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FREE RADICAL PRODUCTION BY CHLORPROMAZINE SULFOXIDE, AN ESR SPIN-TRAPPING AND FLASH PHOTOLYSIS STUDY

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Abstract—Using the spin-trapping technique we have investigated the photolysis of chlorpromazine sulfoxide and promazine sulfoxide. Photolysis of these sulfoxides in aqueous solution resulted in a species which is capable of oxidizing ascorbate, cysteine, glutathione, NADH, and azide by one electron, in addition to extracting hydrogen atoms from ethyl alcohol and dimethyl sulfoxide. These oxidations were not dependent on the presence of dissolved oxygen. The oxidizing species is proposed to be the hydroxyl free radical arising from the homolytic cleavage of the S–O bond of the sulfoxide. Flash photolysis of the chlorpromazine and promazine sulfoxides demonstrated the formation of cation radicals consistent with the loss of the hydroxyl radical from the sulfoxides. In addition we present a simple direct method for the quantitative synthesis of promazine and chlorpromazine sulfoxides from the parent promazine derivatives.

INTRODUCTION

Phenothiazine drugs are widely used in medicine because of their tranquilizing and antihistaminic properties. However, these drugs are sensitive to oxidation by chemical, photochemical and metabolic mechanisms. Numerous side effects following exposure to light include cutaneous photosensitization (Fitzpatrick *et al.*, 1963) and ocular complications (Zelickson and Zeller, 1964; Johnson and Buffaloe, 1966; Siddall, 1965; Clare *et al.*, 1947). The mechanisms for these side effects as well as for the therapeutic action of the phenothiazines are as yet not completely understood; however, free radicals appear to play a role in the observed photo-effects (Chignell *et al.*, 1985).

Oxidation of the phenothiazines produces 1, 2, and 4-electron oxidation products. For example with chlorpromazine (CPZ)[§]:

$$\text{CPZ} \rightarrow \text{CPZ}^{+\cdot} + e^- \quad (1)$$

$$\text{CPZ}^{+\cdot} + H_2O \rightarrow \text{CPZ-SO} + e^- + 2H^+ \quad (2)$$

$$\text{CPZ-SO} + H_2O \rightarrow \text{CPZ-SO}_2 + 2e^- + 2H^+ \quad (3)$$

The two-electron oxidation product is the phenothiazine sulfoxide, which can be obtained either by hydrolysis of the cation radical (Cheng *et al.*, 1978) or by direct reaction with molecular oxygen (Motten *et al.*, 1985).

In the eye, metabolically formed sulfoxide may itself be an active pharmacologic agent which is involved in lens damage (Clare *et al.*, 1947; Carr, 1968). After treatment with phenothiazine, phenothiazine sulfoxide appears in both the blood and aqueous humor of calves, but not in the corresponding fluids from sheep (Whitten and Filmer, 1947; Clare, 1947). A photosensitized keratitis occurs in calves but not in sheep dosed with the customary amount of phenothiazine. Furthermore, Clare *et al.* (1947) demonstrated that phenothiazine sulfoxide will produce keratitis when injected into the anterior chamber of the eyes of normal animals exposed to sunlight, with an action spectrum for the keratitis corresponding to the absorption spectrum of the phenothiazine sulfoxide.

Yeung *et al.* (1983) have reported that more than half of the chlorpromazine found in human plasma after a 50 mg oral dose is present as the sulfoxide. Even though this high level of CPZ-SO has been noted, very little attention has been paid to the possible photoeffects of the phenothiazine sulfoxides. We report here that the photolysis of chlorpromazine and promazine sulfoxides results in the formation of a highly oxidizing species capable of

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[§]*Abbreviations*: ACPZ, acepromazine; BSA, bovine serum albumin; CPZ, chlorpromazine; $CPZ^{+\cdot}$, chlorpromazine radical cation; CPZ-SO, chlorpromazine sulfoxide or chlorpromazine 5-oxide; $CPZ-SO_2$, chlorpromazine sulfone; CPZ-SO*(S), excited singlet chlorpromazine sulfoxide; CPZ-SO*(T), excited triplet state chlorpromazine sulfoxide; DMPO, 5,5-dimethyl pyrroline-N-oxide; DMSO, dimethyl sulfoxide; ESR, electron spin resonance; MTPZ, methoxypromazine; P$^{\cdot}$, 10-[3-(Dimethylamino)-propyl]-10H-phenothiazin-2-yl; PZ, promazine; $PZ^{+\cdot}$, promazine radical cation; PZ-SO, promazine sulfoxide or promazine 5-oxide; SOD, superoxide dismutase; TFPZ, triflupromazine.

oxidizing ascorbate, cysteine, glutathione, NADH, azide, DMSO and ethanol. We currently believe this oxidizing species is the hydroxyl free radical formed by the homolytic cleavage of the sulfur-oxygen double bond of the sulfoxide.

MATERIALS AND METHODS

Chlorpromazine (Smith, Kline and French Labs, Philadelphia, PA, Lot No. N-70-7) and promazine (Wyeth Labs, Philadelphia, PA) were used as received. Chlorpromazine sulfoxide standard was obtained from the Neurosciences Research Branch of the National Institute of Mental Health. Promazine sulfoxide standard was obtained from Smith, Kline and French Labs, Philadelphia, PA. The phenothiazine sulfoxides were prepared using a modification of the method outlined by Leonard and Johnson (1962) as follows. The phenothiazine was added to a slight molar excess of 0.5 *M* solution of sodium metaperiodate at ice temperature. The mixture was stirred overnight at 4°C. The reaction mixture was filtered to remove the salt and the filtrate was extracted with chloroform after making the solution basic (pH 12). The extract was washed twice with water and then the chloroform was removed under vacuum. Product confirmation was obtained using TLC (silica gel and methanol) and mass spectrometry and by comparison of UV and fluorescence spectra with the standards. No starting material remained and no evidence for sulfone, the four electron oxidation product, was observed. This method has the advantage that the phenothiazine is quantitatively converted to sulfoxide with no formation of the sulfone, which is often produced when other oxidizing agents are used.

ESR spin trapping studies were done on a Varian E-109 spectrometer equipped with a TM_{110} cavity. Samples were irradiated in the cavity at 330 nm with a Schoeffel 1000 W arc lamp and monochromator combination described elsewhere (Motten *et al.*, 1985). DMPO (Aldrich Chemical Co., Milwaukee, WI) was purified with charcoal (Buettner and Oberley, 1978) and was stored as an aqueous (1 *M*) frozen solution until use.

Laser flash photolysis experiments were performed at the Center for Fast Kinetic Research, Austin, TX, using an Nd : YAG Q switched laser with a 12 ns pulse. Excitation was accomplished with the 355 nm output, the pulse energy being 22 mJ to the sample. Conventional flash photolysis was performed with a PRA FP-1000 flash photolysis system.

RESULTS

Free radicals as detected by spin trapping

The photolysis of aqueous CPZ-SO or PZ-SO solutions in the presence of the spin trap DMPO resulted in the formation of the hydroxyl radical spin adduct of DMPO (DMPO/OH), Fig. 1A. When cysteine, glutathione, azide, ethanol or DMSO were included in the spin trapping mixture the DMPO/OH ESR signal was replaced by the DMPO/cysteinyl (Fig. 1B), glutathiyl (Fig. 1C), azidyl, α-hydroxyethyl, methyl or methylperoxy radical adduct (not shown) respectively. Figure 1 shows the DMPO/cysteinyl and glutathiyl adducts formed when these naturally occurring radical scavengers react with ·OH. Exactly parallel results (not shown) were obtained when CPZ-SO was replaced with PZ-SO. Ascorbate and NADH were also oxidized by both

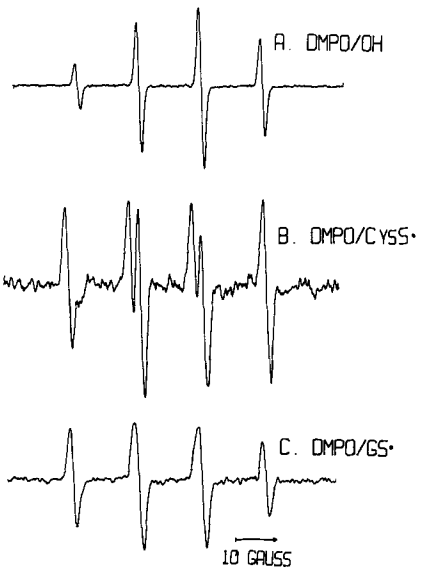


Figure 1. ESR spin trapping with CPZ-SO and DMPO. Photolysis of the samples began approximately 2 min before the first, i.e., low field, line was encountered. Spectrometer settings were: mod. amp., 1 G; scan 100 G/8 min; power, 10 mW. (A.) DMPO-OH spin adduct, a_N = a_H = 14.9 G (Buettner, 1982). The photolysis solution contained 250 μ*M* CPZ-SO, 5 m*M* DMPO in air-saturated pH 7 phosphate buffer. The identical spectrum was obtained in nitrogen-saturated solution with no change in intensity. If CPZ-SO was replaced by PZ-SO the same spectrum was obtained with a 1.8 increase in intensity. Spectrometer gain was 5×10^3, time constant 1/2 s. (B.) DMPO-cysteinyl free radical adduct, a_N = 15.3 G, a_H = 17.25 G(Saez *et al.*, 1982; Harman *et al.*, 1984; Buettner, 1984). The photolysis solution contained 250 μ*M* CPZ-SO, 10 m*M* DMPO and 30 m*M* cysteine. The same spin adduct was observed when CPZ-SO was replaced with PZ-SO. Gain = 5×10^4, time constant 1 s. (C.) DMPO-glutathiyl free radical adduct, a_N = 15.3 G, a_H = 16.2 G (Josephy *et al.*, 1984, report a_N = 14.9 G, a_H = 15.4 G in 3/1 H_2O/MeOH). All conditions were the same as in B above, except cysteine was replaced by glutathione at 30 m*M*. Gain = 2×10^4, time constant 1 s.

CPZ-SO and PZ-SO upon irradiation (data not shown).

The hydroxyl spin adduct of DMPO can arise from processes not involving the ·OH radical itself. To test for the possible participation of dissolved oxygen in the mechanism of DMPO/OH formation, the solution of Fig. 1A was bubbled with nitrogen gas. The photolysis of this deoxygenated solution resulted in the formation of the DMPO/OH spin adduct with no change in intensity from that of the air-saturated solution. Thus, dissolved oxygen appears to play no role in the formation of DMPO/OH. The intensity was also unchanged when SOD or catalase was added to the solution before irradiation, thus DMPO/OH is not formed from superoxide or from photolyzed H_2O_2.

The observation that the DMPO/OH spin adduct is replaced by the radical adducts of cysteine, glutathione, azide, ethanol and DMSO is consistent with the formation of ·OH from the photolysis of CPZ-SO and PZ-SO. Other radicals such as the phenyl-type

Table 1. Effect of ·OH scavengers on DMPO-OH ESR signal intensity. The aqueous solution (50 m*M* phosphate, pH 7.0) contained 250 μ*M* CPZ-SO or PZ-SO, 5 m*M* DMPO and the ·OH competitor at the concentration indicated. The concentrations are those expected from the rate constants to yield a 50% reduction in the ESR signal of the DMPO-OH adduct. For the reaction DMPO + ·OH, the rate constant has been taken as $3.4 \times 10^9\ M^{-1}\ s^{-1}$ (Finkelstein *et al.*, 1980). The rate constants quoted are taken from Farhataziz and Ross (1977). The standard deviation of the values of observed % reduction is ± 5 for all experiments.

Competitor	*k*	Concentration	Observed % reduction	
	$10^9\ M^{-1}\ s^{-1}$	m*M*	with CPZ-SO	with PZ-SO
Ethanol	1.9	8.9	44	51
DMSO	7.0	2.4	75	77
2-propanol	6.5	2.6	25	47
N_3^-	11	1.5	40	33
Cysteine	20	0.85	28	33

radical, P·, could be produced by photolysis and these, too, are capable of abstracting H· from radical scavengers. Intermediates of this type could then give DMPO-OH as the end product, either by decay of the initial adduct or by direct one-electron oxidation of DMPO. Therefore, to gather additional evidence that ·OH is indeed produced, a series of kinetic competition experiments were performed. An ·OH scavenger was included in the spin trapping mixture at a concentration calculated to reduce the intensity of the DMPO/OH signal by 50%, i.e., the rate of the reaction of ·OH with the scavenger is equal to its rate of reaction with DMPO, or:

$$k_{\text{Scavenger}}[\text{Scavenger}][\cdot\text{OH}] = k_{\text{DMPO}}[\text{DMPO}][\cdot\text{OH}]$$

thus

$$[\text{Scavenger}] = \frac{k_{\text{DMPO}}[\text{DMPO}]}{k_{\text{Scavenger}}}$$

The results of these experiments, which are presented in Table 1, are reasonably consistent with the formation of ·OH. The rate constants obtained from the literature, and thus measured under slightly different conditions, give calculated reductions in DMPO-OH formation consistent with the results in Table 1 within a factor of 3. The result most consistent with published rate constants was obtained with ethanol as the scavenger and indeed, Finkelstein *et al.* (1980) originally estimated the rate constant for the reaction of DMPO with ·OH from a kinetic competition experiment with ethanol.

Motten *et al.* (1985) have observed the dechlorination radical, P·, of the parent CPZ using spin trapping. In the experiments reported here no evidence for dechlorination of CPZ-SO was noted, even when the gain of the ESR spectrometer was increased by over 100 as compared to the conditions of Fig. 1A (not shown).

Flash photolysis

In laser flash photolysis experiments with CPZ-SO and PZ-SO the transient absorption spectrum observed, upon excitation at 355 nm, corresponds to that previously assigned to $CPZ^{+\cdot}$ and $PZ^{+\cdot}$; see Figs. 2 and 3 (Borg and Cotzias, 1962; Navaratnam *et al.*, 1978; and Davies *et al.*, 1979). In the wavelength range investigated no features were observed that could be attributed to a triplet–triplet absorption. The initial (1 μs) absorbance of $PZ^{+\cdot}$ at 520 nm is 1.6 times that of $CPZ^{+\cdot}$. (The absorbance of each solution was 1.0 at the exciting wavelength.) In the parallel spin trapping experiments the DMPO/OH signal intensity observed from photolysis of PZ-SO was 1.8 times that observed from CPZ-SO using identical photolysis conditions. Thus the combination of laser flash photolysis and spin trapping results supports homolytic cleavage of the S–O bond to give ·OH and cation radical.

DISCUSSION

Homolytic cleavage of the excited sulfoxide at the sulfur-oxygen bond is consistent with our ex-

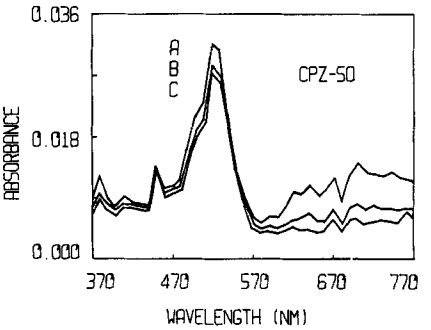


Figure 2. Flash photolysis of chlorpromazine sulfoxide. Transient absorption spectrum of 0.5 m*M* CPZ-SO in nitrogen-saturated pH 6.5 citrate buffer. Excitation was accomplished with a 12 ns, 22 mJ pulse with the 355 nm laser output. The absorbance of the CPZ-SO solution was 1.0 at 355 nm. The spectra presented are at different times after the pulse. (A.) 1 μs, (B.) 8.5 μs, (C.) 67 μs.

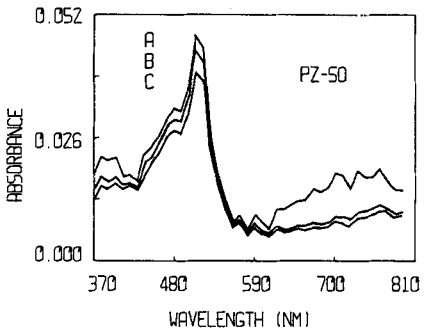


Figure 3. Flash photolysis of promazine sulfoxide. Transient absorption spectrum of 0.5 m*M* PZ-SO in nitrogen-saturated pH 6.5 citrate buffer. The laser flash conditions were the same as in Fig. 3.

perimental results. It is surprising that such a lysis would occur, because the sulfoxide group is commonly portrayed with a double bond between the sulfur and the oxygen (S = O) (see for example Bodea and Silberg, 1968).

However, Szmant (1971) has argued persuasively that the sulfoxide bond order depends strongly upon neighboring groups and degree of solvation. Moreover, electronic excitation is likely to further weaken the sulfur-oxygen bond so that a more accurate representation, at least in the excited state, may be a single bond with charge separation ($^{+}$S-O^{-}) (Shine and Mach, 1965). We can postulate that cleavage takes place after excited-state protonation of the sulfoxide:

$$\text{CPZ-}\overset{+}{\text{S}}\text{OH}^{*}\ (\text{S,T}) \rightarrow \text{CPZ}^{+\cdot} + {}^{\cdot}\text{OH} \qquad (4)$$

We have been unable to obtain conclusive evidence for an excited state protonation. The absorption spectrum of CPZ-SO does not change between pH 7 and pH −0.5, but its fluorescence is quenched below pH 2. The excited-state pK_a could not be determined because the fluorescence lifetime is too short and the protonated species does not fluoresce. The single phosphorescence band found at 77 K for promazine sulfoxide in a 70% glycerol rigid glass does not change position or shape when the room temperature pH is varied from 8.4 to −0.6, although the phosphorescence intensity is decreased in strongly acidic solutions. Neither the phosphorescence lifetime (100 ms) nor the absorption spectrum change over the same pH range. Our results are consistent with the very low pK_s (−2 to −4) normally associated with groundstate sulfoxide-group protonation (Landini *et al.*, 1969) and they suggest that even the triplet-state pK_a may not be high enough to permit excited-state protonation under physiological conditions. However, the triplet-state protonation may proceed more *quickly in fluid* solutions at room temperature. We also note that sulfoxides form strong hydrogen bonds (Szmant, 1961) and these might promote homolysis in either the singlet or triplet excited states.

Tentatively, we propose the following mechanism to account for our observations:

$$\text{CPZ-SO} + h\nu \rightarrow \text{CPZ-SO}^{*}\ (\text{S}) \qquad (5)$$

$$\text{CPZ-SO}^{*}\ (\text{S}) \rightarrow \text{CPZ-SO}^{*}\ (\text{T}) \qquad (6)$$

$$\text{CPZ-SO}^{*}\ (\text{S,T}) + \text{H}^{+} \rightarrow \text{CPZ-}\overset{+}{\text{S}}\text{OH}^{*}(\text{S,T}) \qquad (7)$$

$$\text{CPZ-}\overset{+}{\text{S}}\text{OH}^{*}\ (\text{S,T}) \rightarrow \text{CPZ}^{+\cdot} + {}^{\cdot}\text{OH} \qquad (4)$$

The hydroxyl radical will initiate the following reactions

$$\text{DMPO} + {}^{\cdot}\text{OH} \rightarrow \text{DMPO/OH} \qquad (8)$$

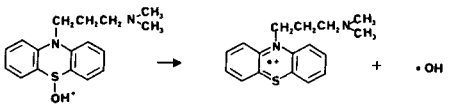
$$\text{CH}_3\text{CH}_2\text{OH} + {}^{\cdot}\text{OH} \rightarrow \text{CH}_3\dot{\text{C}}\text{HOH} + \text{H}_2\text{O} \qquad (9)$$

$$\text{N}_3^{-} + {}^{\cdot}\text{OH} \rightarrow \text{N}_3{}^{\cdot} + \text{OH}^{-} \qquad (10)$$

The radicals generated in equation 9 and 10 compete with ·OH for the spin trap.

The phototoxic effects of CPZ are generally greater than those of PZ except at low doses under aerobic conditions, when phototoxicity is about equal. The more severe photo-effects of CPZ have been ascribed to the dechlorination reaction of CPZ which forms P· and Cl· as well as the possible photoionization forming CPZ$^{+\cdot}$. Motten *et al.* (1985) have unambiguously confirmed the photoformation of P· from CPZ and have demonstrated that it is indeed an extremely reactive species. Motten *et al.* (1985) have also provided evidence that the photoformation of CPZ$^{+\cdot}$ and PZ$^{+\cdot}$ by photoionization may have only a minor role in the phototoxic effects of CPZ and PZ. Photolysis of PZ does not result in the formation of P· as seen by spin trapping (Motten *et al.*, 1985).

Little attention has been given to the photochemistry of the sulfoxides. Our spin trapping experiments with CPZ-SO have demonstrated that dechlorination is a very minor process if it occurs at all. However, these experiments have revealed the formation of an extremely strong oxidizing agent from both PZ-SO and CPZ-SO. This species is capable of oxidizing substrates by either electron transfer or hydrogen atom transfer reactions. The spin trapping data are consistent with the formation of the hydroxyl free radical. The flash photolysis experiments with CPZ-SO and PZ-SO resulted in transient absorption spectra attributable to CPZ$^{+\cdot}$ and PZ$^{+\cdot}$ respectively, with no features that could be assigned to other transient species such as the triplet states of CPZ-SO or PZ-SO. The presence of CPZ$^{+\cdot}$ and PZ$^{+\cdot}$ is consistent with the formation of hydroxyl radical from the sulfoxides.



Over 50% of the CPZ taken orally is metabolized to CPZ-SO during presystemic absorption (Yeung *et al.*, 1983), and irradiation of the parent promazine in

air-saturated aqueous solutions also results in the formation of sulfoxide (Iwaoka and Kondo, 1974). Ascorbic acid, which is at a high level in the eye (Varma *et al.*, 1984) reduces cation radical to the parent promazine (Pelizzetti *et al.*, 1979), which can be oxidized again to the sulfoxide. Thus, the photolysis of the phenothiazine sulfoxide can result in a cycle of ·OH production.

The presence of dissolved oxygen has been shown to be a key factor in some *in vitro* photo-effects of promazine derivatives. For example, Hoffman and Dischер (1968) observed that oxygen was an absolute requirement for the CPZ-sensitized photodynamic oxidation of BSA and GSH. Merville *et al.* (1984) studied the photosensitized cross-linking of bovine lens crystallins by the promazine derivatives PZ, CPZ, TFPZ, MTPZ and ACPZ. For all five drugs, the cross-linking rates were found to depend upon the presence of oxygen and were decreased strongly in its absence. Merville *et al.* (1983) also found that in the absence of oxygen during irradiation, PZ, MTPZ and ACPZ lose their cross-linking activities for erythrocyte ghost membrane proteins.

No studies of this nature have been done with the corresponding sulfoxides, but the *in vivo* experiments of Clare *et al.* (1947) have clearly identified phenothiazine sulfoxide as the photodynamic agent responsible for the photosensitized keratitis in calves dosed with phenothiazine. Our *in vitro* detection of ·OH and the corresponding sulfoxides clearly has implications for the possible mechanism of the photo-effects of these and other phenothiazine drugs.

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