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# VOLATILE AMINE OXIDASE INHIBITOR FROM HYDRAZINE DERIVATIVES

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**Abstract**—Under conditions of pH and temperature compatible with biological systems a volatile substance is produced from iproniazid, benzylhydrazine, and various other hydrazine derivatives that are inhibitors of monoamine oxidase. The volatile material is produced in a nonenzymatic reaction that requires oxygen and whose rate is greatly increased by cyanide or thiourea. The volatile substance inhibits liver and brain monoamine oxudase both *in vivo* and *in vitro*. It also inhibits bacterial tyramine oxidative deaminase.

NUMEROUS derivatives of hydrazine are known to be inhibitors of mitochondrial monoamine oxidase (MAO).[1] These compounds also inhibit soluble tyramine oxidative deaminase (TOD) of bacterial origin, an enzyme that resembles monoamine oxidase in the type of reaction catalyzed.[2] However, it is not clear whether hydrazines are directly responsible for the inhibition attributed to them with either enzyme.

Davison[3] observed that oxygen is required for irreversible inhibition with iproniazid (1-isonicotinyl-2-isopropylhydrazine) (IPR) and that cyanide greatly increases the degree of inhibition. In contrast, cyanide is without influence on the degree of inhibition by nonhydrazine compounds. Davison postulated that IPR forms an irreversible complex with the enzyme only after it is dehydrogenated at the enzyme surface. Cyanide was presumed to sensitize rat liver mitochondria to IPR by an unknown mechanism. Eberson and Persson[4] showed that phenipramine (β-phenylisopropylhydrazine) undergoes auto-oxidation in the presence of cupric ions via a radical mechanism under conditions that might be found in biological systems. They proposed, on the basis of this model system, that intermediate radicals react with the enzyme to produce irreversible inhibition. Pletscher[5] suggested that alkylhydrazides yield alkylhydrazines, which are the active inhibitors. More recently Schwartz[6] showed that isocarboxazid [1-benzyl-2-(5-methyl-3-isoxazolylcarbonyl)hydrazine] is hydrolyzed to benzylhydrazine (BHZ) in the presence of rat liver mitochondria. He concluded that BHZ is the agent responsible for the inhibition observed with isocarboxazid. Oxygen was not required for the inhibitory reaction.

The availability of both mitochondrial and soluble oxidative deaminase prompted a detailed consideration of the inhibition by hydrazines and of the role of cyanide in the inhibitory reaction. A brief report has appeared.[7]

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## MATERIALS AND METHODS

*Enzymes.* The source of monoamine oxidase was rat liver mitochondria prepared in 0·25 M sucrose according to Schneider.[8] The mitochondria were dried from the frozen state and stored for extended periods as a powder at −10° without appreciable loss of activity. When needed, 100 mg of the dried mitochondria was hydrated in 1 ml of water; 0·3–0·4 ml of this suspension was used in each flask. Mitochondria from livers of rats which had received intraperitoneal injections of inhibitor were tested immediately after isolation. Brain mitochondria from such animals were prepared according to Brody and Bain[9] and were also used immediately. Tyramine oxidative deaminase was prepared from *Sarcina lutea* grown in broth with 0·1% tyramine. Washed cells were distrupted by sonic oscillation.[2] After centrifugation the clear enzyme solution was frozen and stored at −10°. The protein content was about 15–20 mg/ml; 0·3–0·4 ml was used per flask.

*Test system.* Amine oxidase activity was estimated manometrically with standard Warburg equipment at a bath temperature of 37°. The standard test system was essentially that of Creasey[10] with minor modifications. It consisted of 200 to 250 μmoles of phosphate buffer (pH 7·0–7·2), 40–50 μmoles of semicarbazide (pH 7·0), either MAO or TOD, and water placed in the main compartment. The side arm contained 20 μmoles of tyramine (pH 7·0). The total fluid volume was 2·2 ml. Additions of other ingredients to any compartments of the flask are noted in the appropriate places. The duration of temperature equilibration was 20 or 30 min, followed by a reaction period of 30 min. The gas was air unless indicated otherwise.

*Chemicals.* All chemicals were of commercial origin except BHZ and isopropylhydrazine (IHZ), which were prepared by Dr. J. Mills of these laboratories, to whom we are grateful.

*Solution of volatile inhibitor.* In some experiments (data of Tables 6, 8, and 9) a solution of the volatile inhibitor was prepared apart from the enzyme. For this purpose it was convenient to use a Warburg flask, although any other vessel with two intercommunicating compartments may be used. The main compartment contained 2 ml of $5 \times 10^{-3}$ M phosphate buffer (pH 12·0), which served as a trapping solution. In the side arm was placed 8 mg BHZ.HCl and 7·6 mg thiourea.HCl. To this, 0·4 ml of 1 M phosphate (pH 7·8) was added and the flask immediately attached to its manometer. If gas other than air was required (i.e. $O_2$ or $N_2$), the flask was first gassed and the phosphate added at the end of the gassing period through the side-arm opening. The side arm was then plugged and the flow of gas stopped. The distillation of volatile inhibitor from the side arm into the main compartment was allowed to proceed for 80 min at 37·5° with shaking. After this period the solution in the main compartment was removed and used immediately.

## RESULTS

### *Inhibition by volatile inhibitor*

The data of experiments 1 and 2 in Table 1 show that inhibition of MAO and TOD by IHZ is increased in the presence of cyanide. Furthermore, preincubation of cyanide with inhibitor (experiment 2), with enzyme (experiment 3), or entirely apart from the reaction mixture (experiment 4) gives equivalent results. Similarly, the reaction is inhibited even though IHZ is confined to the center well of the Warburg flask, as seen in experiments 6, 7, and 8. Here too the inhibition is enhanced by cyanide (experiments

5 and 6), but the flask compartment in which the cyanide is placed is without effect on the degree of inhibition (experiments 6, 7 and 8). Similar results have been obtained with various other hydrazine derivatives, including BHZ and IPR. In contrast, non-hydrazine inhibitors such as pentamidine (1,5-*bis*[*p*-guanylphenoxy]pentane) and parnate (*trans*-2-phenylcyclopropylamine) are not effective when confined in the center well (experiments 9 and 10).

TABLE 1. VOLATILITY OF ISOPROPYLHYDRAZINE AND CYANIDE*

| Expt. | Main compartment | Center well | Side arm | Per cent inhibition TOD | MAO |
|---|---|---|---|---|---|
| 1 | | | IHZ | 11 | 8 |
| 2 | | | IHZ + KCN | 100 | 100 |
| 3 | KCN | | IHZ | 100 | 100 |
| 4 | | KCN | IHZ | 100 | 100 |
| 5 | | IHZ | | 14 | 0 |
| 6 | | IHZ + KCN | | 100 | 100 |
| 7 | | IHZ | KCN | 100 | 100 |
| 8 | KCN | IHZ | | 100 | 100 |
| 9 | | PENT. + KCN | | 0 | |
| 10 | | PAR + KCN | | 0 | |

* Standard test system with tyramine oxidative deaminase (TOD) or mitochondrial monoamine oxidase (MAO). Additions were made to flask compartments as indicated and in the following amounts given in micromoles: KCN, 2·5; isopropylhydrazine (IHZ), 0·5; pentamidine (PENT.), 0·5; parnate (PAR.), 0·5. The final volume of the reaction mixture was 2·1 ml, and of the center well, 0·1 ml. The flasks were equilibrated for 30 min at 37° before contents of the side arm were poured into main compartment. The data are for a 30-min reaction period.

These and similar data indicate that hydrazine MAO inhibitors, in the presence of cyanide, produce volatile substances which inhibit both MAO and TOD; cyanide is also volatile under these experimental conditions.

*pH of inhibitor*

The effect of pH of hydrazine inhibitor, present in the center well, on the inhibition obtained is shown in Fig. 1. In the absence of cyanide the pH of the inhibitor has no effect on the degree of inhibition given by the concentration of inhibitor used. With cyanide present, either in the main compartment or in the center well, maximal inhibition is obtained from about pH 6·5 to pH 9·5. The inhibition decreases rapidly on either side of this span and is negligible at pH 11·0. In other experiments concentrations of inhibitors 20 times greater than these were without effect at pH 11·5. The data are for BHZ, but similar results have been obtained with IHZ and IPR.

*Inhibition in absence of volatile inhibitor*

If the hydrazine inhibitor is at pH 11·5–12·0, volatile inhibitory material is not produced, and the enzyme is not exposed to the inhibitor during equilibration. Recognition of this fact is useful in studying the functions of cyanide. It also permits a comparison of various classes of amine oxidase inhibitors on an equitable basis. After the required period of equilibration, the hydrazine inhibitor is brought from pH 11·5–12·0 to pH 7·0 by mixing it with buffer and substrate; the enzyme is immediately added to this mixture. The effect of cyanide under these conditions, i.e. wherein

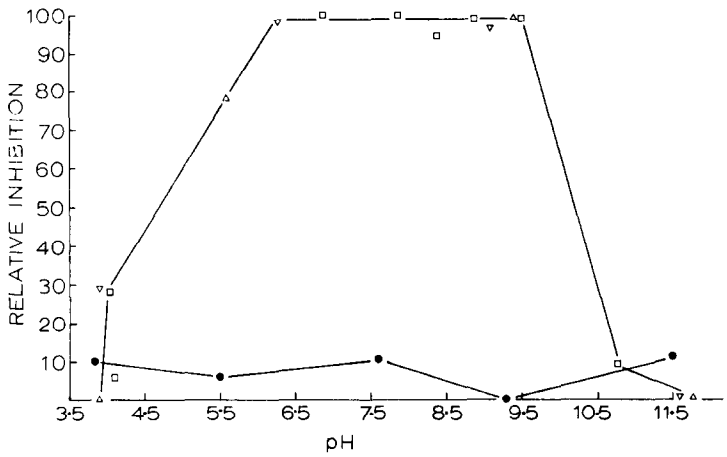


FIG. 1. Effect of pH of inhibitor in center well on degree of inhibition. Standard test system with TOD, except that volume of reaction mixture was 2·05 ml. The center well contained, in a total volume of 0·15 ml, 0·05 μmole BHZ, either with 2·5 μmoles KCN (□), without KCN (●), or 25 μmoles KCN was in the main compartment (△); 25 μmoles of one of the following buffers was in the center well to give approximate pH shown in parentheses: formate (3·7), acetate (5·7), phosphate (7·7), glycine (9·7), or phosphate (12·7); gas, air.

the inhibitor is at pH 11·5–12·0 until exposed to the enzyme simultaneously with the substrate—is given in Table 2.

No inhibition occurs in the absence of cyanide at the indicated inhibitor concentration (experiment 5). However, it is evident that cyanide is equally effective whether preincubated with enzyme, inhibitor, or substrate (experiments 2, 3 and 4). It should be emphasized that only in experiment 3 are inhibitor and cyanide both at pH 11·5–12·0 and therefore confined to this portion of the flask until the reaction is started. In the remaining flasks cyanide is at pH 7·0; hence it distills from one compartment to

TABLE 2. EFFECT OF PREINCUBATION OF CYANIDE WITH VARIOUS REACTION COMPONENTS ON DEGREE OF INHIBITION IN ABSENCE OF VOLATILE INHIBITOR*

| Expt. | Main compartment† | Side arm | | Per cent inhibition | | |
|---|---|---|---|---|---|---|
| | | No. 1‡ | No. 2† | BHZ | IHZ | IPR |
| 1 | | | TOD + KCN | 0 | 0 | 0 |
| 2 | | I§ | TOD + KCN | 76 | 65 | 64 |
| 3 | | I + KCN | TOD | 74 | 63 | 53 |
| 4 | KCN | I | TOD | 61 | 65 | 64 |
| 5 | | I | TOD | 0 | 0 | 0 |
| | | μmoles inhibitor/flask: | | 0·1 | 1·0 | 10·0 |

* The main compartment of double side-arm flasks contained 200 μmoles phosphate buffer, 40 μmoles semicarbazide, 20 μmoles tyramine, 2·5 μmoles KCN where shown (all at pH 6·8), and water to make a fluid volume of 1·6 ml. Side arm 1 contained 1·0 μmole phosphate buffer (pH 12·0), without inhibitor or with 0·1 μmole BHZ, 1·0 μmole IHZ, and 10·0 μmoles IPR, and 2·5 μmoles of KCN where indicated, in a volume of 0·2 ml (pH 12·0). Side arm 2 contained TOD and water or 2·5 μmoles KCN (pH 7·0) as indicated. After 20 min of temperature equilibration, the manometers were read. The contents of side arm 1 were then poured into the main compartment, mixed, and immediately followed by contents of side arm 2. The reaction time was 30 min.

† Contents at pH 7·0.

‡ Contents at pH 12·0.

§ Any one of the hydrazine inhibitors of the last three columns.

all others. However, in all cases the inhibitor is at pH 11·5–12·0 and thus not with either enzyme or substrate until the moment of mixing at the start of the reaction.

Inasmuch as the degree of inhibition is unchanged by preincubation of cyanide with one component more than another, only the hydrazine inhibitor needs to be at pH 11·5–12·0 to suppress formation and distillation of volatile inhibitor from it. Neither the pH of the cyanide nor the component with which it is preincubated has any effect on degree of inhibition.

These data also show that the relative abilities of the three hydrazines to cause inhibition under these conditions are BHZ > IHZ > IPR.

Similar results have been compiled for mitochondrial MAO, except that inhibitory concentrations for all inhibitors tested are somewhat lower. For instance, under experimental conditions described above, 50 per cent inhibition is obtained with BHZ at a concentration of $3{\cdot}2 \times 10^{-6}$ M for MAO and $2{\cdot}3 \times 10^{-5}$ M for TOD.

*Kinetics*

The rate of inhibition of TOD by BHZ decreases with time. If the log of per cent residual activity is plotted against time, the experimentally determined points fall along a straight line, as in Fig. 2, curves A and C, representing two concentrations of inhibitor. These data were obtained with inhibitor at pH 11·5–12·0 until exposed to the enzyme for the times indicated on the abscissa. After these intervals the inhibitory reaction was stopped by addition of substrate, and residual catalytic activity was measured, which is the ordinate on the graph.

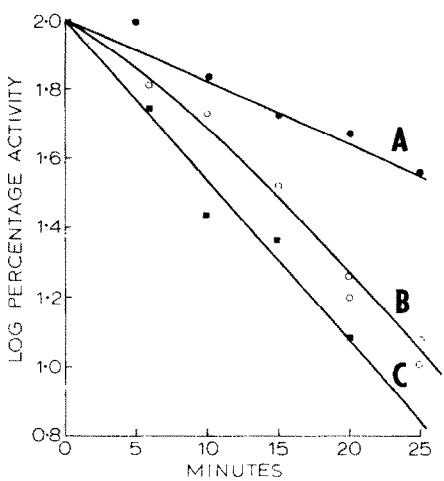


FIG. 2. Kinetics of the inhibitory reaction of BHZ and the volatile inhibitor produced from BHZ. To obtain the data for curves A (●) and C (■), double side-arm flasks were used in which the main compartment contained 200 μmoles phosphate buffer (pH 6·8), 40 μmoles semicarbazide, 2·5 μmoles KCN (both at pH 6·8), TOD, and water to give a total volume of 1·9 ml. In side arm 1 there were 2·5 μmoles KCN, 0·0025 μmole BHZ, 0·25 μmole phosphate buffer (pH 12·0), and water. The fluid volume was 0·2 ml. Side arm 2 contained 20 μmoles tyramine in 0·2 ml volume. For the data of curve B (○), the same reaction components were used, except that contents of side arm 1 were at pH 7·0, reduced in volume to 0·1 ml, and put into the center well of single side arm flasks. For curves A (●) and C (■), the abscissa is the time interval between pouring side arm 1 (inhibitor) and side arm 2 (substrate) into the main compartment. For curve B (○) the abscissa shows the length of time volatile inhibitor is produced and presumably reacting with the enzyme in the main compartment before side arm 1 (substrate) was poured.

The rate at which inhibition increases with time when the inhibitor is the volatile substance from BHZ is given by curve B. It should be mentioned that the rate shown is a combination of rate of production, distillation from the center well into the main compartment, and reaction with enzyme of the volatile inhibitor. Thus, not unexpectedly, the experimentally determined points do not fall on a straight line. The data are useful to show that both rate and degree of inhibition are appreciable even with relatively small amounts of hydrazine inhibitor. Under these conditions BHZ may be more effective when used in the center well as a source of volatile inhibitor than is an equal amount when exposed directly to the enzyme. Although the basis of comparison for the preceding statement (i.e. total amount of inhibition per flask) is open to criticism, the data suggest that if volatile inhibitor is produced, exposure of the enzyme to it even for the time required for equilibration is enough to give an erroneous evaluation of the inhibitory power of hydrazines. Thus, during equilibration the inhibitor must be at pH 11·5–12·0 to suppress its volatilization.

*Effect of semicarbazide*

Semicarbazide interferes with the production of the volatile inhibitor both in the presence and absence of cyanide (Table 3; experiments 1, 2, 3 and 4). However, semicarbazide is without effect on the inhibitory reaction itself. Its presence with the enzyme during the course of the inhibitory reaction (experiment 6) does not change the degree of inhibition compared to that in its complete absence (experiment 5) or when it is present in the side arm with the substrate (experiment 7) and thus out of contact with the enzyme until the moment the reaction is started.

TABLE 3. THE EFFECT OF SEMICARBAZIDE ON PRODUCTION AND REACTION WITH ENZYME OF VOLATILE INHIBITOR FROM BHZ*

| Expt. | Main compartment† | Center well† ‡ | Per cent inhibition |
|---|---|---|---|
| 1 | | 1·5 BHZ | 74 |
| 2 | | 1·5 BHZ + 10 SC§ | 16 |
| 3 | 2·5 KCN | 0·0015 BHZ | 68 |
| 4 | 2·5 KCN | 0·0015 BHZ + 5 SC | 31 |
| 5 | 2·5 KCN | 0·001 BHZ | 34 |
| 6 | 2·5 KCN + 40 SC | 0·001 BHZ | 46 |
| 7 | 2·5 KCN¶ | 0·001 BHZ | 44 |

* The main compartment of single side-arm flasks contained TOD, 200 μmoles phosphate buffer (pH 7·0), other components as shown, and water to a total volume of 1·6 ml. The center well contained 50 μmoles phosphate buffer (pH 7·2), with additions as indicated, and water to make 0·2 ml. The flasks were equilibrated for 20 min before substrate was poured from the side arm into the main compartment.

† Figures are μmoles/flask.

‡ All at pH 7·0.

§ Semicarbazide (SC).

¶ With 40 SC in side arm.

*Site of cyanide reaction*

Cyanide may have one or more functions in the mechanism of hydrazine inhibition. For instance, it may catalyze the production of the volatile inhibitor, it may react with the enzyme in some way, making it more susceptible to inhibition, or it may do both of these.

The data in Table 4 indicate that cyanide is involved in the production of the volatile inhibitor. Cyanide was present with the enzyme in all experiments, yet the degree of inhibition is considerably increased when cyanide is also included in the center well with benzylhydrazine. Such data do not indicate any possible effects of cyanide on the

TABLE 4. THE EFFECT OF CYANIDE ON PRODUCTION OF VOLATILE INHIBITOR FROM BHZ*

| μmoles BHZ in center well | Per cent inhibition | |
|---|---|---|
| | Without $CN^-$ in center well | With $CN^-$ in center well |
| 0·0005 | 9 | 34 |
| 0·0015 | 11 | 68 |
| 0·005 | 67 | 97 |

* Standard test system with TOD and 2·5 μmoles KCN in the main compartment. The center well contained 25 μmoles phosphate buffer (pH 7·2), BHZ in the amounts shown, and either water or 2·5 μmoles KCN; fluid volume, 0·2 ml. The side arm had 20 μmoles tyramine in 0·4 ml. The substrate was poured into the main compartment after 20 min of temperature equilibration.

enzyme. To obtain information on this question, it is necessary to prepare the volatile inhibitor in the absence of cyanide. It was noticed that thiourea (SHU) replaces cyanide reasonably well to give the desired increase in inhibition and is itself not volatile, as is shown in Table 5. In experiments 1 and 2 very little inhibition occurs in the complete absence of thiourea or when it is in the main compartment, indicating that it is not volatile, hence does not distill into the center well as does cyanide. At much lesser amounts of BHZ, but with thiourea, the inhibition is comparable to that with cyanide (experiments 3 and 4).

TABLE 5. REPLACEMENT OF CYANIDE BY THIOUREA*

| Expt. | Main compartment† | Center well† | Per cent inhibition |
|---|---|---|---|
| 1 | | 0·15 BHZ | 14 |
| 2 | 2·5 SHU | 0·15 BHZ | 15 |
| 3 | | 0·0015 BHZ + 2·5 SHU | 37 |
| 4 | | 0·0015 BHZ + 2·5 KCN | 41 |

* Standard test system with TOD, 2·5 μmoles thiourea (SHU) as shown, and water to make 1·65 ml in main compartment. The center well contained BHZ, with or without SHU or KCN, in a total volume of 0·15 ml; 20 μmoles tyramine present in the side arm in 0·4 ml.

† Figures are μmoles/flask.

To test the effect of cyanide on the enzyme, the volatile inhibitor was prepared from BHZ with thiourea and trapped in phosphate buffer, pH 11·5–12·0. The solution of inhibitor was tested both in the presence and absence of cyanide. The data of Table 6 (experiment 1), show that insufficient inhibitor is produced in the absence of SHU to cause inhibition when tested either with or without cyanide. However, when

prepared in the presence of SHU, sufficient amount is produced to cause inhibition with or without cyanide, as in experiment 2, and similarly for MAO, as in experiment 5. In both cases the presence of cyanide in the enzyme increases the degree of inhibition by a slight amount. When the enzyme is treated with cyanide and then collected by precipitation with $(NH_4)_2SO_4$, even this small effect disappears (experiment 3). It would appear the precipitation alone is sufficient to eliminate the small effect cyanide may have on the enzyme (experiment 4).

TABLE 6. EFFECT OF CYANIDE ON DEGREE OF INHIBITION BY PREFORMED VOLATILE INHIBITOR FROM BHZ*

| Expt. | Enzyme and its treatment | Volatile inhibitor formed in: | Per cent inhibition −KCN | Per cent inhibition +KCN |
|---|---|---|---|---|
| 1 | TOD | $PO_4^{=}$ | 0 | 0 |
| 2 | TOD | $PO_4^{=}$ + SHU | 78 | 95 |
| 3 | TOD + KCN + $(NH_4)_2SO_4$ | $PO_4^{=}$ + SHU | 81 | 84 |
| 4 | TOD + $(NH_4)_2SO_4$ | $PO_4^{=}$ + SHU | 86 | 72 |
| 5 | MAO | $PO_4^{=}$ + SHU | 50 | 65 |

* Standard test system with TOD or MAO, 2·5 μmoles KCN, or without KCN, and water to bring final volume to 1·6 ml. The volatile inhibitor was prepared in phosphate ($PO_4^{=}$) alone, or with phosphate and thiourea (SHU), as described under Methods. 0·05 ml of a 1:10 dilution of the volatile inhibitor solution was placed in the center well along with 50 μmoles phosphate buffer (pH 7·0); total volume, 0·2 ml. The side arm contained 20 μmoles tyramine in 0·4 ml. For experiments 3 and 4, respectively, 1·2 ml TOD was added to 1·0 ml M phosphate buffer (pH 6·5) containing 0·5 ml of 0·05 M KCN or the same amount of water. After 30 min at 37°, solid $(NH_4)_2SO_4$ was added to 80% of saturation. The precipitate was collected by centrifugation and resuspended to a volume of 1·2 ml in saline.

*The effect of oxygen on inhibitor production and inhibitor reaction*

Inhibition by volatile inhibitor derived from BHZ requires oxygen, as shown by the data for both TOD and MAO in Table 7. When both the production of volatile inhibitor from BHZ and the inhibitory reaction occur in a $N_2$ atmosphere, lengthening the time that enzyme is exposed to inhibitor does not cause a corresponding increase

TABLE 7. THE EFFECT OF GAS PHASE ON INHIBITION BY VOLATILE INHIBITOR FROM BHZ*

| | | TOD | | | MAO | | |
|---|---|---|---|---|---|---|---|
| Time in min: | | 5 | 10 | 20 | 5 | 10 | 20 |
| Expt. | Gas | | | | | | |
| 1 | $N_2$ | 26 | 31 | 36 | 8 | 9 | 15 |
| 2 | $O_2$ | 25 | 75 | 98 | 14 | 22 | 40 |

* Reaction mixture for TOD was as follows. The main compartment of double side-arm flasks contained 0·012 μmole BHZ, 20 μmoles KCN, 20 μmoles tyramine, 2·5 μmoles phosphate buffer (pH 12·0), and water to make 1·4 ml. Side arm 1 was poured into the main compartment bringing its contents to pH 6·8 to 7, thereby starting the production of volatile inhibitor. The reaction mixture was incubated for the times shown. At the end of each period the measurement of residual enzyme activity was begun by pouring side arm 2 into the main compartment. A flow of $O_2$ (even though $O_2$ may have been the previous gas phase) was maintained for 5 min. Readings were begun after 10 min of equilibration; the experimental period was 30 min.

The reaction mixture for MAO was as described for TOD with the following changes in amounts: 0·0016 μmole phosphate buffer (pH 7·0) in side arm 1, and 100 μmoles semicarbazide in side arm 2. The experimental procedure was as for TOD.

in inhibition (experiment 1). On the other hand, if both reactions are conducted in an atmosphere of $O_2$, the degree of inhibition increases with time (experiment 2). A comparison of the inhibition by volatile inhibitor from BHZ prepared in $N_2$ and $O_2$, but allowed to react with enzyme in air, is shown in Table 8. It is evident that oxygen is needed for inhibitor production (experiments 1 and 2). When the volatile inhibitor is prepared in air, the gas phase ($N_2$ or $O_2$) during the time the inhibitor reacts with enzyme is without effect on the degree of inhibition obtained (experiments 3 and 4). Oxygen is not required for the inhibitory reaction itself.

TABLE 8. EFFECT OF GAS PHASE ON INHIBITOR PRODUCTION AND INHIBITORY REACTION WITH MAO*

| | Experiment: | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Inhibitor prepared in | $N_2$ | $O_2$ | Air | Air |
| Enzyme exposed to inhibitor in | Air | Air | $N_2$ | $O_2$ |
| Inhibition (%) | 0 | 100 | 74 | 66 |

* The volatile inhibitor was prepared under an atmosphere of $N_2$, $O_2$, or air by the procedure described under Methods. In experiments 3 and 4, the volatile inhibitor solution was placed in the main compartment of flasks already containing 5 μmoles KCN, 20 μmoles tyramine, 5 μmoles phosphate buffer (pH 12·0) in a volume of 1·5 ml. In side arm 1 were 400 μmoles phosphate buffer (pH 7·0) in 0·4 ml; 100 μmoles semicarbazide, 100 μmoles phosphate buffer (pH 7·0), and MAO in a volume of 0·3 ml were in side arm 2. The appropriate gas was passed through the flask for 20 min. This was followed by 10 min of equilibration and then side arm 1 was poured. Ten min later side arm 2 was poured, and $O_2$ was passed through the flasks for 5 min. The first reading was made after 10 min of equilibration.

*Effect* in vivo *of volatile inhibitor*

After it was observed that thiourea would substitute for cyanide, the effect of volatile inhibitor *in vivo* was determined. The volatile inhibitor was prepared as described in the methods section. Three doses, consisting of 1 ml each of this solution, were injected intraperitoneally into rats within an 8-hr period. The animals were sacrificed 16 hr later, and mitochondria were prepared from liver and brain. The data of Table 9 indicate that the volatile inhibitor obtained from BHZ is an effective inhibitor of MAO when applied *in vivo*.

TABLE 9. EFFECT *in vivo* ON MAO OF VOLATILE INHIBITOR FROM BHZ*

| | Per cent inhibition |
|---|---|
| Rat liver MAO | 78 |
| Rat brain MAO | 96 |

* The residual MAO activity of mitochondria which were isolated from animals injected i.p. with a solution of volatile inhibitor was estimated in the standard test system. The basis for the calculations was the activity of MAO in mitochondria obtained from animals inoculated with buffer only. The solution of volatile inhibitor was prepared as described under Methods.

*Effect of* 1,2-*naphthoquinone-4-sulfonate* (*NQS*)

When the volatile inhibitor was trapped in a solution of NQS a fluorescent compound was not produced, as occurs with alkylhydrazines. However, the absorption peak at 250 mμ obtained with NQS alone was shifted to 235 mμ. Similar spectra were seen with volatile material from BHZ, IHZ, and IPR (Fig. 3).

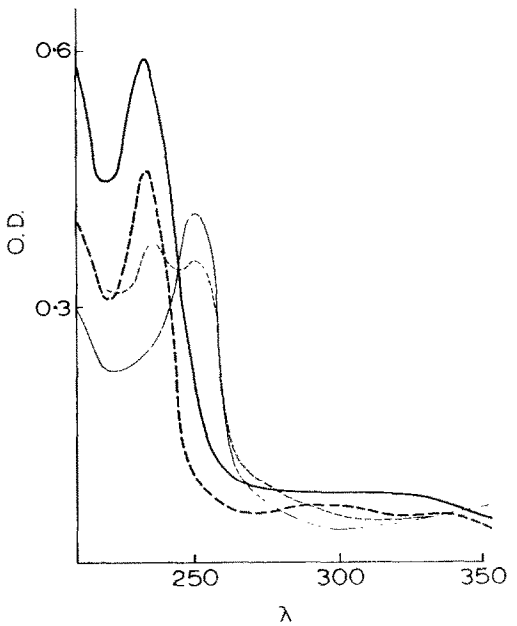


FIG. 3. Spectra of 1,2-naphthoquinone-4-sulfonate (NQS) (———) alone and with volatile inhibitor from BHZ (———), IHZ (— — —), or IPR(– – – –). The volatile material from each of the hydrazines was produced in the presence of KCN and trapped in phosphate buffer, pH 12·0, over a period of 2 hr at 37°. The pH of the solution was then brought to 6·0, NQS added, and the spectrum obtained with a model 14 Cary recording spectrophotometer.

The presence of NQS with TOD protects the enzyme from inhibition by volatile inhibitor derived from BHZ (Table 10). Similar data have been obtained with MAO.

TABLE 10. EFFECT OF 1,2-NAPHTHOQUINONE-4-SULFONATE ON INHIBITION BY VOLATILE INHIBITOR FROM BHZ*

| NQS in main compartment, μmoles | Inhibition, % |
|---|---|
| None | 80 |
| 0·03 | 77 |
| 0·1 | 56 |
| 0·3 | 30 |
| 1·0 | 6 |

* Standard test system with TOD, 2·5 μmoles KCN and addition of 1,2-naphthoquinone-4-sulfonate (NQS) to main compartment as shown, in a volume of 1·65 ml. The center well contained 0·003 μmole BHZ in 0·15 ml; 20 μmoles tyramine was present in the side arm in 0·4 ml. The flasks were equilibrated for 20 min before substrate was tipped into main compartment.

*Effect of* $Cu^{2+}$

The formation of the volatile inhibitor, either in presence or absence of cyanide, is suppressed by $CuSO_4$.

## DISCUSSION

MAO is inhibited by several distinct and unrelated classes of compounds. Some of these compounds are readily reversible inhibitors, others are not. Derivatives of hydrazine, which came into widespread use after Zeller *et al.*[11] discovered iproniazid, are without exception of the irreversible type.

Irreversible inhibition of MAO by hydrazine derivatives has been attributed to formation of a hydrazone. Presumably the hydrazine reacts with an electrophilic center on the enzyme surface, similar to the reaction of a hydrazine with a carbonyl compound.[12]

Unlike any other class of MAO inhibitors, hydrazines have been reported to require oxygen for inhibition; cyanide greatly enhances the degree of inhibition.[3] Furthermore, incubation of inhibitor with enzyme, in the absence of substrate, is generally reported to be necessary for maximal inhibition.[3, 6, 13] None of these characteristics of hydrazine inhibition is readily reconciled with an inhibitory mechanism which depends only upon hydrazone formation.

When it was apparent that a volatile inhibitory substance is produced from hydrazine derivatives under conditions that prevail in biological systems, these characteristics became readily explainable. The data presented establish that oxygen is required for the formation of the volatile inhibitor from a hydrazine derivative, but not for the reaction of inhibitor with enzyme. Cyanide, or thiourea, is involved but only with the formation of the inhibitory material. Thus both oxygen and cyanide are needed for the non-enzymatic production of the volatile inhibitor from hydrazine derivatives, and neither appears to be necessary in any part of the inhibitory reaction itself. Incubation of an inhibitor such as iproniazid with enzyme to obtain maximal inhibition might simply be a means of allowing time to produce an adequate amount of the volatile inhibitor. Some preliminary data indicate that the reaction of enzyme with the preformed volatile inhibitor is very rapid.

Inhibition by hydrazides has been ascribed to hydrazines, which are assumed to be produced by enzymatic hydrolysis.[5] Thus iproniazid[12] and isocarboxazid[6] would give isopropylhydrazine and benzylhydrazine respectively. Although the identity of the volatile inhibitor obtained from hydrazines is unknown, data available at present indicate it is not a hydrazine. Furthermore, when volatile inhibitory material from IHZ, BHZ, or IPR is allowed to react with NQS the spectral changes are similar. This suggests that the functional group may be common in the volatile substance from all three hydrazine derivatives, although the rest of the molecule may be different. Thus it seems unlikely that inhibition by a hydrazide is due to a hydrazine directly. The inhibitory capability of a hydrazine derivative itself, i.e. exclusive of the inhibition produced by the volatile material, is unknown. Under conditions where no volatile inhibitor, even in trace amount, is present, hydrazine derivatives as such may be non-inhibitory. Thus, inhibition of MAO (and TOD) by all hydrazines and hydrazides might be due entirely to a volatile substance that is similar, if not identical, in structure irrespective of source.

Hydrazine derivatives continue to be pharmacologically interesting compounds. The evaluation of their effect on biological systems, both *in vivo* and *in vitro*, is complicated by a degree of instability heretofore unrecognized. At the present time their mechanism of action is uncertain.

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*Note added in press*—The data which we have presented on iproniazid corroborate the observations of T. E. Smith, H. Weissbach, and S. Udenfriend (*Biochemistry*, **2**, 746, 1963) whose paper appeared while the present paper was in press.

## REFERENCES

1. A. E. ZELLER, L. A. BLANKSMA, W. P. BURKARD, W. L. PACHA and J. C. LAZANAS, *Ann. N.Y. Acad. Sci.* **80**, 583 (1959).
2. M. KORY, C. P. WALTERS, E. MINGIOLI, W. C. KENNEY and E. R. DILLER. To be published.
3. A. N. DAVISON, *Biochem. J.* **67**, 316 (1957).
4. L. E. EBERSON and K. PERSSON, *J. med. pharm. Chem.* **5**, 738 (1962).
5. A. PLETSCHER, in Proceedings, Fourth Int. Congr. Biochem., Vienna, vol. 3, p. 124. Pergamon Press, New York (1958).
6. M. A. SCHWARTZ, *J. Pharmacol. exp. Ther.* **135**, 1 (1962).
7. E. MINGIOLI and M. KORY, *Fed. Proc.* **22**, 389 (1963).
8. W. C. SCHNEIDER, *J. biol. Chem.* **176**, 259 (1948).
9. T. M. BRODY and J. A. BAIN, *J. biol. Chem.* **195**, 685 (1952).
10. N. H. CREASEY, *Biochem. J.* **64**, 178 (1956).
11. E. A. ZELLER, J. BARSKY, J. R. FOUTS, W. F. KIRCHHEIMER and L. S. VAN ORDEN, *Experientia (Basel)* **8**, 349 (1962).
12. J. BARSKY, W. L. PACHA, S. SARKAR and E. A. ZELLER, *J. biol. Chem.* **234**, 389 (1959).
13. E. A. ZELLER, J. BARSKY, E. R. BERMAN, M. S. CHESKAS and J. R. FOUTS, *J. Pharmacol. exp. Ther.* **124**, 282 (1958).