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Aniline-, Phenylhydroxylamine-, Nitrosobenzene-, and Nitrobenzene-Induced Hemoglobin Thiyl Free Radical Formation *In Vivo* and *In Vitro*

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SUMMARY

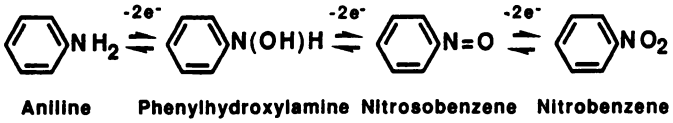
We have employed the ESR spin trapping technique *in vivo* to detect the formation of the 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO)/hemoglobin thiyl free radical adduct in the blood of rats following administration of either aniline, phenylhydroxylamine, nitrosobenzene, or nitrobenzene. This DMPO adduct was a six-line, strongly immobilized, radical adduct. Using rat red blood cells, both phenylhydroxylamine and nitrosobenzene were able to induce the formation of the DMPO/glutathiyl free radical adduct and the same DMPO/hemoglobin thiyl free radical adduct was detected in *in vivo* samples. In experiments using purified rat oxyhemoglobin, a four-line, weakly immobilized, DMPO/hemoglobin thiyl free radical adduct was detected, in addition to the six-line strongly immobilized adduct. When this study was repeated using human red blood cells, we detected only the DMPO/glutathiyl free radical adduct and, when purified human oxyhemoglobin was employed, only the four-line, weakly immobilized, DMPO/hemoglobin thiyl radical adduct could be detected. In a study using reduced glutathione, we found that phenylhydronitroxide free radicals were reduced by glutathione and that glutathione was concomitantly oxidized to its thiyl free radical. We propose that the species responsible for the oxidation of the thiols to yield the thiyl free radicals *in vivo* and *in vitro* was the phenylhydronitroxide radical produced from the reaction of phenylhydroxylamine with oxyhemoglobin.

The ability of aniline to induce methemoglobin formation following aniline poisoning has been linked to the biotransformation of aniline into proximate toxic compounds of the liver. Specifically, aniline is metabolized into *p*-aminophenol, *o*-aminophenol, and phenylhydroxylamine (1, 2). The toxicity of these compounds to the liver is minimized by very rapid conjugation of the aminophenols and by reduction of phenylhydroxylamine back to aniline (3). However, phenylhydroxylamine can also enter RBCs within the liver and react with oxyhemoglobin to yield methemoglobin and nitrosobenzene (1, 2). Nitrosobenzene is a better ligand for the ferrous iron of hemoglobin than is dissolved molecular oxygen (4). Once nitrosobenzene is formed in the RBCs, it can ligate to the iron and escape the liver via the RBC. Nitrosobenzene can be reduced within the RBC nonenzymatically by endogenous reducing agents (5, 6) or enzymatically by NADPH methemoglobin reductase to yield phenylhydroxylamine (7), thus completing a redox cycle shuttling phenylhydroxylamine to nitrosobenzene while converting oxyhemoglobin to methemoglobin.

Although the mechanism for this reaction sequence has not been fully elucidated, free radical formation has been implicated in these reactions. As early as 1948, Heubner (8) proposed the formation of a nitroxide free radical intermediate in the redox cycle of nitrosobenzene and phenylhydroxylamine in RBCs. Using ESR in 1959, Wahler and co-workers (9) claimed the formation of a nitroxide free radical due to the one-electron oxidation of phenylhydroxylamine by oxyhemoglobin. In a recent paper, Takahashi and co-workers (6) were able to show that the direct reaction of nitrosoaromatics with thiol reducing agents resulted in a one-electron transfer to yield the stable nitroxide free radicals. However, there have been no reports using the ESR spin-trapping technique to attempt to detect thiyl free radical formation resulting from this one-electron transfer reaction. Likewise, there have been no reports using this ESR technique to study the *in vivo* formation of thiyl free radicals within RBCs due to the administration of either aniline, nitrosobenzene, phenylhydroxylamine, or nitrobenzene. Although the interconversion of these xenobiotics is normally expressed as two-electron transfers, on the basis of these previous studies it is probable that thiols, including hemoglobin thiols, are oxidized *in vivo* to yield thiyl free radicals during the metabolism of these four compounds.

We have recently reported the detection of the *in vivo* formation of a hemoglobin thiyl free radical adduct of DMPO in rats dosed with either phenylhydrazine (10, 11) or hydrazine-based drugs (11). In the present study, we have applied the

ABBREVIATIONS: RBC, red blood cell; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMSO, dimethyl sulfoxide.



ESR spin-trapping technique *in vivo* to investigate the metabolism of aniline, phenylhydroxylamine, nitrosobenzene, and nitrobenzene. In addition, we have performed spin-trapping experiments *in vitro* using either isolated RBCs, purified oxyhemoglobin, or glutathione.

Materials and Methods

Aniline, DMSO, nitrobenzene, nitrosobenzene, and zinc dust were obtained from the Aldrich Chemical Company. Maleimide, *N*-ethylmaleimide, iodoacetamide, EDTA, Sephadex G-25-80 and G-75-120 gels, reduced glutathione, and DMPO were purchased from Sigma. The DMPO was vacuum distilled in the dark before use. We synthesized β-phenylhydroxylamine [m.p. 80–82°, literature value 82° (12)] from nitrobenzene, using zinc dust, by an established procedure (13) and stored it at −80° until used.

The ESR spectra were recorded at room temperature using a Varian E-104 spectrometer operating at 9.4 GHz with a 100 kHz modulation frequency. All samples were transferred to a quartz flat cell, which was then centered in a TM_{110} microwave cavity for analysis. ESR spectral simulations were performed using an HP 9000-236 computer.

All studies employed male Sprague-Dawley rats (155–288 g), which were allowed free access to both food and water. Following the administration of anesthesia (pentobarbital), the rats were given an intraperitoneal injection of DMPO (0.5 ml/kg), followed by an intragastric injection of either aniline, phenylhydroxylamine, nitrosobenzene, or nitrobenzene (all dissolved in DMSO to equivalent molar concentrations to yield doses ranging from 0.1 to 2 mmol/kg). At the end of 2 hr the rats were sacrificed, employing a lethal dose of pentobarbital, and the blood was collected by heart puncture. Blood samples taken while the rat was alive were obtained from the rat's tail and were taken immediately before dosing and every 20 min after dosing for 2 hr. EDTA was added to all blood samples as an anticoagulant, and the blood was frozen and stored at −80° until examined.

In vitro RBC experiments were performed using RBCs obtained from sacrificed rats or from outdated human blood (Durham County Blood Bank). The RBCs were isolated from the whole blood by centrifugation and washed as before (10). In a typical experiment, 5.5 μl of DMPO (100 mM final concentration) were added to 0.5 ml of a suspension of RBCs in isotonic, pH 7.4, phosphate buffer (0–100% RBC suspension). The reaction was initiated by the addition of 5 μl of a 0.1 M solution of either aniline, phenylhydroxylamine, nitrosobenzene, or nitrobenzene dissolved in DMSO (final drug concentration, 1 mM). Aliquots were examined by ESR within 1 min of mixing. The DMSO was used in these experiments to ensure that the phenylhydroxylamine and nitrosobenzene stock solutions were totally dissolved. Experiments were also performed using ethanol in place of DMSO to confirm that DMSO was not affecting our results.

For experiments involving purified oxyhemoglobin, washed RBCs were lysed by three freeze/thaw cycles and the supernatant was collected by centrifugation. This supernatant was either passed through a Sephadex G-25 column and then a Sephadex G-75 column (both equilibrated with pH 7.4, 100 mM phosphate buffer) or was dialyzed twice against 100 mM phosphate buffer (1:100 dilution factor each time) using dialysis tubing (Spectrum) with a 10,000 molecular weight pore size. When necessary, the oxyhemoglobin solution was concentrated using Spectrum Spectra/Con Concentrator water absorption fingers. When sulfhydryl-blocking agents were used, the oxyhemoglobin solution was mixed with either maleimide (10 mM), *N*-ethylmaleimide (100 mM), or iodoacetamide (10 mM) and allowed to stand for 2 min at room temperature before the addition of other reagents. CO-hemoglobin was prepared by slowly bubbling carbon monoxide through the purified oxyhemoglobin solution for 30 min. During bubbling, samples were monitored by visible spectroscopy for CO-hemoglobin formation; maximal CO-hemoglobin formation was achieved within the 30-min time frame.

Results

***In vivo* rat studies.** Fig. 1 shows the spectra obtained from whole blood 2 hr after the rats were dosed with DMPO (0.5 ml/kg, intraperitoneal) and 0.1 mmol/kg (intragastric) of either aniline (Fig. 1A), phenylhydroxylamine (Fig. 1B), nitrosobenzene (Fig. 1C), or nitrobenzene (Fig. 1D). Under these dosage conditions, only phenylhydroxylamine and nitrosobenzene were able to yield the DMPO/hemoglobin thiyl free radical adduct, which was previously assigned in investigations with phenylhydrazine and hydrazine-based drugs (10, 11). When we repeated the experiment but used 2 mmol/kg of the compounds, the phenylhydroxylamine- and nitrosobenzene-treated rats died within 20 min of dosing. However, the aniline- and nitrobenzene-treated rats survived the higher dosage and, as shown in Fig. 2, the aniline-treated (Fig. 2A) and nitrobenzene-treated (Fig. 2B) rats now yielded the DMPO/hemoglobin thiyl free radical adduct.

The time course of adduct formation was determined by taking blood samples every 20 min after dosing. As shown in Fig. 3, when rats were dosed with nitrosobenzene (0.1 mmol/kg, $n = 3$), the adduct signal intensity maximized much earlier than for the phenylhydroxylamine-treated rats (0.1 mmol/kg, $n = 3$), yet after 2 hr the relative signal intensities were equal. Nitrobenzene- (2 mmol/kg, $n = 3$), and aniline-treated rats (2 mmol/kg, $n = 3$) (Fig. 4) demonstrated a time profile quite

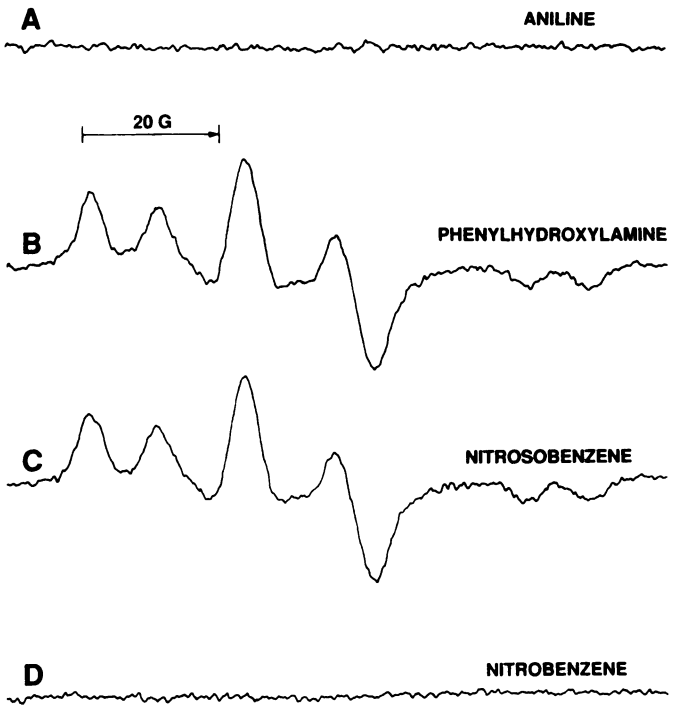


Fig. 1. ESR spectra obtained from whole blood taken two hr after rats were dosed with DMPO (0.5 ml/kg, intraperitoneal) and one of the following drugs (0.1 mmol/kg, intragastric): aniline (A), phenylhydroxylamine (B), nitrosobenzene (C), or nitrobenzene (D). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 3.3 G; time constant, 0.25 sec; scan rate, 50 G/min.

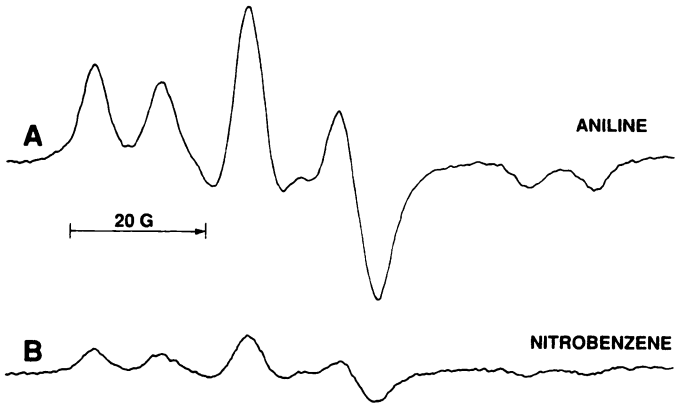


Fig. 2. ESR spectra obtained from whole blood taken two hr after rats were dosed with DMPO (0.5 ml/kg, intraperitoneal) and one of the following drugs (2 mmol/kg, intragastric): aniline (A) or nitrobenzene (B). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 3.3 G; time constant, 0.25 sec; scan rate, 50 G/min.

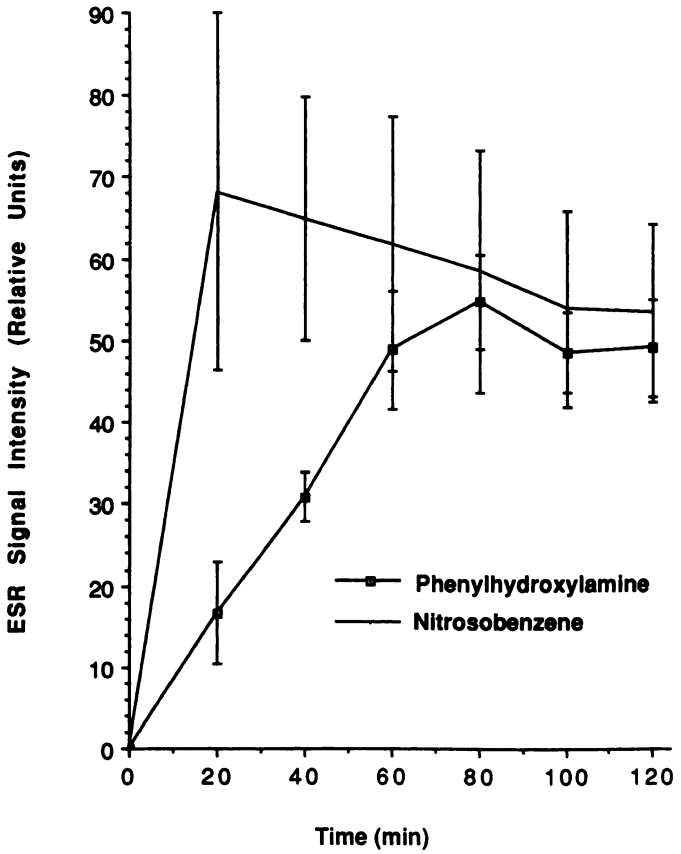


Fig. 3. Time profile for the formation of DMPO/hemoglobin thiyl free radical adduct in whole blood following the dosing of rats with DMPO (0.5 ml/kg, intraperitoneal) and either phenylhydroxylamine (0.1 mmol/kg, intragastric) or nitrosobenzene (0.1 mmol/kg, intragastric). The *error bars* represent the standard errors obtained using three rats/group.

similar to that of the phenylhydroxylamine-treated rats, whereas the nitrobenzene-treated rats showed a continual slow growth in adduct signal intensity. The influence of the route of administration of phenylhydroxylamine on the time profile of adduct formation was determined next. When phenylhydroxylamine was given intraperitoneally, the adduct formation time profile mimicked that of nitrosobenzene given intragastrically (data not shown), suggesting that phenylhydroxylamine is more

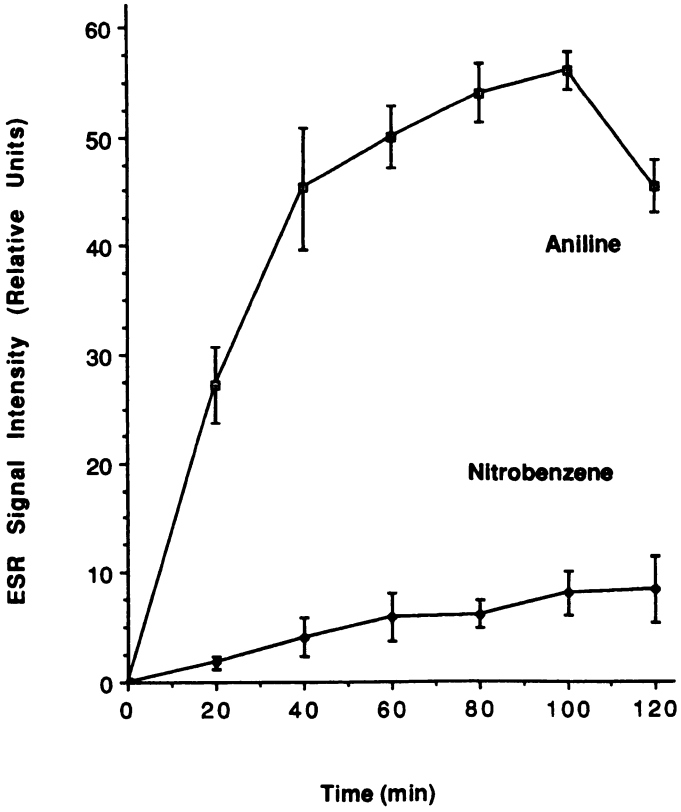


Fig. 4. Time profile for the formation of DMPO/hemoglobin thiyl free radical adduct in whole blood following the dosing of rats with DMPO (0.5 ml/kg, intraperitoneal) and either aniline (2 mmol/kg, intragastric) or nitrobenzene (2 mmol/kg, intragastric). The *error bars* represent the standard errors obtained using three rats/group.

slowly absorbed from the gut than nitrosobenzene. (The time profile for nitrosobenzene was the same whether it was given intraperitoneally or intragastrically).

***In vitro* rat RBC studies.** As shown in Fig. 5, when packed RBCs were given DMPO (100 mM) and 1 mM concentrations of either phenylhydroxylamine (Fig. 5A), nitrosobenzene (Fig. 5B), nitrobenzene (Fig. 5D), or aniline (Fig. 5E), only phenylhydroxylamine and nitrosobenzene reacted to yield a radical adduct. The computer simulation for the initial radical adduct detected was calculated using the hyperfine splitting constants a^N = 15.2 G and a_β^H = 16.1 G, and a broad peak-to-peak linewidth of 1.0 G (Fig. 5C). This radical adduct was assigned to the DMPO/glutathiyl free radical adduct (DMPO/GS·) based upon comparison of these splitting constants and linewidths with those reported in the literature (14). The formation of this adduct depended on the presence of DMPO (not shown), either phenylhydroxylamine or nitrosobenzene (Fig. 5F), and RBCs (Fig. 5, G and H). The residual signal detectable in Fig. 5, G and H, was due to the presence of phenylhydronitroxide, an impurity radical in the stock solutions of both nitrosobenzene and phenylhydroxylamine. Experiments performed using ethanol in place of DMSO yielded analogous results.

The DMPO/GS· adduct was not persistent and, as shown in Fig. 6, it rapidly decayed. Concurrent with the decay of DMPO/GS·, the DMPO/hemoglobin thiyl free radical adduct began to grow to detectable levels and after 28 min (Fig. 6D) was the predominant radical adduct. Nitrosobenzene yielded exactly the same results with packed RBCs as phenylhydroxylamine (data not shown). When the RBC experiments were repeated

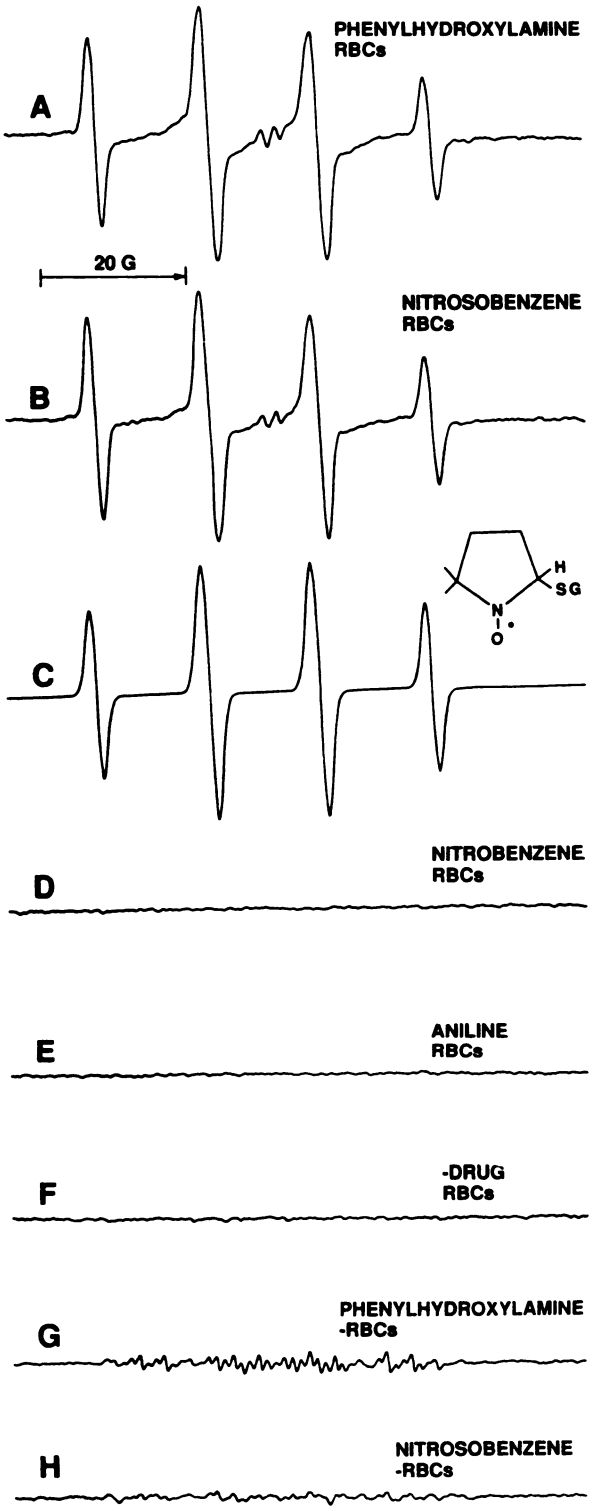


Fig. 5. A, the ESR spectrum resulting from the addition of phenylhydroxylamine (1 mM) to a mixture of packed rat RBCs and DMPO (100 mM). B, As in A, except nitrosobenzene (1 mM) was added in place of phenylhydroxylamine. C, Computer simulation employing the hyperfine splitting constants a^N = 15.2 G and a^H_β = 16.1 G, a linewidth of 1.0 G, and 10% Lorentzian/90% Gaussian lineshape. D, As in A, except nitrobenzene (1 mM) was added in place of phenylhydroxylamine. E, As in A, except aniline (1 mM) was added in place of phenylhydroxylamine. F, As in A, except no drug was added. G, As in A, except pH 7.4, 100 mM buffer was used in place of the RBCs. H, As in B, except pH 7.4, 100 mM buffer was used in place of the RBCs. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 0.67 G; time constant, 0.5 sec; scan rate, 25 G/min.

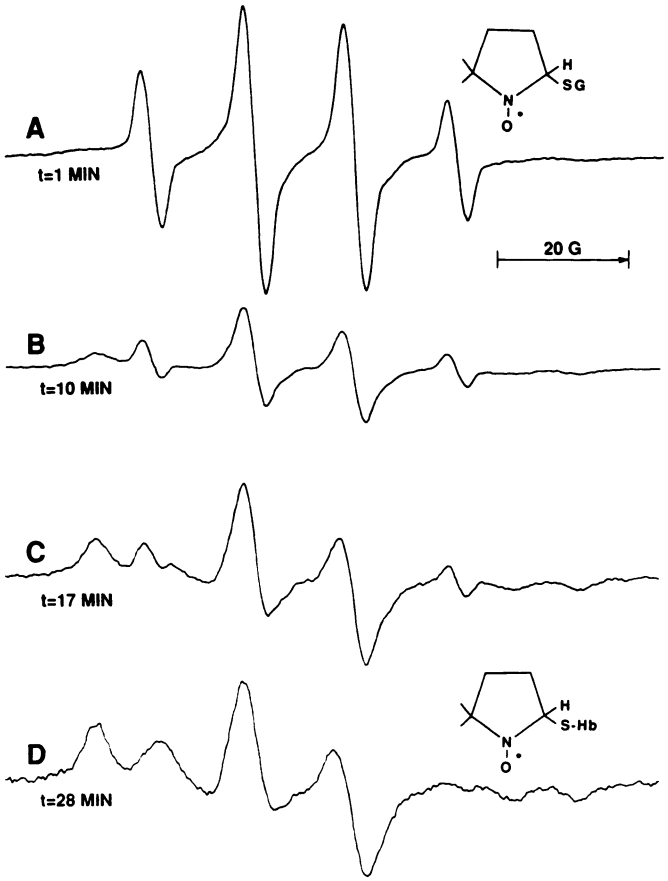


Fig. 6. ESR spectra resulting from the addition of phenylhydroxylamine (1 mM) to a mixture of packed rat RBCs and DMPO (100 mM). A, Scan initiated 1 min after mixing. B, Scan initiated 10 min after mixing. C, Scan initiated 17 min after mixing. D, Scan initiated 28 min after mixing. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 3.3 G; time constant, 0.25 sec; scan rate, 50 G/min.

using various dilutions of RBCs rather than packed RBCs, we found that phenylhydroxylamine yielded higher concentrations of radical adducts with diluted RBC suspensions than did nitrosobenzene. This finding suggests that phenylhydroxylamine either was the active agent in forming the radical adducts or was more readily converted into the active agent than was nitrosobenzene. These data are shown in Fig. 7, where the percentage of dilution of the RBCs was plotted versus the ratio of the phenylhydroxylamine-induced to nitrosobenzene-induced formation of the DMPO/GS· and DMPO/hemoglobin thiyl free radical adducts.

***In vitro* purified rat oxyhemoglobin studies.** The finding that phenylhydroxylamine was more effective than nitrosobenzene in yielding radical adducts with dilute RBCs was consistent with nitrosobenzene being reduced to phenylhydroxylamine within the RBC before adduct formation (Fig. 7). If this was so, then oxyhemoglobin should yield the DMPO/hemoglobin thiyl radical adduct with phenylhydroxylamine to a much greater extent than with nitrosobenzene. As shown in Fig. 8A, when dialyzed hemolysate was reacted with phenylhydroxylamine, a novel four-line, weakly immobilized, radical adduct was detected. Nitrosobenzene yielded a much weaker signal than phenylhydroxylamine (Fig. 8B). Without phenylhydroxylamine, no radical adducts could be detected in DMPO-oxyhemoglobin mixtures (Fig. 8C). In the absence of the he-

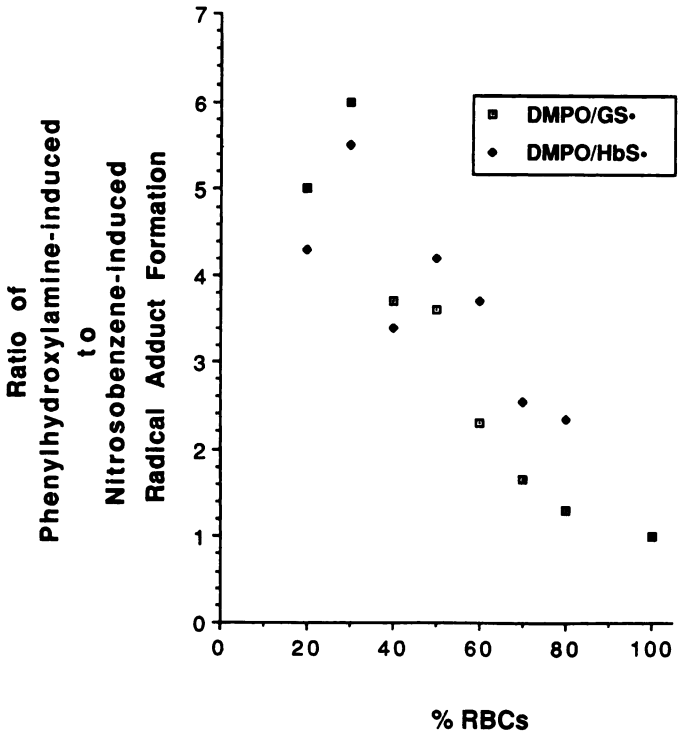


Fig. 7. Effect of dilution of RBCs on the relative ability of phenylhydroxylamine (1 mM) or nitrosobenzene (1 mM) to induce DMPO/hemoglobin thiyl radical adduct (*DMPO/HbS*·) and DMPO/glutathiyl radical adduct (*DMPO/GS*·) formation when added to a mixture of rat RBCs and DMPO (100 mM).

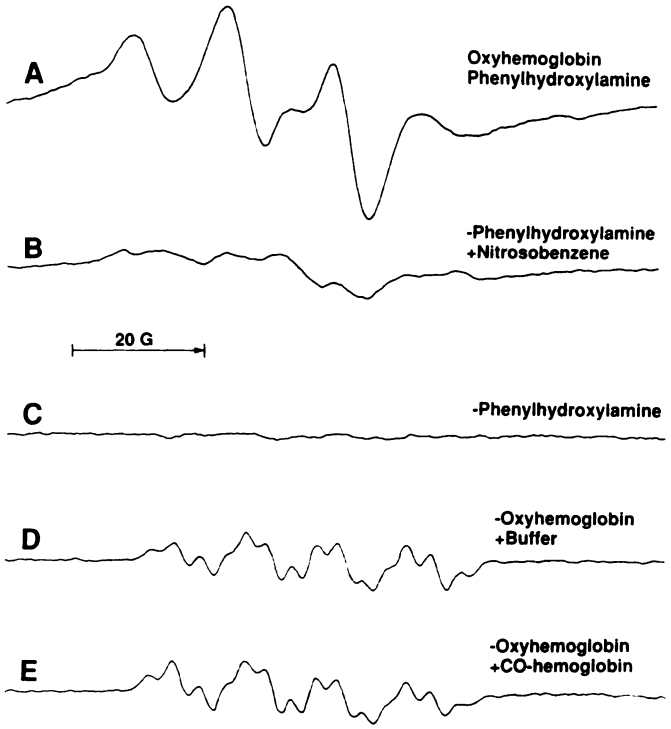


Fig. 8. A, ESR spectrum obtained when phenylhydroxylamine (1 mM) was added to a solution of dialyzed rat hemolysate (5 mM oxyhemoglobin) and DMPO (100 mM). B, As in A, except nitrosobenzene (1 mM) was added in place of phenylhydroxylamine. C, As in A, except phenylhydroxylamine was not added. D, As in A, except pH 7.4, 100 mM phosphate buffer was added in place of the hemolysate. E, As in A, except the hemolysate was bubbled with carbon monoxide for 30 min before the addition of the other reagents. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 3.3 G; time constant, 0.5 sec; scan rate, 25 G/min.

moglobin, only the background phenylhydronitroxide signal from the phenylhydroxylamine solution could be detected (Fig. 8D). When CO-hemoglobin was used, radical adduct formation was not detected (Fig. 8E). Analogous results for these experiments were obtained using oxyhemoglobin that had been purified using gel chromatography (data not shown).

When we monitored the ESR signal from the oxyhemoglobin-phenylhydroxylamine-DMPO mixture, a second radical adduct was detected at later time points. Thus, initially we detected the four-line, weakly immobilized signal (Fig. 9A), but after 18 min the spectral lines of the six-line, strongly immobilized, DMPO/hemoglobin thiyl free radical adduct became apparent (Fig. 9C). We hypothesize that the four-line, weakly immobilized adduct could also be a DMPO adduct of a hemoglobin thiyl free radical but that the thiol being oxidized may be closer to the surface of the hemoglobin protein than the thiol that was oxidized to yield the six-line, strongly immobilized, DMPO/hemoglobin thiyl radical adduct. Purified oxyhemoglobin (data not shown) or dialyzed hemolysate pretreated with the sulfhydryl-blocking agents iodoacetamide (Fig. 10B), *N*-ethylmaleimide (Fig. 10C), or maleimide (Fig. 10D), inhibited the formation of both adducts, consistent with their both being derived from the oxidation of thiol groups.

***In vitro* human RBC-oxyhemoglobin studies.** We became curious as to whether these immobilized adducts could be detected with human oxyhemoglobin. As shown in Fig. 11A, dialyzed human oxyhemoglobin did indeed yield the four-line, weakly immobilized adduct, but, interestingly, failed to yield the six-line, strongly immobilized adduct. When the experiment was repeated using packed human RBCs, only the DMPO/GS· adduct was detected (Fig. 11B). Thus, the six-line, strongly immobilized, DMPO/hemoglobin thiyl free radical adduct detected in the rat appears to be species specific. When 1 mM glutathione was added to the dialyzed human oxyhemoglobin before the addition of phenylhydroxylamine, DMPO/GS· was detected (Fig. 11C). When used in place of phenylhydroxylamine, nitrosobenzene produced less DMPO/GS· in this system (Fig. 11D).

***In vitro* GSH studies.** In the purified oxyhemoglobin studies, we found that GSH was oxidized to yield glutathiyl free radicals. This thiyl radical formation could be due to the direct oxidation of GSH by the nitrosobenzene produced from the reaction of phenylhydroxylamine with oxyhemoglobin or could be due to oxidation of GSH by some other intermediate. Inasmuch as nitrosobenzene was less effective at yielding DMPO/GS· than phenylhydroxylamine in the oxyhemoglobin system, we hypothesized that a different intermediate, the phenylhydronitroxide radical, was the oxidizing agent. To test this, we first examined whether GSH would reduce the phenylhydronitroxide radical. As shown in Fig. 12, when GSH is added to a 1 mM solution of phenylhydroxylamine, the background phenylhydronitroxide radical signal intensity is dramatically decreased. If DMPO is included in the reaction mixture, DMPO/GS· formation can be detected concurrent with phenylhydronitroxide reduction (Fig. 12C), thus suggesting that GSH reduces the phenylhydronitroxide radical to phenylhydroxylamine while being oxidized to GS·.

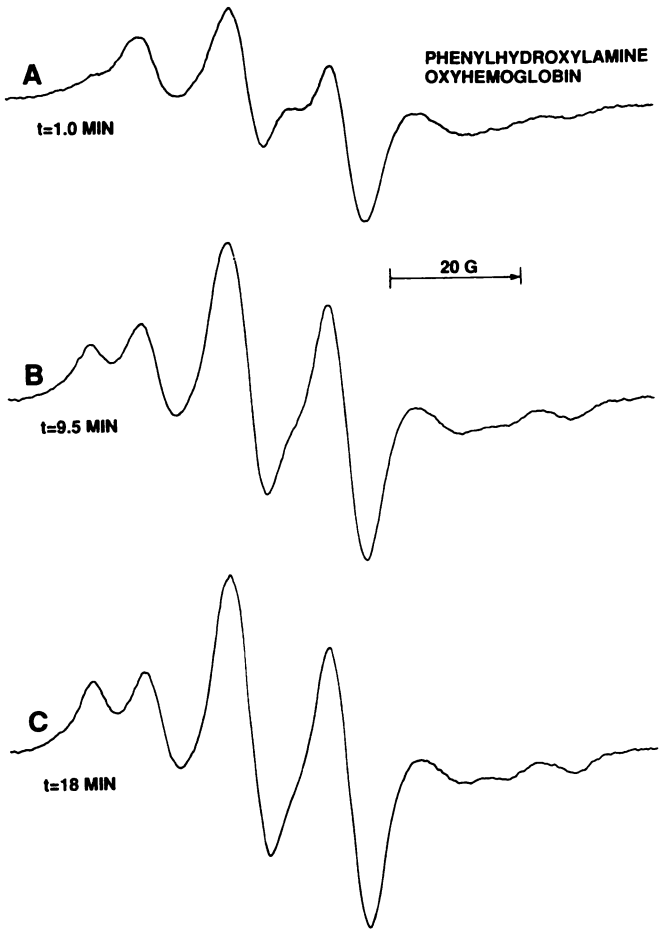


Fig. 9. ESR spectra obtained when phenylhydroxylamine (1 mM) was added to a solution of dialyzed rat hemolysate (5 mM oxyhemoglobin) and DMPO (100 mM). A, Scan initiated 1 min after mixing. B, Scan initiated 9.5 min after mixing. C, Scan initiated 18 min after mixing. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 3.3 G; time constant, 0.5 sec; scan rate, 25 G/min.

Discussion

Our results are the first to use ESR to demonstrate the oxidation of hemoglobin thiols *in vivo* and the oxidation of GSH and hemoglobin thiols *in vitro* to thiyl free radicals by phenylhydronitroxide. Our inability to detect DMPO/GS· and the four-line, weakly immobilized DMPO/hemoglobin thiyl free radical adduct *in vivo* was likely a reflection of the rapid reduction of most radical adducts into ESR-invisible hydroxylamines by ascorbate and other biochemical reducing agents (15, 16). Other decay mechanisms may also be operating, such as the irreversible destruction of radical adducts by other radicals (17, 18).

It was apparent from Fig. 12 that the phenylhydronitroxide radical was capable of oxidizing thiols. We deem it quite likely that both *in vivo* and *in vitro* phenylhydroxylamine reacted with oxyhemoglobin to yield phenylhydronitroxide radicals. These radicals might then oxidize hemoglobin thiols and/or glutathione to yield the thiyl free radicals that we detected. On the basis of our results, we can not state whether we trapped oxyhemoglobin thiyl radicals, methemoglobin thiyl radicals, or both. Thus, we refer to these radicals using the generic term hemoglobin thiyl radicals. The ability of nitrosobenzene to yield thiyl free radicals can also be linked to phenylhydronitroxide

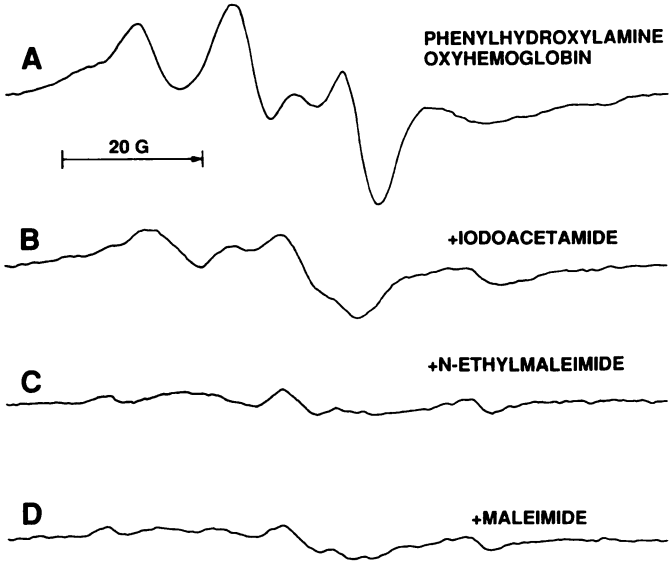


Fig. 10. Effect of thiol-blocking agents on the formation of the four-line, slightly immobilized, DMPO/hemoglobin thiyl radical adduct. A, ESR spectrum recorded immediately after the addition of phenylhydroxylamine (1 mM) to a solution of dialyzed rat hemolysate (5 mM oxyhemoglobin) and DMPO (100 mM). B, As in A, except the hemolysate was first incubated for 2 min with 10 mM iodoacetamide. C, As in A, except the hemolysate was first incubated for 2 min with 100 mM *N*-ethylmaleimide. D, As in A, except the hemolysate was incubated for 2 min with 10 mM maleimide. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 3.3 G; time constant, 0.5 sec; scan rate, 25 G/min.

radical formation. First, it has already been shown that nitrosobenzene will react with thiol reducing agents to yield phenylhydronitroxide radicals (6). Thus, the ability of nitrosobenzene to produce DMPO/GS· in dialyzed human oxyhemoglobin-glutathione mixtures (Fig. 12D) can be explained by this reaction. Second, nitrosobenzene can also be rapidly reduced within the RBC by native reductants (5, 6) or by NADPH methemoglobin reductase (7) to produce phenylhydroxylamine, which can then react with oxyhemoglobin to yield phenylhydronitroxide radicals. The ability of phenylhydronitroxide radicals to oxidize thiols is consistent with the recent report (19) that nitroxide radicals derived from desferrioxamine are capable of oxidizing both protein and non-protein thiols. We propose the following mechanism to account for the formation of thiyl free radicals due to the reaction of phenylhydroxylamine [Ph-N(OH)H] with oxyhemoglobin [$HbFe(II)O_2$], wherein HbSH represents a hemoglobin thiol.

$$HbFe(II)O_2 + \text{Ph-N(OH)H} \rightleftharpoons HbFe(III)OOH + \text{Ph-N(O·)H} \quad (1)$$

$$\text{Ph-N(O·)H} + [HbFe(III)OOH] \rightleftharpoons [HbFe(IV)O] + \text{Ph-N=O} + H_2O \quad (2)$$

$$\text{Ph-N(OH)H} + [HbFe(IV)O] \rightleftharpoons HbFe(III)OH^- + \text{Ph-N(O·)H} \quad (3)$$

$$\text{Ph-N=O} + HbFe(II)O_2 \rightleftharpoons HbFe(II)O{=}N\text{-Ph} + O_2 \quad (4)$$

$$\text{Ph-N=O} + GSH \rightleftharpoons \text{Ph-N(O·)H} + GS· \quad (5)$$

$$2\ \text{Ph-N(O·)H} \rightleftharpoons \text{Ph-N(OH)H} + \text{Ph-N=O} \quad (6)$$

$$\text{Ph-N(O·)H} + GSH \rightleftharpoons \text{Ph-N(OH)H} + GS· \quad (7)$$

$$\text{Ph-N(O·)H} + HbSH \rightleftharpoons \text{Ph-N(OH)H} + HbS· \quad (8)$$

$$DMPO + GS· \rightarrow DMPO/GS· \quad (9)$$

$$DMPO + HbS· \rightarrow DMPO/HbS· \quad (10)$$

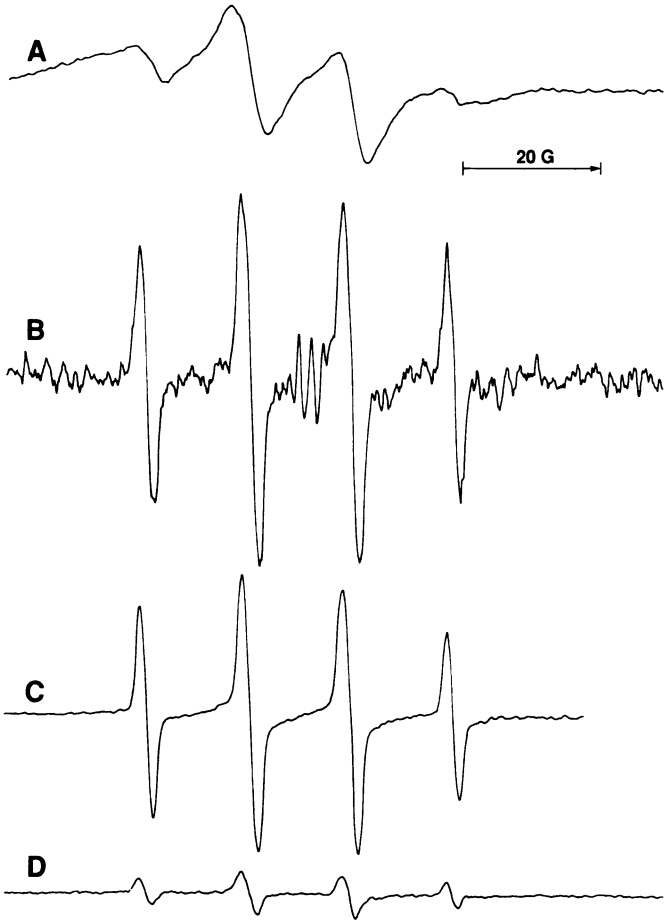


Fig. 11. A, ESR spectrum recorded immediately after phenylhydroxylamine (1 mM) was added to a mixture of dialyzed human hemolysate (9 mM oxyhemoglobin) and DMPO (100 mM). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 3.3 G; time constant, 2 sec; receiver gain, 2×10^4, scan rate, 12.5 G/min. B, ESR spectrum obtained following the addition of phenylhydroxylamine (1 mM) to a mixture of packed, undiluted, human RBCs and DMPO (100 mM). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 0.67 G; time constant, 0.5 sec; receiver gain, 6.3×10^4, scan rate, 25 G/min. C, ESR spectrum obtained after phenylhydroxylamine (1 mM) was added to a mixture of dialyzed human hemolysate (9 mM oxyhemoglobin), glutathione (1 mM), and DMPO (100 mM). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 0.67 G; time constant, 0.25 sec; receiver gain, 4×10^3; scan rate, 50 G/min. D, As in C, except nitrosobenzene (1 mM) was added in place of phenylhydroxylamine.

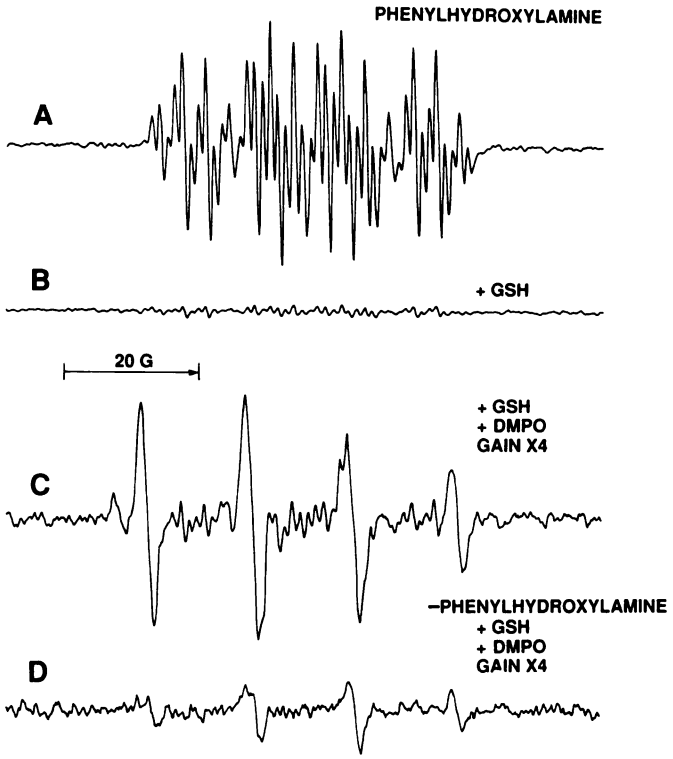


Fig. 12. A, ESR spectrum of the phenylhydronitroxide radical obtained from a 1 mM solution of phenylhydroxylamine in pH 7.4, 100 mM phosphate buffer. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 0.67 G; time constant, 0.5 sec; receiver gain, 8×10^3; scan rate, 25 G/min. B, As in A, except glutathione (1 mM) was added. C, As in A, except both glutathione (1 mM) and DMPO (100 mM) were added and the receiver gain was 3.2×10^4. D, As in C, except phenylhydroxylamine was not added.

The proposed reaction sequence of Eqs. 1 to 4 takes into account that during the co-oxidation of oxyhemoglobin and phenylhydroxylamine, reactive reduced oxygen species were not detected. Neither catalase nor superoxide dismutase had any effect either on the rate of ferrihemoglobin formation or on the pattern of reaction products (20, 21). Meanwhile evidence is growing that myoglobin and hemoglobin are capable of forming compound I- and II-type complexes (22–24). Clearly, there are also many other reactions of nitrosobenzene with thiols by non-free radical processes to yield semimercaptals and other metabolites (2, 25–27) that this mechanism does not take into account.

The facile metabolism of the four compounds examined allowed for each to yield the DMPO/hemoglobin thiyl free radical adduct *in vivo*. Both phenylhydroxylamine and nitrosobenzene reacted directly within the RBCs, whereas aniline required metabolism most likely occurring in the liver (1, 2) to oxidize it into phenylhydroxylamine. The ability of nitrobenzene to induce DMPO/hemoglobin adduct formation *in vivo* is likely a reflection of its reduction by the gut microflora (28) or possibly by hepatic microsomal nitroreductase (29).

The species specificity for the formation of the six-line, strongly immobilized, DMPO/hemoglobin thiyl free radical adduct can be explained by the differences between the rat and human hemoglobin chains. Although the β-chain for both species has the same number and location of cysteine amino acids, the α-chain for the rat has two additional cysteine amino acids (30, 31). It is assumed that the six-line, strongly immobilized, DMPO/hemoglobin thiyl free radical adduct is localized at one or both of these two additional sites on the rat hemoglobin α-chain. The more extensive immobilization of the rat-specific, six-line, DMPO/hemoglobin thiyl free radical implies that this adduct was buried in a deep pocket of the protein. This point is also borne out by our observation that maximal adduct formation of this six-line, strongly immobilized, radical adduct always occurred later than that of DMPO/GS· or the four-line, weakly immobilized, DMPO/hemoglobin thiyl free radical adduct.

To summarize, our results demonstrate that aniline, phenylhydroxylamine, nitrosobenzene, and nitrobenzene are all metabolized *in vivo* to yield the same metabolite, most probably the phenylhydronitroxide radical, which was responsible for the oxidation of thiols within RBCs.

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