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Compartmental Analysis of Amine Storage in Bovine Adrenal Medullary Granules

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SUMMARY

The efflux of ^{14}C-epinephrine from isolated bovine adrenal medullary granules was determined in order to study the storage and distribution of newly incorporated amines. After the granules had been labeled, the efflux of endogenous and ^{14}C-epinephrine was measured for 1 hr at 37°. The efflux of ^{14}C-epinephrine was biphasic: a period of fast efflux (25% of the ^{14}C-epinephrine, $t_{1/2}$ = 5 min) was followed by a period of slow efflux (75%, $t_{1/2}$ = 2–3 hr). There appeared to be no exchange of ^{14}C-epinephrine between the two efflux pools, suggesting that these might represent two populations of granules. By appropriate treatment, each pool could be preferentially loaded with ^{14}C-epinephrine without altering its $t_{1/2}$. The presence of reserpine (50 μM) or *N*-ethylmaleimide (150 μM), during loading, the absence of ATP and $MgCl_2$, and the use of higher epinephrine concentrations or shorter incubation times all increased the percentage of catecholamine in the rapidly releasing pool and decreased its proportion in the slowly releasing pool. The net uptake of epinephrine was reduced in both pools when ATP-Mg^{2+}-stimulated amine uptake was blocked. These observations suggest that epinephrine is taken up into two pools, one having a slower release rate than the other, a greater dependence on ATP-Mg^{2+}-stimulated uptake, and a greater storage capacity. The effluxes of ^{3}H-octopamine and ^{3}H-metaraminol were biphasic, but differed from ^{14}C-epinephrine in distribution and in the half-life of the slow phase: 32% of the ^{3}H-octopamine was released with $t_{1/2}$ = 5 min, and 68% with $t_{1/2}$ = 50 min; 64% of the ^{3}H-metaraminol was released with $t_{1/2}$ = 6 min, and 36% with $t_{1/2}$ = 37 min.

INTRODUCTION

Catecholamines can be taken up and concentrated in adrenal medullary granules by an ATP-Mg^{2+}-dependent mechanism (1, 2); reserpine blocks this uptake. Metaraminol, however, is taken up by an ATP-Mg^{2+}-independent mechanism, which is insensitive to reserpine (3, 4). Thus, two different processes are implicated in amine uptake.

Based on studies of the distribution of catecholamines and ATP in subcellular fractions of adrenal homogenates, Hillarp (5) proposed the existence of two pools for catecholamine storage: a large store of amines, associated with granules of high density and high stability, and a smaller store, bound less stably to granules of lower density. Lishajko (6) demonstrated in sheep medullary granules that norepinephrine was released at a more rapid initial rate than epinephrine, again suggesting the existence of two different amine storage pools.

In the present study, the relationships between the dual-uptake and two-pool con-

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cepts are examined by kinetic analysis of the efflux of newly incorporated amines in bovine adrenal medullary granules.

METHODS

Preparation of storage vesicles. Bovine adrenal glands from freshly killed animals were obtained at a local slaughterhouse and were kept on ice until utilized (about 45 min). All steps were performed at 0–2°. Fat and connective tissue were removed, and the cortex was stripped. The medullae were washed in 0.3 M sucrose (pH 7.0), blotted dry, weighed, and homogenized in an all-glass apparatus in 10 volumes of 0.3 M sucrose. The homogenate was centrifuged at 750 × *g* for 10 min to remove erythrocytes, nuclei, cell fragments, and other large particles. The supernatant fraction was centrifuged at 26,000 × *g* for 20 min; the supernatant solution was discarded, and fat was wiped from the sides of the centrifuge tube. Lighter particles lying on top of the pellet were removed by gently swirling twice with 5 ml of 0.3 M sucrose. The pellet was resuspended by homogenization (Teflon-glass) in one-half the original volume of sucrose and was centrifuged at 26,000 × *g* for 10 min. The supernatant fluid was discarded, and the pellet was resuspended by homogenization (Teflon-glass) in one-tenth the original volume of 0.3 M sucrose, containing 25 mM Tris, pH 7.0 (sucrose-Tris). This suspension contains 455 ± 33 (SE) μg of catecholamine (as epinephrine) per milliliter of suspension.

Labeling of granules with radioactive amines. Four milliliters of granule suspension were used in each incubation. Unless otherwise indicated, 0.5 ml of a solution containing 50 mM ATP and 50 mM $MgCl_2$ was added. Other agents, when added, were present at the following final concentrations: *l*-epinephrine, endogenous levels or 5 mM, as indicated; *dl*-epinephrine-7-^{14}C, 1 μCi/ml; *dl*-epinephrine-7-^{3}H, 20 μCi/ml; *dl*-octopamine, 0.1 mM; *dl*-octopamine-2-^{3}H, 20 μCi/ml; *l*-metaraminol, 0.1 mM; *dl*-metaraminol-7-^{3}H, 20 μCi/ml; tyramine, 0.1 mM; uniformly labeled tyramine-^{3}H 20 μCi/ml; reserpine, 50 μM; and *N*-ethylmaleimide, 150 μM. 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 5 ml with sucrose-Tris. When no additional epinephrine was added, the external catecholamine concentration (as epinephrine) was 0.22 ± 0.03 (SE) mM.

The samples were incubated for 20 min at 30°, chilled in ice, and centrifuged at 26,000 × *g* for 10 min. When *N*-ethylmaleimide was used, the granules were incubated with *N*-ethylmaleimide at 30° for 15 min in sucrose-Tris before any other agents were added;[2] the treatment thereafter was identical. The supernatant solutions were saved for determination of radioactivity and catecholamines, and the pellets were washed by resuspension (Teflon-to-glass homogenization) in ice-cold 0.3 M sucrose. After centrifugation at 26,000 × *g* for 10 min, the wash was discarded, and the pellet was resuspended by homogenization (Teflon-to-glass) in 4 ml of sucrose-Tris and used for studies of efflux.

Efflux. Labeled granule suspension (0.2 ml) was added to each of 15 tubes containing 0.8 ml of sucrose-Tris. The tubes were placed in a water bath at 37° and incubated without shaking. Thirty seconds were allowed to warm the tubes to 37°; one tube was withdrawn at each of the following times thereafter: 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min. To stop the efflux, 2 ml of ice-cold 0.3 M sucrose were added, and the samples were centrifuged at 26,000 × *g* for 5 min. The supernatant fraction was saved for radioactivity and catecholamine determinations; the pellet was resuspended in 3 ml of distilled water and shaken vigorously to lyse the granules. The lysed preparation was centrifuged at 26,000 × *g* for 5 min, and the supernatant fluid was saved for radioactivity and catecholamine determination.

Dopamine β-hydroxylase (EC 1.14.2.1) Activity. Assays of supernatant and lysed fractions were performed in the presence of 10 μM *p*-hydroxymercuribenzoate to inactivate endogenous inhibitors (7). The reaction mixtures contained potassium phosphate buffer (140 mM), sodium ascorbate (1 mM), sodium fumarate (60 mM), ATP (5 mM), tyramine hydrochloride (10 μM), tranylcypromine (0.5

[2] Fifteen-minute incubation of the granules did not in itself alter the uptake and efflux of amines.

mM), catalase (9600 units), ^{3}H-tyramine (100 μCi), and 0.3 ml of supernatant or lysate in a final volume of 1 ml. All solutions were at pH 5.7. After 20 min of incubation at 37° with shaking, the reaction was stopped with 1 ml of 7% perchloric acid. The samples were centrifuged at 26,000 × g for 10 min, and 1 ml of each solution was assayed for the ^{3}H-octopamine formed (8). Glycerol instead of $NaHSO_3$ was used to stop the periodate reaction.

Catecholamine determinations. Unlabeled catecholamines (as micrograms of epinephrine) were determined as described previously (9); purification by alumina adsorption was not performed.

Radioactive amines were determined by mixing a 1-ml aliquot with 10 ml of a 2:1 mixture of toluene and Triton X-100 containing fluors (2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene). The samples were counted in a Packard Tri-Carb liquid scintillation counter.

Uptake and efflux calculations. The uptake of amines was calculated using the equation

$$U = \frac{L_0 C}{S}$$

where U = uptake, L_0 = counts per minute in the lysate before the efflux study was begun, C = concentration of amine in the medium at the completion of the labeling phase, and S = counts per minute in the medium during the labeling phase.

The efflux is expressed as the percentage of labeled amines remaining inside the granules; the presence of amines in the supernatant at zero time has to be taken into account as follows:

$$R = 100\left[\frac{b/(a+b)}{b_0/(a_0+b_0)}\right]$$

where R = percentage remaining, a_0 = counts per minute in the supernatant fraction at zero time, b_0 = counts per minute in the lysate at zero time, and a and b are the counts per minute in the supernatant fraction and lysate at time t, respectively. a and b were measured at each time point to minimize pipetting errors. This equation was used to determine the efflux of unlabeled amines also; in this case, a_0, b_0, a, and b refer to micrograms of catecholamines instead of counts per minute.

Statistical methods. All experiments were repeated at least once; the results were reproducible. The data presented are from individual experiments. The results were fitted to straight lines by the method of least squares (10).

Materials. ATP was obtained from P-L Biochemicals; reserpine phosphate (Serpasil) was obtained from Ciba Pharmaceutical Company; *N*-ethylmaleimide was obtained from Sigma Chemical Company; *dl*-epinephrine-7-^{14}C (0.04 Ci/mmole; radiochemical purity > 99%), *dl*-epinephrine-7-^{3}H (15 Ci/mmole; radiochemical purity > 97%), uniformly labeled ^{3}H-tyramine (7.3 Ci/mmole; radiochemical purity > 97%), *dl*-metaraminol-7-^{3}H (17 Ci/mmole; radiochemical purity > 99%), and *dl*-octopamine-2-^{3}H (3 Ci/mmole; radiochemical purity > 97%) were obtained from New England Nuclear Corporation; tyramine hydrochloride and *dl*-octopamine hydrochloride were obtained from Calbiochem; *l*-metaraminol bitartrate was obtained from Merck Sharp and Dohme; *l*-epinephrine bitartrate was obtained from Winthrop Laboratories.

RESULTS

Efflux of epinephrine. A typical efflux curve for ^{14}C-epinephrine appears in Fig. 1. A period of rapid efflux was followed by a period of slow efflux. Graphical analysis revealed 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 of catecholamine remaining in the granules at time t, n_0 and n_0' represent the proportions remaining at zero time ($n_0 + n_0' = 100\%$), and k and k' are the rate constants for disappearance of amine from the granules. The parameters were evaluated by (a) determining a least-squares line to fit the data points from 25 to 60 min, (b) extrapolating this line to zero time, (c) subtracting the remaining data points from the extrapolated line, and (d) determining a least-squares line to fit the points derived in step (c).

By this method, the data in Fig. 1 could be summarized as follows: 25% of the ^{14}C-

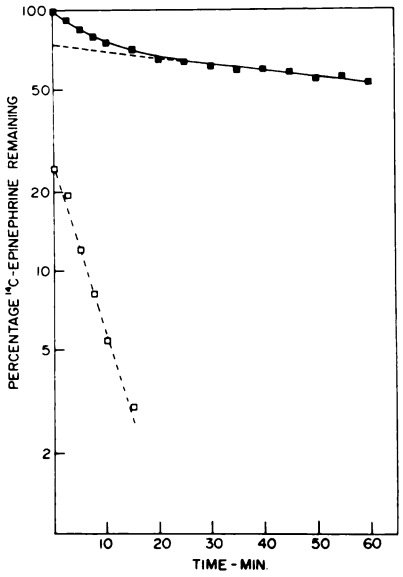

FIG. 1. *Efflux of ^{14}C-epinephrine from labeled storage vesicles.*

■, experimental values; – – –, final slope (determined by least-squares analysis) extrapolated to zero time; □, difference between experimental values and extrapolated line; –·–·–, least-squares fit of difference points; ——, theoretical efflux curve from two-compartment model. Parameters of efflux: 24.7% with $t_{1/2}$ = 4.7 min; 75.3% with $t_{1/2}$ = 117 min.

epinephrine is released with a half-time of 4.7 min, and 75% is released with a half-time of 117 min. The values of these parameters varied from preparation to preparation (10–30% with $t_{1/2}$ = 3–6 min, 70–90% with $t_{1/2}$ = 110–180 min); thus, the determination of an epinephrine control curve was required for each experiment.

The efflux of unlabeled epinephrine corresponds to the efflux of the endogenous compound, since the amount taken up during labeling is negligible. This release followed an equation of the type

$$n_t = n_0 e^{-kt}$$

which indicates a monophasic efflux (Fig. 2). The half-time for the disappearance of endogenous catecholamine from the granules was similar to that of the slow phase of ^{14}C-epinephrine efflux. No dopamine β-hydroxylase was released along with endogenous epinephrine.

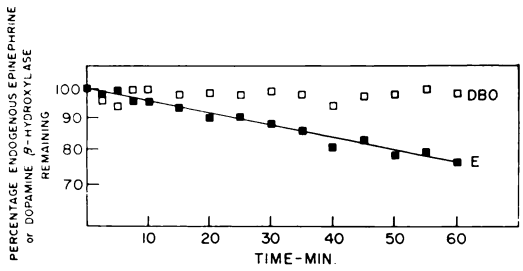

FIG. 2. *Efflux of endogenous epinephrine (E, ■) and soluble dopamine β-hydroxylase (DBO, □) from adrenal medullary granules (logarithmic ordinate).*

The release of the amine was accompanied by little or no release of the enzyme. The line was fitted to the epinephrine data by the method of least squares.

In order to test whether the fast efflux phase is a real phenomenon, and not an artifact resulting from damage to the granules from resuspension prior to the determination of efflux, labeled granules were incubated for 20 min at 37° to allow the fast efflux to occur. Following centrifugation, washing, and resuspension of the granules, efflux was again determined. Only the slow phase of efflux was observed, indicating that the fast phase was not a result of the method of preparation.

To determine whether the rapidly released pool represents a precursor pool for the stably bound epinephrine, an attempt was made to chase the epinephrine from one pool to the other. Labeled granules were washed and centrifuged to form a hard pellet and were incubated at 0° and 37° for 20 min under a solution containing sucrose-Tris, ATP, and $MgCl_2$. The supernatant fluid was removed, and the granules were washed and resuspended; efflux was then measured in the usual manner. The efflux from granules treated in this manner was identical with the efflux from granules which were not previously incubated. No reduction was observed in the size of either pool or in the magnitudes of the rate constants, suggesting that the two pools are independent of each other.

Effect of temperature, ATP-Mg^{2+}, and reserpine on efflux. The efflux of ^{14}C-epinephrine and endogenous epinephrine was temperature-dependent (Table 1). At 0° essen-

TABLE 1

Influence of temperature on epinephrine efflux

The epinephrine concentration during labeling was 0.22 mM. Efflux was studied in sucrose-Tris for 1 hr. Pool sizes are reported in terms of the percentage of total radioactive amine contained in the vesicles. The efflux of endogenous epinephrine is monophasic.

Efflux temperature	^{14}C-Epinephrine				$t_{1/2}$ for endogenous epinephrine
	Pool size	$t_{1/2}$	Pool size	$t_{1/2}$	
	%	*min*	%	*min*	*min*
0°	0		100	877	1600
30°	15	6.1	85	151	260
37°	24	5.3	76	133	148

TABLE 2

Influence of 5 mM ATP, 5 mM $MgCl_2$, and 0.01 mM reserpine on epinephrine efflux

The epinephrine concentration during labeling was 0.22 mM. Efflux was studied for 1 hr at 37° in sucrose-Tris containing the indicated agents. Pool sizes are reported in terms of the percentage of total radioactive amine contained in the vesicles. The efflux of endogenous epinephrine is monophasic.

Efflux conditions	^{14}C-Epinephrine				$t_{1/2}$ for endogenous epinephrine
	Pool size	$t_{1/2}$	Pool size	$t_{1/2}$	
	%	*min*	%	*min*	*min*
Control	24	4.8	76	148	215
Reserpine	20	4.6	80	178	226
ATP-$MgCl_2$	23	4.2	77	453	1033
ATP-$MgCl_2$ + reserpine	23	3.2	77	160	258

tially no efflux occurred; at 30° the efflux rate was lower than at 37°. The apparent change in the distribution of ^{14}C-epinephrine with temperature is unexplained.

The addition of 5 mM ATP-$MgCl_2$ to the incubation medium had no effect on the fast efflux but increased the half-time of the slow efflux substantially; the latter effect could be blocked by the inclusion of 10 μM reserpine (Table 2). Reserpine alone produced no change in the efflux of either ^{14}C-labeled or endogenous epinephrine.

Effect of labeling conditions on uptake and efflux. In these studies, the granules were labeled in the presence of various agents, and the efflux was measured for 1 hr in sucrose-Tris in the absence of these agents. Without the further addition of epinephrine, the external catecholamine concentration during loading averaged 0.22 mM. Increasing the concentration to 5 mM increased the percentage of ^{14}C-epinephrine in the rapidly released ("fast") pool to 34% from a control value of 22% (Table 3). The net uptake increased by a factor of 6; the uptake into the fast pool was increased 10-fold, while the uptake into the slow pool was increased 5-fold. Thus, the fast pool was more than twice as responsive to the increase in exogenous amines as was the slow pool.

In the absence of ATP-Mg^{2+} or in the presence of *N*-ethylmaleimide, the percentage of catecholamine in the fast pool was 44% when the granules were labeled at 5 mM epinephrine (Table 3). Uptake dropped by 50% compared to granules labeled in the presence of 5 mM epinephrine with ATP-Mg^{2+}; uptake into the fast pool was 2–3 times less sensitive to the lack of ATP-Mg^{2+} or to the presence of *N*-ethylmaleimide than was uptake into the slow pool (Table 3).

Reserpine reduced uptake by about 60% at the higher epinephrine concentration, and 90% at the lower epinephrine concentration (Table 3).[3] In the presence of reserpine, about 60% of the radioactive ^{14}C-epinephrine incorporated into the granules was stored in the fast pool at either epinephrine concentration.

Hence, although the percentage in the fast pool was increased by the absence of ATP-Mg^{2+} or by the presence of *N*-ethylmaleimide or reserpine, the net uptake of epinephrine was decreased in both pools. The percentage in the fast pool increased because the uptake into the slow pool decreased to a greater extent than did the uptake into the fast pool. There was no change in the half-time of disappearance from either compartment, regardless of treatment (Table 3).

[3] ^{3}H-Epinephrine was used at the lower epinephrine concentration, because the high specific activity enabled measurements to be made despite a 10-fold decrease in uptake in the reserpine-treated granules.

TABLE 3

Influence of labeling conditions on epinephrine efflux: effects of epinephrine concentration, ATP-$MgCl_2$ (5 mM), N-ethylmaleimide (0.15 mM), and reserpine (0.05 mM)

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. The efflux of endogenous epinephrine is monophasic.

Uptake conditions	^{14}C-Epinephrine				$t_{1/2}$ for endogenous epinephrine	Total uptake[a]
	Pool size	$t_{1/2}$	Pool size	$t_{1/2}$		
	%	min	%	min	min	
0.22 mM epinephrine + ATP-$MgCl_2$	22	4.0	78	131	175	3.1
5 mM epinephrine + ATP-$MgCl_2$	34	4.2	66	141	191	19.6
5 mM epinephrine	44	3.8	56	139	145	10.1
5 mM epinephrine + ATP-$MgCl_2$ + *N*-ethylmaleimide	44	4.1	56	158	142	10.1
5 mM epinephrine + ATP-$MgCl_2$ + reserpine	60	4.2	40	131	182	8.4
0.22 mM epinephrine[b] + ATP-$MgCl_2$ + reserpine	58	4.1	42	141	172	0.3

[a] Nanomoles taken up in 20 min per amount of granules containing 100 μg of catecholamine.
[b] ^{3}H-Epinephrine.

Effect of labeling time on uptake and efflux. The relationship between efflux and the period of incubation during labeling was determining. The shorter the period of labeling, the greater was the percentage found in fast pool (Table 4). With a 20-min incubation, 27% was contained in the fast pool; with a 5-min incubation, this amounted to 42%. Net uptake during this time period was fairly linear, although uptake into the fast compartment appeared to level off after 20 min.

Uptake and efflux of ^{3}H-octopamine, ^{3}H-metaraminol, and ^{3}H-tyramine. The uptake and efflux of octopamine, metaraminol, and tyramine were studied; the concentration of each during loading was 0.1 mM. Efflux was measured at 0° and 37°. With none of the amines was there significant efflux at 0°. At 37° the effluxes of all three were biphasic. The distributions of octopamine and metaraminol differed from that of epinephrine (Table 5). A much larger proportion was found in the fast pool. While the half-times of the fast pool for epinephrine, octopamine, and metaraminol were identical, octopamine and metaraminol were released much more rapidly from the slow pool than was epinephrine.

The uptakes of octopamine and metaraminol into the fast pool were higher than that of epinephrine; the uptakes into the slow pool and the net uptakes were lower than those of epinephrine. The efflux of endogenous epinephrine was unaffected by either amine. It should be noted that endogenous levels of epinephrine (0.1 mM) were present in the medium during the labeling of the granules with other amines.

The determination of the uptake and efflux characteristics of ^{3}H-tyramine was

TABLE 4

Influence of labeling conditions on epinephrine efflux: labeling incubation time

The epinephrine concentration during labeling was 0.22 mM. 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. The efflux of endogenous epinephrine is monophasic.

Labeling incubation time	^{14}C-Epinephrine				$t_{1/2}$ for endogenous epinephrine	Total uptake[a]
	Pool size	$t_{1/2}$	Pool size	$t_{1/2}$		
min	%	min	%	min	min	
5	41	4.7	59	129	129	0.5
10	34	4.5	66	125	148	1.2
20	27	5.0	73	114	152	2.5

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

TABLE 5

Efflux of tritiated epinephrine, octopamine, metaraminol, and tyramine

The epinephrine concentration during labeling was 0.1 mM. 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. The efflux of endogenous epinephrine is monophasic.

Amine (0.1 mM)	Pool size	$t_{1/2}$	Pool size	$t_{1/2}$	$t_{1/2}$ for endogenous epinephrine	Uptake[a]
	%	min	%	min	min	
Epinephrine	10	5.0	90	171	155	6.3
Octopamine	32	5.2	68	49.9	172	3.0
Metaraminol	64	5.8	36	37.3	155	2.4
Tyramine[b]	52	5.2	48	43.1	152	8.2

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

[b] Sixty per cent of the tyramine was converted to octopamine.

complicated by the conversion of tyramine to octopamine by dopamine β-hydroxylase present in the granules. About 60% of the tyramine taken up was converted to octopamine before the measurement of efflux began. The radioactivity was distributed evenly between the two pools (Table 5). The half-time for disappearance and the uptake of radioactivity into the slow pool were intermediate between those of octopamine and metaraminol, while the half-time for the fast pool was the same as that of the other amines. The majority of tritium label in the fast pool was associated with tyramine, while the majority in the slow pool was associated with octopamine. It seems likely, therefore, that tyramine is distributed in a manner more like metaraminol than like octopamine. The uptake of tritium label indicated a greater net uptake for tyramine than for epinephrine; the fast pool accumulated 6 times as much radioactivity as it did for epinephrine, while the amount taken up into the slow pool was lower than for epinephrine.

DISCUSSION

The granule preparation used in these studies is not completely pure, and is contaminated with mitochondria. However, the presence of a monoamine oxidase inhibitor (iproniazid) did not alter the uptake or efflux of amines, indicating that deaminated metabolites resulting from mitochondrial contamination are unimportant in these determinations. It should also be noted that metaraminol, which is not a substrate for monoamine oxidase, is distributed primarily into the fast pool, suggesting that this pool does not represent acid metabolites.

The efflux of newly incorporated ^{14}C-epinephrine was biphasic, suggesting that epinephrine is stored in two pools. One pool, containing about one-fourth of the ^{14}C-epinephrine, released the amine with a $t_{1/2}$ of 5 min at 37°. The other pool, containing three-fourths of the ^{14}C-epinephrine, released it with a $t_{1/2}$ of 2–3 hr.

The release of endogenous stores of epinephrine was monophasic and occurred at a slightly lower rate than that of the larger of the ^{14}C-epinephrine pools. The lower release rate for endogenous stores suggests that equilibration of the newly incorporated epinephrine with endogenous amines was not completed during the 20-min period of labeling. The fast efflux phase for endogenous stores observed by Lishajko (6, 11) is absent, because this pool is emptied during the labeling period. In some experiments, the efflux of endogenous amines was measured during the labeling phase and was found to be biphasic.

The release of soluble dopamine β-hydroxylase may be taken as a measure of the degree of lysis of granules during incubation (12). Since little or no dopamine β-hydroxylase was released during the efflux of epinephrine, the efflux represents leakage from intact granules, rather than release due to lysis. Furthermore, since the efflux was negligible at 0°, and since the granule membrane is permeable to amines at this temperature (13, 14), the epinephrine in both pools must exist in a bound state. The rate of efflux of ^{14}C-epinephrine from the two pools, therefore, is a measure of the stability of the catecholamine-binding complex in each pool.

If the two pools are in series, a net transfer of amine from the fast pool to the slow pool

should occur during the incubation of the labeled pellet. Since the half-time for the fast pool is 5 min, about 85% of the amine in the fast pool should enter the slow pool during the 20-min period during which the pellet was incubated. The two pools appear to be independent of each other; there was no transfer of ^{14}C-epinephrine from the fast pool to the slow pool or vice versa. Therefore the pools are in parallel, not series.

The two pools do not represent differential binding of the *d* and *l* forms of epinephrine to one type of binding site. The release of endogenous *l*-amines is biphasic if the granules are not incubated to label them with radioactive amines (6, 11). Carlsson and coworkers (15) have shown that the uptake of amines into the granules is not stereospecific, and in addition the pools described in the present work differed from each other in the characteristics of uptake. Assuming that any stereospecific uptake mechanism would favor the natural, *l*-isomer, and assuming that there is only one pool with different affinities for the *d*- and *l*-isomers, one would not expect the less stable, fast pool to contain more than 50% of the radioactive amine. Tables 3 and 5 indicate cases in which more than 50% was contained in the fast pool.

It seems likely that the two pools reflect the existence of two populations of particles. Both pools have an ATP-Mg^{2+}-dependent uptake system as well as an ATP-Mg^{2+}-independent system; the uptake into both pools, therefore, is decreased by the lack of ATP-Mg^{2+}, or by agents which block this stimulated uptake, such as reserpine or *N*-ethylmaleimide. The granules representing the slow pool are much more dependent on stimulated uptake than those representing the fast pool. Thus, when stimulated uptake is blocked, the proportion of amine contained in the fast pool is increased, although the net uptake in both pools is decreased. Since the active uptake mechanism causes saturation at low substrate levels (15), an increase in exogenous amine concentration during labeling increases the percentage of ^{14}C-epinephrine found in the fast pool. At low substrate levels, the slow pool will be labeled preferentially. These conclusions are supported by the observation that ATP-Mg^{2+} in the efflux mixture greatly lengthens the half-life of epinephrine in the slow pool but does not change the efflux from the fast pool (Table 2). ATP-Mg^{2+} stimulates the reuptake of epinephrine only into the slow pool during efflux, since the external amine concentration is low, and this uptake is blocked by reserpine.

Because of the disparity in the stability of binding of epinephrine in the two pools (as reflected in their efflux half-times), the rates of equilibration during labeling will differ. The fast pool will equilibrate rapidly, and the slow pool will equilibrate slowly. For this reason, and because the slow pool has a greater storage capacity, the longer the granules are exposed to ^{14}C-epinephrine during labeling, the greater is the percentage found in the more stable pool.

The structure-activity relationships for the binding of amines were examined. Octopamine was distributed into the fast pool to a greater extent than epinephrine on both an absolute and a percentage basis, while the uptake into the slow pool was lower than that of epinephrine. The stability of the octopamine storage complex was equal to that of epinephrine in the fast pool, but was markedly lower in the slow pool. Hence, both ring hydroxyl groups contribute to the uptake and storage by the slow pool. The uptake and the stability of the storage complex in the slow pool were further decreased if metaraminol was substituted for epinephrine, suggesting that side chain modifications also alter uptake and storage in this compartment. The stability of storage of metaraminol in the fast pool was equal to that of epinephrine, but the net uptake of metaraminol was higher. The slow pool, therefore, has more rigid structural requirements for uptake and storage than does the fast pool. The qualitative differences between the two pools are summarized in Table 6.

The uptake of tyramine was complicated by the conversion of 60% of the substance to octopamine. The total uptake of tyramine was higher than that of epinephrine, even though the uptake into the slow pool was lower. The relatively large amount of tyramine in the fast pool suggests that a β-hydroxyl group is not necessary for uptake

TABLE 6
Differences between amine storage pools

Characteristic	Fast pool	Slow pool
Catecholamine capacity	Low	High
Stability of storage complex	Low	High
Dependence on stimulated uptake	Low	High
Sensitivity to reserpine and *N*-ethylmaleimide	Low	High
Rate of equilibration with exogenous amines	Fast	Slow
Uptake of metaraminol	High	Low
Effect of structural change of amine on rate of efflux	Low	High

and storage into this pool. The study of Musacchio and co-workers (16), in which a variety of phenylethylamine derivatives were administered to animals, indicates that both the catechol and β-hydroxyl groups are important for efficiency of binding in nerve vesicles.

The relatively low uptakes of octopamine and metaraminol into the slow pool suggest that these amines might compete for a common uptake system with the exogenous epinephrine present during the labeling of the granules. Since the slow pool exhibits greater structural specificity for uptake than does the fast pool, epinephrine will reduce the uptakes of octopamine and metaraminol into the slow pool but not into the fast pool.

Previous work by Hillarp (5) suggested the existence of two pools for the storage of amines; the two pools differed in the density of the storage granules and in the stability of amine storage. Lishajko (11) proposed the existence of two storage pools in the heavy granule fraction; the two pools differed in the identity of the amine stored, the rate of release of the amine, and the osmotic pressure inside the granule. The two-pool system described in the present study differs from that of Hillarp (5) in that it involves the heavy granule fraction only. While Lishajko (11) proposed a system with segregation of different amines in the two pools, both pools described in the present study were able to take up epinephrine. The rapid initial efflux of catecholamine, which Lishajko ascribed to lysis of the granules, can be demonstrated in the absence of lysis with newly incorporated amines. In addition, Lishajko demonstrated that the rapid release of endogenous amines could be reduced if the efflux occurred in hypertonic (0.5 M) sucrose. In the present study, no change in the rate of release of ^{14}C-epinephrine was observed in hypertonic solutions.

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