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STUDIES ON THE INHIBITION OF HISTIDINE DECARBOXYLASE, AROMATIC-L-AMINO ACID DECARBOXYLASE AND ACID SECRETION BY BROCRESINE AND ITS METABOLITES*

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Abstract—Brocresine (NSD 1055) is rapidly metabolized *in vivo*. The probable metabolites are 4-bromo-3-hydroxy-benzyl alcohol, 4-bromo-3-hydroxy-benzoic acid and 4-bromo-3-hydroxy-hippuric acid. Brocresine and these metabolites inhibit both rat fetal and rat gastric histidine decarboxylase (L-histidine carboxylase, EC 4.1.1.22) *in vitro* with a molar I_{50} of about 10^{-8}, 10^{-4}, 10^{-3} and 10^{-5}, respectively, for both enzymes. Brocresine and the metabolites also inhibit aromatic-L-amino acid decarboxylase (3,4-dihydroxy-L-phenylalanine carboxylase EC 4.1.1.26) from hog kidney and rat gastric mucosa *in vitro* with a molar I_{50} of about 10^{-7}, 10^{-4}, 10^{-3} and 10^{-3}, respectively, for both enzymes. Brocresine, the alcohol metabolite and the acid metabolite inhibited rat gastric histidine decarboxylase after intraperitoneal administration of 200 mg/kg, whereas the hippurate was only weakly inhibitory. All four compounds inhibited gastric acid secretion in the pylorus-ligated rat, but the acid and hippurate were only moderately inhibitory. The reaction of hemoglobin with brocresine to form methemoglobin readily explains the rapid disappearance of the inhibitory activity of the drug.

STUDIES ON histidine decarboxylase and on the physiological importance of histamine have been facilitated by the recent development of more sensitive techniques for measuring histidine decarboxylase activity, and by the discovery of inhibitors of this enzyme. Reid and Shepherd[1] reported that 4-bromo-3-hydroxybenzyloxamine dihydrogen phosphate (brocresine or NSD 1055) was a potent inhibitor of both the specific histidine decarboxylase (L-histidine-carboxylyase, EC 4.1.1.22) and aromatic-L-amino acid decarboxylase, also referred to as dopa decarboxylase (EC 4.1.1.26). These findings were confirmed by Aures and Clark,[2] and by Levine *et al.*[3] The latter investigators also showed that the administration of brocresine to rats resulted in a decrease in the levels of histamine in heart, stomach and urine. Oral administration of brocresine to normal humans and patients with systemic mastocytosis resulted in inhibition of histamine synthesis.[4]

Recent studies indicate some disagreement on the effectiveness of brocresine *in vivo*. Johnston and Kahlson[5] and Mesch and Sewing[6] did not find it to be a potent inhibitor

* A preliminary report of the results of this investigation was presented at the 62nd Annual Meeting of the American Society of Biological Chemists in June, 1971 (*Fedn Proc.* **30**, 1146, 1971).

of histamine formation *in vivo*. In contrast to the studies of Levine[7] and Thayer and Martin,[8] Becker and Sewing[9] found that gastric acid secretion in rats was not effectively inhibited by brocresine.

Studies by Wustrack and Levine,[10] Kobayashi *et al.*[11] and our laboratory[12] have shown that the inhibitory activities of brocresine are transient. This rapid disappearance of inhibitory activity is probably due to metabolism of brocresine *in vivo*;[12] the reaction products have been identified and the schematic representation of the metabolic route is shown in Fig. 1. The details of the isolation and characterization of the metabolic products will be the subject of a separate report. The present communication is concerned with the inhibition of histidine decarboxylase, aromatic-L-amino acid decarboxylase and gastric acid secretion by the alcohol, acid and hippurate metabolites (I, II and III, respectively) of brocresine.

MATERIALS AND METHODS

Materials

L-Histidine-^{14}COOH (4·0–9·1 mCi/m-mole) obtained from CalBiochem and New England Nuclear Corp., was the source of the DL-dopa-^{14}COOH (18 mCi/m-mole). Pyridoxal phosphate and nonradioactive DL-dopa were obtained from Nutritional Biochemicals Corp., and nonradioactive-L-histidine was the product of General Biochemicals Corp. The metabolites were synthesized by the Process and Analytical Research Section of Lederle Laboratories.

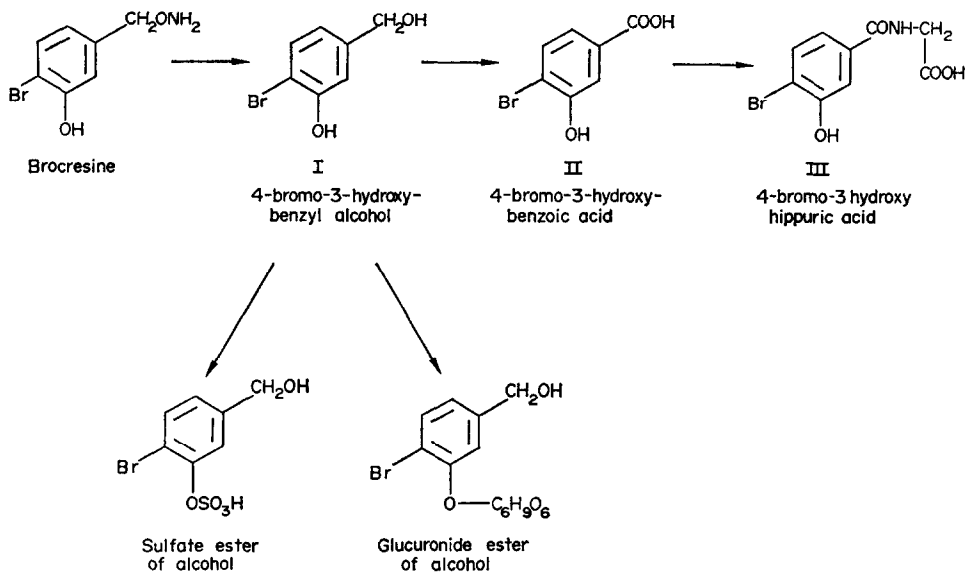

FIG. 1. Metabolism of brocresine.

Animals

Whole fetuses (19–20 days gestation) were obtained from pregnant CFE (Carworth Farms) rats. The pregnant rats were killed by decapitation, and the fetuses, free of any

placental tissue, rapidly removed and frozen in dry ice. In a few experiments, whole rat fetuses were obtained from Pel-Freez Biologicals, Inc., Rogers, Ark.

Adult male CFE (Carworth Farms) rats (200–300 g) were the source of our rat-stomach enzyme preparations.

Freshly frozen whole hog kidneys were obtained from a local slaughterhouse.

Methods

Histidine decarboxylase. Rat fetal histidine decarboxylase was prepared by the Levine and Watts[13] modification of the method of Håkanson,[14] using acetate buffer at pH 5·5 instead of pH 4·5. The supernatant II fractions were used. Rat gastric histidine decarboxylase was prepared as previously described.[15]

Dopa decarboxylase. For the preparation of kidney dopa decarboxylase, hog kidneys were minced and then homogenized at 0° with 2 vol. of 0·005 M phosphate buffer, pH 7·2, containing 0·01 M mercaptoethanol. The homogenate was centrifuged at 30,000 *g* for 30 min. The precipitate was discarded and an equal volume of saturated ammonium sulfate was added slowly to the supernatant with stirring at 0°. The solution was allowed to stand overnight and it was then centrifuged at 16,000 *g* for 30 min. The supernatant was discarded and the precipitate was resuspended in cold 0·01 M phosphate buffer, pH 7·0, and dialyzed against the same buffer at 0°. The dialysate was used as the source of the enzyme.

Rat gastric dopa decarboxylase was prepared from rat pylorus. The tissue was homogenized with 3 vol. of cold saline and centrifuged at 35,000 *g* for 1 hr. The resulting supernatant was used.

Enzyme assays. Fetal histidine decarboxylase was assayed by the procedure of Ellenbogen *et al.*[16] except that the radioactivity was assayed in a methanol toluene phosphor as previously described.[12] Rat gastric histidine decarboxylase was assayed as described.[15]

Dopa decarboxylase was assayed by the same techniques used in the assay of histidine decarboxylase except that the substrate used was DL-dihydroxyphenyl-alanine-carboxyl-^{14}C (DL-dopa-^{14}COOH). The incubation mixture for the assay of kidney dopa decarboxylase contained in a final volume of 2·0 ml (in order of addition) water, 0·4 ml of 0·1 M phosphate buffer, pH 6·8, 0·2 ml of $3{\cdot}7 \times 10^{-4}$ M pyridoxal-5-phosphate (PLP), 1·0 ml (2–3 mg) of 1:10 dilution of enzyme preparation, inhibitor, if any, and 0·1 ml of 1×10^{-2} M labeled dopa (0·5 μCi). The mixture was incubated for 30 min and the CO_2 was collected for 30 min after stopping the reaction. The conditions for assaying rat pylorus dopa decarboxylase were similar to those of the hog kidney assay except that 0·4 ml of PLP, which was found to be optimal, was used.

Measurement of gastric acid secretion. The method was based on the procedure of Shay *et al.*[17] Male CFE (Carworth Farms) rats (200–250 g) were fasted for 48 hr prior to the experiment. During this time they were fed *ad lib.* a solution of 8% sucrose and 0·2% NaCl. On the day of the experiment, the test compounds were administered by intraperitoneal injection. Saline, the vehicle for the compounds, was used in the control animals.

Exactly 1 hr after injection, the rats were lightly anesthetized with ether, the pylorus was exposed by a midline laparotomy and tied off with ordinary white cord. The incision was then closed with "Autoclips", after which the rats were placed in individual cages and allowed to recover. Exactly 3 hr after surgery, the rats were sacrificed

by decapitation. The incision was reopened, the esophagus clamped at the cardiac sphincter and the entire stomach excised. The stomach was cut open and the contents were allowed to drain into a 12-ml centrifuge tube. The gastric mucosa was washed with 2 ml of saline and the wash added to the contents.

The contents were centrifuged, the supernatant fluid was poured off and total acid in the supernatant determined by titration with dilute NaOH.

Determination of protein. Protein determinations were made by the improved phenol procedure of Lowry *et al.*[18]

Determination of methemoglobin formation. Methemoglobin formation was determined by measurement of its spectrum in a Cary model 14 recording spectrophotometer.

RESULTS

Studies in vitro *with histidine decarboxylase.* The three major metabolites of brocresine were studied for their effects on fetal rat and adult rat gastric histidine decarboxylase activity. The alcohol, acid and hippurate metabolites each inhibited histidine decarboxylase activity prepared from both sources (Table 1). The extent of inhibition was markedly less than observed with brocresine. Although PLP stimulated the activity of the enzymes about 3-fold, the inhibition was also demonstrated in the absence of added cofactor. The magnitude of inhibition of each compound was about the same with each of the two sources of enzyme.

TABLE 1. INHIBITION OF HISTIDINE DECARBOXYLASE *in vitro* BY BROCRESINE AND ITS METABOLITES*

Source of enzyme	Molar I_{50}			
	Brocresine	Alcohol metabolite	Acid metabolite	Hippurate mstabolite
Fetal rat				
+ PLP	1×10^{-8}	4×10^{-4}	2×10^{-3}	5×10^{-6}
− PLP	1×10^{-8}	3×10^{-4}	3×10^{-3}	5×10^{-6}
Rat gastric				
+ PLP	1×10^{-8}	4×10^{-4}	3×10^{-3}	4×10^{-5}
− PLP	1×10^{-7}	1×10^{-3}	1×10^{-3}	2×10^{-5}

* The molar I_{50} values were obtained from a plot of the points obtained from the mean of the duplicate analyses of at least three inhibitor concentrations. See text for other details of assay.

Preliminary kinetic studies with the alcohol and acid indicate that these metabolites, as well as brocresine, are competitive with substrate. Similar studies using a Lineweaver–Burk plot of PLP vs activity indicate that the curve obtained in the presence of brocresine or the alcohol or acid metabolites is nonlinear. The reason for the failure to obey Michaelis–Menten kinetics is unknown but marked inhibition of the fetal enzymes in the absence of any inhibitor is observed with high concentrations of PLP (10^{-3}–10^{-4} M).

Studies in vivo *with histidine decarboxylase.* Brocresine and the three metabolites were administered intraperitoneally at a dose of 200 mg/kg to adult male Carworth Farms rats. The rats were sacrificed 30, 60 or 90 min later and their stomachs assayed for histidine decarboxylase activity. As can be seen in Fig. 2, in the presence of PLP in the assay, optimum inhibition by brocresine was observed at 30 and 60 min after its administration. Maximal inhibition was observed at 60 and 90 min after dosage of the alcohol and acid metabolites, respectively (Figs. 3 and 4). Although inhibition was also observed in the absence of PLP, the inhibition was not as marked. No curve for the hippurate is shown since it did not inhibit histidine decarboxylase *in vivo*.

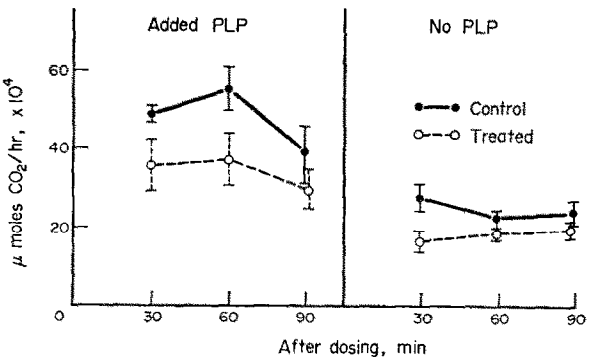

FIG. 2. Effect of brocresine on gastric histidine decarboxylase *in vivo*. Male Carworth Farms rats (nonfasted), five per group, received 200 mg/kg of brocresine or saline (i.p.). Starting 30, 60 and 90 min later, the animals were sacrificed by decapitation and gastric enzyme was prepared and assayed in the absence or addition of $3{\cdot}7 \times 10^{-5}$ M PLP.

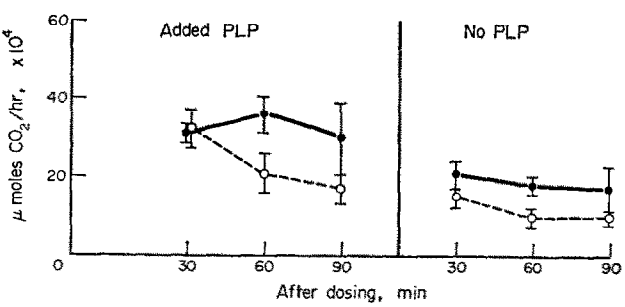

FIG. 3. Effect of alcohol metabolite on gastric histidine decarboxylase *in vivo*. Male Carworth Farms rats (nonfasted), five per group, received 200 mg/kg of alcohol metabolite or saline (i.p.). Starting 30, 60 and 90 min later, the animals were sacrificed by decapitation and gastric enzyme was prepared and assayed in the absence or addition of $3{\cdot}7 \times 10^{-5}$ M PLP.

The results of a dose–response study on the inhibition of gastric histidine decarboxylase *in vivo* are shown in Table 2. The inhibition by the alcohol and acid is somewhat less than that by brocresine, and the hippurate was not inhibitory at any of the dose levels.

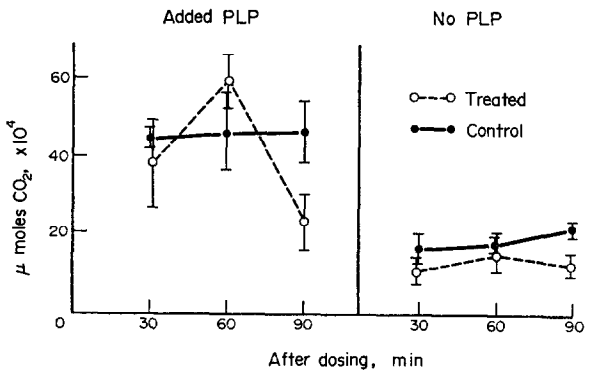

FIG. 4. Effect of acid metabolite on gastric histidine decarboxylase *in vivo*. Male Carworth Farms rats (nonfasted), five per group, received 200 mg/kg of acid metabolite or saline (i.p.). Starting 30, 60 and 90 min later, the animals were sacrificed by decapitation and gastric enzyme was prepared and assayed in the absence or addition of $3{\cdot}7 \times 10^{-5}$ M PLP.

TABLE 2. INHIBITION OF RAT GASTRIC HISTIDINE DECARBOXYLASE*

Dose (mg/kg)	Inhibition (%)			
	Brocresine	Alcohol metabolite	Acid metabolite	Hippurate metabolite
100	37 ± 12	0	0	0
200	53 ± 8	38 ± 17	35 ± 10	0
400	76 ± 6	50 ± 5	38 ± 3	0

* Male Carworth Farms rats (nonfasted), six per group, received drug or saline intraperitoneally. The animals were sacrificed by decapitation 60 min after the dosing. The animals receiving the acid metabolite were sacrificed 90 min after the dosing. The gastric enzyme was prepared and assayed for histidine decarboxylase as described in the Methods section. The values represent average ± S. E. M.

The studies of Wustrack and Levine[10] and Ellenbogen *et al.*[12] suggest an additional method for assaying the histidine decarboxylase inhibitory activity of a drug. Accordingly, the inhibitory activity of rat plasma was determined after dosage of brocresine or its metabolites. As can be seen in Table 3, the inhibitory activity rapidly disappears. The inhibitory activity at 60 min is less than half of that observed 15 min after oral dosing. The inhibitory activity in plasma reaches a peak at 30 min after administration of the alcohol and acid metabolites. Significant inhibitory activity was observed 15 min following oral administration of the hippurate but not after this.

Studies in vitro *with dopa decarboxylase.* Brocresine and the three metabolites were studied for inhibition *in vitro* of rat gastric and hog kidney aromatic-L-amino acid decarboxylase. The results are shown in Table 4.

Each of the four compounds inhibited both enzymes to the same extent. The relative inhibition of aromatic-L-amino acid decarboxylase by brocresine and the alcohol and acid metabolites was approximately the same as was observed with fetal and rat gastric

histidine decarboxylase enzyme, whereas the hippurate inhibited the aromatic-L-amino acid decarboxylase about 100-fold less than it inhibited fetal and rat gastric histidine decarboxylase.

TABLE 3. HISTIDINE DECARBOXYLASE INHIBITORY ACTIVITY OF RAT PLASMA AFTER ORAL DOSING*

Time (min)	Inhibition (%)			
	Brocresine	Alcohol metabolite	Acid metabolite	Hippurate metabolite
15	75 ± 3	5 ± 5	15 ± 5	25 ± 6
30	39 ± 10	16 ± 5	32 ± 10	0
60	32 ± 4	7 ± 2	4 ± 2	0

* Male Carworth Farms rats, six per group, were orally dosed with compound (60 mg/kg). The rats were exsanguinated at 15, 30 and 60 min and the 0·1 ml of plasma was assayed for histidine decarboxylase inhibitory activity as previously described.[12] The plasma of rats orally intubated with water and vehicle for brocresine served as control for each of the time periods. The values represent average ± S. E. M.

TABLE 4. INHIBITION OF AROMATIC-L-AMINO ACID DECARBOXYLASE *in vitro* BY BROCRESINE AND ITS METABOLITES

Source of enzyme	Molar I_{50}			
	Brocresine	Alcohol metabolite	Acid metabolite	Hippurate metabolite
Hog kidney				
+ PLP	5×10^{-7}	5×10^{-4}	5×10^{-4}	1×10^{-3}
− PLP	1×10^{-6}	1×10^{-3}	1×10^{-3}	5×10^{-3}
Rat gastric				
+ PLP	5×10^{-7}	5×10^{-4}	5×10^{-4}	1×10^{-3}
− PLP	1×10^{-6}	1×10^{-3}	1×10^{-3}	5×10^{-3}

* The molar I_{50} values were obtained from a plot of the points obtained from the mean of duplicate analyses of at least three inhibitor concentrations. See text for details of assay.

Effect on gastric secretion. All four compounds were studied for inhibition of gastric acid secretion in the Shay pylorus-ligated rat. The results are shown in Table 5. Marked inhibition of gastric acid secretion was observed with the alcohol and acid metabolites as well as with brocresine, whereas the hippurate displayed only a slight inhibition at a high dose of 400 mg/kg. The alcohol appeared to be as potent as brocresine. None of the compounds inhibited gastric acid secretion when given orally instead of intraperitoneally.

Reaction of brocresine with hemoglobin. Freshly hemolyzed dog blood (0·1 ml) was mixed with 0·1 ml of 1×10^{-4} M solution of brocresine and the visible spectrum was measured. The hemoglobin spectrum was immediately transformed to that of methemoglobin with its typical peak at 630 mμ. Use of the alcohol or the sodium salt of the acid

metabolite at concentrations as high as 0·1 M did not result in the formation of the methemoglobin. The formation of methemoglobin suggests that the overall reaction may be oxidation of hemoglobin accompanied by reduction of brocresine (Fig. 5).

TABLE 5. INHIBITION OF ACID SECRETION (PYLORUS LIGATED RAT)*

Dose (mg/kg)	Inhibition (%)			
	Brocresine	Alcohol metabolite	Acid metabolite	Hippurate metabolite
50		18 ± 4		
100	44 ± 8	26 ± 5	9 ± 12	0
200	88 ± 2	91 ± 11	9 ± 7	0
400	86 ± 4		60 ± 6	25 ± 9

* Male CFE (Carworth Farms) rats were administered compound by intraperitoneal injection. Saline vehicle for the compounds was used in the controls. Total gastric acid was determined as described in the Methods section. Values represent average ± S. E. M. of six animals per group.

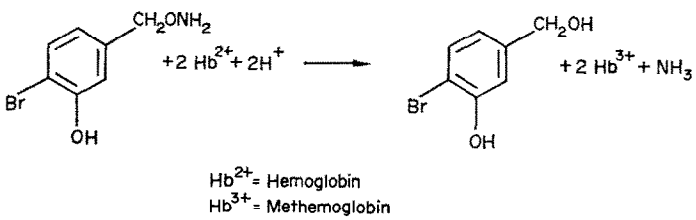

FIG. 5. Reaction of hemoglobin with brocresine.

In order to determine the nature of the breakdown products, brocresine was added to whole blood and the reaction permitted to proceed 2 hr. The sample was extracted with nitromethane, acetone was added to stabilize any remaining brocresine and the extract was submitted to partition chromatography on celite. The degradation product obtained was 4-bromo-3-hydroxy-benzyl alcohol as determined by mass spectroscopy.

DISCUSSION

In contrast to the results of Johnston and Kahlson[5] and Mesch and Sewing,[6] our data demonstrate that brocresine effectively inhibits histidine decarboxylase *in vivo* and are in agreement with the findings of Levine *et al.*[3] The reported lack of inhibition can readily be explained on the basis of the time elapsed before examination of the drug. Johnston actually observed a moderate inhibition of histidine decarboxylase after injection of brocresine three times daily. Brocresine is also a potent inhibitor of rat pyloric and hog kidney dopa decarboxylase. The inhibition of dopa decarboxylase *in vivo* in mice has been previously reported.[19]

Brocresine is rapidly metabolized and the decline in histidine decarboxylase inhibitory activity in blood has been demonstrated by Wustrack and Levine[10] and Ellenbogen *et al.*[12] Studies carried out *in vitro* indicate that brocresine preincubated with blood prior to assay does not retain its inhibitory properties. This fact suggested that the inhibitory properties of brocresine might be due to one or more of its metabolites. The reaction of hemoglobin with brocresine to form methemoglobin readily explains the reason for the rapid disappearance of the inhibitory activity of the drug.

Although the alcohol and acid metabolites inhibit histidine decarboxylase *in vitro* and *in vivo*, they are considerably less potent than brocresine. Since the activity of brocresine is more than 1000-times greater than the alcohol and acid, one cannot rule out that traces of brocresine which might remain could be responsible for the inhibition of histidine or dopa decarboxylase.

It has been established that pyridoxal or pyridoxal phosphate forms an oxime complex with brocresine.[20] Brocresine could exert its inhibitory effect after formation of an oxime with pyridoxal or pyridoxal phosphate. However, it has been shown that these oximes are less inhibitory than brocresine *in vitro*, and in preliminary studies we have found them to be less inhibitory *in vivo* (inhibition of gastric histidine decarboxylase and gastric secretion). It is therefore more likely that the rapid disappearance of brocresine histidine decarboxylase inhibitory activity is a result of the reaction of brocresine with blood and/or the metabolism of brocresine *in vivo*.

The potent gastric inhibition by the alcohol metabolite also explains the fact that not all pharmacological activity disappears or diminishes so quickly. The effects of brocresine on gastric secretion are in agreement with Levine[7] and Thayer and Martin.[8]

The conversion of brocresine to its alcohol metabolite with a subsequent diminished inhibitory activity may be of consequence in the assay of tyrosine hydroxylase under certain conditions. Brocresine, often, is added to inhibit the decarboxylation of dihydroxyphenylalanine in this assay when tritiated tyrosine is used as the substrate.

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