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Inhibition of Bovine Lung Semicarbazide-Sensitive Amine Oxidase (SSAO) by Some Hydrazine Derivatives

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ABSTRACT. Microsomal semicarbazide-sensitive amine oxidase (SSAO) from bovine lung was shown to be inhibited by a number of hydrazine derivatives, but the mechanisms of inhibition were found to differ. Hydralazine behaved as an irreversible and partially time-dependent inhibitor with an IC_{50} value of 1 μM under the conditions used. Phenylhydrazine was found to be a potent irreversible inhibitor of SSAO (IC_{50} 30 nM). Semicarbazide behaved as a specific irreversible inhibitor (active-site-directed irreversible inhibitor) in first forming a non-covalent enzyme-semicarbazide complex (with a K_i value of 85 μM), which then reacted to give an irreversibly inhibited enzyme species in a reaction defined by the first-order rate constant $k_2 = 0.065\ min^{-1}$. Phenelzine behaved as a reversible inhibitor, but dialysis at 37°C was found to be necessary to obtain full recovery of enzyme activity. The dependence of inhibition on phenelzine concentration was complex and consistent with multiple binding sites for this inhibitor. This diversity in the action of a family of compounds with the same functional group must be taken into account in attempts to design more specific inhibitors of this enzyme. BIOCHEM PHARMACOL 52;2:187–195, 1996.

KEY WORDS. β-aminopropionitrile; hydralazine; microsomes; phenelzine; phenylhydrazine; semicarbazide

Several different amine oxidases are inhibited by carbonyl reagents, such as semicarbazide. Although they are all classified as the amine oxidases (copper-containing) (EC 1.4.3.6), they comprise a large group of enzymes with different substrate specificities and tissue distributions. Their sensitivity or inhibition by semicarbazide is commonly used to distinguish these enzymes from monoamine oxidases [amine oxidase (flavin-containing); EC 1.4.3.4] and, unlike the latter enzymes, the semicarbazide-sensitive amine oxidases are resistant to inhibition by acetylenic inhibitors, such as clorgyline, deprenyl, and pargyline.

Semicarbazide-sensitive amine oxidase (SSAO) activities that are active towards monoamines are found in blood plasma and associated with membranes in several mammalian tissues. High activities are present in blood vessels, predominantly associated with the smooth muscle cells [1] and its presence in the smooth muscle layers of rat aorta has been described [2]. A secreted soluble form of SSAO from cultured vascular smooth muscle cells has also been reported [3]. SSAO activity has also been found in nonvascular tissues, such as chondrocytes, in rat articular cartilage [4] and in adipocytes from rat white and brown fat [5, 6]. Its presence has been confirmed in pig dental pulp [7] and it has also been found in different parts of the bovine eye [8]. Despite this widespread tissue distribution, the physiological roles of the SSAOs remain far from clear [9], although the activity has been shown to be affected by development, tissue damage, and cancer in a way that suggests an involvement in cell growth [10, 11].

Both monoamine oxidase (MAO) activity [12, 13] and that of SSAO [14] have been shown to contribute to the metabolism of amines in preparations of lung. Because SSAO has been shown to be active towards some volatile short-chain aliphatic amines, such as methylamine, whereas MAO is not [15], this activity could play a functional role in the deamination of inhaled volatile amines. Attempts to define the functions of SSAO more closely have been hampered by the lack of inhibitors of suitable potency and specificity.

Although the sensitivity to inhibition by carbonyl reagents, such as semicarbazide, is commonly used to differentiate SSAO activity from that of MAO, and the interaction of such compounds with the former enzymes has been used in studies of the nature and binding of the quinone cofactor, there have been few studies on the kinetic nature of the inhibitory processes involved. The aims of the present work were to study the inhibitory effects of some hydrazine derivatives (see Fig. 1) on SSAO from bovine lung microsomes.

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FIG. 1. The structures of the inhibitory compounds used in this work.

MATERIALS AND METHODS

Materials

The radioactive substrate [7-^{14}C]-benzylamine hydrochloride (55 Ci/mol) was obtained from Amersham International (Amersham, U.K.) and [ethyl-1-^{14}C]-2-phenylethylamine hydrochloride (50 Ci/mol) was from New England Nuclear (Stevenage, U.K.). The amine oxidase inhibitors semicarbazide hydrochloride, phenylhydrazine hydrochloride, hydralazine hydrochloride, phenelzine hydrochloride, and β-aminopropionitrile were obtained from Sigma (Poole, U.K.). Clorgyline hydrochloride was a gift from May & Baker Ltd. (Dagenham, U.K.). DEAE-Sephacel and Triton X-100 were from Pharmacia (Uppsala, Sweden) and Sigma, respectively. All other reagents were of analytical grade.

Preparation of Bovine Lung Microsomes

Bovine lung was obtained from the abattoir after slaughter, packed in ice, and transported immediately to the laboratory. After removal of connective tissue, the lung was weighed and washed with saline (0.9% NaCl w/v) to eliminate blood as a potential source of contaminating plasma amine oxidase. The tissue was then homogenised in 1:10 (w/v) 20 mM Tris-HCl buffer, pH 7.2, containing 0.25 M sucrose, by use of a Waring blender, and filtered through 2 layers of cheesecloth. The homogenate was subjected to differential centrifugation and the microsomal fraction was obtained by addition of $CaCl_2$ to the postmitochondrial supernatant and centrifugation, as described previously [14]. The final microsomal pellet was resuspended in 20 mM potassium phosphate buffer, pH 7.2, at a protein concentration of 10 mg/mL and stored in aliquots of −20°C until assay.

Partial Purification of Bovine Lung Microsomal SSAO

The temperature was kept at 4°C throughout. Samples of microsomal membranes (10 mg/mL) were mixed with an equal volume of 1.2% (w/v) Triton X-100 in 20 mM potassium phosphate buffer, pH 7.2, at 4°C for 30 min, before centrifugation at 105,000 *g* for 1 hr. The resulting supernatant, containing approximately 90% of the microsomal SSAO activity, was removed, saved, and the pellet was discarded.

The extracted protein was loaded at 50 mL/hr on to a DEAE-Sephacel column (2 × 2 cm, 6 mL bed-volume) that had been equilibrated with 20 mM potassium phosphate buffer, pH 7.2, containing 0.1% (w/v) Triton X-100. The column was washed with 150 mL of the same buffer, until the unbound protein had eluted, followed by 300 mL of a linear gradient of NaCl (0 to 0.5 M) in the same buffer. Fractions (10 mL) were collected and assayed for protein and enzyme activity. Those that contained SSAO activity (eluting at NaCl concentrations between 0.1 and 0.25 M) were combined and concentrated under a nitrogen atmosphere using an Amicon ultrafiltration cell with a XM-50 membrane. The final concentrate was dialysed overnight against 1000 volumes of 20 mM potassium phosphate buffer, pH 7.2, containing 0.1% Triton X-100, and divided into aliquots that were stored at −80°C until required. This procedure gave a 12-fold purification of the microsomal enzyme (50-fold from tissue homogenate values) with a yield of 70%.

Radiochemical Assay of SSAO Activity

SSAO activity was determined radiochemically at 37°C by the method of Fowler and Tipton [16] using benzylamine (20 μM; 3 mCi/mmol) and 2-phenylethylamine (PEA, 100 μM; 2.5 mCi/mmol) as substrates. The reaction was performed in a final volume of 225 μL of 50 mM potassium phosphate buffer, pH 7.2. After fixed times, reactions were stopped by the addition of 100 μL 2 M citric acid and the products were extracted into toluene/ethyl acetate 1:1 (v/v) containing 0.6% (w/v) 2,5-diphenyloxazole. Radioactivity was measured in a liquid scintillation counter. Blanks were routinely determined by the addition of the 2M citric acid at time zero. Experiments in the absence of the enzyme preparation and with heat-inactivated enzyme preparations showed no nonenzymic product formation to occur under these conditions. All activity determinations were performed over times where product formation was shown to proceed linearly under all conditions used, and where initial rate was a linear function of enzyme concentration.

Inhibition Studies

The concentration of inhibitors giving rise to 50% inhibition under the conditions used in the present work (IC_{50} values) were determined both before and after preincubation of the enzyme with inhibitor for 30 min at 37°C. In the former case, the assay reaction was started by the addition of enzyme to the assay mixture containing substrate and inhibitor. The concentration ranges of the inhibitors used were as follows: 10^{-10}–10^{-6} M for phenylhydrazine and phenelzine, 10^{-9}–10^{-4} M for hydralazine, 10^{-7}–10^{-3} M for semicarbazide, and 10^{-6}–10^{-2} M for β-aminopropionitrile (βAPN). The SSAO activity remaining was determined radiochemically with 20 μM benzylamine as substrate.

The reversibility of inhibition was assessed by dialysis. The enzyme (900 μL) diluted appropriately in 20 mM potassium phosphate buffer, pH 7.2, was incubated at 37°C for 30 min with 100 μL of the corresponding inhibitor at a

concentration that gave approximately 90% inhibition. The mixture was then dialysed overnight against 2000 volumes of the same buffer at 4°C and the enzyme activity determined. Control samples where the inhibitor was replaced by an identical volume of distilled water were treated in the same way.

The nature of the reversible phase of the inhibition by phenelzine and semicarbazide was studied, in the absence of enzyme-inhibitor preincubation, by initial-rate determinations with varying benzylamine concentration, in the range 25–300 μM, and at a series of fixed inhibitor concentrations. Data were obtained from triplicate determinations from three different preparations and analysed by nonlinear regression using the computer program GraphPad Inplot (GraphPad Software Inc, version 4.03, 1992). Results are presented as double-reciprocal plots, from a single set of triplicate determinations in each case, for illustrative purposes only.

The irreversible inhibition of the enzyme by semicarbazide followed specific active-site directed mechanism according to the equation:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \qquad (1)$$

where E and I are the free enzyme and inhibitor, respectively, the noncovalent enzyme-inhibitor complex is EI and EI^* represents the irreversibly inhibited species. The rate of irreversible inhibition in such a process can be described by the equation [17]:

$$\frac{d[EI^*]}{dt} = k_2[EI] = \frac{k_2([E_t] - [EI^*])}{(1 + K_i/[I])} \qquad (2)$$

where $[E_t]$ and $[I]$ are the total enzyme and inhibitor concentrations, respectively, and $[EI]$ and $[EI^*]$ are the concentrations of the noncovalent and irreversibly-bound enzyme-inhibitor complexes, respectively. K_i is the dissociation constant of the EI complex (k_{-1}/k_{+1}).

Time-courses of the irreversible inhibition of SSAO activity by semicarbazide were determined by incubating 25 μL of the microsomal fraction (230 μg of protein) with different concentrations of the inhibitor (0, 25, 50, 75, and 100 μM) for a series of fixed times at 37°C. The substrate (350 μL) to give a final benzylamine concentration of 200 μM was, then, added and enzyme activity was determined. This method allowed the activity remaining to be determined under conditions where the dilution renders the degree of reversible inhibition negligible. Under these conditions, the rate equation can be integrated to give:

$$k't = \ln[E_t] - \ln([E_t] - [EI]) \qquad (3)$$

where the apparent first-order rate constant for activity loss, k', is given by:

$$k' = \frac{k_2}{(1 + K_i/[I])} \qquad (4)$$

Values of k' were obtained at each inhibitor concentration from graphs of the logarithm of the activity remaining against preincubation time. Values of k_2 and k_i were then determined from the hyperbolic dependence of k' on inhibitor concentration using nonlinear regression. The half-time of the enzyme inhibitory reaction ($t_{1/2}$) was calculated from the relationship: $t_{1/2} = (\ln 2)/k_2$.

RESULTS

Sensitivity Towards Carbonyl Reagents

The inhibitory effects of different hydrazine compounds toward SSAO from bovine lung microsomes were determined at a series of different inhibitor concentrations. Curves of inhibition, as a function of inhibitor concentration, described simple sigmoids and total inhibition were observed in all cases (data not shown). Such behavior would be consistent with only a single enzyme (SSAO) being responsible for the metabolism of benzylamine. The corresponding IC_{50} values determined from these experiments are shown in Table 1.

The possible presence of lysyl oxidase in bovine lung microsomes was studied by using β-aminopropionitrile (βAPN), which has been shown to inhibit lysyl oxidase irreversibly and in a time-dependent manner, but to be a reversible and competitive inhibitor of SSAO [18]. When this inhibitor was tested against bovine lung microsomal membranes, the same IC_{50} value (see Table 1) was obtained after 0 and 30-min incubation with the inhibitor. Furthermore, there was complete recovery of activity after dialysis. The reversibility and lack of time-dependence of the inhibition are not consistent with the presence of lysyl oxidase.

Semicarbazide was shown to be a time-dependent inhibitor of SSAO from bovine lung. An inhibitor concentration of 100 μM was necessary to give a 50% inhibition in the absence of prior enzyme-inhibitor preincubation, whereas this value fell to 10 μM after 30-min preincubation (Fig.

TABLE 1. Inhibition of bovine lung microsomal SSAO by hydrazine derivatives and β-aminopropionitrile (βAPN)

Inhibitor	IC_{50} (0 min)*	IC_{50} (30 min)†	Reversibility‡
Semicarbazide	100 ± 12 μM	10 ± 3 μM	None
Phenelzine	20 ± 5 nM	20 ± 4 nM	Partial§
Phenylhydrazine	30 ± 7 nM	25 ± 9 nM	None
Hydralazine	5 ± 2 μM	1.5 ± 0.8 μM	None
βAPN	250 ± 25 μM	250 ± 20 μM	Fully

The IC_{50} value is the inhibitor concentration necessary to give 50% inhibition under the assay conditions described in the text. Each value represents the mean value ± standard deviation of determinations with 3 separate enzyme preparations, each assayed in triplicate. * and † Determined after 0 or 30 min preincubation with the enzyme preparation before assay; ‡ Assessed by dialysis overnight at 4°C; § Fully reversible by overnight dialysis at 37°C.

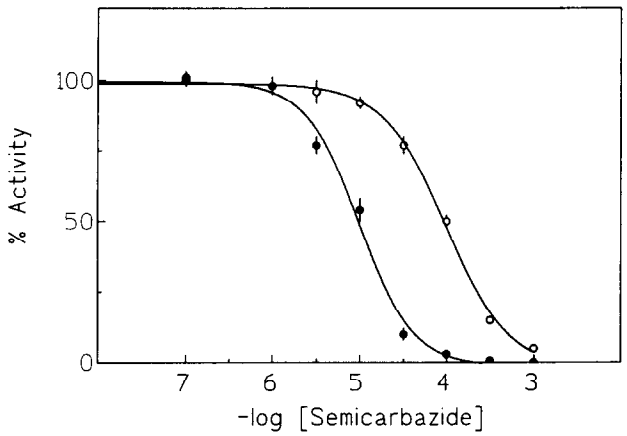


FIG. 2. Inhibition of SSAO activity by semicarbazide. After inhibition of any MAO activity by preincubating for 30 min at 37°C with 1 mM clorgyline, bovine lung microsomal preparations were incubated 0 (○) or 30 min (●) with inhibitor before assay for activity with 20 μM benzylamine. Each point represents the mean ± standard deviation of 3 different determinations.

2). This inhibition was irreversible because it was not relieved by dialysis.

Inhibition by hydralazine increases with time at higher, but not lower (nM range), inhibitor concentrations. Similar behaviour has been reported for SSAO from rat heart [19]. Inhibition was irreversible because it was not relieved by dialysis (see Table 1).

Phenelzine and phenylhydrazine showed the highest potencies as inhibitors of the enzyme with the lowest calculated IC_{50} values (Table 1). No significant time-dependence was observed. Phenylhydrazine was shown to be an irreversible inhibitor of SSAO because dialysis gave no recovery of enzyme activity. In contrast, the inhibition by phenelzine was partially reversed after dialysis at 4°C and was completely reversed by dialysis at 37°C. Similar behaviour has been reported with this compound as an inhibitor of rat lung SSAO [20].

Kinetics of Inhibition by Semicarbazide

In the absence of enzyme-inhibitor preincubation, semicarbazide behaved as a simple linear-competitive inhibitor of the enzyme with respect to benzylamine with a K_i value of 96 ± 8 μM (Fig. 3).

The time-courses of the irreversible inhibition of SSAO activity were determined at a series of different semicarbazide concentrations. Figure 4 shows the semilogarithmic plots of percentage activity remaining against preincubation time from which the first-order rate constants k' for activity loss were obtained. The double-reciprocal plot of

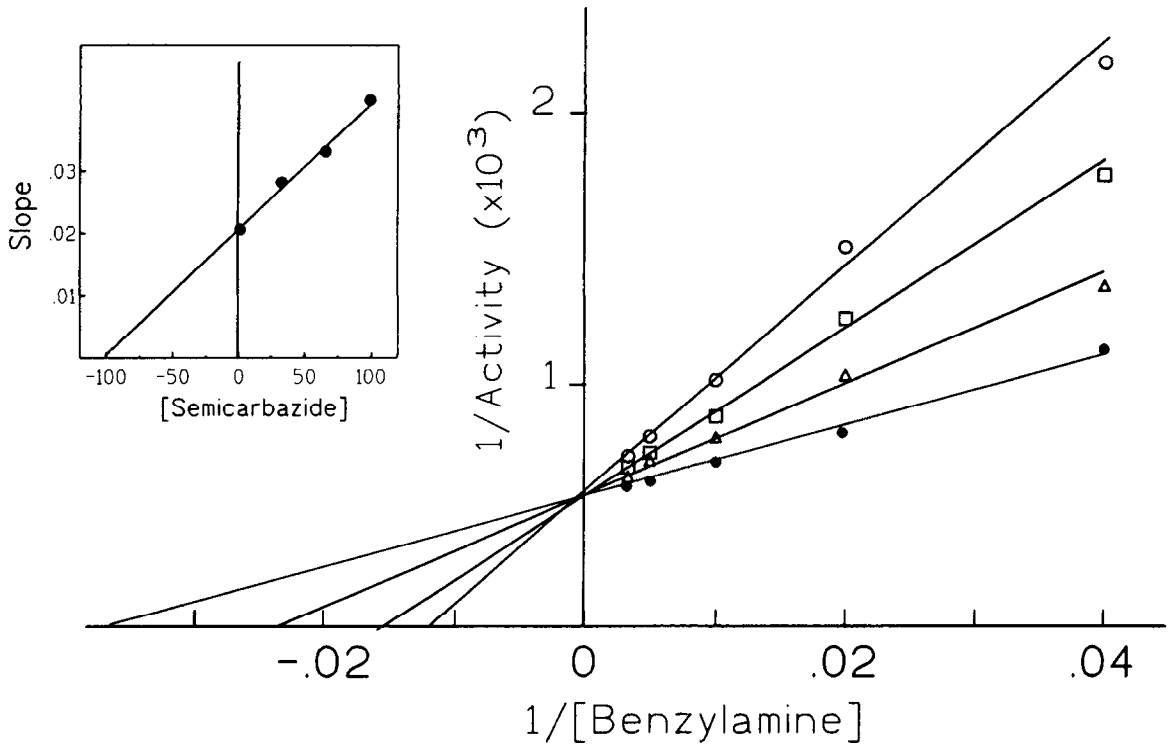


FIG. 3. Double-reciprocal plots for the inhibition of SSAO by semicarbazide. The oxidation of benzylamine (25–300 μM) by samples of bovine lung microsomes, which had been pretreated with clorgyline as described in Fig. 2, was studied in the presence and absence of semicarbazide without preincubation. Final semicarbazide (μM) concentrations were: 0 (●), 33 (△), 66 (□) and 100 (○). Each point is the mean of a triplicate determination. Ranges of individual values were less than 5% in all cases. Error bars are omitted for clarity. The dependence of the slopes of these lines (apparent K_m/V_{max} values) upon the inhibitor concentration, which may be used to determine the K_i value for semicarbazide, is shown in the inset.

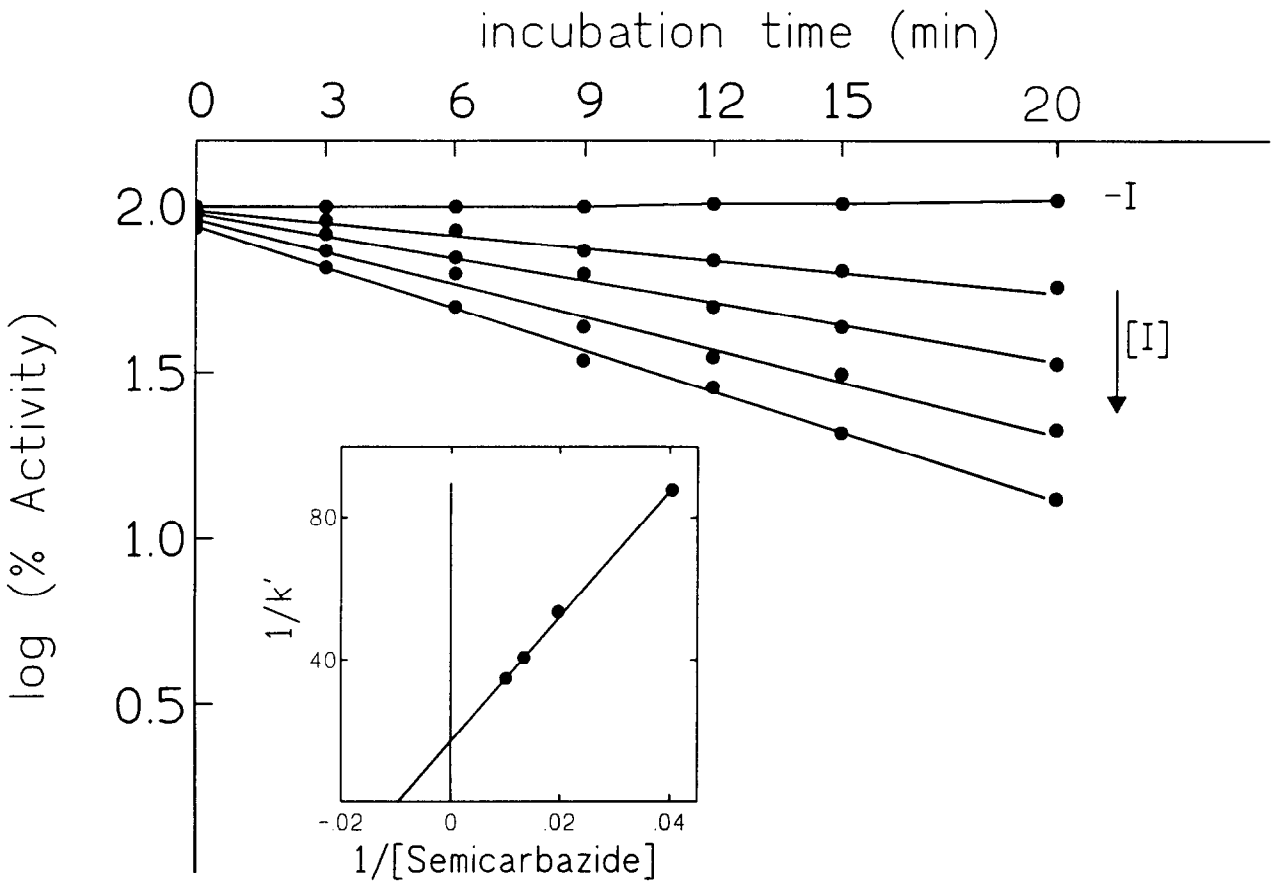
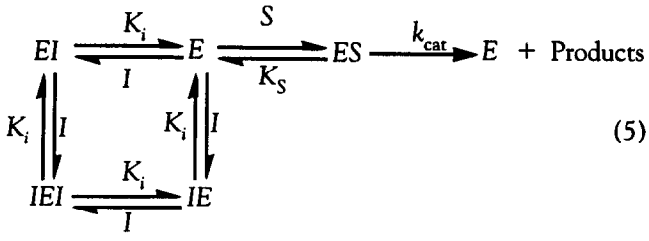


FIG. 4. Time-dependent inhibition of SSAO by semicarbazide assayed with 200 μM benzylamine as substrate. Semicarbazide concentrations were 0, 25, 50, 75, and 100 μM. The results represent the mean of determinations using 3 microsomal preparations. Error bars are omitted for clarity, but ranges of individual values were less than 6% in all cases. The inset shows the plot of 1/apparent first-order rate constant (k') (calculated by linear regression analysis of the slopes, as described in text) vs the semicarbazide concentration.

the k' values against inhibitor concentrations was linear (see inset of Fig. 4) indicating inhibition to be a saturable process. A value of 85 ± 10 μM was determined for K_i, which was consistent with the value determined from the competitive inhibition studies described above. The first-order constant (k_2) for the formation of the irreversibly inhibited species was 0.065 ± 0.009 min^{-1}, which would correspond to the half-life ($t_{1/2}$) of 10.7 ± 1.7 min at saturating concentrations of semicarbazide.

Kinetics of Inhibition by Phenelzine

Initial-rate studies indicated phenelzine to behave as a competitive inhibitor of bovine lung enzyme with respect to benzylamine (Fig. 5). However, when the secondary plots of slopes of the double-reciprocal plot were plotted against the inhibitor concentration, a parabolic relationship was obtained (inset in Fig. 5). Such behaviour suggests the presence of multiple inhibitory binding sites for phenelzine. A simple model that could account for such behaviour would be to assume the enzyme to bind two molecules of the competitive inhibitor, in a random manner, under conditions of rapid equilibrium [21]:



(5)

Where the initial velocity (v) would be given, in terms of the constants shown in the above scheme, by the following equation in which [E] and [S] are the enzyme and substrate concentrations, respectively, and K_i and K_s are the dissociation constants for the equilibria shown.

$$v = \frac{k_{cat}[E][S]}{K_s(1 + [I]/K_i)^2 + [S]} \quad (6)$$

This relationship predicts a nonlinear plot such as that shown in the inset to Fig. 5. The slopes of the lines in the double-reciprocal plot of Fig. 5 would be related to the inhibitor concentration by the relationship:

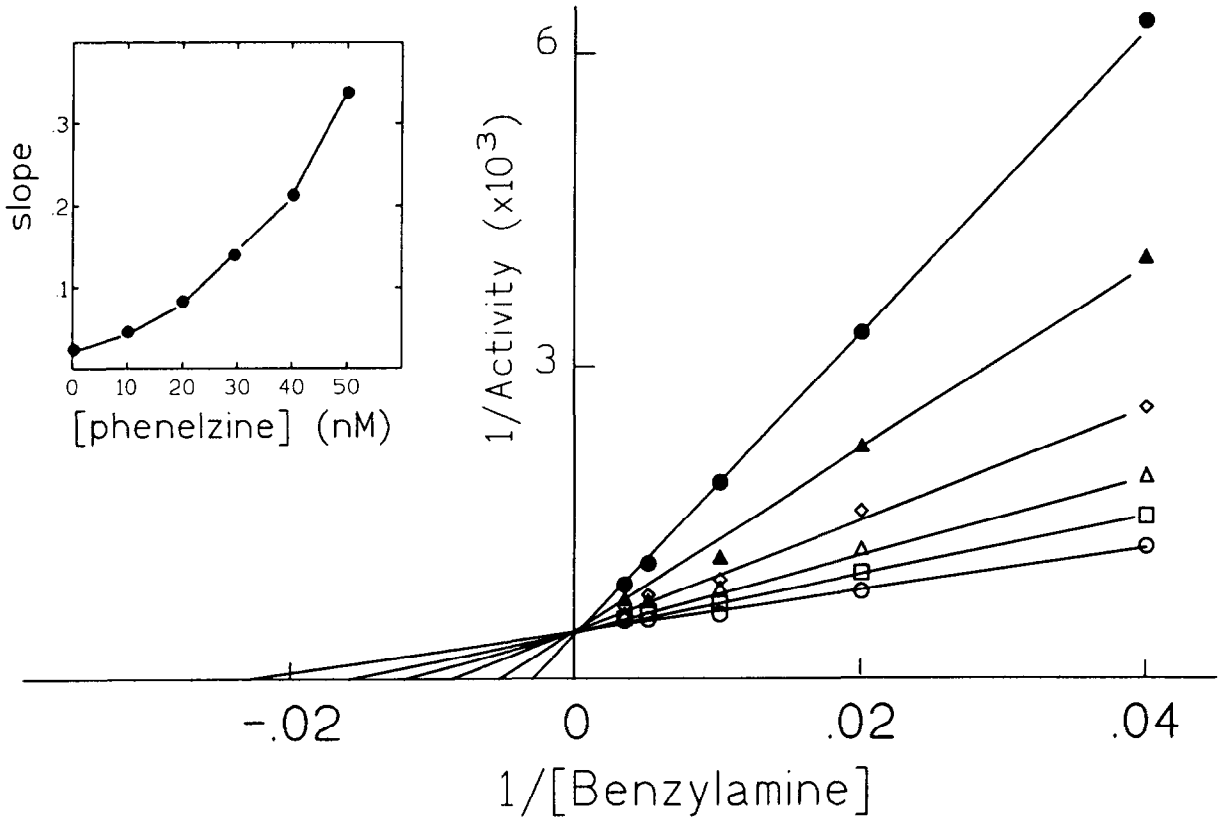


FIG. 5. Double-reciprocal plots for the inhibition of SSAO by phenelzine. The oxidation of benzylamine (25–300 μM) by samples of clorgyline-treated bovine lung microsomes was studied in the presence and absence of phenelzine. Final inhibitor (nM) concentrations were: 0 (○), 10 (□), 20 (△), 30 (◇), 40 (▲), and 50 (●). Each point is the mean of a triplicate determination. Ranges of individual values were less than 5% in all cases. Error bars are omitted for clarity. The slope replot is shown in the inset.

$$\text{slope} = \frac{K_s}{k_{cat}[E]}(1 + [I]/K_{islope}) \quad (7)$$

The values of K_{islope} determined at each inhibitor concentration in this way are not simple enzyme-inhibitor dissociation constants, but are related to K_i by the relationship:

$$K_{islope} = \frac{K_i^2}{(2K_i + [I])} \quad (8)$$

this can be linearised to:

$$\frac{1}{K_{islope}} = \frac{[I]}{K_i^2} + \frac{2}{K_i} \quad (9)$$

A graph of $1/K_{islope}$ against the phenelzine concentration was linear (see Fig. 6), as predicted by the above relationship, and allowed the K_i value to be determined as 27 nM.

The inhibitory behaviour of phenelzine at a fixed concentration of benzylamine (20 μM) was fitted to the Hill [22] equation using nonlinear regression data analysis and a value of 2.90 ± 0.06 was obtained for the Hill constant (Fig. 7a). This behaviour was essentially unchanged if 2-phenylethylamine (100 μM) was used as substrate instead of benzylamine (Hill constant 3.12 ± 0.07, see Fig. 7b). This complex inhibitory behaviour does not appear to result from the use of a membrane-bound enzyme because a similar Hill constant (2.95 ± 0.09) was obtained for phenelzine when the partially purified enzyme, which had been extracted from the membrane with detergent, was used.

DISCUSSION

The microsomal localisation of SSAO from bovine lung and its substrate specificity have been previously demonstrated [14]. A major problem in attempts to elucidate the functions of the SSAO enzymes has been the lack of inhibitors with suitable specificity and potency. The present studies on the mechanisms of action of hydrazine derivatives were envisaged as a step towards the development of compounds.

A characteristic feature of SSAO activity is its sensitivity to inhibition by a variety of carbonyl reagents. For example, hydralazine, a peripheral vasodilator used as antihypertensive agent, irreversibly inhibits SSAO from bovine lung microsomes and showed some dependence upon preincubation time when concentration in the micromolar range were used. The SSAO from rat heart has also been reported to show time-dependent inhibition by higher, but not

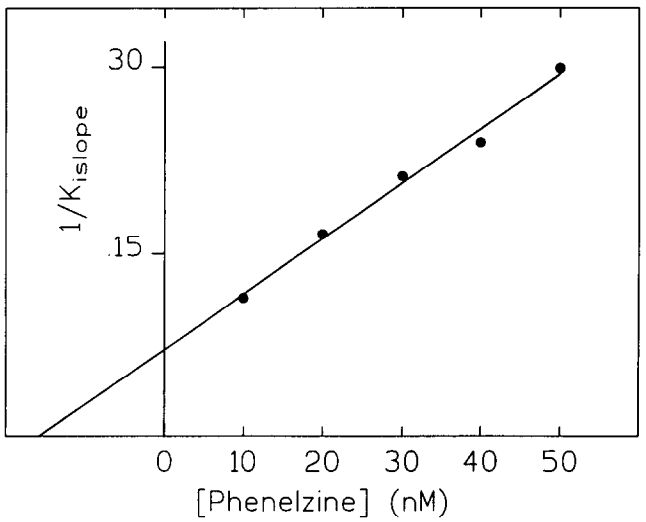


FIG. 6. Treatment of the inhibition of SSAO by phenelzine to determine the K_i value. Reciprocal K_{islope} values (obtained from Fig. 5, as described in text) were plotted vs phenelzine concentration. The line is that of best fit by regression analysis.

lower, concentrations of hydralazine, although the concentrations required to give 50% inhibition of the latter enzyme were about 100 times lower than those reported here for bovine lung SSAO [19]. IC_{50} values depend on the nature of the inhibitory process and, in the case of irreversible inhibitors, on the molar concentration of enzyme. However, they do provide a useful guide to relative potencies and time-dependence under any set of defined conditions, such as those used in the present studies (see [23] for discussion).

Phenylhydrazine irreversibly inhibited bovine lung SSAO with an IC_{50} value in the nanomolar concentration range. These results are in agreement with those previously reported by Falk [24] on amine oxidase activity in pig plasma, which differs in several other respects. The lack of any detectable time-dependence would suggest an extremely rapid reaction to form the inhibitory adduct. Further work using lower temperatures or rapid-reaction techniques would be necessary to study the kinetics of the inhibitory process to ascertain whether or not the formation of a noncovalent enzyme-inhibitor complex precedes the irreversible inhibitory step. Phenylhydrazine has been used for derivatising the TOPA quinone cofactor of several plasma amine oxidases prior to partial digestion and sequencing of the active site of the enzyme (see [25] for review). Thus, the present results suggest that a similar approach might also be used to study the active site of a membrane-bound SSAO enzyme.

Although sensitivity to inhibition by semicarbazide has been used as a method to distinguish between SSAO and MAO activities, the mechanism of inhibition has not previously been studied in detail. The SSAO from rat skull and lung was reported to be inhibited by semicarbazide in a reversible, noncompetitive manner [20], and the bovine and porcine aorta enzymes were irreversibly inhibited [26]. The results obtained in the present work indicated inhibition of the bovine lung microsomal enzyme to be time-dependent and irreversible. One might expect a group-specific reagent, such as this, to react unspecifically with the enzyme in a simple second-order reaction:

$$E + I \xrightarrow{k} EI^* \qquad (10)$$

However, under conditions where the inhibitor concentration was significantly greater than that of the enzyme, such a system would predict *pseudo*-first-order kinetics for the inhibitory process, in which the value of the apparent first-order rate constant would increase linearly with inhibitor concentration. The observations that semicarbazide inhibition gave saturation kinetics in this respect and, also, that inhibition was competitive during the initial phase of the reaction indicate that semicarbazide first forms a noncovalent enzyme-inhibitor complex, in which reaction subsequently takes place to give the irreversibly inhibited species. Such behaviour is shown by active-site directed inhibitors and also by mechanism-based (or k_{cat}) inhibitors. There would appear to be no necessity for postulating the latter type of mechanism in view of the intrinsic reactivity of semicarbazide. An alternative mechanism in which semicarbazide binds to the active site as a simple reversible inhibitor and behaves as a irreversible inhibitor in a separate reaction site [27]:

$$EI \underset{I}{\overset{K_i}{\rightleftharpoons}} E \underset{I}{\overset{k}{\rightarrow}} EI^* \qquad (11)$$

can, however, be excluded, because it would give a nonlinear dependence of $1/k'$ upon $[I]$. All these systems involve semicarbazide initially interacting with the enzyme in a competitive fashion. This observation may prove to be of value in attempts to design more specific SSAO inhibitors. However, competitive inhibition may not necessarily indicate that the inhibitor binds to the active site of the enzyme. Studies on the activation of bovine plasma amine oxidase by chlorpromazine led Mussey and Churchich to suggest that the binding of this effector could induce a conformation change of the plasma enzyme that was transmitted to the catalytic site [28].

Phenelzine has been widely used as an antidepressant drug due to its inhibitory effects on MAO activity [29]. More recently, it has also been shown to be a potent and reversible inhibitor of SSAO from different rat tissues. It was the most potent inhibitor of bovine lung microsomal SSAO among the hydrazine derivatives studied here (Table 1) and behaved as a competitive inhibitor, as has been previously described for different SSAO enzymes from other sources [29, 30]. However, despite the lack of detectable time-dependence of the inhibitory process, complete reversibility could only be demonstrated by dialysis at 37°C. It is possible that phenelzine behaves as a tight-binding inhibitor [31] that dissociates from the enzyme very slowly

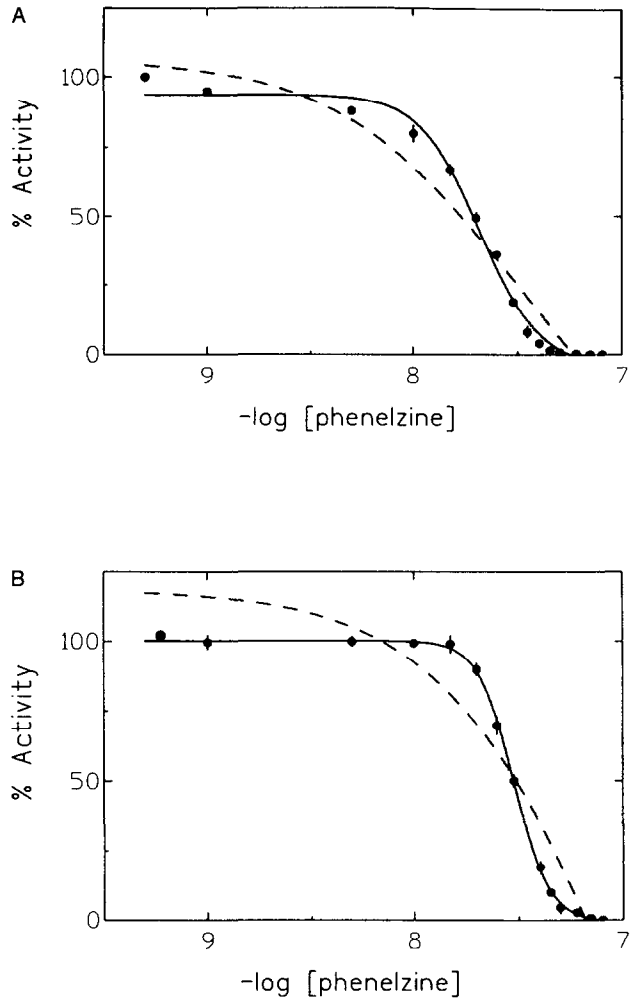


FIG. 7. Inhibition of SSAO activity by phenelzine. Samples of clorgyline-pretreated bovine lung microsomes were assayed for activity with (A) 20 μM benzylamine or (B) 100 μM 2-phenylethylamine in the presence of different phenelzine concentrations (1–100 nM range). Solid lines represent the best curve fits obtained by fitting the data to the Hill equation [22] by nonlinear regression analysis, which gave Hill constant values of 2.90 ± 0.06 and 3.12 ± 0.07 for benzylamine and 2-phenylethylamine, respectively. Broken lines represent the theoretical curve obtained with a value of 1 for the Hill constant. Each point represents the mean ± range of duplicate determinations. In both cases, the solid lines gave a significantly better fit than the broken curves ($P < 0.0001$, Student's *t*-test).

at lower temperatures. An alternative possibility, that a stable, perhaps covalent, intermediate is formed that only breaks down rapidly at elevated temperatures, cannot be excluded. Phenelzine is a substrate as well as an inhibitor of MAO [32] and further work is necessary to see if any transformation of the compound is catalysed by SSAO.

The kinetics of the inhibition were complex and consistent with the presence of multiple binding sites on the enzyme. The fact that the data gave an acceptable fit to a simple model involving two inhibitor binding sites does not, of course, establish the validity of that model, and several more complicated mechanisms are possible. Indeed, the relationship used to describe the dependence shown in Fig. 6 would still give linearity if the inhibitor concentration was raised to higher powers. If the system were at steady-state, for example, rather than at thermodynamic equilibrium for inhibitor binding, higher order effects would be expected. That would be consistent with the Hill constant of 3 obtained. An alternative explanation that cannot be excluded, would be a positively cooperative interaction with phenelzine involving a minimum of 3 interacting sites. Similar upwardly-curving kinetic plots have been observed for the inhibition of pig kidney diamine oxidase by nazlinin and 1-(4-butylamino)-3,4-dihydro-β-carboline [33].

Mussey and Churchich, using fluorescence methods, showed that the activation of bovine plasma amine oxidase by chlorpromazine was due to the presence of a heterogeneous population of binding sites [28]. We have previously reported reversible high-substrate inhibition of bovine lung microsomal SSAO by benzylamine, which could be explained by a simultaneous binding of two substrate molecules at the active centre of the enzyme [14]. In contrast, high-substrate inhibition was not evident when 2-phenylethylamine was used as substrate [14]. However, the complex behaviour of phenelzine inhibition was not a result of the specific substrate used because the inhibition given when 2-phenylethylamine was used as substrate was not significantly different, in terms of Hill constant, from that observed with benzylamine. Neither was the complexity a result of interactions of the enzyme with its membrane environment because extraction with a non-ionic detergent and partial purification did not affect the behaviour.

The sensitivity of SSAO to inhibition by carbonyl reagents, such as hydrazine derivatives, has frequently been ascribed to their covalent binding to a carbonyl-type cofactor that has recently been identified as trihydroxyphenylalanine (TOPA) residue in the peptide chain [25]. The observations in the present paper indicate that the behaviour of these compounds is much more complex and varied than would be expected for simple nonspecific group reactivity. Inhibition ranged from simple competitive, as might be expected for substrate analogues, through apparently tight-binding inhibition (at least at lower temperatures) and the existence of multiple inhibitory binding sites to apparently specific active-site-directed irreversible inhibitory behaviour. These different modes of interaction of relatively simple hydrazine derivatives may open the way to the successful design of potent and selective SSAO inhibitors as an aid to elucidating the functions of these enzymes. However, the results obtained here indicate that the mode of interaction of any new inhibitory compound would have to be studied in detail as an essential part of any structure-activity relationship correlations.

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