were isolated, and the structure and configuration of three hydroxylated products, one from Ⅲ and two from Ⅳ, were clarified; 1β,3β,14,21-tetrahydroxy-14β-pregnan-20-one (Ⅴ), 7β,12β-dihydroxy-4,5-dehydrodigitoxigenone (Ⅷ), and 7β-hydroxy-4,5-dehydrodigitoxigenone (Ⅸ).

Based on these experimental results, the relationships between the structure of substrate and the positions to be hydroxylated in the microbiological transformation of cardiac aglycone derivatives by *A. orchidis* were discussed.

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157. Tadashi Watabe, Hidetoshi Yoshimura, and Hisao Tsukamoto: Metabolism of Drugs. L.*1 The *In Vitro* Study on Metabolism of Brucine and 4-Substituted Veratroles.

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In the previous papers of this series, it was found that one of two adjacent methoxyl groups of brucine and 4-substituted veratroles were selectively removed in rabbits.[1,2] Interesting finding in that study was that the selective demethylation of brucine occurred at the *meta*-position of its lactam group, while of 4-substituted veratroles at the *para*-position of their 4-substituents.

The enzyme systems which catalyze O-demethylation of various foreign compounds have been known to locate in liver microsomes and to require both reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen for their activity.[3] It, therefore, seemed to be of interest to examine whether or not the microsomal enzyme systems were also responsible for the demethylation of above compounds which have two adjacent methoxyl groups attached to an aromatic ring.

The present paper deals with *in vitro* demethylation of brucine and 4-substituted veratroles, using 9000×g. supernatant fractions of rabbit liver homogenates. In addition, demethylation of 4-nitroveratrole has been further studied by the microsomal fractions.

It will be shown that these demethylations are also catalyzed by the microsomal enzyme systems, and that not only NADPH but also reduced nicotinamide adenine dinucleotide (NADH) is an effective cofactor for demethylation of 4-nitroveratrole.

Materials and Methods

Materials——Brucine·HCl was prepared from a commercial sample of brucine, and MDB-I (2-methoxy-3-hydroxystrychnine) and MDB-Ⅱ (2-hydroxy-3-methoxystrychnine) were obtained from the urine of rabbits administered brucine and by partial hydrolysis of brucine, respectively, as described previously.[1,2] 4AV (4-acetamidoveratrole),[4] 4AG (4-acetamidoguaiacol),[5] 5AG (5-acetamidoguaiacol),[6] 4-aminoveratrole,[7]

*1 Part XLIX: Biochem. Pharmacol., in press.
*2 Katakasu, Fukuoka (渡部 烈, 吉村英敏, 塚元久雄).
1) H. Tsukamoto, H. Yoshimura, T. Watabe, K. Oguri: Biochem. Pharmacol., in press.
2) H. Tsukamoto, H. Yoshimura, T. Watabe: *Ibid.*, in press.
3) B. B. Brodie, J. R. Gillette, B. N. LaDu: Ann. Rev. Biochem., **27**, 427 (1958).
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5) E. Mameli, G. Pinna: Chem. Zentr., **1899**, I, 877.
6) W. A. Jacobs, M. Heiderberger: J. Am. Chem. Soc., **39**, 2195 (1917).

4NV (4-nitroveratrole),[8] 4NG (4-nitroguaiacol),[8] and 5NG (5-nitroguaiacol)[9] were prepared by the known methods.

NAD, NADH, NADP, NADPH, and glucose-6-phosphate dipotassium salt were obtained from the Sigma Chemical Company. SKF 525-A (2-diethylaminoethyl diphenylpropylacetate hydrochloride)*3 was kindly supplied by Smith Kline & French Labolatories.

Preparation of Tissue Samples——The preparation of tissue samples was carried out at 0 to 2°. Male albino rabbits weighing 2.5 to 3.0 kg. were stunned, exsanguinated, and the livers were quickly removed and chilled in cold isotonic KCl.

Homogenate was prepared in 2 volumes of isotonic KCl containing 50 μ moles of nicotinamide per 1 g. of liver with a Potter-Elvehjem type homogenizer (teflon pestle). A supernatant fraction was prepared by centrifugation of the homogenate at 9000×g., for 20 min. in a Kubota KR-6LD centrifuge and adjusted with isotonic KCl so that each 3 ml. was equivalent to 1 g. of liver.

To prepare microsomes and the soluble fraction, the supernatant fraction was centrifuged at 108,000×g. for 1 hr. in a Hitachi 40P ultracentrifuge. Microsomes were separated, washed thoroughly with isotonic KCl, and recentrifuged at 108,000×g. for 1 hr. The microsomal pellets were resuspended in a volume of isotonic KCl, equivalent to that of the original homogenate.

Dialysis of soluble fractions were carried out against 50 volumes of deionized H_2O at 3° for 24 hr. on a magnetic stirrer. Dialyzed or non-dialyzed soluble fraction was adjusted with deionized H_2O to a final volume containing soluble fraction from 1 g. of liver in each 3 ml.

Enzyme Assays——In most metabolic studies on brucine, 4AV, and 4NV with liver 9,000×g. supernatant fraction, an incubation mixture consisted of 6 ml. of supernatant fraction, 30 μmoles of each substrate, 100 μmoles of nicotinamide, 100 μmoles of $MgCl_2$, 5 ml. of 0.2*M* phosphate buffer, pH 7.5 and H_2O to make a final volume of 16 ml. (for brucine) or 20.5 ml. (for 4AV and 4NV). Brucine·HCl was added directly to the incubation mixture, and 4AV and 4NV as a solution in 0.5 ml. of MeOH.

When an enzyme inhibitor, SKF 525-A was used, it was added to the mixture so as to give a final concentration of $2\times10^{-4}M$. Various buffer solutions used for investigation of pH effect on 4NV-metabolism were 0.2*M* Na_2HPO_4-0.2*M* NaOH, pH 6.0 to 8.0 and 0.1*M* Na_2HPO_4-0.05*M* Na_2CO_3, pH 9.0. Incubations were carried out aerobically in a metabolic shaker for 1 hr. (brucine and 4NV) or 2 hr. (4AV).

In experiments of 4NV with microsomes, an incubation beaker contained 4 ml. of microsomal preparation, 3 ml. of dialyzed or non-dialyzed soluble fraction, the same amounts of nicotinamide, $MgCl_2$, and 4NV, as described in the experiment with 9,000×g. supernatant fraction, 7 ml. of 0.2*M* phosphate buffer, pH 7.5, certain amounts of cofactors and water to make a final volume of 20.5 ml.

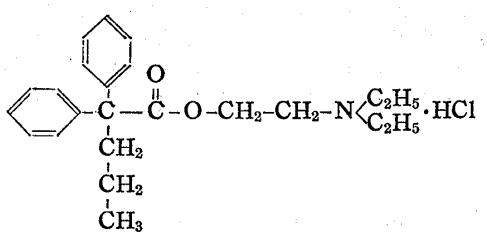
When soluble or microsomal fraction was omitted, instead, 6 ml. or 4 ml. of 0.2*M* phosphate buffer, pH 7.5 was added, respectively. Incubations were carried out aerobically in a metabolic shaker at 37° for 1 hr.

Formed phenolic metabolites were determined quantitatively by the following procedure.

A) Brucine: The incubation was stopped by the addition of 0.5 ml. of conc. HCl and 20 ml. of 40% CCl_3COOH, and the precipitate was removed by centrifugation. An aliquote of 10 ml. of the supernatant was made alkaline with 1 ml. of conc. NH_4OH, shaken with 20 ml. of $CHCl_3$ after addition of 10 g. of NaCl, and centrifuged. To remove nicotinamide from the $CHCl_3$ extract, the 10 ml. portion was washed with equal volume of 0.03*M* phosphate buffer, pH 5.2. Phenolic bases in 8 ml. of this $CHCl_3$ phase was transferred into the equal volume of 0.5*N* NaOH by shaking. The total amount of MDB-I and MDB-II in the aqueous alkaline phase was determined spectrophotometrically at 318 mμ.*4

To estimate the ratio of these two isomers, an aliquote of the above $CHCl_3$ extract after being washed with buffer was dried on Na_2SO_4, concentrated to a small volume, and then submitted to thin-layer chromatography which will be described later. Spots corresponding to MDB-I and MDB-II were visualized under an ultraviolet lamp, and taken out separately from the chromatogram. They were extracted with 5 ml. of 0.2*N* HCl by shaking for 10 min. and centrifuged, respectively. To 4 ml. of each

*3



*4 Both MDB-I and MDB-II had the absorption maximum at 318 mμ, and the same molecular extinction coefficient (see ref. 1).

7) R. G. Farger: J. Chem. Soc., **117**, 869 (1920).
8) D. Cardwell, R. Robinson: *Ibid.*, **107**. 250 (1915).
9) L. Paul: Ber., **39**, 2779 (1906).

HCl extract, 0.5 ml. of 6*N* NaOH was added and centrifuged. The ratio of MDB-I and MDB-II was estimated spectrophotometrically by reading optical density of both alkaline extracts at 318 mμ. From the total amount and the ratio of MDB-I and MDB-II, each amount of these two phenolic metabolites were calculated. In every process mentioned above, shaking was carried out mechanically for 15 min. and centrifugation for 5 min. in 60 ml. glass-stoppered centrifugation tubes. Authentic MDB-I and MDB-II carried through the incubation and analytical procedure served as a blank.

B) 4NV : To 10 ml. portion of incubation mixture 0.5 ml. of conc. HCl and 5 g. of NaCl were added. The mixture was shaken with 20 ml. of isopropyl ether and centrifuged. An aliquote of 10 ml. of the organic phase was then shaken with 10 ml. of 0.1*N* NaOH and centrifuged. Phenolic metabolites, 4NG and 5NG contained in this alkaline extract were determined spectrophotometrically. Since both 4NG and 5NG had the closed absorption maxima around 263 and 435 mμ, and their optical densities at these two wave lengths were 0.0348 and 0.107 (4NG), and 0.068 and 0.0198 (5NG), respectively in the concentration of 1 μg. of substances in 1 ml. of 0.1*N* NaOH, the amount of each metabolites in 0.1*N* NaOH, could be calculated through the equations, (1) and (2).

$$E_{263}=0.0348x+0.068y \quad (1)$$

$$E_{435}=0.107x+0.0198y \quad (2)$$

Where E_{263} and E_{435} are the observed optical densities at 263 and 435 mμ, respectively, and x and y are the concentrations of 4NG and 5NG (μg.) per ml. of 0.1*N* NaOH, respectively. In the analytical procedure mentioned above, shaking and centrifugation were carried out in a similar manner to that employed in the experiment of brucine. Authentic 4NG and 5NG carried through the incubation and analytical procedure served as standards. The incubation mixture containing all components except substrate served as a blank.

C) 4AV : The metabolism of 4AV was not studied quantitatively, but only qualitatively. The reaction mixture was extracted thoroughly with AcOEt after saturation with NaCl. The extract was reduced to a small volume and submitted to paper chromatography. Metabolites on the chromatogram were identified in a comparison with those of authentic samples.

Paper and Thin-layer Chromatographies——Paper chromatography was carried out in the ascending technique using the following solvent systems and Toyo Roshi No. 51A filter paper buffered at pH 10.8 with 0.05*M* carbonate buffer. Only when the solvent system 3 was applied, non-buffered filter paper was used. System 1 : BuOH-pyridine-benzene-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).

Chromatogram was visualized under an ultraviolet lamp (short waved "Manaslu Light," Manaslu Chem. Ind. Co., Ltd., Tokyo). For detection of phenolic metabolites of 4NV and 4AV, 0.2% solution of diazotized sulfanilic acid in 10% Na_2CO_3 and 5% KOH solution were also used, respectively. Ehrlich reagent was used for detection of amino derivatives.

Thin-layer chromatography was carried out by the usual ascending technique with use of silica gel*5 plates, 20×20 cm. in size, 0.2 mm. in thickness, activated at 100° for 1 hr. The solvent system used was $CHCl_3$-diethylamine (9:1). Chromatogram was visualized by spraying with Dragendorff reagent or by an ultraviolet lamp.

Results

Metabolism of Brucine and 4-Substituted Veratroles by Rabbit Liver 9000×g. Supernatant Fraction

A) **Brucine**—— The chloroform extract of the incubation mixture which was freed from nicotinamide, was submitted to thin-layer chromatography for identification of the metabolites. The chromatogram gave four Dragendorff-positive spots at Rf 0.28, 0.58, 0.75, and 0.78. Among them, one having Rf 0.75 was shown to be unchanged brucine. Furthermore the spots at Rf 0.28 and 0.58 were found to be responsible to MDB-I and MDB-II, respectively by the facts that they had the identical Rf values and ultraviolet absorption spectra in aq. NaOH with authentic samples, respectively. The compound which was revealed as a very faint spot at Rf 0.78 was a non-phenolic base, but not investigated further.

The each amount of metabolites formed by this system are shown in Table I. Resembling to the result of the *in vivo* study on brucine,[1] MDB-I was a preferential

*5 "Silica-Rider," Daiich Pure Chemicals Co., Ltd., Tokyo was used.

metabolite, and another isomeric phenol, MDB-II was produced only about one-fourth as much as the former.

B) 4NV——The paper chromatogram (system 2) of isopropyl ether extract from the incubation mixture showed the existence of two metabolites together with unchanged 4NV at Rf 0.47, 0.79, and 0.92, respectively. Both metabolites of Rf 0.47 and 0.79 gave dark spots under an ultraviolet lamp and afforded particular yellow colors of nitrophenol derivatives with 5% potassium hydroxide. They were identified as 4-nitroguaiacol (Rf 0.47) and 5-nitroguaiacol (Rf 0.79) by their Rf values and the ultraviolet absorption spectra of the eluates from the chromatogram with 0.1*N* NaOH. Any other product, for example 4-nitrocatechol which was produced in a small amount by whole animal[2] was not detected in this case.

Results of determination of the metabolites are shown in Table I. It is seen that 4-nitroveratrole was much more active substrate than brucine. This might explain the less formation of *in vivo* demethylation products of brucine than of 4-nitroveratrole.[1,2] Here again, 4-nitroguaiacol was a preferential metabolite and formed about twice as much as 5-nitroguaiacol. It will be described later, however, this demethylation selectivity is dependent on pH of incubation mixture.

Table I. *In Vitro* Demethylation of Brucine and 4-Nitroveratrole by Rabbit Liver 9000×g. Supernatant Fraction[a]

Experiment	Brucine		4NV	
	MDB-I	MDB-II	4NG	5NG
	(μmoles)		(μmoles)	
1	0.85	0.20	4.24	2.23
2	0.90	0.22	4.36	2.25

a) Incubation conditions are described under methods. (Phosphate buffer, pH 7.5 was used.)

C) 4AV——Paper chromatographic examination (system 1 and 3) of the extract from the incubation mixture indicated that there were two metabolites except unchanged 4-acetamidoveratrole. One was visualized as a very large Ehrlich positive spot at Rf 0.85 and 0.48, and the other a very small, light violet colored spot with a diazotized sulfanilic acid reagent at Rf 0.65 and 0.77, with system 1 and 3, respectively. These two metabolites were identified as 4-aminoveratrole and 4-acetamidoguaiacol, respectively, by their Rf values and characteristic color reactions. A spot corresponding to 5-acetamidoguaiacol (Rf 0.50 and 0.77, with system 1 and 3, respectively) was not detected at all. These result suggested that differing from the *in vivo* metabolism, 4-acetamidoveratrole underwent deacetylation rather than demethylation as the major metabolism by the rabbit liver supernatant fraction. A possibility that 4- and 5-amino guaiacols might be produced by deacetylation of the corresponding acetamidoguaiacols or by demethylation of 4-aminoveratrole could not be ruled out, since these aminophenols were found not to be extractable from the incubation mixture by preliminary experiment. It, therefore, is necessary to investigate further for learning the whole metabolic map of this substrate.

In spite of that, if one excludes deacetylation reaction from discussion, it may be again concluded that selective demethylation of this substrate also take place at the methoxyl group on the *para*-position of acetamido substituent.

The incubation for 1 hour gave the similar result to that for 2 hour.

Effect of pH on 4-Nitroveratrole Metabolism by Rabbit Liver 9000×g. Supernatant Fraction

As described above, 4-nitroguaiacol was formed about twice as much as 5-nitro-

guaiacol at pH 7.5. It was found, however, that the ratio between two products formation was variable, depending upon the pH of the incubation mixture (Table II). In the incubation media of pH 7.0 and 8.0, a preferential demethylation occurred at the *para*-position of nitro substituent, while in a more acidic or alkaline medium (pH 6.0 or 9.0), at the *meta*-position although the activity was considerably low. The preferential formation of 4-nitroguaiacol at the physiological pH is well related with the *in vivo* result.[2] Although this characteristic change of the demethylation selectivity by varying pH is very interesting, whether or not demethylation of each methoxyl group may be catalyzed by the different enzyme which has different optimal pH each other, is uncertain.

Table II. Effect of pH on Demethylation of 4-Nitroveratrole by Rabbit Liver 9000×g. Supernatant Fraction[a]

pH	Metabolites formed (μmoles)		Ratio between 4NG and 5NG
	4NG	5NG	
6.0	0.64	2.24	20 : 80
7.0	4.63	3.60	56 : 43
8.0	5.26	2.70	66 : 34
9.0	0.40	1.37	23 : 77

a) Incubation conditions are described under methods.

Effect of SKF 525-A on 4-Nitroveratrole Metabolism by Rabbit Liver 9000×g. Supernatant Fraction

SKF 525-A, a general inhibitor of a wide variety of oxidative metabolism of foreign compounds, also inhibited demetylation of 4-nitroveratrole (Table III).

The inhibition took place about equally (50%) to both demethylations of two methoxyl groups in a concentration of $2\times10^{-4}M$ of the inhibitor.

Table III. Effect of the Inhibitor, SKF 525-A on Demethylation of 4-Nitroveratrole[a]

	Metabolites formed (μmoles)		
	4NG	5NG	4NG+5NG
Control	3.62	0.93	4.55
SKF 525-A ($2\times10^{-4}M$)	1.93	0.42	2.35

a) Incubation conditions are described under methods.

Metabolism of 4-Nitroveratrole by Rabbit Liver Microsomes

Table IV shows representative data on demethylation of 4-nitroveratrole by rabbit liver microsomal fractions. As well as in other oxidative drug metabolism by the previous worker,[3] neither the microsomes nor the soluble fraction alone showed appreciable activity but when added together the activity was markedly restored.

The role of soluble fraction was also suggested to supply the cofactor by the facts that only a slight formation of metabolites could be observed with use of dialyzed soluble fraction, and the activity was restored remarkably with addition of NADPH*[6] and glucose 6-phosphate which served as NADP-reducing system together with glucose 6-phosphate dehydrogenase containing in dialyzed soluble fraction.

*[6] At the time of this experiment, a satisfactory amount of oxydized form of this cofactor was not available, but of reduced form. This system, however, will also serve as that of a continuous supply of NADPH during certain time.

Striking result that about half of the maximum activity obtained by recombination of microsomes and the soluble fraction was restored, was then obtained when microsomes were incubated with substrate, NAD, and NADH-generating system consisting of glucose and the dialyzed soluble fraction as the sourse of glucose dehydrogenase.

Table IV. Intracellular Distribution of Enzyme Activity and Cofactor Requirement in the Demethylation of 4-Nitroveratrole[a)]

	Metabolites formed (μmoles)		
	4NG	5NG	4NG+5NG
Microsome+soluble fraction	4.36	1.10	5.46
Microsomes	0.00	0.00	0.00
Soluble fraction	0.20	0.11	0.31
Microsomes+dialyzed soluble fraction	0.17	0.15	0.32
Microsomes+dialyzed soluble fraction+10 μmoles of NADPH+50 μmoles of G-6-P[b)]	2.65	1.36	4.01
Microsomes+dialyzed soluble fraction+10 μmoles of NAD+100 μmoles of glucose[c)]	1.95	0.73	2.68

a) Incubation conditions are described under methods.
b) Glucose 6-phosphate. *c*) cf. J.R. Gillette: J. Biol. Chem., **234** 139 (1959).

Direct evidence for not only NADPH but also NADH being the effective cofactor was provided by the experiment of their incubation with substrate and microsomes. Representative data given in Table V indicate that NADH was about as effective as NADPH when added separately 10 μmoles each. At a higher level of the cofactors (20 μmoles), NADH was even more effective than NADPH. The effect of the two cofactors was roughly additive at both levels. It was also shown that neither NADP nor NAD was effective as the cofactor.

Table V. Requirement for NADPH and NADH in the Demethylation of 4-Nitroveratrole by Liver Microsomes[a)]

Components	Metabolites formed (μmoles)		
	4NG	5NG	4NG+5NG
Microsomes+10 μmoles of NADPH	0.39	0.27	0.66
Microsomes+10 μmoles of NADH	0.44	0.17	0.61
Microsomes+10 μmoles of NADPH +10 μmoles of NADH	0.99	0.58	1.57
Microsomes+20 μmoles of NADPH	0.69	0.39	1.08
Microsomes+20 μmoles of NADH	1.10	0.30	1.40
Microsomes+20 μmoles of NADPH +20 μmoles of NADH	1.67	0.88	2.55
Microsomes+10 μmoles of NADP	0.0	0.0	0.0
Microsomes+10 μmoles of NAD	0.0	0.0	0.0

a) Incubation conditions are described under methods.

Discussion

A wide variety of oxidative metabolic reactions of foreign compounds has been known to be catalyzed by enzyme systems locating in liver microsomes and requiring both NADPH and oxygen.[3)] Supernatant fraction (9000×g.) of liver homogenate consists of microsomes and soluble fraction, and is often used conveniently for metabolism of foreign

compounds, in which soluble fraction participates its role in supplying the cofactor. In this paper, supernatant fraction has been also used to examine whether or not the above systems are responsible for O-demethylation of brucine and 4-substituted veratroles. In addition, metabolism of 4-nitroveratrole has been studied further by the microsomal fraction as the representative of the compounds which have two adjacent methoxyl groups attached to an aromatic ring.

It has been shown that coinciding with the *in vivo* result,[1,2] one of two methoxyl groups of brucine and 4-nitroveratrole is demethylated predominantly by this enzyme system at physiological pH. On the other hand, 4-acetamidoveratrole undergoes deacetylation rather than demethylation as the major *in vitro* metabolism. A high activity of amidase in the microsomal fraction has been also reported.[10] It therefore is not so surprising that in this specified condition deacetylase suppresses demethylase, differing from the *in vivo* result.

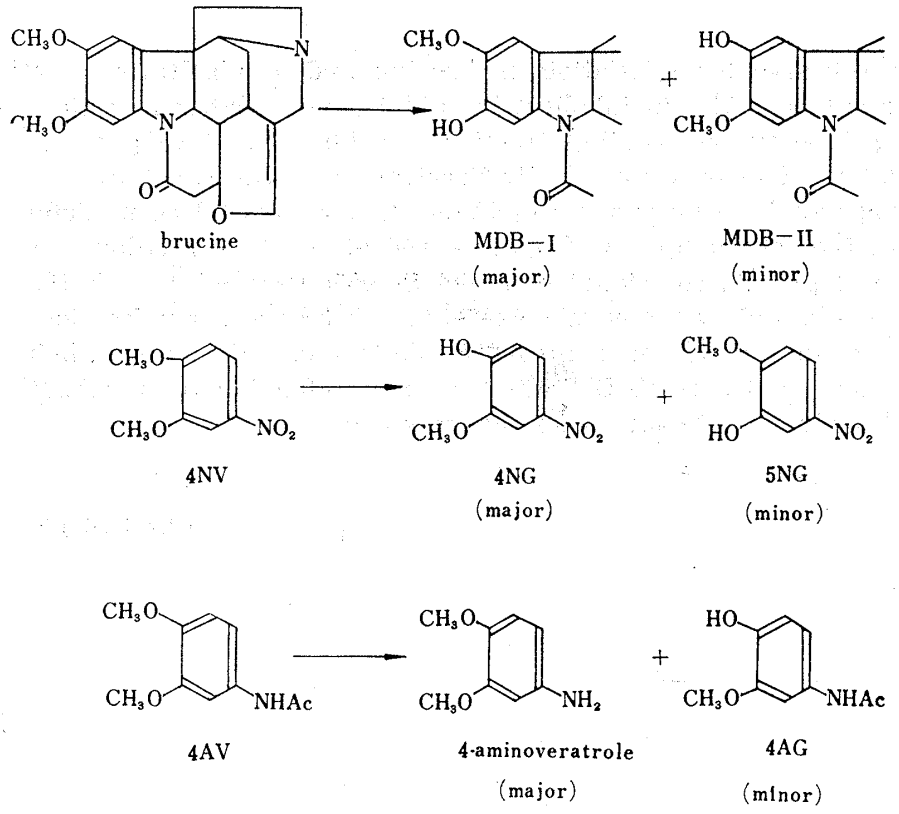


Chart 1.

Very interesting finding in this paper is cofacter requirement of the O-demethylating enzyme system of 4-nitroveratrole. A remarkable effectiveness of NADH as well as of NADPH has been shown in this study. In general, the cofactor, NADPH can not be replaced by NADH in the oxidative metabolism of foreign compounds, however a few exceptional reports has been appeared so far. Axelrod described that NADH was about one-third as effective as NADPH in the enzymatic conversion of codeine to morphine.[11] In the study on enzymatic N-demethylation of 3-methyl-4-methylaminoazobenzene Conney, *et al.* found that both NADH and NADPH were needed for its maximum activity, although NADH alone had little effect.[12] Very recently Nilsson and Johnson studied

10) G. Hollunger, B. Niklasson : Proc. First Inter. Pharmacol. Meeting, **6**, 149 (1962).
11) J. Axelrod : J. Pharmacol. Exptl. Therap., **115**, 259 (1955).
12) A. H. Conney, R. R. Brown, J. A. Miller, E. C. Miller : Cancer Research, **17**, 628 (1957).

extensively on the cofactor requirement for O-demethylation of biochanin A and for other reactions, and described that both NADPH and NADH might be required not only for O-demethylation of biochanin A, but also other oxidative metabolism of foreign compounds.[13]

In addition to these studies, the present investigation has further provided a remarkable evidence that NADH participates an important role in O-demethylating enzyme system as well as NADPH.

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Summary

Interesting selective demethylation of brucine and 4-substituted veratroles, which was found to occur as major metabolism in rabbits, was further investigated by *in vitro* systems. Using 9000×g. supernatant fraction of rabbit liver homogenate, it was shown that demethylation of brucine and 4-nitroveratrole was catalyzed by so called "liver microsomal drug metabolizing enzyme systems," and it took place predominantly at the *meta*-position of the lactam group of brucine and at the *para*-position of nitro group of 4-nitroveratrole at pH 7.5, coinciding with the *in vivo* results.[1,2] On the other hand, 4-acetamidoveratrole underwent mostly deacetylation by the same fraction.

In addition, demethylation of 4-nitroveratrole studied further by rabbit liver microsomes, and it was found that NADH was even more effective than NADPH as the cofactor if added sufficiently to the incubation mixture.

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13) A. Nilsson, B. C. Johnson : Arch. Biochem. Biophys., **101**, 494 (1963).