Biochemical Pharmacology, Vol. 23, pp. 2045–2056. Pergamon Press, 1974. Printed in Great Britain.

PHARMACOLOGICAL AND BIOCHEMICAL ACTIONS OF THE HEMOLYTIC AGENTS ACETYLPHENYLHYDRAZINE AND PHENYLHYDRAZINE ON MONOAMINE OXIDASE IN THE RAT*,†,‡

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(*Received* 25 *June* 1973; *accepted* 2 *November* 1973)

Abstract—The injection of normal rats with the hemolytic agents acetylphenylhydrazine (APHZ) and phenylhydrazine (PHZ) rapidly produces a long-lasting inhibition of monoamine oxidase (MAO). The effect is more pronounced in liver than in brain at the dosage levels used, viz. 80 mg/kg body weight. APHZ inhibits liver MAO more actively than does an equivalent dose of PHZ. The former compound causes 65–75 per cent inhibition of activity by 24 hr after injection, whereas PHZ inhibits by about 40 per cent at 24 hr and 50 per cent at 2 days. On the other hand, PHZ is slightly more active than APHZ against the rat brain enzyme *in vivo*. Equal doses of PHZ and APHZ cause 39 and 30 per cent inhibition, respectively, after 2 days. The inhibitory effects of APHZ *in vivo* are enhanced in riboflavin-deficient rats. Both drugs inhibit MAO *in vitro* in an immediate, irreversible, non-competitive manner; inhibition is independent of pH. PHZ is more active than APHZ against rat liver MAO. Concentrations of the respective chemicals which cause 50 per cent inhibition *in vitro* after 15 min of pre-incubation with the enzyme at 37° and pH 7·0 are about $1{\cdot}6 \times 10^{-5}$ M and $3{\cdot}6 \times 10^{-5}$ M. Kynuramine protects MAO against inhibition by APHZ but not PHZ. Cyanide is active in enhancing the inhibitory action of both substances on MAO *in vitro*.

THE RESULTS of previous studies of nutritional deficiencies of iron and copper in the rat have suggested that iron is essential for the biosynthesis or the action of monoamine oxidase (monoamine:oxygen oxidoreductase (deaminating) EC 1.4.3.4, MAO) *in vivo*.[1–3] Moreover, iron-chelating agents inhibit MAO activity in mitochondrial preparations of rat liver, whereas potent copper chelators do not inhibit the enzyme.[1] Hence, it is possible that other means of interfering with iron metabolism, e.g. its storage and mobilization in the rat, may have some effect on MAO activity. Therefore, it was decided to test the effects of hemolytic agents as a means of accelerating development of the deficiency. The classical hemolytic agents acetylphenylhydrazine (APHZ) and phenylhydrazine hydrochloride (PHZ), as well as allylisopropylacetamide (AIA), were assessed for effects on MAO.

Bernheim[4] and Green[5] had reported that PHZ and arylalkylhydrazines inhibit particulate MAO *in vitro*. Bernheim's study was mainly a comparison of the effects of PHZ on dehydrogenases, and did not include kinetic data.

* This work is part of a thesis submitted by A. L. S. in 1972 to the Faculty of Graduate Studies and Research, McGill University, Montreal, in partial fulfillment of the requirements for the Ph.D. degree.

† A partial account has been presented at the Fifty-fourth Annual Conference of the Chemical Institute of Canada, Halifax, Nova Scotia, 1971.

‡ Supported by a grant of the Medical Research Council of Canada.

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Several other derivatives of hydrazine have been synthesized and tested in clinical studies as MAO inhibitors[6–8] after iproniazid was found to have antidepressive effects.[9–11] The potency of PHZ as an inhibitor of MAO and diamine oxidase (DAO) has been compared *in vitro* with that of several substituted hydrazines.[12] It has also been used as a tool in attempts to elucidate the mechanism of the reaction occurring at the catalytic site of the bovine kidney enzyme.[13] However, neither PHZ nor APHZ has been studied for its anti-MAO properties in any detail, probably because of the high toxicity of these compounds. Because of this neglected aspect of the chemistry of MAO, it seemed important to carry out these investigations. It was expected that the results would provide some knowledge of how these particular substances affect mitochondrial MAO activity.

MATERIALS AND METHODS

Chemicals

Reagent grade chemicals were used. They were obtained from the following suppliers: sucrose, phenylhydrazine HCl, zinc sulfate and semicarbazide HCl, Fisher Scientific Co., Montreal; iso-amylamine, Eastman Organic Chemicals, Rochester, N.Y.; 1-acetyl-2-phenylhydrazine, Matheson, Coleman & Bell, East Rutherford, N.J.; serotonin creatinine sulfate and tyramine HCl, CalBiochem, Los Angeles, Calif.; kynuramine dihydrobromide, Regis Chemical Co., Chicago; sodium cyanide, Merck Chemical Co., Montreal; 4-hydroxyquinoline, K & K Fine Chemicals, New York; allylisopropylacetamide was a gift from Hoffman-LaRoche Inc., Nutley, N.J.

Animals and diets

Male, albino Sprague–Dawley rats were used in all the experiments. Those animals that were used in experiments with inhibitors weighed between 100 and 200 g. They were fed on Purina rat pellets and had tap water to drink.

The rats used in the nutritional deficiency experiments weighed 40–50 g initially. They were fed solid semisynthetic diets designated as control and riboflavin deficient; they were given glass-distilled water to drink. The deficient diet had the same composition as the control diet except for the omission of riboflavin. The diets were prepared as reported previously.[3]

Drug treatments

Allylisopropylacetamide (AIA). This porphyria-producing drug was dissolved in absolute alcohol. Sufficient sodium chloride dissolved in water was added to the AIA solution to make it 40% (v/v) in alcohol and 0·15 M in NaCl. The test animals were given 200 mg/kg, subcutaneously, once daily for each of 5 consecutive days. Control rats received the alcoholic saline injection only. The rats were sacrificed on day 6. The livers were perfused *in situ* with ice-cold 0·25 M sucrose solution, removed and weighed, then homogenized in sucrose. The homogenates were assayed for MAO activity and protein.

Hydrazines. APHZ was prepared for injection as described for AIA, except that the concentration of alcohol required to keep it soluble in the saline medium was 20% (v/v). PHZ was readily soluble in 0·15 M NaCl solution.

Chronic administration

Test animals were given single (s.c.) injections of the drug daily, at various dosage levels, for 6 days. Hemoglobin levels of tail blood were measured on day 8. The dosage level causing a significant decrease in hemoglobin concentration concomitant with survival at day 6 was selected as the minimum effective dose of APHZ to be used in more extensive experiments described below.

Acute administration

Rats injected once (s.c.) with 80 mg/kg of APHZ were sacrificed by decapitation at 3, 6, 12, 24 and 48 hr and at 4, 8, 12 and 22 days after the injections. A similar dose of PHZ (80 mg/kg) in 0·15 M NaCl solution was given subcutaneously to another set of rats. Effects of this drug were studied only at 24 and 48 hr after the injections. All the control animals received injections of the saline solutions. The time course of inhibition and recovery of MAO activity of liver and brain was followed, and the serotonin levels of the brains were also estimated.[14]

Administration in riboflavin deficiency

The interaction of APHZ and riboflavin deficiency (4 weeks' duration) on liver and brain MAO activity was studied. Groups of rats were killed at 24 and 48 hr after they had been injected with 0, 50 and 80 mg/kg of APHZ. Hemoglobin was determined in their blood, and tissues were removed for estimation of enzymic activity.

Preparations of hepatic MAO

The livers of the decapitated animals were perfused *in situ* with freshly prepared ice-cold 0·25 M sucrose, removed quickly, and then washed and chilled thoroughly before being dried and weighed. After weighing, the livers were homogenized in sucrose solution. Brains were also washed in ice-cold sucrose before they were homogenized. Washed mitochondria, prepared from liver homogenates by the method of Hawkins,[15] were resuspended in 10 mM phosphate buffer, pH 7·4, and kept frozen until used in the experiments *in vitro*.

Determination of MAO activity in vitro

MAO activity was estimated by a polarographic method reported previously.[1] The reaction mixtures[16] contained: semicarbazide hydrochloride 20 μmoles, sodium cyanide 2 μmoles, 50 mM phosphate buffer (pH 7·4), 0·5 ml enzyme preparation, equivalent to 50–100 mg fresh weight of liver and water to 3·0 ml. The reaction was started by addition of the substrate: 40 μmoles iso-amylamine or 20 μmoles tyramine. With tyramine, the buffer used was at pH 7·0. All incubations were carried out at 37·5° under air-saturating conditions. Enzyme activity is expressed as μl oxygen consumed/hr.

MAO activity was also determined in the enzyme inhibition experiments *in vitro* by a microfluorometric method with kynuramine as substrate[17] (Aminco–Bowman spectrophotofluorometer, American Instrument Co., Silver Spring, Md.). The incubation mixtures contained in a total volume of 3 ml: 33·3 mM phosphate buffer, pH 7·0 or pH 7·4; kynuramine, 1 mM final concentration unless otherwise indicated, and enzyme. Whole homogenates or nuclei-free homogenates were used for the tests *in*

vivo, and mitochondrial preparations for the tests *in vitro*. Mixtures were incubated at 37° with intermittent shaking for 20 min. The enzyme reaction was stopped by the rapid addition of 5% zinc sulfate solution followed by centrifugation. The supernatant solutions were made alkaline with an equal volume of 2 N sodium hydroxide solution. Fluorescence readings were obtained using excitation and emission wavelengths of 315 nm and 380 nm respectively. Inhibitors were preincubated with the enzyme for 20 min prior to the addition of kynuramine, except where otherwise indicated. Where kynuramine is the substrate, the unit of enzyme activity is 1 nmole 4-hydroxyquinoline formed in 20 min. Specific activity is expressed as units of MAO activity/mg of protein.

Determination of protein and serotonin

The protein concentration of the enzyme preparations was determined by the method of Lowry *et al.*[18] Human plasma albumin (Cutter Laboratories, Berkeley, Calif.) was used as a standard. Serotonin was determined by the method of Snyder *et al.*[14]

RESULTS

Effects of AIA in vivo

The mean MAO activity of livers from rats injected with AIA was 20·94 ± 0·90 units/mg of protein. Control values averaged 22·68 ± 1·67. There were six animals making up each mean; the difference was not statistically significant.

Effects of APHZ in vivo

The minimum effective dose, 80 mg/kg body weight, of this drug was arrived at by the following criteria: that dosage level which was given subcutaneously once daily to test animals for 6 consecutive days, and caused a significant decrease in hemoglobin concentration with survival of the animals for at least 1 day after the last injection.

There was a rapid, long-lasting decline in MAO activity of rats that were given APHZ either as a series of injections or in a single injection only. The loss in activity observed was of the same order when other substrates than kynuramine were tested. Regeneration of the liver enzyme was very slow. Somewhat more than 3 weeks was required for complete restoration of its activity. This is in contrast to brain MAO activity which was inhibited by APHZ to a lesser extent and recovered completely by about 12 days (Fig. 1). A subcutaneous injection of PHZ (80 mg/kg) produced a significant decrease in the MAO activity of rat liver and brain after 24 hr. Rat liver activity toward tyramine and kynuramine declined by 50 and 42 per cent, respectively, while the decrease in brain enzyme activity was 37 per cent (kynuramine). The liver MAO activity was 47 per cent of control activity by 48 hr after the injection of PHZ when isoamylamine was used as substrate.

The effects of APHZ on the activity of liver MAO in normal and riboflavin-deficient rats were dose dependent as well as time dependent, as may be seen in Fig. 2. Moreover, the depression of enzymic activity was much greater in the riboflavin-deficient than in the riboflavin-supplemented group of rats. Mean sp. act. of liver MAO

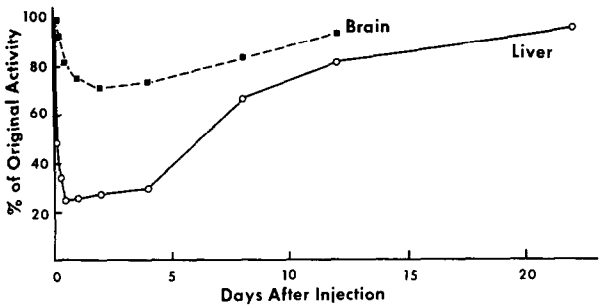

FIG. 1. Recovery of rat MAO activity from inhibition by APHZ. A single (s.c.) injection of APHZ (80 mg/kg) was given to rats. The animals were killed at various intervals afterwards, and homogenates of the tissues were examined for MAO activity using kynuramine as substrate. Experimental points represent the mean ± S.E. of at least six determinations, expressed as the percentage of control activity. Controls varied from experiment to experiment, but over the series the mean (±S. E.) for brain was 10·3 (±0·68) nmoles 4-hydroxyquinoline formed in 20 min/mg of protein; for liver the corresponding value was 34·8 (±4·48).

toward kynuramine from eight normal rats injected with saline was 25·5 ± 0·95 units/mg of protein; for four rats given 80 mg/kg of APHZ, it was 5·98 ± 0·32 units/mg of protein at 48 hr after the injections. With respect to the riboflavin-deficient rats (eight per group), the mean sp. act. was observed to decrease from 5·93 ± 0·68 (saline) to 2·44 ± 0·19 units/mg of protein (APHZ) during the same interval of time. This is similar to the observation made earlier by Distler and Sourkes[19] that MAO from livers of riboflavin-deficient rats was more susceptible to inhibition than the enzyme from control rat livers.

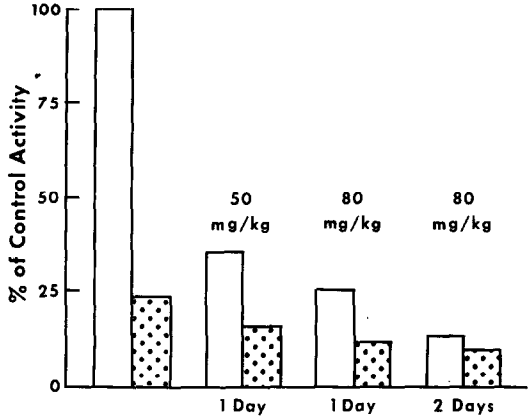

FIG. 2. Effect of APHZ on MAO activity in livers of riboflavin-deficient rats. Groups of rats fed on riboflavin-deficient and -supplemented (control) semisynthetic diets for 28 days were injected with APHZ subcutaneously at dosage levels of 0, 50 and 80 mg/kg. The animals were killed at the indicated times after injection, and homogenates of their tissues were assayed for MAO activity using kynuramine as substrate. Data obtained from six to eight determinations (individual animals) per group were expressed as a percentage of the values found for control rats injected (s.c.) with alcoholic saline and plotted as histograms. The paired bars shown closest to the ordinate represent pooled data (both days of the experiment) from saline-treated control rat livers (open bars) and deficient rat livers (dotted bars). The mean control activity (n = 8) was 25·5 ± 0·95 units of MAO activity.

There was no effect of riboflavin deficiency alone on MAO activity of rat brain. The effect of APHZ in this condition was to depress the activity of the enzyme in brains from the deficient group to the same extent as in the control group.

The concentrations of serotonin were estimated in the brains of twenty-four rats. Six experimental animals were injected with APHZ and six others with PHZ. The remainder served as controls. Although each drug produced a decrease in brain MAO activity when given to rats at 80 mg/kg body weight, neither of them caused any increase in brain serotonin after 24 hr. PHZ caused the level of this amine in brain to increase from the control value of 0·52 ± 0·04 μg/g to 0·63 ± 0·04 μg/g after 48 hr. However, the increase was not statistically significant. Chessin *et al.*[6] and Pletscher[20] have emphasized that 50–100 per cent inhibition of brain MAO may be required to elevate brain serotonin levels significantly. The amounts of APHZ and PHZ administered to the experimental rats were insufficient to cause such an inhibition of enzymic activity, probably because of the nonspecificity of the effects of these two inhibitors toward MAO. Pletscher[20] has also reported that the MAO inhibitor-induced accumulation of monoamines in brain is of shorter duration than inhibition of the enzyme.

Experiments in vitro

Data obtained for MAO activity measured in the presence of differing concentrations of APHZ and PHZ are shown as semi-logarithmic plots of per cent inhibition vs log (inhibitor concentration) in Fig. 3. Concentrations of PHZ and APHZ theoretically capable of producing 50 per cent inhibition of enzyme activity (I_{50} concentrations) were derived from the graphs by interpolation and are $1{\cdot}6 \times 10^{-5}$ M and $3{\cdot}6 \times 10^{-5}$ M respectively. It is evident that PHZ inhibits MAO more actively than does APHZ.

Kynuramine was found to protect MAO against inhibition by APHZ but not PHZ. This difference is illustrated by the data of Table 1. In these experiments, a

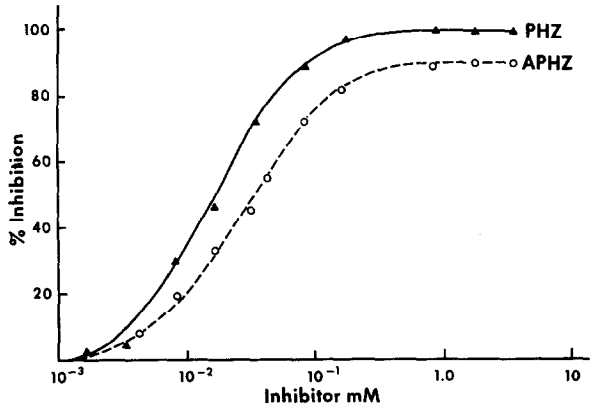

FIG. 3. Inhibition of MAO by APHZ and PHZ *in vitro*. Rat liver mitochondrial preparations containing 0·80 and 0·94 mg protein in separate experiments with the respective inhibitors were incubated with buffered aqueous solutions of the inhibitors for 15 min at 37° and pH 7·0. Kynuramine (1 mM final concentration) was introduced into the incubation media, and the reaction allowed to proceed for 20 min. The complete reaction mixtures contained 33·3 mM phosphate in a total volume of 3 ml. The experimental points represent the mean values (±S. E.) of duplicate determinations. Control values were 62·4 ± 1·04 and 52·7 ± 2·28 units of MAO activity in the APHZ and PHZ experiments respectively.

TABLE 1. EFFECT OF PRIOR ADDITION OF SUBSTRATE (KYNURAMINE) ON THE INHIBITION OF RAT LIVER MITOCHONDRIAL MAO BY APHZ AND PHZ *in vitro**

Inhibitor	Concn (M)	Inhibition (%)
APHZ	$4{\cdot}2 \times 10^{-5}$	2·81 ± 0·0 (2)†
APHZ	$8{\cdot}3 \times 10^{-4}$	49·7 ± 0·48 (3)
PHZ	$1{\cdot}7 \times 10^{-5}$	57·6 ± 0·42 (3)
PHZ	$3{\cdot}3 \times 10^{-4}$	98·7 ± 0·40 (3)

* Kynuramine was added to the reaction mixture just before either inhibitor was added. The incubation was then allowed to continue for 20 min. Reaction mixtures contained 50 mM phosphate buffer, pH 7·0; 0·15 mg mitochondrial protein; 1·0 mM substrate; and the inhibitor as shown in the Table. Final volume was 3·0 ml. Means ± S. E. are shown.

† Figures in parentheses indicate the numbers of determinations that were performed. Activity of the non-inhibited preparation was 40·6 ± 0·77 nmoles 4-hydroxyquinoline formed in 20 min (units)/mg of protein (n = 4).

concentration of APHZ ($4{\cdot}2 \times 10^{-5}$ M) that was expected to cause about 50 per cent inhibition of MAO had only a negligible effect when the substrate, kynuramine, was added immediately before. It required a 20-fold greater concentration of APHZ under this condition to achieve 50 per cent inhibition. On the other hand, the prior addition of kynuramine did not prevent the usual amount of inhibition from taking place when the PHZ was present in its I_{50} concentration ($1{\cdot}7 \times 10^{-5}$ M, Table 1). The reason for this difference in behaviour toward these two structurally similar inhibitors is not immediately apparent. Bernheim[4] and Davison[21] had reported that MAO inhibition by phenylhydrazine could be prevented or reversed if the reaction mixture contained product (aldehyde) or substrate respectively. Green[5] had also found that the prior addition of substrate (tyramine) to incubation mixtures could suppress, but not reverse, the inhibitory action of several hydrazine derivatives toward particle-bound MAO. It is interesting that, despite certain disagreements between these data for PHZ and certain earlier ones, the data of Table 1 are in excellent agreement with values published by Bernheim[4] for MAO inhibition by PHZ with isoamylamine as substrate.

The inclusion of sodium cyanide or semicarbazide in incubation media which contained PHZ or APHZ increased the extent of inhibition of MAO over that observed in their absence (Table 2). Cyanide had the greater potentiating effect for both inhibitors. The effect of semicarbazide was smaller, the net increase which it produced in the action of APHZ being half that observed when it was added to mixtures that contained PHZ. Similar phenomena have been remarked on previously.[5,21]

The effect of pH on inhibition of MAO by APHZ and PHZ was studied. The pH-activity curves of the partially inhibited enzyme were parallel to those of the uninhibited enzymes. Optimal activity of MAO toward kynuramine occurred between pH 8·6 and pH 9·0 whether or not either drug was present in the incubation mixture. The relative inhibition of enzymic activity caused by the respective substances was constant at each pH value tested. The results obtained in these experiments suggest two things: in the first place, binding of the inhibitors to the enzyme did not seriously

TABLE 2. EFFECTS OF CYANIDE AND SEMICARBAZIDE ON THE INHIBITORY ACTION OF APHZ AND PHZ TOWARD MAO ACTIVITY OF RAT LIVER MITOCHONDRIA *in vitro**

Inhibitor	Concn (M)	Inhibition (%)	Net change (%)	P
APHZ	$4{\cdot}17 \times 10^{-5}$	40·1 ± 1·2 (2)		
Plus NaCN	$6{\cdot}7 \times 10^{-4}$	63·6 ± 0·5 (3)	+23·5	< 0·001
Plus semicarbazide	$6{\cdot}7 \times 10^{-3}$	48·9 ± 1·3 (3)	+7·7	< 0·05
PHZ	$1{\cdot}67 \times 10^{-5}$	76·9 ± 0·9 (3)		
Plus NaCN	$6{\cdot}7 \times 10^{-4}$	96·2 ± 1·3 (3)	+19·3	< 0·001
Plus semicarbazide	$6{\cdot}7 \times 10^{-3}$	92·4 ± 0·4 (3)	+15·4	< 0·001

* The enzyme preparations and all test substances were incubated together for 20 min at 37° and pH 7·4 before the reaction was initiated. Means ± S. E. are shown. Figures in parentheses indicate the numbers of replicate determinations that were performed. The non-inhibited enzyme preparation had a sp. act. of 40·6 ± 0·77 units of MAO activity/mg of protein.

alter any of the charge-dependent components of the enzyme molecule that are responsible for maintaining it in an active configuration. Second, binding of PHZ and APHZ to MAO, and consequent inhibition of its activity toward kynuramine, did not depend on the pH value of the reaction media.

Effects of dilution. The data presented in Table 3 are evidence that PHZ and APHZ bind MAO irreversibly, because extensive dilution (30-fold with respect to inhibitor) did not significantly change the degree of inhibition originally observed on 20-min incubation of inhibitors and enzyme.

Effects of incubation time. Linear plots of log (% residual activity) were obtained as a function of incubation time for both inhibitors (Figs. 4 and 5). The time dependence of the inhibitions indicated that APHZ and PHZ bind irreversibly to MAO. The inhibition of MAO observed in both instances is referred to as "immediate" and not "progressive," because the lines, on being produced back to the Y-axis (zero time), do not pass through their origins and indicate very avid instantaneous binding of inhibitor to enzyme, possibly like a chemical reaction gone to completion. When MAO was preincubated with APHZ (final concentration $4{\cdot}31 \times 10^{-5}$ M) for longer than 30 min, the residual activity time line became less steep (Fig. 5). The change

TABLE 3. EFFECTS OF DILUTION ON MAO INHIBITION BY APHZ AND PHZ*

Sample	Inhibitor	Concn (M)	Inhibition (%)
Undiluted experimental	APHZ	$4{\cdot}31 \times 10^{-5}$	70·2 ± 0·83 (2)
Diluted experimental	APHZ	$1{\cdot}44 \times 10^{-6}$	67·51 ± 0·78 (3)
Diluted experimental with additional APHZ	APHZ	$4{\cdot}31 \times 10^{-5}$	72·06 ± 1·39 (3)
Undiluted experimental	PHZ	$2{\cdot}77 \times 10^{-5}$	85·14 ± 0·95 (4)
Diluted experimental	PHZ	$9{\cdot}23 \times 10^{-7}$	83·21 ± 0·76 (4)
Diluted experimental with additional PHZ	PHZ	$2{\cdot}77 \times 10^{-5}$	84·33 ± 0·68 (3)

* The enzyme preparation, consisting of washed mitochondria from rat liver, and the inhibitors were incubated together for 20 min at 37° and pH 7·4. At the end of that interval, aliquots of the mixtures were withdrawn and transferred to vessels containing buffer, 1 mM kynuramine as substrate and either additional inhibitor to maintain the original concentration or no more inhibitor. The reaction was allowed to proceed for another 20 min. Fluorescence of the reaction product was compared with that of control and undiluted inhibited enzyme preparations. Means ± S. E. are shown. The figures in parentheses represent the numbers of determinations made.

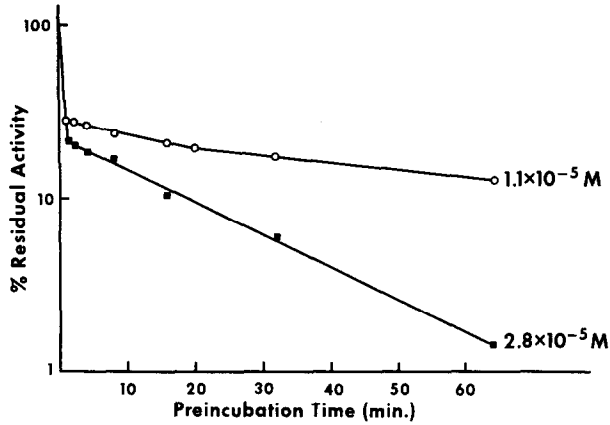

FIG. 4. Effect of preincubation time on PHZ inhibition of MAO *in vitro*. Rat liver mitochondrial preparations (containing 0·59 mg protein) were incubated with buffered aqueous solutions of the inhibitor at the indicated concentrations for various intervals of time at 37° and pH 7·4. Kynuramine (0·51 mM final concentration) was added at the end of each preincubation period and allowed to react with the enzyme for 20 min. The complete reaction mixtures contained 33·3 mM phosphate in a total volume of 3 ml. Under these conditions, there were 59·3 (±1·47) units of MAO activity in the control flasks (no inhibitor). The experimental points represent the mean values of duplicate determinations.

in slope of the APHZ line may be indicative of a mixed, partly reversible association of enzyme and inhibitor beyond that time.

The data obtained in these experiments were subjected to analysis by the method of Lineweaver and Burk.[22] Values derived for the apparent Michaelis constant under the experimental conditions used are in the range of 30–40 μM, in the presence and absence of the respective inhibitors (Table 4). The values shown for the maximal reaction velocities were obtained in separate experiments and do not represent specific activities. Moreover, the difference in uninhibited valued of V_{max} may be ascribed to the different operations followed in their derivation, i.e. fewer concentrations of substrate were used in the experiment in which the effect of APHZ was tested, owing

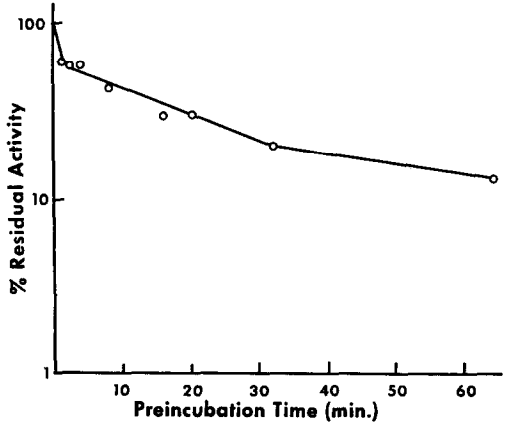

FIG. 5. Effect of preincubation time on APHZ inhibition of MAO *in vitro*. Rat liver mitochondrial preparations containing 0·58 mg protein were incubated with 4·31 × 10^{-2} mM APHZ for various intervals of time at 37° and pH 7·4. Other conditions as in legend to Fig. 4.

TABLE 4. EFFECTS OF APHZ AND PHZ ON "KINETIC CONSTANTS" OF MAO *in vitro**

Inhibitor	Concn (mM)	Apparent K_m $\times 10^{-5}$ M (moles/l)	V_{max} (units)
APHZ	none	4·10	108·9
APHZ	$5{\cdot}39 \times 10^{-3}$	3·58	83·1
APHZ	$1{\cdot}08 \times 10^{-2}$	3·76	81·1
APHZ	$4{\cdot}31 \times 10^{-2}$	3·64	33·8
PHZ	none	2·84	534·8
PHZ	$1{\cdot}75 \times 10^{-3}$	2·78	78·4
PHZ	$8{\cdot}75 \times 10^{-3}$	2·78	24·7

* The enzyme preparation, consisting of washed mitochondria from rat liver, and the inhibitors were incubated together for 20 min at 37° and pH 7·4 before the reaction was initiated. Determinations were performed in duplicate for each concentration of substrate used.

to substrate inhibition being observed at higher concentrations of kynuramine. A wider range of kynuramine concentrations was used in the experiment with PHZ as inhibitor. This experiment seems to give a more reliable value for V_{max} since very high substrate concentrations capable of inhibiting MAO activity were not used. From the results of this set of experiments, both APHZ and PHZ are found to be irreversible, non-competitive inhibitors of MAO, a property which they share with several other hydrazine derivatives.[20,21,23]

DISCUSSION

Zeller[24,25] had based the distinction of MAO from DAO on the inability of the former to be inhibited by carbonyl reagents which inhibit the latter. Most of the common carbonyl reagents, including hydrazine itself, do not inhibit MAO.[26] However, several derivatives of hydrazine, including phenylhydrazine, inhibit MAO *in vivo* as well as *in vitro*.[27] Thus, the inhibitory effects of PHZ and APHZ on MAO reported here seem to be due chiefly to their direct action on the enzyme itself and not to a lower availability of iron for possible use in the synthesis or activity of the enzyme. This seems to be true especially for the brain enzyme. There was no effect of riboflavin deficiency on brain MAO activity, confirming previous findings by Youdim[28] and Leodolter and Genner.[29] Although the vitamin is known to be part of the prosthetic group of several mammalian MAOs, its concentration in rat brain does not decrease appreciably in comparison with heart and liver, in spite of long periods of dietary deprivation.[30,31]

The recovery of brain MAO from inhibition by APHZ occurred much sooner than it did for the liver enzyme (Fig. 1). These results are in contrast to those of Horita[32] who reported that pargyline, in a dose of 20 mg/kg administered to adult rats (i.p.), could cause total inhibition of both the liver and brain enzyme, and that recovery of activity in the liver was 2–3 times as rapid as that of brain. Injection of riboflavin-deficient rats with APHZ caused greater losses of enzymic activity than were due to deficiency alone (Fig. 2). Sourkes[33] and Wiseman–Distler and Sourkes[19,34] had postulated cofactor functions for riboflavin in MAO activity on the basis of similar results. Richter[35] and Hawkins [36] had suggested earlier that riboflavin participates

in the action of MAO. The rat liver enzyme is now known to contain riboflavin,[37,38] a property which it shares with mitochondrial MAOs from other mammalian organs.[39–45]

Hellerman and Erwin[13] found that incubation of a purified preparation of the beef kidney enzyme with limiting amounts of PHZ, pargyline and the *d*-isomer of tranylcypromine (equal concentrations of each inhibitor) resulted in additive inhibition. Initial addition of substrate (benzylamine) could protect the enzyme against inhibition, but its later addition did not reverse the inhibition caused by PHZ and the other inhibitors. In this work, prior addition of kynuramine afforded the rat liver enzyme protection against inhibition by APHZ but not PHZ (Table 1). The differential effects observed for substrate protection of MAO against these agents and in their time courses of inhibition of the enzyme (Figs. 4 and 5) suggest either that APHZ and PHZ bind at different sites on the enzyme molecule or that more than one kind of MAO exists. In regard to rat liver mitochondrial MAO, the evidence for its occurrence in multiple forms that has accumulated since 1963[46] has only recently begun to be strengthened by physical separations of two or more species. The number of such species has been estimated as high as four or five, of which one is considered to be a "dopamine oxidase."[47] Diaz Borges and D'Iorio[48] provide evidence for different enzymes in rat liver mitochondria catalyzing the oxidation of benzylamine and serotonin. Whether APHZ or PHZ is acting on different amine oxidases generally subsumed under the rubric "monoamine oxidase" is not known.

The biphasic nature of the graph shown in Fig. 5 could also be an indication that MAO degrades APHZ to some other reactive substance which inhibits it. The suggestion that biodegradation of hydrazine derivatives and their inhibitory effects on MAO are interdependent has been made earlier by several authors.[49–57] Clineschmidt and Horita[51] have shown that phenelzine is not only an irreversible MAO inhibitor, but also serves as a substrate of MAO in the intact rat. Hucko-Haas and Reed[58] have reported that beef plasma DAO also degrades hydrazine derivatives.

APHZ was a more potent inhibitor of rat liver MAO *in vivo* than was PHZ, while the converse was true for the brain enzyme. With regard to their action *in vitro*, both drugs inhibit the rat liver enzyme non-competitively and irreversibly, PHZ being the more active of the two. The data presented here suggest that each inhibitor forms a non-dissociable complex with the enzyme. Complex formation and MAO inhibition *in vitro* were both independent of pH. The pH independence of MAO inhibition by APHZ and PHZ had been observed for other hydrazine compounds earlier, by Bloom.[54]

Acknowledgement—We thank Mrs. Brenda Ley for her assistance in the preparation of the synthetic diets and caring for the experimental animals.

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