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The relation between the subcellular distribution of [^{3}H]reserpine and its proposed site of action

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The amount of [^{3}H]reserpine retained after injection is greater in the heart, a tissue with a high density of adrenergic innervation than in femoral muscle. Subcellular distribution studies emphasized that 15 and 60 min after injection of [^{3}H]reserpine it could not be shown to be uniquely associated with the noradrenaline-containing granules. The possibility existed that the large amount of reserpine bound to cellular lipids at these times might mask a more specific binding. A reduction in the dose and a lengthening of the time between drug administration and tissue measurement to 18 h revealed a small amount of [^{3}H]reserpine to be bound more firmly to the microsomal or amine granule-containing fraction. Prior saturation of tissue binding sites by treatment with large amounts of unlabelled reserpine or tetrabenazine decreased the retention of subsequently injected [^{3}H]reserpine. This decreased tissue retention resulted in an increase in the formation of [^{3}H]trimethoxybenzoic acid.

A model is proposed relating tissue distribution and binding to reserpine's mechanism of action.

In 1957, Shore, Pletscher & others noted that the ability of reserpine to deplete brain 5-hydroxytryptamine seemed to extend beyond the period that reserpine's presence in the brain was measurable. Subsequently, Shepphard, Tsein & others (1958), using [^{3}H]reserpine, were able to measure trace amounts 72 h after injection, and in 1964, Maggiolo & Haley found traces even after 120 h. Although neither report satisfactorily accounted for the apparent discrepancy between the small amount of reserpine found and the relatively large depletion of amines effected, both experiments suggested that an irreversible damage hypothesis (Shore, Pletscher & others, 1957) need not be the only mechanism of action. Since only whole tissue concentrations of reserpine were measured in the previous work, the possibility exists that reserpine may be concentrated in or bound to a specific subcellular component. As available evidence indicates that reserpine inhibits amine uptake at the adrenergic nerve granule (Bertler, Hillarp & Rosengren, 1961; Stitzel & Lundborg, 1967) it is possible that it is at this subcellular site that a high concentration of reserpine might be found. The present experiments were conducted to test this hypothesis.

METHODS

Male Swiss-Webster mice (18–35 g) were injected via a tail vein with 600 μg/kg of [^{3}H]reserpine (575 mCi/mM) dissolved in 0·25 M sucrose. The animals were killed by a blow on the head either 15 min, 60 min or 18 h after injection. Hearts were

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removed, pooled (6 hearts/group), and homogenized (1000 rev/min, 30 s) using a Teflon pestle (0·005″ clearance) in cold 0·25 M sucrose containing 0·005M phosphate buffer, pH 7·4 and 0·001M $MgCl_2$. Nuclear and mitochondrial fractions were obtained by centrifuging in the cold for 10 min at 600 and 8000 *g*, respectively. The 8000 *g* supernatant was then centrifuged at 105 000 *g* for 60 min in a Spinco model L ultracentrifuge to provide a microsomal and a high speed supernatant fraction. The identification of the fraction was made by standard, albeit arbitrary, designations.

As noradrenaline-containing granules, which are present in the microsomal fraction, can be destroyed by excessive homogenization, the conditions needed to minimize granular loss were assessed. Mice were injected with 1 μg/kg of [^3H]noradrenaline (^3H-NA) (7·5 Ci/mM; i.v.). After 30 min the hearts were removed and homogenized in an ice bath using a Teflon pestle at 1000 rev/min. The extent of homogenization was assessed, after 30, 50, 60 or 70 up-and-down strokes, by differential centrifugation to determine the amounts of noradrenaline contained in the microsomal fraction compared to the amount in the microsomal + supernatant fraction, as this should indicate the extent of granular disruption. The homogenate prepared with 60 strokes yielded the maximum intact granules as judged by a high ratio of microsomal to microsomal and supernatant ^3H-NA and a high total amount of ^3H-NA in these two fractions. All subsequent homogenizations were made using that number of strokes.

Noradrenaline and normetanephrine extraction and analysis

In those experiments in which ^3H-NA was injected, the nuclear, mitochondrial, microsomal and supernatant fractions from mouse heart and femoral muscle were extracted with perchloric acid. Ten ml of 0·4N perchloric acid were added to re-suspend each sediment, while 1 ml of concentrated perchloric acid was added to the high speed supernatant fraction. The suspension was then centrifuged at 9000 *g*, filtered into graduated cylinders, and then stored overnight at −20°. After thawing, each sample was analysed for its ^3H-NA and [^3H]normetanephrine (^3H-NM) content using ion exchange column chromatography (Carlsson & Waldeck, 1963). The columns were eluted with 1N HCl, the first 8 ml was discarded, the next 9 ml contained the ^3H-NA and the third 14 ml fraction contained the ^3H-NM. A 1 ml portion of the eluate was added to a counting vial containing 10 ml of a scintillation mixture [2,5-diphenyloxazol 5 g/litre; 1,4-di-(5-phenyloxazolyl)-benzene 0·9 g/litre, Beckman Bio-Solv 200 ml] and 5 ml of Beckman Triton X-100 and counted using a Packard Tri-Carb scintillation counter. Efficiency was calculated by the use of external standards (approximately 24%).

Reserpine extraction and analysis

Experiments involving the isolation and measurement of [^3H]reserpine utilized a modification of the method of Manara (1967) to extract the reserpine and its principal metabolite, trimethoxybenzoic acid (TMBA). The nuclear, mitochondrial and microsomal sediments were re-suspended twice in 10 ml of acetone and filtered into graduated cylinders and the volume of each fraction determined. Ten ml of chloroform was added to the high speed supernatant fraction which was then shaken for 15 min and centrifuged at 600 g for 10 min to break any emulsion formed. An 8 ml portion of the chloroform phase was then taken for further processing. The filtered acetone or chloroform was then evaporated at 40° under nitrogen and stored at −20°.

The residue was redissolved in 1·1 ml of chloroform containing 2·5 mg/ml of cold reserpine to protect the [^{3}H]reserpine from oxidation and to aid its visualization. 100 μl portions of the chloroform were spotted on silica gel (Warner-Chilcot silica gel G) and developed (approximately 45 min) in cyclohexane–chloroform–diethyl amine (5:4:1; v/v). The reserpine spots were visualized under ultraviolet light and the TMBA (R_F = 0·0) and reserpine (R_F = 0·5) spots were scraped into counting vials. Fifteen ml of a toluene counting solution [2,5-diphenyloxazol 5 g/litre; 1,4-di-2-(5-phenyloxazolyl)-benzene 0·9 g/litre and Beckman Bio-Solv 200 ml/litre] were added. Blanks were from that part of the plate with no radioactivity. External standards were used to calculate the counting efficiency (approximately 45%). The recovery of [^{3}H]reserpine carried through the procedure was approximately 92%. No corrections for recovery have been made.

Portions of each subcellular fraction were analysed for protein (Lowry, Rosebrough & others, 1951) using a Technicon Auto-Analyzer. The level of significance used for all determinations was $P < 0{\cdot}05$ using Student's t-test.

RESULTS

Redistribution of [^{3}H]reserpine due to homogenization. Alpers & Shore (1969) suggested that a redistribution of reserpine may occur during tissue homogenization. To test this we examined the amounts of [^{3}H]reserpine present in each subcellular fraction after both *in vitro* and *in vivo* addition of the labelled compound.

In the *in vitro* experiments, 10 ng of [^{3}H]reserpine (about the amount in heart 18 h after an *in vivo* injection of 600 μg/kg) were added to six mouse hearts before

Table 1. In vitro *and* in vivo *distribution of [^{3}H]reserpine*

Fraction	Percentage distribution *In vitro**	*In vivo*†
Nuclear	45·6 ± 1·5	56·3 ± 3·9 (99)
Mitochondrial	24·0 ± 0·4	15·6 ± 2·0 (15)
Microsomal	8·9 ± 0·1	27·6 ± 3·0 (128)
Supernatant	21·5 ± 1·8	0·5 ± 0·2 (15)

* Values represent the mean percentage of ± s.e. [^{3}H]reserpine found in each subcellular fraction and are based on at least three experiments. 10 ng of [^{3}H]reserpine were added to six mouse hearts before homogenization.

† Values represent the mean percentage of [^{3}H]reserpine found in each subcellular fraction 18 h after the i.v. administration of 600 μg/kg and are based upon three experiments. Figures in parenthesis indicate ng of reserpine retained per gram of protein.

Table 2. *Subcellular distribution of [^{3}H]noradrenaline and [^{3}H]reserpine in cardiac tissue.*

Fraction	[^{3}H]Reserpine 15 min	60 min	% change	[^{3}H]Noradrenaline 15 min	60 min	% change
Nuclear	385 ± 23	143 ± 10	—64	2·08 ± 0·19	1·75 ± 0·16	—15·9
Mitochondrial	86 ± 12	30 ± 2	—65	0·24 ± 0·04	0·25 ± 0·01	+ 4·2
Microsomal	49 ± 3	19 ± 1	—61	0·34 ± 0·03	0·36 ± 0·04	+ 5·8
Supernatant	17 ± 3	8 ± 2	—55	1·54 ± 0·26	1·20 ± 0·07	—22·1

Each value represents the mean content (ng/g) ± s.e. and are based on 5–10 experiments. Mice were killed either 15 or 60 min after the i.v. injection of ^{3}H-NA (1 μg/kg) of [^{3}H]reserpine (600 μg/kg).

homogenization and differential centrifugation. The percentage *in vitro* and *in vivo* distributions differed (Table 1), especially in microsomal and supernatant [³H]reserpine content. The microsomal fraction, i.e. that fraction containing a large amount of catecholamine granules, had three times the [³H]reserpine content after *in vivo* administration than did the same fraction after the *in vitro* addition. If the *in vivo* retention of [³H]reserpine is expressed as amount retained per gram of protein in each fraction then the microsomal fraction contains much more activity than any other subcellular fraction.

Subcellular distribution of [³H]reserpine and ³H-NA in mouse heart 15 and 60 min after injection. [³H]Reserpine was found in all subcellular fractions 15 min after its intravenous administration. The concentration was highest in the nuclear fraction > mitochondrial > microsomal > supernatant fractions (Table 2). During the next 45 min, all fractions showed similar percentage decreases in reserpine content. The total amount of [³H]reserpine remaining in the heart 60 min after injection was about 37% of that present 15 min after injection.

The subcellular distribution of ³H-NA in the mouse heart is also shown in Table 2. Sixty min after injection the nuclear and supernatant fractions showed small but significant decreases in their ³H-NA content. The microsomal and mitochondrial fractions showed no change. Total cardiac ³H-NA concentrations declined by only about 15% between 15 and 60 min post-injection. The relative distribution of ³H-NA in the various fractions differed markedly from that of reserpine: ³H-NA was highest in the nuclear fraction > supernatant > microsomal > mitochondrial fractions.

Retention of [³H]reserpine, ³H-TMBA and ³H-NA in heart and femoral muscle. [³H]Reserpine (600 μg/kg) was administered to groups of 3–10 mice via tail vein. The animals were killed either 15 or 60 min later and the concentrations of [³H]-reserpine and ³H-TMBA were determined in both heart and femoral muscle. Approximately 537 (±36 s.e.) ng/g of [³H]reserpine were found in cardiac tissue within 15 min. This fell to 200 (±18) ng/g at 60 min (63% reduction). In the femoral muscle 44 (±4) ng/g were recovered 15 min after drug administration and only 16 (±3) ng/g after 60 min (a reduction of 64%).

Fifteen min after the injection of [³H]reserpine, 74 ± 2 and 14 ± 2 ng/g of ³H-TMBA were detected in heart and femoral muscle, respectively. After 60 min these values had declined to 61 ± 7 and 6 ± 1 ng/g.

At the 15 and 60 min intervals ³H-NA was taken up and retained in cardiac tissue to a much greater extent (4·1 ± 0·3 and 3·7 ± 0·2 ng/g) than in femoral muscle (0·2 ± 0·01 and 0·1 ± 0·02 ng/g) after injection of 1 ng/g of ³H-NA (i.v.). The rate of disappearance of the ³H-NA was much slower from heart than from femoral muscle, the amount in heart after 60 min was 89% of its 15 min concentration while that in femoral muscle was only 48%.

Distributions of two doses of [³H]reserpine in subcellular fractions of the mouse heart 18 h after administration. It is possible that the relatively large amount of reserpine (600 μg/kg) flooded the tissue and masked a smaller more specific reserpine binding. To test this, groups of mice were injected with either 600 or 300 μg/kg of [³H]reserpine and killed 18 h later. Table 3 shows that not only was the percentage of the dose retained after 300 μg/kg less than that after 600 μg/kg, but also that the subcellular

Table 3. *Subcellular distribution of* 300 *and* 600 *μg/kg of* [^3H]*reserpine* 18 *h after injection.*

Fraction	600 μg/kg*	% of total	300 μg/kg*	% of total
Nuclear	7·42 ± 0·50	82·0	2·81 ± 0·56	69·5
Mitochondrial ..	0·51 ± 0·01	6·0	0·32 ± 0·07	7·9
Microsomal	0·77 ± 0·14	8·5	0·73 ± 0·02	18·0
Supernatant	0·38 ± 0·06	4·2	0·19 ± 0·04	4·7
Total	9·08 ± 1·3		4·05 ± 0·31	

* Each value represents the mean [^{3}H]reserpine content (ng/g) ± s.e. in the mouse heart and is based on at least three experiments.

distribution was changed. An increased relative percentage of [^{3}H]reserpine was found in the microsomal fraction after the low dose.

Effect of pretreatment with reserpine or tetrabenazine on the subcellular distribution of [^3H]*reserpine.* Either reserpine (5 mg/kg, i.p.) or tetrabenazine (100 mg/kg, i.p.) were given 1 h before 300 μg/kg (i.v.) of [^{3}H]reserpine and 18 h later the animals were killed. Pretreatment with unlabelled reserpine or tetrabenazine significantly ($P < 0·05$) reduced the [^{3}H]reserpine content in the nuclear and microsomal fractions (Table 4). No [^{3}H]reserpine could be detected in the supernatant fraction from pretreated animals.

Effect of reserpine pretreatment on ^{3}H-TMBA content of the mouse heart. Results from animals pretreated with unlabelled reserpine (5 mg/kg, i.p.) 1 h before [^{3}H]-reserpine and killed 18 h later showed in a significant ($P < 0·01$) increase in the amount of ^{3}H-TMBA found in the mouse heart (control 1·25 ± 0·02, pretreated 2·2 ± 0·02 ng/g). In control animals the [^{3}H]reserpine/^{3}H-TMBA ratio was 2·6 while in mice given unlabelled reserpine this ratio fell to 0·9, indicating the presence of more metabolite than parent compound in the treated group.

DISCUSSION

Using a procedure minimizing granule disruption we have previously demonstrated that ^{3}H-NA does not undergo redistribution during tissue homogenization (Stitzel & Lundborg, 1967). Reserpine, however, is a more lipid-soluble drug and may not be bound as firmly to tissue component as noradrenaline. The *in vitro* addition of [^{3}H]reserpine to heart shows that only 9% of the total cardiac reserpine is bound

Table 4. *Effect of reserpine or tetrabenazine pretreatment on the cardiac subcellular distribution of subsequently injected* [^3H]*reserpine.*

		Pretreatment	
Fraction	Control	Reserpine	Tetrabenazine
Nuclear	2·58 ± 0·25	1·34 ± 0·16*	1·41 ± 0·14*
Mitochondrial ..	0·29 ± 0·06	0·29 ± 0·08	0·31 ± 0·05
Microsomal ..	0·40 ± 0·03	0·12 ± 0·08*	0·09 ± 0·01*
Supernatant ..	0·06 ± 0·02	—	—
Total..	3·34 ± 0·13	1·84 ± 0·10*	1·81 ± 0·20*

Mice were injected with [^{3}H]reserpine (300 μg/kg) 1 h after the i.p. administration of either reserpine (5 mg/kg) or tetrabenazine (100 mg/kg) and killed 18 h after receiving the labelled drug. Each value is the mean ± s.e. [^{3}H]reserpine content (ng/g) of 4–13 experiments.
* $P < 0·05$ compared with control.

to amine granules. Our *in vivo* studies showed that the granular binding of [^{3}H]-reserpine was some three times that found *in vitro*. This suggests that the *in vivo* distribution of [^{3}H]reserpine cannot be accounted for solely on the basis of redistribution during homogenization and, at least in part, must reflect the actual retention of the tritiated compound.

Wagner & Stitzel (1969) demonstrated that shortly after administration of [^{3}H]-reserpine the *in vivo* distribution closely paralleled the distribution of total lipid in each cardiac subcellular fraction. The microsomal fraction did not contain an unusually high proportion of the total [^{3}H]reserpine present 15 or 60 min after its injection. Alpers & Shore (1969) did not find [^{3}H]reserpine localized in a single subcellular compartment, but because their dose was large, much of the drug was bound to lipid so a smaller more specific binding could have been masked. Therefore, we reduced the dose and waited 18 h before assessing the distribution and demonstrated an increase in the relative percentage retention of [^{3}H]reserpine associated with the microsomal fraction. Thus a small amount did appear to be more tightly bound to the amine storage granules. These findings are consistent with those of Kirshner (1962, 1965), who demonstrated a firm binding of reserpine to adrenal chromaffin granules *in vitro*.

Further support for the contention that a portion of administered [^{3}H]reserpine is associated specifically with adrenergic neurons is found in our comparative studies of the retention of noradrenaline and reserpine in skeletal and cardiac muscle. The retention of ^{3}H-NA and [^{3}H] reserpine in the skeletal muscle was only 5·1 and 8·2% respectively of that in cardiac muscle, while the corresponding lower density of adrenergic innervation in skeletal muscle probably accounts for the decreased binding. Carlsson, Lundborg & others (1967) reasoned similarly for the uptake and disappearance of ^{3}H-α-methylnoradrenaline.

In agreement with Manara (1967) and Alpers & Shore (1969) we found a significant decrease in total tissue [^{3}H]reserpine retention as a result of pretreatment with reserpine. This decrease suggests that a portion of the labelled reserpine is specifically bound. Furthermore, we found that the most pronounced impairment of [^{3}H]-reserpine retention occurred in the nuclear and microsomal fractions. The nuclear fraction is heterogeneous, containing unbroken cells, nuclei and cell membrane fragments (Gösta Jonsson, personal communication). Thus changes in its [^{3}H]reserpine content are difficult to interpret. However, the decrease in microsomal [^{3}H]reserpine content after reserpine and tetrabenazine pretreatment appears to provide direct evidence of a small, but significant, amount of specific binding in this fraction.

Pretreatment of animals with unlabelled reserpine resulted in a doubling of the amount of ^{3}H-TMBA found in the heart after injection of [^{3}H]reserpine. This increase in metabolite formation is likely to be the result of the decreased availability of binding sites in sympathetic nerves which leads to an increase in the amount of [^{3}H]reserpine available for metabolism.

We would like to propose the following model of reserpine's distribution and mechanism of action. After a single intravenous injection, most of the circulating [^{3}H]reserpine is either metabolized by liver enzymes (Stawarz & Stitzel, 1971) or is taken up in the lipid depots of the body. A much smaller amount may associate with the sympathetic granular membrane and bind in a relatively specific manner to the vesicle. It is possible that the lipid and granular pools are in equilibrium

and that the large lipid pool of [^{3}H]reserpine may serve as a source of available reserpine for the continued inhibition of the granular uptake mechanism. In the proposed model, inhibition of amine uptake would continue either until all storage and binding sites had lost their reserpine content, or possibly, until the arrival of sufficient quantities of fresh, uninhibited granules.

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