Release and Characterization of Bound Benomyl and Carbendazim Metabolites in Animal Tissues via Raney Nickel Desulfurization and Acid Dehydration†

K. David Monson‡

Experimental Station, Du Pont Agricultural Products, Wilmington, Delaware 19880-0402

A technique for the release and characterization of sulfide-bound metabolites of [^{14}C]benomyl and [^{14}C]carbendazim in dairy cow, goat, poultry, and rat liver is described. The procedure is based on cleaving of glutathione-derived sulfide bonds of the bound pesticide conjugates utilizing Raney nickel desulfurization chemistry followed by acid dehydration of the released residues. Liver tissue was first homogenized in sodium hydroxide and then treated with nickel–aluminum alloy which generated Raney nickel and hydrogen in situ. Subsequent treatment with hot acid converted the released polar exocons to carbendazim and the 5-hydroxy analogue of carbendazim which could be extracted with ethyl acetate and chromatographed after neutralization of the tissue/reaction mixture. The technique is useful for better evaluation of terminal pesticide residues in animal tissues which otherwise would be uncharacterized and for which the toxicological significance would have remained uncertain.

INTRODUCTION

The nature of nonextractable bound pesticide residues in animal tissues can be of regulatory concern particularly when they comprise the majority of the total terminal residue. Animal metabolism studies by design should characterize the composition of the terminal residue resulting from exposure of the animal to a pesticide.

Bound pesticide residues are those resulting from the bonding of the pesticide or its metabolites with cellular components to yield nonendogenous products which cannot be removed from the sample matrix by exhaustive extraction with apolar and polar solvents (Kovacs, 1986). In an effort to characterize the bound residues, hydrolysis of the sample tissue with hot acid or base at reflux temperatures may render some of the residues extractable into organic solvent. Such harsh chemical treatments, however, may so extensively degrade the pesticide that the subsequently released unbound exocon bears no resemblance to its original structure in the intact untreated sample. The use of enzymes may solubilize the bound residue and allow characterization. Unfortunately, the solubilized pesticide residue may be in a matrix which makes it difficult, if not impossible, to characterize.

This, then, is the dilemma of bound pesticide residue assessment. Nondestructive extraction, or at least well-characterized chemical degradation, is a prerequisite to enable evaluation of the toxicological significance of the bound terminal residue. In regard to the development of a well-characterized chemical degradation, it is important to first consider one of the potential mechanisms for pesticide binding.

Many pesticides are metabolized to glutathione conjugates, which in turn can be catabolized to other peptide conjugates via the mercapturic acid pathway. If the peptide linkages of these conjugates become incorporated into the protein matrix through biosynthetic processes, the result could be a pesticide residue covalently bound to protein through the glutathione-derived sulfide bond. This kind of bound residue would be both unextractable and unhydrolyzable. Enzyme treatments would certainly lead to various oligopeptides and amino acids still bonded to the pesticide residue, making identification extremely difficult.

On the basis of an understanding of this metabolic pathway, a more selective method for releasing the bound liver metabolites of benomyl and carbendazim has been developed in this laboratory by utilizing Raney nickel desulfurization chemistry followed by acid dehydration of the released residues. Raney nickel is generated in situ with sodium hydroxide and nickel–aluminum alloy to reductively cleave the covalently bound sulfide conjugates in animal liver. Subsequent acid treatment of the tissue/reaction mixture serves to convert the released metabolites to a chemical form suitable for extraction and chromatographic characterization. Examples demonstrating the release and characterization of bound benomyl and carbendazim metabolites in dairy cow, goat, poultry, and rat liver are reported.

EXPERIMENTAL PROCEDURES

Materials. [*phenyl*(U)-^{14}C]Benomyl had a specific activity of 3.43 μCi/mg and a radiochemical purity of 99% (Du Pont Co., Wilmington, DE). [2-^{14}C]Benomyl had a specific activity of 3.49 μCi/mg and a radiochemical purity of 98%. [*phenyl*(U)-^{14}C]-Carbendazim had a specific activity of 32.6 μCi/mg and a radiochemical purity of 94% (Du Pont Research NEN Products, Boston, MA). [2-^{14}C]Carbendazim had a specific activity of 51.4 μCi/mg and a radiochemical purity of 99%. Methyl (5-hydroxy-1*H*-benzimidazol-2-yl)carbamate (abbreviated 5-HBC) was synthesized by Du Pont. Nickel–aluminum alloy was obtained from Aldrich Chemical Co. (Milwaukee, WI). All solvents used for extraction and chromatography were of HPLC grade.

Animal Dosing and Liver Collection. A lactating Holstein dairy cow was dosed twice daily for 5 consecutive days with [2-^{14}C]-carbendazim by capsule at a rate equivalent to 50 ppm in the average total daily feed. Two nonlactating, adult, female goats were dosed once daily for 29 consecutive days with [*phenyl*(U)-^{14}C]carbendazim by capsule at a rate equivalent to 54 ppm in the daily feed. Two white Leghorn hens were dosed daily for 3 consecutive days with [2-^{14}C]benomyl by capsule at a rate equivalent to 29 ppm in the daily feed, and two hens were dosed

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‡ Present address: Battelle Memorial Institute, 505 King Avenue, Columbus, OH 43201.

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daily for 3 consecutive days with [*phenyl*(U)-^{14}C]benomyl at a rate equivalent to 27 ppm. Five male and five female young adult Sprague-Dawley rats were each given a single oral dose of [*phenyl*(U)-^{14}C]carbendazim at 50 mg/kg of body weight. Liver tissues were collected from the cow, goats, and hens within 24 h of the last dose. Liver tissues were collected from the rats 72 h postdosing. All liver samples were frozen and stored at approximately −20 °C or lower until analysis.

The diversity of dosing regimens, radiolabel position, and whether benomyl or carbendazim was administered reflects the animal metabolism regulatory requirements encountered while completing benomyl and carbendazim registration studies.

Desulfurization and Dehydration of Liver Tissue. A primary goal of this procedure was to provide a high surface area exposure of the tissue cells to the chemical steps that follow. Two procedures were developed. In the first procedure, 25 g of fresh liver tissue was lyophilized and finely ground with dry ice. The dry material was placed in a 500-mL beaker set on a combination hot plate/magnetic stirrer. Next, 50 mL of 10% sodium hydroxide solution was added to the tissue residue and the mixture stirred until the tissue residue was evenly suspended. Approximately 5 g of nickel–aluminum alloy was added to this mixture over the course of an hour. During this time, a large watchglass was placed over the beaker and the reaction mixture was blanketed with nitrogen to exclude oxygen.

This procedure of reacting nickel–aluminum alloy with sodium hydroxide liberated hydrogen in situ according to

$$NiAl_2 + 6NaOH \rightarrow Ni + 2Na_3AlO_3 + 3H_2$$

leaving a black sponge of nickel atoms interspersed with holes left by the departed aluminum atoms. The sponge nickel, known as Raney nickel, was effective as a hydrogenation catalyst.

The generation of hydrogen was quite evident as the mixture became an expanded foam. Heat generated by the magnetic stirrer usually was sufficient to sustain vigorous hydrogen production; otherwise, mild heating was supplied by the hot plate. The tissue mixture was stirred and digested in the sodium hydroxide solution overnight (approximately 18 h), which also aided in the hydrolysis and breakup of protein.

As an alternative to the use of lyophilized tissue, 25 g of fresh liver tissue was placed in a glass blender jar along with 50 mL of 10% sodium hydroxide solution. The mixture was then blended until no visible hunks of tissue remained. The same procedure with the nickel–aluminum alloy was followed while the mixture was blended on a low setting. Likewise, this mixture was blanketed with nitrogen.

On occasion with both procedures, the hydrogen evolution became too robust and the expanding foam approached the limit of the vessel size. When this occurred, distilled water rinses from a wash bottle helped to collapse the foam.

The next step was dehydration. The reaction tissue slurry was adjusted to pH 1 by the stepwise addition of 85% phosphoric acid. The mixture was then transferred to a 500-mL round-bottom flask, a magnetic stirring bar added, a condenser attached, and the mixture heated to reflux for 1 h by using a heating mantle placed on a stirring plate. This, too, was conducted under a blanket of nitrogen.

Extraction. The extraction procedure was based on the method developed by Kirkland (1973) for the extraction of benomyl residues from animal tissues. After the refluxed tissue mixture had cooled, it was transferred to a 250-mL centrifuge bottle and extracted with 50 mL of hexane to remove lipids. Since emulsions had a tendency to form, the extraction mixture was centrifuged at 2000 rpm for 10 min. The hexane was drawn off with a 50-mL syringe and discarded. The mixture was then adjusted to pH 6.5 with 50% sodium hydroxide solution, cooled to room temperature, and subsequently extracted with three 100-mL aliquots of ethyl acetate. Centrifugation was necessary to break the emulsions formed. The ethyl acetate extracts were combined, dried by filtering through sodium sulfate, and reduced to ~5 mL by rotary evaporation. The remaining aqueous phase was decanted from the pellet of solids and its volume determined. Aliquots of the ethyl acetate and aqueous extracts were counted by liquid scintillation counting (LSC) to determine recovery. The pellet was air-dried, weighed, and homogenized by grinding. Aliquots of the solid residue were combusted and counted by LSC to determine the remaining bound radioactivity.

To facilitate better chromatography of the ethyl acetate extract, a cleanup procedure was used to remove additional lipid interferences. The ethyl acetate extract was back-extracted with two 25-mL portions of 0.1 N hydrochloric acid and centrifuged, and an aliquot of the ethyl acetate phase was analyzed by LSC. This ethyl acetate contained only 1–2% of the recoverable radioactivity and was therefore discarded. The acidic aqueous phase was neutralized (pH 6.5–7.0) with 1 N sodium hydroxide and extracted with two to four 50-mL portions of ethyl acetate. The final ethyl acetate phase was filtered through sodium sulfate and concentrated to 5–10 mL by rotary evaporation. An aliquot of this final ethyl acetate phase was counted by LSC to confirm recovery of the extractable radioactive residues.

Chromatographic Methods. Ethyl acetate extracts were analyzed either by thin-layer chromatography (TLC) or by high-pressure liquid chromatography (HPLC). Extracts were streaked onto precoated 20 cm × 20 cm silica gel TLC plates. Treated plates were developed for 15 cm in a solvent mixture of benzene/ethyl acetate/acetic acid (65/70/10 v/v/v). Radiolabeled compounds were located and quantified by using a Berthold linear analyzer (Berthold Systems, Inc., Pittsburgh, PA). Nonradiolabeled reference standards were located by ultraviolet fluorescence quenching. Alternatively, liver extracts were filtered and injected onto a reverse-phase PRP-1 column (30.5 cm × 7.0 mm, Hamilton Co., Reno, NV) maintained at 40 °C. The flow rate was 3.0 mL/min. The column was eluted with 100% 0.25 M acetic acid for 20 min followed by a linear gradient to 50% acetonitrile/50% 0.25 M acetic acid over the next 30 min. The elution of radioactivity was monitored with a RAMONA-D radiochemical detector (IN/US Corp., Fairfield, NJ). Radiochromatograms were acquired with a VAX Multichrom data acquisition system (VG Laboratory Systems, Ltd., Cheshire, England).

RESULTS AND DISCUSSION

Raney nickel has previously been used in the context of xenobiotic metabolite identifications by Boyland et al. (1961) and Jerina et al. (1970) to reduce isolated and purified *S*-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione to 2-naphthol. Jerina et al. (1970) demonstrated that this same conjugate and a related metabolite, *trans*-1,2-dihydro-1,2-dihydroxynaphthalene, could be dehydrated with warm acid to *S*-naphthylglutathione and 1-naphthol, respectively. Ryan and Hoffman (1978) used Raney nickel to release bound residues of trenbolone acetate from a proteolytic digest of cattle tissue. These researchers were able to release 50% of the bound residues into an organic phase and analyze it by thin-layer chromatography; however, no definitive identifications were made. In this present work, extraction methods were developed that linked desulfurization with dehydration to chemically release and modify sulfide-bound conjugates from intact liver tissue.

The results of extracting dairy cow, goat, poultry, and rat livers according to the Raney nickel reduction techniques described above are shown in Table I. Also reported are the results of extracting cow and poultry livers without use of the reduction step, so as to demonstrate the effect of desulfurization on the release of bound benomyl and carbendazim metabolites.

The reduction step significantly reduced the levels of bound radioactivity, as demonstrated by the dairy cow and poultry liver results (Table I). At the same time, the levels of organic extractable radioactivity increased. Unpublished results (this laboratory) from the extraction of a similarly derived goat liver with organic solvents of successive polarity indicated that >90% of the radioactive residues remained unextractable. As seen from Table I, bound residues in goat liver decreased to 11% with the balance of radioactivity being aqueous and organic soluble.

Table I. Distribution of Radioactive Residues following the Extraction of Liver Tissue from Animals Dosed with Radiolabeled Benomyl or Carbendazim

fraction	% ^{14}C	
	Raney nickel reduction	no reduction
dairy cow[a]		
bound	36	76
aqueous	42	21
ethyl acetate[b]	22	3
goats[a]		
bound	11	
aqueous	49	
ethyl acetate[b]	40	
hens [2-^{14}C][c]		
bound	19	57
aqueous	21	39
ethyl acetate[b]	60	4
hens [*phenyl*(U)-^{14}C][c]		
bound	17	58
aqueous	34	39
ethyl acetate[b]	49	3
rats (female)[a]		
bound	15	
aqueous	22	
ethyl acetate[b]	63	
rats (male)[a]		
bound	13	
aqueous	23	
ethyl acetate[b]	64	

[a] Dosed with [^{14}C]carbendazim. [b] Values reflect the radioactivity determined in the ethyl acetate extract prior to chromatographic cleanup. [c] Dosed with [^{14}C]benonyl.

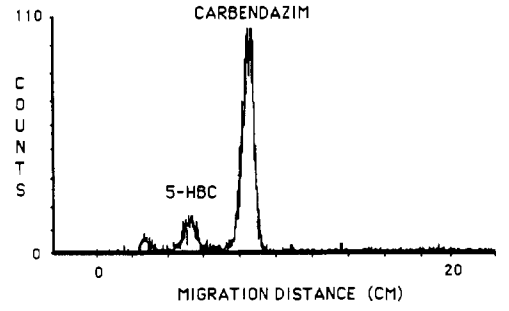

Figure 1. TLC radiochromatogram of cow liver ethyl acetate extract from dairy cow dosed with [^{14}C]carbendazim.

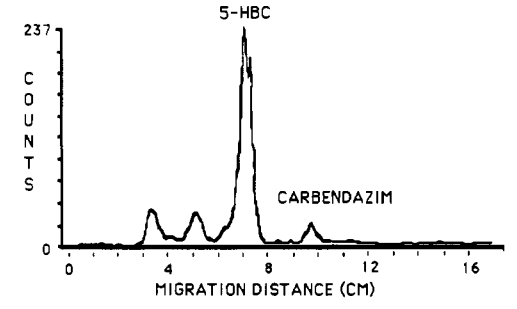

Figure 2. TLC radiochromatogram of goat liver ethyl acetate extract from goats dosed with [^{14}C]carbendazim.

The extraction of rat livers using the reduction and dehydration steps enabled the majority of the radioactive residues to be organic extractable.

The nature of the benomyl and carbendazim metabolites released and extracted into ethyl acetate is indicated by the chromatograms shown in Figures 1–4. The presence of carbendazim in the cow liver extracts (Figure 1) is consistent with the residue having originated as dihydro-

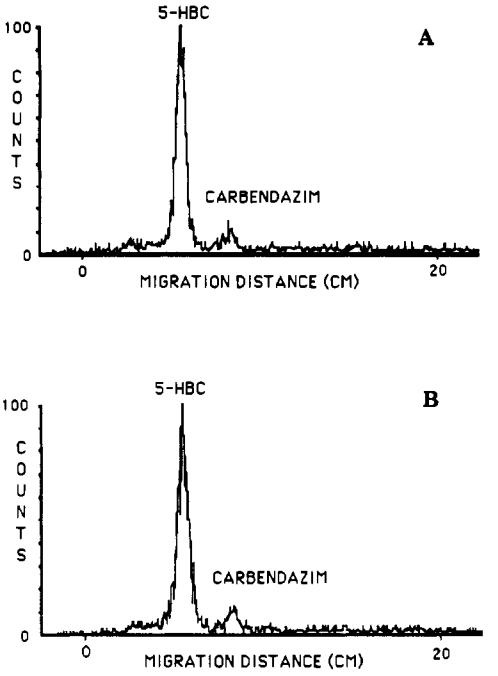

Figure 3. TLC radiochromatogram of poultry liver ethyl acetate extract from laying hens dosed with (A) [2-^{14}C]benomyl and (B) [*phenyl*(U)-^{14}C]benomyl.

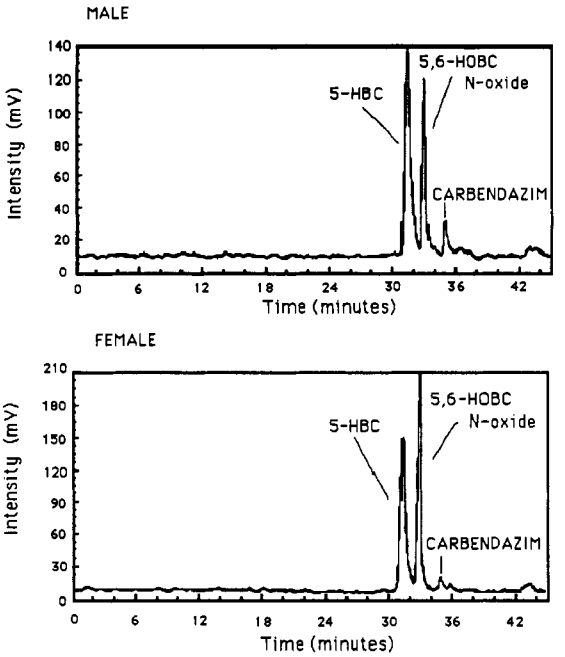

Figure 4. HPLC radiochromatograms of rat liver extracts from rats dosed with [^{14}C]carbendazim.

hydroxycarbendazim sulfide conjugates. The hydrogenation most likely cleaved and reduced the bound carbendazim residues to free dihydrohydroxycarbendazim, which in turn was dehydrated by the hot acid to carbendazim. It is improbable that unmetabolized carbendazim would exist as a bound residue as there is no logical mechanism for binding. Since benomyl is rapidly hydrolyzed to carbendazim in animals (Douch, 1973; Gardiner et al., 1974), the metabolic fate of the benzimidazole portion of benomyl would follow that just described for carbendazim.

The identification of methyl (5-hydroxy-1*H*-benzimidazol-2-yl)carbamate (abbreviated 5-HBC in Figures 1–4) in all of the liver extracts is consistent with the results of

Jerina et al. (1970), who observed that 2-naphthol was produced from the treatment of *S*-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione with Raney nickel. Both 5-HBC and carbendazim would be alternate products resulting from the chemical breakdown of covalently bound dihydrohydroxycarbendazim sulfide conjugates.

5-HBC may also be a direct metabolite in these liver tissues rather than chemically derived as described above. This would be consistent with reports that 5-HBC is a major metabolite of benomyl in rat, mouse, rabbit, dog, chicken, sheep, and dairy cow excreta, present as glucuronide and/or sulfate conjugates (Gardiner et al., 1968, 1974; Douch, 1973). An additional metabolite was tentatively identified (by fast atom bombardment mass spectrometry and NMR) in rat liver as methyl (6-hydroxy-5-oxo-5*H*-benzimidazol-2-yl)carbamate N^3-oxide (abbreviated 5,6-HOBC *N*-oxide in Figure 4).

Although benomyl would be degraded by the procedures described in this paper, no benomyl initially would be present in tissues due to complete metabolism to other products, as previously referenced. Carbendazim, 5-HBC, and 5,6-HOBC *N*-oxide are stable throughout the base and acid treatments and, if initially present in tissue, would remain intact. Identification of radiolabeled products extracted into the aqueous fractions was not feasible due to the low specific activities of the aqueous fractions that resulted.

These results indicate that the bound liver residues derived from benomyl- and carbendazim-dosed animals are for the most part benzimidazole-related rather than natural products resulting from breakdown and reincorporation. Chemical reduction and dehydration of these benomyl- and carbendazim-derived sulfide-bound residues in animal tissues have provided a means for better evaluation of these terminal pesticide residues which otherwise would have been uncharacterized and for which the toxicological significance would have remained uncertain.

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LITERATURE CITED

Boyland, E.; Ramsay, G. S.; Sims, P. The Secretion of Metabolites of Naphthalene, 1:2-Dihydronaphthalene and 1:2-Epoxy-1:2:3:4-Tetrahydronaphthalene in Rat Bile. *Biochem. J.* **1961**, *78*, 376–384.

Douch, P. G. C. The Metabolism of Benomyl Fungicide in Mammals. *Xenobiotica* **1973**, *3*, 367–380.

Gardiner, J. A.; Brantley, R. K.; Sherman, H. Isolation and Identification of a Metabolite of Methyl 1-(Butylcarbamoyl)-2-benzimidazolecarbamate in Rat Urine. *J. Agric. Food Chem.* **1968**, *16*, 1050–1052.

Gardiner, J. A.; Kirkland, J. J.; Klopping, H. L.; Sherman, H. Fate of Benomyl in Animals. *J. Agric. Food Chem.* **1974**, *22*, 419–427.

Jerina, D. M.; Daly, J. W.; Whitkop, B.; Zaltzman-Nirenberg, P.; Udenfriend, S. 1-2-Naphthalene Oxide as an Intermediate in the Microsomal Hydroxylation of Naphthalene. *Biochemistry* **1970**, *9*, 147–156.

Kirkland, J. J. Method for High-Speed Liquid Chromatographic Analysis of Benomyl and/or Metabolite Residues in Cow Milk, Urine, and Tissues. *J. Agric. Food Chem.* **1973**, *21*, 171–177.

Kovacs, M. F., Jr. Regulatory Aspects of Bound Residues (Chemistry). *Residue Rev.* **1986**, *97*, 1–17.

Ryan, J. J.; Hoffman, B. Trenbolone Acetate: Experiences with Bound Residues in Cattle Tissues. *J. Assoc. Off. Anal. Chem.* **1978**, *61*, 1274–1279.

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