

# Spin-trapping and Human Neutrophils

## LIMITS OF DETECTION OF HYDROXYL RADICAL\*

(Received for publication, February 9, 1989)

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Using the spin trap, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and an excess of dimethyl sulfoxide, we previously reported that in the absence of an exogenous iron catalyst, human neutrophils will not generate hydroxyl radical, manifested as the catalase-inhibitable methyl radical spin-trapped adduct, 2,2,5-trimethyl-1-pyrrolidinyloxy (DMPO-CH<sub>3</sub>) (Britigan, B. E., Rosen, G. M., Chai, Y., and Cohen, M. S. (1986) *J. Biol. Chem.* 261, 4426-4431). However, superoxide destroys the preformed hydroxyl radical spin-trapped adduct, 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxy (DMPO-OH), and DMPO-CH<sub>3</sub>. The present study was undertaken to better resolve the limits of sensitivity of the spin-trapping method. Photolytically generated DMPO-CH<sub>3</sub> and DMPO-OH slowly decomposed in the presence of a low flux (1 μM/min) of enzymatically (xanthine/xanthine oxidase)-generated superoxide, but more rapid decomposition of these adducts occurred with higher superoxide flux (5 μM/min). Inclusion of cysteine markedly increased the rate of DMPO-OH and DMPO-CH<sub>3</sub> decomposition, masking the effect of superoxide alone. The addition of varying concentrations of superoxide dismutase did not lead to increased formation of DMPO-OH or DMPO-CH<sub>3</sub>, as should have occurred if these adducts were being destroyed by superoxide. As a positive control, we employed an iron-supplemented system with phorbol 12-myristate 13-acetate-stimulated neutrophils or xanthine/xanthine oxidase to generate DMPO-CH<sub>3</sub>. Addition of superoxide dismutase increased the magnitude of DMPO-CH<sub>3</sub>, primarily by increasing the rate of hydrogen peroxide formation, and to a lesser extent by prolonging the half-life of DMPO-CH<sub>3</sub>. Although spin-trapped adducts can be destroyed by a high concentration of superoxide, or by lower concentrations of superoxide in the presence of thiol-containing compounds, our results demonstrate that such decomposition does not interfere with the ability of the spin-trapping method to detect hydroxyl radical generated by human neutrophils. These data do not support the

## capacity of neutrophils to generate hydroxyl radical in the absence of an exogenous Haber-Weiss catalyst.

As a consequence of activation of both soluble and particulate stimuli, neutrophils undergo a rapid increase in oxygen utilization, termed the "respiratory burst" (1). During the "burst," oxygen metabolism leads to the generation of oxygen reduction products, including superoxide and hydrogen peroxide (2, 3). Although formation of hydroxyl radical by stimulated neutrophils has been reported, problems associated with interpretation of data and limitations of techniques have led several laboratories to question this conclusion (reviewed in Ref. 4). With appropriate controls, spin trapping appears to be one of the most specific procedures for the detection of hydroxyl radical (4, 5). However, when phorbol 12-myristate 13-acetate (PMA)<sup>1</sup> or opsonized zymosan were used as stimuli, we only spin-trapped superoxide (6-8).

Several years ago, we observed that six-membered ring nitroxides could be reduced to their corresponding hydroxylamines in the presence of superoxide and thiols; the absence of either component prevented this reaction (9). We also demonstrated that these otherwise stable nitroxides were rapidly reduced by PMA-activated human neutrophils (8). Neither resting (unstimulated) nor PMA-stimulated neutrophils in the presence of superoxide dismutase led to nitroxide reduction. More recently, Samuni and co-workers (10) reported that superoxide generated by stimulated human neutrophils enhanced the degradation rate of α-hydrogen-containing nitroxides, including DMPO-OH and DMPO-CH<sub>3</sub>. Thus, destruction of these nitroxides by superoxide could decrease the sensitivity of the spin-trapping technique. Therefore, the current study was undertaken to better understand the limits of spin trapping and to examine the formation of neutrophil-derived oxygen-centered free radicals under conditions designed to maximize the sensitivity of this procedure.

## MATERIALS AND METHODS

**Reagents**—Diethylenetriaminepentaacetic acid (DTPA), superoxide dismutase, cysteine, catalase, xanthine oxidase, xanthine, and ferricytochrome c (type VI) were purchased from Sigma. PMA was obtained from Midland Chemical Co., Brewster, NY; dimethyl sulfoxide (Me<sub>2</sub>SO) from Fisher; and Hanks' balanced salt solution from Gibco Laboratories. The spin trap, DMPO, was synthesized according to the procedure of Bonnet *et al.* (11) and was freshly distilled just

<sup>1</sup> The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DMPO-CH<sub>3</sub>, 2,2,5-trimethyl-1-pyrrolidinyloxy; DMPO-OH, 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxy; Me<sub>2</sub>SO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid.

\* This work was supported in part by grants from the National Science Foundation, the Chemistry of Life Processes Program (DCB 8616115), National Institutes of Health (HL 33550 and AI 23939), The Council for Tobacco Research-U.S.A. Inc., Veterans Administration Research Service, and the Pfizer Scholars Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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prior to use. The buffer system used in this work was Hanks' balanced salt solution, pH 7.4, containing DTPA (0.1 mM). PMA was dissolved in  $\text{Me}_2\text{SO}$  (100 ng/ml, final concentration).

**Neutrophil Separation**—Neutrophils were isolated from the whole blood of normal human volunteers as previously described (12). Neutrophils were resuspended in Hanks' balanced salt solution and stored on ice until used.

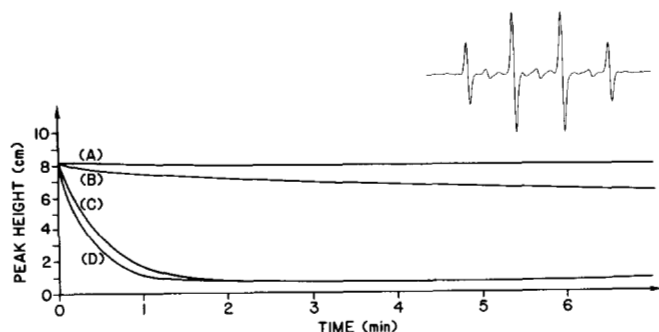
**Superoxide Detection**—Superoxide was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* and monitored spectrophotometrically at 550 nm, as previously reported (13). The rate of superoxide generation was calculated using an extinction coefficient of  $21 \text{ mM}^{-1} \text{ cm}^{-1}$ . For superoxide generated from a xanthine/xanthine oxidase system, various concentrations of the enzyme were added to a solution containing xanthine (400  $\mu\text{M}$ ) and ferricytochrome *c* (80  $\mu\text{M}$ ) to a final volume of 1 ml. To stimulate superoxide production by neutrophils, PMA (100 ng/ml in  $\text{Me}_2\text{SO}$ ) was added to a mixture of cells ( $10^6$ ) and ferricytochrome *c* (80  $\mu\text{M}$ ). Under these experimental conditions, the rate of superoxide production was 1–3  $\mu\text{M}/\text{min}/10^6$  cells. By varying the number of cells obtained from each volunteer, we were able to produce consistent rates of superoxide generation.

**Reduction of DMPO-OH and DMPO-CH<sub>3</sub>**—DMPO-OH was generated by irradiating an EPR flat cell containing a 0.5-ml solution of DMPO (10 mM),  $\text{H}_2\text{O}_2$  (0.3%) and placing the cell 7 cm from the UV light source (Ultra-violet Product, Inc., San Gabriel, CA, model #SCT 1) for 1 min. To 0.1 ml of the irradiated reaction mixture, a solution of xanthine (400  $\mu\text{M}$ ) in Hanks' balanced salt solution and varying amounts of xanthine oxidase to generate the desired flux of superoxide were added. In some experiments, catalase (300 units/ml) was added prior to the addition of cysteine (200  $\mu\text{M}$ ). Reaction mixtures (0.5 ml) were transferred to a flat quartz EPR cell, fitted into the cavity of an EPR spectrometer (Varian Associates, model E-9), and spectra were recorded at 25 °C. For DMPO-CH<sub>3</sub>, DMSO (0.14 M) was added to the reaction mixture prior to photolysis.

**Spin Trapping**—Spin-trapping experiments were performed as previously described (6–8) by mixing neutrophils ( $1\text{--}2 \times 10^4$  cells/ml), DMPO (0.1 M), and PMA (100 ng/ml in  $\text{Me}_2\text{SO}$ , 0.14 M, final concentration) with or without catalase (300 units/ml) or varying concentrations of superoxide dismutase. Reaction mixtures were transferred to a flat EPR quartz cell and fitted into the cavity of the spectrometer (Varian Associates E-9), and the spectra were recorded at 25 °C.

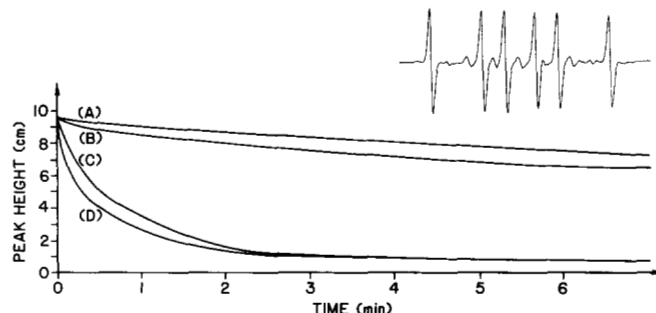
## RESULTS AND DISCUSSION

Nitroxides like DMPO-OH and DMPO-CH<sub>3</sub> rapidly decompose in the presence of superoxide alone (10), suggesting that spin trapping might be of limited value in detecting hydroxyl radical when superoxide is concomitantly generated. To examine this possibility, we measured the rate of DMPO-OH

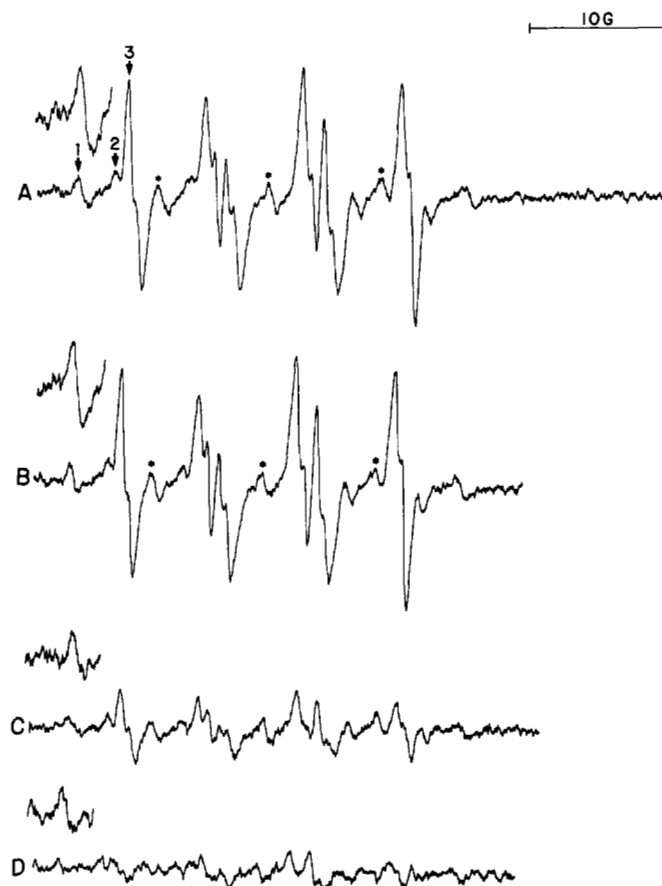


**FIG. 1. Effect of superoxide on the stability of DMPO-OH.** The magnetic field was set at the top of the second low field peak of DMPO-OH. Time zero is the time at which recording began. Tracing A represents the peak height of DMPO-OH in the absence of superoxide. Tracings B and C were generated in the presence of superoxide at 1  $\mu\text{M}/\text{min}$  and 5  $\mu\text{M}/\text{min}$ , respectively. Tracing D was identical with tracing B except that cysteine (200  $\mu\text{M}$ ) was added. Catalase (300 units/ml) was included prior to the addition of cysteine. Microwave power was 20 milliwatts, modulation frequency was 100 kHz with an amplitude of 1 G, sweep time was 12.5 G/min, response time was 1 s, and receiver gain was  $5 \times 10^3$ .

and DMPO-CH<sub>3</sub> decomposition as a function of superoxide flux using the aerobic oxidation of xanthine by xanthine oxidase, as a source of this free radical. DMPO-OH was generated under controlled conditions by the UV irradiation



**FIG. 2. Effect of superoxide on the stability of DMPO-CH<sub>3</sub>.** The magnetic field was set at the top of the lower field peak of DMPO-CH<sub>3</sub>. Tracings A–D were generated as described in Fig. 1. Instrument settings were identical with Fig. 1, except that the receiver gain was  $6.3 \times 10^3$ .



**FIG. 3. EPR spectra from stimulated neutrophils.** Neutrophils ( $10^6$  cells/ml) were stimulated by PMA (100 ng/ml in  $\text{Me}_2\text{SO}$ , 0.14 M) in the presence of DMPO (0.1 M) and DTPA (0.1 mM) (scan A). Spectra were recorded 8 min after the addition of PMA. The EPR spectrum in scan A consists of DMPO-OOH (3), to a lesser extent DMPO-OH (2) and DMPO-CH<sub>3</sub> (1). Scans B–D were recorded under identical conditions, except for the addition of catalase (300 units/ml, scan B), superoxide dismutase (0.1 unit/ml, scan C), and superoxide dismutase (1 unit/ml, scan D). An amplification ( $5 \times 10^4$ ) of DMPO-CH<sub>3</sub> was also included in scans A–D. The triplet (\*) was not present in the absence of PMA and resulted from the aerobic oxidation of DMPO. Instrument settings were identical with those in Fig. 1, except the receiver gain was  $1.25 \times 10^4$ .

of a solution of hydrogen peroxide and DMPO. As shown in Fig. 1A in the absence of superoxide, the signal intensity of the second low field peak of DMPO-OH did not diminish over the period of at least 8 min. In the presence of  $1 \mu\text{M}$  superoxide/min, greater than 80% of the initial peak height remained after 8 min (Fig. 1B). Not surprisingly, when the rate of superoxide generation was increased to  $5 \mu\text{M}/\text{min}$ , the rate of DMPO-OH decomposition was more rapid (Fig. 1C). Inclusion of a thiol such as cysteine ( $200 \mu\text{M}$ ) in the presence of as little as  $1 \mu\text{M}$  superoxide/min enhanced the rate of DMPO-OH decay equal to that observed when the rate of superoxide generation was  $5 \mu\text{M}/\text{min}$  in the absence of the thiol (Fig. 1D). Similar results were obtained when DMPO-CH<sub>3</sub>, generated by the addition of Me<sub>2</sub>SO with DMPO and hydrogen peroxide subjected to UV irradiation, was substituted for DMPO-OH in the above described reactions (Fig. 2). These data support the recent results of Samuni *et al.* (10), but emphasize the important role of a thiol in the destruction of nitroxides (9).

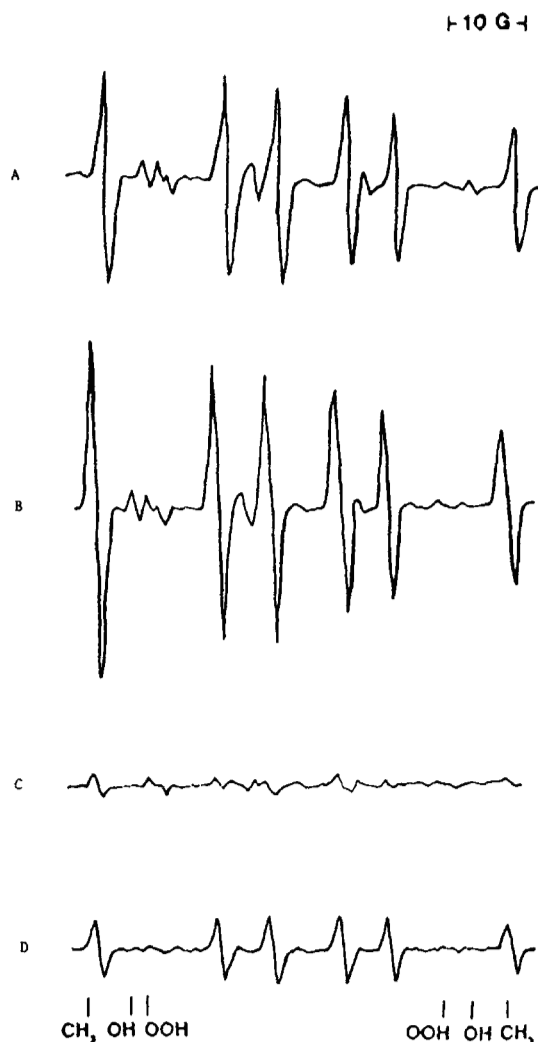


FIG. 4. Effect of superoxide dismutase on spin-trapping hydroxyl radical in a model system. EPR spectra were obtained following the addition of xanthine oxidase (0.2 units/ml) to xanthine ( $400 \mu\text{M}$ ), DMPO (0.1 M), DTPA (0.1 mM), Fe<sup>3+</sup> (0.1 mM), and Me<sub>2</sub>SO (0.14 M). A, control; B, superoxide dismutase (1 unit/ml) added; C, catalase (300 units/ml) added; D, catalase (300 units/ml) added 1 min after the addition of superoxide dismutase. Instrument settings were identical with those in Fig. 1, except that the receiver gain was  $6.3 \times 10^3$ .

The ability of spin-trapping methods to detect hydroxyl radical, as either DMPO-OH or DMPO-CH<sub>3</sub>, is a reflection of the equilibrium between the rate of spin trap adduct formation and decomposition. Accelerated destruction would be expected to occur in the presence of superoxide and thiols. However, when neutrophils were stimulated with PMA in the presence of DMPO and Me<sub>2</sub>SO, we observed DMPO-OOH, DMPO-OH, and DMPO-CH<sub>3</sub> (Fig. 3A). Thus, our ability to detect DMPO-OH and DMPO-CH<sub>3</sub> demonstrates that the experimental conditions were not sufficient to prevent us from observing these nitroxides.

However, it may be possible that the DMPO-OH and DMPO-CH<sub>3</sub> detected represented only a small fraction of all that had formed. To test this hypothesis, we generated hydroxyl radical (reported as DMPO-CH<sub>3</sub>) by addition of ferric salts (0.1 mM) to a xanthine/xanthine oxidase system in the presence of Me<sub>2</sub>SO (Fig. 4A). We found that superoxide dismutase enhanced the magnitude of DMPO-CH<sub>3</sub> (Fig. 4B), while catalase almost completely inhibited the EPR spectrum (Fig. 4C). These observations could have resulted from the increased rate of hydrogen peroxide formation catalyzed by superoxide dismutase (whereby more substrate would be available for reduction by Fe<sup>2+</sup>). Alternatively, a decrease in superoxide concentration, by addition of superoxide dismutase, could have afforded protection to DMPO-CH<sub>3</sub>. To address these possibilities, catalase was added to our model hydroxyl radical generating system 1 min after the addition of superoxide dismutase. Under these experimental conditions, DMPO-CH<sub>3</sub> was markedly inhibited (Fig. 4D), suggesting that much of the superoxide dismutase-mediated increase in DMPO-CH<sub>3</sub> was the result of enhanced rate of hydrogen peroxide formation.

These findings show that some concentrations of superoxide are adequate for formation of hydroxyl radical, via reduc-

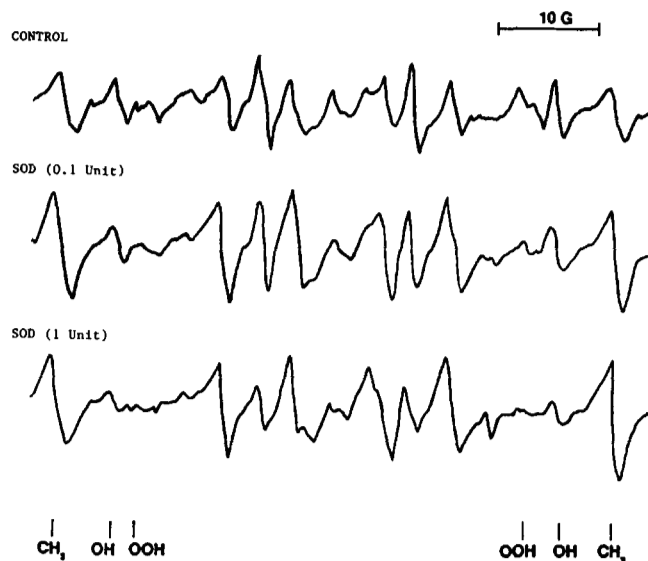


FIG. 5. Effect of superoxide dismutase on spin-trapping hydroxyl radical by neutrophils. Sequential 5-min EPR scans were obtained after the addition of PMA (100 ng/ml) to a suspension of neutrophils ( $10^6/\text{ml}$ ), DMPO (0.1 M), Fe<sup>3+</sup> (0.1 mM), and Me<sub>2</sub>SO (0.14 M) in the absence (control) and presence of superoxide dismutase (0.1 and 1.0 unit/ml). For simplicity, representative spectra ( $N = 3$ ) beginning 10 min after the addition of PMA for each condition are shown, although spectra were continuously monitored throughout the experiment. At either concentration, superoxide dismutase increased DMPO-CH<sub>3</sub> peaks while a concentration-related decrease in both DMPO-OOH and DMPO-OH was observed. Instrument settings were as in Fig. 1, except that receiver gain was  $3.2 \times 10^4$ .

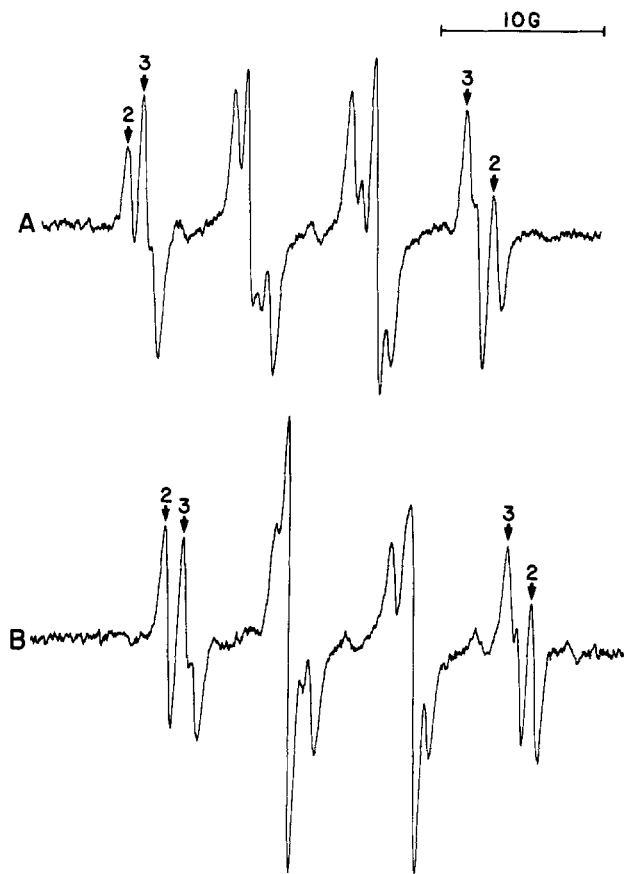


FIG. 6. Effect of DMPO concentration on DMPO-OH formation. EPR spectra were obtained from the aerobic action of xanthine oxidase on xanthine. The rate of superoxide generation was  $1 \mu\text{M}/\text{min}$ . In scan A, DMPO was  $100 \text{ mM}$ ; scan B, DMPO was  $300 \text{ mM}$ . EPR spectra were recorded 8 min after the addition of xanthine oxidase to the reaction mixture, in which DMPO-OH is 2 and DMPO-OOH is 3. Even with the higher concentration of DMPO, we observed no effect on the xanthine oxidase-mediated formation of superoxide. Instrument settings were the same as noted in Fig. 1, except that the receiver gain was  $1.25 \times 10^4$ .

tion of  $\text{Fe}^{3+}$ , but are too low to affect the stability of DMPO- $\text{CH}_3$ . Additionally, the enhanced rate of hydrogen peroxide formation, produced by superoxide dismutase, increases the sensitivity of the spin-trapping method. Therefore, small amounts of superoxide dismutase ( $0.1$ – $1.0 \text{ unit}/\text{ml}$ ) added to stimulated neutrophils should enhance DMPO- $\text{CH}_3$  formed via the Haber-Weiss reaction. When we tested this hypothesis using iron-supplemented neutrophils stimulated with PMA, we observed a significant increase in DMPO- $\text{CH}_3$  (Fig. 5).

Given the critical importance of superoxide to the destruction of DMPO-type spin-trapped adducts, decreasing the flux of superoxide should allow increased detection of DMPO-OH and DMPO- $\text{CH}_3$  if hydroxyl radicals were, indeed, generated. However, as shown in Fig. 3, when neutrophils ( $10^6/\text{ml}$ ) were stimulated with PMA in the presence of superoxide dismutase ranging in concentration from  $0.1$  to  $1.0 \text{ unit}/\text{ml}$ , a proportional decrease in DMPO-OOH, DMPO-OH, and DMPO- $\text{CH}_3$  was observed. These data, combined with those described in Figs. 4 and 5, clearly demonstrate that if a continual flux of hydroxyl radicals were generated during the PMA stimulation of neutrophils, spin-trapping methods could detect its presence.

An alternative method to "protect" DMPO-type spin-trapped adducts might be the addition of high concentrations

of DMPO. Indeed, when  $0.3 \text{ M}$  DMPO was added to PMA-stimulated neutrophils, Samuni and co-workers (10) noted increased peak height of DMPO-OH. However, this type of experiment is difficult to interpret. First, high concentrations of DMPO blocked phagocytic production of superoxide by  $>90\%$ , as measured by the cytochrome *c* reduction method (data not shown). Second, increased formation of DMPO-OH is an inevitable consequence of the first order decay of DMPO-OOH as can be demonstrated by using the xanthine/xanthine oxidase superoxide generating system (Fig. 6).

In the current study, we used a variety of techniques to amplify detection of hydroxyl radical by spin trapping. However, this free radical was formed by human neutrophils only with iron supplementation. In other recent work using mass spectrometry (14, 15), deoxyribose oxidation (16), and aromatic hydroxylation of phenylalanine (17), hydroxyl radical formation by human neutrophils was not observed. Our results show that if hydroxyl radical were generated in a steady state flux above the detection limits of EPR spectroscopy, we could measure its presence. If, however, hydroxyl radical were produced solely as a bolus in the presence of a high flux of superoxide ( $\geq 1 \mu\text{M}/\text{min}$ ), spin trapping might not be able to detect its formation. Since stimulated neutrophils generate a continued, prolonged flux of superoxide (2), formation of physiologic hydroxyl radical (generated through the Haber-Weiss reaction) should also occur at a steady state level, and therefore be spin-trapped at the levels detectable by EPR spectroscopy. Available data do not support the idea that hydroxyl radical is a physiologic oxygen reduction product generated by human neutrophils in the absence of an exogenous Haber-Weiss catalyst.

*Acknowledgments*—We wish to acknowledge the technical assistance of Dr. Harold Goff, Danielle Hamill, and Karen Bean.

#### REFERENCES

- Babior, B. M. (1978) *N. Engl. J. Med.* **298**, 659–668
- Babior, B. M., Kipnes, R. S., and Curnette, J. T. (1973) *J. Clin. Invest.* **52**, 741–744
- Root, R. K., Metcalf, J., Oshino, N., and Chance, B. (1975) *J. Clin. Invest.* **55**, 945–955
- Cohen, M. S., Britigan, B. E., Hassett, D. J., and Rosen, G. M. (1988) *Free Rad. Biol. Med.* **5**, 81–89
- Britigan, B. E., Cohen, M. S., and Rosen, G. M. (1987) *J. Leukocyte Biol.* **41**, 349–362
- Britigan, B. E., Rosen, G. M., Chai, Y., and Cohen, M. S. (1986) *J. Biol. Chem.* **261**, 4426–4431
- Britigan, B. E., Rosen, G. M., Thompson, B. Y., Chai, Y., and Cohen, M. S. (1986) *J. Biol. Chem.* **261**, 17026–17032
- Rosen, G. M., Britigan, B. E., Cohen, M. S., Ellington, S. P., and Barber, M. J. (1988) *Biochim. Biophys. Acta* **969**, 236–241
- Finkelstein, E., Rosen, G. M., and Rauckman, E. J. (1984) *Biochim. Biophys. Acta* **802**, 90–98
- Samuni, A., Black, C. D. V., Krishna, C. M., Malech, H. L., Bernstein, E. F., and Russo, A. (1988) *J. Biol. Chem.* **263**, 13797–13801
- Bonnett, R., Brown, R. F. C., Clark, V. M., Sutherland, I. O., and Todd, A. (1959) *J. Chem. Soc.* 2094–2102
- Cohen, M. S., and Cooney, M. H. (1984) *J. Infect. Dis.* **150**, 49–56
- Kuthan, H., Ullrich, V., and Estabrook, R. W. (1982) *Biochem. J.* **203**, 551–558
- Thomas, M. J., Shirley, P. S., Hedrick, C., and DeChatalet, L. R. (1986) *Biochemistry* **25**, 8042–8048
- Thomas, M. J., Hedrick, C., and Shirley, P. S. (1986) in *Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine* (Rotilio, G., ed) pp. 19–21, Elsevier Science Publishing Co., New York
- Winterborn, C. C. (1986) *J. Clin. Invest.* **68**, 545–550
- Kaur, H., Fagerheim, I., Grootveld, M., Puppo, A., and Halliwell, B. (1988) *Anal. Biochem.* **172**, 360–367