REVERSAL OF HORMONE-ENHANCED PEA EPICOTYL ELONGATION BY ALTERNATE ELECTRON TRANSPORT INHIBITORS AND CONCANAVALIN A

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REVERSAL OF HORMONE-ENHANCED PEA EPICOTYL ELONGATION BY ALTERNATE ELECTRON TRANSPORT INHIBITORS AND CONCANAVALIN A

An Abstract

Presented to

the Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in Biology

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by

William Hunter Atkinson

December, 1977

ABSTRACT

Epicotyl sections excised from the third internode of seven day old dark grown Pisum sativum L. variety "Little Marvel" were studied to determine the effects of electron transport inhibitors, adenosine triphosphate, and lectins on elongation enhanced by the hormones gibberellic acid and indoleacetic acid.

Antimycin A, an inhibitor of cyanide-sensitive electron transport, and 8-hydroxyquinoline, an inhibitor of cyanide-resistant electron transport, inhibit hormone-enhanced elongation of pea epicotyls. The two inhibitors in combination drastically interfere with hormone-enhanced elongation. The data suggest that hormone-enhanced elongation is dependent on both pathways of electron transport.

Adenosine triphosphate does not effect growth of pea stem sections and the energy of this molecule is not a limiting factor in elongation of tissue that is treated with electron transport inhibitors. The data indicate that hormone-enhanced elongation of pea epicotyl sections requires oxidized intermediates that are generated via the alternate pathway of electron transport.

Concanavalin A inhibits hormone-enhanced growth in pea stems. The inhibition of elongation by concanavalin A is reversed with mannose. These results may indicate the existence of mannose

specific receptor molecules on the cell membrane.

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REVERSAL OF HORMONE-ENHANCED PEA EPICOTYL ELONGATION BY ALTERNATE ELECTRON TRANSPORT INHIBITORS AND CONCANAVALIN A

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December, 1977

To the Graduate Council:

I am submitting herewith a Thesis written by William Hunter Atkinson entitled "Reversal of Hormone-Enhanced Pea Epicotyl Elongation by Alternate Electron Transport Inhibitors and Concanavalin A." I recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Biology.

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Gratitude is expressed to my parents, whose encouragement and patience during this study are greatly appreciated.

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INTRODUCTION

Gibberellic acid promotes stem growth in dwarf peas by stimulating both cell division and cell elongation (Arney and Mancinelli, 1966). Auxins in the proper concentration bring about cell division and cell enlargement along the longitudinal axis in Avena coleoptiles (Went and Thimann, 1937). Segments of excised etiolated "Alaska" pea seedlings show a dramatic increase in growth when exposed to auxin (Thimann and Schneider, 1938). The synergistic action of gibberellin and auxin on internode section elongation in the dwarf pea, "Meteor" variety, has been reported by Brian and Hemming (1958). Despite much experimental work during the last forty years, the exact mode of action of these plant growth substances has not been resolved (Thimann, 1974). One possible interaction between gibberellin and auxin has been demonstrated. In dwarf peas the level of diffusible auxin is drastically increased in the presence of added gibberellin (Sastry and Muir, 1963). Auxin-induced elongation in Avena coleoptiles is inhibited by potassium cyanide, a respiratory inhibitor (Bonner, 1933). Cyanide-resistant respiration and the existence of an alternate pathway for electron transport has been demonstrated in several higher plants (Solomos, 1977). The cyanide-sensitive pathway is selectively blocked by

Chapter II

REVIEW OF THE LITERATURE

Gibberellic acid (GA) is a natural plant growth substance that was first reported to occur in the flowering plants by Phinney et al. (1957). Gibberellin promotes stem elongation in a wide variety of plants, especially those of the dwarf variety (Brian and Hemming, 1955). Lockhart (1956) observed that GA caused light grown peas to elongate as rapidly as dark grown peas thus reversing the light inhibition of elongation. Many investigators have noted that GA increases cell division in plant tissues (Sachs, 1961; Arney and Mancinelli, 1966). Kato (1956) observed that GA not only stimulates elongation in pea epicotyl sections but that it also increases respiration and water uptake. Kuraishi and Muir (1962) reported that in both tall and dwarf varieties of pea stems, GA increases the levels of diffusible auxin. Gibberellic acid may act to enhance auxin synthesis in the apex of intact peas (Ockerse and Galston, 1967).

Auxin stimulates cell division and cell elongation in Avena coleoptiles (Went and Thimann, 1937) and enhances elongation in excised pea epicotyl sections (Thimann and Schneider, 1938; Galston and Baker, 1951; Purves and Hillman, 1958; Russell and Galston, 1969). Other investigators have found that indoleacetic acid (IAA), an auxin,

antimycin A, and the alternate cyanide-resistant pathway is blocked by 8-hydroxyquinoline (Bendall and Bonner, 1971).

Hormone action in some mammalian systems involves the binding of hormone to specific receptors on the cell membrane. Lectins can compete with the hormone for the binding sites (Cuatrecasas, 1974). In plant hormone research, binding sites of auxin on the cell membrane rich fraction of suspensions have been demonstrated (Poovaiah and Leopold, 1977; Dollstadt, et al., 1976). No gibberellin has been found to bind to any macromolecular fraction (Kende and Gardner, 1976). The specific receptor molecule involved in auxin binding has not been isolated or identified (Kende and Gardner, 1976).

This study was undertaken to: 1) determine the effects of various concentrations and combinations of gibberellic acid and indole-3-acetic acid on elongation of epicotyl sections in Pisum sativum L. var. "Little Marvel"; 2) study the effects of various inhibitors of electron transport on hormone-induced epicotyl elongation; 3) examine the response of tissue to respiratory inhibitors and added adenosine triphosphate; 4) observe the respiratory rate of tissue treated with combinations of inhibitors and growth substances; 5) analyse the growth response of epicotyl sections to plant hormones and the lectins concanavalin A and wheat germ agglutinin.

stimulates cell division and swelling of parenchyma cells from pea stem sections (Fan and Maclachlan, 1967). These investigators also noted a promotion in the synthesis of the enzymatic protein cellulase in response to IAA but concluded that IAA-enhanced elongation does not require synthesis of DNA, RNA, or protein. Barkley and Evens (1970) and Murayama and Ueda (1973) have also concluded that enzymatic synthesis is not necessary for the initial promotion of growth by IAA. Nooden and Thimann (1963) concluded that nucleic acid synthesis is required for auxin action. Green epicotyl sections from peas require higher IAA concentrations for a maximum growth response than do etiolated epicotyl sections (Galston and Baker, 1951; Purves and Hillman, 1958; Russell and Galston, 1969). Bonner (1933) demonstrated the requirement for respiration in auxin enhanced elongation of Avena coleoptiles by showing that potassium cyanide inhibits elongation of auxin treated tissue. Thimann (1974) has observed that although oxidative phosphorylation may be required for auxin-enhanced elongation, this may not be the primary site of auxin action. Despite much experimentation over the last forty years, the exact mode of action of auxin is not known (Thimann, 1974).

The application of both GA and IAA, to excised pea epicotyl sections, enhances elongation, and growth is greater than that obtained with either of these hormones separately (Brian and Hemming, 1958; Kato, 1958; Purves and Hillman, 1958; Galston and Warburg, 1959).

Combinations of both GA and IAA may act synergistically to enhance elongation. This synergism is more pronounced with sections from light grown dwarf variety peas (Ockerse and Galston, 1967). Purves and Hillman (1958) found that the growth response was not always synergistic but more often simply additive. Kato (1958) also found the elongation response of epicotyl sections to both GA and IAA to be additive. Russell and Galston (1969) proposed that GA may suppress some IAA-oxidase system.

The significance of cellular respiration as a requisite for auxin-induced elongation was demonstrated by Bonner (1933) who found that cyanide blocked this enhanced elongation in Avena coleoptiles. The existence of cyanide-resistant respiration in Arum spadices was reported by James and Beevers (1950). Bonner (1965) suggested that all higher plants may possess the capacity for cyanide-resistant respiration. Cyanide-resistant respiration has been reported in many higher plants (Floyd and Rains, 1971) and in many microorganisms (Sherald and Sisler, 1972; Kawakita, 1970). In plants that exhibit cyanide-resistant respiration, electrons may flow along the regular pathway of electron transport via the cytochromes to oxygen or they may be diverted to the cyanide-resistant pathway through an alternate oxidase to oxygen. Floyd and Rains (1971) observed that the last two sites of oxidative phosphorylation are bypassed when electrons pass through the cyanide-resistant pathway but that the first site of energy

conservation is utilized by both pathways. The branch point, where the two pathways diverge, occurs on the substrate side of cytochrome b (Bendall and Bonner, 1971; Storey, 1972; Solomos, 1977). Tomlinson and Moreland (1975) have indicated that the branch point occurs at ubiquinone or at some non-heme iron-sulfur protein.

Antimycin A is one of many chemicals that block electron transport via the cyanide-sensitive pathway (Bendall and Bonner, 1971). Antimycin A inhibits the cyanide-sensitive pathway at the cytochrome b complex (Storey, 1972).

Hydroxylamine is an inhibitor of photosynthetic electron transport in photosystem II (Cheniae and Martin, 1971). Wilson and Brooks (1970) observed that hydroxylamine inhibits mitochondrial succinate oxidation in rat liver tissue which is a cyanide-sensitive system.

The chelator 8-hydroxyquinoline is a specific inhibitor of the cyanide-resistant pathway of electron transport (Bendall and Bonner, 1971; Sherald and Sisler, 1972).

The physiological role of the cyanide-resistant pathway of electron transport is speculative. Many investigators conclude that the alternate, less phosphorylating pathway is useful for the oxidation of substrates required in the tricarboxylic acid cycle under conditions of high ATP levels which inhibit the cyanide-sensitive pathway of electron transport (Palmer, 1976; Tomlinson and Moreland, 1975; Solomos, 1977). Bendall and Bonner (1971) noted the occurrence of

cyanide-resistant respiration in tissue that was rapidly growing, such as the etiolated bean epicotyl.

The cell membrane has received attention as a possible site of hormone action in both plants and animals. Cell membranes contain glycoproteins that are oriented with the saccharide portion of the molecule projecting on the outside of the membrane (Capaldi, 1974; Roseman, 1975; Marchesi, 1975). In mammalian systems it has been demonstrated that some hormones and biologically active agents act by binding to specific glycoprotein receptors on the cell membrane (Cuatrecasas, 1974; Bengelsdorf, 1976).

Lectins, proteins or glycoproteins, are derived from beans and a variety of other sources. These lectins have the property of binding to specific sugars (Lis, 1973; Liener, 1976; Sharon, 1977). Lectins can compete with certain hormones in mammalian systems for receptor molecules on the cell membrane and may displace the hormone from the receptor (Cuatrecasas, 1974). Concanavalin A, a lectin from the jack bean, binds specifically to glucose, mannose, and n-acetyl-D-glucosamine (Liener, 1976).

In experiments with the mammalian hormone insulin, it has been demonstrated that the hormone binds to saccharide residues on the cell membrane and that this somehow activates the enzyme adenylate cyclase which catalyzes the conversion of ATP to cyclic-AMP. The cyclic-AMP activates a protein kinase which initiates a cascade of metabolic events within the cell (Bengelsdorf, 1976).

Auxin has been found to bind to cell membranes of higher plants (Poovaiah and Leopold, 1976; Dollstadt et al., 1976). Kende (1976) was not able to demonstrate the binding of radioactive GA to membranes of higher plants. Kende and Gardner (1976) stated that no receptor molecule for plant growth hormones has been isolated or identified. Wood and Paleg (1972) found that GA increases the permeability of model membranes. Morré and Bracker (1976) showed that auxin decreases the thickness of cell membranes by 10-15%. Auxin also increases the microviscosity of soybean cell membranes (Helgerson et al., 1976).

Earle and Galsky (1971) have presented results which show that cyclic-AMP mimics the action of GA by stimulating the production of ATPase in barley endosperm. These results imply that cyclic-AMP may mediate plant hormone action as it does in some mammalian hormone systems. However, Yunghans and Morré (1977) have concluded that no cyclic-AMP occurs in soybean cell membranes nor does adenylate cyclase activity occur in this tissue.

Chapter III

MATERIALS AND METHODS

Seedling Growth

Seeds of Pisum sativum L. var. "Little Marvel", obtained from Carolina Biological Supply Company, Burlington, North Carolina, were used throughout this study. Seeds were imbibed in tap water for 12 hours while constantly being aerated. After rinsing in tap water, the seeds were sown in moist vermiculite in 19 x 27 centimeter (cm) plastic flats. The flats were placed on shelves of a Precision Scientific Company model 805 growth chamber and maintained at $25^{\circ} \pm 1^{\circ}$ Celsius for 7 days in darkness. On the seventh day, 10 millimeter (mm) epicotyl segments were excised from the third internode just below the apical hook using an instrument consisting of two razor blades mounted in parallel. All excised segments were randomized in a 9 cm petri dish containing distilled water.

Culture Method

Ten epicotyl sections were distributed to each of the 50 milliliter (ml) Erlenmeyer flasks by using a "card-dealing"

method to maximize randomization. The culture flasks contained 5 ml of the control medium or the appropriate treatment dissolved in the control medium. The control medium contained potassium phosphate 5×10^{-3} Molar (M) pH 6.8 as a buffer, 10^{-4} M penicillin and streptomycin, and 3% sucrose (w/v) in distilled water. The culture flasks were placed on the shelf of a Precision Scientific Company, model 805, growth chamber 16 centimeters (cm) below two 15 watt fluorescent lights and maintained at $25^{\circ} \pm 1^{\circ}$ Celsius for 10, 24 or 48 hours as indicated in the results. The light intensity at shelf level was 352 footcandles. After the appropriate incubation time, the epicotyl sections were measured to the nearest 0.25 mm with the aid of a 10x binocular dissecting microscope.

Respiration Studies

Respiration studies were carried out with a Gilson Differential Respirometer. Epicotyl sections prepared and cultured as previously described were removed from the culture vessels after 10 hours incubation and were placed in Gilson reaction vessels containing 5 ml of fresh test solution. In the center well of the reaction vessel was placed 0.5 ml of 6 N potassium hydroxide and pleated wick made from 2 x 2 cm squares of filter paper. The respirometer water bath was

maintained at 27°Celsius. All reaction vessels were allowed to equilibrate for 15 minutes prior to the measurement of respiration. Respiratory measurements were taken for at least 30 minutes. All gas volumes were converted to conditions of standard temperature and pressure. Immediately after the respiratory studies, the epicotyl segments from each treatment were measured, as previously described, blotted dry, and weighed to the nearest 0.0001 gram on a Mettler Gram-atic analytical balance. Respiration was reported as microliters of oxygen consumed per gram of tissue per 30 minutes.

The chemicals used in the experiments were obtained from the following sources: potassium hydroxide from J. T. Baker Chemical Company, Phillipsburg, New Jersey; 8-hydroxyquinoline from Matheson, Coleman and Bell, Norwood, Ohio and East Rutherford, New Jersey; 3-indole-acetic acid from Eastman Organic Chemicals, Rochester, New York; streptomycin sulfate from Nutritional Biochemicals, Cleveland, Ohio; hydroxylamine, gibberellic acid GA_3, pennicillin-G potassium salt, antimycin A, adenesine 5'-triphosphate, wheat germ agglutin, and concanavalin A, from Sigma Chemical Company, Saint Louis, Missouri; mannose from Fisher Scientific Company, Fair Lawn, New Jersey.

All treatment means that are not significantly different, at the 5% level, are connected with a dashed line in the bar graphs (Figures 1-9).

Chapter IV

RESULTS

Response of Excised Epicotyl Sections to Plant Growth Substances

Gibberellic acid (GA) stimulates internode elongation in the dwarf pea (Brian and Hemming, 1955). The stimulatory effects of GA on elongation in epicotyl sections of dwarf peas are not usually statistically significant at the 5% level (Brian and Hemming, 1958). An auxin, indoleacetic acid (IAA), has also been shown to stimulate elongation in pea stem sections (Galston and Baker, 1951). The synergistic action of these two plant growth substances, GA and IAA, has been shown by several investigators (Ockerse and Galston, 1967; Brian and Hemming, 1958).

Experiments were conducted to determine the growth response of excised epicotyl sections to various concentrations of GA, IAA, and combinations of both GA and IAA. The optimum concentrations of GA and IAA were determined by their effects on elongation. Statistical significance was determined for all results at the 0.05 level using Duncan's New Multiple Range Test (Duncan, 1955; Steel and Torrie, 1960). Figure 1 illustrates that IAA, at concentrations of 10 and 100 µM, stimulates elongation. The combination of IAA 10 µM and GA 1 ppm was stimulatory and significantly different from elongation

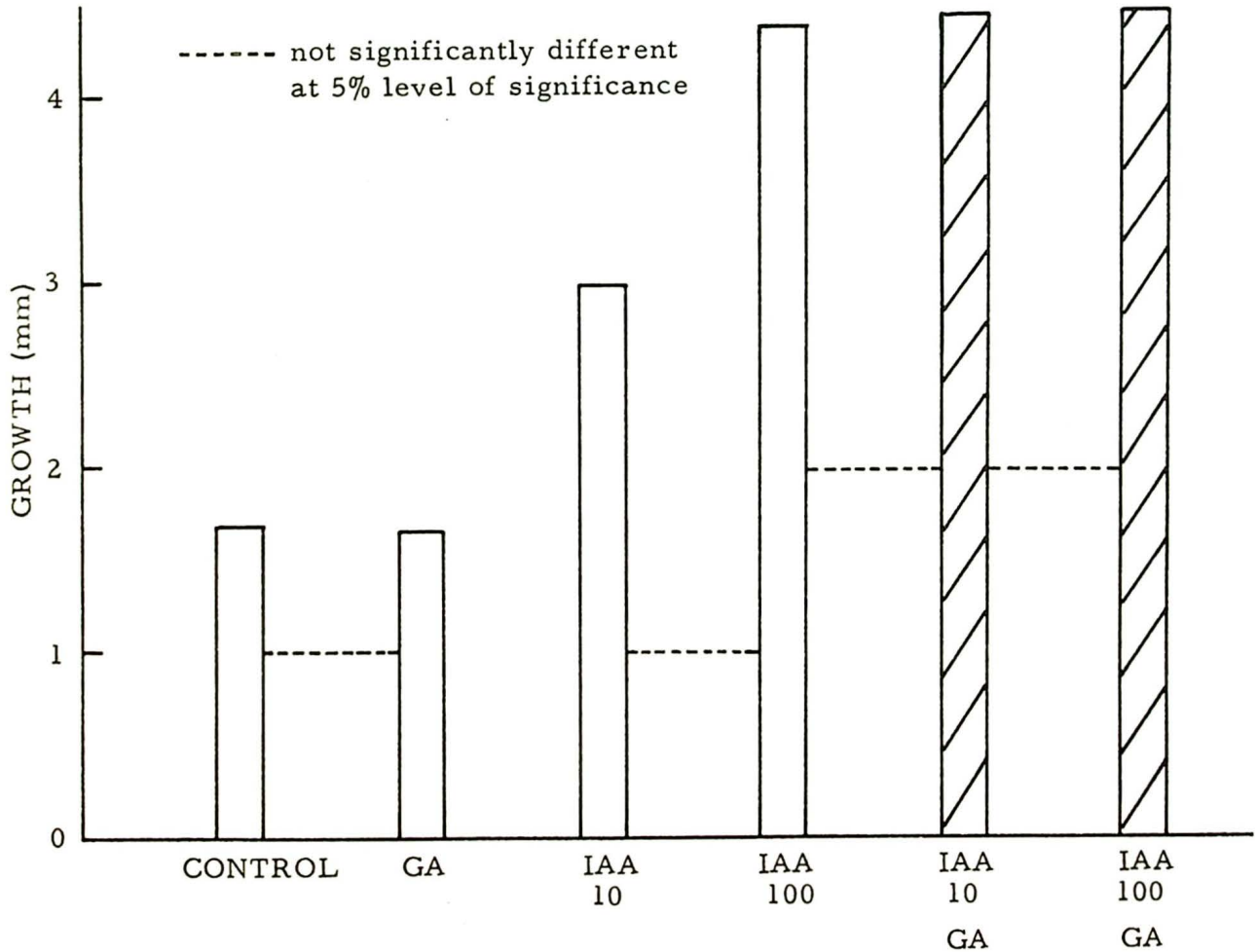

Figure 1. Effect of GA, IAA, and GA + IAA on epicotyl section elongation after 48 hours incubation. Data represents replicate treatments of ten sections per treatment. GA is present as 1 ppm and all IAA concentrations are in uM. Cross-hatched bars represent inclusion of both IAA and GA.

obtained with either IAA 10 μM or GA 1 ppm when used separately.

All tissue treatment combinations of GA and IAA were additive and significantly stimulated elongation when compared to equivalent concentrations of either GA or IAA alone (Figure 2). It was determined that the optimum concentrations of the two growth substances in combination were GA 10 ppm and IAA 10 μM. These concentrations were used throughout in the following experiments. In Table I the extent of this interaction is shown. GA 10 ppm stimulated elongation 168% and IAA 10 μM 247% as compared to the control. However, the combination of the two produced a 324% increase in elongation. With each treatment the growth was significantly different from growth in any of the other treatments.

Effects of Inhibitors of Electron Transport on Elongation

Many inhibitors of plant mitochondrial electron transport are known. These inhibitors may be divided into two classes, those that block the cyanide-resistant or alternate pathway and those that block the cyanide-sensitive pathway of electron transport. Antimycin A is a respiratory inhibitor that acts to block cyanide-sensitive electron transport between cytochromes b and c_1 (Storey, 1972). An experiment was conducted to determine what concentration of antimycin A would inhibit elongation. Concentrations of antimycin A, 10 uM and greater significantly inhibited elongation but 1 μM and 5 μM did not

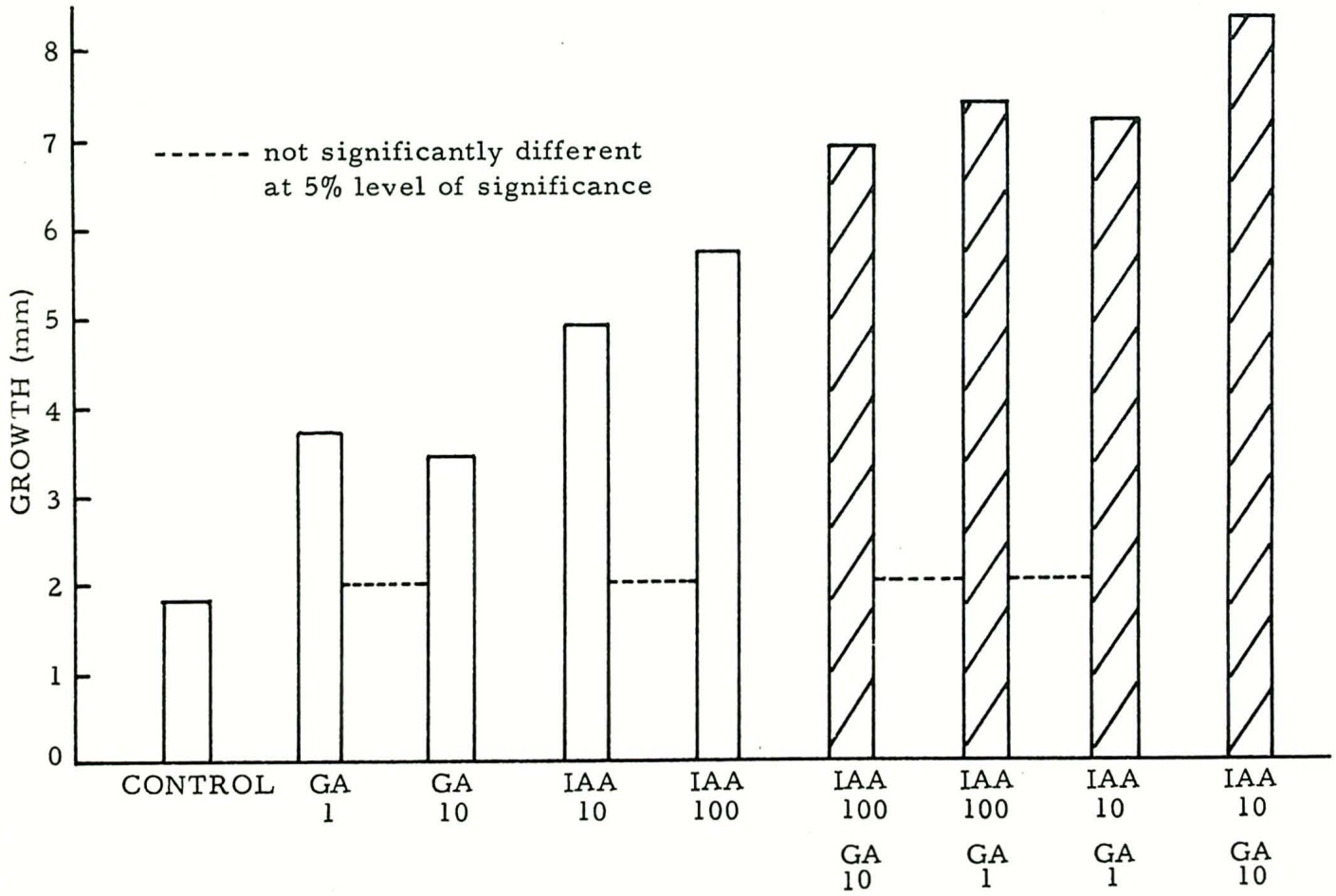

Figure 2. Growth response of epicotyl sections to GA, IAA, and GA + IAA. Data represents an average of replicate experiments after 48 hours growth. All IAA and GA concentrations are in μM and ppm respectively. Cross-hatched bars represent inclusion of both IAA and GA.

Table I

Effects of Gibberellic Acid and Indoleacetic Acid on Epicotyl Section Elongation

Treatment	Percent of Control
CONTROL	100
GA	168
IAA	247
GA + IAA	324

IAA and GA were present at 10 μM and 10 ppm respectively. Epicotyl sections were measured after 48 hours growth. Data is an average of two sets of replicate experiments. All values are significantly different from one another at the 5% level of significance.

(Figure 3). Antimycin A at concentrations of 2 μM and 5 μM did not significantly interfere with elongation (Figure 4). In all further experiments using antimycin A, a concentration of 5 μM was used.

Hydroxylamine is an inhibitor of electron transport in photosystem II (Cheniae and Martin, 1971). Various concentrations of hydroxylamine were used to determine its effect on elongation of excised epicotyl sections (Figure 5). Hydroxylamine at concentrations of 0.5 mM and 5.0 mM had no effect on elongation. At concentrations of 10 mM and 50 mM the hydroxylamine significantly inhibited elongation of the epicotyl sections.

The chelator 8-hydroxyquinoline is a specific inhibitor of the cyanide-resistant pathway of electron transport (Bendall and Bonner, 1971). The effects of various concentrations of 8-hydroxyquinoline on epicotyl elongation are shown in Table II. Concentrations of 0.1, 0.5, and 1.0 mM 8-hydroxyquinoline did not interfere with elongation but the higher concentrations of 5.0, 10.0, and 50.0 mM significantly inhibited elongation of the epicotyl sections. Further work with hydroxylamine was suspended and 8-hydroxyquinoline was used as a specific inhibitor of the cyanide-resistant pathway throughout the following studies.

The effects of inhibitors of both pathways of electron transport on elongation of pea epicotyls and epicotyls in the presence of the growth hormones were investigated (Figure 6). The inhibitors

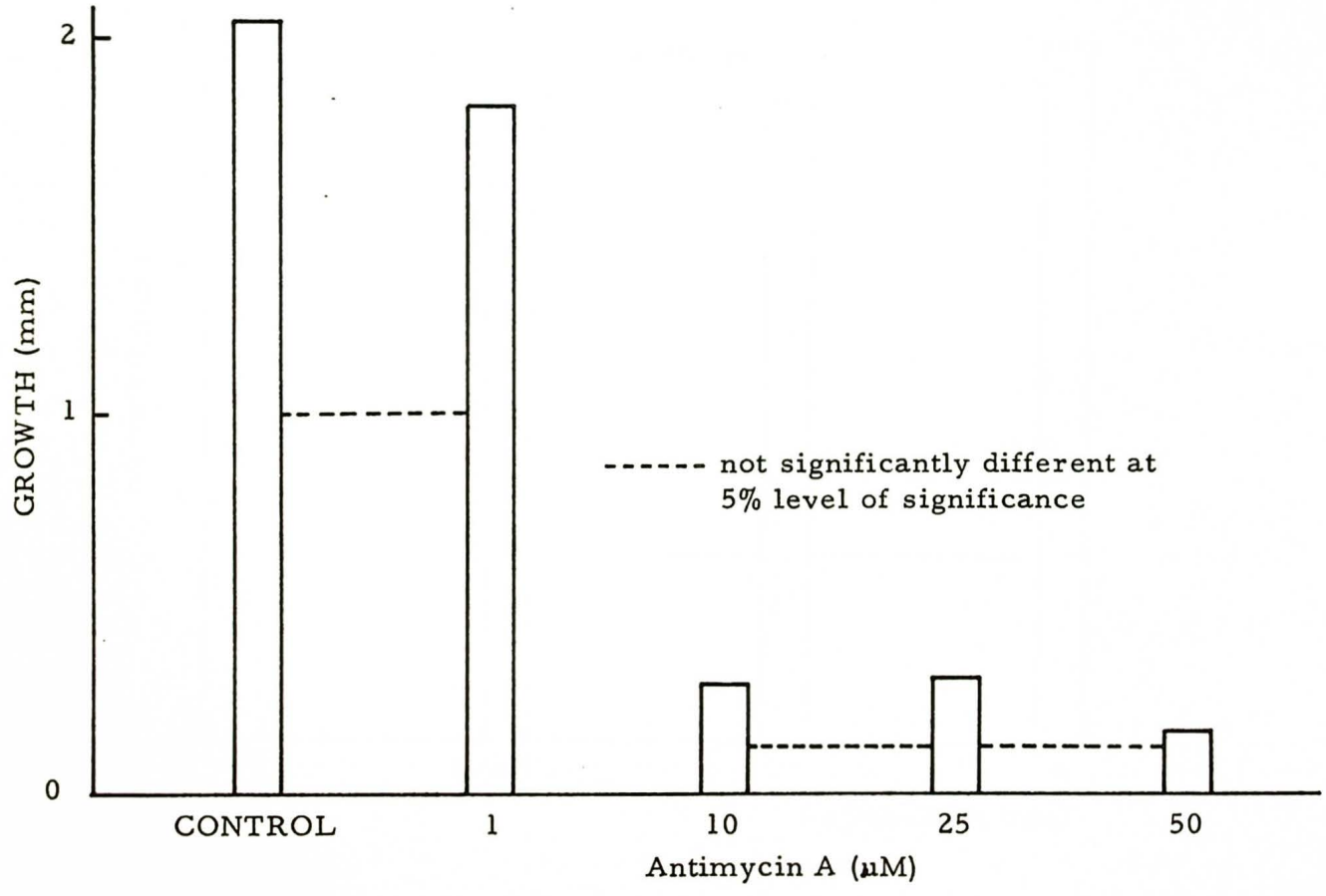

Figure 3. Effect of Antimycin A on elongation of epicotyl sections after 48 hours incubation.

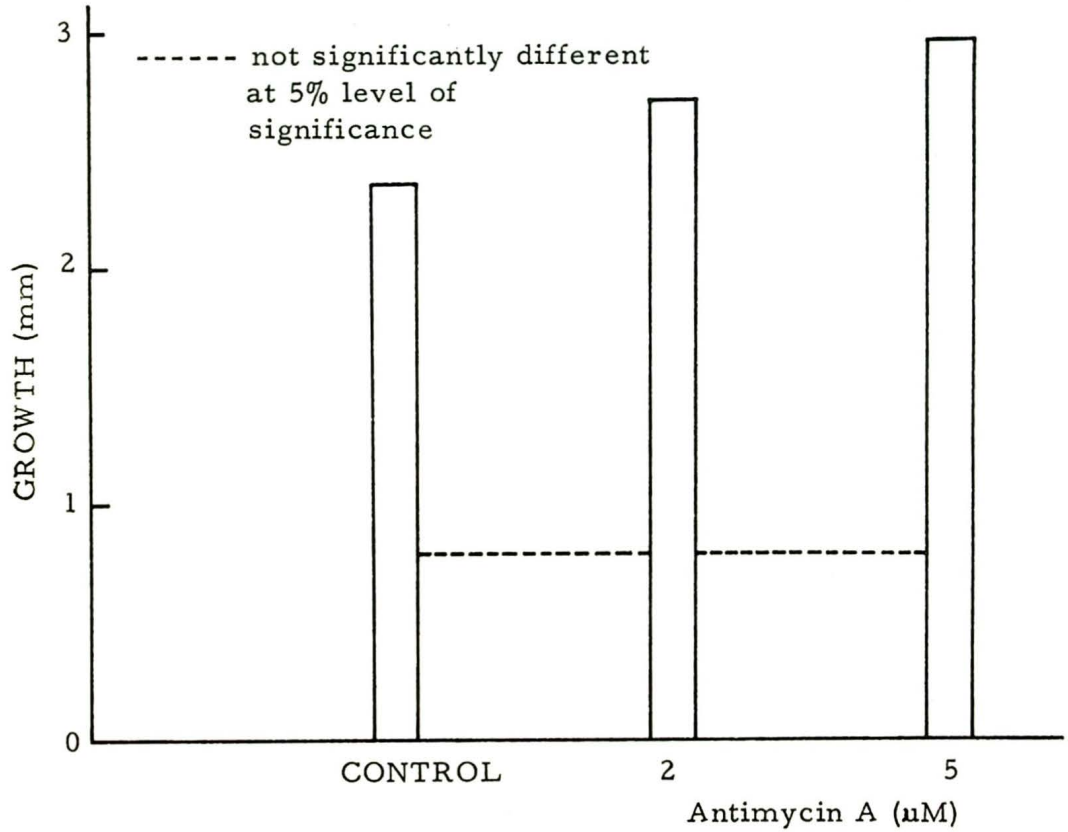

Figure 4. Effect of Antimycin A on elongation of epicotyl sections after 48 hours incubation.

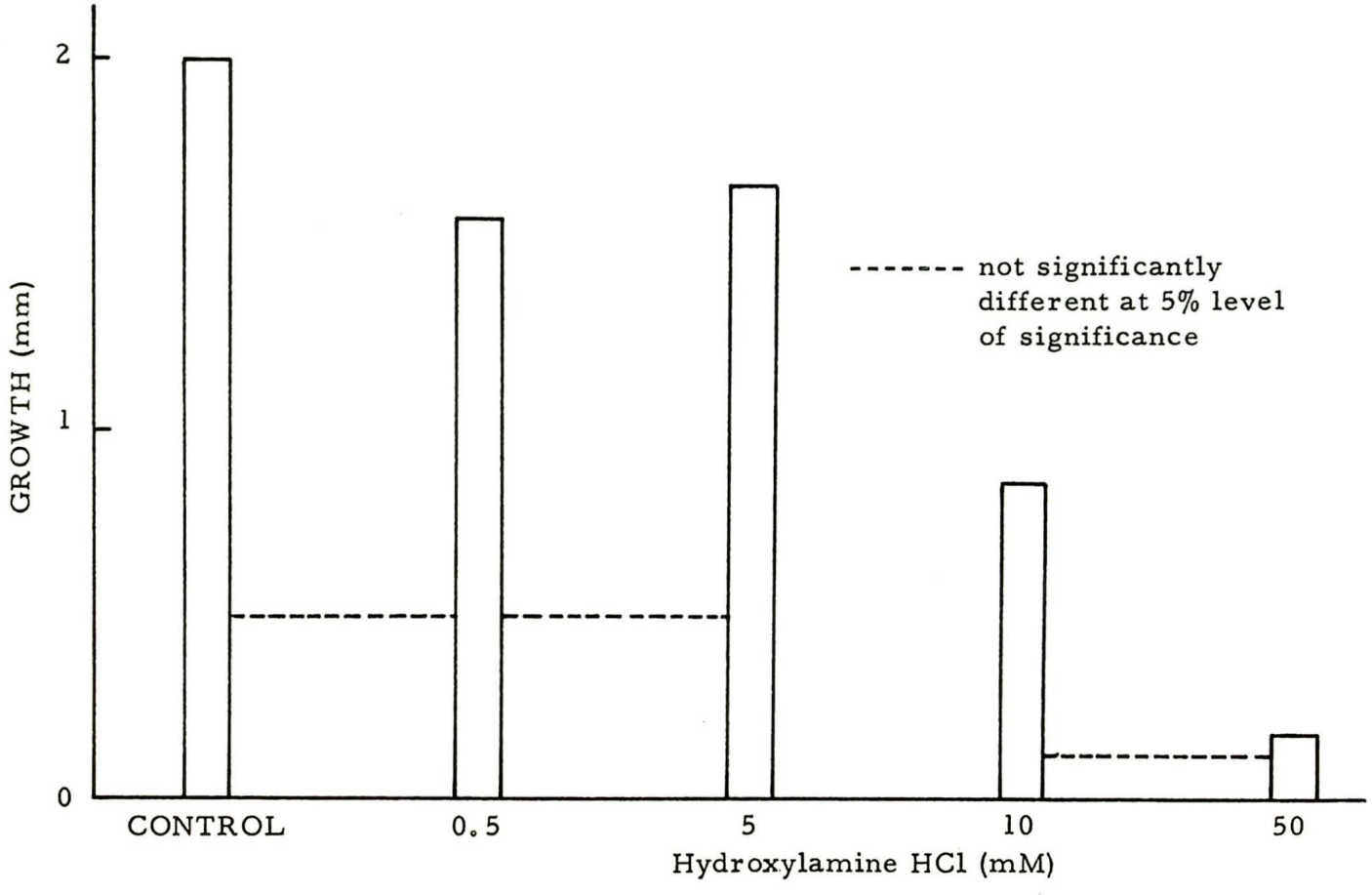

Figure 5. Effect of hydroxylamine on elongation of epicotyl sections incubated for 48 hours.

Table II

Effect of 8-Hydroxyquinoline
on Elongation of
Excised Epicotyl Sections

Treatment	Percent of Control
CONTROL	100
8-HQ 0.1 mM	92*
8-HQ 0.5	69*
8-HQ 1.0	128*
8-HQ 5.0	13
8-HQ 10.0	5
8-HQ 50.0	0

*The means do not differ significantly from the control at the 5% level of significance. Measurements were made after 48 hours growth. 8-Hydroxyquinoline concentration is expressed in mM.

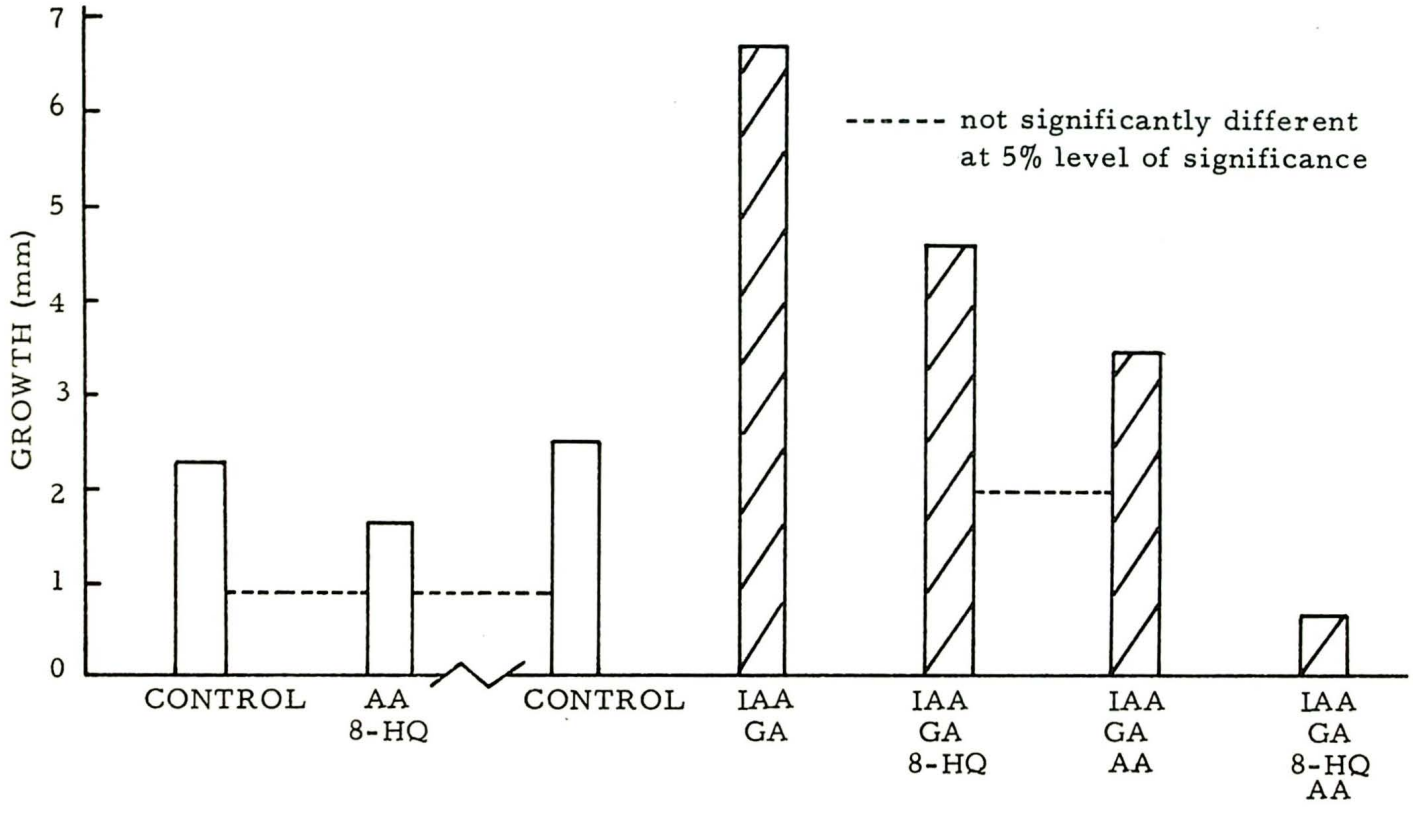

Figure 6. Effect of inhibitors of electron transport on hormone-induced elongation of epicotyl sections incubated for 48 hours. Data is the mean of replicate experiments. IAA and GA were present at 10 μM and 10 ppm respectively. Antimycin A and 8-Hydroxyquinoline were present at 5 μM and 1 mM respectively. Crosshatched bars represent inclusion of both IAA and GA.

antimycin A and 8-hydroxyquinoline, at concentrations of 5 μM and 1 mM respectively, in combination had no effect on elongation when compared to the control. The results observed when including these inhibitors with the growth hormones were quite different. The inhibitors had a profound effect on the hormone-enhanced elongation. Hormone-enhanced elongation was inhibited with 8-hydroxyquinoline 1 mM by 32% and with antimycin A 5 μM by 48%. The presence of either inhibitor of electron transport significantly inhibited hormone-enhanced growth. The inhibition of elongation caused by 8-hydroxyquinoline was not significantly different from the inhibition caused by antimycin A. When both 8-hydroxyquinoline and antimycin A were included, the hormone-enhanced elongation was inhibited by 92%. Growth in hormone treated tissue in the presence of both inhibitors of electron transport was significantly less than growth in tissue treated with either of the inhibitors separately (Figure 5).

Effect of ATP on Elongation in Tissue Treated with Inhibitors of Electron Transport

The electron transport system in the mitochondria is coupled with oxidative phosphorylation. Adenosine triphosphate (ATP) is produced by these reactions. In the next experiment, ATP was added to tissue treated with inhibitors of electron transport to determine the role of oxidative phosphorylation in hormone-enhanced elongation (Figure 7). ATP, at concentrations of 0.01, 0.1, 1.0, and 5.0 mM,

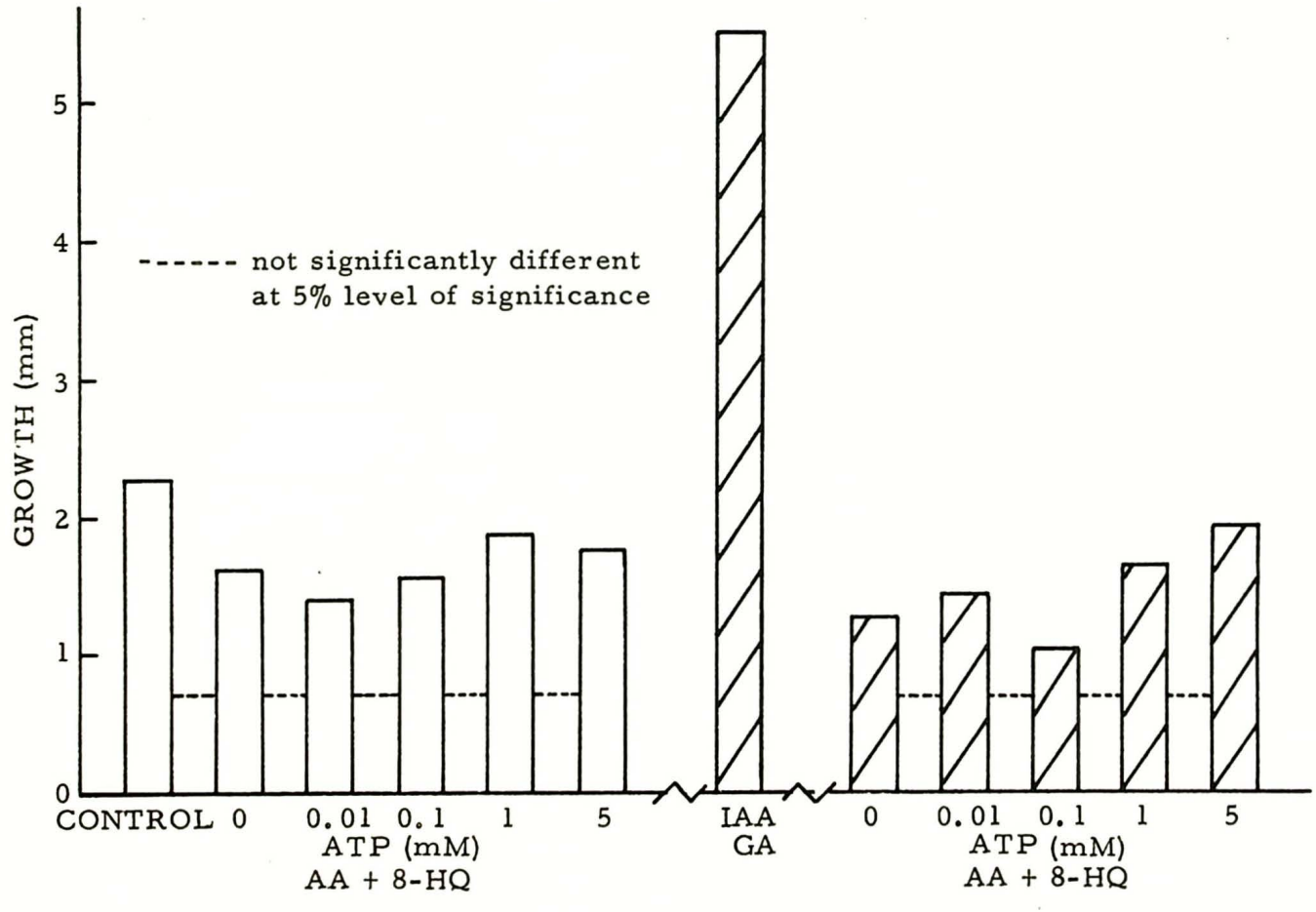

Figure 7. Effect of ATP on elongation of epicotyls with inhibitors of electron transport and plant growth substances. Tissue was incubated for 24 hours. Data is the mean for replicate experiments. Crosshatched bars represent treatments in the presence of IAA and GA at 10 μM and 10 ppm respectively. Antimycin A and 8-Hydroxyquinoline were present at 5 μM and 1 mM respectively.

does not significantly affect elongation in tissue treated with antimycin A and 8-hydroxyquinoline, nor does ATP at these concentrations effect epicotyl elongation in tissue treated with the hormones and inhibitors of electron transport. Adenosine triphosphate, at the concentrations used, does not reinstate elongation in tissue treated with hormones and inhibitors of electron transport.

Time Course Study of Hormone-Enhanced Elongation

A study was conducted to ascertain when hormone-enhanced growth occurs (Figure 8). This experiment was carried out by adding 8-hydroxyquinoline and antimycin A to hormone treated tissue at 0, 1.5, 3, 6, and 10 hours after the start of the experiment. Elongation measurements were made at 24 hours. The data indicates that hormone-enhanced elongation had begun by 6 hours and was essentially complete by 10 hours. No significant growth occurred during the final 14 hours of the experiment. In this 24 hour study the hormone stimulation of growth occurred between 0 and 10 hours.

Respiration Studies of Tissue Treated with Hormones, Inhibitors of Electron Transport, and ATP

This experiment was designed to observe the effects of plant growth substances and inhibitors of electron transport on the rate of respiration. The results in Table III indicate that 10 hours after the start of the experiment, the tissue treated with hormones was respiring

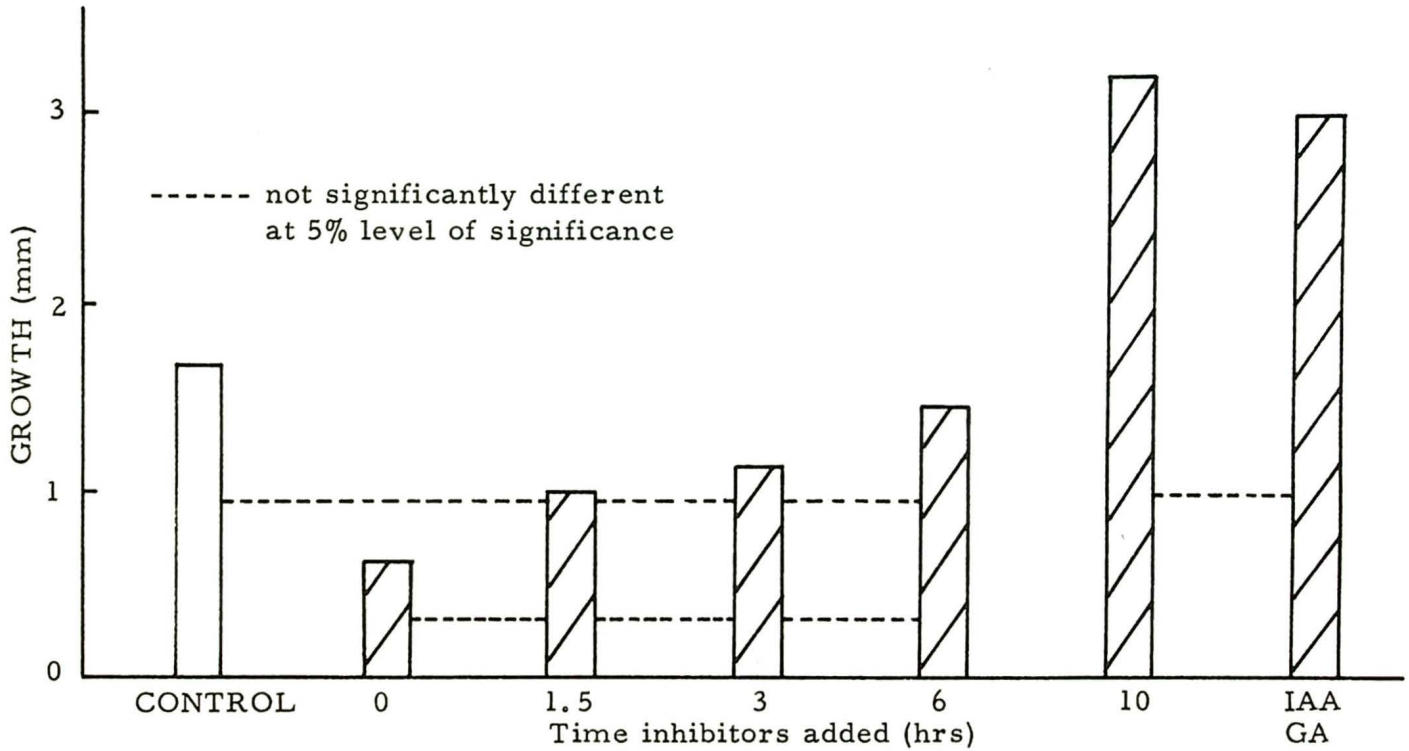

Figure 8. Time course of hormone-induced elongation in 24 hour growth period. Inhibitors of electron transport were added at indicated time after start of experiment. Data is the mean of replicate experiments. All treatments contain IAA 10 μM; GA 10 ppm; AA 5 μM; 8-HQ 1 mM. Cross-hatched bars represent inclusion of both IAA and GA.

Table III

Effects of Plant Growth Substances and Inhibitors of Electron Transport on Rate of Respiration

Treatment	Oxygen Consumption (ul/gram/30 min) 10 hours	24 hours
CONTROL	53.9	90.5
IAA + GA	172	50.9
IAA + GA + AA + 8-HQ	56.4	0
IAA + GA with inhibitors added at indicated time after start of experiment		
1.5 hrs.	39.9	0
3.0 hrs.	116.5	0
6.0 hrs.	160.1	0
10.0 hrs.	102	0

Respiration measurements were taken at 10 and 24 hours after replicate treatments of epicotyl sections had been incubated. Oxygen consumption is based on tissue respiration measured for 60 minutes. IAA and GA were present at concentrations of 10 µM and 10 ppm respectively. AA and 8-HQ were present at concentrations of 5 µM and 1 mM respectively.

faster and consuming 3.25 times as much oxygen as the untreated tissue. At 24 hours oxygen consumption in the control was almost two times higher than in the hormone treated tissue. Although the hormones stimulated the respiratory rate at 10 hours, this stimulation was short-lived and oxygen consumption fell below the control value by 24 hours. In all cases the inhibitors of electron transport decreased oxygen consumption and by 24 hours all tissue respiration had ceased.

An experiment was conducted to determine the effects of ATP on the rate of respiration (Table IV). The results indicate that added ATP, at concentrations of 0.001, 0.01, 0.1, 1.0, and 5.0 uM, does not seem to alter the rate of respiration in tissue when compared to the control. In tissue treated with the hormones and inhibitors of electron transport, added ATP does not affect the depressed respiratory rate. The high respiration rates in tissue treated with hormones alone are not reinstated by the addition of ATP, 0.001-5.0 mM, in tissue treated with hormones and respiratory inhibitors.

The Effects of a Lectin on Hormone-Enhanced Elongation

The cell membrane is a lipid bilayer that contains proteins and glycoproteins with sugar residues that protrude from the outer surface (Marchesi, 1975; Roseman, 1975; Capaldi, 1974). Lectins (phytohemagglutinins) bind with saccharides and saccharide-containing proteins. Each lectin binds specifically to certain sugar residues (Lis,

Table IV

Effect of ATP on Rate of Respiration
in Tissue Exposed to Plant Growth
Substances and Inhibitors of Electron Transport

Treatment	Oxygen Consumption (ul/gram/min)
CONTROL	148.4
ATP 0.001	137.5
ATP 0.01	145.1
ATP 0.1	137.5
ATP 1.0	146.8
ATP 5.0	133.8
IAA + GA	99.1
HORMONES + INHIBITORS and ATP 0.001	31.5
ATP 0.01	27.0
ATP 0.1	32.9
ATP 1.0	15.9
ATP 5.0	34.9
AA + 8-HQ + IAA + GA	28.4

Respiration rates were determined after 10 hours growth in the specified solution. Oxygen consumption is based on measurements made over a 60 minute period of time. All GA and IAA concentrations were 10 μM and 10 ppm respectively. ATP concentration is in mM.

1973; Liener, 1976; Sharon, 1977). Certain lectins have been shown to bind to glycoprotein receptors on the cell membrane in mammalian systems and to displace the hormone insulin which competes for these same binding sites (Cuatrecasas, 1974).

An experiment was set up to determine what effect lectins might have on hormone-enhanced elongation in the pea stem. Some preliminary results suggested that concanavalin A was more effective in pea stem tissue than wheat germ agglutinin. The lectin concanavalin A (Con A) which binds specifically to glucose, mannose, and n-acetyl-D-glucosamine, was used in this experiment. The data indicate that Con A at 50 and 100 ppm does not interfere with epicotyl elongation as compared to the control. In tissue treated with the hormones GA and IAA and Con A 100 ppm, elongation was significantly less than in tissue treated with the hormones alone; whereas, with Con A 50 ppm elongation was not significantly different from the hormone treated tissue (Figure 9). Mannose at concentrations known to inhibit Con A (Goldstein et al., 1965) was added to the incubation medium to observe the effects on hormone-enhanced elongation in the presence of Con A. When mannose 54 mM was included with the hormones and Con A 100 ppm, elongation was not different from that of hormone treated tissue, but was significantly different from tissue treated with hormones and Con A 100 ppm. An enhancement of epicotyl elongation was observed in hormone treated tissue in the presence of mannose (27 and 54 mM)

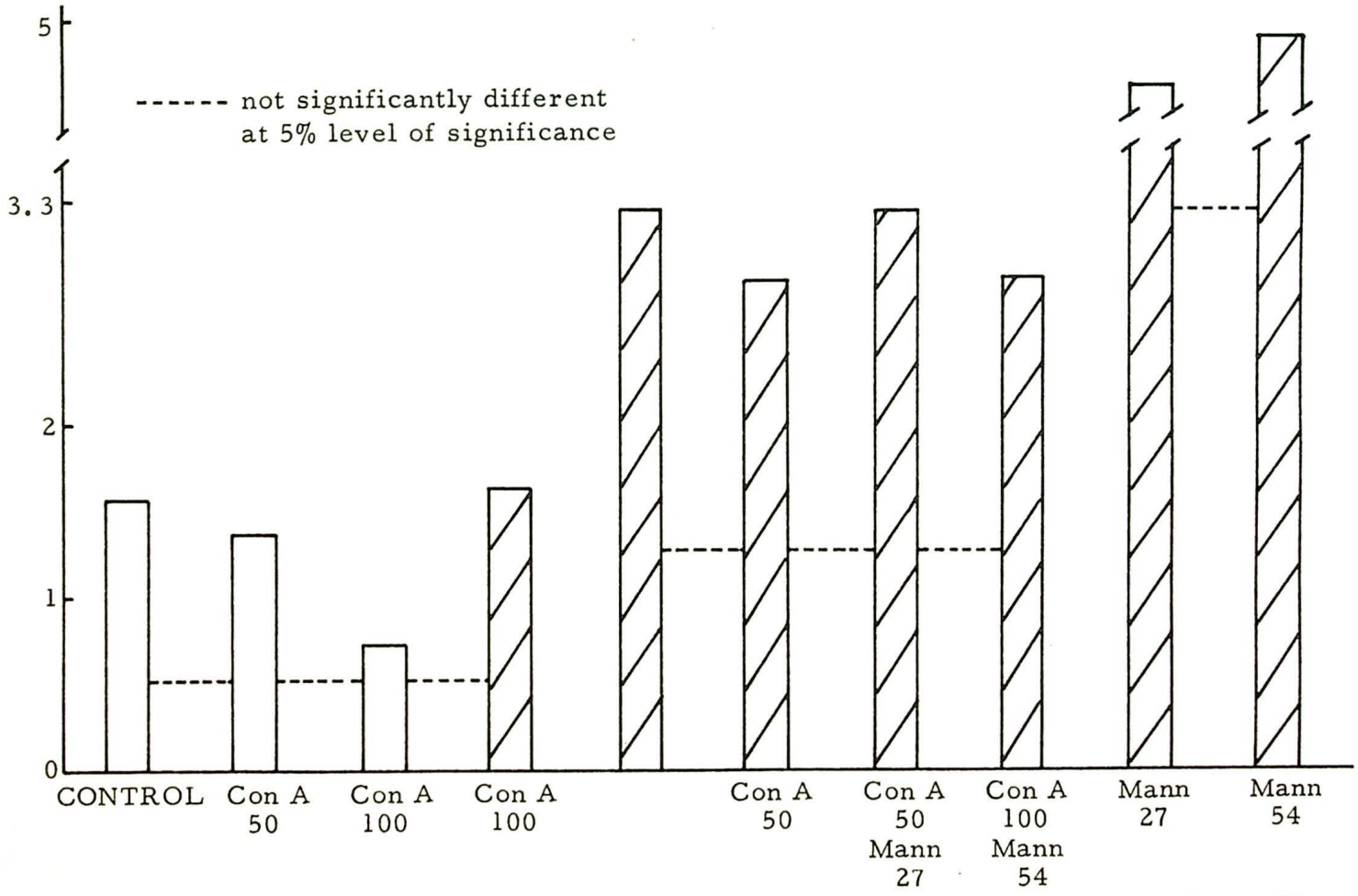

Figure 9. Effect of Concanavalin A on hormone-induced epicotyl elongation and the effect of mannose in reversal of the Concanavalin A inhibition. Data is the mean of replicate treatments incubated 24 hours. Crosshatched bars represent treatment in the presence of IAA 10 μM and GA 10 ppm. Mannose concentration is in mM. Concanavalin concentration is in ppm.

and this enhancement was significantly greater than elongation in tissue treated with hormones alone.

Chapter V

DISCUSSION

The data in Figures 1 and 2 indicate that elongation of excised epicotyl sections from dark grown Pisum sativum variety "Little Marvel" is greatly stimulated by indoleacetic acid (IAA). Indoleacetic acid at both 10 and 100 μM concentrations stimulates growth of epicotyl sections incubated for 48 hours under light in the presence of 3% (w/v) sucrose. This observation is supported by the work of many other investigators although the experimental conditions varied with respect to the variety of pea used, concentration of sucrose in the incubation medium, quality and intensity of light, location on the seedling from which the sections were excised, and section length (Galston and Hand, 1949; Purves and Hillman, 1958; Kato, 1958; Brian and Hemming, 1958; Ockerse and Galston, 1967; Russell and Galston, 1969; Barkley and Evans, 1970; Murayama and Ueda, 1973).

The inclusion of sucrose in the incubation medium enhances elongation of etiolated sections and potentiates IAA-stimulated elongation (Galston and Baker, 1951; Brian and Hemming, 1958). Purves and Hillman (1958) found that the elongation response to sucrose of etiolated sections was greater in short (5 mm) sections than in long 10 mm) sections and all light grown sections responded to sucrose.

The role of light in IAA induced elongation of etiolated epicotyl sections is discussed by Russell and Galston (1969) who found that optimum concentrations of IAA in light-incubated sections is fifty times higher than in dark-incubated sections. They suggest that phytochrome may act to regulate the synthesis of a compound that stimulates the IAA-oxidase system.

Purves and Hillman (1958) found that the length of epicotyl section used and the distance from the apex of that section lead to differential elongation responses induced by IAA. Fan and Maclachlan (1967) showed that IAA stimulates cell division and swelling of parenchyma cells in the pea epicotyl.

Gibberellic acid (GA), at concentrations of 1 and 10 ppm, leads to variable responses in the epicotyl sections (Figures 1 and 2). In no instance was the GA-stimulated elongation near the magnitude of the IAA-enhanced growth. Kato (1956) observed that GA stimulates elongation, increases water uptake and increases respiration in pea stem sections. Ockerse and Galston (1967) found that although both tall and dwarf varieties of peas show a small elongation response to GA, the dwarf variety responds to a lesser concentration. These investigators also found that GA has a greater effect on etiolated sections than on green sections. In intact plants GA stimulates cell division and the resulting elongation is a secondary effect (Arney and Mancinelli, 1966). Gibberellic acid does not lead to great increases in elongation of epicotyl sections because of the absence of meristematic tissue of the

apex. The importance of this was described by Kuraishi and Muir (1962) who found evidence of a promotion of diffusible auxin levels in intact peas treated with GA.

All concentrations of IAA and GA when used in combination stimulated section elongation signficantly when compared with either of the growth substances applied separately (Figures 1 and 2 and Table I). This interaction enhanced elongation of the epicotyl sections and the total growth was simply additive at the most. Synergism was not observed. These results are in agreement with those obtained by Kato (1958), Purves and Hillman (1958), and Galston and Warburg (1959). Other investigators have found that green sections from dwarf varieties of peas respond synergistically to combined GA and IAA. The synergism is not as pronounced in tall variety pea stem sections (Ockerse and Galston, 1967; Brian and Hemming, 1958).

Brian and Hemming (1958) noted that GA would stimulate elongation only if auxin was also present in the tissue and that this might be attributed to the effect on a growth inhibiting factor by the gibberellin. Galston and Warburg (1959) also noted this relationship and proposed that GA may inhibit light-induced auxin destruction by halting an early event in the phytochrome mediated synthesis of compounds which act as cofactors in the IAA-oxidase system.

The work of Fan and Maclachlan (1967) showed that IAA promotes the synthesis of enzymatic protein, specifically cellulase, in

pea epicotyl segments. It would seem likely that the sequence of events in hormone stimulated elongation would be a sparing action by GA on IAA and, by some still unknown mechanism, the regulation of some biosynthetic pathway by IAA. Thimann (1974) has suggested that IAA may effect both the cell membrane and the nucleus or a nuclear product.

The role of the electron transport system in auxin stimulated elongation of Avena coleoptiles was examined by Bonner (1933). He demonstrated that potassium cyanide inhibited auxin stimulated elongation. James and Beevers (1950) observed cyanide-resistant respiration in Arum spadices and since then, the existence of cyanide-resistant respiration has been domonstrated in many higher plants (Floyd and Rains, 1971) and in many microorganisms (Sherald and Sisler, 1972; Sherald and Sisler, 1970; Kawakita, 1970). Bonner (1965) has postulated that perhaps all higher plants have the capacity for cyanide-resistant respiraton. The flow of electrons in the cyanide-resistant pathway is believed to divert from the cyanide-sensitive chain of electron carriers on the substrate side of cytochrome b (Solomos, 1977; Bendall and Bonner, 1971). Tomlinson and Moreland (1975) have suggested that the branch point occurs at ubiquinone or a non-heme iron-sulfur protein. This alternate pathway of electron transport involves an oxidase other than cytochrome oxidase and only the first site of energy conservation is operational in cyanide-resistant respiration (Solomos, 1977).

Antimycin A, a fungal metabolite, blocks the cyanide-sensitive pathway of electron transport at the cytochrome b complex (Storey and Barh, 1969). The inhibition of electron transport by antimycin A is of the same magnitude as the inhibition by cyanide (Bendall and Bonner, 1971). In tissues that exhibit cyanide-insensitive respiration the electrons are diverted, by antimycin A, to the alternate oxidase. The last two sites of oxidative phosphorylation are thus bypassed (Floyd and Rains, 1971).

The data in Figures 3 and 4 indicate that antimycin A at concentrations of 1, 2 and 5 μM does not significantly interfere with pea epicotyl section elongation. Antimycin A at concenerations of 10 μM and greater significantly decreased elongation of the epicotyl sections. These results suggest that the cyanide-sensitive electron transport chain must function for epicotyl section elongation to occur.

In another study the effects of hydroxylamine, an inhibitor of the cyanide-sensitive pathway, on elongation of pea stem sections were observed (Figure 5). Hydroxylamine at concentrations of 10 mM and greater significantly inhibited elongation but at 0.5 and 5 mM concentrations elongation was not inhibited. Further experimentation with hydroxylamine was suspended because of the literature descriptions that questioned if hydroxylamine actually blocked electron flow or if it only inhibited succinate oxidation (Wilson and Brooks, 1970). Antimycin A, a specific inhibitor of electron flow, was used in subsequent studies

when the cyanide-sensitive pathway was to be blocked.

The chelator 8-hydroxyquinoline specifically blocks electron flow through the alternate pathway (Bendall and Bonner, 1971; Sherald and Sisler, 1972). The data (Table III) show that 8-hydroxyquinoline at concentrations of 0.1, 0.5 and 1.0 mM do not inhibit epicotyl section elongation but that concentrations of 5, 10, and 50 mM significantly interfere with elongation.

In subsequent experiments, designed to illustrate the effects of inhibitors of electron transport on hormone-enhanced epicotyl section elongation, the inhibitors of electron transport were used at concentrations that did not interfere with epicotyl section elongation when used singly. These concentrations were, antimycin A 5 µM and 8-hydroxyquinoline 1 mM. These levels would best reveal the role of electron transport in hormone-stimulated elongation of epicotyl sections. The overriding effects of inhibitory concentrations were thus avoided. The results (Figure 6) indicate that both inhibitors together did not inhibit elongation in tissue when compared with the control tissue. However, in tissue incubated in the presence of both plant growth substances, the inhibitors of electron transport had a drastic effect on hormone-enhanced elongation of the stem sections. Hormone-enhanced section elongation was inhibited with antimycin A and 8-hydroxyquinoline by 48% and 32% respectively. In tissue treated with both inhibitors of electron transport, elongation was decreased

by 92% as compared to tissue incubated in the presence of the growth substances alone.

Inhibition of electron transport by antimycin A causes the electrons to move to oxygen via the alternate pathway thus bypassing the last two sites of energy conservation (Floyd and Rains, 1971). Thimann (1974) has stated that oxidative phosphorylation may be required for auxin stimulated elongation but this may not be the primary site of auxin action. The results of an experiment which was carried out to observe the effects of adenosine triphosphate (ATP) on elongation of epicotyl sections in the presence of inhibitors of electron transport and hormones (Figure 7) illustrate that ATP is not effective in reinstating elongation. These data indicate that the energy of ATP, from oxidative phosphorylation, is not a limiting factor required for the hormone-enhanced elongation of epicotyl sections treated with inhibitors of electron transport. Hormone-enhanced elongation of pea stem sections may depend upon metabolic events which require oxidized substrates that are available through operation of the electron transport pathways. The alternate pathway may function to oxidize these intermediates when ATP levels are high. Bendall and Bonner (1971) draw the conclusion that the occurrence of an alternate pathway for electron transport is associated with tissue that is rapidly undergoing growth. Such is the case with the epicotyl sections used in this study as they were excised just below the apical hook and possess a

high potential for rapid growth. Other investigators (Tomlinson and Moreland, 1975; Palmer, 1976; Solomos, 1977) have suggested reasons similar to this when speculating as to the physiological purpose of the less phosphorylating alternate pathway of electron transport. All of these investigators suggest that under conditions of high ATP levels, pathways of biosynthesis require oxidized substrates and that the oxidation of these substrates will not occur via the cyanide-sensitive pathway because ATP levels limit electron flow through this pathway. Consequently, the alternate pathway of electron transport in hormone-enhanced growth response in pea epicotyls may exist as an important alternative for supplying oxidized substrates required for biosynthetic pathways supportive of hormone enhanced growth.

Results of a 24 hour time course study of hormone-enhanced elongation are shown in Figure 8. The data indicate that elongation of tissue treated with both plant growth substances is not significant until sometime after six hours and is essentially complete by ten hours. These findings are in agreement with those of Galston and Hand (1949) and Ockerse and Galston (1967). These experiments were necessary preliminary experiments to identify the effective hormone response time that was used in subsequent oxygen utilization studies.

Experiments were conducted to determine the rate of respiration in tissue treated with plant growth substances and inhibitors of electron transport. The data (Table III) indicate that after ten hours,

tissue incubated in the presence of both plant growth substances was consuming oxygen at a rate 3.25 times as fast as tissue in the control solution. This indicates an accelerated electron flow to oxygen in hormone treated tissue thereby providing oxidized intermediates required for biosynthetic pathways associated with rapid growing tissue. After 24 hours the tissue in the presence of growth substances was respiring at a rate only slightly greater than one-half of that of control tissue. This data is supported by the results of previous experiments involving time course studies where it was shown that hormone-enhanced elongation was complete by ten hours and no further elongation occurred thereafter. All tissues treated with electron transport inhibitors and with hormones consumed less oxygen than tissues incubated in the growth substances alone. After 24 hours all tissue treated with inhibitors of electron transport had stopped respiring.

In experiments designed to determine the effects of ATP on rate of respiration in tissue treated with growth substances and inhibitors of electron transport (Table IV) the results suggest that ATP, at the concentrations used, does not alter the respiratory rate as compared with control tissues. These results contradict the predicted response when viewed with the classical Pasteur effect in mind. This effect accounts for decreased respiratory rates when ATP is present in adequate amounts. Added ATP did not greatly effect the respiratory

rate in tissue treated with hormones and electron transport inhibitors (Table IV). These results suggest that hormone-accelerated electron flow (Table III) is providing oxidized intermediates required for growth rather than energy of ATP required for elongation processes.

In another study the effects of a lectin, concanavalin A, on hormone-enhanced elongation of pea stem sections were observed. The data (Figure 9) indicate that concanavalin A, at a concentration of 100 ppm, significantly inhibits hormone-enhanced elongation but does not interfere with elongation in tissue incubated without growth hormones. Concanavalin A binds specifically to mannose, glucose, and n-acetyl-D-glucosamine (Sharon, 1977; Liener, 1976). Cell membrane receptors in mammalian systems will bind certain lectins and the lectins can be eluted when the lectin specific sugar is added (Cuatrecasas, 1974). In an attempt to study the interaction of plant growth hormones and possible receptor molecules, the sugar mannose was added to tissue incubated in the presence of plant growth substances and concanavalin A. The data indicates that mannose reinstates elongation and the final growth is no different from that of tissue incubated in plant hormones alone. Mannose potentiates the hormone-enhanced elongation of pea stem sections and this stimulation is significant.

In mammalian systems receptor molecules have been demonstrated for polypeptide and catecholamine hormones, acetylcholine, prostaglandins, and many other biologically active chemicals

(Bengelsdorf, 1976). The mode of action of some mammalian hormones has been described (Cuatrecasas, 1974; Bengelsdorf, 1976). When hormone binds to specific receptors on the cell membrane, enzymes located in the membrane are activated. The activation of adenylate cyclase leads to production of cyclic-AMP from ATP inside the cell. Cyclic-AMP then activates a protein kinase which catalyzes other reactions that alter metabolism.

No plant hormone receptor has been isolated (Kende and Gardner, 1976). No gibberellic acid was observed to bind to cell membranes in experiments with dwarf peas (Kende, 1976). Auxin has been found to bind to cell membranes in higher plants (Poovaiah and Leopold, 1976; Dollstadt et al., 1976). Various effects of plant growth substances on cell membranes have been noted. Wood and Paleg (1972) showed that GA increased the permeability of model membranes. Helgerson et al. (1976) demonstrated that auxin application causes increases in soybean membrane microviscosity. Auxin treatments lead to changes in soybean membranes and the result of these changes is that membrane thickness is decreased by 10-15% (Morrè and Bracker, 1976). Earle and Galsky (1971) noted that cyclic-AMP mimics the effect of GA in barley endosperm by stimulating production of ATPase. In the investigations of plant hormone action, experiments have failed to show any activity of adenylate cyclase in plant cell membranes and no cyclic-AMP has been unequivocally

demonstrated to occur in these membranes (Yunghans and Morré, 1977).

The results of these experiments seem to indicate that plant hormone action involves receptor molecules on the membrane that react with concanavalin A and are mannose specific. However, Lis (1973) has stated that lectin inhibition studies with specific sugars do not necessarily indicate that the membrane receptors contain the identical saccharide residues.

Chapter VI

SUMMARY

This study was undertaken to: 1) determine the effects of various concentrations and combinations of gibberellic acid and indoleacetic acid on elongation of epicotyl sections in Pisum sativum L. variety "Little Marvel"; 2) study the effects of various inhibitors of electron transport on hormone-enhanced epicotyl elongation; 3) examine the response of tissue to respiratory inhibitors and added adenosine triphosphate; 4) observe the respiratory rate of tissue treated with combinations of inhibitors and growth substances; 5) analyze the growth response of epicotyl sections to plant growth hormones and the lectins concanavalin A and wheat germ agglutinin.

The data indicate that both gibberellic acid and indoleacetic acid stimulate elongation in pea epicotyl sections. Indoleacetic acid is more effective in enhancing elongation than is GA. When IAA and GA are applied in combination, elongation is stimulated and growth is greater than that obtained with either of the growth substances when applied separately.

Both antimycin A and 8-hydroxyquinoline inhibit hormone-enhanced epicotyl section elongation and a combination of the two is extremely inhibitory. These results indicate that the alternate path-

way of electron transport is required for hormone-enhanced elongation. This pathway may function to oxidize substrates that are required for biosynthetic pathways.

Adenosine triphosphate is not effective in reinstating elongation in tissue treated with plant growth substances and respiratory inhibitors. It would seem that the energy of ATP is not necessary for hormone-stimulated elongation.

Adenosine triphosphate does not effect the rate of respiration in pea epicotyl sections, nor does ATP effect the rate of respiration in tissue treated with hormones and inhibitors of electron transport. The inhibitors of electron transport inhibit oxygen consumption.

The lectin concanavalin A interferes with hormone-enhanced elongation of epicotyl sections. This inhibition is overcome when mannose is included with the concanavalin A. This data indicates that at least one of the plant growth substances is acting on the cell membrane and that a mannose specific receptor molecule may be involved.

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