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STUDIES ON REGULATION OF CELLULASE ACTIVITY AND GROWTH IN HIGHER PLANTS WITH SPECIAL REFERENCE TO EFFECTS OF INDOLEACETIC ACID

Der-Fong Fan

Quantitative methods were developed for extracting and estimating the amount of cellulase (β-1, 4-glucan 4-glucanohydrolase) in tissues from etiolated seedlings of Pisum sativum L. var. Alaska and other higher plants. Factors which regulate the metabolism of this enzyme in the pea epicotyl were studied, with special attention given to effects of indoleacetic acid (IAA) and inhibitors of DNA, RNA and protein synthesis. The results led to conclusions that cellulase:

1. has a relative high turnover rate, due possibly to its denaturation in the cell wall;

2. is only synthesized in the presence of IAA, which appears to be required for transcription of the specific form of ribonucleic acid (e.g., messenger RNA) needed to code for this enzyme;

3. has effects in the primary wall which lead to cell expansion.

STUDIES ON REGULATION OF CELLULASE ACTIVITY AND GROWTH IN HIGHER PLANTS WITH SPECIAL REFERENCE TO EFFECTS OF INDOLEACETIC ACID

by

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PREFACE

This thesis is organized in the following way: after a "GENERAL INTRODUCTION" and a "REVIEW OF LITERATURE", the "EXPERIMENTS AND RESULTS" section consists of three papers incorporated directly in the form in which they were written for publication. The first and second papers (86, 87) have been published and the third paper (88) is in press. In order to simplify organization in this thesis, changes were made in Table, Figure and Reference numbers so that these now appear here consecutively. All references are included in the section "LITERATURE CITED". Supplementary data are given in the "APPENDIX" and new references to these data have been inserted at appropriate points in "EXPERIMENTS AND RESULTS". Some of the data in the "APPENDIX" is due to be published in a fourth paper (186).

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GENERAL INTRODUCTION

The rate of expansion of plant cells can be affected by many substances and conditions in the cell environment. Some of these substances, (e.g., osmotically-active agents) affect turgor pressure in the cell which must, of course, be positive before water uptake can occur (8, 130, 234). Other substances or conditions (e.g., oxygen tension) affect respiration rate and their influence on cell expansion undoubtedly reflects a requirement for an energy source during growth (32,33). A variety of substances (e.g., antibiotics) which inhibit synthesis of protein or ribonucleic acid (RNA) usually also inhibit cell expansion, suggesting that newly-formed enzymes are needed to catalyze reactions essential for growth (63, 162, 202, 224, 226, 245). Plant growth hormones of the auxin type have no effect on osmotic pressure (8) and little effect on respiration rate (33, 58) but recent research has established that such hormones often stimulate the synthesis of both protein and RNA (6, 162, 224, 226, 239). It is widely agreed, therefore, that at least part of the mechanism whereby auxins regulate growth involves enhancement of the synthesis of essential enzymes. The problem is to identify these enzymes and explain the mechanism(s) whereby auxin induces them.

The present study deals mainly with effects of the auxin indoleacetic acid (IAA) on synthesis of cellulase (β-1, 4-glucan 4-

glucanohydrolase EC 3·2·1·4) in young plant tissues. Most of the research was carried out with tissue growing at the apex of etiolated pea epicotyls. At the time when this study was begun it was known that both cellulase (184) and natural auxin (285) are particularly concentrated in this region of the epicotyl. When tissue sections are removed and grown without added substrates, part of the cell wall glucan disappears during incubation (183, 185). When the tissue is left attached to the decapitated seedling and IAA is applied at the apex, the hormone causes swelling and fragmentation of parenchyma cell walls (188, 284). There were grounds, therefore, for suspecting the existence of a relationship between auxin and the amount of active cellulase in this tissue.

There were also grounds for supporting the view that action of cellulase on cellulose microfibrils <u>in vivo</u> could enhance the rate of cell expansion. Primary walls in young plant cells contain a framework of cellulose microfibrils which have a complex orientation that changes during cell expansion (253, 254, 255, 279, 288, 325, 338). The fibrils clearly slide apart when the wall increases in area. Nevertheless, they are so interwoven that it is difficult to visualize how this could occur to the extent it does during growth without some fibril breakage. Hydrolysis of fibrils by cellulase action is well known to be very effective at reducing the tensile strength of cellulose-containing materials [e.g., cotton thread (248, 249, 286) and dead oat coleoptile cell walls (232)].

About 30 years ago it was suggested (130) that growth requires an auxin-induced "loosening" or "plasticization" of the primary cell wall.

The fact that the rigidity and strength of the wall is indeed a major factor in restricting the rate and extent of cell expansion is emphasized by many subsequent studies which demonstrate a relaxing effect of auxin on wall plasticity. Auxin treatment of living tissue rapidly brings about an increase in its extensibility as measured under artificial stress and this reaction occurs before any effects on growth can be detected (62, 210, 260). Although many theories have been advanced since to explain such effects, none has gained wide acceptance.

This thesis presents data which demonstrate that IAA treatment enhances the amount and specific activity of cellulase during growth. Effects of inhibitors leads to the conclusion that IAA regulates synthesis of this enzyme, the activity of which may, in turn, increase wall plasticity and facilitate cell expansion.

REVIEW OF LITERATURE

I. Metabolism of Wall Materials During Growth

A. Changes in Composition

The main categories of substances present in walls of young plant tissues are cellulose, hemicellulose, pectic substances, protein and lipid. Many analyses have been made of the relative amounts of these components, e.g., in tissues from pea epicotyl (185, 204), oat coleoptile (14, 28, 53, 264, 304), maize coleoptile (167), maize mesocotyl (278), onion root (147), maple cambium (306, 307). Except for cellulose, there is little agreement on methods suitable for separating these categories of wall materials from one another or from similar substances in other parts of the cell. It is not surprising, therefore, that wall composition appears to vary widely in different tissues, The most common constituents reported as subfractions of hemicellulose and pectic substances are xylans, glucans, galactans, arabans, polyglucuronates, polygalacturonates. The relative amounts of these materials also appear to vary from one young tissue to another.

α-cellulose is usually isolated as the β-1, 4-glucan insoluble in alkali (approx. 17% NaOH). On analysis this fraction is always contaminated by traces of other polysaccharides and it may contain sugar

units other than glucose covalently bonded within the molecular chains (229, 254). Nevertheless, this has been the most widely reproduced and homogeneous wall fraction examined in the above studies on wall composition in growing tissues. It was generally found to make up from 20 to 40% of the total dry weight of the wall. From studies on wall ultrastructure using techniques of electron microscopy, X-ray diffraction, etc., cellulose is clearly present in the wall in the form of a partially crystalline framework of microfibrils (94, 229, 238, 324, 325). These fibrils are interwoven in young cells with the predominant orientation generally in a transverse direction (253, 255, 278, 279, 288). Hemicellulose appears to be deposited on these fibrils with protein and pectic materials localized mainly in the middle lamella (85).

Enzyme systems have been described which synthesize cellulose (16, 17, 44, 81, 125, 237), β-1, 3-glucan (89, 125), xylan (13, 125), polygalacturonic acid (125, 178, 319, 320) and galactan (206). All of these enzymes require a sugar nucleotide as precursor. In most instances synthesis <u>in vitro</u> is also dependent on the presence of a primer molecule to which sugar units are transferred. Thus, the average molecular chain length of these components would be expected to increase during synthesis. Accurate measurements of molecular weight of wall components of young tissues have only been reported for cellulose. During elongation of cotton seed hairs, cellulose synthesis takes place and the average degree of polymerization increases from values of 2,000 to 3,000 to about 14,000 in the mature hair (198, 199). The site of cellulose synthesis is not known; it may be in the wall or possibly at or near the protoplasm surface in

cytoplasmic microtubules (213, 218, 230, 329). There is clear evidence, e.g., from radioautographic electron microscopy, that synthesis of pectic materials and hemicellulose takes place within the cytoplasm in the golgi apparatus and these products are then transported to the wall within the vesicles (173, 213, 219).

Normal plant growth is always accompanied by an increase in total amount of cell wall material and this increase is stimulated by added auxin (14, 21, 31, 53, 262, 263, 296, 334, 336, 337). Many attempts have been made to specify which of the various wall components are synthesized at rates that correlate with the growth rate. However, there is no agreement between results of such studies on different tissues. Correlation to some degree has been noted between growth and the synthesis of cellulose [e.g., in oat coleoptile (265) and pea epicotyl (185, 334)], pentosans [e.g., in potato tuber tissue (46) and tobacco pith tissue (337)], pectic substances [e.g., in tobacco pith tissue (337) and oat coleoptile (265)] and pectic methyl ester groups (e.g., in oat coleoptile (2, 61, 233, 235)]. The most extensive study of changes in levels of wall materials during normal growth has been carried out with onion root tips (147). The general conclusion of the study was that all major categories of wall materials were synthesized during radial cell enlargement and cell elongation and maturation. Thus, no specific effect of auxin on synthesis of a particular wall component during growth has yet been established.

In view of this apparent variability between tissues, it can be questioned whether there exists any one component of the wall, the synthesis of which is obligatory for growth. Indeed, conditions, admit-

tedly abnormal, have been described where some growth takes place without wall synthesis. Pea epicotyl and oat coleoptile sections floated on buffer without substrates (21, 183, 185, 204) or incubated at low temperature (31, 204, 262) may expand, in some instances considerably, without detectable increase in total wall material. Several of the studies of wall synthesis in growing tissue revealed the fact that the amount of some wall components actually decrease during growth. Thus, there is a fall in galactan level in growing pea epicotyl sections (185, 204), oat coleoptile sections (264), angiosperm xylem (306) and pear parenchyma (149). There is also a decrease in glucan fraction in the first three of the above tissues. The identity of this glucan is unknown. It appears to be alkali-soluble (264, 306) and readily hydrolysable by hot dilute acid (185). It could represent a hemicellulose or possibly amorphous (accessible) regions of cellulose. There have been occasional reports of a fall in level of total hemicellulose and pectin fractions (204) during growth but there is no report of a decrease in total cellulose level.

The mere existence of polysaccharidases in growing tissue which can hydrolyse wall materials provides added evidence that these materials are at least potentially unstable. Thus, cellulases have been found widely distributed in higher plants, including many growing tissues (see Section III). β-1, 3-glucanase (55), pectinase (22, 23) and pectinesterase (105, 106, 107, 217) have also been described in young plant tissues. There are few reports of the existence of xylanase or galactanase in plants and no indication that these enzymes occur in growing

regions. With respect to the intracellular distribution of these enzymes, studies have only been reported on pectinesterase which is found firmly bound to wall materials (105, 106, 107). There is no doubt that other enzymes, e.g., invertase (12, 78, 217), are localized in the wall or outer cytoplasmic membrane.

A few studies have been made of turnover in wall materials using the technique of pulse-labelling or by measuring discrepancies between incorporation of a labelled precursor and change in total concentration of the product. Evidence has been obtained for concurrent synthesis and breakdown of glucan in the youngest and most active growing parts of pea epicotyl sections (183, 185, 204) and wheat coleoptiles (197). Soluble fractions of pectic material may also turn over in the pea epicotyl (204) and oat coleoptile (2, 61). There is considerable evidence for turnover of methyl groups in esterified pectic acid (61, 235). Auxin has been reported to stimulate turnover of total wall material in the pea epicotyl (204) and oat coleoptile (236).

It may be concluded that growth is normally accompanied by synthesis of all wall components and, at the same time, some components are subject to partial degradation. It has not yet been clearly established whether synthesis or breakdown of any one component is essential for growth or specifically affected by auxin.

B. Theories of Wall Loosening

In order for the area of the primary wall to increase as it must do during cell expansion and elongation, it is generally agreed

that reactions variously referred to as loosening (261), relaxing (99), softening (26), plasticizing (130) or leading to increased flexibility (1) must take place. Many observations using a variety of physical methods confirm that auxin treatment very rapidly (i.e., before growth effects occur) causes an increase in extensibility of the primary wall (62, 64, 65, 130, 210, 260). The problem is to identify the events which enhance or restrict wall extensibility.

About 10 years ago, alterations in the gelling properties of pectin appeared to be the most promising mechanism for regulating wall extensibility. It was suggested that the well-known inhibitory effect of calcium ions on growth was due to the formation of rigid (gelled) calcium pectate and that this substance was the main component of the wall which contributed to wall rigidity (70). In this event, auxin would have to remove endogenous calcium from the wall before it could increase the plastic properties of the wall (25, 46). Since the degree of methylation of pectic carboxyl groups could control the degree of calcification, it was proposed that auxin might stimulate growth by increasing the extent of pectic methylation (233, 235).

In intervening years these possibilities were subjected to several experimental tests with generally negative results so that, at present, there are few proponents of pectin methylation or calcification as major growth-regulating reactions. Thus, low calcium levels (10^{-3} M–10^{-5} M) often stimulate growth, e.g., in wheat roots (45). Auxin treatment does not alter the amount of distribution of bound wall calcium (^{45}Ca) in oat and maize coleoptiles (59) in experiments where the hormone

promotes growth. Auxin may actually stimulate calcium uptake during growth in pea epicotyl (204) and oat coleoptile sections (68). In oat coleoptiles careful measurements showed no relationship between rates of pectic methylation and growth (60, 61). Moreover, pectinase solutions containing pectinesterase would be expected to inhibit growth in calcium but, in fact, such solutions cause swelling and deformity [e.g., root hairs (69)]. Finally, it has been objected that the amounts of pectic materials in many young walls are too small for this substance to control wall rigidity (2, 28, 144, 147).

Various theories analogous to the pectin hypothesis have been advanced in which it is suggested that wall rigidity may be regulated by salt-linkages, co-valent bonds or the degree of branching of wall proteins (104, 221) or hemicelluloses (40, 185, 200, 267, 288). Proteins rich in hydroxyproline have been found localized in plant cell walls and it has been proposed that cross-linkages between these proteins and polysaccharides may control wall extensibility (171, 218). At the present time there is insufficient evidence to support or oppose these possibilities.

The above theories are all based on the assumption that cellulose microfibrils slide or slip apart during cell expansion and that matrix wall materials regulate the ease with which this happens, There is an abundance of evidence that microfibrillar orientation does indeed alter during growth and some movement apart undoubtedly occurs (94, 252, 253, 255, 279, 288, 325). However, it is not established that matrix materials control this movement or that a simple sliding mechanism is sufficient to explain the observed degree of fibril re-orientation.

Accordingly, the possibility must be entertained that fibril re-orientation requires breakage or partial degradation of cellulose. Nickerson and Bartnicki-Garcia (221) in a recent (1964) review of cell expansion remarked that "while it is reasonable to accept the mechanical view that osmotic pressure is the driving force for displacement of cell wall molecules during growth, the neglected role of biochemical cleavage of cell wall complexes should be emphasized. It is known that a cell may possess enzymes capable of splitting its own wall, ...action...of these enzymes may well play a decisive role in growth processes." Direct loosening of the integrity of the cellulose framework by the action of cellulase was first proposed at least 15 years ago (83, 310). Moreover, in the first issue (1950) of the Annual Review of Plant Physiology, Frey-Wyssling (95) speculated that "...if plasticizing of the primary wall is due to an enzymatic digestion of microfibrils, ...then one of the secondary effects of auxin may be an activation of the enzymes in this system." Frey-Wyssling has since favored a variety of other explanations for growth and auxin action and, up to the time when the present research was begun, little attention had been given to this proposal. It is still not widely realized that cellulase occurs in many higher plant tissues and its existence in higher plants is occasionally denied even by reviewers today (103). Nevertheless, there is no question that action of this enzyme can rapidly reduce the tensile strength of cellulose (232, 286) and cause the cleavage of microfibrils (248, 249). If such reactions occurred in growing tissues, there could be little doubt that they would contribute to wall loosening.

II. Ribonucleic Acid and Protein Metabolism During Growth

A. Ribonucleic Acid and Protein

It has been repeatedly demonstrated that a close relationship exists in growing tissues between total RNA level and growth rate, e.g., in pea roots (96) and epicotyls (96), corn roots (340, 341), Vicia faba roots (133) and artichoke tubers (203). Since most of the total RNA of young cells is ribosomal RNA, it is generally assumed that growth rate is dependent on the capacity of tissues for synthesizing protein. However, there is generally no close relationship between growth rate and total protein level (35, 54, 159, 224, 303). Therefore, it appears that growth must depend on synthesis of particular proteins.

It has also been shown that there are changes in the kinds of RNA formed during growth. In growing pea roots (128) and peanut cotyledons (49), the base ratios in RNA alter, as do physical properties of the ribosomes in these tissues. On this basis alone it could be predicted that the kinds of proteins synthesized during growth should vary at different growth stages. This has been confirmed, not only by measuring changes in amount of various protein fractions during growth (41, 128) but also by many demonstrations that individual enzymes form or disappear at different times during development (9, 10, 11, 71, 274, 275, 276). There may exist a definite succession of enzymes during growth in any one tissue (128). Except for ribonuclease (18, 19, 174),

however, no single group of enzymes has yet been found to be repeatedly associated with growth in all tissues. The functions of ribonuclease in growth is notunderstood.

Auxin treatment has been shown to increase total RNA levels in soybean tissue, most markedly when the tissue remained attached to the seedling (163) but also when it was excised and floated on solution (161, 162). In excised tissues total RNA levels usually decline during incubation, nevertheless a stimulation of RNA synthesis from labelled precursors has regularly been demonstrated, e.g., in tobacco pith tissue (293), wheat coleoptile (311), oat coleoptile (29, 118, 201), artichoke tuber tissue (202), soybean hypocotyl (161, 162). In attached soybean tissue most of the auxin-induced RNA was ribosomal RNA (163). In detached sections, extra label appears in all RNA sub-fractions, including messenger RNA (160). Particular importance has recently been placed on formation of messenger RNA during growth. This is the main fraction synthesized in growing pea root sections (181) and soybean hypocotyl sections in the presence of 5-fluorouracil (160).

Auxin treatment increases total protein levels in attached soybean tissue (163) but it has little effect on total protein in most detached sections (35, 54, 159, 224, 303). Nevertheless the uptake of labelled amino acids into protein is stimulated by auxin in pea stem sections, artichoke tuber tissue (224) and soybean hypocotyl (162). It has not been clearly established whether this reflects a direct stimulation by auxin of translational steps in protein synthesis or whether it is an indirect consequence of auxin-induced RNA synthesis. Either mechanism

of action could explain the fact that auxin treatment undoubtedly increases the amount and specific activity of particular enzymes. Studies of such effects up to 1961 are reviewed by Cleland (58). Since then, auxin has been reported to have this effect on indoleacetyl aspartate synthestase in pea epicotyls (317, 318), isocitrate lyase in potato tubers (73), invertase in artichoke tubers and chicory roots (93) and peroxidase in pea stems (100). Clear documentation of hormone-induced enzyme synthesis in plants has been reported for α-amylase in gibberellin-treated barley aleurone cells (51, 52, 240, 241, 242, 314, 315) and for lipase in gibberellin-treated germinating cotton seeds (30).

Regardless of the site(s) of auxin action, the view is now widely held that synthesis of messenger RNA and the enzymes for which it acts as a code, is an event essential for growth. Apart from the evidence cited above, there is no doubt that many substances, e.g., antibiotics, which inhibit protein and/or RNA synthesis also inhibit growth. Details of such experiments are discussed below.

B. Inhibitors of Nucleic Acid and Protein Synthesis

A vast number of antibiotics and synthetic chemicals, e.g., precursor analogues, are known to inhibit particular reactions essential for the synthesis of nucleic acid and/or protein. The action mechanisms of many of these inhibitors have often been reviewed (43, 97, 131, 164, 205, 220). The following inhibitors have been used in the present study; discussion is limited to their mechanism of action and their effects on higher plant tissues, where known.

Actinomycin D:

Effects of this antibiotic have been reviewed recently (271). It is a polypeptide-containing pigmented antibiotic (321, 322). In vitro, at low concentrations (10 µg/ml or less) it inhibits transcription of DNA by RNA polymerase and therefore it prevents synthesis of all forms of RNA; higher concentrations (100 µg/ml or more) are required to inhibit replication of DNA by DNA polymerase (109, 111, 136, 150, 153, 166, 272). The inhibitor acts by binding to the guanine residue in DNA (47, 102, 108, 110, 112, 117, 122, 152, 155). These conclusions are based on observations with preparations from micro-organisms and animal tissues. However, they probably also can be applied to plant tissues since actinomycin D has been shown to inhibit the incorporation of nucleotides into RNA by isolated pea nuclei (50, 280).

In vivo, actinomycin D severely inhibits the incorporation of labelled precursors, e.g., orotate, into RNA of the excised pea epicotyl section (65, 210, 317, 318), oat coleoptile (118), soybean hypocotyl (65, 161, 162), potato tuber slice (63, 170) etc.. At the same time, actinomycin D also inhibits incorporation of supplied amino acids, e.g., ^{14}C-leucine, into protein (63, 162, 226). Actinomycin D prevents the action of auxin in stimulating nucleic acid synthesis [e.g., in pea epicotyl (65, 210, 317, 318), oat coleoptile (62, 65, 118, 201, 226), soybean hypocotyl (65, 160, 161, 162) and corn mesocotyl (65, 160)], and protein synthesis [e.g., in oat coleoptile (62, 226) and soybean hypocotyl (162)]. It is usually presumed that the inhibition of protein synthesis is indirect, resulting from dependence of the

process of translation on continued RNA synthesis.

Actinomycin D can also inhibit the transport or absorption into plant tissue sections of nucleic acid precursors (65, 226), amino acids (162, 226) and sugar (15). This raises the possibility that part of the inhibition of nucleic acid and protein synthesis from precursors results from interference with the permeability of tissues and therefore internal precursor pool size.

Actinomycin D reduces endogenous growth and prevents auxin-induced elongation of pea epicotyl and stem sections (226, 245) and many other excised plant tissues (63, 118, 201, 202, 203, 224). Early tests with the oat coleoptile (62) suggested that cell wall plasticity was not affected by concentrations of actinomycin D which inhibited 85% of RNA synthesis. This degree of inhibition was estimated on the basis of the net effect of the inhibitor on incorporation of ^{14}C-orotate. It may be questioned whether the calculation was legitimate in view of the inhibitory effects (noted above) on precursor uptake. When this test was repeated with the pea epicotyl (65, 210), oat coleoptile (65, 201) and other plant tissues (65, 202, 210) actinomycin D did prevent effects of IAA on wall plasticity. In general, these studies have provided some of the strongest evidence available to support the view that plant growth and, in particular, wall loosening, depends on RNA and protein synthesis.

Puromycin:

Effects of puromycin on protein synthesis have been reviewed recently (72). It is a structural analogue of adenosylphenylalanine (323).

It blocks synthesis of protein by ribosomal preparations from lower organisms or animal tissues in vitro (3, 4, 5, 36, 76, 114, 135, 214, 216, 308). Inhibitory action is due to the direct incorporation of puromycin into growing peptide chains which prevents further incorporation of amino acids and causes release of incomplete, soluble peptides (211, 215, 343).

Puromycin inhibits protein synthesis in isolated pea nucleoli (27). It also inhibits incorporation of labelled precursor into both protein and RNA by pea epicotyl sections (317, 318), potato tuber slices (63, 170), soybean hypocotyls (162) and oat coleoptiles (224, 226).

Inhibitory effects on plant growth (224, 226, 245, 318), amino acid uptake (162, 226) and glucose uptake (15) are very similar to those of actinomycin D. There is one report of the stimulation of enzyme synthesis by puromycin in higher plants (318).

Chloramphenicol:

Effects of this antibiotic were reviewed in 1961 (39). It is a nitrophenyl derivative of dichloroacetic acid (277) which prevents protein synthesis by inhibiting the transfer of amino acid from aminoacyl -sRNA to protein. This effect appears to be the result of a chloramphenicol-ribosome complex which interferes with the attachment of m-RNA to the ribosome (57, 79, 115, 208, 266, 316, 327, 339). It prevents protein synthesis in bacteria and animal tissues both in vitro (145, 146, 326) and in vivo (7).

In higher plants, chloramphenicol also inhibits protein synthesis, e.g., in oat coleoptiles (225), carrot roots (142) wheat endosperm (212),

isolated maize microsomes (212, 259) and tobacco cell nuclei (90, 143). In general, higher concentrations are required to inhibit protein synthesis in plants and animal tissues than in bacteria. Chloramphenicol inhibits endogenous and auxin-induced growth in a variety of tissues, e.g., in pea epicotyl and green pea stem sections (224, 225, 245), oat coleoptiles (224, 225) and other plant tissues (225). This growth inhibition closely parallels the inhibition of ^{14}C-leucine incorporation into protein (224, 225). Other inhibitory effects have also been reported, e.g., on salt and water uptake (82, 120, 300, 313), specific amino acid uptake (142), sugar uptake (15, 244) and oxidative reactions (169).

Actidione (cycloheximide):

This antibiotic inhibits both protein and DNA synthesis but not RNA synthesis in mammalian and yeast cells (154, 291, 292, 299). In vitro, it has effects similar to puromycin in that it inhibits protein synthesis by isolated ribosomes. It appears to inhibit the final steps of translation, i.e., the transfer of amino acid from s-RNA to peptides (39, 66, 75, 92). It inhibits the growth of some but not all microorganisms (330).

Effects of actidione on growth of higher plants have not been reported. However, it inhibits formation of fatty acid synthetase in potato tuber slices (335) and nitrate reductase in Brassica leaves (127).

Fluorodeoxyuridine (FUdR):

A review of the effects of fluorinated pyrimidines on nucleic acid and protein synthesis has been published recently (126). FUdR is a structural analogue of thymidine and inhibits specifically the synthesis

of thymidylate (91). In vitro, FUdR can be phosphorylated to the nucleotide analogue of thymidine monophosphate (TMP) which is a powerful inhibitor of thymidylate synthetase (121). Thus, in vivo, FUdR inhibits DNA synthesis and the inhibition can be reversed by adding high concentrations of thymidine or its analogues, e.g., chlorodeoxyuridine, bromodeoxyuridine or iododeoxyuridine (24, 91,222, 223, 243, 301, 302).

FUdR has been shown to inhibit mitosis in Vicia faba root tips (164, 301), tobacco cells (91) and to inhibit cell elongation especially gibberellic acid-induced, of cucumber hypocotyls,wheat coleoptiles (223) and lentil epicotyls (222). It was also reported to cause chromosome breakage in Vicia faba root tips (164).

Mitomycin C:

In animal tissues and bacteria this antibiotic causes inhibition of DNA synthesis (290) and may promote degradation of DNA (156, 157, 158, 231, 273, 289). It has no marked influence on protein or RNA synthesis (140). The antibiotic acts as an alkylating agent (282) to link DNA complementary strands together by covalent bonds (140, 141).

Mitomycin C has been reported to inhibit cell division in Vicia faba root tips (207) and expansion growth of artichoke tuber tissues (203). Aberrations of chromatid structure also occur (282).

Hydroxyurea:

This substance inhibits incorporation of thymidine and phosphate into DNA of regenerating rat liver and HeLa cells (283, 345). It does not affect synthesis of RNA in these cells. It may interfere with DNA metabolism (98, 209) and with the synthesis of pyrimidine deoxyribosides

(209). These effects can be reversed by thymidine, deoxyuridine or deoxycytidine (209). There are no records of the effects of hydroxyurea on plant tissue.

Colchicine:

This substance is well known as a "metaphase poison" which inhibits action of the spindle and separation of chromatids. It appears to act by disorganizing orientation of micelles in the astral rays and spindle fibers (137). This resulting in polyploidy (164, 175). Such cells will resume division if colchicine is removed (164).

8-azaguanine and 6-azathymine:

These structural analogues are incorporated into RNA or DNA of animal (132, 176, 189, 205, 258, 294, 295, 328) and plant tissues (48, 129). Presumably the products have abnormal properties. In addition, 6-azathymidine can be formed from 6-azathymine (256) and this substance inhibits the incorporation of ^{14}C-formate into DNA thymine (257).

8-azaguanine inhibits cell expansion in artichoke tuber tissue (203) and cell division in the pea root (129). It has been reported to stimulate the growth and germination of pollen tubes (312).

III. Cellulase

A. Occurrence

In the literature on plant anatomy (see 67, 85) there are many references to the visible fragmentation, thinning or even complete dissolution of plant cell walls during normal cell differentiation. In many instances, there can be no doubt that cellulose is degraded by endogenous cellulase. This includes the well known disappearance of end walls of tracheids and laticifer elements during formation of vessels and mature laticifers (151, 281). An active cellulase has been extracted from developing vessels of barley roots (281). Similarly, in the abscission zone of bean petioles where cell walls visibly break apart, cellulase can be extracted at a higher activity than in the surrounding tissues (134). Ethylene gas, which promotes abscission in this tissue, also increases extractable cellulase activity. In storage organs (e.g., endosperm) of many grains (e.g., barley), wall dissolution occurs during germination and β-glucanase activity in grain extracts has been shown to increase at this time (38, 187, 242). Gibberellic acid treatment of the grain promotes germination and enhances the activity of many hydrolases, including α-amylase, ribonuclease, protease and β-glucanase (51, 52, 240, 241, 314, 315). The β-glucanase may not be cellulase (251). In grasses, close to the ends of guard-cell pairs, a pore develops during growth providing open connection between the two

cell protoplasts (42). Cellulase activity has been extracted from many grass leaves (55). Pollen tubes grow through many cell walls during their passage down the style and into the ovary and there is electron microscopic evidence (247, 281) that this process involves breakdown of wall cellulose. Cellulase activity has been extracted from pollen grains (305).

In addition to such instances of cellulose breakdown by cellulase, there are many examples of tissues where there is anatomical evidence that this phenomenon probably occurs. Thus, the mechanism of sieve plate pore formation has been a controversial subject for many years but recent electron microscopic studies lead to the conclusion that some cellulose breakdown occurs (84, 281). Lacunae often develop in tissues by the breakdown and disappearance of entire cells, e.g., in pith of hollow stems, seeds, etc. (85) and in the cortex in advance of developing adventitious roots (284). During geotropic curvature of bean epicotyls, measurements of wall thickness suggest dissolution of wall material in cells towards the lower (enlarging) surface (37).

There is ample evidence for the widespread occurrence of active cellulase in extracts of a variety of organs from higher plant tissues. About 50 species have been tested in surveys (55, 195, 309) and cellulase activity was readily detected in most instances. Particularly high cellulase levels were found in the meristematic and growing regions of the pea epicotyl (184) which are the same regions showing most active cell wall turnover (183, 185). Cellulase has also been associated with enlarging cells in tomato fruits (116), bean petioles (134)

and tobacco leaves (309, 310). Tracey's early (1950) results with tobacco leaves led to his suggestion in 1959 (310) that cellulase may "play a role in the remodelling of the primary cell wall during growth."

The above studies lead to the conclusion that cellulose dissolution in higher plants is a highly localized phenomenon, restricted to certain regions or tissues or even to certain cell walls within the tissue. This, in turn, implies that cellulase activity in higher plants must be closely regulated, either by direct inhibitors or activators or by mechanisms which control cellulase metabolism. The existence and action of substances which directly affect cellulase is discussed in the next section. There is no evidence for inducers or repressors of cellulase synthesis in higher plants.

In lower plants, cellulase is well known to be an "adaptive" enzyme in the sense that the ability of many of these organisms to form cellulase has been shown to depend on the composition of the medium on which they are growing. Thus many fungi produce cellulase only when grown on cellulose, cellodextrins or cellobiose (20, 190, 192, 269). Certain other glycosides can replace cellulose or its derivatives in some species. Trichoderma viride and certain Basidiomycetes form cellulase if grown on lactose (190), salicine or sophorose (190, 191, 192, 194). Under different conditions (e.g., temperature, etc.) free glucose or cellobiose may act either as an inducer or as a repressorofcellulase synthesis in T. viride (192). Usually cellulase activity in these organisms is not detectable unless the inducer is present. There are also recorded instances of organisms with low cellulase activity which can be greatly

stimulated *in vivo* by the addition of substances to the outside medium. For example, the production of cellulase by *Trichoderma* is enhanced by providing optimum concentration of cobaltous ions (192). Cellulase synthesis in *Achyla* mycelium appears to be increased by adding one of the sexual hormones for this fungus (305). Whether or not these are examples of enzyme induction remains to be determined.

B. Properties *in Vitro*

Various terms have been used to describe the action of cellulases on β-1, 4-polyglucan substrates. The simplest classification is that which distinguishes between endo- and exo-cellulase according to whether the enzyme attacks the substrate at random points along the molecular chain or specifically at one end to remove cellobiose or glucose units. Most fungal and plant cellulases are probably endo-type (310, 344); exo-cellulases have only been described in a few lower microorganisms (165, 298).

Endo-cellulases can be identified by their effectiveness in reducing the viscosity of carboxymethylcellulose (CMC), an assay dependent on reduction in average chain length. It is probable that cellulases only hydrolyse linkages between unsubstituted anhydroglucose units (227, 228) and, since these are presumed to be distributed at random along the CMC chain, the chain length could be significantly reduced by endo- but not exo-cellulase action. However, in order to prove that endo-hydrolysis occurs, it is necessary to show that the % viscosity loss is greater than the % of total potential reducing power which is

released during hydrolysis. This has been done for pea epicotyl cellulase (184) and cellulases from lower organisms (177, 227, 228). In a definitive study of cellulase from Myrothecium, affinity of the enzyme for substituted cellodextrins decreases with decreasing molecular weight (119, 331). This is also clear evidence for endo-type degradation as is the production of cellodextrins longer than cellobiose (56, 101, 119, 123, 124, 168, 268, 331). These latter techniques have not been employed with higher plant cellulase.

There is considerable controversy over the question of whether or not co-operation of more than one enzyme is required for maximum degradation of native cellulose. Fractionation of crude cellulase preparations have shown the existence of at least two fractions: one, called C_1 (177, 228, 286) or A enzyme (286), will immediately attack fibrous cellulose or hydrocellulose to initiate breakdown and loss of tensile strength; the other, called C_x (177, 228, 286) or B enzyme (286) will not attack fibrous cellulose until after the first fraction has operated for a time or until the cellulose is swollen by acid or alkali treatment. Both fractions are probably glycoproteins (138, 246, 287). The latter has been equated to β-1, 4-glucanase or carboxymethylcellulase (270, 298) while the former may be an enzyme which breaks hydrogen bonds or in some other way increases the accessibility of cellulose chains to hydrolase action. There is no doubt that cellulases act on native cellulose microfibrils at relatively few sites since electron micrographs of partially degraded native cellulose show that dissolution is highly localized (248, 249). Only a small percentage of the total fibril weight

is converted to soluble products, even after repeated cellulase treatments (286).

Some cellulases, all fungal in origin, have been purified to the point where meaningful amino acid analyses could be carried out. Myrothecium cellulase has a molecular weight of about 49,000 and an amino acid composition typical of most enzymes. The presence of glycosides in the enzyme was not investigated. The only unusual property was the absence of free sulfhydryl groups on the active enzyme; 14 to 16 cysteine units were present combined as cystine (74). Cellulases from Trichoderma koningi (138) and Polyporus versicolor (246) are both glycoproteins. Both enzymes have an amino acid composition typical for other enzymes with very low cysteine content. The sugar moeties in Trichoderma cellulase include mainly mannose with a small amount of hexosamine (138).

Many factors influence the activity of endo-cellulases in vitro. The pH optimum for activity is generally below 7.0 for most cellulases. It lies between pH 5.0 and 6.5 for tobacco (310) and pea cellulase (184). Temperature effects on cellulase activity are similar to effects on any enzyme. Animal, bacterial and higher plant cellulases appear to be more labile than fungal cellulases at high temperature (101). Effects of various enzyme inhibitors on cellulase activity have been reviewed recently (101, 195, 196). Most cellulases are inhibited by high concentrations (10^{-4} - 10^{-3} M) of divalent metal ions. Other regeants, [e.g., p-chloromercuribenzoate (PCMB), iodoacetate] which react with sulfhydryl groups may be inhibitory or stimulatory

depending on the enzyme source. Reducing agents (e.g., cysteine, glutathione, ascorbic acid) are also either inhibitory or stimulatory Accordingly, it is impossible to generalize on the question of whether or not cellulase activity requires free sulfhydryl groups. Effects of many other substances (e.g., dyes, sugars, proteins, alkaloids, phenolics, halogens, fungicides, antibiotics) have been tested with widely variable results using different cellulases. Special mention may be made of a report that 10^{-3} M IAA directly inhibits Aspergillus oryzae cellulase (148). There is also an old report of the "unmasking" of Cellulomonas cellulase activity by treatment in vitro with trypsin (250).

The widespread existence of heat-stable natural substances in plant extracts which will inhibit fungal cellulase activity in vitro has been established beyond question. In one survey of methanol, water and aqueous acetone extracts of a variety of organs from about 500 plants, about 100 samples caused substantial inhibition of Trichoderma viride cellulase (193, 195, 196). The inhibition was traced to effects of polymeric leucoanthocyanins in extracts of barberry, perry pears and persimmon fruits and to condensed tannins in extracts of grape leaves and sericea.

EXPERIMENTS AND RESULTS

Paper I. Control of Cellulase Activity by Indoleacetic Acid

Abstract

Apices of etiolated decapitated Alaska pea seedlings were painted with aqueous lanolin ±IAA ± various inhibitors of RNA or protein synthesis. A sub-apical segment from the epicotyl was removed for measurements of growth and the soluble protein content and cellulase activity of enzyme extracts.

During the first 18 hours, the main growth response to IAA was an increase in segment diameter; elongation was inhibited. The amount of extractable cellulase activity per segment and the diameter increased at exactly the same rates relative to controls. In the next 2 days IAA induced rapid cell division and the formation of root primordia. Cellulase activity per segment, per unit fresh weight and per unit soluble protein all increased markedly to levels many times higher than in controls. Chloramphenicol, azaguanine, puromycin and actinomycin D all interfered with protein synthesis, the growth responses and the development of cellulase activity. In the absence of IAA, cellulase activity decayed.

It is concluded that cellulase is subject to turnover in this tissue and that the rate of its synthesis is controlled by auxin concentration. It is proposed that cellulase action on microfibrils *in vivo* plays an essential role in a variety of growth processes.

Introduction

When indoleacetic acid (IAA) stimulates cell expansion in plant tissue, before and during the growth response, there is typically an increase in the rate of synthesis of protein (162, 224, 225) as well as a relaxation of primary wall rigidity (62, 64, 210, 260). Hypothetically, the two events could be related if the hormone were to induce synthesis of one or more enzymes which can catalyze a "loosening" of the primary wall structure. It is generally agreed that the term loosening, in this context, refers to an increase in extensibility of the interwoven wall framework of cellulose microfibrils (278, 288). The problem is to identify such an enzyme.

Cellulase [β-1, 4 glucan 4-glucanohydrolase (EC 3.2.1.4)] has been shown to possess some of the required qualifications. Even slight attack by enzyme on cotton cellulose fibers (286) or on coleoptile cell walls (232) is sufficient to cause a great loss in tensile strength and an increase in extension under stress, i.e., a loosening effect. The enzyme occurs widely in higher plants (55, 270), especially in young tissues (184, 309) where IAA is concentrated (285) and where primary wall glucan is subject to metabolic turnover (185, 197, 264). In this

report we show that IAA applied to the young pea epicotyl can markedly increase the cellulase activity measured in extracts of the tissue. The increase appears to be due to stimulation of enzyme biosynthesis by IAA.

Materials and Methods

Seedlings of Pisum sativum L. var. Alaska were grown in darkness until the third internode was 3 to 5 cm long. They were examined and handled under dim green light. The plumule was cut off just below the hook (183) and a point on the epicotyl 10 mm below the cut apex was marked with ink to delineate a "segment" of tissue (photographs shown in Appendix IA). The apex was painted with an average weight of 2.5 mg of lanolin paste (65-75% water) containing IAA and (or) other substances whose effects on the segment below were to be tested. The concentration of these substances is referred to in this paper as weight per unit weight of wet lanolin, e.g., 0.2 mg puromycin/g represents approximately 0.5 ug applied per apex. The seedlings were then allowed to continue to grow in darkness at 20°.

High concentrations of applied IAA bring about a striking sequence of growth responses within this subapical segment. Our observations confirm those of Scott (284) on the anatomical developments and of MacQuarrie (188) on the changes in segment diameter and fresh weight. After a lag period of 6 to 8 hours, during which lanolin diffuses as much as 10 mm down intercellular spaces and xylem vessels, the diameter of the whole segment begins to increase over that of controls as a result of

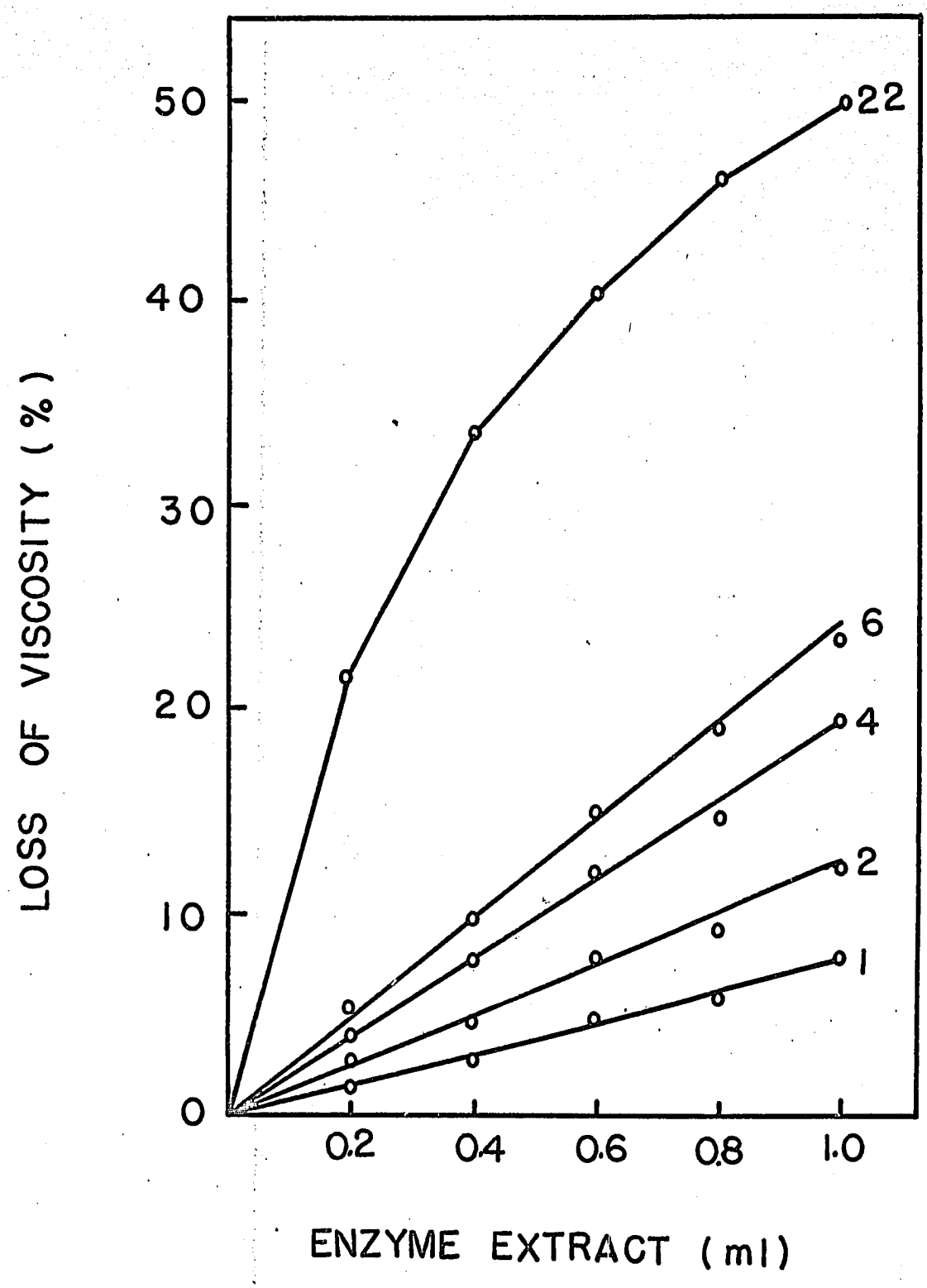
continued lateral expansion of parenchyma cells. At about 16 hours, extensive nuclear and cell division is initiated. Swelling as a result of these two responses continues for 2 to 3 days (see further documentation in Appendices IA and IB, Figures 4 and 5). At this time the beginnings of organized root primordia can be recognized and cortical cells lying between the root primordia and epidermis begin to disintegrate (Appendix IB, Figure 5). The young roots grow through the lacunae so produced, reaching the outside in about a week (Appendices IB and IC). It should be added that in our tests applied IAA always inhibited elongation of the subapical segment whereas MacQuarrie recorded varying degrees of increased length. This difference may be due to a lower endogenous auxin level in the tissue of his tests, which was cut 3 mm lower down the epicotyl from a dwarf (Meteor) variety of peas.

In present tests, 100 segments were removed at intervals of up to 4 days, washed with dilute hypochlorite, and gently blotted with filter paper. The fresh weight and lengths were recorded and immediately the segments were homogenized with 2 volumes of cold 0.1 M phosphate, pH 7.0. The brei was centrifuged at 37,000 X g and the precipitate re-extracted with 2 volumes of buffer. The combined supernatants provided at least 10 ml of crude enzyme extract derived from a known number of segments (5 to 8 segments/ml) and a known fresh weight (approximately 0.2 g/ml). Soluble protein (2 to 5 mg/ml) was estimated in duplicate aliquots with the biuret reagent; purified bovine serum albumin was used as the standard (113, Appendix IIA).

Sufficient extract remained to measure cellulase activity in duplicate samples as described previously (184), i.e., by the initial rate at which aliquots lowered the specific viscosity of solutions of carboxymethylcellulose (CMC). Each value reported in any one experiment is based on the average percentage loss of viscosity brought about in 2 hours at 35° by the addition of 1 ml of extract to 9 ml of 1.1% CMC-0.01 M phosphate (pH 6.0)-0.02% NaF. From these measurements, values were calculated for the amount of cellulase activity per segment of tissue or per unit soluble protein.

A number of tests have been carried out to certify that these methods provide a reliable estimate of the amount of cellulase in this tissue (Appendix III). It was first necessary to boil or autoclave the substrate solution for 30 minutes before use to obtain a reproductible and stable initial viscosity. With this substrate the pH optimum of enzymic attack lay between 5.5 and 6.5 (Appendix IIIB). Figure 1 shows that cellulase activity in a typical crude extract was proportional to enzyme concentration when viscosity losses were measured simultaneously at any time up to 6 hours. Proportionality was not obtained in such tests when longer incubation times were used or when activity was such that viscosity losses were greater than about 40%. Therefore activities were always compared at a time (2 hours) well before this percentage was exceeded by the most active extracts encountered. Boiled enzyme controls were also taken into account in each experiment but generally their values were negligible compared to those of unboiled enzyme because of the relatively short incubation time (184). Further extraction of tissue

Fig. 1. Effect of enzyme concentration on cellulase activity. Losses in viscosity of CMC brought about by aliquots of crude enzyme extract were measured at 1, 2, 4, 6 and 22 hours. See text for detailed methods.


LOSS OF VISCOSITY (%)
50
40
30
20
10
0
0.2
0.4
0.6
0.8
1.0
ENZYME EXTRACT (ml)
22
6
4
2
1

residues with phosphate or with solvents containing chelating agent, IAA, or substances known to release adsorbed protein failed to remove more than a trace of extra cellulase activity. Boiled crude extracts or up to 10^{-3} M IAA, gibberellic acid, or kinetin did not inhibit or stimulate activity of the crude or purified enzyme (Appendix IIIE). Doubling the soluble protein concentration by adding bovine serum albumin during enzyme extraction or during viscometry had no effect on enzyme activity (Appendix IIIE).

Results

A. Growth and Cellulase Activity

Table I records the changes observed over 4 days in the size of the segment and in the amount of its buffer-soluble protein. In controls, the fresh weight approximately doubled as a result of elongation in the first 2 days. The diameter of the segment, measured by the fresh weight per unit length, increased only slightly (by 15-20%) and stopped increasing after the first day. In IAA-treated segments, the fresh weight quadrupled mainly as a result of a great increase in diameter in the first 2 days (the average fresh weight per unit length tripled whereas length increased by only 40-50%). IAA treatment also led to a greater increase in the amount of soluble protein than occurred in controls. (Effects of IAA on growth and protein levels in other plant tissues are shown in Appendix IV. Effects of other hormones on growth and protein levels in the pea epicotyl are shown in Appendix V.

Table I.

Effect of added IAA on growth and soluble protein level in the apical segment* during a 4-day period.

Time (days)	Fr wt (mg/seg)		Length (mm/seg)		Fr wt / length (mg/mm)		Soluble protein (µg/seg)	
	Control	IAA	Control	IAA	Control	IAA	Control	IAA
0	23.0		10.0		2.30		332	
1	40.5	64.1	14.6	13.5	2.77	4.75	386	432
2	50.1	83.9	19.3	14.3	2.60	6.55	478	585
3	55.6	99.1	19.0	15.1	2.92	6.58	456	610
4	51.3	97.3	19.3	14.5	2.62	6.71	512	610

* The apex of etiolated decapitated pea epicotyls was painted with lanolin paste ± 0.5% (w/w) IAA. Length and fresh weight of the tissue which developed from the apical 10 mm of epicotyl was measured at daily intervals. Values are averages for 100 segments. Soluble protein was extracted by 0.1 M phosphate, pH 7.0 (see text).

Effects of the act of decapitation are shown in Appendices VIA and B.)

Figure 2 shows the changes observed in the cellulase activity of extracts from the above segments. In controls, the activity per unit protein or per segment increased slightly at first but then decreased to well below the initial level as the growth rate declined. In IAA-treated segments, cellulase activity also increased only a little during the first day. Subsequently, however, it increased dramatically to reach a maximum level by the third day of about 6 times the initial activity per unit protein and 12 times the initial activity per segment. This experiment has been repeated several times and, thought the absolute levels of initial and final cellulase activities varied (cf. Tables IV and V), there was always a marked response to added IAA after an initial lag period, i.e., a sigmoid progress curve as illustrated in Figure 2. (Appendices IV and V and VI show effects of IAA and other hormones on cellulase activity in the pea epicotyl and other plant tissues.)

Details of the effects of IAA during the first day are shown in Table II and Figure 3. IAA began to inhibit elongation after 12 hours but it began to stimulate growth in fresh weight at 6 hours. Its main effect, therefore, was to prolong the period during which rapid lateral expansion proceeded. In controls, cellulase activity increased rapidly for only the first 6 hours and began to decrease after 18 hours, by which time no readily extractable endogenous auxin can be detected in such tissue (285). With added IAA, both segment diameter and cellulase activity steadily increased at exactly the same rates relative to their respective controls until about 18 hours, when cellulase activity

Fig. 2. Effect of added IAA on relative cellulase activity per unit protein and per segment. Data on growth and soluble protein changes during this experiment are given in Table 1. At zero time 1.0 ml enzyme extract (from 0.2 g fr wt) induced 4.8% loss in CMC viscosity/ 2 hours. This was equivalent to 1.79% loss/2 hours/mg protein or 0.59% loss/2 hours/seg (see Table 1). Cellulase activities in the figure are calculated relative to these initial values.

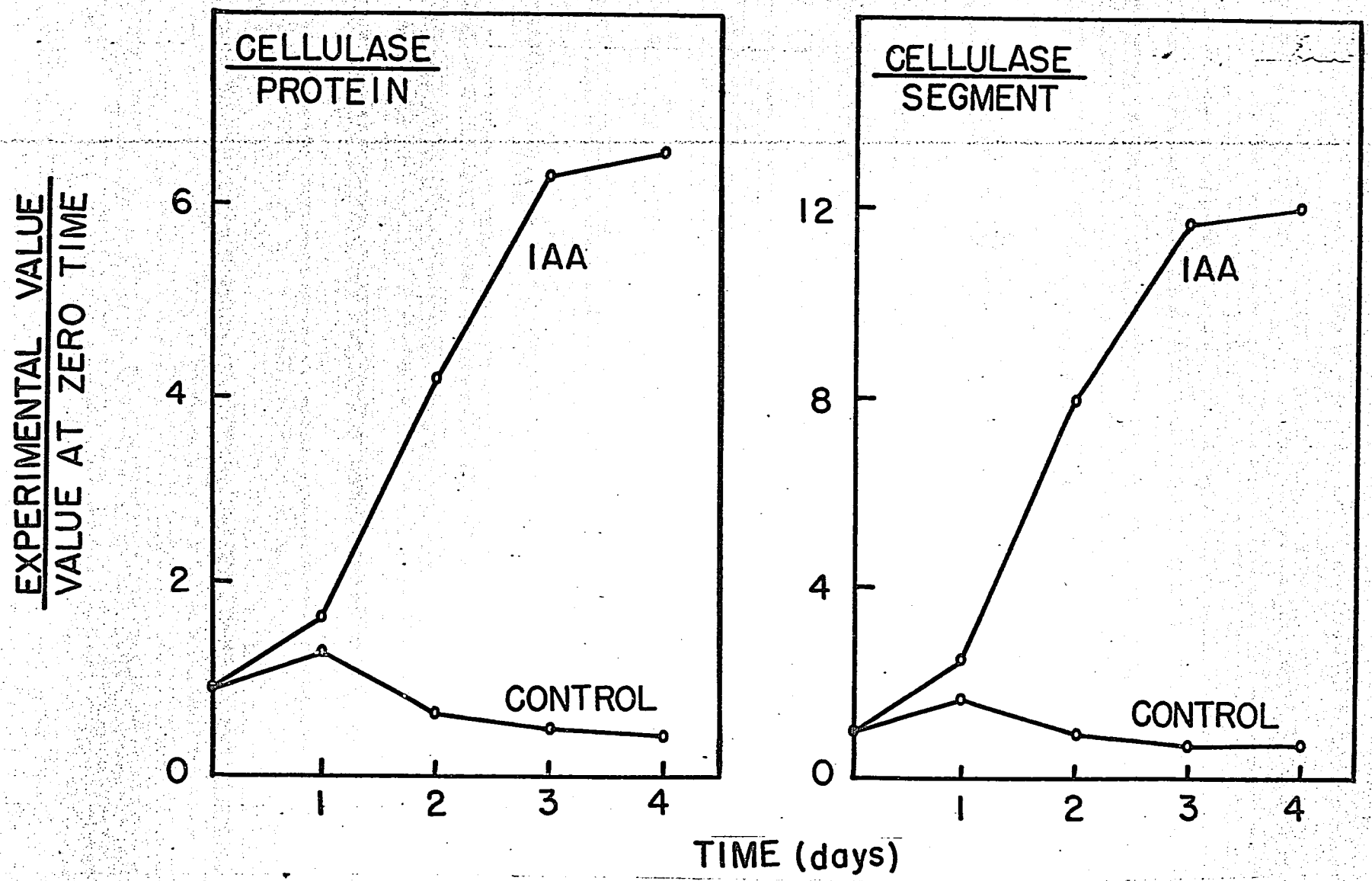

CELLULASE
PROTEIN
CELLULASE
SEGMENT
EXPERIMENTAL VALUE
VALUE AT ZERO TIME
IAA
CONTROL
IAA
CONTROL
0
2
4
6
0
4
8
12
1
2
3
4
1
2
3
4
TIME (days)

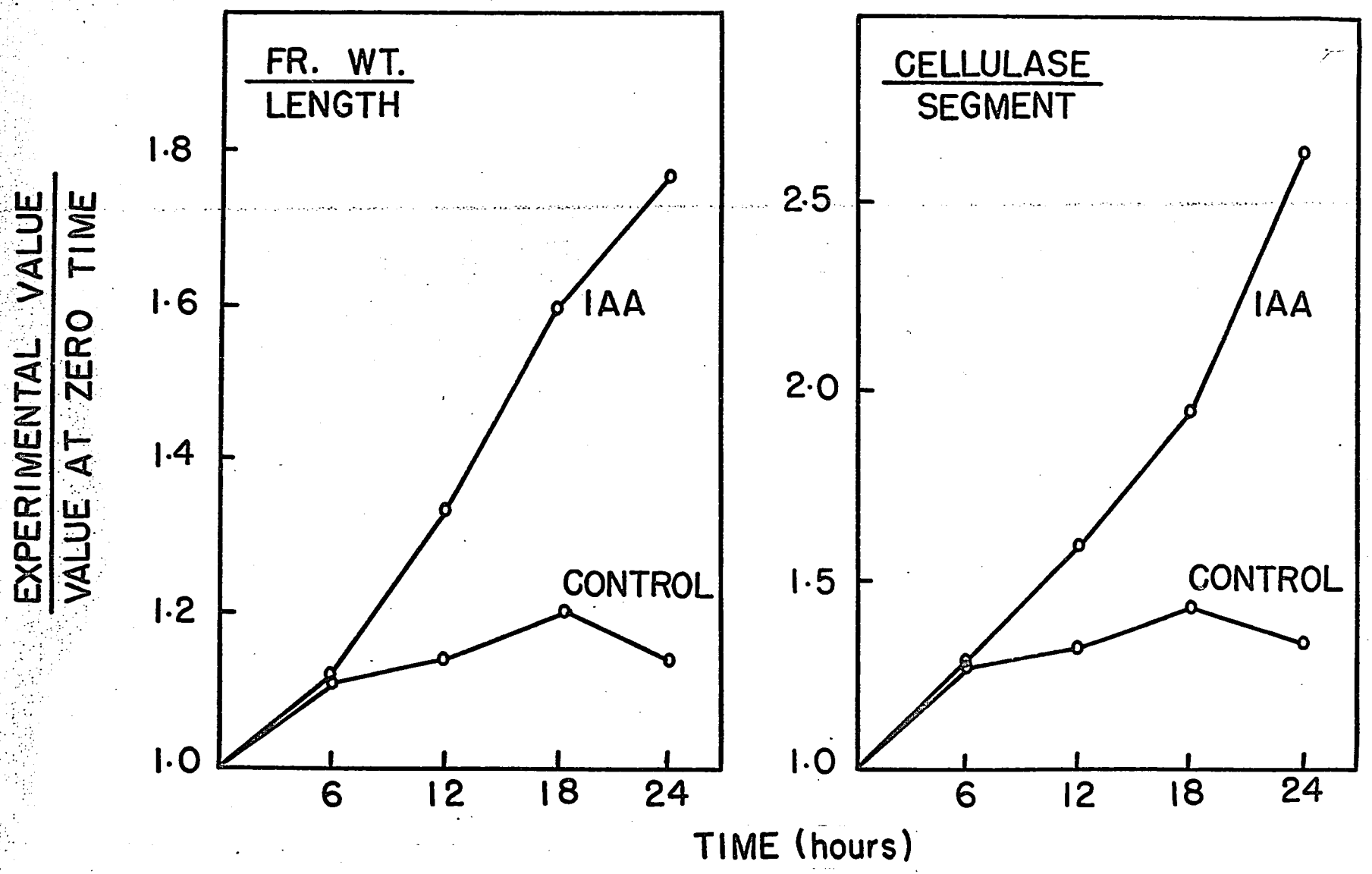
Table 11.

Effect of added IAA on growth and soluble protein level during a 24-hour period.

Time (hrs)	Fr wt (mg/seg)		Length (mm/seg)		Soluble protein (µg/seg)	
	Control	IAA	Control	IAA	Control	IAA
0	22.7		10.0		259	
6	28.1	29.4	11.5	11.4	337	277
12	32.7	38.2	12.5	12.6	297	365
18	38.7	47.8	13.7	12.8	352	428
24	40.4	56.1	15.6	13.6	315	432

Methods are described in Table 1.

Fig. 3. Effect of added IAA on relative fresh weight per unit length (segment diameter) and cellulase activity per segment. Details of length and fresh weight changes are give in Table II. At zero time, 1.0 ml enzyme extract induced 4.2% loss in CMC viscosity/2 hours, equivalent to 0.52% loss/2 hours/seg. Activities in Fig. are calculated relative to this initial value.


EXPERIMENTAL VALUE / VALUE AT ZERO TIME
FR. WT. / LENGTH
1·8
1·6
1·4
1·2
1·0
IAA
CONTROL
6
12
18
24
CELLULASE / SEGMENT
2·5
2·0
1·5
1·0
IAA
CONTROL
6
12
18
24
TIME (hours)

began its unilateral rise. Soluble protein also increased in response to IAA, particularly between 6 and 18 hours, so that there was no marked change until 24 hours in the cellulase activity per unit protein.

Effects of varying the IAA concentration on responses in the first day are shown in Table III. Even low concentrations of IAA (10 ppm) inhibited elongation but stimulated growth in fresh weight and increased the soluble protein level over that in controls. Higher levels of hormone led to correspondingly greater increases in segment diameter and soluble protein. The relative cellulase activity per segment increased with IAA concentration in the same way as relative segment diameter. Cellulase activity per unit protein showed no response.

B. Effect of Inhibitors

The above experiments were repeated with or without added IAA in the presence of substances which inhibit protein or RNA synthesis. Table IV shows the effects of chloramphenicol, an antibiotic known to inhibit growth and amino acid incorporation into protein of sections excised from pea epicotyl or other plant tissue (224, 225). Table V is a summary of results with puromycin, 8-azaguanine, and actinomycin D, which are also known to inhibit plant growth and protein synthesis, the last two by interfering with nucleotide incorporation into RNA (62, 162, 210, 224, 318).

Chloramphenicol, when applied to the apex for 3 days, inhibited elongation and lateral expansion of the segment below, prevented the small increase in soluble protein normally observed (cf. Table I), and

Table III.

Effect of IAA concentration on growth, soluble protein and cellulase activity after 24 hours.

IAA conc.	Fr wt	Length	Soluble protein	Relative Cellulase Activity	
				per unit protein	per seg
(%)	(experimental value/value at zero time)				
0	1.71	1.49	1.61	0.86	1.49
0.001	2.16	1.36	2.08	0.82	1.90
0.01	2.25	1.33	2.18	0.81	2.12
0.1	2.45	1.32	2.01	0.92	2.19
0.5	2.52	1.35	2.76	0.72	2.35

Methods are described in Table I. At zero time, each segment (10 mm) on the average weighed 22.7 mg, contained 257 µg soluble protein and yielded an extract which caused 0.49% loss in CMC viscosity/2 hours. Values in Table are calculated relative to these.

Table IV.

Effect of IAA and chloramphenicol on growth, soluble protein and cellulase activity*

Treatment	Fr wt (mg/seg)	Length (mm/seg)	Fr wt / length (mg/mm)	Soluble Protein (µg/seg)	Relative Cellulase Activity per unit per seg protein	
Zero time	23.6	10.0	2.36	194	1.00	1.00
3-day control	43.2	15.0	2.88	232	0.77	0.88
+ IAA	69.3	12.6	5.51	534	5.92	16.30
+ chloramphenicol	30.8	12.0	2.57	184	0.69	0.66
+ IAA + chloramphenicol	53.8	12.7	4.21	410	3.84	8.12

* IAA (0.5% w/w) and/or chloramphenicol (32 mg/g), suspended/dissolved in lanolin paste, was applied to decapitated epicotyls for 3 days. At zero time, 1.0 ml enzyme extract (from 0.2 g fr wt) induced 2.1% loss in CMC viscosity/2 hours, equivalent to 1.39% loss/2 hours/mg protein or 0.26% loss/2 hours/seg. Values in Table are calculated relative to these initial values.

Table V.

Effect of IAA and puromycin, 8-azaguanine and actinomycin D on growth, soluble protein and cellulase activity*

Treatment	Fr wt (mg/seg)	Length (mm/seg)	Fr wt / length (mg/mm)	Soluble Protein (µg/seg)	Relative Cellulase Activty per unit	Relative Cellulase Activty per seg protein
Zero time	23.5	10.0	2.35	186	1.00	1.00
3 days control	36.0	14.7	2.50	261	0.96	1.30
+ IAA	68.0	13.0	5.23	555	1.96	5.72
+ IAA + puromycin	52.4	18.2	2.88	333	1.05	1.24
+ IAA + 8-azaguanine	62.0	19.8	3.13	420	0.70	1.65
+ IAA + actinomycin D	53.2	16.6	3.21	363	0.83	1.60

* IAA (0.5% w/w) and puromycin (0.2 mg/g), 8-azaguanine (1.0 mg/g) or actinomycin D (0.2 mg/g) suspended/dissolved in lanoline paste, were applied to decapitated epicotyls for 3 days. At zero time, 1 ml enzyme extract (from 0.2 g fr wt) induced 3.2% loss in CMC viscosity /2 hours, equivalent to 2.16% loss/2 hours/mg protein or 0.41% loss/ 2 hours/seg. Values in Table are calculated relative to these initial values.

caused cellulase activity to decrease further than usual (Table IV). Applied to the apex together with IAA, chloramphenicol reduced the stimulatory effects of the hormone on lateral expansion, soluble protein level, and cellulase activity by about half.

The other inhibitors (Table V) interfered even more effectively in IAA-induced swelling and protein synthesis and they virtually prevented the rise in cellulase activity. They also reversed the inhibitory effects of IAA on elongation, i.e., sections grew longer in the presence of IAA plus inhibitors than in controls. (Further studies of effects of these and other inhibitors in this system are shown in Appendix VII.)

Discussion

We interpret the results as follows: cellulase activity, like cell expansion and division, depends on the level of auxin in these tissues whether the auxin is applied as IAA or present as the endogenous hormone.

When the plumule was removed, the auxin level in the segment below was sufficient to maintain rapid growth for only about a day (Table I and II). During this time cellulase activity per segment increased a little (Figures 2 and 3, Table III). But subsequently, when the growth rate declined, cellulase activity also fell, even while the total soluble protein level continued to rise (Table I). Loss of cellulase activity was particularly marked when synthesis of protein was prevented, e.g., by chloramphenicol (Table IV). Apparently the enzyme activity decreases

under conditions where the auxin level can be assumed to have decreased. This may be the reason why cellulase is hardly detectable in segments cut only a few millimeters lower down the epicotyl shaft (184) where the auxin level is definitely lower. The fate of the enzyme has not been studied but it should be noted that, to reach its substrate, cellulase must be transported across the cell membrane. Outside the protoplast any enzyme can be assumed to denature rapidly.

Maximum cellulase activity developed in the presence of the highest concentrations of added IAA (Table III) and reached a peak value after 3 days, during which the most rapid cell expansion and division took place (Figure 2). It is at this time that cells begin to disintegrate in advance of developing root primordia: Scott (284) shows a photograph of a cross section of the apex in which cortical cell walls are obviously breaking into pieces (see also Appendix IB). Presumably this facilitates root emergence. In any event, the evidence is clear that the cellulase activity measured against an artificial substrate *in vitro* bears a real relationship to cellulase activity against cell walls *in vivo*.

There are good reasons, therefore, for assuming that cellulase was also active against cell wall in early stages of these experiments when lateral swelling was the main growth response to added IAA. Young parenchyma cells are well known to have a predominantly transverse orientation of cellulose microfibrils (278, 288). Under the electron microscope, microfibrils of cell walls from the apical pea epicotyl segment resemble thread wound transversely round a spool with only a few longi-

tudinal ribs (unpublished data). It would not be surprising if the main effect of partial enzymic hydrolysis of transverse microfibrils were a loosening of constraint on cell diameter and a consequent lateral swelling of the cell rather than axial extension. In fact, such a reaction has already been recorded (80) for the effect of added fungal cellulase on the development of root hairs. The enzyme causes hairs to grow fatter and shorter than normal. In the early response of the pea segment to added IAA there was always the closest positive correlation between changes in total cellulase activity and segment diameter but not segment length (Tables II-V, Figure 3). The relationship maintained only while swelling was attributable to the expansion of cells, i.e., before cell division was initiated. We propose, therefore, that IAA was instrumental through its stimulatory effect on cellulase activity in dislocating the normal polarity of cell elongation in favor of lateral cell expansion.

These results should not be taken to mean that cellulase activity necessarily has an inhibitory effect on the axial extension of tissues growing normally. It should be emphasized that we were working here with an abnormal system where many metabolic processes were upset by the high levels of IAA employed. The segments at zero time apparently contained levels of natural auxin which were optimal for the elongation process (Table III). It is possible that their levels of cellulase activity, although relatively low, were nevertheless useful in facilitating elongation. The problem requires further study as does the curious situation where elongation was stimulated by adding inhibitors of protein synthesis along with IAA (Table V). Similar phenomena with other anti-

biotics have been described before (139).

As for the mechanism whereby IAA treatment stimulates cellulase activity, we have investigated but found no evidence for direct effects of the hormone on activity, stability or solubility of the enzyme in vitro (see Methods and Appendix IIIE). However, in vivo, IAA treatment always brought about an increase in the amount of soluble protein in the apical segment and the effects of inhibitors leave no doubt, that part of the newly formed protein had cellulolytic activity. The problem, then, appears to be how IAA stimulates cellulase biosynthesis.

The IAA-dependent increase in cellulase activity per segment appeared to take place in two stages. Up to about 18 hours (Figure 3) it was accompanied by a comparable increase in total soluble protein (Tables II and III). During this period of rapid cell expansion, therefore, cellulase was probably only one of many enzymes to be synthesized. It is not necessary to postulate that the whole protein increment or the cellulase component was induced at this time by an interaction of IAA with specific mucleic acid coding systems. IAA can cause a non-specific increase in the rate of anabolism by influencing respiration rate, substrate mobilization, etc. (see 188). Subsequently, during the period of most rapid cell division, cellulase activity increased much more than total protein (Figure 2) and this unilateral increase was opposed by the inhibitors of RNA and protein synthesis (Tables IV and V). This may mean that cellulase was one of few enzymes which were rapidly synthesized at or after cytokinesis, i.e., it may have formed as an indirect consequence of IAA-induced cell division. Certainly any enzyme

which could assist in modelling or re-modelling walls would be useful to newly-formed cells, for example, during insertion of the cell plate into mother cell walls. Alternatively, IAA may have selectively activated coding systems necessary for eventual biosynthesis of cellulase. This could take place independently of any effect the hormone may have on cell division. It appears to happen with the enzyme for indoleacetyl-aspartate formation (317). Further studies to distinguish between these alternatives are in progress.

Whatever the detailed control mechanisms may be, it is clear that pea epicotyl cellulase has the distinction of being subject to a metabolic turnover rate which can be influenced by auxin concentration. Depending on the relative rates of cellulase synthesis and decay, it may be possible for auxin to influence growth by stimulating cellulase formation without at the same time causing much change in the net level of total protein or measurable cellulase activity. Several investigators have recently come to the conclusion that the mechanism of auxin action on growth must involve interaction with such a system. Morré (210), for example, remarked that "...the protein fractions limiting cell expansion in soybean hypocotyl tissue must...be characterized by rapid turnover..." Noodén and Thimann (225) concluded that "auxin promotes directly or indirectly either the synthesis or the turnover of one or more special proteins concerned with cell enlargement". Present results would seem to justify consideration of cellulase as a condidate for this role, at least in so far as its action could account for the swelling component of cell enlargement.

Acknowledgments

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Paper II. Massive Synthesis of Ribonucleic Acid and Cellulase in the Pea Epicotyl in Response to Indoleacetic Acid, with and without Concurrent Cell Division

Summary

Measurements were made over a 4-day period of the effect of added indoleacetic acid (IAA), puromycin, actinomycin D and 5-fluoro-deoxyuridine (FUdR) on growth and the levels of total DNA, RNA, protein and cellulase insegments of tissue at the apex of decapitated etiolated epicotyls of Pisum sativum, L. var. Alaska.

The hormone induced swelling of parenchyma cells and cell division. By 3 days after IAA application, the amounts of DNA and protein were approximately double, RNA triple and cellulase 12 to 16 times the levels in controls. All of these changes were prevented by both puromycin and actinomycin D. FUdR prevented DNA synthesis and cell division but not swelling or synthesis of RNA, protein and cellulase.

It is concluded that IAA-induced RNA synthesis is required for cellulase synthesis and lateral cell expansion, whether or not cell division takes place.

Introduction

When high concentrations of indoleacetic acid (IAA) in lanolin paste are applied to the decapitated apex of etiolated pea epicotyls, in the first 10 mm of tissue below the apex there is a marked increase in amount and specific activity of the enzyme cellulase (β-1, 4 glucan 4-glucanohydrolase, EC 3.2.1.4.) (86). The increase can be inhibited by including in the lanolin any of a number of substances which interfere with protein synthesis. Accordingly, it appears that IAA selectively brings into operation the coding system(s) needed for eventual biosynthesis of cellulase.

Theoretically, a hormone could regulate the synthesis of a specific enzyme by <u>direct</u> interaction with macromolecules which are important in the processes of information transcription (34) or translation (297). In the case of cellulase, however, it is possible that synthesis occurs specifically in cells which are about to divide and in newly-formed cells as an <u>indirect</u> consequence of IAA-induced mitosis. In the pea epicotyl, cellulase is concentrated in the plumule and hook (184) where the endogenous auxin level is high (285) and where cell divisions are most common. When plumule and hook are replaced by IAA, the most rapid increase in epicotyl cellulase begins at about 18 hours (86) which is the time when the first divisions that lead to formation of root primordia become visible (284). Clearly the ability of an epicotyl cell to form cellulase could depend merely on the time elapsed since DNA replication and not on the continued presence of hormone.

A major question, then, in understanding how cellulase synthesis is induced is whether the action of IAA or the division of cells is the more immediate precursor event. This study examines the problem by measuring the degree to which IAA brings about cellulase synthesis in the epicotyl segment when the formation of DNA, RNA and/or protein is inhibited by 5-fluorodeoxyuridine (FUdR), actinomycin D or puromycin.. In epicotyl tissue, both puromycin and actinomycin D inhibit growth and protein synthesis (86, 226, 317, 318) and the latter interferes with RNA synthesis (210, 280, 317). Effects of FUdR on pea tissues have not been reported but this substance prevents thymidylate synthesis (91) and mitosis (164) in a variety of other plant tissues.

Materials and Methods

Etiolated pea seedlings with third internodes 3 to 5 cm long were used throughout this study. Under dim green light the plumule was cut off just below the hook and a point 10 mm below the cut apex was marked with ink to delineate a "segment" of tissue. The apex was painted with about 2.5 mg of lanolin paste (70% w/w water) containing IAA (0.5% w/w) plus or minus FUdR (0.1% w/w), actinomycin D (0.02% w/w) or puromycin (0.02% w/w). At daily intervals 30 to 100 segments from each condition were removed, washed with dilute NaOCl solution and blotted dry. The fresh weight and lengths were recorded. Some of the segments were then used for measurement of cellulase activity and soluble protein in enzyme extracts and others for total protein and nucleic

acid determinations. Two experiments are reported here: one (A) using puromycin and actinomycin D and the other (B) using FUdR.

The methods used for buffer-extraction of soluble protein and cellulase were described in previous papers (86, 184). Cellulase activity in the enzyme extract was measured by the initial rate of loss of viscosity in solutions of carboxymethylcellulose (86). Soluble protein was determined with the biuret reagent standardised against bovine serum albumin (113). Total protein (Appendix IIB), RNA (Appendix IIC) and DNA (Appendix IID) determinations were carried out on homogenized 80% ethanol- and ether-insoluble fractions from the segments. The methods (185) included extraction of total nucleic acid into warm 0.5 N perchloric acid, its estimation by untraviolet absorption and DNA determination with diphenylamine (RNA = total nucleic acid minus DNA). Total protein was determined in the perchloric acid-insoluble residue after Kjehldahl digestion by nesslerization. The yield of buffer-soluble protein accounted for 90% of total segment protein.

Results

A. Growth

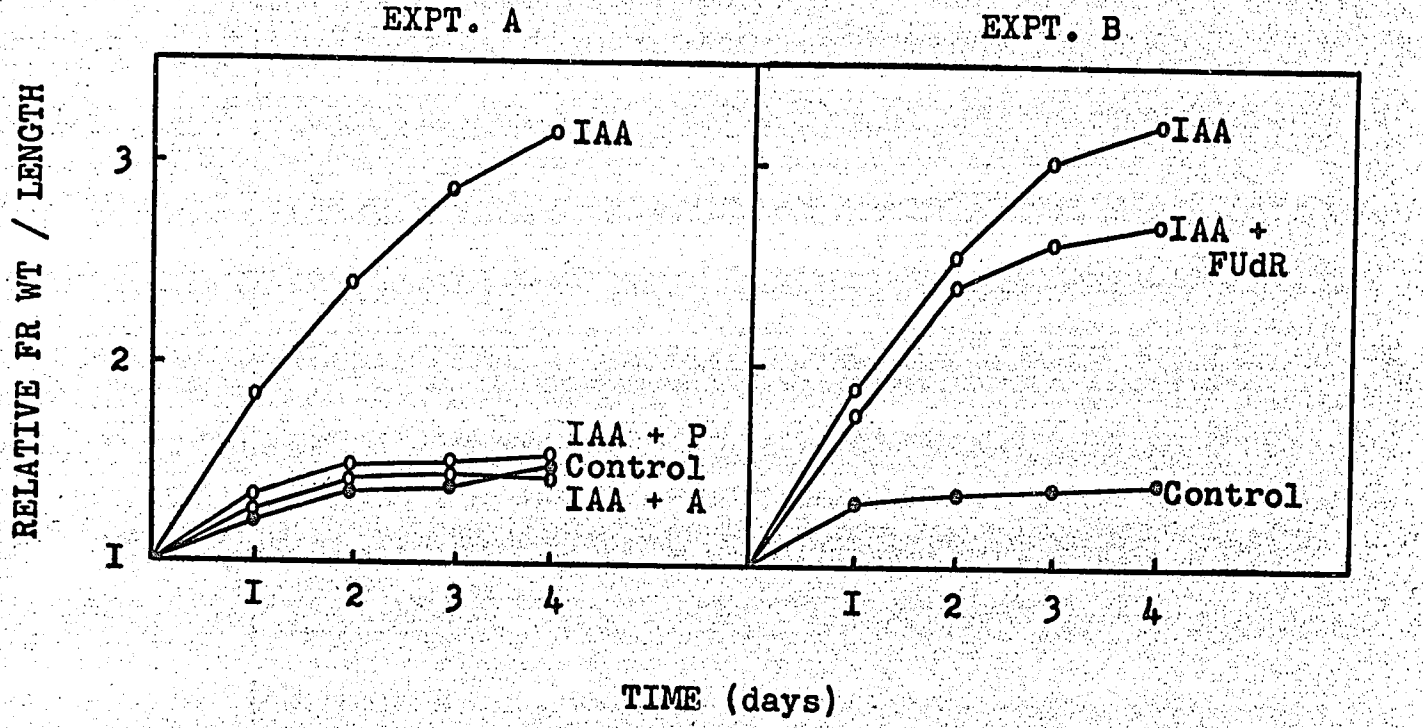
Table VI records the growth in segment length and fresh weight; Figure 4 shows the changes in relative fresh weight per unit length, i.e., a measure of the extent of segment swelling. In untreated segments (control), both length and fresh weight increased mainly during the first 2 days. In IAA-treated segments, elongation was slightly inhibited

Table VI.

Effect of added IAA in combination with puromycin or actinomycin D on the growth of decapitated pea epicotyl tissue segments.

Time (days)	Untreated (control)	+ IAA	+ IAA + puromycin	+ IAA + actinomycin D
		Length (mm/segment)		
0	10.0	10.0	10.0	10.0
1	12.0	12.2	13.1	13.2
2	13.6	12.5	16.4	18.6
3	14.1	14.0	18.6	22.0
4	15.0	14.3	24.0	24.5
		Fr wt (mg/segment)		
0	23.0	23.0	23.0	23.0
1	34.2	53.4	38.8	39.2
2	43.2	69.7	56.8	62.6
3	45.5	92.7	68.5	82.4
4	54.8	106.0	89.0	89.5

Fig. 4. Swelling of segments treated with IAA in combination with puromycin (P), actinomycin D (A) or fluorodeoxyuridine (FUdR). Expts. A and B were carried out on separate occasions. Swelling is indicated by an increase in the fr wt per unit length (see Table VI for original data for Expt. A).


EXPT. A
EXPT. B
RELATIVE FR WT / LENGTH
3
2
I
IAA
IAA + P
Control
IAA + A
IAA
IAA +
FUdR
Control
I
2
3
4
TIME (days)

but the fresh weight increment was much greater than in controls with the result that segments showed marked swelling (3-fold increase in fr wt/length in 4 days). When puromycin or actinomycin D was included with IAA, elongation was greater than that observed after any other treatment given in these tests. The fresh weight kept pace with elongation so that a major effect of these antibiotics was the complete prevention of IAA-induced swelling. In contrast, FUdR had no effect on elongation and it interfered only slightly with swelling.

Figure 5 illustrates the effects of IAA and FUdR on the anatomical structure of segments at 1 and 3 days. In untreated segments some swelling of parenchyma cells but no cell division occurred. Similar results were observed in segments treated with IAA plus puromycin or actinomycin D. In segments treated with IAA alone, marked swelling and some cell division were visible at 1 day. By 2 days many swollen parenchyma cells throughout the cortex had disintegrated to leave lacunae filled with cell and wall debris (284, Appendix 1B). By 3 days recognisable root primordia had been generated in vascular regions. This did not result in much further swelling of the whole epicotyl (cf. Figure 4) because the masses of new cells merely occupied the spaces left by collapsed and disoriented cortical cells. In segments treated with IAA plus FUdR, considerable parenchyma swelling but no visible cell division or wall disintegration took place.

B. Changes in DNA, RNA and Protein

Figure 6 shows effects of IAA and inhibitors on total DNA, RNA

Fig. 5. Anatomical structure of cross sections of segments at 1 and 3 days after treatment with IAA or IAA plus FUdR. Sections (10 μ) were removed from the most swollen regions of tissue after it had been dehydrated in t-butanol and embedded in paraffin wax. Magnification: X 29; stain: saffranin. Note swelling and formation of root primordia in tissue treated with IAA; FUdR prevented cell division.

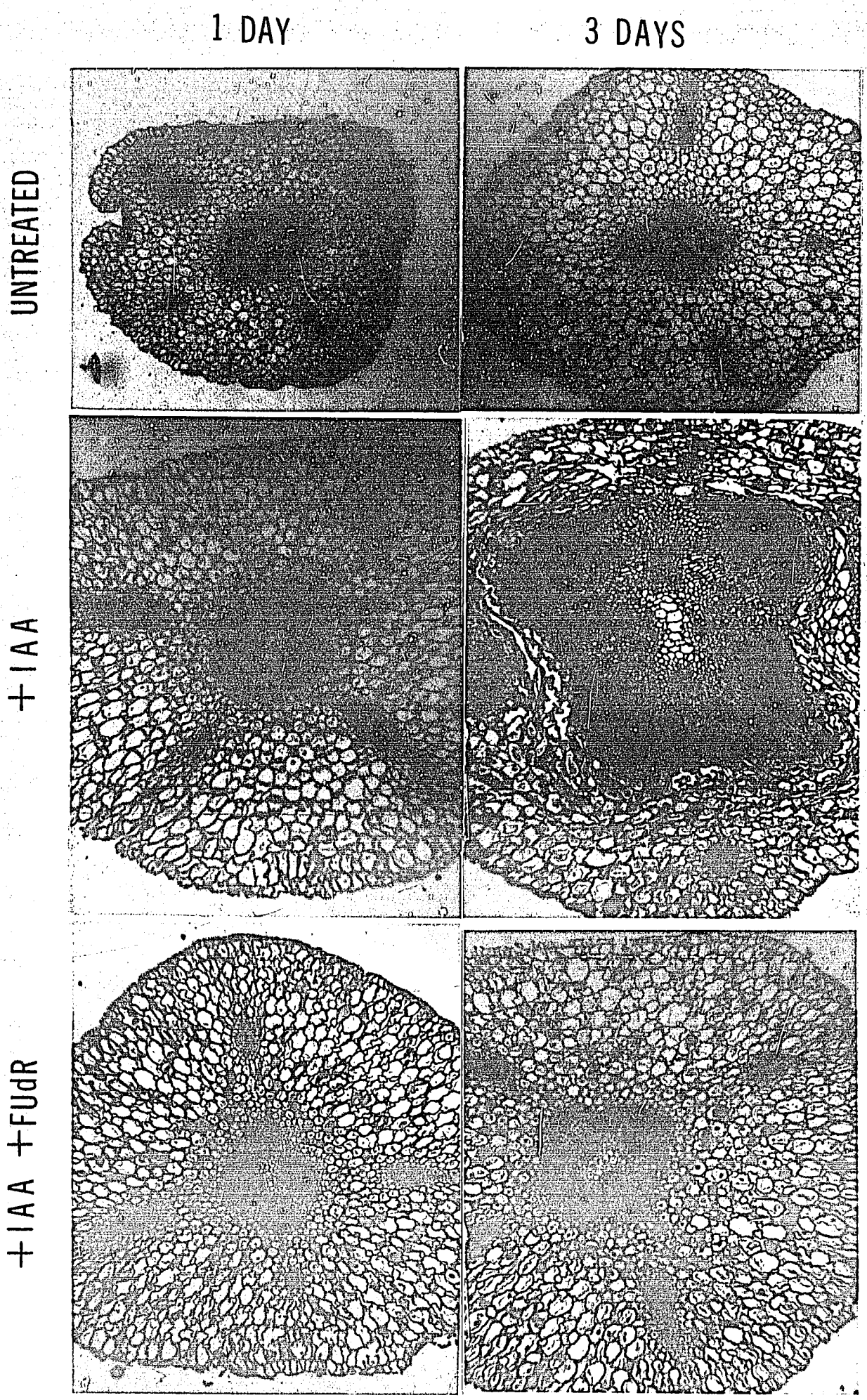
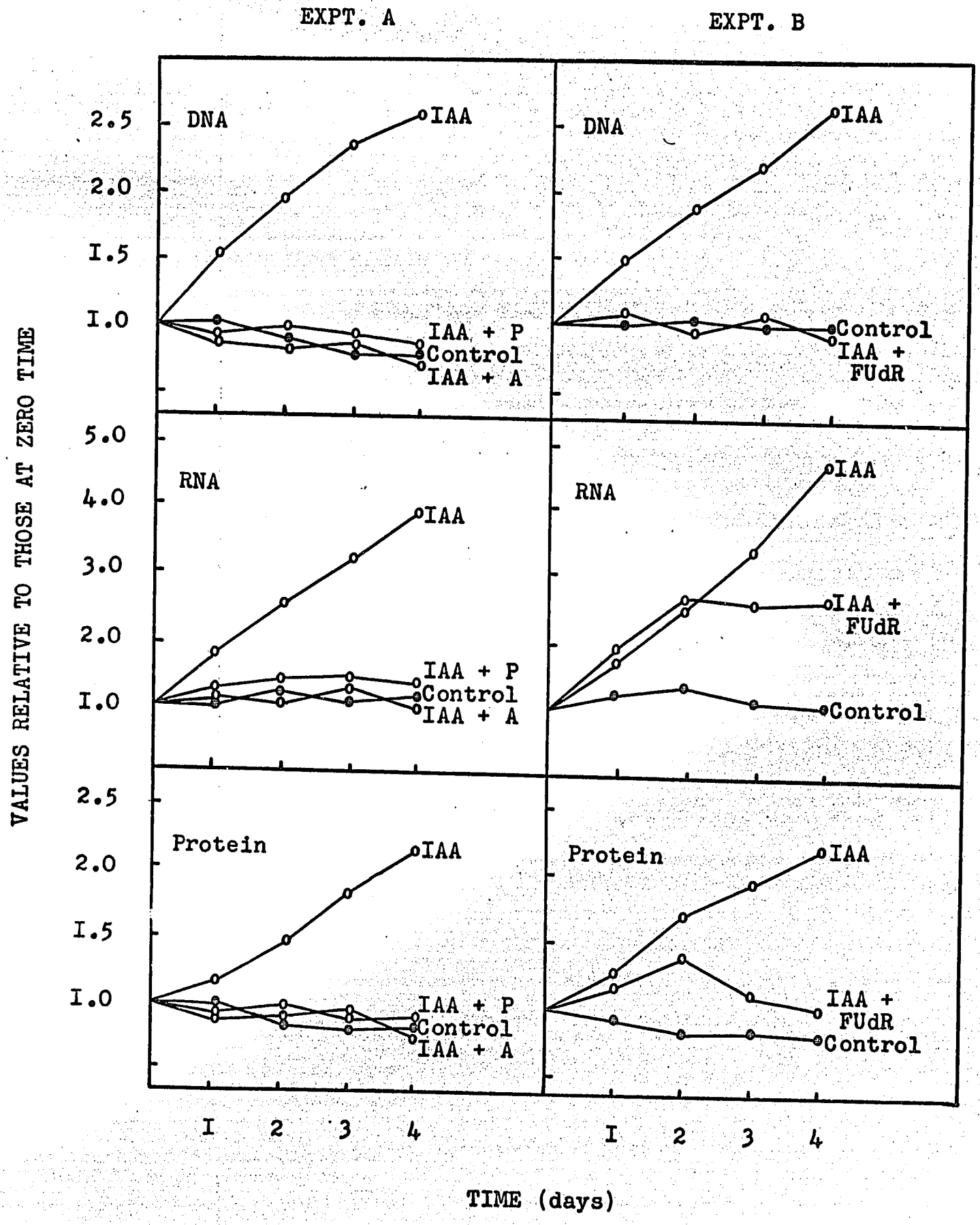

1 DAY
3 DAYS
UNTREATED
+IAA
+IAA +FUdR

Fig. 6. Changes in DNA, RNA and protein levels in segments treated with IAA plus puromycin, actinomycin D or FUdR. Values are calculated relative to those at zero time, namely, for Expt. A and B respectively: DNA = 8.5 and 7.5 μg; RNA = 58.0 and 48.5 μg; total protein = 278 and 238 μg/segment.


EXPT. A
EXPT. B
DNA
RNA
Protein
IAA
IAA + P
Control
IAA + A
IAA + FUdR
2.5
2.0
I.5
I.0
5.0
4.0
3.0
I
2
3
4
VALUES RELATIVE TO THOSE AT ZERO TIME
TIME (days)

and protein levels. Table VII records the ratios of the weight of total RNA/DNA and protein/RNA. In untreated segments, the total and relative amounts of these components showed little change up to 4 days. With IAA treatment, the amounts of each macromolecule increased linearly during the whole experiment. The approximate times necessary for initial levels to double were 2.2 days for DNA, 1.2 days for RNA and 3.4 days for protein. Accordingly, the RNA/DNA ratio steadily increased in the presence of IAA and the protein/RNA ratio decreased. Only the RNA level increased at a rate comparable to the growth rate, i.e., RNA concentration per unit fresh weight was maintained during swelling whereas DNA and protein concentrations decreased (cf. Table VI and Figure 6).

Puromycin and actinomycin D prevented the IAA-induced increases in DNA, RNA and protein (Figure 6). FUdR completely inhibited the increase in DNA which accounts for its effectiveness against cell division (Figure 5). However, FUdR did not interfere seriously with the increase in RNA and protein until after about 2 days incubation. There was therefore a marked increase in the RNA/DNA ratio up to that time (Table VII). Evidently this RNA and protein synthesis, as well as most of the lateral segment expansion (Figure 4), was due to action of IAA in cells which were present in the segment at zero time (pre-existing cells) and was relatively independent of DNA synthesis or cell division.

C. Cellulase Activity.

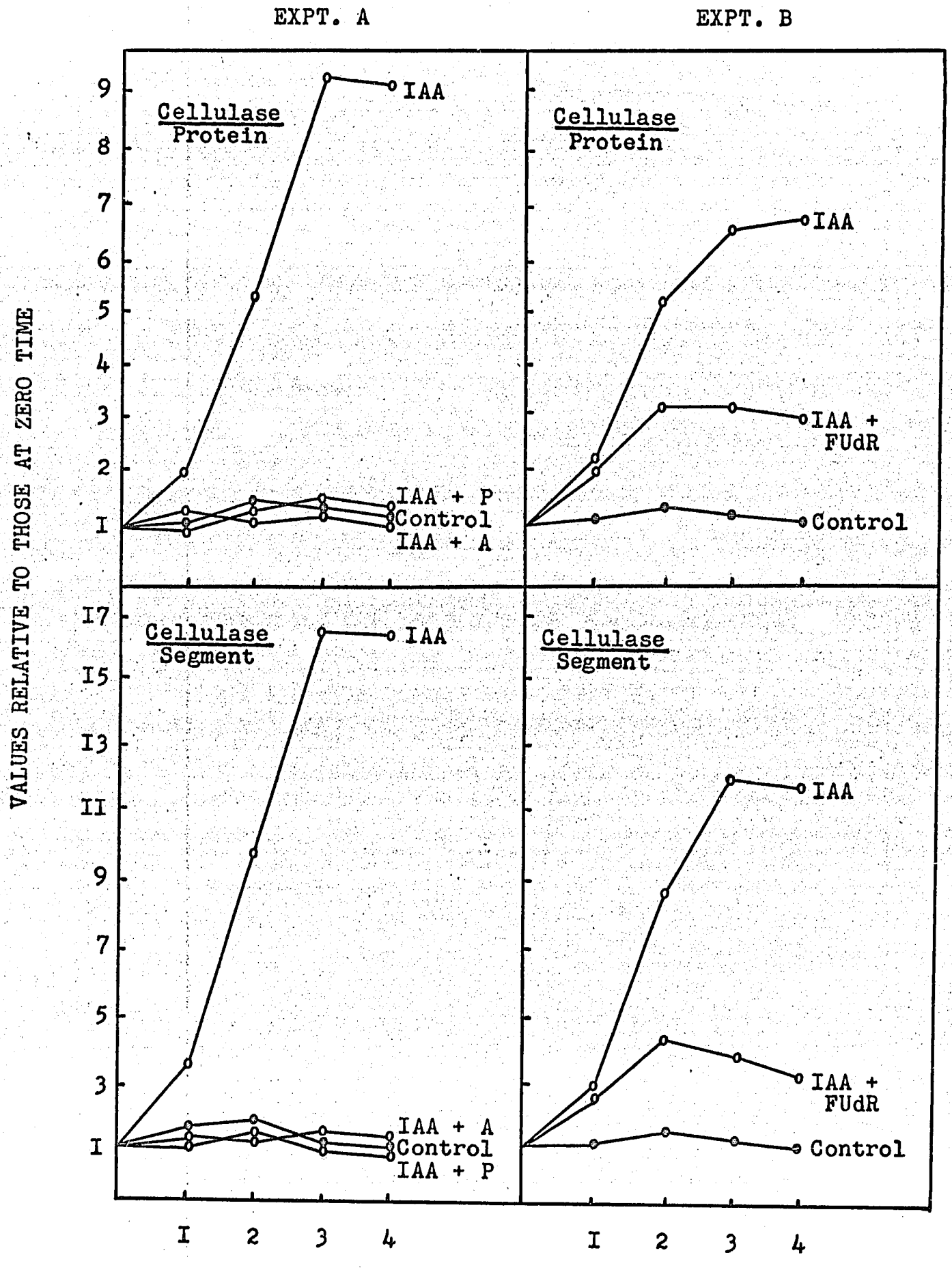
Figure 7 shows the changes observed in cellulase activity. By 3 days, the amount of enzyme per segment reached levels 12 to 16 times

Table VII.

Effect of IAA and FUdR on the weight ratios of RNA/DNA and protein/RNA

Time (days)	RNA/DNA			Protein/RNA		
	Untreated	+ IAA	+ IAA + FUdR	Untreated	+ IAA	+ IAA + FUdR
0	6.5	6.5	6.5	4.9	4.9	4.9
1	7.3	7.8	10.5	3.6	3.5	3.0
2	9.1	8.7	18.5	2.7	3.5	2.7
3	7.1	9.6	16.1	3.5	2.9	2.3
4	6.5	12.0	20.3	4.0	2.2	2.0

Fig. 7. Changes in cellulase activity per unit soluble protein and per segment. Values are relative to those at zero time, namely, for Expt. A and B respectively: cellulase activities = 0.60% and 0.65% loss in viscosity/2 hours/segment; soluble protein = 253 and 228 µg/segment.


EXPT. A
EXPT. B
Cellulase Protein
Cellulase Protein
Cellulase Segment
Cellulase Segment
IAA
IAA + P
Control
IAA + A
IAA + FUdR
VALUES RELATIVE TO THOSE AT ZERO TIME
TIME (days)

the level in controls. This increase was much greater than those in fresh weight (Table VI), total protein, RNA or DNA (Figure 6), i.e., the cellulase level followed a typical sigmoid induction curve regardless of the basis on which it was calculated.

In the presence of IAA plus FUdR, cellulase activity increased rapidly for 2 days, i.e., for as long as rapid synthesis of total RNA and protein continued, and then began to decrease slowly as the total protein level declined (cf. Figures 6 and 7). Since there was no DNA synthesis or cell division in the presence of this inhibitor, the extra cellulase must have formed as a result of direct IAA action in pre-existing cells. The maximum cellulase level reached in these cells (4.5 X zero time) was less than the level reached in segments treated with IAA alone, and the discrepancy between the two increased after the first day as cell division proceeded. Evidently newly-formed cells also synthesized cellulase.

IAA failed to raise cellulase levels in the presence of puromycin or actinomycin D (Figure 7). This cannot have been due to inhibition by these antibiotics of IAA-induced DNA synthesis and cell division since the results with FUdR show that these events were not required for cellulase synthesis. Rather, it may be presumed to result from their effectiveness in preventing RNA and protein synthesis in pre-existing cells.

Discussion

It is clear from the effects of inhibitors (Figures 4 to 7)

that IAA can promote the synthesis of protein in general and cellulase in particular in cells which are in the process of expanding. It is legitimate, therefore, to speculate on the question of where the hormone must act in the sequence of events leading to protein synthesis. Recently it has been suggested that plant hormones promote synthesis of specific enzymes by "unmasking" the appropriate pre formed messenger RNA (297, 345) or by "charging" a particular variety of transfer RNA needed to initiate synthesis (6). Such effects on the process of translation would not necessarily require RNA synthesis. Alternatively, hormones may selectively de-repress part of the genome of maturing cells so that certain species of messenger RNA and eventually the proteins for which they code are synthesized (34, 162, 317). Such control over the transcription of DNA to RNA appears to be the best explanation for nearly all the effects of IAA observed in this study.

IAA induced very great increases in total RNA in the epicotyl segment (Figure 6), not only in time as an eventual result of cell division but from the beginning by direct action in pre-existing cells (cf. RNA/DNA ratios, Table VII). Similarly pronounced and differential effects on RNA synthesis have been reported to follow 2, 4-D-treatment of intact soybean hypocotyls and in this tissue most of the newly-formed RNA was ribosomal (160, 163). In pea seedling tissues, nearly all of the total cell RNA is in microsomes (180) associated with cytoplasm rather than the nucleus (182, 280). Thus, much of the IAA-induced RNA is probably also ribosomal, especially in view of its apparent stability (Figure 6, Experiment B). In this event, merely as consequence of an increased

number of sites where synthesis can proceed, a non-specific increase would be expected in the production of total cell protein. Certainly preferential action of auxin on transcription rather than translation is implied by the fact that auxin causes protein/RNA ratios to decrease in both the pea epicotyl (Table VII) and the soybean hypocotyl (163).

The other main effects of IAA, namely DNA and unilateral cellulase synthesis and growth by cell expansion and cell division, were all closely correlated with the ability of the segments to synthesize RNA. It is difficult to account for these events without supposing that IAA brings about formation of coding varieties of RNA besides ribosomes. In fact, auxins have been shown to stimulate the synthesis of all fractions of RNA including messenger in experiments with attached (163) and detached (160) soybean hypocotyl tissue and with oat coleoptile sections (117). Messenger RNA is the one fraction of total RNA which is still synthesized in detached pea root sections and presumably required in order for growth on solution to proceed (181). It is necessary, of course, to show that only selected messengers are induced in order to explain the specific formation of those enzymes which are needed for growth. To this end, preliminary studies in this laboratory (see 186) have indicated that polyribosomes isolated from pea epicotyl segments after brief IAA treatment are enriched in bound cellulase and appear to have acquired the capacity to synthesize this particular enzyme.

The above considerations apply to reactions brought about by IAA in pre-existing cells. After 2 days of IAA treatment, most or all of the further increases in RNA, protein and cellulase appear to result

from synthesis in newly-formed cells. Even here, however, the continued presence of IAA seems to be needed to promote cellulase synthesis. In these and previous (86) tests, the cellulase level in IAA-treated tissue stopped rising after 3 days although DNA, RNA and protein synthesis continued without abatement. Indeed, when such experiments were carried on for longer time periods, the cellulase level began to decrease while root primordia proliferated and grew through the epidermis. Undoubtedly the concentration of IAA within the segment also decreased with time as a result of translocation, degradation and detoxification reactions, all of which are well known to occur in peas. The implication is that new cells readily form RNA and protein but they lose capacity to form cellulase unless high IAA levels are maintained. Separate tests (Appendix VIC) have confirmed that cellulase activity can be made to increase further after 3 days if fresh 0.5% IAA in lanolin is applied at that time.

It is possible to compare the relative capacities for cellulase synthesis of new and pre-existing cells by calculating the cellulase levels per unit DNA which were reached in segments treated with IAA plus or minus FUdR. The data are assembled in Table VIII. Values close to 5 times initial values were attained in 2 to 3 days whether or not DNA synthesis or cell division took place. After reaching this peak level, the cellulase activity per unit DNA began to decrease under both treatments. It appears, therefore, that cells in this tissue, regardless of age, were capable of generating cellulase at similar high rates provided the genome was fully de-repressed by IAA.

With respect to growth effects in this system, all of the data

Table VIII.

Effect of IAA on the formation of cellulase in new and pre-existing cells

Cell division occurs in IAA-treated segments from the first day unless FUdR is present (Fig.2).

Time (days)	Cellulase activity per unit DNA		
	Untreated	+ IAA	+ IAA + FUdR
0	1.0	1.0	1.0
1	0.8	1.8	2.4
2	1.2	4.8	4.8
3	0.8	5.6	4.0
4	0.6	4.8	4.0

are consistent with the view (86) that IAA-induced cellulase activity helps to bring about lateral swelling of parenchyma cells and fragmentation of their walls (Figure 5). Special attention should be drawn to the fact that greatly enhanced elongation without swelling occurred in segments treated with IAA plus actinomycin D or puromycin (86, Table VI). This result was unexpected since these antibiotics have consistently inhibited auxin-induced elongation in detached pea epicotyl sections (210, 226, 318) and in other excised tissues (160, 162, 181, 226). Nevertheless, several other inhibitors of protein synthesis, e.g. thiouracil, azaguanine, terramycin, etc., have been reported to increase elongation, especially in tissues left attached to the plant (86, 139, 181, 342). Apparently elongation does not require any concurrent increase in the level of RNA, protein or cellulase provided both IAA and some other essential factor derived from elsewhere in the plant are available.

Acknowledgments

We are grateful for gifts of carboxymethylcellulose (type 7MSP) from the Hercules Powder Co., actinomycin D from Merck, Sharp & Dohme Research Laboratories and FUdR from Hoffmann-LaRoche, Canada, Ltd.

Paper III. Studies on the Regulation of Cellulase Activity and Growth in Excised Pea Epicotyl Sections

Abstract

Applied indoleacetic acid (10^{-4} - 10^{-6} M) increased elongation and the amount and specific activity of cellulase in sections detached from the epicotyl of Pisum sativum L. var. Alaska.

Actinomycin D (20 ug/ml) inhibited growth and the capacity of sections for synthesizing protein from absorbed ^{14}C-leucine. At the same time it caused cellulase levels to decrease at a rate which indicated a half-life for the enzyme of less than 24 hours.

Pea cellulase was most stable in vitro between pH 6 and 7; at or below pH 5 its rate of denaturation was comparable to its turnover rate in vivo. Fractionation of sections yielded wall preparations which contained cellulase at a higher specific activity than particles or supernatant.

It is concluded that cellulase is synthesized in excised sections by an auxin-regulated mechanism. It is proposed that the enzyme is transported to the wall where it may promote elongation and eventually become denatured.

Introduction

The application of 0.5% indoleacetic acid (IAA) in lanolin paste to the apex of decapitated etiolated pea epicotyls results in a pronounced increase in cellulase activity in the tissue below (86) as well as marked swelling, cell division and cell wall disintegration (188, 284). All of these responses can be inhibited by including in the lanolin any of a number of substances, e.g., antibiotics, which interfere with protein synthesis (86). Pea cellulase is an endo-glucanase (184), i.e., EC 3.2.1.4. which would be expected to hydrolyse cellulose chains internally wherever amorphous regions occur along the microfibrils. It appears that IAA promotes the formation of cellulase which, in turn, acts to reduce the structural strength and integrity of the primary walls.

Among the questions raised by these observations, the following are examined here in experiments with excised sections of epicotyl tissue:

(a) Can cellulase activity be regulated by endogenous auxin, by physiological levels of applied IAA and by non-toxic levels of antibiotics?

(b) Is cellulase formed directly by action of IAA in pre-existing cells or only indirectly as a consequence of IAA-induced cell division?

(c) Is active cellulase present in the walls of expanding cells?

(d) Does cellulase activity facilitate cell elongation?

The advantages of using excised sections to study these problems include the ease with which the kind and concentration of substances which enter the tissue can be controlled and the fact that pea epicotyl sections respond to low levels of applied IAA by growing in length with little lateral swelling (333). There is no evidence for cell division or DNA synthesis in dark-grown sections (185). Thus any alterations observed in enzyme levels in such sections must proceed independently of mitosis in elongating cells. The main disadvantage of sections is the breakdown of total RNA and protein which begins soon after the tissue is excised (185). As a result, responses which depend on protein synthesis such as growth and enzyme induction, can not be expected to be as pronounced or to continue for as long a period as in the same tissue left attached to the plant.

Materials and Methods

In all tests 10 mm sections were cut with razor blades from the third internode of the epicotyls of 8-day old etiolated pea seedlings. Unless otherwise indicated sections were taken from the apical region of the epicotyl, i.e., from the point where the hook straightens and widens into the epicotyl shaft. Detached sections were either harvested at once (zero time) or after incubation on solutions of 2% sucrose-0.02 M sodium phosphate (pH 6.0) plus or minus various additives. They were handled under dim green light and washed thoroughly in 0.5% NaOCl before their properties were analysed.

At least 80 sections were used to obtain each value reported for length, fresh weight, protein or cellulase content. Procedures for measuring these quantities are described in detail elsewhere (86, 184, 185). One unit of cellulase activity is defined here as that amount of enzyme which brings about 1% loss in viscosity of 10 ml of 1% carboxymethylcellulose (type 7MSP) during 6 hours incubation at 35° and pH 6.0. Each measurement was carried out with 1 ml enzyme extract (from 0.2 g fr wt of tissue) which was derived from at least 5 sections and yielded 5 to 20% loss in viscosity per 6 hours.

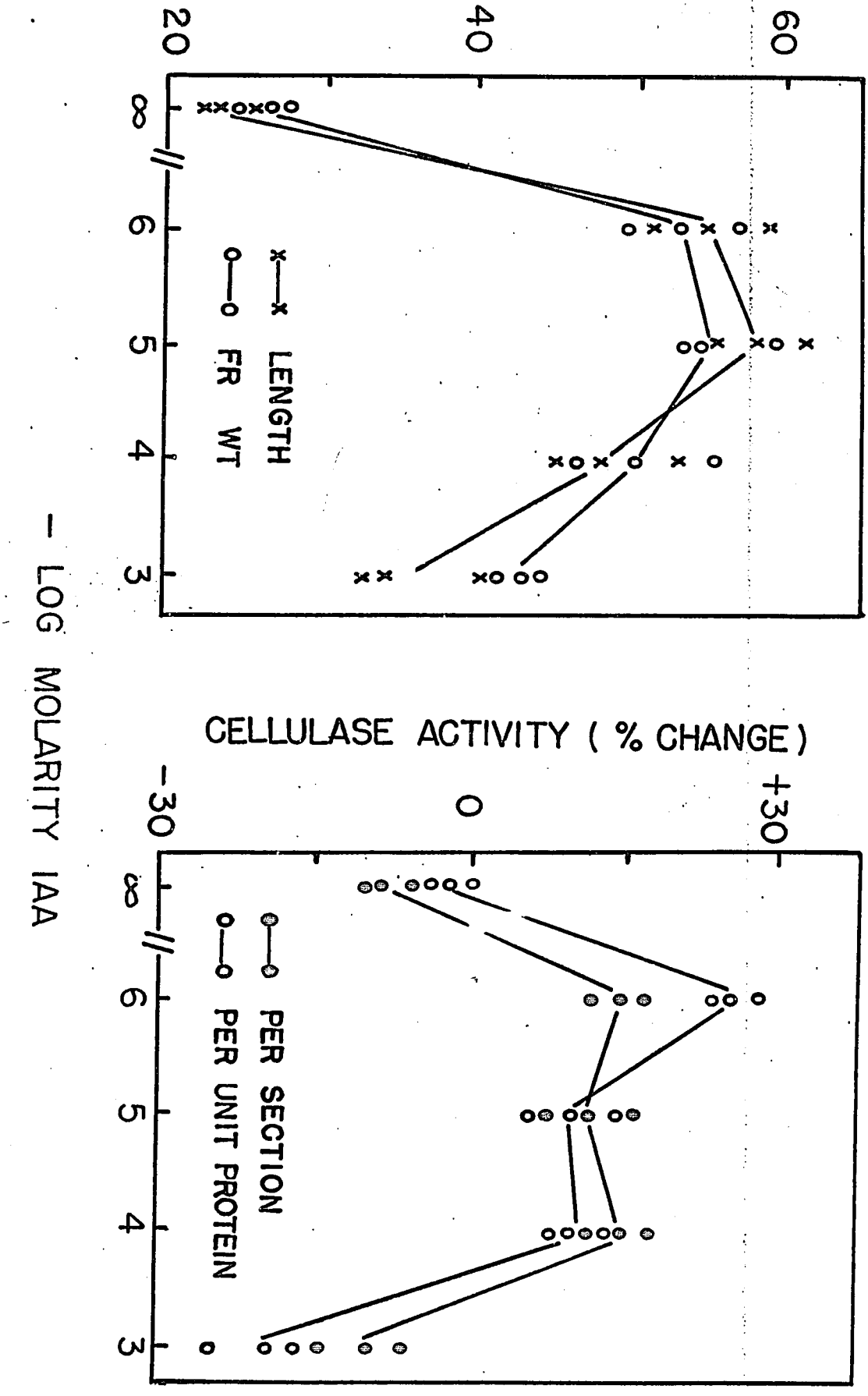
^{14}C-Leucine was obtained from Atomic Energy of Canada. Radioactivity was measured by spreading and drying aliquots on aluminum planchettes and counting at infinite thinness under Q gas with a Nuclear Chicago Model 470 Gas-Flow system. The geometry was such that 1 muc yielded 680 counts/min after correction for background.

Results and Discussion

A. Effects of IAA on Growth and Cellulase Activity

Figure 8 shows the effects in three experiments of IAA added at different concentrations to excised apical sections. After 12 hours incubation, sections in 10^{-6} to 10^{-4} M IAA contained 20 to 30% more cellulase than untreated sections. This difference was comparable to the percentage increase due to IAA in length and fresh weight. IAA at 10^{-3} M was supraoptimal for growth and caused cellulase activity to decrease slightly. The total amounts of buffer-soluble protein also

Fig. 8. Effect of IAA concentration on growth and cellulase activity of excised pea epicotyl sections. Apical sections (80–100, 10 mm long) were floated on 10 ml 2% sucrose – 0.02M sodium phosphate (pH 6.0) with or without added IAA. After 12 hours in darkness at 25°, they were washed in 0.5% NaOCl, weighed and measured for length. In order to extract enzymic protein, sections were homogenized and centrifuged twice in a total of 4 volumes of 0.1M sodium phosphate (pH 7.0). Values given in the Figure are derived from three experiments done on separate occasions. They are calculated relative to values per section measured at zero time, namely, fresh weight = 23.6, 21.8 and 22.6 mg, soluble protein = 212, 192 and 226 µg, and cellulase activity = 1.19, 0.98 and 1.16 units respectively.


GROWTH (% INCREASE)
20
40
60
8
6
5
4
3
x—x LENGTH
o—o FR WT
CELLULASE ACTIVITY (% CHANGE)
-30
0
+30
8
6
5
4
3
PER SECTION
PER UNIT PROTEIN
- LOG MOLARITY IAA

decreased during the experiment but this happened at all IAA concentrations so that changes in cellulase specific activity were similar to those for total cellulase per section. There was little change in fresh weight per unit length in any of the sections indicating that IAA did not cause lateral cell expansion under these conditions. (Effects of IAA on excised sections from other seedlings are shown in Appendices IVF and G.)

In experiments where apical sections were incubated for shorter or longer periods than 12 hours, the cellulase level usually increased in the first few hours when growth was most rapid and then decreased slowly (e.g., see Table XII). Treatment with IAA at 10^{-5} M always led to higher cellulase levels but the magnitude of this increase was not always as great as the growth response.

The rise in cellulase due to added IAA was most pronounced in sections where the level of endogenous auxin could be presumed to be low. Table IX shows the effects of IAA on 10 mm sections cut 0, 5 or 10 mm from the apex. Apical sections contained younger tissue and higher levels of natural auxin than basal sections (285). In this particular test, apical sections increased in fresh weight more than usual and the cellulase level was higher after 24 hours incubation that at zero time (cf. Figure 8). Addition of 10^{-5} M IAA increased the fresh weight by 35% and the cellulase level by 18% over controls. In basal sections the relatively low initial cellulase levels (184) decreased further on incubation except when IAA was added. By 24 hours, IAA-treated basal sections contained nearly twice as much cellulase as controls. The sections appeared to possess a maximum capacity for cellulase synthesis

Table IX.

Effect of IAA on growth and cellulase activity in pea epicotyl sections cut 0, 5 or 10 mm from the apical hook*

Distance from hook (mm)	Fresh** weight		Length**		Cellulase activity** per unit protein		Cellulase activity** per section	
	Absence or presence of IAA (10^{-5} M)							
	−	+	−	+	−	+	−	+
0	165	222	137	145	135	159	124	147
5	127	160	114	125	91	151	100	165
10	127	138	110	117	67	118	70	137

* Sections (10 mm) were incubated for 24 hours as described in Fig. 1. At zero time, for the 3 sections respectively: fr wt = 20.2, 22.0 and 23.6 mg/section, cellulase activity = 1.13, 0.91 and 0.78 units/section, total soluble protein levels = 234, 173 and 138 µg/section.

** Values are expressed as a % of those at zero time.

which could be maintained either by high levels of endogenous auxin or by added IAA.

While it is possible that auxin-induced cellulase activity facilitated cell elongation in the above tests, undoubtedly there are many different processes which can limit growth in excised sections without influencing this enzyme. Other growth regulators were found to have little effect on total cellulase activity (Appendices VC, VIIIA, B and C) even though some (e.g., gibberellic acid, ethylene gas) markedly stimulated section elongation or expansion and others (e.g., Ca^{++}, ascorbate) inhibited growth. Kinetin and benzimidazole caused sections to swell and slightly increased the cellulase level (Appendix VC), especially when added with high levels of IAA (Appendix VD), but this could have resulted from the ability of these substances to protect total protein from breakdown rather than from any specific action on cellulase.

B. Turnover of Cellulase

Cellulase activity often increased or decreased with time independently of measurable changes in total soluble protein, which implies that this enzyme was subject to a higher turnover rate than most other protein in the cell. This was confirmed by studies with actinomycin D, an inhibitor already shown (65, 226, 317) to reduce the amount of growth and the incorporation of labelled precursors into RNA and protein of excised epicotyl sections. It was necessary first to show that under the conditions of present tests actinomycin D directly inhibited proteins synthesis from available (absorbed) substrate, as distinct from any

effects it may have on uptake of substrate into the sections (65, 226).

Table X shows the results of a test in which sections were pre-treated with actinomycin D for 6, 12 or 24 hours and then provided with ^{14}C-leucine for a further 2 hours, after which measurements were made of ^{14}C-incorporation into protein and ^{14}C-uptake into the free-amino-acid pool. The data show that the capacity of sections to take up leucine increased greatly soon after cutting and actinomycin D prevented this increase thereby, in effect, inhibiting uptake. Nevertheless, the incorporation of leucine into protein was inhibited more severly than uptake into the sections: the ratio of combined leucine to free absorbed leucine was reduced by half within 6 hours. Similar inhibitions by actinomycin D were observed in a test of nucleic acid synthesis from ^{14}C-orotate (Appendix VIIA). It is concluded that actinomycin D does inhibit protein and nucleic acid synthesis in detached sections although the inhibition is not complete and is probably not as great as appears from incorporation data alone.

Tables XI and XII show the effects of actinomycin D on growth and cellulase activity. An inhibitor concentration of 20 μg/ml (as used in the isotope experiment, Table X) was sufficient to attain maximum reduction in fresh weight, length and cellulase level. Inhibitory effects were observable within 6 hours and by 12 hours the values for growth and cellulase activity per section or per unit protein were only about half of those in controls. In contrast, actinomycin D had no significant effect on the decrease of total protein during the experiments. Evidently cellulase was one of few proteins which were still synthesized

Table X.

Effect of pretreatment of sections with actinomycin D on subsequent uptake of ^{14}C-leucine and its incorporation into protein*.

Pre-treatment time (hours)	^{14}C-leucine subsequently**				Ratio of ^{14}C in: protein / free amino acid	
	incorporated into protein		absorbed but not in protein			
	Absence or presence of actinomycin D (20 ug/ml)					
	−	+	−	+	−	+
0	1450		700		2.1	
6	4350	650	2400	600	1.8	1.1
12	4450	850	2750	750	1.6	1.1
24	4850	600	2850	800	1.7	0.8

* After pretreatment for the times indicated, 20 sections were transferred to 2.0 ml, 0.02M sodium phosphate (pH 6.0) containing 930,000 c.p.m. in 25.4 mg of L-^{14}C-leucine. After 2 hours, sections were washed in unlabelled leucine and repeatedly homogenized and extracted with 5% trichloroacetic acid and hot 80% ethanol. ^{14}C was measured in the extracts and in the residue. Chromatography confirmed that all of the label was still present in leucine, either free (in extracts) or combined in protein (in residue) (see Appendix VII B).

** Values are expressed as c.p.m. incorporated or absorbed/2 hours/section.

Table XI.

Effect of actinomycin D concentration on growth, protein level and cellulase activity*.

Actinomycin conc. (μg/ml)	Fresh** weight	Length**	Soluble** protein	Cellulase activity** per unit protein	Cellulase activity** per section
0	151	136	75	123	93
10	132	121	84	71	60
20	133	121	72	56	41
40	130	119	75	47	35
100	130	118	73	60	44

* Sections were incubated for 24 hours; at zero time for each 10 mm section: fr wt = 23.5 mg, soluble protein = 258 μg, cellulase activity = 1.39 units.

** Values are expressed as a % of those at zero time.

Table XII.

Progress in the inhibition of growth and cellulase activity by actinomycin D*

Incubation time (hours)	Fresh weight**		Length**		Soluble protein**		Cellulase activity**	
	Absence or presence of actinomycin D (20 µg/ml)							
	−	+	−	+	−	+	−	+
6	118	113	121	113	94	94	127	100
12	137	122	131	117	97	92	178	84
24	160	128	142	119	93	93	159	60
36	158	127	147	124	69	67	110	49

* At zero time, for each 10 mm section, fresh weight = 21.9 mg, soluble protein = 239 µg and cellulase activity = 1.06 units.

** Values per section are expressed as a % of those at zero time.

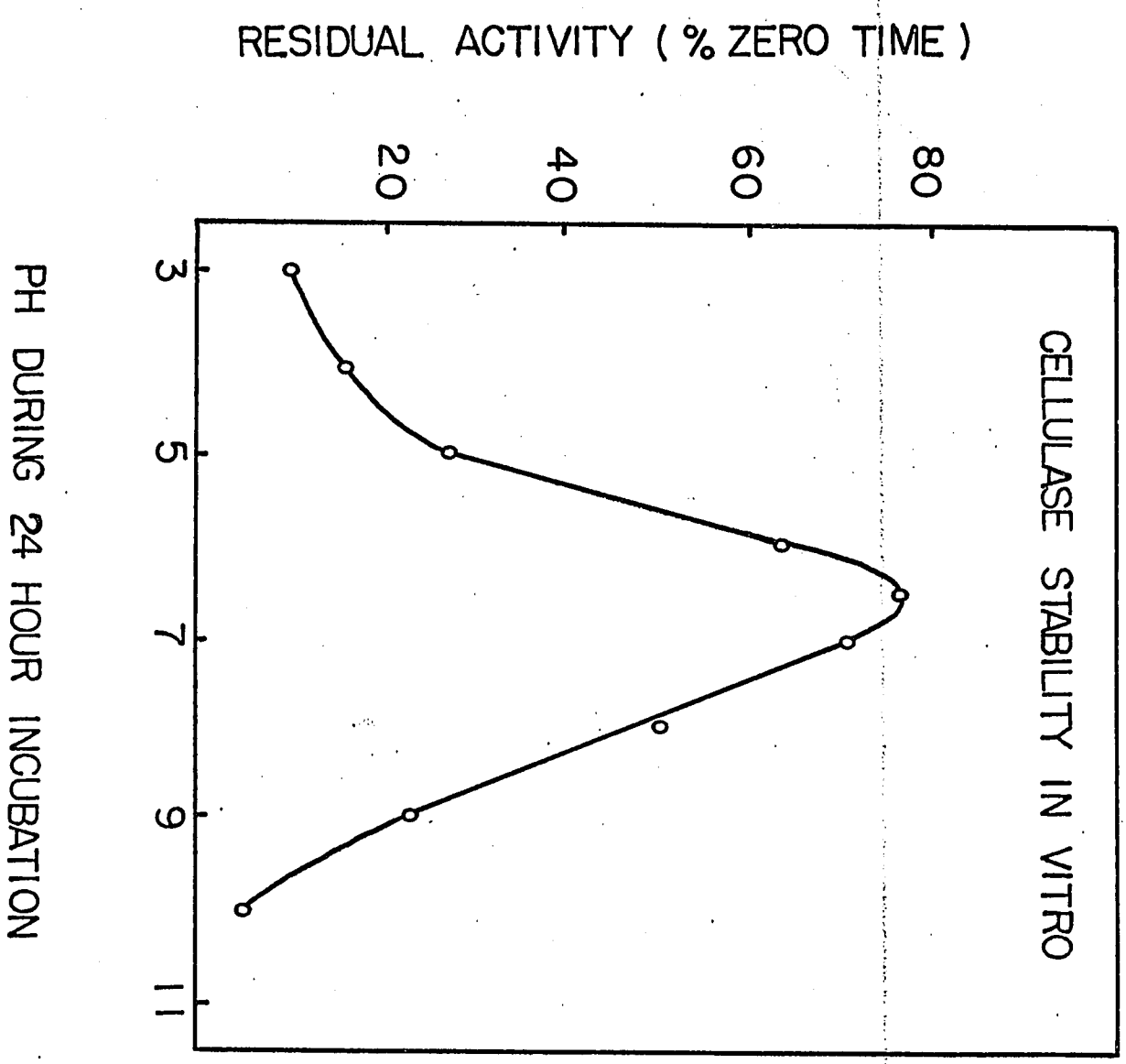
in detached sections and it was also relatively unstable with a half-life of less than 24 hours. Accordingly, in the presence of actinomycin D, it would be possible for the initial amount of active cellulase in the sections and the rate of its inactivation to be major factors in limiting the amount of subsequent growth.

In view of the turnover of cellulase, measurements of increases or decreases in total cellulase level during incubation underestimate the true amounts of this enzyme which have been generated or degraded in that time. Thus, the stimulation of cellulase activity by IAA (Figure 8, Table IX) must be regarded as a minimal estimate of the effect of IAA on rate of cellulase synthesis.

C. Fate of Cellulase

It was suggested earlier (86) that instability of cellulase in vivo may be due to its inactivation in the primary wall. Any extracellular enzyme would probably denature more rapidly than total protoplasmic protein, if only because of the weak buffering capacity which would be expected in fluid bathing the wall. In young pea epicotyl cells, for example, an excreted enzyme could encounter regions of acidity in the wall due to concentrations of polyuronic-acid (185). As shown in Figure 9, soluble pea cellulase is unstable in vitro at pH values much removed from its activity optimum of pH 6 to 7. In particular, over 75% of the activity was lost in 24 hours at pH values below 5, a rate of denaturation which would be sufficient to account for the observed turnover rate of the enzyme in vivo (Tables XI and XII). (Effects of external

Fig. 9. Stability of pea cellulase *in vitro* as affected by pH. Freshly-prepared enzyme extract from apical sections (11.1 units cellulase/ml in 0.02M sodium phosphate, pH 6.5) was adjusted to the pH values indicated in Figure by adding dilute HCl or NaOH. Extracts were incubated at 25° in darkness for 24 hours and the final cellulase activities were compared to those at zero time.


CELLULASE STABILITY IN VITRO
RESIDUAL ACTIVITY (% ZERO TIME)
20
40
60
80
3
5
7
9
11
PH DURING 24 HOUR INCUBATION

pH on cellulase activity in sections *in vivo* are shown in Appendix VIIID.)

Presumably it is only that part of the total cellulase that is active in the primary wall which could be effective in promoting growth and which should be considered in studies of this enzyme's function. It is difficult, however, to separate wall from protoplasmic enzyme without introducing uncertainty over the amount that may have leached from or adsorbed onto the wall during extraction. Only traces of cellulase activity remained associated with insoluble residues when sections were homogenized by methods used routinely in these studies (86, 184). The main problem in this case, therefore, appears to be to devise methods for keepting the wall cellulase *in situ* during fractionation.

Table XIII summarizes data for the distribution of cellulase between wall, particle and supernatant fractions isolated after using different solvents or homogenization methods. When sections were blended with a medium containing 0.6 M sucrose (Experiment A), the wall residue (retained by nylon mesh) yielded only 5% and the particles (pellet from exudate X 50,000 g) 1% of the total cellulase. When sections were frozen and thawed and juices expressed without shearing force by repeated squeezing through nylon mesh and washing with salt-buffer mixtures (Experiment B), the relatively intact wall-residue fraction still contained only 10% and particles 3% of the total cellulase. However, when the initial extraction was performed with a solvent containing Ca^{++} (Experiment C) or Mg^{++} (Experiment D), as much as half of the total cellulase remained associated with wall material at a specific activity per unit protein approaching 3 times that in the supernatant. The ions were

Table XIII.

Effect of various treatments during extraction on the distribution of cellulase activity between wall, particle and supernatant fractions from apical epicotyl sections.

Treatment during extraction	Fraction*	Distribution (% total) of: cellulase activity	soluble protein	Relative cellulase activity per unit protein
A. Homogenized by high-speed blendor twice in 0.6 M sucrose – 0.1 M phosphate (pH 7.0).	Supernatant	94	89	100
	Particles	1	9	15
	Wall	5	2	157
B. Frozen, thawed and juices expressed by repeated washing with 0.3 M NaCl – 0.05 M tris (pH 7.5).	Supernatant	85	89	100
	Particles	3	4	85
	Wall	10	7	183
C. As in B with solvent including 0.01 M $CaCl_2$.	Supernatant	48	64	100
	Particles	3	11	38
	Wall	49	25	256
D. Homogenized by grinding twice in 0.4 M sucrose – 0.005 M tris (pH 7.8) + 0.2% sodium deoxycholate – 0.001 M $MgCl_2$.	Supernatant	78	76	100
	Particles	4	17	19
	Wall	18	7	275

* After all treatments, cellulase was completely removed from the wall fraction (residue retained by nylon mesh) and the particulate fraction (pellet from exudate x 50,000 g) by exhaustive blending in 0.1 M EDTA (pH 7.0).

effective at concentrations which neither precipitated nor activated nor inhibited cellulase activity. They did not increase the amount of specific activity of cellulase in particulate fractions which implies that they did not effect any binding of cellulase to lipo-protein membranes. There is no reason, therefore, for supposing that they introduced ionic linkages between cellulase and protein or polyuronic acid in the wall. Rather, the new cross-linkages which would be expected between structural wall materials may have simply trapped endogenous cellulase in a more than usually gelled or stiffened wall.

The data appear to justify the conclusion that a major part of the extractable cellulase is derived from the wall. Preliminary tests (E. Davies, unpublished) on the fate of the extra cellulase induced by IAA suggest that it is distributed partly in the wall and partly in the protoplasm, as would be expected. Further tests are in progress in an effort to follow the movements of cellulase from the time when it is synthesized until it is inactivated.

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APPENDIX I

Effect of IAA on Anatomical Structure of Pea Epicotyls

I. External Appearance of IAA-Treated epicotyls

The following methods were used for growing pea seedlings: seeds of Pisum sativum L. Alaska were washed with 0.5% NaOCl solution for 10 minutes and soaked in tap water for about 10 hours. They were sown in wet perlite and grown in darkness for 8 days. Seedlings with third internodes 3 to 5 cm long were selected. The plumule was cut off just below the hook. A point on the epicotyl 10 mm below the cut apex was marked with ink. The cut apex was then painted with lanolin paste with or without IAA (0.5% w/w) and the seedlings were allowed to continue growing in darkness at 20°.

Visible effects of the above treatments on growth at the apex of the epicotyl and branching at the second node are shown in Appendix IA. IAA treatment resulted in rapid swelling of the upper part (segment) of the epicotyl which was visible within 1 day. Eventually (in 5 days) adventitious roots broke through the epidermis of the swollen region. In the presence of IAA no branch developed at the node. Control epicotyls showed elongation and branching at the second node but no swelling of the segment. Decapitation is well known to remove apical dominance over

the second node and the influence from the apex can be replaced by added auxin (188).

2. Effect of IAA on Anatomical Structure of Cross-sections of Segments.

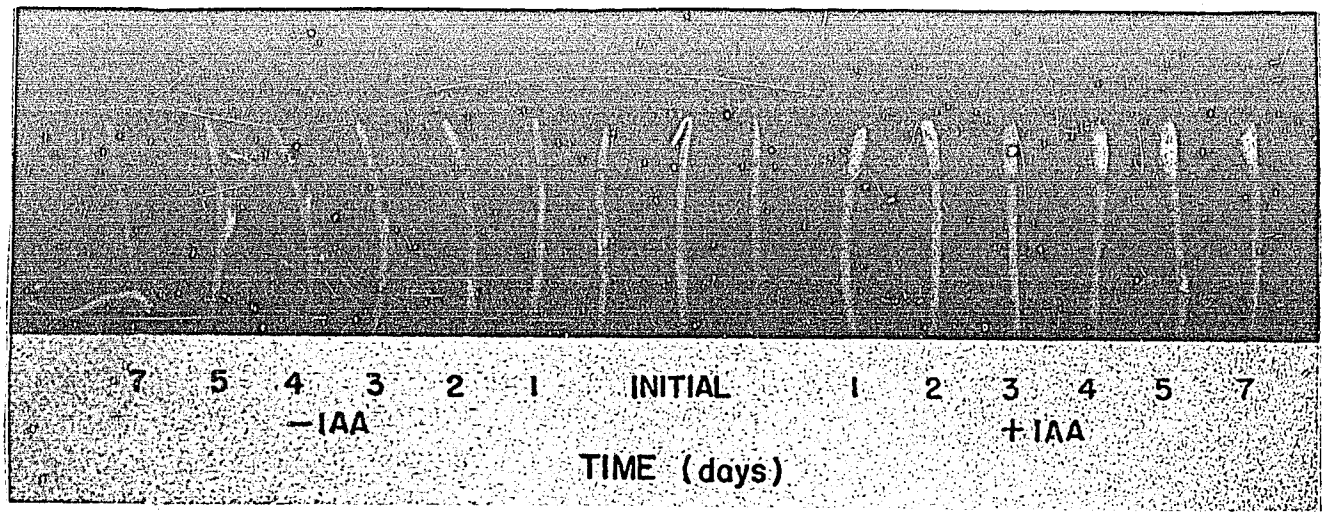
Segments were cut from the apical region of decapitated pea epicotyls treated with or without IAA as described above (Appendix IA). They were fixed in F.A.A. (formalin-acetic acid-alcohol) and dehydrated with a standard t-butanol series. The dehydrated tissues were then embedded in Fisher Tissue Mat. Sections (10 μ) were removed from the most swollen region of tissue with a rotary microtome and mounted on microscope slides. The slides were passed through the following sequence of reagents for staining:

(a) absolute xylene
(b) 50/50 absolute xylene/absolute ethanol
(c) 95% ethanol
(d) 85% ethanol
(e) 70% ethanol
(f) safranin in 70% ethanol
(g) 85% ethanol
(h) 95% ethanol
(i) fast green in 95% ethanol
(j) 95% ethanol

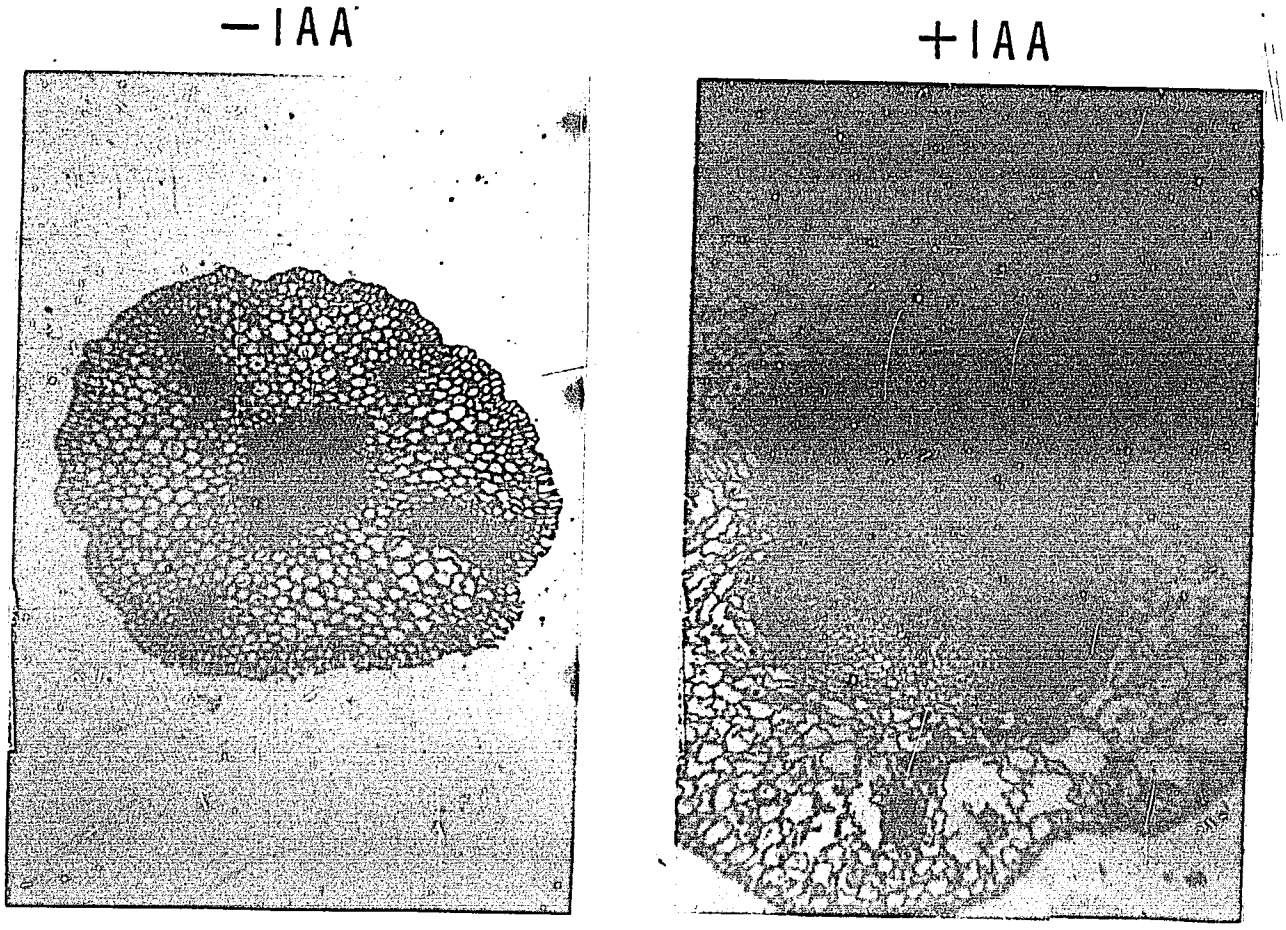
Finally, the tissue was mounted in Permount (Fisher Scientific Company). All photographs of the slides were taken with a Zeiss Jena photomicroscope using Kodak high contrast film.

Appendix IB shows the cellular structure visible after 2 days of treatment. In the presence of added IAA there was extensive cell division, particularly in regions of the central stele and cortical bundles. Parenchyma cells were swollen and cell walls in many cases had disintegrated to form large lacunae. Without added IAA, there was little visible change in segments from zero time (cf. Fig. 5).

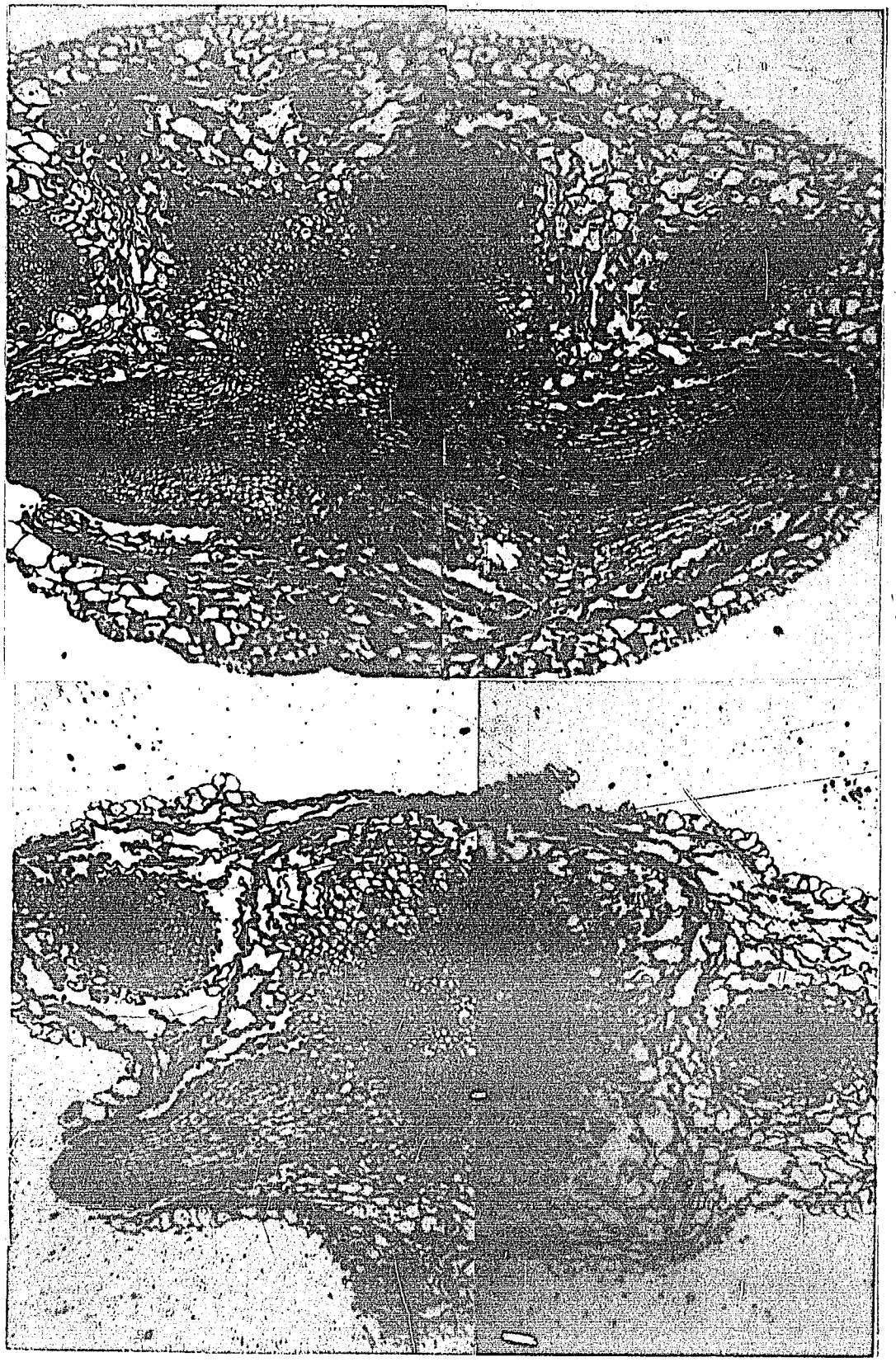
Appendix IC shows the structure at 5 and 6 days. Root primodia were visible microscopically within 3 days of IAA treatment (Fig. 5) and by 5 or 6 days they had grown through the cortex and penetrated the epidermis (see also Appendix IA). The primodia develop from the central stele and there are continuous vascular traces into them. Control segments showed no change from zero time (as in Appendix IB).



Appendix IA. External appearance of decapitated epicotyls after painting apex with lanolin plus or minus 0.5% IAA



Appendix IB. Effect of 2 days treatment at the epicotyl apex with or without IAA on anatomical structure of cross sections of swollen regions of the epicotyl



Appendix IC. Root formation after 5 and 6 days of IAA treatment

APPENDIX II

Methods for Determination of Soluble Protein, Total Protein, RNA and DNA

1. Soluble Protein Determination by the Biuret Method

The biuret method used here was specific for estimating peptide bonds and was the same as that described in the Methods in Enzymology (113, 172). As shown in Appendix IIA, using bovine serum albumin as standard protein, the method accurately measured from 1 to 8 mg protein.

The procedure used with protein extracted from plant sources in this study was as follows: to each 2.5 ml of crude enzyme extract, 0.5 ml of 3 M trichloroacetic acid was added and the mixture was centrifuged. The precipitate was suspended in 3 ml of distilled water and 3 ml of Biuret reagent was then added. The reagent was prepared and stored in the brown bottle. A commercially available reagent (Fisher Scientific Co. So-B-51) was also found to be effective. After stirring and shaking until the protein was totally dissolved in the reagent and incubation at room temperature for at least 30 minutes, the optical density (O.D) of the colored solution was measured at 545 mμ.

2. Total Protein Determination by Nesslerization

A Kjeldahl digestion followed by nesslerization (77) was used

for total nitrogen determination. As shown in Appendix IIB, the method measured from 1 to 8 μg nitrogen as $(NH_4)_2SO_4$, equivalent to from about 6 to 50 μg protein (conversion factor X 6.25). The procedure yields values for bovine serum albumin which are the same as those obtained by the Biuret method.

Details of the procedure used for determining total segment protein are as follows: segments (about 2.5 g) were extracted in succession with 80% ethanol, ethanol-ether (3:1 v/v) and warm 0.5 N $HClO_4$ in order to remove low-molecular weight nitrogenous compounds and nucleic acids. The residue was placed in a Kjeldahl flask with 30 ml of 50% H_2SO_4. The mixture was digested for 2.5 hours over a microburner. The flasks were then cooled and 20 ml of 30% H_2O_2 were added. The mixture was digested for an additional 1.5 hours. The colorless digest was diluted to a total volume of 500 ml. To 1 ml of this digest, 6 ml of Nessler reagent (Fisher Scient. Co. So-N-20) was added. After at least 30 minutes incubation at room temperature, the O.D. of the clear yellow solution was measured at 425 mμ.

3. Total Nucleic Acid Determination by Optical Density Absorption

Appendix IIC shows that the method (77) calibrated with soluble RNA (Nutrition. Biochem. Co. 6164) measured from 10 to 60 μg RNA. Details of the procedure used for determining total segment nucleic acid are as follows: segments (about 2.5 g) were extracted with 80% ethanol and ethanol-ether (3:1 v/v). The residue was then homogenized in 10 volumes of warm 0.5 N $HClO_4$. The mixture was incubated for 1 hour at 70° and

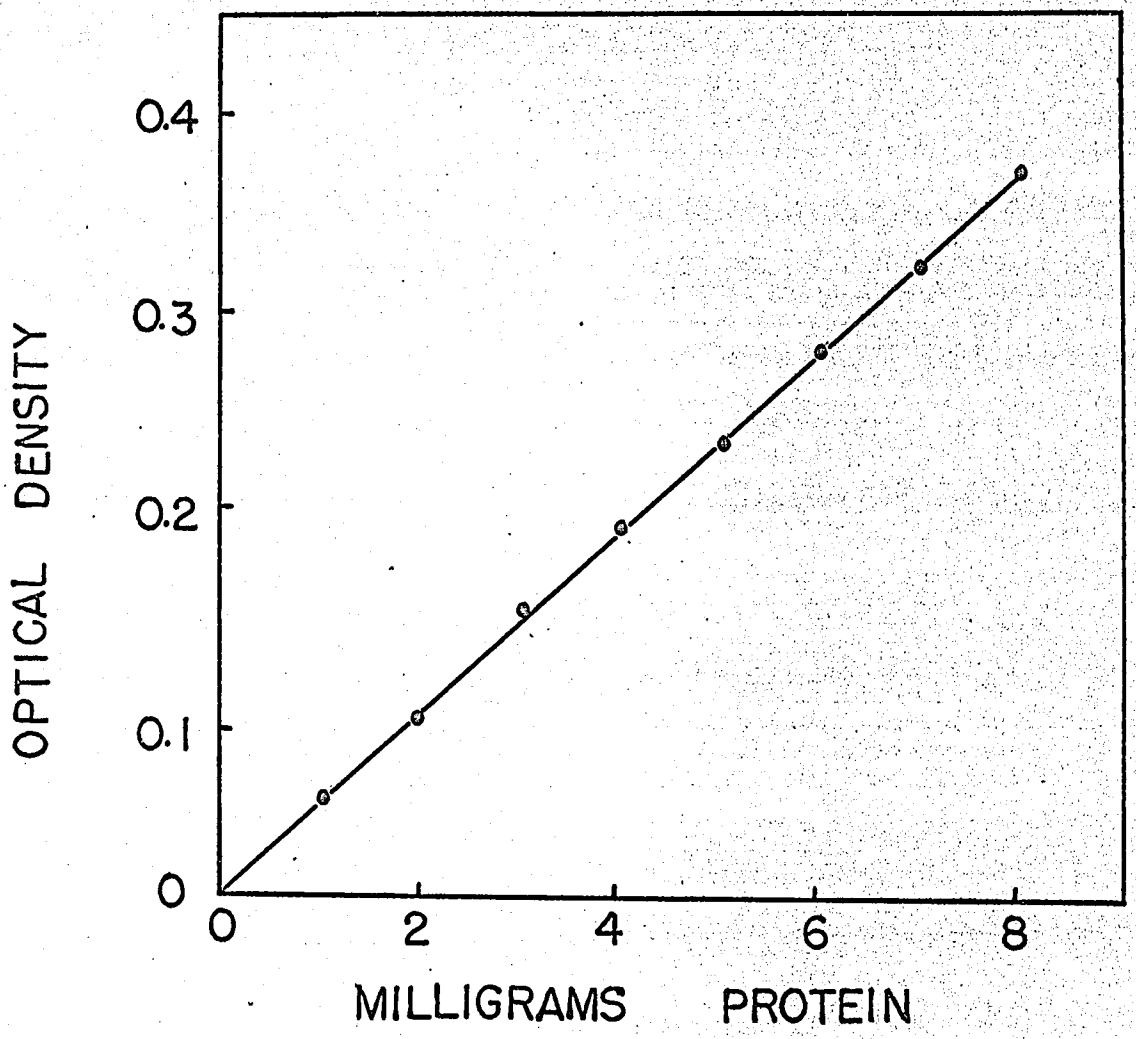
centrifuged. The extraction was repeated once. The combined extracts were diluted to a total volume of 200 ml. Aliquots were used to measure O.D. at 260 mμ and 290 mμ. O. D. values in Appendix IIC equal the difference of readings ($O.D._{260} - O.D._{290}$).

RNA concentration was calculated from values for total nucleic acid (Appendix IIC) by subtracting values for DNA (see Appendix IID).

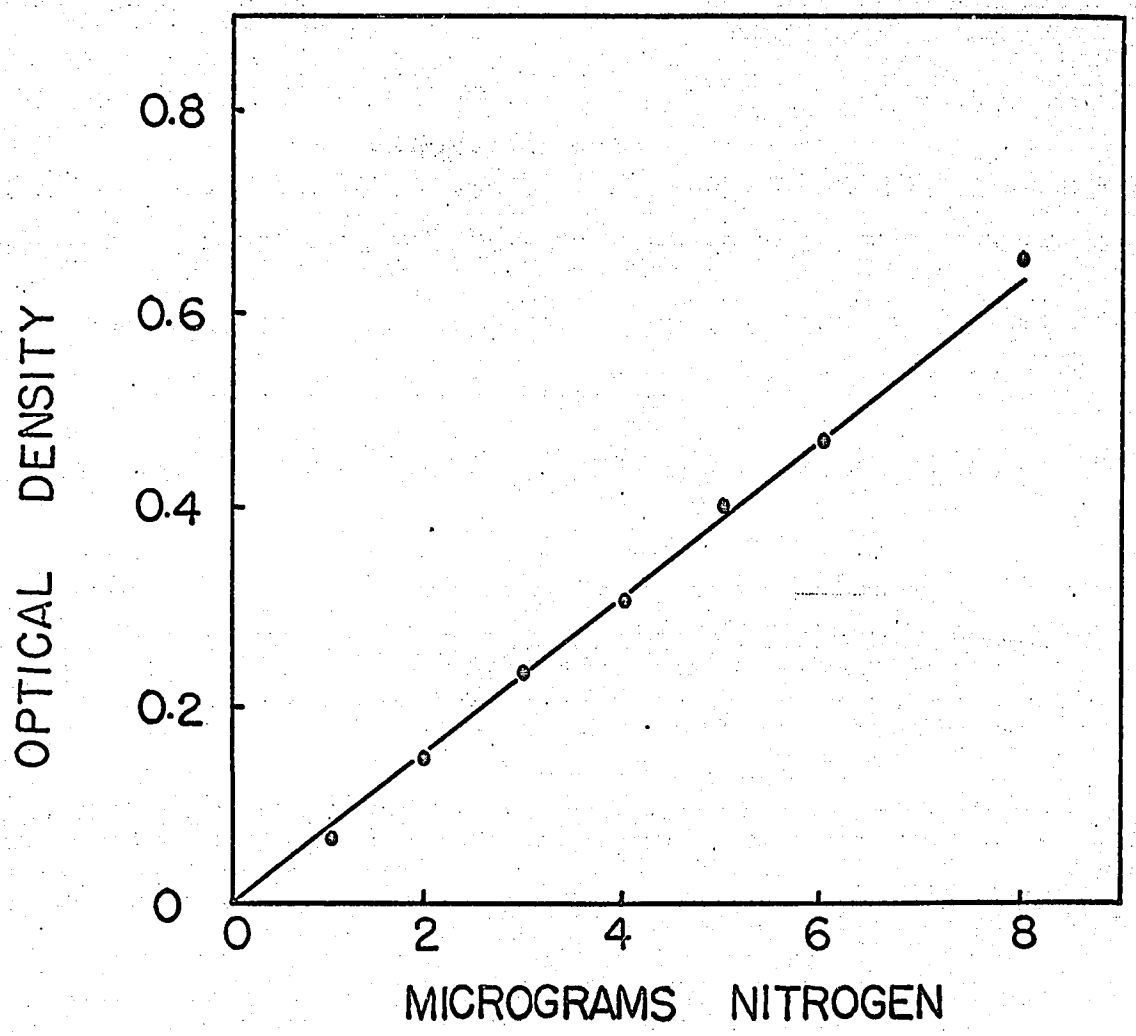
4. Total DNA Determination by Diphenylamine Reaction

As shown in Appendix IID, the method (77) calibrated with sperm DNA (Nutrition. Biochem. Co.) measured from 20 to 120 μg DNA. Details of the procedure used for determining total segment DNA were as follows: to 2 ml of total nucleic acid extracted into perchloric acid (see Appendix IIC), 4 ml of diphenylamine reagent was added. The reagent was prepared by dissolving 1.5 g of diphenylamine in 100 ml of a solution of 1.5% (w/v) H_2SO_4 in glacial acetic acid. Before use, 0.1 ml of aqueous acetaldehyde (16 mg acetaldehyde/ml) was added to each 10 ml of the diphenylamine reagent. The reaction mixture was incubated in a water bath for 18 hours at 30^0. The O.D. was measured at 600 mμ and 540 mμ. The difference between these values ($O.D._{600} - O.D._{540}$) was proportional to DNA concentration (Appendix IID).

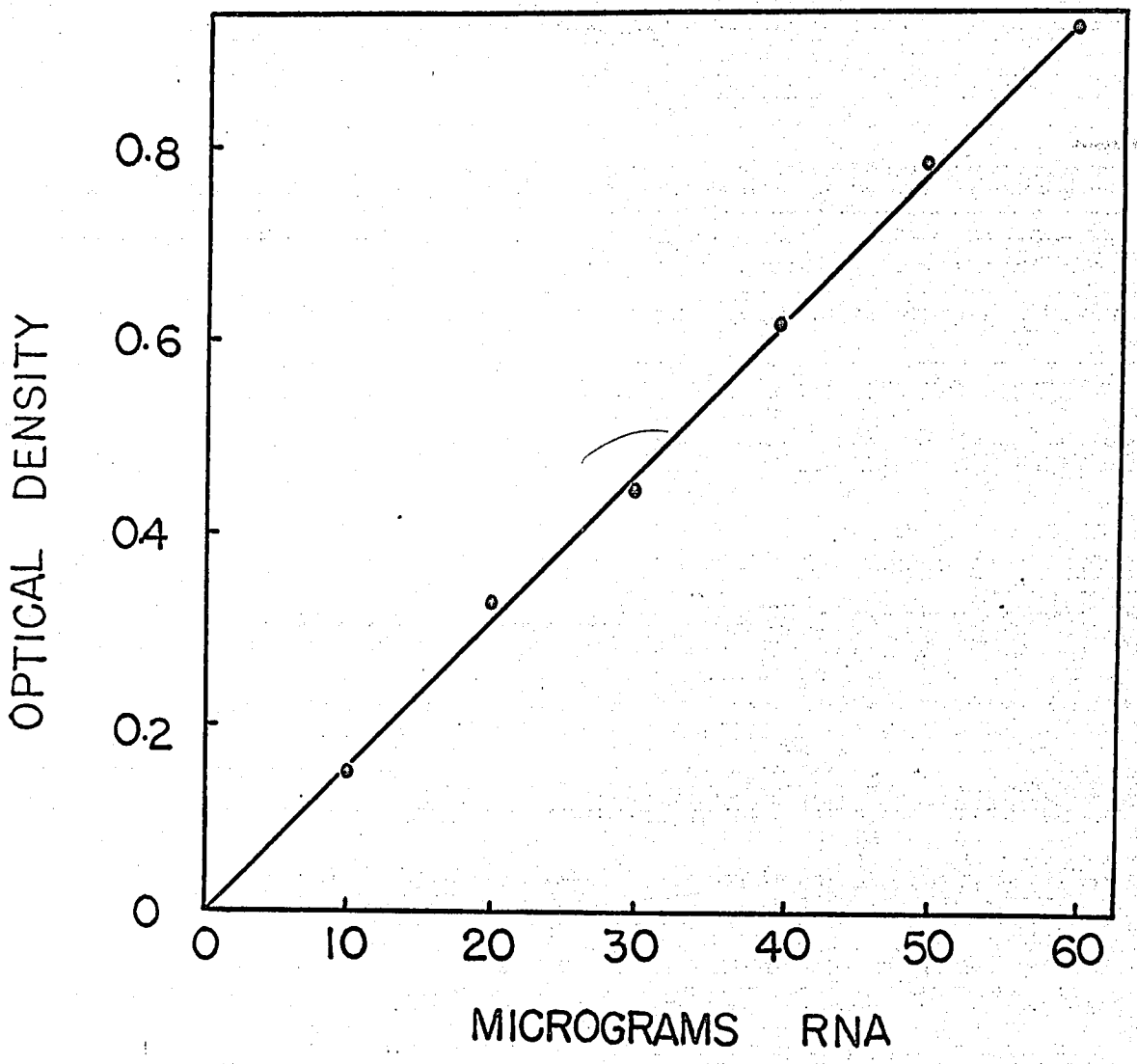
RNA concentration was calculated from values for total nucleic acid (Appendix IIC) minus those for DNA (Appendix IID).



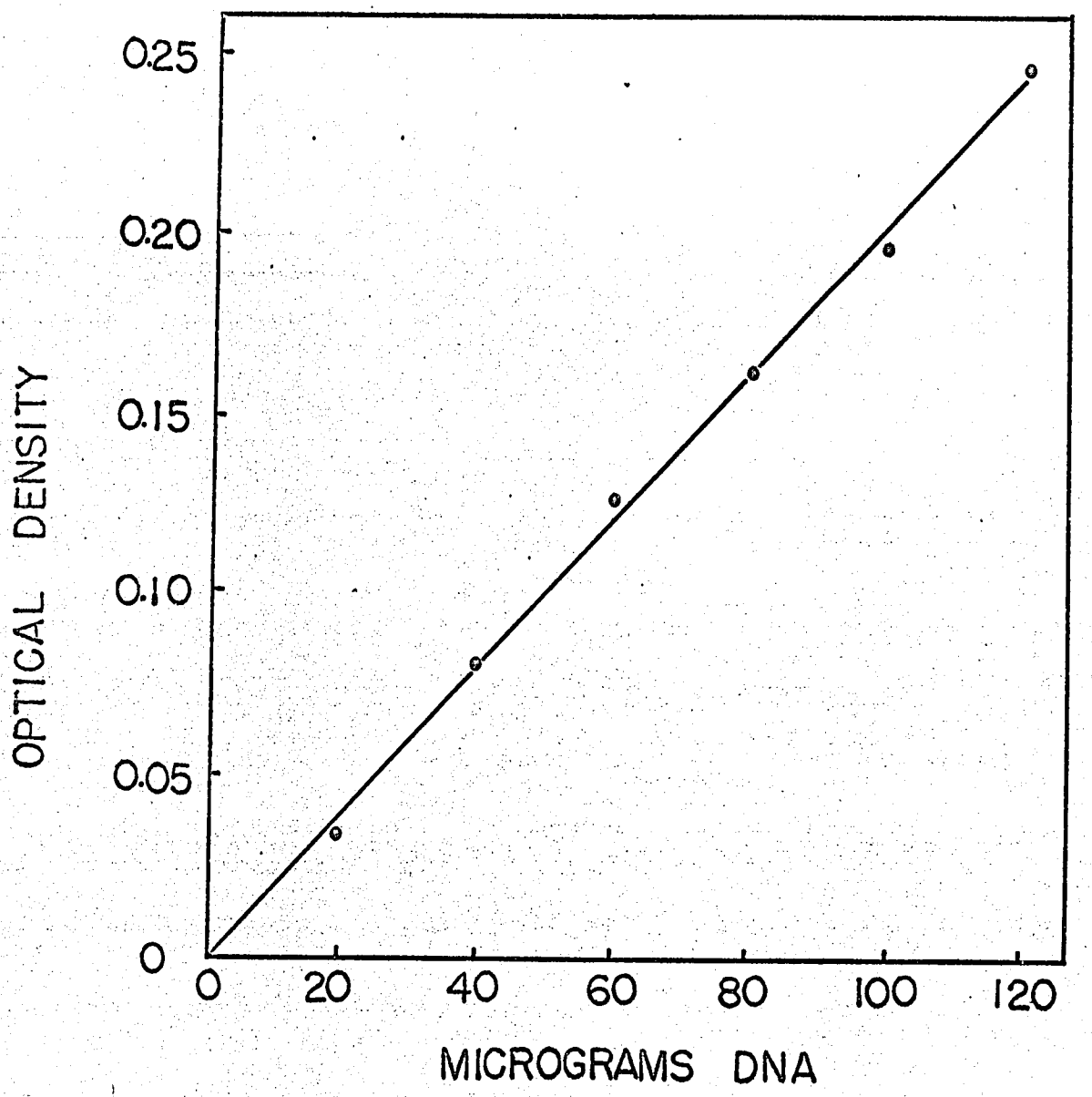
Appendix IIA. Standard curve for total soluble protein determination by the biuret method



Appendix IIB. Standard curve for total nitrogen determination by nesslerization



Appendix IIC. Standard curve for total nucleic acid determination by optical density absorption



Appendix IID. Standard curve for total DNA determination by the diphenylamine reaction

APPENDIX III

Properties of Cellulase *in Vitro*

1. Effect of Substrate Concentration

Throughout these studies a final concentration of 1.0% (w/v) substrate (CMC) has been used. Appendix IIIA shows that this concentration is sufficient to obtain near maximum rates of pea cellulase activity. When the data is plotted according to Lineweaver-Burk (179), a K_m value of 0.23% CMC is obtained. This value is higher than those generally obtained for fungal cellulase (i.e., about 0.14% see 195) which may indicate that pea cellulase has a relatively low affinity for this substrate.

2. Optimum pH for Activity

Over the pH range 5.0 to 8.0 the viscosity of CMC (type 7MSP) varies slightly. Accordingly, activity of pea cellulase was measured at different pH values by calculating the % loss in viscosity at that pH. The results are shown in Appendix IIIB. Pea cellulase has a clear optimum for activity between pH 5.5 and 6.5. The same optimum was found for pea cellulase activity as measured by production of reducing power (184).

3. Effect of Temperature on Stability

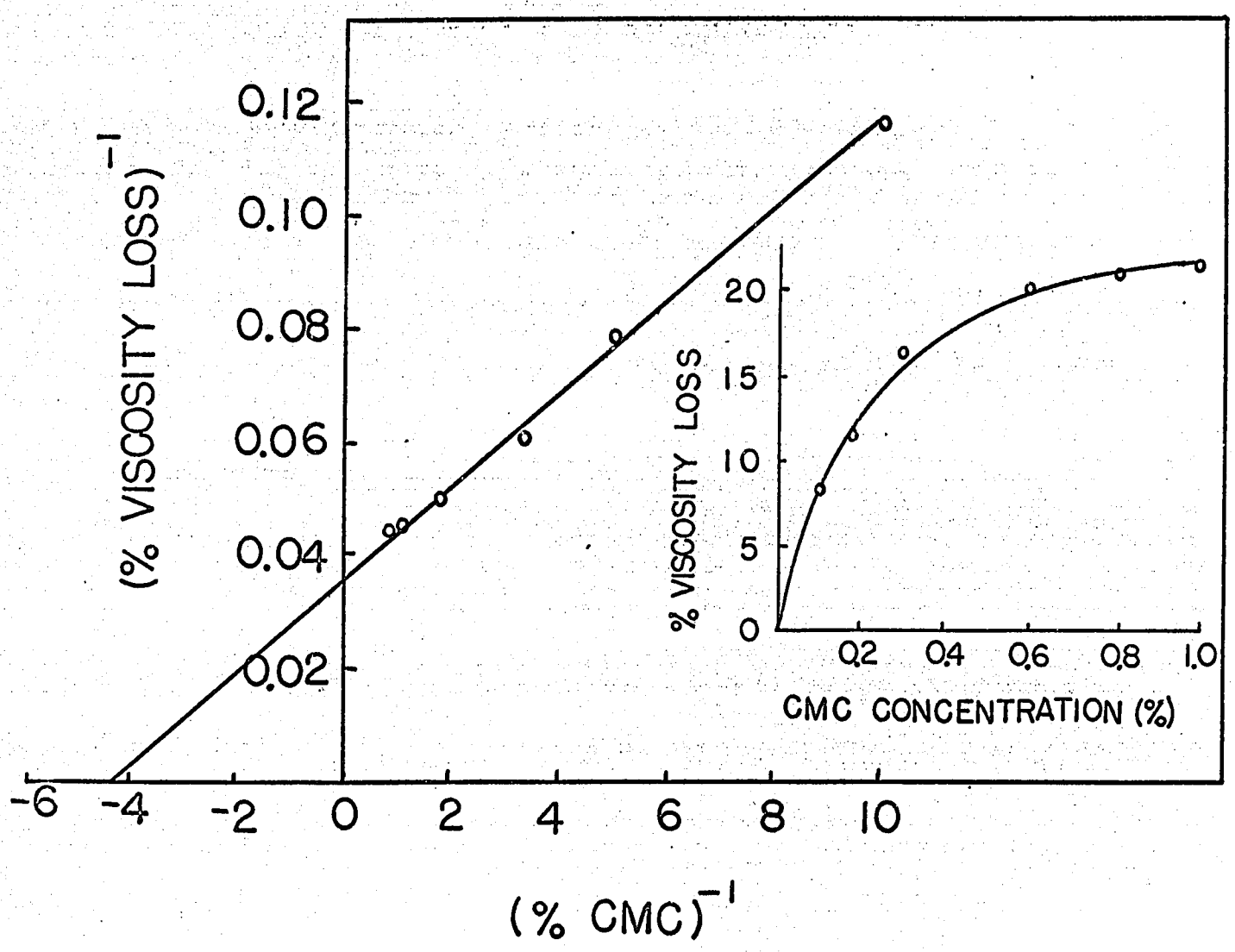
Appendix IIIC shows the effects on cellulase activity of heating crude pea extracts at pH 6.5 in a boiling water bath for 10 to 50 minutes. Over 90% of the activity was lost in 10 minutes. Data in Appendix IIID show that denaturation was also nearly complete when the enzyme was incubated for 10 minutes at 75°. The enzyme was stable for this time at 25°. Eventually, of course, cellulase does denature at room temperature. The rate is approximately 25% activity loss in 24 hours at 25°, pH 6.0 to 7.0 (Figure 9). At 4° activity losses were reduced to 5% or less per day (data not shown here). No activity loss occurred as a result of freezing sections of tissue and storing at -20° for up to one month.

4. Inhibition and Activation

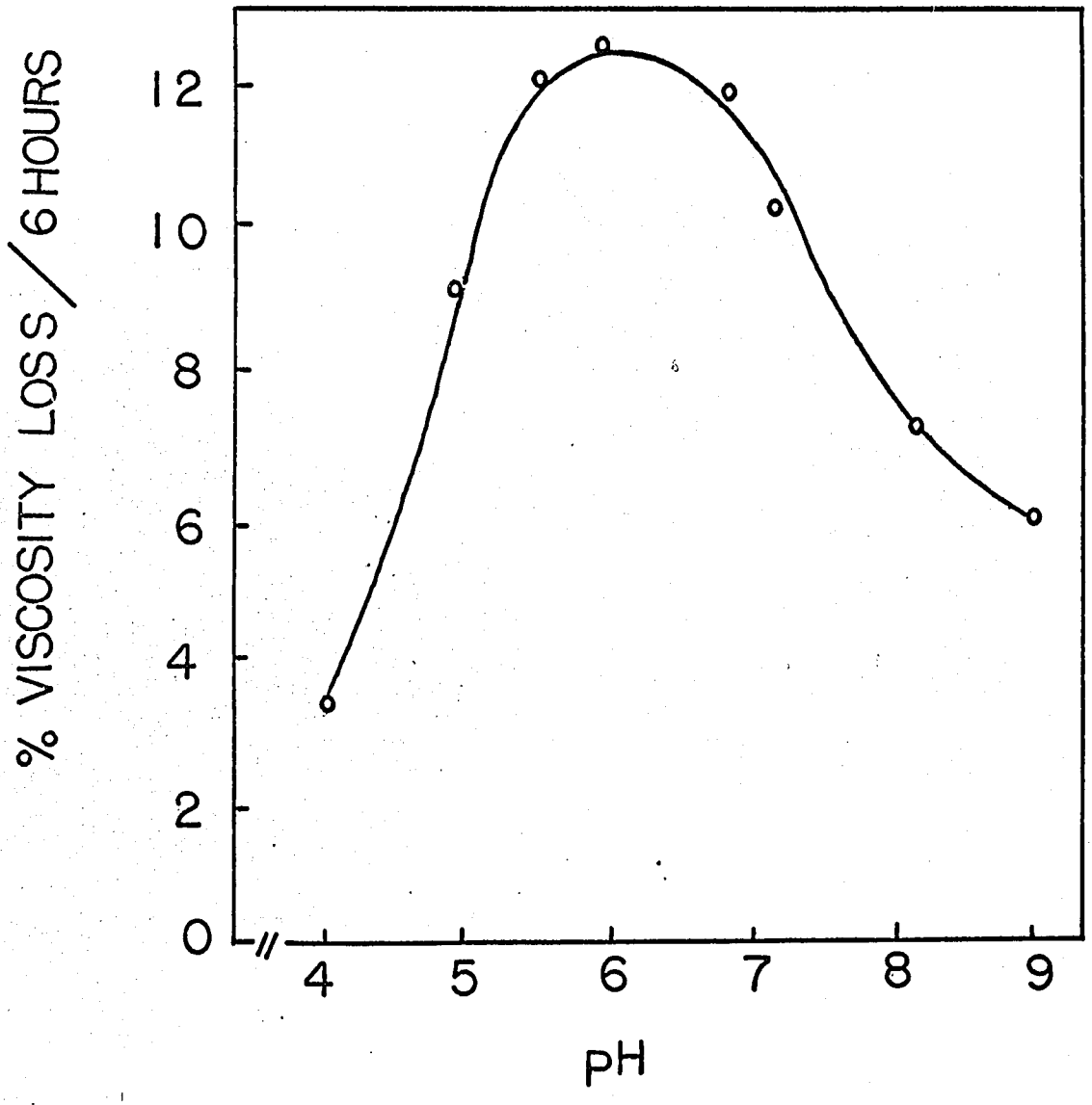
Direct effect of various chemicals on activity of crude pea cellulase <u>in vitro</u> are summarized in Appendix IIIE. Most of the substances tested had no significant inhibitory or stimulatory effect, even at high (unphysiological) concentrations. These included Ca^{++}, Mg^{++} (up to 0.1 M), reducing agents, growth hormones (IAA, GA, kinetin), antibiotics, proteins (proteases, albumin), cellobiose and hydroxyproline, boiled pea extracts. The apparent enhancement of cellulase activity by bromelin was due to the presence of β-1, 4 glucanase in the commercial preparation (270).

The only definite inhibitors of pea cellulase among the substances tested were $HgCl_2$ and p-chloromercuribenzoate (PCMB). At 0.01 M

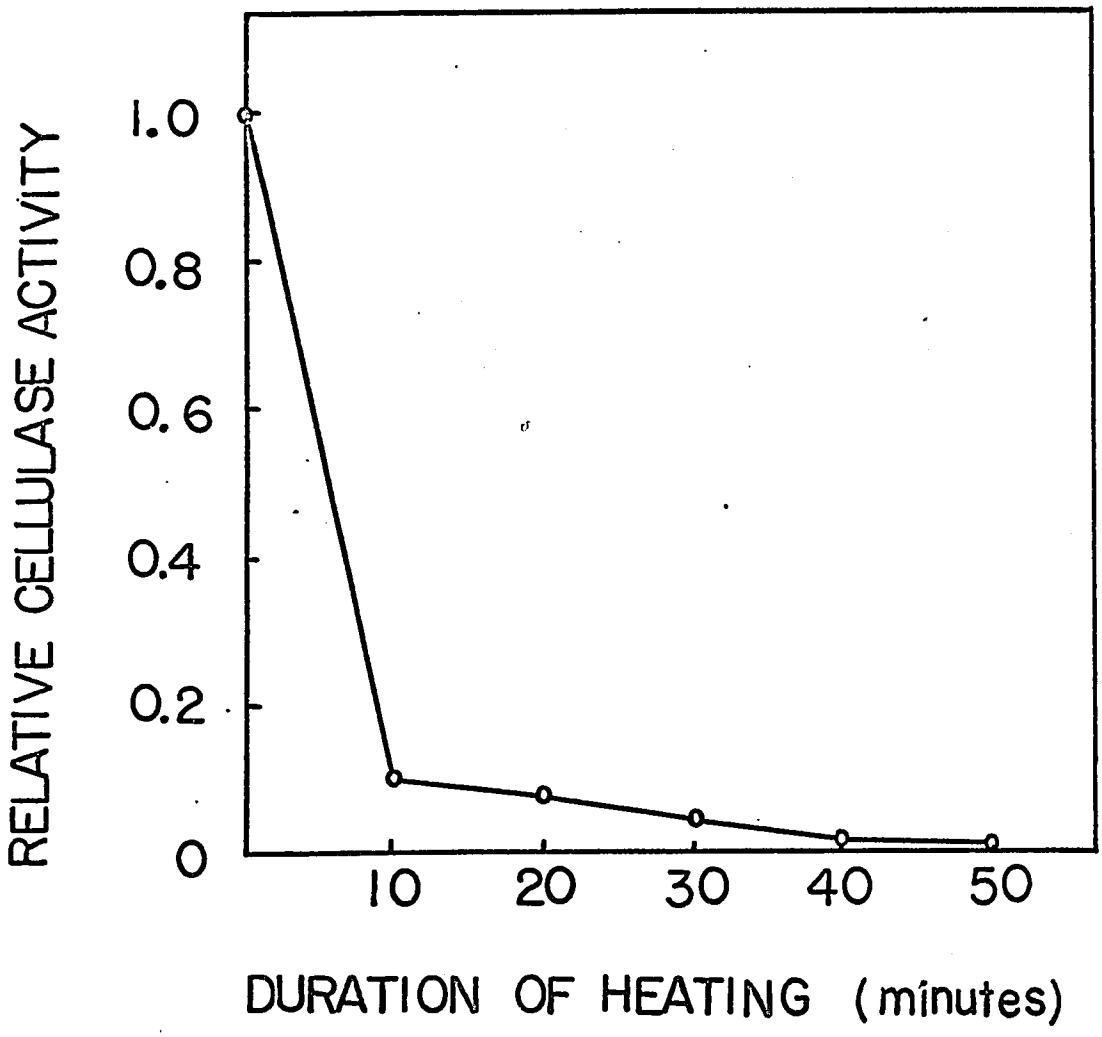
these sulfhydryl alkylating reagents completely prevented activity.



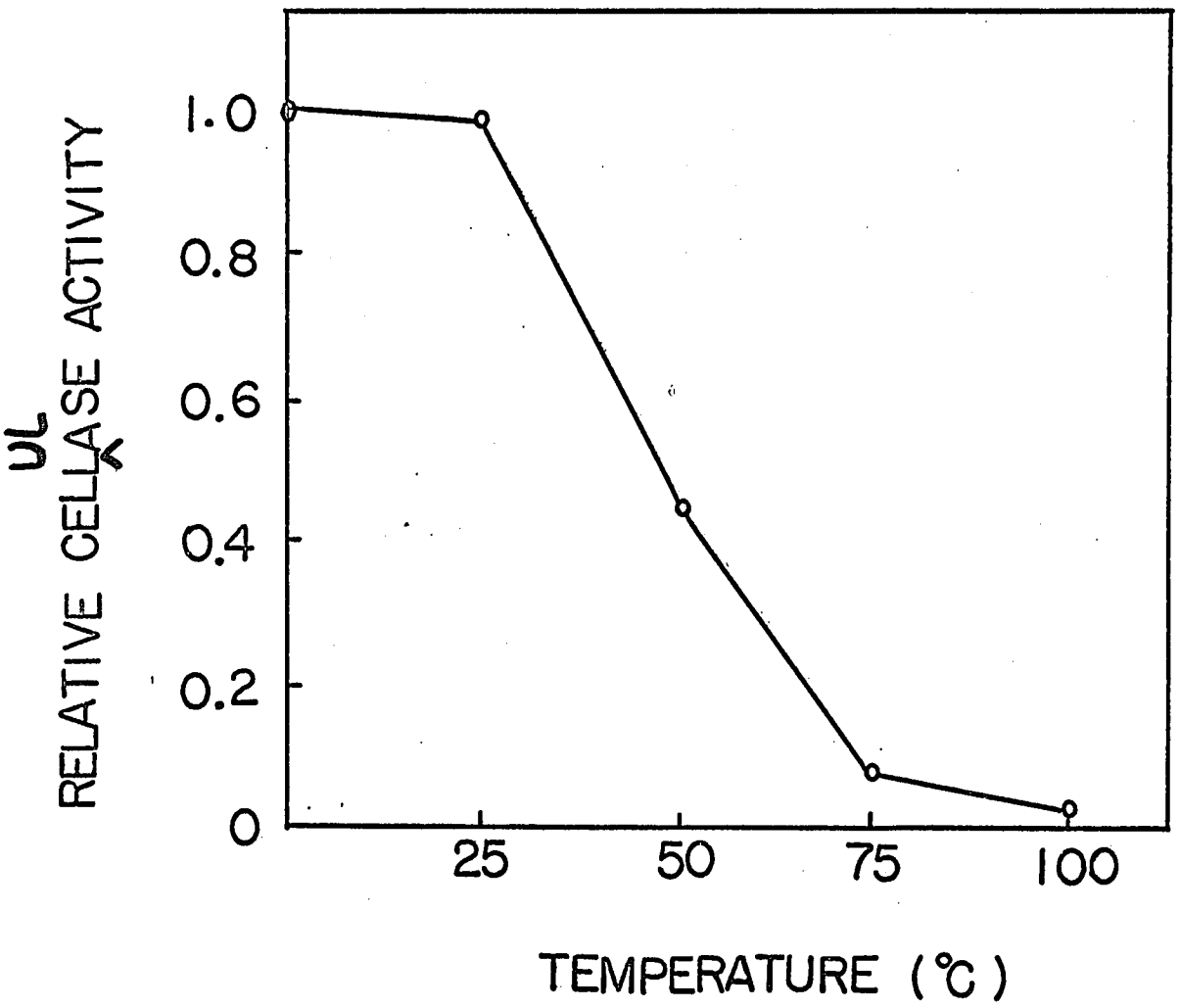
Appendix IIIA. Effect of substrate (CMC) concentration on cellulase reaction rate



Appendix IIIB. Effect of pH on cellulase activity *in vitro*. 1.0 ml of crude pea cellulase extract was mixed with 9.0 ml of 1.1% CMC solution. The CMC solution was prepared with 0.02M sodium acetate-acetic acid buffer between pH 4.0 and 5.5, with 0.02M sodium phosphate buffer between pH 6.0 and 8.0 and with 0.02M glycine-NaOH buffer at pH 9.0



Appendix IIIC. Residual cellulase activity after heating enzyme extract at 100° for different periods in absence of substrate



Appendix IIID. Residual cellulase activity after heating enzyme extract at different temperatures in absence of substrate for 10 min.

Appendix IIIE. Inhibition of cellulase activity by various compounds pre-incubated with pea extract for 1 hour

Compounds	Concentrations	Cellulase activity as % of control
$CaCl_2$	0.01 M	100
$CaCl_2$	0.1 M	95
$MgCl_2$	0.01 M	98
$MgCl_2$	0.1 M	90
EDTA	0.1 M	96
Mercaptoethanol	0.01 M	93
Ascorbic acid	0.01 M	95
Actinomycin D	20 µg/ml	92
Puromycin	40 µg/ml	100
Bovine serum albumin	2.0 mg/ml	100
Chymotrypsin	6.0 mg/ml	89
Trypsin	6.0 mg/ml	90
Pronase	6.0 mg/ml	92
Papain	6.0 mg/ml	84
Bromelin	6.0 mg/ml	430
IAA	10^{-3} M	96
IAA	10^{-5} M	98
Gibberellic acid	10^{-3} M	94
Gibberellic acid	10^{-5} M	95
Kinetin	10^{-3} M	89
Kinetin	10^{-5} M	92
Hydroxyproline	0.01 M	92
Cellobiose	0.1 M	99
$HgCl_2$	0.01 M	0
p-chloromecuribenzoate	0.01 M	0
Boiled pea extract	0.1 ml/ml	99

APPENDIX IV

Effect of Added IAA on Growth, Soluble Protein and Cellulase Activity in Higher Plant Tissues Other than Pea Epicotyl Apex

1. Segments Attached to Growing Seedlings

Appendices IVA to IVE show effects of IAA on various decapitated seedlings. IAA distinctly increased the cellulase level in decapitated bean epicotyls (Appendix IVA), maize shoots (Appendix IVB), pea roots (Appendix IVC) and the second node of decapitated pea epicotyls (Appendix IVD). In all of these tissues IAA inhibited elongation and induced swelling and synthesis of soluble protein. In contrast, IAA treatment did not result in any increase in cellulase or soluble protein levels in hypocotyl tissue of sunflower seedlings (Appendix IVE). In this case, the negative reactions were probably due to the lack of substrate for protein synthesis since IAA was applied after cotyledons had been removed.

2. Sections Detached from Growing Seedlings

Effects of IAA at 10^{-3} M and 10^{-5} M on the growth and cellulase activity of sections excised from the growing regions of etiolated oat seedling shoots and bean epicotyls are shown in Appendices IVF and IVG. At the lower IAA concentration, growth in 12 hours in both length and

fresh weight was greater by 20 to 30% than growth in untreated sections. Cellulase activity per section and per unit soluble protein was also increased by 70 to 80% in the oat shoot and by 10 to 20% in the bean epicotyl. The higher IAA concentration was less effective at promoting growth and less effective at enhancing the cellulase level.

Effects of 0.5% IAA in lanolin on fresh weight and cellulase activity in single cotyledons detached from the embryo of fully-imbibed pea seeds are shown in Appendix IVH. Initial cellulase levels per unit fresh weight (2.3% loss in CMC viscosity/2 hours/100 mg fr wt) were comparable to those in growing parts of the pea epicotyl and root (cf. Figure 2, Appendix IVC). However, calculated on a protein basis, cellulase activity was less than one-tenth than that in other pea tissues. This was due to the very high levels of reserve protein in pea cotyledons. During incubation of the cotyledons for 3 days, there was a small increase in fresh weight, a marked decline in soluble protein (cotyledons were senescing) and cellulase activity almost disappeared. IAA had no significant effect on these changes.

Appendix IVA. Effect of added IAA on growth, soluble protein and cellulase activity in decapitated bean epicotyls.

Segments 10 mm long were marked at the decapitated apex of the first internode of 8-day old etiolated Phaseolus vulgaris epicotyls. They were painted with lanolin ± IAA (0.5%) and grown and extracted as in experiments with the pea epicotyl. At zero time, each segment yielded an extract which caused 3.06% loss in CMC viscosity/2 hours equivalent to 3.15% loss/2 hours/mg soluble protein. Values in the Table are calculated relative to these.

Treatment	Fr wt (mg/seg)	Length (mm/seg)	Fr wt / length (mg/mm)	Soluble protein (mg/seg)	Relative cellulase activity per unit protein	Relative cellulase activity per seg
Zero time	78.6	10.0	7.86	0.97	1.00	1.00
1 day -IAA	179.0	21.4	8.35	1.44	0.93	1.38
1 day +IAA	270.0	18.1	14.90	1.76	1.39	2.50
3 days -IAA	383.0	40.5	9.45	1.98	0.87	1.77
3 days +IAA	526.0	25.9	20.20	2.86	2.42	7.05

Appendix IVB. Effect of added IAA on growth, soluble protein and cellulase activity in decapitated maize shoots

5-day old etiolated Zea mays L. shoots were decapitated about 2 mm below the mesocotyl apex and a point 10 mm below this was marked to delineate a segment of tissue. At zero time, each segment (containing coleoptile plus mesocotyl) yielded an extract which caused 0.74% loss in CMC viscosity/2 hours, equivalent to 0.65% loss/2 hours/mg soluble protein. Values in the Table are calculated relative to these.

Treatment	Fr wt (mg/seg)	Length (mm/seg)	Fr wt / length (mg/mm)	Soluble protein (mg/seg)	Relative cellulase activity per unit protein	Relative cellulase activity per seg
Zero time	34.0	10.0	3.40	1.14	1.00	1.00
1 day -IAA	79.0	27.6	2.86	1.39	1.49	1.81
1 day +IAA	72.5	22.4	3.20	1.37	4.02	4.77
3 days -IAA	251.0	78.0	3.22	2.08	2.95	5.40
3 days +IAA	175.0	59.5	2.94	1.90	6.52	10.90

Appendix IVC. Effect of added IAA on growth, soluble protein and cellulase activity in decapitated pea roots

The apical 2 mm of the primary root of 4-day old etiolated pea seedlings were removed and a 10 mm segment of tissue was delineated at the apex. At zero time, each segment yielded an extract which caused 0.35% loss in CMC viscosity/2 hours, equivalent to 2.16% loss/2 hours/mg soluble protein. Values in the Table are calculated relative to these.

Treatment	Fr wt (mg/seg)	Length (mm/seg)	Fr wt / length (mg/seg)	Soluble protein (µg/seg)	Relative cellulase activity per unit protein	Relative cellulase activity per seg
Zero time	11.0	10.0	1.10	162	1.00	1.00
2 days -IAA	20.5	16.0	1.28	227	3.20	4.40
2 days +IAA	17.0	11.7	1.45	228	3.70	5.20

Appendix IVD. Effect of added IAA on growth, soluble protein and cellulase activity in the second node of decapitated pea epicotyls

8-day old etiolated pea epicotyls were decapitated just below the hook. A 10 mm segment of tissue was removed at zero time and at 3 days from the region which bore the second node (3-5 cm below apex). By 3 days, the node began to develop a lateral branch unless IAA had been added (see Appendix IA). At zero time, each segment yielded an extract which caused 0.45% loss in CMC viscosity/2 hours, equivalent to 2.5% loss/2 hours/mg soluble protein. Values in the Table are calculated relative to these.

Treatment	Fr wt (mg/seg)	Fr wt / length (mg/mm)	Soluble protein (µg/seg)	Relative Cellulase activity per unit protein	Relative Cellulase activity per seg
Zero time	30.4	3.04	179	1.00	1.00
3 days -IAA	41.9	--	572	0.48	1.51
3 days +IAA	35.6	3.56	241	1.84	2.47

Appendix IVE. Effect of added IAA on growth, soluble protein and cellulase activity in sunflower hypocotyls

Segments (10 mm long) of 7-day old etiolated hypocotyls of Helianthus annuus, L. from which cotyledons had been removed were delineated below the cut apex. At zero time, each segment yielded an extract which caused 0.65% loss in CMC viscosity/2 hours, equivalent tp 2.82% loss/2 hours/mg soluble protein. Values in the Table are calculated relative to these.

Treatment	Fr wt (mg/seg)	Length (mm/seg)	Fr wt / length (mg/mm)	Soluble protein (µg/seg)	Relative cellulase activity per unit protein	Relative cellulase activity per seg
Zero time	35.0	10.0	3.50	231	1.00	1.00
1 day -IAA	61.9	18.9	3.26	234	0.65	0.66
1 day +IAA	56.0	15.5	3.62	275	0.61	0.73
3 days -IAA	114.0	39.1	2.91	205	1.24	1.09
3 days +IAA	67.5	17.5	3.85	223	0.90	0.87

Appendix IVF. Effect of added IAA on growth, soluble protein and cellulase activity in detached oat shoot sections

Sections (10 mm long) were removed from the growing region of 4-day old etiolated oat shoots. Each section (containing both mesocotyl and coleoptile) at zero time weighed 10 mg, contained 140 µg soluble protein and yielded an extract which caused 0.54% loss in CMC viscosity /6 hours, equivalent to 3.85% loss/6 hours/mg soluble protein. Values in the Table are calculated relative to these.

	Values at 12 hrs. relative to those at zero time					
Treatment	Fr wt	Length	Fr wt / length	Soluble protein	cellulase activity per unit protein	cellulase activity per sect.
0	1.68	1.44	1.17	1.44	0.40	0.58
10^{-5} M IAA	1.97	1.84	1.07	1.40	0.71	0.97
10^{-3} M IAA	1.85	1.67	1.09	1.34	0.63	0.82

Appendix IVG. Effect of added IAA on growth, soluble protein and cellulase activity in detached bean epicotyl sections

Sections (10 mm long) were removed from the apical region of the first internode of 8-day old etiolated bean epicotyls. At zero time, each section weighed 146 mg, contained 1.59 mg soluble protein and yielded an extract which produced 11.2% loss in CMC viscosity/1 hour, equivalent to 7.1% loss/1 hour/mg soluble protein. Values in the Table are calculated relative to these.

	Values at 12 hrs. relative to those at zero time					
Treatment	Fr wt	Length	$\frac{\text{Fr wt}}{\text{length}}$	Soluble protein	cellulase activity per unit protein	cellulase activity per sect.
0	1.11	1.07	1.03	0.85	0.95	0.81
10^{-5} M IAA	1.35	1.20	1.13	1.01	1.05	1.03
10^{-3} M IAA	1.28	1.20	1.05	0.93	0.97	0.90

Appendix IVH. Effect of added IAA on growth, soluble protein and cellulase activity in de-embryoized pea cotyledons

Surface-sterilized pea seeds were soaked in distilled water for 24 hours (until they were fully imbibed). The embryos were removed and single cotyledons were painted with lanolin ± 0.5% IAA and incubated in sterilized 2% sucrose--0.02 M sodium phosphate (pH 6.0). The medium was changed daily. At zero time, each cotyledon yielded an extract which caused 2.31% loss in CMC viscosity/2 hours.

Treatment	Fr wt (mg/cotyledon)	Soluble protein (µg/cotyledon)	Relative cellulase activity per unit protein	Relative cellulase activity per cotyledon
Zero time	97.5	11,200	1.00	1.00
3 days -IAA	114.2	7,800	0.03	0.21
3 days +IAA	122.8	8,300	0.02	0.19

APPENDIX V

Comparative Effects of Various Hormones on Growth, Soluble Protein and Cellulase Activity in Pea Epicotyl Tissue

I. Segments Attached to Decapitated Seedlings

Effects of applying 0.5% gibberellic acid (GA) or 0.5% kinetin in lanolin paste are compared to effects of IAA in Appendix VA. After 5 days incubation, cellulase activities and soluble protein levels had been increased as usual by IAA over control values but they were not altered significantly by GA or kinetin. GA greatly increased segment elongation and fresh weight but had no effect on swelling. Kinetin had no effect on growth in any of these dimensions. Microscopic examination revealed no cell division in GA- or kinetin-treated tissues.

Appendix VB shows effects of ethylene gas (10 ppm) added to the atmosphere of decapitated seedlings which were treated with lanolin ± 0.5% IAA. Ethylene gas alone had no effect on protein levels or cellulase activity. However, when added together with IAA it reduced the stimulatory effects of IAA on swelling and protein levels by about 50%. It almost abolished the IAA-induced rise in cellulase activity. Ethylene acted here as if it were an inhibitor of protein synthesis.

2. Effect of Swelling Agents on Detached Sections

Appendix VC shows the effects of kinetin (10^{-5} M), benzimidazole (10^{-5} M) and ethylene (10 ppm) added with or without IAA (10^{-5} M) to excised sections for 24 hours. All of these substances inhibited elongation but caused swelling of the sections, especially when added together with IAA. They had little effect on soluble protein levels which generally decreased by about 20% under all treatments. IAA, kinetin and benzimidazole all increased cellulase activity slightly. Ethylene had no effect on cellulase.

Effects of kinetin (10^{-5} M) plus or minus high concentrations of IAA (0.5% in lanolin painted at apical end of sections) are shown in Appendix VD. Excess IAA caused swelling and enhanced the breakdown of total protein and cellulase. Kinetin also caused swelling but it appeared to alleviate the endogenous and IAA-induced breakdown of protein, including cellulase.

Appendix VA. Effect of IAA, GA and kinetin on growth, soluble protein and cellulase activity in decapitated pea epicotyls

At zero time, each segment (10 mm) yielded an extract which produced 0.31% loss in CMC viscosity/2 hours, equivalent to 1.32% loss/ 2 hours/mg soluble protein. Values in the Table are calculated relative to these.

Treatment	Fr wt (mg/seg)	Length (mm/seg)	Fr wt / length (mg/mm)	Soluble protein (µg/seg)	Relative cellulase activity per unit protein	Relative cellulase activity per seg
Zero time	24.1	10.0	2.41	235	1.00	1.00
5 days -IAA	47.1	16.7	2.82	350	1.12	1.64
+ 0.5% IAA	91.3	15.0	6.10	602	2.75	7.10
+ 0.5% GA	74.0	25.8	2.86	435	0.84	1.55
+ 0.5% kinetin	41.1	15.7	2.52	390	0.99	1.64

Appendix VB. Effect of ethylene and IAA on growth, soluble protein and cellulase activity in decapitated pea epicotyls

Decapitated seedlings painted with lanolin +IAA were grown in closed glass chambers into which ethylene gas (10 ppm) was injected daily. At zero time, each segment of epicotyl tissue yielded an extract which caused 0.69% loss in CMC viscosity/2 hours, equivalent to 2.62% loss/2 hours/mg soluble protein. Values in Table are calculated relative to these.

Treatment	Fr wt (mg/seg)	Length (mm/seg)	Fr wt / Length (mg/mm)	Soluble protein (µg/seg)	Relative cellulase activity per unit protein	Relative cellulase activity per seg
Zero time	20.7	10.0	2.07	258	1.00	1.00
3 days						
-IAA	44.7	15.4	2.90	231	1.42	1.29
+IAA	82.8	12.6	6.56	635	7.10	12.30
+ethylene	45.6	13.5	3.37	242	1.13	1.05
+IAA +ethylene	64.0	13.6	4.70	410	1.23	1.92

Appendix VC. Effect of swelling agents on growth, soluble protein and cellulase activity in detached pea epicotyl sections

At zero time, each section (10 mm) weighed 23.8 mg, contained 231 µg soluble protein and yielded an extract which caused 1.18% loss in CMC viscosity/6 hours, equivalent to 5.1% loss/6 hours/mg soluble protein. Values in the Table are calculated relative to these.

	Values at 24 hrs relative to those at zero time					
Treatment*	Fr wt	Length	Fr wt / length	Soluble protein	Cellulase activity per unit protein	Cellulase activity per sect.
Control	1.65	1.50	1.05	0.80	1.35	1.09
+IAA	2.24	1.68	1.23	0.81	1.49	1.21
+K	1.59	1.37	1.16	0.89	1.51	1.37
+IAA +K	1.98	1.35	1.49	0.87	1.71	1.49
+B	1.73	1.42	1.13	0.81	1.48	1.15
+IAA +B	2.22	1.41	1.46	0.76	1.63	1.24
+E	1.45	1.16	1.35	0.80	1.27	1.03
+IAA +E	2.31	1.42	1.65	0.83	1.44	1.17

* Concentrations used in this test were: IAA, 10^{-5} M; kinetin (K), 10^{-5} M; benzimidazole (B), 10^{-5} M; ethylene (E), 10 ppm.

Appendix VD. Effect of kinetin and excess IAA on growth and cellulase activity in detached epicotyl sections

Apical sections were painted at the apical end with about 2.5 mg lanolin paste ± 0.5% IAA and incubated for 24 hours ± 10^{-5} M kinetin. Values per section are expressed as a percentage of those at zero time, namely, fresh weight = 23.8 mg, soluble protein = 231 µg and cellulase activity = 1.18% loss in CMC viscosity/6 hours.

Treatment	Values at 24 hrs relative to those at zero time					
	Fr wt	Length	Weight / length	Soluble protein	Cellulase activity per unit protein	per sect.
No additions	1.65	1.50	1.05	0.85	1.41	1.20
+ kinetin	1.65	1.37	1.16	0.89	1.57	1.40
+ excess IAA	1.89	1.49	1.27	0.79	1.18	0.93
+ both	2.08	1.31	1.50	0.87	1.43	1.24

APPENDIX VI

Changes with Time in Growth and Cellulase Activity in Pea Epicotyl Tissue

Growth effects of decapitation are shown in Appendix VIA and effects on soluble protein levels and cellulase activity are recorded in Appendix VIB. The intact segment growing normally increased in both length and fresh weight 3- to 4-fold within 3 days. There was little swelling up to or after this time. Protein levels and cellulase activity increased a little during the growth period and then declined slowly. Decapitation greatly reduced segment growth (by 70-80%). It prevented any increase in soluble protein levels. Cellulase activity showed a brief (2 days) rise followed by a relatively rapid decline.

Appendix VIC shows effects of adding IAA to the decapitated epicotyl at zero time and again at 3 days. Without the second hormone treatment, cellulase activity reached a maximum value at about 3 days and then declined. Extra hormone caused some further swelling and protein synthesis in the epicotyl segment and it maintained or increased the cellulase activity for a further 2 to 4 days.

Appendix VIA. Effect of decapitation on growth

A 10 mm region of tissue was delineated at the apex of the third internode of 8-day old pea seedlings. Growth of this segment was measured either in intact or decapitated seedlings over a 10-day period. The Table shows results of separate experiments in which, at zero time, the fresh weights of the apical 10 mm segment were: 21.1 mg (intact) and 24.4 mg (decap).

Time (days)	Values per segment relative to those at zero time					
	Fr wt		length		Fr wt/length	
	intact	decap	intact	decap	intact	decap
2	3.70	1.65	2.92	1.52	1.27	1.08
3	3.86	1.55	3.03	1.44	1.32	1.07
5	3.60	1.49	3.10	1.41	1.16	1.05
7	3.30	1.47	3.15	1.42	1.05	1.03
10	3.25	1.40	3.05	1.40	1.08	1.00

Appendix VIB. Effect of decapitation on soluble protein levels and cellulase activity

Data on growth during these experiments are given in Appendix VIA. At zero time, each segment from intact and decapitated seedlings yielded respectively; 197 and 259 µg soluble protein and cellulase activities of 2.70 and 2.02% loss in CMC viscosity/2 hours.

Time (days)	Soluble protein		Relative cellulase activity per unit protein		per seg	
	intact	decap	intact	decap	intact	decap
2	1.63	0.98	1.70	2.05	2.52	1.62
3	1.38	0.95	1.63	1.93	2.27	1.47
5	1.16	1.01	1.67	0.92	1.98	0.75
7	1.03	0.95	1.69	0.71	1.75	0.54
10	1.17	0.73	1.33	0.72	1.55	0.41

Appendix VIC. Effect of IAA added at zero time and again at 3 days on growth, soluble protein and cellulase activity

At zero time, each segment yielded an extract which caused 0.56% loss in CMC viscosity/2 hours, equivalent to 2.42% loss/mg soluble protein. Values in the Table are calculated relative to these.

Time (days)	Fr wt (mg/seg)		Length (mm/seg)		Soluble protein (µg/seg)		Relative cellulase activity per unit protein		per seg	
0	20.6		10.0		252		1.0		1.0	
3	72.0		13.6		568		8.1		20.0	
	Segments freshly treated with lanolin ±IAA at 3 days									
	−	+	−	+	−	+	−	+	−	+
5	74.7	85.0	13.0	13.7	606	662	6.3	8.4	16.5	24.2
7	67.8	90.4	1.28	1.37	560	820	5.1	6.7	12.4	23.7
10	75.0	87.9	1.35	14.0	600	810	5.1	4.7	13.1	16.3

APPENDIX VII

Effect of Inhibitors of Nucleic Acid or Protein Synthesis on Growth and Metabolism in Pea Epicotyl Tissue.

1. Effect of Actinomycin D on Metabolism of ^{14}C-orotate and ^{14}C-leucine

Appendix VIIA shows effects of actinomycin D on uptake of ^{14}C-orotate into trichloroacetic acid- and ethanol-soluble fractions (nucleotides) and into insoluble fractions (nucleic acids) of detached pea epicotyl sections. The experiment was conducted in the same manner as in previous tests using ^{14}C-leucine (Table IX). The results lead to similar conclusions, namely, that actinomycin D inhibits nucleic acid synthesis more severely than uptake of precursor into the sections.

It was important for interpretation of the results of the experiment with ^{14}C-leucine (Table X) to establish that all of the supplied label was still present within the sections as leucine, either free or combined (in protein). Appendix VIIB shows a radioautograph of a chromatograph of combined soluble and hydrolysed fractions from this experiment which confirms that all detected ^{14}C was located in leucine.

2. Effects of Inhibitors on Growth and Cellulase Activity in Detached Sections

Appendix VIIC shows the effects of three inhibitors on properties of IAA-treated detached sections. FUdR, which inhibits DNA synthesis but not RNA or protein synthesis in attached epicotyl segments (Figures 5 and 6) had no effect on growth, protein level or cellulase activity in detached sections by 24 hours. Actinomycin D (at 20 ug/ml) which interferes with protein (Figure 6) and nucleic acid (Appendix VIIA) synthesis in detached sections, also prevented IAA-induced growth and severely reduced the cellulase activity. Chloramphenicol used at such a high concentration (100 ug/ml) that it reduced the solubie protein level in sections, was a very effective inhibitor of growth and cellulase activity.

Appendix VIID shows effects of puromycin added alone to sections at different concentrations. Growth inhibition was detectable at puromycin concentrations of 10 µg/ml. Maximum inhibition occurred at 40 µg/ml or higher. Both total protein levels and cellulase activity per section were lower after treatment with puromycin at concentrations between 10 and 60 µg/ml. However, there was no effect on cellulase activity per unit protein. Unilateral inhibition of cellulase activity only occurred with 100 µg puromycin/ml.

3. Effects of Inhibitors on Growth and Cellulase Activity in Attached Segments.

A number of substances reported (see Literature Review) to interfere with mitosis or synthesis of nucleic acids or protein were added with IAA in lanolin to decapitated epicotyls. Effects are sum-

marised in Appendices VIIE and VIIF. Mitomycin and azathymine, although often used to interfere with DNA metabolism, did not stop cell division in the present tests (observed microscopically) and had no effects on IAA-induced growth or cellulase activity. Hydroxyurea and colchicine were also ineffective in causing visible alterations in cell division but they inhibited the increases due to IAA in fresh weight (by 40-50%), protein synthesis (by 30%) and cellulase activity (by 40%). Acti-dione (cycloheximide) was an extremely effective inhibitor of all IAA-induced processes; it even prevented the normal growth and protein synthesis which took place without added IAA.

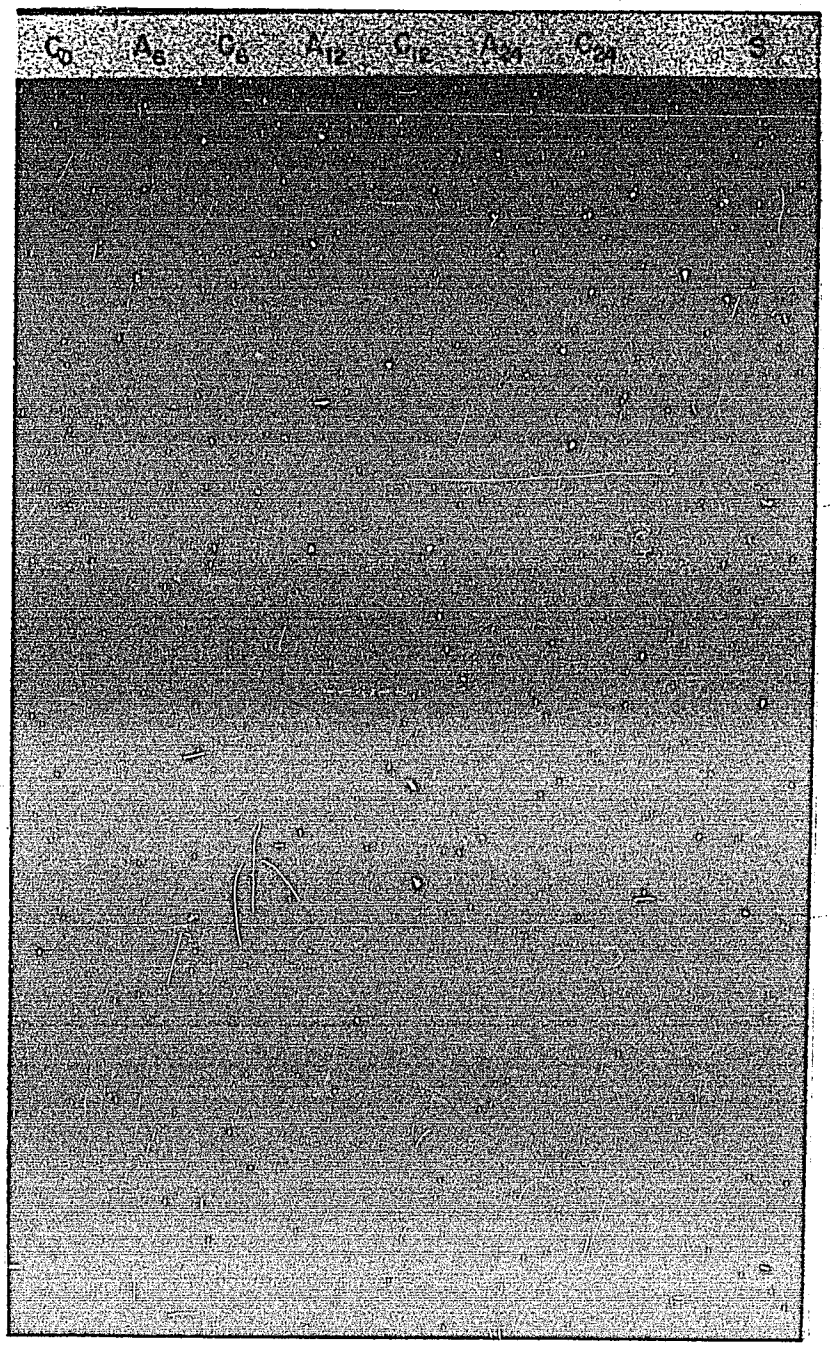
Appendix VIIA. Effect of pretreatment of sections with actinomycin D on subsequent uptake of ^{14}C-orotate and its incorporation into nucleic acid

After pretreatment for the times indicated, 20 sections were transferred to 2 ml 0.02 M sodium phosphate (pH 6.0) containing orotate-6-^{14}C (4.7 ug, 586 000 counts/min.). They were left for 2 hours in darkness at 25°, washed in unlabelled orotate and then homogenised and centrifuged twice, with 80% ethanol. The residue was extracted with a series of solvents, namely, cold 5% trichloroacetic acid, 80% ethanol, absolute ethanol and ethanol-ether. Radioactivity in pyrimidine nucleotides and other low-molecular-weight derivatives was measured in the combined washings; radioactive nucleic acid was measured as total label in the insoluble residue.

Pre-treatment time (hours)	^{14}C-orotate subsequently incorporated into nucleic acid		^{14}C-orotate subsequently absorbed but not in nucleic acid		Ratio of ^{14}C in: nucleic acid / Nucleotide fraction × 100	
	Absence or presence of actinomycin D (20 ug/ml)					
	−	+	−	+	−	+
0	195		6 050		3.2	
6	260	60	6 850	3 600	3.8	1.7
12	285	75	5 300	3 050	5.4	2.5
24	390	60	4 600	2 350	8.5	2.6

Appendix VIIB. Radioautograph of fractions derived from pea sections incubated on ^{14}C-leucine solution

Fractions, including ethanol-soluble ^{14}C and hydrolyzed ^{14}C-protein, were chromatographed with the solvent n-butanol: acetic acid: water (4:4:1 v/v/v). A and C refer to sections pretreated with actinomycin D and to controls, numbers refer to time (hours) of pretreatment, S is supplied ^{14}C-leucine.



Appendix VIIC. Effect of inhibitors on DNA, RNA and protein synthesis in combination with IAA on growth and cellulase activity

Sections were incubated for 24 hrs; concentrations of added substances were: IAA = 10^{-5} M, 5-fluorodeoxyuridine (FUdR) = 100 ug/ml, actinomycin D = 20 ug/ml, chloramphenicol= 1 mg/ml. Values per section at zero time were: fr wt = 23.1 mg, soluble protein = 242 µg, cellulase activity = 1.12% loss in CMC viscosity/6 hours.

	Values at 24 hrs relative to those at zero time				
Treatment	Fr wt	Length	Soluble protein	cellulase activity per unit protein	per sect
No additions	1.64	1.48	0.64	1.89	1.21
+IAA	2.10	1.51	0.61	2.13	1.30
+IAA +FUdR	2.18	1.56	0.65	2.20	1.43
+IAA + actinomycin D	1.57	1.34	0.61	0.93	0.57
+IAA + chloramphenicol	1.24	1.22	0.42	0.63	0.27

Appendix VIID. Effect of puromycin concentrations on growth, soluble protein and cellulase activity

At zero time, each section (10 mm) weighed 25.5 mg, contained 278 µg soluble protein and yielded an extract which caused 1.64% loss in CMC viscosity/ 6 hours, equivalent to 5.9% loss/mg soluble protein.

Puromycin concentration (ug/ml)	Values at 24 hrs relative to those at zero time					
	Fr wt	Length	Fr wt / length	Soluble protein	Cellulase activity per unit protein	Cellulase activity per sect.
0	1.61	1.45	1.04	0.86	1.23	1.07
10	1.56	1.42	1.02	0.76	1.23	0.93
20	1.52	1.38	1.03	0.70	1.22	0.86
40	1.42	1.27	1.04	0.61	1.23	0.80
60	1.43	1.29	1.08	0.67	1.32	0.89
100	1.39	1.30	1.02	0.72	0.90	0.65

Appendix VIIE. Effect of including mitomycin C or azathymine with IAA on growth, soluble protein and cellulase activity in decapitated pea epicotyls

Mitomycin C (M) and azathymine (AT) were added in lanolin (0.02% w/w) with IAA (0.5% w/w). At zero time, each segment (10 mm) yielded an extract which caused 0.86% loss in CMC viscosity/2 hours.

Treatment	Fr wt (mg/seg)	Length (mm/seg)	Fr wt / length (mg/mm)	Soluble protein (µg/seg)	Relative cellulase activity per unit protein	Relative cellulase activity per seg
Zero time	22.4	10.0	2.24	264	1.00	1.00
3 days -IAA	55.6	16.9	3.28	248	0.85	0.78
3 days +IAA	74.2	12.5	5.95	565	5.20	11.10
3 days +IAA +M	78.0	12.1	6.45	578	5.70	12.50
3 days +IAA +AT	81.0	12.5	6.50	580	5.50	11.80

Appendix VIIF. Effect of including hydroxyurea, colchicine or actidione with IAA on growth, soluble protein and cellulase activity in decapitated pea epicotyls

Hydroxyurea (H, 1.0% w/w), colchicine (C, 1.0% w/w) and actidione (A, 0.1% w/w) were added in lanolin with IAA (0.5% w/w). At zero time, each segment (10 mm) yielded an extract which caused 0.45% loss in CMC viscosity/2 hours.

Treatment	Fr wt (mg/seg)	Length (mm/seg)	Fr wt / length (mg/mm)	Soluble protein (µg/seg)	Relative cellulase activity per unit protein	Relative cellulase activity per seg
Zero time	21.5	10.0	2.15	214	1.00	1.00
3 days -IAA	42.4	13.4	3.16	200	1.52	1.43
3 days +IAA	73.6	12.5	5.90	550	8.20	21.20
3 days +IAA +H	60.4	11.8	5.12	464	6.00	13.00
3 days +IAA +C	57.8	11.5	5.02	455	5.90	12.60
3 days +IAA IA	27.1	10.0	2.71	147	1.39	0.96

APPENDIX VIII

Effect of Various Treatments on Growth and Cellulase Activity in Detached Pea Epicotyl Sections

1. Plasmolysing Agents

Appendix VIIA shows effects of incubating sections plus or minus IAA in buffered 0.3 M NaCl or 0.4 M mannitol. These treatments all resulted in flaccid sections which did not grow. Cellulase activity per section fell to about half the level in controls with or without IAA. In the presence of NaCl, there was an increase in cellulase activity per unit protein but this was clearly due to denaturation of much of the cellular protein in salt solution at this strength.

2. Reducing Agents

Appendix VIIIB shows effects of various reducing agents which were added with or without IAA. Added cysteine and ascorbate had little effect on growth or soluble protein levels but both reagents resulted in low cellulase levels. Mercaptoethanol and Cleland's reagent (dithiothreitol) severly inhibited growth and lowered the levels of total protein and of cellulase. IAA did not overcome any of these inhibitory effects.

3. Calcium

Appendix VIIIC shows the effects of $CaCl_2$ added to sections with or without IAA. High Ca^{++} concentration (20 mM) inhibited growth and slightly reduced the cellulase level. Lower Ca^{++} concentrations (10^{-4} M) slightly stimulated both growth and cellulase activity. IAA stimulated growth and enhanced the cellulase level in the presence or absence of Ca^{++}.

4. pH

Appendix VIIID shows the effects of varying the external pH on which sections were incubated. Growth over 24hours in both fresh weight and length and final cellulase activity was greatest at pH 6 to 7. This is the pH range of maximum cellulase activity (Appendix IIIB) and stability (Figure 9) in vitro. Soluble protein levels were low after incubation at pH 4 or 5, presumably because the acetate buffers penetrated and killed some tissue cells. There was no indication of the death of tissue at higher pH values (up to pH 10). It is possible, therefore, that the loss of cellulase activity at high pH was due to extracellular denaturation of the enzyme.

Appendix VIIIA. Effect of plasmolysing agents on growth and cellulase activity

Sections (10 mm) were incubated in 2% sucrose - 0.02 M sodium phosphate, pH 6, ± IAA (10^{-5}), NaCl (0.3 M) and mannitol (0.4 M). At zero time, values per section were: fr wt = 21.9 mg, soluble protein = 237 µg, cellulase activity= 1.11% loss in CMC viscosity/ 6 hours.

	Value at 24 hrs relative to those at zero time				
Treatment	Fr wt	Length	Soluble protein	cellulase activity per unit protein	per sect
Control	1.51	1.34	0.81	1.54	1.25
+ NaCl	0.90	1.00	0.25	2.53	0.63
+ NaCl +IAA	0.95	1.04	0.30	2.63	0.78
+ mannitol	0.96	1.04	0.88	0.65	0.60
+ mannitol +IAA	0.97	1.03	0.88	0.75	0.66

Appendix VIIIB. Effect of reducing agents on growth and cellulase activity

Sections (10 mm) were incubated in 2% sucrose - 0.02 M sodium phosphate, pH 6.0, ± IAA (10^{-5} M), cysteine (C, 0.01 M), B mercaptoethanol (M, 0.01 M), Cleland's reagent (CR, 0.02 M) and ascorbic acid (A, 0.01 M). At zero time, values per section were: fr wt = 22.7 mg, soluble protein = 222 µg, cellulase activity = 1.01% loss in CMC viscosity /6 hours.

	Values relative to those at zero time				
Treatment	Fr wt	Length	Soluble protein	Cellulase activity per unit protein	per sect
Control	1.53	1.31	0.76	1.80	1.40
+C	1.47	1.26	0.88	1.07	0.94
+C +IAA	1.81	1.45	0.83	0.82	0.67
+M	0.98	1.00	0.37	4.10	0.67
+M +IAA	0.99	1.00	0.50	3.72	0.80
+CR	1.18	1.16	0.50	1.93	0.97
+CR +IAA	1.19	1.16	0.46	2.12	1.00
+A	1.41	1.24	0.81	1.32	1.05
+A +IAA	1.95	1.39	0.74	1.30	0.95

Appendix VIIIC. Effect of calcium chloride on growth and cellulase activity

Sections (10 mm) were incubated for 24 hours under different conditions. Values per section at zero time are: fr wt = 23.2 mg, soluble protein = 260 µg, cellulase activity = 1.00% loss in CMC viscosity /6 hours.

	Values at 24 hrs relative to those at zero time				
Treatment*	Fr wt	Length	Soluble protein	Cellulase activity per unit protein	per sect
Control	1.51	1.39	0.80	1.81	1.44
+IAA	1.73	1.42	0.79	2.26	1.78
+ 10^{-4} M $CaCl_2$	1.20	1.10	0.89	1.45	1.27
+IAA + $CaCl_2$	1.39	1.15	0.79	1.93	1.52
+ 20 mM. $CaCl_2$	1.63	1.48	0.75	2.13	1.58
+IAA + $CaCl_2$	1.77	1.50	0.68	2.70	1.87

* IAA concentration used in this test: 10^{-5} M

Appendix VIIID. Effect of external pH on growth and cellulase activity

For tests at each pH, 100 sections were supported with nylon cloth on the surface of 1 liter of buffer which was stirred magnetically for 24 hours at 25°. The buffers (sodium salts, 0.02 M) were acetate (pH 4 and 5), phosphate (pH 6, 7 and 8) and glycinate (pH 9 and 10). At zero time, values per section were: fr wt = 19.6 mg, soluble protein = 242 µg, cellulase activity = 1.21% loss in CMC viscosity/6 hours.

	Values at 24 hrs relative to those at zero time				
pH range (zero time to 24 h)	Fr wt	Length	Soluble protein	cellulase activity per unit protein	per sect
4.0 - 4.3	0.99	1.04	0.09	0.62	0.06
5.0 - 5.2	1.20	1.20	0.53	0.76	0.41
6.0 - 6.0	1.69	1.54	0.72	1.18	0.86
7.0 - 7.0	1.55	1.38	0.67	1.06	0.71
8.0 - 7.7	1.49	1.27	0.63	0.92	0.58
9.0 - 8.6	1.49	1.29	0.72	1.02	0.74
10.0 - 9.2	1.41	1.28	0.69	0.48	0.33