The Biochemistry of Aromatic Amines

9. MERCAPTURIC ACIDS AS METABOLITES OF ANILINE AND 2-NAPHTHYLAMINE*

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The findings of Cramer, Miller & Miller (1960) and of Nelson & Troll (1961) that 2-acetamidofluorene and 2-naphthylamine respectively are metabolized by *N*-hydroxylation, and of Boyland & Manson (1961) that 2-naphthylhydroxylamine sulphate is excreted by dogs dosed with 2-naphthylamine, indicated that arylhydroxylamines are intermediates in the metabolism of arylamines. Arylhydroxylamines are reactive compounds; they react with cysteine to give *S*-(aminoaryl)cysteine derivatives and with acetylcysteine to give aminoarylmercapturic acids (Boyland, Manson & Nery, 1962). Because of this reaction it seemed probable that aromatic amines would be excreted as mercapturic acids, and a product which appears to be (2-amino-1-naphthyl)mercapturic acid has been detected in the urine of dogs and rats treated with 2-naphthylamine.

In addition, by analogy with the metabolism of aromatic hydrocarbons (Knight & Young, 1958; Boyland & Sims, 1958) and because of the presence of a dihydrodiol as a metabolite of 2-naphthylamine (Boyland & Manson, 1958), an acid-labile mercapturic acid might be expected from the metabolism of this amine. Such a compound has been detected in the urine of guinea pigs, rats and rabbits dosed with 2-naphthylamine. The urine of rabbits and rats dosed with aniline has been shown to contain aminophenyl- and acetamidophenyl-mercapturic acids.

EXPERIMENTAL

Animals. Animals were kept in cages designed for the separate collection of urine and faeces. The animals were injected intraperitoneally with 2-naphthylamine in arachis oil unless otherwise stated. Rabbits (approx. body wt. 2 kg.) were given 0·4 g. of amine in 10 ml. of oil; rats (approx. 200 g.), 0·05 g. of amine in 1 ml. of oil; hamsters (approx. 80 g.), 0·02 g. in 1 ml. of oil; guinea pigs (approx. 400 g.), 0·1 g. in 2 ml. of oil. A cat (approx. 2 kg.) was given 0·15 g. of amine in 2 ml. of oil intraperitoneally under ether anaesthesia. Beagles (bitches, approx. 10 kg.) were dosed either by subcutaneous injection in the nape of the neck or by feeding the amine in gelatin capsules in the doses given in the text. Rats were given 0·02 g. of aniline in 1 ml. of oil intraperitoneally, or 0·1 g. in 2 ml. of 0·5N-HCl orally; rabbits were given 0·5 g. of aniline in 5 ml. of oil intraperitoneally, or 0·5 g. in 5 ml. of N-HCl orally.

Paper chromatography. Whatman no. 1 chromatography paper was employed for descending development in the following solvent systems: (*a*), butan-1-ol–propan-1-ol–aq. 0·1N-NH_3 soln. (2:1:1, by vol.); (*b*), butan-1-ol–propan-1-ol–water (2:1:1, by vol.); (*c*), butan-1-ol–acetic acid–water (2:1:1, by vol.); (*d*), ethyl methyl ketone–acetone–formic acid–water (40:2:1:6, by vol.) (Reio, 1960). For the detection of compounds on paper the following reagents were used: (1) N-HCl and $NaNO_2$ (0·5%) followed by hexylresorcinol (0·5%, w/v) in 2N-NaOH; (2) 0·1M-$K_2Cr_2O_7$–acetic acid (1:1, v/v) followed by $AgNO_3$ (0·1M) (Knight & Young, 1958); (3) *p*-dimethylaminocinnamaldehyde (2 g. in 100 ml. of 6N-HCl and 100 ml. of ethanol) (Harley-Mason & Archer, 1958); (4) Na_2CO_3 (10%, w/v) followed by diazotized sulphanilic acid [1·6 ml. of $NaNO_2$ (0·5%) added to 10 ml. of sulphanilic acid (0·2%, w/v, in N-HCl)]; (5) platinic iodide (Toennies & Kolb, 1951); (6) ninhydrin (0·5%, w/v) in butan-1-ol saturated with water; (7) $NaNO_2$ (0·1%) and HCl (0·5N) followed by ammonium sulphamate (0·5%) and *N*-(1-naphthyl)-ethylenediamine hydrochloride (0·1%); (8) *p*-dimethylaminobenzaldehyde (0·5%, w/v) in ethanol containing conc. HCl (1%, v/v). A Chromatolite lamp (Hanovia Ltd.) was used as a source of u.v. light. Hydrolysis of acetamido groups was carried out by spraying the chromatograms with 2N-HCl and heating them between glass sheets in an oven at 70° for 30 min. Radioautographs of chromatograms were prepared by apposition of the chromatograms to Ilfex X-ray film in cassettes for various periods (usually 2–3 weeks). Urine (0·05–0·1 ml.) was applied directly to chromatograms or concentrated before application by adsorption on columns of charcoal deactivated with 7·5% (w/v) stearic acid (Asatoor & Dalgliesh, 1956). The columns were washed with water, the metabolites eluted with aq. 5% (w/v) phenol and the phenol eluate was evaporated almost to dryness under reduced pressure. Residual phenol was removed in a desiccator over NaOH pellets. For 24 hr. specimens of urine from single animals (rat, guinea pig and hamster) the whole amount was passed through charcoal columns (about 2·0 cm. × 3·5 cm.) and the residue from the phenol eluate dissolved in 0·5–1 ml. of water, 0·05 ml. being taken for chromatography. For dog urine 50 ml. portions of each 24 hr. specimen (about 300 ml.) were treated with charcoal. The phenol residue was dissolved in 2–3 ml. of water and 0·05 ml. taken. For rabbit urine 20 ml. portions were used, the phenol residue was dissolved in 3 ml. of water and 0·05 ml. was taken. The

* Part 8: Boyland, Kinder & Manson (1961).

whole of the 24 hr. specimens of cat urine (about 100 ml.) was used, the phenol residue was dissolved in 4 ml. of water and 0·05 ml. was taken for chromatography. For large volumes of urine, columns of untreated charcoal were employed and the metabolites eluted with methanol containing about 5% (v/v) of ammonia solution (sp.gr. 0·88).

Materials. 2-Naphthylamine, aniline and *o*- and *p*-aminophenols were of commercial origin. 2-[8-^{14}C]Naphthylamine (specific activity, 2 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. (2-Amino-1-naphthyl)mercapturic acid, *o*- and *p*-aminophenyl- and *o*- and *p*-acetamidophenyl-mercapturic acids were prepared as described by Boyland *et al.* (1962). 2-Acetamido-6-naphthol was prepared by the method of Booth, Boyland & Manson (1955) and 2-acetamido-5-naphthol by the acetylation of 2-amino-5-naphthol (Light and Co. Ltd., Colnbrook, Bucks.) (Mueller & Hamilton, 1944).

(2-Amino-5-naphthyl)mercapturic acid was prepared as follows. Cuprous oxide (3·6 g.) was added with stirring to L-cysteine hydrochloride (7·2 g.) in N-H_2SO_4 (150 ml.) at about 70°. The solution was cooled to 0° and a solution of diazotized 6-nitro-1-naphthylamine, prepared as follows, was added to it over 30 min. A mixture of finely ground $NaNO_2$ (3·2 g.) and cooled conc. H_2SO_4 (30 ml.) was heated to 70° and cooled again. A solution of 6-nitro-1-naphthylamine (5·3 g.) from the reduction of 1,6-dinitronaphthalene (Hodgson & Turner, 1943) in acetic acid (80 ml.) was added at 10° to the nitrite solution in 20 min. with stirring. When the diazotized amine had been added to the mercaptide solution the whole was stirred for 2 hr. and left overnight. Much tarry material was present. The mixture was filtered and the filtrate treated by shaking successively with two 5 g. portions of charcoal. The charcoal was filtered off and washed well with water followed by methanol containing 5% (v/v) of ammonia solution (sp.gr. 0·88). The methanol washings were evaporated to dryness. The residue was dissolved in a small amount of dilute ammonia solution, the tar filtered off and the filtrate acidified with acetic acid to give a gelatinous precipitate (0·65 g.). This gave a yellow ninhydrin-positive spot at R_F 0·4 in solvent system (*a*). The substance was dissolved in 2N-NaOH (20 ml.) with cooling, and acetic anhydride (2 ml.) was added. After 30 min. the solution was acidified to yield a yellow precipitate (0·55 g.) which gave a ninhydrin-negative spot at R_F 0·5 in solvent system (*a*). The precipitate was dissolved in aq. 60% (v/v) acetic acid (20 ml.) and shaken with zinc dust (0·5 g.). After 1 hr. the solution was treated with charcoal which was washed with water and methanolic ammonia. The methanol extracts were evaporated to dryness and the residue was dissolved in water (10 ml.). Acidification with acetic acid gave a tarry precipitate which was filtered off and the filtrate was evaporated *in vacuo* in a desiccator. More tar was filtered off and the concentration procedure repeated to yield grey plates of *(2-amino-5-naphthyl)mercapturic acid* (19 mg.), m.p. 114–116° (decomp.) (Found: C, 58·7; H, 5·4; N, 9·45. $C_{15}H_{16}N_2O_3S$ requires C, 59·2; H, 5·3; N, 9·2%). The compound darkened on keeping. Several preparations were unsuccessful owing to the formation of large amounts of tar. The compound had R_F 0·32 in solvent system (*a*) and 0·83 in (*c*), giving a bluish white fluorescent spot, which gave no reaction with ninhydrin but gave a positive reaction with the $K_2Cr_2O_7$–$AgNO_3$ reagent. It gave a red colour with *p*-dimethylaminocinnamaldehyde and a reddish orange colour with hexylresorcinol after diazotization. When the compound was treated with acetic anhydride in pyridine, paper chromatography of the solution showed a non-fluorescent spot at R_F 0·4 in solvent system (*a*) and 0·94 in (*c*), which on treatment with ammonia vapour gave an orange–pink fluorescence. It did not react with ninhydrin but gave a positive reaction with the $K_2Cr_2O_7$–$AgNO_3$ reagent. It did not react with the amino group reagents until the spot had been heated at 70° with 2N-HCl. The compound was presumably (2-acetamido-5-naphthyl)mercapturic acid. Some of the substance obtained from the reaction of diazotized 6-nitro-1-naphthylamine with cysteine was reduced by shaking with zinc dust in dilute acetic acid. Paper chromatography of the solution showed the presence of a spot with a bluish white fluorescence at R_F 0·4 in solvent system (*a*) and 0·7 in (*c*), which gave a blue colour with ninhydrin and an orange-red colour with hexylresorcinol after diazotization. The compound was presumably (2-amino-5-naphthyl)cysteine. Several attempts were made to synthesize (6-nitro-2-naphthyl)cysteine but paper-chromatographic examination of the reaction mixtures indicated that little reaction between diazotized 6-nitro-2-naphthylamine and cysteine occurred.

RESULTS

Metabolism of aniline

Rat. Urine (1 l.) was collected from the rats used in this experiment before the dosing. The urine (8 l.) from 30 rats receiving daily intraperitoneal doses of aniline in oil (total dose 6 g.) was collected daily under toluene and stored at −5° in the dark. The combined urine had a dark colour and a pH of 9. The extract obtained after continuous ether extraction of a sample (200 ml.) of the urine for 16 hr. contained mainly *p*-aminophenol [R_F 0·70 in solvent (*a*)] and aniline (as acetanilide, m.p. and mixed m.p. 114°, obtained by treatment of the steam distillate of the ethereal extract with N-sodium hydroxide and acetic anhydride).

The aqueous fraction was combined with the remainder of the urine, acidified to pH 4·0 with acetic acid, stirred with charcoal (15 g.), the charcoal filtered off, washed with water (1 l.), stirred with methanol (150 ml.) containing 5% (v/v) of aq. ammonia (sp.gr. 0·88), filtered and washed with methanol (50 ml.), and the combined filtrate and washing evaporated *in vacuo* to a viscous syrup. This was applied to sheets of 3MM chromatography paper, run in solvent system (*a*), and the sheets were cut into strips corresponding to the R_F regions of *o*- and *p*-aminophenyl- and *o*- and *p*-acetamidophenyl-mercapturic acids. Each strip was eluted with 1% (v/v) ammonia (sp.gr. 0·88) in methanol, and the eluates were concentrated *in vacuo*, applied again on 3MM paper, re-run in solvent system (*c*), and strips cut off and eluted as

before. The solvent from each of the four fractions thus obtained was evaporated *in vacuo* and the residual gums were dissolved in water (10 ml.). Four fractions were similarly obtained from the normal urine collected before the dosing and each was compared with the corresponding metabolic fraction in the following tests: (i) Descending chromatography in solvent systems (*a*) and (*c*). This showed the presence of *p*-aminophenyl-, *p*-acetamidophenyl- and *o*-aminophenyl-mercapturic acids in fractions 1, 2, and 3 respectively. No *o*-acetamidophenylmercapturic acid was detected. (ii) Estimation of mercapturic acid by iodometric titration of the alkaline hydrolysate of 5 ml. samples of each fraction according to the Stekol procedure as modified by Betts, James & Thorpe (1955). The recovery of *o*-aminophenylmercapturic acid under these conditions is quantitative. The value obtained for *p*-aminophenylmercapturic acid has been multiplied by a factor of 4·5 since the recovery of the synthetic compound by this method, when added to urine, was about 22%. That this is due to incomplete oxidation by iodine of the *p*-aminothiophenol produced is shown by the fact that this compound, when added to urine, consumes about 23% of the theoretical amount of iodine. *p*-Acetamidothiophenol and *p*-acetamidophenylmercapturic acid give similar results. Betts *et al.* (1955) obtained similar results for (pentachlorophenyl)mercapturic acid. (iii) Paper chromatography of the acid hydrolysates (hydrolysed in 2N-hydrochloric acid at 100° for 1 hr.). Under these conditions, fractions 1 and 2, containing *p*-aminophenyl- and *p*-acetamidophenyl-mercapturic acids respectively, both gave *S*-*p*-aminophenylcysteine [R_F 0·33 in solvent system (*a*) and 0·71 in (*c*)]. No *S*-*o*-aminophenylcysteine was detected in the hydrolysate of fraction 3 containing *o*-aminophenylmercapturic acid; this is not surprising in view of the ease with which the latter compound is decomposed under acidic conditions (cf. Boyland *et al.* 1962).

The results are summarized in Table 1. The urine of rats dosed orally with aniline contained similar metabolites.

Rabbit. Rabbits tolerated oral doses of aniline (0·5 g.) in N-hydrochloric acid (5 ml.); the same dose administered intraperitoneally caused inertia, loss of appetite and dilation of pupils, but administration of aniline (0·5 g.) in oil (5 ml.) caused no obvious ill effects. The urine (4 l.) collected from three rabbits that had been given a total dose of 15 g. of aniline was treated as described for rat urine. Elution of paper strips cut off in the appropriate regions showed the presence of *p*-acetamidophenol (0·4 g. isolated, m.p. and mixed m.p. 168°), *p*-acetamidophenylmercapturic acid (less than 0·2% of the dose) and an unidentified sulphur-containing metabolite [R_F 0·19 in solvent system (*a*) and 0·72 in (*c*)] which turned brown with reagent (2) and yellow (slowly, or faster if first sprayed with N-hydrochloric acid) with reagent (8). No attempt was made to isolate or identify the metabolites that have already been described in the literature (cf. Parke, 1960).

Metabolism of 2-naphthylamine

Rat. A rat (Marshall strain, female) was dosed with unlabelled 2-naphthylamine (50 mg.) and labelled 2-naphthylamine (0·8 mg.), and the urine was collected over 24 hr. The urine was treated with charcoal. The residue from the phenol eluate was examined by two-dimensional paper chromatography in solvent system (*a*) followed by (*c*). Radioautography showed the presence of many radioactive areas. Some of these metabolites have been described but many still remain unidentified. Two radioactive spots gave a positive reaction with the platinic iodide reagent (a clear space against a pink background) and with the potassium dichromate–silver nitrate reagent (a yellow spot against a reddish brown background). One, a white fluorescent spot [R_F 0·43 in solvent system (*a*) and 0·9 in (*c*)], gave a red colour with *p*-dimethylamino-

Table 1. *Mercapturic acids excreted in the urine of rats dosed with aniline*

Experimental details are given in the text.

Strip region (R_F)			R_F values		Colours with reagent			Percentage of dose converted [based on test (ii)]
First run [in solvent (*a*)]	Second run [in solvent (*c*)]	Compound	Solvent (*a*)	Solvent (*c*)	(2)	(7)	(8)	
0·16–0·24	0·65–0·70	*p*-Aminophenyl-mercapturic acid	0·20	0·67	Yellow	Bluish purple	Yellow	0·9
0·24–0·30	0·78–0·81	*p*-Acetamidophenyl-mercapturic acid	0·29	0·80	Yellow	None	None	Trace
0·30–0·33	0·74–0·78	*o*-Aminophenyl-mercapturic acid	0·32	0·76	Yellow	Purple	Yellow	0·02
0·33–0·36	0·81–0·84	No *o*-acetamidophenyl-mercapturic acid detected	—	—	—	—	—	None

cinnamaldehyde and diazotized and coupled with hexylresorcinol to give a yellow colour. The other, present in greater amount [R_F 0·26 in solvent system (*a*) and 0·8 in (*c*)], was not fluorescent and did not diazotize and couple with hexylresorcinol, but after treatment with cold 2N-hydrochloric acid for a few minutes followed by exposure to ammonia vapour the spot had an orange–pink fluorescence. After treatment of the paper at 70° with dilute hydrochloric acid the spot had a white fluorescence on exposure to ammonia vapour and gave a red colour with *p*-dimethylaminocinnamaldehyde and a reddish orange colour after diazotization and coupling with hexylresorcinol. The first spot corresponded in R_F values and colour reactions to (2-amino-1-naphthyl)mercapturic acid. Acidification of the material applied to the chromatogram did not change the R_F of this metabolite. The second metabolite underwent a change in R_F values after acidification of the phenol eluate residue [R_F then 0·41 in solvent system (*a*) and 0·94 in (*c*)]. On treatment with ammonia vapour the spot had an orange–pink fluorescence. When run on a chromatogram with the product obtained from the acetylation of (2-amino-5-naphthyl)mercapturic acid the two compounds showed identical properties. After treatment with hot acid, followed by neutralization with ammonia, the spot had a white fluorescence and gave positive reactions for an amino group. In view of the established existence of acid-labile mercapturic acids derived from aromatic hydrocarbons the second metabolite appears to be another compound of this type. As it reacts with amino group reagents only after treatment with hot mineral acid it is probably an acetamido derivative. The pink fluorescence of the mercapturic acid derived from the 'premercapturic acid' has been noted for 1-naphthylmercapturic acid (Boyland & Sims, 1958). With the naphthylamine metabolite the pink fluorescence is not seen after the amino group has been freed. When the chromatograms were sprayed with 2N-hydrochloric acid along the line of the metabolites separated in solvent system (*a*), allowed to dry and treated with ammonia vapour before development in solvent system (*c*), the R_F of the presumed premercapturic acid changed to 0·94 in this solvent system, and the spot fluoresced orange–pink. When the chromatogram was sprayed with sodium carbonate and diazotized sulphanilic acid a red colour was produced just above the mercapturic acid spot, suggesting the associated presence of an acetamidonaphthol. Acidification of the premercapturic acid from naphthalene also yielded, by the elimination of *N*-acetylcysteine, 2-naphthol and some 1-naphthol. By acid treatment of the chromatogram similarly reacting phenolic spots were also derived from metabolites identified as 2-acetamido-6-naphthyl hydrogen sulphate and 2-acetamido-5,6-dihydro-5,6-dihydroxynaphthalene and its glucosiduronic acid (Boyland & Manson, 1957, 1958). All the spots giving the colour reactions were radioactive. In another experiment the premercapturic acid was located on several chromatograms by its radioactivity and was eluted with methanolic ammonia. The combined eluates were evaporated to a small volume and the solution was examined by paper chromatography in solvent system (*a*), with and without acidification of the material. The acidified compound had the R_F, fluorescence and colour reactions described above and yielded a spot at R_F 0·92 which was non-fluorescent and not diazotizable but which gave a pale-red colour with diazotized sulphanilic acid. After treatment with hot acid the spot gave a green colour with nitrous acid, a red colour after diazotization and treatment with hexylresorcinol and a mauve colour with diazotized sulphanilic acid, colours characteristic of 2-amino-6-naphthol. The acid-labile precursor of 1-naphthylmercapturic acid, which is probably *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine, yields 1-naphthol as well as 2-naphthol. Hence the naphthylamine metabolite may yield 2-acetamido-6-naphthol and 2-acetamido-5-naphthol. The latter compound was not separable from the 6-isomer in the solvent system employed but its colour reactions were different. It gave a bright-red colour with a mauve border with diazotized sulphanilic acid, changing to a brownish mauve when sprayed with 2N-hydrochloric acid, whereas the pale red of the 6-isomer remained unchanged. 2-Amino-5-naphthol gave a yellow colour with nitrous acid, changing to a brownish red with hexylresorcinol, and a red colour with diazotized sulphanilic acid. Hence 2-acetamido-6-naphthol appeared to be the predominant acetamidonaphthol produced by acidification of the metabolite.

Both the mercapturic acids detected in the urine of Marshall rats were also present in that of the Holland and Chester Beatty albino strains after injection of the amine. In the Marshall rats both mercapturic acids were detected after the administration of a small dose only of 2-naphthylamine (0·9 mg. of labelled amine). When 2-naphthylamine (50 mg. of unlabelled and 0·9 mg. of labelled amine) was administered to rats of the Slonaker strain the compound believed to be (2-amino-1-naphthyl)mercapturic acid was not detected in the urine, but the acid-labile mercapturic acid was present. With these rats the amount of 2-amino-1-naphthyl hydrogen sulphate was also very small compared with that with the other strains although 2-amino-1-naphthyl glucosiduronic acid was readily detected.

An attempt was made to isolate the mercapturic acids from rat urine after the daily intraperitoneal

injection of 50 mg. of 2-naphthylamine to each of 20 rats for 10 days. The combined urines were continuously extracted for 3 hr. at pH 6·0 to remove 2-naphthylamine and 2-acetamido-6-naphthol and for a further 3 hr. at pH 4·0. The second ether extract contained only the mercapturic acid identical in R_F values and colour reactions with (2-amino-1-naphthyl)mercapturic acid. The acid-labile premercapturic acid was not extracted by ether or chloroform at pH 4·0, but after acidification of the urine to pH 2·0 the mercapturic acid could be extracted by chloroform. Thus two mercapturic acid fractions were obtained but subsequent attempts to obtain the pure compounds were unsuccessful.

Dog. The examination by two-dimensional paper chromatography of the urine of beagles undergoing oral dosing with 2-naphthylamine (0·4 g.) five times weekly showed the presence of a compound with the properties of (2-amino-1-naphthyl)mercapturic acid. 2-Amino-1-naphthyl hydrogen sulphate and 2-amino-1-naphthyl glucosiduronic acid were detected. The acid-labile mercapturic acid was not detected at any time either in the urine itself or after concentration by charcoal adsorption. No other metabolites substituted in the ring not bearing the amino group, except for traces of 2-amino-6-naphthyl hydrogen sulphate and the corresponding glucosiduronic acid, were detected in dog urine over a long period of examination. The urine from three dogs dosed over 7 days by the daily oral administration of 0·4 g. of 2-naphthylamine was collected, adjusted to pH 4·0 with concentrated hydrochloric acid and continuously extracted with ether for several hours. The ether extracts were evaporated to dryness and the residues dissolved in dilute sodium hydroxide. The pH was adjusted to 7·0 and the solution extracted several times with ether. Adjustment to pH 6·0 yielded a precipitate which was filtered off and, after alteration of the pH to 4·0, the solution was extracted with ether again. The residue from the ether extract was dissolved in 2N-sodium hydroxide. The addition of ethanol yielded a gelatinous precipitate which was discarded. The filtrate was evaporated in a desiccator to yield a gum (300 mg.), which was dissolved in 5 ml. of water and applied to sheets of 3MM chromatography paper. The compound with the properties of (2-amino-1-naphthyl)mercapturic acid was located and eluted with methanolic ammonia. The methanol extracts were evaporated to dryness and dissolved in the minimum of water. Attempts to precipitate the compound by acidification with acetic acid resulted only in the formation of gummy material. The synthetic compound had also proved difficult to purify. When the metabolite was heated for 30 min. at 100° in 5N-hydrochloric acid paper chromatography showed the presence of a spot [R_F 0·43 in solvent system (*a*) and 0·79 in (*c*)], which in addition to the reactions of the untreated compound gave a blue colour with ninhydrin.

A suspension of the metabolite in water was treated with hydrogen peroxide (6%, v/v) for 10 min. Paper chromatography showed a fluorescent spot at R_F 0·38 in solvent system (*a*) and 0·83 in (*c*), which gave a red colour with *p*-dimethylaminocinnamaldehyde and a pink colour with hexylresorcinol after diazotization. A feeble reaction only was given by the potassium dichromate–silver nitrate reagent. When treated with diazomethane in ether and methanol the metabolite gave a fluorescent spot at R_F 0·97 in solvent system (*a*). The synthetic mercapturic acid behaved similarly in all these reactions. No change of R_F of the metabolite was noticed when the urine or the residues from charcoal concentration procedures were acidified with hydrochloric acid. After complete extraction of samples of urine with ether at pH 4·0, adjustment of the pH to 2·0 and repetition of the extraction did not yield more of the metabolite from the decomposition of an acid-labile precursor. The crude metabolite fraction obtained above was not affected by acidification with hydrochloric acid.

Four beagles, not of the series used above, were dosed at weekly intervals by subcutaneous injection of varied doses of 2-naphthylamine. Labelled 2-naphthylamine (3 mg.) was given with 40, 160, 640 and 1280 mg. of unlabelled amine, and chromatograms of 24 hr. specimens of the urine and of urine concentrated by charcoal adsorption were examined by spray reagents and radioautography. (2-Amino-1-naphthyl)mercapturic acid was detected with all the doses, although 2-amino-1-naphthyl hydrogen sulphate was the predominant and apparently the only other metabolite. 2-Amino-1-naphthylglucosiduronic acid was not detected in any of the urines. When two of the dogs were given the amine orally (640 mg.) this glucosiduronic acid was then found and 2-amino-6-naphthyl hydrogen sulphate and 2-amino-6-naphthylglucosiduronic acid were also detected. Chromatograms of urine from dogs injected subcutaneously with labelled 2-naphthylamine by Twombly, Zomzely & Meislich (1957) showed no activity at the R_F of 2-amino-1-naphthyl glucosiduronic acid in their solvent system. However, in our first series of dogs we did not observe any difference between the metabolites excreted after oral and after subcutaneous administration of the amine.

Rabbit. The urine of rabbits dosed with 2-naphthylamine contained the acid-labile mercapturic acid but not (2-amino-1-naphthyl)mercapturic acid. Six rabbits were dosed with 2-

naphthylamine (0·4 g.) for 10 days and the combined urine was passed through 150 g. of charcoal. The charcoal was washed with water and the metabolites were eluted with methanolic ammonia. The methanol eluate was evaporated to dryness and the metabolites of 2-naphthylamine were separated on a large cellulose column in solvent mixture (*a*) as described by Boyland, Manson & Orr (1957). The acid-labile mercapturic acid fraction was collected, but the acid-labile mercapturic acid itself was not readily detected on a one-dimensional chromatogram in solvent system (*a*) owing to the presence of a normal urine constituent with a white fluorescence which had the same R_F and which also gave a positive reaction to the potassium dichromate–silver nitrate reagent. The two compounds were separated by solvent system (*c*), in which the normal urine constituent had R_F 0·7 and the acid-labile mercapturic acid R_F 0·8. The residue from evaporation of the acid-labile mercapturic acid fraction of the column was dissolved in water and acidified with hydrochloric acid to produce the stable mercapturic acid. This precipitated much of the interfering material, which was collected and the filtrate evaporated to dryness to give a gum. This was resubmitted to column chromatography but the mercapturic acid was not obtained crystalline. The metabolite had the same properties as the compound found in the rat urine after acidification of the precursor and appeared to be identical with the product of acetylation of (2-amino-5-naphthyl)-mercapturic acid. When the metabolite fraction was heated for 1 hr. at 100° in 5N-hydrochloric acid a fluorescent spot was detectable at R_F 0·43 in solvent system (*a*) and at 0·71 in (*c*). The spot gave a blue colour with ninhydrin and a reddish-orange colour after diazotization and coupling with hexylresorcinol, and appeared to be identical with the product obtained by the reduction of (2-nitro-5-naphthyl)cysteine. Fractions from the column were examined for the presence of (2-amino-1-naphthyl)mercapturic acid but none was found. The 2-acetamido-6-naphthol fraction from this column was examined for the presence of 2-acetamido-5-naphthol by hydrolysis with concentrated hydrochloric acid. Paper chromatography in solvent systems (*b*) and (*d*) gave only spots at R_F values of 0·81 and 0·6 respectively with the colour reactions of 2-amino-6-naphthol, and none at 0·88 and 0·80, the R_F values of 2-amino-5-naphthol in these solvent systems.

Guinea pig. No (2-amino-1-naphthyl)mercapturic acid was detected in the urine of guinea pigs after the injection of 2-naphthylamine with 0·8 mg. of labelled amine. 2-Amino-1-naphthyl hydrogen sulphate was not detected, but the corresponding glucosiduronic acid and its *N*-acetyl derivative were present. Only traces of the acid-labile mercapturic acid were present although the structurally related 2-acetamido-5,6-dihydro-5,6-dihydroxynaphthalene and its glucosiduronic acid were readily detected. 2-Acetamido-6-naphthyl glucosiduronic acid was the predominant metabolite with traces of the corresponding sulphuric ester.

Cat. Neither of the mercapturic acids was detected in the 24 hr. specimen of cat urine after dosing with the amine. 2-Amino-1-naphthyl hydrogen sulphate was the major metabolite with traces of 2-amino-6-naphthyl hydrogen sulphate and of other unidentified metabolites. None of the known glucosiduronic acids was found.

Hamster. Neither of the mercapturic acids was detected. The spot due to 2-amino-1-naphthyl hydrogen sulphate was very weak but 2-amino-1-naphthyl glucosiduronic acid was very readily detected. 2-Acetamido-1-naphthyl glucosiduronic acid, 2-acetamido-6-naphthyl glucosiduronic acid and 2-acetamido-6-naphthyl hydrogen sulphate were detected. A trace of the dihydrodiol glucosiduronic acid was present but none of the unconjugated compound was detected.

The results obtained with 2-naphthylamine in the various species are summarized in Table 2.

Table 2. *Mercapturic acids of 2-naphthylamine and their related compounds in the urine of different species*

Experimental details are given in the text.

	Rabbit	Rat (Chester Beatty albino, Marshall and Holland strains)	Rat (Slonaker strain)	Dog	Hamster	Guinea pig	Cat
N-Acetyl-*S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)-L-cysteine	+	+	+	−	−	Trace	−
2-Acetamido-5,6-dihydro-5,6-dihydroxynaphthalene	+	+	+	−	−	+	−
2-Acetamido-5,6-dihydro-5,6-dihydroxynaphthalene glucosiduronic acid	+	+	+	−	Trace	+	−
(2-Amino-1-naphthyl)mercapturic acid	−	+	−	+	−	−	−
2-Amino-1-naphthyl hydrogen sulphate	Trace	+	Trace	+	Trace	−	+
2-Amino-1-naphthyl glucosiduronic acid	+	+	+	+	+	+	−

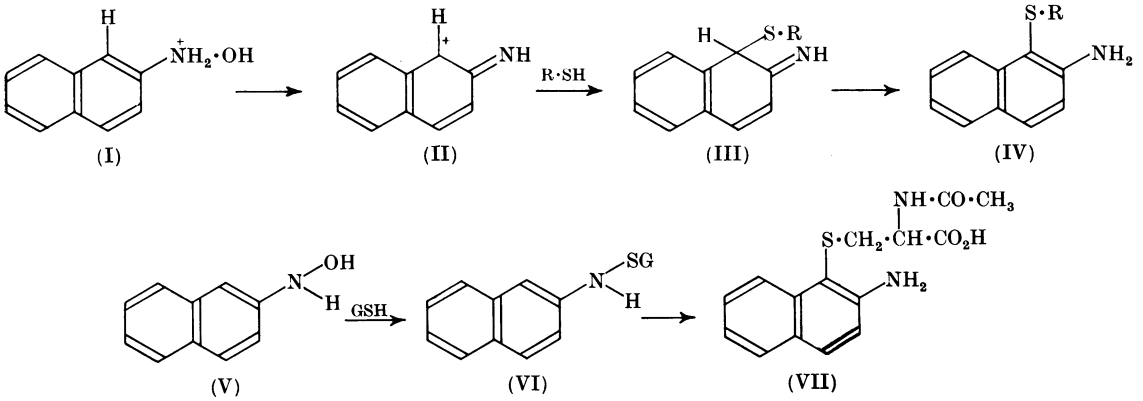
DISCUSSION

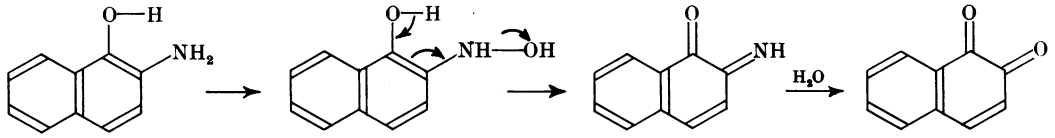
Although the metabolism of aniline has been frequently investigated (Smith & Williams, 1949; Parke, 1960), the formation of (aminophenyl)mercapturic acids does not appear to have been described. Kiese (1959*a*, *b*) has shown that aniline is oxidized to nitrosobenzene, presumably via the formation of phenylhydroxylamine, when it is injected intravenously into dogs. Phenylhydroxylamine itself, when administered to rats, is converted into *p*-aminophenylmercapturic acid, which has been isolated (E. Boyland, D. Manson & R. Nery, unpublished work). A possible pathway of aniline metabolism is via *N*-hydroxylation followed by mercapturic acid formation by some such mechanism as is outlined for 2-naphthylamine.

The detection of an acid-labile mercapturic acid or 'premercapturic acid' as a metabolite of 2-naphthylamine is not unexpected in view of the known existence of such metabolites from the metabolism of aromatic hydrocarbons (Knight & Young, 1958; Boyland & Sims, 1958). Although proof of its structure is incomplete the formation of 2-acetamido-6-naphthol and of another mercapturic acid when the compound is acidified indicates that the metabolite is *N*-acetyl-*S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)-L-cysteine.

Boyland & Sims (1958) and Booth, Boyland & Sims (1959) have suggested that with naphthalene the reactive intermediate in the formation of mercapturic acids is the hypothetical 1,2-epoxynaphthalene. This may then react with the thiol groups of cysteine, glutathione or tissue proteins to form intermediates which, after any necessary breakdown, are acetylated and excreted as the naphthylpremercapturic acid. The other mercapturic acid present in the urine, which has the properties of (2-amino-1-naphthyl)mercapturic acid, does not appear to have an acid-labile precursor in the urine, and it is unlikely that it could be derived from such a precursor.

Cramer *et al.* (1960) have isolated *N*-hydroxy-2-acetamidofluorene from the urine of rats dosed with 2-acetamidofluorene, and Nelson & Troll (1961) have detected 2-naphthylhydroxylamine in the urine of man and dogs as a metabolite of 2-naphthylamine. Boyland *et al.* (1962) demonstrated that phenylhydroxylamine and 2-naphthylhydroxylamine react with thiols to yield aminoaryl derivatives of these compounds. 2-Naphthylhydroxylamine reacts with cysteine, acetylcysteine and glutathione to yield 2-amino-1-naphthyl derivatives. This reaction may proceed through a quinolimide ion from the naphthylhydroxylamine (I–IV) such as is believed to occur in the rearrangement of phenylhydroxylamine to *p*-aminophenol. Hughes & Ingold (1952) consider this reaction to be a nucleophilic intermolecular rearrangement in which the quinolimide ion reacts with any available nucleophilic molecule or anion. After treatment of 2-naphthylhydroxylamine with phosphoric acid or sulphuric acid 2-amino-1-naphthyl dihydrogen phosphate or 2-amino-1-naphthyl hydrogen sulphate respectively may be detected (Boyland, Manson & Nery, 1960). The oxidation of 2-naphthylamine to 2-naphthylhydroxylamine *in vivo* may thus provide a reactive compound from which the 2-amino-1-naphthyl hydrogen sulphate and (2-amino-1-naphthyl)mercapturic acid could be formed. Only 2-amino-1-naphthyl derivatives would be formed in this manner as the acid-catalysed rearrangement of 2-naphthylhydroxylamine yields only 2-amino-1-naphthol (Boyland *et al.* 1962). Alternatively, (2-amino-1-naphthyl)mercapturic acid may arise by the reaction of 2-naphthylhydroxylamine with glutathione (GSH) to give compound (VI), which by loss of glutamic acid and glycine followed by acetylation and coupled at some point by rearrangement, perhaps intramolecular, yields the mercapturic acid (VII). Hence the extent to which 2-amino-1-naphthyl





(VIII) (IX) (X) (XI)

derivatives are formed may depend on the capacity of the animal to carry out *N*-hydroxylation of 2-naphthylamine. Miller & Miller (1960) showed that in the rat the 1-hydroxy metabolite of 2-acetamidofluorene arose largely, if not entirely, from *N*-hydroxy-2-acetamidofluorene. From the chromatograms of the urine the impression was obtained that, in the species (dog; Marshall, Holland and Chester Beatty albino strains of rats) where 2-amino-1-naphthyl hydrogen sulphate was readily detected, (2-amino-1-naphthyl)mercapturic acid could also be found, although it was not detected in the cat urine. In the rabbit, guinea pig, hamster and Slonaker strain of rat, where 2-amino-1-naphthyl hydrogen sulphate was a minor metabolite or was not detectable, no (2-amino-1-naphthyl)mercapturic acid was found. The formation of these two compounds may be dependent on a common precursor. On the other hand, 2-amino-1-naphthyl glucosiduronic acid was present in the urine of rabbits, guinea pigs, hamsters and Slonaker rats. This compound may be formed by a different route, e.g. direct oxidation to the amino naphthol.

The formation *in vitro* from 2-naphthylhydroxylamine of 2-amino-1-naphthyl derivatives of cysteine, acetylcysteine and glutathione may indicate the mode of binding of the amine to tissues. Protein binding of 2-naphthylamine may occur through the formation of compound (X) or of 1,2-naphthaquinone (XI). The results of Belman & Troll (1962) favour the latter derivative as the reactive compound in a study of the reaction *in vitro* of 2-amino-1-naphthol with rat-bladder tissue and bovine serum albumin. The reaction could conceivably proceed via the *N*-hydroxylation of 2-amino-1-naphthol. The long-term retention of 2-naphthylamine or its metabolites in the animal body has been demonstrated by Goldblatt, Henson & Somerville (1960), particularly in the blood and, in the rat, within the red cell rather than in the red-cell membrane. However, as Belman & Troll (1962) point out, 'the relationship of protein binding to carcinogenesis is, at present, correlative and no causal connection has been demonstrated'.

SUMMARY

1. A substance has been detected in the urine of rats, rabbits and guinea pigs dosed with 2-naphthylamine which yields 2-acetamido-6-naphthol and a mercapturic acid when acidified with mineral acid, and which is probably *N*-acetyl-*S*-(2-acetamido-5,6-dihydro-6-hydroxy-5-naphthyl)-L-cysteine.

2. (2-Amino-1-naphthyl)mercapturic acid has been detected in the urine of dogs and rats dosed with 2-naphthylamine. This mercapturic acid is not excreted as an acid-labile precursor.

3. Possible differences in the mode of origin of these two mercapturic acids are discussed.

4. *o*- and *p*-Aminophenyl- and *p*-acetamidophenyl-mercapturic acids have been detected in the urine of rats and rabbits dosed with aniline.

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Structure and Synthesis of Cephalosporidine, a Degradation Product of Cephalosporin C

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Radioactive cephalosporin C which had been formed by a species of *Cephalosporium* in a medium containing [1-^{14}C]acetate yielded a previously unrecognized degradation product on hydrogenolysis with Raney nickel in hot neutral aqueous solution (Trown, Abraham, Newton, Hale & Miller, 1962). The new degradation product was ninhydrin-negative but radioactive and was first detected as a spot on radioautographs after electrophoresis and chromatography on paper of the mixture of substances formed when hydrogenolysis was followed by acid hydrolysis. Subsequently, it was revealed on paper by the chlorination procedure of Rydon & Smith (1952). It showed no net charge at pH 4·5 and its R_F in butan-1-ol–acetic acid–water (4:1:4, by vol.) was 0·78 that of valine. The isolation and nature of this compound are described in the present paper. It has been given the trivial name 'cephalosporidine'.

Although cephalosporidine was first obtained from a mixture of substances formed on hydrogenolysis of cephalosporin C, it was later shown to be formed when cephalosporin C (either sodium salt or free acid) was heated under reflux in aqueous solution. It also appeared to be formed under similar conditions from an impure preparation of the sodium salt of penicillin N (cephalosporin N), and to be identical with a ninhydrin-negative compound which was detected by Trown *et al.* (1962) among the products of hydrolysis of material which probably contained the penillic acid from penicillin N. This material had been separated from cephalosporin C during the purification of the latter on a column of Amberlite XE-58.

Cephalosporidine survived unchanged when the solution in which it had been formed from cephalosporin C was made 2N with respect to hydrochloric acid and then heated under reflux for 16 hr. It was isolated from the acid hydrolysate by adsorption on Dowex 50 (X8) and elution at pH 7, countercurrent distribution in a solvent system composed of phenol and 5% (v/v) acetic acid, and chromatography on Dowex 50 (X8) in N-hydrochloric acid followed by 2N-hydrochloric acid. The purified product separated from ethanol–acetone in orthorhombic crystals.

Cephalosporidine formed a monohydrate in moist air. Elementary analysis and an estimate of molecular weight indicated that the probable molecular formula of the monohydrate was $C_8H_{10}N_2O_2,H_2O$. No *C*-methyl group was revealed by a Kuhn–Roth determination. The compound showed $[\alpha]_D^{20} - 2°$ (*c* 10 in water) and $[\alpha]_D^{20} + 7·3°$ (*c* 10 in 6N-hydrochloric acid). Its ultraviolet-absorption spectrum showed $\lambda_{max.}$ 211 mμ when measured in water and $\lambda_{max.}$ 212 mμ when measured in N-hydrochloric acid (log ϵ, 3·75). When measured in 0·1N-sodium hydroxide it showed $\lambda_{max.}$ 218 mμ (log ϵ, 3·60).

Electrometric titration indicated that cephalosporidine contained two ionizable groups with pK values of less than 2·2 and of 8·1 respectively. These values were consistent with the behaviour of the compound when it was subjected to electrophoresis on paper. It showed no significant net charge at pH 4·5 or 7·0, migrated 0·65 times as far as valine and 0·7 times as far as 1-imidazolylacetic acid towards the cathode at pH 2·2, and migrated