Biochemical Pharmacology, Vol. 25, pp. 2523–2527. Pergamon Press, 1976. Printed in Great Britain.

CATECHOLAMINE SYNTHESIS, STORAGE AND RELEASE IN ADRENAL MEDULLA AND WHOLE BRAIN DURING ACUTE AND CHRONIC METHADONE ADMINISTRATION

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(*Received* 31 *December* 1975; *accepted* 21 *April* 1976)

Abstract—Methadone was administered daily to rats and the adrenals were analyzed for catecholamines (CA), tyrosine hydroxylase (TH) activity and dopamine β-hydroxylase (DBH) activity. Four hr after 2.5 mg/kg, CA and DBH were depleted significantly but recovered to normal by 24 hr. After 6 days of treatment, adrenal catecholamines were elevated above controls; 24 hr after increasing the dose to 10 mg/kg, CA and DBH again declined, and TH was elevated. Chronic administration of 10 or 25 mg/kg led to restoration or elevation of adrenal CA and marked increases in TH and DBH. While methadone increased the rate of formation of new adrenal storage vesicles, it also inhibited CA uptake into the vesicles, an effect which was also observed with methadone *in vitro*. Similarly, methadone *in vitro* inhibited amine uptake into crude whole brain synaptosomes, but the effect was not observed after acute or chronic administration *in vivo*. These data suggest that methadone, like morphine, stimulates the sympatho-adrenal axis, but that unlike morphine, methadone exerts a direct effect on adrenal storage vesicles.

To a large extent, the actions of morphine on the biogenic amines of the peripheral adrenergic system can be explained by its direct and centrally mediated stimulatory effects on the sympatho-adrenal axis [1–4]. Consequently, acute morphine administration results in loss of adrenal catecholamines (CA) followed by compensatory trans-synaptic induction of the CA biosynthetic enzymes, tyrosine hydroxylase and dopamine β-hydroxylase [1, 2, 5]. The latter effect leads to replenishment of adrenal CA stores despite continued stimulation-induced loss during chronic administration.

In addition to effects on CA levels and enzymes, adrenal stimulation by morphine results in increased formation of new CA storage vesicles [1]. Upon prolonged treatment with large doses, vesicles are synthesized which have an altered membrane transport system for CA [1, 6]; this effect appears not to be a direct action of the drug, since morphine *in vitro* is unable to inhibit vesicular uptake effectively [1, 7]. Recent studies by Ciofalo [8, 9] have shown that morphine and methadone may have different actions on biogenic amine uptake systems at the level of synaptic membranes in the central nervous system, suggesting the need for comparative studies of the effects of the two drugs on CA release, biosynthesis and uptake at other sites. In the current study, the actions of methadone administration *in vivo* on preparations of adrenal medulla and whole brain have been examined and contrasted with the direct effects of methadone and morphine *in vitro*.

METHODS

Male Sprague–Dawley rats (Zivic-Miller) weighing 310 g initially were given saline or methadone HCl subcutaneously once daily on the following dosage schedule: first week 2.5 mg/kg, second week 10 mg/kg and third week 25 mg/kg. Animals were weighed and killed by decapitation at 4 hr (2.5 mg/kg only) and at 24 hr and 6 days after initiation of each dosage. With the exception of the 4-hr point, sacrifice took place 24 hr after the last injection.

Studies in adrenals. Adrenals from each animal were homogenized in 2.5 ml of 0.3 M sucrose containing 0.025 M Tris (pH 7.4) and 10^{-5} M iproniazid (irreversible monoamine oxidase inhibitor). Homogenate (0.1 ml) was added to 1.9 ml of 3.5% perchloric acid, centrifuged at 26,000 *g* for 10 min and the supernatant analyzed for catecholamines using an autoanalyzer [10]. Homogenate (0.5 ml) was added to an equal volume of water containing 2000 units/ml of beef catalase and used for duplicate assays of dopamine β-hydroxylase activity by the method of Friedman and Kaufman [11] with 10^{-5} M [^{3}H]tyramine as substrate and 5×10^{-4} M parahydroxymercuribenzoate to inactivate endogenous inhibitors. The remainder of the homogenate was centrifuged at 800 *g* for 10 min and 1 ml of the supernatant used for determination of epinephrine uptake (see below). The rest of the 800 *g* supernatant was centrifuged at 26,000 *g* for 10 min to sediment the catecholamine storage vesicles and the supernatant used for duplicate determinations of tyrosine hydroxylase activity by the method of Waymire *et al.* [12] using 10^{-4} M [^{14}C]-tyrosine as substrate.

The abilities of the storage vesicles to incorporate [^{3}H]epinephrine were determined by standard techniques as described in detail previously [13]. For each adrenal preparation, duplicate tubes were prepared containing 0.5 ml of the 800 *g* supernatant, 5 μmoles ATP and Mg^{2+}, 5 μCi [^{3}H]epinephrine, 0.1 μmole

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epinephrine bitartrate and sucrose–Tris in a final volume of 1 ml. Samples were incubated for 30 min at 30° while the duplicate tubes were kept on ice to serve as blanks. Uptake was stopped by the addition of 2 ml of ice-cold sucrose–Tris and labeled vesicles were sedimented and washed twice; both labeling medium and washed vesicles were analyzed for catecholamines and radioactivity. Although contaminating particles are present, under these conditions labeling occurs solely in the storage vesicles [14, 15]. The temperature-dependent uptake was calculated as described previously [13] and expressed as uptake per unit of endogenous catecholamines.

For studies of the effects of methadone *in vitro* on enzymes and uptake, adrenals from untreated animals were fractionated as described above and incubated with different concentrations of methadone or morphine. In addition to rat adrenal tyrosine hydroxylase and dopamine β-hydroxylase, the effect of methadone *in vitro* on another catecholamine biosynthetic enzyme, dopa decarboxylase, was assessed according to the assay of Waymire *et al.* [12], using 3.3×10^{-5} M [^{14}C]dopa as substrate.

The effect of methadone *in vitro* on β-hydroxylation in isolated adrenal storage vesicles was determined using [^{3}H]tyramine as substrate. The 800 *g* supernatant fraction of adrenals from untreated animals was incubated and washed as described for [^{3}H]epinephrine uptake, except that the incubation mixture contained 5 μmoles ATP–Mg^{2+}, 1 μmole fumarate, 1 μmole ascorbate, 5 μCi [^{3}H]tyramine and 0.1 μmole of unlabeled tyramine, with and without methadone. The washed, labeled vesicles were analyzed both for tyramine uptake and for levels of the β-hydroxylated product, octopamine [16].

Studies in whole brain. Brains from saline- or methadone-treated rats were weighed and homogenized in 9 vol. of sucrose–Tris–iproniazid, and duplicate 0.1-ml aliquots were assayed for tyrosine hydroxylase activity as described for adrenals, except that final concentrations of 0.1% Triton X-100 and

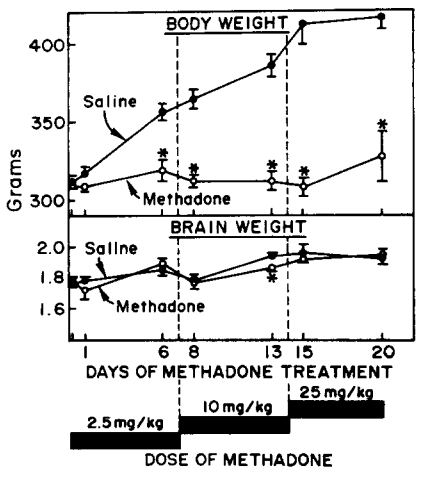

Fig. 1. Body and brain weights in rats treated with saline or methadone (once daily, s.c.) at the dosages indicated. Points and vertical bars represent means ± standard errors of five to twelve animals for each determination; asterisks denote significant differences ($P < 0.05$ or better).

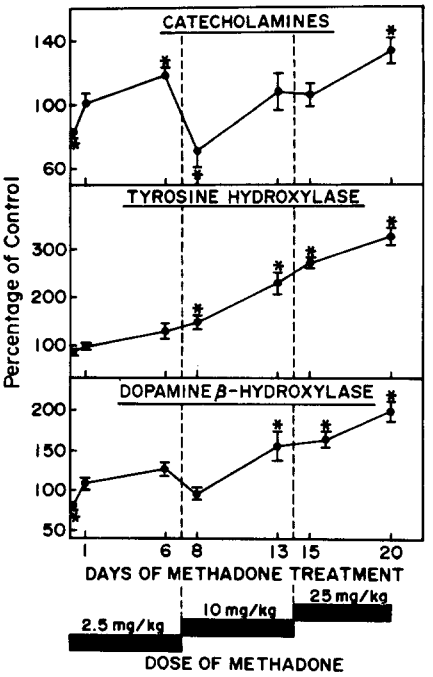

Fig. 2. Adrenal catecholamines, tyrosine hydroxylase activity and dopamine β-hydroxylase activity in rats given methadone (once daily, s.c.) at the dosages indicated. Points and vertical bars represent means ± standard errors of five to twelve animals for each determination as percentage of control; asterisks denote significant differences ($P < 0.05$ or better). Values from saline-treated controls (39 animals) which did not vary significantly over the course of the experiment were: catecholamines, 11.7 ± 0.7 μg/gland; tyrosine hydroxylase, 15.1 ± 1.4 nmoles $^{14}CO_2$ evolved/hr/gland; dopamine β-hydroxylase, 1.46 ± 0.10 nmoles/hr/gland. First time point is at 4 hr.

7×10^{-4} M $CaCl_2$ were added to the assay medium to optimize activity. The remainder was centrifuged at 1000 *g* for 10 min and the supernatants were recentrifuged at 10,000 *g* for 20 min. The crude synaptosomal pellet was resuspended in the original volume of Krebs–Henseleit medium modified by the addition of 1.25×10^{-6} M iproniazid and 2×10^{-6} M ascorbate, and recentrifuged. The washed pellet was resuspended by gentle homogenization (Teflon-to-glass) in half the original volume of Krebs–Henseleit medium and 0.5-ml aliquots were added to an equal volume of medium containing 5 μCi [^{3}H]tyramine (0.75 nmole). Samples were incubated for 5 min at 37° while duplicate tubes were kept on ice to serve as blanks, and tyramine uptake was stopped by the addition of 2 ml of ice-cold Krebs–Henseleit medium; the labeled crude synaptosomes were sedimented at 10,000 *g*, washed and recentrifuged twice, resuspended in 3 ml water, counted for total radioactivity and both 0° and 37° samples analyzed for [^{3}H]octopamine by periodate oxidation as described for the dopamine β-hydroxylase assay. Uptake and synthesis at 37° were generally three to four times the values for the 0° blanks. [^{3}H]tyramine offers the advantage of permitting evaluation of the degree to which uptake is affected specifically in noradrenergic systems [17–19] because dopamine β-hydroxylase is located almost

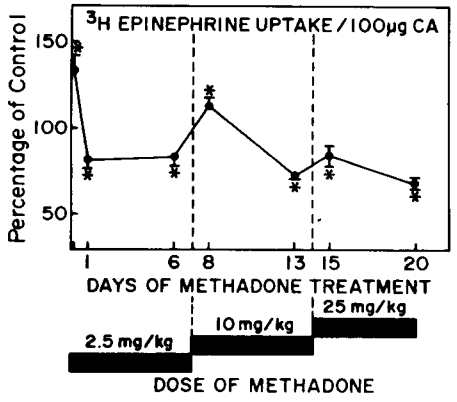

Fig. 3. Uptake of [^{3}H]-epinephrine by isolated adrenal storage vesicles from rats given methadone (once daily, s.c.) at the dosages indicated. Points and vertical bars represent means ± standard errors of five to twelve animals for each determination as percentage of control; asterisks denote significant differences ($P < 0.05$ or better). The value from the saline-treated controls which did not vary significantly over the course of the experiment was 18.8 ± 0.4 nmoles taken up/100 μg of endogenous catecholamines/30 min of incubation (39 animals).

solely within the norepinephrine storage vesicles. The actions of methadone and morphine *in vitro* on brain tyrosine hydroxylase, synaptosomal uptake and β-hydroxylation were evaluated by the same methods, using preparations from untreated rats.

Statistics. Results are expressed as means ± standard errors, and levels of significance are calculated by Student's *t*-test.

Materials. Tyramine[G-^{3}H] (6.67 Ci/m-mole), *dl*-epinephrine-7[^{3}H] (10 Ci/m-mole), l-tyrosine-l[^{14}C] (10 mCi/m-mole) and *dl*-dopa-l[^{14}C] (10 mCi/m-mole) were purchased from New England Nuclear Corp. Epinephrine bitartrate was obtained from Winthrop Laboratories, iproniazid phosphate, tyramine HCl, *p*-hydroxymercuribenzoate and beef liver catalase from Sigma Chemical Corp., and methadone HCl and morphine HCl from Merck, Sharp & Dohme.

RESULTS

Studies in vivo. Over the 3-week period of the study, control rats increased in body weight from 310 g initially to a final value of 420 g, and in brain weight from 1.78 to 1.95 g (Fig. 1). Rats treated with methadone failed to gain body weight, but nevertheless displayed normal brain weights.

Within 4 hr of administration of 2.5 mg/kg of methadone, there was a significant ($P < 0.02$) depletion of adrenal catecholamines (CA), with recovery to normal by 24 hr (Fig. 2). However, after 6 days of treatment, CA levels were elevated by 20 per cent. Subsequent administration of 10 mg/kg resulted again in an initial (24 hr) decline in CA, with recovery after six daily injections. At the completion of 6 days at 25 mg/kg, CA reached 30–40 per cent above control values.

Adrenal tyrosine hydroxylase (TH) showed a dose- and time-dependent increase with methadone treatment, reaching more than three times the control value after chronic administration of the highest dose (Fig. 2). On the other hand, dopamine β-hydroxylase (DBH) activity displayed a pattern qualitatively similar to that of adrenal CA, with initial declines after 2.5 or 10 mg/kg followed by increases during chronic administration; DBH activity reached a maximum of twice normal after 6 days at 25 mg/kg.

To determine the effects of methadone administration on adrenal storage vesicle function, the uptake of [^{3}H]epinephrine was determined. Four hr after administration of 2.5 mg/kg, uptake per unit of endogenous CA was elevated markedly (Fig. 3). However, by 24 hr and after 6 days of treatment, the amine uptake capability of the vesicles was depressed. Upon increasing the dose, this pattern was repeated, with short-term increases in uptake followed by inhibition during chronic administration.

In contrast to the marked effects of methadone treatment on adrenal CA, TH, DBH and uptake, there was little or no effect on whole brain TH, synaptosomal amine uptake or on synaptosomal conversion of [^{3}H]tyramine to [^{3}H]octopamine (Table 1).

Studies in vitro. To determine the extent to which the actions of methadone might be accounted for on

Table 1. Effects of chronic methadone administration on brain tyrosine hydroxylase, synaptosomal [^{3}H]tyramine uptake and [^{3}H]octopamine synthesis*

Dose of methadone	Days of methadone treatment	Tyrosine hydroxylase	[^{3}H]tyramine uptake	[^{3}H]octopamine synthesis	No. of animals
		Percentage of controls*			
2.5 mg/kg (begun day 0)	0.17	108 ± 7	116 ± 9	113 ± 10	6
	1	90 ± 6	97 ± 3	105 ± 6	6
	6	97 ± 8	108 ± 8	97 ± 3	11
10 mg/kg (begun day 7)	8	90 ± 5	99 ± 9	96 ± 7	12
	13	108 ± 4	109 ± 7	106 ± 6	5
25 mg/kg (begun day 14)	15	103 ± 9	106 ± 13	112 ± 12	5
	20	113 ± 4	118 ± 6	107 ± 2	7

* Animals were given methadone (once daily, s.c.) at the dosages and time periods indicated. Values for saline-treated controls (39 animals), which did not vary over the course of the experiment, were: tyrosine hydroxylase, 24.2 ± 1.6 nmoles $^{14}CO_2$ evolved/hr/g; [^{3}H]tyramine uptake, 67.5 ± 3.0 pmoles taken up/g/5 min of incubation; [^{3}H]octopamine synthesis, 10.2 ± 0.4 pmoles synthesized/g/5 min of incubation.

Table 2. Effects of methadone and morphine *in vitro* on uptake of [^{3}H]epinephrine or [^{3}H]tyramine and synthesis of [^{3}H]octopamine in isolated adrenal storage vesicles and whole brain synaptosomes*

Drug concn (mM)	Adrenal vesicles			Synaptosomes	
	[^{3}H]epinephrine uptake	[^{3}H]tyramine uptake	[^{3}H]octopamine synthesis	[^{3}H]tyramine uptake	[^{3}H]octopamine synthesis
	Percentage of control				
0	100 ± 4	100 ± 9	100 ± 8	100 ± 5	100 ± 9
Methadone					
0.01	97 ± 3	74 ± 5†	75 ± 4‡	87 ± 3†	84 ± 6
0.1	80 ± 3†	78 ± 8	72 ± 4§	60 ± 3‖	50 ± 7¶
1	17 ± 5‖	37 ± 5‖	34 ± 2‖	17 ± 1‖	12 ± 3‖
Morphine					
0.1	111 ± 4			107 ± 3	105 ± 4
1	87 ± 2†			88 ± 3	83 ± 14

* Values represent means ± standard errors of five to ten determinations as percentage of control. Control values were: adrenal vesicles, 19.2 ± 0.7 nmoles [^{3}H]epinephrine taken up/100 μg of endogenous catecholamines in a 30-min incubation, 14.4 ± 1.3 nmoles [^{3}H]tyramine taken up and 3.85 ± 0.29 nmoles [^{3}H]octopamine synthesized/100 μg of endogenous catecholamines in a 30-min incubation; whole brain synaptosomes, 70.0 ± 3.5 pmoles [^{3}H]tyramine taken up and 6.88 ± 0.41 pmoles [^{3}H]octopamine synthesized/g of brain in a 5-min incubation. Incubations for adrenal vesicles contained 10^{-4} M epinephrine or tyramine, and those for synaptosomes contained 7.5×10^{-7} M tyramine.

† $P < 0.05$ vs control.
‡ $P < 0.02$ vs control.
§ $P < 0.01$ vs control.
‖ $P < 0.001$ vs control.
¶ $P < 0.002$ vs control.

the basis of direct effects on enzymes or uptake systems, studies were conducted in which methadone or morphine was added directly to the incubation media. With neither opiate was there any marked effect on CA biosynthetic enzymes (TH, dopa decarboxylase, DBH) in drug concentrations up to 1 mM (data not shown). However, the effect of methadone on adrenal vesicle uptake of [^{3}H]epinephrine was more appreciable (Table 2); at 0.1 mM methadone (equimolar with substrate) there was 20 per cent inhibition of uptake and at 1 mM uptake was blocked nearly completely. In contrast, morphine had little or no effect on uptake into adrenal storage vesicles.

Because the uptake of amine substrate into storage vesicles plays a key role in determining the rate of β-hydroxylation [16–19], it was important to determine whether inhibition of vesicular uptake by methadone might influence the intravesicular synthetic step. To measure this effect, the uptake of [^{3}H]tyramine and subsequent conversion to octopamine were assessed. Methadone in concentrations up to 1 mM produced a parallel inhibition of both tyramine uptake and β-hydroxylation, indicating that although the drug does not inhibit DBH directly, it may reduce access of substrate to the enzyme compartment (Table 2). Similarly, methadone *in vitro* produced an inhibition of uptake and β-hydroxylation in isolated whole brain synaptosomes. In contrast to methadone, morphine had little or no effect *in vitro*.

DISCUSSION

The chronic administration of drugs which evoke either direct or reflex stimulation of the sympathoadrenal axis produces a typical pattern of effects on levels of adrenal catecholamines (CA) and CA biosynthetic enzymes. Thus, after treatment with such diverse agents as nicotine [20, 21], insulin [13, 14, 22] or morphine [1, 2] there is an initial depletion of CA and exocytotic loss of soluble dopamine β-hydroxylase (DBH) followed by compensatory trans-synaptic induction of both tyrosine hydroxylase (TH) and DBH and accelerated synthesis of new CA storage vesicles. These changes generally enable prompt restoration of CA stores, even in the face of continued stimulation during chronic treatment.

In the current study, the same pattern was observed during methadone administration, viz. CA and DBH depletion shortly after initiation of 2.5 or 10 mg/kg followed by a return to normal or supranormal CA levels at subsequent times, with induction of TH and DBH. Since DBH is a marker enzyme for storage vesicles, the increment in this enzyme despite increased loss via exocytosis suggests that, as with other stimulatory agents, synthesis of new vesicles has been accelerated by methadone. To test this hypothesis, isolated vesicles from methadone-treated rats were evaluated for their ability to incorporate exogenous [^{3}H]epinephrine; since newly synthesized vesicles have low endogenous CA contents, the ratio of [^{3}H]epinephrine uptake/endogenous CA increases if vesicle turnover is accelerated [1, 6, 13, 14, 16, 20]. Four hr after the first dose of 2.5 mg/kg of methadone and 24 hr after initiation of the higher doses, uptake per unit CA was increased, indicating that a prompt increment occurs in resynthesis of new vesicles.

To the extent that the actions of methadone reflect sympatho-adrenal stimulation, then, its effects resemble closely those of acute and chronic morphine administration [1]. However, this study provides evidence that, unlike morphine, methadone may also exert direct effects on the vesicular amine uptake system. Upon long-term administration of large doses of morphine, storage vesicles are produced which

have a reduced affinity for uptake of CA [1, 6]; however, morphine itself appears to have little effect directly upon the uptake system [1, 7]. In contrast, administration of even a single dose of methadone (2.5 mg/kg) produced inhibition of uptake within 24 hr, and this effect persisted and intensified with chronic treatment at all doses. Since the time of onset of this action was prompt, these data suggested that methadone might block amine uptake directly. Incubation of vesicles from untreated rats with methadone *in vitro* showed that significant inhibition could be obtained with equimolar concentrations of substrate and methadone, while morphine was virtually ineffective. Thus, in addition to actions which reflect sympatho-adrenal stimulation, methadone (but not morphine) exerts a direct effect on adrenal storage vesicles. While methadone does not have direct effects on CA biosynthetic enzymes, it could conceivably exert indirect inhibition of DBH by denying access of substrate to the enzyme compartment (since DBH is located almost solely in the vesicle) in a fashion similar to other inhibitors of vesicular uptake [18]. This action was demonstrated *in vitro* utilizing the uptake of [^{3}H]tyramine and conversion to [^{3}H]-octopamine; further study is required to see if this mechanism plays a role in the actions of methadone *in vivo*.

Methadone *in vitro* also had an effect upon amine uptake mechanisms in whole brain synaptosomal preparations from untreated rats. The high concentrations required to do this confirm (with [^{3}H]tyramine as substrate) the observation of Ciofalo [8] that methadone is a poor inhibitor of synaptosomal uptake in CA neurons. Furthermore, since methadone is a potent inhibitor of uptake into serotonergic neurons [9], and since inhibition of β-hydroxylation (a step specific to noradrenergic neurons) generally paralleled inhibition of [^{3}H]tyramine uptake, these data suggest that under these conditions [^{3}H]tyramine is taken up primarily into CA neurons.

In contrast to the profound effects of methadone *in vivo* on thh adrenal medulla, chronic methadone administration had little effect on the preparations of whole brain homogenates. While adrenal TH activity was increased markedly and adrenal DBH and uptake mechanisms altered substantially by methadone, whole brain TH was virtually unchanged and no effect was noted on either synaptosomal amine uptake or β-hydroxylation; the latter finding suggests that the effects of methadone on synaptosomes *in vitro* do not play a major role in the actions of the drug *in vivo*. It should be noted, however, that regional differences may exist in effects on catecholamine uptake, synthesis, storage or release which can be masked by studies in whole brain.

Acknowledgements—Supported by USPHS DA-00465. Dr. Slotkin is the recipient of Research Scientist Development Award K01-DA-00006 from the National Institute on Drug Abuse.

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