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EFFECTS OF DL-3-(5-BENZIMIDAZOLYL)-2-METHYLALANINE ON BRAIN AND HEART CATECHOLAMINES—I. DEPLETING EFFECTS*

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Abstract The intraperitoneal administration of DL-3-(5-benzimidazolyl)-2-methylalanine (MBA) causes a marked decline in rat brain and heart catecholamines. The release of tritiated norepinephrine from the rat heart by MBA indicates a release component in the action of the compound. The releasing potency of decarboxylated MBA and the inability of MBA to lower heart norepinephrine in the presence of a decarboxylase inhibitor implicate a decarboxylation product of the amino acid as responsible for the release of norepinephrine by MBA. When MBA and then a monoamine oxidase inhibitor are administered to reserpinized rats, the level of brain amines remains constant, indicating inhibition of norepinephrine synthesis *in vivo*. The activity of tissue tyrosine hydroxylase in MBA-treated rats confirms this inhibition.

DL-3-(5-Benzimidazolyl)alanine (BA) has been shown to be a good inhibitor of tyrosine hydroxylase and to release brain biogenic amines and heart norepinephrine (NE) in rats [1]. Because of the different, and at times increased, effects observed in α-methyl amino acids, DL-3-(5-benzimidazolyl)-2-methylalanine (MBA) was synthesized [2]. Preliminary tests indicated that MBA was, as a competitive inhibitor of tyrosine *in vitro* in a bovine adrenal tyrosine hydroxylase system, equal to BA ($K_i = 2 \times 10^{-5}$), but had more pronounced and more prolonged effects on brain catecholamine and on heart NE levels [2]. After the administration of a 0·3 m-mole (87·6 mg)/kg dose of MBA a marked decline followed by a delayed rise in catecholamines occurred [2]. An investigation of the mechanisms leading to the decline in catecholamine levels is presented in this report.

Since BA was shown to be an inhibitor of tyrosine hydroxylase *in vivo* as well as a depletor of biogenic amines [1], attempts were made to ascertain whether the decline in catecholamines caused by MBA was effected by these two mechanisms.

MATERIALS AND METHODS

Heart norepinephrine release assay

Materials. DL-Norepinephrine[7-^{3}H] (5 mCi/μmole) was purchased from New England Nuclear Corp.; sodium heparin and tyramine hydrochloride were obtained from Sigma Chemical Co. Scintillation grade toluene, 2,5-diphenyloxazole (PPO), *p*-bis-[2-(5-phenyloxazolyl)-benzene] (POPOP) and Triton X-100 were purchased from Packard Instrument Co. Whatman No. 4 chromatography paper and other reagent grade chemicals were purchased from Fisher Scientific Corp. MBA was prepared as previously reported [2], and DL-2-amino-1-(5-benzimidazolyl) propane dihydrochloride (MBA-amine) was synthesized in our laboratory [3].

Methods. Tritiated NE (^{3}HNE) was chromatographed and purified according to the methods of Iversen and Whitby [4]. The purity of ^{3}HNE was confirmed by chromatography (*n*-butanol–acetic acid–water, 4:1:1) and by comparison of sp. act. before and after recrystallization. The assay was based upon the uptake and release method of Axelrod *et al.*[5, 6].

^{3}HNE (1 nmole, 5 μCi) in 0·1 ml of isotonic saline containing 5 μg heparin was injected into the tail vein of male Wistar rats. Test compounds were injected intraperitoneally 1 hr before and 1 hr after the injection of ^{3}HNE. Three hr after the injection of ^{3}HNE, the animals were sacrificed by a blow on the head and cervical dislocation. The hearts were excised, chilled on ice, and homogenized in 10 vol. of 0·4 N $HClO_4$. The homogenates were centrifuged, and three 0·2-ml aliquots of the supernatant were added to scintillation vials containing 12 ml of toluene phosphor and counted; a fourth 0·2-ml aliquot was chromatographed in the system described above.

Biogenic amine assay

Materials. Male Wistar rats were sacrificed with a guillotine (Harvard Instrument Co.). A glass and Teflon homogenizer (Duall tissue grinder, Kontes Glass Co.) was used in all homogenization procedures. Fluorescence was measured on an Aminco Bowman spectrophotofluorometer in 1-cm quartz cells. Spectra were recorded on an Aminco model 814 XY recorder and were uncorrected. All reagents and solvents (spectral grade) were obtained commercially. Glass-distilled deionized water was used throughout. Dopamine was obtained from ICN Nutritional Biochemical Corp., L-norepinephrine from Sigma Chemical

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Co., Serpasil from CIBA Pharmaceuticals and α-methyl-*p*-tyrosine (MT) from Aldrich Chemical Co. Tranylcypromine was supplied by Smith, Kline & French, Philadelphia, Pa., and DL-seryl-2,3,4-trihydroxybenzylhydrazine hydrochloride (RO-4-4602) by Roche, Inc., Basel, Switzerland.

Methods. Brain and heart tissues were removed rapidly, rinsed in iced saline, blotted dry, placed in screw-cap vials, frozen on dry ice, and stored at −10° until assayed.

At the time of assay, each brain was homogenized in 10 vol. of cold acidified butanol (0·85 ml conc. HCl/liter) and the homogenate was centrifuged. A 2·5-ml aliquot of the supernatant was added to 5 ml heptane and 0·4 ml of 0·1 N HCl and extracted. After centrifugation, the organic phase and the tissue plug were removed by aspiration. Norepinephrine (NE) and dopamine (DA) were separated from a 0·2-ml aliquot of the aqueous phase by Alumina adsorption as described by Chang [7]. NE was determined by the trihydroxyindole procedure [7] on an aliquot of the Alumina eluate. The remainder of the Alumina eluate was then adjusted to pH 4·0 and heated for 45 min in a 90° water bath to develop DA fluorescence. Heart NE was determined according to the procedure of Maickel *et al.* [8].

Data were analyzed by the Student's *t*-test; *t* values and standard deviations were determined on a Hewlett Packard programmable calculator model 9100 A. The levels of significance (P) were taken from standard tables.

Tissue enzyme assays

Materials. 2-Amino-2-hydroxymethyl-1,3-propanediol (Tris) was obtained from the Aldrich Chemical Co. L-Tyrosine, bovine catalase, NADPH and Dowex 50 W × 8-400 were obtained from the Sigma Chemical Co. 2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) was purchased from Calbiochem. Sheep liver was obtained from Pel-Freeze Biologicals, Rogers, Ark. Male Wistar rats (100–120 g) were obtained from Microbiological Associates of Bethesda, Md. [3,5-^{3}H-L]tyrosine was purchased from Amersham/Searle Corp. 3-Hydroxy-4-bromobenzyloxyamine dihydrogen phosphate (NSD 1055) was a gift of Dr. Solomon Snyder of the Johns Hopkins University. Glass-distilled deionized water was used throughout.

Methods. At the time of sacrifice the rats were anesthetized with ether and their superior cervical ganglia removed via a midline incision in the neck, rinsed in iced saline, and frozen immediately in scintillation vials placed on dry ice. The animals were then decapitated with scissors, their brains immediately removed, rinsed in iced saline, placed in scintillation vials on dry ice, and stored at −70°. Within 3 weeks all specimens were thawed, homogenized in Tris buffer (0·05 M, pH 6·0, containing 0·2% Triton X-100) and divided into aliquots which were frozen for later assays. Brain specimens were homogenized in 10 vol and superior cervical ganglia specimens in 100 vol of the Tris buffer. Protein was assayed according to the procedure of Lowry *et al.* [9].

Tyrosine hydroxylase assay

The method of Nagatsu *et al.* [10] was used; several of the modifications suggested by Coyle [11] were incorporated to increase the sensitivity of the method sufficiently for assay of the enzyme in tissue homogenates. First, Tris buffer containing Triton X-100 was used to improve solubilization of the enzyme and to reduce the need for excess ferrous ion. Second, catalase was added to decrease the blank and to maintain the linearity of the reaction with time for a longer period. Last, dihydropteridine reductase was used instead of mercaptoethanol, since the former has been shown by Shiman *et al.* [12] to be effective in maintaining $DMPH_4$ levels. The dihydropteridine reductase preparation was obtained from sheep liver homogenate purified through the second ammonium sulfate purification step by the method of Kaufman [13].

One hundred μl of thawed homogenate or of purified enzyme [14] was added to the reaction mixture containing 200 μl of acetate buffer (0·2 M, pH 6·0), 50 μl of 2 mM NSD 1055, 100 μl of the dihydropteridine reductase preparation (63 μg protein) dissolved in Tris buffer, 2200 units of catalase in 300 μl water, 100 μl of 0·2 mM NADPH and 100 μl of 10·0 mM $DMPH_4$ which had been freshly prepared in cold 5 mM HCl. The assay mixture was preincubated for 5 min on a Dubnoff metabolic shaker at 37° and the reaction was then initiated by the addition of 50 μl (1 or 2 μCi) of 10^{-3} M tyrosine. After 45 min the reaction was terminated and the mixture processed according to Nagatsu *et al.* [10].

RESULTS

In a first attempt to investigate the effects of MBA, a 1 m-mole/kg (292 mg/kg) dose was injected intraperitoneally into rats. This dose, which was equimolar with the dose of BA used by Johnson *et al.* [1] in evaluating BA and equimolar with the dose of MT shown to inhibit catecholamine biosynthesis completely [15], proved too toxic for pharmacological evaluations. From then on, an MBA dose of 0·3 m-mole/kg (87·6 mg/kg), equimolar with the minimum effective dose of BA [1], was used. At this dose, animals were first depressed for about 48 hr then stimulated, and appeared to have completely recovered at 80 hr. A time course study indicated that heart NE and brain catecholamines were maximally depleted at 12 hr and raised above normal levels at 64 hr (brain NE), 72 hr (heart NE) and 80 hr (brain DA) after the injection of MBA [2].

Axelrod *et al.* [5, 6] have described a rapid and convenient method for the study of uptake and release of NE from storage in the rat heart. Tracer amounts of tritiated NE are taken up by the rat heart, mixed and equilibrated with endogenous NE, and affected in a manner identical to endogenous NE by agents which stimulate or inhibit the uptake or the release of stored amines.

When test compounds (MBA, MBA-amine) were injected 1 hr before the injection of ^{3}HNE and the animals sacrificed 3 hr after the injection of isotope, the amount of isotope in the heart was not affected (Fig. 1). On the other hand, when test compounds (tyramine, MBA, MBA-amine) were injected 1 hr after ^{3}HNE and the animals sacrificed 3 hr after the injection of isotope, a significant decrease of heart ^{3}HNE was observed (Fig. 1). Chromatography of heart homogenates of control and treated animals demon-

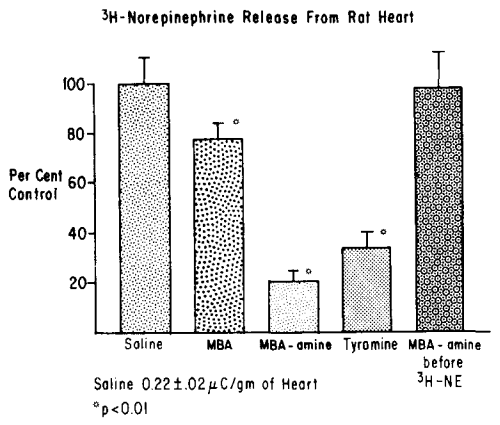

Fig. 1. Effect of DL-3-(5-benzimidazolyl)-2-methylalanine on heart [^{3}H]norepinephrine. Four groups each of six male Wistar rats (90–110 g) were given saline, MBA (0·3 m-mole/kg), MBA-amine (3 μmoles/kg) or tyramine (0·1 m-mole/kg) i.p. 1 hr after an i.v. injection of tritiated norepinephrine (1 nmole, 5 μCi). A fifth group was given MBA-amine 1 hr before tritiated norepinephrine. Results are represented as per cent control (saline group) ± the standard error of the mean.

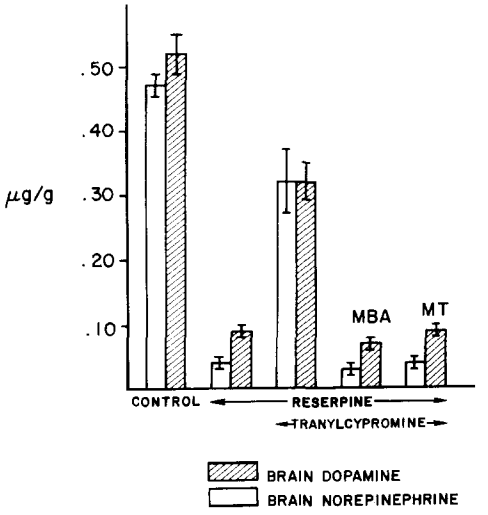

Fig. 2. Effect of DL-3-(5-benzimidazolyl)-2-methylalanine on catecholamine stores after tranylcypromine administration in reserpinized rats. Five groups each of six male Wistar rats (100–120 g) were used in this experiment. When indicated, reserpine (2·5 mg/kg) was administered 48 and 24 hr before sacrifice. Animals were give saline (control groups), MBA (0·3 m-mole; 87·6 mg/kg) or MT (1·0 m-mole; 195 mg/kg) 4 hr before sacrifice. When indicated, tranylcypromine sulfate (10 mg/kg was administered 3 hr before sacrifice. Values represent the mean (μg catecholamine/g of brain) ± the standard error of the mean.

strated that 88·2 ± 2·9 per cent of the recovered radioactivity was associated with a spot of the same R_f as authentic NE. Homogenates from animals treated with MBA or MBA-amine showed a nonradioactive, ultraviolet-absorbing spot with an R_f identical to that of standard MBA-amine. To establish the degree to which the reduction of brain and heart catecholamines was dependent upon MBA decarboxylation, animals were treated with 750 mg/kg of the aromatic amino acid decarboxylase inhibitor, RO-4-4602 [16], 30 min prior to receiving 87·6 mg/kg (0·3 m-mole/kg) of MBA. Three hr after the administration of MBA, the animals were sacrificed and their heart NE and brain DA and NE levels compared to those of a group of animals receiving MBA alone (Table 1). The data presented show that a 750 mg/kg dose of RO-4-4602 was completely effective in blocking the decrease of NE in the heart, but was only partially effective in blocking the decrease of catecholamines in the brain.

To establish whether inhibition of catecholamine biosynthesis occurred *in vivo*, the effect of MBA on the increase in catecholamine levels in reserpinized rats treated with a monoamine oxidase (MAO) inhibitor [1] was assessed. Tranylcypromine was used as the MAO inhibitor and MT as a control. The results of this experiment, represented in Fig. 2, indicate that the biosynthesis of brain catecholamines was effectively blocked 4 hr after the administration of 0·3 m-mole/kg of MBA.

A more direct demonstration of MBA interference with catecholamine biosynthesis by MBA was afforded by measuring tyrosine hydroxylase activity in tissues of animals sacrificed 4 and 12 hr after the administration of MBA. The results obtained (Table 2)

Table 1. Effect of the decarboxylase inhibitor RO-4-4602 on the reduction of heart and brain catecholamines by MBA*

Compounds administered	No. of animals	Heart NE	Brain DA	Brain NE
(A) Saline	6	0·63 ± 0·04	0·56 ± 0·05	0·48 ± 0·03
(B) Saline + RO-4-4602	4	0·49 ± 0·03	0·53 ± 0·06	0·41 ± 0·02
(C) Saline + MBA	4	0·29 ± 0·04	0·09 ± 0·07	0·16 ± 0·03
(D) RO-4-4602 + MBA	5	0·52 ± 0·05	0·14 ± 0·06	0·31 ± 0·02
Significance	A vs C	$P < 0·01$	$P < 0·01$	$P < 0·01$
	A vs B	$P < 0·01$	NS	$P < 0·05$
	D vs C	$P < 0·01$	NS	$P < 0·01$

* Male Wistar rats (130–160 g) were pretreated with saline or RO-4-4602 (750 mg/kg), given saline or MBA i.p. 0·5 hr later, and sacrificed 3 hr later. Each value represents mean (μg/g) ± standard error of the mean.

Table 2. Tyrosine hydroxylase activity in various tissues after MBA treatment*

Time after MBA (hr)	Brain (per g)	Superior cervical ganglia (per pair)
Control	171·7 ± 1·2	24·0 ± 1·7
4	28·0 ± 0·9	4·7 ± 0·4†
12	43·3 ± 1·3	6·1 ± 0·4†

* Male Wistar rats (130–150 g) were given either saline or MBA (0·3 m-mole/kg). At the time of sacrifice superior cervical ganglia and brain were removed under ether anesthesia. Results are expressed as the mean (nmoles Dopa formed per hr per unit of tissue) ± the standard error of the mean; there were seven animals in each group.

† $P < 0·01$ vs control group.

indicate that there was, at these times after MBA, a marked decrease in the tyrosine hydroxylase activity in homogenates of brain and superior cervical ganglia. The tyrosine hydroxylase activity of a sample consisting of an aliquot of a brain homogenate from a control rat combined with an equal aliquot from a rat given MBA 4 hr before sacrifice (Table 3) suggests that the decrease in activity seen in the animal given MBA is due to the presence of an inhibitor. Further, the decrease in inhibition (from 82 to 30 per cent) observed in the brain homogenates of MBA-treated rats when the tyrosine concentration was increased from 5×10^{-5} to $1·4 \times 10^{-4}$ M (data not shown) suggests that the inhibitor present in these homogenates is competitive.

DISCUSSION

The gross physiological effects observed after the administration of MBA (depression followed by stimulation) were analogous to those seen after the administration of reserpine, MT or BA and pointed to the disruption of biogenic amine function as the probable mode of action of MBA. The mechanism and the extent of tyrosine hydroxylase inhibition *in vitro* by BA and MBA were found to be identical [1, 2], but the degree and the duration of catecholamine depletion by 0·3 m-mole/kg of MBA were more intense and more prolonged than those seen after 1·0 m-mole/kg of BA.

Table 3. Tyrosine hydroxylase activity. Effect of mixing a sample of decreased activity with a sample of control activity*

Animals	Found	Calculated
Control 1	173·1 ± 1·3	
Control 2	172·1 ± 1·3	
Expl 1	28·1 ± 0·3	
C. 1 + C. 2	331·9 ± 2·3	345·2
C. 1 + E. 1	33·4 ± 0·7	201·2

* Brain homogenates were prepared from two control animals and from an experimental animal given 0·3 m-mole/kg MBA 4 hr before sacrifice. Tyrosine hydroxylase activity was measured in three 100-μl aliquots of each homogenate diluted with 100 μl of Tris homogenization buffer and in three 200-μl aliquots combined in equal parts from two of the homogenates. Results for triplicate samples are expressed as the mean (nmoles Dopa/hr/g tissue) ± the standard error of the mean.

After MBA, heart NE was depleted to 33 per cent of control after 8 hr and to 16 per cent of control after 12 hr [2]. Since the half-life of heart NE has been estimated to be 9 hr [17], the depletion data suggest a release component in rat heart depletion by MBA. When it was shown that MBA or MBA-amine did not prevent the uptake of ^{3}HNE in the heart but brought about the release of NE from the labeled pool (Fig. 1), the presence of a release component in the mechanism of action of administered MBA was firmly established. The release data indicate that MBA had much less but MBA-amine considerably more (about 50-fold) releasing activity than tyramine.

In order to establish that MBA does not have releasing activity of its own, its ability to deplete catecholamines was tested in the presence of the decarboxylase inhibitor, RO-4-4602. The ability of a small dose (10 mg/kg) of RO-4-4602 to prevent the release of heart norepinephrine by BA [1] is consistent with the extracerebral effectiveness reported for this compound. Since it has been established that a large dose (above 500 mg/kg) of RO-4-4602 completely or almost completely inhibits brain aromatic amino acid decarboxylase activity [18, 19], an attempt was made to see if brain as well as heart catecholamine release by MBA could be prevented in the presence of such a dose. The results obtained (Table 1) indicate that decarboxylation was essential for the release of NE from the heart, was necessary for the release of some NE from the brain, but did not affect the ability of MBA to lower brain dopamine. It may be speculated that the failure of a massive dose of decarboxylase inhibitor to affect brain DA while affecting brain NE is related to differential effects of MBA, or of a derivative thereof, in different nuclei of the brain.

Large doses of reserpine deplete catecholamines, and this depletion in turn abolishes the negative feedback effects of NE, thus increasing tyrosine hydroxylase activity and NE biosynthesis. When MAO activity is blocked in reserpinized animals, the rise in the level of catecholamines is, within limits, proportional to the activity of the catecholamine biosynthetic pathway. When a test compound (e.g. a tyrosine hydroxylase inhibitor) is administered to a reserpinized animal, the MAO activity of which is blocked, any decrease observed in the level of catecholamines represents an inhibition *in vivo* of the biosynthetic pathway. Although one could anticipate the tyrosine hydroxylase inhibitors BA, MBA and MT to be equivalent inhibitors of biosynthesis *in vivo* because of their equipotent effect on tyrosine hydroxylase *in vitro*, MBA (at 0·3 m-mole/kg) appears at least as potent as MT (Fig. 2) or BA [1] at 1·0 m-mole/kg. Further, the tyrosine hydroxylase inhibition observed in homogenates of animals 4 and 12 hr after the administration of MBA appears substantial in view of the dilution effected during the homogenization and the enzyme assay (Table 3); there was, 12 hr after the administration of 0·3 m-mole/kg of MBA, a better than 70 per cent inhibition of tyrosine hydroxylase in a superior cervical ganglion homogenate diluted 950-fold. Whether this effect is related primarily to

an increased affinity of MBA for the enzyme *in vivo* or is due to a metabolite of MBA remains to be elucidated.

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