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MATURATION OF THE ADRENAL MEDULLA—I
UPTAKE AND STORAGE OF AMINES IN ISOLATED STORAGE VESICLES OF THE RAT*

THEODORE A. SLOTKIN

Department of Physiology and Pharmacology, Duke University Medical Center, Durham, N.C. 27710, U.S.A.

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Abstract—Albino Wistar rats were sacrificed at 10-day intervals from birth to 50 days of age. Adrenal storage vesicles were analyzed for catecholamines (CA) and for uptake of ^{14}C-epinephrine (E) and ^{3}H-metaraminol (MA). At birth, CA and E and MA uptakes per gland were 3–8 per cent of 50-day levels, about 20 per cent at 10 days, 25 per cent at 20 days, 40 per cent at 30 days, and 65 per cent at 40 days. Although all three parameters increased approximately in parallel, MA uptake slightly exceeded E uptake and CA from birth to 30 days. As a result, MA uptake per 100 μg of CA in the vesicles was 20 per cent above normal at the early time periods, while E uptake per 100 μg of CA was normal. There was no change in the endogenous norepinephrine/epinephrine ratio during development. The rates of efflux of ^{14}C-E and CA from isolated vesicles was normal except at 10 days, when a more rapid efflux was observed, suggesting that storage stability was impaired at that time. Changes in the density of labeled vesicles were evaluated by centrifugation on continuous sucrose density gradients. At birth, both E- and MA-labeled vesicles were more dense than vesicles from 50-day-old rats. By 10 days, however, E- and MA-labeled vesicles were lighter than normal. E-labeled vesicles returned to normal by 20 days, but MA-labeled vesicles did not approach normal densities until 30–40 days. These data suggest that the rate of vesicle synthesis is low at birth but increases rapidly thereafter. The heavy vesicles at birth may represent "overloaded" vesicles, while the lighter ones at later times may be "underloaded" due to the rapid synthesis of new vesicles.

DURING prenatal and postnatal development, there is a marked increase in catecholamine levels in adrenergic neurons and in the adrenal medulla,[1–4] as well as changes in the levels of catecholamine-synthesizing enzymes.[1,5] In chick embryos, tyrosine hydroxylase is present on the first day of gestation, and all the enzymes are present on the sixth day.[1] In the rat, electron microscopy of adrenal tissue reveals the presence of a small number of storage vesicles 4 days before birth, with the subsequent appearance (2 days before birth) of catecholamines.[6,7] These data suggest that the biogenesis of storage vesicles is a determining factor in the prenatal increase in adrenal catecholamines.

Recent studies[8–10] have examined the properties of newly synthesized storage vesicles in adult rats in which adrenal catecholamine stores had been depleted by insulin or reserpine treatment. These investigations have now been expanded to include the storage vesicles of postnatal developing rats. In the present study, the ability of the vesicles to incorporate catecholamines (epinephrine, uptake predominantly ATP- Mg^{2+}-dependent[11,12]) and non-catecholamines (metaraminol, uptake

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predominantly ATP-Mg^{2+}-independent[11–13]) has been examined, along with the stability of storage of endogenous and incorporated amines. Additionally, the equilibrium densities of labeled vesicles from adult and developing rats have been examined utilizing continuous sucrose density gradient centrifugation.

METHODS

Uptake of amines. Albino Wistar rats (Hilltop Lab Animals, Scottdale, Pa.) were sacrificed 1, 10, 20, 30, 40 or 50 days after birth. Adrenal glands were excised, cleaned of fat and connective tissue, and pooled as follows: at 1 day of age, 4 pairs of adrenals per determination were used; at 10 days, 3 pairs; at 20 days, 2 pairs; at 30 days, 2 pairs; at 40 and at 50 days, 1 pair each. The pooling of glands from younger rats was necessary to achieve measureable degrees of uptake. Each experiment consisted of four determinations of uptake at 50 days along with six determinations of uptake at a younger age.

Each group of glands was homogenized (glass-to-glass) in 2·2 ml of sucrose–Tris [300 mM sucrose, 25 mM Tris, 0·01 mM iproniazid (monoamine oxidase inhibitor) adjusted to pH 7 with sulfuric acid] and a 0·1-ml aliquot was withdrawn for catecholamine analysis. The suspension was centrifuged at 800 *g* for 10 min to remove debris and the supernatant was divided into four 0·4-ml portions. Tubes were prepared in duplicate containing: (a) 0·4 ml supernatant, 0·1 ml of 1 mM epinephrine, 0·1 ml of 50 mM ATP-Mg^{2+}, 1 μCi ^{14}C-epinephrine, and sucrose–Tris to a final volume of 1 ml; or (b) 0·4 ml supernatant, 0·1 ml of 1 mM epinephrine, 0·1 ml of 1 mM metaraminol, 0·1 ml of 50 mM ATP-Mg^{2+}, 5 μCi ^{3}H-metaraminol, and sucrose–Tris to a final volume of 1 ml. The added epinephrine was sufficient to eliminate any differences in extravesicular catecholamine concentrations among samples. One metaraminol- and one epinephrine-containing sample were placed in a water bath at 30° and shaken for 30 min while the duplicate tubes were kept on ice. In all cases, the tubes were open to the air. Uptake was stopped by the addition of 2 ml of ice-cold sucrose–Tris, the mixture was centrifuged at 26,000 *g* for 10 min, and the supernatant was analyzed for catecholamines and radioactivity. The vesicular pellet was washed by resuspension in sucrose–Tris and recentrifuged. The wash procedure was repeated once more and the final pellet was resuspended in 3 ml of 3·5% perchloric acid, centrifuged, and the supernatant analyzed for catecholamines (CA) and radioactivity. Uptake was calculated according to the equations:

$$\text{Gross uptake/gland} = \frac{\text{cpm in vesicles} \times \text{CA content/gland}}{\text{Specific activity of labeling medium} \times \text{CA content of vesicles in sample}}$$

$$\text{Gross uptake/100 } \mu\text{g CA} = \frac{\text{Uptake/gland} \times 100}{\mu\text{g CA/gland}}$$

The uptake at 0° was then substracted from the uptake at 30° to give the temperature-dependent vesicular uptakes. The uptake at 0° was constant throughout development; since incorporation at 0° was independent of the amount of tissue and since uptake into vesicles is negligible at 0°, this indicated that 0° uptake represented contamination or nonspecific binding. In adult rats incorporation at 0° was generally 20 per cent of the uptake at 30°, and the percentage increased with decreasing age (60 per cent in neonates). Results are expressed as the percentage of 50-day values. Calculation of

uptake in terms of catecholamine content corrects for lysis of vesicles during homogenization, since the uptake in an aliquot of homogenate is determined in terms of the proportion of total catecholamines contained in that aliquot.

Results are not reported on a tissue weight basis for two reasons: first, only part of of the adrenal is medulla, and the proportion of the gland weight will vary according to the relative development of both cortex and medulla; second, the small glands in neonatal rats cannot easily be cleaned of all fat and connective tissue, and tissue weight would not adequately reflect adrenal weight.

Efflux of amines. The effluxes of ^{14}C-epinephrine and endogenous catecholamines were determined in two groups of experiments (because of practical experimental limitations, no more than four efflux curves could be run simultaneously) utilizing the following sets of adrenal glands: In the first group, at 1 day of age, 37 pairs of adrenals were used; at 10 days; 21 pairs; and at 50 days, 4 pairs. In the second group, at 20 days of age, 10 pairs were used; at 30 days, 7 pairs; at 40 and at 50 days, 4 pairs each. The adrenals from rats of each age were pooled and homogenized (glass-to-glass) in 4 ml sucrose–Tris, centrifuged at 800 *g* for 10 min, and the supernatant was decanted. The vesicles in the supernatants were then labeled with ^{14}C-epinephrine: 3·5 ml of each supernatant was added to 0·5 ml of 1 mM epinephrine, 0·5 ml ^{14}C-epinephrine (10 μCi/ml) and 0·5 ml of 50 mM ATP-Mg^{2+}. The samples were incubated 30 min at 30°, at which time uptake was stopped by the addition of 5 ml of ice-cold sucrose–Tris. The samples were centrifuged 10 min at 26,000 *g* and the vesicular pellets were resuspended by homogenization (Teflon-to-glass) in 5 ml sucrose–Tris. After recentrifugation, the pellets were washed again, recentrifuged and resuspended (Teflon-to-glass homogenization) in 8 ml sucrose–Tris. Efflux was determined on 1-ml aliquots by incubation at 30° for 0, 5, 10, 20, 30, 40 and 60 min. Efflux was stopped by the addition of 2 ml of ice-cold sucrose–Tris; the samples were then centrifuged and the supernatants analyzed for CA and radioactivity. The vesicular pellets were lysed with 3·5% perchloric acid, centrifuged, and the supernatants analyzed for CA and radioactivity. Efflux was calculated as the percentage of ^{14}C-epinephrine or endogenous CA remaining, as described previously.[11] Each experiment was repeated at least once and the results were in good agreement. Data are reported from single experiments.

Continuous sucrose density gradients. Adrenal glands were pooled as follows: at 1 day of age, 37 pairs were used; at 10 days, 31 pairs; at 20 days, 14 pairs; at 30 days, 10 pairs; at 40 days, 7 pairs; and at 50 days, 4 pairs. The glands from each group were homogenized in 8 ml sucrose–Tris and centrifuged at 800 *g*. Vesicles from each group were labeled as follows: (1) 3·5 ml supernatant from 50-day-old rats, 0·6 ml of 50 mM ATP-Mg^{2+}, 0·6 ml of 1 mM epinephrine, 0·6 ml ^{14}C-epinephrine (10 μCi/ml) and 0·7 ml sucrose–Tris; (2) 3·5 ml supernatant from 50-day-old rats, 0·6 ml of 50 mM ATP-Mg^{2+}, 0·6 ml of 1 mM epinephrine, 0·6 ml of 1 mM metaraminol and 0·7 ml ^{3}H-metaraminol (50 μCi/ml); (3) same as (1), except 3·5 ml supernatant from day "*x*"; (4) same as (2), except 3·5 ml supernatant from day "*x*". Samples were incubated at 30° for 30 min, and uptake was stopped by the addition of 6 ml of ice-cold sucrose–Tris. The vesicles were washed twice as described in the efflux studies and resuspended in 2·2 ml (samples 1 and 2) or 1·1 ml (samples 3 and 4) of sucrose–Tris. Mixtures of vesicles were prepared as follows: tube A, 1 ml of sample 1 and 1 ml of sample 2; tube b, 1 ml of sample 1 and 1 ml of sample 4; tube C, 1 ml of sample 2 and 1 ml of sample 3. The mixtures were then layered on continuous sucrose density gradients

(hyperbolic from 2 to 1 M; total volume, 30 ml[9]) and centrifuged at 90,000 *g* for 3 hr. The tubes were emptied dropwise from the bottom (20 drops/sample) and fractions were analyzed for CA and radioactivity. Each experiment was performed at least twice, and the results were in good agreement. Data are reported from single experiments.

Assays. All samples were deproteinized by the addition of perchloric acid to a final concentration of 3·5%, centrifuged at 26,000 *g* for 10 min, and the supernatants used for analysis of total catecholamines by the trihydroxyindole method,[14] and for ^{14}C and ^{3}H by liquid scintillation spectrometry.[11] In another group of rats, differential analysis of norepinephrine and epinephrine in glands from individual animals was performed by the method of Merrills.[15]

Statistics. Results are presented as means ± standard errors. Levels of significance are calculated by Student's *t*-test, and efflux curves are fitted by the method of least squares,[16] according to a two-compartment model.[11]

Materials. Epinephrine-7-^{14}C and metaraminol-7-^{3}H were obtained from New England Nuclear Corp.; epinephrine bitartrate and norepinephrine bitartrate were obtained from Winthrop Laboratories, and metaraminol bitartrate from Merck, Sharp & Dohme.

RESULTS

During the course of development, total catecholamine levels and uptakes per gland increased from about 5 per cent of the 50-day value at birth to 65 per cent at 40 days (Fig. 1). While the largest absolute increases occurred from 30 to 50 days, the

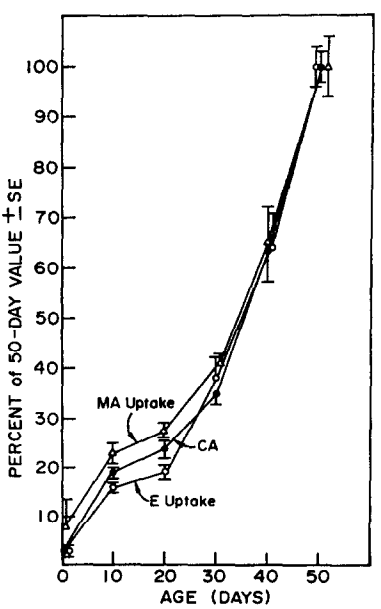

FIG. 1. Catecholamine (CA) levels (●), epinephrine uptake per gland (○) and metaraminol uptake per gland (△) expressed as percentage of 50-day values. Points represent mean ± S.E. of six determinations for ages 0–40 days, 20 determinations for age 50 days. Fifty-day values were: CA, 9·7 ± 0·3 μg/gland; epinephrine uptake, 2·01 ± 0·09 nmoles/gland; metaraminol uptake, 0·32 ± 0·02 nmoles/gland.

TABLE 1. TOTAL CATECHOLAMINE AND NOREPINEPHRINE CONTENTS OF ALBINO WISTAR RAT ADRENALS

Age (days)	Total catecholamines (μg/gland $\pm$ S.E.)	Norepinephrine content (% total amines $\pm$ S.E.)	No. of animals
1	0·28 $\pm$ 0·05	31 $\pm$ 0	3
10	1·30 $\pm$ 0·10	20 $\pm$ 1	3
20	2·63 $\pm$ 0·28	25 $\pm$ 2	3
30	3·54 $\pm$ 0·42	25 $\pm$ 3	3
40	5·60	26	1
50	9·04 $\pm$ 0·40	26 $\pm$ 1	2

largest relative rate of rise occurred between 1 and 10 days, when catecholamine content and uptake per gland increased 3- to 6-fold; later increases were smaller proportionally to the amount already present. The percentage of catecholamines as norepinephrine varied between 20 and 30 per cent from birth to 50 days of age (Table 1).

The incorporations of epinephrine and metaraminol were also calculated on the basis of uptake per 100 μg of CA present in the vesicles. These values should remain constant if there are no changes in the properties of individual vesicles. However, between 10 and 30 days, metaraminol uptake per 100 μg of CA was significantly elevated above 50-day levels, but there was no significant difference for epinephrine (Fig. 2). The values for day 1 have been omitted because of wide variations due to the low CA levels.

At birth, there was little difference in the rates of efflux of endogenous CA and ^{14}C-epinephrine compared to 50-day-old rats (Fig. 3A and B). However, 10 days after birth, the stability of storage was decreased for both endogenous CA and ^{14}C-epinephrine (Fig. 3A and B). The differences disappeared by 20 days, and there were no changes in efflux pattern observed thereafter (Fig. 3C and D).

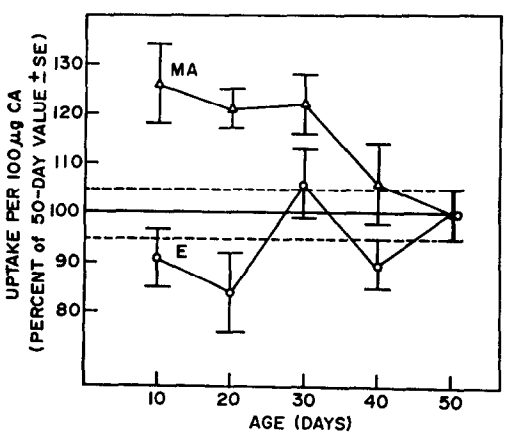

FIG. 2. Epinephrine (E) uptake (○) and metaraminol (MA) uptake (△) per 100 μg of catecholamines in vesicles. Points represent mean $\pm$ S.E. of six determinations for ages 10–40 days, 16 determinations for age 50 days. None of the values for epinephrine is statistically different from the 50-day values, while metaraminol is higher than 50-day values at 10, 20 and 30 days ($P < 0.02$). Fifty-day values were: epinephrine uptake, 22·1 $\pm$ 1·1 nmoles/100 μg CA; metaraminol uptake, 3·3 $\pm$ 0·2 nmoles/100 μg CA.

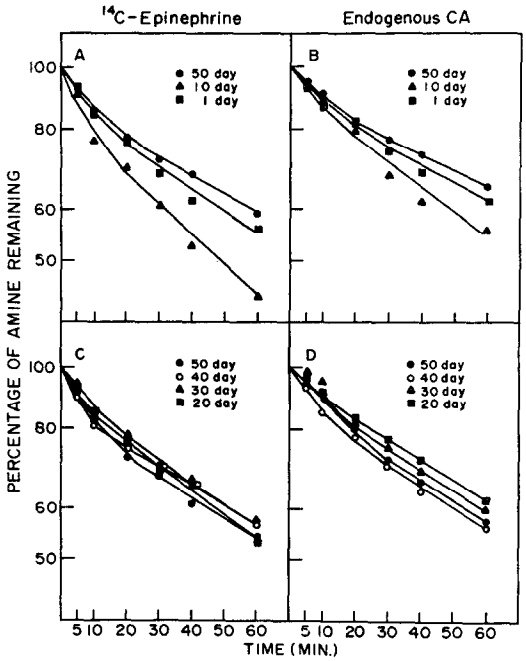

FIG. 3. Efflux of ^{14}C-epinephrine and of endogenous CA from isolated adrenal vesicles. A and B, 1-, 10- and 50-day values; C and D, 20-day to 50-day values. Ordinate is logarithmic.

The distribution of labeled storage vesicles on continuous sucrose density gradients was markedly age-dependent. In 50-day-old rats, both ^{14}C-epinephrine- and ^{3}H-metaraminol-labeled vesicles distributed in a manner identical to each other and to endogenous CA (Figs. 4A and 5A); this indicates that the incorporation of radioactive amines by the 800 *g* supernatant is solely into the storage vesicles. When vesicles from 1-day-old rats were labeled with ^{3}H-metaraminol and run in the same gradient with ^{14}C-epinephrine-labeled vesicles from adult rats, the tritium label was displaced toward higher density particles (Fig. 4B). Similarly, ^{14}C-epinephrine-labeled vesicles from the 1-day-old rats showed higher densities than ^{3}H-metaraminol-labeled vesicles from the 50-day-old rats (Fig. 4C).

By 10 days after birth, this pattern was completely reversed. Metaraminol-labeled vesicles from 10-day-old rats were markedly lighter in density than vesicles from 50-day-old rats (Fig. 5B), while epinephrine-labeled vesicles from the 10-day-old rats were somewhat lighter than in the 50-day-old rats (Fig. 5C). To quantitate the differences in densities, for each gradient a ratio was calculated:

$$\frac{^{3}\text{H}/^{14}\text{C in trailing edge of CA peak}}{^{3}\text{H}/^{14}\text{C in leading edge of CA peak}}$$

where the trailing edge represented the three fractions immediately following the peak and the leading edge represented the peak and two preceding fractions. If metaraminol and epinephrine distributed identically in the 50-day-old rats, the ratio should be close to unity; in ten determinations (tube A of each gradient run), the trailing edge/leading edge ratio averaged 1·08, thus confirming that the two labels distributed into

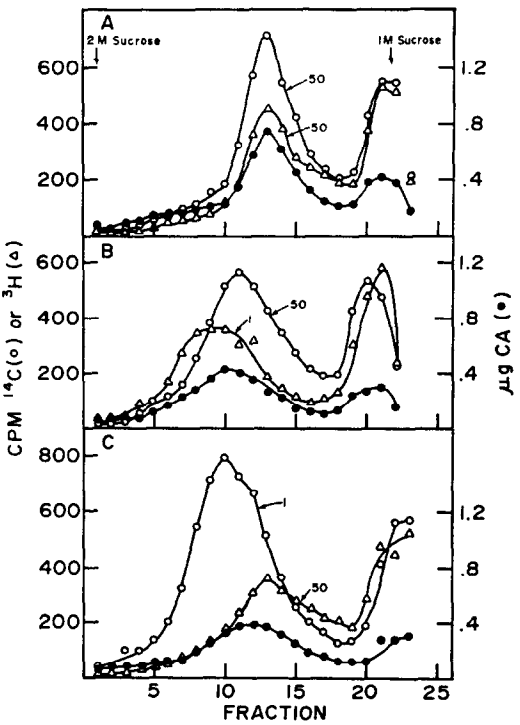

FIG. 4. Continuous sucrose density gradients of mixtures of labeled adrenal storage vesicles. (A) ^{14}C-epinephrine (○) and ^{3}H-metaraminol (△), 50-day-old rats; (B) ^{14}C-epinephrine (○) from 50-day-old rats and ^{3}H-metaraminol (△) from 1-day-old rats; (C) ^{14}C-epinephrine (○) from 1-day-old rats and ^{3}H-metaraminol (△) from 50-day-old rats. In each gradient, solid circles (●) denote catecholamines.

particles with the same density. A shift toward lighter density particles in the younger rats would raise the ratio in tube B and lower it in tube C. The results are shown in Table 2. In the 1-day-old rats, there was a marked decrease in the ratio for tube B and an increase for tube C, reflecting increases in the densities of both epinephrine- and metaraminol-labeled vesicles compared to the 50-day-old rats. By 10 days, however, the metaraminol-labeled vesicles were much lighter than in the 50-day-old rats (ratio = 1·50); the epinephrine-labeled vesicles were also lighter (ratio of 0·92 vs 1·08 in 50-day-old rats). By 20 days, epinephrine label distributed in a nearly normal manner, but metaraminol label was still associated with particles of lighter density. Ratios for both labels approached 50-day levels by 30–40 days.

DISCUSSION

The properties of rat adrenal storage vesicles which accompany development can be summarized as follows: (1) at birth, vesicles have higher than normal densities; (2) at 10–20 days, lower than normal densities, elevated uptake of metaraminol compared to epinephrine uptake in the lighter particles, and increased rate of efflux (10 days); (3) at 30–40 days, densities approach adult (50-day) levels, and relative uptakes of metaraminol vs epinephrine approach adult.

Why should the storage vesicles in the newborn be more dense than in adults? It is not likely that the heavier vesicles represent norepinephrine-containing, rather than

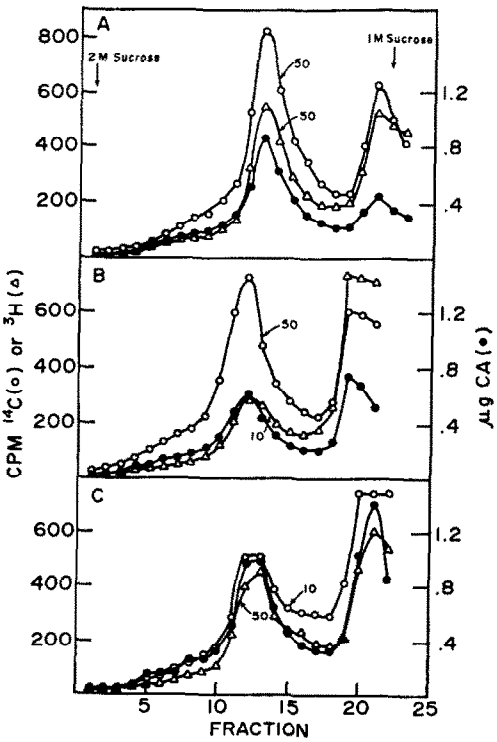

FIG. 5. Continuous sucrose density gradients of mixtures of labeled adrenal storage vesicles. (A) ^{14}C-epinephrine (○) and ^{3}H-metaraminol (△), 50-day-old rats; (B) ^{14}C-epinephrine (○) from 50-day-old rats and ^{3}H-metaraminol (△) from 10-day-old rats; (C) ^{14}C-epinephrine (○) from 10-day-old rats and ^{3}H-metaraminol (△) from 50-day-old rats. In each gradient, solid circles (●) denote catecholamines.

epinephrine-containing particles. Eade,[17] Fortier *et al.*,[18] Schumann,[19] and Hagen and Barrnett[20] have reported that norepinephrine-containing vesicles are more dense, and in several species and some rat strains the relative amount of norepinephrine is at a maximum at birth and declines markedly thereafter.[21] However, determination of relative norepinephrine and epinephrine contents in the present study indicated only small changes with development, confirming an earlier report that the maturing adrenals of albino Wistar rats contain a constant ratio of norepinephrine to epinephrine.[22] Thus, the increased density of storage vesicles at birth cannot be attributed to differences in the nature of the amine stored.

A likely explanation for the existence of high density particles is that the vesicles may contain higher than normal concentrations of catecholamines, ATP and soluble proteins. Catecholamines and ATP alone make up 40 per cent of the dry weight of the storage vesicles,[23] and decreases in the amounts of these components are associated with decreased vesicle densities.[9,10,24,25] Studies in adult rats have shown that the composition and hence the buoyant density of newly synthesized storage vesicles depend on the relative rates of synthesis of catecholamines, ATP and vesicles.[8–10] Thus, an increase in vesicle density in the neonate may reflect increased vesicular catecholamines, ATP or soluble proteins due to a disparity in the relative rates of

TABLE 2. RELATIVE DENSITIES OF LABELED ADRENAL VESICLES FROM DEVELOPING RATS

Age (days)	Ratio of $^3H/^{14}C$ in trailing edge of peak / $^3H/^{14}C$ in leading edge of peak: Tube B (^{3}H-metaraminol in young rats, ^{14}C-epinephrine in 50-day rats)	Tube C (^{3}H-metaraminol in 50-day rats, ^{14}C-epinephrine in young rats)
1	0·50	2·56
10	1·50	0·92
20	1·62	1·04
30	1·16	1·07
40	1·10	1·05
50	1·08 ± 0·05 (10 experiments)	1·08 ± 0·05

synthesis of these components vs. the synthesis of storage vesicles themselves, with the consequent production of "overloaded" vesicles. The later decrease in density may then represent a changeover to higher rates of vesicle synthesis such that the vesicles are "underloaded" relative to the vesicles in adults, a situation which is known to occur in adult rats and rabbits during recovery of their catecholamine stores after depletion with insulin or reserpine.[8–10,24,25] In the light vesicles from these adult rats there is an increase in metaraminol uptake relative to epinephrine uptake.[8,9] Similarly, in the vesicles of developing rats, there was a significant elevation in metaraminol uptake per 100 μg of catecholamines during the period 10–30 days, while there was no change for epinephrine. This implies that there was a decreased specificity for catecholamines vs. non-catecholamines, although the changes were much smaller than in the insulin- or reserpine-treated adult rats. (The adult resynthesizes all the vesicles, ATP and catecholamines in 4 days, while the increase to adult levels is spread out over 50 days in the developing rat.) The smaller shift in specificity and the nearly parallel nature of the increases in catecholamines and uptake per gland compared to the drug-treated adults indicate that, although there is a lag between vesicle synthesis and filling with soluble components in both cases, there is less of a disparity in the rates of assemblage of the various components in developing rats compared to insulin- or reserpine-treated adults.

There is an increased rate of efflux of amines at 10 days, but not at birth. The rate of efflux is actually a measure of the stability of the storage complex,[11] since the vesicle membrane is apparently freely permeable to catecholamines.[26,27] The decrease in stability may reflect a reduced number of binding sites due to a difference in the rate of vesicle synthesis vs. accumulation of binding components. It is noteworthy that the change in efflux occurs at the time when the proportional increase in CA stores is at a maximum (6-fold increase from birth to 10 days) and at a time when amine uptake occurs in lighter vesicles. These data tend to support the hypothesis that vesicle synthesis outstrips synthesis of soluble components at this stage of development.

In constructing a model of this type, it should be kept in mind that the changes observed in vesicular properties represent gross alterations which can be produced by

many different factors. For example, where measurements of vesicle density are made, the ATP, catecholamine and protein contents, the size of the particle, the permeability of the vesicle membrane, and the density and osmolarity of the gradient material all have effects on the observed density of the vesicles.[28,29] The proof of this model requires the direct measurement of concentrations of vesicle components as well as determination of factors, such as neural input, which alter the synthesis of these components during development.

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