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THE METABOLISM OF OXINDOLE AND RELATED COMPOUNDS

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Abstract—The metabolism *in vivo* and *in vitro* of oxindole, N-methyloxindole, 3-methyloxindole and indole by rats, guinea pigs and rabbits is described. The formation of a phenolic metabolite by hydroxylation in the 5-position of the oxindole ring has been shown by comparisons of its physical characteristics with those of standard synthetic hydroxyoxindoles.

INTRODUCTION

AS PART of a wider study on the biotransformation of some indole and oxindole alkaloids, the metabolic products of the parent compounds were determined.

Previous reports indicate that indoles are hydroxylated by liver microsomes, e.g. indole is hydroxylated by rabbit liver microsomes in the 3-position to form indoxyl,[1] which is then conjugated to form urinary indican;[2] naturally occurring lipid-soluble 3-substituted indoles are hydroxylated specifically in the 6-position.[3]

Similarly, skatole is 6-hydroxylated by liver preparations, and is then conjugated to form 6-sulphatoxyskatole—a frequent urine constituent.[4, 5]

The formation, possibly via oxindole as an intermediate, of 6-hydroxyoxindole from indole in rats has been reported.[2]

METHODS

Materials

4-Hydroxyoxindole and 6-hydroxyoxindole were prepared by demethylation of 4- and 6-methoxyoxindole respectively by the method of Wieland and Unger.[6] 5-Hydroxyoxindole was prepared by cyclisation of N-chloroacetyl-*p*-methoxyaniline with aluminium chloride according to the method of Beer *et al.*[7] 5-Hydroxy, and 6-hydroxy-N-methyloxindole were prepared by cyclisation of the chloro-acetyl derivatives of *para*- and *meta*-N-methylanisidine respectively.[8, 9] Nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), adenosine triphosphate (ATP), nicotinamide and glucose-6-phosphate (Sigma Chemical Company). Indole (Mann Chemicals (New York), oxindole (Aldrich Chemical Company). Samples of 5-, 6-, and 7-hydroxyoxindole were also kindly supplied by Professor R. T. Williams (St. Mary's Hospital, London), 3-methyloxindole by Dr. S. Witkop (National Institute of Health, Bethesda), and the hydroxyskatoles by Dr. R. A. Heacock (University of Saskatoon, Canada).

EXPERIMENTS *IN VITRO*

Animals

Male Wistar rats weighing between 250 g and 300 g were used. The rats received intra-peritoneal injections of sodium phenobarbitone (100 mg/kg). 48 hr before being killed. Male Flemish rabbits weighing between 2·5 and 3·0 kg received intra-peritoneal injections of sodium phenobarbitone (40 mg/kg) on consecutive days, 72 and 48 hr before being killed. Unpretreated male albino Wistar guinea-pigs weighing between 450 g and 480 g and unpretreated rats and rabbits were also used.

Microsome preparation

The animals were killed by cervical fracture, and their livers were immediately removed and chilled. The tissues were homogenised with two volumes of 0·1 M phosphate buffer pH 7·6 in a Waring blender, and centrifuged at 9000 *g* for 20 min at 0°. The supernatant, containing microsomes and the soluble fraction, was used in the incubation experiments.

Incubation experiments

The incubations were carried out in 50-ml Erhlenmeyer flasks, each containing 100 μmole nicotinamide, 0·4 μmole NADP, 0·4 μmole NAD, 20 μmole glucose-6-phosphate, 60 μmole of magnesium chloride, 0·1 M phosphate buffer pH 7·6, 6·5 μmole substrate and 2·0 ml of liver homogenate (0·6 g liver) in a total volume of 6·0 ml. The substrates were dissolved in a small volume of ethyl alcohol, and were re-precipitated in a fine amorphous form by adding phosphate buffer solution. The final concentration of ethyl alcohol in the incubation medium was 1% (v/v). The incubations were performed in a Mickle shaking incubator in air at 37° (±0·5°) for 60 min. 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 on untreated animal livers in the same incubation medium plus 70 μmoles of semicarbazide in the final volumes of 6·0 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% (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. Two 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.

EXPERIMENTS *IN VIVO*

Animals

Male unpretreated Wistar rats weighing between 250 g and 300 g were given intraperitoneal injections of indole and oxindole (60 mg/kg) in propylene glycol. The faeces-free urine was collected for 48 hr.

Extraction of metabolites in vivo

An aliquot of the urine was adjusted to pH 4·8 with acetic acid, and incubated with a preparation of bacterial β-glucuronidase (Sigma Chemical Company) in a medium consisting of 10 ml of urine, 30 mg of β-glucuronidase (1000 I.U.), and 2·0 ml of 0·5 M acetate buffer pH 4·8 for 24 hr at 37° (±0·5°). After incubation, 0·5 ml of 40% (w/v) trichloracetic acid was added to the reaction mixture, the precipitate removed by centrifugation, and the supernatant twice extracted with *n*-butanol at pH 7·0. The combined extracts were concentrated by evaporation under reduced pressure and examined by thin layer chromatography.

A further aliquot of 10 ml of urine was heated in a test-tube with 1·0 ml of conc. hydrochloric acid in a boiling water-bath for 30 min. The urine was adjusted to pH 7·0 with ammonia and centrifuged. The supernatant was twice extracted with *n*-butanol and concentrated by evaporation under reduced pressure.

SEPARATION AND ANALYTICAL TECHNIQUES

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 chromatogram thickness was 250μ.

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) Chloroimide (DQC reagent)[12] 2,6-dichloro-quinone chloroimide 0·25% (w/v) in absolute ethanol. Ammonia vapour was used to enhance the colour.

(b) Diazotised sulphanilic acid reagent: (Pauly Reagent)[12]

(c) Diazotised *p*-nitroaniline reagent:[12]

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 S.P. 800 recording spectrophotometer. Control elutions from a corresponding area of a blank chromatogram were used for the reference cell.

Infra red spectra

The i.r. spectra of the reference compounds and the metabolites were determined with a Unicam S.P. 200 recording spectrophotometer.

The *n*-butanol extracts of the incubation media were concentrated to dryness by evaporation under reduced pressure, the residue shaken with N/10 sodium hydroxide solution, and centrifuged at 20,000 *g* for 10 min. The supernatant was adjusted to pH 7·0 with 1N hydrochloric acid and twice extracted with *n*-butanol. The concentrated extract was examined by thin layer chromatography and i.r. spectroscopy.

Spectrophotofluorimetry

For spectrophotofluorimetric analysis, the eluates from u.v. spectral measurements were concentrated by evaporation under reduced pressure, and chromatographed on preparative thin layer chromatograms that had been pre-run in pure methanol. The elutions were performed with dry spectroscopically pure ethanol which had been redistilled over caustic potash. The solutions were centrifuged at 120,000 *g* for 30 min, and the fluorescence spectra of the supernatants were determined on both an Aminco-Bowman and a Zeiss recording spectrophotofluorimeter.

The fluorescence spectra of the reference hydroxyoxindoles and the metabolites were determined in spectroscopically pure ethyl alcohol.

RESULTS

Physical data of reference compounds

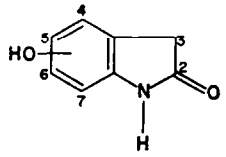
The position of hydroxylation of the oxindole nucleus (see I), may be determined by examination of R_f value and colour reactions on thin-layer chromatograms (see Table 1).

TABLE 1. THIN LAYER CHROMATOGRAPHIC R_f VALUES AND COLOUR REACTIONS OF HYDROXYOXINDOLES

Compound	R_f value solvent system* I	II	III	Colour reactions Chloroimide reagent	Diazotised *p*-nitro-aniline reagent	Diazotised sulphanilic acid
4-hydroxyoxindole	0·23	0·38	0·36	blue	yellow	yellow
5-hydroxyoxindole	0·17	0·28	0·26	red-purple	yellow-brown	brown
6-hydroxyoxindole	0·16	0·34	0·30	grey	orange	yellow
7-hydroxyoxindole	0·24	0·38	0·34	red-purple	yellow	brown
oxindole	0·55	0·56	0·50	red	yellow	yellow
5-hydroxy-N-methyloxindole	0·48	0·37	0·40	purple	light-brown	brown
6-hydroxy-N-methyloxindole	0·27	0·48	0·38	grey	orange-red	yellow
N-methyloxindole	0·64	0·62	0·59	maroon	yellow	yellow
3-methyloxindole	0·62	0·60	0·66	violet	yellow	yellow
oxindole metabolite	0·17	0·28	0·27	red-purple	yellow-brown	brown
N-methyloxindole metabolite (A)	0·47	0·37	0·40	purple	yellow-brown	brown
N-methyloxindole metabolite (B)	0·55	0·57	0·50	red	yellow	yellow
3-methyloxindole metabolite	0·19	0·31	0·45	violet	yellow-brown	brown
indole metabolite	0·56	0·56	0·51	red	yellow	yellow

* *Solvent systems*
Silica gel 'G'
("E. Merck"/Darmstadt)

I chloroform-19/ethyl alcohol-1
II benzene-4/ethyl acetate-6
III chloroform-5/acetone-4



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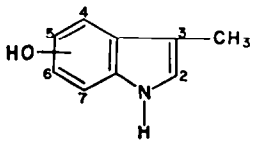


II

The hydroxyoxindoles may also be differentiated by u.v. spectral measurements, and these differences may be increased by the addition of sodium hydroxide to effect bathochromic shifts (see Table 2, Fig. 1).

TABLE 2. ULTRA-VIOLET ABSORPTION MAXIMA OF THE HYDROXYOXINDOLES

	λ_{max} Ethanol mμ	λ_{max} 0·1 N NaOH Ethanol mμ
4-hydroxyoxindole	233, 249, 284, 289	233, 298
5-hydroxyoxindole	214, 256, 302	221, 274, 318
6-hydroxyoxindole	223, 255, 287, (298)	238, 299
7-hydroxyoxindole	220, 247, 292	230, 309
oxindole	214, 248, (282)	—
5-hydroxy-N-methyloxindole	212, 257, 300	221, 278, 320
6-hydroxy-N-methyloxindole	226, 257, 285, (293)	238, 301
N-methyloxindole	213, 250, (281)	—
3-methyloxindole	212, 249, 282	—
indole	217, 287	—

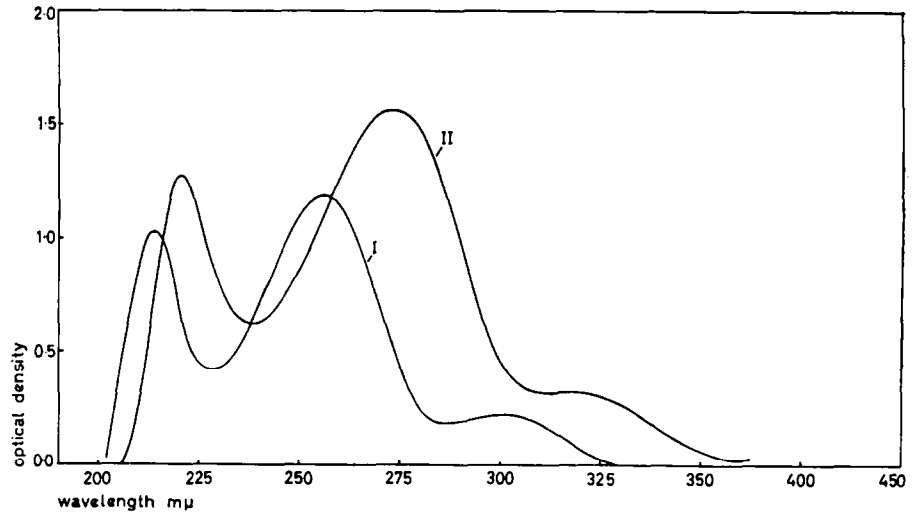


FIG. 1. Ultra-violet absorption spectra of 5-hydroxyoxindole. Curve I. 5-hydroxyoxindole in ethyl alcohol. Curve II. 5-hydroxyoxindole in ethyl alcohol plus 0·1 N sodium hydroxide.

As in the case of the hydroxyindoles,[13] the fluorescence spectra and activation wavelengths of the hydroxyoxindoles and hydroxyskatoles (See II) varied with the position of hydroxylation on the benzene ring (See Table 3), the fluorescence of hydroxyoxindoles was much less than that of the corresponding indoles.

Metabolic results

Thin layer chromatograms of the butanol extracts after the incubation of oxindole with rat, guinea-pig and rabbit liver microsome preparations derived from animals with and without pretreatment with phenobarbitone, showed the presence of one phenolic metabolite with R_f values and chemical colour reactions identical with those

TABLE 3. EXCITATION AND FLUORESCENCE MAXIMA OF HYDROXYOXINDOLES AND HYDROXYSKATOLES

	Excitation maxima [E] (mμ)	Fluorescence maxima [F]* (mμ)
4-hydroxyoxindole	275	311
5-hydroxyoxindole	289	337
6-hydroxyoxindole	297	345
7-hydroxyoxindole	275	322
4-hydroxyskatole	292	320
5-hydroxyskatole	306	343
6-hydroxyskatole	300	355
7-hydroxyskatole	290	322
skatole	290	350
oxindole metabolite	290	336
3-methyloxindole metabolite	292	337

* The fluorescence maxima was corrected for variation of the Aminco-Bowman spectrophotofluorimeter, which had been calibrated against a Pen Ray Quartz Lamp, and against a standard solution of Quinine sulphate 0·1 μg in 0·1 N sulphuric acid. (Sprince and Rowley, 1957).[14]

TABLE 4. INFRA-RED SPECTRA OF HYDROXYOXINDOLES

Compound	Infra-red bands (Nujol) (Wave number)
4-hydroxyoxindole	770, 835, 1040, 1170, 1210, 1235, 1290, 1335, 1610, 1635, 1690, 2900, 3240
5-hydroxyoxindole	675, 708, 755, 817, 850, 905, 940, 1130, 1210, 1260, 1270, 1335, 1660, 2900, 3170
6-hydroxyoxindole	685, 750, 815, 855, 890, 960, 1040, 1105, 1145, 1180, 1200, 1300, 1340, 1520, 1625, 1670, 2900, 3180
7-hydroxyoxindole	770, 870, 960, 1020, 1205, 1320, 1350, 1440, 1595, 1675, 2900, 3180
oxindole	675, 710, 750, 805, 850, 915, 945, 1020, 1090, 1170, 1195, 1235, 1300, 1330, 1615, 1690, 2900, 3180
oxindole metabolite	675, 710, 750, 780, 820, 855, 910, 940, 990, 1135, 1210, 1260, 1280, 1340, 1660, 2900, 3170

of 5-hydroxyoxindole (see Table 1); larger quantities of the metabolites were present using the phenobarbitone pretreated animals.

This metabolite exhibited an absorption spectrum, bathochromic shift and i.r. spectrum identical with that observed with 5-hydroxyoxindole (see Tables 2 and 4, Figs. 1 and 2).

Similarly, 3-methyloxindole gave one phenolic metabolite, when incubated with rat, guinea-pig and rabbit liver microsome preparations, giving colour reactions similar to those of 5-hydroxyoxindole (see Table 1).

The u.v. spectrum of the metabolite of 3-methyloxindole was similar to that of 5-hydroxyoxindole (see Fig. 1) (cf. the absorption spectrum of 3-methyloxindole is identical to that of oxindole).

Further confirmation of the structure of the metabolites was obtained by a study of their fluorescence spectra; in both cases the spectrum was identical to that of 5-hydroxyoxindole (see Table 3).

N-methyloxindole gave two metabolites, the major one phenolic and identical in R_f values, colour reactions and u.v. spectrum with 5-hydroxy-N-methyloxindole (see Tables 1 and 2), and a non-phenolic metabolite characterised as oxindole (see Tables 1 and 2).

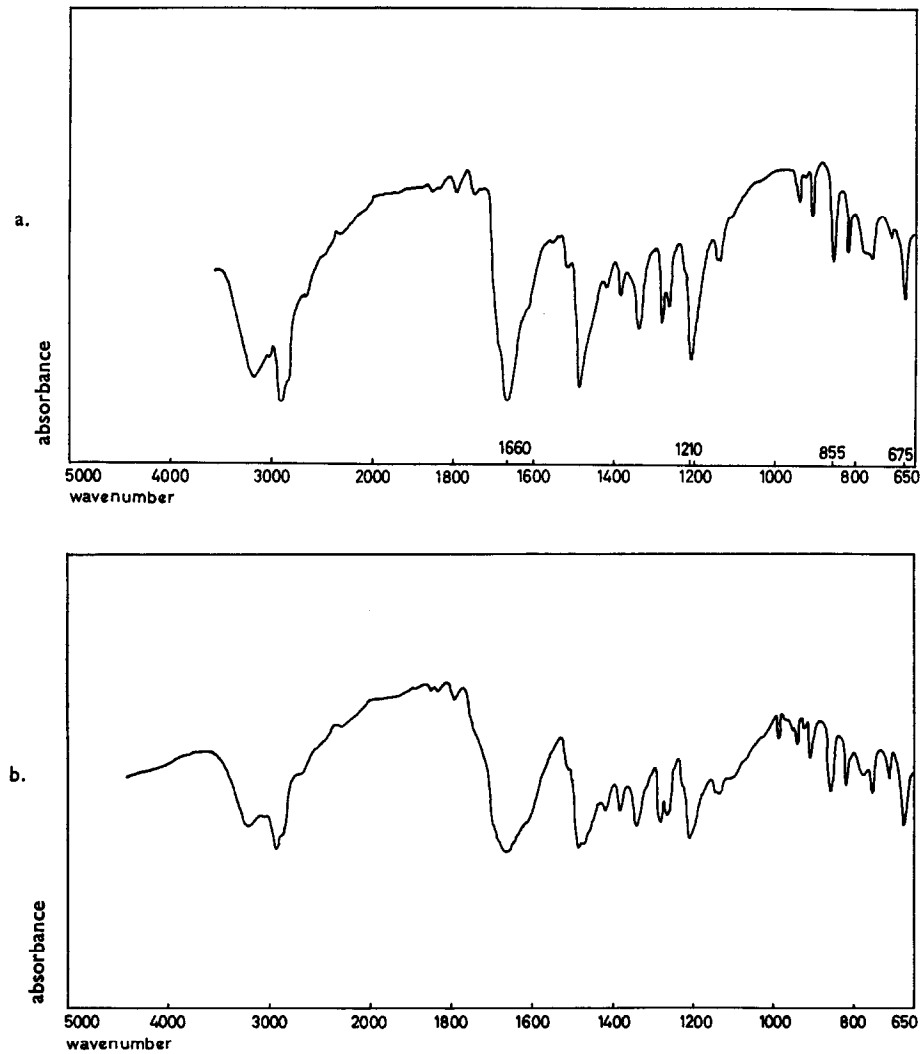


FIG. 2. (a) The i.r. absorption spectrum of 5-hydroxyindole. (b) The i.r. absorption spectrum of oxindole metabolite.

The N-demethylation was also demonstrated by measurement of formaldehyde produced in the reaction; more occurred in guinea-pig liver preparations than in rat and rabbit (see Table 5).

TABLE 5. SPECIES VARIATION IN THE RATE OF N-DEMETHYLATION OF N-METHYLOXINDOLE

Compound	μmoles HCHO produced per g* of liver per hour		
	Rabbit	Rat	Guinea-pig
N-methyloxindole	0·78	0·71	0·89

* The data presented, represents the average from three animals, all results of which were within ± 10 per cent of the recorded values.

Thus, oxindole is metabolised to 5-hydroxyoxindole, N-methyloxindole to 5-hydroxy-N-methyloxindole and 3-methyloxindole to 5-hydroxy-3-methyloxindole by rat, guinea-pig and rabbit liver microsome preparations.

With indoles, work is complicated by their chemical decomposition during the above procedure. The chemical artifacts were disregarded on the evidence of control incubations and extractions.

Incubation of indole with rabbit liver microsomes gave indoxyl, which rapidly oxidised and dimerised to give indigo blue (cf. Posner *et al.* 1961).[1]

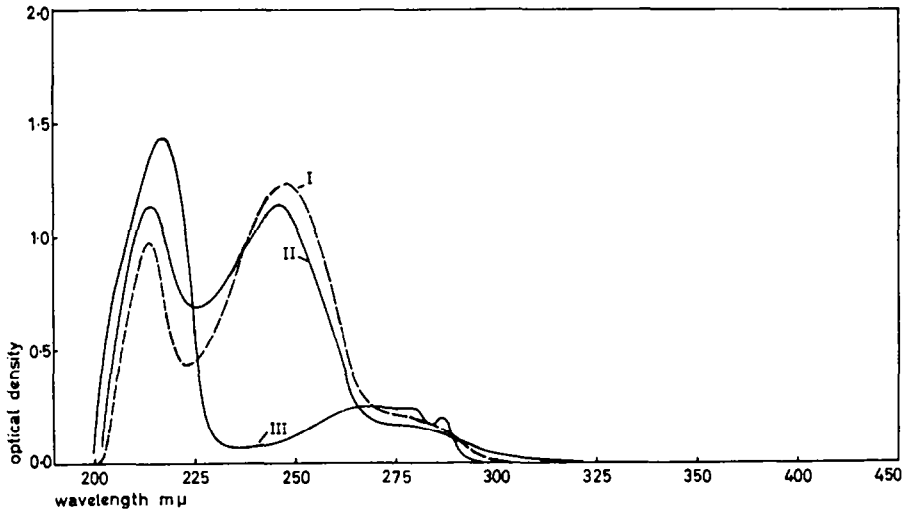


FIG. 3. The u.v. absorption spectra of indole and oxindole. Curve (1)—Oxindole in ethyl alcohol. Curve (II)—Indole metabolite in ethyl alcohol. Curve (III)—Indole in ethyl alcohol.

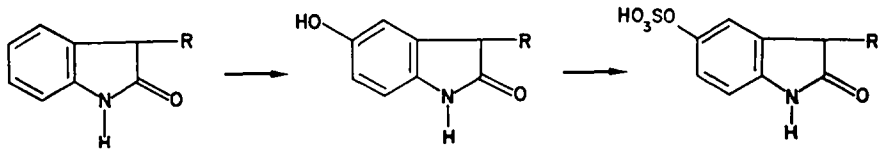
Rat liver microsomes, on the other hand, did not yield indigo blue under the described conditions, but yielded oxindole, the identity of which was proved by R_f values, chemical and colour reactions and u.v. spectroscopy (see Tables 1, 2 and Fig. 3).

After intra-peritoneal injections of oxindole, the urine of rats was found to contain a metabolite which was unchanged by β-glucuronidase enzyme action, but on hydrolysis

yielded ionic sulphate and a phenolic metabolite identical to that formed in the liver microsome preparations and charactertised as 5-hydroxyoxindole. No trace of oxindole, other oxindole metabolites, or the unconjugated phenolic metabolite was found in the urine.

When indole was administered to rats by intra-peritoneal injection, no oxindole could be detected in the urine, but small traces of a phenolic metabolite were observed after acid hydrolysis. This metabolite was characterised as 5-hydroxyoxindole by thin-layer chromatography, colour reactions and the physical evidence described in the work with the microsome preparations.

The biotransformations may be represented by the following scheme:



R = H or CH_3

DISCUSSION

The formation of 6-hydroxyoxindole from indole and oxindole has been reported by King *et al.* (1963),[2] but the results obtained in this present study have not confirmed these findings. The hydroxylation of oxindole, N-methyl-oxindole and 3-methyl-oxindole has been shown to occur at the 5-position of the oxindole ring.

Ichihara *et al.* (1957)[15] reported the 5-hydroxylation of indoles by liver microsomes but no evidence for this conversion was found in later work by Jepson *et al.* (1959),[16] Udenfriend *et al.* (1959)[17] or Posner *et al.* (1961).[1]

The absence of 3-hydroxylation in oxindole may be due to, (a) steric factors, i.e. close proximity of oxygen at position 2 of the nucleus, or (b) possible changes in electron density—due to the oxygen and loss of aromaticity in the heterocyclic ring of the compound. The presence of a 3-substituent in oxindole and a methyl substituent on the nitrogen atom, had no effect on the position of hydroxylation in the aromatic ring.

The formation of oxindole and 5-hydroxyoxindole from indole may indicate a further pathway in the metabolism of indolic compounds by liver enzymes.

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ADDENDUM

Subsequent to the acceptance of the present paper, a publication by King *et al.*[18] supports our findings that hydroxylation of oxindole occurs in the 5-position.

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