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REACTIVITY OF VARIOUS PHENOTHIAZINE DERIVATIVES WITH OXYGEN AND OXYGEN RADICALS*

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Abstract—7,8-Dihydroxychlorpromazine, 7,8-dihydroxyprochlorperazine and 7,8-dihydroxyperphenazine all reacted with O_2 to make hydrogen peroxide (H_2O_2). The rate of reaction between the *ortho*-dihydroxyphenothiazines and O_2 was increased by superoxide dismutase, an enzyme which catalyzes the dismutation of the superoxide radical (O_2^-) to H_2O_2 and O_2. This indicated the formation of O_2^- during the autoxidation of the *ortho*-dihydroxyphenothiazines. Several phenothiazines lacking the *ortho*-dihydroxy groups did not consume O_2 at a detectable rate (did not generate H_2O_2). All *ortho*-dihydroxyphenothiazines tested also reacted with O_2 in the presence of methional to form ethylene. Ethylene formation was inhibited by catalase and hydroxyl radical (·OH) trapping agents, such as sodium benzoate; this indicated ·OH formation from the *ortho*-dihydroxyphenothiazines. In addition, several nonhydroxylated phenothiazines and monohydroxylated phenothiazines inhibited ethylene generation from 6-aminodopamine, which also generates ·OH. This inhibition was probably mediated by a reaction between the phenothiazine and ·OH. All of the above reactions (generation of H_2O_2, O_2^- or ·OH or reaction with ·OH) may be responsible for some of the beneficial and/or adverse effects of administered phenothiazines.

Chlorpromazine (CPZ) or other substituted phenothiazines are used extensively in the treatment of schizophrenic patients. Chloropromazine can be hydroxylated in model systems to form both monohydroxylated and dihydroxylated derivatives [1–3], several of which have been isolated from the urine of schizophrenic patients on CPZ therapy [4]. It has been shown that 7-hydroxychlorpromazine (7-OH-CPZ) possesses pharmacological properties similar to those of CPZ itself [5]. It has been postulated that a hydroxylated derivative like 7,8-dihydroxychlorpromazine (7,8-diOH-CPZ) might play a role in the toxicological manifestations of skin pigmentation [3] and corneal opacity formation [6] from CPZ therapy. The availability of several substituted phenothiazines [CPZ, prochlorperazine (Ppz) and perphenazine (Pz)] and their corresponding 7-hydroxy, 8-hydroxy or 7,8-dihydroxy derivatives led us to investigate some of their biochemical properties. We report that *ortho*-dihydroxylated phenothiazine derivatives can react with oxygen to form the very reactive and potentially toxic chemical species: hydrogen peroxide, the superoxide radical and the hydroxyl radical. In addition, nonhydroxylated or monohydroxylated phenothiazines (both 7-hydroxy and 8-hydroxy) can react with the hydroxyl radical. Perhaps these above properties (generation of hydrogen peroxide, superoxide radical or hydroxyl radical, or reactivity with the hydroxyl radical) might provide clues as to how the therapeutic and/or adverse effects of these phenothiazine-type drugs can be realized.

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MATERIALS AND METHODS

Chemicals and enzymes. CPZ HCl and Ppz dimaleate were generous gifts of Smith Kline & French Labs, Philadelphia, Pa. Other phenothiazine derivatives (7-OH-CPZ, 8-OH-CPZ, 7,8-diOH-CPZ HCl, 7-OH-8-OMe-CPZ, 7,8-dioxo-CPZ HCl, 7-OH-Ppz, 8-OH-Ppz, 7,8-diOH-Ppz diHCl, Pz diHCl, 7-OH-Pz diHCl, 8-OH-Pz diHCl and 7,8-diOH-Pz diHCl) were synthesized and provided by the Psychopharmacology Research Branch, National Institute of Mental Health, Rockville, Md. Professor J. Cymerman Craig, University of California Medical Center, San Francisco, Calif. generously provided 3,7-diOH-CPZ. 6-Aminodopamine diHCl (2-amino-4,5-dihydroxyphenylethylamine dihydrochloride) was a generous gift of Dr. E. Engelhardt of Merck Sharp & Dohme, West Point, Pa. Methional (β-methylthiopropionaldehyde) was purchased from the Sigma Chemical Co., St. Louis, Mo. All other chemicals used were obtained from standard sources and were of the highest available grade.

Superoxide dismutase (3000 units/mg) was purchased from Truett Laboratories, Dallas, Tex. Catalase (54,000 units/mg) was purchased from Worthington Biochemicals, Freehold, N.J. The stock catalase suspension was centrifuged at 700 *g* for 10 min; the supernatant was discarded and the pellet resuspended in distilled water in two times the original volume.

Two buffers were used: 0·2 M acetate at pH 6·4 containing 10^{-4} M EDTA and modified Krebs–Ringer phosphate at 7·4, which consisted of 118 mM NaCl, 15·9 mM sodium phosphate, 4·7 mM KCl, 1·8 mM $CaCl_2$, 1·2 mM $MgSO_4$ containing 1·3 mM EDTA and 5·6 mM glucose.

Reaction of compounds with oxygen (O_2^- and H_2O_2 generation). Oxygen consumption by various compounds was studied on a Biological Oxygen Monitor (Yellow Springs Instruments) connected to a Honeywell Electronik 19 recorder. The temperature of the system was kept at 37° by a circulating water pump. Oxygen consumption was measured in 1·0 ml of the Krebs–Ringer phosphate buffer at pH 7·4. Various compounds were added with a 10- or 20-μl Oxford automatic pipette from N_2-sparged stock solutions.

Ethylene formation ($\cdot OH$ generation). A modification [7] of the system described by Beauchamp and Fridovich [8] was used. The reaction medium consisted of 1·0 ml of 10^{-3} M methional at pH 6·4 and 37°, contained in an 18-ml test tube which was sealed with a screw cap and a silicone septum. The ethylene-generating reaction was initiated by addition (Hamilton syringe) of 100 μl of ice-cold, N_2-sparged aqueous solutions of various compounds. Samples (0·5 to 1·0 ml) of head gas were removed by syringe and injected immediately into a Hewlett–Packard 5750 gas chromatograph, which was equipped with a flame ionization detector, a Beckman recorder and a 6-foot Poropak N column operated at 90°. Zero grade gases were used throughout. The sensitivity of the instrument was 3·09 scale divisions for 1·00 pmole of injected ethylene. The response was linear over the range of ethylene concentrations encountered in this study.

RESULTS

O_2 consumption (O_2^- and H_2O_2 generation). Typical experiments run on an oxygen electrode are shown in Fig. 1. 7,8-diOH-CPZ, 2×10^{-4} M, reacted very rapidly with O_2; catalase, which was added after the reaction had nearly stopped, caused the return to the solution of almost exactly one-half of the consumed O_2 (Fig. 1A). This indicates that consumed O_2 can be equated to H_2O_2 formation.

After the consumption of O_2 by 7,8-diOH-CPZ had nearly stopped, the addition of ascorbic acid caused renewed consumption of O_2 (Fig. 1B). Glutathione and dithiothreitol had the same effect as ascorbic acid (data not shown). 7,8-Dioxo-CPZ, the orthoquinone of 7,8-diOH-CPZ, did not consume O_2 by itself (Fig. 1C). However, the addition of ascorbic acid (Fig. 1C), glutathione or dithiothreitol (data not shown) caused rapid consumption of O_2. Under the conditions of these experiments, none of the above reducing agents (ascorbic acid, glutathione or dithiothreitol) by itself consumed O_2.

Two other dihydroxyphenothiazines, namely 7,8-diOH-Ppz and 7,8-diOH-Pz, also reacted quite rapidly with O_2 (Table 1); the use of catalase again indicated that O_2 consumption could be equated to H_2O_2 formation. The initial rates of O_2 consumption were 57, 133 and 93 nmoles O_2/min for 7,8-diOH-CPZ, 7,8-diOH-Ppz and 7,8-diOH-Pz respectively. Several other phenothiazines, namely CPZ, 7-OH-CPZ, 3,7-diOH-CPZ and 7-OH-8-OMe-CPZ, did not consume O_2 at a detectable rate (Table 1).

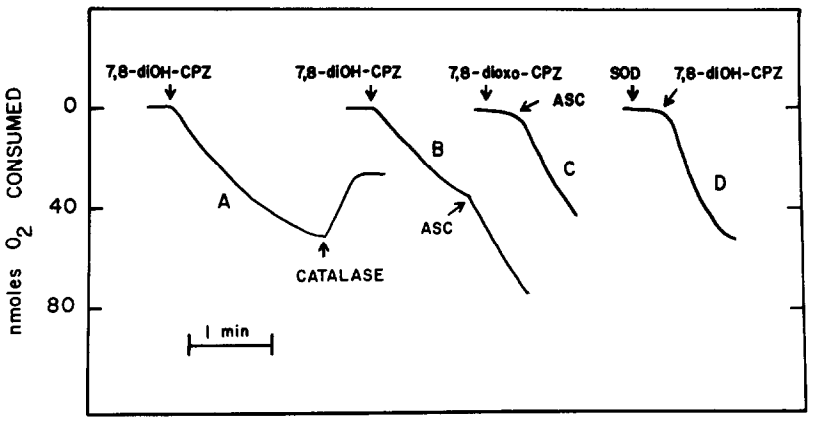

Fig. 1. Consumption of oxygen by 7,8-dihydroxychlorpromazine (7,8-diOH-CPZ) and 7,8-dioxochlorpromazine (7,8-dioxo-CPZ); effects of catalase, ascorbate (ASC) and superoxide dismutase (SOD). Oxygen consumption was measured with a Biological Oxygen Monitor at 37° in 1 ml of Krebs–Ringer phosphate buffer. Points of addition of the various substances are shown by arrows. Final concentrations were: 7,8-diOH-CPZ and 7,8-dioxo-CPZ, 2×10^{-4} M; catalase, 0·2 mg/ml; ascorbic acid, 10^{-2} M; and SOD, 100 μg/ml.

Table 1. Effect of superoxide dismutase (SOD) on the initial rate of oxygen consumption by various phenothiazines (2×10^{-4} M)*

Compound	Initial rate of O_2 consumption† − SOD	+ SOD
Chlorpromazine	< 2 (3)‡	< 2 (3)
7-Hydroxychlorpromazine	< 2 (3)	< 2 (3)
7-Hydroxy-8-methoxychlorpromazine	< 2 (3)	< 2 (3)
3,7-Dihydroxychlorpromazine	< 2 (3)	< 2 (3)
7,8-Dihydroxychlorpromazine	57 ± 4 (19)	138 ± 12 (18)
7,8-Dihydroxyprochlorperazine	133 ± 13 (10)	250 ± 32 (9)
7,8-Dihydroxyperphenazine	93 ± 12 (9)	226 ± 28 (9)

* Conditions are the same as in Fig. 1, except that in some experiments SOD (100 μg/ml) was added just prior to the addition of the phenothiazine derivative. Boiled SOD had no stimulatory effect in this system. The number of determinations is in parentheses.

† nmoles O_2/min ± S.E.M.

‡ Limit of detection.

Superoxide dismutase (SOD) is an enzyme which breaks down the superoxide radical (O_2^-) to H_2O_2 and O_2 [9, 10]. Any effect of SOD in an experimental reaction system suggests the involvement of O_2^- in the reaction. The addition of SOD prior to the addition of any of the 7,8-dihydroxy derivatives resulted in a large increase (an approximate doubling) in the initial rate of O_2 consumption (Table 1; also compare Fig. 1, A and D). Boiled SOD caused no increase in the rate of O_2 consumption.

Ethylene production (·OH generation). Beauchamp and Fridovich [8] showed recently that hydroxyl radicals (·OH) generated from a xanthine oxidase–xanthine experimental system could react with methional to form ethylene. The 7,8-dihydroxyphenothiazines also reacted with methional to form ethylene (Table 2). The addition of catalase to the reaction medium resulted in almost complete inhibition of ethylene formation (Table 2). This indicates some role for H_2O_2 in ethylene production. However, H_2O_2 by itself generates no ethylene under these experimental conditions [7]. There was no consistent effect of SOD on ethylene formation (Table 2). However, sodium benzoate, which is known to react with the hydroxyl radical, was a potent inhibitor of ethylene formation.

Inhibition of ethylene production (reaction with ·OH). In the presence of methional, 6-aminodopamine generates large amounts of ethylene, presumably by a reaction between methional and the hydroxyl radical, which is generated during the autoxidation of 6-aminodopamine [7]. The generation of ethylene from 6-aminodopamine and methional was inhibited by all nonhydroxylated or monohydroxylated phenothiazine derivatives tested (Table 3). The compounds ranged in effectiveness from 8-OH-Pz which caused an 82 per cent inhibition to 7-OH-Pz which caused only a 21 per cent inhibition.

DISCUSSION

All *ortho*-dihydroxy phenothiazines tested rapidly consumed O_2 (Fig. 1, Table 1), while those phenothiazines lacking the *ortho*-dihydroxy substitution (CPZ, 7-OH-CPZ, 3,7-diOH-CPZ and 7-OH-8-OMe-CPZ) did not react with O_2 at a detectable rate. The comparative rates of autoxidation (O_2 consumption) indicated the importance of structure in determining the chemical reactivity of the various substituted phenothiazines. Catalase caused the return to solution of approximately one-half of the consumed O_2 (Fig. 1).

Table 2. Ethylene production measured at 40 min from methional (10^{-3} M) and various dihydroxylated phenothiazines (10^{-3} M)—Effect of catalase, superoxide dismutase (SOD) and sodium benzoate*

Compound	None	Additions Catalase	SOD	Sodium benzoate
7,8-DiOH-CPZ	4·17 ± 0·90	0·07 ± 0·006 (−98)	3·73 ± 1·43 (−11)	1·15 ± 0·06 (−72)
7,8-DiOH-Ppz	14·92 ± 2·19	0·26 ± 0·02 (−98)	16·72 ± 1·93 (+12)	9·60 ± 0·04 (−36)
7,8-DiOH-Pz	13·51 ± 2·40	0·22 ± 0·02 (−98)	19·07 ± 2·30 (+41)	7·25 ± 0·01 (−46)

* To 0·9 ml of 0·2 M acetate buffer at pH 6·4 containing 10^{-4} M EDTA and 10^{-3} M methional was added by injection (Hamilton syringe) 100 μl of concentrated solutions of various compounds in N_2-sparged water. The catalase (16 μg), SOD (50 μg) or sodium benzoate (10^{-2} final concentration) were present prior to addition of the phenothiazine. Data are expressed as nmoles ethylene generated during the 40-min incubation period ± S. D. (n = 3). The percent change from the appropriate control is given in parentheses.

Table 3. Inhibition of ethylene production from 6-aminodopamine (10^{-4} M) by various phenothiazine derivatives*

Phenothiazine	Per cent inhibition of ethylene formation at 40 min
Chlorpromazine	72 ± 6
7-Hydroxychlorpromazine	64 ± 8
8-Hydroxychlorpromazine	37 ± 4
Prochlorperazine†	58 ± 6
7-Hydroxyprochlorperazine	60 ± 14
8-Hydroxyprochlorperazine	45 ± 13
Perphenazine	74 ± 3
7-Hydroxyperphenazine	21 ± 6
8-Hydroxyperphenazine	82 ± 2

* The reaction system is the same as that described for Table 2. The inhibitors (10^{-2} M final concentration) were present before the addition of the buffer. Those inhibitors which were salts (see Materials and Methods) were dissolved in 100 μl water, while those which were free bases were dissolved in 100 μl of 0·1 N HCl. The reaction was started by the addition of 6-aminodopamine and continued for 40 min. The per cent inhibition was calculated by comparison to the 6-aminodopamine sample (1·51 ± 0·29 n-moles ethylene/40 min; n = 10). Data are expressed as per cent inhibition ± S.D. Three to ten determinations were carried out on each phenothiazine.

† Prochlorperazine (dimaleate salt) lowered the pH of the acetate buffer. Since ethylene formation is pH-dependent (unpublished observations), we titrated control samples to the appropriate pH and compared the effects of prochlorperazine to this control.

This indicated that O_2 consumption may be equated to H_2O_2 generation according to the following reaction sequence, using 7,8-diOH-CPZ as an illustration:

$$2(7,8\text{-diOH-CPZ}) + 2\,O_2 \longrightarrow 2(7,8\text{-dioxo-CPZ}) + 2\,H_2O_2 \quad (1)$$

$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2 \quad (2)$$

Thus, of 2 moles of O_2 consumed in the first reaction, 1 mole would be regenerated by reaction 2. We previously showed, using catalase, that 6-hydroxydopamine [11], 6-aminodopamine [12] and dialuric acid [13] all reacted with O_2 to make H_2O_2. The 7,8-dioxo-CPZ, the other endproduct of the autoxidation of 7,8-diOH-CPZ [3] (reaction 1), by itself did not react with O_2. However, the addition of a suitable reducing agent caused the rapid consumption of O_2 by 7,8-dioxo-CPZ. This was probably due to the reduction of 7,8-dioxo-CPZ (reaction 3) to 7,8-diOH-CPZ (or to a semiquinone free radical intermediate) and the subsequent autoxidation (see reaction 1 above):

$$7,8\text{-dioxo-CPZ} + RH_2 \rightarrow 7,8\text{-diOH-CPZ} + R \quad (3)$$

(where RH_2 represents a reducing agent such as ascorbic acid, glutathione or dithiothreitol).

It is generally accepted [14] that in autoxidation reactions H_2O_2 is generated by means of two subsequent one-electron transfers (reaction 4) rather than one simultaneous two-electron transfer:

$$O_2 \xrightarrow{1e} O_2^- \xrightarrow{1e} O_2^{=} \xrightarrow{2H^+} H_2O_2 \quad (4)$$

This initial species, formed by the one-electron reduction of O_2 is O_2^-, the superoxide radical. McCord and Fridovich [9] first isolated and described an enzymatic function for superoxide dismutase, an enzyme which catalyzes the breakdown of the superoxide radical:

$$2\,O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

Superoxide dismutase had a stimulatory effect on the initial rate of O_2 consumption by the 7,8-dihydroxyphenothiazine derivatives (Table 1). This indicated that the superoxide radical was formed during the autoxidation process. Since a stimulatory action of SOD was observed, the implication was that O_2^- slowed the rate of autoxidation of the 7,8-dihydroxyphenothiazine derivatives. It is of interest that in another system, namely the autoxidation of 6-hydroxydopamine, SOD inhibited the rate [15], thereby indicating a role of O_2^- as a catalyst.

Beauchamp and Fridovich [8] previously showed that a xanthine-oxidase experimental system, which also generated H_2O_2 and O_2^-, could react in the presence of methional to form ethylene. This ethylene generation was inhibited by catalase and by superoxide dismutase, as well as by hydroxyl radical (·OH) trapping agents. This led Beauchamp and Fridovich [8] to postulate that ·OH was generated by the Haber–Weiss reaction [16]:

$$H_2O_2 + O_2^- \rightarrow \cdot OH + OH^- + O_2 \quad (6)$$

The ·OH would then, in turn, react with methional to form ethylene:

$$\text{methional} + \cdot OH \rightarrow \text{ethylene} \quad (7)$$

Our data (Table 2) showed that the 7,8-dihydroxyphenothiazines also reacted in the presence of methional to generate ethylene. The inhibitory effect of sodium benzoate (Table 2), a compound known to react with ·OH [17], suggested that ethylene formation was also mediated by ·OH. The almost complete inhibition by catalase indicated that H_2O_2 was required for ·OH generation. We previously showed that catalase also inhibited ethylene formation caused by 6-hydroxydopamine, 6-aminodopamine, dialuric acid and 6,7-dihydroxytryptamine [7]. Hydrogen peroxide by itself was not capable of promoting ethylene generation from methional under these experimental conditions (viz. at pH 6·4) [7].

On theoretical ground, SOD might be expected either to lower or to raise levels of ·OH, depending upon the conditions. SOD might lower the levels of ·OH (and thus inhibit ethylene production) by scavenging O_2^- and thereby slowing the rate of the Haber–Weiss reaction (reaction 6). Alternatively, SOD

might raise levels of ·OH (and thus stimulate ethylene production) by preventing reaction 8 [18].

$$O_2^- + \cdot OH \rightarrow O_2 + OH^- \quad (8)$$

In our experimental system (Table 2), SOD was not consistent in causing either inhibition or stimulation of ethylene production from the *ortho*-dihydroxylated phenothiazines. The lack of a consistent effect of SOD may indicate that both of the above phenomena were occurring simultaneously. In another study [7], we found that SOD inhibited ethylene generation from 6-aminodopamine, dialuric acid or 6,7-dihydroxytryptamine. With 6-hydroxydopamine, a more detailed study of the time course of ethylene generation showed that SOD was inhibitory at early time periods, but actually caused a slight stimulation at later times [7].

If the Haber–Weiss reaction (reaction 6) were the sole source of ·OH radicals during the autoxidation of the *ortho*-dihydroxylated phenothiazines, it seems reasonable to expect that SOD would have had a powerful inhibitory action on ethylene generation, similar to that exhibited by catalase (Table 2). It may be that there were other H_2O_2-dependent (viz. catalase-inhibited) sources of ·OH in the system. The possibilities include the reaction of H_2O_2 with the *ortho*-dihydroxylated phenothiazines themselves, or with free radicals derived from the phenothiazines during the autoxidation process.

The formation of ·OH and the generation of ethylene from methional during autoxidation of 6-aminodopamine have been studied previously [7]. In this system (autoxidation of 6-aminodopamine), a large group of phenothiazine derivatives inhibited ethylene formation (Table 3), presumably by reaction with ·OH. The *ortho*-dihydroxylated phenothiazine derivatives could not be studied in this regard because they generated ethylene in the test system (Table 2).

CPZ readily loses an electron to form a stable free radical [19]. The action of this species in either the beneficial or harmful effects of CPZ therapy is not clear. We have shown that the *ortho*-dihydroxylated phenothiazines react with oxygen to form H_2O_2 and the radicals, O_2^- and ·OH. All of these species are very reactive and have been implicated in a variety of toxic actions. For example, 6-aminodopamine and 6-hydroxydopamine (which cause degeneration of catecholamine nerve terminals), 6,7-dihydroxytryptamine (which causes degeneration of serotonin nerve terminals) and dialuric acid (which causes experimental diabetes in normal animals as well as erythrocyte hemolysis in vitamin E-deficient animals), all make H_2O_2, O_2^- and ·OH [7]. It has been shown that H_2O_2 can inhibit the biogenic amine uptake systems of neuronal membranes [11]. There exists evidence that both H_2O_2 [20] and O_2^- [21] are required for the destruction of microorganisms by phagocytes. In addition, it has been reported that O_2^- is cytotoxic to microorganisms [22]. Hydroxyl radicals have also been implicated in damage to DNA caused by high-energy irradiation [23, 24]. Perhaps some of the adverse effects caused by chlorpromazine treatment, such as corneal and lenticular opacities or hyperpigmentation of the skin, might be caused by H_2O_2, O_2^- or ·OH derived from chlorpromazine metabolites. It is also of interest that schizophrenics maintained on long-term CPZ have a low incidence of cancer [25]; in addition, several commonly used anticancer compounds react quite well with free radicals [26].

All of the above data indicate that some phenothiazines can generate reactive species (H_2O_2, O_2^- or ·OH), while other phenothiazines can react with reactive species (·OH). The extent to which administered phenothiazine might either react with these species or generate them would depend on the degree of metabolism (hydroxylation) of the phenothiazine, as the non-hydroxylated or monohydroxylated derivatives react with ·OH, while the *ortho*-dihydroxylated phenothiazines generate ·OH (as well as H_2O_2 and O_2^-). All this points to a danger in the field of drug therapy, since normal metabolism of administered drugs might well generate harmful products from therapeutic agents.

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