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Charles F. Babbs

Purdue University, babbs@purdue.edu

David W. Griffin

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SCATCHARD ANALYSIS OF METHANE SULFINIC ACID PRODUCTION FROM DIMETHYL SULFOXIDE: A METHOD TO QUANTIFY HYDROXYL RADICAL FORMATION IN PHYSIOLOGIC SYSTEMS

CHARLES F. BABBS and DAVID W. GRIFFIN

Biomedical Engineering Center and Department of Veterinary Physiology and Pharmacology, Purdue University, West Lafayette, Indiana, USA.

ABSTRACT

A major impediment to the confirmation of free radical mechanisms in pathogenesis is a lack of direct, chemical evidence that oxygen centered free radicals actually arise in living tissues in quantities sufficient to cause serious damage. This investigation was conducted to validate the use of dimethyl sulfoxide (DMSO) as a quantitative molecular probe for the generation of hydroxyl radicals (HO^*) under physiologic conditions. Reaction of HO^* with DMSO produces methane sulfinic acid (MSA) as a primary product, which can be detected by a simple colorimetric assay. To develop a method for estimating total HO^* production, we studied two model systems: the superoxide driven Fenton reaction in vitro, using xanthine oxidase as the source of superoxide, and a computer model of Fenton chemistry. Measured MSA production both in vitro and in the computer model was a predictable function of the concentrations of DMSO and competing scavengers of HO^* , according to the principle of competition kinetics. Both experimental results and model calculations showed that Scatchard analysis may be used to infer total HO^* generation, despite the presence of scavengers other than DMSO, such as mannitol. Thus, methane sulfinic acid production from DMSO holds promise as an easily measured marker for HO^* formation in biologic systems pretreated with DMSO, and Scatchard analysis of repeated experiments with varying DMSO concentrations can yield an estimate of total HO^* generation.

Keywords--Hydroxyl radical, Lipid peroxidation, Oxygen toxicity, Reperfusion injury, Superoxide radical, Free radical

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INTRODUCTION

Evidence for the pathogenic role of oxygen derived free radicals in biologic systems is mounting. Oxygen centered free radicals may play a central role in the initiation or exacerbation of tissue damage in heart attack, stroke, organ transplantation, acute pancreatitis, liver cirrhosis, arthritis, Parkinson's disease, and the toxicity of and redox cycling drugs and chemicals.¹⁻¹¹ Perhaps of greatest clinical importance is the amplification of tissue damage during reperfusion of previously ischemic tissues, including heart,¹² kidney,¹³⁻¹⁴ small intestine,¹⁵⁻¹⁷ skin,¹⁸ and skeletal muscle.¹⁹ Widespread acceptance of pathogenic mechanisms invoking formation of oxygen radicals has been limited by their inability of investigators to convincingly detect and quantify such radicals in isolated tissues or in animal models.^{3, 20} This paper describes a new approach to the measurement of HO* radicals in biologic systems by trapping them with DMSO and applying a newly developed and simple colorimetric assay²¹ to detect the methane sulfinic acid produced by the trapping reaction,

described by Lagercrantz²² and Cohen,²³ for which the rate constant is $k = 7 \times 10^9 \text{ l}/(\text{M}\cdot\text{sec})$.²⁴ DMSO has the advantages of being a hydroxyl radical trap that distributes rapidly to all body fluid compartments²⁵ and that can be tolerated in high concentrations in vivo.^{26, 27} Methane sulfinic acid (MSA) is a primary and characteristic product of the trapping reaction; it is poorly metabolized by tissue;²¹ and it is relatively stable as a salt at pH > 4.²⁸ The yield of methane sulfinic acid in gamma irradiated, aqueous solutions of DMSO matches the theoretical radiochemical yield of hydroxyl radicals; and similarly, the yield of methane sulfinic acid when Fenton's reaction is performed in aqueous DMSO solutions matches the amount of hydrogen peroxide reduced.²⁹ Thus, presence of methane sulfinic acid may provide a specific and sensitive marker for generation of HO* in many biologic systems.

If one desires to quantify HO* generation in a biologic system, using this approach, however, one major experimental difficulty remains: the problem of accounting for the HO* radicals that react with endogenous bio-molecules rather than with DMSO. Hydroxyl radicals are so highly reactive that they combine with proteins, nucleic acids, and unsaturated lipids at nearly diffusion controlled rates.²⁴ Thus, low concentrations of DMSO, such as 1 mM, may capture only a tiny fraction of actual HO* generated, and even high concentrations, such as 100 mM, may not capture 100% of nascent HO*.

To obtain an estimate of total generation of HO* radicals, despite their rapid scavenging by components of the test system, we propose a Scatchard-style regression analysis of otherwise identical experiments, in which differing amounts of DMSO are added to the test system to probe for HO*. In this Scatchard analysis the MSA/DMSO concentration ratio is plotted as a function of MSA. For simple competition kinetics³⁰ this function is a downward sloping straight line, as shown formally in the Appendix. The regression function intercepts the horizontal, MSA, axis at infinite DMSO concentration ($\text{MSA/DMSO} \rightarrow 0$). By plotting the MSA/DMSO ratio in this way and extrapolating the Scatchard plot to the horizontal axis intercept, one can, in principle, estimate the total number of hydroxyl radicals in the sample that would have been trapped at "infinite" DMSO concentration, thus correcting for competition of HO* scavengers other than DMSO. The objectives of the present study were to define the experimental conditions under which Scatchard analysis of sulfinic acid production in DMSO containing systems provides a measure of total HO* generation, and to validate the accuracy and practicality of this approach.

METHODS

To study the conditions required for quantitative estimation of HO* production by Fenton chemistry under physiologic conditions, we selected two model systems. The first was the well characterized in vitro xanthine oxidase system, including xanthine oxidase, hypoxanthine, and EDTA chelated iron in phosphate buffer at pH 7.4. The second was an idealized computational model of the kinetics of Fenton chemistry, incorporating 50 simultaneous differential equations, based upon published rate constants for 50 simultaneous, inter-related reactions. In both systems mannitol was added as a prototype hydroxyl radical scavenger other than DMSO to study the effects of competition kinetics. In this way we could identify the utility of Scatchard analysis, the required DMSO concentrations, and other necessary conditions for quantitative determination of HO*, despite the presence of competing scavengers.

Xanthine/xanthine oxidase system

Xanthine, hypoxanthine, and bovine milk xanthine oxidase (E.C. 1.2.3.2) were obtained from Sigma and used without further purification. Reagent grade ethylenediaminetetraacetate (EDTA), ferric chloride, sodium chloride, and sodium phosphate were obtained from standard sources. Xanthine or hypoxanthine was dissolved in 1.0 N NaOH (18 mg/ml) and diluted in phosphate buffered saline (PBS, 100 mM NaCl, 50 mM phosphate buffer, pH 7.5) to a concentration of 132 μM . In a typical experiment 0.2 ml of 1 mM EDTA--Fe, 0.1 ml of 0.001 to 14 M DMSO, and 0.2 ml of xanthine oxidase (0.83 U/ml in PBS) were added to 2.6 ml of 132 μM hypoxanthine at room temperature. Final concentrations were thus 100 μM hypoxanthine, 0 to 1 M DMSO, 48 mU/ml xanthine oxidase. In some experiments mannitol was added in the amounts stated in figure legends to model effects of competition for HO* by radical scavengers other than DMSO. Reactions were initiated by addition of xanthine oxidase and terminated by depletion of hypoxanthine and xanthine substrates after a development time of 20 min.

Methane sulfinic acid assay

The reaction mixtures were assayed for methane sulfinic acid by addition of 1.0 ml of 30 mM Fast Blue BB salt, followed by extraction with 2.0 ml of toluene/butanol 3/1, as described by Babbs and Gale.²¹ Color in the organic phase is caused by generation of the yellow diazosulfone:

Since minimal interference was encountered in this simple system, preliminary extraction of methane sulfinic acid into acidic butanol, as previously described,²¹ was not necessary. The toluene/butanol phase was washed with 5 ml of butanol-saturated water to remove remaining unreacted diazonium salt. The samples were centrifuged at 500 g for 3 min, and the upper phase, containing the diazosulfone, was retained. After addition of 0.5 ml of 19:1 pyridine:acetic acid buffer to prevent fading of color, visible spectra and peak absorbance values at 425 nm were obtained using a Perkin-Elmer Lambda 3B spectrophotometer against blanks containing all reagents except DMSO. Standards were prepared containing all reagents minus DMSO, plus 30 nmol/ml authentic methane sulfinic acid, obtained from Fairfield Chemical Company, Blythewood, South Carolina, 29106, USA.

Computational model

Methane sulfinic acid production by HO* mediated oxidation of DMSO was computed using a kinetic model of 50 relevant enzymatic and free radical reactions, incorporating rate constants obtained from the published literature (Table 1). A "C" language computer program was developed to perform numerical integration of the kinetic equations implied in Table 1, including superoxide production by xanthine oxidase, the Haber-Weiss reaction, the DMSO trapping reaction, and subsequent secondary radical reactions. The kinetic model containing the set of 50 kinetic equations shown in Table 1 was derived from a much larger model describing over 90 reactions from the published literature (available from the authors on request) that could conceivably have played a role in this or similar systems. The list of equations to be included in the present model was pruned by omitting obviously irrelevant ones, such as those dealing with lipid peroxidation, and by eliminating reactions which formed zero or negligible product (less than 0.1 micromolar) during the course of representative test simulations. The input values for the rate constants of these irrelevant reactions were set to zero, and the software was so configured that the corresponding computations were not performed (i.e., arrays of pointers indicating reactions to be included were re-sorted to exclude reactions with zero rate constants).

Using an iterative method, the computer program calculates concentrations of the various species after successive small time steps, dt , as functions of all the other concentrations and the rate constants in Table 1. Numerical values for rate constants were obtained from the published literature or estimated, when necessary, from rate constants of reactions involving similar or analogous substrates. The eleven of fifty rate constants that were estimated describe either radical-radical chain termination reactions, which are in any case very fast, or reactions involving hydroxyl radicals and minor products, which are likely to have little effect on the overall results.

Central to the operation of the kinetic model is a subroutine to compute the concentration changes during a differential time step, dt , produced by a family of competing bi-molecular reactions of the form

for which it is assumed that species A is most reactive (usually a radical). In the case that the consumption of A is rate-limiting, the distribution of products is determined according to competition kinetics, as described by Spinks.³⁰ and presented in detail in the Appendix. The kinetic behavior of xanthine oxidase is modeled as a ping-pong/bi-bi mechanism, as described by Walsh.³²

Simulation parameters, including the duration of the simulation, the sampling interval at which concentration data are recorded, the time step for numerical integration, and the various rate constants for reactions included in the simulation are read from a control file. The output file contains a listing of rate constants actually used in the simulation, and the concentrations of nonradical species as a function of time. Sufficiently small time steps for integration were selected in the present study, such that halving or doubling the time step did not affect the results. The initial conditions for both computer simulations and experiments were: hypoxanthine or xanthine, 100 μM ; xanthine oxidase, 48 mU/ml; oxygen, 200 μM ; EDTA--Fe 57 μM , and DMSO ranging from 0 to 1.0 M. (The oxygen concentration of air-saturated water at one atmosphere is about 200 μM .)

RESULTS

Standard curves generated by the colorimetric assay for authentic methane sulfinic acid were linear, reproducible, and completely unaffected by the presence of from 1 mM to 1 M mannitol. Linear regression analysis of absorbance, y , as a function of the micromolar concentration of standard methane sulfinic acid, x , for 5 pooled standard curves in the presence of varying mannitol concentrations gave the regression function $y = -0.019 + 0.00445 x$, $r = 0.9998$. The detection limit³³ of the colorimetric assay was approximately 10 nanomoles per sample.

Table 1. Rate Constants for Kinetic Model*

Reaction	Rate Constant l/(mole-sec) [†]	References
$\text{H}_2\text{O}_2 + \text{Fe}^{2+}\text{—EDTA} \rightarrow \text{Fe}^{3+}\text{—EDTA} + \text{HO}\cdot$	$k[0]$	$3e + 4$ [52,53]
$\text{HO}\cdot + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{OH}^-$	$k[1]$	$3e + 8$ [54,55]
$\text{HO}\cdot + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{H}^+ + \text{O}_2^-$	$k[2]$	$3e + 7$ [24,54,55]
$\text{HO}\cdot + \text{DMSO} \rightarrow \text{CH}_3\cdot + \text{MSA}$	$k[3]$	$7e + 9$ [24,55]
$\text{O}_2^- + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{O}_2$	$k[4]$	$1e + 6$ [52,56–58]
$\text{CH}_3\cdot + \text{O}_2 \rightarrow \text{CH}_3\text{OO}\cdot$	$k[5]$	$5e + 9$ [59–61]
$\text{CH}_3\text{OO}\cdot + \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{OOH} + \text{H}^+ + \text{O}_2^-$	$k[7]$	$1e + 4$ Estimated
$\text{CH}_3\text{OO}\cdot + \text{Fe}^{3+} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{OOH} + \text{OH}^- + \text{Fe}^{2+}$	$k[8]$	$1e + 7$ [54]
$\text{Fe}^{2+} + \text{CH}_3\text{OOH} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{CH}_3\text{O}\cdot$	$k[9]$	$3.1e - 5$ [62,63]
$\text{Fe}^{2+} + \text{ROOH} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{RO}\cdot$	$k[10]$	$3.1e - 5$ [62,63]
$\text{HO}\cdot + \text{HO}\cdot \rightarrow \text{H}_2\text{O}_2$	$k[14]$	$5e + 9$ [24,54,55]
$\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$	$k[15]$	$6e + 5$ [64–66]
$\text{CH}_3\cdot + \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{OH} + \text{H}^+ + \text{O}_2^-$	$k[21]$	$1.8e + 6$ [60]
$\text{CH}_3\text{O}\cdot + \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{OH} + \text{H}^+ + \text{O}_2^-$	$k[22]$	$1e + 4$ [67]
$\text{R}\cdot + \text{H}_2\text{O}_2 \rightarrow \text{R}'\text{H} + \text{H}^+ + \text{O}_2^-$	$k[23]$	$1e + 4$ [67]
$\text{ROO}\cdot + \text{H}_2\text{O}_2 \rightarrow \text{ROOH} + \text{H}^+ + \text{O}_2^-$	$k[24]$	$1e + 4$ [67]
$\text{RO}\cdot + \text{H}_2\text{O}_2 \rightarrow \text{ROH} + \text{H}^+ + \text{O}_2^-$	$k[25]$	$1e + 4$ [67]
$\text{HO}\cdot + \text{CH}_3\text{OOH} \rightarrow \text{CH}_3\text{OO}\cdot + \text{H}_2\text{O}$	$k[27]$	$1e + 7$ Estimated
$\text{HO}\cdot + \text{RH} \rightarrow \text{R}\cdot + \text{H}_2\text{O}$ (RH = mannitol)	$k[28]$	$1.7e + 9$ [55]
$\text{HO}\cdot + \text{ROOH} \rightarrow \text{ROO}\cdot + \text{H}_2\text{O}$	$k[29]$	$1e + 7$ Estimated
$\text{HO}\cdot + \text{O}_2^- \rightarrow \text{O}_2 + \text{OH}^-$	$k[32]$	$1e + 10$ [24,66]
$\text{CH}_3\text{O}\cdot + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{OH}^- + \text{CH}_3\text{OH}$	$k[33]$	$1e + 7$ [54]
$\text{R}\cdot + \text{O}_2 \rightarrow \text{ROO}\cdot$	$k[37]$	$5e + 9$ [59]
$\text{ROO}\cdot + \text{Fe}^{3+} + \text{H}_2\text{O} \rightarrow \text{Fe}^{2+} + \text{OH}^- + \text{ROOH}$	$k[39]$	$1e + 7$ [54]
$\text{RO}\cdot + \text{Fe}^{3+} + \text{H}_2\text{O} \rightarrow \text{Fe}^{2+} + \text{OH}^- + \text{ROH}$	$k[42]$	$1e + 7$ [54]
$\text{O}_2^- + \text{R}\cdot + \text{H}_2\text{O} \rightarrow \text{ROOH} + \text{OH}^-$	$k[49]$	$1e + 9$ Estimated
$\text{O}_2^- + \text{ROO}\cdot + \text{H}_2\text{O} \rightarrow \text{ROOH} + \text{OH}^- + \text{O}_2$	$k[50]$	$1e + 8$ [68]
$\text{O}_2^- + \text{RO}\cdot + \text{H}_2\text{O} \rightarrow \text{ROH} + \text{OH}^- + \text{O}_2$	$k[51]$	$1e + 8$ Estimated
$\text{HO}\cdot + \text{CH}_3\cdot \rightarrow \text{CH}_3\text{OH}$	$k[53]$	$1e + 10$ [61]
$\text{HO}\cdot + \text{CH}_3\text{O}\cdot \rightarrow \text{CH}_3\text{OOH}$	$k[55]$	$1e + 10$ Estimated
$\text{HO}\cdot + \text{RO}\cdot \rightarrow \text{ROOH}$	$k[58]$	$1e + 10$ Estimated
$\text{CH}_3\cdot + \text{CH}_3\cdot \rightarrow \text{CH}_3\text{—CH}_3$	$k[60]$	$1e + 9$ [60,61,69]
$\text{CH}_3\cdot + \text{ROO}\cdot \rightarrow \text{CH}_3\text{OOR}$	$k[64]$	$3e + 7$ Estimated
$\text{CH}_3\text{OO}\cdot + \text{CH}_3\text{OO}\cdot \rightarrow \text{CH}_3\text{OOCH}_3 + \text{O}_2$	$k[67]$	$6e + 5$ [62,68,69]
$\text{CH}_3\text{OO}\cdot + \text{CH}_3\text{O}\cdot \rightarrow \text{CH}_3\text{OCH}_3 + \text{O}_2$	$k[68]$	$1e + 6$ Estimated
$\text{R}\cdot + \text{R}\cdot \rightarrow \text{R—R}$	$k[78]$	$2e + 7$ [69]
$\text{ROO}\cdot + \text{ROO}\cdot \rightarrow \text{ROOR} + \text{O}_2$	$k[82]$	$9e + 5$ [59,62,68,69]
$\text{RO}\cdot + \text{RO}\cdot \rightarrow \text{ROOR}$	$k[85]$	$1e + 6$ Estimated
$2 \text{O}_2^- + 2 \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 + 2 \text{OH}^-$ (SOD, turnover number l/sec)	$k[88]$	$1.6e + 6$ [32,70–74]
$\text{xanthine} + \text{O}_2 \rightarrow \text{UA} + \text{O}_2^-$ (xanthine oxidase turnover number l/sec)	$k[89]$	$1.6e + 7$ [75–77]
$\text{hypoxanthine} + \text{O}_2 \rightarrow \text{xanthine} + \text{O}_2^-$ (xanthine oxidase turnover number l/sec)	$k[90]$	$1.6e + 7$ [75–77]
$\text{HO}\cdot + \text{CH}_3\text{SOOH} \rightarrow \text{CH}_3\cdot + \text{products}$	$k[91]$	$1e + 9$ Estimated
$\text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \text{O}_2^-$ (reverse of reaction 4)	$k[92]$	$1e + 4$ [52]
$\text{H}_2\text{O}_2 + \text{Fe}^{3+}\text{—EDTA} \rightarrow \text{Fe}^{3+}\text{—EDTA—O}_2^-$ (formation of peroxo complex)	$k[93]$	350 [52,78,79]
$\text{O}_2^- + \text{Fe}^{2+}\text{—EDTA} \rightarrow \text{Fe}^{3+}\text{—EDTA—O}_2^-$ (formation of peroxo complex)	$k[94]$	$7e + 6$ [52,56,58,79]
$\text{Fe}^{3+}\text{—EDTA—O}_2^- \rightarrow \text{Fe}^{3+}\text{—EDTA} + \text{H}_2\text{O}_2$	$k[95]$	$1e + 9$ [52]
$\text{HO}\cdot + \text{Hypoxanthine} \rightarrow \text{products}$	$k[96]$	$6.5e + 9$ [55]
$\text{HO}\cdot + \text{Xanthine} \rightarrow \text{products}$	$k[97]$	$5.2e + 9$ [55]
$\text{CH}_3\cdot + \text{H}_2\text{O}_2 \rightarrow \text{CH}_3\text{OH} + \text{HO}\cdot$	$k[98]$	$3.5e + 7$ [55,61]
$\text{HO}\cdot + \text{Uric Acid} \rightarrow \text{products}$	$k[99]$	$7e + 9$ [55]

*The notation for values of rate constants of the form, $Ae + n$, is equivalent to $A \times 10^{+n}$.[†]Unless otherwise noted.

The mixtures of xanthine oxidase, hypoxanthine, chelated iron, and DMSO readily produced methane sulfinic acid in good agreement with predictions of the kinetic model. No sulfinate was detected in the absence of enzyme, in the absence of xanthine or hypoxanthine substrate, in the absence of iron, or in the absence of DMSO (Fig. 1). The yield of sulfinate depended upon the concentration of the DMSO and the presence of competing HO* trapping agent, mannitol, both in vitro (Fig. 2) and in the computational model (Fig. 3). In the absence of mannitol (dashed curves) 90 to 100% of HO* radicals are trapped by DMSO and converted to methane sulfinic acid when DMSO concentration exceeds 10 mM. In the computer simulations (Fig. 3) the amounts of hydroxyl radical formed in the reaction were identical, to the nearest 0.1 micromolar, to the amounts of DMSO formed in the presence of zero mannitol and ≥ 100 mM DMSO. The plateau value near 35 μ M methane sulfinic acid is close to the theoretical maximum of hydroxyl radical formation, based upon superoxide production. According to Fridovich,³⁴ xanthine oxidase would be expected to produce 40 μ M superoxide and 80 μ M hydrogen peroxide directly at pH 7.8 in the present system. Evidently, most of the superoxide was able to form DMSO-trappable hydroxyl radical in the absence of mannitol.

Fig. 1. Absorbance spectra of the final organic phase in the sulfinic acid assay of the xanthine oxidase test system. Upper curve with peak at 425 nm was obtained for the complete system. Lower four curves, nearly identical, were obtained with system minus enzyme, minus substrate, minus EDTA-iron, and minus DMSO. Production of color, indicating hydroxyl radical trapped as methane sulfinic acid, requires the simultaneous presence of enzyme, substrate, EDTA iron, and DMSO.

Fig. 2, Methane sulfinic acid production by the xanthine oxidase system in the presence of 100 μ M hypoxanthine 57 μ M Fe--EDTA and varying concentrations of DMSO. Concentrations of methane sulfinic acid plotted on the ordinate represent cumulative HO^ trapped by DMSO during the experiment. Upper curve zero mannitol. Remaining curves show influence of varying concentrations of mannitol. Note log scale.*

Fig. 3. Methane sulfinic acid production in the computational model with inputs corresponding to conditions of Figure 2 (100 μ M hypoxanthine 57 μ M Fe--EDTA and varying concentrations of DMSO). Results of the computer model, developed independently from the known rate constants for relevant free radical reactions and the equations of competition kinetics, are similar to those of the in vitro xanthine oxidase system.

In the presence of either millimolar or molar concentrations of mannitol (solid curves) greater DMSO concentrations are required to trap a high percentage of HO* in both model systems. The shapes of the curves can be explained on the basis of competition kinetics for the reactions:

as is confirmed by the regression analysis, presented in Figure 4. Linearity of the Scatchard plots, expected on the basis of competition kinetics (See Appendix), was obtained for systems containing mannitol concentrations varying over three orders of magnitude (Figs. 4 A and C vs. B and D). The horizontal axis intercepts near 35 μM MSA represent the maximal yield of MSA expected at "infinite" DMSO concentration.

Thus, even in the presence of high concentrations of mannitol, maximal conversion of HO* to CH₃SOOH, can be estimated from the horizontal axis intercepts of the Scatchard plots in Figure 4. The results (Table 2) are in reasonable agreement, except for the lower curve in Figure 4 B, which may represent inhibition of xanthine oxidase by 1 M mannitol. Additionally, the less efficient trapping of hydroxyl radicals at low DMSO concentrations observed in the xanthine oxidase system compared to the computer model may have been related to the presence of 2 M ammonium sulfate in the Sigma preparation of xanthine oxidase. Ammonium ions, which are good hydroxyl scavengers could have competed successfully with DMSO under these conditions.

Table 2. Results of Scatchard Analysis of Plots in Figure 4

Mannitol Concentration (mM)	Maximal Methane Sulfinic Acid Production, Extrapolated to "Infinite" DMSO Concentration (nmol/ml)	
	Experimental Model	Computer Model
0	32	36.4
1	28	36.4
500	34	36.5
1000	21	36.1

Fig. 4. Scatchard plots of data in Figs. 2 and 3. Linear plots confirm expectations of competition kinetics for either millimolar (A and C) or molar (B and D) concentrations of mannitol. Horizontal axis intercepts near 35 μM methane sulfinic acid represent trapping of 100% of HO^* radicals at "infinite" DMSO concentration. This 100% yield value can still be inferred from the Scatchard analysis despite the presence of mannitol in concentrations varying over 3 orders of magnitude. Plots A and B are for the experimental, xanthine oxidase system; plots C and D are for the computational model. Plots A and C describe low DMSO concentrations. Plots B and D, to a different scale, describe high DMSO concentrations.

DISCUSSION

Lack of a simple, low cost, and effective method for detecting active oxygen radicals in biologic systems has been a major limitation in the study of free radical mechanisms of pathogenesis. This paper demonstrates one method able to detect hydroxyl radicals produced by amounts of xanthine oxidase and iron similar to those reported in animal models of ischemia and reperfusion,³⁵⁻³⁷ and in the presence of xanthine and hypoxanthine substrate levels approximately one tenth that measured in postischemic kidney and heart.^{38,39} The competition of scavengers other than DMSO for HO* was modeled both in vitro and in computations, based upon published rate constants obtained in pulse radiolysis studies. In both models the fraction of nascent HO* radicals trapped as methane sulfinic acid depends upon the concentration of DMSO and can approach 100% at DMSO concentrations of 1 to 5% (0.15 to 0.7 M), which are tolerable in living tissues.^{26, 40-42} Despite the inevitable reaction of some HO* radicals with molecules other than DMSO, it appears that a reasonable estimate of total HO* production can be obtained from Scatchard analysis of methane sulfinic acid production in otherwise identical experiments with varying DMSO concentration.

Scatchard analysis may also be important in biological studies using DMSO as a molecular probe for HO* for another reason: it permits a quantitative test of the assertion that the DMSO concentrations necessary to trap HO* radicals efficiently may inhibit or potentiate the generation of these radicals. An interaction of this sort would be a significant one, because it would mean that the process of making the measurement was altering the phenomenon under study. The existence of such undesirable interactions may be detectable by Scatchard analysis. In the event that DMSO interferes with radical production at higher concentrations, then one would expect a relatively lower yield than that predicted on the basis of simple competition kinetics. In this case the Scatchard plot would bend downward in a nonlinear fashion at the higher DMSO concentrations. Alternatively, in the event that DMSO potentiates radical production at higher concentrations, then one would expect a relatively greater yield than that predicted on the basis of simple competition kinetics. In this case the Scatchard plot would bend upward in a nonlinear fashion, at the higher DMSO concentrations. In this way Scatchard analysis may provide a useful experimental check for undesirable effects of DMSO upon the model system under study.

We believe that DMSO approximates in many ways an ideal molecular probe for oxygen radicals in vivo, owing to its unique chemical and biological properties. Its small molecular size and hybrid water and lipid solubility enable DMSO to distribute rapidly to all tissue compartments, including both membrane and cytoplasmic phases.^{25, 26} Methane sulfinic acid produced by the trapping of HO* by DMSO is a stable, nonradical compound, not normally found in biological specimens. It is a primary product of the trapping reaction (1) produced in 70 to 100% yield.²⁹ In contrast, aromatic compounds such as salicylic acid and phenols also trap HO* efficiently, but yield multiple hydroxylated isomers upon reaction with HO*, which subsequently must be separated and distinguished from natural products.^{43, 44} Ethanol, for example, gives three intermediate radicals, $\cdot\text{CH}_2\text{-CH}_2\text{-OH}$, $\text{CH}_3\text{-CH}\cdot\text{-OH}$, and $\text{CH}_3\text{-CH}_2\text{-O}\cdot$ upon reaction with HO*, which decay to form a multiplicity of products.²²

The unique chemical properties of dimethyl sulfoxide that give it special advantages as a molecular probe for HO* are complemented by its benign biological effects. The median lethal dose (LD50) of intravenous DMSO in animals ranges from about 4 to 10 g/kg body weight, depending on the species.^{26, 27} The maximum tolerated dose increases to 10 or more g/kg when the compound is given orally. In a series of remarkable experiments Benville, Smith, and Shanks⁴⁰ studied young salmon and trout totally immersed in DMSO solutions. Fish immersed in 2% (0.26 M) DMSO for 100 days exhibited good appetite and normal weight gain. DMSO in concentrations ranging from 1 to 2 molar (7 to 15%) has been used successfully to permit freezing and thawing of blood without hemolysis,⁴⁵ as well in the cryopreservation of cultured cells⁴⁶ and the embryos of mice, rabbits, cows, and sheep prior to transplantation.^{41, 47, 48} Some enzymes, including Na⁺/K⁺-ATPase, are reversibly inhibited by DMSO;⁴² however, there is little evidence for much effect in concentrations below about 5% (0.7 M). For these reasons, we believe that DMSO is likely to be well tolerated by biological systems in the high concentrations required for efficient scavenging of a reasonably high percentage of nascent HO*. Other compounds such as salicylates,^{43, 44} readily react with HO* to form distinctive hydroxylated products, however, the tolerable concentrations of these aromatic molecular probes in vivo are likely to be well below those needed for quantitative estimation of total HO* production.

Previously Cohen²³ had proposed measuring formaldehyde as a marker for hydroxyl radical reaction with DMSO. He proposed that formaldehyde (HCHO) was formed by a Russell reaction mechanism,⁴⁹

and was successful in detecting formaldehyde production in vitro when DMSO was exposed to hydroxyl radical generating systems.²³ We sought an alternative method, since formaldehyde detection in whole tissues would be difficult, owing to rapid reaction of free formaldehyde with tissue proteins. Further, the yield of formaldehyde molecules is small, because the Russell reaction requires the encounter of two CH₃OO* radicals (a relatively unlikely termination reaction). Since CH₃OO* radicals can readily react with free ferrous iron, endogenous antioxidants, unsaturated fatty acids, and other biological species, only a small fraction would be expected to react with each other, making the Russell reaction a relatively inefficient indicator of HO* trapping by DMSO in tissue. The present approach provides an extension of Cohen's method, in which a primary product of the trapping reaction (CH₃SOOH) is detected. In addition, we find it aesthetically appealing that one of the oxygen atoms in each measured molecule of methane sulfinate is the same oxygen atom present in the original trapped hydroxyl radical.

Repine and coworkers⁵⁰ have previously reported the feasibility of using DMSO in concentrations up to 130 mM as a probe for hydroxyl radicals in human phagocytes, in experiments in which methane was measured by gas chromatography as a product of the trapping reaction, based upon the assumption that $\text{DMSO} + \text{HO}^* \rightarrow \text{CH}_3\text{SOOH} + \text{CH}_3^*$ was followed by $\text{CH}_3^* + \text{RH} \rightarrow \text{CH}_4 + \text{R}^*$. The yields of methane so produced are likely to be much greater than the yield of formaldehyde, as previously described. However, there remains the potential in some systems for competition of molecular oxygen for the methyl radicals, namely $\text{CH}_3^* + \text{O}_2 \rightarrow \text{CH}_3\text{OO}^*$ and $\text{CH}_3\text{OO}^* + \text{RH} \rightarrow \text{CH}_3\text{OOH} + \text{R}^*$, followed by reduction of the methyl hydroperoxide to methanol. Thus, there is a slight, at least theoretical, advantage to methane sulfinic acid production, compared to methane production, as an indicator of total hydroxyl radical entrapment by DMSO. Methane production may be the method of choice for investigations of leukocyte function, since we have found that hypochlorous acid, produced in abundance by leukocytes, can degrade methane sulfinic acid (unpublished observation).

The present paper demonstrates that the extremely short-lived hydroxyl radicals can be detected by trapping them with dimethyl sulfoxide (DMSO) and measuring a nongaseous primary product of the trapping reaction by a simple colorimetric assay. Scatchard analysis may be used to correct for the competition between other HO^* scavengers in the sample and added DMSO for reaction with evolving hydroxyl radicals, in order to obtain the total number of hydroxyl radicals produced. Thus, the technique holds promise as a widely applicable tool to detect and quantify oxygen radicals in a variety of in vitro and animal models of pathologic conditions postulated to be caused by overproduction of superoxide and HO^* .

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ABBREVIATIONS

DMSO--dimethyl sulfoxide
 EDTA--ethylenediaminetetraacetic acid
 LD50--median lethal dose
 LOOH--lipid hydroperoxide
 MSA--methane sulfinic acid
 SOD--superoxide dismutase
 RH--generic nonradical species
 UA--uric acid

APPENDIX

This analysis is based upon the principle of competition kinetics.³⁰ Suppose that radical R^* can react with either substance S or competing substance C as follows: $R^* + S \rightarrow \text{product } P_1$ with rate constant, k_1 , and $R^* + C \rightarrow \text{product } P_2$ with rate constant k_2 .

Then the probability that R^* will react with S is given by

$$\frac{k_1[R^*][S]}{k_1[R^*][S] + k_2[R^*][C]} = \frac{k_1[S]}{k_1[S] + k_2[C]}$$

Consider an experiment in which sulfinic acid production is measured in the presence of varying DMSO concentration, other variables being held constant. If S = DMSO and C = a lumped parameter representing all the competing scavengers of HO^* in a given system, then the total molar concentration of nascent HO^* radicals trapped as methane sulfinic acid by DMSO in the system can be written as

$$\begin{aligned}
[\text{CH}_3\text{SOOH}] &= [\text{HO}\cdot] \frac{k_1[\text{DMSO}]}{k_1[\text{DMSO}] + k_2[\text{C}]} \\
&= [\text{HO}\cdot] \frac{[\text{DMSO}]}{[\text{DMSO}] + \frac{k_2}{k_1} [\text{C}]}
\end{aligned}$$

Note that in the case for which $[\text{DMSO}] \gg [\text{C}]$, then $[\text{CH}_3\text{SOOH}] \rightarrow [\text{HO}\cdot]$.

The foregoing is an expression of the form

$$[\text{CH}_3\text{SOOH}] = \frac{[\text{CH}_3\text{SOOH}]_{\text{max}} [\text{DMSO}]}{[\text{DMSO}] + K}$$

reminiscent of classical enzyme kinetics, where $[\text{CH}_3\text{SOOH}] = [\text{HO}\cdot]$ = the total number of hydroxyl radicals evolved during the period of observation per unit volume, and $K = (k_2/k_1)[\text{C}]$, a hybrid constant describing the relative ability of non-DMSO molecules in the sample to react with $\text{HO}\cdot$. Scatchard analysis involves rearrangement of this expression in the form

$$\frac{[\text{CH}_3\text{SOOH}]}{[\text{DMSO}]} = \frac{[\text{CH}_3\text{SOOH}]_{\text{max}}}{K} - \frac{[\text{CH}_3\text{SOOH}]}{K},$$

a linear function of $[\text{CH}_3\text{SOOH}]$ for which the slope equals $-1/K$ and the intercept equals $[\text{CH}_3\text{SOOH}]_{\text{max}}/K$.⁵¹ The value $[\text{CH}_3\text{SOOH}]_{\text{max}}$ at "infinite" DMSO concentration can be estimated from the $[\text{CH}_3\text{SOOH}]$ -axis intercept of this function as $[\text{CH}_3\text{SOOH}]/[\text{DMSO}]$ approaches zero, thus correcting for competition of other molecules in the sample for $\text{HO}\cdot$ radicals.

In practical computation let $y = [\text{CH}_3\text{SOOH}]/[\text{DMSO}]$ and $x = [\text{CH}_3\text{SOOH}]$ for the Scatchard plot. The value of the horizontal, x-axis intercept, is computed from the y-intercept, a_0 , and the slope, a_1 , of the linear regression function for y vs. x, namely $[\text{CH}_3\text{SOOH}]_{\text{max}} = x_{\text{max}} = -y\text{-intercept}/\text{slope} = -a_0/a_1$.