Metabolism of N-[3-Chloro-4-(β-D-glucosylmethyl)phenyl]urea, a Metabolite of Chlorotoluron, in Rat and Japanese Quail†

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The radioactivity administered as a single oral dose of N-[3-chloro-4-(β-D-glucosyl[^{14}C]methyl)phenyl]-urea was rapidly absorbed from the gastrointestinal tract and eliminated in feces and urine by both the rat and the Japanese quail. Within 6 h after treatment 57% and 74% of the dose was excreted by rat and quail, respectively. Of the 78% of the dose excreted by rat within 12 h, 49% was the parent glucoside, 3.6% the aglycon, and 14.6% the aglycon acid (the carboxyphenyl derivative of the aglycon). However, within the same period quail excreted 90% of the dose, of which 25.5% was the parent glucoside, 26.6% the aglycon, and 12% the aglycon acid. Unidentified metabolites, two in rat and seven in quail, formed 10.8% and 25.5% of the dose, respectively. The aglycon in rat and both the aglycon and the aglycon acid in quail formed conjugates.

Procedures for the analysis of pesticide residues in or on raw agricultural commodities involve extraction with organic solvents, cleanup of interfering materials, and quantitative measurement of the pesticide and its biologically active products. Most of these procedures utilize apolar solvents designed to extract the pesticide and its apolar metabolites and leave behind polar compounds, such as glycosides and other conjugates. It has been well established that one of the principal detoxication mechanisms for pesticides in plants is the formation and storage of glycosides, but they may not be detected in most conventional residue analytical procedures. Thus, the food materials of man and other animals may contain undetected quantities of glucoside conjugates of pesticides.

Studies of the fate of 1-naphthyl glucoside (Dorough et al., 1974) and the glucoside of methyl N-hydroxy-N′,N′-dimethyl-1-thiooxamimidate, an oxamyl derivative (Harvey and Han, 1978), showed that the glucoside was absorbed from the gastrointestinal tract of rats and partly hydrolyzed and the aglycon was further metabolized. Marshall and Dorough (1979) reported that in rats 3-hydroxycarbofuran glucoside was directly translocated to the lower small intestine and cecum, where cleavage of the glucosidic linkage occurred, and the freed 3-hydroxycarbofuran was then absorbed.

Since very little information is available on the fate of glucoside conjugates, their bioavailability, and toxicological significance in animals, we have investigated the fate of N-[3-chloro-4-(β-D-glucosylmethyl)phenyl]urea (chlorotoluron glucoside), a glucoside of a chlorotoluron metabolite, N-[3-chloro-4-(hydroxymethyl)phenyl]urea, in rat and Japanese quail. The metabolic fate of chlorotoluron has been reported previously (Muecke et al., 1976; Hinderer and Menzer, 1976a,b).

MATERIALS AND METHODS

Materials. N-[3-Chloro-4-(β-D-glucosyl[^{14}C]methyl)phenyl]-urea was synthesized from [*methyl*-^{14}C]-*o*-toluidine hydrochloride. *o*-Toluidine was first nitrated in the 4-position by the method of Hillers et al. (1950) using fuming nitric–sulfuric acid. The amino group of the 4-nitro-*o*-toluidine was then replaced by a chlorine according to the method of Albert and Linnell (1936) using the Grignard reaction. The product was oxidized with potassium dichromate to yield 2-chloro-4-nitrobenzoic acid. The acid was methylated with methanolic sulfuric acid, the nitro group was reduced to an amino group with ethanolic stannous chloride, the amine was reacted with phosgene to form the isocyanate, and the urea was formed by passing a stream of anhydrous ammonia through a benzene solution of the isocyanate. The ester was then reduced with lithium aluminum hydride to form N-[3-chloro-4-(hydroxymethyl)phenyl]urea, the precursor for glucosylation by the Koenigs-Knorr reaction (Koenigs and Knorr, 1901; Overend, 1972). The glucosylating reagent was 2,3,4,6-tetraacetyl-α-D-glucopyranosyl bromide (acetobromoglucose), prepared according to the method of Lemieux (1963). Since the glucosylation reaction proceeds to equilibrium, the yield was increased by conducting the reaction in three stages. The precursor urea was reacted in the dark with acetobromoglucose, by use of silver carbonate as a catalyst, in methylene chloride. After 24 h, the solvent was removed, additional acetobromoglucose in methylene chloride was added to the reaction mixture, and the reaction was continued for an additional 24 h. This procedure was repeated once more. The methylene chloride extracts were combined, the solvent was evaporated, and the syrupy concentrate was deacetylated with methanolic ammonia (Tsou and Seligman, 1952). The final product was purified on methanol-prewashed silica gel TLC using chloroform–methanol–water (65:25:4). Both the unreacted urea and the glucoside were recovered from the plates. Overall yield from *o*-toluidine to glucoside was 10%. The specific activity of the radiolabeled glucoside used in this study was 4.9 mCi/mmol.

The nonlabeled reference compound N-(3-chloro-4-carboxyphenyl)urea, was supplied by Ciba-Geigy Limited, Basle, Switzerland. Its methylated derivative, prepared by reaction with diazomethane, was also used as a reference compound. Diazomethane was prepared by the method of McKay (1948). Oth-

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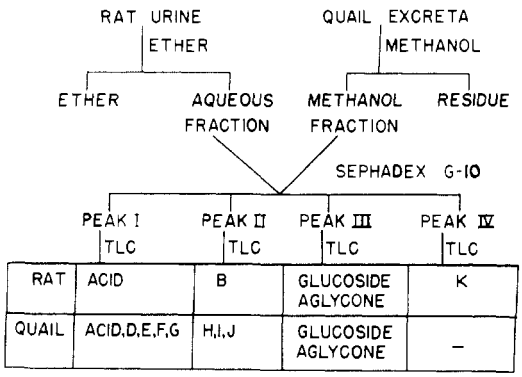

Figure 1. General outline of extraction and analysis of metabolites from rat urine and quail excreta. Rat feces were not extracted.

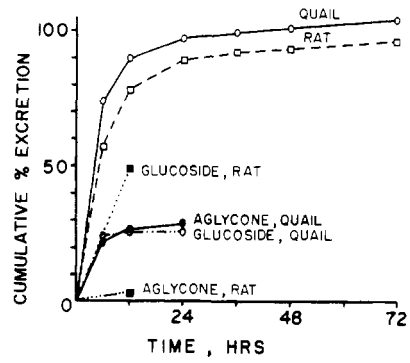

Figure 2. Excretion of total radioactivity by rat and Japanese quail after an oral dose of 1.2 mg of [^{14}C]chlorotoluron glucoside/kg.

er chemicals used were β-glucosidase (almonds) and β-glucuronidase (beef liver) from ICN Nutritional Biochemicals, Cleveland, OH; sulfatase (limpets, type v) and cellulase (*Aspergillus niger*, type I) from Sigma Chemicals Co., St. Louis, MO; silica gel TLC plates from E. Merck AG, Darmstadt, Germany; Sephadex G-10 from Pharmacia Fine Chemicals AB, Uppsala, Sweden; and Soluene 100 sample solubilizer from Packard Instrument Co., Inc., Downers Grove, IL. All solvents and other chemicals used were of reagent grade.

Treatment of Animals and Collection of Excreta. Eight male albino rats, Sprague-Dawley strain (Flow Labs, Dublin, VA), approximately 210 g each, and seven male Japanese quails (obtained from a colony maintained by Dr. J. H. Soares, Department of Poultry Science, University of Maryland, College Park), 6 months old, approximately 110 g each, were given a single oral dose of approximately 1.2 mg of [^{14}C]glucoside/kg. Two hundred microliters of 50% ethanol was used as carrier. The animals were held individually in metabolism cages and had free access to food and water. Rat urine and feces and quail excreta samples were collected at various time intervals up to 72 h.

Radioanalysis. The quail excreta and rat feces samples were allowed to dry at room temperature for 12–24 h. They were then ground by using mortar and pestle. Five to 30 mg of each excreta sample, in duplicate, was used for radioassay. Each sample was placed in a liquid scintillation vial, followed by the addition of 0.1 mL of water and 1.0 mL of Soluene 100. The capped vial was then incubated at 60 °C for up to 6 h. The sample was decolorized at room temperature by adding 0.5 mL of propanol followed by 0.2 mL of H_2O_2. The contents of the vial were mixed by shaking, and the vial was loosely capped. After 10 min, the sample was incubated at 55 °C for up to 6 h. The vial was then allowed to cool to room temperature. Ten milliliters of liquid scintillation counting solution [5.5 g of PPO, 0.2 g of POPOP in 1 L of toluene–Triton X-100 (2:1)] was added. After 24 h, the samples were counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 3375). Quenching was corrected by using [^{14}C]hexadecane as internal standard. In the case of rats 30–300 μL of urine samples was used for radioassay.

Extraction and Isolation of Metabolites. The scheme for extraction and isolation of metabolites is given in Figure 1. The 6-, 12-, and 24-h quail excreta samples were pooled separately and extracted with 80% methanol (5 mL/g) for 16 h with continuous stirring. The extract was filtered off, and the solid residue was exhaustively extracted with methanol. All the methanol extracts for each time interval were pooled, concentrated to a small volume, and radioassayed. The radioactivity present in the dry residue of excreta samples was also determined.

The concentrated methanol extracts were kept in centrifuge tubes at 4 °C for a few days. As a result, large amounts of contaminants crystallized out. The tube contents were centrifuged. The supernatant methanol solution was removed, and the precipitate was washed with a small volume of cold methanol. The process was repeated three times. The precipitate contained less than 1% of the total radioactivity. The methanol washes and extract concentrate were combined and analyzed by TLC.

Table I. TLC R_f Values of Metabolites and Reference Compounds

compound	R_f in solvent system 1	2	3	4	5
glucoside	0.19	0.56	0.22	0.70	0.75
aglycon	0.67	0.80	0.64	0.79	0.87
aglycon acid	0.61	0.79	0.45	0.74	0.75
rat metabolites					
B	0.10	0.37	0.00	0.32	0.49
K	0.72	0.67	0.30	0.77	
quail metabolites					
D	0.28	0.69	0.00	0.63	0.61
E	0.11	0.41	0.00	0.52	0.42
F	0.05	0.36	0.00	0.33	0.45
G	0.03	0.30	0.00	0.12	0.50
H	0.23	0.53	0.25	0.69	0.76
I	0.15	0.48	0.13	0.63	0.69
J	0.05	0.31	0.00	0.10	0.60

The 6-h extract was subjected to methanol–acetonitrile–hexane partitioning. All of the radioactivity remained in the methanol–acetonitrile phase; a portion of the color was removed by hexane. The methanol–acetonitrile phase was evaporated, and the oily residue was dissolved in 2 mL of distilled water. This aqueous solution was then analyzed by Sephadex G-10 column chromatography using distilled water as eluent. The various metabolite fractions thus obtained were lyophilized. The metabolites were partially purified on TLC in at least two solvent systems.

The individual 6- and 12-h rat urine samples were extracted three times with diethyl ether (4 mL/mL of urine) at pH 7.0. The ether extracts were analyzed by TLC. The aqueous fractions were then pooled and analyzed by Sephadex G-10 column chromatography using distilled water as eluent. The rest of the procedure was the same as for quail. Rat feces were not extracted.

Thin-Layer Chromatography. Thin-layer chromatography was accomplished on precoated silica gel 60 F-254 chromatoplates (0.25-mm thickness). Radioactive compounds on the TLC plate were located by radioautography. Nonradioactive reference compounds were detected by short-wavelength (254 nm) UV absorption. The radioactive spots on TLC plates were quantitated by counting the gel scraped from the spot area. The following solvent systems were used: (1) chloroform–methanol–acetic acid 8:2:1; (2) butanol–acetic acid–water 70:15:15; (3) ethyl acetate–propanol–water 18:4:1, (4) methylene chloride–methyl ethyl ketone–formic acid 8:2:1 followed by propanol–water–pyridine 70:15:10; (5) propanol–water–ethyl acetate 7:2:2.

Enzyme Hydrolysis. Various metabolites were incubated with 0.5–1.0 mg of β-glucosidase (2.5 units/mg), β-glucuronidase (70–100 units/mg), sulfatase (11.6 units/mg), and cellulase (1.35 units/mg) in 0.5 mL of 0.5 M sodium acetate–acetic acid buffer, pH 5.0, at 37 °C for up to 20 h. The incubates were

Table II. Reactivity of Metabolic Products[a]

				unknown								
				rat		quail						
treatment	glucoside	aglycon	aglycon acid	B	K	D	E	F	G	H	I	J
methylation	-	-	+	-	-	-	-	-	-	-	-	+
acylation	+	+	-	+	-	-	+	+	+	+	+	+
β-glucosidase	+			-	-	-	-	-	-	-	+	-
β-glucuronidase	+			-	-	-	+	+	-	-	+	-
cellulase	+			-	-	-	±	+	±	+	+	±
sulfatase	+			-	-	-	-	±	±	-	-	-
acid hydrolysis	+			+	+	-	+	+	+	+	+	+

[a] + indicates the production of degradation products, as indicated in the text. [b] - indicates no reaction.

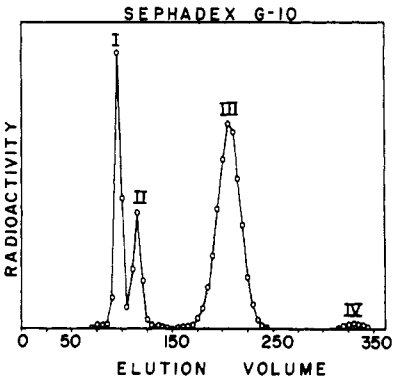

Figure 3. Sephadex G-10 column elution pattern of rat urinary and quail methanol-extractable metabolites of chlorotoluron glucoside. The composition of peaks I-IV is indicated in Figure 1.

then frozen, lyophilized, and analyzed by TLC. These incubations were accompanied by an appropriate control experiment without enzymes. Metabolites were also incubated in 0.1 mL of 1.0 N methanolic hydrochloric acid for 20 h at 37 °C. The hydrolysis products were analyzed by TLC.

The compounds were acylated according to the method of Koenig et al. (1973). Mass spectra were obtained by using a Du Pont Model 21-491B mass spectrometer interfaced with a Hewlett-Packard Model 2100A computer.

RESULTS

The excretion pattern following an oral dose of the glucoside is presented in Figure 2. The glucoside was rapidly absorbed from the gastrointestinal tract of both the rat and the quail. Within 6 h 57% and 74% of the administered radioactivity was excreted by rat and quail, respectively. At the end of 72 h essentially all of the administered radioactivity (97% in rat, 82.5% in urine and 17.5% in feces, and 104% in quail) was excreted by both rats and quail. Excretion was almost complete within 24 h after administration.

Only 1.4% at 6 h and 1.9% at 12 h of the rat urinary radioactivity was ether-extractable and was found to be due mainly to the aglycon. The aqueous fraction was analyzed by Sephadex G-10 column chromatography (Figure 3).

Most of the quail excretory radioactivity was methanol-extractable (89% at 6 h, 79% at 12 h, and 84% at 24 h). TLC analysis (solvent system 1) of this extract showed the presence of at least six metabolite regions in addition to the parent compound. After preliminary cleanup, the methanol extract was analyzed by Sephadex G-10 column chromatography.

Both the rat urine aqueous fraction and the quail methanol extract gave a very similar Sephadex column elution pattern with peaks I–III as shown in Figure 3. Peak IV was present in only 12-h rat urine. The products isolated by TLC from each of these peaks are listed in Figure 1.

The rat urinary radioactivity was found to be due to 5 compounds and the quail methanol-extractable radioactivity due to 10 compounds. Within 12 h about 49% and 25% of the dose was excreted as the parent glucoside by rat and quail, respectively, indicating a greater degree of glucoside metabolism in quail than in rat (Figure 2). Within the same period, however, 3.6% of the dose was excreted as aglycon by rat, compared to 26% by quail, indicating that the aglycon is further metabolized to a greater extent in rat than in quail. The glucoside was identified to be the parent glucoside by TLC and by comparing its mass spectrum with that of a reference compound. The aglycon was tentatively identified by TLC (Table I). Another major product identified by TLC was the aglycon acid (Table I), the carboxyphenyl derivative of the aglycon. It forms about 14.6% and 12% of the dose in rat and quail, respectively. Two products from rat (about 10.8% of the dose) and seven from quail (about 25.5% of the dose) remain unidentified. The R_f values for all the metabolites and reference compounds are given in Table I.

The TLC of metabolic products shows that most of these products are more polar than the parent glucoside, indicating that they are probably conjugates. Attempts were made to characterize the unknown metabolic products by methylation and acylation, by acid hydrolysis, and by enzymatic hydrolysis by β-glucosidase, β-glucuronidase, cellulase, and sulfatase. Data in Table II show that most of these metabolites are probably polysaccharide conjugates with and without uronic acid. TLC analysis of the acid hydrolysis products showed the presence of the aglycon in rat metabolites B and K and quail metabolites E–G and the presence of the acid in quail metabolites H–J. Metabolite D was not hydrolyzed by acid or any enzyme so it is probably not a sugar or sulfate conjugate; no further characterization of this material was attempted.

DISCUSSION

The glucoside conjugate was rapidly absorbed from the gastrointestinal tract of both the rat and the quail. It was partly hydrolyzed to the aglycon. The aglycon was further metabolized to the corresponding carboxyphenyl derivative, the aglycon acid, a route that had been previously shown by Muecke et al. (1976). Both the aglycon and the aglycon acid probably formed glucuronide conjugates which were further converted into polysaccharides. The glucoside itself was possibly converted into polysaccharides. The proposed metabolic pathways are summarized in Figure 4.

It has been reported that 1-naphthyl glucoside (Dorough et al., 1974) and the glucoside of methyl *N*-hydroxy-*N'*,*N'*-dimethyl-1-thiooxamimidate, an oxamyl metabolite (Harvey and Han, 1978), were absorbed from the gastrointestinal tract of rats and partly hydrolyzed, and the aglycons then underwent metabolism as they normally

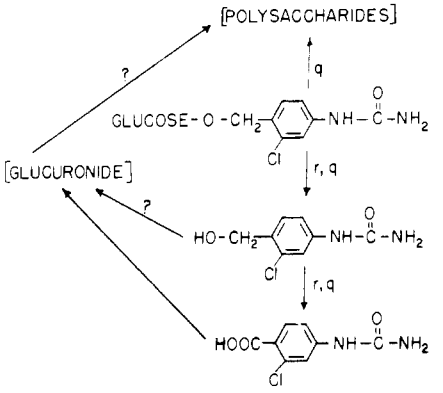

Figure 4. Proposed metabolic pathways for chlorotoluron glucoside in rat (r) and Japanese quail (q).

would, including the formation of conjugates. Part of the radioactivity of the glucoside of the oxamyl metabolite was also incorporated in normal tissue constituents, mainly amino acids. These studies are similar to the results we have reported here. Marshall and Dorough (1979), however, found that in rats 3-hydroxycarbofuran glucoside was directly translocated to the lower small intestine and cecum, where cleavage of the glucosidic linkage occurred and freed 3-hydroxycarbofuran was then absorbed.

Differences were observed in the metabolism of glucoside in rat and quail. Within 6 h the percentage of the administered dose excreted by quail was greater than that excreted by rat; i.e., the excretion in quail was faster than in rat. Quantitative and qualitative differences were also observed in the metabolites produced. Within 12 h rats excreted twice as much unchanged glucoside as quails. Within the same period, however, the quantities of aglycon acid excreted by both rat and quail were almost the same, although quails excreted almost 4 times as much aglycon as did rats.

Several metabolites are present as conjugates, varying in the conjugating moiety. They differ in chromatographic properties and ease of hydrolysis by various enzymes. In rat only two conjugates containing the aglycon were found, whereas in quail at least six conjugates containing the aglycon or the aglycon acid were found. The differences observed in the metabolism of the glucoside in rat and quail are in agreement with previous papers by Hinderer and Menzer (1976a,b) on the metabolism of chlorotoluron, wherein they reported that chlorotoluron was more resistant to in vitro metabolism by various tissue preparations from rat than by similar tissue preparations from quail.

This study shows that the glucoside of this pesticide formed in plants, if consumed in the diet by rat and Japanese quail, may be absorbed from the gastrointestinal tract and provide a source of biologically active compounds. However, bioavailability may differ from species to species.

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