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Effects of hormones, pH, and fluoride on ponderosa pine hypocotyl elongation

Jeanne Marie Lang
The University of Montana

EFFECTS OF HORMONES, pH, AND FLUORIDE ON PONDEROSA PINE

HYPOCOTYL ELONGATION

by

Jeanne Marie Lang

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Approved by:

David E Bilderback
<u>Chairman, Board of Examiners</u>

<u>Dean of Graduate School</u>

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ABSTRACT

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Effects of Hormones, pH, and Fluoride on Ponderosa Pine
    Hypocotyl Elongation (132 pp.)

Director: David E. Bilderback $D.E.B$.

The regulation of elongation in <u>Pinus</u> <u>ponderosa</u> (Laws) was
studied by incubating 5 mm excised hypocotyl segments either
in a buffered solution of hormones at pH 6 or in buffer at a
range of pH from 6 to 3. Naphthalene acetic acid (NAA)
enhanced elongation of hypocotyl segments, with the greatest
response at 10^{-6} M. Gibberellic acid (GA_3) at a range of
concentration from 10^{-8} to 10^{-4} M did not affect elongation.
If segments were slit longitudinally and aspirated or split
in half longitudinally, a response to citrate buffer at a
low pH was also obtained, with the maximal elongation at
pH 3. The data support the theory of acid mediated auxin
action.

The effects of fluoride on hypocotyl elongation was
investigated by incubating excised segments in either buffered
solutions of NaF at a range of pH from 6 to 3 or in solutions
of HF at a range of pH from 6 to 2.5. Growth of intact
plants in agar containing NaF was also studied. Sodium
fluoride inhibited elongation at 10^{-1} M. No effect was
produced at lower concentrations of NaF given alone, although
NaF at 10^{-2} M inhibited auxin enhanced elongation. Hydro-
fluoric acid inhibited growth of excised segments at pH 2.5
or 9×10^{-3} M. No evidence of fluoride stimulation of
elongation was obtained.

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I would like to express my deepest thanks to Dr. David E. Bilderback for his generous help with this thesis.

LIST OF FIGURES

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

Fluoride Induced Elongation

The deleterious effects of fluoride on plants, including inhibition of metabolism, reduction of overall growth, and production of foliar lesions, have been documented in numerous studies (National Academy of Sciences, 1971). Fluoride is specifically known to inhibit stem elongation. In studies on pea stem elongation using excised stem segments, fluoride inhibits auxin induced elongation (Christiansen and Thimann, 1950a). Apparently, fluoride inhibits the auxin stimulated deposition of carbohydrates in the cell wall (Christiansen and Thimann, 1950a) and has extensive effects on the metabolism of elongating stem tissue (Christiansen and Thimann, 1950b). Sodium fluoride inhibits the auxin enhanced release of xyloglucan from the cell wall of elongating pea stem segments (Labavitch and Ray, 1974). This release of xyloglucan may be the method by which auxin causes cell wall loosening, a prerequisite for elongation (Labavitch and Ray, 1974).

1

It therefore seems surprising that stimulation of stem elongation by fluoride has been repeatedly observed (Adams and Sulzbach, 1961; Applegate and Adams, 1960; Gordon, 1969a, 1969b, 1972a, 1972b; Treshow et al., 1967, and references cited therein; Treshow and Harner, 1968). In field studies on Pseudotsuga menziesii in the vicinity of a phosphate reduction plant, Treshow et al. (1967) observed increased needle length and shoot length. The stimulation of needle elongation was significent using a t test (Table 2 in Treshow et al., 1967). Gordon has conducted extensive field studies in areas of fluoride pollution including Garrison, Montana; Columbia Falls, Montana; and Mt. Storm, West Virginia. He has observed increased elongation of the terminal shoot in several species of pine and in spruce (Gordon, 1969a, 1969b, 1972b). This stimulation of growth could be produced under controlled greenhouse conditions in white and scotch pine by applying solutions of HF to the candles of 4 to 5 year old seedlings (Gordon, 1972a). Hydrofluoric acid at a range of pH from 3 to 5 caused increased candle elongation. This effect was not obtained with sulfuric acid solutions.

Adams and Sulzbach (1961) observed that internodal elongation was stimulated in Phaseolus vulgaris L. seedlings fumigated with HF at a concentration of 4 to 7

$\mu g \ F^-/m^3$. The stimulation in <u>Phaseolus vulgaris</u> occurred also at a lower level of HF concentration, 2 $\mu g \ F^-/m^3$ (Applegate and Adams, 1960). Treshow and Harner (1968), using approximately the same HF concentration, also reported an increase in internode elongation in <u>Phaseolus vulgaris</u>.

Brewer et al. (1960a, 1967) have been referred to as providing evidence that fluoride promotes growth (Treshow et al., 1967; National Academy of Sciences, 1971). Although they reported an increase in total linear growth, this growth is merely caused by an increased lateral budding, resulting in the production of many small, weak branches. The control plants, in comparison, produced fewer branches, but each of these was longer than branches of fluoride treated plants. The treated plants were shorter than the controls and had a dwarfed appearance (Figure 3 in Brewer et al., 1960a). Therefore, these experiments show fluoride inhibition of stem elongation rather than stimulation.

Several explanations for the phenomenon of fluoride induced elongation have been suggested. Bennett et al. (1974) noted that stimulation of growth by low levels of pollutants is a general occurrence. They point out that air in a natural environment will seldom be as pure as the filtered air used as a control for most air

pollution experiments. Since plants are adapted to natural air, they may actually be adapted to low levels of pollutants, and may grow better in air with low pollutant levels than in filtered air.

In the case discussed by Bennett et al. (1974), the increased elongation represents a true stimulation of growth. However, when other measurements of growth have been made in studies of fluoride stimulated elongation, a positive correlation between elongation and increase in biomass is not always found. Although Treshow and Harner (1968) reported an increase in fresh and dry weight of fumigated plants as well as an increase in internode length, they found in their earlier study with Pseudotsuga menziesii that needle length and radial growth of the trunk were negatively correlated (Treshow et al., 1967). They suggested that an important part of fluoride damage to forests may be occurring in areas where trees appear healthy. The increased needle length in this case does not represent a true growth stimulation since the longer needles are less effective in increasing radial wood growth than the normal needles.

An interaction of fluoride with growth regulators can be postulated to explain localized fluoride induced elongation of plant organs whether this elongation represents stimulation or inhibition of overall growth of the

plant. Treshow et al. (1967) mentioned the possibility that fluoride may be influencing the response of cells to gibberellin or auxin. Gordon (1972b) noted that long terminal shoots were not found close to the Mt. Storm plant but were most common 14 miles away. He suggested that fluoride might be acting at low concentrations, comparable to effective hormonal concentrations.

A study of fluoride effects on elongation would be aided by a previous knowledge of natural controls on elongation in the system being studied. Areas of natural control are where the effects of fluoride alone or inter-actions of fluoride with endogenous growth regulators could be sought.

Natural Regulators of Elongation

A great deal is already known about the regulation of stem elongation in a wide variety of plants. Much of the work involves auxin effects on elongation. The extensive literature on auxin control of stem elongation has recently been reviewed by Davies (1973) and Evans (1974). These reviews and Evans (1973) also discuss the stimulation of elongation by acid and its possible relation to auxin action. Briefly, acid solutions are known to mimic the rapid auxin effect on elongation and may be duplicating the initial wall loosening effect

caused by auxin. The auxin may produce wall loosening by activating a proton pump, causing H^+ ions from the cytoplasm to be extruded into the cell wall region. There they somehow promote a loosening of the cell wall so that it can extend in response to turgor pressure.

In the auxin and acid studies, the usefulness of excised stem segments in studying effects on elongation has been demonstrated. This system provides the advantage of removing important sources of endogenous hormones, such as the apex and leaves, so that the effects of exogenously applied substances can be studied with a minimum of interference from the endogenous regulators.

Gibberellin is another hormone frequently implicated in elongation of stem segments (P.A. Adams, et al., 1973; Evans, 1974; Goto and Esashi, 1974; Hashizume, 1965; Kamisaka, 1973; Kazama and Katsumi, 1973; Shibaoka, 1974; Silk and Jones, 1975). A factor from cotyledons has been found to synergistically enhance gibberellin induced elongation (Kamisaka, 1973), so the effect of gibberellin on excised segments may be increased by incubating segments with excised cotyledons in the same solution.

Elongation in Ponderosa Pine

The present study resulted from a desire to investigate the effect of fluoride on elongation of ponderosa

pine. Ponderosa pine is known to show increased elongation in response to fluoride pollution in the field (Gordon, 1969a). However, little work has been done on control of elongation in this species.

The young seedling represents a system undergoing dramatic elongation. The hypocotyl increases from a length of approximately 2 mm in the embryo to 30 mm, in a period of 10 days following germination. Since the hypocotyl is actively elongating, it would be expected to be sensitive to substances which promote elongation. Though the hypocotyl is not directly comparable to the candle of a tree or several year old seedling, some similarity of growth regulators controlling elongation in hypocotyls and terminal shoots is suggested by a study in which substances isolated from buds of Pinus resinosa Ait. are able to promote elongation of hypocotyls of the same species (Giertych and Forward, 1966). Three promoters and one inhibitor were found in buds from trees ranging in height from 5 ft to 50 ft. Although different aged trees had different concentrations of the substances, as measured in the hypocotyl straight growth test, the fact that all four substances were present at each stage tested suggests a qualitative similarity of growth regulatory substances in trees of all ages.

Hashizume (1965b) found promoters of elongation in needles from <u>Pinus</u> <u>nigra</u> using the hypocotyl straight growth test. Mirov (1941) used the <u>Avena</u> curvature bioassay to evaluate levels of growth promoters in ponderosa pine. From results of Giertych and Forward (1966) the elongation responses of <u>Avena</u> and red pine hypocotyls are almost identical. Thus, the results obtained by Mirov (1941) with the <u>Avena</u> test can tentatively be applied to ponderosa pine hypocotyls. Mirov (1941) found the <u>Avena</u> bioassay indicated that more growth promotive substance occurred in the terminal shoots of fast growing ponderosa pine than in terminal shoots from slow growing trees. Developing short shoots, which would show little elongation, were found to have a very low hormone level in the <u>Avena</u> bioassay. The ability of regulators to stimulate growth in the tree therefore seems to be the same as their ability to stimulate growth in <u>Avena</u>. The hypocotyl of pine, if anything, would be expected to be even more closely related in response to the shoots of the pine tree (Giertych and Forward, 1966).

The hypocotyl is a convenient system for elongation studies because segments can easily be excised and studied for growth effects. The advantages of the excised segment system have already been discussed. Furthermore, the permeability of this young tissue reduces penetration

problems. There is less chance of finding a lack of growth effect due to the prevention of entry of the test solution by resins when segments of hypocotyl are used rather than segments from candles or tree shoots.

Since evidence presented above suggests a similar response of the hypocotyl and terminal shoots to growth regulators and since the hypocotyl segments are able to be studied with a minimum of interference from permeability problems and endogenous hormone sources, the use of hypocotyl segments for preliminary studies of fluoride and natural growth regulator effects on elongation in ponderosa pine seems justified. Information presented in this study does not, however, apply directly to the control of growth in terminal shoots of trees. The growth effects found in the hypocotyl system are effects which might be found in trees, and the data presented here can only be interpreted as providing evidence that similar effects in trees would be worth investigating.

Although most of the work on stem segment elongation has involved angiosperms (Evans, 1974), there have been several studies on elongation of pine stem segments. Auxin enhances elongation of stem segments from several species of pine (Witkowska-Zuk and Wodzicki, 1970, and references cited therein; Hashizume, 1965a; Giertych and

Forward, 1966; Zakrzewski, 1975, and references cited therein). To my knowledge, the response of ponderosa pine hypocotyl segments to auxin or any other homones has not previously been investigated.

Although Witkowska-Zuk and Wodzicki (1970) found a positive auxin response, they were unable to detect a response of Pinus silvestris hypocotyl segments to citrate-phosphate buffer at low pH. Another tissue which was found to respond to auxin but not acid is the light-grown (green) pea stem segment (Barkley and Leopold, 1973). Although they state that removal of the cuticle had no effect on the lack of response of segments to low pH, evidence exists that a permeability problem was indeed present in their experiments. Yamamoto et al. (1973) found that when green pea segments were superficially slit longitudinally and aspirated in a solution of low pH, an elongation response to the acid buffer could occur. Cleland and Rayle (1975) also slit green pea segments longitudinally, but did not aspirate segments and were also able to obtain an acid response. The response of pine hypocotyls to auxin and acid is therefore of some interest. The question of whether pine hypocotyls represent a tissue responding to auxin but not to acid is an important one for the hypothesis of acid mediated auxin action. A further question is whether

permeability factors are important in the pine hypocotyl acid response, as they seem to be in pea.

Since acid may promote elongation, the fluoride effect may be an acid effect caused by hydrofluoric acid. In order to differentiate between a fluoride effect and an acid effect, the elongation responses to HF and NaF are tested separately in the present study.

Endogenously occurring gibberellins have been isolated from Pinus silvestris, but as yet GA_3 has not been found (Michniewicz et al., 1974; references cited in Zakrzewski, 1975). Pinus silvestris hypocotyl growth is reported to be stimulated by these endogenous gibberellins (Kopcewicz, 1968, cited in Zakrzewski, 1975). However, gibberellic acid applied alone has not been found to enhance elongation in any of the pine species studied (Hashizume, 1965a; Witkowska-Zuk and Wodzicki, 1970; Zakrzewski, 1975, and references cited therein). Hazhizume (1965a) did report an enhanced response to auxin in the presence of gibberellic acid.

To facilitate comparison of the results for the ponderosa pine hypocotyl presented in this study with other stem systems, the general histology of the hypocotyl is illustrated in Figures 1-7. Vascular bundles are present as separate strands (Figure 3). The cortex and pith contain numerous starch grains (Figures 1, 2, 3 and

Figure 1. Cross section of the cortical region of the
hypocotyl of ponderosa pine showing epidermis with
stomate, and large cortical cells containing PAS
positive starch grains (stained red). The cortex
contains large intercellular air spaces. x 40.

Figure 2. A cross sectional view of the vascular region
between the cortex (above) and the pith (below). A
bundle of fibers staining slightly red can be seen to
the left of the xylem strand. The pith contains a few
starch grains. x 40.
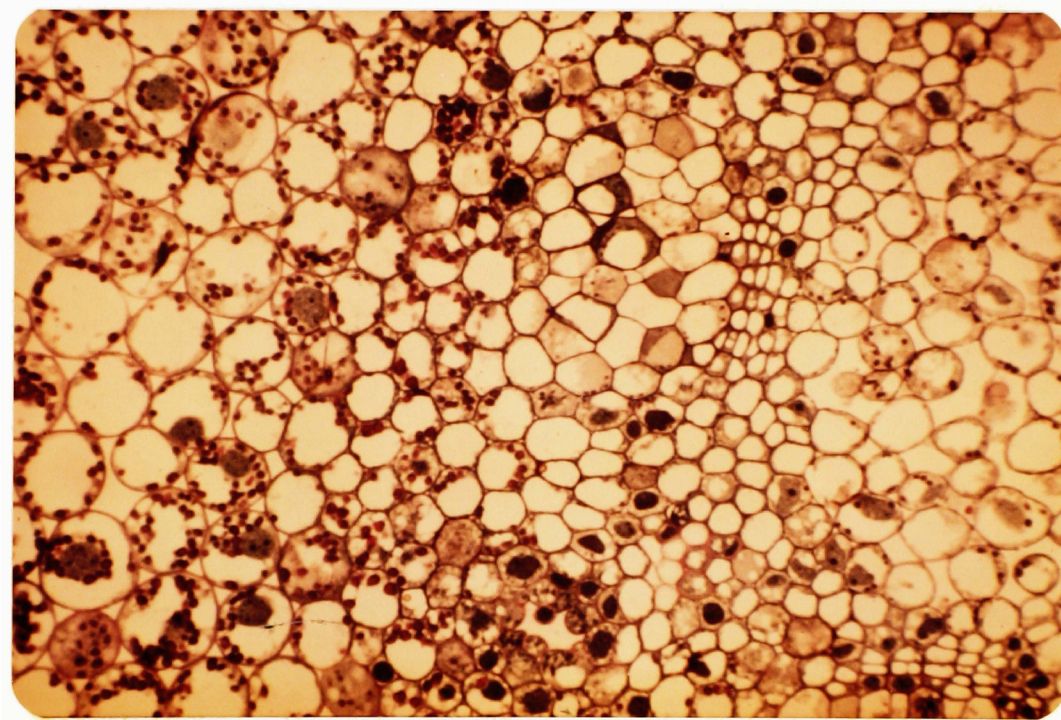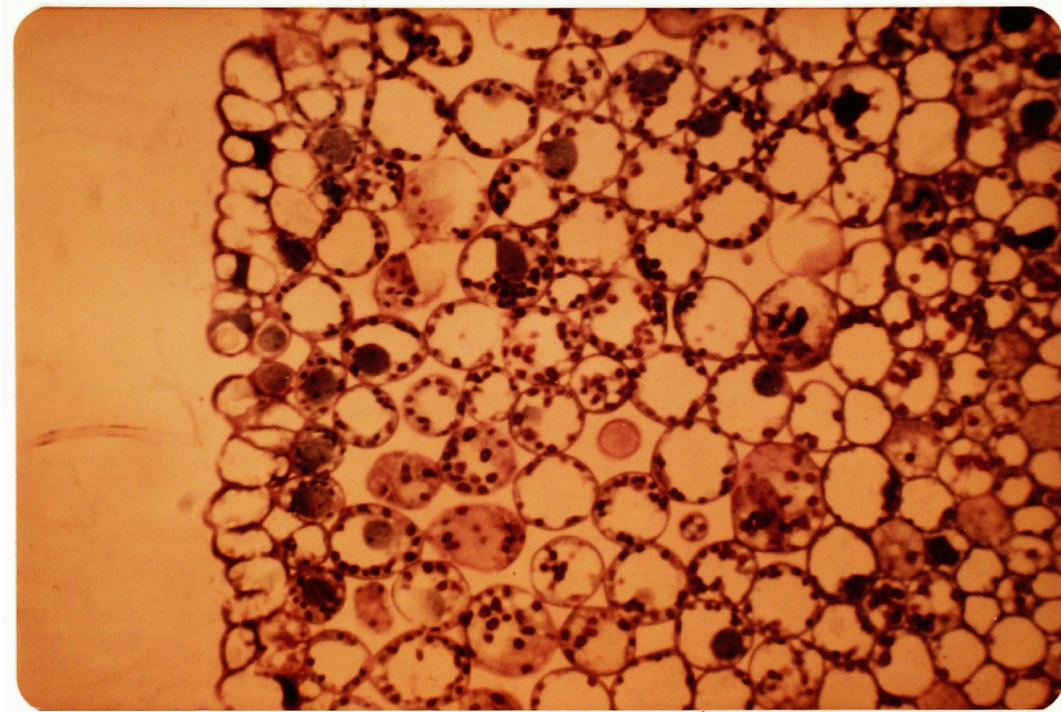
Figure 3. Cross section of the vascular cylinder. The
vascular tissue can be seen to occur as separate bundles.
Starch grains in the pith are visible. Interfascicular
red staining fibers can be seen between the vascular
bundles. x 25.

Figure 4. Epidermal cross section showing epidermal
cells (below) with walls slightly thicker than those
of cortical cells (above). x 125.
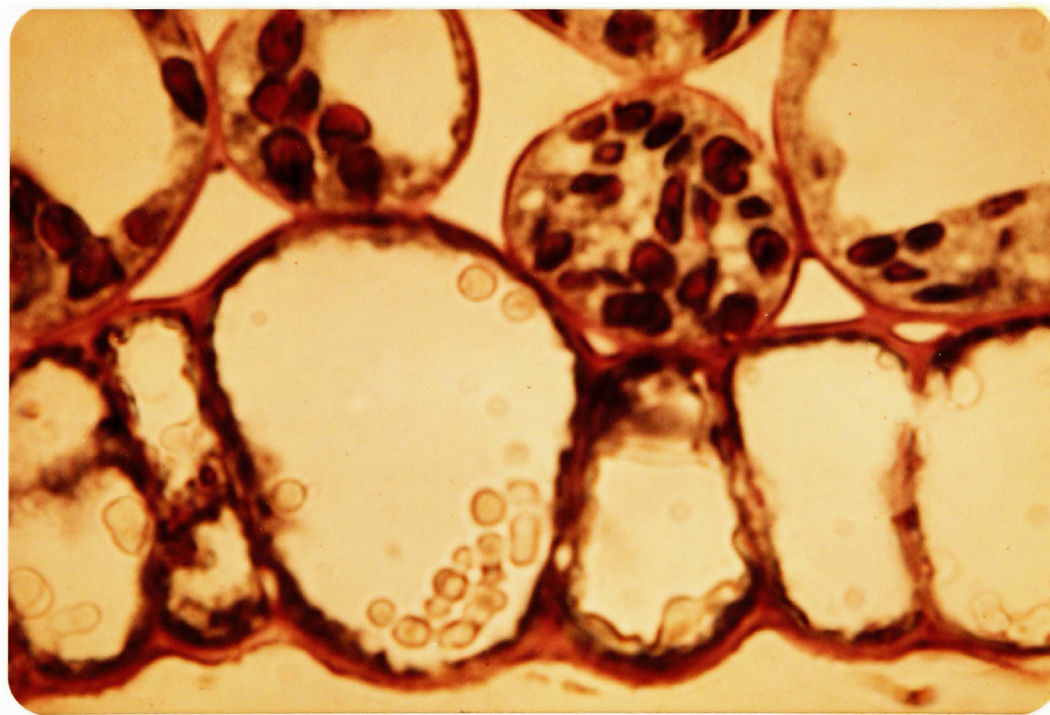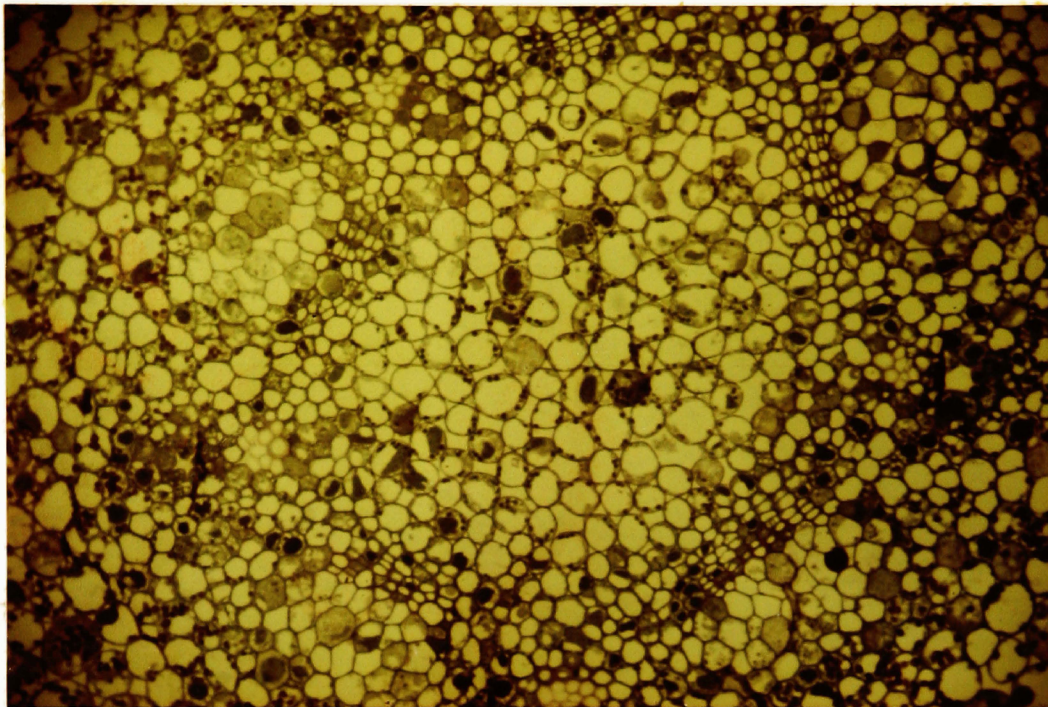
Figure 5. Stomate in cross section, x 125. This is
the same stomate seen in Figure 1.

Figure 6. Cross section of cortical cells showing
starch grains. x 125.
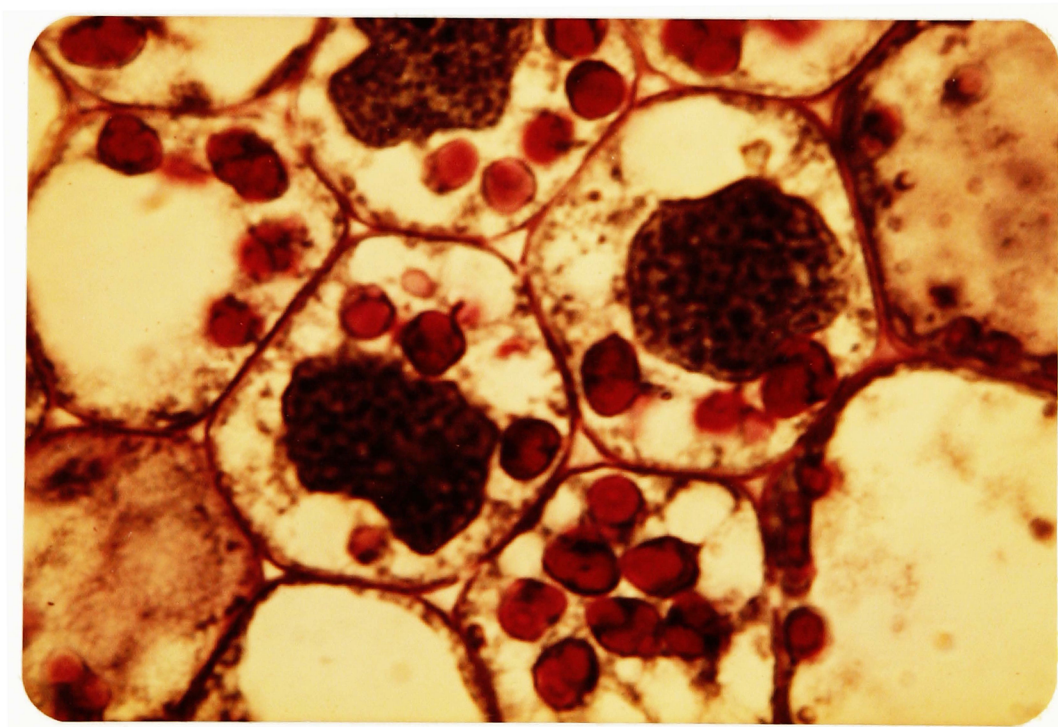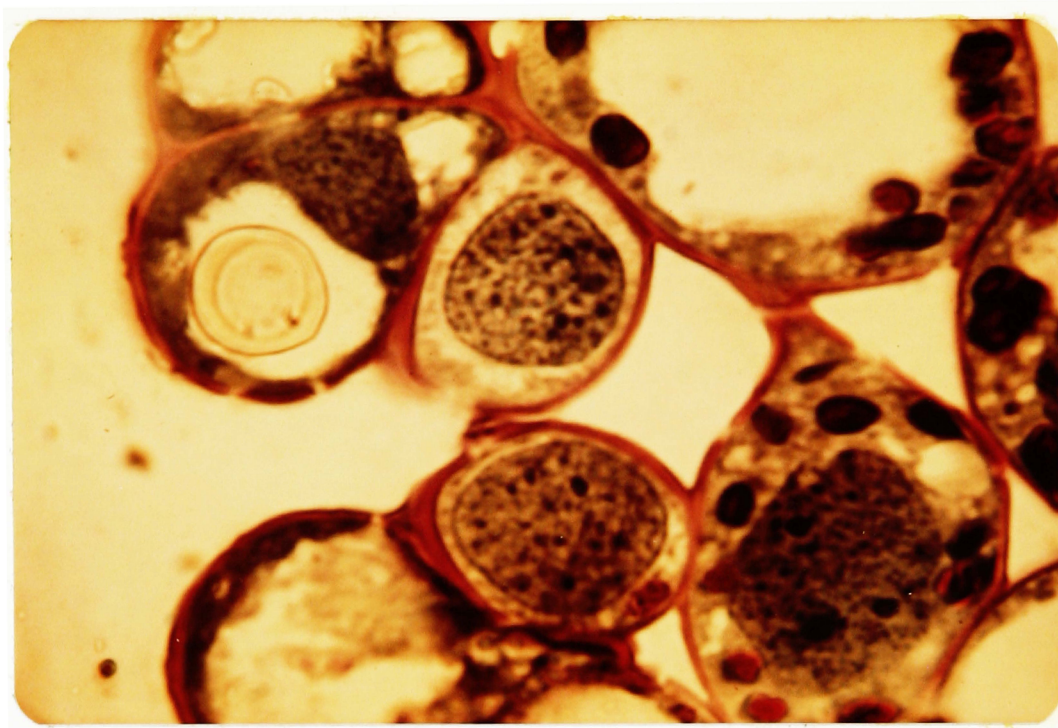
Figure 7. Cross section of vascular tissue with phloem
to the left, xylem in the middle with red staining walls
and pith to the right. x 125.
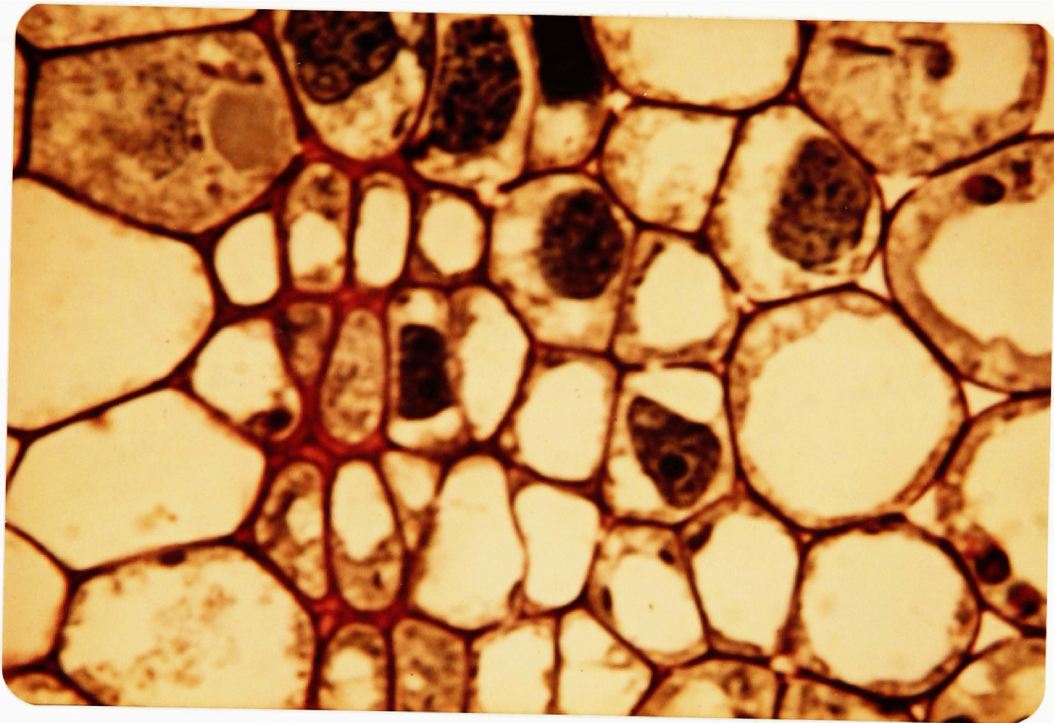
6), and stomates occur in the epidermis (Figures 1 and 5).
Epidermal cell walls appear to be thickened (Figures 4
and 5).

Data are presented in this thesis on response of
hypocotyl segments to auxin, acid and gibberellic acid
as a survey of the natural regulators of growth. The
interaction of gibberellic acid with auxin and with
cotyledons is also presented.

Data on fluoride effects are presented for two types
of fluoride compounds, HF and NaF. Each is tested alone,
and the following combinations are tested: HF with coty-
ledons, NaF with cotyledons, NaF with gibberellic acid,
NaF with auxin, NaF with gibberellic acid and cotyledons,
and NaF with acid buffer.

CHAPTER II

MATERIALS AND METHODS

Unstratified seeds of ponderosa pine (Pinus ponderosa
Laws) were purchased from the Silvaseed Company, Roy,
Washington. The seeds were prepared for germination by
washing for 4 hr under cold running tap water and then
stratified at 4°C for 15 days in moist vermiculite. The
integuments and nucellus were then removed from the
megagametophyte and the micropylar end of the megagameto-
phyte was inserted into moist sterile vermiculite in
three-inch deep Pyrex petri dishes. The dishes were kept
in a growth chamber at 20°C with 12 hr of light per day
provided by fluorescent lights at 1000 ft c.

For the experiments on growth of entire plants,
seedlings were maintained in the petri dishes in the
growth chamber under environmental conditions described
above. To determine the role of the megagametophyte in
hypocotyl elongation, the megagametophyte was removed
from the cotyledons of the seedlings when the hypocotyl
was 1 cm long. To accomplish this, the seedling was
gently removed from the vermiculite. The megagametophyte
was slit longitudinally with a razor blade and then

broken in half manually. The seedling was then replanted in the vermiculite. For the experiments involving removal of the apical meristem and cotyledons, the megagametophyte was removed as described above, and the cotyledons and apical meristem was excised 1 mm below the cotyledonary node. When a drop of agar was to be applied to the cut surface of the hypocotyl, solutions of naphthaleneacetic acid (NAA) at concentrations of 10^{-4}, 10^{-6}, and 10^{-8} M, in 3% agar, were introduced into 5 cc hypodermic syringes. Agar alone was used as a control. The drops were applied to the seedlings using a needle which had been filed off so that the plane of the aperture was perpendicular to the long axis of the needle. This permitted formation of a drop which could be applied easily to a seedling. The drop readily adhered to the cut surface regardless of its orientation. Drops were applied to the seedlings immediately after the apical meristem and cotyledons had been removed, on the third day after germination. Fresh drops were applied on the fourth, sixth, and eighth days after germination.

Measurements of entire plants growing in vermiculite were made by holding the seedlings upright as straight as possible next to a ruler held with the zero point at the level of the base of the hypocotyl. If curves in the hypocotyl were present, the stem was gently straightened

as much as possible by exerting tension along the long
axis. If a curve still remained, the length was estimated
using the ruler.

To determine in vivo elongation of the hypocotyl
region immediately below the cotyledonary node, a mark
was made with a black felt tip pen at a distance of 5 mm
from the cotyledonary node. After 24 hr of growth in the
vermiculite in petri dishes in the same environmental
conditions as used for excised segment experiments (des-
cribed below), the distance from the mark to the cotyle-
donary node was measured with a ruler.

Excised segments, used for most of the experiments,
were prepared from seedlings grown for three days after
germination in conditions described above. The hypocotyls
were approximately 1 cm long at this age. To obtain a
hypocotyl segment, 5 mm of tissue was cut with a razor
blade from the hypocotyl just below the cotyledonary node
(Figure 8). In some experiments, hypocotyl segments with
attached cotyledons were used. These were prepared by
leaving 2 mm of cotyledon tissue attached to the 5 mm
hypocotyl segment. Hypocotyl segments incubated with
unattached cotyledons in the same flask were tested for
an elongation response to GA_3, NaF, and combinations
of GA_3 and NaF. For these experiments, cotyledon segments
were excised just above the cotyledonary node using a

Figure 8. The top of the ponderosa pine hypocotyl and cotyledons, showing the region from which the 5 mm segment was excised.

razor blade. Twenty-five cotyledon segments were incubated with 25 hypocotyl segments. For acid response experiments, hypocotyl segments were slit superficially the length of the hypocotyl at intervals of approximately 0.5 mm and then aspirated with test solution for 1 min using a vacuum pump. In one experiment, hypocotyl segments were cut in half longitudinally to produce split segments.

For all except one of the segment experiments, batches of 25 segments were incubated in a 50 ml Erlenmeyer flask containing 20 ml of test solution. In one of the sodium fluoride experiments, individual segments were placed alone in 10 ml Erlenmeyer flasks containing 2 ml of solution. The flasks were placed on a mechanical shaker. The shaker was placed in a growth chamber at 20°C with 12 hr light provided by fluorescent lights at 170 ft c. The tissues were incubated for 24 hr. An exception was the two experiments on hypocotyl segments with unattached cotyledons. Flasks were incubated for 48 hr in continuous light for one experiment and for 72 hr in continuous dark for the other.

For the first experiments, segments were measured with a ruler. These include experiments on auxin alone, gibberellin alone, and gibberellin and fluoride with unattached cotyledons. For most of the experiments, pictures of the segments were taken before and after

incubation. The segments were photographed in a petri dish with graph paper used as a background to determine the magnification of the slide. Measurements of length of the segments were made by projecting slides of the segments onto a cardboard surface on a wall and determining average lengths of the two sides of the segment with a map measure. These measurements were later converted to millimeters. Measurements were correct to the nearest 0.1 mm. In the case of hypocotyl segments with cotyledons attached, only the hypocotyl portion of the segment was measured.

For the fast auxin response experiment, either cheesecloth or nylon screen was used. If cheesecloth was used, segments were placed on a 14 cm square piece of cheesecloth and photographed before incubation. The cheesecloth was then formed into a bag and lowered into 20 ml of incubation solution in a 50 ml beaker. At 15 min intervals during the first hour, and then at longer time intervals, the cheesecloth and segments were lifted out of the beaker, the segments were arranged to lie flat in a small area, and a photograph of the segments was taken. The segments and cheesecloth were then lowered back into the beaker, and incubation of the segments was resumed. The nylon screen was used with a similar experiment except that segments were incubated alone in

the beaker. Segments were photographed by pouring the
incubation solution containing them over the screen.
The screen with segments on it was placed on a circle
of filter paper moistened with the incubation solution.
The segments were arranged on the screen and then photo-
graphed. The screen was inverted over the beaker with
test solution and tapped, causing the segments to fall
into the test solution rapidly. Incubation of the segments
was then resumed.

Hormone solutions used for the experiments with
excised segments were prepared in 1 mM potassium phosphate
buffer at pH 6, to insure an equal initial pH for all
these tests. The hormones NAA and gibberellic acid (GA_3)
at concentrations of 10^{-4}, 10^{-6}, and 10^{-8} M were used
alone and in combination with NaF at 10^{-1}, 10^{-2}, 10^{-3},
10^{-4}, 10^{-6}, and 10^{-8} M. Combinations of the three con-
centrations of GA_3 with 10^{-6} M NAA were also tested.
Abscisic acid (ABA) also was tested at concentrations of
10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10} M.

For acid response experiments, a pH range from 6 to
3 was obtained by using a 0.01 M sodium citrate buffer.
Various concentrations of NaF were prepared in the
phosphate buffer or the citrate buffer. Hydrofluoric
acid solutions were prepared using Baker analyzed reagent
grade 48% HF. The first stock solution was made by

diluting 1 ml of concentrated HF with distilled water to 100 ml. A more dilute stock solution was then prepared using 1 ml of the first stock solution brought to 100 ml with distilled water. Solutions at pH values of 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, and 2.5 were prepared by adding stock solution to 100 ml of distilled water. The more concentrated stock solution was used only for preparation of the pH 2.5 solution. The molarities of the HF solutions were 2.2×10^{-5}, 3.4×10^{-5}, 5.0×10^{-5}, 8.0×10^{-5}, 2.1×10^{-4}, 1.2×10^{-3}, and 9×10^{-3} M. A Corning Model 5 pH meter was used for preparation of buffer solutions and HF solutions. All solutions were prepared fresh for each experiment.

Treatment of entire plants with NaF was accomplished either by germinating seeds in fluoride agar slants or by transferring seedlings from petri dishes to the fluoride slants two days after germination. Fluoride agar was prepared by autoclaving 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7} M NaF solutions in 1.3% agar. Slants were made with 10 ml of agar in 30 ml test tubes. Plants growing in the slants were maintained in a growth chamber at 20°C with 12 hr light supplied with fluorescent and incandescent lights at 1600 ft c.

For the experiment testing the effect of pretreatment of the seedlings to NaF on subsequent auxin response,

seedlings in petri dishes were transferred to the slants
2 days after germination. The seedlings remained in the
slants for 3 days. They were then removed from the
slants, and hypocotyl segments were cut and incubated
in 10^{-6} M auxin in the usual manner.

The experiments on growth of entire plants in ver-
miculite were analyzed statistically using the Student's
t test. Analysis of the experiments on excised segments
and entire plants grown in slants was somewhat more com-
plicated. For these analyses, nonparametric methods were
chosen, so that it would be unnecessary to assume that
the populations being analyzed were normally distributed.
Generally, the test used when there were more than two
treatments was the Kruskal-Wallis test (Malik and Mullen,
1973). If the Kruskal-Wallis test indicated a difference
between some of the treatments, a multiple comparison
test based on the Kruskal-Wallis test was used to deter-
mine which of the treatments were different from which
others (Hollander and Wolfe, 1973). The computer program
used for the Kruskal-Wallis and multiple comparison tests
and sample output from the program appear in Appendix A I.
Basically, this test compares average rank sums between
every possible pair of treatments. From the test statistic,
q, a value is calculated which must be less than the com-
puted difference in average rank sums if a treatment pair

is to be significant. When only two treatments were to
be compared for an experiment, the Wilcoxon Rank Sum
test was used (Malik and Mullen, 1973). This test is
analogous to the Kruskal-Wallis test used for multiple
populations. The Kruskal-Wallis test is merely a gener-
alization of the Wilcoxon Rank Sum test for two samples
to the case of more than two samples. The level of α
used for the Kurskal-Wallis, multiple comparison, and
Wilcoxon tests was 0.05.

A problem with the nonparametric tests described
above is that they assume that the populations being
compared have equal variances. This was not always the
case in my experiments. Especially, the auxin-treated
populations had a much greater variance than controls
and other treatments. When experiments having populations
with unequal variances were to be analyzed, the popula-
tions were divided into 2 groups, one group with low
variance and one with high variance. The low variance
populations in these cases would always be shorter in
growth than the high variance populations. The highest
ranking low variance population was compared to the
lowest ranking high variance population by means of the
Kolmogorov-Smirnov test (Hollander and Wolfe, 1973).
An example of this test appears in Appendix A II. This
nonparametric test makes no assumption of equal variance,

but only 2 populations can be tested at one time. The high variance populations were then compared to each other using the Kruskal-Wallis and multiple comparison test described above, and the low variance populations were analyzed similarly. If an equal variance group consisted of only 2 populations, the Wilcoxon Rank Sum test was used rather than the Kruskal-Wallis test. Since, in these cases of unequal variance, three non-independent tests were made on the same data, α was set equal to 0.01 for each test. Thus, the combined α for all three tests was less than 0.05.

Another problem of analysis arose from the design of the photographically measured experiments. An ideal experimental design would have been to separate individual hypocotyls receiving the same treatment, so that actual measurements of change in length during the treatment could have been obtained for each individual segment. However, because of the survey nature of this project, involving testing numerous factors and combinations of factors for ability to stimulate elongation, this design was impractical. As an alternative, I used the Kruskal-Wallis test and the multiple comparison test to determine whether any of the initial groups of segments were sig-nificantly different. Unfortunately, in most cases the treatment groups were initially different. This is

because the accuracy of cutting segments to 5 mm was very
low for curved segments. In most of the experiments,
though, the differences between the segments initially
are much less than the final differences in length due
to treatments. Also, in most cases, the final treatment
ranks seem to have no relationship to the initial ranks.
However, because the initial differences may be influencing
the final length differences in some cases, the results
of the initial differences will be discussed separately
for each experiment where they may be important. From a
comparison of the initial and final treatment differences,
it was usually possible to obtain a fairly clear picture
of the response that occurred.

CHAPTER III

RESULTS

Although single experiments often included combin-
ations of fluoride treatments and hormone or acid treat-
ments, for presentation, the results are separated into
2 sections: natural regulators of elongation (non-fluoride)
and fluoride effects on elongation. Therefore, in some
cases, results are presented as though they are the results
of an entire experiment, whereas the actual analysis was
done on these results combined with data from other
treatments. In Appendix A III to F XI, the results of
statistical tests are presented as they were actually
obtained. However, for presentation of the results,
the treatments of interest are considered separately
from the other treatments with which they were analyzed.

Natural Regulators of Elongation

Natural regulators of elongation were studied in
experiments on seedlings and excised segments. Seedlings
were grown in vermiculite throughout the course of the
experiment. These experiments sometimes involved excising
portions of the seedlings and then measuring elongation

of the hypocotyl. During the excised segment experiments, the hypocotyl was excised and then incubated in a test solution.

Entire Plants

Entire plant studies included (1) the time course of elongation in the intact plant, (2) the role of the mega-gametophyte, cotyledons and apical meristem on elongation, and (3) the _in_ _vivo_ elongation of the hypocotyl region immediately below the cotyledonary node.

First, the time course of elongation was determined to provide a basis for the selection of seedlings for experiments on excised hypocotyl segments. Elongation of intact seedlings is great during the period from two to five days after germination (Figure 9). The chosen age for excision of a 5 mm hypocotyl segment was three days after germination, since this was within the period of rapid elongation, and growth of the hypocotyl to at least 5 mm could be expected at this time.

Total growth of seedlings was unaffected by removal of the megagametophyte after the hypocotyl had grown 1 cm (Figure 9). The removal of the cotyledons and apical meristem had no effect on elongation for four days (Figure 2). However, after four days no subsequent growth occurred. An attempt was made to replace the cotyledons and apical meristem with auxin in agar drops

Figure 9. Growth curve for entire seedlings from the
day of germination (Day 0) to 10-11 days after germin-
ation. Hypocotyl length is shown for intact seedlings
(●), seedlings minus the megagametophyte (▲), and
seedlings minus the megagametophyte, apical meristem
and cotyledons (○).
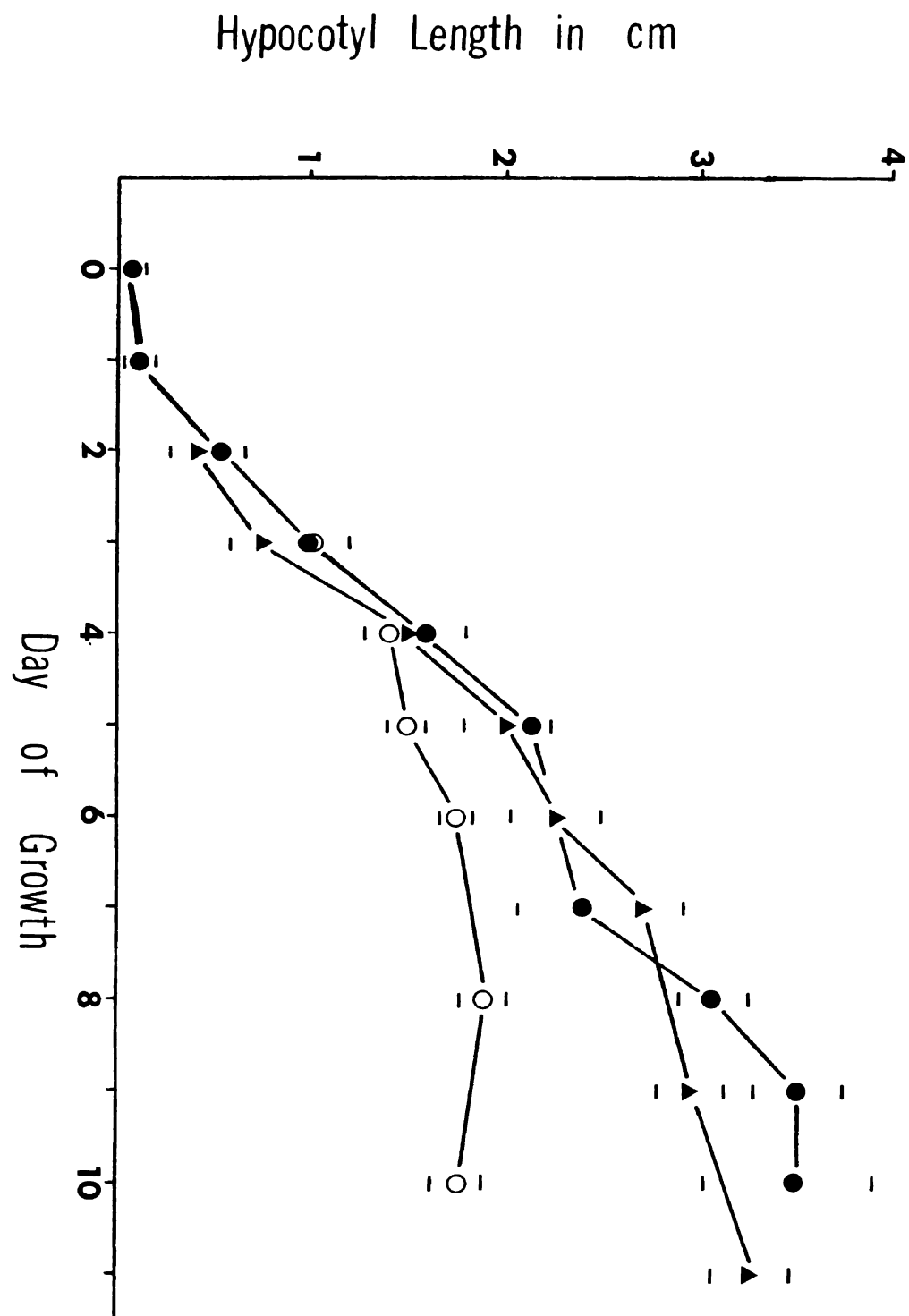
applied to the cut surface of the hypocotyl. No significant difference in hypocotyl elongation was found, using a t test, but this may be due to the inaccuracy of the measuring technique. A dramatic morphological effect was produced by auxin at 10^{-4} M in this experiment. The hypocotyl, instead of growing straight and erect as usual, was more wavy in morphology. It appeared that if the hypocotyl was growing erect at the time of auxin application, it subsequently bent and began to grow downwards. If the hypocotyl hook was still present at the time of auxin application, the hypocotyl responded by bending upwards. Subsequent auxin application resulted in a wavy hypocotyl. The hypocotyls also appeared thicker than those of the other treatments. The lower auxin concentrations, 10^{-6} and 10^{-8} M, and the control did not have any effect on hypocotyl morphology.

The in vivo elongation response of 5 mm of hypocotyl tissue immediately below the cotyledonary node was observed for future comparison with the elongation of excised hypocotyl segments (Figure 10).

Excised Segments

Auxin. Elongation of excised hypocotyl segments was enhanced by NAA (Figures 11 and 12). All auxin treatments were significantly different from the control using the Kolmogorov-Smirnov test (Appendix A III). The

Figure 10.   Histograms showing the elongation _in vivo_ of the 5 mm section of hypocotyl tissue immediately below the cotyledonary node. (A) 5 mm of hypocotyl marked off 3 days after germination and elongation measurement made 4 days after germination. (B) Same as A except elongation measured from day 2 to day 3. Each symbol represents one individual.

A

Length in mm

B
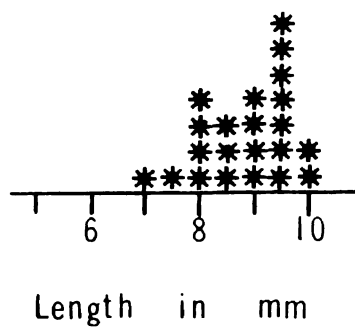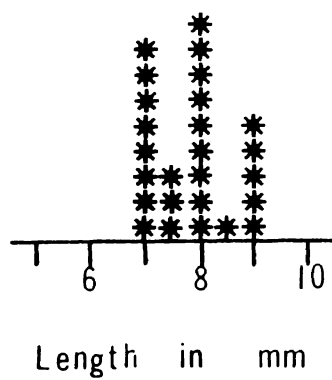
Length in mm

Figure 11. Histograms for hypocotyl segment length after incubation for 24 hrs in 10^{-4}, 10^{-6}, and 10^{-8} M NAA solutions in 0.001 M phosphate buffer, pH 6 and control phosphate buffer alone. Lengths are rounded to the nearest 0.5 mm.
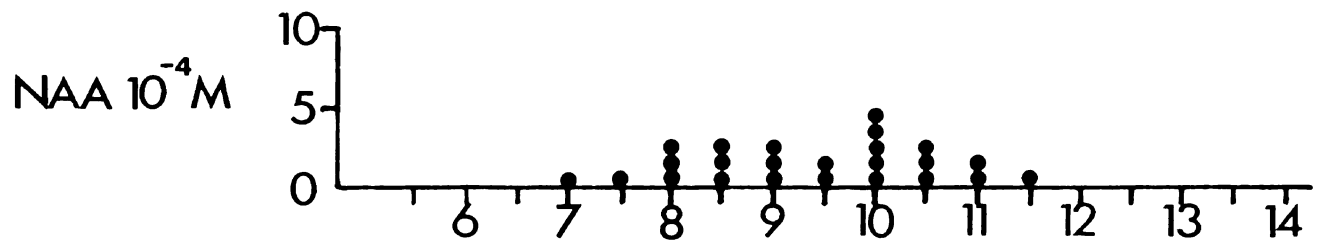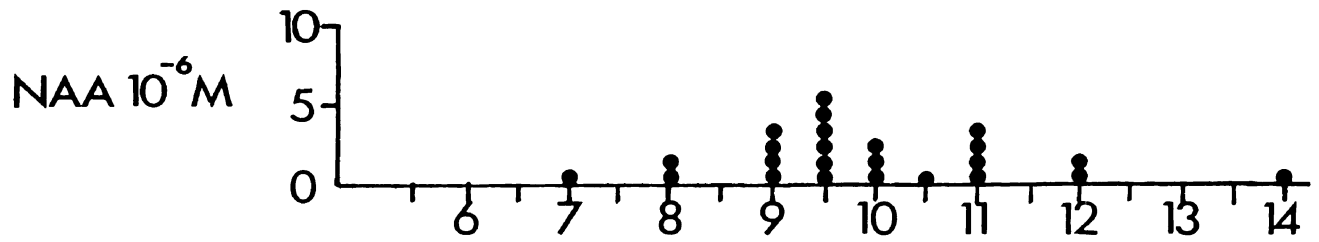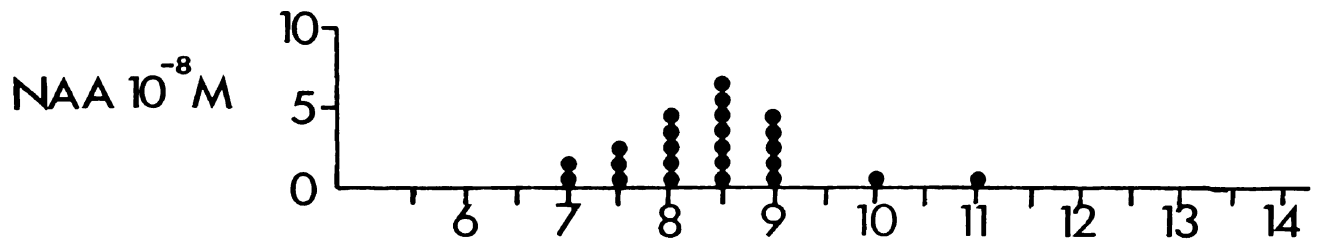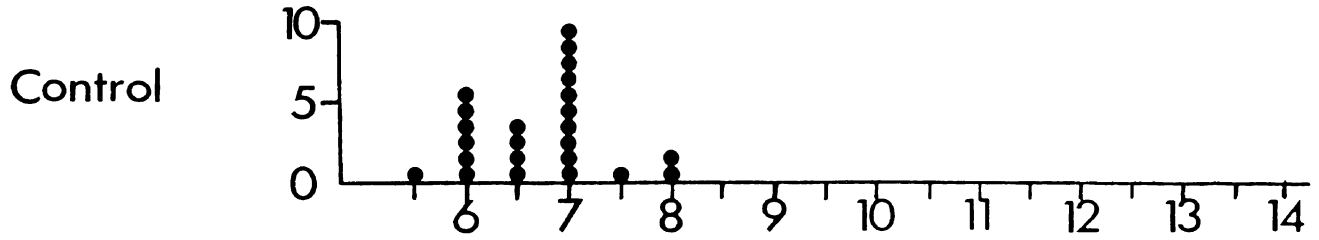
Figure 12.   Segments after 24 hrs incubation in 10^{-6} M
NAA (N24) and 0.001 M phosphate buffer (B24).
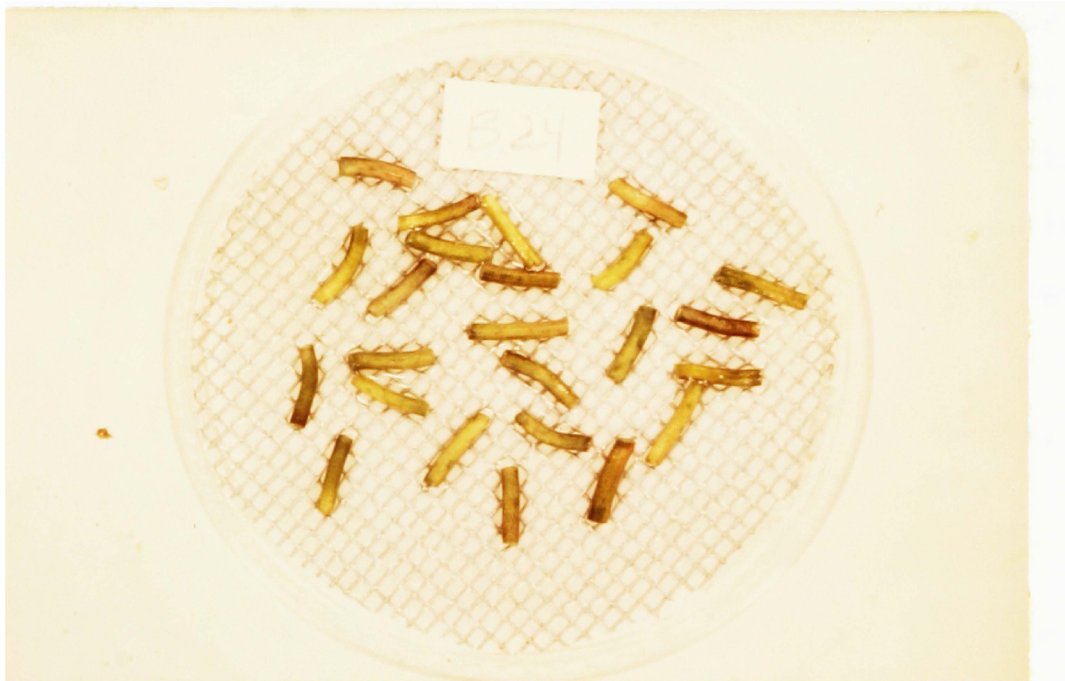
10^{-8} M NAA treatment produced significantly less elongation than the 10^{-6} M NAA treatment using the multiple comparison test. However, the 10^{-4} M treatment was not significantly different from either of the other two auxin treatments. The optimum concentration for elongation in this system appears to be approximately 10^{-6} M. Elongation of the segments incubated in auxin solutions appears to be similar in magnitude to elongation of the corresponding region in the intact plant (Figures 10 and 11). The hypocotyls incubated in auxin solutions seemed to have more of a tendency to curve than those in the control (Figure 12). This curving reduced the accuracy of measurement, since the photographic technique was not used for this experiment. Therefore, it is difficult to compare directly the in vivo hypocotyl elongation with the excised segment elongation in auxin solutions. Also, some variation in elongation occurred between controls from different experiments. Because of this variation, comparison of results from different experiments is only approximate, even when the photographic technique is used.

When hypocotyl segments with attached cotyledons were incubated in auxin solutions, results were similar to those obtained with hypocotyl segments alone. However, no elongation response occurred in the 10^{-8} M NAA solution. The response was again greatest in 10^{-6} M NAA, but, as

with hypocotyls alone, the responses at 10^{-6} M and 10^{-4} M
were not significantly different. The magnitude of elon-
gation in both control and auxin treatments was unchanged
by the cotyledons.

An increase in the rate of elongation in response
to auxin by the excised hypocotyl segments did not occur
until 120 min (2 hr) after the beginning of incubation
in 10^{-6} M auxin (Figure 13).

Acid. Elongation of intact hypocotyl segments was
not stimulated by 0.01 M citrate buffer at low pH. When
segments were split in half longitudinally, they responded
to low pH, with the optimum response at pH 3 (Figure 14).
The response at pH 3 was significantly greater than the
response at pH 5 and 6, using the multiple comparison
test (Appendix B I). The pH 4 response was greater than
that at pH 6. Thus, there seems to be a increasing
stimulation of elongation as the pH is lowered from 6 to
3. Since the initial average rank sums for the treatments
are in the same order as the final ones (Appendix B I)
the validity of these results might be questioned. However,
segments in the pH 5 and pH 3 treatment groups are not
significantly different to begin with, but the segments
in the pH 3 treatment have become significantly longer
than the pH 5 segments after 24 hr of incubation (Appendix
B I). Therefore, there does seem to be a real response
at pH 3.

Figure 13. Time course of elongation of hypocotyl segments in 10^{-6} M NAA in 0.001 M phosphate buffer, pH 6 (◆) and control phosphate buffer alone (●).
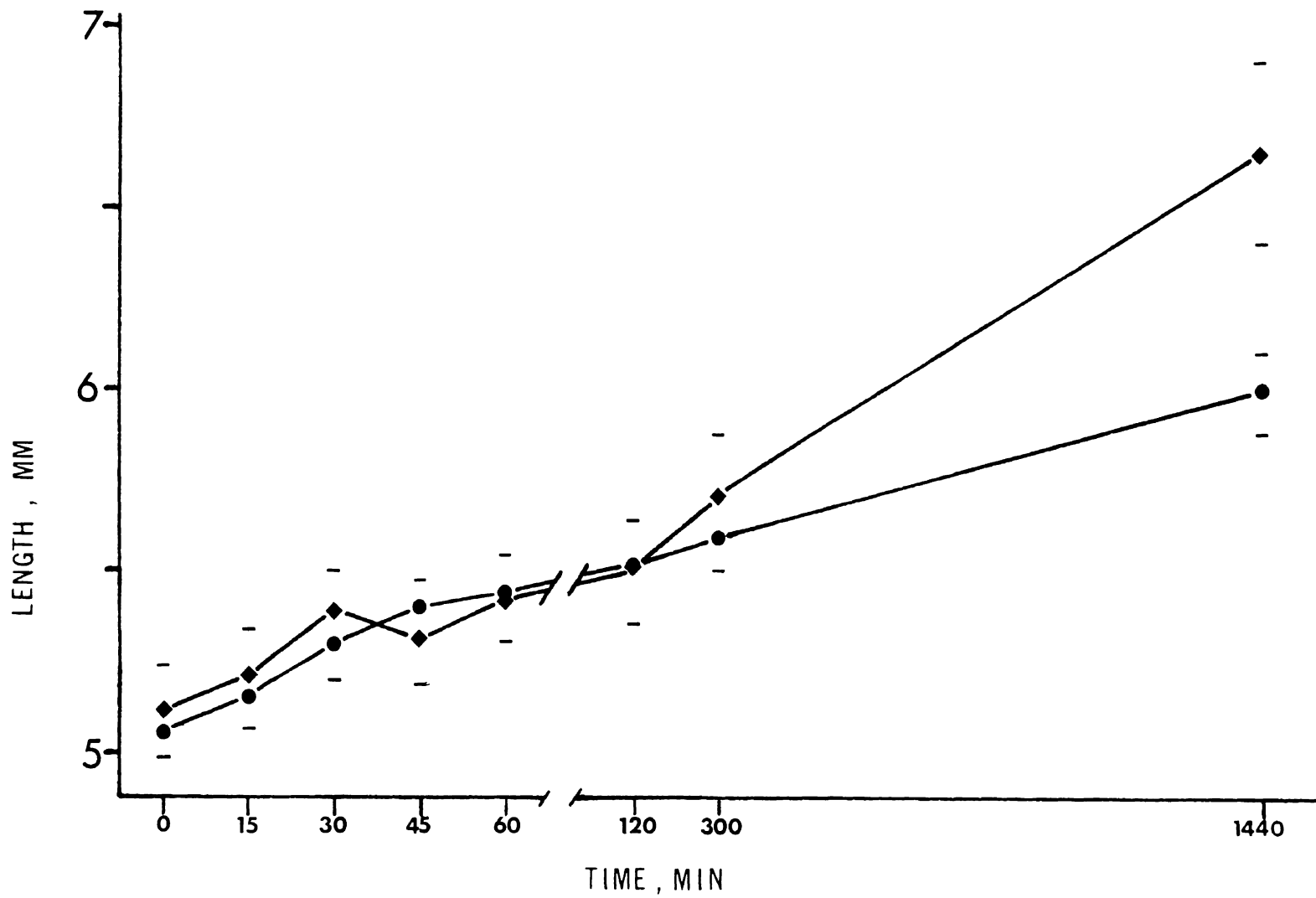
Figure 14. Histograms for lengths of longitudinally split hypocotyl segments incubated in 0.01 M citrate buffer, pH 6, 5, 4, and 3, for 24 hrs. Lengths are rounded to the nearest 0.5 mm. (O) before incubation and (●) after incubation.

Number of hypocotyls

pH 6

pH 5

pH 4

pH 3

LENGTH IN MM
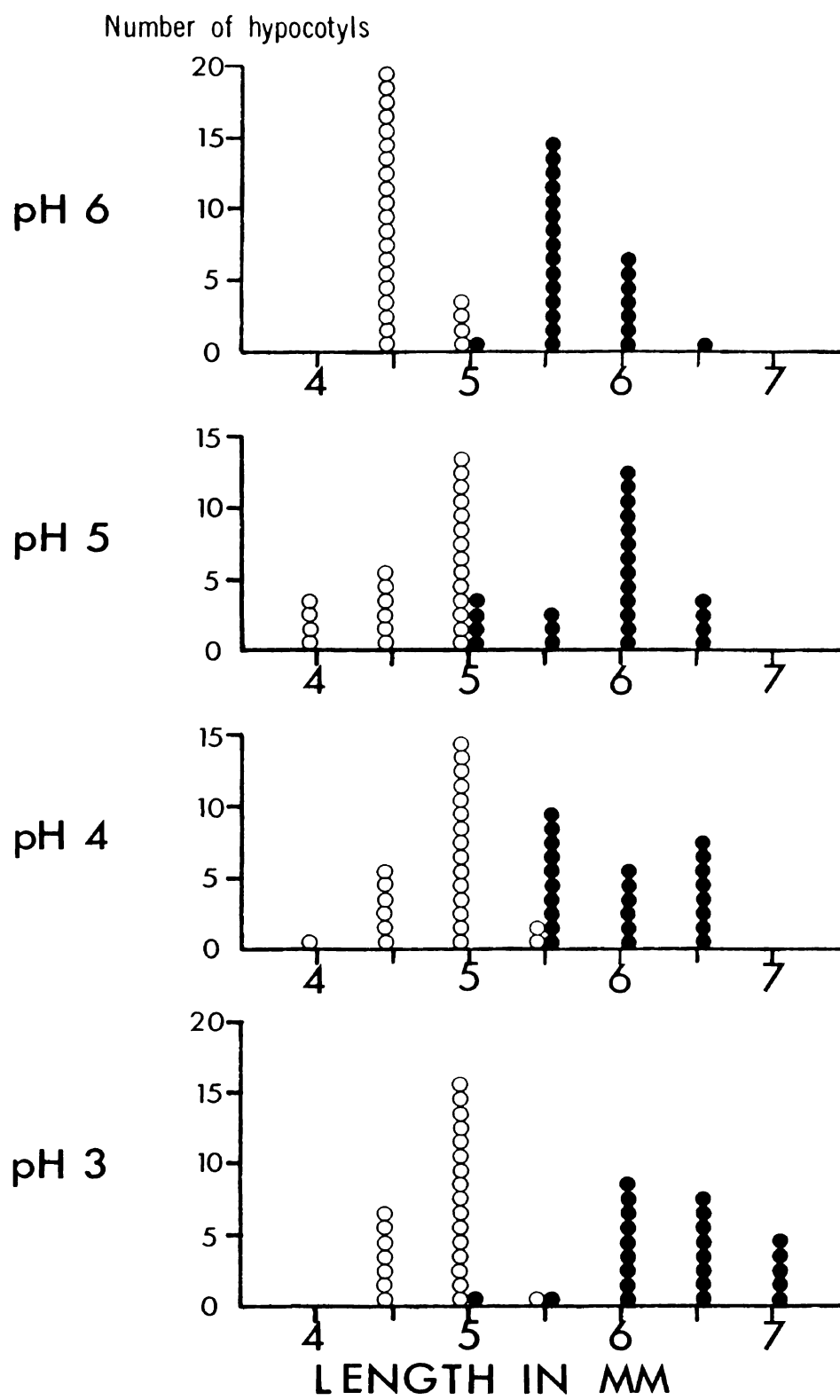
The pH 3 treated split segments, in addition to
showing increased elongation, were yellow rather than
green, like the segments in other treatments. There was
also a loss of turgidity in the pH 3 treatment after
24 hrs.

When hypocotyl segments were slit longitudinally
and aspirated 1 min in the test solution, elongation was
stimulated (Figure 15). Maximum stimulation was at pH 3,
as in the split hypocotyl experiment. This time the
initial lengths did not present a problem, since the pH
3 treatment group was initially the shortest and the pH
6 group initially longest (Appendix B II). Though the
pH 3 segments started out the shortest, they were sig-
nificantly longer, using the multiple comparison test,
than the segments of any other treatment after 24 hr of
incubation. The magnitude of the response, expressed
as increase in mean length of the pH 3 treatment relative
to the control (pH 6) was 1.13 mm. This represented a
118% increase in elongation in the pH 3 treatment, using
the control as 100%. Seedlings from the same planting
were used for an auxin fast response experiment, so the
magnitude of the auxin and acid responses can be compared
approximately. The auxin-treated segments elongate to
an average length 1.9 mm greater than the mean control
length, or 127%. However, the controls themselves are

Figure 15. Histograms for lengths of slit and aspirated
hypocotyl segments at initiation of incubation (O) and
after 24 hours of incubation (●) in 0.01 M citrate buffer
at pH 3, 4, 5, and 6. Measurements were rounded to the
nearest 0.5 mm.

Number of hypocotyls

pH 6

pH 5

pH 4

pH 3

LENGTH IN MM
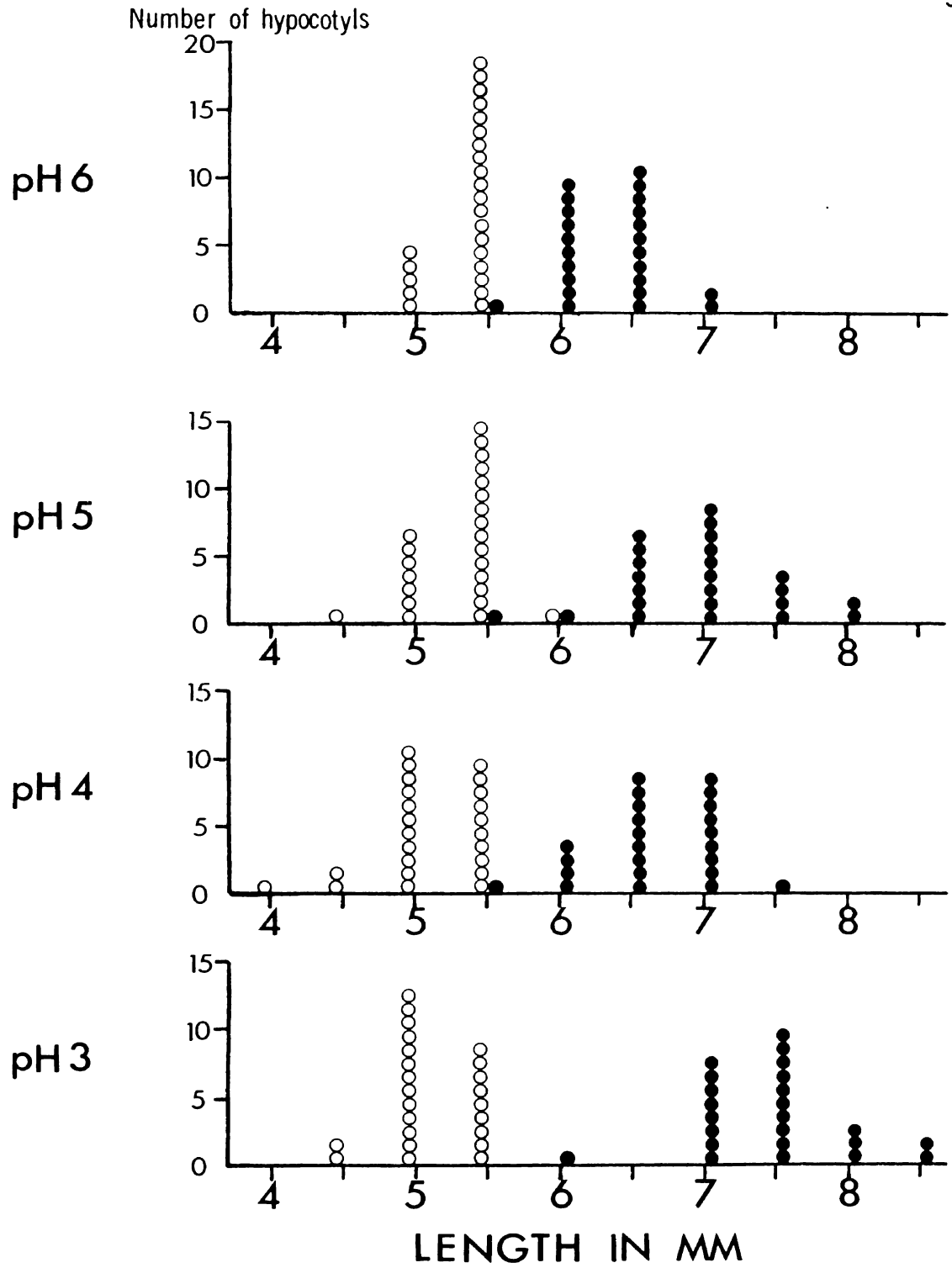
very different. The growth in 0.1 M citrate buffer at
pH 6, used as a control for the acid experiment was
depressed when compared to growth in the 0.001 M phosphate
buffer. In fact, the stimulation at pH 3 produced seg-
ments only slightly longer than those of the phosphate
buffer control, the respective means being 7.4 mm and
7.0 mm.

Slit segments incubated at pH 3 lost turgor and
became discolored. The ends of segments and sometimes
whole segments appeared yellow or yellowish beige in color.

Gibberellin. No effect of GA_3 on elongation of
excised hypocotyls was found (Figure 16, Appendix C I).
The treatments at 10^{-8}, 10^{-6} and 10^{-4} M were not signif-
icantly different from the control using the Kruskal-
Wallis test. No response to GA_3 was obtained in experi-
ments at the same GA_3 concentrations using hypocotyl
segments with attached cotyledons or segments incubated
in the flask with isolated excised cotyledons (Appendix
C II and C III). GA_3 had no effect on the response of
segments to 10^{-6} M NAA (Appendix C I).

Cheesecloth. An unexpected stimulation of elongation
by cheesecloth was found. When the fast auxin response
experiment was performed using cheesecloth incubated
with the hypocotyl segments, the control segments

Figure 16. Histograms for lengths of hypocotyl segments after incubation for 24 hrs in 10^{-4}, 10^{-6}, and 10^{-8} M GA_3 solutions in 0.001 M phosphate buffer and control phosphate buffer alone. Lengths are rounded to the nearest 0.5 mm.

Number of hypocotyls

Control

GA 10^{-8}M

GA 10^{-6}M

GA 10^{-4}M

LENGTH IN MM
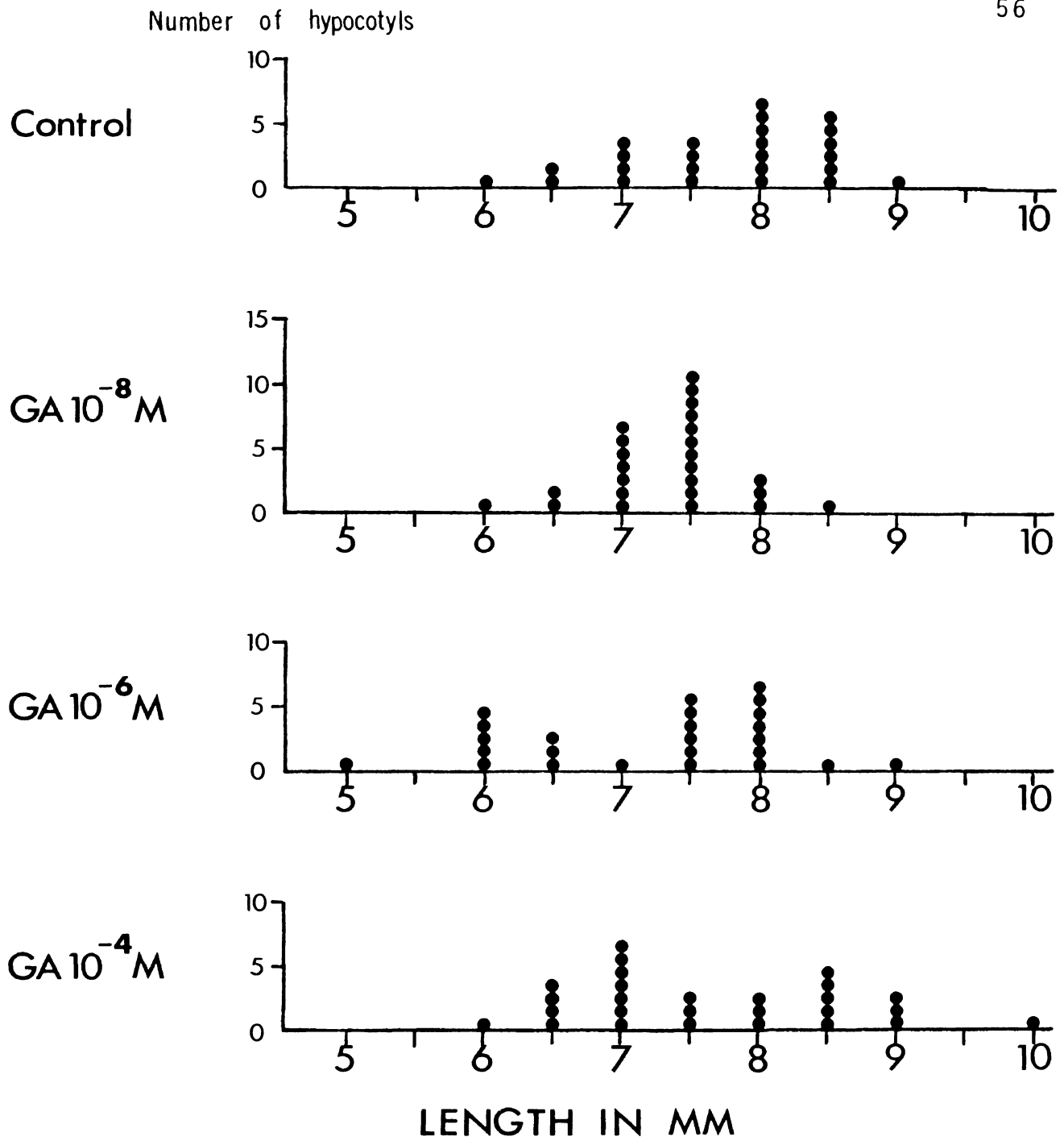
elongated as much as the auxin treated segments. The experiment was repeated twice, and results were the same whether cheesecloth was used for the fast response experiment or merely incubated with the segments for 24 hr. Typical results appear in Appendix D. The mean change in length caused by cheesecloth relative to the control is 2.74 mm, or 149%. An auxin experiment from the same planting showed a 1.91 mm increase over the control, which represented 133%. Thus, the cheesecloth response is at least as large in magnitude as the auxin response and perhaps greater. Any auxin response in the presence of cheesecloth is completely obscured; incubating in cheesecloth with auxin does not produce more elongation than with cheesecloth alone (Appendix D).

Abscisic Acid. In an attempt to determine whether the cheesecloth effect resulted from cheesecloth being a source of ABA, the response of the hypocotyl segments to ABA was tested. Though there was a slight significant stimulation of elongation using the multiple comparison test (Appendix E), the response was far from being as great in magnitude as the cheesecloth effect. In fact, the magnitude of the response, 0.36 mm or 106% mean elongation relative to the control, could easily be a variation due to chance.

Fluoride Effects on Elongation

Sodium Fluoride

Entire Plants. When seedlings were transferred two days after germination to agar slants containing 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7} M NaF, no effect on growth was observed in the range from 10^{-7} to 10^{-3} M NaF. Elongation of hypocotyls, roots, and cotyledons was measured. Only 10^{-1} M NaF significantly inhibited elongation of all parts measured (Figure 17, Appendix F I). The seedlings transferred to 10^{-1} M NaF remained the same length as they were when transferred; no elongation occurred in the slant. Results were similar when seeds were germinated on agar slants with NaF. In this case, the 10^{-1} M seeds failed to germinate, so again no growth at all was observed at this concentration. Other NaF treatments showed no effect on elongation in the germination experiment.

Excised Segments. No effect on elongation was observed with NaF in the range from 10^{-1} M to 10^{-8} M (Figure 11, Appendix F II, Appendix A I). At a concentration of 10^{-1} M, elongation was inhibited (Figure 18). This inhibition was significant using the multiple comparison test. These results agree with results from seedlings grown in slants.

Figure 17. Representative seedlings grown in fluoride agar slants. The first number in the label for each plant is the negative log of the NaF concentration, except for the control numbered 0. The second number is the seedling identification number. Growth was the same as the control in all concentrations of NaF except 10^{-1} M.
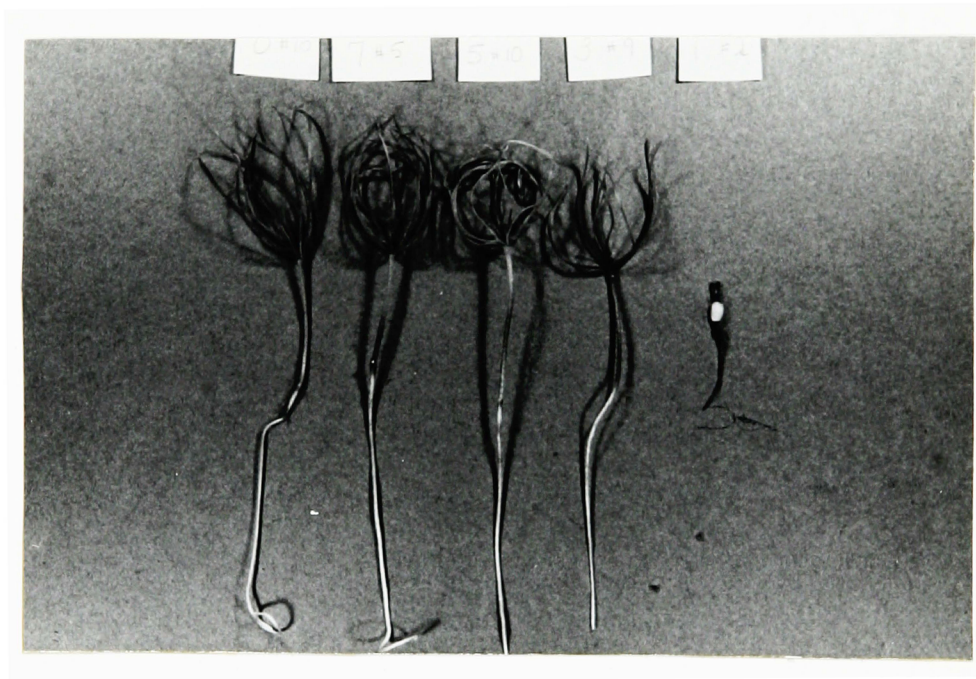
Figure 18.   Histograms for hypocotyl segment lengths
before incubation (O) and after 24 hrs incubation in
10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} M NaF solutions in 0.0001 M
phosphate buffer and control phosphate buffer alone.
Lengths are rounded to the nearest 0.1 mm.
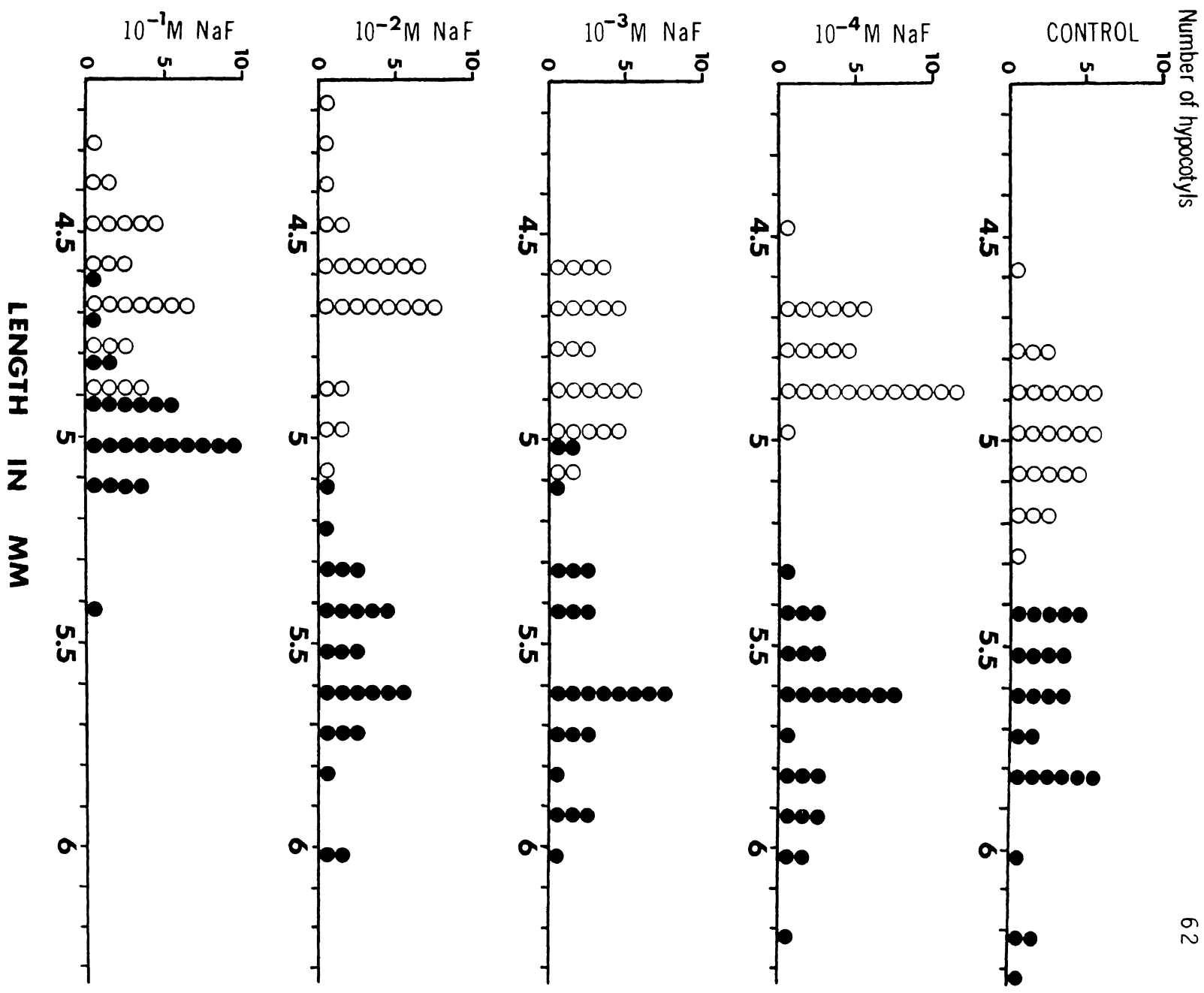
To determine whether the inhibition of elongation at 10^{-1} M NaF might be an osmotic effect, an experiment using NaCl at concentration of 10^{-1}, 10^{-3}, and 10^{-5} M was done. No inhibitory effect was observed with NaCl (Appendix F III). Although the treatment with 10^{-1} M NaCl is significantly longer than the control after incubation, it was also significantly longer before incubation. Therefore, there is assumed to be no real stimulation of elongation by NaCl.

When cotyledons were left attached to hypocotyl segments incubated in NaF, results were generally similar to those without attached cotyledons. At high concentrations of NaF, there is an indication of inhibition. Although the 10^{-2} M NaF treatment is not significantly less than the 5 x 10^{-3} M treatment (Appendix F IV), using the Kolmogorov-Smirnov test at the 0.025 level, the difference is significant at the 0.05 level. Thus, the 10^{-2} M treatment can be assumed to be different at the 0.025 level from the other treatments, which are greater than the 5 x 10^{-3} M treatment. The general pattern of inhibition of elongation with increasing NaF concentration was similar to results from hypocotyls without cotyledons, even though the threshold concentration in this experiment might be higher (10^{-2} M instead of 10^{-1} M).

Since a preliminary experiment had indicated a slight stimulation in elongation by NaF when cotyledons were left attached, in disagreemtnt with the results just described, another experiment was done to deal with this conflict. This time, data were kept on the change in length of individual segments exposed to either NaF or control solutions. This design reduced variation in final length due to initial length differences. The results showed a slight but significant inhibition with NaF at 5×10^{-4} M, using the Wilcoxon Rank Sum test.

Age of seedlings used as sources of hypocotyl segments was a variable which could have been important in the NaF effect. Perhaps younger seedlings could have been more sensitive to NaF. When seedlings two days after germination were used (one day younger than those used for other experiments), no significant effect on elongation was found with NaF at 5×10^{-4} M. The Wilcoxon Rank Sum test was used.

Isolated cotyledons were also incubated with hypocotyls in NaF solutions at 10^{-4}, 10^{-6}, and 10^{-8} M. In both continuous light and continuous darkness, the three concentrations of NaF had no effect on hypocotyl elongation, using the multiple comparison test (Appendix C III and C IV).

When GA_3 at 10^{-4} M was used in combination with NaF as a treatment solution, the results were the same as with NaF alone in the range of concentrations from 10^{-1} to 10^{-8} M (Appendix F VI and C I). When GA_3 at 10^{-6} to 10^{-8} M was added to NaF at 10^{-4} to 10^{-8} M, no effect on elongation was found (Appendix C I). Although the lowest treatment, 10^{-6} M GA_3 with 10^{-4} M NaF was significantly less than two of the treatments and the control, the variation observed in this experiment seems to be due to chance. No gradual change in effect with a change in concentration was found. Treatments very close in concentration were significantly different, while treatments more different in concentration were not. For example, 10^{-6} M GA_3 with 10^{-4} M NaF was different from 10^{-6} M GA_3 with 10^{-6} M NaF. However, the former was not different from GA_3 10^{-8} M without NaF. Any attempt to show a response pattern was futile because of the lack of significance of the differences of almost all responses in this experiment. The same interpretation applied to the experiments with GA_3 and NaF using segments with unattached cotyledons. Although there were several significant differences, the overlap of insignificant differences was too great to show any response (Appendix C III and C IV). These experiments appeared to show no response after 24 hr of incubation, so instead of measuring

at this time, they were allowed to incubate longer to determine whether a response might occur. However, even after the longer incubation time, a response could not be detected.

When NAA at 10^{-6} M was added to NaF at a range from 10^{-1} to 10^{-4} M, an auxin response was obtained with 10^{-3} and 10^{-4} M NaF (Appendix F VII). The auxin response at both of these NaF concentrations was significantly greater than the control, using the Kolmogorov-Smirnov test. The auxin response with 10^{-4} M NaF was significantly greater, however, than that with 10^{-3} M NaF, using the Wilcoxon Rank Sum test at the 0.01 level. An inhibition of the auxin-induced stimulation of elongation occurred at a concentration of 10^{-2} M NaF. This treatment was significantly less than the control, using the multiple comparison test.

Sodium fluoride at 10^{-4} to 10^{-8} M was also tested in combination with 10^{-4} to 10^{-8} M NAA. The NaF had no effect on the NAA response when 10^{-6} and 10^{-4} M NAA were tested. However, the auxin stimulation of elongation observed with 10^{-8} M NAA was lost when NaF was added at 10^{-4}, 10^{-6} or 10^{-8} M (Appendix A I).

The possibility was considered that the plant might require some period of pretreatment to fluoride before a fluoride-induced stimulation of the auxin response

could be observed. When seedlings were grown in 10^{-3}, 10^{-5}, and 10^{-7} M NaF agar slants for three days before the hypocotyl segments were excised and incubated in 10^{-6} M NAA, no change in response was observed (Appendix F VII). The auxin treated segments were different from the control segments using the Kolmogorov-Smirnov test. The auxin treatments were equal using the multiple comparison test. The control segments from slants with different fluoride concentrations were also equal to the control from a slant without fluoride.

The effect of NaF at a range from 10^{-4} to 10^{-8} M on the acid response using citrate buffer was tested. In this experiment, the pH optimum for elongation was 4 (Appendix F IX). No stimulation of elongation by fluoride in combination with acid was observed. Addition of NaF at all three concentrations negated the observed stimulation at pH 3. Also, the pH 4 treatment with NaF at 10^{-8} M was the same as the control and the pH 3 treatments with NaF. At pH 3, 74% of the HF formed by the association of NaF with water will be in undissociated form, as calculated using the Henderson-Hasselbalch equation (Handbook of Chemistry and Physics, 1971; Murphy and Rousseau, 1969; Rendina, 1971). Therefore, the fluoride effect at this pH is due primarily to HF rather than F^-. At pH 4, the HF is 22% undissociated, so HF is important at this concentration also.

Hydrogen Fluoride. When hypocotyl segments with attached cotyledons were tested, a stimulation of elongation by HF was not observed (Figure 19). No significant effect on elongation was obtained from pH 5.5 to pH 4, using the multiple comparison test (Appendix F X). The response at pH 3 was not different from that at pH 5.5, using the Kilmogorov-Smirnov test. The pH 5.5 treatment had the lowest rank sum of all the treatments in the pH range from 5.5 to 3.5. Thus, pH 3 segments may still be shorter than those of other treatments. Using the Wilcoxon Rank Sum test, the segments incubated at pH 2.5 are significantly shorter than the pH 3 segments. Since pH 3 is the shortest of all the other treatments, the pH 2.5 segments must be significantly shorter than all treatments. The histogram (Figure 19) shows elongation to be almost completely inhibited in the pH 2.5 solution. The effect is at least as dramatic as that caused by 10^{-1} M NaF. Thus, pH 2.5 HF, which represents a 9×10^{-3} M concentration is as effective an inhibitor as NaF at approximately 10 times this concentration.

Dramatic color changes were observed in this experiment. The segments incubated at pH 2.5 were entirely discolored to a beige color. A loss of turgor was also evident. The segments treated at pH 3 were yellow at the ends and green in the middle.

Figure 19. Histograms for hypocotyl segments with attached cotyledons before (○) and after 24 hrs (●) of incubation in HF at pH 5, 4, 3, and 2.5, and control distilled water. Lengths are rounded to the nearest 0.2 mm. This figure shows results from representative treatments. For results of the complete experiment, see Appendix F X.
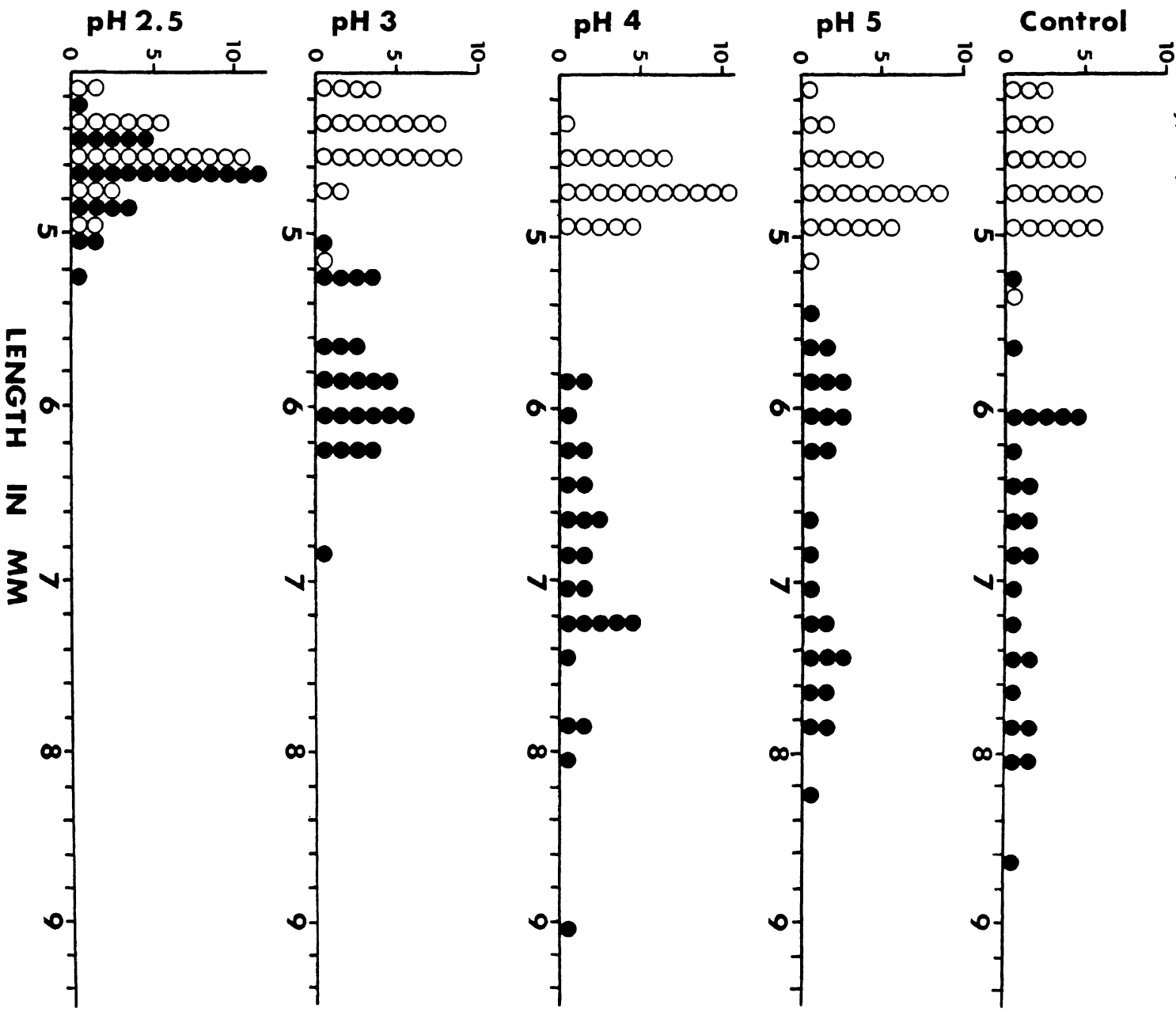
Hypocotyl segments slit longitudinally and aspirated, as for the citrate buffer experiments, were also incubated in HF solutions. A range of pH from 6 to 3 was tested. The elongation at pH 3 was significantly less than for the control using the multiple comparison test (Appendix F XI). The treatments at pH 4 and 4.5 were also shorter than the control. However, the pH 4 treated segments started the experiment significantly shorter than the control (Appendix F XI). This experiment, nonetheless, does indicate that the inhibition response to HF occurs at a pH higher than 2.5 when penetration of the solution is facilitated.

The color change associated with low pH again was observed. The segments at pH 3 were entirely beige-colored or had beige-colored ends. A loss of turgor also occurred.

CHAPTER IV

DISCUSSION

Results of the experiments with intact plants indicate that the megagametophyte has essentially no growth regulatory role after the hypocotyl has achieved a length of 1 cm. The apical meristem and cotyledons, however, are important, since total growth is decreased when they are removed. The growth achieved by decapitated seedlings, however, is almost 2/3 that of the intact seedlings. Thus, the hypocotyl and root system is capable of some growth on its own. The hypocotyl, which is photosynthetic, is a source of nutrients. The roots may be a source of the hormone gibberellin (GA), since there is evidence that GA is synthesized in root tips (Lang, 1970). The effect of the apex and cotyledons does not seem to be replaced by auxin applied as agar drops. It seems possible that the wavy hypocotyl morphology found at the highest auxin concentration might be due to auxin production of ethylene. Some asymmetric distribution of hormones also would have to occur to produce the unequal growth of the two halves of the stem, forming a wave. The indication that ethylene might be involved is that the hypocotyls showing the change

72

in morphology also appeared to be larger in diameter than the other hypocotyls. Ethylene is known to induce lateral swelling, and its inhibition of growth may be a factor in geotropic curvature of roots (Leopold and Kriedemann, 1975).

Excised hypocotyl segments of ponderosa pine elongate in response to auxin, as do hypocotyl segments from other pine species (Giertych and Forward, 1966; Hashizume, 1965a; Witkowska-Zuk and Wodzicki, 1970. and references cited therein; Zakrzewski, 1975, and references cited therein). Calculating from my data and from the published data the percent elongation with auxin relative to the final length of the control, and also relative to the initial length of the segment provides a means for comparing my results with those of other workers. Ponderosa pine segments show approximately a 127% stimulation in growth with auxin relative to the control as calculated from the ratio of average final length after auxin treatment to the average final control length. The change in length during auxin treatment relative to the initial length before treatment is approximately 93%. The segments almost double in length when treated with auxin for 24 hrs. The reason these values are approximate is that different plantings of seeds grew differently, although an auxin response was always obtained relative to the control.

This magnitude of auxin enhanced growth agrees with results obtained by Hashizume (1965a) with Pinus nigra. The growth increase calculated from his data indicates a 97% change in length relative to the initial length at the optimum concentration of indoleacetic acid (IAA). This concentration, 50 mg/l or approximately 5×10^{-4} M, seems quite high relative to my results and other results discussed below. However, tissues may respond differently to various auxins. As an example, Pinus silvestris probably is optimally sensitive to NAA at a lower concentration than to IAA (Zakrzewski, 1975).

Using a hypocotyl segment length of 8 to 12 mm, and incubating 24 hrs, Witkowska-Zuk and Wodzicki (1970) obtained with Pinus silvestris a 121% stimulation by IAA relative to the control (their Table 2) and a 69% IAA mediated change in length relative to initial length, as calculated from their data. Thus, elongation relative to the control is similar to ponderosa pine but the change in length is less. The optimum response to IAA in Pinus silvestris is at a concentration of 1.0 mg/l, or almost 10^{-5} M. This concentration is more than the optimal NAA concentration for ponderosa pine, at approximately 10^{-6} M. More recently, Pinus silvestris has been studied further by Zakrzewski (1975). Since he used NAA as an auxin source and 4.5 mm segments incubated for 20 hrs, his

experiments are almost exactly comparable to mine. The optimum NAA concentration is equal to that for ponderosa pine. The stimulation by auxin relative to the control, calculated from his data, is 125%, which is very close to my value of 127%. However, as in Witkowska-Zuk and Wodzicki (1970), the change in length induced by auxin is less. The change in length calculated from Zakrzewski (1975) is 67%. This is less than my calculated result of 93%. Perhaps the stimulation relative to the control is a more constant value, since variation in growth potential from experiment to experiment will be reflected in the control as well as the treatment. Zakrzewski (1975, Figure 1) does show variation in control growth between different experiments.

Comparison of the ponderosa pine response to auxin with the response in green pea shows some similarity. Cleland and Rayle (1975) present data for 5 mm pea stem segments incubated in IAA for 210 min. Elongation is 19.2% during the total time of incubation, which averages to 5.5%/hr. The response in ponderosa pine, 93%/24 hr, represents an average rate of 3.8%/hr. Thus, the response in ponderosa pine is of a similar order of magnitude to that in pea. Yamamoto et al. (1973) indicate a length increase of 2.5 mm in 7 mm segments incubated for 3 hr with auxin. This is a response of approximately 12%

increase in length per hr, or faster than results obtained
in pea by Cleland and Rayle (1975) or in ponderosa pine.
The fact that both results with pea are faster than with
ponderosa pine could be due to my averaging auxin response
over 24 hrs, while pea tissues were incubating for much
shorter time periods. Although the auxin enhanced rate
of elongation is fairly steady, Zakrzewski (1975, Figure 2)
indicates that this steady rate begins to decline after
12 hrs of incubation in NAA. Thus my values for the
percent elongation per hr are probably somewhat under-
estimated.

The difference between the two pea responses, though,
is difficult to explain. Yamamoto et al. (1973) reported
results for peeled segments incubated with auxin that are
quite similar to Cleland and Rayle's unpeeled segment
response and to my ponderosa pine response. Peeled segments
elongate 1.25 mm in 3 hr, or 6%/hr. Cleland and Rayle
(1975) give data for peeled segments also, which can be
converted to 3.6%/hr. Thus, Yamamoto et al. (1973) con-
clude that peeling segments reduces the response to auxin,
while Cleland and Rayle (1975) maintain that the response
of peeled and unpeeled segments is essentially the same.
The question of whether the epidermis is needed for a
maximal auxin response in pea remains unresolved. It
seems that there is variation in different crops of peas

of the same variety (Cleland and Rayle, 1975). Thus, the different responses might be explained by this variation.

The ponderosa pine hypocotyl response seems to be well within the range of response reported for green pea, considering the amount of variation among reports for pea as discussed above.

Ponderosa pine hypocotyl segments showed a similar response to auxin whether cotyledons were left attached during incubation or not. This may relate to the simi-larity of the magnitude of the 24 hr elongation obtained for the 5 mm area below the cotyledonary node in intact plants and the elongation of excised hypocotyl segments in solution. If auxin is given exogenously at an optimal concentration, the possible source of endogenous auxin, the apex, is not required. Apparently any compounds synthesized by the cotyledons also do not have an effect on elongation in the presence of auxin. It seems strange that auxin could not replace the cotyledons and apex in the decapitated seedling experiments but does seem able to do so in excised segment experiments. For some unknown reason, auxin applied in agar drops to the decapitated hypocotyl produced an abnormal growth response (the wavy hypocotyl) while auxin applied to excised segments shaking in a solution simulated growth in the intact plant. The only condition that seemingly could be different in the

two experiments is aeration. The excised segments were shaken constantly in the solution, and were presumably well aerated. The agar drops were moist and stuck to the cut hypocotyl surface, creating a seal over the surface. Thus, gas exchange in the top region of the hypocotyl may have been impaired. The auxin response is known to require oxygen (Hager et al., 1971). As mentioned earlier in the results, though, some curving was found in excised segments treated with auxin. Thus auxin seems to promote unequal growth of the two sides of the hypocotyl in two different systems. It would be interesting to determine the cause for this auxin promoted curvature in the hypocotyl.

The lack of a fast response in ponderosa pine may be due to the insensitivity of the utilized technique to small increments in growth. Further kinetic studies should be made with pine.

The results obtained with ponderosa pine support the contention by Cleland and Rayle (1975) and by Yamamoto et al. (1973) that the cuticle in light grown stems may be a barrier to H^+ ions. As in _Pinus silvestris_ (Witkowska-Zuk and Wodzicki, 1970), no response to low pH is obtained in ponderosa pine when the cuticle and epidermis are intact. Slitting the epidermis longitudinally and aspirating produced an acid response comparable to that obtained by

Cleland and Rayle (1975). They report a rate of approx-
imately 3.25%/50 min (their Figure 3) which is equal to
3.9%/hr. The ponderosa pine acid response represents
elongation from an initial length of 5.2 mm to a final
length of 7.4 mm, after 24 hours of incubation. This
averages to a rate of approximately 2%/hr. The rate
obtained by Yamamoto et al. (1973) is from 7.0 mm to
7.6 mm in 1 hr (their Figure 1) or 11.7%/hr.

A comparison of the magnitudes of the auxin and acid
responses in ponderosa pine shows the acid response to
be less for 24 hrs than the auxin response. The percent
stimulation by acid relative to the control is 118% as
compared to 127% with auxin. The mean change in length
relative to the initial length is 42% for acid and 93%
for auxin.

That the magnitude of the 24 hr ponderosa pine acid
response is lower than the auxin response and the reported
values for one hour acid responses in pea is not surprising.
The acid response is known to decline after about two hours
(Rayle and Cleland, 1970). Since the rates of the response
to auxin and acid are similar during the first two hours
(Evans, 1974) and the auxin response is maintained at a
steady rate for 24 hrs, the expected result should be an
average extension of acid treated segments at 2/24 or
1/12 of the rate shown by auxin treated segments. However,

the acid rate for ponderosa pine, 42%/24 hr, is almost
half the auxin rate of 93%/24 hr.

Although buffer concentrations at 0.01 M have not
been found to inhibit elongation in other studies (Hashizume,
1965a; Rayle and Cleland, 1970), ponderosa pine hypocotyl
is inhibited by 0.01 M citrate buffer relative to growth
in 0.001 M potassium phosphate buffer. The mean 24 hr
elongation in 0.01 M citrate buffer at pH 3, though much
greater than elongation in the citrate buffer at pH 6,
is only slightly greater than elongation in 0.001 M potas-
sium phosphate buffer, pH 6. The spontaneous growth in
the buffers without exogenous auxin may be due to the
ability of the segments to synthesize their own auxin.
Evans (1973) states that the most likely explanation for
spontaneous growth rate increase in Avena and corn is the
"regeneration of a physiological tip" by the segments,
which occurs about four hours after the segments have
been cut. This physiological tip has the ability to
synthesize auxin. Auxin enhanced elongation is known to
be inhibited in the presence of strong neutral buffers
(Durand and Rayle, 1973). In Avena, they report that a
buffer at pH 6.2 must be at a concentration less than
5 mM if it is to allow the auxin response to occur unim-
paired. Thus, any response to endogenous auxin occurring
in the pine hypocotyl segments would be expected to be

inhibited by the citrate buffer at 0.01 M. The lack of
an inhibition of spontaneous elongation in other studies
mentioned above, using strong buffers, may be explained
by the impermeability of the cuticle to the buffer solu-
tions. With the cuticle protecting the internal tissue,
the auxin would still be able to lower the pH of the
cell wall region sufficiently to induce wall loosening.
However, wall loosening would not occur in slit segments,
since the pH 6 buffer could penetrate the cell wall regions
and prevent a low pH induced wall loosening. This expla-
nation is supported by results with ponderosa pine using
segments with intact cuticle and epidermis in 0.01 M
citrate buffer, pH 6. They have a mean change in length
from 4.92 mm to 6.29 mm, equal to a mean elongation of
1.37 mm. This response is comparable to responses in
other experiments using 0.001 M potassium phosphate buffer,
so 0.01 M citrate buffer does not seem to be able to
inhibit elongation when permeability is not enhanced by
slitting the epidermis. Durand and Rayle (1973) also
report no inhibition with unpeeled segments. If a res-
ponse to endogenous auxin by slit segments is actually
being inhibited in the pH 6 citrate buffer, this response
should still be able to occur in the pH 3 buffer, since
strong buffers at a pH below 6 won't inhibit an auxin
response (Durand and Rayle, 1973). Thus, the endogenous

auxin response would be expected to proceed at a comparable rate as in the 0.001 M phosphate buffer without auxin. If so, then the true acid response would be the difference between the response in the 0.001 M phosphate buffer, pH 6, and the 0.01 M citrate buffer, pH 3, since both would have the endogenous auxin response, but the pH 3 treatment would be expected to produce also an acid response. If the acid response is calculated from the mean citrate buffer, pH 3 response minus the mean phosphate buffer, pH 6 response, the acid response is 0.4 mm in 24 hrs. Comparing this to the 4.3 mm/24 hrs auxin response, the acid response becomes approximately 1/10 that of the auxin response. This is close to the expected 1/12 ratio. As mentioned above, the auxin response may not persist unabated for the full 24 hrs, so this could explain the slightly greater ratio obtained than expected. The fact that the acid response as now calculated is 0.3%/hr, or much less than the values obtained for pea presented above, is still not surprising. Averaging a two hr response over 24 hr would be expected to produce a very low calculation.

Whether the inhibition obtained with 0.01 M citrate buffer is repeatable, is open to some question. There did not seem to be as much inhibition in other experiments with slit and aspirated segments in the citrate

buffer. Notably, the segments split completely in half do not seem to be inhibited in 0.01 M citrate buffer at pH 6 relative to pH 6 0.001 M phosphate buffer. In this treatment, permeability to the buffer would be expected to be great. It is possible that this experiment is not really analogous to the slit and aspirated experiment. Segments split in half still have an intact epidermis. In the area next to the epidermis, a proton pump activated by endogenous auxin might still be able to lower the pH enough to cause cell elongation. An explanation similar to this has been proposed to explain curving in split segments treated with auxin (Durand and Rayle, 1973; Yamamoto et al., 1973). In ponderosa pine, coiling is exhibited in split segments treated with auxin (unpublished observations). The longer outer portion has the epidermis. The curvature should be reversed relative to auxin induced curavture if segments are treated with low pH (Durand and Rayle, 1973; Yamamoto et al., 1973). However, acid-treated split segments of ponderosa pine are curved in the same direction as auxin treated segments although the curvature is lower in magnitude. Therefore, there may be a hydrogen ion gradient within the tissue causing the epidermal side to grow more than the other side. Also, this curvature may indicate that the segments are responding to endogenous auxin. In any case, the fact

that the epidermal side grows more than the inner side implies that there is a lower pH next to the epidermis than next to the acid solution. This could occur if a barrier to permeability through the inner tissue existed. The pith and cortex contain much intercellular air space, and, therefore, may be obstacles to penetration of fluid into the hypocotyl tissue. The problem of air spaces would explain the occurrence of the maximal acid response in segments aspirated in the acid solutions.

Perhaps movement of solution into the pith side of the split segment is easy. In this case the reduced elongation on the pith side could be explained by the pH 6 buffer preventing wall loosening and the low pH buffer acidifying cell contents and damaging metabolic systems and membranes. But then, the pH 5 or 4 buffer would still be expected to promote elongation as pH 5 does in peeled segments of <u>Avena</u> (Rayle, 1973). Since in <u>Avena</u> coleoptile cells, elongation is very sensitive to variation in the water potential gradient between the cell and the surrounding solution (Evans, 1973, and references cited therein), buffers may produce an osmotic inhibition of elongation. Perhaps a slight osmotic inhibition may be occurring in the split ponderosa pine segments on the side without the epidermis. This inhibition would have to be low in magnitude, though, since

length of segments is not inhibited relative to intact segments in 0.001 M phosphate buffer. The osmotic inhibition would explain the lower response at pH 3 in split segments relative to slit and aspirated segments. More work seems to be needed to determine the amount of elongation occurring in buffers of different concentration and at different pH values.

The decreased turgor observed in all experiments with acid treated ponderosa pine segments is expected, since this is a general occurrence (Hager et al., 1971; Rayle and Cleland, 1972). It seems to be partially a result of the loss of turgor that the acid response is of short duration. As the wall is continually loosened by acid, the amount of turgor pressure in the cell promoting extension of the loosened wall becomes less and less. A loosened wall with no force to stretch it will not extend. If an external force is applied to the segment, it will continue elongating for 5 to 6 hrs (Rayle and Cleland, 1972). The reason elongation cannot continue as long as the auxin response even in this case is apparently that low pH causes a loss of membrane integrity and inhibits metabolic processes (Hager et al., 1971).

My results indicating that ponderosa pine responds to both auxin and acid supports the hypothesis of an acid-mediated auxin effect which causes cell wall loosening.

The lack of a GA_3 response in ponderosa pine agrees with results of Witkowska-Zuk and Wodzicki (1970) with Pinus silvestris. Unlike Hashizume (1965a), no stimulation by GA_3 of the auxin effect was observed. Other gibberellins should be tested with ponderosa pine, though, since endogenous gibberellins do promote elongation in Pinus silvestris (references cited in Witkowska-Zuk and Wodzicki, 1970). Michniewicz et al. (1974) indicate that levels of gibberellin are well correlated with stem elongation while auxin levels may be correlated with cell division activity, but are not correlated with stem elongation. Perhaps the gibberellin level in the ponderosa pine hypocotyls is optimal already, and thus no further response to higher exogenous levels occurs. The endogenous auxin level in the intact plant may be suboptimal, since the endogenous level decreases as the hypocotyl maximally elongates (Michniewicz et al., 1974). The hypocotyls would be expected to show a response when a higher concentration of auxin is applied. If this is so, however, then why don't segments in auxin solutions grow to a greater length than the segments left on the intact plant? Perhaps the lack of nutrients or other regulators besides auxin normally obtained from the roots, apex and cotyledons is limiting growth of the excised segments.

This limitation occurring at the same time as an auxin enhanced elongation could produce a final length approximately the same as the intact plant. It also seems possible from my observations that the auxin treated segments may indeed be elongating more than comparable regions in the intact plant. My first auxin experiment (Figure 4) does indeed show longer segment lengths than those in the intact plant (Figure 3). Since there is variation in growth potential between experiments, though, more work needs to be done to determine relative magnitudes of elongation due to different treatments. This applies for all the experiments, since magnitudes have been computed from mean responses. A better measure of length would be to average the responses of known individual segments. Now that acid and auxin responses have been demonstrated for ponderosa pine, more detailed studies on the kinetics of these effects would be fruitful.

The cheesecloth effect remains unexplained. A possible experiment would be incubating the cheesecloth alone in the identical experimental conditions used for segments. If segments then incubated in the solution in which the cheesecloth had been incubated showed enhanced growth, the cheesecloth effect could be shown to be due to a water soluble substance. Heat stability could be tested, and attempts could be made to isolate

the substance. Apparently, the substance is not abscisic acid (ABA), since ABA was far from enhancing growth as much as the cheesecloth, and may not have had any growth-promoting effect at all. The possibility that ABA may enhance growth in some systems should not be eliminated, however, since Gaiter and Lutz (1975) have convincingly shown a stimulation of extension in roots.

In no case was fluoride found to stimulate elongation. An inhibition of elongation was expected from the results with pea (Christiansen and Thimann, 1950a). Although they found an inhibition of auxin induced elongation with 5×10^{-3} M NaF, a concentration at which NaF alone has no effect in my system, my results indicate a greater sensitivity of the auxin response to NaF than the spontaneous growth response of segments without auxin. The auxin response is inhibited in ponderosa pine at a concentration of 10^{-2} M NaF, which is close to the results of Christiansen and Thimann (1950a). Only at 10^{-1} M NaF is the response of segments without auxin inhibited consistently. Perhaps this greater sensitivity of auxin treated tissue is related to the increased metabolic rate of the tissue, which is caused by the auxin treatment and is part of the auxin response (Evans, 1974). Fluoride inhibits enzymes (National Academy of Sciences, 1971), and specifically inhibits cell wall metabolism

(Christiansen and Thimann, 1950). Since auxin promotion of the activity of enzymes may be part of the auxin induced elongation (Evans, 1974), the two factors acting together might result in overall enzyme activity being at the lower control level rather than at the higher level as normally achieved by auxin stimulation. This is actually observed in ponderosa pine. The 10^{-2} M NaF inhibits the increase in length of the auxin treated segments so that they are below the control. Since fluoride is thought to affect membrane permeability (Miller and Miller, 1974), the establishment of a proton gradient may be prevented by flouride. Substances known to promote membrane permeability are able to inhibit the auxin response (Hager et al., 1971).

Although usually NaF applied alone could not inhibit elongation at a concentration lower than 10^{-1} M, in the experiment where individual segments were monitored, a concentration as low as 5 x 10^{-4} M was found to inhibit elongation. Determination of the exact threshold of the response requires further study. It seems possible that problems of cuticle permeability could be involved.

Variations in cuticle permeability have been mentioned by Dowler et al. (1974), in their reinvestigation of the report by Kang and Burg (1971) that auxin increases the membrane permeability to water. Dowler et al. (1974) find the half times for ^{3}HHO efflux (their measure of

water permeability) differ in the three separate labs
where this was tested for the investigation. They conclude
that different cuticular permeability exists in the
seedlings grown in the different laboratories. Cuticular
permeability was not affected by growing seedlings in
conditions of different humidity. However, a seemingly
small modification in technique, handling segments with
forceps with rough gripping faces as opposed to handling
them with butterfly forceps with smooth gripping faces
produced an increase in half-time for ^{3}HHO efflux from
34 to 37 minutes. They conclude that Kang and Burg's
^{3}HHO results were due to differences in cuticle perme-
ability rather than cell membrane permeability. For
instance, they suggest that the cuticle in their segments
might have cracked when the segment elongated in response
to IAA. This would cause an increase in permeability of
the segment to ^{3}HHO. If conditions in the growth chamber
used for my study were different for different plantings,
cuticles might have varied between experiments. Further-
more, the use of different types of forceps for different
experiments may have affected results. Indeed, segments
for the individual segment experiment were handled more
than segments in the other experiments, since the root,
hypocotyl, and cotyledons of each plant were measured,
and data was recorded on the color and general appearance

of the seedling before segments were excised from the seedling. If abrasion by a forceps can alter permeability, this seems to be a factor which great care should be taken to control. In addition to the cuticle preventing passage of solution, the epidermis of ponderosa pine seems to have thickened cell walls and may also be a barrier. Permeability variation, in fact, seems to be the major drawback to the excised segment technique.

Hydrogen fluoride was more toxic to the segments than NaF. At a concentration of 9×10^{-3} M (pH 2.5), HF almost totally inhibited elongation. This effect is comparable to the 10^{-1} M NaF inhibition. Dr. M. Behan (personal communication) suggested that the cell membrane may be more permeable to HF than to the F^- ion. As a general rule, uncharged molecules are able to pass through membranes more easily than ions (Salisbury and Ross, 1969). Membrane permeability to the F^- ion should not be too low, however, since the F^- ion is so small. Unionized HF, though, is also small and is uncharged. Therefore, though both rates may be fast, the rate of HF absorption into the cell may be faster. Furthermore, the F^- ion may be reacting with cell wall material before it is able to penetrate into the cell. For instance, fluoridation of sugars or amino acids in the cell wall could be occurring (Eyer, personal communication).

The above explanation might apply if the amount of fluoride present in unionized form is greater in the HF solutions than in the NaF solutions. This is indeed the case. Since HF is a weak acid in aqueous solution, with a pK_a of 3.45 (Handbook of Chemistry and Physics, 1970), it will be only 10% ionized at pH 2.5. Sodium fluoride, in contrast should be almost completely ionized (Brewer et al., 1960). The F^- will be reacting with H^+ to form HF. The undissociated form of HF will be in equilibrium with ionized HF (Murphy and Rousseau, 1969). At pH 6, as most of the NaF solutions were applied, the HF in the solution will be almost completely dissociated. Therefore, permeability to fluoride might be greater for HF solutions than for NaF solutions. In experiments where NaF solutions were buffered at lower pH values, fluoride was more toxic at pH 3 and 4 than at pH 5 and 6. The fact that at pH 3 and 4 more undissociated HF is present could account for the observed increase in toxicity.

The inhibition of elongation at pH 2.5 by HF might be a pH effect. In pea segments, as the pH is lowered to values below 3, the elongation rate rapidly drops to values below the rate at pH 6 (Rayle and Cleland, 1970). This lack of growth approaches zero below pH 2.6 and shrinkage sometimes occurs, suggesting that the membranes are damaged (Hager et al., 1971; Rayle and Cleland, 1970).

Thus, a pH effect seems sufficient to account for the observed growth inhibition by HF at pH 2.5. However, Cleland and Rayle (1970) obtain a maximal extension response at pH 3 in their experiments with buffers. The HF solution at pH 3 does not stimulate elongation in the slit segment experiment and causes an inhibition with cotyledons attached. Therefore, pH alone is probably not responsible for the toxicity of HF solutions, but rather, fluoride is involved as well.

It is not surprising that fluoride at high concentrations inhibits elongation of hypocotyl segments. However, no stimulation of hypocotyl segment growth is obtained, in contradiction to numerous reports of flouride enhanced elongation. I propose three hypotheses concerning flouride's effect on elongation and will attempt to explain the discrepancy between my results and those of other investigators based on these conjectures. First of all, the fluoride induced stimulation of stem extension may be an effect specifically due to the F^- ion. This would be in agreement with Gordon's (1972a) observation that white and scotch pine stems grew longer than usual when treated with HF but not when treated with H_2SO_4. There are several possible reasons for my failure to observe stimulation of elongation by flouride in the ponderosa pine hypocotyls. Although I designed experiments to test

numerous conditions in which fluoride might produce an
elongation effect, perhaps there are other factors or a
combination of factors which are necessary for flouride
action but were not present in my hypocotyl segment
system. By setting up different conditions for the
hypocotyl experiment, a fluoride enhanced growth might
be obtained in the hypocotyl. Perhaps accumulation of
fluoride was too fast in solutions used for hypocotyl
experiments. Two cut ends were available for penetration
by the solution, unlike the conditions in Gordon's (1972a)
experiment, in which an intact candle received the solutions.
Similarly, if low concentrations of gaseous HF were used,
the rate of entry of fluoride into the plant might be
slow enough to enable the plant to detoxify the fluoride.
The hypocotyl segments might not have shown a stimulation
because a greater toxicity of fluoride resulted from its
faster absorption.

Perhaps the hypocotyl itself is unable to respond
to fluoride, but the candle of a tree is. Factors con-
trolling elongation could be different enough in these
two systems to allow fluoride to be effective in one and
not the other.

A second hypothesis is that the response to HF
observed in the field and laboratory is an acid response.
Since acid solutions are known to promote elongation,

this possibility cannot be ignored. Evidence against this is the report by Gordon (1972a) of no stimulation of elongation using H_2SO_4 at the same pH used to obtain HF stimulated elongation. However, permeability of the cuticle and epidermis to H_2SO_4 and HF may be different. As mentioned earlier in the discussion, membranes are more permeable to uncharged molecules than to ions. The permeability of the cuticle and epidermis to ions may also be lower than their permeability to uncharged molecules, since ions may more easily react with substances in the cuticle and epidermal cell wall. In ponderosa pine and in pea (Yamamoto et al., 1973; Cleland and Rayle, 1975) the cuticle and epidermis have been shown to be relatively impermeable to H^+ ions. In ponderosa pine, elongation is inhibited by approximately the same concentration of HF whether segments are slit and aspirated or not. However, elongation in response to citrate buffer occurs in slit segments but is completely absent in unslit segments. Therefore, penetration of segments by undissociated HF seems to be easier than penetration by H^+ ions from the buffer. Assuming that cuticle and epidermis impermeability to ions is a general phenomenon, permeability to a highly dissociated acid should be lower than permeability to an undissociated acid. In an aqueous solution, as the pH is raised, H_2SO_4 will first dissociate to H^+ and HSO_4^-

(the bisulfate ion). The bisulfate ion will then dis-
sociate to H^+ and $SO_4^=$. The pK_a for the first step is
approximately 0.40, and the pK_a for the second step is
1.92 (Segel, 1968). The pK_a of HF is 3.45 (Handbook of
Chemistry and Physics, 1970). Thus, at any given pH,
HF will be less dissociated than H_2SO_4. Within the range
of pH from 3 to 5 where increased elongation is observed
by Gordon (1972a) with HF, H_2SO_4 is almost completely
dissociated. At pH 3, for instance, HF is 26% ionized
as calculated from the Henderson-Hasselbalch equation
(Rendina, 1971; Segel, 1968), while the ratio of the
$SO_4^=$ concentration to the original H_2SO_4 concentration
is 92%. The remaining H_2SO_4 should be almost entirely
in the form of HSO_4^-. Thus, HF may have entered the
candles treated by Gordon (1972a) more easily than H_2SO_4.
Once inside the tissue, the HF solutions would be diluted
in the tissue fluid, and dissociation would occur. Cell
wall regions could then become acidified. Since the
number of cells in the candle is greater than that in 5 mm
segments, and the amount of solution applied at one time
by Gordon (1972a), 1 ml, is much less than the amount
given to ponderosa pine hypocotyl segments, 20 ml, the
problem of fluoride toxicity may have been less in Gordon's
system than in the hypocotyl segment system. Detoxifi-
cation of the fluoride might have been accomplished in

the interval between the daily applications to the candles.
Candles may also be less sensitive to fluoride than
hypocotyls. Thus, the candles may have been able to
elongate in response to HF because it could penetrate
the tissue and they could detoxify it fast enough, but
they could not respond to H_2SO_4 because of its low
penetration. The results of Gordon (1972a) can thus be
explained in terms of the hypothesis that the HF response
is due to H^+ ions.

If H_2SO_4 were able to penetrate tissue, increased
elongation would be expected. Some evidence exists that
H_2SO_4 may be able to stimulate elongation. Ferenbaugh
(1974) sprayed Phaseolus vulgaris with H_2SO_4 solutions.
He mentions that plants treated at pH 3.5 and 4.5 some-
times were taller than control plants. In his Figure 2,
it can be seen that 45 day old plants sprayed with pH 3
solutions were about an inch taller than controls.
Obviously, more evidence is needed to prove that an acid
response could occur when solutions were applied to intact
plants or when HF gas entered plant tissues and dissolved
in the tissue water. However, negative evidence does
not seem to be too devastating to the hypothesis, since
lack of an acid response could simply mean the tissue
wasn't permeable to the H^+ ions. If so much controversy
and variability in results can result from cuticle and

epidermis differences in the excised segment system, as described earlier, then it would be expected that even more variation and inconsistency would be found in studies depending on the permeability of the relatively more complicated and less permeable coverings of mature plants. Permeability control has been shown to be crucial to the acid response.

The third possibility regarding fluoride enhancement of elongation is that it is nonexistent. The only presentation of data with a statistical treatment appears in Treshow et al. (1967). His conclusion that the significant 2 mm elongation of Douglas fir needles is due to fluoride seems overly confident. Assuming that an elongation difference of that relatively small magnitude is not due to chance alone, an assumption which may in fact not be justified, it seems that there should probably be many other important factors operating in the environment, besides fluoride, which might be able to affect elongation to the degree observed.

In work with segments floating in solutions, the variation due to uncontrolled factors is often quite large. Spontaneous growth increases may be mistaken for true responses to test solutions (Evans, 1973). Cuticle variation may obscure effects or produce an apparent effect of test solutions (Cleland and Rayle,

1975; Dowler et al., 1974). Other causes of variation remain unexplained, for instance variation in spontaneous growth from experiment to experiment observed by the author and by Zakrzewski (1975), and by Rayle and Cleland (1970) for the duration of their acid response. Seasonal variation in the response of hypocotyls used in the hypocotyl straight growth bioassay for auxin has even been postulated (Wodzicki and Wodzicki, 1973). If so little control on variation exists in the excised system where hypocotyls are isolated from the variation found in the rest of the plant, much more variation would be expected in systems using intact plants, each with different growth rates and endogenous levels of growth regulators. Even a further increase in variation occurs in experiments on intact plants in the natural environment. Not only do the plants differ internally, but factors other than those being studied can influence growth. Because of the numerous sources of variation in any experiment, any attempt to show an effect of some factor on plants must involve a careful demonstration that variation caused by the factor in question is greater than that due to chance, or to uncontrolled conditions.

Carlson (personal communication) attempted to measure terminal shoots of trees in areas of fluoride pollution to determine whether an increase in length could be shown

to be statistically significant. He found a great deal of variation among terminal shoot lengths of single trees. It appeared to him from preliminary observations that this variation would be greater than that between fluoride-polluted areas and nonpolluted areas. The study was not continued, however, because of the difficulty of dealing with the large variation due to other factors besides fluoride.

Further investigations on intact plants under controlled conditions are needed to determine which of the three possibilities described above is correct. It is possible, of course, that both fluoride and acid are stimulating elongation, either separately or together. Although a completely defendable selection of one hypothesis is impossible without more data, I prefer the second hypothesis that observed stimulations of elongation are due to an acid response. My failure to get an acid response with HF solutions means merely that acidification of the cell wall must occur without simultaneous poisoning by fluoride. If the HF could get into a stem, and the F^- ion could be detoxified fast enough, acid enhanced elongation should be able to occur.

CHAPTER V

SUMMARY

Control of hypocotyl elongation was studied in ponderosa pine. Investigation of effects of natural regulators of growth included experiments testing effects of NAA, acid, GA_3, cotyledons, cheesecloth, and ABA. Combinations of NAA, GA_3, and cotyledons were also tested. Stem elongation was enhanced by NAA, acid, and cheesecloth. To obtain the acid response, it was necessary to slit segments longitudinally and aspirate them in the test solution. Permeability of the intact cuticle and epidermis to the H^+ ions may thus be quite low. The response in ponderosa pine to both auxin and acid provides support for the theory of acid-mediated auxin response.

The elongation response of the hypocotyl to fluoride was investigated in an attempt to elucidate the role of fluoride in stimulating stem elongation. However, fluoride failed to promote elongation in any of the experimental systems. Fluoride inhibited growth when applied to entire plants or excised segments as NaF at 10^{-1} M. Hydrofluoric acid inhibited growth of excised segments at pH 2.5 or 9×10^{-3} M HF. The HF solutions are thus more toxic to the segments.

101

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EXPLANATION OF APPENDIX

Results of statistical tests are summarized in Appendix A III to F XI. Abbreviations used for treatments are as follows: N (NAA), F (NaF), G (GA_3), Ch (Cheesecloth), Ab (ABA), C (Control). Arabic numerals following hormone and fluoride symbols indicate the negative log of the concentration as molarity. For example, the N4F8 treatment consists of NAA at 10^{-4} M plus NaF at 10^{-8} M.

Lines underneath the treatment symbols indicate the results of the multiple comparison test based on the Kruskall-Wallis test. Treatments connected by a single line are not significantly different from each other. Lines above the treatment symbols indicate results of Kolmogorov Smirnov (KOLMS) and Wilcoxon Rank Sum (WILC) tests, as labelled. Again, treatments connected by lines are not significantly different. Numbers under the treatments compared with the multiple comparison test are average ranks.

For experiments in which measurements of segments were made before and after incubation, TO refers to statistical analysis for treatments before incubation and 24 refers to analysis for treatments after incubation.

Appendix A I.   Computer program for multiple comparison

test based on the Kruskall-Wallis test, and sample output

from program.

```
KWHS                 16:16              25-MAY-73
1 DIM A(500),X(500),K(500),J(500),M(500)
2 DIM B(13),G(13),P(13,13),D(13),G1S(13)
3 READ N1,C
4 DIM DIS(50)
5 READ A1S,BIS
6 FOR I=1 TO C
7 B(I)=N1
8 NEXT I
9 N=N1*C
10 PRINT "KRUS FOR  ";
11 PRINT A1S" ";BIS" ";A2S
12 FOR T=1 TO C
13 READ G1S (T)
14 NEXT T
15 FILES CONF.DAT
16 PRINT "          M. D.       IMPLTABLE"
17 READ D1,W1
18 FOR I=1 TO N
20 READ X1,K(I)
25 A(I)=X(I)
30 NEXT I
50 FOR I=1 TO N-1
60 FOR KI=I+1 TO N
70 IF A(I)<=A(KI) THEN 110
80 F=A(I)
90 A(I)=A(KI)
100 A(KI)=F
110 NEXT KI
130 NEXT I
200 L=1
210 FOR I=1 TO N
220 IF A(I)=A(I+1) THEN 230
221 J(L)=I
222 Y(L)=A(I)
229 L=L+1
230 NEXT I
231 M=L
500 FOR L=1 TO M-1
510 IF J(L)-J(L-1)=1 THEN 550
520 FOR I=1 TO (J(L)-J(L-1))-1
530 Q=(J(L)-1)+Q
540 NEXT I
550 P(L)=Q/(J(L)-J(L-1))
555 H2=H2 +((J(L)-J(L-1))*3-(J(L)-J(L-1)))
561 Q=0
```

Appendix A I (con't.)

```
770 GOTO 520
530 P(L)=J(L)
591 NEXT L
860 FOR I=1 TO C
663 C=C+D(I)
673 E(I)=C
680 NEXT I
700 FOR T=1 TO C
710 FOR I=G(T-1)+1 TO G(T)
720 FOR L=1 TO M-1
730 IF K(I)<>K(L) THEN 760
740 Z=Z+P(L)
750 GOTO 770
760 NEXT L
770 NEXT I
780 D(T)=Z
781 PRINT T")";D(T)/N1;"   ";G1$(T)
793 Z=0
800 NEXT T
810 FOR T=1 TO C
820 X=X+((D(T)^2)/D(T))
830 NEXT T
840 H=((12/(N*(N+1)))*X)-(3*(N+1))
850 H1=H/(1-(N9/(N^3-N)))
870 PRINT "H'=";H1
880 PRINT"REJECT H0 IF H'>=";D1$;"AT ALPHA=";E1
890 FOR T=1 TO C
900 FOR I=T+1 TO C
910 P(T,I)=ABS(D(T)/N1-D(I)/N1)
920 NEXT I
930 NEXT T
940 FOR T=1 TO C
950 FOR I=T+1 TO C
960 PRINT "P(";T;",";I;")=" P(T,I)
970 NEXT I
980 NEXT T
990 READ W
1000 S=W*(SQR((C*(C*N1+1))/12))
1010 PRINT "S=";S
1011 PRINT
1020 FOR L=1 TO N-1
1030 NEXT L
4100 DATA 24,9
4101 DATA "5/13/75 ","FIELD EXPERIMENT"
4110 DATA "I4F6","I4F4","I4F6","I4F5","I5F4"
4120 DATA "I6F4","I6F6","I6F6","I6F ",30.00,0.5
4990 DATA 5.70
99999 END
```

Appendix A I (con't.)

Sample output from program.

```
KRUS FOR '5/18/75' AUXIN EXPERIMENT
        M. R.   TREATMENT
  1 )  118.813    N4F0
  2 )  129.229    N4F4
  3 )  89.7917    N4F6
  4 )  96.3958    N4F8
  5 )  141.354    N6F0
  6 )  131.646    N6F4
  7 )  120.229    N6F6
  8 )  102.708    N6F8
  9 )  66.3333    N8F0
H'= 26.0495
REJECT H0 IF H'>= 20.09 ;ALPHA= 0.01
P( 1 , 2 )= 9.58333
P( 1 , 3 )= 29.0208
P( 1 , 4 )= 22.4167
P( 1 , 5 )= 22.5417
P( 1 , 6 )= 12.8333
P( 1 , 7 )= 1.41667
P( 1 , 8 )= 16.1042
P( 1 , 9 )= 52.4792
P( 2 , 3 )= 19.4375
P( 2 , 4 )= 12.8333
P( 2 , 5 )= 32.125
P( 2 , 6 )= 22.4167
P( 2 , 7 )= 11
P( 2 , 8 )= 6.52083
P( 2 , 9 )= 42.8958
P( 3 , 4 )= 6.60417
P( 3 , 5 )= 51.5625
P( 3 , 6 )= 41.8542
P( 3 , 7 )= 30.4375
P( 3 , 8 )= 12.9167
P( 3 , 9 )= 23.4583
P( 4 , 5 )= 44.9583
P( 4 , 6 )= 35.25
P( 4 , 7 )= 23.8333
P( 4 , 8 )= 6.3125
P( 4 , 9 )= 30.0625
P( 5 , 6 )= 9.70833
P( 5 , 7 )= 21.125
P( 5 , 8 )= 38.6458
P( 5 , 9 )= 75.0208*
P( 6 , 7 )= 11.4167
P( 6 , 8 )= 28.9375
P( 6 , 9 )= 65.3125*
P( 7 , 8 )= 17.5208
P( 7 , 9 )= 53.8958
P( 8 , 9 )= 36.375
S= 64.7818
```

Appendix A II. Sample computations for the Kolmogorov-Smirnov test for the 5/18/75 experiment. In the δ_i column, 1=buffer control and 0=10^{-8} M NAA.

i	$Z_{(i)}$	δ_i	$F_{10}(Z_{(i)})$	$G_{10}(Z_{(i)})$	$\lvert F_{10}(Z_{(i)}) - G_{10}(Z_{(i)})\rvert$
1	5.5	1	1/20	0/20	1/20
2	6	1	2/20	0/20	2/20
3	6	1	3/20	0/20	2/20
4	6	1	4/20	0/20	4/20
5	6	1	5/20	0/20	5/20
6	6	1	6/20	0/20	6/20
7	6	1	7/20	0/20	7/20
8	6.5	1	8/20	0/20	8/20
9	6.5	1	9/20	0/20	9/20
10	6.5	1	10/20	0/20	10/20
11	6.5	1	11/20	0/20	11/20
12	7	0	11/20	1/20	10/20
13	7	1	12/20	1/20	11/20
14	7	0	12/20	2/20	10/20
15	7	1	13/20	2/20	11/20
16	7	1	14/20	2/20	12/20
17	7	1	15/20	2/20	13/20
18	7	1	16/20	2/20	14/20
19	7	1	17/20	2/20	15/20
20	7	1	18/20	2/20	16/20 MAX;J_3=16
21	7.5	0	18/20	3/20	15/20
22	7.5	1	19/20	3/20	16/20
23	8	0	19/20	4/20	15/20
24	8	1	20/20	4/20	16/20
25	8	0	20/20	5/20	15/20
26	8	0	20/20	6/20	14/20
27	8	0	20/20	7/20	13/20
28	8.5	0	20/20	8/20	12/20
29	8.5	0	20/20	9/20	11/20
30	8.5	0	20/20	10/20	10/20
31	8.5	0	20/20	11/20	9/20
32	8.5	0	20/20	12/20	8/20
33	8.5	0	20/20	13/30	7/20
34	9	0	20/20	14/20	6/20
35	9	0	20/20	15/20	5/20

Appendix A II. (con't.)

i	$Z_{(i)}$	δ_i	$F_{10}(Z_{(i)})$	$G_{10}(Z_{(i)})$	$|F_{10}(Z_{(i)}) - G_{10}(Z_{(i)})|$
36	9	0	20/20	16/20	4/20
37	9	0	20/20	17/20	3/20
38	9	0	20/20	18/20	2/20
39	10	0	20/20	19/20	1/20
40	11	0	20/20	20/20	0/20

$J_3 = 16$

From table A.23 in Hollander and Wolfe (1973)

$P(J_3 \geq 10) = .0123$

Therefore, H_o is rejected at the 0.01 level.

Appendix A III. Results of the multiple comparison test for the 5/18/75
auxin experiment with hypocotyl segments.

_____   _____   KOLM-S

Buf	N8F0	N4F6	N4F8	N6F8	N4F4	N4F0	N6F6	N5F4	N6F0
	66.3	89.8	96.4	102.7	109.2	118.8	120.2	131.6	141.4

Buf	F6	N8F6	F8	N8F8	F4	N8F4

Appendix A IV. Statistical analysis for the 9/19/75
NAA with attached cotyledons experiment.

	TO	BUF	N4	N8	N6
		33	57	57	61

		WILC	KOLMS	WILC	
24		BUF	N8	N4	N6

Appendix B I.   Statistical analysis for 9/19/75 experiment with hypocotyl segments split longitudinally and incubated in 0.01 M citrate buffer at pH 6, 5, 4, and 3.

TO	pH6	pH5	pH4	pH3
	700	1096.5	1357.5	1502

24	pH6	pH5	pH4	pH3
	677.5	1057.5	1276.5	1644.5

Appendix B II. Statistical analysis for 10/11/75 experi-
ment with slit and aspirated hypocotyl segments incubated
in 0.01 M citrate buffer at pH 6, 5, 4, and 3.

TO	pH3	pH4	pH5	pH6
	931	983	1499	1637

24	pH6	pH4	pH5	pH3
	580	978	1275	1822

Appendix C I. Statistical analysis for the 5/28/75 experiment with hypocotyl segments
incubated in solutions of GA_3 and NaF.

G6F4	G4F6	G8F4	G6F8	G4F4	G6F0	G8F0	G8F6	G4F8	G8F8	G4F0	G6F6	Buf
76.54	124.54	125.62	140.35	159	160.08	163.02	164.83	166.23	170.92	178.44	193.15	211.77

KOLM-S

Buf	G4N6	G6N6	G8N6
211.77	33.4	37.6	47.5

Appendix C II. Statistical analysis for the 9/19/75
experiment using hypocotyl segments with cotyledons
attached incubated in GA$_3$ solutions.

TO	Buf	G4	G6	G8
	32	50	59	61

24	G4	G8	Buf	G6

Appendix C III. Statistical analysis for the 7/14/75 experiment with hypocotyl

segments and unattached cotyledons incubated in solutions of GA_3 in continuous

darkness.

G8F6 G8F8 F8   G8F4 F4   G6FO G4F4 G8FO G6F6 Buf F6   G6F5 G4F6 G4F8 G6F8 G4FO
127 152 155 155 156 169 183 199 202 208 213 214 223 238 239 246

Appendix C IV. Statistical analysis for the 7/18/75 experiment with hypocotyl
segments and unattached cotyledons incubated in solutions of GA_3 in continuous
light.

Buf	G6F6	G8F8	F6F4	G8F4	G4F6	G8F0	G6F0	F4	G6F8	G4F8	G4F0	F8	F6	G4F4	G8F6
134.5	146.2	149.9	153.8	162.5	172.0	183.9	202.7	204	208.3	218.3	218.5	211.8	223.3	239.5	250.6

Appendix D. Statistical analysis for the 9/19/75 experiment with hypocotyl segments incubated with NAA and cheesecloth.

Buf	N8Ch	N4Ch	Ch	N6Ch
314	1196	1336	1814	2009

Appendix E. Statistical analysis for the 9/19/75 experiment with hypocotyl segments incubated in ABA solutions.

T0	Ab8	Ab4	Buf	Ab10	Ab6
	1060	1462	1559	1866	1928

24	Buf	Ab4	Ab6	Ab8	Ab10
	1222	1360	1640	1668	1984

Appendix F I.   Statistical analysis for 10/10/75 experiment with seedlings grown in slants with fluoride agar. The analysis shown is for hypocotyl growth. Similar results were obtained for root and cotyledon elongation.

		KOLMS		
F1	F7	F3	C	F5
	18.55	19.25	21.3	22.9

Appendix F II. Statistical analysis for the 9/9/75 experiment with hypocotyl segments incubated in NaF solutions.

TO	F1	F2	F4	F3	C
	38	39	68	72	98

24	F1	F2	F3	F4	C
	15	65	70	82	84

.

Appendix F III.   Statistical analysis for the 9/19/75
experiment with hypocotyl segments incubated in NaCl
solutions.

T0	NaCl5	Buf	NaCl3	NaCl1
	31	35	54	73

24	Buf	NaCl3	NaCl5	NaCl1
	32	46	52	64

Appendix F IV. Statistical analysis for the 9/26/75 experiment with hypocotyl segments with attached cotyledons incubated in NaF solutions.

TO	F5X4	F3	F4	F2	F5X3	Buf
	67	69	72	74	78	93

24	F2	F5X3	F3	Buf	F5X4	F4
		40	59	68	78	75

Appendix F VI.   Statistical analysis for the 9/9/75
experiment with hypocotyl segments incubated in solutions
of NaF with 10^{-4} M GA_3.

TO	F3G	F4G	Buf	F2G	
	33	36	57	76	

24	F1G	F3G	F2G	Buf	F4G
	13	69	73	77	83

Appendix F VII. Statistical analysis for the 9/9/75
experiment with hypocotyl segments incubated in solutions
of NaF and 10^{-6} M NAA.

TO	F4N	F3N	Buf	F1N	F2N
	29	42	78	81	85

___ ___ WILC

___ ___KOLMS

24	F1N	F2N	Buf	F3N	F4N
	14	41	59		

Appendix F VIII. Statistical analysis for the 10/21/75 experiment with hypocotyl segments incubated in solutions of 10^{-6} M NAA (N) or phosphate buffer (B). The hypocotyls were from seedlings grown in fluoride agar. The Arabic numerals indicate the negative log of the fluoride concentration in the agar slant.

T0	3B	3N	5B	7N	3N	0N	0B

	3B	7B	0B	5B
	13.60	19.15	21.05	28.20

24

	3N	0N	5N	7N
	17.75	18.35	21.65	24.25

Appendix F IX. Statistical analysis for the 12/19/75 experiment with slit and aspirated hypocotyl segments incubated in 0.01 M citrate buffer at pH values from 6 to 3 with NaF.

T0	pH4F8	pH5F4	pH3F8	pH4F6	pH4F4	pH3F4	pH3F0	pH6F0	pH3F6	pH5F6	pH5F0	pH5F8	pH4F0
	2520	2744	3295	3382	3438	3636	3782	3948	4113	4175	4454	4523	4818

24	pH6	pH3F4	pH3F8	pH3F6	pH4F8	pH3F0	pH5F8	pH5F0	pH5F4	pH5F6	pH4F6	pH4F0	pH4F4
	1522	2984	3021	3200	3574	3620	3801	3870	4434	4434	4521	4748	5097

Appendix F X. Statistical analysis for the 9/26/75

experiment with hypocotyl segments with attached cotyledons

incubated in HF solution at a range of pH from 6 to 2.5

TO	pH3	pH2.5	H_2O	pH3.5	pH5.5	pH5	pH4	pH4.5
	57	68	94	99	104	104	111	133

24	pH2.5	pH3	pH5.5	pH5	H_2O	pH4.5	pH3.5	pH4
			46	71	76	77	81	83

Appendix F XI. Statistical analysis for the 11/15/75 experiment with slit and aspirated hypocotyl segments incubated in HF solutions at a range of pH from 6 to 3.

TO	pH3.5	pH4.0	pH4.5	pH3.0	pH6.0	pH5.0	pH5.5
	58	61	84	88	103	109	112

24	pH3	pH4	pH4.5	pH5	pH3.5	pH5.5	pH6
	26	67	90	95	96	106	136