tative and quantitative effects *in vivo* from those observed *in vitro* and that the conditions used here are not suitable for these substances to act as they would in the intact nerve ending.

The values obtained using Triton X-100 and activating ions on whole homogenates have again shown that the amounts of TH in the caudate nucleus are greater than earlier observations (4.5 compared with 2.5 μmoles/g/hr [3], both of which were greater than values reported by other authors [8, 9], indicating that the caudate nucleus has a considerable capability for the synthesis of L-dopa under optimal conditions. It is, possible however, that the enzyme *in vivo* may not be present in its most active form.

From the results obtained with homogenates and small tissue samples it is clear that the Triton X-100/NaCl incubation mixture is suitable for the assay of TH in all cases where the measurement of maximal activity is required.

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Biochemical Pharmacology, Vol. 29, pp. 1595–1598.

0006-2952/80/0601-1595 $02.00/0

Maturation of sympathetic neurotransmission in the rat heart—III. Developmental changes in reserpine inhibition of norepinephrine uptake into isolated synaptic vesicles*

(*Received* 13 *November* 1979; *accepted* 26 *December* 1979)

Reserpine inhibits the uptake of catecholamines into storage vesicles by competitive, irreversible blockade of the ATP-Mg^{2+}-dependent transport mechanism [1–5], resulting in profound depletion of neurotransmitters from the vesicles and interference with sympathetic neurotransmission. After administration of reserpine to the rat, the drug disappears biphasically: most of the drug is eliminated very rapidly, but a small amount disappears extremely slowly [6, 7] due to irreversible binding of reserpine to sites on the vesicles [7, reviews 8, 9]. Disappearance of the bound reserpine, along with the appearance of new, unoccupied reserpine binding sites, has been used to estimate the rate of synthesis and axonal transport of newly formed vesicles; complete recovery of binding sites in the adult rat heart requires 7–8 weeks [10]. This closely approximates the time required for full recovery of endogenous heart norepinephrine content after reserpine treatment [11].

Another index of new vesicle arrival in the terminal after reserpine treatment [5] is the recovery of vesicular uptake capabilities. Recently, a technique has been developed to measure uptake of norepinephrine into synaptic vesicles isolated from small amounts of rat heart [12]. Using this method, rapid increases in rat heart vesicular uptake sites have been demonstrated during the first few days of postnatal life [13], possibly corresponding to increased rates of vesicle synthesis and/or down-transport in the neonates compared to adults. In support of this explanation of the postnatal increases, studies in rat brain have found that a single injection of reserpine into neonatal rats results in inhibition of norepinephrine uptake lasting only a few days, compared to more than 2 weeks in adults, despite the fact that neonates display a greater initial sensitivity to the drug [5]. In the present study, this approach has been extended to the developing peripheral sympathetic nervous system by measuring the time required for recovery of cardiac vesicular norepinephrine uptake after giving reserpine to rats of different ages. The results confirm that the ontogenetic increases in transmitter vesicular uptake sites are associated with accelerated arrival of new vesicles.

Timed pregnant Sprague–Dawley rats (Zivic-Miller, Allison Park, PA) were housed individually in breeding cages and allowed food and water *ad lib*. Pups from all litters were randomized at birth and redistributed to the nursing mothers, with litter sizes kept at 8–11 pups to maintain a standard nutritive status. Additionally, for each experiment, pups of both sexes were selected from several different cages. Neonates were given 250 μg/kg reserpine subcutaneously on days 1, 9, 17 and/or 30 and were killed at intervals from 3 hr to several days after each reserpine injection. Adult male 250 g rats were given a single subcutaneous injection of reserpine (either 250 or 50 μg/kg) and decapitated 3 hr, 24 hr, 4, 7, 14, 21 or 28 days after the injection. All control animals received equivalent volumes of vehicle (1 ml/kg).

For studies of uptake of norepinephrine into cardiac sympathetic vesicle, hearts were weighed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) immediately in 4 vol. of ice-cold 300 mM sucrose buffered with 25 mM Tris (pH 7.4) containing 10 μM iproniazid. During the first 2 weeks of postnatal development, hearts from several animals were pooled to obtain at least 200 mg of tissue for analysis. A crude fraction containing synaptic vesicles was prepared from the heart homogenate by the method adapted from Seidler *et al.* [14] by Bareis and Slotkin [12]. The homogenate was centrifuged at 1000 *g* for 15 min and the pellet discarded. The supernatant fraction was recentrifuged at 20,000 *g* for 30 min and this supernatant fraction was sedimented at 100,000 *g* for 30 min in a Beckman Type 40 fixed angle rotor. The crude vesicle pellet (from preparation to preparation consistently con-

* Supported by USPHS HD-09713 and HL-24115.

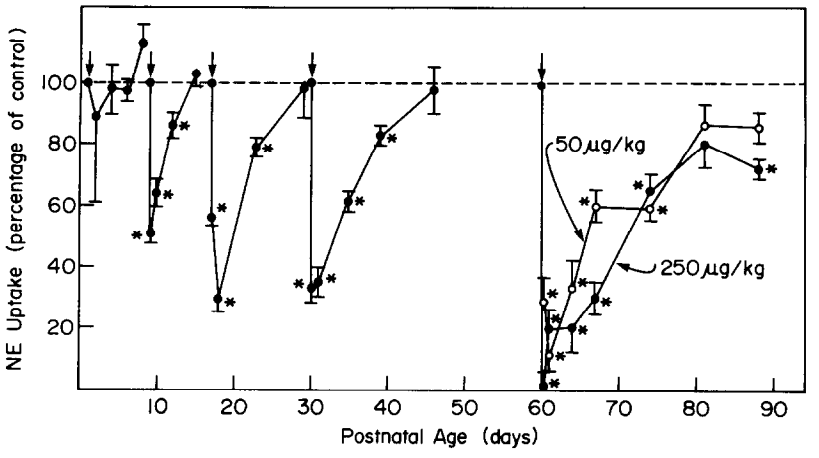

Fig. 1. Effects of reserpine, administered at different ages, on [^{3}H]norepinephrine uptake into rat heart synaptic vesicles. Developing rats received reserpine (arrows) as described in the text. Points and bars represent means and S.E.M. of five to eighteen determinations. Asterisks denote significant differences from controls ($P < 0.05$ or better). Control uptakes in pmoles/g heart were: day 2, 0.38 ± 0.10; day 4, 0.58 ± 0.05; day 7, 1.13 ± 0.10; day 10, 1.20 ± 0.09; day 13, 1.42 ± 0.12; day 17, 1.34 ± 0.07; day 23, 1.43 ± 0.09; day 30, 1.46 ± 0.09; day 37, 1.83 ± 0.07; day 46, 1.97 ± 0.15; and days 60–90, 1.73 ± 0.08.

taining about 30 per cent of the total endogenous heart norepinephrine) was resuspended by means of 3–4 up–down strokes in a Teflon-to-glass homogenizer in 4 vol. (based on original wet tissue weight) of 130 mM potassium phosphate buffer, pH 7.4. This suspension was used for subsequent incubations. Each sample contained 0.67 ml of vesicle suspension (representing 167 mg of heart) in a final incubation volume of 1.7 ml, with final concentrations of 1 mM ATP-$MgSO_4$, 10 μM ascorbic acid, 5 μM iproniazid and 0.1 μM [^{3}H]norepinephrine. Samples were incubated for 4 min at 30° with duplicate samples kept on ice to serve as blanks. Incubations were stopped by adding 1.7 ml of ice-cold phosphate buffer, and the labeled vesicles were immediately vacuum filtered on cellulose acetate filter paper (Gelman GA-8, pore size 0.2 μm). The paper was washed three times with cold phosphate buffer and counted by liquid scintillation spectrometry in a Triton X-100-containing toluene-based scintillation fluid at an efficiency of 39 per cent. Uptake was determined by subtracting the 0° tissue blank from the 30° sample and expressed as pmoles of norepinephrine taken up in 4 min per original g of heart. Although the uptake procedure utilized a crude microsomal preparation, the incorporation of [^{3}H]norepinephrine occurred predominantly into the sympathetic storage vesicles [12] and provided a reliable index of maturation of vesicle function during both normal and drug-perturbed development [13, 15].

Results are reported as means and standard errors with levels of significance calculated by Student's two-tailed, unpaired *t*-test.

l-[7-^{3}H]Norepinephrine (3.80 Ci/mmole) was obtained from the New England Nuclear Corp. (Boston, MA) and reserpine phosphate (Serpasil) from Ciba Pharmaceuticals (Summit, NJ); ATP (disodium salt) and iproniazid phosphate were obtained from the Sigma Chemical Co. (St. Louis, MO).

Control pups increased in body weight from about 9 to 184 g between 2 and 46 days of age; their heart weights

Table 1. Effects of reserpine on body and heart weights of developing rats*

Days of age	Body weights (g) Control	Body weights (g) Reserpine	Heart weights (mg) Control	Heart weights (mg) Reserpine
2	9.5 ± 0.4	9.1 ± 0.3	47 ± 1	46 ± 1
4	12.5 ± 0.2	11.8 ± 0.4	63 ± 2	56 ± 2†
6	15.1 ± 0.7	14.6 ± 0.6	81 ± 3	70 ± 3‡
8	20.0 ± 0.6	18.0 ± 0.6‡	95 ± 2	88 ± 3
10	23.0 ± 0.6	21.0 ± 0.5†	100 ± 5	101 ± 3
12	28.4 ± 0.8	27.0 ± 0.7	124 ± 4	119 ± 4
15	35.8 ± 1.2	30.9 ± 1.6§	184 ± 6	155 ± 6§
18	38.5 ± 2.3	36.9 ± 1.7	197 ± 10	210 ± 8
23	54.4 ± 3.0	48.1 ± 2.7	269 ± 13	234 ± 15
30	94.0 ± 3.6	88.8 ± 4.8	391 ± 14	378 ± 18
38	152 ± 9	134 ± 5	580 ± 32	538 ± 20
46	184 ± 9	165 ± 9	680 ± 30	650 ± 20

* Developing rats were given reserpine injections on days 1, 9, 17 and/or 30 as described in the text. Results represent means ± S.E.M. of six to twelve determinations.

† $P < 0.02$ vs control.

‡ $P < 0.05$ vs control.

§ $P < 0.002$ vs control.

Table 2. Effects of reserpine *in vitro* on uptake of [^{3}H]norepinephrine by heart vesicles from developing rats*

Postnatal age (days)	Uptake in presence of 0.1 μM reserpine (percentage of control)
4	53 ± 3† (4)
10	45 ± 5† (6)
17	37 ± 3† (4)
30	49 ± 5† (6)
90	28 ± 2 (6)

* Results are means ± S.E.M. of the number of determinations in parentheses. Control values are in the legend to Fig. 1; 90-day control is 1.92 ± 0.07 pmoles/g.

† Significant differences ($P < 0.05$ or better vs 90-day value).

went from 47 to 680 mg over the same time period (Table 1). In most cases, pups that received 250 μg/kg reserpine on days 1, 9, 17 and 30 had body and heart weights which were substantially the same as those of controls.

Twenty-four hours after administration of reserpine to 1–7 day-old rats, [^{3}H]norepinephrine uptake into rat heart synaptic vesicles was the same as in control pups (Fig. 1); however, when administered at 9 days of age, reserpine produced an immediate and persistent inhibition of uptake. At subsequent ages, the older the animal at the time of reserpine injection, the more profound and prolonged were the actions of the drug. Treatment at 9 days of age produced 50 per cent inhibition at 3 hr with partial recovery at 24 hr and complete recovery by 1 week after reserpine; the same dose given to 17-day-old rats produced about 70 per cent inhibition of uptake in 24 hr with recovery to control levels requiring 12 days, and reserpine administered at 30 days of age inhibited vesicular uptake about 70 per cent initially with a return to normal 16 days later. Effects were even more pronounced in adult rats (60-days-old), where a single injection of 250 μg/kg reserpine produced 80–95 per cent inhibition in the first 3–24 hr and significant inhibition of uptake was present even 3–4 weeks after the injection. Decreasing the adult dose to 50 μk/kg did not decrease the initial amount of inhibition of norepinephrine uptake and the recovery was substantially the same as at the higher dose.

It should be noted that, in the developing rats, complete recovery of uptake was allowed to occur before administration of a subsequent dose of reserpine. Nevertheless, because of the possibility that a cumulative effect could have occurred in the developing rats in which reserpine was given more than once, studies were also conducted in which previously untreated 30-day-old rats were given reserpine. There was no difference in either the initial amount of inhibition or the rate of recovery between rats that received all four reserpine injections (at days 1, 9, 17 and 30) and rats treated only at 30 days of age; therefore, results from both sets of animals were pooled in Fig. 1.

In addition to age-dependent alterations in reserpine sensitivity *in vivo*, synaptic vesicles prepared from hearts of untreated developing rats were less sensitive than those from adult animals to reserpine *in vitro* (Table 2). From 4 to 30 days of age, 0.1 μM reserpine added to the incubation medium decreased uptake to 40–50 per cent of control while adult heart vesicle uptake in the presence of reserpine was reduced to about 28 per cent of control values.

The normal pattern of postnatal maturation of the uptake of norepinephrine by rat heart synaptic vesicles consists of a rapid increase in vesicular uptake capabilities per g of tissue during the first week of age, followed by a slow increase until about 30 days of age, at which point a second small growth spurt occurs until about 40 days of age when adult levels of vesicular uptake are reached [13]. The present studies tested the hypothesis that the ontogenetic increases in vesicular amine uptake represent proliferation of vesicles in the terminal due to arrival of new vesicles. An index of vesicle replacement rate was obtained by giving a single injection of reserpine at different ages and following the length of time required for recovery of norepinephrine uptake; the dose of reserpine used was sufficient for maximal uptake inhibition of any vesicles present at the time of the injection. Because reserpine binds to vesicles irreversibly [review, 9], restoration of vesicular uptake capabilities requires degradation of reserpine-inhibited vesicles and replacement of these with newly synthesized ones; therefore, the faster that vesicles arrive in the terminal, the more rapid is the rate of recovery. Very little measurable uptake was present on the day after birth, and reserpine administration at this age had no effect by 24 hr post-injection, suggesting that there were very few vesicles in the nerve endings of neonatal rat hearts. Between 9 and 30 days of age, reserpine produced inhibition of uptake lasting no more than 1–2 weeks. However, when adult rats were treated with reserpine, recovery was incomplete even after 4 weeks, and decreasing the dose to 50 μg/kg did not substantially change the rate of recovery, probably because 50–100 μg/kg saturated the binding sites for reserpine to vesicles in the heart [6]. The longer recovery times for adults (weeks) compared to developing rats (days) cannot result from differences in axonal transport time due to neuronal outgrowth; noradrenergic synaptic vesicles travel down the axon at > 100 mm/day [16]. These results thus indicate that the rapid vesicle replacement occurring in the hearts of immature animals probably reflects higher rates of vesicle synthesis. In turn, the normal developmental increases in vesicular uptake capabilities would seem to result from *de novo* synthesis of vesicles and their subsequent arrival in the nerve terminals. Alternatively, reserpine binding might be reversible in the neonate and irreversible binding may then appear by 9 days of age, the point at which a persistent effect on uptake can be obtained; this latter explanation, however, still would not explain the progressive difference in duration of effect at subsequent ages.

Increases in the number of cardiac noradrenergic synaptic vesicles and changes in the rate of vesicle synthesis may not be the only important processes occurring during the postnatal maturation of these vesicles. There is some indication that properties of the uptake system may change with age as well, since synaptic vesicle preparations from developing animals were inherently less sensitive than adult vesicles to inhibition of norepinephrine uptake by reserpine *in vitro*. The existence of a reserpine-resistant vesicular amine uptake site has been demonstrated in vesicle preparations from adrenal medulla and brain [17–19], and uptake by the reserpine-insensitive site may predominate over reserpine-sensitive, ATP-Mg^{2+}-dependent uptake from birth to 30 days of age [20]. Development of cardiac synaptic vesicle norepinephrine uptake capabilities may thus include shifts in the proportion of reserpine-sensitive and reserpine-insensitive types of uptake, in addition to changes in the number and rate of arrival of vesicles in the terminals.

In summary, the rate of synthesis of noradrenergic vesicles and their subsequent arrival in cardiac sympathetic nerve terminals of developing rats was estimated by following the length of time required for recovery of norepinephrine uptake into vesicles isolated from rats which received a single injection of reserpine at different ages; the synthesis of new synaptic vesicles appears to be elevated compared to adult rats through the first 5 weeks of age. Synaptic vesicles prepared from rats from birth to 30 days of age were also less sensitive than adult vesicles to inhibition of norepinephrine uptake by reserpine *in vitro*, suggesting that properties of the uptake system may change during postnatal development.

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Biochemical Pharmacology, Vol. 29, pp. 1598–1601.

0006-2952/80/0601–1598 $02.00/0

A possible role for cyanate in the albumin binding defect of uremia

(*Received* 17 *October* 1979; *accepted* 13 *December* 1979)

The decrease in binding of numerous drugs by uremic albumin or plasma is now widely recognized. Sjöholm *et al.* [1] reported a decrease in binding and affinity constants for both salicylic acid and warfarin in uremic serum. The binding of phenytoin [2], diazoxide [3], furosemide, clofibrate and sulfonamides [4], penicillins [5], diazepam [6], and valproic acid [7] to albumin is also diminished in uremic serum.

The precise nature of the binding defect has not been elucidated, though evidence for at least three separate and perhaps complementary mechanisms has been published. Andreasen [8], on the basis of dialysis studies, has suggested that the binding defect in uremia is attributable to the presence of small molecules which reversibly alter ligand-binding characteristics of albumin and which accumulate in uremia. The dilution studies of others [1] using warfarin and salicylic acid in uremic and normal sera suggest, however, that the substances which mediate the uremic binding defect are irreversibly bound to albumin at normal pH. Craig *et al.* [5] and other investigators [9] have evaluated the possible contribution to the binding defect of small molecules such as urea, guanidinosuccinic acid, creatinine, *p*-aminohippuric acid, and other small molecules which accumulate during uremia. As yet, none have been found to create a binding defect in normal serum or to reinstate the defect in charcoal-treated uremic serum. Changes in the uremic albumin molecule have been proposed. Electrophoretic differences between normal serum albumin and albumin isolated from uremic sera have been reported [10]. Moreover, for uremic and normal serum albumin, a quantitative difference in the amino acid composition of the two albumin bands separated by isoelectric focusing has been demonstrated [11].

Cyanate, a substance which is capable of reacting with albumin via carbamoylation of free amino groups, is a reasonable suspect as a potential contributor to the albumin binding defect. While values for cyanate concentrations in either normal or uremic serum have not, to our knowledge, been reported, cyanate is believed to circulate at excessively high levels in uremia [12]. We undertook a series of experiments to determine the impact of cyanate upon albumin and its warfarin-binding characteristics.

Methods

Derivatization (carbamoylation) of albumin. Bovine serum albumin (BSA, fraction V, electrophoretically pure, CalBiochem, San Diego, CA) was prepared as a 4% solution in a physiologic electrolyte solution (Normosol-R, Abbott Laboratories, North Chicago, IL)* with pH adjusted to 7.3. BSA was incubated with various concentrations of potassium cyanate (200, 250, 500, 1000 and 2000 mg/l) for 4, 24 or 48 hr. All solutions were sterilized by filtration through 0.45 μm membranes (Nalge). Incubations were performed in air under sterile conditions at 37° using a Dubnoff metabolic shaking incubator oscillating at 120 cycles/min. Derivatized albumin was dialyzed for 24 hr against running water at 22° and then lyophilized. Lyophilized samples were reconstituted with distilled water to a 0.4% concentration and electrophoresed on cellulose acet-

* Normosol-R contains the following per liter: sodium chloride, 5.26 g; sodium acetate, 2.22 g; sodium gluconate, 5.02 g; potassium chloride, 370 mg; and magnesium chloride, 140 mg.