The Biochemistry of Aromatic Amines

4. *O*-GLUCOSIDURONIC ACID DERIVATIVES OF 2-NAPHTHYLAMINE*

By E. BOYLAND and D. MANSON
Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3

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The metabolism of 2-naphthylamine is of particular interest because the amine is known to cause cancer of the bladder in man and dogs and the carcinogenic action is undoubtedly due to a metabolite excreted in urine. From the knowledge of the behaviour of the metabolites identified by earlier workers and by Boyland & Manson (1955) and Boyland, Manson & Orr (1957) it was argued (Boyland, 1956) that only one metabolite, 2-amino-1-naphthyl glucosiduronic acid, should be carcinogenic. Bladder cancer in mice has since been induced with this glucuronide (Allen, Boyland, Dukes, Horning & Watson, 1957). The preparation and properties of this and other 2-amino-naphthyl glucosiduronic acid derivatives are described in this paper.

MATERIALS

2-Naphthylamine, 2-acetamidonaphthalene, 2-acetamido-1-naphthol and 2-amino-6-naphthol hydrochloride were prepared as described by Booth, Boyland & Manson (1955). Calf-spleen β-glucuronidase (Viobin Laboratories, Monticello, Ill., U.S.A.) and bacterial β-glucuronidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) were employed for enzymic hydrolyses.

METHODS

Rats and rabbits were kept in metabolism cages and urine was collected daily. Rabbits were fed on cabbage, bran, rat cake and water, and rats on bread, rat cake and water. 2-Naphthylamine or 2-acetamidonaphthalene was injected intraperitoneally in daily doses of 0·5 g. in 10 ml. of arachis oil into rabbits and 0·05 g. in 1 ml. of oil into rats. Paper chromatography was carried out by upward development on Whatman no. 1 paper with butanol–propanol–aqueous 0·1 N-NH_3 (2:1:1, by vol.). Acetamido compounds were hydrolysed to amino compounds on the chromatograms by spraying with N-HCl and heating for 30 min. between glass plates at 70°. The colour reactions of acetamidonaphthyl glucosiduronic acids after hydrolysis under such conditions were characteristic of the aminonaphthyl glucosiduronic acids and not of the aminonaphthols. Reagents used in this work have been described by Booth *et al.* (1955). 2-Amino-1-naphthol was detected in solution by the green-coloured derivative which is formed on addition of NH_3. This derivative is extracted with benzene, in which solvent it is mauve (Liebermann & Jacobson, 1882). A Chromatolite lamp (Hanovia Ltd.) was used as a source of ultraviolet light. R_F values and colour reactions of the compounds isolated are given in Table 1. R_F values obtained by downward development in several solvent systems and colour reactions with nitrous acid followed by hexylresorcinol and with Ehrlich's reagent have been given by Booth *et al.* (1955).

RESULTS

Isolation of glucosiduronic acids

Both 2-amino-1-naphthol hydrochloride and the free base are toxic (LD_{50} in mice 25 mg./kg. body wt.) and so unsuitable as sources of 2-amino-1-naphthyl glucosiduronic acid. The administration of unsubstituted aromatic amines causes excretion of free glucuronic acid, which might interfere with the isolation of glucosiduronic acids. 2-Acetamido-1-naphthol was therefore used and the acetamido group of the glucosiduronic acid was hydrolysed by dilute sulphuric acid. Attempts to isolate 2-amino-1-naphthyl glucosiduronic acid from the urine of rats dosed with 2-naphthylamine were unsuccessful, although the acid could be detected in the urine by paper chromatography. Small amounts of 2-amino-6-naphthyl glucosiduronic acid were isolated from the urine of rabbits dosed with 2-amino-6-naphthol.

2-Acetamido-6-naphthyl glucosiduronic acid was isolated as its sodium salt from rabbit urine after administration of 2-acetamidonaphthalene. Rabbits deacetylate acetyl derivatives of arylamines relatively slowly, and when dosed with 2-acetamidonaphthalene rabbits excrete only small amounts of 2-amino-1-naphthylsulphuric acid and neither 2-naphthylsulphamic acid nor 2-amino-1-naphthyl glucosiduronic acid. 2-Acetamido-6-naphthyl glucosiduronic acid was, however, isolated from rabbit urine as the triacetyl methyl ester after injection of 2-naphthylamine. The glucosiduronic acid fraction was obtained by adsorption on charcoal and elution. If the charcoal was heated before use (cf. Corner, Billett & Young, 1954) the glucosiduronic acid was bound so that only small amounts were removed with cold or hot methanol, but it was eluted with aqueous phenol (cf. Dalgliesh, 1952). If the

* Part 3: Booth & Boyland (1957).

Table 1. *R_F values and colour reactions of 2-amino- and 2-acetamido-naphthyl glucosiduronic acids*

Conditions of hydrolysis are described in the text.

Compound	R_F in butanol–propanol–0·1N-NH_3 (2:1:1, by vol.)	Fluorescence	Colours with diazotized sulphanilic acid	
			Before hydrolysis	After hydrolysis
2-Amino-1-naphthyl glucosiduronic acid	0·22	Blue–white	Pale yellow	Pale yellow
2-Acetamido-1-naphthyl glucosiduronic acid	0·25	None	None	Pale yellow
2-Amino-6-naphthyl glucosiduronic acid	0·15	Blue–white	Orange–red	Orange–red
2-Acetamido-6-naphthyl glucosiduronic acid	0·20	None	None	Orange–red

charcoal was not heated, cold methanol eluted only a little of the glucosiduronic acid; hot methanol removed more, but aqueous phenol was still necessary for complete elution. The glucosiduronic acid fractions contained 2-acetamido-6-naphthyl glucosiduronic acid, with small amounts of what is probably 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid. Evidence for the presence of the latter conjugate is discussed below.

2-*Acetamido*-1-*naphthyl glucosiduronic acid*

2-Acetamido-1-naphthol (0·3 g. in 10 ml. of arachis oil) was administered daily for 3 days to each of five rabbits (total dose 4·5 g.). The urine was adjusted to pH 4·0–5·0 with acetic acid and a saturated solution of lead acetate added. After removal of the precipitate by centrifuging, the supernatant was adjusted to pH 8·0 with aq. NH_3 (sp.gr. 0·88) and a saturated solution of basic lead acetate added until precipitation was complete. The precipitate was centrifuged off, washed with water, suspended in water and H_2S was passed in until precipitation of PbS was complete. The precipitate was filtered off and the filtrate concentrated *in vacuo*. On cooling, plates of 2-*acetamido*-1-*naphthyl glucosiduronic acid* were deposited, which after crystallization from water had m.p. 194–197°, $[\alpha]_D^{23}$ +2·4° in ethanol (*c*, 3·7) (Found: neutralization equivalent, 408; C, 52·4, 52·2; H, 5·8, 5·8; N, 3·4, 3·6%. $C_{18}H_{19}O_8N,2H_2O$ requires neutralization equivalent 413; C, 52·2; H, 5·6; N, 3·4%). On heating with 5N-HCl for 15 min. a compound was formed which after diazotization could be coupled with hexylresorcinol to give a red colour. It gave a positive reaction with naphthoresorcinol. After incubation with calf-spleen β-glucuronidase at pH 4·5 for 16 hr. at 37°, 2-acetamido-1-naphthol was identified by paper chromatography. A total of 1·5 g. of the compound was isolated, equivalent to 16% of the dose. The urine collected for a further 2 days yielded only a few milligrams of the glucosiduronic acid.

Examination of the untreated urine by paper chromatography showed that free 2-acetamido-1-naphthol and traces of 2-amino-1-naphthylsulphuric acid and 2-amino-1-naphthyl glucosiduronic acid were present, but no 2-acetamido-1-naphthylsulphuric acid could be detected.

2-*Amino*-1-*naphthyl glucosiduronic acid*

2-Acetamido-1-naphthyl glucosiduronic acid (1·0 g.) was heated on a water bath with 0·5N-H_2SO_4 (15 ml.) for 30 min., and the solution was treated with a small amount of charcoal, filtered and kept at room temperature overnight. Unchanged acetamido derivative (0·5 g.) separated and was filtered off; the filtrate was adjusted to pH 4·0 and evaporated in a vacuum desiccator until brown crystals began to appear. The solution was cooled to 5° overnight and the product filtered off. Recrystallization from water yielded small rosettes of prisms (100 mg., 11%) of 2-*amino*-1-*naphthyl glucosiduronic acid*, m.p. 178–180° (decomp.), $[\alpha]_D^{23}$ −155° in 0·1N-HCl (*c*, 0·175) (Found: C, 54·4; H, 5·8; N, 4·3, 4·2. $C_{16}H_{17}O_7N,H_2O$ requires C, 54·4; H, 5·4; N, 4·0%). The compound was soluble in water and readily soluble in ethanol. After hydrolysis by calf-spleen β-glucuronidase, 2-amino-1-naphthol was detected by the colour produced on shaking with aq. NH_3.

The acid hydrolysis gave some 2-acetamido-1-naphthol and for this reason the hydrolysis time was limited to 30 min. A guide to the optimum time of hydrolysis was obtained by examining samples by paper chromatography at intervals. Hydrolysis of the glucosiduronic acid linkage was slower than that of the acetamido group. Attempted hydrolysis with 2N-H_2SO_4 or 0·2N-HCl caused blackening and 2N-NaOH gave a red gum from which no 2-amino-1-naphthyl glucosiduronic acid could be isolated.

2-*Amino*-6-*naphthyl glucosiduronic acid*

Each of five rabbits received 2-amino-6-naphthol hydrochloride (0·45 g.), neutralized just before injection, for 4 days (total dose 7·2 g. of 2-amino-6-naphthol). The glucosiduronic acid fraction was separated by the lead acetate method and dissolved in the minimum of water, and the solution adjusted to pH 5·0. On cooling to 5° crystals of 2-*amino*-6-*naphthyl glucosiduronic acid* separated after 2 days. Recrystallization by solution in N-HCl and adjustment of the pH to 5·0 yielded 0·06 g. (0·4% of theory), $[\alpha]_D^{20}$ −76·8° in 0·1N-HCl (*c*, 0·625). The compound blackened and sintered at 250° (Found: C, 54·2; H, 5·55; N, 4·0. $C_{16}H_{17}O_7N,H_2O$ requires C, 54·4; H, 5·4; N, 4·0%). Paper chromatography showed that the mother liquors contained the acetamido derivative in addition to the amino compound.

2-*Acetamido*-6-*naphthyl glucosiduronic acid*

(*a*) *As the sodium salt.* 2-Acetamidonaphthalene was given daily to each of six rabbits for 7 days (total dose 16·8 g.). The collected urine was filtered (Ford's clarifying pad, grade F.C.B.) and adjusted to pH 7·0. Each day's urine was stirred for 0·5 hr. with about 50 g. of charcoal (British Drug Houses Ltd. activated charcoal, heated at 150° for about 0·5 hr. and cooled) and filtered. The almost colourless filtrate contained no detectable metabolites of 2-acetamidonaphthalene, but still gave a strong naphthoresorcinol reaction.

Preliminary experiments showed that elution of the charcoal with water or aq. 0·1N-NH_3 did not remove metabolites but that elution with hot methanol removed 2-acetamido-6-naphthol, 2-acetamido-6-naphthylsulphuric acid, a compound considered to be 2-acetamido-5:6-dihydro-

5:6-dihydroxynaphthalene and a trace of 2-acetamido-6-naphthyl glucosiduronic acid. Elution with 5% aqueous phenol removed the 2-acetamido-6-naphthyl glucosiduronic acid.

The charcoal was stirred with hot methanol (total vol. 3 l.) until all the first series of metabolites were extracted. The charcoal was then packed in a column above a layer of sand and treated with 5% aqueous phenol until the glucosiduronic acids were eluted, as indicated by paper chromatography. The eluate was evaporated to dryness and then kept in an evacuated desiccator over sodium hydroxide pellets for 24 hr. to remove the residual phenol. Solution of the residue in the minimum of water and the addition of several volumes of ethanol gave a hygroscopic precipitate (4 g.). (A sample of this preparation was incubated with bacterial β-glucuronidase in acetate buffer at pH 6·0 for 24 hr. at 37°; paper chromatography showed that 2-acetamido-6-naphthol and a little of the presumed 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene had been liberated.) After three crystallizations by solution in the minimum of hot water and addition of hot ethanol *sodium 2-acetamido-6-naphthyl glucosiduronate* (1·0 g.) was obtained as needles, m.p. 252–254° (decomp.), $[\alpha]_D^{25} - 78{\cdot}4°$ in water (*c*, 1·02) (Found: C, 49·5; H, 5·05; N, 3·2; Na, 5·8. $C_{18}H_{18}O_8NNa,2H_2O$ requires C, 49·65; H, 5·1; N, 3·2; Na, 5·3%). The yield was equivalent to 2·5% of the dose. The compound dissolved readily in water, giving a neutral solution, and was soluble in methanol and insoluble in ethanol. It gave a positive reaction with naphthoresorcinol and diazotized and coupled only after heating with 2 N-HCl. After incubation with bacterial β-glucuronidase at pH 6·0 (acetate buffer) or pH 7·0 (phosphate buffer) 2-acetamido-6-naphthol was detected by paper chromatography but 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene was not present. The hydrolysis product was isolated, m.p. alone and mixed with an authentic specimen of 2-acetamido-6-naphthol, 221–223°.

Sodium 2-acetamido-6-naphthyl glucosiduronate (0·4 g.) on heating for 3 hr. at 100° in 5 N-H_2SO_4 (10 ml.) gave 2-amino-6-naphthol, confirmed by the red colour given on diazotization and coupling with hexylresorcinol. The hydrolysis product was acetylated with acetic anhydride in pyridine and the diacetyl derivative, after recrystallization from ethanol, melted at 217–220°, alone and mixed with an authentic specimen of 2-acetamido-6-acetoxynaphthalene. Acidification of an aqueous solution of sodium 2-acetamido-6-naphthyl glucosiduronate gave no crystalline material and evaporation of the solution gave a yellow gum, soluble in water and ethanol, which could not be crystallized.

p-Toluidine 2-acetamido-6-naphthyl glucosiduronate was prepared by the addition of *p*-toluidine hydrochloride (0·1 g.) to a solution of the sodium salt (0·1 g.) in hot water (5 ml.). On cooling, needles separated which after recrystallization from water melted at 181–182° (decomp.), $[\alpha]_D^{24} - 72{\cdot}5°$ in water (*c*, 1·02) (Found: C, 62·0; H, 5·7; N, 5·8. $C_{25}H_{28}O_8N_2$ requires C, 62·0; H, 5·8; N, 5·8%).

In another experiment charcoal which was not preheated was used for adsorption and exhaustively extracted with hot methanol. The urine from rabbits dosed with 2-acetamidonaphthalene (28 g.) was divided into five portions, each approximately 2 l., which were each passed through columns of charcoal (250 g.). The columns were eluted with water (2 l.) and cold methanol (3 × 500 ml.). Indoxylsulphuric acid was identified in the methanol fractions. The columns were lagged and eluted with hot methanol (about 60°) in 500 ml. portions. Urinary phenols were eluted in the first three fractions. Twelve hot methanol washings were collected; the later washings contained very little 2-acetamido-6-naphthyl glucosiduronic acid. The hot methanol washings were combined, evaporated almost to dryness and the residue was dissolved in water and extracted several times with ether, followed by ethyl acetate. Ethyl acetate removes the compound which is thought to be 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene. The aqueous fraction was again evaporated to dryness and the residue dissolved in the minimum of butanol–propanol–water (2:1:1, by vol.) and sufficient Whatman cellulose powder added to form a paste. The paste was added to the top of a cellulose column (30 cm. × 6 cm.) prepared with the same solvent mixture and was slowly eluted over 5 days. Collection of fractions was guided by paper chromatography. 2-Acetamido-6-naphthol, 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene, 2-acetamido-6-naphthylsulphuric acid, an unidentified sulphuric ester and the glucosiduronic acid fraction were successively eluted from the column. Enzymic hydrolyses showed that this last fraction contained the glucosiduronic acids of 2-acetamido-6-naphthol and 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene, but mainly the former compound. The glucosiduronic acid fractions were combined and evaporated to dryness, the residue dissolved in the minimum of hot water and hot ethanol added until the solution was just cloudy. On cooling, rosettes of needles (3·5 g.) were formed, which after recrystallization gave sodium 2-acetamido-6-naphthyl glucosiduronate (1·5 g.) m.p. 252–254°. A further 1·0 g. of less pure material was obtained from the mother liquors and another 1·0 g. by elution of the charcoal with aqueous phenol. Attempts to isolate 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid by chromatography of the mother liquors or by fractional crystallization of the triacetyl methyl esters were not successful.

(*b*) *As the p-toluidine salt.* Urine from rabbits dosed with 2-acetamidonaphthalene (14 g.) was passed through a column of charcoal. The column was washed with water, with cold methanol which eluted relatively little of the glucuronide, and finally with aqueous phenol. The phenolic eluate was evaporated down, the residue dissolved in the minimum of water and 15 vol. of ethanol added. The precipitate (8·0 g.) was dissolved in water (70 ml.) and *p*-toluidine hydrochloride (12 g.) was added. After cooling overnight the *p*-toluidine salt of 2-acetamido-6-naphthyl glucosiduronic acid was collected and recrystallized twice from water to yield needles (2·3 g.), m.p. 181–182°, which did not depress the m.p. of the derivative prepared in (*a*).

(*c*) *As the triacetyl methyl ester.* Urine of rabbits dosed with 2-naphthylamine (total 10 g.) was passed through a charcoal column (150 g., not pretreated by heat). The column was washed first with water, then with hot methanol until the glucosiduronic acid fraction began to be eluted and then with aqueous phenol. The ethanol-insoluble material from the phenol eluate was dissolved in water, the pH adjusted to 5·0 and the solution evaporated to dryness in a desiccator. The residue was dissolved in methanol (100 ml.), treated with an ethereal solution of diazomethane (from 20 g. of nitrosomethylurea) and kept overnight at 5°. After filtration and concentration *in vacuo* the residue was dissolved in pyridine (10 ml.) and acetic anhydride (10 ml.). The solution was kept overnight and poured into water, and the precipitate

collected. After two crystallizations from aqueous ethanol *methyl (2-acetamido-6-naphthyl tri-O-acetylglucosid)uronate* was obtained as needles (0·7 g.), m.p. 205–206°, $[\alpha]_D^{24}$ – 28·6° in ethanol (*c*, 0·28) (Found: C, 58·1; H, 5·0; N, 2·7. $C_{25}H_{27}O_{11}N$ requires C, 58·0; H, 5·3; N, 2·7%). Although the original crude solid appeared to contain 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid no crystalline triacetyl methyl ester of this could be isolated.

Isolation of allantoin

The first and second cold methanol eluates from charcoal columns (see p. 277) were combined and the solution was concentrated to 30 ml. On cooling, prisms (1·5 g. from 10 l. of urine) separated, m.p. after three crystallizations from water 234–236° (decomp.), alone and mixed with an authentic specimen of allantoin (Found: N, 35·6. Calc. for $C_4H_6O_3N_4$: N, 35·4%). The compound had an R_F of 0·1 in butanol–propanol–0·1 N-NH_3, identical with that of allantoin. The spot gave a yellow colour with Ehrlich's reagent and a green colour with sodium hypochlorite (5% in water) after spraying with phenol (5% in 95% ethanol). On heating either the isolated substance or the authentic allantoin in 5 N-HCl with naphthoresorcinol it gave a cherry-red colour, extractable by ether. Asher (1910) described this colour reaction of allantoin and the adsorption of allantoin by charcoal. Corner & Young (1955) described the formation of a reddish purple colour from an unidentified constituent with naphthoresorcinol during the estimation of glucuronic acid in rat urine, but the colour did not interfere with the estimations. It is possible that allantoin was a contributory factor to the formation of this pigment. Under the conditions of the Dische (1947) method of estimating glucuronic acid, allantoin gave a blue–green colour.

The O-glucosiduronic acids formed in the metabolism of 2-naphthylamine

After rats and rabbits had been dosed with 2-naphthylamine the urine of each species was examined by paper chromatography for the presence of glucosiduronic acids. 2-Amino-1-naphthyl glucosiduronic acid was detected in the urine of both species, but was more abundant in that of the rat. Rat urine (2 ml.), diluted with an equal volume of 0·2 M-acetate buffer (pH 4·5), was incubated with calf-spleen β-glucuronidase at 37° for 2 days. 2-Amino-1-naphthol was liberated and identified by the ammonia–benzene test. A control urine sample gave no colour reaction to this test. A fraction containing the compound could be obtained by precipitation of the urine of rats or rabbits by basic lead acetate.

2-Amino-6-naphthyl glucosiduronic acid was detected in rat urine but not in rabbit urine. The compound was precipitated from rat urine by basic lead acetate. After decomposition of the lead salt by hydrogen sulphide a sample of the glucosiduronic acid fraction so obtained gave 2-amino-6-naphthol on incubation with calf-spleen β-glucuronidase. Rabbit urine, but not rat urine, contained a compound (R_F 0·2) which was non-fluorescent and which was not diazotizable until after treatment with acid at 70°. It corresponded in its reactions to 2-acetamido-6-naphthyl glucosiduronic acid. Paper chromatography of the urine after it had been heated for 15 min. with an equal volume of 5 N-hydrochloric acid gave a new spot which corresponded in R_F and colour reactions to 2-amino-6-naphthyl glucosiduronic acid. Although only one glucosiduronic acid appeared on hydrolysis there are two possibilities for the structure of its precursor. Both 2-acetamido-6-naphthyl glucosiduronic acid and 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid would give the amino compound on acid treatment. The glucosiduronic acid fraction of rabbit urine (50 ml.) was prepared by precipitation with basic lead acetate. After decomposition of the lead salt by hydrogen sulphide excess of the latter was removed by a current of air. The solution was adjusted to pH 6·5 and continuously extracted with ether for 18 hr. and then evaporated to 5 ml. in a desiccator at room temperature. After dilution with an equal volume of acetate buffer (pH 6·0) a sample of the solution was incubated with bacterial β-glucuronidase. Paper chromatography showed that two new spots were present. One corresponded to 2-acetamido-6-naphthol in R_F (0·92) and colour reactions. The other spot (R_F 0·81) was not fluorescent, did not react with diazotized sulphanilic acid, could not be diazotized and coupled with hexylresorcinol, but after treatment with acid at 70° the material in the spot could be diazotized (green colour with nitrous acid) and coupled with hexylresorcinol (red colour), and coupled with diazotized sulphanilic acid (mauve colour)—reactions identical with those of 2-amino-6-naphthol.

The material obtained by enzymic hydrolysis was also examined by two-dimensional chromatography, developing first with butanol–propanol–0·1 N-NH_3, then treating with acid at 70°, drying, and developing in the second direction with butanol–acetic acid–water (2:1:1, by vol.). Two spots corresponding in R_F and colour reactions to 2-amino-6-naphthol were present after the second development, one derived from the 2-acetamido-6-naphthol spot and the other from that of the unknown compound. The properties of this latter compound could be accounted for by the hydrolysis and dehydration of 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene. Acid-catalysed dehydration of cyclic dihydrodiols usually proceeds so that a hydroxyl group remains on the position which is normally attacked by electrophilic reagents (Badger, 1949). For example 3:4-dihydro-3:4-dihydroxychlorobenzene yields 4-hydroxychlorobenzene (Smith, Spencer & Williams, 1950) and 1:2-dihydro-1:2-dihydroxynaphthalene gives 1-naphthol (Young, 1947), although some 2-naphthol is also formed (Boyland & Sims, 1953). In the case

under consideration 2-acetamido-6-naphthol would probably be the major product (yielding the amino compound by simultaneous hydrolysis). The glucosiduronic acid spot with R_F 0·2 therefore represents a mixture of 2-acetamido-6-naphthyl glucosiduronic acid and 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid. 2-Acetamido-1-naphthyl glucosiduronic acid was not detected in either rat or rabbit urines after injection of 2-naphthylamine or 2-acetamidonaphthalene. In support of this 2-acetamido-1-naphthol was not detected by paper chromatography after the enzymic hydrolysis of any of the glucuronide fractions.

DISCUSSION

Two *N*-glucosiduronic acid derivatives (2-naphthylamine *N*-glucosiduronic acid and 2-amino-1-naphthylsulphuric acid *N*-glucosiduronic acid) have been described as metabolites of 2-naphthylamine by Boyland *et al.* (1957). Four *O*-glucosiduronic acid derivatives are now described (three of which are metabolites of 2-naphthylamine) and evidence is given for the presence of a fourth *O*-glucosiduronic acid metabolite. It has been argued (Boyland, 1956) that only 2-amino-1-naphthyl glucosiduronic acid should be carcinogenic because the urinary β-glucuronidase would liberate 2-amino-1-naphthol from this. Bonser, Bradshaw, Clayson & Jull (1956) have shown that 2-amino-1-naphthol is carcinogenic when introduced into the bladders of mice. Using a similar technique Allen *et al.* (1957) have shown that 2-amino-1-naphthyl glucosiduronic acid induces bladder cancer in mice but that other metabolites, including 2-naphthylsulphamic acid, 2-naphthylamine *N*-glucosiduronic acid, 2-amino-6-naphthol and 2-acetamido-6-naphthol, are inactive. The other three of the glucosiduronic acid derivatives which have been isolated are now being tested for carcinogenic activity.

The occurrence of 2-amino-1-naphthyl glucosiduronic acid in urine of rats dosed with 2-naphthylamine is difficult to reconcile with the fact that rats do not develop bladder cancer when dosed with 2-naphthylamine, although rat urine contains much more β-glucuronidase than does human urine (D. C. Williams, personal communication). The β-glucuronidase should liberate the carcinogenic 2-amino-1-naphthol when the urine of rats dosed with 2-naphthylamine is allowed to stand. It is not liberated unless β-glucuronidase is added to this urine. This apparent anomaly is being investigated.

SUMMARY

1. 2-Acetamido-1-naphthyl glucosiduronic acid was isolated from the urine of rabbits dosed with 2-acetamido-1-naphthol.

2. The carcinogenic 2-amino-1-naphthyl glucosiduronic acid was prepared by hydrolysis of 2-acetamido-1-naphthyl glucosiduronic acid.

3. 2-Amino-6-naphthyl glucosiduronic acid was isolated from the urine of rabbits dosed with 2-amino-6-naphthol.

4. 2-Acetamido-6-naphthyl glucosiduronic acid was isolated as the sodium and *p*-toluidine salts from the urine of rabbits dosed with 2-acetamidonaphthalene and as methyl (2-acetamido-6-naphthyl tri-*O*-acetylglucosid)uronate from the urine of rabbits dosed with 2-naphthylamine.

5. Evidence is presented that a glucosiduronic acid of 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene is excreted when rabbits are dosed with 2-naphthylamine or 2-acetamidonaphthalene.

6. Urine from rats and rabbits injected with 2-naphthylamine has been examined by paper chromatography. Rats and rabbits excrete 2-amino-1-naphthyl glucosiduronic acid. Rabbits excrete 2-acetamido-6-naphthyl glucosiduronic acid, but this compound was not detected in rat urine. Rats excrete 2-amino-6-naphthyl glucosiduronic acid, but rabbits do not. 2-Acetamido-1-naphthyl glucosiduronic acid was not detected in the urine of either species.

7. Allantoin (which gives a red colour with the naphthoresorcinol reagents) was isolated by adsorption on charcoal and elution with methanol.

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