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O- AND N-DEMETHYLATION OF METANEPHRINE-7-^{3}H *IN VIVO**

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Abstract—Metanephrine-7-^{3}H, given to rabbits by intravenous infusion, was found to be O- and N-demethylated *in vivo*, as shown by the occurrence of adrenaline, noradrenaline and 3:4-dihydroxymandelic acid in urine and liver.

INTRODUCTION

IT HAS been shown by Bacq and Bacq and Renson[2] that metanephrine and normetanephrine sensitize the nictitating membrane of cats towards both pre- and post-ganglionic sympathetic stimulation and towards the effects of adrenaline, noradrenaline and their own effects. A similar sensitization was also observed in the isolated rabbit uterus, the isolated rat vas deferens and the isolated seminal vesicle of guinea pig.

In discussing the mechanism of action of the sensitizing effect of metanephrine and normetanephrine, various suggestions have been made by these authors.

According to a first suggestion, metanephrine and normetanephrine react with "ineffective" α-receptors, which would account for their poor proper effects, leaving a relatively larger number of "effective" α-receptors available for subsequent reaction with noradrenaline, adrenaline or the methoxy-derivatives themselves.

It has further been suggested that metanephrine and normetanephrine may exert their sensitizing effect by inhibiting monoaminoxydase through substrate accumulation. This point of view is supported by other experiments of Bacq and Renson,[3] in which injection of 5-hydroxytryptamine, which is known to be a good substrate of monoaminoxydase, was shown to enhance the effect of pre- and post-ganglionic stimulation of the nictitating membrane of cats.

Finally, in order to explain the secondary contraction of the nictitating membrane, which occurs upon stimulation of the cervical sympathetic in metanephrine-pretreated animals, the question was raised whether the methoxy-metabolites could not be O-demethylated *in vivo*, with formation of the biologically active catecholamines from which they are derived.

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O-demethylating enzymes requiring both reduced TPN and oxygen and localized in the microsomes of rabbit liver, have been described by Axelrod[4, 5] and by Axelrod and Szara.[6]

It has been shown by Kopin *et al.*,[7] on the other hand, that after intravenous injection of metanephrine-7-^{3}H-methoxy-^{14}C in rats the ratio of ^{14}C:^{3}H was less in the free and combined metanephrine and in its deaminated products, isolated from urine, than in the metanephrine administered, suggesting the occurrence of O-demethylation of this substance *in vivo*.

In order to obtain more direct evidence, the present experiments were performed.

EXPERIMENTAL

Rabbits weighing from 1800 to 2100 g were infused with from 25 to 75 μc of metanephrine-7-^{3}H in 30 ml of physiological saline via the femoral vein over a 30-min period.

The animals were sacrificed 30 min after the end of the infusion. The urinary bladder was punctured and the urine collected in 10 ml of a 10% solution of trichloracetic acid. The liver was removed and homogenized in a 10% trichloracetic acid solution. The homogenate was filtered on a suction flask and the filtrate extracted three times with ether to remove the trichloracetic acid. The residual ether was evaporated *in vacuo* at 35° in a rotating evaporator.

Given the small amount of total radioactivity infused and since the subsequent analysis was restricted to free metanephrine and its free metabolites, the radioactivity recovered in plasma, heart and spleen was found to be too low to give reliable results. Accordingly, analysis was confined to urine and liver.

The separation of free metanephrine-7-^{3}H and its free ^{3}H-metabolites in urine and liver was performed in two different ways.

In the first method of separation 30 ml of concentrated urine or liver extract was passed through a 4 × 1 cm column of Amberlite IRC-50 (Rohm & Haas Co., Philadelphia, Pa.), prepared and buffered at pH 6·1, as previously described by Kirshner *et al.*[8, 9] The column is washed with 15 ml of water and the basic compounds are eluted from the column with 25 ml of 0·5 N acetic acid. The eluate is evaporated to dryness *in vacuo* at 35° using a rotating evaporator. The residue is dissolved in 0·5 ml of a 95% ethanol–0·1 N HCl solution and applied along a 15-cm line on Whatman No. 1 filter paper, together with "cold" reference compounds. After exposure to a water-saturated atmosphere for 15 min, the paper is subjected to ascending chromatography, using as solvent systems n-butanol saturated with normal HCl, or n-butanol–acetic acid–water (4:1:1), or benzyl alcohol–acetic acid–water (5:1:1).

When the liquid front has reached a height of from 20 to 25 cm, the filter paper is dried and a 2·5 cm strip is cut from each side of the chromatogram and sprayed with a 0·44% solution of potassium ferricyanide in phosphate buffer at pH 7·8, in order to locate the positions of adrenaline and noradrenaline. The position of metanephrine is determined by means of a subsequent spray with a 0·5% solution of copper sulphate. The central part of the chromatogram is cut into 1-cm strips parallel to the line of application and the radioactivity of each section measured with the Packard Tri-Carb Liquid Scintillation Spectrometer in 10 ml of a solution of 30% absolute alcohol and 70% toluene containing 4 g of 2:5-diphenyloxazole (PPO) and 100 mg of 1:4-bis-2-(5-phenyl-oxazolyl)-benzene (POPOP) per l. of toluene.

* Metanephrine-7-^{3}H was prepared by Dr. J. Renson at the Laboratory of Clinical Biochemistry, National Heart Institute, National Institutes of Health, Bethesda, Maryland.

The effluent and washing of the Amberlite IRC-50 column are combined and concentrated to 10 ml *in vacuo* using a rotating evaporator. The concentrate is adjusted to pH 1 with 6 N HCl and extracted three times in a double volume of ethyl acetate. The ethyl acetate is then shaken with an aqueous 5% solution of sodium bicarbonate, followed by re-extraction into ethyl acetate at pH 1. The organic phase is evaporated to dryness *in vacuo* using a rotating evaporator. The residue is dissolved in 0·5 ml of absolute ethanol and subjected to ascending chromatography on Whatman No. 1 filter paper, using n-butanol saturated with normal HCl or *iso*propyl alcohol–ammonia–water (40:1:9) as solvent systems. The position of the acidic compounds is determined by means of a spray with diazotized *p*-nitroaniline (25 ml of a 0·3% solution in 10% HCl plus 1·5 ml of 5% sodium nitrite), followed by a 20% sodium carbonate spray.

The second separation procedure is based on the adsorption of free catechols on alumina, prepared as previously described by Weil-Malherbe and Bone,[10] followed by elution with 25 ml of 0·2 N HCl. The eluate is evaporated to dryness *in vacuo* at 35° using a rotating evaporator. The residue is dissolved in a 95% ethanol–0·1 N HCl solution and subjected to ascending chromatography on Whatman No. 1 filter paper using n-butanol saturated with normal HCl, n-butanol–acetic acid–water (4:1:1), or benzyl alcohol–acetic acid–water (5:1:1) as solvent systems. Metanephrine, on the other hand, is extracted from the effluent and washing in ethyl acetate at pH 10. The extract is evaporated to dryness *in vacuo* at 35° using a rotating evaporator. The residue is dissolved in a 95% ethanol–0·1 N HCl solution and subjected to ascending chromatography using *n*-butanol saturated with normal HCl. Extraction of 3-methoxy-4-hydroxymandelic acid is performed as described in the first method of separation.

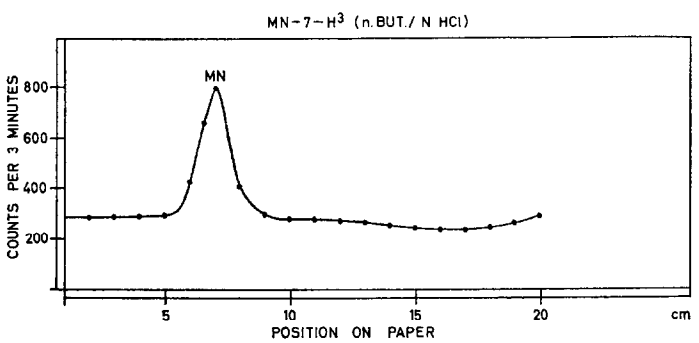

FIG. 1. Chromatogram of metanephrine-7-^{3}H on Whatman No. 1 filter paper: solvent, n-butanol saturated with N HCl. MN = metanephrine.

RESULTS

The purity of the metanephrine-7-^{3}H used in the present experiments was checked by paper chromatography, as shown in Fig. 1.

Using the above described combination of column and paper chromatography, intravenous infusion of this tritiated metanephrine in rabbits was found to be followed

by the occurrence of tritiated metanephrine and 3-methoxy-4-hydroxymandelic acid, as well as of adrenaline, noradrenaline and 3:4-dihydroxymandelic acid in both urine and liver, as illustrated in Figs. 2–8. 3:4-Dihydroxyphenylglycol was tentatively identified in urine on the basis of chromatographic data (Fig. 4).

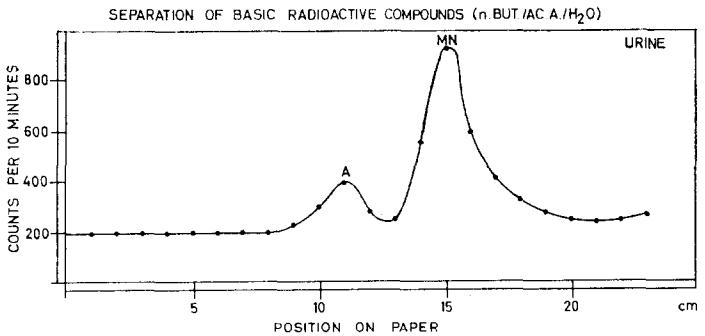

FIG. 2. Separation of basic compounds in urine on Whatman No. 1 filter paper: solvent, n-butanol–acetic acid–water (4:1:1). A = adrenaline; MN = metanephrine.

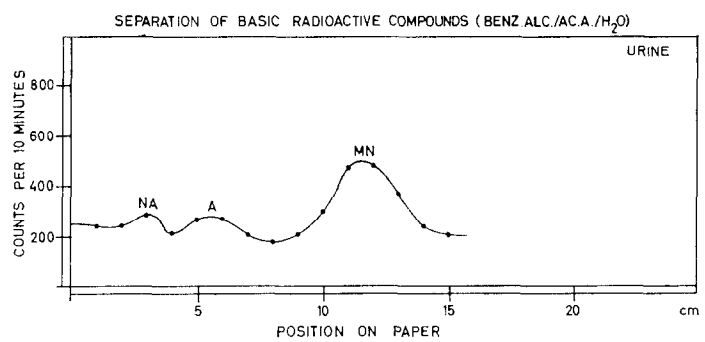

FIG. 3. Separation of basic compounds in urine on Whatman No. 1 filter paper: solvent, benzyl alcohol–acetic acid–water (5:1:1). NA = noradrenaline; A = adrenaline; MN = metanephrine.

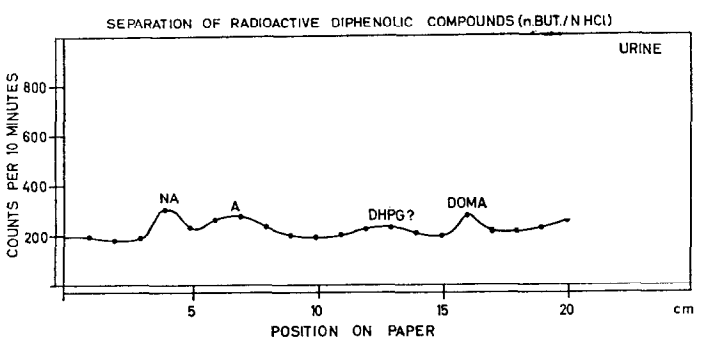

FIG. 4. Separation of diphenolic compounds in urine on Whatman No. 1 filter paper: solvent, *n*-butanol saturated with N HCl. NA: noradrenaline; A = adrenaline; DHPG = 3:4-dihydroxyphenylglycol; DOMA = 3:4-dihydroxymandelic acid.

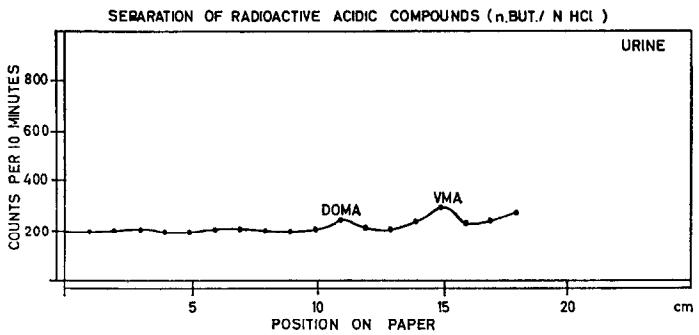

FIG. 5. Separation of acidic compounds in urine on Whatman No. 1 filter paper: solvent, n-butanol saturated with N HCl. DOMA = 3:4-dihydroxymandelic acid; VMA = 3-methoxy-4-hydroxymandelic acid.

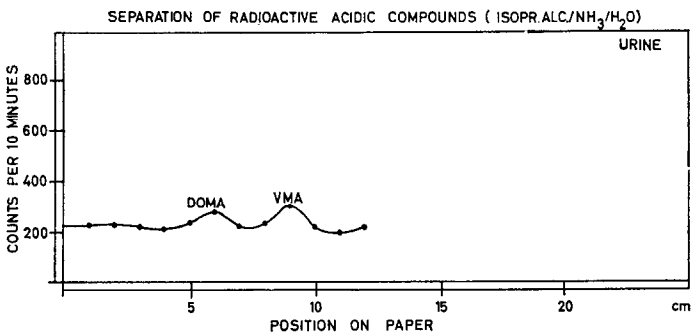

FIG. 6. Separation of acidic compounds in urine on Whatman No. 1 filter paper: solvent, *iso*propyl alcohol–ammonia–water (40:1:9). DOMA = 3:4-dihydroxymandelic acid; VMA = 3-methoxy-4-hydroxymandelic acid.

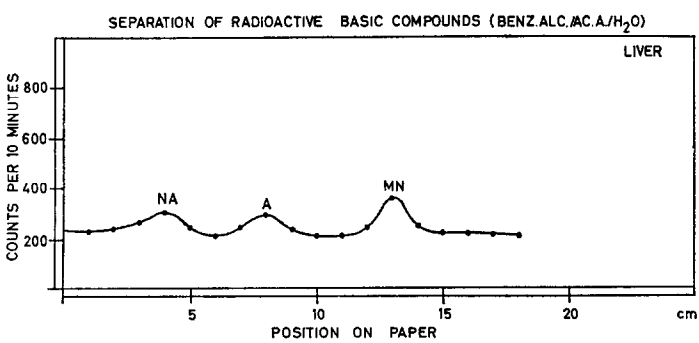

FIG. 7. Separation of basic compounds in liver on Whatman No. 1 filter paper: solvent, benzyl alcohol–acetic acid–water (5:1:1). NA = noradrenaline; A = adrenaline; MN = metanephrine.

DISCUSSION

The present experimental observations produce direct proof of the occurrence of both O- and N-demethylation of metaneprhine *in vivo*, as shown by the presence of adrenaline and noradrenaline in urine and liver, additional evidence being given by the identification of 3:4-dihydroxymandelic acid in urine and liver.

These findings are in accordance with the *in vitro* experiments of Axelrod[4, 5] and Axelrod and Szara,[6] and with recent *in vivo* experiments in cats by Verly *et al.*[11] The fact that no evidence for the presence of normetanephrine in urine or liver was found after administration of metanephrine confirms previous observations in rats by Axelrod.[12]

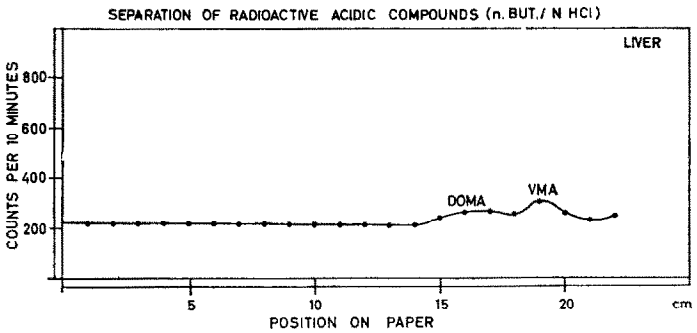

FIG. 8. Separation of acidic compounds in liver on Whatman No. 1 filter paper: solvent, n-butanol saturated with N HCl. DOMA = 3:4-dihydroxymandelic acid; VMA = 3-methoxy-4-hydroxymandelic acid.

These observations lend further support to the hypothesis proposed by Bacq and Renson[2] that O-demethylation of metanephrine and normetanephrine may play a role in the mechanism of the sensitizing effect of these substances towards sympathetic stimulation of some adrenergic structures, such as the nictitating membrane.

Furthermore, *in vivo* demethylation of metanephrine and normetanephrine may be of importance with regard to the development and maintenance of chronic arterial hypertension. It is known that catecholamines and their metabolites are not excreted in the urine in higher amounts than normal in dogs with chronic renal hypertension[13] or in patients with essential arterial hypertension.[14–23] In view of the present experimental findings, it is not excluded that in these cases metanephrine and normetanephrine are O- and N-demethylated, respectively O-demethylated, to a larger extent than in normotensive conditions. This would result in a higher biological activity of the amount of catecholamines liberated at the periphery, without increasing this amount itself and without changing the amounts of catecholamines and metabolites excreted in the urine.

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