The Inhibition of Monoamine Oxidase by Arylalkylhydrazines

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Arylalkylhydrazines, such as β -phenylethylhydrazine and β -phenylisopropylhydrazine, are among the most powerful known inhibitors of monoamine oxidase [monoamine-oxygen reductase (deaminating), EC 1.4.3.4]. The inhibition of monoamine oxidase by these compounds *in vivo*, and its consequent biochemical and pharmacological effects, have been widely investigated (e.g. see Symposium on Monoamine Oxidase Inhibitors, 1959), but little has been reported on the process of inhibition *in vitro*. This paper describes studies on the kinetics of inhibition by benzylhydrazine and some related compounds. Considerable differences have been shown to exist between the kinetic patterns of inhibition by arylalkylhydrazines and inhibition by hydrazides such as iproniazid (1-isopropyl-2-isonicotinylhydrazine). The apparent inhibitory power of these compounds depends on several factors besides the intrinsic reactivity of the inhibitor with the enzyme, and measurements of pI_{50} values under arbitrary conditions may be misleading if taken as criteria of this reactivity.

METHODS

Preparation of hydrazine derivatives. The arylalkylhydrazines were obtained by boiling the corresponding arylalkyl chloride or bromide with 5 molar equivalents of hydrazine hydrate in ethanol. The free bases were then converted into their crystalline hydrochlorides by addition of HCl in ether to a solution of the base in ethanol and ether (Biel *et al.* 1959).

The acylated benzylhydrazines were obtained by heating benzylhydrazine for 24–48 hr. with 2-ethoxyethyl acetate, methyl butyrate, ethyl benzoate or ethyl nicotinate at 140° or at reflux temperature, whichever was the lower (Fox & Gibas, 1953).

Analytical data and melting points for the compounds tested as inhibitors are given in Tables 5 and 6.

Preparation of tissue fractions. Guinea-pig liver was homogenized in ice-cold 0.25 M-sucrose (9 ml./g. of liver) with a Potter-Elvehjem-type glass homogenizer and the homogenate was centrifuged for 10 min. at 600g to remove cell debris. Fractions containing predominantly mitochondria or microsomes were then isolated by spinning at 0–4° for 10 min. at 5000g, then for 30 min. at 70 000g (Hogeboom, 1955). The two sediments were resuspended in 0.1 M-phosphate buffer, pH 7.4 (83 ml. of 0.1 M- Na_2HPO_4 + 17 ml. of 0.1 M- NaH_2PO_4); 1 ml. of suspension was equivalent to 1 g. wet wt. of liver. In one series of experiments

the fractions were isolated as described above from liver homogenized in phosphate buffer instead of in sucrose (cf. Davison, 1957). All suspensions were stored at –10° until required.

The protein concentrations in the mitochondrial and microsomal fractions, determined by the biuret reaction (Aldridge, 1957), were approx. 32 mg./ml. and 17 mg./ml. respectively.

Measurement of monoamine-oxidase activity. The mitochondrial fraction isolated in sucrose was used as monoamine oxidase in all experiments except where otherwise stated. This preparation was normally diluted about tenfold with buffer before use and the monoamine-oxidase activity was determined by the dinitrophenylhydrazine method (Green & Haughton, 1961).

The effects of inhibitors were studied as follows. Mixtures of 1 ml. of diluted enzyme with 1.8 ml. of buffer were placed in loosely stoppered 25 ml. conical flasks, which were shaken in a water bath at 25° for about 10 min. to allow the contents to reach uniform temperature. Aqueous inhibitor (0.4 ml.) was then added to each flask at appropriate time intervals. After the desired time (or times) of contact between enzyme and inhibitor, 0.1 M-tyramine hydrochloride (0.4 ml.) and 0.125 M-semicarbazide hydrochloride (0.4 ml.) were added, both these solutions having been previously neutralized to pH 7.4. The flasks were shaken in the bath for a further 30 min., when 0.5 N-acetic acid (1 ml.) was added to each flask to stop the reaction. The mixtures were then deproteinized and the remaining steps in the determination of the residual monoamine-oxidase activity were carried out as described by Green & Haughton (1961). In each experiment, controls and blanks were run, in which water was added instead of inhibitor or tyramine respectively.

RESULTS

Time-course of inhibition. In order to obtain marked inhibition of monoamine oxidase by low concentrations of hydrazine derivatives it is necessary to allow the inhibitor to react with the enzyme for some time before any substrate is added (Zeller, Barsky & Berman, 1955). Addition of tyramine (to give a final concentration of 0.01 M) at any time after the inhibitor effectively prevents further increase in the degree of inhibition although it does not reverse inhibition that has already occurred. Fig. 1 shows the type of curve obtained when the percentage inhibition produced by benzylhydrazine, α -naphthylmethylhydrazine or iproniazid was plotted logarithmically against the

duration of contact between enzyme and inhibitor before addition of substrate. Curves similar to those shown for benzylhydrazine and α -naphthylmethylhydrazine, in which the rate of inhibition during this preincubation period fell rapidly with

Fig. 1. Time-course of monoamine-oxidase inhibition by: $\square$, benzylhydrazine ($0.4 \mu\text{M}$); $\circ$, α -naphthylmethylhydrazine ($20 \mu\text{M}$); $\triangle$, iproniazid ($30 \mu\text{M}$). Aqueous inhibitor (0.4 ml.) was shaken at 25° with 2.8 ml. of an approx. 25-fold dilution of the mitochondrial enzyme preparation in phosphate buffer (0.1 M , pH 7.4) for various times before the addition of 0.1 M -tyramine (0.4 ml.) and 0.125 M -semicarbazide (0.4 ml.).

Fig. 2. Time-course of monoamine-oxidase inhibition by: $\bullet$, benzylhydrazine ($0.5 \mu\text{M}$); $\circ$, α -naphthylmethylhydrazine ($20 \mu\text{M}$); $\triangle$, iproniazid ($5 \mu\text{M}$). Aqueous inhibitor (0.4 ml.) was shaken at 25° with 2.8 ml. of an approx. 25-fold dilution of the mitochondrial enzyme preparation in phosphate buffer (0.1 M , pH 7.4) for various times before addition of 0.1 M -tyramine (0.4 ml.) and 0.125 M -semicarbazide (0.4 ml.). Residual enzyme activity is calculated as a percentage of the activity of the enzyme ($\square$) in a series of control flasks without inhibitor.

time, were found with all the other arylalkylhydrazines that were tested. Hydrazides gave straight lines, such as that shown for iproniazid, indicating that the rate of inhibition was independent of time, or shallow curves indicating a slight fall in rate with increased duration of contact.

The extent of inhibition found with the same three compounds when the period of preincubation with inhibitor was greatly prolonged is illustrated in Fig. 2. As the enzyme in buffer alone deteriorates to some extent on prolonged shaking at 25° , the degree of inhibition was calculated as the percentage of the activity of control samples containing no inhibitor. The extent of inhibition produced by benzylhydrazine became constant in about 1 hr., then remained constant up to at least 5 hr. With α -naphthylmethylhydrazine, inhibition became maximal in about 30 min., but appreciable recovery had occurred in 5 hr. The rate of inhibition by iproniazid remained constant until inhibition was virtually complete and there was no indication of recovery.

Effect of inhibitor concentration and the presence of substrate. An increase or decrease in inhibitor concentration led to an increase or decrease in the extent of inhibition produced in a given time but did not change the general appearance of the time-dependence curves. The rate of inhibition by iproniazid, calculated directly from the slopes of the lines obtained in logarithmic plots such as those shown in Figs. 1 and 2, was roughly proportional to the iproniazid concentration.

If the substrate was added to the enzyme at the same time as the inhibitor, inhibition was completely prevented unless the inhibitor concentration was greatly increased. Thus if the substrate was added 15 min. after the inhibitor, 50% inhibition was obtained with about $0.3 \mu\text{M}$ -benzylhydrazine or $5 \mu\text{M}$ - α -naphthylmethylhydrazine. If the substrate and inhibitor were added at the same time it was necessary to raise these concentrations to $> 10 \mu\text{M}$ or $> 100 \mu\text{M}$ respectively in order to obtain 50% inhibition.

Inhibition by all the other compounds tested was likewise suppressed in the presence of 10 mM -tyramine.

Dependence of inhibitory action on enzyme concentration. When mixtures containing a constant concentration of inhibitor but different concentrations of enzyme were shaken for a fixed time and were then diluted with buffer, substrate and semicarbazide mixtures to give the same final concentration of enzyme, the degree of inhibition produced by benzylhydrazine and α -naphthylmethylhydrazine was found to decrease strikingly as the initial enzyme concentration was raised, whereas inhibition by iproniazid was barely affected (see Table 1). This effect was not due to the lower final

inhibitor concentration in those mixtures containing the higher initial enzyme concentration since addition of sufficient inhibitor to the buffer-substrate mixture to ensure that the final inhibitor concentration would be the same in each case did not significantly increase the extent of inhibition observed. When mixtures of enzyme with benzylhydrazine or α -naphthylmethylhydrazine were incubated for a fixed time, the degree of inhibition was in fact found to depend approximately on the enzyme:inhibitor ratio. This was not the case for inhibition by iproniazid (see Table 2).

These results suggested that the arylalkylhydrazines were being lost from the solution by interaction with some component of the enzyme preparation and that the cessation of inhibition after contact between enzyme and inhibitor for about 30 min. (Fig. 2) was due to the disappearance of all free inhibitor. This was demonstrated for α -naphthylmethylhydrazine as follows. One flask (A) containing enzyme, buffer and inhibitor ($20\ \mu\text{M}$) and two flasks (B and C) containing enzyme and buffer alone were shaken at 25° for 30 min., when a sample from each flask was added to buffer, substrate and semicarbazide and analysed for monoamine-oxidase activity. The remaining contents of the flasks were chilled and centrifuged at 0° for 30 min. at 40 000g in order to sediment all

the enzymic material. The supernatant solutions were separated from the sediments, which were then resuspended in the supernatant from one of the other flasks, i.e. the sediment from the flask containing inhibitor (A) was suspended in the supernatant from a flask containing no inhibitor (B), the sediment from this flask was suspended in the supernatant from the third flask (C) and the sediment from this flask was suspended in the supernatant from the flask containing inhibitor (A). The three mixtures were returned to the water bath, shaken for 30 min. at 25° , and then sampled and assayed. The results are shown in Table 3. The virtual absence of inhibition in the flask containing uninhibited sediment plus the supernatant from the flask (A) originally containing inhibitor shows that little free inhibitor can have been left in this supernatant. When the preincubation time in this experiment was reduced from 30 to 10 min. some inhibitor was still left, although much had already disappeared (see Table 3). In both cases there appeared to be slight recovery of activity on adding inhibitor-free supernatant to the sedimented inhibited enzyme.

Disappearance of free benzylhydrazine by interaction with the mitochondrial enzyme preparation. The disappearance of free benzylhydrazine when shaken with the mitochondrial enzyme preparation

Table 1. *Effect of enzyme concentration on monoamine-oxidase inhibition by benzylhydrazine, α -naphthylmethylhydrazine or iproniazid*

Various concentrations of mitochondrial suspension (1 ml. equivalent to 1 g. wet wt. of liver) were shaken at 25° in phosphate buffer (0.1M, pH 7.4) with a constant concentration of inhibitor for 10 min. The mixtures were then diluted with buffer, semicarbazide and tyramine to give the same final enzyme concentration, and assayed for monoamine oxidase. Percentage inhibition is based on controls containing no inhibitor.

Vol. of mitochondrial suspension (ml.)	Vol. of enzyme + buffer + inhibitor (ml.)	Relative initial concn. of enzyme	Inhibition (%)		
			With $1\ \mu\text{M}$ -benzyl- hydrazine	With $20\ \mu\text{M}$ - α -naphthyl- methylhydrazine	With $50\ \mu\text{M}$ - iproniazid
0.3	12.8	1	86	72	64
0.6	6.4	4	61	30	64
1.5	3.2	20	<5	<5	58

Table 2. *Effect of enzyme:inhibitor ratio on monoamine-oxidase inhibition by benzylhydrazine, α -naphthylmethylhydrazine or iproniazid*

Mixtures containing a constant ratio of mitochondrial suspension to inhibitor were shaken for 30 min. at 25° and pH 7.4. They were then diluted with buffer, semicarbazide and substrate to give the same final enzyme concentration, and assayed for monoamine oxidase. Percentage inhibition is based on controls containing no inhibitor.

Vol. of mitochondrial suspension (ml.)	Vol. of enzyme + buffer + inhibitor (ml.)	Initial concn. of inhibitor (μM)	Inhibition (%)		
			With benzyl- hydrazine	With α -naphthyl- methylhydrazine	With iproniazid
1.5	3.2	1	50	—	—
0.6	12.8	0.1	45	—	—
1.5	3.2	50	—	60	91
0.6	12.8	5	—	56	47

Table 3. *Loss of α -naphthylmethylhydrazine on contact with the mitochondrial enzyme preparation*

Enzyme (0.5 ml.) was shaken at 25° with buffer (pH 7.4, 10.7 ml.) and inhibitor (0.16 mM, 1.6 ml., flask A) or water (1.6 ml., flasks B and C). After 10 or 30 min., 3.2 ml. from each flask was added to tyramine (0.4 ml.) and semicarbazide (0.4 ml.) and assayed for monoamine-oxidase activity. The remaining flask contents were then spun for 30 min. at 0° and 40 000g, after which the sediments and supernatants were interchanged. After a further 10 or 30 min. at 25°, 3.2 ml. portions were again added to tyramine and semicarbazide and assayed. Activities are corrected for denaturation or loss of enzyme during centrifuging, resuspension and reincubation at 25°.

Incubation time (min.)	Relative enzyme activities				
	Before interchange		After interchange		
	Inhibitor absent (B and C)	Inhibitor present (A)	Uninhibited sediment (B) + supernatant without inhibitor (C)	Uninhibited sediment (C) + supernatant with inhibitor (A)	Inhibited sediment (A) + supernatant without inhibitor (B)
10	100	26	100	68	30
30	100	18	100	94	31

Table 4. *Disappearance of free benzylhydrazine by interaction with the mitochondrial enzyme preparation*

Benzylhydrazine (0.55 ml. of 40, 10 or 4 μ M) was shaken at 25° and pH 7.4 for 30 min. with enzyme [2.2 ml. of a dilution (1:6) of the stock suspension with buffer] or buffer (2.2 ml.). The mixtures were then chilled and spun at 40 000g for 30 min.; 0.4 ml. of each supernatant was then added to 0.5 ml. of diluted enzyme and 2.3 ml. of buffer. After 15 min., tyramine (0.4 ml.) and semicarbazide (0.4 ml.) were added and the mixture was assayed for monoamine oxidase.

Final concn. of benzylhydrazine assuming no loss (μ M)	Inhibition (%)	
	Benzylhydrazine + enzyme	Benzylhydrazine + buffer
1	20	85
0.25	0	67
0.1	0	11

is shown by the experiment summarized in Table 4. After a mixture of enzyme and inhibitor (8, 2 or 0.8 μ M) had been shaken for 30 min. at 25°, all enzymic material was removed by centrifuging for 30 min. at 40 000g. The supernatant was then tested for inhibitory properties against fresh enzyme. In the control samples the benzylhydrazine was mixed with buffer, in which it is stable for at least an hour, instead of enzyme. When the initial benzylhydrazine concentration was raised from 8 to 200 μ M (the supernatant being diluted before assay for inhibitory activity) very little loss of inhibitory action could be detected, hence the rate of disappearance of free inhibitor does not increase linearly with its concentration.

Preincubation for 30 min. of benzylhydrazine (1 μ M) with sucrose (1 mM) or pyridoxal (20 μ M) did not decrease the extent of inhibition observed

when the enzyme was subsequently added. Incubation of the enzyme preparation with ethylenediaminetetra-acetic acid (12.5 mM neutralized to pH 7.4) before addition of benzylhydrazine also had no effect on the extent of inhibition.

Effect of cyanide and semicarbazide on the inhibition of monoamine oxidase by arylalkylhydrazines. The marked potentiating effect of CN⁻ ion on inhibition of monoamine oxidase by iproniazid (Davison, 1957) also appears to occur generally with arylalkylhydrazines. For example, addition of sodium cyanide (1 mM) to the enzyme before addition of 3,4-dichlorobenzylhydrazine (0.5 μ M) or 3,4-dimethoxybenzylhydrazine (1 μ M) increased the extent of inhibition produced in 15 min. from about 40% to well over 90%. Addition of semicarbazide (12.5 mM) to the enzyme before addition of the inhibitor (instead of adding it together with the substrate) also changed the extent of inhibition by most compounds, although not by all. The direction of this effect varied from compound to compound and showed no obvious relationship to the structure of the inhibitor. For example, semicarbazide roughly doubled the concentrations of iproniazid or 3,4-dimethoxybenzylhydrazine required to produce 50% inhibition, but halved the necessary concentrations of 3,4-dichlorobenzylhydrazine or of benzylhydrazine itself. The general appearance of the inhibition-time curves (such as in Fig. 1) was unchanged by the presence of semicarbazide. The magnitude of the semicarbazide effect was generally much less than the magnitude of the cyanide effect. Cyanide and semicarbazide themselves at the concentrations used in these experiments have no direct effect on monoamine-oxidase activity (Blaschko, Richter & Schlossmann, 1937).

Comparative effects of benzylhydrazine and iproniazid on monoamine oxidase in liver homogenate and in mitochondrial and microsomal fractions. It has been shown (Zeller *et al.* 1955; Davison, 1957)

that monoamine oxidase in isolated mitochondria is much more susceptible to inhibition by iproniazid than monoamine oxidase in tissue homogenates. The extent of inhibition produced by benzylhydrazine also varies with the nature of the enzyme preparation but the type of variation differs from that found with iproniazid. In order to test the homogenate, the mitochondrial and the microsomal fractions under comparable conditions, guinea-pig liver was homogenized directly in phosphate buffer instead of in sucrose. Cell debris was removed from the homogenate and the two sub-cellular fractions were separated by centrifuging as described under Methods. The monoamine oxidase in the microsomal fraction was only slightly less sensitive to iproniazid than the enzyme in the mitochondrial fraction, 50 % inhibition being produced in 10 and 7 min. respectively by 50 μ M-iproniazid, but the enzyme in the homogenate was, as expected, much less sensitive, showing only 20 % inhibition in 30 min. even with 100 μ M-inhibitor. In contrast, monoamine oxidase in the homogenate was not much less sensitive to benzylhydrazine than the enzyme in the mitochondrial fraction whereas that in the microsomal fraction was inhibited much less. The concentrations of benzylhydrazine giving 50 % inhibition of approximately equal quantities of monoamine oxidase in the homogenate, mitochondrial fraction and microsomal fraction respectively were 0.4, 0.2 and 2 μ M. Inhibition of all three preparations by benzylhydrazine or iproniazid showed the same type of

time-dependence as illustrated for these two compounds in Fig. 1. Similar results were obtained in comparative experiments with the mitochondrial and microsomal fractions isolated from homogenates in sucrose.

Some variation in susceptibility to inhibition was found in individual batches of the normal mitochondrial enzyme preparation but the concentration of inhibitor required to give 50 % inhibition rarely varied by a factor greater than 2.

Relationship between inhibitory action and chemical structure of substituted hydrazines. The complex kinetic pattern of inhibition found with arylalkylhydrazines precludes measurement of their reactivity with the active centre of monoamine oxidase by any readily determined rate constant. Inhibition by all the arylalkylhydrazines studied showed the same general type of time-dependence, the rate becoming slow or negligible after about 15 min.; therefore the concentration giving 50 % inhibition in 15 min. of an approximately constant quantity of the enzyme preparation was taken as a rough indication of reactivity. Table 5 lists the negative logarithm of the concentration (pI_{50}) for a number of arylalkylhydrazines. Table 6 gives pI_{50} values for iproniazid and some acylated benzylhydrazines but these are not directly comparable with the values in Table 5 since the time-course of inhibition was so different (Figs. 1 and 2). Since the compounds in the two Tables were not all tested against the same batch of enzyme, no significance can be attached to differences in pI_{50} of

Table 5. *Inhibition of monoamine oxidase by arylalkylhydrazines* $R[CH_2]_n \cdot CHR' \cdot NH \cdot NH_2 \cdot HCl$

A portion (2.8 ml.) of an approx. 25-fold dilution of the mitochondrial enzyme preparation was incubated at pH 7.4 and 25° with 0.4 ml. of various concentrations of the inhibitor for 15 min. before addition of 0.1 M-tyramine (0.4 ml.) and 0.125 M-semicarbazide (0.4 ml.). The concentration (M) of inhibitor giving 50 % inhibition was obtained by interpolation.

Compound no.	R	n	R'	M.p.	N (%)		Cl (%)		pI_{50}
					Calc.	Found	Calc.	Found	
1	Phenyl	0	H	112°	—	—	22.4	22.2	6.5
2	Phenyl	0	Me	83–85	—	—	20.5	20.6	6.2
3	Phenyl	0	Ph	211	—	—	15.5	15.1	<3.7
4	Phenyl	1	H	169	—	—	20.5	20.4	6.5
5	Phenyl*	1	Me	—	—	—	—	—	6.2
6	<i>p</i> -Methylphenyl	0	H	162–163	16.2	16.4	—	—	7.0
7	<i>p</i> -Isopropylphenyl	0	H	135–137	14.0	14.3	—	—	5.6
8	<i>p</i> -Methoxyphenyl	0	H	198	14.9	15.3	—	—	6.7
9	<i>p</i> -Chlorophenyl	0	H	168	14.5	14.8	—	—	6.6
10	<i>o</i> -Chlorophenyl	0	H	145	14.5	14.7	—	—	6.7
11	3,4-Dichlorophenyl	0	H	158–160	12.3	12.3	—	—	6.2
12	3,4-Dimethoxyphenyl	0	H	180	12.8	13.2	—	—	5.9
13	α -Naphthyl	0	H	189–190	—	—	17.0	16.9	5.3
14	2-Pyridyl†	0	H	174	21.3	21.5	—	—	5.3
15	3-Pyridyl†	0	H	188	21.3	21.3	—	—	5.8
16	4-Pyridyl†	0	H	199	21.3	21.3	—	—	5.7
17	2,6-Dimethylphenyl	1	H	181–183	13.9	13.6	—	—	6.2

* Compound kindly provided by Benger Laboratories Ltd., Holmes Chapel, Cheshire.

† Isolated as dihydrochloride salts.

Table 6. *Inhibition of monoamine oxidase by 1-acyl-2-benzylhydrazines (Ph·CH₂·NH·NH·CO·R) and iproniazid*

A portion (2.8 ml.) of an approx. 25-fold dilution of the mitochondrial enzyme preparation was incubated at 25° and pH 7.4 with 0.4 ml. of various concentrations of the inhibitor for 15 min. before addition of 0.1 M-tyramine (0.4 ml.) and 0.125 M-semicarbazide (0.4 ml.). The concentration (M) of the inhibitor giving 50% inhibition was obtained by interpolation.

Compound no.	R	M.p.	N (%)		pI ₅₀
			Calc.	Found	
18	Methyl	81–82°	17.1	17.4	4.5
19	n-Propyl	66	14.6	14.6	4.5
20	Phenyl	114	12.4	12.4	5.5
21	Pyrid-3-yl*	199–200	23.6*	23.5*	5.1
22	5-Methylisoxazol-3-yl†	—	—	—	5.5
23	Iproniazid†	—	—	—	4.8

* Obtained as dihydrochloride salt; analysis for Cl, not N.

† Compounds kindly provided by Roche Products Ltd., Welwyn Garden City, Herts.

less than 0.3, this being roughly the range of variation in pI₅₀ found when a single compound was tested against different batches of enzyme.

DISCUSSION

In common with the inhibition of monoamine oxidase by hydrazides, such as iproniazid (Zeller *et al.* 1955; Davison, 1957), inhibition by arylalkylhydrazines is time-dependent, prevented by substrate and reversible only slowly, if at all. In contrast with inhibition by iproniazid, the rate of inhibition by arylalkylhydrazines before addition of substrate falls rapidly with time (Figs. 1 and 2), the extent of inhibition in many cases becoming constant within about 15 min., and the extent of inhibition is dependent not only on the inhibitor concentration, but also, inversely, on the enzyme concentration (Tables 1 and 2). These results are explicable if the observed extent of inhibition is primarily dependent on two competing reactions: first, a bimolecular reaction between the inhibitor and the active centre of monoamine oxidase; secondly, a reaction between the inhibitor and some component of the crude enzyme preparation other than monoamine oxidase. The rapid loss of free inhibitor from enzyme-inhibitor mixtures has been shown directly for benzylhydrazine and α -naphthylmethylhydrazine (Tables 3 and 4). This loss of inhibitor is unlikely to be due to combination of the hydrazine with an aldehyde or ketone group to give a hydrazone, since preincubation of benzylhydrazine with sucrose or pyridoxal did not affect the subsequent extent of inhibition when enzyme was added. The variable effect of semicarbazide also suggests that a carbonyl group is not involved in the disappearance of the inhibitor. As addition of a chelating agent, ethylenediaminetetra-acetic acid, to the enzyme also had no effect on the extent of inhibition by benzylhydrazine, the disappear-

ance of benzylhydrazine is probably not due to catalytic decomposition by trace metals. The appreciably lower sensitivity to benzylhydrazine of the monoamine oxidase in the microsomal fraction of guinea-pig liver compared with the mitochondrial fraction suggests that disappearance of the inhibitor is due not to interaction with monoamine oxidase itself but to interaction with some other cellular constituent localized mainly on the smaller cell particles. This constituent differs from the soluble component of liver homogenates which greatly decreases the sensitivity to iproniazid of monoamine oxidase in homogenates as compared with mitochondrial and microsomal fractions (Zeller *et al.* 1955; Davison, 1957).

The variable effect of semicarbazide on inhibition, especially the opposite effects found on inhibition with such closely related compounds as 3,4-dichlorobenzylhydrazine and 3,4-dimethoxybenzylhydrazine, is puzzling. This action of semicarbazide is almost certainly different in nature from the effect of cyanide, which always appears to increase the sensitivity of the enzyme to the inhibitor.

No extensive generalizations are discernible in the effect of structure on the inhibitory potency of either the arylalkylhydrazines or the hydrazides; however, a few correlations are evident. The pyridylmethylhydrazines (Table 5, compounds 14–16) are all less active than their phenyl counterpart (compound 1). Loading the phenyl ring with bulky substituents or with more than one substituent also tends to reduce the inhibitory power (compounds 7, 11–13 and 17), as does substitution of one of the hydrogen atoms on the α -carbon by methyl, or, more particularly, by phenyl (compounds 2, 3 and 5). A single small substituent in the phenyl ring either has little effect or slightly increases the inhibitory activity (compounds 6 and 8–10). The electronic character of the substituent

appears to bear no relation to the changes that are produced. Among the acylated compounds (Table 6), acyl groups derived from aromatic or heterocyclic acids (compounds 20-22) give more active compounds than aliphatic acyl groups (compounds 18 and 19).

The acylated compounds generally are all much less reactive with monoamine oxidase than benzylhydrazine, but, owing to their greater persistence in the presence of the crude enzyme preparation, the extent of inhibition achieved on long duration of contact between enzyme and inhibitor can approach that found with similar concentrations of the parent hydrazine. An effect of this kind probably accounts for the much greater potency of hydrazides as monoamine-oxidase inhibitors *in vivo* than would be expected from their potency on short duration of contact with the enzyme *in vitro*.

SUMMARY

1. The extent of inhibition of monoamine oxidase by arylalkylhydrazines depends on the duration of contact between enzyme and inhibitor before addition of substrate. The observed rate of inhibition decreased rapidly as this duration of contact was increased. This fall in rate is due, at least partly, to disappearance of free inhibitor from the enzyme-inhibitor mixture.

2. The degree of inhibition produced by a single concentration of benzylhydrazine or α -naphthylmethylhydrazine decreased as the concentration of the crude enzyme preparation was raised. With hydrazides, such as iproniazid, the enzyme concentration had only a minor effect on the extent of inhibition.

3. Cyanide ion greatly potentiated inhibition by arylalkylhydrazines as well as by hydrazides. Semicarbazide had a smaller, more variable effect. Inhibition was generally potentiated but with some hydrazines and most hydrazides a decrease in inhibition was found. Preincubation of the enzyme

with ethylenediaminetetra-acetic acid, or of the inhibitor with sucrose or pyridoxal, had no effect on the extent of inhibition produced by benzylhydrazine.

4. The monoamine oxidase in homogenates of guinea-pig liver was inhibited by benzylhydrazine almost as readily as the enzyme in the mitochondrial fraction, whereas the monoamine oxidase in the microsomal fraction was appreciably less sensitive to inhibition. In these respects benzylhydrazine differs markedly from iproniazid.

5. Inhibition by benzylhydrazine showed no reversal after 5 hr. at 25° but monoamine oxidase inhibited by α -naphthylmethylhydrazine did recover some activity after this time.

6. The effect of structure on the inhibitory power of a number of arylalkylhydrazines and hydrazides is described and discussed. No general correlation was found between structure and inhibitory potency.

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