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THE REACTIONS OF 3,5-DIBROMO-4-NITROSOBENZENESULFONATE AND ITS BIOLOGICAL APPLICATIONS

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Recently, 5,5-dibromo-4-nitrosobenzenesulfonate (DBNBS) has been applied to detect biological free radicals. However, DBNBS has various non-specific reactions which lead to perplexing results. Thus, we investigated some basic reactions of DBNBS in combination of other nitroso spin traps to assign DBNBS spin adducts derived from human platelets which presumeably related to the endothelium-derived relaxing factor (EDRF). The collagen activated platelets yielded four spin adducts (S_T, L_T, S_S, and L_S) in the presence of DBNBS (40 mM). The broad triplet due to S_T was also observed by bubbling NO gas into a DBNBS solution. To identify S_T, nitrosobenzene (NB) in dry dioxane was mixed with NO-saturated dioxane. The NB-NO spin adduct was observed but decomposed into diphenyl aminoxyl by the addition of H_2O indicating that the primary adduct formed by the reaction of NO and DBNBS is unstable and turns into a dimerization product. Although S_T could be eliminated by the inhibitor of EDRF, S_T was shown to be produced by non-specific reactions. Another triplet was assigned to an S-centered radical because thiyl radicals which were generated from either the decomposition of S-nitrosothiol, or glutathione oxidation exhibited almost identical triplet signals. The other two sextets were assigned to C-centered radical adducts. Thus, DBNBS detected NO-related, S-centered, and two C-centered radicals derived from human platelets. Special cautions are necessary for the identification of DBNBS spin adducts in a biological system to exclude artifactual radicals.

KEY WORDS: DBNBS, spin trapping, platelet, EDRF, nitric oxide, S-nitrosothiol

INTRODUCTION

3,5-Dibromo-4-nitrosobenzenesulfonate (DBNBS) was first developed by Perkins *et al.* in 1981 as a trap of C-centered radicals.[1] However, DBNBS studies in biological systems are still limited in number.[2-4] Among them, there are recent works which applied DBNBS to neuroblastoma cells[3] and platelets[4], demonstrated that DBNBS spin adducts derived from the reaction are related to nitric oxide (NO). Although three NO-related DBNBS spin adducts were reported, the identification of these three adducts remains incomplete and the reaction mechanism of DBNBS has not been clarified as yet.

Most cells and tissues such as endothelial cells, neurons, platelets, and white blood

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cells can produce an NO-related compound as a chemical mediator or for cytotoxic activities.[5] This compound was designated as the endothelium-derived relaxing factor (EDRF), since it was first demonstrated in endothelial cells and produced marked vasodilation[6]. EDRF was also found to be released from platelets and has a potent effect on the inhibition of platelet aggregation.[7] EDRF is synthesized through L-arginine/NO pathway using L-arginine as a substrate and most publications in this field regarded EDRF as NO itself.[5] However, other NO-related compounds such as S-nitrosothiol remain candidates of EDRF.[8] The additional uncertainty in this matter is that no radical intermediates have yet been identified even though the L-arginine/NO pathway in theory includes radical reactions.[9] Thus, we used DBNBS spin adducts detected in human platelet system for the identification of EDRF and its intermediates. We also investigated some basic reactions between DBNBS and various NO-related compounds by comparing the reaction between nitrosobenzene, a simple nitrosoaromatic spin trap, and these compounds for the identification of DBNBS spin adducts.

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MATERIALS AND METHODS

Chemicals

DBNBS and L-N^G-monomethylarginine (L-NMMA) were obtained from Sigma and used without further purification. 2,4,6-Tri chlorophenylhydrazine and horseradish peroxidase (Grade I) suspended in 3.2 M $(NH_4)_2SO_4$ were obtained from Aldorich and Boeringer, respectively. Saturated NO solution was prepared by bubbling N_2-flushed HEPES buffer (5 mM, pH 7.4) with 99.9% NO gas (Nihon Sanso, Japan) for 20 min at 25°C in a rubber-sealed test tube. It contained 1.9 mM of NO. Dioxane used for nitrosobenzene was dried over CaH_2 and distilled prior to use. S-nitroso-N-acetylpenicillamine was synthesized from N-acetyl-D, L-penicillamine and HNO_2.[10] Other reagents were purchased from Wako Pure Chemical Co. (Japan).

The isolation of human platelets and its EPR measurement

The preparation of platelet-rich plasma (PRP) and washed platelets (WP) was reported previously[4]. Briefly, blood (60 ml) from healthy volunteers was collected into a plastic tube containing 3.15% sodium citrate (1:9 v/v). Platelet-rich plasma (PRP) was obtained by centrifuging collected blood (800 g, 8 min). To prepare washed platelets (WP, 1.5–3.5 × 10^5/ul), 300 ng/ml of PGI_2 was added to PRP and the mixture was centrifuged at 100,000 g for 18 min. The resultant platelet pellet was washed once and resuspended in 20 ml of calcium-free isotonic buffer (0.32 M sucrose, 10 mM HEPES, 1 mM D,L-dithiothreitol, pH 7.4). PRP and WP were mixed with 40 mM of DBNBS, incubated at 37°C for 2 minutes with Ca^{2+} (1–3 mM), and activated with collagen (17 ug/ml). EPR spectra were recorded 1 min later in the dark.

RESULTS AND DISCUSSION

Figure 1a shows the EPR spectrum obtained from PRP fraction by collagen activation. Figure 1b is the computer simulation of Figure 1a. The broad background

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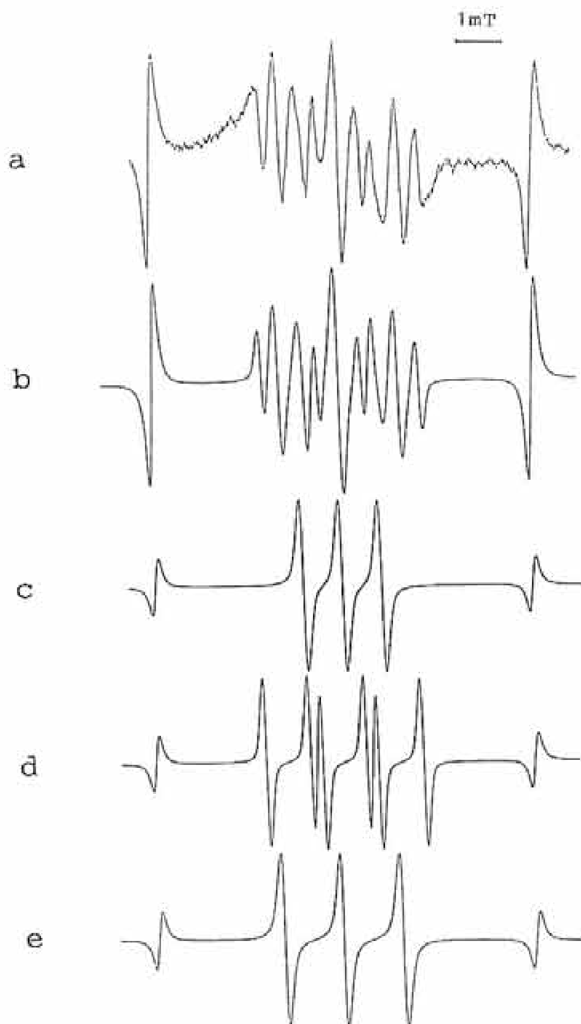

FIGURE 1 *a*) The EPR spectrum obtained from the PRP fraction of human platelet activated by collagen (17 μg/ml) in the presence of DBNBS (40 mM). *b*) Overall computer simulation of *a* (the addition of *c*, *d* and *e* (3:3:5)). *c*) small triplet (S_T: a_N = 0.92 mT, linewidth = 0.20 mT). *d*) small sextet (S_S: a_N = 1.33 mT, a_H = 1.02 mT, and linewidth = 0.16 mT). *e*) large triplet (L_T: a_N = 1.39 mT. linewidth = 0.20). The signals on both sides of the central EPR spectra correspond to a Mn^{2+} internal instrumental standard.

signal appears to be originating from a radical adduct immobilized by a large molecule such as protein. In the simulated process, the background signal was not considered. The simulation spectrum consists of three components as shown in Figures 1c–1e and arbitrarily designated as small triplet S_T, small sextet S_S, and large triplet L_T. These spin adducts were stable for more than ten minutes.

Figure 2a was obtained from the washed platelet activated in the same way. Figure 2b is the computer simulation of Figure 2a, and consists of 2 components as shown

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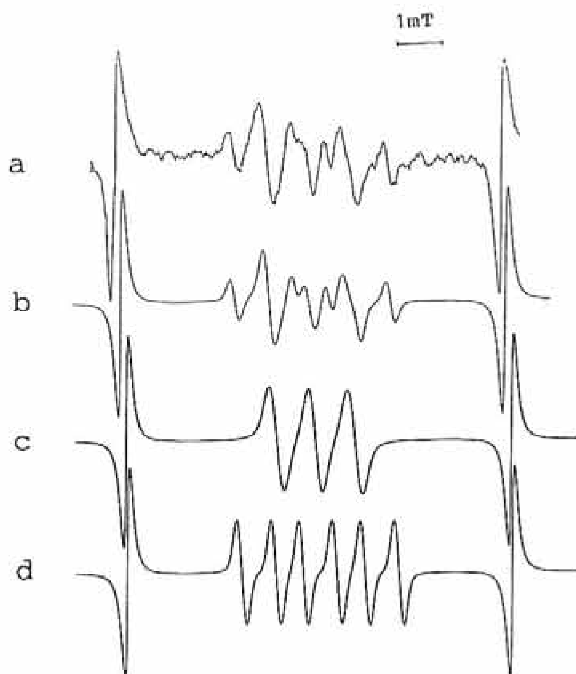

FIGURE 2 *a*) The EPR spectrum obtained from the WP fraction of human platelet activated by collagen (17 μg/ml) in the presence of DBNBS (40 mM). *b*) Overall computer simulation of *a* (the addition of *c* and *d* (0.53 : 0.36)). *c*) small triplet S_T which has the same a_N of Figure 1c. *d*) large sextet (S_S: a_N = 1.40 mT, a_H = 0.78 mT, and linewidth 0.21 mT).

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in Figures 2c and 2d, which were also arbitrarily designated as small triplet S_T and large sextet L_S. Small triplet S_T was an identical spectrum with that obtained from PRP fraction. L_S is a newly obtained stable adduct and its hyperfine splitting constants (hfsc) are 1.40 mT for one nitrogen and 0.78 mT for one hydrogen. Therefore, activated human platelets totally yielded four adducts, S_T, L_T, S_S, and L_S. To ascertain that these spectra are related to the L-arginine/NO pathway, experiments were repeated in the presence of L-NMMA which is a specific inhibitor of the L-arginine/NO pathway.[5] No DBNBS adducts were observed in the presence of L-NMMA.

To identify these four adducts, we first applied NO to a DBNBS solution to test whether DBNBS traps nitric oxide or not. The addition of 100 mM of DBNBS to an NO saturated solution gave a broad triplet spectrum (Figure 3a). The EPR parameters of this broad triplet are identical to the S_T adduct obtained in human platelet. The spectrum shown in Figure 3a has a small shoulder, which became dominant when diluted DBNBS (10 mM) was used (Figure 3b). The hfsc of this shoulder signal are identical to the reported parameters of DBNBS-SO_3^- adduct obtained by SO_3^{2-} oxidation with H_2O_2 and horseradish peroxidase.[11] Thus, the spectrum obtained by the reaction of NO and DBNBS is identified as the mixture of the SO_3^- adduct and an unknown broad triplet.

To clarify the generation mechanism of this broad triplet, a simpler nitroso spin trap, nitrosobenzene, was used instead of DBNBS. Figure 4a shows the EPR

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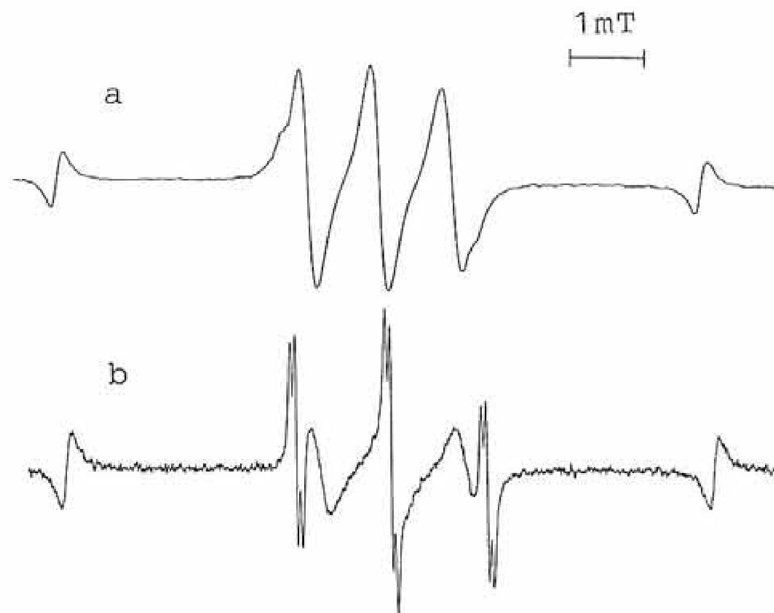

FIGURE 3 The EPR spectra obtained by the reaction between DBNBS and saturated NO (1.9 mM) in deaerated HEPES buffer (5 mM, pH 7.4). *a*) 100 mM of DBNBS, *b*) 10 mM of DBNBS. The sharp triplet of triplet has the hyperfine coupling constants of $a_N = 1.26$ mT and $2a_H = 0.06$ mT, which are identical to those of DBNBS-SO_3^- adduct.[11]

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spectrum obtained by the addition of saturated NO solution to 1 mM of nitrosobenzene in dehydrated dioxane. The adduct was unstable in the presence of trace water or acid, which necessitated an extensive dehydration procedure of dioxane to obtain this spectrum. The addition of triethylamine stabilized this adduct further. A computer simulation study revealed the contribution of 2 kinds of nitrogen splittings, 1.028 mT and 0.386 mT, and 2 kinds of hydrogen splittings, 0.280 mT and 0.098 mT (Figure 4b). We assign that this spectrum to the nitrosobenzene-NO adduct. However, the spectrum of the nitrosobenzene-NO adduct readily collapsed and finally turned into the well-known spectrum of diphenyl aminoxyl radical ($1a_N = 1.15$ mT, $6a_H = 0.21$ mT, $4a_H = 0.10$ mT) by the addition of water. This confirms that the NO-adduct of nitrosoaromatic spin traps are labile and easily decomposes into dimerization product in aqueous media.

To test whether the above-mentioned dimerization reaction occurs in the reaction of DBNBS and NO, the DBNBS-phenyl adduct was produced for comparison of the nitrogen hfsc since the structure of the dimerized product of DBNBS is similar to the spin adduct of aryl radicals. Phenyl radical was generated by Cu^{2+} oxidation of phenylhydrazine and trapped by DBNBS (Figure 5a).[12] The computer simulated spectrum was obtained by assuming $1a_N = 1.142$ mT and $3a_H = 0.310$ mT, which are the contributions from the ortho and para protons of the phenyl group (Figure 5b). Since the nitrogen hfsc (1.142 mT) of this DBNBS phenyl adduct is significantly larger than that of S_T component (0.92 mT), the possible effect of substituents on the phenyl ring was examined. For that purpose, phenylhydrazine was replaced by 2,4,6-trichlorophenylhydrazine. A triplet signal of

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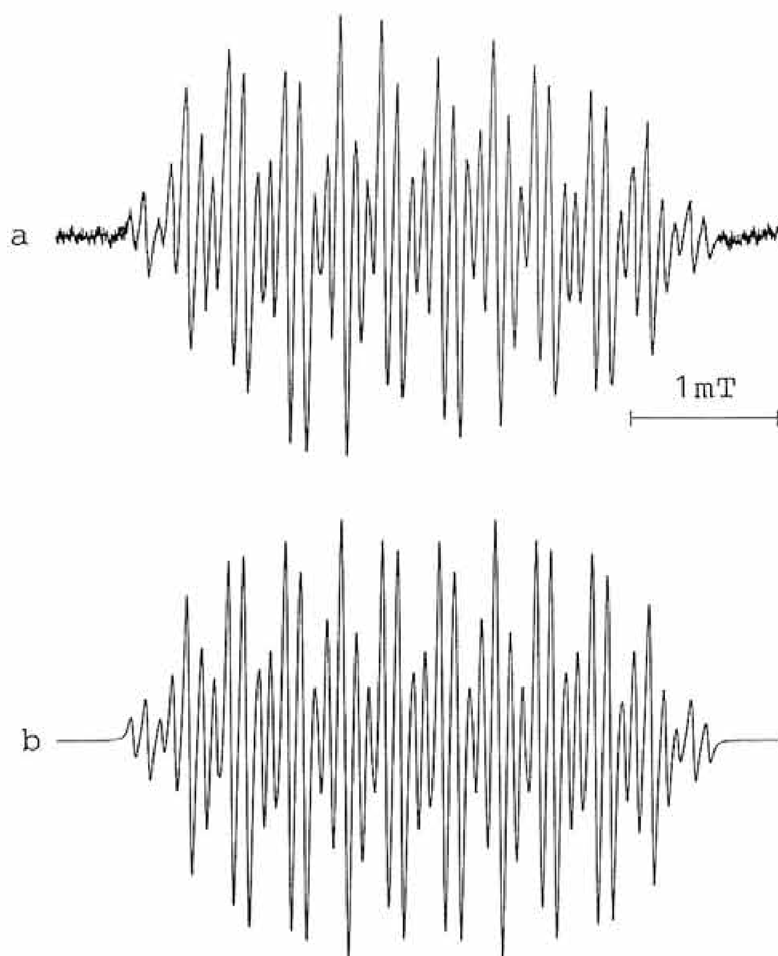

FIGURE 4 *a*) The EPR spectrum obtained by the reaction of nitrosobenzene (1 mM) and saturated NO solution in dried dioxane with trace amount of triethylamine. *b*) The computer simulation of *a*. a_N = 1.028 mT, a_N = 0.386 mT, $3a_H$ = 0.280 mT, and $2a_H$ = 0.098 mT.

DBNBS-trichlorophenyl adduct was also obtained (Figure 5c). The nitrogen hfsc was smaller than that of the DBNBS-phenyl adduct (0.95 mT) and was almost the same as that of S_T component suggesting that electron-withdrawing groups like chlorine and bromine make the nitrogen hfsc smaller. In the DBNBS-trichlorophenyl adduct, a chlorine hyperfine splitting was not observed but the chlorine substituents broadened the line-width significantly compared with that in the DBNBS phenyl adduct.

Since the broad triplet (S_T) did not show other hyperfine splitting than that by α-nitrogen, it is not possible to assign the broad triplet (S_T) to the DBNBS-NO adduct itself. However, the additional splittings can be hidden in the linewidth of S_T. As shown in Figure 4, the β-nitrogen hfsc (0.386 mT) was about one-third of the α-nitrogen hfsc (1.028 mT) in the nitrosobenzene NO-adduct. Thus, the

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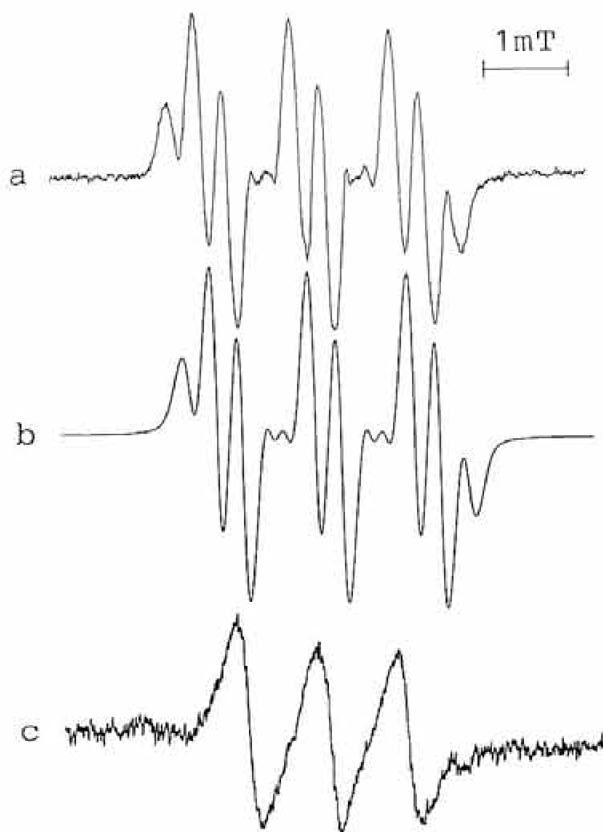

FIGURE 5 *a*) The EPR spectrum obtained by Cu^{2+} (10 μM) oxidation of phenylhydrazine (1 mM) in the presence of DBNBS (7.5 mM) in 50 mM sodium carbonate buffer (pH = 10.0). *b*) The computer simulation of *a*. a_N = 1.142 mT and $3a_H$ = 0.310 mT. *c*) The EPR spectrum obtained under the condition *a* except that saturated 2,4,6-trichlorophenylhydrazine solution was used instead of phenylhydrazine (a_N = 0.95 mT).

β-nitrogen hfsc of the DBNBS-NO adduct should be also in the range of 0.3 mT, which is larger then the observed linewidth (0.2 mT) of the broad triplet (S_T). This excludes the possibility of the concealment of the β-nitrogen hyperfine splitting. Since the nitrosobenzene-NO adduct proved to be unstable in the presence of water, it can be deduced that the DBNBS-NO adduct may be also unstable and decomposes into a dimerization product. Moreover, the bromine nuclei may broaden the spectrum of the dimerized DBNBS like chlorine nuclei did in the DBNBS-trichlorophenyl adduct. Thus, we illustrate the reaction process of NO and DBNBS as in Figure 6. In the platelets experiment, the DBNBS-NO adduct itself was invisible by EPR and only decomposed products from dimerization was observed.

Although S_T in the human platelets system was detected only when the platelets were activated by collagen and S_T could be eliminated by the inhibitor of L-arginine/NO pathway, S_T and DBNBS-SO_3^- adducts can be also observed by non-radical attacks to DBNBS. Ultraviolet light decomposition of DBNBS also

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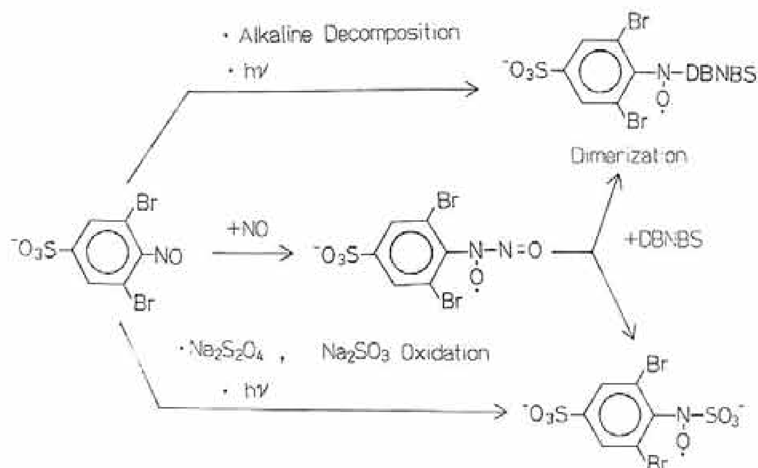

FIGURE 6 The proposed scheme for the reaction of DBNBS and NO. The reaction of DBNBS and NO results in the generation of labile DBNBS-NO adduct, which decomposes into the dimerized product of DBNBS and DBNBS-SO_3^-. These decomposed products can be also generated by the non-specific reactions denoted in this figure.

showed both S_T and DBNBS-SO_3^- (data not shown). NADH reduction of concentrated DBNBS (100 mM) yielded a mixture of S_T and another adduct (data not shown). The DBNBS-SO_3^- adduct has been detected in the following systems and assigned to several species: (1) Horseradish peroxidase catalyzed oxidation of sulfite by H_2O_2 (assignment: DBNBS-SO_3^-).[11,13] (2) Xanthine-xanthine oxidase system containing DMSO. The adduct was first assigned to the superoxide adduct[14]. However, it has been reported recently that this adduct is generated by H_2O_2 oxidation of DBNBS catalyzed by the peroxidase activity of commercially available xanthine oxidase.[15] (4) The Fenton reaction system including H_2O_2 and Fe^{3+} (assignment: DBNBS-OH).[16] (5) Ce^{4+} oxidation of selenite (assignment: DBNBS-SeO_3^-).[17] Since the identical spectrum was obtained in all of these system and with the additional analysis using ^{33}S satellite hfs[11] the spectrum was assigned to DBNBS-SO_3^- adduct. As discussed above, both S_T and DBNBS-SO_3^- can be generated even in non-radical attack of DBNBS. Therefore, extreme care should be taken in the interpretation of the DBNBS adducts generated in biological systems and artifactually produced adducts should be excluded.

Regarding the identification of the L_T adduct, an S-centered radical adduct was considered as the candidate for the L_T adduct based on the following two reasons: Firstly, only a nitrogen hfs and no additional hydrogen hfs was observed. Secondly, S-nitrosothiol is one of the proposed species as EDRF and easily decomposes into S-centered radical and NO. To check this assumption, we synthesized S-nitroso-N-acetyl-D, L-penicillamine (SNAP) which is a relatively stable S-nitrosothiol and can be isolated as powder.[10] The addition of SNAP to DBNBS solution yielded a triplet signal (Figure 7a). This adduct was stable in aqueous solution and the nitrogen hfs was 1.40 mT, which is close to that of L_T adduct (1.39 mT). Glutathionyl radical, which can be the relevant source of S-centered radical in biological system, was also examined. The following two methods were utilized to produce glutathionyl radical: (1) HRP-catalyzed oxidation of glutathione with

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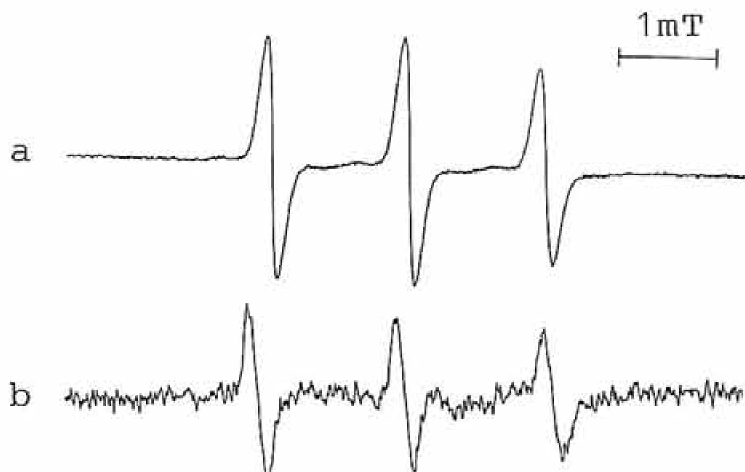

FIGURE 7 *a*) The EPR spectrum obtained by the decomposition of S-nitroso-N-acetyl-D,L-penicillamine (1 mM) in the presence of 10 mM DBNBS (a_N = 1.40 mT). *b*) The EPR spectrum obtained by the DBNBS trapping of glutathionyl radical (a_N = 1.42 mT). The concentration of DBNBS was 50 mM. Glutathionyl radical was generated by the following two methods: (1) the mixing of glutathione (10 mM), H_2O_2 (2 mM), horseradish peroxidase (0.2 mg/ml). (2) the reaction of glutathione (50 mM) and isoamylnitrite (50 mM).

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hydrogen peroxide,[18] and (2) the reaction of glutathione and isoamylnitrite.[19] Both methods produced the spectrum having the nitrogen hfsc of 1.42 mT, which was in the same range as that of the L_T adduct. Thus, as summarized in Table I, the L_T adduct can be assigned to an S-centered radical adduct.

Regarding the assignment of two sextets, S_S and L_S, C-centered radicals are the most plausible candidates since these contain one hydrogen, possibly a β-hydrogen. The hfsc of C-centered radicals reported by Perkins[1] are summarized in Table I for the comparison with our S_S and L_S adducts. The hfsc of S_S and L_S adducts fall into the range of reported C-centered radicals.

In conclusion, firstly, DBNBS trapped four radical species in activated human platelet system and these species are related to the L-arginine/NO pathway. The S_T adduct is derived from the reaction with NO. However, it is not an NO-adduct itself, but a secondary dimerized product. L_T is an S-centered radical, which may be generated through the reaction of NO with a sulfhydryl group. S_S and L_S are C-centered radicals. Further investigations are necessary to determine the underlying mechanisms for the generation of these radicals in activated cells. Secondly, it is clear that there are several pitfalls in DBNBS system and special caution is necessary to exclude non-specific reactions of DBNBS such as dimerization and SO_3^- formation by oxidation, reduction, or decomposition of labile adducts.

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TABLE I
Hyperfine coupling constants of DBNBS spin adducts

Radical	a_N(mT)	a_H(mT)	Reference
S_T	0.92	-	this work
S_S	1.33	1.02 (1H)	this work
L_S	1.40	0.78 (1H)	this work
L_T	1.39	-	this work
(C-centered)			
Me·	1.45	1.35 (3H)[a]	1
$HOCH_2$·	1.37	0.92 (2H)[a]	1
$HOCH_2CH_2$·	1.41	1.13 (2H)[a]	1
MeCHOH	1.40	0.92 (1H)[a]	1
MeCHOEt	1.25	0.71 (1H)[a]	1
Me_2CH·	1.42	0.93 (1H)[a]	1
Ph·	1.142	0.310(3H)[b]	this work
2,4,6-Cl_3Ph·	0.95	-	this work
(S-centered)			
SO_3^-	1.26	0.06 (2H)[c]	11
NAP·	1.40	-	this work
GS·	1.43	-	this work

NAP· : N-acetyl-D,L-penicillamine thiyl radical.
GS· : glutathionyl radical.
[a]β-hydrogen; [b]*o,p*-protons of phenyl radical; [c]two protons on DBNBS

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