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EPR Spin Trapping Study of the Decomposition of Azo Compounds in Aqueous Solutions by Ultrasound: Potential for Use as Sonodynamic Sensitizers for Cell Killing

VLADIMÍR MIŠÍK[1,a], NORIO MIYOSHI[2] and PETER RIESZ[1]

[1]*Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, MD 20892, USA;* [2]*Department of Pathology, Fukui Medical School, Matsuoka, Yoshida-gun, Fukui, Japan*

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Sonodynamic therapy, a promising new approach to cancer treatment, is based on synergistic cell killing by combination of certain drugs (sonosensitizers) and ultrasound. Although the mechanism of sonodynamic action is not understood, the role of free radicals produced from sonosensitizers by ultrasound is implicated. In this work, we studied formation of free radicals during the decomposition of several water-soluble azo compounds by 50 kHz ultrasound in aqueous solutions. Using the spin trap 3,5-dibromo-4-nitrosobenzene sulfonate (DBNBS) tertiary carbon-centered radicals from 2,2'-azobis (N,N'-dimethyleneisobutyramidine) dihydrochloride (VA-044), 2-(carbamoylazo)-isobutyronitrile (V-30), and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and $^{\bullet}CH_3$ radicals from 1,1'-azobis (N,N'-dimethylformamide) (ADMF) were detected in argon-saturated solutions and the corresponding oxygen-centered radicals (alkoxyl and peroxyl) from VA-044, V-30, and AAPH were identified using the spin trap 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) in aerated sonicated solutions. No free radicals from 4,4'-dihydroxyazobenzene-3,3'-dicarboxylic acid, disodium salt (DHAB) could be found in either system. While VA-044 and AAPH could also be readily decomposed by heat (42.5°C and 80°C), V-30 decomposition only occurred in the ultrasound-exposed solutions. The most likely mechanism of decomposition of azo compounds by ultrasound is their thermolysis in the heated shell of the liquid surrounding cavitating bubbles driven by ultrasound and/or by pyrolysis inside these bubbles. Experiments using scavengers of $^{\bullet}OH$ and $^{\bullet}H$, which are produced by sonolysis in aqueous solutions, demonstrated that these radicals are not involved in the ultrasound-mediated radical production from the azo compounds. Due to the known cytotoxic potential of free radicals produced from azo compounds, the use of these compounds as ultrasound sensitizers appears to be a promising approach for sonodynamic cell killing.

Key words: free radicals, azo compounds, ultrasound, sonodynamic therapy, sonosensitization, EPR

Correspondence: Peter Riesz, National Cancer Institute, NIH, Bldg. 10, Room B3B69, Bethesda, MD 20892–1002, USA. Phone: (301)496-4036, fax: (301)480-2238, E-mail: sono@helix.nih. gov

[a]VM is a Fogarty Visiting Fellow on leave from the Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovak Republic.

INTRODUCTION

Synergistic cell killing by a combination of ultrasound and certain drugs (sonosensitizers) is a promising new approach for cancer treatment. Several classes of compounds as diverse as porphyrins,[1,2,3,4] known chemotherapeutic agents (e.g. adriamycin and diaziquone),[5,6,7,8,9] and polar organic solvents such as dimethyl formamide (DMF), methyl formamide (MMF), and dimethylsulfoxide (DMSO)[10] were shown to be useful sonosensitizers. While the requirement of acoustic cavitation (this term refers to the ultrasound-driven growth of microbubbles from tiny gas pockets present in the solution, and their subsequent violent collapse which produces locally extreme temperatures and pressures inside these collapsing bubbles) for the observed synergistic effect has not been demonstrated for all of the drugs, at least in some instances[1–3,10] ultrasonic cavitation seems to be required for the sonodynamic effect. Although currently the mechanism of sonosensitization is not understood, it is likely that reactive radical intermediates formed from these compounds by ultrasound (either as a result of a direct pyrolysis in the hot cavitation bubbles or after reaction with the $^{\bullet}OH$ radicals and $^{\bullet}H$ atoms which are produced by sonolysis of water) are involved in cell killing: formation of peroxyl radicals from DMF, MMF, and DMSO was demonstrated recently in highly diluted air-saturated aqueous solutions of these compounds exposed to 50 kHz ultrasound.[11] Due to the therapeutic potential of sonodynamic therapy for cancer treatment it is of interest to investigate other classes of compounds capable of ultrasound-induced formation of reactive intermediates capable of cell killing.

The chemistry of azo compounds has been studied extensively in the past 20 years (for review see Refs.[12] and [13]). Azo compounds have also been a subject of significant interest for the biochemical community studying effects of free radicals in living systems, due to their ability to decompose both thermally and photochemically in a controlled fashion to yield carbon-centered radicals [Eq.1], which in the presence of oxygen are converted to peroxyl radicals [Eq.2] capable of initiating peroxidation of lipids or of damaging other biologically important cellular sites.[14]

$$R_1{-}N{=}N{-}R_2 \rightarrow {}^{\bullet}R_1 + {}^{\bullet}R_2 + N_2 \qquad [1]$$

$${}^{\bullet}R_{1(2)} + O_2 \rightarrow {}^{\bullet}OOR_{1(2)} \qquad [2]$$

Recently the potential use of an azo compound AAPH in hyperthermia sensitization has been investigated:[15] it was demonstrated that 50 mM AAPH was not cytotoxic to V79 cells at 37°C for exposures up to 3 hours. However, cell survival was reduced markedly when the cells were incubated with 50 mM AAPH at 42°C, while hyperthermia (42°C) alone had only a minor cytotoxic effect. The authors concluded that AAPH may be a useful heat sensitizer and has potential in local hyperthermia treatment.

Ultrasound is one of the methods of producing locally intensive hyperthermia.[16] The heating effect of ultrasound in tissue is due to ultrasound absorption and energy dissipation in tissues[17,18] and is independent of cavitation produced by high intensity ultrasound. Cavitation, which refers to the growth and the violent collapse of gas microbubbles driven by the pressure changes in the sound field, is responsible for the chemical effects of ultrasound and its presence seems to be required for sonodynamic activation of sonosensitizers. In the present work we compared the ability of several water-soluble azo compounds to decompose in aqueous solutions exposed to cavitation-producing levels of 50 kHz ultrasound with the efficiencies of their thermal decomposition.

MATERIALS AND METHODS

Chemicals

The water-soluble azo compounds (Figure 1) 2,2′-azobis (2-amidinopropane) dihydrochloride

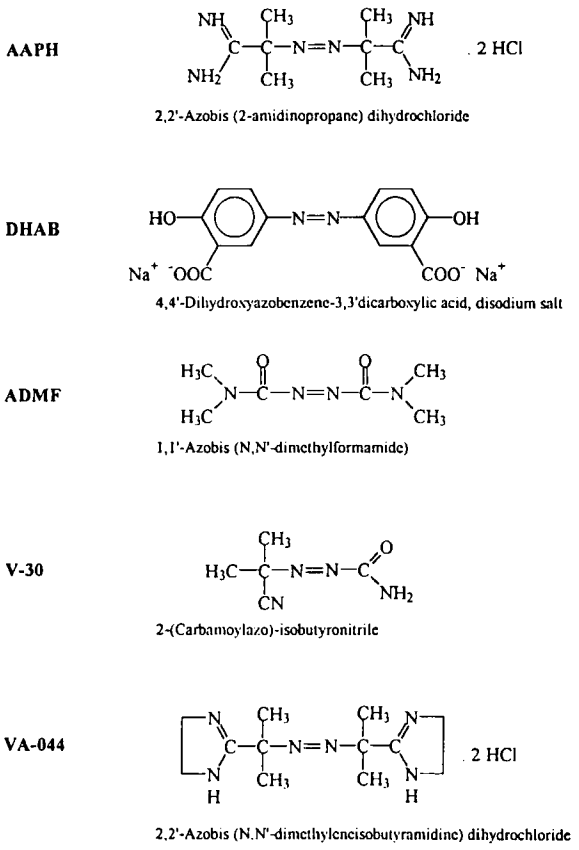

FIGURE 1 Structures of the azo compounds.

(AAPH), 2-(carbamoylazo)-isobutyronitrile (V-30), and 2,2'-azobis (N,N'-dimethyleneisobutyramidine) dihydrochloride (VA-044), were a generous gift by Wako Chemical USA, Inc., Richmond, VA. The other water-soluble azo compounds, 4,4'-dihydroxyazobenzene-3,3'-dicarboxylic acid, disodium salt and 1,1'-azobis (N,N'-dimethylformamide) were obtained from Aldrich (Milwaukee, WI). The spin traps 3,5-dibromo-4-nitrosobenzene sulfonic acid, sodium salt (DBNBS) and 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) were acquired from Sigma (St. Louis, MO). All other chemicals were from commercial sources and of the highest purity available. MilliQ water was used in all experiments.

Sonolysis experiment

Experiments were performed using a 50 kHz bath sonicator (Bransonic) as described previously.[11] Briefly, aqueous samples (1.7 ml) containing 10 mM azo compounds and either 8.2 mM DBNBS or 50 mM DMPO were placed in Pyrex 13 × 100 mm disposable tubes (Corning Inc., New York) fixed in the center of a sonication bath (Bransonic 1200) with ultrasound frequency of 50 kHz. The temperature of the coupling water was 20°C. The samples were sealed with a rubber septum and bubbled with air, or argon through a Teflon tube attached to a fine needle (the argon flow rate was 50 ml/min). After three minutes of ultrasound exposure the samples were transferred into a quartz EPR flat cell and the scan of the first EPR spectrum was started 1 minute after the end of ultrasound exposure.

Thermolysis experiment

Samples containing 10 mM azo compounds and either 8.2 mM DBNBS or 50 mM DMPO were pre-bubbled with gas (air or argon) sealed with parafilm and placed in the incubation bath set at 42.5°C or 80°C. At the end of incubation, samples were briefly cooled on ice and transferred into the flat cell for EPR measurement.

EPR measurement and spectral simulation

A Varian E-9 spectrometer with 100-kHz modulation frequency and a microwave power of 20 mW operating in X-band mode was used to record the spectra for both sonolysis and thermolysis experiments. The EPR software EPRDAP, written by Dr. P. Kuppusamy (U.S. EPR, Inc., Clarksville, MD), was used for acquisition, analysis and simulation of EPR data. All reported hyperfine splitting constants were obtained by successful computer simulations of the EPR spectra; simulations of mixed-component spectra were confirmed by the auto-simulation routine of the EPRDAP program. The yields of the spin trapped radicals were quantified by comparing the double

integrals of the simulated spectra with the double integral of the spectrum of a known concentration of the stable radical 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TEMPONE) obtained under identical experimental conditions.

RESULTS AND DISCUSSION

Results of EPR measurements in air-saturated samples containing 10 mM azo compounds and 50 mM spin trap DMPO exposed to 3 minutes of ultrasound are shown in Figure 2. No radical species originating from the azo compounds were detected for DHAB and ADMF – the EPR spectra C and D shown in Figure 2 are the result of $^{\bullet}OH$ radical trapping by DMPO (DMPO/$^{\bullet}OH$; hyperfine coupling constants $a_N = a_H = 14.9$ G) and their

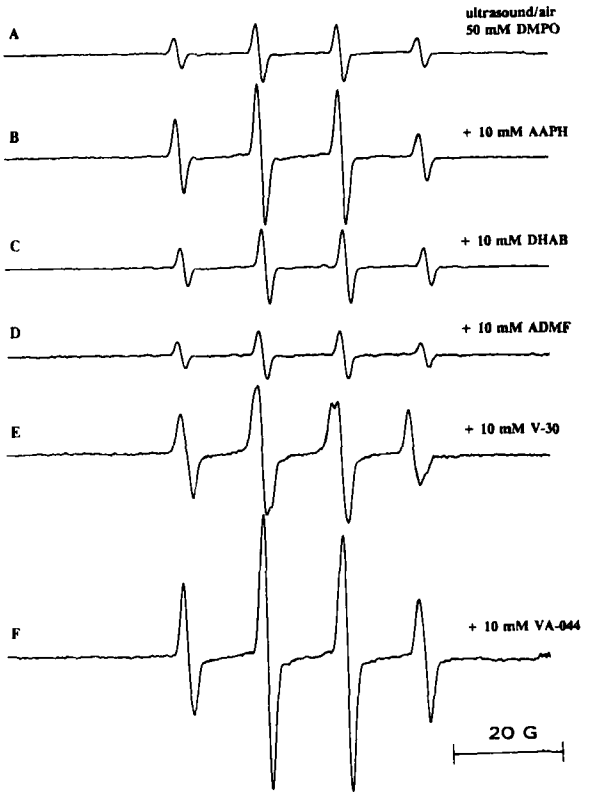

FIGURE 2 EPR spectra of DMPO adducts formed in air-saturated aqueous solutions containing 10 mM azo compounds and 50 mM DMPO exposed to 3 minutes of ultrasound. The instrumental settings were: modulation amplitude 1.25 G, microwave power 20 mW, scan rate 0.42 G/sec, time constant 0.250 sec.

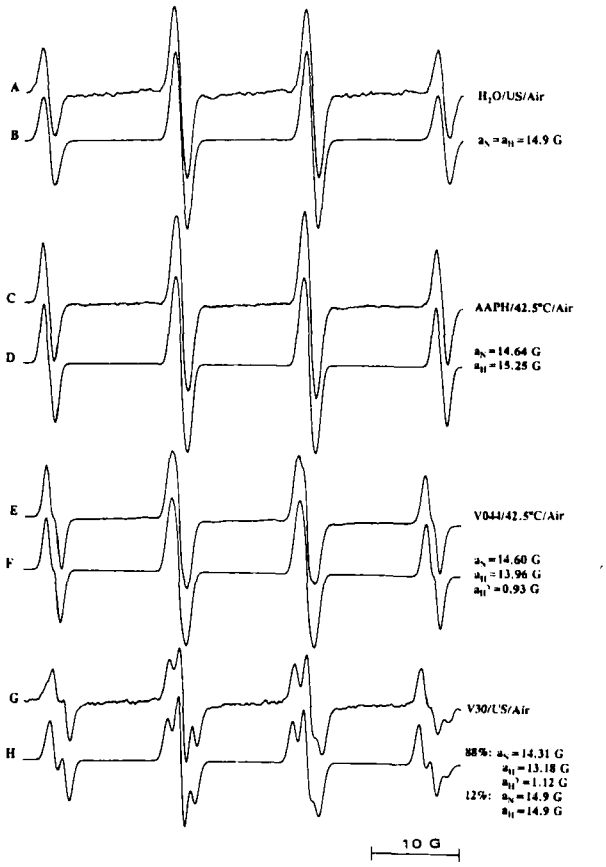

FIGURE 3 EPR spectra of DMPO adducts, along with their computer simulations (the splitting constants used in simulations are listed next to the corresponding spectra), formed in air-saturated aqueous solutions containing 10 mM azo compounds and 50 mM DMPO. A – control, no azo compounds present, 3 minutes of ultrasound; B – simulation of A; C – 10 mM AAPH exposed to 10 minutes of 42.5°C; D – simulation of C; E – 10 mM VA-044 exposed to 10 minutes of 42.5°C; F – simulation of E; G – 10 mM V-30 exposed to 3 minutes of ultrasound, H – simulation of G: the overlapping component (12%) with hyperfine splittings $a_N = a_H = 14.9$ G is the signal of the DMPO/$^{\bullet}OH$ adduct. The experimental spectra were obtained using 0.5 G modulation amplitude. Note that for a better resolution the spectra width is 50 G compared to 100 G used in Figure 2.

yields were not significantly different from controls containing no azo compound (spectrum A). New signals typical of oxygen-centered adducts of DMPO were detected for AAPH, V-30 and VA-044. Figure 3 shows a detailed analysis of the spectra of the radical adducts derived from AAPH, VA-044, and V-30. Because of the similar hyperfine splitting constants of DMPO/$^{\bullet}OH$ and the spin adducts formed from AAPH and VA-044

TABLE 1 DMPO spin adducts from azo compounds exposed to ultrasound or heat in air-saturated aqueous solutions.

		Hyperfine coupling constants (G)			
Azo compound	Experim. conditions	a_N	a_H	a_H^γ	Assignment
AAPH	80°C, air	15.42	25.4	–	DMPO/$^\bullet$C$(CH_3)_2$C(NH)NH_2
	US[a]/42.5°C/80°C, air	14.64	15.25	–	DMPO/$^\bullet$OC$(CH_3)_2$C(NH)NH_2
DHAB	US/42.5°C/80°C, air	–	–	–	no detectable signals
ADMF	80°C/(US)[b], air	15.63	19.53	–	DMPO/$^\bullet$C(O)N$(CH_3)_2$
V-30	US, air	14.31	13.18	1.12	DMPO/$^\bullet$OOC(CN)$(CH_3)_2$(?)
VA-044	80°C, air	15.57	24.92	–	DMPO/$^\bullet$C$(CH_3)_2$C[N$(CH_2)_2$NH]
	US/42.5°C/80°C, air	14.6	13.96	0.93	DMPO/$^\bullet$OOC$(CH_3)_2$C[N$(CH_2)_2$NH] (?)

[a]US = ultrasound
[b]Only traces of this signal detected by ultrasound.

and due to the overlap of these signals with the signal of DMPO/$^\bullet$OH in the sonolysis experiments (~30% of DMPO/$^\bullet$OH in AAPH and ~10% DMPO/$^\bullet$OH in VA-044; Figure 2) the accurate splitting constants of these adducts were determined from the spectra of these radical adducts in pure form, obtained by thermal decomposition of AAPH and VA-044 (Figure 3C and 3E). In the case of V-30 the splitting constants were determined from the spectra obtained by sonolysis (Figure 3G), because of the insufficient rate of thermal decomposition of this compound (Table 3). The EPR splitting constants of these new DMPO adducts obtained by spectral simulation are listed in Table 1 along with their tentative assignments, based on both literature data[19] and on subsequent experiments with another spin trap DBNBS (shown below). As seen from the Table 1 adducts with hyperfine splittings in a range typical of alkoxyl radical adducts in aqueous solutions (a_N = 14.64 G, a_H = 15.25 G) were detected in solutions containing AAPH. The lower value of a_H than usually found in aqueous spin trapping of alkoxyl radicals (typically in a range of 15.7–16.8 G)[19] is most likely due to the presence of the electron withdrawing substituent (-C(NH)NH_2) on the alkoxyl radical derived from AAPH.

Radical formation by thermolysis and photolysis of azo compound azobis (isobutyronitrile) (AIBN) in toluene, benzene and xylene in the presence of oxygen was studied in great detail by Janzen and co-workers.[20] Using EPR spectroscopy and the spin traps PBN (α-phenyl-N-*tert*-butyl nitrone) and DMPO and these authors detected

TABLE 2 DBNBS spin adducts from azo compounds exposed to ultrasound or heat in argon-saturated aqueous solutions.

		Hyperfine coupling constants (G)			
Azo compound	Experim. conditions	a_N	a_H	a_H^m	Assignment
AAPH	US[a]/80°C, argon	13.05	–	–	DBNBS/$^\bullet$C$(CH_3)_2$C(NH)NH_2
DHAB	US/80°C, argon	–	–	–	no detectable signals
ADMF	US, argon	14.4	13.5(3)	0.78(2)	DBNBS/$^\bullet CH_3$
V-30	US/80°C, argon	13.33	–	–	DBNBS/$^\bullet$C(CN)$(CH_3)_2$
VA-044	US/80°C, argon	13.05	–	–	DBNBS/$^\bullet$C$(CH_3)_2$C[N$(CH_2)_2$NH]

[a]US = ultrasound

the formation of 2-cyano-2-propyl radicals (CP$^{\bullet}$) and 2-cyano-2-propyloxyl radicals (CPO$^{\bullet}$) depending on the concentration of oxygen. Formation of 2-cyano-2-propylperoxyl radicals (CPOO$^{\bullet}$), which should be the major product in the presence of oxygen according to the mechanism shown in Equations 1 and 2, could only be detected with PBN when partially oxygen depleted solutions of AIBN were photolyzed in toluene at low temperatures (205 K). At higher temperatures (>230 K) the EPR spectrum of the peroxyl radical adduct disappeared and only alkoxyl radical adducts of PBN and 2-methyl-2-nitrosopropane, produced by the decomposition of PBN/peroxyl adduct, could be detected. At temperatures above 270 K only the signal of PBN alkoxyl adduct was detectable.[20] Using NMR and mass spectroscopy these authors demonstrated formation of non-paramagnetic mixed double adducts with PBN and DMPO (CPO-PBN-CP and CPO-DMPO-CP) formed by the addition of alkyl radicals to the nitroxide function (10^8–10^9 M^{-1} s^{-1}) of alkoxyl adducts to produce alkoxylamines.[20]

Thus, alkoxyl radical adducts detected in our system in AAPH containing solutions could be formed either by decomposition (rearrangement) of DMPO/peroxyl radical adducts or by direct trapping of alkoxyl radicals formed by the reactions of peroxyl radicals [Eq.3]:

$$2\,ROO^{\bullet} \rightarrow 2\,RO^{\bullet} + O_2 \qquad [3]$$

On the other hand, the values of hyperfine splittings obtained when V-30 and VA-044 were decomposed by ultrasound or by heat in our system (Table 1) are more typical of peroxyl radical adducts than of alkoxyl radical adducts (a_N/a_H>1; resolved γ-hydrogen splittings) but the β-hydrogen splittings for these adducts are 1–2 G higher than the reported values of other peroxyl radical adducts of DMPO and are also more stable ($t_{1/2}$>10 minutes) than would be expected for peroxyl radical adducts.[19] Therefore, the assignment of these adducts is not unequivocal.

Concentrations of the spin trapped radicals are shown in Figure 4A. Data in Figure 4A were corrected for the thermal decomposition of azo compounds in the bulk solution, determined by placing the samples for 3 minutes in a bath at 21°C (the temperature of the coupling water in the ultrasound bath increased from 20°C to 21°C during 3 minutes of sonolysis). While AAPH and VA-044 were also the most potent thermally-active compounds (Figure 4B and 3C) V-30 did not show any appreciable rate of thermal decomposition at either 42.5°C or 80°C (Figure 4B and 4C). As seen from Figure 4C carbon-centered radicals from AAPH and VA-044 were also spin trapped at 80°C along with the oxygen-centered radicals, which is a result of a limited oxygen availability

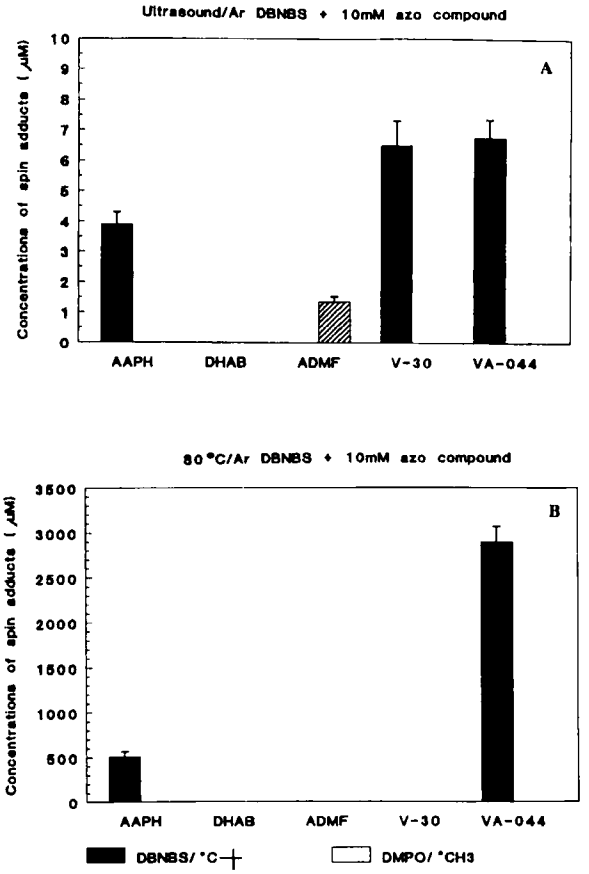

FIGURE 4 The yields of DMPO spin adducts formed in air-saturated aqueous solutions containing 10 mM azo compounds and 50 mM DMPO exposed to 3 minutes of ultrasound (A), 10 minutes of incubation at 42.5°C (B), or 5 minutes of incubation at 80°C (C).

TABLE 3 Thermal stabilities of azo compounds

Compound	$t_{1/2}$(80°C)[a] (minutes)	10 hour halflife decomposition temperature[b]
AAPH	33	56°C water
DHAB	>>10 000[c]	–
ADMF	150	–
V-30	180	104°C toluene
VA-044	13	44°C water

[a]Determined in this work from the disappearance of the optical maxima in aqueous solutions of azo compounds.
[b]Data from Wako catalogue.
[c]Less than 3% decomposed after 24 hours of incubation at 80°C.

in this system. The acyl-radical type adducts [DMPO/$^{\bullet}C(O)N(CH_3)_2$] originating from the thermal decomposition of ADMF could be spin trapped at 80°C (Table 1). No radicals from DHAB could be spin trapped at either temperature. The observed radical yields from thermolysis of azo compounds reflects the thermal stability of these compounds (Table 3). The rate of thermal decomposition of azo compounds increases with increasing stability of the radicals produced and with increasing strain of the azo molecule.[12]

Qualitatively similar results were obtained when sonolysis of 10 mM solutions of azo compounds was performed in the absence of oxygen, in argon-saturated solutions, where carbon-centered radicals are the dominant azo-derived radical species. Therefore, the spin trap DBNBS, which is useful for determination of carbon-centered radicals, was used in these experiments. EPR spectra obtained in this system are shown in Figure 5. Again no radicals from DHAB could be spin trapped but low levels of $^{\bullet}CH_3$ radicals were produced from ADMF. These radicals were not formed according to the mechanism shown in Equation 1 but as a result of the pyrolysis of the C–N bond in the molecule of ADMF:

$$(CH_3)_2N(O)C{-}N{=}N{-}C(O)N(CH_3)_2 \rightarrow (CH_3)_2N(O)C{-}N{=}N{-}C(O)N^{\bullet}CH_3 + {}^{\bullet}CH_3 \quad [4]$$

The most likely reason for the absence of detectable levels of $CH_3O^{\bullet}$ or $CH_3OO^{\bullet}$ radicals (Figure 4A), which should be produced by ultrasound in oxygenated solutions from $^{\bullet}CH_3$ radicals, is that the pyrolysis mechanism (Equation 4) is less effective in aerated solutions due to the lower final temperature of the collapsing cavitation bubbles filled with diatomic gas (oxygen and nitrogen) compared with monoatomic gas (argon). This difference can be attributed to the difference of the ratio of the specific heats, $\gamma = C_p/C_v$, between the monoatomic argon ($\gamma_{Ar} = 1.67$)[21] and diatomic oxygen and nitrogen ($\gamma_{O_2} = 1.385$; $\gamma_{N_2} = 1.41$),[21] which affects the final temperature inside the collapsing cavitation bubbles.[22,23]

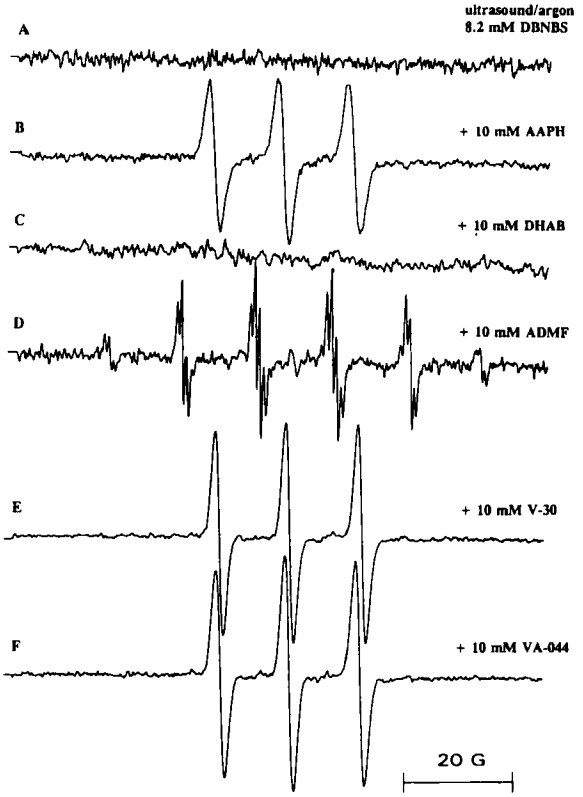

FIGURE 5 EPR spectra of DBNBS adducts formed in argon-saturated aqueous solutions containing 10 mM azo compounds and 8.2 mM DBNBS exposed to 3 minutes of ultrasound. The instrumental settings were: modulation amplitude 0.63 G, microwave power 20 mW, scan rate 0.42 G/sec, time constant 0.250 sec.

It is possible that $(CH_3)_2N(O)C^{\bullet}$ radicals were formed according to the mechanism shown in Equation 1, but did not form sufficiently long-lived

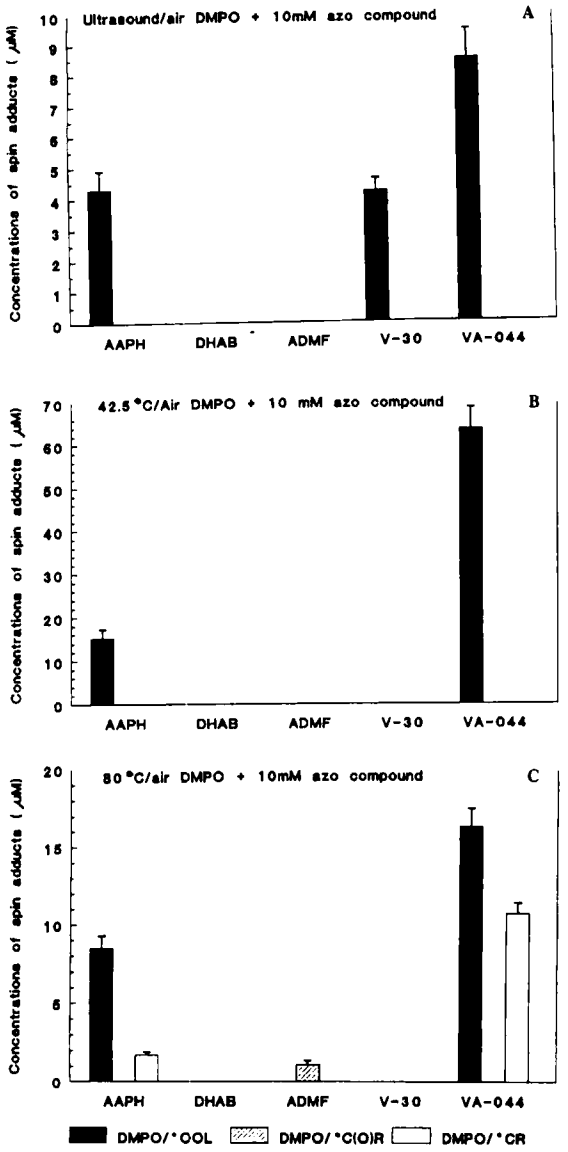

FIGURE 6 The yields of DBNBS spin adducts formed in argon-saturated aqueous solutions containing 10 mM azo compounds and 8.2 mM DBNBS exposed to 3 minutes of ultrasound (A), or 5 minutes of incubation at 80°C (B).

adducts with DBNBS. Indeed, under conditions where formation of carbonyl carbon-centered radicals from ADMF was demonstrated (thermal decomposition of ADMF at 80°C; Figure 4C) no DBNBS adducts could be detected (Figure 6B). To our knowledge, no carbonyl-carbon centered radical adducts of DBNBS have been reported.[19]

We conducted experiments to verify whether the decomposition of azo compounds by ultrasound can be mediated by the addition of $^{\bullet}OH$ radicals or $^{\bullet}H$ atoms followed by a β-cleavage [Equations 5,6]. Beta cleavage of H adducts of the azoalkane 2-(*tert*-butylazo)-2-(dimethylamino)-propane was proposed to lead to the formation of secondary azoalkane-derived radicals.[24]

$$R_1\text{–}N{=}N\text{–}R_2 + {}^{\bullet}OH \rightarrow [R_1\text{–}N^{\bullet}\text{–}N(OH)\text{–}R_2] \rightarrow R_{1(2)}{}^{\bullet} + ? \quad [5]$$

$$R_1\text{–}N{=}N\text{–}R_2 + {}^{\bullet}H \rightarrow [R_1\text{–}N^{\bullet}\text{–}N(H)\text{–}R_2] \rightarrow R_{1(2)}{}^{\bullet} + ? \quad [6]$$

Reaction of $^{\bullet}OH$ radicals with ADMF was investigated ($k_{OH + ADMF} = 6 \pm 2 \times 10^9\ M^{-1}s^{-1}$)[25,26] but the subsequent fate of the radical intermediate formed by this reaction was not studied. Table 4 shows that the presence of the $^{\bullet}OH$ and $^{\bullet}H$ scavengers potassium iodide ($k_{OH + I^-} = 1 \times 10^{10}\ M^{-1}\ s^{-1}$ (pH 7),[27] $k_{H + I^-} = 3.4 \times 10^7\ M^{-1}\ s^{-1}$ (pH 7)),[28] sodium azide ($k_{OH + N_3^-} = 1.2 \times 10^{10}\ M^{-1}\ s^{-1}$ (pH 7.9–13),[29] $k_{H + N_3^-} = 1.9 \times 10^9\ M^{-1}\ s^{-1}$ (pH 6.7)),[30] and sodium formate ($k_{OH + HCOO^-} = 3.2 \times 10^9\ M^{-1}\ s^{-1}$,[31] $k_{H + HCOO^-} = 2.1 \times 10^8\ M^{-1}\ s^{-1}$ (pH 7))[31] during sonication did not decrease the concentrations of spin trapped radicals formed from AAPH, V-30, and VA-044 under conditions where the radical scavengers should protect the azo compounds from $^{\bullet}H$ and $^{\bullet}OH$ radical attack (the concentration of azo compounds was 100 times less than that of the scavengers and the estimated rate constants with $^{\bullet}OH$ radicals and $^{\bullet}H$ atoms are within the same range as those of the scavengers (perhaps with the exception of the rate constant of iodide with $^{\bullet}H$ atoms): $k_{OH + azo}{}^{estim} \approx 10^9\ M^{-1}\ s^{-1}$; based on the known rate $k_{OH + ADMF} = 7 \times 10^9\ M^{-1}\ s^{-1}$;[25,26] $k_{H + azo}{}^{estim} \approx 10^8 - 10^9\ M^{-1}\ s^{-1}$, an estimate based on the rates of additions of $^{\bullet}H$ atoms to double bonds).[31] In fact, as seen from Table 4, addition of the $^{\bullet}OH$ and $^{\bullet}H$ scavengers increased the signal of spin trapped radicals in most cases, probably because of protection against destruction of the spin adducts by

TABLE 4 Effect of $^{\bullet}OH$ and $^{\bullet}H$ scavengers on the yields of DBNBS spin adducts of carbon-centered radicals produced by ultrasound from azo compounds.[a]

azo compound	control	KI	NaN_3	$HCOON_a$
AAPH	2.5[b]	3.5	3.4	2.7
V-30	6.5	6.8	5.9	2.9[c]
VA-044	5.9	23.1	12.2	18.9

[a]Samples containing 8.2 mM spin trap DBNBS, 10 mM azo compound, and 1 M scavenger were exposed to 3 minutes of 50 kHz ultrasound under argon.
[b]Data are expressed in μM of DBNBS spin adducts.
[c]The decay rate of the signal higher in the presence of HCOONa: $t_{1/2}$(control) = 10 minutes, $t_{1/2}$(HCOONa) = 3 minutes.

$^{\bullet}OH$ radicals and $^{\bullet}H$ atoms. Thus, these experiments verified that decomposition of azo compounds during ultrasound exposure was not mediated by $^{\bullet}OH$ radicals and $^{\bullet}H$ atoms produced by ultrasound. Therefore, the probable mechanism of the radical formation from the azo compounds is their thermal decomposition in the heated shell surrounding collapsing cavitation bubbles and/or pyrolysis inside the cavitation bubble. The radical formation cannot be ascribed to the small heating effect of the bulk solution during ultrasound exposure, which by itself was not sufficient to produce the observed levels of azo-derived radicals.

Staško *et al.*[32] reported formation of EPR-observable hydrazyl radicals (R_1 (R_2)N–N$^{\bullet}R_3$ – type) produced by the addition of $NaSO_3^{\bullet}$ (at 275 K in water) and $^{\bullet}H$ (at 320 K in ethanol) to the azo compound PhN = NSO_3Na during its *in situ* photochemical decomposition. When spin traps were omitted from our reaction mixture during sonolysis we were unable to detect formation of the radicals produced by addition of the azo compound-derived radicals to the double bond of the parent azo compound according to the mechanism shown in Equation 7,

$$^{\bullet}X + R_1\text{–}N\text{=}N\text{–}R_2 \rightarrow R_1\text{–}N^{\bullet}\text{–}N(X)R_2 \qquad [7]$$

probably because of the low stability of these types of adducts (the reported halflives of hydrazyl radicals produced by the radical addition to azosulfonates were in the range of seconds).[32]

Our data show that several water soluble azo compounds can be decomposed by ultrasound to their corresponding carbon-centered radicals and the decomposition is largely independent of the heating of the bulk liquid by ultrasound. If the sonolysis is performed in aerated solutions the carbon-centered radicals produced by sonolysis of azo compounds are converted to peroxyl and alkoxyl radicals capable of cell killing by damaging biologically important cellular sites. Thus, our results suggest the potential use of azo compounds as ultrasound sensitizers for cell killing in sonodynamic therapy and hyperthermia cancer treatment. A particularly interesting compound from the mechanistic point of view, for distinguishing between hyperthermia and cavitation-induced azo compound mediated cell killing, is V-30 which appears to be inert within the range of physiological temperatures but is readily decomposed by ultrasound with intensities above cavitation threshold.

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