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INTERACTION OF PHENOTHIAZINE DERIVATIVES WITH HUMAN CERULOPLASMIN

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Abstract—Tranquillizing drugs of the phenothiazine class (promazine, chlorpromazine, triflupromazine and levomepromazine) were oxidized to free radicals by human ceruloplasmin. The blue colour of the enzyme, due to protein-bound cupric atoms, was reduced by addition of phenothiazine derivatives. In the presence of reducing agents (NADH, NADPH, reduced glutathione and ascorbate) the rate of the ceruloplasmin-catalyzed oxidation of phenothiazine derivatives was markedly increased. NADH was oxidized during the process, suggesting that the activating effect is due to a reduction of phenothiazine derivative radicals, which rapidly react with several reducing agents. Straight lines were obtained when the reciprocal rate of NADH oxidation was plotted against the reciprocal phenothiazine derivative concentration ($1/V$ vs $1/[S]$). The V_{max}-values for the four substrates investigated do not vary significantly. At lower substrate concentrations, however, the rate of triflupromazine oxidation was considerably slower than the rates obtained with the other substrates. Phenothiazine derivatives activate the enzymic oxidation of dopamine and dopa, probably by acting as a cycling intermediate between ceruloplasmin and catecholamine.

PHENOTHIAZINE derivatives are readily oxidized to free radicals by several metal ions (Fe^{3+}, Co^{3+}, Mn^{3+}, Ce^{4+}),[1,2] and by the iron-containing enzymes, catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7).[3] A kinetic study of the peroxidase catalyzed oxidation of chlorpromazine has been made by Piette *et al.*[4]

Although free cupric ions are unable to oxidize phenothiazine derivatives,[1] it was of interest to investigate if the serum oxidase, ceruloplasmin (EC 1.12.3.-),* containing cupric atoms, could react with compounds of the phenothiazine class. Ceruloplasmin has oxidase activity towards *p*-phenylenediamine derivatives, producing free radicals as reported by several investigators.[6–8] During the reaction an electron is transferred from the substrate to a protein-bound cupric ion.

In the present communication we report the results of a kinetic study of the interaction between ceruloplasmin and some phenothiazine derivatives.

MATERIALS AND METHODS

Human ceruloplasmin was obtained from AB Kabi and crystallized according to the method of Deutsch.[9] The purified enzyme had an absorbance ratio A_{610}/A_{280} of 0·041. Enzyme concentrations were calculated from the 610 nm absorption ($\epsilon = 10{,}900\ M^{-1}\ cm^{-1}$).[9]

* This code number is proposed by Osaki *et al.*[5]

The phenothiazine derivatives were obtained from different pharmaceutical companies. NADH, NADPH, dopamine,* dopa* and reduced glutathione were purchased from Sigma Chemical Co., ascorbate, $Na_2S_2O_4$ and $(NH_4)_2Fe(SO_4)_2.6H_2O$ from E. Merck and Desferal* from Ciba Pharmaceutical Co. The iron-chelating agent, Desferal, was added to all reaction mixtures in order to prevent the activating effect of iron ions on the ceruloplasmin-catalyzed oxidations.[10] All aqueous solutions were prepared in deionized, glass-distilled water.

Spectrophotometric measurements were carried out with a Beckman DK-1 recording spectrophotometer, equipped with a thermo cell (1-cm light path). The temperature was 30° in all experiments. The rate of oxygen uptake during ceruloplasmin-catalyzed oxidations was measured with an oxygen electrode made at the institute. The electrode was connected to a W + W 3012 recorder.

RESULTS

Immediately after addition of ceruloplasmin to a solution of chlorpromazine a red-coloured compound is formed, displaying an absorption maximum at 530 nm (Fig. 1a). This compound has been identified by Piette *et al.*[4] as a free radical by means of simultaneous electron–paramagnetic–resonance optical–absorption measurements. The reaction between ceruloplasmin and promazine produced a compound with an absorption maximum at 515 nm. The product concentration reached a maximum after about 10 min and then declined (Fig. 1b). The rate of radical formation from promazine was faster than the rate observed with chlorpromazine, indicating that promazine reacts more rapidly with the enzyme.

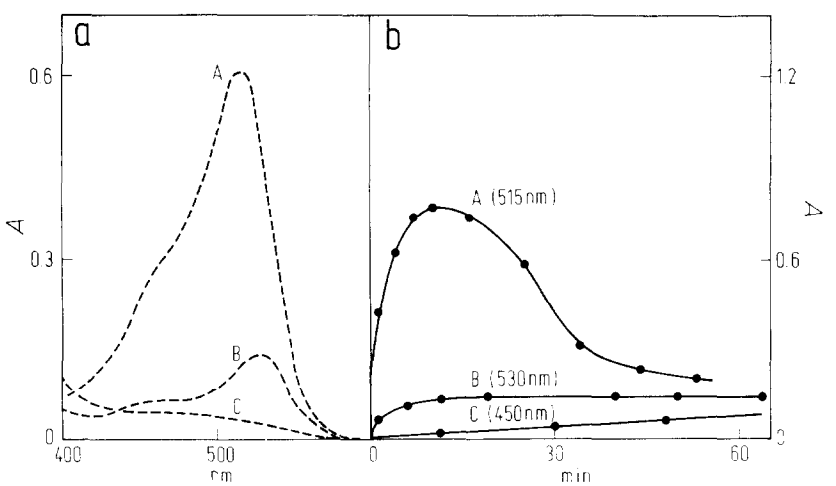


FIG. 1. (a) Oxidation of promazine (A), chlorpromazine (B) and triflupromazine (C) by ceruloplasmin. Spectra were recorded at 3 min (A), 15 min (B) and 20 min (C) after addition of enzyme. The reaction mixture contained 52 μM ceruloplasmin, 2·8 mM phenothiazine derivative, 30 μM Desferal and 2·8 mM Cl^- in 0·6 M sodium acetate buffer, pH 4·8. (b) Time course of the reaction observed on mixing ceruloplasmin and promazine (A), chlorpromazine (B) and triflupromazine (C). Experimental conditions were as described above.

* Dopamine, 3-hydroxytyramine; dopa, D-3,4-dihydroxyphenylalanine; Desferal, deferoxamine B-methane sulfonate.

When triflupromazine acted as substrate a broad band formed slowly in the visible region (Fig. 1a).

The blue colour of ceruloplasmin, due to protein-bound cupric ions (Type-1), decreased when a phenothiazine derivative was added to the enzyme solution as shown in Fig. 2, indicating that a reduction of Type-1 copper atoms occurred. The fastest rate of reduction was obtained with promazine, the order of reducing effectiveness of phenothiazine derivatives being promazine > levomepromazine > chlorpromazine > triflupromazine.

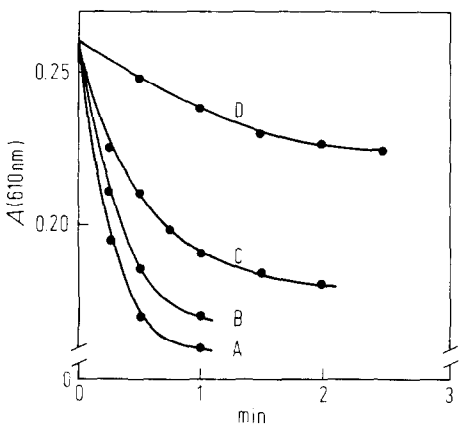


FIG. 2. Effect of promazine (A), levomepromazine (B), chlorpromazine (C) and triflupromazine (D) on the absorption of ceruloplasmin at 610 nm. The reaction mixture contained 24 μM ceruloplasmin, 0·54 mM phenothiazine derivative, 1·1 mM Desferal and 0·54 mM Cl^- in 0·8 M sodium-acetate buffer, pH 5·6.

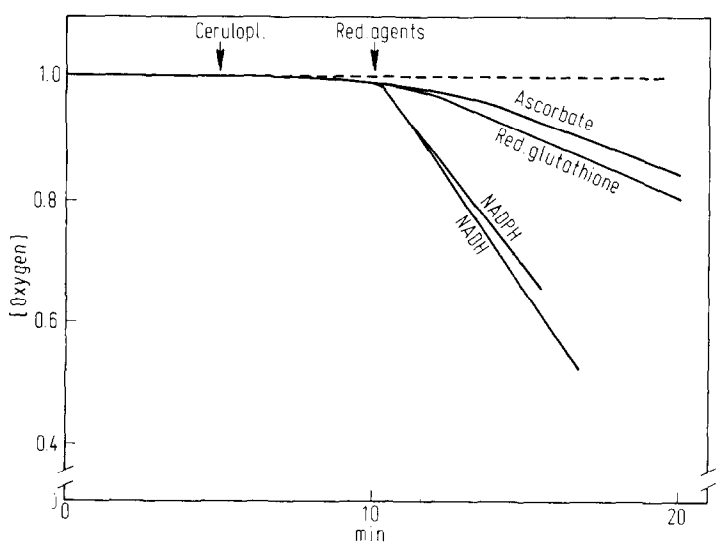


FIG. 3. Effect of reducing agents on the rate of oxygen uptake during the ceruloplasmin-catalyzed oxidation of chlorpromazine. The reaction mixture contained 1·5 μM ceruloplasmin, 2 mM chlorpromazine, 24 μM Desferal and 2 mM Cl^- in 0·25 M sodium-acetate buffer, pH 5·6. The concentration of NADH and NADPH was 0·7 mM, while the concentration of reduced glutathione and ascorbate was 1·75 mM. The oxygen concentration in the air saturated solution was set to 1.

Addition of reducing agents (NADH, NADPH, reduced glutathione and ascorbate) to a reaction mixture of ceruloplasmin and chlorpromazine markedly increased the rate of oxygen consumption (Fig. 3). Oxygen is used for reoxidizing cuprous ions during reaction. In the absence of ceruloplasmin or chlorpromazine no reaction took place. The stronger reducing agents, NADH and NADPH, increased the reaction rate more than the weaker ones (reduced glutathione and ascorbate). Similar results were obtained with the other phenothiazine derivatives investigated. NADH was oxidized in the presence of ceruloplasmin and phenothiazine derivatives as demonstrated in Fig. 4. The time course of the NADH oxidation was recorded

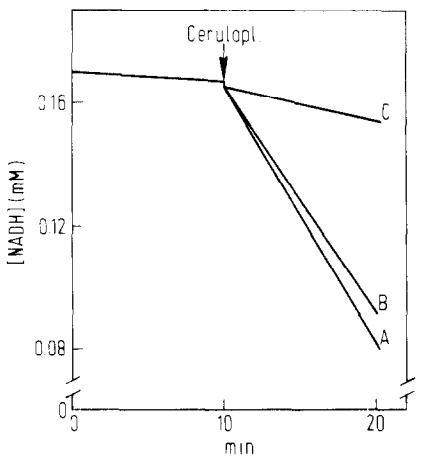


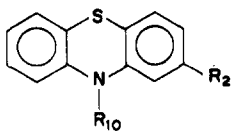
FIG. 4. Effect of promazine (A), chlorpromazine (B) and triflupromazine (C) on the oxidation of NADH in the presence of ceruloplasmin. The reaction mixture contained 0·85 μM ceruloplasmin, 0·4 mM phenothiazine derivative, 0·5 mM Desferal, 0·4 mM Cl^- and 0·17 mM NADH in 0·25 M sodium acetate buffer, pH 5·8.

spectrophotometrically at 340 nm. The fastest reaction was obtained with promazine and the slowest with triflupromazine. In the absence of phenothiazine derivatives only a slight auto-oxidation of NADH took place.

The experiments demonstrate that promazine radicals, generated according to the method of Borg and Cotzias,[1] spontaneously react with reducing agents, like NADH, reduced glutathione, ascorbate, dopamine, dopa, Fe^{2+} and $S_2O_4^{2-}$ (Fig. 5a). Twice as many radicals are reduced by NADH, reduced glutathione, ascorbate, dopamine and dopa as are reduced by the one-electron donors, Fe^{2+} and $S_2O_4^{2-}$ (Fig. 5b). Similar results were obtained with chlorpromazine radicals. The molar absorption of promazine- and chlorpromazine radicals was estimated to 11,500 M^{-1} cm^{-1} (515 nm) and 11,700 M^{-1} cm^{-1} (530 nm), respectively.

The effect of phenothiazine derivative concentration on the initial rate of NADH oxidation in the presence of ceruloplasmin is shown in Fig. 6. Straight lines were obtained in the double reciprocal plots. The apparent Michaelis constants (K_m) and maximum activities (V_{max}) are calculated from the plots in Fig. 6 and listed in Table 1. The V_{max}-value obtained with chlorpromazine is slightly higher than the one obtained with promazine. However, at lower substrate concentrations promazine was more rapidly oxidized than chlorpromazine. The rate of levomepromazine

TABLE 1. LIST OF KINETIC PARAMETERS



Compound	R_2	R_{10}	K_m (mM)	$V_{max}/[CP_0]$* (min^{-1})
Promazine	—H	$-CH_2CH_2CH_2N(CH_3)_2$	1·3	26
Chlorpromazine	—Cl	$-CH_2CH_2CH_2N(CH_3)_2$	2·2	32
Triflupromazine	$-CF_3$	$-CH_2CH_2CH_2N(CH_3)_2$	11·0	20
Levomepromazine	$-OCH_3$	$-CH_2CHCH_2N(CH_3)_2$ (CH$_3$ on CH)	0·9	20

* Total ceruloplasmin concentration.

oxidation was very close to the rates observed with promazine and chlorpromazine (Fig. 6), while triflupromazine was oxidized at a much slower rate. The K_m-value obtained with triflupromazine is considerably higher than the others (Table 1). Kinetic studies of the interaction of catecholamines with ceruloplasmin show that dopa is a poor substrate, inhibiting the enzymic oxidation of dopamine.[11] Dopa was also found to decrease the rate of the ceruloplasmin-catalyzed oxidation of promazine (Fig. 7).

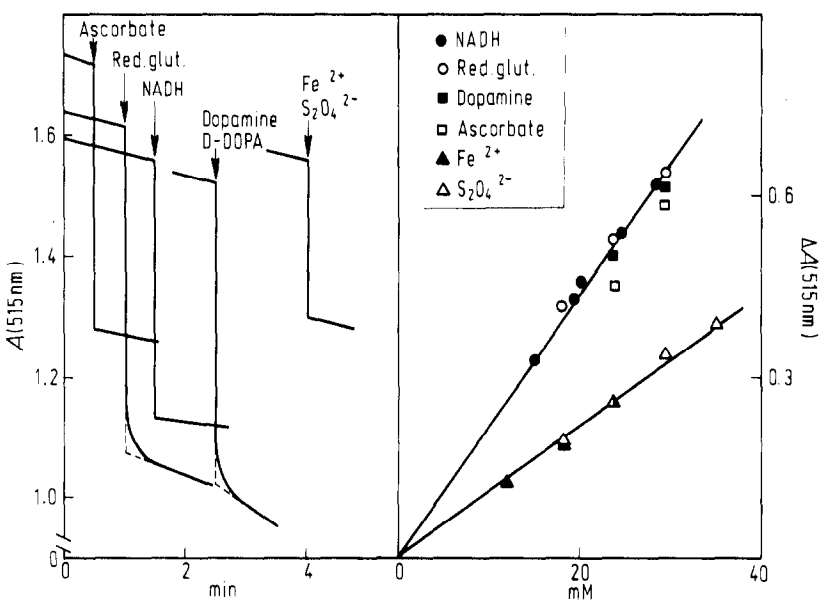


FIG. 5. (a) Effect of NADH (19·3 μM), reduced glutathione (28·4 μM), ascorbate (23·8 μM), dopamine (23·8 μM), dopa (23·8 μM), Fe^{2+} (23·8 μM) and $S_2O_4^{2-}$ (23·8 μM) on the absorption of promazine radicals at 515 nm. Free radicals were obtained in a solution of 6·6 mM $MnCl_2$ and 6·6 mM promazine (0·75 ml) by adjusting the pH to 7·5–8·0 with NaOH and adding 0·25 ml of 1 M sodium–acetate buffer, pH 4·8. The radical solution was diluted four times with water before adding reducing compounds. (b) ΔA (515 nm) plotted against the concentration of reducing agents.

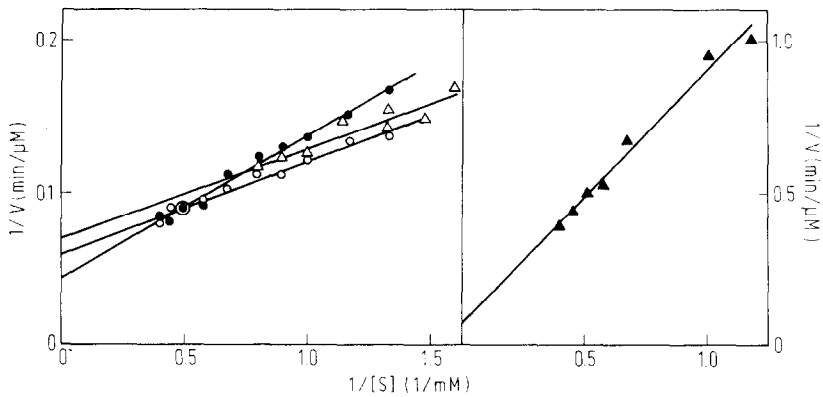


FIG. 6. Effect of promazine (○), chlorpromazine (●), levomepromazine (△) and triflupromazine (▲) on the rate of NADH oxidation in the presence of ceruloplasmin. The reaction mixture contained 0·68 μM ceruloplasmin, phenothiazine derivative (0·63–2·5 mM), 20 μM Desferal, 2·5 mM Cl^- and 0·17 mM NADH in 0·25 M sodium–acetate buffer, pH 5·6.

The effect of promazine and triflupromazine on the enzymic oxidation of dopamine and dopa is shown in Fig. 8. The phenothiazine derivatives increased the rate of catecholamine oxidation as reported by Barrass and Coult.[12] Promazine is a better activator than triflupromazine. It has been shown previously that one molecule of oxygen is consumed when one molecule of dopamine is transformed to chrome, the amount of chrome formed being equal to the total amount of oxygen in the solution.[13] This was also found to be the case in the promazine- and chlorpromazine-stimulated systems in the present study, suggesting that all the oxygen is used for chrome formation.

DISCUSSION

The results show that the phenothiazine derivatives investigated are catalytically oxidized to free radicals by ceruloplasmin. The promazine and chlorpromazine

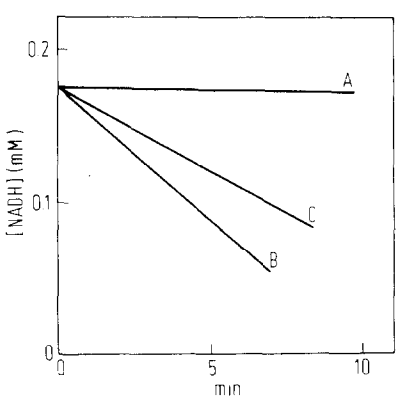


FIG. 7. Effect of dopa (A), promazine (B), and dopa and promazine (C) on the rate of NADH oxidation in the presence of ceruloplasmin. The reaction mixture contained 1 μM ceruloplasmin, 3 mM dopa (2 mM promazine), 0·5 mM Desferal and 0·17 mM NADH in 0·1 M sodium–acetate buffer, pH 5·6.

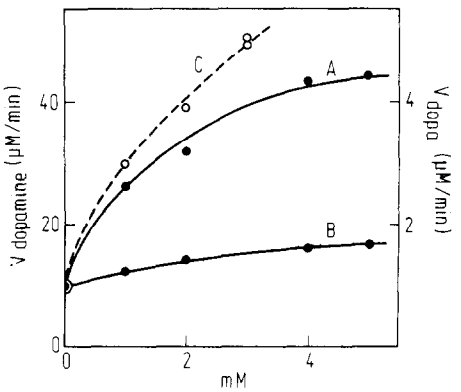
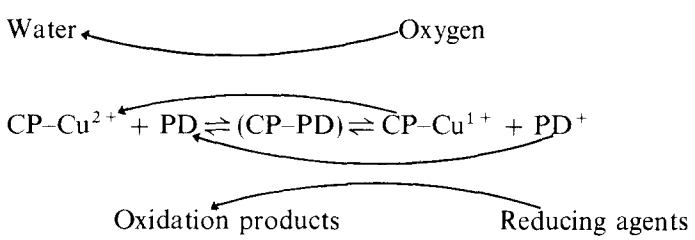


FIG. 8. Effect of promazine (A) and triflupromazine (B) on the rate of chrome formation from dopamine and effect of promazine on the rate of chrome formation from dopa (C). The reaction mixture contained 1 μM ceruloplasmin, phenothiazine derivative (1·5 mM), 0·5 mM Desferal, 1 mM dopamine (2 mM dopa) in 0·1 M sodium–acetate buffer, pH 5·6. The rate of chrome formation was measured spectrophotometrically at 480 nm ($\epsilon = 3500\ M^{-1}\ cm^{-1}$).

radicals are very stable at pH 4·8, the stability decreasing with increasing pH as reported by Piette *et al.*[4], who suggested that the stability of the free chlorpromazine radical may be responsible for the psychotropic activity of the drug.

The rate of reduction of the "blue" (Type-1) cupric atoms by the phenothiazine derivatives (Fig. 2) was found to decrease with increasing electron-attracting power of the substituent in the 2-position of the aromatic ring, the slowest rate obtained with the strongest electron-attracting substituent, $-CF_3$.[14] Levine and Peisach,[15] studying the ceruloplasmin-catalyzed oxidation of aryl polyamines and polyphenols, reported that the reaction rates are directly related to the Hammett sigma value.

The rapid oxidation of NADH in the presence of ceruloplasmin and phenothiazine derivative (Fig. 4) is probably due to the formation of derivative radicals during reaction, since NADH and other reducing agents spontaneously reduce radicals of the phenothiazine class (Fig. 5). The following reaction mechanism is proposed:



CP, PD and PD^+ represent ceruloplasmin, phenothiazine derivative and phenothiazine derivative radical, respectively. This reaction mechanism can account for the activating effect of reducing agents on the reaction rate (Fig. 3), as shown in the case of the ceruloplasmin-catalyzed oxidation of catecholamines.[13]

Plots of $1/V$ against $1/[S]$ in the presence of NADH (Fig. 6) show that the V_{max}-values obtained for the substrates investigated do not vary much, indicating

that the substituent in the 2-position has little effect on the rate of product formation from the enzyme–substrate complex. V_{max} has been shown to be independent of the NADH concentration in a similar system.[13] Young and Curzon,[16] studying the ceruloplasmin-catalyzed oxidation of 37 substrates, also observed a small range of V_{max}-values, suggesting that during cycling of the enzyme a common and rate-limiting change occurs relatively independently of substrate structure. *P*-phenylenediamine derivatives with strong electron-attracting substituents were reported to have higher K_m-values than *p*-phenylenediamine.[16] A similar observation was made in the present study with trifluopromazine, having a strong electron-attracting group, $-CF_3$, in the 2-position (Table 1).

A study of the ceruloplasmin-catalyzed oxidation of dopa shows that all the oxygen in the reaction mixture is used in the formation of dopachrome in the presence of large amounts of promazine, which is a better substrate than dopa (Fig. 7). The most likely explanation is that the phenothiazine derivative acts as a cycling intermediate between ceruloplasmin and catecholamine. As shown in Fig. 5 both dopamine and dopa react spontaneously with promazine radicals. This reaction mechanism also accounts for the activating effect of the phenothiazine derivatives on the rate of catecholamine oxidation (Fig. 8). Curzon and O'Reilly[10] found that inorganic iron ions activate the ceruloplasmin-catalyzed oxidation of *N,N*-dimethyl-*p*-phenylenediamine by acting as a cycling intermediate between the enzyme and the latter compound. Since ferrous ions are rapidly oxidized by ceruloplasmin, Osaki *et al.*[5] have proposed that ceruloplasmin functions as a serum ferroxidase in promoting Fe^{3+}-transferrin formation.

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