age desiccation under field conditions, 33.6 kg/ha (Isenberg and Abdel-Rahman, 1972), the bulb residue is less than 0.4 ppm. The problem of minimum detectable levels of NDA encountered before (Pendergrass *et al.*, 1969) was alleviated by using ^{14}C-labeled material.

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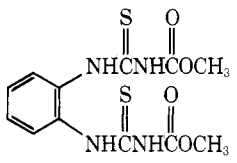
Persistence and Metabolism of Thiophanate-methyl in Soil

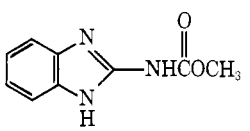
James R. Fleeker,* H. Morgan Lacy, Imogene R. Schultz, and Everin C. Houkom

The fungicide thiophanate-methyl, 1,2-bis(3-methoxycarbonyl-2-thioureido)benzene, underwent rapid conversion in soil to methyl 2-benzimidazolecarbamate (MBC). The rate of conversion was more than four times faster in soil at pH 7.4 than in soil at pH 5.6. The rate of thiophanate-methyl conversion to MBC was also reduced by steam treatment of the soil. Soil incubated 51 days with MBC-2-^{14}C (ring) and MBC-*methyl*-^{14}C released less than 1 and 16%, respectively, of the applied radioactivity as $^{14}CO_2$. The recovery of ^{14}C in acetone extracts of MBC-2-^{14}C treated soils was 79–91% in samples extracted immediately after treatment, and 53–78% 43 days after treatment, depending on soil and a rate of application of 10 or 100 ppm. Nearly all of the extractable ^{14}C chromatographed as MBC.

Thiophanate-methyl (TM) or 1,2-bis(3-methoxycarbonyl-2-thioureido)benzene (I) is a broad-spectrum systemic fungicide. The metabolism of TM has been studied in mice and the bean plant (Noguchi, 1971). A TM metabolite common to all the organisms studied is methyl 2-benzimidazolecarbamate (MBC) (II).



I



II

Reported here are studies on the persistence and metabolism of TM and its metabolite MBC in North Dakota soils.

MATERIALS AND METHODS

The soil types used in these experiments and their pH were Barnes sandy loam, 7.4; Fargo silty clay, 7.7; and Towner loamy fine sand, 7.2. The organic contents of the soils were 4.2, 3.4, and 1.1%, respectively. The soils were air dried and passed through 20 mesh screen before use. In certain experiments the pH of the soil was adjusted with 1 *N* HCl with sufficient time for equilibration.

Extraction of TM and MBC. The procedures of Pennwalt Corporation (1972) were adapted for this study. Each soil sample (25 g, dry weight) was mixed with 125 ml of acetone and 20 g of Na_2SO_4, and refluxed for 1 hr. The mixture was filtered immediately after refluxing by suction filtration through M-sintered glass, the soil residue was washed three times with 25-ml portions of acetone, and the filtrate and washings were concentrated to 5–10 ml at 30° under reduced pressure. When TM was to be determined, 50 ml of freshly prepared 1% $AgNO_3$ in 95% ethanol was added to the concentrate and the mixture was heated 20 min on the steam bath in order to convert TM to MBC quantitatively. To assay MBC, the treatment with alcoholic $AgNO_3$ was omitted and the 1 *N* HNO_3 added directly to the concentrated acetone extract. TM was determined by difference.

The dilute HNO_3 solution was extracted twice by shaking for 1 min periods with two 50-ml portions of chloroform and the chloroform extracts were discarded. The aqueous phase was adjusted to pH 6.5 with 15 *N* NH_4OH. The neutralized solution was extracted by shaking for 1 min periods with three 25-ml portions of chloroform. The chloroform extracts were concentrated at 30° under reduced pressure to a 5–10-ml volume and rinsed with chloroform into a 20-ml, screw-cap vial. The remaining solvent was removed with a stream of air and the residue assayed colorimetrically for MBC.

Determination of MBC. To the vial containing the MBC residue were added 2 ml of chloroform and 3 ml of color reagent (100 mg of Bromocresol Purple, 20 g of Na_2SO_4, and 4 ml of acetic acid diluted to 1 l. with water). The vial was sealed, shaken 1 min, and allowed to stand 20 min. An additional 3 ml of chloroform was added and the mixture was shaken 1 min and allowed to stand 20 min. The chloroform phase was filtered through filter paper into a cuvet and the absorbance read at 410 nm against a blank obtained from untreated soil.

This colorimetric assay is not specific for MBC (Sutherland, 1964; Stansbury, 1964). Therefore, some soil extracts were examined for the presence of compounds other than MBC. The soil samples were extracted as described above and were streaked on silica gel G thin-layer plates containing an inorganic fluorescent indicator. The plates were developed with either ethyl acetate–*n*-hexane–acetic acid (30:70:2), or ethyl acetate. Only areas of the plates corresponding to the R_f of authentic MBC gave a positive colorimetric test when extracted with methylene chloride. At the same R_f, the methylene chloride extracts gave an ultraviolet absorption spectra identical with that of authentic MBC.

Decomposition of TM in Soil. One milliliter of TM dissolved in acetonitrile (250 μg/ml) was added to each soil sample (25 g in a 125-ml flask). After evaporation of the solvent, the soil was stirred 10 min, water was added

Department of Biochemistry, North Dakota State University, Fargo, North Dakota 58102.

until the desired moisture content was reached, and the soil was stirred again. The flasks were incubated in a controlled environment chamber at 20°. Water was added periodically to maintain the moisture content of the soil. At intervals four 25-g samples were extracted and assayed for TM and MBC.

TM Degradation in Steam-Treated Soil. Samples of air-dried soil (25 g) were treated 24 hr with steam at 20 psi. The soil was cooled and mixed with TM (2.5 mg/25 g of soil), and sterile water was added to bring the soil to the desired moisture content. A steam-treated control containing no TM was also prepared. Incubation was at 20° for 24 hr. The samples were then extracted and assayed for MBC.

Synthesis of MBC-^{14}C. The synthesis of ^{14}C-labeled MBC was essentially according to the method of Loux (1961). MBC-2-^{14}C was prepared from thiourea-^{14}C and MBC-*methyl*-^{14}C was prepared from methyl chloroformate-*methyl*-^{14}C (Soeda *et al.*, 1972). Thin-layer chromatography of both MBC-^{14}C compounds gave a single radioactive spot which cochromatographed with authentic MBC and contained more than 98% of the ^{14}C applied to the plates. The ultraviolet and infrared spectra were identical with authentic MBC. The specific activities for MBC-2-^{14}C and MBC-*methyl*-^{14}C were 0.41 and 0.28 μCi/mg, respectively.

Production of $^{14}CO_2$ from MBC-2-^{14}C Treated Soil. The apparatus described by Bartha and Pramer (1965) was used. This consisted, in part, of a 250-ml flask with a large connecting side arm, both sealed separately with rubber stoppers. Each flask was given 5.0 mg of MBC-^{14}C in 50 g of air-dried soil at the desired field capacity in moisture. In the side arm was placed 10 ml of 0.5 *M* NaOH. Incubation was at 20°. Every 6–7 days the alkali was removed for $^{14}CO_2$ analysis. The $^{14}CO_2$ was precipitated from the alkaline solution by addition of an equal volume of 10% $BaCl_2$. The $Ba^{14}CO_3$ was collected by filtration, washed with water and ethanol, dried, weighed, and assayed for ^{14}C by the method of Nathan *et al.* (1958). Counting efficiency was determined with $Ba^{14}CO_3$ of known specific activity.

Recovery of ^{14}C from Soil Treated with MBC-2-^{14}C. Soil (25 g) in 125-ml flasks was treated with a 2.0-ml solution of MBC-2-^{14}C hydrochloride in acetone (1.25 mg/ml or 125 μg/ml). After evaporation of the solvent the soil was mixed 10 min and water was added to the desired field capacity. The samples were incubated at 20° and water was added at weekly intervals to replace evaporated moisture. Samples were extracted by mixing the treated soil with 20 g of Na_2SO_4 and refluxed with 125 ml of acetone for 1 hr. The mixture was filtered with suction, the soil washed twice with acetone, and aliquots were removed from the combined filtrate and washings and transferred to counting vials. The acetone was removed from the aliquots with a stream of air and the residue assayed for ^{14}C by scintillation counting.

The remainder of the acetone extract was concentrated to a small volume by evaporation under reduced pressure and then streaked on silica gel G thin-layer plates. The plates were developed with chloroform–acetone (6:1) or chloroform–methanol–acetone (2:1:1). Carbon-14 on the plates was detected by cutting the chromatograms into cross-sectional strips and assaying individual strips by scintillation counting.

Determination of Radioactivity. The scintillation counting solution used was 5 g of PPO (2,5-diphenyloxazole) and 0.5 g of dimethyl-POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene], in 500 ml of toluene and 500 ml of absolute ethanol. Counting efficiency was determined by the channels ratio method (Bush, 1963).

RESULTS AND DISCUSSION

The lifetime of TM in moist soil was relatively short. In the Towner sand and Fargo clay soils, 80–90% of the applied TM had disappeared from the soil 5 days after application (Figures 1A and 1B). No significant difference was observed in the rate of TM disappearance in soils treated with 10 or 100 ppm of TM. The moisture content of the soil influenced the rate of TM disappearance. In air-dried soil with no added moisture, 10–20% of the applied TM could be detected 30 days after application (Figure 1C).

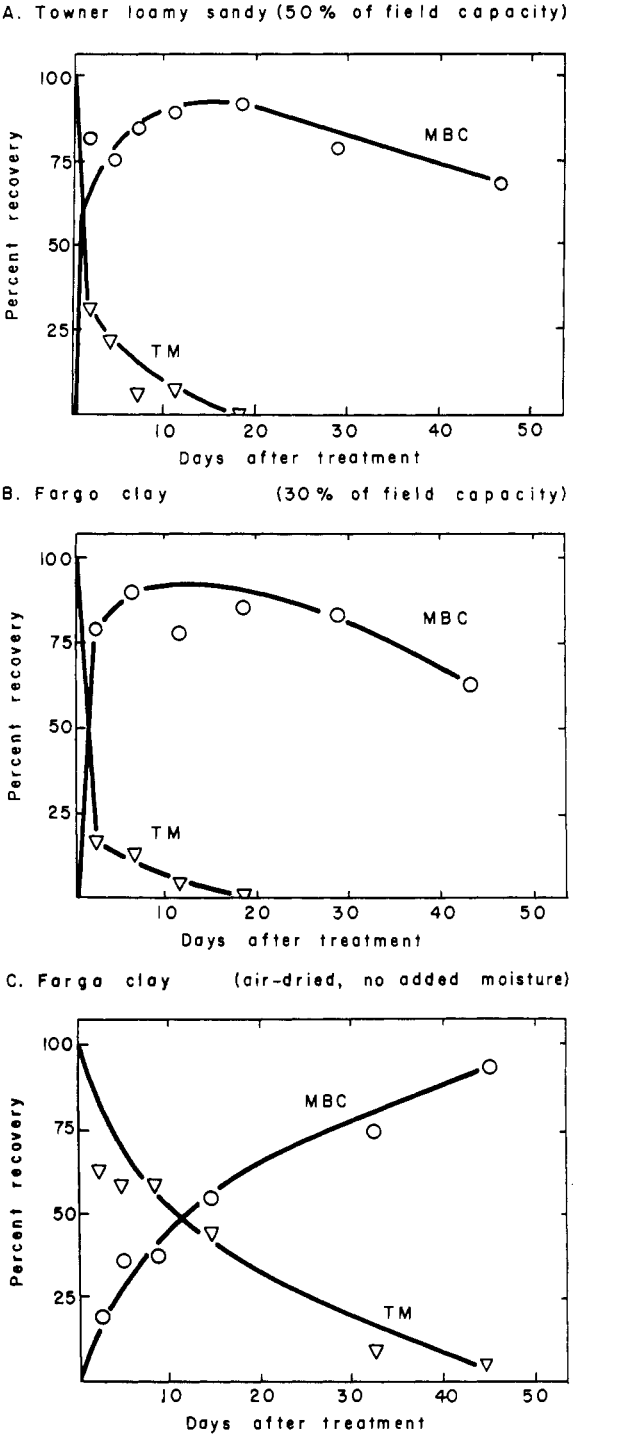


Figure 1. Per cent recovery of TM and MBC from soil after treatment with 10 ppm of TM. Incubation at 20°. Moisture content of soil indicated as per cent of field capacity in A and B. Each point represents the mean of four replicates.

The principal metabolite or degradation product of TM found in the soils was MBC. Figure 1 shows a rapid increase of MBC in soil, proportional with TM loss. Studies on TM metabolism in mice and bean plants have shown

Table I. Relative Rates of Thiophanate-methyl Conversion to MBC in Barnes Loam after Steam Treatment and Acidification of the Soil[a]

Soil condition	Rel rate of conversion
Barnes loam (pH 7.4)	1.00 ± 0.15
Barnes loam (pH 7.4, steam treated)	0.59 ± 0.13
Barnes loam (pH 5.6)	0.21 ± 0.07

[a] Values were relative to Barnes loam at its natural pH of 7.4 and not treated with steam. The data represent means and deviations from the mean.

TM is converted to MBC in tissues of these organisms (Noguchi, 1971). The conversion also occurs slowly in tap water and on silica gel plates (Selling *et al.*, 1970), and rapidly in dilute alkali (Noguchi, 1971).

MBC has fungicidal properties (Clemons and Sisler, 1969) and is a derivative of the fungicide methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl). MBC is a metabolite of benomyl in rats (Gardiner *et al.*, 1968), plants (Peterson and Edgington, 1969, 1970, 1971; Siegel and Zalbia, 1972; Sims *et al.*, 1969), and soil (Kirkland *et al.*, 1973). The data in Figure 1 suggest the fungicidal properties of TM in soil may also be due, in part, to the presence of MBC formed from TM.

The long-term fungicidal properties of benomyl in soil may also be due, in part, to its conversion to MBC. Hine *et al.* (1969) reported that incorporation of benomyl into nonsterile soil at 10 or 100 ppm prevented growth of *Phymatotrichum omnivorum* for up to 16 weeks. In view of the stability of MBC in soil observed in this study, the length of the *P. omnivorum* inhibition may have been due in part to the presence of MBC formed from benomyl.

Table I shows the results of an experiment on TM cyclization in a normally alkaline soil which was acidified. The relative rate of TM conversion to MBC was greater at the alkaline pH. The results suggest that TM would persist longer in acid soils than in similar soils of alkaline pH.

In steam-treated soil MBC was formed from TM at a slower rate than in soil not steam treated (Table I). Although no significant change in pH was observed after the soil was steam treated, changes in the physical and chemical properties of the soil may have had an effect to an unknown degree. Assuming any such effects were minimal, the data suggest that the microbial population of soil, in part, effects the conversion of TM to MBC.

The cumulative production of $^{14}CO_2$ over a 51-day period from soils treated with MBC-2-^{14}C was less than 1% of the applied ^{14}C, while under the same conditions, 16% of the ^{14}C in MBC-*methyl*-^{14}C was recovered as $^{14}CO_2$ (Figure 2). The difference may be due to the hydrolysis of the carbamate moiety of MBC yielding 2-aminobenzimidazole (AB). While no large accumulation of acetone-extractable compounds other than MBC was observed, a small amount of the extracted ^{14}C did not chromatograph as MBC (Figure 3). AB has been found in soil treated with benomyl-2-^{14}C (Kirkland *et al.*, 1973). No attempt was made in this study to identify AB in soil extracts. The slow rate of radioactive carbon dioxide production from MBC-2-^{14}C suggests stability of the benzimidazole ring of MBC to microbial oxidation.

The large amount of MBC used (100 ppm) in the study on $^{14}CO_2$ formation from ^{14}C-labeled MBC (Figure 2) may have affected soil microbial activity. This, in turn,

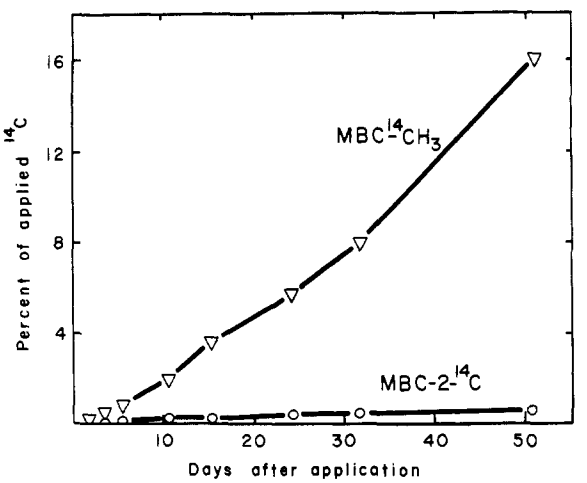


Figure 2. Cumulative per cent recovery of $^{14}CO_2$ from Barnes loam treated with 100 ppm of MBC-2-^{14}C and MBC-*methyl*-^{14}C; moisture content at 50% of field capacity.

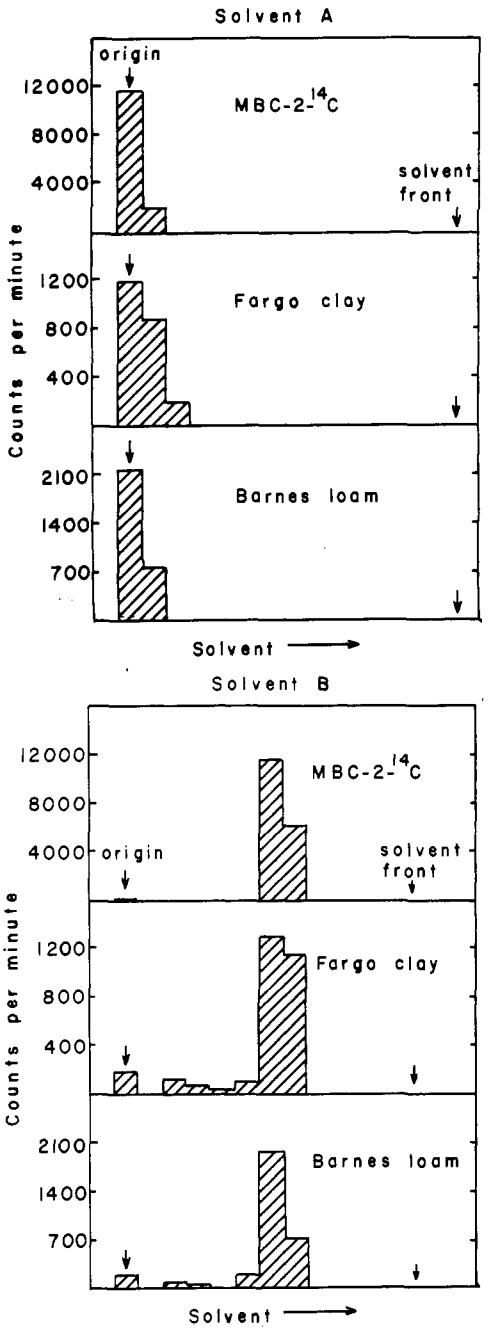


Figure 3. Distribution of ^{14}C on chromatograms of MBC-2-^{14}C and acetone extracts of soil incubated 43 days with MBC-2-^{14}C. Chromatograms were silica gel G thin-layer plates developed with (A) chloroform–acetone (6:1) and (B) chloroform–methanol–acetone (2:1:1).

Table II. Per Cent Recovery of ^{14}C Extracted from Soils Treated with MBC-2-^{14}C[a]

Days after treatment	Fargo clay, μg of MBC-2-^{14}C/g of soil		Barnes loam, μg of MBC-^{14}C/g of soil	
	10	100	10	100
0[b]	79	85	82	91
20	60	72	61	81
43	55	64	53	78

[a] Data represent means from duplicate samples. [b] Extracted immediately after application of MBC-^{14}C.

may have had an effect on the rate of MBC-^{14}C degradation. However, we have found that concentrations of MBC up to 100 ppm did not significantly change the rate of nitrification and carbon dioxide formation in soil (Lacy *et al.*, 1974).

A portion of the ^{14}C applied to soil as MBC-2-^{14}C was not recovered and is assumed to have been retained in the soil in a nonextractable form. The amount not recovered increased with time (Table II) and further extraction with chloroform did not release significant additional amounts of ^{14}C.

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Purification and Some Properties of Miraculin, a Glycoprotein from *Synsepalum dulcificum* Which Provokes Sweetness and Blocks Sourness

Eugene L. Giroux[1] and Robert I. Henkin*[2]

Miraculin, a taste-affecting glycoprotein from miracle fruit, *Synsepalum dulcificum*, has been purified by ion exchange column chromatography. Purified miraculin contains 6.3% carbohydrate and 14.4% nitrogen and has a mol wt of ~45,000. When denatured and reduced it is cleaved into two fragments, of approximately 28,000 and 17,000 mol wt. The amino acid and carbohydrate compositions of miraculin have been investigated. Twenty micrograms of chromatographically purified miraculin produces a marked increase in sweetness of lemon and concomitantly a marked diminution of sourness.

Miraculin is the name given a taste-affecting glycoprotein found in miracle fruit, *Synsepalum dulcificum*. In the (human) oral environment miraculin causes sour tastes to be appreciated as sweet tastes. Two schemes for preparing the active principle from berries of miracle fruit have been reported (Brouwer *et al.*, 1968; Kurihara and Beidler, 1968). The purification procedure we wish to report results in a higher yield of miraculin than noted previously and the final preparation is free of contaminating polyphenols and proteolytic activity. In addition some chemical and physical properties of the purified miraculin are reported.

Section on Neuroendocrinology, Hypertension-Endocrine Branch, National Heart and Lung Institute, Bethesda, Maryland 20014.

[1] Present address: Centre de Recherche Merrell International, 67000 Strasbourg, France.

[2] Present address: National Institutes of Health, Bethesda, Md. 20014.

MATERIALS AND METHODS

Berries of *Synsepalum dulcificum* were obtained from plants cultivated in Florida. The fruits were frozen and shipped by air to our laboratory in Dry Ice. There, berries were stored for periods of as long as 2 years at −40° while the extractability of miraculin apparently remained unaffected.

Insoluble polyvinylpyrrolidone (PVP), "Miracloth," Bio-Gel CM-30, and Bio-Rad ion-exchange resins were obtained from Calbiochem; thin-layer plates of cellulose were obtained from E-M Laboratories; QAE-Sephadex A-50 and other Sephadex materials were obtained from