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The Reduction of a Nitroxide Spin Label as a Probe of Human Blood Antioxidant Properties

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The kinetics of reduction of the radical $R^{\bullet}$, 5-dimethylaminonaphthalene-1-sulfonyl-4-amino-2,2,6,6-tetramethyl-1-piperidine-oxyl, by blood and its components were studied using the EPR technique. The results demonstrate that $R^{\bullet}$ is adsorbed to the outer surface of the membrane and does not penetrate into the erythrocytes. A series of control experiments in PBS demonstrate that ascorbate is the only natural reducing agent that reacts with $R^{\bullet}$. The observed first order rate of disappearance of the nitroxide radical, k, is: $k_{blood} > k_{eryth} > k_{plasma}$ and $k_{blood} \cong k_{eryth} + k_{plasma}$. The results demonstrate that:

a. The erythrocytes catalyze the reduction of $R^{\bullet}$ by ascorbate.

b. The rate of reduction of the radical is high though it does not penetrate the cells.

c. In human erythrocytes there is an efficient electron transfer route through the cell membrane.

d. The study points out that $R^{\bullet}$ is a suitable spin label for measuring the reduction kinetics and antioxidant capacity in blood as expressed by reduction by ascorbate.

Keywords: Erythrocyte; EPR; Nitroxide; Antioxidant capacity; Ascorbic acid

INTRODUCTION

There is a growing interest in developing simple and accurate techniques for the determination of the antioxidant properties of biological samples, especially of blood, as it reflects different oxidative stresses in the body. It was found that stable nitroxide radicals (NRs) are reduced by blood and some other biological materials, to the corresponding hydroxylamines.[1,2] This reduction process can be followed by EPR spectroscopy.[3–8] The kinetics of the nitroxides disappearance, thus measured, provide useful biochemical and biophysical information about the antioxidant properties of biological systems.[3–12] Most NRs are reduced by ascorbate and not by other biological antioxidants.[6] Therefore, NRs were used as models of persistent radicals to study the antioxidant function of ascorbate in human erythrocytes.[8] It has been shown that in erythrocytes the reduction occurs mainly intracellularly, as the lifetime of nitroxides that penetrate the cell is considerably shorter than that of charged not penetrating NRs.[6,12,13] NRs with fatty acid substituents, which are located in the membranes are reduced by intracellular components with lifetimes which are about one order of magnitude longer than the lifetimes of penetrating nitroxides.[3] The kinetics of reduction of NRs, which penetrate the erythrocytes are reported to obey first order rate laws, and the product of their reduction is the corresponding hydroxylamine.[6] The rate of reduction of NRs in whole blood of healthy human adults is the sum of the rates observed in plasma and in erythrocytes suspension in phosphate-buffered saline (PBS).[6] The mechanism of reduction has been

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studied and it was concluded that human erythrocytes regenerate ascorbate from dehydroascorbate in a GSH-dependent mechanism.[10,14–17]

It seemed of interest to check whether a NR, which does not penetrate the cells, but is adsorbed to its membrane is efficiently reduced by the intracellular antioxidants. For elucidating this question, the spin label $R^{\bullet}$, (5-Dimethylaminonaphthalene-1-sulfonyl-4-amino-2,2,6,6,-tetramethyl-piperidine-oxyl), was synthesized.[18] This label, due to its size, is not expected to penetrate into the cells, or to do so very slowly. However, due to its hydrophobic substituent, it is expected to adsorb to the membranes of the erythrocytes.

The reduction of $R^{\bullet}$ by a variety of antioxidants in PBS solutions was studied in order to determine its redox properties. The results clearly demonstrate that though $R^{\bullet}$ does not penetrate the cells, it is an effective label for the estimation of the antioxidant properties of the different blood components.

MATERIALS AND METHODS

Materials

Chemicals

All chemicals were of A.R. grade, purchased from Sigma Chemical Co. unless otherwise stated. All solutions were prepared in water with a resistance of $>10^7\,\Omega$/cm. The initial amount of spin label: 5-Dimethylaminonaphthalene-1-sulfonyl-4-amino-2,2,6,6,-tetramethyl-piperidine-oxyl, R, was a kind gift of professor K. Hideg, Pecs University, Hungary. Further samples of $R^{\bullet}$ were synthesized in our laboratory. In both cases $R^{\bullet}$ was analyzed by NMR and MS spectroscopy and the structure was confirmed. The nitroxide spin label (R) was dissolved in dimethyl-sulfoxide/PBS 1:1 to a concentration of about (less than) 10 mM. Twenty microliter of the solution of the nitroxide were added to 200 μl blood samples, erythrocytes or plasma, to a final concentration of about 1 mM.

Red Blood Cells and Plasma Preparation

Blood samples from normal healthy adults using edta as an anticoagulant were collected. The plasma and buffy coats were removed. Erythrocytes were washed three times in isotonic PBS solution, 0.075 M (NaH_2PO_4/Na_2HPO_4) and NaCl in a concentration of 0.075 M (pH 7.4). Following the third washing the cells were resuspended in PBS to the original hematocrit. Plasma was obtained by the first centrifugation of the blood.

Methods

EPR

EPR spectra were recorded using a Bruker EMX 220 X-band digital EPR spectrometer ($\nu = 9.4$ GHz) at room temperature. Non-saturating microwave power of 20 mW and 100 kHz magnetic field modulation of G were used to obtain correct line shapes and intensity ratios. Spectral acquisition, manipulation and calculation of parameters were done using Bruker's WIN-ACQ and WIN-EPR software.

Immediately following the addition of the spin labels, the EPR spectra were obtained. The kinetics of the NR reduction were monitored by tracking changes in the height of the midfield hyperfine line ($M_s = 0$) of the characteristic 3-line nitroxide spectrum. The magnetic field was fixed at the value which corresponds to the peak of the midfield signal and then the spectrum was run in the Time Scan mode. In order to assure that no changes in the spectrum line shape and ratios between signal intensities occurred, the EPR spectrum was also taken at the end of each kinetic run.

Determination of the Reduced Glutathione (GSH) Concentration[19]:

The measurement is based on the redox reaction between thiol groups ($-$SH) and the reagent DTNB (5,5′-Dithiobis-2-nitrobenzoic acid). DTNB is reduced in the reaction to form a yellow anion, the concentration of which is determined spectrophotometerically at 412 nm. The measurement is performed on the filtrate after protein denaturation.

RESULTS AND DISCUSSION

In order to verify that the spin label $R^{\bullet}$ is adsorbed to the erythrocytes membranes, the EPR spectra of $R^{\bullet}$ in PBS and in PBS containing 1 mM albumin and in solutions containing the different blood components were measured. Typical spectra obtained in these media are presented in Figs. 1 and 2. The spectrum in Fig. 1(b) clearly consists of two overlying spectra indicating that the spin label $R^{\bullet}$ is partially adsorbed to the albumin molecules. A detailed analysis of the spectra enables to calculate the binding ratio, e.g. the ratio between the amounts of $R^{\bullet}$, which are "adsorbed" (bound or immobilized) and "free" (non-immobilized) in the medium. This ratio is calculated using the equation:

$$\text{Ratio} = \left(1 - \frac{3\text{DIN}_3}{\text{DIN}}\right)$$

where DIN_3 is the double integral under the right line B_1 in Fig. 1(b), and DIN is the total double

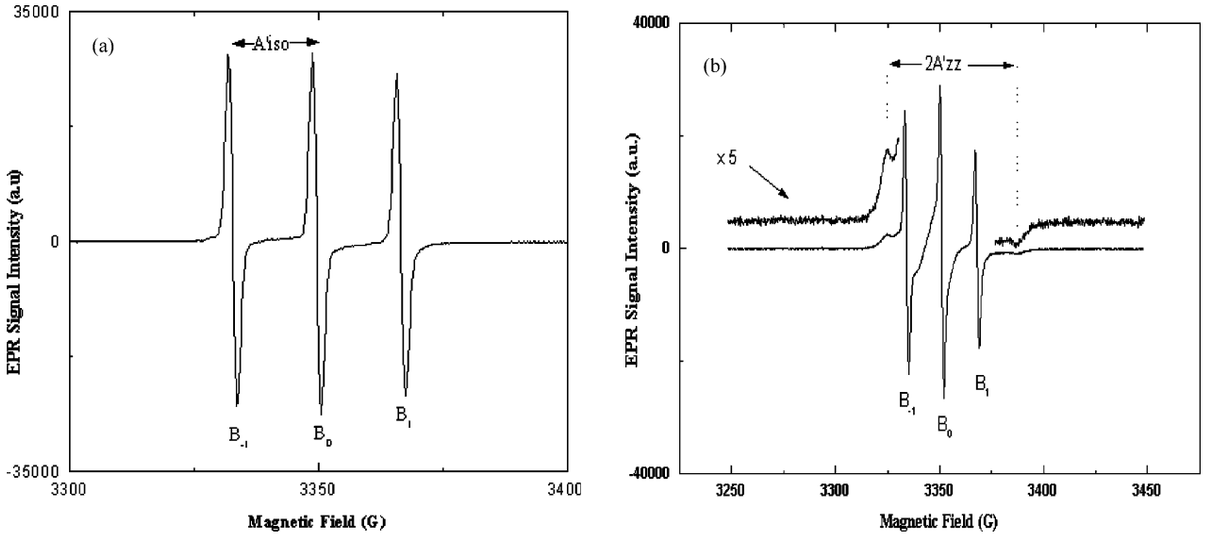

FIGURE 1 (a) EPR spectrum of R• in PBS. (b) EPR spectrum in PBS + 1 mM albumin.

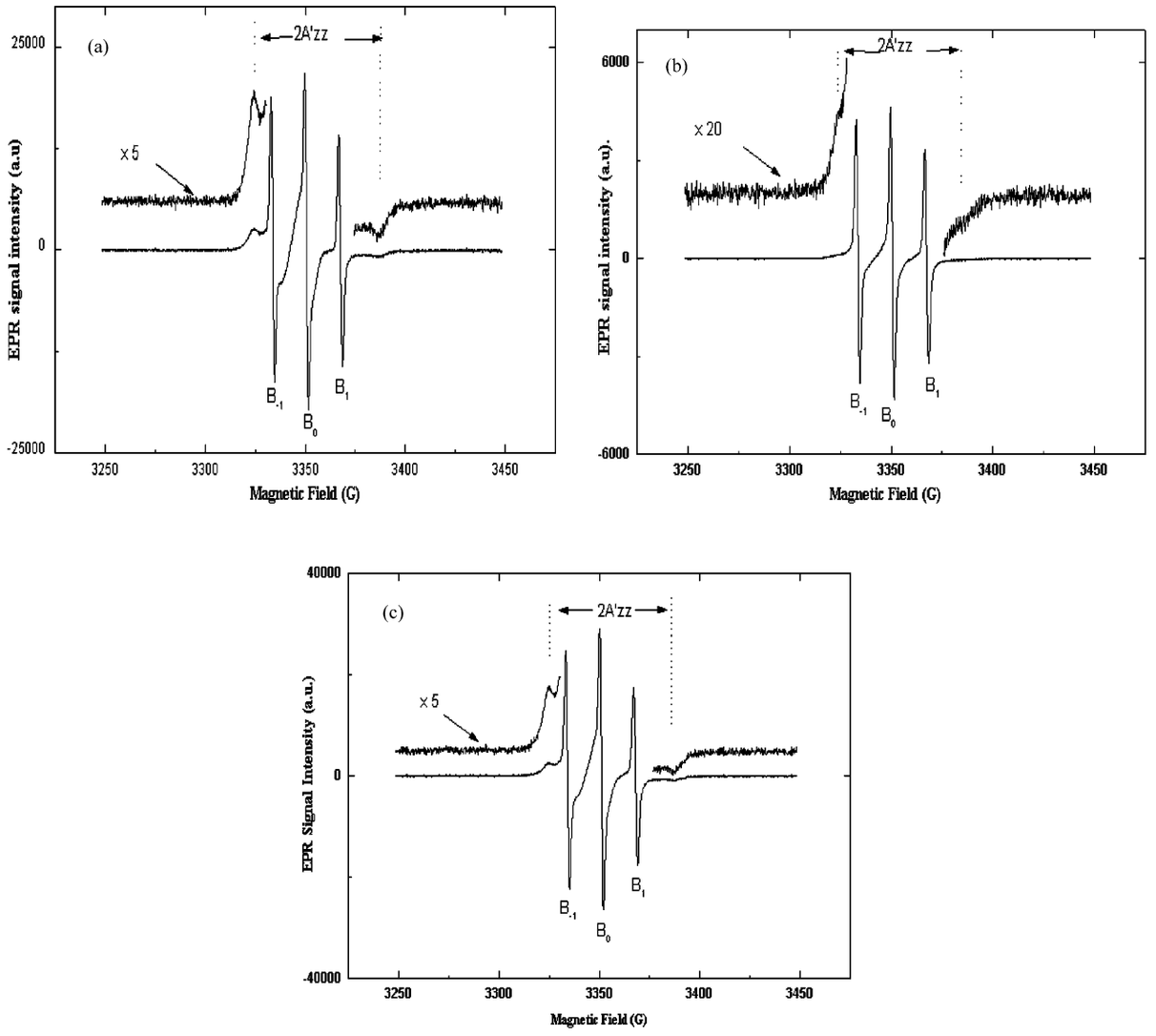

FIGURE 2 (a) EPR spectrum of R• in plasma. (b) EPR spectrum in erythrocytes suspension. (c) EPR spectrum in whole blood.

TABLE I Adsorption and rotation correlation times τ_c(s) of the immobilized labels

Medium	Fraction of adsorbed R	τ_c(s) of the "adsorbed" fraction
PBS + 1 mM Albumin	0.80 ± 0.08	$7.3 \times 10^{-9} \pm 7 \times 10^{-10}$
Plasma	0.88 ± 0.09	$6.8 \times 10^{-9} \pm 7 \times 10^{-10}$
Erythrocytes suspended in PBS, 50% Htc.	0.63 ± 0.06	$5.7 \times 10^{-9} \pm 6 \times 10^{-10}$
Whole blood, 50% Htc.	0.83 ± 0.08	$6.8 \times 10^{-9} \pm 7 \times 10^{-10}$
PBS	No binding	Fast rotation

integral. This equation is based on the observation that the right absorption line is due only to the mobilized fraction of the NR in the solution as its overlapping with the extreme right line is negligible. Therefore the ratio $3DIN_3/DIN$ equals the fraction of the mobilized R. It should be noted that the effect of adsorption on the EPR spectra decreases from the left line to the right line of the spectrum, i.e. the right line is due mainly to the non-adsorbed component of the radical. This observation is in accord with previous observations.[20]

The rotation correlation time (τ_c) of the bound radicals, which reflects the restricted mobility of the label adsorbed to the macromolecules on the membrane, was calculated as well, according to the equation.[20]

$$\tau_c = 5.4 \times 10^{-10}\left(1 - \frac{2A'ZZ}{74.4}\right)^{-1.36}$$

where A′zz- is the distance between the two peaks in the spectrum of the immobilized spin label. The results are summarized in Table I.

The same two forms of the spin label—"adsorbed" and "free"—are clearly distinguished also in the EPR spectra of $R^\bullet$ in the different blood components, (Fig. 2). The fraction of $R^\bullet$ adsorbed to macromolecules in these media and their correlation times are summarized in Table I. The spectra of $R^\bullet$ in plasma, Fig. 2(a), and in whole blood, Fig. 2(c), are similar to the spectrum of $R^\bullet$ in PBS + 1.0 mM albumin, (Fig. 1b). About 80% of $R^\bullet$ is adsorbed to some macromolecules, however, the τ_c values of the adsorbed fraction in these media are somewhat smaller than that of $R^\bullet$ adsorbed to albumin. The spectrum of $R^\bullet$ in erythrocyte suspensions differs significantly from these in the other media. However, in this medium too, the analysis of the spectra indicates that about 77% of $R^\bullet$ are adsorbed to "macromolecules" in the medium and the τ_c value for the adsorbed fraction in these suspensions is even slightly lower than those in the other media.

The results thus clearly demonstrate that the spin label $R^\bullet$ interacts with the erythrocytes. It was therefore decided to determine whether it penetrates the cells or is just adsorbed to the cell membrane. For this purpose the effect of $[Fe(CN)_6^{3-}]$ on the line width of the EPR signal of the NR was measured ($[Fe(CN)_6^{3-}]$ titration). It is well known that paramagnetic species, e.g. $[Fe(CN)_6^{3-}]$, broaden EPR line if they appear in the proximity to the unpaired electron of the species of interest, e.g. the spin label.[20] It is has already been shown that $[Fe(CN)_6^{3-}]$ does not penetrate into erythrocytes.[14,21] It is also not adsorbed to the erythrocytes due to the negative charge of their membranes. To determine whether the observed effect of the line broadening is due to the penetration of spin label into the cells, the same titration was done using the nitroxide label Tempol, since it is known[13,22] that Tempol does penetrate the cell.

The result of the titration experiment of Tempol is presented in Fig. 3. It should be pointed out that the $[Fe(CN)_6^{3-}]$ is not distributed homogenously in the erythrocyte suspensions i.e. the concentration in the intracellular part is zero and in the extracellular solution nearly double the stated concentration.

The slight increase in the line width of the spectra in the erythrocyte suspensions at low $[Fe(CN)_6^{3-}]$ concentrations is due to its effect on the spectrum of the extracellular Tempol. At higher concentrations mainly the spectrum of the intracellular Tempol is observed and this is not affected by the extracellular ferricyanide. In contrast, when the dependence of the central EPR line width of $R^\bullet$ on $[Fe(CN)_6^{3-}]$ is measured, the pattern is totally different. The results are presented in Fig. 4. Two main features are observed:

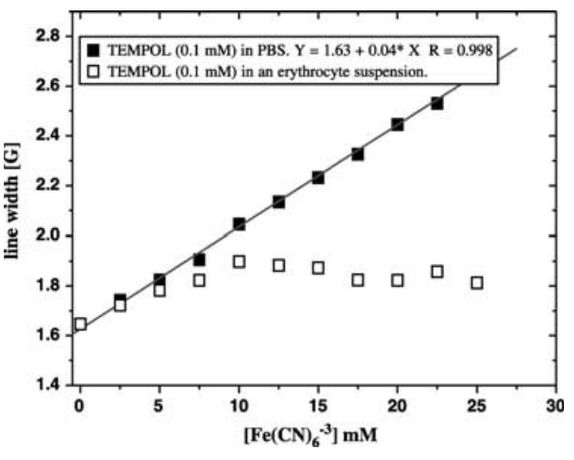

FIGURE 3 Dependence of EPR line width of Tempol on $[Fe(CN)_6^{3-}]$ in PBS and in erythrocyte suspensions.

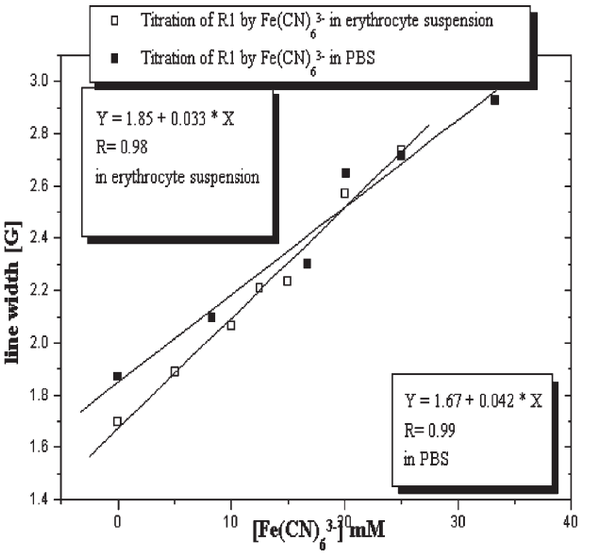

FIGURE 4 Dependence of EPR middle signal width of R• on $[Fe(CN)_6^{3-}]$ concentration in the presence and in the absence of erythrocytes.

a. The central line width of the spectrum increases with the $[Fe(CN)_6^{3-}]$ and does not reach saturation. This observation points out that R• does not penetrate the cells.
b. The slope of the "line" in the erythrocyte suspensions is considerably lower than that in the PBS solutions. This observation is attributed to the fact that the collision frequency of the adsorbed R• with the $[Fe(CN)_6^{3-}]^-$is lower than that of the free R•.[2] This stems from two origins: (1) The diffusion coefficient of the adsorbed R• is very small. (2) The erythrocyte membrane has a negative charge and therefore repels the $[Fe(CN)_6^{3-}]$ anions.

Another way to look at these effects is to measure the effect of $[Fe(CN)_6^{3-}]$ on the relative heights of the central and right lines of the spectrum of R. As the right line B_1 is mainly due to free R• its width increases faster and therefore its height decreases faster.

The ratio between the signals height, B_0/B_1 in the presence and absence of $[Fe(CN)_6^{3-}]$ is presented in Table II. The results clearly demonstrate that the ratio between the central signal height to the right signal height, in the absence of erythrocytes is nearly not affected by $[Fe(CN)_6^{3-}]$. On the other hand, in the erythrocyte suspensions $[Fe(CN)_6^{3-}]$ dramatically affects this ratio.

The results of the $[Fe(CN)_6^{3-}]$ titration thus point out that:

a. R• does not penetrate the erythrocytes in accord with expectations.
b. R• is adsorbed to the outer surface of the erythrocytes membrane.

In order to study the redox properties of R•, it was mixed with a variety of antioxidants dissolved in PBS and the rates of the reduction of R• were followed. The results point out that R• is reduced by ascorbate but not by GSH, Trolox—a soluble derivative of vitamin E—albumin, *N*-acetyl-cysteine or captopril-an −SH containing antihypertensive drug in concentrations of 1.0 mM. This result is in accord with earlier reports on the reactivity of analogous nitroxides.[3,6,9,10] The reaction of excess ascorbate with R• obeys a first order rate law. The observed rate constant is proportional to the concentration of ascorbate. From the slope of the linear line, k (R• + ascorbate) $= 5.0 \pm 0.5\,M^{-1}\,s^{-1}$ is derived, assuming that the ascorbyl radical produced in the reaction reacts in a fast process with a second molecule of R.

The results up to this stage point out that R• is adsorbed to the outer surface of the erythrocyte membrane as well as to albumin and other plasma proteins, and that it is reduced by ascorbate which is present both in the erythrocytes and in the plasma at concentration smaller than 100 μM.[23,24] It seemed therefore of interest to measure the rates of reduction of R• by blood and by its components, plasma and erythrocytes. A typical result is presented in Fig. 5. It should be pointed out that albumin accelerates the rate of reaction of R• with ascorbate.[25] However, the physiological concentration of ascorbate in the plasma is low. It is suggested that the reaction of the ascorbate present in the plasma is often over prior to the start of measurements. After mixing, in some cases a small fast process is observed. The follow up process, which is observed, is very slow, with a large error, and its nature is not clear.

TABLE II Ratios between the signals heights, B_0/B_1 in the presence and absence of $Fe(CN)_6^{3-}$

PBS solution		Erythrocytes suspension in PBS		
+	−	+	−	$Fe(CN)_6^{3-}$ 60 mM
1.07 ± 0.05	0.92 ± 0.05	3.36 ± 0.17	1.47 ± 0.07	B_0/B_1

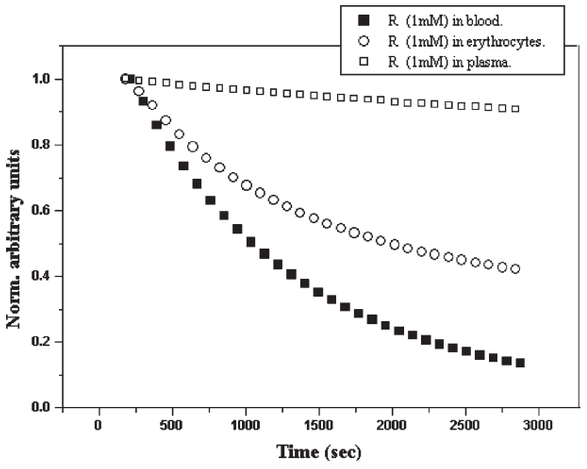

FIGURE 5 Exponential decay of $R^{\bullet}$ (1 mM) in plasma, erythrocyte suspension in PBS and whole blood of healthy adults.

The average rates observed in the different components are summarized in Table III. The data for the plasma are probably not for the first process.

Several conclusions can be drawn from these results:

1. The kinetics of disappearance of $R^{\bullet}$ obeys first order rate laws in all systems.
2. The rate of reduction of $R^{\bullet}$ is always larger in the blood than in erythrocyte suspensions in PBS, which, in turn, is considerably larger than in plasma. Often the rate constant seems to be additive, i.e. $k_{(\text{blood})} = k_{(\text{erythrocytes})} + k_{(\text{plasma})}$. This observation is in accord with studies on other nitroxides.[5,9,10] However, note the question whether the process observed in the plasma is the first one.
3. In whole blood nearly all the $R^{\bullet}$ labels are reduced though clearly the concentration of ascorbate is considerably smaller than 0.5[R].
4. In the plasma only a small portion of $R^{\bullet}$ is reduced. However, the amount of reduced $R^{\bullet}$ seems to be somewhat larger than that which can be attributed to the ascorbate concentration in plasma.[25] In erythrocytes suspensions the amount of $R^{\bullet}$ reduced is considerably larger than that reduced in plasma but significantly smaller than that in whole blood. The amount reduced is additive in most cases, i.e. $\Delta[R^{\bullet}]_{(\text{blood})} = \Delta[R^{\bullet}]_{(\text{erythrocytes})} + \Delta[R^{\bullet}]_{(\text{plasma})}$, Table III.

The results thus suggest that ascorbate is a catalyst for the reduction of $R^{\bullet}$, i.e. that dehydroascorbate is reduced in the presence of erythrocytes and probably even in the plasma to some degree. It was, therefore, decided to check whether the addition of 1–4 mM glutathione, which does not penetrate the cells[24] to whole blood or to erythrocyte suspensions in PBS affects the rate of reduction of R. The results point out that the addition of GSH does not change the rate of reduction of $R^{\bullet}$. Even more surprising is the observation that the addition of 1 mM of $R^{\bullet}$ to whole blood does not affect the concentration of GSH in the blood after 2 h, i.e. the cells are capable of recycling ascorbate without affecting the concentration of GSH. Even the addition of 0.2–2.0 mM GSH to hemolysates does not affect the rate of reduction of $R^{\bullet}$ or its fraction of reduction, thus pointing out that the rate of recycling of ascorbate by GSH is too slow to be detected by this technique. The addition of 1 mM *N*-acetyl cysteine (NAC), which penetrates the cells,[26] under the same experimental conditions also does not affect the reduction of R. Moreover, 1 h preincubation of erythrocytes with NAC, which significantly raised the intracellular thiols,[26] did not have any effect too.

On the other hand, the addition of dehydroascorbate, DHA, to erythrocyte suspensions in PBS considerably accelerates the rate of reduction of $R^{\bullet}$ (see, for example, Fig. 6).

As expected, the addition of DHA to solutions of $R^{\bullet}$ in PBS has no effect on the lifetime of $R^{\bullet}$. The results thus point out that ascorbate plays the role of a catalyst in the reduction of $R^{\bullet}$ by the erythrocytes. This result is in accord with earlier reports for nitroxides, which penetrate into the cells.[8–13]

The results thus point out that the erythrocytes act as a reductant of $R^{\bullet}$, probably via the recycling of ascorbate. Therefore, the effect of the erythrocytes concentration, as expressed by hematocrit, on the rate of reduction of $R^{\bullet}$ was measured. The results are presented in Fig. 7.

These results clearly demonstrate that the rate of reduction of $R^{\bullet}$ is proportional to the hematocrit. It should, however, be pointed out that in principle this could also be interpreted as pointing out that the rate

TABLE III Values of reduction rates and fraction of reduction of $R^{\bullet}$ (in normalized units) observed in plasma, erythrocytes and whole blood presented as means ± SD

Blood component	Whole blood	Erythrocytes	Plasma
k_{obs} (s^{-1}) × 10^3	1.24 ± 0.46	0.86 ± 0.24	0.34 ± 0.22
Fraction of reduction of $R^{\bullet}$ (AU)	0.88 ± 0.07	0.72 ± 0.19	0.15 ± 0.10

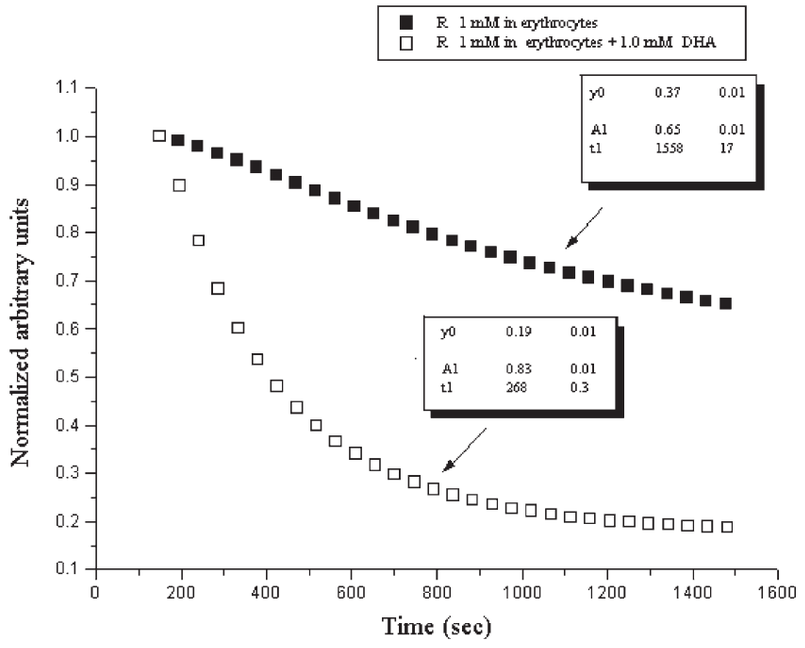

FIGURE 6 Effect of DHA on the kinetics of reduction of $R^{\bullet}$ in suspension of erythrocytes in PBS.

is proportional to the total ascorbate concentration as this is introduced mainly as an intracellular component of the erythrocytes.

CONCLUDING REMARKS

The results obtained can be summarized as follows:

1. Ascorbate is the only natural antioxidant that reduces $R^{\bullet}$ in PBS solutions.
2. The rate of reduction of $R^{\bullet}$ in the presence of erythrocytes is considerably faster than in plasma. This is true though $R^{\bullet}$ is adsorbed to the external side of the cells membrane. This result points out that there is an efficient route for electron transfer through the membrane.
3. The observed rate of reduction of $R^{\bullet}$ in whole blood, or in erythrocyte suspensions, is lower than that calculated from the data obtained from the reduction of $R^{\bullet}$ by an access of ascorbate. Thus, for example, the extrapolation of the observed rate vs. hematocrit, Fig. 7, to 100% yields a rate of $1.9 \times 10^{-3}\,s^{-1}$, which corresponds to an $R^{\bullet}$ concentration of about 200 μM. The reduction yield is considerably larger than that which can be attributed to the actual physiological ascorbate concentration. Thus the reduction observed is a more complex process as its catalysis by DHA points out.

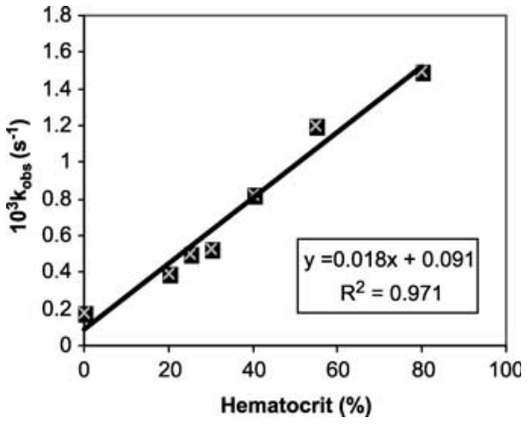

FIGURE 7 Dependence of the apparent rate constant of reduction of $R^{\bullet}$ on the hematocrit in plasma.

Finally the results clearly demonstrate that $R^{\bullet}$ is a suitable spin label for measuring the reduction kinetics and antioxidant capacity in blood, as expressed by reduction by ascorbate. Furthermore, since $R^{\bullet}$ was found to be adsorbed to the membranes, this spin label may also be useful for the study of changes in the state of the erythrocyte membranes.

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