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Analytical Characterization of a Sensitive Radioassay for Tyrosine Hydroxylase Activity in Rodent Striatum

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Several buffer compositions with a wide range of pH values have been reported for radiometric assay of tyrosine hydroxylase (TH) in biological samples. Assay sensitivity becomes a prime concern while analyzing TH in minute samples like tissue biopsies or discrete regions of rodent brain wherein lower enzyme levels are anticipated due to smaller sample sizes. It was therefore rationalized to evaluate relative affinities of three commonly used assay buffers (sodium phosphate, sodium acetate, and Tris-acetate) with TH enzyme activity. The impact of buffer pH and cofactor concentration on the sensitivity of TH assay was also investigated. Striata from rats or mice were homogenized, respectively, with 1.0 or 0.5 ml of the assay buffer containing 0.5% Triton X-100. The supernatants (200 μl) were incubated (20 min, 37°C) with 0.8 μCi [^{3}H] L-tyrosine, 1.5 mM DL-6-methyl-5,6,7,8-tetrahydropterine (6-MPH$_4$), 100 U catalase, and 1.0 μM dithiothreitol in a total volume of 300 μl. The reaction was terminated by 1-ml suspension of activated charcoal in 0.1 M HCl. After centrifugation, 200-μl aliquots were mixed with 5 ml of cocktail for quantitation of [^{3}H] H_2O in supernatant. The results showed significant impact of pH rather than the buffer composition on the sensitivity of TH assay. An optimal pH range was found to be 5.5–6.0, whereas TH activity was significantly inhibited at pH 5.0 and pH 6.8 ($F = 55.09$, $P = 0.000$). A significantly high TH activity was observed with 1.5 mM 6-MPH$_4$, whereas higher concentrations (3.0–4.5 mM) inhibited TH activity ($F = 7.47$, $P = 0.005$). Analysis of serially diluted striatal homogenates showed a significant correlation between TH activity and sample amount. The assay reaction was linear for 20- and 30-min incubation for rat and mice striata, respectively.

KEY WORDS: Assay sensitivity; buffer pH; enzyme activity; incubation time; rodent striatum; tyrosine hydroxylase.

INTRODUCTION

Tyrosine hydroxylase (TH; EC 1.14.16.2) is the primary enzyme involved in the biosynthesis of catecholamine neurotransmitters in the brain. The potential application of TH as a sensitive and reliable marker of neurodegeneration is evidenced by numerous reports (1–8). Due to the biochemical and physiological importance of TH, various methods have been developed for the measurement of this enzyme. The principles and methodologies of these tests are quite diverse and include radiochemistry (9–12), high-performance liquid chromatography (HPLC) (13), enzyme-linked immunosorbent assay (ELISA) (1,14), immunohistochemistry (3,4), mRNA amplification using reverse transcription polymerase chain reaction (RT-PCR) (5–7), and Western blotting of TH protein (6). The techniques of ELISA, immunohistochemistry, Western blotting, and RT-PCR are basically used for the measurement of TH content or expression and not the TH enzyme activity. Although HPLC can be used to measure TH activity,

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its methodology tends to be complex and lengthy. On the other hand, radiometric methods of enzyme assay are comparatively simple, rapid, specific, and sensitive than nonradiometric assays and therefore particularly advantageous for measuring low levels of enzymatic activities in crude extracts (15).

The radiometric method of TH assay is based on assessing the radioactivity of tritiated water produced from tritiated tyrosine substrate in presence of TH (9–12). Different assay buffers including sodium or potassium phosphate buffer (16–22), sodium acetate buffer (8,23,24), and Tris-acetate buffer (8,19) have been reported for tissue homogenization and enzymatic reaction. Because the targeted sample size for TH assay may be as small as a mouse striatum, attainment of high sensitivity is of prime concern particularly when assessing TH in tiny samples. Moreover, a sensitive TH assay would also be useful for studying the effect of drugs on TH inhibition. This investigation was therefore undertaken to examine the effect of buffer composition, pH, cofactor level, and incubation time on TH activity in rodent striatal homogenates.

EXPERIMENTAL PROCEDURE

Wistar male rats (230 ± 20 g) and C57BL male mice (28 ± 3 g) were housed in a temperature-controlled room with 12-h light/dark cycle; laboratory food and water were freely available *ad libitum*. After 1-week acclimatization in the home cages, the animals were sacrificed by decapitation. The brains were dissected on ice; the striata were isolated and rapidly frozen in liquid nitrogen and then stored at −80° until analyzed (within 3 days in this study).

L-Tyrosine-[ring-3,5-^{3}H], 24 Ci/mmol was obtained from ICN (Costa Mesa, CA, USA). Catalase, 1600 U/mg solid; DL-dithiothreitol; DL-6-methyl-5,6,7,8-tetrahydropterine (6-MPH$_4$); Tris-(hydoxymethyl) aminomethane; Triton X-100; and activated charcoal were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Optiphase Hisafe scintillation cocktail was obtained from Wallac (Wallac, Turku, Finland). All other reagents used were of analytical grade. The compositions of various buffers used for TH assay are summarized in Table I.

The TH assay procedure reported by Reinhard et al. (9) was used with some modifications. Striata from rats (25 ± 5 mg) or mice (6 ± 1 mg) were homogenized, respectively, with 1.0 or 0.5 ml of the assay buffer containing 0.5% Triton X-100. The homogenates were centrifuged at 20,000 rpm for 5 min in a refrigerated centrifuge (Hettich, GmbH, Tuttlingen, Germany). Two hundred microliters of supernatant (sample) or respective buffer (blank) were incubated with 0.8 μCi [^{3}H] L-tyrosine, 1.5 mM 6-MPH$_4$, 100 U catalase, and 1.0 μM dithiothreitol in a total volume of 300 μl (including sample) for 20 min (or as specified) at 37°C. The reaction was terminated by adding 1.0 ml of stirred suspension of 7.5% (w/v) activated charcoal in 0.1 M HCl. The mixture was rapidly vortexed and centrifuged at 3000 rpm for 5 min. Two hundred microliters of aliquots were transferred to a scintillation vial containing 5 ml of cocktail for quantification of [^{3}H] H_2O in supernatant. The radioactivity was measured as disintegrations per minute (dpm), while each sample was counted for a period of 60 s using a liquid scintillation counter (Wallac). The enzyme activity was reported as dpm · mg^{-1} · 20 min^{-1}.

Table I. Preparation of Various Buffers Used for Tyrosine Hydroxylase Enzyme Assay

Buffer*	Compound (0.2 M)	pH	A (*x* ml)	B (*y* ml)
Sodium-phosphate	(A) NaH_2PO_4	5.0	99	1
	(B) Na_2HPO_4	5.5	95	5
		6.0	88	12
		6.8	51	49
Sodium-acetate	(A) CH_3COONa	6.0	98	2
	(B) CH_3COOH			
Tris-acetate	(A) $NH_2C(CH_2OH)_3$	6.0	68	32
	(B) CH_3COOH	7.0	71	29

* Mix *x* ml of A with *y* ml of B and make the total volume to 200 ml with distilled water; measure the pH.

Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple-range test for comparing means. *P* values less than 0.05 were considered statistically significant.

RESULTS

Effect of Different Assay Buffers on TH Activity

Among the three assay buffers with earlier reported pH values (Fig. 1), sodium acetate buffer (pH 6.0) showed

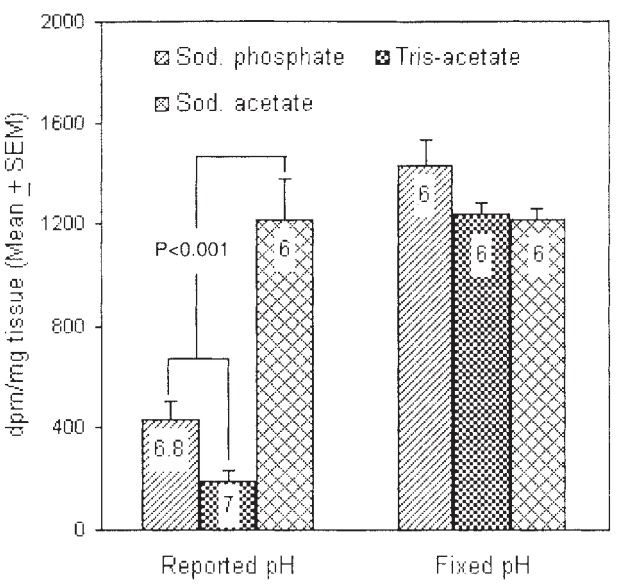

Fig. 1. Assay of tyrosine hydroxylase (TH) in rat striatum using sodium phosphate (pH 6.8 and 6.0), Tris acetate (pH 7.0 and 6.0), and sodium acetate (pH 6.0) buffers. Results are presented as the mean of three samples ± SEM. Values inside the bars indicate the pH values of the respective buffers. Statistical comparisons were performed using Dunnett's multiple-range test.

significantly high TH activity (1218 ± 159 dpm/mg) as compared to sodium phosphate buffer at pH 6.8 (435 ± 72 dpm/mg) and Tris-acetate buffer at pH 7.0 (193 ± 39 dpm/mg) (ANOVA $F_{2,6} = 43.72$, $P = 0.000$). When these assay buffers were tested at a constant pH (6.0), a slightly higher enzyme activity was observed with sodium phosphate buffer (1428 ± 105 dpm/mg) as compared to sodium acetate (1241 ± 43) or Tris-acetate (1215 ± 45) buffers; however, this difference appeared to be statistically insignificant (ANOVA $F_{2,6} = 2.718$, $P = 0.144$) (Fig. 1).

Effect of Assay Buffer pH on TH Activity

A significantly high TH activity was observed using the phosphate buffer of pH 5.5 (1403 ± 77 dpm/mg) and pH 6.0 (1281 ± 125 dpm/mg) (Fig. 2), whereas further decrease (23 ± 6 dpm/mg at pH 5.0) or increase (434 ± 72 at pH 6.8) in pH significantly inhibited TH activity (ANOVA $F_{4,10} = 55.094$, $P = 0.000$).

Effect of Cofactor (6-MPH_4) Concentration on TH Activity

The assay mixture containing 0.75 mM and 1.5 mM of 6-MPH_4 resulted in TH activities of 886 ± 119 dpm/mg and 1139 ± 125 dpm/mg, respectively (Fig. 3), whereas the abolition (421 ± 66 dpm/mg) or usage of higher concentrations of cofactor (3.0 mM, 690 ± 126 dpm/mg; 4.5 mM, 538 ± 158 dpm/mg) significantly inhibited TH activity in rat striatal homogenates (ANOVA $F_{4,10} = 7.437$, $P = 0.005$).

TH Assay Linearity as a Function of Sample Quantity

Aliquots (25, 50, 100, and 200 μl) of rat striatal homogenate were mixed with 175, 150, 100, and 0 μl of sodium phosphate buffer (pH 6.0), respectively. These serially diluted samples were analyzed in triplicate to evaluate the linearity of TH assay with respect to sample concentration. The results showed a direct correlation (R = 0.97) between effective volume of striatal homogenate and TH activity (Fig. 4).

TH Assay Linearity as a Function of Incubation Time

TH assay reaction was performed on striatal homogenates from rats and mice using 10-, 20-, 30-,

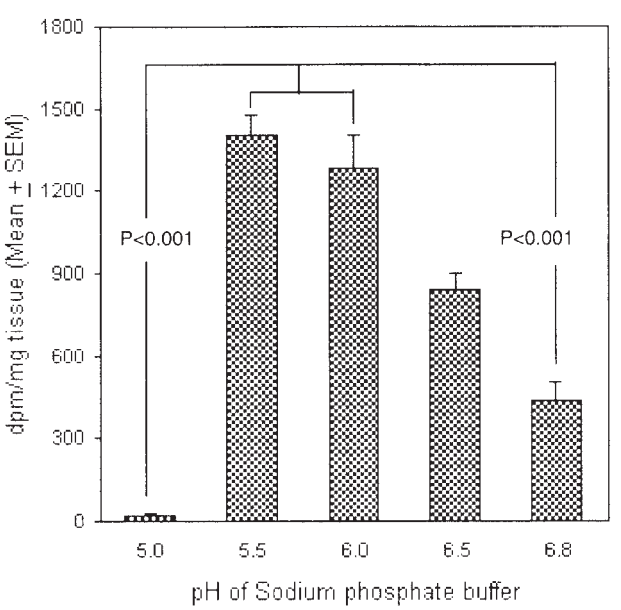

Fig. 2. Effect of phosphate buffer pH on the sensitivity of TH assay in rat striatum (three samples per group). The pH range of 5.5–6.0 was found to be optimum, whereas pH deviations in both directions significantly inhibited TH activity. Statistical comparisons were performed using Dunnett's multiple-range test.

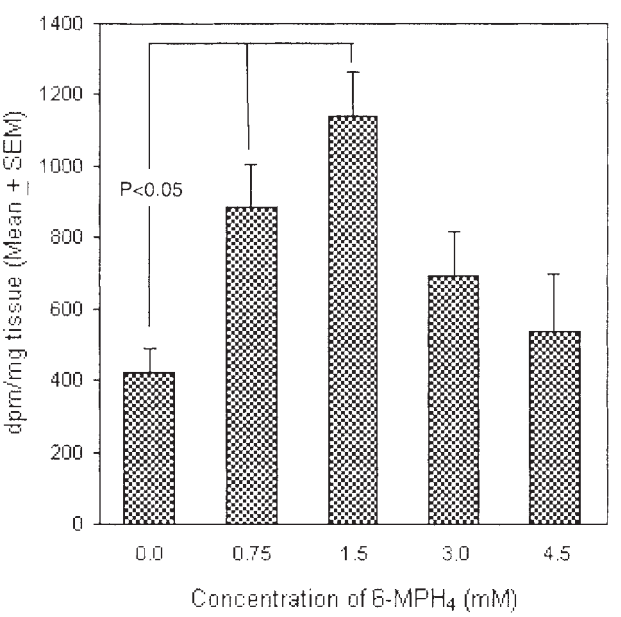

Fig. 3. Effect of cofactor concentration on the sensitivity of TH assay in rat striatum. The reactions were performed in triplicate in phosphate buffer (pH 6.0) using varying concentrations (0.75–4.5 mM) of cofactor (6-MPH_4). Statistical comparisons were performed using Dunnett's multiple-range test.

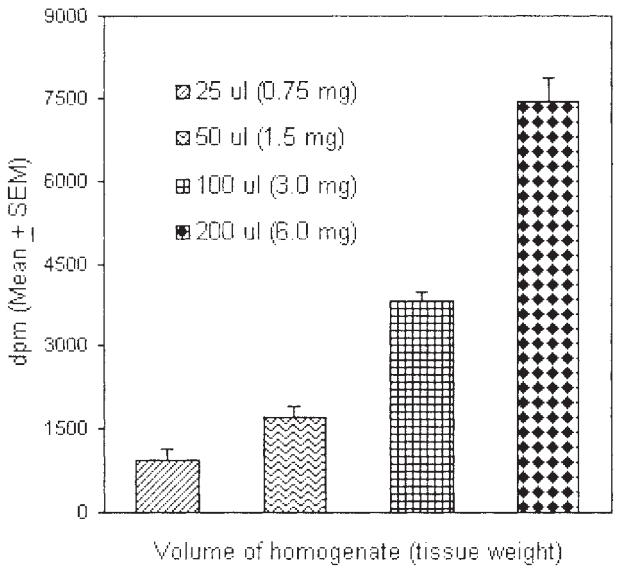

Fig. 4. Evaluation of assay linearity as a function of sample volume. Serially diluted rat striatal homogenates were analyzed in triplicate and the results reported as total dpm counts in specified sample volumes and corresponding tissue weights (correlation coefficient, R = 0.97).

and 40-min incubation times at 37°C. The reaction rate was found to be linear for the first 20 min for rat striata and 30 min for mice striata (Fig. 5).

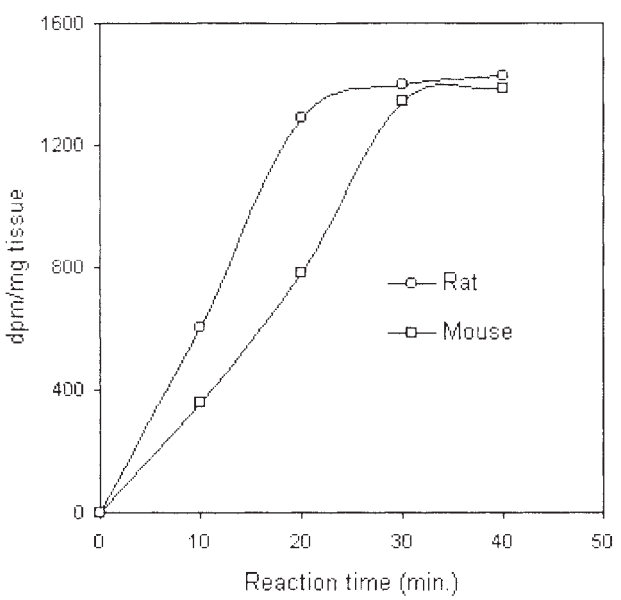

Fig. 5. Evaluation of assay linearity as a function of reaction time (incubation period). Striatal homogenates from rat and mice striata were assayed for TH activity using varying lengths of incubation period at 37°C.

DISCUSSION

The initial batch of experiments primarily dealt with the comparison among three assay buffers at their earlier reported pH values. The findings revealed a decreasing trend in TH activity with sodium acetate (pH 6.0) > sodium phosphate (pH 6.8) > Tris-acetate (pH 7.0) buffers, which pointed toward a possible role of pH rather than buffer composition on TH assay sensitivity (Fig. 1). This notion was confirmed by comparing all three buffers at a constant pH of 6.0, and the resulting TH activities were not statistically significant though a slight edge occurred in favor of phosphate buffer (Fig. 1). Phosphate buffer was preferentially adapted for further experimentation because the pH optima (≈6) of TH assay fell within the recommended working range of phosphate buffer (pH range, 5.0–8.0) as compared to sodium acetate (pH range, 3.8–5.6) or Tris-acetate (pH range, 7.0–9.0) buffers. A pH range of 5.5–6.0 was found to be optimum for TH assay in striatal homogenates (Fig. 2). Although several investigators have performed TH assay at pH 6.0 or 6.1 (9,17–19,24,25) others have also used pH 6.8 (21,22), pH 7.0 (8,20,26), or pH 7.2 (16). A significant inhibition of TH activity was observed at pH 5.0 and pH 6.8 (Fig. 2). Baillien et al. (18) have also reported strong inhibition of TH activity at pH 7.0 and 7.5 while showing maximum TH activity at pH 6.0, though the effects of pH less than 6.0 were not examined in that study.

The exact mechanism of high TH activity at pH 5.5–6.0 and almost complete inhibition at pH 5.0 or partial inhibition at pH 7.0 is not clear. A pH range of 5.4–6.5 has been shown to favor reversible binding of Fe (II) with TH leading to enzyme activation, whereas more acidic pH values are responsible for iron removal from enzyme-iron moiety causing TH inhibition (27). On the other hand, dithiothreitol, which is used to prevent accumulation of quinonoid form of cofactor, increases iron dissociation from TH at neutral pH (27) that may account for reduced TH activity at pH 6.8–7.0 (Figs. 1 and 2). Richtand et al. (28) have suggested that TH enzyme activity in striatal homogenates declines below pH 5.9 for nonphosphorylated TH and below pH 7.4 for phosphorylated TH because of instability of enzyme at more acidic pH, whereas decreases in TH activity at higher pH are supposed to be caused by ionization of either enzyme or substrate, which decreases the rate of catalysis at more basic pH (28,29). Some molecules like heparin and NaCl that present in tissue are known to activate native TH at pH 6.0 but not at pH 7.0 (30). On the other hand, phosphorylated TH is unaffected by heparin or NaCl at pH 6.0 and is inhibited at pH 7.0 (30).

Interestingly, a recombinant rat PC12 TH enzyme (virtually activated and unaffected by further phosphorylation) showed maximal activity over a broader pH range (6.0–7.5) with apparent pH optima of 6.5 (23). Treatment of this recombinant TH with dopamine (DA) showed a sharp pH optima of 5.8, whereas subsequent phosphorylation of the pretreated enzyme reversed the effect of DA on pH optima (23). Furthermore, high concentrations of naturally occurring DA in rodent striatal homogenates (31) might impose detrimental effects on TH assay by inhibiting TH activity at physiological pH (23,32), whereas DA-induced TH inhibition is significantly minimized at pH 6.2 suggesting that a low-pH assay buffer would apparently favor tyrosine hydroxylation reaction in DA-rich samples (32).

A high TH activity while using 0.75–1.5 mM of the cofactor 6-MPH_4 is in agreement with earlier reports (8,24,33), though comparatively higher concentration of 6-MPH_4 (6.6 mM) has also been used for TH assay (25). Increasing the level of 6-MPH_4 (3.0–4.5 mM) caused a concentration-dependent inhibition of TH activity; however, this effect was not statistically significant (Fig. 3). Inhibition of TH activity by excess concentrations of natural cofactor tetrahydrobiopterin (BH_4) has previously been reported (26,34). The detection of TH activity even in the absence of synthetic cofactor 6-MPH_4 points toward some degree of existing autoactivation (autophosphorylation) of TH leading to hydroxylation of tyrosine (28). Although phosphorylation is known to activate TH (35,36), this strategy has probably never been adapted for routine TH assays and is not implied in this study, basically due to instability of phosphorylated TH (28,36,37) that might confer a negative analytical impact rather than outweighing additional time and cost associated with phosphorylation steps.

A direct correlation between TH activity and the volume of striatal homogenate or the amount of brain tissue added in assay tube (Fig. 4) clearly suggests the suitability of this procedure for *in vivo* TH assay in small tissues. TH activity was found to be linear as a function of incubation time for the first 20 (rat striatum) or 30 min (mouse striatum) (Fig. 5). The right shift in the kinetic curve while using mouse specimens was presumably associated with dilution factor; mice striatal homogenates being about two-fold more diluted than the rat striatal homogenates would ultimately result in a slow reaction rate. Thus, appropriate incubation times for rats and mice striata can be regarded as 15 and 20 min, respectively. This is in accordance with several reports (8,9,18,19,23,25,26), though longer incubation times (30–60 min) have also been used in some studies (16,17,24), which may be attributed to differences in buffer composition, pH, and/or the concentrations of substrate and cofactor(s) used. As the selection of incubation time is not arbitrary the linearity of TH assay can also be achieved for longer incubation times by increasing substrate concentration and/or dilution of the sample.

In conclusion, this study clearly demonstrates the crucial roles of assay buffer pH and cofactor concentration on the sensitivity of TH assay in rodent striatum. It is also important to select appropriate incubation time according to sample amount to avoid spurious results.

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