near detection limits. Agreement between the two methods is good. When the level of *N*-nitrosamine in a 200 g sample of fat is below about 5 μg/kg, the colorimetric method may give a false negative result. The limit of detection of the colorimetric method is therefore in the order of 5 μg/kg. The GC–MS results on sample 2 are low, probably due to a poor recovery of distillate.

In Table III, the results of a comparison of the colorimetric and thin-layer densitometric (TLD) methods are given. In these experiments half the final eluate was used for measurement of liberated nitrite and half for the measurement of amines. On the basis of these 19 samples, a correlation coefficient of 0.99 was calculated for the two methods. Table IV compares the results obtained by the GC–MS technique with those obtained with the currently described method using the colorimetric and TLD techniques.

The limited number of samples analyzed does not permit a statistical evaluation of these results but, when taken in conjunction with Tables II and III, results show that the combined methods described form a valid alternative to the GC–MS method for measuring volatile *N*-nitrosamines in cooked bacon fat.

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Behavior and Fate of [^{14}C]Maleic Hydrazide in Tobacco Plants

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The behavior and fate of [4,5-^{14}C]- and [3,6-^{14}C]maleic hydrazide (MH) have been studied in selected varieties of flue-cured and burley tobacco. Foliar-absorbed [^{14}C]MH was translocated rapidly to actively growing tissues in a "source-to-sink" pattern. Twenty-eight days after treatment, 30–40% of the absorbed [^{14}C]MH was translocated to the roots and released into the nutrient solution, 12–22% remained in the plant, 14–18% was extracted as methanol-soluble metabolites, and 25–35% remained in the roots and other tissues as a methanol-insoluble residue. Only 2% of the absorbed ^{14}C was evolved as $^{14}CO_2$. Degradation of the MH heterocyclic ring structure was not a significant metabolic pathway. The major methanol-soluble metabolite in foliar tissues was isolated and identified as the β-D-glucoside of MH. Unchanged MH was isolated and identified as a hydrolysis product following acid and base treatment of isolated methanol-insoluble residues. The distribution of ^{14}C in methanol-soluble and methanol-insoluble residue fractions from freshly harvested young tobacco plants was not altered greatly when the tobacco was flue-cured or air-cured under laboratory conditions.

Maleic hydrazide (1,2-dihydro-3,6-pyridazinedione) is used extensively as a systemic plant growth regulator for the control of axillary bud and sucker growth in tobacco (Tso, 1972). It is applied to the upper portion of the tobacco plant within 24 h after excising the inflorescence (topping). Under physiological conditions, maleic hydrazide (MH) exists as the phenolic tautomer, 6-hydroxy-3-(2*H*)-pyridazinone (Miller and White, 1956) and is apparently quite stable (Smith et al., 1959; Noodén, 1970).

Numerous reports of MH residues in tobacco (Haeberer et al., 1974; Lane, 1965; Hoffman et al., 1962; Liu and Hoffmann, 1973; Davis et al., 1974; Cheng and Steffens, 1976), limited information about MH metabolism in tobacco, and recent concern about the possible health effects of MH residues in tobacco products (Liu and Hoffmann, 1973) prompted the present investigation on the behavior and fate of MH in the tobacco plant. Specific objectives were: (1) to determine quantitatively the behavior and fate

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Table I. Thin-Layer and Gas-Liquid Chromatography of MH, a Major Methanol-Soluble Metabolite and Metabolite Derivatives

Compound	Solvent system ($R_f \times 100$)[a]					GLC retention time,[b] min
	1	2	3	4	5	
MH	14	73	59 (77)[c]			
Major metabolite	0	23	27 (51)[c]			
Acetylated metabolite				54		14
Silylated metabolite					54, 75 [88][d]	11 [13][d]

[a] 1 = ethyl acetate-2-propanol-water (18:2:1); 2 = chloroform-methanol-water (65:25:4) developed 4×; 3 = butanol-acetic acid-water (120:30:50); 4 = diethyl ether-methanol (9:1); 5 = methyl acetate-2-propanol (10:1) developed 2×. All developmental tanks lined with filter paper. [b] Temperature programmed at 4 °C/min (200 to 250 °C). [c] Developed two times. [d] Minor peaks.

of foliar-absorbed MH; (2) to isolate and identify major MH metabolites; (3) to compare the behavior and fate of MH in major flue-cured and burley tobacco varieties used currently in U.S. agriculture; (4) to determine if the tobacco curing process altered the distribution or types of MH residues present in harvested foliar tissues; and (5) to determine the possible nature and extent of "terminal" MH residues that might remain in plant material and be incorporated in the soil.

MATERIALS AND METHODS

Radioactive Chemicals. The [3,6-^{14}C]MH (0.3 mCi/mM) was provided by Uniroyal Inc., Naugatuck, Conn. The [4,5-^{14}C]MH (2.8 mCi/mM) was purchased from Amersham/Searle Corporation, Arlington Heights, Ill. In comparative metabolism studies, [4,5-^{14}C]MH was diluted to the same specific activity as the [3,6-^{14}C]MH with reagent grade MH. All plant treatments were made with the potassium salt of [^{14}C]MH.

Plant Materials. Two flue-cured tobacco varieties, Coker 319 and Speight G-28, a burley variety, Kentucky 10, and a burley hybrid, MS Burley 21 × Kentucky 10, were seeded in vermiculite and grown in the greenhouse. The seeded flats were subirrigated with one-six strength Hoagland's nutrient solution. After approximately 2 months, individual plants were transferred into darkened Mason jars (500 mL) with aerated one-fourth strength Hoagland's nutrient solution and maintained in a growth chamber with a 16-h photoperiod (10–12 klux) at 25 °C and 40% relative humidity. After approximately 2 weeks, five uniform plants of each tobacco variety were treated with [^{14}C]MH. These plants were used in time-course studies on the behavior and fate of foliar-applied [^{14}C]MH, $^{14}CO_2$ evolution, and the effects of tobacco curing on MH residues.

Additional Speight G-28 plants were used for the determination of evolved $^{14}CO_2$, the partial characterization of methanol-insoluble ^{14}C residues, and the isolation and identification of a major methanol-soluble ^{14}C metabolite.

Plant Treatment with [^{14}C]MH. Tobacco plants were treated with either [4,5-^{14}C]- or [3,6-^{14}C]MH. All plants used for time-course and $^{14}CO_2$ evolution studies and some plants used for ^{14}C metabolite isolation were treated by a leaf-flap absorption technique. A leaf-flap (10 by 20 mm) was cut on either side of the mid-rib at the tip of the oldest leaf on each plant. Freshly cut leaf-flaps were immersed in 13 by 15 mm beakers containing 150 μL of 1×10^{-3} M [^{14}C]MH. Initial uptake of the treating solution was monitored and leaf-flaps were kept moist by periodic additions of 0.1 to 0.2 mL of distilled water. Treated leaves were maintained in continuous light for 1.25 days. Other leaves were shaded. After 1.25 days, leaf-flaps were removed from the beakers and rinsed with distilled water. The ^{14}C in the distilled water rinse and the residual ^{14}C in each beaker was quantitated by liquid scintillation counting. The foliar-absorbed [^{14}C]MH was assumed to be the difference between the total ^{14}C present in the initial treating solution and the ^{14}C recovered in the distilled water rinse and treatment beaker.

Time-Course Studies. Four plants of each tobacco variety were treated with [4,5-^{14}C]MH by the leaf-flap method and harvested after 1.25, 7, 14, and 25 days. An additional plant of each variety was used for time course $^{14}CO_2$ evolution studies and harvested after 28 days for tobacco curing studies.

Leaves, apical tissues and stems, and roots of harvested plants were extracted separately with methanol. Unchanged [^{14}C]MH, methanol-soluble ^{14}C metabolites and methanol-insoluble ^{14}C residues were determined in each tissue. Excised tissues were homogenized in ten volumes of methanol with an Omnimixer and vacuum filtered through a 10 μ Millipore Teflon filter. Filtered residues were washed extensively with additional volumes of methanol and air-dried for dry weight determinations and ^{14}C combustion analysis (Oliveria et al., 1962).

Aliquots of methanol extracts were chromatographed directly by TLC to determine the amounts of unchanged [^{14}C]MH and methanol-soluble ^{14}C metabolites in treated tissues. Chromatographic separation was achieved on 250 μ silica gel HF plates with solvent systems 1 and/or 3 (Table I). Separated MH and methanol-soluble metabolites were removed from developed chromatograms and ^{14}C quantitated by liquid scintillation counting. Chromatograms of methanol-soluble ^{14}C metabolites were monitored with a radiochromatogram scanner or compared directly by autoradiography. All chromatograms showed one major methanol-soluble ^{14}C metabolite. Nutrient solutions from each plant were changed every week, counted for ^{14}C, and examined by TLC for ^{14}C metabolites.

Laboratory Simulated Tobacco Curing. Speight G-28 and Coker 319 plants treated with [4,5-^{14}C]MH by the leaf-flap method and used in time-course $^{14}CO_2$ evolution studies were harvested after 4 weeks and subjected to a laboratory simulated flu-curing process (Tso, 1972). Roots were excised and extracted with methanol. Leaves and stems were placed in a forced air oven with the following temperature program: 0 to 8 h (31 °C), 8 to 24 h (36 °C), 24 to 48 h (40 °C), 48 to 60 h (44 °C), 60 to 72 h (52 °C), 72 to 96 h (55 °C), and 96 to 120 h (70 °C). Cured shoot tissues were extracted with 80% methanol to determine the distribution and nature of ^{14}C residues.

Kentucky 10 and MS Burley 21 × Kentucky 10 plants used in the $^{14}CO_2$ evolution studies were also harvested 4 weeks after leaf-flap treatments with [4,5-^{14}C]MH. At harvest, excised roots from the burley variety plants were extracted with methanol. Leaves and stems were air-cured for 1 month at 25 °C and 40% relative humidity and extracted with 80% methanol.

Determination of $^{14}CO_2$. At specified time intervals, selected tobacco plants treated by the leaf-flap method were placed in glass CO_2 collection chambers for 48-h periods. The $^{14}CO_2$ released by [^{14}C]MH-treated plants

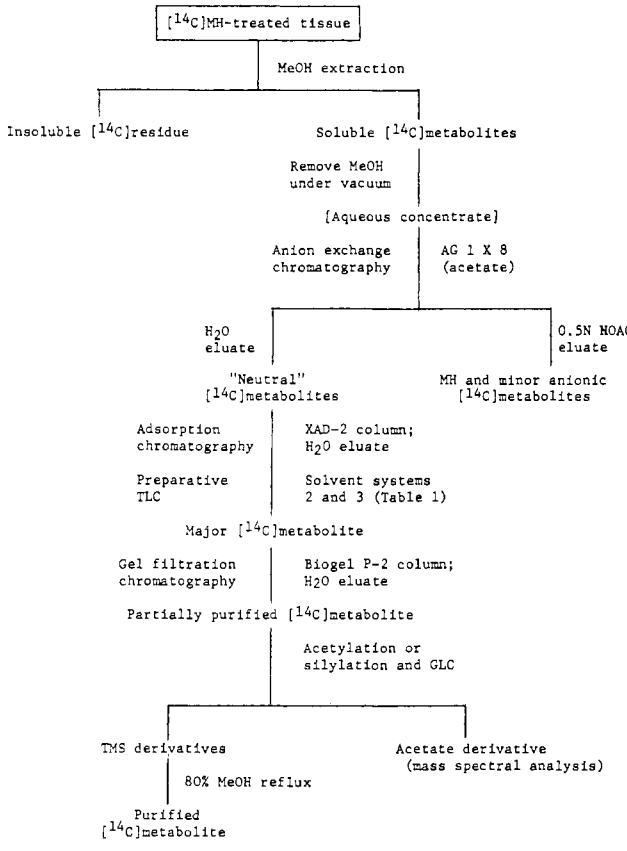

Figure 1. Extraction, separation and purification of methanol-soluble ^{14}C metabolites.

was trapped in either methyl Cellosolve–monoethanolamine (7:1 v/v) or in 1 N NaOH and quantitated by liquid scintillation counting. Initial identification of NaOH-trapped $^{14}CO_2$ was confirmed by precipitation as $Ba^{14}CO_3$.

Isolation and Purification of Major Methanol-Soluble ^{14}C Metabolite. The major methanol-soluble ^{14}C metabolite of MH was isolated and identified from excised shoots of 2.5- to 3-month-old Speight G-28 plants. Plants were grown in the greenhouse in 10-cm plastic pots filled with vermiculite and moistened with one-fourth strength Hoagland's nutrient solution. Shoots were excised above the vermiculite level and the cut stems were immersed in 8 mL of 2×10^{-2} M [^{14}C]MH. Uptake of the [^{14}C]MH was completed in 4 to 5 h, and the cut stems were maintained in distilled water for up to 4 days before harvest and methanol extraction of the tissues.

Procedures used for the isolation and purification of a major methanol-soluble ^{14}C metabolite are shown in Figure 1.

Methanol extracts were concentrated to an aqueous fraction (5–10 mL) and placed on a 1 × 25 cm column of anion exchange resin (AG 1 × 8, 200–400 mesh) in the acetate form. The unknown major metabolite was separated from unreacted MH and other minor anionic metabolites by stepwise elution with 60 mL of distilled water followed by 100 mL of acetic acid. The "neutral" metabolites in the distilled water eluate were placed on a 1.5 × 15 cm XAD-12 column and eluted with 60 mL of distilled water. Many naturally occurring plant impurities were removed by adsorption on the XAD-2 column. The distilled water effluent from the XAD-2 column was lyophilized, dissolved in a small volume of methanol, and purified further by preparative TLC (500 μ silica gel HF) in solvent system 3 developed two times and in solvent system 2 developed four times (Table I). Zones corresponding to the major ^{14}C metabolite were removed from chromatograms and eluted with methanol. After methanol was removed under vacuum, the ^{14}C metabolite was dissolved in 1 to 2 mL of distilled water, placed on a 1 by 90 cm Bio-Gel P-2 column (100–200 mesh) and eluted with distilled water (1 mL/min). The major ^{14}C metabolite was detected with a 254-nm UV monitor and eluted as a single, sharp symmetrical peak (80–105 mL).

Peak fractions were pooled, lyophilized, acetylated, and silylated, and purified as acetate or trimethylsilyl (Me_3Si) derivatives by GLC on a 1.8 m by 0.6 cm glass column packed with 3% OV-1 on 60 to 80 mesh Gas-Chrom Q. Gas–liquid chromatography conditions were as follows: N_2 carrier gas, 60 mL/min; column inlet temperature, 250 °C; column temperature program, 4 °C/min from 200 to 250 °C; flame ionization detector temperature, 275 °C; and column effluent split ratio, 10:1. The purified acetate derivative was trapped from the column effluent in capillary tubes for mass spectral analysis. Purified ^{14}C metabolite was recovered from GLC-trapped Me_3Si derivatives by refluxing for 1 h with 80% MeOH.

Derivatization of Major Methanol-Soluble ^{14}C Metabolite. After gel filtration (Figure 1), 0.5 to 1.0 mg of the partially purified metabolite was acetylated by reaction with 50 μL of dry pyridine and 50 μL of acetic anhydride at 35 °C for 20 h. After removal of excess reactants with a stream of N_2, the acetylated derivative was dissolved in 100 μL of methanol and aliquots (10 to 20 μL) were used for TLC and GLC analysis. Mass spectra of the acetylated ^{14}C metabolite were obtained after GLC separation from remaining plant impurities. Me_3Si derivatives of the partially purified metabolite were obtained by reacting the metabolite (0.5 to 1.0 mg) with 50 μL of Tri-Sil Z reagent (Pierce Chemical Company, Rockford, Ill. at 50 °C for 16 h. Aliquots (5 to 10 μL) of the silylation reaction mixture were used for GLC separation and isolation of purified metabolite.

^{14}C Metabolite Hydrolysis and Glucose Determination. Purified ^{14}C metabolite was hydrolyzed quantitatively with either β-glucosidase (Sigma Chemical Company, St. Louis, Mo.) or HCl. Acid hydrolysis was carried out at 100 °C for 2 h with 1 N HCl. Enzyme hydrolysis was complete after reaction with 0.5 mg of β-glucosidase for 20 h in 0.05 M acetate buffer at pH 4.5. Glucose was identified qualitatively by TLC (Gal, 1968) and determined quantitatively as a reducing sugar by colorimetric analysis (Park and Johnson, 1949) and by specific enzymatic oxidation with glucose oxidase (Frear and Swanson, 1972) [Glucostat (ultra micro), Worthington Biochemical Corp., Freehold, N.J.].

Partial Characterization of Methanol-Insoluble ^{14}C Residues. Methanol-insoluble ^{14}C residues were isolated from root-treated tobacco plants. The roots of 2-month-old Speight G-28 plants were immersed in 20 mL of 1.16×10^{-5} M [^{14}C]MH. Initial uptake of the treating solution ($\simeq$6 h) was followed by a 70-mL distilled water chase. Additional one-fourth strength Hoagland's nutrient solution was added as needed for 7 days before root and shoot tissues were harvested and extracted with methanol.

Methanol-insoluble ^{14}C residues from the roots of either [3,6-^{14}C]- or [4,5-^{14}C]MH-treated Speight G-28 plants were partially characterized by acid and base hydrolysis (Figure 2). The hydrolysis of methanol-insoluble ^{14}C residues from excised shoot tissues gave similar results. Methanol-soluble hydrolysis products were separated by TLC in solvent systems 2 and 3 (Table I). The [^{14}C]MH was tentatively identified as a methanol-soluble hydrolysis product by TLC. Identification was confirmed by GLC–MS analysis of the $(Me_3Si)_2$ derivative (Haeberer et al., 1974) after TLC

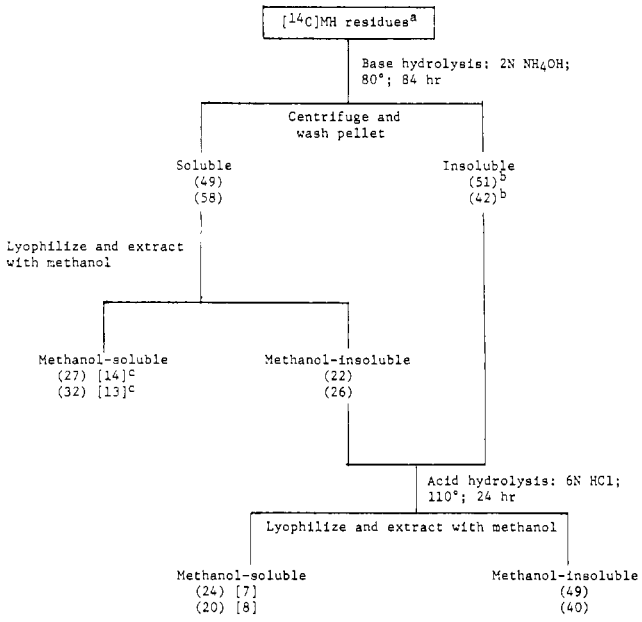

Figure 2. Hydrolysis of methanol-insoluble ^{14}C residues isolated from [^{14}C]MH-treated root tissues. [a]Eight-week-old Speight G-28 seedlings pulse-treated via the roots with 4×10^6 dpm (0.3 mCi/mM) of either [3,6-^{14}C]- or [4,5-^{14}C]MH and harvested 7 days after treatment. Root tissues extracted with methanol (Omnimixer), washed with methanol and acetone, and air-dried. Fifty-milligram samples (dry wt) used for hydrolysis treatments contained 1.2×10^5 dpm and 1.1×10^5 dpm of [3,6-^{14}C]- and [4,5-^{14}C]MH, respectively. [b]Figures in parentheses indicate percent ^{14}C recovered in each fraction for [3,6-^{14}C]- (upper) and [4,5-^{14}C]- (lower) MH treatments, respectively. [c]Figures in brackets indicate percent ^{14}C identified as MH in methanol-soluble fractions from [3,6-^{14}C]- (upper) and [4,5-^{14}C]- (lower) MH treatments, respectively.

separation, absorption on a DE 52 cellulose anion-exchange column (0.5 by 5 cm), and elution with 0.1 N HCl.

General Methods. Mass spectra were obtained either by direct GLC–MS analysis or by solid sample insertion of peak fractions trapped from GLC effluents. Thin-layer chromatograms were developed in several solvent systems. Typical R_f values for MH and the major methanol-soluble metabolite are shown (Table I). The R_f values and GLC retention times are given also for the acetate and Me_3Si derivatives of the isolated ^{14}C metabolite.

RESULTS AND DISCUSSION

Leaf Absorption of [^{14}C]MH. Surface applications of [^{14}C]MH with an air brush or as 10 to 50 μL droplets from a microsyringe resulted in limited and highly variable absorption by treated leaves or axial tissues. The use of different salt forms, surfactants, and treatment under different humidity conditions failed to improve the uniformity or increase the amount of [^{14}C]MH absorbed. The results of these preliminary studies showed that surface absorption was limited and that other treatment methods were necessary to introduce significant and measurable quantities of [^{14}C]MH into foliar tissues. The leaf-flap technique was the most effective means for treatment. Although this method still resulted in variable uptake (Table II), amounts of [^{14}C]MH adequate for quantitation were rapidly absorbed. With this treatment method, 6 to 15 μg (34 to 75%) of the applied [4,5-^{14}C]MH was absorbed by treated leaves in 1.25 days.

Time-Course Studies. Growth of all the [4,5-^{14}C]-MH-treated plants used in the time-course studies was indicated by increases in the dry weights of methanol-extracted plant residues (Table II). Growth of the plants

Table II. [4,5-^{14}C]MH Absorption and Growth of Foliar-Treated Tobacco Plants

Tobacco variety[a]	Posttreatment harvest time, days	MH absorbed, μg	Methanol-insoluble residue dry wt, g
Coker 319	1.25	6.1	0.43
	7	11.8	1.07
	14	9.0	1.77
	28	6.8	4.78
	28[b]	13.5	2.81
Speight G-28	1.25	7.5	0.24
	7	7.7	0.62
	14	13.3	1.94
	28	8.3	4.25
	28[b]	11.1	2.33
Kentucky 10	1	8.4	0.25
	7	12.7	1.17
	14	12.6	2.82
	28	13.5	5.56
	28[b]	10.5	3.26
Kentucky 10 × MS burley 21	1	9.1	0.28
	7	10.9	0.75
	14	10.8	2.11
	28	10.4	7.55
	28[b]	11.5	3.59

[a] Each plant was treated for 1.25 days via a leaf-flap with 1×10^6 dpm (17.9 μg) of [4,5-^{14}C]MH. [b] These plants were used for both $^{14}CO_2$ evolution and tobacco curing studies.

used for $^{14}CO_2$ evolution and tobacco curing studies was reduced, probably because of the stress conditions imposed on these plants during four 48-h $^{14}CO_2$ trapping intervals in glass chambers.

Quantitative data on the time-course distribution of ^{14}C in both the methanol-soluble and methanol-insoluble fractions from leaves, apical tissues and stems, and roots supported earlier reports (Smith et al., 1959; Crafts and Yamaguchi, 1958; Crafts, 1959; Crafts and Yamaguchi, 1960; Crafts and Crisp, 1971) that MH was translocated symplastically or from "source-to-sink" in treated plants. A typical time-course distribution pattern of foliar absorbed ^{14}C in the different tissues of Kentucky 10 plants is illustrated in Table III. Foliar-absorbed ^{14}C was translocated rapidly into younger leaves, apical tissues, and roots. A substantial redistribution of the "mobile" methanol-soluble ^{14}C fraction is supported by the presence of ^{14}C, primarily methanol-soluble ^{14}C, in newly formed foliar tissues and by decreased ratios of methanol soluble ^{14}C to methanol-insoluble ^{14}C in older tissues and plants.

A summary of the total ^{14}C distribution in [4,5-^{14}C]-MH-treated plants and nutrient solutions is shown in Table IV. Total recoveries of leaf-flap absorbed ^{14}C ranged from 81 to 110% over the 28-day treatment period. Lower ^{14}C recoveries at the 28-day posttreatment harvest probably result from quantitation errors due to increased plant mass. Large amounts of ^{14}C were found in the nutrient solutions within 7 days following leaf-flap treatment. The "leakage" of MH from roots into nutrient solutions has been reported (Ashton and Crafts, 1973). After 28 days, 30 to 40% of the absorbed ^{14}C was recovered in the nutrient solutions of tobacco plants treated by the leaf-flap method. Qualitative TLC analysis indicated that almost all of the ^{14}C in the nutrient solution (>95%) was unchanged [^{14}C]MH. These data suggest that substantial quantities (>30%) of foliar absorbed MH may be translocated to the roots and released into the soil environment under field conditions. One of the plants in the time-course study, a Coker 319 plant (Table IV), had a damaged

Table III. Time-Course Distribution of Foliar-Absorbed ^{14}C in Tissues of Kentucky 10 Tobacco Plants[a]

	Posttreatment harvest time, days							
	1.25		7		14		28	
Tissue	MeOH soluble, %	MeOH insoluble, %	MeOH soluble, %	MeOH insoluble, %	MeOH soluble, %	MeOH insoluble, %	MeOH soluble, %	MeOH insoluble, %
Leaf no. 1	0.2		0.5[b]	0.1[b]	} 6.6[b]	} 10.7[b]	} 1.6[b]	} 6.9[b]
Leaf no. 2 (treated)	83.6		5.5	4.8				
Leaf no. 3	0.3		1.0	0.2	} 3.6	} 2.5		
Leaf no. 4			10.6	1.7			} 6.5	} 4.3
Leaf no. 5			29.2	1.7	} 20.7	} 1.5		
Leaf no. 6							} 6.6	} 3.3
Leaf no. 7								
Leaf no. 8							} 10.2	} 2.7
Leaf no. 9								
Shoot tip and/or stem	7.7		23.5	1.4	31.4	2.5	11.6 7.4	1.1 8.3
Shoots (total)	91.8	2.0	70.3	9.9	62.3	17.2	43.9	26.6
Roots	5.8	0.4	8.0	11.9	5.6	15.1	6.0	23.5

[a] Each plant was treated for 1.25 days via a leaf-flap with 1×10^6 dpm (17.9 μg) of [4,5-^{14}C]MH. [b] Senescent leaves.

Table IV. ^{14}C Distribution and Recovery in Tobacco Plants and Nutrient Solutions after Foliar Absorption of [4,5-^{14}C]MH

		Distribution of recovered ^{14}C, %				
		Plant tissues				
		Methanol soluble				
Variety	Posttreatment harvest time, days	MH	Metabolites	Methanol insoluble	Nutrient solution	Total ^{14}C recovery, %
Coker 319	1.25	91.0	5.7	3.3	0	102
	7[a]	41.3	8.2	20.7	29.7	90
	14	42.9	14.2	23.4	19.4	90
	28	17.7	18.2	30.8	33.4	83
	28[b]	17.6	18.0	34.5	29.8	92
Speight G-28	1.25	87.9	7.7	3.2	1.2	102
	7	38.9	13.9	18.4	28.9	92
	14	30.6	13.5	19.4	36.4	88
	28	16.9	14.7	26.8	41.6	81
	28[b]	16.9	15.1	25.3	42.7	89
Kentucky 10	1.25	89.0	7.4	2.3	1.3	110
	7	53.1	10.4	17.8	18.7	96
	14	42.5	11.1	25.4	21.1	86
	28	17.5	13.6	31.2	37.8	85
	28[b]	15.6	16.6	32.9	34.9	84
Kentucky 10 × MS burley 21	1.25	90.1	6.7	3.0	0.3	101
	7	53.1	10.6	14.5	21.8	94
	14	39.4	12.9	20.7	27.0	94
	28	22.3	16.7	32.7	28.2	92
	28[b]	12.2	15.5	34.3	38.0	90

[a] This plant did not have an apical meristem. [b] These plants were used for both $^{14}CO_2$ evolution and tobacco curing studies.

apical meristem. In this plant, 23.5% of the methanol-soluble ^{14}C was present in the roots. Corresponding plants of the other three tobacco varieties contained 8 to 12% of the methanol-soluble ^{14}C in their roots. These results suggested that the absence of a physiologically functional apical "sink" causes a greater translocation of ^{14}C to the roots. A similar situation may occur in the field when plants are topped and then sprayed with MH.

Differences in the behavior and fate of [4,5-^{14}C]MH in flue-cured and burley tobacco varieties were minimal. Overall distribution patterns of ^{14}C in all four tobacco varieties were similar. Likewise, ^{14}C distribution patterns in the methanol-soluble and methanol-insoluble fractions from both freshly harvested and cured tobacco were similar in young, foliar-treated plants. These results suggest that the ^{14}C residues in cured tobacco are similar to those found in the plant at harvest and that only limited autolysis of conjugated metabolites or insoluble residues occurred during the curing process. A recent report (Cheng and Steffens, 1976) showed that MH residues were similar in green and cured leaf of Maryland tobacco.

The methanol-soluble ^{14}C fractions in the tobacco plants decreased from 96% after 1.25 days to an average of 33% of the recovered ^{14}C 28 days after treatment (Table IV). This amounted to an average of 52% of the ^{14}C found in the plant after 28 days. At the same time, the methanol-insoluble ^{14}C residues increased to an average of 31% of the recovered ^{14}C and represented 48% of the ^{14}C found in the plants.

TLC separation of the methanol-soluble fractions showed several minor, highly polar ^{14}C metabolites, a major polar ^{14}C metabolite, and unchanged [^{14}C]MH. Twenty-eight days after treatment, 12 to 22% of the recovered ^{14}C was present in the plants as unchanged [^{14}C]MH (Table IV). This represented 20 to 31% of the ^{14}C that remained in the plants. These data support the findings of extensive MH residues in tobacco (Haeberer et al., 1974; Lane, 1965; Hoffman et al., 1962; Liu and Hoffman, 1973; Davis et al., 1974; Cheng and Steffens, 1976) and reports that MH is stable in plants (Smith et al., 1959; Noodén,

Table V. $^{14}CO_2$ Evolution from Foliar-Absorbed [4,5-^{14}C]MH

Posttreatment $^{14}CO_2$ trapping intervals, h	Absorbed [4,5-^{14}C]MH released as $^{14}CO_2$, %: Coker 319	Speight G-28	Kentucky 10	MS burley 21 × Kentucky 10
30 to 78	0.023	0.134	0.103	0.108
168 to 216	0.073	0.158	0.173	0.125
336 to 384	0.142	0.077	0.185	0.091
504 to 552	0.186	0.105	0.168	0.165
Av $^{14}CO_2$ released/48-h interval	0.106	0.119	0.157	0.122
Estimated $^{14}CO_2$ released over 28-day treatment period (48 h average × 14)	1.5	1.7	2.2	1.7

1970). Methanol-soluble ^{14}C metabolite formation was rapid and accounted for 14 to 18% of the recovered ^{14}C 28 days after leaf-flap treatment. Twenty-eight days after treatment, the methanol-soluble ^{14}C metabolites accounted for 22 to 27% of the ^{14}C that remained in the plants.

Methanol-insoluble ^{14}C residues were found in all plant tissues. Twenty-eight days after leaf-flap treatment, 25 to 35% of the recovered ^{14}C was present in the methanol-insoluble fraction and accounted for 44 to 55% of the ^{14}C that remained in the plants (Table IV). A significant portion (26 to 47%) of this methanol-insoluble ^{14}C residue was found in the roots. In the field, these residues remain in the soil environment and may be degraded further. Similar ethanol-insoluble MH residues have been reported in corn roots (Noodén, 1970, 1975).

$^{14}CO_2$ Evolution Studies. Time-course studies of $^{14}CO_2$ evolution (Table V) suggested that the ring structure of MH was not degraded to any appreciable extent in the plant. Calculated $^{14}CO_2$ evolution over a 28-day posttreatment period was only 2% of the absorbed ^{14}C. Similar results were obtained with [3,6-^{14}C]MH-treated plants. These data are in conflict with other reports (Isenberg, 1964; Biswas et al., 1967) that suggest significant ring degradation in plants. Apparent differences in the stability of the MH ring structure in plants may result from photochemical degradation of MH on leaf surfaces (Povolotskaya, 1961) and in aqueous solution (Stoessl, 1964) or possibly from microbial degradation (Hoffman et al., 1962; Helweg, 1975; Kaufman and Kalayanova, 1976).

Methanol-Insoluble ^{14}C Residues. Methanol-insoluble root residues were fractionated as shown in Figure 2. A combination of base and acid hydrolysis treatments resulted in the release of 50 to 60% of the ^{14}C as methanol-soluble products. Extraction of the methanol-insoluble ^{14}C residue with water, 9:1 dioxane–water (v/v) or Me_2SO at 50 °C and treatment with cellulase, cellulysin (Calbiochem, LaJolla, Calif.), or hesperidinase (Sigma Chemical Company, St. Louis, Mo.) failed to solubilize a significant portion (<10%) of the ^{14}C. TLC separation of the methanol-soluble acid–base hydrolysis products indicated that 21% of the methanol-insoluble ^{14}C was released as [^{14}C]MH. This was confirmed by mass spectral analysis of the $(Me_3Si)_2$ derivative. Major ion fragments were the same as those reported for the $(Me_3Si)_2$ derivative of MH (Haeberer et al., 1974) and included a molecular ion at m/e 256 and a base peak at m/e 241. Other highly polar methanol-soluble and methanol-insoluble hydrolysis products were not identified. The distribution of ^{14}C in the several hydrolysis fractions was similar for both the [3,6-^{14}C]- and the [4,5-^{14}C]MH pulse treatments. This suggests a common metabolic pathway for both labeled forms of [^{14}C]MH and limited cleavage of the heterocyclic ring structure.

Table VI. Quantitative Analysis of ^{14}C Metabolite Hydrolysis Products

Glucose analysis method	Metabolite,[a] nmol	Glucose, nmol	Ratio (MH/glucose)
Reducing sugar	22.7	23.3	0.97
Glucose oxidase	45.5	48.0	0.95

[a] Calculated as MH equivalents based on ^{14}C determination and the specific activity (0.071 μCi/μmol) of the [4,5-^{14}C]MH used in plant treatment.

Isolation and Identification of the Major MH Metabolite in Tobacco Plants. The formation of a MH glycoside in several plant species has been reported (Noodén, 1970; Towers et al., 1958). In the present study, qualitative TLC analysis of methanol extracts from pulse-treated plants (leaf-flap) or excised shoots showed the presence of one major (>90%), highly polar metabolite. Complete removal of plant impurities required derivatization with Tri Sil Z, GLC purification of the major Me_3Si derivatives and methanolysis to recover the purified metabolite (Figure 1). TLC and GLC analysis of the Tri Sil Z reaction products suggested that silylation was incomplete under the reaction conditions used and/or that Me_3Si derivatives of the MH metabolite were unstable (Table I). A single purified metabolite was obtained, however, after both of the GLC effluent peaks were hydrolyzed with 80% methanol.

The purified metabolite was identified as the β-D-glucoside of MH by qualitative and quantitative analysis of hydrolysis products and by mass spectral analysis of an acetylated derivative. Quantitative hydrolysis of the purified metabolite was achieved by treatment with either HCl or β-glucosidase. Qualitative TLC analysis of the hydrolysis products showed the presence of only glucose and MH. Quantitative analysis of the β-glucosidase hydrolysis products showed an equal molar ratio of MH and glucose (Table VI). The acetylated derivative of the purified metabolite appeared as a single spot or peak when chromatographed by TLC or GLC (Table I). Mass spectra of the GLC-purified acetate derivative failed to show a molecular ion, but did confirm the presence of both the MH and the glucose moieties. Characteristic ion fragments for MH at m/e 112, 82, and 55 and for glucose tetraacetate at m/e 331, 169, 127, and 109 were observed.

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Influence of Nitrogen Fertilization on Potato Discoloration in Relation to Chemical Composition. 1. Lipid, Potassium, and Dry Matter Content

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The effect of nitrogen fertilization on enzymatic darkening of potatoes was examined during a 2-year study. Ammonium nitrate was applied at rates of 100, 150, 200, and 250 lb/acre. Tubers were examined for susceptibility to enzymatic discoloration, lipid, potassium, and dry matter contents. Enzymatic darkening increased significantly as the level of nitrogen increased. A significant negative correlation ($r = -0.95$) was found between the degree of enzymatic discoloration and lipid content. Potassium content decreased and dry matter increased with increasing levels of nitrogen fertilization.

Heavy fertilization of potatoes is routinely done since the crop has high nutrient requirement and high gross value per acre. Although response to nitrogen fertilizers varies according to source of nitrogen, method of application, and the amount applied, increases in yields may be realized with increasing nitrogen application up to certain levels in most potato growing areas. An increase in yield, however, may not be accompanied by an increase in quality of the tuber. Early work by Van der Waal (1929), de Bruyn (1929), and Merkenschlager (1929) showed that the incidence of black spot was increased by applications of large amounts of nitrogen fertilizers. Koblet (1947, 1948) found an increase in black spot of 12 and 24% as the nitrogen rate per acre increased from 27 to 81 lb, respectively. Jacob et al. (1950) reported an increase in black spot as nitrogen increased from 50 to 100 lb/acre. de Bruyn (1929) found that high amounts of nitrogen increased the susceptibility of tubers to stem-end darkening.

The tendency of potatoes to enzymatic discoloration has been related to their lipid content (Mondy et al., 1965). These workers found that two varieties of potatoes, Pontiac and Ontario, which differed widely in their lipid content also differed in their susceptibility to discoloration. The variety most resistant to darkening had the highest lipid content. Mondy and Mueller (1977), studying potato discoloration in relation to anatomy, found that enzymatic darkening was always greater and lipid content lower in the stem than in the bud regions of the tuber. Chippewa potatoes had a greater crude lipid and phospholipid content than Katahdin potatoes and were less susceptible to enzymatic darkening. A decrease in tuber lipid content and increase in susceptibility to enzymatic darkening have been observed following the use of some chemical sprout inhibitors (Mondy and Mueller, 1977).

Potassium fertilization also exerts a significant influence on black spot. Oortwijn Botjes and Verhoeven (1927), Van der Waal (1929), and Verhoeven (1929) were among the first to show that potash reduced the amount of black spot in potatoes. Mulder (1949) observed that potassium-deficient tubers were susceptible to discoloration and that the amount of black spot could be estimated from the severity of potassium deficiency of the leaves. Scudder (1951) reported that the percentage of tubers exhibiting black spot was reduced from 63 to 40% as potassium fertilizer applications were increased from 100 to 400 lb/acre. Kunkel et al. (1965) and Mondy et al. (1967) also observed that the discoloration of potatoes decreased as potash applications were increased. Vertregt (1968) found tubers of different black spot susceptibility also differed in potassium content. Black spot incidence in Bintje and Eigenheimer potatoes was under 20% if potassium content in the tubers was over 580 mequiv/kg of dry matter (DM). Black spot was over 50% at potassium content under 500 mequiv/kg of DM.

Potassium content has been correlated with lipid levels in several types of plant tissues. Experiments with Katahdin potatoes revealed that potassium significantly increased the crude lipid and phospholipid content in both pith and cortex tissues and potassium-fertilized tubers discolored less than control tubers (Mueller, 1976). Fabian (1969) reported a similar phenomenon in sunflower plants, where he observed that phospholipid content in leaves and roots was decreased by potassium deficiency. Potassium

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