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A thin-layer chromatographic procedure for the assay of labelled noradrenaline and its metabolites in tissues and in incubation medium

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A method for the extraction, separation and quantitative determination of [^{3}H]noradrenaline [^{3}H-NA] and its five major metabolites has been devised using thin layer chromatography. This procedure was used to study the pattern of ^{3}H-NA and its metabolites in the total radioactivity of the tissues and that released spontaneously from the rat isolated vas deferens. Whereas in tissues ^{3}H-NA represented almost all of the total radioactivity (94·8 ± 0·47%), in the samples of spontaneous outflow it represented only 16·8 ± 2·1%. The rest was mostly accounted for by the five metabolites, primarily ^{3}H-DOPEG and ^{3}H-MOPEG. These findings show that in the rat vas deferens ^{3}H-NA is preferentially metabolized via the two glycol derivatives, i.e. ^{3}H-DOPEG and ^{3}H-MOPEG.

Most procedures described for the extraction, separation and assay of tritiated noradrenaline (^{3}H-NA) and its five major metabolites; namely, normetanephrine (NMN); 3,4-dihydroxymandelic acid (DMA); 3,4-dihydroxyphenylglycol (DOPEG); 3-methoxy-4-hydroxyphenylglycol (MOPEG), and 3-methoxy-4-hydroxymandelic acid (VMA), from various biologic materials either suffer from one or more deficiencies or do not adequately separate all six compounds (Kopin, Axelrod & Gordon, 1961; Schneider & Gillis, 1965; Fleming & Clark, 1970; Langer, 1970; Su & Bevan, 1970; Levin, 1973). Those methods (Rutledge & Weiner, 1967; Taylor & Laverty, 1969; Graefe, Stefano & Langer, 1973) which do separate all six compounds are tedious and time consuming and hence limit the number of samples that can be processed conveniently.

We describe a thin layer chromatographic method by which all six compounds can be separated efficiently and conveniently. This procedure has been used to study the metabolic pattern of ^{3}H-NA released spontaneously from the rat isolated vas deferens and composition of the radioactivity present in the tissues following incubation with ^{3}H-NA.

MATERIALS AND METHODS

Preparation of tissues and collection of samples

Male Wistar rats, 250–300 g, were killed by cervical dislocation. Both vasa deferentia were removed and prepared (Vohra, 1969). Each was slit open longitudinally and its lumen cleaned. Both tissues were then tied to a stainless steel tube (0·64 mm) and placed in a tube (15 × 85 mm) containing 3 ml of Krebs-Ringer bicarbonate solution and allowed to equilibrate at least for 1 h before incubation with ^{3}H-NA. The bathing medium was continuously gassed with 5% CO_2 in oxygen at 37 ± 1° and pH 7·4–7·5. The composition of Krebs-Ringer bicarbonate solution was (in mM): NaCl, 115·5; KCl, 4·63; $CaCl_2$, 2·47; $MgCl_2$, 1·16; $NaHCO_3$, 21·9; NaH_2PO_4, 1·16; glucose, 49·2; Na_2-EDTA, 0·01; ascorbic acid, 0·005.

After equilibration, the tissue was transferred to a tube containing 10 μCi of ^{3}H-NA (±)-7-[^{3}H-]noradrenaline, New England Nuclear Corporation, Boston, Mass., specific activity of 13·6 Ci mmol^{-1}) in 3 ml of incubation medium. The incubation with ^{3}H-NA was terminated after 15 min by transferring the tissue rapidly twice through tubes, which contained 3 ml of amine-free Krebs solution. Unless otherwise stated, the tissue was then washed for 80 min by transferring it every 10 min into new tubes containing 3 ml of fresh medium and thereafter the tissue was washed every 5 min until the end of the experiment (240 min).

Measurement of radioactivity

The total radioactivity of various samples and extracts was determined by liquid scintillation after adding an aliquot of the sample directly into 10 ml of Bray's (1960) scintillation solution at 6–8°. The efficiency of counting for the least quenched samples

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was approximately 40%. Quenching was estimated by the channel ratio procedure and the net disintegration per minute (d min^{-1}) calculated.

Extraction of radioactivity

Tissues. The tissues were collected, blotted dry, weighed and homogenized in a mixture of 2·5 ml ethanol and 0·1 ml of 0·1 N HCl at 0–4° and stood in an ice-bath for 10 min with frequent mixing. The homogenate was then centrifuged at 9000 *g* for 10 min at 4° and the supernatant collected and the pellet re-extracted with 0·5 ml of acid–ethanol mixture and after further centrifugation, the two supernatants were pooled and the total volume noted. An aliquot of 0·1 ml was counted to determine the total radioactivity of the extract. Another aliquot (0·1 ml) of the extract was used for thin-layer chromatographic separation of ^{3}H-NA and its metabolites.

In some experiments, the pellet was digested with 1 ml of NCS (Nuclear Chicago Solubilizer) at 50° for 4 to 6 h with occasional mixing and counted directly by adding 10 ml of Bray's scintillation fluid.

Samples of incubation media. From a 3 ml sample of incubation medium, an aliquot of 0·5 ml was taken to determine the total radioactivity. To the remainder 100 μg of Na_2-EDTA was added and the pH adjusted to 3–4 with 1 N HCl (0·05–0·07 ml). After the volume was reduced at 20° under vacuum over calcium chloride almost to dryness, the residue was taken up in 0·1 ml of 2% sodium metabisulphite solution and 0·2 ml of ethanol added. To this 0·8 ml of cold acetone was added dropwise with constant stirring. The resultant precipitate was centrifuged and 1 ml of acetone added. After centrifugation, the supernatant was collected and dried under vacuum. The residue was resuspended in 0·02 ml of glass distilled water and 1 ml of cold acetone added dropwise. After centrifugation, the supernatant was dried under vacuum. The near dried residue was dissolved in 0·1 ml methanol and used for thin-layer chromatography.

Thin-layer chromatography

Procedure. ^{3}H-NA and its five major metabolites were separated on strips 18 × 3 cm prepared from 20 × 20 cm polygram sheets coated with cellulose MN 300 Ecteola (Mondray Ltd., Montreal) a weak basic anion exchanger (layer thickness 100 μm) using as solvent n-butanol–ethanol (absolute)–0·5 N acetic acid (35:8·5:20 by volume).

An aliquot (100 μl of tissue extract or 20–30 μl of incubation medium extract) was spotted on to a strip along with 15–30 μl of the freshly prepared reference standard mixture under a continuous stream of nitrogen and in the absence of direct light. The reference standard mixture was freshly prepared by mixing 100 μl of a stock solution of each of noradrenaline, NMN, DMA, DOPEG, MOPEG and VMA 5 mg ml^{-1} previously prepared in absolute methanol and stored in brown bottles at −23°. The use of a reference standard mixture served as carrier for various compounds. The strip was placed in a covered jar (20 cm high and 8 cm diameter) containing 20 ml of the solvent system and developed by ascending chromatography for 3–4 h or until the solvent front had risen 15 cm after which it was removed, dried with warm air, sprayed with aqueous ethylenediamine (1:4 v/v) and heated at 50° for 10–20 min and visualized under an ultraviolet lamp (Segura-Cardona & Soehring, 1964). All catechol derivatives exhibited a light to intense green fluorescence whereas non-catechol derivatives appeared as dark spots of purple colour.

Under ultraviolet light each chromatogram was divided and then cut into seven consecutive segments (portions) each being scraped and then eluted with 0·01 N HCl in a vial to which Bray's solution (10 ml) was added to allow the radioactivity to be determined.

Total tritium was calculated as d min^{-1} per 100 mg of tissue (wet weight). For other results, the net d min^{-1} in each segment was expressed as a percentage of the total net d min^{-1} on the chromatogram. From these percentages the radioactivity in the original extract represented by each compound was then calculated from the total radioactivity of the extract (determined by separately counting an aliquot of the extract). The conventional Student's *t*-test was used to determine the significance between any two means.

Drugs and chemicals. Noradrenaline and the five metabolites used as carriers: (±)-noradrenaline HCl, (NA); (±)-3,4-dihydroxymandelic acid (DMA); 3,4-dihydroxyphenylglycol (DOPEG); (±)-normetanephrine HCl (NMN); (±)-4-hydroxy-4-methoxymandelic acid (VMA); bis-4-hydroxy-3-methoxyphenylglycol-piperazine salt (MOPEG) were purchased from Sigma Chemical Company; ethylenediamine, Analyzed Reagent (Baker Chemical Corp.). The solvents and other chemicals were reagent grade and obtained from Fisher Scientific Co. or Baker

Chemical Co. Liquid Scintillation materials were from Packard Instrument Co.

RESULTS

T.l.c. separation of noradrenaline and its metabolites

The relative R_F values of noradrenaline and its major metabolites (NMN, DMA, DOPEG, MOPEG, and VMA), as calculated from the centre of the spots, of various reference standards were DMA, 0·20; VMA, 0·37; noradrenaline, 0·49; NMN, 0·61; DOPEG, 0·74 and MOPEG, 0·89. There was no overlapping of any of the compounds.

Only the tissue extracts could be chromatographed directly. The samples of incubation medium required concentration as well as purification before chromatography.

Recovery of radioactivity

Tissues. To determine the amount of total radioactivity extracted from the tissues, the tissue pellet was digested (see Methods) and counted. The radioactivity of the pooled extract was then expressed as a percent of the total of the radioactivity remaining in the pellet plus the radioactivity in the pooled extract.

The mean recovery of the total radioactivity in the pooled extract from tissues previously incubated with ^{3}H-NA, was 96·8 ± 0·6% (n = 8). On chromatography, 94·5 ± 0·5% (n = 8) of the total radioactivity of the pooled extract was represented by unchanged ^{3}H-NA.

Incubation medium. A series of tubes, each containing 45 nCi of ^{3}H-NA (a quantity equal to that expected in actual samples) in 3 ml of oxygenated incubation medium, were prepared, gassed with 5% CO_2 in oxygen for 5 min at 37°, and processed (see Methods). After t.l.c. of an aliquot of the final methanolic extract, the percentage of unchanged ^{3}H-NA was calculated from the total radioactivity on the chromatogram. 90·8 ± 0·4% (n = 8) of the total radioactivity was unchanged ^{3}H-NA. The recovery of total radioactivity ranged between 68·3 to 85·7% with a mean value of 76·6 ± 2·7% (n = 8).

Effect of acid elution from chromatograms on efficiency of counting

The recovery of radioactivity from the chromatograms was measured by spotting two sets of t.l.c. strips with varying quantities, 0·5–15 nCi of ^{3}H-NA along with the reference standard mixture. The strips were then developed and the noradrenaline spot identified under ultraviolet light after spraying with aqueous ethylenediamine solution. The noradrenaline spot was cut from both sets, one set was placed in scintillation vials containing 10 ml of Bray's solution and counted directly, the second set was eluted with 1 ml of 0·01 N HCl as described in Methods and 10 ml of Bray's solution added for counting. The percent recovery was calculated by dividing the observed d min^{-1} by the total d min^{-1} applied initially and multiplied by 100.

The recovery of radioactivity from the non-eluted segments was only 45·9 ± 5·4% (n = 8) whereas recovery from the eluted segments was 80·8 ± 2·8% (n = 8).

Washout of tritiated compounds from vas deferens

Tissues previously incubated with ^{3}H-NA were transferred every 10 min into new tubes containing non-labelled physiological solution for 240 min and the total radioactivity of each tube monitored. Initially, there was a rapid loss of radioactivity but from 90 min onwards, the loss of radioactivity was minimal and occurred exponentially. At 90 min the half-life of the efflux of spontaneously released radioactivity was 225 ± 20 min (n = 4).

Tissue content of ^{3}H-NA and its metabolites. Since more than 50% of the initial label was retained by the tissues between 90 and 240 min washout, attempts were made to determine the tritiated compounds contributing to the total radioactivity of the tissues. Although the tissue contents of total radioactivity for 90 and 240 min samples were significantly

Table 1. *Proportions of [^{3}H]noradrenaline and its metabolites in the rat vas deferens.*

Substance	Radioactivity (^{3}H) given as percent of total* (means ± s.e.m.) (n = 8)
DMA	1·5 ± 0·1
VMA	0·8 ± 0·06
Noradrenaline	94·8 ± 0·47
NMN	0·4 ± 0·04
DOPEG	1·5 ± 0·18
MOPEG	0·8 ± 0·21
TOP	—

* The tissues were incubated with 10 μCi of (±)-^{3}H-NA (spec. act. 13·6 Ci mmol^{-1}) in 3 ml of Krebs solution at 37 ± 1° for 15 min. The solution was gassed with 5% CO_2 in oxygen. After incubation with ^{3}H-NA, the tissues were washed repeatedly for 90 or 240 min according to the schedule given in Methods. The tissues were then removed, extracted with acid–ethanol mixture and the extract chromatographed to separate various compounds as described in Methods. Results are mean values for 8 experiments and are expressed as percentage of the total radioactivity present in the tissues at the time of their collection.

($P < 0.01$) different from each other, the proportions of various tritiated compounds were essentially similar. Therefore, the data of both sets of tissues have been pooled and are shown in Table 1 and show that ^{3}H-NA and all of the 5 metabolites were present in the tissues. ^{3}H-NA, however, represented over 94% of the total radioactivity in the tissue.

Metabolic pattern of spontaneous outflow. To determine the pattern of ^{3}H-NA and its metabolites in the spontaneous outflow of tritiated compounds and to see if there were marked alterations in this pattern during the course of an experiment, samples of incubation medium which had been bathing the tissues for 5 min (see Methods) were collected at three different points along the washout curve at 1 h intervals, starting after 100 min washout. Table 2 shows that, in contrast with tissues, ^{3}H-NA comprised only 17% of the total radioactivity in the washout medium. The rest consisted of all five metabolites of ^{3}H-NA. Of the labelled metabolites, the deaminated glycol derivatives ^{3}H-DOPEG and ^{3}H-MOPEG represented respectively 38 and 14% of the total radioactivity. The other metabolites were present to a lesser extent, ^{3}H-DMA, ^{3}H-VMA and ^{3}H-NMN accounted for 9,4 and 9% respectively of the total radioactivity. Unlike tissues, some (8·6 ± 1·5%) of the total radioactivity of the spontaneous outflow on t.l.c. was associated with the solvent front. It is shown as TOP in Table 2 as it did not correspond to the position of ^{3}H-MOPEG on the chromatograms. No attempts were made to establish the nature of this radioactivity.

Table 2. *Proportions of [^{3}H]noradrenaline and its metabolites in the spontaneous outflow collected at various times from the rat vas deferens.*

Substances	Radioactivity (^{3}H) given as percent of total* (mean ± s.e.m.) (n = 5) 100 min†	160 min	220 min
DMA	8·6 ± 3·2	4·7 ± 1·8	5·3 ± 1·8
VMA	4·2 ± 0·9	4·7 ± 0·6	4·4 ± 0·8
Noradrenaline	16·8 ± 2·1	16·7 ± 0·7	21·0 ± 3·1
NMN	9·4 ± 1·9	11·4 ± 2·5	7·4 ± 0·2
DOPEG	37·9 ± 5·6	39·4 ± 5·3	38·4 ± 4·3
MOPEG	14·3 ± 1·2	17·2 ± 1·8	15·4 ± 1·3
TOP	8·6 ± 1·5	5·6 ± 1·1	8·3 ± 2·1

* Total tritium in samples of incubation medium collected at 5 min intervals.
† Time of collection of samples of incubation medium after exposure of the tissues to [^{3}H]noradrenaline.
For more details refer to Table 1.

When the samples of incubation medium were collected from the same tissues at three different points (i.e. at 100, 160 and 220 min washout) along the washout curve and assayed, no significant ($P > 0.05$) changes in the proportions of individual metabolites were observed (Table 2), indicating that the metabolic pattern of spontaneously released tritiated compounds remained essentially unaltered during the course of the experiment.

DISCUSSION

The procedure described yields recoveries comparable to other published methods (Fleming & Clark, 1970; Levin, 1973) and loss of ^{3}H-NA due to oxidation is minimal. Unlike other procedures (Langer, 1970; Fleming & Clarke, 1970; Levin, 1973) it uses a single solvent system and one spray reagent for the separation and detection of the various compounds.

In a study of the metabolism of ^{3}H-NA in the isolated rat vas deferens, the results show that whereas most of the radioactivity retained by the tissues consisted of unchanged ^{3}H-NA (95% of the total), the spontaneous outflow from these tissues contained only a small amount (17%) of total radioactivity as unchanged ^{3}H-NA. The latter was mostly composed of metabolites, chiefly ^{3}H-DOPEG (38%) and ^{3}H-MOPEG (14%). The finding of a low ^{3}H-NA content in the spontaneous outflow is consistent with the findings of others (Langer, 1970; Su & Bevan, 1970; Tarlov & Langer, 1971).

Since the tissues contained mostly ^{3}H-NA it is unlikely that preformed metabolites were released directly into the incubation medium. Further, the presence of large quantities of metabolites in the spontaneous outflow cannot be regarded as an artifact of the present methodology since it was shown that the procedure does not appreciably alter ^{3}H-NA added to the incubation medium. Nor can the origin of metabolites in the spontaneous outflow be considered to arise from the metabolism of ^{3}H-NA bound extraneuronally as the samples of medium were collected after a long period of washing (100 to 140 min) following incubation with ^{3}H-NA (Potter, Copper & others, 1965). Consequently it is reasonable to conclude that only when the ^{3}H-NA has been released from its storage sites is it acted upon by the catabolizing enzymes.

Thus, the present results further substantiate that in the rat vas deferens, ^{3}H-NA is preferentially metabolized via the two glycol derivatives (^{3}H-DOPEG and ^{3}H-MOPEG). These results although supporting most of the findings of Langer (1970),

fail to explain one discrepancy. He reported a large proportion of DMA (32% of the total radioactivity) in the spontaneous outflow, whereas, in our study DMA accounted for only 3–9% of the total radioactivity.

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REFERENCES

BRAY, G. A. (1960). *Analyt. Biochem.*, **1**, 279–285.
FLEMING, R. M. & CLARK, W. G. (1970). *J. Chromat.*, **52**, 305–312.
GRAEFE, K. H., STEFANO, F. J. E. & LANGER, S. Z. (1973). *Biochem. Pharmac.*, **22**, 1147–1160.
KOPIN, I. J., AXELROD, J. & GORDON, E. (1961). *J. biol. Chem.*, **236**, 2109–2113.
LANGER, S. Z. (1970). *J. Physiol. (Lond.)*, **208**, 515–546.
LEVIN, J. A. (1973). *Analyt. Biochem.*, **51**, 42–60.
POTTER, L. T., COPPER, T., WILLMAN, V. L. & WOLFE, D. E. (1965). *Circulation Res.*, **16**, 468–481.
RUTLEDGE, C. O. & WEINER, N. (1967). *J. Pharmac. exp. Ther.*, **157**, 290–301.
SCHNEIDER, F. H. & GILLIS, C. N. (1965). *Biochem. Pharmac.*, **14**, 623–626.
SEGURA-CARDONA, R. & SOEHRING, K. (1964). *Medicin. Exp. Basel*, **10**, 251–257.
SU, C. & BEVAN, J. A. (1970). *J. Pharmac. exp. Ther.*, **172**, 62–68.
TARLOV, S. R. & LANGER, S. Z. (1971). *Ibid.*, **179**, 186–197.
TAYLOR, K. M. & LAVERTY, R. (1969). *J. Neurochem.*, **16**, 1361–1366.
VOHRA, M. M. (1969). *Life Sci.*, **8**, part 1, 25–31.