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CATECHOL-*O*-METHYLTRANSFERASE—I

AN ENZYMATIC ASSAY FOR CARDIAC NOREPINEPHRINE

B. NIKODIJEVIC,* J. DALY and C. R. CREVELING

Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U.S. Department of Health, Education and Welfare, Bethesda, Md. 20014, U.S.A.

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Abstract—Nanogram quantities of norepinephrine from heart tissue were assayed enzymatically by using purified catechol-*O*-methyltransferase and S-adenosylmethionine-methyl-^{14}C. Conditions for extraction of tissues and incubations were optimized. The product of the enzymatic reaction, normetanephrine-methyl-^{14}C, was extracted and measured by scintillation spectrometry. Cardiac norepinephrine levels were measured by this method in rat and guinea pig, and also in mouse, both before and after treatment with drugs that alter levels of norepinephrine. The results were in agreement with those obtained by the standard fluorometric assay for norepinephrine. It is suggested that, in combination with thin-layer separation of the *O*-methylated products, the method may be used for the simultaneous assay of dopamine, norepinephrine and epinephrine.

BIOGENIC amines such as serotonin, histamine, epinephrine, norepinephrine and dopamine are present in a variety of tissues and have important physiological roles. Frequently such amines occur in picomolar concentrations and thus inherently sensitive biological or fluorometric techniques have been required for their assay. Radioisotopic techniques in conjunction with specific methyltransferase enzymes have now been developed which also provide sufficient sensitivity for the assay of certain biogenic amines. For example, histamine has been measured by using a double labeling technique with S-adenosylmethionine-methyl-^{14}C and histamine-*N*-methyltransferase.[1] Norepinephrine (NE)[2] and octopamine* have been assayed by using S-adenosylmethionine-methyl-^{14}C and phenethanolamine-*N*-methyltransferase followed by paper chromatographic separation of the products.

The use of catechol-*O*-methyltransferase (COMT) with S-adenosylmethionine-methyl-^{14}C for the assay of the catecholamines, dopamine, epinephrine and NE would also appear feasible. A number of reports have appeared [3–6] in which COMT and S-adenosylmethionine-methyl-^{14}C have been used to measure microsomal formation of catechols from phenols, including the formation of dopamine from tyramine and NE from octopamine.[3, 4] The measurement of NE in human plasma and urine by a double labeling technique utilizing COMT and S-adenosylmethionine-methyl-^{14}C

* Present address: Department of Pharmacology, Medical Faculty, University of Skopje, Skopje, Yugoslavia.

* J. Axelrod and P. Malinoff, personal communication.

has now been reported.[7] The present communication describes application of COMT and S-adenosylmethionine-methyl-^{14}C to the assay of NE in mouse heart. A preliminary report of these studies has appeared.[8]

MATERIALS AND METHODS

S-Adenosylmethionine-methyl-^{14}C (55·0 mc/m-mole) was obtained from New England Nuclear Corp. in solution in dilute sulfuric acid, pH 4·0, and was then diluted with water to a concentration of $1{\cdot}8 \times 10^{-4}$M and stored at −20°. DL-Norepinephrine-7-^{3}H (5 mc/μmole) was obtained from New England Nuclear Corp.

COMT was purified 237-fold from rat liver by a modification[9] of the procedure of Axelrod and Tomchick.[10] The COMT used in this study was obtained after elution from calcium phosphate gel and had a specific activity of 0·2–0·7 μmole/mg/20 min for the conversion of dihydroxybenzoic acid to its *O*-methylated product.

Preparation of tissue extract. Hearts were removed, blotted, frozen on dry ice, weighed and homogenized in 10 vol. of cold, 5% trichloroacetic acid in a conical, ground-glass homogenizer. After standing for 15–30 min in ice, the homogenates were centrifuged (15,000 *g*, 10 min). An aliquot (0·5–2 ml) of supernatant fluid was removed and extracted four times with 10 vol. of ether (presaturated with water) in a conical, shaking tube. Each ether extract containing the trichloroacetic acid was removed by aspiration and, after the final extraction, traces of ether were removed by bubbling nitrogen through the aqueous fraction for 15 min. This aqueous fraction referred to as the extract, had a final pH of 5·7–6·4 and contained the equivalent of 100 mg tissue per ml.

Incubation with COMT. The extract (0·2 ml) was incubated for 20 min at 37° in a reaction mixture containing the following components (in micromoles); $MgCl_2$, 1; S-adenosylmethionine-methyl-^{14}C, 0·018 (180,000 cpm); purified COMT, 5–10 mg; Tris buffer, pH 9·1, 50; in a final volume of 0·5 ml. A duplicate sample was incubated containing an internal standard of 0·1–0·3 nmoles of authentic NE. The reaction mixtures were always prepared in ice with Tris buffer as the last addition. Blanks were prepared either by addition of 250 μg ferric chloride or by preincubating the extract for 10 min with Tris buffer. Either of these treatments destroys the norepinephrine present in the extract. The reaction was stopped by the addition of 0·5 ml of 0·5 M borate buffer, pH 10, and 2·5 μmoles of carrier normetanephrine (NMN) was added. The reaction mixture was washed with 10 ml benzene and was then extracted with 10 ml of a 3:7 (v/v) mixture of *iso*amyl alcohol–toluene. A 5-ml aliquot of the *iso*amyl alcohol–toluene extract was added to 10 ml of Bray's phosphor solution and radioactivity was determined by scintillation spectrometry with a counting efficiency of 38 per cent.

RESULTS

An example of the data obtained by this method is shown in Table 1. Each of the values presented in this table is a mean obtained from eight determinations on an identical sample of heart extract. The observed deviation in each of the three types of values obtained in the assay is small with excellent reproducibility. In practice, a single blank, usually with ferric chloride, was used.

Recovery of NE from tissue and throughout the assay. The efficiency of NE extraction from heart tissue was determined by the recovery of NE-^{3}H from the hearts of mice

prelabeled through the intravenous administration of 10 μc NE-^{3}H 1–3 hr prior to sacrifice. The contribution of tritiated metabolites to the total radioactivity in heart tissue was less than 5 per cent at 1 hr and essentially zero at 3 hr.[11] The radioactivity in the heart homogenate was taken as 100 per cent and compared to the activity found in the supernatant fluid. The extraction obtained with cold 5% trichloroacetic acid was 100 $\pm$ 7 per cent. Other extraction solvents, such as *n*-butanol, methanol and ethanol, gave less efficient extraction of the NE-^{3}H. Efficient extraction was obtained with mineral acids such as hydrochloric and perchloric acids, but it was difficult to remove the acid without large losses of NE. The recovery of NE-^{3}H in the aqueous phase after removal of the trichloroacetic acid by extraction into ether was 98 $\pm$ 8 per cent. Similar recoveries were obtained after the addition of NE-^{3}H directly to the crude homogenate. Thus the extract presented to the enzyme contained. essentially all of the NE from the tissue. The enzymatic conversion of NE to NMN proceeded to 15–25 per cent completion under the conditions of the assay. The efficiency of the final extraction of NMN from the reaction mixture into toluene–*iso*amyl alchohol was 88 $\pm$ 5 per cent. Thus the overall recovery of NE-^{3}H as NMN throughout the entire assay procedure was 10–20 per cent.

TABLE 1. ENZYMATIC ASSAY OF NOREPINEPHRINE*

	Radioactivity extracted (cpm)
A. Heart extract	1130 $\pm$ 18
B. Heart extract + NE (0·25 n-mole)	2800 $\pm$ 150
C. Heart extract + $FeCl_3$	433 $\pm$ 32
D. Heart extract + preincubation (15 min) at pH 9·1	435 $\pm$ 40

* Heart extract was obtained from the hearts of ten animals and 0·2 ml or the equivalent of 20 mg tissue was assayed as described in Methods. Each value, expressed as cpm/5 ml of toluene–*iso*amyl alcohol extract, is the mean obtained from 8 identical incubations. Tissue level of NE was calculated from the above data as follows:
[(A–C) ÷ (B–A)] 0·25 nmole NE = nmoles NE/20 mg heart.

Linearity and time course of the reaction. The reaction was linear with incubation time for 20–25 min, as illustrated in Fig. 1. Variations in the amount of enzyme and other components or the inclusion of ascorbic acid in the reaction mixture did not increase the linear time course.

The conversion of authentic NE to NMN was directly proportional to substrate concentration from 0·05 to 0·3 nmoles, as shown in Fig. 2. A similar linear relationship was obtained with 0·05- to 0·3-ml aliquots of heart extract. However, as illustrated in Table 2, the extract partially inhibited the *O*-methylation of added NE. Therefore, an internal standard was necessary to obtain quantitative measurements. The inclusion of ascorbic acid, mercaptoethanol or α,α-dipyridyl in the reaction mixture did not reverse this inhibition. When the extract was heated for 10 min, 60 min and 18 hr prior to incubation, subsequent inhibition was reduced only slightly, suggesting a heat-stable inhibitor in the extract.

Increasing the concentration of isotopically labeled S-adenosylmethionine 2- to 5-fold resulted in only a minimal increase in conversion. The reaction rate was

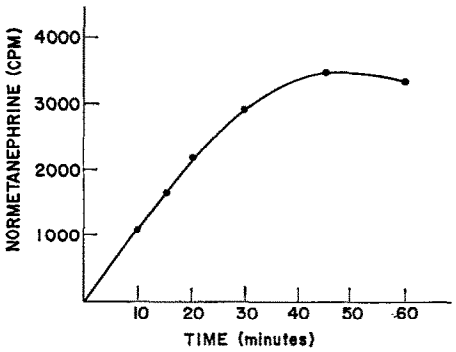

FIG. 1. NMN formation from NE (0·25 nmole) at incubation times from 10 to 60 min. Incubation conditions and assay as described in Methods.

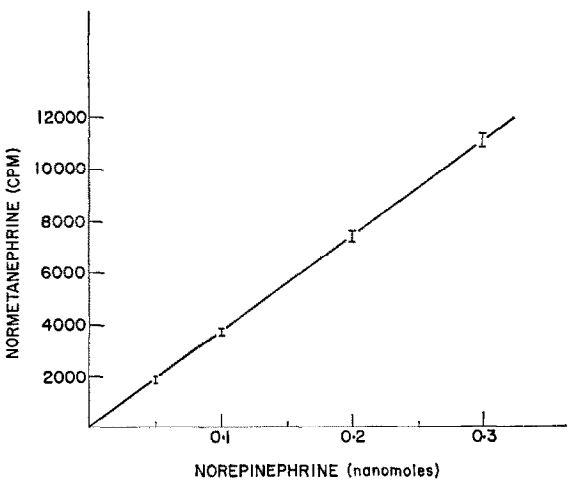

FIG. 2. Rate of NMN formation with NE concentrations from 0·05 to 0·3 nmoles. Incubation conditions and assay as described in Methods.

TABLE 2. ENZYMATIC ASSAY OF NOREPINEPHRINE: INHIBITION BY HEART EXTRACT*

Extract	Normetanephrine formed (cpm)			
	Extract alone	Extract + standard (0·2 nmole NE)	Δ	% Inhibition
0·0	—	7600	—	0
0·1	970	6260	5290	30
0·2	1940	4590	2650	65
0·3	2910	3650	1740	76

* Values, expressed as cpm/5 ml of toluene–isoamyl alcohol extract minus the blank value, were obtained from incubations of heart extracts equivalent to 10, 20 and 30 mg tissue. Incubation conditions as described in Methods.

proportional to enzyme concentration over a very short range, as shown in Fig. 3. A 10-fold increase in enzyme concentration gave no further stimulation of the reaction rate.

The nature of the ionic medium and the pH, as shown in Fig. 4, had a marked effect on the reaction rate. A 3-fold increase in rate was observed at pH 9·1 in Tris buffer over that with phosphate buffer at pH 7·8. This change in rate was accompanied by a change in the apparent K_m from $2{\cdot}6 \times 10^{-4}$ M to $7{\cdot}2 \times 10^{-5}$ M. NE was stable in the presence of the enzyme preparation at pH 9·1, as indicated by the linearity of the reaction, but in the absence of the protein NE was rapidly destroyed at this pH (Table 1). The protection of NE afforded by the enzyme preparation was evident even at pH 11·0.

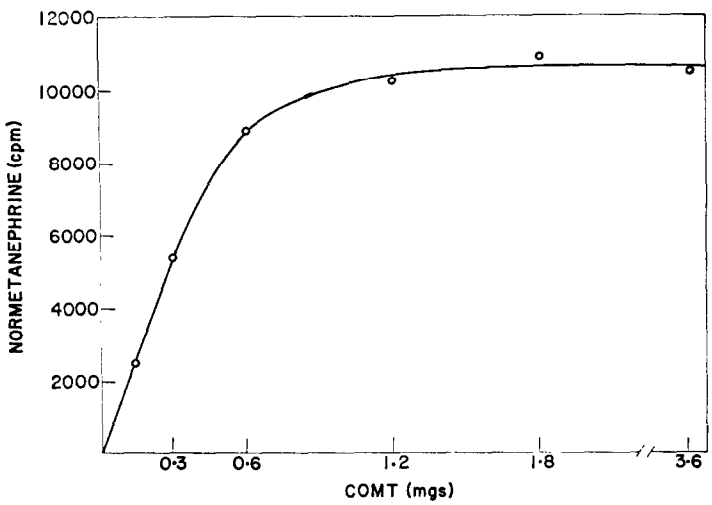

FIG. 3. Formation of NMN with COMT concentrations from 0·3 to 3·6 mg. Incubation conditions and assay as described in Methods.

The formation of the product, NMN, was demonstrated by thin-layer chromatography, as shown in Fig. 5. The major radioactive peak migrated with authentic NMN in several solvent systems. The identity of the material present in blank was not established.

Tissue levels of NE. A comparison of measurement of heart content of NE in several species by this enzymatic assay and the more common fluorometric procedure[12] is presented in Table 3. In the mouse and rat the levels measured were the same, while in the guinea pig the enzymatic method gave a consistently higher result, suggesting the presence of epinephrine or dopamine. A similar comparison was made after drug-induced alterations in the level of NE in mouse heart. As shown in Table 4, both methods gave similar values after drugs which had either lowered or increased the level of heart norepinephrine.

DISCUSSION

This enzymatic assay permits an accurate and sensitive measure of NE in heart tissue. The conditions for the enzymatic reaction are linear with time and NE concentration. The conditions have been maximized with respect to enzyme, buffer and cofactor concentration, and pH. The use of an internal standard for accurate quantitation is necessary because of the presence of an inhibitor present in tissue. While this assay cannot be directly applied to tissues containing relatively large amounts of other catecholamines, such as brain or adrenal glands, it does possess certain advantages over fluorometric procedures. By using this assay, the content of NE can be determined in a single mouse heart (weighing as little as 70 mg) with the same accuracy as that obtained with larger amounts of tissue. The fluorometric assay, on the other hand, necessarily involves the isolation of NE from tissue either by extraction[13] or by alumina adsorption[12] and requires a minimum of six to ten mice for a single determination. The advantage of the enzymatic assay is of particular importance in the screening of compounds that alter NE levels where the amount of compound available might preclude the use of many animals or a larger species.

The sensitivity of this methyltransferase reaction, under the present conditions, permits the accurate measurement of as little as 0·3 ng NE. With a sensitivity of this order of magnitude, many analytical applications may be possible. As shown in Tables 3 and 4, the results obtained with the fluorometric and enzymatic *O*-methyltransferase assay are in good agreement. Preliminary experiments have now indicated

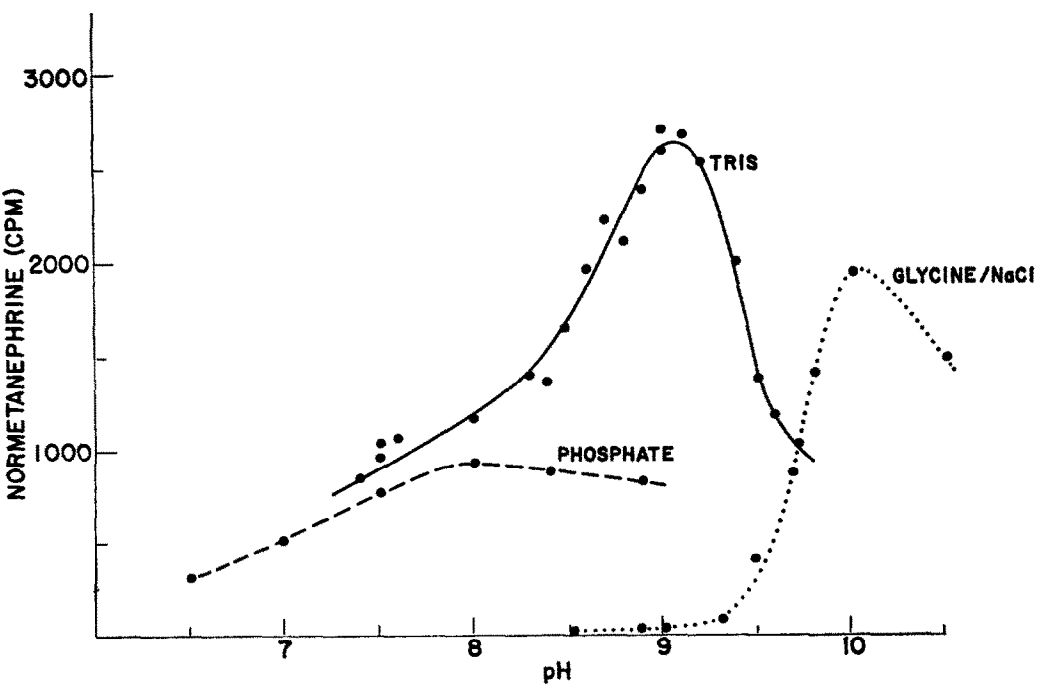

FIG. 4. Reaction rate as a function of pH and type of buffer. Buffer concentrations were 10^{-2}M and NE concentration was 2×10^{-3}M. Similar pH curves were obtained with NE concentration at 3×10^{-7}M. Incubation conditions and assay as described in Methods.

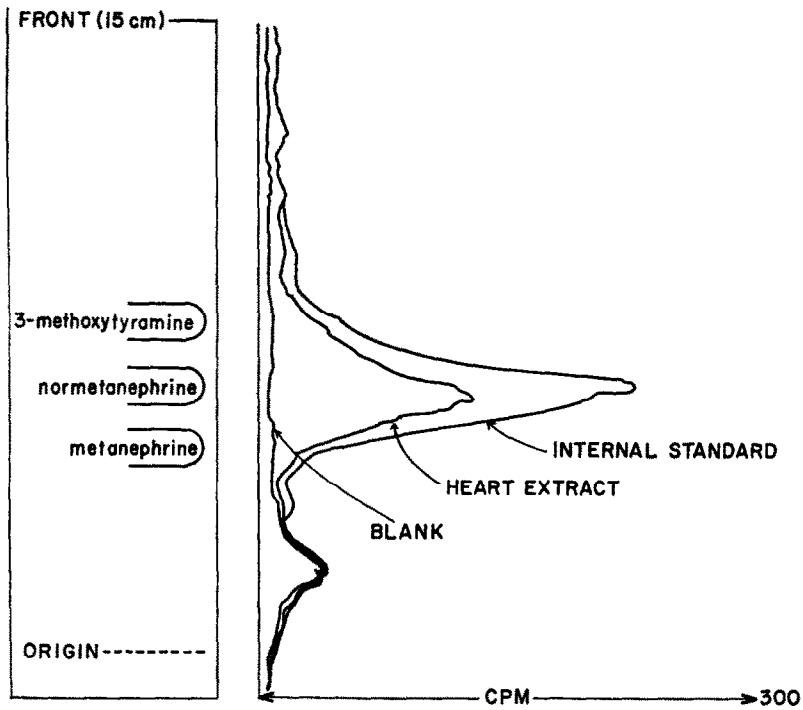

FIG. 5. Incubation mixtures, as described in Methods, were extracted with ethyl acetate–methanol (10:1; 5 ml), the extracts were dried over Na_2SO_4 concentrated with an N_2 stream and the residue was spotted on thin-layer chromatoplates of silica gel G, 250 μ thick. Plates were developed in a solvent system composed of *n*-butanol:formic acid: water: benzene (15:25:1·5:5). Carrier NMN, metanephrine and 3-methoxytyramine were visualized with Gibbs reagent and isotope distribution was obtained by using a Vanguard gas-flow scanner.

TABLE 3. ENZYMATIC ASSAY OF NOREPINEPHRINE: HEART CONTENT IN SEVERAL SPECIES*

Species	Fluorometric (μg NE/g)	Enzymatic (μg NE/g)
Mouse	0·92 ± 0·16	0·90 ± 0·15
Guinea pig	1·85 ± 0·23	2·14 ± 0·23
Rat	0·82 ± 0·13	0·86 ± 0·17

* NE content is expressed as the mean value obtained from the following animals: NIH general purpose mice (male, 20 g), fluorometric assays with five groups of ten animals and enzymatic assays with five groups of two animals; Hartley guinea pigs (male, 250 g), assays with five animals; Sprague–Dawley rats (male, 200 g), assays with five animals.

that it is possible to assay total catechols in as little as 1 ml of rabbit plasma by this procedure. In addition, by using the thin-layer system illustrated in Fig. 5 to separate the *O*-methylated products from the COMT reaction with dopamine, epinephrine and NE, the ratio of catecholamines in a single mouse adrenal gland has been determined.

TABLE 4. DRUG-INDUCED ALTERATIONS IN CARDIAC NOREPINEPHRINE*

Drug	Dose (mg/kg)	Time (hr)	Fluorometric (% of control)	Enzymatic (% of control)
Tyramine	5·0	2	57	56
6-Hydroxydopamine	5·0	2	40	41
L-Metaraminol	5·0	2	19	13
Reserpine	1·0	2	18	26
L-α-Methyltyrosine	50	2	56	53
Pargyline	100	18	—	138
Tranylcypromine	20	18	—	123
Marsilid	200	18	200	207

* Drugs were given by subcutaneous injection at the times indicated prior to sacrifice. Fluorometric assays were performed on pooled tissues from three groups of seven mice each; enzymatic assays were performed on tissues from three groups of two mice each.

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