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Mechanism of Inactivation of Mitochondrial Monoamine Oxidase by *N*-Cyclopropyl-*N*-arylalkyl Amines

Sir:

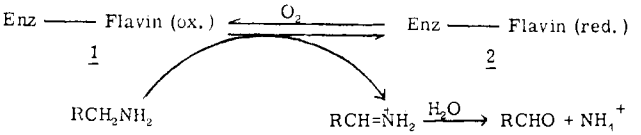
Mitochondrial monoamine oxidase (MAO, E C 1.4.3.4), a flavin-dependent enzyme which catalyzes the oxidative deamination of physiologically active monoamines, has been the target of inhibition for hundreds of potential antihypertensive and antidepressant agents.[1] The reaction catalyzed by mitochondrial MAO is shown in Scheme I.

One class of irreversible MAO inhibitors, the propargyl amines, has been studied extensively[2] and a possible mechanism of action has been proposed.[3] The mechanism of another class of potent and specific MAO inhibitors, the *N*-cyclopropyl-*N*-arylalkyl amines,[4] however, has not been determined. We report here our initial findings on the mechanism of action of this important class of compounds.

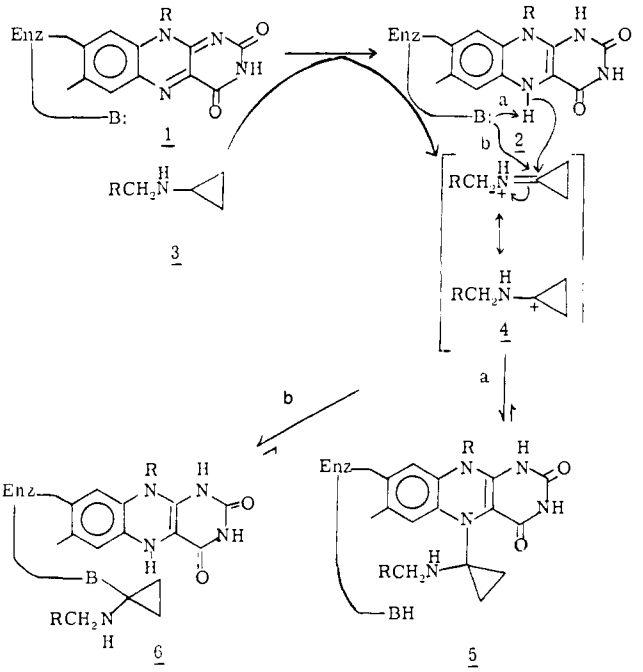
The *N*-cyclopropyl-*N*-arylalkyl amines that we have studied are mechanism-based inactivators of purified mitochondrial MAO from pig liver. A mechanism-based or suicide[5] inactivator is an unreactive compound which is catalytically transformed by the target enzyme into a highly reactive species. While sequestered in the active site, this then reacts with an enzymatic functional group and results in covalent bond formation with the enzyme. Scheme II depicts the proposed mechanism for the inactivation of MAO by *N*-cyclopropyl-*N*-arylalkyl amines. It is postulated that the cyclopropyl carbon attached to the nitrogen is oxidized, yielding the highly reactive cyclopropanone[6] imine (**4**) which reacts either with N-5 of reduced flavin (**2**) (pathway a) to give the mixed diamino ketal of cyclopropanone (**5**)[7] or with an active site nucleophile (pathway b) to give **6**. There is ample precedent for the generation of cyclopropanone imines in the inactivation of amine oxidases by cyclopropylamines.[8] Cyclopropanone (hydrate) generated from coprine recently has been implicated as the reactive species in the inactivation of aldehyde dehydrogenase.[10]

Mechanism-based inactivation of MAO by *N*-cyclopropyltryptamine (**3a**, R = indolylmethyl) and *N*-cyclopropylbenzylamine (**3b**, R = phenyl) was concluded from the following experiments: (1) a first-order time-dependent loss of enzyme activity was observed ($t_{1/2}$ = 1.5 min for 50 μM *N*-cyclopropyltryptamine and $t_{1/2}$ = 2.8 min for 50 μM *N*-cyclopropylbenzylamine at 25 °C, pH 9.0); (2) the rate of inactivation was considerably slower at pH 7.0 than at pH 9.0, the pH optimum of the enzyme; (3) the rate of inactivation was considerably slower in the presence of the substrate, benzylamine; (4) extensive dialysis of inactivated enzyme against pH 7.0 buffer led to recovery of <5% of the enzyme activity; (5) incubation of MAO with [*phenyl*-^{14}C]-*N*-cyclopropylbenzylamine,[11] followed by dialysis, led to association of the radioactivity with the protein to the extent of 1.2–1.4 mol[12] of inactivator/mol of enzyme; and (6) incubation of MAO with *N*-[1-^{3}H]cyclopropylbenzylamine[13] resulted in a time-dependent release of 0.73 mol of ^{3}H/mol of [*phenyl*-^{14}C]-*N*-cyclopropylbenzylamine bound to the active site. Kinetics of inactivation of MAO by *N*-cyclopropylbenzylamine (**3b**) are depicted in Figure 1.

Scheme I. Oxidation of Monoamines by Monoamine Oxidase



Scheme II. Proposed Mechanism for the Inactivation of MAO by *N*-Cyclopropyl-*N*-arylalkyl Amines



The mechanism of the inactivation was deduced from the following results. Using *N*-[1-^{2}H]cyclopropylbenzylamine,[13] a deuterium isotope effect of 1.5 was observed at a concentration of 17 μM (pH 9.0, 0 °C), but, as the concentration of the inactivator was increased, the isotope effect approached 1.0. This is reminiscent of the findings of Belleau and Moran[14] for the isotope effect of deuterated substrates with MAO. In conjunction with the tritiated inactivator experiment, this suggests that the proton is lost in a partially rate-determining step at low concentrations of inactivator. The cyclopropyl group is essential for irreversible inactivation since *N*-isopropylbenzylamine is a competitive (K_i = 320 μM) *reversible* inhibitor of the enzyme. The cyclopropyl group must be attached directly to the nitrogen as evidenced by the observation that *N*-cyclopropylmethyltryptamine is also a competitive (K_i = 100 μM) *reversible* inhibitor. Oxidation of the benzylic methylene of **3b**, as in benzylamine (Scheme I, R = Ph), would lead to *N*-benzylidenecyclopropylamine. To determine whether this compound was responsible for inactivation, MAO was anaerobically reduced (by benzylamine) and then was treated with *N*-benzylidenecyclopropylamine at pH 9.0.[16] No irreversible inhibition of the enzyme occurred. Aerobically, this compound was observed to be a potent noncompetitive (K_i = 56 μM) reversible inhibitor of MAO. During the inactivation of MAO, the optical spectrum of the flavin coenzyme changes

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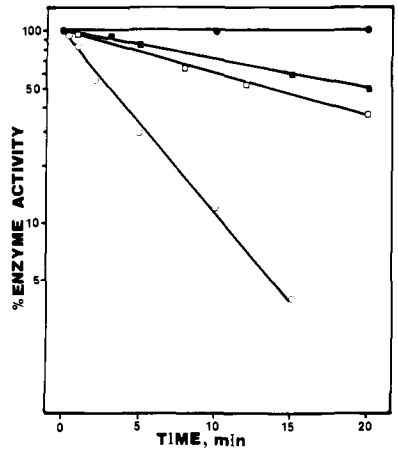

Figure 1. Irreversible inactivation of monoamine oxidase by *N*-cyclopropylbenzylamine. MAO (7.0 μg, specific activity 4657 Tabor units) was incubated at 25 °C in 100 μL of a solution containing 50 μM *N*-cyclopropylbenzylamine and 20 mM Tris-HCl buffer, pH 9.0 (○); 20 mM KP_i buffer, pH 7.0 (□); or 20 mM Tris-HCl, pH 9.0, containing 450 μM benzylamine (■). Periodically, 10-μL aliquots were removed and diluted to 500 μL with 1 mM benzylamine in 20 mM buffer and assayed spectrophotometrically:[15] ● is a control which contained no *N*-cyclopropylbenzylamine.

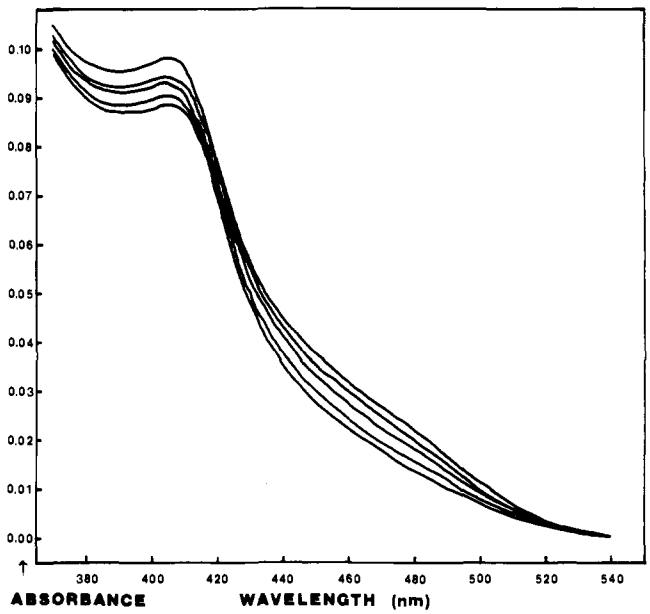

Figure 2. Flavin spectrum change during inactivation of monoamine oxidase by *N*-cyclopropylbenzylamine. MAO (0.25 mg, specific activity 5788 Tabor units) was incubated with *N*-cyclopropylbenzylamine (100 μM) in 20 mM KP_i, pH 7.0 buffer. The five spectra tracings in order of decreasing ε value correspond to enzyme activity of 100 (control), 72, 47, 23, and 4%, respectively.

from that of the oxidized to a modified reduced form (Figure 2)[17] as would be expected from an adduct such as **5**. However, a similar spectral change would be expected if the interaction of the inactivator were with the protein (e.g., **6**) and the entrance of O_2 to the active site were blocked, thus preventing reoxidation of the flavin.

If there exists a small equilibrium between **5** and **6** with **4** plus reduced flavin **2**, it should be possible to trap **4**, thereby liberating the reduced flavin, leading to regeneration of the enzyme activity. Treatment of either *N*-cyclopropyltryptamine- or *N*-cyclopropylbenzylamine-inactivated MAO with benzylamine produced a time-dependent return of enzyme activity ($t_{1/2} \sim 50$ min for *N*-cyclopropyltryptamine and ~ 90 min for *N*-cyclopropylbenzylamine).[19] When this experiment was carried out with MAO which was inactivated with [*phenyl*-^{14}C]-*N*-cyclopropylbenzylamine, all of the radioactivity associated with the protein was released upon dialysis. From high voltage paper electrophoresis and chromatography experiments, we have evidence that a substance released from benzylamine-treated inactivated MAO is different from *N*-cyclopropylbenzylamine, thus suggesting that the inactivator was transformed during inactivation. The stability of the adduct formed between the *N*-cyclopropyl-*N*-arylalkyl amines studied and pig liver mitochondrial MAO suggests that a reversible covalent bond is produced. Walsh and co-workers[21] recently hypothesized that reversible covalent bonds are formed during the inactivation of lysine ε-aminotransaminase by certain unsaturated lysine analogues; the covalent adducts were kinetically labile upon gel filtration and dialysis.

These results suggest that the *N*-cyclopropyl-*N*-arylalkyl amines differ considerably from the propargyl amines in which the resulting covalent bond with the enzyme is stable. The mechanism of inactivation of MAO by *N*-cyclopropyl-*N*-arylalkyl amines as depicted in Scheme II is consistent with the data obtained. We are currently investigating the mechanism in more detail.[22]

Acknowledgments. The authors thank Professor Lars Oreland (University of Umeå, Sweden) for a generous donation of purified mitochondrial pig liver MAO and Dr. Martin Winn (Abbott Laboratories) for samples of *N*-cyclopropyl- and *N*-cyclopropylmethyltryptamine. We are grateful to the National Institutes of Health (MH 33475) for support of this research.

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(11) [*phenyl*-^{14}C]-*N*-Cyclopropylamine was prepared by the sodium cyanoborohydride reduction of [*phenyl*-^{14}C]-*N*-benzylidenecyclopropylamine, which was synthesized by the reaction of [*phenyl*-^{14}C]benzaldehyde (Pathfinder Laboratories, St. Louis, Mo.) with cyclopropylamine.
(12) This number was derived from calculations made using an MAO sample that was ~50% pure and which contained 1 mol of flavin/115 000 g of active enzyme.
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(16) The rate constant for hydrolysis of *N*-benzylidenecyclopropylamine at 25 °C in 20 μM Tris pH 9.0 buffer was found to be 2.0×10^{-2} min^{-1} ($t_{1/2}$ = 35 min). The anaerobic experiment was performed under conditions where enzyme treated with 220 μM *N*-cyclopropylbenzylamine was totally inactivated within a few minutes. The enzyme treated with 220 μM *N*-benzylidenecyclopropylamine showed no irreversible inhibition compared with untreated enzyme even after 20 min.
(17) The small decrease in absorbance in the 430–500-nm region of the flavin spectrum is typical of formation of an alkylated reduced flavin.[3a,18] The lowest spectral scan is the same as that of dithionite-reduced native enzyme.
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(22) NOTE ADDED IN PROOF. From the Swain equation, the 0.73 mol of ^3H released from *N*-[1-^3H]cyclopropylbenzylamine/mol of enzyme inactivated corresponds to 1.00 mol of ^3H released when the primary tritium isotope effect at that concentration of inactivator is considered.

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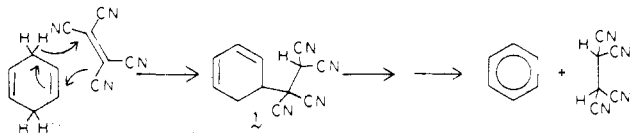
Aromatization of 1,4-Cyclohexadiene with Tetracyanoethylene: An Ene Reaction

Sir:

The aromatization of 1,4-dihydrobenzenes with tetracyanoethylene[1] (TCNE) is a very mild reaction that has seen occasional synthetic use.[2] It has been proposed that the reaction proceeds via hydride abstraction or electron-proton-electron transfer to yield a cyclohexadienyl cation[3] in analogy to quinone-mediated aromatizations, though the latter are themselves the subject of some mechanistic controversy.[4] Evidence is reported here that, at least for 1,4-cyclohexadiene (1,4-CHD) itself, the aromatization is actually initiated via an ene reaction with TCNE to give 5-(1,1,2,2-tetracyanoethyl)-1,3-cyclohexadiene (**1**). Intermediate **1** then decomposes,



perhaps by dissociation to cyclohexadienyl cation and tetracyanoethyl anion and thence to benzene and tetracyanoethane.[5]

In both tetrahydrofuran and acetonitrile-d_3 the disappearance of 1,4-CHD is a second-order reaction, first order in 1,4-CHD and first order in TCNE. The rate constants at 35.3 °C are 5.3×10^{-3} L M^{-1} min^{-1} and 6.4×10^{-2} L M^{-1} min^{-1}, respectively.[7] However, the appearance of benzene is much slower and does not give a good second-order plot. If the reaction is run in a sealed NMR tube, signals can be seen appearing in the region δ 5.8–6.6 in tandem initially with the disappearance of the 1,4-CHD signal (Figure 1a). These new signals reach a maximum and then gradually decrease with a corresponding increase in the benzene and tetracyanoethane signals (Figure 1b,c). Assuming that there are four vinyl hydrogens in the intermediate, the sum of the benzene, 1,4-CHD, and intermediate concentrations remains constant within experimental error throughout the reaction. Once the 1,4-CHD is >90% reacted, the conversion of the intermediate into benzene follows fairly good first-order kinetics with rate constants at 35.3 °C of 3.87×10^{-4} min^{-1} in THF and 1.62×10^{-3} min^{-1} in acetonitrile-d_3.[8] With the hypothesis in mind that the intermediate has structure **1**, a trapping experiment was attempted. Because TCNE is itself a very good dienophile and yet failed to react with the intermediate in Diels–Alder fashion, the more reactive *N*-phenyl triazolinedione (PTAD) was used. However, the reaction could not be run with the PTAD present initially because PTAD and 1,4-CHD themselves give an ene adduct very rapidly.[9] Therefore, a reaction run in an open NMR tube between TCNE and 1,4-CHD was allowed to proceed until most of the diene had reacted. The PTAD was

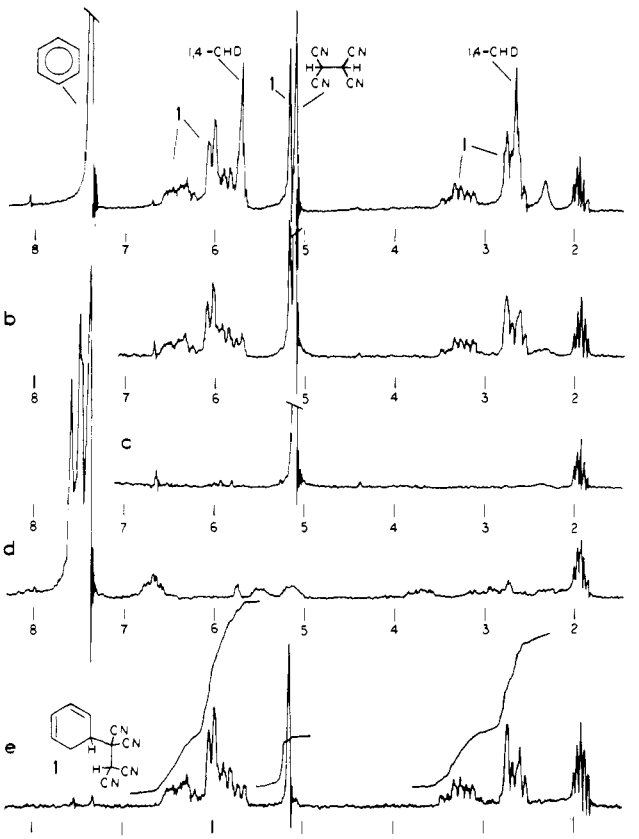


Figure 1. ^1H NMR spectra of (a) TCNE and 1,4-CHD after 25 min at 35 °C; (b) the same mixture after 4 h; (c) the same mixture after 24 h; (d) the same mixture after 4 h after addition of PTAD; (e) the isolated compound **1**. All spectra were in CD_3CN (residual protium in the solvent at δ 1.95) with Me_4Si internal standard. The large benzene signal in (b) and (c) has been omitted for the sake of clarity of the other spectra.

then introduced. The red color of the trapping agent faded in seconds and the spectrum of Figure 1d was observed. Although most of the signals are consistent with those expected for a Diels–Alder adduct of **1** and PTAD, the loss of both the singlets (δ 5.05 and 5.09) for the hydrogens α to the cyano groups in **1** and tetracyanoethane as well as the formation of 4-phenylurazole (isolated) indicated that more than mere trapping by a Diels–Alder reaction was occurring.[11] Because the trapping experiment produced a rather complex mixture and the trapped adduct was also of limited stability, a pure product could not be isolated. The resulting uncertainty in the precise structure of the trapped adduct (beyond reasonably strong assurance of its origin in a Diels–Alder reaction) led to an attempt to isolate the intermediate **1** itself. A solution of 300 mg of 1,4-CHD in acetonitrile was cooled to −20 °C and then added to 240 mg of freshly sublimed TCNE. The mixture was stored under N_2 at −12 °C for 24 h. Without warming, all volatiles were then pumped away at 0.03 mm of Hg. The oily residue was triturated with fresh cold acetonitrile, pumped again, triturated with CCl_4, and pumped dry a third time, all without warming above −10 °C, to yield an off-white solid. This was briefly stirred with a few milliliters of ethanol-free $CHCl_3$ at room temperature, the insoluble TCNE and tetracyanoethane were filtered off, and the filtrate was immediately evaporated (no heating) to give white crystals of **1** in 36% yield (based on initial TCNE) with NMR spectrum as shown in Figure 1e.[12] These crystals are only modestly stable. A sample stored for a week at −12 °C turned pink and showed ~5% conversion into benzene and tetracyanoethane. When dissolved in acetonitrile-d_3, **1** yielded benzene and tetracyanoethane quantitatively with, as expected, a slightly greater rate (k = 1.86×10^{-3} min^{-1} at 35.3 °C) than observed for the first-order

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