Growth Substances from Veratrum tenuipetalum

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Abstract. Young leaves and buds of *Veratrum tenuipetalum* yielded non-indolic growth accelerators and inhibitors in the acidic ether fraction. The titer of accelerators decreased while the inhibitors increased as leaves matured. This was also true when comparing extracts of immature and fruiting inflorescences. Indole 3-acetic acid was at no time detected in leaf, bud, and inflorescence extracts but indole 3-acetyl-aspartic acid was readily recovered from all of these.

The alkaline ether fraction of leaf base meristems, buds, roots, and rhizomes was rich in crystalline and amorphous alkaloids and phenolic acids. Reducing the quantity of major substances through crystallization, precipitation, and filtration permitted thin film chromatographic separation of the minor alkaloids and phenolic acids in the presence of the major ones. The unchromatographed mixture, and also certain of the purified major and minor alkaloids studied, strongly inhibited growth in germinating oat and winter rye seeds. In addition, profound changes in morphology and cytology of the seminal roots resulted. DNA disappeared partially to completely from affected tissue in 2 day germinated seeds.

Western *Veratrum* species have a reputation for toxicity to cattle and sheep (1). When I observed in the Colorado Rockies massive monospecific stands of *Veratrum* and learned that these had developed over the past 25 years through ecological invasiveness this suggested that substances capable of influencing germination, morphogenesis, and growth might be isolated from this genus. At the periphery of these stands species of *Delphinium* and *Aconitum* and even small subalpine fir or Englemann spruce trees had a spindly and pale look. Could this be explained on the basis of *Veratrum* root-secreted products or substances liberated to the soil by frost kill of the above ground parts? I determined to investigate all parts of the plant. The work involved indoles, non-indolic inhibitors and accelerators of growth, alkaloids, and phenolic acids. I hoped to isolate one to several substances exhibiting deleterious morphogenetic action as an answer to the invasiveness and toxicity of *Veratrum*. I clearly recognized, however, that to isolate such substances from the plant would be one thing, but to verify the action of such substances in an ecological sense would necessitate extensive field research.

Materials and Methods

Source and Storage of Plants. Plants were obtained from the Black Mesa Experimental Range, Crawford, Colorado. They were wrapped in plastic sheeting with sphagnum moss and were shipped by air express arriving usually unwilted. Pre-flowering and flowering (or late season when flowering did not occur) shipments were received during 4 years. On arrival the plants were separated into inflorescences, leaves, leaf bases, buds, rhizomes, and roots. These were separately packed in polyethylene bags and stored in a freezer until taken for extraction.

Extraction: Acid Ether Fraction. Leaves, buds, and inflorescences were minced while frozen and extracted 4 days in a 5°C refrigerator, inverting the containers several times a day. In one extraction procedure 50% isopropyl alcohol was used; the alcohol was removed with a flash evaporator setting the bath temperature at 35 to 37°C. The remaining aqueous material was acid (about pH 5.6, the native pH) and was extracted with an equal amount of peroxide-free diethyl ether. The aqueous layer was then brought to pH 3.0 with HCl and again extracted with ether; these were kept separate. In some experiments the acid aqueous layer was brought to pH 7.5 to 8.0 with solid sodium bicarbonate and again extracted with ether. The ether

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1 This work was supported through Grants-In-Aid from The Research Corporation.
was removed after which the residues were taken up with a minimum of isopropyl alcohol. A second extraction method employed ether over water. The native pH ether was separated after which the aqueous layer was adjusted to pH 3.0 and re-extracted as above. These were assayed separately.

**Chromatography and Bioassay: Acid Ether Fraction.** Extract residues were purified by paper chromatography so that both color reactions and bioassays could be employed in identifying active substances. Whatman No. 1 chromatographic paper was used in ascending apparatus and developed with isopropyl alcohol : NH₂OH : H₂O (10:1:1). A modified Ehrlich reagent (9) was the primary color test used for locating indoles in all of this work.

In preparation for bioassays, leaf, bud, and inflorescence extract residues representing approximately 5 grams fresh weight were strip started on 56 mm wide paper and developed with 10:1:1. A strip 12 mm wide was cut from the edge of the chromatogram and dipped through the Ehrlich reagent so as to locate indolic materials. The remainder of the chromatogram was cut into equal Rₚ zones 1 through 10. The papers were folded and placed in Beckman 5 ml micro beakers. To these 0.3 ml Knop's solution including sucrose at 0.5% was added. Some experiments were also run using this mixture to which indole-3-acetic acid (IAA) was added to yield a concentration of 10 μM.

Hulled Clinton oats were germinated 3 days between filter papers moistened with distilled water. Light tight containers were used and all handling outside the 25° incubator was done in weak red light. Seedlings withcoleoptiles ranging from 2 to 3 cm were selected for cutting. The cutting tool discarded a tip of 1.88 mm and cut 1 section of 3.76 mm immediately behind this. Five of these sections were placed in each micro beaker. A control using plain chromatographic paper was used in each experiment. The micro beakers were placed in opaque microscope slide boxes to which 3 ml water was added. These were incubated at 25° for 24 hours after which the sections were measured by projection. The growth increments were calculated and plotted as percent of control (6). Some germination experiments were also run using Lepidium (Cress) seeds. Zones from the paper chromatograms were placed in 8 cm crystallizing dishes and wetted with Knop's solution. The dishes were covered with Petri dish halves and incubated in a humidifier in the dark at 25°.

**Isolation of Principal Indole: Acid Ether Fraction.** In confirming the identity of the principal indole Whatman No. 3MM chromatographic paper was folded so that a 100 mm short arm was turned up over the main sheet and supported by a thread. Leaf and inflorescence extract residues were strip started in the short arm 25 mm above the fold. The chromatograms were developed in 10:1:1 until the solvent front had progressed 50 mm above the starting line. The chromatograms were then removed from the tank and dried after which the short arm was cut half way between the starting line and the solvent front thus discarding high Rₚ pigment and other interfering substances. The paper was then straightened at the fold and re-chromatographed in 10:1:1. A strip was cut from the edge of the finished chromatogram and dipped through the Ehrlich reagent. The principal indole was located and the zone marked off on the remainder of the chromatogram, cut out, and eluted with hot isopropyl alcohol or 12 parts n-hexyl alcohol: 3 parts acetic acid: 5 parts water (12:3:5). The eluate was dried, taken up with isopropyl alcohol and either spotted or strip started with pure indole 3-acetyl aspartic acid (IAAP) included for a test of identity. The developers 12:3:5 and equal parts of n-hexyl alcohol, pyridine, and water (1:1:1) were used in confirming the plant extracted principal indole.

**Extraction and Purification: Alkaline Ether Fraction.** In extracting leaf bases, buds, roots, and pieces of rhizome these were crushed by folding them in heavy aluminum foil and using a large vise. The macerate was then placed in jars containing 50% isopropyl alcohol: 4 to 5 times as much liquid as plant material was used. Rhizome pieces weighing 250 grams were thus extracted with about 700 ml 50% isopropyl alcohol. The jars were refrigerated at 5° for 5 days inverting the jars twice daily. The isopropyl alcohol was then removed with a flash evaporator and the remaining aqueous material brought to pH 7.5 to 8.0 with solid sodium bicarbonate. An equal amount of peroxide-free diethyl ether was then used to extract the alkaline aqueous layer. Approximately three quarters of the ether was removed by distillation the remainder was removed with a stream of air.

The alkaline ether residues from leaf base meristems, buds, roots, and rhizomes contained a large amount of crystalline and amorphous material which was taken up in hot isopropanol after which the major alkaloids and phenolic acids were frozed out of solution as much as possible. These were then removed by gravity filtration in the freezer with the use of pre-chilled fritted glass filter funnels. The filtrate was evaporated at room temperature in the dark and the solid residues were dissolved in 80% (v/v) acetone. This solution was evaporated at room temperature in the dark: a brown film was removed from time to time from the mostly aqueous residue. This aqueous residue, a yellow suspension, was collected from all alkaline ether residues and held in the freezer. From the pooled aqueous residue a crystalline precipitate was separated by cold filtration: the crystalline materials were pooled with major alkaloids and phenolic acids previously removed.

Removal of pigments from the yellow aqueous residue and especially from the resinous film was
accomplished by the use of neutral alumina activity grade one. A slurry was formed, a little acetone being used to wet up the brown resinous material. The slurry was extracted 3 times with a mixture (a chromatographic solvent) of 40 parts petroleum ether (boiling range 37.9–55.5°): 9.5 parts chloroform: and 0.5 parts 2-butyl alcohol. The supernatant was collected by centrifuging and was concentrated by evaporation after which the residue was taken up in hot isopropanol and stored in the freezer.

Considerably better than 1 mg per gram fresh weight yields of both the principal alkaloid (m.p. 238-240°) and 2 phenolic acids (m.p. 265-263.5° and 272-274°) were obtained from rhizome, leaf base meristems, and leaf buds. Roots yielded about one.

Thin Film Chromatography: Alkaline Ether Fraction. Separation of the alkaloids and phenolic acids in this final fraction, as well as the major ones removed earlier, was accomplished through the use of aluminum oxide G and silica gel G. Films were 250 microns in thickness; 20 by 20 cm and 5 by 20 cm glass plates were used. Where it was intended to recover subfractions 20 by 20 cm plates were strip started by using a miniature glass sprayer and a spray guard of poly(ethylene terephthalate) having a 4 mm gap. When using the principal system, aluminum oxide G, a developer consisting of 95 parts chloroform and 5 parts methanol was used initially; somewhat better resolution was obtained by using 96 parts chloroform and 4 parts 2-butyl alcohol.

Substances were located on the chromatoplates through the use of long and short wave ultra violet light; the latter is particularly helpful in locating phenolic acids which appear dark blue to blue-purple. A Dragendorff spray extended with ethyl acetate was used to locate alkaloids which appear as persisting orange to orange-red areas (7). Diazotized p-nitraniline was used as it gives a range from deep yellow to dark brown with phenolic acids and is quite specific (14). I developed a new reagent which detected one of the most biologically active fractions. This consisted of 1 ml concentrated HCl, 4 ml acetone, and 1 drop congo red in saturated acetone solution (CR-HCl). A strong pink to red transient color is given by this reagent in the presence of certain alkaloids.

After locating the principal substances the plates were marked into appropriate bands which were then scraped off into separate containers. The adsorbed substances were then eluted from the alumina with methanol which was rapidly evaporated at room temperature; the residues were then taken up in isopropanol.

Round spot and strip start tests of the eluates were run on aluminum oxide G or silica gel G using a variety of developers. Two very useful systems consisted of 90 parts chloroform to 10 parts absolute ethanol used with aluminum oxide G (5), and 90 parts benzene, 16 parts methanol, and 8 parts acetic acid used with silica gel G (15).

The Bioassay: Alkaline Ether Fraction. Biological activity of the eluted fractions was tested with whole Clinton oat or Balboa winter rye seeds. Strips of filter paper 1 cm by 7.5 cm were used with test tubes of the same dimensions. The material to be tested was applied with a calibrated micropipette; dry weights of substances assayed on the papers varying between 0.13 and 0.66 µg/mm². The dried papers were inserted into the test tubes and a single seed was pushed between the paper and the wall of the tube. The strips were moistened with 0.15 ml distilled water and the tubes were placed in 2.5 by 20 cm test tubes to which 0.5 ml water had been added. The outer tubes were stoppered, stood upright, and incubated in the dark at 25° for 2 or 3 days. Growth inhibition was judged with respect to controls by measuring to the nearest mm the lengths of the coleoptiles and roots. More than 25 experiments were performed in which from 1 to 7 separate substances were tested in duplicate. A sample seedling assay in which coumarin was tested with relation to an active Veratrum fraction is given in table III.

Normal and deanged roots were fixed in formalin-acetic-alcohol and were sectioned and stained in safranin and fast green. Parallel studies were made by fixing in 3:1 alcohol-acetic acid followed by Feulgen staining. In this case roots were squashed after partial dehydration in 50% ethanol. This insured that the flattened tissues were sufficiently intact to permit following files of cells from the apical meristem back into the cortex or stele.

Results

Acid Ether Fraction. Oat coleoptile section straight growth assays of the acidic ether fraction of leaf, bud, and inflorescence extracts revealed the presence of growth accelerators and growth inhibitors active both in the presence of added IAA or in its absence (fig 1). The titer of accelerator substances was lower while the amount of inhibitor was higher when lower (older) leaves were extracted (fig 1B) as compared with the assays of the 5 uppermost leaves (fig 1A, D). This was also true in comparing fruiting with immature inflorescence (fig 1F, E). The inhibitors and accelerators located by bioassay procedures did not give color reactions with the Ehrlich reagent and were thus considered to be non-indolic. Tests with xanthodrol, diazotized sulfanilic acid, and other location reagents (9,16) were not conclusive as a number of reacting substances overlapped the Rf zones in which accelerators and
Fig. 1. Non-indolic accelerators and inhibitors of growth from the acidic ether fraction. Bud assay contains 10 μM IAA in growth medium; all other assayed with Knop’s plus sucrose only. Growth response due to 10 μM IAA indicated in A. In A the extract residue was dewaxed while in D no attempt was made to remove the wax. Confidence limits extend to plus and minus 20% of control.
inhibitors were located. Distinct fluorescence was not associated with these substances.

Wax extracted from the leaves and inflorescences interfered with chromatographic separation of inhibitors. When wax was not precipitated by storing the isopropyl alcohol solution of ether residues in the freezer at least overnight, some inhibitor was adsorbed on the waxy material at the starting line. If coleoptile sections were placed directly on the original strip at the starting line very strong inhibition resulted (Fig. 1D). Placing coleoptile sections in the same assay beaker (RF 0.0-0.1) but at some distance from the starting line did not result in inhibition (fig 1A). Cress seeds placed directly on the starting line failed to germinate or epicotyls only germinated, while in the same dish seeds a short distance from the starting line germinated normally. This suggested that the waxy material was itself active. I collected precipitated wax using pre-chilled centrifuge tubes. This was dissolved in hot methanol and obtained in relatively pure form by slowly evaporating the solvent so as to allow pseudocrystalline growth of the wax upon the wall of the container. This material was harvested free from sediments and purified by the same technique. It was in the form of spherules which melted on a warm slide to yield a transparent oil. The purified wax was completely inactive. I concluded that the wax held by adsorption part of the inhibitors present in applied extract residues. From this point on residues were taken up without warming of the glassware thus leaving the wax to be discarded.

Three Ehrlich positive substances appeared on the chromatograms of leaf, bud, and inflorescence acidic ether fractions. Two faint bands appeared at or just below the solvent front when very heavy loads were applied to the paper; such overloading, however, prohibited good resolution especially of slower running substances. One Ehrlich positive band was constant and of sufficient titer to warrant further study: this substance occurred at RF 0.03 to 0.10 using 10:1:1. It was present in the native as well as the pH 3.0 fractions. The cleanest isolation of this indole was obtained by the ether over water extraction method which took up considerably less pigment and other interfering substances than the 50% isopropyl alcohol extraction. With the Ehrlich reagent the first color of the principal indole band was apricot to tan depending upon the amount of interfering material present. The final color was grey blue to royal blue as with pure IAA.

By using the fold over and repeat run technique described it was possible to obtain relatively clean bands of the principal indole. Recchromatography of eluted product against a pure sample of IAAP was done with 12:3:5 and 1:1:1 using Whatman No. 1 paper (Table I). Recchromatography with 10:1:1 did not give closely repeatable RF values when prior elution was done with 12:3:5 due to the presence of interfering pigment and a product formed during elution. This product could be clearly separated from the principal indole with the 1:1:1 developer; it reacted with the Ehrlich reagent to yield an orange spot.

Tests for biological activity of the principal indole were done through the use of the standard assay with the exception that Whatman No. 3MM paper was employed so as to increase the titer of the indole 4- to 5-fold. Band areas in the RF 0.0 to 0.1 zone were equivalent to those obtained with the No. 1 paper, however, growth acceleration up to 250% of control resulted. The principal indole after elution from chromatograms and also pure synthetic IAAP were likewise active.

As further confirmation of the identity of the *Veratrum* principal indole a sheet of Whatman 3MM paper was slotted vertically to divide the paper into 3 equal tabs. To the starting line of 1 part only *Veratrum* leaf extract was added, to another third *Veratrum* leaf extract plus pure IAAP were added, to the remaining part only pure IAAP was added. The fold over and repeat run technique was used, developing first in 10:1:1 then in 1:1:1. As the latter developer runs chlorophyll and other pigments to the solvent front and this technique discards much pigment initially, the resulting Ehrlich positive bands were unusually clean. The 3 bands were identical as to RF, color immediately on spraying, and color on standing for a day at room temperature. I concluded that the *Veratrum* principal indole was IAAP.

Tests of the acidic ether fraction at no time revealed IAA although it was very easy to recover added IAA from leaf and inflorescence materials. The RF of IAA in pure form compared very favorably with that of IAA running in the mixture of *Veratrum* products. Also the presence of *Veratrum* materials did not interfere with the formation of the first and final colors with the Ehrlich reagent as IAA ran well above the slower moving pigments in 10:1:1.

**Alkaline Ether Fraction.** The solubility fraction selected for chromatographic separation contained all of the major and minor alkaloids and phenolic

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Pure standard</th>
<th>Veratrum product</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>RF</td>
<td>First color</td>
</tr>
<tr>
<td>12:3:5</td>
<td>0.83</td>
<td>Magenta</td>
</tr>
<tr>
<td>1:1:1</td>
<td>0.52</td>
<td>Magenta</td>
</tr>
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</table>

Table I. Chromatographic Identification of Indole 3-acetyl Aspartic Acid
acids detected in this study. Through prior crystallization, precipitation, and filtration the major alkaloids and phenolic acids had been diminished to such a degree that the minor substances could be detected in their presence. Much pigment and also very small amounts of alkaloids and phenolic acids had been discarded through purification with active alumina. In all fractionation and purification procedures color reactions had been used along with bioassays to follow the active fractions as the final mixture was prepared for chromatographic separation. A general analysis of the substances separated from the final mixture by thin film chromatography is given in Table II. Additional data pertaining to the substances present in eluates from the 5 bands is given:

Band 1) Consists primarily of a minor alkaloid which dries to a crumpled sheet plus a yellow varnish.

Band 2) Consists of 3 major products: A) A major alkaloid crystallizing from acetone or ethanol in 2 forms: flat needles m.p. 222.5 to 224.5°, and solid hexagons m.p. 239.5 to 241.5°. Both of these give a vivid spectral display in polarized light: they are only slightly inhibitory in the seedling bioassay. B) A major alkaloid crystallizing from acetone or ethanol as slender needles m.p. 219.5 to 221°. Crystals are bright in polarized light: this alkaloid is very strongly inhibitory as well as producing abnormalities in seedling root tips. C) A major phenolic acid crystallizing as druses from ethanol and giving a slight display of color in polarized light.

Band 3) Consists of 2 minor alkaloids easily separated on aluminum oxide G with the developer 90 parts chloroform and 10 parts ethanol. The upper spot is blue-purple in shortwave ultra violet light, gives a red color with CR-HCl, and is strongly inhibitory causing morphological abnormalities in seedling root tips. The lower spot does not fluoresce, gives no color with CR-HCl, but likewise strongly inhibits seedling growth with the appearance of abnormalities.

Band 4) Consists of several minor alkaloids recrystallizing from ethanol as druses which give a spectral display in polarized light. Small quantities of phenolic acids are also present in this band.

Band 5) Consists of a major phenolic acid, several minor phenolic acids, and a trace of alkaloid. The major constituent crystallizes from ethanol as rhombs which give a vivid spectral display in polarized light.

Gross morphological alterations in roots of seedlings were observed in testing the unchromatographed yellow aqueous fraction with seedlings of cress, oat, sunflower, and winter rye. After separating this mixture the highest activity on a dosage basis resided in band 3. Study of eluates from this band and from its 2 constituents provided several interesting observations. A general shortening and thickening of the root tips by the cessation of mitosis in the major meristem, plus enlargement of cortical cells already differentiated, was coupled with an excessive development and crowding of root hairs. Some root tips were strongly reduced in diameter at the apex (fig 2A). In this case not only was there a reduction in the dimensions of the individual cells but also the number of cell files was diminished. Staining response pointed to a reduction in vacuolation.

Swelling, bending, and partial to complete necrosis of tissues resulted from low to high dosages of the unchromatographed mixture as well as band 3 alkaloids. Figure 2B demonstrates a frequently observed abnormality: an excess of root hairs arise from a swollen but more or less normal region well behind the necrotic root apex. Accessory meristems were formed in this case both near the apex and well basipetal to it. The more basipetal ones appeared to be branch root origins and included normal mitotic figures showing no evidence of chromosome damage. Those close to the apex, while not showing mitotic figures were disarranged (fig 2B, D) and in early stages of necrosis.

Cells adjacent to necrotic areas developed secondary walls prematurely: nuclei appeared to be absent from these cells which was confirmed through the use of Feulgen staining without sectioning. This approach revealed that in slightly brown (partially necrotic) tissue nuclei were either very small or stained very lightly with the Feulgen technique. In more necrotic areas there was no

Table II. Analysis of Bands from Aluminum Oxide G Plates

<table>
<thead>
<tr>
<th>Band No.</th>
<th>R_F</th>
<th>CR-HCl</th>
<th>Diazotized p-nitraniline</th>
<th>Dragendorff</th>
<th>Growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.91-SF</td>
<td>Negative</td>
<td>Negative</td>
<td>Yellow</td>
<td>Slight</td>
</tr>
<tr>
<td>2</td>
<td>0.68-0.91</td>
<td>Red pink to blue</td>
<td>Red</td>
<td>Orange</td>
<td>Strong</td>
</tr>
<tr>
<td>3</td>
<td>0.43-0.68</td>
<td>Red above negative</td>
<td>Tan above yellow</td>
<td>Orange</td>
<td>Strong</td>
</tr>
<tr>
<td>4</td>
<td>0.15-0.43</td>
<td>Negative</td>
<td>Pale tan</td>
<td>Pale orange bands</td>
<td>Moderate</td>
</tr>
<tr>
<td>5</td>
<td>0.00-0.15</td>
<td>Faint pink to blue</td>
<td>Tan above brown SS¹</td>
<td>Pale orange</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

¹ SF is used for solvent front, and SS for starting strip.
Fig. 2. Abnormalities from treatment with band 3 alkaloids, 210×. A and B are median longitudinal sections. In A the number of files of cells is reduced in the narrowed tip; B represents more gross damage with complete necrosis of the apical meristem, premature secondary wall thickening in cells adjacent to this, and an accessory or branch root meristem which is deranged. C is a cross section of a control, and D of an abnormal root tip; these are taken basipetal to the apex and show an immature metaxylem vessel at the center. D also illustrates an acropetal extension of abnormal meristem.
Feulgen response whatever (fig 3). Root cap cells were resistant to the effects of the band 3 alkaloids; stele and pro-stele were less seriously damaged than cortex and pro-cortex.

**Discussion**

**The Acid Ether Fraction.** The role of IAAP in the absence of free IAA is not entirely clear. Formation of IAAP during the exposure of excised *tomato* roots to IAA (17) suggests that this complex may be a detoxifying product. This view appears in a review by Kefford (11) in which it is reported that in pea roots IAAP regulates the uptake of exogenous IAA. By this means the amount of free IAA may be closely regulated in *Veratrum tenuifolium*. The unidentified non-indolic accelerators suggest that as in Maryland Mammoth Tobacco (2) not all growth regulation in the shoots is dependent upon IAA.

One *Veratrum* acidic inhibitor appears to be dormin as reported by Lipe and Crane (12). This judgment is made on the basis of solubility, Rf in 10:1:1 (0.65-0.75), and the absence of color re-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root lengths (mm)</th>
<th>Total (mm)</th>
<th>% Of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.13 µg/mm²</td>
<td>6.5, 8.5, 7.0</td>
<td>22.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0, 8.5, 7.5, 7.5</td>
<td>28.5</td>
<td>24.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coumarin</td>
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<tr>
<td>0.66 µg/mm²</td>
<td>1.0, 2.0, 0.5</td>
<td>3.5</td>
<td></td>
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<tr>
<td></td>
<td>1.0, 2.5, 0.5</td>
<td>4.0</td>
<td>3.7</td>
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<tr>
<td>Band 3 red</td>
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<tr>
<td>0.13 µg/mm²</td>
<td>6.0, 3.0, 2.0, 18.0</td>
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<tr>
<td></td>
<td>6.0, 15.0, 16.0</td>
<td>37.0</td>
<td>32.3</td>
</tr>
<tr>
<td>Control</td>
<td>16.0, 28.0, 35.0, 22.0</td>
<td>101.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.5, 29.0, 47.0, 21.5</td>
<td>103.0</td>
<td></td>
</tr>
</tbody>
</table>

1 Indicates necrotic root tips.
actions. Earlier studies of changes in growth substances during after-ripening and germination in *Punica granatum* (unpublished master’s thesis) and *Lindera benzoin* (unpublished doctoral research) yielded a closely similar pattern of inhibition.

I heartily agree with Crosby and Vitos (2) that RF data, “specific” color tests, and bioassay information may be deceptive. Though one may give careful attention to extraction, separation, and chromatographic methods it is necessary to admit that substances may overlap so as to either fortify or cancel one another. Fluctuations in RF values may also be expected to occur due to qualitative and quantitative changes in pigments, lipids, and other nuisance substances. The *Veratrum* materials, however, handled very nicely as compared with lipid rich extracts from seeds and seedings.

**Alkaline Ether Fraction.** To avoid the suggestion that the *Veratrum* substances produce morphogenetic changes simply because they were used at excessive concentrations I set up a test with coumarin substituted at the lowest and highest concentrations used with *Veratrum* materials (table III). Feulgen preparations of roots strongly inhibited by coumarin did not reveal morphogenetic changes, necrosis, or the disappearance of DNA while the band 3 *Veratrum* alkaloids caused all of these changes in several root tips. It should be made clear, however, that the effective titer of coumarin cannot be realistically compared with the *Veratrum* substances since the latter are very much less soluble in water than coumarin is.

In assaying the *Veratrum* substances it is necessary that the roots lie directly upon the treated papers. Some roots do not do so and as a consequence grow considerably in advance of those which come into direct contact with applied alkaloids and phenolic acids. Papers treated with the *Veratrum* materials were sometimes used 2 or 3 times in which case seedling inhibition did not diminish. The papers were then tested with color reagents and gave strong reactions confirming the remainder of a considerable quantity of the original load. The above considerations indicate the difficulty in estimating effective dose and also suggest that a very small percent of the applied *Veratrum* material produced the effects.

Results of the Feulgen staining suggest that the active *Veratrum* products when used in concentrations sufficient to bring about necrosis in root tips may do so through autolysis of the cells. The partial or complete disappearance of DNA from cells formed and altered in 2 days germination time cannot be considered as the cause any more than the result of cell death. It is interesting to speculate, however, as to whether abnormal cells behaved so through derangement of their genetic components. Differences in susceptibility may have been due to the establishment of diffusion gradients or a gradient of inactivation of the toxic principles.

The external features of affected roots resembled the findings of Ennis (3) while the underlying cytological explanation differed strongly. In his work swollen oat root tips possessing excessive root hairs frequently develop from several days exposure to a-isopropyl N-phenyl carbamate. Upon cytological investigation it was found by him that cortical cells of the primary root were greatly enlarged. Adventitious root initials composed of abnormally large cells and anaphase bridges, blocked metaphases, nuclear fragments, binuclear cells plus giant vesiculate nuclei were observed by Ennis. In their work with protoanemonin Erickson and Rosen (4) showed the development of patches of necrotic cells in roots of corn. Here, however, the meristem remained nearly normal; the absence of mitochondria, marked chromosome contraction, and arrest of mitosis at interphase or early prophase characterize the effects of protoanemonin.

Binns *et al.* (1) have worked with the closely related *Veratrum californicum* and report extensive morphological derangement in developing sheep fetuses. Their most active fraction (10) approximates the one found in this work which suggests that morphogenetic changes induced in sheep fetuses may be a response to active principles similar to those isolated in this work.

One inescapable impression is this: that large quantities of inhibitory alkaloids and phenolic acids are readily extracted from rapidly growing tissues such as leaf primordia or leaf base meristems. Extraction of leaf bases immediately above the intercalated meristem yielded negative results both with color reagents as well as in bioassays. It would appear that where physiological activity is very high these substances are formed in quantity in the sense of metabolic end products (13). Due to their toxicity these substances must be isolated in the cells through the formation of insoluble crystals or complexes, or by their inclusion within selectively permeable membranes as the older literature maintains (8). Upon frost killing of *Veratrum* above ground parts are these substances liberated to the soil? Do they act there as inhibitors and morphogenetic substances? These questions remain to be answered.

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Literature Cited


