Chapter 1. Introduction

1.1. Monoamine Oxidase

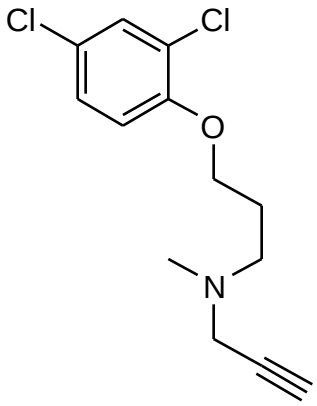
1.1.1. Characteristics

Monoamine oxidase [EC 1.4.3.4. (MAO)] is a flavin adenine dinucleotide (FAD) containing enzyme which catalyzes the oxidation of amines to the corresponding aldehyde and ammonia using O_2 as an electron acceptor. The stoichiometry of this oxidation reaction is as shown. The pathway will be discussed later.

$$RCH_2NH_2 + FAD + O_2 + H_2O \longrightarrow RCHO + FADH_2 + HOOH + NH_3$$

MAO is an integral protein found in the outer mitochondrial membrane of both neuronal and nonneuronal tissue, e.g. platelets, placenta, liver, heart and lung.[1] One of its primary roles is the regulation of the levels of biogenic amines in the brain and peripheral tissues by catalyzing their oxidative deamination. It occurs in two immunologically and catalytically distinct forms, MAO-A and MAO-B. In some tissues a single form is present. In human, most tissues express both MAO-A and MAO-B, although placenta contains predominantly MAO-A[2] and platelets[3,4] and lymphocytes[4] express only MAO-B. Both forms of MAO seem to be present in most areas of the brain. Monoclonal antibodies specific for each of the forms have shown that MAO-A is located in catecholaminergic cells, including those present in the substantia nigra, while MAO-B is located in serotonergic regions.[5,6] MAO-B was also found in astrocytes, a neuronal helper cell population found in all parts of the brain.

The two forms are distinguished by their sensitivity to highly selective inhibitors. MAO-A is inhibited by nanomolar concentrations of clorgyline[7] (**1**) while MAO-B is inhibited by nanomolar concentrations of *R*-(-)-deprenyl[8] (**2**).



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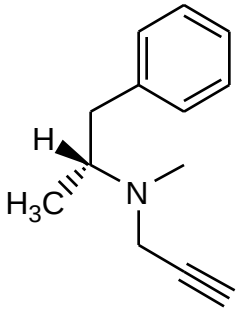

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The notion was once held that MAO-A and MAO-B were the same protein with different lipids attached. However, Pearce and Roth[9a] have demonstrated that both the A and B forms of human brain MAO are separable with retention of enzymatic activity by simple chromatographic techniques. Further studies where monoclonal antibodies have been raised against each form of the enzyme have provided additional evidence indicating that the two major enzyme sub-types are different proteins.[9b] The most decisive evidence came from the isolation of cDNA clones that encode MAO-A and MAO-B and determination of their nucleotide sequences.[10] The amino acid sequence deduced from these data show a 70% homology. Both sequences contain the pentapeptide Ser-Gly-Gly-Cys-Tyr in which the obligatory co-factor FAD is covalently linked via a thioether bond between the cysteine residue and the C_8-methyl substituent of the flavin moiety (Fig. 1).[11]

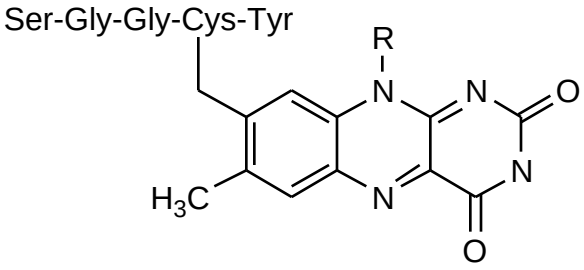
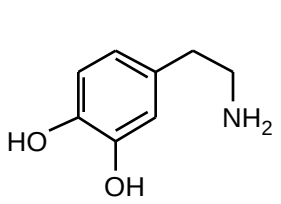


Fig. 1. Partial Amino Acid Sequence of MAO Containing the Flavin Moiety

The subunit molecular weights of the A and B forms have been calculated to be 59,700 and 58,800 daltons, respectively.[12] Although the enzymes have not been crystallized, studies show a ratio of one FAD per 63 kDa (MAO-A) and 57 kDa (MAO-B) human enzyme.[13] The above data, in conjunction with the molecular weights obtained from cDNA studies, indicate that MAO consists of either one or more subunits, each of which binds one FAD molecule.[14]

1.1.2. MAO Substrates

Monoamine oxidase catalyzes the oxidative deamination of a variety of biogenic amines[7,15,16] including dopamine (**3**), noradrenaline or norepinephrine (**4**), adrenaline or epinephrine (**5**), serotonin (**6**) and 2-phenylethylamine (**7**). MAO-A preferentially metabolizes serotonin[7] while MAO-B shows a preference for 2-phenylethylamine.[16] In addition to the endogenous substrates of MAO, numerous substances of exogenous origin have been shown to be oxidatively metabolized by MAO. Benzylamine[17a] (**8**) is a preferred MAO-B substrate while kynuramine[17b] (**9**) is a preferential MAO-A substrate.



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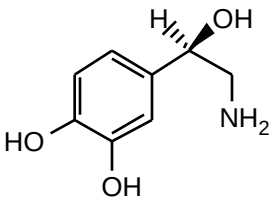

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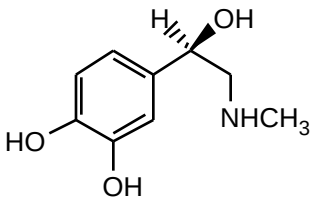

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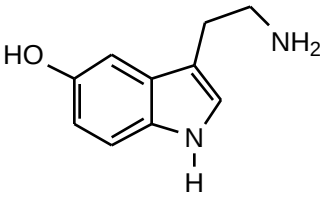

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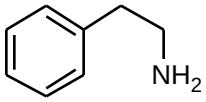

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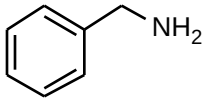

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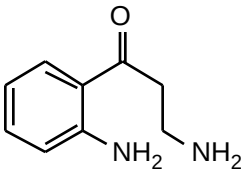

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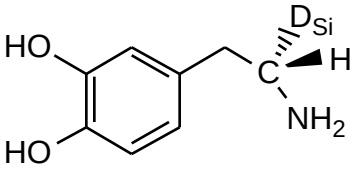
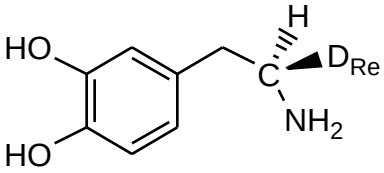
MAO is thought to catalyze amine oxidation by a single electron transfer pathway (discussed in detail in section 1.4). Abstraction of an α-proton from the intermediate cation radical was suggested to be the rate-limiting step in the oxidation process.[25] The absolute stereochemistry of the abstraction of a hydrogen atom from the prochiral methylene group has been determined. Yu *et al.*[18] using dopamine or benzylamine as the substrate have established that both MAO-A and B exhibit the same stereospecificity, that is, the pro-*R*-hydrogen is abstracted by both forms of MAO.



S-[α-$^{2}H_{1}$]-dopamine

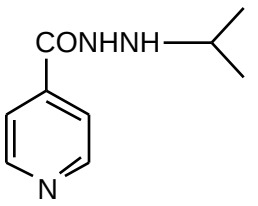


R-[α-$^{2}H_{1}$]-dopamine

1.1.3. MAO Inhibitors

The discovery of the therapeutic activity of monoamine oxidase inhibitors was due to the recognition of an important clinical side effect. Patients who had been treated with a tuberculosis fighting drug, iproniazid, displayed a profound mood elevating side effect. After five years of clinical observations in tuberculosis patients, iproniazid was introduced into clinical medicine as the first antidepressant drug.[19] Biochemical studies indicated that iproniazid (**10**) was a specific and potent inhibitor of MAO.[20] The discovery that iproniazid is an inhibitor of MAO activity paved the way to the synthesis and pharmacological assessment of inhibitors of the enzyme.

Some of the MAO inhibitors currently in clinical use in certain European countries for treatment of depression include phenelzine (**11**), tranylcypromine (**12**), pargyline (**13**) and isocarboxazid (**14**). The MAO inhibitors can be divided into three structural classes, namely the acetylenes, the cyclopropylamines and the hydrazines. They exhibit their inhibition properties by a variety of mechanisms including irreversible inhibition and mechanism-based inactivation. A brief review of enzyme inhibition is provided in the next section.



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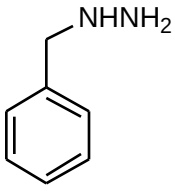

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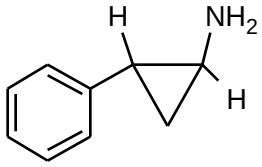

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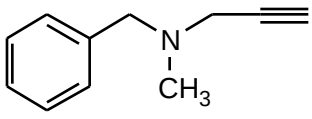

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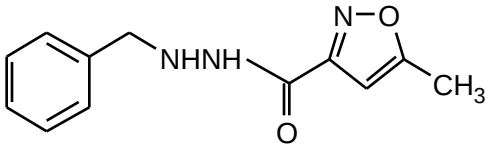

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1.2. Enzyme Inhibition

Enzyme inhibition is a phenomenon of great practical importance as well as a useful probe for characterizing the molecular features of the active site of the enzyme and other factors that influence enzyme activity. The active site of an enzyme represents a small part of the whole enzyme molecule. Characterization of active centers for enzymes for which no X-ray structure is available is dependent on structure activity relationship (SAR) studies including those utilizing specific enzyme inhibitors which may act reversibly or irreversibly. Before proceeding to a discussion of enzyme inhibition it is important to understand how enzymes function as catalysts in the metabolism of substrate molecules in biochemical reactions and therefore a brief description of enzyme catalysis is included.

1.2.1. Enzyme Catalysts

Enzymes are proteins that catalyze biochemical reactions. Enzymes in general tend to be highly selective for the type of reaction they catalyze. The "catalytic site" on the enzyme contains functionalities that are directly involved in the catalysis. The catalytic site is usually a small, localized region of the enzyme surface. The substrate binding site and the catalytic site are usually in close proximity to each other and frequently overlapping. Together they are referred to as the "active site".

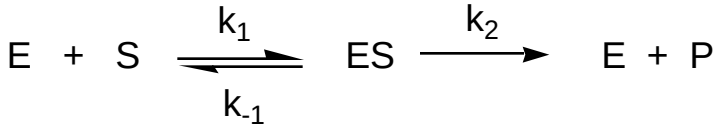
1.2.2. Kinetics of Enzyme Catalyzed Reactions

As a rule kinetic analysis is done on purified or partially purified enzymes in vitro. Kinetic analysis is a major tool used to investigate the mechanism of enzyme catalysis. One of the main features of kinetic analysis involves the variation in the rate of reaction with substrate concentration. For this purpose a

fixed low concentration of the enzyme is used in a series of parallel experiments in which only the substrate concentration is varied. Under these conditions the initial reaction velocity increases until it reaches a maximum value at high substrate concentrations reflecting that all enzyme binding sites are occupied with substrate.

Initially it may be assumed that the reaction between enzyme (E) and substrate (S) proceeds through the formation of an enzyme-substrate complex (ES) and that the (ES) is in equilibrium with the free enzyme (E) and substrate (S). The formation of products proceeds only through the (ES) complex. The steady-state kinetic condition may be introduced to eliminate the requirement that the (ES) complex be in equilibrium with the free enzyme and substrate. The steady state evaluation assumes a fixed level of (ES) complex (Scheme 1). The steady state, which is achieved shortly after mixing enzyme and substrate, constitutes the time interval when the rate of reaction is constant with time. The concentration of the (ES) complex is also a constant during this time period. Usually the substrate is present in much higher molar concentrations than the enzyme. Since the initial period of the reaction only is being examined, the free substrate concentration is approximately equal to the total substrate added to the reaction mixture.

Scheme 1. Enzyme Catalyzed Reaction of Substrate (S) to Product (P)



For the purpose of graphical representation of experimental data in an enzyme catalyzed reaction it is convenient to use the Lineweaver-Burke equation derived using the steady state approximation which states:

$$1/V = (K_m/V_{max})\cdot(1/[S]) + (1/V_{max}).$$

The constant K_m is called the Michaelis constant. One of the important relationships is that between K_m and the substrate concentration (S). K_m can be defined as the concentration of the substrate when the maximum rate of enzyme catalyzed reaction is 50%, i.e. half V_{max}. From the Lineweaver-Burke equation, a plot of 1/V (the reciprocal of the initial rate) vs 1/S (the reciprocal of the substrate concentration) should give a straight line. The slope of that line is K_m/V_{max}, the intercept on the ordinate is $-1/V_{max}$, and the intercept on the abscissa is $-1/K_m$.

Another important aspect of enzyme catalysis is the turnover number of an enzyme (k_{cat}) which is defined as the maximum number of substrate molecules converted to product per active site per unit time. Since the rate limiting step in Scheme 1 is assumed to be the formation of product P from (ES), the initial velocity of the reaction is given by

$$V_i = k_2(ES).$$

The maximum velocity should be obtained when the total enzyme (E_t) is in the form of the enzyme substrate complex (ES):

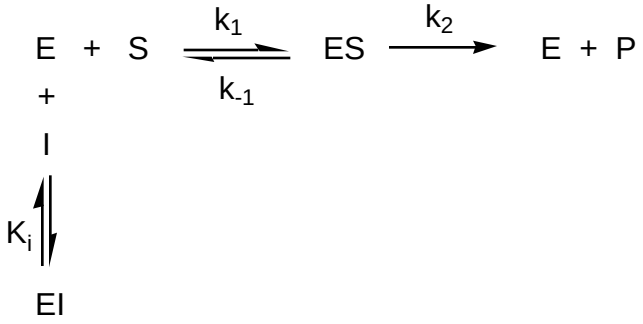
$$V_{max} = k_2(E_t) \text{ where } E_t = E + ES.$$

Thus for the situation represented in Scheme 1, in which there is only one (ES) complex and the conversion of (ES) complex to product is rate limiting, k_{cat} is equal to the kinetic constant k_2. Thus the turnover number is a direct measure of the catalytic efficiency of the active site of the enzyme.

1.2.3. Reversible Inhibitors

Reversible inhibitors are classified into competitive, noncompetitive, and uncompetitive inhibitors. They work by a variety of mechanisms that frequently can be distinguished by kinetic analysis. In competitive inhibition, the binding of an inhibitor and substrate is mutually exclusive. The inhibitor is a substance that combines with free enzyme in a manner that prevents substrate binding (Scheme 2). Usually, but not always, competitive inhibition occurs because the inhibitor structurally resembles the substrate and binds at the active center. A competitive inhibitor acts only to increase the apparent K_m for the substrate. As [I] increases, K_{mapp} increases. The V_{max} remains unchanged, but in the presence of a competitive inhibitor a much greater substrate concentration is required to attain any given fraction of V_{max}. However, greater concentrations of the substrate overcome the negative effects caused by the inhibitor because both substrate and inhibitor molecules are competing for binding to the same site (Scheme 2).

Scheme 2. Enzyme Inhibition by Competitive Inhibitors



As in the case of the interaction of a substrate with an enzyme the inhibitor can also form a complex with the enzyme (see Scheme 2). The equilibrium constant K_i (= k_{off}/k_{on}) is the dissociation constant for the

breakdown of the EI (enzyme-inhibitor) complex, i.e. $K_i = [E][I]/ [E{\cdot}I]$. Therefore, the smaller the value of K_i for I, the better the inhibitor. The equilibrium depends upon the concentrations of the enzyme, the inhibitor, and the substrate. Since the enzyme concentration is low and fixed, the EI concentration will principally depend upon the concentrations of the substrate and inhibitor.

Interactions of the inhibitor with the enzyme can occur at a site other than the substrate binding site and still result in inhibition of substrate turnover. When this occurs, usually as a result of an inhibitor induced conformational change in the enzyme to give a form of the enzyme that does not bind to the substrate properly, then the inhibitor is known as a noncompetitive inhibitor. These types of inhibitors have no effect on the K_m value but decrease the V_{max} of the reaction. The net effect of a noncompetitive inhibitor is to make it seem as if less enzyme were present.

An uncompetitive inhibitor is a compound than binds reversibly to the enzyme-substrate complex yielding an inactive ESI complex. It does not bind to the free enzyme. Uncompetitive inhibition is less common than the previous two but when it does occur, it decreases the V_{max} of the reaction and also increases the apparent K_m of the substrate.

1.2.4. Irreversible Inhibitors

All competitive and some non-competitive inhibitors are reversible. If the inhibitor concentration is lowered, the enzymatic activity increases. If the inhibitor is removed, the enzyme activity returns to its normal value. In the case of irreversible inhibition, the inhibitor binds so strongly to the enzyme (usually via a covalent bond) that it will not dissociate when the inhibitor concentration is lowered. Irreversible inhibition can occur at the active site or elsewhere. The

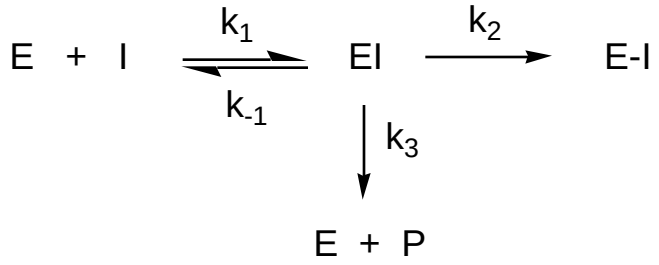
site of binding of the irreversible inhibitors is best determined by direct chemical methods. Irreversible inhibitors often provide valuable information about the active site of the enzyme. For example, certain amino acid residues that are part of the active site may undergo covalent adduct formation with specific inhibitors.

1.2.5. Mechanism-based Inactivators

Mechanism based inactivators generally are relatively unreactive compounds with structural similarities to the substrate or product of a particular enzyme. The normal catalytic mechanism of action converts the inactivator molecule into a species which, without prior release from the active site, binds covalently to that enzyme.

Generally in enzyme catalyzed reactions, an equilibrium is established rapidly between the enzyme bound and free form of the inactivator. The on and off rates for this process are governed by the rate constants k_1 and k_{-1}, respectively (Scheme 3). When an enzyme catalyzes the conversion of a mechanism based inactivator into its reactive form there are two processes that can follow. The altered form can be released as product (k_3) or it can react with the enzyme to form a covalent linkage (k_2) as shown is Scheme 3.

Scheme 3. Irreversible Mechanism-Based Inactivation



On the assumption that the equilibrium for reversible EI complex formation is rapid and the rate of dissociation of the EI complex (k_{-1}) is fast relative to covalent bond formation, then k_2 will be the rate determining step. This reaction scheme (see Scheme 3) is similar to that for the conversion of substrate to product (see Scheme 1) but instead of a k_{cat}, a catalytic constant (turnover number) for product formation, there is a k_{inact}, a first order rate constant for enzyme inactivation. In this case, unlike simple reversible inhibition, there will be a time-dependent loss of enzyme activity. The rate of inactivation is proportional to low concentrations of inhibitor but becomes independent at high concentrations.

An important concept related to mechanism based inactivation is the partition ratio which describes the ratio of the number of latent inactivator molecules converted and released as product relative to each turnover leading to enzyme inactivation. According to Scheme 3 this ratio would be k_3/k_2. The partition ratio is, therefore, the measure of the efficiency of the inactivator and depends upon the reactivity of the activated intermediate, the rate of diffusion from the active site, and the proximity of an appropriate nucleophile, radical or electrophile on the enzyme for covalent bond formation.

Mechanism based inactivators have proven to be useful in the study of enzyme mechanisms. The value of these inactivators in the study of enzyme mechanisms is derived from the fact that they are processed by the enzyme by the same catalytic pathway followed in the turnover of a substrate. Acting like a true substrate, they are converted by the catalytic mechanism of the enzyme into products. However, the products of these enzyme mediated reactions are generally reactive species that then become attached covalently to the enzyme.

The important event is that the conversion to the activated form is at least initiated by the same catalytic steps involved in the reaction with normal substrates, only the products happen to be more reactive than those generated from normal substrates. Therefore, whatever mechanistic information that can be gained from inactivation studies is directly linked to the catalytic mechanism of the enzyme.

1.2.6. Criteria for Mechanism-Based Inactivation

A set of criteria has evolved for the evaluation of mechanism based inactivation. In order to characterize unambiguously an inactivator falling into this class, most or all of the criteria should be satisfied. The criteria are as follows:

1) A time dependent loss of enzyme activity is observed.

2) The rate of inactivation is proportional to low concentrations of inhibitor but independent at high concentrations (saturating conditions).

3) The rate of inactivation is slower in the presence of substrate than its absence.

4) Enzyme activity does not return upon dialysis or gel filtration.

5) A 1:1 stoichiometry of radio-labeled inactivator to active site usually results after inactivation followed by dialysis or gel filtration.

1.3. Kinetic Isotope Effects

Over the last two decades the measurement of isotope effects has emerged as one of the most powerful techniques available for the elucidation of the chemical mechanisms of enzyme catalyzed reactions. The major reason for determining kinetic isotope effects in enzymology has been to determine whether the maximal velocity is a measure of the rate of the step in which

carbon-hydrogen bond cleavage takes place. Such a step is normally identified as the "rate-determining step" of the enzymatic reaction. An enzyme catalyzed reaction usually consists of a large number of separate reaction steps of similar energy, the rate of only one of which is affected by isotopic substitution. Therefore, it is rare for the isotopically sensitive step to be cleanly rate limiting. For example, the binding and release of substrates and products, further chemical steps, and possibly conformational changes in the protein may all be partially rate limiting.

The fundamental equation of enzyme kinetics is the Michaelis-Menten equation (eq 1), relating the rate of an enzyme catalyzed reaction to the substrate concentration. The equation contains two fundamental parameters. The first is V_{max}, the maximum rate of reaction at infinite substrate concentration. The second parameter is V_{max}/K_m, the apparent first order rate constant for the reaction at low substrate concentration.

$$v = (V_{max}[S]) / (K_m + [S]) \quad \text{eq. 1}$$

The following model will be used to define the kinetic parameters:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + P$$

where, under initial velocity conditions, k_4 and k_6 are equal to zero. The steady-state rate equation for this mechanism is given in eq. 2, from which kinetic expressions for V_{max}, K_m and V_{max}/K_m may be derived (eq 3-5).

$$v = \{k_1k_3k_5[S]\} / \{k_5(k_2 + k_3) + k_1(k_3 + k_5)[S]\} \quad \text{eq. 2}$$

$$V_{max} = (k_3k_5) / (k_3 + k_5) \quad \text{eq. 3}$$

$$K_m = k_5(k_2 + k_3) / k_1(k_3 + k_5) \quad \text{eq. 4}$$

$$V_{max}/K_m = k_1k_3 / (k_2 + k_3) \quad \text{eq. 5}$$

Notice that the equation for K_m contains all the rate constants for the enzyme catalyzed reaction, while V_{max} includes only the catalytic step and product release step and V_{max}/K_m includes the binding step and the catalytic step. Because K_m depends on all the rate constants of the reaction, an isotope effect associated with K_m is not informative.[21]

The isotope effects associated with V_{max} and V_{max}/K_m are as follows:

$$^{D}V_{max} = (^{D}k_3 + k_3/k_5) / (1 + k_3/k_5)$$

$$\text{and } ^{D}(V_{max}/K_m) = (^{D}k_3 + k_3/k_2) / (1 + k_3/k_2).$$

If binding of the substrate to the enzyme is rate determining, an isotope effect on V_{max}/K_m but not on V_{max} will be observed. If the rate determining step includes product release, an effect on only V_{max} is seen. An effect on both V_{max} and V_{max}/K_m indicates that the catalytic step is rate determining.

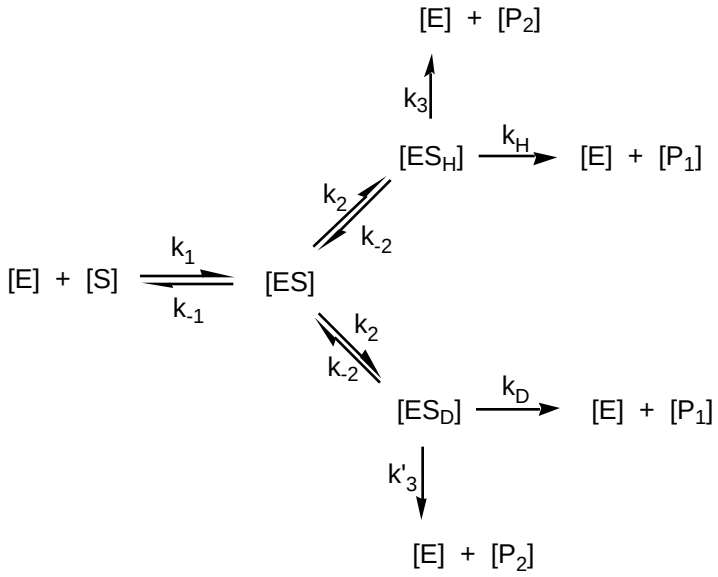
Isotope effect measurements may be estimated by intermolecular or intramolecular experiments. However, Northrop[21] has shown that the magnitudes of isotope effects on enzyme catalyzed reactions are subject to unknown degrees of suppression from numerous pre-catalytic steps when they are determined by intermolecular competition (separate incubations of deuterated and nondeuterated substrates). Measurements of isotope effects by this method may result in a substantial underestimation of the intrinsic isotope effect. This effect is often referred to as "masking".

Alternatively, isotope effects can be measured by intramolecular experiments.[22,23] In such an experiment, a molecule that has two positions that are equivalent in all respects except for isotopic substitution ideally is used as the substrate. The observed isotope effect then reflects the intramolecular competition between two otherwise equivalent sites. In most cases an

intramolecular isotope effect more nearly approximates the intrinsic isotope effect since it depends primarily upon the product-determining step rather than other potential rate-limiting steps, e.g., the magnitude of an observed intramolecular isotope effect will be independent of product release but the magnitude of an intermolecular isotope effect will not.

Another factor that may influence the magnitude of the observed isotope effect is "metabolic switching".[24] This phenomenon is seen primarily in a branched reaction sequence, i.e., one in which more than one product can arise from an enzyme-substrate complex (Scheme 3a). Metabolic switching or isotopically sensitive branching can be defined as a change in the relative ratios of products, due to isotopic substitution, arising from the same enzyme-substrate complex. The pathway that leads to a product where no carbon-hydrogen bond cleavage occurs will be the favored pathway. An inverse isotope effect is often observed.

Scheme 3a. Kinetic Model for Isotopically Sensitive Branched Reaction Pathways

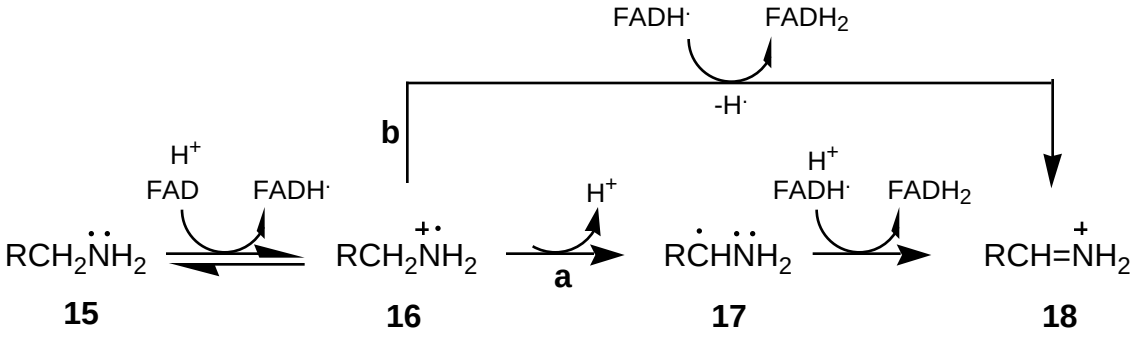


1.4. Proposed Catalytic Mechanism

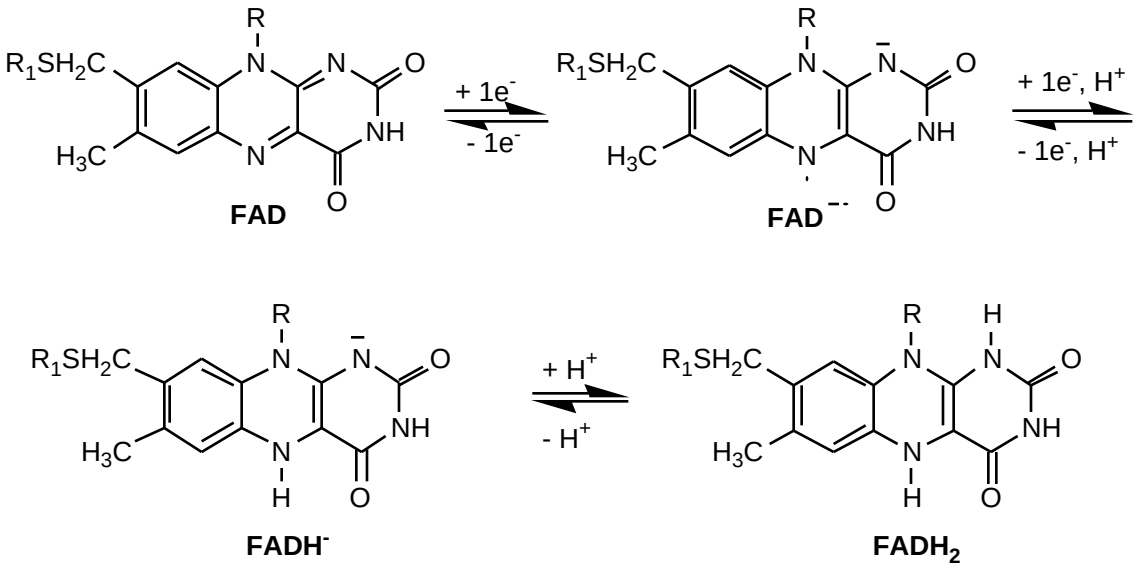
1.4.1. Single Electron Transfer

Our current knowledge regarding the mechanism of MAO-catalyzed amine oxidation has come mainly from studies on the interaction of bovine liver MAO-B with mechanism-based inactivators. From these studies, Silverman and colleagues have proposed a pathway for MAO that proceeds by a one-electron mechanism.[25] One-electron transfer from the substrate amino group to the oxidized flavin (FAD) gives the amine radical cation (**16**) and the flavin semiquinone ($FADH^{\cdot}$) (Scheme 4). Loss of a proton gives the α-amino radical (**17**), which can transfer the second electron to the flavin semiquinone to give the reduced flavin ($FADH_2$) and the iminium product (**18**). A potential alternative to proton transfer (pathway a) followed by electron transfer is hydrogen atom transfer (pathway b), which bypasses the α-amino radical intermediate (**17**). Scheme 4a shows the proposed fate of the FAD. In its oxidized form FAD accepts an electron from the amine substrate to form the semi-reduced radical anion $FAD^{\cdot -}$. A second electron transfer and protonation converts the FAD- to the reduced flavin ion $FADH^-$. Finally protonation of $FADH^-$ yields $FADH_2$.

Scheme 4. Proposed Radical Mechanism for MAO Catalysis



Scheme 4a. Reduction of the Flavin

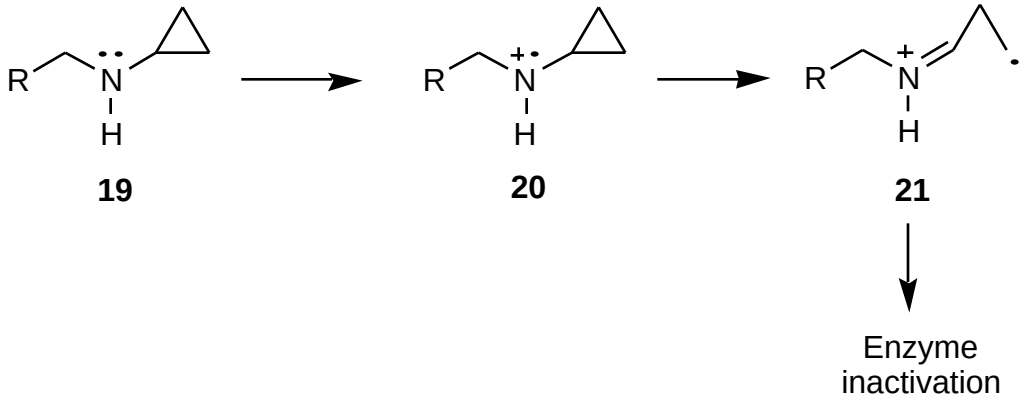


However, there has been no evidence provided for radical intermediates. Tan *et al.*[26] found no evidence for radicals by EPR spectroscopy during substrate turnover and stopped-flow kinetics experiments have failed to provide evidence for a flavin semiquinone intermediate.[27] Therefore, if radical intermediates are formed, they must be very short-lived, low in concentration, or spin paired with another radical.[28] Because of the lack of spectral evidence for radical intermediates, Silverman and colleagues took a chemical approach to the detection of a radical intermediate. They attached a cyclopropyl group to primary and secondary amine substrates. The selection of this group was based on the laser flash photolysis work of Maeda and Ingold[29] who found that secondary aminyl radicals could be generated and observed by low-temperature EPR spectroscopy. When the corresponding cyclopropylaminyl radical was generated by the same method, however, it could not be observed.

Instead, the ring-cleaved radical product was detected. The rate of cyclopropyl ring opening was too fast to measure and was estimated at >5 x 10^8 s^{-1}.

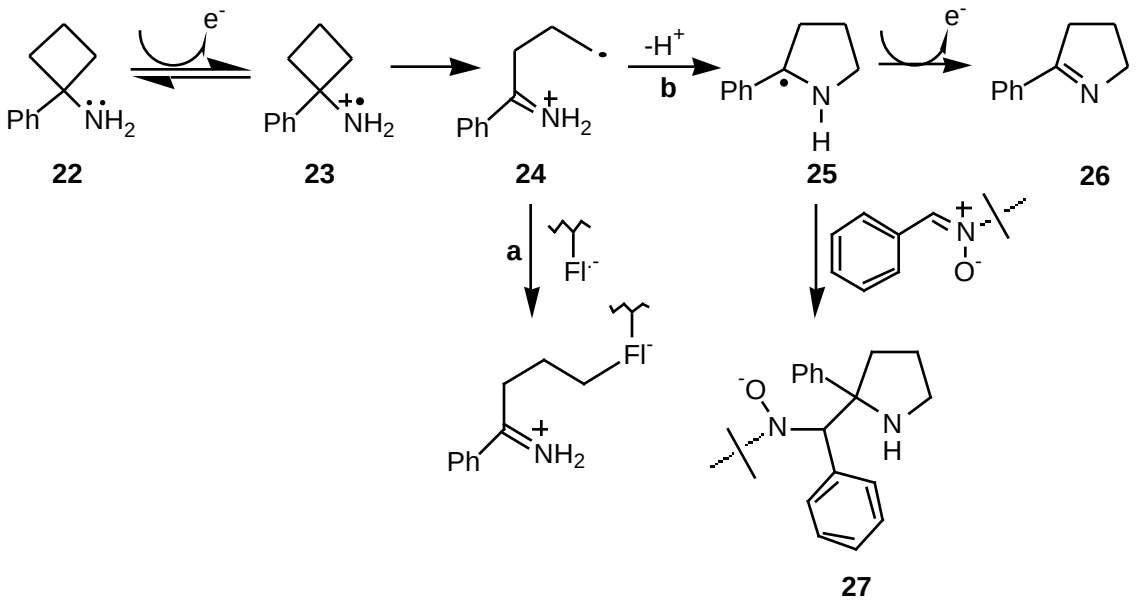
Many cyclopropyl-containing substrate analogs have been examined as potential mechanism-based inactivators of MAO[30-35] such as N-benzylcyclopropylamine (**19**). Upon incubation of MAO with each as these compounds, the enzyme became inactivated (irreversible loss of enzyme activity), as would be expected for oxidation to the aminyl radical cation (**20**) followed by cyclopropyl ring opening to give a reactive primary radical (**21**) (Scheme 5). Covalent attachment of the primary radical to either the flavin semiquinone or an amino acid radical is thought to mediate the inactivation.

Scheme 5. Proposed MAO Inactivation Pathway for Cyclopropylamines



More direct evidence for a radical intermediate in the MAO-catalyzed amine oxidations was sought by carrying out the reaction of MAO with 1-phenylcyclobutylamine (**22**). This compound was shown to be both a substrate and a time-dependent irreversible inactivator of MAO-B.[36] A mechanism (Scheme 6) similar to that proposed for cyclopropylamines was proposed for the inactivation process.

Scheme 6. Evidence for Electron Transfer Mechanism Using Spin Traps



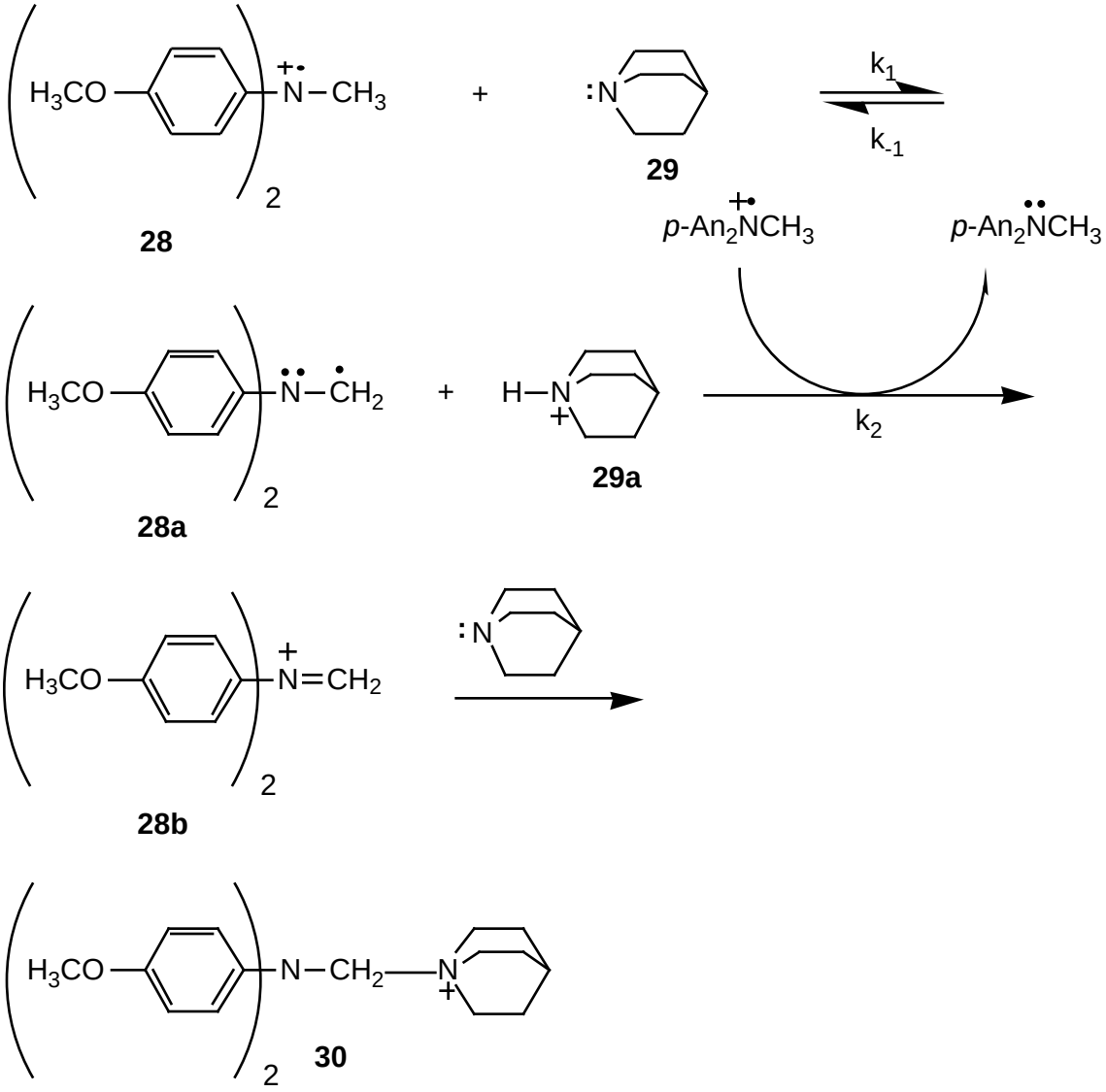
MAO-catalyzed one-electron oxidation of **22** would generate **23**, which would trigger cyclobutyl ring opening leading to **24**. The time-dependent inactivation of MAO is thought to occur from trapping of the radical intermediate **24** by the flavin semiquinone (pathway a). The radical **24** has another possible fate; it can undergo endo-cyclization followed by one-electron loss to generate 2-phenyl-1-pyrroline (**26**, pathway b). Incubation of **22** with MAO in the presence of the radical spin trap α-phenyl-N-*tert*-butylnitrone resulted in the time-dependent formation of the stable nitroxy radical (**27**). This evidence supports an initial one-electron transfer from the amine to the flavin.

The deprotonation step of the amine radical cation **16** as suggested in Scheme 4 is a commonly proposed reaction for many organic cation radical intermediates. For example this is a generally accepted fate of alkylaromatic as well as tertiary amine cation radicals generated in chemical,[37,38] photochemical[39] and enzymatic oxidation reactions.[40,41] Nevertheless the

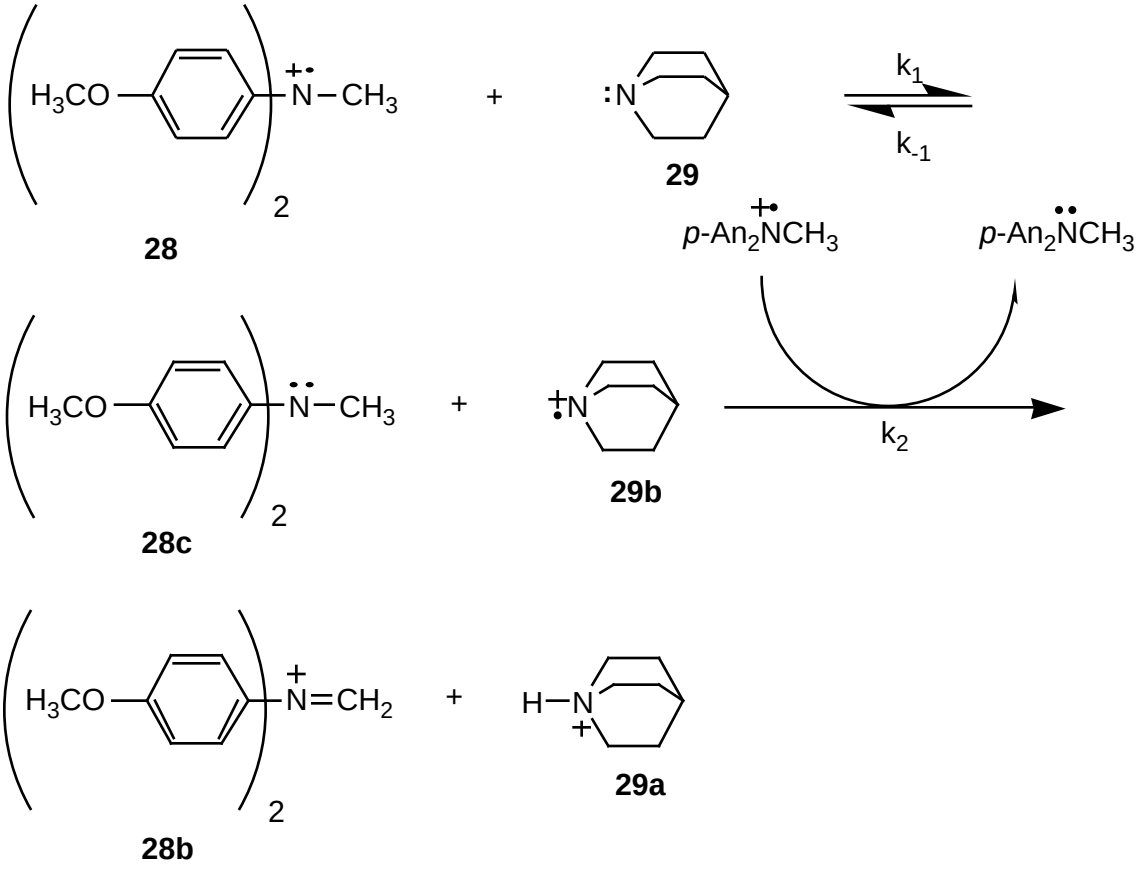
step remains controversial and an alternative pathway also has been proposed in which loss of a hydrogen atom rather than a proton takes place (Scheme 4, pathway b).

Tertiary amine cation radical deprotonations have been the subject of considerable discussion in the recent literature. Nelson and Ippoliti[42] have argued that the high kinetic acidity previously proposed for tertiary amine radical cations might be incorrect and on the basis of thermodynamic acidity estimates they argued that cation radicals are not extremely acidic and therefore α-deprotonation of an amine radical cation is not as favorable a pathway as hydrogen atom abstraction. Dinnocenzo and coworkers[43] presented arguments based on model studies that the thermodynamic acidity is irrelevant and that the true pK_a for amine radical cations determined by direct measurements is no more than 10 which makes amine radical cations weaker acids than originally thought. Consequently they suggested that α-deprotonation is a favorable and rate-limiting pathway. Studies with the tertiary amine cation radical salt[43] **28** and its subsequent reaction with quinuclidine (**29**) which led to the adduct **30** (Scheme 7) indicated the reaction to be first order in both the reactants and to proceed with an isotope effect of 7.68 (for the disappearance of the aminium radical when comparing the reaction rate between **28** and **28-d$_3$**). This is clearly a primary isotope effect and requires that cleavage of the C-H(D) bond be rate determining and suggests a deprotonation mechanism (Scheme 7, pathway a) rather than a second electron transfer (Scheme 7, pathway b) leading to adduct **30**.

Scheme 7 (Pathway a). Proton Transfer Mechanism



Scheme 7 (Pathway b). Electron Transfer Mechanism



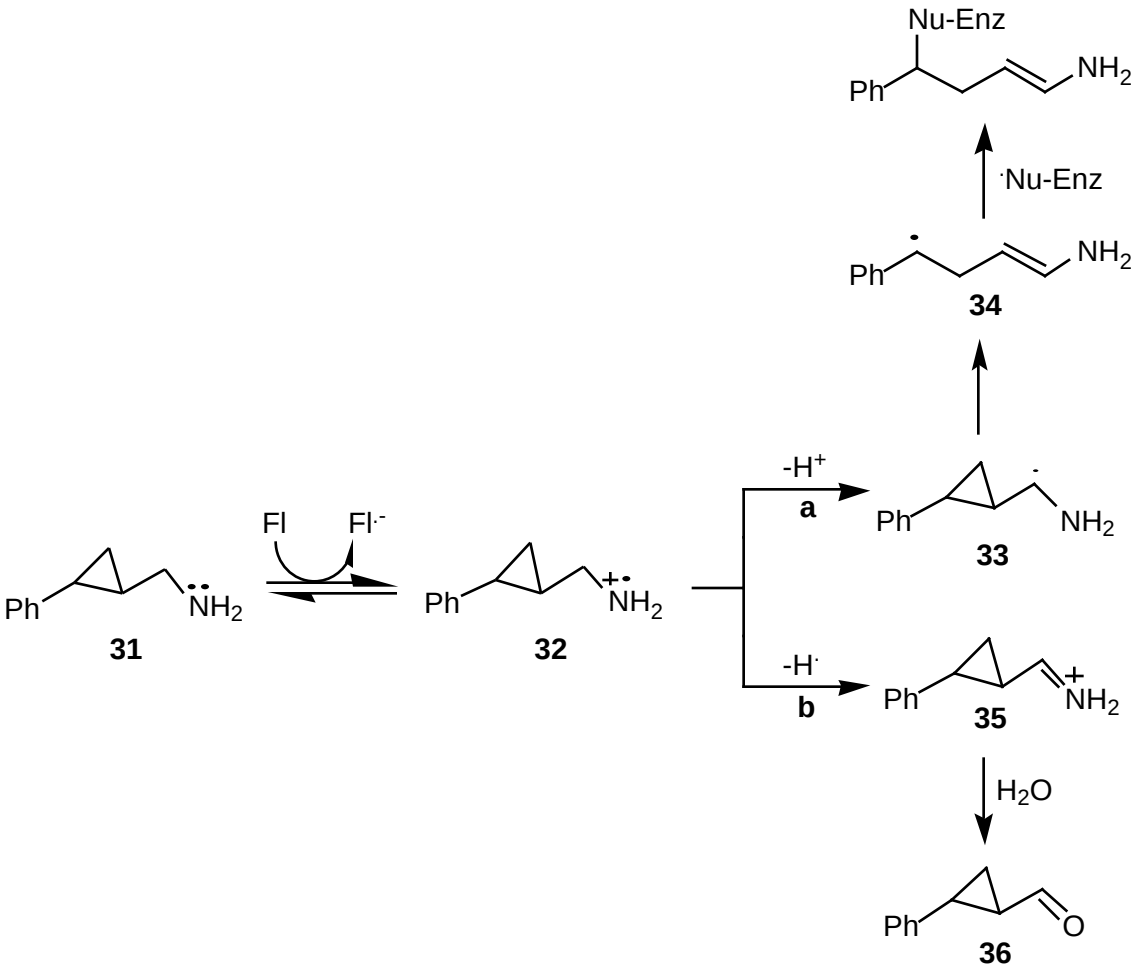
Thus the two mechanisms which were put forward consisted of the deprotonation pathway and the electron transfer pathway. The proton transfer mechanism consisted of deprotonation of **28** (isotope effect of 7.68) to produce an α-amino radical **28a** and the quinuclidinium ion **29a**. The α-amino radical **28a** is strongly reducing and would be expected to be oxidized by **28** to form the iminium ion **28b** and **29**. The iminium ion **28b** is then transformed into **30** by addition of **29** and **28b**. The susceptibility of **28b** towards nucleophilic attack by **29** to generate **30** was confirmed by reacting synthetic **28b** with **29**. The electron transfer mechanism involves an initial electron transfer from **28** to **29** to generate the corresponding amine **28c** and the quinuclidinium radical

cation **29b**. Abstraction of a hydrogen atom by **29b** from the starting material **28** generates **28b**. Alternatively this hydrogen atom abstraction can also occur from **28c** to form the same intermediate **28a** as in the deprotonation pathway.

Recently Silverman[44] has provided evidence for a hydrogen atom transfer mechanism or a proton/fast electron transfer mechanism as plausible pathways of deamination by monoamine oxidase. It is known that cyclopropylcarbinyl radicals rearrange to the corresponding 3-butenyl radical at a rate estimated to be 10^8 sec^{-1} or faster.[45] This rate increases when the cyclopropyl ring is substituted with radical stabilizing groups. For example, the 2-phenylcyclopropylcarbinyl radical opens to the 1-phenyl-3-butenyl radical at a rate of approximately 10^{11} sec^{-1}.[46] Silverman argues that such fast rates of ring opening could compete with electron transfer mechanisms and designed *trans*-1-(aminomethyl)-2-phenylcyclopropane (Scheme 8, **31**) as a test compound to study whether an α-deprotonation/electron transfer mechanism (Scheme 4, pathway a) or hydrogen atom transfer (Scheme 4, pathway b) operates during the oxidation process. The principal difference in these pathways is the presence or absence of intermediate **17**.

If deprotonation (Scheme 8, pathway a) occurred following initial electron transfer, the unstable cyclopropylcarbinyl radical intermediate (**33**) would result. Ring opening of **33** should lead to enzyme inactivation as depicted in pathway a. However, if hydrogen atom transfer (pathway b) occurred, the cyclopropylcarbinyl radical would be bypassed and the corresponding cyclopropylimine **35** would result.

Scheme 8. Monoamine Oxidase Catalyzed Oxidation of *trans*-1-(Aminomethyl)-2-phenylcyclopropane (**31**)

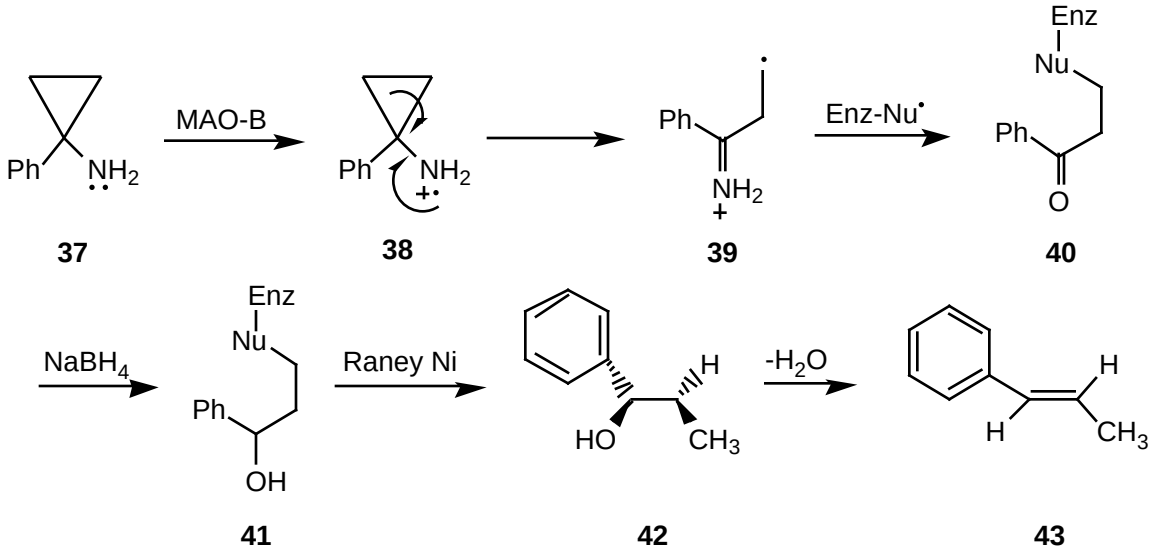


Experimental results indicated that incubation of MAO with **31** produced a single product, namely *trans*-2-phenylcyclopropanecarboxaldehyde (**36**), the oxidation and hydrolysis product without ring cleavage; no inactivation was observed. These results suggest that either the α-carbon radical is not an intermediate in the oxidation process or, if it is, second electron transfer to the flavin semiquinone occurs at a rate faster than cyclopropyl ring opening. Possible explanations for the lack of ring opening are (1) steric constraints at the enzyme active site may lead to improper orbital overlap between the α-

carbon radical and the cyclopropyl ring and (2) that the amino group stabilizes the radical, thereby slowing down the rate of ring cleavage.

Mechanistic studies by Silverman[35] also were carried out with 1-phenylcyclopropylamine (PCPA, **37**), a known mechanism-based inhibitor of MAO-B, to identify the active site residue which is covalently modified. It was shown that eight molecules of 1-[phenyl-^{14}C]PCPA were needed to inactivate irreversibly one molecule of the enzyme with the incorporation of one equivalent of radioactivity. An N-5-(3-oxo-3-phenylpropyl)flavin adduct (**40**) was identified as the product of irreversible inactivation. Seven of the eight molecules of the radio-labeled material were shown to form covalent bonds to active site amino acid residues. However these adducts decomposed with a half-life of 65 minutes to regenerate the active enzyme. This process explains the requirement of 8 labeled molecules per irreversible inactivation event as the rate of irreversible inactivation was found to be seven times slower than the rate of this reversible adduct breakdown. It was thus proposed that this irreversible adduct had the same structure as the reversible ones except that the site of covalent modification was different.

Scheme 9. Identification of the Amino Acid Residue Modified by 1-Phenylcyclopropylamine



37 **38** **39** **40** **41** **42** **43**

The irreversible adduct was a result of covalent modification at N-5 of the flavin whereas the reversible adducts were the result of covalent modification of amino acid residues. The amino acid was identified as a cysteine residue based on chemical treatment of the inactivated enzyme. Treatment of the inactivated enzyme-inhibitor adduct (**40**, Nu = amino acid, Scheme 9) with $NaBH_4$ led to a stabilized adduct (**41**) and prevented its decomposition (reduction of the keto group to an alcohol). Treatment of **41** with Raney nickel (a specific reagent for cleavage of carbon-sulphur bonds) led to the formation of a single compound identified as *trans*-β-methylstyrene (**43**) which results from the dehydration of 1-phenylpropanol (**42**), the actual product of Raney nickel catalyzed cleavage of **41**. A second experiment consisted of a determination of the effect of the reversible inactivation on the total number of cysteine residues in MAO. Thus in the presence of the cyclopropylamine only 5.2 cysteine residues could be detected whereas in a control experiment with no inhibitor,

6.2 cysteine residues could be detected. These results were found to be consistent with cysteine being the amino acid residue to which PCPA reversibly binds.

1.4.2. Hydrogen Atom Abstraction

In an effort to test critically the proposed SET mechanism, Edmondson and coworkers[47] have studied a series of MAO-B substrates rather than mechanism-based inactivators. Before discussing studies on the enzyme, it is instructive to review the known properties of chemical amine oxidation systems employing various oxidants. A compilation of these data is shown in Table 1. In three separate systems where secondary or tertiary benzylamine oxidation occurs via a SET mechanism with an aminium cation radical as the first reaction intermediate, the reactions exhibit negative ρ values of approximately -1, which indicates electron donating groups facilitate the rate-limiting formation of the positively charged intermediate. The electrochemical data also show that electron donating groups also facilitate the ease of electron removal since the potential for this process is lowered. Consistent with the assignment of electron transfer as being rate limiting in the chemical reactions with ClO_2 or with alkaline ferricyanide are the low ^{D}k's (the ratio of the rate constant measured with α-^{1}H amines divided by the rate constant measured with α-^{2}H amines). All oxidative reactions of amines that follow a SET mechanism exhibit reaction rates in the order of tertiary>secondary>primary. This behavior is a consequence of the greater ease in $1e^-$ oxidation of alkylated amines due to the lowering of oxidation reduction potentials with increasing levels of alkylation (~0.5 V lower potential for tertiary than for primary amines).[48]

Table 1. Characteristics of Model Amine Oxidation Reactions[a]

Oxidant	Amine	Mechanism	^{D}k[e]	ρ[f]
ClO_2	*N*-*t*-But-$BzNH_2$[b]	SET	1.8	-0.92
ClO_2	*N*-*t*-But-$BzNH_2$[b]	$H^{\cdot}$	5.0	----
$Fe(CN)_6$	*N*,*N*-diMe$BzNH_2$[c]	SET	1.0	-0.99
Electrode	*N*,*N*-diMe$BzNH_2$[c]	SET	1.0	-0.94
$KMnO_4$	$BzNH_2$[d]	$H^{\cdot}$ (?)	7.0	-0.28

[a]taken from ref. 49.

[b]*N*-*t*-But-$BzNH_2$, *N*-t*ert*-butylbenzylamine.

[c]*N*,*N*-diMe$BzNH_2$, *N*,*N*-dimethylbenzylamine.

[d]$BzNH_2$, benzylamine.

[e]^{D}k is the ratio of the measured rate constant for α-^{1}H amine oxidation divided by the measured rate constant for α-^{2}H amine oxidation.

[f]ρ is the slope measured from a plot of the logarithm of the observed rate of oxidation of para- and meta-ring substituted amines versus σ of the substituent.

In contrast with the properties of chemical SET oxidations of amines, $H^{\cdot}$ abstraction mechanisms may exhibit more pronounced ^{D}k's and, at least in the case of $KMnO_4$ oxidation (Table 1), exhibit less of an influence of electronic effects on rate (ρ = -0.28). Any observed influence of substituent on rate could reflect an electronic effect on benzyl C-H bond dissociation energies as observed in model $H^{\cdot}$ abstractions from substituted toluenes.[50] It should also be noted that no systematic influence of the level of amine alkylation on rate of $H^{\cdot}$ abstraction has been observed in contrast with the reactivity observed in SET oxidations. Whereas the observation of the absence or presence of large ^{D}k's has been used to distinguish the two mechanisms of amine oxidation,

particularly in biological systems,[51] recent studies have shown that the deprotonation of the α-hydrogen in aminium cation radicals can exhibit large ^{D}k's[52-54] and model H˙ abstraction reactions from amines have been observed to exhibit rather small ^{D}k's.[55] These considerations result in the conclusion that no single experimental approach (such as measuring the deuterium isotope effect) can distinguish between an SET and a H˙ abstraction mechanism.

The studies of chemical oxidation of amines show that electron-donating substituents accelerate the initial one-electron oxidation of amines when *N*,*N*-dimethylbenzylamines with *para* or *meta* substituents are examined since ρ~ -1. This situation would hold when one-electron oxidation of the amine is rate limiting in the overall sequence of steps, which is also supported by the low ^{D}k observed.

To probe the mechanism of MAO-B catalysis, Walker and Edmondson[47] investigated the influence of electron withdrawing or donating substituents on the kinetic parameters of the MAO-B catalyzed oxidation of a series of *para* and *meta* substituted benzylamine analogs. Both α,α-diprotio and α,α-dideuteriobenzylamine analogs were compared in steady-state as well as stopped-flow kinetic experiments in order to determine the extent to which carbon-hydrogen bond breakage is rate limiting during catalysis.

The results from this study showed that the binding of *para*-substituted benzylamine analogs to MAO-B increases in affinity with increasing hydrophobicity of the substituent, while the binding affinity of *meta*-substituted analogs decreases with increasing molecular volume of the substituent. The limiting rate for flavin reduction is invariant with the nature of the *meta* substituent, whereas the rate of MAO-B reduction is decreased with increasing

values of the E_S (steric influence) parameter of the *para* substituent. No evidence was found for any electronic contribution to the rate of MAO-B flavin reduction by any of the analogs tested (*meta* or *para*). All the analogs tested exhibited $^{D}k_{cat}$ values ranging from 5.5 to 8.9. No spectral evidence was found for any flavin radical intermediates during the time course of MAO-B flavin reduction in anaerobic reductive half-reduction stopped-flow experiments.

They made several conclusions from these data. Both *para*- and *meta*-substituted benzylamines showed large deuterium kinetic isotope effects on k_{cat} and the magnitude of ^{D}k is independent of the nature of the substituent. Model system studies of aminium cation radical deprotonation[43,53,54] have also displayed reasonably large deuterium isotope effects with the magnitude dependent on the nature of the abstracting base and on the nature of the *para* substituent. Thus, the observation of large ^{D}k values with MAO-B does not disagree with the proposed mechanism in Scheme 4 but should also not be construed as support since other model amine oxidation reactions (e.g., the permanganate oxidation of benzylamine)[56] also exhibit large deuterium kinetic isotope effects.

Model studies of amine oxidations have shown that C-H bond cleavage (either $H^{\cdot}$ abstraction or H^{+} abstraction) is facilitated when overlap of the C-H bond with the amine nitrogen lone pair orbital occurs.[55,57] The influence of steric effects on rate with the *para*- but not with the *meta*-substituted analogs suggests the pro-*R* α-CH of the substrate is optimally oriented with the amine nitrogen lone pair orbital in the case of *meta*- but not with *para*-substitution. These data demonstrate the steric orientation of the substrate to be important in

the rate of amine oxidation by MAO-B and that *meta* ring substituents favor this orientation.

The absence of a significant electronic influence on the rate of C-H bond cleavage is suggestive of little charge build up in the transition state, which would appear to argue against C-H bond cleavage occurring by H^+ or H^- abstraction. It should be noted that Hartmann and Klinman[58] observed a ρ = 1.47 for the C-H bond cleavage step in the plasma amine oxidase reaction in which good evidence exists for a H^+ abstraction in this quinoprotein mechanism. It could be reasonably argued that if C-H bond cleavage occurred early in the transition state in MAO-B catalysis, then one might expect to observe little or no electronic influence on the rate. Recent kinetic isotope effect data, however, provide evidence that the C-H bond cleavage occurs late in the transition state in MAO-B catalysis.[59] If C-H bond cleavage were to occur early in the transition state, then the secondary isotope effect observed should be close to unity, whereas in the case of a late transition state, the value for the secondary isotope effect should approach the equilibrium value for an sp^3 to sp^2 rehybridization.[60] Jonsson *et al.*[59] measured the primary and secondary isotope effects for the oxidation of *para*-methoxybenzylamine by MAO-B by competition experiments. The primary k_H/k_T = 22, and the secondary k_H/k_T = 1.33. The value for the secondary isotope effect is close to the equilibrium value which argues that C-H bond cleavage occurs late in the transition state. Thus, if MAO-B catalysis were to operate by an aminium cation radical mechanism as depicted in Figure 4, the C-H bond cleavage step should exhibit a substituent electronic effect dependence reflecting the degree of buildup of electron density on the benzyl carbon on deprotonation.

A further consideration of the operative mechanism is thermodynamic permissibility. For a SET mechanism to be thermodynamically permitted, $\Delta G^{\ddagger} > \Delta G_{et}$,[52] where $\Delta G^{\ddagger}$ is the free energy required to go over the transition state of the rate-determining step in the catalytic reaction and ΔG_{et} is the free energy change associated with one-electron transfer from the amine to the flavin as calculated from the Nernst equation. From stopped flow kinetic data of *meta*-substituted benzylamine analog reduction of MAO-B (which is identical with the rate of enzyme reduction by *para*-substituted benzylamines when the rates are corrected for the E_S contribution), $\Delta G^{\ddagger}$ is calculated (using the Eyering equation) to be ~12 kcal/mol. If the oxidation reduction potential for primary amine oxidation is ~1.5 V versus the standard hydrogen electrode[61] and the one-electron couple for the MAO-B flavin is 0.045 V[62], then ΔG_{et} is calculated to be in the range of 20-30 kcal/mol given any uncertainties in potential on substrate binding. From these considerations, the enzymatic oxidation of the amine by a SET mechanism where flavin is the oxidant is considered unlikely.

An alternative possibility to a SET mechanism for amine oxidation by MAO is a $H^{\cdot}$ abstraction mechanism (Scheme 10). If MAO-B catalysis were to proceed by a radical mechanism, the evidence above would support a rate-limiting $H^{\cdot}$ abstraction from the α-carbon to generate **17** followed by rapid electron transfer from the aminyl substrate radical to form the flavin hydroquinone and the protonated imine product (**18**). The lack of any observable substituent electronic effect on the rate of MAO-B reduction by the benzylamine analogs together with the failure to observe any flavin radical intermediates on reduction are supportive of a $H^{\cdot}$ abstraction mechanism. An argument against this mechanism is the lack of any known groups on the

enzyme sufficiently reactive to abstract a $H^{\cdot}$ from the benzyl carbon. Flavins have been shown to abstract $H^{\cdot}$ only in their photoexcited triplet states and no direct evidence is available to suspect that ground state flavin would exhibit this reactivity. Protein-bound amino acid radicals could function as the $H^{\cdot}$ abstracting agent; however, to date there has been no definitive demonstration of the presence of an amino acid radical in MAO-B but only suggestive evidence.[63]

Scheme 10. Proposed Hydrogen Atom Abstraction Mechanism for MAO Catalysis