Assay of Phenylethanolamine *N*-Methyltransferase[1] (37146)

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(Introduced by W. H. Elliott)

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The currently used assay system for the measurement of phenylethanolamine *N*-methyltransferase (EC 2.1.1.) (PNMT), the enzyme which converts norepinephrine to epinephrine, was developed by Axelrod in 1962 (1). Since that time few improvements in the assay procedure have been made. Wurtman, *et al.* (2) and Kitabchi and Williams (3) showed that 40 m*M* phosphate buffer used in the original assay is inhibitory. Addition of 2-mercaptoethanol provided enhanced activities of partially purified enzyme preparations (3).

Potential inhibitors of this enzyme include substances known to be present in the adrenal in high concentration. Among these are steroids (4), epinephrine (5), and the cations of copper and zinc (3). Since monoamine oxidase further alters the product of PNMT action, a falsely low apparent conversion of norepinephrine to epinephrine could be inferred from the presence of this catabolic enzyme.

Lack of a uniform method for removing these substances prior to assay may account for the wide variation in values reported for the activity of PNMT by various investigators (4, 6, 7). Procedures have been developed to remove potentially inhibitory substances from homogenates of adrenal gland. The effects of these procedures on the activity of adrenal PNMT are reported in this communication.

Materials and Methods. Preparation of tissue. Male rats obtained from the St. Louis University colony were killed by a blow on the head. The excised adrenal glands from one animal were homogenized in 1.8 ml of 0.15 *M* KCl containing 2 m*M* mercaptoethanol. Three types of enzyme preparation were assayed: (a) untreated homogenates; (b) soluble fraction (supernatant liquid obtained after centrifugation of homogenates at 100, 000*g* for 20 min); and (c) dialyzed soluble fraction (soluble fraction dialyzed overnight against 1 m*M* potassium phosphate buffer (pH 7.7) containing 2 m*M* 2-mercaptoethanol).

Enzyme assays. PNMT activity was measured in 50 μl aliquots by the method of Wurtman and Axelrod (4) to which modifications were added during the course of these investigations. The basal medium for each assay contained 171 nmoles DL-normetanephrine · HCl, and 1 μmole of *S*-adenosylmethionine (methyl-^{14}C) (50 μCi/μmole), in a total volume of 255 μl of 4 m*M* phosphate buffer (pH 7.9) (1/100 the concentration originally used) (4). The reaction was initiated by addition of the enzyme source. After incubation for 30 min at 37°, the reaction was stopped by addition of 0.5 ml of 0.5 *M* sodium borate buffer (pH 10). The ^{14}C-metanephrine formed was extracted into 6 ml of toluene–isoamyl alcohol (3:2, v/v). The ^{14}C in 4 ml portions of the extract was determined by use of a Packard Tri-Carb, liquid scintillation spectrometer after addition of 1 ml of absolute ethanol and 10 ml of phosphor (4 g PPO and 0.1 g POPOP in 1 liter of toluene).[2] Corrections for ^{14}C-*S*-adenosylmethionine and endogenously formed radioactive epinephrine were determined in experiments identical to those described ex-

[1] This work was supported in part by Training Grant Number GM 446 from the National Institutes of Health.

[2] Abbreviations used are: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazolyl-2)benzene; TCA, trichloroacetic acid.

cept that normetanephrine was omitted. The efficiency with which ^{14}C was determined was approximately 80%. The monoamine oxidase activity of the tissue preparations was measured by the method of Wurtman and Axelrod (8).

Copper assay. The copper concentration of various extracts of the adrenal gland was measured using glassware washed in hot nitric acid and rinsed in 6 washes of distilled, deionized water. Reagent grade chemicals and distilled deionized water were used in the preparation of all solutions. Male rats (180–200 g each) were decapitated and the adrenal glands quickly removed. Five pairs of adrenals were combined and homogenized in 10 ml of 0.15 *M* KCl. Adrenal homogenate and fractions derived from it were treated with 5% TCA to release any protein-bound copper. The protein-free solutions were treated with 1% $LaCl_3$ to precipitate phosphates which interfere with analysis of copper by atomic absorption spectrometry. Samples were analyzed using a Perkin-Elmer atomic absorption spectrophotometer, Model 303.

The standard against which samples were measured was prepared from pure copper metal dissolved in concentrated nitric acid and diluted to appropriate concentrations. Efficiency of measurement was determined using dilute copper solutions prepared in the same media as the homogenate fractions.

Effect of insulin on PNMT activity of rat adrenal. Six rats were injected with 8 units of regular insulin/day for 6 days; controls received an equal volume of isotonic saline. Animals were sacrificed on the sixth day, 6 hr after the final injection of insulin. The enzyme activity in the dialyzed soluble fraction and the catecholamine content of the whole homogenate were measured (9).

Results. In preliminary studies, a marked improvement in PNMT activity was noted with the addition of 2 m*M* 2-mercaptoethanol during homogenization of adrenal tissue. Similar stimulation in activity has previously been described for addition of 10 m*M* 2-mercaptoethanol to assays of this enzyme (3). With a view toward providing maximum activity, 2 m*M* 2-mercaptoethanol was therefore present during homogenizations and subsequent dialysis of tissue extracts.

Removal of 97% of the catecholamine deaminating enzyme, monoamine oxidase, by separating mitochondria from the remaining soluble fraction provided an insignificant improvement in PNMT activity (Fig. 1). Dialysis of the soluble fraction, however, produced a 70% greater activity of the enzyme. To determine whether the higher activity after dialysis was due to removal of inhibitory substances constitutive to adrenal gland, the effects of addition of corticosterone and epinephrine were determined. Corticosterone was added to dialyzed soluble fraction at concentrations as high as 10^{-4} *M* with no significant effect on the enzyme activity (Fig. 2). Epinephrine at a concentration of 2.5×10^{-4} *M* provided an inhibition of some 45% which was fully reversed by dialysis.

Other experiments were designed to test the effect of removal of monoamine oxidase on the activity of PNMT. In these, addition of 20 m*M* KCN to tubes in which the whole homogenate was assayed, produced only slight

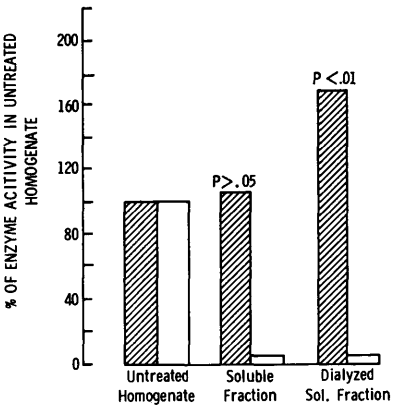

FIG. 1. Effect of treatment of adrenal homogenate: Two adrenals were homogenized in 1.8 ml of 0.15 *M* KCl. The PNMT and monoamine oxidase activities were determined on untreated homogenate, a 100,000*g* supernatant fraction, and a 100,000*g* supernatant fraction dialyzed overnight against 1 m*M* PO_4, 2 m*M* mercaptoethanol (pH 7.7). The height of each bar represents enzyme activity expressed as percentage of that activity found in the untreated homogenate; the open bars designate relative activity of monoamine oxidase and the hatched bars that of PNMT; p = probability that value obtained is not different from the control value represented by the left bar.

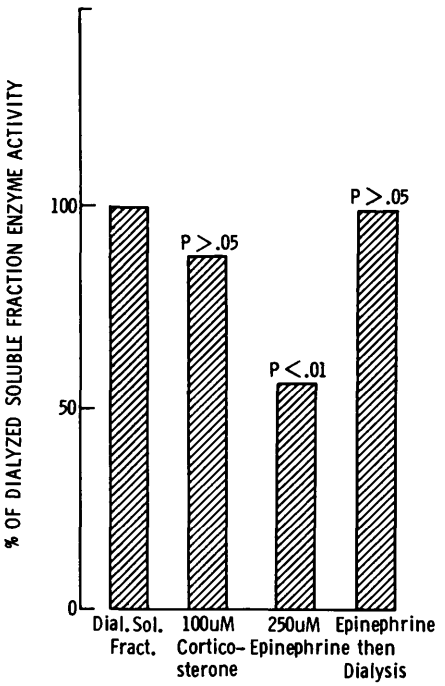

FIG. 2. Effects of inhibitors added to incubation medium: Two adrenal glands were homogenized in 1.8 ml of 0.15 *M* KCl, 2 m*M* 2-mercaptoethanol; dialyzed soluble fraction was prepared as described in the text. PNMT activity was then determined with the indicated substances added to the assay mixture. A separate experiment was performed (right bar) in which 1 m*M* epinephrine was added to the soluble fraction and this mixture was dialyzed overnight at 4° against 3 liters of 1 m*M* PO_4, 2 m*M* 2-mercaptoethanol (pH 7.7). The height of each bar represents the enzyme activity under each condition relative to 100% for the dialyzed soluble fraction; p = probability that value obtained is not different from the control value represented by the left bar.

inhibition of monoamine oxidase activity. Under the same conditions, however, an improvement of 30% was noted in the activity of the epinephrine-forming enzyme (Fig. 3). That the effect of cyanide may be due to its capacity to chelate heavy metals was indicated by its effect in reversing inhibition with 10^{-5} *M* Cu^{2+}.

The copper concentration of a typical homogenate of adrenal tissue was found by atomic absorption spectrophotometry to be 1.06×10^{-1} μg/ml (Table I). This value corresponds to a tissue level of approximately 7–10 μg/g. The copper content of the soluble fraction was somewhat less than that of the homogenate but even after dialysis, the copper concentration was 7.4×10^{-2} μg/ml.

Introduction of the modifications of homogenization and dialysis in the presence of 2-mercaptoethanol, and addition of KCN to the assay system, provided higher activities of PNMT than observed previously. Under these conditions there is a linear production of metanephrine during the first 45 min (Fig. 4). These modifications, taken together, improved the activity of the enzyme approximately twofold (9) over that reported for similar animals (4) where adrenal extracts were assayed without prior dialysis or the addition of KCN. Slightly greater precision was observed between duplicate measurements on a given biological sample assayed

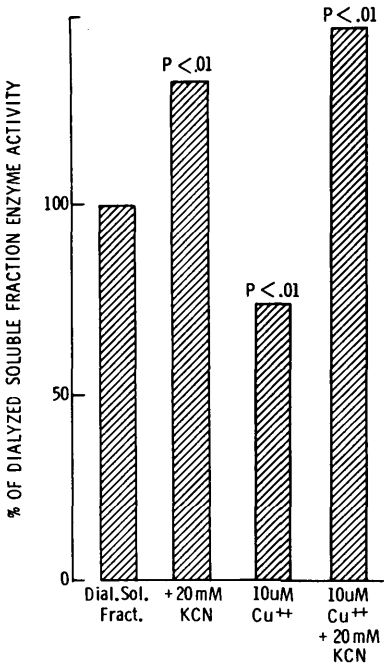

FIG. 3. Effect of KCN on dialyzed soluble fraction: Two adrenal glands were homogenized in 1.8 ml of 0.15 *M* KCl, 2 m*M* mercaptoethanol and dialyzed soluble fraction was prepared. PNMT activity was determined with the indicated substances added to the enzyme assay mixture. The height of each bar represents the enzyme activity under each of these conditions relative to 100% for the dialyzed soluble fraction; p = probability that value obtained is not different from the control value represented by the left bar.

TABLE I. Copper Concentration of Fractions of Rat Adrenal Homogenate.[a]

Fraction	Concn (μg/ml)
Whole homogenate	0.106 ± 0.01
Soluble fraction	0.069 ± 0.001
Pellet (100,000 g, 20 min)	0.062 ± 0.005
Dialyzed soluble fraction	0.074 ± 0.0005

[a] The adrenals from 3 groups of 5 rats each were homogenized in 10 ml of 0.15 *M* KCl, 2 m*M* mercaptoethanol. The homogenate was centrifuged 20 min at 100,000*g* to provide soluble fraction and pellet. A portion of the soluble fraction was dialyzed against 1 m*M* phosphate buffer, 2 m*M* mercaptoethanol (pH 7.7) overnight. Results are expressed as mean ± SE.

by the modified procedure than was obtained on a similar sample not subjected to further treatments.

To study the adequacy of the new assay system under condiitons in which one of the constitutive inhibitory substances was diminished in a group of animals, insulin, a catecholamine-releasing agent, was used. Results show that insulin caused a significant increase of 14% in PNMT activity as well as a 49% depletion of adrenal catecholamines (Table II).

Discussion. The inhibitory effect of *p*-chloromercuribenzoate (1) and the stimulatory effect of 2-mercaptoethanol (3) are evidence for the necessity of SH groups for the activity of PNMT. More recently, purified bovine PNMT has been found to contain 8 SH groups/molecule (10). In view of this evidence, it is surprising that in the routine measurement of PNMT, most investigators have not added compounds to protect thiol groups during preparation and incubation of tissue extracts.

TABLE II. Effect of Insulin on PNMT Activity in Rat Adrenal Tissue.[a]

Treatment	Enzyme activity (units/pair adrenals)	Catecholamines (μg/pair adrenals)
Control	7.94 ± 0.39	37.81 ± 1.64
Insulin	9.06 ± 0.36[b]	19.21 ± 0.65[c]

[a] Groups of six rats each were injected intraperitoneally with 8 units of insulin (zinc, crystalline, 80 units/ml) or equal volume of 0.154 *M* NaCl once per day for 6 days. Animals were sacrificed 6 hr following the final administration of insulin and the enzyme activity and catecholamine content were measured. Results are expressed as mean ± SE.

[b] $p < .05$.

[c] $p < .001$.

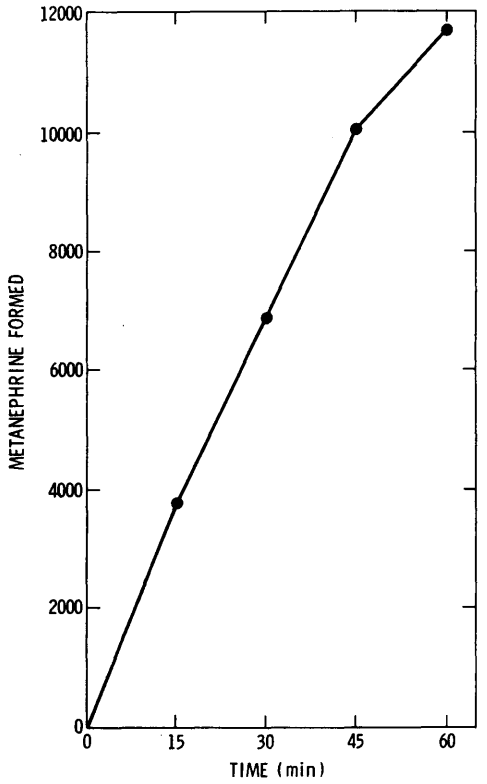

FIG. 4. Rate of metanephrine production by PNMT: One pair of adrenals from a 250 g rat was homogenized in 1.8 ml of 0.15 *M* KCl, 2 m*M*-mercaptoethanol; the homogenate was centrifuged and dialyzed. Aliquots of 50 μl were taken for measurement of enzyme activity under the normal assay conditions described in the text. The relative amount of radioactive metanephrine formed (dpm/adrenal pair) is plotted against time.

Among the potentially inhibitory substances in rat adrenal tissue, corticosterone was without effect in the enzyme assay even if added to dialyzed soluble fraction at concentrations 100 times that expected in the homogenate (11).

The apparent K_i for epinephrine on rat PNMT is 95 μM (5). From epinephrine content of rat adrenal tissue (9), one may calculate that as much as 33 μM epinephrine

might be present in incubations of undialyzed soluble fraction. Thus, one of the beneficial effects of dialysis may be the removal of this and other catecholamines. The essentiality of thiol compounds during lengthy treatments of PNMT is amply demonstrated by failure of other preparations (2) to be stimulated by dialysis in the absence of these agents.

The activity of partially purified extracts of PNMT with addition of enzyme to the incubation, have been shown to deviate from linearity (1), suggestive of simultaneous addition of bound inhibitor. An additional enhancement of activity upon addition of cyanide to dialyzed enzyme (Fig. 3) was not found in highly purified preparations of PNMT (10). This effect is in accord with the presence of a heavy metal inhibitor which is not removed entirely by dialysis in the presence of 2-mercaptoethanol but which has a high association constant with cyanide ion (12). Both copper and zinc are inhibitory (3) and both are present in relatively high concentration in adrenal tissue of rats (13). Atomic absorption spectroanalysis provided evidence that copper ion is present in extracts dialyzed overnight in the presence of mercaptoethanol.

In the intact adrenal gland compartmentation exists between the enzyme of interest and some or all of the inhibitory substances. Epinephrine, for example, is largely restricted to storage granules while the known association of copper and zinc with specific proteins suggests a nonrandom distribution of these metal ions within a cell. Homogenization disrupts compartmentation thus making inhibitors newly available to some enzyme surfaces. Misleading inferences about changes in enzyme activity could result from treatments which alter the concentrations of inhibitors without having any effect on the activity of the enzyme *per se*. For example, consider the stimulation by insulin of PNMT activity (7). Inasmuch as insulin is known to promote release of catecholamines, the effect of this hormone might alternatively be interpreted as due to diminution in concentration of an inhibitory substance. By the methods reported in this paper it was possible to show that increase in PNMT activity probably results from an increase in amount of enzyme and does not occur because of diminished levels of epinephrine or other inhibitory substance following insulin treatment.

Summary. PNMT is inhibited by a number of compounds and ions which occur in adrenal tissue. Removal of some of the inhibitors could be achieved by dialysis of tissue extracts in the presence of 2-mercaptoethanol to provide 70% stimulation in activity. Additional 30% enhancement of activity was provided by addition of 20 m*M* KCN to the dialyzed extracts. Since copper ion, a known inhibitor of the enzyme, is present in the dialyzed extracts it is likely that the effect of KCN involves removal of this and other inhibitory cations. The effect of administration of insulin in providing higher activities of PNMT has been evaluated as one of increased amount of enzyme rather than alterations in activity due to loss from the tissue of the inhibitory catecholamines.

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Received Nov. 27, 1972. P.S.E.B.M., 1973, Vol. 142.