Metabolic Fate of Methyl-2-Benzimidazole Carbamate in Melon Plants

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ABSTRACT

The metabolism of methyl-2-benzimidazole carbamate (MBC) was studied in melon plants grown in a liquid nutrient solution containing benomyl. The plants were treated for 2 months with a 20 μg/ml of benomyl; seven metabolites were identified in the leaves by gas-liquid chromatography: MBC, 2-aminobenzimidazole (2-AB); benzimidazole; *o*-aminobenzonitrile; aniline; the beta-glycosidic conjugates of MBC and 2-AB. Some other metabolites could not be identified. A metabolic pathway for benomyl in melon plants is suggested.

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Benomyl rapidly decomposes to methyl-2-benzimidazole carbamate (MBC) in aqueous solution and generally is not recovered from treated plants (2, 4, 5, 6, 7). This suggests that only MBC is taken up by plants (11).

Siegel et al. (9, 10), working with dwarf peas and strawberries, demonstrated that MBC accumulates principally in leaves produced before and during treatment; less is present in fruits and leaves formed after treatment. In pea plants they identified only MBC. In strawberry plants they detected MBC and 2-AB and observed unidentified metabolites, both water-soluble and bound to the plant material after exhaustive organic solvent extraction. In the present work, the distribution and the metabolic fate of MBC in melon plants are investigated, using ^{3}H MBC and gas-liquid chromatography.

MATERIALS AND METHODS.—*Plant culture.*—Melon plants (*Cucumis melo* L. 'Noir des Carmes') were grown in sandy loam containing 15% organic matter, until they reached the two-leaf stage. Sets of five plants were placed in 5-liter black plastic containers. These were filled with Shive and Robbins solution 1 (3), (0.2 g/liter of ferric monosodium salt of ethylenediaminetetraacetic acid was used in the place or iron sulfate). The pH of the solution was 6.3. The solution was aerated by forced-air at about one liter per minute. The plants were grown at 28 C by day with 16 hours of light (11,000-13,000 lux) and at 24 C at night. The relative humidity of the air ranged 60-90%. The nutrient solution was changed every five days and tap water was added each day to maintain the initial volume.

Fungicides and chemicals.—Benomyl and MBC were supplied by E. I. du Pont de Nemours & Co., Wilmington, Del., and by Chimac, Brussels, Belgium. Randomly tritium-labelled MBC (specific activity = 4,300 mCi/mmole) was purchased from the Institut National des Radioéléments, Mol, Belgium. The labelled MBC was dissolved in methanol (0.75 mCi/mmole) and its purity was verified by thin-layer chromatography. The analytical grade trifluoroacetic anhydride and benzimidazole were from Aldrich and Serva respectively. The liquid phases and the support used for the gas-liquid chromatography were from Varian. β-Glucosidase was from BDH Chemicals (Poole, England). The other chemicals were of analytical grade and obtained from Merck.

Treatment of the plants with benomyl.—In one treatment group, each plant was treated with 50 μg/ml of benomyl containing ^{3}H MBC (5.85 μg ^{3}H MBC plus 250 mg benomyl in 5.0 liters of liquid culture medium). Treatment was applied at the five-leaf stage and was stopped after five days by replacing the growth medium with a new solution which contained neither benomyl nor ^{3}H MBC. Plants were analyzed for radioactivity incorporation at five, ten, fifteen, and twenty days after the treatment was started. In a second treatment group, plants were exposed to 20 μg/ml of nonradioactive benomyl during two months. Then leaves were collected and analyzed for decomposition products of benomyl.

In both treatments, phytotoxic effects were noted; namely, leaf malformations along with progressive yellowing, and necrosis of the leaf margins. These symptoms appeared on the sixth day after the treatment was started.

Extraction and distribution of ^{3}H MBC label.—Each plant was divided into three fractions: roots, stems, and leaves. Each fraction was homogenized for two min in a Waring Blendor, with distilled water (1:10, wet weight:volume). The pH of the homogenate was 6.1. Two-ml aliquots were removed from each homogenate, and filtered on Gelman type-A glass-fiber filters (pore size: 0.3 μm). The solid residues were washed with distilled water to remove any unbound label, dried under an infra-red lamp and measured for ^{3}H with a liquid scintillation counter. The liquid fraction was counted separately. For both parts, a Bray's scintillation mixture was used. The mean radioactivities of the solid residue and of the filtrate were added together.

Extraction and preparation of the samples for gas-liquid chromatography analyses.—After harvest, the leaves were frozen in plastic bags with an ethanol-solid CO_2 mixture and stored at −20 C. From these samples, two different extracts were prepared. The first step was applied similar for both.

A benzene extraction was carried out as follows. One-hundred grams of leaves were homogenized during fifteen min with 400 ml of benzene at 8,000 rpm in a Sorvall

Omnimixer (5). The homogenate was centrifuged at 4,500 rpm for 15 minutes, and the supernatant filtered through anhydrous sodium sulfate. The precipitate resulting from this centrifugation was further used for aqueous extraction as described below. The volume of the filtrate was reduced to 50 ml in a rotatory vacuum concentrator at 40 C. The concentrate was transferred to a 500-ml separatory funnel, and extracted two times with 50 ml of 0.1 N HCl. The acidic fractions were pooled and washed three times with 50 ml of chloroform. The aqueous phase was adjusted to a pH of 7.8-8.2 with 1.0 N NaOH and 0.1 N NaOH consecutively and extracted three times with 50 ml of ethyl acetate. The ethyl acetate extracts were filtered through anhydrous sodium sulfate, and the residual sodium sulfate was washed with an additional 50 ml of ethyl acetate.

The aqueous extraction was carried out as follows. The solid residue left after the centrifugation of the benzenic homogenate was added with 300 ml of distilled water and homogenized for fifteen min at 8,000 rpm in the Omnimixer. The suspension was centrifuged at 4,500 rpm for twenty min, the supernatant was filtered through glass-wool and centrifuged again at 4,500 rpm for twenty min. The supernatant was filtered on Whatman No. 5 paper and freeze-dried, to give a powder which was submitted to two kinds of hydrolysis. One gram of

TABLE 1. Distribution of ^3H originating from radioactive MBC from liquid nutrient medium into the different part of melon plants treated for five days with the fungicide

Time[a] (days)	Radioactivity (specific activity)			
	Roots	Stems	Leaves	Whole plants
5	68.4[b] (22.8)[c]	24.3 (12.6)	299.1 (64.6)	68.0 (100)
10	29.1 (12.6)	10.3 (11.4)	148.0 (76.0)	40.5 (100)
15	14.1 (10.9)	7.3 (13.2)	61.9 (75.9)	23.8 (100)
20	8.0 (9.7)	2.7 (8.1)	34.5 (82.2)	14.3 (100)

[a]Expressed as days following the addition of the fungicide.
[b]Specific activity ($\mu Ci.10^3$/g fresh weight).
[c]The data in brackets indicate the percent of total radioactivity calculated for each fraction, with 100% as the average value for the whole plant. The data have been calculated as the mean value obtained from analyses of five different plants.

TABLE 2. Some typical gas chromatographic characteristics of identified metabolites of benomyl in melon leaves

Conditions	Compounds				
	MBC	2-AB	Benzimidazole	*o*-Aminobenzonitrile	Aniline
NON-TFA (a)					
1) See 30 (b)					
Column temp (C)		170	150	120	
Retention time (minutes)		2.4	2.3	2.5	
Lower limit of sensitivity (μg/g) relative to 100 g of fresh leaves		0.2	0.2	0.2	
2) OV 17 (b)					
Column temp (C)		180	170	145	
Retention time (minutes)		2.8	3.3	3.2	
3) OV 225 (b)					
Column temp (C)		175	165	125	
Retention time (minutes)		2.9	3.4	3.1	
TFA (a)					
1) SE 30 (b)					
Column temp (C)	140	110	130	120	90
Retention time (minutes)	3.1	2.2	2.7	2.6	3.0
Lower limit of sensitivity (μg/g) relative to 100 g of fresh leaves	0.02	0.07	0.05	0.03	0.03
2) OV 17 (b)					
Column temp (C)	160	120	145	130	100
Retention time (minutes)	3.0	2.4	3.2	2.2	2.9
3) OV 225 (b)					
Column temp (C)	150	110	140	120	90
Retention time (minutes)	3.5	3.4	2.6	2.9	3.5

[a]Nontrifluoroacetylated (NON-TFA) and trifluoroacetylated (TFA) samples.
[b]Silicone liquid phases for gas-liquid chromatography.

powder was dissolved in twelve ml of 2.0 N HCl and kept at 100 C for 1 hour in a sealed tube. Another one-gram of powder was dissolved in 20 ml of 0.02 M citrate-phosphate buffer (pH 4.8) containing one drop of toluene and 50 mg of β-glucosidase (2×10^4 EU). The mixture was incubated for 20 hours at 35 C. Both hydrolysates were diluted to thirty ml with distilled water, centrifuged at 4,500 rpm for twenty min and brought to pH 7.8-8.2 with 1.0 N and 0.1 N NaOH. The aqueous extract was partitioned in ethyl acetate as described above.

Gas-liquid chromatography (GLC) analyses.—The benzenic and aqueous extracts recovered in ethyl acetate were evaporated to dryness at 40 C in a vacuum evaporator. Two kinds of samples were prepared for GLC analyses. For the first one, the residue was dissolved in 1.0 ml of acetone; this was the nontrifluoroacetylated (nonTFA) GLC sample. For the second sample, the trifluoroacetylated sample (TFA), the residue was treated in a sealed tube at 100 C for 35 min with five ml of dried ethyl acetate and 0.6 ml of pure trifluoroacetic anhydride. The cooled mixture was then evaporated to dryness by a flow of dry nitrogen and by heating at 40 C. Finally, the

TABLE 3. Concentration and distribution of the identified metabolites of benomyl in melon leaves. Plants were exposed to 20 μg/ml benomyl during two months, and the treatment was started at the five-leaf stage

Metabolites	Concentration in melon leaves (μg/g of fresh weight)			Distribution, mole %	
	Free compounds (in benzenic extract)	Conjugated compounds (in aqueous extract)			
		Acidic hydrolysis	Enzymatic hydrolysis	Free compounds	Conjugated compounds released by acidic hydrolysis
MBC	11.0	0.5	0.2	51.2	2.3
2-AB	3.0	1.1	0.4	20.1	7.4
Benzimidazole	1.2	0.0	0.0	8.8	0.0
o-Aminobenzonitrile	1.1	0.0	0.0	8.4	0.0
Aniline	0.2	0.0	0.0	1.8	0.0

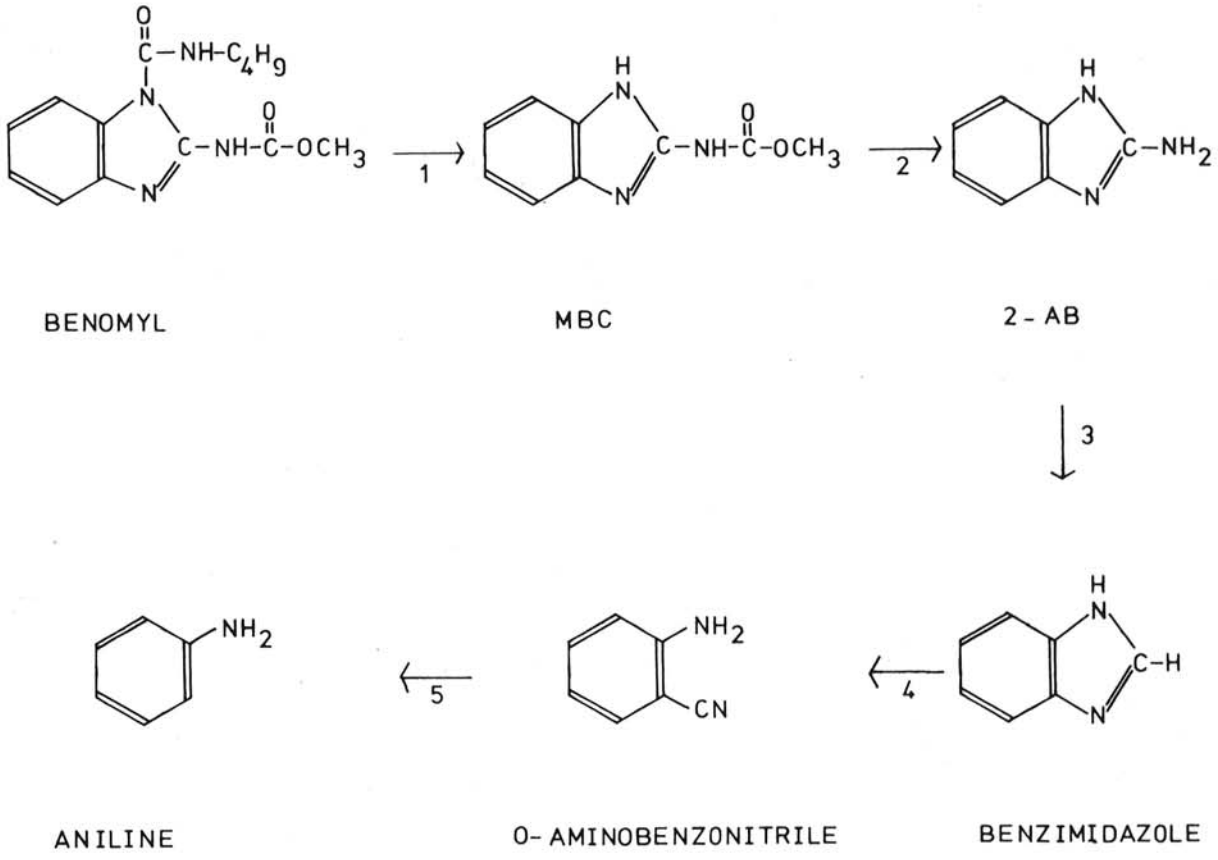

Fig. 1. Suggested metabolic pathway for benomyl in melon plants.

residue was dissolved in 1.0 ml of dried acetone.

A 1.0 μliter "plug" of the final GLC sample was injected in the Varian Model 2700 gas chromatograph under the following conditions. Detector: ^{3}H electron capture. Recorder: Varian Model A 25, 1 mV. Chart speed: 50 cm/hr. Column: glass, 1.50 m × 2.2 mm internal. Liquid phases: SE 30, OV 17, OV 225, Carbowax 20 M. Support: Chromosorb R 80 to 149 μm (100-mesh) particle size. Concentration of the liquid phases: 5 g %. Injection temperature: 250 C. Detection temperature: 225 C. Carrier gas: nitrogen at 40 ml/min. The peaks of the chromatograms were measured with a planimeter. The μg/g of residue in a sample was calculated by comparing the response obtained for a known amount of the same standard derivative.

RESULTS.—*Translocation and distribution of label in roots, stems, and leaves.*—In the experiments with melon plants treated with ^{3}H MBC and benomyl for five days, it appeared that most of the radioactivity was recovered in the leaves produced before and during treatment (Table 1). Fifteen days after the fungicide had been removed from the nutrient solution, the specific radioactivity of the leaves that were present at the beginning of the treatment was 30.4×10^{-3} μCi/g fresh weight, whereas the leaves formed afterwards were characterized by a specific radioactivity of 4.1×10^{-3} μCi/g fresh weight. But, since after twenty days, the weight of the plants increased about four-fold, the results of incorporation of ^{3}H MBC had to be corrected for the dilution of the chemical. The total amount of radioactivity recovered from each plant was however quite stable during the fifteen days after the treatment was terminated.

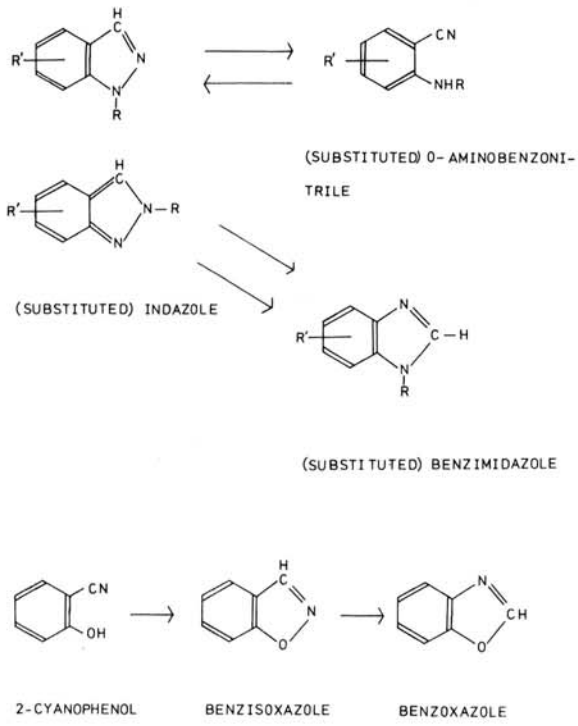

Fig. 2. Photochemistry of the benzimidazoles (1, 12).

Characterization of benomyl and its metabolites by gas-liquid chromatography.—The metabolites of benomyl were analyzed in the leaves of plants treated for 2 months with 20 μg/ml benomyl. Seven metabolites have been identified (Tables 2 and 3). Five were recovered in the benzene extract, and two conjugated compounds in the aqueous extract. The gas chromatograms of extracts from treated plants were compared with those of extracts from untreated plants. The peaks present in the first and absent in the second were considered as possible metabolites. The identification of some metabolites was possible because, at a given column temperature, the number of significant peaks was usually not greater than five during the first seven minutes of elution. The identification of the metabolites was accomplished by comparing the retention times with those of both trifluoroacetylated and nontrifluoroacetylated pure chemicals. Their identity was confirmed by analysis with four different liquid phases, at several temperatures and flows of the carrier gas, and with both the trifluoroacetylated and the nontrifluoroacetylated extracts. The suggested identities of the metabolites were accepted as such when the corresponding peaks were observed in each of all the enumerated chromatographic conditions. Table 2 gives some typical gas chromatographic characteristics of the identified metabolites.

Recovery experiments were run on a number of samples by adding known amounts of metabolites at the benzenic blending step. The results for the different compounds were: MBC, 80-100%; 2-AB, 76-100%; benzimidazole, 83-100%; *o*-aminobenzonitrile, 78-100%; and aniline, 75-100%. MBC, 2-AB, benzimidazole, *o*-aminobenzonitrile, and aniline were stable: none of them degenerated into the others during extraction, work-up, or GLC sample preparation. This was shown by separately adding known amounts of each metabolite at the benzenic blending step of control extracts. Except for MBC, the nonconjugated metabolites observed in the benzenic extract were thus truly present as such in the plant. Benomyl was completely hydrolyzed to MBC during the work-up.

In the nonhydrolyzed aqueous extracts from treated plants, neither MBC nor 2-AB were observed. MBC was stable during the acidic and enzymatic hydrolysis steps of control extracts. The MBC (or benomyl) and 2-AB observed in the aqueous extract were thus the aglycones of conjugated compounds actually present in the plant. More MBC and 2-AB were freed by the acidic hydrolysis than by the enzymatic one (Table 3). This suggests that there were other conjugated products besides the β-glycosides.

The concentration of the seven compounds that were recognized in the plant are given in Table 3.

Some unidentified trifluoroacetylated metabolites were detected in treated plant extracts. Their gas chromatographic characteristics on SE 30 [column temperature, (C)/retention time, min] for those in the benzenic extract: 120/3.8 and 140/2.1; and for those in the aqueous extract: 120/3.0 and 140/7.1. Since these metabolites could not be identified, their exact concentration was not determined; however, the GLC results suggest that they were formed in only small quantities in the plants.

DISCUSSION.—After melon plants were treated for five days with the ^{3}H MBC-benomyl mixture, most of the radioactivity (82%) was recovered after 3 weeks in the leaves. If the total radioactivity measured in all the leaves was counted as 100, the greatest part (70-90%) was recovered in the leaves that were present at the start of the treatment. The slight accumulation of label into the newly formed leaves originated primarily from roots and stems in which the concentration of labelled products decreased after the cessation of the treatment. This suggested a low rate of radial distribution of the fungicide. These results are in close agreement with those obtained with other plant species (2, 6, 7, 10, 11). Since only leaves were analyzed, and some radioactivity was associated with the solid residue after the benzenic and aqueous extractions, a balance-sheet for the different metabolites found in the plants is not possible. Siegel (9) suggested that the labelled products in the solid residue might be associated with hemicellulose of the cell walls.

Among the metabolites recovered from the plants treated continuously for 2 months, MBC and 2-AB represent 51.2 and 20.1 mole % respectively of the total amount of the identified metabolites. These two compounds, and the conjugates of MBC and possibly of 2-AB, have been identified previously as a result of MBC degradation (9, 10). The other metabolites identified by GLC are reported for the first time from benomyl-treated plants. It is suggested that these compounds originate from benomyl according to the sequence represented in Fig. 1. The results obtained provide no proof of the reactions included in the suggested sequence, only analogies may be reported. The formation of 2-AB (reaction 2) is very slow in acidic medium, but fast in a basic medium (7). Thus, in plants, at a pH of 6.1, enzymatic hydrolysis is likely. Deamination occurring in reaction 3 is common during metabolism of drugs, and is catalyzed by deaminases.

As far as the opening of the benzimidazole nucleus is concerned (reaction 4), the photochemistry of the benzimidazoles offers some analogies (Fig. 2). Under irradiation, indazoles are reversibly transformed into substituted *o*-aminobenzonitrile. The reversibility of the photochemical isomerisation of indazoles into benzimidazoles has not yet been reported. If that reversibility could be observed, reaction 4 could be a photochemical process. There are photosensitizers in plants which could accelerate the photoreaction. The formation of an intermediate indazole in reaction 4 is not necessary. It is known that the photochemical cyclization of 2-cyanophenol into benzoxazole can proceed directly or through the intermediate benzisoxazole, according to the experimental conditions (1). On the other hand, an enzymatic mechanism cannot be excluded for reaction 4.

Reaction 5 is not necessarily performed in a single step. Hydrolysis of the cyano group to an amido group and eventually to a carboxylic group, followed by decarboxylation may be suggested. However, up to now, intermediates have not been detected.

As a conclusion, although MBC is generally regarded as a stable compound, particularly in the cultural conditions used in these experiments, it was subject to a certain decomposition which resulted in the opening of the benzimidazole nucleus. The fungitoxicity of MBC is well known. There is much less information concerning the fungitoxicity and the stability of the other derivatives. These two aspects are, however, among the most important, not only for benomyl, but for all systemic pesticides.

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