbonate (100 mM) solutions in 1 M phosphate buffer, pH 6.9; B, same as A but with the spectrum scanned after stopping the flow; C, peroxynitrite (20 mM) mixed with 1 M phosphate buffer, pH 7.1; D, bicarbonate (100 mM) mixed with water, pH 12. The spectra were recorded 15 ms after mixing (flow of 7 ml/min) as described under “Experimental Procedures” except for the spectrum (B) that was scanned immediately after stopping the flow. The final concentrations of peroxynitrite, bicarbonate, and phosphate buffer were 10, 50, and 500 mM; the final pH was 6.9. Instrumental conditions: microwave power, 2 mW; modulation amplitude, 5G; time constant 0.16 s; scan rate 0.84 G/s. Each spectrum is the accumulation of two scans.
6.8) (3) and carbon dioxide ($pK_a$ apparent = 6.4) (28) to produce the nitrosoperoxocarboxylate adduct whose homolysis would generate the carbonate radical (Scheme 1A).

To better characterize the radical detected during fast flow mixing of the peroxynitrite and bicarbonate solutions, we tried to increase the detectable radical concentrations under our instrumental conditions. As expected, the EPR signal shown in Fig. 1 increased with the flow in the 4–8 ml/min range, but the available instrumentation only permitted flows of up to 8 ml/min. The EPR signal did not change significantly when the buffer solutions were purged free of oxygen but grew when the concentrations of both peroxynitrite or bicarbonate were increased. Consequently, to obtain higher radical concentrations, we took advantage of the relatively slow carbon dioxide hydration-dehydration equilibrium as compared with peroxynitrite protonation and decay to nitrate (Scheme 1B) (7). For this purpose, solutions of 100 mM bicarbonate in 0.1 M phosphate buffer, pH 6.4, were fast mixed with alkaline solutions of 40 mM peroxynitrite to give solutions of pH 8.4 whose transient carbonate radical anion was assumed to decay via three major pathways, including $O_2^*$ (reaction 7), dismutation (reaction 8), and oxidation of residual nitrite to nitrate (Scheme 1B). In this case, the singlet signal of the unlabeled carbonate radical (Fig. 3A) because of the contribution of the $^{13}C$ atom ($I = 1/2$). The obtained hyperfine splitting constant of $^{13}C$ is 11.7 G (Fig. 1B), a value in excellent agreement with those previously reported as experimental (Table I) (29, 30) and calculated values (11 G) (31) for the labeled carbonate radical.

The concentrations of the carbonate radical were estimated to be about $3 \times 10^{-6}$ M under nonequilibrium conditions at pH 6.9 (Fig. 1A) and $5 \times 10^{-6}$ M under nonequilibrium conditions at pH 8.4 (Fig. 3A). These values should be taken as rough estimates, because the experiments were run close to the detection limits of the instrument, and EPR itself presents inherent quantification problems (32). Still, the obtained values are of the same order of magnitude as those calculated by computer simulation considering reactions 2–12 (Table II) in which the decomposition of nitrosoperoxocarboxylate produces the carbonate radical anion with 35% yield (reaction 5a, Table II) and with $k_f$ assumed to be $1 \times 10^8$ s$^{-1}$ (7, 17). In this scheme (Table II), the carbonate radical anion was assumed to decay via three major pathways, including $O_2^*$ transfer to nitrogen dioxide (reaction 7), dismutation (reaction 8), and oxidation of residual nitrite to nitrate (Scheme 1B).

**Table I**

| Media            | $g$ value | Line width | $a(^{13}C)$ Reference |
|------------------|-----------|------------|-----------------------|
| KCl matrix       | 2.0113    | 4.4        | 11.2                  | 29 |
| KBr matrix       | 2.0021    | 4.8        | 11.3                  | 29 |
| (KHCO$_3$)$_2$ crystal | 2.0066*   | 5.5        | 11.7                  | 30 |
| Aqueous solutions| 2.0113    | 5.5        | 11.7                  | This work |

* Principal $g$ value ($g_2$).

**Fig. 2.** Representative plot of the effects of pH on the yield of the carbonate radical anion produced in fast flow mixtures of peroxynitrite and bicarbonate. The yields of the radical were expressed as the peak height of the EPR signal in arbitrary units (a.u.). Final concentrations of peroxynitrite, bicarbonate and phosphate buffer were 10, 50, and 500 mM, respectively. Bicarbonate solutions were prepared in 1 M phosphate buffer of various pHs, and the pH was checked for each mixture before and after mixing with peroxynitrite solutions (pH = 12). The changes in pH before and after mixing were ±0.2 pH units, and the average pH values were used in the plot. The spectra were recorded 15 ms after mixing (flow of 7 ml/min) as described under “Experimental Procedures” with the same instrumental conditions as shown in Fig. 1. The data represent a series of experiments obtained on the same day with the same peroxynitrite preparation. The same profile was obtained in a different set of experiments; the data were not presented as the average ± S.D. because of the difficulties in obtaining exactly the same final pH values in different sets of experiments.

**Fig. 3.** EPR continuous flow spectra of the carbonate radical produced from mixing peroxynitrite and bicarbonate solutions at room temperature under nonequilibrium conditions. A, peroxynitrite (40 mM in water, pH = 12) and bicarbonate (100 mM) solutions in 0.1 M phosphate buffer, pH 6.4; B, same as A but with bicarbonate labeled with $^{13}C$. The spectra were recorded 15 ms after mixing (flow of 7 ml/min) as described under “Experimental Procedures.” The final concentrations of peroxynitrite, bicarbonate, and phosphate buffer were 20, 50, and 50 mM; the final pH was 8.4. Instrumental conditions: microwave power, 2 mW; modulation amplitude, 5G; time constant, 0.16 s; scan rate, 0.84 G/s. Each spectrum is the accumulation of two scans.

**Fig. 4.** EPR continuous flow spectra of the carbonate radical produced from mixing peroxynitrite and bicarbonate solutions at room temperature under nonequilibrium conditions. A, peroxynitrite (40 mM in water, pH = 12) and bicarbonate (100 mM) solutions in 0.1 M phosphate buffer, pH 6.4; B, same as A but with bicarbonate labeled with $^{13}C$. The spectra were recorded 15 ms after mixing (flow of 7 ml/min) as described under “Experimental Procedures.” The final concentrations of peroxynitrite, bicarbonate, and phosphate buffer were 20, 50, and 50 mM; the final pH was 8.4. Instrumental conditions: microwave power, 2 mW; modulation amplitude, 5G; time constant, 0.16 s; scan rate, 0.84 G/s. Each spectrum is the accumulation of two scans.
nitrite (reaction 9) (33–35). Under the same conditions as shown in Fig. 1A, the simulation showed that the transient concentration of the carbonate radical anion is 6 μM at 15 ms (Fig. 4).

It is important to note that the one-line signal shown in Fig. 3A did not visibly split when the pH was changed over the 6–9 range or by the use of modulation amplitudes as low as 1 G (data not shown). This indicates that either the proton hyperfine splitting constant of the carbonate radical is lower than 1 G or that the species is not protonated (see Reaction 11) over the studied pH range of 6–9. Although the literature contains conflicting evidence regarding the protonated state of the carbonate radical over the 6–12 pH range (36, 37), recent studies by time resolved RAMAN spectroscopy have found no evidence for its protonation at pHs between 7.5 and 12.3 (38). These studies are in agreement with our EPR data indicating that the radical is not protonated at physiological pHs.

\[
\text{CO}_3^- + \text{H}^+ \rightarrow \text{HCO}_3^- \\
\text{REACTION 11}
\]

DISCUSSION

A role for the carbonate radical in peroxynitrite-mediated processes was first suggested by Radi and co-workers (39), who demonstrated that bicarbonate greatly increased luminol chemiluminescence triggered by peroxynitrite. The direct reaction between peroxynitrite and carbon dioxide was then characterized by Lyman and Hurst (7). That this reaction should be a significant fraction of the biological reactivity of peroxynitrite and that it produces reactive oxidizing and nitrating species is currently supported by the work of many investigators (8–13). The chemical nature of these reactive intermediates has also been proposed, but this is the first report presenting direct spectroscopic evidence for one of these intermediates, the carbonate radical (Figs 1 and 3, Table I). The proposed mechanisms of carbonate radical formation (reactions 3 and 5a) and consumption (reactions 7–9) (Fig. 4; Table II) are consistent with the carbonate radical levels (~3–5 × 10^{-6} M) quantified experimentally under equilibrium and nonequilibrium conditions.

Our results confirmed that the carbonate radical is not protonated at physiological pHs (Figs. 1–3) (38). As discussed by Bisby et al. (38), these results indicate that the carbonate radical anion is slightly more oxidizing in neutral solutions (E° = 1.80 V) than previously assumed considering a pK_a of 9.6 (E° = 1.64 V) (38). Although local concentrations of carbon dioxide and biological targets will determine the decomposition pathway of peroxynitrite that might be formed in a particular physiological compartment, a significant fraction of peroxynitrite (35%) will produce reactive free radicals such as nitrogen dioxide and carbonate radical anion (Scheme 1A). Formation of hydroxyl radical is also possible (Scheme 1B) (19, 20) but unlikely to be significant under physiological conditions, because the rate of peroxynitrite homolysis is too slow to efficiently compete with peroxynitrite attacking the many available biological targets (5, 13). Both the hydroxyl radical and the carbonate radical anion can oxidize substrates by hydrogen atom abstraction, but in the case of the hydroxyl radical, the faster addition reactions would be likely to predominate in most conditions (40). This can explain why peroxynitrite-mediated nitration of aromatic amino acids is increased in the presence of carbon dioxide (5, 13).

Direct EPR detection of the carbonate radical anion is important to unravel mechanistic details of oxidative damage inflicted not only by peroxynitrite but also by other oxidizing species such as hydrogen peroxide and the hydroxyl radical. On the one hand, normal plasma bicarbonate and carbon dioxide concentrations are 25 and 1.3 mM, but higher levels could be achieved during pathologic events such as respiratory distress syndrome or ischemia reperfusion (41). On the other hand, the carbonate radical has been proposed to be responsible for the effects of bicarbonate increasing photodamage to erythrocytes (42) and the bactericidal activity of the hydroxyl radical (5, 23). The carbonate radical, however, has never been characterized in these systems. In a recent paper published by Hurst and co-workers (23), a role for the carbonate radical in the bactericidal effects of the hydroxyl radical (5, 13). Both the hydroxyl radical and the carbonate radical anion can oxidize substrates by hydrogen atom abstraction, but in the case of the hydroxyl radical, the faster addition reactions would be likely to predominate in most conditions (40). This can explain why peroxynitrite-mediated nitration of aromatic amino acids is increased in the presence of carbon dioxide (5, 13).


table

| Reaction | Constant | Reference |
|----------|----------|-----------|
| 1. ONOO^− + H^+ \rightarrow ONOOH | pK_a = 6.8 | 3 |
| 2. ONOO^− + CO_2 \rightarrow ONOOOCO_2^- | k = 3 × 10^9 M^{-1} s^{-1} | 7 |
| 3. ONOO^- \rightarrow NO_3^- + H^+ | k_a = 1.3 s^{-1} | 14 |
| 4. NO_2^- + NO_3^- \rightarrow ONOO^- | k = 0.35 k_a | 7, 17 |
| 5a. ONOOOCO_2^- \rightarrow CO_2^- + NO_3^- | k = 0.65 k_a | 7, 17 |
| 5b. ONOO^- + CO_2 \rightarrow CO_2^- + NO_3^- | pK_a = 6.4 | 26 |
| 6. CO_2 + H_2O \rightarrow HCO_3^- + H^+ | h = 1 × 10^9 M^{-1} s^{-1} | 33 |
| 7. CO_2^- + NO_3^- \rightarrow CO_3^- + NO_2^- | h = 1 × 10^9 M^{-1} s^{-1} | 34 |
| 8. 2 CO_3^- \rightarrow products | h = 4 × 10^8 M^{-1} s^{-1} | 33 |
| 9. CO_2^- + NO_3^- + H_2O \rightarrow HCO_3^- + NO_2^- + OH^- | k = 4.7 × 10^7 M^{-1} s^{-1} | 35 |
| 10. 2 NO_2^- + H_2O \rightarrow NO_3^- + NO_2^- + 2 H^+ | | |
whereas in the carbonate radical, most of the electron density is on the oxygen atoms (30, 38), and adducts of these radicals with DMPO are unlikely to have similar EPR parameters. In addition, to the best of our knowledge, nobody has been able to trap the carbonate radical with DMPO. There is a report of trapping of the carbonate radical with nitromethane by irradiation at alkaline pHs, and as expected, the nitromethane-carbonate radical adduct has EPR parameters similar to those of the hydroxyl radical (22), an oxygen-centered radical. The carbonate radical adduct has EPR parameters similar to those of the nitromethane-carbonate radical adduct (Reactions 12–14). In our experience, it is common to detect DMPO-carbon dioxide radical adduct from air-equilibrated buffer solutions in the presence of reducing agents such as hydrazine (43).

\[
\text{CO}_2 + \text{H}^+ \rightarrow \text{CO}_2^+ + \text{H}^+ \\
\text{CO}_2^+ + \text{DMPO} \rightarrow \text{DMPO} - \text{CO}_2^2 \\
\text{H}^+ + \text{DMPO} \rightarrow \text{DMPO} - \text{H}^+ 
\]

**Reactions 12–14**

In summary, our results represent the first detection of the carbonate radical anion in aqueous solutions at physiological pHs. Detection and characterization of the radical as negatively charged at neutral pHs should contribute to the understanding of the roles of ubiquitous carbon dioxide in modulating the pathogenic mechanisms of peroxynitrite and other oxidizing intermediates.

**REFERENCES**

1. Huie, R. E., and Padmaja, S. (1993) *Free Radic. Res. Commun.* 18, 195–199
2. Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. (1997) *Chem. Res. Toxicol.* 10, 1285–1292
3. Beckman, J. S., Beckman, T. W., Marshall, P. A., and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 1620–1625
4. Beckman, J. S. (1996) *Chem. Res. Toxicol.* 9, 836–844
5. Lymar, S. V., and Hurst, J. K. (1996) *Chem. Res. Toxicol.* 9, 845–850
6. Augusto, O., Linares, E., and Giorgio, S. (1996) *Braz. J. Med. Biol. Res.* 29, 853–862
7. Lymar, S. V., and Hurst, J. K. (1995) *J. Am. Chem. Soc.* 117, 8867–8868
8. Uppa, R. M., Squadrito, G. L., and Pryor, W. A. (1996) *Arch. Biochem. Biophys.* 327, 335–343
9. Dencila, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996) *Arch. Biochem. Biophys.* 333, 40–58
10. Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996) *Biochemistry* 35, 7855–7861
11. Lymar, S. V., and Hurst, J. K. (1998) *Inorg. Chem.* 27, 394–301
12. Goldstein, S., and Czapski, G. (1998) *J. Am. Chem. Soc.* 120, 3458–3463
13. Squadrito, G. L., and Pryor, W. (1998) *Free Radic. Biol. Med.* 25, 392–403
14. Koppenol, W. H., Moreno, J. J., Pryor, W., Ischiropoulos, A. H., and Beckman, J. S. (1992) *Chem. Res. Toxicol.* 5, 834–842
15. Augusto, O., Gatti, R. M., and Radi, R. (1994) *Arch. Biochem. Biophys.* 510, 118–125
16. Pryor, W. A., and Squadrito, G. L. (1995) *Am. J. Physiol.* 1069–1722
17. Merényi, G., and Lind, J. (1997) *Chem. Res. Toxicol.* 10, 1216–1220
18. Merényi, G., Lind, J., Golstein, S., and Czapski, G. (1998) *Chem. Res. Toxicol.* 11, 712–713
19. Gatti, R. M., Alvarez, B., Vasquez-Vivar, J., Radi, R., and Augusto, O. (1998) *Arch. Biochem. Biophys.* 349, 36–46
20. Richeson, C. E., Mulder, P., Bowry, V. W., and Ingold, K. U. (1988) *J. Am. Chem. Soc.* 10, 2711–2719
21. Buettner, G. R., and Mason, R. P. (1990) *Methods Enzymol.* 186, 127–133
22. Behar, D., and Peschenda, R. W. (1972) *J. Chem. Phys.* 67, 1710–1721
23. Woelct, R. G., Franks, B. S., Hannum, D. M., and Hurst, J. K. (1994) *J. Biol. Chem.* 269, 9721–9728
24. Saha, A., Golstein, S., Cabelli, D., and Czapski, G. (1996) *Free Radic. Biol. Med.* 24, 653–659
25. Mehllhorn, R. J., and Keith, A. D. (1972) in *Membrane Molecular Biology* (Fox, C. F., and Keith, A. D., eds) pp. 392–403, Sinauer Association, Stamford, CT
26. Mendes, P. (1993) *Comput. Appl. Biosci.* 9, 563–571
27. Mendes, P. (1997) *Trends Biochem. Sci.* 22, 361–363
28. Dean, J. A. (1979) in *Lange’s Handbook of Chemistry* (Bors, W., Saran, M., and Tait, D., eds) pp. 92–121, McGraw-Hill Book Co., New York
29. Hisatune, I. C., Adl, T., Beahm, E. C., and Kempf, R. J. (1970) *J. Phys. Chem.* 74, 3225–3231
30. Chantry, G. W., Horsfield, A., Morton, J., and Whiffin, D. H. (1962) *Mol. Phys.* 5, 569–599
31. Symons, M. (1987) in *Chemical and Biochemical Aspects of Electron Spin Resonance*, pp. 16–25, John Wiley & Sons, Inc., New York
32. Wertz, J. E., and Bolton, J. R. (1972) in *Electron Spin Resonance: Elementary Theory and Applications*, pp. 450–457, McGraw-Hill Book Co., New York
33. Liiie, J., Hanrahan, R. J., and Henglein, A. (1987) *Radiat. Phys. Chem.* 11, 225–227
34. Simic, M. G., and Hunter, E. P. L. (1984) in *Oxygen Radicals Chemistry and Biology* (Bors, W., Saran, M., and Tait, D., eds) pp. 109–121, Walter de Gruyter & Co., Berlin
35. Treinin, A., and Hayen, E. (1976) *J. Am. Chem. Soc.* 92, 5821–5828
36. Chen, S., and Hoffman, M. Z. (1973) *J. Phys. Chem.* 77, 1111–1116
37. Eriksson, T. E., Lind, J., and Merényi, G. (1985) *Radiat. Phys. Chem.* 26, 197–199
38. Bihary, R. H., Johnson, S. A., Parker, A. W., and Tavender, S. M. (1998) *J. Chem. Soc. Faraday Trans.* 94, 2069–2072
39. Radi, R., Cosgrove, T. P., Beckman, J. S., and Freeman, B. A. (1993) *Biochem. J.* 290, 51–57
40. Pryor, W. A. (1988) *Free Radic. Biol. Med.* 4, 219–223
41. Levinson, N. G. (1994) in *Harrison’s Principles of Internal Medicine* (Harrison, T. R., Isselbacher, K. J., Braunwald, E., Wilson, J. D., Martin, J. B., Fawcett, A. S., Petersen, R. G., eds) pp. 253–262, 13th Ed., McGraw-Hill, Inc., New York
42. Michelon, A. M., and Maral, J. (1983) *Biochimie (Paris)* 65, 95–104
43. Gomes, L. F. (1996) *The Role of Carbon-centered Radicals on Hydrazine Toxicity*. Ph.D. thesis, Instituto de Quimica, Universidade de Sao Paulo