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Catecholamine and 5-hydroxyindole metabolism in immunosympathectomized rats

P. M. CEASAR, C. R. J. RUTHVEN AND M. SANDLER

Bernhard Baron Memorial Research Laboratories and Institute of Obstetrics & Gynaecology, Queen Charlotte's Maternity Hospital, London, W.6

1. The daily urinary excretion of normetadrenaline, metadrenaline, 4-hydroxy-3-methoxyphenylglycol, 4-hydroxy-3-methoxymandelic acid, dopamine, homovanillic acid, 5-hydroxytryptamine and 5-hydroxyindolylacetic acid has been estimated in the urine of immunosympathectomized and control rats.

2. A method is given which allows separate spectrophotometric determination of 4-hydroxy-3-methoxyphenylglycol and 4-hydroxy-3-methoxymandelic acid in the same sample of rat urine. Metadrenaline and normetadrenaline were estimated by a modification of the method of Anton & Sayre (1966).

3. Approximately 17% of the 4-hydroxy-3-methoxymandelic acid in rat urine is excreted in the free state, over 60% as a glucuronide conjugate and the remainder as a sulphate conjugate.

4. Urinary excretion of 4-hydroxy-3-methoxyphenylglycol was approximately halved in immunosympathectomized rats ($P<0.001$). 4-Hydroxy-3-methoxymandelic acid output was significantly increased in treated animals ($P<0.05$) when expressed in terms of body weight. No significant difference was detected for any of the other compounds investigated.

5. The results are discussed in the light of previous knowledge of the effect of immunosympathectomy on catecholamine metabolism.

Immunosympathectomy (Levi-Montalcini & Booker, 1960), which results in permanent degeneration of a major part of the peripheral sympathetic nervous system, provides conditions uniquely suited to the study of many aspects of sympathetic function in the living animal (for review, see Levi-Montalcini & Angeletti, 1966). Observations on its effect on the metabolism of biologically active monoamines have been mainly confined to individual tissues where sharp decreases in catecholamine concentration (Levi-Montalcini & Angeletti, 1962; Hamberger, Levi-Montalcini, Norberg & Sjöqvist, 1965; Klingman, 1965; Visscher, Lee & Azuma, 1965; Zaimis, Berk & Callingham, 1965) are observed after immunosympathectomy. Uptake of radioactive noradrenaline is reduced at many sites but unchanged at others, while an increase of uptake has been reported in the vas deferens (Sjöqvist, Titus, Michaelson, Taylor & Richardson, 1965; Iversen, Glowinski & Axelrod, 1965, 1966; Zaimis *et al.*, 1965).

Apart from some radioactive studies (Iversen *et al.*, 1966 ; Sjöqvist, Taylor & Titus, 1967) and some data on the urinary output of free noradrenaline and adrenaline (Carpi & Oliverio, 1964 ; Brody, 1964 ; Schönbaum, Johnson & Sellers, 1966), however, little is known of the overall effect of immunosympathectomy on catecholamine production and metabolism. The present work compares the urinary excretion of some catecholamine metabolites in normal and immunosympathectomized rats, in addition to providing similar data on dopamine excretion. Urinary output of 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindolylacetic acid (5-HIAA), has also been measured in these two groups of animals, because an increase in the number of 5-HT containing cells is found in the intestinal mucosa of immunosympathectomized rats and mice (Hamberger *et al.*, 1965 ; Iversen *et al.*, 1966 ; Thompson & Campbell, 1966).

Methods

Animals

Male (455–775 g body weight) and female (210–585 g body weight) histologically proven immunosympathectomized (nerve growth factor antiserum obtained from Abbott Laboratories, North Chicago, Illinois) Wistar rats and their litter-mate controls, all aged 18–20 months, were put at our disposal for urine collection by Professor Eleanor Zaimis at the Royal Free Hospital School of Medicine, London. We are greatly indebted to her for facilities provided, and for allowing us to use the valuable colony of animals which she had prepared.

Urine collection

Immunosympathectomized or control rats were placed separately in cylindrical (26·5 cm diameter) Perspex metabolic cages fitted with floors of stainless steel mesh resting on large glass funnels. The mesh was siliconized by treating with polymethylhydrogen siloxane (Hopkin and Williams, Ltd.), thus preventing possible catalytic oxidation of catecholamines on contact with the metal. Faeces were separated from urine by interposing a conical glass bulb, apex downwards, between the tip of the large glass funnel and the urine collection vessel. Urine was collected for 24 hr into 6 N HCl (1 ml.) during which time the animal was fasted from solid food, but allowed water *ad libitum*. At the end of the collection period, any urine on the funnel was washed into the collection vessel with distilled water. Specimens were stored at $-15°$ C until required.

Determination of 4-hydroxy-3-methoxyphenylglycol (HMPG) and 4-hydroxy-3-methoxymandelic acid (VMA)

Two 24 hr urine collections from the same rat were pooled and the resulting mixture divided equally. One half was estimated directly and the other after the addition of internal standards (20 μg HMPG and 20 μg VMA). Samples were brought to pH 11 and a slight excess (about 0·4 ml.) of saturated $BaCl_2$ added to precipitate inorganic phosphate and sulphate which might inhibit the enzymatic hydrolysis. After centrifugation, the decanted supernatant solution was brought to pH 6·4–6·5 and incubated for 18 hr at 37°C with 0·2 ml. of a β-glucuronidase-sulphatase preparation (Suc d'*Helix pomatia*, Industrie Biologique Francaise, Gennevilliers, Seine, France).

The hydrolysed urine was passed through a column (10 mm bore and 6 cm bed) of 4 g anion-exchange resin, AG1-X2 (200–400 mesh, acetate form) (Bio-Rad Laboratories, Richmond, California). The effluent was discarded. HMPG, weakly bound to the resin, was eluted with deionized water (25 ml.), thus separating it from VMA, which is held more firmly. VMA was eluted from the resin with 30 ml. of 1 M ammonium acetate buffer (pH 4·8) (Kirshner, Goodall & Rosen, 1958).

The water eluate was acidified (HCl) to pH 4 and extracted twice with 30 ml. of ethyl acetate. These, and subsequent extractions in this procedure, were made by shaking for 5 min on a mechanical shaker. After centrifugation, portions (25 ml.) of organic phase from each extract were combined and evaporated under vacuum at 35° C using a "Rinco" rotating evaporator. The dry residue was dissolved in 4N-NH_4OH (11 ml.) and portions (5 ml.) were treated for test and blank as described by Ruthven & Sandler (1965). HMPG in the test was converted to vanillin by the action of periodate. After removing excess reagent with sodium meta-bisulphite and adjusting the pH to 7·4–7·5, the vanillin was extracted with toluene and back-extracted into 1M K_2CO_3, where it was measured spectrophotometrically against water at 360 mμ and 380 mμ. The blank was treated as the test except that water was substituted for the periodate reagent.

The ammonium acetate eluate was brought to pH 1 with 50% v/v H_2SO_4 (2 ml.) and 15 ml. aliquots were taken for test and blank determination. The test solution was saturated with NaCl, extracted twice (2×20 ml.) with ethyl acetate and a portion (15 ml.) of each extract combined and evaporated under vacuum at 35° C using a rotary evaporator (Rotary Evapo-Mix, Buchler Instruments, N.Y.). The residue was dissolved in 1M K_2CO_3 (1 ml.) and VMA assayed by the method of Pisano, Crout & Abraham (1962), starting after the first carbonate extraction. This method also involves conversion of the compound to vanillin with periodate, then extraction from neutral solution into toluene and spectrophotometric measurement at 360 mμ after re-extraction into carbonate. A blank was obtained similarly but omitting periodate.

To check the specificity of these estimations, spectrophotometric readings were made at four different wavelengths, 330, 350, 360 and 380 mμ. The spectral curves obtained in the assay of HMPG were characteristic of those given by pure vanillin but some distortion between 330 and 350 mμ was observed in the curves obtained during the assay of VMA (Sandler and Ruthven, 1961). *p*-Hydroxybenzaldehyde, produced in the estimation by the oxidation of urinary *p*-hydroxymandelic acid, was the probable cause of this interference, and was corrected for by solving simultaneous equations as described by Pisano, Oates, Karmen, Sjoerdsma & Udenfriend (1961), incorporating the extinctions at 330 and 350 mμ.

In these procedures, normetadrenaline or metadrenaline, which also yield vanillin on oxidation, did not contribute to the final extinction when added (100 μg) to the urine hydrolysates.

Conjugation of VMA in rat urine

Two experiments were performed. In the first, urine from fifteen rats (220 ml.) was pooled and in the second from fourteen rats (231 ml.). Three duplicated 25 ml. portions of the pooled urine specimen were used in each experiment, one sample of each pair containing 20 μg VMA as internal standard. Free VMA was estimated

in a pair of unhydrolysed urine samples. Total VMA was measured in the second pair after hydrolysis with the β-glucuronidase-sulphatase enzyme preparation. Finally, determination of free VMA plus VMA glucuronide only was carried out in the rat urine after preliminary hydrolysis with a β-glucuronidase preparation (Type II, Sigma Chemical Company, St. Louis, U.S.A.) at pH 6·8. VMA was estimated quantitatively as in the method described above.

Differential determination of normetadrenaline and metadrenaline (modification of the method of Anton & Sayre, 1966)

Rat urine (3 ml. of a 24 hr collection) was hydrolysed with acid and extracted as described by Anton & Sayre (1966): after washing with isoamyl alcohol to remove a fluorescent contaminant, the urine was saturated with dipotassium hydrogen phosphate powder and extracted with ether. The metadrenaline and normetadrenaline in the ether phase were extracted with 0·1N HCl and then extracted back into ether again after first saturating the aqueous phase with dipotassium hydrogen phosphate as before. Finally the second ether extract was shaken with 0·01N HCl, which was separated and retained for spectrofluorimetry.

To overcome inaccuracies due to erratic recoveries which the original method gave in our hands, labelled ^{14}C-metadrenaline (0·3 ml. of solution giving 25,000 d.p.m.) and ^{3}H-normetadrenaline (0·3 ml. of solution giving 115,000 d.p.m.) were added as internal standards to the hydrolysed urine before extraction. An insufficient weight of non-radioactive metadrenaline was present in these standards to affect the accuracy of the assay. The overall recovery of the procedure was calculated by comparing the counts added with those from each isotope measured in an aliquot (0·1 ml.) of the final 0·01N HCl extract. Radioactivity was measured with a " Tri-Carb " liquid scintillation spectrometer set for double-labelled counting.

After hydrolysis, specimens were mixed with a vortex mixer during each extraction. Vigorous mixing is imperative since the overall recovery dropped from 60–70% to values as low as 5% when a horizontal mechanical shaker was used for equilibration.

Fluorophor formation in aliquots of the final extract in 0·01N HCl was carried out essentially as described by Anton & Sayre. Aliquots (0·1 ml.) diluted to 0·4 ml. with 0·01N HCl were assigned to test, test plus external standard (non-radioactive metadrenaline (10 ng) or normetadrenaline (30 ng) as appropriate) and blank tubes respectively. Two sets were prepared, one for periodate oxidation after adjusting to about pH 5·0 to determine metadrenaline plus normetadrenaline, and the other for oxidation at about pH 1·5 for the estimation of metadrenaline alone. The reaction was stopped with sodium sulphite and alkaline ascorbate. Measurements of relative fluorescence intensity (RFI) were made at activation/fluorescent wavelengths of 409/519 mμ at about pH 5·0 and at 422/531 mμ at about pH 1·5, using an Aminco-Bowman spectrophotofluorimeter with slit arrangement No. 4. Neglecting any fluorescence due to normetadrenaline in the sample oxidised at pH 1·5, the amount of metadrenaline or normetadrenaline in the test aliquot was calculated from the RFI of the appropriate external standard and test after correction for blank. Urinary levels of metadrenaline and normetadrenaline were calculated from these values and the overall recovery determined radioisotopically.

Determination of dopamine (3-hydroxytyramine), homovanillic acid (HVA) 5-hydroxytryptamine and 5-hydroxyindolylacetic acid

Free dopamine was estimated in unhydrolysed urine by the method of Sourkes & Murphy (1961), which involved adsorption of urinary dopamine on to alumina at pH 8·4 followed by elution with acetic acid. After iodine oxidation and rearrangement of the product in the presence of alkali and sulphite, the fluorophor which developed was stabilized and intensified with 5N HCl containing ascorbic acid added before measurement.

As described by Sato (1965), free HVA in the urine was absorbed on Dowex AG1 anion exchange resin, eluted with NaCl solution, extracted with organic solvent and oxidized to a fluorescent product by potassium ferricyanide in ammonium hydroxide.

Urinary 5-HT was estimated by the method of Oates (1961). The base was adsorbed on a column of Amberlite IRC-50 cation-exchange resin ; the column was washed with buffer and the 5-HT eluted with 1N HCl. A portion of the eluate was made 3N with concentrated HCl and measured spectrophotofluorimetrically.

By the assay procedure of Contractor (1966), 5-HIAA in acidified urine was selectively adsorbed on Sephadex G-10. After interfering fluorophors had been leached out, 5-HIAA was eluted with 0·02N NH_4OH. The eluate was adjusted to 3N with concentrated HCl and measured spectrophotofluorimetrically. During this determination, blanks were read 20 min after the addition of saturated potassium persulphate, since 15 min were required to attain steady blank readings with rat urine.

Materials

3-Hydroxytyramine hydrochloride (Koch-Light Laboratories Ltd.); bis (4-hydroxy-3-methoxyphenylglycol) piperazine salt (Regis Chemical Co., Chicago, Ill.); (±)-4-hydroxy-3-methoxymandelic acid (British Drug Houses Ltd.); (±)-metanephrine ((±)-metadrenaline) hydrochloride and (±)-normetanephrine ((±)-normetadrenaline) hydrochloride (Calbiochem, Los Angeles, Calif.); L-adrenaline (Koch-Light Laboratories Ltd.); homovanillic acid (Calbiochem); serotonin (5-hydroxytryptamine) creatinine sulphate (Koch-Light Laboratories Ltd.); 5-hydroxyindolyl-3-acetic acid (Koch-Light Laboratories Ltd.); (±)-normetanephrine-7-H^3 ((±)-normetadrenaline-7-^3H) hydrochloride (3,700 mc/m-mole) in 0·01N acetic acid and S-adenosyl-(−)-methionine (S-methyl-^{14}C) (approx. 40 m/m-mole) in dil. H_2SO_4 (New England Nuclear Corp., Boston, Mass.).

Preparation of ^{14}C-methoxy-labelled metadrenaline

^{14}C-Methoxy-labelled metadrenaline was prepared by a procedure based on the work of Axelrod, Albers & Clemente (1959) and Inscoe, Daly & Axelrod (1965). Rat liver (5 g) was homogenized in cold isotonic KCl (5 vol.), centrifuged (14,000 *g* for 20 min) and a portion (0·1 ml.) of the supernatant added to the following mixture; water (0·5 ml.), 0·5M phosphate buffer (pH 7·8) (0·2 ml.), 0·1M $MgCl_2$ (0·1 ml.), 0·1% w/v adrenaline in 0·01N HCl (0·1 ml.) and ^{14}C-S-adenosyl-(−)-methionine (approx. 1 μc). After incubation at 37° C for 90 min, the labelled metadrenaline was extracted into *iso*amyl alcohol (2 × 5 ml.) from the mixture, which had been previously made alkaline with 0·5M borate buffer (0·5 ml., pH 10). The

combined extracts, acidified with glacial acetic acid (0·05 ml.), were evaporated under vacuum at 40° – 45° C. ^{14}C-Metadrenaline was isolated from the residue by paper chromatography (*n*-butanol-glacial acetic acid-water, 12:3:5). It appeared to be chromatographically pure in this solvent and in *tert.*-amyl alcohol-0·880 ammonia, 4:1. The product was stored in solution (0·01N HCl) at – 15° C.

Results

The urinary excretion of normetadrenaline, metadrenaline, HMPG and VMA was determined on male rats only, whereas animals of both sexes were used for the measurement of dopamine, HVA, 5-HT and 5-HIAA. Results are given in terms of μg/24 hr and μg/kg per 24 hr (Table 1).

A comparison of the urinary excretion of metabolites in immunosympathectomized and control rats shows that only HMPG output differed significantly ($P<0·001$) in the two groups (Table 1), excretion being approximately halved in treated animals. In contrast, VMA output, when expressed as μg/kg per 24 hr, was slightly increased in the treated animals although this difference is significant only at the 5% level. The increase in VMA output is not significant when expressed as μg/24 hr.

There was a tendency for the animal to animal variation in metabolite levels to be less in immunosympathectomized animals than in controls: 5-HIAA (μg/kg

TABLE 1. *Excretion of catecholamine and 5-hydroxyindole metabolites in the urine of immunosympathectomized and control rats*

	Immunosympathectomized rats Mean excretion ± S.E.M.			Control rats Mean excretion ± S.E.M.		
	No. of expts.	μg/24 hr	μg/kg per 24 hr	No. of expts.	μg/24 hr	μg/kg per 24 hr
Dopamine	10	2·90 ± 0·29	5·47 ± 0·44	8	3·62 ± 0·42	6·62 ± 0·79
HVA	9	25·35 ± 3·15	52·77 ± 9·32	9	31·97 ± 4·07	71·62 ± 11·69
Normetadrenaline ‡	6	2·18 ± 0·13	3·89 ± 0·35	7	2·54 ± 0·54	4·02 ± 0·87
Metadrenaline	6	0·77 ± 0·08	1·36 ± 0·16	7	0·87 ± 0·15	1·36 ± 0·21
HMPG ‡	7	30·32 ± 1·11*	58·97 ± 2·71 *	6	67·73 ± 3·73	108·00 ± 4·19
VMA ‡	6	10·53 ± 0·84	19·99 ± 1·54 †	6	9·20 ± 0·77	14·88 ± 1·40
5-HT	5	1·39 ± 0·39	2·30 ± 0·15	5	1·60 ± 0·22	3·04 ± 0·39
5-HIAA	7	44·48 ± 5·09	87·77 ± 6·69	7	48·4 ± 8·93	86·31 ± 13·73

* $P < 0·001$ compared with controls.
† $P < 0·05$ compared with controls.
‡ Free plus conjugated.

TABLE 2. *Conjugation of VMA in rat urine*

	Urine No. 1		Urine No. 2	
	μg/25 ml.	% total VMA	μg/25 ml.	% total VMA
Total	28·4	100·0	18·3	100·0
Free VMA	4·4	15·6	3·4	18·5
Free VMA + VMA glucuronide	22·1	78·0	14·9	81·4
VMA glucuronide (by difference)	17·7	62·4	11·5	62·9
VMA sulphate (by difference)	6·3	22·0	3·4	18·6

Total VMA was estimated after enzymatic hydrolysis with a β-glucuronidase-sulphatase preparation. Free VMA was measured in unhydrolysed urine and free VMA plus VMA glucuronide assayed after hydrolysis with a β-glucuronidase preparation.

per 24 hr), normetadrenaline (μg/24 hr) and HMPG (μg/24 hr) show a significant reduction in variation ($P<0.05$) and all the other compounds measured exhibit this trend.

The results of the VMA conjugation studies are shown in Table 2, which shows that the major part of the VMA excreted is in the form of a glucuronide conjugate.

Discussion

HMPG is the predominant catecholamine metabolite in rat urine (Kopin, Axelrod & Gordon, 1961). During the present investigation, it was the only compound in either metabolic pathway studied, including normetadrenaline, to show a significant alteration in urinary output after immunosympathectomy. The observed decrease tended to run in parallel with the fall in output of free noradrenaline previously reported in immunosympathectomized animals (Brody, 1964; Carpi & Oliverio, 1964; Schönbaum *et al.*, 1966). Measurement of such a selective reduction in urinary HMPG excretion may thus provide a convenient index during life of the extent to which sympathetic nerve growth has been retarded in the immunosympathectomized rats.

Apart from providing information on the effect of immunosympathectomy on urinary output of certain catecholamine and 5-hydroxyindole metabolites, this study has established a set of normal values for the adult rat where previous firm data had been scanty. Apart from a passing reference by Armstrong & McMillan (1959), the fact that VMA is excreted in conjugated form in the rat has completely escaped attention. Our observations have shown however, unlike Armstrong & McMillan who were unable to detect any unconjugated VMA in rat urine, that a minor, but significant, proportion is excreted in a free form. Whereas the major proportion is conjugated as a glucuronide, our results indicate that a small amount of sulphate conjugation of VMA also occurs in the rat. The comparatively low total output is a further indication that reduction of the aldehyde formed by oxidative deamination of the amine, rather than oxidation as in the human, is the predominant metabolic route in this species. Whatever the species, there is evidence from direct study that HMPG rather than VMA may be formed primarily in many tissues such as rabbit atrium (Rutledge & Weiner, 1967), brain of rabbit (Rutledge & Jonason, 1967), rat (Glowinski, Kopin & Axelrod, 1965; Schanberg, Breese, Schildkraut, Gordon & Kopin, 1968), dog (Maas & Landis, 1968) and cat (Mannarino, Kirshner & Nashold, 1963), guinea-pig vas deferens (Weiner & Rabadjija, 1968), human neural crest tumour in tissue culture (La Brosse, Belehradek, Barski, Bohuon & Schweisguth, 1964), and guinea-pig and human placenta (Morgan, 1968, personal communication). The predominant output of VMA in human (Armstrong, McMillan & Shaw, 1957; La Brosse, Axelrod & Kety, 1958) and guinea-pig urine (Smith, 1961) may thus represent a secondary conversion of HMPG to VMA (La Brosse, 1966; La Brosse, Schweisguth & Bohuon, 1968).

The output of the noradrenaline precursor and third endogenous catecholamine, dopamine and its main O-methylated deaminated metabolite, HVA, has not previously been reported in the immunosympathectomized animal. There was no significant change compared with controls in either case. These results are compatible with the view that the amount of dopamine associated with the sympathetic nervous system is at most a small percentage of the total body depot. It is likely that the study of animals treated with 6-hydroxydopamine which appears to provide

a more complete chemical sympathectomy (Thoenen & Tranzer, 1968) will enable any minor variations in this pathway to be detected.

The increased intestinal 5-HT levels in immunosympathectomized animals (Hamberger *et al.*, 1965; Iversen *et al.*, 1966; Thompson & Campbell, 1966) were not reflected in these studies by an increased excretion of 5-HT or its major oxidatively deaminated metabolite, 5-HIAA, despite the fact that the gastrointestinal tract of the rat contains about 60% of total body 5-HT (Erspamer, 1954). Presumably the elevated gut levels are associated with a relatively low turnover. There was, however, a wide scatter in excretion values of 5-HIAA in both sets of rats, a factor which might mask any change occurring in treated animals.

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