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SPECIFIC BINDING OF RESERPINE—

ASSOCIATION WITH NOREPINEPHRINE DEPLETION*

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Abstract—Small doses of ^{3}H-reserpine (10–100 μg/kg) were injected i.v. into rats and the drug concentration in heart, spleen and liver measured at various intervals. Eighteen hours after a dose of 25 μg per kg, concentrations of the tritiated compound were: 4·98 ng per g heart, 6·39 ng per g spleen, and 1·48 ng per g liver. The biologic half-life of ^{3}H-reserpine in heart and spleen was about 18 hr; that of liver was about 4 hr. Specificity studies showed that the labeled material extracted from rat heart 18 hr after administration of the tritiated compound behaved similarly to authentic reserpine.

Pretreatment of rats with tetrabenazine (20 mg/kg) or unlabeled reserpine (0·5 mg/kg) significantly reduced the amount of ^{3}H-reserpine found in heart and spleen 18 hr after administration of the radioactive drug, while administration of either tetrabenazine or unlabeled reserpine after the injection of ^{3}H-reserpine had no significant effect on levels of the labeled drug.

The concentration of norepinephrine remaining in heart 18 hr after the administration of small doses of reserpine was inversely related to the concentration of persisting drug and the correlation was highly significant. It is concluded that the persistent depletion of norepinephrine by small doses of reserpine is induced and maintained by the physical presence of minute amounts of very highly bound reserpine.

Calculations reveal that each molecule of reserpine persisting in heart is associated with a deficit of about 500 norepinephrine molecules. It is also calculated that about 20 molecules of reserpine persist for each affected catecholamine-containing granule.

AFTER administration of radioactive reserpine, small amounts of radioactivity remain in mammalian brain for relatively long periods of time. Radioactivity was found in guinea pig brain for 48 hr[1] and for 72 hr[2] and in mouse brain for 5 days[3] after administration of labeled reserpine.

Although it has thus been suggested that small amounts of residual drug may be associated with the prolonged actions of reserpine, the validity of this suggestion has not been established. The low specific activity of the labeled compounds used in the earlier investigations made specificity studies and quantitation difficult.

With the availability of radioactive reserpine of high specific activity, it became

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feasible to undertake a rigorous examination of this hypothesis. The results of this investigation are presented in this communication.

MATERIALS AND METHODS

Drugs. Radioactive reserpine (277 mc/mM) with tritium in positions 2 and 5 of the trimethoxybenzoyl portion of the molecule was obtained from the New England Nuclear Corp. Unlabeled reserpine for treatment of animals was Serpasil (Ciba). Unlabeled reserpine for specificity studies was obtained from Mann Research Laboratories. Tetrabenazine was kindly donated by Roche Laboratories.

Animals. Female Sprague–Dawley rats weighing between 150–200 g were used throughout this study. Intravenous injections were made via the tail vein.

Extraction of ^{3}H-reserpine from rat tissues. Animals were killed by chloroform asphyxiation, tissues were removed, rinsed in saline and blotted dry. Tissues were weighed and homogenized in 5 vol. of 0·01 N HCl using a motor-driven glass homogenizer. To a 1·5-ml aliquot of homogenate, 0·5 ml of 0·5 M borate buffer, pH 9, was added. If necessary, the pH was adjusted to 9 with the dropwise addition of 0·1 N NaOH. The alkalinized homogenate was then extracted with 5 ml toluene containing 1·5% isoamyl alcohol (v/v). The samples were shaken for 15 min in a shaking apparatus and then centrifuged for 10 min. Aliquots of the toluene layer were used for the determination of radioactivity.

Determination of radioactivity. The counting solution contained 5 g 2,5-diphenyloxazole (PPO) and 0·3 g 1,4-*bis*-2(5-phenyloxazoly)-benzene (POPOP) per l. toluene. Each aliquot of toluene (2–4 ml) was added to 10 ml of scintillation solution and counted in a Beckman liquid scintillation spectrometer. Each vial was counted to a statistical accuracy of ± 5 per cent or less; the counting efficiency ranged from 59 to 63 per cent. Counts were at least five times background.

As controls for the extraction procedure and count, each group of ^{3}H-reserpine determinations included the following controls and blanks: (1) a known amount of labeled reserpine added to tissue homogenates of untreated rats which were then subjected to the same extraction procedures as tissues from the reserpinized animals; (2) a known amount of labeled reserpine in toluene added to scintillation fluid to serve as a standard; (3) a toluene extract from tissue homogenates of untreated rats which provided a background count reference; and (4) a check for quenching on each sample by the external standard technique which was automatically performed by the instrument.

Norepinephrine determinations. The method of Shore and Olin[4] was used for the norepinephrine determinations.

Procedure for the differential centrifugation of rat heart homogenates. Rat hearts trimmed of atria were homogenized in 9 vol. of ice-cold sucrose, 0·25 M. The homogenates were centrifuged at approximately 1000 *g* for 10 min at 5°. The sediment from this centrifugation was designated the "coarse" fraction and the supernatant fluid was centrifuged at approximately 10,000 *g* for 15 min at 5°. The sediment from this second centrifugation was designated the "mitochondrial" fraction and the supernatant fluid was centrifuged at approximately 100,000 *g* for 30 min at 5° in a Spinco model L ultracentrifuge. This sediment was designated the "microsomal" fraction and the supernatant fluid as the supernatant fraction. Each fraction was then analyzed for ^{3}H-reserpine as described above.

RESULTS AND DISCUSSION

Specificity of the reserpine extraction procedure

Evidence for the specificity of the method was obtained by comparing the partition ratios of apparent ^{3}H-reserpine from rat heart with authentic reserpine. Unlabeled reserpine, about 600 μg, was added to pooled heart tissue from rats that received ^{3}H-reserpine 18 hr previously. The tissue was homogenized and extracted with toluene as described above. The toluene extract of heart tissue was divided into five equal portions. One portion received no further treatment and served as a reference. Each of the other portions was re-extracted with one of four aqueous solutions: 0·1 N H_2SO_4, 1 N H_2SO_4, 1 N HCl, or 0·5 M borate buffer, pH 9. The radioactivity and total reserpine concentration of each of the five toluene portions were then determined. Radioactivity was measured as described above and total reserpine concentration was determined by u.v. absorption spectrophotometry at 300 mμ, the peak absorption exhibited by reserpine in toluene. Absorption, as determined over a range of 10–75 μg/ml, was proportional to reserpine concentration. The concentration used in these determinations fell within that range.

The specific activity, i.e. the ratio of radioactivity to total reserpine concentration as measured spectrophotometrically, was calculated for each portion (Table 1).

TABLE 1. COMPARISON OF EXTRACTION CHARACTERISTICS OF APPARENT ^{3}H-RESERPINE FROM RAT HEART AND AUTHENTIC RESERPINE

Experiment No.	Ratio of specific activity before re-extraction of toluene to specific activity after re-extraction			
	0·1 N H_2SO_4	1 N H_2SO_4	1 N HCl	0·5 M borate buffer, pH 9
1	1·03	1·31	0·94	1·12
2	0·96	0·78	0·97	0·96
3	1·00	0·89	0·89	0·80
(Mean)	(1·00)	(0·98)	(0·93)	(0·96)
Approximate amount of reserpine removed by aqueous solvent	50%	60%	0%	0%

The specific activity of the reference toluene portion of each experiment was taken as unity and the specific activity of each re-extracted toluene portion compared to this value. As shown in Table 1, the apparent ^{3}H-reserpine from rat heart showed extraction characteristics similar to authentic reserpine.

As all of the tritium was present in the trimethoxybenzoyl moiety of the reserpine molecule and none in the methyl reserpate moiety, and as trimethoxybenzoic acid does not extract under the conditions described above, it is clear that the material measured was not trimethoxybenzoic acid, methylreserpate or reserpic acid. The possibility exists, however, that the radioactive material is a slightly altered reserpine molecule such as the syringoyl derivative. A minor change in the large reserpine molecule might not have altered its solubility characteristics sufficiently to distinguish it from the unchanged reserpine molecule by the technique described above.

Rate of disappearance of ^{3}H-reserpine from rat tissues after intravenous administration

Concentrations of the labeled drug in heart, spleen and liver were determined at

3, 6, 18 and 30 hr after i.v. administration of the drug (25 μg/kg) (Table 2). Levels in heart and spleen decreased relatively rapidly during the interval between 3 and 6 hr and then decreased more slowly over the remaining period of tissue examination (Fig. 1). Levels in liver declined rapidly during the entire period of examination and at 30 hr after drug administration were less than 15 per cent of the concentrations remaining in heart and spleen. The biologic half-life of reserpine in heart and spleen was approx. 18 hr, but was much less in liver, about 4 hr.

TABLE 2. RAT TISSUE CONCENTRATIONS OF ^{3}H-RESERPINE AT VARIOUS TIMES AFTER THE INTRAVENOUS ADMINISTRATION OF 25 μg per kg

Tissue	^{3}H-reserpine (ng/g ± S.E.M.)			
	3 hr (4)*	6 hr (6)*	18 hr (8)*	30 hr (6)*
Heart	14·74 ± 1·05	8·40 ± 0·83	4·98 ± 0·25	3·01 ± 0·44
Liver	17·10 ± 1·50	11·55 ± 2·30	1·48 ± 0·17	0·40 ± 0·13
Spleen	18·46 ± 1·59	12·77 ± 1·55	6·39 ± 0·39	3·85 ± 0·67

* Numbers in parentheses refer to number of animals used.

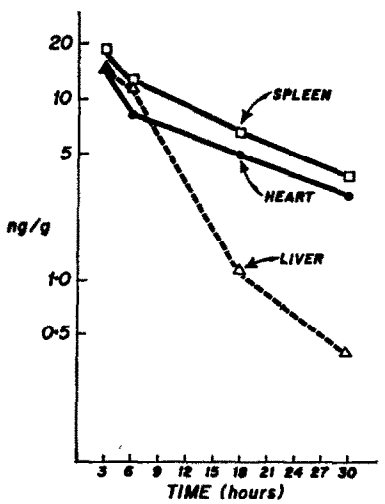

FIG. 1. Rate of disappearance of ^{3}H-reserpine from heart, spleen and liver of rats after a dose of 25 μg per kg injected i.v. Each point represents the mean of four to eight experiments.

Effect of dose on 18-hr levels of ^{3}H-reserpine

Doses of 10, 25, 50 or 100 μg per kg were given i.v. and concentrations of ^{3}H-reserpine were determined 18 hr later (Table 3). At this time, levels of radioactivity in heart and spleen were greater than those in liver at all dosages used.

The capacity of the examined organs to persistently hold reserpine is clearly saturable, as little more ^{3}H-reserpine was found 18 hr after injection of 100 μg ^{3}H-reserpine than after only half this dose.

Effect of tetrabenazine administration on tissue levels of ^{3}H-reserpine

There is evidence that tetrabenazine and reserpine have similar modes of action.

TABLE 3. RAT TISSUE CONCENTRATIONS OF ^{3}H-RESERPINE 18 hr AFTER VARIOUS INTRAVENOUS DOSES

Tissue	^{3}H-reserpine (ng/g ± S.E.M.)			
	10 μg/kg (4)*	25 μg/kg (8)*	50 μg/kg (6)*	100 μg/kg (5)*
Heart	2·54 ± 0·28	4·98 ± 0·29	5·51 ± 0·28	7·14 ± 0·38
Liver	0·65 ± 0·09	1·48 ± 0·17	3·07 ± 0·59	3·37 ± 0·31
Spleen	3·01 ± 0·18	6·39 ± 0·39	10·60 ± 0·71	11·38 ± 1·05

* Numbers in parentheses refer to number of animals used.

Experiments *in vivo* have indicated that they compete for the same pharmacological receptors[5] and both compounds inhibit the uptake of catecholamines by the Mg^{++}-ATP-dependent uptake mechanism of adrenal medullary storage granules.[6] Reserpine and tetrabenazine are long and short-acting blocking agents, respectively, of the intracellular amine storage function.[7–9]

Pretreatment of rats with tetrabenazine (20 mg/kg i.p.) 30 min before administration of ^{3}H-reserpine resulted in markedly reduced concentrations of the labeled compound in heart and spleen 18 hr later (Table 4). The concentration of reserpine in liver of the pretreated animals was not significantly different from control values.

Administration of tetrabenazine (20 mg/kg i.p.) 30 min after ^{3}H-reserpine injection had no effect on the concentration of tritiated reserpine in heart, liver or spleen 18 hr later (Table 4).

TABLE 4. EFFECT OF TETRABENAZINE ON RAT TISSUE CONCENTRATIONS OF ^{3}H-RESERPINE 18 hr AFTER ADMINISTRATION OF ^{3}H-RESERPINE*

Tissue	^{3}H-reserpine (ng/g ± S.E.M.)		
	Control	Tetrabenazine 30 min before ^{3}H-reserpine	Tetrabenazine 30 min after ^{3}H-reserpine
Heart	4·98 ± 0·29 (8)	2·02 ± 0·40 (6)†	4·34 ± 0·25 (4)‡
Spleen	6·38 ± 0·39 (8)	2·77 ± 0·58 (4)†	4·55 ± 0·77 (4)‡
Liver	1·48 ± 0·17 (8)	1·17 ± 0·31 (6)‡	1·25 ± 0·14 (4)‡

* Rats were given tetrabenazine (20 mg/kg i.p.) 30 min before or 30 min after ^{3}H-reserpine (25 μg/kg i.v.). Control rats were given ^{3}H-reserpine only. Animals were killed 18 hr after reserpine administration. Figures in parentheses represent number of experiments.
† Highly significant difference from control ($P < 0·001$).
‡ Not significantly different from control.

Effect of administration of unlabeled reserpine on tissue levels of ^{3}H-reserpine

Pretreatment with unlabeled reserpine (500 μg/kg i.p.) 18 hr before injection of tritiated reserpine (25 μg/kg i.v.) resulted in markedly lowered concentrations of ^{3}H-reserpine in heart and spleen 18 hr after injection of the labeled compound (Table 5). Lower concentrations were also found in liver, but in this tissue the reduction was not significant.

When unlabeled reserpine was given 18 hr after the labeled compound, no difference

TABLE 5. EFFECT OF 18-hr PRETREATMENT WITH UNLABELED RESERPINE ON TISSUE CONCENTRATIONS OF ^{3}H-RESERPINE*

Tissue	^{3}H-reserpine (ng/g ± S.E.M.)	
	Control (No. = 8)	Pretreated (No. = 4)
Heart	4·98 ± 0·25	0·74 ± 0·14†
Spleen	6·39 ± 0·39	1·87 ± 0·27†
Liver	1·48 ± 0·17	0·88 ± 0·18‡

* Rats received unlabeled reserpine (500 μg/kg i.p.) and 18 hr later received ^{3}H-reserpine (25 μg/kg i.v.). Controls received ^{3}H-reserpine only. Tissues were analyzed 18 hr after the administration of ^{3}H-reserpine.
† Highly significant difference from control ($P < 0·001$).
‡ Not significantly different from control.

TABLE 6. EFFECT OF UNLABELED RESERPINE GIVEN 18 hr AFTER ^{3}H-RESERPINE ON CONCENTRATIONS OF ^{3}H-RESERPINE*

Tissue	^{3}H-reserpine (ng/g ± S.E.M.)	
	Control (No. = 8)	Treated (No. = 6)
Heart	2·39 ± 0·24	2·83 ± 0·45†
Spleen	3·61 ± 0·22	3·57 ± 0·39†
Liver	0·56 ± 0·13	0·60 ± 0·13†

* Rats received ^{3}H-reserpine (25 μg/kg i.v.) and 18 hr later received unlabeled reserpine (500 μg/kg i.p.). Control rats received ^{3}H-reserpine only. Tissues were analyzed 36 hr after ^{3}H-reserpine administration.
† Not significantly different from control.

was seen in the 36-hr levels of ^{3}H-reserpine in these and control rats given only ^{3}H-reserpine (Table 6).

The extremely low levels of labeled reserpine in heart and spleen after pretreatment with unlabeled reserpine are interpreted as indicating prior occupation of a readily saturated site by unlabeled reserpine. The second set of experiments, in which the order of administration is reversed, supports this interpretation. In this instance, the ^{3}H-reserpine is firmly attached to the limited number of susceptible sites and unlabeled reserpine cannot displace the radioisotope.

Reserpine-induced depletion of heart norepinephrine as a function of dose

Rats were given unlabeled reserpine intravenously in doses of 10, 25 or 100 μg/kg. Hearts were analyzed 18 hr later for norepinephrine. Hearts from untreated rats were analyzed with each group of treated animals. It was found that a 10 μg/kg dose of reserpine depleted approx. 48 per cent of heart norepinephrine, the 25 μg/kg dose approximately 60 per cent and the 100 μg/kg dose approximately 86 per cent (Table 7).

The concentrations of norepinephrine found in the heart after each of these three doses and that of the control group were plotted against ^{3}H-reserpine concentrations found in the heart (see Table 3) after the same three doses of labeled reserpine had been given. The association of these two groups of data is indicated by the regression line shown in Fig. 2. The apparent correlation is real ($P < 0.05$) and very high ($r = 0.97$).

TABLE 7. EFFECT OF DOSAGE OF RESERPINE ON NOREPINEPHRINE CONCENTRATION IN RAT HEART 18 hr AFTER DRUG ADMINISTRATION*

	Reserpine-treated group of rats			Untreated control group
	Dosage of reserpine			
	10 μg/kg (No. = 6)	25 μg/kg (No. = 6)	100 μg/kg (No. = 6)	(No. = 14)
Norepinephrine concn (μg/g ± S.E.M.)	0·61 ± 0·03	0·47 ± 0·03	0·17 ± 0·01	1·17 ± 0·04
% Depletion	47·9	59·8	85·5	

* Rats received unlabeled reserpine in intravenous doses of 10, 25 or 100 μg per kg. Hearts were analyzed for norepinephrine 18 hr after the administration of the drug. Untreated rats were used as controls.

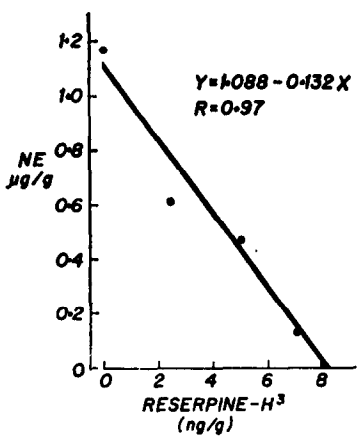

FIG. 2. Regression line correlating norepinephrine concentration and ^{3}H-reserpine concentration in rat heart 18 hr after various i.v. doses of the drug. Each point represents the mean concentration of norepinephrine present plotted against the mean ^{3}H-reserpine concentration.

Using the equation for the regression line from Fig. 2 ($Y = 1{\cdot}0877952 - 0{\cdot}1320569X$) as a base of calculations, a numerical relationship between the "missing" norepinephrine and tritiated reserpine can be obtained. Each ng per g ^{3}H-reserpine (1·616 picomoles) is associated with a norepinephrine deficit of 0·128 μg per g (757·4 picomoles). The ratio of the molar values is 469. Thus, each molecule of reserpine present in rat heart is associated with a deficit of approximately 500 molecules of norepinephrine.

Norepinephrine in rat heart at various times after a 25 μg/kg dose of reserpine

Rat hearts were analyzed for norepinephrine at 6, 18, 30, 42 and 60 hr after administration of a single dose of reserpine (25 μg/kg i.v.). Hearts from untreated animals were analyzed with each group of treated animals (Table 8). The lowest concentration of norepinephrine (0·47 μg/g) was found at 18 hr after the administration of the drug. Thereafter, norepinephrine concentration increased gradually until at 60 hr it was approximately 64 per cent of control values.

TABLE 8. NOREPINEPHRINE LEVELS IN RAT HEART AT DIFFERENT TIMES AFTER RESERPINE ADMINISTRATION*

	Reserpine-treated group of rats					Untreated control group
	No. = 7	No. = 6	No. = 6	No. = 6	No. = 6	No. = 14
Time after administration of reserpine (hr)	6	18	30	42	60	
Norepinephrine concn (μg/g ± S.E.M.)	0·64 ± 0·06	0·47 ± 0·04	0·60 ± 0·04	0·68 ± 0·03	0·75 ± 0·03	1·17 ± 0·03

* Rats received a single dose of unlabeled reserpine (25 μg/kg i.v.). Hearts were analyzed for norepinephrine at various times later. Untreated rats were used as controls.

It is to be noted that the rate of disappearance of reserpine from rat heart is more rapid than the rate of repletion of norepinephrine. This is to be expected as the normal half-life of norepinephrine in rat heart is about 9–12 hr,[10, 11] and each granule, once freed of the presence of reserpine, then can only slowly recoup its amine content by biosynthesis. The rate of norepinephrine repletion after reserpine is thus a complex function of the rates of reserpine disappearance and norepinephrine biosynthesis. The above data suggest that norepinephrine-containing granules are reutilized after reserpine has disappeared, as norepinephrine restoration in heart after even a large dose of reserpine (5 mg/kg) occurs within 10–14 days,[12] while the calculated life-span of amine storage granules in the rat is about 35 days.[13] A different view has been presented by Dahlstrom and Haggendal[14] who suggest that norepinephrine repletion must await the formation of new storage granules.

Distribution of ^{3}H-reserpine after differential centrifugation of heart homogenates

Hearts of rats that had received tritiated reserpine 18 hr previously were homogenized and subjected to the schedule of differential centrifugation outlined in Materials and Methods. In another set of experiments, small amounts of the labeled drug were added directly to heart homogenates of untreated animals, followed by differential centrifugation. The similarity of distribution shown by these two sets of data (Table 9) suggests that to a large extent the apparent subcellular distribution of the intravenously administered drug may be artifactual, i.e. that a redistribution of the drug may occur during the homogenation and centrifugation procedures. Tetrabenazine pretreatment of the animals receiving ^{3}H-reserpine was without effect on the relative subcellular distribution of radioactivity. This finding also suggests that the

subcellular distribution obtained by this technique does not reflect the situation *in vivo*.

The results of this study are thus in general agreement with the reports of others[1-3] regarding the persistence of radioactivity in mammalian tissue after administration of labeled reserpine. In addition, evidence is presented here that the small amount of reserpine which remains has pharmacological significance in that it is highly and specifically bound in a persistent manner, as evidenced by the experiments with tetrabenazine and unlabeled reserpine. Furthermore, the strong correlation between catecholamine depletion and the amount of ^{3}H-reserpine remaining provides evidence

TABLE 9. SUBCELLULAR DISTRIBUTION OF ^{3}H-RESERPINE IN RAT HEART*

Cell fraction	Per cent total ^{3}H-reserpine	
	^{3}H-reserpine injected into rat	^{3}H-reserpine added to heart homogenate
Coarse	28·3	41·2
Mitochondrial	20·9	20·8
Microsomal	37·1	21·2
Supernatant	13·7	16·7

* Rats received ^{3}H-reserpine intravenously and were killed 18 hr later. Hearts were removed, homogenized and subjected to differential centrifugation in the cold. ^{3}H-reserpine (3 ng/g) was added to heart homogenates of untreated rats and differential centrifugation performed. Each figure shows the mean value of four experiments.

for the highly significant nature of the reserpine which persists in several organs. In view of this relationship, it seems unnecessary to assume, as has been done previously,[15] that reserpine exerts its effects through injury persisting in the absence of the drug. When small doses are given, reserpine acts *per se* in inducing and maintaining norepinephrine depletion in rat heart. However, the possibility of an irreversible injury when large doses of reserpine are given is not precluded by the findings reported here.

Although ^{3}H-reserpine could not be localized in a single subcellular compartment for reasons described above, it is well established that this drug affects the storage of catecholamine at an intraneuronal site. It is to be recognized that the site of ^{3}H-reserpine binding does not of necessity coincide with the site where its action becomes manifest, i.e. the storage granule. This consideration aside, the following calculations may be made regarding the number of reserpine molecules persistently bound per affected catecholamine storage granule, based on the assumption that this organelle represents the exclusive localization of the tritiated reserpine found in rat heart.

Dahlsrom *et al.*[16] have estimated that each varicosity of the adrenergic neuron contains approximately 5×10^{-3} picogram norepinephrine and that there are about 1500 granules per varicosity. From this information it can be estimated that each granule contains $3{\cdot}3 \times 10^{-18}$ g norepinephrine. This amount, expressed as molecules of the amine, is approximately 10,000 molecules of norepinephrine in each granule. Our finding that each molecule of reserpine is associated with a deficit of about 500 molecules of norepinephrine would indicate that approximately 20 molecules of reserpine are bound to each affected granule.

Independently of these studies, Manara and Garattini[17] have recently reported somewhat similar findings in that they found unchanged ^{3}H-reserpine in rat brain 12 hr after injection of the drug and that pretreatment with tetrabenazine resulted in lower brain reserpine levels.

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