The Metabolism of [2-¹⁴C]Indole in the Rat

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1. [2-¹⁴C]Indole has been synthesized from [¹⁴C]formate and *o*-toluidine via *N*[¹⁴C]-formyltoluidine. 2. When fed to rats, the ¹⁴C of [¹⁴C]indole (dose 70–80 mg./kg. body wt.) is fairly rapidly excreted, and in 2 days an average of 81% appears in the urine, 11% in the faeces and 2.4% as carbon dioxide in the expired air. 3. Radioactivity is excreted in the urine as indoxyl sulphate (50% of the dose), indoxyl glucuronide (11%), oxindole (1.4%), isatin (5.8%), 5-hydroxyoxindole conjugates (3.1%), *N*-formylanthranilic acid (0.5%) and unchanged indole (0.07%). The faeces contain indoxyl sulphate (0.4% of the dose) and indole (0.2%), but the major metabolites have not been identified. 4. Fed to rats with biliary cannulae an average of 5.6% of a dose of [¹⁴C]indole (20–60 mg./kg. body wt.) is excreted in the bile in 2 days. Radioactivity is present as indoxyl sulphate (0.8% dose) and 5-hydroxyoxindole conjugates (0.6%). 5. Rats further metabolize indoxyl into *N*-formylanthranilic acid and anthranilic acid, and oxindole into 5-hydroxyoxindole. 6. With rat-liver microsomes plus supernatant under aerobic conditions, indole gives indoxyl, oxindole, possibly isatin, *N*-formylanthranilic acid and anthranilic acid, but under anaerobic conditions gives only oxindole. Similarly, under aerobic conditions, oxindole gives 5-hydroxyoxindole, anthranilic acid and *o*-aminophenylacetic acid. 7. Indole is metabolized by two pathways, one via indoxyl to isatin, *N*-formylanthranilic acid and anthranilic acid, and the other via oxindole to 5-hydroxyoxindole and possibly to *o*-aminophenylacetic acid and anthranilic acid. 8. The following new compounds are described: 4-hydroxy-2-nitrophenylacetic acid, 3-, 4- and 5-benzoyloxy-2-nitrophenylacetic acid, 5- and 7-hydroxyoxindole and 5-aminoacridine indoxyl sulphate.

The indole nucleus is a fundamental structure in a number of biologically important compounds, but the metabolic fate of indole itself has not been fully elucidated. It has been known for a considerable time that indole is converted into 3-hydroxyindole (indoxyl) in the animal body (Baumann, 1877; Mester, 1888) and this has been repeatedly confirmed (see Williams, 1959). The indoxyl formed is excreted in the urine primarily as an ethereal sulphate (urinary indican) and to a smaller extent as a glucuronide. Preliminary studies from this Laboratory (Kaighen, 1961) had shown that indoxyl was not the only metabolite of indole in the rabbit, and evidence was obtained which suggested that indole was hydroxylated to a small extent in the 5-position. Further, it was reported by Terashima (1957) that anthranilic acid was a metabolite of indole in the rabbit.

By using [2-¹⁴C]indole in the rat, we have shown that, although indoxyl is, in fact, the major metabolite of indole, there are other metabolites in urine, and these have been identified as oxindole, isatin, *N*-formylanthranilic acid, anthranilic acid and the

ethereal sulphate and glucuronide of 5-hydroxyoxindole. There is also a small output of ¹⁴CO₂ in the expired air of rats receiving [¹⁴C]indole and this appears to be derived from indole via indoxyl and *N*-formylanthranilic acid.

EXPERIMENTAL

Materials

Synthesis of [2-¹⁴C]indole. The synthesis was based on the pyrolysis of *N*-formyl-*o*-toluidine in the presence of potassium *tert*-butoxide (Tyson, 1955). Sodium [¹⁴C]formate (11.1 mg., 1 mc), 99% (w/v) formic acid (0.65 ml.) and 85% (w/v) H₃PO₄ (0.011 ml.) were heated together in a 5 ml. flask and the mixture was distilled. The distillate of [¹⁴C]-formic acid (0.73 g., 0.57 mc) was collected in a 25 ml. flask cooled in acetone–solid CO₂; *o*-toluidine (1.8 g.) was added and the mixture was heated under reflux on a water bath for 3 hr. After cooling, the mixture was seeded with a crystal of *N*-formyl-*o*-toluidine and left overnight to crystallize. The solid product was filtered, washed with 0.1N-HCl (10 ml.) and then water (10 ml.), dried *in vacuo* over P₂O₅ and twice recrystallized from benzene–light petroleum (b.p. 40–60°) (1:2, v/v) to give *N*[¹⁴C]-formyl-*o*-toluidine, m.p. 61°

(1.57g., 0.62mc). This was added to a freshly prepared solution of potassium *tert.*-butoxide (2.0g.) in 2-methylpropan-2-ol (50ml.) in a 100ml. flask and the mixture distilled to remove the solvent. The solid residue was then heated in a metal bath, the temperature of which was slowly raised to 250°, when the residue melted and excess of potassium *tert.*-butoxide distilled over. This slow heating prevented decrepitation of the material and increased the final yield of indole. The temperature of the metal bath was then raised to 350–360° for 20min., after which the flask was removed from the bath and the contents were cooled in a stream of N₂. The pyrolysis product was decomposed by treatment with 0.1N-HCl (10ml.) and the mixture was then extracted with four 100ml. portions of ether. The combined ether extracts were concentrated *in vacuo* at room temperature to about 3ml., which was then chromatographed on a column (1cm. diam.) of silica gel (Florisil, 50g.). The column was developed with chloroform–light petroleum (b.p. 40–60°) (1:2, v/v) and the first 600ml. of eluate was collected and evaporated to dryness *in vacuo* to give crude [2-¹⁴C]indole (0.445g.). This was further purified by chromatographing similarly on a second column of silica gel. Recrystallization from light petroleum (b.p. 40–60°) containing a little ether gave [2-¹⁴C]indole as white plates, m.p. 53° (0.174g., 97μc, equivalent to 19.4% of the theoretical radiochemical yield).

Further elution of the silica-gel columns gave unchanged *N*-formyl-*o*-toluidine, which was recovered and converted into indole by pyrolysis as before. Additional radioactive indole was also obtained by entrainment of radioactive residues in repeated syntheses starting with unlabelled formic acid. In this way two further batches of [2-¹⁴C]indole (total of 1.43g. and 66μc) and two batches of *N*[¹⁴C]-formyl-*o*-toluidine (total of 3.47g. and 92μc) were recovered. The remainder of the original radioactivity (about 50% of the theoretical radiochemical yield) was accounted for as polymerized end products.

The [2-¹⁴C]indole was chromatographed on paper in the solvent systems described under 'Chromatography'. Development of the chromatograms with the different detecting reagents and determination of the radioactivity by an end-window Geiger–Müller counter tube scanner revealed no impurities and showed that radioactivity was associated only with the indole.

6-Hydroxyoxindole. This compound, m.p. 243° (decomp.), has been obtained previously from 4-methoxy-2-nitrotoluene by the Reissert synthesis (Wieland & Unger, 1963) but the yields were poor. Better yields were obtained by catalytic reduction of 4-hydroxy-2-nitrophenylacetic acid. 4-Amino-2-nitrophenylacetic acid (Gabriel & Meyer, 1881) (1g.) in 8N-H₂SO₄ (30ml.) was cooled to 0° and treated with NaNO₂ (0.4g. in 2ml. of water) with stirring. The diazotized mixture was left for 1hr. at 0° and then added slowly to 150ml. of 25N-H₂SO₄ at 110° and kept at this temperature for 5min. The solution was cooled rapidly, diluted to 250ml. with water and extracted with ether (3 × 200ml.). The combined extracts were dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo* to an orange semi-crystalline residue, which was dissolved in hot ethyl acetate (charcoal) and reprecipitated by the dropwise addition of light petroleum (b.p. 60–80°). After leaving overnight the 4-hydroxy-2-nitrophenylacetic acid was filtered off, dried and recrystallized from benzene–light petroleum (b.p. 60–80°) to give pale-yellow needles (0.1–0.5g.), m.p. 180° (decomp.)

(Found: C, 48.6; H, 3.9; N, 6.6. C₈H₇NO₅ requires C, 48.7; H, 3.6; N, 7.1%). The acid (1g.) in acetic acid (40ml.) was hydrogenated at 40lb./in.² for 2hr. with 10% palladium-charcoal (0.1g.) as catalyst. The catalyst was filtered off and the solvent removed to leave a white crystalline residue, which was recrystallized from hot water (charcoal). 6-Hydroxyoxindole was filtered off, washed with water and dried to give white needles (0.35g.), m.p. 265° (decomp.) (Found: C, 64.0; H, 5.0; N, 9.4. Calc. for C₈H₇NO₂: C, 64.4; H, 4.7; N, 9.4%).

Better yields of 6-hydroxyoxindole were obtained by catalytic reduction of 4-benzyloxy-2-nitrophenylacetic acid. A solution of 1.5g. of 4-benzyloxy-2-nitrophenylpyruvic acid (Stoll, Troxler, Peyer & Hofmann, 1955) in 15ml. of 0.05N-NaOH was treated dropwise at 0° with H₂O₂ (6%, w/v) until the solution no longer gave a red colour with excess of NaOH. The resulting solution was then acidified with 2N-HCl and the precipitate that formed was filtered off, washed with water, dried and recrystallized from benzene to give pale-yellow needles (1.25g.) of 4-benzyloxy-2-nitrophenylacetic acid, m.p. 157° (Found: C, 62.9; H, 4.6; N, 5.0. C₁₅H₁₃NO₅ requires C, 62.7; H, 4.6; N, 4.9%). This material (2.0g.) was then converted into 6-hydroxyoxindole by catalytic reduction as described above. The 6-hydroxyoxindole (yield, 0.5g.) prepared by this method had m.p. 265° (decomp.) and was shown by thin-layer chromatography on alumina to be identical with that prepared by reduction of 4-hydroxy-2-nitrophenylacetic acid.

5-Hydroxyoxindole. This compound was prepared similarly by the reduction of 5-benzyloxy-2-nitrophenylacetic acid. A solution of 5-benzyloxy-2-nitrophenylpyruvic acid (Stoll *et al.* 1955) (8g. in 80ml. of 0.05N-NaOH) was treated at 0° with H₂O₂ (6%, w/v). The solution was acidified with 2N-HCl and the precipitate was filtered off and recrystallized from benzene to give colourless needles (6.8g.) of 5-benzyloxy-2-nitrophenylacetic acid, m.p. 168° (Found: C, 62.9; H, 4.7; N, 4.9. C₁₅H₁₃NO₅ requires C, 62.7; H, 4.6; N, 4.9%). This material (6.7g.) was then hydrogenated in the presence of 10% palladium-charcoal catalyst to give 5-hydroxyoxindole (1.75g.), m.p. 269° (decomp.), which formed colourless needles from water (Found: C, 64.4; H, 4.7; N, 9.5. C₈H₇NO₂ requires C, 64.4; H, 4.7; N, 9.4%).

7-Hydroxyoxindole. This compound was prepared similarly by the reduction of 3-benzyloxy-2-nitrophenylacetic acid. 3-Benzyloxy-2-nitrophenylpyruvic acid (Beer, Clarke, Khorana & Robertson, 1948) (4g. in 50ml. of 0.05N-NaOH) was treated at 0° with H₂O₂ as above to give colourless needles (3.1g.) of 3-benzyloxy-2-nitrophenylacetic acid, m.p. 161°, after recrystallization from benzene (Found: C, 62.25; H, 5.1; N, 5.0. C₁₅H₁₃NO₅ requires C, 62.7; H, 4.6; N, 4.9%). This (1.2g.) was then hydrogenated in the presence of 10% palladium-charcoal catalyst to give 7-hydroxyoxindole as colourless needles (0.4g.) (m.p. 251°, decomp.) from water (Found: C, 64.3; H, 4.8; N, 9.6. C₈H₇NO₂ requires C, 64.4; H, 4.7; N, 9.4%).

5- and 6-Hydroxyindole. These were prepared as required by reductive debenzoylation of the corresponding benzyloxyindoles. 5-Benzyloxyindole, m.p. 102°, or 6-benzyloxyindole, m.p. 116° (50mg.; Koch–Light Laboratories Ltd., Colnbrook, Bucks.), in 30ml. of methanol was hydrogenated at 40lb./in.² for 2hr. with 0.01g. of 10% palladium-charcoal as catalyst. The catalyst was filtered off and the solvent

removed *in vacuo* at 25°. The colourless solid residues of 5-hydroxyindole, m.p. 116° [Stoll *et al.* (1955) give m.p. 108°], and 6-hydroxyindole, m.p. 125°, were used without further purification since they became oxidized and darkened rapidly on exposure to air.

5-Aminoacridine indoxyl sulphate. This was prepared by treating a solution of potassium indoxyl sulphate (0.50 g.) in water (25 ml.) with a solution of 5-aminoacridine hydrochloride (0.46 g.) in hot water (20 ml.). The salt, which separated immediately, was filtered off, washed with cold water and recrystallized from water. 5-Aminoacridine indoxyl sulphate formed yellow crystals (0.6 g.), m.p. 215° (decomp.) [Found, SO₄, 23.3; C₁₃H₁₀N₂ (5-aminoacridine), 48.2%. C₂₁H₁₇N₃O₄S requires SO₄, 23.5; C₁₃H₁₀N₂, 47.7%].

Other materials. Potassium indoxyl sulphate (Boyland, Sims & Williams, 1956), *N*-formylanthranilic acid, m.p. 168° (Meyer & Bellmann, 1886), oxindole, m.p. 126° (Di Carlo, 1944), indirubin (sublimes at 244°) (Forrer, 1884), indigotin (Thomas, Bloxam & Perkin, 1909) and isatin (Hansen, 1924) were synthesized and purified. Indole, m.p. 54°, and isatin, m.p. 201°, were purchased (Koch-Light Laboratories Ltd.) and purified.

Chromatography

Samples of urines (0.2 ml.) and of concentrates (0.02 ml.) prepared from the urines by adsorption on to partially deactivated charcoal (Asatoor & Dalglish, 1956) were chromatographed on sheets of Whatman no. 1 paper, 23 cm.

square, in solvent *A* (propan-2-ol-aq. NH₃ (sp.gr. 0.88)-water; 8:1:1, by vol.), and then at right-angles in solvent *B* (butan-1-ol-acetic acid-water; 4:1:1, by vol.). Paper chromatograms were also prepared in solvents *C* (benzene-propionic acid-water; 20:14:1, by vol.), *D* (butan-1-ol-pyridine-water; 1:1:1, by vol.), *E* (phenol-aq. NH₃ (sp.gr. 0.88)-water; 160:1:40, by vol.) and *F* (aq. 20% KCl). Radioautographs were taken from the paper chromatograms, which were also examined in u.v. light (254 mμ; Hanovia Chromatolite lamp), and developed by spraying with the following reagents: 2% (w/v) *p*-dimethylaminobenzaldehyde in 2*N*-HCl (Ehrlich's reagent); 0.05% (w/v) 2,6-dichloroquinonechloroimide in ethanol followed by aq. saturated NaHCO₃ (Gibbs's reagent). Radioactive spots on radioautographs were identified by the *R_F* values and colour reactions of corresponding spots on paper chromatograms (see Table 1).

5- and 6-Hydroxyoxindole were separated on thin-layer chromatoplates (De Saga) of alumina (E. Merck A.-G., Darmstadt, Germany) developed in solvent *G* (*n*-hexane-propanol-1-ol; 10:1, v/v) (see Table 2).

Animal experiments

Collection of excreta. Female albino rats [Wistar strain, weight about 200 g.; Animal Supplies (London) Ltd.] were maintained on 25 g./day of Diet 41B (Joseph Rank Ltd.), with free access to water. [2-¹⁴C]Indole and unlabelled indole were administered orally as solutions in arachis oil (5%, w/v). Oxindole, 5- and 6-hydroxyoxindole, indoxyl acetate, indoxyl sulphate, *N*-formylanthranilic acid and

Table 1. *Chromatography and colour reactions of possible metabolites of indole*

Ascending chromatograms were run on square sheets of Whatman no. 1 paper in solvent *A* and then at right-angles in solvent *B* (see the text). Metabolites were detected by spraying with Ehrlich's reagent or Gibbs's reagent followed by aq. saturated NaHCO₃, or by the fluorindal reaction, i.e. Ehrlich's reagent followed by aq. saturated sodium acetate solution, with which indoxyl derivatives give a red colour. Fluorescence was observed in u.v. light (254 mμ; Hanovia Chromatolite lamp).

Metabolite	<i>R_F</i> in		Colour with			
	Solvent <i>A</i>	then solvent <i>B</i>	Ehrlich's reagent	Gibbs's reagent	Fluorindal reaction	Fluorescence
Indole	0.94	0.95	Purple	Purple	—	None
Indoxyl sulphate	0.52	0.44	Brown	Purple	+	Blue
Indoxyl glucuronide	0.21	0.42	Purple → brown	Purple	+	Purple
5-Hydroxyindole	0.85	0.85	Purple	Purple	—	—
6-Hydroxyindole	0.73	0.87	Blue	Purple	—	—
Oxindole	0.79	0.81	Orange*	Red	—	q†
5-Hydroxyoxindole sulphate	0.35	0.21	Orange*	Red	—	q†
5-Hydroxyoxindole glucuronide	0.07	0.20	Orange*	Red	—	q†
5-Hydroxyoxindole	0.58	0.65	Orange*	Purple-red	—	q†
6-Hydroxyoxindole	0.58	0.68	Orange*	Purple-red	—	q†
7-Hydroxyoxindole	0.85	0.80	Orange*	Blue	—	q†
Indirubin	0.63	0.81	—	—	—	q†
Indigotin	0.60	0.75	—	—	—	q†
Isatin	0.66	0.81	—	—	—	q†
<i>N</i> -Formylanthranilic acid	0.52	0.87	Yellow	Blue-green	—	Blue
Anthranilic acid	0.41	0.87	Yellow	Blue-green	—	Blue

* Colour developed after 48 hr.

† q means quenching of background fluorescence.

Table 2. *Thin-layer chromatography of some hydroxyoxindoles and bis-indoles*

Alumina chromatoplates were used. For solvent *G*, Ehrlich's reagent and Gibbs's reagent see the text. DSA is 1% (w/v) diazotized sulphanilic acid to which an equal volume of 2*N*-Na₂CO₃ was added just before spraying.

Compound	<i>R_F</i> values in		Colour with		
	Solvent <i>G</i>	CHCl ₃	Ehrlich's reagent	Gibbs's reagent	DSA
5-Hydroxyoxindole	0.61		Orange	Greyish violet	Orange
6-Hydroxyoxindole	0.35				
7-Hydroxyoxindole	0.07				
Hydroxyoxindole fraction from indole urine	0.61				
Hydroxylation products of oxindole by ascorbic acid-iron system*	0.61 0.30				
Isatin†	0.21	0.22	—	Red	—
Isatan†	0.38	0.35	—	—	—
Indirubin†	0.40	0.45	—	—	—
Oxindole	0.52	0.50	—	Red	—

* Oxindole (240 mg.) in phosphate buffer, pH 6.7 (250 ml.), containing EDTA (disodium salt) (7.6 g.), ascorbic acid (3 g.) and FeSO₄ (0.2 g.) was shaken with O₂ for 6 hr. (see Udenfriend *et al.* 1954). The solution was extracted with ether (3 × 100 ml.). The extract was dried (anhydrous Na₂SO₄) and evaporated to dryness, and the excess of oxindole removed from the residue with benzene (25 ml.). The residue was dissolved in a little methanol for chromatography.

† These compounds are coloured.

anthranilic acid were administered as aqueous suspensions; 5- and 6-hydroxyindole were administered as 5% solutions in propylene glycol.

Rats given [2-¹⁴C]indole were placed in a plastic metabolism chamber through which a current of air was drawn. The outgoing air was passed through two wash-bottles in series containing acetone at -30° to trap volatile organic compounds and then through two wash-bottles containing 4*N*-NaOH to remove respiratory CO₂. Urine and faeces were collected daily. After 3 days the animals were killed and the radioactivity of the tissues was determined. The urine was examined for metabolites qualitatively by chromatography and radioautography, and quantitatively by reverse isotope dilution.

Collection of bile. Polythene cannulae were fitted into the common bile duct of rats and the animals were then allowed to recover from the operation for 24 hr. (Stewart & Harrison, 1961). They were then dosed orally with [2-¹⁴C]indole and bile was collected for 48 hr. into a tube kept at -10° to minimize any possible action of biliary enzymes on conjugates. The total radioactivity of the bile was estimated as described below and that of any metabolite present by reverse isotope dilution.

Determination of radioactivity. Radioactive materials were counted as samples of infinite thickness with high-sensitivity low-background counting equipment (Panax Equipment Ltd.) incorporating the MX 157 anti-coincidence Geiger Counter assembly (Mullard Ltd.). Urine and bile were counted as such, tissues and faeces as homogenates in water, and the ¹⁴CO₂ in the expired air as Ba¹⁴CO₃. Samples were counted for periods of time sufficient to record at least 10³ counts net and to obtain a standard error of the mean less than 2%.

Qualitative identification of metabolites

A portion (5 ml. containing about 1.5 μC) of the 24 hr. urine from a rat dosed orally with [2-¹⁴C]indole (15.75 mg.,

9.0 μC) was diluted to 20 ml. with water acidified to pH 3 with acetic acid, and then shaken with 1 g. of charcoal that had been partially deactivated with *n*-octadecylamine. The charcoal was filtered off and then washed with water (3 × 20 ml.) to remove urea and electrolytes, and the adsorbed aromatic compounds were eluted from it with aq. 50% (v/v) acetone (3 × 40 ml.). The eluate was concentrated to 1 ml. at 35° *in vacuo*. A portion of this (0.2 ml., containing about 0.3 μC) was then chromatographed on Whatman no. 1 paper, first in solvent *A* and then at right-angles in solvent *B*. A further portion of the radioactive urine (0.05 ml., 0.015 μC) was applied directly to Whatman no. 1 paper and chromatographed as before. Normal rat urine was similarly treated with charcoal and the concentrated eluate from it was chromatographed as above. The chromatograms and radioautographs of the urine from rats dosed with indole revealed the presence of several metabolites, but most of the radioactivity was accounted for by six metabolites, namely indoxyl sulphate, indoxyl glucuronide, *N*-formylanthranilic acid, 5-hydroxyoxindole sulphate, 5-hydroxyoxindole glucuronide and an unidentified metabolite (see Table 1 for *R_F* values).

Indoxyl sulphate. This was identified by its *R_F* values, its strong blue fluorescence in u.v. light and by colour reactions. With Ehrlich's reagent followed by aq. saturated sodium acetate it gave a red colour, with a strong orange fluorescence in u.v. light (the fluorindal reaction), which is indicative of indoxyl derivatives. With acidified potassium persulphate it gave a blue colour due to the formation of indigotin, and with 0.1% (w/v) isatin in *N*-HCl followed by heating at 110° for 2 min. it gave the magenta colour of indirubin. These reactions were similar to those given by synthetic potassium indoxyl sulphate, and a mixture of the radioactive metabolite and the unlabelled synthetic indoxyl sulphate could not be separated by paper chromatography in the six solvents *A-F*.

Indoxyl glucuronide. This was identified by its *R_F* values and colour reactions (Jepson, 1960). It gave the same

colour reactions as indoxyl sulphate with acidified potassium persulphate and isatin, a positive fluorindal reaction, and a positive naphtharesorcinol reaction for glucuronic acid. Since all indoxyl derivatives give a similar blue colour (due to indigotin) on treatment with acids alone, the effect of β -glucuronidase on the metabolite was studied. The urine (12 ml.) of a rat given indole was applied as a strip on four 50 cm.-long sheets of Whatman 3MM paper and chromatographed in solvent *A*. The indoxyl glucuronide area from each sheet was eluted with water and purified by two-dimensional chromatography on Whatman 3MM paper in solvents *A* and *B*. The purified material was eluted with water (25 ml.) and the eluate concentrated *in vacuo* to 3 ml. A portion (1 ml.) of the eluate was incubated with 5 ml. of 5 mM-acetate buffer, pH 3.95, and 240 Fishman units (Talalay, Fishman & Huggins, 1946) of β -glucuronidase (0.8 mg.) (Koch-Light Laboratories Ltd.) at 37° for 4 hr. Paper chromatography of the material incubated with β -glucuronidase and of an ether extract of the incubated material showed that only indigotin and indirubin were present, indicating that the metabolite was a glucuronide of a precursor of these substances, most probably indoxyl glucuronide. Chromatography of the metabolite similarly incubated at pH 3.95 without β -glucuronidase showed only the presence of the original conjugate.

Anthranilic acid. This was identified by its R_F values, colour reactions and its strong blue fluorescence in u.v. light. Since the 2-¹⁴C atom of the radioactive indole would be eliminated in forming anthranilic acid, this metabolite was not radioactive and therefore was not detectable in radioautographs. Although this compound was also present in the chromatograms of normal rat urine, the chromatograms of the urine of rats dosed with indole showed a larger spot. The urine obtained from the rats given a total of 0.3 g. of indole was acidified to pH 2 and continuously extracted with ether for 5 hr. The extract was concentrated to 5 ml. and then purified by two-dimensional chromatography on Whatman 3MM paper in solvents *A* and *B*. The purified material was eluted with water and 2 mg. of authentic anthranilic acid was added to the eluate. Chromatography of this material in six different solvents *A-F* always produced only a single spot with the R_F values of anthranilic acid.

***N*-Formylanthranilic acid.** This was readily detected by its blue fluorescence in u.v. light and was identified by its R_F values and colour reactions. The latter are similar to those given by anthranilic acid but develop more slowly. Since the 2-¹⁴C atom of the indole nucleus is still present in *N*-formylanthranilic acid, it was readily detected on radioautographs. It was present together with anthranilic acid in the ethereal extract of urine from rats given indole and was separated and purified by the chromatographic procedure used for the purification of anthranilic acid. However, repeated chromatography caused slight hydrolysis and *N*-formylanthranilic acid and anthranilic acid were always present together in chromatograms.

The *N*-formylanthranilic acid was eluted from the chromatogram with ethanol. The eluate was evaporated to dryness *in vacuo* at 37° and the residue was refluxed for 15 min. in 0.1N-HCl. Paper chromatography of an ethereal extract of the hydrolysed material in solvents *A* and *B* revealed only a single spot, which from its R_F values and colour reactions was identical with anthranilic acid.

5-Hydroxyoxindole sulphate. The radioactive spot on

two-dimensional paper chromatograms, with R_F values 0.35 and 0.21 in solvents *A* and *B* respectively, appeared as a dark spot when examined in u.v. light. With Ehrlich's reagent it gave an orange colour after 48 hr., which suggested that the pyrrole ring of the indole molecule had been modified. The fluorindal reaction was negative and no blue colour was obtained when the compound was treated with 0.1% (w/v) isatin in N-HCl and heated at 110° for 2 min. This indicated that the compound was not an indoxyl derivative. With Gibbs's reagent the compound gave a red colour, which suggested that it was not a 4- or 7-hydroxyindole derivative since these would probably give a blue colour with this reagent. No colour was obtained on diazotization and coupling with *N*-(1-naphthyl)ethylene-diamine dihydrochloride, showing the absence of free aromatic amino groups. When the paper was treated with Cl₂ and then sprayed with a solution of starch and KI (Rydon & Smith, 1952) a blue colour was obtained, suggesting the presence of an amide group, but after treatment with Altman's reagent [4% (w/v) *p*-dimethylaminobenzaldehyde in acetic anhydride] and heating at 140° for 5 min. no orange colour was obtained, showing that the amide group was not present in a glycine conjugate. No blue colour was obtained with indoxyl sulphuric acid, and 2,4-dinitrophenylhydrazine did not give an orange colour, showing that the compound is not a simple derivative of isatin. Treatment with rhodizonic acid-acid BaCl₂ (Parke, 1960) showed that the compound was a sulphate ester.

The urine obtained from ten rats, each dosed orally with indole (30 mg.), was evaporated *in vacuo* at 37°. The resultant gum was extracted with three 100 ml. portions of methanol-light petroleum (b.p. 40–60°) (3:1, v/v) and the extract evaporated to dryness *in vacuo* at 37°. The residue was dissolved in water (5 ml.) and the solution chromatographed on Whatman 3MM paper in solvent *B*. The material of R_F 0.21 was eluted with water and purified by further chromatography first in solvent *B* and then in solvent *A*. The purified material in water (5 ml.) was acidified to pH 2 with 2N-HCl and refluxed under N₂ for 1 hr. The hydrolysed material was continuously extracted with ether and the extract dried (anhydrous Na₂SO₄). Chromatography of the extract on Whatman no. 1 paper in solvents *A* and *B* revealed a single new spot, which was found to be chromatographically identical with a product obtained by the oxidation of oxindole with the ascorbic acid-iron hydroxylating system of Udenfriend, Clark, Axelrod & Brodie (1954). Chromatography on paper in solvents *A-F* and colour reactions with Ehrlich's and Gibbs's reagents and alkaline diazotized sulphanilic acid did not distinguish this compound from 5- or 6-hydroxyoxindole. A separation of 5- and 6-hydroxyoxindole, however, could be effected by thin-layer chromatography on alumina in solvent *G* and the hydrolysis product was finally identified as 5-hydroxyoxindole, the original metabolite being 5-hydroxyoxindole sulphate (see Table 2).

5-Hydroxyoxindole glucuronide. The radioactive spot with R_F values 0.07 and 0.20 in solvents *A* and *B* appeared as a dark spot in u.v. light. The colour reactions were identical with those given by 5-hydroxyoxindole sulphate except that the rhodizonic acid-acid BaCl₂ test for ethereal sulphate was negative. The naphtharesorcinol test for glucuronic acid was negative at this stage.

The rat urine extract that had been used for the chromatographic separation of 5-hydroxyoxindole sulphate was also

used for that of 5-hydroxyoxindole glucuronide. The band of R_F 0.0-0.15 of the paper chromatogram developed in solvent *A* was cut out and eluted with water (5ml.). The eluate was applied to a column (20 cm. $\times$ 0.6 cm.) of the anion-exchange resin De-Acidite G (X2-3; 100-200 mesh) prepared in the formate form. The column was washed with water (50 ml.) and the metabolite was eluted with water saturated with SO₂ (50 ml.), followed by water until the eluate was nearly neutral (100 ml.). The eluate was concentrated *in vacuo* to 5 ml., and was shown to give a strongly positive naphtharesorcinol test for glucuronic acid and a single spot of R_F values 0.07 and 0.20 when chromatographed on Whatman no. 1 paper in solvents *A* and *B* respectively.

The eluate was refluxed with conc. HCl (0.2 vol.) for 1 hr. under N₂. A sample of the hydrolysed solution was continuously extracted with ether for 4 hr. and the extract dried over anhydrous Na₂SO₄. Chromatography of the ethereal extract on thin-layer plates of alumina in solvent *G* (Table 2) showed one spot, which was identical with 5-hydroxyoxindole. The remainder of the hydrolysed solution was absorbed on a column (7 cm. $\times$ 1 cm.) of activated charcoal (Ultrabsorb S.C. 120/240; British CarboNorit Ltd.) (10 g.). The column was washed with water until neutral, and then eluted with 30 ml. of aq. 5% (v/v) ethanol to remove sugar derivatives. The eluate, after concentration *in vacuo* at 37° to 2 ml., was chromatographed on Whatman no. 1 paper in three different solvent systems, namely propan-2-ol-water (4:1, v/v), propan-2-ol-butan-1-ol-water (7:1:2, by vol.) and butan-1-ol-acetic acid-water (12:3:5, by vol.). The three chromatograms were developed by spraying with the naphtharesorcinol reagent and then heated at 100° for 10 min. Two spots corresponding in R_F values to D-glucuronic acid and D-glucuronolactone were detected on all three chromatographs and it was therefore concluded that the original metabolite was the glucuronide of 5-hydroxyoxindole.

Oxindole and isatin. Although these two compounds are possible metabolites of indole, they were not detected on paper chromatograms or radioautographs of the urine of rats dosed with [2-¹⁴C]indole. Since 5-hydroxyoxindole is a metabolite of indole, then its precursor might be oxindole, for the feeding of oxindole to rats results in the excretion of 5-hydroxyoxindole and its conjugates (see Table 3). Both oxindole and isatin are readily converted into coloured bis-indoles. Isatin is readily reduced to the yellow isatan, and can condense with oxindole to give isatan or the red isoindigotin. Oxindole or isatin can condense with indoxyl to give indirubin. When oxindole (0.1 mg./ml.) is added to normal rat urine it can be partly recovered by CHCl₃ extraction and subsequent thin-layer chromatography on alumina with solvent *G*. Isatin (0.1 mg./ml.), however, was largely recovered as a yellow pigment, probably isatan. When added to the fresh urine of rats dosed with indole, both could be detected in thin-layer chromatograms of CHCl₃ extracts of the urine, but attempts at purification by elution and further chromatography resulted in their conversion into pigments including isatan and indirubin.

The urine (pH 8.0-8.2) of six rats, which had collectively received orally 90 mg. of indole in arachis oil, was adjusted to pH 3 with 0.1N-HCl as soon as it was passed during 24 hr. Each sample was immediately extracted twice with CHCl₃ (0.5 vol.), and the combined extracts (30 ml.) were dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The con-

centrate was chromatographed as a band on a thin-layer plate of alumina in CHCl₃ and run with reference spots (20 μ g.) of isatin and oxindole. Many coloured bands were formed, the most prominent of which were the following (R_F values given in parentheses): deep blue (origin); buff (0.1-0.12); blue (0.12); violet (0.14); yellow, several bands (0.14-0.35); violet (0.35-0.65); violet-grey (0.65-0.80); blue (0.80-0.95); buff (0.95). The areas (R_F 0.1-0.22 and 0.4-0.6) containing isatin and oxindole respectively were cut out and eluted with CHCl₃ (2 $\times$ 2 ml.). After centrifuging, the extract was concentrated and chromatographed on thin-layer alumina in solvent *G*. The material from the first area gave bands of R_F 0.21 (isatin), 0.38 (isatan) and 0.40 (indirubin). Further chromatography of this isatin band after elution resulted in the complete loss of the isatin, and only isatan and indirubin was found. Similar treatment of the second area above yielded several coloured bands on rechromatographing, including indirubin, but there was one colourless spot (R_F 0.52, oxindole), which gave a red colour with Gibbs's reagent and strongly absorbed u.v. light. Elution of this spot and further chromatography resulted in its disappearance and the simultaneous appearance of coloured bands of bis-indoles.

These experiments show that oxindole and isatin are detectable as metabolites of indole in fresh urine. It is probable that these compounds are reasonably stable in urine, but as soon as the urine is acidified indoxyl is released from its conjugates and the formation of bis-indoles can occur. Oxindole and isatin also disappear when chromatographed in small quantities. These points had to be carefully considered when isotope dilutions were carried out for oxindole and isatin.

Isotope dilution procedures

Total indoxyl. Carrier potassium indoxyl sulphate (0.5 g.) was added to urine (6 ml.) or bile (2 ml.) or faecal homogenate (30 ml.). Isatin (0.35 g.) and 5N-H₂SO₄ (40 ml.) were then added and the mixture was heated under reflux for 4-5 hr. After cooling the indirubin formed was filtered off, washed with water, with 2N-NaOH and again with water, and finally recrystallized from aqueous acetone to constant specific activity. The indirubin was then converted into its oxime (Lucius & Brünig, 1914-1916), which was recrystallized from aqueous acetone to constant specific activity (m.p. 246°, decomp.).

Indoxyl sulphate. A solution of potassium indoxyl sulphate (0.5 g. in 20 ml. of water) was added to urine (6 ml.) or bile (2 ml.) or faecal homogenate (30 ml.). The mixture was then poured into a solution of 5-aminoacridine hydrochloride (0.5 g. in 20 ml. of hot water). After cooling, the 5-aminoacridine salt of indoxyl sulphate was filtered off, washed with cold water, dried and recrystallized from hot water to constant specific activity (m.p. 215°, decomp.).

N-Formylanthranilic acid. N-Formylanthranilic acid (0.5 g.) dissolved in saturated NaHCO₃ (25 ml.) was added to urine (6 ml.) and reprecipitated by acidifying with 2N-HCl. It was filtered off, washed with water and then hydrolysed by heating in a micro-distillation apparatus with H₃PO₄ (sp.gr. 1.75) (1 ml.) and water (1 ml.). The radioactive formic acid liberated was then distilled into a receiver cooled to -30°, and neutralized with 10N-NaOH and finally adjusted to pH 6.4 with 0.01N-HCl. The solution was then refluxed for 0.5 hr. with 0.8 g. of 4-bromophenacyl bromide and 10 ml. of ethanol. After cooling, the crystalline

4-bromophenacyl formate was filtered, dried and recrystallized from light petroleum (b.p. 60–80°) to constant specific activity (m.p. and mixed m.p. 95°).

Total 5-hydroxyoxindole. The carrier (0.3 g.) was added to urine (6 ml.) or bile (2 ml.) and the mixture refluxed with 12 ml. of 3*N*-HCl for 1.5 hr. under N₂. The solution was cooled and continuously extracted with ether for 48 hr. The extract was dried over anhydrous Na₂SO₄, evaporated to dryness *in vacuo* and then recrystallized from water (charcoal) to constant activity (m.p. 269°, decomp.). This material was further purified by chromatography on thin-layer plates of alumina in solvent *G*. The 5-hydroxyoxindole found at *R_F* 0.6 was cut out, eluted with methanol and the eluate evaporated to dryness *in vacuo*. The 5-hydroxyoxindole purified in this way contained no 6-hydroxyoxindole and had the same specific activity as the material crystallized from water.

Total 6-hydroxyoxindole. 6-Hydroxyoxindole (0.3 g.) was added to urine or bile, and the procedure described for 5-hydroxyoxindole was followed. The recrystallized 6-hydroxyoxindole (m.p. 265°, decomp.) contained considerable radioactivity, which was found to be due to the presence of traces of the 5-isomer. Chromatography of the recrystallized material on thin-layer plates of alumina in solvent *G* separated these isomers. The 6-hydroxyoxindole was eluted with methanol and the specific activity of material purified in this way was negligible.

Indole. Carrier indole (0.5 g.) was added to urine (6 ml.) or homogenized faeces (60 ml., equivalent to 30 g. of faeces) and refluxed with 200 ml. of water for 0.5 hr. The mixture was then steam-distilled and the distillate (500 ml.) extracted with ether (3 × 50 ml.). The combined extracts were dried (anhydrous Na₂SO₄) and evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in CCl₄ (1–2 ml.) and the solution chromatographed on a column (20 cm. × 1 cm.) of alumina (100 g.; Spence grade H) previously deactivated by shaking for 24 hr. with 10% (v/v) acetic acid. The material was eluted with CCl₄, and the first 500 ml. of eluate, which contained most of the indole, was evaporated to dryness at room temperature *in vacuo*. The residue dissolved in 5 ml. of ethanol was then treated with a hot solution of picric acid (1 g.) in ethanol (20 ml.). On cooling, the crystalline indole picrate was filtered off and recrystallized from ethanol to constant specific activity (m.p. 184°).

Oxindole. Oxindole (0.5 g.) was added to a mixture of the urine (2 ml.) and 2*N*-HCl (25 ml.) and the whole refluxed under N₂ for 0.5 hr. The solution was cooled and continuously extracted with ether for 4 hr. After drying over anhydrous Na₂SO₄, the extract was evaporated to dryness. The residue in benzene (2 ml.) was chromatographed on a column (20 cm. × 1 cm.) of alumina (20 g.) partially deactivated with 10% acetic acid. The column was eluted with benzene, and the first 150 ml. of eluate, which contained most of the oxindole, was evaporated to dryness *in vacuo*. The residue of oxindole, m.p. 128°, was repeatedly recrystallized to constant specific activity from water (charcoal).

Isatin. Isatin (0.3 g. in 100 ml. of water) was added to urine (2 ml.) and the solution left for 5 min. to equilibrate. The solution was extracted with CHCl₃ (3 × 50 ml.) and the combined extracts were dried (anhydrous Na₂SO₄) and evaporated to dryness *in vacuo*. The residue was chromatographed on thin-layer chromatoplates of alumina in CHCl₃, and the band of isatin (*R_F* 0.0–0.25) was removed and eluted

with methanol. The eluate was evaporated to dryness *in vacuo* and the residue of isatin recrystallized from water and then benzene-methanol (4:1, v/v) to constant specific activity, m.p. 211°. The purified isatin (0.15 g. in 50 ml. of hot water) was heated on a water bath for 20 min. with phenylhydrazine hydrochloride (0.16 g.) and sodium acetate (0.1 g.). The mixture was cooled, and the product of isatin-3-phenylhydrazone filtered off, washed with water and recrystallized from methanol to constant specific activity (m.p. 212°). The isatin-3-phenylhydrazone was further purified by chromatography on thin-layer chromatoplates of alumina first in CHCl₃ (*R_F* 0.92) and then at right-angles in solvent *G* (*R_F* 0.85). The purified material was eluted with methanol and determination of its specific activity showed that this was unchanged.

Metabolism of indole and oxindole in vitro

Aerobic oxidation of indole. The microsome plus supernatant fraction (10000 g) of liver homogenates of female albino rats containing the equivalent of 0.25 g. of liver/ml. was prepared (see Creaven, Parke & Williams, 1965). Indole (112 μmoles or 13.1 mg. plus 250 mg. of Tween 80 to solubilize it) with 28 ml. of the 10000 g supernatant, 14 ml. of 0.1*M*-phosphate buffer, pH 7.4, glucose 6-phosphate (168 μmoles), nicotinamide (280 μmoles) and NADPH (7 μmoles, as the tetrasodium salt), in a total volume of 56 ml. of 1.15% (w/v) KCl, was incubated in fourteen 100 ml. beakers in air at 37° for 1 hr. in a Mickle shaking incubator. The incubation mixtures were combined, 1 ml. of 10*N*-HCl was added and the mixture was extracted with ether (2 × 100 ml.). The extract was dried over anhydrous Na₂SO₄, concentrated *in vacuo* at room temperature to 2 ml. and then chromatographed on paper in solvents *A* and *B*.

Seven spots were detected on the chromatograms, and by comparison with the *R_F* values and colour reactions of authentic materials they were identified as indole, indigotin, indirubin, oxindole, *N*-formylanthranilic acid and anthranilic acid. The seventh spot had *R_F* values 0.71 and 0.73 in solvents *A* and *B* respectively and gave a yellow colour with Ehrlich's reagent and purple fluorescence in u.v. light, but it was not identified.

The ethereal extract of a similar incubation of indole with rat-liver 10000 g supernatant preparation was dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml. of benzene and chromatographed on a column (20 cm. × 0.6 cm.) of alumina (Spence grade H, deactivated with 10% acetic acid). The column was developed with benzene (30 ml.), to elute the unchanged indole, indigotin and indirubin, and then with benzene-ether (10:1, v/v) (30 ml.) to elute oxindole, which was detected by the red colour it gave with Gibbs's reagent. The solution of oxindole was evaporated to dryness *in vacuo*, and the residue, dissolved in butan-1-ol (3 ml.), was heated in a water bath for 5 hr. with 1.0 ml. of a solution of *p*-dimethylaminocinnamaldehyde in butan-1-ol (250 μg./ml.) and 1 drop of pyridine. The solution was then diluted to 25 ml. with butan-1-ol and the u.v.-absorption spectrum of the solution determined. The absorption spectrum (λ_{max} , 460) of the condensation product of *p*-dimethylaminocinnamaldehyde with the oxindole formed enzymically from indole was identical with that prepared similarly from authentic oxindole (λ_{max} , 460 mμ).

Anaerobic oxidation of indole. The microsomal oxidation of indole was also studied anaerobically. A 10000g supernatant preparation (28 ml.) of rat-liver homogenate with phosphate buffer, pH 7.4, glucose 6-phosphate, nicotinamide and NADPH, in the same quantities as before, was distributed equally in 12 Thunberg tubes. The tubes were evacuated and then flushed with N₂ three times before finally filling with N₂ and introducing the substrate, indole (13.1 mg. plus 250 mg. of Tween 80). The tubes were then incubated at 37° for 1 hr. in a Mickle shaking incubator. The incubation mixtures were combined, 1 ml. of 10N-HCl was added and the solution was extracted with ether (2 × 100 ml.). The extract (dried over anhydrous Na₂SO₄) was concentrated *in vacuo* at room temperature to 2 ml. and the residue chromatographed on paper in solvents *A* and *B*.

Two spots were detected on the chromatograms and, by comparison with the *R_F* values and colour reactions of authentic compounds, these were shown to be indole and oxindole. Indigotin, indirubin, *N*-formylanthranilic acid and anthranilic acid were not detected.

Oxindole. Oxindole (112 μmoles) was substituted for indole plus Tween 80 in the above incubation mixture and the whole incubated at 37° for 1 hr. in air. Extracts were prepared as above and chromatographed on paper in solvents *A*, *B* and *C*. Four spots were detected, which were identified as oxindole, 5-hydroxyoxindole, anthranilic acid and *o*-aminophenylacetic acid. The identity of 5-hydroxyoxindole was confirmed by chromatography of the ethereal extract on thin-layer plates of alumina in solvent *G*. 6-Hydroxyoxindole was not detected.

Metabolism of indole metabolites

The metabolism in the rat of some of the possible metabolites of indole, namely indoxyl, oxindole, *N*-formylanthranilic acid, anthranilic acid, 5- and 6-hydroxyindole and 5-, 6- and 7-hydroxyoxindole, was studied qualitatively. These compounds were administered orally and samples of the 24 hr. urines were chromatographed on paper in solvents *A* and *B* as described above. The excretory products in urine were detected and identified by *R_F* values and colour reactions (see Table 3).

Table 3. *Fate of some possible metabolites of indole in the rat*

The compounds were given orally, and the 24 hr. urine was examined chromatographically on Whatman no. 1 paper. The metabolites were identified by *R_F* values and colour reactions (see the text). Abundance: + + +, major metabolite; + +, smaller amounts; +, minor metabolite.

Compound	Dose (mg./rat)	<i>R_F</i> in		Identity	Abundance
		Solvent <i>A</i>	then solvent <i>B</i>		
Indoxyl acetate or potassium indoxylsulphate	30	0.54	0.40	Indoxyl sulphate	+ + +
		0.19	0.44	Indoxyl glucuronide	+ + +
		0.43	0.90	Anthranilic acid	+
		0.52	0.90	<i>N</i> -Formylanthranilic acid	+
Oxindole	30	0.35	0.21	5-Hydroxyoxindole sulphate	+ + +
		0.09	0.20	5-Hydroxyoxindole glucuronide	+
		0.58	0.65	5-Hydroxyoxindole	Trace
5-Hydroxyindole	20	0.49	0.40	5-Hydroxyindole sulphate	+ + +
		0.15	0.31	5-Hydroxyindole glucuronide	+ + +
		0.85	0.85	5-Hydroxyindole	+
6-Hydroxyindole	20	0.50	0.40	6-Hydroxyindole sulphate	+ + +
		0.23	0.34	6-Hydroxyindole glucuronide	+ + +
5-Hydroxyoxindole	30	0.35	0.21	5-Hydroxyoxindole sulphate	+ + +
		0.09	0.20	5-Hydroxyoxindole glucuronide	+
		0.58	0.65	5-Hydroxyoxindole	Trace
6-Hydroxyoxindole	30	0.36	0.21	6-Hydroxyoxindole sulphate	+ + +
		0.08	0.21	6-Hydroxyoxindole glucuronide	+
		0.57	0.63	6-Hydroxyoxindole	Trace
7-Hydroxyoxindole	30	0.62	0.36	7-Hydroxyoxindole sulphate	+ + +
		0.15	0.30	7-Hydroxyoxindole glucuronide	+
<i>N</i> -Formylanthranilic acid	100	0.28	0.38	Anthranilyl glucuronide	+ + +
		0.38	0.74	Anthranilyl glycine	+
		0.43	0.91	Anthranilic acid	+
		0.52	0.91	<i>N</i> -Formylanthranilic acid	+
Anthranilic acid	100	0.28	0.38	Anthranilyl glucuronide	+ + +
		0.38	0.74	Anthranilyl glycine	+
		0.43	0.91	Anthranilic acid	+

Indoxyl was administered either as indoxyl acetate (30mg.) or potassium indoxyl sulphate (100mg.). The output of indoxyl glucuronide after feeding with these compounds appeared to be greater than after feeding with indole. Small amounts of anthranilic acid and *N*-formylanthranilic acid were detected but 5-hydroxyoxindole and its conjugates were not found.

Oxindole (30mg.) gave rise to the excretion of 5-hydroxyoxindole and its conjugates, principally the sulphate. Oxindole, 6-hydroxyoxindole, *N*-formylanthranilic acid, anthranilic acid and their conjugates were not detected.

Anthranilic acid (100mg.) was excreted principally as such and as two compounds, which were identified as anthranilyl glucuronide and anthranilyl glycine, the known metabolites of anthranilic acid (Charconnet-Harding, Dalglish & Neuberger, 1953). The spots on the chromatogram corresponding to these compounds gave a violet colour when diazotized and coupled with *N*-(1-naphthyl)-ethylenediamine (1% in water), thus indicating a free aromatic amino group in each. A spot with R_F values 0.28 and 0.38 in solvents *A* and *B* respectively gave a positive naphtharesorcinol test for glucuronic acid, whereas a spot with R_F values 0.38 and 0.74 gave the characteristic orange colour of a glycine conjugate after spraying with a solution of *p*-dimethylaminobenzaldehyde (4% in acetic anhydride) and heating at 140° for 3min. Free glucuronic acid was also detected on the chromatograms; this could arise from anthranilyl glucuronide or possibly from an *N*-glucuronide of anthranilic acid.

N-Formylanthranilic acid (100mg.) administered orally or intraperitoneally gave similar results. Some unchanged *N*-formylanthranilic acid was excreted but the major metabolites were anthranilic acid and its glycine and glucuronide conjugates.

5- and 6-Hydroxyindole (20mg.) each gave rise to the excretion of their respective ester sulphates and glucuronides as the major metabolites. Free 5-hydroxyindole was also excreted in small amounts but free 6-hydroxyindole was not detected in the urine. 5- and 6-Hydroxyoxindole and their conjugates were not found.

5- and 6-Hydroxyoxindole (30mg.) each gave three spots on chromatograms of the urine excreted. These were identified as the corresponding ethereal sulphates, which were the major metabolites, the glucuronides and

small amounts of the free hydroxyoxindoles. No spots corresponding to *N*-formylanthranilic acid or anthranilic acid were detected.

RESULTS AND DISCUSSION

Qualitative chromatographic studies of the urine of rats dosed with [2-¹⁴C]indole show that the major metabolites of indole are 3-hydroxyindole sulphate and glucuronide, and that the minor metabolites are 5-hydroxyoxindole sulphate and glucuronide, *N*-formylanthranilic acid and anthranilic acid. Isotope dilution experiments showed that oxindole and isatin were also minor metabolites but these two compounds were only present, as shown by thin-layer adsorption chromatography, in the fresh urine, for they were rapidly converted into coloured bis-indole compounds in the presence of conjugates of indoxyl. The anthranilic acid found in the urine may have been formed by spontaneous decomposition of *N*-formylanthranilic acid, since the oral administration of anthranilic acid results largely in its excretion as the glucuronic acid and glycine conjugates.

When [2-¹⁴C]indole (70–80mg./kg. body wt.) is administered orally to rats, the radioactivity is fairly rapidly excreted in the urine, faeces and expired air. The major route of excretion is the urine, for 75% of the dose is excreted by this route in 24hr. and nearly 80% in 2 days. About 10% is eliminated in the faeces and 2% in the expired air as ¹⁴CO₂. Relatively small amounts of radioactivity (0.4% of dose) were found to remain in the tissues 2 days after dosing (Table 4). Of the radioactivity in the urine, an average of 72% of the dose is accounted for as indoxyl sulphate (50% of the dose), indoxyl glucuronide (11%), 5-hydroxyoxindole conjugates (3.1%), isatin (5.8%), oxindole (1.4%) and *N*-formylanthranilic acid (0.5%) (Table 5). The nature of the faecal radioactivity

Table 4. *Distribution of ¹⁴C in the excreta and tissues of rats receiving [2-¹⁴C]indole orally*

—, Not determined.

	Percentage of dose in 2 days									Average for rats 4, 6 and 7
	Rat no.	...	...	1	2	3	4	5	6	7
Weight (g.)	...	...	...	230	170	180	200	200	220	240
Dose of indole (mg./kg.)	...	...	...	76	70	80	80	74	70	64
Dose of ¹⁴ C (μC)	...	...	...	0.5	0.34	0.5	9.0	8.2	8.8	8.8
Urine				65.6	86.0	58.0	92.3	90.3	72.1	77.9
Faeces				12.4	11.7	9.1	7.2	3.7	15.4	10.7
Expired air				—	—	—	3.1	—	1.9	2.1
Tissues				—	—	—	0.0	0.4	0.4	0.2
Total accounted for				78.0	97.7	67.1	102.6	94.4	89.8	90.0

Table 5. *Quantitative aspects of the metabolism of [2-¹⁴C]indole in rats*

Details of the methods used are given in the text. The dose of indole and of ¹⁴C are given in Table 4, and the values quoted are for excreta collected for 2 days after dosing with indole.

	Percentage of dose of ¹⁴ C		
	Rat no.....5	6	7
Expired air			
CO ₂	—	1.9	2.1
Urine			
Indole	0.0	0.06	0.07
Total indoxyl	67.6*	60.8	59.9
Indoxyl sulphate	49.8	53.7	45.2
Indoxyl glucuronide	17.8*†	7.1†	14.7†
Total 5-hydroxyoxindole	—	3.0	3.1
Total 6-hydroxyoxindole	—	0.0†	0.0†
Oxindole	—	1.9	0.9
Isatin	—	5.5	6.1
<i>N</i> -Formylanthranilic acid	0.63	0.44	0.53
Total of above metabolites in urine	68	72	71
Total ¹⁴ C in urine	90	72	78
Faeces			
Indole	0.09	0.13	0.19
Total indoxyl	—	0.68	0.59
Indoxyl sulphate	—	0.43	0.37
Total of above metabolites in faeces	0.09	0.81	0.78
Total ¹⁴ C in faeces	3.7	15.4	10.7

* Includes isatin.

† Difference between total indoxyl and indoxyl sulphate.

‡ Limit of detection is 0.3% of dose.

Table 6. *Excretion of ¹⁴C in the bile of rats receiving [2-¹⁴C]indole*

Rats were cannulated as described in the text and given [2-¹⁴C]indole orally. Bile was collected for 2 days. —, Not determined.

		Percentage of dose of ¹⁴ C in the 2-day bile			
Rat no.		8	9	10	11
Weight (g.)		200	200	200	210
Dose of indole (mg./kg.)		21	21	63	49
Dose of ¹⁴ C (μC)...		2.3	2.3	7.3	5.9
Total indoxyl		—	—	0.85	0.78
Indoxyl sulphate		—	—	0.84	0.75
5-Hydroxyoxindole		—	—	0.52	0.60
Total of above		—	—	1.37	1.38
Total ¹⁴ C in bile		5.4	7.1	4.65	5.2
					(average 5.6)

(average 5.6)

(10% of dose) has not been elucidated, but indole (0.2% of dose) and indoxyl sulphate (0.4% of dose) are only minor components of it (Table 5). Radioactivity is also excreted in the expired air as ¹⁴CO₂ (2% of the dose), so that at least an equivalent amount of indole must have been metabolized into non-radioactive metabolites by scission of the pyrrole ring and loss of the 2-¹⁴C atom. Neither indole nor any other volatile metabolite was detected in the expired air. When [¹⁴C]indole

is given orally to biliary-cannulated rats an average of 5% of the administered radioactivity is eliminated in the bile in 48 hr., of which 0.8% of the dose is indoxyl sulphate and 0.6% is conjugated 5-hydroxyoxindole (Table 6).

To elucidate the routes of metabolism of indole in the rat, studies were made of the metabolism of indole and its possible metabolites *in vivo* and *in vitro*. When indoxyl is given orally, it gives rise to the excretion of *N*-formylanthranilic acid and

anthranilic acid, whereas oxindole gives rise to 5-hydroxyoxindole and its conjugates. When 5- and 6-hydroxyindole are administered to rats they are excreted in the urine as the sulphate and glucuronic acid conjugates, and oxidation of the pyrrole ring to give hydroxyoxindoles does not occur. When indole is incubated aerobically with rat-liver microsomes plus supernatant, there are formed indigotin, indirubin, oxindole, *N*-formylanthranilic acid and anthranilic acid, whereas under anaerobic conditions only oxindole is formed. The occurrence of indigotin and indirubin in the incubation mixtures suggests that indoxyl and possibly isatin are formed from indole under aerobic conditions. When oxindole is incubated aerobically with similar rat-liver preparations, 5-hydroxyoxindole, anthranilic acid and *o*-aminophenylacetic acid, but not *N*-formylanthranilic acid, are formed. It thus appears that initially indole undergoes two separate hydroxylations, one in the 3-position to give indoxyl and one in the 2-position to give oxindole. The oxidation in the 2-position can proceed under aerobic or anaerobic conditions and possibly the oxygen introduced is derived from water. The oxidation in the 3-position, however, occurs only under aerobic conditions, and presumably, like other microsomal hydroxylations of foreign compounds, it requires molecular oxygen and NADPH.

The initial oxidation of indole occurs mainly in the 3-position, the position of highest electron density, and to a smaller extent in the 2-position, the position of second highest electron density. The indoxyl formed is now mainly conjugated with sulphate and glucuronic acid and excreted. How-

ever, some indoxyl is further oxidized to formylanthranilic acid, possibly via 2,3-dihydroxyindole, dioxindole and isatin. To a small extent formylanthranilic acid probably yields anthranilic acid and formate, the latter probably being the source of the $^{14}\text{CO}_2$ in the expired air. The oxindole formed does not appear to be able to conjugate owing to lack of suitable groupings. It therefore undergoes further hydroxylation in the 5-position to 5-hydroxyoxindole. The hydroxylation of oxindole in the 5-position is to be expected since this position is *para* to the NH group. 5-Hydroxyoxindole is then largely disposed of by conjugation with sulphate and glucuronic acid. The experiments with oxindole *in vitro* suggest that it could break down to *o*-aminophenylacetic acid and possibly to anthranilic acid. Most of these suggested pathways require further detailed examination.

The metabolism of indole is reminiscent of that of quinoline, in which the major initial hydroxylations occur in the 2- and 3-positions. 2-Hydroxyquinoline or 2-quinolone is then further hydroxylated to 6-hydroxy-2-quinolone, position 6 in quinoline being the equivalent of position 5 in indole (see Smith & Williams, 1955; Sax & Lynch, 1964).

The metabolism of indole as elucidated so far is shown in Scheme 1.

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Scheme 1. Hypothetical metabolites are in brackets and the percentage of dose found in the urine (except CO_2) as a particular metabolite is given under its formula.

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