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STUDIES OF FLAVIN-ADENINE DINUCLEOTIDE-REQUIRING ENZYMES AND PHENOTHIAZINES—III

INHIBITION KINETICS WITH HIGHLY PURIFIED D-AMINO ACID OXIDASE

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Abstract—The inhibition of a very highly purified D-amino acid oxidase apoenzyme preparation (specific activity: 300–330 μl O_2/min/mg protein) by a variety of phenothiazine derivatives has been studied. Only derivatives possessing antipsychotic activity were found to be inhibitors. The inhibition was coenzyme-competitive and the following K_i's could be calculated: (1) chlorpromazine, 5×10^{-5}M, (2) triflupromazine, 3×10^{-5} M; (3) perphenazine, $2{\cdot}7 \times 10^{-5}$M; (4) fluphenazine, $2{\cdot}4 \times 10^{-5}$M; (5) trifluoperazine, $2{\cdot}0 \times 10^{-5}$M; and (6) thioridazine, $1{\cdot}6 \times 10^{-5}$M. The inhibitory capacities of these compounds were generally in good agreement with their relative clinical efficacy and potency in antipsychotic therapy. Phenothiazine derivatives with little or no clinical efficacy as tranquilizing agents (promazine, trimeprazine, promethazine, and the sulfoxides of chlorpromazine and thioridazine) did not inhibit D-amino acid oxidase even in concentrations as high as 4×10^{-4}M; neither did imipramine, an antidepressant which is structually similar to the phenothiazines. However, chlorprothixene, a thiaxanthene derivative with definite utility in antipsychotic management, was also a coenzyme-competitive inhibitor and had a K_i of 4×10^{-5}M. Since the K_i's were low enough to represent concentrations that might be encountered *in vivo*, the hypothesis that phenothiazines could act by inhibiting flavoenzymes appears to gain further significance.

PREVIOUS studies, initiated in order to investigate the possibility that phenothiazines might act by inhibiting flavoenzymes, have shown that certain phenothiazine derivatives inhibited a partially purified D-amino acid oxidase preparation.[1, 2] Although the inhibition involved competition between these agents and flavin-adenine dinucleotide (FAD) for the apoenzyme, the relative impurity of the enzyme preparation and the possible involvement of nonspecific binding of phenothiazines by foreign protein precluded the possibility of determining the mechanism of inhibition precisely.

Since there appeared to be a definite relationship between the inhibitory potencies of these drugs and their efficacy in antipsychotic therapy, it was considered essential to study the kinetics of the reaction with a very highly purified apo-oxidase preparation. This report presents the results of such a study.

MATERIALS AND METHODS

Enzyme purification

The apoenzyme of D-amino acid oxidase (D-AAO) [D-amino acid: O_2 oxidoreductase

(deaminating), E.C.1.4.3.3.] was prepared directly from hog kidneys obtained immediately after the animals were dispatched.* The purification procedure, which involved the utilization of benzoate (a potent substrate-competitive inhibitor of D-AAO), chromatography on a hydroxylapatite column, and removal of FAD by $(NH_4)_2\ SO_4$ precipitation at pH 2·8, was essentially the same as that reported by Yagi and Ozawa.[3, 4] No attempts were made to crystallize the enzyme. Twelve apoenzyme preparations thus purified had almost identical specific activities (18–22 μl O_2/μg protein/hr) when assayed at pH 8·3 with saturating concentrations of FAD (4×10^{-6} M) and D-alanine (0·089 M). The apoenzyme was stable for at least 3 months when stored at −15°.

Partial physical characterization of the enzyme

1. *Ultracentrifugal behavior.* In an attempt to determine the homogeneity of the enzyme preparation, it was subjected to ultracentrifugal analysis in the Spinco model E. The apoenzyme, after dialysis against 0·15 M pyrophosphate buffer (pH 8·3), gave three peaks with observed sedimentation coefficients of 1·2S, 6·8S, and 10·0S at a protein concentration of 10·8 mg/ml (Fig. 1, upper). However, the holoenzyme (Fig. 1, lower) sedimented as one peak with an observed sedimentation coefficient of 5·6S at a protein concentration of 6 mg/ml. Although no attempt was made to determine the effect of varying the protein concentration, the behaviour agrees qualitatively with that reported by Massey *et al.*[5] and Charlwood *et al.*[6] The latter have discussed this in terms of association and dissociation and have suggested that the presence of FAD facilitated the polymerization of the enzyme. Recent reports from Massey's laboratory[7, 8] have shown that the concentration-dependent equilibrium is further affected by temperature, pH, and the form of the enzyme (apo-, holo-, or benzoate complex).

These findings are in marked contrast to those of Yagi and Ozawa[9] and Miyake *et al.*,[10] which indicate that both the apo- and the holoenzymes sediment as a single peak. The large differences in S_{20w} between the two forms that Yagi and Ozawa have reported have been ascribed by these authors to drastic conformation changes undergone by the apoenzyme when it is converted to the holoenzyme. They have recently reported, however, that the α-helix is not affected during this transformation.[11]

Dixon and Kleppe,[12] on the other hand, have observed two peaks with both the apo- and holoenzymes and could find no differences in the S_{20w}'s of these peaks when the apoenzyme was converted to the holoenzyme. It is apparent, therefore, that further investigation is necessary to ascertain the causes of these inconsistencies between laboratories.

2. *Absorption spectrum.* Although no activity was obtained with the apoenzyme when FAD was omitted from the usual systems, an endogenous content of FAD equal to about 10^{-8} M could be detected if much larger concentrations of enzyme were assayed. Trace amounts of FAD are also demonstrated by the slight absorbance at 450 mμ in the visible region of the absorption spectrum in Fig. 2.

3. *Molecular weight.* The molecular weight of the D-AAO preparations was estimated by the gel-filtration method proposed by Andrews.[13] In this procedure various proteins of known molecular weight were used to calibrate a Sephadex G-100 column (1·9 × 80 cm) by plotting their elution volumes against the log of their molecular

* Obtained from North East Packing Co., Somerville, Mass.

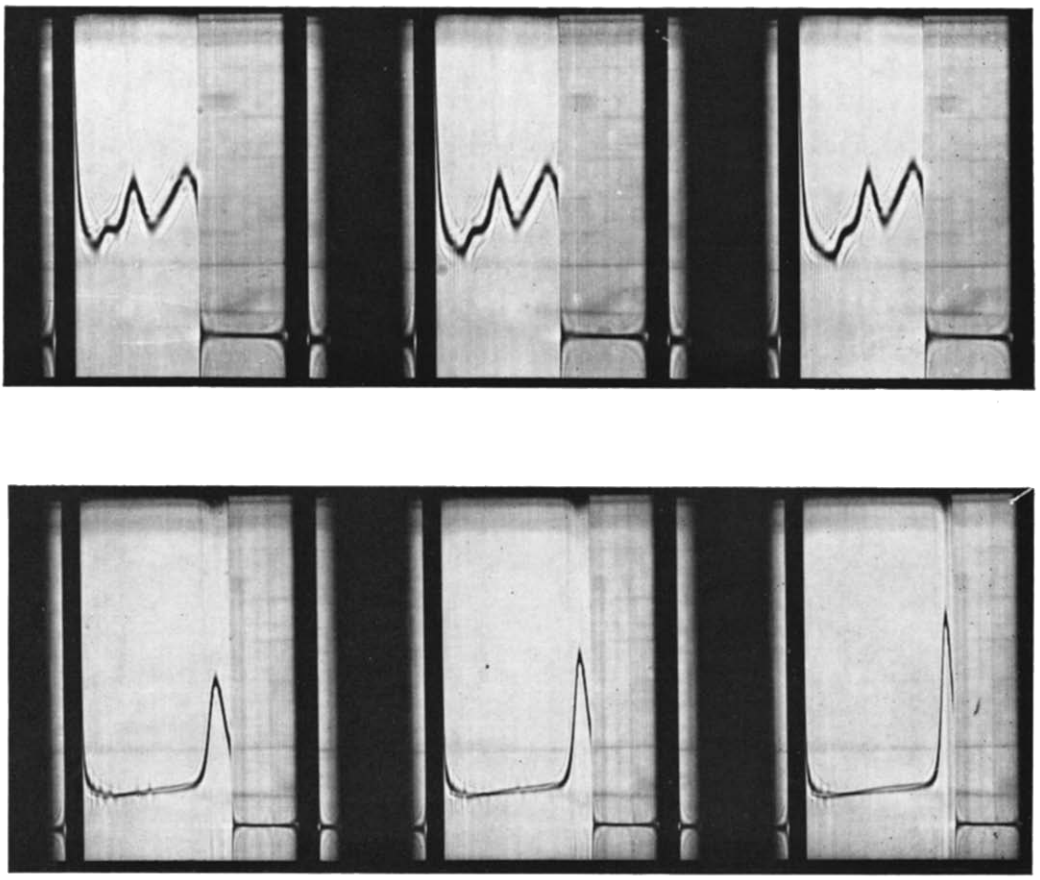


FIG. 1. Upper: *Representative ultracentrifuge patterns of* D-*AAO apoenzyme.* The centrifugal direction is from right to left. Photographs were taken at 4-min intervals starting 40 min after reaching speed. Bar angle, 40°; speed, 50,740 rev/min; rotor temp.: 20°. Protein concentration; 10·8 mg/ml in 0·05 M pyrophosphate buffer, pH 8·3.

Lower: *Representative ultracentrifuge patterns of* D-*AAO holoenzyme.* The centrifugal direction is from right to left. Photographs were taken at 4-min intervals starting immediately after reaching speed. Speed, 50,740 rev/min; rotor temp., 20°; bar angle, 60°. Protein concentration, 6 mg/ml in 0·05 M pyrophosphate buffer, pH 8·3.

weights. The proteins used for this purpose were trypsin, ovalbumin, bovine serum albumin, rabbit muscle lactic dehydrogenase, aldolase, and beef liver catalase. In each case 2 ml (4 mg) was placed on the column and eluted with 0·05 M pyrophosphate buffer, pH 8·3, in 1-ml fractions.

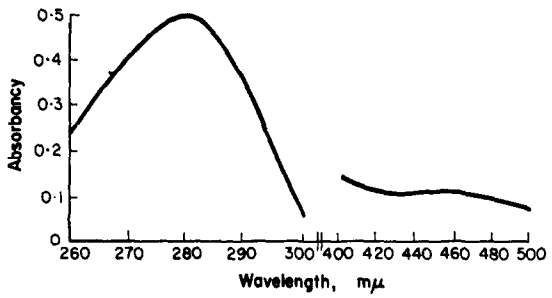


FIG. 2. Absorption spectrum of D-AAO. Original scans were made with Beckman DB in 1-cm cuvettes. Protein concentration, UV, 0·53 mg/ml; visible, 5·3 mg/ml.

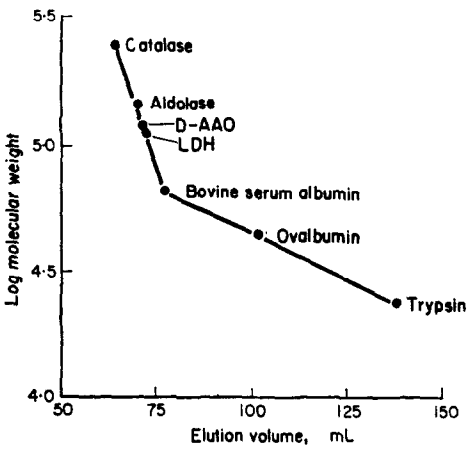


FIG. 3. Estimation of molecular weight by gel filtration.

As can be seen in Fig. 3, D-AAO was eluted in a volume which would correspond to a molecular weight of approximately 115,000. This agrees quite well with the molecular weights estimated by a variety of methods by several authors.[9, 10, 14]

Kinetics studies

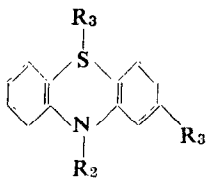
The enzymatic reaction was assayed manometrically at pH 7·3 and 37° as previously described.[1] Preliminary studies permitted the calculation of the following Michaelis constants: K_f (apoenzyme and FAD) $= 1 \times 10^{-7}$ M; K_m (apoenzyme and D-alanine) $= 3{\cdot}3 \times 10^{-3}$ M. Since most studies with D-AAO and D-alanine are performed at pH 8·3, the constants at this pH were determined for comparison and were found to be 1×10^{-7} M (K_f) and $1{\cdot}8 \times 10^{-3}$ M (K_m).

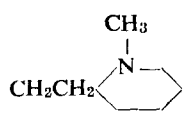
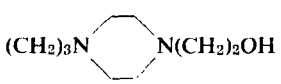
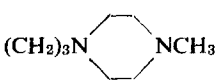
Although there was considerably more inhibition when the apoenzyme and the phenothiazine derivatives were preincubated, it was felt that the kinetic studies would be more meaningful if the apoenzyme were exposed to the FAD and the

inhibitor simultaneously. Therefore, in the inhibition studies, the apoenzyme was always pipetted last into the main compartment, and the reactions were always initiated by the addition of substrate from the side arm of the Warburg flask.

The structures of the phenothiazine derivatives used in this study are given in Table 1.

TABLE 1. STRUCTURE OF PHENOTHIAZINE DERIVATIVES



	R_1	R_2	R_3
Aliphatic series			
Promazine	H	$(CH_2)_3N(CH_3)_2$	
Chlorpromazine	Cl	idem	
Chlorpromazine sulfoxide	Cl	idem	=O
Triflupromazine	CF_3	idem	
Trimeprazine	H	$CH_2CH(CH_3)CH_2N(CH_3)_2$	
Promethazine	H	$CH_2CH(CH_3)N(CH_3)_2$	
Piperidine series			
Thioridazine	SCH_3		
Thioridazine sulfoxide	SCH_3	idem	=O
Thioridazine disulfoxide	$S(O)CH_3$	idem	=O
Piperzine series			
Perphenazine	Cl		
Fluphenazine	CF_3	idem	
Trifluoperazine	CF_3		

RESULTS

The results obtained with one representative from each of the three series of phenothiazine derivatives are presented in the standard Lineweaver–Burk plots of Figs. 4–6. The inhibition by chlorpromazine (CPZ), thioridazine, and trifluoperazine can thus be seen to be FAD-competitive. Triflupromazine, fluphenazine, and perphenazine behaved in a qualitatively similar fashion. The K_i's for these inhibitory derivatives are given in Table 2.

Promazine, promethazine, trimeprazine, and the sulfoxides of CPZ and thioridazine had no effect on D-AAO even in concentrations as high as 4×10^{-4} M.

In order to investigate further the specificity of this system, imipramine and chlorprothixene (Fig. 7) were also examined. Imipramine (4×10^{-4} M) did not

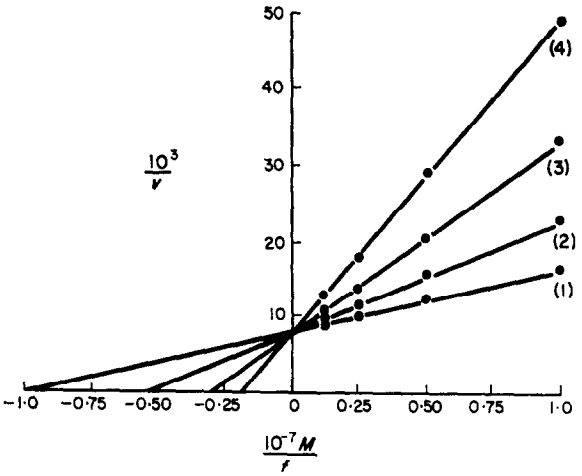


FIG. 4. Coenzyme-competitive inhibition of D-AAO by chlorpromazine. The reaction mixture contained 1 ml pyrophosphate buffer (0·1 M, pH 7·3), 18μg apoenzyme, and the indicated final concentrations of FAD (f) and chlorpromazine. D-alanine (0·2 ml) was added from the side arm to give a final concentration of $4{\cdot}45 \times 10^{-2}$M. The final chlorpromazine concentrations were: (1) none, (2) 4×10^{-5}M, (3) 10^{-4}M, and (4) 2×10^{-4}M. Final reaction volume, 2·5 ml; gas phase, air; temp., 37°. Velocity is expressed as μlO_2 in 30 min.

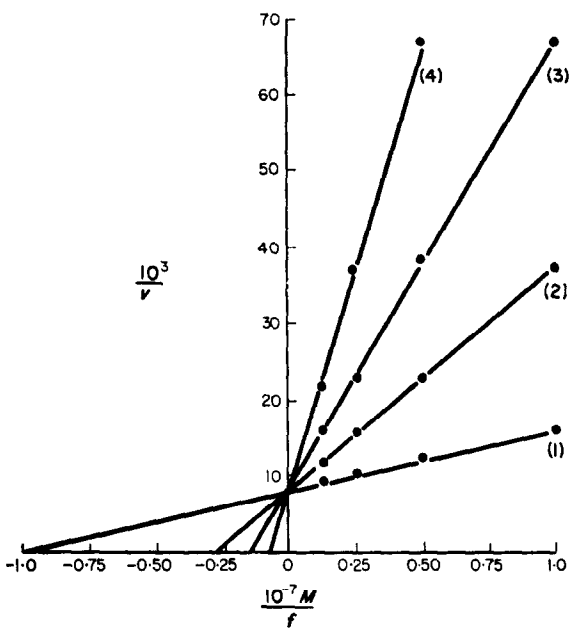


FIG. 5. Coenzyme-competitive inhibition of D-AAO by thioridazine. The reaction mixtures and conditions were identical with those in Fig. 4. Final thioridazine concentrations were: (1) none. (2) 4×10^{-5}M, (3) 10^{-4}M, and (4) 2×10^{-4}M.

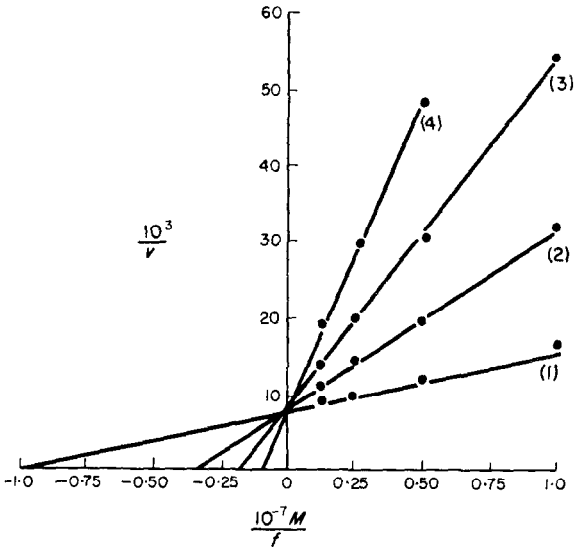


FIG. 6. Coenzyme-competitive inhibition of D-AAO by trifluoperazine. The reaction mixtures and conditions were identical with those in Fig. 4. Final trifluoperazine concentrations were: (1) none, (2) 4×10^{-5}M, (3) 10^{-4}M, and (4) 2×10^{-4}M.

TABLE 2. COMPARISON OF INHIBITORY POTENCIES AND RELATIVE ACTIVITY OF PHENOTHIAZINE DERIVATIVES (CPZ = 1·0)

	K_i*	Pharmacological tests† Reduction of: CAR‡	SMA‡	Clinical efficacy/ Side-effects†	Antipsychotic daily dosage (mg)§
Chlorpromazine	5×10^{-5}M	1·0	1·0	1·0/mild	120–300
Triflupromazine	3×10^{-5}M	2·5		10·0/severe	60–150
Perphenazine	$2{\cdot}4 \times 10^{-5}$M	7·4	6·3		16–64
Fluphenazine	$2{\cdot}3 \times 10^{-5}$M				2·5–15
Trifluoperazine	$2{\cdot}0 \times 10^{-5}$M	10·0	11·0	15–20/readily occur	2–4
Thioridazine¶	$1{\cdot}6 \times 10^{-5}$M	<1·0	<1·0		40–120

* Determined in present study.

† Taken from M. W. Parkes' data (Ref. 35), who calculated from figures by original authors. References to original paper are given in his paper.

‡ Abbreviations: CAR, conditioned avoidance response; SMA, spontaneous motor activity.

§ Taken from Ref. 17 (pp. 134–39).

¶ N.B.: Thioridazine does not fit this pattern.

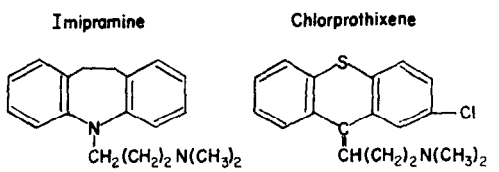


FIG. 7. Structures of imipramine and chlorprothixene.

inhibit D-AAO even when only 10^{-7} M FAD was present in the reaction mixture. Chlorprothixene, however, was found to be an effective FAD-competitive inhibitor of D-AAO (Fig. 8) with a K_i of 4×10^{-5} M.

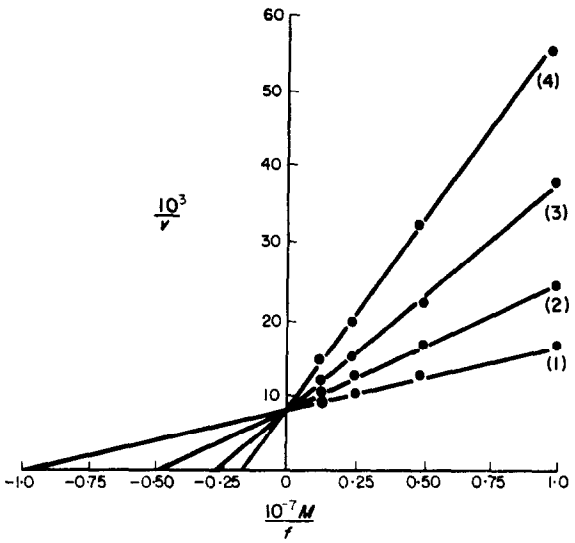


FIG. 8. Coenzyme-competitive inhibition of D-AAO by chlorprothixene. The reaction mixtures and conditions were identical with those in Fig. 4. Final chlorprothixene concentrations were: (1) none, (2) 4×10^{-5}M, (3) 10^{-4}M, and (4) 2×10^{-4}M.

DISCUSSION

In regard to the relative inhibitory capacities of the various phenothiazine derivatives, the results of this study are in good agreement with those previously reported with a relatively less pure enzyme.[2] Since trifluprομazine had a lower K_i than CPZ, and since promazine was completely ineffective in the concentrations tested, the substitutions on carbon-2 may be arranged in the following order of decreasing inhibitory power: $CF_3 > Cl > H$. This relationship is in very good agreement with results obtained from studies of suppression of conditioned responses in rats and taming action in monkeys.[15] Activity was reported to increase in the following order: H = OMe < Ac < Cl < CF_3. The 2-substituents influence properties of the trifluoromethyl and chloro groups, and this is also reflected somewhat similarly throughout the piperazine series of derivatives in which fluphenazine was found to have a slightly lower K_i than perphenazine. Since the difference in K_i's between the CF_3 and Cl analogs is not so well marked in the piperazine group as it is in the aliphatic series, there is an indication that the nature of the side chain on the ring nitrogen atom is a more important determinant of inhibitory capacity than is the nature of the group substituted at carbon-2. In this regard, it should also be noted that even the 2-chloro piperazine derivative (perphenazine) exhibited a lower K_i than the 2-trifluoromethyl aliphatic compound (trifluprομazine).

It is not possible from these studies to assess the relative contributions of the thiomethyl group or the piperidine side chain in thioridazine. It would be necessary

to test not only the 2-Cl and 2-CF_3 analogs of thioridazine, but also the 2-SCH_3 analogs with an aliphatic or piperazine side chain to obtain this information. In a similar manner, the effects of branching or shortening the aliphatic chain could not be assessed, since both trimeprazine and promethazine are unsubstituted at carbon-2.

Since chlorprothixene did inhibit D-AAO, the ring nitrogen atom apparently is not a specific structural requirement for D-AAO inhibition by these compounds. The failure of imipramine to exhibit any inhibitory properties cannot be properly evaluated, since it also is unsubstituted at carbon-2. Because of this it is impossible to delineate the effect of replacement of the ring sulfur atom with two methylene groups.

The behavior of these two compounds, however, lends support to the original hypothesis that phenothiazine tranquilizers might act by inhibiting flavoenzymes.[1] Although both are structurally quite similar to the phenothiazines, chlorprothixene's tranquilization properties are approximately equal to those of CPZ,[16-18] while imipramine has exactly the opposite clinical effect, i.e. it is an antidepressant. In regard to the relationship between the antipsychotic efficacy of the phenothiazine derivatives and their ability to inhibit D-AAO, three particularly relevant observations may be made: (1) only those compounds with definite clinical usefulness were found to inhibit D-AAO, (2) their relative inhibitory capacities are generally lower for those compounds that require larger dosages for their efficacy and potency in clinical use,[17] and (3) the K_i's represent concentrations that might be expected pharmacologically.[19, 20] Paradoxically, CPZ is still by far the most widely used compound in antipsychotic therapy even though it requires considerably higher dosages to achieve its effect. However, it is a fairly well-established fact that the increase in clinical effectiveness and the incidence of side effects tend to be parallel (cf. Table 2). If the various types of side effects associated with the use of these agents could be properly quantitated in terms of severity, perhaps this could be resolved. Indeed, flavoenzyme inhibition might be intimately associated with certain of these secondary effects.

A correlation between the inhibitory potencies of phenothiazine derivatives and their clinically effective daily dosages, as outlined in Table 2, was attempted. As can be seen, some derivatives withstand the comparison. This discrepancy, however, deserves a few comments. (1) Antipsychotic daily dosages, unfortunately, have not been rigorously quantitated[17, 18] (likewise, dosages as a continued use for optimum effects have not been agreed upon). (2) The range of dosages so far established (by clinicians or on recommendation by manufacturers) have been very arbitrarily selected, and routinely divided into daily dosages in "mild states" or "severe states". (3) A further complication arises when daily dosages, depending on the indications, are recommended as b.i.d., t.i.d., or q.i.d. Thus, owing to these uncertainties we have indicated the lower and higher dosages, for an average daily dosage range would not do justice to this comparison (cf. Table 2). Since a uniformity in administering these drugs is deemed necessary, this problem of clinically effective doses should be resolved. Perhaps, as opposed to the general practice, dosages expressed as milligrams per kilogram body weight per day would be a welcome standard criterion of safety as well as effectiveness. In this connection, it should be remembered that the judicious use of these antipsychotic agents should not only involve proper selection of drug and doses but also appropriate measures for reducing or counteracting side effects.

Since the inhibition is coenzyme-competitive, presumably the drugs bind to the same sites on the apoenzyme as does the FAD. In this connection it would be desirable if the exact mode of attachment of FAD to the apoenzyme were known. Walaas and Walaas[21] have suggested that FAD was bound to the apoenzyme via its phosphoryl, ribosyl, and adenine moieties but that the 6-amino group of adenine was not involved. Yagi and Harada,[22] however, have proposed that this 6-amino group was hydrogen-bonded to an –SH group of protein. Frisell and Hellerman[23] have shown that pre-exposure of the apoenzyme to FAD protected it from –SH reagents. Preliminary attempts to implicate an –SH group in the present study have thus far been inconclusive. Reduced glutathione protected D-AAO slightly when present in a 10 : 1 molar ratio to triflupromazine, but cysteine and mercaptoethanol were completely ineffective. Reduced glutathione and cysteine have been shown to protect some enzymatic systems against CPZ[24–26] but to be ineffective in other systems.[27, 28]

In regard to other possible means of binding, a hydrogen bond between the 3-imino group of FAD and a tyrosyl residue of the protein has been proposed,[29, 30] while other authors[31, 32] have suggested that a charge transfer complex is involved as a major interaction in association of FAD and apoenzyme. This latter is especially interesting in view of the fact that Szent-Györgyi and his co-workers,[33, 34] on the basis of molecular orbital calculations, have suggested that CPZ was an unusually effective electron donor and was capable of forming stable charge-transfer complexes with a variety of electron acceptors.

Although the differences in inhibitory potency cannot be explained yet in terms of binding sites, the available data concerning the approximate correlation between K_i and the relative efficacy in clinical use of these compounds in antipsychotic therapy, the failure of phenothiazines with little (if any) tranquilization properties to inhibit D-AAO, and the fact that the concentrations of drug required for inhibition (K_i) are well within the range that may be encountered *in vivo* all offer additional clues to the hypothesis that the phenothiazine tranquilizers might exert their primary or secondary effects by inhibiting flavoenzymes.

Confirmation of this hypothesis must await investigation of the interactions of these compounds with flavoenzymes of definite biochemical and physiological significance (e.g. xanthine oxidase, diaphorases), and particularly with those of the central nervous system (e.g. succinic dehydrogenase). In the absence of such studies, a generalization or full interpretation of the present study is obviously difficult. These points will have to be dealt with in future work.

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