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STIMULATORY EFFECT OF RESERPINE ON MONOAMINE OXIDASE IN GUINEA PIG HEART

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Abstract—(1) In slices, homogenates or mitochondrial fractions of the hearts of guinea pigs after reserpine treatment, the amounts of deaminated metabolites of ^{14}C-norepinephrine were more than in the controls. This seems to be because heart mitochondrial monoamine oxidase (MAO) is activated by reserpine. So far activation of MAO by reserpine could only be demonstrated *in vivo*, or in slices *in vitro*.

(2) When ^{14}C-dopamine or ^{14}C-tyramine, better substrates for mitochondrial MAO than norepinephrine, were used as substrates for MAO activity, there was little increase in their deamination after reserpine-treatment.

(3) Even when ^{14}C-norepinephrine was used as a substrate for MAO, there was little or no increase in its deamination after reserpine treatment when measured in hypotonic medium. The increase seen with normal heart preparations was probably caused by swelling or structural changes of heart mitochondria leading to increased deamination of ^{14}C-norepinephrine.

(4) These findings suggest the interesting possibility that reserpine may act on the mitochondrial membrane or structure to increase the penetration of norepinephrine to mitochondrial MAO. In this way the deamination of norepinephrine would be increased in hearts after reserpine treatment.

BOTH pharmacological and biochemical evidence has been presented to show that there are at least two pools of bound norepinephrine (NE) in sympathetic nervous tissues;[1–8] one is a small "available pool" which can be released by tyramine and by nerve stimulation (tyramine-sensitive pool) and the other is larger, "firmly bound pool" which can be released by reserpine (tyramine-resistant pool). Moreover, the metabolic fates of the NE released from these two pools have been suggested to be different;[3, 9] the NE released from the "available pool" is primarily *O*-methylated by catechol-*O*-methyl-transferase (COMT), whereas the NE released from the "firmly bound pool" is predominantly deaminated by monoamine oxidase (MAO). This suggestion is supported by the experiments of Kopin and Gordon[10–12] showing that the NE released by tyramine mostly entered the circulation as NE, though some was *O*-methylated in the tissues. On the other hand, the NE released by reserpine was predominantly deaminated in the tissues, and only a little is released as NE.

Recently, however, the presence of two bound pools of NE in the heart (the tyramine-sensitive and tyramine-resistant pools) has become doubtful. Neff *et al.*[13–15] and Gutman and Weil-Malherbe[16] found that, when a high concentration of tyramine is maintained by continuous infusion, the NE in the heart decreases at a single exponential rate and NE stores are lowered by a maximum of 95 per cent. Jonsson *et al.*[17] also reported similar results with phenylethylamine. From these findings it

seems questionable whether NE stores can be divided into tyramine sensitive and tyramine resistant pools.

Furthermore, the finding that the NE released by reserpine is predominantly deaminated, while the NE released by tyramine is not, may also be simply explained as being the result of activation of mitochondrial MAO by reserpine, rather than representing a true difference in the metabolic fates of two actual NE pools released by reserpine and tyramine, respectively.

From this point of view, the present studies were undertaken to investigate the effect of reserpine on MAO activity in guinea pig heart. A preliminary report of this work has already been presented.[18]

METHODS

The activity of MAO in the heart was estimated by measurement of radioactivity in deaminated products of ^{14}C-labeled catecholamines. These were NE-7-^{14}C (bitartrate), Dopamine (DA)-7-^{14}C (HCl) and tyramine-7-^{14}C (HCl) and they were used at final concentrations of $5{\cdot}3 \times 10^{-6}$M (6×10^4cpm), $5{\cdot}3 \times 10^{-6}$M ($4{\cdot}8 \times 10^4$cpm), and $4{\cdot}4 \times 10^{-5}$M ($10{\cdot}9 \times 10^4$cpm), respectively.

Guinea pigs (250–300g) were used throughout the study. A single intraperitoneal (i.p) injection of 5 mg/kg of reserpine (Serpasil, Ciba), was given and animals were killed by decapitation 2·5 hr later. Their hearts were pooled and chilled in ice cold Ringer solution (154 mM NaCl, 5·6 mM KCl, 2·2 mM $CaCl_2$, 1·3 mM $MgSO_4$, 10 mM glucose, 40 mM Tris maleate buffer, pH 6·8).

In experiments with slices, hearts were sliced with a Stadie–Rigg slicer and weighed immediately on a torsion balance. About 200 mg of slices (each slice weighed about 50 mg) were incubated in 3 ml of Ringer solution as described in the footnote of Table 1. The reaction was stopped by addition of perchloric acid at a final concentration of 0·4 N. Slices were homogenized in a glass Potter homogenizer and ^{14}C-deaminated amine compounds were extracted by standing the homogenate at 0° for

TABLE 1. INCREASE OF MAO ACTIVITY BY RESERPINE TREATMENT (GUINEA PIG HEART SLICES)

MAO activity (cpm $\times 10^{-2}$*/100 mg wet tissue wt./hr)		
Control	Reserpinized	% Increase
129 ± 5 (15)	183 ± 12 (5)	40 ± 15

* cpm of deaminated metabolites.
Values are the means of five or fifteen experiments ± S.D. Guinea pigs were treated with reserpine (5 mg/kg, i.p.) 2·5 hr previously. Heart slices (200 mg) were incubated with $5{\cdot}3 \times 10^{-6}$M of *dl*-^{14}C-NE (6×10^4 cpm) in normal Ringer solution (pH 6·8). The final volume was 3 ml. The incubation was carried out at 37° for 1 hr with shaking.

1 hr. Then the homogenate was centrifuged and the precipitate was washed once with 2 ml of 0·4 N perchloric acid. The washings and supernatant were pooled and charged onto a Dowex 50 column (H^+ form, 0·4 × 4 cm) to absorb the ^{14}C-amines. In this way, the radioactivity of ^{14}C-amine was absorbed onto the Dowex 50 column. The

radioactivity in the fractions of eluate containing the deaminated products of ^{14}C-amine was measured in Tricarb scintillation spectrophotometer.

For experiments in which heart homogenates were used for estimation of MAO activity, hearts were homogenized using a glass Potter homogenizer in Ringer solution containing 40 mM Tris maleate buffer, pH 6·8. The homogenates were filtered through gauze to remove coarse debris. The homogenate was incubated with ^{14}C-amine without shaking, as shown in the footnote of Table 2. Subsequent procedures were the same as those used with slices.

TABLE 2. INCREASE OF MAO ACTIVITY BY RESERPINE TREATMENT (GUINEA PIG HEART HOMOGENATE)

MAO activity (cpm*/mg protein/hr)		
Control	Reserpinized	% Increase
237 ± 11 (20)	425 ± 84 (20)	79 ± 39

* cpm of deaminated metabolites.
Values are the means of twenty experiments ± S.D.
Guinea pigs were treated with reserpine (5 mg/kg, i.p.) 2·5 hr previously. The homogenate (about 50 mg of protein) was incubated with $5{\cdot}3 \times 10^{-6}$M of *dl*-^{14}C-NE (6×10^{-4} cpm) in normal Ringer solution (pH 6·8). The final volume was 3 ml. Incubation was carried out at 37° for 1 hr without shaking.

When the mitochondrial fraction of heart was used as the MAO preparation, hearts were homogenized in ten volumes of 0·25 M sucrose containing 40 mM Tris maleate buffer, pH 6·8. The homogenate was centrifuged at 600 *g* for 10 min to remove the debris and the supernatant was then centrifuged at 8000 *g* for 20 min. The resulting sediment was suspended in Ringer solution containing 40 mM Tris maleate buffer, pH 6·8. Incubations of the mitochondrial fraction with ^{14}C-amine were carried out without shaking as described in the footnote of Table 3. Subsequent procedures for estimation of MAO activity were the same as in experiments with slices.

TABLE 3. INCREASE OF MAO ACTIVITY BY RESERPINE TREATMENT (GUINEA PIG HEART MITOCHONDRIA)

MAO activity (cpm*/mg protein/hr)		
Control	Reserpinized	% Increase
451 ± 19 (15)	643 ± 68 (15)	45 ± 23

* cpm of deaminated metabolites.
Values are the means of fifteen experiments ± S.D.
Guinea pigs were treated with reserpine (5 mg/kg, i.p.) 2·5 hr previously. Heart mitochondria (about 10 mg of protein) was incubated with $5{\cdot}3 \times 10^{-6}$M of *dl*-^{14}C-NE (6×10^{4} cpm) in normal Ringer solution (pH 6·8). The final volume was 3 ml. Incubation was carried out at 37° for 1 hr without shaking.

RESULTS

(1) *Deamination of ^{14}C-norepinephrine in normal and reserpine pretreated heart slices, homogenates or mitochondrial fractions.*

Heart slices from normal guinea pig and those treated 2·5 hr previously with reserpine were incubated with ^{14}C-NE in Ringer medium at 37° for 1 hr. As shown in Table 1, in the latter slices, the amount of deaminated metabolites of ^{14}C-NE was more than in the controls.

Table 2 shows the deamination of ^{14}C-NE in heart homogenates of normal guinea pigs and those pretreated with reserpine. After pretreatment with reserpine, the deaminated products of ^{14}C-NE were also significantly more than normal.

In the following experiments, MAO activity in the mitochondrial fractions of hearts from normal and reserpine-treated guinea pigs was measured. The mitochondrial fractions isolated from hearts of normal animals and those treated 2·5 hr previously with reserpine were incubated with ^{14}C-NE as described in the footnote of Table 3. As shown in Table 3, more deaminated products of ^{14}C-NE were also produced by the mitochondrial fraction from reserpine pretreated guinea pig hearts than by mitochondrial fraction of normal guinea pig hearts.

(2) *Deamination of ^{14}C-NE at various times after reserpine treatment*

The time course of the effect of reserpine on MAO activity in the heart was investigated. Heart homogenates of guinea pigs were prepared and incubated with ^{14}C-NE at various times after the administration of a single dose of reserpine (5 mg/kg). Five animals were used in each group of experiments. As shown in Table 4, the amount

TABLE 4. INCREASE OF MAO ACTIVITY BY RESERPINE TREATMENT (GUINEA PIG HEART HOMOGENATE)

	MAO activity (cpm*/mg protein/hr)	% Increase
Control	237 ± 11 (20)	0
Reserpinized 1 hr	340 ± 40 (4)	43 ± 19
Reserpinized 2·5 hr	425 ± 84 (20)	79 ± 39
Reserpinized 5 hr	387 ± 61 (4)	63 ± 24
Reserpinized 24 hr	327 ± 38 (4)	38 ± 18

* cpm of deaminated metabolites.
Values are the means of four or twenty experiments ± S.D. Guinea pigs were treated with reserpine (5 mg/kg, i.p.). Substrate; *dl*-^{14}C-NE ($5·3 \times 10^{-6}$M, 6×10^4 cpm).

of deaminated products of ^{14}C-NE increased rapidly 1 hr after reserpine-treatment. This increase in deamination of ^{14}C-NE was maximal 2·5 or 5 hr after reserpine treatment. However, even 24 hr after reserpine-treatment increased deamination of ^{14}C-NE was still observed, though to a lesser degree.

(3) *Deamination of ^{14}C-DA or ^{14}C-tyramine in normal and reserpine-pretreated heart homogenate*

^{14}C-DA or ^{14}C-tyramine was used instead of ^{14}C-NE as a substrate for estimation of MAO activity. Heart homogenates of normal guinea pigs and those treated with reserpine 2·5 hr previously were incubated with ^{14}C-DA or ^{14}C-tyramine, as shown in Table 5. Deaminations of DA and tyramine were found to be much more rapid

TABLE 5. INCREASE OF MAO ACTIVITY BY RESERPINE TREATMENT (GUINEA PIG HEART HOMOGENATE)

	MAO activity (cpm*/mg protein/hr)		
Substrate	Control	Reserpinized	% Increase
Noradrenaline	237 ± 11 (20)	425 ± 84 (20)	79 ± 39
Dopamine	620 ± 50 (5)	770 ± 112 (5)	24 ± 4
Tyramine	1600 ± 125 (5)	1605 ± 117 (5)	0

* cpm of deaminated metabolites.
Values are the means of five or twenty experiments ± S.D.
Guinea pigs were treated with reserpine (5 mg/kg, i.p.) 2·5 hr previously. Incubation was carried out in normal Ringer solution at pH 6·8 (40 mM tris maleate buffer) with ^{14}C-NE ($5{\cdot}3 \times 10^{-6}$M, 6×10^4 cpm), ^{14}C-DA ($5{\cdot}3 \times 10^{-6}$M, $4{\cdot}8 \times 10^4$ cpm) or ^{14}C-Tyramine ($4{\cdot}4 \times 10^{-5}$M, $10{\cdot}9 \times 10^4$ cpm) as substrate. The final volume was 3 ml and incubation was at 37° for 1 hr without shaking.

than that of NE. However, deaminations of DA and tyramine in reserpine treated hearts were similar to those in the controls.

(4) *Effect of tonicity on MAO activity of heart homogenates or mitochondrial fraction*

Hearts from normal animals and those treated 2·5 hr previously with reserpine were homogenized in isotonic (NaCl 154 mM, KCl 5·6 mM) or hypotonic (NaCl–KCl free) medium, containing 40 mM Tris maleate buffer, pH 6·8. These isotonic and hypotonic heart homogenates were used as enzyme preparations (Table 6).

TABLE 6. EFFECT OF TONICITY ON MAO ACTIVITY OF GUINEA PIG HEART HOMOGENATE

	MAO activity (cpm*/mg protein/hr)		
Tonicity	Control	Reserpinized	% Increase
Isotonic	194 ± 15 (5)	350 ± 27 (5)	80 ± 38
Hypotonic	291 ± 12 (5)	312 ± 19 (5)	7 ± 2

* cpm of deaminated metabolites.
Values are the means of five experiments ± S.D.
Guinea pigs were treated with reserpine (5 mg/kg, i.p.) 2·5 hr previously. Hearts were homogenized in isotonic (NaCl 154 mM, KCl 5·6 mM) or hypotonic (NaCl–KCl-free) medium both containing 40 mM of tris maleate buffer (pH 6·8). Homogenates were used as enzyme preparations. *dl*-^{14}C-NE ($5{\cdot}3 \times 10^{-6}$M, 6×10^4 cpm) was used as substrate. Incubation was carried out at 37° for 1 hr without shaking.

The heart mitochondrial fractions from normal and reserpine treated animals were isolated as described in the Methods for use as MAO preparations and divided into two parts. One part was suspended in isotonic Ringer solution and the other in hypotonic (NaCl–KCl free) solution.

As shown in Tables 6 and 7, deamination of ^{14}C-NE by normal homogenates or mitochondrial fractions increased in hypotonic medium, probably because of swelling

TABLE 7. EFFECT OF TONICITY ON MAO ACTIVITY OF GUINEA PIG HEART MITOCHONDRIA

	MAO activity (cpm*/mg protein/hr)		
Tonicity	Control	Reserpinized	% Increase
Isotonic	370 ± 18 (5)	537 ± 48 (5)	45 ± 19
Hypotonic	507 ± 29 (5)	500 ± 23 (5)	0

* cpm of deaminated metabolites.
Values are the means of five experiments ± S.D.
Guinea pigs were treated with reserpine (5 mg/kg, i.p.) 2·5 hr previously. Hearts were homogenized in NaCl (154 mM)–KCl 5·6 mM) solution containing 40 mM of tris maleate buffer (pH 6·8) and mitochondria were isolated by the routine method. Mitochondria were suspended in isotonic (NaCl 154 mM, KCl 5·6 mM) or hypotonic (NaCl–KCl-free) solution contained 40 mM of tris maleate buffer (pH 6·8). *dl*-^{14}C-NE ($5{\cdot}3 \times 10^{-6}$ M, 6×10^4 cpm) was used as substrate. Incubation was carried out at 37° for 1 hr without shaking.

or structural changes of the heart mitochondria. However, even when ^{14}C-NE was used as substrate for MAO, there was little or no increase of deamination of NE in reserpinized guinea pig hearts in hypotonic medium.

(5) In vitro *effect of reserpine on the deamination of ^{14}C-NE in heart slices, homogenates and mitochondrial fractions.*

Experiments were carried out on whether the stimulatory effect of reserpine on the MAO activity described above could be observed *in vitro*. Heart slices from normal guinea pigs were incubated with ^{14}C-NE in the presence or absence of reserpine phosphate. As shown in Table 8, increased amounts of deaminated products of ^{14}C-NE were observed in the presence of reserpine (5×10^{-6}M).

TABLE 8. *IN VITRO* EFFECT OF RESERPINE ON MAO ACTIVITY OF GUINEA PIG HEART SLICES

MAO activity (cpm × 10^{-2}*/100 mg wet tissue wt./hr)		
Control	Reserpinized	% Increase
130 ± 8 (10)	179 ± 25 (10)	37 ± 13

* cpm of deaminated metabolites.
Values are the means of ten experiments ± S.D.
Incubation medium contained about 200 mg of heart slices, $5{\cdot}3 \times 10^{-6}$M of *dl*-^{14}C-NE (6×10^4 cpm), 40 mM of tris maleate buffer and normal Ringer solution. Reserpine phosphate (5×10^{-6}M) was present or absent. The final volume was 3 ml. Incubation was carried out at 37° for 1 hr with shaking.

However, it was found that when a homogenate or mitochondrial fraction from normal guinea pig hearts was incubated with 5×10^{-6}M reserpine, there was little or no increase in deamination of ^{14}C-NE, as shown in Table 9.

TABLE 9. *IN VITRO* EFFECT OF RESERPINE ON THE CARDIAC MAO ACTIVITY

	MAO activity (cpm*/mg protein/hr)		
	Control	Reserpinized	% Increase
Homogenate	237 ± 11 (20)	248 ± 8 (5)	0
Mitochondria	451 ± 19 (15)	438 ± 15 (5)	0

* cpm of deaminated metabolites.
Values are the means of five to twenty experiments ± S.D.
Incubation was carried out in normal Ringer solution (pH 6·8) at 37° for 1 hr without shaking. $5{\cdot}3 \times 10^{-6}$M of *dl*-^{14}C-NE (6×10^4 cpm) was used as substrate. Reserpine phosphate (5×10^{-6}M) was present or absent.

Next, heart slices of guinea pig hearts were preincubated with 5×10^{-6}M reserpine at 37° for 1 hr, and then homogenates or mitochondrial fractions were prepared from these slices. With these homogenates or mitochondrial fractions, increased amounts of deaminated products of ^{14}C-NE were observed relative to those with homogenates or mitochondrial fractions of slices preincubated in the absence of reserpine (Table 10).

TABLE 10. PRETREATMENT OF HEART SLICES WITH RESERPINE

MAO activity (cpm*/mg protein/hr)		
Control	Reserpinized	% Increase
216 ± 10 (5)	270 ± 21 (5)	25 ± 3

* cpm of deaminated metabolites.
Values are the means of five experiments ± S.D.
Guinea pig heart slices were preincubated at 37° for 1 hr with shaking in normal Ringer containing 40 mM of tris maleate buffer (pH 6·8). Reserpine phosphate (5×10^{-6}M) was present or absent. After preincubation, the slices were homogenized in normal Ringer solution containing 40 mM of tris maleate buffer (pH 6·8). This homogenate was used as the enzyme preparation. $5{\cdot}3 \times 10^{-6}$M of *dl*-^{14}C-NE (6×10^4 cpm) was used as substrate. Incubation was carried out at 37° for 1 hr without shaking.

DISCUSSION

Kopin and Gordon[3, 9] found that the NE released by tyramine was metabolized by COMT, whereas the NE released by reserpine was mainly metabolized by MAO in the tissues. Accordingly, it was supposed that the NE released from the "tyramine-sensitive pool" was destroyed enzymatically by *O*-methylation, whereas the NE from the "firmly bound pool" released by reserpine was predominantly deaminated by MAO. However, this finding that the NE released by reserpine is mainly deaminated in the tissues may be simply because mitochondrial MAO is activated by reserpine. It has already been reported from our laboratory that MAO activity in the brain

stem of guinea pigs is stimulated by reserpine administration.[19] In this work, therefore, the effects of reserpine on MAO activity in guinea pig hearts were examined in further detail.

In heart slices of animals 2·5 hr after reserpine treatment, ^{14}C-NE was found to be deaminated much more than in control slices. This agrees with the findings of Iversen *et al.*[20] that on perfusion of hearts from animals after treatment with reserpine, ^{3}H-NE taken up from the perfusion medium is mainly metabolized by MAO. When homogenates of hearts from animals after reserpine-treatment were incubated with ^{14}C-NE, it was also observed that the deaminated products of ^{14}C-NE was significantly more than normal.

This increase in deamination of ^{14}C-NE in slices or homogenates of hearts of animals after reserpine treatment is probably due to activation of MAO by reserpine. However, it is also possible that reserpine inhibits the binding of ^{14}C-NE to intracellular storage granules so that less is bound and more free ^{14}C-NE is metabolized by mitochondrial MAO. This possibility, however, was excluded by results of experiments on MAO activity in mitochondrial fraction of hearts from animals after reserpine treatment, since the major storage sites for exogenous and endogenous NE are known to be localized, not in the mitochondrial fraction, but in the microsomal fraction in the heart.[21–24] The mitochondrial fraction from hearts after reserpine treatment deaminated more ^{14}C-NE, than the mitochondrial fraction of normal guinea pig hearts.

Therefore, these results clearly show that reserpine treatment activates heart mitochondrial MAO activity, resulting in increased deamination of ^{14}C-NE in the heart after reserpine treatment.

The activation of heart mitochondrial MAO activity induced by reserpine administration was observable after 1 hr and was maximal after 2·5 or 5 hr. Even 24 hr after reserpine administration the activation of MAO was still observable. These data suggest that there is some relationship between the depletion of tissue NE and the increase of MAO activity induced by reserpine. Further studies are required on this problem, but an interesting possibility is that the action of reserpine in increasing deamination of NE by mitochondrial MAO may contribute largely to its effect in NE depletion. This possibility is supported by the previous findings[25–29] that the initial effect of reserpine in decreasing tissue NE is found, not in the particulate fraction, but in the supernatant fraction, and also that pretreatment with MAO-inhibitors prevents the depletion of tissue NE caused by reserpine administration.[30–34]

The activation of heart mitochondrial MAO activity caused by reserpine has only been demonstrated so far *in vivo*, and in slices *in vitro*. When heart slices of guinea pigs were incubated with ^{14}C-NE in the presence of reserpine, the amounts of deaminated products of ^{14}C-NE increased more than in the absence of reserpine. However, the deamination of ^{14}C-NE in heart homogenates or mitochondrial fractions was not increased by the addition of reserpine. It is interesting that deamination of ^{14}C-NE in homogenates or mitochondrial fractions prepared from slices which had been incubated with reserpine *in vitro*, was increased in the same way as when reserpine was given *in vivo*. These results suggests that in *in vitro* systems, cell structures or membranes are essential for demonstration of the action of reserpine in increasing the deamination of ^{14}C-NE by mitochondrial MAO.

The mechanism by which reserpine activates heart mitochondrial MAO is not yet

known. However, it was observed that when ^{14}C-DA or ^{14}C-tyramine (far better substrates for mitochondrial MAO than NE) was used as a substrate for estimation of MAO activity, there was little increase in the deamination of DA or tyramine in hearts after reserpine treatment relative to that in the controls. Furthermore, it was found that even when ^{14}C-NE was used as a substrate for MAO, there was little or no increase in deamination of NE in hearts after reserpine treatment when measured in hypotonic medium, though normal hearts showed an increase presumably due to swelling or structural changes of the heart mitochondria leading to increased deamination of ^{14}C-NE. The increase in NE deamination under hypotonic conditions seems to be in accordance with our previous results[35] and those of others[36] showing that the activity of mitochondrial MAO is closely related to the structural state of the mitochondria.

Therefore, an interesting possibility based on these findings is that reserpine may act, not on the amount of MAO enzyme in mitochondria, but on the mitochondrial membrane or structure to increase the penetration of NE into mitochondrial MAO. In this way the deamination of NE would be increased in reserpinized heart. This possibility is strongly supported by recent morphological evidence[37, 38] showing that the structure of the heart mitochondria is clearly changed by administration of reserpine and that this structural change may be related to the effect of reserpine in depleting tissue NE.

The well known fact that the NE released by reserpine is mainly metabolized to deaminated compounds has been explained by the attractive hypothesis proposed by Kopin *et al.*[10–12] that reserpine can release NE from the "firmly bound" NE-pool and so this NE is primarily deaminated. However, this phenomenon can also be explained as simply due to the action of reserpine in facilitating the deamination of NE by mitochondrial MAO, as described above. The finding that the NE released by tyramine is primarily metabolized by COMT might also be explained on the ground that tyramine competitively inhibits the deamination of released NE by mitochondrial MAO, since it is known to be a better substrate of MAO than NE. In this way, much more NE is inactivated by COMT than by MAO.

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