J. Pharm. Pharmac., 1966, **18**, *Suppl.*, 82S–87S Received May 2, 1966

The *in vivo* metabolism of isomeric methoxyoxindoles

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Isomeric methoxyoxindoles are metabolised by rats, guinea-pigs and rabbits to phenolic metabolites by *O*-demethylation and hydroxylation. This has been shown by comparison of the physical characteristics of the metabolites with those of standard synthetic hydroxyoxindoles. Negligible *O*-demethylation of methoxyoxindoles occurs with rat or guinea-pig liver microsome preparations, when compared with those of rabbit, but significant *O*-demethylation occurs with rat liver slices.

A MAJOR route of metabolism of many aromatic alkyl ethers involves cleavage to the corresponding phenols (Axelrod, 1956) by those enzyme systems, located in the microsomes of liver, requiring both reduced nicotinamide adenine dinucleotide phosphate and oxygen.

It has been shown previously (Beckett & Morton, 1966b) that methoxyoxindoles are *O*-demethylated by rabbit liver microsome preparations, but not by rat or guinea-pig liver microsome preparations.

As part of a wider study on the biotransformation of oxindole alkaloids, the dealkylation of isomeric methoxyoxindoles *in vivo* has been examined.

Since conjugated *O*-demethylated metabolites are present in the urine of rats and guinea-pigs after parenteral administration of methoxyoxindoles, further experiments with rat liver homogenates and slices are described.

Experimental

MATERIALS AND METHODS

The preparation of standard synthetic hydroxyoxindoles has been previously described (Beckett & Morton, 1966a).

In vivo experiments. Male Wistar rats weighing 300–350 g, male Flemish rabbits weighing 2·5–3·0 kg and male albino Wistar guinea-pigs weighing 450–500 g were used. The animals received intraperitoneal injections (1·0 ml) of 4-, 5-, 6- or 7-methoxyoxindole (60 mg/kg) in propylene glycol. The faeces-free urine was collected for 96 hr and the metabolites extracted as previously described (Beckett & Morton, 1966a).

In vitro experiments. The animals used were similar to those previously described (Beckett & Morton, 1966b). Some of the rats received intraperitoneal injections of 3,4-benzpyrene (40 mg/kg) 48 hr before being killed.

Tissue preparation. The microsome fraction and microsome plus soluble fractions of liver were prepared as described by Beckett & Morton (1966a). Liver slices, approximately 250 μ in thickness, were prepared by the freehand method using a razor blade, and immediately placed in isotonic phosphate Ringer solution (Dickens & Šimer, 1930) maintained at 37° ($\pm$0·5°).

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Incubation experiments. The incubation experiments using liver homogenates were made in 50 ml Erhlenmeyer flasks, each containing 100 μmoles nicotinamide, 0·8 μmoles NADP, 0·8 μmoles NAD, 20 μmoles glucose-6-phosphate, 60 μmoles magnesium chloride, 0·1M phosphate buffer pH 7·6, 12·0 μmoles substrate and 2·0 ml of liver homogenate in a total volume of 7·0 ml.

Experiments using liver slices were made in 50 ml Erhlenmeyer flasks, each containing 1·0 g (wet weight) of liver slices, 6·0 μmoles of substrate and isotonic phosphate Ringer solution pH 7·4 in a total volume of 10·0 ml. Flasks were shaken (60 min) in air at 37° ($\pm$0·5°). Controls with inactivated tissue, and controls in which the substrate was omitted were also used.

In vitro *demethylation.* The demethylations were made in the same incubation medium to which semicarbazide hydrochloride (70 μmoles) had been added. The resulting incubate was assayed for formaldehyde by the method of Cochin & Axelrod (1959).

Extraction of in vitro *metabolites.* After completion of the incubation period, the liver slices were homogenised in an all-glass Potter-Elvehjem homogeniser, and the protein was precipitated immediately by adding to each flask zinc sulphate solution (20% w/v) (2·0 ml) and a saturated solution of barium hydroxide (2·0 ml). The mixtures were centrifuged (5 min) at 10,000 *g*. Extractions of the supernatant with 2 volumes of n-butanol were made at pH 7·0. The combined extracts were evaporated almost to dryness under reduced pressure, and examined by thin-layer chromatography. The control incubations were extracted and examined in the same way.

Thin-layer chromatography and ultraviolet spectroscopy of the metabolites and reference compounds were as described by Beckett & Morton (1966b).

TABLE 1. URINARY METABOLITES AFTER INTRAPERITONEAL INJECTION OF 4-METHOXYOXINDOLE TO RATS. (Identical results were obtained with the urine extracts of guinea-pig and rabbit)

Metabolite	Rf value*		Chemical colour reactions			
	System A	System B	DQC reagent	Diazotised *p*-nitroaniline reagent	Naphthoresorcinol reagent	Rhodizonic acid/acid $BaCl_2$ reagent†
4-Methoxyoxindole metabolite A	28	38	blue	—	—	+++
Metabolite A after hydrolysis	81	85	blue	yellow	—	—
4-Methoxyoxindole Metabolite B	26	33	magenta	—	—	+++
Metabolite B after hydrolysis	76	79	magenta	brown	—	—
4-Methoxyoxindole Metabolite C	3	9	blue	—	blue	—
Metabolite C after hydrolysis	82	85	blue	yellow	—	—
4-Methoxyoxindole Metabolite D	2	7	magenta	—	blue	—
Metabolite D after hydrolysis	75	78	magenta	brown	—	—
4-Hydroxyoxindole	82	84	blue	yellow	—	—
5-Hydroxyoxindole	75	72	magenta	brown	—	—
6-Hydroxyoxindole	77	76	grey	orange-red	—	—
7-Hydroxyoxindole	85	86	red-brown	brown	—	—

* *Solvent systems:* A. Chloroform-methanol (4:1). B. Chloroform-methanol-acetone (2:1:7). Silica Gel "G" ("E. Merck"/Darmstadt). Chromatogram thickness 250 μ.
† Parke, 1960.

Results

IN VIVO

After intraperitoneal administration of the isomeric methoxyoxindoles to rats, the metabolites shown in Tables 1, 2 and 3 were detected in urine. These metabolites were characterised as shown in the Tables. In addition, the ultraviolet absorption spectra of the metabolites were identical with authentic samples. The metabolites of the isomeric methoxyindoles are listed in Table 4.

TABLE 2. URINARY METABOLITES AFTER INTRAPERITONEAL INJECTIONS OF 5- AND 6-METHOXYOXINDOLE TO RATS. (Identical results were obtained with the urine extracts of guinea-pig and rabbit)

Metabolite	Rf value*		Chemical colour reactions			
	System A	System B	DQC reagent	Diazotised *p*-nitro-aniline reagent	Naphtho-resorcinol reagent	Rhodizonic acid/acid $BaCl_2$ reagent
5-Methoxyoxindole Metabolite E	23	37	magenta	—	—	+++
Metabolite E after hydrolysis ..	75	73	magenta	brown	—	—
5-Methoxyoxindole Metabolite F	2	10	magenta	—	blue	—
Metabolite F after hydrolysis ..	76	72	magenta	brown	—	—
6-Methoxyoxindole Metabolite G	26	36	grey	—	—	+++
Metabolite G after hydrolysis ..	78	76	grey	orange-red	—	—
6-Methoxyoxindole Metabolite H	3	10	grey	—	blue	—
Metabolite H after hydrolysis ..	78	76	grey	orange-red	—	—
4-Hydroxyoxindole	82	84	blue	yellow	—	—
5-Hydroxyoxindole	75	72	magenta	brown	—	—
6-Hydroxyoxindole	77	76	grey	orange-red	—	—
7-Hydroxyoxindole	85	86	red-brown	brown	—	—

Examination of the spot area and density of colour on thin-layer chromatograms, showed that approximately equal quantities of the *O*-demethylated and hydroxylated metabolites were formed after administering 4- and 7-methoxyoxindole to rats. (In the absence of authentic

TABLE 3. URINARY METABOLITES AFTER INTRAPERITONEAL INJECTIONS OF 7-METHOXYOXINDOLE TO RATS. (Identical results were obtained with the urine extracts of guinea-pig and rabbit)

Metabolite	Rf value*		Chemical colour reactions			
	System A	System B	DQC reagent	Diazotised *p*-nitro-aniline reagent	Naphtho-resorcinol reagent	Rhodizonic acid/ $BaCl_2$ reagent
7-Methoxyoxindole Metabolite J	27	36	red-brown	—	—	+++
Metabolite J after hydrolysis ..	86	87	red-brown	brown	—	—
7-Methoxyoxindole Metabolite K	21	31	magenta	—	—	+++
Metabolite K after hydrolysis ..	79	81	magenta	brown	—	—
7-Methoxyoxindole Metabolite L	7	11	red-brown	—	slight blue	—
Metabolite L after hydrolysis ..	86	86	red-brown	brown	—	—
7-Methoxyoxindole Metabolite M	4	5	magenta	—	slight blue	—
Metabolite M after hydrolysis ..	80	81	magenta	brown	—	—

samples of 5-hydroxy-4-methoxyoxindole and 5-hydroxy-7-methoxyoxindole, it is assumed that equi-molecular quantities of these compounds give spot sizes and colour intensities similar to those exhibited by 5-hydroxyoxindole.)

TABLE 4. RAT URINARY METABOLITES OF ISOMERIC METHOXYOXINDOLES

Compound administered	Metabolites detected	
	Ethereal sulphate conjugate	B-Glucosiduronic acid conjugate*
4-Methoxyoxindole	4-Hydroxyoxindole 5-Hydroxy-4-methoxyoxindole	4-Hydroxyoxindole 5-Hydroxy-4-methoxyoxindole
5-Methoxyoxindole	5-Hydroxyoxindole	5-Hydroxyoxindole
6-Methoxyoxindole	6-Hydroxyoxindole	6-Hydroxyoxindole
7-Methoxyoxindole	7-Hydroxyoxindole 5-Hydroxy-7-methoxyoxindole	7-Hydroxyoxindole 5-Hydroxy-7-methoxyoxindole

* Minor metabolites.

Similar results were obtained with guinea-pigs, but with rabbits greater amounts of the *O*-demethylated and less of the hydroxylated metabolites were formed.

IN VITRO

Liver microsome preparations. Previous experiments have shown that 4-, 5-, 6- and 7-methoxyoxindoles are significantly *O*-demethylated by rabbit liver microsome preparations, but not by the liver microsome preparations of rats and guinea-pigs (Beckett & Morton, 1966b). Formaldehyde production observed with the rabbit liver microsome preparations may be substantially increased by pretreatment of the rabbits with phenobarbitone.

TABLE 5. FORMALDEHYDE PRODUCTION AFTER INCUBATION OF 4-METHOXYOXINDOLE WITH THE INTRACELLULAR FRACTIONS OF RAT AND RABBIT LIVER

Intracellular fraction	Formaldehyde produced per hr per g of liver (μmoles)*
Rat microsomes in phosphate buffer 7·6	0·03
Rat microsomes plus rat soluble fraction	0·09
Rat soluble fraction	0·0
Rat whole homogenate	0·05
Rat microsomes plus rabbit soluble fraction	0·10
Rabbit microsomes in phosphate buffer 7·6	0·84
Rabbit microsomes plus rabbit soluble fraction	3·75
Rabbit soluble fraction	0·0
Rabbit whole homogenate	2·43
Rabbit microsomes plus rat soluble fraction	1·20

* 12 μmoles of 4-methoxyoxindole were added per g of liver. The data presented represent the average for three animals, all results of which were within ±10% of the recorded values.

Although negligible amounts of formaldehyde were observed in the present experiments, during the incubation of 4-methoxyoxindole with rat liver microsomal or soluble fractions or with the whole liver homogenate (see Table 5), a metabolite formed by hydroxylation in the 5-position of the

aromatic ring of the oxindole nucleus was present in the incubation extracts. The *O*-demethylating activity of the rat microsome preparations was not increased by pretreatment of the rat with phenobarbitone or 3,4-benzpyrene, or by adding excess quantities of NADP or sodium pyrophosphate to the incubation medium (see Table 6).

TABLE 6. FORMALDEHYDE PRODUCTION AFTER INCUBATION OF 4-METHOXYOXINDOLE WITH NORMAL AND PRETREATED RAT AND RABBIT LIVER MICROSOME PREPARATIONS

Liver fraction and additions	Formaldehyde produced per hr per g of liver (μmoles)*
Untreated rat microsomes plus soluble fraction	0·10
Untreated rat microsomes plus soluble fraction plus 2 μmoles NADP	0·12
Phenobartitone pretreated rat microsomes plus soluble fraction	0·12
Phenobarbitone pretreated rat microsomes plus soluble fraction plus 2 μmoles NADP	0·15
Phenobarbitone pretreated rat microsomes plus soluble fraction plus 50 μmoles sodium pyrophosphate	0·12
3,4-Benzpyrene pretreated rat microsomes plus soluble fractions	0·10
3,4-Benzpyrene pretreated rat microsomes plus soluble fraction plus 2 μmoles NADP	0·12
Untreated rabbit microsomes plus soluble fraction	2·30
Untreated rabbit microsomes plus soluble fraction plus 2 μmoles NADP	3·75
Phenobarbitone pretreated rabbit microsomes plus soluble fraction	5·48
Phenobarbitone pretreated rabbit microsomes plus soluble fraction plus 2 μmoles NADP	5·72

* 12 μmoles of 4-methoxyoxindole were added per g of liver. The data presented represent the average for three animals, all results of which were within ±10% of the recorded values.

The formaldehyde production observed during the incubation of 4-methoxyoxindole with rabbit liver microsome preparations was markedly reduced when the rabbit microsomes were incubated in the presence of rat liver soluble fractions, but there was no increase in the *O*-demethylating activity of the rat microsomes when they were incubated in the presence of rabbit liver soluble fractions (see Table 5).

Liver slices. Thin-layer chromatograms of the n-butanol extracts after the incubation of 4-methoxyoxindole with rat liver slices, showed the presence of a metabolite with Rf values in different systems and chemical colour reactions and an ultraviolet spectrum, after elution from the chromatogram, identical to those of an authentic sample of 4-hydroxyoxindole (see Beckett & Morton, 1966b). However, no measurable amounts of formaldehyde were produced during the incubations of 4-methoxyoxindole with the rat liver slices. A second metabolite identical in chemical colour reaction and ultraviolet spectrum with an authentic sample of 5-hydroxyoxindole, but differing in Rf values was also present in the extracts of the incubation medium.

Discussion

The metabolic products formed after intraperitoneal injections of 4-, 5-, 6- and 7-methoxyoxindole to rats, guinea-pigs and rabbits are summarised in Tables 1–3. The corresponding unconjugated metabolites have been reported for the same compounds after incubation with rabbit liver microsome preparations (Beckett & Morton, 1966b).

O-Demethylation of the methoxyoxindoles occurs in rats and also in rat liver slices, but not with rat liver microsome preparations. It is unlikely that the *O*-demethylating enzyme systems were inhibited by the homogenisation of the rat liver since *O*-demethylation of codeine and *p*-nitroanisole has been reported with similarly prepared rat liver microsome preparations (Axelrod, 1955; Netter & Seidel, 1964).

Jacobson & Kaplan (1957a,b) have demonstrated that concentrations of pyrophosphatases which cleave NADP and NADPH are higher in rat liver microsome preparations than in similar rabbit liver microsome preparations, but the negligible *O*-demethylating activity of the rat liver preparations, when compared with that of rabbit liver microsome preparations, cannot be attributed solely to the action of pyrophosphatases because the addition of excess quantities of NADP or sodium pyrosphosphate (an alternative substrate) to the incubation media, did not increase the formaldehyde production from the methoxyoxindoles.

It has been shown that pretreatment of rats with 3,4-benzpyrene increases the *O*-demethylation of methoxyacetanilide and codeine by rat liver microsome preparations (Conney, Miller & Miller, 1956; Henderson & Mazel, 1964). Similar pretreatment of rats with 3,4-benzpyrene and phenobarbitone in this present study did not increase the *O*-demethylation of 4-methoxyoxindole.

Failure to observe formaldehyde production despite *O*-demethylation during the metabolism of 4-methoxyoxindole by rat liver slices, may be due to the further incorporation or metabolism of the formaldehyde within the cells, or the inability of the semicarbazide reagent to penetrate the cell membranes and complex with the formaldehyde.

Acknowledgements. We thank the Science Research Council for a research scholarship to D.M.M. We are indebted to Mr. R. W. Daisley of the Department of Pharmacy, Chelsea College of Science and Technology, for the preparations of the hydroxyoxindoles and methoxyoxindoles.

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