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Hydroxylamine and Phenol-Induced Formation of Methemoglobin and Free Radical Intermediates in Erythrocytes

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ABSTRACT. As previously shown with isolated oxyhemoglobin, methemoglobin formation can also be induced in intact erythrocytes by hydroxylamine compounds and substituted phenols such as butylated hydroxyanisole (BHA). Electron spin resonance investigations revealed that, accordingly, free radical intermediates were formed in erythrocytes from hydroxylamine, *N,N*-dimethylhydroxylamine, and *N*-hydroxyurea. Due to the low stability of the dihydronitroxyl radicals, their detection required the use of a continuous flow system and relatively high amounts of the reactants. As has already been demonstrated with the solubilized hemoglobin system, hemoglobin of intact erythrocytes also reacts with the more hydrophilic xenobiotics such as hydroxylamine. However, the reaction rate was slightly reduced, indicating the existence of an incomplete permeability barrier for these compounds. The limited solubility of phenolic compounds in the aqueous buffer of suspended erythrocytes (in combination with the strict requirement of osmolarity in order to prevent hemolysis) impeded the direct detection of the respective phenoxyl radicals previously reported in hemoglobin solutions. However, in accordance with earlier findings in homogeneous reaction systems, chemiluminescence was observed as well, indicating the existence of a further reaction intermediate, which was also obtained in pure hemoglobin solutions when mixed with the respective reactants. As has recently been demonstrated, this light emission is indicative of the existence of highly prooxidative compound I intermediates during methemoglobin formation. Prooxidant formation in erythrocytes is reflected by a significant decrease in thiol levels even with those compounds where free radical formation was not directly detectable by ESR spectroscopy. The use of the spin-labeling technique revealed membrane effects as a result of oxidative stress. Oxidative metabolism of hemoglobin with hydroxylamine caused a release of low molecular weight iron. The marked hemolysis observed in the presence of BHA results from a direct membrane effect of this compound rather than a consequence of free radical-induced oxidative stress. A correlation of the different results is discussed in terms of possible toxicological consequences. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;12:1821–1829, 1996.

KEY WORDS. erythrocytes; methemoglobin; free radicals; iron release; phenols; hydroxylamines

Toxic actions of pollutants or harmful side effects of pharmaceuticals on erythrocytes may be triggered by different mechanisms. Basically, three major aspects can be distinguished. First, direct oxidation of the heme moiety to methemoglobin affects the total oxygen transport capacity and can also give rise to the formation of highly toxic reaction intermediates such as free radicals or ferryl heme species [1, 2]. Second, interaction with the erythrocyte membrane may lead to partial or complete hemolysis as well as release of prooxidants such as iron [3] and hematin [4]. Third, interaction with functional groups of erythrocyte proteins such as thiol groups [5] or sugar moieties [6] may render the erythrocyte susceptible to secondary damage. Finally, a particular substance can act simultaneously by two or more different mechanisms with the possibility of potentiating damage.

In several previous papers, we have reported on the formation of reactive intermediates evolving from the reaction of various xenobiotics such as hydroxylamines [7–9] or phenolic compounds [10, 11] with oxyhemoglobin or oxymyoglobin [12]. Apart from different types of free radical species detected by ESR† [7–12], we also observed low-level chemiluminescence [13–15] resulting from the transient formation of compound I type ferryl heme species. The latter are formed via the reduction of heme-oxygen to the redox state of hydrogen peroxide. In contrast to isolated heme compounds where water-soluble xenobiotics have direct access

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† *Abbreviations:* BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; compound I, ferryl ion + protein radical or ferryl ion + porphyrin cation radical; compound II, ferryl ion=$[Fe^{IV}=O]^{2+}$ or (oxo)iron(IV); ESR, electron spin resonance; GSH, glutathione; HPLC, high performance liquid chromatography; HbO_2, oxyhemoglobin; *p*-HyAn, *p*-hydroxyanisole; HyUr, hydroxyurea; Me_2NOH, *N,N*-dimethylhydroxylamine; MetHb, methemoglobin; SOD, superoxide dismutase.

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to the reactive heme-moiety of oxyhemoglobin, the reactivity of these compounds towards oxyhemoglobin enclosed in intact erythrocytes is also determined by their capability to permeate the erythrocyte membrane. Furthermore, a direct influence on membrane properties can be expected on the part of membrane-active antioxidants such as BHA or BHT, which, in addition, have recently been shown both to induce severe alterations of membrane potential in rat heart mitochondria [16] and to trigger methemoglobin formation from isolated oxyhemoglobin. Recent investigations by Butterfield *et al.* [4–6, 17, 18] have emphasised the existence of different susceptible sites of the erythrocyte membrane. In addition to the lipid bilayer itself [17], possible targets are SH-groups on the spectrin web [5], areas close to the polar headgroup section [18], and sugar moieties such as sialic acid and galactose residues [6] located on transmembrane proteins such as glycophorin and band 3. Early events of membrane alterations prior to any other change detectable were made visible by the use of spin-labeling techniques. In the case of reducing MetHb-generators, the prooxidant formation recently reported [7–12] may be envisaged as a primary underlying toxic mechanism possibly triggering membrane alterations. Because thiols are highly susceptible to "oxidative stress," measurement of changes in the thiol redox status [19, 20] in erythrocytes would be helpful in differentiating between direct membrane effects due to a membrane association of the respective MetHb-generator and indirect membrane effects evolving from the establishment of oxidative stress. A possible route leading to the formation of reactive oxygen species is the release of redox-active iron from the heme-moiety. Recently, Ferrali *et al.* reported on the release of low molecular weight, dialyzable iron in an incubation consisting of phenylhydrazine and erythrocytes [3] and also in the respective oxyhemoglobin systems [21].

The aim of the present study was to investigate (i) whether reactive metabolites recently detected in homogeneous oxyhemoglobin/xenobiotic incubation systems are also formed in intact erythrocytes, and (ii) whether these reactive compounds may exert damaging effects in erythrocytes such as oxidation of thiol groups, release of iron, physical and/or chemical membrane alterations, or even hemolysis. Furthermore, it was of interest whether toxic effects attributed to these compounds are due to a single underlying mechanism or whether different mechanisms act together, leading to damage of the function and structure of erythrocytes.

MATERIALS AND METHODS

Chemicals

BHA (2- and 3-*tert*-butyl-4-hydroxyanisole), Ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt monohydrate), *p*-hydroxyanisole (4-methoxyphenol), *N*-hydroxyurea, and *N,N*-dimethylhydroxylamine hydrochloride were purchased from Aldrich (Steinheim, Germany). BHT (2,6-di-tert-butyl-4-methylphenol) was from Fluka (Buchs, Switzerland). L(+)-Ascorbic acid, ethanol, and hydroxylamine hydrochloride were from Merck (Darmstadt, Germany); Sephadex G 25 came from Pharmacia (Uppsala, Sweden); Celite and $DEAE_{25}$-cellulose were from Serva (Heidelberg, Germany); and 4-amino-TEMPO, 4-maleimido-TEMPO, and 5-doxyl-stearic acid were from Sigma (Deisenhofen, Germany).

The thiol-specific spin-label *bis*-(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl)-disulfide (biradical, ·RSSR·) was obtained from the Institute of Organic Chemistry (Novosibirsk, Russia).

Isolation of Erythrocytes

Bovine erythrocytes were prepared by a modification of the procedure described by Eyer *et al.* [22] for human hemoglobin. The cells were washed five times with twice the amount of phosphate-buffered saline (140 mM NaCl, 10 mM sodium/potassium phosphate, pH 7.4) and centrifuged for 10 min at 2200 × *g*. The hemoglobin content was determined as follows: 100 μL of homogeneous cell suspension was hemolyzed in hypotonic buffer (5 mM sodium/potassium phosphate, pH 7.4) and the absorbance was measured at 540 nm according to Grisk [23]. The erythrocytes were stored at 4°C and used within the first 4 days. Glucose (5 mM) was added to the storage buffer, except for the ESR and chemiluminescence samples, where glucose interferes with the free radicals formed. These experiments were therefore performed immediately after the preparation.

Isolation of Hemoglobin

For the isolation of hemoglobin [22], the erythrocytes were hemolysed in distilled water, and 10 g of Celite was added to 250 mL of the hemolysate. The mixture was stirred for 20 min and then centrifuged for 30 min at 15,000 × *g*. Purified hemoglobin was prepared by chromatography of the hemolysate on $DEAE_{52}$-cellulose. The fractions were tested for catalase [24] and SOD activity [25]: only those with catalase activity $k < 1$ and no detectable SOD activity were pooled. The pooled fractions were dialyzed several times against 1.5 mM phosphate buffer, pH 6.8, until no traces of glutathione were detectable in the dialysis buffer. Oxyhemoglobin was determined at its absorption maximum at 540 nm as described above.

ESR Spectroscopy

The ESR experiments were carried out in a Bruker ER 200 D-SRC 9/2.7 spectrometer operating at 9.6 GHz with 100 kHz modulation frequency equipped with a rectangular TE_{102} or a cylindrical TM_{110} microwave cavity. For the detection of the short-lived dihydronitroxyl radical (NH_2O·) derived from hydroxylamine, a flow system had to be used: A solution of hydroxylamine hydrochloride (150 mM, adjusted to pH 7.4 with NaOH) was mixed with an erythrocyte suspension (13.5 mM heme content) immedi-

ately before entering the ESR cavity with an approximate flow rate of 7.2 mL/min.

Spin-labeling experiments were performed according to Butterfield *et al.* [17]. The spin label 5-doxylstearic acid (0.1 mM) was incorporated into erythrocyte (2 mM) or ghosts (1–2 mg/mL), and the excess of spin label was washed out after 30 min and incubated with the respective xenobiotic (2 mM) for at least 15 min. Changes of linewidth (half-width at half-height) of the low field line were taken as an indicator of relative fluidity according to Giavedoni *et al.* [26].

Thiol level measurements were performed according to Weiner *et al.* [19]. Erythrocytes were diluted to 2 mM (with respect to heme groups) and incubated for 2 hr at 37°C in the presence of the respective xenobiotic (2 mM). Samples were then diluted 1:10, and 0.2 mM RSSR was added. ESR spectra were taken after 30 min equilibration at ambient temperature. To account for the loss of free SH-groups due to autoxidation, all values were calculated relative to a control sample incubated under the same conditions (2 hr/37°C), except that no xenobiotic was added.

Chemiluminescence Measurements

Chemiluminescence measurements were carried out on a red-sensitive photomultiplier (EMI 9658 AM, Thorn EMI Electron Tubes Inc., Fairfield, NJ, U.S.A.) cooled to −25°C and connected to an amplifier discriminator (model 1121; Princeton Applied Research, Princeton, NJ, U.S.A.) adapted for single photon counting (model 1109; Princeton Applied Research).

For the measurements, 500 μL of a 100 mM stock solution of the respective xenobiotic in buffer or ethanol (*p*-HyAn, BHA, BHT) was added to 6 mL of a continuously stirred erythrocyte suspension (4 mM HbO_2) in isotonic phosphate buffer (0.2 M, pH 7.4). Light emission was integrated during the 2-hr incubation time, and the observed values were corrected for the respective dark counts.

Methemoglobin Formation

Methemoglobin formation was measured at 540 nm using a Hitachi model 150-20 UV-VIS spectrometer. After 2 hr incubation of erythrocytes (2 mM HbO_2) with the respective xenobiotic (2 mM), a 100-μL sample was withdrawn, hemolysed with 900 μL MQ-water, and centrifuged for 5 min at 4500 × *g*. The optical density of the solution was adjusted with the necessary amount of buffer, and the absorbance was read at 540 nm. Calibration was carried out after the addition of potassium hexacyanoferrate(III), which stoichiometrically converts hemoglobin to the ferric state.

Hemolysis

Hemolysis was determined as the amount of hemoglobin released from the erythrocytes into the buffer relative to a calibration standard (complete hemolysis = 100%). The erythrocyte suspension was centrifuged for 5 min at 4500 × *g*, and the absorbance of the supernatant was read at 540 nm after the addition of a moderate excess of $K_3[Fe(CN)_6]$ to convert traces of nonoxidised oxyhemoglobin to methemoglobin. The hemoglobin content of the packed red cells was determined after complete hemolysis induced by the addition of distilled water.

Determination of Iron

Iron determination was performed spectrophotometrically by the Ferrozine method according to Carter [27] with a modification described by Ferrali *et al.* [21] for the separate detection of dialyzable iron. An aliquot (10 mL) of an erythrocyte suspension or hemoglobin solution containing 2 mM oxyhemoglobin and 2 mM of the respective xenobiotic was incubated for 2 hr at 37°C in an Erlenmeyer flask into which a dialysis tube containing 5 mL of the same buffer (140 mM NaCl, 10 mM sodium citrate, pH 7.4) was dipped. At the end of the incubation period, ascorbate (1 mM) and Ferrozine (1 mM) were added to the dialysate, and iron was determined spectrophotometrically at 562 nm [27]. In addition, 500 μL of the remaining erythrocyte suspension was hemolysed upon the addition of 1.5 mL distilled water and subsequently ultrafiltered through a low-binding cellulose ultrafiltration cone with an exclusion limit of 10,000 Da (Millipore, Molsheim, France) for 30 min at approximately 5000 × *g*. Iron was determined as above, after addition of ascorbate (1 mM) and Ferrozine (1 mM).

RESULTS

ESR Spectroscopy

Rapid mixing of erythrocytes with hydroxylamine hydrochloride resulted in the existence of a transient ESR spectrum of the dihydronitroxide radical ($NH_2O\cdot$) (Fig. 1a). Spectral parameters of this paramagnetic species ($a_N = a_H$ = 12.6 G; $g = 2.0062$) are in agreement with those recently described in homogeneous solution of oxyhemoglobin and hydroxylamine [7]. In contrast to the earlier experiments, maximal ESR signal intensities were only obtained in the system under study when the mixing flow rate between hydroxylamine and erythrocytes was low. This observation indicates a decrease in the overall reaction rate, probably due to accessibility barriers of hydroxylamine to hemoglobin enclosed within the erythrocytes. In this case, not only the rate of reaction (Eqn 1)

$$NH_2OH + HbO_2 \rightarrow NH_2O\cdot + [MetHb(III)\text{-}O\text{-}O\text{-}H] \quad (1)$$

$$[MetHb(III)\text{-}O\text{-}O\text{-}H] \rightarrow [Prot\cdot\ Hb(IV)=O] + H_2O \quad (2)$$

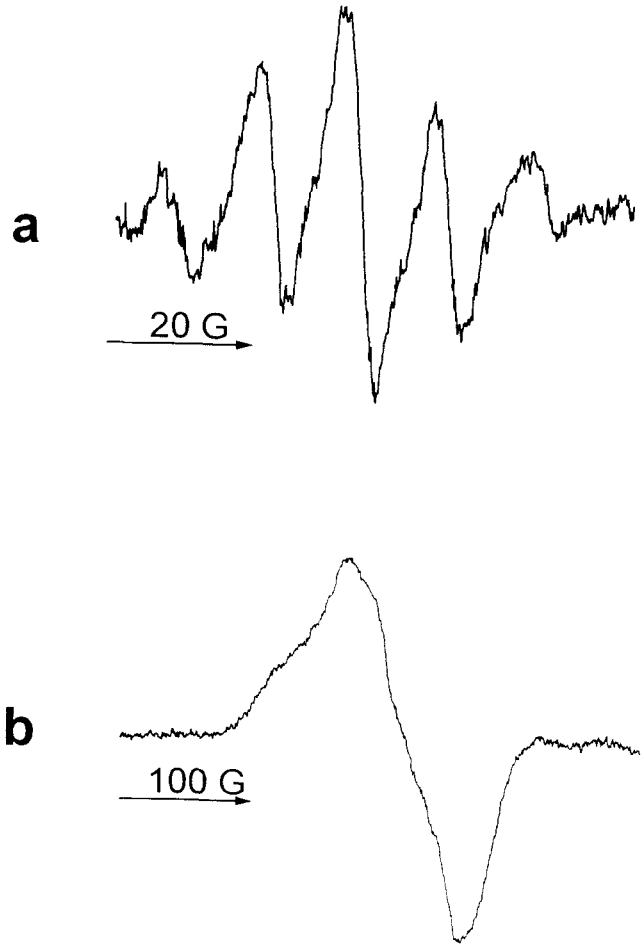

FIG. 1. Formation of xenobiotic-derived free radicals. (a) Erythrocytes (13.5 mM heme content, suspended in isotonic phosphate buffer, 150 mM, pH 7.4) and NH_2OH (150 mM, adjusted to pH 7.4 with NaOH and balanced with 50 mM phosphate buffer to give an isotonic solution) were mixed in a flow cell at a total flow rate of 7.2 mL/min. ESR settings: sweep width, 100 G; modulation amplitude, 4.0 G; microwave power, 20 mW; time constant, 0.16 sec; receiver gain, 1×10^5; scan rate, 143.0 G/min. (b) Same incubation as in (a) but recorded after 60 min at 110 K. ESR settings: sweep width, 400 G; modulation amplitude, 1 G; microwave power, 5 mW; time constant, 0.33 sec; receiver gain, 1×10^5; scan rate, 71.5 G/min.

has to be considered, but also the diffusion step of reaction (Eqn 3) across the erythrocyte membrane:

$$NH_2OH \text{ (solution)} \rightarrow NH_2OH \text{ (erythrocyte)} \qquad (3)$$

The clearly weaker ESR signal intensities observed with HbO_2 associated with erythrocytes are a consequence of the slower reaction rate (see above) and possibly also the presence of antioxidants within the erythrocytes.

When the flow was stopped, the ESR signal disappeared almost immediately (within a few seconds). However, after an incubation time of several minutes, a further paramagnetic species was found with a broader ESR spectrum. Its maximum intensity was reached after 60 min. The identification of this paramagnetic species required low temperatures (Fig. 1b). Based on its ESR characteristics at 110 K ($g_{shoulder} = 2.060$, $g_{min} = 1.986$), this spectrum was assigned to the hemoglobin–nitric oxide adduct previously shown to exist in homogeneous oxyhemoglobin solution [7].

When the dimethyl-derivative of hydroxylamine (Me_2NOH) was added to erythrocytes instead of the parent compound, a relatively persistent ESR signal was obtained (Fig. 2a). Due to its spectral characteristics ($a_N = 17.0$ G, $a_{H(6)} = 14.8$ G, $g = 2.0054$), the signal can clearly be assigned to the dimethylnitroxyl radical ($Me_2NO\cdot$). The intensity of the signal was slightly lower compared to the one obtained with the solubilized HbO_2/Me_2NOH system [8].

N-hydroxyurea, which was previously shown to initiate a radical reaction sequence in the presence of HbO_2 [9], was incubated with erythrocytes in a stationary system. Due to the slow reaction rate of *N*-hydroxyurea with oxyhemoglobin, 100 repetitive scans had to be accumulated to obtain a sufficiently resolvable signal/noise ratio (Fig. 2b). According to its spectral parameters ($a_H = 11.7$ G, $a_N = 8.05$ G, $g = 2.0063$), the product was identified as the aminocarbonylaminooxyl radical ($H_2N-CO-NHO\cdot$) previously detected in homogeneous solution [9] and identified according to Lassmann and Liermann [28], who reported similar values. The penetration across the erythrocyte membrane was not the rate-limiting step, because the reaction rate was equally low in the homogeneous HbO_2/*N*-hydroxyurea system [9].

Incubation of erythrocytes with the phenolic com-

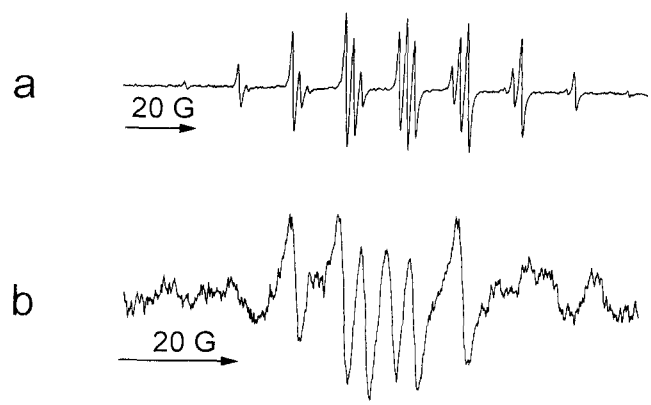

FIG. 2. Formation of xenobiotic-derived free radicals. (a) Erythrocytes (3.4 mM heme content, suspended in isotonic phosphate buffer, 150 mM, pH 7.4) and Me_2NOH (7.7 mM, adjusted to pH 7.4 with NaOH in isotonic phosphate buffer) were mixed in a stationary system. ESR settings: sweep width, 160 G; modulation amplitude, 0.5 G; microwave power, 10 mW; time constant, 0.33 sec; receiver gain, 1×10^5; scan rate, 57.2 G/min. (b) Erythrocytes (6.8 mM heme content) were suspended in isotonic phosphate buffer, pH 7.4 containing *N*-hydroxyurea (100 mM). 100 ESR scans were accumulated under the following conditions: sweep width, 100 G; modulation amplitude, 2.0 G; microwave power, 20 mW; time constant, 0.16 sec; received gain, 4×10^5; scan rate, 71.5 G/min.

pounds, *p*-hydroxyanisole, BHA, and BHT did not result in the detection of any ESR signal. In previous experiments using homogeneous oxyhemoglobin systems with *p*-hydroxyanisole or BHA, the detected ESR signals of the respective phenoxyl radicals were very weak [10, 11]; with BHT, whose poor solubility in aqueous solution is a limiting factor, no signal was detected at all.

It was, however, possible to detect a BHT-derived phenoxyl radical [11] using a model system (MetHb/H_2O_2) based on the formation of the highly reactive compound-I type ferryl species [1], which has previously been reported as a reaction intermediate in MetHb formation according to Eqns 1 and 2: Starting from a mixture of MetHb and H_2O_2, much higher concentrations of compound I can be obtained, so that more BHT-derived phenoxyl radicals are formed according to Eqn 4:

$$[\text{Prot}^{\cdot}\ \text{Hb(IV)=O}] + 2\ \text{BHT} \rightarrow \text{MetHb(III)–OH} + 2\ \text{BHT}^{\cdot} \quad (4)$$

Adaptation of this model reaction to intact erythrocytes (preincubation of erythrocytes with excess H_2O_2) was not successful. Even the compounds with higher reactivity and better solubility (*p*-hydroxyanisole, BHA) did not form the expected phenoxyl radicals in concentrations high enough for ESR detection, possibly because the diffusion barrier of the erythrocyte membrane decreases the reaction rate.

Correlation of Chemiluminescence Measurements and Methemoglobin Formation

We have recently shown that activated heme intermediates exhibiting an excited state are formed when xenobiotics under study are incubated with oxyhemoglobin in homogeneous solution [13–15]. Spectral resolution of the emitted light [12] indicated that lipid peroxidation does not play a major role in this process. Experiments in D_2O buffer and the use of a specific $^1O_2$ trap revealed that singlet oxygen can also be excluded as the source of chemiluminescence. When a model system consisting of MetHb/H_2O_2 or MetMyo/H_2O_2 was used, a good correlation between the disappearance of the ESR spectrum of a globin-related protein radical and the intensity of the emitted light was obtained [13]. Because emission occurs predominantly in the red (between 600 and 800 nm), an excited porphyrin species is most likely responsible for light emission. This conclusion is based on recent studies by Vasváry *et al.* [29], in which hematoporphyrin systems in DMSO solution were found to exhibit an emission maximum in the red above 650 nm.

Chemiluminescence detection from erythrocyte suspensions required significantly higher concentrations of the reactants as compared with the MetHb/H_2O_2 model system because, due to the turbidity of the erythrocyte suspension, considerable absorption and diffraction of the emitted photons occur. Experiments with whole erythrocytes were performed (i) to detect activated oxo-heme intermediates with phenolic compounds as well (where one-electron oxidation products of the xenobiotics were not detectable by ESR spectroscopy), and (ii) to determine possible involvement of membrane lipid peroxidation in the observed light emission.

In the incubations containing NH_2OH, *p*-HyAn, and BHA, MetHb formation was virtually 100% after 2 hr. Approximately 50% methemoglobin was formed in the presence of Me_2NOH and very little with BHT and HyUr within this time period.

A correlation of MetHb formation with integrated chemiluminescence counts is shown in Fig. 3 (values corrected for background noise). The correlation (R = 0.991) is highly significant, indicating that in all cases chemiluminescence is linked to the reaction leading to MetHb formation.

In contrast to the homogeneous HbO_2/NH_2OH system [12], where two maxima were obtained (in the 660–715-nm and the 750–800-nm ranges), a complete spectral resolution of the erythrocyte/NH_2OH system was not possible due to the low intensity of the emitted light. However, the use of a 600-nm cut-off filter reduced light intensity by at least 70%. This indicates that most of the emitted light stems from the same reaction pathway found in hemoglobin and myoglobin solutions [14], i.e. compound I decomposition via excited porphyrin states [15]. Taking into account the lower sensitivity of the spectrometer above 600 nm, 70% of light corresponds to approximately 80–95% of spectral distribution. The remaining 5–20% cannot be assigned unequivocally, but a small contribution of wavelengths of approximately 450 nm makes the participation of membrane lipid peroxidation reasonable.

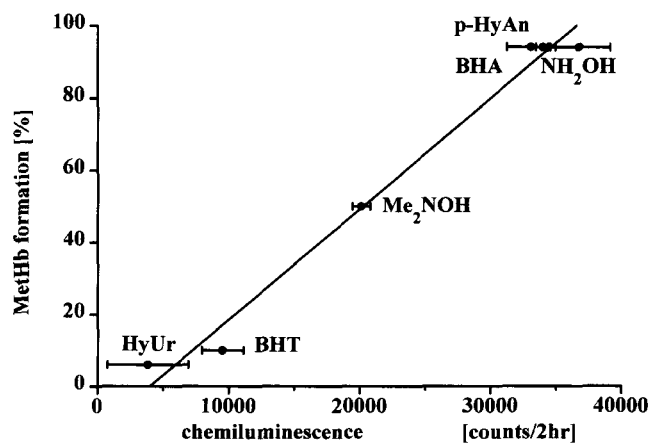

FIG. 3. Correlation of chemiluminescence and methemoglobin formation. Correlation of MetHb formation (%, after 2 hr) and integrated light emission (2 hr, background subtracted) of erythrocytes in the presence of the xenobiotics NH_2OH, Me_2NOH, HyUr, *p*-HyAn, BHA, and BHT. The indicated value is the mean of three independent measurements ± SD. The correlation coefficient was R = 0.991.

Hemolysis

Virtually no hemolysis was found in any incubation system containing the respective xenobiotic and suspended erythrocyte, except for the system with BHA, where almost complete hemolysis was observed within the first 2 hr (not shown). No correlation was observed between hemolysis and MetHb formation rates in this case. This indicates that the formation of MetHb is not directly linked to the process leading to hemolysis.

Spin-Labeling Experiments

To clarify the mechanism leading to hemolysis in the incubation system containing erythrocytes and BHA, we tested the influence of the xenobiotics on membrane fluidity (Fig. 4a) using the spin-labeling technique described by Butterfield *et al.* [17]. An additional control was performed with erythrocyte ghosts to estimate a direct effect of xenobiotics on the membrane structure that is not mediated by the redox activity of oxyhemoglobin present in whole erythrocytes. The results are shown in Fig. 4b (erythrocyte ghosts). In the case of BHA, the increase in the width of the low-field ESR line clearly indicates higher mobility of the erythrocyte membrane lipids. This effect is solely dependent on BHA but independent of the presence of oxyhemoglobin. This effect must therefore be explained in terms of a direct hydrophobic interaction with the membrane bilayer. A similar, but weaker, increase of membrane fluidity was observed with BHT, although hemolysis in the presence of BHT was not significantly increased above the control level. Physical membrane changes detected with the spin-labeling technique reveal that large-scale effects are required to allow hemolysis.

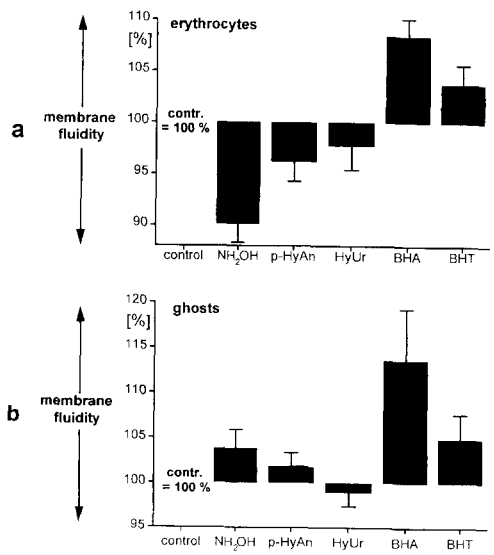

FIG. 4. Membrane fluidity changes in erythrocytes and ghosts. Spin-labeling of (a) erythrocytes (11 mM) and (b) erythrocyte membrane ghosts (2 mg/mL) with 5-doxylstearic acid (4 μg/mL) after the addition of various xenobiotics (2 mM). ESR spectra were taken after 15 min incubation under the following conditions: sweep width, 100 G; modulation amplitude, 2.0 G; microwave power, 5 mW; time constant, 0.66 sec; receiver gain, 1 × 10^5; scan rate, 17.9 G/min. Values are expressed as line width of the low-field ESR line minus the respective control values.

With hydroxyurea, only minor membrane changes were observed. For NH_2OH and *p*-HyAn, two different mechanisms seem to coexist: (i) a large decrease in membrane fluidity when measured in whole erythrocytes (Fig. 4a), most likely the consequence of oxidative changes in membrane constituents (such as cross-linking), and (ii) a small, but significant, fluidity increase measured with membrane ghosts in the absence of HbO_2 (Fig. 4b), which indicates a direct membrane effect.

Iron Liberation

Previous reports on oxidative heme degradation [30, 31] described iron releases as a consequence of porphyrin ring opening. Because iron ions play a significant role as prooxidant catalysts in the presence of reactive oxygen species such as superoxide or hydrogen peroxide, the ability of xenobiotics to release iron from erythrocytes is also an important aspect of their prooxidant characteristics. In the presence of hydroxylamine, significant liberation of iron from hemoglobin was observed. In an erythrocyte suspension containing 2 mM HbO_2, approximately 18.3 μM iron was released, i.e. almost 1% of total heme iron. Approximately 11.2 μM remained within the cells and could only be observed after complete hemolysis and ultrafiltration of erythrocytes (Fig. 5a). A second fraction of released iron (approx. 7.1 μM) was detected outside of the erythrocytes, measured as the proportion that permeated a dialysis membrane under the experimental conditions chosen, i.e. during the 2-hr incubation period (Fig. 5b).

With all other investigated xenobiotics (Me_2NOH, HyUr, *p*-HyAn, BHA, and BHT), the observed iron release was not significantly different from the respective control value (0.40 μM). Similar results were obtained with homogeneous HbO_2/xenobiotic systems, where practically no iron release was observed except in the case of hydroxylamine (14.4 μM total iron released).

Thiol Redox Status Experiments

During xenobiotic MetHb formation, a series of potent prooxidants are formed: xenobiotic-derived free radicals, globin-derived radicals, ferryl heme compounds, and iron ions released from hemoglobin, which can also be converted to a prooxidant in the presence of hydroperoxides. Thiol groups are very sensitive to oxidative stress: their decrease can indirectly indicate the formation of prooxidants at an early stage, before other compounds are affected. In the case of reduced glutathione, SH groups even play a vital role in protecting membrane lipids and proteins from oxidative degradation. Prooxidants such as hydrogen peroxide or lipid hydroperoxide are reduced by the GSH/

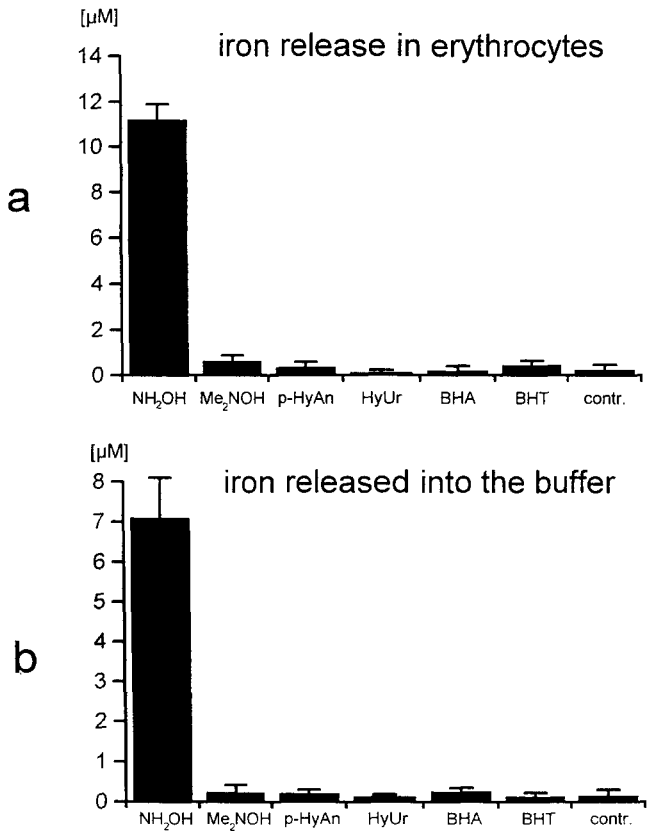

FIG. 5. Xenobiotic-induced release of low molecular weight iron. Erythrocytes (10 mL; 2 mM) and xenobiotics (2 mM) were gently shaken for 2 hr at 37°C in an Erlenmeyer flask. Inside the flask was a dialysis tube containing 5 mL of Ferrozine (1 mM) in PBS buffer, pH 7.4. After 2 hr, the content of the dialysis tube was mixed with ascorbic acid to convert all iron to the ferrous state and absorption was then measured at 562 nm. Erythrocytes were hemolysed upon the addition of distilled water, mixed with mM Ferrozine, and ultracentrifuged at 25,000 × g for 25 min. After the addition of Ferrozine and ascorbic acid, the absorption was read at 562 nm.

GSH-peroxidase system, and free radicals can directly react with GSH. In the early phase of oxidative stress, prooxidants are scavenged immediately and do not accumulate. We monitored changes in reduced SH groups (mainly GSH) using the biradical technique of Nohl *et al.* [20]. This ESR method allows the noninvasive measurement of thiols in intact erythrocytes. The results are shown in Fig. 6. Because thiol levels decrease even in the absence of xenobiotics due to autoxidation, values are expressed as differences versus the respective control (erythrocytes [2 mM], 2 hr/37°C, no xenobiotic). A significant decrease in thiol levels was observed in all cases except for BHA, where only a small decrease was observed, and for BHT, where the reduced thiol groups remained approximately 5% above the respective control value. This indicates that the antioxidant properties of BHT are predominant. With Me_2NOH, ESR-based thiol level measurements were not possible due to strong interference with the very persistent $Me_2NO\cdot$ radical.

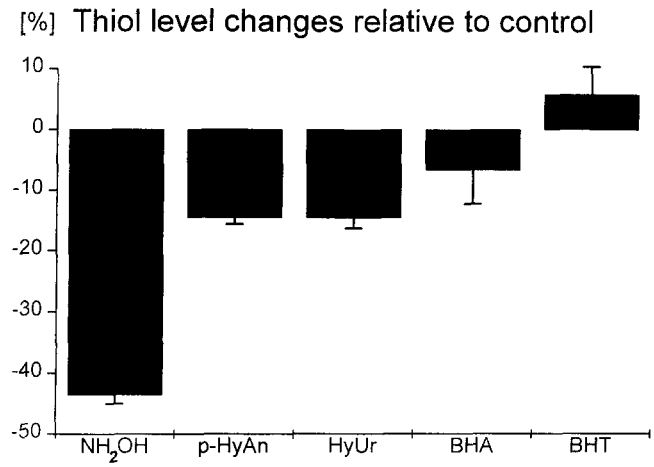

FIG. 6. Xenobiotic-induced thiol level changes in erythrocytes. Thiol redox changes were measured as follows: erythrocytes (2 mM) and the respective xenobiotic (2 mM) were incubated for 2 hr at 37°C. An aliquot (0.2 mM) was mixed with the biradical RSSR (0.2 mM) and incubated for 30 min at ambient temperature before the ESR spectrum was recorded. A calibration standard was taken in the expected concentration range, where the peak height/concentration dependence was found to be nearly linear. ESR spectrometer settings: sweep width, 50 G; modulation amplitude, 0.17 G; microwave power, 5 mW; time constant, 0.33 sec; received gain, 2.5 × 10^3; scan rate, 17.9 G/min.

DISCUSSION

The present study provides experimental evidence that xenobiotics react with oxyhemoglobin of intact erythrocytes in a manner similar to that previously shown in a homogeneous solution of isolated HbO_2. Antioxidants present in erythrocytes such as catalase, SOD, or GSH did not inhibit the formation of methemoglobin and xenobiotic-derived free radicals. This observation is consistent with a mechanism involving three different steps.

The first step (Eqn 5) is a one-electron transfer from the xenobiotic (Xen-OH) to oxyhemoglobin (HbO_2):

$$HbO_2 + Xen\text{-}OH \rightarrow [MetHb(III)\text{-}OOH] + Xen\text{-}O\cdot \quad (5)$$

The hypothetical reaction intermediate [MetHb(III)-OOH], where oxygen has the formal oxidation state of hydrogen peroxide, is not stable at room temperature [32]. In previous experiments starting from $MetHb^{3+} + H_2O_2$, we observed the formation of a compound I-type ferryl intermediate [Prot· Hb(IV)=O] and concomitant emission of low-level chemiluminescence [13, 14]. From this, we conclude that [Prot· Hb(IV)=O] is also formed in the erythrocyte/Xen-OH systems (Eqn 2). A reaction mechanism involving ·OH radicals has also been discussed (Eqn 6), but side reactions of ·OH with quenchers have not been detected [14]

$$[MetHb(III)\text{-}OOH] \rightarrow [Prot\text{-}H\ Hb(IV)=O] + \cdot OH \quad (6)$$

$$[\text{Prot–H Hb(IV)=O}] + \cdot OH \rightarrow [\text{Prot}\cdot \text{ Hb(IV)=O}] + H_2O \quad (7)$$

The high reactivity of [Prot· Hb(IV)=O] prevents its accumulation to concentrations detectable by UV-VIS (interference with MetHb) or ESR (interference with Xen–O·). Due to its high reactivity, the reaction of [Prot· Hb(IV)=O] with excess amounts of the xenobiotic will lead to the formation of additional Xen–O· radicals according to Eqns 8 and 9:

$$[\text{Prot}\cdot \text{ Hb(IV)=O}] + \text{Xen–OH} \rightarrow [\text{Prot–H Hb(IV)=O}] + \text{Xen–O}\cdot \quad (8)$$

$$[\text{Prot–H Hb(IV)=O}] + \text{Xen–OH} \rightarrow \text{MetHb(III)–OH} + \text{Xen–O}\cdot \quad (9)$$

In our experiments, the steady-state concentrations of free radicals were considerably reduced when compared with the results obtained in homogeneous solution. Our observation that the maximum of the paramagnetic intermediate ($NH_2O\cdot$) was only obtained at slow mixing rates of hydroxylamine with erythrocytes (see Fig. 1a) strongly suggests that the free radical formation rate is low rather than that decay rates are high (in this case, the flow rate would have to be increased). The diffusion barrier across the erythrocyte membrane, which remained intact throughout the experiment (except for the BHA system, where complete hemolysis was observed), may very well be implicated.

In the case of the hydrophilic hydroxylamine, the diffusion barrier could be overcome by increasing the xenobiotic concentration so that a well-resolved ESR spectrum was obtained. The phenolic compounds, on the other hand, were only poorly water-soluble. Even in the presence of EtOH (7.7%), the solubility of *p*-HyAn, BHA, and BHT was not sufficient to increase paramagnetic reaction intermediates up to the ESR sensitivity limit.

In contrast, measurement of MetHb formation and chemiluminescence experiments with phenols revealed results similar to those observed with homogeneous oxyhemoglobin solution, strongly suggesting analogous reaction steps involving the same paramagnetic species.

Due to considerable changes in the hemoglobin molecule following interaction with xenobiotics, the release of iron from the heme group was expected. Significant iron release was, however, only found with unsubstituted hydroxylamine, the compound with the highest reactivity towards HbO_2. Hydroxylamine-induced iron release was observed in homogeneous HbO_2 solution as well as in whole erythrocytes, indicating that the released iron is the result of heme degradation. All other xenobiotics investigated showed little or no iron release. As a consequence, secondary oxidative damage due to Fenton-type chemistry is not likely to play a significant role.

We also considered the possibility that the formation of xenobiotic-derived free radicals will ultimately lead to destruction of the erythrocyte membrane by direct free radical attack. However, a clear correlation between the reactivity of the respective xenobiotic (MetHb formation rate, ESR, and chemiluminescence intensities) and the extent of membrane alteration was not found. The only compound that significantly induces hemolysis is BHA; all other chemicals, even the highly reactive hydroxylamine, show little or no effect at all. The increased fluidity observed in the erythrocyte/BHA (or BHT) systems strongly suggests that BHA-induced hemolysis is a direct BHA effect on the erythrocyte membrane rather than an indirect effect through free radical-induced lipid peroxidation. Furthermore, hydroxylamine, which is the strongest prooxidant, slightly increases membrane fluidity when inserted into the membrane of erythrocyte ghosts (see Fig. 4b). In whole erythrocytes, where this compound exerts an oxidative stress, membrane fluidity was found to decrease, possibly due to peroxidation of membrane lipids (see Fig. 4a). These unexpected membrane alterations resulting from a direct membrane-destabilizing effect and an indirect membrane-stabilizing effect provide a rationale as to why hemolysis is not induced by hydroxylamine despite its great impact on thiols and membrane lipids (fluidity decrease).

A rather good correlation of methemoglobin formation and integrated light emission (see Fig. 3) indicates that chemiluminescence is directly linked to the redox process leading to MetHb formation and not to an insignificant side reaction. Iron release, on the other hand, which is only significant in the case of hydroxylamine, seems to be linked to the high reactivity of NH_2OH towards HbO_2, leading to oxidative stress. Furthermore, the final reaction product of the HbO_2/NH_2OH system, nitric oxide, has been reported to form stable complexes $[Fe(NO)_2X_2]$ with low molecular weight iron (X = inorganic anions) as well, thereby solubilizing the released iron [33].

In summary, free radical formation in erythrocytes proceeds at a rate similar to that in homogeneous hemoglobin systems and is not prevented by the antioxidant systems of the cell. On the other hand, the release of iron, which can trigger or increase the process of lipid peroxidation, plays only a minor role, and hemolysis, which was only significant in the presence of BHA, is not directly linked to free radical attack.

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