VESPER, MARY JO STAGGENBORG
STUDIES ON THE ROLE OF PROTONS IN THE CONTROL
OF CELL ELONGATION BY AUXIN.

THE OHIO STATE UNIVERSITY, PH.D., 1978
STUDIES ON THE ROLE OF PROTONS IN THE
CONTROL OF CELL ELONGATION BY AUXIN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Mary Jo Vesper, B.A., M.Sc.

* * * * *

The Ohio State University
1978

Reading Committee:
Michael L. Evans
Carroll A. Swanson
Gary L. Floyd

Approved By
Michael L. Evans
Adviser
Department of Botany
ACKNOWLEDGMENTS

With sincere and grateful appreciation, I acknowledge the advice and encouragement that my adviser, Dr. Michael L. Evans, has given me which has made the preparation of this dissertation very rewarding. I would also like to thank Dr. Evans for giving me a special enthusiasm for research in plant physiology.

I am deeply indebted to my husband, Steve, for his patience and perseverance. He has given me the motivation necessary for completion of this undertaking and he has always strived to make the path easier to tread.

The help and guidance extended to me by my reading committee is gratefully acknowledged. I thank them for carefully reading this dissertation and for their helpful comments concerning its preparation.
December 7, 1948 ................ Born - Covington, Kentucky


1973–1974, 1977 ........... University Fellow, The Ohio State University, Columbus, Ohio

1974–1976, 1978 ........... Teaching Associate, Department of Botany, The Ohio State University, Columbus, Ohio

1975 ....................... M.Sc., The Ohio State University, Columbus, Ohio

PUBLICATIONS


FIELDS OF STUDY

Major Field: Botany, Plant Physiology

Ca$^{2+}$ binding to plant cell membranes. Professor Michael L. Evans

Nature of endogenous changes in growth patterns of excised coleoptile segments. Professor Michael L. Evans

Mechanism of action of auxin. Professor Michael L. Evans
**TABLE OF CONTENTS**

<table>
<thead>
<tr>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS....................................................... ii</td>
</tr>
<tr>
<td>VITA ............................................................... iii</td>
</tr>
<tr>
<td>LIST OF TABLES ........................................................... v</td>
</tr>
<tr>
<td>LIST OF FIGURES .......................................................... vi</td>
</tr>
<tr>
<td>INTRODUCTION ............................................................ 1</td>
</tr>
</tbody>
</table>

**Chapter**

I. SIMULATION OF AUXIN-INDUCED PROTON SECRETION 
   BY INTRACELLULAR HYDROLYSIS OF NAPHTHYL ACETATE ....... 9

   Introduction ........................................... 9
   Materials and Methods. ....................................... 12
   Results. .................................................. 15
   Discussion .............................................. 39

II. FURTHER TESTS OF THE CORRELATION BETWEEN 
   H⁺ SECRETION AND GROWTH .................................. 47

   Introduction ........................................... 47
   Materials and Methods. ....................................... 49
   Results. .................................................. 50
   Discussion ............................................. 86

III. TIME-DEPENDENT MODIFICATION OF THE H⁺ 
   SECRETION AND GROWTH RESPONSES TO AUXIN: 
   EVIDENCE FOR A SENSORY ADAPTATION ......................... 94

   Introduction ........................................... 94
   Materials and Methods. ....................................... 96
   Results. .................................................. 98
   Discussion ............................................. 122

SUMMARY ............................................................... 126

LIST OF REFERENCES .......................................................... 127
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Esterase Localization in Coleoptile Segments</td>
<td>23</td>
</tr>
<tr>
<td>2. Acidification of External Medium upon Withdrawal</td>
<td>83</td>
</tr>
<tr>
<td>of 10 μM Auxin and Auxin Analogues</td>
<td></td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chemical structures of α- and β-naphthalene acetic acid compared with the structures of α- and β-naphthyl acetate.</td>
<td>17</td>
</tr>
<tr>
<td>2.</td>
<td>Diagramatic illustration of the hydrolysis of NA occurring inside of a cell.</td>
<td>20</td>
</tr>
<tr>
<td>3.</td>
<td>Promotion of growth and acidification of the external medium by NA in corn and oat coleoptile segments and in lentil roots.</td>
<td>22</td>
</tr>
<tr>
<td>4.</td>
<td>Effect of α-naphthol and Tween 80 on the elongation of corn coleoptile segments.</td>
<td>26</td>
</tr>
<tr>
<td>5.</td>
<td>Growth response to NA compared with response to acidic buffers, pH 4.0.</td>
<td>28</td>
</tr>
<tr>
<td>6.</td>
<td>The lack of effect of pretreatment with either 10 μg/ml CHI or 1 mM KCN on a subsequent decrease in external pH caused by 0.8 mM α-NA.</td>
<td>31</td>
</tr>
<tr>
<td>7.</td>
<td>Response of corn coleoptile segments to 0.8 mM NA alone and in the presence of 10 μg/ml CHI and 1 mM KCN.</td>
<td>33</td>
</tr>
<tr>
<td>8.</td>
<td>Inhibition of an acid growth response by 1 mM KCN.</td>
<td>36</td>
</tr>
<tr>
<td>9.</td>
<td>Dose-response curves for α-NA effects on growth and acidification of the external medium.</td>
<td>38</td>
</tr>
<tr>
<td>10.</td>
<td>Comparative effects of 80 μM α-NA and 10 μM IAA on elongation and decrease in pH of the external medium.</td>
<td>41</td>
</tr>
<tr>
<td>11.</td>
<td>IAA-induced growth in peeled and non-peeled coleoptile segments of oats and corn.</td>
<td>53</td>
</tr>
<tr>
<td>12.</td>
<td>Dose-response curves for IAA-induced growth and H⁺ secretion.</td>
<td>55</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>13.</td>
<td>$H^+$ secretion response to increasing concentrations of $\alpha$-NAA</td>
<td>58</td>
</tr>
<tr>
<td>14.</td>
<td>Growth responses to increasing concentrations of $\alpha$-NAA.</td>
<td>60</td>
</tr>
<tr>
<td>15.</td>
<td>Response of segments to 10 $\mu$M IAA and 10 $\mu$M $\alpha$-NAA given 90 min after excision</td>
<td>63</td>
</tr>
<tr>
<td>16.</td>
<td>Effect of gassing of the external medium with various gases compared with a non-gassing condition on IAA-induced $H^+$ secretion</td>
<td>66</td>
</tr>
<tr>
<td>17.</td>
<td>The time course of IAA-induced $H^+$ secretion under the condition of oxygenation of the external medium compared with the condition of non-gassing.</td>
<td>69</td>
</tr>
<tr>
<td>18.</td>
<td>Effect of gassing of the growth medium with various gases on IAA-induced growth</td>
<td>71</td>
</tr>
<tr>
<td>19.</td>
<td>Reversal of inhibition caused by gassing of the external medium with $N_2$ upon return to $+O_2$ conditions</td>
<td>73</td>
</tr>
<tr>
<td>20.</td>
<td>The effect of a 35 min pulse of $N_2$ during IAA-induced $H^+$ secretion and growth</td>
<td>75</td>
</tr>
<tr>
<td>21.</td>
<td>A comparison of the kinetics of an endogenous growth response (SGR) with the kinetics of endogenous $H^+$ release into the external medium</td>
<td>79</td>
</tr>
<tr>
<td>22.</td>
<td>Effect of withdrawal of $\alpha$-NAA on acidification of the external medium and growth</td>
<td>81</td>
</tr>
<tr>
<td>23.</td>
<td>Effect of various solution changes on the acidification of the external medium</td>
<td>85</td>
</tr>
<tr>
<td>24.</td>
<td>Dependence of the growth response to IAA on time after excision.</td>
<td>100</td>
</tr>
<tr>
<td>25.</td>
<td>Dependence of the growth response to IAA on time after prior exposure to IAA</td>
<td>103</td>
</tr>
<tr>
<td>26.</td>
<td>Time–dependent change in sensitivity to IAA shown in IAA-induced $H^+$ secretion</td>
<td>106</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>27. Uptake of $^3$H-IAA from 0.5 µM IAA was followed over a 30-min period using segments placed in uptake medium either 0.5 hr or 2.5 hr after excision</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>28. IAA dose-response curves for segments 0.5 hr and 2.5 hr after excision</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>29. Concentration dependence of latent period of IAA response in segments 0.5 hr and 2.5 hr after excision</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>30. Growth response to 0.5 µM IAA applied 0.5 hr (0) and 2.5 hr (●) after excision expressed as % of control growth rate</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>31. Growth response to 0.1 µM (0) and 10 µM (●) IAA applied 1.5 hr after excision expressed as % of control growth rate</td>
<td>121</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION

When auxin is applied to subapical coleoptile segments growing at a basal rate in water or weak buffer, a latent period of about 10-20 min generally precedes the enhancement of growth rate (as much as ten times greater than the endogenous rate). The initial auxin-induced growth response has been likened to an acid-growth response (Rayle and Cleland, 1972a; Hager et al., 1971). This connection has generated much interest and has provided the impetus for renewed efforts in uncovering the mechanism of action of auxin, a problem unsolved after more than 50 years of research since this plant hormone was first defined.

One of the primary actions of auxin is to induce an increase in the extensibility of the rigid cell wall (Cleland, 1968). However, auxin does not act on the cell wall directly since auxin applied to cells which have had their protoplasts disrupted by freezing and thawing ("in vitro" cell walls), does not cause an increase in wall extensibility (Rayle and Cleland, 1972a). However, H+ (hydrogen ion[s]) can act on the wall and cause wall loosening even in in vitro cell walls (Rayle and Cleland, 1970). Cleland and Rayle (1972a) have shown that auxin-induced wall loosening is inseparable from wall extension, i.e. wall loosening does not proceed in the presence of either auxin or acid if wall extension does not occur simultaneously (there is
no "stored growth"). Both H⁺-induced and auxin-induced enhancement of wall extensibility depends on tension whether extensibility is tested in vivo (turgor provides tension) or in vitro (a constant force applied to the tissue provides tension) (Rayle and Cleland, 1972a).

After detailed experiments on Avena coleoptile segments and Helianthus hypocotyl segments, Hager et al. (1971) also proposed that protons mediate the effect of auxin and they presented a model in which auxin activates an anisotropic ATPase acting as a proton pump. On finding that protons, like auxin, induce an increase in wall extensibility, Rayle and Cleland (1972a) concluded that auxin must induce the appearance in the wall of some factor which initiates changes there and results in wall loosening. They suggested that hydrogen ions might be involved.

The acid growth hypothesis of auxin action was then proposed and supported by the observation that auxin induces H⁺ extrusion from Avena coleoptile segments (Cleland, 1973; Rayle, 1973). Further support was gained from the observations that enhancement of H⁺ secretion shows auxin specificity and is inhibited by cycloheximide (CHI), 2,4-dinitrophenol (DNP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP), compounds which also inhibit auxin-induced growth (Cleland, 1973; Rayle, 1973). Ilan (1973) and Marré et al. (1973a & b) also showed auxin-induced H⁺ secretion. Marré et al. (1973a) were the first to describe this in a dicot tissue (pea internode segments).

The acid growth hypothesis states that auxin causes cell elongation by inducing the efflux or pumping of protons from the protoplast. The protons enter the cell wall where they act as second messengers to
cause wall loosening and therefore cell extension.

Since the time of the initial studies on the relationship between acid-induced growth and auxin-induced growth, all tissues tested that respond to auxin by enhanced cell elongation also respond similarly to acidic buffers when care is taken to sufficiently remove or abrade the cuticle. (The cuticle has been shown to offer considerable resistance to movement of H⁺ [Rayle, 1973].) Thus, attempts have been made to determine whether or not a correlation exists between auxin-induced growth and auxin-induced H⁺ secretion in dicot and monocot tissues. Reports of a positive correlation are numerous (Cleland, 1973, 1975, 1976a,b&c; Cleland and Rayle, 1978; Jacobs and Ray, 1976; Marré et al., 1973a&b; Mentze et al., 1977; Rayle, 1973), but difficulties in finding a significant effect of auxin on H⁺ release have also been reported (Ilan, 1973; Parrish and Davies, 1977; Penny et al., 1975; Vanderhoef et al., 1977). Most of the difficulties can probably be obviated by sufficient abrasion of the cuticle (Cleland and Rayle, 1978; Mentze et al., 1976; Rayle and Cleland, 1977).

Efforts have been made recently to determine exactly how auxin interacts with the protoplast to cause the efflux of protons. In 1972, Hertel et al. reported in vitro binding of auxin to particulate fractions from coleoptiles. Since this initial report several groups have more carefully defined auxin binding to specific subcellular fractions (Batt and Venis, 1976; Batt et al., 1976; Dohrmann et al., 1978; Ray, 1977; Ray et al., 1977a&b). Ray (1977) has established that the bulk of the binding sites for auxin are concentrated in the microsomal fraction (endoplasmic reticulum); however, the plasmalemma and Golgi
membranes have a limited number of binding sites as well (Batt and Venis, 1976; Ray, 1977). Auxin-binding proteins have also been solubilized (Cross and Briggs, 1978; Cross et al., 1978; Venis, 1977).

The suggestion that auxin activates an ATPase (Hager et al., 1971) has been tested in membrane preparations and with solubilized auxin-binding proteins. Although there has appeared one report that auxin enhances the activity of a membrane-bound ATPase from mung bean hypocotyls (Kasamo and Yamaki, 1974), it appears that auxin-binding proteins from corn coleoptiles do not stimulate ATPase activity in the microsomal fraction (Cross et al., 1978), a fraction containing most of the binding sites for auxin in this tissue (Ray, 1977). This weakens but does not conclusively eliminate the possibility that an ATPase can be influenced by auxin.

From kinetic analysis of auxin-induced $H^+$ efflux and $K^+$ influx, Cleland and Lomax (1977) have concluded that auxin activates an electrogenic $H^+$ pump, such as a membrane-bound ATPase. Marré (1977) has reached the same conclusion. However, he proposes that fusicoccin (FC, a fungal toxin which stimulates $H^+$ secretion and rapid growth in plant tissues) acts by the same mechanism as auxin. Cleland and Lomax (1977, and Cleland, 1976a) have suggested that FC operates by a different mechanism, i.e. electrogenic $K^+/H^+$ exchange.

Another model for the mechanism of $H^+$ movement in response to auxin has appeared recently which does not rely on a membrane-bound ATPase (Ray, 1977). This model proposes that auxin, in binding to sites at the endoplasmic reticulum (ER), induces the packaging of protons into ER vesicles which then move to the plasmalemma, fuse with
it, and expel their contents into the cell wall. This model is inviting because it is the only one which includes an explanation for the latent period to the auxin growth response and $\text{H}^+$ secretion response. This model can also account for the rapid inhibitory effect of CHI on hormone-induced $\text{H}^+$ secretion since the formation and movement of ER vesicles may depend on continuous protein synthesis. However, there are difficulties with this model, especially with respect to the number and internal pH of the vesicles needed to account for the magnitude of $\text{H}^+$ release encountered in response to auxin.

Whatever the method is by which auxin induces $\text{H}^+$ efflux, a working model must account for the fact that auxin causes hyperpolarization of the membrane potential (Cleland et al., 1977). This implies that more protons exit the tissue than are balanced by cation influx. The presence of cations in the external medium (notably $\text{K}^+$ and $\text{Ca}^{2+}$) has been shown to enhance auxin-induced $\text{H}^+$ secretion (Cohen and Nadler, 1976; Lado et al., 1976).

Some differences between the auxin growth response and the acid growth response still have to be dealt with. Among these is the nearly immediate response of peeled tissue to acidic buffers compared to an optimal auxin response with a latent period of up to 20 min or more. The fungal toxin, FC, also induces an almost immediate (latent period 1-2 min) enhancement of growth and $\text{H}^+$ secretion. FC is known to bind primarily to the plasmalemma (Dohrmann et al., 1977), while auxin may be most effective when it reaches receptor sites at the ER (Ray et al., 1977a).
The duration of an acid growth response is transient, while an auxin growth response is long-lived. However, even after the termination of a transient acid growth response, while the tissue is still in the presence of external acid, auxin can cause an increase in rate of elongation, indicating that the low pH has not altered the cell membrane to such an extent that it can no longer sustain the turgor necessary to drive cell elongation. In Chapter I, I show that it is possible to obtain an acid response of longer duration by non-hormonally generating a source of H⁺ inside the cell (a simulation of the auxin-induced H⁺ secretion). Such a source of H⁺ is created by hydrolysis of naphthyl acetate (NA) through the action of esterases to give acetic acid. Another non-hormonal agent, FC, has been used by others to induce H⁺ secretion and growth (Marre et al., 1973a; Cleland, 1976b; Yamagata and Masuda, 1975). While the action of FC differs from that of auxin in many respects (Marre, 1977; Yamagata and Masuda, 1975), it has been a useful tool in investigating the acid growth hypothesis. NA's action in inducing growth and H⁺ release is very dissimilar from the action of either FC or auxin, but it also is a tool which can be used to the same end.

Although the rate of auxin-induced H⁺ secretion has been reported to correlate well with the rate of auxin-induced growth, this has been tested only over a narrow range of auxin concentrations (Cleland, 1973; Rayle, 1973). Such studies were done using Avena coleoptile segments. With respect to the auxin dose-response relationship, Avena coleoptile segments show an increasing growth rate with increasing auxin concentration only over the range of about 0.01 to 1 μM (Cleland,
1972; Durand and Zenk, 1972; Tietze-Hass and Dörffling, 1977). The shape of the dose-response curve is sigmoidal over this lower concentration range. This is followed by a plateau region which indicates that the growth rate does not decline from the maximum at higher auxin concentrations. The auxin dose-response relationship in corn coleoptiles, however, is different. When tested 1.5 hr after excision, corn coleoptile segments give a bell-shaped auxin dose-response curve (Durand and Zenk, 1972; Vesper and Evans, 1978). This makes the corn coleoptile a more suitable material than the *Avena* coleoptile for testing the dose-response relationship in auxin-induced growth and $H^+$ secretion. In Chapter II, I present evidence that with two different auxins, indoleacetic acid (IAA) and $\alpha$-naphthalene acetic acid ($\alpha$-NAA), the dose-response relationship for growth closely parallels that for $H^+$ secretion. In addition, auxin-induced $H^+$ secretion is shown to be sensitive to anoxia, as is auxin-induced growth. This evidence along with other presented in Chapter II seems to support the acid growth hypothesis of auxin action.

In the final chapter, Chapter III, the dose-response relationship is analyzed in a different way. Qualitative as well as quantitative changes occur in the auxin-induced growth response as higher levels of auxin are applied. A large step-up in auxin concentration produces a change in magnitude of both the latent period and the growth rate as well as a change in the quality of the latent period, i.e. large steps-up in auxin concentration lead to transient inhibition of growth during the latent period preceding growth enhancement. This effect is seen not only by examining the dose-response relationship, but it is also
evident when one applies a constant low level of auxin to corn coleoptile segments at increasing periods of time after excision from the seedling.

The qualitative and quantitative changes in the response to auxin that are determined by the length of time the tissue has been without a source of the hormone can be interpreted as evidence of an adaptive sensory mechanism operating in the coleoptile tissue. Auxin given soon after excision is effectively a lower dose due to the high level of adaptation in the tissue which has just been removed from its endogenous auxin source. With increasing time after excision, the same auxin concentration becomes an effectively larger dose since the level of adaptation to the now-diminished endogenous auxin supply is low. These qualitative and quantitative differences between a large and a small step-up in auxin concentration are also reflected in such auxin-mediated responses as changes in the lateral electrical potential (Morath and Hertel, 1978) and in the curvature induced by unilateral application of auxin (Ullrich, 1978), as well as in $\text{H}^+$ secretion (Chapter III).

In the chapters that follow, the phenomena of the nature of the acid-induced growth response relative to auxin action, $\text{H}^+$ secretion, dose-response relationships, and apparent adaptation are examined in detail. The results presented herein are discussed in relation to the information they provide toward the ultimate elucidation of the mechanism of auxin action on cell elongation.
CHAPTER I. SIMULATION OF AUXIN-INDUCED PROTON SECRETION BY INTRA-CELLULAR HYDROLYSIS OF NAPHTHYL ACETATE

INTRODUCTION

Although Bonner reported in 1934 (Bonner, 1934) that coleoptile segments grew more at low pH (4.1) than at neutral pH, it was not until more recently that this effect was characterized in detail (Evans, 1967; Rayle and Cleland, 1970), especially with respect to the rapidity and duration of the response. The enhancement of cell wall extension and therefore growth by acid solutions became of interest because the effect was similar in some respects to auxin-induced growth. Further studies of the acid-growth response in coleoptiles were undertaken in an attempt to arrive at an understanding of the mechanism of auxin-induced plant cell enlargement (Hager et al., 1971; Rayle and Cleland, 1972a&b; Rayle et al., 1970). The additional finding that auxin-induced acidification of the external medium bathing peeled (cuticle and part of the epidermis removed with fine-tipped forceps) coleoptile segments (Cleland, 1973; Rayle, 1973) indicated that auxin-induced cell wall loosening and acid-induced wall loosening may involve the same biochemical events. These workers had already suggested that H⁺ were the wall loosening factors in auxin-induced growth (Rayle and Cleland, 1972a).
On the basis that addition of ATP could allow expression of a growth response to auxin under anaerobic conditions, together with other evidence, Hager et al. (1971) proposed that auxin may activate some membrane-bound ATPase, which, acting as a proton pump, functions to increase the H\(^+\) concentration in the cell wall region. The acid growth hypothesis for the mechanism of action of auxin was thus born and has been tested for its universal application using tissues which respond to auxin by rapid cell enlargement. Evans (1974) has outlined criteria which should be met in all systems (dicot as well as monocot) if this theory of the action of auxin is tenable.

Although there are some inconsistencies encountered in studies of the acid response in dicots (Parrish and Davies, 1977; Penny et al., 1975; Perley et al., 1975; Vanderhoef et al., 1977), it is generally accepted that peeled coleoptile segments respond to acidic buffers (pH 4.0 to 5.0) with a growth rate similar to an auxin-induced growth rate (Jacobs and Ray, 1976; Rayle and Cleland, 1970, 1977), and they secrete H\(^+\) into the tissue free space (Jacobs and Ray, 1976) and then into the external medium (Cleland, 1976c) in response to auxin. The lag period for this H\(^+\) secretion response was found to be shorter than or equal to the lag period preceding the onset of growth. Auxin-induced H\(^+\) secretion is inhibited by metabolic inhibitors which also stop growth (Cleland, 1973; Rayle, 1973) and the rate of H\(^+\) secretion can be varied to a limited extent by altering the auxin concentration (Cleland, 1973; Rayle, 1973). Durand and Rayle (1973) also provide evidence that strong buffers (10 mM K-phosphate, pH 6.2) inhibit the response to auxin in peeled coleoptiles where the buffers can readily penetrate the
tissue.

With all of this supporting evidence for the acid-growth theory from studies using coleoptiles, there are some points which must be clarified before this theory is accepted, even for monocot tissues. One objection to the acid-growth theory is that there are definite differences in the growth responses to acidic buffers and auxin, most notably the difference in the duration of the two responses. Hydrogen ion-induced growth persists for only 1-2 hr (Rayle, 1973; Rayle and Cleland, 1972a), while auxin-induced growth remains steady for many hours (Jacobs and Ray, 1976; Rayle and Cleland, 1970, 1972a).

There have been some explanations proposed for the lack of a long-lived acid response in coleoptile segments. These are summarized by Rayle and Cleland in a recent review (1977). They suggest that the growth response to acidic buffers may be transient because 1) external \( \text{H}^+ \) cannot cause, as auxin can, an internal increase in solute concentration (osmoregulation) so that turgor becomes limiting during \( \text{H}^+ \)-induced rapid cell expansion, and, 2) high concentrations of \( \text{H}^+ \) are toxic to cells (Rayle and Cleland, 1977). However, it is not likely that the concentration of \( \text{H}^+ \) used becomes toxic because the cells remain responsive to auxin following pretreatment at an acidic pH. There is still a need to determine why the acid growth response is so short-lived, or to find a manner in which to extend the response. Since the application of acidic buffers does not closely simulate the auxin-induced release of \( \text{H}^+ \) from the protoplasts, I have attempted to devise a condition which would allow for a triggered release of \( \text{H}^+ \) from the protoplasts in order to simulate auxin-induced \( \text{H}^+ \) efflux while avoiding
the other effects that auxin may have on the growth process.

By use of an esterase substrate, naphthyl acetate (NA) (Gomori, 1953; Gahan and McLean, 1969), an "artificial" source of H⁺ is generated within the cells. NA is not an auxin and causes no growth promotion at hormonal concentrations (i.e. 1-80 μM). Assuming that hydrolysis of NA occurs primarily in the protoplast (and not in the cell wall), acetic acid, an hydrolysis product of NA, would serve as an internally generated source of H⁺. These H⁺ leave the cell passively, flowing down a concentration gradient. The protons expelled can be monitored by placing a pH electrode in the external medium. If this scheme is correct, one would predict that a drop in the external pH should occur and should be accompanied by a rapid growth response. I have tested these ideas and found that addition of NA to various plant tissues leads to rapid acidification of the external medium and growth promotion. This chapter deals with the use of this NA-induced proton release to re-examine the correlation between acid-induced growth and auxin-induced growth.

MATERIALS AND METHODS

Plant Material

In most of the experiments coleoptiles from 4 to 5 day old corn (Zea mays L., hybrid WF9X38, Bear Hybrid Corn Co., Decatur, IL) seedlings were used. The corn grains were soaked overnight then placed embryo-up on three layers of moist paper towelling in closed plastic trays and were given red light for the next 24 hr. The seedlings were then kept in darkness at 25 C until used, at which time the coleoptiles
were 30-40 mm long. For experiments using oat (*Avena sativa*, L. "Victory") coleoptiles, the husked grains were grown as described previously (Evans and Ray, 1969). Lentil seedlings (*Lens culinaris*, Med.) were obtained as described by McBride and Evans (1977).

**pH Experiments**

Coleoptiles were prepared for pH experiments by first peeling excised coleoptiles, floating them on buffer, cutting 1-cm segments and deleafing the segments (oat coleoptiles were deleafed prior to cutting segments) before placing them into a 5-ml beaker containing 1 mM K-phosphate buffer, initial pH 6.2-6.3. One-cm apical root segments from lentil seedlings were cut, floated on buffer and then placed into a beaker containing K-phosphate buffer. A semi-micro combination pH electrode was inserted into the beaker for recording of pH. The tissue:volume ratio was 45 segments:3.0 ml for corn, 60 segments:3.2 ml for *Avena*, and 60 segments:3.4 ml for lentil. For all experiments, the basal medium was 1 mM K-phosphate buffer (pH 6.2-6.3).

The beaker used was specially designed so that a teflon-coated spin bar was isolated from the segments by mounting a disk of plastic screen about 3 mm from the bottom of the beaker. The screen was held in place by silicone caulking compound. This beaker design allowed for rapid mixing of the solution but prevented clumping of the segments near the electrode tip. By preventing excessive contact between the electrode tip and the segments, I was able to obtain essentially noise-free traces with the pH probe output recorded on a Sargent-Welch Model SLRG recorder adjusted to 0.6 pH unit full scale.
Growth Experiments

Growth experiments involving corn and oat coleoptile segments were performed using a shadowgraphic growth recording device (Evans and Ray, 1969) to record the combined elongation of 12 1-cm segments in about 20 ml of growth medium. Coleoptile segments were prepared for growth experiments as described by Evans and Hokanson (1969), modified according to Vesper and Evans (1978).

Lentil root growth experiments were done using intact seedlings with the root auxanometer developed and described by Evans (1976). The growth medium for lentil roots was half-strength Meyer's Solution (Meyer et al., 1955) at pH 6.2, while the growth medium for coleoptile elongation was 1 mM K-phosphate buffer, pH 6.3.

Esterase Assay

NA and naphthol were held in solution by using Tween 80, final concentration of 0.24% (v/v). Esterase activity in intact segments and in homogenized tissue was assayed according to Gomori (1953) by measuring α-naphthol release following incubation in 0.4 mM α-NA (in 1 mM K-phosphate buffer, pH 6.3) for 45 min at 26-27 C. At the end of the incubation a small sample of the incubation medium was diluted with K-phosphate buffer and 0.3 ml of 0.5% Fast Garnet (prepared in 10% sodium lauryl sulfate) added to give a final volume of 2.5 ml. The reaction mixture was allowed to stand at room temperature for 35 min before absorbance at 560 nm was read on a Gilford Model 240 Spectrophotometer. Addition of Fast Garnet has been shown to stop the enzyme reaction (Gomori, 1953). The fresh tissue:volume ratio was maintained at 3 segments:6 ml for the assay.
Tissue Fractionation

To obtain a fraction from corn coleoptile segments which is enriched in cell wall material, a procedure outline by Johnson et al. (1974) was followed. Deleafed 1-cm segments were frozen in a refrigerator freezer overnight in 1 mM K-phosphate buffer, pH 6.3 (3 segments /6 ml). The tissue was warmed to room temperature before being ground using mortar and pestle. The resultant brei was homogenized in a tissue homogenizer using a teflon pestle. The homogenate was centrifuged at 500g for 5 min. The supernatant (500g supernatant) was saved. The pellet (500g pellet) was washed four times in ice-cold buffer (centrifugation 500g for 5 min), the final centrifugation being 10 min in length. The supernatants from the washes were discarded. The final wash gave a clear supernatant following centrifugation.

RESULTS

Characterization and Localization of Esterase Activity in Coleoptiles

The chemical structures of α- and β-NA are compared with those of α-naphthalene acetic acid (α-NAA) (active auxin) and β-naphthalene acetic acid (β-NAA) (inactive auxin analogue) in Figure 1. These four compounds have the same molecular weight. However, the esters, α- and β-NA, have no dissociable H⁺, that is, they are not themselves acids as are α- and β-NAA. Alpha-NAA is effective in promoting growth in coleoptiles at concentrations as low as 1 μM (β-NAA causes no enhancement of growth in coleoptiles), while no significant growth promotion is obtained in the presence of α-NA or β-NA until the concentration is above 80 μM. Therefore, any promotion of growth by NA is probably
Figure 1. Chemical structures of α- and β- naphthalene acetic acid compared with the structures of α- and β-naphthyl acetate.
Figure 1.

α-Naphthaleneacetic acid

β-Naphthaleneacetic acid

α-Naphthyl acetate

β-Naphthyl acetate
not due to its action as a "hormone." In fact, the levels of NA used which give strong growth responses (4-8 mM) are indicative of its acting as a substrate in an enzymatic reaction, yielding a product which is itself the growth promoter.

Figure 2 diagramatically illustrates the probable action of cellular esterases on NA, giving the hydrolysis products of naphthol and acetic acid. It is suggested that the enzymatic production of the organic acid ultimately leads to an enhanced growth rate via the release of H⁺ upon dissociation of the acid. Hydrogen ions, generated internally, leave the cell protoplast and enter the cell wall region where they are effective agents of wall loosening. A rapid decrease in pH of the external medium is recorded almost immediately after addition of NA (Figure 3). The nature of the release of this acid makes endogenous enzymatic hydrolysis of NA a useful tool to imitate one proposed action of auxin—that action being the initiation of release of protons from the protoplast into the cell wall.

NA is primarily hydrolyzed within the protoplast itself and not in the cell wall. The results given in Table 1 indicate that upon homogenization of corn coleoptile segments and subsequent centrifugation at 500g, most of the esterase activity remains in the supernatant. The low speed pellet is taken to be largely cell wall material (Johnson et al., 1974). Likewise, no appreciable esterase activity is found in the medium itself in which coleoptile segments have been previously incubated (Table 1, in vivo, -segments).
Figure 2. Diagramatic illustration of the hydrolysis of NA occurring inside of a cell (top portion of figure). The chemical reaction scheme is shown in the lower portion of the figure.
Figure 2.

[Diagram showing the chemical reaction of naphthol acetate, N-acetyl
catechol and naphthol with esterase.]
Figure 3. Promotion of growth (upper curves) and acidification of the external medium (lower curves) by NA in corn and oat coleoptile segments and in lentil roots. NA (0.8 mM) was added at the arrows in each case. Elongation curves for corn and oat represent the sum of 12 1-cm coleoptile segments and the elongation curve for lentil is that for 1 intact root. (pH experiments for lentil were done with 1-cm apical root segments.) Marker bars for corn and oats indicate elongation of 1 mm, for lentil the marker bar is 0.5 mm.
Figure 3.
Table 1.

ESTERASE LOCALIZATION IN COLEOPTILE SEGMENTS

<table>
<thead>
<tr>
<th>Fraction</th>
<th>μmole α-naphthol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
</tr>
<tr>
<td>500g Pellet</td>
<td>0.30</td>
</tr>
<tr>
<td>500g Supernatant</td>
<td>3.24</td>
</tr>
<tr>
<td>Total Homogenate</td>
<td>3.48</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
</tr>
<tr>
<td>Incubation Medium -Segments</td>
<td>0.05</td>
</tr>
<tr>
<td>Incubation Medium +Segments</td>
<td>1.26</td>
</tr>
</tbody>
</table>

*Fractions obtained from three 1-cm segments, assay by incubation in 12 ml of 0.4 mM α-NA, 27°C, 45 min
Growth Response and Acidification of External Medium Caused by NA

While the data in Table 1 indicate that increased naphthol levels can be easily detected in the external medium surrounding corn coleoptile segments (in vivo, +segments), pH experiments show that an increased concentration of $\text{H}^+$ in the external medium parallels the appearance of the naphthol (Figure 3).

Figure 3 shows the immediate and dramatic effect of 0.8 mM NA on growth (upper curves) and pH of the external medium (lower curves) in corn coleoptile segments, as well as in oat coleoptile and lentil root segments. All three tissues are known to undergo increased extension in response to acidic buffers (Jacobs and Ray, 1976; McBride and Evans, 1977; Rayle and Cleland, 1970, 1972a).

The hydrolysis product, naphthol, was determined to cause no significant change in the pH of the medium bathing corn coleoptile segments; Tween 80 was also found to have no effect (data not shown). Parallel measurement of growth (Figure 4) shows that 0.8 mM $\alpha$-naphthol induces a weak growth response which is transient. Segments in the presence of either $\alpha$-naphthol or Tween 80 undergo a normal auxin growth response when given 10 $\mu$M indoleacetic acid (IAA) (Figure 4).

Comparison of Growth Responses Induced by NA and Acidic Buffers

Figure 5 shows the time course of elongation of corn coleoptile segments given 0.8 mM NA at pH 6.3, or treated with one of two different acidic (1 mM acetate or 1 mM citrate-phosphate) buffers (pH 4.0). Peeled corn coleoptile segments appear to have an optimum acid growth response at about pH 4.0; a response to pH 4.5 is considerably weaker.
Figure 4. Effect of α-naphthol and Tween 80 on the elongation of corn coleoptile segments. Both α-naphthol (0.8 mM) and Tween 80 (0.24%) were applied 90 min after excision. IAA (10 μM) was applied 45 min following addition of either α-naphthol or Tween 80.
Figure 4.
Figure 5. Growth response to NA compared with response to acidic
(Ac: acetate; Cit-phos: citrate phosphate) buffers, pH 4.0. All
segments were held in 1 mM K-phosphate, pH 6.3, for 90 min before
solutions were changed. NA was applied in 1mM K-phosphate, pH 6.3.
Figure 5.
(data not shown). This observation is in agreement with data of Jacobs and Ray (1976). As can be seen in Figure 5, α-NA elicits a stronger growth response than does acetate alone at a pH which is optimal for acid-induced growth. Both the α-NA and the acetate, pH 4.0, responses are stronger than the response to citrate-phosphate, pH 4.0. The duration of the α-NA response is about three times longer than that of the acetate response. At the end of a 3.5 hr incubation in α-NA having pH 6.3 at the start, the pH of the solution had dropped to about pH 6.05.

The initial growth rate induced by 0.8 mM α-NA is over three times the magnitude of an optimum auxin growth response. Reducing the NA concentration to 0.4 mM results in a growth rate still about twice that of the response to auxin.

Effect of Inhibitors

That the growth response induced by α-NA is really an acid response is indicated by the lack of sensitivity of the NA-induced growth and acidification to the inhibitors CHI and KCN (Figures 6 and 7). CHI is known to cause cessation of auxin-induced growth and $H^+$ secretion within 5 min (Cleland, 1973, 1975). I also found that 10 μg/ml CHI rapidly (in about 2-3 min) inhibited auxin-induced $H^+$ secretion (Figure 6), but even when CHI was given as a 30 min pretreatment, it did not affect the acidification (Figure 6) or growth (Figure 7) caused by α-NA. Rayle and Cleland (1972a) also noted that CHI was without effect on an acid growth response. Apparently the turnover of esterase is not so rapid as to be markedly affected by the pretreatment with CHI.
Figure 6. The lack of effect of pretreatment with either 10 μg/ml CHI or 1 mM KCN on a subsequent decrease in external pH caused by 0.8 mM α-NA. For comparison the effect of 10 μg/ml CHI on IAA-induced H⁺ secretion is shown. The equilibrium pH is given by each curve.
Figure 6.
Figure 7. Response of corn coleoptile segments to 0.8 mM NA alone (curve A) and in the presence of 10 µg/ml CHI (curve B) and 1 mM KCN (curve C). All solutions were prepared in 1 mM K-phosphate buffer and the pH was adjusted to 6.3 just prior to use. The time in min is time from excision.
Figure 7.
KCN, given as pretreatment, did not inhibit NA-induced acidification of the external medium (Figure 6); the slight reduction in NA-induced growth following KCN pretreatment (Figure 7) was about the same seen for KCN inhibition of an acid response (about 25% inhibition) (Figure 8). Acid growth responses have been shown to be essentially insensitive to KCN (Evans et al., 1971). Auxin-induced growth (Evans et al., 1971; Gillbank et al., 1973) and H⁺ secretion (Cleland, 1976b&c) on the other hand, are very sensitive to 1 mM KCN.

**NA Dose-Response Relationship**

The growth rate and drop in pH induced by α-NA is plotted over a limited concentration range in Figure 9. The induced drop in pH increases nearly in direct proportion to the log of the molar concentration of α-NA from 8 μM to 0.8 mM (limit of solubility). The growth induced over this concentration range does not increase in the same fashion as does the H⁺ release. While there is a nearly linear increase in H⁺ efflux with increasing concentration of NA, growth is distinctively not linear over this concentration range but increases sharply between 80 μM and 0.4 mM. This observation may point to a potential inconsistency in the acid-growth hypothesis. It has been repeatedly stated that growth rate corresponds closely to the magnitude of pH drop in the external medium (Cleland, 1975; Rayle, 1973; Rayle and Cleland, 1977). This has been tested until now only during auxin-induced H⁺ secretion. However, NA also causes release of H⁺ from the cells and the cell wall region (which is directly responsive to H⁺) should respond to any source of H⁺ regardless of whether they are generated by
Figure 8. Inhibition of an acid growth response by 1 mM KCN. KCN was given as a 40 min pretreatment (+KCN) before the pH was lowered from 6.3 to 4.0 (using 1 mM citrate-phosphate buffer). KCN was present during treatment at pH 4.0.
Figure 8.
Figure 9. Dose-response curves for α-NA effects on growth and acidification of the external medium. Values indicated for IAA are the growth (■) and H⁺ secretion (□) responses to 10 μM IAA. The pH decrease was determined after 20 min for 800 μM α-NA (maximum effect) and after 60 min for 8 μM and 80 μM α-NA as well as for 10 μM IAA. Growth rates are the initial accelerated rates.
Figure 9

GROWTH RATE (mm·hr⁻¹·seg⁻¹)

CONC α-NA (µM)

pH DECREASE

0.1 0.2 0.3 0.4 0.5 0.6

0.0 0.2 0.4 0.6 0.8
auxin action or by other means (e.g. α-NA hydrolysis). There can be no qualitative difference in the protons themselves released from the protoplast via auxin action or via NA hydrolysis.

Figure 10 illustrates this inconsistency further by showing that the magnitude of acidification caused by 80 μM α-NA is larger than that caused by optimal IAA (10 μM) over the same time period (lower portion of figure). Nevertheless, there is no significant growth response to this concentration of α-NA but a substantial growth response to auxin (upper portion of figure). Failure of the segments to respond to 80 μM α-NA is not likely due to toxicity of this concentration of α-NA since segments do respond to higher concentrations of α-NA. Also, segments treated with 80 μM α-NA are still capable of showing a normal auxin response.

These observations show that there may exist in the cell wall a threshold for an acid growth response which is high with respect to the rate of entry of H⁺ into the wall. Therefore, the data indicate that the magnitude of auxin-induced H⁺ secretion is below the threshold required to elicit an acid growth response.

DISCUSSION

NA can be used to cause release of H⁺ from cells in a manner which closely simulates auxin-induced proton release. Addition of NA is, in a sense, like simply lowering the pH of the external medium, except that the protons in this case are coming from the protoplast, thus simulating the proposed action of auxin in inducing H⁺ secretion. The direction from which the H⁺ enter the wall region seems to make a great difference in the overall response.
Figure 10. Comparative effects of 80 μM α-NA (-----) and 10 μM IAA (······) on elongation (upper portion of figure) and decrease in pH of the external medium (lower portion of figure). Addition of each compound was made at the arrow 90 min after excision.
Figure 10.
Using NA to mimic endogenous secretion of $H^+$, I have made three important observations. 1. Stimulation of elongation by endogenously generated $H^+$ is greater than stimulation by exogenous application of optimal levels of $H^+$, i.e. the direction from which $H^+$ enter the cell wall appears to be important. 2. Stimulation of elongation by endogenously generated $H^+$ is long-lived compared to that induced by comparable exogenous acidic buffers. The response to exogenous acid declines after about 1 hr whereas the response to NA continues unabated for about 3 hr. 3. The threshold for the acid growth response appears to be high in terms of the rate of entry of $H^+$ into the cell wall.

Generation of $H^+$ inside the cell (following application of 0.8 mM $\alpha$-NA) and subsequent release to the outside allows for a stronger and more prolonged acid growth response than when the cells are bathed in an external solution of acidic pH. In both cases the cell wall must experience a pH low enough to cause wall loosening. Rayle and Cleland (1977) have argued that a low external pH can be toxic to the tissue and therefore growth would stop due to such toxicity, accounting for the transient growth response to acidic buffers. In my experience, external pH values below 4.0 often induce shrinkage in peeled corn coleoptile segments following an initial rapid burst of growth. This shrinkage may be assigned to the toxic effect of the high $H^+$ concentration. Shrinkage induced by NA was also observed on one occasion but only after 4 hr of exposure to NA. In this case the rapid growth phase was followed directly by shrinkage, indicating that the $H^+$ concentration had perhaps reached toxic levels near the plasmlemma. However, the eventual slowing of the NA-induced growth after about 3 hr may be
due to toxic levels of naphthol accumulating in the tissue (vascular bundles are considerably darkened following even 1 hr incubation in NA).

Rayle and Cleland (1977) have also suggested that the transient nature of acid-induced growth can be accounted for by the absence of osmoregulation when the tissue is given acid externally. They argue that auxin-induced growth is sustained by hormone-induced osmoregulation. It may be suggested that the growth response to NA is sustained over that due to the external presence of acid because the hydrolysis of NA may in fact cause an elevation of solute concentration inside the cells. The production of acetate within the protoplast may effectively increase the osmotic concentration there, allowing turgor to keep up with rapid cell expansion. In this respect the acid growth response to the internally generated acid differs from that of externally applied acid.

The data presented here can be used to support the idea that such osmoregulation plays an important part in rapid cell expansion. Perley et al. (1975) found that external H+ caused lateral shrinkage in lupin hypocotyl segments accompanying the increase in longitudinal extension. Although these workers used this as evidence that auxin-induced growth and acid-induced growth are intrinsically different, Taiz and Métraux (1978) have shown that lateral shrinkage in response to acidic buffers can be greatly reduced or prevented by a short pretreatment with 10 mM KCl or 2% glucose, indirectly indicating that internal osmoregulation may lag behind the turgor drop in rapidly extending segments. Since a smaller amount of lateral shrinkage is observed during auxin-induced growth (Taiz and Métraux, 1978), auxin probably induces osmoregulation
in cells expanding in its presence thus allowing a sustained growth response. That the short duration of the acid growth response may be accounted for by a decrease in turgor is shown in experiments in which *Avena* coleoptile sections were allowed to extend under a constant applied tension (20 g) in the presence of acidic buffers (Rayle and Cleland, 1972a). Under these conditions turgor is replaced by the constant tension and the segments are observed to extend in acid for longer periods of time, i.e. 4-8 hr (Rayle and Cleland, 1972a, 1977). Malate levels are reported to increase in coleoptile segments treated with IAA (Haschke and Luttge, 1975; Johnson and Rayle, 1976; Stout et al., 1978).

The implication is made here that auxin-induced $H^+$ secretion may actually be below the threshold for acid-induced growth. This would pose a serious problem for the acid growth hypothesis. If, in fact, the magnitude of $H^+$ secretion caused by auxin is too weak to generate a growth response, then the $H^+$ released in the presence of auxin must have some function other than being the second messengers of auxin action, or may be entirely incidental to the growth process.

If much emphasis is to be placed on the correlation between the growth rate and the $H^+$ efflux rate (as is done by supporters of the acid growth hypothesis [Cleland, 1975]), then the enzymatic generation of internal protons is a good test for this correlation because almost any rate of $H^+$ release could be obtained by just varying the substrate (NA) concentration. In the presence of 0.8 mM NA, the rate of pH drop in the external solution is faster than any recorded in our laboratory (see Chapter II) in response to auxin. Likewise, the growth response
to 0.8 mM NA is the fastest growth response yet encountered in corn coleoptile segments (nearly three times greater than a response to optimal IAA). As a rate of H⁺ secretion is approached (by lowering the concentration of NA) which is close to an optimum auxin-induced rate, the growth rate should vary accordingly. But as shown above, 80 μM NA elicits a stronger rate of acidification of the external medium than does optimal auxin with little or no accompanying elongation.

It could be argued that in lower concentrations of α-NA (80 μM), all of the α-NA is hydrolyzed by the outermost layer of cells, the first to come in contact with the substrate. (The inner cylinder of a coleoptile segment is most often isolated from the external medium by an air bubble trapped there.) H⁺ released may be expelled from these outermost cells so rapidly that only a very low percentage of the cell walls in the segment are exposed to sufficient acid to cause wall loosening. If such conditions prevailed, an increased growth rate might not develop. There is, however, circumstantial evidence that most cells (and therefore cell walls) in the segment are exposed to the substrate and/or products of hydrolysis. The two vascular bundles of a coleoptile segment appear darkened following incubation in 80 μM α-NA. In fact, the darkened strands are more visible if viewed from the inner part of the cylinder, indicating penetration deep into the tissue.

Additional alternative explanations can be suggested to explain the lack of correlation between NA-induced H⁺ efflux and growth. Auxin may act in some way which sensitizes the wall to the protons coming from the protoplast and therefore the tissue responds to a lower rate
of H\(^+\) secretion ("sub-threshold") in +IAA than in -IAA. Or perhaps more likely, the protons secreted during an auxin response may simply be secondary or incidental to the increased growth rate.

Lastly, some comment is warranted concerning the rapidity with which the NA-induced drop in external pH is detected. H\(^+\) released on hydrolysis of NA within the cells are measured almost immediately in the external medium. This counters arguments appearing in the literature (Cleland, 1976c) that diffusion of H\(^+\) from the tissue to the electrode is the limiting step in the detection of the H\(^+\) (assuming a sufficiently large tissue:volume ratio), and therefore, in assessing the lag period for auxin-induced H\(^+\) secretion. It still remains desirable to measure the actual pH in the cell wall, but the system used here for measuring proton release from tissues can be validly used in comparative studies. While the tissue:volume ratio can be expected to influence the total magnitude of the change in pH, the time course of change should not be altered greatly from that in the cell wall itself.
CHAPTER II. FURTHER TESTS OF THE CORRELATION BETWEEN H⁺ SECRETION AND GROWTH

INTRODUCTION

Among the earliest reports on the nature of auxin-induced H⁺ secretion were those in which a correlation between the magnitude of auxin-induced H⁺ secretion and elongation was suggested (Cleland, 1973; Rayle, 1973). Such a correlation is an essential test of the validity of the acid growth hypothesis of auxin action. A limited proportionality between growth and H⁺ secretion in Avena coleoptile segments was shown by Cleland (1975). He used three different methods to obtain a variation in growth rate, namely, 1) varying the auxin concentration, 2) use of inhibitors of growth, and, 3) varying the age of the segments. A positive correlation was found using the first two methods but not with the third. Therefore, this proportionality needs further investigation before a real correlation between auxin-induced H⁺ secretion and growth can be established.

The auxin concentration range over which a variation in growth rate in Avena coleoptile segments is obtained is very limited and restricted to the lower (0.01 to 0.3 μM) concentrations (Cleland, 1972). However, the auxin dose-response curve for growth in corn coleoptile segments generally shows a bell-shaped relationship, that is, growth varies with auxin concentration over a broad range (Durand and Zenk,
1972; Vesper and Evans, 1978) with an intermediate concentration of auxin providing maximum promotion. It appears then that corn coleoptile tissue would be a better material to use in testing a correlation between auxin-induced H⁺ secretion and growth. Such a test was one objective of this study.

Previously only the naturally occurring auxin, IAA, was used to test the dose-response relationship for H⁺ secretion in coleoptiles (Cleland, 1975; Rayle, 1973), yet synthetic auxins have a potency like that of IAA in inducing elongation (Audus, 1972). Therefore, I have used varying concentrations of both IAA and α-NAA in testing the correlation between growth and H⁺ secretion.

The withdrawal of auxin from corn coleoptile segments has been shown to be followed by a period during which the auxin-induced growth rate is maintained (dela Fuente and Leopold, 1970; Evans and Hokanson, 1969). The time for the decay of the growth response is directly dependent on the concentration of auxin used (Evans and Hokanson, 1969). I attempted to determine if such a concentration-dependent decay phase would be found in the H⁺ secretion response upon withdrawal of auxin. Unfortunately, overriding complications associated with solution changes made it unfeasible to record a decay phase in auxin-induced H⁺ secretion. The expected gradual decline in H⁺ secretion rate upon withdrawal of auxin was completely masked by a large efflux of H⁺ at the time of withdrawal. However, the results of withdrawal experiments have given evidence that the cell wall acts as a cation exchange surface, and that it may store H⁺ when other exchange cations are not available. The effect of application and withdrawal of the inactive
auxin analogues benzoic acid (BA), indolecarboxylic acid (ICA), and
2-chlorophenoxyacetic acid (2-CPA) was also tested.

Inhibition of auxin-induced $H^+$ secretion by various inhibitors,
which also inhibit growth, has been demonstrated (Cleland, 1975,
1976b&c; Marré et al., 1973b; Marré et al., 1974; Rayle, 1973). In
this study I present data showing that anoxia inhibits both auxin-
induced $H^+$ secretion and growth to about the same extent. The effects
of anaerobiosis on auxin-induced $H^+$ secretion have not previously been
directly tested. Data presented in this chapter indicate that segments
may be $O_2$-limited under conditions normally prevailing in studies in
which the acidification of the external medium is measured.

MATERIALS AND METHODS

Plant Material

Corn and Avena coleoptiles were obtained from seedlings grown as
described in Chapter I.

pH Experiments

Measurement and recording of the pH of the external medium was
conducted as described in Chapter I. Gassing with $O_2$, air or $N_2$ was
done in some experiments and this is noted in the text or figure leg­
ends.

When addition of auxin or auxin analogues was made during a pH
experiment, a concentrated solution was used so that delivery of 0.3 ml
would give the desired final concentration (0.3 ml of the bathing med­
ium was removed before addition of the concentrate). In cases where
auxin was withdrawn, a Pasteur pipette was used to remove the solution from the beaker. The segments were rinsed four times with buffer solution, the pH of which had been adjusted to equal that of the solution being withdrawn. The beaker was then filled with buffer solution of the same volume as that removed. Recording was interrupted during this procedure.

Growth Experiments

The procedure outlined in Chapter I was followed for measurement of elongation.

RESULTS

Choice of Experimental Material

There is only one study reported in the literature that has described in detail auxin-induced H⁺ secretion in corn coleoptile segments. This study (Jacobs and Ray, 1976) employed a technique using a micro pH electrode of a sufficiently small tip diameter that the free space pH of the coleoptile was recorded, a technique which does not involve the measurement of the pH of an external solution. In their report, Jacobs and Ray (1976) showed a response to auxin much more rapid and stronger than any published previously for Avena. There are no published data on the measurement of H⁺ secretion in corn coleoptile segments using the method employed for the studies reported here (by recording decrease in pH of the external medium).

To be assured that the results I obtained with corn were acceptable, I attempted to repeat some basic experiments showing auxin
induction of H\(^+\) secretion in \textit{Avena}, which is well-documented (Cleland 1973, 1975, 1976b; Rayle, 1973). Parallel growth experiments with peeled \textit{Avena} coleoptile segments were also done. During the course of these experiments, it became apparent that corn coleoptile tissue was the preferable material to use with respect to general viability of peeled segments. This is illustrated in Figure 11 which shows a typical auxin-induced growth response in peeled and non-peeled \textit{Avena} segments compared with one in peeled and non-peeled corn segments. The process of removing the cuticle by stripping away the epidermal cell layer probably inflicts greater damage on the more fragile \textit{Avena} coleoptiles. Peeled \textit{Avena} coleoptiles nearly lose their ability to respond to auxin while peeled corn coleoptiles display a normal auxin response (Figure 11). The typical kinetics of endogenously controlled growth in corn, i.e. occurrence of the spontaneous growth response (SGR) about 3.5 to 4 hr after excision (Evans and Schmitt, 1975), is likewise apparent in peeled corn coleoptile segments (data not shown), an indication that peeled corn coleoptile segments behave very similarly to intact segments in terms of growth performance.

**Dose-Response Relationship for Auxin-Induced H\(^+\) Secretion and Growth**

The dose-response curves for IAA-induced growth and H\(^+\) secretion are shown in Figure 12. The growth response appears to peak at about 1 \(\mu\text{M}\); however, the H\(^+\) secretion response shows a maximum at a much higher concentration (10 to 100 \(\mu\text{M}\)). The dose-response curve for elongation in corn coleoptile segments changes with time from excision (Chapter III). A maximum in the dose-response relationship occurs at
Figure 11. IAA-induced growth in peeled and non-peeled coleoptile segments of oats and corn. Curve A in each case represents elongation of non-peeled segments; curve B represents elongation of peeled segments. IAA (10 μM) was added at arrows, 90 min after excision.
Figure 11.
Figure 12. Dose-response curves for IAA-induced growth and H⁺ secretion. The hormone was applied 90 min after excision. The pH decrease indicated in this figure is that which occurs during the first 60 min of H⁺ secretion (i.e. following the latent period). The growth experiments and pH experiments were performed in parallel and each point is the average of at least three experiments. The pH reached an equilibrium between 6.38 and 6.42 before addition of IAA. All experiments were performed under conditions of oxygenation.
Figure 12.

GROWTH RATE

- pH CHANGE AT 60 MIN

- LOG IAA CONC
1 µM IAA in segments used 2.5 hr after excision, as it does here for segments used 1.5 hr after excision. In spite of the disparity between the doses of IAA giving the maximum responses in growth and H⁺ secretion, both dose-response curves are bell-shaped.

The dose-response relationships obtained using α-NAA are similar to those shown for IAA. The concentration dependence of α-NAA effects is shown in Figure 13 for H⁺ secretion and in Figure 14 for elongation. At either extreme of the concentration range growth as well as H⁺ secretion is reduced. In both cases the maximum response is induced by 10 to 50 µM α-NAA. A difference in the two responses is most evident at 0.1 mM. At this concentration growth is strong (Figure 14, curve B) but H⁺ secretion (Figure 13, curve B) is barely above the control. In the IAA concentration series, H⁺ secretion is relatively greater than growth at 0.1 mM; in 0.1 mM α-NAA growth is relatively greater than H⁺ secretion.

Corn coleoptile tissue appears more sensitive to high levels of α-NAA than IAA, as indicated by the absence of growth and H⁺ secretion in the presence of 1 mM α-NAA (Figures 13 and 14, curve A). Growth and especially H⁺ secretion are strong in the presence of 1 mM IAA (Figure 12). At the other extreme of the concentration series, the tissue appears more sensitive to IAA. Only very weak responses are seen upon application of 1 µM α-NAA (Figures 13 and 14, curve E); however, this concentration of IAA results in a maximum growth response (Figure 12).
Figure 13. H⁺ secretion response to increasing concentrations of α-NAA. α-NAA was applied in each case at zero time (90 min after excision). The equilibrium pH for all experiments was between 6.38 and 6.43. Concentrations of α-NAA applied are: A, 1 mM; B, 0.1 mM; C, 10 μM; D, 50 μM; E, 1 μM; F, no α-NAA. The external medium was oxygenated during the experiments.
Figure 13.
Figure 14. Growth responses to increasing concentrations of α-NAA. α-NAA was added at zero time, which was 90 min after excision. These experiments were performed in parallel with those shown in Figure 13. Concentrations of α-NAA applied are: A, 1 mM; B, 0.1 mM; C, 10 μM; D, 50 μM; E, 1 μM; F, no α-NAA.
Figure 14.
The increase in pH upon application of α-NAA (Figure 13) is consistent and is stronger at higher concentrations. This increase may be due to preferential uptake of the protonated form of the acid (Rubery, 1977), thus removing H⁺ from solution. However, this increase in pH is paralleled by an inhibition of growth during the latent period of the growth response. This inhibition is seen by comparing curves B, C and D of Figure 13 with the control curve (F). Application of 1 μM α-NAA seems to cause an immediate, although weak, decrease in pH and there also is no inhibition apparent during the latent period of the growth response (Figure 14, curve E). Such changes in growth rate during the latent period will be discussed further in Chapter III.

Influence of Gassing with Oxygen, Air, and Nitrogen on Auxin-Induced H⁺ Secretion

In Figure 15 is shown the total time course of auxin-induced acidification of the medium bathing corn coleoptile segments. In this figure the responses to both 10 μM IAA and 10 μM α-NAA are shown. The pH of the external medium equilibrates at about pH 5.9 by 90 min after the beginning of the experiment (zero time in Figure 15). In response to subsequent application of IAA the pH drops to about 5.5 in a little more than 3 hr. These experiments were performed with rapid stirring of the medium but in the absence of any external gassing. This condition is the one most frequently used in H⁺ secretion studies (Cleland, 1973, 1975; Marré et al., 1973a&b; Marré et al., 1974; Vanderhoef et al., 1977). All studies with Avena coleoptile segments have been performed without gassing (Cleland, 1973, 1975, 1976b&c;
Figure 15. Response of segments to 10 μM IAA and 10 μM α-NAA given 90 min after excision. The external medium received no gassing during the experiments.
Figure 15.
Rayle, 1973). However, Parrish and Davies (1977) noted that pea stem segments require vigorous bubbling with air or O$_2$ to maintain their H$^+$ efflux capability.

The initial experiments I performed on auxin-induced H$^+$ secretion in corn coleoptile segments were done using methods outlined in published accounts of H$^+$ secretion in *Avena* (Cleland, 1973, 1975; Rayle, 1973). The buffer, buffer strength and pH used in these studies were duplicated in my experiments, but the tissue:volume ratio was increased because of the difficulty in detecting H$^+$ secretion in corn using a lower tissue:volume ratio. Nevertheless, the H$^+$ secretion response to auxin remained weak (Figure 15). Using similar methods with *Avena* Rayle (1973) reported an auxin-induced pH drop from about 6.0 to about 5.0 in 140 min with merely "gentle stirring" of the external medium. Stirring of the external medium is assumed to bring the buffer solution into equilibrium with the atmosphere. However, I suspected that the tissue may be somewhat oxygen-limited under these conditions. I therefore tested the effect on H$^+$ secretion of gassing the medium with either O$_2$, air, or N$_2$. The pH response to 10 μM IAA under such conditions is shown in Figure 16 and compared with condition normally used in H$^+$ secretion studies, that of no external gassing.

As illustrated in Figure 16, segments held in buffer without gassing equilibrate at a pH lower than the initial pH (6.3) of the buffer and lower than the equilibrium pH of segments receiving aeration, oxygenation, or gassing with N$_2$. I also found that termination of gassing at any time results in an immediate decrease in the external
Figure 16. Effect of gassing of the external medium with various gases compared with a non-gassing condition on IAA-induced H⁺ secretion. IAA was added at the arrows (90 min after excision). The condition noted by each curve was the condition prevailing throughout the experiment.
Figure 16.
pH. These observations can be taken as indirect evidence that a lack of vigorous gassing results in a drop in pH of the external medium due to an accumulation of respiratory CO$_2$ in solution.

The time course of auxin-induced H$^+$ secretion in the presence of O$_2$ is shown in Figure 17. The rate of the pH drop and the total magnitude of acidification is greater in the presence of O$_2$. The kinetics of acidification is very similar when air is used instead of O$_2$. Such an equivalency of oxygenation to aeration in an auxin response is also evident in the elongation response (Figure 18).

Gassing with N$_2$ is observed to nearly eliminate auxin-induced elongation (Figure 18) and H$^+$ secretion (Figure 16). Although the effect of anoxia has not been directly tested in auxin-induced H$^+$ secretion before now, it was expected that oxygen-limiting conditions should inhibit growth as well as H$^+$ secretion. This has been shown here. Both auxin-induced H$^+$ secretion and growth are dependent on the availability of metabolic energy and inhibitors such as KCN and DNP also quickly inhibit both of these auxin-induced responses (Cleland, 1975, 1976b&c; Marré et al., 1973b; Rayle, 1973).

The inhibition of an auxin response by gassing with N$_2$ (Figure 18) can be reversed if O$_2$ is subsequently given. As soon as gassing with N$_2$ is stopped and oxygenation started, the growth rate and H$^+$ secretion rate increase (Figure 19).

A pulse of N$_2$ 35 min in length given during an auxin-induced growth response causes a gradual decline in the elongation rate and is followed immediately by a burst of growth (Figure 20). This burst of growth is similar to but not nearly as strong as "emergent growth"
Figure 17. The time course of IAA-induced H⁺ secretion under the condition of oxygenation of the external medium (curve A) compared with the condition of non-gassing (curve B). IAA (10 μM) was added 90 min after excision.
Figure 17.
Figure 18. Effect of gassing of the growth medium with various gases on IAA-induced growth. IAA was applied at the arrows, 90 min after excision. The gas conditions indicated by each curve were maintained throughout the experiment.
Figure 18.
Figure 19. Reversal of inhibition caused by gassing of the external medium with $N_2$ upon return to $+O_2$ condition. Gassing of the external medium with $N_2$ began 1 hr before addition of IAA in both $H^+$ secretion (curve A) and growth (curve B) experiments. IAA was added 90 min after excision and remained on the segments from then on. After the segments were in the presence of IAA for 1 hr (and in $N_2$ for 2 hr), gassing with $O_2$ replaced gassing with $N_2$. 
Figure 19.
Figure 20. The effect of a 35 min pulse of N$_2$ during IAA-induced H$^+$ secretion (curve A) and growth (curve B). IAA was added 90 min after excision and the segments were in oxygenated media until the N$_2$ pulse began 40 min after application of IAA.
Figure 20.
described by Davies and Parrish (1977) in studies using pea stem segments. The emergent growth response occurs upon restoration of \( O_2 \) following a \( N_2 \) pulse and involves enhancement of elongation 100-200\% over that of a continuously oxygenated control. Return of corn coleoptile segments to aerobic conditions also causes a burst of \( H^+ \) to be released from the tissue (Figure 20). Emergent growth is not accompanied by an enhancement of \( H^+ \) efflux into the external medium (Davies and Parrish, 1977). It should be noted that gassing with \( N_2 \) during an auxin-induced \( H^+ \) secretion response causes an immediate increase in pH although growth is not immediately inhibited (Figure 20).

**Kinetics of the Endogenous Acidification of the External Medium**

The endogenous (i.e. not induced by any external factors) growth of corn coleoptile segments remains at a low rate following excision from seedlings until about 3.5 to 4 hr later when a spontaneously-occurring increase (3- to 10-fold) in growth rate begins. This new growth rate is strong and remains steady for at least several hours. Evans and Schmitt (1975) have described this as the "spontaneous growth response" (SGR). As with growth, the rate of endogenous acidification of the external solution remains low in the first hours following excision. In the presence of \( O_2 \) the pH stabilizes (at about pH 6.35) rapidly and remains very steady for about 3 hr. At that time a decrease in the pH of the medium begins. (An endogenous drop in pH occurring at about the same time as also observed in the non-gassing condition but it was weaker.) The pH drops at an increasing rate until a steady strong rate of decrease is attained (similar to an optimal IAA
response). The kinetics of the SGR and the kinetics of the endogenous drop in pH are compared in Figure 21. These endogenous changes in growth rate as well as $H^+$ secretion rate occur even if the segments are held for 2.5 hr in a large volume of oxygenated buffer and then transferred to the respective systems for measuring growth and $H^+$ secretion (data not shown).

Withdrawal of Auxin and Auxin Analogues

The kinetics of decay of auxin-induced $H^+$ secretion upon withdrawal of the hormone is completely masked by the occurrence of a rapid and large decrease in the pH (about 0.3 pH units) of the external medium apparently triggered by solution change. In Figure 22 (curve A) the rapid drop in pH upon withdrawal of auxin is shown to occur during the succeeding 12-15 min. The growth rate of segments from which auxin is withdrawn begins to decline about 20 min after withdrawal (Figure 22, curve B). No increase in growth rate is observed to parallel the rapid decrease in pH. This effect of withdrawal of auxin is consistently observed. A pulse of auxin as short as 20 min is likewise followed by a rapid decrease in pH when auxin is removed.

Since this response was unexpected, various other conditions under which solutions were changed were tested, including, a change from buffer to buffer, a change from auxin to auxin, application and withdrawal of auxin analogues, and experiments using acetone-treated segments. The results of these experiments are presented below. Each time a solution was removed to be replaced by another, the procedure followed was the same as that described earlier for auxin withdrawal,
Figure 21. A comparison of the kinetics of an endogenous growth response (SGR) (curve B) with the kinetics of endogenous H\(^+\) release into the external medium (curve A). Growth medium was oxygenated throughout the experiments. In each case recording began as soon after excision as possible.
Figure 21.
Figure 22. Effect of withdrawal of α-NAA on acidification of the external medium (curve A) and growth (curve B). α-NAA was applied 90 min after excision and withdrawn 60 min later. The method of solution withdrawal is presented in "Materials and Methods." The pH experiment was performed under a non-gassing condition; withdrawal of auxin has the same effect under conditions of oxygenation.
Figure 22.
i.e. once the solution was removed, the segments were rinsed in the beaker 3 to 4 times with the next solution and then the new solution was added to the beaker. The pH of this solution was adjusted prior to addition to the pH of the solution being replaced. This procedure took about 3 min to complete and recording was necessarily suspended during this time.

The auxin specificity of the withdrawal effect was tested by using applications of 10 μM BA, ICA and 2-CPA followed after 1 hr by withdrawal of the compounds. Table 2 shows the magnitude of the pH drop on withdrawal of the auxin analogues compared with that for the withdrawal of IAA. While segments were in solutions containing the analogues, no significant decrease in pH was observed. Thus the H⁺ secretion induced by IAA or α-NAA appears to be specific for active auxins. Similar results were obtained by others (Jacobs and Ray, 1976; Marré et al., 1973a; Rayle, 1973) using different auxin analogues. No growth enhancement was observed in response to either application or withdrawal of the analogues.

Since the rapid decline in pH occurred on withdrawal of all of the solutions listed in Table 2, the possibility that the pH drop was an artifact of solution change was examined. Results of solution changes from buffer to buffer and from IAA to IAA are shown is Figure 23. Replacing buffer with buffer (Figure 23, curve C) produced a weak and probably insignificant change in pH of the external medium. Because this solution change produced no large shift in pH, it was expected that a change from auxin to auxin would produce similar negative results. However, when 10 μM IAA was replaced by 10 μM IAA, a
Table 2.

ACIDIFICATION OF EXTERNAL MEDIUM UPON WITHDRAWAL OF 10 μM AUXIN AND AUXIN ANALOGUES

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH Drop on Withdrawal</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAA</td>
<td>6.25 - 5.94</td>
<td>0.31</td>
</tr>
<tr>
<td>ICA</td>
<td>6.30 - 6.15</td>
<td>0.15</td>
</tr>
<tr>
<td>2-CPA</td>
<td>6.31 - 6.10</td>
<td>0.21</td>
</tr>
<tr>
<td>BA</td>
<td>6.39 - 6.20</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*All solutions were gassed with O₂ while in contact with segments
Figure 23. Effect of various solution changes on the acidification of the external medium. Segments in the experiment shown by curve B were pretreated with 100% acetone for 7 min, then rinsed thoroughly before being placed in the beaker for a pH experiment (segments were no longer turgid). In curves A and D, IAA was added 90 min after excision and replaced by fresh IAA solution 50-60 min later. Curve C shows an experiment in which 1 mM K-phosphate buffer was replaced by fresh K-phosphate buffer. The pH values at zero time were: A, 6.37; B, 6.25; C, 6.32.
Figure 23.
rapid decrease in the external pH again resulted (Figure 23, curve A). Figure 23 also shows that when the same experiment was performed with segments which were pretreated in acetone (curve B), there was no indication of either an auxin-induced or solution change-induced modification of the external pH. In addition, manipulations involved in solution changes caused no change in pH when performed in the absence of segments (data not shown). The withdrawal effect is evidently found only when live tissue is used.

It was noted that when IAA was replaced with IAA, the pH leveled off following the rapid decrease upon solution change but then began to drop again. Under these conditions, the total time course for acidification of the external medium was similar to that for control segments in which IAA remained on the segments without a solution change.

**DISCUSSION**

For the acid growth hypothesis to be widely accepted, it must be shown that auxin induces a drop in the pH of the cell wall to a wall loosening level (below about pH 5.5, see Rayle, 1973) before the onset of increased elongation. It is most important to know the pH of the cell wall so that an accurate comparison of the timing of cell wall acidification and growth enhancement can be obtained. Using a much reduced external solution volume, Cleland (1976c) has measured an auxin-induced decrease in the pH of the external medium which begins at about the same time (in some cases shortly before) the increase in growth begins. Jacobs and Ray (1976) have measured the pH
in the tissue free space (not involving measurement of any external solution) and found that auxin-induced H⁺ secretion begins just before the growth rate increases. There are some technical obstacles which have still to be overcome so that measurement of the actual pH in the cell wall can be accomplished. Therefore, the actual change in pH of the cell wall cannot be determined by the technique I have used in these experiments nor by either of the techniques used in the time course studies just described. Because of the physical distance between the cell walls and the pH probe in the external medium and because of the large Donnan phase in the wall, errors may be expected in estimating the wall pH (Cleland, 1976c; Jacobs and Ray, 1976; Rubery and Sheldrake, 1974). However, I have shown that relative changes in the rate of H⁺ efflux can be easily detected with the technique employed in this study.

In general, there appears to be positive correlation between the magnitude of auxin-induced H⁺ secretion and growth. This correlation is evident: 1) in the auxin dose-response relationships, 2) in the change induced by gassing with O₂, air and N₂, and, 3) in the lack of response to inactive auxin analogues. A correlation is also suggested by the endogenous increase in H⁺ secretion which occurs at about the time of the spontaneous growth response.

There are some instances when H⁺ secretion does not seem to parallel growth. One noticeable example is that the rapid decrease in pH observed upon withdrawal of auxin or auxin analogues is not accompanied by a promotion of growth. This may or may not have relevance to the correlation between H⁺ secretion and growth as discussed below.
That both the IAA and α-NAA dose-response curves for H+ secretion and growth are bell-shaped indicates that there is some correlation between the two responses. This is the first report of a correlation in reduction in H+ secretion and growth at higher auxin concentrations. This study also corroborates evidence in the literature that auxin induces an increasing amount of H+ efflux from coleoptile segments with increasing auxin concentrations at the lower end of the effective concentration range (Cleland, 1975; Rayle, 1973). It might be noted that although no further increases in H+ secretion rate appear at concentrations above about 0.3 μM IAA in Avena coleoptile segments, investigators using Avena segments routinely use 10 μM IAA to elicit "normal" auxin-induced H+ efflux (Cleland, 1973, 1975, 1976a,b,c; Rayle, 1973).

The decline in magnitude of the pH drop at higher auxin levels may be associated with removal of H+ from the solution as large amounts of the undissociated acid (either IAA or α-NAA) move into the tissue. However, this would be expected to cause a decrease in the pH of the cytoplasm since the pH there is normally neutral (Rubery, 1977) and the acid should dissociate (Goldsmith, 1977) making H+ available for export. The fact that growth and H+ secretion are completely inhibited by 1 mM α-NAA but remain accelerated in 1 mM IAA indicates that uptake of auxin does not itself create a condition under which H+ secretion should be prevented.

The observations made here on the effect of gassing the external medium are interesting in several respects. It can now be suggested that respiratory CO₂ must have influenced the pH at which coleoptile
segments equilibrated with their external medium in studies which involved no gassing of the external medium (Cleland, 1973, 1975, 1976b&c; Rayle, 1973). Rayle (1973) reported an equilibrium pH value of between 5.8 and 6.0 for Avena coleoptile segments in K-phosphate buffer (8 segments/ml), initially at pH 6.3. This pattern of pH change during the equilibration period is the same as reported here for non-gassing conditions. Cleland (1975) reported that Avena segments lowered the pH of the external medium from pH 6.0 to 5.7 during equilibration.

In view of the fact that the external medium equilibrated at pH 6.0 to 5.7 in Cleland's experiments, the technique by which Cleland (1975, 1976b) determined the amount of auxin-induced H⁺ secretion may be open to some criticism. After addition of auxin, Cleland simply used titration with NaOH to bring the pH of the external medium back to pH 6.0 every 30 min, and the µmole of H⁺ released was determined from this titration (Cleland, 1975, 1976b). My results indicate that considerable error may be introduced during such determinations. I observed that when gassing (whether with O₂ or air) was stopped, the pH of the external medium began to fall immediately and within about 10 min was observed to have decreased by about 0.3 pH unit. Since Cleland (1975, 1976b) was titrating to a pH value (pH 6.0) above the equilibrium value (pH 5.7), under non-gassing conditions a considerable portion of the decrease in pH of the medium may have been due to increased CO₂ in solution. There is one report (Sloane and Sadava, 1975) that implicates respiratory CO₂ as the causal agent in the pH decline they found in media bathing auxin-treated pea stem segments.
(Cleland [1975, 1976b] did note that corrections were made for CO$_2$ uptake above pH 6.0.)

The inhibition of auxin-induced H$^+$ secretion and growth by N$_2$ is reversed by subsequent oxygenation. This occurs whether the gassing with N$_2$ begins 1 hr before auxin is applied or N$_2$ gassing is given as a pulse some time during the auxin response. Following a N$_2$ pulse there is an appearance of "stored growth" (Ray, 1961) upon return to aerobic conditions. Stored growth is a period of rapid growth which follows a period of growth inhibition so that the total amount of elongation is equal to a control. This indicates that wall loosening potential (e.g. H$^+$) was stored while the tissue was unable to expand. Whenever aerobic conditions are restored during a pH experiment, a burst of H$^+$ is detected. While the growth response following transient anaerobic conditions is not nearly as strong in corn as is emergent growth in pea stem segments upon return to aeration (Davies and Parrish, 1977), a drop in pH of the external medium parallels the increased growth rate in corn. No such drop in pH accompanies emergent growth in pea stem segments. Krauss and Hager (1976) have shown that an acid response in *Avena* coleoptile segments is inhibited by a pulse of N$_2$. When the segments were returned to aerobic conditions, a growth burst ("ANA-effect") was also recorded (Krauss and Hager, 1976).

The correlation between H$^+$ secretion and growth is less well-defined during the actual N$_2$ pulse. Auxin-induced growth is not immediately affected but the pH of the external medium reverses its downward trend and increases. Davies and Parrish (1977) show that a similar increase in the pH of the external medium occurs during a 1 hr
pulse with \( N_2 \) in pea stem segments which are acidifying the external medium. Recording, however, was not continued during their \( N_2 \) pulse. The immediate cessation of the decrease in pH may suggest that some component of the plasmalemma involved in \( H^+ \) secretion is extremely sensitive to anaerobiosis. However, growth may continue for a while if the level of \( H^+ \) in the wall is sufficient to allow extension and turgor is not immediately affected by anaerobiosis.

There are at least two potential explanations for the lack of correlation between pH drop and growth rate upon withdrawal of solutions. One explanation assumes the decrease in pH is a result of \( H^+ \) leaving the protoplast, passing through the cell wall to be detected in the external medium. No enhancement of growth is seen on solution change or on removal of the auxin or auxin analogues, yet the segments undergo similar mechanical manipulations as those in the pH experiments (such as brief periods during which they are not submerged). This non-specifically induced pH drop may be of a magnitude too low to allow for increased cell extension, that is, it may be below the threshold for acid-induced extension (see Chapter I). Supporting this is the fact that the pH drop on withdrawal is about equal to the drop in pH induced by 10 \( \mu M \) IAA in 1 hr (with oxygenation). If the level of \( H^+ \) released is below the threshold for increased growth, then one could assume that auxin in some way "sensitizes" the cell wall, enabling it to respond to lower levels of \( H^+ \) than non-treated segments. Alternatively, auxin-induced \( H^+ \) release could be viewed as incidental or secondary to the growth response.
Another explanation assumes that the $H^+$ released during the withdrawal effect are not coming from the protoplast, but are already occupying the Donnan space in the cell wall. Perhaps during incubation the $K^+$ concentration in the buffer is decreased substantially. This would more likely occur in cases where the external pH is dropping, such as during an auxin response. An observation consistent with this explanation is that the pH decrease upon withdrawal of auxin is greater than upon withdrawal of auxin analogues (Table 2). Adding fresh $K$-phosphate buffer might then allow a rapid exchange between the replenished $K^+$ and the $H^+$ held in the wall resulting in release of previously bound $H^+$ into the medium. A similar rapid pH drop was noted by Parrish and Davies (1977) when $K^+$ were added to a K-free medium bathing pea stem segments. This explanation is also supported by evidence that the cell wall acts as a cation exchanger (Rubinstein et al., 1977). It is widely recognized that there are a large number of fixed anionic groups in the wall which act as exchange sites for cations, and it is known that calcium ions can induce a release of $H^+$ from cell walls of frozen-thawed coleoptile segments (Rubinstein et al., 1977). However, if the $K^+$ in my experiments were simply exchanging and replacing $H^+$ in the cell wall, one would expect the same thing to occur (i.e. rapid drop in pH of the external medium) in the acetone-treated segments, but it does not. The withdrawal effect as described here requires that the tissue be alive. However, this effect is insensitive to KCN and CHI (data not given), indicating that the process of $H^+$ release on solution exchange is not an active pumping. The exact mechanism of this effect may be difficult to uncover since inhibitors
are ineffective at preventing it, yet the tissue must be viable for it to be expressed.

The ion exchange explanation for the withdrawal effect is supported by the observation that when auxin is re-added, the decline in pH is followed by a period during which $H^+$ efflux appears to stop before the pH continues to decrease at an auxin-induced rate. This period of pH stabilization may be the time that is required for the Donnan phase to again become saturated with $H^+$ before freely mobile protons can exit the wall. If this explanation is acceptable, it is obvious that $H^+$ released from the Donnan phase of the wall (evidenced by the rapid pH drop on solution change) are not effective in causing wall loosening. This, of course, is expected since the wall pH would be increased by the release of the bound $H^+$. 
CHAPTER III. TIME-DEPENDENT MODIFICATION OF THE H⁺ SECRETION AND GROWTH RESPONSES TO AUXIN: EVIDENCE FOR A SENSORY ADAPTATION

INTRODUCTION

There are several descriptions in the literature of spontaneously occurring changes in endogenous growth rates of isolated coleoptile segments (Cline et al., 1974; Evans and Ray, 1969; Evans and Schmitt, 1975; Evans et al., 1977; Macdowall and Sirois, 1976; van Overbeek, 1941). These endogenously controlled changes in growth rate occur within 5 hr following excision, i.e. well within the normal time span of short term growth experiments. In subapical corn coleoptile segments, a strong increase in the endogenous growth rate occurs about 3.5 hr after excision and has been designated as the SGR (Evans and Schmitt, 1975). They observed that the SGR rate in corn coleoptiles is 3 to 10 times greater than the very low basal growth rate which immediately precedes it.

Previous attempts to uncover the nature of the SGR in corn coleoptile segments have indicated that the response may arise, in part, from an increased capacity for auxin biosynthesis throughout the subapical segments with increasing time after excision (Evans and Schmitt, 1975). The occurrence of the SGR appears not to be exactly the same as the phenomenon of regeneration of the physiological tip which occurs...
in decapitated coleoptiles (especially *Avena*) since the increase in production of auxin in corn segments is apparently not limited to the apical portion of the segment (Evans and Schmitt, 1975). No single enzyme in the pathway from tryptophan to IAA has yet been found to be limiting during the period preceding the SGR (Evans et al., 1977).

An increase in auxin biosynthesis appears in the coleoptile segments by 5 hr after excision (Evans and Schmitt, 1975). I present evidence here that there is also a time-dependent increase in sensitivity of the coleoptile tissue to exogenous auxin during the period preceding the SGR. This indicates that although there may be an increase in auxin biosynthetic activity during this time, the actual level of the endogenous hormone is probably greatly reduced compared to the level found in an intact coleoptile. The SGR then may not be so much an indication of an increasing concentration of auxin in the excised segment, but rather it may be evidence of a greatly increased sensitivity to the hormone.

This time-dependent increase in sensitivity is evident in the $H^+$ secretion response as well as the elongation response to exogenous auxin. In Chapter II, I presented data showing that an endogenous $H^+$ efflux begins about the time of the SGR. Results of experiments described here indicate that the increased sensitivity to auxin is identified by: 1) an increasing magnitude of the $H^+$ secretion and growth responses to IAA during the 3 hr period following excision, 2) a decrease in the latent period of the IAA-induced growth response over this same period, and, 3) a qualitative change in the effect of auxin on growth rate during the latent period preceding enhancement of growth.
The effect of auxin on growth rate during the latent period changes from non-inhibition to one of inhibition with increasing time from excision. The same time-dependent changes in auxin sensitivity can be seen if auxin is applied at various times following removal of 1 μM IAA. The time-dependent modification of the response to IAA can be viewed as a type of sensory adaptation.

That the degree of adaptation to auxin is reflected in the rate of the response to auxin as well as in the latent period of this response indicates that the level of adaptation controls the tissue's "perception" of an auxin dose. The change in response to a constant IAA concentration that occurs with increasing time from excision is shown to be similar to the change in response to increasing concentration of IAA given at a constant time after excision.

MATERIALS AND METHODS

Plant Material

All experiments were done using subapical coleoptile segments from etiolated corn (Zea mays L., hybrid WF9X38 from Bear Hybrid Corn Co., Decatur, IL) seedlings grown as previously described (Evans and Hokanson, 1969; Evans and Ray, 1969). One-cm segments were cut beginning 3 mm from the tip of coleoptiles 3-3.5 cm long. The segments were deleafed and a column of 12 1-cm segments was mounted immediately after excision in a shadowgraphic growth-recording device as described previously (Evans and Ray, 1969; Evans et al., 1977). Recording of growth of the segments began no longer than 10 min after excision of the first segment. The expression "time of excision" as
used here refers to the time at which half of the segments were cut.

**IAA Solutions**

The pH of IAA solutions was adjusted to pH 6.3 or 6.8. Potassium phosphate buffer (1 mM or 3 mM) was used to maintain the pH of the solutions containing high concentrations of IAA. This buffer has no effect on the occurrence of the SGR (Evans and Schmitt, 1975), or on auxin-induced growth responses. The buffer concentration for all pH experiments was 1 mM.

**$^3$H-IAA Uptake**

Uptake of IAA was measured by incubating segments in 0.5 $\mu$M IAA (plus 3 mM K-phosphate, pH 6.5) containing $^3$H-IAA at a specific radioactivity of 500 $\mu$Ci/µmol. Coleoptile segments were mounted as for recording of elongation and uptake was monitored over a 30-min period beginning either 0.5 or 2.5 hr after excision. At prescribed intervals, the uptake of $^3$H-IAA was terminated by removing the segments from the uptake medium and immediately rinsing them in three changes of distilled $H_2$O. Each 1-cm segment was flushed with a stream of deionized $H_2$O for 10 sec. The segments were blotted briefly before each was placed into a test tube containing 2 ml of 80% ethanol (v/v). The segments were extracted overnight and 1 ml of the ethanolic extract was added to a scintillation vial containing 9 ml of Bray's scintillation cocktail. Determinations of radioactivity were made with a Beckman Model LS-230 scintillation spectrometer. An external standard was used to correct for sample quenching. Counting efficiency was about 26%.
pH Experiments

All pH experiments were performed in dim red light and all included oxygenation. The basic procedure given in Chapter I was followed. Recording of pH began about 10 min after time of excision.

RESULTS

Dependence of the Auxin Growth Response on Time from Excision or Time after Withdrawal of Exogenous IAA

The possible existence of a time-dependent increase in sensitivity to auxin in corn coleoptile segments was tested using two experimental protocols: 1) a low concentration of IAA (0.5 μM) was applied at increasing times from excision, and 2) 0.3 μM IAA was applied at increasing times from the withdrawal of a higher auxin concentration (1 μM). The effect of the time of IAA application was discerned by measuring the magnitude of the IAA response and the duration of the latent period associated with the response. The latent period is defined here as the period following application of auxin during which there is no measurable increase in growth rate (or H⁺ secretion rate for the pH experiments).

Growth curves of segments exposed to a low concentration of IAA at increasing times after excision are shown in Figure 24. There is a dramatic increase in the magnitude of the response to IAA and a dramatic decrease in the latent period of the response with increasing times from excision. The average growth rate induced by IAA given 0.5 hr after excision is about 0.17 mm/hr·segment, and the latent period is about 60 min. However, when IAA is applied 2.5 hr after
Figure 24. Dependence of the growth response to IAA on time after excision. Segments were held in water prior to exposure to auxin. The time in hr by each curve indicates the time after excision at which IAA (0.5 μM) was applied to that set of segments. Each experiment was repeated at least four times with representative curves displayed.
Figure 24.
excision, the auxin-enhanced growth rate is about 0.52 mm/hr·segment and the latent period is reduced to 14 min.

In other experiments, the coleoptile segments were placed in 1 μM IAA immediately after escision and left there for 45 to 60 min. (This treatment did not lead to an enhanced growth rate relative to the control until about 50 min had elapsed.) The segments were then rinsed thoroughly and transferred to water. A low concentration of IAA (0.3 μM) was then applied at increasing times from withdrawal of the 1 μM auxin and the time-dependent changes in the magnitude and latent period of the auxin response were measured. Again there was strong evidence for enhanced sensitivity to auxin with time, as measured by the increased magnitude and decreased latent period of the response to 0.3 μM IAA. Figure 25 shows the relationship of both the auxin-induced growth rate and the latent period of the auxin response to time of application of IAA following termination of the 1 μM IAA pulse.

In Figure 25 the auxin-induced growth rate is not significantly greater when IAA is applied 3.0 hr compared to 2.5 hr after the auxin pulse. However, at the same time, the latent period is considerably reduced at 3.0 hr relative to 2.5 hr (Figure 25). This observation, plus other factors considered below, indicates that the latent period and the actual increased elongation rate are not obligatorily coupled quantitatively. This is also indicated by the observation that the strongest increase in magnitude of the response to IAA does not correspond to the period of strongest decrease in latent period.
Figure 25. Dependence of the growth response to IAA on time after prior exposure to IAA. The time on the abscissa is time from withdrawal of 1 μM IAA. Following withdrawal of IAA the segments were thoroughly rinsed and placed in water until 0.3 μM IAA was added. Left ordinate: growth rate in response to 0.3 μM IAA. Right ordinate: latent period of response to 0.3 μM IAA. Standard deviations are given for each point. Experiments were performed a minimum of four times each. (SGR had begun just prior to addition of IAA at 3.0 hr; growth rate expressed at 3.0 hr is total rate.)
Figure 25.
Dependence of the Auxin H+ Secretion Response on Time from Excision

Like the growth response, the H+ secretion response exhibits a change in sensitivity to auxin with time. The correlation between growth and H+ secretion is very close in this respect. Auxin (0.5 \( \mu \)M) applied to corn coleoptile segments approximately 30 min after excision induces a weak rate of H+ secretion and the latent period to the response is about 45 min. This response is compared in Figure 26 with a response to 0.5 \( \mu \)M IAA given 2 hr 20 min after excision. The rate of H+ secretion induced at this time is almost three times greater than the one induced 30 min after excision. This increase in rate is directly comparable to that observed in the growth response where there is also a 3-fold increase in the auxin-induced rate over this same time period. The latent period to the H+ secretion response is reduced to about 17 min when auxin is given 2.3 hr after excision. Because of the difficulties encountered during IAA withdrawal in pH experiments (Chapter II), the nature of the auxin-induced H+ secretion response was not determined as a function of time after a pulse of weak auxin.

Uptake of \(^3\)H-IAA

\(^3\)H-IAA uptake was followed for 30 min beginning either 0.5 hr or 2.5 hr after excision. The uptake kinetics for these periods is shown in Figure 27. There appears to be no significant difference in the capacity for IAA uptake measured shortly after excision as compared to 2.5 hr after excision. Therefore, differential hormone uptake cannot explain the 2- to 3-fold increase in auxin responsiveness that occurs
Figure 26. Time-dependent change in sensitivity to IAA shown in IAA-induced H⁺ secretion. IAA (0.5 μM) was added either 30 min (curve A) or 140 min (curve B) after excision. Addition of IAA is indicated by the arrows. The external medium was oxygenated throughout these experiments.
Figure 26.
Figure 27. Uptake of $^3$H-IAA from 0.5 μM IAA was followed over a 30-min period using segments placed in uptake medium either 0.5 hr (o—o) or 2.5 hr (●—●) after excision. Each point represents the average of at least eight segments. Variation between segments was not larger than 25%.
Figure 27.
during the same time span.

Differential IAA uptake has been shown by Macdowall and Sirois (1977) to be largely responsible for variation with time from excision in the IAA growth response of wheat coleoptile segments. As additional support of the controlling effect of uptake, these workers have presented evidence that as the number of transverse cuts per unit length of coleoptile tissue increases, the latent period in response to IAA decreases. However, when I subsectioned 1-cm corn coleoptile segments, no change was observed in the latent period of the response to either 0.5 μM or 1 μM IAA measured from applications either 0.5 hr or 2.5 hr after excision (data not shown). A similar lack of dependence of the latent period of the auxin response on segment length in corn coleoptiles was demonstrated by dela Fuente and Leopold (1970) and in detail by Durand and Zenk (1972). Also in contrast to wheat coleoptiles in which there was an inverse relationship between segment length and magnitude of the auxin response, increasing the number of cut ends per unit length of corn coleoptile tissue led to no increase in the IAA-induced growth rate and, in some cases there was a measurable decrease relative to the auxin-induced growth rate of control segments. Likewise, the response of peeled coleoptiles to IAA did not deviate from that observed in non-peeled segments.

Changes in IAA Dose-Response Relationship with Time from Excision

The shape of the dose-response curve differs significantly in segments to which auxin is applied 0.5 hr after excision compared to 2.5 hr after excision (Figure 28). Segments used shortly after
Figure 28. IAA dose-response curves for segments 0.5 hr and 2.5 hr after excision. Curve A: IAA applied 0.5 hr after excision; curve B: IAA applied 2.5 hr after excision. In controls the SGR rate ranged from 0.12 to 0.17 mm/hr·segment. Each concentration of IAA was tested at least three times in both cases.
Figure 28.
excision display a definite sigmoidal pattern in their response to increasing IAA concentrations over the lower end of the range of concentrations used. A dose-response relationship similar to this has been described for Avena (Cleland, 1972a; Nissl and Zenk, 1969). A maximum rate of elongation in corn coleoptile segments given IAA 0.5 hr after excision was first attained in 5 μM IAA (Figure 28, curve A). In Avena, the maximum rate of elongation was seen beginning at 0.3 μM IAA (Cleland, 1972a).

When the IAA dose-response curve for corn coleoptiles is determined using segments 2.5 hr after excision, the curve is not sigmoidal (Figure 28, curve B). Instead, over the low range of auxin concentrations, there is a linear rise in response to increasing concentrations of IAA. The maximum rate occurs in concentrations 0.5 to 5 μM IAA. Further increases in concentration from this point result in decreased auxin responses. The shape of the curve generated with segments 2.5 hr after excision could be called a hyperbolic or a modified bell curve. This dose-response curve (Figure 28, curve B) for corn coleoptile segments agrees with that of Durand and Zenk (1972). Dose-response curves for wheat coleoptiles show no evidence of a sigmoidal curve at low IAA concentrations (Macdowall and Sirois, 1977).

It has been shown in Avena that the shape of the dose-response curve changes with time, and that a bell-shaped curve is only obtained in the presence of sucrose (Cleland, 1972a) or when determined about 24 hr after excision (Nissl and Zenk, 1969). My data indicate that the IAA dose-response curve in corn also changes with time, but that the changes occur between 0.5 hr and 2.5 hr after excision, that
is, within the latent period of a normal SGR in this tissue. Although a dose-response curve was not completed for the H⁺ secretion response with segments 0.5 hr after excision (see Chapter II for dose-response curve at 1.5 hr after excision), a change in the H⁺ secretion response to 1 μM IAA, as well as 0.5 μM IAA, is also evident with increasing time from excision.

Concentration Dependence of Latent Period as a Function of Time from Excision

The latent period of the response to IAA decreases with increasing concentrations of IAA whether the auxin is applied to segments 0.5 hr or 2.5 hr after excision (Figure 29). This is true up to 50 μM IAA in segments used 2.5 hr after excision and up to 0.5 mM in segments used 0.5 hr after excision. As higher concentrations are applied, the latent period again increases, but the point of inflection differs in segments tested 0.5 hr and 2.5 hr after excision (Figure 29). The latent period in response to a given concentration of IAA is always longer in segments used 0.5 hr after excision.

Using wheat coleoptiles, Macdowall and Sirois (1977) have shown that a plot of the inverse of the intercept time versus the negative log of the IAA concentration gave a linear curve. This observation indicated to them that the intercept time is the reflection of the rate-limiting penetration of IAA into the segments from media containing low concentrations of IAA. A similar plot for corn coleoptiles (using inverse of the latent period instead of inverse of intercept time) is not linear whether determined using segments 0.5 hr or 2.5 hr
Figure 29. Concentration dependence of latent period of IAA response in segments 0.5 hr and 2.5 hr after excision. Data shown were taken from the same experiments used to generate dose-response curves of Figure 28.
Figure 29.
after excision (Figure 29).

**Qualitative Changes in the Latent Period Associated with Large Step-up in Auxin Concentration**

Recently published reports indicate that a large step-up in auxin concentration causes an immediate reaction in a direction opposite to the direction of the later response. In a study in which lateral electrical potential changes were measured following unilateral application of auxin to corn coleoptiles there was an initial negative potential followed by a larger positive potential generated in response to a large step-up in auxin concentration (Morath and Hertel, 1978). Similarly, a large step-up in auxin concentration again applied unilaterally to corn coleoptiles produced an initial inhibition of growth on the side of application followed by a strong promotion of growth (Ullrich, 1978). In both of the above studies a small step-up in auxin concentration did not produce the initial negative or inhibitory responses. A transient inhibition of growth during the latent period of a response to auxin has been described in straight growth experiments as well (Rayle et al., 1970; Tietze-Hass and Dörrfling, 1977). Still others have shown such transient inhibitions to occur following application of auxin (Barkely and Evans, 1970; Lager et al., 1971; Krauss and Hager, 1976). Not all workers have been able to detect a growth inhibition during the latent period to an IAA response in corn coleoptile segments (Durand and Zenk, 1972; Nissl and Zenk, 1969). However, Tietze-Hass and Dörrfling (1977) have defined such an inhibition in *Avena* segments.
I have found that an inhibition of growth during the latent period of an auxin elongation response can be induced by a large step-up in auxin concentration. A large step-up can be obtained in two different ways: 1) by applying an "optimal" concentration of auxin (i.e. one which produces a maximum growth response), and, 2) by applying a low concentration of auxin at a time when the sensitivity to auxin is highest (i.e. about 2.5 hr after excision in corn coleoptile segments).

Figure 30 shows the pattern of reduction in growth rate that accompanies application of 0.5 μM IAA given 2.5 hr after excision (curve B). Application of this same concentration of IAA at 0.5 hr after excision causes no apparent reduction in growth rate (Figure 30, curve A).

Figure 31 illustrates that transient inhibition can be obtained by applying a large step-up in auxin concentration (to 10 μM) at a particular time after excision (here 1.5 hr), while a smaller step-up (to 0.1 μM) causes no inhibition. The lower concentration of IAA (0.1 mM) (Figure 13, curve A) is known to be suboptimal at this time (Figure 12) and represents a small step-up in auxin. No inhibition is noted during the latent period to this weak growth response. However, 10 μM IAA (Figure 31, curve B) induces inhibition during the latent period and induces subsequently a strong growth response.

By comparing the rate curves in Figures 30 and 31, it can be seen that varying the auxin concentration induces a qualitative as well as a quantitative change in the latent period (illustrated in Figures 24, 25 and 29). Furthermore, the similarity in the two figures indicates that the effective concentration of IAA can be changed in two ways. In
Figure 30. Growth response to 0.5 μM IAA applied 0.5 hr (○) and 2.5 hr (●) after excision expressed as % of control growth rate. IAA was added at the arrow.
Figure 30.

GROWTH RATE, % of CONTROL [x10^-2]

TIME IN MINUTES

IAA

A

B
Figure 31. Growth response to 0.1 µM (○) and 10 µM (●) IAA applied 1.5 hr after excision expressed as % of control growth rate. IAA was added at the arrows.
other words, a constant concentration of IAA can be at one time a small step-up (0.5 hr after excision) and at another time can be a large step-up (2.5 hr after excision) in the auxin dose.

**DISCUSSION**

The experimental evidence presented here indicates that there is a time-dependent increase in sensitivity of corn coleoptile segments to auxin. A 2- to 3-fold increase in the magnitude of the growth response to IAA with increasing time from excision or time from withdrawal of exogenous IAA accompanies a 3-fold decrease in the latent period of the response to auxin. This change in sensitivity to auxin is also directly reflected in auxin-induced H⁺ secretion as a 3-fold increase in magnitude with increasing time from excision. It has been shown that these changes in response to auxin cannot be attributed to a time-dependent change in the IAA uptake ability of the segments. The increase in auxin sensitivity is also reflected in a change in the auxin dose-response relationship of corn coleoptile segments between 0.5 and 2.5 hr after excision. The inhibition during the latent period that appears as auxin is applied further from excision is like that caused by higher concentrations of auxin. This supports the idea that shortly after excision a dose of exogenous IAA is recognized as effectively lower than it would be if applied some time later.

This measurable increase in sensitivity to exogenous auxin is a good indication that a type of sensory adaptation modifies the response of excised coleoptile segments to auxin. In the intact seedling, the coleoptile tip is considered a source of growth-promoting auxin for the
remainder of the coleoptile (Anker, 1973; van Overbeek, 1941). Therefore, excision of a subapical segment results in its removal from an immediate supply of active auxin. In corn coleoptile segments there is a period of rapid elongation which follows excision and persists for about 35 to 40 min. This initial period of rapid growth has been attributed largely to residual auxin, indicating that the coleoptile had been experiencing near optimal levels of IAA just prior to excision. In fact, the effect of the endogenous auxin supply on setting the level of sensitivity (or adaptation) to exogenous auxin can be mimicked by supplying the excised segments with 1 µM IAA (Figure 25).

There are reports of other adaptation phenomena in plants. List (1969) has studied the ability of intact primary roots of corn to adapt to growth-inhibiting levels of IAA. He noted that roots inhibited by 0.1 µM IAA begin to adapt after a period of 1 hr and resume growth. Sporangiophores of Phycomyces show adaptation in their light-induced growth response. In a classical study, Delbrück and Reichardt (1956) showed that the sporangiophore growth response is dependent on the light intensity to which the specimen is adapted before a new light stimulus is given. Hild and Hertel (1973) followed sensory adaptation of corn coleoptiles to a geostimulus. They found that repeated brief exposures of the coleoptiles to a horizontal position (prestimulation) prior to the final geostimulus results in an alteration of the normal pattern of geotropic curvature in corn coleoptiles.

In both the Phycomyces light-induced growth system and the corn coleoptile geotropic curvature system a time constant was determined
which characterized the half-time of sensory adaptation. Experiments to derive this value in the study of coleoptile geostimulus adaptation involved the monitoring of the level of adaptation (expressed as the amount of geotropic curvature) following increasing increments of prestimulation. In my corn coleoptile system the stimulus is the application of IAA and the response to this stimulus indicates the level of adaptation. Levels of adaptation in any system are set by the intensity of prestimulation (which in this case is the amount of endogenous active auxin), and there should be a time constant associated with the adaptation. However, the level of prestimulation cannot be controlled by the experimenter in my system, but depends on events of auxin metabolism in the tissue. It is known that IAA-synthetase as well as IAA-oxidase increase in activity between 1 and 5 hr after excision (Evans and Schmitt, 1975). Therefore, one cannot assume that prestimulation is constant during the time course of the experiments, and an estimate of a time constant for adaptation is not warranted at this time.

It has been proposed that events during the latent period of an auxin response represent a separate reaction to the hormone (Tietze-Hass and Dörffling, 1977; Ullrich, 1978). The total response to auxin has been divided into two phases: Phase I indicates that portion of the response that is immediate (such as inhibition of growth during the latent period), while Phase II indicates the positive response (such as the increased growth rate) (Tietze-Hass and Dörffling, 1977; Morath and Hertel, 1978). The results of my experiments support such a separation. Whether the time-dependent change in sensitivity to
auxin is tested following excision or following auxin pretreatment, the period of strongest increase in the magnitude of the growth response to IAA does not correspond to the period of strongest decrease in the latent period. Also, the latent period is not only quantitatively but also qualitatively different in response to a large step-up compared to a small step-up in auxin concentration.

Ullrich (1978) reports that α-NAA causes a stronger inhibition during Phase I than does IAA. In Chapter II it was shown that an increase in pH during the latent period (Figure 13) accompanied α-NAA application at all concentrations promoting a strong growth response as well as those that are completely inhibitory (1 mM) (Figure 13). This may be evidence that a separation of the two phases of the auxin response is seen upon application of 1 mM α-NAA. (Segments in 1 mM α-NAA respond to acidic buffers, but lose turgor shortly thereafter.) Separation of two responses to auxin in another tissue (soybean hypocotyl segments) has been achieved by differential inhibition using cytokinin (Vanderhoef and Stahl, 1975; Vanderhoef et al., 1976).

A more detailed study of the changes in Phase I could aid in the understanding of the sensory adaptation reported here. It has already been suggested that adaptation is expressed through Phase I of the auxin response (Hild and Hertel, 1972; Morath and Hertel, 1978).
SUMMARY

There have been several pieces of evidence presented in these three chapters which further our understanding of the mechanism of action of auxin. A close correlation between $H^+$ secretion and growth is shown in auxin dose-response studies, in inhibition by $N_2$ and its reversal by subsequent gassing with $O_2$, and in a time-dependent change in sensitivity to auxin.

However, it is also pointed out here that auxin may induce a rate of $H^+$ secretion below the threshold for acid-induced growth. Experiments with NA, which have given results indicating that such a threshold may exist, are especially suited for the study of the $H^+$ dose-response relationship. This is true because NA causes release of $H^+$ from the protoplast, simulating $H^+$ release caused by auxin.

Also in these studies insight is gained into how auxin controls its own effectiveness. In the investigation of the change in sensitivity of coleoptile segments to auxin, it is shown how auxin finely tunes the responsiveness of a plant tissue to changes in its own concentration, thereby providing a regulatory control for hormone action. This extends a similar concept proposed earlier from observations that auxin influences its own polar transport in auxin sensitive tissues (Hertel and Flory, 1968; Rayle et al., 1969).


