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THE METABOLISM OF ISOMERIC METHOXYOXINDOLES

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Abstract—The *in vitro* metabolism of isomeric methoxyoxindoles and N-methyl-methoxyoxindoles by rats, guinea-pigs and rabbits is described.

The formation of phenolic metabolites by O-demethylation and hydroxylation has been shown by comparison of their physical characteristics with those of standard synthetic hydroxyoxindoles.

The extent of dealkylation has been found to vary with the position of substitution on the aromatic nucleus, and on methylation of the oxindole nitrogen atom.

A MAJOR route of the metabolism of many aromatic alkyl ethers by enzyme systems, localized in the microsomes of liver and requiring both reduced NADP and oxygen, involves cleavage to yield phenolic metabolites.[1]

Examples of these reactions are, cleavage of *p*-nitrophenyl ethers (Huggins *et al.*[2]), phenacetin and *p*-phenetidine (Brodie and Axelrod[3]) and several nuclear-substituted anisoles (Bray *et al.*[4]).

It has been suggested[5] that more than one enzyme system is involved, and that ethers substituted in the *para*-position are cleaved more easily than the corresponding *meta*- or *ortho*-substituted compounds.

The present paper presents studies on the dealkylation of isomeric methoxyoxindoles, as part of a wider study on the metabolism of aromatic-substituted indole and oxindole alkaloids.

METHODS

Materials

The preparation of 4-, 5- and 6-hydroxyoxindole has been previously described.[6] 5-Hydroxy-N-methyloxindole and 6-hydroxy-N-methyloxindole were prepared by cyclization of the chloro-acetyl derivatives of para- and meta-N-methyl-anisidine respectively.[7, 8] 4-hydroxy, and 7-hydroxy-N-methyloxindole were prepared by demethylation of 4-, and 7-methoxy-N-methyloxindole by the method of Loudon and Ogg.[9]

Nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide and glucose-6-phosphate were supplied by The Sigma Chemical Company.

Experiments in vitro

Animals. Male Wistar rats weighing between 300 and 350 g, male Flemish rabbits weighing between 2·5 and 3·0 kg, and male albino Wistar guinea-pigs weighing

between 450 and 500 g were used. These animals received intra-peritoneal injections of sodium phenobarbitone (40 mg/kg) on consecutive days, 72 and 48 hr before being killed.

Microsome preparation. The microsome plus soluble fractions of liver were prepared according to the method described by Beckett and Morton.[6]

The microsomal fraction was prepared by centrifugation of the "10,000 *g* supernatant" at 120,000 *g* for 1 hr at 0° (MSE Super Speed 40 refrigerated centrifuge). The microsomal fraction was resuspended in the original volume of ice-cold 0·1 M phosphate buffer pH 7·6 in an all-glass Potter-Elvehjem homogenizer.

Incubation experiments. The incubation experiments were carried out in 50 ml Erhlenmeyer flasks, each containing 100 μmoles nicotinamide, 0·8 μmoles NADP, 0·4 μmoles NAD, 20 μmoles glucose-6-phosphate, 60 μmoles magnesium chloride, 12·0 μmoles substrate all dissolved in phosphate buffer (0·1 M) pH 7·6 (5·0 ml) and 2·0 ml of liver homogenate to give a total volume of 7·0 ml. The substrates were dissolved in a small volume of ethyl alcohol, and were reprecipitated in a fine amorphous form by adding phosphate buffer solution. The incubations were performed in a Mickle shaking incubator in air at 37° ($\pm$ 0·5°) for 1 hr. Controls with inactivated tissue, and controls in which the substrate was omitted were also used.

Demethylation studies in vitro. The demethylation studies were carried out in the same incubation medium plus 70 μmoles of semicarbazide in the final volume of 7·0 ml. The resulting incubate was assayed for formaldehyde by the method of Cochin and Axelrod[10] as follows.

The incubation mixture was added to 2·0 ml of a 20 per cent w/v solution of zinc sulphate and, after stirring, 2·0 ml of a saturated solution of barium hydroxide was added. After centrifugation, a 6·0 ml sample of supernatant was added to 2·0 ml of double strength Nash reagent.[11] The colour was developed by heating at 60° for 30 min, and the tubes assayed spectrophotometrically at 412 mμ. Known concentrations of formaldehyde treated in the same way were used as standards. Control incubations in the absence of the substrates were also used.

Extraction of metabolites in vitro. After completion of the incubation, the protein was precipitated by the addition to each flask of 2·0 ml of a zinc sulphate solution (20 % w/v) and 2·0 ml of a saturated solution of barium hydroxide. The mixtures were centrifuged at 10,000 *g* for 5 min. Extractions of the supernatant with 2 volumes of *n*-butanol were carried out 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.

Assay procedure for 5-hydroxyoxindoles. Samples of 6·0 ml of the protein-free incubation medium, containing the 5-hydroxyoxindoles and unchanged 5-methoxyoxindoles, were twice shaken at pH 7·0 with equal volumes of ether to remove the unchanged 5-methoxyoxindoles and other impurities from the incubation medium. The ether extracts were rejected, and the aqueous solutions containing the 5-hydroxyoxindoles were twice shaken with an equal volume of *n*-butanol. The combined extracts were evaporated to dryness under reduced pressure, and the residue was dissolved in 2·0 ml of absolute ethanol. A freshly prepared solution (1·0 ml) of 2-chloro-4-nitrobenzene-diazonium naphthalene-2-sulphonate (NNCD reagent) (0·2 % w/v) in 10 per cent v/v hydrochloric acid were added, and suitable dilutions of the tubes were assayed spectrophotometrically at 440 mμ after 30 min.

The protein-free incubation medium, from incubations in the absence of the substrates, carried through the same assay procedure was used for the reference beam. The concentration of 5-hydroxyoxindoles were read from a concentration–absorbance calibration graph which was linear between 0·1 and 2·0 μmoles of 5-hydroxyoxindole.

Partition coefficient determination. $0{\cdot}75 \times 10^{-5}$ g mole samples (approx 1 mg) of the compounds were shaken horizontally in glass-stoppered tubes with 10 ml samples of Sørensons Phosphate Buffer (pH 7·6) and 10 ml samples of ethylene dichloride and ethyl acetate for 24 hr at 37° ($\pm$ 0·5°), immersed in a water bath. At the end of the incubation period, the layers were separated before cooling, and the absorption spectra of suitable dilutions of the organic and aqueous phase were recorded with a Unicam SP.800 spectrophotometer, with the appropriate solvent in the reference beam. The solutions were read at the wavelengths recorded in Table 3, measuring at the maximum absorbance in the 250 mμ and 280 to 300 mμ regions.

Corrections were made for the extinctions of solvent samples prepared without a solute, and the partition coefficients were determined as the ratio of the concentrations in the two layers.

Chromatography. Thin layer chromatography and preparative chromatography was carried out on silica-gel "G" ("E.Merck"/Darmstadt). All plates were heated at 100° for 30 min and cooled before being used.

The plates were viewed under an u.v. lamp (365 mμ), and the metabolites and standard reference compounds located by the use of spray reagents.

(a) Ferric chloride/Perchloric acid reagent (Salkowski reagent[12]).
(b) Chloroimide (DQC reagent[13]) 2,6-dichloro-quinone-chloroimide 0·25 per cent w/v in absolute alcohol. Ammonia vapour was used to enhance the colour.
(c) Diazotised sulphanilic acid reagent (Pauly Reagent[13]).
(d) Diazotised *p*-nitroaniline reagent.[13]

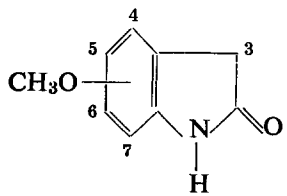
The metabolites were eluted from the excised areas of silica-gel with spectroscopically pure ethyl alcohol.

Ultra-violet spectra. The u.v. absorption spectra of the standard reference compounds and eluted metabolites were determined with a Unicam SP.800 recording spectrophotometer. Control elutions from a corresponding area of a blank chromatogram were used for the reference cell.

RESULTS

Qualitative metabolic results

The metabolites formed when the isomeric methoxyoxindoles (See I) were incubated with liver microsome preparations were characterized by their R_f values and chemical colour reactions on thin-layer chromatograms (see Tables 1 and 2).



I

TABLE 1. THIN LAYER CHROMATOGRAPHIC R_f VALUES AND CHEMICAL COLOUR REACTIONS OF METHOXYOXINDOLES AND THEIR METABOLITES AFTER INCUBATION WITH RABBIT LIVER MICROSOME PREPARATIONS

Compound	R_f value*			Colour reactions				Suggested metabolite structure
	System I	System II	System III	Ferric chloride/ perchloric acid reagent	D.Q.C. reagent	Diazotized sulphanilic acid reagent	Diazotized paranitraniline reagent	
4-Methoxyoxindole	65	56	52	brown	orange-red	yellow	yellow	—
Metabolite A	23	37	36	blue	blue	yellow	yellow-brown	4-Hydroxyoxindole
Metabolite B	36	28	37	brown	magenta	brown	brown	4-Methoxy-5-hydroxyoxindole
5-Methoxyoxindole	66	48	47	brown	red	yellow	yellow	—
Metabolite A	18	28	25	brown	magenta	brown	brown	5-Hydroxyoxindole
6-Methoxyoxindole	67	53	51	brown	red	yellow	orange	—
Metabolite A	16	33	30	maroon	grey	yellow	orange-red	6-Hydroxyoxindole
7-Methoxyoxindole	67	57	52	brown	red	yellow	yellow	—
Metabolite A	24	38	33	red-brown	maroon	brown	yellow	7-Hydroxyoxindole
Metabolite B	39	32	36	brown	magenta	brown	brown	7-Methoxy-5-Hydroxyoxindole
4-Hydroxyoxindole	23	38	36	blue	blue	yellow	yellow-brown	—
5-Hydroxyoxindole	17	28	26	brown	magenta	brown	brown	—
6-Hydroxyoxindole	16	34	30	maroon	grey	yellow	orange-red	—
7-Hydroxyoxindole	24	38	34	red-brown	maroon	brown	yellow	—

Chromatogram thickness 250μ.

* Solvent system

I	Chloroform	19	Silica Gel 'G' (E. Merck/Darmstadt)
	Ethanol	1	
II	Benzene	4	
	Ethylacetate	6	
III	Chloroform	5	
	Acetone	4	

TABLE 2. THIN-LAYER CHROMATOGRAPHIC R_f VALUE AND CHEMICAL COLOUR REACTIONS OF N-METHYL-METHOXYOXINDOLES AND THEIR METABOLITES AFTER INCUBATION WITH RABBIT LIVER MICROSOME PREPARATIONS

Compound	R_f value, Solvent system* I	II	III	Colour reactions: Ferric chloride/perchloric acid reagent	D.Q.C. reagent	Diazotized *p*-nitraniline reagent	Suggested metabolite structure
4-Methoxy-N-methyloxindole	84	80	76	brown	red	yellow	—
Metabolite A	65	56	52	brown	orange-red	yellow	4-Methoxyoxindole
Metabolite B	57	52	47	brown	magenta	brown	4-Methoxy-5-hydroxy-N-methyloxindole
Metabolite C	48	50	45	blue	blue	yellow-brown	4-Hydroxy-N-methyloxindole
5-Methoxy-N-methyloxindole	81	79	73	brown	red	yellow	—
Metabolite A	67	48	47	brown	red	yellow	5-Methoxyoxindole
Metabolite B	48	37	40	brown	magenta	brown	5-Hydroxy-N-methyloxindole
6-Methoxy-N-methyloxindole	79	78	77	brown	red	yellow	—
Metabolite A	67	53	51	brown	red	orange	6-Methoxyoxindole
Metabolite B	27	48	38	maroon	grey	orange-red	6-Hydroxy-N-methyloxindole
7-Methoxy-N-methyloxindole	80	81	75	brown	red	yellow	—
Metabolite A	67	56	52	brown	red	yellow	7-Methoxyoxindole
Metabolite B	54	54	49	brown	magenta	brown	7-Methoxy-5-hydroxy-N-methyloxindole
Metabolite C	46	49	47	red-brown	maroon	yellow	7-Hydroxy-N-methyloxindole
4-Hydroxy-N-methyloxindole	48	51	45	blue	blue	yellow-brown	—
5-Hydroxy-N-methyloxindole	47	37	40	brown	magenta	brown	—
6-Hydroxy-N-methyloxindole	27	49	38	maroon	grey	orange-red	—
7-Hydroxy-N-methyloxindole	46	50	47	red-brown	maroon	yellow	—

Metabolic results

Thin layer chromatograms of the *n*-butanol extracts after the incubation of the isomeric methoxyoxindoles and N-methyloxindoles with rabbit liver microsome preparations, showed the presence of phenolic metabolites with R_f values and chemical colour reactions identical with those of the corresponding hydroxyoxindoles (see Tables 1 and 2).

These products of O-demethylation, which were not formed by the liver microsome preparations of rat and guinea-pig under the same incubation conditions, exhibited an absorption spectrum and bathochromic shift upon making the solutions alkaline with sodium hydroxide, identical to those observed with the corresponding hydroxyoxindoles (see Table 3).

TABLE 3. ULTRA-VIOLET ABSORPTION MAXIMA OF METHOXYOXINDOLES AND THEIR METABOLITES

Compound	λmax Ethanol mμ	λmax Ethanol containing 0·1N NaOH mμ
4-Methoxyoxindole	227, 250, 283, 289	—
Metabolite A	235, 249, 284, 289	233, 299
Metabolite B	215, 257, 304	223, 275, 319
5-Methoxyoxindole	213, 257, 303	—
Metabolite A	215, 256, 303	221, 275, 318
6-Methoxyoxindole	222, 255, 283, (293)	—
Metabolite A	225, 255, 283	239, 299
7-Methoxyoxindole	222, 248, 292	—
Metabolite A	220, 248, 293	231, 309
Metabolite B	216, 256, 302	222, 274, 319
4-Hydroxyoxindole	233, 249, 284, 289	233, 298
5-Hydroxyoxindole	214, 256, 302	221, 274, 318
6-Hydroxyoxindole	223, 255, 283, (294)	238, 299
7-Hydroxyoxindole	220, 247, 292	230, 309

4-Methoxyoxindole, 7-methoxyoxindole and their N-methyl derivatives were further metabolized by the liver microsome preparations of rabbit, rat and guinea-pig to give phenolic metabolites with chemical colour reactions, absorption spectra and bathochromic shifts similar to that observed with 5-hydroxyoxindole (see Tables 1, 2 and 3). The results, therefore, indicated that these compounds were probably hydroxylated in the 5-position of the oxindole nucleus.

When 5-methoxyoxindole and 6-methoxyoxindole were incubated with rat and guinea-pig liver microsome preparations, no metabolites were formed.

A non-phenolic metabolite with R_f values, chemical colour reactions and absorption spectrum identical with those of the isomeric methoxyoxindoles was formed when each of the corresponding N-methylmethoxyoxindoles was incubated with rat, rabbit and guinea-pig liver microsome preparations (see Tables 2 and 3).

The metabolic reactions observed with the N-methyl-methoxyoxindoles are summarized in Table 4.

Quantitative determination of the demethylation of methoxyoxindoles

The O- and N-demethylation of the methoxyoxindoles and N-methyl-methoxyoxindoles, measured by the determination of formaldehyde produced, was found to vary with the position of substitution on the oxindole nucleus (see Table 5).

O-demethylation reactions were observed with liver microsome preparations of rabbit, but not those of rat or guinea-pig. The most actively O-demethylated compound was the 4-methoxyoxindole isomer.

When the oxindole nitrogen atom was substituted by a methyl group, there was increased formaldehyde production with the 5- and 7-methoxy isomers, but not with the 4- and 6-methoxy isomers (see Table 5).

TABLE 4. METABOLIC REACTIONS OBSERVED WITH METHOXY-N-METHYLOXINDOLES WITH RABBIT LIVER MICROSOME PREPARATIONS

4-Methoxy-N-methyloxindole	5-Methoxy-N-methyloxindole	6-Methoxy-N-methyloxindole	7-Methoxy-N-methyloxindole
4-O-Demethylation	5-O-Demethylation	6-O-Demethylation	7-O-Demethylation
N-Demethylation	N-Demethylation	N-Demethylation	N-Demethylation
5-Hydroxylation	—	—	5-Hydroxylation

TABLE 5. DEMETHYLATION *in vitro* OF METHOXYOXINDOLES USING LIVER MICROSOME PREPARATIONS FROM SODIUM PHENOBARBITONE PRETREATED ANIMALS

Compound	Formaldehyde produced in 1 hr per g liver (μmoles)*		
	rabbit	rat	guinea-pig
4-Methoxyoxindole	5·48	0	0
5-Methoxyoxindole	3·08	0	0
6-Methoxyoxindole	1·32	0	0
7-Methoxyoxindole	0·98	0	0
4-Methoxy-N-methyloxindole	5·62	0·72	0·81
5-Methoxy-N-methyloxindole	5·92	0·66	0·70
6-Methoxy-N-methyloxindole	1·28	0·46	0·42
7-Methoxy-N-methyloxindole	2·42	0·38	0·42

* 12 μmoles of each compound was added per g of liver.
The data presented represents the average for three animals, all results of which were within ±10% of the recorded values.

Since 5-methoxyoxindole and its N-methyl derivative were not ring-hydroxylated, it was possible to measure the O-demethylation reaction, as distinct from the combined O- and N-demethylation, i.e. formaldehyde production, by the formation of phenolic 5-hydroxyoxindole and 5-hydroxy-N-methyloxindole (see Methods section). The formation of 5-hydroxy-N-methyloxindole from 5-methoxy-N-methyloxindole was greater than the formation of 5-hydroxyoxindole from 5-methoxyoxindole (see Table 6). The increased formaldehyde production observed with 5-methoxy-N-methyloxindole was, therefore, mainly due to an increase in the O-demethylation. Since no measurable amounts of 5-hydroxylated oxindoles were found when 5-methoxyoxindole and 5-methoxy-N-methyloxindole were incubated with guinea-pig

liver microsome preparations, the formaldehyde produced using the N-methyl derivative came from N-demethylation reactions.

N-demethylation of the methoxy-N-methyloxindoles, as measured by formaldehyde production, and shown by the presence of N-demethylated derivatives on thin-layer chromatograms of incubation extracts, occurred with the liver microsome preparations of rat, rabbit and guinea-pig (see Tables 2 and 5).

TABLE 6. THE DEMETHYLATION OF 5-METHOXYOXINDOLE AND 5-METHOXY-N-METHYLOXINDOLE BY RABBIT AND GUINEA-PIG LIVER MICROSOME PREPARATIONS

Substrate	Rabbit		Guinea-pig	
	Formaldehyde produced in 1 hr per g liver (μmoles)*	5-Hydroxylated oxindole formed in 1 hr per g liver (μmoles)*	Formaldehyde produced in 1 hr per g liver (μmoles)*	5-Hydroxylated oxindole formed in 1 hr per g liver (μmoles)*
5-Methoxyoxindole	1·66	1·75	0	0
5-Methoxy-N-methyloxindole	3·49	2·80	0·63	0

* 12 μmoles of each compound was added per g of liver.

TABLE 7. THE DEMETHYLATION *in vitro* OF METHOXYOXINDOLES WITH THE LIVER MICROSOME PREPARATIONS OF NORMAL AND SODIUM PHENOBARBITONE PRETREATED RABBITS

Compound	Formaldehyde produced in 1 hr per g liver (μmoles)*		Partition coefficients	
	Phenobarbitone pretreated animals	Normal untreated animals	Ethylene dichloride/phosphate buffer	Ethylacetate/phosphate buffer pH 7·6
4-Methoxyoxindole	5·48	2·30	7·3	51·6
5-Methoxyoxindole	3·08	1·74	6·3	34·6
6-Methoxyoxindole	1·32	0·58	4·2	28·2
7-Methoxyoxindole	0·98	0·46	7·4	51·7
4-Methoxy-N-methyloxindole	5·62	2·82	10·1	65·2
5-Methoxy-N-methyloxindole	5·92	3·78	8·9	41·7
6-Methoxy-N-methyloxindole	1·28	0·72	8·4	36·9
7-Methoxy-N-methyloxindole	2·42	1·27	9·3	69·4

* 12 μmoles of each compound was added per g of liver.

The data presented represents the average for three animals, all results of which were within ±10% of the recorded values.

The pattern of O- and N-demethylation within this series of methoxyoxindoles, was found to be very similar when incubations were carried out with rabbit liver microsome preparations obtained from normal animals and those pretreated with sodium phenobarbitone (see Table 7). Thus, the stimulation of enzyme activity probably represents quantitative increase in enzymes rather than change in enzyme characteristics.

The difference in formaldehyde production from the various structural isomers does not appear to be merely a function of difference in the partition coefficients

between the aqueous incubation medium and the lipid enzyme surface, as represented by the organic phase, for similar partition coefficients were obtained for the 4-methoxy and 7-methoxyoxindoles, which showed a marked difference in formaldehyde production (see Table 7).

DISCUSSION

The metabolic reactions observed with the four isomeric methoxyoxindoles have been found to vary greatly with the position of substitution on the aromatic nucleus.

Hydroxylation of the aromatic ring at the 5-position, as observed with oxindole and N-methyloxindole (Beckett and Morton[6]), occurred with the 4- and 7-methoxyoxindoles in all species examined.

O-demethylation, as measured by formaldehyde production, was observed with the liver microsome preparations of rabbit, but not those of rat or guinea-pig, a phenomenon which may be explained by (a) the presence of heat labile inhibitors in the liver extracts of rat and guinea-pig, but not in those of rabbits (Axelrod[5]), or (b) the instability of the O-demethylation enzyme systems of rat and guinea-pig. For example, Leadbeater and Davies,[14] have demonstrated the striking instability of the microsomal enzymes that O-demethylate codeine when compared with the stability of the enzymes which N-demethylate the same compound.

The extent of the O-demethylation of the methoxyoxindoles, shown to be in the order $4 > 5 > 6 > 7$, is probably due to steric factors in enzyme–substrate association rather than changes in partition characteristics of these molecules.

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