Biochemical Pharmacology, Vol. 24, pp. 1469–1474. Pergamon Press, 1975 Printed in Great Britain.

MATURATION OF THE ADRENAL MEDULLA—IV

EFFECTS OF MORPHINE*

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(*Received* 4 *November* 1974; *accepted* 31 *January* 1975)

Abstract—Chronic morphine administration in adult rats results in neurogenic secretion of adrenal catecholamines and compensatory increases in basal catecholamine levels, in activities of catecholamine biosynthetic enzymes (tyrosine hydroxylase and dopamine β-hydroxylase) and in the number of storage vesicles in the tissue. Perinatally addicted developing rats demonstrated changes completely different from those seen in adults; catecholamine levels and dopamine β-hydroxylase activity were reduced compared to controls and no induction of tyrosine hydroxylase was observed. The time course of adrenomedullary maturation was delayed through the first 10–20 days of age, with reduced numbers of storage vesicles and larger proportions of partially filled vesicles. On exposure to morphine, continued until weaning, perinatally addicted rats did not display any of the changes in catecholamine synthesis or vesicular uptake seen in adult rats. Developing rats treated only *in utero* or only postnatally demonstrated different types of biochemical deficits which appeared at different times during development. The effects of morphine in developing rats vs adult rats can be partly explained by the absence of functional innervation of the neonatal adrenal medulla; however, other factors may also operate.

Administration of morphine to adult rats produces a biphasic change in adrenal catecholamines; in the acute phase there is a reflex stimulation-induced depletion and this is followed by increases in catecholamines during chronic administration [1, 2]. Recent studies from our laboratory [1, 3, 4] have demonstrated that the rise in catecholamines results from long-term increases in the number of storage vesicles accompanied by induction of catecholamine biosynthetic enzymes, and that the amine uptake system of the new vesicles is altered by chronic morphine administration.

During maturation of the rat adrenal medulla, the catecholamine synthetic enzymes and vesicular uptake and storage systems undergo a series of changes which appear to be in part dependent upon the level of neural input to the gland [3,5–8]. The nature of the age-dependent alterations in catecholamine disposition suggested that drugs (such as morphine), which themselves produce changes in these parameters, might have different effects in developing rats from those in adults; additionally, the neural stimulation evoked by drugs like morphine could conceivably alter the subsequent maturation of the tissue. In the present study, the effects of morphine on the synthesis, uptake and storage of catecholamines in developing adrenal medulla have been examined.

METHODS

Treatment of rats. Timed pregnant rats (Zivic-Miller) were housed in individual cages and given morphine HCl subcutaneously twice daily in increasing doses (2·5 mg/kg, 7 and 8 days prepartum; 5 mg/kg, 5 and 6 days prepartum; 10 mg/kg, 3 and 4 days prepartum; 20 mg/kg, until 1 day postpartum; 40 mg/kg, thereafter). Morphine both crosses the placenta [9] and appears in milk [10]; thus, pups were exposed to morphine *in utero* and subsequently postnatally via the milk, and exposure was terminated by weaning. In an additional set of experiments, pups born of morphine-treated mothers were nursed by control mothers and pups from control mothers were nursed by morphine-treated mothers. This sorting gave three groups of pups; those never exposed to morphine (C-C), those exposed to morphine prenatally only (M-C), and those exposed to morphine postnatally only (C-M). Pups were weighed and killed by decapitation at intervals of several days from birth until 30 days of age.

Enzyme activities and catecholamines. Adrenal glands were excised and pooled as follows: at 1 day of age, five pairs of glands were used for each determination; at 5 days, four pairs; at 10 days, three pairs; at 18 or 20 days, two pairs; at 23, 26 and 30 days, one pair each. The pooling of glands was necessary to obtain sufficient material for analysis.

The pooled glands were homogenized (glass-to-glass) in 1·5 ml of 0·15 M KCl. Aliquots (0·1 ml) of the homogenates were removed, deproteinized with perchloric acid (final concentration, 3·5%) and centrifuged for 10 min at 26,000 *g*. The supernatants were analyzed for catecholamines by the trihydroxyindole method using an autoanalyzer [11]. Duplicate 0·4-ml portions of the homogenates were used for analysis of dopamine β-hydroxylase (periodate oxidation method [12]), using ^{3}H-tyramine (10 μM) as substrate. *Para*-hydroxymercuribenzoate was used to inactivate endogenous inhibitors [13]; optimal concentration

* Portions of this research were presented at the Perinatal Addiction Research Conference held at Vanderbilt University, Nashville, Tenn., September 5–6, 1974.

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at all ages was 0·5 mM. The remainder of the homogenates was centrifuged at 26,000 *g* for 10 min, and duplicate 0·1-ml portions of the supernatants were assayed for tyrosine hydroxylase activity by the method of Waymire *et al.* [14], using ^{14}C-tyrosine (100 μM) as substrate.

Uptake of amines. Adrenal glands were pooled as follows: at 1 and 5 days of age, six pairs of adrenals were used for each determination; at 10 days, five pairs; at 18 or 20 days, three pairs; at 23 days, two pairs; at 26 and 30 days, one pair each.

Each group of glands was homogenized (glass-to-glass) in 3 ml sucrose–Tris (300 mM sucrose, 25 mM Tris, and 0·01 mM iproniazid) at pH 7·4 and an aliquot was removed for catecholamine assay. The suspension was centrifuged at 800 *g* for 10 min, and 0·5 ml of the supernatant was added to each of four tubes containing (final concentrations) 5 mM ATP-Mg^{2+}, 0·1 mM epinephrine and either 5 μCi of ^{3}H-epinephrine or 5 μCi of 0·1 mM ^{3}H-metaraminol. The unlabeled epinephrine was added to obviate any differences in extravesicular catecholamine concentrations among the samples. Sucrose–Tris was added to bring the volume of each tube to 1 ml. One epinephrine- and one metaraminol-containing sample were brought to 30° for 30 min; the duplicate tubes were kept on ice. Uptake was stopped by adding 2 ml of ice-cold sucrose–Tris. The samples were centrifuged at 26,000 *g* for 10 min, and aliquots of the supernatants were assayed for catecholamines and radioactivity after deproteinization with 3·5% perchloric acid. The vesicular pellets were washed and recentrifuged twice with sucrose–Tris, and the final pellets were deproteinized and analyzed for catecholamines and radioactivity. Radioactive amines were measured by liquid scintillation spectrometry [15]. Under these conditions, labeling occurs solely in storage vesicles [16]. The temperature-dependent component of uptake in each sample was calculated as described previously [15].

Statistics. Results are presented as means ± standard errors, and levels of significance were calculated by Student's *t*-test [17].

Materials. Epinephrine-7-^{3}H (10 Ci/m-mole), metaraminol-7-^{3}H (10 Ci/m-mole), tyramine-G-^{3}H (10 Ci/m-mole) and tyrosine-1-^{14}C (50 mCi/m-mole) were obtained from New England Nuclear Corp.; epinephrine bitartrate was obtained from Winthrop Laboratories, and metaraminol bitartrate and morphine hydrochloride from Merck, Sharp & Dohme.

RESULTS

Effects of morphine on body weight. Pups exposed to morphine throughout development showed retardation in weight gain which persisted even after termination of exposure to morphine at weaning (Fig. 1). Pups exposed to morphine prenatally only had low birth weights but displayed normal body weights by 10 days of age (Fig. 2); however, after weaning, gains in body weights were again slowed. Rats born to normal mothers and nursed by morphine-treated mothers displayed normal body weights at 1 day of age, but a marked deficiency in weight gain throughout subsequent development and after weaning (Fig. 2). Despite the retardation in weight gain, poor nutrition *per se* could not account for the changes seen in catecholamine disposition, since there was no statistical correlation between body weight and any other parameter within age and treatment groups; however, a specific nutritional defect in the nervous system or adrenal medulla cannot be ruled out.

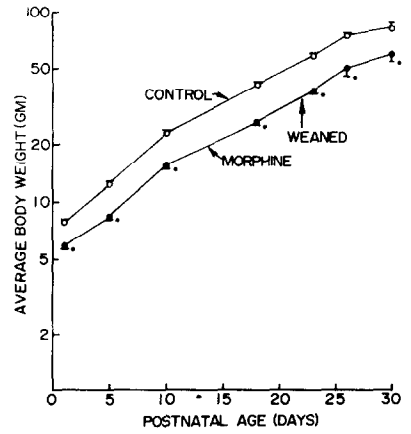

Fig. 1. Body weights of developing control rats (○) and rats born to and nursed by morphine-treated mothers (●). Points and bars represent means ± standard errors of five to six determinations at each age; asterisks denote significant differences ($P < 0.05$ or better). Ordinate is logarithmic.

Effects of morphine on catecholamines, dopamine β-hydroxylase and tyrosine hydroxylase. Neonates exposed to morphine throughout development exhibited a reduction in catecholamines at birth compared to controls, which persisted through 10 days of age, but disappeared by 18 days despite the continued exposure to morphine (Fig. 3). In pups exposed to morphine prenatally only (M-C rats), the marked deficiency in catecholamines at birth disappeared by 5 days of age but reappeared at weaning (Fig. 4). Exposure to morphine postnatally only (C-M rats)

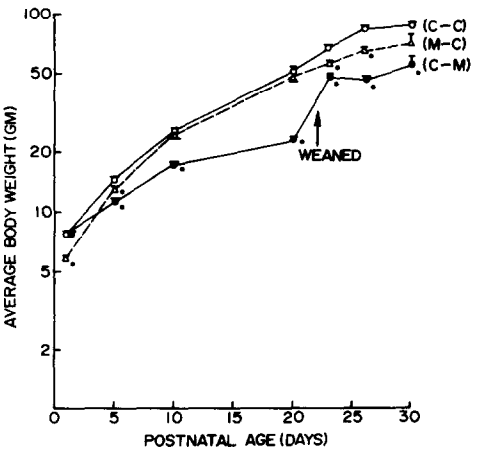

Fig. 2. Body weights of control rats born to and nursed by control rats (C-C, ○), rats born to morphine-treated mothers but nursed by control mothers (M-C, △), and rats born to control mothers but nursed by morphine-treated mothers (C-M, ●). Points and bars represent means ± standard errors of five to six determinations at each age; asterisks denote significant differences from C-C rats ($P < 0.05$ or better). Ordinate is logarithmic.

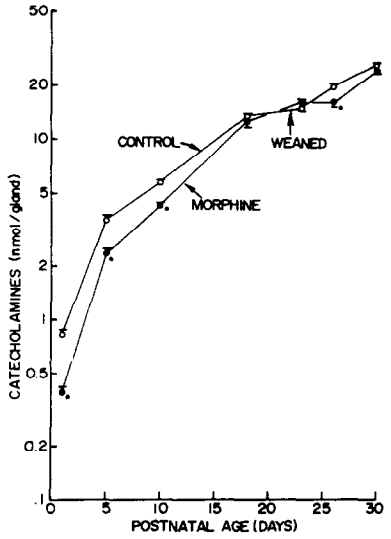

Fig. 3. Development of adrenal catecholamines in control rats (○) and rats born to and nursed by morphine-treated mothers (●). Points and bars represent means ± standard errors of five to six determinations at each age; asterisks denote significant differences ($P < 0.05$ or better). Ordinate is logarithmic.

resulted in little change in adrenal catecholamine content until 18–25 days of age, when levels were less than in controls (Fig. 4).

Tyrosine hydroxylase (TH) activity was not affected in pups exposed to morphine throughout development (Fig. 5). However, the development of dopamine β-hydroxylase (DBH) activity was retarded from birth until 5 days of age (Fig. 5). In pups exposed to morphine prenatally only (M-C), TH activities equalled or exceeded controls (C-C) through 10 days of age, but were lower than controls thereafter (Fig. 6); DBH activity was below normal at birth, above or equal to normal from 5 to 20 days of age and somewhat below or equal to normal thereafter (Fig. 6). In pups exposed to morphine postnatally only (C-M), the age-dependent increases in TH and DBH activity were significantly retarded compared to controls (C-C) beginning at 10 days of age for DBH and 20 days of age for TH (Fig. 6); activities of both enzymes were markedly below normal in C-M rats after weaning.

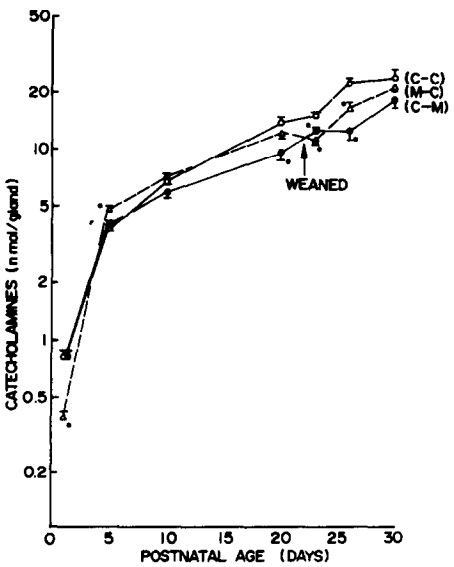

Fig. 4. Development of adrenal catecholamines in control rats born to and nursed by control rats (C-C, ○), rats born to morphine-treated mothers but nursed by control mothers (M-C, △), and rats born to control mothers but nursed by morphine-treated mothers (C-M, ●). Points and bars represent means ± standard errors of five to six determinations at each age; asterisks denote significant differences from C-C rats ($P < 0.05$ or better). Ordinate is logarithmic.

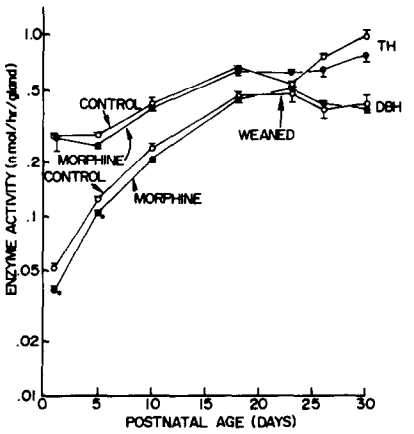

Fig. 5. Development of adrenal tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) in control rats (○) and rats born to and nursed by morphine-treated mothers (●). Points and bars represent means ± standard errors of five to six determinations at each age; asterisks denote significant differences ($P < 0.05$ or better). Ordinate is logarithmic.

Effects of morphine on amine uptake. The abilities of isolated storage vesicles to incorporate exogenous amines was determined to assess changes in uptake

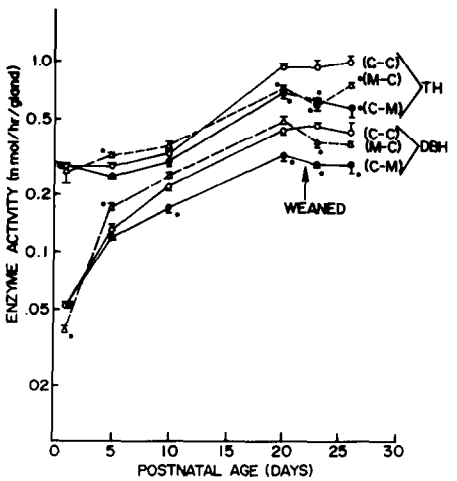

Fig. 6. Development of adrenal tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) in control rats born to and nursed by control rats (C-C, ○), rats born to morphine-treated mothers but nursed by control mothers (M-C, △), and rats born to control mothers but nursed by morphine-treated mothers (C-M, ●). Points and bars represent means ± standard errors of five to six determinations at each age; asterisks denote significant differences from C-C rats ($P < 0.05$ or better). Ordinate is logarithmic.

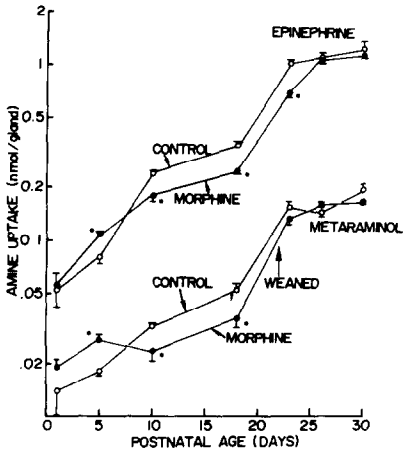

Fig. 7. Uptake of epinephrine and metaraminol per gland in isolated adrenal vesicles from developing control rats (○) and rats born to and nursed by morphine-treated mothers (●). Points and bars represent means ± standard errors of five to six determinations at each age; asterisks denote significant differences ($P < 0.05$ or better). Ordinate is logarithmic.

or storage functions. Pups exposed to morphine throughout development showed a significantly increased uptake per gland of both epinephrine and metaraminol 5 days after birth (Fig. 7). However, the uptakes of both amines from 10 days of age until weaning was significantly less than that of controls. Because uptake per gland depends in part on the number of vesicles as well as their uptake capabilities, the uptake was also evaluated per unit of endogenous catecholamines, which provides a measure of the abilities of individual vesicles to incorporate exogenous amines relative to endogenous content (independently of the number of vesicles). Metaraminol showed a marked elevation of uptake/100 μg of catecholamines at 1 and 5 days of age, while epinephrine uptake tended toward elevation (Fig. 8). At subsequent ages, the uptake/100 μg of catecholamines of both metaraminol and epinephrine showed little or no difference from normal. Amine uptake per gland

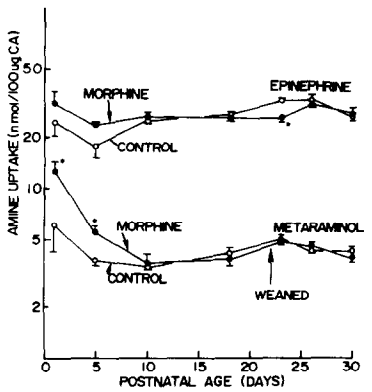

Fig. 8. Uptake of epinephrine and metaraminol/100 μg of endogenous catecholamines in isolated adrenal vesicles from developing control rats (○) and rats born to and nursed by morphine-treated mothers (●). Points and bars represent means ± standard errors of five to six determinations at each age; asterisks denote significant differences ($P < 0.05$ or better). Ordinate is logarithmic.

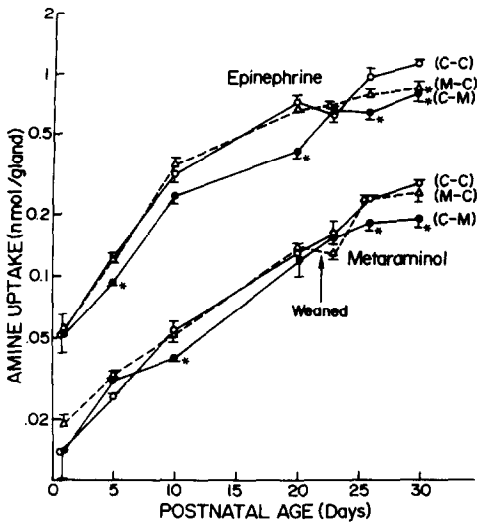

Fig. 9. Uptake of epinephrine and metaraminol per gland in isolated adrenal vesicles from developing control rats born to and nursed by control rats (C-C, ○), rats born to morphine-treated mothers but nursed by control mothers (M-C, △), and rats born to control mothers but nursed by morphine-treated mothers (C-M, ●). Points and bars represent means ± standard errors of five to six determinations at each age; asterisks denote significant differences from C-C rats ($P < 0.05$ or better). Ordinate is logarithmic.

in pups subjected to morphine prenatally only (M-C) showed little or no differences compared to controls, except for epinephrine at 30 days of age (Fig. 9). When the uptake data for M-C rats were related to the endogenous catecholamine content (Fig. 10), there was a slight elevation in uptake/100 μg of catecholamines at 23 days and of metaraminol uptake at 26 days of age, but there was no consistent pattern of

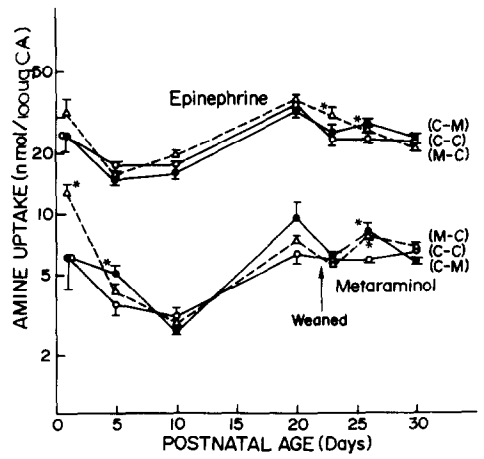

Fig. 10. Uptake of epinephrine and metaraminol/100 μg of endogenous catecholamines in isolated adrenal vesicles from developing control rats born to and nursed by control mothers (C-C, ○), rats born to morphine-treated mothers but nursed by control mothers (M-C, △), and rats born to control mothers but nursed by morphine-treated mothers (C-M, ●). Points and bars represent means ± standard errors of five to six determinations at each age; asterisks denote significant differences from C-C rats ($P < 0.05$ or better). Ordinate is logarithmic.

difference from controls. The uptake of epinephrine per gland in pups exposed to morphine postnatally only (C-M) was significantly less than controls at most points from 5 to 30 days of age (Fig. 9); the effect on metaraminol uptake per gland was less consistent. When amine uptake was related to the endogenous catecholamine content in pups exposed to morphine postnatally (Fig. 10), epinephrine showed a significant increase in uptake at 26 days of age and metaraminol uptake was significantly elevated at 5 and 26 days of age, but again no consistent pattern was observed.

DISCUSSION

Chronic morphine administration to pregnant rats resulted in a lower level of adrenal catecholamines (CA) in the neonates. This alteration did not appear to reflect a deficiency in the CA biosynthetic capability, since the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in norepinephrine synthesis, was normal at birth in the perinatally addicted rats. However, the activity of dopamine β-hydroxylase (DBH), a marker enzyme for storage vesicles, was lower in the morphine-exposed neonates, indicating that the reduction in CA resulted in part from deficiency in the number of vesicles present in the tissue.

Because the relative difference between morphine-treated and control pups was larger for CA than for DBH, these data further suggested that those vesicles which were present in the treated pups were only partially filled with CA. To test this hypothesis, the abilities of the vesicles to incorporate amines were evaluated; earlier studies had shown that partially filled vesicles (which have reduced soluble contents) exhibit a reduction in the normal preference for epinephrine vs metaraminol [1, 5, 7, 13, 18]. In the treated neonates, there was a significant elevation in metaraminol uptake/100 μg of CA but no elevation for epinephrine, confirming the existence of large numbers of partially filled vesicles. The enhancement of uptake/100 μg of CA early in development also explains in part the increase in uptake per gland of metaraminol (and to a lesser extent, of epinephrine) despite the deficiency in the number of storage vesicles; however, it is difficult to evaluate in detail the significance of changes in uptake per gland because the number and content of the vesicles, and the kinetic parameters for uptake are themselves changing during early development [5–7], and the effects of morphine are thus superimposed on an already complex pattern. It is evident, though, that continual pre- and postnatal exposure to morphine does produce developmental alterations in the vesicular uptake system, as well as in catecholamine content and in the number of vesicles, and that by weaning all parameters are no longer abnormal despite continued administration of the drug.

These results are in marked contrast to the effects of chronic morphine administration in adult rats, where intense neurogenic secretion is rapidly compensated by trans-synaptic induction of TH and increased vesicle synthesis [1], resulting in marked increases in CA, TH, DBH and uptake per gland. In adult rats, chronic morphine also leads to production of "defective" vesicles with reduced uptake/100 μg of CA [1, 4]; however, at no time was such a defect in uptake/100 μg of CA noted in developing rats exposed to morphine, further indicating the disparity between adult and immature rats. A number of these differences may be explainable by the fact that innervation of the adrenal medulla does not take place until several days after birth [6]. Morphine can evoke non-neurogenic secretion from denervated adult adrenals [19], and non-neurogenic responses also can be elicited in neonates by a number of other agents which ordinarily evoke a neurogenic discharge [20]. Prior to the establishment of innervation, morphine may cause direct, non-neurogenic secretion with a consequent reduction in the number and content of storage vesicles but no compensatory increases in TH and vesicle synthesis (which are neurally dependent). However, it should be noted that an adult pattern of effect of morphine was not observed during development even after innervation of the adrenal medulla, indicating that other age-dependent factors also play important roles in producing different drug effects in the immature rats.

The time course of morphine administration during development appeared to influence the type and degree of both immediate and delayed effects of the drug. In animals exposed only *in utero*, the depression of CA and DBH seen at birth was no longer evident by 5 days of age; however, shortly before or after weaning the deficiency of CA reappeared, accompanied by a reduction in TH activity relative to controls. These data suggest the development of a delayed defect in maturation of the adrenal medulla related to deficient CA biosynthetic capabilities. In rats who received morphine only postnatally, there was little or no alteration in development until about 10 days of age, after which point maturational deficits in CA, TH, DBH and amine uptake per gland were all in evidence. Since rats who received morphine throughout development (both *in utero* and postnatally) did not demonstrate the later defects seen in rats exposed only prenatally or only postnatally, a form of tolerance or "protection" seems to occur if exposure is maintained. This is again in marked contrast to the chronic effects of morphine in adult rats, where neurogenic secretion, enzyme induction and the formation of defective vesicles continue for as long as the drug is administered [1].

It is of additional interest that in control rats the development of adrenal CA generally paralleled that of DBH but not that of TH. These data suggest that the formation of storage vesicles, rather than CA biosynthesis, plays a determining role in the age-dependent increases in CA stores, an hypothesis which is supported by earlier biochemical and ultrastructural studies [5–7, 21, 22]. The same sequence does not appear to be true in the resynthesis of pharmacologically depleted CA stores in adult rats, where CA synthesis, and not vesicle formation, is rate-limiting [16, 18].

In conclusion, the effects of morphine on the adrenal medullae of developing rats are different and often opposite from those seen in adult rats. The net effect is a delay in the normal pattern of maturation of CA stores; however, the time of the appearance of biochemical defects, the type of defect seen and alterations in subsequent development during the post-exposure period, all depend upon the age at

which exposure commences and the duration of exposure.

Acknowledgements—Supported by USPHS DA-00465 and by a faculty development award from the Pharmaceutical Manufacturers Association Foundation. The authors thank Mrs. M. D. Abou-Donia and Mr. C. Lau for their technical assistance.

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