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Original Contribution

HORSERADISH PEROXIDASE-CATALYZED SULFOXIDATION OF PROMETHAZINE AND PROPERTIES OF PROMETHAZINE SULFOXIDE

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Abstract—Promethazine sulfoxide was obtained with a quantitative yield in a horse radish peroxidase-catalyzed reaction of promethazine and hydrogen peroxide and was also prepared by direct chemical synthesis. The enzymatic sulfoxidation of promethazine was studied in vitro as a function of pH, promethazine, and hydrogen peroxide concentration. Promethazine sulfoxide inhibits with an apparent K_i of 59.7 μM at pH 5.5 the enzymatic reaction, followed spectrophotometrically, polarographically, potentiometrically, and luminometrically. The reaction was also inhibited by ascorbic acid (K_i 26.8 μM) and glutathione (K_i 41.8 μM). The spectrophotometric techniques employed, together with ESR spectrometry, allowed the identification of at least three radical species formed in the course of the reaction. Promethazine sulfoxide is devoid of the antioxidant effect exhibited by promethazine on rat brain synaptosomes. The sulfoxide also lacks photosensitizing action, while retaining the neuroleptic effect of the parent compound.

Keywords—Promethazine, Promethazine sulfoxide, Horse radish peroxidase, Synaptosomes, Photosensitization, Neuroleptic action, Free radicals

INTRODUCTION

The sulfoxide derivative is one of the main metabolites of chlorpromazine,[1] and phenothiazine drugs, in general, lose their activity after sulfoxidation.[2–4]

Both enzymatic and nonenzymatic oxidation of phenothiazines involves a variety of oxidizing agents and occur via radical intermediates.[5] A quenching action of the phenothiazine ring on radical formation is responsible of inhibitory effects on lipid peroxidation[6,7] with IC_{50} values ranging between 100 and 200 μM.[8]

In a previous article[9] we described the reduction of the antioxidant and photosensitizing properties of chlorpromazine after quaternization of the side chain nitrogen.

The enzymatic oxidation of phenothiazines has been extensively studied, but several questions are still open on this reaction mechanism.[10,11]

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We have taken promethazine as an example of phenothiazine, and in this article we describe its peroxidase-catalyzed sulfoxidation together with the direct chemical synthesis of promethazine sulfoxide, to compare its characteristics with those of the parent compound. The identification of various intermediate radical species and the kinetic characteristics of the enzymatic reaction were utilized to propose a mechanistic view of it.

MATERIALS AND METHODS

Chemical synthesis

Promethazine hydrochloride (5 g) (Aldrich Chem. Co., Milwaukee, WI) dissolved in 100 ml of b. d. water. at 4°C, and 10 ml of hydrogen peroxide 30% (Merck, Darmstadt, Germany) were added dropwise within 15 min.

The product promethazine sulfoxide was crystallized and its structure confirmed by TLC, NMR, and elemental analysis.

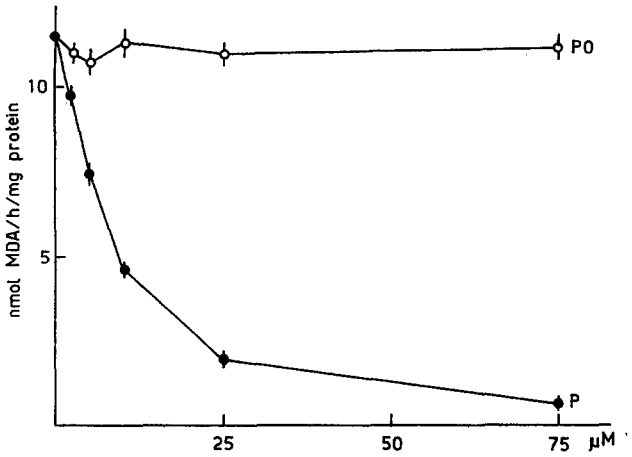


Fig. 1. Effect of promethazine (P) and promethazine sulfoxide (PO) on peroxidation of 0.5 mg synaptosomal protein at 37°C for 1 h. The points are mean ± SD values from four experiments.

Methods

Liposomes were prepared from brain of Wistar male rats (250 g) as described by Nakano and colleagues[11] suspended and sonically irradiated in 0.125 M KCl and 20 mM Tris/*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) pH 7.4, as described previously.[9] Rat brain synaptosomes were prepared as described by Musacchio and colleagues[12] by the Ficoll gradient procedure of Nicholls,[13] and peroxidation was induced with 20 μM Fe (II) and 1 mM ascorbate. Oxygen uptake was detected polarographically at 25°C with a Clark's electrode assembly (Yellow Springs Instrument Co., OH), and photosensitizing effects were evaluated after an UV irradiation carried out with a 320–400 nm emitting tube (Sylvana F6T 5-BL, UV Products Inc., S. Gabriel, CA).

Horseradish peroxidase (Boehringer–Mannheim, Germany) was used to catalyze promethazine sulfoxidation and the reaction was followed with the absorbance increases at 335 nm and 530 nm by a Perkin–Elmer Lambda 5 spectrophotometer (Uberlingen, GFR), at 25°C. The incubation mixture contained in 1 ml different concentrations of promethazine hydrochloride in 0.25 M acetate (or other) buffer pH 5.5, 4.5 μg peroxidase and 100 μM H_2O_2. Alternatively, it contained different concentrations of H_2O_2 and a fixed concentration of 75 μM promethazine hydrochloride. The effect of various concentrations of promethazine sulfoxide, glutathione (Sigma, St. Louis, MO), ascorbic acid (Merck, Darmstadt, Germany, or superoxide dismutase (Boehringer–Mannheim, Germany) was studied.

Total glutathione was determined according to Tietze[14] and protein according to Gornall and colleagues.[15] The neuroleptic activity was assayed on Wistar male rats (250 g) as a length of sleep induced by IP sodium 5,5-diethylbarbiturate (Merck, Darmstadt) after an IP treatment with the phenothiazines.[9] The enzymatic sulfoxidation reaction was also followed by a luminometer LKB-Wallac 1250 (Bromma, Sweden) and ESR spectra were detected with a Bruker 200 D spectrometer (Karlsruhe, Germany) interfaced with a Digital DECpc computer.

RESULTS AND DISCUSSION

Sulfoxidation induces the disappearance of the 300 nm peak of the UV spectrum of promethazine with a new absorption peak appearing at 335 nm (ϵ_M of 5,000 M^{-1} cm^{-1}).

Figure 1 summarizes the effect of promethazine and promethazine sulfoxide on the Fe(II)/ascorbate-induced peroxidation of rat synaptosomes. An IC_{50} value of 7.5 μM can be calculated for promethazine, while its sulfoxide is totally ineffective up to concentrations of 0.5 mM.

Figure 2 shows the effect of sulfoxidation on the photosensitizing action of promethazine. The sulfoxide is devoid of photodynamic action within the first 10 min. The initial oxygen consumption, in the presence of promethazine was 59 nmol/min, while in the presence of promethazine sulfoxide it was 2 nmol/min.

Figure 3 shows a typical first order kinetic of the peroxidase-catalyzed formation of promethazine sulfoxide followed spectrophotometrically at 335 nm and, in parallel, those of the luminescence emission and absorbance change at 530 nm due to the production of decaying intermediates.

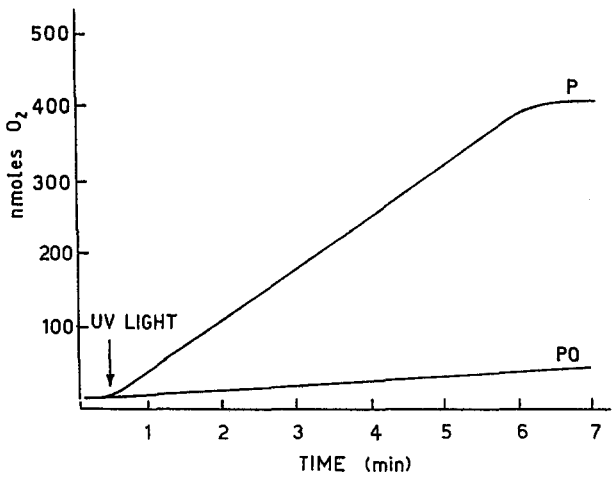


Fig. 2. Oxygen uptake of rat brain liposomes incorporated with promethazine (P) and promethazine sulfoxide (PO). Both compounds were 1 mM in KCl/Tris-HEPES buffer pH 7.4.

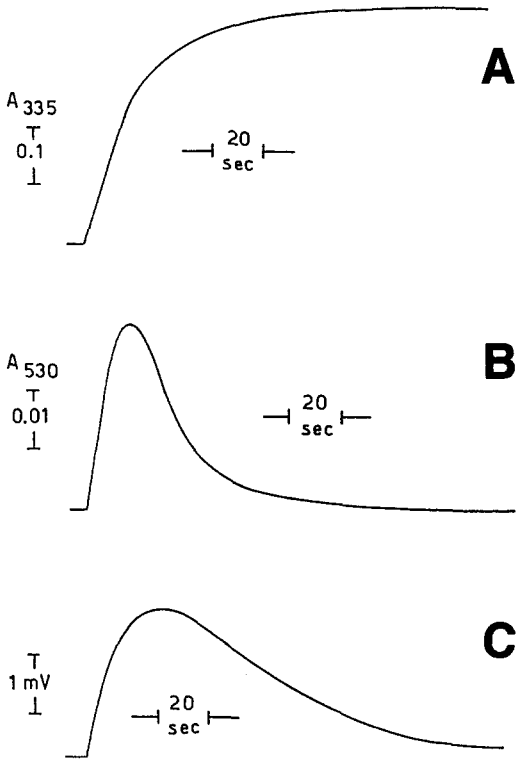


Fig. 3. Typical kinetic of promethazine sulfoxide formation followed by increase of absorbance at 335 nm (A) and 530 nm (B) or luminescence emission (C). One milliliter of final volume of 0.25 M acetate buffer pH 5.5 contained 75 mM promethazine, 100 mM H_2O_2, and 4.5 mg peroxidase. In (C) concentrations were 10 times higher.

The pH velocity profile of the reaction at 335 nm is a bell-shaped curve with a pH optimum around 6. The peroxidase-catalyzed sulfoxidation occurs with a Km of 110 ± 5 μM (mean ± SD) for promethazine, 111 ± 6 for H_2O_2, and K_i of 26.8 ± 3.1 for ascorbic acid, 41.8 ± 6.2 for glutathione, and 59.7 ± 4.1 for promethazine sulfoxide. Velocity increased linearly with increasing concentrations of the enzyme up to 10 μg/ml.

Glutathione acts as a sulfoxidation inhibitor being completely oxidized at completion of the reaction (data not shown). The incubation of promethazine sulfoxide with glutathione induces a slow oxidation of the latter, in the absence of peroxidase, with a half-life of 100 min. Superoxide dismutase has no effect on the peroxidase-catalyzed sulfoxidation of promethazine.

The peroxidase-catalyzed sulfoxidation of promethazine requires oxygen, and the reaction velocity decreased three to four times in a medium deaerated by nitrogen bubbling.

Figure 4 shows the consumption of oxygen that parallels the formation of promethazine sulfoxide detected with the oxygraph.

Figure 5 shows the ESR spectrum of the free radical cation generated in the peroxidase-catalyzed sulfoxidation of promethazine.

The radical concentration was monitored by acquiring the ESR spectra in a short time, with a sweep width of 50 G, sweep time of 1000 s, and modulation field 0.4 G_{pp}. The reaction mixture contained in 1 ml of 0.1 M acetate buffer pH 3.5, 0.9 mM H_2O_2, 0.65 mM promethazine, and 270 μg peroxidase. The ESR experiments were performed at acid pH to increase the half-life time of the cation radical.[16] The rate of sulfoxide generation was followed spectrophotometrically at 335 nm under the conditions used to acquire the ESR spectra and a first order rate constant of 2.0×10^{-3} s^{-1} was obtained, while that of the radical decay was 1.9×10^{-3} s^{-1}.

Table 1 summarizes the neuroleptic activity of both promethazine and its sulfoxide in the rat. The difference between the effect of the compounds is not significant.

The protective effect of promethazine on lipid peroxidation can be ascribed to its electron donor properties. In fact, when promethazine is sulfoxidized, its antioxidant characteristics disappear (Fig. 1). The same seems to be true for the reaction in which the phenothiazine ring forms a free radical upon UV irradiation.[11]

The peroxidase-catalyzed oxidation of promethazine (P) yields promethazine sulfoxide (PO) as a final product with a nearly quantitative yield. The intermediate free radical cation ($P^{+\bullet}$) together with other species is produced in the course of the reaction. ESR documents the formation of $P^{+\bullet}$ while the behavior of the luminescence indicates the formation and decay of a luminescent intermediate. If we indicate with P* a luminescent excited singlet species and with P^{2+} the red phenazathionium detected at 530 nm, the following may be a plausible mechanism for the reaction:

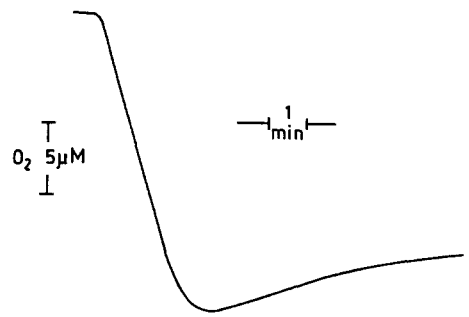


Fig. 4. Oxygraph tracing of a peroxidase-catalyzed sulfoxidation of promethazine. One milliliter of 0.25 M acetate buffer pH 5.5 contained 4.5 μg enzyme, 100 μM H_2O_2, 80 μM promethazine, and 2 mM EDTA.

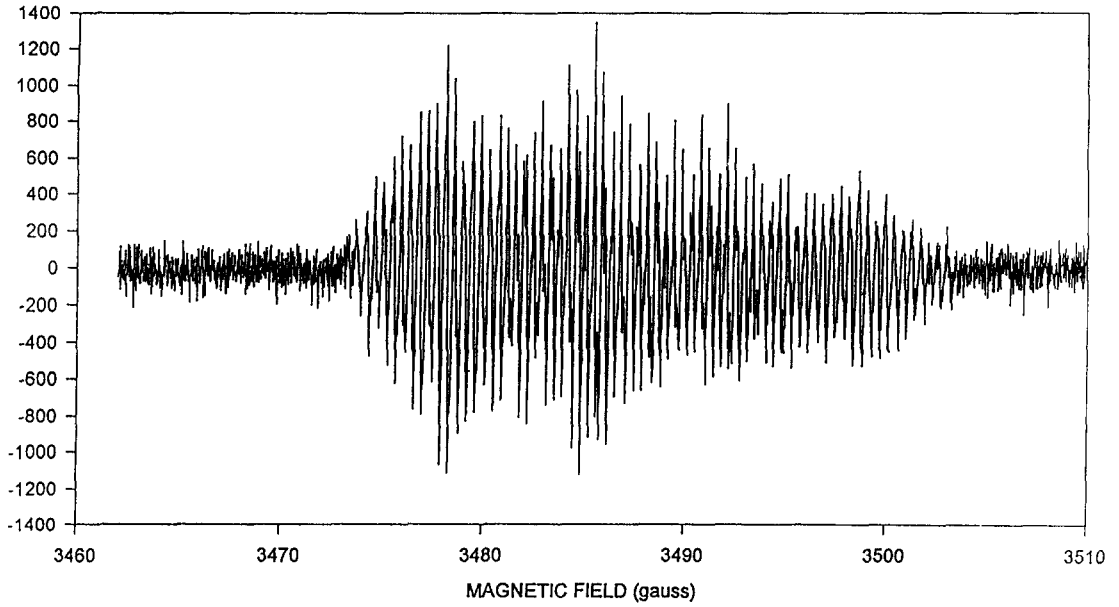


Fig. 5. ESR spectrum of the cation radical generated by addition of peroxidase to a solution deaerated with nitrogen containing the reagents described in Materials and Methods. Conditions were: 50 G sweep width, 1000 s sweep time, 40 mGpp modulation field, 20 mW microwave power, 9.82 GHz microwave frequency, and 200 ms integration constant.

$$1.\ 4P + 2H_2O_2 + 4H^+ \xrightarrow{\text{peroxidase}} 4P^{+\bullet} + 4H_2O$$

$$2.\ 4P^{+\bullet} + O_2 + 4H^+ \rightarrow 4P^{2+} + 2H_2O$$

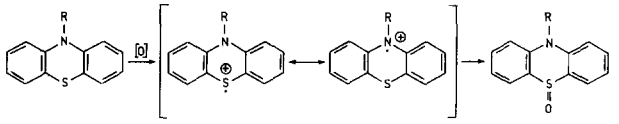
$$3.\ 4P^{2+} + 4H_2O \rightarrow 4PO + 8H^+$$

$$\text{Overall: } 4P + 2H_2O_2 + O_2 \rightarrow 4PO + 2H_2O$$

In addition to the main reaction, side-reactions can be considered, such as:

$$P^{+\bullet} + P^{+\bullet} \rightarrow P^{2+} + P^* \ (\rightarrow P + h\nu)$$

From a structural viewpoint, we may describe the reaction as:



where

$$R = -CH_2-CH(CH_3)-N(CH_3)_2$$

According to the proposed reaction scheme, if the $P^{+\bullet}$ radical disappears mostly by reaction 2, the decay should follow an apparent first-order kinetic rate law, because under our experimental conditions $(O_2) \gg (P^{+\bullet})$. The ESR data show that the radical decay is first order and the kinetic constant measured is similar to that of sulfoxide formation monitored at 335 nm.

The consumption of oxygen documented with the oxygraph supports the stoichiometric ratios of the reagents in the overall reaction, which is compatible with the classical view of the peroxidase-catalyzed oxidations.[17]

Glutathione and ascorbic acid may act as competitors of promethazine, while the lack of effect of superoxide dismutase may indicate that superoxide formation is not involved in the sulfoxidation reaction or in the formation of the $P^{+\bullet}$ radical (not shown).

The inhibitory action of promethazine sulfoxide may be related to its direct interaction with the peroxi-

Table 1. Action of Promethazine

	Sleeping Time (min)
Control	80 ± 30
+ P	140 ± 10
+ PO	125 ± 35[a]

HCl (P) and promethazine sulfoxide. HCl (PO) on the length of sleep induced by sodium 5,5-diethylbarbiturate. The rats were injected IP with the phenothiazines (5 mg/kg) and after 30 min sleep was induced with IP sodium 5,5-diethylbarbiturate (40 mg/kg). Means ± SD of five experiments.

[a]Difference with P not significant by Student's *t*-test.

dase molecule, documented by hyperchromic and hypochromic effects induced by promethazine sulfoxide on the absorbance peaks of the protein (data not shown).

Glutathione induces a lag time in the kinetic of increase of absorbance at 335 nm and at 530 nm, and the same glutathione-dependent lag time is observed in oxygen consumption.

The sulfoxidation of promethazine does not significantly affect its apparent neuroleptic effect, as it was observed with phenothiazines chemically modified in their alkylamminic chain.[9] We suggest that promethazine sulfoxide may be transformed in vivo to the same active species responsible for the neuroleptic effect of promethazine.

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