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Reserpine-Like Effects of Harmine on Isolated Adrenal Medullary Vesicles

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SUMMARY

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Because of its structural similarities to reserpine, the actions of harmine on adrenal medullary vesicles were examined in order to clarify the mechanisms involved in drug effects on amine uptake and storage. Harmine markedly inhibited the ATP-Mg^{2+}-dependent incorporation of epinephrine into isolated rat adrenal medullary vesicles. The inhibition was both competitive and reversible; K_m for epinephrine was 37 μM and K_i for harmine was 3.2 μM. Metaraminol incorporation, which is predominantly ATP-Mg^{2+}-independent and reserpine-insensitive, was decreased only 23% by equimolar concentrations of harmine (which caused 85% inhibition of epinephrine incorporation). Harmine had no effect on efflux of epinephrine from adrenal medullary vesicles, indicating that the effect on incorporation was due solely to inhibition of uptake. No temperature-dependent uptake of harmine into the vesicles was detected, implying that the effects on the uptake system are purely inhibitory. Harmine in equimolar concentrations inhibited binding of epinephrine and serotonin to bovine adrenal vesicle membranes by 43% and 13%, respectively, but did not inhibit binding of metaraminol. Harmine strongly inhibited monoamine oxidase but had no effect on the activities of the catecholamine-synthesizing enzymes tyrosine hydroxylase, dopa decarboxylase, and dopamine β-hydroxylase. The data suggest that the β-carboline section of the harmine and reserpine structures is responsible for affinity for the amine uptake mechanism and for inhibition of uptake, while the remainder of the reserpine molecule confers irreversibility. A mobile carrier in the adrenal vesicle membrane may be the site of action.

INTRODUCTION

Isolated adrenal medullary vesicles incorporate catecholamines by at least two temperature-dependent mechanisms, one of which is stimulated by ATP-Mg^{2+} and inhibited by reserpine (1–4), and a second mechanism which is neither stimulated by ATP-Mg^{2+} nor inhibited by reserpine (3–6). Epinephrine is incorporated by the reserpine-sensitive system, whereas most non-catecholamines (such as metaraminol) are incorporated by the insensitive system (3–6). However, serotonin, an indoleamine, displays a higher affinity for ATP-Mg^{2+}-stimulated incorporation than epinephrine, despite the fact that its stability of storage within the vesicle is less than that of the

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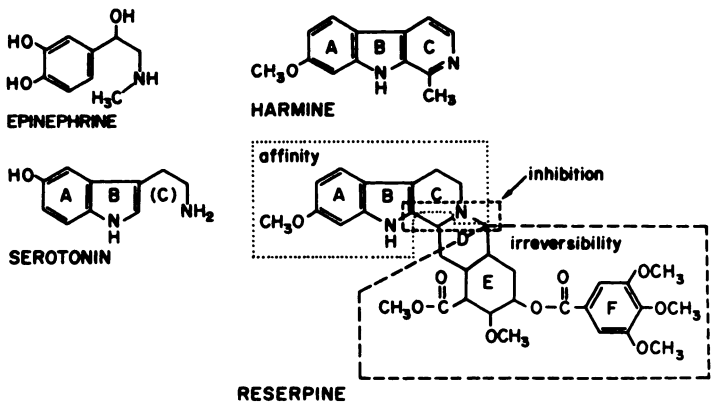

FIG. 1. *Structures of some substances with high affinity for vesicular uptake system, showing relative contributions of different parts of reserpine molecule to actions of reserpine*

catecholamines (4). These and additional results suggest that catecholamine uptake into adrenal storage vesicles proceeds via carrier-mediated transport (7–11).

Thus, although the adrenal storage vesicles normally contain catecholamines, the uptake mechanism shows a preference for indoleamines. Reserpine, which inhibits uptake in a competitive but irreversible manner (1, 2, 12), is also an indoleamine structurally related to serotonin (Fig. 1). In order to examine the characteristics which determine the affinity of a given indoleamine for uptake or its ability to inhibit uptake, as well as its degree of reversibility, it is necessary to look at molecules which have characteristics in between those of serotonin and reserpine. One substance with these characteristics is the monoamine oxidase inhibitor (13) harmine (7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole) (Fig. 1), an alkaloid obtained from *Banisteria caapi* (14). Harmine also has physicochemical characteristics (lipid solubility and pK_b) which approximate those of reserpine. This report describes the effects of harmine on the uptake and efflux of amines in rat adrenal medullary vesicles, effects on the binding of amines to vesicle membranes, and effects on several of the enzymes involved in epinephrine biosynthesis and degradation. These data provide useful information about the structural components of the amine molecule which determine whether the substance will attach to the putative carrier, whether it will utilize or inhibit the transport system, and whether inhibition is likely to be reversible or irreversible.

METHODS

Male Wistar rats (Hilltop Lab Animals) weighing 200–300 g were killed by decapitation. Their adrenal glands were excised, cleaned of fat and connective tissue, and homogenized in ice-cold 300 mM sucrose in an all-glass apparatus containing 25 mM Tris, pH 7, and 0.01 mM iproniazid, an irreversible monoamine oxidase inhibitor.

Amine uptake. The adrenal glands from eight rats were homogenized in 20 ml of ice-cold sucrose–Tris–iproniazid and centrifuged at 800 × *g* for 10 min. The pellet was discarded, and 0.5-ml aliquots of the supernatant fraction were added to tubes containing 0.1 mM epinephrine, 5 mM ATP-Mg^{2+}, various concentrations of harmine, and either 1 μCi of [^{14}C]epinephrine or 5 μCi of 0.1 mM [^{3}H]metaraminol. Sucrose–Tris was added to give a final volume of 1 ml. Tubes were incubated in a water bath at 30° for 30 min and shaken gently while duplicate tubes were kept on ice. Under these conditions uptake occurs solely into storage vesicles (15). Uptake was stopped by addition of 2 ml of ice-cold sucrose–Tris, and the samples were centrifuged for 10 min at 26,000 × *g*. The supernatant fractions were decanted, added to an equal volume of 7% perchloric acid, centrifuged to remove precipitated proteins, and analyzed for radio-

activity and catecholamines (15). The vesicular pellets were washed and recentrifuged twice with fresh sucrose–Tris, and the final pellet was resuspended by homogenization with a glass pestle in a glass tube in 3 ml of 3.5% perchloric acid. After centrifugation to remove insoluble material, the samples were analyzed for catecholamines and radioactivity. The temperature-dependent amine uptake was calculated as described previously (15). For K_m and K_i determinations, the harmine concentration was 5 μM and the epinephrine concentration was varied from 5 to 80 μM.

The uptake of harmine was determined by direct fluorometric measurement as described by Slotkin and DiStefano (16). The excitation wavelength was 365 nm, and the emission wavelength 419 nm.

Efflux of epinephrine. The efflux of endogenous catecholamines and of newly incorporated [^{14}C]epinephrine was measured by the procedure of Slotkin and Kirshner (15). Washed, [^{14}C]epinephrine-labeled vesicles were prepared as described previously (15), and two sets of eight 0.5-ml aliquots in sucrose–Tris, with or without 0.1 mM harmine (final volume, 1 ml), were brought to 30° to allow efflux to occur. Efflux was stopped by addition of 2 ml of ice-cold sucrose–Tris after 0, 5, 10, 20, 30, 40, and 60 min. Samples were centrifuged, and the supernatant solutions were decanted, acidified with 0.02 ml of 3 N HCl, and analyzed for catecholamines and radioactivity. The vesicular pellets were lysed and analyzed as described above, and efflux was calculated (3).

Reversibility of harmine inhibition. The 800 × *g* supernatant fraction was divided into four aliquots; in the first two, uptake of [^{14}C]epinephrine in the presence and absence of 0.1 mM harmine was determined as described above, while the other two were incubated with no [^{14}C]epinephrine added to either the harmine-free or harmine-containing samples. Vesicles from the latter incubations were washed twice and resuspended in fresh sucrose–Tris, and uptake of [^{14}C]epinephrine was determined in the absence of any additional harmine. The initial inhibition of uptake calculated from the first two samples was then compared with the inhibition in the vesicles in the last two, in which the preparation was exposed to harmine and washed prior to determination of uptake.

Binding of amines to adrenal medullary vesicle membranes. Purified bovine adrenal medullary vesicle membranes were prepared according to Slotkin and Kirshner (8). Binding of 0.1 mM [^{14}C]epinephrine, [^{3}H]metaraminol, and [^{14}C]serotonin was determined in the presence of 5 mM ATP-Mg^{2+}, with and without 0.1 mM harmine.

Enzyme activities. Monoamine oxidase and dopamine β-hydroxylase were determined in water homogenates of rat adrenals, while tyrosine hydroxylase was determined in the 26,000 × *g* supernatant fraction of glands homogenized in isotonic KCl. Hog kidney dopa decarboxylase (obtained from Dr. N. Kirshner) was prepared by the method of Waymire *et al.* (17). Harmine concentrations in each assay varied from 0.1 to 100 μM. Monoamine oxidase activity was measured by the procedure of Laduron and Belpaire (18), using [^{3}H]tyramine (10 μM) as substrate. Dopamine β-hydroxylase was assayed by the method of Friedman and Kaufman (19), using [^{3}H]tyramine (10 μM) as substrate (3, 20). Tyrosine hydroxylase and dopa decarboxylase activity were measured by the method of Waymire *et al.* (17), using [^{14}C]tyrosine (100 μM) or [^{14}C]dopa (33 μM) as substrate.

Analytical procedures. Catecholamine analyses were done by the trihydroxyindole method, using a Technicon AutoAnalyzer (3). Radioactive amines were measured by liquid scintillation spectrometry as described previously (4).

Statistical analyses. Data are reported in terms of control values and percentages of control. Levels of significance were calculated by Student's *t*-test, and straight lines were determined by the method of least squares (21).

Materials. [^{14}C]Epinephrine, [^{14}C]serotonin, [^{14}C]dopa, [^{3}H]metaraminol, [^{3}H]tyramine, and [^{14}C]tyrosine were obtained from New England Nuclear Corporation. Epinephrine bitartrate was purchased from Winthrop Laboratories, and metaraminol bitartrate from Merck Sharp & Dohme. Serotonin

TABLE 1

Effect of harmine on amine uptake into rat adrenal medullary vesicles

The Epinephrine and metaraminol concentrations were 100 μM. Values are means ± standard errors of the number of determinations shown in parentheses.

Harmine concentration	Epinephrine uptake[a]	Inhibition	Metaraminol uptake[a]	Inhibition
μM		%		%
0	15.6 ± 0.5 (3)		3.5 ± 0.2 (3)	
1	16.4 ± 0.4 (2)		4.0 ± 0.2 (3)	
10	8.7 ± 1.1 (3)	44 ± 7[b]	3.4 ± 0.2 (3)	
100	2.4 ± 0.5 (3)	85 ± 4[c]	2.7 ± 0.04 (3)	23 ± 1[d]

[a] Nanomoles taken up in 30 min per 100 μg of endogenous catecholamines.
[b] $p < 0.005$.
[c] $p < 0.001$.
[d] $p < 0.02$.

creatinine phosphate was obtained from Calbiochem, and harmine hydrochloride was purchased from Aldrich Chemical Company.

RESULTS

The effect of harmine on the temperature-dependent uptake of epinephrine and metaraminol into rat adrenal medullary vesicles in the presence of ATP and Mg^{2+} is shown in Table 1. Harmine in a concentration of 10 μM produced approximately half-maximal inhibition of epinephrine uptake but no inhibition of metaraminol uptake, while 100 μM harmine caused nearly complete inhibition of epinephrine uptake but inhibited metaraminol uptake only 23%.

The efflux of [^{14}C]epinephrine and endogenous catecholamines is shown in Fig. 2. Harmine had no effect on efflux, indicating that blockade of epinephrine uptake by harmine results solely from reduced influx rather than accelerated efflux.

The inhibition of epinephrine uptake by harmine was competitive, as shown by the Lineweaver-Burk plot (22) in Fig. 3. Since harmine and epinephrine compete for the same site involved in uptake, the degree of inhibition is a function of the ratio of harmine and epinephrine concentrations. For epinephrine, K_m was (37 ± 2) μM, which is somewhat lower than K_m values determined for epinephrine uptake into bovine adrenal medullary vesicles (5, 12). For harmine, K_i was (3.2 ± 0.3) μM, indicating

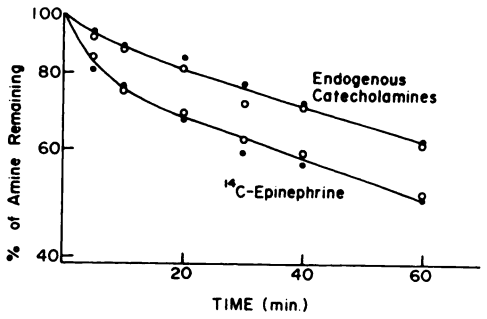

FIG. 2. *Efflux of* [^{14}C]*epinephrine and endogenous catecholamines from rat adrenal medullary vesicles (logarithmic ordinate) in the presence* (○) *and absence* (●) *of 100 μM harmine*

At zero time, endogenous levels were 3.52 μg of catecholamines and 11,709 cpm, and exogenous levels were 0.4 μg and 3680 cpm. Specific activity during labeling was 0.1 μCi/μg of catecholamines.

that harmine has 10 times the affinity of epinephrine for the site involved in ATP-Mg^{2+}-stimulated uptake.

When adrenal medullary vesicles were exposed to harmine and then washed prior to determination of epinephrine uptake, most of the inhibition was lost (Table 2), indicating that the inhibition was reversible. In contrast, inhibition of uptake by reserpine cannot be reversed by washing the treated vesicles (1, 2, 23). Since harmine competitively inhibited the incorporation of epinephrine, it was important to determine whether harmine itself utilizes the uptake system.

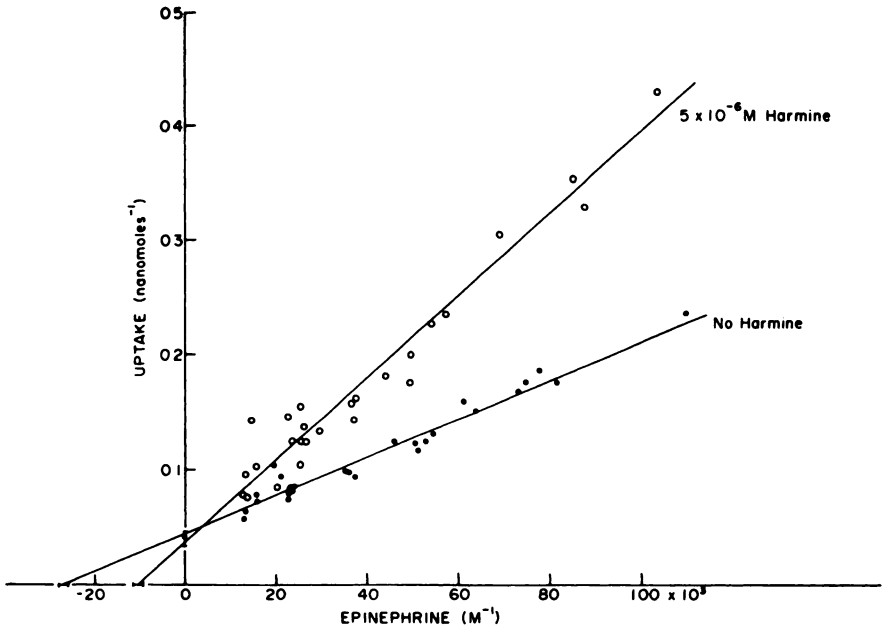

FIG. 3. *Lineweaver-Burk plot of epinephrine uptake into rat adrenal medullary vesicles*
●, no harmine; ○, 5 μM harmine. Bars indicate the standard errors of the intercepts. Lines were fitted by the method of least squares. Intercepts on the ordinate are not significantly different ($p > 0.05$); intercepts on the abscissa are significantly different ($p < 0.001$).

Table 3 shows that, in contrast to epinephrine, there was no temperature-dependent uptake of harmine into the vesicles. Thus harmine produces a purely inhibitory effect.

Besides inhibiting catecholamine uptake, harmine is a well-known inhibitor of monoamine oxidase (13). In order better to define its range of actions, its effects on dopamine β-hydroxylase, tyrosine hydroxylase, and dopa decarboxylase activities were compared with those on monoamine oxidase (Table 4). Harmine did not inhibit the enzymes involved in epinephrine synthesis.

The binding of amines to purified bovine adrenal vesicle membranes was studied under conditions identical with those used during determinations of uptake, except that the medium was 10 mM Tris. Amine concentration was 0.1 mM, and ATP-Mg^{2+} was 5 mM. The binding followed the order serotonin > epinephrine > metaraminol (Table 5). Harmine in equimolar concentrations had no effect on the binding of metaraminol, a small inhibitory effect (13%) on serotonin, and a larger inhibitory effect (43%) on epinephrine.

TABLE 2
Reversibility of harmine inhibition
Values are means ± standard errors of four determinations.

Treatment	Epinephrine uptake	
	Initial	Washed twice
	nmoles/100 μg catecholamine	
Control	17.1 ± 0.4	15.2 ± 0.3
Harmine (100 μM)	2.9 ± 0.1	11.8 ± 0.4
Inhibition	83 ± 0.5%	22 ± 2%

DISCUSSION

Harmine is structurally related to reserpine (Fig. 1) and is similar in its effects on isolated adrenal medullary vesicles, in that it competitively inhibits epinephrine uptake but has little effect upon uptake of the non-catecholamine, metaraminol. Since metaraminol is taken up predominantly by a non-stimulated mechanism (3–6), harmine, like reserpine, only inhibits uptake which proceeds via the ATP-Mg^{2+}-stimulated pathway. Neither harmine nor reserpine (3) affects the efflux of amines, indicating that

TABLE 3

Incorporation of harmine into adrenal medullary vesicles.

Values are means ± standard errors of the number of determinations shown in parentheses.

Amine	Uptake		Temperature-dependent uptake
	0°	30°	
	nmoles/30 min/100 μg endogenous catecholamines		
Epinephrine (100 μM)	2.7 ± 0.3 (2)	22.4 ± 1.0 (4)	19.7 ± 1.0
Harmine (30 μM)	10.0 ± 0.4 (2)	9.7 ± 0.2 (4)	−0.3 ± 0.3

TABLE 4

Effect of harmine on enzymes involved in catecholamine synthesis and degradation

Values are means ± standard errors of five determinations.

Enzyme	Activity at various harmine concentrations				
	0	0.1 μM	1 μM	10 μM	100 μM
	%	%	%	%	%
Monoamine oxidase[a]	100 ± 7	18 ± 1	13 ± 1	12 ± 1	11 ± 1
Dopamine β-hydroxylase[b]	100 ± 1	103 ± 1	102 ± 1	102 ± 1	103 ± 1
Tyrosine hydroxylase[c]	100 ± 6	113 ± 2	107 ± 3	110 ± 2	93 ± 3
Dopa decarboxylase[d]	100 ± 3	103 ± 3	102 ± 3	99 ± 6	94 ± 5

[a] Rat adrenal; control activity was 0.29 ± 0.02 nmole/gland/hr.
[b] Rat adrenal; control activity was 0.62 ± 0.01 nmole/gland/hr.
[c] Rat adrenal; control activity was 6.9 ± 0.4 nmoles/gland/hr.
[d] Hog kidney; control activity was 30 ± 1 nmoles/hr/10 μl of enzyme preparation.

the reduced incorporation of catecholamines is due to an effect on influx. Furthermore, because the rate of efflux is a measure of stability of amine storage (3), the reduced influx must represent an effect prior to storage. Several observations in this study suggest that the system involved is a mobile carrier in the vesicle membrane.

1. Uptake is saturable despite the fact that the total storage capacity is far greater than the amount taken up. Thus the limiting factor in incorporation is not storage but is probably the translocation of the amine to the inside of the vesicle, and it is this process which is inhibited by harmine. Since the limiting process is saturable and competitive and displays energy (ATP) dependence, a carrier process is likely to be involved.

2. The binding of amines to purified vesicle membranes followed the same order of specificity as affinity for uptake into intact vesicles (serotonin > epinephrine > metaraminol), and harmine, which inhibited uptake of epinephrine but not of metaraminol, reduced the binding of epinephrine but not of metaraminol. Harmine reduced the binding of serotonin to a small extent, which is consistent with earlier reports (4, 8) that serotonin has a higher affinity for uptake than epinephrine. Other inhibitors of uptake (reserpine and *N*-

TABLE 5

Effect of 100 μM harmine on binding of amines to purified bovine adrenal medullary vesicle membranes

Values are means ± standard errors of four determinations.

Amine (100 μM)	Binding		Inhibition
	−Harmine	+Harmine	
	nmoles/mg protein		%
Epinephrine	9.7 ± 1.0	5.5 ± 0.7[a]	43 ± 7
Serotonin	103 ± 4.5	89.1 ± 2.7[b]	13 ± 2
Metaraminol	4.5 ± 0.6	3.8 ± 0.4[c]	

[a] $p < 0.02$.
[b] $p < 0.05$.
[c] Not significant.

ethylmaleimide) have effects upon binding which are similar to those of harmine (8). In addition, the K_{diss} of binding is close to the K_m of uptake (8).

These data suggest that the first step in amine incorporation is binding to a carrier and that it is this process for which epinephrine, serotonin, harmine, and reserpine compete. Why are epinephrine and serotonin taken up by the vesicles while harmine and reserpine, which also have high affinities, are inhibitors? An examination of the structures of these molecules (Fig. 1) suggests that specific molecular features are responsible for affinity, inhibition, and irreversibility. Serotonin has a higher affinity for uptake than any of the catecholamines but is not stored as stably; thus the indoleamine nucleus, which is also present in harmine and reserpine, is probably responsible for the high affinity of these compounds for the putative carrier.

Two characteristics differentiate the structures of serotonin and harmine: alteration of the location and nature of the A-ring substituent, and completion of and substitution on the C-ring (Fig. 1). While both factors may play a role in converting a compound which utilizes the carrier (serotonin) to one which inhibits it (harmine), the C-ring alterations are probably the most important. Deserpidine, which lacks any A-ring substituent, is equipotent with reserpine, which has the methoxy group in the same position as in harmine (24, 25). Furthermore, studies with phenethylamines have shown that ring substituents generally do not alter affinity for uptake, but affect stability of storage (3, 4). Thus the major determinant in the inhibitory actions of harmine is probably closure and substitution of the C-ring.

Reserpine can be viewed as an extensively substituted harmine derivative (Fig. 1). The effects of reserpine and harmine on storage vesicles are strikingly similar with one notable difference: harmine demonstrates reversible inhibition while reserpine is apparently irreversible. The irreversibility of reserpine action may be attributed to the D-E-F system (Fig. 1), which is spatially removed from the indoleamine moiety (24). The importance of this portion of the reserpine molecule is emphasized by the lack of pharmacological effect of stereoisomers in the D-E-F system (24). This suggests that D-E-F binding occurs at a specific site which is near, but not identical with, the binding site for the indoleamine moiety. There may be an "accessory" site for binding of the D-E-F system analogous to the accessory sites proposed for binding of pharmacological antagonists to receptors (26). The importance and specificity of this type of binding in compounds with reserpine-like actions are further illustrated by the diminished effect of reserpinine (25), which has a harmine-like A-B-C structure but is very different in the D-E-F system. It appears that unless the "fit" of the accessory D-E-F portion is correct, there is hindrance of the reserpine-like effect.

Additional factors may play roles in the effects *in vitro* and *in vivo* of compounds resembling reserpine. Alterations in charge, pK_b, or lipid solubility act to reduce the activity *in vitro* of compounds like reserpic acid and serpentine (25), while alterations in distribution, excretion, and metabolism may account for the lack of activity *in vivo* of methyl reserpate, which is effective *in vitro* (24, 25). Similar factors probably limit the duration of the reserpine-like actions of harmine *in vivo*; it is rapidly metabolized and excreted, and since the action is reversible, the rapid destruction of harmine would result in a short-lived effect (27, 28). It is difficult to evaluate the extent of reserpine-like action of harmine in the intact animal for several reasons. First, inhibition is reversible. Vesicular uptake measured *in vitro* after harmine administration *in vivo* is normal, because the drug is washed out during preparation of the vesicles. Second, precursor studies are confounded by the many other actions of harmine (monoamine oxidase inhibition, ganglionic blockade cholinesterase inhibition, cardiovascular effects, etc.) which affect distribution and disposition of radiolabeled precursors (29). However, there is indirect pharmacological evidence that harmine may indeed exhibit a reserpine-like action *in vivo* (29). It is well known that the combined administration of reserpine and monoamine oxidase inhibitors

results in signs of profound sympathetic stimulation such as hypertension and cardiac arrythmias (30). The administration of harmine produces a reflexly mediated decrease in blood pressure, but this is followed by a prolonged period of hypertension, cardiac arrhythmias, and extreme sensitivity to small doses of catecholamines (29), which perhaps reflects the combination of reserpine-like actions and inhibition of monoamine oxidase.

The K_m for uptake of epinephrine reported here is considerably below the estimated values of 400–800 μM in bovine adrenal vesicles (5, 12). A species difference seems unlikely, since the K_m calculated from the data of Viveros *et al.* (31) in rabbit adrenal vesicles is close to that in the present work (40 μM). The K_m values calculated for bovine vesicles are almost certainly too high, owing to the high concentrations of catecholamines utilized in the determinations; the unstimulated uptake mechanism, which saturates at higher concentrations (3), probably contributed significantly to the uptake in the earlier studies, resulting in an apparent K_m in between those of the two uptake processes.

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