Biochemical Pharmacology, 1964, Vol. 13, pp. 1499–1505. Pergamon Press Ltd., Printed in Great Britain.

METABOLISM OF DRUGS—XLIX. THE STUDY OF SELECTIVE DEMETHYLATION OF 4-ACETAMINO- AND 4-NITRO-VERATROLES *IN VIVO*

H. TSUKAMOTO, H. YOSHIMURA and T. WATABE

Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan

(*Received* 14 *April* 1964; *accepted* 19 *May* 1964)

Abstract—Both 4-acetamino- and 4-nitro-veratroles undergo an interesting selective demethylation as their major metabolic pathways in rabbits and produce 4-acetamino- and 4-nitro-guaiacols as the predominant metabolites, respectively. These findings, together with the preceding study on brucine, suggest that electronic configuration is not an important factor for occurrence of selective demethylation of two adjacent methoxyl groups attached to an aromatic ring system. No other reaction, including deacetylation or nitro reduction, has been demonstrated in either case except a slight formation of 4-nitrocatechol in the latter.

IN THE preceding paper of this series, very interesting selective demethylation of brucine has been found to occur in rabbits as the major metabolic pathway.[1]

The O-demethylation reaction in mammals has been understood to be a hydroxylation reaction on a methyoxyl carbon atom, followed by cleavage of unstable intermediate to phenol and formaldehyde. It has also been known that this demethylation is catalyzed by liver microsomal enzyme systems and requires oxygen and reduced nicotinamide adenine dinucleotide phosphate as a cofactor, as well as many other metabolic oxidations of drugs.[2]

Since these oxidations take place preferentially on a carbon atom with higher electron density, the authors have considered that selective demethylation of brucine might be caused by its electronic configuration. If this is true, one could suggest which of two adjacent methoxyl groups attached to an aromatic ring would predominantly cleave in the animal body.

The present investigation has been undertaken in order to decide whether or not this consideration would be acceptable in general, by use of 4-acetamino- and 4-nitro-veratroles. The former compound has an electronic configuration similar to that of brucine; the latter has a substituent with a reverse electronic property.

METHODS

Materials

4-Acetaminoveratrole (m.p. 133°),[3] 4-nitroveratrole (m.p. 96°),[4] 4-aminoveratrole (m.p. 87°),[3] 4-acetaminoguaiacol (m.p. 117°),[5] 5-acetaminoguaiacol (m.p. 171°),[6] 4-nitroguaiacol (m.p. 102°),[4] and 5-nitroguaiacol (m.p. 104°)[7] were prepared by the known methods and used after confirmation of their purity by elementary analysis, u.v. and i.r. absorption spectra, and paper chromatography. β-Glucuronidase preparation was obtained from preputial glands of adult female rats by its homogenation

in 200 volumes of deionized water which showed the activity of 7×10^4 *p*-nitrophenyl-β-D-glucuronide units[8] per ml.

Administration of 4-acetamino- and 4-nitro-veratroles

The compounds were suspended in 10% gum arabic (100 mg/ml), and 10 ml (4-acetaminoveratrole) and 9 ml (4-nitroveratrole) were given by stomach tube to male albino rabbits weighing 2·5–3·0 kg.

Determination of the urinary metabolites of 4-acetaminoveratrole

A 20 ml portion of 24-hr urine from a rabbit which received 1 g of 4-acetaminoveratrole was adjusted to pH 4·8 with acetic acid and incubated with 2 ml of β-glucuronidase preparation at 38° for 24 hr. After incubation, the mixture was made alkaline with concentrated NH_4OH, saturated with NaCl, and extracted three times with 50-, 50-, and 25-ml portions of ethyl acetate under mechanical shaking for 20 min each. The organic solvent layers separated by centrifugation were combined and dried over anhydrous Na_2SO_4. After removal of the solvent from the extract, the residual gum was completely dried *in vacuo* for 6 hr and dissolved in methanol to make a concentration of 1 mg/0·12 ml. An aliquot (0·03 ml) of the solution was submitted to paper chromatography; the sample was applied in three spots to the paper, 6 × 40 cm, and developed with system 1 as described below. Spots corresponding to 4-and 5-acetaminoguaiacol on the chromatogram were cut out separately after marking under an ultraviolet lamp, eluted with 5 ml of 0·1 N NaOH, and centrifuged. The amounts of 4- and 5-acetaminoguaiacols in each eluate were determined spectrophotometrically at 264 and 296 mμ respectively. The extract from the filter paper which was run without compounds served as a blank solution. Recovery of the metabolites in this procedure was found to be quantitative.

For the conjugated metabolites which were not hydrolyzed by β-glucuronidase the following further procedure was performed. To urine remaining after β-glucuronidase treatment and ethyl acetate extraction was added 3 ml of conc. HCl, and the mixture was heated at 60–70° for 40 min. The hydrolysate was made alkaline with 1·5 ml of conc NH_4OH and shaken three times with 50-, 50-, and 25-ml portions of ethyl acetate for 20 min each. Combined extracts were then subjected to the same determination procedure as described above.

Determination of the urinary metabolites of 4-nitroveratrole

The first 48-hr urine from a rabbit which received 900 mg of 4-nitroveratrole was heated on a boiling water bath for 30 min in a 5% HCl concentration. The hydrolysate was made alkaline with conc. HN_4OH and extracted continuously with ether for 20 hr. After removal of the solvent from the extract, the residual gum was dried *in vacuo* for 7 hr and dissolved in methanol to make a concentration of 1 mg/0·1 ml. An aliquot (0·03 ml) of the solution was submitted to the same determination procedure as was used in the 4-acetaminoveratrole experiment except that system 2 was used as a developing solvent system for paper chromatography, and 435 and 263mμ were the wavelengths of the spectrophotometric determinations of 4- and 5-nitroguaiacols respectively.

Paper chromatography

The filter paper used was Toyoroshi no. 51A buffered with 0·05 M carbonate buffer, pH 10·8. Only when solvent system 3 was applied was nonbuffered filter paper used. The development was performed in ascending technique with the following solvent systems. System 1, BuOH:benzene:pyridine:conc. NH_4OH (8:8:5:3); system 2, BuOH:pyridine:benzene:10% NH_4OH (8:8:5:3); system 3, BuOH:AcOH:H_2O (4:1:5).

The chromatogram was visualized under an ultraviolet lamp (short-wave "Manaslu Light," Manaslu Chemical Industries Co. Ltd., Tokyo).

Phenolic metabolites of 4-acetamino- or 4-nitro-veratroles were also visualized by spraying 0·2% solution of diazotized sulfanilic acid in 10% Na_2CO_3, or 5% KOH solution respectively. Ehrlich reagent was used for detection of amino derivatives.

RESULTS

Detection of the urinary metabolites of 4-acetaminoveratrole

The ethyl acetate extract of 24-hr urines after treatment with β-glucuronidase was investigated by paper chromatography with system 1. With diazotized sulfanilic acid reagent, a light violet and a bright orange spot were detected on the chromotogram having *Rf*s of 0·65 and 0·50 respectively. The same spots were also visualized under an ultraviolet lamp. Any other spots due to unchanged 4-acetaminoveratrole and 4-aminoveratrole were not detected by these or Ehrlich reagents.

The spot at *Rf* 0·65 was much larger than that at *Rf* 0·50.

Their *Rf* values and colorations with diazotized sulfanilic acid were the same as authentic 4- and 5-acetaminoguaiacols respectively.

TABLE 1. R_F VALUES AND COLOR REACTIONS OF 4-ACETAMINOVERATROLE AND RELATED COMPOUNDS

Compound	*Rf*		Color reaction	
	System 1	System 2	Diazotized sulfanilic acid	Ehrlich reagent
4-Acetaminoveratrole	0·85	0·71		
4-Aminoveratrole	0·85	0·48	Dark orange	Yellow
4-Acetaminoguaiacol	0·65	0·77	Light violet	
5-Acetaminoguaiacol	0·50	0·77	Bright orange	

The urine before treatment with β-glucuronidase showed no spots except a very small and faint one corresponding to 4-acetaminoguaiacol.

Isolation, characterization, and identification of the metabolites of 4-acetaminoveratrole

Combined 24-hr urines from four rabbits, each of which was given 1 g of 4-nitroveratrole, were heated at 60–70° on a water bath for 30 min at a 5% HCl concentration. The hydrolysate was made alkaline with conc. NH_4OH and extracted continuously with ethyl acetate for 20 hr. The extract was shaken with N NaOH and the resulting aqueous alkaline layer, after washing with ethyl acetate, was saturated with NH_4Cl

and extracted with ethyl acetate. After drying over anhydrous Na_2SO_4 and evaporation of the solvent, the extract gave a reddish-brown gum (0·7 g). It was dissolved in a small quantity of ethyl acetate, poured onto a column of silica gel (35 g) and eluted with benzene, and benzene:ethyl acetate (2:1, and 1:1). The fractions that were positive with diazotized sulfanilic acid reagent were collected, and the solvent was removed to give a red gum (0·55 g). A part of this gum (200 mg) was dissolved in acetone, spotted on 20 sheets of the filter paper, 40 × 40 cm, and developed with system 1.

The areas corresponding to 4- and 5-acetaminoguaiacols were cut out and extracted separately with acetone in a Soxhlet apparatus for 30 hr. Acetone was removed from each extract to give a slightly colored gum. The gum obtained from the 4-acetaminoguaiacol fraction was recrystallized from benzene–acetone to colorless prisms (102 mg), m.p. 117°. (Found:C, 59·40; H, 6·22; N, 7·45. $C_9H_{11}O_3N$ requires C, 59·66; H, 6·12; N, 7·73.) It revealed a light violet color with diazotized sulfanilic acid reagent and was shown to be identical with authentic 4-acetaminoguaiacol by the mixed melting-point test, and the comparison of u.v. and i.r. absorption spectra.

$$\text{u.v. } \lambda_{max} \text{ 0·1 N NaOH m}\mu \text{ (log } \epsilon\text{):263 (3·97)}$$

The other gum obtained from the 5-acetaminoguaiacol fraction was also recrystallized from benzene–acetone to colorless prisms (41 mg), m.p. 171°. (Found: C, 59·91; H, 6·29; N, 7·80. $C_9H_{11}O_3N$ requires C, 59·66; H, 5·12; N, 7·73.) This sample was identical with authentic 5-acetaminoguaiacol in coloration with diazotized sulfanilic acid (bright orange), in melting point, and in u.v. and i.r. absorption spectra.

$$\text{u.v. } \lambda_{max} \text{ 0·1 N NaOH m}\mu \text{ (log } \epsilon\text{): 297 (3·75)}$$

These two metabolites were proved not to be formed artificially during the course of hydrolysis and extraction by the fact that 4-acetaminoveratrole added to normal urine was not converted to these phenols after hydrolysis and extraction in the same conditions described above.

Determination of the urinary metabolites of 4-*acetaminoveratrole*

The metabolites in the 24-hr urine were determined after treatment with β-glucuronidase. The result is shown in Table 2. It was demonstrated that about half the administered material was excreted almost exclusively as 4-acetaminoguaiacol. Another metabolite did not exist in a detectable amount. Since 0·1 mg of this metabolite in 20 ml of urine could be determined with good precision by this method, the amount should be less than 0·1% of the administered substance.

The urine that remained after β-glucuronidase treatment and extraction seemed to contain additional metabolites and was therefore hydrolyzed again with HCl. Considerable amounts of metabolites were detected after this treatment (Table 2). A total of about 12% of the administered substance was excreted in the urine as 5-acetaminoguaiacol and about 70% as 4-acetaminoguaiacol.

Detection of the urinary metabolites of 4-*nitroveratrole*

The ether extract of 48-hr urine after hydrolysis with HCl was investigated by paper chromatography with system 2. On the chromatogram two yellow spots and a very

small, reddish-violet one were revealed by spraying with KOH solution; they had R_fs of 0·47, 0·79, and 0·52 respectively. Coloration of the first two suggested that these were nitrophenol derivatives. Their R_f values were identical with those of authentic 4- and 5-nitroguaiacols respectively. The last compound (R_f 0·52) seemed to be 4-nitrocatechol by its coloration (which had been reported by Benedikt[9]). Unchanged 4-nitroveratrole and amino derivatives were not detected.

TABLE 2. EXCRETION OF THE METABOLITES IN 24-HOUR URINES AFTER 4-ACETAMINOVERATROLE

(percentage of drug administered)

Exp. no.	β-Glucuronidase treatment		HCl hydrolysis after β-glucuronidase treatment		Total	
	5-Ag (%)	4-Ag	5-Ag (%)	4-Ag	5-Ag (%)	4-Ag
1	<0·1	45·5				
2	<0·1	51·6	12·0	13·6	12·0	65·2
3	<0·1	42·9	11·7	26·7	11·7	69·3

Isolation, characterization, and identification of the metabolites of 4-nitroveratrole

Ether extract (1·8 g), which was obtained from 48-hr urines from three rabbits according to the determination procedure, was dissolved in a small quantity of methanol and poured onto a column prepared from cellulose powder (100 g, 200–300 mesh) which was washed thoroughly with the solvent of system 2 before use. It was then eluted with solvent system 2 and separated into 5-ml fractions immediately after the first elution of the yellow band of nitroguaiacols. Fractions 1 to 32 were shown to be a mixture of 4- and 5-nitroguaiacols; 35 to 50 consisted only of 4-nitroguaiacol by paper chromatography.

The latter fractions were combined and the solvent removed under reduced pressure. The residue was dissolved in isopropyl ether and filtered. The brown gum (0·32 g) obtained after removal of the solvent from the filtrate was dissolved in a small quantity of benzene, poured onto a silica gel column (15 g), and eluted with benzene:petroleum ether (2:1). Crystals from the yellow effluent were recrystallized from benzene:petroleum ether to bright yellow needles (0·25 g), m.p. 102°. (Found; C, 49·96, H, 4·38; N, 8·43. $C_7H_7O_4N$ requires C, 49·71; H, 4·17; N, 8·28.) The melting point of this metabolite was not depressed on admixture with authentic 4-nitroguaiacol. The u.v. and i.r. absorption spectra were also identical with those of 4-nitroguaiacol.

u.v. $\lambda_{max}^{0.1\,N\,NaOH}$ mμ (log ε): 266 (3·73), 435 (4·26)

The former fractions gave a brown gum (0·33 g) on evaporation of the solvent. It was dissolved in a small quantity of methanol, spotted on 16 sheets of the buffered filter paper, 40 × 40 cm, and developed with solvent system 2. The area corresponding to 5-nitroguaiacol on the chromatogram of each sheet which was visualized under an ultraviolet lamp was cut out, and the combined scraps were extracted with ether in a Soxhlet apparatus for 20 hr. The residue remaining after evaporation of ether from

the extract was dissolved in a small quantity of benzene and purified through silica gel (0·5 g) column with benzene:petroleum ether (2:1) as an effluent solvent. Crystals obtained after evaporation of the solvent from the yellow eluate were recrystallized from benzene–petroleum ether to slightly yellow needles (85 mg), m.p. 104°. (Found: C, 49·89; H, 4·30. $C_7H_7O_4N$ requires C, 49·71; H, 4·17.) The melting point and u.v. and i.r. absorption spectra were identical with those of authentic 5-nitroguaiacol.

u.v. $\lambda_{max}^{0.1\ N\ NaOH}$ mμ (log ε); 228 (3·98), 263 (4·06), 232 (3·68), 418 (3·54)

It was also proved that the above metabolites were not produced during the course of hydrolysis and extraction (Fig. 1).

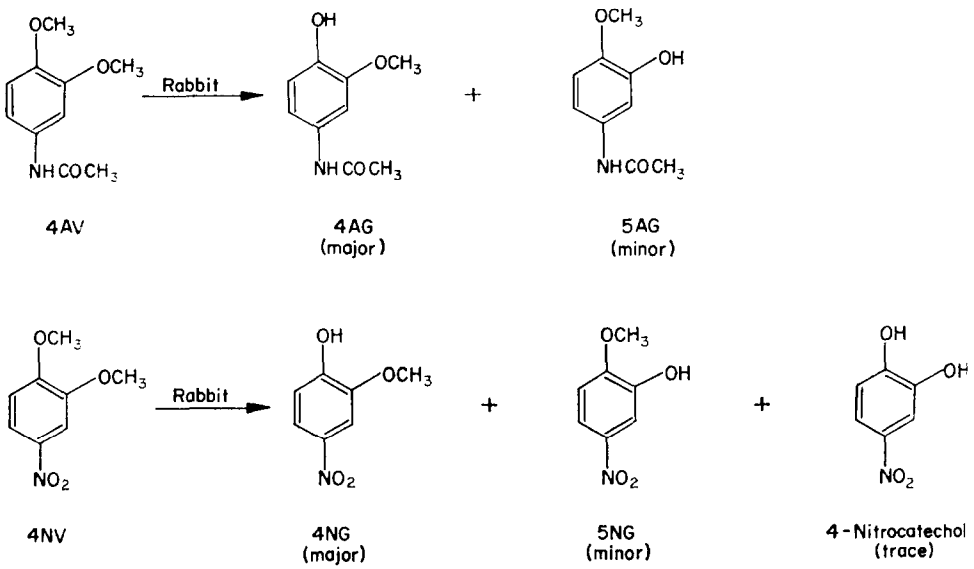

FIG. 1.

Determination of the urinary metabolites of 4-nitroveratrole

The determination was made on the combined 48-hr urines from three rabbits after acid hydrolysis. It was indicated that about half the 4-nitroveratrole was excreted as 4-nitroguaiacol and less than 10% as isomeric monophenol (Table 3).

TABLE 3. EXCRETION OF THE METABOLITES IN 48-HOUR URINES AFTER 4-NITROVERATROLE

(percentage of drug administered)

Metabolite	Metabolite excreted (%)
4-Nitroguaiacol	54·0
5-Nitroguaiacol	8·4

DISCUSSION

The purpose of this study was to determine the correlation between the electronic configuration and the selective demethylation of two adjacent methoxyl groups attached to an aromatic ring. The data obtained here, however, did not afford a simple correlation. In brucine the methoxyl group attached to the *meta*-position of its lactam nitrogen was demethylated predominantly,[1] whereas in both 4-acetamino- and 4-nitroveratroles, the methoxyl group attached to the *para*-position of its substituents, whose electronic properties are the complete reverse of each other, was cleaved at a much higher rate than the first. It is therefore concluded that occurrence of demethylation selectivity *in vivo* is not due to electronic configuration but to other circumstances.

McMahon *et al.*[10] reported recently cleavage of a series of alkyl *p*-nitrophenyl ethers *in vivo* and *in vitro*. Using the alkyl group varied over a wide range, they found that the dealkylation rate *in vivo* of longer alkyl ethers (*n*-butyl, *n*-hexyl) markedly decreased in comparison with less long ones (methyl, ethyl, *n*-and *iso*-propyl). An interesting result in relation to our study was that cyanomethyl or allyl ether showed an excretion rate similar to that of the parent alkyl ether (methyl or propyl), although the electron density at the α-methylene carbon, adjacent to ether oxygen of the cyanomethyl ether or allyl is much lower than the parent alkyl ether.

Apart from this disussion, it is also interesting to report that in both 4-acetamino- and 4-nitro-veratroles demethylation reaction was found to be the major *in vivo* metabolic pathway in rabbit, and no deacetylation or nitro reduction was observed' Excretion of catechol in a small amount was suggested in the case of 4-nitroveratrole, but this was apparently only a slight contribution to the metabolism and did not warrant further study.

The major metabolite of 4-acetaminoveratrole (4-acetaminoguaiacol) was excreted in the urine mostly as β-glucuronide. On the other hand, isomeric 5-acetaminoguaiacol conjugates were not hydrolyzed with β-glucuronidase but only with HCl. The possibility that it might be another type of conjugate, for example sulfate, has not been confirmed.

Acknowledgements—This work was supported partially by a Grant-in-Aid for Scientific Research provided by the Ministry of Education to which the authors are indebted. The authors wish to acknowledge the excellent technical assistance of Miss E. Kirino in this research and to thank the members of the analytical group of this Institute for the elemental analysis and for the determination of i.r. and u.v. spectra.

REFERENCES

1. H. Tsukamoto, H. Yoshimura, T. Watabe and K. Oguri, *Biochem. Pharmacol.* **13**, To be published. (1964).
2. B. B. Brodie, J. R. Gillette and B. N. LaDu, *Ann. Rev. Biochem.* **27**, 427 (1958).
3. P. Jacobson, M. Jaenicke and F. Meyer, *Chem. Ber.* **29**, 2690 (1890).
4. D. Cardwell and R. Robinson, *J. chem. Soc.* **107**, 256 (1915).
5. E. Mameli and G. Pinna, *Chem. Zentr.* **1**, 877 (1899).
6. W. A. Jacobs and M. Heiderberger, *J. Amer. chem. Soc.* **39**, 2195 (1917).
7. L. Paul, *Chem. Ber.* **39**, 2779 (1906).
8. K. Kato, K. Yoshida, H. Tsukamoto, M. Nobunaga, T. Masuya and T. Sawada, *Chem. pharm. Bull. (Tokyo)* **8**, 329 (1960).
9. R. Benedikt, *Chem. Ber.* **11**, 362 (1878).
10. R. E. McMahon, H. E. Culp, J. Mills and F. J. Marshall, *J. med. Pharm. Chem.* **6**, 343 (1963).