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A Highly Sensitive Assay for Phenylethanolamine *N*-Methyltransferase in Human Brain[1] (42225)

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Abstract. A rapid, highly sensitive assay for phenylethanolamine *N*-methyltransferase in brain using the natural substrate, norepinephrine, is described. The method is based on the selective adsorption and elution of the reaction product, epinephrine, from alumina. A small but important further lowering of blanks and increase in sensitivity is attained by removal of the radiolabeled substrate, [*methyl*-^{3}H]-*S*-adenosylmethionine by precipitation as the reineckate prior to adsorption of norepinephrine to alumina. The assay has a sensitivity of 30 fmole and the PNMT activity could be measured in as little as 1 mg (wet wt) of human locus coeruleus tissue. The sensitivity is enhanced by homogenizing tissue in small volumes and removing potential inhibitors by dialysis. We report for the first time PNMT activity in specific regions of the human cerebral and cerebellar cortex.

Phenylethanolamine *N*-methyltransferase (PNMT, EC 2.1.1.28), the enzyme which catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to phenylethanolamines, occurs in highest concentration in the adrenal medulla (1). In the medulla, the enzyme converts norepinephrine (NE) to epinephrine (Epi). PNMT has also been detected in low concentrations in specific regions of brain (2–9) where its product Epi may act as a neurotransmitter (10, 11).

The current methods for assay of PNMT involve either radiochemical techniques (12–15) or high-performance liquid chromatography with electrochemical detection (HPLC-EC) (16, 17). The initial radiometric assays, used to measure PNMT in the medulla (1, 18, 19), were found to be inadequate for brain PNMT because they lacked either specificity (9) or sensitivity (13). Later radiometric assays (13, 14) used *methyl*-^{3}H-labeled SAM in place of [^{14}C]SAM and employed extensive extraction and evaporation procedures (4, 12, 13). These changes improved the specificity of the assay, increased its sensitivity by 50-fold and allowed measurement of PNMT in brain. The HPLC-EC assays of PNMT (16, 17) further increased the specificity of the assay by using NE instead of phenylethanolamine as substrate and by specific measurement of Epi. However the HPLC-EC assay is limited by the presence of small amounts of Epi as a contaminant in the NE substrate and by the difficulty of separating large amounts of NE from Epi (6, 17). The results obtained from a variety of assays are in agreement for areas of brain, such as hypothalamus, which contain large amounts of PNMT. However, either because of low sensitivity or lack of specificity, there have been significant differences in the reported distribution of PNMT in areas of relatively low activity in human brain (2–6). Even with the most sensitive previous methods for measuring brain PNMT (2, 4–6) there have been no reports of significant PNMT activity in specific regions of human cerebral or cerebellar cortex.

In this paper we describe a new radiochemical assay procedure for PNMT using NE as substrate. This assay is both specific and highly sensitive. Using this method we were able to measure PNMT in specific areas of human cerebral and cerebellar cortex.

Materials and Methods. *Chemicals.* (−)-Norepinephrine hydrochloride, catalase, L-epinephrine bitartrate, Na ascorbate, 2-mercaptoethanol, harmaline hydrochloride, *S*-adenosyl-L-methionine iodide salt and reinecke salt were purchased from Sigma Chemical Company (St. Louis, Mo.). Woelm brand of alumina was prepared by Bioanalytical Systems (West Lafayette, Ind.). Perchloric acid,

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Ultrex, and HPLC grade water were obtained from J. T. Baker Chemical Company (Phillipsburg, N.J.). Heptane sulfonic acid was from Taylor Chemical Company (St. Louis, Mo.). *S*-[*methyl*-^{3}H]-adenosyl-L-methionine 12.8 Ci/mmol was purchased from New England Nuclear Corporation (Boston, Mass.). Phase combining system (PCS) liquid scintillation fluid was from Amersham (Arlington Heights, Ill.). All other chemicals were analytical grade.

Preparation of tissue. Autopsy material was used in this study. The interval between death and refrigeration at 4°C was no longer than 3 hr. Death autopsy interval was no longer than 24 hr. The tissue was dissected at the time of autopsy and stored at −70°C prior to assay. The tissue was routinely homogenized in 5 vol of 0.15 M KCl containing 0.2% Triton X-100 and 2 m*M* mercaptoethanol. Where higher sensitivity was required, tissue was homogenized in 2 vol. The homogenate was centrifuged 27,000*g* for 30 min at 4°C. The supernatant was either assayed immediately or dialyzed overnight against 1 m*M* Na-phosphate buffer (pH 7.7) containing 2 m*M* mercaptoethanol (17).

Assay procedure. The assay was carried out in 12-ml plastic conical tubes (Sarstedt, Princeton, N.J.). The standard incubation mixture, in a total volume of 250 μl, consisted of the following components: 0.195 nmole containing 2.5 μCi of *S*-[*methyl*-^{3}H]-adenosyl-L-methionine, (taken to dryness with a stream of N_2 gas prior to assay); 180 μl of 5 m*M* Na-phosphate, pH 7.9; 10 μl of 1 *M* KCN, pH 7.9; 10 μl of 1.9 m*M* norepinephrine in 0.126 *M* Na ascorbate; and 50 μl of dialyzed supernatant. Blanks contained 10 μl of H_2O in place of NE. The reaction was initiated by the addition of 50 μl of supernatant and was stopped after 30 min by addition of 100 μl of 25 m*M* nonradioactive SAM in 2.0 *N* HCl as a carrier for [^{3}H]SAM in the reineckate precipitation. This was followed immediately by 1.0 ml of ice-cold, saturated aqueous solution of reinecke salt (0.2 *N*). The 25 m*M* SAM and solutions of NE and Epi were prepared immediately prior to use because of instability of these compounds (20). The assay tubes were allowed to stand in ice for 5 min then filtered thru 0.2-μm filters (Gelman No. 4192; Gelman Inc., Ann Arbor, Mich.). The supernatant liquid was decanted into clean tubes and 5 ml of 0.5 *M* K-phosphate, pH 9.3, containing 30 mg $Na_2S_2O_5$, 100 mg Na_2 EDTA, 1 μg Epi, and 125 μg of Na ascorbate was added. The final pH of this mixture was 8.5. After checking the pH, immediately 200 mg of alumina was added to the mixture which was then shaken on a chemistry mixer Model 346 (Fisher, St. Louis, Mo.) for 10 min. The alumina was washed three times by shaking 10 sec with 10-ml aliquots of H_2O. To reduce radioactivity in the blanks the plastic caps were changed and the alumina was eluted with 3 ml 0.1 *N* perchloric by shaking on the mixer for 15 min. Two milliliters of each eluate was added to 15 ml of PCS counting fluid and the radioactivity content determined using a Beckman scintillation spectrometer.

Measurement of epinephrine. Experiments were performed to determine the recovery of Epi from alumina under various conditions and to identify it as the product of the reaction. In these experiments high-pressure liquid chromatography was used to measure Epi by a method previously described (21). The mobile phase contained 1% acetic acid, 5 m*M* heptane sulfonic acid, 1 m*M* EDTA with pH adjusted to 3.0, run at a flow rate of 1 ml/min through a C18 reverse-phase column (25 × 4.6 cm i.d.) packed with 5 μ*M* ultrasphere ODS. The electrochemical detector potential was set at 0.74 V. Enzyme activities were corrected for recovery of Epi from alumina under optimum conditions.

Results and Discussion. The concentration of PNMT in various regions of brain (2–9) is several hundred times less than that of the adrenal medulla (1). Previously described assays for PNMT in the adrenal medulla (1, 18, 19) are not useful in brain because they lack sensitivity due to substances (9, 12) which afford excessively high and variable blanks. A sensitive and specific assay is therefore required to measure brain PNMT. Previous radiometric and HPLC-EC assays both demonstrate the presence of PNMT in regions of high PNMT activity in human brain (2–6). However, even with the most sensitive of these methods no significant amounts of PNMT have been reported in specific regions of human cerebral or cerebellar cortex (2, 4–6). We have sought to maximize both the specificity and sensitivity

of the PNMT assay to determine if this marker enzyme for the neutrotransmitter Epi could be detected in these regions.

Previous assays have utilized alumina columns to adsorb the product, Epi, to make the assay more specific, however, the recovery of Epi from the columns has been low (9). We tested a number of conditions including amount and type of alumina, adsorption time, and normality of the eluting PCA to determine the optimum conditions for recovery of Epi. Under the optimum conditions, we experienced 73% recovery of Epi, a 50% increase over that of previous PNMT methods (9). To confirm that the product of the reaction was only Epi, a portion of each eluate from alumina was put into vials and counted and an equal portion was injected with carrier Epi onto an HPLC column and the Epi peak collected. Results showed that 100% of the radioactive product was Epi (control: 514 ± 16 $\times 10^3$ dpm/LC vs HPLC collected: 531 ± 2 $\times 10^3$). The specificity of the assay was further demonstrated by the fact that there was no PNMT activity when dopamine, a catechol phenylethylamine, was used as substrate (our unpublished observation). This indicates that the nonspecific phenylethylamine *N*-methyltransferase does not interfere with the assay. The K_m for NE (20.8 μM) in our assay is the same as that previously reported for brain PNMT (5).

TABLE I. EFFECT OF EXTRACTION PROCEDURES ON RADIOACTIVITY REMAINING IN BLANKS

Procedure	Radioactivity in blanks (dpm/tube)
Reinecke precipitation	865,918.8 ± 256
Alumina adsorption	9,029.0 ± 36[a]
Reinecke + alumina	428.1 ± 17[a,b]

Note. Each reaction mixture contained 2.5 μCi (5.55 $\times 10^6$ dpm) of [^{3}H]SAM in a cocktail, 50 μl of dialyzed supernatant of one locus coeruleus and 10 μl of H_2O in place of NE. The tubes were incubated at 37°C for 30 min. The reactions were stopped and processed as described in the text with the following exceptions. Aliquots of supernatant from the "reinecke tubes" were counted after centrifugation without the addition of pH 9.7 buffer or alumina. In tubes where alumina adsorption only was used, the reaction was stopped with pH 8.5, 0.5 *M* K-phosphate buffer and immediately adsorbed onto alumina. Results are expressed as the means of two assays ± SE.

[a] $P < 0.001$ vs reinecke.

[b] $P < 0.001$ vs alumina.

TABLE II. PNMT ACTIVITY IN VARIOUS REGIONS OF HUMAN BRAIN

Brain region	PNMT activity (pmole/hr/g)
Inferior frontal gyrus	39.3 ± 1.80
Middle frontal gyrus	23.3 ± 0.02
Superior frontal gyrus	19.1 ± 1.71
Amygdala	50.5 ± 3.13
Hippocampus	25.7 ± 2.47
Cerebellum	40.9 ± 1.91

Note. Tissue from various regions of human brain was homogenized in 2 vol of 0.15 *M* KCl, 0.2% Triton X-100, 2 m*M* mercaptoethanol; the homogenates were centrifuged and dialyzed. Aliquots of 50 μl were assayed for enzyme activity as described in the text. Results are expressed as the mean of two assays ± SE.

To increase the sensitivity of the assay we combined selective adsorption of Epi onto alumina (22) with the selective precipitation of [^{3}H]SAM by reinecke salt to reduce nonspecific adsorption of [^{3}H]SAM to alumina (19, 20). Results in Table I indicate that reinecke salt alone removes 84% of the radioactivity due to nonspecific adsorption of unreacted [^{3}H]SAM in the blank. The alumina extraction eliminates 99.84% and the combination of the two procedures eliminates 99.99% of unreacted [^{3}H]SAM or its metabolites (12) from the blank. While the addition of reineckate provided only a small (0.15%) improvement this was important for the sensitivity of the assay. This low blank together with the use of high specific activity [^{3}H]SAM gives the assay a sensitivity (i.e., twice the blank value) of 30 fmole (Table I). This is 10–65 times the reported sensitivity of previous brain PNMT assays (13–17). Because dialysis, rather than large homogenizing volumes, was used to remove potential inhibitors from the supernatant prior to assay, tissue could be homogenized in as low as 2 vol (Table II) instead of 5–30 vol. This increases the effective sensitivity by another 2.5- to 15-fold over other assays (4, 5, 6, 16). The overall sensitivity of the assay is at least 100 times that of the most sensitive assays previously used to measure PNMT in human brain (2, 4–6). In addition the low standard errors associated with the blanks (Table I) provide evidence that the procedure

is highly reproducible. Other radiometric assays have reported fourfold variation in blank values, which necessitated running individual blanks for each sample of low activity analyzed (4). The low, reproducible blanks in the present assay will allow measurement of PNMT in regions of low enzyme activity using a single blank.

Further improvement in this assay was achieved by employing procedures which increase the amount of [^{3}H]Epi formed. High speed centrifugation of the homogenate to remove the catabolic enzyme, the mitochondrial monoamine oxidase (MAO), (18) provided a 3.3-fold apparent increase in PNMT (Table III). Addition of the MAO inhibitor harmaline to the reaction mixture did not further enhance PNMT (our unpublished observation). Dialysis increased the apparent enzyme activity by twofold over that measured in the undialyzed supernatant fraction (Table III). This enhancement in activity by dialysis may have been due to either removal of inhibitors (18) or removal of tissue SAM which would dilute the [^{3}H]SAM (22). The pH optimum, 7.9, determined using this assay, was the same for brain as previously reported for adrenal medullary PNMT (1, 18). This assay can be completed in approximately 3 hr.

Under optimum conditions, the rate of Epi formation by human LC PNMT was linear for 30 min at 37°C (Fig. 1). PNMT activity could be measured in a little as 1 mg (wet wt) of LC and was linear up to 15 mg of tissue

TABLE III. EFFECT OF CENTRIFUGATION AND DIALYSIS ON PNMT ACTIVITY IN HUMAN LOCUS COERULEUS

Source of enzyme	PNMT activity (pmole/hr/g)
Homogenate	20.6 ± 0.31
Supernatant	69.3 ± 0.55[a]
Dialysate	137.9 ± 2.04[b]

Note. One locus coeruleus was homogenized in 5 vol of 0.15 *M* KCl, 0.2% Triton X-100, 2 m*M* mercaptoethanol. PNMT activity was determined on untreated homogenate, a 27,000 *g* supernatant fraction and a 27,000*g* supernatant fraction dialyzed overnight against 1 m*M* PO_4, 2 m*M* mercaptoethanol (pH 7.7) as described in the text. Results are expressed as the mean of two assays ± SE.

[a] $P < 0.001$ vs homogenate.

[b] $P < 0.005$ vs supernatant.

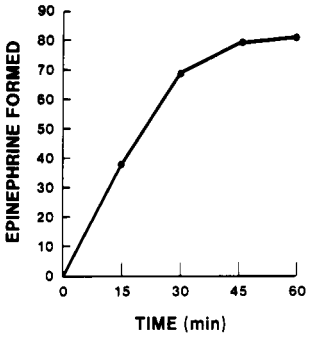

FIG. 1. Rate of epinephrine production by PNMT. One pair of locus coerulei was homogenized in 5 vol of 0.15 *M* KCl, 0.2% Triton X-100, 2 m*M* mercaptoethanol; the homogenate was centrifuged and dialyzed. Aliquots of 50 μl were taken for measurement of enzyme activity as described in the text. The amount of epinephrine formed (pmole/g of tissue) is plotted against time.

(Fig. 2). To further demonstrate the sensitivity of the assay we measured PNMT in regions of human brain where, with other assays, no significant enzyme activity had been reported (2, 4–6). PNMT activities in the human frontal cortex, amygdala, hippocampus, and cerebellum are listed in Table II. These results demonstrate the presence of PNMT in specific regions of the human cerebral and cerebellar cortex. Because PNMT is the marker enzyme

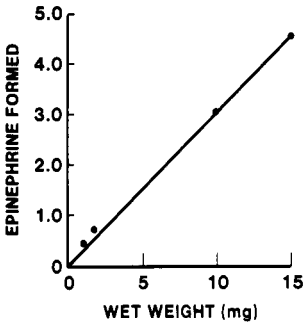

FIG. 2. The linearity of the PNMT assay in locus coeruleus extracts. One locus coeruleus was homogenized in 5 vol of 0.15 *M* KCl, 0.2% Triton X-100, 2 m*M* mercaptoethanol. The homogenate was centrifuged and dialyzed. Increasing amounts of dialyzed supernatant (equivalent to 1–15 mg of tissue) were added to 275 μl of reaction mixture and assayed for PNMT as described in the text. The picomoles of epinephrine formed/hr in the 275 μl reaction mixture is plotted against the amount of tissue added.

for the neurotransmitter Epi (11), these results imply a greater function for this neurotransmitter in human brain than was previously known.

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