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Modification of an enzyme radiochemical assay procedure for noradrenaline

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SAELENS, Schoen and Kovacsics[1] described an assay procedure for noradrenaline (NA) based on the transfer of radioactively labelled methyl groups from S-adenosylmethionine to unlabelled noradrenaline, catalysed by the enzyme phenylethanolamine-*N*-methyl transferase (PNMT). We have tested this method and offer some comments on the technique and its application to the assay of NA in tissue extracts. The following modified procedure is, in our hands, the most satisfactory:—

A reaction mixture is freshly prepared at the start of an experiment from ingredients stored at −20°; 100 μl bovine PNMT in 0·2 M sodium phosphate buffer pH 7·6 (protein content approx. 20 mg/ml; prepared from bovine adrenal glands according to the method of Saelens *et al.*[1]); 1 μl 2-mercaptoethanol, 5% (v/v); 25 μl EDTA disodium salt, 1% w/v; 10 μl pargyline hydrochloride, 40 mM; 10 μl pyrogallol 40 mM; 5 μl S-adenosyl methionine 1 mM; 50 μl ^{3}H-methyl-S-adenosyl methionine, 80 μc/ml (S.A. 4·2 c/m-mole, Radiochemical Centre, Amersham, England). Ten μl of this reaction mixture was added to a 0·2 ml microtube placed in an ice-water bath and followed by 10 μl of freshly prepared tissue homogenate (1:10 in ice-cold 5 mM sodium phosphate buffer pH 7·0). Internal standards of 1 μl (4 ng) of a freshly prepared solution containing 4 μg L-NA/ml were added to duplicate tubes containing tissue homogenates, and reagent blanks were prepared by substituting 10 μl of water for the tissue homogenate. After incubation at 37° for 60 min, the tubes were returned to an ice-water bath and the entire contents (20 μl) were transferred to the origin of a Whatman 3 MM paper, previously spotted with 20 μl of carrier L-adrenaline (1 mg/ml + 1% sodium metabisulphite). The chromatograms were developed overnight with *n*-butanol saturated with 1 N hydrochloric acid. The papers were air dried, and the adrenaline spots were visualized by spraying with ethanolic ammonia solution (ammonium hydroxide:ethanol 1:10 v/v), followed by 0·25% w/v potassium ferricyanide solution. The pink adrenaline spots were cut out, shredded and extracted with 4 ml of 95% ethanol in counting

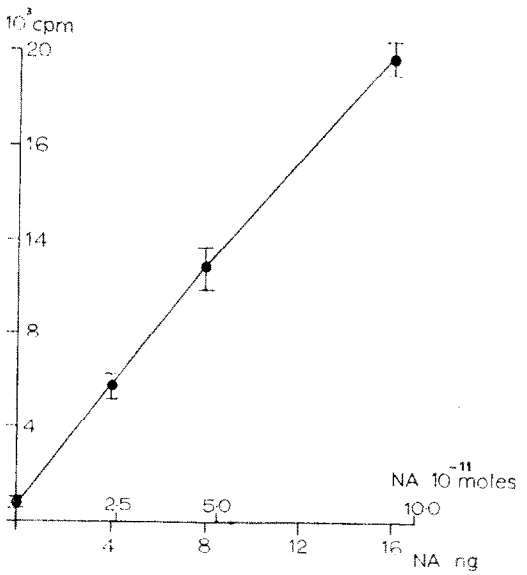

FIG. 1. Yield of radioactive adrenaline from various amounts of standard L-NA. Each point is the mean ± S.E. Mean for five determinations, except for the 16 ng standard, which is the mean ± range for two experiments.

vials. After allowing at least 4 hr for complete elution of adrenochrome, 10 ml of toluene phosphor [0·4% butyl-PBD (CIBA)] was added.

Figure 1 illustrates results obtained with NA standards indicating that the sensitivity of the method is approximately 0·5 ng of L-NA. (3×10^{-12} moles). When homogenates of vas deferens, nictitating membrane, auricle, iris or salivary glands were used, internal standards yielded the same amount of radioactivity as aqueous solutions of standard NA, and the results obtained for such tissues agreed well with the values obtained previously in this laboratory by fluorometric assay procedures (Table 1).

TABLE 1. APPLICATION OF ENZYME ASSAY METHOD TO MEASUREMENT OF ENDOGENOUS NA CONTENT OF PERIPHERAL TISSUES—COMPARISON WITH FLUOROMETRIC ASSAY DATA

Tissue	Endogenous NA content (μg/g)	
	Fluorometric assay	Enzyme method
Rat vas deferens	9·56 ± 0·52 (6)	8·92 ± 0·45 (6)
G. Pig vas deferens	9·57 ± 0·75 (6)	9·10 ± 0·63 (6)
Rat submaxillary gland	1·68 ± 0·09 (6)	2·07 ± 0·10 (3)
Rat iris/ciliary body	3·80 ± 0·15 (3)	3·74 ± 0·05 (3)
Rat auricle	1·70 ± 0·11 (4)	1·41 ± 0·06 (3)

Values are means ± S.E. Mean for no. of observations indicated in parentheses.

TABLE 2. APPLICATION OF ENZYME ASSAY TO MEASUREMENT OF OCTOPAMINE LEVELS IN RAT TISSUES

Tissue	NA Content (μg/g)	Octopamine content (μg/g)
Heart	1·36 ± 0·03	0·68 ± 0·12
Brain	1·48 ± 0·07	0·35 ± 0·06
Vas deferens	15·82 ± 1·64	1·71 ± 0·39
Submaxillary glands	2·81 ± 0·14	0·95 ± 0·12

NA and octopamine (norsynephrine) were assayed simultaneously by the enzyme method in homogenates of rat tissues 96 hr after the administration of the first of four doses of pheniprazine (10 mg/kg, i.p.) at 24-hr intervals. In normal tissues the levels of octopamine were too low to be accurately measured by this method. Results are mean values ± S.E. Mean for four animals.

We have found bovine adrenal medullary PNMT preferable to the enzyme prepared from rabbit adrenal glands.[1] The bovine enzyme, apart from being more readily available, has a lower K_m for L-NA (5·9 μM by our measurements) than the rabbit enzyme (10 μM), and hence offers higher sensitivity. The purification procedure described by Saelens *et al.*[1] proved satisfactory for the isolation of the enzyme from bovine adrenal glands. A further 10-fold purification could readily be achieved by chromatography on Sephadex G-100, but the purified enzyme did not have any obvious advantages over partially purified preparations. Under the conditions of our assay the reaction proceeds quantitatively to completion within 30–60 min at 37°, and we believe this to be an important feature of any assay of this nature. In the original method[1] the assay depended on measuring the *rate* of formation of product, rather than on a complete conversion of NA to adrenaline. In order to protect the substrate NA and the product adrenaline from spontaneous or enzymic degradation during the incubation, we have found it necessary to add stabilizing agents (2-mercaptoethanol and EDTA) and enzyme inhibitors (pargyline and pyrogallol) to the incubation mixture. None of these substances interfered with the enzyme reaction. The conversion of NA to adrenaline was not inhibited by dopamine or 5-hydroxytryptamine at concentrations up to 5 μg/ml, nor did these substances themselves give rise to detectable amounts of labelled products. L-adrenaline, DL-octopamine, DL-normetanephrine and D-NA, however, are all substrates for PNMT and gave rise to ^{3}H-methylated products which could be recovered from

appropriate regions of the chromatograms. Provided that none of these compounds was present in excess, their presence did not interfere with the assay of NA. The enzyme procedure can in principle be applied to the assay of any of these alternative substrates; a method for the assay of octopamine in this way has been described.[2] We have also applied the present method to octopamine assays with satisfactory results (Table 2). The assay of NA in freshly prepared tissue homogenates is simpler and more reliable than the use of perchloric acid extracts.[1] In our hands, it has proved unsatisfactory to use perchloric acid extracts because of the difficulty of neutralizing and removing the perchloric acid prior to assay. The use of magnesium carbonate for this purpose, as advocated by Saelens *et al.*,[1] tended to produce samples with an alkaline pH in which the catecholamines were extremely unstable. Potassium phosphate or TRIS were not suitable as neutralizing agents because these substances when present in high concentrations interfered with the enzyme reaction. The presence of catecholamine metabolizing enzymes in freshly prepared tissue homogenates does not interfere with the assay since the reaction mixture contains inhibitors of both monoamine oxidase and catechol-*O*-methyltransferase.

The method described is suitable for the assay of NA in small aliquots of homogenates of tissues which contain relatively high concentrations of endogenous NA or in aliquots of samples from sucrose density gradients.[3, 4] For such samples this method has the advantage of allowing NA to be assayed on a small aliquot, leaving material which can be used for the parallel assay of other components (enzymes etc.). In other tissues, such as brain, however, in which the reaction did not consistently proceed to completion the method yielded unreliable results.

By reducing the volumes of incubation mixture and sample, it has proved possible to increase the sensitivity of the radiochemical assay procedure to less than 0·1 ng for L-NA, so that for the micro-assay of NA in very small samples of tissue the enzyme assay has obvious advantages and possibilities for future use. Preliminary results suggest that it may be possible to control to some extent for the inconsistent completion of the reaction in certain tissues by incorporating a double isotope modification, in which ^{14}C-NA is added to the tissue samples before assay, and the ratio of ^{3}H/^{14}C is measured in the product adrenaline.

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The effect of antihistamines on red blood cell acetylcholinesterase activity *in vitro*

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Several reports concerning the pseudocholinesterase-inhibitor effect of certain antihistamines have appeared; Vincent[1] reported the inhibitory effect of Antergan, Todrick[2] that of promethazin. Benstz, observed the same effect of Phenergan and chlorpromazine, and also determined their influence on