0026-895X/88/030344-07$00.00/0

MOLECULAR PHARMACOLOGY, 33:344–350

In Vivo Rat Hemoglobin Thiyl Free Radical Formation Following Phenylhydrazine Administration

KIRK R. MAPLES, SANDRA J. JORDAN, and RONALD P. MASON

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

Received September 18, 1987; Accepted November 30, 1987

SUMMARY

The reaction of oxyhemoglobin with phenylhydrazine has received considerable attention for many decades. The basis for this interest stems from the ability of phenylhydrazine and hydrazine-based drugs to induce hemolytic anemia. Considerable evidence obtained from *in vitro* ESR experiments implicates free radicals in the events leading to red blood cell hemolysis. However, until this report, no corroborating ESR evidence for *in vivo* free radical formation has been presented. We have successfully employed ESR to detect the formation of a radical adduct in the blood of rats which received an intragastric dose of phenylhydrazine followed by an intraperitoneal injection of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). An immobilized radical adduct was detected by ESR when phenylhydrazine was administered in a dosage comparable to that prescribed for currently employed hydrazine-based drugs. We were also able to detect this immobilized DMPO adduct when hydrazine was employed in place of phenylhydrazine in the rat studies. The results of a series of experiments led us to ascribe this DMPO radical adduct to the trapping of a hemoglobin-derived thiyl free radical.

For more than 100 years the reaction of phenylhydrazine with RBCs has been receiving attention (1–28). This reaction has been shown to induce the oxidative denaturation of hemoglobin. When such denaturation takes place in the RBC, the intracellular aggregates of denatured hemoglobin are called Heinz bodies (1, 15, 24). Their formation initiates a series of processes leading to premature destruction of the cell and resulting in hemolytic anemia. The tendency of a wide variety of hydrazine derivatives used in medicine, agriculture, and industry to induce hemolytic anemia has intensified research efforts in the last 15 years to unravel the mechanistic aspects of this toxicity (4–28).

The deleterious effects of phenylhydrazine on RBCs had long been hypothesized as being due to intracellular free radical formation. However, no direct evidence of radical formation was obtained until 1979 when Goldberg *et al.* (9) detected the superoxide anion radical in frozen samples of phenylhydrazine and hemoglobin. Subsequent to this work, the phenyl radical was detected via ESR spin-trapping techniques in mixtures of phenylhydrazine with oxyhemoglobin (10, 14, 20) or RBCs (10, 16, 20). Unequivocal identification of this phenyl radical adduct of DMPO was achieved by mass spectral analysis of the purified adduct (10). The phenyl radical derived from this reaction has also been shown to attack the heme moiety at the iron (14). More recently, Smith and Maples (23) used direct, fast-flow ESR to detect the phenylhydrazyl radical produced when phenylhydrazine reacts with oxyhemoglobin.

Although free radical formation due to the reaction of phenylhydrazine with RBCs has been established *in vitro*, analogous ESR spectroscopic evidence for *in vivo* radical formation has not yet been reported. Based on the recent success in this laboratory (29) and others (30–35) of applying the spin-trapping technique to detect free radical metabolite formation *in vivo*, and encouraged by the apparent stability of the phenyl radical adduct of DMPO (10), we decided to apply this ESR technique to study the *in vivo* reaction of phenylhydrazine with blood. We were able to demonstrate that, indeed, free radicals are formed in rats treated with phenylhydrazine. Direct examination of blood samples from DMPO/phenylhydrazine-treated rats with ESR revealed an immobilized nitroxide spectrum. We were also able to detect this adduct in the blood of rats which were dosed with hydrazine rather than phenylhydrazine. Our studies show that this adduct is immobilized in the hemoglobin protein and is most likely a DMPO/hemoglobin-derived thiyl free radical adduct. When we extracted the blood with either chloroform or a 2:1 chloroform/methanol solution, we could also detect a weak signal from the DMPO/phenyl radical adduct.

Materials and Methods

Phenylhydrazine hydrochloride, hydrazine hydrate, sodium dithionite, chloroform, copper(II) sulfate, urea, EDTA, DTPA, guanidine hydrochloride, iodoacetamide, maleimide, *N*-ethylmaleimide, rat he-

ABBREVIATIONS: RBC, red blood cell; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid.

moglobin, G-25 Sephadex gel, and G-75 Sephadex gel were purchased from Sigma. DMPO was purchased from Sigma and vacuum distilled prior to use.

The ESR spectra were obtained at room temperature using either a Varian E-104 or a Varian Century series E-109 spectrometer operating at 9.5 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.

Rat blood was obtained from sacrificed animals via heart puncture. The blood was centrifuged at 3000 × *g* for 5 min at 0–5° to pack the RBCs. The supernatant blood plasma and buffy coat were removed; then, the cells were washed three times with isotonic saline and centrifuged at 3000 × *g* for 5 min at 0–5° to repack the RBCs before removal of each wash solution. The RBCs were then stored at 0–5° until use.

Purified rat oxyhemoglobin was prepared using standard procedures (14, 20, 36), as follows. Sigma rat hemoglobin was dissolved in isotonic (pH 7.4) phosphate buffer. To this solution, 1 mg/ml of sodium dithionite was added and the solution stirred for ~5 min. The resultant deoxy- and oxyhemoglobin were then isolated from 1 ml of this solution via gel exclusion chromatography at 0–5° using a 2.5 × 50.0 cm column of Sephadex G-25 gel preequilibrated with isotonic (pH 7.4) phosphate buffer. The hemoglobin so obtained was then bubbled with oxygen to convert deoxyhemoglobin to oxyhemoglobin. The concentration of oxyhemoglobin was measured spectroscopically using a Hewlett-Packard 8451A Diode Array Spectrophotometer, based on an extinction coefficient of 1.53×10^4 M^{-1} cm^{-1} at 540 nm (6, 37). When necessary, this solution was concentrated using Spectrum Spectra/Con Concentrator water absorption fingers.

Adduct elution profiles were performed using a 2.5 × 50.0 cm column of Sephadex G-75 gel equilibrated with isotonic (pH 7.4) phosphate buffer at 0–5°. Before being applied to the column, the RBCs in the adduct solution were lysed by equal admixture of deionized water. An aliquot of this solution, ~1 ml, was then applied to the column, and the eluting fractions were analyzed by ESR for relative adduct concentration and by visible spectroscopy for oxyhemoglobin concentrations.

In Vivo Studies

Anesthetized male Sprague-Dawley rats (195–643 g) were given 500 μl/kg intraperitoneal injections of DMPO followed by intragastric administration of either phenylhydrazine hydrochloride or hydrazine hydrate in deionized water. The phenylhydrazine dosage ranged from 1 mg/kg up to its LD_{50} value, 188 mg/kg (38); the hydrazine dosage was 129 mg/kg. Blood samples were collected from the tip of the tail of the rat at 15-min intervals for the first hour following phenylhydrazine administration. At the end of 2 hr the rats were sacrificed using a lethal dose of pentobarbital, and blood was collected via heart puncture. EDTA was added to all blood samples as an anticoagulant.

In Vitro Studies

RBC dilution effect. Packed RBCs were diluted with varying amounts of isotonic (pH 7.4) phosphate buffer to yield a total volume of 2 ml. To each solution, 40 μl of 0.05 M DTPA (1 mM final concentration) and 22 μl DMPO (100 mM final concentration) were added and mixed. The reaction was initiated by addition of 4 μl of 0.5 M phenylhydrazine (1 mM final concentration) and aliquots were examined by ESR within 2 min of mixing.

Protein denaturation effect. Phenylhydrazine (1 mM), DMPO (100 mM), and DTPA (1 mM) in undiluted RBCs were added to either solid urea (6 M) or guanidine hydrochloride (6 M, pH 7.4) and then stirred. The ESR spectra of these solutions were obtained within 2 min of mixing. To serve as a blank for the guanidine hydrochloride experiment, RBCs were also mixed with isotonic (pH 7.4) phosphate buffer to an equivalent dilution and examined by ESR.

Purified rat oxyhemoglobin/phenylhydrazine studies. Purified rat oxyhemoglobin was mixed with DTPA (1 mM) and DMPO (100 mM). To this solution, phenylhydrazine was then added to yield an oxyhemoglobin/phenylhydrazine concentration ratio of 20:1. This concentration ratio was essentially equal to that obtained using undiluted, packed RBCs with 1 mM phenylhydrazine. Samples were analyzed by ESR within 2 min of mixing.

Thiol-blocking agents effect. Either iodoacetamide (10 mM), maleimide (10 mM), or *N*-ethylmaleimide (10–100 mM) was mixed with either purified rat oxyhemoglobin or RBCs for ~10 min at room temperature. Following this incubation, DTPA, DMPO, and phenylhydrazine were added to the hemoglobin solution in the same manner as in the Purified Rat Oxyhemoglobin/Phenylhydrazine experiments. For some experiments involving RBCs, the excess thiol-blocking agent was removed prior to addition of reagents by centrifugation (3000 × *g* for 5 min)-isotonic phosphate buffer wash cycles.

Adduct studies. The DMPO/phenyl adduct was prepared as follows. To a solution of copper(II) sulfate (10 μM) and DMPO (100 mM) in pH 7.4 phosphate buffer, phenylhydrazine (1 mM) was added with stirring. Chloroform was then added and, following mixing, the aqueous layer was discarded. The chloroform solution was washed with concentrated HCl to convert any excess phenylhydrazine into its acid salt, which was discarded with the acid wash solution. The resulting chloroform solution was washed twice more with pH 7.4 phosphate buffer to remove residual HCl and then transferred into a clean, dry test tube. Aliquots of this solution were evaporated dry using nitrogen gas to yield the DMPO/phenyl radical adduct. This adduct was then dissolved in either RBC solutions or phosphate buffer and examined by ESR.

Results

When we used ESR to examine blood collected 2 hr after the administration of both phenylhydrazine and DMPO to the rat, we obtained a six-line spectrum, due to an immobilized DMPO/free radical adduct (Fig. 1A). This adduct could be detected within 15 min of dosing and the relative adduct concentration continually increased over the 2-hr time frame. As evidenced

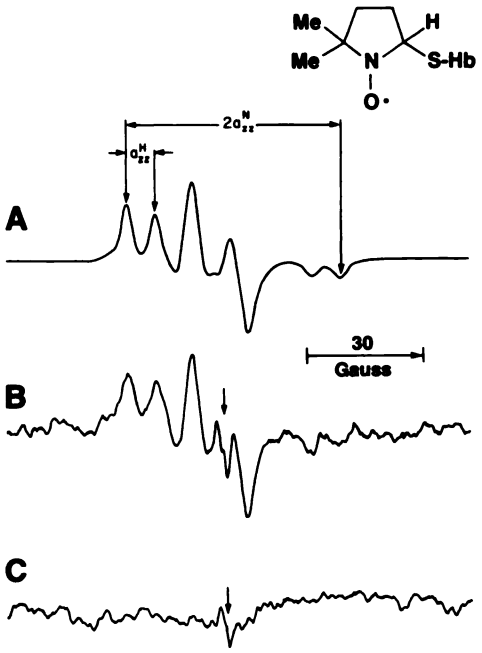

Fig. 1. A. The ESR spectrum obtained from the blood of a rat 2 hr after being given 188 mg/kg phenylhydrazine per os and 500 μl/kg DMPO intraperitoneally. Instrumental conditions: 30 mW microwave power, 2.1 G modulation amplitude, 16 sec time constant, 5×10^3 receiver gain, and 2 G/min scan rate. B. Same as in A, but the phenylhydrazine dosage was 1 mg/kg and the receiver gain was 8×10^5. C. Same as in A, but the rat was given only DMPO and the receiver gain was 8×10^5. In both B and C the *arrow* marks the location of the small doublet due to the presence of the ascorbate radical.

in Fig. 1B, a phenylhydrazine dosage of only 1 mg/kg was sufficient to yield this spectrum from blood samples. As shown in Fig. 1C, administration of DMPO alone did not yield a detectable adduct signal. The coupling constants for this adduct, a_{xx}^{N} and a_{xx}^{H}, are 31.8 G and 9.5 G, as indicated in Fig. 1A.

In order to determine the site of the immobilized signal, we first separated the RBCs from the blood plasma by centrifugation and examined both fractions. Only the RBC fraction evidenced this signal. We next lysed the RBCs with deionized water and, following centrifugation, examined the stroma-free solution fraction. If the immobilized radicals were within the cell membrane itself, we would expect the signal intensity of the stroma-free solution to be diminished compared to equally diluted RBCs. However, no such diminution of signal intensity was found. This implied that the primary site of immobilization was within the cell. As the major protein component of RBCs is hemoglobin, and since hemoglobin is involved in the reaction with phenylhydrazine (1–28), this protein appeared to be a likely candidate for the site of intracellular immobilization. Therefore, in order to test this hypothesis, we first employed a G-75 Sephadex molecular sieve to separate the various protein components of RBCs and examined the eluting fractions for oxyhemoglobin and immobilized adduct content. As illustrated in Fig. 2, oxyhemoglobin eluted concurrently with the immobilized adduct. Second, we reacted purified oxyhemoglobin with phenylhydrazine, DMPO, and DTPA. We were able to detect the immobilized adduct in Fig. 3, the spectrum of which is equivalent to that obtained *in vivo*. To further test this hypothesis, we examined the consequences of the addition of a protein denaturation agent on the immobilized signal. As shown in Fig. 4B, addition of urea produced a spectrum of much lower signal intensity which shows the presence of an almost freely rotating DMPO adduct, as well as the immobilized adduct signal. However, when guanidine was employed to denature the protein,

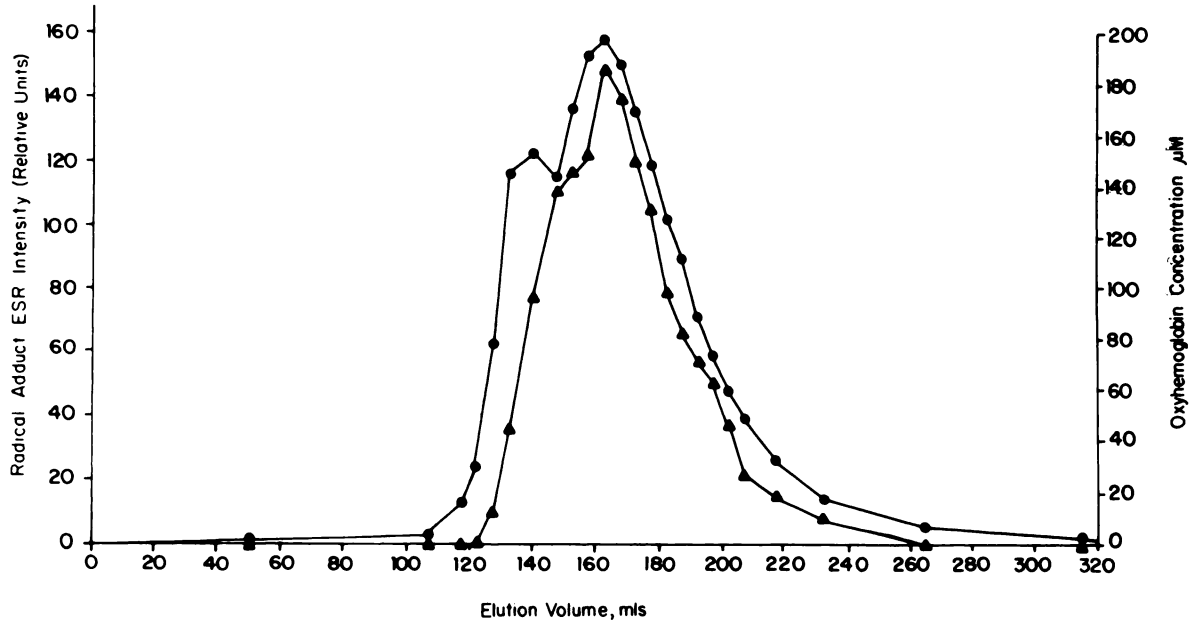

Fig. 2. Elution profile of lysed RBCs from rats given 188 mg/kg phenylhydrazine per os and 500 μl/kg DMPO intraperitoneally. Eluting fractions were analyzed by ESR for relative DMPO/hemoglobin thiyl free radical adduct concentration and by visible spectroscopy at 540 nm for oxyhemoglobin concentration (▲ = DMPO/hemoglobin thiyl free radical adduct, ● = oxyhemoglobin). Instrumental conditions: 20 mW microwave power, 1.67 G modulation amplitude, 4 sec time constant, and 12.5 G/min scan rate.

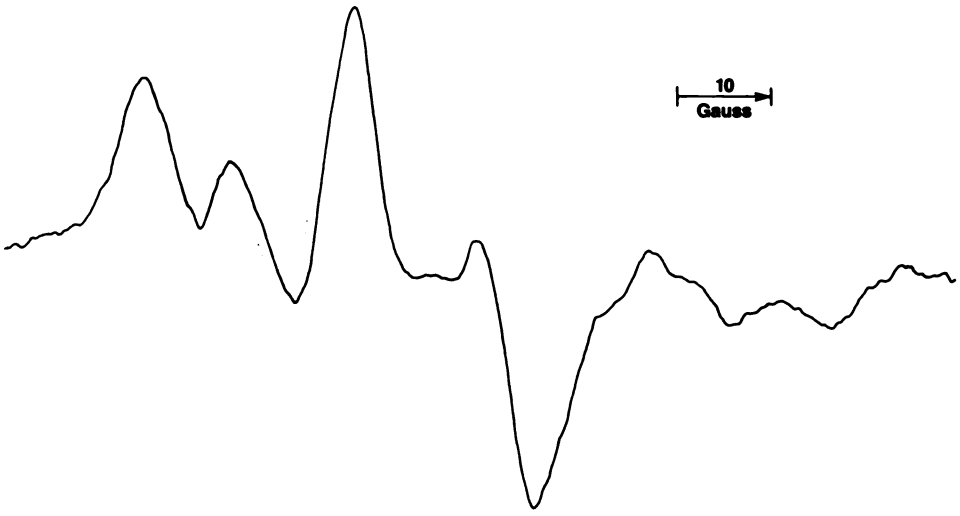

Fig. 3. The ESR spectrum obtained from a mixture of phenylhydrazine (20 μM), DMPO (100 mM), DTPA (1 mM), and purified rat oxyhemoglobin (1 mM). Instrumental conditions: 20 mW microwave power, 0.33 G modulation amplitude, 2 sec time constant, and 12.5 G/min scan rate.

the ESR signal shown in Fig. 4C showed evidence of only a weak, freely rotating DMPO adduct. The diminution of the amplitude of the fourth and sixth high field lines ($M_I = -1$ for nitrogen) with respect to the other lines suggests that there still exists a slight degree of immobilization. The ability of these protein denaturation agents to affect the degree of immobilization of the adduct supports the concept that this adduct is indeed immobilized by an intracellular protein. Based on our experiments, this protein is hemoglobin.

When Hill and Thornalley (10, 16, 20) and others (14) examined the phenylhydrazine/oxyhemoglobin reaction, only freely rotating DMPO/phenyl radical adducts were detected. In their studies, Hill and Thornalley (10, 16, 20) employed 1% suspensions of human RBCs in buffer, rather than essentially undiluted RBC mixtures. When we repeated their experiment employing a 1% rat RBC solution, we obtained the freely rotating phenyl radical adduct of DMPO (Table 1). However, when we used undiluted rat RBCs, an immobilized spectrum identical to the *in vivo* results was obtained. As shown in Fig. 5, we found that the concentration of the immobilized DMPO adduct was dependent on the extent of dilution of the rat RBCs prior to addition of phenylhydrazine and DMPO. Dilution of the immobilized adduct samples with buffer did not affect the degree of immobilization. Therefore, the ratio of DMPO to rat RBCs must determine the concentration of the immobilized radical adduct.

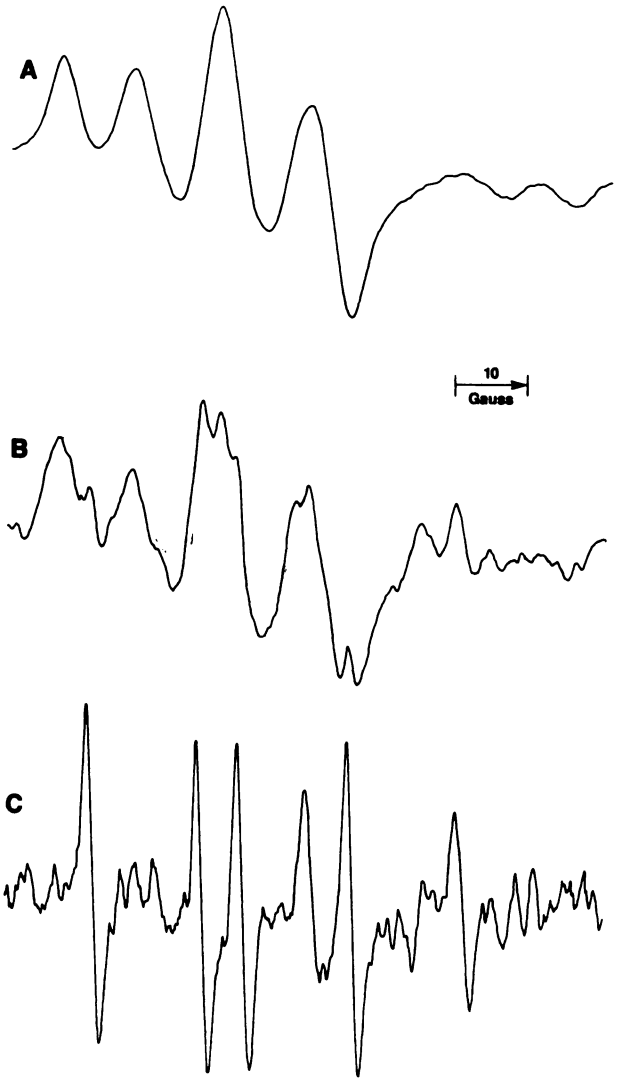

Fig. 4. A. The ESR spectrum resulting from the addition of DTPA (1 mM), DMPO (100 mM), and phenylhydrazine (1 mM) to undiluted rat RBCs. Instrumental conditions: 20 mW microwave power, 1.3 G modulation amplitude, 2 sec time constant, 1.6×10^4 receiver gain, and 12.5 G/min scan rate. B. The spectrum resulting from the addition of urea (6 M) to the mixture in A. Instrumental conditions: Same as in A, except the receiver gain was 1.25×10^5. C. The spectrum resulting from the addition of guanidine (6 M) to the mixture in A. Instrumental conditions: same as in A, except the receiver gain was 3.2×10^5 and the modulation amplitude was 0.67 G.

When we extracted solutions of immobilized or freely rotating radical adducts with either chloroform or a 2:1 chloroform/methanol solution, we were able to detect DMPO/phenyl radical adduct signals in the chloroform fraction from all samples (Table 1). The adduct signal intensity of the chloroform fractions of both samples was only marginally improved by bubbling the solution with oxygen for 5 min followed by purging with nitrogen. The identical spectrum was also obtained by chloroform extraction from aqueous solutions of the DMPO/phenyl radical adduct prepared by oxidizing phenylhydrazine with Cu^{2+} (Table 1).

In order to test the hypothesis that the immobilized radical was probably a DMPO/phenyl radical adduct trapped within the hemoglobin protein, we added rat RBCs to a dry test tube containing the DMPO/phenyl radical adduct (prepared from Cu^{2+}/phenylhydrazine/DMPO reaction mixtures). The resulting solution yielded the ESR spectrum in Fig. 6B, which is similar to that obtained following guanidine denaturation of the immobilized adduct solutions (Fig. 4C), demonstrating that the DMPO/phenyl radical adduct would not incorporate itself within hemoglobin following formation. Thus, if the immobilized signal were due to the immobilization of a DMPO/phenyl radical adduct in hemoglobin, this immobilization would need to occur before the adduct leaves the heme pocket.

To test whether the DMPO/phenyl radical adduct was indeed the immobilized radical we detected in rat blood, we employed hydrazine in place of phenylhydrazine *in vivo*. As shown in Fig. 7, hydrazine was able to induce the formation of the immobilized adduct, albeit at a much lower concentration. Thus, this

TABLE 1
Hyperfine splitting constants of radical adducts

Source	Structure	Hyperfine splittings (gauss)		Source
		a_β^H	a^N	
Phenylhydrazine/DMPO/rat RBCs	DMPO/·C_6H_5	24.5	16.0	Fig. 5F
Phenylhydrazine/DMPO/human RBCs	DMPO/·C_6H_5	24.4	15.8	Ref. 10
Chloroform extraction of blood from phenylhydrazine/DMPO rats	DMPO/·C_6H_5	20.8	14.6	This work
Chloroform extraction from phenylhydrazine/DMPO/rat RBCs	DMPO/·C_6H_5	20.7	14.6	This work
Chloroform extraction from phenylhydrazine/DMPO/Cu^{2+}	DMPO/·C_6H_5	20.7	14.6	This work
Benzene extraction from phenylhydrazine/DMPO/Cu^{2+}	DMPO/·C_6H_5	19.2	13.8	Ref. 10

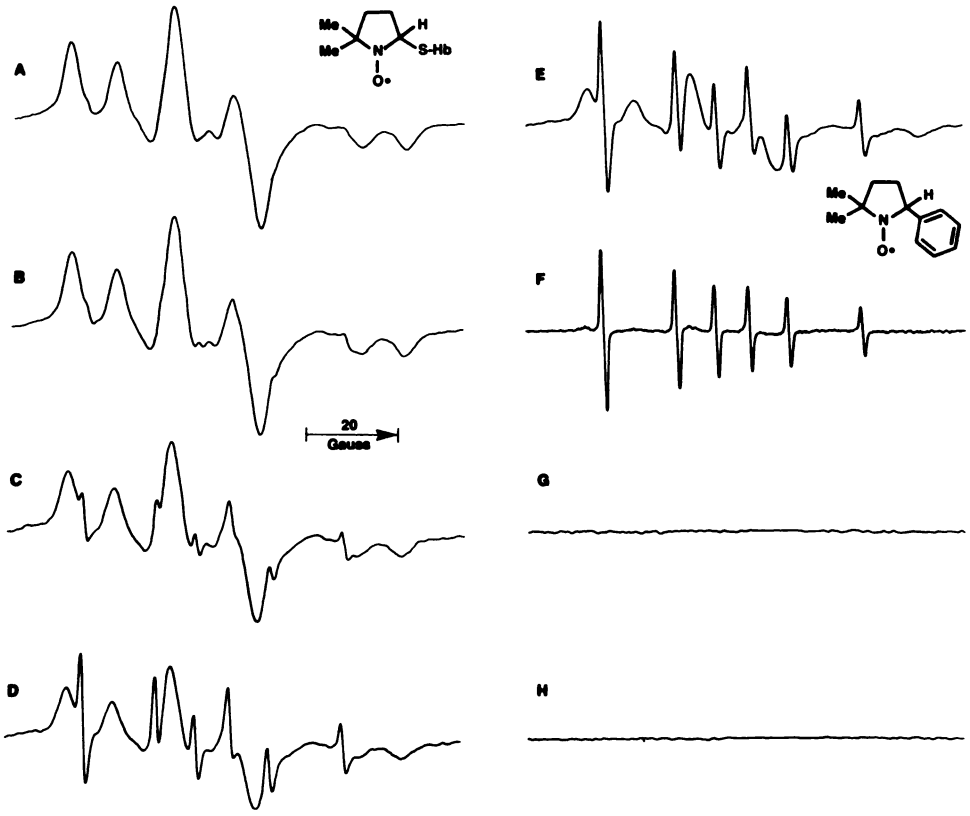

Fig. 5. The effect of diluting rat RBCs with isotonic (pH 7.4) phosphate buffer prior to addition of DTPA (1 mM), DMPO (100 mM), and phenylhydrazine (1 mM). A. Undiluted RBCs. B. RBCs (50%) in buffer. C. RBCs (25%) in buffer. D. RBCs (10%) in buffer. E. RBCs (5%) in buffer. F. RBCs (1%) in buffer. G. Only buffer, no RBCs. H. Undiluted RBCs, DMPO, and DTPA, no phenylhydrazine. Instrumental conditions for A and B: 20 mW microwave power, 0.67 G modulation amplitude, 2 sec time constant, 1.0×10^4 receiver gain, and 12.5 G/min scan rate. Instrumental conditions for C–E, G, and H: Same as for A and B, except the receiver gain was 1.25×10^4. Instrumental conditions for F: 20 mW microwave power, 0.33 G modulation amplitude, 0.5 sec time constant, 6.3×10^3 receiver gain, and 12.5 G/min scan rate.

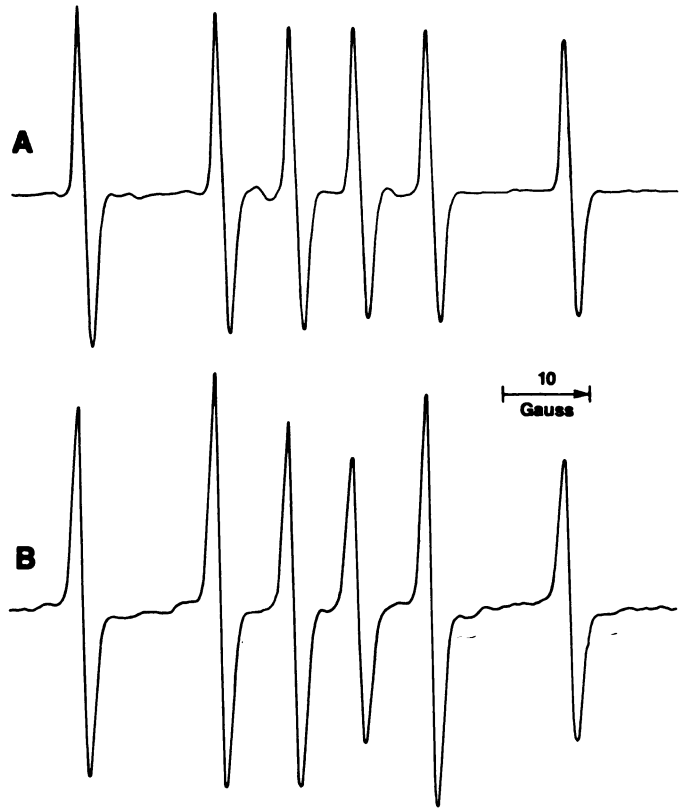

Fig. 6. A. The ESR spectrum resulting from the addition of phosphate buffer to a test tube containing the purified DMPO/phenyl radical adduct. Instrumental conditions: 20 mW microwave power, 0.67 G modulation amplitude, 2 sec time constant, 3.2×10^3 receiver gain, and 12.5 G/min scan rate. B. The ESR spectrum obtained from the addition of rat RBCs to a test tube containing the purified DMPO/phenyl radical adduct. Instrumental conditions: same as in A, except the receiver gain was 4.0×10^3.

radical species is not an immobilized DMPO/phenyl radical adduct.

Of the choices left for the identity of the radical which reacted with DMPO to yield the immobilized signal, a hemoglobin-derived free radical seemed the next likely candidate. Of the amino acids which compose hemoglobin, cysteine is the most easily oxidized. It is known that with the use of thiol-blocking agents, e.g., iodoacetamide, maleimide, or *N*-ethylmaleimide, the number of free sulfhydryl groups in a protein can be markedly diminished (39, 40). Therefore, treatment of rat hemoglobin with such agents should reduce the number of free cysteine sulfhydryl groups. If cysteine is involved in the immobilized adduct formation, such pretreatment of rat oxyhemoglobin prior to the addition of DMPO and phenylhydrazine should reduce the relative immobilized adduct concentration. As shown in Fig. 8, this was indeed the case. Pretreatment of rat RBCs with either iodoacetamide (Fig. 8B), maleimide (Fig. 8C), or *N*-ethylmaleimide (Fig. 8D) markedly lowered the normal adduct concentration (Fig. 8A). Analogous results were obtained when we used purified rat oxyhemoglobin in place of RBCs in these experiments and when we removed the excess thiol-blocking agent from the RBCs before the addition of the other reagents.

Discussion

Although the formation of free radicals from the reaction of phenylhydrazine with oxyhemoglobin has been demonstrated in *in vitro* systems, this current study is the first to report radical adduct formation *in vivo*. When we started this work, we were concerned that any radical adduct (nitroxides) formed in blood *in vivo* would be rapidly reduced by native reductants such as ascorbate or glutathione and, thus, adduct levels might never reach detectable levels. Apparently, the trapping of a

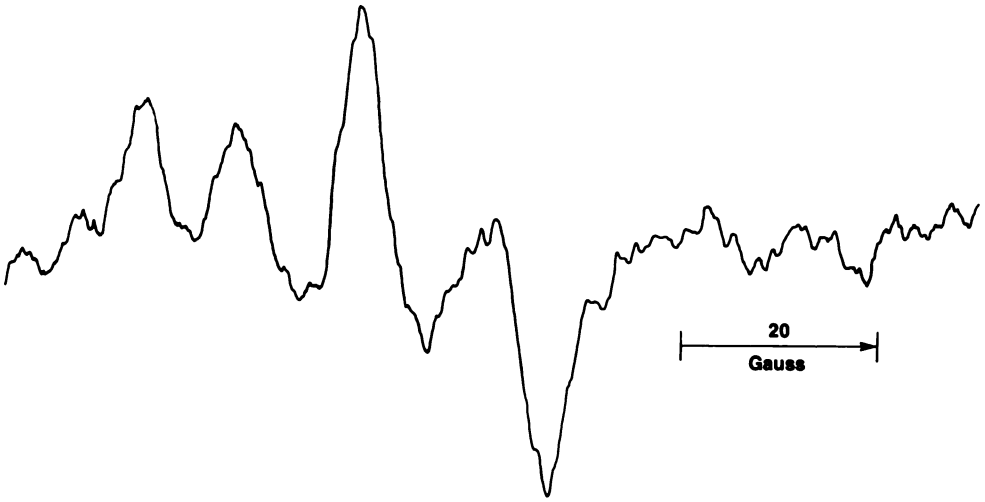

Fig. 7. The ESR spectrum obtained from the blood of a rat 2 hr after being given 129 mg/kg hydrazine per os and 500 μl/kg DMPO intraperitoneally. Instrumental conditions: 20 mW microwave power, 1.67 G modulation amplitude, 4 sec time constant, and 6.25 G/min scan rate.

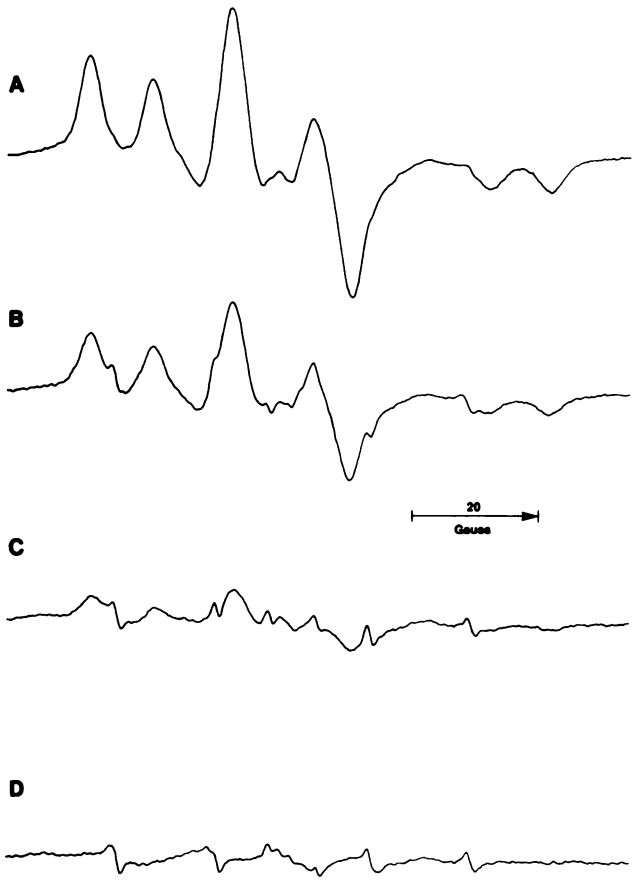

Fig. 8. The effect of pretreatment of rat RBCs with a thiol-blocking agent prior to addition of DTPA, DMPO, and phenylhydrazine. A. No agent added. B. Iodoacetamide (10 mM) added. C. Maleimide (10 mM) added. D. *N*-Ethylmaleimide (100 mM) added. Instrumental conditions: 20 mW microwave power, 0.67 G modulation amplitude, 1 sec time constant, and 12.5 G/min scan rate.

thiyl free radical by DMPO bound to rat hemoglobin protein protected the adduct from reduction and allowed the achievement of very high radical adduct concentrations. Consequently, even at phenylhydrazine dosages which approximate those prescribed for hydrazine-derived pharmaceuticals (41, 42), we were still able to detect this adduct in blood samples. This is the first report of target macromolecule free radical formation as a consequence of xenobiotic metabolism.

Our ability to detect the DMPO/phenyl radical adduct in the chloroform fraction from *in vivo* blood extractions implies that the rate of adduct formation must have been higher than the rate of adduct decay. However, the weak signal intensity of these extracts suggests that the reaction of the phenyl radical with oxyhemoglobin is limiting the steady state concentration of this adduct.

The reactions which lead to the formation of the detected adducts are likely to involve

$$C_6H_5N\dot{H}NH_2 + HbFe(II)\text{-}O_2 \rightarrow C_6H_5\dot{N}NH_2 + HbFe(III)\text{-}O_2H \quad (1)$$

$$2C_6H_5\dot{N}NH_2 \rightarrow C_6H_5N{=}NH + C_6H_5NHNH_2 \quad (2)$$

$$C_6H_5N{=}NH + O_2 \rightarrow C_6H_5N{=}N\cdot + (H^+ + O_2^{\cdot-}) \quad (3)$$

$$C_6H_5N{=}N\cdot \rightarrow \cdot C_6H_5 + N_2 \quad (4)$$

$$\cdot C_6H_5 + DMPO \rightarrow DMPO/\cdot C_6H_5 \quad (5)$$

$$HbSH + R\cdot \rightarrow HbS\cdot + RH \quad (6)$$

$$HbS\cdot + DMPO \rightarrow DMPO/HbS\cdot \quad (7)$$

where $R\cdot$ could be any of the free radicals produced in the reaction. The marked diminution of the DMPO/HbS· signal in the blood of hydrazine-treated rats suggests that the phenyl radical may make an important contribution to the thiol free radical formation depicted in Eq. 6 following phenylhydrazine administration. Apparently, at *in vivo* oxyhemoglobin concentrations, DMPO cannot successfully compete with oxyhemoglobin for the phenyl radical, as indicated by Fig. 5. Thus, when we employed 100% RBCs *in vitro* we detected the DMPO/thiyl free radical adduct, not the DMPO/phenyl radical adduct obtained using 1% RBCs.

Although this study is the first to report the *in vivo* trapping of a protein thiyl free radical, such reactions have already been demonstrated *in vitro*. Graceffa (40) demonstrated, using phenyl-*N*-*t*-butylnitrone, the trapping of thiyl radicals generated by the oxidation of bovine serum albumin and myosin by Ce^{4+} (40). As in our case, his adduct signals were immobilized, and *N*-ethylmaleimide pretreatment of bovine serum albumin prior to Ce^{4+} oxidation decreased the resulting spectral intensity.

Our tentative assignment of the immobilized radical adduct to a DMPO/hemoglobin thiyl radical adduct is based on two points. First, we have demonstrated that if the RBCs are pretreated with sulfhydryl blocking reagents, the immobilized

radical adduct concentration decreases. Thus, sulfhydryl groups appear to be involved in the processes leading to this radical adduct. Second, the site of immobilization was shown to be the hemoglobin protein. Finally, we have eliminated the only radical adduct previously detected *in vitro*, the DMPO/phenyl radical adduct, from being the species responsible for our immobilized adduct signal. Admittedly, this assignment is indirect and does not definitely establish the structure of this radical adduct. However, based on our results, a DMPO/hemoglobin thiyl radical adduct appears to be the most logical candidate for the radical we have detected.

The application of the ESR spin-trapping technique to *in vivo* metabolism has been limited to the study of halogenated hydrocarbons (29–35). However, based on our results, the *in vivo* spin-trapping technique appears to be very applicable to the study of the metabolism of at least some other xenobiotics. Clearly, the *in vivo* detection of proposed free radical metabolites in intact animals is of crucial importance to the confirmation of the relevance of reaction mechanisms which are based on *in vitro* studies.

References

1. Heinz, R. Morphologische Veränderungen der rothen Blutkörperchen durch Gifte. *Virchows Arch. Pathol.* **122**:112–116 (1890).
2. Hoppe-Seyler, G. Ueber die Wirkung des Phenylhydrazins auf den Organisms. *Z. Physiol. Chem.* **9**:34–39 (1885).
3. Beaven, G. H., and J. C. White. Oxidation of phenylhydrazines in the presence of oxyhaemoglobin and the origin of Heinz bodies in erythrocytes. *Nature (Lond.)* **173**:389–391 (1954).
4. Goldberg, B., and A. Stern. The generation of O_2^- by the interaction of the hemolytic agent, phenylhydrazine, with human hemoglobin. *J. Biol. Chem.* **250**:2401–2403 (1975).
5. Goldberg, B., A. Stern, and J. Peisach. The mechanism of superoxide anion generation by the interaction of phenylhydrazine with hemoglobin. *J. Biol. Chem.* **251**:3045–3051 (1976).
6. Misra, H. P., and I. Fridovich. The oxidation of phenylhydrazine: superoxide and mechanism. *Biochemistry* **15**:681–687 (1976).
7. Goldberg, B., and A. Stern. The mechanism of oxidative hemolysis produced by phenylhydrazine. *Mol. Pharmacol.* **13**:832–839 (1977).
8. Castro, C. E., R. S. Wade, and N. O. Belser. Conversion of oxyhemoglobin to methemoglobin by organic and inorganic reductants. *Biochemistry* **17**:225–231 (1978).
9. Goldberg, B., A. Stern, J. Peisach, and W. E. Blumberg. The detection of superoxide anion from the reaction of oxyhemoglobin and phenylhydrazine using EPR spectroscopy. *Experientia* **35**:488–489 (1979).
10. Hill, H. A. O., and P. J. Thornalley. Phenyl radical production during the oxidation of phenylhydrazine and in phenylhydrazine-induced hemolysis. *FEBS Lett.* **125**:235–238 (1981).
11. Ortiz de Montellano, P. R., and K. L. Kunze. Formation of *N*-phenylheme in the hemolytic reaction of phenylhydrazine with hemoglobin. *J. Am. Chem. Soc.* **103**:6534–6536 (1981).
12. Saito, S., and H. A. Itano. *β-meso*-Phenylbiliverdin IX*α* and *N*-phenylprotoprophyrin IX, products of the reaction of phenylhydrazine with oxyhemoproteins. *Proc. Natl. Acad. Sci. USA* **78**:5508–5512 (1981).
13. Mansuy, D., P. Battioni, J.-P. Mahy, and G. Gillet. Comparison of the hemoglobin reactions with methyl- and phenylhydrazine: intermediate formation of a hemoglobin Fe(II)-methyldiazene complex. *Biochem. Biophys. Res. Commun.* **106**:30–36 (1982).
14. Augusto, O., K. L. Kunze, and P. R. Ortiz de Montellano. N-Phenylprotoporphyrin IX formation in the hemoglobin-phenylhydrazine reaction. *J. Biol. Chem.* **257**:6231–6241 (1982).
15. Itano, H., and J. L. Matteson. Mechanism of initial reaction of phenylhydrazine with oxyhemoglobin and effect of ring substitutions on the bimolecular rate constant of this reaction. *Biochemistry* **21**:2421–2426 (1982).
16. Hill, H. A. O., and P. J. Thornalley. Free radical production during phenylhydrazine-induced hemolysis. *Can. J. Chem.* **60**:1528–1531 (1982).
17. Mansuy, D., J.-P. Battioni, D. Dupre, E. Sartori, and G. Chottord. Reversible iron-nitrogen migration of alkyl, aryl, or vinyl groups in iron porphyrins: a possible passage between *α* Fe^{III}(porphyrin)(R) and Fe^{II}(N-R) (porphyrin) complexes. *J. Am. Chem. Soc.* **104**:6159–6161 (1982).
18. Mason, R. P. Free-radical intermediates in the metabolism of toxic chemicals. *Free Rad. Biol.* **5**:161–222 (1982).
19. Ortiz de Montellano, P. R., K. L. Kunze, and O. Augusto. Hemoprotein destruction. Iron-nitrogen shift of a phenyl group in a porphyrin complex. *J. Am. Chem. Soc.* **104**:3545–3546 (1982).
20. Hill, H. A. O., and P. J. Thornalley. The effect of spin traps on phenylhydrazine-induced haemolysis. *Biochim. Biophys. Acta* **762**:44–51 (1983).
21. Kunze, K. L., and P. R. Ortiz de Montellano. Formation of a *σ*-bonded aryliron complex in the reaction of arylhydrazines with hemoglobin and myoglobin. *J. Am. Chem. Soc.* **105**:1380–1381 (1983).
22. Mason, R. P. Spin trapping free radical metabolites of toxic chemicals, in *Spin Labeling in Pharmacology* (J. L. Holtzman, ed.). Academic Press, New York, 87–129 (1984).
23. Smith, P., and K. R. Maples. EPR study of the oxidation of phenylhydrazine initiated by the titanous chloride/hydrogen peroxide reaction and by oxyhemoglobin. *J. Magn. Reson.* **65**:491–496 (1985).
24. Shetlar, M. D., and H. A. O. Hill. Reactions of hemoglobin with phenylhydrazine: a review of selected aspects. *Environ. Health Perspect.* **64**:265–281 (1985).
25. Kalyanaraman, B., and B. K. Sinha. Free radical-mediated activation of hydrazine derivatives. *Environ. Health Perspect.* **64**:179–184 (1985).
26. Imanishi, H., T. Nakai, T. Abe, and T. Takino. Role of free radical scavengers on phenylhydrazine induced hemolysis in rat. *Acta Vitaminol. Enzymol.* **7**:71–76 (1985).
27. Arduini, A., Z. Chen, and A. Stern. Phenylhydrazine-induced changes in erythrocyte membrane surface lipid packing. *Biochim. Biophys. Acta* **862**:65–71 (1986).
28. Einsele, H., M. R. Clemens, U. Wegner, and H.-D. Waller. Effect of free radical scavengers and metal ion chelators on hydrogen peroxide and phenylhydrazine induced red blood cell lipid peroxidation. *Free Rad. Res. Commun.* **3**:257–263 (1987).
29. Connor, H. D., R. G. Thurman, M. D. Galizi, and R. P. Mason. The formation of a novel free radical metabolite from CCl_4 in the perfused rat liver and *in vivo*. *J. Biol. Chem.* **261**:4542–4548 (1986).
30. McCay, P. B., E. K. Lai, J. L. Poyer, C. M. DuBose, and E. G. Janzen. Oxygen- and carbon-centered free radical formation during carbon tetrachloride metabolism. Observation of lipid radicals *in vivo* and *in vitro*. *J. Biol. Chem.* **259**:2135–2143 (1984).
31. Lai, E. K., P. B. McCay, T. Noguchi, and K.-L. Fong. *In vivo* spin-trapping of trichloromethyl radicals formed from CCl_4. *Biochem. Pharmacol.* **28**:2231–2235 (1979).
32. Poyer, J. L., P. B. McCay, C. C. Weddle, and P. E. Downs. *In vivo* spin-trapping of radicals formed during halothane metabolism. *Biochem. Pharmacol.* **30**:1517–1519 (1981).
33. Fujii, K., M. Morio, H. Kikuchi, S. Ishihara, M. Okida, and F. Ficor. *In vivo* spin-trap study on anaerobic dehalogenation of halothane. *Life Sci.* **35**:463–468 (1984).
34. Poyer, J. L., P. B. McCay, E. K. Lai, E. G. Janzen, and E. R. Davis. Confirmation of assignment of the trichloromethyl radical spin adduct detected by spin trapping during ^{13}C-carbon tetrachloride metabolism *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.* **94**:1154–1160 (1980).
35. Albano, E., A. K. Lott, T. F. Slater, A. Stier, M. C. R. Symons, and A. Tomasi. Spin-trapping studies on the free radical products formed by metabolic activation of carbon tetrachloride in rat liver microsomal fractions, isolated hepatocytes and *in vivo* in the rat. *Biochem. J.* **204**:593–603 (1982).
36. Augusto, O., and G. Cilento. The effect of diphenols upon the autoxidation of oxyhemoglobin and oxymyoglobin. *Arch. Biochem. Biophys.* **168**:549–556 (1975).
37. Lemberg, R., and J. W. Legge. *Hematin Compounds and Bile Pigments.* Interscience, New York, 392 (1949).
38. Windholz, M. (Ed.). *The Merck Index*, Tenth Edition. Merck and Co. Inc., Rahway, NJ, 1051 (1983).
39. Webb, J. L. *Enzyme and Metabolic Inhibitors*, Vol. III. Academic Press, London, 1-18, 337–365 (1966).
40. Graceffa, P. Spin labeling of protein sulfhydryl groups by spin trapping a sulfur radical: application to bovine serum albumin and myosin. *Arch. Biochem. Biophys.* **225**:802–808 (1983).
41. Reynolds, J. E. F. (Ed.). *Martindale, The Extra Pharmacopoeia.* The Pharmaceutical Press, London, 122 (1982).
42. *AMA Drug Evaluations*, Fifth Edition. American Medical Association, Chicago, 264 (1983).

Send reprint requests to: Dr. Kirk R. Maples, Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, P. O. Box 12233, Research Triangle Park, NC 27709.