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SPECTROSCOPIC STUDIES OF CUTANEOUS PHOTOSENSITIZING AGENTS—VIII. A SPIN-TRAPPING STUDY OF LIGHT INDUCED FREE RADICALS FROM CHLORPROMAZINE AND PROMAZINE

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Abstract—The clinically important phenothiazine drugs, particularly chlorpromazine, often elicit phototoxic and photoallergic reactions. We have used the spin traps 2-methyl-2-nitrosopropane (MNP) and 5,5-dimethyl-pyrroline-N-oxide (DMPO) to define the radical photolysis pathways of chlorpromazine and promazine. In the absence of oxygen the dechlorination product of chlorpromazine is trapped by MNP. The reactivity of the dechlorination product is similar to that of the phenyl radical as shown by its ability to extract hydrogen atoms from donors. Our results suggest that the dechlorination product is sufficiently reactive to account for the observation that chlorpromazine is more phototoxic than its parent promazine. In the presence of oxygen both chlorpromazine and promazine form a superoxide-dismutase-insensitive oxygen-centered intermediate which, when trapped by DMPO, rapidly decays to DMPO-OOH and subsequently to DMPO-OH. In addition, chlorpromazine readily undergoes photoelectron ejection only when it is excited into the second excited singlet state ($\lambda < 280$ nm). This previously unknown wavelength dependence of photoionization should be considered in establishing the mechanism of chlorpromazine photosensitization.

INTRODUCTION

The clinically important phenothiazine drugs, particularly chlorpromazine, often elicit undesirable side effects including phototoxic and photoallergic reactions (Fitzpatrick *et al.*, 1963) and ocular complications (Zelickson and Zeller, 1964). In a quantitative *in vivo* measurement of phototoxicity in mice, Ljungren and Möller (1976) found chlorpromazine to be an order of magnitude more phototoxic than its parent promazine. Chlorpromazine is also more effective than promazine in inducing photomutagenesis in bacteria (Jose, 1979) and causing protein cross-linking in erythrocyte ghost membranes (Merville *et al.*, 1983). Chlorpromazine produces corneal stromal granularity in dogs, while promazine has no detectable ocular effects (Barron *et al.*, 1972).

For other drugs with similar side effects, free radicals have been proposed as the toxic species (Harber and Baer, 1969). The difference between chlorpromazine and promazine in photoreactivity has been attributed to the formation of radicals by dechlorination of chlorpromazine (Jose, 1979), a pathway not available to promazine. The cation radical has also been implicated in photosensitization (Merville *et al.*, 1984; De Mol *et al.*, 1983).

In this study we have used the spin traps MNP† and DMPO to elucidate the radical photolysis pathways of chlorpromazine and promazine. The complex photochemistry of chlorpromazine can be largely explained by the radical intermediates we detected, while the lower activity of promazine is consistent with its reduced ability to form such radicals.

MATERIALS AND METHODS

Chlorpromazine (Smith, Kline and French Labs) and promazine (Wyeth Labs) were used without further purification. DMPO† (Aldrich) was purified with charcoal (Buettner and Oberley, 1978) and was stored as an aqueous frozen solution (under nitrogen) until use. Aqueous MNP (Aldrich) stock solutions were prepared with mild heating and stirring immediately before use. Anaerobic samples were bubbled with N_2 for 5–10 min; N_2O saturated solutions were bubbled with N_2O for 20 min.

Electron spin resonance spectra were run at 9.5 GHz on a Varian E-109 spectrometer equipped with a TM_{110} cavity. Samples were irradiated in the cavity with a Schoeffel 1000 W Xe arc lamp and monochromator combination (bandpass 10 nm, unless stated otherwise) with an intensity of 4–20 J m^{-2} s^{-1}, depending on the wavelength, as measured 1 cm in front of the cavity grid using a Yellow Springs Instrument Model 65 radiometer.

SOD (Sigma, bovine blood) containing solutions were at 100 units/mℓ final concentration; the activity of the SOD was verified immediately after use with a xanthine (Sigma)-xanthine oxidase (Sigma) system. Absorbance measurements were made on a Gilford spectrophotometer.

RESULTS

Anaerobic photolysis of chlorpromazine at 330 nm

When MNP was used as the spin trap, one

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†*Abbreviations*: BSA, bovine serum albumin; CPZ, chlorpromazine; DMPO, 5,5-dimethyl pyrroline-N-oxide; DTBN, di-*tert*-butyl nitroxide; ESR, electron spin resonance; MNP, 2-methyl-2-nitrosopropane; PZ, promazine; SOD, superoxide dismutase, EC 1.15.1.1.

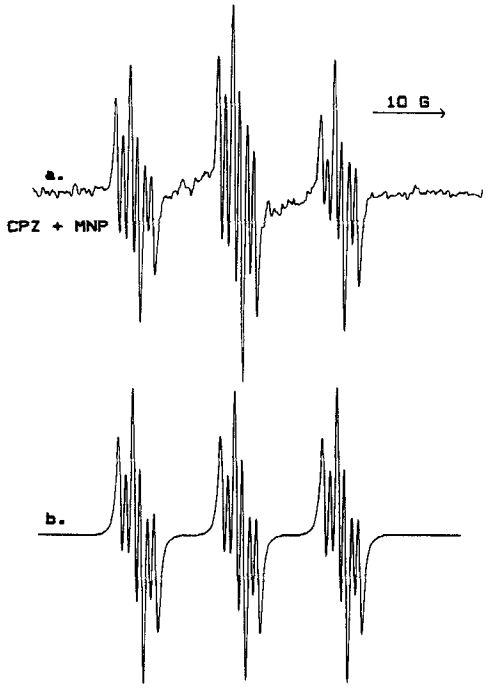


Figure 1. (a) ESR spectrum of MNP adduct of P· radical from CPZ (5 mg/mℓ) in H_2O adjusted to pH 4.0, λ_{ex} = 330 nm. Receiver gain was 2.5 × 10^4; mod. amp., 0.33 G; power, 20 mW; 4 min scan; time constant 0.25 s. Hyperfine splittings from the spin trap, a^N = 14.1 G, and from three protons from the dechlorinated ring, a^H = 0.92 G, 1.95 G, and 1.99 G, can be seen. (b) Computer simulation of (a) using a Lorentzian lineshape, linewidth = 0.4 G, and the above hyperfine splitting constants.

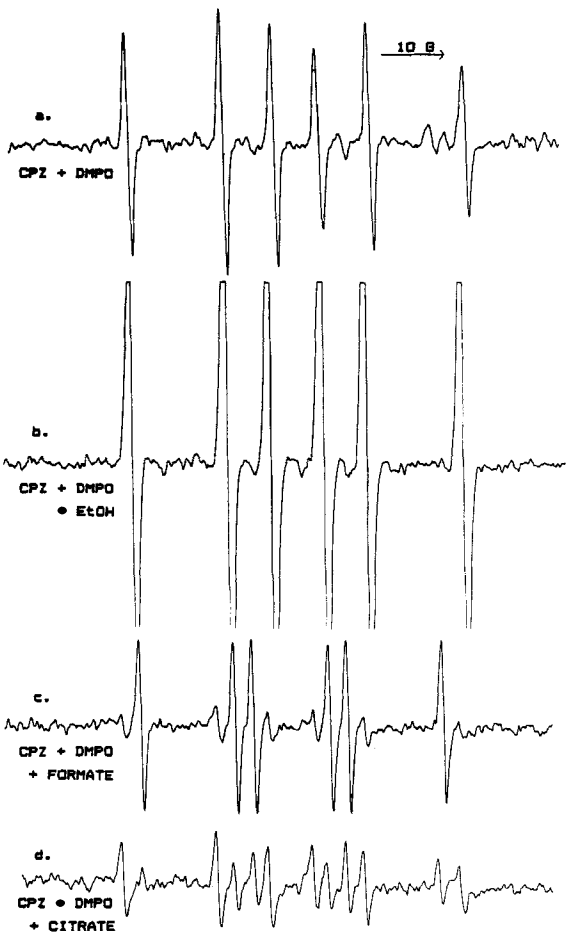


Figure 2. ESR spectra of DMPO adducts of CPZ with H-atom donors, pH 6.5. (a) CPZ + DMPO in phosphate buffer. DMPO-P, a^N = 15.9 G, a^H = 24.3 G. (b) Same as (a) with 1 *M* EtOH. DMPO-CH(CH_3)OH, a^N = 15.9 G, a^H = 23.1 G. (c) Same as (a) with 150 m*M* sodium formate. DMPO-CO_2^-, a^N = 15.8 G, a^H = 18.8 G. The small peaks are DMPO-P. (d) CPZ + DMPO in 50 m*M* citrate buffer. A mixture of DMPO-P and DMPO-citrate, a^N = 15.3 G, a^H = 18.6 G. All samples were bubbled with N_2 before irradiation. CPZ was 0.2 m*M*, DMPO 5 m*M*; gain, 8 × 10^4; mod. amp., 0.5 G; microwave power, 10 mW; 4 min scans with 0.5 s time constant. λ_{ex} was 310 nm with a 10 nm bandpass.

carbon-centered adduct was detected from CPZ over the range pH 3.5 to pH 6.5 (Fig. 1) (some spectra also had the three-line spectrum of DTBN present as an impurity). The hyperfine splitting constants (a^N = 14.1 G; a^H = 0.92 G, 1.95 G, 1.99 G) of the adduct are consistent with a structure containing three aromatic ring hydrogens derived from the reaction of MNP with the dechlorination product, P· (see Scheme 1).

In nitrogen-bubbled citrate buffer, 50 m*M* pH 6.5, with DMPO as the spin trap, a carbon-centered radical from CPZ was trapped, as well as a carbon-centered radical from the citrate buffer (DMPO-citrate) (see Fig. 2d). To test the ability of P· to extract a hydrogen atom, CPZ was photolyzed in 50 m*M* pH 6.5 phosphate buffer in the presence of different donors. Only the carbon-centered adduct, DMPO-P (a^N = 15.9 G, a^H = 24.3 G), was observed in phosphate buffer (Fig. 2a). When 1 *M* EtOH was added, the DMPO-P disappeared and a different DMPO adduct (a^N = 15.3 G, a^H = 23.1 G) was readily observed (Fig. 2b). Addition of 150 m*M* formate generated the DMPO-CO_2 adduct (Fig. 2c), while at the same time suppressing the DMPO-P adduct. Since an adduct with nearly the same splittings as DMPO-CO_2 was observed in

Table 1. Guanine reduction of DMPO-P at 330 nm*

[guanine] (m*M*)	% of Control
0	100
2.5	31
5	24
10	12
20	6

*The solutions contained DMPO at 10 m*M* and CPZ at 0.25 m*M* in nitrogen bubbled pH 6.5 phosphate buffer.

citrate buffer, P· must also abstract H· from citrate to allow formation of DMPO–citrate.

The guanine bases in DNA appear to be particularly susceptible to photosensitized damage by promazines (Merville *et al.*, 1984). The introduction of guanine into the CPZ-DMPO spin trapping solution produced a concentration dependent reduction of the DMPO-P ESR signal (Table 1). However, this reduction in DMPO-P was not accompanied by the appearance of a new DMPO spin adduct as was observed with EtOH and formate.

No DMPO-H was observed in pH 6.5 citrate when exciting at 330 nm. However, in phosphate buffer at pH 6.5 a trace of DMPO-H was sometimes observed but the signal was always very weak and was seen only after prolonged irradiation.

Aerobic photolysis of chlorpromazine at 330 nm

When the CPZ concentration was less than the O_2 concentration, a superoxide or peroxy radical (a^N = 14.2 G, a^H = 11.3 G, a^H_γ = 1.3 G) was trapped with DMPO (DMPO-OOCPZ, DMPO-OOP, or DMPO-OOH) (citrate buffer, pH 4.0) (Fig. 3a) on photolysis with 330 nm light. The adduct decayed to DMPO-OH. At pH 6.5 the peroxy spin adduct was too short-lived to detect, but the DMPO-OH product was readily observed, in agreement with the observations of Decuyper *et al.* (1984) at pH 7.0. Since superoxide and peroxy-DMPO adducts have very similar hyperfine-splittings (Kalyanaraman, 1982) SOD was used to test for the presence of superoxide (Fig. 4). In citrate buffer pH 6.5, SOD caused no reduction in the DMPO-OH signal height. Nonetheless, the asymmetry in the outside lines of the peroxy radical (Fig. 3a) is characteristic of the DMPO-OOH adduct (R. P. Mason, unpublished, see also Buettner, 1982). Irradiation of SOD in the presence of 0.1 m*M* CPZ did not affect the activity of this enzyme in suppressing DMPO-OOH formation from a xanthine-xanthine oxidase system. It is possible that DMPO-OOH is itself a decay product of a very short-lived peroxy radical intermediate. Two such intermediates are plausible (Scheme 1), one a product of P· reacting with oxygen, P-OO·, and the other a peroxy group covalently bound to the sulfur atom, CPZ-SOO·. At present we cannot distinguish between these two possibilities; a mixture is also possible. Once oxygen was nearly depleted, the carbon-centered DMPO adducts could also be observed if there was an excess of CPZ.

Photolysis of chlorpromazine at 270 nm

When chlorpromazine was photolyzed using 270 nm radiation, i.e. into the S_2 absorption band, the carbon-centered and oxygen-centered DMPO adducts previously observed were still present. In addition, a strong signal corresponding to DMPO-H (a^N = 16.4 G, a^{2H} = 22.7 G) was observed (Fig. 5a). The presence of oxygen did not significantly reduce the DMPO-H ESR signal intensity (not shown). The

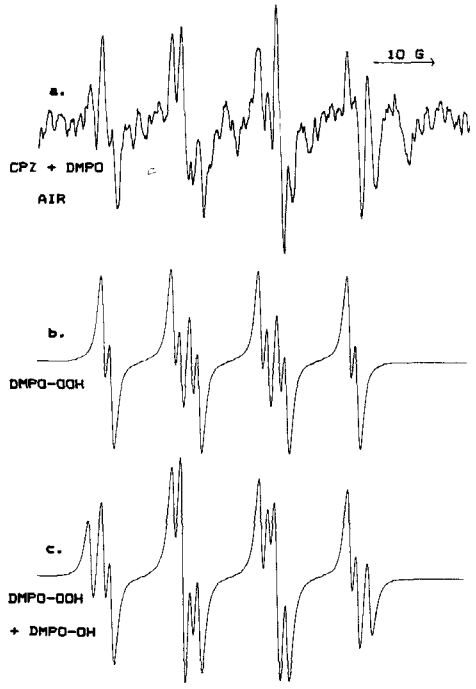


Figure 3. (a) An ESR spectrum of DMPO-peroxy adduct from CPZ (0.45 m*M*) in pH 4.0 citrate buffer in the presence of O_2 (0.45 m*M*). Two components are present: DMPO-OOH, a^N = 14.2 G, a^H = 11.3 G, a^H_γ = 1.3 G; and DMPO-OH, a^N = 15.0 G, a^H = 15.0 G. The proportion of DMPO-OH increases during the scan. Gain 2 × 10^5, mod. amp. 0.8 gauss, power 10 mW, 4 min scan with 0.5 s time constant; λ_{ex} = 330 nm with a 10 nm bandpass. (b) Computer simulation of DMPO-OOH using the above hyperfine splittings with a Lorentzian lineshape and linewidth of 0.5 G. (c) Computer simulation of DMPO-OOH plus DMPO-OH in a ratio of 3:1.

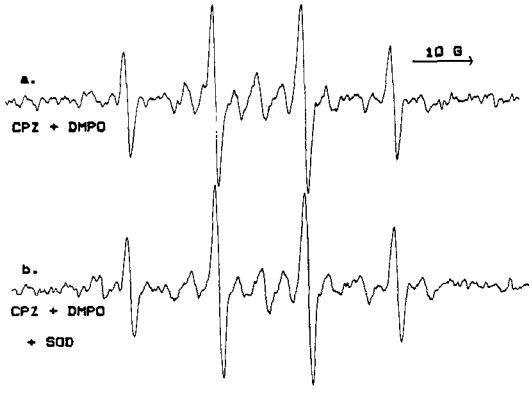
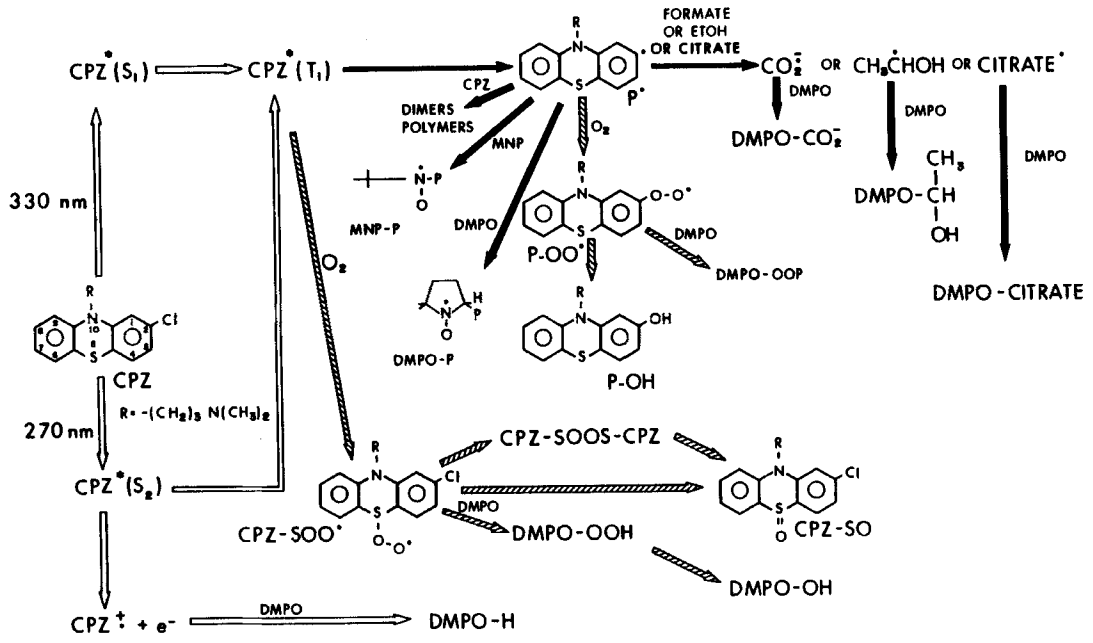


Figure 4. ESR of air-saturated CPZ solution and effect of SOD. (a) CPZ (0.2 m*M*) in pH 6.5 citrate buffer with DMPO (50 m*M*). The DMPO-OH spectrum (a^N = 15.0 G, a^H = 15.0 G) dominates. (b) Same as (a) with 100 units/mℓ SOD added. Irradiation at 330 nm (16.5 nm bandpass); gain, 3.2 × 10^4; mod. amp., 1G; 4 min scan with 0.5 s time constant; microwave power 10 mW.

DMPO-H adduct may be formed either by reaction of DMPO with H· or by addition of an electron followed by protonation. Since ejection of an electron from chlorpromazine would form the stable



Scheme 1.

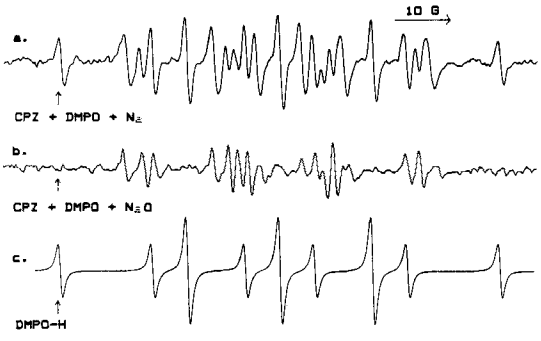


Figure 5. (a) ESR spectrum of CPZ (0.1 m*M*) in pH 6.5 citrate buffer with 15 m*M* DMPO, bubbled with N_2 and excited at 270 nm (20 nm bandpass). The outer lines of the DMPO-H adduct can be seen clearly in the wings (arrows). (b) Same as (a) but saturated with N_2O. DMPO-H adduct has disappeared (arrows). Gain, 2.5×10^4; mod. amp., 0.5 G; 8 min scan with 1 s time constant; 10 mW microwave power. (c) Computer simulation of ESR spectrum of DMPO-H, $a^{2H} = 22.7$ G, $a^N = 16.4$ G, linewidth = 1 G.

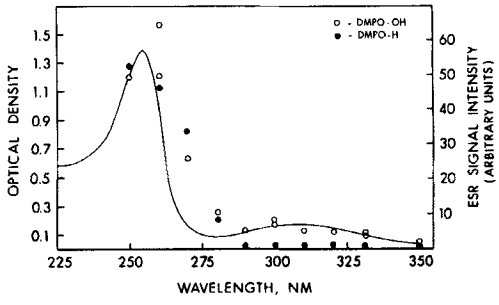


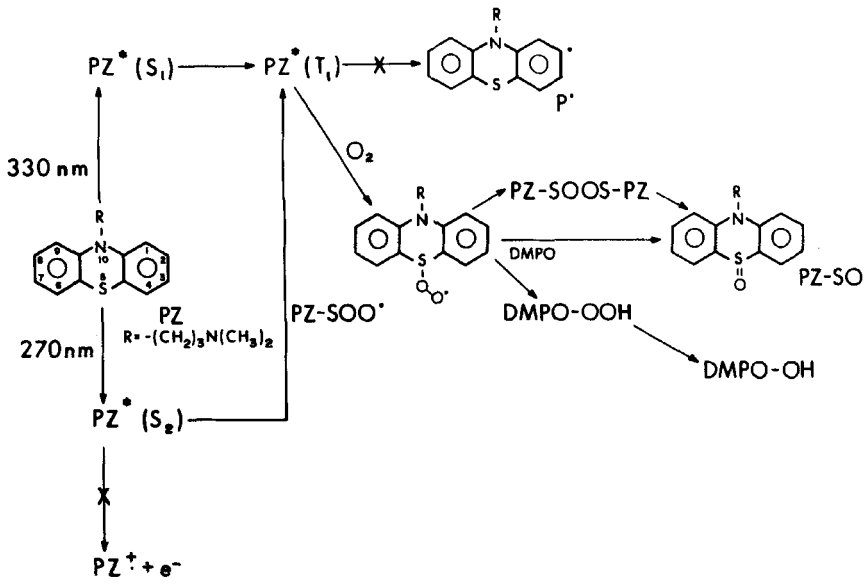
Figure 6. ESR signal intensity of DMPO-OH (○) and DMPO-H (●) compared with the absorption spectrum (——) of CPZ. Absorption of 4×10^{-5} *M* CPZ in citrate buffer, pH 6.5. ESR signal intensities were measured on the leftmost (low-field) line for DMPO-H immediately following an 8 min scan, while amplitudes for DMPO-OH were measured at the third (next to highest-field) line during an 8 min scan started when the light was turned on. Mod. amp., 0.5 G; scan width, 100 G; time constant 2 s. Amplitudes were corrected for lamp intensity which was measured as in Methods. DMPO-H amplitudes at 290 nm and longer λ_{ex} were not distinguishable from the noise.

cation radical (Scheme 1) we used N_2O to determine the source of the DMPO-H adduct. N_2O reacts with electrons but not with H· at neutral pH (Anbar *et al.*, 1973). In N_2O-saturated solution the DMPO-H adduct signal was suppressed (Fig. 5) suggesting that the observed DMPO-H adduct is formed entirely from trapped electrons and not hydrogen atoms.

In Fig. 6, the absorption spectrum of chlorpromazine is shown with the magnitudes of DMPO-OH and DMPO-H as a function of excitation wavelength. While DMPO-OH follows the absorption curve fairly closely, the excitation curve for DMPO-H can best be explained if excitation into S_2 is required for electron ejection to compete successfully with dechlorination.

Photolysis of promazine

Excitation into S_1 (330 nm) produced no significant radical adducts in nitrogen bubbled solutions. Excitation into S_2 (270 nm) produced an unidentified carbon-centered radical which was trapped by DMPO ($a^H = 22.8$ G, $a^N = 15.5$ G; at pH



Scheme 2.

6.5 in citrate buffer). This adduct has splittings similar to DMPO-P produced from chlorpromazine, but is probably not DMPO-P since neither the MNP-P adduct, hydrogen abstraction from EtOH, nor DMPO-H was observed.

In the presence of O_2, promazine produced an oxygen-centered radical which was trapped to give a DMPO-peroxy adduct at pH 4 and the DMPO-OH decay product at pH 6.5 (Fig. 7a). The signal intensity of the DMPO-OH adduct was decreased in the presence of SOD by about 50% (Fig. 7b). Bovine serum albumin at the same protein concentration had no significant effect (Fig. 7c). In contrast to chlorpromazine, promazine thus appears to produce roughly half superoxide and half peroxy radical. This reaction occurs when exciting into S_1 (330 nm), where no carbon-centered radicals are trapped, suggesting that the sulfur-peroxy radical PZ-SOO· is formed from the excited state reaction with oxygen.

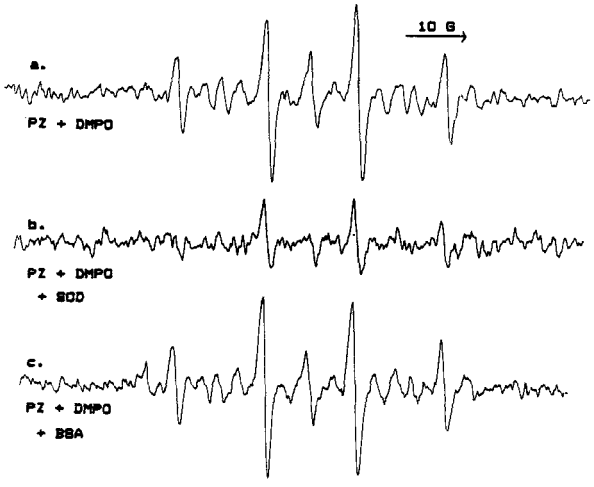


Figure 7. ESR spectra of photolyzed promazine. (a) Promazine (0.2 m*M*) in air-saturated pH 6.5 citrate buffer with DMPO (50 m*M*). The DMPO-OH spectrum dominates. (b) Same as (a) with 100 units/mℓ SOD added prior to irradiation. (c) Same as (b) with equivalent protein concentration of BSA instead of SOD. Irradiation was at 330 nm with 20 nm bandpass; spectrometer gain, 3.2×10^4; modulation amplitude, 1 gauss; microwave power, 10 mW; scan time 4 min with a 0.5 s time constant.

DISCUSSION

We have found evidence for three primary photoreactions in chlorpromazine: (a) dechlorination to form the promazine radical, P·; (b) addition of oxygen, ultimately forming the sulfoxide CPZ-SO; and (c) electron ejection. All of the above radical intermediates will form further photoproducts, as outlined in Scheme 1, some of which may also be radical intermediates. Non-radical pathways may also occur but will not be observed by ESR.

The CPZ dechlorination radical P· is generally acknowledged to form from the lowest excited triplet state T_1 (Navaratnam *et al.*, 1978; Iwaoka and Kondo, 1974). This radical is able to extract a hydrogen atom from a variety of donors such as ethanol, citrate and formate to yield promazine, a known photoproduct of CPZ (Grant, 1974). Previous reports of hydroxyl radical production from CPZ may be artifactual (Lion *et al.*, 1982), as hydrogen atom extraction was used to test for its presence. Chlorine atom may also be able to extract hydrogen atom from some donors. However, DMPO

and the hydrogen atom donors both react with P· since the donors inhibit the formation of DMPO-P. The promazine radical may also react with another promazine radical or with CPZ to give dimers and higher polymers. These photoproducts have been observed, particularly in more concentrated solutions (Kochevar and Hom, 1983). As the CPZ concentration was increased above 0.5 m*M*, less P· was trapped by DMPO.

In the presence of oxygen the DMPO–OH adduct dominates. The origin of this adduct need not be direct spin-trapping of OH·; DMPO–OH adduct can also be obtained as a decay product of DMPO-OOH and possibly as a decay product of DMPO-OOR. In fact, at pH 4 the DMPO-OOH adduct is observed first and can be seen to decay to DMPO-OH. Thus the DMPO-OH observed at pH 6.5 is also most likely to be a decay product of DMPO-OOH. Since the DMPO-OOH adduct formed in the presence of CPZ is SOD-insensitive (see Scheme 1), the DMPO–OOH may itself be the decay product of an intermediate peroxy adduct such as the product of P· and oxygen (a 2-peroxy CPZ radical, POO·), or the result of addition of oxygen to the sulfide bridge (CPZ-SOO·) as suggested by Iwaoka and Kondo (1974). They have shown that the oxygen incorporated into CPZ to form CPZ-SO comes from dissolved oxygen as opposed to solvent oxygen. The CPZ-SOO· intermediate would lead to CPZ-sulfoxide (Grant, 1974), while POO· would decay to 2-hydroxypromazine, P-OH, which has also been observed as a CPZ photoproduct (Grant, 1974). A mixture of these products is also possible. The observation of SOD-insensitive peroxy radicals from promazine as well as chlorpromazine strongly suggests that the sulfur-peroxy intermediate is a significant component of the peroxy intermediates. The observation that other carbon-peroxy DMPO adducts analogous to DMPO-OOH do not decay to DMPO-OOH or DMPO-OH (Kalyanaraman, *et al.*, 1983; Sinha, 1983), further supports CPZ-SOO· rather than P-OO· as a possible intermediate trapped by DMPO which could decay to DMPO-OOH.

The lifetime of the CPZ excited singlet state, S_1, is only 1.3 ns in air-saturated H_2O (R. D. Hall, unpublished), which is too short for a diffusion-controlled reaction with O_2 to be limiting. Thus, formation of the sulfur-peroxy radical must occur from the triplet state T_1. Sysak *et al.* (1977) and Liang *et al.* (1983) have proposed that singlet oxygen can react with (singlet) sulfur compounds to produce RSOO. Could it be then, that triplet CPZ and (triplet) O_2 could react to produce CPZ-SOO·? The detailed mechanism of the reaction of CPZ with O_2 is a subject for further study.

The formation of superoxide during the photolysis of promazine is in sharp contrast to its absence as a major photolysis product from chlorpromazine. An electron must be lost to form the peroxy radical from PZ or CPZ and O_2, and it is possible that in PZ, O_2 also serves as the acceptor, forming O_2^-, while in CPZ solutions Cl· and P· are the acceptors. Since little or no P· radical was trapped until dissolved oxygen was nearly used up, either the rate constant for CPZ-SOO· formation must be greater than that for dechlorination, or the P· that does form must then react with O_2 or CPZ-oxygen intermediates. Formation of O_2^- and subsequent photolysis of H_2O_2 to form ·OH may explain the activity of PZ in phage deactivation (Merville *et al.*, 1983) at low concentrations (~0.1 m*M*).

Ejected electrons were only trapped when excitation was into S_2 ($\lambda_{ex} < 280$ nm), the second excited singlet state. This was surprising since the hydrated electron and cation radical have been observed in laser flash photolysis with 347 nm excitation (Navaratnam *et al.*, 1978) for both CPZ and PZ. At a 50 m*M* DMPO concentration, N_2O did not quench all the electrons before they could be trapped, thus if hydrated electrons were produced at 330 nm excitation we should have been able to trap them. We suggest that at the very high light levels used in laser flash photolysis a substantial steady state triplet population can be produced during the length of a flash (25 ns in the case of Navaratnam *et al.* (1978) compared with the singlet state lifetime of only 1.3 ns). Triplet-triplet absorption and subsequent electron ejection could under these conditions be pseudo-first order with respect to light intensity, in agreement with what was observed (Navaratnam *et al.*, 1978). Both Iwaoka and Kondo (1972) and Decuyper *et al.* (1984) detected photoejected electrons upon near-UV irradiation, but both groups used lamp-filter combinations which would admit some light of 280 nm or shorter wavelengths to the sample. Our results using monochromatic light demonstrate the importance of controlling the excitation wavelength when studying photolytic processes.

At present we are unable to account for our observation of electron ejection from chlorpromazine but not from promazine under the same conditions. The ionization potentials of CPZ and PZ free base are nearly equal in chloroform (Fulton and Lyons, 1968).

The cation radical formed on electron ejection has been considered an obligate intermediate in the formation of chlorpromazine sulfoxide (Iwaoka and Kondo, 1974). However, we have observed the formation of the peroxy intermediate under conditions where the cation radical is not formed. Preliminary results obtained by monitoring fluorescence suggest that quenching the cation radical with ascorbate has only a minimal effect on the rate of formation of sulfoxide (R. D. Hall, unpublished). The cation radical is, in fact, relatively stable even in the presence of oxygen, and produces sulfoxide slowly (Borg and Cotzias, 1962; Cheng *et al.*, 1978) compared to direct reaction of O_2 with triplet CPZ.

Our observation that photoionization of CPZ only occurs with excitation into the S_2 level (< 280 nm) suggests that photoformation of phenothiazine cation radicals by sunlight will not be important in cutaneous and ocular photosensitivity. The *in vivo* action spectrum of CPZ in hairless mice follows the absorption spectrum of CPZ from 330–370 nm (Hunter *et al.*, 1970). The P· radical (and possibly Cl·) from CPZ could initiate lipid peroxidation even at low CPZ concentrations, and at high CPZ concentrations P· could form dimers and higher polymers which are known to cause membrane damage (Kochevar and Lamola, 1979; Kochevar and Hom, 1983). The formation of P· could thus account for the difference in phototoxicity between CPZ and PZ. The P· radical may also be the active intermediate in the mutagenicity of chlorinated phenothiazines (Jose, 1979).

This study has clearly demonstrated the photoproduction of P· from CPZ. In addition we have found that the reactivity of P· is similar to that of hydroxyl or phenyl radical in its capability to abstract hydrogen atoms from a variety of substrates. We have also discovered a previously unknown wavelength dependence of the photoionization of CPZ. This wavelength dependence will need to be considered in establishing the mechanism of CPZ photosensitization.

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