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PREFERENTIAL METABOLISM OF (—)-^{3}H-NOREPINEPHRINE THROUGH THE DEAMINATED GLYCOL IN THE RAT VAS DEFERENS

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Abstract—A method for separation of norepinephrine (NE) and its metabolites by the combined use of Alumina and Dowex columns is described. The most commonly used eluents, 0·2 N acetic acid, 0·04 N perchloric acid or 0·2 N HCl, were found to elute 3,4-dihydroxyphenylglycol (DOPEG) together with NE from Alumina. The use of Dowex columns for further separation of NE from DOPEG was essential to measure labeled NE without contamination. With the use of the method described in this publication, the metabolism of (—)-^{3}H-NE released spontaneously from the rat vas deferens was studied. ^{3}H-DOPEG was the main metabolite in the spontaneous outflow, and it accounted for 70 per cent of the total radioactivity. In the presence of cocaine (10^{-5} M), there was a decrease in the percentage of DOPEG while the proportion of NE rose significantly. Phenoxybenzamine (3×10^{-5} M) elicited an increase in total outflow of radioactivity which was mainly composed of the deaminated glycol, DOPEG.

AS PREVIOUSLY reported,[1] when Alumina columns are used for separation of ^{3}H-norepinephrine from its metabolites, the deaminated catechol ^{3}H-DOPEG (3,4-dihydroxyphenylglycol) contaminates the norepinephrine fraction. The deaminated glycol represents an important proportion of the radioactivity in the spontaneous outflow from the guinea-pig atria[2,3] and the vas deferens of the rat[4] labeled with ^{3}H-norepinephrine. Furthermore ^{3}H-norepinephrine released by reserpine-like agents or by phenoxybenzamine is to a large extent metabolized selectively via DOPEG.[2]

These reports[2–4] were based on the use of paper chromatography for the separation of norepinephrine and its metabolites. Under these conditions, recoveries of the deaminated metabolites have not been determined. However, it is known that recoveries of the deaminated metabolites can be rather low and different from each other with the use of other paper chromatographic systems.[5,6] Consequently, it was considered of interest to re-examine the column chromatographic methods available to achieve adequate separation of the norepinephrine metabolites under conditions of high recoveries and with low elution volumes.

A satisfactory method has been obtained. With the use of this procedure the metabolic pathway of spontaneous and drug-induced release of ^{3}H-norepinephrine was studied in the rat vas deferens.

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MATERIALS AND METHODS

Alumina (Aluminiumoxid standardisiert, Aktivitatsstufe II–III; Merck-AG) was prepared according to Crout[7] and washed, before drying, with 0·2 M sodium acetate.

Dowex 50 W × 4 (200–400 mesh) was treated in bulk. It was washed several times with 2 N NaOH (containing 1% Na_2–EDTA) at 50° until the supernatant was colorless. Then, the resin was washed successively with twice distilled water, 2 N HCl, twice distilled water and finally equilibrated with 0·01 N HCl (pH 2). Before use the Dowex columns were washed with 20 ml of 6 N HCl–ethanol (1:1, v/v) and then equilibrated with 0·01 N HCl (pH 2). The washing with 6 N HCl–ethanol was necessary for the removal of a highly fluorescent material with a broad emission peak (maximum at 430 nm) which was eluted when 6 N HCl–ethanol was used for the first time.

Columns for chromatography were of 0·5 cm diameter and stoppered with glass wool. Two-hundred mg of activated Al_2O_3 was used throughout and washed before use with 5 ml of 0·2 N sodium acetate (adjusted to pH 8·2). Dowex columns were filled to 1·5 cm height with the resin at pH 2. The columns were washed with 2 ml of twice distilled water prior to use. As a rule, the resin columns were reused. Regeneration was achieved by successive washings with 10 ml of 6 N HCl–ethanol (1:1, v/v), 20 ml of 2 N HCl, 20 ml of twice distilled water and finally with 20 ml of 0·01 N HCl.

Fluorimetric determinations. The recovery of norepinephrine (NE) and its metabolites was measured with the use of the native fluorescence (280/340 nm, uncorrected). Samples of 50 μg of NE and of each metabolite were used throughout for recoveries. The fluorescence yield was near the maximum at a pH below 3 for: NE, NMN (normetanephrine), DOPEG (3,4-dihydroxyphenylglycol) and MOPEG (3-methoxy-4-hydroxyphenylglycol). Therefore, these metabolites were measured directly or after acidification of the different fractions of the columns. However, for VMA (3-methoxy-4-hydroxymandelic acid) and DOMA (3,4-dihydroxymandelic acid), maximal fluorescence was achieved by adjusting the pH to 5–6. The native fluorescence was shown to be linear for concentrations of up to 5 μg/ml for the metabolites tested. Consequently, appropriate dilutions had to be made. The standards and blanks were prepared from the respective fractions of blank columns. The lowest level of sensitivity of the method was about 250 ng/ml (1·5 times the blank).

The endogenous content of NE in the tissue was determined with the use of the trihydroxyindole method described by Laverty and Taylor.[8]

Tissue incubations. Male rats of 200–250 g were killed by neck dislocation and both vasa deferentia were freed of surrounding tissue, excised and about 7 mm each of the epididymal and prostatic ends of the organ were discarded. The portion of the vas deferens which was used was about 25 mm long.

The vas was cut open by a longitudinal incision and two tissues were mounted together on the tip of a plastic pipette through which O_2 was bubbled into the incubation medium. The composition of the incubation medium was the following (mM concentration): NaCl, 140·0; KCl, 5·0; $CaCl_2$, 2·5; Tris–HCl, pH 7·8, 10·0; glucose, 1·1; Na_2–EDTA, 0·04 and ascorbic acid, 0·11. The final pH of the solution at 37° was 7·5.

After preincubation for 10 min, the tissue was transferred into 2·5 ml of medium containing (−)-^{3}H-NE (190 ng/ml) and incubated for 30 min at 37°. Then, the tissue was washed for 120 min with amine-free medium by transferring the vasa deferentia

every 10 min into new tubes each containing 2 ml of medium. After the 2-hr washing period was completed, the tissues were transferred to amine-free media every 2 min. Control efflux of radioactive products was measured prior to the addition of the drugs in four consecutive 2-min periods by collecting 1-ml samples from each tube. The 4 ml was pooled and acidified to pH 2 for temporary storage by the addition of 1 N HCl. Subsequently, the samples were analyzed by column chromatography (for details see Results). When the effects of phenoxybenzamine (PBA) (3×10^{-5} M) or cocaine (10^{-5} M) on the efflux of radioactivity were studied, the tissue was placed in tubes to which the drug had been added. The samples corresponding to the first 4 min of exposure to the drug were used for total counts only. Then, aliquots from four consecutive 2-min samples in the presence of the drug were collected and processed as described for the control samples. Aliquots of 0·2 ml for total radioactivity were taken from every sample.

At the end of the experiment, the tissues were blotted dry, weighed and homogenized in 5 ml of cold 0·4 N perchloric acid containing 1 mg/ml of EDTA and 1·25 mg/ml of Na_2SO_3. Aliquots of tissue extracts were analyzed for labeled compounds as described in Results. Another aliquot was purified on Alumina columns (200 mg) and the endogenous NE was measured fluorimetrically in the 0·2 N acetic acid eluate (2 ml) of the column by using the trihydroxyindole method.

Radioactivity was measured by scintillation counting. The composition of the scintillation medium was the following: toluene, 600 ml; Triton X-100, 300 ml; absolute ethanol, 100 ml; 1 N HCl, 20 ml; 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene (POPOP), 0·1 g and 2,5-diphenyloxazole (PPO), 5 g.

The following drugs were used: (−)-7-^{3}H-norepinephrine (New England Nuclear, 6·4 Ci/m-mole), (−)-norepinephrine bitartrate monohydrate, cocaine hydrochloride, phenoxybenzamine hydrochloride, 3,4-dihydroxymandelic acid (Sigma), 3,4-dihydroxyphenylglycol (Sigma), 3-methoxy-4-hydroxymandelic acid (Sigma), bis-(3-methoxy-4-hydroxyphenylglycol) piperazine salt (Sigma), and normetanephrine (Calbiochem). Stock solutions of norepinephrine and its metabolites were kept in 0·01 N HCl.

Statistical calculations were performed according to conventional procedures.[9] Throughout the text values are given as mean ± S.E.M.; *n* indicates the number of observations.

RESULTS

(A) *Pattern of elution of catechols from Alumina columns*

Norepinephrine and the deaminated catechols (DOPEG and DOMA) were added to Alumina columns in samples adjusted to pH 8·1 to 8·3 and containing 10 mg of EDTA and 12·5 mg Na_2SO_3. Figure 1 shows the elution pattern for NE, DOPEG and DOMA when 0·04 N perchloric acid or 0·2 N acetic acid were used as first eluents. Under these experimental conditions, neither perchloric acid nor acetic acid were able to separate NE from the deaminated glycol, DOPEG. In fact, the pattern of elution for these two catechols was almost identical, when both acids were compared (Fig. 1). On the other hand, the deaminated acid, DOMA, was not eluted by 0·2 N acetic acid and only 9·3 ± 0·65 per cent ($n = 4$) of this metabolite was eluted by the 6 ml of

0·04 N perchloric acid. When 0·2 N HCl followed either perchloric acid or acetic acid, the deaminated acid, DOMA, was efficiently extracted (Fig. 1).

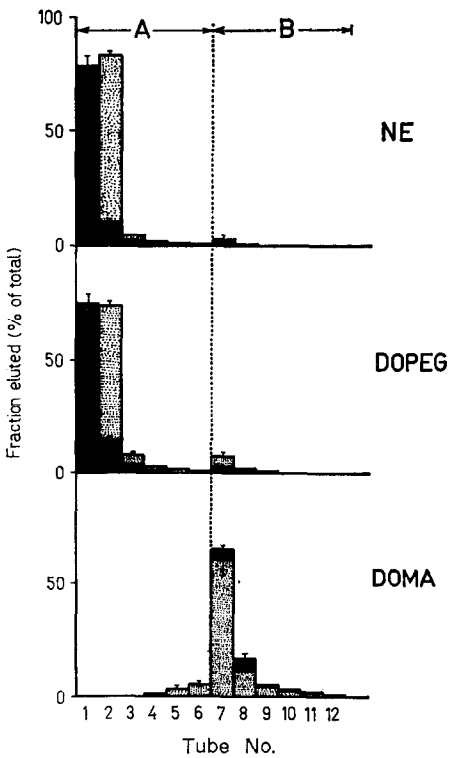

FIG. 1. Elution pattern of the catechols from Alumina. Ordinates: recovery in per cent of the amount applied to the column. Abscissae: number of the tube in which 1-ml fractions were collected. Fifty μg of each catechol was added at pH 8·1 to 8·3 to different alumina columns. (A) (■): elution with 0·2 N acetic acid; (▦): elution with 0·04 N perchloric acid. (B) Elution with 0·2 N HCl; (■) or (▦) refer to the acid used in the same column as eluent in step 1. Shown are mean recoveries in per cent ± S.E. of four measurements. Abbreviations used are: NE, norepinephrine; DOPEG, 3,4-dihydroxyphenylglycol and DOMA, 3,4-dihydroxymandelic acid.

Neutralization of the samples in which elution of DOMA was investigated led to precipitation of Al $(OH)_3$ (particularly in the 0·2 N HCl fractions). Therefore, before fluorescence readings were taken, the samples were centrifuged (2000 g; 10 min).

The elution pattern from Alumina columns for the three catechols was not altered when the volume of the sample added to the column was increased up to 20 ml.

These results indicate that under our experimental conditions separation of NE from the deaminated glycol, DOPEG, cannot be achieved with the use of column chromatography on Alumina. However, when 0·2 N acetic acid is employed to elute DOPEG and NE, the deaminated acid, DOMA, can be separated from the former compounds and subsequently eluted with 0·2 N HCl. Under these conditions the contamination by NE or DOPEG in the DOMA fraction is rather low (Fig. 1).

Several experiments were carried out in an attempt to diminish the extent of the cross-contamination in the DOMA fraction. After a standard elution of NE and

DOPEG with two consecutive 1-ml volumes of 0·2 N acetic acid, different strengths of acid were used to complete the elution from the Alumina.

It was found that 4 ml of either 1 N acetic acid or 0·5 N HCl decreased the contamination by NE and DOPEG in the DOMA fraction to less than 1 per cent. However, under these conditions both acetic acid and HCl eluted a rather high proportion of DOMA (20 per cent for 1 N acetic acid and 90 per cent for 0·05 N HCl). In contrast with the former procedures, the addition of 4 ml of 0·5 N acetic acid after the 2 ml of 0·2 N acetic acid was found to diminish the contamination by NE and DOPEG in the DOMA fraction without eluting significant amounts of DOMA (less than 3 per cent).

The recovery of the three catechols was closely related to the rate of flow of the eluent through the Alumina column. For instance, the recovery of DOPEG, when eluted with 4 ml of 0·2 N acetic acid, was 85·3 $\pm$ 0·5 per cent (n = 11), and it rose to 92·2 $\pm$ 1·8 per cent (n = 8) when the elution was carried out at a slower rate by using 4 times 1 ml of the acid. The difference between these two values of recoveries was significant ($P < 0{\cdot}01$). Recoveries for NE and DOMA were also significantly higher when the elution was performed at the slower rate of flow by adding 1 ml at a time of 0·2 N acetic acid for NE or 0·2 N HCl for DOMA.

When the recoveries and the pattern of elution of NE from Alumina were determined with the trihydroxyindole method,[8] the results were identical with those obtained with the use of native fluorescence.

(B) *Separation of NE and its metabolites*

The information described in section (A) was applied in the development of a method for the separation of NE from its metabolites. The procedure is outlined in

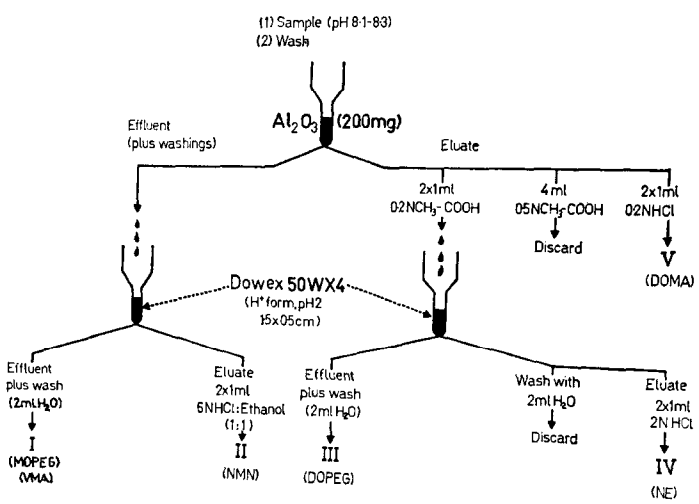

FIG. 2. Column chromatographic system for the separation of ^{3}H-norepinephrine and its metabolites. Samples of up to 20 ml, containing 12·5 mg of Na_2SO_3 and 10 mg of EDTA, were adjusted to pH 8·1 to 8·3 and applied to the Alumina column. Fraction I, MOPEG = 3-methoxy-4-hydroxyphenylglycol, VMA = 3-methoxy-4-hydroxymandelic acid; fraction II, NMN = normetanephrine; fraction III, DOPEG = 3,4-dihydroxyphenylglycol; fraction IV, NE = norepinephrine and fraction V, DOMA = 3,4-dihydroxymandelic acid.

Fig. 2. The initial step was the adsorption of the catechol compounds onto Alumina columns at pH 8·1 to 8·3. The effluent and subsequent washings from the column (1 ml of 0·2 M sodium acetate followed by 2 ml of H_2O) contained NMN, VMA and MOPEG. The effluent and washings containing the three metabolites dripped directly into a Dowex 50 W × 4 column. The *O*-methylated deaminated metabolites, OMDA (i.e. VMA and MOPEG), passed through the column and were collected in the effluent and washings (Fig. 2, I). NMN was retained by the Dowex column and subsequently eluted with 2 times 1 ml of 6 N HCl–ethanol (v/v) (Fig. 2, II).

After washing, the Alumina column was placed over a second Dowex 50 W × 4 column and NE together with DOPEG was eluted into the resin by the addition of 2 times 1 ml of 0·2 N acetic acid (Fig. 2). The neutral catechol, DOPEG, was then recovered in the effluent and the first washing (2 ml of H_2O) from the Dowex column (Fig. 2, III). After washing the resin with 2 additional ml of H_2O, NE was eluted with 2 times 1 ml of 2 N HCl (Fig. 2, IV). Subsequently the Alumina column was washed with 4 ml of 0·5 N acetic acid. This washing eluted approx. 5 per cent of both NE and DOPEG (Table 1). Therefore it was discarded in order to minimize the contamination of the DOMA fraction by these two catechols. Finally the deaminated acid, DOMA, was eluted by 2 times 1 ml of 0·2 N HCl (Fig. 2, V).

(C) *Recoveries of NE and its metabolites*

The samples containing the outflow of radioactive products from the vas deferens and the aliquots of the tissue extracts were analyzed with the column chromatographic technique described in section (B). Ten μg of NE and of each of the NE metabolites were added as carriers to every sample. In addition, recoveries of NE and of the NE metabolites were determined in a parallel set of columns in each experiment. The recoveries for each fraction are shown in Table 1. These values show little scatter indicating the high reproducibility of the method.

The 2-ml volumes used to elute the columns were sufficiently small to allow direct collection of fractions II, IV and V (Fig. 2) in the counting vials. In spite of these low volumes, the recoveries for NE and all of its metabolites exceeded 80 per cent.

According to these results (Table 1), the amount of labeled compounds found in each fraction was corrected by the recovery determined in the corresponding fractions. The intermediate washing of the Alumina column with 4 ml of 0·5 N acetic acid reduced the overlapping of the catecholamine and the deaminated glycol into the DOMA fraction. However, it was consistently found that the radioactivity recovered from the DOMA fraction was almost halved, when appropriate corrections for the cross-contaminations were carried out. The extent of the contamination of the DOPEG fraction (III) by NE and VMA was sufficiently low to be neglected.

The consistency of the whole chromatographic procedure and the accuracy of the corrections made were tested by comparing the total radioactivity in the sample with the sum of the radioactivity found in the five fractions. The radioactivity recovered from the fractions amounted to 98·1 ± 1·43 per cent ($n = 24$) of the total radioactivity added to the Alumina column.

(D) *Metabolism of (—)-^{3}H-NE released spontaneously from the rat vas deferens*

As described in Methods, the NE stores of the vas deferens were labeled with (—)-^{3}H-NE. After 2 hr washout, the spontaneous outflow of radioactive products had

TABLE 1. RECOVERY OF NOREPINEPHRINE AND ITS METABOLITES IN THE DIFFERENT FRACTIONS OF THE COLUMN CHROMATOGRAPHIC SYSTEM*

Norepinephrine and metabolites	I	II	III	IV	V	0·5 N acetic acid
MOPEG	96·0 ± 1·05 (6)	B.S.				
VMA	91·3 ± 1·09 (4)		2·1 ± 0·43 (3)			
NMN	B.S.†	96·0 ± 1·46 (6)				
DOPEG			87·2 ± 1·38 (8)		4·5 ± 0·44 (7)	5·8 ± 0·45 (5)
NE			0·5 ± 0·26 (3)	80·6 ± 1·31 (8)	3·3 ± 0·26 (7)	4·6 ± 0·51 (5)
DOMA			B.S.	B.S.	88·7 ± 1·64 (6)	2·6 ± 1·07 (5)

* The roman numerals refer to the fractions obtained with the combined column chromatographic procedure shown in Fig. 2. I, MOPEG (3-methoxy-4-hydroxyphenylglycol), VMA (3-methoxy-4-hydroxymandelic acid); II, NMN (normetanephrine); III, DOPEG (3,4-dihydroxyphenylglycol); IV, NE (norepinephrine) and V, DOMA (3,4-dihydroxymandelic acid). Acetic acid (0·5 N) was used to wash the Alumina columns in between 0·2 N acetic acid and 0·2 N HCl (for details see Results, section B). Number of observations is shown in parentheses. The values are given as mean recovery in per cent ± S.E.

† B.S. = below the sensitivity of the method (250 ng/ml). Fifty μg of each compound was applied to different Alumina columns and estimated where indicated by their native fluorescence.

leveled off to a single exponential decline. At this time the half life of the efflux of labeled compounds was 223·5 ± 13·5 min, and the (−)-^{3}H-NE retained in the tissue was 46·1 ± 0·77 per cent ($n = 6$) of the amount accumulated by the vas deferens at the end of the incubation period. (−)-^{3}H-NE accounted for 95·1 ± 0·53 per cent ($n = 6$) of the total radioactivity retained by the tissue. Therefore, under these experimental conditions the spontaneous release of radioactive products can be considered to be of neuronal origin.

The composition of the spontaneous outflow of radioactivity was examined with the use of the column chromatographic system described in section (B). Table 2 shows the

TABLE 2. METABOLIC PATHWAY OF ^{3}H-NOREPINEPHRINE IN SPONTANEOUS OUTFLOW FROM THE ISOLATED RAT VAS DEFERENS*

	(nCi/min × g^{-1})
Total radioactivity	63·2 ± 7·94
NE	6·0 ± 0·96
DOPEG	44·3 ± 5·53
OMDA	8·9 ± 0·93
DOMA	3·1 ± 0·49
NMN	0·9 ± 0·14

* The spontaneous outflow of radioactivity was analyzed 120 min after the end of the incubation with (−)-^{3}H-NE (190 ng/ml, 7·4 μCi/ml). At this time, the tissue contained 25·4 ± 1·51 μCi/g of labeled products; 95·1 ± 0·53 per cent of the radioactivity was found to be NE. Shown are mean values ± S.E. of six observations each. Abbreviations used are: NE, norepinephrine; DOPEG, 3,4-dihydroxyphenylglycol; OMDA, *O*-methylated-deaminated metabolites; DOMA, 3,4-dihydroxymandelic acid and NMN, normetanephrine.

absolute values for the spontaneous outflow of labeled products under control conditions. While NE represented less than 10 per cent of the outflow of radioactivity, the deaminated glycol, DOPEG, was the main metabolic pathway for the labeled transmitter released spontaneously. The deaminated glycol represented more than 70 per cent of the total radioactivity in spontaneous outflow (Table 2, Fig. 3, A). On the other hand, the deaminated acid, DOMA, accounted for less than 5 per cent of the total outflow of radioactivity (Table 2, Fig. 3, A). Metabolism through catechol-*O*-methyltransferase (COMT) alone appeared to be unimportant in this tissue, because NMN represented only 1·3 per cent of the total amount of labeled products (Table 2, Fig. 3, A). However, the OMDA fraction (MOPEG plus VMA) was found to represent 14 per cent of the total radioactivity.

(E) *Effects of phenoxybenzamine or cocaine on the metabolism of* (−)-^{3}*H-NE released from the rat vas deferens*

When 3×10^{-5} M phenoxybenzamine (PBA) was added to the incubation medium (for details, see Methods), a gradual increase in the outflow of radioactivity was observed. The enhancement in release of radioactivity induced by PBA reached a

maximum 6–8 min after addition of the drug. During exposure to PBA, the efflux of radioactivity rose to 2·32 ± 0·20 times the control outflow ($n = 3$; $P < 0{\cdot}05$). In contrast to the results obtained with PBA, in the presence of 10^{-5} M cocaine the outflow of total radioactivity remained almost unchanged (the ratio of drug-induced over control outflow was 1·06 ± 0·03; $n = 4$).

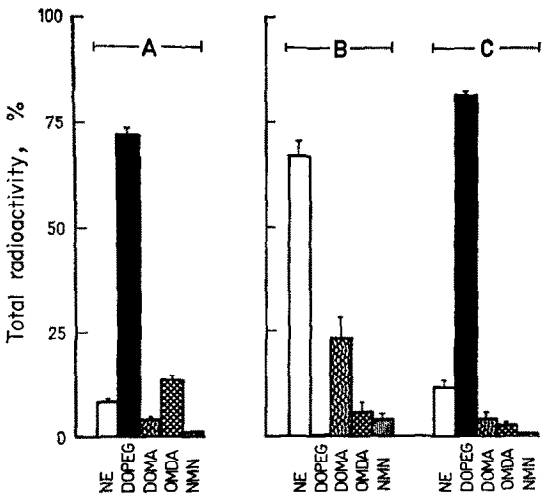

FIG. 3. Percentage distribution of ^{3}H-norepinephrine and its metabolites in the outflow of radioactivity from the isolated rat vas deferens. Ordinate: release of labeled products in per cent of total radioactivity. Tissues were incubated with (−)-^{3}H-NE (190 ng/ml, 7·4 μCi/ml) for 30 min. In each group one experiment was carried out with half the (−)^{3}H-NE concentration. Since the percentage values of the metabolites in these experiments did not differ from those experiments performed with 190 ng/ml of (−)^{3}H-NE, the results were pooled. After washing in amine-free medium for 128 min, the tissue was transferred into a medium containing cocaine (10^{-5} M) or phenoxybenzamine (3×10^{-5} M). (A) Spontaneous outflow in the controls; (B and C) Increase in outflow above the basal levels (delta) induced by cocaine (B) or phenoxybenzamine (C). Shown are means ± S.E. of eight (A), four (B) and three (C) observations each. Abbreviations used are: NE, norepinephrine; DOPEG, 3,4-dihydroxyphenylglycol; DOMA, 3,4-dihydroxymandelic acid; OMDA, *O*-methylated-deaminated metabolites and NMN, normetanephrine.

Figure 4 shows the absolute values of the release of labeled compounds obtained in four paired experiments. For PBA, there was an increase in the total tritium released and for each of the labeled compounds the values exceeded their basal levels (Fig. 4). However, this increase was of unequal magnitude in the different fractions, and ^{3}H-DOPEG accounted for most of the radioactivity released by PBA. On the other hand, in the presence of cocaine an actual decrease in the outflow of the main metabolite, DOPEG, was consistently observed in all experiments. The ratio of outflow of DOPEG in the presence of cocaine over the corresponding control value was 0·89 ± 0·02 ($n = 4$; $P < 0{\cdot}02$). During exposure to cocaine the outflow of NE rose to 2·17 ± 0·36 times the basal value.

A more thorough and sensitive analysis of the differences between the metabolism of the labeled transmitter released by either PBA or cocaine was obtained as follows. The sum of the positive changes in each fraction (black part of the columns in Fig. 4) was defined as drug-induced "delta". The percentage distribution of NE and its metabolites

in the PBA or cocaine-induced "delta" was calculated (Fig. 3, B and C) and compared with the percentage values in the spontaneous outflow (Fig. 3, A).

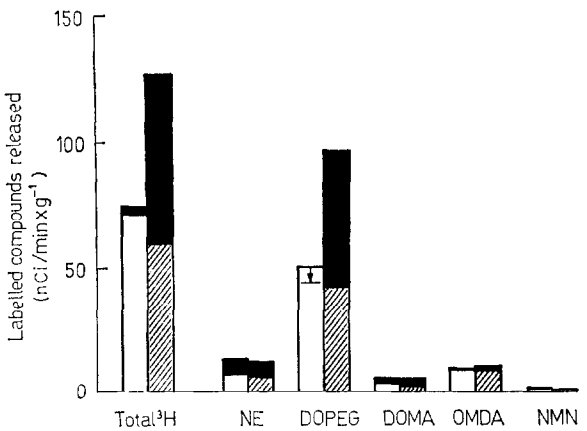

FIG. 4. Effects of cocaine or phenoxybenzamine on the spontaneous outflow of labeled compounds from the isolated rat vas deferens. Ordinate: efflux of total radioactivity and of labeled products in nCi/min × g^{-1}. The tissue was incubated with (−)-^{3}H-NE (190 ng/ml, 7·4 μCi/ml) for 30 min. After 128 min of washings, the tissue was transferred into a medium containing cocaine (10^{-5} M) or phenoxybenzamine (3×10^{-5} M). The white columns represent the spontaneous outflow of ^{3}H-NE and its metabolites in the absence of the drug (□, prior to the addition of cocaine; ▨, prior to the addition of phenoxybenzamine). The black columns indicate the outflow of radioactivity in excess of the corresponding control values for each fraction. Note the decrease in outflow of ^{3}H-DOPEG in the presence of cocaine. Shown are means of two paired experiments each. Abbreviations used are: NE, norepinephrine; DOPEG, 3,4-dihydroxyphenylglycol; OMDA, *O*-methylated-deaminated metabolites; DOMA, 3,4-dihydroxymandelic acid; and NMN, normetanephrine.

Figure 3, B shows that while NE was the main fraction in the cocaine-induced "delta", the deaminated acid, DOMA, accounted for nearly 25 per cent.

In contrast to these results, in the presence of PBA the deaminated glycol, DOPEG, was responsible for more than 80 per cent of the tritium released by the drug (Fig. 3, C). Both NE and DOMA contributed, although only to a small extent, to the release induced by PBA. The percentage of NE or DOMA in the enhanced efflux induced by PBA differed significantly from the corresponding values in the presence of cocaine ($P < 0{\cdot}001$ for NE and $P < 0{\cdot}05$ for DOMA). While NMN accounted for nearly 5 per cent of the "delta" induced by cocaine, the proportion of this metabolite in the increase in outflow caused by PBA was negligible. The difference between the contribution of NMN in the "delta" obtained in the presence of cocaine or PBA was significant ($P < 0{\cdot}025$).

The most striking difference between the metabolic pattern in the spontaneous outflow and that observed in the release induced by cocaine was the reversal of the relative importance of NE and the deaminated glycol, DOPEG, in these groups (Fig. 3, A and B).

Analysis of the total outflow of radioactivity rather than the increase induced by the drugs revealed that in the presence of cocaine the proportion of NE in the resting outflow rose from $8{\cdot}6 \pm 0{\cdot}61$ to $17{\cdot}8 \pm 1{\cdot}23$ ($P < 0{\cdot}001$), while the percentage of

DOMA increased from 4·3 ± 0·45 to 7·2 ± 0·89 ($P < 0·01$); the contribution of DOPEG, on the other hand, dropped significantly from 72·3 ± 1·46 to 60·8 ± 2·09 per cent ($P < 0·005$). During exposure to PBA, the proportion of DOPEG rose to 77·4 ± 1·4 ($P < 0·05$), while the OMDA fraction was reduced from 13·6 ± 0·77 in the controls to 7·82 ± 0·02 ($P < 0·005$).

The endogenous NE content of the tissues was determined at the end of the experiments (NE measured fluorimetrically minus labeled amine). In the tissues exposed to PBA, the endogenous NE was 9·1 ± 1·00 μg/g ($n = 3$), while in the cocaine group the NE level was 11·1 ± 1·16 μg/g ($n = 4$). The difference between these two groups was not significant.

DISCUSSION

The column chromatographic technique described in the present publication is a simple and efficient method for the separation of NE and its metabolites. The use of short columns and therefore rather small volumes for the washings and elutions increases the sensitivity of the method. In addition, separation with this technique can be achieved in a rather short time, thus ensuring optimal stability of the compounds. Another advantage of our procedure resides in the high recoveries for NE and all the metabolites with rather small cross-contaminations for which adequate corrections can be made.

The first step in this method involves the use of Alumina to separate non-catechols from catechols. The NE and DOPEG fractions which were eluted together from Alumina columns were subsequently separated on Dowex 50. A number of methods have been described for the isolation and determination of NE with the use of Alumina columns.[7,10–13] The results of the present publication clearly demonstrate that, when labeled compounds are dealt with, the contamination of ^{3}H-NE by ^{3}H-DOPEG is inevitable unless separation from Alumina is followed by an additional step. Several authors[14–17] have used eluates from Alumina for the estimation of radioactive NE or for the determination of the specific activity of the amine.[18–20] In view of the finding that the deaminated glycol, DOPEG, is eluted together with NE under these experimental conditions, several factors deserve careful analysis: (1) labeled NE can be grossly overestimated if metabolism via the deaminated glycol is significant; (2) determination of specific activities for NE can also lead to overestimations; since the magnitude of the error will be least pronounced for tissue estimations, a comparison of specific activities for NE in the tissue and in the efflux medium can be distorted; (3) inhibition of metabolism through MAO may remain undetected or it could lead to an apparent decrease in the specific activity of NE; (4) increased metabolism through MAO can easily be overlooked. In view of these sources of error, the results obtained with eluates from Alumina should be considered cautiously, particularly under experimental conditions in which metabolism of NE is important, as in spontaneous or induced release of the labeled transmitter (either by drugs or by nerve stimulation).

While metabolism of NE is important for the conditions mentioned above, the situation differs when the radioactivity retained in the tissue is considered. Under these conditions we found that more than 90 per cent of the radioactivity retained in the tissue is accounted for by the unchanged amine. Yet, it has been reported[21] that the ^{3}H-3,4-dihydroxyphenylglycolic aldehyde can be present in rather high concen-

trations in certain subcellular fractions. The deaminated aldehyde behaved like ^{3}H-NE on Alumina columns.[21]

For further separation of the labeled compounds in the Alumina eluate, the complete elution of all the catechols is essential. With the use of 0·2 N acetic acid, 0·04 N perchloric acid or 0·2 N HCl as eluents of the Alumina column, variable amounts of DOMA are extracted. The size of the column used might influence the extent of the contamination by DOMA. It has been reported, for instance, that 40 per cent of DOMA was recovered when eluted from the Alumina column with 0·04 N perchloric acid.[22] This value is rather high when compared with our results. However, differences in the size of the column and the volume of the eluent might be responsible for this discrepancy. The importance of different methodological conditions in the application of column chromatographic techniques should be illustrated by another example. In the method used by Rutledge and Weiner,[6] ^{3}H-DOPEG and ^{3}H-DOMA are eluted separately from Alumina with 1 N acetic acid followed by 0·2 N HCl. In our procedure, 1 N acetic acid eluted almost 20 per cent of the DOMA retained by the Alumina. The large size of the column used by Rutledge and Weiner[6] (4 × 0·8 cm) is most likely the reason for this difference. The same reason might be responsible for the retention of VMA by Alumina as reported by these authors. In our conditions, VMA appeared in the effluent (80 per cent) and was efficiently washed from Alumina with only 2 ml of water because of the small size of our column.

By employing Dowex × 50 W (H^+ form) equilibrated at pH 2, it was possible for us to use a cascade system both for the effluent and for the eluate (0·2 N acetic acid) from Alumina. In the cascade system, NE and NMN were efficiently retained by the resin in spite of being applied at different pH values (approx. 3 and 8 respectively). The use of small columns (0·5 × 1·5 cm) and of the × 4 type of the resin permitted elution in small volumes and direct collection in counting vials. If the separation of the *O*-methylated acid, VMA, from the *O*-methylated glycol, MOPEG is desired, an anion-exchange resin as described by Taylor and Laverty[23] can be used.

The metabolic pattern of the radioactivity released spontaneously from the rat vas deferens labeled with ^{3}H-NE has been determined by Langer[4] and by Tarlov and Langer[3] with the use of paper chromatographic techniques. These authors found that DOPEG was the main metabolite in the spontaneous outflow from this tissue. Yet, there are some quantitative differences between their results and the present observations. In our experimental conditions, DOPEG accounted for more than 70 per cent of the spontaneous outflow of radioactivity, while it was about 40 per cent in the paper by Langer.[4] On the other hand, DOMA was found to be less than 5 per cent while in the previous publication[4] it was reported to account for 30 per cent of the total radioactivity. The levo isomer of ^{3}H-NE was employed in the present experiments while Langer[4] used the labeled racemate. Consequently these quantitative differences may not be due entirely to differences in recoveries of the metabolites by the two methods but, in addition, to the differences in the metabolic pattern between (−) NE and (±) NE in this tissue (F. J. E. Stefano and K. H. Graefe, unpublished observations).

The concentrations of cocaine and PBA used in the present experiments were 3-fold higher than the ED_{50} for inhibition of neuronal uptake by these drugs in this tissue.[24] In spite of the use of nearly equipotent concentrations in their effect on neuronal uptake, the total release of tritium and the metabolic pattern of the transmitter re-

leased differed when both drugs were compared. With PBA an enhanced release of radioactivity was observed, while in the presence of cocaine there was no significant increase in the total outflow of tritium. Similar results have been reported for isolated guinea-pig atria.[2] As far as the metabolism is concerned, after PBA, DOPEG was the main metabolite released, while during exposure to cocaine the deaminated glycol consistently fell below the control values. This striking difference between the effects of the two drugs can be explained by assuming that, in addition to inhibiting the transport of NE across the neuronal membrane, PBA has an intraneuronal site of action. The release of ^{3}H-NE as deaminated metabolites has been used as a marker for the granular action of a drug.[25–27] Moreover, it has been shown that NE released by RO 4-1284 or PBA in guinea-pig atria is metabolized selectively to DOPEG.[2] Therefore, our results are compatible with the view put forward by Adler–Graschinsky *et al.*[2] that PBA has a reserpine-like action and that the deaminated glycol, DOPEG, is predominantly of presynaptic origin.

In contrast to the small effect on total outflow of radioactivity, cocaine caused a significant increase in the release of NE. Therefore, the simultaneous reduction in the outflow of DOPEG in the presence of cocaine appears to be a consequence of the inhibition of neuronal reuptake caused by the drug. According to this view, presynaptic deamination of NE to DOPEG would originate from: (a) NE leaking from the granules into the axoplasm and (b) NE recaptured through the neuronal uptake mechanism. The reduction of the DOPEG fraction may be a useful tool for the measurement of inhibition of neuronal reuptake provided that the drug tested has no effects on the storage granules.

The comparison of the distribution of the metabolites in the drug-induced outflow of labeled products above the basal values gave further insight into the origin of the metabolites. In the increased outflow obtained in the presence of cocaine, the proportions of both DOMA and NMN were at least 5-fold higher than the corresponding values in the enhanced release induced by PBA. It has been reported that a similar concentration of PBA inhibits extraneuronal uptake of NE in the rat heart.[24,28] Therefore, both metabolites appear to be of extraneuronal origin.

The finding that DOPEG is the main metabolite in the spontaneous outflow of the labeled transmitter is not restricted to the vas deferens of the rat. In several organs of different species, this metabolite represents an important proportion of the outflow both under resting conditions and during release induced by nerve stimulation or by drugs.[2–4] In the isolated perfused cat's spleen labeled with ^{3}H-NE, the deaminated glycol accounts for 60 per cent of the total radioactivity in spontaneous outflow (M. Dubocovich and S. Z. Langer, unpublished observations). In addition, recent results from our laboratory obtained in the isolated nictitating membrane of the cat show that the deaminated glycol is the main metabolite in the spontaneous outflow of radioactivity and in the increase induced by nerve stimulation. The latter can be reduced in the presence of cocaine (M. A. Enero and S. Z. Langer, unpublished observations). Since the method described in the present publication allows adequate separation and estimation of NE and its deaminated metabolites, it offers a useful tool in studies involving the metabolism of the adrenergic neurotransmitter.

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