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Monoamine Oxidase Inhibitors and Procedures for their Evaluation *In Vivo* and *In Vitro*

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Early studies on the inhibition of the enzyme, monoamine oxidase, suggested that certain sympathomimetic drugs owed their pharmacological activity to their ability to inhibit this enzyme. Drugs such as amphetamine, cocaine, and ephedrine were considered to stimulate the actions of epinephrine by blocking the destruction of this hormone through inhibition of monoamine oxidase.[1] These ideas concerning the mechanism of action of the sympathomimetic agents are no longer held, but were prevalent until quite recently. The demonstration of truly potent monoamine oxidase inhibitors, such as iproniazid, by Zeller and his collaborators,[2,3] as well as the finding of new amine substrates for the enzyme, rekindled interest in monoamine oxidase inhibitors as pharmacological agents. At present, the pharmaceutical industry is actively engaged in developing monoamine oxidase inhibitors, and clinicians are applying them in the treatment of mental depression, hypertension, angina pectoris, etc. Apart from their immediate clinical use, these agents are of great importance in elucidating the metabolism of the known amines and in detecting amines which are otherwise too rapidly metabolized, such as tryptamine, phenylethylamine, *p*-tyramine, *o*-tyramine and *m*-tyramine.[4,5]

Since Zeller's original work many compounds have been shown to inhibit monoamine oxidase. Although attempts have been

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made to relate structure to enzyme inhibition, they have been of use only in a particular series of compounds.[6,7] However, since widely diverse classes of compounds possess this activity, a more thorough investigation of the many different structures was warranted.

Studies in these laboratories on the biochemistry of endogenous amines necessitated the need for simple procedures to determine the relative *in vitro* and *in vivo* activities of compounds suspected to be monoamine oxidase inhibitors. A screening procedure was developed that readily answered the following questions:

(1) Is the compound an inhibitor of monoamine oxidase?
(2) Does it inhibit monoamine oxidase both *in vitro* and *in vivo*?
(3) Is the inhibition of long or short duration?
(4) Is monoamine oxidase inhibited in the central nervous system, as well as peripherally?

Details of these procedures are presented together with their application to the detection and study of several new classes of inhibitors and a comparison with known monoamine oxidase inhibitors.

Materials

Serotonin (5HT) creatinine sulphate, 5-hydroxy-DL-tryptophan (5HTP) and 5-hydroxyindoleacetic acid (5HIAA) were obtained from the California Corp. for Biochemical Research.

(1) *In vitro.* Rats were sacrificed, and the livers were quickly removed and homogenized in two volumes of cold distilled water. Incubations were performed in air in 20-ml beakers in a Dubnoff metabolic shaker. The standard incubation mixture contained 1 ml of liver homogenate, 0·5 ml of 0·5 M phosphate buffer, pH 7·4, 1 mg of serotonin and water to add up to a final volume of 3·5 ml. At 0, 20 and 40 min 0·5-ml aliquots were removed and assayed for serotonin. The rate of serotonin destruction was essentially linear over this time period and for routine procedure 40-min incubations were employed. The potential inhibitors were pre-incubated for 20 min with the tissue preparation before the addition of substrate.

Serotonin was measured by a modification of the colorimetric

method of Udenfriend *et al.*[8] The 0·5-ml aliquot removed from the incubation beaker was transferred to a 40-ml glass-stoppered bottle containing 2 ml of 0·5 M borate buffer, pH 10·0 and 2 g of NaCl salt. Fifteen millilitres of butanol was added and the reagents were thoroughly mixed. After centrifugation 10 ml of the butanol phase was transferred to a 40-ml glass-stoppered shaking tube containing 10 ml of heptane and 2·5 ml of 0·1 N HCl. After shaking and centrifugation 2 ml of the final acid was assayed colorimetrically.

The compounds to be tested for *in vivo* activity were injected intraperitoneally (i.p.) into rats and the livers removed 2 and 14 h later. Homogenates were prepared as described above, and serotonin disappearance was determined and compared with control liver preparations (untreated animals).

(2) *Effect on endogenous brain levels.* Compounds to be tested were administered i.p. to rats. Brains were removed 2 and 14 h later, and assayed for serotonin according to the fluorometric method of Bogdanski *et al.*[9] Values were compared with control levels.

Brain levels after 5HTP. Two hours after i.p. administration of an inhibitor, 200 mg/kg of DL-5HTP was administered (i.p.) and the animals were sacrified 2 h afterwards. The brain levels of serotonin were determined and compared with those of untreated controls and of animals receiving only 5 HTP. Marked excitement in the animals receiving both the compound and 5HTP also indicated that monoamine oxidase was being inhibited.

(3) *Effect on the conversion of serotonin to 5HIAA in whole mice.* One hour after i.p. administration of the compound to mice, 2 mg of serotonin was administered (also i.p.). The individual animals were kept in beakers and after 2 h each mouse (including any excreta) was homogenized in 100 ml of 0·1 N HCl. The homogenate was passed through a thin layer of cheese cloth and an aliquot corresponding to about 1 g (6 ml of homogenate) was assayed for 5HIAA employing solvent extraction.[10] The aliquot removed was made up to a volume of 12 ml by the addition of water, and 2 ml of a 10 per cent solution of zinc sulphate was added. After careful mixing, 1 ml of 1 N NaOH was added to precipitate the proteins, and the tube was centrifuged at high speed. Ten millilitres of the supernatant was transferred to a

40-ml glass-stoppered shaking tube containing 4 g of sodium chloride and 0·5 ml of 6 N HCl. Twenty millilitres of ether was added and the tubes were shaken for 5 min. After centrifugation 15 ml of the ether phase was transferred to another glass-stoppered centrifuge tube containing 1·5 ml of 0·5 M phosphate buffered at pH 7·0. After agitation and centrifugation 1 ml of the aqueous buffer was transferred to a test tube containing 0·3 ml of 12 N HCl. The 5HIAA was then assayed spectrophotofluorometrically. Standards and blanks were carried throughout the entire procedure.

Results and Discussion

Of over eighty compounds tested for *in vitro* activity those listed in Tables I–III are representative examples of various groups of related compounds. As shown in Table I, many harmala alkaloids were found to be active and some of these compounds, harman and the harmines (Table I, Nos. 1–3), demonstrated activity at concentrations of 10^{-5}–10^{-6} M. Data on harmaline, which has an activity of the same order, have already been presented.[11] Slight changes in structure, as shown in the subsequent compounds, invariably lowered monoamine oxidase inhibition markedly. As has been reported previously,[7] phenylisopropylhydrazine (Table III, No. 21) and phenylisobutylhydrazines (Table III, No. 23) are also potent inhibitors. We have been able to corroborate the reported[6] marked differences of *in vitro* activities between the L- and D-isomers of *N*-acetylalanylisopropylhydrazine (Table III, No. 24).

A large number of very active inhibitors of the hydrazine type are now available, and many of these are undergoing clinical trials. That active inhibitors (10^{-5} M) can be found among classes of compounds not related to hydrazine, emerges as a fact of great interest. Thus, the amphetamine analogue, phenylcyclopropylamine (tranylcypromine) (Table III, No. 22), is highly active, as are some deoxyephedrine analogues (Table III, No. 27). Procaine amide (Table III, No. 30) is active and some of its congeners (Table III, No. 26) were found to be even more potent.

Some well-known drugs which were found to have no activity at 10^{-3} M were atropine, diphenhydramine, chlorothiazide,

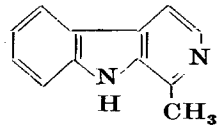
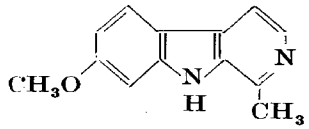
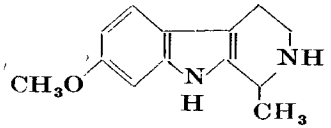
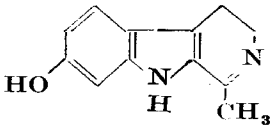
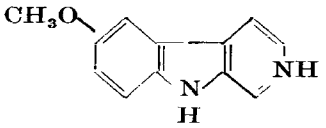
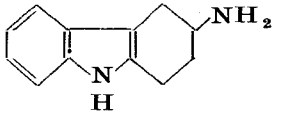
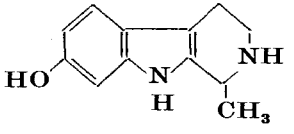
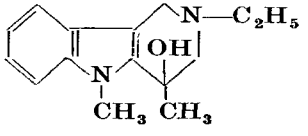
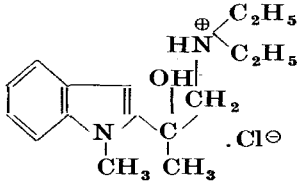
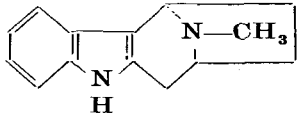
No.	Formula	Name	Final molar concentration at which 40–60% inhibition occurs	Remarks
1		Harman	2×10^{-6}	m.p. 238° Inhibits 66% at [10^{-5}], 79% at [2×10^{-5}]
2		Harmine	2×10^{-6}	m.p. 256° Inhibits 70% at [10^{-5}], 94% at [2×10^{-5}]
3		Tetrahydroharmine	2×10^{-5}	m.p. 199° Inhibits 82% at [10^{-4}]
4		Harmalol	10^{-4}	
5		6-Methoxy-1,2,3,4-tetrahydronorharman	10^{-4}	m.p. 212° (Obtained from Labatec, Geneva) Needles (from EtOH). m.p. 214–216°. *Anal.* Calcd. for $C_{12}H_{14}N_2O$: C, 71·26; H, 6·98; N, 13·85. Found: C, 71·02; H, 6·68; N, 13·40. Obtained by $LiAlH_4$ reduction from the lactam, a degradation product of hortiamine and hortiacine, placed at our disposal through the courtesy of Dr. G. E. Ullyot, Smith Kline & French Laboratories

Table I—*continued*

No.	Formula	Name	Final molar concentration at which 40–60% inhibition occurs	Remarks
6		3-Amino-1,2,3,4-tetrahydrocarbazole	10^{-4}	Obtained through the courtesy of Koppers Company Inc., Research Division
7		Tetrahydroharmol	10^{-3}	m.p. 264–265° (from EtOH) (prepared by Dr. J. W. Daly by catalytic hydrogenation (Pd/C) of harmalol)
8		1,9-Dimethyl-1-hydroxy-3-ethyl-1,2,3,4-tetrahydro-γ-carboline	10^{-3}	*cf.* Kebrle, Rossi and Hoffmann[15]
9		1-Methyl-2-(1-diethylamino-2-hydroxy-2-propyl)-indole hydrochloride	(9% inhibition at [10^{-3}])	m.p. 127°[15]
10		Indolo-[2,3-b]-tropane	(7% inhibition at [10^{-3}])	Obtained through the courtesy of Dr. Karl Hoffmann, Ciba Limited, Basel

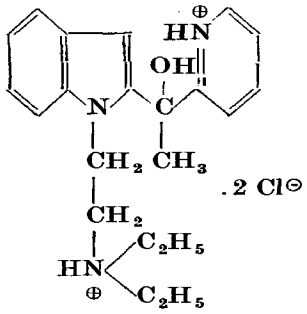
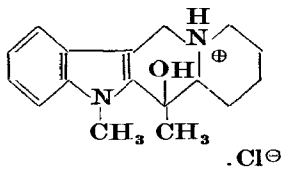
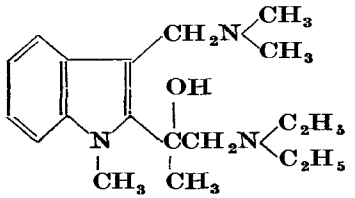
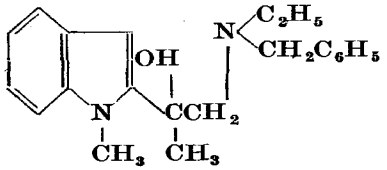
11		1-(Diethylaminoethyl)-2-[methyl-(2-pyridyl)-hydroxymethyl]-indole dihydrochloride	(6% inhibition at [10^{-3}])	Obtained through the courtesy of Dr. Karl Hoffmann, Ciba Limited, Basel
12		11,12-Dimethyl-12-hydroxy-1,2,3,4,12,-12a-hexahydrochinolizino-[2,3-b]-indole hydrochloride	inactive	m.p. 320° (d.)[15]
13		1-Methyl-2-(1-diethylamino-2-hydroxy-2-propyl)-3-(dimethylaminomethyl)-indole	inactive	*cf.* Kebrle, Rossi and Hoffmann[15]
14		1-Methyl-2-[1-benzylethylamino-2-hydroxy-2-propyl]-indole	inactive	m.p. 71°[15]

Table II. Some heterocyclic inhibitors of monamine oxidase

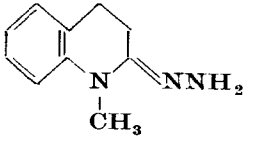
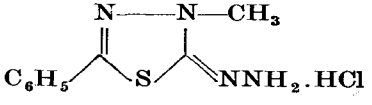
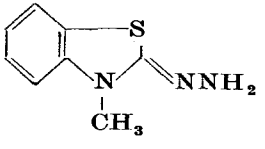
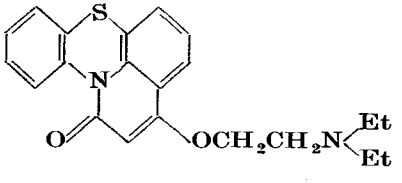
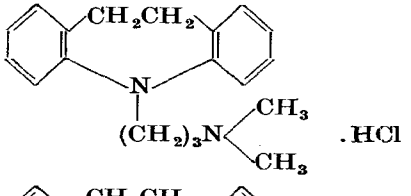
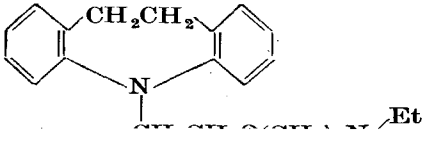
No.	Formula	Name	Final concentration at which 40–60% inhibition is observed	Remarks
15		*N*-Methyl-2-quinolone hydrazone	2×10^{-4}	Obtained through the courtesy of Prof. S. Hünig, Marburg[16]
16		3-Methyl-5-phenyl-1,3,4-thiadiazolone hydrazone	10^{-3} (68% inhibition)	Obtained through the courtesy of Prof. S. Hünig, Marburg[17]
17		*N*-Methylbenzothiazolone hydrazone	10^{-3}	Obtained through the courtesy of Prof. S. Hünig, Marburg[18]
18		Benzophenothiazine derivative code G-32	10^{-3}	Obtained through the courtesy of I. R. Geigy
19		*N*-(γ-Dimethylaminopropyl)-10,11-dihydrodibenzazepine (Tofranil®)	$< 10^{-3}$ (20% inhibition at $[10^{-3}]$)	Obtained through the courtesy of I.R. Geigy
20		*N*-(β-Diethylaminoethoxyethyl)-10,11-dihydrodibenzazepine (G-33)	inactive	Obtained through the courtesy of I. R. Geigy

Table III. Inhibitors of monoamine oxidase derived from phenylalkylamines, hydrazones and ethylenediamines

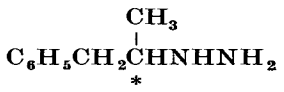
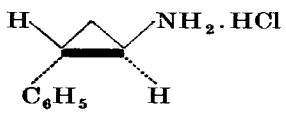
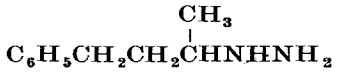
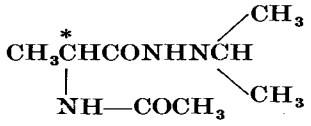
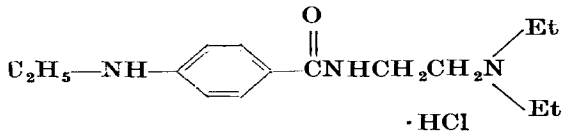
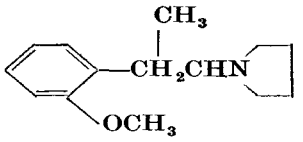
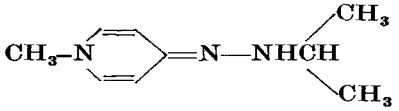
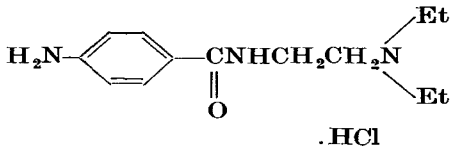
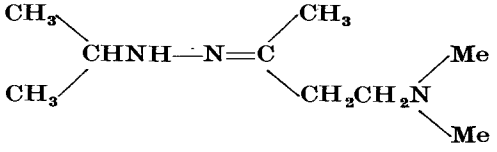
No.	Formula	Name	Final molar concentration at which 40–60% inhibition occurs	Remarks
21		DL-Phenylisopropylhydrazine (JB-516, Catron®)	10^{-6}	Obtained through the courtesy of Dr. J. H. Biel, Lakeside Laboratories[19]
22		*trans*-DL-2-Phenylcyclopropylamine (tranylcypromine)	10^{-5}–10^{-6}	The compound was obtained through the courtesy of Smith Kline & French Laboratories[20]
23		DL-Phenyl-isobutylhydrazine	10^{-5}–10^{-6}	Obtained through the courtesy of Dr. J. H. Biel, Lakeside Laboratories
24		*N*-Acetyl-L-alanine-isopropylhydrazide	2×10^{-5}	The L-isomer still showed 10% inhibition at [10^{-6}] and 96% inhibition at [2×10^{-5}]. The D-isomer was inactive at [10^{-3}]
		N-Acetyl-D-alanine-isopropylhydrazide	inactive	
25		1-(α-Picoloyl)-2-benzoylhydrazine	10^{-5}–10^{-6}	Inhibition at [10^{-6}] was 25% and at [2×10^{-5}] 90%. Obtained through the courtesy of Charles Pfizer and Co.
26		1-(*p*-Ethylaminobenzoylamino)-2-diethylaminoethane hydrochloride	10^{-5}	Obtained from the Squibb Institute for Medical Research

Table III—*continued*

No.	Formula	Name	Final molar concentration at which 40–60% inhibition occurs	Remarks
27		1-(2-Methoxyphenyl)-2-*N*-pyrrolidylpropane	10^{-5}	Obtained through the courtesy of the Upjohn Company (the parent ethane compound has 6% inhibition at [10^{-3}])
28		1-γ-Picoloyl-2-benzylhydrazine (nialamide)	10^{-5}	Obtained through the courtesy of Chas. Pfizer & Co.
29		*N*-Methyl-4-piperidone-isopropylhydrazone	10^{-4}–10^{-5}	Inhibition at [10^{-5}] was 24% and at [10^{-4}], 100%
30		1-(*p*-Aminobenzamido)-2-diethylaminoethane (Procaineamide)	10^{-4}	Obtained through the courtesy of the Squibb Institute for Medical Research
31		1-Dimethylaminobutan-3-one-isopropylhydrazone	10^{-4}	Inhibition at [10^{-5}] was negligible, at [10^{-4}], 46%

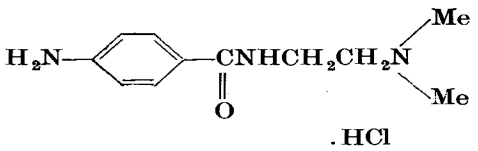
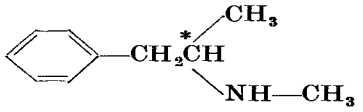
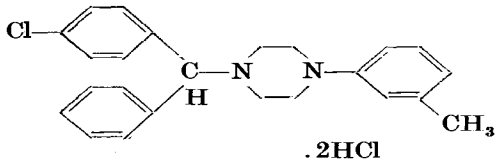
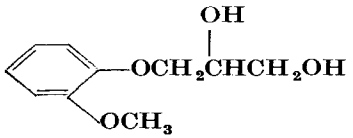
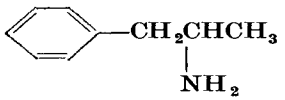
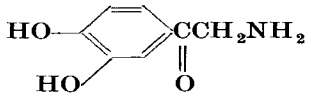
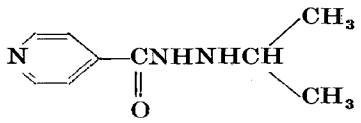
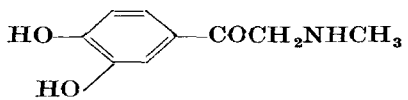
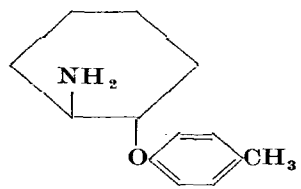
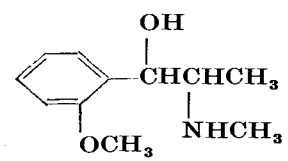
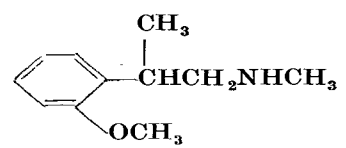
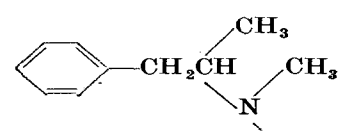
32		1-(*p*-Aminobenzamido)-2-dimethylaminoethane	10^{-4} (35% inhibition)	Obtained through the courtesy of the Squibb Institute for Medical Research
33		Deoxyephedrine	10^{-4}	Obtained through the courtesy of the Upjohn Company
34		*N*-Benzhydryl-*N'*-*m*-tolyl-piperazine (meclizine)	10^{-3} (100% inhibition)	Inhibition was 11% at [10^{-4}]. The related cyclocyclizin (*N'*-methyl instead of *N'*-*m*-tolyl) was inactive at [10^{-3}]
35		3-*o*-Toloxy-1,2-propanediol (mephenesin)	10^{-3} (70% inhibition)	No inhibition at [10^{-4}]. Obtained through the courtesy of the Squibb Institute for Medical Research
36		Amphetamine	10^{-3} (65% inhibition)	Obtained through the courtesy of the Upjohn Company
37		Arterenone	10^{-3} (62% inhibition)	Obtained from the Sterling-Winthrop Research Institute through the courtesy of Dr. S. Archer
38		Isonicotinic acid-*N*-isopropylhydrazide (iproniazid)	10^{-3}	

Table III—*continued*

No.	Formula	Name	Final molar concentration at which 40–60% inhibition occurs	Remarks
39		Adrenalone	10^{-3}	Obtained from the Sterling-Winthrop Research Institute through the courtesy of Dr. S. Archer. (Adrenaline shows 11% inhibition at [10^{-3}])
40		*trans*-2-Aminocyclohexyl-*p*-tolyl ether	10^{-3} (41% inhibition)	Obtained through the courtesy of Dr. F. Häfliger, Geigy, Basel (the *N*-methyl homologue has 10% inhibition at [10^{-3}], the quarternary has only 6%)
41		*o*-Methoxyephedrine	10^{-3} (30% inhibition)	Obtained through the courtesy of the Upjohn Company. Ephedrine shows no inhibition at [10^{-3}]
42		2-(*o*-Methoxyphenyl)-1-methylaminopropane	$< 10^{-3}$ (23% inhibition)	Obtained through the courtesy of the Upjohn Company
43		*N*-Benzyl-*N*-methyl-amphetamine	$< 10^{-3}$ (13% inhibition)	Obtained through the courtesy of the Upjohn Company

cocaine, ephedrine, ethionine, hyoscine, niacinamide, lysergic acid diethylamide, penicillin-G, phentolamine, methyl phenidate, and streptomycin.

Of the large number of compounds screened for *in vitro* activity only the most active were subjected to further tests. As shown in Table IV, all the active compounds inhibited monoamine oxidase in tissues when administered to rats *in vivo*. The fact that harmine at 10 mg/kg did not seem to inhibit very well after two hours, is due to its being a reversible and competitive inhibi-

Table IV. Monoamine oxidase inhibition in rat tissues following administration of various compounds[a]

Compound	mg/kg	Percentage inhibition	
		2 h	14 h
Iproniazid	100	100	100
p-*N*-Ethylprocaine amide	50	22	13
Phenylcyclopropylamine	50	100	86
Nialamide	50	100	92
Thyroxine	50	4	0
Harmine	10	36	0
DL-Phenylisopropylhydrazine	25	100	100

[a] Details of the procedure used are presented in Methods, Section 1B.

tor.[11] Metabolism of the drug, and dilution of tissue fluids during the process of homogenization would be expected to lower the effective concentration and thereby reverse the inhibition. Thyroxine, which has been shown to lead to a decrease of monoamine oxidase in tissues following prolonged administration,[12] had no observable effects *in vivo* following a large single dose. Since thyroxine *per se* had no effects *in vitro*, one has therefore to agree with the conclusions of Zile and Lardy[12] that thyroxine affects the formation of the enzyme.

That these compounds also influence the metabolism of endogenous amines is shown in Table V. Apparently not all the compounds have similar activity. Thus, harmaline elevated the level of serotonin in the brain most rapidly, but, because of the reversible nature of its inhibition, the effect had disappeared after 14 h.

In this test, too, a single dose of thyroxine was inactive. The *N*-ethyl homologue of procaine amide (Table III, No. 26) also showed little activity.

Table V. Effect of monoamide oxidase inhibition of endogenous brain serotonin levels[a]

Compound	mg/kg	5HT μg/g	
		2 h	14 h
Control	0	0·42	0·40
Iproniazid	100	0·56	1·02
p-*N*-Ethyl procaine amide	50	0·35	0·59
Phenylcyclopropylamine	50	0·64	0·84
Nialamide	50	0·56	0·69
Thyroxine	50	0·35	0·52
Harmaline	50	0·75	0·40
DL-Phenylisopropylhydrazine	10	0·80	1·24

[a] Details of procedure used are presented in Methods, Section 2.

By prior administration of 5HTP, the precursor of serotonin, the sensitivity of the brain serotonin procedure for detecting monoamine oxidase inhibition is markedly increased. When this

Table VI. Effect of monoamine oxidase inhibitors on 5HT levels in rat brain after administration of 5HTP[a]

Compound[b]	mg/kg	Brain level μg/g
Normal (no 5HTP)	—	0·33
Control	—	1·36
Iproniazid	100	3·77
p-*N*-Ethylprocaine amide	50	1·32
Phenylcyclopropylamine	50	7·92
Thyroxine	50	0·99
Nialamide	50	5·95
Harmine	50	4·20
DL-Phenylisopropylhydrazine	10	4·52

[a] Details of procedure discussed in Methods, Section 2.
[b] All animals received 200 mg/kg of 5HTP i.p. unless otherwise indicated.

is done to normal animals, brain serotonin levels rise due to the presence, and subsequent decarboxylation, of 5HTP in brain.[13] While the activity of monoamine oxidase is so great that even

large doses of 5HTP increase brain serotonin to only about 3–4 times the normal levels, 7–10 fold increases are observed after administration of an active monoamine oxidase inhibitor. As seen in Table VI, phenylcyclopropylamine, nialamide, iproniazid, harmine and DL-phenylisopropylhydrazine were extremely active in this test. Again thyroxine and the *N*-ethyl homologue of procaine amide exhibited little activity.

Another way of demonstrating the inhibition of monoamine oxidase *in vivo* is based on the procedure previously reported by

Table VII. Effect of monoamine oxidase inhibitors on conversion of serotonin to 5HIAA in the intact mouse[a]

Compound	Dose mg/kg	5HIAA % of administered serotonin	Percentage inhibition
Control	—	31	0
Iproniazid	25	4	88
p-N-Ethylprocaine amide	25	30	0–4
Phenylcyclopropylamine	10	2	94
Nialamide	25	3	76
Thyroxine	25	45	0
Harmine	5	5·2	83
DL-Phenylisopropylhydrazine	10	3·5	91

[a] Details of procedure described in Methods, Section 3.

Udenfriend *et al.*[14] When serotonin is administered to a normal mouse, a certain percentage appears as 5HIAA in homogenates of the whole animals (including excreta) following a given period of time. In the presence of an active monoamine oxidase inhibitor the amount of 5HIAA formed is markedly diminished. As shown in Table VII, all the previously active compounds (Tables III–VI) were active in this test. Thyroxine and the *N*-ethyl homologue of procaine amide were inactive.

Many of the procedures presented here are now being used in a number of laboratories for the detection and evaluation of inhibitors of monoamine oxidase. The *in vitro* procedure, together with one or two of the *in vivo* assays, should prove quite satisfactory for successful screening. Another important use of the *in vivo* evaluation of monoamine oxidase inhibition is the

correlation of the enzymatic effects with pharmacological or behavioural studies. Such relationships between the biochemical and pharmacological effects of these drugs may lead to a better understanding of their mechanism of action. In fact, it has now become possible to evaluate the biochemical effects of monoamine oxidase inhibitors in man *in vivo*.[5] These new concepts and techniques offer a rational approach to the determination of effective dosages for new drugs, to the detection of differences in sensitivity to a given drug from patient to patient, and to the comparative pharmacology of drugs in general.

Summary. Procedures have been presented to detect inhibitors of monoamine oxidase *in vitro* and *in vivo* and to determine the duration of their action centrally and peripherally. These procedures have been applied to the detection and study of several new classes of inhibitors. Potent inhibitors of this enzyme were found among many different classes of compounds, yet within a given class slight changes in structure modified activity markedly. A number of potent *in vitro* agents possessed little activity in the intact animal.

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