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STUDIES ON THE MECHANISM OF INHIBITION OF MONOAMINE-OXIDASE BY HYDRAZINE DERIVATIVES

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Abstract—A comparative study has been made of the inhibition of monoamine-oxidase by hydrazine derivatives and of their cupric-ion catalysed decomposition. Both processes require oxygen and both are potentiated by low concentrations of cyanide ions. Several compounds which chelate with cupric ions suppress the catalysed decomposition of hydrazines and reduce the extent of inhibition of monoamine-oxidase produced by 2-phenylisopropylhydrazine or iproniazid. Hydroquinone and 1,2-naphthaquinone-4-sulphonic acid also protect monoamine-oxidase from inhibition by iproniazid but accelerate its cupric-ion catalysed decomposition. Some of these chelating agents and related compounds themselves inhibit monoamine-oxidase. 8-Hydroxyquinoline behaves as a typical competitive inhibitor but inhibition by hydroquinone is largely non-competitive. Inhibition of monoamine-oxidase by hydrazines may also be prevented by 2-phenylisopropylamine, a reversible, competitive monoamine-oxidase inhibitor, which does not affect the catalysed decomposition of hydrazines.

The inhibitory activity of 2-phenylisopropylhydrazine is considerably reduced by N-methylation and almost abolished by N′-methylation. N-Alkylation of benzylhydrazine also reduces its inhibitory action. In contrast, N-methylation, N-ethylation or N, N-dimethylation of 2-phenylisopropylamine do not appreciably diminish its monoamine-oxidase inhibitory properties. *p*-Tolylhydrazine, *tert.*-butylhydrazine and N-benzylhydroxylamine are irreversible inhibitors of monoamine-oxidase, similar in their kinetic behaviour to arylalkylhydrazines, but less potent.

The suggestion that monoamine-oxidase is a copper enzyme in which the copper catalyses the decomposition of hydrazine derivatives in a similar manner to free cupric ions, would explain many of these characteristic features of monoamine-oxidase inhibition.

DESPITE many studies on factors influencing the inhibition of monoamine-oxidase [monoamine-oxygen reductase (deaminating), EC 1.4.3.4] by hydrazine derivatives, and on the variation of inhibitory activity with molecular structure,[1, 2] the chemical mechanism of the inhibitory process is still obscure. In order to obtain marked inhibition with low concentrations of hydrazine derivatives it is necessary to allow the inhibitor to react with the enzyme for some time before substrate is added, but once inhibition has been produced it can be reversed only with difficulty, if at all. These and other observations imply that during inhibition one or more strong covalent bonds are formed between inhibitor and enzyme.[3] Davison[3] suggested that the hydrazine derivative was initially dehydrogenated to give a substituted hydrazone which then reacted with some unspecified part of the enzyme's active centre, while Carbon, *et al.*[4] postulated a direct nucleophilic attack by the hydrazine derivative on an activated

amide group; but, as discussed later, both these hypotheses are open to serious objections. More recently, substituted hydrazines were shown to be readily decomposed by cupric ions with the formation of free radicals,[5] and it was suggested that inhibition of monoamine-oxidase by hydrazines might result from a catalysed decomposition of this type. As shown in the present paper, some striking similarities exist between the cupric-ion catalysed decomposition of hydrazine derivatives, and the inhibition by these same compounds of monoamine-oxidase. The mechanism of inhibition of monoamine-oxidase by hydrazine derivatives is discussed in the light of these results.

MATERIALS

The benzylhydrazine and 2-phenylisopropylhydrazine derivatives were prepared according to the general methods given by Biel *et al.*[6] N-*Benzyl*-N-*methylhydrazine hydrochloride*, m.p. 106° (Found Cl: 20·7; $C_8H_{13}N_2Cl$ requires Cl, 20·5%) and N-*methyl*-N-2-*phenylisopropylhydrazine oxalate*, m.p. 169–171° (Found: N, 10·9; $C_{12}H_{18}N_2O_4$ requires N, 11·0%) have not been previously reported. N-Benzylhydroxylamine was obtained from acetoxime, benzyl chloride and acetic acid[7] and *tert*-butylhydrazine from *tert*-butyl chloride and anhydrous hydrazine.[8] Methylhydrazine, *p*-tolylhydrazine and 2-phenylisopropylamine salts were obtained from British Drug Houses Ltd., and N-methyl-2-phenylisopropylamine hydrochloride from L. Light & Co. 2-Phenylisopropylamine was N, N-dimethylated with formaldehyde and formic acid[9] and N-ethylated by acetylation followed by reduction with lithium aluminium hydride.[10] Iproniazid (1-isopropyl-2-isonicotinylhydrazine) phosphate and isocarboxazid [1-benzyl-2-(5-methyl-3-isoxazolylcarbonyl)hydrazine] were donated by Roche Products Ltd., Welwyn Garden City. Iproniazid base was isolated from the phosphate by addition of two equivalents of N-NaOH followed by repeated extraction with ether.

The enzyme preparation was a suspension of guinea-pig liver mitochondria in phosphate buffer.[11]

METHODS

Measurement of inhibition of monoamine-oxidase

The monoamine-oxidase activity was determined by the dinitrophenylhydrazine method with tyramine as substrate.[12] The inhibitory potencies of the hydrazine derivatives were measured by the negative logarithm of the concentration (pI_{50}) giving 50 per cent inhibition when allowed to react with an approximately constant quantity of the enzyme preparation at 25° and pH 7·4 for 15 min before addition of excess substrate.[11] Where protecting agents were included (e.g. see Table 3), these, in buffer, were added to the enzyme–buffer mixture about 5 min before the hydrazine derivative.

Two procedures were used in studying reversible inhibitors. In the first, 0·4 ml of various concentrations of inhibitor were added to the enzyme (2·8 ml of an approximately 25-fold dilution of the mitochondrial suspension in 0·1 M-phosphate buffer, pH 7·4) and 0·125 M-semicarbazide (0·4 ml) about 5 min before addition of a constant concentration of tyramine (0·4 ml). In the second, a single concentration of inhibitor (0·4 ml) was added to the enzyme–buffer–semicarbazide mixture at the same time as various concentrations of tyramine (0·4 ml). Otherwise the experiments with reversible inhibitors were carried out in the same manner as those with hydrazine derivatives.

The effect of oxygen exclusion on the extent of inhibition was studied as follows. A mixture of approximately 0·1 ml enzyme with 2·7 ml of 0·1 M-phosphate buffer (pH 7·4) was placed in a Warburg flask and 0·4 ml of aqueous inhibitor was placed in the single side-arm. Nitrogen containing less than 10 ppm of oxygen (British Oxygen Co.) was passed through an oxygen trap containing sodium anthraquinone-β-sulphonate in alkaline sodium dithionite.[13] The Warburg flask was flushed with the purified nitrogen for 15 min in a water bath at 25° and sealed. The inhibitor was tipped into the enzyme and the flask was shaken at 25° for a further 15 min. The flask was then unstoppered and the contents added rapidly (approx. 2 sec) to 0·1 M-tyramine (0·4 ml) and 0·125 M-semicarbazide (0·4 ml) in a 25-ml conical flask. The mixture was shaken at 25° in air for a further 30 min when 0·5 N-acetic acid (1 ml) was added to terminate the reaction. The remaining steps in determining the monoamine-oxidase activity were as already described.[12]

Measurement of the rate of the cupric-ion catalysed decomposition of hydrazine derivatives

The method is essentially a small scale adaptation of Eberson and Persson's procedure[5] using manual instead of automatic titration. A 4-mM solution of the hydrazine derivative (4 ml), 1·2 M-sodium perchlorate (1 ml) and water (2·5 ml) were stirred magnetically at 37° in a small water-jacketed beaker containing a glass electrode. A calomel electrode immersed in saturated potassium chloride, also in a jacketed beaker, was linked to the reaction vessel by a salt bridge. The two electrodes were connected to a pH meter. Oxygen was bubbled through a sintered disc into the stirred solution. The pH was adjusted to the required value, usually 6·5, with 0·1 N-NaOH from an 'Agla' microsyringe fitted with a Polythene outlet tube dipping into the reaction mixture. Aqueous copper sulphate (0·5 ml, 0·16–3·2 mM) was then added. The pH was rapidly restored to 6·5 and alkali was then added at a rate sufficient to keep the pH constant. The first-order rate constant for the decomposition was calculated graphically from the relation $k_{exp} = [2{\cdot}303/(CuSO_4)\ t]\ \log [a/(a - x)]$ where a was the volume of alkali required to neutralize all the acid formed and x the volume added after time t. A typical graph of $\log [(a - x)/a]$ against t is shown in Fig. 1.

Ionization constants were determined by titration of a solution of the hydrazine salt (about 3 mM) in aqueous sodium perchlorate (0·15 M) with 0·1 N-NaOH. The pK_a values were calculated by the Henderson–Hasselbalch equation from pH's around the half-neutralization points.

RESULTS

Relationship between the chemical structure of substituted hydrazines and their potency as monoamine-oxidase inhibitors

In order to obtain maximal inhibition of monoamine-oxidase by arylalkylhydrazines, the inhibitor must be pre-incubated with the enzyme before addition of substrate.[11] However, the increase in the extent of inhibition with increasing pre-incubation time does not follow a simple rate law. During the first few minutes of contact between inhibitor and enzyme, the extent of inhibition increases rapidly with time, but the rate of increase then tails off until ultimately the extent of inhibition becomes constant. This stage is generally reached after about 30 min pre-incubation. As a

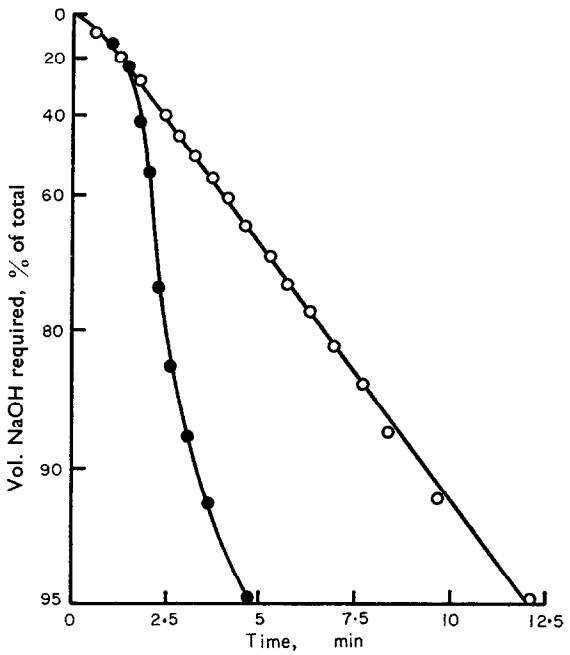

FIG. 1. Effect of cyanide on the copper-catalysed decomposition of 2-phenylisopropylhydrazine. 2-Phenylisopropylhydrazine (2 mM) and copper sulphate (40 μM) were stirred at 37° in aq. $NaClO_4$ (0·15 M) in the presence (filled circles) or absence (open circles) of KCN (0·1 mM). Oxygen was bubbled through the solution and 0·1 N-NaOH was added to keep the pH at 6·5.

TABLE 1. INHIBITION OF MONOAMINE-OXIDASE BY HYDRAZINE DERIVATIVES

0·1 ml of the mitochondrial enzyme preparation diluted with 0·1 M-phosphate buffer (2·7 ml) was shaken for 15 min at 25° and pH 7·4 with 0·4 ml of various concentrations of the inhibitor. 0·1 M-tyramine (0·4 ml) and 0·125 M-semicarbazide (0·4 ml) were then added and the mixture was assayed for monoamine-oxidase. The concentration (M) of inhibitor producing 50 per cent inhibition was obtained by interpolation.

Inhibitor*	pI_{50}
Benzylhydrazine	6·7
N, N-Dibenzylhydrazine	4·3
N-Benzyl-N-methylhydrazine	3·6
2-Phenylisopropylhydrazine	6·4
N-Methyl-N-2-phenylisopropylhydrazine	5·3
N-Methyl-N′-2-phenylisopropylhydrazine	$< 2·7$
N, N-Dimethyl-N′-2-phenylisopropylhydrazine	$< 2·7$
N, N′-Di-2-phenylisopropylhydrazine	3·0
p-Tolylhydrazine	5·0
tert-Butylhydrazine	2·6
Methylhydrazine	3·9
Hydrazine	$\leqslant 2·3$
N-Benzylhydroxylamine	3·3
Hydroxylamine	$\leqslant 2·3$

* All the hydrazine derivatives were used as their hydrochlorides except for methylhydrazine sulphate and N-methyl-N-2-phenylisopropylhydrazine oxalate.

result, the relative reactivities of arylalkylhydrazines with monoamine-oxidase cannot be assessed by any readily determined rate constant. However, pI_{50} values measured as described, give an approximate and fairly reproducible measure of relative reactivity.[11] Values of pI_{50} for a number of di- and tri-substituted derivatives of benzylhydrazine and of 2-phenylisopropylhydrazine are given in Table 1. Included in the Table are pI_{50} values for hydrazine itself, methylhydrazine, *p*-tolylhydrazine and *tert*-butylhydrazine; also for hydroxylamine and N-benzylhydroxylamine. The results in Table 1 were obtained on a single batch of enzyme, and all the inhibitors, except methylhydrazine, showed the same type of kinetic behaviour as reported earlier for benzylhydrazine. With the weaker inhibitors the constant level of inhibition was sometimes reached only after 1–2 hr contact between enzyme and inhibitor. Methylhydrazine behaved like iproniazid,[11] the rate of inhibition remaining constant with increasing pre-incubation time. With different batches of enzyme, pI_{50} values for a single compound rarely varied by more than 0·3 units. The only exception was N, N-dibenzylhydrazine, for which pI_{50} values ranged from 4–6. This variation appeared to be unrelated to the size, sex or colour of the guinea-pig from which the liver was obtained.

Inhibition of monoamine-oxidase by hydrazine derivatives in an atmosphere of nitrogen

The effect of treating monoamine-oxidase with hydrazine derivatives in an atmosphere of nitrogen is illustrated in Table 2. In every case the extent of inhibition was

TABLE 2. INHIBITION OF MONOAMINE-OXIDASE BY HYDRAZINE DERIVATIVES IN THE PRESENCE AND ABSENCE OF OXYGEN

Inhibitor (0·4 ml) was shaken for 15 min at 25° and pH 7·4 with an approx. 25-fold dilution of the mitochondrial enzyme preparation (2·8 ml) in an atmosphere of air or nitrogen. The mixture was then added rapidly to 0·1 M-tyramine (0·4 ml) and 0·125 M-semicarbazide (0·4 ml) and assayed for monoamine-oxidase.

Inhibitor	Conc. μM	% inhibition under, Air	Nitrogen
N-Isopropyl-N′-isonicotinyl-hydrazine (Iproniazid)	30	44	3
N-Benzyl-N′-(5-methyl-3-isoxazolyl-carbonyl)hydrazine (Isocarboxazid)	4	51	13
Methylhydrazine	200	68	32
Benzylhydrazine	1	84	25
2-Phenylethylhydrazine	1	57	8
2-Phenylisopropylhydrazine	1	62	15
N-Methyl-N-2-phenylisopropylhydrazine	10	54	15
p-Tolylhydrazine	20	52	14
N-Benzylhydroxylamine	1000	55	31

much less than was observed when the reaction took place in air. The residual inhibitory activity under nitrogen varied with the care expended on eliminating as much oxygen as possible from the system. Shaking the enzyme–buffer mixture alone under nitrogen for 15 min had no effect on its monoamine-oxidase activity when this was measured immediately afterwards in an atmosphere of air.

Effect on monoamine-oxidase of compounds chelating or reacting with cupric ions

Gorkin[14] reported that a number of metal-chelating agents were moderately active, reversible inhibitors of monoamine-oxidase, 8-hydroxyquinoline being about the most potent.

Figure 2 shows Lineweaver–Burk plots (reciprocal of the enzyme activity (1/v) against reciprocal of the substrate concentration (1/S)) for monoamine-oxidase and tyramine in the presence and absence of 8-hydroxyquinoline (0·2 mM). The intersection approximately on the 1/v axis shows that inhibition is competitive.[15] From the plot in the absence of inhibitor, the Michaelis–Menten constant K_m was found to be approximately 0·22 mM, which gives a value of K_i for 8-hydroxyquinoline of about 40 μM. Straight lines were also obtained when the reciprocal of the enzyme activity was plotted against the inhibitor concentration at constant tyramine concentration.

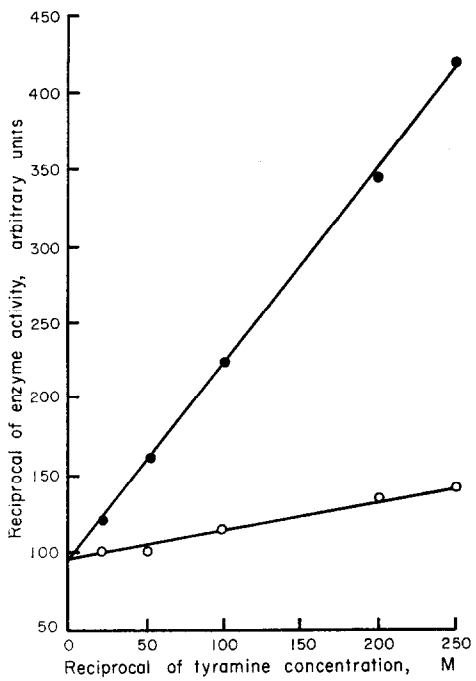

FIG.2. Inhibition of monoamine-oxidase by 8-hydroxyquinoline. 8-Hydroxyquinoline (0·4 ml, 2 mM) and various concentrations of tyramine (0·4 ml) were added at the same time to 0·1 ml of the enzyme preparation diluted with 0·1 M-phosphate buffer (2·7 ml, pH 7·4) and 0·125 M-semicarbazide (0·4 ml) and the mixture was incubated for 30 min at 25°. Filled circles—inhibitor present, open circles—inhibitor absent. The reciprocal in the abscissa is that of the tyramine concentration in the 0·4 ml added.

Other compounds which react with cupric ions were also tested as inhibitors, mostly in the presence of 1 mM-tyramine.

Apparent weak inhibition (about 10 per cent) was found with 1 mM-potassium cyanide in the presence of 1 mM, but not 10 mM-tyramine. However, this may have been an artefact since cyanide, being a good aldehyde reagent, may interfere with the assay procedure.

Appreciable inhibition was found with polyphenols, particularly hydroquinone. Lineweaver–Burk plots from experiments in which tyramine and hydroquinone (1 mM) were added together to the enzyme indicated that inhibition was partly competitive and partly non-competitive. When the hydroquinone was added a few min before the tyramine, the extent of inhibition increased somewhat and became almost wholly non-competitive. 1 mM-Hydroquinone produced about 45 per cent inhibition with tyramine concentrations of 0·4–10 mM. When the reciprocal of the enzyme activity was plotted against the inhibitor concentration at constant tyramine concentration (10 mM), a straight line was obtained giving an I_{50} value of 1·1 mM. Catechol and pyrogallol also inhibited, but more weakly than hydroquinone or 8-hydroxyquinoline. 1 mM-Solutions produced 10–20 per cent inhibition in the presence of 1 mM-tyramine. As with hydroquinone a non-competitive component appeared to be present in this inhibition.

No significant inhibition was found with 1 mM-cysteine, glutathione or ethylene diamine.

Inhibition of monoamine-oxidase by N-alkylated 2-phenylisopropylamines

2-Phenylisopropylamine is a well-known, reversible inhibitor of monoamine-oxidase,[16] but doubt has been cast on whether inhibition is of the competitive type.[17]

Lineweaver–Burk plots for monoamine-oxidase and tyramine in the presence and absence of 2-phenylisopropylamine (0·1 mM) gave straight lines intersecting approximately on the 1/v axis, similar to those in Fig. 2; but, owing to experimental scatter it was impossible to be sure that no small non-competitive component was present.

A straight line was also obtained when 1/v was plotted against the 2-phenylisopropylamine concentration at constant tyramine concentration (1 mM). Similar plots were obtained for N-methyl, N-ethyl, and N, N-dimethyl-2-phenylisopropylamines. In the presence of 1 mM-tyramine, 50 per cent inhibition was produced by 0·15 mM-2-phenylisopropylamine and by 0·25–0·3 mM concentrations of the N-alkylated compounds.

Modifying effect of other compounds on the inhibition of monoamine-oxidase by iproniazid and 2-phenylisopropylhydrazine

The marked potentiating effect of cyanide ions on inhibition of monoamine-oxidase by iproniazid or arylalkylhydrazines has already been described.[3, 11] This effect appears to occur with all hydrazines but not with other types of inhibitor,[18] and is detectable with concentrations of cyanide as low as 5μM.

Other compounds are known which can reduce the inhibitory action of hydrazine derivatives, both *in vitro*, for example glutathione[3] and 1,2-naphthaquinone-4-sulphonic acid,[19] and *in vivo*, for example the reversible monoamine-oxidase inhibitor harmine.[20] This protective action is shared by various types of compound, as shown by the results in Table 3 for inhibition by iproniazid. Some of the active compounds themselves inhibit monoamine-oxidase, but there is little correlation between the two effects.

A comparable protective effect against inhibition by 2-phenylisopropylhydrazine (0·5–1 μM) was shown by all the active compounds in Table 3 except glutathione.

TABLE 3. PROTECTIVE EFFECT OF OTHER COMPOUNDS AGAINST THE INHIBITION OF MONOAMINE-OXIDASE BY IPRONIAZID

The protecting compound (0·4 ml) was added to 0·1 ml of the mitochondrial enzyme preparation and 0·1 M-phosphate buffer (pH 7·4, 2·3 ml), about 5 min before 0·8 mM-iproniazid (0·4 ml). After 15 min at 25°, 0·1 M-tyramine (0·4 ml) and 0·125 M-semicarbazide (0·4 ml) were added and the mixture was assayed for monoamine-oxidase. Percentage inhibition is based on controls containing protecting agent but no iproniazid.

Compound	Conc. mM	% Inhibition produced by protecting agent alone	% Inhibition produced by 0·1 mM-iproniazid
None	—	—	90–95
Ethylene diamine	1	0	>90
Cysteine	1	0	>90
Glutathione	1	0	24
Hydroquinone	0·1	10	61
	0·5	31	21
	1	45	6
Catechol	0·2	9	5
Pyrogallol	0·2	5	14
8-Hydroxyquinoline	1	31	0
2-Phenylisopropylamine	0·2	9	37
1, 2-Naphthaquinone-4-sulphonic acid	0·1	0	20

The catalytic decomposition of hydrazine derivatives by cupric ions

In the presence of oxygen and catalytic concentrations of cupric ions, substituted hydrazines decompose approximately according to the equation:[5]

$$RNHNH_2.HCl + 1{\cdot}5\ O_2 \longrightarrow \text{products} + N_2 + HCl$$

One mole of acid is liberated per mole hydrazinium salt decomposed, and the rate at which it is liberated provides a direct measure of the rate of decomposition. The decomposition should be carried out at a pH less than the pK_a of the hydrazine in order that the predominant species present will be the hydrazinium cation rather than the free base. Following Eberson & Persson,[5] a pH of 6·5 was found to be satisfactory. Sodium perchlorate (0·15 M) was included to maintain constant ionic strength. Its presence did not greatly affect the decomposition rates. In order to obtain a maximal and steady rate of decomposition the solution had to be saturated with oxygen. When nitrogen was bubbled through the solution instead of oxygen the decomposition became very slow. With most of the compounds studied the decomposition followed a first order rate law with respect to the hydrazine concentration, as illustrated in Fig. 1, and to the cupric ion concentration. In Table 4, rate constants are given for the decomposition of various hydrazine derivatives. The three experiments with 2-phenylisopropylhydrazine show the first order dependence of the rate on the cupric ion concentration. As the pK_a values of N-benzylhydroxylamine and *p*-tolylhydrazine are 5·1 and 5·3 respectively, it was necessary to follow the decomposition of these compounds at pH 4·5.

Methylhydrazine and iproniazid also decomposed rapidly in the presence of cupric ions but the decomposition was not a first order reaction. As decomposition proceeded the rate increased appreciably. Methylhydrazine decomposed a little faster than

2-phenylisopropylhydrazine whereas iproniazid decomposed rather more slowly. Although none of the iproniazid would have been present as a cation at pH 6·5, 1 mole of acid was still produced per mole iproniazid destroyed. This is presumably isonicotinic acid.

Low concentrations of potassium cyanide have a marked potentiating effect on the rate of decomposition. The experiment illustrated in Fig. 1 showing the effect of 0·1 mM-potassium cyanide on the decomposition of 2-phenylisopropylhydrazine is

TABLE 4. DECOMPOSITION OF HYDRAZINE DERIVATIVES BY CUPRIC IONS

Oxygen was bubbled through a stirred solution of the hydrazine hydrochloride (2 mM), sodium perchlorate (0·15 M) and cupric sulphate (10–200 μM) at 37°. The pH was maintained at 6·5 by addition of 0·1 N-NaOH and the rate constant for decomposition obtained from the rate at which the alkali was added.

Rate constants for compounds other than 2-phenylisopropylhydrazine are the means of 3 experiments.

Compound	pK_a	$CuSO_4$, μM	k,* l./mmole/min
2-Phenylisopropylhydrazine	7·2	20	7·7
		40	6·7
		80	7·8
N-Methyl-N-2-phenylisopropylhydrazine	6·8	40–100	5·5
N-Methyl-N′-2-phenylisopropylhydrazine	7·0	10–20	30
N, N-Dimethyl-N′-2-phenylisopropyl-hydrazine	6·1	100–200	3·0
Benzylhydrazine	6·8	40	7·7
N-Benzyl-N-methylhydrazine	6·4	80–100	4·0
p-Tolylhydrazine†	5·3	10–40	23
N-Benzylhydroxylamine†	5·1	100–200	1·4

* Eberson & Persson[5] give k = 8·8 for benzylhydrazine and 7·7 for 2-phenylisopropylhydrazine.
† Results at pH 4·5 not 6·5.

typical. After a short induction period, which was often found even without cyanide, decomposition became extremely rapid. With 1 mM-potassium cyanide, decomposition was almost instantaneous.

Eberson and Persson[5] showed that the first step in the decomposition was the rapid formation of a complex between the hydrazine and cupric ions followed by the rate-limiting decomposition of this complex. Strong chelating agents for cupric ions might consequently be expected to lower the decomposition rate. This has been found. At 1 mM concentration, catechol, pyrogallol, cysteine, glutathione, 8-hydroxyquinoline, ethylene diamine and oxalate ions retarded or suppressed the decomposition of 2-phenylisopropylhydrazine in the presence of 80 μM-copper sulphate. 1, 2-Naphthaquinone-4-sulphonic acid (0·1 mM) also lowered the decomposition rate, possibly by being first reduced to a 1,2-dihydroxynaphthalene derivative[19] which then behaves like catechol.

The decomposition of iproniazid was generally affected in much the same way. 1 mM-ethylene diamine, glutathione, catechol and phosphate ions all lowered the rate of decomposition in the presence of 0·2 mM-copper sulphate, whereas 0·2 mM-potassium cyanide increased the rate, although not to the same extent as with 2-phenylisopropylhydrazine. However, hydroquinone (1 mM), which marginally accelerated the decomposition of 2-phenylisopropylhydrazine, caused a marked acceleration in

the decomposition of iproniazid, and 1,2-naphthaquinone-4-sulphonic acid (0·2 mM), which retarded decomposition of 2-phenylisopropylhydrazine, accelerated that of iproniazid.

DISCUSSION

Hydrazines are nucleophilic reagents and reducing agents, and it is likely that they owe their inhibitory action on monoamine-oxidase to a reaction of one of these types. Carbon *et al.*[4] have attributed inhibition to nucleophilic attack by the hydrazine on some unspecified carbonyl group, possibly part of an amide link. This hypothesis has several weaknesses. Firstly, it is difficult to see why simple alkylhydrazines such as methylhydrazine (Table 1) and ethylhydrazine[21] are strong inhibitors whereas conventional carbonyl reagents like hydrazine and hydroxylamine are inert. Secondly, it is difficult to explain why inhibition is invariably less rapid in the absence of oxygen (Table 2). Thirdly, it is difficult to account for the strong inhibitory properties of the weakly nucleophilic 1-acyl-2-alkylhydrazines, like iproniazid, unless it is assumed that the hydrazide is first hydrolysed to the corresponding hydrazine. There is considerable evidence against this possibility for iproniazid,[3] but Schwartz[19] showed that 1,2-naphthaquinone-4-sulphonic acid, which reacts rapidly with hydrazines but not with hydrazides, reduces the extent of monoamine-oxidase inhibition produced by both types of compound. It was deduced from this that the hydrazide must first be hydrolysed before inhibition can occur. However, since this protective action is shared by other compounds (Table 3) which would not be expected to react either with hydrazines or hydrazides, this evidence is not conclusive.

The decrease in the extent of inhibition found when enzyme and inhibitor were incubated under nitrogen instead of air or oxygen was originally noticed by Davison[3] both for iproniazid and for isopropylhydrazine; Schwartz, however, although confirming this result for iproniazid failed to detect any effect with isopropylhydrazine or with isocarboxazid.[19] The effect of replacing air by nitrogen has been re-investigated for nine hydrazine derivatives of different structural types, and a reduction was always found in the extent of inhibition (Table 2). However, great care was needed in these experiments and even in the presence of a very small amount of oxygen, the reduction in inhibition was, with many compounds, barely perceptible. In order to explain the need for oxygen, Davison[3] suggested that inhibition resulted from dehydrogenation of the hydrazine to a hydrazone which then combined with the enzyme by an addition reaction across the new double bond. Since hydrazones themselves are not inhibitors[21] the dehydrogenation and addition steps would need to be simultaneous without the incipient double bond ever fully forming. This hypothesis would account for inhibition by both hydrazines and alkylated hydrazides and is additionally attractive in that the mechanism is analogous to that normally postulated for the oxidative deamination of substrates.[16] However, if this is the mechanism, there must be a hydrogen atom on the α-carbon before dehydrogenation can occur; but in fact the presence of such a hydrogen atom is not essential for inhibitory activity. *Tert*-butylhydrazine and particularly *p*-tolylhydrazine are inhibitors whose kinetic behaviour is similar to that found with arylalkylhydrazines and which, consequently, are likely to inhibit by the same mechanism. The alternative possibility, namely that the incipient double bond is formed between the two nitrogen atoms, fails to explain the lack of inhibitory properties of hydrazine itself or of non-alkylated hydrazides.[21]

Hydrazine bases are not particularly stable substances but undergo slow autoxidation. This autoxidation is markedly accelerated by cupric ions, although not to any great extent by ferric, cobalt, nickel or manganous ions, and leads to the formation of free alkyl or aryl radicals.[5] It was suggested[5] that if a similar decomposition of hydrazines could occur in the immediate vicinity of the active centre of monoamine-oxidase, free radicals liberated in this way could readily and irreversibly inhibit the enzyme by combination with some other group, perhaps a thiol. also located at the active centre.

There are many striking similarities between the inhibition of monoamine-oxidase by hydrazine derivatives and their cupric-ion catalysed decomposition. Both processes require oxygen and both are markedly potentiated by cyanide ions. Both can be retarded or suppressed by compounds capable of chelating with, or being oxidized by cupric ions, such as 8-hydroxyquinoline, catechol, pyrogallol or glutathione. The catalysed decomposition and the enzyme inhibition occur with both hydrazines and hydrazides, but only those hydrazine derivatives whose decomposition would give rise to alkyl or aryl radicals are monoamine-oxidase inhibitors. Hydrazine itself and non-alkylated hydrazides do not inhibit.[21] N-Benzylhydroxylamine which decomposes like benzylhydrazine also inhibits monoamine-oxidase, whereas hydroxylamine itself does not.

The marked stereospecificity shown by optically active hydrazines towards monoamine-oxidase,[22] and the correlation which exists between the inhibitory potencies of homologous alkyl- and arylalkylhydrazines and the susceptibility to enzymic oxidation of amines isosteric with them,[23] make it unlikely that inhibition results from a non-specific liberation of free radicals by cupric ions not directly associated with the enzyme itself. Monoamine-oxidase is not a metal activated enzyme,[2] consequently, if inhibition by hydrazine derivatives is due to a metal-ion catalysed liberation of free radicals, the metal must be a permanent part of the enzyme's active centre. Any certainty whether mitochondrial monoamine-oxidase is a metalloenzyme must await its purification, however, copper is known to be present in mitochondria,[24] and in certain soluble amine-oxidases.[25, 26] Indirect evidence suggesting that mitochondrial monoamine-oxidase may be a copper-enzyme is provided by the inhibitory action of chelating agents,[14] and of compounds such as hydroquinone which can be oxidized by cupric ions without the prior formation of chelates.[27]

It is also uncertain whether cupric ions bonded on one side by chelation with an enzyme would behave towards hydrazines in the same way as cupric ions in solution, but several examples can be quoted of copper-enzymes, such as ascorbic acid oxidase, ceruloplasmin and tyrosinase, where enzyme catalysed reactions can also be catalysed by free cupric ions.[28] In these instances both the enzyme catalysed and cupric-ion catalysed reactions can be suppressed by suitable chelating agents.[28]

Although many of the compounds which can protect monoamine-oxidase against inhibition by iproniazid or 2-phenylisopropylhydrazine also suppress their copper-catalysed decomposition, there are exceptions. Hydroquinone and 1,2-naphthaquinone-4-sulphonic acid protect against inhibition by iproniazid but accelerate its copper-catalysed decomposition. The absence of any direct correlation between these two actions could reflect differences between the chelating power and oxidation-reduction potential of the free cupric–cuprous system compared with cupric ions already partially bonded by chelation on one side with the enzyme.

It is more surprising, if monoamine-oxidase is a copper enzyme, that little correlation exists between this protecting action against inhibition by hydrazines and the direct inhibitory action of these protective compounds themselves. This might arise if the copper had some secondary enzymic function and did not participate directly in the oxidative deamination of substrates. This might also explain the weakness of the very small cyanide ion as a monoamine-oxidase inhibitor. Cyanide is a strong inhibitor of the well-established copper enzymes,[28] but in all of these the copper is involved in the direct transfer of electrons to atmospheric oxygen.

One problem which arises whichever theory of inhibition is accepted is to account for the fall in inhibitory activity which occurs when benzylhydrazine and 2-phenylisopropylhydrazine are N-alkylated, and more particularly when N′-alkylated. This latter effect has been reported for many ring-substituted 2-phenylisopropylhydrazines[29] and for benzylhydrazine.[30] This fall is not accompanied by any corresponding fall in the rate of the copper-catalysed decomposition. Although the decompositions were studied at pH 6·5 instead of 7·4, the pH of the inhibition experiments, over the pH range 5·5–7·5 the rate of decomposition of 2-phenylisopropylhydrazine is linearly related to the hydroxide ion concentration.[5] Assuming that this is also true for the methylated derivatives of 2-phenylisopropylhydrazine, the relative decomposition rates would be the same at pH 7·4 as at pH 6·5. Nor is steric hindrance likely to account for the profound decrease in inhibitory activity. N-Methyl,N-ethyl and N,N-dimethyl-2-phenylisopropylamines, which are isosteric with 2-phenylisopropylhydrazine and its N′-methyl and N-methyl derivatives, are equi-active as reversible monoamine-oxidase inhibitors. The possibility that inhibition involves hydrazone formation is difficult to reconcile with the activity of N-benzylhydroxylamine in which the terminal amino group is absent and with the inactivity of hydrazine and non-alkylated hydrazides.

One possible explanation is that cupric-ion catalysed decomposition of all types of disubstituted hydrazine would give two free radicals instead of one and that these may sometimes be liberated in such a manner that they combine more readily with each other than with the enzyme.

It is evident from the above discussion that the theory outlined in this paper, namely that monoamine-oxidase is a copper enzyme and that inhibition by hydrazine derivatives results from a copper-catalysed liberation of free radicals in the vicinity of the enzyme's active centre, is by no means proven. However, it can account in terms of a known chemical reaction for many of the more puzzling features of monoamine-oxidase inhibition by substituted hydrazines, and in this respect at least it would seem to be an improvement on any of the mechanisms proposed previously.

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