Mitochondrial Monoamine Oxidase

II. ACTION OF VARIOUS INHIBITORS FOR THE BOVINE KIDNEY ENZYME. CATALYTIC MECHANISM*

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SUMMARY

Inhibitions of the relatively highly purified flavoenzyme, bovine kidney mitochondrial monoamine oxidase, previously described in Paper I (Erwin, V. G., and Hellerman, L., J. Biol. Chem., 242, 4230 (1967)), by several agents of current interest have been investigated. The inhibitors include N-methyl-N-(2-propynyl)benzylamine (pargyline), (+)-trans-phenylcyclopropylamine (d form of tranylcypromine), as well as various substituted hydrazines including l-hydrazino-phthalazine (hydralazine) and β-phenylethylhydrazine (phenelzine). With the exception of hydralazine which produced a reversible, substrate-competitive inhibition (K_i, 2 x 10^{-5} M), the inhibitory hydrazine derivatives investigated caused irreversible inhibitions, differing primarily in the rate of attack on the enzyme; aromatic hydrazines, e.g. phenylhydrazine, were more aggressive than aliphatic hydrazines, e.g. methylhydrazine, members of either class being more active than hydrazides. When graded amounts of arylhydrazine, tranylcypromine, or pargyline were allowed to react with the enzyme, the activity of the enzyme was observed to decrease as a linear function of the amount of inhibitor added; complete inhibition was attained at a ratio of 1 mole of inhibitor per 10^5 g of protein. It is proposed that these results represent titration at the catalytic site of monoamine oxidase.

(+)-Tranylcypromine is known to act as a substrate-competitive inhibitor of monoamine oxidase; for our preparation, K_i was determined as 5.8 x 10^{-5} M and inhibition was seen to be only partially reversed by dialysis in the presence of substrate.

Inhibition by pargyline was not reversible and studies performed with 14C-pargyline (labeled at C-7) indicated that reaction of this inhibitor with monoamine oxidase included ultimate formation of a bond to the flavoenzyme that appeared to be essentially covalent in character. Evidence is presented that suggests that pargyline interacts initially with monoamine oxidase as an electron donor for this flavoenzyme; in a nitrogen atmosphere. reduction of the flavoenzyme by pargyline is followed by or accompanies the irreversible binding step. It was observed that pargyline did not inhibit after the enzyme was first reduced anaerobically with excess benzylamine or with sodium dithionite (followed by addition of benzylamine before the introduction of air). Tranylcypromine, a reversible, nonreducing inhibitor, was found capable of inhibiting the enzyme under such conditions.

Analytical determination establishing persistence of approximately 8 sulfhydryl residue equivalents per 10^5 g of protein in the enzyme initially inhibited by either pargyline or phenylhydrazine (identical with the sulfhydryl content of the native enzyme in our uninhibited preparation) established that these compounds had not effected inhibition of monoamine oxidase through a process involving irreversible interaction with these protein sulfhydryl residues. A mechanism for inhibition of monoamine oxidase by pargyline is proposed.

Evidence is cited suggesting that the catalytic mechanism for mitochondrial monoamine oxidase follows a pattern closely related to the mechanism (Neims, A. H., De Luca, D. C., and Hellerman, L., Biochemistry, 5, 203 (1966); Hellerman, L., and Coffey, D. S., J. Biol. Chem., 242, 582 (1967)) recently proposed for the enzyme, D-amino acid oxidase.

In recent years it has become evident that mitochondrial monoamine oxidase may play an important role in the metabolism of catecholamines and serotonin (1, 2). Administration of certain inhibitors of this enzyme to individuals has been found to produce characteristic pharmacological responses and some of these inhibitors are rather extensively used in treatment of hypertension and central nervous system depression (3, 4).
Studies concerning the inhibition in vitro of monoamine oxidase by various compounds have been the subject of a number of review articles (5-7). However, as a result of use of diverse procedures, embodying variations in time of incubation of inhibitor with enzyme, in the source of the enzyme and its state of purity, and the substrate oxidized, conflicting results on the mechanistic and quantitative aspects of monoamine oxidase inhibition have been reported (9).

In Paper I of this series (8), we reported on the purification of bovine kidney mitochondrial monoamine oxidase and its characterization as a flavoenzyme. By extension of these observations particularly to rat tissue oxidases it was inferred that mitochondrial monoamine oxidases generally are of the flavoenzyme category. In this paper we present our recent observations on the mechanism of inhibition of the kidney enzyme by various compounds, including certain agents of therapeutic interest. In addition, some experiments and concepto bearing on the mechanism of catalysis by monoamine oxidase are presented.

EXPERIMENTAL PROCEDURE

Materials—Monoamine oxidase was prepared from bovine kidney cortex as previously described (8). N-Methyl-N-(2-propynyl)benzylamine (2-14C-pargyline), d-transphenylethylcyclopropylamine hydrochloride, β-phenylethylhydrazine (phenelzine) sulfate, and 1-hydrasinophthalalazine hydrochloride (hydrasalazine) were supplied to us as the pure, powdered, or crystalline substances. Phenylhydrazine hydrochloride and α-naphthylhydrazine hydrochloride were purified by recrystallization, decomposition points being 250-250° and 250-255°, respectively. m- and p-Substituted benzylamines and their hydrochlorides were kindly prepared by Dr. D. C. De Luca1; the hydrochlorides were threex recrystallized from isopropyl alcohol.

Procedure—The kinetic measurements (based on initial rates) and determination of the molar absorptions of substituted benzaldehydes were accomplished spectrophotometrically with a Spectronic 600 Bausch and Lomb spectrophotometer equipped with a Sargent SRL recorder and AT-20 digital readout attachment. Monoamine oxidase activity was determined by the method of Tabor, Tabor, and Rosenthal (9) with benzylamine as substrate, or by the method of Weissbach et al. (10) with kynurenamine dihydromonod as substrate. The various inhibitors studied did not interfere in the enzyme assay methods.

In the investigations involving labeled materials, radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer or with a low background Nuclear-Chicago model 181A gas flow counter.

Estimation of sulfhydryl residues of monoamine oxidase was performed by use of the sensitive, essentially specific radioassay method of Neims, Coffey, and Hellerman (11). The following procedure also was used.

**Determination of Protein —SH Groups by Chromatographic Separation (Brief Outline)—** Enzyme was incubated for 1 hour with a 10-fold excess of 14C-p-chloromercuribenzoate, the mixture then being passed through a Sephadex G-25 column (1 × 30 cm); equivalents of 14C-mercurobenzoate that were bound to the protein that eluted in the void volume of the column were calculated. Details of this method will be elaborated on later. The radioassay method with tetraethylthiuram disulfide (11) is considered to be the more widely applicable and objective procedure.

Protein determinations were performed by the Folin-biuret method with use of crystalline bovine serum albumin as standard.

**DISCUSSION OF RESULTS**

Effects of Certain Hydrazines on Mitochondrial Monoamine Oxidase—Zeller, Barsky, and Berman (12) reported that various hydrazides, such as iproniazid, produce an irreversible inhibition of monoamine oxidase. It has been suggested that this compound is hydrolyzed to give isopropylhydrazine, here the actual inhibitor of the enzyme (13). As a result of the use of diverse experimental conditions and also the use of enzyme preparations of low and varying purity, conflicting results concerning the quantitative aspects of monoamine oxidase inhibition by the hydrazines have been reported (compare Reference 14). Therefore, we have used our relatively highly purified mitochondrial monoamine oxidase (8) in determination of the characteristics of inhibition of this enzyme by various hydrazines. As shown in Fig. 1, the (initial) rate of inhibition by the aromatic hydrazine α-naphthylhydrazine and phenylhydrazine was 1.6 to 2.15-fold greater than the rate of inhibition with methylhydrazine. However, after any of these hydrazines had been incubated with the enzyme (1 eq of inhibitor per 100 g of protein) for approximately 2 hours, complete inhibition was observed with each of the compounds studied except 1-hydrasinophthalalazine. This compound was observed to have produced at 37° a competitive inhibition of monoamine oxidase, $K_i, 2.0 \times 10^{-4}$ M.

(Figs. 2 and 3), with either benzylamine or kynurenamine as substrate, and the inhibition was found to be reversible either by

1 Unpublished data.
2 We are deeply appreciative of generous donations of valuable research samples of certain compounds required in this investigation. The samples were provided as follows: pargyline, 14C-labeled pargyline salt by Dr. K. G. Wiegand, Chemical Pharmacology Section, Scientific Division, Abbott Laboratories; d-transphenylethylcyclopropylamine hydrochloride and several other isomers related to tranylcypromine, Dr. C. L. Zirkle, Research and Development Division, Smith, Kline and French Laboratories; p-phenylethylhydrazine (phenelzine) sulfate, Mr. L. N. Starker, Warner-Lambert Research Institute, research affiliate of Warner-Chilcott Laboratories; 1-hydrasinophthalalazine (hydrasalazine) hydrochloride, Dr. A. J. Plummer, Research Department, Ciba Pharmaceutical Company.
3 Present address, Department of Biochemistry, University of Arkansas, School of Medicine, Little Rock, Arkansas 72201.

The data of Reference 8 indicate that on an equivalent weight basis with respect to flavin content and enzyme protein (1 residue weight flavin per 100 g) this preparation may be assumed to have been 50% "purified." Although sedimentation data suggested "homogeneity," other characteristics contradicted any assumption that the enzyme preparation in fact consisted of a homogeneous protein. However, our data generally, and especially the data of Table VI, suggest that the presence of nonenzyme impurities has not altered the binding and stoichiometric relationships for various inhibitors.
Fig. 1. Inhibition of monoamine oxidase activity by hydrazines. Sufficient a-naphthylhydrazine hydrochloride (Δ—Δ), phenylhydrazine hydrochloride (●—●), or methylhydrazine hydrochloride (○—○) was added to 360 units of enzyme (specific activity 12,000) to give 1 eq of inhibitor per 10^6 g of protein in a volume of 0.2 ml (final inhibitor concentration, 1.5 × 10^{-7} M) and a final concentration of 0.1 M potassium phosphate buffer, pH 7.6. After incubation at 37° for the periods shown, 1.7 ml of 0.1 M phosphate buffer, pH 7.6, and 0.1 ml of 0.05 M benzylamine were added to each sample and the enzyme activity was determined spectrophotometrically as described in the text. For each time interval, enzyme samples were incubated as above without added inhibitor, and the activity was compared to samples containing inhibitor; under these conditions, incubation of the enzyme without inhibitor for 140 min at 37° resulted in a loss of approximately 20% of the original enzyme activity.

addition of excess substrate or by dialysis (Table I). All other hydrazines studied produced irreversible inhibitions (cf. Table I).

When monoamine oxidase was incubated with varying amounts of phenylhydrazine in discontinuous "titrations," the activity was observed to decrease as a linear function of the amount of inhibitor added; as shown in Fig. 4, complete inhibition was obtained at approximately 1 eq of phenylhydrazine per mole eq of flavonucleotide (10^6 g of protein). These results indicate that the hydrazines react stoichiometrically with a specific site on the flavoenzyme molecule.

Linear Inactivation of Monoamine Oxidase by Pargyline and (+)-Transphenylcyclopropylamine—Although it has been shown that pargyline and (+)-transphenylcyclopropylamine (14) are highly effective inhibitors of mitochondrial monoamine oxidase, certain aspects of the mechanism of inhibition have not previously been described. As shown in Fig. 4, pargyline and (+)-trans-2-phenylcyclopropylamine produced complete inhibition at a ratio of 1 mole of inhibitor per 10^6 g of protein. Also, the activity decreased linearly when increasing amounts (serially) of either inhibitor were incubated with the enzyme. Interaction was rapid at 37°. Inhibition of monoamine oxidase by pargyline was irreversible while inhibition by (+)-trans-2-phenylcyclopropylamine was partially reversed by prolonged dialysis against benzylamine (Table I). Zeller and Sarkar (13) observed that inhibition of monoamine oxidase by (+)-trans-2-phenylcyclopropylamine (d form of tranylcypromine) was partially reversed by benzylamine and stated also that this inhibition was fully reversed by addition of phenylbutylamine. Linear titration of enzymatic activity with (+)-transphenylcyclopropylamine, a reversible inhibitor, apparently to complete inactivation at a ratio of 1 eq of inhibitor per 10^6 g of protein, most probably depends on the significantly high affinity of this inhibitor for the enzyme catalytic site. The latter assumption is consistent with observations of the substrate-competitive nature of inhibition of the enzyme by (+)-transphenylcyclopropylamine with K_I, 5.8 × 10^{-5} M (Fig. 5). Belleau and Moran (15) and Guha...
have reported similar results concerning competitive inhibition by tranylcypromine.

As described above, phenylhydrazine, pargyline, and (+)-transphenylcyclopropylamine effectively inactivate monoamine oxidase at a ratio of 1 mole of the respective inhibitor per 10^5 g of protein. In previous studies we reported that our concentrated enzyme preparation contained 1 mole eq of catalytically active flavin group per 10^5 g of protein (8). Therefore, it was of interest to determine whether these inhibitors were possibly acting at the "catalytic site" of the enzyme. When limiting amounts of varying mixtures of these agents were incubated with the enzyme, the percentages of inhibition were found to be additive (Table II); this suggests that these inhibitors are acting independently, but at the same site, i.e. the catalytic site of the enzyme. In support of this suggestion, it was observed that the substrate, benzylamine added initially, has the capability of protecting against inhibition by these inhibitors (Table III). It may be noted that benzylamine had afforded but little protection against inhibition by phenylhydrazine (9) but did protect the enzyme from inhibition by the other compounds referred to above, suggesting that pargyline and phenylhydrazine are not reacting with a sulfhydryl residue of the enzyme.

**Table I**

**Reversal of inhibition of monoamine oxidase by dialysis against substrate**

Phenylhydrazine, pargyline, or d-trans-2-phenyleicyclopropylamine was added to 0.1 mg of enzyme (specific activity 12,000) in sufficient quantity to give 2 mole eq of inhibitor per 10^5 g of protein. The final concentration of 1-hydrazinophthalazine was 5 × 10^{-5} M. Each sample contained a final concentration of 0.05 M phosphate buffer, pH 7.6, in a total volume of 3 ml, after dialysis at 4°C against 100 volumes of 1 mM benzylamine hydrochloride in 0.05 M phosphate buffer, pH 7.6, for 4, 12, and 12 hours successively, aliquots of each sample were removed and monoamine oxidase activity was determined as described in the text.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition</th>
<th>Reversal of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylhydrazine</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Pargyline</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>d-trans-2-Phenylcyclopropylamine</td>
<td>50%</td>
<td>90%</td>
</tr>
</tbody>
</table>

* Pargyline, N-methyl-N-(2-propynyl) benzylamine.

**Fig. 5.** Kinetics of monoamine oxidase inhibition by d-trans-2-phenyleicyclopropylamine. The reaction mixture contained 3 μg of enzyme protein (specific activity 12,000). Assay conditions are described in the text. The ordinate gives the reciprocal of the change in absorbance at 250 μm per min at 37°C, and the abscissa gives the reciprocal of the molarity of benzylamine, K_I for the d form of tranylcypromine was 5.8 × 10^{-5} M. In similar experiments conducted with kynuramine as substrate, the K_I was determined as 5.9 × 10^{-6} M.

**Fig. 4.** Titration of monoamine oxidase activity with pargyline, d-trans-phenyleicyclopropylamine, and phenylhydrazine. Varying amounts of pargyline ( ), d-trans-phenyleicyclopropylamine ( ), or phenylhydrazine ( ) were added to 3 μg portions of monoamine oxidase (specific activity 12,000) in 0.05 M phosphate buffer, pH 7.6, and the mixtures were held at 37°C for 30 min. The enzyme-catalyzed reaction was initiated by addition of sufficient benzylamine hydrochloride to give 3 μM final concentration in 2 ml. Enzyme activity was assayed spectrophotometrically as described in the text. (For structure of pargyline, see "Discussion")
Enzyme activity was assayed spectrophotometrically after the addition of sufficient benzylamine hydrochloride to give 3 mM final concentration in 2 ml.

**TABLE II**

Additive effect of pargyline, d-trans-phenylcyclopropylamine, and phenylhydrazine in relation to oxidase inhibition

Varying amounts of inhibitors were added to 3-μg portions of enzyme protein (specific activity 12,000) in 0.05 M sodium phosphate buffer, pH 7.6, and the mixtures were held at 37° for 2 hours. Enzyme activity was assayed spectrophotometrically after the addition of sufficient benzylamine hydrochloride to give 3 mM final concentration in 2 ml.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Total inhibitor per 10⁵ g of protein</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Pargyline</td>
<td>0.25</td>
<td>24.8</td>
</tr>
<tr>
<td>+ d-trans-2-Phenylcyclopropylamine</td>
<td>0.25</td>
<td>25.2</td>
</tr>
<tr>
<td>+ Phenylhydrazine</td>
<td>0.25</td>
<td>26.0</td>
</tr>
<tr>
<td>+ Pargyline + d-trans-2-phenylcyclopropylamine</td>
<td>0.50</td>
<td>51.0</td>
</tr>
<tr>
<td>+ Pargyline + phenylhydrazine</td>
<td>0.50</td>
<td>50.2</td>
</tr>
<tr>
<td>+ Pargyline + d-trans-2-phenylcyclopropylamine + phenylhydrazine</td>
<td>0.75</td>
<td>74.0</td>
</tr>
</tbody>
</table>

* The amount of inhibitor added to enzyme was 0.25 mole in each case.

b Pargyline, N-methyl-N-(2-propynyl)benzylamine.

c d-trans-2-Phenylcyclopropylamine is the d form of tranelypromine.

**TABLE III**

Protection from inhibition of monoamine oxidase by substrate and reduced glutathione

Enzyme and reagents were added in the order shown. Of the enzyme-protein, 3 μg (specific activity 12,000) were incubated for 30 min at 37° with 0.05 M phosphate buffer, pH 7.6, with or without 1 mM substrate; then there was added 3 mole eq of pargyline or phenylhydrazine per 10⁵ g of protein or 16 mole eq of p-chloromercuribenzoate (CMR) per 10⁵ g of protein. Sufficient freshly prepared, neutralized glutathione or benzylamine was added to afford a final concentration of 1 mM in a volume of 2 ml in 0.05 M phosphate buffer, pH 7.6. Sufficient benzylamine then was added to final concentration, 3 mM, and the enzyme activity assayed spectrophotometrically as described in the text.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Inhibition</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ CMR</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>+ GSH; CMR</td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>+ Benzylamine; CMR</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>+ Pargylineb</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>+ GSH; pargyline</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>+ Benzylamine; pargyline</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>+ Phenylhydrazine</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>+ GSH; phenylhydrazine</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>+ Benzylamine; phenylhydrazine</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

* Pargyline, N-methyl-N-(2-propynyl)benzylamine.

Reported by Gorkin (17) on the basis of observations with a less purified enzyme preparation from liver mitochondria.

**Action of Pargyline on Absorbance of Monoamine Oxidase—**

The above results fortify the postulate that pargyline, like (+)-transphenylethylene, may indeed be acting at the catalytic site of monoamine oxidase. Accordingly, owing particularly to a certain similarity of this compound to the substrate, benzylamine (see "Discussion"), the ability of pargyline to reduce this flavoenzyme under anaerobic conditions was investigated. As shown in Fig. 6, pargyline (at a ratio of 1 mole per 10⁵ g of protein) increased the absorption maximum at 412 mp and decreased the flavin absorption at 400 mp. When air was admitted to the mixture, the increase in absorption at 412 mp remained unchanged; however, the flavin (400 mp) was reoxidized to the extent of 50%. The reduction by pargyline of the flavin at 460 mp accounted for approximately 50% of the total flavin reducible by excess benzylamine under similar conditions. However, when benzylamine was added similarly to the enzyme in a ratio of 1 mole eq per 10⁵ g of protein under conditions paralleling those used with pargyline, only 60% reduction of the flavin was obtained; thus, the system in these experiments had held sufficient oxygen to reoxidize 40 to 50% of the reduced flavin. Nevertheless, the results suggest that although pargyline acts to reduce and also to inactivate irreversibly monoamine oxidase, reduction of a flavin component (possibly altered) in the product is not irreversible.

Additional evidence, presented in Table V, indicates that pargyline in fact acts as an electron donor for monoamine oxidase and that this inhibitor is oxidized before or simultaneously with accomplishment of irreversible inhibition of the enzyme. When a 5 molar excess of pargyline was incubated with the enzyme under aerobic conditions, inhibition, when observed after 10 min, was found to be complete; similar results were obtained when the incubation mixture included also a 5 molar excess of benzylamine under these conditions. On the contrary, pargyline in this concentration produced little inhibition of monoamine oxidase when incubated with the enzyme that had been initially reduced with a 5 molar excess of benzylamine under anaerobic conditions. Moreover, pargyline was seen to cause but little inhibition of the enzyme when the initial reduction had been

**TABLE IV**

Inhibitory effect of pargyline and phenylhydrazine in relation to sulphydryl residues of monoamine oxidase

Of the enzyme-protein, 50 μg (specific activity 12,000) were incubated for 1 hour at 37° with sufficient pargyline or phenylhydrazine to provide 2 mole eq of inhibitor per 10⁵ g of protein in 0.5 ml of 0.05 M phosphate buffer, pH 7.6. After incubation with inhibitor, a 10-fold excess of [14C]-p-chloromercuribenzoate ([C-CMB]) (specific activity 0.38 mC per mmol) or [3H]-tetraethylthiuram disulfide ([H-TT]) (specific activity 1.27 mC per mmol) (11) was added and the mixture was allowed to stand at 25° for 1 hour; the number of reactive sulphydryl residues was then determined as described in the text.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Method of assay</th>
<th>No. of sulphydryl residues per 10⁵ g of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Pargylinea</td>
<td>[14C]-CMB</td>
<td>7.88 (7.80-8.05)</td>
</tr>
<tr>
<td>+ Phenylhydrazine</td>
<td>[14C]-CMB</td>
<td>6.10 (7.90-8.20)</td>
</tr>
<tr>
<td>+ Phenylhydrazine</td>
<td>[3H]-TT</td>
<td>7.95 (7.80-8.17)</td>
</tr>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Pargylinea</td>
<td>[3H]-TT</td>
<td>7.90 (7.70-8.10)</td>
</tr>
<tr>
<td>+ Phenylhydrazine</td>
<td>[3H]-TT</td>
<td>7.85 (7.70-8.10)</td>
</tr>
<tr>
<td>+ Phenylhydrazine</td>
<td>[3H]-TT</td>
<td>7.90 (7.80-8.22)</td>
</tr>
</tbody>
</table>

* Values represent the averages of three experiments with the range observed.

b Pargyline, N-methyl-N-(2-propynyl)benzylamine.
similarly accomplished alternatively by the use of 0.5 mg of sodium dithionite (Table V). As anticipated, the inhibitory capability of pargyline was unaffected in a control involving initially incubation of this inhibitor alone with a similar concentration of sodium dithionite before addition to the enzyme-substrate reaction mixture. On the other hand, the nonreducing inhibitor, (+)-tranzsphenylcyclopropylamine, exhibited its characteristic inhibitory capacity irrespective of any preliminary reduction of the enzyme by dithionite.

**TABLE V**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Benzylamine per 10⁶ g of protein</th>
<th>Sodium dithionite</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pargyline</td>
<td>0 moles eq</td>
<td>0 mg</td>
<td>100%</td>
</tr>
<tr>
<td>Pargyline</td>
<td>5 moles eq</td>
<td>0 mg</td>
<td>15%</td>
</tr>
<tr>
<td>Pargyline</td>
<td>0 moles eq</td>
<td>0.5 mg</td>
<td>10%</td>
</tr>
<tr>
<td>None</td>
<td>0 moles eq</td>
<td>0.5 mg</td>
<td>8%</td>
</tr>
<tr>
<td>d-trans-2-Phenylcyclopropylamine</td>
<td>0 moles eq</td>
<td>0 mg</td>
<td>100%</td>
</tr>
<tr>
<td>d-trans-2-Phenylcyclopropylamine</td>
<td>5 moles eq</td>
<td>0 mg</td>
<td>80%</td>
</tr>
<tr>
<td>d-trans-2-Phenylcyclopropylamine</td>
<td>0 moles eq</td>
<td>0.5 mg</td>
<td>100%</td>
</tr>
</tbody>
</table>

*Pargyline, N-methyl-N-(2-propynyl)benzylamine.

b. d-trans-2-Phenylcyclopropylamine is the d form of tranleypromine.

**Nature of Binding of Pargyline to Monoamine Oxidase**—The oxidase was incubated with 1 mole eq of 7-¹⁴C-pargyline per 10⁶ g of protein, and the solution then was passed through a Sephadex G-25 column (1 × 30 cm) that had been equilibrated with a 10⁴-fold excess of unlabeled pargyline. As shown in Fig. 6, the radioactivity was tightly associated with the protein; the protein and radioactivity were eluted in the void volume of the column. The labeled flavoprotein was dialyzed three times and then precipitated with trichloroacetic acid. The yellow precipitate was washed with 0.05 M potassium phosphate buffer, pH 7.6. The resulting precipitate contained radioactivity equivalent to 1 mole of 7-¹⁴C-pargyline per 10⁶ g of protein (Table VI). After washing of this precipitated protein three times with ether and three times with chloroform-methanol (2:1), approximately 98% of the radioactivity was bound to the denatured protein; the counts were equivalent to 0.9 mole of pargyline per 10⁶ g of protein. These results strongly suggest that the radioactive material had become tightly (covalently) bound to the

Fig. 6. Effect of pargyline on the absorption spectrum of monoamine oxidase. Upper, Curve I represents the native enzyme, 1.2 mg of protein (specific activity 12,000) per ml of 0.05 M phosphate buffer, pH 7.6. Curve II was obtained under anaerobic conditions (see note below) after addition of 1 mole of pargyline per 10⁶ g of protein. Curve III was obtained by passing a stream of oxygen over the anaerobic sample which had provided Curve II. The enzyme solution had been made anaerobic by flushing thoroughly with nitrogen 20 min with stirring of the sample at 2°. Nitrogen was passed through Fieser's solution, 1% lead acetate, and 0.1 M sodium phosphate buffer before use. The cuvette was tightly sealed with a rubber stopper and addition of pargyline was made with a hypodermic needle and syringe. Lower, the difference spectra between native enzyme (I) and enzyme reduced with pargyline (II) as well as the reoxidized enzyme (III) are shown. (Conditions as described above.)

Fig. 7. Binding of pargyline-¹⁴C-(7). Of the enzyme protein, 150 µg (specific activity 12,000) were incubated with sufficient ¹⁴C-pargyline (specific activity 1.05 mCi per mmole) to give 1 mole of inhibitor per 10⁶ g of protein in 0.2 ml of 0.05 M phosphate buffer, pH 7.6, at 25°. After 30 min incubation, the sample was placed on a Sephadex G-25 column (1 by 30 cm) which was equilibrated with a 10⁴-fold excess of unlabeled pargyline in 0.05 M phosphate buffer, pH 7.6. Approximately 0.6 ml of eluate was collected in each fraction and the absorbance at 280 m (---) and radioactivity (-----) of each fraction were determined. Protein which eluted in the void volume of the column contained 0.9 to 1.0 mole eq of labeled residue from pargyline-¹⁴C-(7) per 10⁶ g of protein.
were combined, and 1.0 mg of bovine serum albumin was added.

An enzyme preparation of specific activity 4000 (arbitrary units
incubated with a slight excess of 74C-pargyline only 0.32 mole
of radioactive material was bound per 105 g of protein. Thus,
monoamine oxidase presumably with accompanying reduction
of flavonucleotide at the active site is also shown in Table VI.

Thus, when a monoamine oxidase preparation containing one-


the respective specific activities of the preparation used above was


to hydrogen (H—). See Reference 18 and Table VII.

(8)) was completely inactivated by pargyline at a molar ratio
of 0.30 mole of pargyline per 104 g of protein. The “addition
reaction” may be specific for monoamine oxidase since it was
observed also that two other flavoenzymes, crystallized d-amino
acid oxidase and isoply dehydrogenase, are not inhibited by
pargyline. An interpretation is presented under “Discussion.”

Hammett Effect; m- or p-Substituted Benzylamines as Sub-
strates—To aid in the elucidation of the mechanism of catalysis
by monoamine oxidase and to better understand the mechanism
of inhibition of the enzyme e.g. by pargyline, we have examined
catalytic capability for oxidation of several substituted benzyl-
amines. As shown in Table VII, the maximal velocity was
found to be enhanced markedly through substitution on the
ring of suitable electron-attracting groups. Conversely, the
rate was decreased when substituent groups were electron-


donating. When the log maximal velocity was plotted as a
function of the σ values (σ < 0) for the substituents according
to the procedure of Hammett (19) a rather large σ value, approxi-
mately 2.5, was suggested (Fig. 8). Possibly biphasic character
is indicated at the σ value, 0; this will require more extensive
exploration (compare References 20 and 21 and the discussions
therein). These preliminary results5 support the hypothesis
that abstraction of a proton from the methylene carbon atom
may initiate the rate-limiting step in the catalysis of oxidation
of benzylamine and other substrates (see below). Such a
hypothesis would be based on an argument paralleling the treat-
ment presented by Neims, De Luca, and Hellerman (20) who
conducted an extensive investigation of substituent effects in
the oxidation of substrate α-aminophenylacetic acids in the
presence of crystallized d-amino acid oxidase.6 Furthermore,

5 It is anticipated that a more complete investigation of the
Hammett series for monoamine oxidase will be undertaken by
Professor De Luca and one of us.

6 Compare also an investigation of Hammett effects with re-
spect to L-amino acid oxidase by Radda (22).

---

**Table VI**

Evidence indicating stoichiometric covalent binding of 14C-residue
from pargyline-14C-7 to monoamine oxidase

Conditions were the same as described in Fig. 7. Enzyme
samples were adjusted to 0.5 mg of protein per ml and 0.3 ml was
incubated with 14C-pargyline and placed on a Sephadex G-25
column as in Fig. 7. The fractions containing labeled protein
were combined, and 1.0 mg of bovine serum albumin was added.

The protein was then precipitated by addition of sufficient tri-
chloracetic acid to give a final concentration of 7% followed by
gentle heating in a water bath at 100° for 3 min. The trichlor-
acetic acid precipitates were washed successively three times with
2.5 ml of 0.05 M phosphate buffer, pH 7.6, three times with 2.5
ml of ether, three times with 2.5 ml of chloroform-methanol
(2:1), and three times with 2.5 ml of distilled water.

![Fig. 1](http://www.jbc.org/) Logarithm of the maximal rate (V_m) for the substituted
benzylamines as substrates for mitochondrial monoamine oxidase
plotted against σ (see below). The respective maximal rates,
taken from Table VII, refer to micromoles of products formed per
min per mg of protein at 37° in air. Initial rates of production of
the respective aldehydes were used (See "Results"). The designa-
tion, σ, relates to an experimentally derived measure of the
electron-attracting (σ > 0) or electron-donating (σ < 0) property
of a particular substituent (e.g. p-Cl—, m-CH3—) relative to
hydrogen (H—). See Reference 18 and Table VII.

![Fig. 8](http://www.jbc.org/) Logarithm of the maximal rate (V_m) for the substituted
benzylamines as substrates for mitochondrial monoamine oxidase
plotted against σ (see below). The respective maximal rates,
taken from Table VII, refer to micromoles of products formed per
min per mg of protein at 37° in air. Initial rates of production of
the respective aldehydes were used (See "Results"). The designa-
tion, σ, relates to an experimentally derived measure of the
electron-attracting (σ > 0) or electron-donating (σ < 0) property
of a particular substituent (e.g. p-Cl—, m-CH3—) relative to
hydrogen (H—). See Reference 18 and Table VII.

---

**Table VII**

Maximal rates and Michaelis constants for substituted benzylamine
series as substrates for monoamine oxidase

Reaction rates were determined by spectrophotometric assay
of the product aldehydes at the wave lengths shown. Maximal
velocities and Michaelis constants were estimated graphically,
each value representing the average obtained from at least three
independent experiments at 37° in air. The molar absorbances
of the product aldehydes at the wave lengths shown. Maximal
rates and Michaelis constants were estimated graphically,
each value representing the average obtained from at least three
independent experiments at 37° in air. The molar absorbances
of the product aldehydes at the wave lengths shown. Maximal
rates and Michaelis constants were estimated graphically,
each value representing the average obtained from at least three
independent experiments at 37° in air. The molar absorbances
of the product aldehydes at the wave lengths shown. Maximal
rates and Michaelis constants were estimated graphically,
this proposal is in harmony with the conclusions of Belleau and Moran (15) who observed that substitution of deuterium atoms for hydrogen at the \( \alpha \)-carbon atom of tyramine resulted in a decrease in the rate of monoamine oxidase-catalyzed oxidation of this substrate. The isotope effect was marked.

It is clear from the foregoing discussion (and references) that whereas substrate-bound flavonucleotide (8) obviously is "at the active site" of monoamine oxidase, other aspects of the catalytic site require consideration. Certainly investigation of the mode of "activation" of the sterically conforming substrate amine (virtually, transition state of oxidation) is essential.

**DISCUSSION**

It is known that various arylalkylamines are substrates for mitochondrial monoamine oxidase, whereas short chain aliphatic amines are not efficiently oxidized in presence of this enzyme. Zeller (23) and Belleau and Moran (19) have suggested that the aromatic ring system is involved in the formation of the enzyme-substrate complex. In itself this apparently would have little direct bearing on observations concerned with the inhibitory behavior of various hydrazines with respect to this enzyme. We have found (cf. "Results") that the rates of inhibition of the aromatic hydrazines, phenyl- and \( \alpha \)-naphthyl-hydrazines, are 1.6- to 2.15-fold faster than the rate of inhibition in the presence of methylhydrazine (Fig. 1), but this may be a function of the superior reducing capability of the aromatic agents, as suggested by their more general behavior. Since the simple aryl and alkyl hydrazines inhibit irreversibly, it might be assumed that although they may "fit" the enzyme catalytic center sufficiently well, the irreversible action is related to electronic aspects in the inhibitors themselves. If, however, reducing action for such hydrazines is to be considered at least in part a component of their irreversible action upon monoamine oxidase, it should be recalled that reductions by hydrazines can be complex (particularly in the presence of oxygen). Certainly their action upon electron acceptor groups (e.g. flavin) in susceptible enzymes requires additional study. It may be noted that complete inhibition of the enzyme was attained here with 1 mole eq of methylhydrazine per lo5 g of protein (Fig. 4). Inasmuch as it was shown in Paper I (8) that our enzyme preparation bears 1 mole eq of catalytically active flavin component per lo5 g of enzyme, it appears that each of these inhibitors has reacted stoichiometrically with the active catalytic site of monoamine oxidase. Additional evidence is presented in Table II (cf. "Results") which may be taken to indicate that each of these agents acts independently upon the catalytic site, the inhibitory effects, as observed, being additive.

**Pargyline**—The data of Fig. 6 and Table V indicate that pargyline can act, initially at least, as an electron donor for monoamine oxidase, with capability of reducing the flavin moiety. Increase in absorbance of the treated enzyme at 412 m\( \mu \) may point to reaction of pargyline with some flavin position or with another protein group substituted close to the flavin component. Certain interactions, probably of the charge-transfer type, between flavins, and e.g. barbiturate compounds, are known to produce such spectral changes (26). As shown in Fig. 7 and Table VI, pargyline forms an extremely tight, probably covalent, bond with the flavoenzyme, this bonding being possibly specific, among flavoenzymes, for the catalytic site of monoamine oxidase (cf. "Results").

A hypothesis for the mechanism of inactivation, at least in its initial steps, by pargyline, a tertiary amine (Scheme 1), may be considered. We have observed that the purified monoamine oxidase flavoenzyme (8) can act as catalyst for the oxidation of

![Scheme 1](http://www.jbc.org/)

\[
\begin{align*}
\text{C}_6\text{H}_4\text{N}^+ & \quad \text{CH}_2^+ \\
\text{CH}_3 & \quad \text{C}-\text{O}=\text{CH} \\
\text{A} & \quad \text{B}
\end{align*}
\]
some tertiary amines, in addition to substrate primary and secondary amines; furthermore, its protein thiol groups apparently are not involved in the action of several structurally differing inhibitors, including pargyline (cf. "Results"). The pattern of enzyme interaction with substrate amines probably includes over-all abstraction of a proton (see "Results") and transfer of 2 electrons from the methylene carbon atom vicinal to amino nitrogen to the flavin moiety with subsequent hydrolysis of a presumed intermediate amidino group to give the related aldehyde and ammonium ion (e.g. in the case of substrate primary amines). Such a pattern is reflected in analytical data in the older literature and especially in data defining stoichiometry of monoamine oxidase-catalyzed processes presented in Paper I (8). Although the general pattern of electron transfer may be applicable for pargyline, it seems possible from data referred to below that the initial step in the pargyline-enzyme interaction involves electron transfer to flavin chiefly from the methylene carbon atom designated B (Scheme 1). Activation at carbon B would be determined by the characteristic properties of the propynyl group carrying B and possibly also by proton abstraction preferentially from B. Attachment of carbon B (after electron displacement) to a nucleophilic enzyme site, probably coincident with or subsequent to reduction of the flavin would be consistent with the data reported under "Results," including the observation that the radioactivity of carbon 7\(^4\)C, A, is recovered quantitatively in the adduct (Fig. 7 and Table VI). Benzaldehyde apparently is not a product here. Furthermore, the increase in absorbance of the pargyline-treated enzyme at 412 mp adds weight to the conclusion that the formation of the adduct may involve rather specifically the active catalytic site of monoamine oxidase. It is appreciated that the nucleophilic acceptor for formation of the pargyline-enzyme adduct could be a protein group such as e-amino of lysine (see below) or tyrosine phenolic —O\(^-\); but presumably not —SH which would be unavailable here (Table IV).

Although a structural relationship in pargyline to a substrate, benzylamine, is apparent, electron transfer predominantly from the N-propynyl group rather than from carbon 7, A, would imply here the relatively superior role of the former entity as the locus of activation of the inhibitor molecule. Indeed, Swett et al. (27) synthesized various analogues of pargyline and observed that the acetylenic component and also the substituent hydrogen atoms of the adjacent methylene group of this active moiety, i.e. the intact propynyl group, are essential for inhibitory action. Conversely, only relatively small decreases in inactivating potency were observed when the hydrogen atoms of A were replaced by methyl groups.

Catalytic Mechanism—Cogent evidence permitting classification of monoamine oxidase as a flavoenzyme (8) allows formulation of a more valid and useful concept of the catalytic mechanism than was hitherto available. Our quantitative data bearing on the enzymatic oxidation of a variety of substrates (8), in addition to older data, point to the following conventional formulation depicting the oxidation of a number of amines of physiological interest, and in addition of the benzylamines, and others:

\[
\begin{align*}
R-C=CNH_2 + 1/2 O_2 & \quad \text{(flavoenzyme)} \rightarrow R-C=NH + H_2O \\
R-C=NH + H_2O & \rightarrow R-C=O + NH_3
\end{align*}
\]

This formalism is reminiscent of the pattern of oxidation of p- and L-\(\alpha\)-amino acids in the presence of the respective specific oxidases. Suggestions and evidence concerning the mechanisms involved in the action particularly of the \(\alpha\)-amino acid oxidase, through recent close mechanistic studies from this laboratory (20, 25, 26), have extended the data in the older literature. Descriptive conventional equations had proposed that a susceptible \(\alpha\)-amino acid, for example, is oxidized by the flavoenzyme, \(\alpha\)-amino acid oxidase, to yield imino acid and reduced (leuco) flavoenzyme, which is reoxidized by \(O_2\) with production of \(H_2O\) (in the presence of catalase), the imino acid being hydrolyzed subsequently to produce the end products, keto acid and \(NH_3\).

More recent investigations of amino acid oxidases offer suggestions applicable also to amine oxidation catalyzed by mitochondrial monoamine oxidase. In Scheme 2, which is based upon arguments presented in the amino acid oxidase work (20, 25), it is hypothesized that the enzyme-substrate combination has been attained through addition of the substrate amino group to a position on the isocitroxazine nucleus with formation reversibly of a carbinalamine (hydrated Schiff's base) or equivalent. Activation of the substrate through proton abstraction at the hand of a protein nucleophilic site, strategically located, favors release\(^11\) of 2 electrons to isocitroxazine, a reduced form of which in this instance (as with amino acid oxidases) is reoxidized by oxygen. There results a flavoenzyme-product "complex," in which "product" has the configuration of an aldime. The latter, displaced by substrate amine, is withdrawn (presumably in a stabilized form) from the complex to a more polar environment, being hydrolyzed to give the corresponding aldehyde and ammonium ion. The withdrawal and hydrolysis of the product aldines must involve a rather subtle control mechanism. Little is known of the properties of the simple aldines, their stability, sensitivity to polymerization, etc. It is postulated here that the aldinic configuration is preserved and protected within the field of the catalytic site. By analogy with the postulated amino acid oxidase mechanism (25), withdrawal and controlled hydrolysis might be dependent upon the functioning of a protein

\(^1\) The scheme is utilized for illustrative purposes. Details of electron transfer and other mechanistic aspects are not evaluated

\(^2\) The available evidence, pertinent references, and discussion are presented in the last section of "Results" (see also Reference 26).

\(^3\) Reference 25 presents certain aspects of electron exchange. A complete elucidation is not yet available on an experimental basis.
be anticipated that such a fragment, by a process of controlled electron repulsion (Scheme 2), and avert simultaneously liberation of the labile free imine by elaboration instead of an azo-imino-nitrogen, might facilitate initially electron exchange by hydrolysis, should yield ultimately the products, R–CH(0)lysyl residue" which, by close approach, through its nucleophilic substrate, D- or L-alanine (carbonyl-I%), it was found that suitable methine-ammono addition product (cf. Reference 25). It would actual appearance of a reactive aldimine (cf. Reference 25).

In a recent communication Massey et al. (29), outlined certain observations leading them to conclude that our "derivatized" L-amino acid oxidase and also derivatized D-amino acid oxidase (after removal of altered FAD and replacement by authentic FAD) are themselves enzymatically active with identical activity of the respective native enzymes concerned. However, these authors have neglected to include an essential control distinguishing between the presence of regenerated native enzymes and the labeled derivatized enzymes in the final reaction mixtures.
Mitochondrial Monoamine Oxidase: II. ACTION OF VARIOUS INHIBITORS
FOR THE BOVINE KIDNEY ENZYME. CATALYTIC MECHANISM
Leslie Hellerman and V. Gene Erwin


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