The Biochemistry of Aromatic Amines

6. THE METABOLISM OF 3:4-DIMETHYLANILINE IN RATS*

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3:4-Dimethylaniline is one of the simplest aromatic amines with carcinogenic properties. Morris, Lombard, Wagner & Weisburger (1957) showed that increases in the incidence of pituitary gland tumours occur when the amine is fed to rats, and Woolley (1953) and Woolley & Schaffner (1954) showed that the related *o*-aminophenol, 2-amino-4:5-dimethylphenol, causes a transient regression of spontaneous mammary tumours in mice. Allen, Boyland, Dukes, Horning & Watson (1957) showed that tumours are produced when pellets of the aminophenol and cholesterol are implanted in the bladders of mice. The metabolism of 3:4-dimethylaniline and of its *N*-acetyl derivative in rats is described below.

EXPERIMENTAL

Melting points are uncorrected.

Materials

3:4-Dimethylaniline had m.p. 53° and 3:4-dimethylacetanilide formed needles from benzene–light petroleum (b.p. 60–80°), m.p. 98°. *Potassium 3:4-dimethylphenyl sulphamate*, prepared by the action of chlorosulphonic acid in pyridine on 3:4-dimethylaniline (Boyland, Manson & Orr, 1957), separated from aq. ethanol in plates (Found: N, 6·0: $C_8H_{10}O_3NSK$ requires N, 5·85%).

Potassium 2-amino-4:5-dimethylphenyl sulphate was prepared by the action of alkaline $K_2S_2O_8$ on 3:4-dimethylaniline (Sims, 1958) and formed plates from aq. ethanol. 2-Amino-4:5-dimethylphenol was obtained from the sulphuric ester by hydrolysis with 6N-HCl and formed plates from light petroleum (b.p. 80–100°), m.p. 169–171° raised to 174–175° by sublimation at 150° and 0·5 mm. Hg. Diepolder (1909) gives m.p. 173–175° (turning brown at 165°). The aminophenol could be detected in aqueous solution at concentrations greater than 0·5 mg./ml. by shaking the solution with a little 2N-NaOH and ether, when a cherry-red ether layer separated.

2-Methyl-4-nitrobenzoic acid was prepared in poor yield by the oxidation of 3:4-dimethylnitrobenzene with HNO_3 as described by Jacobsen (1884). The nitro compound was reduced with hydrogen and Adams catalyst to yield 4-amino-2-methylbenzoic acid, which formed flat needles from water, m.p. 161–162° (decomp.). The acid was treated with acetic anhydride in 2N-NaOH, yielding 4-acetamido-2-methylbenzoic acid, which was purified by precipitation with 2N-HCl from a solution in aq. saturated $NaHCO_3$; it formed needles, m.p. 249° (decomp.). 4-Acetamido-2-methylbenzoic acid was also prepared by heating 3:4-dimethylacetanilide (2 g.) under reflux with water (200 ml.) whilst $KMnO_4$ (4 g.) was added during 10 min. The mixture was cooled and filtered and the filtrate was washed with ether (2 × 250 ml.), acidified to pH 4·0 with 2N-H_2SO_4 and extracted with ether for 6 hr. The product (950 mg.) obtained on evaporation of the ether had m.p. 228–240° (decomp.), which was raised, after repeated dissolution in aq. saturated $NaHCO_3$ and precipitation with 2N-HCl, to 249° (decomp.), undepressed in admixture with the product obtained by the first method. The acetamido compound was heated to 100° with an excess of 6N-HCl for 1 hr., when the product, extracted with ether after the addition of an excess of sodium acetate to the reaction mixture, formed flat needles from water, m.p. 162° (decomp.), undepressed in admixture with 4-amino-2-methylbenzoic acid prepared as described above.

* Part 5: Boyland & Manson (1958).

Paper chromatography

Downward development on Whatman no. 1 paper was carried out with the solvents indicated in Table 1. The chromatograms were sprayed with either Ehrlich reagent (*p*-dimethylaminobenzaldehyde, 0·5% in ethanol containing 1 ml. of concn. HCl/100 ml.), a solution of *p*-dimethylaminocinnamaldehyde (2 g.) in 6N-HCl (100 ml.) and ethanol (100 ml.), as described by Harley-Mason & Archer (1958), or successively with aq. $NaNO_2$ (0·5%), 0·5N-HCl and 2-naphthol (0·5%) in ethanol–2N-NaOH (1:1, v/v). Other chromatograms were sprayed with 2N-HCl and heated in an oven to 70° for 15 min. before being sprayed with the above-mentioned reagents. The properties of the various metabolites on such paper chromatograms are listed in Table 1.

Isolation experiments

Eight rats (approx. 200 g.) were each given 3:4-dimethylaniline (50 mg.) in arachis oil (1 ml.) by forced feeding on alternate days until 7 g. of the amine had been administered. The urine was collected daily, pooled and stored at 0°. The preliminary treatment of the urine, the absorption of the aromatic compounds on activated charcoal and their subsequent elution was carried out as previously described (Boyland & Sims, 1958), except that the preliminary washing of the charcoal with aq. 2N-NH_3 soln. was omitted and the elution was carried out with methanol containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88). The material obtained on evaporation of the eluate was chromatographed on a column of cellulose powder (500 g., Whatman standard grade) with butanol–*cyclo*hexane–aq. 2N-NH_3 soln. (9:2:1, by vol.) as developing solvent. Fractions (200 ml.) were

Table 1. *Properties on paper chromatograms of compounds related to 3:4-dimethylaniline*

Solvent systems and time of run: 1, butanol saturated with aq. 2N-NH_3 soln, 15 hr.; 2, butanol–propanol–water (2:1:1, by vol.), 15 hr.; 3, butanol–acetic acid–water (2:1:1, by vol.), 15 hr. Colours given in parentheses refer to those obtained after acid treatment of the paper chromatogram (see text). When the chromatograms were viewed under ultraviolet light all the compounds appeared as dark absorbent spots.

Compound	R_F in solvent 1	2	3	Ehrlich reagent	*p*-Dimethylamino-cinnamaldehyde reagent	Diazotization and coupling with 2-naphthol
3:4-Dimethylaniline	0·93	0·91	0·83	Yellow	Red	Pink
3:4-Dimethylacetanilide	0·88	0·88	0·92	Yellow* (yellow)	Red* (red)	—
2-Amino-4:5-dimethylphenol	0·82	0·78	0·84	Yellow turning brown	Red	Pale red
2-Amino-4:5-dimethylphenyl sulphate	0·42	0·51	0·65	Yellow turning brown	Red	Red turning brown
3:4-Dimethylphenyl sulphamate	0·37	0·45	0·59	Yellow* (yellow)	Red	Pink
4-Amino-2-methylbenzoic acid	0·11	0·86	0·87	Deep yellow	Violet	Pink
4-Acetamido-2-methylbenzoic acid	0·22	0·88	0·90	Yellow* (yellow)	Violet* (violet)	—
Conjugate, probably 4-amino-2-methylbenzoyl-glucosiduronate	0·07	0·20	0·53	Deep yellow	Violet	Pink

* Colour develops during 24 hr.

collected and separately evaporated to about 10 ml. under reduced pressure and the solutions were examined on paper chromatograms. The fractions were all tested for glucosiduronic acids with naphtharesorcinol: all tests were negative. The subsequent treatment of these solutions is described below.

Six rats were each given 3:4-dimethylacetanilide (75 mg.) in arachis oil (1 ml.) by intraperitoneal injection and the urines were collected for 24 hr. and treated as described below. Two pairs of rats were given 3:4-dimethylaniline (50 mg.) in arachis oil (1 ml.), either orally or intraperitoneally, and the urines, collected for 24 hr., were examined on paper chromatograms after a preliminary charcoal treatment as indicated above.

RESULTS

Fractions 1–3 from the cellulose column, which contained a little of two substances resembling 3:4-dimethylaniline and 3:4-dimethylacetanilide on paper chromatograms, were combined and evaporated; the residue was dissolved in 2N-H_2SO_4 and the solution extracted with ether (3 × 25 ml.) The ether was removed and the residue recrystallized from benzene–light petroleum (b.p. 60–80°) to yield 3:4-dimethylacetanilide (22 mg.), m.p. and mixed m.p. 95–97°.

Fraction 10 deposited a solid on standing which had m.p. 242° (decomp.), undepressed in admixture with 4-acetamido-2-methylbenzoic acid. Fractions 4–9 and the filtrate from fraction 10 were combined and evaporated, and the residue was dissolved in water (25 ml.) and extracted with ether for 4 hr. The ether-soluble material contained small amounts of compounds indistinguishable from 3:4-dimethylaniline and 4-acetamido-2-methylbenzoic acid on paper chromatograms. The pH of the aqueous layer was adjusted to 10 with 2N-KOH, the solution was evaporated to about 10 ml. under reduced pressure and an equal volume of ethanol was added. The crystals which separated overnight formed from aq. ethanol plates of potassium 2-amino-4:5-dimethylphenylsulphate (370 mg.) (Found: N, 5·35; S, 12·7. Calc. for $C_8H_{10}O_4NSK$: N, 5·5; S, 12·6%). The infrared spectrum was identical with that of the synthetic material. On hydrolysis with 2N-HCl at 100° the ester yielded 2-amino-4:5-dimethylphenol as plates from light petroleum (b.p. 80–100°), m.p. and mixed m.p. 169–171°. Paper chromatography showed that the mother liquors contained more of the sulphuric ester, together with a compound which was indistinguishable from 3:4-dimethylphenylsulphamic acid on all paper chromatograms.

Fraction no. 11 also deposited 4-acetamido-2-methylbenzoic acid, m.p. and mixed m.p. 241–243° (decomp.), on standing. The mother liquors from this fraction were combined with fractions 12–14 and evaporated to dryness and the residue was

warmed to 60° with 50 ml. of water. The solid was filtered off and the filtrate extracted with ether for 4 hr. The ether was evaporated, the residue was warmed with water (5 ml.) and the solid collected. The two solids were combined and purified by solution in aq. saturated $NaHCO_3$ and precipitation with 2N-HCl to yield 4-acetamido-2-methylbenzoic acid in needles, m.p. and mixed m.p. 249° (decomp.) (Found: C, 62·0; H, 6·0; N, 7·3. Calc. for $C_{10}H_{11}O_3N$: C, 62·2; H, 5·7; N, 7·25%). The infrared spectrum was identical with that of the compound obtained by the permanganate oxidation of 3:4-dimethylacetanilide. A total of 2·37 g. of 4-acetamido-2-methylbenzoic acid was obtained from the various fractions. A sample of acetamido compound was hydrolysed with 6N-HCl at 100° for 30 min. to yield 4-amino-2-methylbenzoic acid, m.p. and mixed m.p. 161–162° (decomp.). The mother liquors from fractions 11–14 were shown by paper chromatography to contain 2-amino-4:5-dimethylphenyl sulphate.

Fractions 15–19 were evaporated to dryness under reduced pressure, the residue was dissolved in water and the solution extracted with ether for 6 hr. Removal of the ether and recrystallization of the residue from water yielded 4-amino-2-methylbenzoic acid (152 mg.) in plates, m.p. and mixed m.p. 162–163° (decomp.).

Fractions 19–24 were found by paper chromatography to contain small amounts of 4-amino-2-methylbenzoic acid and traces of a substance which, for reasons presented below, is believed to be a conjugate of this acid with glucuronic acid. Further elution of the column did not yield any more metabolites.

The pooled urines from the rats given 3:4-dimethylacetanilide (450 mg.) were acidified with HCl and extracted with ether for 6 hr. Removal of the ether and purification of the residue by solution in aq. $NaHCO_3$ and precipitation with acid as described before yielded 4-acetamido-2-methylbenzoic acid (150 mg.), m.p. and mixed m.p. 247–249° (decomp.). The aqueous layer was treated with charcoal and the products obtained on elution of the charcoal were examined on paper chromatograms together with the products obtained from the rats dosed orally and intraperitoneally with 3:4-dimethylaniline. Examination of the chromatograms showed no qualitative and little quantitative difference in metabolism in the three cases. All the metabolites mentioned above were detected except 3:4-dimethylphenylsulphamic acid and 4-amino-2-methylbenzoic acid, whilst the substance believed to be a glucuronic acid conjugate of the latter acid was much more prominent. Evidence for the structure of this compound was obtained by eluting spots from paper chromatograms with methanol and hydrolysing the recovered materials with 2N-HCl at 100° for 10 min. The hydrolysates, on paper chromatograms, contained 4-amino-2-methylbenzoic acid, but no glycine could be detected with ninhydrin. The unhydrolysed extracts gave positive naphtharesorcinol tests. A comparison on paper chromatograms of the compound detected in the urine with the product from the reaction of 4-amino-2-methylbenzoic acid with glucuronic acid showed that the compounds differed, the *N*-glucosiduronic acid being completely decomposed by solvent 3 and partially decomposed by solvent 2 (Table 1). It is probable therefore that the compound in the urine is 4-amino-2-methylbenzoylglucosiduronic acid.

DISCUSSION

There appears to be little difference in the metabolites of 3:4-dimethylaniline and 3:4-dimethylacetanilide, the metabolism of both compounds proceeding by two main routes. One involves an oxidation of the methyl group in the position *para* to the amino group, followed by either acetylation of the amino group or conjugation of the carboxyl group with glucuronic acid. The amino group may be acetylated before oxidation occurs, in which case conjugation of the carboxyl group with glucuronic acid must be accompanied by deacetylation. There was no evidence of conjugation of 4-acetamido-2-methylbenzoic acid with glucuronic acid. The second route involves a hydroxylation *ortho* to the amino group, followed by conjugation to give 2-amino-4:5-dimethylphenyl sulphate.

In the formation of 4-acetamido-2-methylbenzoic acid, the metabolism of the compounds resembles that of *p*-acetotoluidide, which Jaffe & Hilbert (1888) and Bray & Thorpe (1948) showed was converted by rabbits into *p*-acetamidobenzoic acid. Bray, Lake & Thorpe (1949) found that the methyl group of *N*-*p*-tolylurea is similarly oxidized. None of these workers, however, found evidence for hydroxylation and sulphuric acid conjugation, and Hildebrandt (1907) was unable to detect any sulphuric ester formation with *p*-toluidine in rabbits.

The absence of a glycine conjugate of 4-amino-2-methylbenzoic acid is not surprising: Quick (1932) found that substituents *ortho* to the carboxyl group inhibit glycine conjugation and Bray, Thorpe & Wood (1949) and Bray, Humphris & Thorpe (1949) found that little glycine conjugation occurs when *o*-toluic acid and *o*-xylene are given to rabbits. Bray, Lake, Neale, Thorpe & Wood (1948) isolated 4-aminobenzoylglucosiduronic acid from the urine of rabbits receiving *p*-aminobenzoic acid. Since 4-amino-2-methylbenzoic acid could not be detected in freshly voided urine, it is probable that the acid isolated by means of the cellulose column arose by the decomposition of the glucuronic acid conjugate.

There was no evidence for *N*- or phenolic *O*-conjugation with glucuronic acid; in this respect the results differ from those of Elson, Goulden & Warren (1958), who found that rats tend to excrete amines which have carcinogenic activity as glucuronic acid conjugates whereas non-carcinogenic amines are excreted as sulphuric esters. The presence of only small amounts of 3:4-dimethylphenylsulphamic acid is not unexpected since Boyland *et al.* (1957) found that rats usually excrete little of this type of metabolite after treatment with aromatic amines. The sulphamate was detectable only after repeated dosing with 3:4-dimethylaniline.

The two sites of biological oxidation (the methyl group attached to C-4 and the carbon atom at position 6) are the same as those in which chemical reactions occur with permanganate and persulphate respectively. There is no evidence of oxidation of the methyl group attached to C-3 or of hydroxylation at C-2 or C-5 in either the biological or chemical reactions. Bray & Thorpe (1948) found that with *m*-acetotoluidide, oxidation to the carboxylic acid occurred to a lesser extent than with *p*-acetotoluidide.

Which, if either, of the two metabolic routes is involved in carcinogenesis is not known, although 2-amino-4:5-dimethylphenyl sulphate, which is hydrolysed by a number of sulphatases (Boyland, Manson, Sims & Williams, 1956), could give rise to 2-amino-4:5-dimethylphenol (which is known to possess carcinogenic activity) on hydrolysis. The free aminophenol was not detected in the urine of rats treated with 3:4-dimethylaniline. Little is known of the carcinogenic activity of compounds such as 4-acetamido-2-methylbenzoic acid although simpler compounds such as *p*-toluidine are inactive. The results give no indication why 3:4-dimethylaniline is active mainly in the pituitary gland.

SUMMARY

1. 3:4-Dimethylaniline and 3:4-dimethylacetanilide are excreted by rats as 4-acetamido-2-methylbenzoic acid, 2-amino-4:5-dimethylphenyl sulphate and probably 4-amino-2-methylbenzoylglucosiduronic acid. Small amounts of 3:4-dimethylsulphamic acid and 3:4-dimethylacetanilide were formed from 3:4-dimethylaniline.

2. The behaviour on paper chromatograms of a number of these compounds is described.

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