The Movement and Fate of (2-Chloroethyl)phosphonic Acid in Walnut

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Abstract. $^{14}$C-ethephon applied to a walnut leaflet penetrated and translocated rapidly in young plants, but more slowly in older plants. The compound translocated to the kernel at higher levels when applied to a leaflet than when applied to the hull but in both cases levels of activity were low. Between 5 and 7 days after application the radioactivity in the kernel decreased markedly. $^{14}$C-ethephon in the leaves, hull, shell, and kernel was metabolized, but no side products remained in the plant tissue that could be detected by the techniques employed.

As early as 1950 it was known that ethylene gas could induce hull dehiscence in walnut and thus aid harvest operations (3), however, practical application of this knowledge was limited. The advent of (2-chloroethyl)phosphonic acid (ethephon), a compound which releases ethylene upon decomposition (1,4), has provided a practical means for regulating use of ethylene as a harvest-aid for walnut (2). Information pertinent to the movement and fate of ethephon would enhance its ultimate use on walnut.

The purpose of these studies was to examine the translocation, breakdown, and fate of ethephon in walnut.

Materials and Methods

Mature trees of Juglans regia L. 'Hartley' and 'Franquette', growing in the University of California orchard at Davis, were used. 'Hartley', an early-maturing cultivar, was used in the first 2 experiments, and 'Franquette', a late-maturing cultivar was used in the later experiments. No differences in results were noted between the 2 cultivars therefore they are not referred to individually in the text. Uniform shoots containing 1 leaf and the terminal 2 fruits were tagged and hand-sprayed with freshly prepared ethephon solution containing 0.05% X-77. All treatments were duplicated and the values presented in tables and figures are averages. After 30 min all leaflets, except 1, were removed. $^{14}$C-ethephon was then applied with a micro-syringe at 0.5 to 1.0 μl to the upper surface of the leaflet or the surface of the hull. Additionally, a leaflet flap prepared by trimming the leaflet blade to the midrib, was submerged in a vial containing $^{14}$C-ethephon for 24 hr.

Preparation of tissue for extraction. On designated days leaflets and fruits were harvested and immediately brought to the laboratory. There the fruits were separated into different parts that were either stored in small, closed, plastic vials at -18°C and freeze-dried before extraction, or were immediately extracted for determination of total $^{14}$C activity. For extraction, the frozen tissues were ground in a small mortar with a pestle and quantitatively transferred to plastic centrifuge tubes containing 25 ml absolute methanol. The tubes and contents were mechanically shaken for 48 hr after which the material was centrifuged at 12,000 rpm for 30 min. The supernatant fluid was then decanted into a 100-ml volumetric flask, and the pellet was resuspended in 15 ml absolute methanol and reextracted 4 times for 48 hr each. Ethephon at 250 ppm in absolute methanol was used in the final 2 extractions and in pellet and tube washings. Also, all containers and filter papers were pretreated with 250 ppm ethephon to facilitate extraction and minimize adsorption of $^{14}$C-ethephon. Determination of total $^{14}$C activity. One ml or 5 ml of the methanolic extract were transferred to plastic scintillation vials to which 15 ml of scintillation fluid was then added (44 ml absolute methanol whenever 1 ml of extract was used). Radioactivity was measured at least 3 times for 10 min each.
Fig. 3. Autoradiograph of TLC plates spotted with extracts from walnuts treated with \(^{14}\text{C}-\text{ethephon}\). Plates were developed for 10 cm in benzene, concd acetic acid, and water (8:3:5, v/v/v). From left to right the plates are standard \(^{14}\text{C}-\text{ethephon}\) and extracts from leaf, hull and kernel. The outlined section includes the radioactive area of each plate.

using a liquid scintillation spectrometer (Packard Tri Carb Model 3003). The liquid scintillation fluid consisted of 500 ml 1,4-dioxane; 100 ml 2-ethoxyethanol; 6.0 g PPO; 0.3 g POPOP; and 30.0 g naphthalene.

**Metabolic studies.** To minimize loss of \(^{14}\text{C}\) from ethephon during subsequent handling, the methanolic extract was acidified with concd HCl to a pH of 1.0. The acidified extract was then dried under vacuum at 30\(^\circ\)C. The dried material was dissolved in 4 ml of absolute methanol for analysis. Depending on the amount of radioactivity, 25 or 50 \(\mu\)l of the solutions

Table 1. Total \(^{14}\text{C}\) radioactivity recovered from different portions of walnut fruits treated with \(^{14}\text{C}-\text{ethephon}\).a

<table>
<thead>
<tr>
<th>Method of (^{14}\text{C}-\text{ethephon}) application</th>
<th>Hull cpm</th>
<th>% of activity applied</th>
<th>Shell cpm</th>
<th>% of activity applied</th>
<th>Kernel cpm</th>
<th>% of activity applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaflet flap (0.5 (\mu)c)</td>
<td>1433</td>
<td>0.121</td>
<td>337</td>
<td>0.028</td>
<td>1040</td>
<td>0.088</td>
</tr>
<tr>
<td>Fruit stem (peduncle) flap (0.1 (\mu)c)</td>
<td>400</td>
<td>0.169</td>
<td>233</td>
<td>0.099</td>
<td>180</td>
<td>0.076</td>
</tr>
<tr>
<td>Overspotting on the hull (0.5 (\mu)c)</td>
<td>21800</td>
<td>1.843</td>
<td>3615</td>
<td>0.306</td>
<td>1920</td>
<td>0.162</td>
</tr>
<tr>
<td>Overspotting on the leaflet (1.0 (\mu)c)</td>
<td>5500</td>
<td>0.233</td>
<td>1107</td>
<td>0.047</td>
<td>3740</td>
<td>0.158</td>
</tr>
</tbody>
</table>

*aLeaves and fruits were sprayed with ethephon at 1000 ppm before treating with the radioactive chemical. Treatments were applied on July 31, 1970 and harvested after 5 days.
Table 2. Radioactivity recovered from different portions of walnut fruits at 2 stages of maturity treated with $^{14}$C-ethephon.a

<table>
<thead>
<tr>
<th>Method of $^{14}$C-ethephon application</th>
<th>Hull</th>
<th>$^{14}$C activity recovered</th>
<th>Shell</th>
<th>$^{14}$C activity recovered</th>
<th>Kernel</th>
<th>$^{14}$C activity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maturity</td>
<td>cpm</td>
<td>% of activity applied</td>
<td>cpm</td>
<td>% of activity applied</td>
<td>cpm</td>
</tr>
<tr>
<td>Overspotting on the hull (1.0 μc)</td>
<td>Immature</td>
<td>43600</td>
<td>1.843</td>
<td>7230</td>
<td>0.612</td>
<td>3840</td>
</tr>
<tr>
<td></td>
<td>Mature</td>
<td>30240</td>
<td>1.279</td>
<td>1551</td>
<td>0.066</td>
<td>1500</td>
</tr>
<tr>
<td>Overspotting on the leaflet (1.0 μc)</td>
<td>Immature</td>
<td>5500</td>
<td>0.233</td>
<td>1107</td>
<td>0.047</td>
<td>3740</td>
</tr>
<tr>
<td></td>
<td>Mature</td>
<td>6290</td>
<td>0.029</td>
<td>262</td>
<td>0.011</td>
<td>5150</td>
</tr>
</tbody>
</table>

a Treated were harvested after 5 days.

b Immature - 62 days before harvest, nuts were inedible. Mature - 14 days before harvest, nuts were edible.

containing the hull and shell extracts were streaked on thin-layer chromatographic (TLC) Analtech plates coated with Avicel 250 μ in thickness. Because of the low level of radioactivity in the kernel extracts after concentrating, up to 250 μl of solution were streaked on the plates. The plates were developed for 10-cm in: methanol, iso-propanol, concd ammonium hydroxide, and water (9:6:2:3, v/v/v/v); benzene, concd acetic acid, and water (8:3:5, v/v/v) or n-butanol, iso-propanol, concd ammonium hydroxide, and water (6:2:1:2, v/v/v/v). A TLC scanner (Actigraph III, Nuclear-Chicago) was used to locate radioactivity on the developed plates.

Autoradiography. After TLC scanning, the TLC plates were autoradiographed (5).

Translocation. $^{14}$C-ethephon at 1.0 μc was applied to a leaflet or hull of an immature (62 days before harvest) or mature (14 days before harvest) walnut. Samples were taken 5 days later for comparison of total radioactivity recovered from hull, shell and kernel extracts as previously described. Also, at 14 days before harvest 1.0 μc of $^{14}$C-ethephon was applied to the leaflet closest to the fruit. Fruit and leaflet samples were taken after 1, 3, 5, or 7 days for analysis of total radioactivity. To examine the distribution pattern of $^{14}$C-ethephon 2 μc were applied to a leaflet of a walnut seedling and to a leaflet of a mature tree. After 2 days the intact seedling was harvested; after 5 days the leaf was harvested from the mature tree. The seedling and the mature leaf were then pressed, freeze-dried, and autoradiographed (5).

Results and Discussion

Total $^{14}$C activity recovered. Overspotting $^{14}$C-ethephon on the leaflet or the hull resulted in the highest recovery of radioactivity in the kernel (Table 1). Irrespective of the method of application, highest $^{14}$C activity was always found in the hull extracts. In 2 of 4 cases the lowest $^{14}$C activity was recovered in the kernels despite girdling the treated branch and removing all but the treated leaflet to insure preferential movement of $^{14}$C-ethephon to the kernel.

In general, $^{14}$C activity was greater in immature tissues (Table 2). When $^{14}$C-ethephon was overspotted on the leaflet, more $^{14}$C activity was recovered from the hull of immature
fruit. This would reflect that either less translocation of 14C-ethephon to the hull occurred as the nuts matured or that more breakdown of the translocated 14C-ethephon occurred in the hull of mature nuts.

In a study of translocation to mature fruit 14C-ethephon was applied to the leaflet closest to the fruit and samples were harvested after 1, 3, 5 or 7 days (Fig. 1). One day after treatment 14C activity was present in the hull, shell, and kernel. Activity in the hull decreased only slightly with time, which may indicate low rates of translocation or metabolism or both of 14C-ethephon. In contrast, 14C activity in the kernel reached a max 5 days after application and thereafter declined. The amount of 14C activity recovered from the shell rose sharply between the 5th and 7th days after treatment, possibly an indication that the kernels were no longer competing for the translocating 14C-ethephon.

Metabolic studies. To investigate the metabolic fate of 14C-ethephon in leaves, hulls, and kernels, samples were selected among those of the highest recovered radioactivity. Concentrated methanolic extracts of those plant parts were streaked on TLC plates and developed in 3 solvent systems. Only those extracts from the leaves and hulls were sufficiently radioactive to provide a signal above background on the TLC plate scanner. No radioactive metabolites were detectable in the extracts of hull or leaves (Fig. 2).

Even though kernel extracts from all the experiments were pooled and concd to a small vol, there was insufficient radioactivity to project a TLC scanner signal above background, therefore, autoradiographs were made of the TLC plates. After 6 weeks exposure, the developed autoradiographs showed a barely detectable outline of the 14C activity in the leaves and hull, which coincided with that of standard 14C-ethephon. The activity for the kernel extract was faintly visible within the outlined area for one solvent system (Fig. 3). However, metabolites of 14C-ethephon were not evident.

Autoradiographs of intact seedlings and mature leaves. 14C-ethephon applied to a walnut seedling penetrated the tissue and translocated throughout the plant (Fig. 4). In contrast, 14C-ethephon applied to a leaflet on a mature tree resulted in a marked decrease in penetration and subsequent translocation compared to the seedling (Figs. 4 and 5). Penetration in the seedling leaflet was probably more rapid than in the mature plant because of the presence of less cuticle on the seedling. It is not clear why the distribution of 14C-ethephon from the source leaflet would be less in the mature plant than in the seedling. This evidence coincides with that of Table 2 which showed less radioactivity in mature than immature tissues following 14C-ethephon treatment.

The data in this report do not preclude the presence of radioactive metabolites of ethephon below the sensitivity of the assay techniques used. Further, no accounting of released ethylene was made as this aspect has been previously documented (4). No doubt the prominent decrease in total activity recovered was as a result of released 14C-ethylene. Of practical significance, it is interesting to note that due to the enclosure of the kernel in a shell and hull and to rather slow translocation of ethephon, little applied chemical was found in the edible product. Further, the slow translocation shown in mature tissues (Fig. 5) would indicate the importance of thorough spray coverage during field use.

Literature Cited