M.Sc. ABSTRACT Biology

HORMONAL SPECIFICITY FOR REGULATION OF CELLULASE ACTIVITY AND GROWTH IN THE PEA EPICOTYL

Beverley Dorothy Bonn

Various potential plant growth regulators were added at different concentrations, alone and in combinations, to the apex of decapitated pea epicotyls (Pisum sativum L. var. Alaska). At suitable time intervals, measurements were made in the tissue immediately below the apex of length, fresh weight and levels of protein, nucleic acids and the enzyme cellulase (β-1,4 glucan 4-glucanohydrolase, EC 3.2.1.4).

The results led to the conclusions that cell expansion in the pea epicotyl is limited by at least four hormone-regulated reactions: one controlled by gibberellic acid is necessary for elongation, and three others controlled by auxin, cytokinin and ethylene gas, all lead to lateral expansion. Swelling is always accompanied by some increase in cellulase activity, but massive unilateral increments (induction) of this enzyme only occur following treatments with active auxins. Herbicides (e.g. 2,4-D) are especially effective at evoking cellulase activity.

M.Sc. Biology

Beverley Dorothy Bonn

Suggested Short Title:

"Hormonal Specificity for Growth and Cellulase Induction"

HORMONAL SPECIFICITY FOR REGULATION OF CELLULASE ACTIVITY AND GROWTH IN THE PEA EPICOTYL

by

Beverley Dorothy Bonn

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Department of Biology
McGill University
Montreal, Canada

July, 1970

ACKNOWLEDGMENTS

I wish to express my sincere thanks and appreciation to Dr. G.A. Maclachlan for his guidance and enthusiastic encouragement during these studies and also for his help in preparing this manuscript. Thanks are also due to Dr. M. V. Roscoe for her continued interest in my progress and to my fellow graduate students and summer students for their valuable suggestions, assistance and friendship.

I wish also to thank McGill University for the award of McConnell Memorial Fellowships in 1968-1970. Financial support for supplies and equipment used in this research was provided by the National Research Council of Canada.

TABLE OF CONTENTS

LIST OF TABLES

CONTENTS OF APPENDIX

INTRODUCTION

The enzyme, cellulase (β-1,4 glucan 4-glucanohydrolase, EC 3.2.1.4) occurs widely in higher plants where it is highly localized in particular tissues (Tracey, 1950; Clarke and Stone, 1962, Reese and Mandels, 1963; Fan, 1967). Thus, for example, the level of extractable cellulase activity is especially high in young tissues, such as in the apex of the pea epicotyl (Maclachlan and Perrault, 1964), where natural auxin-type growth hormones, e.g. indoleacetic acid (IAA), are concentrated (Scott and Briggs, 1963). This distribution may not be coincidental. Studies over the past five years have established clearly that this particular hormone can regulate the amount of cellulase activity in the epicotyl and probably also in other young tissues.

The addition of IAA to the apex of decapitated pea epicotyls results in swelling and, concurrently, a great increase occurs in the amount and specific activity of cellulase (Fan and Maclachlan, 1966). Other glucanases and pectic enzymes are not affected to the same degree (Datko and Maclachlan, 1968). The cell expansion and increase in cellulase activity are both prevented by inhibitors of RNA or protein synthesis, but not by inhibitors of DNA or cell division (Fan and Maclachlan, 1967b). Cellulase increments after IAA treatment are detectable before swelling occurs in a subfraction of the epicotyl tissue which

contains microsomes (Davies and Maclachlan, 1968). This fraction is capable of carrying out protein synthesis *in vitro* and, during the reaction, cellulase activity rises in the preparation (Davies and Maclachlan, 1969). Accordingly, it has been suggested that IAA induces cellulase in etiolated peas by a derepression mechanism, and that activity of this enzyme in cell walls *in vivo* is required for cell expansion (Fan and Maclachlan, 1967a; Maclachlan *et al.*, 1968).

The present study was concerned with the question of whether or not swelling in the pea epicotyl and high cellulase activity were necessarily related events, and also whether or not these responses were specific to the auxin-type of hormone. Growth reactions and enzyme levels were examined after treatments with a variety of indole derivatives and other auxins, both naturally-occurring and synthetic. Some of these substances, e.g. 2,4-dichlorophenoxyacetic acid, were herbicides and lethal to peas. Tests were also carried out on effects of other classes of plant growth regulators, added alone or in combinations. These included gibberellic acid (GA), which generally causes elongation, cytokinins, which usually stimulate cell division, and ethylene gas, which evokes destructive or aging processes, e.g. during fruit ripening, senescence, and abscission. The latter regulator is generated in plants, including the pea epicotyl, wherever auxin levels are high (Abeles and Rubinstein, 1964). Several authors have suggested that ethylene may affect growth by stimulating activity of cellulase and other poly-

saccharidases (Horton and Osborne, 1967; Abeles, 1969). It has even been proposed (Burg and Burg, 1968) that all effects of IAA in the pea epicotyl are mediated via ethylene.

Preliminary investigations in this laboratory on effects of GA, kinetin and ethylene gas in the decapitated pea epicotyl (Fan, 1967; Datko, 1968; Davies and Maclachlan, 1968) failed to demonstrate any inductive-type reactions by the enzyme cellulase. A recent study using this same tissue system (Ridge and Osborne, 1969) showed that ethylene (500 ppm) inhibited the development of IAA-evoked cellulase activity. No efforts were made, however, to study effects of various concentrations of these regulators, or to combine them with one another or with IAA, in spite of the fact that optimal concentrations have been noted for growth responses in other systems, and complex interactions between regulators are well established (Galston and Davies, 1969). Accordingly, in the present study, a survey has been made of interactions and concentration requirements of the main classes of plant growth hormones for effects on length, fresh weight and cellulase activity in the decapitated pea epicotyl.

LITERATURE REVIEW

A. The decapitated pea epicotyl

The upper region of the etiolated pea seedling (Pisum sativum L.) is characterized by a slim shaft terminated by a meristematic plumule and hook. The shaft after about nine days growth contains two dormant nodes and three internodes. Priestly (1926), Gourley (1931), Scott (1938) and Fan (1967) have all described the microscopic anatomy of such seedlings.

Throughout the epicotyl, the epidermis and cortex surround an inner stele of four vascular bundles. Two separate vascular bundles in the cortex supply the nodes. Two strands of sclerenchyma also occur in the cortex. The first three internodal areas consist of a root-stem transition zone, i.e. the shaft structure is an epicotyl and not a stem due to the presence of exarch, as well as endarch, vascularization and the possession of an endodermis. The exarch bundles in the third internode supply the scale leaves at the node and the plumular apparatus. The endodermis is not fully developed in the youngest (third) internode where the vascular bundles are surrounded by a starch sheath. During maturation the starch disappears and Casparian strips appear.

The elongation zone of the pea epicotyl is confined to a region about 10 mm long just below the apical hook (Young, 1962). Early work by Thimann and Skoog (1933) showed that removal of the plumule and hook ("decapitation") resulted in

early cessation of growth of the internode. They concluded that natural hormones were generated by meristematic cells in the apex and transported to the epicotyl shaft where they were required for cell elongation.

The meristematic tissue in the plumule and hook is not only the site of cell division, but also the site of the highest accumulation and synthesis of natural auxin (van Overbeek, 1938; Scott and Briggs, 1963). Gibberellins are also found in apical regions of etiolated pea seedlings, but it has not been established that their synthesis is confined to meristematic regions (Jones, 1968; Jones and Lang, 1968). Natural cytokinins are sometimes considered to be produced mainly by meristematic regions of roots (Street, 1966) but they definitely occur in, and may be formed by, other plant organs (Letham, 1967). A cytokinin has been detected in extracts of pea seedlings attached to t-RNA (Hall, 1968). Ethylene gas is unquestionably formed by pea epicotyls, particularly in meristematic regions (Goeschl and Pratt, 1968). The gas is generated by apical sections of pea epicotyls following treatment with high concentrations of auxins (Burg and Burg, 1968) or kinetin but not GA (Fuchs and Lieberman, 1968). Nevertheless, GA treatment can sometimes stimulate release of ethylene gas from other tissues, e.g. bean explants (Abeles and Rubinstein, 1964). In fact, there is evidence (Goeschl et al., 1966; Imaseki et al., 1968) that any treatment that caused "stress" or wounding of plant tissue can lead to release of ethylene.

Treatment of the cut pea epicotyl apex with auxins in lanolin paste at concentrations of from 5 to 10,000 ppm in the paste evokes a large increase in fresh weight per unit length (swelling) of the tissue immediately below compared to responses in untreated controls (Scott, 1938; MacQuarrie, 1965; Fan, 1967). There is no enhancement of elongation under these conditions, even at low levels of applied auxin. Most of the initial swelling occurs in the parenchyma cells of the cortex. By the end of one day of treatment, if relatively high levels of IAA had been used, (e.g. 5,000 ppm = approx. 10 µg/epi), cell divisions can be seen in the vascular system. By three days it is clear that the masses of new cells are organizing into root primordia. After two to four days of treatment, "lacunae" develop in the cortex as a result of cell wall fragmentation and protoplast dissolution (Fan and Maclachlan, 1967b). Root primordia grow through these lacunae, breaking to the outside of the epicotyl in about 7 days. None of these events occurs in the intact untreated seedling, nevertheless, the responses should not be regarded as abnormal since they occur in many other tissues after treatment with physiological levels of auxin.

Attempts have been made to reproduce the hormonal contributions normally made to the epicotyl by the plumule and hook by adding growth regulators other than auxin to the decapitated epicotyl apex. It has been reported that treatments with kinetin (Datko, 1968; Davies and Maclachlan, 1968) or

ethylene gas (Fan, 1967; Ridge and Osborne, 1969) are not effective at evoking any major change in length or fresh weight or protein levels of the tissue below. Considerable elongation has been obtained by adding GA at a concentration of 10 µg per epicotyl (Datko, 1968; Davies and Maclachlan, 1968). It may be, therefore, that the decapitated epicotyl normally contains adequate or optimal levels of auxins and cytokinins, but not the gibberellins, and that the latter must be added in order to maintain elongation of cells in the epicotyl. The above studies could not be considered as definitive, however, because only one series of measurements at a single concentration of the regulators was made in each instance.

With respect to biochemical changes in the decapitated epicotyl, previous work in this laboratory (Fan, 1967; Datko, 1968; Davies, 1968) shows that IAA-evoked lateral expansion and cell division is accompanied by massive increments in protein, RNA and DNA, as well as a great rise in specific activity of the enzyme cellulase. Inhibitors of protein and RNA synthesis, e.g. chloramphenicol, puromycin, cycloheximide, actinomycin D, etc. all interfere with the development of cellulase activity. An inhibitor of DNA synthesis (FUdR), which prevents cell division, only partially inhibits the development of cellulase activity. Evidently, IAA can evoke an increase in cellulase activity in pre-existing cells, as well as in newly-divided cells. In so far as tests were made, the reaction was specific to IAA, i.e. treatments with GA, kinetin or

ethylene did not enhance cellulase activity per unit protein, and other polysaccharidases were relatively unaffected.

B. Natural and synthetic auxins

The growth-stimulating activities of most of the compounds related to IAA which were tested in this study are reviewed by Thimann (1951), Audus (1959), and Fawcett (1961). They are also referred to by many authors in past proceedings of the International Conferences on Plant Growth Substances, (e.g. Vol 6, 1968). The following brief comments deal mainly with known effects and metabolism of these substances in peas.

Tryptophane-^{14}C is converted readily to IAA in pea shoot tips (Morré and Shaner, 1967) and in many other plants (Wightman and Cohen, 1968). The exact reaction pathway between the two is in dispute, but enzyme systems which carry out the overall reaction have been extracted from pea seedlings (Muir and Lantican, 1968) and various components of the system have been identified in peas. The latter include:- enzyme(s) which bring about tryptophane decarboxylation (Reed, 1968); indole-3-acetaldehyde, a probable intermediate of the reaction sequence, (Larsen and Aasheim, 1959); and enzyme(s) which form this aldehyde from tryptamine (Clarke and Mann, 1957). Indole acetaldehyde is so labile that it oxidizes spontaneously in water solutions to IAA (Larsen and Rajagopal, 1964), which is probably why this intermediate has not been identified with certainty in plant extracts. Despite this evidence for the

conversion of tryptophane and indoleacetaldehyde to IAA, neither precursor has much stimulatory effect on growth of excised plant tissue (Bentley and Housley, 1952), unless added at high concentration (Larsen and Rajagopal, 1964).

Indoleacetylnitrile occurs naturally in some plants (Jones et al., 1952) and it promotes growth, probably after conversion to IAA (Thimann, 1954; Thimann and Mahadevan, 1958). With excised pea sections, however, it does not stimulate growth even at high concentrations (Larsen and Rajagopal, 1964). A recent study (Evans and Rayle, 1970) has confirmed that indoleacetylnitrile is not converted to IAA in peas, although it is an excellent precursor in tissues (from corn and wheat) where it stimulates growth.

When IAA-^{14}C is supplied to pea epicotyl sections, the main products formed in short-term experiments (hours) are CO_2 and indoleacetylaspartate (Andreae and Ysselstein, 1956). The latter is regarded as a detoxification product, for various reasons, including the fact that it has no effect on growth of pea sections (Andreae and Good, 1955), and enzyme(s) to catalyse its formation appear to be induced by high concentrations of IAA (Venis, 1964; Sudi, 1966).

A number of other indole derivatives which might be convertible to IAA are found in plants. These include indole-glyoxylate, indolepropionate and indolebutyrate (see review by Fawcett, 1961). Little work has been done using the glyoxylate derivative, but the other derivatives are effective growth promoters in peas and other plants (Fawcett et al., 1960).

In some tests, e.g. the split pea test, these derivatives are even more stimulatory than IAA, possibly because they enter tissues more rapidly than IAA.

With respect to artificial auxins, α-naphthaleneacetic acid (NAA) is the substance which is probably used commercially most often in place of IAA as a growth stimulant. It is at least as effective as IAA in tests on elongation growth of pea tissue (Zenk, 1962), and when applied in lanolin paste to the apex of light-grown red kidney beans, it causes tumour-like growth responses and root formation (Hamner and Kraus, 1937; Audus, 1959). Extensive tests on pea growth with substituted phenyl derivatives (Thimann, 1951) showed that chlorine substitution in the ortho- or para- positions increased the auxin activity of low concentrations of these substances. The two most active compounds in the phenoxy series were 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). These latter substances are more stable than IAA in plants (van Overbeek, 1966). Used at relatively high concentrations they are effective herbicides, especially for dicotyledonous plants. The mechanism of herbicidal action is not known. All of the metabolic responses which occur after IAA or NAA treatment (e.g. RNA synthesis) also appear to occur after 2,4-D or 2,4,5-T treatments, though often to a greater degree (Moreland, 1967).

C. Gibberellic acid (GA)

The occurrence and activity of the gibberellins is

reviewed by Leopold (1964), Paleg (1965), Brian (1966) and Cleland (1969). The best known effect of GA is its ability to stimulate elongation growth, especially of some varieties of dwarf peas (Brian and Hemming, 1955; Gorter, 1961) and of seedlings (Russell and Galston, 1969) or sections of tissue (Lockhart, 1960) from red-light-grown plants. GA treatment also greatly stimulates elongation in the decapitated pea epicotyl, even when the seedlings have never been exposed to red light (Datko, 1968; Davies, 1968).

From studies using light-grown plant tissues, it has been concluded that gibberellin activity is mediated through auxin (Kuse, 1958; Kuraishi and Muir, 1962; Kuraishi and Muir, 1964) and that the action of GA and auxin are synergistic (Brian and Hemming, 1958; Galston and Warburg, 1959; Ockerse and Galston, 1967). However, Purves and Hillman (1958; Purves and Hillman, 1959) supplied GA and IAA simultaneously to etiolated pea sections and found no synergism but, rather, an inhibition of GA-evoked elongation by IAA. Kaufman and co-workers (1969) also failed to find synergism when they studied the effects of GA and IAA on excised Avena stem sections. Sections treated with both IAA and GA exhibited lateral expansion instead of elongation. In the tests with pea and oat tissue, a concentration of IAA at least as great as that of GA was required to suppress the elongation due to GA.

In recent years, it has come to be widely accepted that GA, like auxin, exerts control over plant growth by regulating formation of nucleic acid and protein (Key, 1969). It is well

established that elongation growth following GA treatment is inhibited by actinomycin and puromycin (Nitsan and Lang, 1965). There is also no doubt that GA treatment can increase the amounts of both DNA and RNA in some varieties (i.e. dwarf) pea seedlings (Broughton, 1968; Giles and Myers, 1966). Both responses are generally greater in light-grown plants and RNA synthesis precedes any increase in DNA synthesis.

With respect to the question of which enzyme(s) may be induced by GA, the best documented system comes from a series of studies by Varner and colleagues (reviewed by Filner et al., 1969) on the snythesis of α-amylase in the aleurone layer of barley endosperm. By use of density-gradient fractionation techniques it has been shown that GA brings about de novo synthesis of this enzyme in this particular tissue. Varner's group also detected substantial increments in activity of protease and RNAase in this system, and other workers (Macleod and Millar, 1962) have noted a rise in endo-β-glucanase (acting on a glucan containing mixed β-1,3- and β-1,4-linkages). The only other enzyme activity which has been observed to increase greatly after GA treatment is invertase (in Jerusalem artichoke tissue, Edelman and Hall, 1965). In the GA-treated decapitated pea epicotyl, invertase activity rises only slightly during elongation (Datko, 1968) and cellulase does not respond (Davies and Maclachlan, 1968).

D. Ethylene

The most recent review on the physiological roles of

ethylene gas is that by Pratt and Goeschl (1969).

It is firmly established that ethylene gas inhibits elongation, both endogenous growth and that due to added IAA, when supplied in air at concentrations above 1 ppm. Such effects have been observed with excised pea stem (green) or pea epicotyl segments (Burg and Burg, 1966), and also with the decapitated pea epicotyl (Michener, 1938; Fan, 1967; Ridge and Osborne, 1969). In most of these studies, it was noted that ethylene caused some swelling, though not as much as that which could be evoked by the addition of high concentrations of IAA. Indeed, ethylene generally inhibited the swelling due to IAA. Accordingly although IAA treatment of pea epicotyls does evoke release of ethylene gas (Burg and Burg, 1968), the growth responses due to IAA cannot entirely be mediated by ethylene. For similar reasons, Andreae et al., (1968) concluded that mechanism of the well-known inhibition of root growth by IAA could not have depended entirely on mediation by ethylene.

Swelling after ethylene treatment is not due to cell division. In the decapitated epicotyl, ethylene does not alter the cell number but instead increases cortical cell volume (Ridge and Osborne, 1969). Indeed, ethylene is well known to inhibit proliferation of cambium (e.g. in radish roots, Radin and Loomis, 1969) and other cells (e.g. in pea buds, Burg and Burg, 1968). It also inhibits the RNA synthesis which follows 2,4-D treatment (e.g. in soybean hypocotyls, Holm et al., 1970). In all of these respects, therefore, ethylene has a different or opposite effect to that of auxin.

Finally, opposing effects of treatments with auxins and with ethylene gas are perhaps best documented in connection with the process of abscission. Ethylene initiates reactions leading to abscission of leaves and fruits while auxin, applied to the distal end of the abscission zone, generally delays these same events. An entire issue of the journal plant Physiology (vol. 43, No. 9, Part B, 1968) has been devoted to these phenomena. It is established that when ethylene promotes abscission, it also enhances RNA and protein synthesis in the abscission zone (Abeles and Holm, 1966; Abeles, 1968). Cellulase may be one of the proteins generated in this zone after ethylene treatment because its activity increases by as much as two-fold over untreated controls (Horton and Osborne, 1967). This modest effect can hardly have resulted from a specific induction, as suggested by Abeles (1969), when protein levels increased to a similar extent.

E. Cytokinins

The occurrence, chemistry and physiological activities of some natural and synthetic cytokinins are reviewed by Letham (1967), Helgeson (1968) and Fox (1969). A series of articles on these substances occur in the 6th International Conference on Plant Growth Substances (1968).

The discovery of kinetin (6-furfurylaminopurine) in tobacco callus tissue and the demonstration that it stimulates cell division (Miller et al., 1955) prompted many subsequent studies on the physiological effects of natural and synthetic analogues of this adenine derivative (collectively referred to

as cytokinins). The most active cytokinins are those which are substituted in the N^6 position of adenine, e.g. N^6-benzyladenine (Helgeson, 1968; Skoog and Leonard, 1968). Such substances occur in the free (uncombined) state naturally in pea seedlings (Zwar and Skoog, 1963). Also, a cytokinin attached to r-RNA has been reported in pea extracts (Hall, 1968).

Most studies on the effects of cytokinins on etiolated peas have used excised epicotyl sections. Kinetin was observed to inhibit elongation of these sections (Forsyth and Samborski, 1960; Katsumi, 1963), and to promote an increase in their diameter (Sommer, 1961; Katsumi, 1962). Sommer compared the effects of kinetin, IAA and GA alone and in combination on longitudinal and lateral development of such sections. Using mainly the upper 0.4 cm of epicotyl below the plumular-hook he observed that application of 10 ppm kinetin plus 0.1 to 1.0 ppm IAA gave the greatest increase in diameter (+40% to +50%). Swelling effects due to these two hormones were approximately additive. Kinetin inhibited the elongation due to either GA or IAA treatments. Sprent (1968), using a light-grown pea system, also demonstrated a suppression of GA-stimulated elongation by benzyladenine.

Two brief tests of the effects of kinetin on the decapitated etiolated pea were conducted by Datko (1968) and Davies and Maclachlan (1968). They found that treatment at the apex with 10 µg kinetin per epicotyl resulted in no increase in elongation or swelling of the decapitated epicotyl, and

only a slight rise in total protein and cellulase activity. Effects of other cytokinins were not measured, although those which penetrated into or moved within the tissue more rapidly than kinetin (see McCready, 1966) might well be expected to influence growth and synthetic reactions, since in many other plant systems, e.g. in tobacco callus cultures, senescing detached leaves, etc., cytokinins have marked stimulatory effects on the synthesis of proteins, RNA and DNA.

METHODS

A. Growth and treatment of pea epicotyls

Pea seeds (Pisum sativum var. 'Alaska') were obtained from W. H. Perron Co. Ltd., Chomedy, Quebec and W. A. Jenkins Mfg. Co. Ltd., London, Ontario. Before planting, the peas were surface sterilized with 0.5% NaOCl for 20 min, washed thoroughly with water and soaked overnight (approx. 16 hr) under tap water.

For treatment with most growth regulators, peas were planted on wet vermiculite (2 volumes vermiculite/volume water) in plastic trays, dusted lightly with dry vermiculite and grown in a dark cupboard at 20° to 23° for 8 or 9 days until the third internode was 2 to 4 cm long (Fan, 1968). Seedlings were then decapitated (i.e. the plumule and hook was removed) and a mark was made with a water-soluble marking pen one cm below the cut apex (Datko, 1968). This delineated an amount of tissue which shall be referred to here as a "segment". Growth regulators in solid or liquid form were mixed in lanolin paste (30% water wt/wt) and approx. 2 mg of lanolin was applied to each decapitated epicotyl apex. At desired time intervals, peas were harvested, surface lanolin was wiped away, segments were excised and fresh weight and length were recorded. Fresh weight was measured to the nearest 0.1 mg. Length was estimated to the nearest 0.5 mm with a ruler.

At least 20 segments were used for every value recorded in this thesis.

In experiments involving ethylene gas (tests numbered 1 to 13 in original records), peas were planted in round chromatography jars (vol approx. 8,500 ml, $8\frac{3}{4}$" diam. x 10" height). They were grown in darkness for 7 days or until the second internode was 2 to 4 cm long (apex about 10 cm from top of jar). Seedlings were then decapitated, segments delineated and apices treated with lanolin paste ± regulators as described above. Jars were covered with plexiglas and sealed with silicone grease. At zero time, air was withdrawn and ethylene gas at the appropriate concentration was injected into the jars with microsyringes through vaccine caps which had been fitted into holes in the covers.

In early experiments utilizing jars (tests 1 and 2), pea seedlings were left to grow without replacing the atmosphere until harvest at 3 days. However, as reported by Holm et al. (1970), continuous enclosure of the seedlings with or without ethylene gas was found to result in severe fungal infections and very poor growth responses. For example, segments from such seedlings which had been treated with IAA failed to show the usual swelling, protein increment or rise in cellulase activity. If however, jars were opened twice daily (at 9:00 a.m. and 6:00 p.m.) and flushed with air or other gas mixtures, fungal growth was much reduced and ability to respond to IAA was restored fully (see Appendix I for

representative data). Accordingly, in all tests reported here where seedlings were enclosed, gas mixtures were replaced twice daily.

B. Enzyme preparation

Segments for enzyme or protein assays were homogenized for 3 min in 2 vol of cold 20 mM sodium phosphate buffer (pH 7.0) in a "Virtis 45" homogenizer run at top speed. The homogenate was centrifuged at 37,000 x g for 20 min and the residue was re-extracted with 2 vol of the same buffer. Supernatant extracts were combined and this fraction was used for many of the assays reported in this paper, i.e. measurements were made on that part of the total protein and cellulase activity which remained soluble.

Fan and Maclachlan (1967a) reported that the fraction of pea epicotyl segments which was soluble in phosphate buffer normally retained about 90% of the protein and cellulase activity. Nevertheless, their experiments also showed that addition of small amounts of divalent metal ions to isolation medium could seriously reduce solubility (e.g. to 50%). Indeed, only about two-thirds of total protein was extracted routinely by phosphate buffer in experiments conducted later with a different batch of Alaska peas (Datko, 1968; Datko and Maclachlan, 1970).

In the present study, at one period when the pea

seed which was used was about a year old, protein and cellulase levels in soluble enzyme extracts were found to decrease gradually from experiment to experiment. On investigation, the missing protein and cellulase activity was located in insoluble fractions. The reason(s) for this phenomenon were not investigated but in subsequent experiments, protein and cellulase assays were carried out on initial homogenates in order to be sure that total amounts were being measured. In all Tables and in the text where results of such measurements are described, values are referred to as "total" in contrast to values for "soluble" fractions only.

C. Cellulase assay

Pea cellulase is an endo-hydrolase, i.e. β-1,4-glucan, 4 glucanohydrolase, EC 3.2.1.4, (Maclachlan and Perrault, 1964) with a pH optimum close to 6.0 (Fan and Maclachlan, 1966). Activity of this enzyme can be measured readily using a viscosimetric assay. Fan (1968) observed that the rate of loss of viscosity of the soluble substrate carboxymethylcellulase (CMC) following addition of pea cellulase is linear up to 20% loss and proportional to enzyme concentration provided readings are taken before this point is reached. This assay was used with minor alterations by Datko (1968) and Davies (1968) and it was the basis for the assay used in the present study.

Viscosimetric assays used Ostwald-Cannon-Fenske (10-ml) or Cannon-Manning Semi-micro (1-ml) viscometers, capillary

size 200, incubated with enzyme plus substrate at 35°. Flow-through time was measured with a stopwatch to the nearest 0.2 seconds. For both types of viscometers, flow-through times were about 10 sec for water and from 120 to 200 sec for initial reaction mixtures. Routinely flow-through times were measured following incubation for 1, 2, 4 and 6 hr and average rates of viscosity loss per 2 hr were taken from the time intervals during which this value was linear.

The substrate, CMC, was a commercial variety manufactured by Hercules Powder Co. Early experiments in this study employed the particular CMC sample (Type 7MSP, lot no. 38999, prepared April 1, 1963) which was used in previous experiments in this laboratory, e.g. by Fan (1968), Datko (1968) and Davies (1968). Later tests (i.e. those with ethylene) employed a fresh sample of CMC (Type 7M8SF, Lot no. 67847, prepared December 4, 1967) which had the same degree of substitution (70%) and a very similar viscosity when dissolved in phosphate buffer as the previous sample. The two samples of CMC lost viscosity in the presence of Aspergillus niger cellulase at almost exactly the same rate, (e.g. by 6 hr in one test, Type 7MSP had lost 64.0% and Type 7M8SF 67.2% of initial viscosity).

Substrate was prepared by homogenizing CMC at a concentration of 1.1% (wt/vol) in 20 mM sodium phosphate buffer (pH 6.0) containing 0.22% NaF for 10 min at top speed in a "Virtis 45" homogenizer at room temperature. The viscous solution was boiled for 30 min and stored at 2°. For enzyme

assays, 9.0 ml (or 0.9 ml) of this substrate was equilibrated at 35° in 10-ml (or 1.0-ml) viscometers. Reaction was initiated by mixing 1.0 ml (or 0.1 ml) of enzyme with the substrate.

One unit of cellulase activity is defined here as that amount of enzyme which gives 1% loss in viscosity/2hr under the above conditions.

D. Protein assays

Three methods of protein determination were used following the procedures described by Fan (1968) and Datko (1968). The biuret method was reported to assay accurately from 1 to 6 mg protein in soluble extract of peas. The Lowry method was used for 10 to 50 µg protein and Nesslerization for 10 to 50 µg protein in insoluble residues.

Biuret reagent is specific for compounds containing 2 or more peptide bonds; dipeptides and most free amino acids (exceptions are histidine, serine and threonine) do not react with the reagent (White et al., 1964). In present tests, bovine serum albumin (BSA) was used as a reference standard. As shown in Appendix 2, this method gives a linear relationship between O.D. and protein levels up to at least 5 mg protein. The procedure was follows (see Garnall et al., 1949; Layne, 1957):

Protein, dissolved or suspended in 2.0 ml 20 mM sodium phosphate buffer (pH 7.0), was precipitated by adding 0.35 ml 3M trichloroacetic acid (TCA), incubating at 2°

for several hours, and centrifuging at approx. 3,000 x g. The precipitate was shaken and suspended in 3.0 ml water plus 3.0 ml biuret reagent at room temp for 30 min. By this time, protein from soluble enzyme extracts was completely dissolved. Turbidity (due to wall materials, lanolin, etc.) was removed by centrifugation. O.D. was read at 545 mμ.

The Lowry method is 100 times more sensitive than the biuret method, it depends on reactions with phenolic amino acids and it gives more colour with proteins than with free amino acids (Lowry et al., 1951). In present tests, BSA was used as a reference standard. As shown in Appendix 3, this method gives a linear relationship between O.D. and protein level up to 30 μg protein. The procedure was as follows:

Soluble protein in 0.4 ml was added to 3.6 ml of Lowry's reagent "C" (50 ml 2% Na_2C0_3 plus 1.0 ml 0.5% $CuS0_4.5H_20$ in 1% NaK tartrate). After 10 min at room temp, 0.4 ml Lowry's reagent "E" (0.1N Folin reagent, Fisher Scientific Co.) was added with rapid mixing. After exactly 30 min, O.D. of the samples was read at 750 mμ.

The amount of protein in insoluble residues was determined by estimating the amount of ammonia nitrogen with Nessler's reagent following digestion of the protein with conc. sulphuric acid. Anhydrous ammonium sulphate was used as a reference. As shown in Appendix 4, this method gives a linear relationship between O.D. and protein level up to 18 μg

nitrogen. A multiplication factor of 6.25 was used to convert µg nitrogen to µg protein. The procedure was as follows (see Middleton, 1960):

> Samples were digested in conc. H_2SO_4 in Kjeldahl flasks, (with H_2O_2 added if necessary) until solutions were clear. After cooling, 1.0 ml digestion mixture was added to 1.0 ml Nessler's solution "A" (0.6M NaK tartrate) plus 1.0 ml Nessler's solution "B" (0.2% gum arabic solution) plus 1.0 ml Nessler's solution "C" (0.9% HgI_2-1.2% KI). The solutions were mixed and 2.0 ml Nessler's solution "D" (1.0N NaOH final concentration) was shaken into the mixture rapidly. O.D. was read at 425 mµ after 30 min.

E. Nucleic acid assays

Estimations of the amount of RNA and DNA in soluble extracts of etiolated pea epicotyls were determined by the procedures described by Duda (1964), Fan (1968) and Davies (1968).

Nucleic acids were precipitated from soluble extracts or total homogenates by mixing with an equal volume of cold 10% TCA and incubating at 2° for several hours. Mixtures were centrifuged at approx. 3,000 x g, washed once with cold 10% TCA and once with cold acetone. The pellets were then suspended in 0.5N perchloric acid and extracted 1 hr at 70°. Insoluble material was removed by centrifugation and re-extracted with perchloric acid. Supernatants containing DNA and RNA were combined.

Total nucleic acid in perchloric acid was determined as described by Holdgate and Goodwin (1965), by measuring the value for $O.D._{260} - O.D._{290}$ using as standard purified wheat germ RNA (Calbiochem). The method avoids interference by protein and is accurate for 5 to 50 µg RNA (Appendix 5).

DNA was estimated with diphenylamine following the procedure of Burton (1956). Reaction mixtures containing 1 vol of DNA in 0.5N perchloric acid plus 2 vol diphenylamine reagent were incubated at 30° for 18 hrs. The diphenylamine reagent was prepared by dissolving 1.5 g diphenylamine in a solution containing 1.5 ml conc. H_2SO_4 plus 100 ml glacial acetic acid. Immediately before use, 0.1 ml aqueous acetaldehyde solution (16 mg acetaldehyde/ml water) was added to each 20 ml of the reagent. DNA concentration in the final reaction mixture was estimated by measuring the value for $O.D._{600} - O.D._{540}$. As shown in Appendix 6, the method is measuring DNA in samples containing 10 to 100 µg/ml.

RESULTS

A. Effects of auxins

1. Indoleacetic acid (IAA)

Table I shows effects of IAA added to the apex of decapitated pea epicotyls on the growth of an apical segment of tissue (0-10 mm at zero time), and on the levels of soluble cellulase activity in protein extracted from that segment. Each apex was painted with approx. 2.0 mg lanolin paste containing no IAA (for untreated controls) or 0.01, 0.1, 1.0, or 10 µg IAA. Effects were measured at daily intervals for 4 days.

None of the IAA concentrations caused significant elongation. Fresh weight was distinctly increased, however, even by the lowest levels of IAA employed; i.e. segments exhibited greater lateral expansion than untreated controls. The extra swelling (increase in fr wt/length over controls) was apparent within one day and was complete by 3 days. Maximum swelling occurred with as little as 0.1 µg IAA added per epicotyl. Higher concentrations were not inhibitory.

Marked increases occurred during this experiment in the amounts of extractable cellulase activity in segments which had been treated with the highest IAA concentrations. For example, after 2 to 4 days treatment with 10 µg IAA per epicotyl, cellulase levels were about 20 times those at day

Table I

Title and Legend

Effects of Indoleacetic Acid (IAA) on Elongation, Swelling and the Development of Buffer-soluble Cellulase Activity in Apical Segments of Decapitated Pea Epicotyls*

(see following page for data)

---

*"Segment" refers to the tissue which developed from the apical 10 mm of third internode while it was still attached to the pea seedling but after removal of plumule and hook (decapitated). Each cut apex was painted with approx. 2.0 mg lanolin paste (30% water) containing IAA at the concs. indicated. 100 seedlings were treated at each IAA conc. and at daily intervals 20 were harvested and the length and fr wt of segments were measured. Swelling was indicated by the ratio fr wt/length. Soluble cellulase activity was assayed in protein extracted with 20 mM sodium phosphate buffer (pH 7.0) following centrifugation at 37,000 x g.

At zero time, average measured length was 10.1 mm (S.E. ± 1.5 mm), fr wt was 2.3 mg/mm and soluble cellulase activity was 0.3 units/epicotyl.

Table I

Effects of Indoleacetic Acid (IAA) on Elongation, Swelling and the Development of Buffer-soluble Cellulase Activity in Apical Segments of Decapitated Pea Epicotyls*

Time (days)	IAA (µg/epi) 0	0.01	0.1	1.0	10
	Length (mm)				
1	11.4	11.5	12.8	10.1	10.4
2	11.5	13.2	11.7	11.9	11.4
3	12.7	14.7	12.3	12.8	12.2
4	10.9	13.7	12.9	11.9	11.8
	Fr wt/length (mg/mm)				
1	3.3	3.9	4.4	3.8	4.1
2	3.9	4.9	5.7	5.4	5.3
3	3.5	4.7	6.3	5.8	6.1
4	3.2	4.8	6.1	5.8	5.8
	Soluble cellulase activity (units/seg)				
1	0.6	0.6	0.6	0.8	1.1
2	0.6	0.8	1.0	1.9	7.6
3	0.5	0.8	0.8	2.4	5.9
4	0.3	0.3	-	1.9	6.8

*See legend for Table I on preceding page.

zero and at least 10 times those in segments which received no IAA (controls). Increments in cellulase activity were much less pronounced, however, after treatments with lower IAA concentrations. Following addition of 0.1 and 0.01 µg IAA per epicotyl, for example, soluble cellulase levels never increased to levels which were more than twice those in untreated controls.

These results (Table I) indicate that low IAA concentrations can cause substantial swelling of the pea epicotyl without any great accumulation of soluble cellulase activity during the progress of expansion. A second test was carried out to measure effects on total cellulase activity by assaying buffer-insoluble (residual or wall-bound) cellulase activity as well as the soluble fraction. Results are shown in Table II.

Growth responses to IAA in this test (Table II) were similar to those observed previously except that a conc. of 0.1 µg IAA per epicotyl was required before fr wt per unit length increased to values distinctly above those in untreated controls. Total cellulase levels also began to rise (slightly) above control levels after treatment with 0.1 µg IAA. Dramatic increments were only attained at higher IAA levels. Throughout the test buffer-insoluble fractions contained 25 to 40% of total cellulase activity and changes in levels within the insoluble fraction were no more marked than those in soluble fraction.

2. Other auxins and herbicides

A number of indole derivatives which could be regarded as potential auxins were tested for their effects on growth,

Table II

Effects of 3 Days Treatment with IAA on Elongation, Swelling and the Development of Buffer-soluble and -insoluble Cellulase Activity in Pea Epicotyl Segments*

Component per seg	IAA (µg/epi) 0	0.01	0.1	1.0	10
Fr wt (mg)	43.8	42.1	57.6	70.0	63.4
Length (mm)	11.1	11.1	11.6	10.6	11.6
Fr wt/l (mg/mm)	3.9	3.8	4.7	6.6	5.5
Cellulase activity (units)					
Buffer-soluble	0.62	0.64	0.65	2.14	4.21
Buffer-insoluble	0.23	0.23	0.42	0.68	2.70

*Methods were the same as those described in Table I except that from 85 to 100 segments (rather than 20) were used for each measurement, and cellulase activities were assayed in both supernatants and in centrifuged pellets derived from homogenized segments.

At zero time, average measured length was 10.3 ± 1.5 mm, fr wt was 2.6 mg/mm and cellulase activity was 0.40 and 0.16 units/seg in soluble and insoluble fractions respectively.

soluble protein levels and amount of soluble cellulase activity. Tests involved painting decapitated epicotyls with lanolin paste ± approx. 10 µg per epicotyl of additive. Responses in the tissue segment below were measured after 3 days. In another experiment carried out 10 days later the same parameters were measured, but several other indole derivatives were tested, as well as the auxin α-naphthaleneacetic acid (NAA) and two herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The results, given in Tables III and IV, indicate that the growth responses with and without IAA, as well as the changes in protein levels and cellulase activities, were similar in the two experiments. Accordingly, it was considered legitimate to make direct comparisons of data from these two experiments.

With respect to elongation, none of the additives evoked a lengthening of the segment which was comparable to the 2- to 3-fold increases observed in this tissue when it remained in intact epicotyls (Datko, 1968) or when segments were treated with GA (Davies and Maclachlan, 1968). In segments treated with most of the indoles and herbicides and in controls, length increased by only about 20%. The greatest elongation in these tests followed treatment with indoleacetylaspartate (+59%), tryptophane (+47%) and indoleacetaldehyde (+43%).

The fresh weight of control segments doubled during the experiment and most of the additives, including the herbicides, caused no more than a 2.5-fold rise; i.e. they had little effect on swelling. These same additives also had no great effect on soluble protein levels, which generally changed little during

Table III

Effects of Various Potential Auxins (Indoles) on Decapitated Pea Epicotyl Segments*

Additive (10µg/epi)	Length (mm)	Fr wt (mg/seg)	Soluble protein (µg/seg)	Cellulase activity (units/seg)
Zero Time	10.0	22.1	210	0.17
3 Days				
None (Control)	11.8	41.3	177	0.35
Indoleacetaldehyde	14.3	44.3	166	0.33
Indoleacrylate	11.9	36.8	196	0.34
Indoleacetyl-aspartate	15.9	52.0	244	0.35
Indolebutyrate	13.0	70.0	480	4.40
Indoleacetate	12.5	72.5	560	5.10
Indolepropionate	13.6	88.5	720	8.20

*Methods were the same as described in Table I except that 80 to 100 segments were used for each treatment.

Table IV

Effects of Various Potential Auxins (Indoles and Herbicides) on Decapitated Pea Epicotyl Segments*

Additive	Length (mm)	Fr wt (mg/seg)	Soluble protein (µg/seg)	Cellulase activity (units/seg)
Zero Time	10.0	21.0	234	0.25
3 Days				
None (Control)	12.3	41.0	215	0.19
Tryptophane	14.7	51.0	275	0.27
Indoleglyoxylate	13.2	48.0	234	0.47
Indoleacetonitrile	10.9	39.0	213	0.59
Indoleacetate	11.8	68.0	400	3.24
Naphthaleneacetate	11.3	60.0	399	4.86
2,4-D	12.4	55.0	355	11.20
2,4,5-T	11.4	46.0	362	11.30

*Methods were the same as those described in Tables I and III.

the experiment. However, 3 auxins, namely: IAA, indole-butyric, and NAA, evoked a 3-fold to 3.5-fold rise in fresh weight and a 2- to 2.5-fold rise in protein levels. Indolepropionic acid caused the greatest increment in fresh weight (4-fold) and protein level (3.5-fold). Evidently there was a correlation between the ability of these potential auxins to cause swelling and their ability to evoke accumulation of protein.

Cellulase activity increased dramatically following treatment with those 3 auxins which caused distinct swelling and protein accumulation. The increases were at least 10 times values observed at zero time or in control (untreated) segments, i.e. much greater than increases in total protein. The other indole derivatives (which did not cause swelling or protein synthesis) evoked no more than a doubling of the cellulase level. The most marked effects on cellulase activity followed treatment with 2,4-D or 2,4,5-T both of which brought about 40- to 50-fold increments. This effect of herbicides was relatively specific to cellulase since concomitant increases in protein levels were modest compared to those with natural indole hormones.

B. Effects of gibberellic acid (GA)

Table V shows values for length and fr wt of the epicotyl segment at 1 to 4 days following treatment with different amounts of GA (0.01 - 10 μg per epicotyl). In contrast to effects of IAA (Table I), GA stimulated elongation

Table V

Effects of Gibberellic Acid (GA) on Decapitated Pea Epicotyl Segments*

Time (days)	GA (µg/epicotyl) 0	0.01	0.1	1.0	10
	Length (mm)				
1	11.8	13.6	13.1	13.7	13.8
3	11.9	14.7	17.4	14.5	18.3
4	11.2	15.2	16.7	16.2	17.7
	Fr wt/length (mg/mm)				
1	3.2	3.6	3.2	2.9	3.2
3	3.1	3.6	3.4	2.9	2.7
4	3.2	3.8	3.4	3.2	3.2
	Total cellulase activity (units/seg)				
1	0.9	1.1	1.5	1.1	0.8
3	1.1	0.8	0.7	1.1	0.9
4	1.0	0.7	1.0	1.0	-

*Methods were the same as those described in Table I. At zero time, average length was 10.6 ± 1.5 mm, fr wt was 2.1 mg/mm and cellulase activity was 0.5 units/seg.

in the segment and had no significant effect on swelling. By the end of the experiment, even the lowest levels of GA employed in this test had caused length to increase four times as much as in untreated controls. At higher GA levels, the initial rate of elongation was no greater than at low levels, but elongation continued for longer periods of time.

Table V includes results of measurements of total cellulase activity in the segments. GA treatment failed to result in any substantial rise in cellulase level at any time during the elongation response. Evidently elongation did not require an overall enhancement of this enzyme activity beyond the levels present in the untreated tissue.

In later experiments (see Table XIV), measurements were made of effects of GA ± IAA on nucleic acid and protein levels. With GA alone, a small increment in total protein over control values occurred during elongation (see also Davies and Maclachlan, 1968) but there was no change in total RNA or DNA content and presumably no cell division in the segments. When GA and IAA were combined (each at 10 µg/epi) the responses were similar to those following treatment with IAA alone, i.e. swelling rather than elongation, and massive accumulation of protein, RNA and DNA. Even very low levels of IAA (e.g. 5 mµg) were sufficient to severely reduce the elongation response to GA (see Appendix 7). Indeed, the only apparent modification in the IAA-treated segment due to inclusion of GA was a small reduction in the amount of evoked cellulase activity.

C. Effects of ethylene gas (E)

Table VI shows swelling and total cellulase activity of decapitated pea epicotyls following 3 days treatment with concentrations of ethylene from 0.1 to 1000 ppm in the presence or absence of IAA. The results in this Table were derived from a single experiment which was designed to provide an overall view of the actions and interactions of these two growth regulators.

Effects in 5 earlier tests of adding 10 ppm ethylene ± 10 µg IAA per epicotyl are summarized in Tables VII and VIII. Table IX shows similar data from two other experiments in which 1000 ppm ethylene was employed. In these early tests only soluble cellulase activity was assayed.

Segment elongation was not much affected by ethylene but fr wt was enhanced, i.e. like IAA, ethylene caused swelling (Tables VI and VII). An increase in fr wt per unit length was evident at gas concentrations as low as 1 ppm, and maximum effects were achieved by 10 ppm. Even at 1000 ppm ethylene, however, the response was never as pronounced as the swelling which followed treatment with 10 µg IAA, and when the two regulators were added together, their effects on swelling were not additive. Indeed, in many tests (Table VII), ethylene inhibited the swelling due to IAA. Thus, naturally-occurring ethylene gas, which undoubtedly forms in the epicotyl following IAA treatment (Burg and Burg, 1966), could hardly have been

Table VI

Effects of 3 Days Treatment with Ethylene Gas ± IAA on Decapitated Pea Epicotyl Segments*

10 µg IAA per epicotyl	Ethylene concentration (ppm) 0	0.1	1.0	10	100	1000
			Length (mm)			
-	11.2	11.8	12.2	12.5	11.7	13.2
+	13.8	12.4	12.9	12.9	11.8	13.7
			Fr wt/length (mg/mm)			
-	3.2	3.2	4.0	4.9	-	4.7
+	5.5	5.0	5.4	5.5	5.5	5.4
			Total cellulase activity (units/seg)			
-	0.21	0.28	0.40	0.49	0.54	0.56
+	3.1	3.7	1.9	1.6	1.7	1.5

*At zero time average length was 10.0 ± 1.5 mm, fr wt was 2.8 mg/mm and cellulase activity was 0.16 units per segment. Gas mixtures were replaced twice daily (test no. 11 in original reports).

Table VII

Swelling as a Result of 3 Days Treatments with 10 ppm Ethylene (E) ± 10 µg IAA per Epicotyl*

Treatment	Experiment number				
	3	4	6	7	8
	Fr wt/length (mg/mm)				
Control	2.8	3.5	3.1	2.8	2.9
+ E	3.9	4.0	3.0	3.8	3.2
+ IAA	5.0	6.2	5.0	5.0	5.8
+ E + IAA	4.0	5.3	3.6	4.7	4.4
	Ratio of values				
E/control	1.4	1.1	1.0	1.4	1.1
E/IAA	0.8	0.6	0.6	0.8	0.6
[E + IAA]/IAA	0.8	0.9	0.7	0.9	0.8

*At zero time, fr wt/length of segments from experiments 3 to 8 respectively were 2.3, 2.6, 2.4, 2.2 and 2.4 mg/mm.

responsible for the whole of the swelling response to IAA.

In later experiments (see Table XV), measurements were also made of effects of 10 ppm ethylene ± IAA on nucleic acid and protein levels. With ethylene alone, the increments in protein and RNA that occurred during swelling were as substantial as those that followed IAA treatment. There was no DNA synthesis after ethylene treatment, however, so that it is unlikely that cell division accompanied the swelling due to this regulator. When both ethylene and IAA were added together, the main effect was a reduction on the amount of RNA accumulated; DNA and protein levels were about the same as those following IAA treatment.

Cellulase activity levels generally increased following treatment with ethylene alone at concentrations above 1 ppm (Tables VI, VIII, IX and XV). Nevertheless, increments in 3 days resulted in only 2 to 3 times the activity levels observed in untreated controls. Such responses were modest compared to the marked increments in cellulase activity following IAA treatment. Evidently the stimulatory effects of IAA on development of this particular enzyme could not be attributed to ethylene. Indeed 10 ppm ethylene, added together with 10 µg IAA, severely inhibited (by 50-70%) the IAA-evoked rise in cellulase activity. Similar inhibitions were noted by Ridge and Osborne (1969) using 500 ppm ethylene + IAA in short term (24 hr.) tests.

Table X shows results of an experiment to test effects

Table VIII

Change in Soluble Cellulase Activity as a Result of Treatment with 10 ppm Ethylene or 10 µg IAA per epicotyl*

Time and treatment	Experiment number 3	4	6	7	8
	units/segment				
Zero time	0.29	0.28	0.22	0.21	0.25
3 days, control	0.22	0.22	0.39	0.29	0.47
3 days, +E	0.48	0.31	0.49	0.35	1.00
3 days, +IAA	2.85	5.90	5.28	0.84**	1.44**

*Data for swelling in these tests is given in Table VII.

**These activity values were much lower than usual for extracts of IAA-treated tissue probably because substantial activity was discarded in insoluble fractions.

Table IX

Change in Soluble Cellulase Activity as a Result of Treatment with 1000 ppm Ethylene ± IAA

Time and treatment	Experiment number 9	11
	units/segment	
Zero time	0.29	0.16
3 days, control	0.38	0.20
3 days, +E	0.72	0.56
3 days, +IAA	4.73	4.39
3 days, +E +IAA	2.70	2.82

Table X

Effects of 3 Days Treatment with 10 ppm Ethylene ± 10 µg GA per Epicotyl*

Time and treatment	Length (mm/seg)	Fr wt / length (mg/mm)	Total cellulase activity (units/seg)
Zero time	10.8	2.5	0.34
3 days, control	12.6	3.8	0.82
3 days, +E	11.6	4.3	0.32
3 days, +GA	22.4	3.6	1.10
3 days, +E +GA	12.8	3.2	0.49

*Test no. 13 in original reports.

Table XI

Effect of 100 ppm Ethylene on Swelling and Development of Cellulase Activity in the Apical 10 mm of Intact Pea Epicotyls*

Time (days)	Fr wt (mg/10 mm)		Total cellulase (units/10 mm)	
	Control	+E	Control	+E
0	23.8		0.2	
1	28.4	34.8	0.3	0.2
2	26.8	53.5	0.8	0.3
3	29.6	66.7	0.2	0.7
4	29.3	75.3	0.7	0.6

*Test no. 10 in original reports. Sections 10 mm long were excised from the region immediately below the hook of intact seedlings. Swelling due to ethylene under these conditions was comparable to that due to IAA (i.e., decapitated segments treated with 10 µg IAA and grown in open trays during this experiment weighed 62.6 mg at 3 days but contained 3.54 units of cellulase activity).

of combining 10 ppm ethylene gas and 10 μg GA on responses in the decapitated pea epicotyl segment. In the presence of both regulators, growth responses differed little from those in untreated controls, i.e. ethylene abolished the elongation due to GA, and GA prevented the swelling response to ethylene. Apparently these two regulators were mutually antagonistic in this system. Neither regulator altered cellulase levels.

During these tests, it was noted that marked swelling occurred in the epicotyl region just below the hook in seedlings of peas which had not been decapitated but which had been enclosed in jars and exposed to ethylene. These intact and swollen seedlings did not resume elongation growth until after the ethylene experience. Details of a test to record this phenomenon are given in Table XI. The data include the observation that cellulase levels in the swollen tissue were not consistently greater than levels in controls. This result agrees with that recorded by Ridge and Osborne (1969).

D. Effects of the cytokinins, kinetin (K) and benzyladenine (BA)

Effects of kinetin (K), with and without IAA or GA, are shown in Tables XII and XIII respectively. Effects of benzyladenine (BA) ± IAA ± GA ± ethylene are summarized in Tables XIV and XV.

With respect to growth responses, BA, and to a lesser extent K, caused distinct swelling of the epicotyl. N^6 - adenine derivatives are also more effective than kinetin at evoking

Table XII

Elongation, Swelling, and Cellulase Activity in Etiolated Pea Epicotyls Treated ± Kinetin (K) ± IAA

Treatment (10 µg/epi)	Length (mm)	Fr wt / length (mg/mm)	Cellulase activity (units/seg)
Zero time	10.0	1.89	0.1
3 days			
None (control)	11.7	2.86	0.3
IAA	11.4	4.80	5.8
K	11.6	3.36	0.3
IAA + K	10.6	4.40	4.1

Table XIII

Elongation, Swelling and Cellulase Activity in Etiolated Pea Segments Treated ± K ± GA

Treatment (10 µg/epi)	Length (mm)	Fr wt / length (mg/mm)	Cellulase activity (units/seg)
Zero time	10.0	2.28	0.3
3 days			
None (control)	11.6	3.29	0.4
GA	20.0	2.97	0.3
K	12.9	3.43	0.8
GA + K	16.2	4.73	0.3

cell division in other systems (Helgeson, 1968). Both cytokinins also evoked swelling when added together with GA; indeed, they inhibited the elongation response to the latter hormone. When BA and ethylene were added together, swelling was greater than that due to each regulator added alone. However, the greatest swelling responses obtained throughout this study were observed following treatments with BA plus IAA. Further tests in this laboratory by Birmingham (unpublished data) have confirmed repeatedly that swelling responses to these last two regulators are additive (e.g. see Table XIV).

Biochemical changes in the segment during its swelling response to BA (or K) differed from those following treatment with IAA (or E). IAA treatment caused great accumulation of protein and nucleic acid, and swelling was due in part to cell division (Fan and Maclachlan, 1967b). These observations were confirmed in the present study (Tables XIV and XV). After BA treatment, the protein increments (+ 35-50%) were less pronounced than those following IAA treatment (+ 130%), and cellulase activity did not accumulate. Increases in total RNA were about the same after BA and after IAA treatments. However, BA did not evoke DNA synthesis and no cell divisions could be detected visually (in free-hand sections).

With respect to biochemical changes which resulted from combining hormone treatments, no marked interactions were detected between BA (or K) and either GA or ethylene. None of these regulators alone or in combinations evoked DNA synthesis

Table XIV

Effects of 10 µg per Epicotyl of Benzyladenine (BA), IAA and GA, Alone and in Combination*

Treatment	Length (mm)	Fr wt / length (mg/mm)	Total protein (µg)	Total DNA (µg)	Total RNA (µg)	Total cellulase (units)
Zero time	10.5	2.6	295	7	17	0.3
3 days, control	12.0	3.4	191	5	19	0.5
+BA	10.8	5.6	456	7	37	1.2
+IAA	11.3	6.8	710	13	39	10.5
+GA	20.9	3.7	268	5	20	1.2
+BA + IAA	11.2	10.3	1,120	22	61	11.8
+BA + GA	13.7	4.6	305	4	15	0.6
+IAA + GA	11.9	6.6	605	10	38	7.0

*Values for all measured quantities are expressed per segment of tissue. Protein estimates were by the biuret test.

Table XV

Effects of 10 µg per Epicotyl of BA and IAA and 10 ppm Ethylene, Alone and in Combination*

Treatment	Length (mm)	Fr wt / length (mg/mm)	Soluble protein (µg)	Total DNA (µg)	Total RNA (µg)	Total cellulase (units)
Zero time	10.0	3.0	190	10	16	0.5
3 days, control	11.9	2.7	207	9	12	1.3
+BA	12.2	4.8	256	10	21	1.1
+IAA	12.3	7.3	475	16	24	15.1
+E	13.3	5.1	444	10	24	2.5
+BA + IAA	13.7	8.0	570	16	36	11.6
+BA + E	12.6	6.2	386	-	34	1.8
+IAA + E	13.5	7.0	390	-	16	6.5

*Values for all measured quantities are expressed per segment of tissue which was cut from the 2nd internode. Protein estimates were by Lowry's method. Those treatments which included ethylene gas were carried out in closed jars; others were in open trays.

or any great rise in cellulase activity. RNA and protein levels increased modestly in the presence of all the combinations. The most marked increases in RNA and protein that were observed throughout these experiments, occurred following treatment with BA plus IAA. Here, the increments noted in tissue treated with each regulator alone were clearly additive when the regulators were combined.

DISCUSSION

This study repeatedly confirmed the basic observations made on decapitated pea epicotyls by others in this laboratory (Fan, 1968; Datko, 1968; Davies, 1968), namely, that the application of 10 µg IAA per apex results in lateral expansion and accumulation of large amounts of RNA, DNA, protein and cellulase activity in the tissue which develops below. The rise in cellulase activity occurred after a lag period and was proportionately much greater than the rise in total protein level, i.e. it showed characteristics of a typical induction (see also Maclachlan et al., 1968).

Experimental data which bear upon the specificity of these reactions towards the auxin type of hormone are summarized in Table XVI. Effects of other growth regulators, alone or in combination with IAA, are surveyed in Table XVII. Three main conclusions can be drawn from these data:

1. The unilateral increase in cellulase activity was the only one of the responses to auxin which could not be reproduced by treatments with other types of growth regulator. The minor increases in cellulase activity which sometimes followed treatments with ethylene (see Tables VI, VIII, IX, and XV) or cytokinins (see Tables XII to XV), were no greater in magnitude than the concurrent increases in total protein. They were not unilateral and, therefore, they could not be regarded as results

TABLE XVI

Summary of Effects of Various Potential Auxins on Decapitated Pea Epicotyl Segments*

Additive**	Value per seg at 3 days/value at zero time			
	Length	Fr Wt / length	Soluble protein	Cellulase activity
None (control)	1.2	1.5	0.9	0.8
Indoleacetaldehyde	1.4	1.5	0.7	1.0
Indoleacrylate	1.2	1.5	0.8	1.0
Indoleacetyl-aspartate	1.6	1.3	1.0	1.0
Tryptophane	1.5	1.6	1.1	1.1
Indoleglyoxylate	1.3	1.8	1.0	1.9
Indoleacetonitrile	1.1	1.8	0.9	2.4
Indolebutyrate	1.3	2.1	1.9	13.0
Indoleacetate	1.2	2.7	2.3	15.0
Naphthaleneacetate	1.1	2.3	1.6	19.5
Indolepropionate	1.4	3.2	2.9	24.1
2,4-D	1.2	2.1	1.0	44.8
2,4,5-T	1.1	1.6	1.4	45.2

*Ratios are calculated from values obtained in two experiments: data reported in Tables III and IV. Ratios for control and IAA-treated segments are averages.

**Auxins are arranged in three groups according to whether they had: no major effect on growth or cellulase; or effects comparable to IAA; or little effect on growth but massive stimulation of cellulase activity.

TABLE XVII

Survey of Effects of Growth Regulators, Alone and Combined, on Decapitated Pea Epicotyl Segments: Stimulation (+), No Effect (0), Inhibition (-)

Additions	Changes in:						Table References
	Length	$\frac{\text{Fr wt}}{\text{Length}}$	Protein	RNA	DNA	Cellulase	
None	0	0+	0	0	0	0	All
IAA (or 2,4-D, etc.)	0	++	++	++	++	++	Most
GA	++	0	0+	0	0	0	V, XIV, Appendix 7
BA (or K)	0	+	+	++	0	0+	XII, XIII, XIV, XV
E	0	+	+	+	0	0+	VI, VII, VIII, IX, XV
IAA + GA	0	++	++	++	?	+	XIV
IAA + BA	0	+++	++	++	++	++	XIV, XV
IAA + E	0	++	++	?	?	+	XV
GA + BA	0	0	0	0	0	0	XIV
GA + E	0	0	?	?	?	0	XV
BA + E	0	++	++	++	?	0	XV

of derepression (see also Datko and Maclachlan, 1968; Davies and Maclachlan, 1968).

It is concluded that in the pea epicotyl the generation of cellulase activity requires an auxin as inducing agent.

2. Lateral cell expansion in the epicotyl segment was stimulated by treatment with any one or combination of IAA, BA, or ethylene. GA evoked elongation, and each of the swelling agents could prevent this. Clearly, the critical growth-promoting reactions which were regulated by IAA, BA and ethylene must have differed from the reaction(s) promoted by GA. Also, since the swelling effects due to IAA and BA were additive (Tables XIV and XV), these regulators must have controlled different limiting reactions. Finally, the swelling due to IAA and ethylene appear to have been different reactions because, while the latter was readily reversed by treatment with GA (see Tables X and XIV), the former proceeded in the presence of GA (Table XV), even when the IAA/GA ratio was only 1:1000 (Appendix 7).

It is concluded that cell expansion in the pea epicotyl is limited by at least four reactions: one regulated by GA (necessary for elongation), and three others regulated by IAA, BA and ethylene (all leading to lateral epicotyl expansion).

3. The auxins that were most effective in evoking cellulase activity (and protein synthesis) were, in general,

those which were most effective in causing lateral expansion. The relationship between cellulase activity and swelling was not one of simple proportionality, however. Swelling did not necessarily follow from a massive accumulation of cellulase, e.g. extremely high levels of cellulase activity developed after treatment with the auxin herbicides, yet the epicotyl was visibly injured by such agents and growth (and protein synthesis) did not continue for long. Also, substantial swelling could ensue from treatments with low auxin levels and these conditions evoked only modest increases in cellulase activity. Nevertheless, some cellulase increment was detected whenever swelling occurred in every instance in the present series of tests, even after treatments with BA or ethylene and in untreated controls.

Accordingly, some increase in cellulase activity may be required for lateral cell expansion in the pea epicotyl, but concomitant accumulation of the enzyme is not essential.

In addition to these conclusions, a number of problems were raised by present observations which require further investigation:

Detailed studies of anatomical changes in the pea epicotyl, combined with measurements of growth effects in the presence of antibiotic inhibitors, could answer several outstanding questions about the mechanism of action of the regulators in the decapitated pea epicotyl. They could indicate, for example, whether 2,4-D, ethylene and BA all caused swelling

and fragmentation of the same cortical cells as were affected by IAA, whether these regulators evoked cell division as did IAA, and whether their action on growth required DNA, RNA and/or protein synthesis. Ethylene is well known to inhibit cell division in roots (Radin and Loomis, 1969), and it may be that its inhibitory effects on IAA-stimulated swelling and cellulase activity in the pea epicotyl (e.g. Table XV) were due to the prevention of cytokinesis. In contrast, cytokinins are well known to stimulate rooting and cell division (Fox, 1969); and the additive effects of BA and IAA noted throughout this study (e.g. Table XIV) could be due to this phenomenon.

Finally, this study dealt only with one plant tissue system and responses in it of only one enzyme, albeit to a variety of hormones and regulators. A complete picture of specificity should include, of course, a variety of tissues and enzymes. Responses of some 30 other hydrolase activity levels in the pea epicotyl have been tested in the system following IAA treatment, but none of these activities increased to the extent that cellulase activity increased, and none could be considered inducable by this hormone (Datko, 1968; Maclachlan et al., 1970). Such studies need to be done with GA, BA and ethylene. With respect to the auxin herbicides, there is no reason to suspect that they would influence any enzyme that IAA does not affect. However, the mechanism of herbicidal action is unknown and there is a possibility that treatments with 2,4-D etc. are lethal to particular plants (dicots) because of the relative efficiency with which they generate and

accumulate destructive enzyme activities. This could be readily tested by studying responses in a variety of susceptable and resistant plants.

LITERATURE CITED

Abeles, F. B. 1968. Role of RNA and protein synthesis in abscission. Plant Physiol. 43: 1577-86.

Abeles, F. B. 1969. Abscission: role of cellulase. Plant Physiol. 44: 447-52.

Abeles, F. B. and R. E. Holm. 1966. Enhancement of RNA synthesis, protein synthesis and abscission by ethylene. Plant Physiol. 41: 1337-42.

Abeles, F. B. and B. Rubinstein. 1964. Regulation of ethylene evolution and leaf abscission by auxin. Plant Physiol. 39: 963-69.

Andreae, W. A. and N. E. Good. 1955. The formation of indolylacetylaspartic acid in pea seedlings. Plant Physiol. 30: 380-82.

Andreae, W. A., M. A. Venis, F. Jursis and T. Dumas. 1968. Does ethylene mediate root growth inhibition by indole-3-acetic acid? Plant Physiol. 43: 375-7.

Andreae, W. A. and M. W. H. van Ysselstein. 1956. Studies on 3-indoleacetic acid metabolism. III. The uptake of 3-indoleacetic acid by pea epicotyls and its conversion to 3-indoleacetylaspartic acid. Plant Physiol. 31: 235-40.

Audus, L. J. 1959. Plant Growth Substances. Interscience Publ. Inc., New York.

Bentley, J. A. and S. Housley, 1952. Biological activities

of 3-indolylacetaldehyde and 3-indolylacetonitrile. J. Exp. Botany 3: 393-405.

Brian. P. W. 1966. The gibberellins as hormones. Int. Rev. Cytol. 19: 229-266.

Brian, P. W. and H. G. Hemming. 1955. The effect of gibberellic acid on shoot growth of pea seedlings. Physiol. Plant. 8: 669-81.

Brian, P. W. and H. G. Hemming. 1958. Complementary action of gibberellic acid and auxins in pea internode extension. Ann. Botany N. S. 22: 1-17.

Broughton, W. J. 1968. Influence of gibberellic acid on nucleic acid synthesis in dwarf pea internodes. Biochim. Biophys. Acta. 155: 308-10.

Burg, S. P. and E. A. Burg. 1966. The interaction between auxin and ethylene and its role in plant growth. Proc. Natl. Acad. Sci. U. S. 55: 262-92.

Burg, S. P. and E. A. Burg. 1968. Auxin stimulated ethylene formation; its relationship to auxin inhibited growth, root geotropism and other plant processes. In: Biochemistry and Physiology of Plant Growth Substances. Ed. F. Wightman and G. Setterfield. The Runge Press Ltd., Ottawa, Canada. pp. 1275-1294.

Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62: 315-23.

Clarke, A. E. and B. A. Stone. 1962. β-1,3 glucan hydrolases

from the grape vine (Vitis vinifera) and other plants. Phytochem. 1: 175-88.

Clarke, A. J. and P. J. G. Mann. 1957. The oxidation of tryptamine to 3-indolylacetaldehyde by plant amine oxidase. Biochem. J. 65: 763-74.

Cleland, R. E. 1969. The gibberellins. In: Physiology of Plant Growth and Development. Ed. M. B. Wilkins. McGraw-Hill, New York. pp. 49-77.

Datko, A. H. 1968. Development of glucoside hydrolase and pectic enzyme activities in growing pea epicotyl tissue. Ph. D. thesis. McGill University.

Datko. A. H. and G. A. Maclachlan. 1968. Indoleacetic acid and the synthesis of glucanases and pectic enzymes. Plant Physiol. 43: 735-42.

Davies, E. 1968. Formation of cellulase activity by pea ribosomes both in vivo and in vitro. Ph. D. thesis. McGill University.

Davies, E. and G. A. Maclachlan. 1968. Effects of indoleacetic acid on intracellular distribution of β-glucanase activities in the pea epicotyl. Arch. Biochem. Biophys. 128: 595-600.

Davies, E. and G. A. Maclachlan. 1969. Generation of cellulase activity during protein synthesis by pea microsomes in vitro. Arch. Biochem. Biophys. 129: 581-7.

Duda, C. T. 1964. Metabolism of polymeric material during growth of etiolated pea epicotyl sections. M. Sc. thesis. McGill University.

Edelman, J. and M. A. Hall. 1964. Effect of growth hormones on the development of invertase associated with cell walls. Nature 201: 296-7.

Evans, M. L. and D. L. Rayle. 1970. The timing of growth promotion and conversion to indole-3-acetic acid for auxin precursors. Plant Physiol. 43: 240-43.

Fan, D. F. 1967. Studies on regulation of cellulase activity and growth in higher plants with special reference to effects of indoleacetic acid. Ph.D. thesis. McGill University.

Fan, D. F. and G. A. Maclachlan. 1966. Control of cellulase activity by indoleacetic acid. Can. J. Botany 44: 1025-34.

Fan, D. F. and G. A. Maclachlan. 1967a. Studies on the regulation of cellulase activity and growth in excised pea epicotyl sections. Can. J. Botany 45: 1837-44.

Fan, D. F. and G. A. Maclachlan. 1967b. Massive synthesis of ribonucleic acid and cellulase in the pea epicotyl in response to indoleacetic acid, with and without concurrent cell division. Plant Physiol. 42: 1114-22.

Fawcett, C. H. 1961. Indole auxins. Ann. Rev. Plant Physiol. 12: 345-68.

Fawcett, C. H., R. L. Wain and F. Wightman. 1960. The metabolism of 3-indolylalkanecarboxylic acids, and their amides, nitriles and methyl esters in plant tissues. Proc. Roy. Soc. (London), B. 152: 231-54.

Filner, P., J. L. Wray, and J. E. Varner. 1969. Enzyme

induction in higher plants. Science 165: 358-67.

Forsyth, F. R. and D. T. Samborski. 1960. Effects of kinetin and benzylimidazole on the growth of etiolated pea stems and barley coleoptiles. Can. J. Botany 38: 875-81.

Fox, J. E. 1969. The cytokinins. In: Physiology of Plant Growth and Development. Ed. M. B. Wilkins. McGraw-Hill, New York. pp. 85-114.

Fuchs, Y. and M. Lieberman. 1968. Effects of Kinetin, IAA and gibberellin on ethylene production, and their interactions on growth of seedlings. Plant Physiol. 43: 2029-36.

Galston, A. W. and P. J. Davies. 1969. Hormonal regulation in higher plants. Science 163: 1288-97.

Galston, A. W. and H. Warburg. 1959. An analysis of auxin-gibberellin interaction in pea stem tissue. Plant Physiol. 34: 16-22.

Giles, K. W. and A. Myers. 1966. The effects of gibberellic acid and light on RNA, DNA and growth of the three basal internodes of dwarf and tall peas. Phytochem. 5: 193-6.

Goeschl, J. D. and H. K. Pratt. 1968. Regulatory roles of ethylene in the etiolated growth habit of Pisum sativum. In: Biochemistry and Physiology of Plant Growth Substances. Ed. F. Wightman and G. Setterfield. The Runge Press Ltd., Ottawa, Canada. pp. 1229-1242.

Goeschl, J. D., L. Rappaport and H. K. Pratt. 1966. Ethylene as a factor regulating the growth of pea epicotyls subjected to physical stress. Plant Physiol. 41: 877-84.

Gornall, G., C. J. Bardwell and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751-66.

Gourley, J. H. 1931. Anatomy of the transition region of Pisum sativum. Botan. Gaz. 92: 367-83.

Gorter, C. J. 1961. Dwarfism of peas and the action of gibberellic acid. Physiol. Plant. 14: 332-43.

Hall, R. H. 1968. Cytokinins in the transfer-RNA; their significance to the structure of t-RNA. In: Biochemistry and Physiology of Plant Growth Substances. Ed. F. Wightman and G. Setterfield. The Runge Press Ltd., Ottawa, Canada. pp. 47-56.

Hamner, K. C. and E. J. Kraus. 1937. Histological reactions of bean plants to growth promoting substrates. Bot. Gaz. 98: 735-807.

Helgeson, J. P. 1968. The cytokinins. Science 161: 974-81.

Holdgate, D. P. and T. W. Goodwin. 1965. Quantitative extraction and estimation of plant nucleic acids. Phytochem. 4: 831-43.

Holm, R. E., T. J. O'Brian, J. L. Key and J. H. Cherry. 1970. The influence of auxin and ethylene on chromatin-directed ribonucleic acid synthesis in soybean hypocotyl. Plant Physiol. 45: 41-5.

Horton, R. F. and D. J. Osborne. 1967. Senescence, abscission and cellulase activity in Phaseolus. Nature 214: 1086-8.

Imaseki, H., I. Vritani and M. Stahmann. 1968. Production of ethylene by injured sweet potato root tissue. Agr. Biol. Chem. 32: 387-89.

Jones, E. R. H., H. B. Henbest, G. F. Smith and J. A. Bentley. 1952. 3-Indolylacetonitile: A naturally occurring plant growth hormone. Nature 169: 485.

Jones, R. L. 1968. Agar diffusion techniques for estimating gibberellin production by plant organs; the discrepancy between extractable and diffusible gibberellins in pea. In: Biochemistry and Physiology of Plant Growth Substances. Ed. F. Wightman and G. Setterfield. The Runge Press Ltd., Ottawa, Canada. pp. 73-84.

Jones, R. L. and A. Lang. 1968. Extractable and diffusible gibberellins from light- and dark-grown pea seedlings. Plant Physiol. 43: 629-34.

Katsumi, M. 1962. Physiological effects of kinetin. Effect on the thickening of etiolated pea stem sections. Physiol. Plant. 15: 115-21.

Katsumi, M. 1963. Physiological effects of kinetin. Effect of kinetin on the elongation, water uptake and oxygen uptake of etiolated pea stem sections. Physiol. Plant. 16: 66-72.

Kaufman, P. B., L. B. Petering and P. A. Adams. 1969. Regulation of growth and cellular differentiation in

in developing Avena internodes by gibberellic acid and indole-3-acetic acid. Am. J. Botany 56: 918-27.

Key, J. L. 1969. Hormones and nucleic acid metabolism. Ann. Rev. Plant Physiol. 20: 449-584.

Kuraishi, S. and R. M. Muir. 1962. Increase in diffusible auxin after treatment with gibberellin. Science 137: 760-61.

Kuraishi, S. and R. M. Muir. 1964. The relationship of gibberellin and auxin in plant growth. Plant and Cell Physiol. 5: 61-9.

Kuse, G. 1958. Necessity of auxin for the growth effect of gibberellin. Botan. Mag. Tokyo 71: 151-59.

Larsen, P. and T. Aasheim. 1959. The occurrence of indole-3-acetaldehyde in certain plant extracts. In: International Conference on Plant Growth Regulation (Boyce Thomp. Inst.) N.Y. pp. 43-55.

Larsen, P. and R. Rajagopal. 1964. The activity of indole-3-acetaldehyde in relation to that of indole-3-acetic acid in various auxin tests. In: Régulateurs Naturels de la Croissance Végétale. C.N.R.S., Paris. pp. 221-34.

Layne, E. 1957. Methods in Enzymology. Eds. Colowick and Kaplan. 3: 450-1.

Leopold, A. C. 1964. Plant Growth and Development. McGraw-Hill Co., New York. pp. 116-32.

Letham, D. S. 1967. Chemistry and physiology of kinetin-like compounds. Ann. Rev. Plant Physiol. 18: 349-64.

Lockhart, J. A. 1960. Intracellular mechanism of growth inhibition by radiant energy. Plant Physiol. 35: 129-35.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-75.

McCready, C. C. 1966. Translocation of growth regulators. Ann. Rev. Plant Physiol. 17: 283-94.

Maclachlan, G. A., A. H. Datko, J. Rollit and E. Stokes. 1970. Sugar levels in the pea epicotyl: regulation by invertase and sucrose synthetase. Phytochem. (In press).

Maclachlan, G. A., E. Davies and D. F. Fan. 1968. Induction of cellulase by 3-indoleacetic acid. In: Biochemistry and Physiology of Plant Growth Substances. Ed. F. Wightman and G. Setterfield. The Runge Press Ltd., Ottawa, Canada. pp. 443-53.

Maclachlan, G. A. and J. Perrault. 1964. Cellulase from pea epicotyls. Nature 204: 81-2.

MacLeod, A. M. and A. S. Millar. 1962. Effects of gibberellic acid on barley endosperm. J. Inst. Brewing 68: 322-32.

MacQuarrie, I. G. 1965. Some responses of etiolated seedlings of Pisum sativum L. to applied 3-indoleacetic acid in relation to apical dominance. Can. J. Botany 43: 29-38.

Michener, H. D. 1938. The action of ethylene of plant growth. Am. J. Botany 25: 711-20.

Middleton, K. R. 1960. New Nessler reagent and its use in the direct Nesslerisation of Kjeldahl digests. J. Appl.

Chem. 10: 281-6.

Miller, C. O., F. Skoog, M. H. Von Saltza, and F. M. Strong. 1955. Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc. 77: 1329.

Moreland, D. E. 1967. Mechanisms of action of herbicides. Ann. Rev. Plant Physiol. 18: 365-86.

Morré, T. C. and C. A. Shaner. 1967. Biosynthesis of indoleacetic acid from tryptophan-^{14}C in cell-free extracts of pea shoot tips. Plant Physiol. 42: 1787-96.

Muir, R. M. and B. P. Lantican. 1968. Purification and properties of the enzyme system forming indoleacetic acid. In: Biochemistry and Physiology of Plant Growth Substances. Ed. F. Wightman and G. Setterfield. The Runge Press Ltd., Ottawa, Canada. pp. 259-72.

Nitsan, J. and A. Lang. 1965. Inhibition of cell division and cell elongation in higher plants by inhibitors of DNA synthesis. Develop. Biol. 12: 358-76.

Ockerse, R. and A. W. Galston. 1967. Gibberellin-auxin interaction in pea stem elongation. Plant Physiol. 42: 47-54.

van Overbeek, J. 1938. Auxin distribution in seedlings and its bearing on the problem of bud inhibition. Botan. Gaz. 100: 133-66.

van Overbeek, J. 1966. Plant hormones and regulators. Science 152: 721-31.

Paleg, L. G. 1965. The physiological effects of gibberellins.

Ann. Rev. Plant Physiol. 16: 219-322.

Pratt, H. K. and J. D. Goeschl. 1969. Physiological roles of ethylene in plants. Ann. Rev. Plant Physiol. 20: 541-84.

Priestly, J. H. 1926. On the anatomy of etiolated plants. New Phytol. 25: 144-70.

Purves, W. K. and W. S. Hillman. 1958. Response of pea stem sections to indoleacetic acid, gibberellic acid and sucrose as affected by length and distance from the apex. Physiol. Plant. 11: 29-35.

Purves, W. K. and W. S. Hillman. 1959. Experimental separation of gibberellin and auxin actions in etiolated pea epicotyl sections. Physiol. Plant. 12: 786-98.

Radin, J. W. and R. S. Loomis. 1969. Ethylene and carbon dioxide in the growth and development of cultured radish roots. Plant Physiol. 44: 1584-9.

Reed, D. J. 1968. Tryptophan decarboxylation by cell-free extracts of Alaska pea epicotyls. In: Biochemistry and Physiology of Plant Growth Substances. Ed. F. Wightman and G. Setterfield. The Runge Press Ltd., Ottawa, Canada. pp. 243-58.

Reese, E. T. and M. Mandels. 1963. Enzymic hydrolysis of β-glucans. In: Advances in Enzymic Hydrolysis of Cellulose and Related Materials. Ed. E. T. Reese. Macmillan Company, New York. pp. 197-234.

Ridge, I. and D. J. Osborne. 1969. Cell growth and cellulases:regulation by ethylene and indole-3-acetic

acid in shoots of Pisum sativum. Nature 223: 318-19.

Russell, D. W. and A. W. Galston. 1969. Blockage by Gibberellic acid of phytochrome effects on growth, auxin responses, and flavenoid synthesis in etiolated pea internodes. Plant Physiol. 44: 1211-16.

Scott, F. M. 1938. Anatomy of auxin treated etiolated seedlings of Pisum sativum. Botan. Gaz. 100: 167-85.

Scott, T. K. and W. R. Briggs. 1963. Recovery of native and applied auxin from the dark-grown Alaska pea seedling. Am. J. Botany 50: 652-57.

Skoog, F. and N. J. Leonard. 1968. Sources and structure: activity relationships of cytokinins. In: Biochemistry and Physiology of Plant Growth Substances. Ed. F. Wightman and G. Setterfield. The Runge Press Ltd., Ottawa, Canada. pp. 1-18.

Sommer, N. F. 1961. Longitudinal and lateral response of etiolated pea sections to indoleacetic acid, gibberellin, kinetin, sucrose and cobaltous chloride. Physiol. Plant. 14: 741-9.

Sprent, J. I. 1968. Effects of benzyladenine on the growth and development of peas. Planta 81: 80-7.

Street, H. E. 1966. The physiology of root growth. Ann. Rev. Plant Physiol. 17: 315-44.

Sudi, J. 1964. Induction of the formation of complexes between aspartic acid and indolyl-3-acetic acid or 1-naphthalene acetic acid by other carboxylic acids. Nature 201: 1009-10.

Thimann, K. V. 1951. The synthetic auxins: relation between structure and activity. In: Plant Growth Substances. Ed. F. Skoog. University of Wisconsin Press. pp. 21-36.

Thimann, K. V. 1954. The physiology of growth in plant tissues. Amer. Sci. 42: 589-606.

Thimann, K. V. and S. Mahadevan. 1958. Enzymatic hydrolysis of indoleacetonitrile. Nature 181: 1466-67.

Thimann, K. V. and F. Skoog. 1933. Studies on the growth hormone of plants. III. The inhibiting action of the growth substance on bud development. Proc. Natl. Acad. Sci. U.S. 19: 714-16.

Tracey, M. V. 1950. Cellulase from leaves and roots of tobacco. Biochem. J. 47: 431-3.

Venis, M. A. 1964. Induction of indoleacetylaspartate synthesis. Nature 202: 900.

Wightman, F. and D. Cohen. 1968. Intermediary steps in the enzymatic conversion of tryptophan to IAA in cell-free systems from mung bean seedlings. In: Biochemistry and Physiology of Plant Growth Substances. Ed. F. Wightman and G. Setterfield. The Runge Press Ltd., Ottawa, Canada. pp. 273-88.

Young, M. R. 1962. Cell wall metabolism and growth of etiolated pea epicotyls. M. Sc. thesis. University of Alberta.

Zenk, M. H. 1962. Aufnahme und stoffwechsel von α-naphthyl-Essigsäure durch Erbsenepicotyle. Planta 58: 75-94.

Zwar, J. A. and F. Skoog. 1963. Promotion of cell division by extracts from pea seedlings. Aust. J. Biol. Sci. 16: 129-39.

Appendix I

Inhibition of Growth, Protein Synthesis and Cellulase Induction by Enclosure of Seedlings in 8.5 litre Jars*

Treatment* and time	Expt. number	Fr wt / length (mg/mm)	Soluble protein (µg/seg)	Cellulase activity (units/seg)
Jars closed continuously				
Zero time	2	2.56	160	0.08
3 days, +IAA		3.48	250	0.16
Jars opened twice daily, flushed with air				
Zero time	5	1.88	150	0.33
3 days, +IAA		5.12	980	5.12

*Treatment of pea seedlings and protein and cellulase assays are described in Methods.

Appendix 2

Relationship between Protein Concentration and Optical Density as Measured by the Biuret Method*

Protein (mg/ml stock solution)	$O.D._{545}$	$O.D._{545}$ per mg protein
1.0	0.040	0.040
2.0	0.080	0.040
3.0	0.120	0.040
4.0	0.174	0.044
5.0	0.240	0.048

Average value = 0.042 O.D. units/mg protein.

*For assay, 1.0 ml stock solution containing bovine serum albumin was mixed with 3 ml water plus 3 ml biuret reagent, see Methods, Section D. Values are corrected for reagent blanks.

Appendix 3

Relationship between Protein Concentration and Optical Density as Measured by the Lowry Method*

Protein (mg/ml stock solution)	$O.D._{750}$	$O.D._{750}$ per µg protein
5.0	0.094	0.019
10.0	0.168	0.017
15.0	0.268	0.017
20.0	0.308	0.015
25.0	0.395	0.016
30.0	0.449	0.015

Average value = 0.017 O.D. units/µg protein.

*For assay, 0.4 ml stock solution containing bovine serum albumin was mixed with 3.6 ml Lowry's reagent "C" and 0.4 ml Lowry's reagent "E", see Methods, Section D. Values were corrected for reagent blanks.

Appendix 4

Relationship between Nitrogen Concentration and Optical Density as Measured by Nessler's Reagent*

Nitrogen (µg/ml stock solution)	$O.D._{425}$	$O.D._{425}$ per µg Nitrogen
1.0	0.040	0.04
2.0	0.115	0.06
3.0	0.190	0.06
5.0	0.298	0.06
8.0	0.383	0.05
10.0	0.465	0.05
12.0	0.593	0.06
15.0	0.685	0.05
17.0	0.895	0.05

Average value = 0.054 O.D. units/µg nitrogen.

*For assay 1.0 ml stock solution containing ammonium sulphate was mixed with 1.0 ml Nessler's "A", 1.0 ml Nessler's "B", 1.0 ml Nessler's "C", and 2.0 ml Nessler's "D", see Methods, Section D. Values were corrected for reagent blanks.

Appendix 5

Relationship between RNA Concentration and Optical Density

RNA (µg/ml stock solution)	$O.D._{260}$	$O.D._{290}$	$O.D._{260-290}$	$O.D._{260-290}$ per µg RNA
5.0	0.148	0.073	0.075	0.015
10.0	0.240	0.105	0.135	0.014
15.0	0.348	0.138	0.210	0.014
20.0	0.465	0.195	0.270	0.014
30.0	0.688	0.283	0.405	0.014
40.0	0.940	0.370	0.570	0.014
50.0	1.160	0.420	0.740	0.015

Average value = 0.014 O.D. units/µg RNA.

*For assay, stock solution of wheat germ RNA in 0.5N perchloric acid was used directly for O.D. measurements in a Beckman DB Spectrophotometer.

Appendix 6

Relationship between DNA Concentration and Optical Density as Measured After Reaction with Diphenylamine*

DNA (µg/ml stock solution)	$O.D._{600}$	$O.D._{540}$	$O.D._{600-540}$	$O.D._{600-540}$ per mg DNA
21.6	0.145	0.067	0.078	3.61
54.0	0.315	0.145	0.170	3.15
108.0	0.655	0.305	0.350	3.24

Average value = 3.33 O.D. units/mg DNA.

*For assay, DNA in 0.5N perchloric acid was used following reaction with diphenylamine for O.D. measurements in a Bausch and Lomb "Spectronic 20" Spectrophotometer (see Methods, Section E).

Appendix 7

Interference by IAA in the Elongation Response to GA*

Untreated	Treated with 5 µg GA ± IAA (µg/epi)				
(controls)	0	0.005	0.05	0.5	5.0
	Length (mm)				
13.5	17.7	13.7	12.3	12.8	11.9
	Fr wt/length (mg/mm)				
3.5	3.5	3.7	3.9	4.4	4.7

*At zero time, average length measured 10.8 ± 1.5 mm, and fr wt was 1.9 mg/mm; above measurements were made after 3 days treatment. Note that there was considerable swelling in the segment even in untreated controls in this particular experiment, suggesting that endogenous auxin levels were unusually high.