Studies in Detoxication

84. THE METABOLISM OF [¹⁴C]ANILINE IN THE RABBIT AND OTHER ANIMALS*

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The fate of aniline in the rabbit was investigated by Smith & Williams (1949), who showed that glucuronides and ethereal sulphates of *o*- and *p*-aminophenol, and possibly 4-aminoresorcinol, were metabolites accounting for 40–45% of the aniline fed; but the main metabolite, more than 50% of the dose, was considered to be a labile glucuronide of aniline itself. This labile *N*-glucuronide was also thought to be the principal metabolite of aniline by Ishidate, Hogiya, Hashimoto & Takitani (1958); and another conjugate of aniline, phenylsulphamic acid, was detected in the urine of rabbits dosed with aniline (Boyland, Manson & Orr, 1957). The metabolism of aniline by liver slices from rat, rabbit, dog and cat has yielded *o*- and *p*-aminophenol and 4-aminoresorcinol (Sato, Suzuki, Fukuyama & Yoshikawa, 1955); rat-liver and cat-liver microsomes in the presence of reduced triphosphopyridine nucleotide and oxygen gave *o*- and *p*-aminophenol (Booth & Boyland, 1957; H. S. Posner, unpublished results), and rabbit-liver microsomes under the same conditions gave only *p*-aminophenol (Mitoma, Posner, Reitz & Udenfriend, 1956). The hydroxylation of aniline to *p*-aminophenol has been effected by ascorbic acid in the presence of ferrous iron, ethylenediaminetetra-acetic acid and oxygen (Brodie, Axelrod, Shore & Udenfriend, 1954), and it appears likely that ascorbic acid is involved in the hydroxylation of aniline *in vivo* (Axelrod, Udenfriend & Brodie, 1954; Volkova, 1958). The present work with [¹⁴C]aniline has shown that the principal fate of aniline in mammals is hydroxylation to *o*- and *p*-aminophenol, with the *N*-glucuronide of aniline as only a minor metabolite.

The use of [¹⁴C]aniline has made possible the accurate estimation in urine of the isomeric aminophenols, aniline *N*-glucuronide, phenylsulphamic acid and acetanilide, and of the amounts exhaled in the breath and retained in the faeces and tissues. In this way some 80–100% of a single dose of aniline has been accounted for. In addition, the principal metabolites have been estimated in several animal species and the proportion of *o*- to *p*-aminophenol excreted by different animals has been shown to vary widely.

MATERIALS AND METHODS

Melting points are corrected.

Preparation of [¹⁴C]aniline. [¹⁴C]Aniline randomly labelled with ¹⁴C in one carbon atom was prepared from [¹⁴C]nitrobenzene by catalytic transfer-hydrogenation with cyclohexane and palladium black according to Braude, Linstead & Wooldridge (1954) (yield 91%, b.p. 184°).

Aniline *N*-glucuronide. Salts of aniline-*N*-glucosiduronic acid have been previously described (Thierfelder, 1889; Bergmann & Wolff, 1923; Ishidate *et al.* 1958), but unequivocal characterization of these unstable compounds was first established by Heyns & Baltes (1958), who described a sodium salt, C₁₂H₁₄O₆NNa₂· $\frac{1}{2}$ C₆H₇N, m.p. 201–203° (decomp.), and a potassium salt, C₁₂H₁₄O₆NK₂·C₆H₇N·H₂O, m.p. 175° (decomp.). An hydrated ammonium salt, m.p. 130° (decomp.), [α]_D²⁴ –55° → –15° (c, 2.3 in water), was described but not adequately characterized by Ishidate *et al.* (1958). The ammonium salt used in experiments described in this paper was prepared as follows: to a solution of 5 g. of glucuronic acid in 15 ml. of water was added 1.9 ml. of aq. NH₃ soln. (sp.gr. 0.88) followed by 5 ml. of aniline in 10 ml. of ethanol. After mixing, the solution was left at room temperature until a precipitate began to form. The mixture was then kept at 0° for 8 hr. and the precipitate was filtered off and washed with a little cold ethanol. Recrystallization from aq. 50% (v/v) ethanol gave *ammonium aniline-N-glucosiduronate*, containing 1 mol.prop. of aniline of crystallization, as colourless needles, m.p. 122° (decomp.); [α]_D²⁵ –77° → –17° ± 1° (c, 2.0 in water) (Found: C, 54.7; H, 6.65; N, 10.4; aniline, 47.3. Calc. for C₁₂H₁₈O₆N₂·C₆H₇N·H₂O; C, 54.4; H, 6.85; N, 10.6; aniline, 46.9%). The total aniline content was estimated by refluxing 0.05 g. of the salt with 10 ml. of 2*N*-HCl for 1 hr., steam-distilling the solution after adjusting to pH 10 with 5*N*-NaOH, and determining the difference in light absorption of the steam-distillate at 280 m μ in 0.1*N*-HCl and 0.1*N*-NaOH (aniline in 0.1*N*-NaOH showed light-absorption at 280 m μ , ϵ 1300; in 0.1*N*-HCl at 280 m μ , ϵ 0). The ammonium salt is readily soluble in cold water, less soluble in ethanol and insoluble in ether and ethyl acetate. It slowly reduces Benedict's reagent but reduces more readily after preliminary treatment with *N*-HCl. It gives the naphtharesorcinol reaction for glucuronic acid and shows the colour reactions characteristic of free aniline. The structure of the ammonium salt is similar to that of the potassium salt and not the sodium salt, as would be expected, since the radius of the ammonium ion, 1.48 Å, is closer to that of potassium (1.33 Å) than that of sodium (0.95 Å).

Other compounds. 3-Aminocatechol-HCl and 7-hydroxy-2-phenylbenzoxazole, m.p. 192° (Henrich, 1921); 4-aminocatechol-HBr (Jacobs, Heidelberg & Rolf, 1919) and

* Part 83: Baldwin, Robinson & Williams (1960).

4-benzamidocatechol, m.p. 169° (Kehrmann & Hoehn, 1925); 2-aminoresorcinol-HCl and 2-benzamidoresorcinol, m.p. 190° (Kauffmann & dePay, 1906); 4-aminoresorcinol-HCl and 4-benzamidoresorcinol dibenzoate, m.p. 172° (Henrich & Wagner, 1902); 5-aminoresorcinol-HCl and 5-acetamidoresorcinol diacetate, m.p. 121° (Pollack, 1893); aminoquinol-HCl (Henrich, 1921) and acetamidoquinol, m.p. 100° (Kehrmann & Bahatryan, 1898); *p*-aminothiophenol, m.p. 46° (Hinsberg, 1906) and 4:4'-diaminodiphenyl disulphide, m.p. 81° (Hodgson & Dix, 1914); *p*-aminophenylglucuronide, m.p. 215° (Williams, 1943) and *p*-acetamidophenylglucuronide, m.p. 193° (Smith & Williams, 1948); and barium phenylsulphamate (Traube, 1890) were also prepared. Other compounds required for isotope-dilution experiments were purchased or prepared by standard methods and purified to constant m.p.

Animals. The [¹⁴C]aniline contained about 100 µC/ml. and was administered, alone or suitably diluted with inactive aniline (A.R.), b.p. 184–185°, by stomach tube to gerbils, guinea pigs, hamsters, rabbits, rats, mice, ferrets, cats, dogs and hens. The mice, rats, gerbils, rabbits and hamsters were maintained on Diet 41 B (E. Dixon and Son, Ltd., Ware, Herts.); guinea pigs on diet 18 (E. Dixon and Son, Ltd.) and cabbage; ferrets and dogs on milk together with raw beef and wholemeal bread in equal amounts; cats on milk and Kit-e-Kat; and hens on SPBRA 'Battery intensive mash'. Both food and water were available without restriction to all animals. All animals were female except where stated to the contrary.

The rabbits, after dosing, were placed in a metabolism chamber and the expired air was collected and analysed for unchanged aniline and CO₂ as described previously (Parke, 1956). They were removed from the chamber 3 or 4 days after dosing, killed and their tissues were analysed for radioactivity. The daily volume of urine was approximately 100 ml., and portions of 1–10 ml./day, according to the doses of ¹⁴C, were taken for the isotope-dilution procedures. The other animals were placed in open metabolism cages after dosing and their urine was collected and analysed daily.

Measurement of radioactivity. Measurements were carried out on solid and liquid samples of infinite thickness on nickel-plated iron planchets (General Electric Co., Ltd.) with an end-window counter tube, the background of which was 7–9 counts/min. The specific activities were determined by comparison with a stable polymer reference (The Radiochemical Centre, Amersham, Bucks.), which was standardized against [¹⁴C]acetanilide, prepared from the labelled aniline. A sample of 1.75 cm.² containing 1 µC of ¹⁴C/g. of substance gave approximately 1080 counts/min. The variation in disk size and preparation of sample gave an error of ±5%.

Urines and tissues. The total radioactivity of the urines and tissues were measured at BaCO₃ obtained by wet combustion (see Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). In some experiments the urine was counted directly as a liquid and the tissues were counted as homogenates. There were no significant differences between the results from these two methods. The limits of detection of metabolites in the urine were of the order of 0.02% of the dose.

Analytical methods. Glucuronic acid and ethereal sulphate in urine were determined as described by Mead, Smith & Williams (1958).

Estimation of metabolites

Carbon dioxide. Each 4 hr. fraction of the CO₂ absorbed in the NaOH was converted into BaCO₃ and the radioactivity measured as described by Calvin *et al.* (1949). Radioactivity was detected in trace amounts in the expired CO₂ only on the second day after dosing and in 3 or 4 days amounted to less than 0.4% of the dose.

Aniline in the expired air. The aniline eliminated in the expired air was estimated spectrophotometrically with a Unicam spectrophotometer SP. 500. The selected u.v.-absorption maximum of aniline in ethanol was 233 mµ, where ϵ was 11 500 (slit width 0.5 mm.). By this means it was shown that the amount of aniline excreted in the expired air was less than 0.02% of the dose/day. Moreover it is unlikely that any other metabolite of aniline was eliminated in the expired air since the absorption spectra of the ethanolic solutions showed no significant maxima, and the radioactivity of the ethanol corresponded to less than 0.05% of the dose/day.

Aniline in the urine. (i) Free aniline was estimated by adding aniline-HCl (250 mg.) to a portion of the urine (5–10 ml.), adjusting the solution to pH 8.0 with NaHCO₃ and extracting the aniline with ether (2 × 10 ml.). The ethereal extracts were washed with water and the aniline was extracted into 2N-HCl (10 ml.). The acid extract was made alkaline (pH 10) with 2N-NaOH and steam-distilled, and the steam-distillate treated with acetic anhydride and 2N-NaOH to yield acetanilide. This was filtered off, washed with water and recrystallized from water to constant specific radioactivity (m.p. and mixed m.p. 114°). (ii) Total aniline was estimated by refluxing a portion of the urine (5–10 ml.) with aniline (250 mg.) and 0.5 vol. of conc. HCl for 3 hr. The solution was made alkaline with 2N-NaOH, steam-distilled and the aniline in the distillate was converted into acetanilide and recrystallized to constant specific radioactivity as before.

Aniline conjugates. The urines were examined for aniline-N-glucuronide, phenylsulphamic acid, acetanilide, formanilide, succinanilide and succinanic acid. Acylations other than acetylation were considered since the transfer of formyl groups is well known (Huennekens, Osborn & Whiteley, 1958), and acyl esters of coenzyme A other than acetyl have been demonstrated (see Williams, 1959).

Aniline-N-glucuronide. Aniline glucuronide (1 g.) was added to a portion of the urine (about 5 ml.), an equal volume of ethanol was added and the mixture was heated to dissolve the glucuronide. On cooling, the glucuronide separated and this was repeatedly recrystallized from aq. ethanol to constant specific radioactivity (m.p. 122°, decomp.). The pure aniline glucuronide was then hydrolysed by refluxing with 2N-H₂SO₄ (10 ml.) for 0.5 hr., and the aniline was steam-distilled, after it was made alkaline with 2N-NaOH, and converted into acetanilide (m.p. and mixed m.p. 114°) as described previously.

Phenylsulphamic acid. Barium phenylsulphamate (2 g.) was dissolved in 10 ml. of ice-cold N-HCl, a portion of the urine (5–10 ml.) was then added and the solution was adjusted to pH 8.0 by dropwise addition of ammonia solution (sp.gr. 0.88). The solution was heated, filtered hot and cooled to allow the barium phenylsulphamate to crystallize. The barium salt was filtered off, washed with cold ethanol and recrystallized twice from water, then repeatedly from aq. ethanol (1:1) until the specific radioactivity was constant. The purified barium phenylsulphamate was then

hydrolysed by refluxing in 2*N*-HCl (10 ml.) for 0.5 hr. and the aniline was steam-distilled and converted into acetanilide (m.p. and mixed m.p. 114°) as previously described.

Anilides. Acetanilide, formanilide, succinanilide or succinanic acid (250 mg.) dissolved in 10 ml. of hot water was added to a portion of the urine (10 ml.). After 1 hr. the mixture was extracted three times with 20 ml. portions of ether (acetanilide and formanilide) or CHCl_3 (succinanilide and succinanic acid), the extracts were bulked, washed with 2*N*-HCl and then water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residues were recrystallized to constant specific radioactivity; acetanilide from water (m.p. and mixed m.p. 114°), formanilide from light petroleum (b.p. 60–80°) (m.p. and mixed m.p. 50°), succinanilide from ethanol (m.p. and mixed m.p. 230°) and succinilic acid from ethanol and then from water (m.p. and mixed m.p. 145°).

Aminophenols. *o*- and *p*-Aminophenol were estimated as described by Parke (1956). *m*-Aminophenol (500 mg.) was added to a portion of the urine (10–50 ml.), which was then refluxed with an equal volume of conc. HCl for 3 hr. The solution was cooled, adjusted to pH 8.0 with solid NaHCO_3 and extracted three times with an equal volume of ether. The ethereal extracts were bulked, washed with a little water and the *m*-aminophenol was transferred into 20 ml. of 2*N*- H_2SO_4 . The acid solution was again adjusted to pH 8.0 with NaHCO_3 and extracted three times with an equal volume of ether; the ethereal extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue of *m*-aminophenol was dissolved in 2 ml. of ethanol and filtered to remove the less soluble *o*- and *p*-aminophenol. The solution was then applied to Whatman no. 1 paper and chromatographed in wet diisopropyl ether (see Table 1); the *m*-aminophenol band, R_F 0.60, was cut out and eluted with acetone. The eluate was evaporated to dryness and the residue benzoylated to yield the *ON*-dibenzoyl derivative of *m*-aminophenol, which was recrystallized from ethanol, toluene and carbon tetrachloride to constant specific radioactivity and had m.p. and mixed m.p. 156°.

Acetamidophenols. *o*- or *p*-Acetamidophenol (250 mg.), dissolved in 10 ml. of hot water, was added to 10 ml. of urine. The mixture was continuously extracted with ether for 6 hr. and the ethereal solution washed with *N*-HCl, *N*- Na_2CO_3 and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue of *o*-acetamidophenol, m.p. and mixed m.p. 203°, was recrystallized from ethanol and then methanol to constant specific radioactivity. The residue of *p*-acetamidophenol, m.p. and mixed m.p. 168°, was recrystallized from aq. ethanol to constant specific radioactivity.

was recrystallized from aq. ethanol to constant specific radioactivity.

***p*-Aminophenylglucuronide.** The glucuronide (1 g.) dissolved in 5 ml. of saturated NaHCO_3 soln. was added to a portion of urine (5–10 ml.). The mixture was then acidified to pH 4.0 with 2*N*-HCl and the precipitated *p*-aminophenylglucuronide was filtered off, washed with water, dissolved in saturated NaHCO_3 and reprecipitated at pH 4.0. The glucuronide was finally recrystallized from water (charcoal) to constant specific radioactivity and had m.p. and mixed m.p. 215°.

***p*-Acetamidophenylglucuronide.** The glucuronide (1 g.) was dissolved in a portion of the urine (5–10 ml.), which was then evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in 10 ml. of 95% ethanol, the solution was filtered, treated with 1 ml. of benzylamine and then diluted with 100 ml. of ethyl acetate. After being left at 0° for 1 hr. the crystalline precipitate of the benzylamine salt of *p*-acetamidophenylglucuronide was filtered off and was recrystallized from 95% ethanol to constant specific radioactivity (m.p. 196°).

Dihydroxyanilines. 3-Aminocatechol-HCl, 4-amino-catechol-HBr, 2-, 4- and 5-aminoresorcinol-HCl and aminoquinol-HCl (500 mg. each) were added separately to portions of the urine (5–10 ml.), which were refluxed for 3 hr. with an equal volume of conc. HCl and then evaporated to dryness *in vacuo*. The residue of 3-aminocatechol-HCl was refluxed with 3 g. of benzoyl chloride for 1 hr., and the product, the benzoyl derivative of 7-hydroxy-2-phenylbenzoxazole, was distilled, the fraction above 300° being collected. This was then refluxed for 1 hr. with 5 ml. of ethanolic 10% KOH, and the ethanol was distilled off. The residue was washed with water and the insoluble 7-hydroxy-2-phenylbenzoxazole was filtered off and recrystallized from ethanol and then benzene to constant specific activity (m.p. and mixed m.p. 192°). The residues of 4-aminocatechol and 2-aminoresorcinol were redissolved in water (2–5 ml.) and treated with 1 equiv. of benzoyl chloride and excess of sodium acetate to give the *N*-benzoyl derivatives, 4-benzamidocatechol, m.p. and mixed m.p. 169°, and 2-benzamidoresorcinol, m.p. and mixed m.p. 190°, which were recrystallized from ethanol and aq. ethanol to constant specific radioactivity. The residue of 4-aminoresorcinol was dissolved in 5 ml. of water and treated with excess of benzoyl chloride and NaOH to give 4-benzamidoresorcinol dibenzoate, which was recrystallized from light petroleum (b.p. 60–80°) (m.p. and mixed m.p. 172°). The residue of 5-aminoresorcinol was refluxed for 2 hr. with 2 ml. of acetic

Table 1. R_F values and colour reactions on paper of aminophenols

R_F values are for descending chromatography on Whatman no. 1 paper in diisopropyl ether saturated with water, run for 6 hr. The diisopropyl ether was washed with a saturated aq. soln. of KMnO_4 , a saturated aq. soln. of FeSO_4 , aq. 2*N*- Na_2CO_3 and then water, and it was dried over anhydrous Na_2SO_4 and redistilled (b.p. 68°). Detecting reagents used were Gibb's reagent (consisting of ethanolic 2% soln. of 2:6-dichloroquinonechloroimide followed by 2*N*- Na_2CO_3) and diazo reagent consisting of a 1% soln. of NaNO_2 in 0.5*N*-HCl followed by a 1% soln. of dimethyl- α -naphthylamine in ethanol and then 2*N*- Na_2CO_3 .

	R_F	Colour reactions		
		Gibb's reagent	Diazo reagent	
			Acid	Alkaline
<i>o</i> -Aminophenol	0.79	Blue	Yellow	Green
<i>m</i> -Aminophenol	0.60	Violet	Red	Yellow
<i>p</i> -Aminophenol	0.38	Blue-brown	Violet	Green

anhydride and the product of 5-acetamidoresorcinol diacetate (m.p. and mixed m.p. 121°) was recrystallized from benzene and then water (charcoal). The residue of aminoquinol-HCl was dissolved in 5 ml. of water, the solution was filtered and adjusted to pH 8.0 with 2N-NH₃ soln. to precipitate the free aminoquinol, which was filtered off and redissolved in 2N-HCl. This process was repeated except that the free aminoquinol was finally redissolved in the least quantity of hot 2N-H₂SO₄. On cooling, aminoquinol sulphate separated and this was filtered off and redissolved in 5 ml. of water and acetylated with 1 equiv. of acetic anhydride and excess of sodium acetate. The product was extracted with ether, the ethereal solution was dried over anhydrous Na₂SO₄ and evaporated to dryness to give acetamidoquinol, m.p. and mixed m.p. 100°, which was recrystallized from ether to constant specific activity.

Other possible metabolites

Oxidation products. Aniline is oxidized to nitrosobenzene when it is injected intravenously into dogs (Kiese, 1959*a, b*). Phenylhydroxylamine is probably an intermediate in this oxidation and could react with the nitrosobenzene to form azoxy-, azo- and hydrazo-benzene. Further oxidation to nitrobenzene would give rise to *m*- and *p*-nitrophenol (Robinson, Smith & Williams, 1951). The urines were therefore examined for *m*- and *p*-nitrophenol, nitrosobenzene, azoxybenzene, azobenzene and benzidine, since hydrazobenzene forms benzidine on treatment with acids, by the isotope-dilution techniques previously described (Parke, 1956). None of these compounds was found to entrain radioactivity.

Deamination products. The deamination of aromatic amines is unknown, and oxidative deamination of aniline by amine oxidase or by the rat-liver microsomal deaminase (Axelrod, 1955) is most improbable. However, hydrolytic deamination as occurs in the conversion of adenine into hypoxanthine or guanine into xanthine, or reductive deamination similar to that which occurs with mepacrine (Scudi & Jelinek, 1944), might occur with aniline, giving rise to phenol or benzene respectively. The urines were therefore examined for phenol and quinol, the principal metabolites of benzene, by the isotope-dilution procedures previously described (Parke & Williams, 1953). Neither of these compounds was found to entrain radioactivity.

Methylation products. *N*-Methylation is a minor metabolic pathway of the tertiary aromatic bases, pyridine (Tomita, 1921), quinoline and isoquinoline (Tamura, 1924). Methylation of a primary aromatic amino group occurs in the metabolism of 4'-fluoro- and 3'-methyl-aminoazobenzene, which, to a small extent, are converted into their *N*-monomethyl derivatives (Miller & Miller, 1952). It was therefore desirable to examine the urines for the *N*-methylation products, methylaniline and dimethylaniline.

Methylaniline (500 mg.) was added to a portion of the urine, which was then refluxed for 3 hr. with 0.5 vol. of conc. HCl; the hydrolysed solution was made alkaline with 5N-NaOH and steam-distilled. The steam-distillate (10 ml.) was acidified with 5 ml. of 2N-HCl and diazotized at 0° by addition of NaNO₂ (0.5 g. in 2 ml. of water). The oily nitrosoamine which separated was extracted into ether (three portions of 10 ml.), the extract was washed with water to remove the benzenediazonium sulphate and then dried over anhydrous Na₂SO₄. Evaporation of the ethereal solution gave the *N*-methyl-*N*-nitrosoaniline, which was

converted into methylaniline by warming with 5 ml. of a solution of Cu₂Cl₂ in conc. HCl (17.5%, w/v) (Jenes & Kenner, 1932). The resulting mixture was diluted with water (10 ml.) and the white precipitate of the copper salt of methylaniline was removed by filtration, washed with a little 2N-HCl, suspended in water and treated with an excess of 2N-NaOH. The methylaniline which was liberated was steam-distilled and the toluene-*p*-sulphonate was prepared, which after recrystallization from ethanol contained no radioactivity (m.p. and mixed m.p. 95°).

Dimethylaniline (500 mg.) was added to a portion of the urine and refluxed for 3 hr. with 0.5 vol. of conc. HCl. The hydrolysed solution was made alkaline with 5N-NaOH and steam-distilled. The dimethylaniline in the steam-distillate was extracted into ether (three portions of 20 ml.), the extract was dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue was dissolved in 5 ml. of 5N-HCl and treated with a solution of 0.5 g. of NaNO₂ in 1 ml. of water. The *p*-nitrosodimethylaniline hydrochloride which separated was filtered off, washed with a little 2N-HCl and suspended in 10 ml. of *N*-NaOH. The free base which was liberated was collected in ether, the ethereal solution was dried over anhydrous MgSO₄ and evaporated to dryness. The residue of *p*-nitrosodimethylaniline had m.p. and mixed m.p. 85° after recrystallization from aq. ethanol and was not radioactive.

Mercapturic acids. Aromatic amines are not known to form mercapturic acids, but the urines were nevertheless examined for the presence of *p*-aminophenylmercapturic acid, *p*-aminothiophenol being used for the isotope-dilution technique, since it was assumed that if aminophenylmercapturic acid was present it would be hydrolysed by alkali to the aminothiophenol. 4,4'-Diaminodiphenyl disulphide (500 mg.) was reduced to *p*-aminothiophenol by refluxing for 15 min. in 10 ml. of ethanol with 2 g. of KOH. A portion of the urine (5–10 ml.) was then added and the mixture was refluxed for a further 1 hr. The solution was then diluted with 10 ml. of water and the ethanol rapidly removed by distillation. After adjusting to pH 6.0 with 2N-H₂SO₄ the solution was steam-distilled and the aqueous distillate of *p*-aminothiophenol was oxidized by the addition of 1 ml. of 20 vol. H₂O₂ and 2 ml. of 2N-NaOH. The product, 4,4'-diaminodiphenyl disulphide, was filtered off and recrystallized from ethanol until finally free from radioactivity (m.p. and mixed m.p. 80°). The rabbit urines were also examined for the presence of mercapturic acids by refluxing for 1 hr. with 0.2 vol. of 10N-NaOH to hydrolyse mercapturic acids to substituted thiophenols. The hydrolysed urines were then adjusted to pH 6.0 with 10N-H₂SO₄ and steam-distilled. The distillates contained no appreciable thiophenol, as determined by iodometric titration, and oxidation with H₂O₂ did not yield any diphenyl disulphide.

Chromatographic detection of metabolites

Aminophenols. The urine from a rabbit dosed with [¹⁴C]-aniline (Expt. 3) which remained after the isotope-dilution experiments, was hydrolysed by refluxing with an equal volume of conc. HCl for 3 hr. The hydrolysed solution was cooled, adjusted to pH 8.0 with Na₂CO₃ and continuously extracted with ether for 8 hr. The ethereal extract was dried over anhydrous Na₂SO₄ and chromatographed on paper in wet diisopropyl ether (see Table 1). Radioautographs were subsequently developed from the chromatogram before detection of the metabolites by colour tests. The chromato-

Table 2. *R_F* values and colour reactions on paper of *N*-benzoyl derivatives of aminophenols

R_F values are for descending chromatography on Whatman no. 1 paper. Solvent mixtures: A, CHCl₃-formic acid-methanol-water (1000:4:100:96, by vol.) (Reio, 1958) run for 8 hr.; B, benzene-ethyl methyl ketone-formic acid-water (900:100:2:98, by vol.) (Reio, 1958) run for 6 hr. Detecting reagents used were: Gibb's reagent (see Table 1); aq. 0.5% FeCl₃ followed by saturated NaHCO₃ (pH values are in parentheses); aq. 0.2% soln. of Brentamine Fast Blue 2B salt followed by aq. 2*N*-NH₃ soln.; aq. 0.2% soln. of Brentamine Fast Garnet GBC salt followed by aq. 2*N*-NH₃ soln.

Benzoylated aminophenol	<i>R_F</i>		Colour reactions			
	A	B	Gibb's reagent	FeCl ₃	Brentamine Fast Blue 2B salt	Brentamine Fast Garnet GBC salt
<i>o</i> -Aminophenol	0.93	0.96	Blue	Brown	Yellow	Yellow
<i>m</i> -Aminophenol	0.81	0.89	Violet	None	Orange	Red
<i>p</i> -Aminophenol	0.85	0.98	None	None	Violet	Violet
3-Aminocatechol	0.90	0.96	Blue	Violet (7) Violet (9)	Yellow	Yellow
4-Aminocatechol	0.33	0.46	Blue	Green (7) Violet (9)	Violet	Violet
2-Aminoresorcinol	0.87	0.87	Violet	Pink (7) Buff (9)	Red	Red
4-Aminoresorcinol	0.44	0.80	Violet	Orange (7) Violet (9)	Violet	Red
5-Aminoresorcinol	0.01	0.02	None	None	Violet	Red
Aminoquinol	—	—	None	None	None	None

Table 3. *R_F* values and colour reactions on paper of aniline conjugates

R_F values are for descending chromatography on Whatman no. 1 paper in solvent mixture C, ethanol-butan-1-ol-aq. 3*N*-NH₃ soln./3*N*-ammonium carbonate buffer (11:40:19, by vol.) (cf. Fewster & Hall, 1951) run for 16 hr. Detecting reagents were used after hydrolysis by spraying with *N*-HCl and heating at 100° for 10 min. Aniline was detected with diazo reagent (see Table 1); sulphate was detected with an aq. 0.1% soln. of BaCl₂ followed by an aq. 0.1% soln. of rhodizonic acid sodium salt (sulphate gives a white spot on a red background); glucuronic acid was detected by spraying with a 1% soln. of naphtharesorcinol in aq. 20% trichloroacetic acid and heating the paper at 100° for 20–30 min. (glucuronic acid gives a blue spot on a pink background).

Aniline conjugates	<i>R_F</i>	Colour reactions		
		Diazo reagent acid	Naphtha-resorcinol	Rhodizonic acid
Aniline- <i>N</i> -glucuronide	0.09	Violet	Blue	None
<i>p</i> -Aminophenyl glucuronide	0.02	Brown	Brown	None
Glucuronic acid	0.02	None	Blue	None
Phenylsulphamic acid	0.26	Violet	None	White
Acetanilide	0.83	Violet	None	None
Aniline*	0.87	Violet	None	None

* Volatile, and therefore only detected when amounts of 200 μg. or more are used.

grams showed six radioactive spots which were identified by *R_F* values and colour reactions as *p*-aminophenol (*R_F* 0.39, 50% of dose), *m*-aminophenol (*R_F* 0.58, 0.1% of dose), *o*-aminophenol (*R_F* 0.83, 5% of dose), polymeric oxidation products of aminophenols (*R_F* 0.00, 10% of dose) and unidentified spots at *R_F* 0.89 (yellow, 0.2% of dose) and *R_F* 0.95 (pink, 0.1% of dose). Both spots changed to blue-green on spraying with Gibb's reagent. The same spots were also found in chromatograms and radioautographs of ethereal extracts of the hydrolysed urine collected from dogs that had been dosed with [¹⁴C]aniline.

Dihydroxyanilines. Urine from a rabbit dosed with [¹⁴C]aniline (Expt. 6) which remained from isotope-dilution experiments was hydrolysed with conc. HCl as

before and then evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml. of water, treated with 1 equiv. of benzoyl chloride and an excess of sodium acetate and evaporated to dryness *in vacuo* at room temperature. (Free dihydroxyanilines were too susceptible to atmospheric oxidation to permit chromatography.) The residue was extracted with ethanol and the extract chromatographed on paper in solvents A and B (see Table 2). Spots corresponding to *o*- and *p*-aminophenol only were seen, and the aminocatechols and aminoresorcinols were not present in amounts greater than 0.05% of the dose.

Aniline conjugates. The urine from a rabbit that had been dosed with aniline (250 mg./kg.) was collected, for the first day only, in a flask containing 1 ml. of aq. *N*-NH₃ soln. to

prevent decomposition of aniline conjugates. One-half of the urine (pH 8.0) was treated with an excess of a saturated aq. soln. of basic lead acetate, and the glucuronides were separated as described by Kamil, Smith & Williams (1951). This glucuronide fraction was dissolved in ethanol and chromatographed on paper in solvent C (see Table 3). The other half of the urine was treated with 20 ml. of an aq. 10% (w/v) soln. of $\text{Ba}(\text{OH})_2$, the excess of barium was removed with solid CO_2 and the solution was filtered and concentrated to 10 ml. *in vacuo*. The concentrated solution was then treated with 50 mg. of $(\text{NH}_4)_2\text{SO}_4$, the BaSO_4 was filtered off and the filtrate (the phenylsulphamate fraction) was chromatographed on paper in solvent C. Both fractions showed spots corresponding to glucuronic acid (R_f 0.02), aniline-*N*-glucuronide (R_f 0.08, equivalent to 5% of the dose) and phenylsulphamic acid (R_f 0.26, 10% of dose).

RESULTS AND DISCUSSION

The quantitative results for the metabolism of aniline in the rabbit are shown in Table 4. In Expts. 3, 6 and 7 some 80–100% of the dose of radioactivity was accounted for. In 3–8 days after oral dosage with [^{14}C]aniline an average of 70% of the radioactivity is eliminated from the body in the urine: 1% is in the faeces, less than 0.5% is in the expired air and about 5% or more remains in the body, from which it is slowly eliminated via the urine and respiratory carbon dioxide. The urinary excretion of metabolites is fairly rapid; in Expt. 3

over 80% was eliminated in the first 12 hr. after dosing (see Fig. 1); it is usually complete in the first 2 days, but the urines are still slightly radioactive after even 8 days. The radioactivity remaining in the carcass (see Table 5) was evenly distributed throughout the tissues except for a slightly higher concentration in the spleen and fat, and in one case in the gut contents.

Expired air. The radioactivity eliminated in the expired air was very small. Aniline could not be detected within the limits of the method (less than 0.02% of the dose/day) and no other organic metabolites were present. Radioactive carbon dioxide was present in the expired air on the second and subsequent days after dosing, but this amounted to less than 0.4% of the dose in 3 or 4 days (see Fig. 1), although at the termination of the experiments the excretion of $^{14}\text{CO}_2$ was still maximal at about 0.2% of the dose/day. Since aniline (b.p. 184°) itself is not excreted in the expired air, it is unlikely to occur in the free state in the blood, for iodobenzene (b.p. 189°) and 1:3:5-trichlorobenzene (b.p. 209°) after oral administration are exhaled in the breath of rabbits to the extent of 3% and 10% of the doses respectively (Azouz, Parke & Williams, 1952; Parke & Williams, 1960).

Metabolites in urine. The metabolites of aniline excreted by the rabbit are shown in Table 4; *p*-

Table 4. *Elimination of metabolites by rabbits receiving [^{14}C]aniline*

Doses were given orally. Metabolites were determined in bulked urines collected for 3 days. Tissues were examined after 3, 5 and 8 days in Expts. 3, 6 and 7 respectively. See Table 7 for Expts. 8–10. The s.e.m. is given in parenthesis.

Expt. no.	...	...	1	2	3	4	5	6	7	
Dose of aniline (mg./kg.)	...	...	160	200	200	250	250	250	500	
Dose of ¹⁴ C (μC/animal)	...	...	9	34	35	41	15	67	109	
Duration of expt. (days)	...	...	3	3	3	6	4	5	8	
	Percentage of dose									Average of 10 expts.
Respiratory CO ₂	—	—			0.4	—	—	< 0.2	< 0.2	
Aniline (total)	1.2	10.8			19.2*	11.7	6.5	14.5*	4.3*	8.2 (1.8)
Aniline (free)	0.8	2.5			6.4	1.6	—	—	1.0	1.8 (0.7)
Phenylsulphamic acid	—	7.6			8.9	8.2	—	8.0	2.4	5.4 (1.0)
Aniline- <i>N</i> -glucuronide	—	4.6			10.8	3.3	—	4.2	1.9	3.5 (1.3)
Acetanilide	0.07	0.2			0.2	—	0.3	0.1	0.3	0.2 (0.03)
<i>o</i> -Aminophenol (total)	5.5	10.1			11.0	9.6	8.4	12.8	9.8	9.1 (0.7)
<i>m</i> -Aminophenol (total)	< 0.1	< 0.1			0.16	—	< 0.1	0.06	0.09	0.1 (0.03)
<i>p</i> -Aminophenol (total)	43	58			66	56	46	47	56	51 (2.6)
<i>p</i> -Aminophenylglucuronide	—	—			9.0	—	—	—	22.2	—
<i>p</i> -Acetamidophenylglucuronide	—	—			17.2	—	—	—	24.6	—
Total metabolites in urine	50	81			97	77	61	72	71	68 (4.7)
Radioactivity in urine	60	80			90	73	69	77	73	70 (4.1)
Radioactivity in faeces	—	—			0.7	—	—	1.1	1.5	—
Radioactivity in tissues	—	—			3.0	—	—	6.3	6.7	—
Total accounted for	—	—			94	—	—	85	81	—

* Less than 0.2% in the expired air.

Table 5. Occurrence of radioactivity in the tissues of rabbits receiving [14C]aniline

Expt. no.	6			7		
	67			109		
Dose of 14C (μC)	5			8		
	8			8		
Time of death (days after dosing)	Wt. of wet tissue (g.)	Specific activity of tissue (μC/g.)	Percentage of dose present in tissue	Wt. of wet tissue (g.)	Specific activity of tissue (μC/g.)	Percentage of dose present in tissue
Lungs	15	0.001	0.02	14	0.001	0.01
Liver	70	0.001	0.10	53	0.003	0.15
Kidney	11	0.002	0.03	12	0.004	0.05
Heart	7	< 0.001	< 0.01	7	< 0.001	< 0.01
Spleen	2	0.005	0.02	1.5	0.004	0.01
Blood	350	0.002	1.1	350	0.003	1.0
Muscle, voluntary	700	0.001	1.1	750	0.001	0.8
Muscle, involuntary	200	0.001	0.3	200	0.001	0.2
Fat	470	0.004	2.8	550	0.005	2.5
Brain	35	< 0.001	< 0.05	40	0.002	0.8
Ovary	1	0.001	< 0.01	1	< 0.001	< 0.01
Bile	—	—	—	1	< 0.001	< 0.01
Stomach contents	105	0.002	0.3	70	0.005	0.4
Intestinal contents (small intestine)	130	0.002	0.4	210	0.004	0.8
Intestinal contents (caecum)	60	0.002	0.2			
Faeces	75	0.010	1.1	60	0.027	1.5
Total in tissues and faeces	—	—	7.4	—	—	8.2
Total in expired air	—	—	< 0.4	—	—	< 0.4
Total in urine	—	—	77	—	—	73
Total accounted for	—	—	85	—	—	81

Table 6. Elimination of metabolites in the urines of dogs receiving [14C]aniline orally

Expt. no.	1	2
Dose of aniline (mg./kg.)	175	200
Dose of 14C (μC/animal)	12.5	15
Duration of expt. (hr.)	16*	41*
Aniline (total)	2.8	12.8†
Aniline (free)	0.4	8.9†
Acetanilide	< 0.1	< 0.1
o-Aminophenol	10.7	25.2
m-Aminophenol	< 0.1	< 0.1
p-Aminophenol	7.1	11.3
Sum of metabolites	20.6	49.3
Total radioactivity in urine	18.6	48.5

* Both animals showed symptoms of methaemoglobin-aemia and were killed when distressed.
† Some of this was from vomit.

aminophenol accounts for 51 % of the dose. Other metabolites are o-aminophenol (9 %), phenylsulphamic acid (5.5 %), aniline-N-glucuronide (3.5 %), m-aminophenol (0.1 %) and acetanilide (0.2 %). 4-Aminoresorcinol, which was tentatively suggested by Smith & Williams (1949) as a metabolite of aniline, was not found, and none of the other dihydroxyanilines was detected. No anilide other than acetanilide was present and no evidence of methylation or deamination was obtained. There was no evidence of the formation of a mercapturic acid since the isotope-dilution procedure for p-

Fig. 1. Excretion of aniline (●) and CO₂ (○) in the expired air, and total radioactivity (○) in the urine, of rabbit no. 3 dosed orally with [14C]aniline (200 mg./kg.).

aminothiophenol was negative, and steam-distillation of alkali-hydrolysed urines did not yield any thiophenols. No evidence of nitrosobenzene, azoxybenzene, azobenzene or benzidine was found in the urines, but two unidentified metabolites, equivalent to about 0.1 and 0.2 % of the dose, were found by paper chromatography in the urines of both rabbits and dogs. The same two compounds have also been found in chromatograms of the urines of rabbits dosed with nitrosobenzene and with azoxybenzene (D. Robinson, personal communication) and this therefore gives some support to the claim of Kiese (1959a, b) that aniline is oxidized to nitroso-

benzene. The metabolites of aniline in dog urine have also been estimated (Table 6); 4-amino-resorcinol, phenol, quinol, methylaniline and dimethylaniline were not metabolites.

Aminophenol conjugates. The metabolism of the aminophenols in rabbits was studied by Bray, Clowes & Thorpe (1952*a*), who showed that 4% and 25% of a dose (about 400 mg./kg.), of *o*- and *p*-aminophenol respectively, were excreted as free acetamidophenols. In contrast to this, no free acetamidophenols were found in the urine of rabbits dosed with aniline. Bray *et al.* (1952*a*) also found that 61% of the total *p*-aminophenol was excreted as glucuronides, 45% as *p*-aminophenylglucuronide and 16% as *p*-acetamidophenylglucuronide. In the present experiments the fraction of the total *p*-aminophenol which was excreted as glucuronides varied with the dose. At an oral dose of aniline of 200 mg./kg. rabbits yield 9% of *p*-aminophenylglucuronide and 17% of *p*-acetamidophenylglucuronide, which are equivalent to 14% and 26% of the total *p*-aminophenol. At a dose of 500 mg./kg. the corresponding figures are *p*-aminophenylglucuronide 22% and *p*-acetamidophenylglucuronide 25%, equivalent to 40% and 44% of the total *p*-aminophenol. This is in agreement with Smith & Williams (1949), who, although unable to estimate precisely the total amounts, found by isolation approximately equal amounts of both glucuronides in the urine of rabbits dosed with aniline. This increase of *p*-aminophenylglucuronides with increased dose is in agreement with the increased

excretion of glucuronide conjugate at high doses (see Table 7). The principal fact that emerges from this table is that the percentage conjugation with sulphuric acid decreases as the dose increases so that the total amount of aniline metabolites conjugated by this mechanism is approximately constant at 40–70 mg./kg. Glucuronide conjugation on the other hand increases at higher doses. These findings might have been expected from the kinetic studies of Bray, Humphris, Thorpe, White & Wood (1952*b*), who found that the conjugation of phenols with glucuronic acid is a first-order reaction whereas conjugation with sulphuric acid is limited by the availability of sulphate precursors.

Excretion of free glucuronic acid. At all doses the total conjugates are in excess of the total radioactivity in the urine (Table 7), particularly at the toxic dose of 500 mg./kg., when the total conjugates are two to three times as great as the total metabolites. This is probably due to the excretion of free glucuronic acid, which was detected in paper chromatography of the urines, and was in fact isolated by Smith & Williams (1949) from the urines of rabbits dosed with aniline. Free glucuronic acid has been demonstrated in the urines of rabbits dosed with hexachlorobenzene (Parke & Williams, 1960), and in the urines of rats, guinea pigs and dogs dosed with barbital (Burns, Evans & Trousof, 1957), and is probably due to a toxic effect on carbohydrate metabolism. This excretion of free glucuronic acid in aniline urine is responsible for the reducing action of the urines upon Benedict's solu-

Table 7. *Excretion of conjugates and principal metabolites in the urine of rabbits receiving [¹⁴C]aniline at varying oral doses*

The figures for conjugates are the means for three animals; ranges are in parentheses. The figures for metabolites were obtained by isotope-dilution experiments on one animal only.

Dose of aniline (mg./kg.)	100	300	500*
Dose of ¹⁴ C (μc/animal)	2.9	2.6	2.4
Duration of expt. (days)	5	5	3–5
	Percentage of dose		
Glucuronic acid (G)	64 (51–87)	68 (44–86)	122 (98–156)†
Ethereal sulphates‡ (E)	41 (26–50)	17 (10–25)	14 (14–16)
Total conjugates (E + G)	105 (101–113)	85 (69–101)	136 (112–170)
Total radioactivity in urine	80 (77–85)	63 (54–77)	50 (45–60)
Expt. no.	8	9	10
Aniline (total)	4.4	5.0	3.6
Aniline (free)	0.3	1.2	0.5
Aniline- <i>N</i> -glucuronide	0.5	1.8	1.1
Phenylsulphamic acid	3.2	2.8	2.2
Acetanilide	0.2	0.2	0.3
<i>o</i> -Aminophenol	8.5	8.5	6.5
<i>p</i> -Aminophenol	52	46	38
Total metabolites in urine	64 (78)§	59 (58)§	48 (45)§

* All animals died from 3 to 5 days after dosing.

† These urines reduced Benedict's solution.

‡ Including phenylsulphamate.

§ Figures in parentheses are for the total radioactivity in the urine.

tion which was observed by Smith & Williams (1949), and was thought to indicate the presence of a labile glucuronide of aniline. In the present experiments, the urines of rabbits dosed with aniline showed a marked reduction of Benedict's solution only at the higher doses (400–500 mg./kg.), i.e. doses at which there is a marked excretion of free glucuronic acid.

Aniline conjugates. The urines of rabbits dosed with aniline contain an average of 8 % of the dose as phenylsulphamic acid (5·5 %) and aniline-*N*-glucuronide (3·5 %). Since one-half of the total aniline of the ammonium salt of aniline-*N*-glucuronide is labile at pH 8·0 (Axelrod, Inscoe & Tomkins, 1958) the estimate of 'free' aniline should be equivalent to half the *N*-glucuronide if this is present in urine as either the ammonium or potassium salt. This is found to be approximately true (see Table 4). The biological syntheses of both aniline conjugates have been effected *in vitro*. Roy (1958) demonstrated the synthesis of phenylsulphamate from aniline by enzyme preparations of guinea-pig and rabbit livers in the presence of adenosine triphosphate and sulphate; and Axelrod *et al.* (1958) demonstrated the transfer of glucuronic acid from uridine diphosphate glucuronic acid to aniline by a microsomal enzyme of guinea-pig liver to yield an *N*-glucosiduronic acid which was identical with the aniline-*N*-glucuronide described in this paper. The acetylation of aniline to acetanilide has been shown to occur in the rabbit, but only a very small amount of acetanilide (0·2 % of dose) was found, which is in keeping with the findings of Smith & Williams (1948) that acetanilide is extensively metabolized to *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid.

m-Aminophenol. The *m*-aminophenol may arise from direct hydroxylation or from a premercapturic

acid, e.g. *N*-acetyl-*S*-(4-amino-1:2-dihydro-2-hydroxy-1-phenyl)cysteine by elimination of *N*-acetylcysteine (see Knight & Young, 1958; Parke & Williams, 1958). The excretion of traces of the *meta* isomer as a metabolite of an aromatic compound with an *ortho/para*-directing substituent is of particular interest since Gessner & Smith (1960) have shown that in addition to *o*- and *p*-chlorophenol, *m*-chlorophenol also occurs in the urine of rabbits dosed with chlorobenzene.

Ratio of p- to o-aminophenol in different species. The ratio of the total *p*-aminophenol to total *o*-aminophenol was determined in several animal

Fig. 2. Ratio of *p*- to *o*-aminophenol excreted in the urines of rabbits and dogs receiving [¹⁴C]aniline orally. (A) Rabbit 3 (dose, 200 mg./kg.); (B) rabbit 5 (dose 250 mg./kg.); (C) dog 1 (dose 175 mg./kg.); (D) dog 2 (dose, 200 mg./kg.). The mean *para/ortho* ratios on the bulked urines were: 6·0 for (A), 5·5 for (B), 0·66 for (C) and 0·45 for (D).

Table 8. *Metabolic hydroxylation of aniline by various animal species*

Species	Sex	Oral dose (mg./kg.)	Percentage of dose in 2 days*				Ratio* <i>para/ortho</i>	Lethal dose† (g. of aniline/ kg. body wt.)
			Total metabolites	Aminophenol		Aniline (total)		
				<i>ortho</i>	<i>para</i>			
Gerbil	F	250	57	3·2	48	5	15 (12–17) ³	—
Guinea pig	F	250	55	4·2	46	4·5	11 (9–12) ³	2·5
Golden hamster	F	250	64	5·5	53	4·5	10 (9–11) ³	—
Rabbit	F	160–500	71	8·8	50	9	6 (4–9) ¹⁰	1·0
Rat	M	250	52	7·2	42	3	6 (5–6) ³	—
	F	250	71	19	48	3·5	2·5 (2–3) ³	—
Chicken	F	50	61	10·5	44	4	4 (4) ²	—
Mouse‡	M	250	20	4	12	3	3 (2–4) ³	—
Ferret	F	250	61	26	28	3	1 (1) ²	—
Dog‡	F	200	33	18	9	8	0·5 (0·4, 0·6)	0·5
Cat‡	F	200	41	32	14	4	0·4 (0·4, 0·5)	0·2

* Figures are mean values; range is given in parenthesis and the number of expts. as indices.
† See Spector (1956).
‡ Animals died or were killed during the first day after dosing.

species with [^{14}C]aniline and was found to vary from 15:1 in the gerbil to 0.4 in the cat (see Table 8). It is significant that in the hydroxylation *in vivo* with liver microsomes of dog, which produces approximately equal amounts of *ortho* and *para* isomers, both isomers were obtained, whereas with liver microsomes from rabbit, which produces an excess of the *para* isomer, only *p*-aminophenol was detected. This increase of *ortho* hydroxylation in the series of animals from gerbil to cat approximately parallels the increase in toxicity of aniline, which is possibly due to *o*-aminophenol being more toxic than the *para* isomer. A similar species difference in hydroxylation has been observed in the metabolism of *N*-2-fluorenylacetamide (Weisberger, Weisberger & Morris, 1957) and β -naphthylamine (Bonser, Clayson & Jull, 1951). Furthermore, the higher production of *ortho*-hydroxylated metabolites in certain species, e.g. dog, has been correlated with a greater incidence of carcinomas, and the carcinogenic activity of aromatic amines may be due to their metabolism to *o*-aminophenols (Clayson, 1953) or to the formation of *is*phenoxazines by the enzymic oxidation of *o*-aminophenols (Nagasawa & Gutmann, 1959).

One possible explanation for this species difference of the *para/ortho* ratio is that the species with a higher ratio, e.g. rabbit, may acetylate the amino group more extensively than species with a lower ratio. The acetanilide so formed would then be hydroxylated preferentially in the *para* position (see Smith & Williams, 1948). A second explanation is that two enzymes are responsible and that these enzymes occur to different extents in different species. From Fig. 2 it can be seen that with the dog the *para/ortho* ratio changes markedly during the first day. Since the dog, unlike the rabbit, does not readily acetylate aromatic amino compounds (Marshall, 1954), this change in the *para/ortho* ratio could be evidence of the two hydroxylation processes proceeding at different rates.

SUMMARY

1. The fate of [^{14}C]aniline has been studied in the rabbit, dog and other animals.
2. With the rabbit, an average of 70% of the aniline is eliminated in the urine in 3 days as *p*-aminophenol (51%), *o*-aminophenol (9%), *m*-aminophenol (0.1%), phenylsulphamic acid (5.5%), aniline-*N*-glucuronide (3.5%) and acetanilide (0.2%).
3. The isomeric dihydroxyanilines and the free *o*- and *p*-acetamidophenol do not occur in the urine.
4. In 3–4 days the expired air contains no detectable aniline and up to 0.4% of the dose as carbon dioxide. The elimination of carbon dioxide begins

on the second day and continues beyond the fourth day.

5. The total excretion of radioactivity in 3 days in the urine, expired air (up to 1%) and faeces (1%) amounts to about 75–90% of the dose. The remainder is present in the tissues and is slowly eliminated as carbon dioxide and as metabolites in the urine. Rabbits killed 5–8 days after dosing contained about 6% of the dose in their tissues. These experiments account for 80–100% of the dose.

6. Free glucuronic acid is excreted in the urine of rabbits after oral administration of aniline, particularly at high doses (500 mg./kg.).

7. With the dog, about 20% of the aniline is eliminated in the urine in 16 hr. and about 50% in 2 days. The principal metabolites are *o*-aminophenol (25%), *p*-aminophenol (11%) and conjugates of aniline (5%).

8. In both rabbit and dog there was no evidence for the *N*-methylation or deamination of aniline, nor for mercapturic acid formation.

9. Nitrosobenzene, azoxybenzene, azobenzene and benzidine, the presence of which would have confirmed the oxidation of aniline to nitrosobenzene, did not occur in the urine. However, two unidentified metabolites, which have also been found in the urines of rabbits dosed with nitrosobenzene or azoxybenzene, were detected in trace amounts by paper chromatography.

10. The ratio of *p*- to *o*-aminophenol excreted in the urines of various animal species dosed with aniline was: gerbil, 15; guinea pig, 11; golden hamster, 10; rabbit, 6; rat, 6 (male) and 2.5 (female); chicken, 4; mouse, 3; ferret, 1; dog, 0.5; cat, 0.4.

11. With the dog, the *para/ortho* ratio changes with time and it is suggested that at least two different enzyme systems are involved in the hydroxylation of aniline.

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REFERENCES

- Axelrod, J. (1955). *J. biol. Chem.* **214**, 753.
 Axelrod, J., Inscoc, J. K. & Tomkins, G. M. (1958). *J. biol. Chem.* **232**, 835.
 Axelrod, J., Udenfriend, S. & Brodie, B. B. (1954). *J. Pharmacol.* **111**, 176.
 Azouz, W. M., Parke, D. V. & Williams, R. T. (1952). *Biochem. J.* **50**, 702.
 Baldwin, B. C., Robinson, D. & Williams, R. T. (1960). *Biochem. J.* **76**, 600.
 Bergmann, M. & Wolff, W. W. (1923). *Ber. dtsch. chem. Ges.* **56**, 1060.
 Bonser, G. M., Clayson, D. B. & Jull, J. W. (1951). *Lancet*, **ii**, 286.
 Booth, J. & Boyland, E. (1957). *Biochem. J.* **66**, 73.
 Boyland, E., Manson, D. & Orr, S. F. D. (1957). *Biochem. J.* **65**, 417.

- Braude, E. A., Linstead, R. P. & Wooldridge, K. R. H. (1954). *J. chem. Soc.* p. 3586.
- Bray, H. G., Clowes, R. C. & Thorpe, W. V. (1952a). *Biochem. J.* **51**, 70.
- Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952b). *Biochem. J.* **52**, 416.
- Brodie, B. B., Axelrod, J., Shore, P. A. & Udenfriend, S. (1954). *J. biol. Chem.* **208**, 741.
- Burns, J. J., Evans, C. & Trousof, N. (1957). *J. biol. Chem.* **227**, 785.
- Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. H. & Yankwich, P. F. (1949). *Isotopic Carbon*. New York: John Wiley and Sons Inc.
- Clayson, D. B. (1953). *Brit. J. Cancer*. **7**, 460.
- Fewster, M. E. & Hall, D. A. (1951). *Nature, Lond.*, **168**, 78.
- Gessner, T. & Smith, J. N. (1960). *Biochem. J.* **75**, 172.
- Henrich, F. (1921). *Ber. dtsh. chem. Ges.* **54**, 2507.
- Henrich, F. & Wagner, B. (1902). *Ber. dtsh. chem. Ges.* **35**, 4200.
- Heyns, K. & Baltes, W. (1958). *Ber. dtsh. chem. Ges.* **91**, 622.
- Hinsberg, O. (1906). *Ber. dtsh. chem. Ges.* **39**, 2427.
- Hodgson, H. H. & Dix, A. G. (1914). *J. chem. Soc.* **105**, 954.
- Huennekens, F. M., Osborn, M. J. & Whiteley, H. R. (1958). *Science*, **128**, 120.
- Ishidate, M., Hogiya, S., Hashimoto, Y. & Takitani, S. (1958). *Pharm. Weekbl.* **93**, 216.
- Jacobs, W. A., Heidelberger, M. & Rolf, I. P. (1919). *J. Amer. chem. Soc.* **41**, 467.
- Jones, E. C. S. & Kenner, J. (1932). *J. chem. Soc.* p. 711.
- Kamil, I. A., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 235.
- Kauffmann, H. & dePay, E. (1906). *Ber. dtsh. chem. Ges.* **39**, 323.
- Kehrmann, F. & Bahatryan, G. (1898). *Ber. dtsh. chem. Ges.* **31**, 2400.
- Kehrmann, F. & Hoehn, E. (1925). *Helv. chim. acta*, **8**, 222.
- Kiese, M. (1959a). *Arch. exp. Path. Pharmacol.* **235**, 354, 360.
- Kiese, M. (1959b). *Arch. exp. Path. Pharmacol.* **236**, 19.
- Knight, R. H. & Young, L. (1958). *Biochem. J.* **70**, 111.
- Marshall, E. K., jun. (1954). *J. biol. Chem.* **211**, 499.
- Mead, J. A. R., Smith, J. N. & Williams, R. T. (1958). *Biochem. J.* **68**, 61.
- Miller, J. A. & Miller, E. C. (1952). *Cancer Res.* **12**, 283.
- Mitoma, C., Posner, H. S., Reitz, H. C. & Udenfriend, S. (1956). *Arch. Biochem. Biophys.* **61**, 431.
- Nagasawa, H. T. & Gutmann, H. R. (1959). *J. biol. Chem.* **234**, 1593, 1600.
- Parke, D. V. (1956). *Biochem. J.* **62**, 339.
- Parke, D. V. & Williams, R. T. (1953). *Biochem. J.* **54**, 231.
- Parke, D. V. & Williams, R. T. (1958). *Rep. Progr. Chem.* **55**, 386.
- Parke, D. V. & Williams, R. T. (1960). *Biochem. J.* **74**, 5.
- Pollack, J. (1893). *Mh. Chem.* **14**, 419.
- Reio, L. (1958). *J. Chromat.* **1**, 338.
- Robinson, D., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 228.
- Roy, A. B. (1958). *Biochim. biophys. Acta*, **30**, 193.
- Sato, T., Suzuki, T., Fukuyama, T. & Yoshikawa, H. (1955). *Seitai no Kagaku*, **6**, 225. (*Chem. Abstr.* **52**, 20534).
- Scudi, J. V. & Jelinek, V. C. (1944). *J. biol. Chem.* **152**, 27.
- Smith, J. N. & Williams, R. T. (1948). *Biochem. J.* **42**, 541.
- Smith, J. N. & Williams, R. T. (1949). *Biochem. J.* **44**, 242.
- Spector, W. S. (1956). *Handbook of Toxicology*, vol. 1, p. 26. London: W. B. Saunders Co. Ltd.
- Spicer, S. S. (1950). *J. Pharmacol.* **99**, 185.
- Tamura, S. (1924). *Acta Sch. med. Univ. Kioto*, **6**, 449, 459.
- Thierfelder, H. (1889). *Hoppe-Seyl. Z.* **13**, 277.
- Tomita, M. (1921). *Biochem. Z.* **116**, 55.
- Traube, W. (1890). *Ber. dtsh. chem. Ges.* **23**, 1654.
- Volkova, S. A. (1958). *J. Hyg. Epidem. Microbiol. Immunol., Prague*, **2**, 375.
- Weisberger, J. H., Weisberger, E. K. & Morris, H. P. (1957). *Science*, **125**, 503.
- Williams, R. T. (1943). *Biochem. J.* **37**, 329.
- Williams, R. T. (1959). *Detoxication Mechanisms*, 2nd ed. p. 13. London: Chapman and Hall Ltd.

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The Chemical Composition and Structure of the Cell Wall of *Hydrodictyon africanum* Yaman

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The coenobium of the green alga *Hydrodictyon africanum* consists in a single net of about 500 cells or coenocytes of similar size and stage of development. The net-like colonies can easily be grown in culture, the individual cells reaching a size of 2 mm. or more in diameter. The organism thus gives material which is very well suited to a study of

plant cell growth and in particular to the development of the cell wall. The walls are large and can be obtained free from the cell contents by a variety of techniques including microdissection, which is both mechanically and chemically very gentle. The work reported here is a microscopical and chemical study of the cell walls prepared from fully developed cells.