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On the spin trapping and ESR detection of oxygen-derived radicals generated inside cells

(hydroxyl radical/superoxide radical/mammalian cells)

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ABSTRACT **Recently several attempts to identify oxygen-derived radicals in whole cells by spin trapping and electron spin resonance have been reported by using 5,5-dimethyl-1-pyrroline-*N*-oxide as the spin trap. In the present study, the feasibility of this method is examined. Chinese hamster V79 cells and human erythrocytes served as the test systems, while OH radicals were generated by γ radiolysis. Several spin traps were used to scavenge the radicals and a distinction between exo- and endocellular ESR observable species was achieved using tri(oxalato) chromiate(III) as a line broadening agent. To distinguish between exo- and endocellular sites of radical formation, we studied the effects of high molecular weight scavengers (polyethylene glycols), which do not enter the cell. Various possible obstacles associated with trapping and detecting the radicals inside the cells were examined. The results indicate that the primary radicals react with the spin traps. However, these spin adducts decayed within the cells. Cellularly induced decay of 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxyl radical presented the major difficulty in detecting the endogenous radicals, and potential experimental approaches to overcome this difficulty are discussed.**

Oxygen-derived radicals in various biological systems under physiological conditions have been identified by using spin-trapping and electron spin resonance (ESR) spectrometry mainly in solutions or in *in vitro* systems containing subcellular components as with tissue homogenates (1–3). By contrast, few attempts were made to use this technique with whole cells. With this technique, OH and $O_2^{-\cdot}$ radicals, which cannot be detected by ESR under physiological conditions, react with spin traps, usually nitrones or nitroso compounds, yielding long-lived radicals observable by ESR. Initially, the spin trapping of radicals within cells might appear inapplicable in view of the high concentrations of the DNA, RNA, proteins, amino acids, etc. Analysis of competition kinetics indicated that despite the high concentrations of certain cellular constituents and large rate constants of their reactions with free radicals (4), various scavengers, including spin traps, can scavenge at least part of the radicals within whole cells (5). Even though formation under normal physiological conditions of oxygen-derived radicals by cells is well established, their detection by spin trapping might be difficult for several possible reasons.

(*i*) The steady-state concentrations of free radicals within the cell might escape detection if their production rate is too low or decay rate is too high. (*ii*) A significant fraction of these radicals might be produced site specifically near their biological targets. Such radicals would therefore be almost nonscavengeable by radical scavengers in general, including spin traps. (*iii*) Insufficient as well as slow uptake of the spin trap into the cells or facilitated metabolic removal of the spin trap might result in a failure to effectively compete for the free radicals. (*iv*) The spin trap might accumulate in cellular compartments other than those in which the radicals are produced. (*v*) Spin adducts formed within the cell might disappear via metabolic or other pathways that effectively shorten their life time. (*vi*) Restricted motion of the spin adduct inside the cell might cause broadening of the ESR lines and consequently reduce the signal intensity. In spite of these potential limitations, several reports have been published recently describing the cellular generation of OH and $O_2^{-\cdot}$ radicals and their subsequent detection and identification by using spin-trapping techniques (6–11). Spectral characteristics of the spin adduct and the competitive effect exerted by radical scavengers were used to identify and locate the radicals. Yet, conclusive evidence establishing endocellularly formed radicals is indeed desired.

Various normal metabolic processes are assumed to produce radicals; moreover, under pathogenic conditions or drug-induced conditions, a distinction between radical species formed inside or outside the cell (or spilled out) is important in determining their role and location in cellular systems. Although spin trapping is generally a powerful tool for tracing the participating free radicals (3), we have encountered difficulties when attempting to reproduce previous experiments spin trapping OH radicals in erythrocytes. In view of the experimental barriers listed above and because of the wide interest in spin trapping of free radicals within cells, we examined the general feasibility of applying this technique to whole cells. To that end, we studied several cellular test systems and various spin traps. The radicals were generated by γ radiation, and the distinction between the endo- and exocellular radicals was achieved by using line broadening agents and high molecular weight scavengers that are practically excluded from the cell.

MATERIALS AND METHODS

Chemicals. α-Phenyl-*N*-*tert*-butylnitrone (PBN), α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), α-(4-*N*-methylpyridinium-*N*-*tert*-butylnitrone (PyBN), and the stable nitroxide 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) were purchased from Aldrich. Polyethylene glycols (PEG) of various sizes (Sigma), oxalic acid, potassium oxalate, potassium dichromate, and $K_3[Fe(CN)_6]$ (Fisher) were used without further purification. DMPO was purified before use by distillation or activated charcoal. The DMPO concentration in aqueous solutions was

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Abbreviations: PBN, α-phenyl-*N*-*tert*-butylnitrone; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; PyBN, α-(4-*N*-methylpyridinium)-*N*-*tert*-butylnitrone; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; Me_2SO, dimethyl sulfoxide.

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determined spectroscopically. Tri(oxalato) chromiate(III) $[K_3[Cr(C_2O_4)_3]\cdot 3H_2O]$ (chromium oxalate) was prepared as described (12). Solutions were freshly prepared with milli-Q reagent water (18 MΩ), and the experiments were conducted aerobically at room temperature.

Radiation. The samples were irradiated at room temperature using a ^{60}Co γ ray source with a dose rate of 51 Gy·min^{-1}. After irradiation, the samples were scanned for their ESR spectra.

ESR Measurements. Unless otherwise stated, spectra were taken with a Varian E-9 X-band spectrometer (9.5 GHz) with a field modulation frequency of 100 KHz, using microwave power of 10 mW and modulation amplitude of 0.4 G.

Cells. Chinese hamster V79 cell lines and human erythrocytes were used. Logarithmic-phase V79 cells were harvested shortly before the experiment from monolayers and washed four times with and suspended in phosphate-buffered saline (PBS). Subsequently, the spin trap was added and the cell suspension was γ-irradiated in glass vessels for 1 min. Immediately afterward, aliquots were taken and their ESR spectra were monitored. Fresh erythrocytes were prepared similarly.

RESULTS

To distinguish between factors affecting the spin trapping of radicals within the cell, we chose to produce the primary radicals radiolytically. This technique has several advantages: (*i*) it ensures the generation of the water-derived primary free radicals homogeneously throughout the cell rather than in certain subcellular compartments; (*ii*) it is possible to modify the ratio between endo- and exocellular formation of the radicals by varying the cell concentration; (*iii*) the radiolytic generation of the radicals also makes it possible to control the production rate as well as the spectrum of the predominant species (by appropriate scavengers).

Effect of Line Broadening Agents. In principle, a distinction between the ESR signals arising from endo- and exocellular species can be achieved (13) with selective line broadening agents that only very slowly enter the cells. In the present study, we have used chromium oxalate, which was previously found to be superior to ferricyanide or Ni(II) (14). Immediately after the addition of 30 mM chromium oxalate to 29 μM TEMPO, the ESR signal disappeared. Since the line broadening effect is due to spin–spin interaction, the ESR signal reappeared upon suitable dilution. Thus, we added to a PBS solution of the stable free radical nitroxide, TEMPO, either erythrocytes, chromium oxalate, or both, and we monitored the ESR spectra obtained. The results presented in Fig. 1 indicate that in the absence of cells chromium oxalate fully abolished the TEMPO signal (Fig. 1*c*). By contrast, in the presence of both 5.6×10^9 erythrocytes per ml and 30 mM chromium, ≈50% of the signal persisted (Fig. 1*b*), indicating that this fraction was inaccessible to line broadening. In the presence of the cells the TEMPO radicals, which otherwise survive for days, decayed with a half-life of ≈2 hr at room temperature. Evidently, as was found previously for bacterial cells (5), the TEMPO radicals entering the cells became shielded from the line broadening effect but also decayed faster. Similar results with erythrocytes and chromium were observed for the stable 3-carbamoyl-2,2,5,5-tetramethyl pyrrolidine-1-yloxy free radical (data not shown). The same procedure was repeated with a spin adduct rather than a stable nitroxide. Since spin adducts of carbon-centered radicals are generally more stable than the adducts of oxygen-derived radicals, we used the methyl adduct of the spin trap 4-POBN. A solution containing both 1 M dimethyl sulfoxide (Me_2SO) and 0.1 M 4-POBN was γ-irradiated and subsequently frozen. Later it was thawed, mixed with either 5.1×10^9 erythrocytes per ml or 30 mM chromium oxalate,

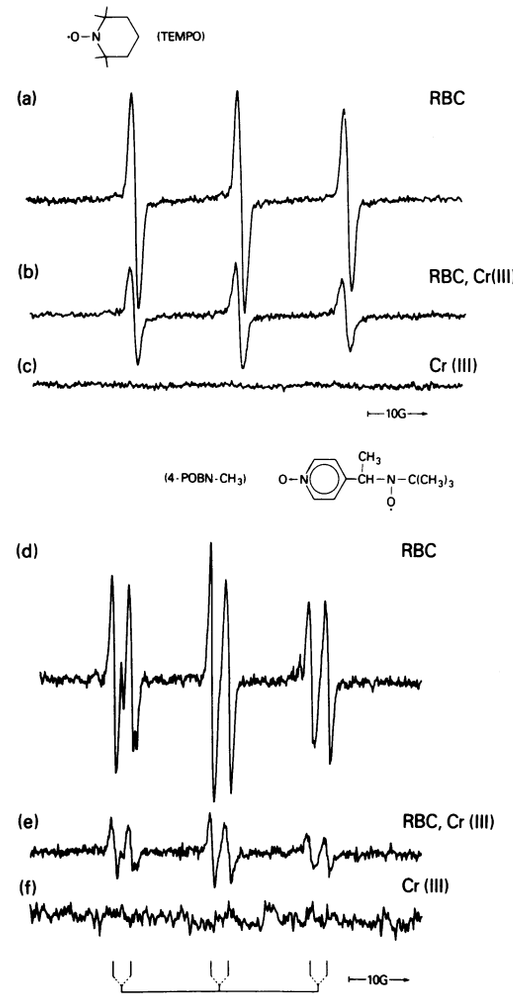

FIG. 1. The ESR spectra of 29 μM TEMPO in PBS measured in the presence of 5×10^9 erythrocytes (RBC) per ml (*a*); 30 mM chromium oxalate and erythrocytes (*b*); 30 mM chromium oxalate (*c*); the ESR spectra for 0.1 M 4-POBN in PBS γ-irradiated for 1 min at room temperature in the presence of 1 M Me_2SO, measured after addition of 5×10^9 erythrocytes per ml (*d*); both erythrocytes and 30 mM chromium oxalate (*e*); 30 mM chromium oxalate (*f*).

or both, and the ESR spectra were recorded. The results in Fig. 1 *d* and *e* show a six-line spectrum having the hyperfine coupling constants a_N = 15.9 G and a_H = 2.65 G, characteristic of 4-POBN-CH_3. The radiolytically formed OH radicals reacted with Me_2SO, yielding methyl radicals. A certain fraction of the methyl radicals in turn add to 4-POBN, yielding ESR observable adducts. These relatively stable nitroxides, unlike 4-POBN-OH, which is short-lived (15), persisted as seen in Fig. 1. The presence of the cells did not alter the half-life of the 4-POBN-CH_3 ($t_{1/2}$, ≈2h) significantly. On the other hand, ≈30% of the ESR signal was shielded from the broadening agent when both cells and chromium oxalate were present. The magnitude of the residual signal is consistent with a calculated ≈44% volume fraction occupied by 5.1×10^9 erythrocytes per ml (the mean corpuscular volume is 86 ± 8 μm^3 for erythrocytes). The residual signal of 4-POBN-CH_3 increased with an increase in cell concentration but did not depend on the incubation time of the cells with the spin adduct. This indicates the ease with which the spin adducts cross the cell wall. The above results indicate (*i*) the spin adducts readily partition between the exo- and the endocellular environments; (*ii*) the width of the ESR spectral lines, beside the high-field doublet, due to endocellular species was not broadened; (*iii*) the relative populations of

exo- and endocellular probes were readily accessible; (*iv*) the 4-POBN-CH_3 spin adducts maintained their stability within the cells.

Similar results (data not shown) were observed when PBN or PyBN were used as spin traps. These results also indicate a limitation of the use of line broadening agents. Chromium oxalate discriminates between exo- and endocellular free radicals according to their location rather than site of formation. Spin adducts formed outside can diffuse into the cell and become shielded from the line broadening. A residual ESR signal observable in the presence of a line broadening agent does not prove endocellular formation of radicals, whereas total elimination of an ESR signal by the chromium oxalate, as was found for activated polymorphonuclear leukocytes, does not exclude endocellular formation of the spin adducts (11). The relative contribution of the volume of the cells to the overall volume in dilute suspension is so small that the free radicals present could escape detection. This may be true, provided that a rapid dynamic exchange of the probe across the cell wall prevails and the partition of the radicals occurs according to the relative endo- and exocellular total volumes.

Spin Trapping of OH and Superoxide Radicals in Cells. Despite numerous experimental artifacts associated with the use of DMPO as a "universal" spin trap (16), it is still widely used. DMPO is a particularly useful probe for trapping oxygen-derived radicals because of the relative stability and the diverse spectral characteristics of the spin adducts (6–11). In addition, DMPO appeared to be an optimal choice in view of its low cytotoxicity, accessibility to the cell, and high rate constant of reaction with OH radicals (15, 17). For these reasons, we examined it in our system. The reaction of DMPO with superoxide has a low rate constant [≈ 10 $M^{-1}\cdot sec^{-1}$ (18)] and yields an unstable spin adduct (19). Nevertheless, with DMPO the trapping and detection of superoxide radicals appeared feasible since superoxide radicals have a longer half-life and the spin adduct is transformed into the more stable DMPO-OH adduct (19).

To compete effectively for the OH or $O_2^{\cdot-}$ inside the cell, it was desirable to use high concentrations (≈ 0.1 M) of the spin trap. A comparison of rate constants of $H^{\cdot}$ and hydrated electrons (e_{aq}^-) with DMPO [4×10^9 $M^{-1}\cdot sec^{-1}$, 2×10^{10} M^{-1} (20)] and with oxygen implied that practically all H atoms and hydrated electrons would react with DMPO (100 mM), yielding DMPO-H, rather than with oxygen (0.28 mM) to form superoxide. Therefore, no difference was expected between systems irradiated in the presence or absence of air. Since only 35% of the OH radicals reacting with DMPO lead to an ESR observable spin adduct (21), a buildup of ≈ 5 μM DMPO-OH following 60 sec of irradiation was expected, provided this spin adduct would remain long-lived (18, 22).

To maximize further the chances of detecting radicals in the cell, we used concentrated cell suspensions. Chinese hamster V79 cells were suspended in PBS (2.2×10^8 cells per ml; mean corpuscular volume, 1100 μm^3) and γ-irradiated for 60 sec in the presence of 0.2 M DMPO. A scan of the cell suspension (3 min after irradiation) showed no detectable ESR signal. The results of an analogous experiment with fresh human erythrocytes are presented in Fig. 2 *a–c*. The ESR spectra of a 0.2 M DMPO solution obtained 2 min after irradiation in the absence (Fig. 2*a*) and in the presence (Fig. 2*b*) of 4.1×10^9 erythrocytes per ml are shown. The ESR signal consisted of the 1:2:2:1 quartet typical of the DMPO-OH spin adduct with $a_N = a_H = 14.9$G (15, 18, 23), and the triplet of triplets having $a_N = 16.5$G, $a_{H(2)} = 22.6$G identified as the DMPO-H spin adduct (24). Fig. 2*b* illustrates that in the presence of erythrocytes the signal of DMPO-OH was markedly decreased when compared to the cell-free system. In addition, the experiment was repeated with 30 mM chromium oxalate added to the cells immediately after irradiation. Under these conditions, the cells constituted $\approx$35% of the total volume of the sample. Therefore, $\approx$35% of the radiolytically formed radicals originate inside the cells. Since chromium oxalate (within the time range of the experiment) selectively eliminates the ESR signal of the exocellular species, a residual signal due to the endogenous radicals was anticipated. Yet, even with a 10-fold higher spectrometer gain, no ESR signal was detected. This failure to observe any residual signal in the presence of the line broadening agent indicates that the ESR signal is due to the exocellular species. In other words, neither DMPO-OH nor DMPO-H adducts located in or outside the cell were detected.

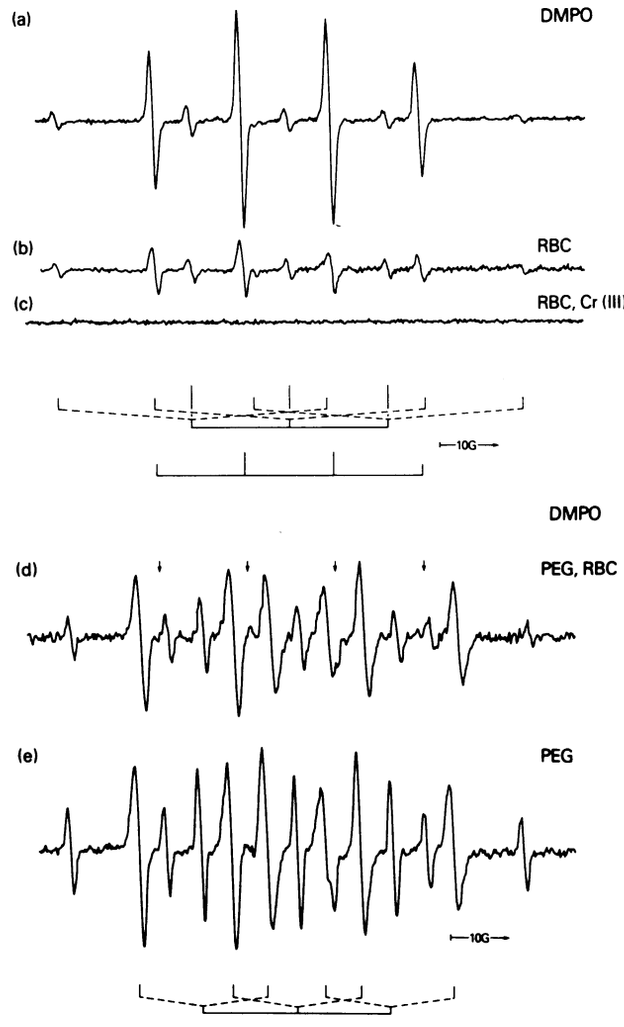

FIG. 2. The ESR spectra of 0.1 M DMPO in PBS γ-irradiated for 60 sec at room temperature in the absence (*a*) and in the presence (*b*) of 4.1×10^9 erythrocytes (RBC) per ml, and measured 2 min after irradiation. (*c*) Same as *b*, but with 30 mM chromium oxalate added after irradiation. (*d*) ESR spectrum obtained for 0.1 M DMPO in PBS after γ-irradiation of 0.1 M DMPO in PBS for 1 min in the presence of 0.1 M PEG 3500; (*d*) with 5.1×10^9 erythrocytes per ml; (*e*) control. Arrows designate locations of the 1:2:2:1 quartet due to DMPO-OH.

To substantiate the above finding, we used a high molecular weight scavenger of free radicals to distinguish between the endo- and the exocellular formation of the radicals.

Effect of High Molecular Weight Scavengers. Polyethylene glycols (PEG) rapidly react with OH and H radicals, primarily through H abstraction ($k = 2.4 \times 10^8$ $M^{-1}\cdot sec^{-1}$), on a monomer basis (25). These polymers, $H(OCH_2CH_2)_nOH$, PEG 3500 (n = 68–84), or PEG 8000 (n = 125–180) are incapable of penetrating into the cell, and therefore only scavenge radicals outside the cell. PEG 3500 (0.05 M) was γ-irradiated for 2 min in the presence of the spin trap PBN.

The resulting ESR signal consisted of triplet of doublets, having a_N = 15.85G and a_H = 2.9G attributable to the PEG spin adduct.

The subsequent introduction of 4.1×10^9 erythrocytes per ml had no significant effect on the signal. On the other hand, 30 mM chromium oxalate, even in the presence of the cells, fully eliminated the signal. Therefore, the PBN-PEG adducts were confined to the exocellular environment. Thus, in the presence of DMPO and excess PEG, DMPO is expected to trap OH radicals inside the cell but to react with the PEG radicals outside the cell.

After the γ-irradiation of a solution of 0.1 M DMPO in the presence of 0.1 M PEG 3500, the ESR spectra seen in Fig. 2*e* were observed. The nine-line signal of the DMPO-H adduct (Fig. 2 *a* and *b*) resulting from H· and e^-_{aq} is observed, whereas the 1:2:2:1 quartet of the DMPO-OH is replaced by the six-line spectrum of DMPO-PEG spin adduct having hyperfine coupling constants of a_N = 15.75G and a_H = 21.6G. These values were not that different from the DMPO spin adduct of the monomer $(CH_2OH)_2$, a_N = 15.6G, a_H = 22.5G. The ESR spectra of DMPO-PEG and DMPO-OH are easily distinguishable and offer a measure of the relative contributions of endo- and exocellular spin adducts to the overall ESR signal. When the concentrated erythrocyte suspension was γ-irradiated in the presence of both 0.1 M DMPO-OH and 0.1 M PEG 3500, the ESR signal of DMPO-PEG was observed, but the DMPO-OH could not be detected (Fig. 2 *d* and *e*). In fact, even in the absence of PEG the signal of the DMPO-OH adduct (Fig. 2*b*) was much smaller than that observed in a cell-free system (Fig. 2*a*). This could be due to a failure of the spin trap to enter the cells or to a decrease in the half-life of the spin adduct. To determine whether the spin trap is indeed taken up by cells without being metabolized, 0.15 M DMPO was incubated for 40 min in the absence or in the presence of 5.1×10^9 erythrocytes per ml (≈50% packed cells) and centrifuged at 800 rpm for 10 min. Subsequently, the two systems were spectrophotometrically assayed for DMPO. No difference was observed in the shapes and intensities of the absorption peaks (227 nm) from DMPO. In addition, the cell pellet was diluted 1:1 with buffer, resuspended, recentrifuged, and assayed for DMPO. After accounting for dilution, the resulting absorption spectrum of DMPO remained unchanged, which indicates that DMPO reversibly partitions between the endo- and exocellular environment.

Effect of Cells on the Lifetime of Exocellular Spin Probes. Cellularly induced decay of spin labels has been previously investigated because it interferes with their function as radiosensitizers (26) and as cellular probes (27). Probably by metabolizing the free radicals, various cells or cellular components were reported to hasten the decay of ESR signals from stable nitroxides and spin adducts (26–31). Therefore, we examined the effect of cells on the DMPO-OH spin adduct. DMPO solutions were γ-irradiated for 60 sec, diluted to keep the DMPO-OH concentration below 10 μM; then cells were added to the sample and the decay of the ESR signal was followed. The DMPO-OH adduct at concentrations <10 μM and in the absence of impurities is relatively long-lived (18, 22). By contrast, DMPO-OH decayed rapidly upon addition of cells. This decay rate increased with cell concentration. Fig. 3 displays decay curves of the ESR signal monitored for increasing cell concentrations and indicates the marked decrease in the half-life of DMPO-OH in the presence of cells.

Factors Affecting the Decay of the Spin Adduct. It has previously been suggested for Ehrlich ascites cells that the conversion of spin label into diamagnetic species, probably through reduction, might occur at the cell surface (31). An alternative explanation is that the radicals might penetrate into the cells and there undergo bioreduction (26–28, 32). The chemical reaction in both cases might be mediated enzymatically. An enzymatic bioreduction would depend on the physiological state of the cell. Hence, we examined the possible effects of cyanide, azide, and heat-inactivation of the cells on the decay of the spin adducts. Preincubation of the erythrocytes (≈50%) for 10 min with 1–10 mM cyanide increased the half-life of the spin adducts ≈2-fold. Conversely, exposure of the erythrocytes to 10 mM azide for 10 min had no effect on the decay rate of DMPO-OH. Preheating the erythrocytes at 60°C for 5 min had no significant effect; however, a prolonged incubation of the cells (50% erythrocytes) at 60°C for 30 min immediately prior to their addition to the DMPO-OH solution, increased the half-life of the spin adduct 2- to 3-fold as compared to intact cells. This indicated that both enzymatic and nonenzymatic pathways contribute to the disappearance of the spin adducts. In addition, since previous studies implicated sulfhydryl groups in the reduction of the radicals (27, 31, 32), we studied the effects of sulfhydryl blockers. To that end, the erythrocytes were preincubated with 10 mM diamide or *N*-ethylmaleimide for 10 min with hardly any effect on the cell-induced decay of DMPO-OH. Therefore, no evidence for the involvement of SH groups in the decay of the spin adducts could be established.

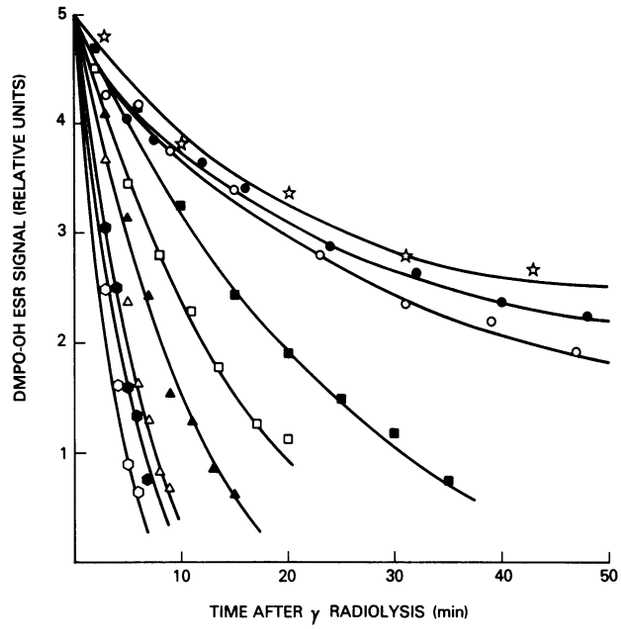

FIG. 3. DMPO solutions (0.1 M) were γ-irradiated for 1 min and then diluted to give ≈10 μM DMPO-OH spin adduct; erythrocytes at various concentrations were added and the decay of the ESR signal at room temperature was followed. ☆, No cells; ●, 0.5%; ○, 1%; ■, 5%; □, 10%; ▲, 20%; △, 30%; ◐, 40%; ◑, 50% packed cells.

In analogous experiments, erythrocytes were preincubated with 5 mM diethylenetriaminopentaacetic acid for 20 min before adding DMPO-OH to the cells. No effect of the chelating agent on the decay of the spin adduct was observed, indicating that transition metal ions were not mediating this decay.

Reversibility of the Reaction. The rapid disappearance, in the presence of cells, of the ESR signal of the paramagnetic species could result from the reduction of the spin adduct or by other forms of chemical destruction. The reversibility of the process by an oxidizing agent would indicate the former alternative. To examine this possibility, 4×10^9 erythrocytes per ml were added to a solution of preformed DMPO-OH adduct. After total disappearance of the spin adduct signal, 1 mM ferricyanide was added: the signal was not restored.

DISCUSSION

Radical Production in the Cell. In the present study, high yields of OH radicals were radiolytically formed throughout the cell. Therefore, the first two of the six main obstacles listed above for spin trapping and detection of radicals within the cells did not exist, and the probability of trapping and detecting them was maximized.

Spin-Trap Permeation into Cells. The quantitative determination of DMPO in cell-free and cell-containing systems indicated that this spin trap partitioned roughly equally between the cellular and the exocellular domains. In addition, no significant decomposition of DMPO in the presence of erythrocytes was observed. Furthermore, the experiments depicted in Fig. 1 *d–f* suggested that the spin adducts are rapidly taken up by cells. This can be concluded because of the inaccessibility of the spin adducts to the exocellular line-broadening agent.

Spin Trapping. The average concentration of the principal cellular constitutents, including nucleic acids, nucleotides, proteins, and amino acids, were evaluated in the past assuming their homogeneous distribution throughout the cell (4). The respective rates of reaction (k times concentration) of the primary water-derived radicals OH, H, and e^-_{aq} with these cellular constituents have been considered and compared with the respective rates of reaction with various radical scavengers (5). This comparison indicated that scavengers such as *tert*-butyl alcohol, EtOH, 2-PrOH, and mannitol, within the concentration range commonly used for radioprotection, would trap a significant fraction of the radicals (5), provided that the endocellular concentration of the scavenger was comparable to that of the exocellular medium. Similar consideration of the reaction rate constant of DMPO with OH radicals indicates that it would trap a significant fraction of them in spite of their very short half-life [$\approx$1 nsec (4)] within the cell. With superoxide, on the other hand, DMPO reacts very slowly [$k = 10\ sec^{-1} \cdot M^{-1}$ (18)]; however, effective spin trapping is still anticipated in view of the relatively long half-life of superoxide radicals and their low reactivity toward most cellular constituents.

The Decay of the Spin Adduct. Evidently, in the present study the conversion of the spin adducts within the cells to diamagnetic species occurred prior to any observable accumulation. The failure, therefore, to detect the spin adduct in the irradiated cell seems to result primarily from the rapid decay of the spin adduct. This is corroborated by the present observation that even the exocellular spin adducts rapidly disappeared with a decay rate that increased with the concentration of the cells (Fig. 3), suggesting that spin adducts inside the cell would be removed much faster. The heating of the cells partially reduced the rate of the cellularly induced decay of the spin adduct but did not eliminate it. This implies that in addition to metabolic pathways by which it can be blocked as discussed (26–32), the spin adduct might be converted into an ESR-silent species via some nonenzymatic mechanism. Supporting this conclusion is the lack of protection by azide and the finding that cyanide provided only a small protective effect. Also, the failure of ferricyanide to restore the spin adducts suggests that their chemical modification exceeded a mere reduction to the corresponding hydroxylamine.

The present results clearly indicate that the main difficulty in applying the spin-trapping technique to oxygen-derived radicals formed in cells stems from the chemical instability of the resulting spin adducts rather than from ineffective trapping of the primary radicals of interest. This implies that failure to detect the spin adduct (11) does not necessarily exclude the participation of the OH or $O_2^{\cdot-}$ radicals, as the resulting spin adducts might escape detection. On the other hand, cases of cellular systems in which DMPO adducts are observed should be carefully checked. For instance, it is unlikely that DMPO-OH adducts formed inside cells will survive detection (6–11), although cells other than V79 or erythrocytes might have a smaller effect. Therefore, a careful and critical review of spin trapping of OH or $O_2^{\cdot-}$ radicals by DMPO inside the cells would be desirable.

To improve this technique for study of cellular systems, it is imperative to increase the stability of the resulting spin adduct. This might be achieved either by paralyzing the reductive capability of the cells or by using spin traps whose adducts are more stable in the cellular milieu. The first approach may not be easy, as the nonenzymatic removal of the spin adduct, unlike its bioreduction, is less controllable. On the other hand, the use of more suitable spin traps, such as 5,5,2-trimethyl-1-pyrroline-1-oxide, appears more promising. The spectral discrimination achievable by the spin adducts of this trap is poor, but the lack of a β-hydrogen in the resulting spin adducts makes them more stable toward reduction. An alternative experimental approach would be to convert OH to methyl radicals (e.g., by Me_2SO) and to trap the carbon-centered radicals inside the cell (10). These spin adducts are far more stable than DMPO-OH and their detection, although not without difficulties, is feasible.

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