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BIOCHEMICAL PROPERTIES OF N-BENZYL-N′-*ISO*PROPYLHYDRAZINE

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Abstract—N-Benzyl-N′-*iso*propylhydrazine (BIH), taken as an example of several N:N′-disubstituted hydrazines, was inactive as an inhibitor of monoamine oxidase (MAO) *in vitro*; however, it was converted by the body into a potent inhibitor of this enzyme system. The potency of the disubstituted hydrazine *in vivo* was estimated by determining its ability to potentiate tryptamine-induced convulsions in mice and by measuring the enzymic activity of livers and brains of the treated mice. These tests demonstrated BIH to be from three to twenty times as potent as iproniazid and, in contrast to iproniazid, to be more effective against brain than against liver MAO. The chemical nature of the active inhibitor derived from the inactive compound is unknown.

DERIVATIVES of hydrazine have been widely studied in recent years as potent inhibitors of monoamine oxidase (MAO). Those compounds demonstrated to have both clinical utility and enzyme inhibitory properties have been either hydrazides or mono-substituted hydrazines, especially hydrazine analogues of phenylethylamines. It may be suggested that a mono-substituted hydrazine moiety is the potent enzyme inhibitor and that the remainder of the molecule serves as a carrier which may impart some pharmacologic and toxicologic differences to the molecule. Thus, phenylethylhydrazine and *iso*propylhydrazine are potent MAO-inhibitors, but differ pharmacologically because of the differences imparted by the phenylethyl and *iso*propyl groups acting as carriers.[1] Iproniazid, on the other hand, may have weak intrinsic activity, and within the body may be activated by hydrolysis, with the release of *iso*propylhydrazine.[2] The other hydrazine and hydrazide compounds can be described in similar manner.

This report describes the properties of N-benzyl-N′-*iso*propylhydrazine (BIH), as a representative of several N:N′-disubstituted hydrazines which appear to differ biochemically from either mono-substituted hydrazines or hydrazides.

METHODS

Enzyme inhibition

The inhibition of monoamine oxidase (MAO) was assayed *in vitro* by determining the relative ability of liver homogenates to destroy serotonin (5-HT) in the presence and absence of the test compound.[3] Inhibitory activity for MAO was also determined *in vitro* by the use of the spectrophotometric assay of kynuramine destruction, as described by Weissbach *et al*[4].

An *in vitro*-measurement of *in vivo*-activity was made by determining the ability of livers and brains of treated mice to metabolize 5-HT,[1, 5] using the same assay as that employed in the *in vitro*-studies. Preliminary indirect measurements of MAO-activity *in vivo* were made by determinations of 24-hr urinary output of tryptamine by rats.[6]

The potency and duration of action of the inhibitors of MAO were estimated by determining the ability of the agents to potentiate a threshold convulsant dose of tryptamine. In this modification of the technique described by Tedeschi *et al*,[7] at an appropriate interval after treatment with an MAO-inhibitor, mice were given 30 mg of tryptamine hydrochloride per kg, by injection into the tail vein, and watched for typical tryptamine convulsions. At this dosage level of tryptamine, 10 per cent of the control mice convulsed. Preliminary experiments with groups of three mice, dosage levels at geometric progression, and pre-treatment intervals of 1, 5, and 18 hr, respectively, were used to suggest appropriate dosage ranges and intervals between administration of the MAO-inhibitor and challenge with tryptamine. At the time of apparent peak activity, using groups of ten mice, the dose of inhibitor inducing convulsions in 50 per cent of the animals (ED_{50}) was determined by the method of Litchfield and Wilcoxon.[8]

Chemical analysis

N-Benzyl-N′-*iso*propylhydrazine was assayed by employing the color formed through the reaction of a hydrazine and phosphomolybdic acid. To determine the BIH-content of blood serum, the serum proteins were precipitated by the addition of 1·5 vols of 5% trichloroacetic acid; of the protein-free filtrate, 3 ml were alkalinized by the addition of 2 ml of 5% $NaHCO_3$ and the BIH was extracted with 10 ml of chloroform. Nine milliliters of the chloroform solution were shaken for 20 min with 4 ml of 1% phosphomolybdic acid. Color was developed in 3 ml of the aqueous phase by the addition of 0·5 ml of 1 N NH_4OH. The intensity of the color was determined at 400 mμ exactly 5 min after addition of the ammonia. A serum blank contributed only about 1 μg/ml to the assay procedure. Urine was assayed in a similar manner and with the omission of the protein precipitation step. Assays of control urine indicated the presence of a blank amounting to about 5 μg/ml. This method was not specific for BIH but measured other hydrazine derivatives as well.

Materials

N-Benzyl-N′-*iso*propylhydrazine, *iso*propylhydrazine, and *sym*-di*iso*propylhydrazine were synthesized by the Allard Laboratories, Nogent-sur-Marne, France, and supplied as the hydrochloride salts*. Benzylhydrazine hydrochloride was prepared by the Department of Organic Chemistry of the Mead Johnson Research Center. Iproniazid phosphate and iscarboxazid, as "Marsilid" and "Marplan", respectively, were supplied through the courtesy of Hoffmann-La Roche, Inc. Phenethylhydrazine sulfate as "Nardil" and phenyl*iso*propylhydrazine as "Catron" were furnished through the courtesy of Warner-Lambert Research Institute and Lakeside Laboratories, Inc, respectively.

* N-Benzyl-N′-*iso*propylhydrazine hydrochloride was synthesized and its biological properties were described by C. H. Hoffmann, J. Frossard, K. M. Xuan, and I. Karadavidoff of the Allard Laboratories (*Fr. pat.* 93 M, 9 January, 1961).

RESULTS

Although the N:N′-disubstituted hydrazines were reported to be active in preventing reserpine ptosis (Dr. C. H. Hoffmann, personal communication), they were found to be inactive *in vitro*. In Table 1 are given the results of comparisons of the *in vitro*-activities of several N:N′-disubstituted hydrazines with those of known MAO-inhibitors.

TABLE 1. ACTIVITY OF SEVERAL MONOAMINE OXIDASE INHIBITORS, *in vitro*, WITH EITHER SEROTONIN OR KYNURAMINE USED AS A SUBSTRATE

Compound	AIC_{50}* (Molarity) Serotonin	Kynuramine
N-Benzyl-N′-*iso*propylhydrazine	$>1 \times 10^{-4}$	1×10^{-3}
Benzylhydrazine	—	$1{\cdot}3 \times 10^{-6}$
sym-Di*iso*propylhydrazine	$>1 \times 10^{-4}$	—
*iso*Propylhydrazine	$1{\cdot}5 \times 10^{-6}$	$1{\cdot}2 \times 10^{-5}$
Iproniazid	$2{\cdot}1 \times 10^{-4}$	8×10^{-5}
Phenyl*iso*propylhydrazine	$1{\cdot}5 \times 10^{-6}$	$1{\cdot}5 \times 10^{-5}$
Phenethylhydrazine	—	$2{\cdot}5 \times 10^{-5}$
Isocarboxazid	—	$3{\cdot}0 \times 10^{-5}$

* Approximate concentration at which 50 per cent inhibition of MAO is observed.

TABLE 2. RELATIVE EFFECTS OF MAO INHIBITORS ON BRAIN AND LIVER ENZYME FOLLOWING INTRAPERITONEAL INJECTION

Compound	Dose ((mg/kg)	% Inhibition Brain	Liver	AID_{50}* (mg/kg) Brain	Liver
N-Benzyl-N′-*iso*propylhydrazine HCl	3·0	41	13		
	5·0	50	22	4	7·4
	10·0	100	67		
Benzylhydrazine HCl	0·2	22	12		
	1·0	52	63	0·8	0·8
	5·0	86	90		
Iproniazid	14	—	20		
	37·5	12	95		
	75	52	82	74	21
	100	61	97		
	150	73	—		
sym-Di*iso*propylhydrazine HCl	0·75	3	14		
	1·5	29	16	—	—
*iso*Propylhydrazine HCl	5·0	67	100	—	—

* The approximate dose causing a 50 per cent inhibition of MAO-activity.

As a first step in resolving this discrepancy, we determined the effect of intraperitoneal injections of these hydrazines on the urinary excretion of tryptamine by rats. At a dosage level of 10 mg/kg, *iso*propylhydrazine hydrochloride, *sym*-di*iso*propylhydrazine hydrochloride and N-benzyl-N′-*iso*propylhydrazine hydrochloride caused marked increases in urinary tryptamine excretion. Accordingly, the relative

effects of BIH and several other agents on the MAO of the liver and brain were determined. In all cases the mice were sacrificed 1 hr following intraperitoneal injection of the MAO-inhibitor and the enzymic activity of liver and brain homogenates was determined immediately. The data in Table 2 indicate that the two monosubstituted hydrazine derivatives, *iso*propylhydrazine and benzylhydrazine, were the most potent of the five hydrazine derivatives tested. The disubstituted compound, BIH, was the only one to show a definitely greater activity against the MAO of the brain than against that of the liver.

The simplest and most reliable method for estimating the duration of action of BIH and obtaining meaningful potency ratios appeared to be through determination of the ability of MAO-inhibitors to potentiate the convulsant activity of tryptamine.[7, 9] In our hands the use of mice rather than rats afforded convenience and economy in materials, time and space. Mice proved to be less sensitive to tryptamine than rats and thus required a higher threshold dose of this compound. The relative potencies of

TABLE 3. POTENTIATION OF TRYPTAMINE CONVULSIONS BY SEVERAL HYDRAZINE DERIVATIVES

Agent	Intraperitoneal administration ED_{50}*	Time† (hr)	Oral administration ED_{50}*	Time* (hr)
N-Benzyl-N′-*iso*propylhydrazine HCl	1·0 (0·5–2·6)	5	3·8 (3·3–4·4)	5
Benzylhydrazine HCl	0·8 (0·4–1·5)	5	1·6 (1·0–2·5)	5
sym-Di*iso*propylhydrazine HCl	0·9 (0·4–1·9)	5	2·6 (1·8–3·8)	5
*iso*Propylhydrazine HCl	0·8 (0·4–1·6)	5	2·7 (2·1–3·6)	5
Iproniazid phosphate	3·6 (2·1–5·6)	18	18·0 (8·0–40·0)	18
Phenyl*iso*propylhydrazine	0·9 (0·6–1·4)	5	2·0 (1·5–2·7)	5
Phenethylhydrazine sulfate	5·2 (2·6–10·4)	5	9·8 (6·9–13·8)	5
Isocarboxazid	2·8 (1·5–5·2)	5	4·5 (4·0–5·0)	5

* Dose in mg/kg causing convulsions in 50 per cent of mice. Ninety-five per cent confidence limits are indicated.

† Time between treatment with the hydrazine derivative and challenge with tryptamine.

several hydrazine derivatives, as determined by this method, are given in Table 3. By the intraperitoneal route, BIH, benzylhydrazine, di*iso*propylhydrazine and phenyl-*iso*propylhydrazine were of similar high potency. Benzylhydrazine and phenyl*iso*-propylhydrazine were the most potent agents by the oral route and were about one-half as active by this route as by intraperitoneal injection. BIH exhibited about

one-half the milligram potency of benzylhydrazine and phenyl*iso*propylhydrazine upon oral administration and the potency by this route, as related to the intraperitoneal route, decreased by a factor of 4.

The onset and duration of action of BIH, benzylhydrazine, and *iso*propylhydrazine following oral administration were estimated with the aid of the tryptamine test. At stated intervals following the administration of the 5-hr oral ED_{50} (Table 3) of the hydrazine, groups of twenty mice each were challenged with tryptamine. As seen in Fig. 1, BIH appears to have the most rapid onset of action. All three compounds exhibit very long lasting effects, with those of BIH and *iso*propylhydrazine being definitely reduced at 24 and 48 hr, respectively; benzylhydrazine, however, was as active at 48 hr as it was at 5 hr following administration.

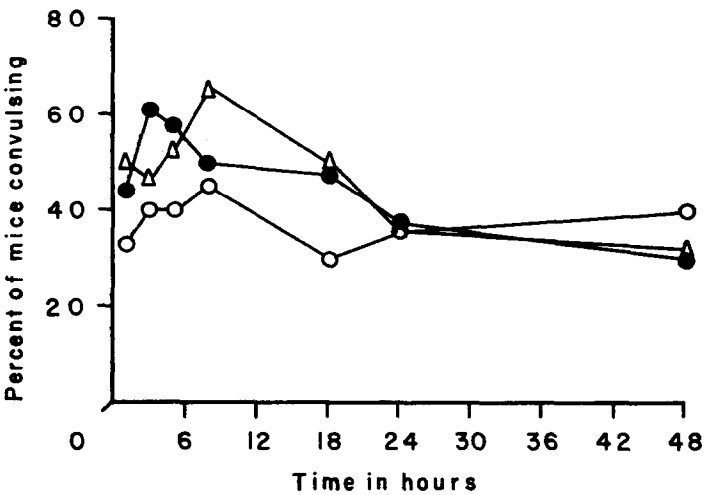

FIG. 1. Onset and duration of action of three hydrazine derivatives following oral administration. ● N-Benzyl-N′-*iso*propylhydrazine. ○ Benzylhydrazine. △ *iso*Propylhydrazine.

The apparent activation *in vivo* of a compound inactive *in vitro* necessitated at least a preliminary examination of the metabolic fate of BIH. Adult, male rats were used to seek an active MAO-inhibitor in the serum of rats treated with BIH. One hour following administration, per kg, of 100 mg of the hydrochloride salt of BIH, rats were killed by decapitation and the blood was collected. The serum was removed and diluted with either 1 or 2 vols of a 0·9% solution of sodium chloride. One milliliter of the diluted serum was tested for its ability to alter the rate of destruction of kynuramine by liver homogenates. Although there was some variability in the data obtained, two findings were unequivocal. First, serum from untreated rats caused a slight, yet consistent, augmentation of the rate of destruction of the amine. Second, rats treated with BIH provided serum which inhibited the MAO-activity in all cases. Slight inconsistencies in the effects of dilution of serum upon its inhibitor potency and in the augmentation properties of control serum made precise calculations of the amount of inhibitor in the serum impossible, but it is estimated that a final dilution of 1 ml of serum in a reaction mixture of 6 ml caused an inhibition of MAO of 30 to 60 per cent. The potency of BIH in this system was such (see Table 1) that more than 200 μg/ml

would be necessary to cause 50 per cent inhibition. However, the amount of BIH and closely related metabolites present at 1 hr following oral administration of 100 mg/kg of the agent to rats was estimated by chemical analysis to be only about 3 μg/ml.

The instability of BIH within the organism was further indicated by the finding of only 1–5 per cent of an oral dose of 100 mg/kg in the 24 hr urine samples from male rats; also, an active inhibitor of MAO could not be extracted with chloroform from such urine. Ether extracts of acidified urine were examined by paper chromatography and failed to exhibit any spots which might represent benzoic or hippuric acids. Such acids would be expected if the biotransformation proceeded through benzylhydrazine or benzylamine.[10]

DISCUSSION

Even if we do not consider the question of the pharmacologic mechanism by which MAO inhibitors achieve their therapeutic ends, we find that the laboratory evaluation of the biochemical properties of these compounds is not simple or routine. Both the necessity for and the gross inadequacies of *in vitro*-techniques of screening and evaluation are pointed up by the present study. Although the hydrazide compounds such as iproniazid and isocarboxazid are said to owe at least part of their potency to activation within the organism,[2, 10] they can be shown to be potent inhibitors of MAO *in vitro* using liver homogenates as the crude enzyme preparation. The ability to inhibit the enzyme system may be due to an intrinsic activity of the molecule itself or to the formation of an active derivative during the incubation of the hydrazid and the liver homogenate; thus, the liberation of little more than 1 per cent of the *iso*propylhydrazine present in iproniazid would cause a similar degree of inhibition to that observed.

The N:N′-disubstituted hydrazines, as exemplified by N-benzyl-N′-*iso*propylhydrazine, are somewhat different. The *in vitro*-potency of BIH, despite its being incubated with liver homogenates prior to addition of the substrate, is so slight that it would be neglected in an *in vitro*-screen. Less than 0·1 per cent of the benzylhydrazine present in BIH would inhibit MAO to a degree similar to that observed by BIH itself.

BIH and its relatives are shown to be without question potent inhibitors of MAO *in vivo*. When administered to mice, they decreased the ability of liver and brain homogenates obtained from the treated mice to destroy serotonin—the same system in which they were inactive if added *in vitro*. These disubstituted hydrazines potentiated the convulsant activity of tryptamine in a manner similar to that of known inhibitors of MAO. Moreover, BIH, when given orally to rats, caused the appearance in the blood serum of an inhibitor of MAO which could not be attributed to the amount of BIH in the serum. The degree of inhibitory activity in the serum could be accounted for if the hydrazine, determined as BIH, represented instead an agent with an intrinsic activity similar to that of benzylhydrazine. And, although benzylhydrazine is indeed found within the structure of BIH, there is nothing either within the known literature or devolving from our preliminary metabolic studies to suggest a biotransformation route in which either benzylhydrazine or *iso*propylhydrazine might be liberated. It is also significant that BIH exhibited a more rapid onset of action than either benzylhydrazine or *iso*propylhydrazine.

In evaluating the significance of the two methods of testing *in vivo* which were used, it is difficult to say which is a more accurate representation of the extent of enzyme

activity within the living animal or which is more truly indicative of therapeutic potential. It has been suggested that the inhibitory potency of an agent may be exaggerated in removing tissues of treated animals and preparing homogenates, since the homogenization treatment may make the enzyme more accessible to the inhibitors.[9] It also may be argued that the tryptamine test is not a direct measurement of MAO and may reflect other physiological and biochemical changes. If one compares the data from the present study in terms of the potency in the homogenate method (ID_{50}) and that indicated by the tryptamine test (ED_{50}), a wide discrepancy among the several hydrazine derivatives will be suggested. Benzylhydrazine can be given a similar potency rating by either criterion. BIH will be judged four times as potent by the tryptamine potentiation method as by the method of direct measurement of MAO-activity of the tissues of treated animals, and iproniazid appears to be twenty times as potent by the former as by the latter method. In the cases just cited, the ID_{50} for brain enzyme was used for comparison; if that for the liver enzyme is used, the ratios would be 1, 7·5 and 6, respectively, for benzylhydrazine, BIH and iproniazid. In no case, however, can it be seen that the use of the tryptamine test would provide a more conservative measure of absolute MAO-inhibitor activity.

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