Physiological Studies of a Synthetic Gibberellin-Like Bioregulator

II. EFFECT OF SITE OF APPLICATION ON BIOLOGICAL ACTIVITY

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ABSTRACT

The biological activity of the synthetic gibberellin agonist AC-94,377 (1-[3-chlorophthalimido]-cyclohexanecarboxamide) in certain plants is strictly dependent on the site of application. Root application of AC-94,377 at concentrations greater than or equal to 1 micromolar to seedlings of dwarf corn (Zea mays L. var d3), dwarf rice (Oryza sativa L. cv Tan-ginbozu), and sunflower (Helianthus annuus L. cv NK265) seedlings resulted in readily measurable gibberellin-like biological activity. Application of up to 10 micrograms of AC-94,377 to the shoots of these same species had no effect. AC-94,377 was metabolized to more polar products in both dwarf corn and sunflower seedlings. After 4 days of continuous root treatment with [14C]AC-94,377, greater than 70% of the recovered 14C was found in the form of unmetabolized AC-94,377. In contrast, only 30 to 40% of the recovered 14C was unmetabolized 4 days after shoot treatment. Translocation studies demonstrated that the movement of [14C]AC-94,377 was limited and occurred almost exclusively in an apoplastic fashion. Four days after leaf treatment, less than 1.5% (corn) or 4% (sunflower) of the recovered radioactivity had moved away from the treated area. It was concluded that the lack of biological activity of AC-94,377 following shoot treatment resulted principally from limited phloem mobility and to a lesser extent from accelerated metabolic breakdown.

The observed biological activity of any externally applied compound ultimately depends on the ability of that compound to reach the appropriate target site (i.e. enzyme or receptor) in sufficient quantity. Many interrelated biological and physical factors, including absorption (uptake), translocation, and metabolism may intercede and thereby alter the activity of any xenobiotic (5). These considerations apply to both naturally occurring and synthetic compounds including metabolic intermediates and growth regulators. With agricultural chemicals (notably herbicides), the alteration in biological activity can be both beneficial (i.e. differential toxicity) or detrimental (loss of efficacy).

Despite encouraging projections, plant growth regulator usage in agricultural situations has lagged considerably behind that of other types of agents (i.e. herbicides, fungicides, and so forth). Inconsistent response is often cited as a principal factor in this situation. In spite of this, there have been very few systematic studies examining the behavior and fate of growth regulators under conditions of variable response.

The substituted phthalimide AC-94,3771 (henceforth AC-94; Fig. 1) has been shown to elicit GA-like activity in a number of specific bioassay systems (12). Besides having value in agricultural situations, this family of synthetic bioregulators could be of use to physiologists exploring the molecular bases of hormone action (for review see Ref. 11). Although generally active in GA bioassays, AC-94 elicited no response in the standard dwarf maize (d3) bioassay. Subsequent studies found that AC-94 elicited considerable GA-like activity when supplied to the roots of the d3 seedling via the nutrient solution.

This type of differential biological activity afforded a unique opportunity to examine the relationship between biological activity and the factors listed above versus site of application. Portions of these studies have been presented in abstract form (13).

MATERIALS AND METHODS

Plant Material and Experimental Protocol. Seeds of dwarf corn (Zea mays L., d3 variety); dwarf rice (Oryza sativa L. cv Tan-ginbozu); and sunflower (Helianthus annuus L. cv NK265) were germinated in vertically oriented cylinders of moist paper toweling and were subsequently transferred to foil-wrapped glass jars (about 500-ml volume) that contained nutrient solution (1). Seedlings were raised in a growth chamber under a 14-h photoperiod provided by a mixture of cool-white fluorescent and incandescent bulbs (light intensity at plant height: 300 μE m–2 s–1; PAR). Day/night temperatures were 25°C/23°C, respectively, and a relative humidity of roughly 50% was maintained. All experiments described in this paper were conducted a minimum of three times and, where possible, each treatment within an experiment was replicated. Data from typical experiments are presented.

Growth Response Studies. For shoot treatment studies, the test compounds were dissolved in 30% (v/v) aqueous aceton containing 0.05% (v/v) Tween 20. In the case of root treatment, the test compounds were dissolved in the nutrient solution. Dwarf corn seedlings were transferred to the hydroponic system 7 d after sowing and were treated the same day. For shoot treatment, 100 μl of solution was applied directly to the inner leaf whorl. Leaf sheath elongation was measured after 7 d.

![Chemical structure of AC-94,377](https://example.com/structure.png)


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Sunflower growth studies were conducted similarly except that only 75 μl of treatment solution were applied to the apex of each seedling. Dwarf rice seedlings were transferred and treated 12 to 14 d after sowing. For shoot treatment, a 2 μl droplet of acetone containing the test compounds was placed between the inside of the first leaf sheath and the emerging second leaf sheath. Leaf sheath elongation was measured 7 d after treatment.

**Metabolism Studies.** Dwarf corn seedlings were transferred and treated as described above. For shoot treatment, a total of 5 seedlings were each treated with 10 μg AC-94 containing 0.23 μCi of [14C]AC-94. Root treatment was conducted using a total of 20 seedlings that were exposed to a 10 μM solution of AC-94 (500 ml) containing 21 μCi of [14C]AC-94. Shoot-treated seedlings were harvested 4 d after treatment and, initially, the treated area was rinsed alternately with 30% (v/v) acetone and water (3 times). Root-treated seedlings were also harvested after 4 d. In this case, roots of the treated seedlings were immersed in distilled water containing 100 μM unlabeled AC-94 for 2 h. The seedlings were frozen in liquid N2 and were homogenized in 80% (v/v) aqueous acetone. The extracts were clarified by filtration and the resultant filtrate was slurried with a 1:1 (w/w) mixture of charcoal and celite in order to remove pigments. Preliminary experiments demonstrated that this step did not influence the quantitative pattern of metabolites recovered. The slurry was stirred for 1 h (4°C) and was then filtered. This filtrate was reduced in volume by rotary film evaporation. Where necessary, these extracts were further concentrated by evaporation (40°C) under a stream of N2. The extracts were then fractionated on 250 μm silica plates (Silica Gel HF; Analtech, Inc.) using 1:1 (v/v) acetone-dichloromethane as the developing solvent. After drying, the plates were scanned for radioactivity, the silica was removed from the plates, placed in scintillation vials, and counted. Sunflower metabolism studies were conducted similarly except that for shoot treatment studies a total of 3 seedlings were each treated with 1 μg AC-94 containing 0.23 μCi [14C]AC-94 and root treatment (3 seedlings) used 10 μg AC-94 containing 2.7 μCi [14C]AC-94 (500 ml total volume).

**Translocation Studies.** Dwarf corn seedlings were transferred and treated 7 d after sowing. In the case of leaf treatment, 1 μg AC-94 containing 0.05 μCi [14C]AC-94 or 100 ng GA3 containing 0.05 μCi [14C]GA3 was applied in a 50 μl droplet (30% [v/v] aqueous acetone plus 0.05% Tween 20) to the distal portion of the first leaf. A lanolin barrier was placed between the treatment zone and the remainder of the leaf to prevent movement of the treatment solution. Root treatment studies were conducted using 6-d-old seedlings. The test compounds (10 μg AC-94 containing 0.5 μCi [14C]AC-94 or 0.1 μM GA3 containing 0.5 μCi [14C]GA3) were dissolved in the nutrient solution (500 ml). Seedlings were exposed to this solution for 24 h and were then transferred to fresh nutrient solution lacking the 14C. In both cases, the seedlings were harvested 4 d after the start of treatment. After dissection into respective tissues, the plant material was lyophilized and the 14C content was determined after combustion by scintillation spectroscopy. Sunflower translocation studies were conducted in a similar fashion using 9-d-old seedlings. In the case of root treatment, sunflower seedlings were treated with [14C]AC-94 for 2 d. Plants were harvested 4 d after treatment.

**Exudation Studies.** Methodologies used here are essentially those originally described by King and Zeevaart (6). Two small areas on fully expanded leaves from 16-d-old sunflower seedlings were lightly abraded using jewelers abrasive. The test compounds (0.24 μCi [14C]AC-94 or 0.26 μCi [14C]GA3; both carrier-free) were applied to the abraded areas in 10 μl droplets (30% [v/v] aqueous acetone + 0.05% Tween 20). One h later, the treated leaves were excised, the petioles were rinsed with water and placed in beakers (25 ml) containing 2 ml glass-distilled water ± 20 mM EDTA (pH 7.0). At the indicated times, the leaves were transferred to fresh solutions. The EDTA (where used) was only present during the first collection period (1 h total). The radioactivity in the exudation solutions was determined by scintillation spectroscopy.

**Partitioning Behavior.** The test compounds (0.02 μCi) were dissolved in 5 ml of buffer solution (universal buffer: 1 mM each of phosphoric, boric and phenylacetic acids titrated with NaOH). To this, 5 ml of octanol were added. These mixtures were shaken in closed vials for 0.5 h. After standing for an additional h, the [14C] in each phase was determined.

**Chemicals.** AC-94, 377 (1-[3-chlorophthalimido]-cyclohexanecarboxamide) was kindly provided by American Cyanamid Co. 1-Amino-[carboxyl-14C]-1-[3-chlorophthalimido]cyclohexane (58 mCi/mmol) and [1,7,12,18-14C] GA3 (10 mCi/mmol) were obtained from Amersham Corp. These compounds were purified by HPLC prior to use. All other chemicals used in this study were obtained from commercial supply houses.

**RESULTS**

Application of up to 10 μg of AC-94 to the leaf whorl (shoot treatment) of dwarf corn seedlings resulted in no measurable increase in subsequent leaf sheath elongation (Figure 2, upper left panel). In contrast, applications of AC-94 to the root system via the nutrient solution at concentrations in excess of 1 μM elicited measurable increases in subsequent leaf sheath elongation (Fig. 2, upper right panel). GA3 effectively stimulated growth in either situation. A similar phenomenon with respect to site of application was observed using sunflower seedlings (Fig. 2, lower panels). We have previously reported (12) that the dwarf rice cultivar Tan-ginbozu responded to AC-94 if the entire seed was treated (i.e., the immersion assay). When care was taken to treat the root and shoot systems separately, only root treatment with AC-94 was effective (Table 1). In both sunflower and rice seedlings, root-applied AC-94 was nearly as active as GA3 (on a concentration basis). In contrast, AC-94 exhibited a 100-fold higher threshold concentration in dwarf seedlings. At a concentration of 100 μM, both compounds elicited comparable effects.

In attempting to identify the physiological bases for the divergent behavior of this compound with respect to treatment site, our attention was directed to differences in metabolism and translocation of AC-94. These two processes have been shown to influence the biological activity of a variety of xenobiotics. Due to the relative ease of manipulation, the remainder of this study will focus on dwarf corn and sunflower seedlings.

[14C]AC-94 was readily metabolized by dwarf corn seedlings (Fig. 3). As judged by TLC, the metabolism of AC-94 by the dwarf corn seedlings resulted in the formation of more polar materials. When root versus shoot treatments were compared, nearly twice as much metabolism of AC-94 occurred when this material was applied to the shoot. In the case of root treatment, 80% of the applied [14C] was found associated with unmetabolized AC-94 4 d after application.

The situation was much the same when [14C]AC-94 was applied to sunflower seedlings (Fig. 4). As with corn seedlings, metabolism of shoot-applied AC-94 was nearly twice that of root-applied material. Again, metabolism of AC-94 resulted in the generation of more polar compounds.

In addition to metabolism, the degree of translocation of externally applied compounds may have a profound impact on their resultant biological activity (5, 7). For this reason, the movement of [14C]AC-94 was followed in both corn and sunflower seedlings. In these studies the movement of [14C]GA3 was
Table 1. Effect of Shoot versus Root Application of GA₃ or AC-94 on Subsequent Leaf Sheath Elongation in Tan-ginbozu Dwarf Rice Seedlings

<table>
<thead>
<tr>
<th>Dose/Concentration</th>
<th>Leaf Sheath Length (cm)</th>
<th>GA₃</th>
<th>AC-94</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.0 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001ᵇ</td>
<td>6.7 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>9.0 ± 0.3</td>
<td>7.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>13.7 ± 0.5</td>
<td>7.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>14.7 ± 0.6</td>
<td>6.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>13.9 ± 0.8</td>
<td>6.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Root treatment</td>
<td>7.7 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.1 ± 0.2</td>
<td>7.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>0.01ᵇ</td>
<td>9.3 ± 0.5</td>
<td>7.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>14.1 ± 1.4</td>
<td>11.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>17.6 ± 0.6</td>
<td>16.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>100.0</td>
<td>19.1 ± 1.5</td>
<td>17.5 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Mean ± SE (n = 6). ᵇ Dosages in μg/seedling. ᵇᵦ Concentrations in nutrient solution (μM).

FIG. 2. Effect of shoot or root treatment with AC-94 or GA₃ on subsequent leaf sheath elongation in dwarf corn seedlings (upper panels) and internode elongation in sunflower seedlings (lower panels). Solid lines, GA₃-treated seedlings; dashed lines, AC-94-treated seedlings. Bars indicate SE.

FIG. 3. Radiochromatogram scans of extracts prepared from dwarf corn seedlings treated with [¹⁴C]AC-94 and fractionated by TLC. Upper panels, extracts from shoot-treated seedlings; lower panel, extracts from root-treated seedlings. Percentage figures indicate fractions of total [¹⁴C] found associated with various regions on each chromatogram. Bar indicates position of AC-94 standard.
applied [14C]GA3 exhibited greater mobility. Greater than 5% of the 14C was recovered in tissues distant from the treatment zone. Of this, some 3% was found in the younger leaf tissues.

A different pattern emerged from the studies on root supplied material. In this case, only 20% of the recovered 14C from AC-94 treatment was found associated with the root tissues 4 d after pulse treatment (Fig. 5, lower). The bulk of the 14C was found in the growing regions. In contrast, nearly half of the 14C from GA3 treatment was still found in the root tissues. The remaining half was distributed throughout the growing regions.

The situation was much the same in sunflower seedlings (Fig. 6). When AC-94 was applied to a leaf, greater than 96% of the 14C was still associated with the treated zone 4 d after treatment. The remaining 4% of the 14C was equally distributed throughout the seedling. As with corn, GA3 was more mobile in these seedlings. Greater than 18% of the applied 14C was found in sites distant from the treatment zone. The bulk of this radioactivity was found in the growing region (internode and second leaves).

Once again, root treatment resulted in a completely different pattern of movement (Fig. 6, lower). Greater than 95% of the recovered 14C from AC-94 was found outside of the root tissues. The majority of the translocated 14C was found in the cotyledons and first true leaves. In the case of GA3, approximately 60% of the recovered 14C was found in the root system 4 d after treatment. The remaining radioactivity was distributed throughout the other seedling tissues.

These results suggest that while AC-94 readily translocates via the apoplast, little symplastic movement occurs. As symplastic movement occurs primarily in the phloem tissues, these results can be interpreted as demonstrating limited phloem mobility. In
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an attempt to more closely examine phloem mobility per se, the efflux of [14C]AC-94 and [14C]GA₃ was studied in sunflower leaves using the EDTA-enhanced phloem exudation technique originally described by King and Zeevaart (6).

Recovery of [14C] in the exudation media following leaf treatment with [14C]-GA₃ was greatest during the first two collection periods and was strictly dependent of EDTA pretreatment (Table II). A similar situation was found when the movement of [3H]-sucrose was monitored (data not presented). In contrast, exudation of [14C] from leaves treated with [14C]AC-94 and EDTA was minimal.

The observed differences in phloem mobility of AC-94 versus GA₃ could be the result of many factors. However, in studies with other xenobiotics the lipophilicity of the test compounds has been shown to be a significant determinant of overall phloem mobility (7). This characteristic is usually determined by measuring the octanol/water partitioning behavior as a function of pH. The partitioning behavior of GA₃ was found to be typical of that of a weak acid (Fig. 7). As the pH is increased, an increasing amount of GA₃ was found in the aqueous phase. In contrast, at pH values between 2 and 8, essentially all of the AC-94 remained in the organic (octanol) phase. In the case of AC-94, pH values in excess of 8 were not studied as we have found rapid decomposition of this material in mildly alkaline solutions (JC Suttle, JF Hultstrand, unpublished data).

DISCUSSION

The results presented in this manuscript demonstrate that, if a suitable application method is used, the N-substituted phthalimide AC-94 elicits GA-like agonist activity in sunflower, dwarf rice, and dwarf corn seedlings (Fig. 2; Table I). The ability to elicit GA-like activity in the GA-deficient dwarf rice and corn seedlings supports the earlier contention (11, 12) that this class of bioirregulator possesses intrinsic GA-like activity and does not simply stimulate endogenous gibberellin biosynthesis.

Not all species examined exhibit this type of differential response. Treatment of intact seeds with AC-94 elicits GA-like activity (14, 16). In some species such as cucumber, tomato, chrysanthemum, and strawberry, foliar application of AC-94 elicits biological activity (3, 8, 9, 15). At present, it is not clear how these species differ from those in the present study. It is possible that the spray treatments used resulted in direct contact of the phthalimide with the growing zone thereby circumventing the limited phloem mobility of this compound (see below).

While not excluding other possible factors, the data presented in this study suggest two underlying physiological mechanisms for the differential biological activity as affected by treatment site. When AC-94 is applied to the shoot or leaf, little biological activity is observed (Fig. 2; Table I). This is associated with a greater degree of metabolic conversion (Figs. 3 and 4) and with limited movement away from the treatment zone (Figs. 5 and 6). In contrast, application of AC-94 to the root system (via the hydroponic medium) elicits considerable biological activity that is associated with a greater percentage of unmetabolized bioirregulator and a higher degree of translocation. Of these two processes, we feel that the limited phloem mobility of AC-94 is of greater importance in determining the final level of biological activity. Our reasoning is as follows: (a) when applied to the shoot, there is still a fair percentage (34-47%) of unaltered AC-94 present after 4 d, and (b) in contrast, there appears to be essentially no phloem movement of this compound.

With respect to metabolism, the picture presented in Figures 3 and 4 is oversimplified. When extracts from treated plants are fractionated by reverse-phase HPLC a more complex pattern can be demonstrated (JC Suttle, JF Hultstrand, unpublished data). The low Rₚ peak observed following TLC separation (Figs. 3 and 4) readily resolves into at least five distinct peaks. The pattern of metabolites is qualitatively similar in the three species examined. Due to the chemical instability of these metabolites, their identities and biological activities are presently unknown.

The phloem mobility of many xenobiotics is thought to be inversely related to their hydrophobicity (5, 7). One possible exception concerns those compounds that are weakly acidic (7). It is generally accepted that weak acids readily enter the phloem in an undissociated state and, once in the phloem, are repelled out of the xylem by an electrostatic force. For example, weakly acidic molecule such as phosphoric acid and acetic acid are rapidly transported into the phloem. However, for weak acids such as malic acid, which is a weakly acidic molecule, there is still a fair percentage of the compound present after 4 d. The chemical nature of the [14C]AC translocated after the application of the test compounds was not investigated.

AC-94 is labile in mildly alkaline solution (pH ≥ 8) and the breakdown products lack biological activity (JC Suttle, JF Hultstrand, unpublished data). Given the alkaline nature of the phloem symplast (roughly 8), any AC-94 that enters this compartment would be subject to rapid decomposition and loss of activity.

Aside from its effects on phloem mobility, the apolar nature of AC-94 may favor immobilization of the compound within the cuticle (10). Due to the lack of consensus concerning the validity of previously used methodologies to study cuticular absorption (2), this possibility was not explored further.

Presumably, the physiological impediments that preclude the expression of biological activity of this bioirregulator will only
manifest themselves during in vivo studies. Therefore, we wish to reemphasize that AC-94 and related chemistries should be valuable probes of GA action at the molecular level (i.e. in vitro studies).

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