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Metabolism of Polycyclic Compounds

8.* ACID-LABILE PRECURSORS OF NAPHTHALENE PRODUCED AS METABOLITES OF NAPHTHALENE

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Baumann & Herter (1877) observed that crystals of naphthalene appeared in the distillate when the acidified urine of rabbits dosed with naphthalene was heated. Bourne & Young (1933) noticed that crystals of naphthalene separated from such acidified urine in the cold. They suggested that this might be due to the dehydration of 1:2-dihydro-2-naphthol, which is known to give naphthalene with cold mineral acid (Bamberger & Lodter, 1895), but dihydronaphthol does not appear to have been isolated from urines of animals dosed with naphthalene. Chang & Young (1943) and Young (1947) estimated that the acid-liberated naphthalene accounted for from 4 to 14 % of the dose fed to rats or rabbits. The liberation of anthracene on acidification of the urine of rats and rabbits dosed with anthracene was noticed by Boyland & Levi (1936). Chang & Young (1943) also found that small amounts of hydrocarbons were liberated on acidification of urines of rats dosed with phenanthrene and anthracene. Acid-labile precursors could not be detected in the urines of rats dosed with higher polycyclic hydrocarbons.

* No. 7 of this series: Boyland, E. & Wiltshire, G. M. (1953).

Studies on 1:2:3:4-tetrahydronaphthalene (tetralin) metabolism by Pohl & Rawicz (1919) and by Rockemann (1922) indicated that tetralin was converted into 1- or 2-tetralyl glucosiduronic acids by dogs and rabbits. When these tetralyl glucosiduronic acids were steam-distilled from acid solution, 1:2-dihydronaphthalene and naphthalene were produced. Pohl & Rawicz (1919) also dosed rabbits with 1:2-dihydronaphthalene and found that naphthalene was produced on acidification of the urine; they suggested that a 1:2-dihydronaphthyl glucosiduronic acid had been formed. Corner, Billett & Young (1954) have recently shown that 1:2-dihydronaphthalene-1:2-diol is excreted as a glucosiduronic acid derivative by rabbits dosed with naphthalene.

Evidence that naphthalene is metabolized to 1:2-dihydro-1-naphthyl glucosiduronic acids, which readily yield naphthalene in acid solution, is presented in this paper.

EXPERIMENTAL

Fifty rats (average body weight 250 g.) maintained on rat cake and water, were each injected intraperitoneally with naphthalene (100 mg.) dissolved in arachis oil (1 ml.) on

5 consecutive days. Four rabbits (body weight approximately 2 kg.) maintained on oats, rat cake, bread and water, were each dosed *per os* with naphthalene (1 g.) dissolved in arachis oil (10 ml.) on 4 consecutive days. The animals were housed in metabolism cages and the urine was collected during the period of dosing, and for 2 days after the last dose.

The melting points given in this paper are uncorrected.

Detection of naphthalene precursors

Samples of naphthalene urine were made alkaline and steam distilled for 5 min. to remove free naphthalene. The solutions were acidified (pH 2) and slowly steam distilled, when crystalline naphthalene separated in the distillate. The naphthalene precursor was also detected by paper chromatography. Solutions were applied to Whatman no. 1 (special chromatographic) paper which was developed downwards with the upper layer of a *n*-butanol-ethanol-water mixture (17:3:20) for 16 hr. This system separated the naphthalene precursor (approximate R_F 0.22), 1-naphthyl glucosiduronic acid (R_F 0.15) and 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid (R_F 0.08). The naphthalene precursor was seen on paper as a dark absorbent region under ultra-violet light (mainly 2536 Å) from a Hanovia chromatolite (Chance OX 7 filter).

Isolation of naphthalene precursors

The 'naphthalene urine' was centrifuged to remove solid material and evaporated under reduced pressure to a brown gum which was extracted repeatedly with boiling ethanol until the extracts were colourless, and the residue became granular. The ethanolic extracts were cooled, filtered and evaporated under reduced pressure to a brown gum which was dissolved in a small volume of water. A mixture of equal parts by weight of charcoal (activated, British Drug Houses Ltd.) and kieselguhr was stirred with water to form a slurry, which was poured into a glass tube (diameter 3 cm.) with a sintered disk covered with sand (about 1 cm.) as a support for the adsorbent. The column (15 cm. long) was washed with water (1 l.). The aqueous solution of the gum (20 ml.), which gave a positive naphthoresorcinol reaction, was allowed to percolate down the column. The column was treated with distilled water (*ca.* 4 l.) using mild suction, until all the naphthalene precursor was eluted. This eluate (*A*) also contained urea, inorganic salts and a little 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid. When the charcoal column was subsequently eluted with 50% (v/v) aqueous acetone, a fraction (*B*) which contained mainly this glucosiduronic acid was obtained.

The aqueous eluate *A* was concentrated under reduced pressure and extracted with hot dioxan, which removed large quantities of urea and traces of 1:2-dihydronaphthalene-1:2-diol (1:2-diol), 1-naphthyl sulphuric acid and another unidentified metabolite of naphthalene. The residual gum was dissolved in water (2 ml.) and passed

through a cellulose partition column prepared by carefully dry-packing (Hough, Jones & Wadman, 1949) Whatman cellulose powder into a glass tube (27 × 7 cm.) and equilibrating by washing with 1 l. of isopropanol-water (9:1). When the top of the column was dry, the solution containing the glucosiduronic acids was poured on, and allowed to soak in before development with isopropanol-water (9:1). The naphthalene precursor was present in the first three fractions (100 ml. each); continued development with this solvent did not elute 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid which was eluted with isopropanol-water (8:2). The fractions containing the naphthalene precursor were evaporated under reduced pressure to a clear yellow gum, which gave a strongly positive naphthoresorcinol test, effervesced in NaHCO_3 solution, produced naphthalene on acidification with mineral acid and did not couple with diazotized *p*-nitraniline. This gum still contained urea and other unidentified impurities and could not be crystallized from any of a number of solvent mixtures. As no water-insoluble salt of the glucosiduronic acid could be obtained, the clear gum was dissolved in hot methanol (20 ml.), cooled, filtered and treated with an excess of diazomethane. Removal of the solvent under reduced pressure afforded the methyl ester of the glucosiduronic acid as a brown gum, which was dissolved in hot methanol (10 ml.) and treated with a mixture (50 ml.) of equal volumes of pyridine and acetic anhydride. After 30 min. the mixture was poured into water and allowed to stand at 4° overnight, when a white powder separated, which was crystallized from warm aqueous ethanol as small needles which were dried *in vacuo* over P_2O_5 at 100°. Yields of this derivative from rat and rabbit urines were 100 mg. (0.4% dose) and 80 mg. (0.5% dose) respectively. This appeared to be methyl (1:2-dihydro-1-naphthyl tri-*O*-acetylglucosid)uronate. The derivative isolated from rat urine had m.p. 203°; $[\alpha]_D^{25} - 84 \pm 3^\circ$ in CHCl_3 (*c.* 1). (Found: C, 59.9; H, 6.1. $\text{C}_{23}\text{H}_{35}\text{O}_{10}$ requires C, 59.7; H, 5.7%.) And that from rabbit urine had m.p. 203°, unchanged by admixture with the rat derivative, and $[\alpha]_D^{25} + 23 \pm 1^\circ$ in CHCl_3 (*c.* 1). (Found, C, 59.8; H, 5.9%.) Both derivatives were soluble in CHCl_3 , acetone, ethyl acetate and warm ethanol but were insoluble in water. Comparative data for triacetyl methyl esters of the glucosiduronic acids of the 1:2-diol and 1- and 2-naphthol (Corner *et al.* 1954) are given in Table 1.

The rat and rabbit triacetyl methyl esters (5 mg. each) were separately shaken with water at pH 2 for 16 hr. Each solution was then made alkaline and steam distilled, when naphthalene collected in the condenser. The naphthalene was washed into the steam distillate with ethanol until the distillate contained 60% ethanol (100 ml.) and estimated spectrophotometrically at 275 $\mu\mu$. with a Unicam spectrophotometer. The derivative obtained from rat urine yielded 1.0 mg. naphthalene and that from the rabbit urine 1.1 mg. naphthalene (theory 1.38 mg.). The residual solution after steam distillation from acid gave a strongly positive

Table 1. Properties of certain glucosiduronic acid derivatives

Derivative	Animal	$[\alpha]_D^{25}$ (°)	M.p. (°)
*Methyl (1:2-dihydro-2-acetoxy-1-naphthyl tri- <i>O</i> -acetylglucosid)uronate	Rabbit	+94	209
*Methyl (1-naphthyl tri- <i>O</i> -acetylglucosid)uronate	Rabbit	-79	160
*Methyl (2-naphthyl tri- <i>O</i> -acetylglucosid)uronate	Rabbit	-35	127-8
Methyl (1:2-dihydro-1-naphthyl tri- <i>O</i> -acetylglucosid)uronate	Rabbit	+23	203
	Rat	-84	203

* Corner *et al.* (1954).

Fig. 1

naphthoresorcinol reaction. Three control experiments using solutions containing known amounts of naphthalene gave recoveries of 67, 72 and 76 %.

Determination of structure

Possible formulae for acid-labile naphthalene precursors include formulae I-V (Fig. 1). 1:2-Dihydro-1-naphthol (I) has not been described, but 1:2-dihydro-2-naphthol (II) is dehydrated very rapidly to naphthalene in cold acid: naphthalene is not produced when 1:2:3:4-tetrahydronaphthalene-2:3-diol (V) is treated with boiling acid (Bamberger & Lodter, 1895).

Ultraviolet absorption spectra. The structure of the tri-acetyl methyl esters was further elucidated by a study of their ultraviolet absorption spectra. The rat and rabbit derivatives (in ethanol) had identical absorption spectra similar to that of 1:2-dihydronaphthalene (Fig. 2 and Table 2). The compounds possessing a 1:2:3:4-tetrahydronaphthalene structure (III-V) would be expected to have characteristic double peaked spectra with $\lambda_{\max}$ 267 and 275 m μ . and $\epsilon_{\max}$ (267 m μ) 740. 1:4-Dihydronaphthalene has a very similar $\lambda_{\max}$ and $\epsilon_{\max}$ values to 1:2:3:4-tetrahydronaphthalene (Morton & de Gouveia, 1934), whereas 1:2-dihydronaphthalene has $\lambda_{\max}$ 262 m μ . and $\epsilon_{\max}$ 10 230. Beale & Roe (1951) observed that the optically active 1:2-dihydronaphthalene-1:2-diol had $\lambda_{\max}$ 265 m μ . and $\epsilon_{\max}$ 8250 ($\pm 2\%$). This indicates that the naphthalene precursors possess a 1:2-dihydronaphthalene structure.

Action of β -glucuronidase

When the crude dihydronaphthyl glucosiduronic acid fraction obtained from the partition column during the isolation was incubated at 37° with bacterial β -glucuronidase (Sigma Chemical Co., Missouri, U.S.A.), a steam-volatile oil was liberated which readily changed to naphthalene. To hinder this decomposition, solutions containing about 200 mg. of each crude naphthalene precursor were incubated at 37° with bacterial β -glucuronidase (1000 Sigma units/100 ml.) under benzene at pH 6.0 for 8 hr. The benzene did not inhibit the enzyme action but reduced bacterial contamination and stabilized the product. The benzene layer, after drying over anhydrous Na_2SO_4 and subsequent evaporation, yielded a trace of oil, which was steam distilled from alkaline solution. The steam distillate was extracted with ether, the ether was removed and the residue was steam distilled from acid when naphthalene was deposited in the condenser.

Identification of 1:2-dihydronaphthyl glucuronides

Bamberger & Lodter (1895) synthesized 1:2-dihydro-2-naphthol (m.p. about 35°) by treating 3-chloro-1:2:3:4-tetrahydro-2-naphthol with alkali; 1:2-dihydro-1-naphthol

Fig. 2. The ultraviolet absorption spectra of: —, 1:2-dihydronaphthalene; ----, methyl (1:2-dihydro-1-naphthyl tri-*O*-acetyl-glucosid)uronate;, 1:2:3:4-tetrahydronaphthalene.

Table 2. Ultraviolet absorption data for some hydrogenated naphthalene derivatives

Compound	$\lambda_{\max}$ (m μ)	$\epsilon_{\max}$
*1:2-Dihydronaphthalene	262	10 230
*1:4-Dihydronaphthalene	267	800
*1:2:3:4-Tetrahydronaphthalene	267	740
†1:2-Dihydronaphthalene-1:2-diol (synthetic racemate)	263	6 830
Crude barium salt of 1:2-dihydro- 2-hydroxy-1-naphthyl glucosid- uronic acid (rat)	264	—
Methyl (1:2-dihydro-1-naphthyl tri- <i>O</i> -acetylglucosid)uronates (rat and rabbit)	263	6 770

* Morton & de Gouveia (1934).

† Beale & Roe (1951).

has not yet been synthesized. Alkaline permanganate oxidation of 1:2-dihydro-2-naphthol yielded products which were unsuitable for characterizing 1:2-dihydro-2-naphthol, of which no derivatives have been described. Attempted catalytic reduction of the 3:4 double bond of crude dihydronaphthyl glucosiduronic acid under various conditions to the corresponding tetralol derivative was unsuccessful; no decrease in absorption at 264 m μ . was observed.

Hydroxylation of the methylene group of 1:2-dihydro-1-naphthyl glucosiduronic acid would yield 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid which is readily converted into 1-naphthyl glucosiduronic acid in boiling acid (Corner *et al.* 1954). The dihydronaphthyl glucosiduronic acid was not oxidized with SeO₂ in boiling ethanol. Bromination in the 2-position of the crude glucosiduronic acid in water with *N*-bromosuccinimide (2%) and benzoyl peroxide (0.1%) for 3 hr. at room temp. followed by hydrolysis with 2% aqueous KOH for 4 hr. yielded sufficient 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid to be detected by paper chromatography. No naphthyl glucosiduronic acid could be detected after acid treatment of the solution.

Catalytic dehydrogenation. Wieland (1912) obtained naphthalene and tetralin by the disproportionation of 1:4-dihydronaphthalene at room temp. in the presence of palladium black. Similar experiments using a hydrogen donor as solvent have been made by Linstead, Braude, Mitchell, Woolridge & Jackman (1952), who obtained equal amounts of naphthalene and tetralin by heating 1:2-dihydronaphthalene at 65° with palladium-charcoal catalyst in tetrahydrofuran.

As a control experiment, 1:2-dihydronaphthalene (200 mg.) in ethanol (100 ml.) was shaken with 5% palladium on charcoal (500 mg.) at room temp. (the catalyst having been well washed with warm ethanol to remove impurities). The reaction was followed by plotting the ultraviolet absorption curves of suitably diluted samples of the reaction mixture at various time intervals. After 22 hr. about 75% of the dihydronaphthalene appeared to be converted into naphthalene. The palladium-charcoal catalyst was filtered off, and the solution evaporated to small volume and kept at 4°. After 2 days the solid was recrystallized 3 times from aqueous ethanol to yield naphthalene, m.p. 79°.

Under the same dehydrogenation conditions the dihydronaphthyl glucosiduronic acid fraction showed no appreciable reaction at room temp. after 6 days. When the experiment was repeated in boiling ethanol, a considerable drop in optical density at 264 m μ . occurred after 6 days, but no naphthyl glucosiduronic acid could be detected in the reaction mixture by paper chromatography. The lack of reactivity might have been due to impurities in the glucosiduronic acid fraction. However, when small quantities of pure methyl (1:2-dihydro-1-naphthyl tri-*O*-acetylglucosiduronate (rat metabolite) in ethyl acetate were treated in the same way, only a slight fall in optical density was observed. It was impossible in any of these experiments to obtain a characteristic curve of either the 1- or 2-naphthyl glucosiduronic acids (Fig. 3).

Dehydrogenation of dihydronaphthyl glucosiduronic acid fractions from rat and rabbit urines was eventually effected by using higher temperatures. The aqueous glucosiduronic acid fractions (20 ml. in each case) were adjusted to pH 8 and heated with palladium-charcoal catalyst (10 mg./ml.) in a sealed tube at 150° for 6 hr. The reaction mixtures, after

filtering to remove the catalyst, showed a marked decrease in absorption at 264 m μ . Paper chromatograms of these solutions revealed that most of the dihydronaphthyl glucosiduronic acid had disappeared and that some 1-naphthol and 1-naphthyl glucosiduronic acid had been formed. The reaction mixtures were extracted with ether for 16 hr. to remove 1-naphthol and a portion of each solution was adjusted to pH 6.0 with acetate buffer and incubated at 37° for 5 hr. with bacterial β -glucuronidase (100 Sigma units/10 ml.). Samples of the ether-extracted enzyme hydrolysates were applied to Whatman no. 3MM paper and developed upwards for 2 hr. with 0.1N-NH₄OH. This procedure, which completely separates 1- and 2-naphthol, showed the presence in each case of only 1-naphthol, which was detected by its characteristic fluorescence under ultraviolet light and colour reaction with diazotized *p*-nitraniline.

The *p*-toluidine salt of 1-naphthyl glucosiduronic acid (95 mg.) was isolated from the dehydrogenation reaction mixture of the rat metabolite using the method of Berenbom & Young (1951). (No *p*-toluidine salt could be obtained from the reaction mixture from the dehydrogenation of the rabbit metabolite.) When the *p*-toluidine salt was dissolved in 6N-HCl (1 ml.) and the solution cooled to 4°, 1-naphthyl glucosiduronic acid separated. It crystallized from water in needles (10 mg.) which after drying at 100° *in vacuo* over P₂O₅ had m.p. 200° (decomp.), unchanged in admixture with authentic 1-naphthyl glucosiduronic acid (m.p. 198–200°) prepared by the method of Berenbom & Young (1951); $[\alpha]_D^{25} -84.3 \pm 2^\circ$ in water (c. 1). (Found: C, 55.6; H, 5.8. Calc. for C₁₆H₁₆O₇, 1.5 H₂O: C, 55.3; H, 5.5%.) The ultraviolet absorption spectra (Fig. 3) corresponded to that of pure 1-naphthyl glucosiduronic acid (λ_{max} . 282 and 319 m μ .; ϵ_{max} . 5650 and 1920). It thus appears that the two optically active dihydronaphthyl glucosiduronic acids are 1:2-dihydro-1-naphthyl glucosiduronic acids.

Fig. 3. The ultraviolet absorption spectra of: ---, 1-naphthyl glucosiduronic acid obtained by dehydrogenation of 1:2-dihydro-1-naphthyl glucosiduronic acid and —, 2-naphthyl glucosiduronic acid obtained from the urine of rats dosed with 2-naphthol.

*Possible precursors of
1:2-dihydro-1-naphthyl glucosiduronic acid*

Alcohols administered to animals are often excreted as glucosiduronic acids. The chemical processes involved in the production of dihydronaphthol by animals dosed with naphthalene are as yet unknown, but free dihydronaphthol is probably first formed in the body from naphthalene and then conjugated with glucuronic acid. 1:2-Dihydro-1-naphthol might be formed in the body either by the addition of the elements of water to the 1:2-bond of naphthalene or by the monohydroxylation of 1:2-dihydronaphthalene.

1:2-Dihydronaphthalene was purified by the method of Rowe (1921) and was shown to be free of naphthalene by infrared analysis. A 50% (v/v) solution of 1:2-dihydronaphthalene in arachis oil (0.5 ml./rat) was administered to rats (200 g.) daily for 3 days. The rat urine was examined by paper chromatography and found to contain relatively more dihydronaphthyl glucosiduronic acid and much less free diol than did rat naphthalene urine. These urines yielded naphthalene and an oil when steam distilled from acid solution.

DISCUSSION

Many workers have shown that in the metabolism of naphthalene an acid-labile metabolite was formed which gave the parent hydrocarbon on subsequent acidification of the urine. The isolation of methyl (1:2-dihydro-1-naphthyl tri-*O*-acetylglucosid)uronate, which yields naphthalene on treatment with acid, has revealed a new class of compound formed during the metabolism of naphthalene (Boyland & Solomon, 1953). Neither 1:2-dihydro-1-naphthol nor any of its derivatives appear to have been previously reported.

1:2-Dihydronaphthalene-1:2-diol is a well established metabolite of naphthalene (Young, 1947; Booth & Boyland, 1949), yet nothing is known of the mechanism of its formation in the body. The occurrence of both mono- and di-hydroxy derivatives of 1:2-dihydronaphthalene in the urine of animals dosed with naphthalene suggested that an initial reduction of the 1:2-position in the naphthalene structure may be followed by hydroxylation at the reduced positions. This theory has been tested by injection of 1:2-dihydronaphthalene into rats when the glucosiduronic acid of 1:2-dihydro-1-naphthol and free 1:2-dihydronaphthalene-1:2-diol were detected in the urine. As free 1:2-dihydronaphthalene-1:2-diol was only present in small amounts in these urines, it seems probable that this metabolite is mainly formed from a precursor other than 1:2-dihydronaphthalene, and lends support to the 'perhydroxylation' theory. The dihydronaphthol and the diol are probably formed by separate metabolic routes. Although the 1:2-diol is excreted both in free and conjugated forms in the urines of

rats dosed with naphthalene, 1:2-dihydro-1-naphthol is excreted only in the form of glucosiduronic acid.

SUMMARY

1. Acid-labile hydrocarbon precursors excreted in the urine of rats and rabbits dosed with naphthalene have been isolated as methyl (1:2-dihydro-1-naphthyl tri-*O*-acetylglucosid)uronates. The product from rat urine had $[\alpha]_D^{25} - 84^\circ$ and that from rabbit urine $+ 23^\circ$.

2. The position of the hydroxyl group of these glucosiduronic acids was determined by catalytic dehydrogenation to 1-naphthyl glucosiduronic acid.

3. 1:2-Dihydronaphthalene also appeared to be excreted as a dihydronaphthyl glucosiduronic acid and so may be an intermediate in the metabolic production of this glucosiduronic acid from naphthalene.

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