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Chemical and Metabolic Characteristics of 1-Naphthyl β-D-Glucoside

H. Wyman Dorough,* Joseph P. McManus,[1] Sham S. Kumar,[2] and Raymond A. Cardona[3]

1-Naphthyl β-D-glucoside was stable under a variety of conditions encountered in metabolism studies. No degradation occurred when stored in methanol or in Tris-HCl buffer (pH 7.0) for 1 week at −20, 0, and 25°. The glucuronide of 1-naphthol was degraded by about 30% after 1 week in the buffer at 25°, but was equal in stability to the glucoside in all other experiments. When 1-naphthyl-^{14}C glucoside was given orally to rats, 67% of the radiocarbon was eliminated in urine after 24 hr. Of this, 28% was the administered compound, 35% 1-naphthyl glucuronide, 15% 1-naphthyl sulfate, and 14% was 1-naphthol. Cleavage of the glucoside linkage was possibly the sole initial step in the metabolism of the compound. However, 1% of a 1-naphthyl glucoside-*glucose*-^{14}C dose was tentatively identified as 1-naphthyl glucuronide, indicating that oxidation of the sugar moiety of intact 1-naphthyl glucoside may have taken place. 1-Naphthol-^{14}C given as a single oral dose was eliminated from rats more rapidly, 90% of the dose in the 0–24-hr urine, than was its glucoside conjugate. 1-Naphthyl glucuronide constituted 81% of the radiocarbon while 17% was 1-naphthyl sulfate and only 1.6% was free 1-naphthol.

Defining the metabolic pathways of pesticides in various biological systems is essential if proper evaluations of their safety are to be conducted. This has long been recognized and has been attempted at varying degrees since the introduction of synthetic organic pesticides. Major emphasis has been placed on metabolites formed by oxidation and/or hydrolysis, and which exist in the free form. Other metabolites, however, are biosynthesized from pesticides which also are potentially toxicologically significant. Conjugation is one of the more important mechanisms of pesticide metabolism, especially in the substituted phenyl carbamate insecticides (Dorough, 1970; Kuhr, 1970). With these chemicals, the majority of the terminal residues in animals and plants may exist as conjugates, many of which contain the intact carbamic acid ester.

Glucuronidation is the most important conjugation mechanism in animals while glucosylation is a major conjugation reaction in plants. Insects, unlike other animals, form glucosides rather than glucuronides. These reactions have been recently investigated in our laboratory using 1-naphthol-^{14}C as a substrate, and insect and rat liver homogenates as enzyme sources (Mehendale and Dorough, 1972a,b). The current study is an extension of these studies, but rather than being concerned with the biosynthesis of glycosides, this report deals with the fate of 1-naphthyl glucoside in rats and with the development

Department of Entomology, University of Kentucky, Lexington, Kentucky 40506.
[1] Present address: Amchem Products, Inc., Ambler, Pa. 19002.
[2] Present address: Syracuse University Research Corporation, Life Sciences Laboratory, University Heights, Syracuse, N. Y. 13210.
[3] Present address: Division of Cancer Research, Department of Medicine, Michael Reese Hospital and Medical Center, Chicago, Ill. 60616.

of techniques basic to the study of the metabolism of any glycosidic conjugates.

EXPERIMENTAL SECTION

Chemical Synthesis of 1-Naphthyl Glucoside. The glucoside of 1-naphthol was prepared using the basic procedure of Helferich and Schmitz-Hillebrecht (1933). Glucose pentaacetate (3.0 g, 0.01 mol) was mixed with 4.3 g (0.03 mol) of 1-naphthol and 0.2 g of *p*-toluenesulfonic acid. The mixture was heated, under vacuum, for 1 hr in an oil bath at 100°. Following cooling to room temperature, the melt was dissolved in 100 ml of benzene and extracted twice with 20-ml portions of 2% sodium hydroxide. The benzene was dried with anhydrous sodium sulfate and then concentrated to dryness. Crystallization from ethanol and water gave 4.5 g of the 1-naphthyl tetra-*O*-acetyl-β-D-glucopyranoside, mp 177–178°. Deacetylation was accomplished by passing dry ammonia gas through a methanol solution of the product (2.0 g/10 ml) for 1 hr at 0° and then holding the solution at 5° for an additional 3 hr. The methanol was evaporated and the residue crystallized from a mixture of ether and ethanol. 1-Naphthyl β-D-glucopyranoside, 1.2 g, was recovered as a white powder, mp 171–175°. The mass spectrum (Finnigan Model 1015C) contained a weak molecular ion at *m/e* 306 while the base peak occurred at *m/e* 144 due to 1-naphthol. Peaks at *m/e* 116 and 115 represented the base peak minus CO and CHO, respectively. The sugar moiety was evidenced by peaks at *m/e* 163 ($C_6H_{11}O_5^+$) and 162 ($C_6H_{10}O_5^+$).

Biosynthesis of Radioactive 1-Naphthyl Glucoside and Glucuronide. For the synthesis of 1-naphthyl-*1*-^{14}C glucoside, an *in vitro* glucosylation system using house fly enzyme was employed (Mehendale and Dorough, 1972a). Each incubation mixture consisted of 0.5 μCi of 1-naphthol-*1*-^{14}C (sp act. 19.6 mCi/mmol), 2.4 ml of Tris-HCl buffer (pH 7.2), 1 ml of 0.1 *M* magnesium chloride, 0.5 ml of a 5 mg/ml solution of uridine 5′-diphosphoglucose dissolved in buffer, and 1 ml (100 mg of tissue equivalents) of a 9000*g* supernatant of a house fly homogenate. Incubations were for 15 min at 37°.

The incubation mixture was extracted thoroughly with ether to remove the unreacted 1-naphthol and the water layer was concentrated to a volume suitable for application to thin layer chromatograms. After development, the 1-naphthyl-*1*-^{14}C glucoside was detected on the chromatograms by radioautography and the compound recovered by extraction of the gel with methanol. Approximately 75% of the radioactive 1-naphthol was converted to the glucoside.

1-Naphthyl glucoside-^{14}C also was synthesized enzymatically using the basic procedure just described. However, an excess of 1-naphthol, 1 mg/flask, was used to achieve maximum reaction with the 1 μCi of uridine diphosphate glucose [D-glucose-*U*-^{14}C, sp act. 227 mCi/mmol] added to each incubation mixture. Under these conditions, 35% of the radiocarbon was recovered as 1-naphthyl glucoside-^{14}C.

Radioactive 1-naphthyl glucuronide was prepared enzymatically using rat liver homogenates as the enzyme source and UDPGA as the cofactor (Mehendale and Dorough, 1972b). The 1-naphthol-^{14}C used in these experiments was the same as that used to prepare the glucoside. 1-Naphthyl glucuronide-^{14}C was synthesized from uridine diphosphate glucuronic acid [D-glucuronic-*U*-^{14}C] having a sp act. of 238 mCi/mmol.

Acetylation of Biosynthesized 1-Naphthyl Glucoside. 1-Naphthyl-^{14}C glucoside was synthesized using house fly enzymes as described earlier and the unreacted 1-naphthol-^{14}C removed by extracting the incubation mixture with ether. The water phase, containing the radioactive 1-naphthyl glucoside, was concentrated to dryness on a rotary evaporator. The dried residue was dissolved in 1 ml of acetic anhydride and cooled to 0° in an ice bath. Two drops of 70% perchloric acid were added and the solution allowed to return to room temperature slowly over a period of 2 hr. At this stage, the solution was held overnight and then transferred to a separatory funnel containing 4 ml of cold water. The water was extracted twice with 4-ml portions of ether which removed 85% of the radioactivity, presumably the acetylated naphthyl glucoside. Its identity was confirmed by cochromatography on tlc with the chemically synthesized material.

Deacetylation of the product with ammonia converted 93% of the radioactivity to a water-soluble material. Tlc analysis demonstrated that this material was identical with an authentic sample of 1-naphthyl glucoside.

Chromatography. 1-Naphthol-*1*-^{14}C was purified on silica gel F-254 chromatoplates (Merck) using a 7:3 mixture of chloroform and acetone as the developing solvent. In addition to providing a means of purification, this system afforded the separation of 1-naphthol, 1,3-naphthalenediol, 1,4-naphthalenediol, and 1,5-naphthalenediol. These compounds constituted the more likely free metabolites of 1-naphthol resulting from treating rats with 1-naphthyl glucoside. Other solvent systems used to establish cochromatography of an unknown with one of these metabolite standards were petroleum ether–ethyl ether (2:1), methylene chloride–ethyl acetate (2:1), and hexane–acetone (7:3).

Chromatographic isolation of acetylated 1-naphthol glucoside (1-naphthyl tetra-*O*-acetyl-β-D-glucopyranoside) was accomplished on silica gel chromatoplates developed in 10:1 petroleum ether–acetone. The compound had an R_f of approximately 0.6 while the 1-naphthyl glucoside remained at the origin. Changing the solvent to 8:2:1 chloroform–methanol–acetic acid moved the acetylated 1-naphthyl glucoside to the solvent front but resulted in an R_f of 0.6 for the 1-naphthyl glucoside. 1-Naphthol glucuronide had an R_f of 0.4 in this system while UDPG and UDPGA remained at the origin. This allowed the separation of the ^{14}C-labeled biosynthesized glycosides from their corresponding radioactive cofactors.

Nonradioactive 1-naphthol and its hydroxylated analogs were detected on the tlc plates by viewing under ultraviolet light. To locate the glycosides, the chromatograms were sprayed with concentrated sulfuric acid and heated at 110° for 5 min. Radioautography was used to detect radioactive areas on the gel. Quantitative radioassays were conducted by liquid scintillation counting.

Stability of 1-Naphthyl Glucoside and Glucuronide. The stabilities of 1-naphthyl-^{14}C glucoside and 1-naphthyl-^{14}C glucuronide were evaluated under various conditions commonly used for evaluating the chemical and/or biological fate of insecticidal compounds. Each of the radioactive glycosides was purified on tlc prior to use in these experiments. The situations under which the glycosides were held for stability determination were: (1) in citrate–phosphate buffer, pH 2.2–8.0, for up to 6 hr at 25°; (2) in Tris-HCl buffer, pH 7.0, and in methanol at temperatures of 25, 0, and −20° for up to 1 week and at 90° for 30 min. At the designated time, the incubation mixtures were concentrated for tlc analysis.

Enzyme Studies. The ability of β-glucosidase and β-glucuronidase (almond and Type B-1 bovine liver, respectively, Sigma Chemical Corp., St. Louis, Mo.) to hydrolyze 1-naphthyl glucoside and glucuronide was determined. The naphthyl-^{14}C glycosides were incubated separately with each of the enzymes in citrate–phosphate buffer (pH 5.0). The incubation mixtures, containing approximately 1.0×10^6 dpm of the glycoside and 4 ml of buffer, were preincubated with shaking for 10 min at 37° and then 0.4 mg of enzyme in 0.2 ml of buffer was added. To achieve maximum cleavage, the same amount of enzyme was added four more times at 6-min intervals with the total incubation time being 30 min. Each reaction mix-

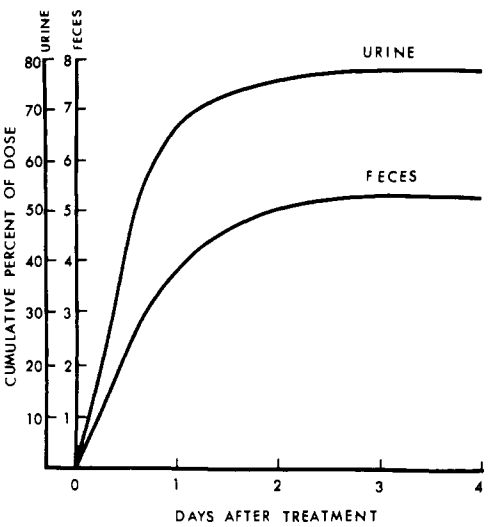

Figure 1. Excretion of radiocarbon from rats treated orally with a single oral dose of 1-naphthyl-^{14}C glucoside.

ture was extracted twice with 5-ml portions of ether and both the aqueous and organic solvent phases were radioassayed. The quantity of radiocarbon in the ether was indicative of the efficiency of the enzyme to cleave the glycoside, thus yielding radioactive 1-naphthol.

To determine if whole rat urine affected the enzymatic cleavage of 1-naphthyl glucoside or 1-napthyl glucuronide, the reactions described above were run in the presence of 0.5 ml of urine. In this case, however, the initial buffer volume was increased to 5 ml and the pH adjusted to 7.0 if required after adding the urine. In addition, each of the glycosides was treated in succession with the two enzymes, first with glucosidase and then with glucuronidase and *vice versa*. This was done to determine if the sequential treatment of a single urine sample with the enzyme would produce results comparable to treatment of the sample with either of the enzymes alone.

Metabolism Studies. Female rats weighing 200–250 g were treated orally with a single dose of the radioactive 1-naphthyl glucoside or 1-naphthol. Four animals were given water solutions of 1-naphthyl-^{14}C glucoside and the excreta collected daily for 4 days. The same number of animals were treated with 1-naphthyl glucoside-^{14}C and 2 rats were administered corn oil soilutions of 1-naphthol-^{14}C. In the latter studies, the urine was collected for 24 hr and the experiments terminated. All animals received approximately 10^6 dpm of the appropriate radioactive preparation.

The urine was concentrated to about 0.5 vol and applied as a band to 20 × 20 cm silica gel chromatoplates. On either side of the band was applied a mixture of authentic standards (1-naphthyl glucoside, 1-naphthyl glucuronide, 1-naphthyl sulfate, 1-naphthyl phosphate, and 1-naphthol) and the plates were developed in the 8:2:1 chloroform–methanol–acetic acid solvent system. After exposure to X-ray film for 7 days, the radioactive bands were extracted individually with methanol and rechromatographed in the 8:2:1 system along with standards having similar R_f values. Radiocarbon chromatographing similar to 1-naphthol was rechromatographed with a mixture of the various naphthalenediols in solvent systems designed to separate these apolar products. Similar tlc analysis of the ring-^{14}C-labeled materials released from the polar metabolites by acid treatment (1 *N* HCl, 90° for 1 hr) was also conducted.

RESULTS AND DISCUSSION

Stability of 1-Naphthol Glucoside and Glucuronide. Both 1-naphthyl-^{14}C glucoside and glucuronide were stable when held at 25° in citrate–phosphate buffer at pH values ranging from 2.2 to 8.0 for 6 hr and at a pH of 7.0 when heated in a water bath at 90° for 30 min. The same was true when the compounds were dissolved in methanol or Tris-HCl buffer (pH 7.0) and stored at −20, 0, and 25° for 7 days, or when the solutions were refluxed for 30 min. The glucuronide derivative was similarly stable under all conditions except when stored in Tris-HCl buffer where 30% degradation occurred at 25°. That radiocarbon not associated with the glucuronide was identified by tlc analysis as 1-naphthol.

Table I. Nature of Residues in the 0–24 hr Urine of Rats Treated Orally with 1-Naphthol and 1-Naphthyl Glucoside

	% of dose when treated with		
		1-Naphthyl glucoside	
Metabolites	1-Naphthol-*I*-^{14}C	Naphthyl-^{14}C	Glucose-^{14}C
1-Naphthyl glucoside	0.1	18.7	15.9
1-Naphthyl glucuronide	73.2[a]	23.6	1.1
1-Naphthyl sulfate	14.9	10.3	0
1-Naphthol	1.4	9.5	0
Unknown (tlc origin)	0.7	5.2	3.1
Total	90.3	67.3	20.1

[a] Trace amounts of 1,4- and 1,5-naphthylenediol were generated from this product when treated with 1 *N* HCl for 1 hr at 90°.

The data indicate that no particular problems would be encountered insofar as stability is concerned during extraction of the compounds from biological media. Their stability in methanol is important since this is an excellent solvent for extracting polar insecticide metabolites from plant and animal tissues. Stability in buffer solutions is essential if enzyme studies such as the cleavage of the conjugates to form the aglycones are to be useful in determining the nature of the intact metabolite. Since some cleavage of the naphthyl glucuronide did occur when held in Tris-HCl buffer (pH 7.0) at 25° nonenzymatic cleavage must be considered a possibility during incubation, especially if the incubation time is of long duration. The citrate–phosphate buffer may be the preferred buffer in such incubations since both glycosides were stable in this buffer for 6 hr at 25°.

Enzyme Studies. Incubation of 1-naphthyl-^{14}C glucoside with β-glucosidase for 30 min at 37° cleaved 91% of the conjugate. β-Glucuronidase treatment yielded only 3% cleavage which was comparable to a control incubation containing all constituents except the enzyme. 1-Naphthyl-^{14}C glucuronide was hydrolyzed in excess of 95% by β-glucuronidase and 7% by β-glucosidase. The latter enzyme did not cleave the conjugate to any greater degree than that observed during incubation with no enzyme.

Whole rat urine, 0.5 ml, added to the enzyme preparation did not decrease the amount of substrate hydrolyzed, nor did it alter the specificity of the enzymes. Also, β-glucuronidase in the incubation mixture did not affect the action of β-glucosidase on 1-naphthyl glucoside. The same was true when the situation was reversed.

From this study, it appears that β-glucosidase and β-glucuronidase treatment of a biological extract could be used as a convenient tool for determining the ability of an organism to metabolize a glucoside to a glucuronide. For example, a rat fed 1-naphthyl glucoside might be expected to convert the material to a glucuronide and excrete the compound in the urine. Treatment of the urine with β-glucosidase, followed by β-glucuronidase, would allow one to rapidly establish the quantity of the dose eliminated as the administered compound and the quantity metabolized to the glucuronide. This would be an immediate indication of the animal's ability to attack, biochemically,

plant-derived glucosides of the chemical in question if consumed in the diet. Identification of the resulting aglycones would give virtual proof of the intact conjugates as they exist in the plant or animal system.

Metabolism. When 1-naphthyl-^{14}C glucoside was administered as a single oral dose to rats, 78% of the dose was eliminated in the urine and 5.2% in the feces after 4 days. Excretion of the radiocarbon was most rapid during the first 24 hr and was essentially complete after 48 hr (Figure 1).

A comparison of the levels and nature of ^{14}C residues in the 0–24-hr urine of rats treated with ^{14}C-labeled 1-naphthyl glucoside and 1-naphthol is shown in Table I. The data clearly demonstrate that 1-naphthyl-^{14}C glucoside metabolism was quantitatively quite different than free 1-naphthol-^{14}C. While 90% of the 1-naphthol-^{14}C dose was eliminated in the urine, only 67% of the 1-naphthyl-^{14}C glucoside dose was excreted *via* this route. This decrease in the rate of excretion could have very significant toxicological implications should the same situation exist where the aglycone was highly toxic inherently. Such concern is amplified by the fact that more free 1-naphthol was present in the urine of rats treated with 1-naphthyl glucoside than those treated with 1-naphthol, 9.5% *vs.* 1.4% of the dose. The data (Table I) suggest that slower excretion rates and the higher level of 1-naphthol observed with the 1-naphthyl glucoside were associated with the rate of 1-naphthyl glucuronide formation. This metabolite accounted for only 35% of the radiocarbon in the urine of 1-naphthyl-^{14}C glucoside treated animals but was 81% of the total urinary ^{14}C in rats treated with 1-naphthol. 1-Naphthol sulfate concentrations were similar in the urine of both groups of animals. Although specifically considered in these studies, no 1-naphthyl phosphate was detected in any of the urine samples.

A rather surprising aspect of the study with 1-naphthyl-^{14}C glucoside was that 19% of the dose was eliminated as the administered compound. One might have suspected that the glucoside linkage would not withstand the acid conditions of the stomach and the biochemical mechanisms of degradation in the body. That the sugar moiety was the same as administered as opposed to cleavage of the conjugate to yield 1-naphthol and reconjugation as a glucoside in the animal was confirmed in two ways. First, 1-naphthol-^{14}C treatment yielded only trace amounts of radioactivity in the urine which corresponded to 1-naphthyl glucoside. Secondly, 16% of a dose of 1-naphthyl glucoside-^{14}C was in the 0–24-hr urine as the administered compound. These data clearly showed that the rats did not form the glucoside, at least to any appreciable extent, and that the 1-naphthyl glucoside in the urine represented the administered compound.

Treatment of rats with 1-naphthyl glucoside-^{14}C confirmed that the 1-naphthyl glucuronide in the urine of rats administered 1-naphthyl glucoside did not result to any major extent from the oxidation of the glucose moiety. Only a small amount, 1% of the dose, of radioactivity corresponded to 1-naphthyl glucuronide, a level so small that confirmation of its identity was not possible. This demonstrated that the glucoside was first cleaved to yield 1-naphthol, which was then conjugated as a glucuronide in the animal system. Totally, approximately 50% of the

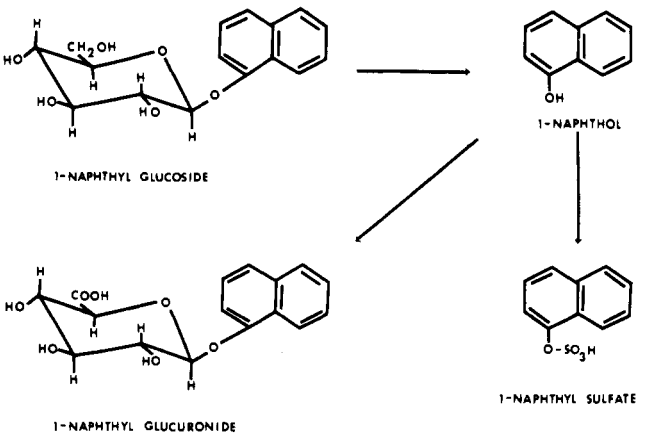

Figure 2. Metabolic pathway of 1-naphthyl glucoside in rats.

1-naphthyl glucoside dose was cleaved in the rats and 1-naphthol eliminated in the urine as the glucuronide, sulfate, or free aglycone. The pathway for 1-naphthyl glucoside metabolism in rats is shown in Figure 2.

With the exception of the radioactive material tentatively identified as 1-naphthyl glucuronide in urine of rats treated with 1-naphthol-^{14}C, acid treatment of all conjugates listed in Table I yielded only 1-naphthol. The exception involved trace amounts (0.1% or less of dose) of radioactive components which corresponded on tlc to 1,4- and 1,5-naphthalenediol. Similar concentrations of these materials also were detected in the free form in the urine of animals dosed with 1-naphthyl-^{14}C glucose. While these data do suggest that ring hydroxylation takes place in the rats, they clearly show that this pathway is extremely minor in the metabolism of 1-naphthol, *per se*, or of 1-naphthyl glucoside.

This study with 1-naphthyl glucoside has allowed us to develop techniques that will assist in studying even more potentially significant metabolites such as glucosides of metabolites of carbamates containing the carbamate moiety (Cardona and Dorough, 1973). More important, the study established that there is a definite need to evaluate the metabolic fate of the conjugates, *per se*, rather than relying entirely on information obtained with the corresponding aglycone.

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