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SHORT COMMUNICATIONS

Characteristics of tyrosine hydroxylation in isolated nerve endings*

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We developed [1] and have been using [2, 3] a very simple, sensitive and rapid semi-micro method to measure the synthesis of catechols in isolated nerve endings prepared from rat brain, which utilizes the rate-limiting endogenous tyrosine hydroxylase and abundant dopa-decarboxylase activity and only endogenous pteridine cofactors to follow the rate of production of $^{14}CO_2$ from [^{14}C]dopa newly formed from L-[1-^{14}C]tyrosine. We now report that this method, in addition to its simplicity, advantageously reflects characteristics of tyrosine hydroxylase that are seen with the purified enzyme only in the presence of biopterin, which is presumably similar or identical to its natural pteridine cofactor [4]. Thus, we have found that tyrosine hydroxylation, as evaluated in synaptosomes by the $^{14}CO_2$ assay, has several characteristics that probably reflect physiologically important attributes of catecholamine synthesis, including stereoselectivity, acceptance of phenylalanine as substrate, low K_m for substrate, nearly neutral pH optimum, inhibition by low concentrations of catechols and some degree of substrate inhibition, as well as loss of the activity after selective destruction of catecholamine nerve endings.

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Validity of our $^{14}CO_2$-trapping assay of tyrosine hydroxylation by rat brain synaptosomes, the methods for which have been reported in detail elsewhere [1–3], was supported by comparison with three other independent methods of assay. In one, [^{14}C]dopa was formed in the presence of a decarboxylase inhibitor (NSD-1055) from L-[U-^{14}C]tyrosine (New England Nuclear), recovered by alumina column chromatography and counted [1, 3]; in other assays, ^{3}H-catechols were similarly recovered after incubating with L-[3, 5-^{3}H]tyrosine (New England Nuclear), or else [^{3}H]water was recovered [5]. The mean agreement (±S.E.M.) with the $^{14}CO_2$ assay for the three other methods (activity as pmoles/min/mg of protein) was: 105 ± 1·5, 90 ± 0·5 and 94 ± 0·7 per cent of the activity estimated by $^{14}CO_2$ trapping, respectively (N = four to six assays). A comparison of all four methods demonstrated similar results at 2, 10 or 20 μM tyrosine in striatal as well as brainstem-diencephalic synaptosomes.

Production of $^{14}CO_2$ from L-[1-^{14}C]tyrosine was temperature-dependent, stereospecific (Table 1), optimal at pH 6·7, and saturable at about 20 μM substrate (apparent K_m = 3·5 ± 0·8 μM, in good agreement with other assays [4, 6, 7]), although at higher substrate concentrations there was evidence of inhibition by tyrosine (Table 1), including upward deviation of the double reciprocal plot of (velocity)$^{-1}$ vs [substrate]$^{-1}$ at concentrations above 20 μM; this phenomenon was reported previously for purified tyrosine hydroxylase only in the presence of biopterin [4]. The activity was also strongly inhibited by isosmotic hydraulic

Table 1. Conditions which inhibit synaptosomal tyrosine hydroxylase activity*

Condition (N = 4–9)	Per cent of control activity ± S. E. M.
Control	100 ± 1·8
4°	< 1 ± 1·0†
Broken synaptosomes	4 ± 0·6†
D-[1-^{14}C]tyrosine as substrate	< 1 ± 1·0†
3-Iodotyrosine (5 μM)	30 ± 5·0†
(50 μM)	< 1 ± 2·0†
Dopamine (0·1 μM)	60 ± 1·5†
(1·0 μM)	39 ± 5·0†
Apomorphine (0·1 μM)	80 ± 1·0†
(1·0 μM)	41 ± 0·5†
(10·0 μM)	11 ± 2·4†
Tyrosine (50 μM)	78 ± 0·4†
(100 μM)	68 ± 0·4†
(250 μM)	67 ± 0·4†
$DMPH_4$ (1 mM)	2 ± 1·7†
$DMPH_4$ + CH_3CH_2SH (25 mM)	15 ± 1·2†
$DMPH_4$ + CH_3CH_2SH + Fe^{2+} (2 mM)	101 ± 0·5

* Tyrosine hydroxylase activity was measured by $^{14}CO_2$ production in homogenates of striatum (0·3 mg protein/assay) after 5-min preincubation ± drugs and 20-min incubation started with L-[1-^{14}C]tyrosine (20 μM) at 37°. Control activity with striatal tissue was 330 ± 6 (S.E.M.) pmoles $^{14}CO_2$/hr/mg of protein. Broken synaptosomes were prepared isosmotically from a P_2 fraction of brainstem-diencephalon in the French press. With dopamine and its control, 10 μM iproniazid was added to inhibit monoamine oxidase.

† Significant by *t*-test ($P < 0·01$, or less).

Table 2. Effects of pretreatment with 6-hydroxydopamine *in vivo* on hydroxylation of aromatic amino acids *in vitro**

Substrate	Hydroxylase activity†	
	Control activity	Pretreated (% of control)
L-[1-^{14}C]tyrosine	29·8 ± 0·8	10·0 ± 0·4 (33%)‡
L-[1-^{14}C]phenylalanine	24·2 ± 0·4	6·8 ± 0·2 (34%)‡
L-[1-^{14}C]tryptophan	13·8 ± 0·2	13·6 ± 1·0 (99%)

* Labeled substrates (20 μM) were incubated with P_2 preparations (1·25 mg protein) of cerebral cortex, and $^{14}CO_2$ was recovered and counted. Rats were pretreated [11] with 1 mg 6-OH-dopamine-HBr (Sigma) intracisternally in two divided doses within 48 hr, 2 weeks previously, or with only the injection vehicle (artificial cerebrospinal fluid with ascorbic acid, 1 mg/ml, added as antioxidant).

† Expressed as pmoles/hr/mg of protein ± S. E. M.; N = 4.

‡ Significant by *t*-test ($P < 0{\cdot}01$). Values for phenylalanine were doubled, assuming that the $^{14}CO_2$ produced from it represented two ring hydroxylations to yield L-dopa.

breakage [8] of the synaptosomes in the French press at 450 kg/cm^2 in the presence of [^{14}C]-labeled tyrosine (Table 1) or phenylalanine, presumably due to dilution of tyrosine hydroxylase and endogenous pteridine cofactors as there was no corresponding loss of activity of dopa-decarboxylase on incubation with L-[1-^{14}C]dopa (Amersham).

The reaction was 70 per cent inhibited by the tyrosine hydroxylase inhibitor L-3-iodotyrosine (CalBiochem) (Table 1) at concentrations (5 μM) that are 40 times lower than the apparent K_m for tyrosine uptake by synaptosomes (200 μM) [9], and did not affect uptake or $^{14}CO_2$ production from [^{14}C]dopa (J. Harris and M. Karobath, unpublished observations). These effects of low concentrations of iodotyrosine probably reflect an action at tyrosine hydroxylase and not on tyrosine uptake, although several analogues of tyrosine, including iodotyrosine ($K_i = 20\ \mu$M), can also inhibit tyrosine uptake into some neuroblastoma clones [10] which have a higher affinity for tyrosine transport ($K_t = 68\ \mu$M) than that reported for synaptosomes [9].

The synthesis of catechols was also decreased by low concentrations of the catechols dopamine and apomorphine (Table 1). This effect is probably due to a direct action of catechols as end-product inhibitors of tyrosine hydroxylase, and not to a receptor-mediated mechanism, since the dopamine-receptor blocking agents chlorpromazine and trifluoperazine (1–10 μM) failed to diminish the effects of 1–10 μM apomorphine (J. Harris, unpublished observation).

The commercially available unphysiological [4] methylated pteridine $DMPH_4$ (CalBiochem) in the presence of reducing agents evidently remained unavailable to the membrane-enclosed synaptosomal enzyme system; alone, it even inhibited the reactions (Table 1), possibly by the formation of peroxide which was apparently decomposed by Fe^{2+}. The lack of a synthesis-enhancing effect of $DMPH_4$ in the presence of reducing agents, and of an inhibitory effect of $DMPH_4$, alone, is consistent with other studies of CNS particulate fractions [11, 12], although at variance with results with the intact vas deferens [13], possibly due to differences in the permeability of pterins in different tissues. The inaccessibility of the cofactor further suggests that membrane-limited synaptosomes are not appropriate for estimates of total tyrosine hydroxylase levels in brain tissue or for analyses of substrate kinetics in the presence of excess cofactor.

The reactions leading to $^{14}CO_2$ production do not seem to involve direct decarboxylation of tyrosine or prior transamination, nor does substrate uptake appear to be rate limiting [1, 9, 12]. The reactions are apparently selectively localized in catecholamine-containing nerve endings, as pretreatment with 6-OH-dopamine strongly reduced the production of $^{14}CO_2$ from L-[1-^{14}C]-labeled tyrosine or phenylalanine, but had no effect on that from labeled tryptophan (Table 2), which presumably was due to the activity of tryptophan 5-mono-oxygenase and its associated decarboxylase [5, 14]. In contrast, pretreatment with 5,7-dihydroxytryptamine [15] led to the selective loss of $^{14}CO_2$ production from tryptophan (S. Gerson and R. Baldessarini, unpublished observation). The apparent acceptance of phenylalanine as a substrate in this reaction accords well with its ability to serve as substrate for purified tyrosine hydroxylase in the presence of biopterin [4] and its ability to produce catechols in isolated nerve endings [2, 6].

Thus, the present findings lend further support to our proposal [1–3] that the $^{14}CO_2$-trapping technique provides a valid and very simple assay of tyrosine hydroxylase activity under conditions that permit its manifestation of physiologically important characteristics in isolated nerve endings in the presence of natural endogenous cofactors.

Department of Pharmacology, Emory University, Atlanta, Ga., U.S.A. — JANE E. HARRIS

Department of Experimental Psychiatry, Psychiatrische Universitätsklinik, University of Vienna, Vienna, Austria — MANFRED KAROBATH

Psychiatric Research Laboratories, Massachusetts General Hospital, Department of Psychiatry, Harvard Medical School, Boston, Mass., U.S.A. — ROSS J. BALDESSARINI

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Acute and chronic ethanol-induced alterations in brain norepinephrine metabolites in the rat*

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Ethanol, in a large single dose, has been shown to cause a decrease in urinary output of 5-hydroxyindolacetic acid (5-HIAA) [1] and 3-methoxy-4-hydroxy-mandelic acid (VMA) in man [2]. Studies using [^{14}C]norepinephrine (NE) [3, 4] and [^{14}C]serotonin (5-HT) [2, 5] also revealed a decreased excretion of labeled VMA and 5-HIAA, respectively, after ethanol ingestion. These changes in the oxidative pathway of biogenic amines were accompanied by an increase in urinary excretion of labeled 3-methoxy-4-hydroxyphenylglycol (MHPG) and 5-hydroxytrypotophol (5-HTP). Similar alterations in NE metabolism have been shown in rats after acute acetaldehyde administration [6].

Since both the brain [7] and peripheral sympathetic nervous system contribute to urinary MHPG, ethanol-induced changes in the latter may or may not provide an index for the synthesis and release of NE in the brain. Ethanol, administered acutely, has been reported to decrease brain endogenous NE and 5-HT levels [8, 9]; however these results have not been confirmed by other investigators [10–13]. Studies using [^{3}H]NE have revealed that acute ethanol administration caused a decrease in NE turnover in the rat brain [14],† whereas chronic ethanol intake in a nutritionally complete diet accelerated the disappearance rate of [^{3}H]NE in the brain [14]. The present study was undertaken to extend these investigations and to determine whether ethanol does, in fact, produce a change in brain NE metabolism.

Male, Wistar rats, weighing 170–190 g, were divided into seven groups with 6–14 rats per group. Two of these groups were maintained on Purina Chow diet and then used for acute ethanol experiments, while rats in the remaining five groups were housed individually and given a nutritionally complete liquid diet (commercial Metrecal) for 2 days before being used for chronic or acute ethanol experiments.

For chronic ethanol experiments, two groups of Metrecal-fed rats were given ethanol (6%, w/v) in their liquid diet for either 4 or 8 days, while the control group was maintained on Metrecal supplemented with an isocaloric amount of sucrose for 6 days. The remaining two groups were also maintained on Metrecal supplemented with sucrose for 6 days and then used for acute ethanol experiments. The food consumption was measured daily for all groups and the animals were weighed every third day. We chose these two days for chronic ethanol because our previous studies have shown that the dramatic changes in NE uptake occur between days 5 and 9 of chronic ethanol ingestion [14].

On the scheduled days, rats were lightly anesthetized with ether and injected intracisternally with 6·6 μg *dl*[7-^{3}H]NE (0·12 μg, sp. act. 10 Ci/m-mole, obtained from New England Nuclear Corp.) in 20 μl Merle's solution by the method of Schanberg *et al.* [15]. All animals used for acute ethanol experiments were fasted for 2 hr prior to injection of [^{3}H]NE and then received either ethanol, 4 g/kg (p.o.), or saline, 30 min later. All animals were killed by decapitation 90 min after [^{3}H]NE. The brains were quickly removed, rinsed in cold saline, blotted dry on filter paper, and then frozen in liquid nitrogen. The blood was collected during decapitation, in cold heparinized centrifuge tubes for plasma ethanol determination.

The frozen brains were weighed and homogenized in cold 0·4 N perchloric acid. After centrifugation, an aliquot of supernatant fluid was counted for total radioactivity in 10 ml Bray's solution on a Beckman model no. LS230 liquid scintillation counter. Additional aliquots were analyzed for [^{3}H]NE and [^{3}H]normetanephrine (NMN) by a dual column technique [3]; labeled glycols (both bound and free) were separated by the method of Eccleston and Ritchie [16]. The results were not corrected for percentage recoveries of NE, NMN (Dowex recoveries varied from 60 to 75 per cent) and MHPG (hydrolysis was 55–70 per cent complete). The total radioactivity for 3,4-dihydroxyphenyl glycol was less than 1 per cent in the brain. Plasma ethanol was determined by gas–liquid chromatography [17]. An unpaired Student *t*-test was used to calculate the level of significance.

Table 1 shows the effect of acute and chronic ethanol on brain NE metabolism. Acute ethanol administration (4 g/kg) to rats increased the accumulation of [^{3}H]MHPG and [^{3}H]NMN in the brain with a concurrent decrease in the disappearance rate of [^{3}H]NE.

To determine whether the Metrecal liquid diet (used for chronic ethanol experiments) had any effect on the brain NE metabolism, the rats fed this diet were given either an oral dose of saline or ethanol (4 g/kg). The pattern of [^{3}H]NE metabolism after ethanol was similar to that observed in rats maintained on Purina Chow. The mean plasma ethanol level after an oral dose of ethanol was 363 ± 29 mg/100 ml. Two-way analysis of variance showed that the diet had no significant effect on brain NE metabolism, whereas on either diet acute ethanol administration did.

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