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# Tyrosine hydroxylase assay for detection of low levels of enzyme activity in peripheral tissues

Douglas Hooper[a], Minoru Kawamura[a,b], Beth Hoffman[c], Irwin J. Kopin[a], Bela Hunyady[a], Eva Mezey[a], Graeme Eisenhofer[a,*]

[a]*Clinical Neuroscience Branch, National Institute of Neurological Disorders and Stroke, Building 10, Room 4D20, National Institutes of Health, Bethesda, MD 20892, USA*

[b]*Institute of Bioactive Science, Nippon Zoki Pharmaceutical Co., Ltd., Kinashi, Yashiro-Cho, Kato-Gun, Hyogo 673-14, Japan*

[c]*Laboratory of Cell Biology, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892, USA*

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**Abstract**

A nonisotopic assay for tyrosine hydroxylase, with optimized signal-to-noise ratios, enables determination of low levels of enzyme activity in peripheral tissues. DOPA produced by the enzyme is measured using HPLC with electrochemical detection. Increased signal-to-noise ratios are obtained by including in the reaction mixture glycerol for reduction of blank values and dihydropteridine reductase and NADPH for regeneration of the tetrahydropteridine cofactor. With this method, tyrosine hydroxylase activity can be detected in as few as 200 PC12 cells and in peripheral tissues at levels as low as 4.5 fmol/min/mg wet weight. The assay permits activity to be assessed in a variety of peripheral tissues.

*Keywords:* Tyrosine hydroxylase; Enzymes; DOPA

## 1. Introduction

The enzyme tyrosine hydroxylase (L-tyrosine, tetrahydropteridine:oxygen oxidoreductase, 3-hydroxylating; EC 1.14.16.2) catalyzes the rate-limiting step in the biosynthesis of catecholamines, formation of 3,4-dihydroxyphenylalanine (DOPA) from L-tyrosine [1]. According to existing dogma, the enzyme is localized exclusively in catecholamine-synthesizing neurons in the brain, sympathetic nerves and peripheral chromaffin tissue such as the adrenal medulla [2]. However, new sensitive histochemical methods for the cellular localization of tyrosine hydroxylase immunoreactivity, or of the mRNA encoding the enzyme, have suggested that the enzyme may be more widely distributed among tissues and cell types than previously believed. These methods have indicated the presence of the relevant translation and transcription products in cell bodies of the ovary [3], pancreas [4], heart [5] and in immunocytes and other cells of the gastric mucosa [6,7]. However, the above studies did not establish enzymatic activity, a necessary step to demonstrate functional significance.

Existing methods to measure tyrosine hydroxylase activity were largely developed to quantitate high levels of enzyme activity in brain, adrenal tissue or pheochromocytoma cell lines [8–16]. Application of these methods to peripheral tissues with lower levels of enzyme activity is limited by practical problems

*Corresponding author.

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inherent in assays of tyrosine hydroxylase activity, notably high blank values due to nonenzymatic hydroxylation of tyrosine to DOPA [1]. The present assay was developed to maximize the ratio of sample-to-blank values, thereby providing a method suitable for measuring tyrosine hydroxylase activity in a wider variety of peripheral tissues than previously reported.

## 2. Experimental

### 2.1. Reagents

Dihydropteridine reductase (55 U/mg protein, EC 1.6.99.7), NADPH, DL-6-methyltetrahydropterine dihydrochloride (6-$MPH_4$), L-tyrosine hydrochloride, D-tyrosine hydrochloride, *m*-hydroxybenzylhydrazine dihydrochloride, diethyldithiocarbamic acid, 1-octanesulfonic acid and the catechol standards (DOPA, α-methyDOPA, norepinephrine, epinephrine, dopamine and dihydroxybenzylamine) were from Sigma (St. Louis, MO, USA). 6-FluoroDOPA was synthesized and provided as a gift by Dr. Kenneth Kirk (NIDDK, NIH, Bethesda, MD, USA). Bovine liver catalase (65 000 U/mg protein, EC 1.11.1.6) was from Boehringer Mannheim (Indiannapolis, IN, USA); 3-iodo-L-tyrosine was from Aldrich (Milwaukee, WI, USA). All other reagents were analytical grade.

### 2.2. Preparation of tissue samples

Rat PC12 cells (a gift from Dr. Lee E. Eiden, NIMH, NIH) were grown as a monolayer in Dulbecco's minimal essential medium supplemented with 7% fetal calf serum, 7% horse serum and 1% penicillin/streptomycin. Cells were harvested using trypsin, counted, and collected by centrifugation at 800*g* for 10 min. The cell pellet was washed, resuspended in phosphate-buffered saline (pH 7.2) and disrupted using a Kontes micro ultrasonic cell disrupter (settings: 30 amp, 50 switch and 5 tune). Cell debris were removed by centrifugation at 15 000 *g* (4°C), and the resulting cleared lysate was stored at −80°C. For assay of tyrosine hydroxylase activity, cell lysates were thawed and diluted in 0.05 *M* sodium acetate buffer (pH 6.0) to a concentration equivalent to 20 000 cells per ml.

Peripheral tissue samples (adrenals, duodenum, heart, lungs, kidneys, pancreas, salivary glands, superior cervical ganglia, thymus, testes and vas deferens) were harvested from male Sprague–Dawley rats (270–300 g) and frozen immediately on dry ice. Tissues were weighed frozen and homogenized in 4 to 10 volumes of ice-cold 0.05 *M* sodium acetate buffer (pH 6.0). Homogenates were centrifuged (3000 *g*) and the supernatant removed and stored frozen at −80°C until assayed for tyrosine hydroxylase activity.

### 2.3. Reaction procedure

The incubation mixture contained 100 μl of appropriately diluted sample preparation (i.e. tissue or PC12 extracts), and included the following components in a total volume of 200 μl: 100 m*M* acetate buffer (pH 6.0), 200 μ*M* L-tyrosine, 1.25 m*M* *m*-hydroxybenzylhydrazine, 1.0 m*M* 6-$MPH_4$, $19.5\times 10^3$ U/ml catalase, 3.8 U/ml dihydropteridine reductase, 1 m*M* NADPH and 0.2 *M* glycerol. Reaction blanks for each sample consisted of identical components as described above, but also included 3-iodo-L-tyrosine (0.45 m*M*) to inhibit tyrosine hydroxylase.

Incubations were carried out at 37°C in a shaking water bath and were initiated by addition of L-tyrosine, 6-$MPH_4$, dihydropteridine reductase and NADPH formulated and mixed immediately before reactions were commenced. Reactions typically lasted 10 min and were terminated by adding 100 μl of 0.4 *M* perchloric acid.

### 2.4. Preparation of incubates for HPLC analysis

The DOPA produced in incubates was extracted onto alumina, based on a previously described method [17]. In brief, 40 μl of two catechol internal standards (12 pmol α-methyDOPA and dihydroxybenzylamine in 0.2 *M* acetic acid), 500 μl Tris–EDTA buffer [1 *M* tris(hydroxymethyl)aminomethane and 0.05 *M* EDTA adjusted to pH 8.6 with 5 *M* HCl] and 10 mg alumina were added to samples of the acidified reaction mixture or to an equivalent volume of catechol standards (5.08

pmol DOPA, 1.48 pmol norepinephrine, 1.37 pmol epinephrine and 1.57 pmol dopamine). After mixing, the alumina was washed and catechols were eluted with 100 μl of 0.2 *M* acetic acid or a mixture of 0.16 *M* acetic acid and 0.02 *M* phosphoric acid. A 90-μl aliquot of the eluent was injected onto the HPLC apparatus.

### 2.5. HPLC with electrochemical detection

The HPLC apparatus included a Model 510 solvent delivery system and a Model 717 refrigerated autosampler (both from Waters Associates, Milford, MA, USA). Chromatographic separation was achieved using a 5-μm particle size Axxi-chrom $C_{18}$ reversed-phase column (250×4.6 mm I.D.) obtained from Axxiom Chromatography (Moorpark, CA, USA). The column temperature was maintained at 18°C using a Model 1166 refrigerated water circulator (PolyScience, Niles, IL, USA) that pumped a chilled equivolume mixture of water and ethylene glycol through a water jacket (also from Axxiom Chromatography) surrounding the column.

A Model 5100A coulometric detector from Environmental Sciences Associates (Bedford, MA, USA) with a triple electrode system was used to quantify the compounds as they eluted from the column. The first electrode in the Model 5021 conditioning cell was set at a potential of +0.35 V. The second and third electrodes in the Model 5011 analytical cell were set at potentials of +0.15 and −0.35 V. The output from the third electrode was recorded with a Macintosh SE computer (Apple Computer, Cupertino, CA, USA) and Dynamax Method Manager hardware and software package (version 1.4; Rainin Instruments, Woburn, MA, USA). The mobile phase (0.1 *M* $NaH_2PO_4$, 0.13 m*M* EDTA, 0.22 m*M* octane sulfonate, 2.5% acetonitrile, pH 3.35) was filtered through a 0.22 μm pore size filter and pumped through the chromatographic system at a rate of 1.0 ml/min.

### 2.6. Calculations

Data from chromatograms were downloaded into a specially formulated spreadsheet program in which DOPA values were automatically adjusted for recovery of the appropriate internal standard and then converted into values for tyrosine hydroxylase activity ($TH_{ACT}$, fmol/mg wet weight tissue/min) according to the formula

$$TH_{ACT} = (DOPA_S - DOPA_B)/(W \cdot T),$$

where $DOPA_S$ and $DOPA_B$ are the respective amounts of DOPA (fmol) produced during incubation of the sample and sample blank, where *W* represents the weight of tissue in the incubation mixture (mg) and where *T* is the time of incubation (min). For assays of tyrosine hydroxylase activity in PC12 cells, data is expressed in fmol/min/1000 cells.

### 2.7. Assay development and validation

Initial experiments were carried out to determine the most appropriate internal standard (dihydroxybenzylamine, 6-fluoroDOPA or α-methylDOPA) to use for correction of analytical recoveries. These experiments were carried out in conjunction with others that compared analytical recoveries with two different agents (0.2 *M* acetic acid or 0.16 *M* acetic acid–0.02 *M* phosphoric acid) used to desorb catechols from the alumina.

Experiments to optimize the enzyme assay included efforts to reduce sample-to-blank ratios, thereby improving the sensitivity of the assay and enabling measurement of the low levels of tyrosine hydroxylase activity present in most peripheral tissues. The influence of several compounds on nonenzymatic production of DOPA was examined. These experiments used water blanks (i.e. no tissue or PC12 samples) and examined the effects of omitting or adding catalase ($19.5\times10^3$ U/ml) or ferrous ammonium sulfate (100 μ*M*) with and without glycerol (0.2 *M*) and diethyldithiocarbamic acid (100 m*M*). Other experiments examined whether boiled samples or use of D-tyrosine (which is not a substrate for tyrosine hydroxylase) provided lower blank values than obtained with the use of L-tyrosine and 3-iodo-L-tyrosine (the latter an inhibitor of tyrosine hydroxylase). Additional experiments, carried out in PC12 cell samples and replicated in pancreas tissue samples, assessed whether tyrosine hydroxylase activity could be increased by the addition of dihydropteridine reductase (3.8 U/

ml) and NADPH (1 m*M*) to the incubation system as previously described [8].

Linearity of the reaction was established with respect to time of incubation and amount of tyrosine hydroxylase enzyme activity (determined with PC12 cell samples). Intra-assay coefficients of variation were determined from repeated determinations of tyrosine hydroxylase activity in PC12 cell samples carried out within the same assay. Interassay coefficients of variation were determined from repeated determinations of tyrosine hydroxylase activity in PC12 cells carried out in different assays.

## 3. Results and discussion

### *3.1. Extraction and chromatography*

As illustrated from chromatographic recordings (Fig. 1), the present tyrosine hydroxylase assay provides adequate sensitivity to measure low levels of enzyme activity in as few as 200 PC12 cells or in most peripheral tissues. Chromatography permitted separation of DOPA and internal standards from the endogenous catecholamines present in tissue samples. Initial studies indicated that α-methylDOPA was the most appropriate internal standard and that recovery of DOPA could be optimized using a mixture of phosphoric and acetic acids rather than acetic acid alone to elute the catechols from alumina (Table 1). The extraction step ensured a clean chromatogram free from interfering contaminants, permitting a shorter chromatographic run for each sample than was otherwise possible.

### *3.2. Optimization of signal-to-blank values*

Determination of tyrosine hydroxylase activity is complicated by nonenzymatic production of DOPA from tyrosine during the incubation step. As pointed out by Nagatsu et al. [1], this explained why the presence of the enzyme was initially so difficult to demonstrate. Non-enzymatic production of DOPA from tyrosine was evident during development of the present assay from a time-dependent increase in DOPA concentrations in water blank reaction mixtures containing all reagents, but not in those in which tyrosine was absent. Nonenzymatic production

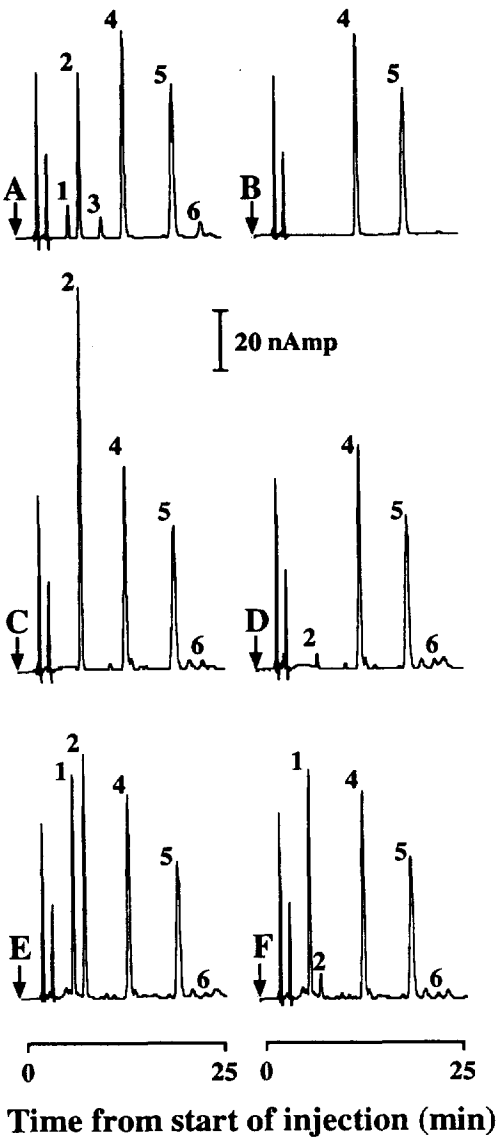


Fig. 1. Chromatographic recordings after HPLC injection (marked by the arrows) of alumina-extracts of all catechol standards (A), internal standards alone (B), an incubate of 1000 PC12 cells (C), the corresponding blank incubate of 1000 PC12 cells (D), an incubate of pancreatic tissue (E) and the corresponding blank incubate of pancreatic tissue (F). Key for identification of chromatographic peaks: 1 = norepinephrine, 2 = DOPA, 3 = epinephrine, 4 = dihydroxybenzylamine, 5 = α-methylDOPA and 6 = dopamine. Amounts in catechol standard mixture (A): norepinephrine (1.48 pmol), DOPA (5.08 pmol), epinephrine (1.37 pmol), dopamine (1.57 pmol) and dihydroxybenzylamine and α-methylDOPA (12 pmol).

Table 1
Recoveries of DOPA and internal standards from alumina for HPLC

| Catechol | Recovery (%) | |
|---|---|---|
| | Acetic acid | Acetic–phosphoric acid |
| DOPA | 51±1 | 70±1 |
| α-MethylDOPA | 52±1 | 71±1 |
| 6-Fluoro-DOPA | 44±1[a] | 69±1 |
| DHBA | 72±2[a] | 78±1[a] |

[a] Significantly ($P<0.05$) different recovery from DOPA (by analysis of variance with Sheffe's post-hoc test).
Analytical recoveries were estimated using two different acids (0.2 *M* acetic acid or a mixture of 0.16 *M* acetic acid and 0.02 *M* phosphoric acid) to elute catechols from the alumina.

of DOPA was reduced substantially in the presence of catalase and reduced further by the addition of glycerol (Fig. 2A).

The presence of $Fe^{2+}$ resulted in substantially increased blank values, an effect that could be largely reversed by glycerol (Fig. 2B). Blank values in incubates containing glycerol and $Fe^{2+}$ were three-fold higher than in those containing only glycerol. The chelating agent, diethyldithiocarbamic acid, also substantially reduced nonenzymatic production of DOPA, but glycerol was more effective (Fig. 2C).

The finding of increased blank values in the presence of $Fe^{2+}$ is an established observation [14,18]. Although addition of $Fe^{2+}$ is apparently required for optimal activity of tyrosine hydroxylase in some tissues [16,19], our observations indicate that increased blanks in the presence of $Fe^{2+}$ obscure any benefit in enzyme activity. This conclusion is in agreement with that of Lee et al. [14]. The findings that glycerol largely reversed the increased blank values observed in the presence of $Fe^{2+}$ and that diethyldithiocarbamic acid had similar effects to glycerol, suggest that reduced blank values might be secondary to chelation or scavenging of free radicals.

Additional nonenzymatic production of DOPA was also apparent from iodotyrosine. Thus, methods of producing tissue blanks, other than using enzyme inhibition with iodotyrosine, were also explored. Blank values were not improved by boiling tissue samples or substituting L-tyrosine with D-tyrosine. In fact, nonenzymatic production of DOPA was almost 2-fold higher in the presence of D-tyrosine than in the

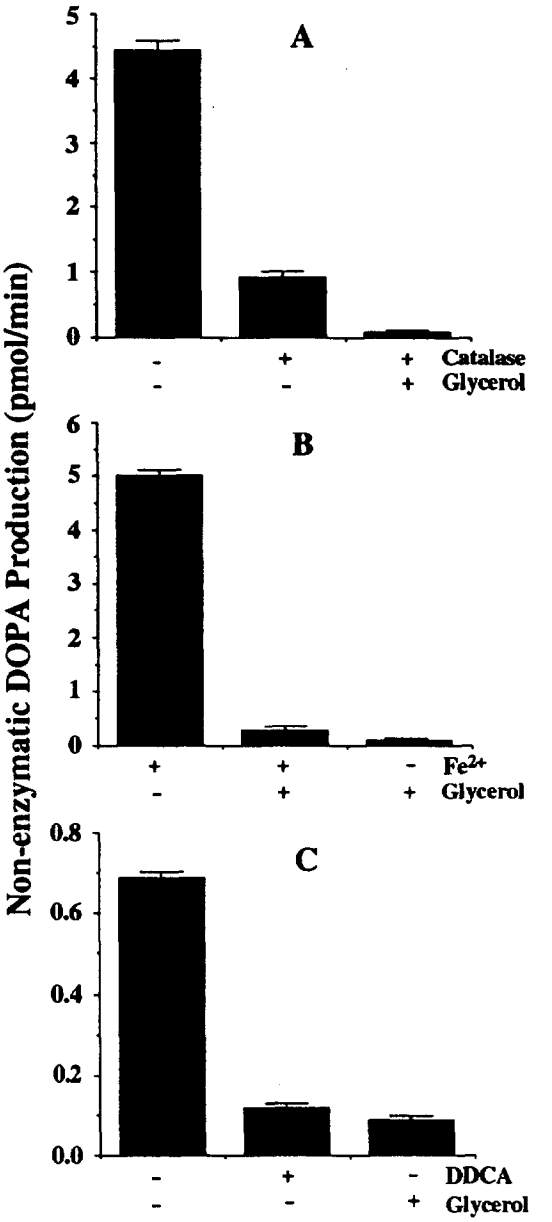


Fig. 2. Nonenzymatic production of DOPA from L-tyrosine. Results represent rates of DOPA production during incubation of water blanks (i.e. no enzyme present) with (+) and without (−) addition of various compounds (no $Fe^{2+}$). (A) The effects of catalase with and without glycerol. (B) The effects of $Fe^{2+}$ with and without glycerol (catalase included throughout). (C) The effects of diethyldithiocarbamic acid (DDCA) compared to glycerol (catalase included throughout, no $Fe^{2+}$). Results represent mean±S.E.M. values ($n=6$–8 for each determination).

presence of L-tyrosine (170±4 vs. 87±3 fmol/min). Use of iodotyrosine in combination with glycerol and exclusion of $Fe^{2+}$ provided optimal blank values.

Incorporation of NADPH and dihydropteridine reductase into the assay was based on the finding of Shiman and Kaufman [8] that use of this enzyme system, for regeneration of the reduced form of tetrahydrobiopteridine, improves activity determinations of tyrosine hydroxylase. In agreement with this finding [8], but not that of Lerner and colleagues [10], we found that combined addition of NADPH and dihydropteridine reductase to reaction mixtures containing PC12 cells dramatically increased sample-to-blank ratios, due largely to a 6.2-fold increase in measureable enzyme activity (Fig. 3). Some increase in activity was also apparent when NADPH was included in reaction mixtures alone, but the full effects required the additional presence of dihydropteridine reductase. Similar results were obtained with samples of pancreatic tissue (data not shown).

### 3.3. Linearity, precision and sensitivity

Enzymatic production of DOPA showed the curvilinear relationship with time of incubation (Fig. 4)

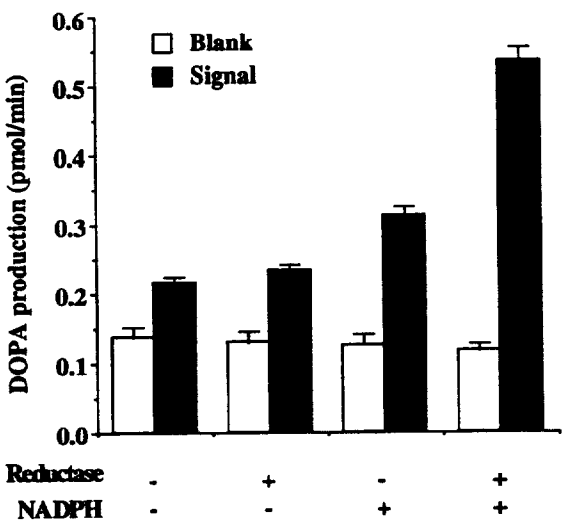


Fig. 3. Effects of addition (+) or omission (−) of NADPH and dihydropteridine reductase on production rates of DOPA in blank (iodotyrosine present) and sample (iodotyrosine absent) incubates of 1000 PC12 cells. Experiments were carried out in the presence of glycerol and catalase. Results represent mean±S.E.M. values ($n=6$ for each determination).

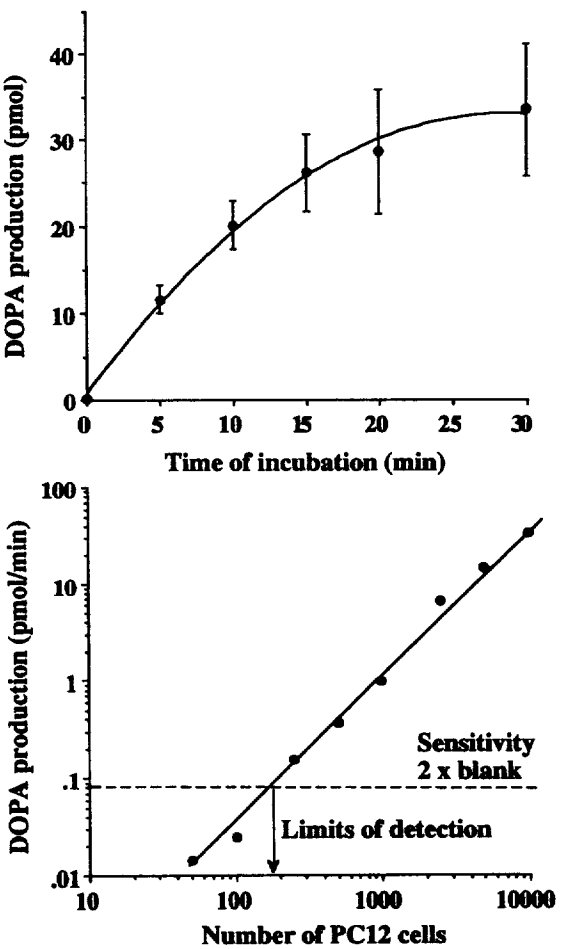


Fig. 4. Relationships of enzymatic DOPA production with time of incubation (top) or amount of enzyme (bottom). Enzymatic DOPA production was calculated after subtraction of blank values. The relationship with time represents the accumulated total production of DOPA at each time point ($n=4$ for each time point). The relationship with amount of enzyme was established from variable dilutions of the PC12 preparation corresponding to 50 to 10 000 cells per sample assayed ($n=4$ for each determination). The sensitivity of the assay determined as twice blank values indicated that tyrosine hydroxylase enzyme activity could be determined in a sample corresponding to as few as 200 PC12 cells.

expected from other studies of tyrosine hydroxylase [14–16]. The nature of the relationship indicated that the reaction was best terminated within 10 min for appropriate estimation of tyrosine hydroxylase activity. There was a linear relationship between the amount of protein in the reaction mixture and estimated enzyme activity up to at least 30 000 fmol/min (Fig. 4). The nature of the relationship

indicated that about 200 PC12 cells produce a signal twice that of the corresponding blank value.

The intra-assay coefficient of variation for the assay was 13% and the inter-assay coefficient of variation was 21.8%. These values are about 2-fold higher than those employing a similar HPLC procedure to measure plasma or tissue DOPA [17]. This is an unavoidable consequence of the additional variation introduced by the incubation step, as well as the necessity to calculate activities after subtraction of blank values. This limitation of enzyme assays that involve multiple sequential procedures indicates some need for within-assay comparisons in studies of tyrosine hydroxylase activity.

With blank values of about 90 fmol/min and the ability to assay up to 20 mg wet weight of tissue per sample (i.e. 20% of sample volume), it can be estimated – at a sample-to-blank ratio of 2 – that 4.5 fmol/mg/min represents the minimum level of enzyme activity that can be reliably assayed in samples of tissue using the present method. This level of sensitivity is largely dependent on blank values (about 900 fmol/sample) and is not restricted by the detection limits of the HPLC procedure (about 50 fmol/sample).

### 3.4. Comparison with existing methods

Early determinations of tyrosine hydroxylase activity involved cumbersome radiotracer techniques that required measurement of radioactive $H_2O$ production from hydroxylation of radioactive tyrosine or radioactive $CO_2$ production during subsequent decarboxylation of DOPA [8–11]. These techniques have largely been superceded by methods in which the direct product of tyrosine hydroxylase, DOPA, is measured by HPLC with electrochemical or fluorometric detection [12–15]. The present method combines some of the features of an earlier radiotracer technique [8] with direct determination of DOPA by HPLC and use of glycerol in the reaction mixture to reduce blank values. The resulting assay is more sensitive than previously described methods [8–15], which were developed to measure high levels of enzyme activity in brain, adrenal tissue or PC12 cells. One exception is the method described by Mandai et al. [20], a procedure for measurement of tyrosine hydroxylase activity in bovine retina. Although comparably sensitive to the present method, no data were provided to establish whether this other method could be used to determine tyrosine hydroxylase activity in the variety of peripheral tissues described here.

### 3.5. Enzyme activities in rat peripheral tissues

In rat peripheral tissues, tyrosine hydroxylase activity varied widely from a low value of 20.4±3.8 fmol/mg/min in the thymus to an expected high value of 20 208±4573 fmol/mg/min in the adrenal glands (Table 2). These values compare with previously reported levels of activity in brain tissue of 2000–6000 fmol/mg/min and in the adrenal medulla of above 40 000 fmol/mg/min [14]. The testes were the only peripheral tissue examined in which tyrosine hydroxylase activity could not be reliably measured (i.e. the sample value was less than twice the blank value). Rat testes are innervated by sympathetic nerves [21], an observation supported here by the low but detectable levels of norepinephrine in this tissue. Thus, failure to detect enzyme activity in this tissue most probably reflects the sensitivity limitations of the assay, rather than a lack of tyrosine hydroxylase enzyme.

## 4. Conclusions

As illustrated by the range of enzyme activities in Table 2, the present assay is sufficiently sensitive to measure tyrosine hydroxylase activity in most peripheral tissues. The assay may be particularly useful to confirm the presence of functional tyrosine hydroxylase in lymphocytes [22,23] and other non-neuronal or extra-adrenal cell types [3–7] where the enzyme may occur. Supporting this conclusion, we recently demonstrated relatively high levels of tyrosine hydroxylase activity remaining in the pancreas of 6-hydroxydopamine sympathectomized rats; this together with the presence of tyrosine hydroxylase mRNA and associated histochemical findings of tyrosine hydroxylase immunoreactivity in certain cell types provided strong evidence for a novel non-neuronal catecholaminergic system [24]. Apart from questions of functional significance, the presence of tyrosine hydroxylase in certain cells of the gastrointestinal tract raises the possibility of an alternative source of enzyme to the fetal brain or adrenal

Table 2
Tyrosine hydroxylase activity and tissue norepinephrine levels in rat peripheral tissues

| Tissue | Tyrosine hydroxylase activity (fmol/mg/min) | Norepinephrine level (pmol/mg) |
|---|---|---|
| Adrenal gland | 20 208±4573 | n.a. |
| Superior cervical ganglia | 9272±2163 | n.a. |
| Vas deferens | 3399±626 | 45.6±8.3 |
| Salivary gland | 1068±129 | 7.6±0.5 |
| Pancreas | 281±33 | 2.3±0.1 |
| Spleen | 186±18 | 5.4±0.6 |
| Heart | 154±21 | 5.0±0.2 |
| Duodenum | 142±41 | 3.5±0.4 |
| Stomach | 121±12 | 1.6±0.2 |
| Kidney | 43±7 | 1.0±0.1 |
| Lung | 25±9 | 0.7±0.1 |
| Thymus | 20±4 | 0.4±0.03 |
| Testis | <4.5 | 0.02±0.005 |

Tyrosine hydroxylase enzyme activities are expressed in fmol DOPA/minute/mg wet weight of tissue. Tissue norepinephrine levels are in pmol/mg wet weight of tissue, and were determined by a previously described method [16]. All results are expressed as means±S.E.M. ($n=6$). Abbreviation: n.a. = not assayed.

medullary tissue currently used for transplantation in the treatment of Parkinson's disease [25].

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