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# PHENYLHYDRAZINE: SELECTIVE INHIBITION OF HUMAN BRAIN TYPE B MONOAMINE OXIDASE

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**Abstract**—Phenylhydrazine ($PhNNH_2$) was found to irreversibly inhibit both human brain type A and type B monoamine oxidase (MAO) *in vitro*. $PhNNH_2$ was a more effective inhibitor of the B form of the oxidase in that 50 per cent inhibition of phenylethylamine (PEA) deamination occurred at approximately 4 $\mu$M, whereas the equivalent inhibition of 5-hydroxytryptamine (5-HT) deamination required almost 200 $\mu$M of the inhibitor. Similarly, inhibition of PEA deamination occurred at a faster rate than that of 5-HT metabolism. Evidence is presented that suggests that $PhNNH_2$ inhibits both forms of MAO by two distinct mechanisms, a temperature-sensitive and a temperature-insensitive process. The temperature-insensitive component proceeded extremely rapidly, compared to the temperature-dependent component. For example, at 4° inhibition of PEA deamination by $PhNNH_2$ attained its maximum within a 30-sec preincubation period. Both inhibition processes were diminished when $PhNNH_2$ was preincubated in a nitrogen atmosphere compared to that in air. Fifty per cent inhibition of PEA and 5-HT deamination by the $PhNNH_2$ temperature-insensitive component occurred at 20 $\mu$M and 1 mM respectively. The presence of two distinct inhibition processes is consistent with prior studies that have demonstrated that more than 1 mole of $PhNNH_2$ is bound per mole of MAO.

Numerous papers over the past decade and a half have demonstrated that there are at least two functionally distinct forms of monoamine oxidase (MAO) that exist in mitochondria both *in vitro* and *in vivo* [1–5]. Although all biogenic monoamines are substrates for both forms of the oxidase, it is generally accepted that the A form of MAO displays greater affinity for the amine neurotransmitters, norepinephrine and 5-hydroxytryptamine (5-HT), whereas the B form of MAO binds more selectively to the naturally occurring amine, phenylethylamine (PEA). The neurotransmitter, dopamine, and the sympathomimetic amine, tyramine, are deaminated *in vitro* by both the A and B forms of the oxidase [6, 7].

Just as there are selective substrates for MAO, it also has been shown that there are selective inhibitors of the two enzyme species. In general, the inhibitors of the A form of MAO are considerably more selective than the inhibitors of the B isoenzyme. For example, the selective A MAO inhibitors clorgyline and Lilly 51641 are approximately 1000 times more effective at inhibiting the A form than the B form of the oxidase [8, 9]. In contrast, the best selective type B MAO inhibitor available, Deprenyl, is only, at best, 100 times more potent an inhibitor of the B oxidase [10].

Since highly selective type B MAO inhibitors do not exist, we thought it would be interesting to examine whether the hydrazine analogs of the selective B MAO substrates, PEA and benzylamine, would act as selective inhibitors of the type B enzyme. In a previous paper [11] from this laboratory it was reported that the structural hydrazine analog of PEA, benzylhydrazine, was indeed a selective inhibitor of human and rat brain type B MAO. Prior studies have demonstrated that phenylhydrazine ($PhNNH_2$)*, a structural derivative of benzylamine, inhibits partially purified beef liver and kidney MAO [12, 13]. In this paper we report on the ability of this hydrazine to selectively inhibit human brain mitochondrial MAO.

## METHODS

Frontal lobes of human brain were obtained at autopsy usually within 8–12 hr after death. Mitochondria were isolated by differential centrifugation, and types A and B MAO were assayed using the weak cation exchange resin, Bio-Rex 70 as described previously [10]. [$^{14}$C]-5-Hydroxytryptamine and [$^{14}$C]-phenylethylamine were employed as types A and B MAO substrates at their respective apparent $K_m$ concentrations in air [14, 15]. Blank values were obtained from reactions containing $10^{-3}$ M pargyline.

To measure the extent of inhibition of both forms of MAO by $PhNNH_2$, reactions containing human brain mitochondria in 0.05 M potassium phosphate buffer, pH 7.4, were preincubated with various amounts of $PhNNH_2$ for an appropriate length of time, as indicated, at either 37° or 4°. Reaction mixtures were immediately centrifuged in the cold at 15,000 *g* to precipitate the mitochondria. The mitochondrial pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, and recentrifuged as before. This washing procedure, which

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† The term $PhNNH_2$ used throughout the text is synonymous with the formula $PhNHNH_2$.

removed unbound $PhNNH_2$, was normally repeated twice, though in several instances it was repeated three times without any altered effects. The resulting pellet was resuspended in phosphate buffer and assayed as described above for A and B MAO activities. Two types of blanks were employed. Both blank reactions were preincubated in the absence of $PhNNH_2$ but to one $PhNNH_2$ was added at the end of the preincubation just prior to the washing procedure described above. This latter blank reaction will be referred to in the text as the "O time" blank. Activity is expressed as nmoles of product formed per mg of protein. Protein concentrations were measured by the method of Lowry *et al.* [16].

To determine the oxygen requirement of $PhNNH_2$ inhibition of MAO, reactions were carried out as described above except that the total assay volume was 2 ml. Flasks containing the reaction mixtures were sealed with rubber septa and flushed for 3 min with nitrogen prior to the addition of $PhNNH_2$. After a 10-min preincubation period, $^{14}C$-labeled substrate was added and the rubber septum was removed from each flask. Blanks containing no $PhNNH_2$ were similarly preincubated for 10 min in an atmosphere of air or nitrogen.

Radioactively labeled [$^{14}C$]-5-hydroxytryptamine (58.0 mCi/mmole) and phenylethylamine (48.25 mCi/mmole) were obtained from the New England Nuclear Corp. Boston, MA; phenylhydrazine–HCl, 5-HT, and PEA from the Sigma Chemical Co., St. Louis, MO; and pargyline from Abbott Laboratories, Chicago, IL. Bio-Rex 70 (100–200 mesh) was obtained from Bio-Rad Laboratories, Richmond, CA. All chemicals used were the purest available from commercial sources. [$^{14}C$]-5-HT was purified by cation-exchange chromatography (Bio-Rex 70) prior to use.

## RESULTS

Our initial studies demonstrated that $PhNNH_2$ interfered with the 5-HT assay. In this regard, it was consistently found that the blank values when $PhNNH_2$ was used as inhibitor were lower than blank values that contained $10^{-3}$ M pargyline or clorgyline. Therefore, it was necessary to first remove any excess unbound phenylhydrazine to measure type A MAO activity. Results of experiments performed in this manner, as illustrated in Fig. 1, indicate that $PhNNH_2$ was indeed a selective inhibitor of the B form of human brain MAO. Fifty per cent inhibition of PEA deamination was achieved at a $PhNNH_2$ concentration of approximately $4 \times 10^{-6}$ M, whereas 50 per cent inhibition of 5-HT deamination occurred at a concentration approximately fifty times greater (around $2 \times 10^{-4}$ M).

The effect of various preincubation times on $PhNNH_2$ inhibition of PEA and 5-HT deamination is shown in Fig. 2. Again, the data indicate that $PhNNH_2$ was a more selective inhibitor of the B form of human brain MAO. Fifty per cent inhibition of PEA deamination was observed within a preincubation period of approximately 5 min whereas it took almost 20 min to inhibit 5-HT metabolism to a similar extent. The data in this figure were obtained using a blank value derived from the "O-time" reaction that contained $10^{-4}$ M $PhNNH_2$. When the same experiment was performed, but the blank values were obtained from reactions containing no $PhNNH_2$, considerably different results were

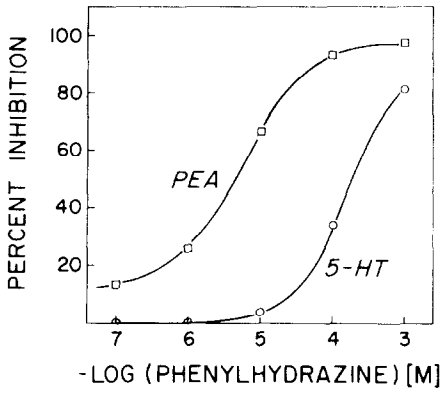


Fig. 1. Effect of various phenylhydrazine ($PhNNH_2$) concentrations on deamination of phenylethylamine (PEA) and 5-hydroxytryptamine (5-HT) at 37°. $PhNNH_2$, at the concentration indicated, was preincubated for 10 min with human brain mitochondria (0.03 to 0.1 mg protein) at 37°. Reactions were centrifuged at 4° and the precipitate was resuspended and recentrifuged two times to remove unbound $PhNNH_2$. Mitochondrial pellets were resuspended in buffer and incubated in the presence of 2 μM PEA or 0.1 mM 5-HT for 10 or 60 min, respectively, at 37°. Blanks did not contain any $PhNNH_2$. Values presented are the average of two experiments in which each reaction was performed in duplicate. The average number of nmoles of product formed per mg of protein from deamination of PEA and 5-HT in the absence of $PhNNH_2$ was 4.10 and 21.6 respectively.

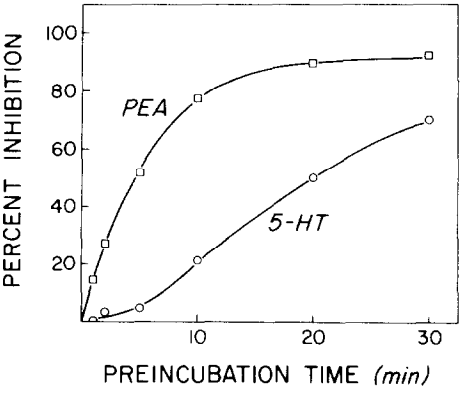


Fig. 2. Effect of preincubation times on $PhNNH_2$ inhibition of PEA and 5-HT deamination. Phenylhydrazine ($PhNNH_2$, 0.1 mM) was preincubated with human brain mitochondria (0.04 to 0.17 mg protein) at 37° for the times indicated. The mitochondria were then washed two times to remove unbound $PhNNH_2$ and incubated in the presence of 2 μM PEA or 0.1 mM 5-HT for 10 or 60 min, respectively, at 37°. Blanks contained $PhNNH_2$, but preincubation time was 0 min. Values presented are the average of three experiments in which each reaction was performed in duplicate. The average number of nmoles of deaminated product formed per mg of protein from deamination of PEA and 5-HT in the absence of $PhNNH_2$ was 1.29 and 27.2 respectively.

Table 1. Inhibition of human brain deamination of phenylethylamine by phenylhydrazine at 37°*

| Preincubation time (min) | $PhNNH_2$ (M) | Product formed† (nmoles/mg protein) | % Inhibition ± S.E. No-$PhNNH_2$ control | % Inhibition ± S.E. 0-Time blank |
|---|---|---|---|---|
| 10 | – | 7.29 | – | – |
| 0 | $1 \times 10^{-5}$ M | 3.94 | 37.4 ± 5.2 | – |
| 10 | $1 \times 10^{-5}$ M | 1.55 | 76.5 ± 1.3 | 62.3 ± 1.1 |
| 10 | – | 4.49 | – | – |
| 0 | $5 \times 10^{-4}$ M | 0.69 | 82.8 ± 2.0 | – |
| 10 | $5 \times 10^{-4}$ M | 0.12 | 96.4 ± 0.5 | 78.6 ± 4.1 |

* Phenylhydrazine, at the concentrations indicated, was preincubated with human brain mitochondria at 37°. Reactions were centrifuged at 4° and the precipitate was resuspended and recentrifuged three times to remove unbound phenylhydrazine. The mitochondria were resuspended in buffer and assayed for MAO activity at 37°. The concentration of phenylethylamine was 2 μM. Values for per cent inhibition are the average of three separate experiments in which each reaction was performed in duplicate.

† Results of a typical experiment in which all reactions were performed in duplicate.

Table 2. Inhibition of human brain deamination of 5-hydroxytryptamine by phenylhydrazine at 37°*

| Preincubation time (min) | Phenylhydrazine | Product formed† (nmoles/mg protein) | % Inhibition ± S.E. No-$PhNNH_2$ control | % Inhibition ± S.E. 0-Time blank |
|---|---|---|---|---|
| 10 | – | 38.89 | – | – |
| 0 | + | 25.11 | 42.5 ± 5.2 | – |
| 10 | + | 12.80 | 66.0 ± 1.1 | 39.5 ± 8.0 |

* Phenylhydrazine ($5 \times 10^{-4}$ M) was preincubated with human brain mitochondria at 37°. Reactions were centrifuged at 4° and the precipitate was resuspended and recentrifuged two times to remove unbound phenylhydrazine. The mitochondria were resuspended in buffer and assayed for MAO activity at 37°. The concentration of 5-hydroxytryptamine was 0.1 mM. Values for per cent inhibition are the average of three separate experiments in which each reaction was performed in duplicate.

† Results of a typical experiment in which all reactions were performed in duplicate.

Table 3. Inhibition of human brain deamination of phenylethylamine by phenylhydrazine at 4°*

| Preincubation time (min) | Phenylhydrazine | Product formed† (nmoles/mg protein) | % Inhibition ± S.E. No-$PhNNH_2$ control | % Inhibition ± S.E. 0-Time blank |
|---|---|---|---|---|
| 10 | – | 5.05 | – | – |
| 0 | + | 1.03 | 79.7 ± 0.3 | – |
| 10 | + | 1.00 | 80.1 ± 0.1 | 1.9 ± 1.0 |

* Phenylhydrazine ($5 \times 10^{-4}$ M) was preincubated with human brain mitochondria at 4°. Reactions were centrifuged at 4° and the precipitate was resuspended and recentrifuged two times to remove unbound phenylhydrazine. The mitochondria were resuspended in buffer and assayed for MAO activity at 37°. The concentration of phenylethylamine was 2 μM. Values for per cent inhibition are the average of three separate experiments in which each reaction was performed in duplicate.

† Results of a typical experiment in which all reactions were performed in duplicate.

Table 4. Inhibition of human brain deamination of 5-hydroxytryptamine by phenylhydrazine at 4°*

| Preincubation time (min) | Phenylhydrazine | Product formed† (nmoles/mg protein) | % Inhibition ± S.E. No-$PhNNH_2$ control | % Inhibition ± S.E. 0-Time blank |
|---|---|---|---|---|
| 10 | – | 37.66 | – | – |
| 0 | + | 25.92 | 34.8 ± 7.5 | – |
| 10 | + | 28.42 | 32.4 ± 7.9 | 0.17 ± 0.2 |

* Phenylhydrazine ($5 \times 10^{-4}$ M) was preincubated with human brain mitochondria at 4°. Reactions were centrifuged at 4° and the precipitate was resuspended and recentrifuged two times to remove unbound phenylhydrazine. The mitochondria were resuspended in buffer and assayed for MAO activity at 37°. The concentration of 5-hydroxytryptamine was 0.1 mM. Values for per cent inhibition are the average of three separate experiments in which each reaction was performed in duplicate.

† Results of a typical experiment in which all reactions were performed in duplicate.

observed. In the latter case, greater inhibition was consistently seen at each time interval, and each curve in the figure was essentially shifted to the left. To clarify this discrepancy, we examined PEA and 5-HT deamination using the two different blanks, with and without $PhNNH_2$.

The data in Table 1 are the per cent inhibitions of PEA deamination determined with $10^{-5}$ and $5 \times 10^{-4}$ M $PhNNH_2$; when the "no-$PhNNH_2$" control was employed, 76 and 96 per cent inhibitions of PEA deamination were seen respectively. Comparing these values with those obtained when the "0-time" blank was employed demonstrates that the per cent inhibition decreased almost 20 per cent in both cases. In addition, the presence of $PhNNH_2$, even at 0 min of preincubation, resulted in significant inhibition of PEA deamination (37 and 83 per cent respectively).

As noted in Table 2, similar experiments with 5-HT also demonstrated that the per cent inhibition

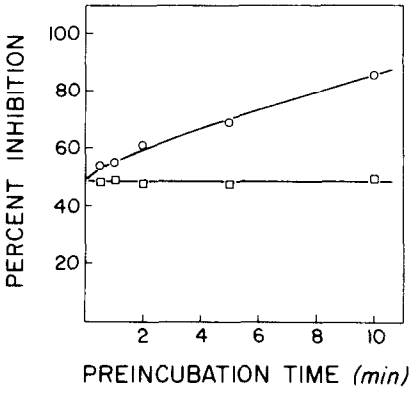


Fig. 3. Effect of preincubation temperature on inhibition of phenylethylamine (PEA) deamination by phenylhydrazine ($PhNNH_2$). $PhNNH_2$ (50 μM) was preincubated with human brain mitochondria (0.03 mg protein) at 37° (–○–○–) or 4° (–□–□–) for the times indicated. Reactions were initiated with the addition of 2 μM PEA and were continued for an additional 10 min at 37°. Values presented are the average of two experiments in which each reaction was performed in duplicate. The average number of nmoles of product formed per mg of protein from deamination of PEA at 4° and 37° in the absence of $PhNNH_2$ was 5.62 and 5.97 respectively.

(39.5 per cent) obtained with the "0-time" blank was lower when compared to the corresponding value seen with the "no-$PhNNH_2$" control (66 per cent). As noted with PEA, the presence of $PhNNH_2$ caused substantial inhibition (42.5 per cent) of 5-HT deamination even though preincubation time was 0 min.

We considered the possibility that the inhibition of PEA and 5-HT deamination that occurred with the "0-time" blank may have been caused by $PhNNH_2$ interacting with the oxidase as the temperature of the reaction mixture was cooling from 37° to 4°. Therefore, additional experiments were performed, identical to those described above, in which the preincubation temperature was 4°. Results of these experiments, shown in Table 3, demonstrate that per cent inhibition values based on the "no-$PhNNH_2$" control remain essentially unchanged (79.7 vs 80.1 per cent). Thus, at 4° inhibition of PEA deamination was independent of the length of time of preincubation since per cent inhibition at 10 min was identical to the "0-time" blank reaction. This is also substantiated by the fact that per cent inhibition for the "0-time" blank at 4° was almost zero. Accordingly, the differences in per cent inhibition observed at 4° and 37° suggest that $PhNNH_2$ most likely inhibited PEA deamination by both a temperature-sensitive and -insensitive process.

The results reported in Table 4 further demonstrate that $PhNNH_2$ interacted with the A form of MAO in much the same manner. It can be seen that per cent inhibition was independent of the time of preincubation at 4° and that inhibition using the "0-time" $PhNNH_2$ blank was essentially zero at 4°. These data are also consistent with $PhNNH_2$ inhibition of 5-HT deamination by a temperature-sensitive and -insensitive process.

At this point in our study, it was considered possible that the temperature-sensitive and -insensitive components may not have actually represented different inhibition processes. There was still the possibility that inhibition of the A and B forms of MAO by the temperature-insensitive component may have occurred during the centrifugation and resuspension procedures. To resolve this problem, experiments were performed in which the $PhNNH_2$ was not washed out of the enzyme preparation prior to addition of the PEA. Results of these experiments

Table 5. Protection of human brain MAO by phenylethylamine against inhibition by phenylhydrazine*

| Phenylhydrazine (M) | Phenylethylamine (M) | Product formed† (nmoles/mg protein) | Temp. | % Inhibition ± S.E. |
|---|---|---|---|---|
| – | – | 6.53 | 37° | – |
| – | $2 \times 10^{-5}$ | 5.96 | 37° | 14.0 ± 3.4 |
| $10^{-5}$ | – | 1.77 | 37° | 73.8 ± 0.6 |
| $10^{-5}$ | $2 \times 10^{-5}$ | 4.91 | 37° | 22.2 ± 1.7 |
| $10^{-5}$ | – | 4.48 | 4° | 31.2 ± 4.2 |
| $10^{-5}$ | $2 \times 10^{-5}$ | 5.98 | 4° | 2.3 ± 2.1 |

* Phenylhydrazine at the concentration indicated, was preincubated for 10 min with human brain mitochondria at 37°. Reactions were centrifuged at 4° and the precipitate was resuspended and recentrifuged two times to remove unbound phenylhydrazine. The mitochondria were resuspended in buffer and assayed for MAO activity at 37°. The concentration of phenylethylamine for this assay was 2 μM. Values for per cent inhibition are the average of three separate experiments in which each reaction was performed in duplicate.

† Results of a typical experiment in which all reactions were performed in duplicate.

are shown in Fig. 3. The data indicate that per cent inhibition of PEA deamination by $5 \times 10^{-5}$ M $PhNNH_2$ at 37° was greater than 50 per cent within 30 sec of preincubation. This inhibition increased consistently at 37° over time to approximately 85 per cent by 10 min of preincubation. In contrast, the reactions performed at 4° remained at slightly less than 50 per cent regardless of the preincubation time. This experiment was also carried out to 30 min without any change in per cent inhibition. The fact that the inhibition did not increase even after a 30-min preincubation period strongly implies that the inhibition observed at 4° in the prior washout experiments most likely did not occur during the centrifugation and resuspension procedures.

It was also considered possible that two or three washings of the mitochondria had not been sufficient to remove excess unbound $PhNNH_2$ and that unbound hydrazine might have subsequently inhibited MAO during the incubations with substrate at 37°. To determine whether this could account for the inhibition obtained, experiments were performed in which washed $PhNNH_2$-treated mitochondria were preincubated for 0 and 10 min at 37° prior to addition of PEA. If unbound $PhNNH_2$ had been present it would be expected that per cent inhibition for the 10-min preincubation period would have been greater than that for the "0-time" blank. The per cent inhibition at both time periods was identical and suggests that all free unbound $PhNNH_2$ was removed by the washing procedures employed.

In addition to the above experiments it was also of interest to determine how variation in the preincubation concentration of $PhNNH_2$ at 4° would affect PEA and 5-HT deamination. The results of these experiments demonstrate that the temperature-insensitive inhibition component was also selective for the B form of human brain MAO. Fifty per cent inhibition of PEA and 5-HT deamination was seen at approximately $2 \times 10^{-5}$ and $1 \times 10^{-3}$ M respectively.

To determine whether the temperature-insensitive interaction of $PhNNH_2$ with both forms of human brain MAO was related to binding at the active site on the oxidase, experiments were performed in which PEA was added to the preincubation mixtures to determine whether substrate could block this inhibition process. The data presented in Table 5 indicate that both the $PhNNH_2$ temperature-insensitive and -sensitive inhibition components of PEA deamination was diminished by addition of PEA during the preincubation period. At both 37° and 4°, $2 \times 10^{-5}$ M PEA protected type B MAO against inhibition by $PhNNH_2$ by about 80–90 per cent.

According to Kenney *et al.* [12], oxygen is required for the irreversible binding of $PhNNH_2$ to MAO. Therefore, it was of interest to determine whether oxygen was necessary for $PhNNH_2$ temperature-sensitive and -insensitive inhibition of MAO. The results presented in Table 6 reveal that per cent inhibition produced by both the temperature-sensitive and -insensitive processes was diminished when preincubations were performed in a nitrogen atmosphere. The temperature-insensitive reaction was decreased approximately 50 per cent and that for the temperature-sensitive process about 65 per cent.

Table 6. Effect of nitrogen on phenylhydrazine inhibition of phenylethylamine deamination*

| Atmosphere | Preincubation temperature | % Inhibition ± S.E. |
|---|---|---|
| Oxygen | 37° | 53.3 ± 1.9 |
| Nitrogen | 37° | 18.1 ± 1.9 |
| Oxygen | 4° | 25.6 ± 2.7 |
| Nitrogen | 4° | 12.7 ± 1.6 |

* Phenylhydrazine (10 μM) was preincubated for 10 min at the temperatures indicated prior to addition of 2 μM phenylethylamine. Reactions were incubated for an additional 10 min and the deaminated product formed was determined as described in Methods. Values are the average for three separate experiments in which each reaction was performed in triplicate.

## DISCUSSION

The data presented in this paper demonstrate that $PhNNH_2$ is a selective inhibitor of human brain type B MAO. This is really not surprising since $PhNNH_2$ is structurally similar to the highly specific B MAO substrate, benzylamine. The distances between the para-carbon atom of the phenyl ring and the primary amine, based on a computer-derived structure of the lowest energy conformation for $PhNNH_2$ and benzylamine, are almost identical (4.87 and 4.97 Å respectively). Relative to other selective B inhibitors of human brain MAO, $PhNNH_2$ then is slightly more specific than pargyline and almost equal to that of Deprenyl. In terms of the concentration of $PhNNH_2$ required to inhibit this form of the oxidase, however, $PhNNH_2$ is approximately 100 times less potent than Deprenyl or pargyline [17]. It can also be inferred from the data that the length of the hydrazine side chain emanating from the phenyl ring helps modulate the abilities of the compounds to inhibit MAO. For example, 50 per cent inhibition of PEA deamination with benzylhydrazine and phenelzine occurs at $2 \times 10^{-8}$ M [10] and $1 \times 10^{-6}$ M [17], respectively, as compared to that for $PhNNH_2$, which is observed at $4 \times 10^{-6}$ M. Thus, the affinities of these hydrazine analogs for the B form of human brain MAO parallel those of the substrates phenylethylamine ($K_m = 1.8 \times 10^{-5}$ M) and benzylamine ($K_m = 2.4 \times 10^{-4}$ M) [18].

In addition, the data herein reveal that $PhNNH_2$ inhibits MAO by two distinct mechanisms, a temperature-sensitive and a temperature-insensitive process. The temperature-insensitive component is extremely rapid and is concentration- and oxygen-dependent. This inhibition process most likely involves interaction at the catalytic binding site since the substrate PEA protects the enzyme from inhibition. Our results agree with those of Patek and Hellerman [13], who have shown previously that $PhNNH_2$ rapidly interacts irreversibly with purified bovine kidney cortex MAO in air at 12°. In addition, our results reveal that there is a slow irreversible or pseudo-irreversible component associated with $PhNNH_2$ inhibition of both the A and B forms of membrane bound human brain MAO. Though not shown, similar results were also obtained with a solubilized preparation of human brain MAO.

Kenney *et al.* [12] have reported previously that inhibition of purified bovine liver MAO by $PhNNH_2$ was accompanied by bleaching of the 450 nm absorbance of the flavin co-factor. The reduction of this absorption band, however, was found to proceed at 30° even after the enzyme was maximally inhibited. These authors concluded that inactivation of MAO cannot be quantitatively ascribed to formation of an adduct with the isoalloxazine moiety of the flavin. This is in agreement with the results of Patek and Hellerman [13] who found that 1.4 to 1.9 moles of $PhNNH_2$ were irreversibly bound per mole of enzyme when incubations were performed at 25°. As demonstrated herein, at the temperatures employed in the above experiments both the temperature-sensitive and -insensitive irreversible components of $PhNNH_2$ inhibition of MAO are likely to occur. Therefore, the results of Kenney *et al.* [12] and Patek and Hellerman [13] are consistent with a bimodal inactivation of MAO as described in this paper. Accordingly, one can speculate that either the temperature-sensitive or -insensitive component may be exclusively associated with formation of a covalent adduct between $PhNNH_2$ and the flavin ring system. This may account for the observation that more than 1 mole of $PhNNH_2$ is bound per mole of enzyme.

Studies are presently underway to determine which of the two inhibition components is involved in binding of $PhNNH_2$ to the 4α-position of the isoalloxazine moiety of the flavin cofactor. Based on the studies of Kenney *et al.* [12] which demonstrated that $PhNNH_2$ inactivation of MAO is considerably more rapid than bleaching of the flavin spectra, it may be predicted that the slow temperature-sensitive inhibition process involves binding to the flavin, and the more rapid temperature-insensitive component involves irreversible binding at other sites on the enzyme.

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