Studies on Monoamine Oxidase: The Mechanism of Inhibition of Monoamine Oxidase by Iproniazid

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Received February 15, 1963

The mechanism of inhibition of monoamine oxidase by iproniazid has been investigated. Studies with iproniazid labeled with C^{14} in the isopropyl group have shown that the inhibitor combines with partially purified monoamine oxidase in an irreversible and specific manner. Data have been obtained indicating that iproniazid is cleaved nonenzymatically to yield a potent inhibitor of monoamine oxidase. The inhibitor formed is a volatile compound and is either isopropylhydrazine or a more highly oxidized product derived from isopropylhydrazine. Oxygen is required for the nonenzymatic formation of the inhibitor from iproniazid and cyanide, and boiled tissue extracts enhance its formation. The formation of such a product by a nonenzymatic mechanism may precede the irreversible inhibition of monoamine oxidase by iproniazid and related hydrazine derivatives.

The inhibition of monoamine oxidase by substituted hydrazines such as 1-isonicotinyl-2-isopropylhydrazine (iproniazid) presents several problems not common to the other known inhibitors of this enzyme (Zeller, Barsky, and Berman, 1955). Iproniazid gives maximal inhibition of enyzme activity only after a suitable period of preincubation in oxygen (Davison, 1957). Systematic alteration of the iproniazid molecule has shown that the isopropylhydrazine moiety is the essential part of the molecule for inhibitory action against monoamine oxidase (Barsky *et al.*, 1959). These findings have led to speculation that iproniazid is first cleaved to free isopropylhydrazine which is the active monoamine oxidase inhibitor (Pletscher, 1959; Schwartz, 1962). It is also known that the degree of inhibition of monoamine oxidase activity obtained with iproniazid is enhanced in the presence of cyanide (Davison, 1957), although cyanide itself does not inhibit monoamine oxidase.

Iproniazid labeled with C^{14} in the isopropyl moiety has made it possible to investigate directly the above observations and presumptions. The initial part of this paper is a re-evaluation of the work by Davison (1957) and shows directly that iproniazid, in some form, is bound to monoamine oxidase in an irreversible manner. Data have been obtained which show that iproniazid can be cleaved nonenzymatically to form a potent inhibitor of monoamine oxidase. The inhibitor is a volatile compound obtained from the breakdown of iproniazid and is either isopropylhydrazine or a product derived from isopropylhydrazine. Oxygen is required for the nonenzymatic formation of the inhibitor from iproniazid and cyanide enhances its formation. These findings allow logical explanations for the observed properties of the inhibition of monoamine oxidase by iproniazid.

Materials and Methods

1-Isonicotinyl-2-(2-C^{14}-isopropyl)hydrazine, specific activity 4.46×10^5 cpm/μmole, and 1-benzyl-2-(5-methyl-3-isoxazolylcarbonyl)hydrazine (isocarboxazid) labeled with C^{14} in the benzyl carbon, specific activity 1.5×10^5 cpm/μmole were kindly supplied by Dr. B. A. Koechlin and Dr. M. Schwartz of Hoffmann-La Roche, Inc. Cyanide-C^{14}, 6×10^6 cpm/μmole, was purchased from the New England Nuclear Corporation. Monoamine oxidase was prepared by treatment of guinea-pig liver mitochondria with digitonin as described by Smith, Weissbach, and Udenfriend (1962). One unit of monoamine oxidase activity is defined as that amount of enzyme necessary to catalyze the oxidation of 0.01 μmole of kynuramine per minute under previously described conditions (Weissbach *et al.*, 1960). Protein was determined by the method of Lowry *et al.* (1951).

Isopropylhydrazine was determined in the absence of other reducing materials as follows: To 0.2 ml of solution containing from 0.02 to 3 μmoles of isopropylhydrazine was added 2 ml of 0.1 N NH_4OH and 0.2 ml of Folin and Ciocalteu phenol reagent, diluted with 1 volume of water. The solution was allowed to stand for 10 minutes after which time 2 ml of 2% sodium carbonate in 0.1 N NaOH was added. After an additional 20 minutes, the optical density of the resulting chromophore was measured at 750 mμ.

Most of the incubations were carried out in Warburg vessels. The inhibitor was placed in the side arm and cyanide, when used, was in the center well. In the studies with iproniazid-C^{14} the enzyme was first incubated for 10 minutes with isoniazid, a hydrazine which does not inhibit monoamine oxidase, but reacts with nonspecific groups which bind hydrazines. Unless isoniazid is used, the relationship of the binding of iproniazid to monoamine oxidase activity is obscured. Iproniazid-C^{14} was then added and the incubation was continued for an additional 60 minutes. Enzymatic activity was determined manometrically after the addition of tyramine to the main compartment from a second side arm of the Warburg flask. Where nitrogen was the gaseous atmosphere for the preincubation, substrate was added and the flask flushed with oxygen for about 2 minutes before enzyme assay. Manometric changes were noted after an additional 2 minutes and compared with those of a thermobarometer which had been flushed in the same manner as the experimental flask. Under these conditions the reaction was linear for at least 20 minutes. In experiments carried out in air, where this type of comparison was not necessary, aliquots were removed and enzymatic activity was determined by the kynuramine method of Weissbach *et al.* (1960). Phosphate buffer at *p*H 8 was used in all experiments.

A typical incubation mixture consisted of 5×10^{-2} M buffer and enzyme in the main compartment. One side arm contained measured amounts of iproniazid-C^{14}

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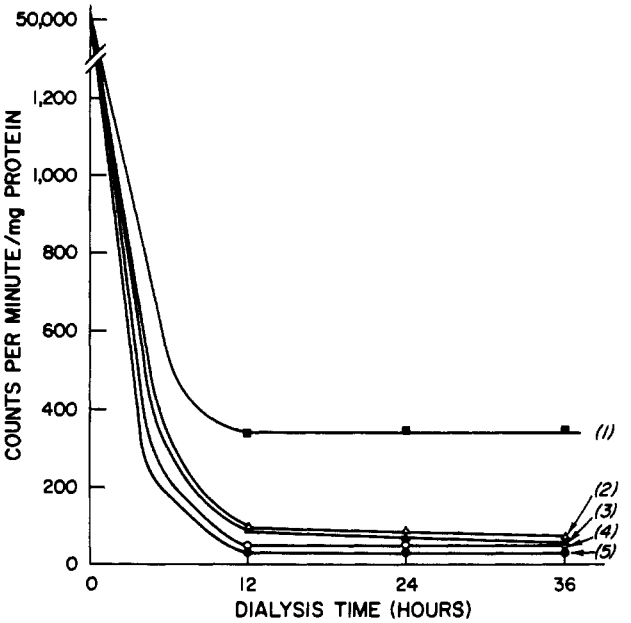

FIG. 1.—Specificity of the binding of iproniazid to proteins. The incubation mixtures contained 100 μmoles of phosphate buffer pH 8, 20 μg of iproniazid-C^{14}, 2 μmoles of isonicotinylhydrazine, and 2 mg of all the proteins except enzyme, of which there was 2.8 mg. The final volumes were 2.2 ml. All flasks were preincubated with the isonicotinylhydrazine for 10 minutes prior to the addition of iproniazid. The incubation was for 60 minutes. (1) Monoamine oxidase; (2) gamma globulin; (3) monoamine oxidase control, the enzyme pretreated with unlabeled iproniazid before the addition of labeled iproniazid. Similar results were obtained with heat inactivated enzyme: (4) albumin; (5) snake venom.

and 50 μmoles of buffer, and the other side arm contained 10–20 μmoles of tyramine. The center well contained 50 μmoles of buffer and 1–10 μmoles of potassium cyanide.

In some instances, after the incubation was completed and the residual enzymatic activity had been determined, the mixture was dialyzed for 12, 24, or 36 hours against running tap water and against several changes of demineralized water. The radioactivity remaining after dialysis (nondialyzable) is referred to as protein-bound radioactivity. Control experiments, run simultaneously, contained the same basic components. However, prior to incubation of the control flasks nonradioactive iproniazid was added to inhibit the enzyme or the enzyme was inactivated by heating before the addition of iproniazid-C^{14}. Experimental values were corrected for the small amount of radioactivity bound in the control experiments.

All radioactivity determinations were made in an automatic Tri-Carb Liquid Scintillation Counter using a *p*-dioxane-methanol counting fluid as described by Bray (1960).

RESULTS

Binding of Iproniazid to Monoamine Oxidase.—When monoamine oxidase was incubated with iproniazid-C^{14} and dialyzed as described under Methods, radioactivity was found to be associated with the protein fraction. The binding of iproniazid to protein under these conditions was dependent upon monoamine oxidase activity as shown in Figure 1. The small amounts of radioactivity bound by gamma globulin, serum albumin, and snake venom (which is rich in L amino acid oxidase) were comparable to the amounts bound by a sample of previously inactivated monoamine oxidase (control).

TABLE I

EFFECT OF UREA ON THE BINDING OF IPRONIAZID TO MONOAMINE OXIDASE

Enzyme preparations (experimental and control) obtained as outlined in Figure 1 were exposed to 8 M urea for 3 hours at room temperature. Dialysis was repeated for 24 hours and the nondialyzable radioactivity was again determined.

	Radioactivity in the Enzyme Solution	
	Before Urea (cpm)	After Urea (cpm)
Enzyme control + iproniazid-C^{14}	81	76
Enzyme + iproniazid-C^{14}	461	420

The data in Table I show that once the inhibitor was bound to the protein it could not be removed by treatment with 8 M urea.

Effect of Substrate and Other Inhibitors on the Binding of Iproniazid to Monoamine Oxidase.—Previous experiments indicate that iproniazid competes with substrate for the active site of monoamine oxidase and that enzyme inhibition by this compound can be prevented or delayed by the presence of large amounts of substrate (Davison, 1957). An unequivocal demonstration of the protective effect of substrate on the binding of iproniazid by monoamine oxidase may be seen in the data shown in Table II. In the presence of substrate essentially no radioactivity from iproniazid-C^{14} was bound to the protein. Also shown here are the effects of other monoamine oxidase inhibitors on the binding of iproniazid to the enzyme. The hydrazine compound, β-phenylisopropylhydrazine, prevented the binding of iproniazid to monoamine oxidase, whereas the nonhydrazine inhibitors, β-phenylcyclopropylamine and *N*-methyl-*N*-benzylpropynylamine, had only a slight effect on the binding of radioactivity, although each of them inhibited enzyme activity. It should be pointed out that in each case excess inhibitor was re-

TABLE II

EFFECT OF SUBSTRATE AND OTHER INHIBITORS ON THE BINDING OF IPRONIAZID BY MONOAMINE OXIDASE

Two-ml aliquots of an enzyme preparation containing about 10 units of activity per ml was incubated for 10 minutes with 2 μmoles of the indicated monoamine oxidase inhibitors (tubes 3–5). Following this the mixtures were dialyzed for 4 hours against large volumes of 0.01 M phosphate buffer to remove excess inhibitor and the activity was determined (column A). To tube no. 2 five μmoles of substrate (tyramine) was added at zero time and every 15 minutes thereafter. Forty μgrams of iproniazid-C^{14} was added to each tube and the incubation continued for 60 minutes. The contents of each tube were again dialyzed, as described, and the protein-bound radioactivity was determined (column B). Enzyme activity is expressed as the change in absorbancy at 360 mμ due to the oxidation of kynuramine.

Tube No.	Additions	Enzyme Activity/ml after Dialysis	cpm/ml
		A	B
1	None	0.290	496
2	Substrate	0.230	33
3	β-Phenylisopropylhydrazine	0.000	49
4	*N*-Methyl-*N*-benzylpropynylamine	0.000	419
5	β-Phenylcyclopropylamine	0.000	386

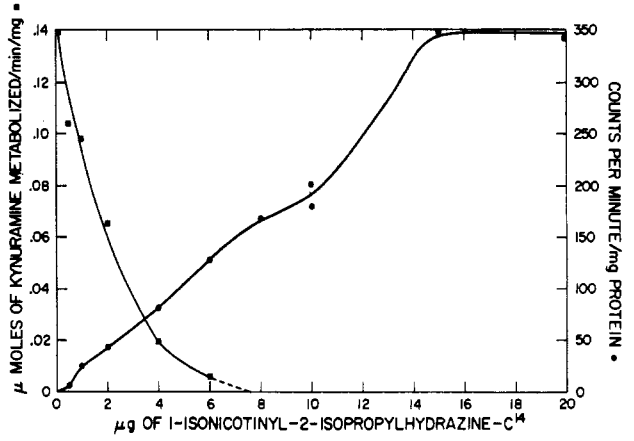

FIG. 2.—Relationship between the binding of iproniazid to protein and the inhibition of monoamine oxidase activity. Each flask contained 5 units of monoamine oxidase and measured amounts of iproniazid-C^{14} in a total volume of 1 ml.

moved by dialysis before the addition of iproniazid-C^{14}.

Stoichiometry of the Binding of Iproniazid to Monoamine Oxidase.—Since the binding of iproniazid to monoamine oxidase appeared to be specifically related to enzyme activity, it seemed possible to investigate some aspects of the stoichiometry between this binding and enzymatic activity. To do this, measured amounts of iproniazid-C^{14} were incubated with a monoamine oxidase preparation as described under Methods. At the end of the incubation, residual enzyme activity and protein-bound radioactivity were determined. A plot of these data versus the amount of iproniazid-C^{14} used is shown in Figure 2. It can be seen that more labeled iproniazid (about twice as much) was bound to the enzyme than was required for complete inhibition. Regardless of the initial number of units of monoamine oxidase used, or the relative purity of monoamine oxidase, when inhibition was complete the ratio of bound radioactivity (cpm) to initial enzyme units was usually about 25:30.

Effect of Cyanide and Anaerobiosis on the Interaction Between Iproniazid and Monoamine Oxidase.—One of the unique characteristics of monoamine oxidase in comparison with other amine oxidases is that it is not inhibited by cyanide. The presence of cyanide in an incubation mixture, however, makes iproniazid a more effective monoamine oxidase inhibitor (Davison, 1957). Davison (1957) has suggested that this effect is on the mitochondria, making the enzyme more sensitive and also more susceptible to interaction with the inhibitor molecule.

When monoamine oxidase was incubated with iproniazid-C^{14} and nonradioactive cyanide in an atmosphere of oxygen, a considerable amount of radioactivity was found attached to the protein after extensive dialysis. There was more protein-bound radioactivity in the presence of cyanide than in its absence (Table III) and in both cases the enzyme was completely inhibited. However, in an atmosphere of nitrogen, or if substrate were added at zero time, much less radioactivity from the iproniazid-C^{14} was attached to the protein both in the presence or absence of cyanide; in both cases a large amount of enzymatic activity was retained. What was of most interest was the finding that in the presence of cyanide inhibition of monoamine oxidase could be demonstrated under conditions in which iproniazid was never in contact with the enzyme. In such an experiment, shown as the control in Table III, the oxygen uptake without cyanide, and with iproniazid

TABLE III

EFFECT OF ANAEROBIOSIS AND CYANIDE ON THE BINDING OF IPRONIAZID TO MONOAMINE OXIDASE

Effect of oxygen and cyanide on enzyme inhibition and binding of iproniazid to monoamine oxidase. All incubations were carried out in Warburg vessels essentially as described under Methods. One side arm of each flask contained 20 μg of iproniazid-C^{14} and the other contained 10 μmoles of tyramine. After equilibrium had been obtained with the indicated gaseous phase, inhibitor was added from the side arm and the flask was incubated for 60 minutes. Substrate was added from the other side arm after the preincubation period and oxygen uptake was determined. The flasks which were preincubated under nitrogen were flushed with oxygen, as described, prior to determination of enzyme activity. The control consisted of enzyme and buffer preincubated in oxygen for 60 minutes (with iproniazid in the side arm) before the addition of substrate. One μmole of cyanide, when used, was in the center well.

	Cyanide	Enzyme Activity (O_2 uptake) (μl)	Inhibitor Bound to Protein (cpm/mg)
Oxygen	+	0	550
	−	0	300
N_2	+	80	61
	−	125	36
Control	−	158	0
	+	0	620

still in the side arm, was 158 μliters/hour. On the other hand, the oxygen uptake of another flask identical to the one just described but with cyanide in the center well was zero. In neither of these flasks had the iproniazid been in direct contact with the enzyme prior to determining the activity of the enzyme preparation. This indicated that under the influence of cyanide a volatile inhibitory compound had been formed from iproniazid. Dialysis of the enzyme preparation which had been inhibited by the volatile inhibitor revealed the presence of at least as much radioactivity in a nondialyzable form as was in those incubations in which the iproniazid-C^{14} was added directly to the enzyme. This type of experiment was repeated several times with and without shaking, and each time essentially the same results were obtained. The effect could not be duplicated with cyanide alone nor with cyanide in the presence of isonicotinylhydrazine.

In an attempt to see whether cyanide itself would bind to the enzyme and also whether the enzyme would bind cyanide more in the presence of iproniazid than in its absence, an experiment was set up as described above but with cyanide-C^{14} in the center well and unlabeled iproniazid in the side arm. The nonspecific binding of cyanide to protein, however, was so high as to make it impossible to obtain significant results.

Requirements for Formation of the Volatile Inhibitor from Iproniazid.—The formation of a volatile inhibitor from iproniazid was found to be oxygen dependent (Table IV). Furthermore, formation of volatile inhibitor occurred in the absence of an enzyme preparation, and enzyme preparations denatured by boiling were slightly more effective in releasing volatile radioactivity and inhibitor than "native" enzyme preparations. These findings show that monoamine oxidase itself is not responsible for the degradation of iproniazid which led to the formation of the inhibitor. Furthermore, significant amounts of the inhibitor were formed in the presence of a monoamine oxidase preparation which had previously been inhibited by β-phenylisopropylhydrazine.

TABLE IV

REQUIREMENTS FOR THE FORMATION OF THE VOLATILE INHIBITOR FROM IPRONIAZID

Reactions were carried out in Warburg flasks. All flasks contained 10 μg of iproniazid-C^{14} in the side arm. The main compartment contained 0.5 ml of enzyme, 100 μmoles of phosphate buffer *p*H 8, and water to 1 ml.

Gas Phase	Additions to the Side Arm in Addition to Iproniazid During Preincubation	Cyanide[a]	Enzyme Activity[b]	Radioactivity Found in the Main Compartment (cpm)
Oxygen				
	None	−	0.350	568
	None	+	0.000	1425
	Enzyme	−	0.000	832
	Enzyme	+	0.000	1944
	Heated enzyme	−	0.000	1075
	Inhibited enzyme[c]	−	0.050	695
Helium				
	None	−	0.530	80
	Enzyme	−	0.450	120

[a] Cyanide, 10 μmoles, was in the center well with 100 μmoles of phosphate buffer *p*H 8. [b] Determinations of enzyme activity were made on aliquots of the enzyme solution contained in the main compartment after the preincubation period (60 minutes). Activity is expressed as change in absorbancy at 360 mμ/ml with kynuramine as substrate. [c] This enzyme was inhibited by prior treatment with β-phenylisopropylhydrazine.

Attempts to Identify the Volatile Monoamine Oxidase Inhibitor Formed from Iproniazid.—Examination of several possible degradation products of iproniazid and of iproniazid itself for their ability to inhibit monoamine oxidase by distillation from the side arm of Warburg vessels revealed that, in addition to iproniazid,[1] only isopropylhydrazine would inhibit enzyme activity (Table V). It therefore appeared important to determine the extent of inhibition of monoamine oxidase by isopropylhydrazine when the latter compound was added directly to an active enzyme solution. Figure 3 shows the results of such an experiment run in both the presence and the absence of cyanide without preincubation of enzyme and inhibitor. The sequence of additions to the enzyme solution, however, was inhibitor then substrate (kynuramine). Fifty per cent inhibition was obtained at concentrations of approximately 4×10^{-7} M and 1.1×10^{-6} M in the presence and absence of cyanide, respectively. These data are compatible with the postulate that either isopropylhydrazine and/or a further oxidation product of it formed during the decomposition of iproniazid is the active inhibitor.

TABLE V

EFFECT OF POSSIBLE DEGRADATION PRODUCTS OF IPRONIAZID ON MONOAMINE OXIDASE ACTIVITY

The main compartment contained 8 units of enzyme, 100 μmoles of phosphate buffer *p*H 8, and water to 1 ml. One μmole of each of the compounds listed was placed in the side arm with 100 μmoles of buffer at *p*H 8. There was no intracompartmental mixing. Enzyme activity was determined by the spectrophotometric method (Weissbach *et al.*, 1960) after a 1-hour preincubation.

Compound Added to the Side Arm	Per Cent Inhibition
None	0
Acetone	0
Isopropylamine	3
Isopropylalcohol	0
Isopropylhydrazine	100
Iproniazid	68

[1] It must be pointed out that the inhibition obtained under these conditions by the relatively large amount of iproniazid, 10–20 times that used in most of the other experiments, probably reflects the spontaneous breakdown of iproniazid to the volatile compound prior to distillation. No evidence for the presence of the isonicotinic acid moiety of iproniazid in the main compartment of the flask could be obtained.

In an attempt to detect isopropylhydrazine formation from iproniazid, experimental flasks were set up with iproniazid-C^{14} as described under Methods, except that the main compartment contained, instead of enzyme, 2–4 μmoles of nonradioactive isopropylhydrazine in 0.1 N HCl. When the materials from the main compartments and the side arms of the flasks were combined and subjected to ion exchange chromatography on IRC-50 resin, two radioactive peaks were revealed (Fig. 4). The first of these was largely unchanged iproniazid and neutral metabolites; the second coincided with the added isopropylhydrazine. The amount of this basic material trapped was small, only about 1% of the radioactivity. It was found, however, that recoveries of carrier isopropylhydrazine were less than 50%. From the data presented (Fig. 4), cor-

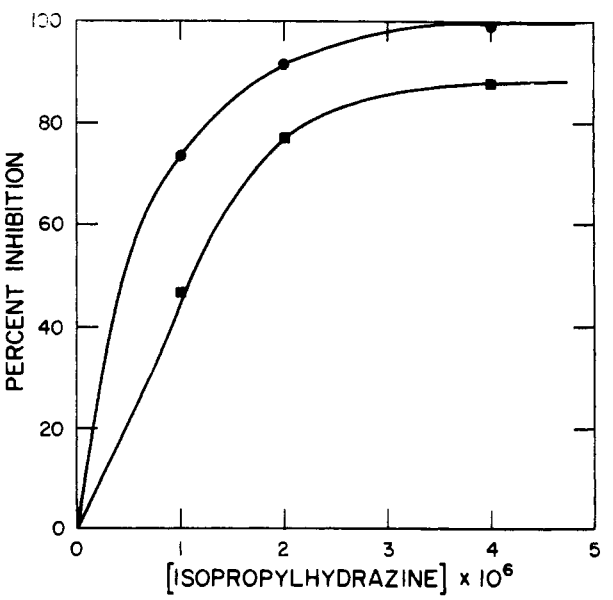

FIG. 3.—The inhibition of monoamine oxidase by isopropylhydrazine in the presence and absence of cyanide. The rate of oxidation of kynuramine was determined in the presence of measured amounts of isopropylhydrazine and 1.6 units of monoamine oxidase. The squares represent the inhibition obtained in the absence of cyanide. The circles represent the inhibition obtained in the presence of cyanide.

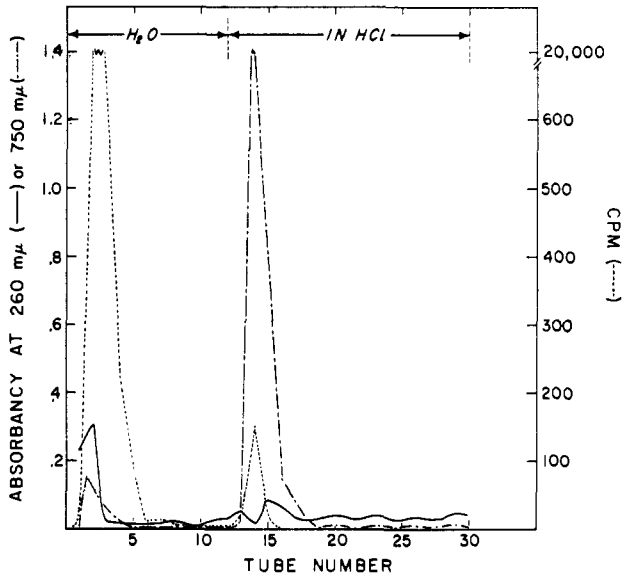

FIG. 4.—Chromatographic separation of iproniazid and isopropylhydrazine from an incubation mixture. The incubation mixture, obtained as described in the text, was subjected to ion exchange chromatography on a 1 × 4.5-cm column of IRC-50 resin which had been equilibrated with acetate buffer at *p*H 6.0–6.5. The column was eluted first with water and then with 1 N HCl. Two-ml aliquots were collected. Cpm represents the total radioactivity per tube. Absorbancy at 260 mμ was used to determine iproniazid and the absorbancy at 750 mμ of the chromophore developed with Folin's reagent, to detect isopropylhydrazine.

rected for recovery, 287 cpm were present in the isopropylhydrazine fraction. This amount of radioactivity would be equivalent to a concentration of about 6×10^{-7} M isopropylhydrazine in the solution in the main compartment.

The Formation of Other Metabolites from Iproniazid.—The data of Figure 4 and Table IV show that significantly more radioactivity can distill from the side arm into the main compartment of a Warburg flask than can be accounted for as isopropylhydrazine-like material. These findings suggest that some other compound(s) containing the isopropyl group are formed from iproniazid. The addition of carrier acetone to the distilled radioactive material and isolation of the 2,4-dinitrophenylhydrazone of acetone containing C^{14} confirmed previous *in vivo* experiments (Koechlin and Iliev, 1959) which showed that acetone is formed from iproniazid. About 49% of the volatile nonisopropylhydrazine radioactivity could be accounted for in this manner. The other radioactive product(s) has not been identified.

Studies with Isocarboxazid-C^{14}.—The type of reaction reported here is not unique for iproniazid. Preliminary experiments with C^{14}-labeled isocarboxazid, another substituted hydrazine inhibitor of monoamine oxidase, indicate that this compound also is bound to monoamine oxidase under conditions similar to those required for the binding of iproniazid (Table VI). This binding is also enhanced by cyanide. It is not unreasonable to assume then that the cleavage of this compound to the active inhibitor, benzylhydrazine (Schwartz, 1962) or a higher oxidized derivative, can proceed nonenzymatically.

DISCUSSION

With the availability of iproniazid-C^{14}, data were obtained which show that iproniazid, in some form, is bound irreversibly to monoamine oxidase. This

TABLE VI

BINDING OF ISOCARBOXAZID-C^{14} TO MONOAMINE OXIDASE

All flasks contained 6.7 units of monoamine oxidase activity and 2.5 μg of isocarboxazid-C^{14} (16,790 cpm) in 50% methanol (0.005 ml). The incubation, carried out exactly as for iproniazid, Figure 1, was for 10 minutes. One μmole of cyanide, where indicated, was in the center well with phosphate buffer at *p*H 8.

Conditions	Net cpm/ml
Enzyme	270
Enzyme + cyanide	1110
Enzyme heated at 100° for 1 minute	0
Enzyme + 4 μmoles of tyramine	10

observation and the observation that the binding is oxygen dependent and competitive with substrate were expected in view of earlier work on monoamine oxidase and iproniazid. The observation that iproniazid would become bound to monoamine oxidase after the enzyme had been inhibited by β-phenylcyclopropylamine and *N*-methyl-*N*-benzylpropynylamine was surprising since all these compounds, presumably, react with the same active center of the enzyme. The fact that β-phenylisopropylhydrazine will prevent the binding of iproniazid to monoamine oxidase might suggest that iproniazid and β-phenylisopropylhydrazine react at the same site on the enzyme which is different from the site attacked by the nonhydrazine inhibitors. In fact, such an idea has been advanced to explain why *in vivo* administration of harmaline will prevent the long-lasting effect of β-phenylisopropylhydrazine, but not that of β-phenylcyclopropylamine, on rat brain amine oxidase activity (Horita and McGrath, 1960). An equally likely explanation for the findings reported in this paper, however, is that iproniazid can displace the nonhydrazine inhibitors but not the hydrazine inhibitors from the active site of the enzyme. All these compounds may react at the same site, but the complex formed between iproniazid and monoamine oxidase is thermodynamically more stable and therefore is favored under the conditions of these experiments.

The possibility should not be overlooked that the mechanism of action of these compounds as inhibitors differs. It has been shown that the complex between β-phenylcyclopropylamine and monoamine oxidase is less firm than the one with iproniazid and can be reversed with substrate, whereas the complex with iproniazid cannot be reversed (Zeller *et al.*, 1962a; Zeller *et al.*, 1962b).

The inhibition of monoamine oxidase by iproniazid and isopropylhydrazine is markedly enhanced by cyanide; this effect of cyanide also requires oxygen. Autoxidation of hydrazines is known to occur (Degering *et al.*, 1950; Lesbre, 1948; Eberson and Persson, 1962), and this reaction can be catalyzed by certain metal ions, potassium ferricyanide, and other materials. It is not surprising, therefore, that during the preincubation of iproniazid a volatile compound is formed, which is the active inhibitor of monoamine oxidase. It should be stressed, however, that monoamine oxidase is not necessary for the formation of this inhibitor since it is readily formed in the absence of active enzyme or of protein. This inhibitor could be isopropylhydrazine, a volatile compound formed during the breakdown of iproniazid (Fig. 4) and known to be a potent inhibitor of monoamine oxidase. However, the finding that cyanide also increases the degree of inhibition of monoamine oxidase by isopropylhydrazine, in spite of the fact that isopropylhydrazine is considerably less stable

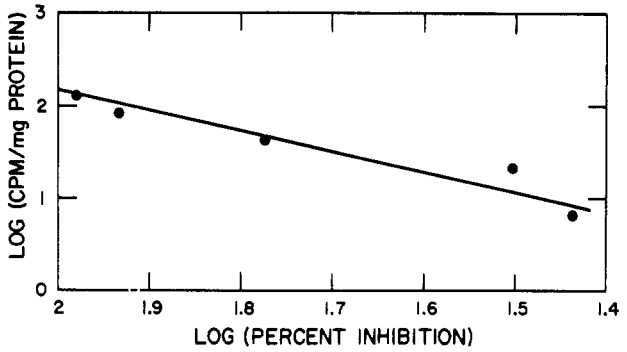

FIG. 5.—A logarithmic plot of the data from Figure 2.

under these conditions, suggests that some more highly oxidized derivative of isopropylhydrazine may be the active inhibitor. There are some experimental data to support this. Experiments have shown that the volatile inhibitor from iproniazid is, for the most part, unstable. When this volatile inhibitor was collected in the presence of enzyme, complete inhibition was obtained. On the other hand, when the volatile material was allowed to distill in the absence of enzyme, and the enzyme was added after a comparable amount of radioactivity had been collected and the remaining iproniazid had been removed, less than 20% inhibition was obtained. This was true even if the mixture was then incubated for an additional hour. The data also suggest that cyanide in some undefined form catalyzes both the cleavage of iproniazid to isopropylhydrazine and the subsequent oxidation of isopropylhydrazine to the more potent and labile inhibitor. Davison (1957) has suggested that the effect of cyanide is on the enzyme, making it more sensitive to the inhibitor. It remains to be seen whether cyanide exerts effects other than the one described here.

In any event, it is clear that there is a specific binding of the active inhibitor derived from iproniazid to monoamine oxidase. This complex is stable to prolonged dialysis and urea treatment. These facts have allowed an approximation of the purity and turnover rate for the enzyme. If the assumption is made that upon complete inhibition of enzyme activity one equivalent of iproniazid is bound to the enzyme,[2] and that there is one catalytic site per mole of enzyme, then it should be possible to estimate the moles of enzyme present in a preparation, and therefore its purity. In a typical experiment utilizing enzyme containing 2.8 mg of protein, 175 cpm of inhibitor was bound at the point of complete inhibition. This corresponds to 0.14 mμmole of inhibitor per milligram of total protein. Assuming a molecular weight of 100,000 for monoamine oxidase, the preparation contained 1.4 mg of enzyme per 100 mg of total protein. The turnover rate can also be calculated. Since the inhibition of monoamine oxidase by iproniazid obeys first order kinetics (Davison, 1957), a plot of the log of the per cent inhibition against the log of the nondialyzable radioactivity (in cpm) should give a straight line. The point on this line corresponding to 100% inhibition should be the log of the cpm attached to the enzyme. The data of Figure 2 have been treated in this manner and are shown in Figure 5. At 100% inhibition 136 cpm was bound to the enzyme, or 27.2 cpm per unit of activity. From the specific activity of the starting labeled compound and the definition of a unit of activity, the turnover rate, in accordance with the assumptions outlined above, may be calculated:

$$\frac{(10^{-6}\ \text{mole min}^{-1}\ \text{unit}^{-1}) \times (4.46 \times 10^{11}\ \text{cpm mole}^{-1})}{27.2\ \text{cpm unit}^{-1}}$$

$$= 164\ \text{min}^{-1}$$

It is hardly necessary to emphasize that the enzyme system is a crude one containing several undefined variables. Therefore this figure is, at best, a rough estimate.

[2] This assumption seems valid from the data of Figure 2 and would hold regardless of what the active species is so long as it contains the isopropyl group.

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