Distribution and Metabolic Fate of the Fungicide Benomyl in Dwarf Pea

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ABSTRACT

Dwarf pea plants growing in nutrient solutions were root-treated with ^{14}C benomyl for 4 days, then harvested at various intervals. No ^{14}C benomyl was recovered from plants at any time. A fungitoxic derivative, ^{14}C methyl 2-benzimidazolecarbamate (MBC), was present in large quantities in organic solvent extracts from treated plants. At senescence (52 days after treatment), 78% of the label was present in MBC, 5% in water-soluble metabolites, and 14% bound to the plant residue. Treatment of residue with hot NaOH released 50% of the bound label, a portion of which was present in 2-aminobenzimidazole.

The labeled products were translocated rapidly from the roots to foliage of the plant. Fifty-two days after treatment, 92% of the label was in nonroot portions, concentrated primarily in the foliage which was present during the treatment period. Even when plant roots were continuously exposed to the labeled fungicide, the seeds accumulated only small amounts of radioactivity. The data suggest that long-term chemotherapeutic effects of MBC will be dependent on the mode of application, distribution, and concentration of the fungicide in plants.

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Benomyl is one of a group of benzimidazole compounds with antifungal activity (1, 7, 8, 9). Benomyl rapidly decomposed in aqueous (4, 18) and nonaqueous solutions (15) to methyl 2-benzimidazolecarbamate (MBC), a fungitoxic breakdown product. By thin-layer chromatography and bioassay, it was determined that MBC was the only fungitoxic compound detected in plants treated with benomyl (18, 19, 20, 21). Methyl 2-benzimidazolecarbamate was translocated apoplastically, and exhibited only slight breakdown in bean leaves (19). The compound moved through the xylem and acropetally in leaves, accumulating at the margins and tips (2, 19, 21); however, accumulation in fruit was considerably less than that in foliage (12, 20). Accumulation in various plant tissue was thought to be dependent on transpiration (12, 20).

The purpose of this study was to determine the distribution and metabolic fate of ^{14}C benomyl in dwarf pea plants.

MATERIALS AND METHODS.—*Plant culture.*—Seeds of dwarf pea (*Pisum sativum* L. 'Laxton Progress') were germinated in running tap water at 24 C. Three-day-old germinated seeds were transferred onto cheesecloth screens placed on the surface of gently stirred, half-strength Knop's solution (16). After 3 days' growth at 21-23 C with a day-length of 14 hr (600-800 ft-c), individual seedlings were placed in blackened glass containers (80 × 100 mm) with 75 ml of full-strength Knop's solution. Plants were grown in controlled environment cabinets under conditions of 20 C, day-length of 14 hr (1,200-1,400 ft-c), and relative humidity of 30-50%. The cotyledons were supported at the surface of the nutrient solution by a plastic holder. Aluminum foil was placed in the containers so that only the cotyledons and green tissue received light. Knop's solution was changed every 4 days, and water was added to replace that lost by evaporation and transpiration. Cotyledons were excised after 21 days of growth.

Fungicide.—Benomyl and MBC were supplied by E. I. du Pont de Nemours & Co., Wilmington, Del. ^{14}C benomyl labeled in the 2 position of the imidazole ring with a specific activity of 2.01 mc/mmole was purchased from New England Nuclear Corp., Boston, Mass. Purity of the fungicide was determined by thin-layer chromatography (TLC) and bioassay. The labeled product contained 85% of the radioactivity in benomyl and 15% of that in MBC. ^{14}C benomyl was converted to 100% MBC by incubating ^{14}C benomyl in methanol at 30 C for 72 hr. ^{14}C 2-aminobenzimidazole (2AB) was made from labeled benomyl by treatment of the fungicide in hot (100-C) 2 N NaOH for 30 min, followed by extraction of 2AB with ethyl acetate.

Distribution and metabolic fate of ^{14}C benomyl and MBC.—Three-week-old plants were placed in 75 ml of nutrient solution which contained 5.2 μmole of ^{14}C benomyl (10.43 μc). Benomyl was added from a methanol stock solution. Tween 80 (polyoxyethylene sorbitan monooleate) (0.2 ml diluted 1:1,000 with water) was added to the nutrient solution to disperse the fungicide which precipitated. After 4 days, the plants were removed from the radioactive solutions and the roots washed in running water for 15 min; they were then placed in new containers with Knop's solution. Plants were harvested from 4 to 52 days after treatment with the fungicide. The roots were washed and blotted gently, and the plants divided into four samples: roots; foliage present during treatment; foliage; and fruit (pods and seeds) developed after treatment. These samples were quick-frozen in plastic bags in an alcohol-dry ice mixture, and stored at −20 C.

To determine whether fruits accumulate fungicide when the roots of the plant are continuously exposed to ^{14}C MBC, 34-day-old plants, with the first flower in full petal, were placed in Knop's solution (75 ml)

containing $0.52\,\mu$mole ($1.04\,\mu$c) of ^{14}C MBC. The Knop's solution and fungicide were changed every 4 days. After 20 days, the roots were washed and the plants divided into four samples: roots, foliage, pods, and seeds. These were freeze-dried, ground to a fine powder in a Wiley mill, and stored in a desiccator in -20 C. Radioactivity in the freeze-dried samples was determined by oxygen combustion (6), modified in that combustion flasks were 1 liter in size, baskets were nichrome wire gauze, and the samples (10-50 mg) were wrapped in black paper for infrared ignition.

Distribution and identification of ^{14}C metabolites in the plant tissue exposed for 4 days to labeled benomyl were determined as follows. The tissue was ground in a Virtis homogenizer in an ice bath with 50 ml of acetone, containing 10% water, for 2-3 min. The slurry was centrifuged, the pellet homogenized twice in 50-ml portions of acetone-water, and the residue refluxed for 5 hr with a benzene-methanol-chloroform (BMC) mixture (1:1:1, v/v). The tissue residue was dried under vacuum, and the amount of radioactivity remaining determined by oxygen combustion. Water, 15% by volume, was added to the combined acetone-water extracts, and an equal volume of chloroform was added; the two phases then separated. The water phase was extracted twice with an equal volume of chloroform. Radioactivity in the aqueous and nonaqueous fractions was determined by liquid scintillation spectrometry. We calculated total radioactivity in the entire or any section of the plant by adding the amounts in the acetone-water, BMC extracts, and the extracted residue. We determined radioactivity in the organic extracts by adding the amounts in the BMC and chloroform fractions.

The chloroform and BMC fractions were taken to dryness and resuspended in 1-2 ml of chloroform. The water phase was reduced to dryness and extracted 5 times with methanol. The various metabolic components in the aqueous and nonaqueous extracts were resolved by TLC. The solvents and R_F values of benomyl and two related compounds are listed in Table 1. The TLC plates were analyzed for radioactivity using a Nuclear-Chicago Actigraph III radiochromatography system.

Bioassay of TLC plates involved incubating a strip of solidified agar medium (5) containing conidia of *Neurospora crassa* Shear & Dodge on the surface of the chromatogram for 24 hr.

The nature of the radioactive material remaining in the residue after extraction was further analyzed. The dried residues from each section of the plant were combined, ground in a Wiley mill, and stirred magnetically for 4 hr in 30 ml of 2 N NaOH in a centrifuge tube immersed in boiling water. A watch glass was placed on top of the tube to prevent evaporation. The mixture was centrifuged, the residue washed once with hot water, and the wash water combined with the NaOH solution. The solution was extracted 3 times with equal volumes of ethyl acetate. The organic extract was reduced to dryness, the remaining residue was brominated (17), and the products were analyzed by TLC (Table 1).

RESULTS.—Plants grown under the described environmental conditions flowered on the 30th-36th day after germination, and at senescence (71st day after germination) were 22-24 cm in height with two pods each (7.0-7.4 cm long) containing three or four seeds. Plants exposed to ^{14}C benomyl for 4 days were harvested, starting on the last day of treatment

TABLE 1. Components and chromatographic characteristics of the benzimidazole compounds studied

System	Development solvent[a]	R_F		
		Benomyl	MBC[b]	2AB[c]
A	CHCl$_3$:acetone (6:1)	0.70	0.14	0.00
B	CHCl$_3$:acetone (3.7:0.3)	0.52	0.06	0.00
C	CHCl$_3$:methanol:acetone (2:1:1)		0.68	0.31
D	CHCl$_3$:ethyl acetate: acetic acid (1:1:0.04)	0.60	0.36	0.03
E	Acetone		0.57	0.10
F	Hexane:ethyl acetate: methanol (10:10:1)		0.62	0.71[d]

[a] Thin-layer glass plates (5 × 20 cm) coated 0.250 mm thick with Silica Gel Hf-254 (Brinkman Instrument, Inc., Westbury, N.Y.) and developed to 16 cm in the various solvents.

[b] MBC = methyl 2-benzimidazolecarbamate.

[c] 2AB = [14]C 2-aminobenzimidazole.
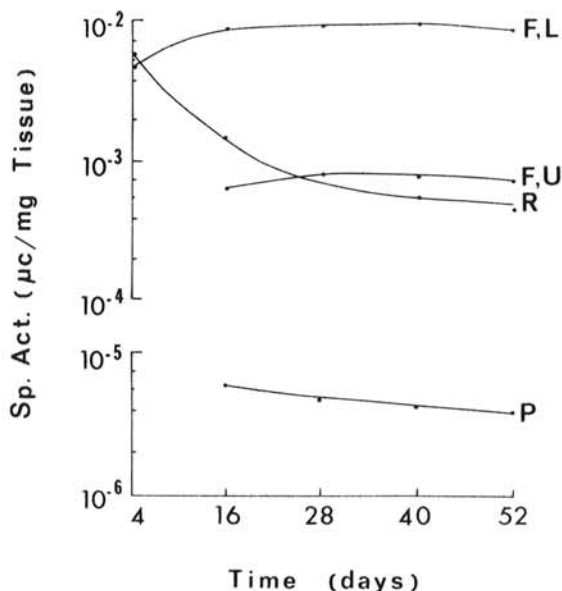
[d] Brominated 2-aminobenzimidazole.

Fig. 1. The amount of radioactivity in dwarf pea plants grown for 4 days in Knop's solution containing ^{14}C benomyl. [14]C benomyl (5.2 μmole-10.43 μc) added to Knop's solution (75 ml) containing 21-day-old plants. Plants were harvested at various intervals starting from the last day of treatment with benomyl (4th day). F,L = foliage present during treatment period; F,U = foliage grown after treatment period; R = roots; P = pods and seeds. Sp. Act. = specific activity - μc/mg dry weight of tissue; the average of three plants.

TABLE 2. The distribution of radioactivity in dwarf pea plants grown for 20 days in Knop's solution containing ^{14}C methyl 2-benzimidazolecarbamate (MBC)[a]

Tissue	%^{14}C[b]	Specific activity[c]
Foliage	86.7	1.2×10^{-3}
Roots	9.3	3.9×10^{-4}
Pods	3.4	9.4×10^{-5}
Peas	0.1	8.1×10^{-6}

[a] Knop's solution (75 ml) containing 0.52 μmole (1.04 μc) ^{14}C MBC added to 34-day-old plants. Solutions changed at 4-day intervals.

[b] Per cent distribution of the label from ^{14}C MBC in the plant.

[c] μc/mg dry wt tissue. Average of six plants.

TABLE 3. Percentage distribution of label in tissue extracts and residue from ^{14}C benomyl-treated dwarf pea plants[a]

Time (days)	Organic-soluble		Water-soluble		Residue	
	Foliage	Root	Foliage	Root	Foliage	Root
4	71.3	20.6	1.7	0.4	4.3	0.7
16	81.3	4.2	3.7	0.8	5.6	3.8
52	78.9	1.3	4.4	0.7	9.2	4.4
	80.2[b]		5.1[b]		13.6[b]	

[a] Conditions the same as in Fig. 1.

[b] Per cent ^{14}C in foliage and roots at 52 days.

TABLE 4. Identification and percentage distribution of labeled compounds in extracts and residue tissue from ^{14}C benomyl-treated dwarf pea plants[a]

Compound[b]	Distribution ^{14}C (%)		
	Organic-soluble	Water-soluble	Residue
MBC	98		
	(80.2)[c]		
2AB	2		10
			(13.6)
Unidentified		100 (5.1)	90

[a] Plants harvested 52 days after treatment with ^{14}C benomyl. Conditions the same as in Fig. 1.

[b] MBC = methyl 2-benzimidazolecarbamate. 2AB = ^{14}C 2-aminobenzimidazole.

[c] Figures in parentheses represent per cent ^{14}C at 52 days in foliage and roots in the various fractions from Table 3.

(4th day), and the amount of radioactivity (specific activity, μc/mg dry wt of tissue) was determined in the various parts of the plant (Fig. 1). The specific activity was divided almost equally between the roots and foliage of plants harvested on the 4th day. The level of activity rapidly declined in the roots in those plants harvested after the removal of the radioactive fungicide. In the interval between the 4th and 16th day, the label in the roots was translocated primarily to the lower foliage (Fig. 1-F, L) that was present during the period when the plant was exposed to the fungicide. Label in the new or upper foliage (Fig. 1-F, U) and in pods and peas (Fig. 1-P) grown after treatment with benomyl was, respectively, 10 and 1,500 times less than the amount in the lower foliage. The ^{14}C present in the lower foliage did not appear to be remobilized and translocated to the upper foliage or fruit. The small amount of label in these tissues could have been translocated from the roots during the 16th through 28th days.

Radioactivity accumulated primarily in the foliage of plants continuously fed ^{14}C MBC, but not in the pods and seeds (Table 2). The specific activities in roots, pods, and seeds were, respectively, 3, 13, and 148 times less than that in the foliage.

When tissue from plants exposed to ^{14}C benomyl for 4 days was extracted, 92% of the label (71% in foliage and 21% in roots) was in the organic-soluble fraction (Table 3). The water-soluble phase contained 2.1%; and the residue, 5%. Fifty-two days after incubation with ^{14}C benomyl, almost all the organic-soluble label was in the foliage; only 1.3% was in the roots. The water phase contained 5.1%, most of which was in the foliage. The amount of label bound to the plant residue increased to 13.6%, of which 9.2% was in the foliage.

The nature of the labeled products in the organic phase, water phase, and residue was characterized (Table 4). No ^{14}C benomyl was recovered at any time interval from any fraction or residue. Solvent systems A-C (Table 1) effectively separated known benomyl from MBC. Methyl 2-benzimidazolecarbamate was identified only in the organic phase, and represented 98% of the total label in that fraction. The area of radioactivity identified as MBC by TLC was fungitoxic when bioassayed. A

small percentage was identified as 2AB in this fraction. The 2AB was not present at the 4-day interval. At the 4- and 52-day intervals, MBC represented 92 and 78% of the total label in the plant, respectively.

The ^{14}C compounds found in the water phase (5.1%) were not identified. One area of radioactivity was found by TLC in solvents B-E (Table 1), and two-three areas were resolved in methanol:acetone (1:1) and methanol:ethyl acetate (1:1) solvents. The R_F values of the ^{14}C compound(s) did not consistently match those values for known 2AB or MBC.

When the residue containing 13.6% of the total radioactivity in the plant was subjected to the hot NaOH-bromination treatment, a portion of the label (10%) was found in 2AB (solvents C-F) (Table 1). Approximately 50% of the label from the residue was solubilized by NaOH treatment.

DISCUSSION.—*Metabolism.*—Failure to recover benomyl from treated pea plants could be due to its rapid decomposition to MBC (4, 15, 18) either in the nutrient medium, in the plant, or during the extraction procedure. The fungitoxic derivative was rather stable in pea plants with only 22% of the total label in products other than MBC 52 days after treatment.

After exhaustive organic solvent extraction had taken place, the residue contained 14% of the label;

after NaOH solubilization and bromination of the products had been completed, a portion of the label was identified as 2AB. It is unknown how or in what form the labeled molecule was attached to the residue plant material; however, a portion must have existed which contained the 2-aminobenzimidazole moiety. Since the fungicide moves primarily in the apoplast (19), the compound(s) probably was bound to cell wall material. ^{14}C 2-(4-thiazolyl)-benzimidazole (TBZ) has also been reported (10) to bind to plant constituents; however, TBZ was hydrolyzed from the residue by hot HCl treatment.

A number of metabolites of benzimidazole pesticides have been recovered as water-soluble products from plants and animals. One of the major products of Fenazaflor (5,6-dichloro-1-phenoxycarbonyl-2-trifluoromethylbenzimidazole) metabolism in mice is a N-glucuronide derivative (3). The N-glucoside product has been recovered from apples (3). Benzimidazole is converted to a nucleotide by extracts from wheat tissue and incorporated into the nucleic acid fraction of wheat tissue (13, 14). Hydroxylation of the benzine ring and conversion to the O-glycoside or sulfate conjugates occur. Animals fed TBZ excrete 5-hydroxythiabendazole free or as the glucuronide or sulfate conjugate (22). Rats metabolize benomyl in a similar manner, excreting methyl 5-hydroxybenzimidazolecarbamate and O-glucuronide and sulfate conjugates (11). Hydroxylation of Fenazaflor by plants and animals and the formation of derivatives have also been reported (3).

The water-soluble ^{14}C metabolites extracted from benomyl-treated plants represented only a small per cent (5%) of the total label from the fungicide. Since these metabolites were not identified, it is unknown whether the N-glucoside and N-glucuronide derivatives or O-glycoside and sulfate conjugates were present in the water-soluble fraction.

Distribution.—Methyl 2-benzimidazolecarbamate was rapidly translocated from the roots to the green portions of the plant. When the supply of the fungicide was discontinued, the level of MBC decreased in the roots to the point where 99% of the fungicide was in nonroot portions of the plant. The fungicide accumulated primarily in the foliage that was present during the treatment period. Only small amounts of MBC were translocated to the new foliage or fruit once the roots were removed from benomyl. The low level of radioactivity present in the new plant tissue indicates that MBC was not translocated in appreciable amounts from the older foliage. Movement of the fungicide in the apoplast would suggest this type of distribution (2, 19).

The lack of accumulation of significant amounts of radioactivity in the fruit (seeds), even when the roots were continuously in the presence of MBC, supports the hypothesis of Peterson & Edgington (19) that the concentration of the fungicide is dependent on the ability of the various plant organs to transpire. When the fruit has few or no functional stomates, the amount of chemical present will be lower than in those organs which have greater transpiration rates.

The level of fungicide in the fruit is probably dependent on the amount of water required for growth and not on transpiration (12).

Data presented in this paper are in close agreement with reports by other workers who have used bioassay or conventional chemical methods to indicate movement and identification of the fungitoxic derivative MBC. The data suggest that long-term chemotherapeutic effects of MBC will be dependent on the mode of application, distribution, and concentration of the fungicide in plants.

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