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INTERACTION OF PHENOTHIAZINE DRUGS WITH HUMAN CERULOPLASMIN

RELATION BETWEEN ACTIVATION OF CATECHOLAMINE NEUROTRANSMITTER OXIDATION AND ELECTRON DONATING ABILITY OF PHENOTHIAZINE DRUGS

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Abstract—The influence has been studied of 11 phenothiazine drugs on the oxidation of the catecholamine neurotransmitters noradrenaline and dopamine, catalyzed by human ceruloplasmin. The phenothiazine drugs were not transformed by the enzyme. This makes participation of free phenothiazine radical cations in the oxidation of the catecholamines unlikely. A relation between the electron donating capacity of the phenothiazine drugs and the activating effect on the enzyme has been observed. It is proposed that in the interaction of phenothiazine drugs with ceruloplasmin, charge transfer complexes between the phenothiazines and Cu^{2+} of the enzyme, are involved. The possible relevance of charge transfer complexes as a model for the receptor interactions of phenothiazine drugs is discussed.

The phenothiazine tranquillizers exist as a wide range of structurally related compounds. They are quite extraordinarily strong electron donors [1], leading to the formation of relatively stable free radical cations [2].

Phenothiazine drugs exhibit a wide range of biological effects, which are probably not the result of only one mechanism. Through the years since the introduction of these compounds many authors have tried to connect the electron donating capacity with the biological activity [1–8]. However, no direct relation exists between, e.g. the neuroleptic activity of a phenothiazine drug and its electron donating capacity [3, 4].

It has been reported that tranquillizers of the phenothiazine class accelerate the ceruloplasmin (CE) catalyzed oxidation [9–12]. It has been suggested that in this latter process free cation radicals derived from the phenothiazine drugs act as cycling intermediates [9].

Ceruloplasmin [1.16.3.1] (CE) is a blue copper-containing α-glycoprotein occuring in human serum. The biological role of this enzyme is not yet fully understood. It is assumed that the enzyme plays a role in the transport of Fe^{2+} by oxidation of Fe^{2+} to Fe^{3+} before combining with apotransferrin [12]. CE also catalyzes the oxidation of aryldiamines and catechols and is of importance as a copper storage protein in serum: in human serum 98% of the copper content is bound to ceruloplasmin. One molecule of CE contains eight atoms of copper. These copper atoms are not identical. Actually the blue colour of the enzyme arises from two so-called blue Cu^{2+} ions and the strong absorption at 610 nm is caused by certain distortions in the coordination structures of the protein [13].

Løvstad [9, 10] reported that the presence of reducing agents (a.o. NADH) markedly increases the CE-catalyzed oxidation of some neuroleptic drugs, including phenothiazine derivatives. The oxidation of the neuroleptic drugs was not monitored by means of quantitative assay of the drugs but only by means of the oxidation of NADH, assuming that NADH is oxidized by reduction of radical cations enzymatically formed from the drugs.

The aim of this study was to investigate the possibility that phenothiazine drugs themselves are substrates for human CE. Furthermore, in view of the reported activation of the human CE-catalyzed catecholamine oxidation, the effects of 11 therapeutically used phenothiazines were studied. Especially the possible formation of phenothiazine radical cations from the interaction with CE is interesting, as these are intermediates in the formation of the sulfoxide metabolites.

MATERIALS AND METHODS

The following phenothiazine compounds were used as purchased: chlorpromazine–HCl, Specia; promethazine–HCl, Brocacef. Promazine–HCl was a gift of Wyeth. Fluphenazine-2 HCl and triflupromazine-HCl were gifts of Squibb. Prochlorperazine-bimethanesulfonate, cyamemazine- and levomepromazine base were gifts of Rhône–

Abbreviations: CE, human ceruloplasmin; NaOAc, sodium acetate; NA, noradrenaline-HCl; DA, dopamine-HCl.

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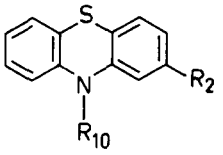
Poulenc. The bases were converted into the maleate salts. Thioridazine and mesoridazine were gifts of Sandoz. Chlorpromazine–methoiodide was a gift of Smith, Kline and French. DL-noradrenaline–HCl (NA) and dopamine–HCl (DA) were obtained from Fluka AG. Human ceruloplasmin (CE) (1.16.3.1) was from Sigma Chemical Co. (Type X, a 5% solution in 0.25 M sodium chloride–0.05 M sodium acetate). Bi-distilled water was used as a solvent.

CE catalyzed reactions. The reaction mixtures consisted of 10 μl Ce (5% solution) 1 ml 0.25 M NaCl–0.05 M NaOAc, 100 μl solution of the phenothiazine derivative 6.2 mM in 0.25 M NaCl–0.05 M NaOAc and 100 μl solution of NA or DA 10.0 mM in 1.25 M NaCl–0.05 M NaOAc. In the case where catecholamine or phenothiazine drug was omitted, 100 μl 0.25 M NaCl–0.05 M NaOAc was added instead. The reaction mixtures in glass vials of 2 ml were placed in a waterbath thermostatted at 30.0° ± 0.05 (Haake F3 thermostat). In each experiment as a reference the oxidation rate of NA or DA was assayed without the presence of phenothiazine drug. The rate constants of NA and DA oxidation were assayed in duplicate in independent experiments. The deviation between the two values was generally less than 5%. In the experiments for the Michaëlis–Menten kinetic analysis reaction mixtures were made in the same way with a variable concentration of substrate i.c. NA. These experiments were performed at 37.0 ± 0.05°. In the course of time samples were taken and analyzed fluorimetrically and with HPLC.

Fluorimetrical assay of catecholamines. Samples of 100 μl from the above described reaction mixtures were diluted down to 25.0 ml with 0.001 N HCl which stopped the reaction. The fluorescence is linear with the catecholamine concentration in this range and oxidation products do not interfere [14]. The assays were performed using the native fluorescence of catecholamines with excitation wavelength at 282 nm and emission at 316 nm on a Perkin–Elmer fluorescence spectrometer model 3000. The cell-holder of the fluorescence spectrometer was thermostatted at 20.0 ± 0.1°. As a reference, for each sample the fluorescence was compared to that of a known catecholamine concentration. It was necessary to make a correction for CE fluorescence. With excitation at 282 nm CE shows fluorescence with maximum emission at 327 nm. This fluorescence remained constant during the reaction.

HPLC assay of phenothiazines. Samples of 20 μl were injected on a Spectra Physics SP 3500 B liquid chromatograph equipped with a (10 × 0.4 cm i.d.) column packed with 10 μ RP-2 (MPLC Brownlee

Table 1. Structure of the investigated phenothiazine derivatives and their activating effect on the CE-catalyzed NA and DA oxidation (reaction mixture: phenothiazine drug 0.52 mM, NA or DA 0.83 mM, CE 2.6 μM in 0.25 M NaCl–0.05 M NaOAc, temperature is 30.0°)



	NAME	R_2	R_{10}	$\frac{K_{NA}}{K_{NA_0}}$	$\frac{K_{DA}}{K_{DA_0}}$
1	PROMAZINE	H	$-(CH_2)_3-N(CH_3)_2$	1.90	1.80
2	CHLORPROMAZINE	Cl	$-(CH_2)_3-N(CH_3)_2$	1.55	1.40
3	PROMETHAZINE	H	$-CH(CH_3)-CH_2-N(CH_3)_2$	1.15	1.15
4	FLUPHENAZINE	CF_3	$-(CH_2)_3-N\square N-CH_2-CH_2OH$	1.20	1.30
5	PROCHLORPERAZINE	Cl	$-(CH_2)_3-N\square N-CH_3$	1.80	1.70
6	THIORIDAZINE	SCH_3	$-(CH_2)_2-\square N-CH_3$	2.02	1.77
7	MESORIDAZINE	$S(O)CH_3$	$-(CH_2)_2-\square N-CH_3$	1.24	1.23
8	LEVOMEPROMAZINE	OCH_3	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	2.28	2.25
9	CHLORPROMAZINE-METHOIODIDE	Cl	$-(CH_2)_3-N^+(CH_3)_3 . I^-$	1.40	1.40
10	CYAMEMAZINE	CN	$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	1.35	1.34
11	TRIFLUPROMAZINE	CF_3	$-(CH_2)_3-N(CH_3)_2$	1.30	1.27

* K_{NA} and K_{DA} are the pseudo first-order rate constants for oxidation of NA and DA respectively in the presence of the phenothiazine derivative, K_{NA_0} and K_{DA_0} those in the absence of phenothiazine drug.

Labs) and connected to a detector LKB 2138 Uvicord S with a wavelength filter of 254 nm. The eluent was acetonitrile/0.04% aqueous $(NH_4)_2CO_3$(70/30) with a flow rate of 3.2 ml/min. Retention times of the phenothiazines were in the range of 3–18 min. The peak area was obtained with a Hewlett–Packard 3390A Integrator.

RESULTS AND DISCUSSION

The structures of the phenothiazine drugs used in this study are shown in Table 1. For the study of the activating effects on catecholamines the neurotransmitters NA and DA were chosen. In the presence as well as in the absence of phenothiazines NA and DA oxidation appeared to proceed according to first order kinetics if the amount of oxidation was less than 30%. The reaction rate constants of NA and DA oxidation in the presence of 0.52 mM phenothiazine drug, relative to those without phenothiazine are also included in Table 1. The activating effect of the phenothiazine drug is similar for both catecholamine neurotransmitters. NA oxidation is ~15% faster than DA oxidation. The influence of the phenothiazine drug concentration on the catecholamine oxidation was studied with promazine. From Fig. 1 it appears that in a concentration range up to 2.0 mM the reaction rate of NA oxidation increases linearly with promazine concentration. Figure 2 shows the Lineweaver–Burk plots of the CE-catalyzed NA oxidation without and in the presence of promazine. The enzymatic nature of the reaction, also in the presence of promazine, appears from the linearity of the curves in the Lineweaver–Burk plot. Within the applied range V_{max} increases linearly with promazine concentration. The K_m-value (0.25 mM) is not significantly changed in the presence of promazine. This indicates that association of the substrate i.c. NA with the enzyme is not affected by promazine.

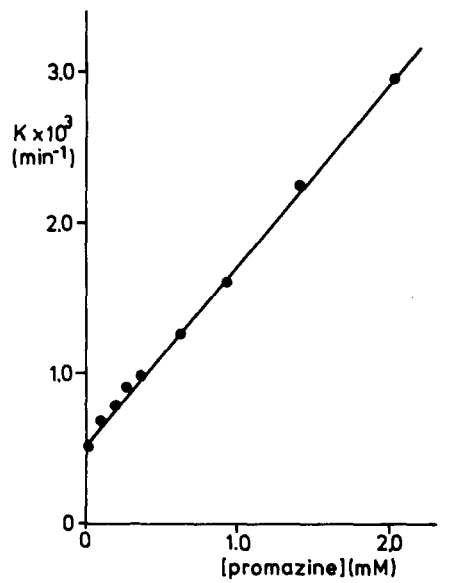


Fig. 1. Effect of promazine concentration on the CE-catalyzed NA oxidation. Reaction conditions: NA 0.83 mM, CE 2.6 μM in 0.25 M NaCl–0.05 M NaOAc. Temperature is 37.0°.

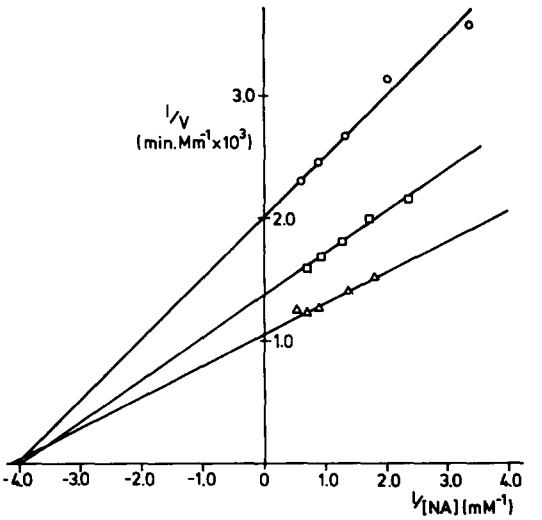


Fig. 2. Lineweaver–Burk plots of the CE-catalyzed NA oxidation. ○, no promazine; □, 0.07 mM promazine; △, 0.14 mM promazine. CE 2.6 μM in 0.25 M NaCl–0.05 M NaOAc. Temperature is 37.0°

An important finding in this study is the fact that neither in the presence, nor in the absence of catecholamine can any significant transformation of the phenothiazine drugs be observed with HPLC analysis. Løvstad [9] proposes that phenothiazine-derived free radical cations generated by CE act as cycling intermediates in the oxidation of catecholamines. There is proof that such a reaction is possible [15].

However, particularly when no catecholamines are present to reduce the free phenothiazine cation radicals, they will react further and loss of phenothiazine compound with concomitant formation of sulfoxides should be observed. The reaction medium containing the nucleophilic NaOAc is such that eventually generated fenothiazine radical cations should react further and form sulfoxides [16]. We actually observed sulfoxide formation upon generation of chlorpromazine radical cation by horseradish peroxidase/H_2O_2 in the NaCl/NaOAc medium. The fact that no sulfoxides are found upon reaction with CE strongly indicates that the activation of the CE-catalyzed oxidation of catecholamines does not proceed via free phenothiazine radical cations. In Fig. 3 the activating effect of the phenothiazine drugs on the CE-catalyzed NA oxidation is plotted against the Hammett σ_{para}-values for the substituents in the 2-position of the phenothiazine ring system. Generally it appears that an electron donating substituent in the 2-position enhances the activating effect on the catecholamine oxidation. The electron donor ability of phenothiazine drugs decreases with increasing σ_{para}-value [6]. The fact that the electron donating ability is an important factor in the enhancement of CE-activity by phenothiazine compounds suggests that in the interaction of phenothiazines with CE formation of charge transfer complexes is involved.

Upon the interaction of phenothiazine drugs with CE the absorption at 610 nm of the blue Cu^{2+}

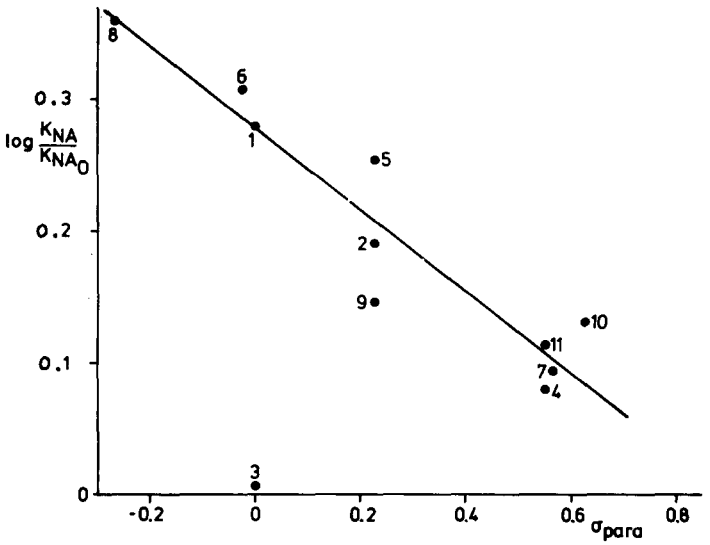


Fig. 3. Relation between activating effect of phenothiazine drugs on the CE-catalyzed NA oxidation and the Hammett σ_{para}-constant [25]. Reaction conditions as in Table 1. The number of the compounds also refer to Table 1, K_{NA} and K_{NA_0} see Table 1. Regression line: log $K_{NA}/K_{NA_0} = 0.275 - 0.302\ \sigma_{para}$. Correlation coefficient = 0.943 (N = 10). Promethazine (3) is not included in the regression analysis.

decreases [17]. The finding of Løvstad that the decolorization rate increases with increasing electron-donating ability [17] parallels the enhancing effect of the phenothiazine drug on CE activity reported now. Assuming that the strong extinction at 610 nm is caused by specific coordination conditions of Cu in the protein, this changes upon formation of the charge transfer complex of phenothiazines with CE. The formation of complexes between chlorpromazine and Cu^{2+} ions has been reported [18]. The spectroscopic properties of these complexes, which could be isolated in pure crystalline form, resembled that of the free radical cation of chlorpromazine [18]. However, from ESR measurements the complex was observed not to be in the strict sense of the word, a free radical species [18].

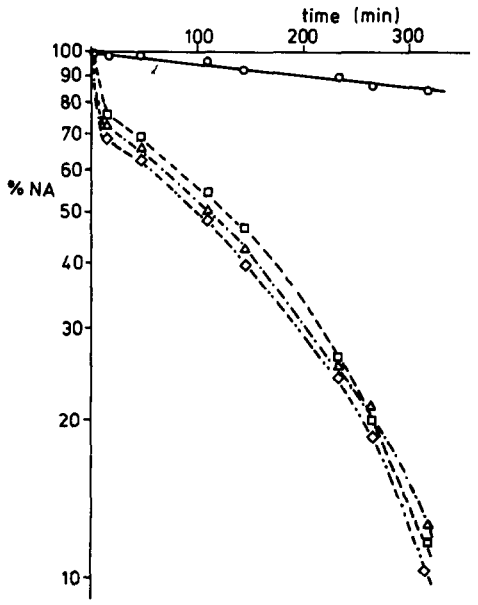


Fig. 4. Cu^{2+}-catalyzed NA oxidation. Reaction conditions: NA 1.19 mM, promazine 1.10 mM; —○—, no $CuCl_2$; —.△—., no promazine, $CuCl_2$, 2.12 mM; --□--, $CuCl_2$, 1.06 mM; –··◇–··, $CuCl_2$, 2.12 mM. Reactions in 0.25 M NaCl – 0.05 M NaOAc, temperature is 37.0°.

The possibility was also investigated whether Cu^{2+} ions not coordinated in a protein structure like CE, were also able to oxidize catecholamines and whether phenothiazines could activate this oxidation. From the results in Fig. 4 it appears that initially Cu^{2+} rapidly oxidizes NA, however, no activation by phenothiazine drug is observed in this case. The presence of 1 mM promazine has also no effect on NA autoxidation, which is small compared to the oxidation in the presence of Cu^{2+}. Increase of the Cu^{2+} concentration from 1 to 2 mM has no effect on the rate of NA oxidation.

Remarkably, in the reaction with free Cu^{2+} HPLC analysis revealed some oxidation of the phenothiazine drug to sulfoxide: loss of ~15% promazine, in the presence of 2.12 mM Cu^{2+} in 5 hr reaction time. For this process the presence of NA is essential. Possibly intermediates formed in the catecholamine oxidation react with phenothiazine derivatives. It can be concluded that in the activation of the CE oxidizing ability the coordination of Cu^{2+} in the protein structure is important. From Fig. 3 it appears that the electron donating capacity of the 2-substituent is not the overall governing factor in the activation process. Also the 10-substituent influences the extent of activation. It can be assumed that electrostatic and steric properties of the side chain influence the orientation and the binding of the phenothiazine compound to the enzyme. The lipophilicity of the phenothiazine drugs, which has been reported to be an important factor in the protein binding of these drugs [19, 20] does not seem to play

an important role here: the difference in activating effect between the quaternairy compound chlorpromazinemethoiodide [9] and chlorpromazine [2] is not large. The exceptional position of promethazine [3] in Fig. 3 is interesting. In promethazine the electron attracting protonated side chain nitrogen (pK_a-value ~9.0 [20]) is only two carbon atoms from the phenothiazine nucleus. This diminishes the electron donating ability of promazine, as also appears from the relative high half-wave potential for voltammetric oxidation [21]. This explains the small activating effect of promethazine, which is not active as a neuroleptic agent.

Concluding, the phenothiazine drugs are not transformed by the enzyme, this makes the involvement of free radical cations derived from the phenothiazine compounds unlikely. In the activation of CE-catalyzed catecholamine oxidation the coordination of Cu^{2+} ions in the protein structure is essential. In the interaction of phenothiazine drugs with CE the electron donating ability of the phenothiazines is an important factor. Electron donating ability may also be important for the neuroleptic activity of these drugs: promethazine and also other phenothiazine drugs with two carbon atoms between the side-chain nitrogen and the ring nitrogen have poor neuroleptic properties and are mainly used as antihistaminics. Therefore, the formation of charge transfer complexes with CE might be a model for such interactions with other copper or suitable metal containing (receptor) proteins. In relation to the neuroleptic activity of the phenothiazine drugs and the central role of catecholamine neurotransmitter metabolism for psychotropic conditions the enhanced CE-catalyzed catecholamine oxidation by phenothiazines is interesting: melanin deposits formed by catecholamine oxidation have been found with patients on prolonged phenothiazine therapy [22, 23]. Furthermore, phenothiazine treatment can also result in Parkinsonian conditions [24].

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