J. Pharm. Pharmac., 1974, **26**, 179–185 *Received October* 8, 1973

In vivo estimation of tyrosine hydroxylation in the dopaminergic terminals of the rat neostriatum

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An approach has been developed to distinguish dopamine synthesis from its release and utilization in dopamine terminals of the rat neostriatum (NCP). It enables a direct *in vivo* estimation of tyrosine hydroxylation, a rate limiting step in dopamine synthesis. Locally injected L-3,5[^{3}H]tyrosine is converted to [^{3}H]dopamine in the NCP. The conversion of the labelled tyrosine into [^{3}H]dopa leads to local accumulation of [^{3}H]water (^{3}H-H_2O). The initial accumulation (3 min) of the ^{3}H-H_2O levels in tissues gives a good reflection of the rate of tyrosine hydroxylation occurring in the dopamine terminals of the nigro-striatal pathway. In effect, ^{3}H-H_2O formation (1) disappeared after elective degeneration of the nigro-neostriatal dopamine neurons, (2) was inhibited by α-methyl-*p*-tyrosine, (3) was not affected by intraperitoneal injection of urea, which is known to reduce brain water content, (4) moreover, ^{3}H-H_2O accumulation was enhanced by various neuroleptic drugs and inhibited by (+)-amphetamine. Finally, Ro 4-4602 an inhibitor of dopa-decarboxylase reduced ^{3}H-H_2O formation 30 min after its injection.

A number of investigations has been concerned with the regulation of dopamine synthesis in dopamine terminals of the neostriatum (NCP) (caudate nucleus + putamen) in the rat. For this purpose, in most cases, changes in its synthesis were estimated *in vivo* by following the conversion of [^{3}H]tyrosine into [^{3}H]dopamine Persson, 1970; Javoy, Hamon & Glowinski, 1970; Nybäck, 1971). In such experiments, the accumulation of the [^{3}H]dopamine does not represent an absolute measurement of synthesis, since part of it, newly synthesized from [^{3}H]tyrosine, is rapidly inactivated enzymatically (Javoy, Agid & others, 1972). Furthermore it has been demonstrated that, in some cases, an enhanced initial accumulation of [^{3}H]dopamine can be related to a simultaneous increased synthesis and decreased utilization of the transmitter. This has been observed in the NCP, immediately after interruption of the nerve impulse activity in the dopamine nigro-neostriatal neurons (Carlsson, Kehr & others, 1972a; Javoy, Agid & others, 1973a; Roth, Walters & Aghajanian, 1973). Moreover, local application of carbachol in the substantia nigra (SN) increased the initial accumulation of newly formed [^{3}H]dopamine in the NCP only by inhibiting the amine utilization (Javoy, Agid & Glowinski, 1973b). Thus it seems necessary to accurately distinguish synthesis from release and utilization processes.

It is generally assumed that dopamine synthesis is mainly regulated at the level of hydroxylation of tyrosine into dopa (dihydroxyphenylalanine). Therefore, it is important to estimate changes affecting this step of its synthesis directly. A technique has previously been developed (Besson, Cheramy & Glowinski, 1971) to measure the

rate of tyrosine hydroxylation in neostriatal slices. Tritiated water (^3H-H_2O) formed during the conversion of L-3,5-[^{3}H]tyrosine into [^{3}H]dopa (Nagatsu, Lewitt & Udenfriend, 1964; Lewitt, Gibb & others, 1967) was used to assess the rate of this important step of the amine synthesis. However, in such conditions normal nerve impulse activity in the dopamine neurons is impaired.

Recently, a new technique has been described by Carlsson, Davis & others (1972b). Using inhibitors of the aromatic amino-acid decarboxylase, these authors measured the rate of tyrosine hydroxylation *in vivo*, by following the initial accumulation of dopa in brain. However, possible interference of such drugs on dopamine transmission and turnover cannot be excluded. Thus, a complementary method, avoiding the use of drugs and allowing the *in vivo* estimation of tyrosine hydroxylation in the dopamine terminals of the NCP, has been developed.

Advantage was taken of two experimental approaches developed previously, the first step of dopamine synthesis was estimated *in vivo* by the method of Besson & others (1971) after the local application of [^{3}H]tyrosine in the NCP (Javoy & others, 1970).

From the present work: (1) ^3H-H_2O accumulated in the NCP is formed in the dopamine terminals and is specific for tyrosine hydroxylation; (2) changes in dopamine synthesis are easily detected by this new approach; (3) the method does not give an absolute rate of the *in vivo* tyrosine hydroxylation, but useful conclusions can be drawn from the relative changes.

METHODS

Physiological procedure

Male Charles River rats (200–250 g) were each positioned in a stereotaxic apparatus (Horsley-Clarke) after being anaesthetized by a mixture of oxygen, nitrous oxide and halothane. Holes were drilled in the skull, and the dura mater was incised. A stainless steel cannula (0·2 mm diam.) was introduced in each NCP using the coordinates from the stereotaxic atlas of Albe-Fessard, Stutinski & Libouban (1966) (in mm: A = 9·5; L = 2·7; H = 6·5). Each cannula was connected to a Hamilton syringe (10 μl) fixed on a variable speed Brown pump. Syringes were filled before each injection with a physiological solution containing purified L-3,5[^{3}H]tyrosine (49 Ci mM^{-1}, CEA France). One μl of this was infused locally (as described previously by Agid, Javoy & others, 1973) in both caudate nuclei simultaneously over 160 s. The animals were then killed at various times after a short infusion and brains were immediately removed and each NCP was dissected in the cold, homogenized, and assayed for its content in ^3H-H_2O. Animals were in groups of 7–10.

Biochemical procedure

L-3,5[^{3}H]Tyrosine purification. The labelled amino-acid was purified just before each experiment to avoid contamination by ^3H-H_2O or [^{3}H]catechol compounds. [^{3}H]Tyrosine was passed through three alumina columns successively. Effluents were adjusted to pH 2 and the [^{3}H]amino-acid was retained on a Dowex AG 50 WX4 column (buffered at pH 7·4). [^{3}H]Tyrosine was finally eluted by NaCl (145 mM). KCl (4·5 mM), $CaCl_2$ (1·3 mM) were added to the solution, which was adjusted to pH 7·4 with 0·2 M Na_2HPO_4. Thus an isotonic solution was locally injected.

Biochemical analysis. NCP were homogenized in 0·4 N perchloric acid (3 ml) with a Polytron apparatus. Ethylenediamine tetra-acetic acid (EDTA) (10 mg) and $Na_2S_2O_5$ (5 mg) were added as stabilizers before homogenization.

An aliquot of the solution (1·5 ml) was kept in the cold for 12 h for the analysis of dopamine and tyrosine (endogenous and labelled). After centrifugation, dopamine and [^{3}H]dopamine (in supernatants) were separated by ion exchange chromatography on Amberlite CG-50, and adsorbed on alumina as described previously (Javoy & others, 1972). The effluents of the Amberlite column were passed through Dowex AG 50 WX4 (H^+). Tyrosine and [^{3}H]tyrosine retained on the column were eluted by 1·5 ml of N perchloric acid. Dopamine and tyrosine were determined by the spectrofluorimetric techniques of Laverty & Taylor (1968) and Udenfriend (1962) respectively.

The remaining 1·5 ml of each homogenate was used for the immediate estimation of ^3H-H_2O. Samples were distilled under vacuum (Besson & others, 1971). Distillation was stopped after condensation of 0·5 ml of water. Radioactivity corresponding to ^3H-H_2O was estimated on 0·2 ml aliquots. Blanks were obtained by distilling samples of boiled tissue homogenates containing the same amounts of [^{3}H]tyrosine as those present in the NCP after the *in vivo* infusion of [^{3}H]tyrosine.

All radioactivities were estimated on aliquots of the collected fractions by liquid scintillation counting (Javoy & others,1972). All data were corrected for respective recoveries.

Experimental values were statistically evaluated with the Student's *t*-test. Differences were accepted as statistically significant for all P values $<0{\cdot}05$.

Drugs. The following drugs were injected intraperitoneally (1 ml): (+)-amphetamine (sulphate, 4 mg kg^{-1}), (±)-α-methyl-*p*-tyrosine (methyl ester hydrochloride, 150 mg kg^{-1}), thioproperaine (methane sulphonate, 4 mg kg^{-1}), haloperidol (15 mg kg^{-1}) and chlorpromazine (hydrochloride, 75 mg kg^{-1}) generously supplied by Rhône-Poulenc; Ro 4-4602 (chlorhydrate, 800 mg kg^{-1}) generously supplied by Hoffman LaRoche. All doses are expressed as base. Urea (2 mg kg^{-1}) was dissolved in a 5% dextrose solution. Control animals received 1 ml of isotonic NaCl solution.

6-Hydroxydopamine (8 μg) was unilaterally injected in the right substantia nigra. Thirty days later, L-3,5[^{3}H]tyrosine (1·45 μCi) was locally infused (160 s) in both NCP simultaneously. Animals were killed 20 s later. Each NCP was analysed separately for its ^3H-H_2O, dopamine and tyrosine (endogenous and ^{3}H) content.

RESULTS

Time course of the accumulation of ^3H-H_2O, [^3H]dopamine and [^3H]tyrosine in the NCP after the local injection of L-*3,5*-[^3H]*tyrosine*

Groups of 5 rats (10 NCP) received a bilateral injection of 1·7 μCi of L-3,5[^{3}H]-tyrosine in the NCP. The animals were killed at various times after the beginning of the local 160 s infusion of the labelled precursor. [^{3}H]Tyrosine, ^3H-H_2O and [^{3}H]-dopamine accumulated in the NCP were estimated at these different times (Fig. 1).

Three min after the beginning of [^{3}H]tyrosine infusion, ^3H-H_2O levels in the NCP were about six to eight times those of blank values. The intratissular levels of ^3H-H_2O increased for 5 min and then declined as a function time. As soon as 3 min after the onset of [^{3}H]tyrosine infusion, [^{3}H]dopamine levels exceeded those of ^3H-H_2O, they increased rapidly until 10 min and then stabilized. [^{3}H]Tyrosine levels in tissues declined rapidly during the first 10 min (at 10 min they were 55% of those found at 3 min) and remained almost unchanged from 10 to 20 min.

The effects of drugs interfering with dopamine metabolism were tested during the initial increase of ^3H-H_2O formation by measuring ^3H-H_2O levels in tissues 3 min after the beginning of [^{3}H]tyrosine infusion.

Effect of pharmacological treatments on the initial accumulation of ^3H-H_2O in the NCP

The different drugs, and the time schedule used in the following experiments, are summarized in Tables 1 and 2. L-3,5[^{3}H]Tyrosine (1·3–1·5 μCi) was locally injected in both NCP at various times after treatments. In all cases, animals were killed 3 min after the 160 s infusion of the labelled tyrosine. ^3H-H_2O, dopamine and tyrosine (endogenous and tritiated) levels were determined in each NCP.

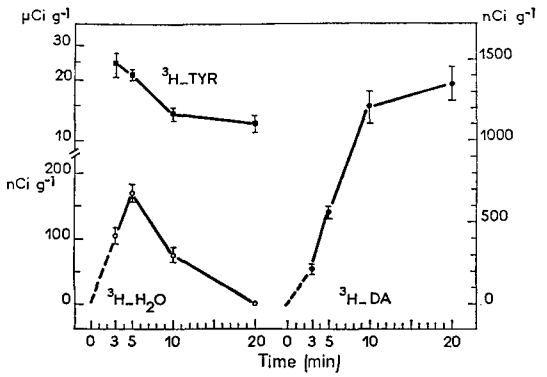

FIG. 1. Time course of the accumulation of ^3H-H_2O, ^3H-DA and [^{3}H]tyrosine in the NCP after local injection of L-3,5-^{3}H-tyrosine. L-3,5[^{3}H]Tyrosine (1·7 μCi) was injected locally, in both NCP simultaneously. The animals were killed at various time intervals after the onset of the 160 s infusion of the tritiated amino-acid. ^3H-H_2O (○) ^3H-DA (●) and [^{3}H]tyrosine (^{3}H-TYR) (■) were estimated in each NCP. Results represent the mean values of 8 determinations ± s.e.

Table 1. *Effect of drugs on the* in vivo *^3H-H_2O accumulation in the NCP.* Each value represents the mean ±s.e. of eight striata corresponding to four animals. (*Statistically significant for all P values <0·05).

	Dose (mg kg^{-1})	Time (min)	[^{3}H]Tyrosine μCi g^{-1}	^{3}H-H_2O nCi g^{-1}	[^{3}H]Dopamine nCi g^{-1}	Dopamine μg g^{-1}	Tyrosine μg g^{-1}
Saline			18·7 ± 1·6	80 ± 3	164 ± 5	9·2 ± 0·4	16·5 ± 1·0
α-M*p*Tyrosine	200	120	17·6 ± 1·2	4·9* ± 1·4	3·4 ± 0·5	3·9* ± 0·2	
Urea	2000	15	16·7 ± 0·8	78 ± 6	160 ± 4		
Thioproperazine	5	60	18·2 ± 0·4	194* ± 28	382* ± 42	9·6 ± 0·3	18·7 ± 0·7
Chlorpromazine	75	60	18·3 ± 1·3	115* ± 8	242* ± 19	9·7 ± 0·4	14·7 ± 1·6
Haloperidol	15	60	19·9 ± 1·3	175* ± 32	354* ± 43	8·4 ± 0·4	15·5 ± 1·3
Amphetamine	5	90	21·7 ± 0·8	32* ± 3	53* ± 6	10·4 ± 0·7	17·5 ± 0·7
Ro 4-4602	800	30	17·2 ± 1·0	35* ± 3	4·9 ± 0·9	7·6* ± 0·3	21·5* ± 1·1

Table 2. *In vivo estimation of the ^3H-H_2O formed from* L-*3,5[^3H]tyrosine after elective degeneration of the dopamine terminals with 6-hydroxydopamine (6-OH-DA) in the NCP.* Control = NCP contralateral to the lesioned side; 6-OH-DA = NCP ipsilateral to the lesioned side. (Means ± s.e. of data with groups of eight rats.* Represents significant statistical difference P <0·05.)

	[^{3}H]Tyrosine μCi g^{-1}	^{3}H-$_2$O nCi g^{-1}	[^{3}H]Dopamine nCi g^{-1}	Dopamine μg g^{-1}	Tyrosine μg g^{-1}
Control ..	25·8 ± 2·2	90 ± 9	209 ± 6	11·3 ± 0·7	16·5 ± 1·0
6-OH-DA ..	24·3 ± 1·9	6·3* ± 1·8	14* ± 3	1·3* ± 0·3	15·5 ± 2·9

*Inhibition of tyrosine hydroxylation by α-methyl-*p*-tyrosine (α-MpT)*

Pretreatment with α-MpT reduced the normal accumulation of ^3H-H_2O and [^3H]-dopamine to 6 and 2% respectively. α-MpT also decreased dopamine levels in the NCP (Table 1).

Elective degeneration of the nigro-neostriatal dopamine pathway

To test the specificity of the experimental approach, ^3H-H_2O formation was estimated in the NCP, after the degeneration of the dopamine neurons. For this purpose a unilateral injection of 6-hydroxydopamine (8 μg) was made in the right SN (Agid & others, 1973).

A month later, the dopamine neurons had degenerated (Sotelo, Javoy & others, 1973) as revealed by the marked decrease of the dopamine content in the right NCP when compared to that of the left NCP (Table 2) (Agid & others, 1973). The initial accumulation of ^3H-H_2O was reduced by 93% (as was that of [^3H]dopamine) in the right NCP, when compared to the NCP of the unlesioned side. In some samples, ^3H-H_2O was even undetectable.

Changes in brain water content

As reported by Reed & Woodbury (1964), injection of highly concentrated solutions of urea induced a maximal decrease of brain water content within 15 min. Therefore, the accumulation of ^3H-H_2O was estimated in rats injected with urea to examine if ^3H-H_2O levels were dependent on water movements. No significant changes in the accumulation of ^3H-H_2O or [^3H]dopamine could be observed in the NCP when compared to that of saline-treated animals.

Other pharmacological treatments

Psychotropic drugs, known to interfere with dopamine metabolism in the NCP, were also tested for their ability to change ^3H-H_2O accumulation: neuroleptics (thioproperazine, haloperidol, chlorpromazine) markedly increased the initial accumulation of tritiated water as they did for that of [^3H]dopamine. The endogenous levels of dopamine were not significantly modified. (+)-Amphetamine inhibited both the initial accumulation of ^3H-H_2O and [^3H]dopamine but did not affect the dopamine content of the NCP. None of these drug treatments induced changes in tyrosine or [^3H]tyrosine levels in the NCP.

As Ro 4-4602, the inhibitor of dopa decarboxylase, has been used to estimate central changes occurring at the first step of dopamine synthesis (Carlsson & others, 1972a; Javoy & others, 1973b), we sought to find if this treatment interfered with tyrosine hydroxylation. The decreased dopamine levels in the NCP, as well as the expected reduction of [^3H]dopamine accumulation, indicated an effective inhibition of dopamine formation in treated animals (Table 1). At the same time, a significant inhibition of the initial accumulation of ^3H-H_2O was observed when compared to normal. Although the tyrosine content of the NCP was increased, the rate of tyrosine hydroxylation was reduced as shown by the ^3H-H_2O/tyrosine specific activity ratio (control: 40·5 ± 4·9; Ro 4-4602: 25·8 ± 3·9 $P < 0{\cdot}05$).

DISCUSSION

Previously (Besson & others, 1971), the rate of L-3,5[^{3}H]tyrosine hydroxylation into [^{3}H]dopa was estimated by measuring the total accumulation of ^{3}H-H_2O formed in striatal slices. *In vitro* the total amount of ^{3}H-H_2O accumulated both in tissues and their incubating medium provided an absolute measurement of this rate-limiting step of [^{3}H]dopamine synthesis. In effect, in these conditions, identical total [^{3}H]dopamine and ^{3}H-H_2O levels were observed at the end of short-incubation periods.

A completely different finding was observed *in vivo*. ^{3}H-H_2O levels in the NCP fell below those of [^{3}H]dopamine rapidly after the local infusion of L-3,5[^{3}H]tyrosine. Indeed, the [^{3}H]dopamine/^{3}H-H_2O ratio calculated from Fig. 1 at different times after [^{3}H]tyrosine infusion was always in favour of [^{3}H]dopamine (=2 at 3 min; =3·3 at 5 min). These data indicate that ^{3}H-H_2O rapidly diffuses out of the NCP (since for each molecule of [^{3}H]dopamine synthesized one molecule of ^{3}H-H_2O is formed) whereas [^{3}H]dopamine, in a large part, remains stored in dopamine terminals. Consequently, *in vivo*, the initial accumulation of ^{3}H-H_2O underestimates the absolute rate of L-3,5[^{3}H]tyrosine hydroxylation. However, the accumulation of ^{3}H-H_2O was not altered by the injections of a hypertonic solution of urea which decreases brain water content (Reed & Woodbury, 1964). On the other hand, the rapid decrease of ^{3}H-H_2O levels observed after 5 min suggests that locally infused [^{3}H]tyrosine is immediately available for [^{3}H]dopamine synthesis but only for a short time.

Despite these limitations, the results obtained in the present study clearly indicate the specificity of the technique. This method represents a valid approach to estimate regulatory processes affecting dopamine synthesis when ^{3}H-H_2O levels are measured shortly (20 s) after the local infusion of L-3,5[^{3}H]tyrosine.

The labelled tyrosine was administered locally in the NCP. This route of injection was chosen since under the present conditions, radioactivity was restricted to the NCP (Javoy & others, 1970; Agid & others, 1973). Therefore, the hypothesis of a contamination of the NCP by ^{3}H-H_2O originating from other structures cannot be sustained. This is further supported by the data reported in Table 2: ^{3}H-H_2O initial accumulation was estimated simultaneously in both NCP of animals in which the right dopamine nigro-neostriatal pathway had degenerated. Thus an almost complete inhibition (93%) of the ^{3}H-H_2O formation was observed in the lesioned NCP, when compared to the NCP of the opposite side. This suggests that more than 90% of the ^{3}H-H_2O detected in the normal NCP was synthesized locally. This also demonstrates that ^{3}H-H_2O formation is linked to the presence of dopamine neurons in the NCP. Consequently, contamination by ^{3}H-H_2O originating from noradrenergic fibres is of no account.

A marked reduction of ^{3}H-H_2O (and [^{3}H]dopamine) formation was observed in NCP of animals pretreated with the inhibitor of tyrosine hydroxylase, α-MpT. Thus the tritiated water was detected in normal conditions, resulted from the conversion of L-3,5[^{3}H]tyrosine into [^{3}H]dopa and did not originate from another metabolic pathway. Consequently, the local accumulation of ^{3}H-H_2O in the NCP gives a measure of tyrosine hydroxylation in dopamine terminals.

The validity of the method is further supported by the pharmacological studies (Table 1). Neuroleptics appeared to activate ^{3}H-H_2O formation while (+)-amphetamine decreased the accumulation of ^{3}H-H_2O in the NCP. Similar experiments performed *in vitro* have demonstrated an increased or a reduced synthesis of ^{3}H-H_2O by neuroleptics and (+)-amphetamine respectively (Besson & others, 1971). In

these pharmacological situations, [^{3}H]dopamine accumulation (estimated at 3 min) fluctuated in the same manner as did that of ^3H-H_2O. However, as mentioned in the introduction, this may not always be the case. Therefore, the *in vivo* estimation of ^3H-H_2O accumulation, which provides direct information about the rate-limiting step of the amine synthesis, may offer some advantages. For example, it is interesting to see that inhibition of dopa-decarboxylase with Ro 4-4602, a drug used to study tyrosine hydroxylation *in vivo* (Carlsson & others, 1972a, b; Javoy & others, 1973b) rapidly induced an inhibition of the tyrosine-dopa conversion. However, the drug effect on dopa-decarboxylase activity was much more pronounced than that observed on tyrosine hydroxylase activity since [^{3}H]dopamine formation was reduced to a much greater extent (−98%) than that of ^3H-H_2O (−57%). It cannot be excluded that Ro 4-4602 directly inhibits tyrosine hydroxylase as well as dopa decarboxylase activity. More likely, the inhibition of the *in vivo* conversion of tyrosine into dopa is induced by the accumulation of large quantities of dopa in dopamine terminals, as a result of dopa-decarboxylase inhibition. Indeed dopa has been shown to be a potent inhibitor of tyrosine hydroxylation activity *in vitro* (Udenfriend, Zaltzmar-Nirenberg & Nagatsu, 1965).

Despite its limitations this rapid method used to assess the *in vivo* rate of tyrosine hydroxylation should aid the elucidation of the regulatory processes of dopamine synthesis occurring in *in vivo* states in neostriatal dopamine terminals.

Acknowledgements

The authors wish to thank Mr. Antoine Chenon for his helpful collaboration.

This research was supported by grants from INSERM, DRME and les Usines Chimiques Rhône-Poulenc.

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