Isolation of 2-Amino-1-naphthyl Hydrogen Sulphate with Cetylpyridinium Bromide from Metabolic and Chemical Oxidations of 2-Naphthylamine

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1. 2-Amino-1-naphthyl hydrogen sulphate can be rapidly isolated from the urine of dogs dosed with 2-naphthylamine by precipitation with cetylpyridinium bromide. 2. No evidence was obtained for the presence of 2-naphthylhydroxylamine-*O*-sulphonic acid, noteworthy as a possible source of the carcinogens, 2-naphthylhydroxylamine and 2-amino-1-naphthol. 3. Treatment of neutral persulphate oxidations of 2-naphthylamine with the reagent gave only the cetylpyridinium salt of 2-amino-1-naphthyl hydrogen sulphate.

2-Naphthylhydroxylamine is a metabolite of the bladder carcinogen 2-naphthylamine excreted by dogs (Troll & Nelson, 1961; Boyland & Manson, 1966*b*; Uehleke & Brill, 1968). A source of the free arylhydroxylamine might be the unknown 2-naphthylhydroxylamine-*O*-sulphonic acid. This, by analogy with the properties of *N*-formyl-2-naphthylhydroxylamine-*O*-sulphonic acid (Boyland & Manson, 1966*a*) could give mainly 2-amino-1-naphthyl hydrogen sulphate by rearrangement and also break down to 2-naphthylhydroxylamine and 2-amino-1-naphthol. Some of the 2-amino-1-naphthyl hydrogen sulphate, the major metabolite excreted by dogs, may be formed in this way. 2-Amino-1-naphthyl hydrogen sulphate has always been isolated after prolonged procedures and by precipitation with mineral acid, methods which cause the breakdown and rearrangement of the known arylhydroxylamine-*O*-sulphonic acids (Boyland & Nery, 1962; Boyland & Manson, 1966*a,b*). These esters are of interest in carcinogenesis by aromatic amines because of the reactivity with cell components that has been demonstrated for similar derivatives of *N*-hydroxy-2-acetamidofluorene (Miller & Miller, 1969). Precipitation with cetylpyridinium bromide was employed to obtain the sulphuric ester fraction rapidly from catheter specimens of dog urine. The technique was also used to investigate the persulphate oxidation of 2-naphthylamine. The *ortho*-orientation of the sulphatoxy group in this method of oxidation of aromatic amines suggests that arylhydroxylamine sulphuric esters might be intermediates (Boyland,

Manson & Sims, 1953; Behrman, 1967; Venkatasubramanian & Sabesan, 1969).

The persulphate oxidation was carried out under neutral conditions. Both the metabolic and chemical experiments gave only the cetylpyridinium salt of 2-amino-1-naphthyl hydrogen sulphate. The persulphate oxidation of 2-naphthylsulphamic acid (Boyland, Manson & Orr, 1957) was also re-examined, but a 2-amino-1-naphthol derivative was the only product isolated.

MATERIALS AND METHODS

Chromatography. Whatman no. 1 chromatography paper was used for descending chromatograms with the following solvent systems (a) butan-1-ol-propan-1-ol-water (2:1:1, by vol.); (b) butan-1-ol-propan-1-ol-0.1M-NH₃ (2:1:1, by vol.); (c) as (a) with 3g of cetylpyridinium bromide per 100ml; (d) butan-1-ol-ethanol-water (3:1:1, by vol.) with 3g of cetylpyridinium bromide per 100ml (Rees, 1960). T.l.c. was carried out on glass plates coated with silica gel G (E. Merck A.G., Darmstadt, West Germany) (0.25mm thick) with the following solvent mixtures: (a) above; (e) light petroleum (b.p. 40-60°C)-acetone (7:3, v/v); (f) light petroleum (b.p. 40-60°C)-acetone (4:1, v/v). Solvent (a) was used in an attempt to identify 2-naphthylhydroxylamine-*O*-sulphonic acid on silica gel plates and on paper, running the latter for only 3h as chromatography of the *N*-acyl derivatives showed that this type of compound is unstable (Boyland & Manson, 1966*a,b*). Solvents (e) and (f) were used for separation of 2-naphthylhydroxylamine and oxidation products of 2-amino-1-naphthol. The compounds used in this work, with their chromatographic properties and colour reactions, have been described by Boyland &

Manson (1966*a,b*). 2-Naphthylhydroxylamine and its *N*-acyl-*O*-sulphonic acids and *O*-glucosiduronic acids give a bluish-green colour with alkaline hexylresorcinol solution. This colour reaction was used in an attempt to locate 2-naphthylhydroxylamine-*O*-sulphonic acid on chromatograms but it is possible that this derivative may not have the same properties.

2-Amino-1-naphthyl cetylpyridinium sulphate. This was prepared by the addition of saturated aqueous cetylpyridinium bromide to 2-amino-1-naphthyl ammonium sulphate (0.5 g) in water (10 ml). Precipitation was complete and the product was collected, washed with water and crystallized from acetone to yield plates, m.p. 128–130°C (decomp.) (Found: C, 69.0; H, 8.15; N, 5.1; S, 6.0. $C_{31}H_{46}N_2O_4S$ requires C, 68.6; H, 8.5; N, 5.2; S, 5.9%). The product was soluble in cold methanol or chloroform. When run on chromatograms in solvent (a) or solvent (b) the compound dissociated into the sulphuric ester (with some streaking) and the cetylpyridinium component, which ran near the solvent front.

2-Formamido-1-naphthyl cetylpyridinium sulphate. This was prepared from 2-formamido-1-naphthyl potassium sulphate (0.3 g) in water (15 ml) and cetylpyridinium bromide (1 g) in water (10 ml). A gel was formed that crystallized after 3 h at room temperature, but precipitation was not complete as with the amino compound. Crystallization from ethanol gave plates, m.p. 76–78°C, soluble in chloroform (Found C, 66.9; H, 8.3; N, 4.6; S, 5.3. $C_{32}H_{46}N_2O_5S$ requires C, 67.3; H, 8.1; N, 5.0; S, 5.6%).

The potassium salts of *N*-formyl- and *N*-acetyl-2-naphthylhydroxylamine-*O*-sulphonic acid in acetate buffer, pH 7.0, gave water-insoluble precipitates with cetylpyridinium bromide. Colour tests and chromatography confirmed that the precipitates were those of the arylhydroxylamine esters. 2-Naphthylsulphamic acid, 2-amino-6-naphthyl hydrogen sulphate, bis-(2-amino-1-naphthyl) hydrogen phosphate, 2-amino-1-naphthyl dihydrogen phosphate and 2-amino-1-naphthyl hydrogen sulphate *N*-sulphonic acid were also precipitated from 1% aqueous solutions by 2% aqueous cetylpyridinium bromide but 2-amino-1- and 2-amino-6-naphthyl glucosiduronic acids, 2-acetamido-1- and 2-acetamido-6-naphthyl glucosiduronic acids, and 2-amino-1-naphthyl mercapturic acid were not.

2-Amino-1-naphthyl triethylammonium sulphate. This was prepared from 2-amino-1-naphthyl hydrogen sulphate (0.6 g) in warm acetone (10 ml) and triethylamine (1 ml). After 30 min at room temperature the product was collected, washed with acetone and ether, giving the triethylamine salt as cubic crystals (0.3 g), m.p. 92–93°C (decomp.) (Found: C, 56.5; H, 6.8; N, 8.4; $C_{16}H_{24}N_2O_4S$ requires C, 56.5; H, 7.1; N, 8.2%). A further 0.2 g was obtained from the mother liquid by precipitation with ether. The compound was soluble in water but could be extracted from it by chloroform. It gave a dumb-bell-shaped spot in solvent (a), presumably because of only partial dissociation of the salt, R_F values 0.56 and 0.5, the lower value being the same as alkali metal salts of the ester in this solvent. It gave a single spot in the ammoniacal solvent (b). The trimethylammonium salt (0.25 g, m.p. 144–146°C, decomp.) was obtained in the same way (Found: C, 52.0; H, 6.05; N, 8.9; $C_{13}H_{18}N_2O_4S$ requires C, 52.3; H, 6.1; N, 9.4%). This salt gave one

spot, at the lower R_F value in solvent (a). The sulphuric ester was precipitated from solutions of both salts by cetylpyridinium bromide.

2-Amino-1-naphthyl hydrogen sulphate and its trimethylamine, triethylamine and ammonium salts were slowly hydrolysed at room temperature in dry pyridine to 2-amino-1-naphthol and very rapidly at 100°C. The sodium and potassium salts were also hydrolysed but more slowly, perhaps partly because of their slight solubility in pyridine. None of the salts was hydrolysed in aq. (50%, v/v) pyridine. The reaction provides an alternative means of hydrolysis to aqueous mineral acid. However, the cetylpyridinium salt, although readily soluble in pyridine, was not noticeably hydrolysed in this solvent even after heating at 100°C for 3 h.

RESULTS

Isolation of 2-amino-1-naphthyl hydrogen sulphate from dog urine. A dog (female mongrel, weight 7 kg) was dosed daily by mouth with 2-naphthylamine (400 mg) contained in a gelatine capsule. On the third day the animal was anaesthetized 1 h after dosing by the injection of Pentothal. Water (100 ml) was given by stomach tube and a catheter inserted into the urinary bladder where it was retained for 4 h. Immediately after insertion of the catheter a sample of urine (50 ml) was obtained, filtered and 20 ml of 5% (w/v) cetylpyridinium bromide added. The precipitate (0.34 g) was collected, washed with water and dried over phosphorus pentoxide. Urine, which was spasmodically voided, was then collected at intervals in flasks containing aqueous cetylpyridinium bromide. The precipitates were collected and treated as before. Untreated urine was examined by t.l.c. in solvent (a) but no evidence for the presence of arylhydroxylamine conjugates was obtained when the plates were sprayed with alkaline hexylresorcinol. Ether extracts of the untreated urine were subjected to t.l.c. in solvents (e) and (f). 2-Naphthylhydroxylamine was detected throughout the collection of urine by catheterization. The precipitates of the cetylpyridinium salt (total 0.32 g) were examined by i.r. spectroscopy the same day without further purification. They were identical with the synthetic cetylpyridinium salt of 2-amino-1-naphthyl hydrogen sulphate, melting point and mixed melting point, 128–130°C. After crystallization from acetone the material yielded plates, melting point unchanged (Found: C, 68.3; H, 8.2; N, 5.3; S, 6.0; $C_{31}H_{46}N_2O_4S$ requires C, 68.6; H, 8.5; N, 5.2; S, 5.9%). Subsequent examination of the urine samples by paper chromatography and t.l.c. showed that precipitation of 2-amino-1-naphthyl hydrogen sulphate was complete. Experience with t.l.c. of the *N*-acylarylhydroxylamine-*O*-sulphonic acids indicated that the unacylated derivative would have a close relationship to 2-amino-1-

naphthyl sulphate. Minor metabolites of 2-naphthylamine remained in solution. Urine collected in a metabolism cage before catheterization yielded 0.32g of the salt and urine collected two days subsequently (after two more doses of 0.4g of amine) gave 1.2g of cetylpyridinium salt. The total yield (2.18g) of salt was equivalent to 0.97g of 2-amino-1-naphthyl hydrogen sulphate, representing approx. 29% of the total dose of amine. Repetition of the experiment with another dog gave 0.85g of cetylpyridinium salt from 1.2g of amine, equivalent to approx. 19% of the dose. The material from the urine collected by catheter was formylated. Formic acid (0.2ml) was added to the cetylpyridinium salt (0.3g) and dicyclohexylcarbodiimide (0.3g) in chloroform (20ml). After 1h ether (50ml) was added to yield plates that were recrystallized from ethanol, to give the *N*-formyl derivative (cetylpyridinium salt) (0.1g) m.p. and mixed m.p. 76–77°C, identical in i.r. spectrum with the synthetic material. *N*-Formyl-2-naphthylhydroxylamine-*O*-sulphonic acid (Boyland & Manson, 1966a) was not a product.

Double-spot effect given by chromatography of urine concentrates containing 2-amino-1-naphthyl hydrogen sulphate. In earlier work on the isolation of 2-amino-1-naphthyl hydrogen sulphate from dog urine paper chromatography of the urine or of phenol eluates from charcoal columns run in solvent (a), but not solvent (b), gave dumb-bell-shaped spots of the sulphuric ester. The synthetic ester did not behave in this way. The colour reactions of the two parts of the spot were the same but it was possible that two different but related compounds were present. The double-spot effect persisted on passing the residue from evaporation of the phenol eluate through a cellulose column developed in solvent (a). Collection of the two fractions and isolation of the metabolite from each by (i) acidification with hydrochloric acid or acetic acid in aqueous solution or (ii) addition of 10M-sodium hydroxide to an alcoholic solution, gave the free acid or sodium salt of 2-amino-1-naphthyl hydrogen sulphate respectively from each fraction. It seemed unlikely that one of the fractions could be 2-naphthylhydroxylamine-*O*-sulphonic acid because of the instability of the *N*-acyl derivatives in water (Boyland & Manson, 1966a,b), their rearrangement *in vivo* (Boyland & Manson, 1966b) and the results of the isolation experiments described above. A chromatographic artifact due to the use of a neutral solvent was more probable. The isolation by charcoal adsorption, elution with aqueous phenol and separation on a cellulose column with solvent (a) was carried out on urine collected from a dog dosed for two periods of 3 days with daily doses of 0.4g of 2-naphthylamine. The sulphate ester fraction gave the double-spot effect

on paper chromatograms developed in solvent (a). The fraction was applied to sheets of Whatman 3MM paper as streaks. After separation by solvent (a) the upper and lower parts of the presumed 2-amino-1-naphthyl hydrogen sulphate fraction were eluted separately with aqueous methanol; an area between them was rejected. The eluates were evaporated to dryness *in vacuo*, the residues were dissolved in water and aqueous cetylpyridinium bromide was added. The precipitates (0.3g from the lower part of the spot and 0.28g from the upper part) were identical in m.p. and in i.r. spectra. It had been found earlier that the 2-amino-1-naphthyl sulphate fraction from the cellulose columns gave an alkylamine-like odour on the addition of strong alkali. On paper chromatograms 2-amino-1-naphthyl triethylammonium sulphate also gave a double spot in solvent (a). Although there was no direct evidence that an organic base in the urine was the cause of the divided spot the differences observed between chromatography in neutral and alkaline solvents resembled those seen after similar treatment of 2-amino-1-naphthyl triethylammonium sulphate.

Isolation of 2-amino-1-naphthyl hydrogen sulphate from neutral persulphate oxidations of 2-naphthylamine. (i) Without precipitant. Potassium persulphate (5g) in water (100ml) was added over 2h with stirring to 2-naphthylamine (5g) in M-potassium phosphate buffer, pH 7.5 (600ml) and acetone (200ml). Acetone was added during the addition of the persulphate solution to retain homogeneity as far as possible. Samples taken during the experiment did not give a colour reaction with hexylresorcinol alone. Ether extracts did not contain 2-amino-1-naphthol, 2-naphthylhydroxylamine or 2-nitrosonaphthalene. The reaction mixture was evaporated under reduced pressure to remove acetone. Unchanged 2-naphthylamine was collected and the filtrate extracted with ether. Evaporation of the aqueous fraction was continued at 50°C, until crystallization of the product began. The product (2.3g) was identical in i.r. spectrum with 2-amino-1-naphthyl potassium sulphate prepared by sulphation of 2-amino-1-naphthol (Boyland & Manson, 1958).

(ii) With cetylpyridinium bromide. The experiment was begun as above with cetylpyridinium bromide (5g) dissolved in the acetone-phosphate buffer mixture. Potassium persulphate solution was added over 30min. After 5min a precipitate formed that gave a colour reaction for 2-amino-1-naphthyl hydrogen sulphate but did not give any colour reaction with alkaline hexylresorcinol alone. After 30min the precipitate (3.2g) was collected and washed with ethanol and ether. It was extracted several times with hot methanol, the methanol eluate was cooled to room temperature,

filtered and the filtrate evaporated under reduced pressure until material came out of solution. The product (1g), m.p. 128–130°C, was identical with the cetylpyridinium salt of 2-amino-1-naphthyl hydrogen sulphate. By dilution of the liquors of the reaction mixture with half its volume of water more material was obtained: 0.5g after recrystallization from acetone. The methanol extract developed a mauve colour like that of 2-amino-1-naphthol, but when subjected to t.l.c. with solvents (e) and (f), none of the coloured decomposition products (Boyland & Manson, 1966b) of this aminonaphthol were found. The methanol-insoluble material was suspended in boiling methanol and acetone was added until it dissolved. On cooling orange-yellow plates (0.5g) were formed, m.p. 192–194°C, undepressed by admixture with 2,2'-azobenzophenone (Found: C, 84.9; H, 5.3; N, 9.8. Calc. for $C_{20}H_{14}N_2$: C, 85.1; H, 5.0; N, 9.9%).

The reaction mixture in oxidation (ii) was much less coloured than in (i) and the yield of sulphuric ester was less. Oxidation (ii) was also carried out in aqueous methanol with similar results.

Persulphate oxidation of 2-naphthylsulphamic acid in neutral solution. (i) Without precipitant. Potassium persulphate (4g) in water (100ml) was added over 2h with stirring to potassium 2-naphthylsulphamate (3g) in M-phosphate buffer, pH 7.0 (100ml). After a further hour the solution, which had become only pale brown in colour unlike the amine oxidation, was evaporated to dryness under reduced pressure. The residue was extracted with hot methanol and the extract evaporated to dryness. The residue was extracted several times with hot ethanol to remove potassium 2-naphthylsulphamate, although the procedure also dissolved some *O,N*-disulphate. The undissolved material was crystallized from aqueous ethanol to yield the dipotassium salt of 2-amino-1-naphthyl hydrogen sulphate *N*-sulphonic acid (0.8g) indistinguishable in chromatographic properties and in i.r. spectrum from material prepared by Boyland *et al.* (1957) by alkaline persulphate oxidation of the sulphamate or from *O,N*-sulphation of 2-amino-1-naphthol.

(ii) With precipitant. Cetylpyridinium bromide could not be added at the beginning of the oxidation as with the amine because of precipitation of the 2-naphthylsulphamate. The oxidation was carried out as above on 1g of potassium 2-naphthylsulphamate. After 5h cetylpyridinium bromide (2g) was added. The product (0.4g after crystallization from ethanol) consisted of a mixture of the cetylpyridinium salts of 2-naphthylsulphamic acid and of the *O,N*-disulphate. The i.r. spectrum was identical with the material obtained by adding cetylpyridinium bromide to an aqueous solution of the two compounds.

Table 1. R_F values on paper chromatograms of some 2-naphthylamine derivatives in solvent (b) and solvent (c), showing the effect of cetylpyridinium bromide added to the solvent mixture (16h run)

	Solvent	
	(b)	(c)
2-Naphthylsulphamic acid	0.40	0.90
2-Amino-1-naphthyl hydrogen sulphate*	0.42	0.90
2-Amino-1-naphthyl hydrogen sulphate- <i>N</i> -sulphonic acid	0.12	0.90
2-Formamido-1-naphthyl hydrogen sulphate*	0.50	0.90
2-Acetamido-1-naphthyl hydrogen sulphate	0.58	0.90
2-Amino-6-naphthyl hydrogen sulphate*	0.31	0.90
2-Amino-1-naphthyl mercapturic acid*	0.38	0.86
Bis-(2-amino-1-naphthyl) hydrogen phosphate*	0.55	0.90
2-Amino-1-naphthyl dihydrogen phosphate	0.09	0.40
2-Amino-1-naphthyl glucosiduronic acid*	0.17	0.61
2-Acetamido-1-naphthyl glucosiduronic acid	0.23	0.73
2-Amino-6-naphthyl glucosiduronic acid*	0.09	0.46
2-Acetamido-6-naphthyl glucosiduronic acid	0.12	0.63
Unidentified glucosiduronic acid*†	0.0	0.09

* Metabolites of 2-naphthylamine excreted by the dog.

† Boyland & Manson (1966b), p. 92.

2-Amino-1-naphthol or 2-naphthylhydroxylamine were not detected in the oxidations nor did the reaction mixtures give any reaction with alkaline hexylresorcinol. Attempts to prepare 2-naphthylhydroxylamine *N,O*-disulphonic acid by treatment of 2-naphthylhydroxylamine with excess pyridine-sulphur trioxide were unsuccessful; only the *N*-sulphonic acid (Boyland & Manson, 1966b) was obtained.

Paper chromatography of polar 2-naphthylamine derivatives in a solvent system containing cetylpyridinium bromide. Rees (1960), extending the work of Bera, Foster & Stacey (1955), increased the R_F values of sugar sulphates by the addition of cetylpyridinium chloride to the solvent systems. Table 1 shows the effect of replacing the aqueous ammonia of solvent (b) with aqueous cetylpyridinium bromide on polar derivatives of 2-naphthylamine, including the metabolites excreted by the dog. Solvent (d) gave similar R_F values. The value of solvent (b) as a means of separating the sulphuric esters was lost but the addition of the quaternary ammonium salts may aid the separation of the glucosiduronic acids from urinary constituents.

The last compound in Table 1 was detected in dog urine after dosing with 2-naphthylamine

and gave 2-amino-1-naphthol, 2-amino-1-naphthyl glucosiduronic acid and glucuronic acid after treatment with β -glucuronidase (Boyland & Manson, 1966b). Its low R_F value in solvent (b) compared with 2-amino-1-naphthyl glucosiduronic acid suggested that the carbohydrate component might carry sulphate or phosphate groups which were hydrolysed by contaminating enzymes in the glucuronidase preparation, but addition of 1:4-saccharolactone inhibited the formation of any product. Cetylpyridinium bromide had little effect on the mobility of the metabolite on chromatograms and did not precipitate it from aqueous solution. A similar metabolite was excreted by rabbits after dosing with 2-formamido-1-naphthol (Boyland & Manson, 1966a), which probably indicates that the above metabolite is a 2-amino-1-naphthol conjugate and not a 2-naphthylhydroxylamine derivative.

DISCUSSION

The use of cetylpyridinium bromide made possible the rapid precipitation of 2-amino-1-naphthyl hydrogen sulphate from urine. Conditions for this are favourable in dog urine because it is the predominant metabolite. No evidence for the excretion of 2-naphthylhydroxylamine-*O*-sulphonic acid was obtained. The *N*-acyl 2-naphthylhydroxylamine-*O*-sulphonic acids are unstable in aqueous solution, giving mainly the corresponding *N*-acyl 2-amino-1-naphthyl sulphates (Boyland & Manson, 1966a,b). After the intraperitoneal injection of *N*-acetyl 2-naphthylhydroxylamine-*O*-sulphonic acid into rats, Boyland & Manson (1966b) found only the 2-acetamido-1-naphthyl isomer in the urine. Attempts to synthesize 2-naphthylhydroxylamine-*O*-sulphonic acid were not successful. Catalytic hydrogenation of *N*-benzyloxy-carbonyl-2-naphthylhydroxylamine-*O*-sulphonic acid (Boyland & Manson, 1966b) gave 2-amino-1-naphthyl hydrogen sulphate but not the arylhydroxylamine ester. 2-Amino-1-naphthol was not detected but 2-naphthylamine was present and may have been produced from the release and reduction of 2-naphthylhydroxylamine but it is possible that the ester breaks down in a different way to the *N*-acyl derivatives. Hence it might not give a colour reaction with alkaline hexylresorcinol as this depends on the release of 2-naphthylhydroxylamine. An attempt was also made to obtain it by the treatment of 2-naphthylhydroxylamine-*N*-sulphonic acid with acetone (Boyland & Manson, 1967). The *N*-sulphonic acid rearranged to 2-amino-1-naphthyl hydrogen sulphate and presumably passed through the *O*-sulphonic acid stage but this intermediate was not obtained. These results suggest that this compound has a very transient

existence, and is unlikely to be present in the urine found in the bladder. Although the results of the persulphate oxidations are equivocal they could support this view. Investigations of the mechanism of the persulphate oxidation of amines favour the arylhydroxylamine-*O*-sulphonic acids as intermediates (Behrman, 1967; Venkatasubramanian & Sabesan, 1969).

2,2'-Azonaphthalene which is formed in the persulphate oxidation of 2-naphthylamine may be derived from several sources, e.g. nitrene or amino radicals, 2-nitrosonaphthalene or 2-naphthylhydroxylamine (although the two last named compounds were looked for and not found) or possibly from the breakdown of 2-naphthylhydroxylamine-*O*-sulphonic acid. The absence of 2-naphthylhydroxylamine and 2-amino-1-naphthol may indicate that 2-naphthylhydroxylamine-*O*-sulphonic acid, if this is an intermediate, breaks down in a different way to the *N*-acyl derivatives. However, the presence of persulphate is probably unfavourable to these readily oxidized compounds.

The question of whether 2-naphthylhydroxylamine is excreted in the free or conjugated state may have some bearing on the carcinogenic action of 2-naphthylamine because of possible differences between the reactivity of free and conjugated 2-naphthylhydroxylamine. 2-Naphthylhydroxylamine, which is carcinogenic when implanted into the bladders of mice (Bonser *et al.* 1963) or injected intraperitoneally into rats (Boyland, Dukes & Grover, 1963), reacts with sulphhydryl groups *in vitro* under both acid and neutral conditions (Boyland, Manson & Nery, 1962). Kriek (1965) did not observe any reaction with nucleic acids *in vitro* at pH 5.2, only at pH 4.0. Reaction with guanylic acid *in vitro* at pH 4.0, but not at pH 7.0, has been observed whereas a sulphuric ester, *N*-formyl-2-naphthylhydroxylamine-*O*-sulphonic acid, reacts at neutral pH with guanylic acid and thiol groups (Boyland & Manson, 1967). This *N*-formyl compound does not appear to be present in the urine as a metabolite of 2-naphthylamine although 2-formamido-1-naphthyl sulphate was found (Boyland & Manson, 1966a). The presence of this does not necessarily indicate an *N*-formyl-2-naphthylhydroxylamine precursor as 2-amino-1-naphthyl sulphate can be formylated *in vivo* (Boyland & Manson, 1966a). 2-Amino-1-naphthyl hydrogen sulphate itself shows an unexpected reactivity in that it gives 2-amino-1-naphthyl mercapturic acid with *N*-acetylcysteine at neutral pH by replacement of the sulphatoxy group and behaves like an arylhydroxylamine derivative in this reaction (Boyland & Manson, 1967). No evidence has been obtained in our work for the excretion of a sulphuric ester or an *N*- or *O*-glucosiduronic acid of 2-naphthylhydroxylamine

but it was apparent that the synthetic *N*-glucosiduronic acid was very unstable (Boyland & Manson, 1966b). However, these attempts to distinguish between the potential effects of the conjugated and unconjugated arylhydroxylamine may not be justified if there is esterification of 2-naphthylhydroxylamine in the bladder tissue, or if dehydroxylation of 2-naphthylhydroxylamine to a reactive intermediate can occur *in vivo* at pH values near neutrality (cf. Booth & Boyland, 1964; Gutmann & Erickson, 1969).

The solubility of the amine salts of 2-amino-1-naphthyl hydrogen sulphate in chloroform suggests that the ester might be made lipid-soluble *in vivo* by similar salt formation. Brill & Radomski (1965) have shown that the sulphuric ester, although a polar compound, is present in the bladder wall of the dog after oral administration of the amine. Goldblatt, Henson & Somerville (1960) found that the ester was absorbed by the cat bladder on perfusion of the lumen. However, the triethylamine salt of 2-amino-1-naphthyl hydrogen sulphate, like the free acid and alkali metal salts (Boyland, Busby, Dukes, Grover & Manson, 1964), has not given a significantly higher yield of tumours compared with the controls when tested in the bladders of mice by the pellet implantation technique.

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