Inhibition of Monoamine Oxidase by Substituted Hydrazines

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1. The initial rate of inhibition of monoamine oxidase by phenethylhydrazine was shown to be similar, in pH-dependence and kinetic properties, to the oxidation of that compound by monoamine oxidase. 2. The time-course of irreversible inhibition of monoamine oxidase by phenethylhydrazine lags behind that of reversible inhibition. 3. Hydralzine was shown to be a reversible competitive inhibitor of monoamine oxidase, but phenethylhydrazine is an irreversible inhibitor. Inhibition by the latter compound is not affected by the absence of oxygen, and the presence of substrate exerts no protective action. 4. Hydrazine does not inhibit monoamine oxidase unless a substrate and oxygen are present. 5. Phenethylidenhydrazine was found to be a time-dependent inhibitor of monoamine oxidase and the rate of inhibition was hindered by increasing oxygen concentration. 6. A mechanism for the inhibition of the enzyme by phenethylhydrazine is proposed in which the product of oxidation of this compound is a potent reversible inhibitor and an irreversible inhibitor of the enzyme. A computer simulation of such a mechanism predicts time-courses of inhibition that are in reasonable agreement with those observed experimentally.

A wide variety of substituted hydrazines have been shown to be inhibitors of monoamine oxidase [monoamine-oxygen oxidoreductase (deaminating), EC 1.4.3.4] and have been used as antidepressant drugs (see Biel *et al.*, 1964, for review). The mechanism of inhibition of monoamine oxidase by these compounds has so far not been elucidated. Davison (1957) demonstrated an oxygen requirement for the inhibition of monoamine oxidase by iproniazid (1-isonicotinoyl-2-isopropylhydrazine); however, Smith *et al.* (1963) suggested that the presence of oxygen may be required for the non-enzymic conversion of iproniazid into isopropylhydrazine, which might be the actual inhibiting species. However, the presence of oxygen has been shown to be necessary for the inhibition of monoamine oxidase by hydrazine derivatives by Green (1964) and Tipton (1971*a*).

Clineschmidt & Horita (1969*a,b*) showed that the inhibitor phenethylhydrazine was a substrate as well as an inhibitor for monoamine oxidase in rat liver mitochondria, and Tipton & Spires (1972) showed that the immediate product of this oxidation was the corresponding hydrazone, phenethylidenhydrazine. In the present paper evidence is presented that indicates that the hydrazone formed by the monoamine oxidase-catalysed oxidation of phenethylhydrazine may represent the true inhibitory species. A preliminary account of part of this work has appeared (Tipton, 1971*b*).

Materials and Methods

Monoamine oxidase was purified from pig brain mitochondria by the method of Tipton (1968) and a partly purified preparation of rat liver monoamine oxidase was made by the procedure, adapted from the more complete purification method of Youdim & Sourkes (1966), previously reported (Tipton & Spires, 1971). Monoamine oxidase activity towards benzylamine was determined by the spectrophotometric method of Tabor *et al.* (1954) and the oxidation of phenethylhydrazine was followed spectrophotometrically at 235 nm (Tipton & Spires, 1971). Assays were carried out at 30°C in 0.2M-sodium phosphate buffer, pH 7.2, containing 100 units of catalase/ml. Extinction changes were measured in either a Hilger-Gilford or a Gilford 240 recording spectrophotometer. The oxygen concentration in the assay media was varied by the addition of solutions of sodium dithionite or H₂O₂ to the buffer mixture (Tipton & Spires, 1971). Protein concentrations were measured by the microbiuret method (Goa, 1953) with bovine serum albumin as a standard.

Catalase was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., and phenethylhydrazine (Phenelzine, Nardil) was obtained from Fluka A.G. Chemische Fabrik, Buchs, Switzerland. Hydralzine (1-hydrazinophthalazine) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and phenethylidenhydrazine was

prepared as described by Tipton & Spires (1972). All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of the highest purity available from this source. Distilled water was passed through a Permutit Mark II deionizer before use.

Results

pH-dependence of monoamine oxidase activity and inhibition

The effect of pH on the oxidative activity of monoamine oxidase preparations from rat liver and pig brain towards phenethylhydrazine and benzylamine are compared with the pH-dependence of the initial rates of inhibition of these preparations by phenethylhydrazine in Figs. 1(a) and 1(b) respectively. These results show that, although the pH-dependencies are widely different for the two enzyme preparations, the effects of pH on activity and inhibition are similar in each case.

Kinetics of inhibition by phenethylhydrazine

The effect of phenethylhydrazine concentration on the initial rate of activity loss is shown for the rat liver enzyme in the form of a reciprocal plot in Fig. 2. A linear plot was also obtained for the pig brain enzyme and values for the K_m for irreversible inhibition obtained in this way were $55\mu\text{M}$ for the rat liver enzyme and $30\mu\text{M}$ for the pig brain enzyme. These results are close to the calculated K_m values determined for these monoamine oxidase preparations with phenethylhydrazine as the substrate (Tipton & Spires, 1971).

Reversibility of the inhibition by phenethylhydrazine

Fig. 3 shows a comparison of the time-course of the inhibition of pig brain monoamine oxidase by phenethylhydrazine with the time-course for irreversible inhibition by this compound. The extent of irreversible inhibition was determined by subjecting samples of an enzyme-phenethylhydrazine incubation mixture to gel filtration through a column of Sephadex G-25 before the activity was assayed. The irreversible inhibition appears to lag behind the total inhibition. Qualitatively similar results were obtained with the rat liver enzyme.

Inhibition of monoamine oxidase by hydralzine

The structure of hydralzine (1-hydrazinophthalazine) is shown in formula (I). When pig brain and rat liver monoamine oxidase were incubated with $250\mu\text{M}$ -hydralzine in 0.2M -sodium phosphate buffer, pH 7.2, at 30°C for periods of up to 60 min, no time-

Fig. 1. Effect of pH on the inhibition and oxidative activity of monoamine oxidase

The activities of monoamine oxidase towards benzylamine (●) and 2-phenethylhydrazine (Δ) were measured at 30°C by observing the changes in extinction at 250 and 235 nm respectively. The substrate concentrations used were 0.1 mM in both cases. The initial rate of inhibition of the activity of monoamine oxidase towards benzylamine was determined at 30°C by drawing tangents to curves of activity against preincubation time (○). The buffer used throughout was 0.2M -tris-HCl. Activities and initial rates of inhibition are expressed in arbitrary units. Fig. 1(a) shows the results with the rat liver enzyme and the results obtained with the pig brain enzyme are shown in Fig. 1(b).

dependent inhibition was observed. The apparently instantaneous inhibition that occurred could be completely reversed by gel filtration of the enzyme-hydralzine mixture through a column of Sephadex

Fig. 2. Effect of 2-phenethylhydrazine concentration on the inhibition of rat liver monoamine oxidase

A reciprocal plot of the initial rate of loss of activity towards benzylamine against 2-phenethylhydrazine concentration is shown. Preincubation was carried out at 30°C in 0.2M-sodium phosphate buffer, pH 7.2, in a total volume of 1.9ml. Activity was determined after incubation by the addition of 100 μ l of 10mM-benzylamine.

G-25 equilibrated with the phosphate buffer. Kinetic studies indicated that the reversible inhibition of the pig brain and rat liver enzymes by hydralazine was competitive with respect to benzylamine. A Dixon (1953) plot for the inhibition of the pig brain enzyme is shown in Fig. 4. From this plot a K_i value of 12 μ M was determined. The K_i value for inhibition of the rat liver enzyme was calculated to be 42 μ M.

Inhibition of monoamine oxidase by phenylhydrazine

Inhibition was time-dependent, as shown in Fig. 5. The presence of the substrate benzylamine at concentrations of up to 500 μ M exerted no protective effect, and the time-course of inhibition was insensitive to depletion of oxygen from the medium. The inhibition could not be reversed by gel filtration at any stage of the incubation.

Inhibition of monoamine oxidase by hydrazine

Progress curves for the oxidation of benzylamine by pig brain monoamine oxidase in the presence and the absence of 5mM-hydrazine hydrochloride are shown in Fig. 6. The presence of hydrazine causes a pronounced curvature of the progress curve, but the time-course is unaffected by preincubation of the enzyme with hydrazine for periods of up to 20min

Fig. 3. Time-course of inhibition of pig brain monoamine oxidase by phenethylhydrazine

Enzyme (200 μ g) was incubated at 30°C with 1mM-phenethylhydrazine in a total volume of 10ml of 0.2M-sodium phosphate buffer, pH 7.2. Samples of the mixture were removed at fixed time-intervals and the activity was assayed. Other samples of the mixture were passed through a column (10cm $\times$ 1cm) of Sephadex G-25 (coarse grade) equilibrated with 0.2M-sodium phosphate buffer, pH 7.2, before the activity was assayed. The activity was assayed by adding the enzyme solution to a cuvette containing 500 μ M-benzylamine in the sodium phosphate buffer to give a final volume of 2ml, and measuring the oxidation of benzylamine spectrophotometrically (Tabor *et al.*, 1954). The degree of dilution of the enzyme sample that occurred on gel filtration was found by determination of the protein concentration by the microbiuret method (Goa, 1963) with bovine serum albumin as a standard. Activity of the mixture before gel filtration ($\bullet$); activity after gel filtration ($\circ$).

(I) Hydralazine (1-hydrazinophthalazine)

before the addition of substrate. No inhibition occurred when monoamine oxidase was incubated with 5mM-hydrazine and 500 μ M-benzylamine in the absence of oxygen for 60min.

Inhibition of monoamine oxidase by phenethylidenehydrazine

The inhibition of pig brain monoamine oxidase by phenethylidenehydrazine was studied at a series of oxygen concentrations. Fig. 7 shows that this compound is a time-dependent inhibitor of the enzyme and that the presence of oxygen appears to exert a protective action. The inhibition of the enzyme by

Fig. 4. Inhibition of pig brain monoamine oxidase by hydralazine (1-hydrazinophthalazine)

A Dixon (1953) plot of the initial rate of benzylamine oxidation, measured at 30°C in 0.2M-sodium phosphate buffer, pH 7.2, against inhibitor concentration is shown. The benzylamine concentrations used were: 100 μM (▲), 50 μM (●), 25 μM (○) and 12.5 μM (Δ).

phenethylidenhydrazine could not be reversed by gel filtration through a column of Sephadex G-25.

Simulation of the inhibitory process

A computer simulation study was done to find the time-course of reversible and irreversible inhibition of monoamine oxidase that would be given by the overall reaction pathway shown in Scheme 1.

The simulation studies were carried out by using computer programs written for the University of Cambridge Titan computer by Hulme (1971) and J. A. Illingworth (unpublished work: details of this computer program, which is written in machine code for the University of Cambridge Titan computer, may be obtained from Dr. J. A. Illingworth, Department of Biochemistry, Cambridge, CB2 1QW). The K_m and V_{max} values for phenethylhydrazine oxidation that are required to characterize reactions (1) and (2) were taken to be 55 μM and 51 μmol of substrate oxidized/min per μmol of enzyme respectively (Tipton & Spire, 1971), assuming the molecular weight of the enzyme to be 102000 (Tipton, 1968). Because of the irreversible inhibition of the enzyme by phenethylidenhydrazine it was difficult to get an accurate assessment of the K_i value of this compound

Fig. 5. Inhibition of pig brain monoamine oxidase by phenylhydrazine

Samples of the enzyme were incubated at 30°C in mixtures containing 50 μM -phenylhydrazine hydrochloride and 200 units of catalase in 0.2M-sodium phosphate buffer, pH 7.2, in a final volume of 1.9 ml. After fixed times 100 μl of 10 mM-benzylamine was added for activity determination. The loss of activity with time is shown as a percentage of the initial activity (▲). In another series of experiments (●), the oxygen concentration of the mixtures was decreased to zero by the addition of $\text{Na}_2\text{S}_2\text{O}_4$ and incubations were done under an atmosphere of N_2 . The mixtures were re-equilibrated with oxygen, after the addition of benzylamine, by shaking them with a fixed amount of H_2O_2 (Tipton & Spire, 1971). The solutions of $\text{Na}_2\text{S}_2\text{O}_4$ and H_2O_2 used were standardized by titration of the oxygen concentration of a sample mixture in a Clark oxygen electrode. The time-course of inhibition of the enzyme in the presence of 500 μM -benzylamine (○) was determined by drawing tangents to a progress curve for the oxidation of this substrate in the incubation mixture.

acting as a reversible inhibitor of the enzyme (Tipton & Spire, 1971). This K_i value is, however, needed to characterize reaction (3), and an approximate value for this constant was calculated to be 8 μM from Dixon (1953) plots of results obtained with benzylamine as the substrate. The rate constant required for reaction (4) was taken as being equal to the apparent

Fig. 6. Inhibition of pig brain monoamine oxidase by hydrazine

The activity of monoamine oxidase was assayed in a mixture containing 5mM-hydrazine hydrochloride, 500 μ M-benzylamine and enzyme in 0.2M-sodium phosphate buffer, pH 7.2, in a total volume of 2ml. The time-course of the reaction (○) is compared with time-courses in which the enzyme-hydrazine-buffer mixture had been incubated at 30°C for 6 min (▲) and 30 min (●) and for the time-course in the absence of hydrazine (Δ). Activity was assayed with an oxygen electrode (Tipton, 1968).

first-order rate constant, calculated from a semi-logarithmic plot of the curve shown in Fig. 7 to be 0.132 min^{-1} . All values were determined at an oxygen concentration of 0.23mM and at 30°C in 0.2M-sodium phosphate buffer, pH 7.2. The phenethylhydrazine concentration in the reaction mixture was 100 μ M and was kept constant at that value throughout the simulation.

The simulation of these data is shown in Fig. 8. The program prints out the variations in concentration of all reactant species with time. Thus the time-course of irreversible inhibition will parallel the time-course of formation of the E-phenethylidenhydrazine complex. The time-course of reversible inhibition is slightly more complicated in that the enzyme species which can give rise to activity are the free enzyme and the E-phenethylidenhydrazine and E-phenethylhydrazine complexes. Since the enzyme-inhibitor mixture is diluted 20 times for the assay of the activity towards benzylamine (500 μ M), it can be calculated that the amount of inhibition caused by competition of phenethylhydrazine for the enzyme will be negligible. The extent of reversible inhibition of the enzyme by phenethylidenhydrazine was calculated by assuming it to be a competitive inhibitor with respect to benzylamine and by using a K_m value for

Fig. 7. Inhibition of monoamine oxidase by phenethylidenhydrazine

Pig brain monoamine oxidase (40 μ g) was incubated in a total volume of 1.9ml, with 88 μ M-phenethylidenhydrazine at 30°C in 0.2M-sodium phosphate buffer, pH 7.2, containing 100 units of catalase/ml for the indicated times, before the activity was assayed by the addition of 100 μ l of 10mM-benzylamine. The oxygen concentrations of the samples were adjusted by the addition of a standardized $\text{Na}_2\text{S}_2\text{O}_4$ solution. After the addition of substrate the oxygen concentration was readjusted to 230 μ M by the addition of a standardized H_2O_2 solution. Oxygen concentrations were used as follows: 155 μ M (■), 200 μ M (●), 230 μ M (▲). The time-course of inhibition when the phenethylidenhydrazine was replaced by phenethylhydrazine (250 μ M) is also shown (○) for an oxygen concentration of 230 μ M.

Scheme 1. Reaction pathway for the inhibition of monoamine oxidase by phenethylhydrazine

the pig brain enzyme oxidizing benzylamine of 165 μ M.

The simulated time-course for the inhibition of the enzyme by phenethylhydrazine is compared with values for the time-courses of irreversible and total inhibition in Fig. 9.

Fig. 8. Computer simulation of the inhibition of monoamine oxidase by phenethylhydrazine

The time-courses of variation of the concentrations of the reactants for the reaction scheme shown in Scheme 1 were calculated by using the University of Cambridge Titan computer. E-phenethylidene hydrazine (μM) (Δ); E-phenethylhydrazine (μM) ($\square$); phenylethylidene hydrazine ($\text{mM} \times 2$) ($\bullet$); E-phenylethylidene hydrazine (μM) ($\blacktriangle$); free enzyme ($\times 10^{-8} \text{M}$) ($\circ$).

Fig. 9. Time-courses for reversible and irreversible inhibition of monoamine oxidase calculated from the simulated data shown in Fig. 8

Inhibition is expressed as a percentage of the initial activity. Curve (a), irreversible inhibition; curve (b), total inhibition (reversible plus irreversible). Experimentally determined results taken from Fig. 3 are shown for comparison. Total inhibition ($\bullet$); irreversible inhibition ($\circ$).

Discussion

Although the pig brain and rat liver monoamine oxidase preparations exhibit markedly different pH

optima, the similarity between the pH-dependencies of the oxidation and inhibition reactions in each case suggests that the oxidation of, and inhibition by, phenethylhydrazine may be intimately linked phenomena. This view is supported by the close agreement between the apparent K_m values calculated for phenethylhydrazine oxidation and inhibition, and between the apparent K_m value for oxygen calculated from the initial rate of inhibition (Tipton, 1971a) and the K_m value for oxygen calculated for the oxidation of phenethylhydrazine (Tipton & Spires, 1971).

The studies on the reversibility of inhibition indicated that the time-course of irreversible inhibition lags behind the time-dependent inhibition. This observation can be explained in terms of the reaction system shown in Scheme 1 in which the enzymic oxidation of phenethylhydrazine produces a powerful competitive inhibitor of monoamine oxidase (phenethylidenehydrazine), which is also acting as a time-dependent irreversible inhibitor of the enzyme. That such a mechanism can account for the observed time-courses of total inhibition and irreversible inhibition is shown by the simulation studies illustrated by Figs. 8 and 9. These studies make a number of simplifying assumptions about the system and can only be regarded as being approximate, but nevertheless a comparison between the curves simulated and the experimentally determined inhibition curves indicates a satisfactory agreement.

If the oxidation of the hydrazine to the corresponding hydrazone represents the generation of the inhibitory species, it would follow that substituted hydrazines that could not act as substrates for monoamine oxidase would not act as irreversible inhibitors of the enzyme in this way. One such hydrazine is hydralazine (I). This compound would not be capable of undergoing dehydrogenation at the carbon-nitrogen bond of the hydrazine, and the failure of this compound to act as an irreversible inhibitor of the enzyme is thus in accord with the mechanism of inhibition shown in Scheme 1. The observation that hydralazine is a reversible inhibitor of monoamine oxidase is in agreement with the results of Hellerman & Erwin (1968), which were obtained with the enzyme purified from ox liver. The failure of acylhydrazines to act as irreversible inhibitors of monoamine oxidase (Barsky *et al.*, 1959) is also in accord with this view.

Phenylhydrazine would be expected not to act as an irreversible inhibitor of monoamine oxidase, since, like hydralazine, it cannot undergo dehydrogenation at the carbon-nitrogen bond. This compound has, however, been shown to act as a time-dependent inhibitor of the enzyme from ox liver (Hellerman & Erwin, 1968) and the results in Fig. 5 show that it is also a time-dependent inhibitor of the pig brain enzyme. The properties of the inhibitory process are, however, different from those shown for the inhibition of the enzyme by phenethylhydrazine.

There is no significant difference between the time-course of total inhibition and reversible inhibition, the presence of benzylamine at a concentration of 500 μ M gives no detectable protection against inhibition, and the inhibition does not appear to require the presence of oxygen. Thus it would appear that inhibition of the enzyme by phenylhydrazine differs considerably in mechanism from the inhibition by phenethylhydrazine and perhaps other aliphatic hydrazines.

The proposed mechanism of inhibition shown in Scheme 1 would predict that free hydrazine would be capable of acting as an inhibitor of monoamine oxidase if the enzyme were producing an aldehyde product that could react with the hydrazine to produce an inhibitory hydrazone. Fig. 6 shows that hydrazine itself is not a time-dependent inhibitor of monoamine oxidase, but it does cause time-dependent inhibition of an enzyme-benzylamine mixture, which is in accord with this prediction.

It has been previously suggested that the inhibition of monoamine oxidase by hydrazine derivatives may involve a catalytic effect of the enzyme (Davison, 1957; Zeller & Sarkar, 1962; Bloom, 1963; Smith *et al.*, 1963; Green, 1964; McEwen *et al.*, 1969). The mechanism proposed here is similar to that proposed by Davison (1957) and is in accord with the observations that the efficiency of hydrazine inhibition parallels the efficiency with which the parent amines are oxidized by monoamine oxidase (Zeller *et al.*, 1960; Bloom, 1963). An exception to this general parallel is the compound 1-methyl-2-phenethylhydrazine, which has been found to be a potent inhibitor of monoamine oxidase (Horita, 1958), whereas the parent amine, amphetamine, is attacked either feebly or not at all by brain monoamine oxidase (see, e.g., Guha & Mitra, 1971). Belleau & Moran (1963) have suggested that the failure of amphetamine to act as a substrate for the enzyme may be due to steric interference by the α -methyl group. The failure of amphetamine to act as a substrate for the enzyme could be due either to a failure to dehydrogenate the amine, or to a failure to hydrolyse the resulting imine.

Chessin *et al.* (1959) found that phenethylidenehydrazine was active as a monoamine oxidase inhibitor *in vivo* with a potency, when administered orally or intraperitoneally to mice, which appeared to be somewhat greater than that of iproniazid. The observation that oxygen appears to protect monoamine oxidase against time-dependent inhibition by phenethylidenehydrazine may be interpreted in the light of the kinetic mechanisms which appear probable for the oxidation of 2-phenethylidenehydrazine (Tipton & Spires, 1971), since in such mechanisms

the binding of oxygen is necessary for the displacement of phenethylidenehydrazine from the enzyme.

Since the generation of the inhibitory species involves the catalytic action of the enzyme, it may be possible to utilize hydrazine derivatives of specific compounds as selective inhibitors of monoamine oxidase in tissues where multiple forms of the enzyme with differing substrate specificities appear to exist (see Youdim *et al.*, 1970, for review).

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