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AN ENZYME ASSAY FOR NOREPINEPHRINE IN BRAIN TISSUE

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Abstract—An enzymatic assay for norepinephrine has been developed in which the methyl-^{14}C group of S-adenosylmethionine-methyl-^{14}C is transferred to the primary amine nitrogen of norepinephrine by the enzyme phenylethanolamine-*N*-methyltransferase. The rate of formation of the product, epinephrine-*N*-methyl-^{14}C, was linearly related to the concentration of substrate norepinephrine up to 5·0 μg/ml. The assay is specific for norepinephrine and can be used to measure this amine in as little as 10 mg of brain tissue.

THE MEASUREMENT of brain levels of norepinephrine (NE) spectrofluorimetrically requires relatively large samples of tissue as well as rather cumbersome extraction procedures. Many times the tissue is taken from animals which have been pretreated with drugs having a fluorescence spectrum similar to NE. Dependent on the methods of extraction and conversion of NE to the measured fluorescent product, other endogenous amines (e.g. epinephrine, dopamine, normetanephrine) may contribute to the fluorescence of the sample.

In view of the above difficulties with spectrofluorimetric methods, different assay systems were considered with the hopes of finding one that was more specific, more sensitive, and less restricted in application. One radiometric enzyme assay system showed some promise. The assay is based on the observations of Axelrod[1] that in the presence of phenylethanolamine-*N*-methyltransferase, the methyl-^{14}C group of S-adenosylmethionine-methyl-^{14}C is transferred to the primary amine nitrogen of norepinephrine. Upon further investigation it was found that the rate of formation of epinephrine-*N*-methyl-^{14}C is linearly related to the concentration of the substrate norepinephrine over a relatively wide concentration range, making the assay reasonable and applicable from 0·1 to 5·0 μg norepinephrine/ml.

EXPERIMENTAL

Preparation of phenylethanolamine-N-methyl transferase

All procedures were carried out at 0–5°. Rabbit adrenal gland tissue (commercially available from Pel-Freeze Biologicals, Inc., Rogers, Arkansas; adult, stripped type, individually frozen), weighing 8 g, was homogenized in 10 vol. of 0·1 M sodium phosphate buffer (pH 7·6) in a mortar and pestle with washed sea sand or fine glass beads. The homogenate was centrifuged for 1 hr at 15,000 rpm in a Spinco L-2 centrifuge with the no. 19 head. The fatty layer that floats to the top of the centrifuge tube was gently removed with a spatula, and the supernatant was poured into a

graduated cylinder and diluted to 100 ml with the 0·1 M phosphate buffer. The solution was fractionated with 0° saturated ammonium sulfate (Mann assayed, special enzyme grade) twice. The first fractionation was from 0 to 60 per cent saturation. The precipitate was taken up in 20 ml of the 0·1 M phosphate buffer. The second fractionation was from 0 to 30 per cent and 30 to 60 per cent saturation. The 30–60 per cent saturation precipitate was taken up in 2 ml of the 0·1 M phosphate buffer and dialyzed twice for 16 hr each against 1 l. of the same buffer. In most cases 20 μl of S-adenosyl-l-methionine-methyl-^{14}C/ml (0·1 mc/ml, 29·9 mg/m-mole; Nuclear Research Chemicals, Inc.) was added to the enzyme and the mixture stored at −70° in 15-μl aliquots in disposable polyethylene microcentrifuge tubes (Beckman).

Enzyme assay system. Phenylethanolamine-*N*-methyl transferase activity was determined by incubating the enzyme with NE and S-adenosylmethionine-methyl-^{14}C. The epinephrine-methyl-^{14}C formed was isolated by chromatography and measured by liquid scintillation spectrometry.

Preparations of brain samples. Individual mouse brains were weighed and then homogenized with 500 μl of an ice-cold homogenizing solution containing 0·2 N perchloric acid, 1 mM MO 911, 1 mM pyrogallol, and 1 mM Na_2 EDTA. When discrete areas of the brain were analyzed, whole brains were placed under a dissecting microscope in a petri dish surrounded with crushed ice. The brains were kept moist by occasionally applying a few drops of ice-cold saline. The area of interest was excised as rapidly as possible and transferred to microhomogenizers (Micro-Metric Instrument Co.). The volume of the sample was roughly estimated by cubing the length of the longest dimension, and 2 vol. of the same homogenizing fluid described above were added. The whole-brain samples were large enough to homogenize with a VirTis blade homogenizer (1 min at the lowest speed in a 15-mm diameter, 50-mm high glass vessel). The smaller samples were homogenized by hand with the glass pestles supplied with the microhomogenizers. A 5-μl aliquot of the suspensions of the smaller samples was used to determine the protein.[4] The NE values of these samples are expressed in terms of micrograms per gram protein instead of milli-micrograms per gram wet weight of tissue, because the amount of tissue was too small to weigh accurately.

Either type of sample suspension was allowed to sit for 1 hr in an ice bath. Excess $MgCO_3$ was added, and each sample was mixed thoroughly with a Vortex mixer. The samples were then centrifuged at 3000 rpm for 10 min in a refrigerated Servall preparative centrifuge. The supernatant was taken for analysis. Volume corrections for the brain solids assumed 90 per cent water by weight.

Preparation of standards. A stock solution of NE containing 10 μg of norepinephrine bitartrate/ml in 0·1 mM HCl was prepared periodically. Appropriate dilutions were made with the homogenizing fluid. From this point on, the standards were treated in the same manner as tissue samples. Blanks were prepared from the homogenizing fluid alone. Standard curves were run after each batch of enzyme was prepared.

A typical reaction mixture in a microcentrifuge tube contained 15 μl of the enzyme-S-adenosylmethionine-methyl-^{14}C mixture and 15 μl of a known concentration of norepinephrine or brain extract. After 1 hr of incubation at 37°, the reaction was stopped by placing the samples in ice. A 20-μl aliquot of the reaction mixture was spotted on Whatman no. 1 chromatography paper previously spotted with 20 μl of

carrier epinephrine (1:1000 as the hydrochloride in 0·1% sodium bisulfite). The samples were air dried and immediately placed in the chromatography tanks. At least one standard sample and two blanks were run on each sheet of chromatography paper along with one to five tissue samples. The unreacted S-adenosyl-methionine-methyl-^{14}C and epinephrine were separated by ascending chromatography with 4:1 butanol and 1·0 N HCl. The epinephrine was located by alternate exposure of the dried chromatograms to NH_3 and I_2 vapors. The stained areas were cut out and placed in 4 ml of 100% ethanol and 10 ml of toluene-based phosphor for counting in a Packard Tri-Carb liquid scintillation spectrometer. The *N*-methylated products of substrates other than norepinephrine were located in similar fashion with appropriate carriers in place of epinephrine. In all cases, the radioactive product formed enzymatically corresponded in R_f value to carrier samples of the *N*-methylated products.

Chemicals. Reserpine was kindly supplied by the Ciba Pharmaceutical Co. and MO 911 (Pargyline, Eutonyl) by Abbott Laboratories. All other chemicals were obtained from commercial sources.

RESULTS

Substrate specificity

Various precursors and products of norepinephrine metabolism were examined for their ability to serve as substrates for the phenylethanolamine-*N*-methyl transferase. These include most of the normally occurring compounds such as norepinephrine itself, normetanephrine, epinephrine, metanephrine, and dopamine. The results are illustrated in Fig. 1. Norepinephrine was *N*-methylated to a greater extent and at

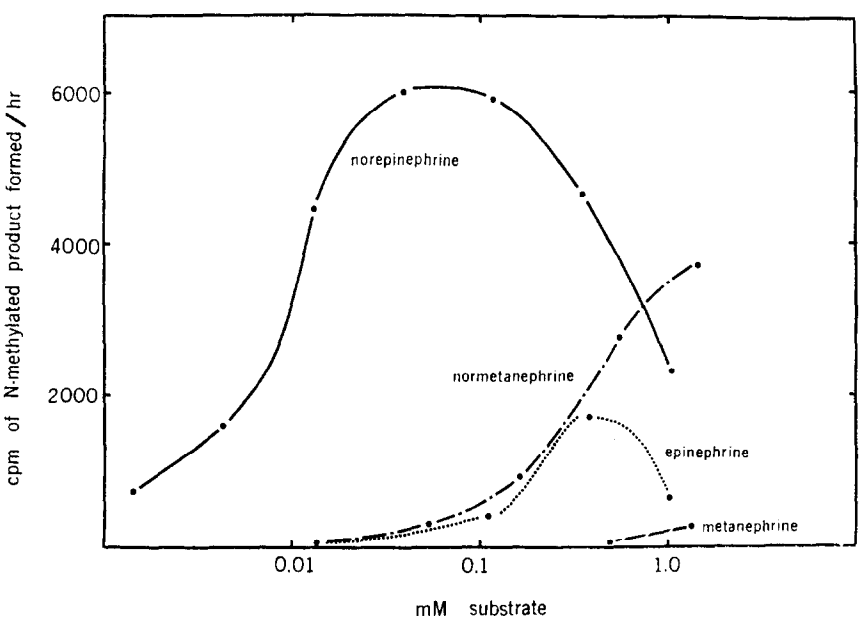

FIG. 1. Substrate specificity of phenylethanolamine-*N*-methyl transferase. Each point represents the average of five estimates.

lower concentrations than any of the other amines. Both norepinephrine and epinephrine exhibited substrate inhibition in the concentration range studied. Tissue concentrations of the amines rarely go above 20 μM. Norepinephrine is the only amine which is able to serve as substrate at that concentration level. Therefore, the enzyme specifically *N*-methylates norepinephrine in the range of interest. The Michaelis-Menten constants for the amines and for S-adenosylmethionine are shown in Table 1. The final concentration of S-adenosylmethionine-methyl-^{14}C used in the

TABLE 1. MICHAELIS-MENTEN CONSTANTS FOR VARIOUS SUBSTRATES

Substrate	K_m (M)
l-Norepinephrine	$9{\cdot}1 \times 10^{-6}$
l-Epinephrine	$2{\cdot}1 \times 10^{-4}$
dl-Normetanephrine	$1{\cdot}3 \times 10^{-3}$
dl-Metanephrine	$>10^{-2}$
Dopamine	$>10^{-2}$

assay was suboptimal (36·2 μM), since higher concentrations gave unwieldy blank values.

Enzymatic N-*methylation of norepinephrine*

Partially purified phenylethanolamine-*N*-methyl transferase was found to require no substances or cofactors other than its substrates and buffer for full activity (Table 2). Copper ions markedly inhibited its activity, suggesting the presence of essential

TABLE 2. EFFECT OF VARIOUS COMPOUNDS ON THE ENZYMATIC *N*-METHYLATION OF NOREPINEPHRINE*

Compound	Millimolar concentration				
	100	10	1 (%)	0·1	0·01
$CaCl_2$	112	112	110		
KCl	83	88	92		
$MgCl_2$	128	99	97		
$CuSO_4$	0	0	1		
Pyrogallol			99	96	101
MO 911			100	90	101
Reserpine			94	105	104

* All values expressed as per cent of control and represent the average of three estimates.

sulfhydryl groups on the enzyme. Pyrogallol, MO 911, and reserpine also did not affect enzyme activity and therefore would not be expected to influence artifactually the measurement of NE in tissue of animals treated with these drugs.

The high affinity of NE for the enzyme as shown by the low K_m suggested that the enzyme might be used for the measurement of this substrate. Another desirable aspect was the linear relationship between the rate of formation of epinephrine and the concentration of substrate NE from 0 to 5 μg/ml. Figure 2 shows typical results with 1:1 serially diluted norepinephrine bitartrate.

Recovery of norepinephrine added to tissue samples

In order to be sure that NE was not destroyed or preferentially bound to the precipitated proteins during the preparation of tissue samples, the recovery of known amounts of NE added to tissue samples was estimated. A homogenate was prepared

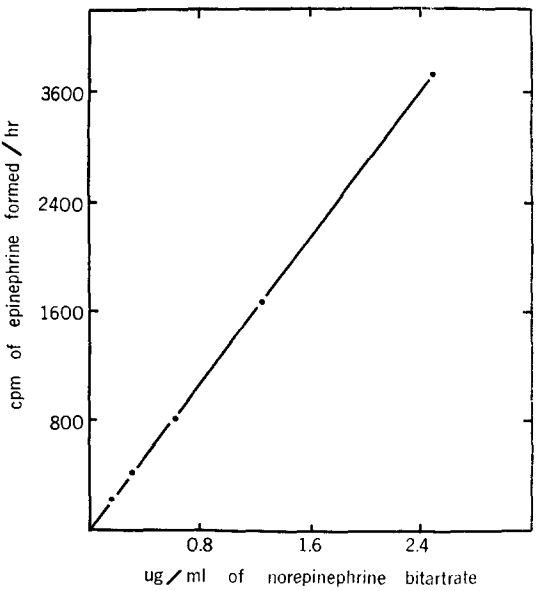

FIG. 2. Typical calibration curve for enzymatic assay system. Each point represents the average of three estimates.

with four mouse brains and 2 ml homogenizing fluid and mixed or compared with amounts of NE within the same order of magnitude of the NE known to be found in brain tissue. The results are shown in Table 3. The cpm of ^{14}C-epinephrine formed are expressed per 10 μl of sample, since only 10 of the 20 μl spotted on the chromatogram are derived from the sample itself. The other 10 μl are derived from the

TABLE 3. RECOVERY OF NOREPINEPHRINE ADDED TO TISSUE SAMPLES

Sample	Homogenate (ml)	NE* Added in 0·5 ml (μg)	Homogenizing solution added (ml)	^{14}C-Epinephrine formed/10 μl† (cpm)	Recovery (%)
1	0·5		0·5	198	
2		0·5	0·5	257	
3		1·0	0·5	541	
4		2·0	0·5	1070	
	sum of sample 1 plus sample 2			455	
5	0·5	0·5		459	101
	sum of sample 1 plus sample 3			739	
6	0·5	1·0		772	104
	sum of sample 1 plus sample 4			1268	
7	0·5	2·0		1459	115

* NE = norepinephrine bitartrate.

† Each value represents the average of three estimates.

enzyme–tracer solution. The recovery was 100 per cent for all practical purposes. Therefore, no correction for recovery in tissue samples was necessary.

Norepinephrine content of discrete areas of brain

The method has been applied to discrete areas of the mouse brain. The major problem here was in the clean dissection and rapid excision of the samples, not in the assay. The results in Table 4 represent preliminary data and may change after more

TABLE 4. NOREPINEPHRINE CONTENT OF DISCRETE AREAS OF MOUSE BRAIN*

Area	(μg NE/g protein)
Cerebellum	3·49
Lower brain stem (exit of cranial nerves)	7·39
Upper brain stem (including pons and tegmentum)	6·26
Hypothalamus (anterior and posterior)	13·2
Olfactory bulb	11.2

* Each value represents the average of two or more estimates of samples taken from the left half of one mouse brain.

experience in dissection and better knowledge of exact location of nuclei are accumulated. As in most other mammals, the hypothalamus was the highest and the cerebellum was the lowest. The olfactory bulb was also quite high.

Effect of drugs

Table 5 shows the effects of two known drugs on whole-brain NE as measured by the enzyme system. Reserpine (5 mg/kg) caused severe depletion of whole-brain NE.

TABLE 5. EFFECT OF TWO DRUGS ON WHOLE-MOUSE BRAIN LEVELS OF NOREPINEPHRINE*

Drug	(mμg/g NE)
Control	621, 629, 583, 532, 604, 591
MO 911 (200 mg/kg; killed 4 hr after injection)	940, 801, 834, 869
Reserpine (5 mg/kg, killed 18 hr after injection)	225, 265, 130, 189
Control (spectrofluorimetric)†	620, 720, 630, 630, 820, 770, 700, 690, 640, 620

* Assays were performed on individual whole mouse brains. Each figure represents the average of five estimates.

† Obtained by the method of Shore and Olin[2] as modified by Mead and Finger.[3]

However, the levels were still measurable. MO 911 (200 mg/kg) caused an increase in whole-brain NE. The lack of direct effect of these drugs of phenylethanolamine-*N*-methyl transferase has already been established (see Table 2).

DISCUSSION

The two main requirements for an assay, specificity and sensitivity, have been fulfilled.

Phenylethanolamine-*N*-methyl transferase will catalyze the transfer of methyl-^{14}C groups only to norepinephrine in the concentration range of interest. Physiological concentrations of dopamine, metanephrine, normetanephrine, and epinephrine do not interfere. This is particularly useful in studies of NE levels of specific brain nuclei which have high concentrations of precursors (e.g. dopamine in the caudate nucleus) or when drugs have been used which alter the brain levels of precursors and metabolites of norepinephrine. Spectrofluorimetric assays require extensive sample manipulation before NE alone can be measured. Because the assay system is done on a microscale, the NE content of as little as 10 mg of brain tissue can be measured (approximately 0·001 μg). It must be understood, however, that this is because such a small sample volume (15 μl) is required. The concentration of NE in that volume must be at least 0·1 μg/ml. Spectrofluorimetric methods usually require a 1-ml sample volume with roughly the same concentration of NE.

The ease of sample preparation is clearly an advantage. The tissue is homogenized in acid, the pH adjusted with solid $MgCO_3$, and the supernatant taken for analysis after centrifugation. The lack of effect of Ca^{++}, K^{+}, and Mg^{++} is convenient, as these ions are endogenous in most biological tissue. Heavy metal ions such as Cu^{++} are sequestered by the high concentration of Na_2 EDTA in the homogenizing buffer. Although monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT) are destroyed by the 0·2 N perchloric acid, pyrogallol and MO 911 were added as an extra precaution to inhibit metabolism of the NE during homogenization. The enzyme preparation also contained varying amounts of MAO and COMT. Without pyrogallol and MO 911, NE estimates were about 40 per cent lower and extremely variable.

Control values of whole-brain NE were slightly lower than the values obtained for total catecholamines with a spectrofluorimeter. This was to be expected from the greater specificity of the enzyme system for NE. The spread of values around the mean was approximately the same for the two methods, as can be seen in Table 3. Reserpine- and MO 911-treated mice had greater variations in whole-brain NE than had control mice.

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