in the reaction medium incubated under aerobic conditions. Nitrosoparathion was rather reduced to aminoparathion *via* hydroxylaminoparathion in the presence of spinach homogenate even under aerobic conditions. This finding indicates that the requirement for anaerobic conditions is not for the entire sequence of reactions, but for the initial stage of reactions, *i.e.*, the reduction of parathion to nitrosoparathion or hydroxylaminoparathion.

Since it is unlikely that anaerobic conditions prevail in live higher plants or mammals, with the exception of ruminants, the biological significance of reductive metabolisms described above may be minor. On the other hand, it has been clarified that the microsomal electron-transport system contributes to the microsomal nitro-reducing system (Gillette *et al.*, 1968; Sazame and Gillette, 1969; Yoshida and Kumaoka, 1969). Therefore, nitro-reducing activity is one of the indexes of microsomal drug-metabolizing enzymes. For a routine assay of nitro-reducing activity, the method of Fouts and Brodie (1957) has been so far used by which the amino analog alone is measured colorimetrically as a reaction product. Although hydroxylamino compounds are usually determined by the complex formation with sodium pentacyanoammine ferroate, nitroso compounds also form a similar complex with it. Therefore, hydroxylamino compounds are indistinguishable from nitroso compounds by such methods. In contrast with them, amino, hydroxylamino, and nitroso analogs of parathion were separately determined by the method described here. The present analytical method will be useful as a sensitive and selective assay method of nitro-reducing systems in various organisms. Gas–liquid chromatography of oxidative metabolites of parathion was also available as a sensitive and precise index of induction of liver microsomal drug-metabolizing enzymes (Davis *et al.*, 1973).

Experiments are now in progress to study the relationship between the nitro-reducing and the photosynthetic electron-transport systems in spinach chloroplasts.

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Metabolism of Siduron in Kentucky Bluegrass (*Poa pratensis* L.)

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Kentucky bluegrass "Merion" (*Poa pratensis* L.) growing in nutrient solution was treated with 1-(2-methylcyclohexyl)-3-phenylurea (siduron) and 1-(2-methylcyclohexyl)-3-phenyl-2-^{14}C-urea. Siduron and its metabolites were extracted, partitioned between chloroform and water, purified by column and thin-layer chromatography (tlc), separated by tlc, and characterized by infrared and mass spectrometry. Conjugates were hydrolyzed by enzymes. Four metabolites were isolated by tlc, three of which were characterized as 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea, 1-(2-hydroxymethylcyclohexyl)-3-phenylurea, and 1-(4-hydroxy-2-methylcyclohexyl)-3-phenylurea. The fourth metabolite was not identified.

The herbicide 1-(2-methylcyclohexyl)-3-phenylurea (siduron) may be used for preemergence control of certain grass weeds in newly seeded or established plantings of some cool-season turf grasses such as Kentucky bluegrass. De Mur (1971) reported on the absorption and translocation of siduron and inhibition of the Hill reaction in resistant Kentucky bluegrass "Merion" (*Poa pratensis* L.) and susceptible bermuda grass "Santa Ana" (*Cynodon dactylon* L.). He proposed that the differential rate of metabolism is the basis of selectivity of siduron. Splittstosser and Hopen (1968) reported that siduron was not metabolized in barley (*Hordeum vulgare* L.). The metabolism of siduron has been studied in animals and soils by Belasco and Reiser (1969) and Belasco and Langsdorf (1969). They also found and identified three metabolites, which were conjugates of hydroxylated siduron. The objectives of the research reported here were to study the metabolism of siduron in Merion Kentucky bluegrass and to identify the major metabolites.

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MATERIALS AND METHODS

Chemicals. The herbicide 1-(2-methylcyclohexyl)-3-phenyl-2-^{14}C-urea and potential metabolite reference compounds were supplied by E. I. DuPont, Wilmington, Del. The purity of the ^{14}C-labeled compound was greater than 99%. This was determined by spotting 2 μl of a 20 μCi/ml solution of ^{14}C-labeled herbicide on silica gel G thin-layer plates. The plates were then developed in two dimensions with chloroform–ethyl acetate (8:9, v/v) and chloroform–methanol (9:1, v/v). A Berthold thin-layer radioscanner was used to determine the position of radioactive spots. The labeled siduron compound migrated as a single radioactive spot. The enzymes α-glucosidase, β-glucosidase, and hesperidinase were purchased from Sigma Chemical Co.

Treatment of Plants. Merion Kentucky bluegrass seeds were germinated in sand culture in a greenhouse. After germination, seedlings were watered daily with half-strength Hoagland solution. When the plants were approximately 5 weeks old, they were transferred in groups

of 4 into 125-ml erlenmeyer flasks containing 125 ml of half-strength Hoagland solution. Each flask was wrapped with aluminum foil. The solution was maintained constantly aerated and its level was adjusted daily by adding either water or nutrient solution. After the plants became established in the new conditions (3–4 days), the solution in which the plants were growing was discarded and a new solution (125 ml) was added which contained siduron-2-^{14}C. Treatment consisted of 2 μCi per culture (specific activity 1.16 μCi/μmol), which was 3.2 μg/ml (1.38×10^{-5} *M*).

Bulk treatments were also made with nonlabeled siduron (3.2 mg/l.) to obtain sufficient material for analysis. These treatments were carried out in 4-l. porcelain jars lined with polyethylene bags and filled with half-strength Hoagland solution. Each jar was covered with a 3-mm sheet of fiberboard with 22 holes. Four plants were inserted in each hole with the roots in the solution and the shoots above the fiberboard sheet. The plants were supported in the holes by wrapping with pieces of polystyrene foam. Aeration, conditioning, and treatment with unlabeled siduron were as described for flask treatments.

Extraction Procedures. The plants were harvested 4 days after treatment. The roots of each plant were washed with distilled water, and the washings added to the treatment solution. The plants were extracted and the extracts processed as indicated in Figure 1. The plants were divided into shoots and roots, after which each plant part was cut and put in 80% aqueous ethanol. The shoots and roots were then extracted by homogenizing with a high speed Virtis blender for 3 min. The homogenates were then filtered and the residues rinsed with more 80% ethanol and homogenized again. The operation was repeated twice. The total volume of the filtrate was measured and recorded. The treatment solution was also filtered and the volume measured.

Radiochemical Assays. The radioactivity of the different extracts was determined by placing aliquots into scintillation vials containing 15 ml of Bray's solution (Bray, 1960). The ^{14}C activity was determined with a liquid scintillation spectrometer. Toluene-^{14}C was used as an internal standard to determine the counting efficiency.

Ten-milliliter aliquots of each crude extract (from shoots and roots) and also of the treatment solution were partitioned between chloroform and water. Each 10-ml aliquot was placed in a vial and evaporated to near dryness on a warm water bath (at 50°) under a stream of nitrogen. Ten milliliters of water and 10 ml of chloroform were added to the residue and the vial shaken vigorously. The radioactivity of each phase was then determined. The total radioactivity in each extract was also determined prior to partitioning. An aliquot of each extract was concentrated and streaked on thin-layer plates and developed as described below. After development, the radioactive streaks on the plates were scraped, extracted, and the extracts radioassayed for ^{14}C. The relative amounts of radiocarbon-containing compounds were estimated by this method.

Purification of Plant Extracts. The crude extracts from shoots and roots were concentrated and partitioned between chloroform and water (Figure 1). The chloroform phase was concentrated to near dryness and the residue taken up in 50% aqueous acetone. The precipitated chlorophylls were filtered off. The filtrate was then concentrated and run through a polyethylene column to separate the rest of the chlorophylls, xanthophylls, and carotenes.

The water phase was incubated with β-glucosidase and hesperidinase in sodium acetate buffer (0.1 *M*, pH 4.8) at 35° for 24 hr. The enzyme-treated solution was then extracted with chloroform and the radioactivity in each phase determined. This new chloroform-soluble fraction was again concentrated for further purification by tlc.

Column Chromatography. The columns were made up

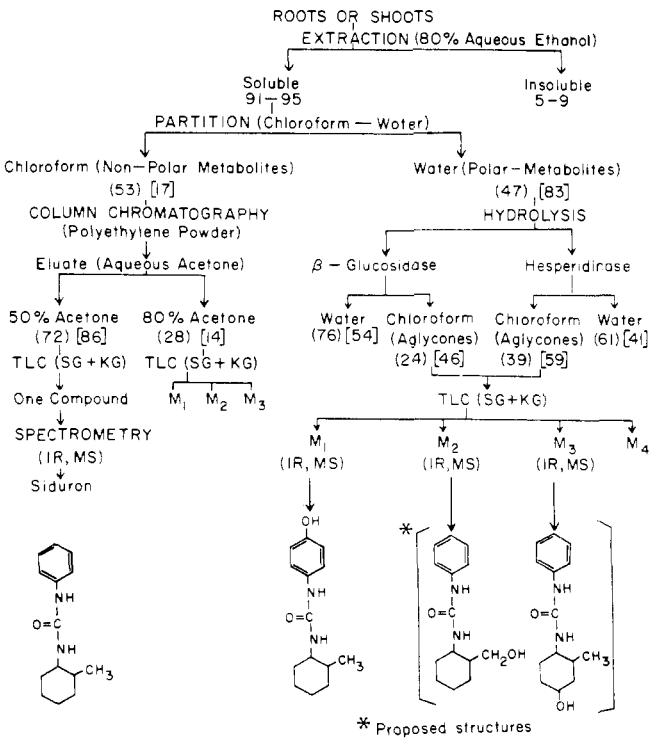

Figure 1. Schematic diagram for extraction, separation, and characterization of siduron, one definite metabolite (M_1), and two proposed metabolites (M_2 and M_3) from Merion Kentucky bluegrass treated 4 days with culture solutions containing siduron-2-^{14}C. The percentages of the ^{14}C-labeled compounds from the roots are enclosed by parentheses while the per cent located in the shoots are enclosed by brackets.

of polyethylene powder (FN-510 from U.S. Industrial Chemicals; particle size 30 μ, melt index 5) which was tightly packed into a column of 2.5 cm i.d. and to a height of 22 cm. The polyethylene powder had been washed previously three times with acetone, once with hexane, and then vacuum dried. The samples were eluted with 50% aqueous acetone until the first dark band (the xanthophylls) was 3–4 cm from the bottom of the column. An aqueous acetone (80%) was used to elute the remaining chlorophylls. Finally, hexane was applied to the top of the column to elute the carotenes. Several fractions were collected and assayed for radioactivity. All of the radioactive compounds were found in the first 10–15 ml of the eluate. The fraction containing the radioactivity was concentrated for further purification on tlc.

Thin-Layer Chromatography. Thin-layer plates 20 × 20 cm were prepared using a mixture of silica gel G and Kieselguhr G (2:3, w/w), to which a small amount of inorganic fluorescent material was added. The plates were developed in methanol and activated at 100° for 30 min before use. The developing solvent systems were benzene–ethyl acetate (1:4, v/v) and chloroform–methanol (9:1, v/v). The final solvent front was 15 cm from the origin. After scanning, the radioactive spots were scraped and extracted with acetone–methanol (1:1, v/v). An aliquot was taken for counting and the rest was concentrated and respotted repeatedly to obtain maximum purity. The above purification procedure was sufficient in most cases. Sometimes, however, a yellow pigment chromatographed with the metabolites, and a final purification procedure was needed. Purification was performed with silica gel plates developed in two solvent systems, first with benzene–ethyl acetate (1:1, v/v) up to the 10-cm level, and second with chloroform–methanol (9:1, v/v) to the 15-cm level. The yellow pigment separated into two spots (bands) with metabolite between them.

Table I. Distribution of Radioactivity from Siduron-2-^{14}C after Chloroform–Water Partition and Tlc Separation of Crude Extracts from Merion Kentucky Bluegrass and Treatment Solution

	Radiocarbon location				
			Tlc separation, %		
	Solvent partition, %			Metabolites	
Source	Chloroform	Water	Siduron-2-^{14}C	Rel nonpolar	Polar
Shoots	17.1	82.9	12.9	2.1	85.0
Roots	53.4	46.6	36.2	14.1	49.7
Treatment solution	99.1	0.9			

Table II. Distribution of Metabolites of Siduron-2-^{14}C after Treatment of the Water-Soluble Fraction from Merion Kentucky Bluegrass with β-Glucosidase

		Metabolites, %			
Source	Enzyme	M_1	M_2	M_3	M_4
Shoots	β-Glucosidase	23.1	45.5	31.5	0
Roots	β-Glucosidase	11.8	40.5	11.9	35.8

RESULTS

Plant Extracts. Merion Kentucky bluegrass plants absorbed 20% of the radioactive siduron from a nutrient solution in which they were grown for 4 days. The efficiency of extraction of ^{14}C-labeled compounds from plant tissue with 80% aqueous ethanol ranged from 91 to 95% as determined by combustion (Davidson and Oliverio, 1967) and liquid scintillation spectrometry (Figure 1). The shoots contained 75% and the roots 25% of the absorbed radioactivity. Tlc of plant extracts showed the presence of three types of radiocarbon substrates: siduron, relatively nonpolar metabolites, and polar metabolites (Table I). Most of the radioactivity in the shoots and about 50% in the roots was polar material which remained at the origin of the plate. Partition of the extracts between chloroform and water indicated that approximately the same amount of radioactivity that remained at the origin was now found in the water phase (Figure 1 and Table I). This indicated that the major metabolites were polar conjugates which did not chromatograph when developed in nonpolar organic solvents. The chloroform phase contained mainly siduron with 28 and 14% relatively nonpolar metabolites in the roots and shoots, respectively (Figure 1). Tlc resulted in three spots with the same R_f values as the three principal metabolites discussed below.

The water-soluble metabolites from shoots and roots were partially hydrolyzed with β-glucosidase and hesperidinase. The free metabolites (aglycones) were now extractable with chloroform. A comparison of aglycone formation after hydrolysis with β-glucosidase and hesperidinase is shown in Figure 1.

Tlc of the chloroform-soluble metabolites revealed three metabolites in the shoots and four in the roots. The relative amount of each metabolite in the chloroform fraction is presented in Table II.

The R_f values for the four metabolites are given in Table III. It is evident that the three major metabolites in the shoots and roots were similar; these will be referred to as M_1, M_2, and M_3 in this article. The fourth metabolite (M_4) occurred in low concentrations, and could be found only in the roots and the treatment solution.

Table III. R_f Values[a] for Siduron and Metabolites Obtained from Shoots and Roots of Merion Kentucky Bluegrass

	R_f	
Substrate	Shoots	Roots
Siduron, standard	0.90	0.90
extracted	0.90	0.90
M_1, standard[b]	0.75	0.76
extracted	0.75	0.75
M_2	0.60	0.58
M_3	0.50	0.48
M_4		0.17

[a] On silica gel G and Kieselguhr G (2:3, w/w) developed with benzene–ethyl acetate (1:4, v/v). [b] 1-(*p*-Hydroxyphenyl)-3-(2-methylcyclohexyl)urea.

Treatment Solution Extracts. After partition of the treatment solution between chloroform and water, most of the radioactivity was detected in the chloroform phase (Table I). All of the chloroform-soluble radiocarbon substrate was determined to be siduron by infrared and mass spectrometry. The water-soluble metabolites were treated with β-glucosidase and extracted with chloroform. Tlc of this chloroform extract revealed low concentrations of M_1, M_2, M_3, and M_4.

CHARACTERIZATION OF METABOLITES

After repeated purification on tlc, enough M_1, M_2, and M_3 were obtained for further identification by infrared and mass spectrometry. The pure sample was incorporated into a KBr pellet, and the pellet was scanned on a Beckman (IR-12) infrared spectrometer. A portion of the same sample was dissolved in acetone and analyzed in a Finnigan 1015 S/L mass spectrometer.

Metabolite M_1 had an R_f value identical with the authentic compound 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea. Metabolite M_1 and the standard decomposed on tlc plates after 3–4 hr. All metabolites were extracted from the plates immediately after development to avoid decomposition. M_1 also had mass and infrared spectra (Figure 2) identical with the standard. Thus, M_1 is 1-(*p*-hydroxyphenyl)-3-(2-methylcyclohexyl)urea. Its structure is shown in Figure 1.

Metabolites M_2 and M_3 had the following fragments in their mass spectra (*m/e*, 70 eV at 150°); M_2, 18 (28), 28 (16), 29 (11), 41 (16), 43 (29), 55 (10), 57 (10), 77 (7), 93 (100), 119 (3), 135 (2), 208 (0.3), 230 (0.2), 248 (2); M_3, 18 (21), 28 (19), 29 (12), 41 (30), 43 (31), 55 (14), 57 (18), 77 (7), 93 (100), 135 (3), 221 (2), 248 (2). The molecular weights of M_2 and M_3 were 248 compared to a molecular weight of 232 for siduron and 248 for M_1. No hydroxylation occurred on the phenyl ring since both spectra showed a strong peak at mass 93, which corresponds to aniline. Peaks at mass 119 and 135 are due to the phenyl isocyanate and phenylureido fragments. The additional oxygen atom in these two metabolites would be located in the cyclohexyl portion of the molecule. The difference between M_2 and M_3 would be the position of the hydroxyl group on the methylcyclohexyl moiety.

The infrared spectra of M_2 and M_3 differed only in some regions of the spectra (Figure 2). For purposes of comparison, the ir spectra of siduron and M_1 are indicated in Figure 2. The main differences are the two absorption bands at 810 and 1380 cm^{-1} which are present in the spectrum of M_3 and absent in that of M_2. The band at 1380 cm^{-1} is characteristic for δ values of the methyl group. This may indicate that the hydroxyl group in M_2 is

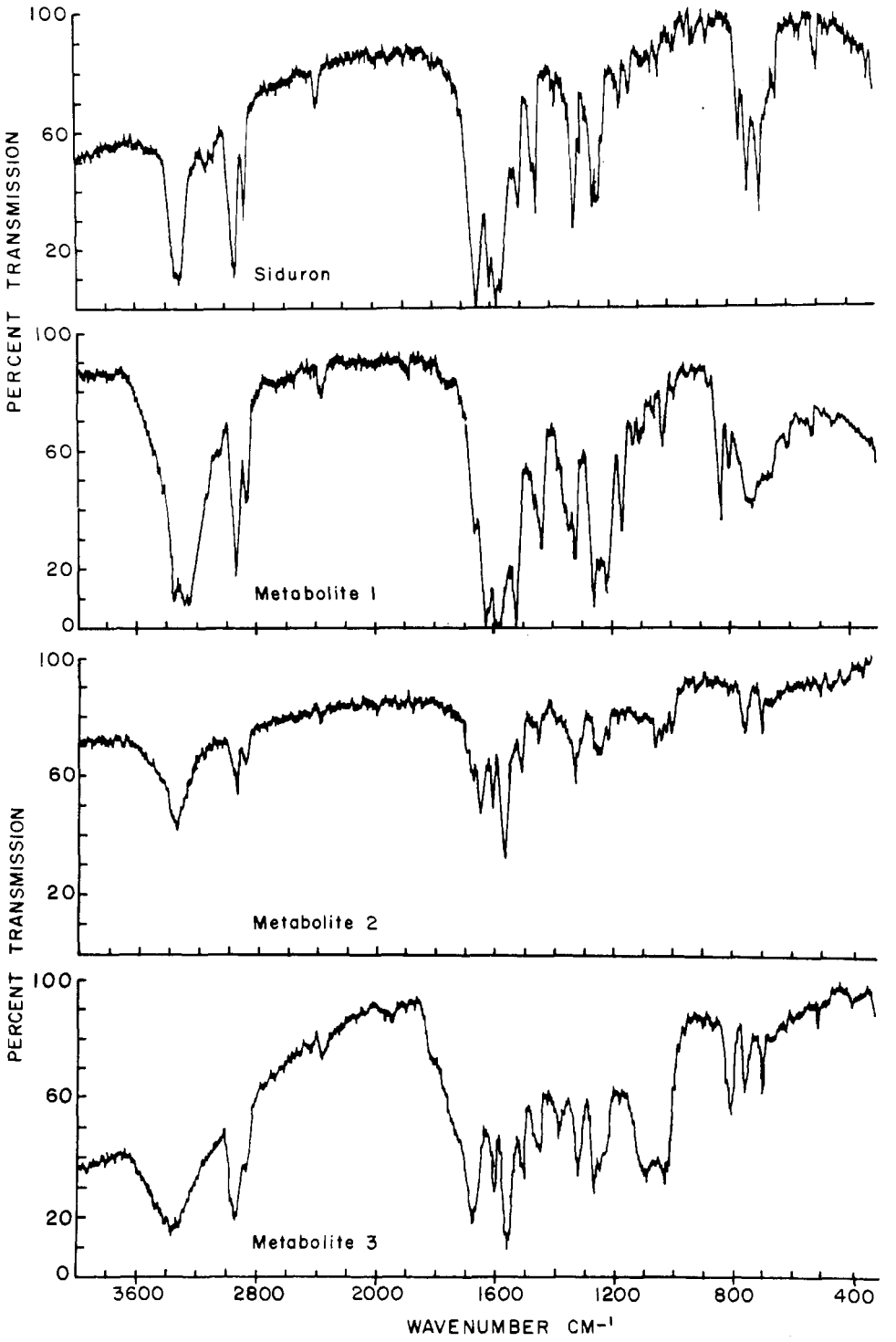

Figure 2. Infrared spectra (KBr pellet) of siduron and metabolites M_1, M_2, and M_3 isolated from Merion Kentucky bluegrass (*Poa pratensis* L).

on the 2-methyl group of the cyclohexyl substituent and in M_3 on the 4 position of the cyclohexyl ring (Belasco and Reiser, 1969). Therefore, metabolites M_2 and M_3 may be 1-(2-hydroxymethylcyclohexyl)-3-phenylurea and 1-(4-hydroxy-2-methylcyclohexyl)-3-phenylurea, respectively (Figure 1). We did not have enough of these metabolites for further analysis by nmr spectrometry; therefore we could not verify the exact location of the hydroxyl groups on the methylcyclohexyl moiety. The metabolite M_4 found in the roots and treatment solution remains unidentified.

DISCUSSION

Siduron was absorbed by Merion Kentucky bluegrass plants from the nutrient culture in which they were grown. It is evident that siduron is hydroxylated on both the phenyl and methylcyclohexyl substituents. On the latter, hydroxylation may occur on either the ring or methyl group. The hydroxylation of siduron is followed by conjugation; on the basis of enzyme hydrolysis, the major conjugates are most likely glucosides. Hydroxylation of the methyl group on the cyclohexyl substituent was not reported by Belasco and Reiser (1969) in the metabolism of siduron in dogs. Metabolites containing two hydroxyl groups on the phenyl and methylcyclohexyl substituents were not formed in Merion Kentucky bluegrass or were found in such minute amounts (*e.g.*, M_4 in roots) that we could not identify them.

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Metabolism Studies with Ethephon in Cherry Leaves

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The presence of a metabolite of [^{14}C]ethephon in leaves of Montmorency cherry was indicated by thin-layer and anion exchange chromatography and autoradiography. The metabolite may contain an intact phosphonic acid group. Matrix interferences prevented its further characterization by mass spectrometry.

Ethephon, (2-chloroethyl)phosphonic acid, is a growth regulator which elicits a variety of responses in fruit, vegetable, and certain other plants. It was initially available as a formulation designated as 66-329, and more recently as 68-240. The trade name for the commercial formulation is Ethrel. Ethephon accelerates color development and hastens fruit maturity of cherries (Bukovac *et al.*, 1969). It may also be a potential aid to mechanical harvesting of cherries by accelerating fruit abscission (Bukovac *et al.*, 1969; Edgerton and Hatch, 1969). Residues of intact ethephon are detectable in treated cherry fruit at harvest (Edgerton and Hatch, 1972). Using ^{14}C-labeled ethephon, thin-layer chromatography, and autoradiography, evidence for the production of metabolites of ethephon has been observed in cherry leaves (Edgerton and Hatch, 1972; Monselise, 1973) and peach fruit (Abdel-Gawad and Martin, 1973; Lavee and Martin, 1974). Evidence for the presence of ethephon metabolites in cherry fruit has not been published. In the work reported, an investigation was undertaken to determine the presence and character of possible metabolites of ethephon in cherry leaves.

EXPERIMENTAL SECTION

Leaf Treatment. Leaves of Montmorency cherry trees grown either under greenhouse conditions or in an orchard receiving standard management practices were thoroughly covered with an aqueous solution of [^{14}C]ethephon (500 ppm, 1.5–4.0 μCi of ^{14}C) according to the procedure of Edgerton and Hatch (1969). Tween 20 (0.1% v/v) was added as a wetting agent. The ethephon-treated leaves were harvested at 3 and 11 days following application and stored at −18° until use. Control leaves were collected from appropriate untreated cherry trees and stored in a similar fashion.

Extraction and Sample Cleanup. The frozen leaf samples were freeze-dried for 18–24 hr prior to grinding in a Wiley mill. About 1 g of the green powder was Soxhlet-extracted for 8 hr with 150 ml of benzene. The benzene extract was discarded. After drying the thimble contents, the residue was Soxhlet extracted with 150 ml of methanol for 8 hr. The methanol solution was evaporated to 10 ml with the aid of a rotary evaporator. The methanol extract was stored at −10° until assay.

Thin-Layer Chromatography. Leaf extracts (100–200 μl) were spotted on either 5 × 20 cm Eastman cellulose sheet (No. 6040; Eastman Organic Chemicals, Rochester, N.Y.) or on 5 × 20 cm silica gel plates (SilicAR, TLC-4G; Mallinckrodt Chemical Works, New York, N.Y.) and developed in either methanol–isopropyl alcohol–ammonia–water (9:6:1:3, v/v) or water–isopropyl alcohol–ethanol–ammonia, 100:50:50:10 (v/v). On one occasion, leaf extract (0.25 ml) was treated with diazomethane (Schlenk and Gellerman, 1960) prior to development in chloroform–ethyl acetate–acetone (18:4:1, v/v).

Radioactive materials were located on the chromatogram by scraping off consecutive zones of adsorbent and measuring their radioactivity by liquid scintillation counting using a Packard Model 3310 Tri-carb liquid scintillation counter (LSC), and by autoradiography of the intact thin-layer plate. Plates and sheets were prepared for LSC assay by dividing each chromatogram into ten equal regions such that zone "0" contained materials remaining at the origin, zone "1" held compounds with an average R_f value of 0.1, etc. The appropriate region from silica gel plates was assayed by LSC after scraping the gel into 10 ml of toluene scintillation mixture containing PPO (0.5% w/v) and POPOP (0.01% w/v). Sections from cellulose chromatograms were assayed in a similar manner after cutting each zone into four pieces of equal size. In the case of autoradiography, the plates were exposed to Kodak medical X-ray film (Royal X-mat) for 4 weeks prior to film development.

Gas Chromatography. The chromatograms were prepared for gas chromatographic analysis by eluting gel previously scraped from silica gel plates with 0.5 ml of methanol. The methanol solutions were treated with diazomethane by the procedure of Schlenk and Gellerman (1960). Following esterification, the volume was adjusted to 1 ml and 1-μl aliquots were injected into a Varian Aerograph Model 705 gas chromatograph (Varian Aerograph, Walnut Creek, Calif.). The gas chromatograph was equipped with a cesium bromide thermionic detector. The borosilicate glass column (4 ft × $^5/_{32}$ in. inside diameter) was packed with 5% Carbowax 20M on 80–100 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Gas flow rates for hydrogen, nitrogen, and air were 25, 35, and 200 ml/min, respectively. The injector, column, and detector were operated at 215, 145, and 235°, respectively.

Ion Exchange Chromatography. One milliliter of the

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