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Abstract

This investigation was conducted to validate the use of dimethyl sulfoxide (DMSO) as a quantitative molecular probe for the generation of hydroxyl radicals (HO) in aqueous systems. Reaction of HO with DMSO produces methane sulfinic acid as a primary product, which can be detected by a simple calorimetric assay. To evaluate this method for estimating total HO production, we studied three model systems, including the Fenton reaction, γ irradiation of water, and ultraviolet photolysis of hydrogen peroxide, for which the theoretical maximum yield of HO could be calculated and compared to measured DMSO oxidation. The results confirm that 0.05 to 1 M DMSO may be used to capture nearly all of the expected HO radicals formed. Thus, methane sulfinic acid production from DMSO holds promise as an easily measured marker for HO formation in aqueous systems pretreated with DMSO.

Introduction

In this paper we describe validation of a simple, low cost method for trapping and quantifying hydroxyl radicals (HO) in aqueous systems [1, 2]. The method is based on the reaction of hydroxyl radicals with dimethyl sulfoxide (DMSO) [2], namely

$$CH_3\text{–}SO\text{–}CH_3 + HO^{\bullet} \rightarrow CH_3\text{–}SOOH + CH_3^{\bullet},$$

to produce methane sulfinic acid (MSA) and the methyl radical [1, 3, 4]. The accumulation of methane sulfinic acid may be measured by a calorimetric assay, recently developed by the authors [4, 5], to quantify the HO radicals formed during a given time period in DMSO-containing systems. A potentially important property of DMSO for possible future studies of biological systems is its remarkably low toxicity [6-8], which permits administration of sufficient amounts of the molecular probe to trap a large fraction of nascent HO, despite the presence of competing scavengers for hydroxyl radicals [2]. The ability of many biological systems to withstand up to molar concentrations of DMSO without detrimental effects [6, 7] means that the molar fraction of HO reacting with DMSO to form MSA can approach unity in practical experiments.

To determine the validity of the MSA assay as a measure of the cumulative HO generation in the presence of DMSO, we investigated three simple non-biological test systems known to produce hydroxyl radicals, in which the number of HO radicals expected to be generated can be specified quantitatively: the Fenton reaction, γ irradiation of water, and ultraviolet photolysis of hydrogen peroxide.

Materials and methods

Materials

Acetic acid, dimethyl sulfoxide, ferrous sulfate, hydrogen peroxide, n-butanol, pyridine, sulfuric acid, and toluene were obtained from Fisher Scientific Company (Fair Lawn, New Jersey). Fast Blue BB salt was obtained from Aldrich Chemical Company (Milwaukee, Wisconsin). Standard curves were prepared using authentic methane sulfinic acid, sodium salt, obtained from Fairfield Chemical Company (Blythewood, South Carolina) and used without further purification.

Fenton reaction **(** $H_2O_2 + Fe^{++} \longrightarrow Fe^{+++} + OH^- + HO$ **)**

Hydroxyl radicals were generated quantitatively by the Fenton reaction in unbuffered, aqueous DMSO by adding, at a rate of 0.5 ml/min, 250 μM H_2O_2 in 50 mM DMSO to a flask containing 80 ml of 1 mM $FeSO_4$ in 50 mM DMSO, freshly prepared and saturated with argon gas to minimize autoxidation of the ferrous iron. On the basis of computational modeling previously described [2], it was expected that the slow addition of H_2O_2 to $FeSO_4$ over a 40 min period would yield 1 mol of HO for each mole of H_2O_2 added. These conditions minimized the reaction of HO with H_2O_2, as well as the reaction of methyl radicals with H_2O_2, which otherwise tended to reduce the yield of MSA. The slow addition of hydrogen peroxide to stirred flasks of $FeSO_4$-DMSO was easily accomplished with a motor-driven syringe (Harvard Apparatus). Aliquots of a mixture of 80 ml of the $FeSO_4$-DMSO solution plus 20 ml of 50 mM DMSO were used as the blanks; reference samples representing 100% yield were obtained from a mixture of 80 ml of the $FeSO_4$-DMSO solution plus 20 ml of 250 μM standard methane sulfinic acid, sodium salt (final concentration 50 μM).

Gamma radiolysis of water **(** $H_2O \longrightarrow H + HO + e^-_{aq}$ **)**

Capped 25-ml vials of unbuffered 50 mM DMSO in distilled water were irradiated in a U.S. Nuclear Model GR-12 Cobalt-60 irradiator for a given duration, and the amount of trapped HO was subsequently measured by the colorimetric assay for methane sulfinic acid. The γ source had been previously calibrated by means of Fricke chemical dosimeters [9] and found to have a dose rate in the center of the irradiation chamber of 28.6 J/kg-min (2860 rads/min) and a "transient dose" during the in/ out cycle of 7.87 J/kg (787 rads).

Recovery of HO in irradiated DMSO solution was compared with literature values for the expected radiochemical yield of hydroxyl radicals, G_{HO}, during γ irradiation of water, 2.92 molecules per 100 eV [9, 10]. After substituting 1 rad = 0.01 J/kg, 1 eV = 1.602 x 10^{-19} J and Avogadro's number, the expected yield, y, of HO radicals in picomoles per gram was computed as y = 3.01 × radiation dose in rads.

The vials of 50 mM DMSO were placed in a circular array in the 6-inch diameter chamber and were irradiated for either 6 or 12 min to produce expected cumulative yields of approximately 50 or 100 μM hydroxyl radicals. After the 6-min period was complete, half of the samples were removed from the chamber and the remaining samples were irradiated for another 6-min period, thus the samples receiving a total of 12 min irradiation also incurred two transient doses. The calculated radiation dose was 163 J/kg for the 6-min samples and 326 J/kg for the 12-min samples. Controls included nonirradiated solutions of 50 mM DMSO, plus or minus standard methane sulfinic acid.

Ultraviolet photolysis of hydrogen peroxide ($H_2O_2 \longrightarrow 2\,HO$).

Ultraviolet exposure of hydrogen peroxide was used to create hydroxyl radicals from the cleavage of H_2O_2 molecules as previously described [11, 12]. A Mineralight lamp at 254 nm wavelength (Ultra-violet Products, Inc., San Gabriel, CA) was used to irradiate 0.5 to 1.5 mM unbuffered aqueous solutions of hydrogen peroxide for periods of 0 to 15 min. The initial and post-exposure concentrations of H_2O_2 were determined from a titration with 0.0085 N potassium permanganate (standardized against sodium oxalate) as described by Skoog and West [13]. To assess the formation of hydroxyl radicals by DMSO oxidation, solutions of 0.5 to 1.5 mM H_2O_2 plus 700 mM DMSO were irradiated with the ultraviolet source for periods of 0 to 2 min, and 2-ml aliquots were assayed for methane sulfinic acid. The irradiation period in the presence of DMSO was limited to a maximum of 2 min, because significant degradation of standard methane sulfinic acid was observed with ultraviolet exposure times greater than 2 min. Therefore, to compare calculated versus observed HO formation, we determined the initial rate of H_2O_2 disappearance and the initial rate of MSA formation by extrapolation.

Initial rate of H_2O_2 photolysis

The photolysis of hydrogen peroxide can be described by the equation

$(dh/dt) = -k_3h,$

where $h = [H_2O_2]$, t = time, and k_3 = rate constant for H_2O_2 photolysis. Upon integration

$\ln(h) = \ln(h_0) + k_3t$,

where the subscript 0 indicates time zero, and the slope of the plot of the natural logarithm of hydrogen peroxide remaining, determined from the permanganate titration in the absence of DMSO, is a measure of k_3. The initial rate of photolysis of H_2O_2, $(dh/dt)_0$, is $-k_3h_0$, and in turn, the expected initial rate of HO formation from ultraviolet photolysis is $2k_3h_0$.

Initial rate of appearance of MSA

Assuming the concentration of hydrogen peroxide remains nearly constant during initial ultraviolet irradiation, the rate of production of methane sulfinic acid may be expressed as

$$\frac{dy}{dt} = k_1 - k_2 y ,$$

where y = [MSA], t = time, k_1 = rate of DMSO oxidation by HO, and k_2 = rate of MSA degradation by ultraviolet light. The solution of this differential equation is

$$y = \frac{k_1}{k_2}\left(1 - e^{-k_2 t}\right),$$

and in turn, the rate of MSA accumulation versus time may be written as

$$\frac{dy}{dt} = k_1 e^{-k_2 t} \quad \text{or} \quad \ln(dy/dy) = \ln(k_1) - k_2 t .$$

To determine k_1 and k_2 experimentally, one may plot the natural logarithm of the measured slope of cumulative MSA production versus time, which is a linear function, having a vertical axis intercept at t = 0 equal to $\ln(k_1)$ and a slope equal to $-k_2$. In particular, we were interested in the initial rate of methane sulfinic acid formation, $(dy/dt)_0 = k_1$, which we determined from linear regression analysis of the semilog plot.

Methane sulfinate assay

Solutions containing methane sulfinic acid (pK_a = 2) [14, 15] were adjusted to pH 2.5 with 0.1 N sulfuric acid. Color development was then caused by the addition of 0.2 ml of 15 mM diazonium salt, Fast Blue BB salt, to form a diazosulfone,

$$CH_3SOOH + Ar{-}N{=}N^+ \rightarrow Ar{-}NN{-}SO_2{-}CH_3 + H^+,$$

as previously described [2, 4].

Results

Visible absorbance spectra for diazosulfones derived from the radiolysis of water, the ultraviolet photolysis of hydrogen peroxide, and the Fenton reaction are shown in Fig. 1. A spectrum of 50 μM standard sulfinate solution (upper curve) is also shown for comparison. The visible absorbance spectra of the MSA assay from all three studies are virtually identical in shape to the spectrum of authentic methane sulfinic acid standard. Since all compounds presently known to intefere with this assay have absorption maxima at shorter wavelengths [4, 16], the spectral

results are consistent with the interpretation that methane sulfinate was indeed produced from DMSO in the three known HO-generating systems.

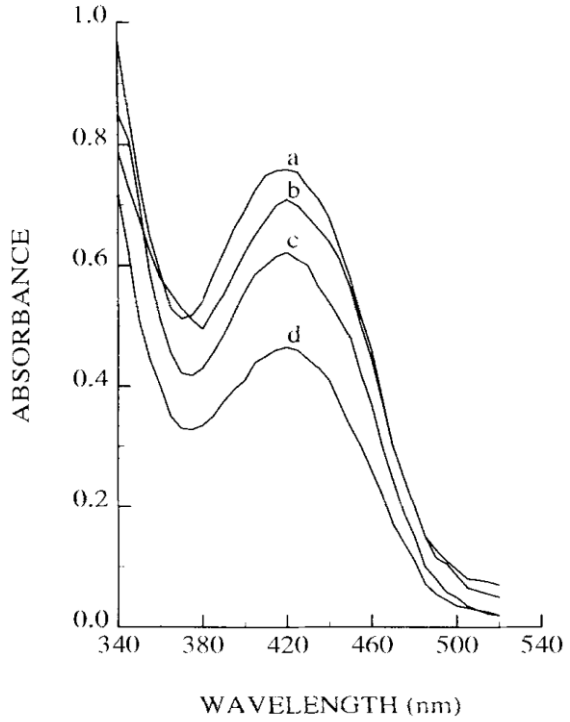

FIG. 1. Visible absorbance spectra for colored MSA reaction product derived from radiolysis of water (curve b), from the Fenton reaction (curve c), and from ultraviolet photolysis of hydrogen peroxide (lower curve d). A spectrum of 50 μM standard methane sulfinic acid (upper curve a) is provided for reference.

Table 1 summarizes the cumulative MSA recovered from four trials of the Fenton reaction and from four trials of γ irradiated DMSO solutions, compared to the calculated cumulative generation of hydroxyl radicals. In the absence of competing scavengers, methane sulfinic acid was the major product of the DMSO trapping reaction in both of these simple radical generating systems.

TABLE 1
MSA Generated by the Fenton Reaction and y Irradiation of Water

Source	Methane sulfinic acid (μM)	
	Calculated	Found
Fenton reaction	50	41 ± 1
6-min irradiation	49	54 ± 0.6
12-min irradiation	98	85 ± 4

During the photolysis of hydrogen peroxide, the ultraviolet-dependent disappearance of hydrogen peroxide, measured by $KMnO_4$ titration, was a decaying exponential function of time, and the ultraviolet-dependent production of methane sulfinic acid was a rising exponential function of time for periods ranging from 0 to 15 min. The initial rate of HO formation was determined from regression analysis of $KMnO_4$ titration of H_2O_2 remaining after various periods of ultraviolet exposure:

$\ln[H_2O_2]$ (mM) = 0.0452 - 397 × 10^{-6} t(sec) (n = 6, r = -0.996).

In turn, the expected initial rate of HO formation, twice the initial rate of H_2O_2 disappearance, was –2 (slope) exp (intercept) or 0.79 μM/S. The measured initial rate of MSA formation, determined by the calorimetric assay after various irradiation times, revealed that

In[MSA formation] (μM/sec) = –0.371 – 1.28 × 10^{-3} t (sec).

Therefore, the initial rate of MSA formation was exp(–0.371) = 0.69 μM/sec.

The ratio of measured MSA formation to the theoretical HO generation (0.69/0.79) during ultraviolet photolysis of hydrogen peroxide again suggested that methane sulfinic acid is the primary product of DMSO trapping of hydroxyl radicals. More generally, we found in all three systems that the measured yield of methane sulfinic acid was approximately equal to the expected yield, supporting the assertion that DMSO traps hydroxyl radicals, forming methane sulfinic acid as the major product.

Discussion

Highly reactive radical species like the hydroxyl radical may be quite difficult to detect, due to their extremely short lifetimes and extremely low steady-state concentration [10]. One effective approach, demonstrated in the present paper, is to "trap" HO radicals by allowing them to react with preexisting DMSO to form a stable, covalently bonded compound, the accumulation of which is related to the time integral of the rate of HO generation. To validate this approach, we created known amounts of HO in three simple systems, in which the expected HO generation can be calculated or measured independently. The results in all three systems demonstrate that DMSO is able to trap almost all of the nascent HO as measurable methane sulfinic acid.

Quantitation of hydroxyl radical by DMSO oxidation may be especially promising for use with biological systems, because DMSO is relatively nontoxic, well tolerated by living systems in up to 1 M concentrations [6-8], and rapidly absorbed and distributed to all tissue compartments [17]. Some enzymes, including Na^+/K^+-ATPase, are reversibly inhibited by DMSO [18]; however, there is little evidence for much effect in concentrations below about 5% (0.7 M). A further advantage in using DMSO to trap hydroxyl radicals in biological systems is that one can estimate actual numbers of HO radicals produced, as opposed to simply detecting the presence of HO radicals without being able to specify the total number generated.

Hydroxyl radicals are so reactive that they readily combine with any molecules in the vicinity at frequencies within one or two orders of magnitude of their collision frequency [19]. Unless the molecular probe is present in high concentration, all but a tiny percentage of HO radicals will react with neighboring biomolecules, rather than with the introduced molecular probe. In complex systems, therefore, one has no way of knowing what fraction of the HO radicals was actually trapped, or in turn the total number of HO radicals produced. Thus, to quantify the number of HO radicals created, the concentration of the trapping agent must be relatively high, in practice about 0.1 M or greater [2, 20, 21] so that the probability that an HO will react with the trapping agent, rather than a host biomolecule, is large. The low toxicity of DMSO makes such experiments possible [1, 16, 22]. In the present study, DMSO concentrations ranging from 0.05 to 1 M were employed in the absence of competing scavengers, and so the probability of quantifying the number of HO radicals created was essentially unity. However, Griffin and co-workers have shown similar trapping efficiency can be approached in physiologic systems, as well as in the presence of up to molar concentrations of competing scavengers [2].

There is evidence that sulfinic acids are not degraded by biologic systems (rat liver) [4], and so would accumulate sufficiently to be detected. The accumulated methane sulfinic acid ($pK_a \sim 2$) can be separated from biological material by acid butanol extraction, followed by back extraction into pH 4 buffer [4], and then submitted to the calorimetric assay herein described, which has been shown to give similar results in the presence and absence of rat liver homogenate [4]. An alternative extraction procedure has been described [5] and shown to give satisfactory results for plant material [22].

Previously Cohen [3] had proposed measuring formaldehyde as a marker for hydroxyl radical reaction with DMSO. He suggested that formaldehyde (HCHO) was formed by a Russell reaction mechanism [23]

$$DMSO + HO^{\bullet} \rightarrow CH_3SOOH + CH_3^{\bullet}$$

$$CH_3^{\bullet} + O_2 \rightarrow CH_3OO^{\bullet}$$

$$2CH_3OO^{\bullet} \rightarrow HCHO + CH_3OH + O_2,$$

and was successful in detecting formaldehyde production in vitro when DMSO was exposed to hydroxyl radical-generating systems [3]. However, the yield of formaldehyde molecules is small, because the Russell reaction requires the encounter of two CH_3OO radicals (a relatively unlikely termination reaction). Since CH_3OO 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 in biological systems, making the Russell reaction a relatively inefficient indicator of HO trapping by DMSO. The present approach provides an extension of Cohen's method, in which a primary product of the trapping reaction (CH_3SOOH) is detected.

Repine and co-workers [24] 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 DMSO + HO $\rightarrow$ $CH_3SOOH + CH_3$ was followed by $CH_3 + RH \rightarrow CH_4 + R$. 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 $CH_3 + O_2 \rightarrow CH_3OO$ and $CH_3OO + RH \rightarrow CH_3OOH + R$, followed by reduction of the methyl hydroperoxide to methanol. Thus, there is at least a theoretical advantage to methane sulfinic acid production, compared to methane production as an indicator of total hydroxyl radical entrapment by DMSO.

Practical applications of the calorimetric assay of methane sulfinic acid as an indicator of hydroxyl radical generation in DMSO pretreated biological systems, including ischemic and reoxygenated rat kidney [1, 16] and paraquat poisoned plants [22], have been described. The demonstration in the present paper that this approach can recover a high percentage of HO radicals in known hydroxyl radical-generating systems may provide further support for the use of this relatively simple and inexpensive method. Although the possibility exists that DMSO may be oxidized to methane sulfinic acid by strong oxidants other than the hydroxyl radical, in our view such oxidants are unlikely to be present in many systems of interest. Hydrogen peroxide alone, for example, does not oxidize DMSO to methane sulfinic acid; samples of 1 M hydrogen peroxide and 0.7 M DMSO produce no color development in the calorimetric assay for methane sulfinic acid (unpublished observation).

Moreover, we find it aesthetically appealing that when HO indeed attacks DMSO, one of the oxygen atoms in each measured molecule of methane sulfinic acid is the same oxygen atom as that in the original trapped hydroxyl radical. For these reasons we suggest that the DMSO trapping approach and measurement of the sulfinic acid product may provide a convenient and inexpensive means to capture and count nascent hydroxyl radicals in a wide variety of practical experiments.

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