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N-ACETYL,4-*O*-METHYLDOPA, A MAJOR METABOLITE OF L-4-*O*-METHYLDOPA IN MAN AND RAT

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Abstract—*N*-Acetyl,4-*O*-methyldopa was identified as a major urinary metabolite of L-4-*O*-methyldopa, both in man and in the rat. The urinary metabolites were examined in man after oral and in rat after intraperitoneal administration of L-4-*O*-methyldopa, L-3-*O*-methyldopa, L-dopa and *N*-acetyl,4-*O*-methyldopa. 4-Mdopa was found to be converted mainly to *N*-acetyl,4-Mdopa and iso-HVA and very little to the corresponding pyruvic and lactic acids, whereas 3-Mdopa was metabolized to its pyruvic, lactic and acetic (HVA) derivatives and practically not acetylated. It is suggested therefore that the 3-hydroxy,4-methoxy group (the iso-vanyl structure) prevents transamination and that *N*-acetylation represents an alternative metabolic pathway. The lack of *N*-acetyl,4-*O*-methyldopa after the L-dopa loads shows that L-dopa is not 4-*O*-methylated and therefore that 4-*O*-methyldopa is not, or only in a minute amount if any, an *in vivo* metabolite of L-dopa. *N*-Acetyl,4-Mdopa administration to rats resulted in excretion of the compound almost unchanged. These results agree with a previous suggestion by the authors of partly distinct metabolic routes for the *O*-methyl catecholamines according to whether the methyl group is bound on the *meta* or on the *para* position and raises the question as to what extent iso-HVA levels in body fluids are representative of a *para*-*O*-methylation of dopamine, implicated in neuropsychiatric disorders.

Recently, Mathieu *et al.* studied the urinary vanyl (3-methoxy,4-hydroxyphenyl-) and iso-vanyl (4-methoxy,3-hydroxyphenyl-) carboxylic acids in rats after intraperitoneal loads with dopamine and related precursors and derivatives [1]. Following L-4-*O*-methyldopa administration, homo-iso-vanillic acid (iso-HVA) was found to be the most prominently elevated urinary metabolite, but two other compounds were present, corresponding to iso-vanyllactic acid and to an unknown iso-vanyl compound. This unknown compound disappeared completely after hydrolysis of the urine with HCl at pH 1, for 10 min. Following the intraperitoneal administration of L-3-*O*-methyldopa, and in agreement with other reports [2, 3, 4], the levels of homovanillic and vanyllactic acids appeared to be elevated but only traces of the vanyl counterpart of the unknown spot after 4-Mdopa were seen.

These observations led us to suggest the existence of partly distinct metabolic routes for the *O*-methyl catecholamines according to whether the methyl group was bound on the *meta* or on the *para* position.

Confirming this suggestion, we now report the identification of this unknown iso-vanyl urinary metabolite of 4-Mdopa and present some different aspects of the excretion of vanyl and iso-vanyl compounds in man and rats.

Abbreviations: 3-Mdopa, 4-Mdopa = L-3- and L-4-*O*-methyldopa; HVA = 4-hydroxy,3-methoxyphenylacetic acid, homovanillic acid; iso-HVA = 3-hydroxy,4-methoxyphenylacetic acid, homo-iso-vanillic acid; *N*-Ac,4-Mdopa = *N*-acetyl,4-*O*-methyldopa; VLA, iso-VLA = vanyl- and iso-vanyllactic acid; 4-MDA = 4-*O*-methyldopamine.

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EXPERIMENTAL

Reagents. L-3- and L-4-*O*-methyldopa were the generous gifts of Hoffmann-LaRoche, Basle (Switzerland) and Weiders Farmasöytiske a/s, Oslo (Norway). Homo-iso-vanillic acid was synthesized according to a previously described procedure [6]. The preparation of *N*-acetyl,4-*O*-methyldopa was carried out by *N*-acetylation of 4-Mdopa, according to Kemp [7]: 4-Mdopa (50 mg) was dissolved in a mixture of acetic anhydride and glacial acetic acid (2 ml of each) and refluxed for 10 min. The reaction mixture was then added to dilute sodium hydroxide and extracted, at pH 1, with ethyl acetate. The dried residue after evaporation of the solvent was purified by preparative chromatography on Whatman 3MM paper, previously washed with acetic acid using isopropanol–ammonia–water (8:1:1) as solvent. *N*-Ac,4-Mdopa was eluted from the paper with ethyl acetate and recrystallized from the cooled concentrated solution. All other compounds were obtained from commercial sources.

Assays on rats. Assays were performed according to the procedure previously described in detail by the authors [1]. Briefly, the 24 hr urines following intraperitoneal injection of 4-Mdopa, 3-Mdopa, L-dopa or *N*-Ac,4-Mdopa (each at a dose of 1 mg/100 g body wt) to albino, Sprague–Dawley strain, rats of both sexes, were collected in HCl (1·0 N, 2 ml) in glass metabolic cages. The phenolic acids were extracted with ethyl acetate and separated by bi-dimensional

paper chromatography (see Fig. 1). The spots were detected by u.v. light (250 nm) and diazotized *para*-nitroaniline. As the vanyl and iso-vanyl isomers exhibit identical chromatographic mobilities (R_f values), elution and re-chromatography of their azo-derivatives, according to Mathieu *et al.* [8, 9], was useful for their separation and identification. The amines were determined according to Kakimoto and Armstrong [10].

In order to get a sufficient amount of the unknown urinary metabolite following 4-Mdopa administration, the 24 hr urine of three rats loaded with 4-Mdopa was pooled, extracted and submitted to preparative chromatography on Whatman 3 MM paper, previously washed with acetic acid, using: isopropanol–acetic acid–water (8:1:1) as solvent, ascending development. The compound was eluted from the paper with ethyl acetate and, after evaporation of the solvent, submitted to mass spectrometry (AEI MS 902 mass spectrometer) and infrared spectroscopy (Perkin Elmer instrument, model 452). Mass and infrared spectra of the parent compound 4-Mdopa were first recorded for comparison.

Human assays. Fasting healthy adult volunteers received orally either 4-Mdopa (500 mg, three assays), 3-Mdopa (500 mg, three assays) or L-dopa (3 g, three assays). The 24-hr urines were collected (in one case after L-dopa, at different time intervals: 0–3 hr, 3–6 hr, 6–9 hr, 9–12 hr and 12–24 hr) and analyzed by paper chromatography as outlined above.

Enzymatic hydrolysis of the urine (from man and rats) was done with a preparation of β-glucuronidase–arylsulfatase from *Helix pomatia* (Industrie Biologique Française, 92-Gennevilliers, France). 10 ml of urine was adjusted at pH 5·5–2 with a 10% $KHCO_3$ aqueous solution, then 1 ml of 1·0 M acetate buffer, pH 5·2 and 0·1 ml of the enzyme preparation were added. The mixture was incubated at 37° for 24 hr. Absence of catecholamine metabolites in the enzyme preparation was checked by paper chromatography.

The metabolites HVA, iso-HVA, VLA and *N*-Ac,4-Mdopa were estimated quantitatively as previously described [1], by visual comparison with standards (from 0·05 to 20 μg) chromatographed in the same way as the biological extracts. Aliquots of the urinary extracts were measured within the standard range.

RESULTS AND DISCUSSION

Identification of the unknown urinary metabolite of L-4-O-*methyldopa.* Mass spectra were recorded at an electron energy of 70 eV, an accelerating voltage of 8 kV and an ionizing current of 100 μA. The sample temperature was 150°. The mass spectrum of 4-Mdopa shows the presence of a molecular ion peak at *m/e* 211 and fragment ion peaks at *m/e* 166, 137, 122, 94, corresponding to the fragmentation pattern shown in the upper part of Fig. 2. The mass spectrum of the unknown metabolite shows the presence of a molecular ion peak at *m/e* 253 and fragment ion peaks at *m/e* 236, 208, 194, 179, 166, 137, 122, 94. The finding of an increase of 42 a.m.u. in the value of the molecular ion in relation to 4-Mdopa, as well as the occurrence of fragment ions at *m/e* 43 and *m/e* 194 (corresponding to $M^+ - 59$) were consistent with the hypothesis that the unknown metabolite was the

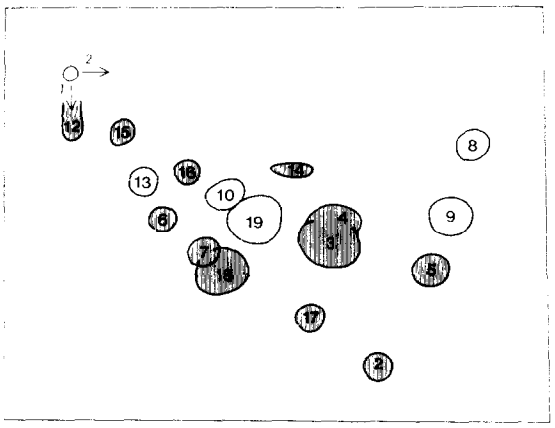

Fig. 1. Chromatographic location of the main phenolic acids with vanyl and isovanyl structures following intraperitoneal administration to the rat of L-3-methyldopa and of L-4-methyldopa. Bidimensional chromatogram on Whatman No. 20 paper; solvent 1, isopropanol–ammonia–water (8:1:1, by vol.) descending development; solvent 2, benzene–propionic acid–water (100:70:4, by vol.) ascending development. Spots were detected under u.v. light (254 nm) and with diazotized *p*-nitroaniline. 8 = vanillic acid (violet) after 3-Mdopa load; 9 = homovanillic acid (grey-blue) and homo-iso-vanillic acid (violet); 10 = vanyllactic acid (grey-blue) after 3-Mdopa load and iso-vanyllactic acid (violet), traces after 4-Mdopa load; 13 = vanylmandelic acid (violet) after 3-Mdopa load and iso-vanylmandelic (violet), traces after 4-Mdopa load; 19 = unknown iso-vanyl compound (violet) after 4-Mdopa load, traces of the vanyl counterpart (grey-blue) after 3-Mdopa load. Outlined spots and corresponding numbers = usual free phenolic acids in rat urine, as previously described and listed by the authors [1, 5].

N-acetylated derivative of 4-Mdopa. This was confirmed by high resolution mass determinations of the fragment ions at *m/e* 253, 194, 179 and 166, the values of which were 253.095794, 194.058203, 179.034529 and 166.087194 respectively, corresponding to the elementary formulas $C_{12}H_{15}NO_5$, $C_{10}H_{10}O_4$, $C_9H_7O_4$ and $C_9H_{12}NO_2$. Identical mass spectrometric data were obtained with authentic *N*-Ac,4-Mdopa. The fragmentation pattern of *N*-Ac,4-Mdopa is presented in the lower part of Fig. 2.

Infrared spectroscopy of the unknown metabolite and of authentic *N*-Ac,4-Mdopa confirmed the identity of the two compounds. It can be concluded, therefore, that spot No. 19 on the chromatogram following L-4-*O*-methyldopa administration to rats (Fig. 1) is the *N*-acetyl derivative of the amino acid.

Urinary iso-vanyl metabolites following L-4-O-*methyldopa,* L-3-O-*methyldopa,* L-*dopa and* N-*acetyl*,4-O-*methyldopa loads in man and in rats.* The urinary patterns of the metabolites found after the different loads to man and rats are presented in Table 1. In rat, following intraperitoneal administration of 4-Mdopa, the urinary output of *N*-Ac,4-Mdopa, excreted wholly unconjugated, was about 1·4 of that of iso-HVA, while iso-VLA was estimated to be less than 1·10 of iso-HVA. Neither iso-vanylpyruvic acid, nor the amino acid (4-Mdopa) itself was detected. Following the 3-Mdopa loads, the relatively poor excretion pattern of the drug and its metabolites is in agreement with other reports [2, 11, 12, 13]. In addition to the

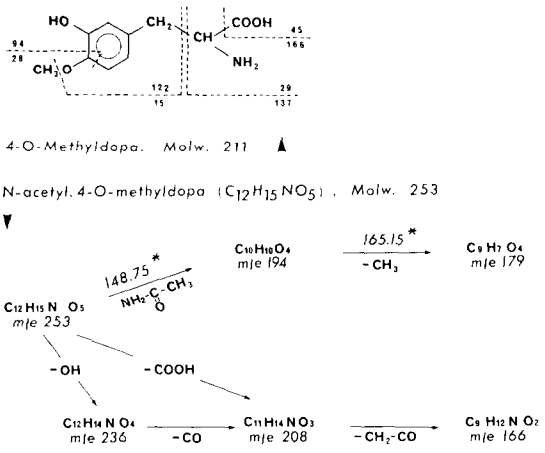

Fig. 2. Fragmentation patterns proposed for 4-*O*-methyldopa (upper part) and for *N*-acetyl,4-*O*-methyldopa (lower part). Asterisks indicate metastable peaks. The appearance of the $M^+ - 59$ ion by loss of a neutral fragment $NH_2\text{-}CO\text{-}CH_3$ results from a MacLafferty fragmentation process with a proton rearrangement.

amino acid (3-Mdopa), vanylpyruvic acid, VLA and HVA, traces of a compound having the same chromatographic characteristics (R_f values) as *N*-Ac,4-Mdopa, except for the colour with diazotized *para*-nitroaniline, which was blue, was observed. This minor urinary metabolite of 3-Mdopa is most probably *N*-acetyl,3-*O*-methyldopa.

N-Acetyl,4-*O*-methyldopa was also found in human urine after administration of 4-Mdopa (500 mg, orally). It was excreted in relatively larger amounts than in the rat. This is probably due to the different routes of administration, oral administration favouring an increased formation in the liver, rather than to a species difference. Tyce [14] noted, in liver perfusion experiments, a significant production of *N*-acetyl derivatives of dopamine. Enzymatic hydrolysis of the urine, with β-glucuronidase–arylsulfatase from *Helix pomatia*, resulted in an increase in the *N*-Ac,4-Mdopa level giving evidence of partial conjugation of the compound on the 3-hydroxy group. The ratio of free:total *N*-Ac,4-Mdopa in urine was about 3:4. Very small amounts of iso-VLA, but no iso-vanylpyruvic acid was detected. The amino acid (4-Mdopa) however was seen on the chromatograms. Following 3-Mdopa administration to man the results were similar to those from rats, with poor excretion of the metabolites, compared with the elimination after the 4-Mdopa and the L-dopa loads. Traces of *N*-Ac,3-Mdopa were always found after 3-Mdopa administration in human subjects.

Thus, the main difference between 4-Mdopa and 3-Mdopa metabolism is that 4-Mdopa is mainly converted to *N*-Ac,4-Mdopa and iso-HVA with very little to the corresponding pyruvic and lactic acids whereas, 3-Mdopa is metabolized to its pyruvic, lactic and acetic derivatives and almost not acetylated. Since it is generally accepted that the slow metabolism of 3-Mdopa is a consequence of being a poor substrate for dopa decarboxylase [15, 16], the formation of VLA takes place after transamination to vanylpyruvic acid [16, 17]. Our results suggest, in addition, that the 3-hydroxy,4-methoxy group (the iso-vanyl structure) prevents transamination and therefore that *N*-acetylation represents an alternative metabolic pathway.

We suggested previously [1] that, due to the lack of any iso-vanyllactic acid on our chromatograms following intraperitoneal administration of L-dopa to rats, both L-dopa and its catabolic derivatives leading to VLA (i.e. 3,4-dihydroxyphenylpyruvic and 3,4-dihydroxyphenyllactic acids) do not undergo *para*-*O*-methylation, and therefore that it was unlikely that 4-Mdopa would be an *in vivo* metabolite of L-dopa. From the present results, which show that iso-VLA is only a very minor whereas *N*-Ac,4-Mdopa is a major derivative of 4-Mdopa, it appears that our previous discussion was erroneous but that its conclusion remains true, due to the absence of *N*-Ac,4-Mdopa on the chromatograms from rats given L-dopa. Also in man *N*-Ac,4-Mdopa was not detected in 24-hr ur-

Table 1. Urinary metabolites of L-4-*O*-methyldopa, L-3-*O*-methyldopa, L-dopa and *N*-acetyl,4-*O*-methyldopa in man and in rat

Substance	Amino acid	*N*-acetylated amino acid	Corresponding pyruvic acid	Corresponding lactic acid	Homovanillic acid	Homo-iso-vanillic acid	Dopac	Corresponding amine
Assays on rats								
4-Mdopa	0	+ – (4)	0	+ (1)		+ + – (16)	0	(+)
3-Mdopa	+	(+)	+	+ + (5)	+ – (3)	(+)	0	(+)
L-Dopa	(+)	0	(+)	(+)	+ – + (8)	+ (0·5)	– –	(+)
N-Ac,4-Mdopa	0	+ – + (21)	0	0		– + (5·5)	0	‡
Human assays								
4-Mdopa	+	+ + + (8·5)*	0	+ (0·5)	(+)	– + (6)	0	(+)
3-Mdopa	–	(–)	+	– – (3·5)	+ + (5·5)	0	0	(–)
L-Dopa	+	0*	(+)	(+)	+ + + (25)	– (2·5)	– –	(–)

Assays on rats. All compounds were given by intraperitoneal injection, at a dose of 1 mg/100 g body wt. Each assay was done in duplicate.

Human assays. All compounds were given orally; 4-Mdopa and 3-Mdopa at a dose of 500 mg, L-dopa at a dose of 3 g. Three assays were done with each compound.

The metabolites were estimated in the 24 hr urines following the loads, using paper chromatography: 0 = not detected; (+) = traces; + = present to + + + = larger amounts. Numbers in parentheses indicate recovery levels of some of the main metabolites, in per cent of the compound given.

* Total recovery: free + conjugated.

† In one assay *N*-acetyl,4-*O*-methyldopa and *N*-acetyl,3-*O*-methyldopa were detected in urine 6–9 hr following the ingestion. The total level of both compounds was less than 0·1 per cent of the given L-dopa.

‡ *N*-Acetyl,4-*O*-methyldopamine: standard in course of synthesis.

ine samples following L-dopa administration. When urine was collected at various time intervals, *N*-Ac,3-Mdopa + *N*-Ac,4-Mdopa, both at a very low level (see Table 1) were detected in the 6–9 hr post-ingestion urine. This observation, which indicates possible formation in man of a minute amount of 4-Mdopa from L-dopa, when given orally, is to be compared with the finding of Karoum *et al.* that the VLA output after L-dopa administration reaches its maximum in the same 6–9 hr urine sample, due to the slow metabolism of 3-Mdopa formed from L-dopa [18].

N-Acetylation of normally occurring aromatic amino acids (phenylalanine, tyrosine, histidine) as well as foreign aromatic amino acids is a well known pathway in mammals [19, 20, 21], but even aliphatic amino acids, like cystathionine in cystathioninuria, can be acetylated [22]. Different *N*-acetylated amines have also been detected in urine from patients with pheochromacytoma, neuroblastoma and carcinoid tumors as well as after mescaline and dopa loads or monoamine oxidase inhibitors [23, 29]. However, we do not know whether the *N*-acetylated amino acids can be decarboxylated or the *N*-acetylated amines can be deaminated before *N*-deacetylation.

In this study (see Table 1), *N*-acetyl,4-*O*-methyldopa administration to the rat resulted in an excretion of the compound mostly unchanged, being almost four times greater than that of iso-HVA; iso-VLA was not detected on the chromatograms. This indicates that the *N*-acetylated amino acid is not transaminated and is, in addition, a poorer substrate for dopa decarboxylase than the *O*-methylated compound. With respect to the increase in iso-HVA, it is not possible to state whether it accounts for a partial decarboxylation and further *N*-deacetylation of *N*-Ac,4-Mdopa to give 4-*O*-methyldopamine (4-MDA) and iso-HVA, or if the *N*-acetylated amine (i.e. *N*-acetyl,4-*O*-methyldopamine formed through decarboxylation of *N*-Ac,4-Mdopa) itself may be deaminated, being a substrate for some of the amine oxidases.

Evidence has already been given that 4-MDA may be formed *in vitro* from dopamine with purified catechol-*O*-methyltransferase [30] as well as *in vivo* in man [31]. In similitude with 4-Mdopa, a possible pathway of 4-MDA may be through *N*-acetylation. If such was the case, it would be important to test to what extent *N*-acetyl,4-*O*-methyldopamine is a substrate for amine oxidase and thus for the formation of iso-HVA (whereas 3-*O*-methyldopamine almost exclusively leads to HVA). This is of interest as we know that iso-HVA is present in brain [9] and in body fluids such as urine and cerebrospinal fluid [6, 8, 9, 31] and that *para*-*O*-methylation of dopamine may be implicated in some neuropsychiatric disorders. It has been claimed that while 3,4-dimethoxyphenylethylamine produces hypokinesis in rats and has pharmacological effects in other species, its *N*-acetylated derivative is ten times more potent than the amine in producing these effects [32, 33]; contrarily in man *N*-acetylated mescaline and *N*-acetyl,dimethoxyphenylethylamine are non-hallucinogenic and inactive, respectively, compared with mescaline and dimethoxyphenylethylamine [34, 35].

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