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Effects of Application Rates on Maleic Hydrazide Residues in Burley Tobacco

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The fate and stability of maleic hydrazide (MH) applied to Burley Tobacco and soil was examined. Four dosages of MH, from one-tenth to twice the recommended amount, were applied to tobacco at various stages of maturity. Residual quantities of MH in green and cured plants, as well as in soil, were determined by our derivatization–gas chromatography method. The effects of different application rates to tobacco are discussed.

Maleic hydrazide (MH, 1,2-dihydro-3,6-pyridazinedione), a systemic plant growth regulator in worldwide use as a tobacco sucker inhibitor, has generated recent interest because of its almost ubiquitous presence in tobacco and tobacco products. These concerns have been stimulated to a great extent by possible European Economic Community import restrictions on tobacco with high MH residues and by the possible health-related effects of MH in test animals (Epstein et al., 1967; Epstein and Mantel, 1968).

The fate of MH in tobacco has been examined by a number of workers over a period of about 20 years (Anglin and Mahon, 1958; Lane, 1965; Davis et al., 1974; Cheng and Steffens, 1976). All of these used the analytical method of Wood (1953) with modifications by Anglin and Mahon (1958), Lane et al. (1958), and Hoffman (1961). This analysis is based on the hydrolytic reduction of MH to hydrazine by zinc in aqueous sodium hydroxide solution. The hydrazine is subsequently steam distilled into an acidic solution of ρ-dimethylaminobenzaldehyde to form an azine which has an absorption maximum at 455 nm. This method, when applied to tobacco, suffers from interferences caused by pyrrole, resorcinol, tryptophan, and possibly other leaf constituents. Because of these interferences and the ambiguous nature of photometric determinations, we felt that a more comprehensive study of residual MH in the tobacco plant and soil was necessary using the gas chromatographic method developed at our laboratory (Haeberer et al., 1974; Haeberer and Chortyk, 1974). In the study of the stability and fate of MH applied to tobacco and soil, the following questions require elucidation: How much MH remains in harvested tobacco leaves, stalks, and roots from a standard application? Will a twofold application be reflected in a twofold increase in MH residue? Does the tobacco plant absorb MH from the soil? How much persists in the soil after 100 days? Will the next crop absorb soil MH? How much MH remains in cured leaves? To answer these questions, several experiments were conducted. Various quantities of MH were applied to soil and to growing Burley tobacco at various stages of maturity. Subsequently, residual MH was determined and the significance of these findings are discussed.

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EXPERIMENTAL SECTION

Reagents. The agricultural formulation of maleic hydrazide as the diethanolamine salt, MH-30 (UniRoyal), was obtained commercially. It was applied without further refinement after dilution with water (35 L of water/liter of MH-30). Maleic hydrazide for standards was obtained from Eastman Kodak Co. as the practical grade. It was recrystallized twice from distilled water. *N,O*-Bis(trimethylsilyl)acetamide (BSA) was obtained from Analabs, Inc. as the pure reagent and used without further refinement. Methyl alcohol (Burdick and Jackson Laboratories, Inc.) was the "distilled-in-glass" grade. Ethyl

Table I. Maleic Hydrazide Contents of Cured Burley Tobacco Leaves and Harvested, Uncured Leaves, Stalks, and Roots

Suckering treatment	MH treatment		MH Content (ppm)										
			Stalks[c]				Harvested leaves[c]			Cured leaves[d]			
	Rate	Days to harvest[a]	Soil[b]	Top 1/2	Bottom 1/2	Roots[c]	1-4	5-8	9-12	1-4	5-8	9-12	Remainder
1. Hand-suckered	100 mg/ft² to soil	214	0	0	0	0	0	0	0	0	0	0	0
2. Hand-suckered	100 mg/ft² to soil	97[e]	45	0	0	0	0	0	0	0	0	0	0
3. Hand-suckered	0		0	0	0	0	0	0	0	0	0	0	0
4. MH to upper third of plant	170 mg/plant	24[f]	0	129	42	14	251	215	133	127	123	73	68
5. MH to upper third of plant	17 mg/plant	24[f]	0	0	0	0	53	50	15	23	21	21	7
6. MH to upper third of plant	340 mg/plant	24[f]	0	184	110	21	274	290	151	103	104	44	73
7. MH to upper third of plant	170 mg/plant	24[f]	0	321	462	18	315	233	197	168	122	55	110
	170 mg/plant	10[g]											

[a] Harvest, Sept 2, 1975. [b] Sampled at planting, May 28, 1975. [c] Sampled at harvest. [d] Samples at 90 days after harvest. [e] At planting, May 28, 1975. [f] At topping, Aug 8, 1975. [g] 10 days before harvest, Aug 22, 1975.

acetate (Mallinckrodt Chemical) was purchased as the analytical reagent grade. Alumina was obtained from Bio-Rad Laboratories in 100/120 mesh size in the neutral (AG-7) fully activated form.

Application and Sampling. In 1975, seven 4- × 5-m plots were planted with 40 plants each of Burley tobacco variety Va. 509. Two-meter balks were maintained between the plots. The soil, Dunmore silt loam, in Greenville, Tennessee, had not been cultivated in at least 30 years. In two of the plots, only the soil was treated with MH at 1 g/m². In four plots, MH was applied to the plants at dosages ranging from one-tenth to twice the normal dosage (170 mg/plant) (Table I). One plot was used as the control with no MH applied to soil or plants. Soil samples, consisting of several 2.5-cm diameter × 15-cm deep cores were taken randomly over each plot on the day of planting. All applications were made with a 1-L capacity hand sprayer. Solutions of the plant growth regulator were applied to the upper third of the tobacco plants at topping, when the plants were in half to full flower. A total of seven different treatments were used in the 1975 experiments. Treatments 3, 4, and 7 were repeated in 1976 using identical agronomic practices.

Treatment 1: 1.08 g of MH/m² applied to soil 117 days before sampling soil and 125 days before analysis of sample.

Treatment 2: 1.08 g of MH/m² applied to soil on day of sampling and 8 days before analysis.

Treatment 3: No MH applied to tobacco plants or soil.

Treatment 4: 170 mg of MH applied to upper third of each plant.

Treatment 5: 17 mg of MH applied to upper third of each plant.

Treatment 6: 340 mg of MH applied to upper third of each plant.

Treatment 7: 170 mg of MH applied to upper third of plant at topping plus 170 mg of MH applied 14 days later.

Plants in the outer perimeter of the plot were discarded. Half of the harvested plants were quick-frozen and half were cured in the conventional Burley manner. Leaves were grouped according to stalk position and numbered from the top downward (top leaf = 1). The average number of leaves per plant was 19. The stalks were cut into two portions, top half and bottom half, and the root system was cut from the stalks at the junction of the uppermost root.

Extraction, Cleanup, and Derivatization. All analysis were carried out in triplicate. All samples (soil, leaf, stalk, roots, and cured leaf) were dried at 100 °C for 10 h. Soil samples (20 g from each plot) were extracted three times with 60-mL portions of boiling methyl alcohol for 1 h. The combined soil extracts were reduced in volume on a rotary evaporator and transferred to 1-mL micro-reaction vessels, equipped with a Teflon-lined cap. The remaining solvent was removed with a stream of dry nitrogen while the vessels were heated to 70 °C. The residue was heated to 100 °C for 1 h with 0.200 mL of BSA in the sealed vessels to form trimethylsilyl (Me_3Si) derivatives of the MH.

The stalks and roots were ground to 40 mesh size. The leaf samples, after the removal of the midribs, were pulverized in a ball-mill. Ground root samples (4 g), stalk samples (8 g), and leaf samples (4 g) were extracted three times with 60 mL of boiling methyl alcohol for 1 h. The combined extracts were reduced to 10.00 mL. A 100-μL sample of the methyl alcohol extract was applied to a microcolumn consisting of a disposable transfer pipet plugged with glass wool and charged with 0.2 g of activated, neutral alumina. The microcolumn was heated at 140 °C for 1 h for volatilization of the solvent, cooled to room temperature, and a second 100-μL portion of extract was applied. After reheating and cooling, the microcolumn was washed with 3 mL of ethyl acetate and redried at 140 °C for 30 min. The alumina was then transferred to a 1.0-mL micro-reaction vessel. BSA (250 μL) was added and the vessel was sealed, vigorously shaken, and heated to 100 °C for 1 h, with repeated shaking. It was then cooled and the supernatant liquid analyzed by gas chromatography.

Gas Chromatography (GC). A length of glass tubing, serving as a demisting trap, was installed into the injection port of a Tracor Model 222 gas chromatograph equipped with flame ionization detectors. The instrument was also fitted with a four-port valve so that excess BSA could be vented. This prevented excessive buildup of silica on the collector of the flame ionization detector. The GC glass column, 2 m × 2 mm i.d. Pyrex, was packed with 20% OV-11 coated on 100/120 mesh Chromosorb W-HP. The column was partially conditioned with temperature programming from ambient to 300 °C at 6 °C/min. Conditioning was completed with 15-μL portions of BSA injected at 30-min intervals until the baseline stabilized. The analyses were carried out isothermally at 130 °C; the injector and detector were maintained at 230 and 320 °C, respectively; the flow rate of the carrier gas, helium, was

set at 40 mL/min. Five-microliter portions of standards or samples were analyzed. The $MH(Me_3Si)_2$ peak eluted with a retention time of 20.5 min (Kovats retention index (1462) and was quantitated with a Hewlett Packard Model 3380A recording integrator.

RESULTS AND DISCUSSION

We analyzed MH residues in Burley tobacco by our method, which consisted of simultaneous extraction–silylation of the MH followed by GC analysis. The soil sample extracts did not require cleanup before GC analysis. The GC chromoatograms of the Me_3Si derivatives had relatively few peaks and good baseline resolution facilitated MH quantitation without any undue hinderance. However, in order to determine low residue levels (<50 ppm), the extracts of the Burley samples required cleanup. This was accomplished simply by microcolumn chromatography. GC of the derivatized refined samples resulted in chromatograms with few peaks and good baseline resolution. This permitted the quantitation of the $MH(Me_3Si)$ peak with greater precision and accuracy than had previously been possible.

Soil analyzed 117 days after application of treatment 1 showed no MH (limit of detection in soil, 0.1 ppm MH), nor was MH detected in any of the tobacco grown on that plot (limit of detection in tobacco, 1 ppm). Treatment 2 soil, sprayed with MH and disked on the day of soil sampling, just before planting, clearly showed the presence of MH (21 ppm MH); but none was found in the tobacco from this plot. Apparently MH applied to soil breaks down rapidly since none could be detected 117 days after an application as heavy as 1.08 g/m^2. At normal application rates of 170 mg of MH/plant, normal spacing of 40 cm between plants in rows 107 cm apart (0.44 m^2/plant), and provided that MH is not absorbed by the plants, the soil would receive at most 0.393 g of MH/m^2. The absence of MH in the soil 117 days after treatment and in the tobacco plants grown on either of the MH-treated soils rules out the absorbance of MH by tobacco plant roots from applications of the previous year. The apparently rapid breakdown of MH in soil also rules out the absorbance of MH by crops rotated with tobacco.

MH treatments 4 to 7 were designed to elucidate the effects of different application rates on final residues. The standard application rate was 170 mg of MH/plant (treatment 4). Treatments 5 and 6 were included as possible extremes. Treatment 7, for a total of 340 mg of MH, was designed to demonstrate the stability of MH on the plant. Upon analysis of the data, the following conclusions were apparent:

(1) MH was degraded in soil in 120 days or less.

(2) Due to the rapid breakdown of MH in soil, MH was not absorbed by the tobacco plant. Plant parts (roots, stalks, leaves) at harvest, 97 days from treatment 2, contained no MH.

(3) In a normal MH treatment (treatment 4), about 15% of the applied MH remained in the harvested leaves of the plant, 4% in the stalk, and about 0.6% in the roots.

(4) MH residues of cured tobacco samples appeared to be lower than those of harvested (uncured) plants (treatment 4, average of 200 ppm for harvested vs. average of 108 ppm for cured leaves). This decrease may have been due to enzymatic activity, which is known to occur in the leaf during the air-drying period.

(5) Single MH treatments at one-tenth the standard dose (treatment 5) yielded cured leaves with a 20 ppm MH content.

Table II. MH Contents of Harvested Plant Portions as Percentage of Applied MH

Tobacco treatment	Leaves 1-12	Remainder of leaves	Stalk	Roots	Total % MH recov
4	10.8	3.7	3.7	0.6	18.8
5	17.9	12.4	0	0	30.3
6	5.7	2.1	3.5	0.4	11.7
7	6.1	2.4	7.0	0.4	15.9

Table III. Maleic Hydrazide Content of Harvested and Cured Burley Leaf, 1976

	Leaves					
	Harvested			Cured		
Treatment	1-4	5-8	9-12	1-4	5-8	9-12
Hand-suckered	0	0	0	0	0	0
170 mg of MH/plant[a]	234	199	124	146	164	111
(170 + 170) mg of MH/plant[b]	357	251	217	249	118	150

[a] Applied at topping, Aug 4, 1976. [b] Applied Aug 4 and 25, 1976.

(6) The higher levels of MH found with treatment 7, compared to those with treatment 6, indicate a decrease of residue with time while the tobacco plant is actively growing.

The 1975 and 1976 growing seasons were plagued by a lack of seasonal rainfall. This is evidenced by the fact that only an average of 20 and 19 leaves/tobacco plant were produced while normally an average of 22 may be expected. This drought may also have resulted in MH residue levels higher than generally reported (Anglin and Mahon, 1958; Cheng and Steffens, 1976; Davis et al., 1974; Lane, 1965). Rainfall shortly after MH applications would be expected to wash away much of the applied MH before it could be absorbed into the plant.

It was also of interest to calculate the total percent of MH recovered in the harvested, uncured plant (Table II). The values were less than 20%. With the normal dosage level (treatment 4), 15% remained on the leaves, 4% in the stalk, and 0.6% in the roots. The double dosage applications still gave recoveries of less than 16%.

The apparent reduction of MH in the curing process prompted us to repeat treatments 3, 4, and 7 during the 1976 growing season. The results from the harvested uncured leaves compared with those from 1975 are presented in Table III. There is very excellent agreement between the residue data from both years. As with the results from the previous crop, a marked decrease in residual MH is noted. The maleic hydrazide could either be undergoing degradation or conversion to a compound that is resistant to derivatization by BSA. Many compounds with a similar adjacent nitrogen moiety such as found in MH will degrade to form hydrazine when the harsh conditions of the Wood (1953) method are employed, precluding any distinction between MH and its reaction products. Whether the MH is being degraded or converted to a compound that is not extracted and derivatized by BSA is currently under investigation at our laboratory and will shortly be reported.

The second application of 170 mg of MH to each tobacco plant was made 10 days before harvest in the 1975 experiment and 1 day before harvest in 1976. This did not alter the MH residue levels in the harvested leaves appreciably. We are encouraged to note the very good reproducibility of the data, indicating the reliability of our rapid and quantitative methodology.

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Photomirex: Synthesis and Assessment of Acute Toxicity, Tissue Distribution, and Mutagenicity

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Photomirex (8-monohydro mirex), the major photodecomposition product of mirex, was synthesized by reductive dechlorination of mirex and the compound characterized by MS, NMR, and GC. The acute oral toxicity of photomirex was determined in rats given single oral dose of 0, 50, 100, 150, and 200 mg/kg of body weight. The 200 mg/kg dose caused 80% mortality in males and 40% mortality in females. The compound accumulated to high levels in adipose tissue and ovaries and to lower levels in liver, kidney, spleen, heart, brain, and testes. Livers and kidneys were mottled and congested in all animals treated with photomirex. Mirex, photomirex, and kepone were not mutagenic in a standard Ames test including liver microsomal activation.

Photomirex (8-monohydro mirex, 1,2,3,4,5,5,6,7,-9,10,10-undecachloropentacyclo[5.3.0.$0^{2,6}$.$0^{3,9}$.$0^{4,8}$]decane) was recently identified as the fourth highest organochlorine pollutant (after PCBs, DDE, and mirex) in the body lipid and eggs of herring gulls breeding in colonies on Lake Ontario (Hallett et al., 1976). Photomirex was also found to be present at similar ratios to the other major contaminants in coho salmon muscle and liver and in alewives and smelt taken from this lake (Norstrom et al., 1977).

Gibson et al. (1972) showed that approximately 5% of mirex was converted to the 8-monohydro derivative after being exposed to sunlight for 3 months as deposits on silica gel plates. Mirex has also been shown to undergo photolytic dechlorination in cyclohexane and 2,2,4-trimethylpentane (Dilling and Dilling, 1967; Alley et al., 1973; Alley and Layton, 1974) and in egg solids (Lane et al., 1976) when irradiated with UV light. The primary photodegradation product was 8-monohydro mirex with lesser amounts of 5,8-dihydro mirex. Carlson et al. (1976) showed that from 16 to 19.5% of the total mirex-related residues recovered from soil samples recovered 12 years after treatment at 1.12 kg/ha was photomirex. Lesser amounts of kepone (3.1 to 6.3%), 10-monohydro mirex, and two isomers of dihydro mirex were also present. When 4X mirex bait was exposed to intense UV irradiation in a Rayonet-type RS reactor for 19.5 h, similar degradation patterns were found with photomirex being the major degradation product (19.9%), along with lesser amounts of kepone (0.2%), and the other derivatives (Carlson et al., 1976).

The half-life of mirex dispersed in water under intense UV light at 95 °C is 48.4 h, as measured by formation of CO_2. This is similar to DDT (42.1 h) and rather long relative to dieldrin (11.5 h) (Knoevenagel and Himmelreich, 1976). Mirex is very resistant to metabolic attack being slowly dechlorinated to a monohydro derivative by anaerobic microbial action in sewage sludge (Andrade et al., 1975) and likely by enteric bacteria in monkeys as evidenced by the formation of a fecal metabolite (Stein et al., 1976). There has been no reported evidence of metabolic degradation by soil microorganisms (Jones and Hodges, 1976) or in mammals (Gibson et al., 1972). It has been shown to accumulate unaltered in both terrestrial and aquatic ecosystems (Mehendale et al., 1972; Metcalf et al., 1973; Pritchard et al., 1973; Collins et al., 1974). Residues of mirex have been detected in human adipose tissue samples taken in Georgia and Louisiana (Kutz et al., 1974).

An acute oral LD_{50} of mirex was reported as 365 mg/kg for female rats (Gaines and Kimbrough, 1970) and 2400 mg/kg for mallard ducks (Tucker and Crabtree, 1970). Reproductive effects of mirex in the diet of rats include reduced survival rate of progeny and a high incidence of cataracts at 25 mg/kg (Gaines and Kimbrough, 1970). Mirex administered to pregnant female rats on days 6 to 15 of gestation at 6 and 12.5 mg/kg resulted in maternal toxicity, pregnancy failure, decreased fetal survival, reduced fetal weight and increased incidence of visceral anomalies (Khera et al., 1976). Male rats were shown to accumulate mirex from daily oral dosages of 1.5 to 6.0 mg/kg in a dose-related manner. Highest concentrations were found in adipose tissue with lower concentrations in liver and testes although this did not affect reproduction parameters in subsequent mating trials. The distribution

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