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STUDIES OF FLAVIN-ADENINE DINUCLEOTIDE-REQUIRING ENZYMES AND PHENOTHIAZINES—II.

STRUCTURAL REQUIREMENTS FOR D-AMINO ACID OXIDASE INHIBITION

S. GABAY and S. R. HARRIS

Biochemical Research Laboratory, Veterans Administration Hospital, Brockton, Mass., and Department of Biochemistry, Boston University School of Medicine, Boston, Mass., U.S.A.

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Abstract—The effect of various phenothiazines on the activity of D-amino acid oxidase of purified hog kidney was studied in a manometric system established at pH 7·3. Analysis of the parallel dose–effect curves showed the order of inhibitory potency to be as follows: trifluoperazine > thioridazine and fluphenazine > perphenazine > chlorpromazine. This order is related to the clinical efficacy and potency of these drugs in antipsychotic treatment. Phenothiazines with little or no antipsychotic activity failed to inhibit D-amino acid oxidase. Kinetic analysis demonstrated that the phenothiazines competed with flavin–adenine dinucleotide for the apoenzyme.

ALTHOUGH the pharmacology and clinical effects of various phenothiazines, particularly chlorpromazine (CPZ), have been extensively studied, the biochemical mechanisms by which they exert these effects remain to be elucidated. Of special importance in this regard is the delineation of the diverse pharmacological effects of various phenothiazines in terms of their relatively subtle structural differences.

The partial structural analogy between the phenothiazine nucleus and the isoalloxazine moiety of flavin-adenine dinucleotide (FAD) has suggested that the phenothiazines might act by inhibiting flavoenzymes.[1] This study presents the results of a systematic comparison of the interactions of several phenothiazine derivatives with a single FAD-requiring enzyme, D-amino acid oxidase (D-AAO) [D-amino acid: O_2 oxidoreductase (deaminating), E.C. 1.4.3.3.].

MATERIALS AND METHODS

Enzyme purification and assay. Hog kidney D-AAO, obtained commercially as an acetone powder (Mann Research Labs.), was purified and assayed manometrically at pH 7·3 as described previously.[1]

Experimental design. In preliminary studies various systems were employed in which the order of addition and length of exposure of the reactants to each other were varied. Since the most marked inhibition was observed when the apoenzyme and the phenothiazine were preincubated together, this system was employed throughout these experiments.

The apoenzyme was diluted in 0·002 M pyrophosphate buffer (pH 7·3) so that the mixture of the apoenzyme and the aqueous solutions of the phenothiazines in the side

arm would attain a pH of 6·2 which is the pH of optimum stability for D-AAO.[2] The components of the reaction mixture and assay conditions are presented in the appropriate figure legends. Activity was recorded for a period of at least 1 hr, during which the reaction was always linear. In any single experiment each set of conditions was run at least in triplicate.

Protein determinations. Protein concentrations were measured by the A_{215}–A_{225} method of Waddell,[3] the validity of which was periodically verified on the basis of micro-Kjeldahl nitrogen determination and the Lowry modified biuret procedure[4] as we previously reported.[1, 5]

Phenothiazine derivatives. Table 1 depicts the structures of the phenothiazine derivatives used in these studies.

RESULTS

Structural relationship and D-AAO inhibition

The inhibitory effect of trifluoperazine on D-AAO in the present studies is shown in Fig. 1. The reaction was found to be linear over a period of 60 min. Similar effects were obtained with other inhibitors. Preincubation of the enzyme preparation with these inhibitors for longer periods (up to 60 min) was found to have no influence on their inhibitory properties.

The relative efficiency of the phenothiazines tested as D-AAO inhibitors is shown in Table 1 for two sets of experiments where FAD was used at final concentrations of

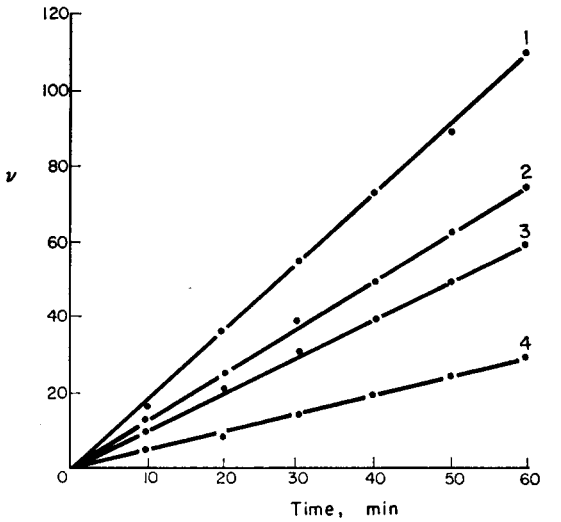
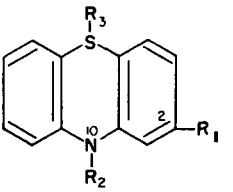
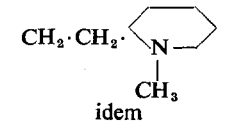
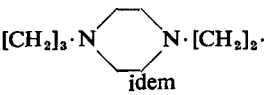
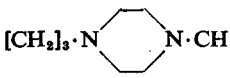


FIG. 1. Inhibition of D-AAO by trifluoperazine as a function of time. The reaction mixture contained 1 ml 0·1 M pyrophosphate buffer (pH 7·3), 0·2 ml apoenzyme solution (100 μg), 0·1 ml FAD (4×10^{-7} M final concentration), 0·2 ml DL-alanine ($4{\cdot}49 \times 10^{-2}$ M final concentration), and 0·9 ml H_2O. Trifluoperazine (0·1 ml) was added to give the following final concentrations: (1) none, (2) 6×10^{-5} M, (3) 8×10^{-5} M, (4) 10^{-4} M. The centre well contained 0·2 ml 2 N NaOH and Whatman 1 filter paper (1 cm^2). Velocity (v) is expressed as $\mu l/O_2$/hr at 37°. Gas phase: air. Total reaction volume: 2·5 ml.

TABLE 1. PHENOTHIAZINE DERIVATIVES: STRUCTURE AND D-AMINO ACID OXIDASE INHIBITION*



	R_1	R_2	R_3	% Inhibition† FAD Concentration 2×10^{-7} M	% Inhibition† FAD Concentration 4×10^{-7} M
Aliphatic series					
Promazine		$[CH_2]_3\cdot N(CH_3)_2$	—	0	0
Chlorpromazine	Cl	$[CH_2]_3\cdot N(CH_3)_2$	—	26	15
CPZ sulfoxide	Cl	idem	=O	0	0
Trimeprazine		$CH_2\cdot CH\cdot(CH_3)\cdot CH_2\cdot N\cdot(CH_3)_2$	—	0	0
Piperidine series					
Thioridazine	SCH_3		—	57	49
Thioridazine sulfoxide	idem	idem	=O	0	0
Thioridazine disulfoxide	$S(O)\cdot CH_3$	idem	=O	0	0
Piperazine series					
Perphenazine	Cl		—	39	26
Fluphenazine	CF_3	idem	—	57	47
Trifluoperazine	CF_3		—	82	71

* The reaction mixture was the same as in Fig. 1.
† Final inhibitor concentration was 10^{-4} M.

2×10^{-7} M and 4×10^{-7} M respectively. As is evident, the inhibitory power of these derivatives was found to be as follows: trifluoperazine > thioridazine = fluphenazine > perphenazine > chlorpromazine. No inhibition was observed with trimeprazine, promazine, or the sulfoxides of CPZ and thioridazine in concentrations even as high as 4×10^{-4} M.

As emphasized in a recent review,[6] unless a parallelism of the dose–response curves is established, it is of questionable validity to say that a certain inhibitor is several times more potent than another. The per cent inhibitions of the various phenothiazines studied were subjected to such an analysis. As indicated in the dose–response curves (Figs. 2a and 2b) an increase in the concentrations of these derivatives was found to increase their inhibition in a similar way *in vitro*. When a two-part analysis of variance was performed on the data,[7] the lines 2, 3, and 4 in Fig. 2a were found to be parallel ($P < 0{\cdot}01$; $F = 1{\cdot}86$). In Fig. 2b, however, only lines 3 and 4 were parallel ($P < 0{\cdot}01$; $F = 1{\cdot}24$). CPZ (line 1) was omitted from the statistical computations because of its insignificant inhibition at the concentrations tested. The deviation of perphenazine

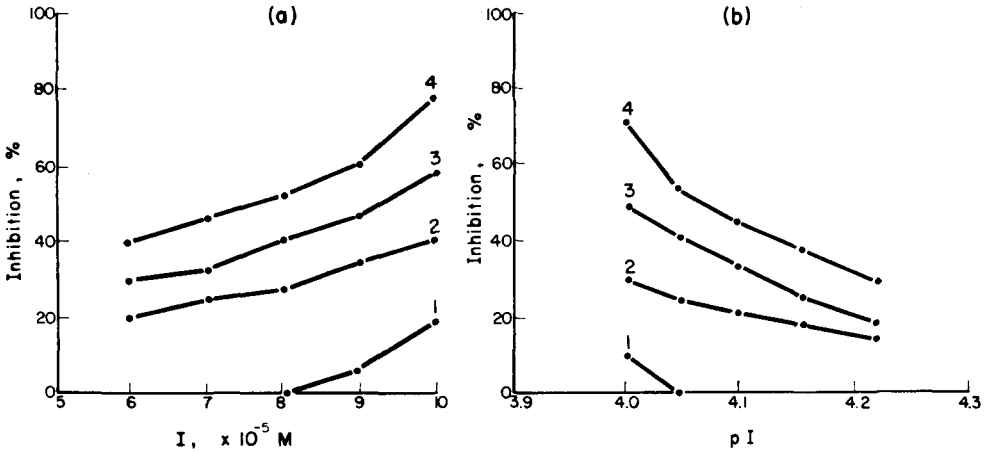


FIG. 2. Inhibition of D-AAO by various phenothiazines. The reaction mixture was the same as in Fig. 1. Experiments were run for 1 hr. The phenothiazines used were: (1) chlorpromazine, (2) perphenazine, (3) fluphenazine and thioridazine, and (4) trifluoperazine. In (a) the abscissa (I) is the final concentration of the drug; in (b) it is the negative log (pI) of this concentration. The final FAD concentrations were 2×10^{-7} M (a) and 4×10^{-7} M (b).

(Fig. 2b, line 2) is believed due to the limitation of manometric techniques in measuring 3% to 5% differences in oxygen uptake. Since a parallelism exists, it is apparent that the drugs exhibit a common mode of action.

Kinetic studies

In order to investigate the nature of the inhibition, data were examined by the graphical method of Lineweaver and Burk.[8] As indicated in Fig. 3, thioridazine was found to compete with FAD for the apoenzyme. The K_f for FAD ($1{\cdot}88 \times 10^{-7}$ M) in the present study was found to be identical with the value reported earlier.[1] However, with the system used throughout these studies, K_i values were found to vary with

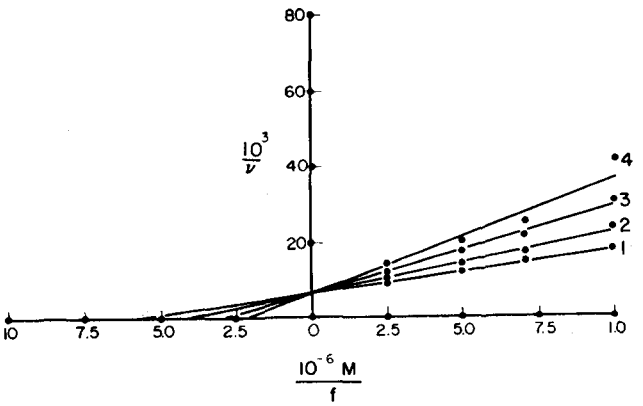


FIG. 3. Coenzyme-competitive inhibition of D-AAO by thioridazine. The reaction mixture was the same as in Fig. 1. Final FAD concentrations (f) were as indicated. Final thioridazine concentrations were: (1) none, (2) 4×10^{-5} M, (3) 6×10^{-5} M, and (4) 8×10^{-5} M. Velocity (v) is expressed as $\mu l/O_2/hr$ at 37°.

thioridazine concentration. This sort of variation could occur if more than one method of inhibition were involved. Although 8×10^{-5} M thioridazine (cf. Fig. 3, line 4) seems to fit poorly, it does not significantly deviate from linearity ($t = 7{\cdot}5$; $P < 0{\cdot}025$). However, when the same data were plotted by the method reported by Dixon,[9] in which the reciprocal of the velocity is plotted against inhibitor concentration, a markedly significant deviation from linearity was obtained. This precluded the possibility of calculating the K_i. Similar results were observed with the other inhibitory phenothiazines. At present, assay systems are being developed for determining inhibitor constants for these derivatives with the use of more highly purified enzyme preparations.

DISCUSSION

The significance of the present results on the biochemical mechanisms of phenothiazines should be evaluated only in the light of the well-established clinical observations with this group of drugs. The clinical pharmacology, particularly the effects of substituents in the 2 and 10 positions of the phenothiazine nucleus, have been recently surveyed in a number of reviews.[10–12] From an analysis of the results presented above and in a previous report,[1] it is evident that minor alterations in the phenothiazine structure markedly influenced their affinities for D-AAO. In the aliphatic series (cf. Table 1) of phenothiazine derivatives, promazine and trimeprazine were found to possess no inhibitory properties. Introduction of a halogen atom at carbon 2, as in CPZ, significantly inhibited D-AAO, indicating that halogenation of position 2 conferred an inhibitory power on otherwise inactive compounds. On the other hand, sulfoxidation of CPZ completely abolished this effect, thus giving evidence that the "ring-sulfur" is important for the inhibitory mechanism.

This was also observed with the piperidine derivatives, where the marked inhibitory power of thoridazine could not be seen with its corresponding sulfoxide and disulfoxide. Replacement of the N-dimethyl portion of the side chain of CPZ with a substituted piperazine ring, as in perphenazine, enhanced the inhibitory power of CPZ. A trifluoromethyl substitution at carbon 2 engendered an even greater inhibitory power (fluphenazine). Furthermore, it is evident that the length of the N-alkyl-substituted piperazine chain plays an important role in the inhibition of D-AAO, since trifluoperazine exhibited greater inhibition than both fluphenazine and perphenazine.

On the basis of these observations it can be concluded that structural alterations of phenothiazine with various substitutions in the 2 and 10 positions are most critical for their inhibitory properties. Thus, there is a high correlation between the ability of these phenothiazines to inhibit D-AAO *in vitro* and their actions *in vivo*, where clinical potency and efficacy were dependent on such substitution at positions 2 and 10.[10–13] It is also interesting to note that the average daily recommended dosage[14] of these drugs in antipsychotic treatment is inversely related to their ability to inhibit D-AAO. It seems, therefore, that our studies have provided a possible biochemical delineation of the mechanism of action of phenothiazine derivatives. Such an explanation gains further substance in that phenothiazines with little (e.g. promazine, trimeprazine) or no antipsychotic activity (e.g. the sulfoxides of CPZ and thioridazine) failed to inhibit D-AAO.

It may further be noted that studies of phenothiazine–enzyme interactions, in which drug concentrations of 10^{-3} M or higher have been used, are still being reported.

Their conclusions are more perplexing than enlightening. The tacit assumption that phenothiazines inhibit enzyme systems has proved to be accurate, but the continued use of concentrations higher than the pharmacological dosage is incorrect.

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REFERENCES

1. S. GABAY and S. R. HARRIS, *Biochem. Pharmac.* **14,** 17 (1965).
2. K. BURTON, *Methods in Enzymology*, vol. 2, p. 199. Academic Press, New York (1955).
3. W. WADDELL, *J. Lab. clin. Med.* **48,** 311 (1956).
4. O. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
5. S. GABAY and D. JORDAN, *J. appl. Spectros.* **17,** 132 (1963).
6. E. A. ZELLER and J. R. FOUTS, *Ann. Rev. Pharmac.* **3,** 12 (1963).
7. G. W. SNEDECOR, *Statistical Methods*, p. 338. Iowa State College Press, Ames (1965).
8. H. LINEWEAVER and D. BURK, *J. Am. chem. Soc.* **56,** 658 (1934).
9. M. DIXON, *Biochem. J.* **55,** 170 (1953).
10. J. KINROSS-WRIGHT, *J. Am. med. Ass.* **170,** 1283 (1959).
11. M. W. PARKES, *Progress in Medicinal Chemistry*, G. P. ELLIS and G. B. WEST, Eds., p. 97. Butterworth, London (1961).
12. E. F. DOMINO, *Clin. Pharmac. Ther.* **3,** 599 (1962).
13. R. M. CORES and C. BUCKMAN, *Dis. Nerv. Syst.* **24,** Suppl. 92 (1963).
14. E. REMMEN, S. COHEN, K. S. DITMAN and J. R. FRANTZ, *Psychochemotherapy*, p. 135. Western Medical Publications, Los Angeles (1962).