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# Limitations of Electron Spin Resonance (ESR) Spectroscopy in the Superoxide Dismutase Assay of Blood

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**ABSTRACT.** We have employed the electron spin resonance spin-trapping method to determine the superoxide dismutase (SOD) activity in plasma and erythrocytes, and investigated various possible obstacles associated with detection of the superoxide radical derived from the hypoxanthine-xanthine oxidase system. With the exception of SOD itself, ascorbate, present in plasma at 40-140 $\mu$M, diminished the peak for the DMPO-OOH spin adduct most remarkably. Its 50% inhibitory concentration was 115 $\mu$M. Cysteine, reduced glutathione (GSH), urate, NADH and 5'-ATP also showed remarkable diminishing effects, although their concentrations in blood were too low to affect the DMPO-OOH spin adduct. Sugars had little or no effect. Hemoglobin and serum albumin showed a significant effect above a concentration of 1.5 and 5 mg/ml, respectively. In addition, ascorbate and albumin each showed a dose-dependent diminishing effect when 1.5 units/ml of SOD was used simultaneously with ascorbate or albumin. Therefore, the presence of ascorbate and albumin was not negligible when a slight amount of SOD activity in plasma was determined directly. The effect of hemoglobin, however, was overcome by dilution because the activity in erythrocytes was sufficiently high.

**Key words : superoxide dismutase — electron spin resonance spectroscopy — radical scavenger**

Currently, there is much interest in the role of free radicals, especially oxygen free radicals, in human disease and in the toxic process of various xenobiotics. A wide variety of mammalian cells have developed defense mechanisms against the potentially deleterious effects of these radicals. Superoxide dismutase (SOD), which was first identified and purified from erythrocytes and which has recently been reviewed extensively, catalytically scavenges the superoxide radical and thereby acts as a first line of defense against free radical damage in living cells.[1,2] Recently, a cyanide-sensitive SOD has been reported to be present in extracellular fluids, although the level is very low.[3,4] Since SOD diminishes the magnitude of the spin-trapped superoxide signal dose-dependently, an electron spin resonance (ESR) method for the analysis of SOD activity has been proposed. This ESR method is especially useful for measuring SOD activity in small crude biological samples for which purification processes, such as column chromatography, cannot be employed. As is the case with all other methods, however, any entity which scavenges

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the superoxide radical or changes the rate of formation of the superoxide radical will lead to erroneous determinations.[5,6]

The present study was undertaken to examine in vitro the obstructive effects of some substances which are present in blood. Using ESR spectrometry, we directly measured the magnitude of the DMPO-spin adduct of the superoxide radical generated in a hypoxanthine (HPX)-xanthine oxidase (XOD) system in coexistence with each substance.

## MATERIALS AND METHODS

*Reagents.* Hypoxanthine (HPX), diethylenetriaminepentaacetic acid (DETAPAC), bovine serum albumin (BSA), ascorbate, superoxide dismutase (SOD from bovine erythrocytes) and hemoglobin containing up to 75% methemoglobin were obtained from Sigma Chemical Co. (St. Louis, Mo). Xanthine oxidase (XOD) was purchased from Boehringer (Mannheim, Germany). The spin trapping reagent, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was obtained from Labotech Co. (Tokyo, Japan). All other chemicals employed were of analytical grade and were used without further purification.

*Preparation of fresh hemoglobin.* Blood was withdrawn from healthy persons and erythrocytes obtained by centrifugation at 600 g for 10 min were washed three times with isotonic solution. They were immediately hemolized and fresh hemoglobin was separated from erythrocyte SOD by chromatography on DEAE-Sephadex at pH 6.5 according to the method of Gärtner *et al.*[7] The hemoglobin concentration was determined by the cyanmethemoglobin method.

*Measurement of the scavenging activity.* ESR spectroscopy was performed according to the method of Hiramatsu and Kohno as follows[8]: Fifty $\mu$l of 2 mM HPX, 35 $\mu$l of 5.5 mM DETAPAC, 50 $\mu$l of aqueous solution or diluted hemolysate, 10 $\mu$l of DMPO and 50 $\mu$l of XOD (0.272 unit/ml) were put into a test tube and mixed. The aqueous solution was one of the substances, including SOD itself, diluted at the desired concentration with 0.1 M phosphate buffer (pH 7.8). At 105 sec after enzyme addition, the DMPO-adduct in the reaction mixture (1.95 ml final volume) was analyzed.

The ratio of peak height for the first signal of the DMPO-OOH spin adduct against that for the standard signal of manganese oxide was measured. The scavenging activity was indicated as the percent inhibition against the production of the spin adduct derived from the HPX-XOD system without any substances.

*ESR spectroscopy.* ESR spectra were recorded with a JES-FE2XG spectrometer (JEOL, Japan) in a flat cell for aqueous solutions mounted in a $TM_{110}$ cavity. The conditions for ESR spectrometry were as follows: magnetic field; $337 \pm 5$ mT, power; 8.0 mW, response; 0.1 sec, modulation; 0.063 mT, temperature; room temperature, amplitude; $4 \times 10^2$, sweep time; 2 min.

*Assay for XOD activity.* XOD activity in the ESR assay system was determined in 33 mM phosphate buffer (pH 7.8) at 37°C using hypoxanthine as a substrate following the formation of uric acid from changes in $OD_{290}$.

## RESULTS AND DISCUSSION

The use of nitrone spin traps for the detection of active radicals in aqueous systems is becoming increasingly common. However, are the values for SOD activity in crude biological materials, especially in small samples, obtained directly by the ESR method accurate? A very serious question arises as to how muh some substances present in these samples, such as proteins, sugars and vitamins, interfere with accurate measurement by the ESR method. The lower the SOD activities are in such samples, the more critical the problem becomes. Therefore, we decided to examine whether or not the method could be used for the SOD assay in blood, namely in erythrocytes and plasma. The ESR spectrum of the free radical derived from the HPX-XOD system is shown in Fig. 1A. The peak height for the DMPO-OOH spin adduct decreased in proportion to the SOD added to the reaction mixture (Fig. 1B). As shown

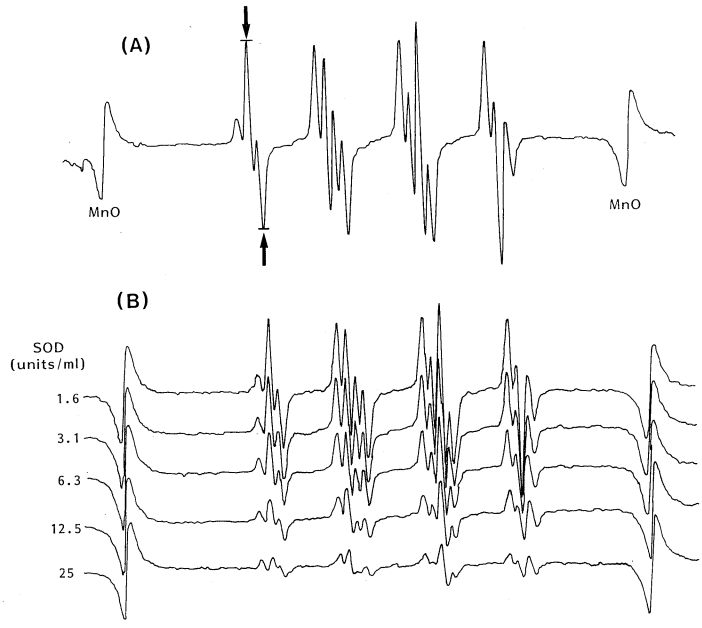


Fig. 1. ESR spectra obtained as a consequence of the reaction of hypoxanthine with xanthine oxidase. Incubations were performed in the presence of DMPO and DETAPAC in 0.1 M phosphate buffer (pH 7.8) for 105 sec (A). Arrow shows the first signal of the DMPO-OOH spin adduct. (B) was performed under identical conditions except that SOD (1.6, 3.1, 6.3, 12.5 and 25.0 units/ml) were present.

in Fig. 2, a standard curve was obtained up to 50 units/ml of SOD by measuring the ratio of the peak height between the first signal of the DMPO-OOH spin adduct and that of manganese oxide. Between-run CVs for the assay using the same lot number of DMPO were 2.8% at a concentration of 12.5 units/ml, 3.1% at 3.1 units/ml and 7.4% at 1.6 units/ml. Many of the substances examined showed diminishing activities to DMPO-OOH at various concentrations (Fig. 3). For each concentration of the majority of substances,

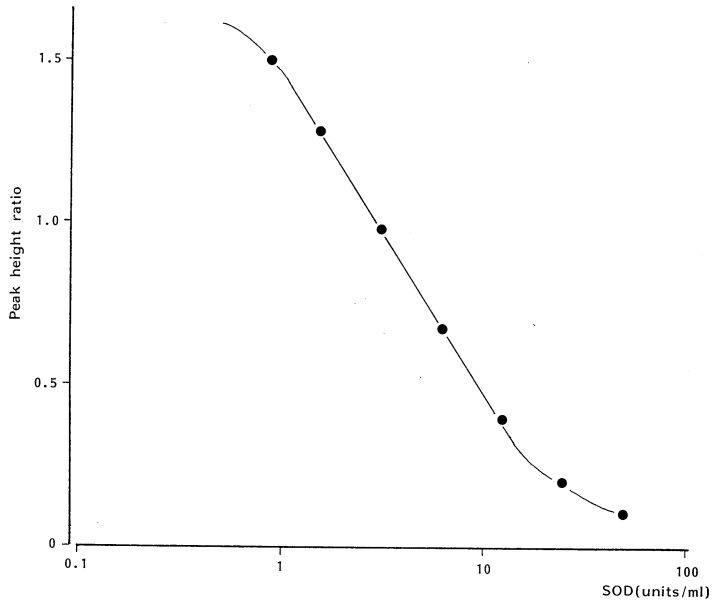


Fig. 2. A standard curve for superoxide dismutase activity. The peak height ratio was calculated from the peaks between the first signal of the DMPO-OOH spin adduct (arrow shown in Fig. 1A) and that of manganese oxide.

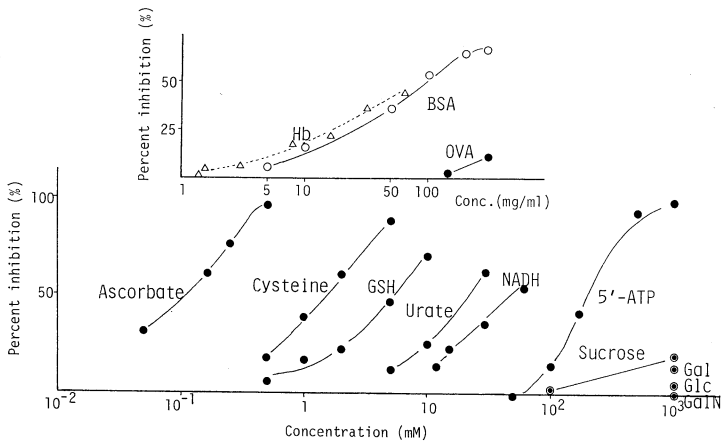


Fig. 3. Scavenging activity to the superoxide radical generated by the hypoxanthine-xanthine oxidase system. The abbreviations used are as follows: Hb: Hemoglobin, BSA: Bovine serum albumin, OVA: Ovalbumin, GSH: Reduced glutathione, NADH: Nicotinamide-adenine dinucleotide (reduced form), 5'-ATP: Adenosine-5'-triphosphate, Gal: Galactose, Glc: Glucose, GalN: Galactosamine.

a corresponding increase in the percent inhibition was observed. Excluding SOD itself, ascorbate diminished the peak for the DMPO-OOH spin adduct most remarkably in our experiment. We found that 0.5 mM ascorbate almost completely inhibited spin-trapping of the superoxide radical by DMPO. The XOD activity used for superoxide radical formation was preserved at the

vitamin concentrations used, which indicated that the decrease in the peak height for DMPO-OOH was a genuine scavenging effect of the vitamin. This vitamin is present in plasma at concentrations of 40–140 $\mu$M[9] and the 50% inhibitory concentration obtained in our experiment was 115 $\mu$M. Considering the high level in plasma and the high reactivity with the superoxide radical, $k = 2.7 \times 10^5$ $M^{-1}s^{-1}$,[10] it is evident that one of the extracellular defenses against the superoxide radical depends on ascorbate as described elsewhere.[11] Since SOD activity in normal human plasma, if any, has been found in very low amounts,[3] the presence of ascorbate should be considered when we determine the enzyme activity in plasma. However, the rate constant for the reaction between SOD and superoxide radical is reported to be $2 \times 10^9$ $M^{-1}$ $s^{-1}$,[12] which is about $10^4$ greater than that between ascorbate and the radical. Therefore, we investigated the scavenging activity of ascorbate in coexistence with 1.5 units/ml of SOD (Fig. 4). As shown in Fig. 4, the activity of the vitamin was dose-dependent, even though SOD showed high reactivity to the radical. Cysteine and reduced glutathione (GSH) also showed inhibitory effects on DMPO-OOH. On the other hand, a marked increase in the peak for DMPO-OH was obtained. When a large excess of thiol compounds, for example 100 mM GSH or 10 mM cysteine, was added to the system, only the ESR spectrum corresponding to DMPO-OH was detected under our experimental conditions (data not shown), although there is no appreciable amount of GSH and cysteine in plasma. Urate, with a plasma level of about 300 $\mu$M,[9] did not inhibit the DMPO-OOH spin adduct until it reached a concentration of more than 2 mM. NADH and 5'-ATP also only affected the DMPO-OOH adduct at high concentrations. The magnitude of the DMPO-OOH adduct was slightly affected or unaffected by the presence of 1 M sugars such as glucose, galactose, galactosamine and sucrose.

Proteins are important antioxidants in plasma and the inhibitory effect of albumin has been reported in some peroxidation systems.[13] It is reasonable to suppose that the high concentration of albumin in plasma (about 40 mg/ml) makes it one of the major antioxidants. As shown in Fig. 3, BSA was sufficiently sensitive to the detection of the superoxide radical above a concentration of about 5 mg/ml. Ovalbumin, on the contrary, showed little effect in our

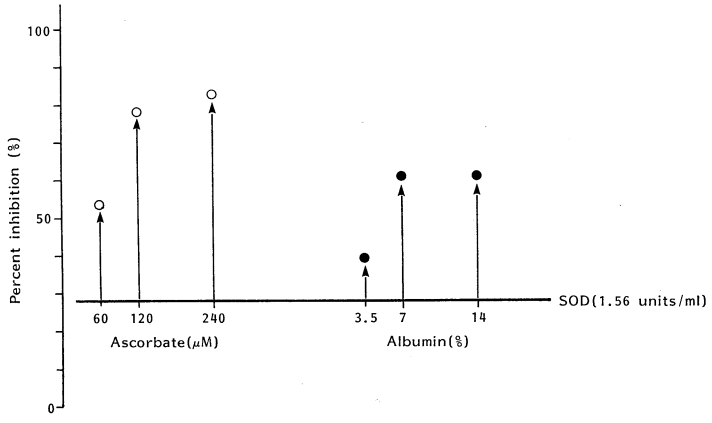


Fig. 4. Diminishing effect of ascorbate and albumin for the DMPO-OOH spin adduct in coexistence with 1.5 units/ml SOD.

experiment. The effect of albumin was also observed when it coexisted with 1.5 units/ml of SOD (Fig. 4). Our results indicate that the extracellular defense against "active oxygen" possibly depends on proteins as well as on a low level of SOD and low molecular weight substances such as ascorbate. When SOD activity in plasma is assayed by the ESR method, the level of activity must be just within the standard range for SOD activity as shown in Fig. 2. Therefore, we found it impossible to dilute the plasma to such a degree that the albumin concentration would be negligible and SOD activity could also be measured.

On the other hand, it is well-known that a high and relatively constant amount of SOD exists in the erythrocytes of many vertebrates.[14] In assays for SOD in erythrocytes, many other methods require a removal step for hemoglobin. In general, this is accomplished by treatment of the hemolysate with chloroform/ethanol. The removal of hemoglobin, however, is a rather laborious procedure and the final yield of SOD obtained is relatively poor. As shown in Fig. 3, freshly obtained human hemoglobin did not diminish the magnitude of the peak for DMPO-OOH until it reached a concentration of more than about 1.5 mg/ml. Hemoglobin containing up to 75% methemoglobin obtained commercially showed a 50% inhibitory effect at 0.33 mg/ml (data not shown). Therefore, it was concluded that the inhibitory effect of hemoglobin could be clearly overcome by diluting the hemolysate 50-100 fold when we wished to determine the SOD activity in freshly obtained erythrocytes. The SOD activity in rat erythrocytes obtained by the ESR method was $2780 \pm 360$ units/g hemoglobin ($n = 10$).

The ESR method appears to be the most suitable method for measuring the SOD activity in small crude samples in which there are significant amounts of SOD activity, such as erythrocytes. It was not necessary to purify the samples and interference by reactants could be overcome easily by dilution. The level of SOD activity, however, could not be accurately assayed by this method in crude samples such as plasma containing a slight amount of SOD activity and some scavengers having a high concentration and high reactivity.

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