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# The Pseudoirreversible Inhibition of Monoamine Oxidase by Allylamine

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SUMMARY

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Allylamine was shown to inhibit flavin-linked monoamine oxidase in a pseudoirreversible manner. The enzyme was irreversibly inhibited with incorporation of radioactivity from [$^{14}$C]allylamine. Inhibition and incorporation of radioactivity were time-dependent, and the radioactivity and inhibition persisted during dialysis and gel filtration. Enzymatic turnover of allylamine preceded the inactivation step. Reaction of the allylamine-inactivated enzyme with benzylamine resulted in the formation of 3-benzylaminopropionaldehyde. Simultaneously with inactivation, the ultraviolet spectrum of the holoenzyme changed to one indicative of the reduced form of the coenzyme. In the presence of a substrate, benzylamine, the ultraviolet spectrum of the enzyme reverted to normal, the radioactivity was removed, and activity was restored. A mechanism for the inactivation and reactivation by benzylamine is presented.

## INTRODUCTION

The enzyme-catalyzed conversion of a substrate to a highly reactive product can lead to irreversible inactivation of the enzyme. This principle is recognized as important in the design of specific enzyme inhibitors (1). The irreversible inhibition of flavin-linked monoamine oxidase by $\beta,\gamma$-acetylenic amines served as one of the early examples of this mode of inhibition (2). The enzymatically activated amines react with the reduced form of the flavin cofactor, resulting in irreversible inhibition of the enzyme (3). We report here that $\beta,\gamma$-olefinic amines are also potent inhibitors of monoamine oxidase, but that the mode of inhibition differs from that observed with the acetylenic amines. Specifically, we show that allylamine is a pseudoirreversible inhibitor of flavin-linked monoamine oxidase. By pseudoirreversible inhibition we mean that the enzyme can apparently be irreversibly inhibited by allylamine (i.e., there is a time course for inactivation, radioactive allylamine is incorporated into the enzyme, and both the inhibition and radioactivity are stable to gel filtration and dialysis) but is reactivated under specific circumstances. In this particular instance the enzyme was reactivated by incubation with benzylamine, a substrate.

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## METHODS

Rat liver mitochondria were prepared by a published procedure (4). The mitochondria were disrupted, and the monoamine oxidase activity was assayed with





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benzylamine as substrate (5). One unit of activity is defined as the amount of enzyme required to cause a change in absorbance of 0.001 unit/min at 25° in potassium phosphate buffer, pH 7.5 (5). Allylamine, allyl alcohol, acrolein, and amino-3-butene were products of Aldrich Chemical Company. All chemicals were distilled before use.

[1-$^3$H]Allylamine-labeled enzyme was first precipitated with 10 ml of cold trichloracetic acid. The precipitates were then transferred, using 0.25 ml of $H_2O$, to a glass counting vial containing 2.0 ml of Protosol (New England Nuclear), and the samples were digested at 50° until clear. Then 10 ml of toluene containing 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter were added, and the samples were counted. At zero time 164 cpm were incorporated into the enzyme. The enzyme was solubilized and purified by a published method (6).

The kinetics of inactivation was determined on a Gilford model 240 spectrophotometer, and ultraviolet spectra were recorded on a Cary 118 spectrophotometer. Radioactivity was measured with Beckman LSC-330 liquid scintillation counter. Protein concentrations were determined by the method of Lowry *et al.* (7).

### *Syntheses*

*[1-$^{14}$C]Allylamine.* One millimole (121 mg) of [1-$^{14}$C]allyl bromide (ICN) (0.55 mCi) was dissolved in 5 ml of dry chloroform. Then 1.2 mmoles (170 mg) of hexamine were added, and the solution was stirred at room temperature overnight (8). The cloudy solution was evaporated to dryness and taken up in 5 ml of saturated ethanolic HCl. This solution was stirred overnight, filtered, and evaporated to dryness. The hydrochloride of [1-$^{14}$C]allyl amine was recrystallized from ethanol–ether. A 78% yield of [1-$^{14}$C]allylamine hydrochloride was obtained after crystallization. The specific activity of a dry sample was 0.55 mCi/mmole.

*[1-$^3$H]Allylamine.* Acrolein (460 mg, 10 mmoles) was dissolved in 3 ml of cold methanol. Then 5 mmoles of $NaB^3H_4$ (New England Nuclear) (20 mCi) were slowly added at 5° with stirring. The solution was stirred at 5° for 30 min and then at room temperature for 2 hr. The methanol was removed by fractional distillation. Further distillation afforded 350 mg (7.3 mmoles) of [1-$^3$H]allyl alcohol. This material was taken up in 5 ml of cold ether, and 10 $\mu$l of dry pyridine were added, followed by 660 mg of phosphorus tribromide in 1 ml of ether. The solution was stirred for 2 hr at 5° and then for 4 hr at room temperature. Water (2 ml) was carefully stirred in, and the aqueous extract was discarded. The extraction process was repeated, and the ethereal layer was dried over $MgSO_4$ and decanted. The ether was removed under a mild vacuum, affording 508 mg (4.2 mmoles) of [1-$^3$H]allyl bromide. This material was converted to [1-$^3$H]allylamine hydrochloride with a specific activity of 0.8 mCi/mmole by the procedure used in the synthesis of [1-$^{14}$C]allylamine hydrochloride.

*[1-$^2H_2$]Allylamine.* [1-$^2H_2$]Allyl alcohol was prepared by a published procedure (9) and converted to [1-$^2H_2$]allylamine by the procedures described above. The final product contained 70% deuterium in position 1 as judged by NMR analysis.

## RESULTS

*Inactivation of mitochondrial bound monoamine oxidase by allylamine.* When rat liver monoamine oxidase was incubated at 25° with allylamine, the enzyme appeared to suffer rapid, irreversible inactivation (Fig. 1). In these experiments the enzyme was incubated with pure allylamine, and aliquots were removed at the indicated times and assayed with benzylamine as substrate (5). The activity of the inactivated enzyme could not be recovered by continued dialysis, which suggests that the mode of inhibition is irreversible. The solubilized, purified enzyme was completely inhibited when incubated with allylamine, as opposed to the approximately 80% inhibition found with the membrane-bound enzyme. Furthermore, when [1-$^3$H]allylamine was used as the inhibitor, labeling of the enzyme occurred at the same rate as inhibition (Fig. 2). These experiments were performed by incubat-

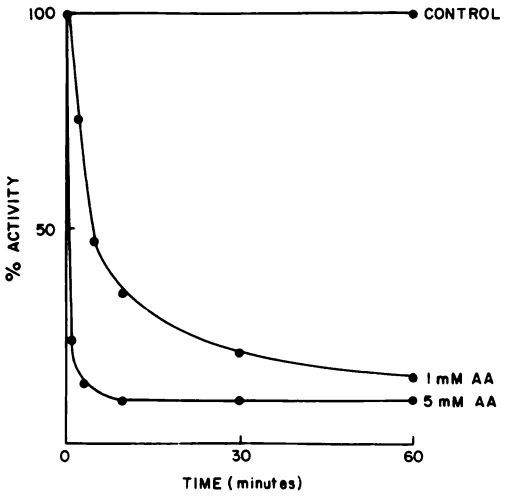



FIG. 1. *Inactivation of monoamine oxidase by allylamine*

Seven units of the enzyme were incubated in 0.2 M potassium phosphate, pH 7.5, with either 5 or 1 mM freshly distilled allylamine (AA). A control with the same amount of enzyme but containing 1 mM benzylamine was also included. The activity remaining after various time intervals was measured by diluting the enzyme from the incubation tubes 50-fold in 3.3 mM benzylamine in the phosphate buffer and measuring the increase in absorbance at 250 nm (benzaldehyde $\lambda_{max}$ = 250 nm). The graph shows the activity of allylamine-treated samples as a percentage of that of the uninhibited control. The concentration of allylamine remaining after dilution of the enzyme was not high enough to cause observable competitive inhibition. The activity of the enzyme could not be restored by dialysis overnight against the 0.2 M potassium phosphate buffer, pH 7.5. The uninhibited control enzyme lost about 20% of its activity as a consequence of the dialysis procedure.

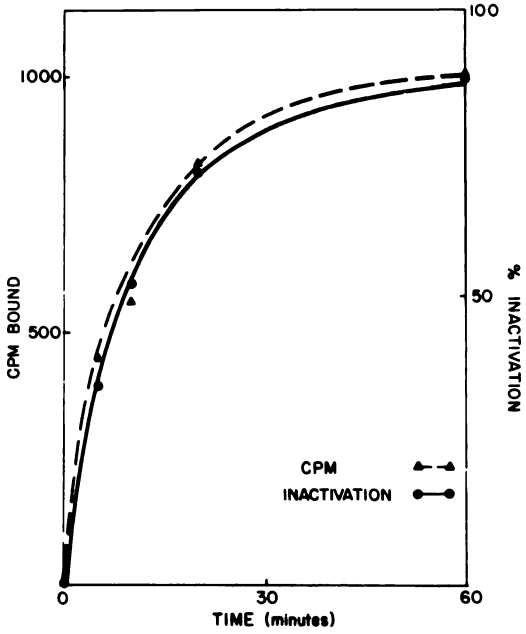



FIG. 2. *Inactivation of monoamine oxidase by [1-$^3$H]allylamine*

Enzyme (2.7 ml containing a total of 370 units) was incubated at 25° in the presence of 1.0 mM mercaptoethanol with 0.7 mM [1-$^3$H]allylamine (specific activity, 0.8 mCi/mmole). At the indicated times, 40 μl (5.4 units) of enzyme were removed and its activity was determined by the standard benzylamine assay. At the same times 0.5-ml aliquots were also removed and counted by the procedure described METHODS. At zero time 164 cpm were incorporated into the enzyme.

ing the enzyme with radioactive allylamine and removing samples at the indicated times to assay both enzymatic activity and radioactivity incorporation. The latter was determined after precipitation of the enzyme with trichloracetic acid. At this point, of course, the enzyme was completely denatured. The finding that radioactivity was retained suggests that the irreversible inhibition observed was a consequence of some sort of covalent interaction between allylamine and the enzyme (or cofactor). Neither radioactivity incorporation nor the inactivation rate was affected by the inclusion of 1 mM mercaptoethanol in the medium. We have also found that *N*-benzylallylamine is an irreversible inhibitor of the enzyme. This is to be expected, since a host of *N*-benzylpropargylamines (i.e., pargyline) are also powerful irreversible inhibitors of monoamine oxidase (10). This enzyme has a binding site for an aromatic grouping, as evidenced by its selectivity of action for aromatic amines.

*Protection against inactivation by the substrate benzylamine.* Prior incubation of the enzyme with the substrate benzylamine competitively protected it against inactivation by allylamine (Fig. 3). This result is consistent with the notion that allylamine is an active site-directed inhibitor of the enzyme.

*Deuterium isotope effect on rate of inhibition.* [1-$^2H_2$]Allylamine was synthesized

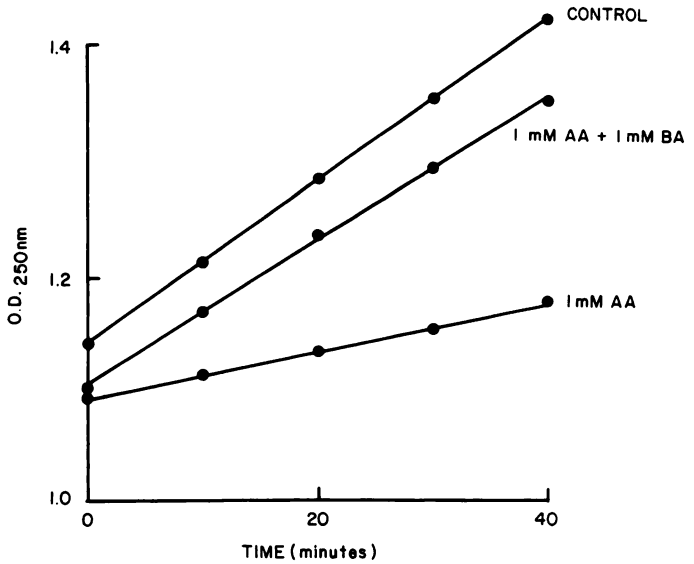



FIG. 3. *Allylamine inactivation of monoamine oxidase in the presence of benzylamine*

Seven units of the enzyme were placed in each of three test tubes containing 1 ml of the standard phosphate buffer. To tube 1 was added 1 mM benzylamine (BA); to tube 2, 1 mM benzylamine and 1 mM allylamine (AA); and to tube 3, 1 mM allylamine. After 10 min at room temperature, the activity of 50-$\mu$l samples was assayed with 3 mM benzylamine. The figure shows the activity of each of the three samples after the 10-min preliminary incubation.

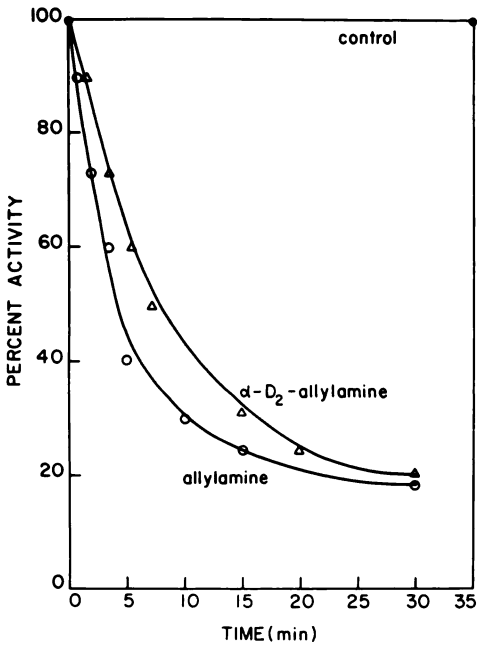



FIG. 4. *Inactivation of monoamine oxidase by [1-$^2H_2$]allylamine*

Seven units of enzyme were incubated in 0.2 M potassium phosphate buffer, pH 7.5, with either 1 mM allylamine or 1 mM [$^2H_2$]allylamine. At the indicated times, aliquots of the enzyme were removed and the activity remaining was measured by the usual technique. A deuterium isotope effect of 2.35 was calculated on the basis of there being 70% deuterium in the $\alpha$-position of the allylamine.

and tested as an inhibitor of the enzyme. A deuterium isotope effect of 2.35 on the rate of inhibition was observed (Fig. 4), suggesting that the $\alpha$-C–H bond must be partially broken in the transition state of the inactivation process. Hence enzymatic turnover of allylamine is a prerequisite for inactivation.

*Incubation of monoamine oxidase with acrolein, allyl alcohol, 1-amino-3-butene, and 2-amino-3-butene.* Incubation of the enzyme for 1 hr in 0.2 M potassium phosphate, pH 7.5, with acrolein, allyl alcohol, or 1-amino-3-butene (10 mM each) did not lead to diminution of activity, although 10 mM 2-amino-3-butene produced a 25% decrease. These results show that the mode of inactivation with allylamine is specific in nature. The following requirements for an effective inhibitor are apparent: the double bond must be $\beta,\gamma$ to a nitrogen

atom in order for inactivation to occur, and the putative inhibitor must be a good substrate for the enzyme. α-Methylamines are very poor substrates for monoamine oxidase (11). The finding that acrolein was not inhibitory suggests that the mode of inhibition does not involve simply the generation of an affinity-labeling agent by the enzyme. Acrolein is, of course, the putative product of the oxidation of allylamine by monoamine oxidase.

*Ultraviolet-visible spectra of monoamine oxidase in the presence and absence of allylamine.* The ultraviolet-visible spectrum of the solubilized, purified monoamine oxidase is shown in Fig. 5. In the presence of allylamine, the spectrum changed to that of a reduced flavin (Fig. 5). This change occurred gradually and simultaneously with the inhibition of the enzyme. Neither extended dialysis nor gel filtration (Sephadex G-15 column) of the inhibited enzyme altered the ultraviolet-visible spectrum.

*Reactivation of allylamine-inhibited monoamine oxidase by benzylamine.* When the inactivated enzyme was incubated with benzylamine, activity was slowly recovered after a lag period of 30–40 min (Fig. 6). This extended lag period did not occur with the solubilized, purified enzyme. The same reactivation phenomenon could also be demonstrated with the 1-$^3$H-labeled inactivated enzyme (Fig. 7); in this instance the radioactivity was lost from the enzyme at the same rate as activity was regained. Also, upon reactivation with benzylamine the ultraviolet spectrum of the enzyme reverted to normal. Nucleophiles such as 2-mercaptoethanol or 1,5-diaminopentane, which are not substrates for the enzyme, were incapable of reactivating it. Furthermore, *N*-benzylallylamine activated enzyme could not be

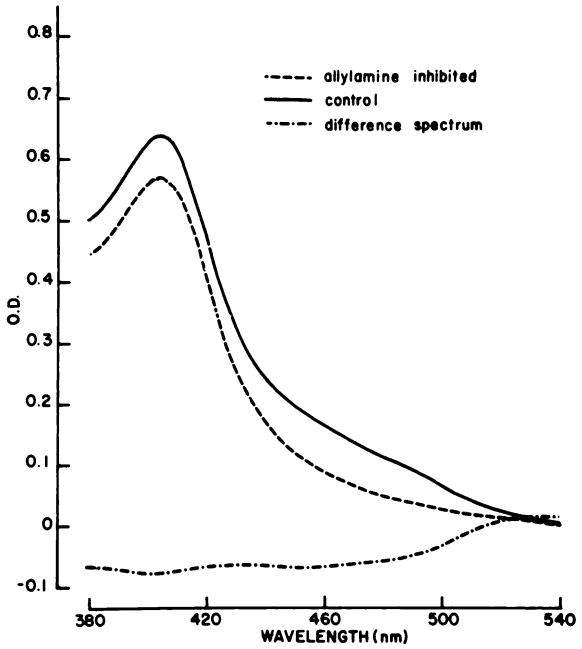



FIG. 5. *Ultraviolet-visible spectra of purified monoamine oxidase in the presence and absence of allylamine*
The purified enzyme (0.5 mg/ml; 25% pure based on flavin concentration) was dissolved in 0.2 M phosphate buffer, and its ultraviolet-visible spectrum (——) was recorded on a Cary 118 instrument against the buffer. (Allylamine (10 mM) was added to an identical enzyme preparation and the enzyme was inhibited to completion. The ultraviolet visible spectrum was again recorded.) Removal of the allylamine at this point by dialysis did not alter the spectrum. The difference spectrum is also shown (– · – · –). When the bleached, allylamine-inhibited enzyme was treated with benzylamine, its spectrum returned to that of the normal, oxidized enzyme.

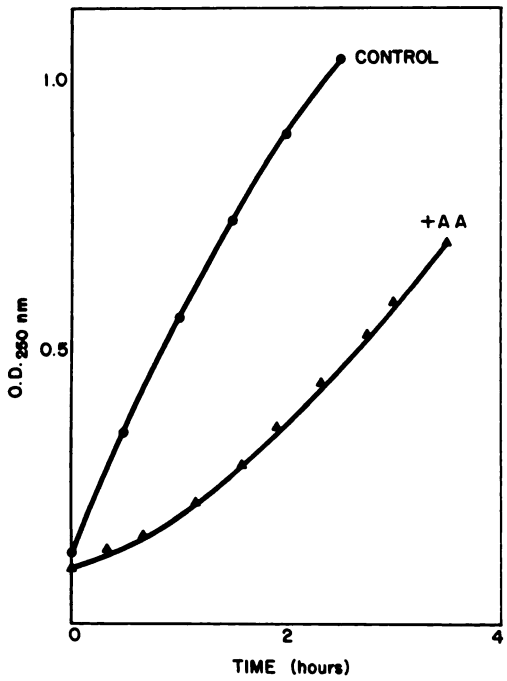


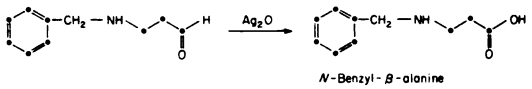

FIG. 6. *Reactivation of inhibited enzyme by substrate*

Ten units of enzyme were inactivated with 1 mM allylamine (AA) for 1 hr in 100 μl of 0.2 M potassium phosphate buffer, pH 7.5. Seven units of this enzyme were removed and added to 3.0 ml of buffer containing 3 mM benzylamine, and the rate of reactivation was followed as usual (5). The initial rate of this reaction was 20% of the control.

reactivated with benzylamine.

*Ultraviolet-visible spectrum of 3-chloroallylamine-inhibited monoamine oxidase.* In the presence of *cis*-3-chloroallylamine, a true irreversible inhibitor of the enzyme (12), a dramatic spectral change occurred (Fig. 8). This change was identical with that obtained when the enzyme is inhibited with β,γ-acetylenic amines (13). When the enzyme was inhibited by *cis*-3-chloroallylamine or by the acetylenic amines, reactivation with benzylamine did not occur.

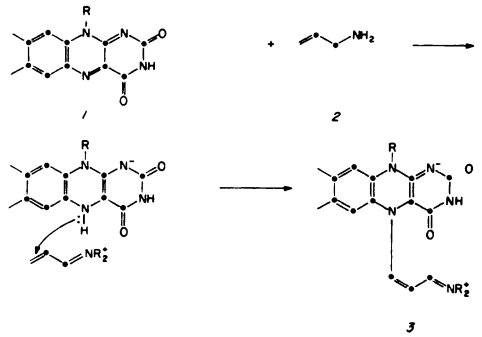
*Identification of adduct between benzylamine and enzyme-allylamine transformation product.* In these experiments we labeled the enzyme with [1-$^{14}$C]allylamine and reactivated the inhibited enzyme with benzylamine. The radioactive product of the reaction process was separated from the protein and oxidized with $Ag_2O$, an oxidant capable of oxidizing aldehyde functional groups (14). The oxidation product proved to be *N*-benzyl-β-alanine (Table 1). This means that the adduct formed




between the allylamine-inactivated enzyme and benzylamine was 3-benzylaminopropionaldehyde. We made no attempt to isolate this compound directly because it is not stable to concentration and chromatographic procedures.

## DISCUSSION

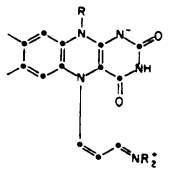
We have shown here that allylamine inhibits monoamine oxidase in a time-dependent and apparently irreversible manner. Enzymatic turnover of allylamine precedes the inhibition step. We established this by demonstrating that (a) a deuterium isotope effect of 2.35 on the rate of inhibition is manifest when [1-$^2H_2$]allylamine is the inhibitor and (6) a change in the ultraviolet-visible spectrum of the flavin cofactor occurs concomitant with inactivation. The spectrum of the inactivated enzyme is very similar to that of the reduced flavin (15). After enzymatic turnover of the allylamine occurs, a chemical reaction must ensue between the activated substrate and the holoenzyme. We propose that the inhibition process can be accounted for as shown in Scheme 1. Once



SCHEME 1

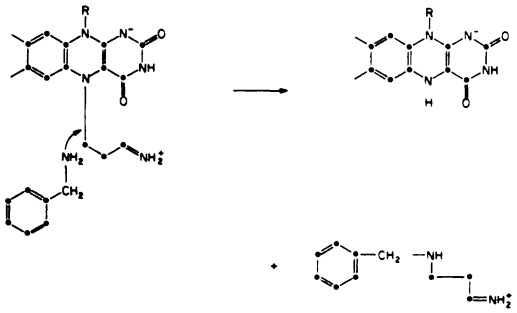
compound *3* is formed, the enzyme is rendered inactive, since the flavin cofactor is "trapped" in the reduced form. This mechanism is in accord with the observations reported here and is very similar to the mechanism proposed by Abeles and co-

workers (3) for the mechanism of the β,γ-acetylenic amine-induced irreversible inhibition of the enzyme. The end product is *4* in this case (3).



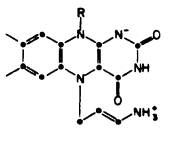
*4*

The reactivation of the allylamine-inhibited enzyme with benzylamine is the most novel feature of the inactivation process reported here. To study this phenomenon we reactivated the [1-$^{14}$C]allylamine-labeled enzyme with benzylamine. After oxidation with $Ag_2O$, we isolated *N*-benzyl-β-alanine. $Ag_2O$ is known to oxidize aldehydic groups to acids efficiently (14). The isolation of *N*-benzyl-β-alanine suggests that the precursor in the $Ag_2O$ oxidation is 3-benzylaminopropionaldehyde. This compound must be the product of the reaction between enzyme-bound benzylamine and *3*. A possible mechanism for this reaction is shown in Scheme 2.



SCHEME 2

We have no way of ruling out the possibility that a tautomeric form of *3*, such as *5*, is actually involved in the reaction with benzylamine. In any case, the benzylamine is envisaged to bind at the aromatic



*5*

binding site of the enzyme and engaged in a nucleophilic attack at the terminus of the 3-carbon inhibitor fragment, with the

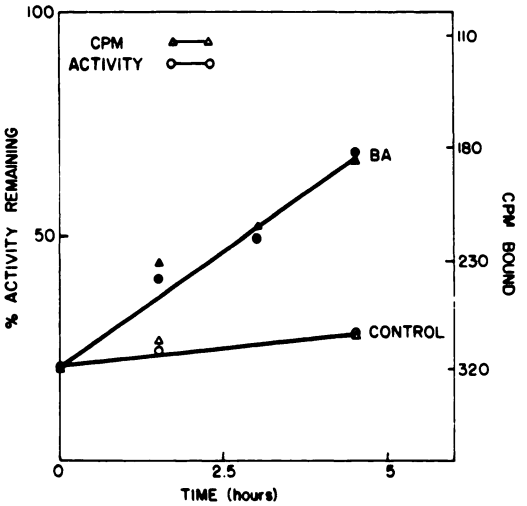
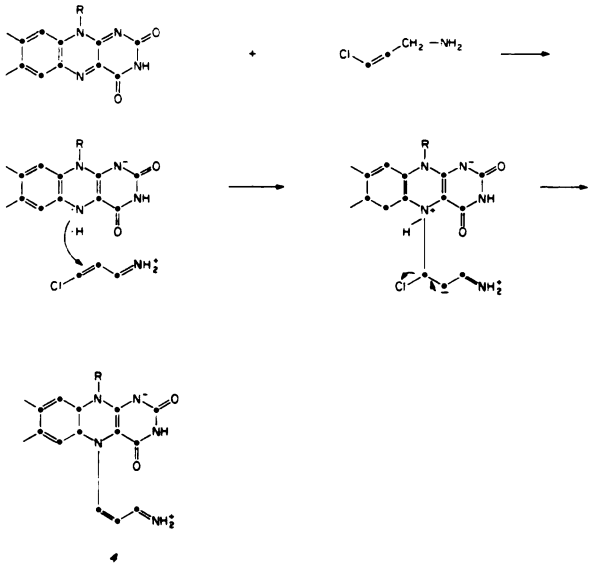



FIG. 7. *Reactivation of [1-$^3$H]allylamine-labeled enzyme by benzylamine*

Enzyme (10 ml, 56 units/ml) in 0.2 M potassium phosphate buffer, pH 7.4, was labeled with [1-$^3$H]allylamine at a final concentration of 0.5 mM. The enzyme was then exhaustively dialyzed to remove the unbound counts. A 3.5-ml sample of the enzyme was diluted 1:5 with the phosphate buffer, and a second aliquot was diluted 1:5 with the phosphate buffer containing 50 mM benzylamine (BA). Both samples were dialyzed against the phosphate buffer at room temperature. At the times indicated, 0.375 ml of each sample was removed and assayed for activity, and at the same time 5 ml of each were removed and counted by the usual technique.

subsequent release of this fragment from the flavin cofactor. The finding that *N*-benzylallylamine is a true irreversible inhibitor of the enzyme is consistent with this mechanism, since the aromatic binding site would already be occupied. Whether the activation process is enzymatically assisted or not was not determined. The observation that nonsubstrate nucleophiles were ineffective reactivating agents compared with benzylamine suggests that some enzymatic assistance may have been involved.

As mentioned under RESULTS, *cis*-3-chloroallylamine is a true irreversible inhibitor of the enzyme. The ultraviolet spectral change that accompanied this inhibition was different from that observed with allylamine. In fact, it is identical with that observed with β,γ-acetylenic amines. Adduct *4* can be arrived at with this compound as shown in Scheme 3.



SCHEME 3

TABLE 1

*Identification of adduct between benzylamine and enzyme-allylamine transformation product*

Enzyme (2.0 ml containing 250 units of activity) was incubated for 1 hr at 25° in the presence of 1.0 mM mercaptoethanol with 1.0 mM [1-$^{14}$C]allylamine (specific activity, 0.55 mCi/mmole). At the end of this incubation period the preparation was centrifuged at 17,000 × *g* for 20 min. The supernatant was discarded, and the pellet was taken up in 10 ml of phosphate buffer and centrifuged again. This process was repeated four times, after which background counts were found in the wash. The pellet was resuspended in 1 ml of phosphate buffer, and 0.1 ml of this suspension was taken up in Protosol and counted as the tritiated samples were. A total of 1062 cpm was then treated with 3 μmoles of benzylamine. The remaining 0.9 ml, containing 9557 cpm, were then treated with 3 μmoles of benzylamine. After 3 hr at 37°, a maximum of 78% of the original activity was regained. This sample was then centrifuged at 12,000 × *g*, and the supernatant (0.8 ml) was collected. The supernatant contained a total of 6212 cpm. Sodium hydroxide was added to a final concentration of 3.8%; then 4 μmoles of $Ag_2O$ were added, and the suspension was stirred for 2 hr at room temperature. The silver and unreacted $Ag_2O$ were removed by filtration. The filtrate was lyophilized for 2 days. The precipitate was taken up in 0.2 ml of $H_2O$, and 10 mg of *N*-benzyl-β-alanine were added as a carrier. Aliquots of the resulting material were then subjected to several chromatographic systems. The high-pressure liquid chromatographic studies were run on a Waters Associates analytical instrument. The *N*-benzylalanine spot or peak was scraped off (thin-layer chromatography), cut out (paper electrophoresis), or collected (high-pressure liquid chromatography) and counted in Aquasol (New England Nuclear).

| Support | Eluent | Radioactivity | |
|---|---|---|---|
| | | Applied | Co-chromatographing with *N*-benzyl-β-alanine |
| | | *cpm* | *cpm* |
| Silica gel thin-layer chromatography | Butanol-water-acetic acid (2:2:1) | 500 | 350 |
| Paper electrophoresis | Formic acid-acetic acid, pH 1.8 | 500 | 400 |
| | | 500 | 375 |
| $C_{18}$ Bondapak column high-pressure liquid chromatogrpahy | 50% methanol in water | 500[a] | 300 |
| | | 500[b] | 320 |

[a] In tetrabutylammonium phosphate.

[b] In heptanesulfonic acid.

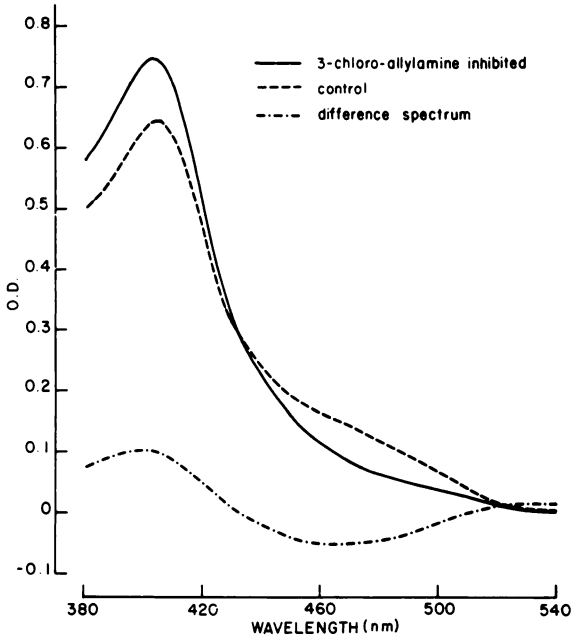



FIG. 8. *Visible spectra of purified monoamine oxidase in the presence of cis-3-chloroallylamine*

The purified enzyme (0.5 mg/ml) was treated with *cis*-3-chloroallylamine, and its spectrum was recorded (——). Benzylamine had no effect on the visible spectrum or activity of the enzyme. The spectrum of the control enzyme (- - -) is also shown.

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