Metabolism of *N'*-(4-Chloro-*o*-tolyl)-*N,N*-dimethylformamidine by Apple Seedlings

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Degradation of the systemic acaricide *N'*-(4-chloro-*o*-tolyl)-*N,N*-dimethylformamidine (Galecron or Fundal) occurred at a slow rate in and on apple seedlings. When Galecron was applied to the upper surface of apple leaves *in situ* and the leaves were analyzed after 20 days, 41% of the total applied radioactivity was recovered as the parent compound. Concentrations of Galecron in the leaves and stems (including main stems, branches, and roots) were 17 and 24%, respectively, in terms of total applied radioactivity recovered 20 days after the compound was injected into the main stem. Galecron metabolites characterized from apple seedlings included *N'*-(4-chloro-*o*-tolyl)-*N*-methylformamidine, *N*-formyl-(4-chloro-*o*-toluidine), and 4-chloro-*o*-toluidine; the glucoside *N*-(2-methyl-4-chlorophenyl)-D-glucosylamine was tentatively identified.

N'-(4-Chloro-*o*-tolyl)-*N,N*-dimethylformamidine (CIBA, Galecron; Morton, Fundal) is a novel acaricide with both ovicidal and adulticidal action (Dittrich, 1966). The compound has a highly active vapor-phase, the ability to penetrate leaves, and capability of translocation in the plant's system (Dittrich, 1967). In laboratory tests the material was equally effective against the organophosphate-tolerant carmine spider mite, *Tetranychus telarius* (L.), and the susceptible two-spotted spider mite, *Tetranychus urticae* (Koch) (Dittrich, 1966). Field evaluation of Galecron for acaricidal activity on apple trees revealed excellent control of the apple rust mite, *Aculus schlechtendali* (Nalepa), in northern Italy (Dittrich, 1967) and the European red mite, *Panonychus ulmi* (Koch), in Missouri (Enns, 1968).

The metabolic fate and associated residues of Galecron must be evaluated before the compound can be used extensively for mite control on apple trees. The metabolism of radioactive Galecron in apple seedlings was accordingly examined.

MATERIALS AND METHODS

Plants and Chemicals. Dormant apple seedlings (Brookings variety), provided gratuitously by Inter-State Nurseries, Inc., Hamburg, Iowa, were stored at 5° C. When required for experimental use, the plants were taken from the freezer and potted individually in greenhouse soil. Leaves appeared on the plants after approximately one week in the greenhouse. The total length of the plants ranged from 40 to 50 cm., and the average weight was 3 to 5 grams during the test period; the height of the plants above the soil was approximately 15 cm. The average number of leaves per plant was nine and the average weight of leaves per plant ranged from 400 mg. at the initial sampling period to 600 mg. at 20 days after treatment.

^{3}H-Galecron (random ring-labeled, synthesized by Schering AG, Berlin) having a specific activity of 94 mc. per mmole and ^{14}C-Galecron (tolyl-labeled) having a specific activity of 4.55 mc. per mmole (diluted to 1.96 mc. per mmole) were provided by CIBA Agrochemical Co., Vero Beach, Fla. When received for experimental use, the compounds were radiochemically pure, as judged by thin-layer chromatography (TLC) and radioautography. In addition to Galecron, the following compounds were also provided by CIBA Agrochemical Co.: *N'*-(4-chloro-*o*-tolyl)-*N*-methylformamidine or demethyl-Galecron (m.p. 90° C.), *N*-formyl-4-chloro-*o*-toluidine (m.p. 119° C.), and 4-chloro-*o*-toluidine. The *N*-(2-methyl-4-chlorophenyl)-D-glucosylamine was synthesized from 4-chloro-*o*-toluidine and D-glucose according to a procedure used to prepare other *N*-arylglucosylamines (Kadunce, 1967); the compound was recrystallized three times from methanol (m.p. 115° C.: per cent calculated for $C_{13}H_{18}NO_5Cl$, N = 4.60, Cl = 11.61; found N = 4.43, Cl = 11.28).

Chromatography and Radioautography. TLC was accomplished on 20- × 20-cm. glass plates coated with a 0.5-mm. layer of silica gel GF_{254} (Brinkmann Instruments, Inc., Westbury, N.Y.); the chromatograms were developed with benzene-diethylamine (95 to 5) (Geissbühler and Gross, 1965). Galecron and degradation products on developed chromatograms were detected by their ability to quench short wavelength ultraviolet light. The following R_f values were obtained: Galecron, 0.85; demethyl-Galecron, 0.41; *N*-formyl-4-chloro-*o*-toluidine, 0.18; 4-chloro-*o*-toluidine, 0.51; and *N*-(2-methyl-4-chlorophenyl)-D-glucosylamine, 0.00.

To locate the radioactive areas on the developed chromatograms from ^{14}C-Galecron experiments, the plates were placed in contact with no screen x-ray film (Eastman Kodak Co., Rochester, N.Y.); the exposure time was usually 10 days.

Estimation of Radioactivity. A Packard Model 3003 liquid scintillation spectrometer was used to quantitate the radioactivity. The scintillation liquid used for all samples was 15 ml. of a mixture of 2,5-diphenyloxazole (12 grams) and 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl) benzene (120 mg.) dissolved in toluene (2 liters) and methyl cellosolve (1 liter).

Persistence on Glass Surface. ^{14}C-Galecron was applied in 10 μl. of acetone to clean glass microscope slides (18-mm. circles) at a radioactivity level of 35,000 c.p.m. per slide; the slides were placed in a laboratory fume hood illuminated with fluorescent light. At selected intervals, the material remaining on the slides was eluted with acetone into a scintillation vial, the acetone evaporated, and the radioactivity counted. All analyses were conducted in duplicate.

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Topical Leaf Treatment and Analysis. In August, 1967, an ethanolic solution of ^{3}H-Galecron was applied as uniformly as possible to the upper surface of eight leaves of 12 apple seedlings that had been potted for 20 days. Each plant received approximately 500,000 c.p.m. of the compound, divided equally among the eight leaves. In March, 1968, another 12 30-day-old apple seedlings were treated topically on the leaves with ^{14}C-Galecron at a radioactivity level of 100,000 c.p.m. per leaf; six leaves were treated per plant.

At posttreatment intervals of 0, 4, 8, 12, 16, and 20 days (^{3}H-treatment) and 0, 8, 12, 16, and 20 days (^{14}C-treatment), the treated leaves from two plants were cut at the petiole, and the combined leaves from each plant were analyzed separately as described below.

For the leaf rinse, the leaves were placed in a beaker containing 5 ml. of chloroform, and the beaker was shaken for 10 minutes. This procedure was repeated, the two chloroform rinses were combined, and the volume was reduced to 5 ml.

To determine the amount of radioactive material remaining after the chloroform rinses, the leaves were homogenized (Lourdes Multimixer, Lourdes Instrument Corp., Brooklyn, N.Y.) in 10 ml. of acetone, followed by two 10-ml. washes of the residue with chloroform. The solvents were separated from the leaf residue by filtration; the filtrates were combined and extracted five times with 10-ml. portions of 1*N* sulfuric acid. (Radioactivity in the chloroform-acetone fraction after acid extraction was negligible.) The acid extracts were combined, made alkaline with 5 ml. of 10*N* sodium hydroxide, and partitioned five times against 10-ml. portions of chloroform. The chloroform was dried over anhydrous sodium sulfate, filtered, and concentrated to a volume of 5 ml.

A 2-ml. aliquot of the chloroform from the leaf rinse and leaf extract was added to scintillation vials, the solvent removed with the aid of a stream of dry air at room temperature, and the residue was assayed to determine the total radioactivity. The remaining 3 ml. of chloroform from the leaf rinse and extract were concentrated to about 0.5 ml. and spotted on a thin layer plate. For the tritium experiment, a mixture of nonlabeled Galecron and suspected degradation products was spotted on the plate adjacent to the leaf rinse and extract. After development of the chromatogram with benzene-diethylamine, the reference compounds were located with ultraviolet light. The zones from the leaf rinse and extract corresponding to the reference compounds and a sample from the origin were scraped into scintillation vials, and the radioactivity was counted. In the ^{14}C experiment, the nonlabeled compounds were added directly to the leaf rinse and extract for purposes of cochromatography, and the mixture was spotted on the chromatoplate. After development in benzene-diethylamine, the radioactive areas were located by radioautography and counted.

Stem Injection and Analysis. In September, 1967, 18 15-day-old apple seedlings were injected in the main stem approximately 2 cm. below the first axillary branch with 20 μl. of acetone-water (1 to 1) containing about 500,000 c.p.m. of ^{3}H-Galecron. The following January, another 12 apple seedlings were injected with 10 μl. of acetone-water containing about 60,000 c.p.m. of ^{14}C-Galecron. Three plants from the tritium treatment and two plants from the ^{14}C treatment were taken for analysis at 0, 4, 8, 12, 16, and 20 days posttreatment, and each plant was processed as follows: The stems (including the main stems, branches, and roots) and leaves were separated, cut into small pieces with a scalpel, and homogenized for 5 minutes in 20 ml. of acetone. The tissue was then homogenized two times with 20-ml. portions of chloroform, and the acetone and chloroform extracts were combined. Saturated sodium chloride solution (7 ml.), 2*N* ammonium hydroxide (2 ml.), and distilled water (20 ml.) were added to the organic extract; the pH of the aqueous solution was approximately 8. The organic and aqueous phases were shaken vigorously and separated. The aqueous phase was washed with 20 ml. of chloroform, and the chloroform wash was added to the initial organic extract. The aqueous phase was not radioassayed in the tritium treatment, but was analyzed in the ^{14}C-Galecron study.

The organic phase was extracted five times with 10 ml. of 1*N* sulfuric acid. The acid extracts were combined and made alkaline with 5 ml. of 10*N* sodium hydroxide. This solution was partitioned five times against 10 ml. of chloroform; the chloroform extracts were combined and dried over anhydrous sodium sulfate. (This aqueous phase contained no radioactivity and was discarded.) The chloroform phase was concentrated to 5 ml. on the rotary evaporator. A 2-ml. aliquot was placed in a scintillation vial, the chloroform evaporated to dryness, and the radioactivity counted. The remainder of the chloroform was reduced to about 0.5 ml. and subjected to TLC as described previously. In the tritium experiment the portion of the silica gel corresponding to the reference compounds was removed from the plate and eluted with 4 ml. of acetone into a scintillation vial. The acetone was evaporated, and the radioactivity was counted. With the ^{14}C experiment, the procedure was similar to that described above, except that the metabolites were located by radioautography and cochromatography and radioassayed.

Experimental results indicated that greater than 95% of Galecron, demethyl-Galecron, and 4-chloro-*o*-toluidine was recovered from stems and leaves with the above procedure; however, only 40% of *N*-formyl-4-chloro-*o*-toluidine was recovered. Therefore, the organic phase, after the acid extraction described above, was cleaned up by column chromatography and analyzed for the remaining *N*-formyl-4-chloro-*o*-toluidine as follows: The solution from the leaves and stems was passed separately through the sodium sulfate–animal charcoal–Florisil column described by Abdel-Wahab *et al.* (1966). After the extract had passed through the column, the column was washed with 15 ml. of chloroform-acetone (2 to 1). The original eluate and wash were combined and concentrated to 10 ml. on the rotary evaporator. A 2-ml. aliquot was counted for total radioactivity, and the remaining 8 ml. was concentrated and spotted on a thin-layer plate along with a mixture of nonlabeled reference compounds. After development of the chromatogram, the area corresponding to *N*-formyl-4-chloro-*o*-toluidine (R_f 0.18) was scraped and eluted with acetone into a scintillation vial, and the radioactivity was counted. The remaining portion of *N*-formyl-4-chloro-*o*-toluidine (*ca.* 60%) was in the chloroform-acetone.

The Schöniger flask combustion technique (Buyske *et al.*, 1961; Kelly *et al.*, 1961) was used to estimate the radioactivity remaining in the leaf and stem residue after solvent extraction. A 50-mg. aliquot of the dried plant tissue in a black paper wrapper was placed in the platinum sample carrier. The 500-ml. flask was purged with oxygen, and the addition funnel head (A. H. Thomas Co., Philadelphia, Pa.) with the sample was clamped in place. After ignition of the tritium-containing samples, the flask was placed in a shallow dry ice–chloroform bath so that only the bottom was immersed. Upon condensation of the water vapor, 20 ml. of the scintillation liquid was added, and the flask was swirled several times; 15 ml. of the liquid was placed in a scintillation vial for counting. The combustion procedure was identical for the ^{14}C-containing samples, except that after combustion the flask was cooled with a stream of water, and 6 ml. of a monoethanolamine–methyl cellosolve (1 to 2) mixture was added through the addition funnel. After swirling the flask, 3 ml. of the flask contents was added to a scintillation vial containing 15 ml. of scintillation liquid, and the radioactivity was counted. Duplicate analyses were performed in all cases. Efficiency experiments indicated that greater than 95% of ^{14}C-Galecron and ^{3}H-Galecron were recovered from fortified plant tissues utilizing this technique.

A procedure similar to that described by Kuhr and Casida (1967) was used to study the nature of the water-soluble radiocarbon-containing metabolites. The aqueous fractions from the two 20-day stem samples were combined, concentrated to 8 ml. on the rotary evaporator, and 2-ml. aliquots were added to four 25-ml. Erlenmeyer flasks. Four milliliters of citrate-phosphate buffer (pH 4.6) were added to each flask, followed by one of the following: β-glucosidase (3 mg.), β-glucuronidase (3 mg.), or hydrochloric acid, to yield a final pH of 1. The other flask, containing only buffer and water-soluble conjugates, served as the control. Following incubation with shaking for 4 hours in air at 38° C., the flask contents were extracted three times with 5-ml. aliquots of chloroform. The chloroform extract was dried over sodium sulfate, and the radioactivity in the chloroform and water fractions was counted. From these determinations the percentage cleavage of the water-soluble conjugates by the two enzymes and acid was calculated.

To study the nature of the aglycone moieties, the following procedure was used: the two 16-day aqueous stem fractions were combined, concentrated to dryness, and hydrolyzed for 16 hours with 9% (w./v.) hydrochloric acid at 38° C. The mixture was extracted three times with 5-ml. aliquots of chloroform, and the chloroform layer was dried with sodium sulfate and concentrated almost to dryness. The chloroform extract and authentic nonlabeled compounds were spotted on the thin-layer plate; the plate was developed with benzene-diethylamine, and a radioautograph was made.

Identification of Metabolites. To obtain sufficient quantities of the metabolites for positive identification, 10 plants were injected with pure nonlabeled Galecron. Each plant received 50 mg. of the compound dissolved in 20 μl. of acetone-water (2 to 1). After 12 days, five whole plants (leaves and stems) were cut into small pieces, homogenized, and extracted as described above.

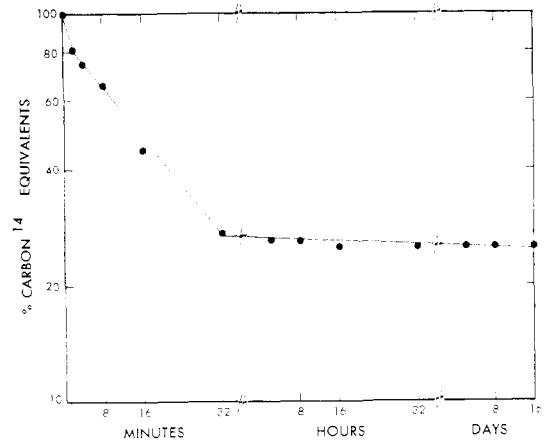

Figure 1. Disappearance of ^{14}C-Galecron when applied to a glass surface and placed under fluorescent light in the laboratory

The other five plants were analyzed at the same time, and the extracts from the plants were combined and subjected to TLC. Infrared analysis, melting point determination, dye formation, and cochromatography with authentic standards were used to establish the identity of the metabolites after extraction from the thin-layer plate.

RESULTS AND DISCUSSION

Before applying Galecron to apple leaves, glass microscope slides were treated to determine the role that volatilization might play in the fate of the compound. After evaporation of the acetone, the disappearance rate of ^{14}C-Galecron equivalents from the glass surface was linear for about 30 minutes, indicating that the decomposition products formed, if any, did not differ significantly in volatility from the parent compound (Figure 1). However, the 25% of the applied radioactivity remaining after 30 minutes was persistent, and there was no appreciable decrease in radioactivity for 16 days. Geissbühler (1966) sprayed glass plates with an aqueous emulsion of Galecron and found that the bulk of the compound disappeared during evaporation of the water, leaving a residue of approximately 25% in terms of applied material. We conclude, therefore, that the linear decline of ^{14}C-Galecron equivalents from 2 minutes through 32 minutes (Figure 1) occurred during the evaporation of traces of water present in the acetone.

The fate of Galecron applied to the surface of apple leaves is given in Table I. It is apparent that the behavior of Galecron on the leaves differs considerably from that on the glass surface. If a comparison is made between the total recovery at zero time for the tritium treatment and the total recovery at zero time (actually 1 hour after treatment) for the ^{14}C treatment, there is a decrease in total radioactivity of 13% during the first hour after application to the leaves. Assuming that little or no translocation into the stems occurred during this period, the loss could be attributed to evaporation. Therefore, the Galecron equivalents were much more persistent on the apple leaves treated *in situ* than on the glass surface. This result is in agreement with that of Geissbühler (1966), who sprayed bean leaves (in vitro) and glass plates with an aqueous emulsion of Galecron

Table I. Fate of Galecron Applied to the Surface of Apple Leaves

	R_f Values for TLC	Per Cent of Applied Radioactivity Recovered at Indicated Days After Treatment[a]										
		^{3}H-Galecron						^{14}C-Galecron				
		0[b]	4	8	12	16	20	0[c]	8	12	16	20
						Leaf Rinse						
Galecron	0.85	99.0	49.0	44.7	41.2	32.2	29.1	82.2	43.3	38.4	33.2	28.7
Metabolite A	0.41	0	1.0	0.8	1.0	1.0	0.9	0	0.6	0.8	0.9	1.2
Metabolite B	0.18	0	0.7	0.6	0.4	0.6	0.9	0.2	0.7	0.9	0.9	1.1
Metabolite C	0.51	0	0.5	0.5	0.8	1.0	0.9	0	0.2	0.4	0.5	0.7
Metabolite D	0.95	0	0	0	0	0	0	0	0	0.2	0.1	0.2
Origin[d]	0.00	0	0.7	0.5	0.4	0.6	0.3	0	0.2	0.2	0.2	0.2
						Leaf Extract						
Galecron	0.85	0.3	6.1	7.0	9.6	8.0	11.6	3.8	6.7	7.9	8.1	12.3
Metabolite A	0.41	0	0.6	0.5	1.0	0.7	0.8	0	0.3	0.6	0.7	0.7
Metabolite B	0.18	0	0.4	0.2	0.3	0.2	0.3	0.1	0.4	0.6	0.7	0.7
Metabolite C	0.51	0	0.2	0.5	0.7	0.5	0.5	0	0.1	0.2	0.2	0.3
Origin[d]	0.00	0	0.2	0.1	0.3	0.3	0.3	0	0.1	0.1	0.1	0.1
Recovery		99.3	59.4	55.4	55.7	45.1	45.6	86.3	52.6	50.3	45.6	46.2
Lost		0.7	40.6	44.6	44.3	54.9	54.4	13.7	47.4	49.7	54.4	53.8

[a] Average of duplicate analyses.
[b] Analyzed immediately after treatment.
[c] Analyzed one hour after treatment.
[d] Radioactive material(s) that would not migrate in the TLC solvent system.

Table II. Nature of Radioactive Materials Resulting from Injection of Galecron into Apple Seedlings

	R_f Values for TLC	Per Cent of Applied Radioactivity Recovered at Indicated Days after Injection											
		^{3}H-Galecron[a]						^{14}C-Galecron[b]					
		0	4	8	12	16	20	0	4	8	12	16	20
							Stems						
Galecron	0.85	98.5	95.8	80.2	54.0	41.2	23.2	98.2	95.4	81.2	53.6	40.3	24.8
Metabolite A	0.41	<0.1	0.5	1.0	1.1	1.2	1.2	<0.1	0.5	1.3	1.6	1.6	1.6
Metabolite B	0.18	<0.1	0.3	0.6	0.5	0.5	1.0	<0.1	0.3	0.7	0.7	1.1	1.1
Metabolite C	0.51	<0.1	<0.1	0.2	0.2	0.3	0.1	<0.1	<0.1	0.2	0.2	0.2	0.3
Origin[c]	0.00	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.1	0.1	0.1
Water Extract		—	—	—	—	—	—	0.1	0.1	1.1	3.5	8.6	10.7
Residue		0.2	0.2	2.8	26.2	28.6	29.9	0.1	0.2	2.2	22.9	28.9	33.0
							Leaves						
Galecron	0.85	<0.1	2.7	8.7	7.4	7.2	17.3	<0.1	3.0	9.0	7.8	7.9	17.9
Metabolite A	0.41	<0.1	<0.1	<0.1	0.7	0.6	1.2	<0.1	<0.1	0.2	0.8	1.0	1.1
Metabolite B	0.18	<0.1	<0.1	<0.1	0.5	0.1	1.1	<0.1	<0.1	0.2	0.4	0.9	1.0
Metabolite C	0.51	<0.1	<0.1	<0.1	0.3	<0.1	0.1	<0.1	<0.1	<0.1	0.2	0.2	0.3
Origin[c]	0.00	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	0.1	<0.1	0.1	0.1
Water Extract		—	—	—	—	—	—	<0.1	0.1	0.1	0.1	0.2	0.3
Residue		<0.1	0.2	0.3	2.7	2.8	3.0	<0.1	0.2	0.3	2.5	3.0	4.7
Recovery		98.7	99.7	93.9	93.6	82.5	78.2	98.4	99.8	96.7	94.4	94.1	97.0

[a] Average of triplicate analyses.
[b] Average of duplicate analyses.
[c] Radioactive material(s) that would not migrate in the TLC solvent system.

and found that the Galecron equivalents remained fixed on the leaf surfaces at higher levels than on the glass plates. Geissbühler (1966) suggests that either the base form of Galecron is rapidly dissolved or adsorbed in the waxy coating of the cuticular layer, or plant exudates —e.g., calcium oxalate—present on the leaf surface buffer the spray solution at a pH at which a considerable portion of the free base is converted to a salt form that does not volatilize.

The Galecron level in the leaf rinse decreased during the 20-day test period; the greatest decrease, 50%, occurred during the initial four days (Table I). However, a different picture was observed in the leaf extract with regard to the behavior of Galecron. The compound gradually increased in concentration to a level of 12% of the total applied radioactivity by 20 days.

The metabolites in the leaf rinse and extract were qualitatively similar with one exception; metabolite D was detected only in the leaf rinse. Generally, the concentration of the metabolites was slightly higher in the leaf rinse than in the extract; however, at none of the sampling intervals did the metabolites A, B, C, and D in or on the leaves account for more than 5% of the total applied radioactivity (Table I).

The loss of applied radioactivity increased with time. However, 46% still remained at 16 and 20 days after treatment; 41% was the parent compound (Table I). Much of the loss of radioactivity resulted from evaporation of the Galecron from the leaf surface during the initial four days after treatment; however, some of the compound could have been translocated into other plant tissues.

The nature and magnitude of the radioactive materials resulting from injection of Galecron into the main stem of apple seedlings are given in Table II. During the first four days after injection of the compound,

greater than 95% of the total radioactivity in the plant was localized in the stems and was the parent compound. Twenty days after treatment, the Galecron level in the stems had decreased to approximately 24%. The greatest decrement of Galecron in the stems was about 26%, and occurred between eight and 12 days. Concurrently, a 22% increase of radioactivity was observed in the stem residue. The relationship between these two events is not apparent at present.

Galecron was translocated into the apple leaves to the extent of 3% during the initial four days. By eight days the Galecron level was 9% and remained constant until the sixteenth day. Twenty days after treatment, the Galecron in the leaves constituted 18% of the total applied radioactivity in the plants (Table II). One explanation for this occurrence is that degradation was in equilibrium with translocation from days 8 through 16. However, after 16 days, translocation exceeded degradation, and this accounted for the 10% increase in Galecron concentration in the leaves observed at 20 days. Alternatively, it is possible that in the xylem there was a more favorable acropetal movement of Galecron than for its metabolites.

Galecron metabolites were detected in the stems by four days and in significant levels in the leaves by 12 days (Table II). Although the metabolites in the stems and leaves were similar qualitatively, quantitative differences were evident, with metabolites A, B, and C occurring in the stems in somewhat higher concentrations than in the leaves. However, the total amount of metabolites A, B, and C in the stems and leaves never exceeded 5.5% of the total radioactivity in the plants during the 20-day experimental period. Metabolite D was not detected in the stems or leaves when the plants were injected with Galecron. A small amount of radioactivity in the stems and leaves remained at the origin during chromatography and the identity of this material(s) is not known.

As was expected, the unextractable radioactive material(s) in the plant increased with time; 20 days after injection of the Galecron, approximately 35% of the total radioactivity was unextractable from the plants (Table II). Although the nature of this radioactive material in the residue is unknown, it seems unlikely that any free Galecron and/or metabolites were present, since the extraction procedure was very efficient in removing Galecron and certain metabolites from the plants. This material possibly represents Galecron degradation products that were complexed with polymeric cell constituents. In this regard, Yih *et al.* (1968) have demonstrated that 3,4-dichloroaniline, a metabolite of the herbicide 3',4'-dichloropropionanilide, complexed with lignin in the rice plant.

It is apparent that the lower total recoveries for the tritium than for the ^{14}C occurred because the water fractions from the stems and leaves in the tritium experiment were not radioassayed. That little, if any, loss of the injected radioactivity occurred by volatilization or expiration during the experimental period is evidenced by the high average recoveries of ^{14}C-containing material (97%).

The total radioactivity in the aqueous fraction increased with time, reaching a maximum of 11% in the stems and 0.3% in the leaves by 20 days (Table II).

Table III. Cleavage of Conjugates Recovered from Stems of Apple Seedlings 20 Days after Injection with ^{14}C-Galecron by Enzymes and Acid

	pH	Per Cent Cleavage[a]
β-Glucosidase	4.6	64.2
β-Glucuronidase	4.6	71.3
HCl	1.0	34.8
Control	4.6	3.0

[a] Calculated as % radioactivity in chloroform phase relative to that in the water plus chloroform fraction.

When an aliquot of the water fraction from the 20-day stems was incubated with enzymes capable of cleaving certain glycosidic bonds, a considerable portion of the radioactivity partitioned into the organic solvent (Table III). This indicated that portions of some or all of the radioactive degradation products were conjugated in the plant. Incubation of radioactive compounds in the 16-day water-soluble fraction with hydrochloric acid for 16 hours followed by TLC and radioautography of the chloroform extract revealed radioactive material that cochromatographed with Galecron, metabolites A, B, C, and D, and origin, plus six additional minor spots. However, metabolite C was present in greatest quantity, as judged by the intensity of the darkened area on the film.

One of the conjugates having metabolite C as an aglycone is probably *N*-(2-methyl-4-chlorophenyl)-D-glucosylamine, although the evidence is not decisive. (The identity of metabolite C as 4-chloro-*o*-toluidine was established.) Cochromatography of a radioactive plant conjugate with the synthetic glucoside was achieved in *n*-butanol–acetic acid–water (60:30:10). Moreover, in experiments in vitro, the synthetic glucoside is hydrolyzed by β-glucosidase or β-glucuronidase (Sen Gupta and Knowles, 1968), and several pesticides containing a free amino moiety or capable of being metabolized to a compound with a similar group are precursors of *N*-arylglucosylamines in certain plants (Colby, 1965; Ries *et al.*, 1968; Still, 1968).

Sufficient amounts of metabolites A, B, and C for positive identification were obtained from 10 apple seedlings injected with pure nonlabeled Galecron. Infrared spectra (Figure 2), melting point, mixed melting point, and cochromatography established the identity of metabolite A as demethyl-Galecron and metabolite B as *N*-formyl-4-chloro-*o*-toluidine. Infrared analysis and cochromatography indicated that metabolite C was 4-chloro-*o*-toluidine. Metabolite C also yielded an azo dye when reacted with sodium nitrite followed by *N*-ethyl-1-naphthylamine; Galecron, demethyl-Galecron, and *N*-formyl-4-chloro-*o*-toluidine yielded the dye only after hydrolysis. The infrared spectrum of Galecron isolated from apple seedling also was identical to the authentic standard. Infrared spectral analysis of these materials isolated from apple seedlings indicated that few, if any, impurities were present, thus providing evidence of the homogeneity of the zones on the TLC plates.

The major pathways for the metabolism of Galecron by apple seedlings are shown in Figure 3. The compound is *N*-dealkylated to demethyl-Galecron; this reaction is not a detoxication, since demethyl-Galecron possesses appreciable acaricidal activity (Boyd, 1968). Presum-

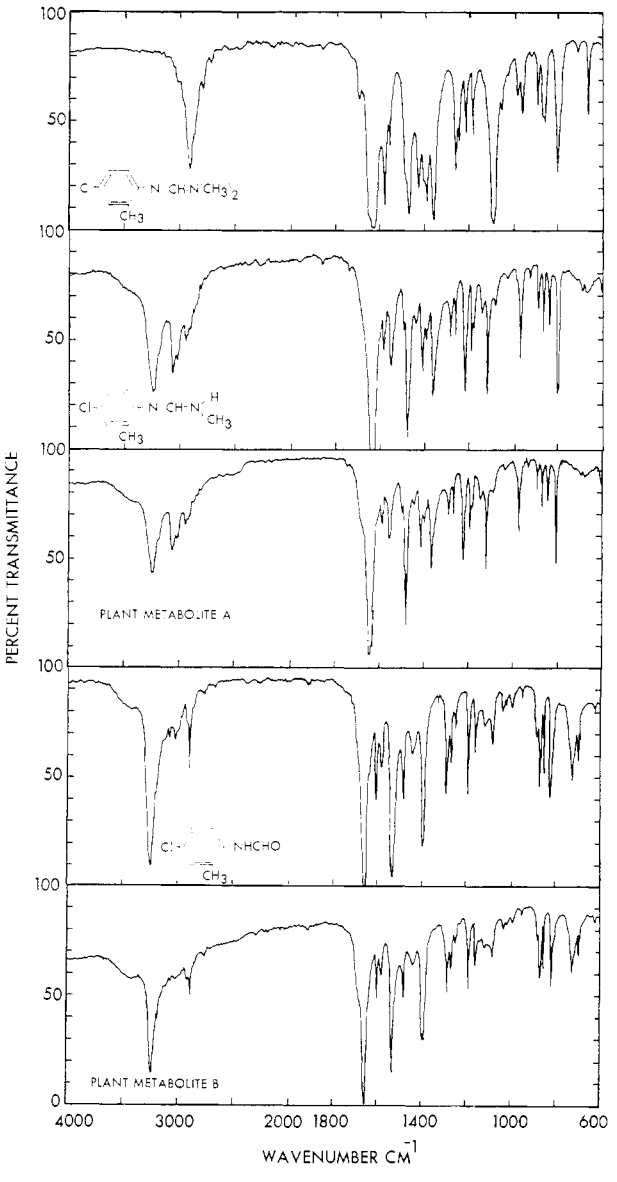

Figure 2. Infrared absorption spectra of authentic Galecron, demethyl- Galecron, and *N*-formyl-4-chloro-*o*-toluidine along with metabolites isolated from apple seedlings

Spectra were taken on a Beckman IR-10 infrared spectrophotometer. All samples were prepared as KBr pellets except Galecron, which was a neat liquid between salt plates

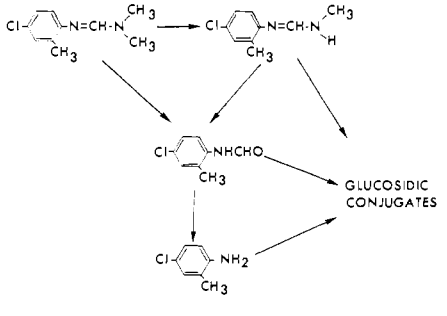

Figure 3. Proposed pathway of Galecron degradation in apple seedlings

ably, the formation of demethyl-Galecron occurred via a hydroxymethyl intermediate; however, a stable free *N*-methylol derivative was not identified. *N*-Hydroxymethyl compounds have been suggested as intermediates in a variety of *N*-demethylation reactions, and Menzer and Casida (1965) stated that these intermediates are probably more stable in the case of amides than with amines. *N*-Formyl-4-chloro-*o*-toluidine can be produced by cleavage of the carbon-nitrogen bond of Galecron and demethyl-Galecron, and the reaction proceeds rapidly in the presence of hydrogen ions (Knowles and Sen Gupta, 1969). Consequently, this compound was expected to be present in higher quantities than the levels that were actually found. In addition, hydrolysis of an aqueous fraction from the plants indicated that only small amounts of *N*-formyl-4-chloro-*o*-toluidine were conjugated. Perhaps the compound with its reactive formyl moiety combined rapidly with naturally occurring plant constituents—e.g., condensation with long-chain aldehydes—and was unextractable from the plant by the procedures used in this study. The 4-chloro-*o*-toluidine also was present in low amounts in the free form; however, the data indicated that the small quantities which actually occurred were readily conjugated.

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LITERATURE CITED

Abdel-Wahab, A. M., Kuhr, R. J., Casida, J. E., J. AGR. FOOD CHEM. **14,** 290 (1966).

Boyd, V. F., CIBA Agrochemical Co., Vero Beach, Fla., private communication, 1968.

Buyske, D. A., Kelly, R., Florini, J., Gordon, S., Peets, E., *Atomlight* (Issued by New England Nuclear Corp.) No. **20,** 1 (1961).

Colby, S. R., *Science* **150,** 619 (1965).

Dittrich, V., *J. Econ. Entomol.* **59,** 889 (1966).

Dittrich, V., *J. Econ. Entomol.* **60,** 558 (1967).

Enns, W. R., Dept. of Entomology, University of Missouri, Columbia, private communication, 1968.

Geissbühler, H., CIBA Ltd., Basle, Switzerland, private communication, 1966.

Geissbühler, H., Gross, D., CIBA Ltd., Basle, Switzerland, Analytical Bulletin, March 30, 1965.

Kadunce, R. E., *J. Chromatog.* **30,** 204 (1967).

Kelly, R. G., Peets, E. A., Gordon, S., Buyske, D. A., *Anal. Biochem.* **2,** 267 (1961).

Knowles, C. O., Sen Gupta, A. K., *J. Econ. Entomol.*, in press (1969).

Kuhr, R. J., Casida, J. E., J. AGR. FOOD CHEM. **15,** 814 (1967).

Menzer, R. E., Casida, J. E., J. AGR. FOOD CHEM. **13,** 102 (1965).

Ries, S. K., Zabik, M. J., Stephenson, G. R., Chen, T. M., *Weed Sci.* **16,** 40 (1968).

Sen Gupta, A. K., Knowles, C. O., Dept. of Entomology, University of Missouri, Columbia, unpublished data, 1968.

Still, G. G., *Science* **159,** 992 (1968).

Yih, R. Y., McRae, D. H., Wilson, H. F., *Science* **161,** 376 (1968).

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