Biochemical Pharmacology, Vol. 24, pp. 89–97. Pergamon Press, 1975. Printed in Great Britain.

MATURATION OF THE ADRENAL MEDULLA—III

PRACTICAL AND THEORETICAL CONSIDERATIONS OF AGE-DEPENDENT ALTERATIONS IN KINETICS OF INCORPORATION OF CATECHOLAMINES AND NON-CATECHOLAMINES*

THEODORE A. SLOTKIN†

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

(*Received* 28 *December* 1973; *accepted* 17 *May* 1974)

Abstract—Rats were sacrificed at 10-day intervals from birth to 50 days of age and the kinetics of uptake of epinephrine (E) and metaraminol (MA) into isolated adrenal medullary storage vesicles were measured. The K_m for epinephrine remained at about 30 μM throughout development, but the maximal uptake (U_{max}) at birth was 30 nmoles/30 min/100 μg of endogenous catecholamines compared to 20 nmoles at subsequent ages. The K_m for MA was about 4 mM at birth, 0·7 mM at 10–20 days of age and 1·2 mM thereafter, while U_{max} was approximately 170 nmoles initially, 50 nmoles at 10–20 days and 70 nmoles thereafter. The preference of the vesicles for uptake of E vs MA (both at 0·1 mM) was approximately 6 to 1 at birth, 1 to 1 at 10 days, 3·5 to 1 at 20 days and 5 to 1 thereafter, while the preference for E vs tyramine was 2 to 1, except at 10 days, when the ratio was 1 to 1. These data suggest that the two vesicular uptake mechanisms, typified by incorporations of E and of MA, develop independently of each other and that the age-dependent alterations are present both at the level of the vesicle membrane and intravesicular storage. The development of a kinetic model aids in the identification of probable sites of specific age-dependent alterations. Maturational changes in uptake and specificity can produce parallel changes in synthesis of catecholamines as determined by conversion of newly incorporated tyramine to octopamine.

On the biochemical level, the development of sympathetic neurons and the adrenal medulla is a complex process involving changes in levels of catecholamine-synthesizing enzymes [1–3] and in the number and properties of storage vesicles [3–6]. These alterations appear to a large extent to be dependent upon the degree of neural input to the tissue [2, 3, 7]; thus maturational increases in catecholamines, tyrosine hydroxylase and dopamine β-hydroxylase (DBH) can be retarded by denervation of the adrenal medulla [2] or in sympathetic ganglia by chronic administration of ganglionic blocking agents[7]. In several species, including the cow and the rat, innervation of the adrenal is absent or nonfunctional at birth and a normal secretory response is not obtained in response to stimulation of the splanchnic nerve [3, 8]. In the rat adrenal medulla, neural tissue proliferates rapidly in the first week after birth [9], and by 10 days of age a normal secretory response is obtainable [3].

The appearance of neural input between birth and 10 days results in marked changes in the content and properties of adrenal storage vesicles [3, 6]. Initially, the vesicles have a higher equilibrium density than vesicles from adult rats and there are few "immature" vesicles (vesicles which are usually associated with neural stimulation and which have below normal catecholamine and ATP contents). By 10 days of age, immature vesicles are present and there are alterations in the proportions of catecholamines, ATP and dopamine β-hydroxylase [3]. Furthermore, these immature vesicles appear to have altered capacities to take up and store amines as well as altered specificities for epinephrine and metaraminol [6]. The two amines utilize two uptake systems, one of which operates at low concentrations, is stimulated by ATP–Mg^{2+} and prefers catecholamines, and the other of which is not stimulated at ATP–Mg^{2+}, is not specific for catecholamines and operates at higher concentrations [10–15]. Because uptake is a complex process involving inward transport, outward leakage (efflux) and storage in at least two intravesicular binding pools, it is difficult to determine which alterations on the molecular scale might be responsible for age-dependent changes in amine uptake. In the current study, the kinetics of uptake of epinephrine and metaraminol have been examined in vesicles from developing rats, as well as

* Supported by grants from the United States Public Health Service DA-00465-01, the Council for Tobacco Research-U.S.A., Inc., and by a Grant-in-Aid from the American Heart Association with funds contributed in part by the North Carolina Heart Association.

† Recipient of Faculty Development Award in Pharmacology from the Pharmaceutical Manufacturers Association Foundation.

the ability of epinephrine to compete with other amines. By utilizing a model kinetic system, these data can identify probable loci of maturational changes in the function of adrenal storage vesicles.

METHODS

Sprague–Dawley rats (Zivic-Miller) were sacrificed at 10-day intervals from birth to 50 days of age. The adrenal glands were removed, cleaned of fat and connective tissue, and pooled as follows: for neonates, 60 rats for each experiment involving determination of K_m and maximal uptake (U_{max}) and 25 rats for each competition or synthesis study; at 10 days, 45 rats for K_m and U_{max} determinations and 25 for competition or synthesis, at 20 days, 25 and 12; at 30 days, 11 and 7; at 40 days, 7 and 3; and at 50 days, 7 and 2.

Measurements of uptake kinetics. The adrenal glands from each group were homogenized in an all-glass apparatus containing 13·5 ml of ice-cold 0·3 M sucrose buffered at pH 7·4 with 0·025 M Tris–sulfate and containing 10^{-5} M iproniazid (irreversible monoamine oxidase inhibitor). The homogenate was centrifuged at 800 *g* for 10 min, the pellet was discarded and 0·5-ml aliquots of supernatant were added to tubes containing sucrose–Tris with 5 mM ATP and Mg^{2+} and either 1 μCi ^{14}C-epinephrine + unlabeled epinephrine (varying concentrations from 5–80 μM) or 5 μCi ^{3}H-metaraminol + unlabeled metaraminol (varying concentrations from 0·1 to 1 mM); total volume was 1 ml for each tube. Tubes were incubated in a water bath at 30° for 30 min, while duplicate tubes were kept on ice. Under these conditions, uptake occurs solely into storage vesicles [15]. The uptake was stopped by the addition of 2 ml of ice-cold sucrose–Tris and the samples were centrifuged at 26,000 *g* for 10 min. The supernatant was deproteinized with 3·5% perchloric acid and analyzed for catecholamines and radioactivity to determine the specific activity of the incubation medium. The labeled pellet was washed twice, resuspended and deproteinized as described previously [6], and analyzed for catecholamines and radioactivity. The temperature-dependent component of uptake was then calculated by the method of Slotkin *et al.* [14] and expressed as nmoles taken up per 100 μg of endogenous catecholamines. For epinephrine, the uptake at 0° was 10–20 per cent of the 30° uptake, while for metaraminol, 0° uptake represented 15–25 per cent. Because of small degrees of lysis of and leakage from vesicles, catecholamine concentrations in the medium always exceeded the added epinephrine; in every case the actual catecholamine concentration was measured and used in the determination of specific activity. Previous studies have shown that, in the presence of ATP–Mg^{2+}, less than 10–15 per cent of endogenous amines leaks out in the 30-min time period, and most of this amount leaks out within the first 5 min [14]; thus, the measurement of supernatant catecholamines reflected the true concentration throughout most of the incubation. Supernatant catecholamines averaged 3–10 μM in samples in which metaraminol uptake was measured.

It is important to note that "uptake", by this definition, represents a net incorporation, which depends on at least three different processes: inward transport, subsequent binding to storage sites, and efflux from the vesicles. In these preparations, it is not possible to measure a true initial velocity of uptake which would reflect the inward transport component only. First, there is not enough material present in preparations in developing rats to achieve measurable uptakes over short time periods. Second, the efflux half-life of one of the storage pools is less than 5 min [6, 14], indicating that even short incubations would be insufficient to give a true initial velocity. Third, when short incubations are used in preparations with larger amounts of tissue, the distribution of incorporated amines does not reflect endogenous stores [14]. Furthermore, since these studies were intended to determine functional changes both in transport and subsequent storage as well as in efflux, it was desirable to incubate the vesicles for a sufficient time to allow storage and efflux to contribute significantly to the uptake. Earlier studies with cow vesicles indicated that, although uptake was linear for at least 20 min, the distribution pattern of incorporation was distinctly time-dependent, demonstrating that storage and efflux were affecting the observed uptake despite the apparent linearity [14]. In the present study, the time course of uptake into adult rat adrenal vesicles was found to be linear for 30 min and fell off from linearity only slightly at 40 min. The contributions of storage and efflux to uptake are detailed in the kinetic discussion below, and it is important to keep in mind that, although Lineweaver–Burk plots of the data give straight lines, the kinetic parameters are not identical to those of simple enzyme kinetics.

Competition studies. Adrenal glands were homogenized in 5·2 ml sucrose–Tris–iproniazid and centrifuged at 800 *g*; 0·5-ml aliquots of the supernatant were added to tubes containing 5 mM ATP–Mg^{2+}, 0·1 mM epinephrine, 0·1 mM metaraminol or tyramine, 1 μCi ^{14}C-epinephrine and 5 μCi ^{3}H-metaraminol or ^{3}H-tyramine. After incubation at 30° (0° for blanks) for 30 min, vesicles were washed and analyzed as described above to determine the relative uptakes of epinephrine vs. metaraminol and epinephrine vs tyramine. In the concentrations utilized, none of the competing amines influences efflux [15].

Synthesis studies. Vesicles were prepared and analyzed as described for competition studies; incubations contained 1 mM ascorbate, 1 mM fumarate, 5 mM ATP–Mg^{2+}, 0·1 mM epinephrine (to obviate differences in supernatant catecholamines), 0·1 mM tyramine and 5 μCi of ^{3}H-tyramine. The washed vesicles were deproteinized with perchloric acid and the octopamine formed was measured by the periodate oxidation method of Friedman and Kaufman [16] utilizing 5% glycerol instead of bisulfite to stop the periodate reaction.

Assays. Catecholamine analysis was done by the trihydroxyindole method using an autoanalyzer [17].

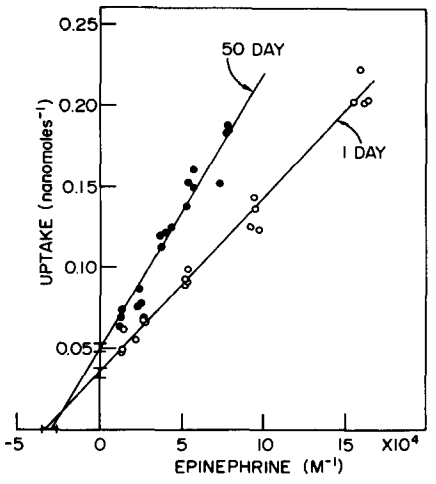

Fig. 1. Lineweaver–Burk plot for uptake of epinephrine into isolated adrenal storage vesicles of 1-day-old (○) and 50-day-old (●) rats. Bars denote standard errors of intercepts. Intercepts on the ordinate are significantly different from each other ($P < 0.001$), while intercepts on the abscissa are not ($P > 0.05$).

Radioactive amines were measured by liquid scintillation spectrometry [14]. Radioactive parahydroxybenzaldehyde formed in the periodate oxidation of octopamine was extracted into toluene before counting.

Statistical analyses. Uptake data are presented as double-reciprocal plots as described by Lineweaver and Burk [18]. The slopes, intercepts and standard errors are calculated by the method of least-squares [19] using 15–57 individual uptake determinations. Levels of significance are calculated by Student's *t*-test [19].

Materials. Epinephrine-7-^{14}C, tyramine-G-^{3}H and metaraminol-7-^{3}H were obtained from New England Nuclear Corp. Epinephrine bitartrate, tyramine hydrochloride and metaraminol bitartrate were obtained from Winthrop, Sigma, and Merck, Sharp & Dohme respectively. Iproniazid phosphate was also obtained from Sigma.

RESULTS

In adult (50-day) animals, a double-reciprocal plot of uptake vs epinephrine concentration indicated a K_m of 35 μM and a U_{max} of 20 nmoles taken up in 30 min per 100 μg of endogenous catecholamines (Fig. 1). These data agree closely with earlier determinations in a different rat strain [20]. In neonates, the U_{max} was 50 per cent higher than in adult animals, but the K_m was the same (Fig. 1). Kinetic constants were determined in the same manner for rats at 10, 20, 30 and 40 days of age and the results are summarized in Table 1. Throughout the course of development, there was no change in K_m for epinephrine, and between 10 and 40 days the U_{max} was the same as in 50-day animals. Thus, the storage vesicles of neonates were unique in having a greater uptake capacity.

To examine the properties of the nonstimulated uptake system, K_m and U_{max} were determined for metaraminol at higher concentrations than in the

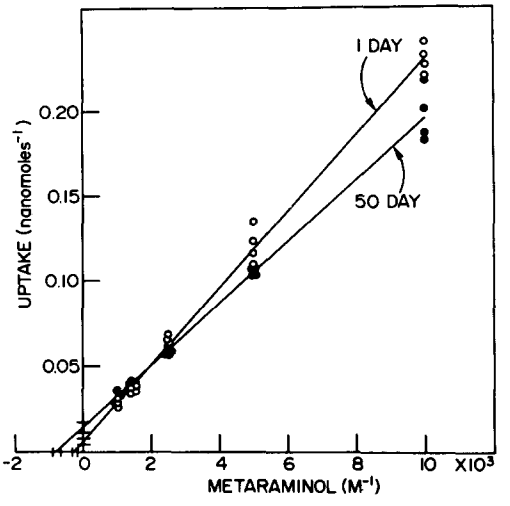

Fig. 2. Lineweaver–Burk plot for uptake of metaraminol into isolated adrenal storage vesicles of 1-day-old (○) and 50-day-old (●) rats. Bars denote standard errors of intercepts. Intercepts on both the ordinate and abscissa are significantly different ($P < 0.001$).

Table 1. Michaelis constants (K_m) and maximal uptake (U_{max}) for epinephrine incorporation into isolated adrenal storage vesicles of developing rats

Age (days)	K_m (μM ± S.E.)	U_{max} (nmoles/100 μg endogenous catecholamines ± S.E.)	No. of uptake points used in determination
1	31·0 ± 1·5	29·3 ± 1·5*	19
10	34·9 ± 2·0	20·2 ± 1·2	19
20	31·6 ± 2·0	21·2 ± 2·3	19
30	38·6 ± 2·2	22·6 ± 1·4	19
40	39·0 ± 3·1	21·7 ± 1·8	19
50	35·7 ± 2·1	19·6 ± 1·1	56

* $P < 0.001$ vs 50-day value.

Table 2. Michaelis constants (K_m) and maximal uptake (U_{max}) for metaraminol incorporation into isolated adrenal storage vesicles of developing rats

Age (days)	K_m (mM ± S.E.)	U_{max} (nmoles/100 μg endogenous catecholamines ± S.E.)	No. of uptake points used in determination
1	3·78 ± 0·43*	169 ± 19*	19
10	0·73 ± 0·06†	52 ± 5‡	15
20	0·63 ± 0·07§	44 ± 5§	18
30	1·17 ± 0·15	57 ± 7	15
40	1·20 ± 0·20	73 ± 14	19
50	1·26 ± 0·21	70 ± 7	57

* $P < 0·001$ vs 50-day value. † $P < 0·005$. ‡ $P < 0·05$. § $P < 0·01$.

epinephrine study. In adult animals, U_{max} for metaraminol was considerably above that for epinephrine (Fig. 2) but the K_m was higher, implying that the affinity for metaraminol was much lower (Fig. 2). In neonates, both U_{max} and K_m for metaraminol were elevated compared to adults, indicating that, depending upon concentration, metaraminol uptake could either exceed or be exceeded by the uptake in adults (Fig. 2). Thus, while in adults K_m and U_{max} were about 1·3 mM and 70 nmoles respectively, in neonates, the values were 3·8 mM and 170 nmoles (Table 2). By 10 days of age, the characteristics of metaraminol uptake were markedly altered; both K_m and U_{max} were lower than in adults, indicating higher affinity but lower capacity for uptake (Table 2). These values persisted through 20 days of age but were at or near adult levels by 30–40 days.

To examine the specificities of the two uptake-systems, vesicles were incubated with ^{14}C-epinephrine and ^{3}H-metaraminol in equimolar concentrations (0·1 mM) and the relative uptakes were compared during development. In adult animals, the uptakes of both amines were essentially the same as when vesicles were incubated with either amine separately, and about 5 molecules of epinephrine were taken up for each metaraminol molecule (Table 3). At birth, there was a 30 per cent elevation in epinephrine uptake compared to that of adults but no change in metaraminol uptake; consequently the preference for epinephrine vs metaraminol was closer to 6 to 1 (Table 3). By 10 days of age, however, metaraminol appeared to compete effectively with epinephrine, and epinephrine uptake was only one-third of adult values while metaraminol uptake was increased. As a result, the vesicles exhibited no preference for epinephrine vs metaraminol (preference ratio of 1). The epinephrine uptake was still somewhat depressed by 20 days of age and metaraminol uptake was still elevated, but by 30 days all parameters were normal (Table 3).

When tyramine was used as the competing amine instead of metaraminol, the results were considerably different. In adults, the uptake of epinephrine was depressed 50 per cent by equimolar concentrations of tyramine, and the preference for epinephrine over tyramine was only 2 to 1 (Table 4). In neonates, both epinephrine and tyramine uptakes were higher than those in adults, but the preference was unaltered. At 10 days of age, tyramine uptake was still elevated above adult levels, but epinephrine uptake was lower than that in adults; consequently, the vesicles exhibited no preference for epinephrine vs tyramine (Table 4). At 20 days of age, both epinephrine and tyramine uptakes

Table 3. Competition between 10^{-4} M epinephrine and 10^{-4} M metaraminol for uptake into storage vesicles of developing rats

Age (days)	Uptake (nmoles/100 μg endogenous catecholamines ± S.E.) Epinephrine	Metaraminol	Preference ratio ± S.E. (Epinephrine/Metaraminol)	No. of determinations
1	27·3 ± 0·2*	4·77 ± 0·23	5·73 ± 0·25†	6
10	7·10 ± 0·63*	6·35 ± 0·20*	1·12 ± 0·11*	6
20	17·9 ± 0·9‡	5·12 ± 0·05§	3·51 ± 0·15*	6
30	20·7 ± 1·1	4·59 ± 0·15	4·51 ± 0·23	6
40	19·3 ± 0·8	4·24 ± 0·13	4·56 ± 0·22	6
50	21·0 ± 0·6	4·28 ± 0·19	4·92 ± 0·19	6

* $P < 0·001$ vs 50-day value. † $P < 0·05$. ‡ $P < 0·02$. § $P < 0·002$.

Table 4. Competition between 10^{-4} M epinephrine and 10^{-4} M tyramine for uptake into storage vesicles of developing rats

Age (days)	Uptake (nmoles/100 μg endogenous catecholamines ± S.E.) Epinephrine	Tyramine	Preference ratio ± S.E. (Epinephrine/Tyramine)	No. of determinations
1	15·0 ± 1·8*	7·42 ± 0·49†	2·02 ± 0·29	5
10	6·34 ± 0·52‡	7·67 ± 0·30§	0·83 ± 0·06‖	6
20	6·23 ± 0·28‡	3·19 ± 0·17†	1·95 ± 0·12	6
30	8·88 ± 0·36	4·91 ± 0·27	1·82 ± 0·05	6
40	8·72 ± 0·46	4·81 ± 0·55	1·83 ± 0·21	6
50	9·66 ± 0·51	5·18 ± 0·72	1·88 ± 0·27	6

* $P < 0.02$ vs 50-day value † $P < 0.05$. ‡ $P < 0.001$. § $P < 0.01$. ‖ $P < 0.005$.

were below adult levels and the preference was once again normal. By 30–40 days, both uptakes approached values found in 50-day-old animals.

To determine whether age-dependent alterations in amine uptake could affect the rate of catecholamine synthesis, the octopamine formed from the β-hydroxylation of tyramine incorporated into the vesicles was measured. At ages at which tyramine uptake was elevated above adult levels (1 and 10 days), octopamine synthesis was similarly increased (Table 5). At 20 days, when tyramine uptake was depressed, octopamine synthesis was decreased. At each age, the ratio of precursor to product was the same as that in adults (Table 5); since the ratio is dependent upon the intravesicular concentration of DBH, these data imply that age-dependent alterations in total DBH activity probably reflect changes in the number of vesicles and vesicle membranes rather than in the amount of DBH per vesicle.

DISCUSSION

This study details age-dependent alterations in K_m and maximal uptake (U_{max}) for two separate systems in the adrenal storage vesicle, one of which operates at low amine concentrations and is stimulated by ATP–Mg^{2+} (epinephrine) and one which operates at higher concentrations and is for the most part unaffected by ATP–Mg^{2+} (metaraminol). In order to understand the significance of the changes in the kinetic constants, it is first important to establish what these parameters represent. Although the uptake systems appear to follow Michaelis–Menten kinetics, the actual situation is considerably different from simple enzyme–substrate interactions [21]; a minimal model would have to include the following processes:

$$A_0 + C \underset{k_2}{\overset{k_1}{\rightleftarrows}} AC \xrightarrow{k_3} C + A_i \underset{k_5}{\overset{k_4}{\rightleftarrows}} A_b$$

$$A_i \xrightarrow{k_6} A_0$$

where A_0 is amine in the medium, C is a mobile carrier in the vesicle membrane, AC is the amine–carrier complex, A_i is free amine inside the vesicle, and A_b is amine bound within the vesicle. The rate constants are all first order. There is ample evidence to support the concept of carrier-mediated transport in this system [21]; however, the following simplifications have been made: (1) Transport is unidirectional. (2) Leakage of amine back into the medium is passive and proportional to free amine inside the vesicle. (3) A single binding pool is indicated, although at least two pools have been identified [14, 15]. The subsequent mathematics would become considerably more complex if the second pool were included, but the interpretation would be qualitatively identical; for that reason, bound amine will be treated as a single pool.

Thus, an amine molecule combines with the carrier, the complex moves to the inside surface of the membrane where the complex is dissociated with the utilization of energy in the form of ATP [21–26]. The

Table 5. Conversion to octopamine of tyramine incorporated into storage vesicles of developing rats

Age (days)	(nmoles/100 μg endogenous catecholamines ± S.E.) Tyramine	Octopamine	Precursor/product ratio ± S.E. (Tyramine/Octopamine)	No. of determinations
1	4·31 ± 0·36*	3·11 ± 0·29*	1·39 ± 0·12	5
10	4·48 ± 0·15†	3·19 ± 0·11‡	1·41 ± 0·06	6
20	1·72 ± 0·11‡	1·47 ± 0·09*	1·18 ± 0·07	6
30	2·94 ± 0·13	1·97 ± 0·09	1·49 ± 0·08	6
40	2·52 ± 0·38	2·29 ± 0·34	1·10 ± 0·17	6
50	2·97 ± 0·35	2·21 ± 0·26	1·34 ± 0·16	6

* $P < 0.05$ vs 50-day values. † $P < 0.005$. ‡ $P < 0.01$.

amine can then either leak outward again or become bound. The rate of uptake (dU/dt) can be defined as:

$$dU/dt = d(A_i + A_b)/dt \quad (1)$$

Since

$$dA_i/dt = k_3AC + k_5A_b - (k_4 + k_6)A_i, \quad (2)$$

and

$$dA_b/dt = k_4A_i - k_5A_b, \quad (3)$$

substituting into equation 1,

$$dU/dt = k_3AC - k_6A_i \quad (4)$$

If k_4 and $k_5 \gg k_3$, then A_i and A_b will equilibrate; this probably occurs in the 30-min incubations used in this study, since after this time period the radioactive epinephrine appears to distribute in a manner similar to endogenous stores [6, 27].

Thus,

$$k_4A_i = k_5A_b. \quad (5)$$

Rearranging

$$A_b = k_4A_i/k_5 \quad (6)$$

Since

$$U = A_i + A_b, \quad (7)$$

from equation 6, then

$$A_i = U/(1 + k_4/k_5). \quad (8)$$

Substituting into equation 4,

$$dU/dt = k_3AC - k_6U/(1 + k_4/k_5) \quad (9)$$

Since A_0 is a fixed concentration throughout the experiment, AC is in steady state if k_1 and $k_2 \gg k_3$. This is the same assumption made in the derivation of the Michaelis–Menten equation [28], and is justified in this case by the observation that amines equilibrate rapidly with binding sites in the vesicle membrane [26], whereas the rate of transport (determined by k_3) is much slower. Thus,

$$dAC/dt \simeq 0 \quad (10)$$

Since

$$dAC/dt = k_1A_0C - (k_2 + k_3)AC, \quad (11)$$

then

$$AC = k_1A_0C/(k_2 + k_3). \quad (12)$$

Substituting into equation 9,

$$dU/dt = k_1k_3A_0C/(k_2 + k_3) - k_6U/(1 + k_4/k_5) \quad (13)$$

Since by definition,

$$K_m = (k_2 + k_3)/k_1, \quad (14)$$

then

$$dU/dt = k_3A_0C/K_m - k_5k_6U/(k_4 + k_5). \quad (15)$$

Since a steady state exists, C is a constant for any given A_0, and if we define

$$x = k_3A_0C/K_m \quad (16)$$

$$y = k_5k_6/(k_4 + k_5), \quad (17)$$

then

$$dU/dt = x - yU. \quad (18)$$

Integrating both sides,

$$[-\ln(x - yU)]/y = t + \text{constant}. \quad (19)$$

When

$$t = 0, \quad U = 0, \quad \text{and constant} = (-\ln x)/y. \quad (20)$$

Substituting into equation 19 and transposing,

$$\ln[(x - yU)/x] = -yt. \quad (21)$$

Raising each side to an exponent,

$$(x - yU)/x = \exp(-yt). \quad (22)$$

Thus,

$$U = \frac{k_3A_0C(k_4 + k_5)(1 - \exp[-k_5k_6\,t/(k_4 + k_5)])}{K_mk_5k_6} \quad (23)$$

When A_0 saturates C, the maximal rate of uptake is proportional to C_t (total carrier):

$$dU_{max}/dt = k_3C_t - k_5k_6U/(k_4 + k_5) \quad (24)$$

But,

$$C_t = C + AC. \quad (25)$$

Substituting into equation 12,

$$C_t = C(1 + A_0K_m). \quad (26)$$

Using steps 18–23,

$$U_{max} = \frac{k_3C_t(k_4 + k_5)(1 - \exp[-k_5k_6\,t/(k_4 + k_5)])}{k_5k_6} \quad (27)$$

From equation 26,

$$U_{max} = \frac{k_3C(1 + A_0/K_m)(k_4 + k_5)(1 - \exp[-k_5k_6\,t/(k_4 + k_5)])}{k_5k_6} \quad (28)$$

Then,

$$U = A_0U_{max}/(A_0 + K_m) \quad (29)$$

This is similar to the integrated form of the Michaelis–Menten equation, indicating that the model will follow a rectangular hyperbola with an intercept on the ab-

scissa of $-K_m^{-1}$ and on the ordinate of U_{max}^{-1}. If equation 29 is rearranged,

$$K_m = A_0(U_{max}/U - 1) \quad (30)$$

all exponential terms and terms containing k_4, k_5 and k_6 drop out. The significance of the K_m value is then the same as the K_m of enzyme kinetics and depends primarily upon the affinity of the amine for the carrier on the outside of the membrane (affinity $= k_1/k_2$) and upon the dissociation of the amine–carrier complex on the inside surface (k_3).

However, from equation 27, it is apparent that U_{max} is much more complex than in the Michaelis–Menten situation. If "V_{max}" is substituted for k_3C_t (as in the usual kinetic treatment),

$$U_{max} = V_{max}(k_4 + k_5)(1 - \exp[-k_5k_6t/(k_4 + k_5)])/k_5k_6 \quad (31)$$

Thus, U_{max} is a function of the time at which the measurement is taken as well as k_4, k_5 and k_6. In the current studies, all determinations were made at 30 min to eliminate the time factor. Age-dependent changes in U_{max} could be produced in the following manner: (1) Alterations in V_{max} (k_3 or C_t) will yield proportional changes in U_{max}. Since uptake is measured relative to endogenous catecholamine content, the expression for concentration of carrier is inversely proportional to catecholamine content per vesicle. (2) Alterations in the binding constants (k_4 and k_5) will markedly alter U_{max}: an increase in k_5 will tend to decrease U_{max}, while an increase in k_4 will tend to increase U_{max}. (3) An increased rate of leakage (k_6) will tend to decrease U_{max}.

This model can now be used to identify specific subvesicular sites which are affected by the maturation process. Throughout development there was no alteration in K_m for epinephrine, indicating that qualitatively there is no change in the properties of the membrane-bound carrier. The higher U_{max} at birth could be explained in several ways. First, it might be due to an increase in V_{max}, that is, in k_3 or C_t. The former is unlikely, since a large change in k_3 would also produce a change in K_m, since $K_m = (k_2 + k_3)/k_1$. While there is no direct way of measuring C_t, the amount per vesicle of another membrane-bound protein, dopamine β-hydroxylase (DBH), does not appear to be altered, since the precursor/product ratio, which depends on DBH activity, was not age-dependent. Since U_{max} and C_t are expressed in terms of nmoles/100 μg of endogenous catecholamines, an increased C_t could result from a decreased catecholamine content per vesicle; however, measurements of the subcellular distribution of catecholamines indicate that the number of "light" vesicles (vesicles with below normal catecholamine contents) is lower than that in adults [3], suggesting that C_t is not in fact elevated in neonates.

A second explanation of the increased U_{max} in neonates may be that the capacity for storage of amines is enlarged, that is, an increase in k_4 or a decrease in k_5 or k_6. The effluxes of endogenous and newly incorporated amines from neonatal storage vesicles are not altered compared to adults [6], indicating that k_5 and k_6 are probably not changed. On the other hand, previous studies have indicated that neonatal vesicles are more dense than those in adults [3], implying increases in the concentrations of soluble vesicle constituents, which include catecholamine-binding components such as ATP. These data would suggest that U_{max} is increased due to an increase in k_4, the value of which is related to the concentration of the binding constituents.

At first glance, the fact that U_{max} and K_m for epinephrine are normal at 10 days would seem to indicate that the uptake properties of the vesicles are the same as those in adults; however, this is not so. Earlier studies have shown that at 10 days of age the vesicles are deficient in catecholamines and ATP [3]; hence, C_t, which is inversely proportional to catecholamine content per vesicle, should be increased, resulting in an increase in V_{max}. The reason that U_{max} is unaltered again rests on the storage parameters. The deficiency of ATP at this age is even greater than that of catecholamines [3], resulting in accelerated efflux of bound amines (increased k_5) as well as a probable decrease in binding ability (k_4). The latter effect follows from the dependence of k_4 on the availability of free binding sites for the newly taken-up amines. The increase in k_5 and the decrease in k_4 will then oppose the increase in V_{max} and result in no change in U_{max}. Similarly, at 20 days of age, U_{max} is unchanged despite the fact that catecholamine content per vesicle is still decreased, because the vesicles are still deficient in ATP [3]; at this age there is no increase in efflux [6], indicating that the compensating defect is solely in k_4. The confirmation of the hypothesis that uptake properties are altered at 10 and 20 days despite the absence of changes in K_m or U_{max} lies in the altered efflux pattern [6] at 10 days as well as in the increased susceptibility to blockade of uptake by other amines (Tables 3 and 4) at either age.

While the effects of development on the uptake of epinephrine appear to rest solely on alterations in storage, the K_m for metaraminol was elevated at birth but was also significantly lower at 10–20 days than in adults, indicating an alteration at the level of the vesicle membrane. This in turn suggests that metaraminol and epinephrine do indeed use separate uptake systems as has been indicated [12–15], since K_m alterations appear for one amine but not for the other. It is difficult to determine whether the alteration in K_m for metaraminol is due to a change in k_1, k_2 or k_3, but the larger value of U_{max} at birth suggests that the increase in K_m may result in part from an increase in k_3, since U_{max} is proportional to k_3. It is unlikely that the intravesicular concentration of ATP affects metaraminol uptake, since metaraminol is bound primarily to other vesicle components and does not appear to enter the storage complex [14, 15, 27, 29]; in fact, vesicles which are deficient in ATP appear to incorporate

metaraminol, but not epinephrine, in a normal manner [27, 29]. This does not rule out the possibility that the binding of metaraminol to other vesicular components (chromogranins, vesicle membranes) may be age-dependent. However, it should not be overlooked that the changes in K_m and U_{max} for metaraminol are always parallel: both are increased at birth and decreased at 10 and 20 days. The only parameter both have in common is k_3.

The proposed model should be able to account for situations in which epinephrine and metaraminol are placed in competition for uptake into the same vesicle. Since the two uptake systems appear to be separate, no competition should be observed when there is an adequate storage capability for each amine. As shown in Figs. 1 and 2 and Table 3, this is exactly the case for ages 30–50 days; the uptakes of the amines together were the same as when either amine was used separately. If, as at birth, the concentration of ATP binding sites for epinephrine is increased, epinephrine uptake should increase, metaraminol uptake should be unaltered or slightly elevated (see Fig. 2 for 10^{-4} M uptake point) and the preference ratio (epinephrine/metaraminol) should then be elevated. On the other hand, a large deficiency of ATP, as at 10 days, would result in a greater proportion of incorporated epinephrine being bound to nonspecific sites for which metaraminol can compete [14, 15]. This would yield a decreased uptake of epinephrine in a situation in which metaraminol is also present, and a decrease in the preference ratio. The effect would not be as pronounced at 20 days, where the ATP deficiency is not as severe[3]. These predictions from the K_m, U_{max}, ATP and flux measurements and based upon the model proposed here, are all verified in Table 3.

An important corollary of these observations is that when metaraminol and epinephrine do compete, it is not at the level of the carrier, but rather at the site of storage. Consequently, even when saturating concentrations of epinephrine are used (10^{-4} M) and $U \simeq U_{max}$, uptake is greatly decreased, indicating a picture similar to noncompetitive kinetics (alteration in U_{max}).

In the case of tyramine, an amine whose uptake and storage characteristics are midway between those of epinephrine and metaraminol [14, 15], competition should occur at both the carrier and storage levels. Even when storage capacity is adequate, the presence of tyramine results in a reduction in epinephrine uptake, as confirmed by Table 4, which shows that in adults epinephrine uptake in the presence of tyramine is one-half the value predicted from uptake with epinephrine alone (Fig. 1). When storage capacity is inadequate (10–20 days), tyramine should exert a metaraminol-like effect, resulting in a decreased preference ratio. However, when binding sites are in excess (1 day), the preference ratio should be nearly normal and uptake of both amines should be increased because both tyramine and epinephrine can take advantage of the increased U_{max} of the stimulated uptake system. Again, these predictions from the model are verified in Table 4. Because of competition at both the carrier and storage sites, tyramine should display mixed competitive–noncompetitive kinetics at 10 days of age (when storage sites are inadequate). The tyramine-induced decrease in epinephrine uptake at 50 days vs. epinephrine alone represents the competitive component, and the further decrement at 10 days represents the metaraminol-like, noncompetitive component (Table 4).

It is important to establish whether the age-dependent alterations in vesicular uptake and storage mechanisms are of functional significance. One function of the vesicles is that of synthesis of norepinephrine from dopamine utilizing the vesicular enzyme dopamine β-hydroxylase. Hydroxylation was evaluated in the current study by measuring the conversion of tyramine to octopamine (Table 5). In every case, the amount of octopamine formed was directly proportional to the amount of tyramine taken up into the vesicles, indicating that maturational changes in uptake produce parallel changes in hydroxylation by altering the accessibility of the precursor to the enzyme. Since β-hydroxylation is not generally considered to be the rate-limiting step in catecholamine synthesis, the alteration may be of limited significance; however, it is noteworthy that the levels of tyrosine hydroxylase and DBH are both markedly age-dependent [1–3] and the limiting factors in catecholamine synthesis may also then be age-dependent.

The developmental changes in uptake are of obvious significance in dealing with the actions of false transmitters (such as metaraminol) which displace catecholamines from the storage vesicles. In the rat adrenal, metaraminol would be less effective a displacer in neonates but more effective at 10–20 days of age.

These studies also indicate interpretational pitfalls which can result from simple measurements of uptake. First, because two uptake systems exist, the observed values of K_m and U_{max} will be dependent upon the concentration of amine. If the epinephrine concentration is high enough, the uptake begins to assume the characteristics of metaraminol uptake (lack of dependence on ATP–Mg^{2+}, resistance to reserpine [14, 15]. As a result, the K_m values for epinephrine concentrations above 10^{-4} M may lie between those of the two uptake systems [12, 30]; in order to measure the K_m of the stimulated system, concentrations below 10^{-4} M must be used. The second problem lies in interpreting the meaning of U_{max}: it does not represent simply the concentration of carrier sites but rather includes terms for storage and outward leakage of amines. Thus, a drug- or age-induced change in U_{max} does not necessarily indicate noncompetitive inactivation of carrier sites or altered number of sites, but may in fact represent competitive interaction at the level of storage or an altered number of storage sites. The interpretation of U_{max} necessitates measurements of efflux as well as the concentrations of binding substituents such as ATP.

The third problem is essentially a methodological one in the measurement of K_m and U_{max}. Because of partial lysis of vesicles during homogenization, the extravesicular concentration of catecholamines is invariably higher than expected from the amount of amine added to the uptake incubation medium, especially at low concentrations. Thus, the external amine concentration must always be measured to calculate the true specific activity of the incubation medium and to obtain thereby the correct uptake at that actual concentration. It is also evident from Figs. 1 and 2 that the variations in amine concentration and uptake are sufficiently large, even in duplicate samples, to dictate that at least triplicate assays at four to five different concentrations of added amines be obtained for each curve.

Despite the inherent limitations in measurement and interpretation of K_m and U_{max} for uptake systems of this type, these data impart much more information than measurements of uptake at a single concentration. For example, if metaraminol uptake were measured solely at 10^{-3} M, the uptake in neonates would be higher than in adults; on the other hand, if 10^{-4} M were used, uptake in adults would be higher (Fig. 2). These apparent contradictions are resolved by measurements over a range of concentrations.

Finally, it is of some interest that there are two apparently independent carrier-mediated transport systems in the vesicle membrane, one with a high affinity and one with a low affinity. Recent studies on the binding of amines to purified bovine adrenal vesicle membranes indicate two sites, also with high and low affinities [26]. The high affinity site has the characteristics (K_{diss}, specificity, drug sensitivity) of a carrier for the stimulated uptake system. While the low affinity site has not yet been fully characterized, its K_{diss} is about 55 times higher than the K_{diss} of the high affinity site [26]. In the present study, the K_m for metaraminol was approximately 40 times higher than that of epinephrine. Furthermore, U_{max} for metaraminol was higher than U_{max} for epinephrine despite the fact that storage of metaraminol is much less stable [14, 15], suggesting that the capacity of the metaraminol transport system is much higher than that of the epinephrine system. Similarly, the binding capacity of the low affinity site in vesicle membranes is substantially greater than that of the site which has the characteristics of epinephrine transport [26]. While these parallels are suggestive of a relationship between the two carrier sites and the two binding sites, additional characterizations of the transport and binding system are necessary before identify is established.

Acknowledgement—The author thanks Mrs. Martha D. Abou-Donia for her excellent technical assistance.

REFERENCES

1. L. J. Ignarro and F. E. Shideman, *J. Pharmac. exp. Ther.* **159,** 38 (1968).
2. R. L. Patrick and N. Kirshner, *Devl Biol.* **209,** 24 (1972).
3. T. A. Slotkin, *Biochem. Pharmac.* **22,** 2033 (1973).
4. S. Daikoku, O. Takashi, A. Takahashi and M. Sako, *Tokushima J. exp. Med.* **16,** 153 (1969).
5. L. G. Elfvin, *J. Ultrastruct. Res.* **17,** 45 (1967).
6. T. A. Slotkin, *Biochem. Pharmac.* **22,** 2023 (1973).
7. I. B. Black, *J. Neurochem.* **20,** 1265 (1973).
8. R. S. Comline and M. Silver, *J. Physiol., Lond.* **183,** 305 (1966).
9. Y. Mikhail and Z. Mahran, *Anat. Rec.* **152,** 431 (1965).
10. N. Kirshner, *J. biol. Chem.* 237, 2311 (1962).
11. A. Carlsson, N.-Å. Hillarp and B. Waldeck, *Medna exp.* **6,** 47 (1962).
12. P. Lundborg, *Acta physiol. scand.* **67,** 423 (1966).
13. P. Lundborg and R. Stitzel, *Br. J. Pharmac. Chemother.* **29,** 342 (1967).
14. T. A. Slotkin, R. M. Ferris and N. Kirshner, *Molec. Pharmac.* **7,** 308 (1971).
15. T. A. Slotkin and N. Kirshner, *Molec. Pharmac.* **7,** 581 (1971).
16. S. Friedman and S. Kaufman, *J. biol. Chem.* **240,** 4763 (1965).
17. R. J. Merrills, *Analyt. Biochem.* **6,** 272 (1963).
18. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56,** 658 (1934).
19. R. L. Wine, *Statistics for Scientists and Engineers*, pp. 499 and 250. Prentice-Hall, Englewood Cliffs, New Jersey (1964).
20. H. O. Green and T. A. Slotkin, *Molec. Pharmac.* **9,** 748 (1973).
21. T. A. Slotkin, *Life Sci.* **13,** 675 (1973).
22. G. Taugner, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **270,** 392 (1971).
23. G. Taugner, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **274,** 299 (1972).
24. A. Pletscher, M. DaPrada, H. Steffen, K. H. Berneis and B. Lutold. *Biochem. Pharmac.* **23,** Suppl. 1, 1 (1974).
25. N. Kirshner, in *Pharmacology of Cholinergic and Adrenergic Transmission* (Eds. G. B. Koelle, W. W. Douglas and A. Carlsson), p. 225. Czech. Med. Press, Prague (1965).
26. T. A. Slotkin and N. Kirshner, *Biochem. Pharmac.* **22,** 2492 (1973).
27. T. A. Slotkin and N. Kirshner, *Biochem. Pharmac.* **22,** 205 (1973).
28. L. Michaelis and M. L. Menten, *Biochem. Z.* **49,** 333 (1913).
29. T. A. Slotkin and N. Kirshner, *Molec. Pharmac.* **9,** 105 (1973).
30. J. Jonasson, E. Rosengren and B. Waldeck, *Acta physiol. scand.* **60,** 136 (1964).