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BIOLOGICAL REDUCTION OF AROMATIC NITROSO COMPOUNDS: EVIDENCE FOR THE INVOLVEMENT OF SUPEROXIDE ANIONS

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The *in vitro* formation of phenylhydronitroxide and 2-methylphenylhydronitroxide free radicals from nitrosobenzene (NB) and 2-nitrosotoluene (NT), respectively, in either red blood cells (RBC) or RBC hemolysates, was confirmed by electron spin resonance spectroscopy (ESR). Free radicals were generated nonenzymatically from reaction of the respective nitroso compounds with a number of biological reducing agents as corroborated by model studies of NB or NT with NAD(P)H. Under aerobic conditions, phenylhydronitroxide and 2-methylphenylhydronitroxide underwent a subsequent one-electron transfer to oxygen, which then resulted in the formation of superoxide anion (O_2^-). The latter product was confirmed by the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c (cyt c). Apparently, oxygen is needed for continuous formation of the hydronitroxide radical derivatives. On the other hand, under anaerobic conditions, no phenylhydronitroxide radical was generated from NB in the presence of NADH, but the formation of phenylhydroxylamine from NB was detected by the absorption spectrometry. These results suggest that oxygen is a preferential electron acceptor for hydronitroxide radical derivatives.

KEY WORDS: 2-nitrosotoluene, nitrosobenzene, hydronitroxide radical, superoxide radical, superoxide dismutase (SOD), ESR.

Abbreviations: NB, nitrosobenzene; NT, nitrosotoluene; RBC, red blood cells; RNO nitroso compound; RNHO·, hydronitroxide free radical derivative; RNH(OH), hydroxylamine derivative; cyt c, cytochrome c; DCIP, 2,6-dichlorophenol-indophenol; SOD, superoxide dismutase.

INTRODUCTION

Nitroso compounds are among the most potent chemical carcinogens and are widely distributed in the environment. These compounds are also recognized as potentially reactive metabolites of a variety of toxicologically interesting compounds which, after covalently binding to proteins and DNA, become carcinogenic and/or mutagenic.[1,2] Many nitroso compounds are reduced nonenzymatically by several natural products, such as NAD(P)H, glutathione, cysteine and hemoglobin with concomitant formation of free radical derivatives, as confirmed by their corresponding

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ESR spectra.[3,4] For example, nitrosobenzene was reported to be reduced to phenylhydronitroxide radicals by a one-electron reduction with NADPH, as confirmed by ESR.[4] However, there have been no reports to date of ESR detection of phenylhydronitroxide free radicals in blood or *in vivo* systems.[5-8]

In this report, we show ESR evidence that when several nitroso compounds are mixed with blood, the corresponding hydronitroxide free radicals are formed, followed by generation of superoxide anion radicals (O_2^-).

MATERIALS AND METHODS

Materials: Nitrosobenzene (NB), 2-nitrosotoluene (NT) and 1-nitroso-2-naphthol-3,6-disulfonic acid disodium salt were purchased from Aldrich Chem. Inc., and NADH and NADPH from Oriental Yeast, Tokyo. Superoxide dismutase (SOD), cytochrome c (type VI, from horse heart; cyt c) and glucose oxidase were purchased from Sigma. All other reagents were of analytical grade.

Biological samples: Red blood cells (RBC) were collected by centrifugation of heparinized human blood; the neutrophil-rich, buffy-coat layer was centrifuged out in dextran.[9]

Methods: ESR spectra were recorded in a JEOL (JES-FE) X-band ESR spectrometer. Typical conditions were follows; microwave power, 5 mW; modulation amplitude, 0.1 gauss at 100 KHz; response, 0.1 s; sweep time, 4 min. All nitroso compounds were dissolved in 50% ethanol. In the RBC experiments, 10 μl of either 30 mM nitrosobenzene, 2-nitrosotoluene or 1-nitroso-2-naphthol-3,6-disulfonic acid disodium salt, in 50% ethanol were added to 100 μl of a suspension of RBC in Krebs-Ringer phosphate buffer [KRP: 122 mM NaCl, 4.9 mM KCl, 1.2 mM $MgCl_2$, 50 mM phosphate buffer (pH 7.4)]. After mixing, aliquots of the mixture transferred to a glass capillary (25 μl volume) were examined. A hemolysate was prepared from RBC by repeated freeze-thawing, followed by a 100-fold dilution with 50 mM phosphate buffer, pH 7.4. The absorption spectra were measured by Hitachi 557 spectrophotometer.

O_2^- detection: O_2^- generation was assayed by measuring the SOD-inhibitable reduction of cyt c on a Hitachi 556 dual-wavelength spectrophotometer. The reaction mixture contained 30 μM cyt c, 30 μM NADH and 30 μM NB (or NT) in 50 mM phosphate buffer (pH 7.0). Direct reduction of cyt c under anaerobic conditions was measured as previously reported.[10]

Oxygen consumption: Oxygen concentration was measured with a Clark-type oxygen electrode (OBH-100, Otsuka Electronics Co., Ltd.) at 30°C.

RESULTS

Reduction of Nitroso Compounds in Blood

We studied the chemical fate of nitroso compounds with blood components. The nitroso compounds, nitrosobenzene (NB), 2-nitrosotoluene (NT) and 1-nitroso-2-

naphthol-3,6-disulfonic acid disodium salt, were mixed with RBC or hemolysate, and ESR spectra of these mixtures were measured. Obtained ESR spectra for the hemolysate system are shown in Figure 1. The identical ESR spectra were also observed in RBC mixed with nitroso compounds used in Figure 1.

In control experiments, however, RBC, hemolysates, or nitroso compounds alone did not produce an ESR signal. In all cases in Figure 1, complex multi-line free radical spectra were observed, which were especially pronounced in the case of NB and NT. These spectra remained stable for relatively long periods (20 to 60 min) depending on the relative amounts of nitroso compound and hemolysate (or RBC).

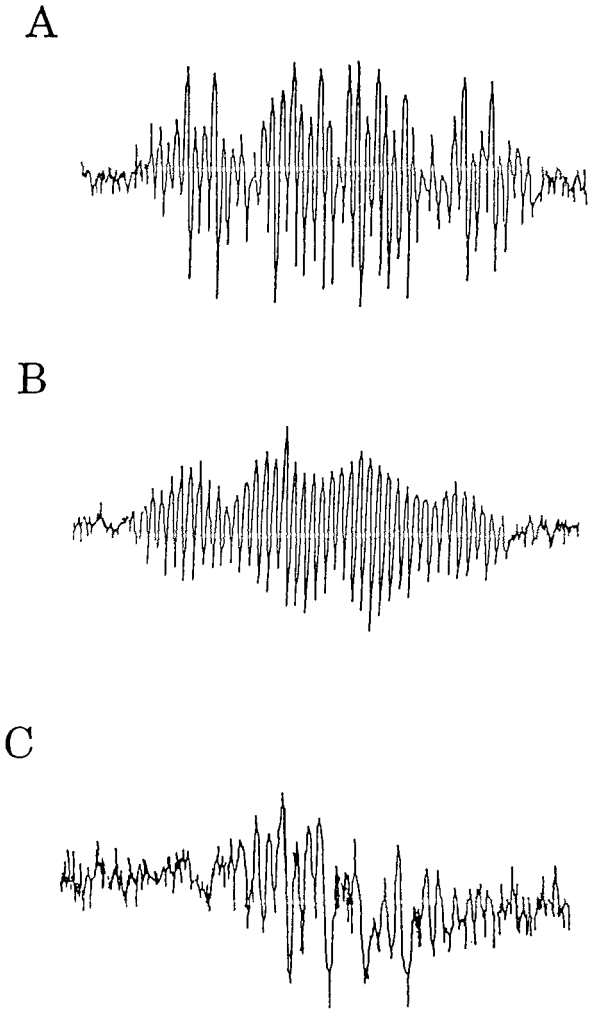

FIGURE 1 Typical X-band ESR spectra obtained from incubating RBC hemolysates with 3 mM (A) nitrosobenzene, (B) 2-nitrosotoluene, or (C) 1-nitroso-2-naphthol-3,6-disulfonic acid disodium salts. Instrumental conditions were: microwave power, 5 mW; modulation amplitude, 0.1 gauss; time constant, 0.1 s; scan rate, 50 gauss/min. Note that the receiver gain in C is five-fold higher than those in A and B.

For example, ESR signals under the conditions of Figure 1 lasted for at least 30 min. However, in the case of 1-nitroso-2-naphthol-3,6-disulfonic acid disodium salt, the steady-state free radical concentration was much less than those of NB and NT, as evidenced by the much weaker ESR signal. When the same experiment (Figure 1) was carried out using heat-treated hemolysate (5 min at 100°C) with NB or NT, identical ESR spectra were observed, suggesting that ESR signals in Figure 1 were generated by a nonenzymatic reaction of nitroso compounds with low MW substances in the hemolysate.

These nitroso compounds are known to undergo one-electron reduction by electrochemical treatment[11] or via reaction with reducing agents,[4] such as ascorbic acid and NAD(P)H. Therefore, we studied reactions of NB and NT with NADH, NADPH, and ascorbic acid, in both the absence and presence of hemolysate. The ESR spectra obtained for the NB/NADH or NT/NADH system in the absence of hemolysate were identical to those in the presence of hemolysate (Figure 2). Both the measured (Figures 2A and B) and simulated spectra (Figures 2C and D) are in good agreement, suggesting that both NB and NT convert to the corresponding hydronitroxide free radical with NADH nonenzymatically. The hyperfine coupling constants used for the simulated hydronitroxide free radical spectra in Figure 2C, D agreed with those in previous report.[11] The generation of phenylhydronitroxide from NB and other reducing agents, such as NADPH and ascorbic acid, were also confirmed by ESR. The life time of the radical generated in these systems (30–60 min) was the same as that in the NB/NADH system.

Non-biological Model Reduction System

In non-biological model system of NB or NT with NADH, we measured the steady-state concentrations of the corresponding hydronitroxide free radical generated in the range of pH 3 to 11 by ESR. Its concentration was unchanged over the pH range of 3 to 11 (data not shown). In another approach, the steady-state production of corresponding hydronitroxide radicals was measured under anaerobic conditions. After generating phenylhydronitroxide radicals from the NB/NADH system in phosphate buffer containing 20 mM glucose (Figure 3A), glucose oxidase was added and ESR spectra were again recorded. The ESR signal disappeared within 30 s after addition of glucose oxidase (Figure 3B), suggesting that oxygen is necessary for the continuous generation of phenylhydronitroxide radicals and that phenylhydronitroxide radicals have converted to diamagnetic forms. Also in the hemolysate or RBC mixed with NB, no ESR signal was detected under anaerobic conditions. In contrast to these results, Takahashi *et al.*[4] reported the generation of phenylhydronitroxide radicals by reacting NB with NADPH which were purged with nitrogen to remove oxygen. Only difference between the both experiments might be a possibility of residual oxygen in the system: A trace of oxygen still might exist in the system purged with nitrogen. When oxygen concentration was less than 1–5 μM in our system, no ESR signal was observed, suggesting that most of phenylhydronitroxide radical converted to a diamagnetic substance after consumption of the trace oxygen. From these results, in aerobiosis, phenylhydronitroxide radicals seem to react with oxygen, that is, consume oxygen. The same results were also obtained in the NT/NADH system.

In order to prove if oxygen is consumed along with the generation of the hydronitroxide radical derivatives, we measured the oxygen concentration in the NB/NADH system with time. The oxygen in the system was found to be depleted as

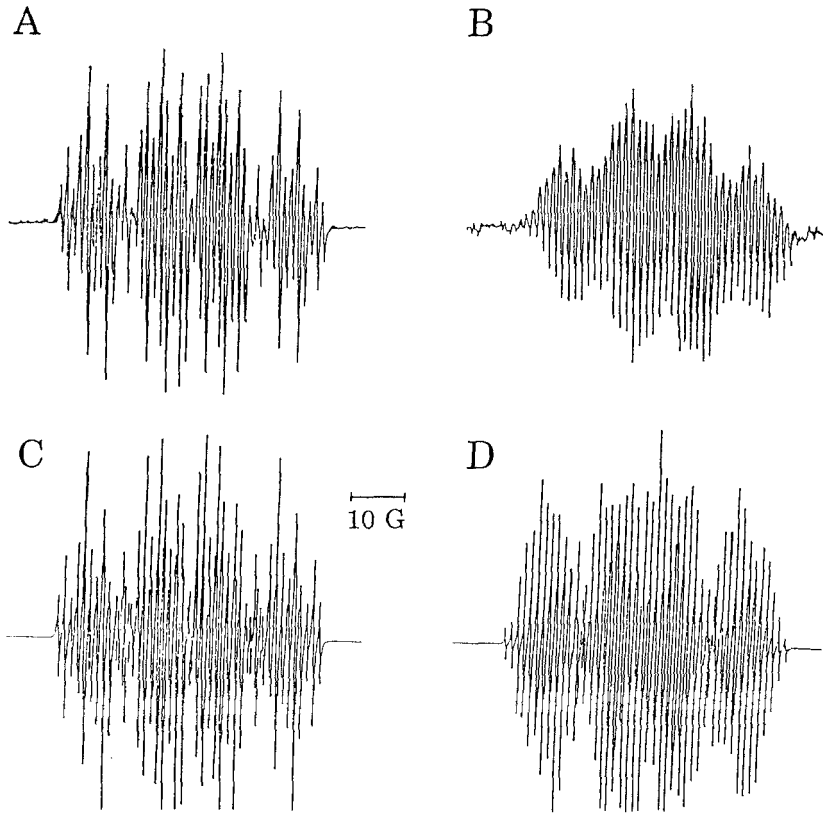

FIGURE 2 Typical X-band ESR spectra resulting from incubating 1 mM NADH with 2 mM (A) nitrosobenzene, or (B) 2-nitrosotoluene, and computer-simulated spectra for (C) nitrosobenzene and (D) 2-nitrosotoluene hydronitroxide radicals. The computer simulations used a 0.1 gauss linewidth. The instrumental conditions were identical to those in Figure 1.

phenylhydronitroxide radicals were generated, and the rate of oxygen consumption increased with increasing concentration of NADH. The question then arises as to how oxygen is reduced, i.e. conversion to superoxide anion or to hydrogen peroxide by a one-electron or two-electron reduction mechanism, respectively. It is most likely that these free radicals transfer spontaneously one electron to oxygen, followed by the generation of superoxide anions.[12] In order to confirm this mechanism, the SOD-inhibitable reduction of cyt c was measured in the NB/NADH system to see whether O_2^- was formed. As shown in Figure 4, the reduction of cyt c was completely inhibited by SOD (15 units/ml), which indicates that O_2^- is formed in the NB/NADH system along with the generation of phenylhydronitroxide radicals. When 2,6-dichlorophenolindophenol (DCIP) was substituted instead for cyt c, its reduction was also completely inhibited by SOD. These results suggest that O_2^- is generated by a direct one-electron transfer from phenylhydronitroxide radicals to the molecular oxygen. On the other hand, the reduction of cyt c was measured in the NB/NADH system under anaerobic conditions (Figure 4C). Direct reduction of cyt c took place under anaerobic conditions, but its rate was 6 to 7

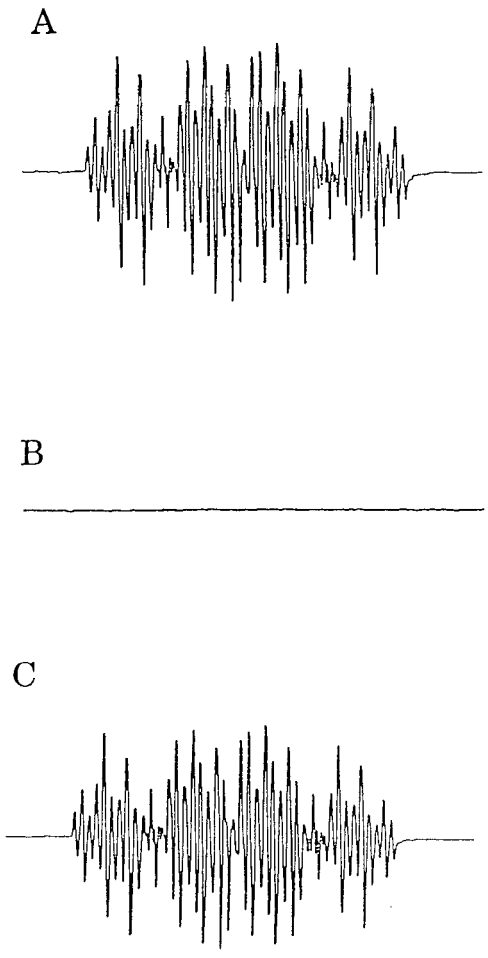

FIGURE 3 Effects of glucose oxidase on the nitrosobenzene/NADH model system before (A) and after (B) its addition. (A) 2 mM nitrosobenzene was reacted with 1 mM NADH at 25°C in 50 mM phosphate buffer containing 20 mM glucose. (B) Glucose oxidase (40 U/ml) was added to the reaction mixture (A). (C) Control experiment: In the absence of glucose oxidase, ESR spectrum was recorded 10 min after the reaction in (A).

times slower than that in aerobiosis. No ESR signal was detected from the NB/NADH system either in the presence or absence of cyt c under anaerobic conditions.

In both aerobiosis and anaerobiosis, the absorption spectra of the NB/NADH system were measured with time. Under aerobic conditions, the absorbance peak at 307 nm of NB decreased with time, and no new peak appeared during ESR spectra of phenylhydronitroxide radicals were detectable (until 30–60 min after addition of NADH to NB). Under anaerobic conditions, however, the peak of NB disappeared within 30 s after addition of NADH, instead a new peak at 233 nm, which is due to phenylhydroxylamine, appeared. This indicates that under anaerobic conditions NB is directly reduced to phenylhydroxylamine, consequently no ESR signal

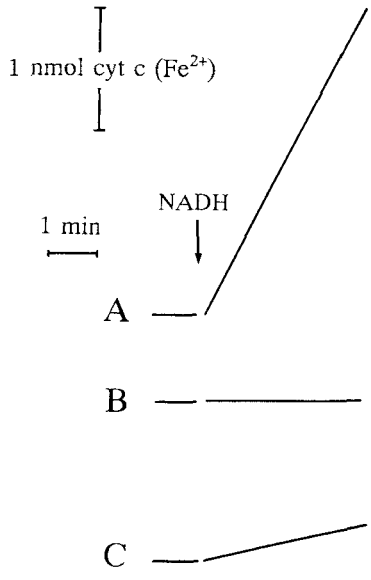

FIGURE 4 Reduction of cyt c in the NB/NADH system in the absence (A) and presence (B) of SOD in aerobiosis and anaerobiosis (C). (A) 30 μM NADH was added to the reaction mixture containing 30 μM cyt c in 50 mM phosphate buffer (pH 7.0) in aerobiosis. (B) Same as (A), except 15 U/ml SOD was present. (C) Same as (A), except in anaerobiosis. Glucose oxidase (40 U/ml) and 20 mM glucose were added to the reaction mixture in (A).

was observed in anaerobiosis. From these results, the reduction of nitroso compounds under aerobic and anaerobic conditions seems to proceed through different pathways.

DISCUSSION

Under aerobic conditions, phenyl- or 2-methylphenylhydronitroxide free radicals were generated from NB or NT in RBC and blood hemolysates. The generation of these radicals would be due to nonenzymatic reactions with several reducing components, such as NAD(P)H and ascorbic acid, in blood. The physiological concentrations of NAD(P)H,[13] ascorbic acid,[14] and glutathione[15] are reported to be 0.04, 0.02, and 2 μmol per ml of RBC, respectively, which are sufficient to explain nonenzymatic generation of hydronitroxide radical derivatives in the mixture of nitroso compounds and blood shown in Figure 1. Furthermore, as phenylhydronitroxide radicals were generated in the system of NB/NADH, O_2^- was generated, which was confirmed by the SOD-inhibitable reduction of cyt c. Under anaerobic conditions, however, no ESR signal of hydronitroxide derivatives was detected in both biological and non-biological model systems, suggesting that oxygen is required for the steady-state formation of hydronitroxide radical derivatives.

Nitroso compounds can be reduced to the corresponding hydronitroxide radicals via a one-electron reduction or to the corresponding hydroxylamine via two-electron reduction. These pathways are summarized in Figure 5. Under aerobic conditions

(Figure 5A), nitroso compounds (RNO) were reduced to RNHO· by the reductant (AH_2), then the generated RNHO· reacted with oxygen, followed by the generation of O_2^-. Therefore, ESR spectra of RNHO· lasted for a relatively long period (20 to 60 min) after addition of reductant to RNO, until most of oxygen molecules in the system were consumed by RNHO·. This view is supported by the spectral data that the absorption peak due to phenylhydroxylamine (RNH(OH)) did not appear during ESR spectra of RNHO· were detectable in NB/NADH system (at least 30 min). On the other hand, under anaerobic conditions (Figure 5B), RNO seems to be directly reduced to RNH(OH) via two-electron reduction, since no ESR signal of RNHO· was detected and the absorption peak due to RNH(OH) appeared immediately after addition of reductant to RNO. Therefore, no RNHO· is generated as a transient intermediate. Becker and Sternson[16] reported that phenylhydroxylamine was stoichiometrically generated from NB, when NADH is in excess with respect to NB (its molar ratio > 10). However, our results indicate that the generation of phenylhydroxylamine from NB with NADH is also dependent on the oxygen concentration in the NB/NADH system.

It should be noted that immediately after injection of NB to mice muscle, ESR signals of phenylhydronitroxide radicals, which were the same as those observed in RBC and hemolysate systems (Figure 1), were detected in L-band *in vivo* ESR studies. However, these ESR signals rapidly underwent further chemical species, and the broad-line moderately immobilized ESR spectrum was observed.[17] We are in the process of characterizing these free radical species from the points of carcinogenic effects of nitroso compounds.

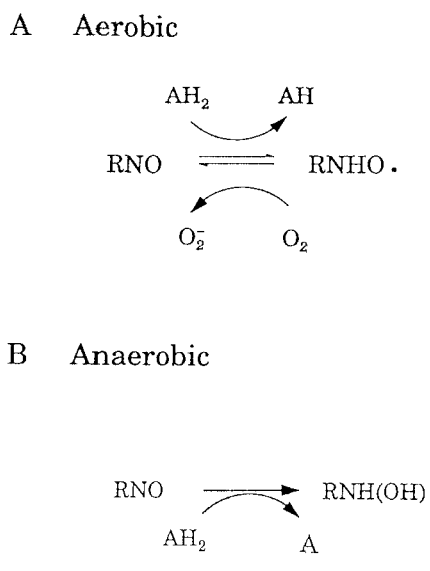

FIGURE 5 Schematic representation of the oxidation/reduction equilibria of nitroso compounds under aerobic (A) and anaerobic conditions (B). Abbreviations used are: RNH(OH), hydroxylamine; RNHO·, hydronitroxide radical derivative; RNO, nitroso compound; AH_2, reductant. Under aerobic conditions (A), RNO is reduced to RNHO· via a one-electron reduction, then RNHO· was reacted with oxygen, following generation of O_2^-. Under anaerobic conditions (B), RNO is directly reduced to RNH(OH) via two-electron reduction, but no RNHO· is generated as an intermediate.

Acknowledgements

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References

1. E.C. Miller, J.A. Miller and H.A. Hartmann (1961) N-Hydroxy-2-acetylaminofluorene with increased carcinogenic activity in the rat. *Cancer Research*, **21**, 815–824.
2. R.M. Hodgson, M. Wiessler and P. Kleihues (1980) Preferential methylation of target organ DNA by the oesophageal carcinogen n-nitrosomethylbenzylamine. *Carcinogenesis*, **1**, 861–865.
3. B. Kalyanaraman, E. Perez-Reyes and R.P. Mason (1979) The reduction of nitroso-spin traps in chemical and biological systems. A cautionary note. *Tetrahedron Letters*, **50**, 4809–4812.
4. N. Takahashi, V. Fischer, J. Schreiber and R.P. Mason (1988) An ESR study of nonenzymatic reactions of nitroso compounds with biological reducing agents. *Free Radical Research Communications*, **4**, 351–358.
5. L.J. Berliner and H. Fujii (1985) Magnetic resonance imaging of biological specimens by electron paramagnetic resonance of nitroxide spin labels. *Science*, **227**, 517–519.
6. L.J. Berliner, H. Fujii, X. Wan and S.J. Lukiewicz (1987) Feasibility study of imaging a living murine tumor by electron paramagnetic resonance. *Magnetic Resonance in Medicine*, **4**, 380–384.
7. L.J. Berliner and J. Koscielniak (1991) L-band microwave bridges. In *EPR imaging and in-vivo ESR*, CRC Press, Fl, pp. 65–72.
8. L.J. Berliner (1991) Applications of *in vivo* ESR. In *EPR imaging and in vivo ESR*, CRC Press, Fl, pp. 291–305.
9. P.D. Morse (1977) Use of spin label tempamine for measuring the internal viscosity of red blood cells. *Biochemical and Biophysical Research Communications*, **77**, 1486–1491.
10. H. Fujii and K. Kakinuma (1991) Electron transfer reactions in the NADPH oxidase system of neutrophils - Involvement of an NADPH-cytochrome c reductase in the oxidase system. *Biochimica et Biophysica Acta*, **1095**, 201–209.
11. D.H. Levy and R.J. Myers (1964) Electron spin resonance spectra of the radical anions of nitrosobenzene and nitrobenzene in liquid ammonia. *Journal of Chemical Physics*, **41**, 3731–3733.
12. V. Berlin and W.A. Haseltine (1981) Reduction of adriamycin to a semiquinone-free radical by NADPH cytochrome P-450 reductase produces DNA cleavage in a reaction mediated by molecular oxygen. *Journal of Biological Chemistry*, **256**, 4747–4756.
13. H. Yoshikawa, M. Nakao, K. Miyamoto, and M. Tachibana (1960) Phosphorus metabolism in human erythrocyte. II. Separation of acid-soluble phosphorus compounds incorporating p. 32 by column chromatography with ion exchange resin. *Journal of Biochemistry*, **47**, 635–641.
14. P. Barkhan and A.N. Howard (1958) Distribution of ascorbic acid in normal and leukaemic human blood. *Biochemical Journal*, **70**, 163–168.
15. E. Beutler (1975) *in* Red Cell Metabolism, 2d ed. Grune & Stratton, New York.
16. A.R. Becker and L.A. Sternson (1980) Nonenzymatic reduction of nitrosobenzene to phenylhydroxylamine by NAD(P)H. *Bioorganic Chemistry*, **9**, 305–312.
17. H. Fujii, B. Zhao, J. Koscielniak and L.J. Berliner (1994) *In vivo* EPR studies of the metabolic fate of nitrosobenzene in the mouse. *Magnetic Resonance in Medicine*, **31**, 77–80.

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