Biochemical Pharmacology, Vol. 31, No. 5, pp. 825–830, 1982.
Printed in Great Britain.

0006-2952/82/050825-06 $03.00/0

CHARACTERISTICS AND SPECIFICITY OF PHENELZINE AND BENSERAZIDE AS INHIBITORS OF BENZYLAMINE OXIDASE AND MONOAMINE OXIDASE*

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(*Received* 7 *May* 1981; *accepted* 20 *August* 1981)

Abstract—The selectivity of benserazide and phenelzine toward inhibition of benzylamine oxidase (BzAO) and monoamine oxidases (MAO-A and MAO-B) was studied in homogenates of rat skull and lung. In addition, the kinetic interaction and reversibility of BzAO inhibition were assessed. Both drugs inhibited BzAO but only phenelzine inhibited MAO, whether tested *in vitro* or *in vivo*. Neither compound acted as an irreversible inhibitor of BzAO. Benserazide was found to be a noncompetitive inhibitor. Phenelzine acted as a substrate for BzAO followed by product-induced noncompetitive inhibition which was labile at 37° but not at 4°. A reversible component in phenelzine-induced inhibition of MAO-A and -B is also suggested from *in vivo* studies.

Monoamine oxidase [MAO; monoamine:oxygen-oxidoreductase (deaminating); EC 1.4.3.4] plays an important physiological role in the deamination of certain endogenous monoamines and exists in two forms, MAO-A and MAO-B. MAO-A activity is inhibited preferentially by clorgyline [1, 2], whereas MAO-B is inhibited preferentially by the related acetylenic inhibitors, (−)-deprenyl and pargyline [3, 4]. Differential classification on the basis of substrate specificity alone is more difficult. Recent studies show that several factors, in addition to relative K_m values, can influence whether a particular amine is deaminated by the A or B form [2, 5–9]. However, with the use of the acetylenic MAO inhibitors it is possible to assay MAO-A and MAO-B activities selectively. 5-Hydroxytryptamine can be used for MAO-A while benzylamine is used frequently for MAO-B.

Another amine oxidase, distinct from MAO but also involved in the deamination of monoamines, has been described in the tissues of animals and man [6, 7–17]. This amine oxidase also prefers benzylamine as substrate but is resistant to inhibition by clorgyline, (−)-deprenyl and pargyline [12–14] at concentrations which inhibit completely MAO-A and MAO-B. Because of resistance to inhibition by clorgyline, this enzyme has been termed "clorgyline-resistant amine oxidase" [13] while the name "benzylamine oxidase" (BzAO) has been used by others [15]. The latter terminology stems from the original work by Blaschko and coworkers [10] on horse plasma amine oxidase. Unlike MAO, which is a flavoprotein and does not contain copper [18, 19], BzAO may be a copper and pyridoxal-dependent enzyme [17, 18].

In vitro BzAO has been shown to be sensitive to inhibition by semicarbazide [12–14], the DOPA-decarboxylase inhibitor benserazide, and the MAO inhibitor phenelzine [6, 15, 20]. Whether these hydrazines are active *in vivo* is at present uncertain.

The purpose of the present study was to examine the properties of benserazide and phenelzine as BzAO inhibitors both *in vitro* and *in vivo* and to determine their selectivity for BzAO compared with effects on MAO-A and MAO-B. Rat skull and lung were used as the enzyme sources since these tissues have been shown to contain good BzAO activity [7, 15]. In addition, experiments were undertaken to ensure the selective assay of BzAO, MAO-A and MAO-B.

MATERIALS AND METHODS

Male rats (Sprague–Dawley descendants) weighing 150–250 g were obtained from TIMCO Breeding Laboratories, Houston, TX. The rats received food and water *ad lib.* and were housed in air conditioned animal quarters on a 12 hr light–dark cycle.

Preparation of tissues. Rats were killed by decapitation. The skull (carefully scraped free of all adhering tissue) and lung were rinsed thoroughly in saline (0.9% NaCl, w/v), blotted dry, and weighed. The lung was minced with scissors and the skull was ground-up with a mortar and pestle. The tissues were homogenized (Ultra Turrex, model SDT, for 1 min at setting number 4.5) using a tissue:buffer ratio (g/ml) of 1:10 in 1 mM phosphate buffer, pH 7.8. Crude homogenates were centrifuged at 700 *g* for 10 min and supernatant fractions of 1 or 2 ml were frozen in vials at −25° for no longer than 5 days before assay.

Dialysis. Inhibitors or an equivalent volume of water were added to homogenates and incubated at 37° for 20 min. Two ml samples of these homogenates were dialyzed against two changes of 2 liters of 1 mM phosphate buffer (pH 7.8), at either 4° or 37°. Control, non-dialyzed homogenates were incubated at 4° or 37° for 19 hr in glass vials.

* This work was presented at the annual meeting of the Federation of American Societies for Experimental Biology, Atlanta, GA, April 1981.

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Enzyme assays. Activities were determined radiochemically by the method of McCaman *et al.* [21] as modified by Callingham and Laverty [22]. Unless stated otherwise, 25 μl inhibitor was preincubated with 25 μl homogenate for 20 min at 37° prior to the addition of 50 μl substrate in 0.2 M phosphate buffer (pH 7.8). [^{14}C]Benzylamine (5 or 20 μM; 10 μCi/μmole) was used to measure BzAO activity after inhibition of both MAO-A and -B with pargyline (10^{-4} M). MAO-B activity was assayed using [^{14}C]benzylamine (150 or 600 μM; 2 μCi/μmole) after inhibition of BzAO with semicarbazide (10^{-4} M). [^{14}C]-5-Hydroxytryptamine (100 or 400 μM; 2 μCi/μmole) was used for the assay of MAO-A. Each reaction was run in triplicate for 15 min and stopped by cooling the tubes on ice followed by acidification with 10 μl of 3 N HCl. Deaminated products were extracted in 600 μl of toluene:ethylacetate [1:1 (v/v) saturated with water] and a 400 μl aliquot was taken for liquid scintillation counting with quench correction. Blank values constituted less than 3% of control counts and were obtained by adding 10 μl of 3 N HCl to homogenate prior to the addition of substrate. None of the drugs used altered extraction efficiency of the deaminated products which was calculated to be in excess of 98%.

Kinetic analysis. The effect of hydrazine inhibitors on the deamination of [^{14}C]benzylamine (10 μCi/μmole) by BzAO was studied in homogenates in which MAO activity was inactivated by prior incubation with pargyline (10^{-4} M). The concentrations (μM) of benzylamine used were: 1.66, 2.0, 2.5, 5.0 and 10.0. Initial velocity rates were obtained by incubating homogenates for 5 min. Linearity of the reaction with time and protein concentration was ensured in all assays. K_m and V_{max} values were calculated by computer program according to the method of Wilkinson [23].

In vivo *experiments.* Rats were injected intraperitoneally with the hydrazine inhibitor or an equivalent volume of saline (0.9% NaCl, w/v) and were killed after 1 or 19 hr. The doses of phenelzine and benserazide used refer to the respective salts. The lungs were removed for assay of BzAO, MAO-A and MAO-B.

Protein. Protein content of all samples was measured by the microbiuret method of Goa [24] with bovine serum albumin (fraction V) as standard.

Chemicals. [^{14}C]Benzylamine hydrochloride was purchased from ICN Pharmaceuticals, Inc., Irvine, CA (12.5 mCi/mmole), and [^{14}C]-5-hydroxytryptamine binoxolate from the New England Nuclear Corp., Boston, MA (51.5 mCi/mmole).

Benzylamine hydrochloride was purchased from ICN Pharmaceuticals, Inc. Plainview, NY, and 5-hydroxytryptamine binoxolate from the Sigma Chemical Co., St. Louis, MO. Pargyline hydrochloride was purchased from the Regis Chemical Co., Morton Grove, IL, and semicarbazide hydrochloride from the Fisher Scientific Co., Fair Lawn, NJ. Clorgyline was a gift from May & Baker Ltd., Dagenham, U.K. Phenelzine sulfate was a gift from Warner–Lambert Research Institute, Morris Plains, NJ, as was benserazide hydrochloride from Hoffmann-La Roche, Inc. Nutley, NJ.

Statistical tests. The significance of differences between mean values was determined using Student's *t*-test. P values are expressed as two-tailed.

RESULTS

Due to the complexity of factors which influence the selectivity of substrates for BzAO, MAO-A and MAO-B (see beginning of paper), it was necessary to validate our assay methods. The MAO inhibitors, clorgyline and pargyline, were used as suggested by previous workers [2, 5, 9]. According to accepted criteria, BzAO is resistant to inhibition by clorgyline at 10^{-4} M, whereas MAO-A and -B are inhibited. MAO-A is inhibited at 10^{-8} to 10^{-7} M clorgyline while MAO-B activity should remain. Semicarbazide (10^{-4} M) was used to inhibit BzAO [7]. Table 1 shows the results obtained using saturating concentrations of substrate for each activity. Ninety to one

Table 1. Effects of clorgyline and pargyline on the deamination of benzylamine and 5-hydroxytryptamine by homogenate of rat skull and lung*

Inhibitor (M)	Percent activity remaining (control = 100%)					
	Benzylamine (20 μM) plus pargyline (10^{-4} M) BzAO		Benzylamine (600 μM) plus semicarbazide (10^{-4} M) MAO-B		5-Hydroxytryptamine (400 μM) MAO-A	
	Skull	Lung	Skull	Lung	Skull	Lung
Clorgyline, 10^{-8}	100	103	97	104	15	16
Clorgyline, 10^{-7}	101	110	103	99	9	8
Clorgyline, 10^{-4}	102	106	5	10	4	0
Pargyline, 10^{-4}	NA	NA	2	4	3	0

* Homogenates were first incubated at 37° for 20 min with pargyline for BzAO, semicarbazide for MAO-B and water for MAO-A, followed by cooling on ice. Clorgyline or pargyline was then added to the appropriate tubes and water to the controls. All tubes were incubated a second time at 37° for 20 min and cooled on ice, and substrate was added. The enzymatic reactions were then run at 37° for 15 min and deaminated products were measured. NA equals not applicable, since pargyline had been added previously. The respective control specific activities [nmoles deaminated · (mg protein)$^{-1}$ · 15 min^{-1}] for skull and lung were: 1.72 and 1.88 (BzAO), 4.0 and 5.0 (MAO-B), and 3.33 and 5.25 (MAO-A).

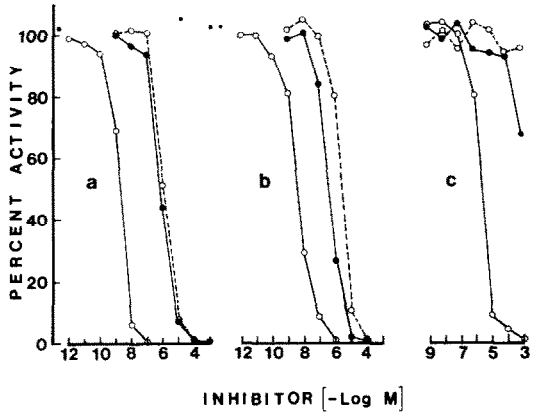

Fig. 1. *In vitro* inhibition of benzylamine oxidase (BzAO) and monamine oxidase (MAO) type A and B by phenelzine in rat skull (a) and lung (b) and by benserazide in lung (c). Remaining activity after preincubation with phenelzine or benserazide for 20 min at 37° was assayed with benzylamine, 5 μM for BzAO (○——○), and 150 μM for MAO-B (○- - -○). 5-Hydroxytryptamine, 100 μM, was used for MAO-A (●——●). Each point is the mean of triplicate determinations expressed as a percentage of control activity. Control activity was determined from the means of six replicate assays for each enzyme.

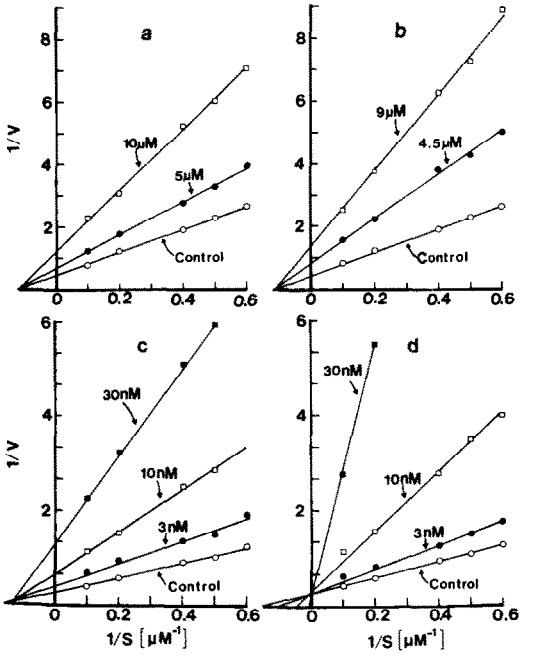

Fig. 2. Double-reciprocal plots of benzylamine deamination by benzylamine oxidase in the presence of semicarbazide (a), benserazide (b), and phenelzine (c and d). Abscissa: reciprocal of benzylamine concentration (μM); ordinate: reciprocal of velocity (v) of enzyme reaction in arbitrary units. Plots a and b were with homogenate of rat skull and plots c and d with homogenate of lung. In a, b and c, the inhibitors were preincubated for 20 min at 37° before addition of benzylamine. In d, benzylamine and phenelzine were added at the same time. Each point is the mean of triplicate determinations. The mean control K_m (μM) and V_{max} [nmoles deaminated·(mg protein)$^{-1}$·min^{-1}] values were: 8.2 and 176.9 for skull (a and b) and 5.8 and 193.5 for lung (c and d) respectively.

hundred percent of the deamination was shown to proceed through the enzymatic activities designated in Table 1 and in Materials and Methods.

The effect of phenelzine (10^{-12} to 10^{-3} M) on BzAO, MAO-A and MAO-B was studied in homogenates of skull and lung. The homogenates were treated as described in Table 1, except phenelzine or water was added for the second incubation. Unless stated otherwise, in these and other experiments, the concentrations of benzylamine and 5-hydroxytryptamine were adjusted to approximate reported K_m values [7, 25] of the substrates for the three activities (5 μM benzylamine for BzAO; 150 μM benzylamine for MAO-B; and 100 μM 5-hydroxytryptamine for MAO-A).

In Fig. 1a and b, it can be seen that phenelzine acted as a potent inhibitor of BzAO giving 50% inhibition at 2 nM (skull) and 3.75 nM (lung). MAO-A and -B were clearly less susceptible to inhibition by phenelzine. At 10^{-4} M, benserazide produced selective inhibition of BzAO in lung giving 50% inhibition at 3.0 μM (Fig. 1c).

In Fig. 2a, b and c, we show double-reciprocal plots for the inhibition of BzAO activity by semicarbazide, benserazide and phenelzine following preincubation at 37° for 20 min. Noncompetitive

Table 2. Effect of dialysis at 4° on hydrazine-induced inhibition of enzyme activities in homogenate of rat skull and lung

Tissue	Enzyme and inhibitor*	% Control activity† Non-dialyzed	Dialyzed
Skull	BzAO		
	Semicarbazide (2×10^{-5} M)	52	116
	Benserazide (10^{-5} M)	58	97
	Phenelzine (10^{-8} M)	36	32
Lung	BzAO		
	Phenelzine (10^{-8} M)	45	47
	Phenelzine (10^{-6} M)	6	10
	MAO-A		
	Phenelzine (10^{-6} M)	12	12
	MAO-B		
	Phenelzine (10^{-6} M)	44	36

* The inhibitors were preincubated with homogenate at 37° for 20 min before dialysis against two 2 liter changes of 1 mM phosphate buffer (pH 7.8) for 19 hr.

† Control (no drug) and non-dialyzed homogenates (hydrazine-inhibited) were kept at 4° for 19 hr.

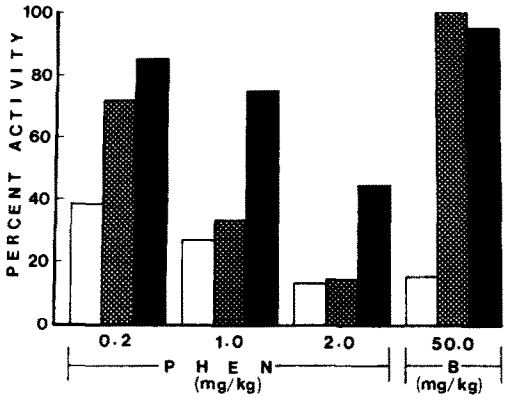

Fig. 3. *In vivo* inhibition of benzylamine oxidase (BzAO) and monoamine oxidase (MAO) type A and B by phenelzine (PHEN) and benscrazide (B) in rat lung. Percentage activity remaining after assay *in vitro* with 20 μM benzylamine for BzAO (open columns), 400 μM 5-hydroxytryptamine for MAO-A (hatched columns), and 600 μM benzylamine for MAO-B (closed columns). Rats were injected i.p. with the inhibitor and killed at 1 hr. Each column is the mean result from two rats. Control activity was determined from four rats. Individual enzymatic activities were assayed in triplicate.

inhibition resulted with each compound. Since phenelzine may act as a substrate for MAO [26, 27], this possibility was tested for BzAO. When phenelzine and substrate were added at the same time, competitive inhibition resulted (Fig. 2d). Identical results were obtained in skull homogenate with phenelzine. Competitive inhibition changed to noncompetitive inhibition following a 20-min preincubation.

In other experiments, we showed phenelzine to be a time-dependent inhibitor of BzAO, reaching a steady state after 20 min of incubation. Between 20 and 45 min no further inhibition resulted (data not shown). Inhibition with semicarbazide and benserazide was also complete after 20 min of incubation (earliest time tested). Dilution of homogenate previously inhibited by semicarbazide or benserazide failed to show recovery of enzymatic activity when assayed at 37° for 15 min. However, both semicarbazide and benserazide were reversed completely when dialyzed against phosphate buffer at 4° for 19 hr (Table 2). In the same experiment, the inhibitory effect of phenelzine on BzAO, MAO-A and MAO-B was not altered by dialysis.

The lack of reversible inhibition by phenelzine with dialysis prompted studies *in vivo*. In an initial experiment, various doses of phenelzine were injected intraperitoneally in rats, the animals were killed 1 hr later, and lung BzAO, MAO-A and MAO-B activities were assayed. A 50 mg/kg dose of benserazide was given intraperitoneally to other rats. The lowest dose of phenelzine (0.2 mg/kg) inhibited BzAO, MAO-A and MAO-B by 62, 32 and 15% respectively (Fig. 3). The two higher doses of phenelzine (1 and 2 mg/kg) failed to discriminate between BzAO and MAO-A but MAO-B was clearly less inhibited. Benserazide acted as a selective inhibitor of BzAO, a result which agrees well with Fig. 1c. Inhibition with phenelzine (2 mg/kg) was studied further in rats at 1 and 19 hr after injection. The results are shown in Fig. 4. In this experiment, the 2 mg/kg dose of phenelzine gave less inhibition at 1 hr than obtained previously (compare with Fig. 3). Qualitatively, however, the relative susceptibilities of the three activities to inhibition by phenelzine

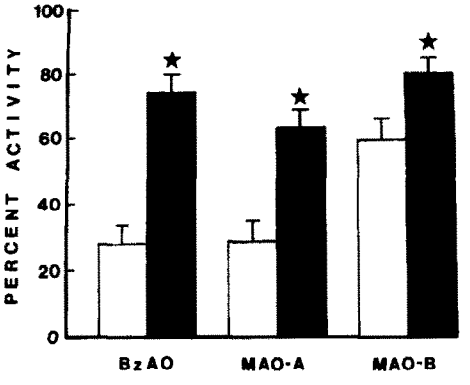

Fig. 4. *In vivo* inhibition of benzylamine oxidase (BzAO) and monoamine oxidase (MAO) type A and B by phenelzine (2 mg/kg, i.p.) in rat lung. Percentage activity remaining after treatment for 1 hr (open columns) and 19 hr (closed columns). Each column is the mean ± the S.E. of the ratio for six rats. Control activities were also determined from six rats. Key: (*) significantly different from 1 hr, $P < 0.05$. All columns are significantly different from control ($P < 0.05$) with the exception of MAO-B at 19 hr. For other details, see the legend to Fig. 3.

Table 3. Effect of heating at 37° for 19 hr on phenelzine-induced inhibition of lung BzAO *in vitro*

Treatment	Phenelzine* (M)	Specific activity†	% Change
4° for 19 hr	None	1.63	
	10^{-8}	0.75	−54
	10^{-6}	0.13	−92
37° for 19 hr	None	1.98	
	10^{-8}	2.04	+3
	10^{-6}	0.73	−63
37° + Dialysis for 19 hr	None	1.62	
	10^{-6}	1.76	+9

* Phenelzine was preincubated with homogenate at 37° for 20 min prior to the treatments.

†nmoles benzylamine deaminated · (mg protein)$^{-1}$ · 15 min^{-1}.

remained the same. At 19 hr, there was a significant recovery of BzAO and MAO-A activity to 75 and 63% of control, respectively. Although MAO-B showed less recovery, the change was sufficient to render the difference from control non-significant.

The recovery of enzymatic activity *in vivo* (Fig. 4) could have resulted from enzyme synthesis and/or dissociation of the enzyme–inhibitor complex at the body temperature of the rat. The latter possibility was tested for BzAO by heating lung homogenate (previously inhibited with phenelzine) at 37° for 19 hr. Heating increased the specific activity of BzAO compared to that obtained at 4° and reversed completely the inhibitory effect of phenelzine (10^{-8} M) as shown in Table 3. At 10^{-6} M phenelzine, 63% inhibition remained, compared with 92% at 4°. Thirty-seven degrees plus dialysis restored BzAO activity with the high concentration of phenelzine (10^{-6} M). A reliable determination of MAO-A and MAO-B activities was not possible in the experiment due to considerable thermal inactivation.

DISCUSSION

We have found that benserazide and phenelzine inhibit BzAO *in vitro* and *in vivo* but that neither compound does so irreversibly.

Inhibition of BzAO *in vitro* by benserazide and phenelzine confirms work by Lewinsohn *et al.* [6, 15] done primarily with homogenates of human tissues. In addition, we found benserazide to be a highly preferential BzAO inhibitor, compared with effects on MAO-A and -B. This preference was retained *in vivo*. MAO-A and MAO-B activities were not altered 1 hr after a 50 mg/kg dose of benserazide whereas BzAO was inhibited by 85%. Lack of MAO inhibition with benserazide *in vivo* agrees well with results obtained following acute [28] and chronic [29] administration of this agent to rats. *In vitro*, phenelzine also acted as a preferential inhibitor of BzAO but, unlike benserazide, this preference was not well retained *in vivo*. BzAO and MAO-A were inhibited equally by the 1 and 2 mg/kg doses of phenelzine. In contrast, inhibition of MAO-A and -B by phenelzine *in vivo* showed the same general preference as obtained with homogenates of lung *in vitro* in that MAO-A was inhibited to a greater extent than MAO-B. This result is in agreement with recent findings by Miller *et al.* [30] in rat striatum and rest of the brain. Overall, it suggests that the accessibility of phenelzine to BzAO and MAO may differ *in vivo*. This is supported by evidence indicating a different subcellular localization of BzAO from MAO [13].

Kinetic analysis showed benserazide and semicarbazide to be noncompetitive inhibitors of BzAO. McEwen [31] and others [20] have found the same type of interaction for semicarbazide on BzAO activity in different tissues. The result is consistent with a role for pyridoxal phosphate as cofactor for BzAO [17, 18]. Neither semicarbazide nor benserazide acted as irreversible inhibitors, however, since activity was recovered fully by dialysis at 4° for 19 hr. Although the rate of recovery was not studied, time-dependency seems likely since dilution of homogenate (previously inhibited with semicarbazide or benserazide) failed to restore enzymatic activity. From similar kinetic and dialysis experiments (4° for 19 hr) with phenelzine this compound seemed to act as an irreversible suicide inhibitor of BzAO, in that initial competitive inhibition changed to noncompetitive inhibition after 20 min of incubation and the noncompetitive phase was not reversed by dialysis at 4°. This interpretation, however, was not substantiated. The recovery of BzAO activity *in vivo* 19 hr after the administration of phenelzine (Fig. 4) suggested that the duration of phenelzine-induced inhibition might be temperature-dependent. This notion was confirmed *in vitro* by incubating homogenate, previously inhibited with phenelzine, at 37° for 19 hr. Under these conditions, a complete recovery of BzAO activity was obtained with phenelzine (10^{-8} M) and with the addition of dialysis the inhibition by 10^{-6} M phenelzine was reversed totally. These experiments were made possible by the remarkable stability of BzAO activity over 19 hr at 37°. In view of these results, the recovery of BzAO activity *in vivo* cannot be concluded to represent new enzyme synthesis. In fact, the turn-over rate of BzAO is unknown due to the lack of a proven irreversible inhibitor of the enzyme.

Analysis of the kinetic interaction of phenelzine with BzAO suggests that the compound acts as a substrate for the enzyme followed by product-induced non-competitive inhibition. A similar conclusion can be drawn from the work of Lindström *et al.* [32, 33] for hydrazine-induced inhibition of pig plasma BzAO and from that of others for MAO [34]. Lindström and Petterson [32] consider arylalkylhydrazines to act as irreversible inhibitors of pig plasma BzAO, since inhibition with phenylhydrazine was not reversed by dialysis at 4°. As shown in the present study, however, this test does not provide conclusive proof. The failure of the active intermediate of phenelzine to bind irreversibly to the enzyme cofactor only became evident by heating at 37°. The nature of this intermediate is not resolved for BzAO but the formed diazene and hydrazone from phenelzine are likely candidates. With MAO, the diazene intermediate is believed to be the active metabolite [34–36]. According to Patek and Hellerman [34], phenelzine is rather unique among arylalkylhydrazines in that over 90% of the formed diazene undergoes intramolecular rearrangement to the hydrazone, which is inactive as an MAO inhibitor.

Whereas the recovery of BzAO activity *in vivo* can be ascribed to reversible inhibition by phenelzine at 37°, we were unable to apply the same test for MAO due to marked thermolability. However, a significant recovery of MAO-A activity and, to a lesser extent, MAO-B activity was observed *in vivo* between 1 and 19 hr (Fig. 4). This return in MAO activity appears too rapid to be due entirely to new enzyme synthesis. Calculated half-lives for MAO-A range from 3.05 and 3.44 days in rat liver [37, 38] to 6.9 days in rat skeletal muscle [9] with corresponding values of 2.5, 2.64 and 6.4 days for MAO-B. Half-lives of 8.8 and 11 days have been found for total MAO activity in rat heart [39] and brain [40] respectively. While it is possible that rat

lung MAO exhibits a faster rate of turn-over, it is also possible that MAO inhibition by phenelzine (2 mg/kg) at 1 hr included a reversible component under the experimental conditions prevailing *in vivo*. As indicated above, MAO contributes to the elimination of phenelzine, most probably via hydrazone formation with the subsequent production of phenylacetic acid [26, 27]. *In vivo*, as much as 87% of administered phenelzine is recovered as this metabolite [27]. Thus, one possibility is that, at 1 hr, the injected phenelzine was still being metabolized by lung MAO without maintaining sufficient levels of formed diazene for an equivalent degree of irreversible inactivation. Other explanations are also possible since the reaction of phenylhydrazine with MAO has been reported to entail a mixed mechanism involving a site in addition to the FAD cofactor [41]. Furthermore, the rate of interaction with FAD is biphasic and does not parallel the inhibition [41]. Some evidence for a reversible component in the inhibition of mouse brain MAO by a 2 mg/kg dose of phenelzine can be deduced from the recent work of Green and El Hait [42]. A larger dose of phenelzine (4 mg/kg), however, acted as an irreversible inhibitor [42] suggesting that possible reversible versus irreversible interactions with MAO may be dose-dependent.

In conclusion, we have shown that two drugs used clinically, beserazide and phenelzine, can inhibit BzAO *in vivo*. The significance of this observation, however, is not readily apparent since the physiological role of BzAO is unknown. A systematic comparison of the toxicities and side-effects of these two drugs, which cannot be explained readily by decarboxylase or MAO inhibition, may shed some light on the possible role of BzAO in man.

Acknowledgements—This study was supported, in part, by NIH Grant GM07405. We would like to thank Sanu Lokhandwala for typing this manuscript.

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