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BIOGENIC AMINE-DEPLETING EFFECTS OF BENZIMIDAZOLE-5(6)-DL-ALANINE*

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Abstract—Studies *in vivo* on benzimidazole-5(6)-DL-alanine were carried out by administering the compound in aqueous solutions intraperitoneally to male Wistar rats. Benzimidazole alanine (278 mg/kg) decreased heart norepinephrine (80 per cent), brain norepinephrine (58 per cent) and brain dopamine (70 per cent) within 3 hr and brain serotonin (70 per cent) within 1 hr of administration. Benzimidazole ethylamine (100 mg/kg) produced a 60 per cent decrease in heart norepinephrine within 3 hr of administration and benzimidazole alanine was not able to reduce heart norepinephrine in animals pretreated with the decarboxylase inhibitor, DL-seryl-2,3,4-trihydroxybenzylhydrazine hydrochloride (Ro-4–4602 before benzimidazole alanine administration). Benzimidazole alanine, administered prior to tranylcypromine completely blocked the increase in brain norepinephrine and dopamine after monoamine oxidase inhibition and reduced the increase in brain serotonin by 65 per cent.

INHIBITORS of catecholamine and serotonin biosynthesis have proven useful in pathological conditions such as pheochromocytoma[1] and certain serotonin (5-HT) secreting carcinoid tumors.[2] It is now recognized that a variety of psychoactive drugs can evoke changes in brain amines. Hence modifiers of biogenic amine concentrations in brain are of interest as potential pharmacological agents or as potential tools in approaching the study of the relationships between brain chemistry and function.

A preliminary screening of the dihydroxyphenylalanine (dopa) analog benzimidazole-5(6)-DL-alanine (BA),[3] revealed BA as an inhibitor of bovine adrenal tyrosine hydroxylase and of rat liver phenylalanine hydroxylase, an enzyme similar in many respects to the rate-limiting step of brain serotonin biosynthesis, tryptophan hydroxylase. BA is a competitive inhibitor of tyrosine hydroxylase ($K_i = 2 \times 10^{-5}$) and was found effective in blocking the tyrosine hydroxylase obtained from a human adrenal neuroblastoma to a degree similar to that of α-methyltyrosine.[4] This report describes the effects of BA *in vivo* on biogenic amine biosynthesis and depletion.

MATERIALS AND METHODS

Compound studies in vivo. Benzimidazole-5(6)-alanine dihydrochloride (BA) was prepared as previously described.[3] Benzimidazole-5(6)-ethylamine hydrochloride (BEA) was synthesized by Dr. Jeremy Wright and will be the subject of another report.

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Tetrabenazine hydrochloride and DL-seryl-2,3,4-trihydroxybenzylhydrazine hydrochloride (Ro-4-4602) were supplied by Roche Inc., Basel, Switzerland. Tranylcypromine was supplied by Smith, Kline & French Laboratories, Philadelphia, Pa. *p*-Chlorophenylalanine (*p*CPA) and reserpine were obtained commercially from Aldrich Chemical Company.

Apparatus and reagents. Male Wistar rats were sacrificed with a guillotine (Harvard Instrument Company). 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 (norepinephrine, dopamine) or in 0·5 ml quartz tubes (serotonin). All spectra were recorded on an Aminco model 814 XY recorder and were uncorrected. All reagents and solvents (spectral grade) were obtained commercially. Glass-distilled water was used throughout.

Procedure for biogenic amine assay. 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/l.) and the homogenate was centrifuged. A 2·5 ml aliquot of the supernatant was added to 5 ml heptane and 0·4 ml 0·1 N HCl and extracted. After centrifugation to separate the phases, the organic phase and the tissue plug were removed by aspiration. A 0·05 ml aliquot of the aqueous phase was condensed with *o*-phthaldialdehyde for the determination of serotonin (5-HT) as described by Maickel *et al.*[5] Norepinephrine (NE) and dopamine (DA) were separated from another 0·2 ml aliquot of the aqueous phase by alumina adsorption as described by Chang.[6] NE was determined by the trihydroxyindole procedure[6] on an aliquot of the alumina eluate which was then adjusted to pH 4·0[7] 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.*[5]

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

RESULTS

The results of a time course study of the effects of BA on brain NE and 5-HT, and on heart NE when administered at a dose of 278 mg/(m-mole)/kg, i.p., to male Wistar rats are shown in Fig. 1. Brain 5-HT reached a maximal depletion (70 per cent) within 1 hr of administration, remained at this level until 3 hr after BA administration and returned to control levels within 24 hr. Brain NE levels were reduced 40 per cent within 1 hr and reached maximal depletion (58 per cent) 3 hr after BA administration. Brain NE also returned to control levels within 24 hr. Brain DA showed a similar pattern, being reduced 70 per cent within 3 hr (data not shown). Heart NE decreased 45 per cent in 1 hr and reached a maximal depletion of 80 per cent 3 hr after BA administration. Heart NE remained at this level until 6 hr after BA administration and also returned to control levels within 24 hr.

The relationship between the dose of BA administered and the depletion of biogenic amines 3 hr after administration of a given i.p. dose of the compound is shown in Fig. 2. The relationship between the doses of BA administered and the degree of

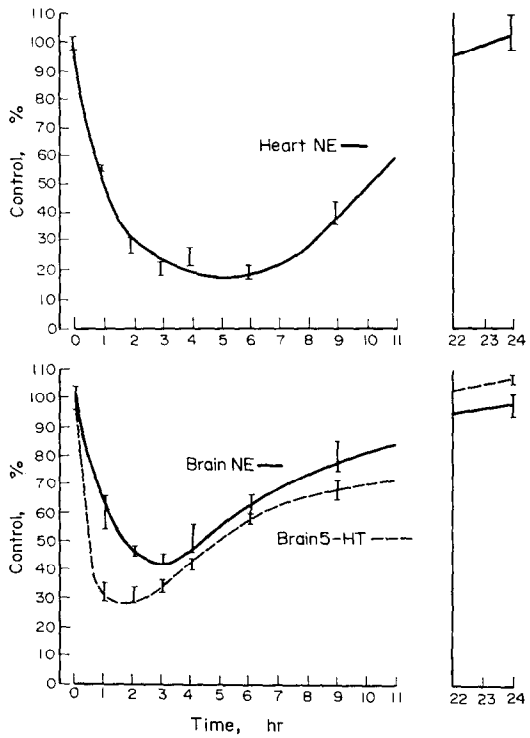

FIG. 1. Effect of 1 mmole (278 mg)/kg BA, i.p., on tissue NE and 5-HT. Each point represents the mean ± S.E.M. of three to nine rats;

amine depletion indicated a "leveling off" of the response to BA in brain above 84 mg (0·3 m-mole)/kg, which is more marked than that observed in the heart and may be due to a saturation of blood–brain transport mechanisms. The rapid decrease observed in heart NE cannot be explained solely on the basis of inhibition of biosynthesis because of the relatively slow turnover of NE in that tissue.[9] This suggests that BA or a metabolite of BA acts at the storage granules to cause a release of biogenic amine. The rapid decrease in brain 5-HT and in brain NE suggests that release occurs to some extent in that tissue. The biogenic amines released from the storage granule would be rapidly metabolized by monoamine oxidase or catechol-*O*-methyl transferase or removed into the circulation, resulting in decreases in tissue levels of amines.

The ability of a given compound to protect against reserpine-induced depletion of biogenic amines has been ascribed to occupation of the site of the granule at which reserpine acts.[8] Protection against reserpine depletion has been demonstrated for tetrabenazine (TB),[8] which brings about a lowering of brain biogenic amines by reversibly disrupting the storage granule, and for aromatic amino acids such as *m*-tyrosine[9] and α-methyl-5-hydroxytryptophan,[10] which displace biogenic amines by way of the decarboxylation products *in vivo*, *m*-tyramine and α-methylserotonin.

The results obtained by pretreating a group of animals with BA (278 mg/kg, i.p.) or with tetrabenazine (50 mg/kg, i.p.) 15 min prior to the administration of reserpine and 24 hr before sacrifice are shown in Table 1. Tetrabenazine effectively protected

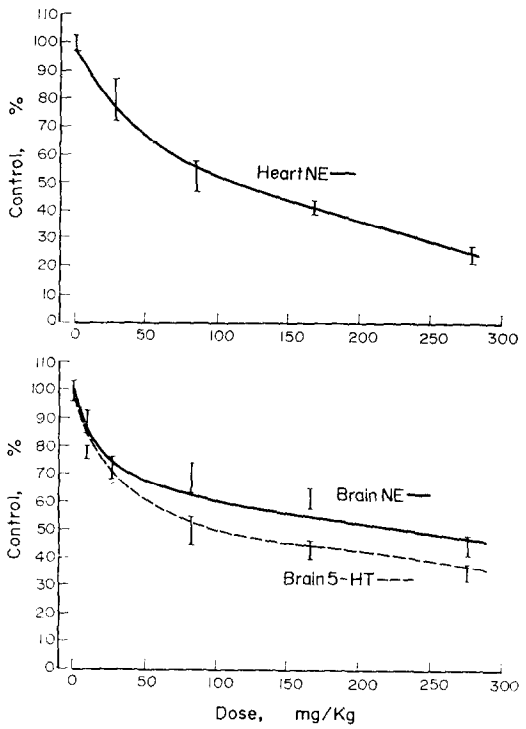

FIG. 2. Effect of varying doses of BA on tissue NE and 5-HT 3 hr after i.p. administration. Each point represents the mean ± S.E.M. of three to nine animals.

TABLE 1. EFFECT OF BENZIMIDAZOLE ALANINE AND TETRABENAZINE PRETREATMENT ON THE DEPLETION OF BIOGENIC AMINES BY RESERPINE*

Compounds administered	Brain NE	Brain DA	Brain 5-HT	Heart NE
(A) Saline	0·315 ± 0·03 (3)	0·52 ± 0·04 (3)	0·47 ± 0·04 (3)	0·50 ± 0·06 (3)
(B) Saline + reserpine	0·14 ± 0·01 (4)	0·32 ± 0·02 (4)	0·295 ± 0·01 (4)	0·06 ± 0·01 (4)
(C) TB + reserpine	0·25 ± 0·01 (4)	0·45 ± 0·03 (4)	0·45 ± 0·04 (4)	0·31 ± 0·04 (4)
(D) BA + reserpine	0·14 ± 0·01 (3)	0·35 ± 0·02 (3)	0·38 ± 0·02 (3)	0·08 ± 0·02 (4)
Significance B vs. C	$P < 0·01$	$P < 0·05$	$P < 0·01$	$P < 0·01$
Significance B vs. D	P NS	P NS	$P < 0·01$	P NS

* Male Wistar rats (90–110 g) were pretreated with TB (50 mg/kg, i.p.) or BA (278 mg/kg, i.p.) 15 min prior to the administration of reserpine (1 mg/kg) and 24 hr before sacrifice. Tissue levels are expressed as micrograms per gram ± S.E.M. The number in parentheses is the number of animals in the group. NS = not significant. See text for other abbreviations.

all of the amine stores from reserpine depletion, whereas BA protected only the brain 5-HT stores. The selective protection afforded by BA to a single amine store is not inconsistent with observations by other authors.[9, 10]

The decarboxylation products of α-methyl-dopa and of α-methyl-5-hydroxytryptophan have been shown to release catecholamines *in vivo*.[11, 12] The ability of BA

to release biogenic amines from storage granules may be caused by its decarboxylation product, BEA.

BEA produced a 60 per cent decrease in heart NE 3 hr after its administration. No decrease in brain 5-HT and only a slight decrease in brain NE were observed, indicating that BEA probably does not readily cross the blood–brain barrier. The data of Table 2 suggest that the NE depletion which follows BA administration is due, to some degree (in the brain) or mostly (in the heart), to BEA or to the β-hydroxylated metabolite of BEA. The weak activity of BEA as an inhibitor of tyrosine hydroxylase (21 per cent inhibition at 10^{-4} M) argues against inhibition *in vivo* of NE biosynthesis by BEA.

TABLE 2. EFFECT OF BENZIMIDAZOLE ETHYLAMINE ON BIOGENIC AMINES*

Compound	Brain NE	Brain 5-HT	Heart NE
(A) Saline (5)	0·42 ± 0·03	0·52 ± 0·04	0·56 ± 0·05
(B) BEA (4)	0·31 ± 0·02	0·58 ± 0·02	0·23 ± 0·02
Significance A vs. B	P < 0·05	P NS	P < 0·01

* Male Wistar rats (90–110 g) were injected i.p. with saline or BEA (100 mg/kg) as an aqueous solution and sacrificed 3 hr later. Tissue levels are expressed as micrograms per gram ± S.E.M. The number in parentheses is the number of animals per group. NS = not significant. See text for other abbreviations.

In order to determine whether the decrease in heart NE after BA administration could be explained solely on the basis of a releasing mechanism by BEA, animals were treated with the decarboxylase inhibitors Ro-4–4602 at a dose of 10 mg/kg, i.p., 30 min prior to BA injection (100 mg/kg, i.p.) and their heart NE levels compared to those of a group given the same dose of BA but no Ro-4–4602 (Table 3). The decarboxylase inhibitor blocked the decrease in heart NE, indicating that, in the heart at least, most or all of the activity of BA *in vivo* was due to BEA.

TABLE 3. EFFECT OF PRETREATMENT WITH A DECARBOXYLASE INHIBITOR (Ro-4–4602) ON THE REDUCTION OF HEART NOREPINEPHRINE BY BENZIMIDAZOLE ALANINE*

Compounds administered	Heart NE
(A) Saline	0·68 ± 0·04
(B) Ro-4–4602 + saline	0·56 ± 0·02
(C) Saline + BA	0·29 ± 0·02
(D) Ro-4–4602 + BA	0·55 ± 0·06
Significance C vs. D	P < 0·01

* Male Wistar rats (120–150 g) were pretreated 30 min prior to administration of BA (100 mg/kg, i.p.) with Ro-4–4602 (10 mg/kg, i.p.) and sacrificed 3 hr later. Each value represents the mean (micrograms per gram) ± S.E.M. of tissue for four animals. See text for abbreviations.

The activity demonstrated by BEA presented the possibility that all the effects of BA *in vivo* were due to a releasing mechanism and that neither tyrosine hydroxylase nor tryptophan hydroxylase inhibition was occurring *in vivo*. Since monoamine oxidase (MAO) represents the primary route of metabolism of catecholamines and 5-HT in brain,[13] inhibition of this enzyme leads to increases in brain levels of amines as long as the biosynthetic machinery is not disrupted. Disruption of amine storage function with high (2·5 mg/kg daily for 3 days) doses of reserpine does not lessen the rise in brain biogenic amines after MAO inhibition.[14] The effect of BA (278 mg/kg i.p.) pretreatment 15 min prior to the administration of the MAO inhibitor tranylcypromine (10 mg/kg, i.p.) and 3 hr prior to sacrifice on the increase in biogenic amines in reserpinized rats is shown in Table 4. BA completely inhibited the biosynthesis of brain NE and DA and reduced the increase in brain 5-HT by 65 per cent.

TABLE 4. EFFECT OF BENZIMIDAZOLE ALANINE ON THE INCREASE IN BIOGENIC AMINE STORES AFTER TRANYLCYPROMINE ADMINISTRATION IN RESERPINIZED RATS*

Compounds administered	Brain 5-HT	Brain NE	Brain DA
(A) Saline (4)	0·40 ± 0·03	0·12 ± 0·04	0·42 ± 0·10
(B) Saline + tranylcypromine (5)	0·94 ± 0·09	0·254 ± 0·04	0·81 ± 0·14
(C) BA + tranylcypromine (5)	0·60 ± 0·07	0·08 ± 0·01	0·26 ± 0·07
Significance C vs. B	$P < 0·05$	$P < 0·01$	$P < 0·01$

* Male Wistar rats (130–160 g) were given reserpine (1·5 mg/kg, i.p.) 24 hr before and BA (278 mg/kg, i.p.) 15 min before tranylcypromine (10 mg/kg, i.p.) and sacrificed 3 hr later. Values represent the mean (micrograms per gram) ± S.E.M. The number in parentheses is the number of animals per group. See text for abbreviations.

DISCUSSION

Based upon studies of BA action *in vitro*[3, 4] and *in vivo*, the mechanism of the depleting effect of BA on brain NE and DA and on heart NE appears to be two-fold. It acts as a potent tyrosine hydroxylase inhibitor, completely inhibiting biosynthesis at doses comparable to those of α-methyltyrosine required to prevent NE formation. In addition, the product of BA decarboxylation, BEA (or its β-hydroxylated metabolite), acts at the storage granule inducing the release of biogenic amine either by a reversible disruption or by a stoichiometric displacement mechanism. This releasing activity is clearly demonstrated by the rapid disappearance in heart (first-order rate constant = approx. 0·62 hr^{-1}), which is ten times the rate (0·05 hr^{-1}) obtained after inhibition of biosynthesis by α-methyltyrosine in rats.[15]

The mechanism by which BA reduces brain 5-HT levels also is likely to be two-fold. First, a degree of inhibition of synthesis *in vivo* is indicated by the reduction of the 5-HT increase after administration of BA and MAO inhibitor. The inhibition of biosynthesis is consistent with the ability of BA to inhibit rat liver phenylalanine hydroxylase,[3] an enzyme very similar to brainstem tryptophan hydroxylase in requirements for O_2, cofactor ferrous ion, and susceptibility to inhibition.[16]

Second, interaction of BA with the storage granules is indicated by the protection it

affords reserpine-induced depletion and by the very rapid decrease in brain 5-HT after BA administration.

The data in this study do not permit a conclusion as to whether the release of biogenic amines caused by BEA is due to a reversible disruption of storage granules or to a stoichiometric displacement mechanism. Because of the lack of specificity of the biogenic amine storage granule,[13] a stoichiometric displacement mechanism would seem more likely.

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REFERENCES

1. K. ENGLEMAN and A. SJOERDSMA, *Circulat. Res.* **18,** suppl., 101 (1966).
2. K. ENGLEMAN, W. LOVENBERG and A. SJOERDSMA, *New Eng. J. Med.* **21,** 1103 (1967).
3. J. D. MILKOWSKI, F. M. MILLER, E. M. JOHNSON, JR. and N. ZENKER, *J. med. Chem.* **13,** 740 (1970).
4. S. IMASHUKU, E. H. LABROSSE, E. M. JOHNSON, JR., V. H. MORGENROTH, III and N. ZENKER, *Biochem. Med.* **5,** 32 (1971).
5. R. P. MAICKEL, R. H. COX, J. SAILLANT and F. P. MILLER, *Int. J. Neuropharmac.* **7,** 275 (1968).
6. C. C. CHANG *Int. J. Neuropharmac.* **3,** 643 (1964).,
7. A. S. WELCH and B. L. WELCH, *Analyt. Biochem.* **30,** 161 (1969).
8. G. P. QUINN, P. A. SHORE and B. B. BRODIE, *J. Pharmac. exp. Ther.* **127,** 103 (1960).
9. A. CARLSSON and M. LINDQUIST, *Eur. J. Pharmac.* **2,** 187 (1967).
10. R. A. LAHTI and P. A. PLATZ, *Biochem. Pharmac.* **18,** 2363 (1969).
11. I. J. KOPIN, *A. Rev. Pharmac.* **8,** 377 (1968).
12. G. A. JOHNSON, R. A. LAHTI, T. L. LEMKE and R. V. HEINZELMAN, *Biochem. Pharmac.* **18,** 1593 (1969).
13. F. E. BLOOM and N. J. GIARMAN, *A. Rev. Pharmac.* **8,** 229 (1968).
14. B. K. KOE and A. WEISSMAN, *J. Pharmac. exp. Ther.* **154,** 499 (1966).
15. B. B. BRODIE, E. COSTA, A. DLABAC, N. H. NEFF and H. H. SMOOKLER, *J. Pharmac. exp. Ther.* **154,** 493 (1966).
16. E. JEQUIER, D. S. ROBINSON, W. LOVENBERG and A. SJOERDSMA, *Biochem. Pharmac.* **18,** 1071 (1969).