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Uptake, Storage, and Distribution of Amines in Bovine Adrenal Medullary Vesicles

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SUMMARY

The biphasic nature of the efflux of amines from isolated adrenal storage vesicles suggests that there are two storage pools. The uptake, storage, and distribution of 11 amines were determined to elucidate the structural specificity of the uptake mechanism and storage system in each pool. The half-times for efflux of newly incorporated amines from the two pools were 5 min ("fast pool") and 0.5–3 hr ("slow pool"). Both pools were located in the heavy vesicle fraction, as demonstrated by density gradient centrifugation of vesicles labeled with ^{14}C-epinephrine (a slow pool seeker) and ^{3}H-metaraminol (a fast pool seeker). The following moieties increased fast pool uptake when substituted on the basic β-phenylethylamine structure: *p*-hydroxyl, *m*-methoxy, and α-methyl. Slow pool uptake was increased by *p*- and *m*-hydroxyl substitution and by replacement of the phenyl ring with 5-hydroxyindole. β-Hydroxyl and catechol groups contributed to the stability of storage in the slow pool, but not in the fast pool. Amines which enter the slow pool were more dependent on ATP-Mg^{2+}-stimulated uptake (and therefore more sensitive to blockade of uptake by reserpine) than were amines which primarily enter the fast pool. Other amines inhibited the uptake of epinephrine in both pools, and the slow pool uptake mechanism was saturated at lower substrate concentrations. β-Phenylethylamine was not taken up into either pool, but inhibited epinephrine incorporation, suggesting that the uptake process is independent of the storage process. Binding of amines to chromogranins cannot account for the storage of amines in either pool, but may contribute to the specificity of binding in the slow pool.

INTRODUCTION

Independent studies by Kirshner (1) and by Carlsson and co-workers (2) demonstrated the ability of isolated catecholamine storage vesicles from the adrenal medulla to incorporate catecholamines, utilizing an ATP-Mg^{2+}-stimulated system. The stimulated uptake is blocked by reserpine. Subsequent studies of metaraminol uptake by Lundborg (3) and Lundborg and Stitzel (4) revealed that there is a second mechanism of amine uptake, which is not stimulated by ATP and Mg^{2+} and is not blocked by reserpine.

Recent work from this laboratory has demonstrated that there are at least two pools for newly incorporated amines in the storage vesicles (5). One pool had a high turnover rate ("fast pool") and little dependence on stimulated uptake (and was

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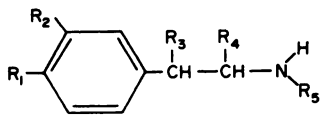
therefore relatively insensitive to reserpine blockade), and it incorporated metaraminol to a greater extent than it incorporated epinephrine. The second pool had a low turnover rate ("slow pool") and considerable dependence on stimulated uptake (and was therefore sensitive to reserpine), and it incorporated epinephrine to a greater extent than metaraminol. Chase experiments indicated that the two pools were not interconnected. These observations strongly suggested the existence of two populations of storage vesicles with different uptake and storage systems. The concept of populations of granules with differing capabilities for amine storage has also been suggested by the studies of Hillarp (6) and Lishajko (7). Hillarp proposed a scheme with a large store of amines associated with granules of high density and stability, and a smaller store, bound less stably to granules of lower density. Lishajko (7) demonstrated a more rapid initial release of norepinephrine than of epinephrine, and suggested that this difference in rates represented sequestration into different vesicle populations.

The current study was undertaken to obtain additional information on the characteristics of the two pools. These investigations include a study in detail of the relationships among structure, uptake, distribution, and storage of amines in isolated vesicles; examination of the ability of ATP and Mg^{2+} to stimulate, and of reserpine to inhibit, the uptake of the various amines; determination whether the uptake of epinephrine into either storage pool can be blocked with an appropriate amine; and examination of the binding of the amines to chromogranins and its relationship to storage specificity in the two pools.

The structures of the amines studied appear in Fig. 1.



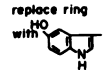
Compound	R_1	R_2	R_3	R_4	R_5
β-phenylethylamine	H	H	H	H	H
Tyramine	OH	H	H	H	H
Octopamine	OH	H	OH	H	H
Norepinephrine	OH	OH	OH	H	H
Epinephrine	OH	OH	OH	H	CH_3
Isoproterenol	OH	OH	OH	H	i-C_3H_7
Normetanephrine	OH	OCH_3	OH	H	H
Amphetamine	H	H	H	CH_3	H
Metaraminol	H	OH	OH	CH_3	H
DOPA	OH	OH	H	COOH	H
Serotonin	replace ring HO with 		H	H	H

FIG. 1. *Structures of amines*

METHODS

Preparation of storage vesicles. Catecholamine storage vesicles were isolated by a modification of the method of Kirshner (1) as previously described (5). The washed vesicles were suspended in a volume of 0.3 M sucrose and 25 mM Tris (pH 7) (sucrose–Tris) equivalent to the original weight of adrenal medullae. The final catecholamine concentration was 445 ± 35 μg/ml of suspension (mean ± standard error).

Uptake studies. Each incubation utilized 0.2 ml of vesicle suspension. Unless otherwise indicated, 0.1 ml of 50 mM ATP and 50 mM $MgCl_2$ was added. Other agents, when added, were (final concentrations in parentheses) *l*-epinephrine (10^{-4} M), *dl*-epinephrine-7-^{3}H (20 μCi/ml), *l*-metaraminol (10^{-4} M), *dl*-metaraminol-7-^{3}H (20 μCi/ml), tyramine (10^{-4} M), uniformly labeled tyramine-^{3}H (20 μCi/ml), β-phenylethylamine (10^{-3}, 10^{-4}, or 10^{-5} M), and reserpine (5×10^{-5} M). All drugs were dissolved in 0.3 M sucrose, and the pH was adjusted to 7. The final volumes of the incubation mixtures were adjusted to 1 ml with sucrose–Tris. The extravesicular catecholamine concentration averaged 35 ± 5 μM in the absence of added epinephrine.

Duplicate samples were incubated at 0° or 30° for 20 min. Uptake was stopped by the addition of 2 ml of ice-cold 0.3 M sucrose containing 2 mM EDTA (pH 7). The samples were centrifuged at 26,000 × *g* for 10 min; the supernatant fluid was analyzed for radioactivity and catecholamines. The pellet was washed with 3 ml of sucrose, centrifuged, and resuspended in 3 ml of distilled water to lyse the vesicles. The lysed preparation was centrifuged as before, and the supernatant solution was analyzed for radioactivity and catecholamines.

Labeling of vesicles and studies of efflux. Each incubation mixture contained 4 ml of vesicle suspension, to which was added 0.5 ml of 50 mM ATP and 50 mM $MgCl_2$. Other agents, when added, were (final concentrations in parentheses) tyramine (10^{-3} or 10^{-4} M), uniformly labeled tyramine-^{3}H (20 μCi/ml), *l*-metaraminol (10^{-4} M), *dl*-metaraminol-7-^{3}H (20 μCi/ml), *l*-norepinephrine (10^{-4} M), *dl*-norepinephrine-7-^{3}H (20 μCi/ml), *dl*-isoproterenol (10^{-4} M), *dl*-isoproterenol-7-^{3}H (20 μCi/ml), *dl*-epinephrine-7-^{3}H (20 μCi/ml), *dl*-normetanephrine (10^{-4} M), *dl*-normetanephrine-7-^{3}H (20 μCi/ml), *d*-amphetamine (10^{-4} M), uniformly labeled *d*-amphetamine-^{3}H (20 μCi/ml), β-phenylethylamine (10^{-4} M), β-phenylethylamine-1-^{14}C (2 μCi/ml), *dl*-dopa (3,4-dihydroxyphenylalanine) (10^{-4} M), *dl*-dopa-2-^{14}C (2 μCi/ml), serotonin (10^{-4} M), serotonin-2-^{14}C (2 μCi/ml), and reserpine (5×10^{-5} M). All drugs were dissolved in sucrose, and the pH was adjusted to 7. The final volumes of the incubation mixtures were adjusted to 5 ml with sucrose–Tris; the external catecholamine concentration (as epinephrine) averaged 104 $\pm$ 4 μM. The uptake and subsequent efflux of the amines were determined as described previously (5).

Sucrose density gradient studies. Sucrose density gradients were prepared using a modification of the apparatus described by Ayad and co-workers (8) to produce hyperbolic gradients. Sucrose (1 M) was pumped into a mixing chamber containing 2.25 M sucrose (15 ml/gradient) at a rate equal to that at which it was pumped out. The total volume of each gradient was 30 ml.

Vesicles were doubly labeled in the presence of *l*-metaraminol (10^{-4} M), *dl*-metaraminol-7-^{3}H (1 μCi/ml), *l*-epinephrine (10^{-4} M), and *dl*-epinephrine-7-^{14}C (1 μCi/ml) as described in the preceding section, and the uptake and efflux of the two amines were determined. Vesicle suspension (1 ml) was layered on the gradient and centrifuged for 3 hr at 100,000 $\times$ *g* in the SW 25.1 rotor of a Beckman model L-2 ultracentrifuge. The tube was then punctured and the solution was allowed to drain from the bottom; 0.6ml fractions were collected. To lyse the vesicles 2 ml of 7% perchloric acid were added to each fraction, and the lysates were centrifuged at 26,000 $\times$ *g* for 10 min. The supernatant solutions were analyzed for radioactivity and catecholamines.

Isolation of chromogranins. The medullae from 100 fresh bovine adrenal glands were homogenized in an all-glass apparatus in 5 volumes of ice-cold 0.3 M sucrose (pH 7), and the storage vesicles were prepared as before. The vesicles were lysed in 5 volumes of distilled water, and insoluble material was removed by centrifugation at 26,000 $\times$ *g* for 20 min. The supernatant solution was lyophilized and resuspended in 40 ml of distilled water, and catecholamines and other small molecules were removed by passing the solution through a Sephadex G-25 column as described by Smith and Kirshner (9). The protein fraction was exhaustively dialyzed against distilled water and lyophilized. After resuspension in distilled water, the solution was dialyzed overnight against 0.01 M phosphate buffer (pH 6.7). The chromogranins were separated from other proteins by chromatography on DEAE-cellulose as described previously (9); the solution was exhaustively dialyzed against distilled water, lyophilized, resuspended, and dialyzed against 0.3 M sucrose and 25 mM Tris (pH 7). The final protein concentration (as determined by measurements of absorbance at 280 nm) was 6.6 mg/ml.

Binding of amines to chromogranins. Dialysis cells with a 1-ml capacity in each chamber were used. One milliliter of chromogranin solution (6.6 mg/ml in sucrose–Tris), containing 0.1 μCi of radioactive amine and various concentrations of unlabeled amine, was placed in one chamber. The other chamber was filled with sucrose–Tris, and the two solutions were stirred until equilibrium was reached (about 24 hr at 5°). Aliquots were withdrawn from both sides of the membrane for determination of radioactivity. The concentrations studied ranged from 5×10^{-7} to 10^{-3} M for epinephrine, and from 5×10^{-5} to 10^{-3} M for other amines.

Catecholamine determinations. Unlabeled catecholamines were determined by the trihydroxyindole method (10); purification by alumina adsorption was not performed.

Radioactive amines were determined after mixing a 1-ml sample with 10 ml of a 2:1 mixture of toluene and Triton X-100 con-

taining 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene. The samples were counted in a Nuclear-Chicago mark III liquid scintillation spectrometer.

Uptake and efflux calculations. The uptake and efflux of amines were calculated as described previously (5).

Statistical methods. All experiments were repeated at least once; the results were reproducible. The data presented are from individual experiments. Data were fitted to straight lines by the method of least squares (11).

Materials. ATP was obtained from P-L Biochemicals; reserpine phosphate (Serpasil) from Ciba Pharmaceutical Company; radioactive amines, from New England Nuclear Corporation; *l*-epinephrine bitartate, *l*-norepinephrine bitartate, and *dl*-normetanephrine hydrochloride, from Winthrop Laboratories; β-phenylethylamine, *dl*-isoproterenol sulfate, *d*-amphetamine sulfate, and *dl*-dopa, from Sigma Chemical Company; tyramine hydrochloride, *dl*-octopamine hydrochloride, and serotonin creatinine phosphate, from Calbiochem; and *l*-metaraminol bitartrate, from Merck Sharp & Dohme.

RESULTS

Efflux of newly incorporated amines. Efflux curves for newly incorporated amines were typically biphasic, consisting of a rapid and a slow component. Two efflux curves, one for ^{3}H-epinephrine and one for ^{3}H-metaraminol, appear in Fig. 2. Graphical analysis of these curves indicates a fit to an equation of the type

$$n_t = n_0 e^{-kt} + n'_0 e^{-k't}$$

where n_t is the percentage remaining in the vesicles at time t, n_0 and n'_0 represent the percentages at zero time ($n_0 + n'_0 = 100\%$), and k and k' are the rate constants for disappearance of the amines from the vesicles.

Thus the data in Fig. 2 indicate that 22% of the labeled epinephrine was released with a half-time of about 5 min, and 78% with a half-time of about 160 min; 75% of the labeled metaraminol was released with a half-time of about 5 min, and 25% with a half-time of about 42 min. The pool with a half-life of 5 min is designated the "fast" pool, and the pool with the relatively long half-life, the "slow" pool. Metaraminol, then, is distributed primarily into the fast pool, while epinephrine is distributed primarily into the slow pool. The percentages and half-lives varied from preparation to preparation, thus requiring that an epinephrine control curve be plotted with each set of experiments. Unless otherwise indicated, there was no efflux of amines at 0°.

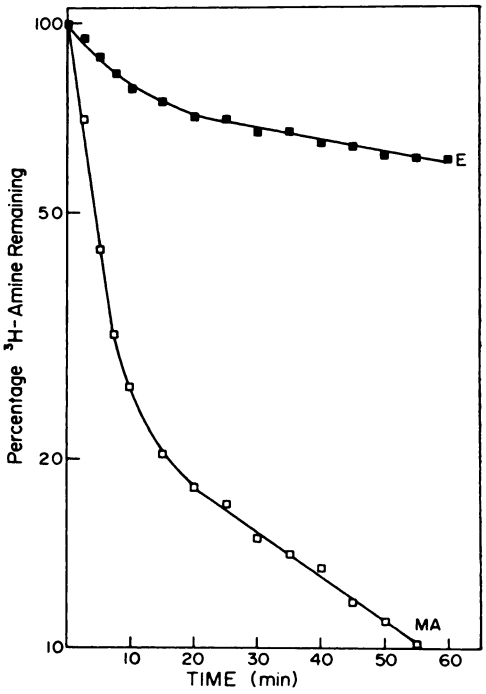


FIG. 2. *Efflux of ^{3}H-epinephrine (E) and ^{3}H-metaraminol (MA) from labeled storage vesicles*
Each curve can be resolved into two first-order components: epinephrine—25% with $t_{1/2}$ = 5 min, 75% with $t_{1/2}$ = 160 min; metaraminol—75% with $t_{1/2}$ = 5 min, 25% with $t_{1/2}$ = 42 min.

In all experiments, the half-life of endoggenous catecholamines was measured and was found to be similar to that of the slow phase of newly incorporated epinephrine or norepinephrine (5).

Sucrose density gradient studies. Vesicles prepared by the method utilized are known to be contaminated with mitochondria, lysosomes, and other particles. It therefore seemed possible that the fast pool might represent uptake by these contaminants. To test this possibility, the preparation was

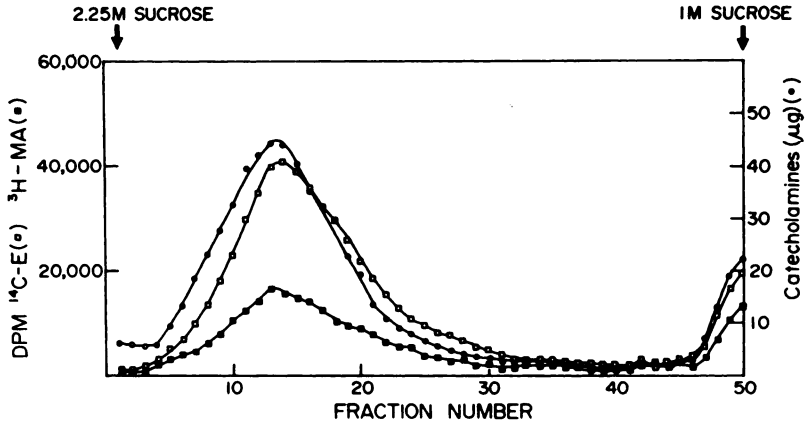


FIG. 3. *Distribution of storage vesicles labeled with ^{14}C-epinephrine (^{14}C-E) or ^{3}H-metaraminol (^{3}H-MA) on continuous sucrose density gradient (hyperbolic from 1 to 2.25 M).*

labeled in the presence of ^{3}H-metaraminol and ^{14}C-epinephrine. Efflux studies of the preparation showed that most of the metaraminol was contained in the fast pool while most of the epinephrine was in the slow pool. One milliliter of the doubly labeled preparation was placed on a sucrose density gradient and centrifuged at 100,000 × *g* until equilibrium was reached (about 3 hr). Fractionation of the gradient (Fig. 3) indicated that both ^{14}C-epinephrine and ^{3}H-metaraminol were contained within the storage vesicles.

Effect of N-substitution. The uptake and distribution of norepinephrine, epinephrine, and isoproterenol are summarized in Table 1. There was little difference between norepinephrine and epinephrine in uptake, distribution, or half-life, although in all experiments there was a trend toward an increased half-life for the slow pool of norepinephrine. When a compound with a bulky *N*-substituent (isoproterenol) was utilized, the uptake into both pools and the half-time of efflux from the slow pool were markedly decreased. The relative distribution of isoproterenol was quite similar to those of norepinephrine and epinephrine, but a consistent trend toward an elevated percentage in the fast pool could be detected.

Effect of hydroxyl groups. The uptake and distribution of epinephrine, octopamine, tyramine, and β-phenylethylamine are presented in Table 1. Removal of the hydroxyl group at the *meta* position relative to the side chain (octopamine) reduced the net uptake of amine and also increased its incorporation into the fast pool, so that more octopamine than epinephrine was taken up by the fast pool while more epinephrine than octopamine was incorporated into the slow pool. The stability of storage of octopamine in the fast pool was the same as that of epinephrine, but the stability of the former in the slow pool was less.

Removal of the β-hydroxyl group (tyramine) from octopamine restored the net uptake to an amount even greater than that of epinephrine. The uptake into each pool was increased over that of octopamine. The distribution was even further displaced in favor of the fast pool, so that tyramine was incorporated into the fast pool to a much greater extent than epinephrine, but to a lesser extent in the slow pool. The stability of storage in the fast pool was unchanged, but in the slow pool the half-time was even shorter than that of octopamine, indicating decreased stability in that pool. Measurements made with tyramine were complicated by the partial conversion of tyramine to octopamine by the dopamine β-hydroxylase present in the vesicles. In all cases, the data are reported in terms of total radioactivity (tyramine plus octopamine).

With all hydroxyl groups removed (β-phenylethylamine), the net uptake of amine into the vesicles was barely measurable, and

TABLE 1

Amine uptake and distribution

The epinephrine concentration during labeling averaged 10^{-4} M. Efflux was studied in sucrose–Tris for 1 hr at 37°. Pool sizes are reported in terms of the percentage of total radioactive amine contained in the vesicles.

Amine (10^{-4} M)	Pool size	$t_{1/2}$	Pool size	$t_{1/2}$	Uptake[a]
		min		*min*	
N-Substitution					
Norepinephrine	16	4	84	177	4.4
Epinephrine	21	5	79	150	4.7
Isoproterenol	23	5	77	46	1.5
Hydroxyl groups					
Epinephrine	10	5	90	171	6.3
Octopamine	32	5	68	50	3.0
Tyramine	52	5	48	43	8.2
β-Phenylethylamine[b]					0.2
Methyl groups					
Epinephrine	15	4	85	156	3.6
Normetanephrine	86	5	14	114	1.8
Metaraminol	75	4	25	42	1.4
Amphetamine[b]					0.5
Other compounds					
Epinephrine	25	5	75	149	2.1
Dopa[b]					0.06
Serotonin	8	5	92	63	8.1

[a] Nanomoles taken up in 20 min per amount of vesicles containing 100 μg of catecholamine.

[b] Uptake was too low for accurate efflux determinations.

the distribution could not be determined accurately. When the uptake of β-phenylethylamine was determined and the vesicles were washed and resuspended in sucrose at 0° to measure efflux, 95% of the radioactivity was found in the medium at zero time. This phenomenon was noted with only one other amine studied, amphetamine, and suggested loose binding of the amine to a site on or in the vesicle.

Effect of methyl groups. The uptake and distribution of epinephrine, normetanephrine, metaraminol, and amphetamine are shown in Table 1. Replacement of one of the catechol hydroxyl groups with a methoxy group (normetanephrine) reduced the net uptake by about 50%. The relative distribution was altered drastically: 86% of the normetanephrine was associated with the fast pool, compared to 15% for epinephrine. Thus uptake into the fast pool was much higher for normetanephrine than for epinephrine, while uptake into the slow pool was much lower. There was no change in the stability of storage of amine in the fast pool, but normetanephrine was stored less stably than epinephrine in the slow pool.

Insertion of a methyl group on the α-carbon of the side chain reduced the net uptake of amine in the slow pool (compare metaraminol with octopamine), but increased the uptake into the fast pool. The distribution of metaraminol favored the fast pool 75%:25%. There was no change in the half-life of the fast pool, but that of the slow pool was reduced to 42 min.

Methylation of β-phenylethylamine on the α-carbon (amphetamine) raised net uptake above that of the parent compound, but the amount incorporated was too small for measurements of efflux.

Other structural changes. The uptake and efflux of epinephrine, dopa and serotonin are shown in Table 1. Addition of a carboxyl group at the α-position (dopa) reduced the uptake nearly to zero. Unlike amphetamine and β-phenylethylamine, both of which had similarly low uptakes, no dopa was found loosely associated with the vesicles.

The uptake of serotonin was nearly 4 times that of epinephrine, and all the additional uptake was associated with the slow pool. Only 8% of the serotonin was found in the fast pool, an amount in nanomoles approximately equal to the epinephrine taken up into this pool. As was the case with all the other amines, the half-life of serotinin was 5 min in the fast pool. Unlike epinephrine, serotonin was stored with a half-life of only 63 min in the slow pool, indicating that replacement of the catechol ring with a hydroxyindole ring decreases the strength of binding.

Effect of reserpine. Inclusion of 5×10^{-5} M reserpine during labeling of the vesicles altered the uptake and distribution of epinephrine (Table 2). Ninety per cent of the uptake of epinephrine was inhibited by reserpine, and 56% of the amount taken up was distributed into the fast pool, compared to 15% in the controls. The uptake of tyramine, on the other hand, was inhibited

TABLE 2

Effect of 5 × 10⁻⁵ M reserpine on amine uptake and distribution

The epinephrine concentration during labeling averaged 10^{-4} M. Efflux was studied in sucrose–Tris for 1 hr at 37°. Pool sizes are reported in terms of the percentage of total radioactive amine contained in the vesicles. Each pair of experiments utilized a separate preparation. Reserpine was present during labeling of the vesicles as indicated below.

Amine (10^{-4} M)	Pool size	$t_{1/2}$	Pool size	$t_{1/2}$	Uptake[a]
		min		*min*	
Epinephrine	15	4	85	135	3.1
Epinephrine + reserpine	56	4	44	147	0.3
Tyramine	49	4	51	38	3.7
Tyramine + reserpine	50	4	50	37	2.2
Metaraminol	81	3	19	43	4.7
Metaraminol + reserpine	81	3	19	42	2.9
Serotonin	8	5	92	59	8.1
Serotonin + reserpine	22	3	78	63	0.7

[a] Nanomoles taken up in 20 min per amount of vesicles containing 100 μg of catecholamine.

only 40% by reserpine, and there was no change in distribution. Metaraminol resembled tyramine in its sensitivity to reserpine. Serotonin, on the other hand, resembled epinephrine, with 90% of the uptake inhibited by reserpine. The distribution into the fast pool increased from a control value of 8% to 22% for the reserpine-treated vesicles. Reserpine did not alter the half-lives of any of the amines.

Effect of ATP and Mg^{2+}. Addition of ATP and Mg^{2+} during uptake increased the uptake of epinephrine by a factor of 12; this effect could be blocked by reserpine (Table 3). ATP and Mg^{2+} produced a doubling of tyramine uptake and a 35–40% stimulation of metaraminol uptake. Both these effects were blocked by reserpine.

Inhibition of epinephrine uptake by other amines. The uptake and efflux of epinephrine were measured in the presence of various concentrations of tyramine. With 10^{-4} M tyramine present during labeling of the vesicles with epinephrine, the uptake of epinephrine was inhibited 50%, although there was little change in relative distribution (Table 4). At higher tyramine concentrations (10^{-3} M), the inhibition of epinephrine uptake was nearly complete, and the distribution of the small amount of epinephrine incorporated was shifted more toward the fast pool than in the controls.

β-Phenylethylamine also inhibited epinephrine uptake, even though it was hardly taken up itself. When the external epinephrine concentration was 3.5×10^{-5} M, 10^{-5} M β-phenylethylamine produced 25% blockade of uptake (Table 4). β-Phenylethylamine at 10^{-4} M produced 45% blockade, and at 10^{-3} M β-phenylethylamine the uptake of epinephrine was only 10% of control values.

Binding to chromogranins. The binding of epinephrine, isoproterenol, β-phenylethylamine, tyramine, octopamine, metaraminol, and serotonin to chromogranins was measured by equilibrium dialysis. A Scatchard plot (12) for epinephrine is shown in Fig. 4. Extrapolation of the line to the y-axis in-

TABLE 3

Effect of ATP, Mg^{2+}, and reserpine on amine uptake

Each group of experiments utilized a separate preparation. The concentration of ATP and Mg^{2+} was 5 mM; reserpine, 5×10^{-5} M; epinephrine (endogenous), 3.5×10^{-5} M.

Amine (10^{-4} M) and additions	Uptake[a]
Epinephrine	
No addition	0.15
ATP, Mg^{2+}	1.89
ATP, Mg^{2+}, reserpine	0.09
Tyramine	
No addition	0.85
ATP, Mg^{2+}	1.96
ATP, Mg^{2+}, reserpine	0.88
Metaraminol	
No addition	1.23
ATP, Mg^{2+}	1.68
ATP, Mg^{2+}, reserpine	1.16

[a] Nanomoles taken up in 20 min per amount of vesicles containing 100 μg of catecholamine.

TABLE 4

Effect of other amines on epinephrine uptake and distribution

Efflux was studied in sucrose–Tris for 1 hr at 37°. Pool sizes are reported in terms of the percentage of total radioactive amine contained in the vesicles.

Amine	^{3}H-Epinephrine pool size	$t_{1/2}$	^{3}H-Epinephrine pool size	$t_{1/2}$	Epinephrine uptake[a]
		min		*min*	
Control[b]	15	5	85	135	3.5
Tyramine[b] (10^{-4} M)	17	4	83	127	1.8
Tyramine (10^{-3} M)	32	4	68	148	0.05
Control[c]	Efflux not measured				4.0
β-Phenylethylamine (10^{-5} M)					3.1
β-Phenylethylamine (10^{-4} M)					2.3
β-Phenylethylamine (10^{-3} M)					0.5

[a] Nanomoles taken up in 20 min per amount of vesicles containing 100 μg of catecholamine.
[b] Epinephrine concentration during labeling = 10^{-4} M.
[c] Epinephrine concentration during labeling = 3.5×10^{-5} M.

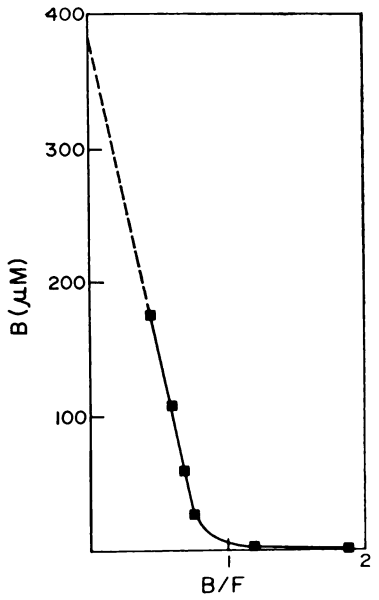


FIG. 4. *Scatchard plot showing binding at 5° of epinephrine to a mixture of chromogranins*
The protein concentration was 6.6 mg/ml.

dicated that the concentration of binding sites in the solution (6.6 mg of protein per milliliter) was 381 μM. The slope of the plot gives the dissociation constant for epinephrine as 4.6×10^{-4} M. Since the other amines were bound to a lesser extent than epinephrine, the slopes of the Scatchard plots were even steeper, and the scattering of points about the lines rendered accurate plotting impractical. Instead, the dissociation contants were calculated from the equation

$$K_{diss} = \frac{n - B}{B/F}$$

where n is the concentration of amine-binding sites on the protein (381 μM), assuming that n is the same for all amines tested; B is the concentration of bound amine; and F is the concentration of free amine. Four concentrations were studied for each amine (5×10^{-5}, 10^{-4}, 5×10^{-4}, and 10^{-3} M), and the dissociation constant was calculated for each point. The values of the constants were then averaged for the four concentrations; the results are shown in Table 5. Because of the magnitudes of the standard errors, few significant differences in binding constants were observed.

DISCUSSION

The biphasic efflux of newly incorporated amines from isolated adrenal storage vesicles indicates that there are two storage pools. Some of the characteristics of these two pools have been described by Slotkin and coworkers (5), who suggested that the two pools might reflect the existence of two populations of storage vesicles or two pools inside the same vesicle which do not exchange amines with each other. Models

TABLE 5

Binding of amines to chromogranins as determined by equilibrium dialysis

Binding was studied at 5°. The protein concentration was 6.6 mg/ml.

Amine	K_{diss}
	$mM \pm SE$
Epinephrine	0.46 ± 0.001
Isoproterenol	0.61 ± 0.09
Serotonin	0.88 ± 0.13
Octopamine	1.0 ± 0.3
Tyramine	1.5 ± 0.4
β-Phenylethylamine	2.0 ± 0.2
Metaraminol	2.4 ± 0.2

invoking two populations of vesicles have been proposed by Lishajko (7) and Hillarp (6). Our system differs from that of Lishajko in that both epinephrine and norepinephrine have the same efflux pattern. The two pools described in the current study cannot represent epinephrine- and norepinephrine-specific vesicles; since the ratio of epinephrine to norepinephrine in bovine adrenal glands is about 3:1, the uptake of epinephrine would exceed that of norepinephrine by that proportion if the two pools represented vesicles specific for those amines. The fact that the uptakes and effluxes of the two amines are nearly identical militates against a model invoking epinephrine- and norepinephrine-specific uptake and storage systems.

In the system described by Hillarp (6), catecholamines were bound less stably in vesicles of low density than in vesicles of high density. In the present study, attempts to separate the fast and slow pools by density gradient centrifugation of doubly labeled vesicles gave equivocal results. ^{3}H-Metaraminol consistently formed a peak one fraction earlier than ^{14}C-epinephrine, and the ^{14}C:^{3}H ratio in the trailing edge was consistently higher than in the leading edge of the peak. These observations suggest that ^{3}H-metaraminol might be contained in vesicles of slightly higher density than the vesicles which contain ^{14}C-epinephrine. In addition, the specific activity of epinephrine was not constant throughout the storage vesicle peak of the gradient. This phenomenon may reflect the existence of storage vesicles with a distribution of catecholamine contents such that vesicles of high catecholamine content have higher densities than vesicles of low catecholamine content. The catecholamine content per vesicle would therefore be lower in a fraction from the trailing edge of the peak, and the number of vesicles would be higher than in a fraction from the leading edge which has the same catecholamine content. If the number of vesicles per microgram of catecholamine is higher in the trailing edge, the uptake and specific activity of ^{14}C-epinephrine would be higher in the trailing edge, provided that uptake per vesicle is the same throughout the peak. Depending on catecholamine content per vesicle, the same results would be observed whether or not the uptake per vesicle was constant throughout the peak, or even if the uptake per vesicle were lower in the trailing edge than in the leading edge. Observations similar to those made here for ^{14}C-epinephrine have been reported for the distribution of dopamine β-hydroxylase (10).

A qualitative summary of the relationships among amine structure, uptake, and storage in the fast and slow pools appears in Table 6. Comparisons based on differences between metaraminol and octopamine are open to question because of the difference in location of the phenolic hydroxyl group.

Study of the uptake and subsequent efflux of amines from adrenal medullary vesicles provides further insight into the nature of the incorporation processes. Thus, if uptake is studied alone, a decrease in incorporation might be due either to an actual decrease in affinity for the uptake system or to a decrease in the stability of the storage complex. The study of efflux determines the latter factor, so that the two effects can be separated. For example, the uptake of serotonin into the slow pool is higher than that of epinephrine, yet its rate of efflux is higher than that of epinephrine. Therefore, serotonin must have a higher affinity for the uptake process than does epinephrine, in order to account for the observed increase in incorporation despite a decrease in storage stability. β-Phenylethylamine is taken up to a very small extent, but large amounts of this amine are loosely associated with the

TABLE 6

Summary of structure-activity relationships

	Uptake		Stability		Binding to chromogranins	Basis of comparison
	Fast	Slow	Fast	Slow		
N-Alkylation						
Add small group	—[a]	—	—	—		E vs. NE
Add large group	↓	↓	—	↓	↓	IPR vs. E
Hydroxyl groups						
Add *p*-OH	↑	↑			↑	T vs. β-PE
Add *m*-OH	↓	↑	—	↑	↑	E vs. O
Add β-OH	↓	↓	—	↑	↑	O vs. T
Methyl groups						
Replace *m*-OH with *m*-OCH_3	↑	↓	—	↓		NM vs. NE
Add α-CH_3	↑	↓	—	↓	↓	MA vs. O
Miscellaneous						
Add α-COOH	↓	↓				Dopa vs. NE
Replace ring with 5-hydroxyindole	—	↑	—	↓	↓	S vs. E

[a] Key: — = little or no change; ↑ = increase; ↓ = decrease; E = epinephrine; NE = norepinephrine; IPR = isoproterenol; T = tyramine; β-PE = β-phenylethylamine; O = octopamine; NM = normetanephrine; MA = metaraminol; dopa = 3,4-dihydroxyphenylalanine; S = serotonin.

vesicles; this suggests that β-phenylethylamine might have an affinity for the uptake process but does not become stably bound. That β-phenylethylamine has an affinity for the uptake system is confirmed by the observation that it can inhibit epinephrine uptake (Table 4). The observations made with serotonin and β-phenylethylamine suggest strongly that the uptake of amines into the storage vesicles and their subsequent binding are two separate processes.

The number and location of hydroxyl groups on the phenylethylamine structure appear to be critical in determining the uptake, storage, and distribution of amines in the vesicles. Addition of the *p*-hydroxyl group has the greatest relative effect on the net uptake; thus, while β-phenylethylamine is not taken up in significant amounts, the total uptake of tyramine exceeds that of epinephrine. The addition of a β-hydroxyl group decreases net uptake but increases the storage stability of the amine in the slow pool. This probably has functional significance, since the vesicles ordinarily take up dopamine and convert it to norepinephrine; the uptake of dopamine would be high because of the absence of the β-hydroxyl group and the presence of the catechol hydroxyls, while storage of the norepinephrine subsequently synthesized would be stabilized by the presence of the β-hydroxyl group. Both catechol hydroxyl groups appear to be necessary for maximal storage stability, since the half-life of epinephrine is the slow pool is much higher than that of octopamine. Furthermore, the replacement of one catechol hydroxyl with a methoxy group reduces both uptake and storage stability in the slow pool.

Hydroxyl groups also play a role in uptake into the fast pool. In this case, although one phenolic hydroxyl group appears to be required, the addition of the second phenolic hydroxyl or the β-hydroxyl reduces uptake. The observation that the half-lives for all amines in the fast pool are the same indicates that differences in uptake are due to differences in affinity for the uptake system.

Lundborg (3) and Lundborg and Stitzel (4) have reported that metaraminol is taken up into the vesicles by an ATP-Mg^{2+}-independent system which could not be blocked by reserpine. The observations reported in Tables 2 and 3 suggest that metaraminol uptake is stimulated by ATP and Mg^{2+}, but to a lesser extent than epinephrine. Thus metaraminol has approximately a 25% contribution from stimulated uptake, while epinephrine owes more than 90% of its in-

corporation to the stimulated system. Part of the reason for this is that most of the metaraminol is taken up into the fast pool, which is much less dependent on stimulation by ATP and Mg^{2+} than the slow pool (5). In addition, metaraminol has a much lower affinity for the stimulated system than epinephrine. It seems likely, then, that all amines which are incorporated will have some degree of dependence on stimulated uptake, which will depend in part on the pool into which the amine is taken up. For example, tyramine enters the fast and slow pools equally, and depends on stimulated uptake for about half its incorporation. Serotonin, 92% of which enters the the slow pool, is even more sensitive to reserpine blockade of stimulated uptake than is epinephrine (Table 2).

A corollary of the differential effect of reserpine on the fast and slow pools is that the presence of reserpine during labeling of the vesicles will not only decrease the total uptake but shift the distribution of amine in favor of the fast pool; this shift will be largest for amines which enter the slow pool, and smallest for amines which enter the fast pool. Hence the percentage of incorporated epinephrine found in the fast pool increases from a control of 15 to 56 in the reserpine-treated vesicles (Table 2). Although there is a similar shift for serotonin, there is no significant shift in distribution when vesicles are labeled with tyramine or metaraminol in the presence of reserpine (Table 2). This confirms the concept of a differential effect of reserpine on the uptake and distribution of amines, caused by differential effects on the slow and fast pools. Furthermore, the observation that reserpine does not alter the half-times of either the fast or the slow pool (5) suggests that the effect of reserpine on incorporation lies in its ability to block the uptake process, rather than in disruption of the storage complex.

If the uptake processes involve transport systems, the uptake of epinephrine should be inhibited by other amines. This has already been described for epinephrine and β-phenylethylamine (Table 4). In addition, tyramine has the ability to inhibit epinephrine uptake into both the fast and slow pools (Table 4); in equimolar concentrations (10^{-4} M), tyramine inhibits epinephrine uptake 50%, with no apparent change in the distribution of the incorporated epinephrine. In higher concentrations (10^{-3} M), tyramine blocks almost all the epinephrine uptake, and the distribution of the epinephrine that is incorporated is shifted in favor of the fast pool, suggesting that the fast pool uptake system is saturated at higher substrate concentrations than the slow pool uptake system. This is consistent with the observation that high substrate concentrations favor incorporation into the fast pool (5). Attempts to measure the K_m and K_i for the various amines were unsuccessful because the endogenous extravesicular catecholamine concentration was higher than the K_m for epinephrine and because of the difficulties in interpreting a four-uptake system (fast and slow, stimulated and unstimulated) in terms of Michaelis-Menten kinetics.

The binding of amines to chromogranins cannot explain the differences in vesicular uptake; the concentration of binding sites is 381 μM for a protein concentration of 6.6 g/liter, or 0.06 mmole of amine bound per gram of protein. About 35% of the dry weight of the adrenal vesicles is protein, and 80% of the protein is soluble after lysis of the vesicles (13); 21% of the dry weight is catecholamines (14). Therefore the intravesicular catecholamine to protein ratio is 4 mmoles/g of protein. The binding of amines to chromogranins could account for only 1.5% of the total catecholamine content of the vesicles. The same conclusion was reached by Smith and Kirshner (9), working with purified S_1 (chromogranin A) protein from bovine adrenal medullary vesicles. The qualitative correlation between the strength of binding of amines to chromogranins and the stability of their storage in the slow pool (Table 6) might suggest that chromogranins play a secondary role in the binding of the amines to ATP in the storage complex, perhaps by stabilizing the complex and contributing to the structural specificity of binding of the various amines in the complex.

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