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The Biochemistry of Aromatic Amines

THE METABOLISM OF 2-NAPHTHYLAMINE AND 2-NAPHTHYLHYDROXYLAMINE DERIVATIVES

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1. 2-Naphthylhydroxylamine and 2-nitrosonaphthalene were present in urine of dogs but not of guinea pigs, hamsters, rabbits or rats dosed with 2-naphthylamine. *N*-Acetyl-2-naphthylhydroxylamine and its *O*-sulphonic acid and *O*-glucosiduronic acid were not detected in the urine of any of these species. 2. Bile from rats dosed with 2-naphthylamine contained (2-naphthylamine *N*-glucosid)uronic acid and 6- and 5,6-substituted derivatives of 2-acetamidonaphthalene. 2-Amino-1-naphthyl and 2-acetamido-1-naphthyl derivatives, 2-naphthylhydroxylamine and its *N*-acetyl derivative or conjugates of these were not detected. Bile from a dog dosed with 2-naphthylamine contained no 2-amino-1-naphthyl derivatives. 3. 2-Naphthylhydroxylamine was metabolized by the dog, rat and guinea pig to the same products as those formed by these species from 2-naphthylamine. Rabbits formed mainly 2-amino-1-naphthyl derivatives; these are minor metabolites of 2-naphthylamine in this species. 4. (*N*-Acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid was excreted in the urine and the bile of rats and in the urine of guinea pigs and rabbits dosed with *N*-acetyl-2-naphthylhydroxylamine. 5. After the administration of 2-acetamidonaphthalene, (*N*-acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid was detected in the urine of dogs, but not in the urine of other species. The dog excreted an acid-labile cysteine derivative of 2-acetamidonaphthalene, but only traces of the corresponding mercapturic acid. 6. After dosing with *N*-acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid, rats excreted derivatives of 2-amino-1-naphthol. 7. 2-Nitrosonaphthalene, *N*-acetyl-2-naphthylhydroxylamine, *N*-acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid, 2-naphthylhydroxylamine-*N*-sulphonic acid, *N*-benzyloxycarbonyl-2-naphthylhydroxylamine and *N*-benzyloxycarbonyl-2-naphthylhydroxylamine-*O*-sulphonic acid were synthesized.

The isolation of *N*-acetyl-2-fluorenylhydroxylamine as a metabolite of 2-acetamidofluorene from rat urine (Cramer, Miller & Miller, 1960) demonstrated a new route of metabolism of aromatic amines. *N*-Hydroxylation of 2-naphthylamine (Troll & Nelson, 1961) and 2-acetamidonaphthalene (Poirier, Miller & Miller, 1963) has been reported. The formation of 2-nitrosonaphthalene in the blood of cats and dogs dosed with 2-naphthylamine has been demonstrated (Heringlake, Kiese, Renner & Wenz, 1960). The present paper describes the metabolism of 2-naphthylamine and some derivatives with reference to *N*-hydroxylation.

METHODS AND MATERIALS

Animals. Animals were kept in cages designed for the separate collection of urine and faeces. Bile fistulae were established in rats by Mr G. S. Ramsay according to the method described by Boyland, Ramsay & Sims (1961*b*), and the bile was collected in saddle-shaped glass vessels strapped to the animals by adhesive tape. A dog with a bile fistula was prepared by Mr W. J. Dempster of the Postgraduate Medical School, Hammersmith, London, W. 12, and the bile was collected from the cannula by a plastic bag. The animal was restrained by a Pavlov stand for part of the experiment but was provided with water. The approximate body weights of the animals were: (*a*) beagle, terrier and mongrel bitches, 10kg.; greyhound bitch, 23kg.; (*b*) rabbits, 2·5kg.; (*c*) guinea pigs, 800g.; (*d*) Chester Beatty albino rats, 150–200g.; (*e*) hamsters, 80g. Compounds were administered by mouth in gelatin capsules to dogs, except for the greyhound, which was dosed by intramuscular injection. Other animals were dosed by intraperitoneal injection of the compounds in arachis oil (1ml. of oil for each 50mg. of compound), except for rats with bile fistulae, which were dosed orally. The following daily doses were given: (*a*) 2-naphthylamine: dog, 400mg.; rabbit, 400mg.; guinea pig, 50mg.; hamster,

20mg.; (*b*) 2-naphthylhydroxylamine: dog, 250mg.; rabbit, 100mg.; guinea pig, 50mg.; rat, 30mg.; hamster, 20mg.; (*c*) *N*-acetyl-2-naphthylhydroxylamine: rabbit, 200mg.; guinea pig, 50mg.; rat, 30mg.; (*d*) 2-acetamidonaphthalene: dog, 400mg.; rabbit, 500mg.; guinea pig, 50mg.; rat, 50mg.; hamster, 50mg.

Chromatography. Whatman no. 1 chromatography paper was employed for descending development in the following solvent systems: (*a*) butan-1-ol–propan-1-ol–aq. 0·1 N-NH_3 soln. (2:1:1, by vol.); (*b*) butan-1-ol–acetic acid–water (2:1:1, by vol.); (*c*) butan-1-ol–acetic acid–water (12:3:5, by vol.); (*d*) butan-1-ol saturated with 0·1 N-NH_3; (*e*) butan-1-ol–benzene (4:1, v/v) saturated with 0·1 N-NH_3 (Brill & Radomski, 1965). Two-dimensional paper chromatography was carried out by successive use of solvents (*a*) and (*b*). Thin-layer chromatography was carried out on glass plates coated with silica gel (E. Merck A.-G., Darmstadt, W. Germany) of 0·25mm. thickness with the following solvent mixtures: (*f*) butan-1-ol–propan-1-ol–water (2:1:1, by vol.); (*g*) light petroleum (b.p. 40–60°)–acetone (7:3, v/v); (*h*) light petroleum (b.p. 40–60°)–acetone (4:1, v/v); (*i*) chloroform–ethyl acetate–acetic acid (6:3:1, by vol.); (*j*) benzene–ethanol (19:1, v/v); (*k*) chloroform–methanol (49:1, v/v); (*l*) chloroform–methanol (19:1, v/v); (*m*) butan-1-ol–acetic acid–water (10:1:1, by vol.). Solvents (*f*) and (*m*) were used for thin-layer chromatography of water-soluble compounds, e.g. sulphuric esters. Better separations were usually obtained by paper chromatography but *N* - acetyl - 2 - naphthylhydroxylamine - *O* - sulphonic acid could not be run on paper chromatograms overnight without rearrangement to 2-acetamido-1-naphthyl hydrogen sulphate, and only thin-layer chromatography could be employed in the examination of urine for this compound. *N*-Benzyloxycarbonyl-2-naphthylhydroxylamine-*O*-sulphonic acid was also too unstable for prolonged paper chromatography.

Chromatography of 2-naphthylhydroxylamine was only successful by the thin-layer technique with quick-running solvents (*g* or *h*), although two-dimensional chromatography showed that some 2-naphthylamine was formed.

2-Amino-1-naphthol hydrochloride was not sufficiently stable to run on paper chromatograms except in butan-1-ol–acetic acid mixtures in the presence of ascorbic acid (Boyland & Manson, 1958*a*). A freshly prepared solution of the free base (Boyland, Manson & Orr, 1957) gave a single spot on thin-layer chromatograms in fast-running solvent systems (*g* or *h*) if it was run immediately after spotting and for a short distance (about 5cm.) instead of the customary 10cm. If the spot was left for a few minutes before running, 2-amino-1-naphthol oxidized and thin-layer chromatography showed the presence of four coloured substances (green, mauve, green and mauve) at R_F values 0·0, 0·06, 0·18 and 0·42 respectively in solvent (*h*). Little of the aminonaphthol remained. 2-Amino-1-naphthol was identified in aqueous solution by the green colour obtained on adding aq. NH_3 soln. On shaking with benzene or ether the green oxidation product gave a mauve colour in the organic phase. The R_F values and colour reactions of some 2-naphthylamine metabolites and other derivatives are given in Tables 1, 2 and 3. Details of reference compounds that are not described in these Tables have been given by Booth, Boyland & Manson (1955), Boyland & Manson (1957*a*, 1958*a*) and Boyland, Manson & Nery (1963*b*).

For the detection of compounds on chromatograms most of the reagents employed were as described by Boyland *et al.* (1963*b*). Aq. sodium amminoprusside (5%, w/v) (Boyland & Nery, 1964), aq. $FeCl_3$ (5%, w/v), ammoniacal $AgNO_3$ and aq. $TiCl_3$ (15%, w/v) were also used. Hydrolysis of compounds with acetamido groups was carried out by spraying the paper chromatograms with 2 N-HCl and heating them between glass sheets at 70° for 30min. Radioautographs were prepared as described by Boyland *et al.* (1963*b*). Urine was applied directly to chromatograms or concentrated by adsorption at pH 5·0 on charcoal, from which the metabolites were eluted with methanol containing about 5% (v/v) of aq. NH_3 (sp.gr. 0·88). Bile was treated in the same way except that 5% (w/v) phenol was used for elution, as it was more efficient for the elution of some metabolites from charcoal.

Detection of 2-naphthylhydroxylamine and some of its derivatives. 2-Naphthylhydroxylamine gives a bluish-green colour with hexylresorcinol (0·5%, w/v, in 2 N-NaOH) alone, i.e. not preceded by $NaNO_2$ and HCl as employed for the diazotization of aromatic amino groups. This colour reaction was specific for 2-naphthylhydroxylamine and its alkali-labile derivatives. *N*-Acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid and (*N*-acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid behaved in this way. This colour was not given by other 2-naphthylamine derivatives nor by any normal urinary constituent excreted by the species used in this work.

Enzymic hydrolyses. The following enzyme preparations were used: Taka-diastase (Parke, Davis and Co. Ltd., Hounslow, Middlesex), Ketodase (Warner–Chilcott Laboratories, Morris Plains, N.J., U.S.A.) and acid phosphatase (Sigma Chemical Co. Ltd., London, S.W. 6).

Materials. 2-Naphthylamine was of commercial origin. 2-[8-^{14}C]Naphthylamine (specific activity 2mc/m-mole) and [1-^{14}C]naphthalene (specific activity 2mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Salts of 2-amino-1-naphthyl hydrogen sulphate were synthesized by the persulphate oxidation of 2-naphthylamine (Boyland, Manson & Sims, 1953) and by the sulphation of 2-amino-1-naphthol (Boyland & Manson, 1958*b*). 2-Amino-1-naphthol, 2-acetamido-1-naphthol and 2-acetamido-6-naphthol were prepared as described by Booth *et al.* (1955). The glucosiduronic acids of 2-amino-1-naphthol, 2-acetamido-1-naphthol, 2-amino-6-naphthol and 2-acetamido-6-naphthol were obtained biosynthetically as described by Boyland & Manson (1957*a*). 2-Acetamido-1-naphthyl sodium sulphate was prepared by treatment of 2-amino-1-naphthyl sodium sulphate with acetic anhydride in pyridine for 8hr. at room temperature in preference to the acetylation with thioacetic acid described by Booth *et al.* (1955). The solution was evaporated *in vacuo* and excess of acetic anhydride removed over NaOH pellets in a desiccator. The product crystallized as needles from ethanol containing a trace of water. The ammonium salt was obtained by dissolving the sodium salt in water, adjusting the pH to 5·0 and adsorbing the compound on charcoal. The charcoal was washed with water and the sulphuric ester was eluted with ammoniacal methanol. The ammonium salt, which crystallized from ethanol in rosettes of needles, was more stable than the sodium or potassium salts, both of which slowly decomposed to 2-acetamido-1-naphthol. Treatment of 2-acetamido-1-naphthyl ammonium sulphate with cold N-HCl yielded a trace of 2-amino-1-naphthyl hydrogen sulphate, but the

main product was 2-acetamido-1-naphthol. Treatment with cold 2N-NaOH yielded a trace of the free amino compound, but no 2-acetamido-1-naphthol.

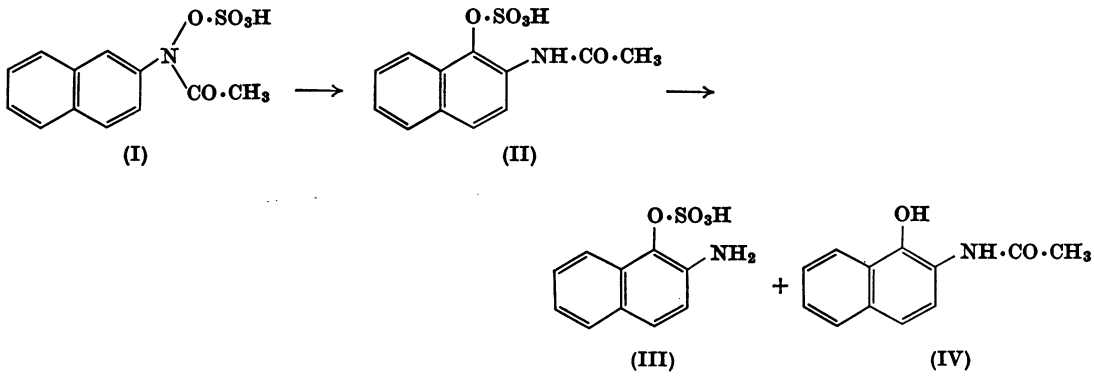
2-Naphthylhydroxylamine (Boyland, Manson & Nery, 1962), 2-amino-1-naphthylmercapturic acid (Boyland *et al.* 1962), 2-amino-5-naphthylmercapturic acid, 2-acetamido-5-naphthylmercapturic acid and *S*-(2-amino-5-naphthyl)-cysteine (Boyland *et al.* 1963*b*) and 2-amino-1-naphthyl dihydrogen phosphate (Boyland & Manson, 1957*b*) were prepared as described in the references quoted.

Synthesis of N-*acetyl*-2-*naphthylhydroxylamine*. Treatment of 2-naphthylhydroxylamine with acetic anhydride, with or without pyridine, yielded only 2-acetamidonaphthalene, and the procedure of Müller & Lindemann (1933) for the acetylation of phenylhydroxylamine was used. Acetyl chloride (0·6ml.) was added with stirring to 2-naphthylhydroxylamine (2·0g.) in dry ether (100ml.). Stirring was continued for 15min. and the precipitate, mainly 2-naphthylamine hydrochloride, was filtered off. The ether was washed with 2N-NaOH (2 × 20ml.), and the alkaline washings were acidified with conc. HCl and cooled to yield an oil, which gradually solidified. *N-Acetyl-2-naphthylhydroxylamine* crystallized from a mixture of light petroleum (b.p. 40–60°) and benzene as rosettes of needles (0·9g.), m.p. 76–78° (Found: N, 7·0. $C_{12}H_{12}NO_2$ requires N, 7·0%). The compound was unaffected by 2N-HCl at room temperature, but at 100° it yielded 2-amino-1-naphthol (detected by the NH_3–benzene test) and a trace of 2-naphthylamine, but no 2-acetamido-1-naphthol. When treated with phosphoric acid in aq. 50% (v/v) acetone at room temperature for 4hr. it did not yield 2-acetamido-1-naphthyl dihydrogen phosphate, although 2-naphthylhydroxylamine gave 2-amino-1-naphthyl dihydrogen phosphate under these conditions (Boyland, Manson & Nery, 1960). After 24hr. a trace of 2-amino-1-naphthyl dihydrogen phosphate was detected. No reaction occurred with cysteine, *N*-acetylcysteine or glutathione, in contrast with the reactions of 2-naphthylhydroxylamine described by Boyland *et al.* (1962).

Synthesis of N-*acetyl*-2-*naphthylhydroxylamine*-O-*sulphonic acid*. Pyridine–SO_3 (3g.) was added to *N*-acetyl-2-naphthylhydroxylamine (1·4g.) in dry benzene (100ml.) and the mixture stirred for 3hr. The precipitate was filtered off and dissolved in methanol (100ml.). Dry NH_3 gas was passed into the methanol solution to precipitate $(NH_4)_2SO_4$. This was filtered off and potassium acetate (2g.) added to the filtrate, which was evaporated to 50ml. and cooled. The product formed small needles (0·6g.) that were recrystallized from hot ethanol, with just sufficient water to bring the material into solution, to yield *potassium* N-*acetyl*-2-*naphthylhydroxylamine*-O-*sulphonate*, m.p. 148–150° (decomp.) (Found: C, 44·9; H, 3·3; N, 4·1. $C_{12}H_{10}KNO_5S$ requires C, 45·1; H, 3·2; N, 4·4%). Paper chromatography of the mother liquors of the recrystallization showed that 2-amino-1-naphthyl hydrogen sulphate and 2-naphthylamine were present. The benzene solution from the reaction was washed with 2N-NaOH, and *N*-acetyl-2-naphthylhydroxylamine (0·8g.) was recovered by acidification of the alkaline extract. Potassium *N*-acetyl-2-naphthylhydroxylamine-*O*-sulphonate in water or acetate buffer, pH7·0, at room temperature gave 2-acetamido-1-naphthol and 2-acetamido-1-naphthyl hydrogen sulphate but not *N*-acetyl-2-naphthylhydroxylamine. The *O*-sulphonic acid was not stable on paper chromatograms and gave 2-acetamido-1-naphthol and its sulphuric ester. On treatment with aq. NH_3 soln. it became green; on shaking with benzene the organic phase became mauve, indicating the presence of 2-amino-1-naphthol. In 2N-NaOH the ester quickly gave a greenish-brown colour and paper chromatography showed the presence of 2-amino-1-naphthyl hydrogen sulphate and 2-naphthylamine. The last-named compound probably arose from 2-naphthylhydroxylamine. In 2N-HCl or H_3PO_4 soln. it gave mainly 2-acetamido-1-naphthol (IV) and some 2-amino-1-naphthyl hydrogen sulphate (III). The treatment with H_3PO_4 did not yield 2-amino-1-naphthyl dihydrogen phosphate, unlike its effect on 2-naphthylhydroxylamine. This may indicate that the rearrangement of *N*-acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid is intramolecular, unlike that of the arylhydroxylamines (Hughes & Ingold, 1952; Boyland *et al.* 1962). The products of the treatment with acid indicate that the rearrangement probably proceeds according to Scheme 1.

2-Acetamido-1-naphthyl hydrogen sulphate readily decomposes in acid solution to yield mainly compound (IV), with some compound (III). *N*-Acetyl-2-naphthylhydroxylamine does not rearrange on treatment with acid to yield compound (IV).

Synthesis of 2-*naphthylhydroxylamine*-N-*sulphonic acid*. 2-Naphthylhydroxylamine (1·5g.) was stirred for 2hr.



Scheme 1.

with pyridine–SO_3 (1·5g.) in dry benzene (150ml.). The product was isolated by the method of Boyland & Nery (1962). The solid that separated from the reaction mixture was dissolved in methanol (200ml.) and dry NH_3 gas was passed to precipitate $(NH_4)_2SO_4$, which was filtered off. Potassium acetate (2g.) was added to the filtrate and the solution was cooled to yield crystals (plates, 0·5g.) that were washed successively with methanol and ether. Recrystallization from aq. ethanol gave *potassium 2-naphthylhydroxylamine-N-sulphonate*, m.p. 170–172° (decomp.) (Found: C, 43·5; H, 3·3; N, 4·8; S, 11·1. $C_{10}H_8KNO_4S$ requires C, 43·3; H, 2·9; N, 5·05; S, 11·6%). The addition of ether (100ml.) to the mother liquors yielded a further 0·25g. of the compound. After standing with H_3PO_4 soln. for 1hr. 2-amino-1-naphthyl dihydrogen phosphate and 2-amino-1-naphthol were formed. These reactions are similar to those of 2-naphthylhydroxylamine (Boyland *et al.* 1960). The compound remained unchanged on incubation with Taka-diastase. It could not be isolated by the charcoal adsorption procedure and only a compound with the chromatographic properties of 2-naphthylsulphamic acid (Boyland *et al.* 1957) was present in the eluate.

Attempted synthesis of 2-naphthylhydroxylamine-O-sulphonic acid. Boyland & Nery (1962) prepared phenylhydroxylamine-*O*-sulphonic acid by the catalytic reduction of *N*-benzyloxycarbonylphenylhydroxylamine-*O*-sulphonic acid, although the main product was *o*-aminophenyl hydrogen sulphate. Attempts to synthesize 2-naphthylhydroxylamine-*O*-sulphonic acid by this method were unsuccessful.

Synthesis of N-benzyloxycarbonyl-2-naphthylhydroxylamine. This compound was prepared in the same way as the phenylhydroxylamine analogue and crystallized from benzene–light petroleum (b.p. 40–60°) to yield needles, m.p. 82–84° (Found: C, 73·8; H, 5·3; N, 5·0. $C_{18}H_{15}NO_3$ requires C, 73·7; H, 5·15; N, 4·8%). *N*-Benzyloxycarbonyl-2-naphthylhydroxylamine (0·5g.) and pyridine–SO_3 (0·6g.) were stirred for 4hr. in dry pyridine (20ml.). Dry ether (100ml.) was added to give a precipitate that was collected and dissolved in methanol (100ml.). Dry NH_3 gas was passed into the solution to precipitate $(NH_4)_2SO_4$, which was filtered off. The filtrate was evaporated to 20ml. and potassium acetate (1g. in 10ml. of methanol) was added. After cooling to 5° needle-shaped crystals were formed that, after recrystallization from aq. ethanol with the minimum of heating, yielded *potassium N-benzyloxycarbonyl-2-naphthylhydroxylamine-O-sulphonate* (30mg.), m.p. 97–101° (decomp.) (Found: C, 52·8; H, 3·3; N, 3·65; S, 8·2. $C_{18}H_{14}KNO_6S$ requires C, 52·55; H, 3·4; N, 3·4; S, 7·8%). After treatment with 2N-HCl for 1hr. the compound gave a blue colour with aq. $NaNO_2$. This may be due to conversion into *N*-benzyloxycarbonyl-2-amino-1-naphthyl hydrogen sulphate followed by hydrolysis to *N*-benzyloxycarbonyl-2-amino-1-naphthol (cf. the blue colour given by 2-acetamido-1-naphthol with HNO_2). *N*-Benzyloxycarbonyl-2-naphthylhydroxylamine did not give this reaction (cf. the stability of *N*-acetyl-2-naphthylhydroxylamine in acid solution). Solutions of the compound became blue on standing. When run on thin-layer chromatograms (solvents *a* and *b*) the compound was sufficiently stable to yield a bluish-green colour with alkaline hexylresorcinol. Some decomposition took place and a blue compound was found at the solvent front. On paper chromatograms *N*-benzyloxycarbonyl-2-naphthylhydroxylamine-*O*-sulphonic acid gave a spot that reacted slightly or not at all with alkaline hexylresorcinol although it gave a strong blue colour with aq. $NaNO_2$ after treatment with 2N-HCl for 1hr. It is probable that the compound underwent rearrangement during paper chromatography (cf. *N*-acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid). Attempts to synthesize 2-naphthylhydroxylamine-*O*-sulphonic acid by removal of the benzyloxycarbonyl group with H_2 in the presence of catalysts (Adams catalyst and 5% palladium on charcoal or on $BaSO_4$) were not successful. Only materials identical in chromatographic properties and infrared spectra with salts of 2-amino-1-naphthyl hydrogen sulphate and 2-naphthylamine were obtained. Samples removed during the reductions were negative to the hexylresorcinol test but this colour reaction may be confined to the *N*-acyl-2-naphthylhydroxylamine-*O*-sulphonic acids and (*N*-acyl-2-naphthylhydroxylamine *O*-glucosid)uronic acids, e.g. the *N*-acetyl and *N*-benzyloxycarbonyl derivatives described in this paper and the *N*-formyl derivatives (Boyland & Manson, 1966). All these give 2-naphthylhydroxylamine in alkaline solution.

Treatment of 2-naphthylhydroxylamine with pyridine–SO_3 in benzene yielded the *N*-sulphonic acid (described above). With pyridine–SO_3 in acetone 2-amino-1-naphthyl hydrogen sulphate was formed.

Synthesis of 2-nitrosonaphthalene. A method devised by Bamberger (1898) for the synthesis of nitrosobenzene was used. Aq. $KMnO_4$ (40ml.; 3%, w/v) was added dropwise over 30min. to the ammonium salt of *N*-nitroso-2-naphthylhydroxylamine (1g.) (Baudisch & Fürst, 1917) in water (100ml.) and 2N-NaOH (3ml.). The solution was stirred for 1hr. and extracted with ether (3 × 50ml.). The ether extracts were dried (Na_2SO_4) and evaporated to dryness *in vacuo* below 100°. The residue was suspended in light petroleum (b.p. 40–60°) (5ml.), warmed and dissolved by the addition of ether. On cooling, yellowish-brown crystals of *2-nitrosonaphthalene* (0·29g.), m.p. 62–64° (green melt), were formed (Found: C, 76·6; H, 4·4; N, 8·85. $C_{10}H_7NO$ requires C, 76·4; H, 4·5; N, 8·9%). By adding a further 10ml. of aq. $KMnO_4$ to the reaction mixture and allowing the solution to stand for 16hr. another crop of nitroso compound (90mg.) was obtained. 2-Nitrosonaphthalene reacted slowly with sodium amminoprusside on thin-layer chromatograms to give a mauve colour. Other colour reactions are described in Table 1. 2-Nitrosonaphthalene (50mg.) and 2-naphthylhydroxylamine (50mg.) in ether (5ml.) on standing for 6hr. at room temperature formed a yellow solid that, after recrystallization from ethanol, gave material (30mg.), m.p. 160–162°, that did not depress the m.p. of 2,2′-azoxynaphthalene (Cumming & Ferrier, 1924) and that had the same infrared spectrum. Oxidation of 2-naphthylhydroxylamine with Ag_2O in dry ether or with CrO_3 in aq. ethanol also gave the nitroso compound but were not practicable methods for its synthesis.

Reaction of 2-naphthylhydroxylamine with glucuronic acid. 2-Naphthylamine and glucuronic acid gave (2-naphthylamine *N*-glucosid)uronic acid (Boyland *et al.* 1957). When 2-naphthylhydroxylamine was added to ammonium glucuronate in aq. 50% (v/v) ethanol examination of the solution by thin-layer chromatography in solvent (*a*) showed a spot with the colour reactions of 2-naphthylhydroxylamine but with a different R_F value (0·33). The product was not detected after paper chromatography in

Table 1. *R_F values of 2-naphthylamine and some derivatives on paper and thin-layer chromatograms*

Compositions of solvents and other details are given in the text.

	R_F on paper chromatograms					R_F on thin-layer chromatograms							
Compound Solvent.....	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)	(*i*)	(*j*)	(*k*)	(*l*)	(*m*)
2-Naphthylamine	0·84	0·86	0·93	0·80	0·92	0·95	0·80	0·60	0·50	0·63	0·80	0·78	—
2-Acetamidonaphthalene	0·95	0·92	0·91	0·91	0·90	0·85	0·57	0·25	0·87	0·35	0·38	0·70	—
2-Naphthylhydroxylamine	—	—	—	—	—	0·95	0·70	0·35	—	0·38	0·33	0·62	—
N-Acetyl-2-naphthyl-hydroxylamine	0·90	0·95	0·92	0·83	0·85	0·95	0·51	0·20	0·08	0·34	0·29	0·45	—
2-Amino-1-naphthol	—	0·85	—	—	—	—	0·65	0·30	—	—	—	—	—
2-Acetamido-1-naphthol	0·87	0·88	0·95	0·77	0·95	0·95	0·65	0·30	0·75	0·32	0·30	0·60	—
Oxidation product of 2-acetamido-1-naphthol*	0·87	0·88	0·95	0·77	0·95	0·75	0·30	0·00	0·50	0·18	0·07	0·45	—
N-Benzyloxycarbonyl-2-naphthylhydroxylamine	0·94	0·96	0·92	0·92	0·92	0·98	0·75	0·45	0·92	0·50	0·63	0·90	—
2-Nitrosonaphthalene†	—	—	—	—	—	0·90	0·95	0·75	0·95	0·98	0·98	0·98	—
2-Amino-1-naphthyl hydrogen sulphate	0·44	0·71	0·65	0·24	0·24	0·57	—	—	—	—	—	—	0·60
2-Acetamido-1-naphthyl hydrogen sulphate	0·60	0·71	0·73	0·52	0·49	0·66	—	—	—	—	—	—	0·66
N-Acetyl-2-naphthyl-hydroxylamine-*O*-sulphonic acid‡	0·60	—	—	—	—	0·66	—	—	—	—	—	—	—
N-Benzyloxycarbonyl-2-naphthylhydroxylamine-*O*-sulphonic acid	—	—	—	—	—	0·77	—	—	—	—	—	—	—
2-Naphthylhydroxylamine-*N*-sulphonic acid	0·50	0·56	0·52	0·22	0·24	0·51	—	—	—	—	—	—	0·55
2-Amino-1-naphthyl dihydrogen phosphate	0·07	0·70	0·65	0·06	0·05	0·40	—	—	—	—	—	—	0·70
2-Amino-1-naphthyl-mercapturic acid	0·43	0·92	0·91	0·18	0·16	0·36	—	—	—	—	—	—	0·68
Metabolite, probably *S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)-cysteine:													
Before treatment with acid	0·20	0·70	0·48	0·09	0·05	—	—	—	—	—	—	—	—
After treatment with acid	0·34	0·73	0·57	0·20	0·16	—	—	—	—	—	—	—	—
(2-Amino-1-naphthyl glucosid)uronic acid	0·19	0·65	0·56	0·02	0·02	0·31	—	0·00	—	—	—	—	0·36
(2-Acetamido-1-naphthyl glucosid)uronic acid	0·28	0·81	0·74	0·07	0·06	0·35	—	0·00	—	—	—	—	0·45
(*N*-Acetyl-2-naphthyl-hydroxylamine *O*-glucosid)-uronic acid	0·30	0·78	0·76	0·08	0·07	0·31	—	0·00	—	—	—	—	0·40
(2-Naphthylamine *N*-glucosid)uronic acid	0·28	—	—	0·06	0·04	0·33	—	—	—	—	—	—	—
(2-Naphthylhydroxylamine *N*-glucosid)uronic acid§	—	—	—	—	—	0·33	—	—	—	—	—	—	—

* This substance, blue in colour, formed during chromatography of 2-acetamido-1-naphthol. On paper chromatograms it did not separate from 2-acetamido-1-naphthol.

† On paper chromatograms it gave an unidentified substance that did not react with sodium amminoprusside.

‡ Converted into 2-acetamido-1-naphthyl hydrogen sulphate and 2-acetamido-1-naphthol on paper chromatograms during the usual length of run (16 hr.); the conversion was least with solvent (*a*). The compound also decomposed on thin-layer chromatography in solvents (*i*) and (*m*).

§ On paper chromatograms it was converted into (2-naphthylamine *N*-glucosid)uronic acid.

solvents (*a*), (*d*) and (*f*) and only (2-naphthylamine *N*-glucosid)uronic acid was identified. Attempts to isolate (2-naphthylhydroxylamine *N*-glucosid)uronic acid gave only (2-naphthylamine *N*-glucosid)uronic acid.

RESULTS

Metabolism of 2-naphthylamine

Identification of 2-naphthylhydroxylamine as a metabolite of 2-naphthylamine in urine in the dog. 2-Naphthylamine was administered by mouth to a mongrel bitch after the emptying of the bladder. After 3hr. the animal was anaesthetized with pentobarbitone and a sample of urine (22ml.) was obtained by catheterization. Seven more specimens (mean volume 7ml.) were obtained during the next 4hr. All were extracted immediately with ether at pH7·0. The ether extracts were combined and evaporated to dryness in a stream of air at room temperature while shielded from the light. After thin-layer chromatography of the residue, three metabolites were detected that were identical in chromatographic properties with 2-naphthylamine, 2-nitrosonaphthalene and 2-naphthylhydroxylamine respectively (see Tables 1 and 2). On repetition of the experiment the same metabolites were observed. *N*-Acetyl-2-naphthylhydroxylamine and 2-amino-1-naphthol were not detected.

After the ether extracts were exposed to daylight for a few minutes 2-naphthylhydroxylamine was no longer detectable. When 2-naphthylhydroxylamine was added to an ether extract of dog urine, it was converted into 2-naphthylamine, whereas a solution in pure ether was stable for several hours. 2-Naphthylhydroxylamine could occur as an *N*-glucosiduronic acid, but neither (2-naphthylhydroxylamine *N*-glucosid)uronic acid nor (2-naphthylamine *N*-glucosid)uronic acid was detected by thin-layer chromatography of the urine in solvent (*a*).

When samples of urine were exhaustively extracted with ether and then left for 20hr. no more 2-naphthylamine or 2-naphthylhydroxylamine was formed. In another experiment a series of catheter specimens was obtained over 6hr. and each was extracted until 2-naphthylamine and 2-naphthylhydroxylamine were not detectable in the ether extracts. The specimens were each divided into two and one sample was incubated with Ketodase at pH5·0 for 16hr. and extracted with ether. No evidence for the release of 2-naphthylhydroxylamine in the enzyme-treated samples was obtained and only 2-amino-1-naphthol and 2-amino-6-naphthol were formed.

2-Naphthylhydroxylamine was not detected in 24hr. specimens of urine. After the addition of 2-naphthylhydroxylamine (50mg.) to normal dog urine (50ml.) it could not be detected in ether extracts made 24hr. later, when only 2-naphthylamine was found.

Another dog (female wire-haired terrier) was anaesthetized with pentobarbitone and the bladder emptied by catheterization. A suspension of 2-naphthylamine in water (100ml.) was given by stomach tube. After 1hr. a specimen of urine was obtained and an ether extract was examined by thin-layer chromatography. Only 2-naphthylamine was detected, but further specimens of urine that were obtained each hour for 4hr. also contained 2-nitrosonaphthalene and 2-naphthylhydroxylamine.

Examination of dog urine for conjugates of 2-naphthylhydroxylamine. Urine from eight beagles undergoing daily dosing with 2-naphthylamine was collected until a total dose of 25·6g. of amine had been given. The urine was adjusted to pH5·0 and treated with 20g. portions of charcoal until it was colourless. The charcoal was washed with water and was then stirred with several portions of methanol containing about 5% (v/v) of aq. ammonia (sp.gr. 0·88). The combined methanol extracts were evaporated to dryness at 40° under reduced pressure. The residue (approx. 22g.) was dissolved in solvent (*a*) and made into a paste with cellulose powder. The paste was added to the top of a cellulose-powder column (6cm. × 55cm.) prepared in the same solvent mixture. The column was developed and the eluate collected in suitable fractions, guided by paper-chromatographic examination. All the eluates containing 2-amino-1-naphthyl hydrogen sulphate were combined and examined by two-dimensional paper chromatography (solvents *a* and *b*), when 2-amino-1-naphthylmercapturic acid was also detected. The eluate was evaporated under reduced pressure after the addition of aq. ammonia, when 2-amino-1-naphthyl ammonium sulphate (total 8·5g.) separated. The chromatographic properties and infrared spectrum of this were identical with those of a synthetic specimen of 2-amino-1-naphthyl ammonium sulphate. It was possible that this fraction might contain the unknown 2-naphthylhydroxylamine-*O*-sulphonic acid, although the failure to synthesize it (see the Methods and Materials section) indicated that it was unstable and unlikely to withstand this method of isolation. The catheter specimens obtained in the experiments for the detection of 2-naphthylhydroxylamine were examined at the time of collection by thin-layer chromatography in solvent (*a*). No evidence was obtained of such a sulphuric ester and the spot of 2-amino-1-naphthyl hydrogen sulphate, which 2-naphthylhydroxylamine-*O*-sulphonic acid might resemble in colour reactions, did not behave differently from the authentic compound.

Table 2. *Colour reactions of 2-naphthylamine and some derivatives on chromatograms*

Details of the tests are given in the text.

Compound	HCl, $NaNO_2$ and hexylresorcinol	Red colour with *p*-dimethylaminocinnamaldehyde	Reduction of ammoniacal $AgNO_3$	Sodium ammino-prusside	Alkaline hexylresorcinol	Other tests
2-Naphthylamine	Yellow	Immediate	Slow	Green	None	
2-Acetamidonaphthalene	None	None	None	None	None	Gives the reactions of 2-naphthylamine after acid hydrolysis
2-Naphthylhydroxylamine	Yellow	Immediate	Immediate	Mauve	Blue–green	Yellow with 2N-NaOH
N-Acetyl-2-naphthylhydroxylamine	Transient green, becoming grey	Slow	Slow	None	None	Mauve with $FeCl_3$
2-Amino-1-naphthol	Blue*	Immediate	Immediate	None	Blue*	Blue with 2N-NaOH or NH_3 vapour
2-Acetamido-1-naphthol	Blue†	None	Immediate	Blue	None	Red with diazotized sulphanilic acid; blue with $FeCl_3$ or $HCl + NaNO_2$
N-Benzyloxycarbonyl-2-naphthylhydroxylamine	Transient green becoming grey	Slow	Slow	None	None	Mauve with $FeCl_3$
2-Nitrosonaphthalene	None	Slow	Slow	Mauve	Grey–green	Mauve with $TiCl_3$; brownish red with aniline
2-Amino-1-naphthyl hydrogen sulphate	Mauve	Immediate	Slow	Green	None	Spot turns reddish brown in sunlight
2-Acetamido-1-naphthyl hydrogen sulphate	None	None	None	None	None	Stable in 2N-NaOH; decomposes in 2N-HCl to give 2-acetamido-1-naphthol (see the Methods and Materials section)
N-Acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid	Blue–green‡	Slow	None	None	Blue–green	For breakdown in acid and alkali see the Methods and Materials section
N-Benzyloxycarbonyl-2-naphthylhydroxylamine-*O*-sulphonic acid	Blue–green‡	Slow	Slow	None	Blue–green	After treatment with 2N-HCl for 1 hr. gives blue colour with aq. $NaNO_2$.

2-Naphthylhydroxylamine-*N*-sulphonic acid	Yellow	Immediate	Immediate	Mauve (slow)	None	Yellow colour a few minutes after spraying with 2N-HCl
2-Amino-1-naphthyl dihydrogen phosphate	Reddish brown (mauve if diazotized with $NaNO_2$ and acetic acid)	Immediate	Slow	Green	None	
2-Amino-1-naphthylmercapturic acid	Yellow	Immediate	Slow	None	None	Positive reaction to $K_2Cr_2O_7$–$AgNO_3$ and platinic iodide reagents
Metabolite, probably *S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)-L-cysteine	None	None	None	None	None	Mauve with ninhydrin; pink fluorescence after treatment with 2N-HCl at room temperature followed by NH_3 vapour; diazotizes and couples with hexylresorcinol (orange–red colour) after treatment with hot 2N-HCl; positive reaction to $K_2Cr_2O_7$–$AgNO_3$ and platinic iodide reagents
(2-Amino-1-naphthyl glucosid)uronic acid	Reddish brown (mauve if diazotized with $NaNO_2$ and acetic acid)	Immediate	Slow	Green	None	
(2-Acetamido-1-naphthyl glucosid)uronic acid	None	None	None	None	None	Gives the reactions of the amino compound after treatment with hot 2N-HCl
(*N*-Acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid	Blue–green‡	Slow	None	None	Blue–green	Yellow with 2N-NaOH; after treatment with 2N-NaOH or 2N-HCl the spot is diazotizable owing to the formation of 2-naphthylamine
(2-Naphthylamine *N*-glucosid)uronic acid	Yellow	Immediate	Slow	Green	None	
(2-Naphthylhydroxylamine *N*-glucosid)-uronic acid	Yellow	Immediate	Immediate	Mauve	Blue–green	

* This colour is due to oxidation in the presence of alkali.

† This colour is due to the action of nitrous acid and no further colour is given with hexylresorcinol.

‡ This colour is due to breakdown to 2-naphthylhydroxylamine, which reacts with hexylresorcinol in alkaline solution (see the penultimate column of this Table and the Methods and Materials section).

Elution of the column was continued and fractions that contained (2-amino-1-naphthyl glucosid)-uronic acid were collected and evaporated to dryness. The residue was dissolved in water and treated with basic lead acetate at pH 7·5. The precipitate obtained was treated with hydrogen sulphide and, after removal of lead sulphide, the filtrate was evaporated to 3ml. and adjusted to pH 4·0. The solution was evaporated in a desiccator until a brown amorphous solid appeared, which was filtered off and crystallized from hot water to yield (2-amino-1-naphthyl glucosid)uronic acid (9mg.), m.p. 182–184°. It did not depress the m.p. of the compound isolated by Boyland & Manson (1957*a*) and had the same colour reactions and R_F values. When incubated with Ketodase at pH 5·0 or bacterial β-glucuronidase at pH 7·0 it yielded 2-amino-1-naphthol, which was identified by the green colour given with ammonia.

After the further elution of the column a fraction was collected that contained the metabolite, detected by Boyland, Kinder & Manson (1961*a*), that had R_F 0·07 in solvent (*a*) and R_F 0·42 in solvent (*b*). The eluate was evaporated to dryness and the residue was dissolved in the minimum of water. The solution was applied to Whatman 3MM chromatography paper and the chromatograms were developed in solvent (*b*). The metabolite was eluted from the paper with aq. 80% (v/v) methanol. The methanol extracts were evaporated to dryness and the residue was dissolved in a few drops of water and ethanol added. After cooling, a brown solid (60mg.) was collected that gave a positive reaction for glucuronic acid when heated with 5N-hydrochloric acid and naphtharesorcinol. It was unaffected by 2N-sodium hydroxide or 2N-hydrochloric acid at room temperature, but in 5N-hydrochloric acid at 100° for 30min. it gave 2-amino-1-naphthol. After heating for 10min. in N-hydrochloric acid (2-amino-1-naphthyl glucosid)-uronic acid was detected. Incubation with Ketodase gave 2-amino-1-naphthol and (2-amino-1-naphthyl glucosid)uronic acid but not 2-naphthylhydroxylamine. 2-Naphthylhydroxylamine was not changed into 2-amino-1-naphthol when incubated under the same conditions.

A solution of (2-amino-1-naphthyl glucosid)-uronic acid and glucuronic acid adjusted to pH 7·0 yielded a spot at the same R_F as the metabolite in solvent (*a*). In solvent (*b*), however, it hydrolysed to (2-amino-1-naphthyl glucosid)uronic acid, whereas the metabolite was stable.

Attempts to identify N*-hydroxy derivatives in the urine of rodents dosed with 2-naphthylamine.* (*a*) Rat. Four rats were dosed for 12 days with 2-naphthylamine. The urine was collected in vessels cooled with solid carbon dioxide and ethanol and extracted with ether as soon as it was passed. The extract was examined by thin-layer chromatography. 2-Naphthylhydroxylamine, 2-nitrosonaphthalene and *N*-acetyl-2-naphthylhydroxylamine were not detected, although 2-naphthylamine was always present. The ether extracts of the urine of both normal and 2-naphthylamine-treated rats contained substances that gave mauve colours with the sodium amminoprusside reagent but no colours with hexylresorcinol. The urine was treated with charcoal and the metabolites were eluted with ammoniacal methanol. (*N*-Acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid (properties described below) was not detected.

2-Naphthylhydroxylamine (20mg.) in ether (1ml.) was shaken with normal rat urine (50ml.), kept at room temperature for 15hr. and extracted with ether. Thin-layer chromatography of the ether extracts showed the presence of 2-nitrosonaphthalene and 2-naphthylamine.

(*b*) Guinea pig, hamster and rabbit. 2-Naphthylamine was administered to two of each of these species for 8 days. (*N*-Acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid was not detected.

Metabolites of 2-naphthylamine in bile. (*a*) Rat. Four rats with bile fistulae were dosed with 2-naphthylamine. The collecting vessels for the bile contained 0·5ml. of M-acetate buffer, pH 5·0. The pH of the bile after collection for 2hr. was approx. 6·5. Extraction of the bile with ether, evaporation of the extract at room temperature and examination of the residue by thin-layer chromatography showed the presence of 2-naphthylamine only. 2-Naphthylhydroxylamine was stable in bile at pH 6·5, and 25mg. added to 10ml. of bile and extracted with ether after 24hr. in the dark showed little conversion into 2-naphthylamine.

The bile from 15 rats after dosing with 2-naphthylamine was concentrated by the charcoal adsorption procedure. The metabolites were eluted with aq. phenol. 2-Amino-1-naphthyl hydrogen sulphate, (2-amino-1-naphthyl glucosid)uronic acid, (2-acetamido-1-naphthyl glucosid)uronic acid, 2-amino-1-naphthylmercapturic acid and (*N*-acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid were not detected in the residue when it was examined by paper chromatography. The first four metabolites were present in the urine. A rat dosed orally with 2-amino-1-naphthyl sodium sulphate (25mg.) excreted the sulphuric ester in the urine but not in the bile. The following *N*-acetyl derivatives were detected in both urine and bile: 2-acetamido-6-naphthol, 2-acetamido-5,6-dihydro-5,6-dihydroxynaphthalene, 2-acetamido-6-naphthyl hydrogen sulphate and (2-acetamido-6-naphthyl glucosid)uronic acid. (2-Naphthylamine *N*-glucosid)uronic acid was detected in the bile before charcoal adsorption.

The acid-labile mercapturic acid identified as

N-acetyl-*S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)cysteine by Boyland *et al.* (1963*b*) was found in most samples. Two other metabolites with similar properties to the acid-labile mercapturic acid were detected when two-dimensional paper chromatography of the residue was carried out in solvent (*a*) followed by solvent (*b*). The chromatograms were sprayed with 2N-hydrochloric acid after development in solvent (*a*), allowed to dry at room temperature, neutralized with ammonia vapour and developed in solvent (*b*). After this treatment with acid both metabolites had an orange–pink fluorescence. 2-Acetamido-6-naphthol was also detected on the chromatograms as a product of acidification of the metabolites. These chromatographic properties were similar to those of the glutathione and cysteinylglycine derivatives detected by Boyland *et al.* (1961*b*) as metabolites of naphthalene in bile. The major one (R_F 0·12 in solvent *a*) was further investigated in bile obtained after the administration of a total of 400mg. of 2-naphthylamine to four rats. The bile was treated with charcoal and the residue from evaporation of the phenol eluate was applied as streaks to 12 sheets of Whatman 3MM chromatography paper. The chromatograms were developed in solvent (*a*) and the areas with the metabolite were cut off and extracted with methanol containing 5% (v/v) of aq. ammonia (sp.gr. 0·88). The extracts were evaporated to a small volume and the residue was applied to Whatman 3MM chromatography paper. The chromatograms were developed in solvent (*b*) and the metabolite was eluted and heated in conc. hydrobromic acid (10ml.) under reflux. After 2hr. the solution was examined by paper chromatography in solvent (*b*). Two fluorescent spots that gave orange–red colours with hexylresorcinol after diazotization were present at R_F 0·5 and 0·55. Two ninhydrin-positive spots at R_F 0·38 and 0·28 were indistinguishable from glycine and glutamic acid when run in solvent (*b*) or in solvent (*c*), where they had R_F values 0·23 and 0·26 respectively. The metabolite originally present was probably *S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)glutathione, which gave glycine and glutamic acid on hydrolysis. The diazotizable products were probably *S*-(2-amino-5-naphthyl)glutathione and *S*-(2-amino-5-naphthyl)-cysteinylglycine. After further heating (4hr.) the amount of these decreased but the final product that would be expected, *S*-(2-amino-5-naphthyl)-cysteine, was not detected because it is destroyed by prolonged heating with acid. Normal bile subjected to the same procedure yielded only traces of glycine and glutamic acid. From the relationships of its R_F values to those of the other conjugates (cf. Boyland *et al.* 1961*b*) the second metabolite was probably the cysteinylglycine derivative, but insufficient was obtained for further examination.

A rat was dosed with 2-[8-^{14}C]naphthylamine (20μc) and unlabelled 2-naphthylamine (50mg.). The bile was collected for 48hr. Two-dimensional paper chromatograms of the bile and of the charcoal concentrates of the bile were prepared and radioautographs made. The radioautographs showed the metabolites described above but revealed that most of the dose was present as other metabolites that occupied a region between the base line to R_F 0·1 in solvent (*a*) (Table 3). They were eluted from charcoal with aq. phenol but not with ammoniacal methanol. These metabolites and the compounds that were probably the glutathione and cysteinylglycine conjugates were not detected in urine.

A rat was dosed with [1-^{14}C]naphthalene (20μc) and unlabelled naphthalene (50mg.). The bile was collected for 48hr. and treated with charcoal. The metabolites were eluted with aq. phenol and radioautographs were prepared of two-dimensional paper chromatograms. The metabolites described by Boyland *et al.* (1961*b*) were present but slow-running compounds comparable with those from 2-naphthylamine were not.

(*b*) Dog. The bile duct of a cholecystectomized female greyhound was catheterized and 2-naphthylamine (100mg.) and 2-[8-^{14}C]naphthylamine (50μc) in oil (2·5ml.) were injected intramuscularly into a hind leg. Bile was removed from the plastic bag at hourly intervals for 4hr. (total 18ml.). Urine was collected by catheterization after 4hr. The animal was released from the Pavlov stand and urine and bile (150ml.) were collected during 20hr. None of the bile samples contained (2-amino-1-naphthyl glucosid)uronic acid or 2-amino-1-naphthyl hydrogen sulphate, but these were detected in the urine. Traces of radioactivity were present near the base line of paper chromatograms developed in solvent (*a*) but nothing was detected after the chromatograms were developed in solvent (*b*).

Metabolism of 2-naphthylhydroxylamine

Dog. A beagle bitch was dosed with 2-naphthylhydroxylamine. Paper-chromatographic examination of the urine showed the same metabolites that were detected after the administration of 2-naphthylamine (Boyland *et al.* 1961*a*, 1963*b*). Subcutaneous injection of 2-naphthylhydroxylamine gave the same metabolites in the urine.

Rat. (*a*) Urine. Four rats given 2-naphthylhydroxylamine excreted the same metabolites in the urine as those found after the administration of 2-naphthylamine, i.e. a mixture of 1-substituted

Table 3. *Paper chromatography of some metabolites of 2-naphthylamine in bile in the rat*

Compositions of solvents and other details are given in the text.

Compound	R_F Solvent (*a*)	R_F Solvent (*b*)	Fluorescence	Reaction with ninhydrin	Reaction with $K_2Cr_2O_7$–$AgNO_3$	
N-Acetyl-*S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)-L-cysteine:						
Before acid treatment	0·26	0·80	None	None	+	These metabolites give 2-acetamido-6-naphthol on treatment with 2N-HCl at room temperature; after heating with 2N-HCl they diazotize and couple with hexylresorcinol to give orange–red colours
After acid treatment*	0·41	0·94	Orange–pink	None	+	
Metabolite, probably *N*-acetyl-*S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)-glutathione:						
Before acid treatment	0·12	0·65	None	+	+	
After acid treatment*	0·24	0·66	Orange–pink	+	+	
Metabolite, probably *N*-acetyl-*S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)-cysteinylglycine:						
Before acid treatment	0·16	0·65	None	+	+	
After acid treatment*	0·30	0·70	Orange–pink	+	+	
Unidentified metabolites:						
Major spots	0·00	0·07	None	+	+	These metabolites do not give 2-acetamido-6-naphthol or show a change in R_F value or the development of an orange–pink fluorescence on treatment with 2N-HCl; after heating with 2N-HCl they diazotize and couple with hexylresorcinol to give orange–red colours
	0·03	0·15	None	+	+	
	0·09	0·30	None	+	+	
Minor spots	0·03	0·14	None	+	+	
	0·06	0·30	None	+	+	
	0·06	0·40	None	+	+	

* The spots were treated for a few minutes with 2N-HCl at room temperature and then neutralized with NH_3 vapour.

derivatives of 2-naphthylamine and 6- and 5,6-substituted derivatives of 2-acetamidonaphthalene. Larger doses or an equal dose on the next day caused collapse and death of some of the animals.

Potassium 2-naphthylhydroxylamine-*N*-sulphonate (30mg.) given intraperitoneally in water (0·5ml.) to each of four rats was excreted unchanged. 2-Naphthylhydroxylamine-*N*-sulphonic acid was not detected in the urine of dogs, rats and rabbits after the administration of either 2-naphthylamine or 2-naphthylhydroxylamine, although the two latter species excrete 2-naphthylsulphamic acid as a metabolite of 2-naphthylamine (Boyland *et al.* 1957).

(*b*) Bile. Bile from two rats dosed with 2-naphthylhydroxylamine contained the same metabolites as were found after the administration of 2-naphthylamine.

Guinea pig. Four guinea pigs were dosed for 4 days with 2-naphthylhydroxylamine and after an interval of 3 days two further doses were given. The urine contained the same metabolites as were identified after dosing with 2-naphthylamine (Boyland *et al.* 1963*b*), i.e. mainly 6- and 5,6-substituted derivatives of 2-acetamidonaphthalene. The animals were killed 16hr. after the last dose. The peritoneal cavities contained oil in which 2-naphthylamine, but not 2-naphthylhydroxylamine, was detected by thin-layer chromatography.

Hamster. Single doses of 2-naphthylhydroxylamine given to each of two hamsters caused prostration of the animals. The urine contained the same metabolites as were found after the administration of 2-naphthylamine (Boyland *et al.* 1963*b*) and also 2-amino-1-naphthylmercapturic acid. The acid-labile mercapturic acid was not detected.

Rabbit. A rabbit was injected with 2-naphthylhydroxylamine. 2-Naphthylamine, but not 2-naphthylhydroxylamine, was detected in ether extracts of the urine collected 4hr. after the dose. (2-Naphthylamine *N*-glucosid)uronic acid and 2-naphthylsulphamic acid were detected in the urine. Treatment of the urine with charcoal and examination of the methanol eluate showed that 2-amino-1-naphthyl derivatives were the predominant metabolites. They included 2-amino-1-naphthylmercapturic acid, which was not found as a metabolite of 2-naphthylamine in this species (Boyland *et al.* 1963*b*).

Metabolism of N-*acetyl*-2-*naphthylhydroxylamine*

Rabbit. A rabbit was injected with *N*-acetyl-2-naphthylhydroxylamine for 5 days and the urine treated with lead acetate at pH 4·0. The precipitate was discarded and the filtrate was treated with basic lead acetate at pH 8·0; the precipitate was treated with hydrogen sulphide, lead sulphide removed by filtration and the filtrate evaporated to dryness *in vacuo* at 40°. The gum was dissolved in water and applied to Whatman 3MM chromatography paper with a camel-hair brush. The chromatograms were developed in solvent (*a*). (*N*-Acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid, the principal metabolite, was detected by the bluish-green colour it gave after a few minutes with alkaline hexylresorcinol. The areas with the metabolite were cut out and extracted with aq. 90% (v/v) methanol. The eluate was evaporated to a gum (90mg.) that gave a naphtharesorcinol reaction. The gum was dissolved in water, acidified with acetic acid and evaporated to dryness in a desiccator. The residue was dissolved in methanol and treated with an excess of diazomethane in ether for 16hr. Two treatments were necessary for complete methylation and some 2-naphthylamine was formed during the procedure. After removal of the ether–methanol mixture the methyl ester was treated with acetic anhydride (1ml.) in pyridine (1ml.) for 16hr. The reaction mixture was diluted with water and cooled. The precipitate was collected, dried and crystallized from aq. ethanol to yield the *triacetyl methyl ester of* (N-*acetyl*-2-*naphthylhydroxylamine* O-*glucosid*)-*uronic acid*, m.p. 124–125° (Found: C, 57·9; H, 5·5; N, 3.2. $C_{25}H_{27}O_{11}N$ requires C, 58·0; H, 5·3; N, 2·7%).

The gum obtained from the separation by paper chromatography yielded an ether-soluble product on treatment with Ketodase at pH 5·0 for 6hr. that was indistinguishable from *N*-acetyl-2-naphthylhydroxylamine in its behaviour on thin-layer chromatography. 2-Acetamido-1-naphthol was not detected, indicating that (2-acetamido-1-naphthyl glucosid)uronic acid was not present and that (*N*-acetyl-2-naphthylhydroxylamine *O*-glucosid)-uronic acid did not rearrange during hydrolysis. When sprayed with hexylresorcinol in M-sodium carbonate the metabolite gave no colour reaction and remained unchanged, as spraying with 2N-sodium hydroxide 24hr. later elicited the colour reaction. The colour reaction given by the glucosiduronic acid was identical with that given by 2-naphthylhydroxylamine with hexylresorcinol and the delay before the appearance of the colour is presumably due to the time required for liberation of the arylhydroxylamine. *N*-Acetyl-2-naphthylhydroxylamine is unaffected by 2N-sodium hydroxide and does not give the hexylresorcinol colour reaction. The metabolite broke down rapidly in 2N-hydrochloric acid or 2N-sodium hydroxide to yield 2-naphthylamine and a trace of 2-acetamido-1-naphthol. *N*-Acetyl-2-naphthylhydroxylamine was not detected. One preparation of the basic lead acetate precipitate from the urine

was dissolved in dilute acetic acid and treated with charcoal. The metabolite was eluted with methanol–aq. ammonia solution, after washing the charcoal with water to remove lead acetate. After incubation with Ketodase for 16hr. the residue from the eluate gave only *N*-acetyl-2-naphthylhydroxylamine, although much of the metabolite remained unchanged.

Neither *N*-acetyl-2-naphthylhydroxylamine nor 2-acetamido-6-naphthol was detected in ether extracts of the rabbit urine. *N*-Acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid was not detected on thin-layer chromatograms of the urine. Treatment of the urine with charcoal and examination of the methanol by two-dimensional paper chromatography in solvent (*a*) followed by solvent (*b*) showed the presence of the metabolites listed in Table 4. Detection of (2-acetamido-1-naphthyl glucosid)uronic acid in the presence of (*N*-acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid required the removal of the latter compound, as they could not be separated. After development in solvent (*a*) the chromatogram was sprayed with 2N-hydrochloric acid and left for 2hr. to decompose the *N*-acetyl-2-naphthylhydroxylamine conjugate. The paper was neutralized with ammonia vapour and developed in solvent (*b*), but (2-acetamido-1-naphthyl glucosid)uronic acid was not found.

Guinea pig. Two guinea pigs were injected with *N*-acetyl-2-naphthylhydroxylamine for 4 days and the urine was collected. Urine (10ml.) was incubated with Ketodase (1ml.) at pH5·0 for 16hr. and extracted with ether; the ether extract contained 2-acetamido-6-naphthol, but neither *N*-acetyl-2-naphthylhydroxylamine nor 2-acetamido-1-naphthol. Paper chromatography of the urine and of charcoal concentrates showed that (*N*-acetylnaphthylhydroxylamine *O*-glucosid)uronic acid, (2-amino-1-naphthyl glucosid)uronic acid and 2-amino-1-naphthyl hydrogen sulphate were present. 2-Amino-1-naphthyl hydrogen sulphate was not detected in guinea-pig urine as a metabolite of 2-naphthylamine or 2-acetamidonaphthalene. Other metabolites are given in Table 4.

Rat. Four rats were dosed with *N*-acetyl-2-naphthylhydroxylamine for 6 days. No *N*-acetyl-2-naphthylhydroxylamine was detected in ether extracts of the urine, but (*N*-acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid was identified in the urine. As with guinea-pig urine, the metabolite did not appear to be hydrolysed in urine by Ketodase or bacterial glucuronidase. Concentrates of the urine prepared by charcoal adsorption or by lead acetate precipitation did not yield *N*-acetyl-2-naphthylhydroxylamine on treatment with glucuronidase, but gave 2-acetamido-1-naphthol and traces of 2-acetamido-6-naphthol. Only after the glucosiduronic acid fraction had been purified by separation on Whatman 3MM paper in solvent (*a*) did treatment with Ketodase or bacterial glucuronidase yield *N*-acetyl-2-naphthylhydroxylamine.

2-Acetamido-1-naphthyl hydrogen sulphate and traces of the 6- and 5,6-substituted derivatives of 2-acetamidonaphthalene were also present (Table 4), but not *N*-acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid.

Two rats with bile fistulae were given *N*-acetyl-2-naphthylhydroxylamine for 4 days. The bile contained (*N*-acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid and a trace of 2-naphthylamine, but neither (2-naphthylamine *N*-glucosid)-uronic acid nor 2-acetamido-1-naphthyl hydrogen sulphate was found. The slow-running compounds detected on chromatograms of bile from rats dosed with 2-naphthylamine were not present.

Metabolism of potassium N-*acetyl*-2-*naphthylhydroxylamine*-O-*sulphonate in rats*

Two rats were each injected with potassium *N*-acetyl-2-naphthylhydroxylamine-*O*-sulphonate (30mg.) in water (1ml.). The urine was collected 1½, 3½ and 5½hr. after injection and extracted with ether and examined by thin-layer chromatography. 2-Naphthylhydroxylamine and *N*-acetyl-2-naphthylhydroxylamine were not detected. Paper and thin-layer chromatography showed that the urine contained 2-amino-1-naphthyl hydrogen sulphate, 2-acetamido-1-naphthyl hydrogen sulphate and (2-acetamido-1-naphthyl glucosid)uronic acid. Conjugates of 2-naphthylhydroxylamine and *N*-acetyl-2-naphthylhydroxylamine were not detected.

Metabolism of 2-acetamidonaphthalene

Dog. A mongrel bitch was dosed for 3 days with 2-acetamidonaphthalene. *N*-Acetyl-2-naphthylhydroxylamine was not detected in ether extracts of the urine. Three portions (30ml.) of the combined urine specimens were incubated with Takadiastase, acid phosphatase and Ketodase separately for 16hr. Ether extracts of the preparations incubated with or without the enzymes contained 2-acetamido-6-naphthol, but neither *N*-acetyl-2-naphthylhydroxylamine nor 2-acetamido-1-naphthol. The remaining urine was concentrated by the charcoal procedure and shown to contain (*N*-acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid.

Paper chromatograms of the urine and of charcoal concentrates showed a different pattern of metabolites from those of 2-naphthylamine in the dog and the only 1-substituted compound detected was 2-amino-1-naphthyl hydrogen sulphate (see Table 4). The urine contained a substance that was similar to the acid-labile mercapturic acid

found as a metabolite of 2-naphthylamine and 2-acetamidonaphthalene from rats and rabbits (Boyland *et al.* 1963*b*). After paper chromatography with solvent (*a*) and treatment with 2N-hydrochloric acid followed by solvent (*b*) the compound yielded 2-acetamido-6-naphthol and a compound that gave a pink fluorescence in ultraviolet light after treatment with ammonia vapour. It could be diazotized and coupled only after treatment with hot 2N-hydrochloric acid. The metabolite differed from the acid-labile mercapturic acid in giving a positive reaction with ninhydrin and the compound was probably *S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)-L-cysteine. A trace of the acid-labile mercapturic acid was also detected.

Rat, guinea pig, hamster and rabbit. Rats, guinea pigs, hamsters (two of each species) and one rabbit were each dosed with 2-acetamidonaphthalene for 5 days. Neither *N*-acetyl-2-naphthylhydroxylamine nor its glucosiduronic acid was detected in the urine. The metabolites that were detected were the same as those found after the administration of 2-naphthylamine (Boyland & Manson, 1957*a*, 1958*a*; Boyland *et al.* 1963*b*).

Two rats with bile fistulae were dosed for 4 days with 2-acetamidonaphthalene. The bile contained the same metabolites as were found after dosing with 2-naphthylamine.

DISCUSSION

The detection of 2-naphthylhydroxylamine as a metabolite of 2-naphthylamine in urine in dogs agrees with the findings of Troll & Nelson (1961), who also identified it in urine from human subjects. 2-Naphthylhydroxylamine (Boyland, Dukes & Grover, 1963*a*; Bonser *et al.* 1963) and *N*-acetyl-2-naphthylhydroxylamine (Bryan, Brown & Price, 1963) are carcinogenic. The occurrence of 2-naphthylhydroxylamine as a metabolite of 2-naphthylamine in men and dogs may account for the carcinogenic action of 2-naphthylamine on the bladders of these species.

N-Acetyl-2-naphthylhydroxylamine is not excreted in the free state or as a sulphuric ester, and its glucuronic acid conjugate was hydrolysed with difficulty by glucuronidase. 2-Naphthylhydroxylamine is unstable in urine and, although it appears to be present as the free compound, it may be excreted as a labile *N*-glucosiduronic acid or a similar derivative. Boyland *et al.* (1961*a*) detected four compounds in the urine of dogs after the administration of 2-naphthylamine that yielded 2-amino-1-naphthyl hydrogen sulphate on treatment with acid. One of these was (2-amino-1-naphthyl hydrogen sulphate *N*-glucosid)uronic acid, which can be readily detected, probably because it is in equilibrium with a large amount of amino compound, whereas the amount of 2-naphthylhydroxylamine is small. Although 2-naphthylamine also occurred in dog urine no *N*-glucosiduronic acid of this amine was detected. Hueper, Wiley & Wolfe (1938) found free glucuronic acid in urine from dogs dosed with 2-naphthylamine.

There was no evidence of a sulphuric ester of 2-naphthylhydroxylamine. *N*-Acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid was excreted, by rats as 2-amino-1-naphthol derivatives, and if 2-naphthylhydroxylamine-*O*-sulphonic acid is formed from 2-naphthylamine by dogs it probably undergoes rearrangement before excretion.

2-Amino-1-naphthyl hydrogen sulphate is the main metabolite of 2-naphthylamine in dogs, which excrete a higher proportion of 2-naphthylamine as this metabolite than do other species. This may be due to the absence of acetylation, so that more *N*-hydroxylation occurs, followed by the excretion of 2-amino-1-naphthyl hydrogen sulphate and 2-naphthylhydroxylamine.

2-Naphthylhydroxylamine and 2-nitroso-naphthalene were not detected as metabolites of 2-naphthylamine in rats although the dose given to rats was relatively higher than that given to dogs. The rat excretes a greater range of metabolites, of which many are substituted in the ring remote from the amino group as a consequence of acetylation. 2-Naphthylamine was always present in the urine.

2-Naphthylhydroxylamine was excreted by hamsters and rabbits as 2-amino-1-naphthyl hydrogen sulphate, (2-amino-1-naphthyl glucosid)-uronic acid and 2-amino-1-naphthylmercapturic acid; the last-named compound has not been identified as a metabolite of 2-naphthylamine in hamsters or rabbits (Boyland *et al.* 1963*b*). In contrast with the metabolism of 2-naphthylamine and 2-acetamidonaphthalene, rabbits excreted only traces of the 6- and 5,6-substituted derivatives of 2-acetamidonaphthalene. The predominance of 2-amino-1-naphthyl derivatives supports the hypothesis that they are derived from 2-naphthylhydroxylamine. As *N*-acetyl-2-naphthylhydroxylamine or its glucuronic acid conjugate was not detected it appears that 2-naphthylhydroxylamine is not acetylated *in vivo*.

In guinea pigs 2-naphthylhydroxylamine appeared to be reduced and acetylated before the formation of 2-amino-1-naphthyl hydrogen sulphate or mercapturic acid could take place. In dogs and rats no difference between the metabolism of 2-naphthylamine and 2-naphthylhydroxylamine was found.

2-Acetamidonaphthalene was excreted as (*N*-acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid by dogs but not by the other species. Poirier

Table 4. *Metabolites of 2-naphthylamine and some derivatives detected in urine of different species*

The Table does not include 2-formamido-1-naphthyl hydrogen sulphate (Boyland & Manson, 1966), 2-nitrosonaphthalene (this paper), (2-naphthylamine *N*-glucosid)uronic acid and 2-naphthylsulphamic acid (Boyland *et al.* 1957) and bis-(2-amino-1-naphthyl) hydrogen phosphate (Boyland *et al.* 1961*a*). The *N*-acetyl compounds in the '6- and 5,6-' columns include conjugates of both 2-acetamido-6-naphthol and 2-acetamido-5,6-dihydro-5,6-dihydroxynaphthalene (found in rat, rabbit, hamster and guinea-pig urine after dosing with 2-naphthylamine or 2-acetamidonaphthalene; Boyland & Manson, 1958*a*; Boyland *et al.* 1963*b*) and in dog urine after 2-acetamidonaphthalene only (this paper). 2-Acetamido-6-hydroxy-5-naphthyl hydrogen sulphate was found in rat and rabbit urine after dosing with 2-naphthylamine or 2-acetamidonaphthalene (Boyland & Manson, 1958*a*) and in dog urine after 2-acetamidonaphthalene (this paper). ++ indicates the principal metabolites or groups of metabolites when compared with those marked +; tr. indicates spots that gave only faint colour reactions; 0 indicates metabolites that were looked for with the aid of reference compounds but were not detected. Vacant spaces have been left where reference compounds were not available for comparison but no evidence was obtained to indicate the presence of these metabolites. Dogs and hamsters were not dosed with *N*-acetyl-2-naphthylhydroxylamine.

Compound administered ...		2-Naphthylamine						2-Acetamidonaphthalene					
	Position of hydroxyl or thio ether group in the metabolite...	*N*-		1-		6- and 5,6-		*N*-		1-		6- and 5,6-	
	Group in 2-position of metabolite ...	Hydroxyl-amino	*N*-Acetyl-hydroxyl-amino	Amino	Acet-amido	Amino	Acet-amido	Hydroxyl-amino	*N*-Acetyl-hydroxyl-amino	Amino	Acet-amido	Amino	Acet-amido
Species	Metabolite												
Dog	Free hydroxyl	+	0	0	0		0	0	0				+
	Sulphuric ester			++	0	tr.	0			tr.	0	0	+
	Glucosiduronic acid		0	+	0	tr.	0	0	+	0	0	0	++
	Mercapturic acid			+			0			0			++*
Guinea pig	Free hydroxyl		0				+		0				+
	Sulphuric ester			0	0	0	+			0	0	0	+
	Glucosiduronic acid		0	+	+	0	++	0	0	+	+	0	++
	Mercapturic acid			0			tr.			0			tr.
Hamster	Free hydroxyl		0				0		0				+
	Sulphuric ester			tr.	0	0	+			tr.	0	0	+
	Glucosiduronic acid		0	+	+	0	++	0	0	+	+	0	++
	Mercapturic acid			0			0			0			0
Rabbit	Free hydroxyl		0				+		0				+
	Sulphuric ester			tr.	0	0	+			tr.	0	0	+
	Glucosiduronic acid		0	+	0	0	++	0	0	+	0	0	++
	Mercapturic acid			0			++			0			++
Rat	Free hydroxyl	0	0				+	0	0				+
	Sulphuric ester			+	0	tr.	++			+	0	tr.	++
	Glucosiduronic acid		0	+	+	tr.	++	0	0	+	+	tr.	++
	Mercapturic acid			+			++			+			++

Compound administered ...		2-Naphthylhydroxylamine						*N*-Acetyl-2-naphthylhydroxylamine					
	Position of hydroxyl or thio ether group in the metabolite...	*N*-		1-		6- and 5,6-		*N*-		1-		6- and 5,6-	
	Group in 2-position of metabolite ...	Hydroxyl-amino	*N*-Acetyl-hydroxyl-amino	Amino	Acet-amido	Amino	Acet-amido	Hydroxyl-amino	*N*-Acetyl-hydroxyl-amino	Amino	Acet-amido	Amino	Acet-amido
Species	Metabolite												
Dog	Free hydroxyl		0				0						
	Sulphuric ester			++	0	tr.	0						
	Glucosiduronic acid		0	+	0	tr.	0						
	Mercapturic acid			+			0						
Guinea pig	Free hydroxyl						tr.		0				+
	Sulphuric ester			0	0	0	tr.		0	+	0	0	tr.
	Glucosiduronic acid		0	+	+	0	++		+	+	0	0	++
	Mercapturic acid			0			tr.						tr.
Hamster	Free hydroxyl						+						
	Sulphuric ester			tr.	0	0	+						
	Glucosiduronic acid		0	+	+	0	++						
	Mercapturic acid			+			0						
Rabbit	Free hydroxyl	0	0				tr.		0				0
	Sulphuric ester			++	0	0	tr.		0	tr.	0	0	tr.
	Glucosiduronic acid		0	+	0	0	tr.		++	tr.	0	0	tr.
	Mercapturic acid			+			tr.						0
Rat	Free hydroxyl						+		0				tr.
	Sulphuric ester			+	0	0	++		0	tr.	+	0	tr.
	Glucosiduronic acid		0	+	+	0	++		+	tr.	+	0	tr.
	Mercapturic acid			+			++						0

* Principally the cysteine derivative; only traces of the *N*-acetylcysteine derivative (mercapturic acid) were detected.

et al. (1963) found only a small amount of *N*-acetyl-2-naphthylhydroxylamine as a metabolite of 2-acetamidonaphthalene from dogs. Enomoto, Lotlikar, Miller & Miller (1962) detected only traces of *N*-acetyl-2-naphthylhydroxylamine in the urine of monkeys after the ingestion of 2-naphthylamine or 2-acetamidonaphthalene. Troll, Belman & Rinde (1963) detected *N*-acetyl-2-naphthylhydroxylamine in some human urines as a metabolite of 2-acetamidonaphthalene. *N*-Hydroxylation does not seem to be a major reaction in the metabolism of 2-acetamidonaphthalene, and Booth & Boyland (1964) were unable to demonstrate *N*-hydroxylation of 2-acetamidonaphthalene by rabbit-liver microsomes. If the amounts of 2-amino-1-naphthyl derivatives excreted depend on *N*-hydroxylation, then this reaction must proceed to a greater extent with 2-naphthylamine than with 2-acetamidonaphthalene. Acetylation may deactivate the nitrogen atom and the 1-position of the nucleus through the inductive effect of the carbonyl group. Bonser, Clayson & Jull (1951) showed that the dog excretes 30–70% of administered 2-naphthylamine as 2-amino-1-naphthol conjugates (mainly sulphate), with only small amounts of conjugates of 2-amino-6-naphthol. Clayson (1950) found that after administration of 2-acetamidonaphthalene less than 5% of the dose was excreted as 2-amino-1-naphthol conjugates, but large amounts of 2-acetamido-6-naphthol derivatives (measured as 'diazotizable amine' after acid hydrolysis) were excreted. (*N*-Acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid would have contributed to the 'diazotizable amine', as this conjugate yields 2-naphthylamine. Similarly, 2-amino-1-naphthylmercapturic acid would be diazotizable under the conditions of estimation, but the amounts of these metabolites are small. *N*-Acetyl-2-naphthylhydroxylamine was excreted only as a glucosiduronic acid derivative. Unlike acetanilide (Bray, James, Thorpe & Wasdell, 1950), 2-acetamidonaphthalene was only slightly deacetylated in the dog and was oxidized mainly on the ring remote from the acetamido group. It was excreted as an acid-labile cysteine derivative rather than an acid-labile mercapturic acid.

Booth & Boyland (1964) showed that *N*-acetyl-2-naphthylhydroxylamine was converted into 2-acetamido-1-naphthol by the soluble fractions of rat or rabbit liver. In the present work the administration of *N*-acetyl-2-naphthylhydroxylamine to a rabbit gave only traces of 2-amino-1-naphthol conjugates in the urine, and (*N*-acetyl-2-naphthylhydroxylamine *O*-glucosid)uronic acid was the principal metabolite.

In guinea pigs 2-amino-1-naphthyl hydrogen sulphate was formed as a metabolite of *N*-acetyl-2-naphthylhydroxylamine, but this sulphuric ester was not excreted after the administration of 2-naphthylamine or 2-naphthylhydroxylamine (probably owing to reduction to the amine), although (2-amino-1-naphthyl glucosid)uronic acid was found. The observation that 2-amino-1-naphthyl hydrogen sulphate was excreted only after the administration of *N*-acetyl-2-naphthylhydroxylamine may indicate that this species does not *N*-hydroxylate 2-naphthylamine. Among the species so far investigated only the guinea pig and the steppe lemming are unable to *N*-hydroxylate 2-acetamidofluorene (Weisburger *et al.* 1964). The glucosiduronic acids of 2-amino-1-naphthol and 2-acetamido-1-naphthol excreted by the guinea pig (Boyland *et al.* 1963*b*) may be formed by ring-hydroxylation of 2-naphthylamine followed by conjugation with glucuronic acid.

The metabolism of *N*-acetyl-2-naphthylhydroxylamine was similar in rats, except that 2-acetamido-1-naphthyl hydrogen sulphate and (2-acetamido-1-naphthyl glucosid)uronic acid were also excreted. *N*-Acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid was converted into 2-acetamido-1-naphthyl hydrogen sulphate and (2-acetamido-1-naphthyl glucosid)uronic acid by rats.

N-Acetyl-2-naphthylhydroxylamine yields a glucosiduronic acid that is readily detected by its characteristic colour reaction with hexylresorcinol. The failure to identify this glucosiduronic acid as a metabolite of 2-naphthylamine or 2-acetamidonaphthalene (except in small amounts in dog urine with the latter compound only) indicates that *N*-acetyl-2-naphthylhydroxylamine is not a major metabolite of these compounds.

Three metabolites found in dog urine, 2-naphthylhydroxylamine, (2-amino-1-naphthyl glucosid)uronic acid and bis-(2-amino-1-naphthyl) hydrogen phosphate, are carcinogenic when implanted in the bladders of mice (Allen, Boyland, Dukes, Horning & Watson, 1957; Bonser *et al.* 1963). 2-Naphthylamine induces tumours of the bladder in dogs and men. Bis-(2-amino-1-naphthyl) hydrogen phosphate has been found as a metabolite in the dog (Troll, Belman & Nelson, 1959; Boyland *et al.* 1961*a*) and in man (Troll, Tessler & Nelson, 1963). (2-Amino-1-naphthyl glucosid)uronic acid is a metabolite of 2-naphthylamine in the dog (Boyland *et al.* 1961*a*, 1963*b*; Troll, Belman, Nelson, Levitz & Twombly, 1959), in the rat, rabbit, guinea pig, hamster and mouse (Boyland *et al.* 1963*b*; Dewhurst, 1963) and in man (Levitz, Seif & Twombly, 1959). Both the phosphoric ester and the glucosiduronic acid could yield 2-amino-1-naphthol by enzymic hydrolysis in the urine or in the bladder wall (Boyland, 1963). Boyland *et al.* (1961*a*) were unable to demonstrate the enzymic hydrolysis of the phosphoric ester by dog urine, although it hydrolyses spontaneously at pH 5·0. (2-Amino-1-

naphthyl glucosid)uronic acid is hydrolysed by glucuronidase, but the hydrolysis is slow at the pH of normal urine (Boyland & Williams, 1960) and (2-amino-1-naphthyl glucosid)uronic acid has a lower affinity for the enzyme than (2-acetamido-1-naphthyl glucosid)uronic acid or (2-acetamido-6-naphthyl glucosid)uronic acid. The two last-named glucosiduronic acids may be competing substrates in the urine of rodents, but not in dog urine where they are not found as metabolites. In man, the metabolites excreted appear to be similar to those of rodents (Twombly, Zomzely & Meislich, 1957; Levitz *et al.* 1959). After a study of the metabolism of 1-naphthylamine, Clayson & Ashton (1963) suggested that excess of 1-amino-4-naphthol conjugates could prevent the release of the carcinogenic 1-amino-2-naphthol. This could account for the fact that 1-naphthylamine is less carcinogenic than 2-naphthylamine. The cat does not excrete (2-amino-1-naphthyl glucosid)uronic acid as a metabolite of 2-naphthylamine (Boyland *et al.* 1963*b*), and G. M. Bonser (unpublished work cited by Clayson, 1962) failed to obtain tumours in this species with 2-naphthylamine.

2-Amino-1-naphthyl hydrogen sulphate is the major metabolite of 2-naphthylamine in the dog and the cat. The effect of this compound on the bladder of the dog is not known but it is not carcinogenic when implanted in the bladders of mice (Bonser, Bradshaw, Clayson & Jull, 1956); the inactivity may be due to the inability of sulphatase to hydrolyse the ester to 2-amino-1-naphthol (Boyland, Manson, Sims & Williams, 1956; Boyland & Williams, 1960). Nevertheless, as the injection of 2-amino-1-naphthyl hydrogen sulphate into rats is followed by the excretion of (2-amino-1-naphthyl glucosid)uronic acid (Boyland & Manson, 1963), the sulphate group can be removed *in vivo*.

More than one metabolite may be involved in aromatic amine carcinogenesis. Arylhydroxylamines can be precursors of aminophenols, as indicated by the metabolism of 2-naphthylhydroxylamine in the rabbit. *o*-Aminophenols cannot be excluded as possible carcinogens, as 3-hydroxyanthranilic acid and 3-hydroxykynurenine give tumours (Allen *et al.* 1957; Bryan *et al.* 1963).

The absence of 2-amino-1-naphthol conjugates from the bile of the rat or of the dog may be the reason that 2-naphthylamine does not induce intestinal tumours. The compounds with low R_F values in solvent (*b*) present in rat bile that can be diazotized only after acid hydrolysis may be peptide derivatives of 2-acetamidonaphthalene analogous to the mercapturic acid detected by Boyland *et al.* (1963*b*) and the glutathione conjugate.

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