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The Mechanism of the Irreversible Inhibition of Rat-Liver Monoamine Oxidase by Iproniazid (Marsilid)

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The physiological role of monoamine oxidase in the oxidation of adrenaline, 5-hydroxytryptamine and related amines is still the subject of controversy (Furchgott, 1955). Many investigators have used monoamine oxidase inhibitors such as choline *p*-tolyl ether, and particularly iproniazid (1-*iso*-nicotinoyl-2-*isopropylhydrazine* phosphate, Marsilid), as tools in studies of this important problem (e.g. Zeller, Barsky & Berman, 1955*a*; Schayer, Smiley & Kaplan, 1952; Brodie, Pletscher & Shore, 1956; Griesmer & Wells, 1956). Despite the widespread use of iproniazid as an irreversible inhibitor, both *in vivo* and *in vitro*, little is known about the mechanism of the inhibition reaction.

In this paper the original observations of Zeller *et al.* (1955*a*), showing that pre-incubation of liver monoamine oxidase with iproniazid is necessary to obtain maximum inhibition, have been extended. Inhibition is easily prevented by the addition of substrate. In the absence of substrate, the irreversible inhibition reaction was found to be progressive and first-order, to require oxygen, and to have a high apparent energy of activation. These findings suggest that iproniazid reacts chemically at or near the enzyme active centre, and a hypothesis is advanced which accounts not only for this possibility but also for the structural requirements for inhibitors as shown by Zeller, Barsky, Fouts & Lazanas (1955*b*).

EXPERIMENTAL

Monoamine oxidase determinations. Livers from adult albino rats were chilled with ice, washed and suspended in Sørensen's 0.067 M-phosphate buffer, pH 7.2 (20%, w/v), by means of a Potter-Elvehjem glass homogenizer. The suspension was centrifuged at room temperature for 2 min. at the maximum speed (approx. 4000 g) of a bench centrifuge (Baird and Tatlock Ltd.) and the resultant supernatant was then recentrifuged at 8500 g for 10 min. at 10° in a refrigerated centrifuge. The mitochondria so obtained were resuspended in the 0.067 M-phosphate buffer [equivalent to 300 mg. (wet wt.) of liver/ml.]. The enzyme activity was determined on 1 ml. of mitochondria suspension by using tyramine (0.01 M final concentration) as a substrate and adjusting the volume in the flasks to 4 ml. with buffer. Substrate and inhibitors were dissolved in the phosphate buffer. The flasks were flushed with oxygen before equilibration (total time 5 min.) and addition of the substrate. The bath temperature was 37°. Potassium hydroxide was not present in the centre well.

Kinetic measurements. The methods were similar to those of Davison (1956). The standard technique was to place 0.5 ml. of inhibitor solution and 0.25 ml. of tyramine solution in side arms of double-armed flasks and, after flushing with oxygen and equilibrating (5 min.), to add inhibitor from the side arm at different times. In these experiments the flasks containing enzyme were shaken at 37° for the same time and only the time of addition of inhibitor was varied. At the end of the incubation the substrate was added to all the flasks and manometer readings were taken every 5 min. for 35 min. The mean oxygen uptake was calculated by the method of least squares (Aldridge, Berry & Davies, 1949). Residual enzyme activity was expressed as a percentage of a control suspension

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without added inhibitor. Oxygen uptake was found to be directly proportional to ammonia liberation.

Inhibitors. *iso*Propylhydrazine was prepared by refluxing 10 g. of iproniazid, 10 ml. of conc. HCl and 10 ml. of water for 1 hr. The mixture was steam-distilled. The distillate was neutralized with HCl and evaporated to dryness; the *iso*propylhydrazine hydrochloride, crystallized from an ethanol-ether mixture and recrystallized from dry acetonitrile, had m.p. 114° (cf. Lochte, Noyes & Bailey, 1922) (Found: C, 32.6; H, 9.84; N, 26.93; Cl, 31.8. $C_3H_{11}N_2Cl$ requires C, 32.7; H, 10.0; N, 25.5; Cl, 31.8%). 1-*iso*Propylidene-2-isonicotinoylhydrazine was obtained from Roche Products Ltd.

RESULTS

Iproniazid and tyramine in various concentrations were added together to mitochondria. The reciprocal of the rate of oxygen uptake was plotted against the reciprocal of the tyramine concentration for each concentration of inhibitor and the dissociation constant for the iproniazid-enzyme complex calculated by the method of Lineweaver & Burk (1934). Under these conditions iproniazid was found to be a competitive inhibitor of monoamine oxidase (Table 1).

Pre-incubation of iproniazid at 37° in oxygen with the enzyme resulted in progressive and irreversible inhibition (Zeller *et al.* 1955*a*, and Table 4). Addition of high concentrations of substrate (0.01 M) stopped further inhibition, thus allowing activity determinations to be made. It was therefore possible to investigate the kinetics of inhibition by incubating iproniazid for different times at 37° with mitochondria before adding substrate and determining the residual enzyme activity. The kinetics of the reaction showed the characteristics of a bimolecular reaction with one component, presumably the inhibitor, in excess and obeyed the first-order equation (Fig. 1). The rate constant and values for 50% inhibition in 30 min. are given in Table 1. Since temperature had a pronounced effect on inhibition by iproniazid, the rates of in-

hibition at 22°, 30°, 37° and 45° were determined and the apparent energies of activation calculated from the Arrhenius equation (Table 1). High values were obtained for both the oxidation of tyramine and the inhibition reaction. These experiments suggested that irreversible inhibition of monoamine oxidase by iproniazid was the result of a chemical reaction probably at the enzyme active centre.

Progressive and irreversible inhibition did not occur if iproniazid was added to mitochondria under anaerobic conditions. Thus in one experiment Warburg flasks containing mitochondria with iproniazid in the side arm were gassed with nitrogen for 5 min. at 37°. The iproniazid (final concentration 10^{-4} M) was then tipped from the side arm and incubated with the mitochondrial suspension for 30 min. Tyramine was then added and the flasks were flushed with oxygen for 2 min. No inhibition of enzyme activity resulted, although in a control experiment with oxygen present throughout nearly complete inhibition was obtained.

Fig. 1. Inhibition of monoamine oxidase activity by iproniazid. For details see the text. x, Iproniazid, 6.6 μ M; $\blacktriangle$, 20 μ M; and $\bullet$, 100 μ M.

Table 1. Constants for inhibition of monoamine oxidase

The constants have been determined with mitochondria as a source of monoamine oxidase and tyramine as substrate. For details see text.

Constant	Substrate (tyramine)	Inhibitors	
		Iproniazid	<i>iso</i> Propylhydrazine
Dissociation constant of inhibitor and substrate-enzyme complex determined by the Lineweaver & Burk (1934) method	3×10^{-8}	2×10^{-4}	3×10^{-5}
Approximate apparent energies of activation for the inhibitory reaction (cal./mole.)	17 000	30 000	26 000
Rate of reaction of inhibitor and enzyme at 37°, pH 7.2, (l. mole. ⁻¹ min. ⁻¹)	—	1.65×10^8	1.55×10^4
Concn. producing 50% inhibition at 37°, pH 7.2, on incubation for 30 min. (M)	—	1.4×10^{-5}	1.5×10^{-6}

Progressive inhibition of enzyme activity by iproniazid was found to be less for liver suspensions (25 %, w/v, in 0.067 M-phosphate buffer prepared as described above) than for mitochondrial preparations. This suggested that the supernatant was responsible for the insensitivity. It was found that if glutathione (0.8 mM) was added to mitochondria, the sensitivity was decreased and the inhibition was similar to that found on addition of the supernatant obtained by centrifuging an equivalent amount of liver suspension. Glutathione by itself had no effect on monoamine oxidase activity.

It has been shown that oxygen and iproniazid are necessary for irreversible inhibition of mono-

amine oxidase and that inhibition can be blocked by addition of substrate. Further, shaking iproniazid with mitochondria in an atmosphere of nitrogen does not result in inhibition of monoamine oxidase activity. It therefore seemed possible that iproniazid acted initially as a substrate for monoamine oxidase itself. This possibility is supported by the finding that the susceptibility of monoamine oxidase to inhibition by iproniazid remains the same after partial inactivation of the enzyme by heat treatment (Table 2). Similarly, inhibition of monoamine oxidase activity by iproniazid is affected by pH in the same way as tyramine oxidation (Fig. 2).

Possible conversion of iproniazid into isopropylhydrazine. Since alkylhydrazines have been shown to be powerful inhibitors of monoamine oxidase (Zeller *et al.* 1955*b*) another possibility was that iproniazid was converted into the potentially much more reactive isopropylhydrazine before inhibition of monoamine oxidase could occur. *iso*Propylhydrazine was found to be an even stronger competitive inhibitor of monoamine oxidase than iproniazid (Table 1).

A study of the kinetics of inhibition of monoamine oxidase revealed that *iso*propylhydrazine, like iproniazid, progressively and irreversibly inhibited the enzyme when incubated in oxygen, but not in nitrogen. The rate of the inhibition reaction was calculated at different temperatures and the value of the apparent energy of activation was similar to that of iproniazid (Table 1). It therefore seemed unlikely that irreversible inhibition by iproniazid was due to prior formation of *iso*propylhydrazine. Additional evidence has been obtained in support of this view.

The reactivation of monoamine oxidase after inhibition by various substituted hydrazines was examined. In these experiments 1-*isonicotinoyl*-2-methylhydrazine and methylhydrazine sulphate were also used, since both compounds are powerful monoamine oxidase inhibitors and resemble *iso*propylhydrazine and iproniazid in possessing a terminal alkylhydrazine group. Mitochondria, prepared in 0.25 M-sucrose, were incubated with the inhibitors for 30 min. at 37°, washed and reactivated.

Fig. 2. Effect of pH on tyramine oxidation (●) and inhibition (x) by iproniazid of mitochondrial monoamine oxidase. The rate of oxidation of tyramine (0.01 M) by mitochondria (300 mg. wet wt. of liver) in Clark & Lub's borate (pH 10), Sørensen's (pH 6–8) and Walpole's acetate (pH 5) buffers was determined. 100 % activity was obtained at pH 8. Mitochondrial suspensions (0.5 ml., equivalent to 600 mg. wet wt. of liver) were incubated with iproniazid (0.1 mM) for 20 min. at 37° in each of the buffers (3 ml.). After incubation, 3 ml. of ice-cold saline was added to the Warburg flasks and the contents were centrifuged at 0°. The precipitated mitochondria were resuspended in 6 ml. of phosphate buffer, pH 7.2, and 3 ml. was taken for enzyme-activity determinations. Uninhibited controls were run. 100 % inhibition was taken as the inhibition obtained at pH 8.

Table 2. *Effect of heat on inhibition of monoamine oxidase by iproniazid*

Suspensions of liver mitochondria (250 mg./ml. wet wt. of liver) in 0.067 M-phosphate buffer, pH 7.2, were heated for 10 min. at different temperatures. A portion (1 ml.) of each sample was incubated for 30 min. at 37° with 6.6 μ M-iproniazid and tyramine oxidase activity determined as a percentage of an uninhibited control. Values given are the mean of two experiments.

Temp. ...	37°	47.5°	50°	52.5°	55°	60°
Activity of suspension without inhibitor (% of activity at 37°)	100	79	53	44	35	20
Activity after treatment with iproniazid (% of uninhibited activity)	67	61	50	65.5	52	35.5

vated either by dialysis or by addition of dimercaptopropanol. Table 3 shows that although some return of monoamine oxidase activity was obtained after inhibition by iproniazid and 1-isonicotinoyl-2-methylhydrazine, there was much less reactivation after inhibition by methyl- and isopropylhydrazines. Therefore the inhibition of the monoamine oxidase by the alkylhydrazines appears to differ in nature from that brought about by the two isonicotinoyl derivatives.

Inhibition of mitochondrial monoamine oxidase by iproniazid in the presence of cyanide. When iproniazid was added directly to mitochondria in the presence of cyanide and the flasks were incubated at varying times before addition of substrate, the inhibition obtained was progressive and the reaction was first-order (Fig. 3). The inhibition was also greater than that obtained in the absence of cyanide. Thus in the presence of cyanide mitochondrial monoamine oxidase activity was inhibited 81% by 20 μ M iproniazid (20 min. at 37°), whereas in the absence of cyanide only 28% of enzyme activity was inhibited. Monoamine oxidase activity could not be restored by diluting either of the two inhibited preparations with ice-cold saline plus tyramine, centrifuging down the mitochondria at 0° and resuspending the mitochondria before determining their tyramine oxidase activity compared with uninhibited mitochondria similarly treated.

The possible reversibility of inhibition of mitochondrial monoamine oxidase by iproniazid in the presence of cyanide was also examined by a dilution technique (Aldridge, 1950). This technique depends on incubating a concentrated enzyme preparation with inhibitor and then diluting with buffer and substrate, or with buffer, substrate and inhibitor of the initial concentration (the substrate prevents further progressive inhibition). Table 4 (Expts. 1

and 2) shows that in the absence of cyanide the inhibition by iproniazid was not reversed on dilution. In the presence of cyanide, however, and under the conditions of this experiment, the additional inhibition obtained can be reversed by dilution (Expts. 3 and 4). Under anaerobic conditions in the presence of cyanide, inhibition by iproniazid can be completely reversed on dilution (Expts. 5 and 6).

In other experiments cyanide was placed with phosphate buffer and mitochondria in the centre compartment with tyramine and iproniazid in the

Fig. 3. Effect of cyanide on inhibition of monoamine oxidase by iproniazid. Each flask contained 1 ml. of mitochondrial suspension (equivalent to 300 mg. wet wt. of liver), KCN (0.002M) and 0.067M-phosphate buffer, pH 7.2, to give a final volume of 4 ml. Tyramine (0.01M final concn.) and iproniazid (4 or 20 μ M final concn.) were in each side arm. The incubation time represents the time of incubation of iproniazid with the enzyme. x, Iproniazid was added immediately to the enzyme; ●, after 0, 10, 25, 30, 35 and 40 min.

Table 3. *Reaction of mitochondrial monoamine oxidase after inhibition by substituted hydrazines*

A suspension of mitochondria was prepared in cold 0.25M-sucrose from rat liver (500 mg. wet wt. of original liver/ml.). Suitable volumes were inhibited by 5 mM-iproniazid, mM-isopropylhydrazine, mM-methylhydrazine, and 2 mM 1-isonicotinoyl-2-methylhydrazine for 30 min. at 37°. The mitochondria were resedimented at 8000 g at 0° and resuspended in Sørensen's 0.067M-phosphate buffer, pH 7.2, to give their original volume. After dialysis the enzyme activity was determined directly; after treatment with dimercaptopropanol (BAL) the mitochondria were centrifuged down and resuspended. Inhibited and uninhibited controls were run in all cases. The numbers of experiments are shown in brackets and mean values given. In the dialysis experiments two series of experiments were run as shown.

Monoamine oxidase inhibited by	Reactivation (%)		
	Dialysis of 5 ml. of mitochondrial suspension for 18 hr. at 20° against 2 l. of mM Sørensen's phosphate buffer, pH 7.2		BAL, final concn. 0.01M
Iproniazid	53 (2)	10 (2)	23 (3)
isoPropylhydrazine hydrochloride	16 (2)	4 (2)	1 (3)
1-isoNicotinoyl-2-methylhydrazine hydrochloride	45	10	24
Methylhydrazine sulphate	10	0	2

Table 4. *Reversibility of inhibition of monoamine oxidase of mitochondria*

Mitochondrial suspensions (0.5 ml., equivalent to 300 mg. of liver/ml.) were incubated with 50 μ M-iproniazid at 37° in O₂ or N₂ (Expts. 5 and 6) in the side arm of a double-armed Warburg flask. In Expts. 3-6 KCN (0.002M) was present (in both side arm and central compartment). After 5, 10, 20 and 40 min. incubation the mitochondria were tipped into the central compartment containing eight times its volume of either buffer containing tyramine (0.01M) or buffer containing tyramine plus iproniazid (50 μ M). Where nitrogen was used, the flasks were then flushed with oxygen before addition of substrate. After addition of tyramine the oxygen uptake was determined and the enzyme activities were calculated as a percentage of uninhibited controls.

Enzyme activity on dilution into buffer with tyramine (0.01M) plus	Enzyme activity (%) after incubation of iproniazid and enzyme for			
	5 min.	10 min.	20 min.	40 min.
1. Iproniazid	71.5	74	51	21
2. —	78.5	78.5	63.5	23
3. Cyanide	78	71	55	20
4. Cyanide + iproniazid	13	>5	>5	20
5. Cyanide (shaken in N ₂)	100	100	100	100
6. Cyanide + iproniazid (shaken in N ₂)	27	54	60	52

Fig. 4. Effect of shaking mitochondrial monoamine oxidase in oxygen with cyanide on inhibition by iproniazid. Each flask contained 1 ml. of mitochondrial suspension (equivalent to 300 mg. wet wt. of liver). Tyramine (0.01M) and iproniazid (4 μ M) were present in the side arms and KCN (0.002M) and 0.067M-phosphate buffer, pH 7.2, in the central compartment to give a final volume of 4 ml. After gassing with oxygen the enzyme preparation was shaken for various times before incubation with iproniazid for 10 min. and determining the resultant inhibition.

side arms. After gassing with oxygen, the inhibitor was added to the centre compartment at different times (0, 10, 20, 30 and 40 min.) and then tyramine added for enzyme-activity determination. In this way the monoamine oxidase preparation was shaken for a constant 40 min. with cyanide and buffer but for different times of incubation with the inhibitor. Under these conditions, instead of progressive inhibition, there was an immediate and greatly increased inhibition which apparently became less with increased time of incubation of enzyme and iproniazid (Fig. 3). The inhibition could not be reversed by washing the inhibited

mitochondria. Shaking the inhibitor in phosphate buffer with or without 0.002M-potassium cyanide had no influence on the degree of inhibition obtained with an unshaken mitochondrial preparation. Fig. 4 shows the effect of shaking liver mitochondria, in oxygen and with cyanide, for different times in Warburg flasks before addition of iproniazid to the enzyme, incubating for 10 min. and subsequently determining enzyme activity. It will be noted that the longer the pre-shaking of the mitochondria in oxygen with cyanide the greater was the inhibition produced by the iproniazid. This sensitizing effect did not increase after 50-60 min. It seemed, therefore, that pre-shaking liver mitochondria under these conditions increased the susceptibility of the monoamine oxidase to inhibition by iproniazid. This was substantiated by determining the approximate dissociation constant of the enzyme inhibitor complex for mitochondria under different conditions of pretreatment. The dissociation constant for the combination of iproniazid and monoamine oxidase in mitochondria was not changed by prior shaking in nitrogen or oxygen. But in the presence of cyanide the approximate affinity constant (1/dissociation constant) increased a hundred times on prior shaking with oxygen for 30 min. before addition of iproniazid and tyramine (Table 5).

Rat-liver mitochondria were shaken in oxygen, at 37° with potassium cyanide for 50 min., to sensitize them fully to inhibition by iproniazid. The kinetics of inhibition of this enzyme preparation by iproniazid (1 μ M) were examined. Inhibition was immediate, not progressive and irreversible. The same sensitized mitochondria were diluted with saline, centrifuged down and resuspended in phosphate buffer. No inhibition could be obtained on incubating with iproniazid (4 μ M) for 10 min. Thus sensitization by cyanide can be readily reversed by washing.

Table 5. *Effect of pre-shaking in oxygen and nitrogen on the affinity of iproniazid for mitochondrial monoamine oxidase*

Mitochondrial suspensions (1 ml., equivalent to 300 mg. wet wt. of liver/ml.) in phosphate buffer were placed in the central compartment of Warburg flasks with or without KCN (0.002M). Suitable concentrations of tyramine and iproniazid were placed in the side arms. Sufficient phosphate buffer was added to give a final volume of 4 ml. The approximate inhibitor dissociation constant was calculated by the Lineweaver & Burk (1934) method.

Prep.	Approximate dissociation constant calc. after pre-shaking in		
	Nitrogen (10 min.)	Oxygen (5 min.)	Oxygen (30 min.)
No KCN	1×10^{-4}	2×10^{-4}	2×10^{-4}
KCN	6×10^{-5}	6×10^{-6}	5×10^{-7}

Preliminary experiments indicate that inhibition of monoamine oxidase by *isopropylhydrazine* can also be increased by adding cyanide.

Possible dehydrogenation of iproniazid as a pre-requisite for inhibition. It has been shown that oxidation of iproniazid is necessary for irreversible inhibition of monoamine oxidase, and it therefore seemed possible that iproniazid acted initially as a substrate for the enzyme. It has been suggested (Blaschko, 1952) that amines are first dehydrogenated to give an imino compound; thus dehydrogenation of iproniazid to 1-*isopropylidene-2-isonicotinoylhydrazine* would be a necessary step in forming an irreversible inhibitor. 1-*iso*Propylidene-2-*isonicotinoylhydrazine*, however, did not inhibit monoamine oxidase at mM *in vitro* or in mice *in vivo*. Mice were given 250 mg. of 1-*isopropylidene-2-isonicotinoylhydrazine*/kg. by intraperitoneal injection. After 1 and 5 hr. pairs of animals were killed. Liver suspensions (25 %, w/v) were prepared in phosphate buffer and monoamine oxidase activity was determined on 1 ml. with tyramine (0.01M) as substrate. There was no inhibition of enzyme activity when compared with liver preparations from control animals. It is also of interest that *isoprenaline sulphate* [1-(3:4-dihydroxyphenyl)-2-*isopropylaminoethanol sulphate*], which possesses the $\cdot\text{NH}\cdot\text{CH}(\text{CH}_3)_2$ terminal group, at a concentration of 0.01M had little effect on the rate of tyramine oxidation and was not itself actively oxidized by monoamine oxidase.

DISCUSSION

In the presence of cyanide mitochondrial monoamine oxidase activity, as measured by oxidation of tyramine, is inhibited by iproniazid at low concentrations (μM). In the absence of cyanide these concentrations of iproniazid have little effect on enzyme activity. Since prior incubation of iproniazid

with cyanide in oxygen or with cyanide and mitochondria anaerobically at 37° does not effect the degree of inhibition of the monoamine oxidase activity subsequently obtained, it seems improbable that cyanide reacts in any way with iproniazid. However, it has been shown that inhibition by iproniazid can be greatly increased by shaking the mitochondria with cyanide in oxygen before adding the inhibitor. This sensitization reaches a maximum in 50–60 min., can be readily reversed by washing, requires the presence of oxygen and does not occur in the presence of iproniazid. Inhibition of fully sensitized liver mitochondrial monoamine oxidase by iproniazid is immediate and irreversible.

Since pre-shaking mitochondria with cyanide in oxygen increases the affinity constant of iproniazid for the enzyme, it appears that the site of attachment of the inhibitor on the enzyme is influenced by this treatment. The sensitization can be easily reversed by washing, which suggests that cyanide reacts at the site of attachment and not by the removal of possible interfering factors, such as metal ions.

When iproniazid is added to mitochondrial suspensions containing cyanide in the presence of oxygen, the inhibition which occurs on incubation is greater than in the absence of cyanide. This inhibition is progressive and first-order and cannot be reactivated by washing the mitochondria. However, the results from a dilution experiment suggest that some part of the inhibition may be reversible. In this method of dilution the incubation of iproniazid has to be performed with concentrated mitochondria in the side arm of a Warburg flask and these different conditions may explain the apparent anomaly. Glutathione or the supernatant prepared from rat-liver suspensions when added to mitochondrial suspensions decreases the inhibition of monoamine oxidase obtained by incubation with iproniazid. This effect can be prevented by adding cyanide. These observations suggest that a thiol group may be involved in the attachment of iproniazid to the enzyme. It is therefore of interest that Friedenwald & Herrmann (1942) have shown that monoamine oxidase is a thiol enzyme and that inhibition by mercurials can be reversed by glutathione only in the presence of cyanide.

Higher concentrations of iproniazid (10^{-5}M), in the absence of cyanide, also irreversibly inhibit monoamine oxidase activity both *in vivo* and *in vitro* (Zeller *et al.* 1955a). It has been found that for irreversible inhibition to occur *in vitro* pre-incubation of liver mitochondrial monoamine oxidase with iproniazid in oxygen is necessary. The inhibition is first-order, and since it has a high energy of activation it seems likely that a chemical reaction of some kind occurs. Zeller *et al.* (1955b) have pointed out that the requisite structure for an inhibitor of monoamine oxidase is $\text{R}_2\text{N}\cdot\text{NHR}'$

(where R is H or other substituent and R' is an aryl or alkyl group). Since there is evidence to suggest that iproniazid may be metabolized to an inhibitor of a pyridoxal phosphate-requiring enzyme (Davison, 1956), it seemed possible that iproniazid was hydrolysed to *isopropylhydrazine*, and that this substance inhibited both monoamine oxidase and pyridoxal phosphate-requiring enzymes. It was therefore interesting to find that *isopropylhydrazine* is a more powerful inhibitor of monoamine oxidase than iproniazid; however, like iproniazid, *isopropylhydrazine* also requires the presence of oxygen to act as an irreversible inhibitor, and the progressive inhibition reaction has an apparent energy of activation of the same order as for iproniazid. This suggests that the mechanism of the inhibition by iproniazid and *isopropylhydrazine* are similar and that iproniazid is not necessarily converted into *isopropylhydrazine*. This view is confirmed by the finding that the degree of inhibition of monoamine oxidase is not altered by shaking iproniazid in buffer at 37° or with mitochondria in an atmosphere of nitrogen. The susceptibility of monoamine oxidase activity to inhibition by iproniazid is not affected by partial thermal inactivation. Thus if alteration of iproniazid does occur, the system that brings this about must have the same sensitivity to heat as monoamine oxidase. Finally it has been shown that monoamine oxidase inhibited by methyl- or *isopropyl*-hydrazine is not easily reactivated, whereas enzyme inhibited by iproniazid or 1-*isonicotinoyl*-2-methylhydrazine can be partially reactivated by dialysis or by the addition of dimercaptopropanol.

Another possibility is that iproniazid is converted into *isopropylhydrazine* by monoamine oxidase itself. This would seem unlikely, because Zeller *et al.* (1955*b*) have shown that such substances as 1:2-disubstituted *isonicotinoylhydrazines* are inhibitors of monoamine oxidase; secondly, *isopropylhydrazine* shows increased inhibition in the presence of cyanide.

An alternative hypothesis for the mechanism of irreversible inhibition of monoamine oxidase activity by iproniazid is that dehydrogenation of iproniazid to 1-*isopropylidene*-2-*isonicotinoylhydrazine* occurs in the presence of oxygen at the enzyme active centre, in a similar way to that postulated for the first stage in amine oxidation.

Evidence that iproniazid reacts at the active centre is provided by our finding that inhibition is competitive and sensitive to pH change and heat in a similar way to substrate oxidation. Zeller *et al.* (1955*b*) have shown that there must be a free hydrogen atom on the terminal nitrogen bearing the alkyl group of the substituted hydrogen; it therefore seems probable that, once attached to the active centre, the iproniazid undergoes dehydrogenation and reacts further with the enzyme.

SUMMARY

1. Inhibition of rat-liver monoamine oxidase by iproniazid is first-order and irreversible and has a high energy of activation.

2. Since the inhibition reaction has also been found to require oxygen, it is postulated that irreversible inhibition is the result of dehydrogenation of iproniazid at the active centre of the enzyme.

3. In the presence of potassium cyanide inhibition of mitochondrial monoamine oxidase is much increased. This effect is augmented by prior shaking of the mitochondria in oxygen with potassium cyanide.

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