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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 con-

tain 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 \times $\frac{5}{32}$ 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 $^\circ$, respectively.

Ion Exchange Chromatography. One milliliter of the

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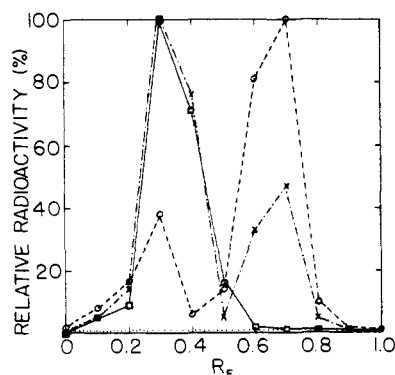


Figure 1. Thin-layer chromatogram showing ethephon and a metabolite in cherry leaves 3 days after treatment with [^{14}C]ethephon (X-.-), the same 11 days after treatment (O-.-), [^{14}C]ethephon fortified to control cherry leaf extract (□-), and control leaf (·-·). Support was Eastman No. 6040 cellulose and solvent system was methanol-isopropyl alcohol-water-ammonia (9:6:3:1, v/v).

methanol leaf extract was mixed with 1 ml of water prior to introduction into 14×0.8 cm (i.d.) columns packed with anion exchange resin (AG1-X10, 100-200 mesh, Calbiochem, Los Angeles, Calif.). The sample was subsequently washed with 30-50 ml of water before elution with 150 ml of 0.12 M lithium chloride. Fractions (5 ml) were collected and later assayed for radioactivity in 10 ml of Aquasol (New England Nuclear, Boston, Mass.).

RESULTS AND DISCUSSION

Prior to the treatment of cherry leaves, ethephon standards (both ^{14}C labeled and "cold") were assayed for purity by several methods. A single peak having a retention time of 5.9 min was obtained by gas chromatography. In contrast, thin-layer chromatographic analysis of [^{14}C]ethephon standard developed using methanol-isopropyl alcohol-water-ammonia (9:6:3:1, v/v) and examination by autoradiography revealed three spots suggesting traces of impurities in the radioactive standard (see Figure 2). The presence of small amounts of impurities in the labeled standard, some having molecular weights greater than ethephon, was subsequently also verified by electron impact mass spectrometry using a Finnigan Model 1015 instrument. The source pressure was 2×10^{-7} Torr.

[^{14}C]ethephon was quantitatively recovered by Soxhlet extraction of control cherry leaf extract fortified with 1 and 0.1 ppm of ethephon. The respective recoveries were 94 and 98%. No appreciable radioactivity was measured in the benzene rinse.

The results in Figure 1 indicate that two radioactive materials are present in extracts from cherry leaves harvested at 3 and 11 days after application of [^{14}C]ethephon. The radioactive peak (R_f 0.3) corresponds to that of standard [^{14}C]ethephon developed under identical conditions. A more rapidly eluting material was exhibited at R_f 0.7 and may have represented either a metabolite or a physicochemical breakdown product of [^{14}C]ethephon. Radioactive materials were not present in the control. The relative quantity of the radioactive materials represented by the earlier eluting peak (R_f 0.3) was twofold greater in the 3-day as compared to that obtained from the 11-day harvest sample, suggesting either that ethephon is more rapidly lost from the leaf than the later eluting compound (R_f 0.7) or that the latter compound accumulates with time in the treated cherry leaves.

The presence of these spots was revealed when the sample extract was developed by thin-layer chromatography and then examined by autoradiography (see Figure 2). The spot at the origin may have represented an artifact of the experimental procedure since corresponding spots were observed after development of either the control ex-

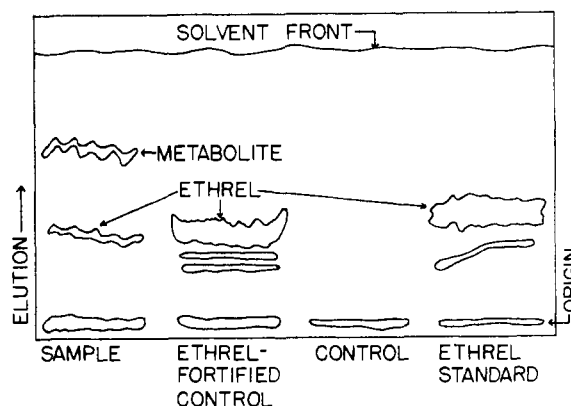


Figure 2. Autoradiogram of thin-layer chromatogram showing the metabolite of ethephon 11 days after treatment with [^{14}C]ethephon. Support was silica gel H and solvent system was methanol-isopropyl alcohol-water-ammonia (9:6:3:1, v/v). Quantities of radioactive material in nanocuries spotted on the plate were: sample (0.05), fortified control (45), and standard (9).

Table I. Thin-Layer Chromatographic Analysis of Extracts of Leaves Treated 11 Days Earlier with [^{14}C]ethephon and the Control Fortified with [^{14}C]ethephon

Solvent system	R_f	
	Control + [^{14}C]Sample	[^{14}C]ethephon
Methanol-isopropyl alcohol-water-ammonia (9:6:3:1, v/v) ^a	0.9, 0.7	0.7
Water-isopropyl alcohol-ethanol-ammonia (100:50:50:10, v/v) ^a	0.9	0.7
Chloroform-ethyl acetate-acetone (18:4:1, v/v) ^b	0.0, 0.3	0.3

^a Chromatography on silica gel (pH 4) followed by scraping off and liquid scintillation counting. ^b Development on silica gel (pH 4) after methylation.

tract or of the control fortified with [^{14}C]ethephon (Figure 2). It has been reported (Williams, 1951) that this artifact can be due to mechanical pressure. The additional spot (the first above the origin) appearing in the fortified control and not in the sample or standard was very faint and presumably due to an impurity in the [^{14}C]ethephon standard which was detected only because the amount of [^{14}C]ethephon represented (45 nCi) was much larger than that in the sample (0.05 nCi) and in the standard (9 nCi).

Additional evidence supporting the contention that a radioactive compound in addition to ethephon is present in the treated leaf extracts is reported in Table I. Radioactive material that eluted more rapidly than standard [^{14}C]ethephon was present on cellulose sheets to which the sample was applied and subsequently developed either in methanol-isopropyl alcohol-water-ammonia (9:6:3:1, v/v) or in water-isopropyl alcohol-ethanol-ammonia (100:50:50:10, v/v). The occurrence of only one radioactive spot on chromatograms developed in the latter solvent suggested that [^{14}C]ethephon and the compound of unknown identity were poorly resolved in the latter solvent system. Diazomethane treatment of the 11-day leaf extract prior to thin-layer chromatography in chloroform-ethyl acetate-acetone (18:4:1, v/v) also resulted in two ra-

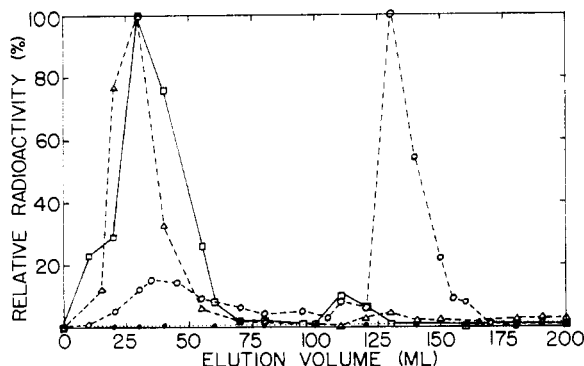


Figure 3. Ion exchange chromatograms showing elution of ethephon and a metabolite in cherry leaves 11 days following application (O ---), [^{14}C]ethephon fortified to control cherry leaf extract (□ -), control leaf extract (· · ·), and [^{14}C]ethephon standard (Δ ---).

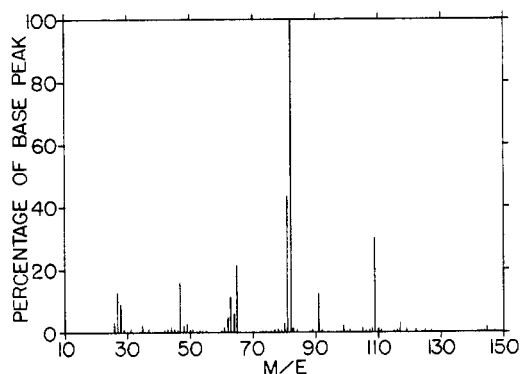


Figure 4. Mass spectrum of standard unlabeled ethephon after its chromatography and collection from the anion exchange column.

radioactive spots; one at the origin and another at an R_f of 0.3.

Attempts were made to isolate and characterize metabolites of ethephon using anion exchange chromatography and mass spectrometry. Residues in the 11-day sample of the raw cherry leaf extract were chromatographed through the anion exchange resin. The recovery of 1 ppm of ethephon from control cherry leaves following extraction and anion exchange chromatography was 87%.

As shown in Figure 3, [^{14}C]ethephon was eluted as a single peak whether added to the column as a pure standard or as the standard fortified to control cherry leaf extract. Radioactive materials were also eluted, however, in the eluate fraction of [^{14}C]ethephon standard from 110 to 140 ml and in the ethephon fortified control from 100 to 130 ml. The elution of the 11-day leaf extract from the anion exchange column also yielded radioactive materials with elution volumes of 15–55 and 110–170 ml presumed to be, respectively, [^{14}C]ethephon and a metabolite of it. These latter two fractions as well as the 15–55-ml column fraction of pure standard [^{14}C]ethephon were freeze-dried and subsequently assayed by mass spectrometry. Mass spectrograms of unlabeled standard ethephon before and after anion exchange chromatography were identical indicating that the compound was not chemically altered on the column. Thus, both spectra showed the presence of a chloroethyl fragment at m/e 63, a phosphonic acid moiety at m/e 82, a fragment at m/e 109 (probably ethephon minus chlorine), and an $(M + 1)^+$ ion at m/e 145. The mass spectrum of standard ethephon after anion exchange chromatography is shown in Figure 4. Mass spectra of the isolated fraction (110–170 ml) believed to contain the metabolite could not be interpreted, however owing to the presence of matrix interferences. The fact that this metabolite was able to be chromatographed on the anion exchange resin column indicates that it could contain an intact phosphonic acid or other anionic group.

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