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 (K₁, 2 × 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 10⁵ 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, Kᵢ was determined as 5.8 × 10⁻⁷ 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 the 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 the enzyme 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 10⁵ 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 variation 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 (8).

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 concepts 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 (714C-pargyline), d-transphenylethylcyclopropylamine hydrochloride, β-phenylethylhydrazine (phenelzine) sulfate, and 1-hydrizinophthalalazine hydrochloride (hydralazine) were supplied to us as the pure, powdered, or crystalline substances. Phenylhydrazine hydrochloride and α-naphthylhydrazine hydrochloride were purified by recrystallization, decomposition points being 225-240° and 250-255°, respectively. m- and p-Substituted benzylamines and their hydrazides were kindly prepared by Dr. D. C. De Luca; the hydrazides were thiamethylated from isopropyl alcohol.

**Procedure**—The kinetic measurements (based on initial rates) and determination of the molar absorbances of substituted benzylhydrazides were accomplished spectrophotometrically with a Spectronic 200 Bausch and Lomb spectrophotometer equipped with a Sargent SHL recorder and AT-20 digital readout attachment. Monoamine oxidase activity was determined by the 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 (19). 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 10^5 g of protein) for approximately 2 hours, complete inhibition was observed with each of the compounds studied except 1-hydrizinophthalalazine. 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 kynuramine 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, N-methyl-N-(2-propynyl)benzylamine hydrochloride, and the 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; β-phenylethylhydrazine (phenelzine) sulfate, Mr. L. N. Starker, Warner-Chilcott Research Institute, research affiliate of Warner-Chilcott Laboratories; 1-hydrizinophthalalazine (hydralazine) 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.
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 10^10 g of protein in a volume of 0.2 ml (final inhibitor concentration, 1.5 × 10^-5 M) and a final concentration of 0.1 M potassium phosphate buffer, pH 7.6. After incubation at 37°C 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°C resulted in a loss of approximately 20% of the original enzyme activity.

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 of flavonucleotide (10^10 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 (+)-trans-Phenylcyclopropylamine—Although it has been shown that pargyline and (+)-trans-phenylcyclopropylamine (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 10^10 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°C. 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 (+)-trans-phenylcyclopropylamine, a reversible inhibitor, apparently to complete inactivation at a ratio of 1 eq of inhibitor per 10^10 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 (+)-trans-phenylcyclopropylamine with K_I, 5.8 × 10^-4 M (Fig. 5). Belleau and Moran (15) and Guha

![Graph showing inhibition of monoamine oxidase activity by hydrazines.](http://www.jbc.org/)

**Fig. 2.** Kinetics of inhibition of oxidation of benzylamine by 1-hydrazinophthalazine (hydralazine). The reaction mixture contained 3.0 μg of enzyme protein (specific activity 12,000). Assay conditions are described in the text. Abscissa, reciprocal of the molarity of benzylamine; ordinate, reciprocal of the change in absorbance at 250 nm per min; temperature, 37°C; K_I, 1.7 × 10^-4 M and 2.0 × 10^-3 M at the concentrations shown.

![Graph showing kinetics of inhibition of kynuramine oxidation by hydralazines.](http://www.jbc.org/)

**Fig. 3.** Kinetics of inhibition of kynuramine oxidation by hydralazines. The reaction mixture contained 6 μg of enzyme protein (specific activity 12,000). Assay conditions are described in the text. Ordinate, reciprocal of the change in absorbance at 360 nm per min at 37°C; abscissa, reciprocal of the molarity of kynuramine; K_I for hydralazine, 2.05 × 10^-4 M.
trunsphenylcyclopropylamine 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 p-chloromercuribenzoate (8). On the other hand the thiol, glutathione, did protect monoamine oxidase effectively from inhibition by p-chloromercuribenzoate but did not 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.

Persistence of Sulfhydryl Groups of Monoamine Oxidase in Presence of Pargyline and Phenylhydrazine—As shown in Table IV, the native enzyme preparation used contained approximately 8 (7.9) sulfhydryl residues per 10^5 g of protein as now determined by the radioassay method of Neims et al. (11) or by a radioassay procedure with "C-p-chloromercuribenzoate (see "Experimental Procedure"). These results are similar to those reported previously by Erwin and Hellerman (9) for the native enzyme and for the enzyme denatured with sodium dodecyl sulfate or guanidine hydrochloride. After addition of sufficient pargyline or phenylhydrazine to completely inactivate the enzyme, the number of sulfhydryl residues per 10^5 g of reacted protein, determined with these methods, was found to be 7.9, indicating that these inhibitors had not attacked irreversibly sulfhydryl residues of the protein. A similar conclusion was

### Table I

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition Before dialysis</th>
<th>Inhibition After dialysis</th>
<th>Reversal of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylhydrazine</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Pargyline</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>50</td>
<td>5</td>
<td>90</td>
</tr>
<tr>
<td>d-trans-2-Phenylcyclopropylamine</td>
<td>100</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

* Pargyline, N-methyl-N-(2-propynyl)benzylamine.
* Hydralazine, 1-hydrazinophthalazine.
* d-trans-2-Phenylcyclopropylamine is the d form of tranylcypromine.

#### Fig. 1

Persistence of Sulfhydryl Groups of Monoamine Oxidase in Presence of Pargyline and Phenylhydrazine—As shown in Table IV, the native enzyme preparation used contained approximately 8 (7.9) sulfhydryl residues per 10^5 g of protein as now determined by the radioassay method of Neims et al. (11) or by a radioassay procedure with "C-p-chloromercuribenzoate (see "Experimental Procedure"). These results are similar to those reported previously by Erwin and Hellerman (9) for the native enzyme and for the enzyme denatured with sodium dodecyl sulfate or guanidine hydrochloride. After addition of sufficient pargyline or phenylhydrazine to completely inactivate the enzyme, the number of sulfhydryl residues per 10^5 g of reacted protein, determined with these methods, was found to be 7.9, indicating that these inhibitors had not attacked irreversibly sulfhydryl residues of the protein. A similar conclusion was
Mitochondrial Monoamine Oxidase. II

Additive effect of pargyline, d-trans-phenylcyclopropylamine, and phenylhydrazine in relation to oxidoase 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 10⁶ g of protein</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Pargylinea</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.

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

‡ d-trans-2-Phenylcyclopropylamine is the d form of transleypromine.

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 10⁶ g of protein or 16 mole eq of p-chloromercuribenzoate (CMR) per 10⁶ 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>+ CMB</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>+ GSH; CMB</td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>+ Benzylamine; CMB</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>+ Pargylinea</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>+ GSH; pargyline</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>+ Benzylamine; pargyline</td>
<td>10</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.

The above results fortify the postulate that pargyline, like (+)-transphenylethanolamine, 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 flavonoenzyme 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 μm and decreased the flavin absorption at 400 μm. When air was admitted to the mixture, the increase in absorption at 412 μm remained unchanged; however, the flavin (460 μm) was reoxidized to the extent of 50%. The reduction by pargyline of the flavin at 460 μm 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...
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, (+)-trans-2-phenylcyclopropylamine, exhibited its characteristic inhibitory capacity irrespective of any preliminary reduction of the enzyme by dithionite.

**Nature of Binding of Pargyline to Monoamine Oxidase**—The oxidase was incubated with 1 mole eq of 7-14C-pargyline per 10^6 g of protein, and the solution then was passed through a Sephadex G-25 column (1 x 30 cm) that had been equilibrated with a 10-fold excess of unlabeled pargyline. As shown in Fig. 7, 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 thrice 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 10^6 g of protein (Table VI). After washing of this precipitated protein three times with ether and three times with chloroform : methanol (2:1), approximately 96% of the radioactivity remained bound to the denatured protein; the counts were equivalent to 0.9 mole of pargyline per 10^6 g of protein. These results strongly suggest that the radiactive material had become tightly (covalently) bound to the protein.

The enzyme mixture containing 3 μg of enzyme-protein in 1.0 ml of 0.05 M phosphate buffer, pH 7.6, was made anaerobic as described in Fig. 6, and then reduced by addition of 5 mole eq of benzylamine hydrochloride per 10^6 g of protein or by addition of 0.5 mg of sodium dithionite. To these samples sufficient pargyline or d-trans-2-phenylcyclopropylamine were added to provide 5 mole eq of inhibitor per 10^6 g of protein. After 10 min, sufficient oxygenated benzylamine hydrochloride was added to give 3 mM final concentration in 2.0 ml with 0.05 M phosphate buffer, pH 7.6, and the enzyme activity was determined spectrophotometrically at 37° as described in the text.

**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 10^6 g of protein. Curve III was obtained by passing a stream of oxygenated benzylamine hydrochloride per 10^6 g of protein through a Sephadex G-25 column (1 by 30 cm). The enzyme solution had been made anaerobic by flushing with nitrogen 20 min with stirring of the sample at 2°. Nitrogen was passed through a 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 μCi per mmole) to give 1 mole of inhibitor per 10^6 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 10-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 μm (— — ) 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 10^6 g of protein.

**TABLE V**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Benzylamine per 10^6 g of protein</th>
<th>Sodium dithionite</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mole eq</td>
<td>mg</td>
<td>%</td>
</tr>
<tr>
<td>Pargyline*</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Pargyline</td>
<td>0.5</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>d-trans-2-phenylcyclopropylamine*</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>d-trans-2-phenylcyclopropylamine</td>
<td>5</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>d-trans-2-phenylcyclopropylamine</td>
<td>0</td>
<td>0.5</td>
<td>100</td>
</tr>
</tbody>
</table>

* Pargyline, N-methyl-N-(2-propynyl)benzylamine.
* d-trans-2-phenylcyclopropylamine is the d form of tranylcypromine.
Evidence indicating stoichiometric covalent binding of $^{14}$C-residue from pargyline-$^{14}$C-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 $^{14}$C-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 trichloroacetic acid to give a final concentration of 7% followed by gentle heating in a water bath at 100° for 3 min. The trichloroacetic 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.

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 observed also that two other flavoenzymes, crystallized n-amino acid oxidase and lipoyl dehydrogenase, are not inhibited by pargyline. An interpretation is presented under “Discussion.”

Hammett Effect: m- or p-Substituted Benzylamines as Substrates—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 benzylamines. 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 $\sigma$ values ($\sigma < 0$) for the substituents according to the procedure of Hammett (19) a rather large $\rho$ value, approximately 2.5, was suggested (Fig. 8). Possibly biphasic character is indicated at the $\sigma$ value, 0; this will require more extensive exploration (compare References 20 and 21 and the discussions therein). These preliminary results 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 treatment presented by Neims, De Luca, and Hellerman (20) who conducted an extensive investigation of substituent effects in the oxidation of substrate $\alpha$-aminophenylacetic acids in the presence of crystallized $\alpha$-amino acid oxidase. Furthermore, 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.

Compare also an investigation of Hammett effects with respect to L-amino acid oxidase by Radda (22).
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 enzyme-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.7

**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 differences in 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 $10^4$ g of protein after 2-hours action of the inhibitor. Phenylisothiourhodanilide (phenelzine) appeared to attack the enzyme even more slowly than methylhydrazine.

In marked contrast to the over-all behavior of the hydrazines discussed above is the effect displayed by 1-hydrazonephthalazine (hydralazine). With this agent we observed a strictly competitively inhibitory behavior for our more highly purified monoamine oxidase when benzylamine was the substrate. There is formal analogy between the structure of $\alpha$-naphthylhydrazine and 1-hydrazonephthalazine, but this provides no clue concerning the sparing effect (cf. "Results") of the phthalazine hydrazine, the substrate-competitive action of which ($K_i = 2 \times 10^{-3} \text{ M}$) is reminiscent of the behavior of naphthols and hydroxyquinolines with monoamine oxidase (8, 24). Furthermore, possible existence of weak hydrogen bonding involving the hydrazino group and position N-2 of the phthalazine nucleus may not contribute significantly to the lesser degree of aggressive action of this hydrazine as an inhibitor. It would seem, therefore, that the origin of the "controlled" effect of this particular hydrazine on kidney monoamine oxidase in vitro is to be sought in a rather marked electron-withdrawing capability characteristic of the phthalazine nucleus, "deactivating" in this instance the hydrazino group so far as its electron-donating capacity or conceivably its ability to coordinate with an electrophilic site on the enzyme (25) is concerned. This assumption obviously invites further investigation of purely chemical aspects. The rather unique behavior of hydralazine among the hydrazines that have been studied as monoamine oxidase inhibitors might have consequences related to certain actions in vivo; this aspect need not be developed here.

We have shown that excess substrate (benzylamine) protects, in a general sense, against inactivation of purified monoamine oxidase by several different agents (Table III). Similar observations have been reported (17) for less purified mitochondrial enzyme preparations, and it has been suggested that these inhibitors act at the active site of the enzyme. In the current study, we have found that our enzyme is inactivated linearly in discontinuous titrations (and suitable periods of incubation) with any one of several structurally differing inhibitors. The latter agents include the tightly bound but substrate-competitive amine, (+)-transphenylcyclopropylamine, certain hydrazines, and pargyline (Table III); complete inhibition of monoamine oxidase is observed after 1 mole eq of any one of these compounds has been used per $10^4$ 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 $10^4$ g of protein, it appears that each of these inhibitors has reacted stoichiometrically with the active catalytic site of monoamine oxidase.4 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

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Zeller (23) has interpreted his findings concerning differences in the rates of oxidation of certain $m$- and $p$-substituted benzyamines with major emphasis upon steric effects. Apparent discrepancies in the results of investigations from the two laboratories undoubtedly will be resolved in the future; they may be attributed in part to the use of enzyme preparations of widely varying purity and to differences in kinetic evaluation. Zeller's (23) emphasis on the importance of certain gross steric effects generally appears to be well taken.
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 7,4C, 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 mJ 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 ε-amine 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. synthesized various analogues of pargyline and observed the acetylenic component and also the substituent hydrogen atoms of the adjacent methylene group of this active moiety, i.e. the intact propynyl grouping, are essential for inhibitory action. Conversely, only relatively small decreases in inactivator 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:

The scheme is utilized for illustrative purposes. Details of electron transfer and other mechanistic aspects are not evaluated.

\[
\begin{align*}
R-C-NH_2 + 1/2 O_2 & \quad (\text{flavoenzyme}) \rightarrow R-C=NH + H_2O \\
R-C=NH + H_2O & \rightarrow R-C=O + NH_3
\end{align*}
\]

This formalism is reminiscent of the pattern of oxidation of n- and l-α-amino acids in the presence of the respective specific oxidases. Suggestions and evidence concerning the mechanisms involved in the action particularly of the n-amino acid oxidase, through recent close mechanistic studies from this laboratory (20, 25, 28), have extended the data in the older literature. Descriptive conventional equations had proposed that a susceptible n-amino acid, for example, is oxidized by the flavoenzyme, n-amino acid oxidase, to yield imino acid and reduced (leuco) flavoenzyme, which is reoxidized by O₂ with production of H₂O (in the presence of catalase), the imino acid being hydrolyzed subsequently to produce the end products, keto acid and NH₃.

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 amidino group to a position on the isoalloxazine nucleus with formation reversibly of a carbinolamine (hydrated Schiff's base) or equivalent. Activation of the substrate through proton abstraction at the hand of a protein nucleophile site, strategically located, favors release of 2 electrons to isoalloxazine, 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 aldimine. 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 aldimines must involve a rather subtle control mechanism. Little is known of the properties of the simple aldimines, their stability, sensitivity to polymerization, etc. It is postulated here that the aldimine 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
lysin residue which, by close approach, through its nucleophili-
cation, to the substrate carbon atom ultimately bearing
mimo-nitrogen, might facilitate initially electron exchange by
electron repulsion (Scheme 2), and avert simultaneously libera-
tion 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, \( R-CH(\equiv) \)
lysyl residue which, by close approach, through its nucleophilic
substrate, D- or L-alanine (carbonyl-\( \equiv \)), it was found that suitable
substrate reaction mixture might introduce a relatively stable
actual appearance of a reactive aldimine (cf. Reference 25).

From the foregoing discussion it might be anticipated that
introduction of sodium borohydride to an active enzyme-sub-
strate reaction mixture might introduce a relatively stable\( \equiv \)
\( e-N \)-benzyl adduct to a critically functioning lysyl residue. However, interpretations based upon such a result might be
inconclusive here. Our preliminary observations with respect to
this matter require amplification. Further investigation
with inhibitors of the pargyline type and with appropriate sub-
strates from the benzylamine series could be enlightening (see
"Results").

\( ^{11} \) In the studies with amino acid oxidases and the appropriate
substrate, d- or L-alanine (carbonyl-\( \equiv \)), it was found that suitable
action of sodium borohydride upon actively functioning enzyme-
substrate reaction mixtures gave labeled protein products, which
after acid hydrolysis yielded a somewhat novel amino acid, \( e-N-\)
(1-carboxyethyl)-L-lysine. In the work with the "n-enzyme,"
aminoc acid analysis indicated that as derivatized lysine appeared
presumably through reduction of the lysyl addition product, a
portion of protein lysine decreased.

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 distinguish-
ing between the presence of regenerated native enzymes and the
labeled derivatized enzymes in the final reaction mixtures for
kinetic study. It is quite conceivable that the effects observed by
Massey et al. may be attributable to restoration of the parent
oxidases through oxidation of the \( e-N \)-adducts. The \( e \)-derivatized
lysyl group, itself constitutes a secondary amino acid entity, that
might be subject to oxidation (and hydrolysis) with reversion to
the parent enzyme. In any case, our over-all evidence points
strongly to the presence of a protein lysyl group (or groups) in
proximity to the catalytic center of d- or of L-amino acid oxidase.
In the event that authentic evidence should establish that our
derivatized enzymes in fact function as efficient catalysts with the
specificities and efficiency of the parent enzymes, this still would
not foreclose an essential role of protein-lysyl groups in the cata-
litic mechanism itself (see "Discussion").

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