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METABOLISM OF DRUGS—XLVIII. THE STUDY OF SELECTIVE DEMETHYLATION OF BRUCINE *IN VIVO*

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Abstract—One of the two adjacent methoxyl groups attached to the aromatic ring of brucine was found to be demethylated selectively in rabbits. The predominant monophenolic metabolite was identified as 2-methoxy-3-hydroxystrychnine and excreted mostly as conjugated forms in the urine, mainly the β-glucuronide. The isomeric monophenol, 2-hydroxy-3-methoxystrychnine, was excreted in only a negligible amount. In addition to these phenolic metabolites, existence of a nonphenolic base in the urine was demonstrated, together with a very small amount of unchanged brucine, by paper chromatography. All of these metabolites and unchanged brucine were also excreted in feces, although the amounts were very small.

EXTENSIVE studies of the dealkylation *in vivo* and *in vitro* of a wide variety of alkylaryl ethers have been reported by a number of workers;[1, 2] however, very few have been investigated in which more than one alkoxyl group were attached to an aromatic ring. Axelrod *et al.*[3] discovered the interesting fact that only one of four methoxyl groups of papaverine was removed in man and guinea pig. Another selective demethylation which occurred as a major metabolic pathway in mammals was demonstrated in the metabolic study of griseofulvin by Barnes and Boothroyd.[4]

Since brucine, one of the Strychnos alkaloids, possesses two adjacent methoxyl groups on its substituted benzene ring, it seemed of interest to investigate whether it undergoes a selective demethylation in mammal similar to that described above. In this paper the authors wish to report that brucine has been demethylated predominantly to 2-methoxy-3-hydroxystrychnine in rabbits.

MATERIALS

Synthesis of the minor phenolic metabolite, 2-hydroxy-3-methoxystrychnine (MDB-II)

A. Synthesis from previously known 2-acetoamino-3-hydroxystrychnine perchlorate. Preparation of 2-acetamino-3-hydroxystrychnine: an aqueous solution of perchlorate of this base, which was prepared from brucine by the method of Leuchs *et al.*,[5] was made alkaline with potassium bicarbonate and extracted with chloroform containing 5% volume of isoamyl alcohol. The residue remained after evaporation of the solvent from the extract was recrystallized from chloroform–isopropyl ether to slightly pale yellow needles, m.p. 225–228° (decomp.).

Preparation of 2-acetamino-3-methoxystrychnine: to a solution of 2-acetamino-3-hydroxystrychnine (7·0 g) in methanol (80 ml), an ethereal solution of diazomethane prepared from nitrosomethylurea (20 g) was added, and the mixture was set aside at room temperature. After 72 hr the solvent was evaporated, leaving a reddish-brown

gum. It was dissolved in a small volume of chloroform and poured onto an alumina column (100 g). It was developed with benzene (150 ml) and then with a mixture of benzene and chloroform (4:1, 900 ml). The residue from this eluate was recrystallized from chloroform–benzene to leaflets (2·4 g), m.p. 235°. (Found: C, 68·11; H, 6·75; N, 9·84. $C_{24}H_{27}O_4N_3$ requires C, 68·39; H, 6·46; N, 9·99%).

i.r. λ_{max}^{KBr} μ: 2·96, 5·90, 6·08, 6·21, 6·57, 6·74

u.v. λ_{max}^{EtOH} mμ (log ϵ): 221 (4·40), 270 (4.17), 280 (4·11), 309 (4·10)

Preparation of 2-amino-3-methoxystrychnine: a solution of 2-acetoamino-3-methoxystrychnine (0·9 g) in 1·5 N HCl (20 ml) was refluxed mildly for 1 hr, then made alkaline with ammonia, and extracted with chloroform. Evaporation of the solvent gave a yellow solid. It was recrystallized from chloroform–acetone to yellow-colored prisms (0·78 g), which were used in the next reaction without further purification.

i.r. λ_{max}^{KBr} μ: 2·96, 6·05, 6·17, 6·25, 6·67

The structure of this compound was reconfirmed by its conversion to α-colubrine as follows: a solution of 2-amino-3-methoxystrychnine (0·45 g) in 2 N H_2SO_4 (20 ml) was treated with a solution of sodium nitrite (0·09 g) in water (5 ml) at room temperature. The resulting diazonium salt solution was poured into a boiling solution of sodium hypophosphite (2·5 g) in water (15 ml), and the reaction mixture was heated until it showed no color with a 1% solution of β-naphthol in 4% NaOH (about 10 min). The mixture was then immediately cooled to room temperature, made alkaline with ammonia, and extracted with chloroform. Evaporation of the solvent left a brown gum (0·36 g). It was dissolved in a small volume of chloroform, poured onto an alumina column (15 g), and eluted successively with mixtures of benzene and chloroform (4:1, 200 ml; 2:1, 100 ml; 1:1, 100 ml; then 1:2, 100 ml). Eluates were evaporated to leave a colorless gum (0·32 g), which was recrystallized from aqueous ethanol to plates, m.p. 183–185°, after drying at 100° *in vacuo* over P_2O_5. (Found; C, 72·72; H, 6·92; N, 7·80. $C_{22}H_{24}O_3N_2$ requires C, 72·51; H, 6·64; N, 7·69%.) This melting point and the coloration with 25% nitric acid were the same as in the preceding description of α-colubrine.[6] A mixed melting point of this specimen with β-colubrine[7] (m.p. 222°) prepared from 2-hydroxystrychnine[8] was depressed. Its i.r. and u.v. absorption spectra were also different from those of β-colubrine.

i.r. $\lambda_{max}^{CHCl_3}$ μ: no OH, 6.00, 6.20, 6·25, 6·67

u.v. λ_{max}^{EtOH} mμ (log ϵ): 221 (4·43), 256 (4·03), 264 (3·93), 294 (3·82), 299 (3·80)

Preparation of 2-hydroxy-3-methoxystrychnine (MDB-II): a solution of 2-amino-3-methoxystrychnine (0·34 g) in 4 N H_2SO_4 (40 ml) containing cupric sulfate (0·54 g) was kept at 0 to 5°. To this mixture a solution of sodium nitrite (0·074 g) in water (8 ml) was added by drops under mechanical stirring over a period of 30 min. Stirring was continued for another half hour at 10°. Excess of nitrite was then decomposed with a small amount of solid urea. The resulting diazonium salt solution, which gave a violet color with a 1% solution of β-naphthol in 4% NaOH, was poured into a boiling solution of cupric sulfate (40 g) in water

(40 ml), and the reaction mixture was heated until no color showed with β-naphthol reagent (about 10 min). The solution was immediately cooled to room temperature, made alkaline with ammonia, and extracted with chloroform containing 5% volume of isoamyl alcohol. The organic solvent layer was shaken with N NaOH. Phenolic material was re-extracted with chloroform containing 5% volume of isoamyl alcohol after addition of excess solid ammonium chloride to the separated aqueous alkaline layer. A crystalline residue obtained on evaporation of the solvent was washed with a small volume of methanol and recrystallized from chloroform–methanol to minute prisms (0·06 g), m.p. 278–280° (decomp.). (Found: C, 69·19; H, 6·52; N, 7·25. $C_{22}H_{24}O_4N_2$ requires C, 69·45; H, 6·36; N, 7·36%.)

i.r. $\lambda_{max}^{KBr}\mu$: 2·92, 6·05, 6·25, 6·67
u.v. $\lambda_{max}^{0.5N\ HCl}$ mμ (log ε): 262 (4·05), 300 (3·91)
u.v. $\lambda_{max}^{0.5N\ NaOH}$ mμ (log ε): 283 (4·05), 318 (4·06)

Methylation of a small amount of this compound with diazomethane yielded the parent compound, brucine, which was proved by paper and thin-layer chromatography, and also by u.v. absorption spectra (Fig. 3). All reaction processes are shown in Fig. 1.

B. Synthesis by partial hydrolysis of brucine with pyridine hydrochloride. A mixture of brucine hydrochloride (5 g), pyridine hydrochloride (1.7 g), and acetic acid (1·6 ml) was kept at 165 to 170° on an oil bath under nitrogen gas for 5 hr. After dissolution of the reaction mixture in diluted hydrochloric acid, it was shaken with N NaOH and chloroform. The aqueous alkaline layer was separated and extracted with chloroform in the presence of excess solid ammonium chloride. On evaporation of the solvent a crystalline phenolic residue (0·62 g) was obtained. It was washed with a small volume of methanol and recrystallized from chloroform–methanol to minute prisms, m.p. 278–280° (decomp.), which were proved to be identical with MDB-II prepared by method *A* above.

The paper and thin-layer chromatograms of the mother liquor, using systems A and 1, respectively, described in the last section of Methods, showed the existence of a small amount of another monodemethylated brucine (MDB-I) together with MDB-II. As mentioned in Results, MDB-I was the major metabolite of brucine in the rabbit. Although this chemically synthesized MDB-I was not isolated from the mother liquor, the identity of this compound with the metabolite MDB-I was confirmed by paper and thin-layer chromatography and by u.v. absorption spectra in both 0·5 N HCl and 0·5 N NaOH (Fig. 2).

METHODS

Administration of brucine

Brucine hydrochloride (150 mg/kg) was given to male albino rabbits weighing 2·5 to 3·0 kg as 3·8% aqueous solution by stomach tube; their 24-hr or 48-hr urines were collected under toluene.

Extraction of metabolites from urine

Combined 24-hr urines (4·5 liters) from animals which had received brucine hydrochloride (10 g) were heated on a boiling water bath with concentrated hydrochloric

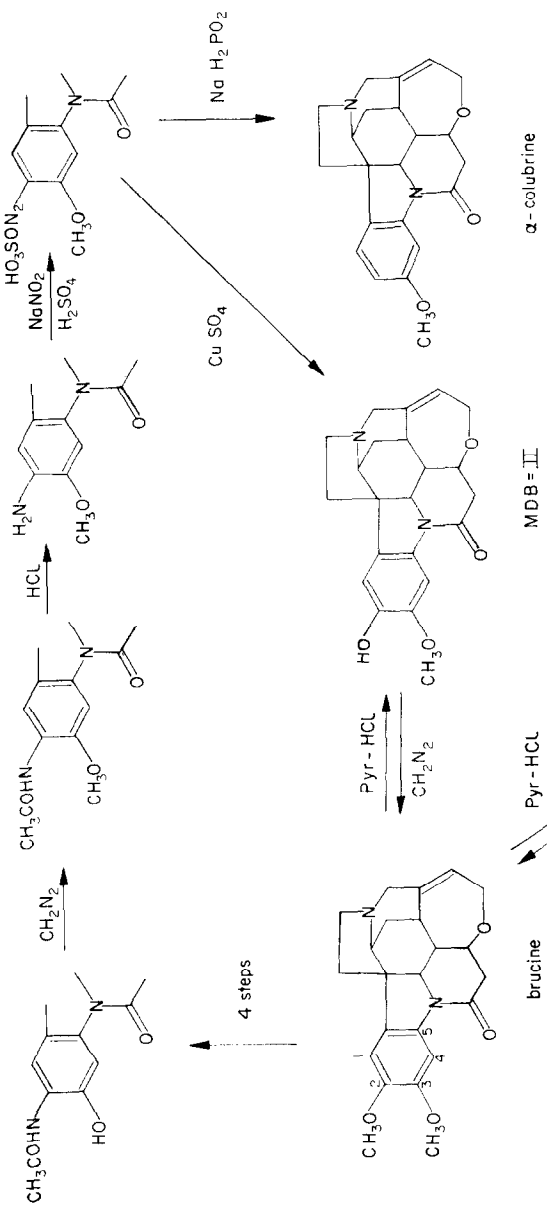

FIG. 1. The synthetic scheme of brucine metabolites.

acid (725 ml) for 30 min and immediately cooled to room temperature. The acid-treated urine was filtered and the clear filtrate adjusted to pH 9·0 with ammonia. It was then extracted twice with two volumes of chloroform after its saturation with NaCl. From the chloroform extracts a brown gum (2·4 g) was obtained. This was dissolved again in chloroform and shaken with N H_2SO_4. The aqueous acidic layer was made alkaline with 5 N NaOH and extracted with chloroform. From the chloroform extract, nonphenolic bases were obtained as a brown gum (0·45 g). The aqueous alkaline phase was saturated with ammonium chloride and extracted with chloroform. The chloroform extract, after evaporation of the solvent, gave a brown gum (0·91 g; phenolic bases).

Quantitative determination of urinary phenolic metabolites

A. After acid hydrolysis. An aliquot of 10 ml of urine excreted in the first 24 hr after dosing was pipetted into a test tube containing 0·9 ml of concentrated hydrochloric acid which was then immersed in a boiling water bath. After heating for 1 hr, water was added to make original volume. Five ml of this hydrolyzed urine was made alkaline with 0·5 ml of concentrated ammonia. After addition of 20 ml of chloroform to this mixture, it was shaken and centrifuged. An aliquot of 10 ml of chloroform layer was then shaken with 20 ml of 0·5 N HCl and centrifuged. Finally, 10 ml of the acidic aqueous layer was transferred to a tube containing 2 ml of 5 N NaOH and 20 ml of chloroform, shaken, and centrifuged. The concentration of phenolic metabolites in this aqueous alkaline layer was determined spectrophotometrically at 318 mμ. In all of these processes shaking was carried out mechanically for 15 min and centrifugation was done for 3 min in 60-ml glass-stoppered centrifuge tubes as vessels. If acid hydrolysis was not required, instead of concentrated hydrochloric acid 0·9 ml of water was added to a 10-ml aliquot of urine and run through the same procedure without heat treatment. A blank value for the zero setting was obtained from a control experiment with normal urine from each rabbit before dosing. Recovery of phenolic metabolites in this procedure was within good precision (95 $\pm$ 2%). The first 48-hr urine after dosing was also examined by the same procedure.

B. After treatment with β-glucuronidase. The urine was adjusted to pH 4·8 with acetic acid and incubated with a β-glucuronidase preparation obtained from adult male rabbit liver by the method of Fishman and Bernfeld,[9] which was purified to the stage of Step 2 in their text by the following procedure. An incubation mixture consisted of a 5-ml portion of urine; 1 ml ($1{\cdot}85 \times 10^4$ units, determined by the method described previously[10]) of enzyme preparation; 1 ml of 0·5 M acetate buffer, pH 4·8; and, if necessary, 0·5 ml of a freshly prepared solution of potassium hydrogen saccharate equivalent to a final concentration of 10^{-3} M, which was heat treated at pH 3·5 as previously described;[10] and water to make a final volume of 7·5 ml. It was incubated at 38° for 5 hr. After incubation, 0·5 ml of 40% trichloroacetic acid was added to the reaction mixture. The precipitate was removed by centrifugation. Five ml of the supernatant solution was subjected to the same determination procedure as that after acid hydrolysis.

Paper and thin-layer chromatography

Paper chromatography was carried out in the ascending technique with the following solvent systems and buffered Toyoroshi no. 51A filter paper (Toyoroshi Co., Ltd.,

Tokyo). System A, BuOH:AcOH (50:1) saturated with water and filter paper buffered at pH 6·0 (0·1 M citrate buffer). System B, BuOH:AcOH:H_2O (4:1:5) and filter paper buffered at pH 6·0 (0·1 M citrate buffer). System C, AcOEt:pyridine:H_2O (15:5:3) and filter paper buffered at pH 6·0 (0·1 M citrate buffer). System D, AcOEt: pyridine:H_2O (15:5:3) and filter paper buffered at pH 9·0 (0·1 M Tris buffer).

Thin-layer chromatography was carried out by use of silica gel plate ("Silica-Rider," Daiichi Pure Chemicals Co., Ltd., Tokyo), 0·2 mm thick, activated at 100° for 1 hr. The solvent systems used are: system 1, chloroform:acetone:diethylamine (5:4:1); system 2, chloroform:diethylamine (9:1).

Paper and thin-layer chromatograms were visualized under an ultraviolet lamp (shortwave "Manaslu-Light," Manaslu Chemical Industries Co., Ltd., Tokyo) or by spraying with Dragendorff reagent.

RESULTS

Isolation, characterization, and identification of the metabolites

The thin-layer chromatograms of crude phenolic fractions in systems 1 and 2 showed the existence of two phenolic metabolites which, as described later, were isomeric monodemethylated brucines, MDB-I and MDB-II. Their R_f values were 0·37 and 0·58 (MDB-I), and 0·23 and 0·28 (MDB-II) in systems 1 and 2 respectively. On the paper chromatograms using system A, MDB-I was visualized at *Rf* 0·30 and MDB-II at 0·38.

The spot of MDB-II was so faint and tiny that it seemed to be negligible. It was, however, confirmed that MDB-II was identical with synthetic 2-hydroxy-3-methoxystrychnine by paper and thin-layer chromatography in the above systems. Further u.v. spectra of MDB-II in 0·5N HCl and 0·5 N NaOH were also identical with those of 2-hydroxy-3-methoxystrychnine (Fig. 2).

Upon treatment of 0·8 g of crude phenolic fraction of metabolite with methanol, only the major metabolite, MDB-I, was crystallized out. It was recrystallized from methanol containing a few drops of water to yield 0·56 g of minute needles, free from MDB-II, m.p. 267–268°. (Found: C, 69·21; H. 6·38; N, 7·23. $C_{22}H_{24}O_4N_2$ requires C, 69·45; H, 6·36; N, 7·36%.

i.r. $\lambda_{max}^{CHCl_3}$ μ: 2·76, 6·01, 6·17, 6·67
u.v. $\lambda_{max}^{0.1\ N\ NaOH}$ mμ (log ϵ): 228 (4·33), 273 (3·92), 318 (4·06)
u.v. $\lambda_{max}^{0.1\ N\ HCl}$ mμ (log ϵ): 262 (4·04), 300 (3·90)

Methoxyl group determination of MDB-I showed clearly that it has one methoxyl group in the molecule. Its u.v. absorption spectrum is illustrated in Fig. 2.

Treatment of MDB-I with diazomethane yielded brucine. Identity of this product with brucine was confirmed by paper and thin-layer chromatography. The *Rf* values in paper chromatography with systems A, B, C, and D were 0·49, 0·73, 0·23, and 0·90, respectively, and in thin-layer chromatography using systems 1 and 2 were 0·47 and 0·65 respectively. In addition, its u.v. absorption spectrum was identical with that of authentic brucine (Fig. 3). This result suggests that any structural change other than demethylation has not taken place in MBD-I.

In considering these facts, it was concluded that MDB-I and MDB-II were isomers of each other. Therefore, the structure of MDB-I should be 2-methoxy-3-hydroxy-strychnine.

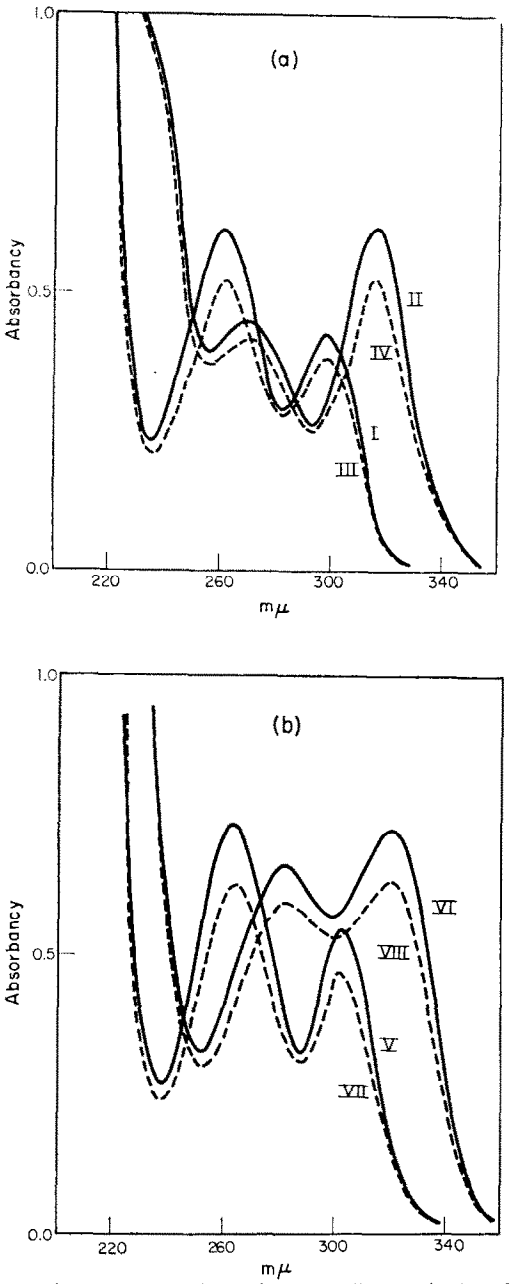

FIG. 2. A: Ultraviolet absorption spectra of the major phenolic metabolite of brucine, MDB-I, and the minor phenolic product obtained by the treatment of brucine with pyridine hydrochloride. Curves I and II: metabolic MDB-I in 0·5 N HCl and 0·5 N NaOH respectively. Curves III and IV: synthetic MDB-I in 0·5 N HCl and 0·5 N NaOH respectively.

B: Ultraviolet absorption spectra of the minor phenolic metabolite of brucine, MDB-II, and the major phenolic product obtained by the reaction of brucine and pyridine hydrochloride. Curves V and VI: metabolic MDB-II in 0·5 N HCl and 0·5 N NaOH respectively. Curves VII and VIII: synthetic MDB-II in 0·5 N HCl and 0·5 N NaOH respectively.

A paper chromatogram of the fraction of nonphenolic bases (system A) showed the presence of one metabolite at R_f 0·44 in addition to a very small amount of unchanged brucine at R_f 0·49. Further study of this nonphenolic metabolite has not been undertaken.

Any possibility that these metabolites might be produced artificially during the course of the extraction procedure, including acid hydrolysis, was excluded by the

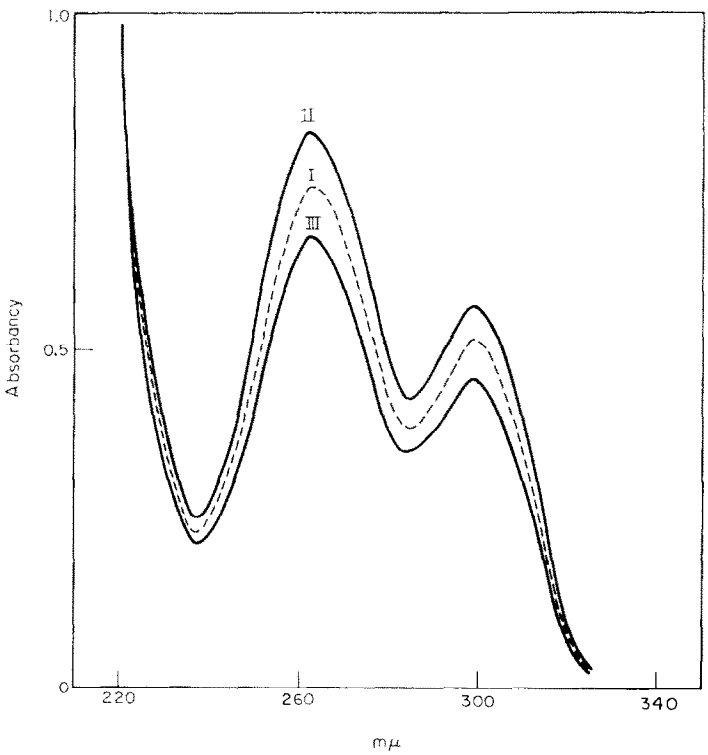

FIG. 3. Ultraviolet absorption spectra of methylated products of metabolic MDB-I and synthetic MDB-II in 0·5 N HCl. Curve I: authentic brucine. Curve II: product derived from MDB-I with CH_2N_2. Curve III: product derived from MDB-II with CH_2N_2. Each reaction mixture of MDB-I and MDB-II with diazomethane was evaporated to dryness and the residue was chromatographed on paper with system A. The area corresponding to brucine was cut from the paper chromatogram and eluted with 0·5 N HCl.

following facts. None of the spots corresponding to metabolites, except that of unchanged brucine, was detected on paper chromatograms when a mixture of 100 ml of normal rabbit urine and 100 mg of brucine hydrochloride was heated with 16 ml of hydrochloric acid for 1 hr and extracted by the procedure described under Methods.

Quantitative determination of urinary phenolic metabolites

Urinary excretion rate of the phenolic metabolites in the first 24 hr after dosing with brucine hydrochloride is summarized in Table 1. It is assumed that each value in the table is the rate for MDB-I because the amount of MDB-II was negligible, as mentioned above.

As seen in the table, 10 to 14% of ingested brucine was detectable as MDB-I after acid hydrolysis, whereas before the treatment MDB-I existed only in a negligible amount. From this observation it was considered that most of MDB-I appeared in the conjugated form in the urine. In addition, the values after treatment with β-glucuronidase in the presence or absence of its inhibitor showed clearly that a large part of the conjugates should be β-glucuronide.

The amount of MDB-I in the first 48-hr urines was also determined after acid hydrolysis, but no significant increase was observed in comparison with the 24-hr urines.

Excretion of metabolites in feces

Collected 72-hr feces from rabbits after dosing were crushed in five volumes of 5% hydrochloric acid. The slurry was heated on a boiling water bath for 1 hr and centrifuged. The separated supernatant was made ammonia-alkaline and extracted with chloroform. On evaporation of the solvent from the chloroform extract a very small amount of gum was obtained. It was subjected to paper chromatography in system

TABLE 1. EXCRETION RATE OF MDB-I IN RABBIT URINE*

Rabbit no.	Untreated urine (%)	HCl-treated urine (%)	β-Glucuronidase-treated urine (%)	β-Glucuronidase-treated urine in the presence of PHS† (10^{-3} M)
1	0·8	14·1	12·3	
2	0·5	12·4	9·5	
3	0·6	10·2	6·2	0·96

MDB-I in the 24-hr urines from rabbits after doses of 150 mg of brucine hydrochloride per kg were determined before or after hydrolysis with HCl or β-glucuronidase.

* It includes a negligible amount of MDB-II.

† PHS: freshly prepared solution of potassium hydrogen saccharate, which was heated for 30 min at pH 3·5.

A, which showed that all metabolites excreted in urine were also detected in feces, and that their relative ratio was nearly the same as in urine, although the amounts were very small. The possibility that the feces were contaminated with urine was excluded because rabbits used in this experiment excreted no feces in the metabolism cages over a period of two days.

Whether these metabolites were produced by intestinal micro organisms or excreted via bile into the intestine has not been confirmed.

DISCUSSION

In addition to the metabolic studies on papaverine by Axelrod *et al.*[3] and on griseofulvin by Barnes and Boothroyd,[4] very interesting selective demethylation has been found to occur in rabbit after administration of brucine. It was cleaved almost exclusively to 2-methoxy-3-hydroxystrychnine, although a trace amount of another

isomeric monodemethylated brucine could be detected (only by paper or thin-layer chromatography).

A minor monodemethylated metabolite, 2-hydroxy-3-methoxystrychnine, was unequivocally synthesized from brucine via the previously known 2-acetamino-3-methoxystrychnine perchlorate by several steps. Furthermore, the structure of one of the intermediates, 2-amino-3-methoxystrychnine, was rechecked by its conversion to the known alkaloid, α-colubrine. The structure of the major monodemethylated metabolite therefore requires no further discussion. These phenolic metabolites were mainly excreted as β-glucuronides, with only a trace as free forms.

The course of biological O-dealkylation has been suggested by Brodie *et al.*[11] Alkylaryl ether first undergoes hydroxylation at the α-carbon atom of the alkyl group, adjacent to the oxygen atom. This very labile intermediate was immediately cleaved to aldehyde and phenol. It is also known that hydroxylation occurs, in general, on a carbon atom with the higher electron density.

From these facts, the authors considered that the lactam group attached to the 5-position in brucine might affect the electron density around a carbon atom of one of its methoxyl groups and cause selective demethylation. This was, however, shown not to be true by the metabolic study on the model compounds, 4-substituted veratroles.[12]

In this study occurrence of a non-phenolic base, the third metabolite, was also demonstrated in addition to a small amount of unchanged brucine by paper chromatography. The authors have not investigated the structure of this metabolite.

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