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APPEARANCE OF ESR SIGNALS BY THE REACTION OF 3,5-DIBROMO-4-NITROSOBENZENESULFONATE (DBNBS) AND NON-RADICAL BIOLOGICAL COMPONENTS

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The reaction of 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS) with non-radical biological components produced spin adducts with ESR signals. The reactions of DBNBS with Trp, Gly-Trp, Trp-Gly, Pro, Cys and glutathione at pH 7.5 and room temperature for more than 1 hour gave the nitroxyl free radicals with ESR signals, whereas the reactions with other amino acids and bovine serum albumin did not. Among the amino acids and the peptides, Trp and Trp-containing peptides gave the most intense signals. The reactions of DBNBS with unsaturated fatty acids, *i.e.*, linoleic acid and oleic acid, gave weak ESR signals, whereas the reaction with stearic acid did not. While DBNBS gave no ESR signals by the reactions with DNA, nucleosides and nucleobases, it caused strand breaking in supercoiled DNA. DBNBS also gave ESR signals by the reaction with human plasma similar to those from the reaction with Trp. It was suggested that the nitroxyl free radicals were produced by the addition of DBNBS to the amino acids and unsaturated fatty acids followed by oxidation in the presence of DBNBS. Hence, the use of DBNBS spin trap to detect free radicals in systems containing these biological components after long incubation may give misleading results.

KEY WORDS: Amino acids, 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS), DNA strand breaking, ESR signal, plasma, unsaturated fatty acids.

INTRODUCTION

Spin trapping-ESR is a useful technique to detect and characterize short-lived free radicals. Usually, appearance of ESR signals from spin trapping agents indicates the presence of free radicals which have been trapped by the spin traps, and the ESR spectra can be used for qualitative and quantitative estimation of the free radicals. 3,5-Dibromo-4-nitrosobenzenesulfonate (DBNBS) (Figure 1) is a water-soluble nitroso-aromatic spin trap, which has been introduced as a useful trap for carbon-centered radicals,[1-4] sulfur-centered radicals,[5-7] oxygen-centered radicals[2,8] and inorganic radicals.[9,10] Recent studies by Kalyanaraman *et al.*[12] have shown that the reaction of DBNBS with low density lipoprotein (LDL) gave spin adducts.

In the present investigation, it was found that ESR signals were observed in the reactions of DBNBS with non-radical biological components such as amino acids, peptides and unsaturated fatty acids. Hence, much caution should be paid for the use of DBNBS to detect free radicals in biological systems.

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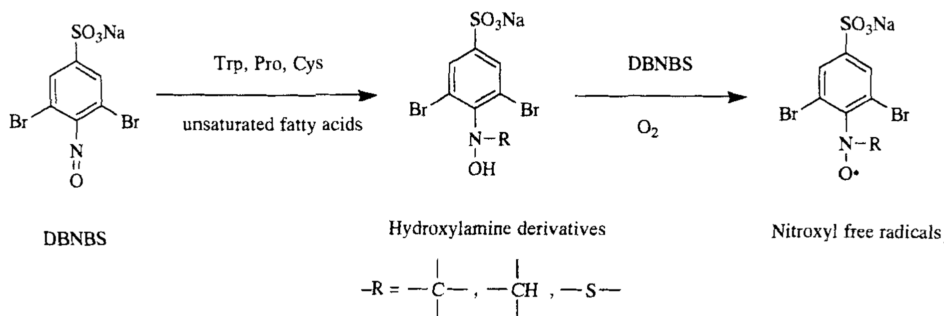

FIGURE 1 Formation of nitroxyl free radicals in the reaction of DBNBS with non-radical biological components.

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MATERIALS AND METHODS

3,5-Dibromo-4-nitrosobenzenesulfonic acid sodium salt hydrate (DBNBS) was obtained from Labotec Company (Tokyo, Japan). Tryptophan (Trp) was obtained from Wako Pure Chemical Industries (Osaka, Japan), Kanto Chemical Company (Tokyo, Japan) and Ajinomoto Company (Tokyo, Japan). Alanine (Ala) and leucine (Leu) were from Nippon Rikagakuyakuhin Kabushikigaisha (Tokyo, Japan). Arginine (Arg), aspartic acid (Asp), cystine (Cys-Cys), glycine (Gly), histidine (His), methionine (Met), phenylalanine (Phe), proline (Pro) and tyrosine (Tyr) were obtained from Wako. Asparagine (Asn), glutamine (Gln), glutamic acid (Glu), isoleucine (Ile), serine (Ser), threonine (Thr) and valine (Val) were from Ajinomoto. Cysteine (Cys) was from Nacalai tesque (Kyoto, Japan). Tryptophanylglycine (Trp-Gly), glycyltryptophan (Gly-Trp), glutathione (GSH) and bovine serum albumin (BSA) were from Sigma Chemical Company (St. Louis, MO, USA). Indole and pyrrolidine were from Nacalai tesque and Kanto Chemical Company, respectively. Linoleic acid and oleic acid were obtained from Nippon Oil and Fats Company (Tokyo, Japan). Stearic acid was from Wako. Human plasma obtained from venous blood collected in acid-citrate-dextrose from a healthy donor was frozen at −30 °C for a year for use. Thymidine was a product of Sigma. Adenine and 2′-deoxyguanosine hydrate was obtained from Wako. Supercoiled plasmid pBR322 DNA (1 mg/ml in 10 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA)) and calf thymus DNA sodium salt were obtained from Takara Shuzo Company (Kyoto, Japan) and Sigma, respectively. Catalase (EC 1.15.1.6) from bovine liver (2000–5000 U/mg protein) and superoxide dismutase (SOD) (EC 1.15.1.1) from bovine erythrocytes (2500–7000 U/mg protein) were the products of Sigma.

ESR Spectra of the Reaction Mixtures of DBNBS with Biological Components

DBNBS (0.1 M) was incubated with 4 mM Tyr, 2.5 mM Cys-Cys, 1% BSA, 1 mg/ml calf thymus DNA and 10 mM other amino acids, peptides, fatty acids, nucleosides and nucleobases in 50 mM phosphate buffer (ph 7.5) at room temperature for 5 h. In the test of plasma, a solution of 0.2 M DBNBS in water was mixed with an equal volume of plasma and the mixture was incubated similarly. For the investigation

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of the effect of ferricyanide, 10 mM potassium ferricyanide was added to the incubation mixture and the mixture was allowed to stand at room temperature for 30 min. ESR spectra were obtained on a Varian E-4 EPR spectrometer in a capillary tube at room temperature. The instrumental conditions were field setting at 338.5 mT, scan range of 10 mT, microwave power at 10 mW and modulation amplitude at 0.1 mT.

DNA Breaking Activity of DBNBS

The mixture of 10 μg/ml supercoiled pBR322 DNA and 10 mM DBNBS in 0.1 M phosphate buffer (pH 7.4) with or without SOD and catalase (0.1 mg/ml each) or 50 mM EDTA was incubated at 37 °C overnight, and the mixture was subjected to agarose gel electrophoresis as described previously.[11]

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RESULTS AND DISCUSSION

When DBNBS was treated with Trp from Wako at pH 7.5 and room temperature, relatively intense ESR signals of a triplet with a hyperfine splitting constant (hfsc) of $a_N = 1.38$ mT gradually appeared during the period of 5 h (Figure 2A). At first we doubted if the spin signals were derived from the impurity of the Trp preparation. We examined the signals from the Trp preparations obtained from Kanto and Ajinomoto, but the same signals with the same intensities were observed under the conditions. The signals were characteristic to the known nitroxyl free radicals bearing no β-hydrogens with a hfsc of $a_N = 1.196$–1.432 mT formed from alkyl carbon-centered radicals.[1-4] The splittings of two protons at 2- and 6-positions of DBNBS moiety is presumably small and lost within the line-width. For instance, $a_H{}^{\delta}$ values of known DBNBS-adducts are very small: 0.063 mT for DBNBS-OH,[2] 0.07 mT for DBNBS-Me[3] and 0.06 mT for DBNBS-CH_2 OH.[2] It was found that the reactions of DBNBS with dipeptides containing Trp, Gly-Trp (Figure 2B) and Trp-Gly (Figure 2C), gave ESR signals identical with those obtained from Trp. Thus Trp in dipeptides can react with DBNBS to give the spin adducts. Indole, a structural component of Trp, gave ESR signals of triplets of a doublet with hfsc of $a_N = 1.38$ mT and $a_H = 1.00$ mT (Figure 2D). The a_N value was identical with that from Trp, and the a_H value indicated the presence of a β-hydrogen with a hfsc of $a_H = 0.89$–1.22 mT for alkyl carbon-centered radical adducts.[1-4] The signals from indole disappeared after 5-h incubation, indicating that the spin adduct was unstable as compared with that from Trp.

Pro gave weak ESR signals after the reaction with DBNBS for more than 3 h. The signals were composed of triplets of a doublet with hfsc of $a_N = 1.31$ mT and $a_H = 0.40$ mT (Figure 3A), indicating the formation of the nitroxyl free radical bearing a β-hydrogen. The a_N value was identical with that of the nitroxyl free radical formed from alkyl carbon *h* centered radicals.[1-4] Reaction of DBNBS with pyrrolidine, a structural component of Pro, gave ESR signals immediately after mixing with DBNBS (Figure 3B). The signals were composed of triplets of a doublet with hfsc of $a_N = 1.36$ mT and $a_H = 0.35$ mT. The signals from pyrrolidine were unstable and disappeared after 5-h incubation. Cys gave weak complex ESR signals just after mixing with DBNBS, but the signals were transformed after 5 h into those composed of a triplet with a hfsc of $a_N = 1.25$ mT (Figure 4A), characteristic to the nitroxyl free radical with a hfsc of $a_N = 1.27$–1.43 mT formed from sulfur-

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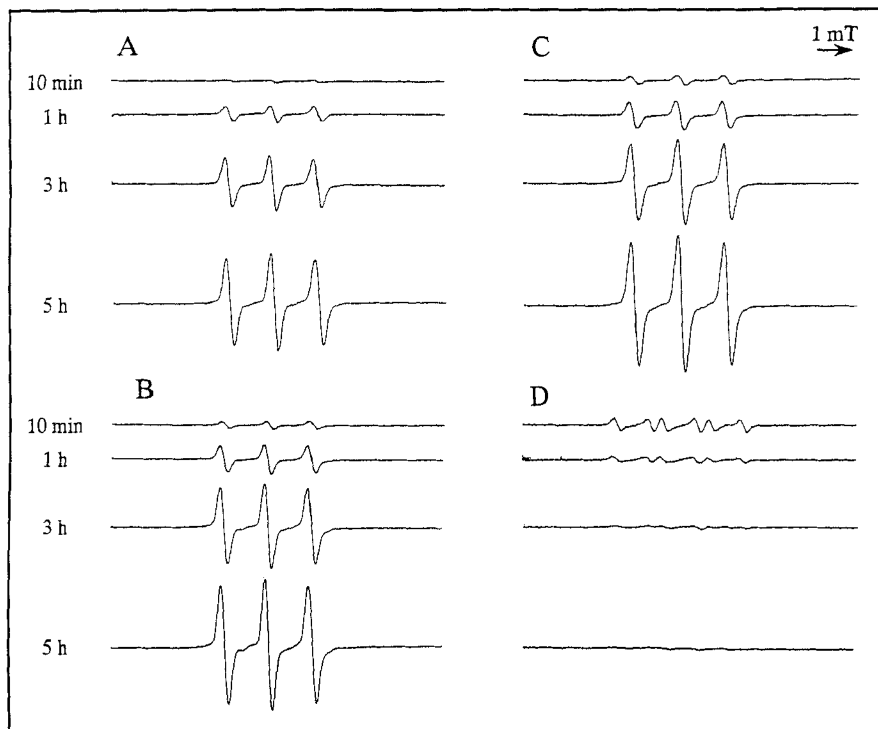

FIGURE 2 ESR spectra obtained from the reaction of DBNBS with Trp (from Wako) (A), Gly-Trp (B), Trp-Gly (C) and indole (D) at pH 7.5 and room temperature for the indicated periods. Receiver gain was set at 500.

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containing radicals.[6,7] The reaction of DBNBS with GSH, a Cys-containing tripeptide, gradually produced weak ESR signals identical with those from Cys (Figure 4B). Other amino acids such as Ala, Arg, Asn, Asp, Cys-Cys, Gln, Gly, His, Ile, Leu, Met, Phe, Ser, Tyr and Val gave no ESR signals by the reactions with DBNBS under the conditions. The appearance of ESR signals depended on the side chain of each amino acid. The reaction of DBNBS with BSA did not give any ESR signals. Trp, Pro and Cys residues in BSA may be buried so as not to react with DBNBS, or the residues inhibitory against the formation of the spin adducts may be present in BSA.

Linoleic acid (Figure 5A) and oleic acid (Figure 5B) gave weak ESR signals by the reactions with DBNBS. The ESR signals from the reaction of DBNBS and linoleic acid or oleic acid were very complicated and/or broad, indicating that the spin adducts were not single. In contrast, stearic acid gave no detectable ESR signals (Figure 4C). Double bonds in the fatty acids seemed to be essential for the production of the spin adducts.

While DBNBS gave no ESR signals after incubation with calf thymus DNA, adenine, thymidine and 2′-deoxyguanosine, DBNBS showed significant breaking activity in supercoiled plasmid DNA (Figure 6). DBNBS effectively converted the supercoiled form I DNA into the nicked open circular form II DNA (lane 2),

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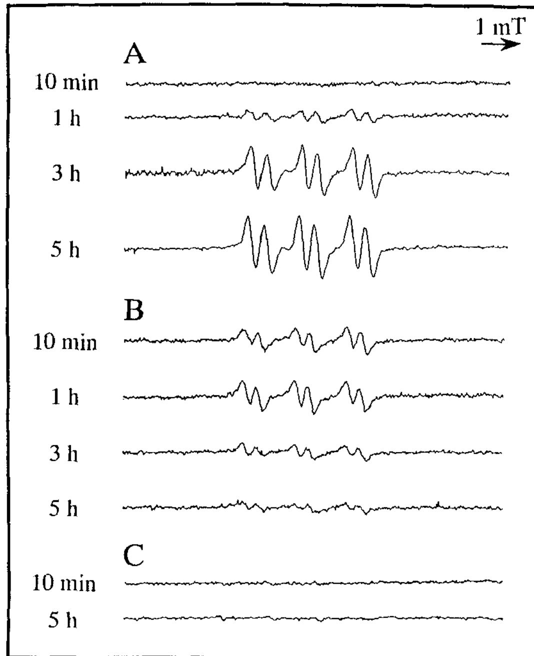

FIGURE 3 ESR spectra obtained from the reaction of DBNBS with Pro (A), pyrrolidine (B) and DBNBS alone (C) at pH 7.5 and room temperature for the indicated periods. Receiver gain was set at 2000.

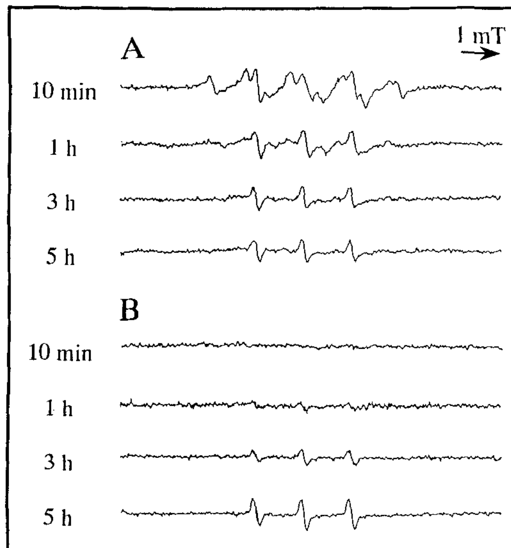

FIGURE 4 ESR spectra obtained from the reaction of DBNBS with Cys (A) and GSH (B) at pH 7.5 and room temperature for the indicated periods. Receiver gain was set at 2000.

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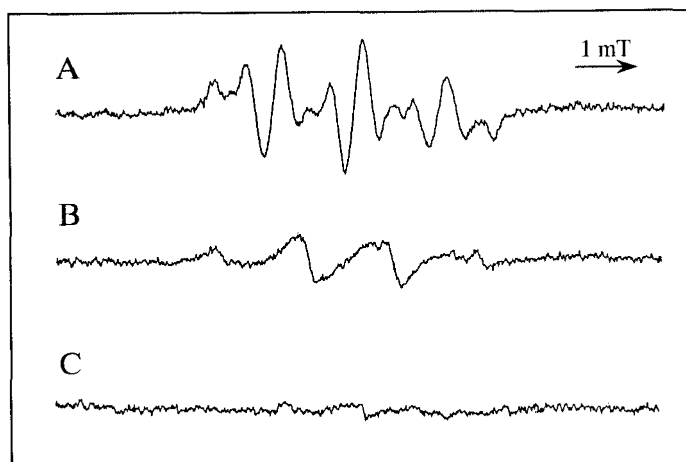

FIGURE 5 ESR spectra obtained from the reaction of DBNBS with linoleic acid (A), oleic acid (B) and stearic acid (C) at pH 7.5 and room temperature for 5 h. Receiver gain was set at 2000.

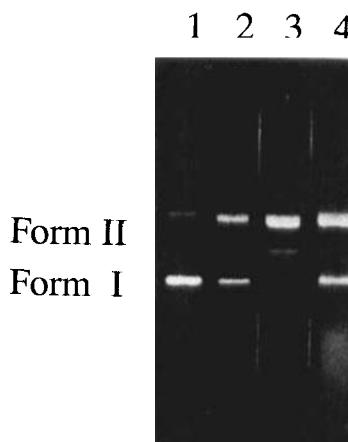

FIGURE 6 Agarose gel electrophoresis of supercoiled pBR322 DNA (10 μg/ml) in phosphate buffer incubated at 37 °C overnight (lane 1) with DBNBS (10 mM) (lane 2) in the presence of SOD and catalase (0.1 mg/ml each) (lane 3) or EDTA (50 mM) (lane 4). Positions of a supercoiled DNA and a nicked open circular DNA are indicated as Form I and Form II, respectively.

indicating that DBNBS induced single strand breaks. The DNA breaking activity was not inhibited by addition of SOD and catalase (lane 3) or EDTA (lane 4), indicating that no active oxygen species were included in the breaking. DBNBS itself or certain radicals generated by the interaction with DNA may participated in the breaking. While DNA and its components did not give any spin adducts with DBNBS, the reagent can react with DNA molecule to induce strand breaks.

Finally, we examined whether ESR signals were detectable in the incubation of

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DBNBS and human plasma. The incubation mixture showed weak ESR signals of a triplet with a hfsc of $a_N = 1.38$ mT, which was similar to those obtained from Trp (Figure 7). It is likely that non-radical components including Trp-containing components in plasma gave ESR signals after the reaction with DBNBS.

So far, it has been known that nitrosobenzene reacts with unsaturated hydrocarbon, 2,3-dimethylbutene, to yield nitroxyl free radical *via* a 'pseudo Diels-Alder mechanism'.[12] Floyd and his coworkers[13-15] have reported that 2-nitrosofluorene, a metabolic intermediate of carcinogenic 2-aminofluorene, reacts with and covalently binds to unsaturated lipid molecules to form nitroxyl free radicals with a hfsc of $a_N = 1.14$ mT. They have suggested the reaction mechanisms as follows. 2-Nitrosofluorene directly adds to the carbon-carbon double bond of the lipid molecule producing a hydroxylamine intermediate which is then oxidized with 2-nitrosofluorene into the nitroxyl free radical. More recently, Kalyanaraman *et al.*[12] have reported the appearance of the ESR signals by the reaction of DBNBS with LDL. The ESR signals are composed of some spin adducts, one with hfsc of $a_N = 1.475$ mT and $a_H = 1.05$ mT is extracted with chloroform/methanol (2:1), and others with $a_N = 1.45$ mT and $a_H = 0.975$ mT remain in aqueous fraction. In this study, pure linoleic acid and oleic acid reacted with DBNBS to give spin adducts *via* double bond-dependent reaction, whereas the ESR signals were so broad and/or complicated and their hfsc values were not determined. Hence, it is established that DBNBS reacts with unsaturated fatty acids to give spin adducts.

Formation of the nitroxyl free radicals by the reactions of DBNBS with Trp, Pro and Cys may similarly proceed as shown in Figure 1. Because the DBNBS/Trp adduct had no β-hydrogens (Figure 2A) and the DBNBS/indole adduct had a β-hydrogen (Figure 2D), the carbon atom at the 3-position of the indole skeleton of Trp or indole may be the binding site of DBNBS. It is likely that the nitrogen atom of DBNBS binds to the carbon atom of Trp or indole by a "pseudo Diels-Alder mechanism" to form corresponding hydroxylamine derivatives as illustrated in Figure 1. The hydroxylamines were in turn oxidized into nitroxyl free radicals in the presence of DBNBS or dissolved oxygen, because nitroso-aromatics including nitrosobenzene[17] and 3-nitroso-1-methyl-5*H*-pyrido[4,3-b]indole[18] act as oxidizing agents. Because both the DBNBS/Pro and DBNBS/pyrrolidine adducts had β-hydrogens, DBNBS might bind to the carbon atoms of Pro or pyrrolidine other than those of the 2-position of the pyrrolidine skeleton, followed by oxidation with DBNBS or oxygen to nitroxyl free radicals. The formation of the spin adduct of DBNBS/Cys may be caused by the multi-step-reaction, because the signals changed during the incubation. The reaction of DBNBS and Cys finally gave the nitroxyl spin adduct in which the nitrogen atom of DBNBS might bind to the sulfur atom of Cys. In these reaction mechanisms, oxidation process of the intermediates may

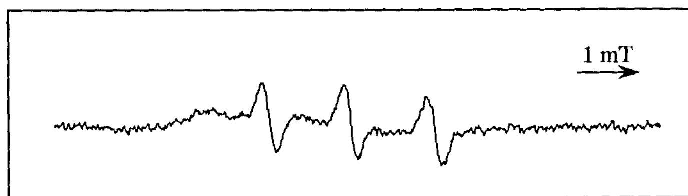

FIGURE 7 ESR spectrum obtained from the reaction of DBNBS with human plasma at pH 7.5 and room temperature for 5 h. Receiver gain was set at 2000.

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be requisite for the formation of the nitroxyl free radicals. When ferricyanide was added to the reaction mixtures of DBNBS/Trp (Figure 2A, 5 h) and DBNBS/Cys (Figure 4A, 5 h) and the mixture was allowed to stand for 30 min, the intensities of the corresponding ESR signals were greatly enhanced. The nitroxyl free radicals were readily produced under the oxidative conditions. Hence, the non-radical hydroxylamine intermediates may be oxidized with DBNBS to produce the nitroxyl free radical as shown in Figure 1.

In conclusion, it was found that DBNBS readily reacted with the biological components to give nitroxyl free radicals by formation of intermediates followed by oxidation in the presence of DBNBS. The use of DBNBS spin trap to detect free radicals in biological systems containing amino acids and other biological components after long incubation may give misleading results.

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References

1. H. Kaur, K.H.W. Leung and M.J. Perkins (1981) A water-soluble, nitroso-aromatic spin-trap, *Journal of the Chemical Society, Chemical Communications*, 142–143.
2. M. Kohno, M. Yamada, K. Mitsuta, Y. Mizuta and T. Yoshikawa (1991) Spin-trapping on the reaction of iron complexes with peroxides and the effects of water-soluble antioxidants, *Bulletin of the Chemical Society of Japan*, **64**, 1447–1453.
3. T. Ozawa and A. Hanaki (1987) Spin-trapping of alkyl radicals by a water-soluble nitroso aromatic spin-trap, 3,5-dibromo-4-nitrosobenzenesulfonate, *Bulletin of the Chemical Society of Japan*, **60**, 2304–2306.
4. P. Smith and J.S. Robertson (1988) Electron paramagnetic resonance study of the reactions of the spin trap 3,5-dibromo-4-nitrosobenzenesulfonate, *Canadian Journal of Chemistry*, **66**, 1153–1158.
5. K. Ichimori, C.M. Arroyo, L. Prónai, M. Fukahori and H. Nakazawa (1993) The reactions of 3,5-dibromo-4-nitrosobenzene sulfonate and its biological applications, *Free Radical Research Communications*, **19**, S129–S139.
6. T. Ozawa and A. Hanaki (1987) Spin-trapping of sulfite radical anion $SO_3^{\cdot -}$, by a water soluble, nitroso-aromatic spin-trap, *Biochemical and Biophysical Research Communications*, **142**, 410–416.
7. K. Stolze, D.R. Duling and R.P. Mason (1988) Spin adducts formed from nitroso spin traps and dithionite, *Journal of the Chemical Society, Chemical Communications*, 268–271.
8. T. Ozawa and A. Hanaki (1991) The first ESR spin-trapping evidence for the formation of hydroxyl radical from the reaction of copper(II) complex with hydrogen peroxide in aqueous solution, *Journal of the Chemical Society, Chemical Communications*, 330–333.
9. T. Ozawa and A. Hanaki (1987) Spin-trapping of phosphorous-containing inorganic radicals by a water-soluble spin-trap, 3,5-dibromo-4-nitroso benzenesulfonate, *Chemistry Letters*, 1885–1888.
10. T. Ozawa and A. Hanaki (1991) Spin-trapping of selenium-containing inorganic radicals in aqueous solutions, *Bulletin of the Chemical Society of Japan*, **64**, 1976–1978.
11. K. Hiramoto, H. Johkoh, K. Sako and K. Kikugawa (1993) DNA breaking activity of the carbon-centered radical generated from 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH), *Free Radical Research Communications*, **19**, 323–332.
12. B. Kalyanaraman, J. Joseph, N. Kondratenko and S. Parthasarathy (1992) Direct modification of low density lipoprotein by the spin trap 3,5-dibromo-4-nitrosobenzenesulfonic acid, *Biochimica et Biophysica Acta*, **1126**, 309–313.
13. A.B. Sullivan (1966) Electron spin resonance studies of a stable arylnitroso-olefin adduct free radical, *Journal of Organic Chemistry*, **31**, 2811–2817.
14. R.A. Floyd (1977) Free radicals produced in a nitrosofluorene-unsaturated lipid reaction, *Experimentia*, **33**, 197–198.
15. R.A. Floyd, L.M. Soong, M.A. Stuart and D.L. Reigh (1978) Free radicals and carcinogenesis. Some properties of the nitroxyl free radicals produced by covalent binding of 2-nitrosofluorene to unsaturated lipids of membranes, *Archives of Biochemistry and Biophysics*, **185**, 450–457.
16. R.A. Floyd (1982) Observations on nitroxyl free radicals in arylamine carcinogenesis and on spin-trapping hydroxyl free radicals, *Canadian Journal of Chemistry*, **60**, 1577.

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17. A.R. Becker and L.A. Sternson (1980) Nonenzymatic reduction of nitrosobenzene to phenylhydroxylamine by NAD(P)H, *Bioorganic Chemistry*, **9**, 305–312.
18. K. Saito, Y. Yamazoe, T. Kamataki and R. Kato (1983) Activation and detoxication of *N*-hydroxy-Trp-P-2 by glutathione and glutathione transferase, *Carcinogenesis*, **4**, 1151–1157.

Accepted by Professor E. Niki

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