in $CHCl_3$) [ref. $[\alpha]_D^{30}+20.1°$ ($CHCl_3$)],[6] yield 3.2 g. *Anal.* Calcd. for $C_{16}H_{14}O_5$: C, 67.12; H, 4.93. Found: C, 67.23; H, 5.22. The IR and NMR spectra are superimposable to those of oxypeucedanin.

Oxypeucedanin Hydrate——Recrystallized from EtOH–H_2O to colorless needles, mp 136—137°, yield 0.1 g. *Anal.* Calcd. for $C_{16}H_{16}O_6$: C, 63.15; H, 5.30. Found: C, 63.29; H, 5.37. The melting point was not depressed on admixture with the authentic sample of oxypeucedanin hydrate.

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Quantitative Determination of 2-Amino-1-naphthyl Glucosiduronic Acid in Urine of the Dog dosed with 2-Naphthylamine

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2-Naphthylamine (I) is a potent bladder carcinogen in the dog and man, the metabolism of which has been studied extensively in several species.[2,3] Among many metabolites so far isolated or detected, 2-amino-1-naphthol (IIa) and N-hydroxy-2-naphthylamine (IIIa) are regarded as the proximal carcinogen directly responsible for the production of bladder cancer, since they have been shown to be much more carcinogenic in the mouse[4] and rat[5] than the parent amine I. These carcinogenic metabolites of I are said to be excreted into urine mainly in the form of conjugates; *i.e.* O-glucuronide, O-sulfate, *etc.*[2a] While the O-glucuronide of IIa, 2-amino-1-naphthyl glucosiduronic acid (IIb), was unequivocally identified in urine of the dog, rat, or rabbit dosed with I, no definite evidence has been found[2d] for the presence of the O-glucuronide of IIIa, N-hydroxy-2-naphthylamine-O-glucosiduronic acid (IIIb), as a metabolite of I or IIIa in urine of the dog, guinea pig, hamster, rabbit, or rat. It was reasonably postulated [2a,6] concering the mechanism of bladder carcinogenesis by I in the dog

1) Location: *a*) *Takada 3-chome, Toshima-ku, Tokyo*; *b*) *Takada 3-chome, Toshima-kn, Tokyo.*
2) *a*) E. Boyland, "The Biochemistry of Bladder Cancer," Charles C. Thomas Publisher, Springfield, Illinois, 1963; *b*) E. Boyland, D. Manson and R. Nery, *Biochem. J.*, **86**, 263 (1963); *c*) E. Boyland and D. Manson, *ibid.*, **99**, 189 (1966); *d*) *Idem*, *ibid.*, **101**, 84 (1966).
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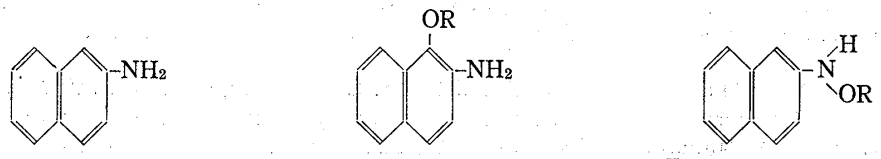
that IIb or IIIb excreted in urine is hydrolyzed by urinary β-glucuronidase liberating the carcinogenic metabolites, IIa or IIIa.



I

IIa : R=H
IIb : R=$C_6H_9O_6$
IIc : R=SO_3H

IIIa : R=H
IIIb : R=$C_6H_9O_6$

$C_6H_9O_6$: β-D-glucopyranosiduronic acid residue

Chart 1

In connection with a work carried on with a potent inhibitor of urinary β-glucuronidase,[7] it was required to develope a method for the quantitative determination of IIb in urine of dogs dosed with I. Determination of the O-glucuronide is usually made by estimating the aglycone after acid or enzymatic hydrolysis. As it was found that IIa was very unstable and underwent rapid decomposition during the hydrolytic procedure, a colorimetric method for the determination of IIb without prior hydrolysis was worked out, which is described in this paper.

Urine from dogs dosed with I was heated at pH 4 to inactivate β-glucuronidase and to hydrolyse N-glucuronides, and then it was passed through an anion exchanger column. Non-conjugated aminonaphthols including IIa were removed by washing the column with 0.2N acetic acid in 20% ethanol. Aminonaphthol glucuronides adsorbed on the column, whose principal component was shown to be IIb as described below, were eluted with 0.1N hydrochloric acid in 4N acetic acid, while sulfates were not eluted under this condition. The glucuronides thus eluted were determined colorimetrically after condensing with *p*-dimethylaminocinnamic aldehyde (DACA). When normal dog urine was treated as above, no color development was observed.

Seven spots were detected by thin-layer chromatography (TLC) in urine from dogs dosed with I after spraying with DACA. The major metabolite was found to be 2-amino-1-naphthyl hydrogen sulfate (IIc). The glucuronide fraction described above, on the other hand, revealed two spots, a deep- and a very faint-colored one, on TLC. The former corresponded to IIb and the latter was thought to be 2-amino-6-naphthyl glucosiduronic acid.

Satisfactory recovery of IIb added to normal dog urine was obtained with the present method, which was 90—98%.

Material and Method

Chemicals——2-Naphthylamine (I) and its hydrochloride were of commercial origin. 2-Amino-1-naphthol (IIa) (as hydrochloride) and 2-acetamido-1-naphthol were prepared as described by Booth, *et al.*[8] 2-Amino-1-naphthyl glucosiduronic acid (IIb) was obtained biosynthetically from rabbit urine after intraperitoneal injection of 2-acetamido-1-naphthol as described by Boyland, *et al.*[9] while 2-amino-1-naphthyl hydrogen sulfate (IIc)[10] and 2-naphthylamine-N-glucuronide[11] were synthesized according to the methods reported previously.

Urine Samples——Female beagle biches (8—12 kg) were kept in cages designed for separate collection of urine and faeces. I (500 or 250 mg) was administered orally in gelatin capsules. Collected urine was immediately frozen.

7) M. Matsui, M. Okada and M. Ishidate, *Chem. Pharm. Bull.* (Tokyo), **17**, 1064 (1969).
8) J. Booth, E. Boyland and D. Manson, *Biochem. J.*, **60**, 62 (1955).
9) E. Boyland and D. Manson, *Biocem. J.*, **67**, 275 (1957).
10) E. Boyland and D. Manson, *J. Chem. Soc.*, **1958**, 532.
11) E. Boyland, D. Manson and S.F.D. Orr, *Biochem. J.*, **65**, 417 (1957).

Thin-Layer Chromatography——TLC plates were prepared using Silica gel G (E. Merck AG) as adsorbent. The solvent system used was *n*-butanol–acetic acid–water (2:1:1) and the spots were revealed by spraying DACA.

Preparation of Anion Exchanger Columns——Amberlite CG 400 (type I, Cl^--form, 100—200 mesh) (50 ml) was washed with water in a beaker and packed in a column (2×40 cm). The column was washed with 2N NaOH (200 ml), water, 2M NaCl (50 ml), 2N HCl (200 ml) and water consecutively. After replacing the resin in a beaker it was washed with 20% ethanol. The resin suspended in 20% ethanol was pipetted into tubes (6 mm in diameter) to form a packed layer 8 cm long.

Separation and Determination Procedures——Urine (10 ml) was adjusted to pH 4 with 2N HCl and made a volume of 20 ml with water. The diluted urine was heated on a boiling water bath for 5 min. After cooling, 2 ml of the treated urine was put on a resin column cited above. The column was washed with 0.2N acetic acid–20% ethanol and then the glucuronides were eluted with 0.1N HCl–4N acetic acid (20 ml). To a 2 ml portion of the effluent was added 4 ml of DACA solution (0.1% DACA in ethanol). The mixture was allowed to stand for 15 min and the optical density was measured at 540 mμ.

Result

Separation of IIb

After adding IIb (25 μ moles) to 10 ml of normal dog urine, it was treated as described above. Normal dog urine was also treated similarly as a control. The effluent from the column was fractionated in 2 ml portions and the fractions were assayed. Elution curve is shown in Fig. 1. About 96% of the added IIb was recovered, while the control showed no color developement.

TLC of Urine from Dogs Dosed with I

Urine from dogs dosed with I (500 mg) and its resin column treated fraction were examined by TLC. As indicated in Fig. 2, the major metabolite was IIc. The column treated fraction was shown to contain mainly IIb. The extremely minor spot detected in the fraction was considered to be 2-amino-6-naphthyl glucosiduronic acid,[9] which gave a positive naphthoresorcinol reaction for uronic acid, although no definite identification was made.

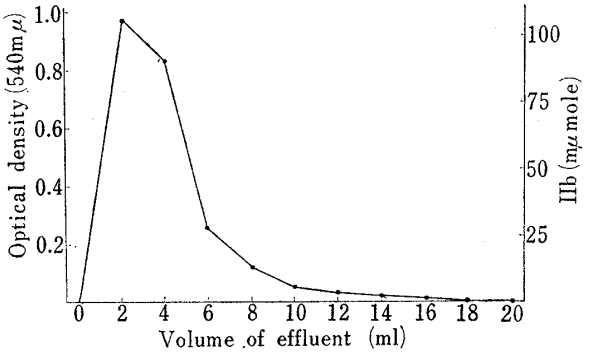


Fig. 1. Elution Curve of 2-Amino-1-naphthyl Glucosiduronic Acid (IIb) Added to Dog Urine

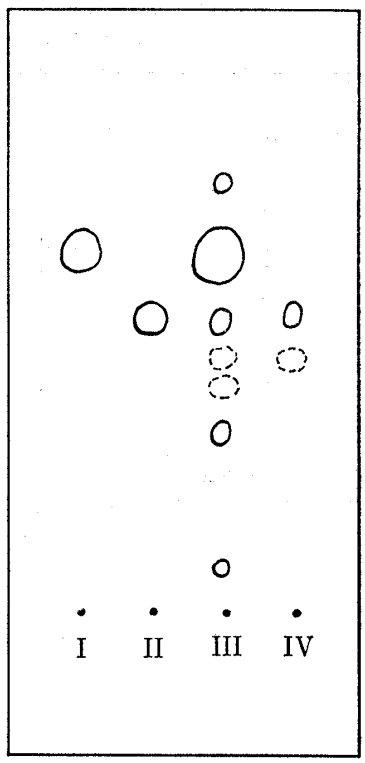


Fig. 2. Thin-Layer Chromatography of Urine from Dogs Dosed with 2-Napthylamine (I)

I: 2-amino-1-naphthyl hydrogen sulfate (IIc)
II: 2-amino-1-naphthyl glucosiduronic acid (IIb)
III: urine from dogs dosed with I
IV: column treated urine

Calibration Curve for IIb

To each 2 ml of IIb in 0.1N HCl–4N acetic acid solution was added 4 ml of DACA solution and the optical density at 540 mμ was determined. Linear proportionality between optical

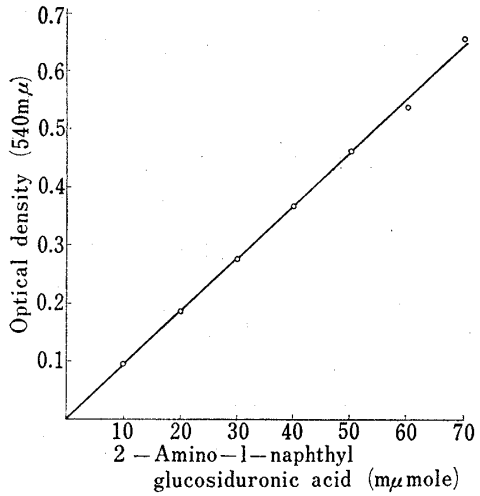


Fig. 3. Calibration Curve for 2-Amino-1-napthyl Glucosiduronic Acid

density and concentration was obtained for IIb at 4—25 μg/2 ml (10—70 mμmoles/2 ml) (Fig. 3).

Recovery of IIb Added to Dog Urine

Each 1,2,4 or 6 μmoles of the equimolar mixture of I hydrochloride, IIa hydrochloride, 2-naphthylamine-N-glucuronide, IIb and IIc was added to each 10 ml of normal dog urine and treated as described in the separation and determination procedures. Recovery of IIb was 90—98%.

Excretion Rate of Dosed I as IIb into Urine[12]

Urinary excretion of IIb was determined after oral administration of I (500 or 250 mg) in dogs. As shown in Table I, about 7% of the dose was excreted as IIb in the case of 500 mg dosage, while about 4% in the case of 250 mg dosage.

Table I. Urinary Excretion of 2-Amino-1-naphthyl Glucosiduronic Acid (IIb) after Oral Administration of 2-Naphthylamine (I) to Dogs[12]

Dog No.	Dose (mg)	IIb in urine (mg)			Excretion rate (%) of dose
		0—12 hr	12—24 hr	Total	
1	500	80.7	9.8	90.5	8.6
2	500	54.8	10.1	64.9	6.1
3	500	55.8	10.0	65.8	6.4
4	500	69.0	10.8	79.8	7.6
5	500	64.0	14.1	78.1	7.3
6	250	6.0	13.3	19.3	3.7
7	250	12.0	9.1	21.1	4.0

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12) As cited above, the glucuronide fraction separated by the resin column contained two aminonaphthol glucuronides, IIb and possibly 2-amino-6-naphthyl glucosiduronic acid. Since the latter was found to be extremely minor component, the glucuronide fraction could be regarded and estimated as IIb.