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METABOLISM OF OF CATECHOLAMINES— IDENTIFICATION AND QUANTIFICATION OF 3-METHOXY-4-HYDROXYPHENYLGLYCOL GLUCURONIDE IN HUMAN URINE*

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Abstract—The glucuronic acid conjugate of 3-methoxy-4-hydroxyphenylglycol (MHPG) has been isolated from human urine and its identification is described. This compound has been found to be a metabolite of DL-MHPG-^{3}H and of DL-norepinephrine-^{14}C. The quantities of MHPG, MHPG sulfate and MHPG glucuronide which are present in normal human urine, and in the urine of patients with neuroblastoma, ganglioneuroma and pheochromocytoma, indicate that the glucuronic acid conjugate is a major metabolite of MHPG. In addition, MHPG and its conjugates are markedly increased in the urine of most patients with tumours of neural crest origin. This finding provides further evidence that MHPG is the major metabolite formed within these tumours, and the assay of urinary MHPG, especially after enzymatic hydrolysis of the glucuronide and sulfate conjugates, should be of value in the diagnosis of tumors of neural crest origin.

3-METHOXY-4-hydroxyphenylglycol (MHPG)‡ was first found to be a catecholamine metabolite by Axelrod *et al.*[1] when they identified its sulfate conjugate in rat urine after the intraperitoneal (i.p.) injection of norepinephrine-7-^{3}H. In 1960, free MHPG was identified by Kopin and Axelrod[2] in pheochromocytoma tumor tissue. In 1963, MHPG was found to be the major metabolite when ^{3}H-norepinephrine (^{3}H-NE) was incubated with neuroblastoma tumor in tissue culture,[3] and subsequent study of the metabolism of DL-MHPG-7-^{3}H, after its intravenous (i.v.) administration to the same patient (P. A.) from whom the neuroblastoma tumor had been almost completely removed, revealed that the MHPG was largely metabolized to 3-methoxy-4-hydroxymandelic acid (VMA); (34 per cent), to the sulfate conjugate of MHPG (7·3 per cent), 15·4 per cent of the tritium was found as tritium-labeled water, and the largest fraction of the labeled material (39·7 per cent) appeared to be a single compound more polar than the sulfate conjugate.[4] To determine whether this highly

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‡ The abbreviations used are: MHPG, 3-methoxy-4-hydroxyphenylglycol; NE, norepinephrine; VMA, 3-methoxy-4-hydroxymandelic acid.

polar compound was also a metabolite of NE, MHPG-7-^{3}H and NE-2-^{14}C were administered to another patient with a "secreting" neural crest tumor and urine was collected for 96 hr. The same highly polar compound was isolated by paper chromatography and was found to contain tritium and ^{14}C in constant ratio after repeated chromatography. This result indicated that the labeled compound is a metabolite of both NE and MHPG.

This paper reports the isolation of this metabolite and its identification as the glucuronic acid conjugate of MHPG. Some quantitative results of MHPG, as the free compounds, as the sulfate and as the glucuronic acid conjugate in the urine of normal individuals and patients with tumors of neural crest origin are also presented.

MATERIALS AND METHODS

DL-MHPG-7-^{3}H was synthesized from 3-methoxy-4-hydroxyphenacyl alcohol by catalytic reduction with tritium.* DL-Norepinephrine (carbinol-^{14}C) DL-bitartrate (DL-NE-2-^{14}C) was obtained commercially.†

DL-MHPG-7-^{3}H (126·9 μc, 2·7 μg) was administered intravenously to a patient with a retroperitoneal ganglioneuroma; 4 hr and 37 min later, DL-NE-2-^{14}C (10·4 μc, 415 μg) was also administered i.v. and individual urine specimens were collected for 96 hr. In addition, urine collected from this patient before the injections was used for the isolation of the glucuronic acid conjugate. The details of the procedure for the isolation are described below.

The solvent systems used for paper chromatographic separation were as follows:

I. *n*-butanol–ethanol–water (4:1:1), descending;
II. *n*-butanol–acetic acid–water (4:1:1), descending;
III. isopropanol–aqueous ammonia–water (8:1:1), descending;
IV. benzene–propionic acid–water (8:1:1), ascending;
V. pyridine–acetone–aqueous ammonia–water (10:6:1:4), ascending;
VI. isopropanol–formic acid–water (8:1:1), ascending;
VII. ethyl acetate–acetic acid–formic acid–water (18:3:1:4), ascending;
VIII. acetone–ethanol–isopropanol–0·05 M borate buffer, pH 10·0 (3:1:1:2), descending.

Whatman No. 3MM filter paper was used for paper chromatography throughout this investigation. For the detection of MHPG and glucuronic acid on chromatograms, diazotized *p*-nitroaniline[5] and aniline-citrate[6] reagents were used respectively.

Creatinine content in urine was determined by a modification of Jaffe's method.[7] MHPG was assayed colorimetrically as described previously[4] and linearity was obtained over the range of 0·5–10 μg MHPG. Glucuronic acid was assayed by Dische's method.[8] Elementary analysis was carried out by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

The radioactivity was assayed in a fluor solution, containing 10 ml toluene, 0·4% diphenyloxazole and 0·01% 1,4-*bis*-2(4-methyl-5-phenyloxazolyl)-benzene and 2 ml ethanol, in a liquid scintillation spectrometer.‡ Corrections were made for quenching and for spill-over of ^{14}C into the ^{3}H count by using internal standards of toluene-^{3}H and benzene-^{14}C.

* The labeling with tritium was carried out by The New England Nuclear Corp., Boston, Mass.
† Nuclear Chicago Corporation, Des Plaines, Ill.
‡ Tri-Carb liquid scintillation spectrometer, model 3002, Packard Instrument Co., Downers Grove, Ill.

Isolation of MHPG glucuronide. Of the urine excreted between 2·5 and 3·5 hr after MHPG-^{3}H administration, 100 ml was mixed with 2·0 l. of pooled urine collected from the same patient before the injection. The mixed specimen contained 0·76 mg creatinine per ml, and paper chromatographic examination of the specimen revealed that the radioactivity was distributed among four compounds: free MHPG, MHPG sulfate, VMA and an unidentified compound with a low R_f value in each solvent system. The urine was adjusted to pH 6·5 by adding 5N aqueous ammonia, filtered, and passed through a 6·5 × 25 cm column of Dowex 1-X8 (100–200 mesh), acetate form. The column was washed with 1 l. of water and with 2 l. of 0·2 N acetic acid. The unidentified compound was eluted from the column with 4 N acetic acid at a volume between 450 and 1100 ml. The eluate in this range was concentrated by lyophilization to 60 ml, adjusted to pH 7·0 with 5 N aqueous ammonia, and applied on a 2·7 × 17 cm column of Dowex 1-X8 (200–400 mesh), acetate form. The column was washed by 20 ml water; 2 N acetic acid was passed through the column and the radioactivity of the unidentified compound emerged in a fraction of the eluate between 920 ml and 1160 ml. This portion of the eluate was lyophilized; the dry residue was dissolved in 1·6 ml water and applied on a strip of filter paper 27 × 57 cm, and the strip was chromatographed in solvent system II. A radioactive area was located at R_f 0·23 and the compound was eluted from this area by 48 ml water. To remove yellow pigment in the eluate, it was further applied on a 1 × 11 cm column of Dowex 1-X8 (200–400 mesh), acetate form, and 5 ml of water, 30 ml of 1 N acetic acid and 60 ml of 2·5 N acetic acid, were passed through the column successively. The radioactive compound was then eluted from the column by an additional 40 ml of 2·5 N acetic acid and this eluate was lyophilized to dryness. The dry residue was dissolved in 5·0 ml water and streaked on two strips of filter paper (6 × 57 cm) for paper chromatography in solvent system III. The strips used for this particular purpose were washed by water before use to eliminate possible contaminates. The chromatograms were scanned, the radioactive area was cut out at R_f 0·19, and the compound was eluted from the paper by a total of 4·2 ml water.

This eluate was passed through a short column of Dowex 50 H^+ form, to remove cations. The effluent from the column was lyophilized, and the residue was dried in a desiccator *in vacuo* over anhydrous $CaSO_4$ overnight. The dried residue weighed 30·3 mg. The residue was dissolved in 0·16 ml of 0·5 M $NaHCO_3$ solution, and further addition of 1·2 ml ethanol yielded an amorphous precipitate, 19·5 mg. Recrystallization from water–ethanol (1:12) yielded 15·1 mg of colorless powder.

Assay of free and conjugated MHPG in urine. Twenty ml urine was adjusted to pH 6·5 to 7·0, shaken three times with 2 vol. ethyl acetate, and the organic phase was pooled for assay of MHPG (sample A). Under these conditions approximately 86 per cent of free MHPG is recovered in the organic phase. Phosphate and sulfate ions were removed from the aqueous phase by adding excess saturated $BaCl_2$ and centrifuging the precipitate. The excess barium remaining in the supernatant solution was precipitated as Ba_2CO_3 by adding 35% K_2CO_3, dropwise in excess, i.e. until the supernatant solution remained clear on further addition; the resulting precipitate was discarded. The sulfate-free urine was divided into two equal portions, and 0·2 ml of 2% spermidine-3 HCl* and 1·0 ml of 5 M sodium acetate buffer, pH 5·0, were added

* Spermidine was added as an activator of β-glucuronidase.[9]

to each portion. One portion was incubated with 2·0 ml Ketodase* (sample B), and the other portion with 1·0 ml Ketodase* plus 0·1 Glusulase† (sample C) for 16–17 hr at 45 °. The hydrolysates were adjusted to pH 6·5 to 7·0 with 2 N NaOH, and the MHPG was extracted three times with 3 vol. ethyl acetate. Under these conditions of extraction, 92 per cent of the released MHPG is recovered. A known amount of MHPG-7-^{3}H (30–33 mμc, 0·62–0·71 mμg) was added to each of the three ethyl acetate extracts from above (samples A, B and C) and the extracts were dried by using a flash evaporator. The MHPG in each extract was quantitatively transferred to a small test tube with 8 ml ethanol and the ethanol was evaporated to dryness under N_2 gas. Each one of the dried residues was dissolved with 0·2 ml ethanol and streaked on a strip of filter paper (6 × 57 cm) along a starting line 8·5 cm from one end. The strips were chromatographed in solvent system IV, and the developed chromatograms were scanned on a paper chromatogram scanner‡ to locate the radioactivity (R_f 0·44). The MHPG was eluted from the radioactive area with 5 ml water and the eluates lyophilized. Each lyophilizate was dissolved in 0·2 ml ethanol and streaked on another strip of filter paper (4 × 57 cm). The strips were developed in solvent system I, dried and scanned for radioactivity (R_f 0·84). The radioactive zone was cut out and immersed in 2·2 ml of 75% ethanol in water for 1 hr. One ml of the solution was used for the colorimetry of MHPG[4] and 0·5 ml for the radioactivity assay. The amount of MHPG determined by the colorimetry was corrected for the recovery during the ethyl acetate extraction and for recovery of the radioactivity added after extraction. The recovery of standard ^{3}H-MHPG in the 2·2 ml ethanol solution was between 9·9 and 42·9 per cent. The corrected amount for sample A is the amount of free MHPG in 20 ml urine. The corrected amount for MHPG conjugated with glucuronic acid, in 10 ml urine, was calculated from the results of sample B, and that for sample C was the total amount of MHPG in both conjugated forms in 10 ml urine; the amount of MHPG sulfate was calculated by subtracting the MHPG in sample B from that in sample C.

Urine specimens from patients with pheochromocytoma were kindly supplied by Dr. John G. Wiswell, Division of Endocrinology, Department of Medicine, University of Maryland School of Medicine. These were acidified during collection by addition of HCl, and were kept frozen until assayed. Urine specimens from patients with neuroblastoma were stored at 4°.

RESULTS

Identification of MHPG glucuronide. The isolated compound was not soluble in ethyl acetate and did not react with diazotized *p*-nitroaniline. However, hydrolysis of the compound with Ketodase gave rise to a *p*-nitroaniline reactive substance which showed identical paper chromatographic behaviour with authentic MHPG when chromatographed in solvent systems I, II, III and IV. A control tube in which the isolated compound was incubated with inactivated enzyme, prepared by heat-denaturation of the Ketodase, did not give rise to free MHPG. Ketodase does not

* Ketodase contains 5000 units of β-glucuronidase per ml and is derived from beef liver; Warner-Chilcott, Morris Plains, N. J.

† Glusulase contains 100,000 units of β-glucuronidase and 50,000 units of sulfatase per ml; Endo Laboratories Inc., Garden City, N.Y.

‡ Radiochromatogram scanner, model 7201, Packard Instrument Co., Downers Grove, Ill.

hydrolyze the sulfate conjugate under the conditions employed.* The radioactivity in the isolated compound was found to be in the MHPG moiety after the hydrolysis. The presence of glucuronic acid in the hydrolysate was confirmed paper chromatographically by comparison with authentic glucuronic acid by using solvent systems II, VII and VIII. Quantitative assays of free MHPG and glucuronic acid in the mixture after incubation with Ketodase revealed that equimolar quantities of these compounds were realeased.

The isolated compound was dried at 80° *in vacuo* and elementary analysis gave: C, 47·32; and Na, 6·34. (C_{15},-H_{19}-O_{10} Na requires: C, 47·12; H, 5·01; Na, 6·01.) A suitable result for the analysis of hydrogen could not be obtained because the anhydrous compound is very hygroscopic; this characteristic has also been found for the glucuronides synthesized by Helferich and Berger.[10]

Formation of ^{14}C-MHPG glucuronide from ^{14}C-norepinephrine in vivo. From a urine specimen collected between 40 and 160 min after an NE-^{14}C infusion and 6·3 to 9·2 hr after an MHPG-^{3}H infusion, 40 ml was lyophilized and the residue was dissolved in water and applied on four strips of filter paper. The strips were developed by chromatography in solvent system I. After drying, the strips were rechromatographed in solvent system II and again rechromatographed in solvent system III. The procedures separate all other tritiated metabolites of MHPG from the glucuronide, but separation of metabolites of NE-^{14}C required further chromatography. The strips were scanned for the radioactivity and the glucuronide was eluted. The eluate was

TABLE 1. CHANGE IN ^{3}H/^{14}C RATIOS IN MHPG GLUCURONIDE DURING CHROMATOGRAPHIC PURIFICATION AFTER INJECTION OF ^{3}H-MHPG AND ^{14}C-NOREPINEPHRINE

Purification step	Procedure	Solvent used†	R_f‡	Radioactivity in aliquots (mμc)		^{3}H/^{14}C
				^{3}H	^{14}C	
	Starting urine			23·9	7·28	3·29
1	PCG	I	0·10			
2	PCG (δ)§	II				
3	PCG (δ)	III		0·967	0·082	11·8
4	PCG (s)§	II	0·22	1·09	0·057	19·2
5	PCG (s)	III	0·19	0·278	0·0133	20·9
6	PCG (s)	III	0·21	0·256	0·0128	20·0
7	PCG (s)	V	0·64	0·339	0·0180	19·7
8	PCG (s)	VI	0·38	0·450	0·0224	20·9
9	Hydrolysis‖ and PCG of the hydrolyzate	I	(0·84)	1·18	0·0603	19·6

† Details are described in Materials and Methods.

‡ R_f values of radioactive MHPG glucuronide, except a figure in parentheses which is an R_f value of MHPG liberated after hydrolysis.

§ PCG (δ) indicates that the same strip was rechromatographed in a different solvent system; PCG (s) indicates that the compound was eluted from a strip and the eluate was applied to another strip for paper chromatography.

‖ Hydrolyzed with β-glucuronidase under the condition described in text.

* A sulfate conjugate of MHPG [the potassium salt of 3-methoxy-4-(hydroxysulfonyloxy)-phenylglycol, Ro4-6028] was generously provided by F. Hoffman-LaRoche and Company, Ltd., Basle, Switzerland.

assayed for ^{3}H and ^{14}C content. An aliquot of the eluate was spotted on a separate strip of filter paper and developed in solvent system II. A major radioactive peak was located by scanning, and the radioactive compound was eluted. The double-labeled compound was chromatographed and transferred from one sheet of filter paper to another, using five different solvent systems, and the $^{3}H/^{14}C$ ratios of the eluted compound were analyzed at each step of this procedure. The results are shown in Table 1 along with R_f values of the radioactive glucuronide in the solvent systems used. After the purification step 4, only a single radioactive area was present on the chromatogram and the $^{3}H/^{14}C$ ratio remained constant (19·6 to 20·9), indicating the formation of MHPG-^{14}C glucuronide from NE-^{14}C *in vivo*. Finally, the double

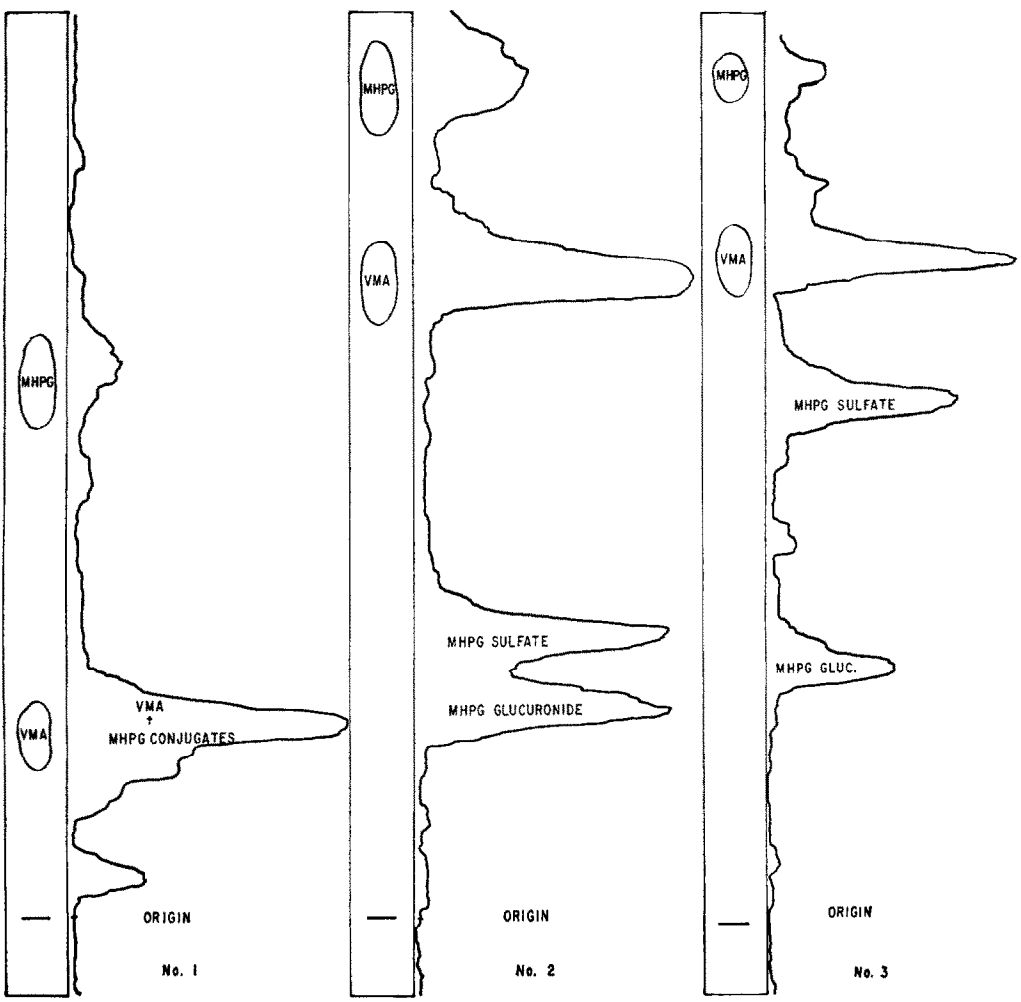

FIG. 1. Radiochromatograms of urine after injection of MHPG-^{3}H. MHPG-^{3}H was administered i.v. to the patient with ganglioneuroma and the urine, collected 95 min after the injection, was used for paper chromatography. Chromatogram 1 was developed in butanol–ethanol–water (4:1:1) followed by rechromatography (No. 2) in butanol–acetic acid–water (4:1:1). The paper was again rechromatographed (No. 3), this time in isopropanol–ammonia–water (8:1:1). It can be seen that this chromatographic series completely separated the glucuronide from the sulfate conjugate.

labeled glucuronide was hydrolyzed with Ketodase and the radioactivity in the liberated MHPG again showed the same $^{3}H/^{14}C$ ratio.

Time course for the rates of enzymatic hydrolysis of the two conjugates of MHPG. Since acid hydrolysis of the MHPG conjugates causes destruction of the liberated MHPG,[11] it has been essential to use enzymatic hydrolysis to release MHPG from its conjugated forms. To examine the time-course and completeness of hydrolysis of the conjugates, MHPG-^{3}H glucuronide and MHPG-^{3}H sulfate were isolated as described above. The isolation was accomplished by paper chromatography in the three different solvent systems described in the above section, and the radiochromatograms are shown in Fig. 1. Specific activity of the conjugates purified in this way was in the range of 100 to 500 mμc/μmole.

For the time-course study, the tritiated glucuronide and sulfate conjugates were incubated with β-glucuronidase and sulfatase, respectively, and portions of the incubation mixtures were removed at intervals. The liberation of free MHPG-^{3}H, measured as the radioactivity found in a pooled ethyl acetate extract (3 × 3 vol.), was assayed over a period of 20 hr (Fig. 2). The results indicate that incubation for at least 15 hr is required to obtain complete hydrolysis of both conjugates.

Completeness of hydrolysis under the conditions used for the routine assay of MHPG conjugates was examined with radioactive and non-radioactive substrates (Table 2). Ten-ml portions of normal urine, treated to remove free MHPG and

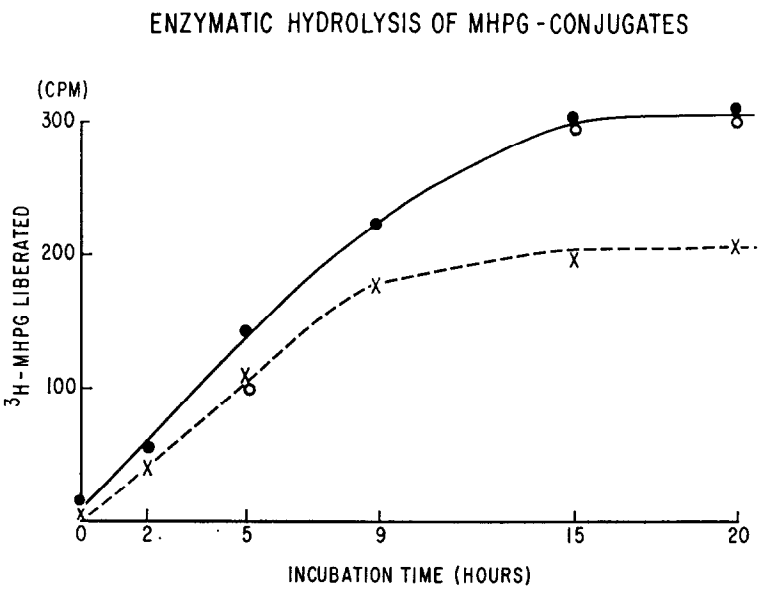

FIG. 2. Enzymatic hydrolysis of MHPG glucuronide and MHPG sulfate. MHPG glucuronide ●——●; MHPG sulfate, ×----×. MHPG glucuronide (366 cpm) was incubated in a mixture of 1·0 ml urine treated to remove sulfate ions, 0·1 ml of 5 M sodium acetate, 0·02 ml of 2% spermidine 3 HCl, and 0·2 ml Ketodase; samples in which the MHPG glucuronide was incubated without adding the spermidine solution are designated, ○ ○. MHPG sulfate (245 cpm) was incubated in a mixture of 1·0 ml urine treated to remove sulfate ions, 0·1 ml of 5 M sodium acetate, 0·02 ml of 2% spermidine 3 HCl 0·1 ml Ketodase and 0·01 ml Glusulase. Incubations were at 45°. Free MHPG was counted after extraction with ethyl acetate and is corrected for recovery (92%).

TABLE 2. COMPLETENESS OF THE ENZYMATIC HYDROLYSIS OF TWO CONJUGATES OF MHPG*

Expt. No.	Conjugates added		MHPG found after hydrolysis		Completeness of hydrolysis (%)	
		Boiled enzymes†	Glucuronidase†	Sulfatase+ glucuronidase§	Glucuronidase‡	Sulfatase+ glucuronidase§
	Glucuronide (mμc)	(mμc)	(mμc)	(mμc)		
1	10·3	0·08	9·91	9·80	96·1	95·0
2	5·10		4·66	4·94	91·3	96·8
	10·2	0·06	9·24	9·65	90·7	94·7
	Mean for glucuronide				92·7	95·5
	Sulfate (mμmoles)	(mμmoles)	(mμmoles)	(mμmoles)		
3	none	3	82	192	—	—
	400	4	83	501	0·3	77·2
	600	4	95	788	2·6	99·5
4	none	2	76	238	—	—
	400	2	67	647	0	102
	800	3	75	945	0	88.1
	Sulfate (mμc)	(mμc)	(mμc)	(mμc)		
5	4·70	0·13	0·13	4·55	2·8	96·8
	4·70		0·12	4·43	2·6	94·2
	Mean for sulfate				1·4	93·0

* Ten ml of normal urine, treated to remove free MHPG and free sulfate ions, was mixed with 1 ml of 5 M sodium acetate, 0·2 ml of 2% spermidine 3 HCl, and 0·1 of substrate in water. The mixture was incubated with enzyme preparations as noted in the table at 45° for 16 hr.
† Heat-denaturated enzymes of Ketodase (1·0 ml) and Glusulase (0·1 ml).
‡ Ketodase (2·0 ml).
§ Ketodase (1·0 ml) and Glusulase (0·1 ml).

sulfate ions, were added to each incubation mixture. After incubation, the liberated MHPG was extracted with ethyl acetate (3 × 3 vol.). The radioactivity found in the ethyl acetate extract was taken as the liberated MHPG-^{3}H after correction for the recovery of free MHPG (92 per cent). Nonradioactive MHPG was determined by colorimetry after purification of the ethyl acetate extract by paper chromatography (solvent system I). Corrections were necessary for the recovery of 92 per cent during the extraction and for a loss during paper chromatographic purification, as measured by a recovery of standard MHPG-^{3}H which was added before the chromatography. More than 90 per cent of both the glucuronide and the sulfate conjugates was hydrolyzed under these conditions.

Excretion of free and conjugated MHPG in urine. The free and conjugated MHPG in urine were determined in the urine of five normal individuals, four patients with pheochromocytoma, one patient with ganglioneuroma, and seven patients with neuroblastoma. The results, expressed in terms of micrograms of MHPG per milligram of creatinine, are presented in Table 3. The amounts of the glucuronide and sulfate conjugates of MHPG are about equal in two of the normals, the glucuronide is about 50 per cent higher in two other cases, and in the final normal subject the glucuronide conjugate is quite low.

Three of the patients with pheochromocytomas had levels of MHPG and its conjugates which were twice the normal levels or greater, and one patient had levels within the normal range.

TABLE 3. EXCRETION OF MHPG IN URINE (μg MHPG/mg CREATININE)

Subjects	Age (yrs)	Free	Glucuronide	Sulfate	Total
Normal individuals					
M. S.	3	0·24	1·52	0·97	2·73
C. A.	6	0·29	1·52	1·71	3·52
R. S.	28	0·17	1·04	1·07	2·28
H. S.	31	0·09	1·61	0·99	2·69
R. P.	45	0·25	0·36	1·78	2·39
Average		0·208	1·21	1·304	2·72
Pheochromocytoma patients					
D. R.	18	0.14	1·98	1·76	2·38
G. G.	20	0·67	4·23	2·39	7·29
L. B.	21	0·42	2·60	2·03	5·05
E. W.	53	0·69	16·1	5·12	21·9
Average		0·48	6·23	2·83	9·53
Ganglioneuroma patient					
R. F.	58	3·75	40·5	22·4	66·7
Neuroblastoma patients					
S. S.	4 mo.	18·5	40·1	30·6	89·2
D. E.	1	5·08	34·5	43·0	82·6
L. W.	3	0·48	5·82	2·46	8·76
D. B.	5	4·02	17·7	75·5	97·2
C. S.	6½	1·68	5·69	4·53	11·9
K. H.	7	3·29	4·49	29·1	36·9
R. Z.	10	5·80	13·1	14·0	32·9
Average		5·55	17·34	28·46	51·35

The patients with neuroblastoma had levels of urinary MHPG and its conjugates which averaged between 10 and 20 times the levels in normal individuals and, in the one patient whose free MHPG was nearer the normal range, the conjugates were still four times and twice the normal average for glucuronide and sulfate conjugates respectively. In the urine of most patients, the glucuronide and sulfate conjugates were nearly equal or the glucuronide was about 25 per cent higher, but in the case of two patients the sulfate conjugate was considerably elevated and was four and six times the level of the MHPG glucuronide.

DISCUSSION

The report by Axelrod *et al.*[1] on the identification of the sulfate conjugate of MHPG as a major urinary metabolite of ^{3}H-NE in the rat was the first paper which indicated that 3-methoxy-4-hydroxymandelaldehyde may be reduced *in vivo*. The earlier report by Armstrong *et al.*[12] on the urinary excretion of elevated levels of VMA by patients receiving norepinephrine and those with norepinephrine-secreting tumors had virtually established the oxidation of 3-methoxy-4-hydroxymandelaldehyde as a metabolic transformation *in vivo*. The findings of free MHPG in pheochromocytoma tumor by Kopin and Axelrod[2] and the demonstration that ^{3}H-MHPG (free and conjugated) was present in the urine of normal volunteers and schizophrenic patients[13] provided evidence that this route of metabolism was present in humans as well as in rats.

Because VMA is excreted very largely in the free form in human urine and its assay is relatively easily accomplished, this acidic metabolite is one of the most extensively studied urinary catecholamine metabolites. However, the finding that only a small fraction of intravenously administered ^{3}H-MHPG was excreted unchanged

in the human (ca. 4 per cent), coupled with the fact that a significant fraction ($\frac{1}{3}$) of the ^{3}H-MHPG was converted to ^{3}H-VMA,[4] has strongly suggested that, *in vivo*, MHPG might be a more important catecholamine metabolite than has been recognized previously. For example, in the case of patient P.A.,[4] ^{3}H-MHPG was the major metabolite of ^{3}H-NE added to his neuroblastoma tumor in tissue culture; the preoperative urinary excretion of VMA by this patient was high, and a significant fraction (34·2 per cent) of ^{3}H-MHPG administered i.v. to this patient was excreted in the urine as ^{3}H-VMA.[4] In addition, levels of MHPG were higher than those of VMA in the tumor and in the blood.[4] These facts all suggest that the tumor was forming a relatively large quantity of MHPG, which was released into the blood stream, further metabolized to VMA, and the latter metabolite was then excreted into the urine at elevated levels.

Previous studies have identified VMA and MHPG sulfate as metabolites of MHPG-^{3}H in patients with neuroblastoma.[4] This paper completes the identification of the major ^{3}H-labeled metabolites in the urine of patient P.A. by providing evidence that the major metabolite is the glucuronic acid conjugate of MHPG. The absence of a color reaction with diazotized *p*-nitroaniline before hydrolysis and the susceptibility of the isolated compound to hydrolysis by beef liver β-glucuronidase, with the equimolar formation of MHPG and glucuronic acid, plus the results of elementary analysis, indicate that the isolated compound is the sodium salt of 3-methoxy-4-*O*-(β-glucuronido) phenylglycol. It has been reported that β-glucuronidase can also hydrolyze β-galacturonides;[14] however, the paper chromatographic behavior of the hydrolysate of the isolated compound corresponded to that of authentic glucuronic acid and was different from that of galacturonic acid.

Since metanephrine and normetanephrine are present in the urine as both glucuronide and sulfate conjugates and since glucuronide formation is a common type of conjugation of phenolic compounds in animals, it is not surprising that a part of the MHPG is excreted in the urine as the glucuronide conjugate. A few reasons can be mentioned to explain why only the sulfate conjugate of MHPG has previously been reported. Both conjugates have similar R_f values on paper chromatograms developed in solvent systems I and II. Glusulase, the commercially available enzyme which has been most commonly used as the source of sulfatase, also has glucuronidase activity; therefore, both conjugated forms are hydrolyzed simultaneously by this preparation. In rats, the animal species which has been used most extensively for isotopic studies of MHPG metabolism,[1, 15–17] most of the urinary MHPG appears to exist as the sulfate conjugate and the urinary MHPG glucuronide seems to be negligible in this species. In the urine of patients with tumors of neural crest origin, however, a considerable amount of MHPG is excreted as the glucuronide in the urine.

Total urinary excretion of MHPG was found to be higher in patients with neural crest tumors than in healthy controls, as may be expected from results reported by other investigators.[11, 18–20] Wilk *et al.*[11] assumed that most of the MHPG which they found in human urine was derived from the sulfate conjugate, but the inadequacy of this assumption is clear from the results presented in this paper.

The assay method for the urinary MHPG used in this study was especially developed to examine the amounts of the two conjugates separately, and such an elaborate differential determination may not be necessary for clinical purposes. However, in order to estimate the total amount of urinary MHPG, it is important to employ

proper conditions for the enzymatic hydrolysis of the conjugates. Sulfate and phosphate ions are known to inhibit sulfatase activity and these ions should be removed. Urine specimens, especially those collected under pathological conditions, may contain inhibitors and inactivators of β-glucuronidase,[14] and removal of these inhibitory factors may be difficult. Addition of an activator, as has been employed for the assay of β-glucuronidase activity,[9] may be of value in routine hydrolysis.

The usual temperature for enzymatic reactions is 37·5; however, Fromageot[21] has recommended incubation at 50° for the arylsulfatase from takadiastase, and β-glucuronidase has been shown to be stable up to 50°.[14] Since the enzymatic activity approximately doubles with every 10° rise, we used 45° for incubation in this study and obtained hydrolysis of the MHPG sulfate and glucuronide conjugates which was more than 90 per cent complete in 20 hr (Fig. 2).

The MHPG colorimetric assay is based on the formation of a purple dye-product (maximum absorbancy at 525 mμ) on reaction of the MHPG (free) with diazotized *p*-nitroaniline in ethanol–K_2CO_3 (2:1). This method is a quantitative adaptation of the dilute form. This reagent has been described by Acheson *et al.*[5] as a spray reagent for VMA and other phenolic acids and by Kraupp *et al.*[22] for quantitative estimation of metanephrine and normetanephrine. The method is sensitive; 0·100 O. D. is equivalent to 1·76 μg MHPG in 3 ml of solution with a 10 mm light path, over the range of 0·5 to 10 μg. The specificity of this method is achieved by extracting the solution to be assayed at pH 7, and the ethyl acetate extract is evaporated to dryness and chromatographed in two dimensions on filter paper. The chromatography separates the MHPG from the small amounts of VMA and other potential interfering compounds, such as metanephrine and normetanephrine, which are extracted at pH 7 and give the same dye-product with alkaline diazotized *p*-nitroaniline.

We recognize that the racemic DL-H^3-MHPG is not an ideal tracer for use in studies *in vivo*, as has been mentioned previously.[4] However, the DL-H^3-MHPG contains 50 per cent of the natural isomer, and therefore we have reported results obtained with the racemic mixture as a basis for comparison with later results when stereospecific labeled isomers become available. In addition, in this study the DL-H^3-MHPG has been used in an isotope dilution assay by a simple procedure such that separation of the stereoisomers seems unlikely. For the reasons noted above, we believe that the results reported here may be of value; they suggest that assay of total urinary MHPG (both free and conjugated) may be of aid in the diagnosis of neuroblastoma in patients whose urinary excretion of norepinephrine, VMA and homovanillic acid are in the range of normal.

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