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STUDIES OF FLAVIN ADENINE DINUCLEOTIDE-REQUIRING ENZYMES AND PHENOTHIAZINES—I. INTERACTIONS OF CHLORPROMAZINE AND D-AMINO ACID OXIDASE

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Abstract—The hog kidney D-amino acid oxidase used in this study was purified approximately 200-fold. The apooxidase, devoid of FAD, exhibited no glycine oxidase activity and was stable over a period of at least 180 days when maintained at -15°.

The limited solubility of phenothiazines at pH 8·3 required that a reliable system be obtained at pH 7·3. That this was accomplished is evidenced by kinetic studies which demonstrated the dependence of enzymic activity on substrate ($K_m = 1{\cdot}37 \times 10^{-2}$ M), FAD ($K_f = 1{\cdot}88 \times 10^{-7}$ M), and enzyme concentrations.

Chlorpromazine was found to inhibit D-amino acid oxidase in competition with its coenzyme, FAD; the K_i for the reaction being $2{\cdot}5 \times 10^{-4}$ M at pH 7·3. The degree of inhibition varied with the method of exposure of the apooxidase, FAD, and chlorpromazine to each other. In addition, this inhibition was found to be a function of the apooxidase protein concentration; i.e. as the protein concentration increased, the extent of inhibition decreased. This was also confirmed by a decreased inhibition when albumin was added to the reaction mixture. Therefore, it is suggested that this nonspecific complexing between chlorpromazine and protein might explain the large number of diverse effects attributed to chlorpromazine and has undoubtedly complicated inhibition studies.

THE pioneers of drug enzyme chemistry have shown in a number of instances that drug actions could be explained on the basis of specific reactions with individual enzymes or a group of enzymes involved in the same function[1–4]. In no instance, however, have comprehensive studies of a class of enzymes requiring the same prosthetic group ever been reported. From the as yet scanty literature available on the mechanism of action of phenothiazine derivatives, it is evident that the tacit assumption underlying much of this work is that these tranquilizing agents specifically inhibit some enzyme system.[5–9]

The biochemical mechanism by which chlorpromazine (CPZ)[10 — (3-dimethylaminopropyl)-2-chlorphenothiazine] exerts its pharmacologic effect is still unknown, although a wide variety of biochemical effects has been attributed to it. Thus, it has been found to inhibit respiratory and glycolytic enzymes[3, 7 10–13] and cholinesterase,[14–16] to uncouple oxidative phosporylation,[6, 17, 18] and to inhibit and stimulate ATP-ase.[7]

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One could visualize the limited structural analogy between the N-alkyl-substituted phenothiazine moiety of chlorpromazine and the N-ribityl-substituted isoalloxazine component of FAD as the basis for an inhibition of FAD-requiring enzymes by phenothiazine derivatives. Yagi *et al.* have shown that CPZ inhibits D-amino acid oxidase (D-AAO) [D-amino acid: 0_2 oxidoreductase (deaminating), E.C. 1. 4. 3. 3] in dog brain.[19] Lazzlo and Meyer,[20] however, have reported that CPZ both inhibits and potentiates the activity of D-AAO. In addition, Kurokawa *et al.*[11] have found that another flavin enzyme, succinic dehydrogenase, is also both inhibited and potentiated by CPZ.

It is believed that a systematic comparison of the interactions of flavoenzymes with phenothiazine derivatives not only might elucidate metabolic reaction sequences and mechanisms but also might lead us to the understanding of the mode of action of psychoactive drugs and perhaps may provide the ground rules for the systematic attempt in producing agents designed to change the functioning enzymes.

The reasons for beginning these studies with the selection of D-AAO as a representative of the flavoenzymes are that: (1) its coenzyme, FAD, may be easily cleaved off from its apoprotein and thus an apooxidase devoid of FAD may be prepared; (2) the apoxidase is instantaneously associable with FAD so that the holoenzyme can be easily reconstituted and its activity restored with exogenous FAD; (3) its catalytic efficiency may be conveniently assayed.

This communication describes the interactions of CPZ and D-AAO in isolated systems.

MATERIALS AND METHODS

Reagents

All reagents were prepared in doubly glass-distilled water and tested as stated below:

1. DL-Alanine was purchased from Mann Research Laboratories and stored as a 0·562 M (5%) stock solution.

2. Pyrophosphate buffer was prepared as a 0·2 M stock solution (pH 10·3) and diluted as needed to 0·1 M. The pH was adjusted with HCl to pH 7·3 or 8·3.

3. FAD was obtained as the sodium salt from California Biochemical Corp. and used with no further purification. Massey and Swoboda[21] have recently reported that this FAD has no flavin mononucleotide, but does contain 9% riboflavin, which is a competitive inhibitor of D-AAO. However, this trace amount of riboflavin contaminant is not of sufficient magnitude to influence the kinetics.

The absorption spectrum of the FAD was similar to that described by Warburg and Christian[22] and the ratio of the absorbance at 260 mμ to that at 450 mμ of a 2×10^{-5} M solution, pH 7·0 was 3·27, which indicated a purity of about 93%[23, 24].

A 10^{-3} M stock solution was prepared and stored at − 15° in small individual flasks which were screened from daylight to prevent photolysis. Fresh dilutions were prepared for each assay and the concentrations determined on the basis of a molar extinction coefficient of $11{\cdot}3 \times 10^3$ M^{-1} cm^{-1} at 450 mμ, pH 7·0.[25]

4. Chlorpromazine, HCl (lot 1091–TZ) was generously supplied by Dr. E. J. Van Loon of Smith, Kline & French. A 10^{-2} M solution was always freshly prepared prior to its use. The concentration of the subsequent dilutions used throughout these

experiments was estimated on the basis of a molar extinction coefficient of $3{\cdot}55 \times 10^3\ M^{-1}\ cm^{-1}$ at 306 mμ.

Enzyme purification

Hog kidney D-AAO was obtained commercially (Mann Research Laboratories) as an acetone powder and purified, with minor modifications, through the fifth step of the procedure of Negelein and Brömel,[26] as described by Burton.[27] The purified apooxidase was stored undialyzed* at −15° in 2-ml aliquots which could then be individually diluted for daily activity and protein determinations. Under these conditions the specific activity of the apooxidase, which was increased approximately 200-fold, did not vary from one preparation to another and was stable for at least 6 months. The final apooxidase solution derived from each of seven different batches of acetone powder was found to have a pH 6·1–6·4, which corresponds to the pH of optimum stability.[27]

Determination of activity

The kinetic studies were performed manometrically in the GME-Lardy circular Warburg apparatus (WB3) with an attached gassing distributor. The rate of shaking was 104 excursions/min with a stroke of 4 cm at the center of the vessel. Except for some preliminary studies with oxygen, air was used as the gas phase. All reactions were run at 37°. The center well contained 0·2 ml of 2 N NaOH and Whatman 1 filter paper ($1 \times 1\ cm^2$).

The reaction mixtures were first allowed to equilibrate for 5 min at 37°. During the next 5 min the flasks were ground in and allowed to equilibrate further. The contents of the side arm were tipped with a triple rinse (3 min) and, after an additional 5-min equilibration, the manometers were closed and the zero time read. Readings were taken every 10 min for at least an hour, during which time the rate was always linear. No change in pH was noted at the end of the reaction.

Since the order of addition and the method of exposure of the reactants to each other were expected to affect the kinetics of the reaction, three experimental designs were employed in which the compounds added from the side arm were varied as follows: (1) CPZ and FAD, (2) apooxidase and CPZ, and (3) FAD and substrate. The details for each system are presented below in the appropriate figure legends. In all cases, the CPZ was pipetted into the flask 10 min before the start of the thermal equilibration period in the Warburg apparatus.

Protein determination

Protein concentrations were determined spectrophotometrically (Beckman DU and DB) on the purified apooxidase prior to each assay. The $E_{215\ m\mu}$–$E_{225\ m\mu}$ method of Waddell[29] was used, since intensive studies in this laboratory have shown that this method agrees quite closely with both the Lowry modified biuret procedure[30] (on dialyzed samples) and micro-Kjeldahl nitrogen determinations with crystalline bovine albumin as the standard. We have found, as Yagi and Ozawa[31] have also recently reported, that Kalckar's method[32] ($1{\cdot}45\ E_{280\ m\mu} - 0{\cdot}74\ E_{260\ m\mu}$) gives protein values about twice those obtained by Waddell's method. A preliminary report of these protein studies has already appeared.[33]

* $(NH_4)_2SO_4$ does not interfere with enzymic reaction.[28]

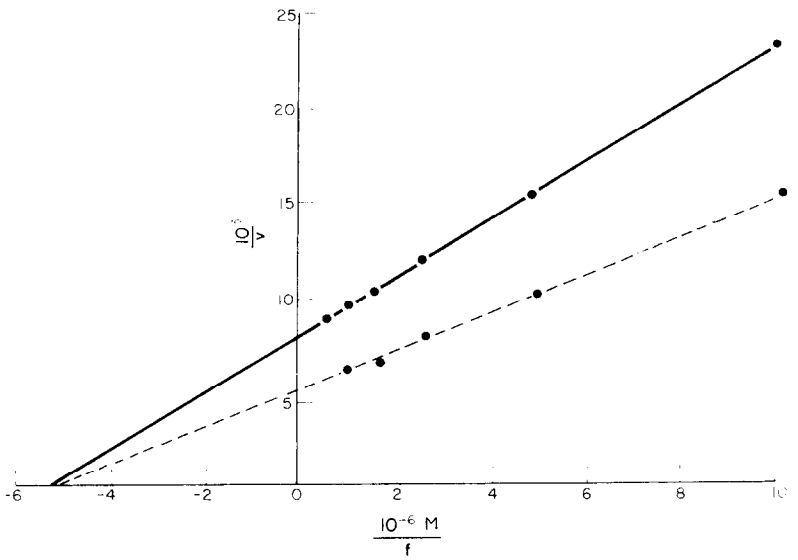

FIG. 1. Enzymic activity of D-AAO as a function of FAD concentration. The reaction mixture contained 1 ml 0·1 M pyrophosphate buffer (pH 7·3 or 8·3), 1 ml enzyme solution (125 μg/ml), 0·1 ml FAD to give the indicated final concentration (f), and 0·2 ml H_2O. DL-Alanine (0·2 ml) was added from the side arm to give a final concentration of $4{\cdot}49 \times 10^{-2}$ M. Velocity (v) is expressed as μl. O_2/hr at 37° at pH 7·3 (——) and pH 8·3 (– – –). Gas phase: air. Total volume: 2·5 ml.

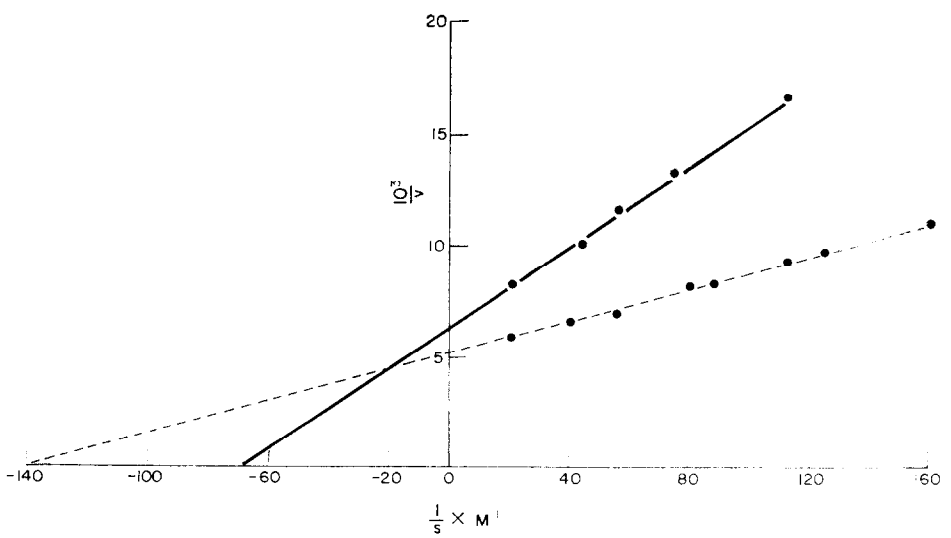

FIG. 2. Enzymic activity of D-AAO as a function of substrate concentration. The reaction mixture was the same as in Fig. 1. The final FAD concentration was 2×10^{-6} M, and the final substrate concentrations (s) were as indicated. Velocity (v) is expressed as μl. O_2/hr at 37° at pH 7·3 (——) and pH 8·3 (– – –). Gas phase: air. Total volume: 2·5 ml.

RESULTS AND DISCUSSION

Characterization of the apooxidase

1. *Kinetics.* Although the pH optimum [34]of D-AOO is about 8·8, the limited solubility of most phenothiazine derivatives at this pH and the desirability of using a more physiological pH necessitated the establishment of a workable system at pH 7·3. That this was accomplished is evidenced by the standard Lineweaver-Burk[35] plots in Figs. 1 and 2 which respectively demonstrate the dependence of velocity on FAD and substrate concentrations. The proportionality between enzymic activity and apooxidase concentration is shown in Fig. 3. These data were derived from approximately 40 assays in which each set of conditions was run at least in triplicate. In these studies

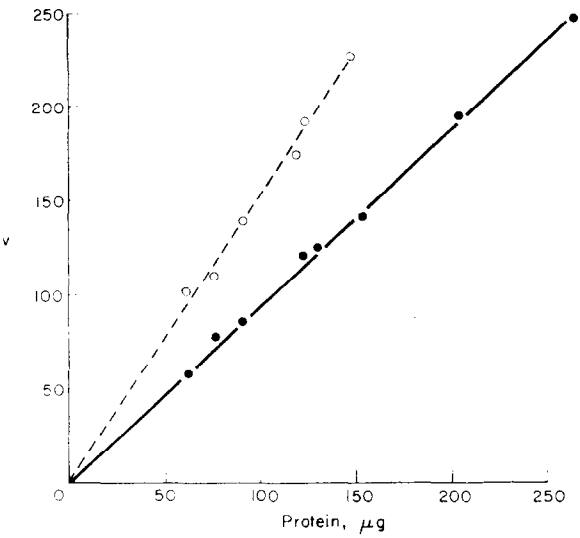

FIG. 3. Enzymic activity of D-AAO as a function of enzyme concentration. The reaction mixture was the same as in Fig. 1. The final concentrations of FAD and DL-alanine were 2×10^{-6} M and 4·49 $\times 10^{-2}$ M respectively. Velocity (v) is expressed as μl. O_2/hr at 37° at pH 7·3 (——) and 8·3 (– – –). Gas phase: air. Total volume: 2·5 ml.

DL-alanine was tipped from the side arm, since no differences were observed when the substrate, FAD, or apooxidase was thus added to the reaction mixture. The addition of catalase to the reaction mixture did not alter the reaction velocity.

The following values have been calculated from the conventional Michaelis equations: (a) K_m (apooxidase and DL-alanine)$=1{\cdot}37 \times 10^{-2}$ M (pH 7·3) and $7{\cdot}14 \times 10^{-3}$ M (pH 8·3); (b) K_f (apooxidase and FAD) $= 1{\cdot}88 \times 10^{-7}$ M (pH 7·3) and $1{\cdot}82 \times 10^{-7}$ M (pH 8·3). The values obtained at pH 8·3 are in good agreement with those reported by others.[36–39]

2. *Absence of FAD.* In order to study the interactions of FAD with phenothiazines, an enzyme devoid of FAD is essential. The preparations used here were found to have no activity without the addition of exogenous FAD. The absence of an absorption maximum at 450 mμ (Fig. 4) also demonstrated the complete removal of FAD during the purification.

3. *Analytical ultracentrifugation.* Figure 5 presents the results of the analytical ultracentrifugation of a typical apooxidase preparation. The apooxidase was found to sediment as one major, symmetrical boundary with a sedimentation coefficient of 4·5 S. This agrees quite well with the value found by Yagi and Ozawa[40] for this concentration of apooxidase.

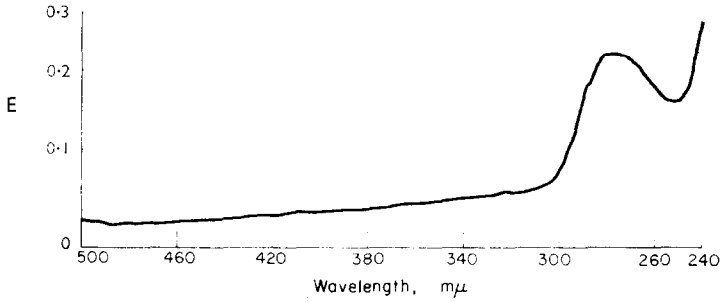

FIG. 4. Absorption spectrum of purified D-AAO. The protein content of the sample was 150 μg/ml in 1·3 × 10^{-3} M pyrophosphate buffer, pH 8·3. The scan was made in the Beckman DB spectrophotometer in 1-cm cuvets.

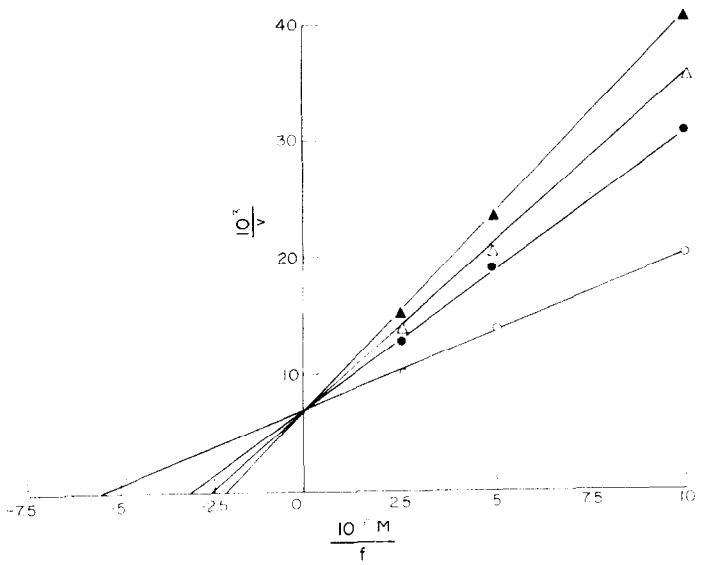

FIG. 6. Coenzyme-competitive inhibition of D-AAO by CPZ. The main compartment contained 1 ml 0·1 M pyrophosphate buffer (pH 7·3), 125 μg apooxidase protein, 4·49 × 10^{-2} M DL-alanine (final concentration). The FAD (f) and CPZ were added from the side arm (0·1 ml of each) to give the indicated final concentrations. Total reaction mixture volume: 2·5 ml. Gas phase: air. Velocity (v) is expressed as μl. O_2/hr at 37°. Final CPZ concentrations: ○, none and 10^{-4} M; ●, 2 × 10^{-4} M; △, 3 × 10^{-4} M; ▲ 4 × 10^{-4} M.

4. *Substrate specificity.* No difference could be found between the rates of oxidation of DL-alanine and D-alanine. D-Methionine was oxidized approximately twice as fast as DL-alanine at pH 7·3 and 25% faster at pH 8·3. The apooxidase used in these studies exhibited no glycine oxidase activity at all at pH 7·3 or 8·3. This is in disagreement with the results recently reported by Neims and Hellerman.[41]

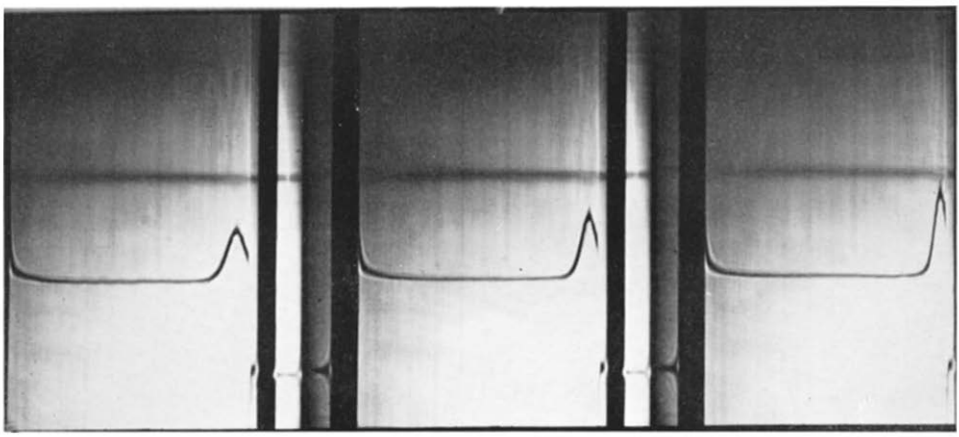

FIG. 5. Representative ultracentrifuge patterns of the purified apooxidase. The Beckman model E analytical ultracentrifuge was used, and the centrifugal direction is to the left. Photographs were taken every 4 min. Speed, 50,740 rev/min; bar angle, 50°; rotor temperature, 20°; protein concentration 1·76 mg/ml.

Inhibition studies

Figure 6 presents the standard Lineweaver-Burk[35] plot of the data from approximately fifty assays in which each set of conditions was at least in triplicate. The inhibition of D-AAO by CPZ is thus shown to be coenzyme-competitive and have a K_I of $2{\cdot}5 \times 10^{-4}$ M, as calculated by the standard methods. The K_I did not vary with CPZ concentration. Yagi *et al.*[42] have reported a K_I of $2{\cdot}3 \times 10^{-5}$ M at pH 8·3.

The inhibition could be demonstrated only over a narrow range of CPZ concentrations, since CPZ precipitates at pH 7·3 in final concentrations over 4×10^{-4} M. Final FAD concentrations slightly above 4×10^{-7} M completely reversed the inhibition. Below 10^{-7} M FAD, with the low apooxidase concentrations used, the oxygen uptake was considered too low for accurate measurement.

The effect of varying the side arm contents is illustrated in Fig. 7. The most marked inhibition occurred when the apooxidase and CPZ were preincubated together in the side arm. A detailed study of this system is now in progress, since the inhibition did not follow strictly competitive kinetics.

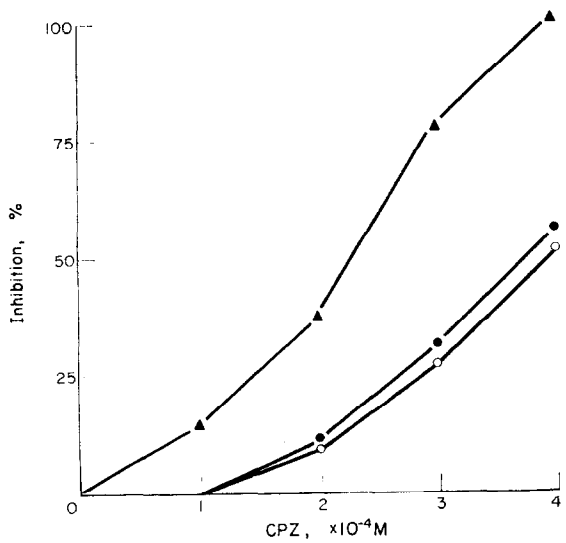

FIG. 7. Effect of different methods of exposure of the D-amino acid apooxidase, FAD, and CPZ to each other. The reaction mixtures were essentially the same as in Fig. 6. A final FAD concentration of 2×10^{-7} M and 115 μg protein was used in all three systems. The contents of the side arms were: ○, FAD, and DL-alanine; ●, CPZ and FAD; ▲, CPZ and enzyme (0·1 ml of each).

What was of more interest here was the inverse relationship (Fig. 8) that was found to exist between protein concentration and the degree of inhibition. A similar phenomenon has been described in the interaction of thioperazine* with glucose-6-phosphate dehydrogenase.[12] The influence of protein concentration was further confirmed by the observation that the addition of 100 μg crystalline bovine albumin (Sigma Chemical Co.) into the side arm with the apooxidase and CPZ reduced the amount of inhibition

* N,N-dimethyl-10-[3-(1-methyl-4-pyrazinyl)-propyl]-2-phenothiazine sulfonamide dimethane sulfonate.

by approximately 60%. This marked decrease cannot be explained on the basis of a simple volumetric dilution of the CPZ by the additional albumin. Obviously, the effective concentration of CPZ (i.e. the concentration of the apooxidase–CPZ complex) must have been lowered to cause this drastic reduction of inhibition. Albumin alone did not potentiate the enzymic activity.

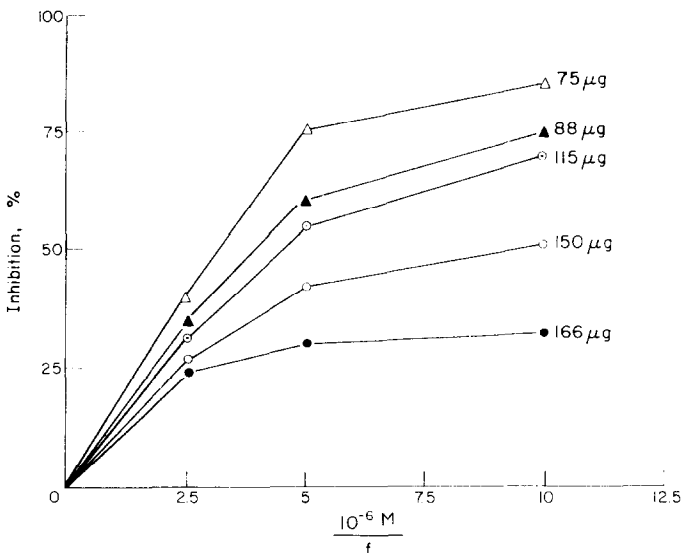

FIG. 8. Relation between apooxidase protein concentration and degree of inhibition. The reaction mixtures were the same as in Fig. 6 except for the indicated protein concentrations. The final concentration of CPZ was 4×10^{-4} M; that of FAD (f) was as indicated.

Further evidence for this protection of D–AAO from CPZ by foreign protein was obtained from studies with the hog kidney acetone powders obtained commercially from both Mann Research Laboratories and Worthington Biochemical Corp. These crude preparations contained a very high concentration of protein other than D-AAO, and it was impossible to demonstrate inhibition even under the most traumatic conditions; i.e. with the CPZ and enzyme in the side arm. Lazzlo and Meyer,[20] on the other hand, have reported a slight potentiation of crude D-AAO preparations by CPZ and believe it to be caused by a complex formation between CPZ and FAD which, consequently, removed an inhibitory effect imposed by excess FAD. It seems more likely that the high foreign protein content of their enzyme preparation (Worthington's acetone powder) and the large amounts of FAD used were responsible for their failure to demonstrate any inhibition. Any real potentiation in this case could easily be the result of the removal of a natural inhibitor present in the preparation by complexing with CPZ. It is significant to note that a nonspecific binding by CPZ to various enzymic proteins could explain a large number of diverse effects attributed to it. These studies also point out the inadvisability of employing benzoate[43] (a most selective inhibitor[44, 45]) or albumin[46] as a stabilizer during the purification of the apooxidase when inhibition studies are to be carried out. No such preservatives were used in the process of our purification.

Attempts employing spectrophotometric and spectrofluorometric methods to demonstrate complex formation between CPZ and the apooxidase remain inconclusive. To date we have been able to find no changes in the absorption spectrum of the apooxidase with the various concentration of CPZ and apooxidase protein used in the kinetic studies.

Ultracentrifugal analysis of our apooxidase preparations has indicated that aggregation may be occurring and that the preparation may not be completely homogenous. Disk electrophoresis has separated three very minor bands from the major apooxidase band. Undoubtedly, differences in purity have caused the disagreement between Yagi *et al.*[47] and Massey *et al.*[48] as to the actual molecular weight of the apooxidase.

Further elucidation of the mechanism of inhibition of D-AAO by CPZ must await the complete characterization of the purified apooxidase.

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