BIOCHEMISTRY OF CELL EXTENSION

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D. James F. Morre

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Abstract

By subjecting various plant tissues to conditions which influence growth, such as treatment with the growth hormone, 3-indoleacetic acid (IAA), changes in certain cytoplasmic constituents were observed which parallel simultaneous changes in the mechanical properties of the cell wall.

Treatment of maize roots for one hour in the presence of concentrations of IAA that normally promote shoot growth and, therefore, inhibit root growth followed by measurement of the deformability of the rapidly elongating region under artificially imposed load, demonstrated a concentration-dependent, IAA-induced plasticity component, not observable in untreated roots. The response is transient; half-maximal plasticity is induced by ca. 5×10^{-7} M IAA, one hour after treatment (a concentration comparable to that of Avena sections). Increased deformability is paralleled by increased growth of root sections, also transient, with maximum IAA-induced increase in growth rate coinciding with the maximum for increased wall plasticization. The initial response of maize roots to IAA, therefore, resembles that of Avena coleoptiles both qualitatively and quantitatively. The end result, however, is to effectively shorten the period over which the root can elongate.

Associated with increased plasticity and growth rate of maize roots is rapid formation (or maintenance) of a protein-bound carbohydrate fraction. Disruption of the complex by such agents as extremes of pH or organic solvents results in increased turbidity of aqueous solutions and acquisition of solubility properties characteristic of lipides. Results

of preliminary characterization studies also suggest that material is of lipoprotein origin.

Evidence for a similar fraction, increased in amount by treatment of the tissue with IAA, has been extended to include Avena coleoptiles, pea epicotyls and pea embryo axes. In addition to lipide-soluble components, these fractions contain approximately equimolar quantities of carbohydrate (including hexose) and of esterified phosphate. The material, therefore, has been designated as a protein-bound glycolipide (PGL).

A cytoplasmic origin of PGL is suggested. Also associated with the 2- to 4-fold increase in amount of protein-bound PGL is a decrease in the heat coagulability of cytoplasmic proteins. Similarily, a portion of an apparent IAA-induced increase in acid phosphatase activity may be attributable to increased stabilization as well. An electron microscopic survey of IAA effects on the fine structure of subcellular organelles revealed no major structural changes, however, the number of vesicles associated with the central Golgi structure of both maize roots and Avena sections may be increased by IAA treatment.

A study of cell wall pectic constituents has revealed that although versene-soluble pectin does represent a solubility class distinct from hot water-soluble pectin, the conditions for extraction do not correspond to those of the classical residual pectin fraction. The existence of pectin in the cold buffer-soluble, 70% ethanol-insoluble fraction of Avena coleoptiles (cold water-soluble pectin) seems doubtful, however.

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This work I, respectfully,
dedicate to my father and mother.

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I. INTRODUCTION

The main conceptual features of cell extension have been known for some time, cf. Heyn (1), and there is now general agreement on many of the physiological processes involved (1-9). Two such features which form an integral part of all discussions of cell extension are: 1) that a cell will not grow without absorbing water and 2) that cell extension, defined as any permanent increase in cell size (1), is related to an irreversible increase in the surface of the cell wall.

Studies on water relations during cell extension have been recently reviewed by Bonner (2), Galston and Purves (3), Thimann (9) and Ordin (10) and the experimental evidence indicates that osmosis rather than "active" water uptake is concerned during these water movements. Furthermore, experiments with certain plant growth regulators suggest that hormonal control of growth by direct osmoregulation is unlikely, although these processes generate turgor pressure which could serve as the driving force in growth (11). According to Bonner (2), other factors being equal, "...the rate of cell extension depends upon the tension to which the wall is subjected by the osmotic pressure of the cell contents." Since water enters the cell during growth, it must do so under the influence of a diffusion pressure deficit gradient resulting from changes other than in osmotic concentration. The responsible physiological changes have been sought through a consideration of the processes of cell wall deposition and expansion.

Chemical analysis of the primary walls of a number of plant tissues suggest that α-cellulose constitutes about 25 to 35% of the cell wall; pectic substances, 1 to 10%; protein, 5 to 10% and lipide, 2 to 20%; the remainder designated as non-cellulosic polysaccharide or hemicellulose, cf. Setterfield and Bayley (4). These constituents are further considered to exist as two phases (12): a discontinuous phase of cellulose microfibrils and a continuous phase formed of an amorphous matrix in which the cellulose microfibrils are imbedded. The microfibrils, being relatively inert and rigid, are thought to be largely responsible for determining the form and structural characteristics of the wall (4, 7). Matrix materials, although contributing to structure as well, are generally considered to be of a more reactive nature and most likely to influence growth by controlling cell wall rigidity (7, 12, 13).

Heyn (1) outlined three possible mechanisms of wall expansion based on cell wall properties: 1) an increase in surface area by active synthesis of new wall; 2) elastic extension of the wall by turgor pressure, the latter being made permanent by deposition of new wall materials and 3) direct plastic stretching of the wall resulting from turgor pressure.

The first mechanism requires a direct correspondence between wall deposition and cell extension, a relationship which has not been shown to be absolute (1, 12, 13, 15, 16). It is possible that enlargement by active wall growth occurs in some cells (17), although passive growth by stretching of existing cell walls would appear to be the more generally favored mechanism.

During the 1930's, Heyn (1) conducted an extensive series of experiments bearing on the validity of the remaining two proposals. He demonstrated, for decapitated Avena coleoptiles, that the plastic (irreversible) extensibility of the wall closely parallels the growth rate, while the elastic (reversible) extensibility shows no apparent relationship to growth rate but rather reflects the growth history of the tissue. The original observations of Heyn have been confirmed and extended (18-21), and the results obtained indicate that cell expansion is initiated through an increase in plasticity of the cell wall. Cell expansion, according to Cleland (20), is then the result of a continual process of cell wall loosenings and expansions.

Although plastic stretching appears to limit cell extension under conditions where net synthesis of wall materials and turgor pressure are non-limiting, wall deposition normally accompanies cell elongation and it has been impossible to separate the two processes for extended periods, cf. Setterfield and Bayley (4). Thus Avena coleoptiles have been observed to elongate 5 to 20%, under conditions where cell extension was uncoupled from wall deposition by incubating the sections at low temperature (15) or in the absence of oxygen (19). Sustained growth, however, would appear to be restricted to situations where non-limiting conditions for wall synthesis are maintained (4, 10, 20, 22).

This interdependency between cell elongation and wall synthesis has been visualized on the basis of an addition of new materials at active sites created during stretching (13). This is evidenced by the fact that simple osmotic inhibition of elongation will prevent wall deposition (10, 13, 22, 23, 24) and that reduced growth in the absence of endogenous

auxins may be associated with reduced wall synthesis, cf. Setterfield and Bayley (4). Furthermore, it is possible that new materials capable of being stretched must be added in order to sustain unlimited growth. According to Setterfield and Bayley (4), the plastic materials would presumably be found as components of the matrix, added by intussusception throughout the wall. On the other hand, the transverse microfibrils would be apposited on the inner surface in order to maintain cell wall mechanical strength. Autoradiographic evidence indicates that cell wall deposition does occur uniformly over the surface of the existing cell wall (25, 26).

Burström (17) has suggested that the order of processes in cell extension would involve first a loosening of the cell wall and a plastic stretching followed by an active wall formation by intussusception. The possibility that both a plastic component and materials capable of cell wall stiffening are added during growth has been discussed by Busse (27); the rate of extension being dependent upon a balance between the two types of materials.

Brown (28) has recently proposed a relationship between the intercellular cohesion and extension growth based on measurements of the area to which fragments of pea root tissue could be compressed after treatment with hydrochloric acid (29). He distinguishes two types of bonds, one being acid-labile and electro-valent, the other acid-stable and possibly covalent. The results indicate that the acid-resistant component is greatly increased when the tissue is incubated under nitrogen or with various respiratory inhibitors, including cyanide, azide, iodoacetate or N-ethylmaleimide. Thus, the acid-resistant component is considered to be the

result of a spontaneous exothermic reaction not dependent upon respiratory energy. Conditions which sustain vigorous growth, such as addition of carbohydrate to the growth medium and aeration, lead to a reduced development of the acid-resistant bonds. Although the discussion is confined to intercellular cohesion within the middle lamella of roots, similar wall stiffening or crosslinking reactions could explain the reduced deformability of Avena coleoptiles grown in the absence of auxin (30, 31). This condition apparently is not developed in the presence of auxin, even when elongation is prevented by incubation at low temperatures or under nitrogen (30).

The actual microfibrillar organization of primary walls has been described in recent reviews by Frey-Wyssling (32, 33), Roelofsen (34) and Wardrop (7), whereas the more general aspects of primary wall structure have been the subject of a review by Setterfield and Bayley (4).

In the case of young parenchyma cells of oat coleoptiles, a net transverse orientation of microfibrils can be observed. This has led to the suggestion by Frey-Wyssling (35) of a tubular structure of the walls. Closer examination of such walls under the electron microscope, reveals a difference in orientation of the microfibrils of the inner and outer surfaces (36). Although the microfibrils are somewhat interwoven throughout the thickness of the cell, on the inner surface they are more or less transversely oriented in a fairly regular fashion. Toward the outside they show greater dispersion and an increasing tendency toward longitudinal orientation. This type of structure has come to be referred to as simple multinet (30). It has been proposed that, in effect, the cell during elongation continuously lays down microfibrils on the inner surface of the

wall in a direction approaching transverse, ideally representing a helix of zero pitch. Extension of the wall causes these transverse microfibrils to be drawn out so as to increase the pitch of the helix and produce a gradiation of microfibril orientations from transverse on the inner surface to approaching longitudinal on the outer (37, 38).

Bonner (39) has arrived at certain definite conclusions regarding the nature of the orientation of cell wall microfibrils through a study of changes in double refraction. Avena coleoptile sections, from which the epidermis had been stripped, were stretched longitudinally under the polarizing microscope. As the tissue is stretched, the microfibrils are observed to re-orient from a position statistically at right angles to the shear axis to one in which the microfibrils are statistically parallel to the shear axis. The re-orientation in the direction of shear is interpreted as the result of interaction between individual microfibrils. If, however, the plastic extensibility of the tissue is increased by treatment with auxin, the interaction appears to be much reduced and re-orientation is replaced by a tendency for the microfibrils to slip past each other. These findings clearly indicate a profound influence of auxin-induced cell wall plasticization of the ability of cellulose microfibrils to orient under stress and are in agreement with more recent modifications of the multinet theory of growth (40).

The multinet theory, although taking into account many of the observations relating to microfibrillar orientation, remains primarily a description of events following deposition of cell wall materials rather

than an explanation of the processes of cell extension (4, 7). Increased cell wall deformability would appear as a necessary, although perhaps not a sufficient, condition and could serve as the basis for at least one control mechanism. However, the chemical nature of cell wall plasticization remains obscure despite numerous and extensive research efforts.

Plasticization through a direct effect on cellulose microfibrils has been discounted in most discussions of the chemical nature of cell wall plasticization and growth (4, 13, 24, 41, 42). The effects of auxin treatment on incorporation of radioactive carbohydrate substrates into cellulose are usually small (21, 24, 43, 44, 45). Although changes in the polysaccharide matrix remain as possible factors in controlling cell extension, unfortunately no major change in any one fraction can be consistently related to growth. This conclusion is supported by observations which show that alterations in all major wall components can be associated with changes in growth or with responses resulting from auxin treatment. These components affected include cellulose (47, 48, 50), hemicellulose (43, 44, 48-51), pectin (24, 44-52), protein (49) and lipide (47, 49). That auxin does not directly increase cell wall synthesis is also evident from experiments in which incorporation of C^{14}-labeled sugars into cell wall materials were studied during incubations of several hours duration (22, 43, 44, 45, 46, 53). In these experiments, incorporation is unaffected or even slightly decreased by the presence of auxin. Somewhat less is known concerning a possible role of lipides in cell wall plasticization. Blank and Frey-Wyssling (47) have observed that IAA-induced elongation coincides with an increase in the concentration of lipides; however, pectins and cellulose

are similarily affected. Experiments on the utilization of acetate by both roots and shoots have indicated that IAA may inhibit incorporation of radioactivity from acetate-1-C^{14} into various lipide fractions (54, 55) not known to be associated with the cell wall.

A more definite role for pectic substances in the control of cell wall plasticization has come from studies concerning growth inhibition by multivalent ions (16, 18, 23, 56). The theory involves changes in the amount of cross-linking between carboxyl groups of pectic substances, primarily through methyl esterification and calcium bridges. The hypothesis has received support from the fact that: 1) added calcium inhibits cell expansion (16, 56) and reduces wall plasticity (18); 2) chelating agents may stimulate cell expansion (16); 3) activity of the enzyme pectin esterase may be influenced as a result of auxin treatment (57, 58) and 4) the rate of incorporation of the methyl group of methionine into hot water-soluble pectin of the cell wall may be increased in the presence of auxins (44, 45, 46). Jansen et al. (46) have shown that the increased methyl incorporation is primarily associated with the pectin fraction soluble in hot water. This effect of auxin occurs rapidly; with inhibition by antiauxins, high auxin concentration, and ethionine. In general, it parallels the influence of auxin on cell wall plasticity; and is considered a true auxin response. However, the auxin-induced incorporation into pectin methyl-ester from methionine does not appear to be a general reaction (59) and no consistent relation to growth has been demonstrated (60). Furthermore, the calcium levels in walls are unaffected by either auxin (61) or chelating agents (62) and the binding of pectin esterase in Avena coleoptiles is not influenced by auxin (63). Both the calcium ions responsible for growth

inhibition, as well as for wall stiffening, appear to be exchangeably bound (2, 56), however, and a reduction of cell wall plasticity through increased ionic binding between pectic carboxyl groups remains an attractive mechanism for controlling cell extension.

The experimental attack of the problem of cell wall plasticization is greatly facilitated by the fact that it may be induced by application of the auxin, 3-indoleacetic acid (IAA). The ability of IAA to regulate the growth rate of plant tissue through increased plasticization of the cell wall is now well documented (1, 2, 3, 17, 18, 20), although a direct action of IAA on the cell wall is not implicit in these observations.

It was clear from the original work of Heyn (64) that auxin cannot produce its effects on dead cell walls, and that some portion of the protoplasm is required. The effect requires an active metabolic system, since IAA produces no increased plasticity of the cell wall under anaerobic conditions (15, 19) or in the presence of various metabolic inhibitors including KCN or dinitrophenol (19, 20, 50, 65, 66). Furthermore, it would appear that some form of cytoplasmic contact between the cell wall and the protoplast is a necessary requisite for IAA-induced cell wall plasticization since, in the case of plasmolyzed cells, in which wall and cytoplasm are not in contact, cell wall softening under the influence of IAA cannot occur (66).

Evidence supporting the concept that auxins combine with a receptor entity within the cell is derived from kinetic analyses (67, 68) and from structure-activity relationships (69-72). The receptor has been assumed to be a protein although an unambiguous IAA-protein complex has not

been isolated. Various auxin-protein combinations have been reported, although their significance in relation to control of growth is uncertain (73, 74, 75). Among the various effects on the cytoplasm that have been observed following IAA treatment are a decrease in structural viscosity of the protoplasm (3, 76, 77), a temporary acceleration of the rate of protoplasmic streaming (77, 78, 79), an alteration in the heat stability of proteins (80, 81), increased oxygen consumption (3, 71), effects on nucleic acid metabolism (82) and possibility altered permeability patterns (3, 69).

The fact that auxins produced changes in the absorption of various materials into plant cells, at one time suggested a direct effect on cell membranes as the primary mode of auxin action. This concept was particularly advocated by Veldstra (83) and received support from studies of the relations between structure and activity of various growth substances. The studies were extended to include a possible direct effect on oleate coacervates (based on the model experiments of Bungenberg de Jong and colleagues (84, 85) with phosphatide coacervate membranes)). Although the turgescent (or opening) action of the growth substances paralleled their activity in the split pea curvature test for auxins in a regular manner (86), the results were in opposition to what would have been predicted if the growth promotion resulted from increased membrane permeability (85). Even though direct effects on membrane permeability have been discounted (3), there is still evidence for a hydrophylic-lipophylic interfacial orientation as being important in growth regulator action (69). A modified proposal along these same lines has been discussed by Van Overbeek (87).

A direct effect of IAA on isolated protoplasts of tomato cotyledons has been reported by Cocking (88). At 0.1 ppm of IAA, the formation of small vacuoles is observed along with an increase in size of the protoplasts and a rapid movement of granules within the cytoplasm. Similar responses were observed in isolated root protoplasts but at a concentration of 10^{-5} ppm of IAA or lower (89). These changes were not observed in untreated protoplasts under similar conditions.

Thus far, there are no direct experimental findings relating to the manner in which the protoplasm controls either microfibril orientation or the more general aspects of cell wall synthesis (4, 41, 90, 91), although the possible involvement of a portion of the endoplasmic reticulum in cell wall synthesis has been indicated (92). In order to further investigate the nature of cell extension and the role of IAA in regulating this process, it is assumed: 1) that an experimental approach to the problem of auxin-induced cell wall plasticization can be achieved through studies of cytoplasmic changes which occur during growth and 2) that by subjecting the tissue to conditions which influence growth, such as treatment with IAA, it will be possible to create observable changes in certain cytoplasmic constituents which can be interpreted in terms of simultaneous alterations in wall structure.

Four types of studies have been pursued in an effort to determine the correctness of these assumptions:

1) Correlations between changes in cell wall deformability and growth rate and the appearance or disappearance of distinct cytoplasmic fractions.

2) Evidence for the general occurrence of auxin-induced changes in cytoplasmic components.

3) Characterizations of these fractions particularly with reference to their possible functions in the control of cell wall properties.

4) Assessment of parallel changes in the cell fine structure associated with IAA-induced growth.

II. A CHEMICAL AND MECHANICAL ANALYSIS OF ROOT GROWTH

The growth response of roots to varying concentrations of IAA is different from that of stems in the following respects (93, 94, 95):

1) In general, concentrations of IAA which promote stem growth lead eventually to an inhibition of root growth. This inhibition has been shown to result primarily from a shortening of the time over which the root elongates.

2) Very low concentrations, i.e., 10^{-9} to 10^{-11} M of IAA may increase root elongation. These concentrations normally have no measurable effect on the growth of stems.

3) Certain types of anti-auxins which competitively interfere in the action of auxins and, therefore, inhibit shoot growth are likewise reported to promote the growth of roots.

As inhibitors of root growth, auxins are extremely potent. They are effective at concentrations 100 to 1000 times lower than those employed with the usual metabolic inhibitors (94). On the other hand, the validity, as well as the interpretation, of growth increases resulting from IAA treatment have been questioned (95). The most frequently cited criticisms are 1) that the response of biological assays to low concentrations of a toxicant are often highly variable (96); 2) that growth increments of the same order of magnitude (ca. 10 to 100% increase in length) are obtainable by such seemingly unrelated substances as respiratory inhibitors, certain

surface active agents and other miscellaneous reagents (94, 97) and 3) positive responses to added IAA and other auxins are noted only after several hours, usually 17 hours or more after treatment (98). However, auxins are still the most effective agents, on a molar basis, reported to increase the growth of roots.

Thus, the fundamental difference between root and stem tissue response to auxin was tentatively based on sensitivity. The representation proposed by Thimann (93) is in the form of optimum curves with intermediate concentrations increasing growth and higher concentrations reducing growth. According to this interpretation, the auxin concentrations required for initiation of the growth response, as well as for growth inhibition, would be approximately 10^5 times greater for stems than for roots.

Growth inhibition induced by high auxin concentrations in stems can now be understood on the basis of two-point attachment. According to Foster, et al. (67), growth inhibition by auxin in shoots possesses kinetics which would be expected on the basis of a monofunctional combination of two individual auxin molecules with a single receptor entity. On the other hand, growth promotion would be possible only under conditions of bifunctional attachment. A similar interpretation of root inhibition could be reconciled with early claims that a growth acceleration was produced following removal of the root tip, the supposed source of natural auxin, cf. Audus (99), page 36. In intact roots, the amount of auxin was thought to vary within the supra-optimal range and that exogenous supplies could only serve to further reduce growth. Recent repetitions of these decapitation experiments have failed to reveal increased growth (100,

Appendix A) or restoration of the capacity of roots to respond to gravity by replacement of the tip (100) as was the case for the early experiments. On the other hand, an occasional acceleration of root extension by added auxin does not necessarily imply a sub-optimal auxin level comparable to that which exists in decapitated Avena coleoptiles (99). Thus, many authors have been content to consider the classical concepts of auxin regulation of root growth as still being valid (94, 95, 98, 101).

However, it must be considered that the concentrations of auxin resulting in complete inhibition of root growth are still well within the range of concentrations associated with hormonal function in shoots. This is true even in the case of excised and/or decapitated roots (94, 95) which, according to classical concepts, would be deprived of a supply of natural auxins. Similarily, results of investigations where root extension is measured over short time intervals (1 hour or less) indicate that growth is either unaltered or inhibited by auxins (95, 98). Apparently increases in cell extension are not characteristic of the short-time response of roots to low concentrations of added auxin. Therefore, the possibility that both stem promotion and root inhibition are both manifestations of the same basic underlying mechanism of IAA action will be considered.

As part of the following analysis of root growth, preliminary evidence will be presented for the IAA-induced formation (or maintenance) of a specific cytoplasmic component. Although the initial changes which occur as a result of IAA-treatment are essentially complete within the first

hour after their initiation, the nature of the response is sufficiently clear and dramatic to permit a definite correlation between increased growth, cell wall softening and an alteration in the level of a carbohydrate-containing fraction localized in the cytoplasm.

Materials and Methods

Plant material.--Seeds of a single cross hybrid strain of white maize, H-23 X H-22, were obtained from the Agricultural Alumni Seed Improvement Association, Lafayette, Indiana and germinated between sheets of filter paper moistened with distilled water. Details concerning the conditions of germination are given as part of Appendix A.

Preparation of IAA solutions.--Stock solutions of IAA were prepared by dissolving a weighed quantity of 3-indoleacetic acid (Calbiochem) into distilled water without aid of cosolvent to yield a final concentration of 10^{-4} M (or 10 ppm, as in initial experiments). The final concentrations were obtained by adding an appropriate amount of 0.01 M potassium maleate buffer, pH 4.5. This concentration of buffer is near the optimum for growth of intact maize roots (97).

Nature of IAA inhibition.--The results of experiments outlined in Appendix A show that growth of the primary root is limited to the first 5 mm of the root tip. Growth of this region was followed during a 12-hour period in the presence and absence of varying concentrations of IAA. These measurements were facilitated through the use of India ink marks placed on the root epidermis at 0.8 mm intervals, beginning 2.4 mm from the distal end of the root cap as described in Appendix A.

Evaluation of changes in cell wall deformability.--The mechanical analysis of wall deformability is similar to that applied by Tagawa and Bonner (18) to a study of the mechanical properties of Avena coleoptiles. The procedure involves measurement of rate of bending of tissue sections fixed at one end in a rigid support and loaded with a suitable weight on the other.

Maize seeds were germinated as previously described and, after 60 hours, the seedlings were sized according to length of primary root. Five seedlings were then transferred to each of an appropriate number of 20 X 100 mm disposable plastic petri plates (Falcon Plastics Co., Los Angeles, Calif.) fitted with circles of Whatman No. 1 filter paper to which 4 ml of 0.01 M potassium maleate buffer, pH 4.5 were added. The petri plates were stacked in enamel instrument pans tilted at an angle of 5° from the horizontal with all roots oriented downward and preincubated in a saturated atmosphere for 12 hours. During the preincubation period, each primary root produced slightly in excess of 12 mm of new growth (steady state growth rate = 1.05 $\pm$ 0.05 mm per hour) suitable for deformability measurements. Reproducable results could not be obtained when roots were treated immediately after transfer from germination trays.

Initial experiments demonstrated that roots respond to extremely low concentrations of IAA during a 12-hour incubation period. Since the IAA concentrations used were of the order of 10^3 greater than the range of concentrations necessary to elicit the long term response, disposable petri plates were used routinely to avoid completely the possible complications arising from incomplete removal of residual IAA.

IAA treatment was initiated at the end of the 12-hour preincubation period by adding 1 ml of buffered IAA solution to the 4 ml of buffer already present. The added solution was distributed uniformly over the petri plate and mixed by gentle rocking.

The deformability of root tissue under external load as a function of IAA concentration was measured with the device depicted in figure 1. The apparatus consists of a series of rigid supports with a common base providing measurements from seven roots during each determination. At appropriate time intervals, 12-mm tip sections were excised and the cut end of the tissue was inserted a distance of 5 mm into a hole (ca. 1 mm in diameter) drilled at right angles to the edge of the support. A 0.170 ± 0.005 mg weight was fitted over the root cap and meristematic region at the distal end of the root tip for a distance of 3 mm. In this manner only the deformability of a 4 mm zone of the rapidly elongating region was measured, the remainder of the 12-mm root tip being contained within the apparatus. Weights were fashioned from fine glass tubing (ca. 1 mm, inside diameter) fitted at one end with a lead cylinder cut from rosin core solder. Measurements were obtained in the presence of low intensity red light and at a temperature of 25° C. In order to maintain the tissue in a turgid condition, the entire apparatus was kept in a water-saturated atmosphere.

Initial curvature of each root tip (minus weight) was determined after applying slight pressure to insure that the tissue segment was securely fixed within the support. Load was applied for exactly 2 minutes and then removed. After one minute, final curvature (minus weight) was

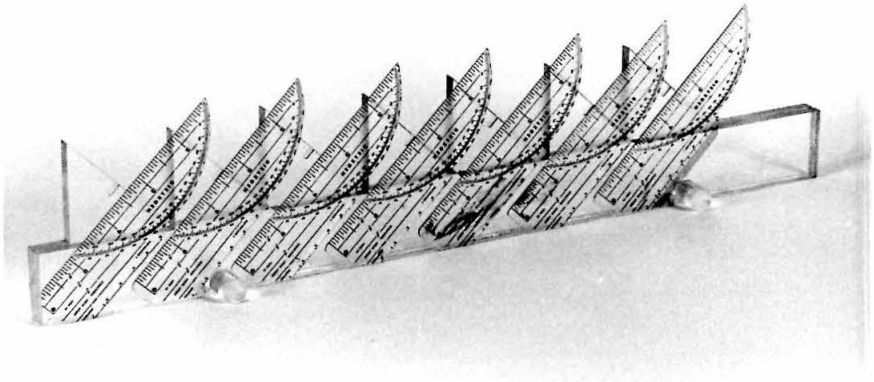


Figure 1. Multiple device for measuring the deformability of tissue sections under external imposed load. The bending angle is estimated from the protractors mounted behind the support.

recorded and the residual angle (final curvature minus initial curvature) was taken to be a measure of irreversible deformability or plasticity.

The apparatus shown in figure 2 was used to determine the rate of deformation as a function of time. As described by Lockhart (102), the tissue section is inserted in a 5-mm deep hole drilled perpendicular to the axis of a rotatable horizontal shaft which serves as the support. Following application of load, the course of deformation is determined from the position of the indicator after restoration of the weight to the initial position. With this device, it was possible to accurately record small deformations obtained under relatively constant imposed force. The angle through which the tissue was deformed was measured within a few seconds after application of the weight and as soon as the initial displacement had occurred (noting time). Subsequent measurements were obtained at 10-second intervals. At the end of 300 seconds, the weight was removed and the course of recovery followed for an additional 100 seconds.

The primary roots of maize decrease in diameter during normal growth giving rise to an increased deformability as illustrated in figure 3. This fact made it necessary to include an untreated control with each treated sample in comparisons of roots of different ages.

Preparation and incubation of root sections.--Subapical 5-mm root sections were cut from primary roots of 48- to 60-hour old seedlings. Cuts were made approximately 2.5 mm behind the root cap using a double bladed cutting tool. Sections were cut directly into 125 ml Erhlenmeyer flasks containing 10 ml of 0.01 M potassium maleate buffer, pH 4.5.

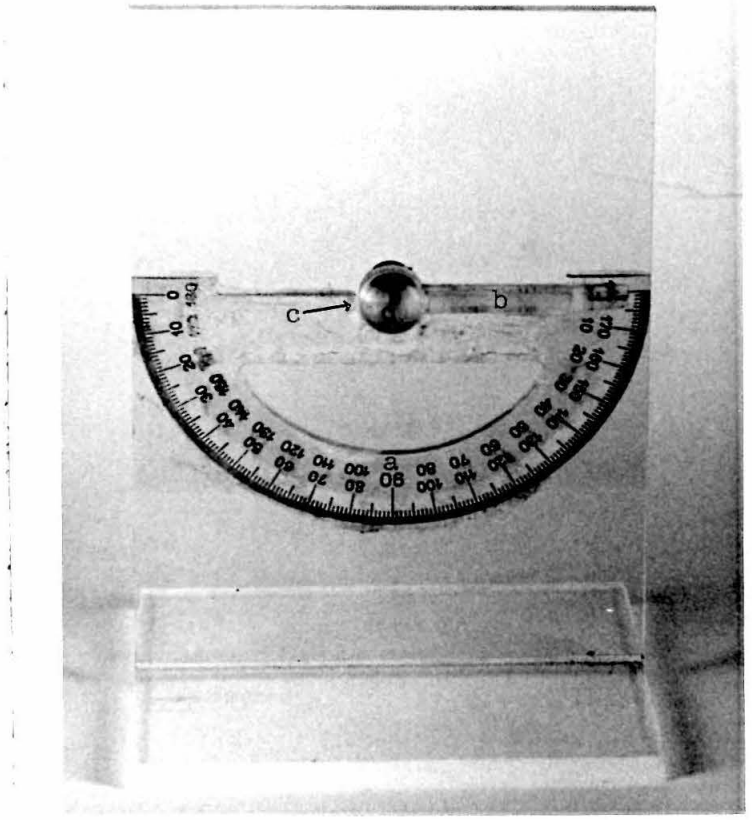
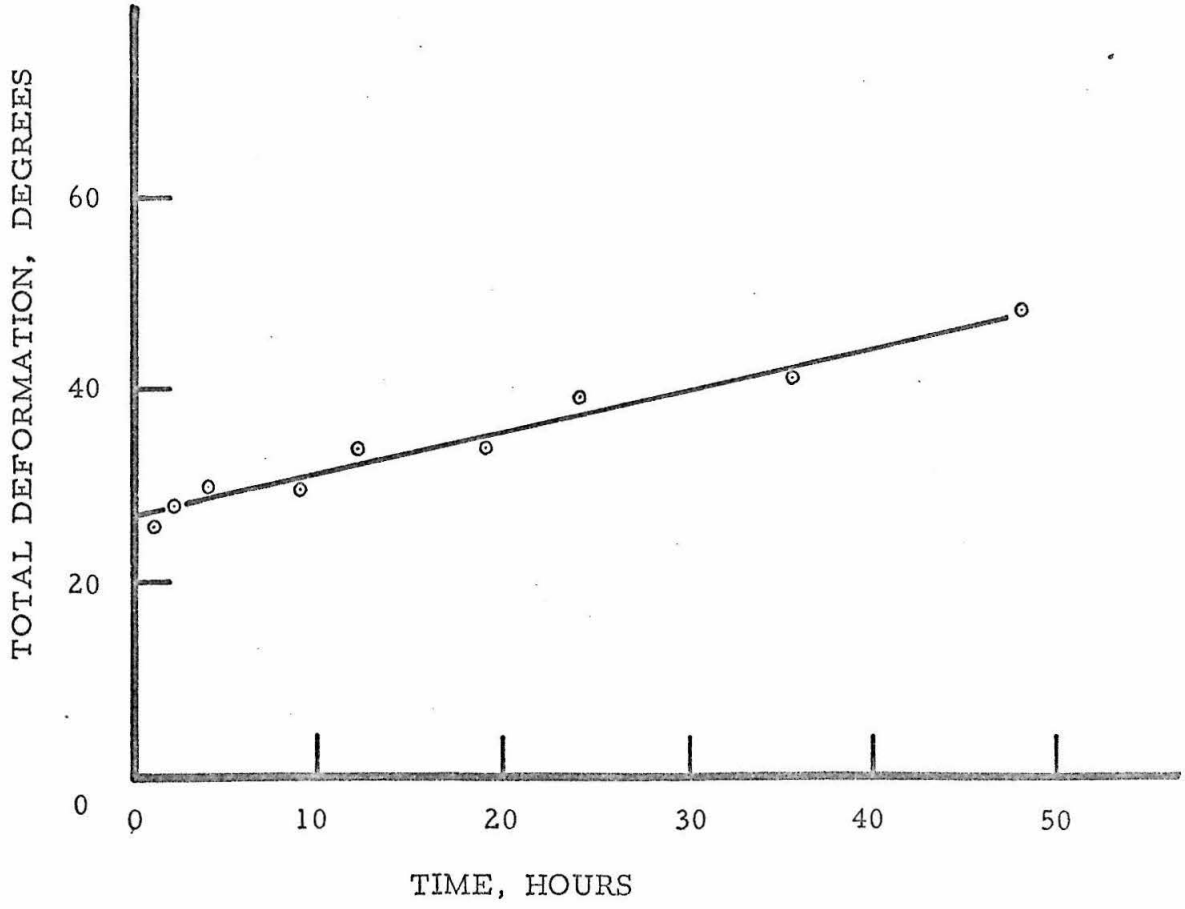


Figure 2. Front view of the device for measuring tissue deformability as described by Lockhart (102). After application of load, the course of deformation is measured from the protractor (a) mounted behind the handle-pointer (b). Tissue sections are inserted into a hole (c) drilled at right angles to the central shaft (d).

Figure 3. Increase in deformability of the elongation zone of intact maize roots as a function of incubation time. Seedlings, germinated for 60 hours, were grown on filter paper moistened with 0.01 M potassium maleate buffer, pH 4.5. Measurements are based on two lots of seven seedlings each with a deformation time of two minutes.



After sufficient sections were obtained in this manner, an additional 10 ml quantity of buffer with and without IAA was added at the beginning of the incubation period. The final IAA concentration in all experiments with root sections was 5×10^{-7} M. Each analysis is based on parallel samples of 500 sections each (fresh weight, ca. 3 g). All incubations were conducted in the dark for the time periods indicated.

Estimation of root section growth during short term experiments.--The growth in length of excised root sections as a function of time is discussed in Appendix A. Since the problem is complicated by an inherently slow rate of growth, coupled with the necessity for measurements at short time intervals, changes in fresh weight are taken to reflect changes in growth rate of the sections. By this method it was possible to obtain an essentially instantaneous evaluation of growth based on the average of a sample of 500 sections.

After incubation, sections were washed with water, dried by centrifuging over cotton for exactly one minute (2,400 rpm, clinical centrifuge) and transferred to tared containers. The fresh weight was then determined to the nearest milligram. Samples were frozen immediately over dry ice and stored in the freezer prior to analysis. Comparative samples were washed, dried and weighed consecutively, following the same time schedule for each sample.

Isolation of a pH 4.4-soluble, carbohydrate-containing fraction from roots, increased in amount in the presence of IAA.--Frozen 5-mm root

sections (ca. 3 g fresh weight) were ground with a pre-chilled mortar and pestle in 12 ml of 0.15 N sodium acetate buffer, pH 4.4. The pH 4.4-insoluble material and cellular debris were removed by centrifugation at 3,000 X g for 35 minutes. These operations were conducted at 0 to 4° C. The supernatent liquid was decanted and the pellet was retained, where applicable, for isolation of cell walls according to the procedure of Jansen, Jang, Albersheim and Bonner (46).

The pH 4.4-soluble extract was then heated to near boiling to denature a fraction of the remaining protein. This denatured protein was removed by low-speed centrifugation and the extracts were concentrated in vacuo to approximately 0.5 ml using a Rinco rotary evaporator connected with a water aspirator. The concentrated supernatent was then transferred quantitatively to a 15 ml conical centrifuge tube with several rinses of acetate buffer to yield a final volume of 3 ml. Absolute ethanol was added to 70% with respect to ethanol and the carbohydrate containing complex was obtained as a flocculant white precipitate. Precipitations were conducted in the cold, usually for periods of 8 to 12 hours.

Incorporation of radioactive metabolites.--Five hundred 5-mm root sections were incubated for one hour in 20 ml of 0.01 M potassium maleate buffer, pH 4.5 containing ca. 1 μc of the radioactive substrate in the presence and absence of 5×10^{-7} M IAA. At the end of the incubation the sections were washed with water, dried by centrifugation over cotton, weighed and fractionated as described. Radioactive compounds were obtained from the California Corporation for Biochemical Research, Los Angeles, California (Calbiochem).

a) Methionine-$C^{14}H_3$.--Methionine-$C^{14}H_3$ was supplied at a final concentration of 5×10^{-6} M with respect to methionine. In addition to the 70% ethanol-insoluble fraction of the pH 4.4 supernatent, cell walls were prepared according to the method of Jansen, et al. (46). Hot water-soluble and hot versene-soluble pectin fractions were obtained by extracting the dry cell walls in turn with two 10 ml portions of hot 0.5% disodium EDTA. The extractions were conducted at 100° C for a period of 30 minutes. Combined extracts from each fraction were concentrated to ca. 0.5 ml and the pectin precipitated from 70% ethanol in the cold. An aliquot of each of the various fractions was saponified with 0.5 N ammonia in a sealed tube for 24 hours at room temperature. Parallel samples were also saponified by treatment with 0.5 N sodium hydroxide at room temperature for 30 minutes. The ammonia was removed by evaporation and samples for counting were evaporated onto glass planchets. Radioactivity before and after saponification was determined to ± 3% accuracy by means of a Nuclear Chicago D-47 gas flow, proportional counter fitted with a "micromil" window. Absolute counting efficiency was approximately 25%. Radioactivity lost as a result of saponification and evaporation of the methanol formed was taken to be an indication of the incorporation of radioactivity from methionine-$C^{14}H_3$ into alkali-labile methyl groups of the various fractions. Data reported is based on two such experiments. As a parallel control experiment, incorporation of isotope from methionine-$C^{14}H_3$ into the pH 4.4-soluble fraction was also measured using an equivalent quantity (by weight) of 5-mm Avena coleoptile sections, primary leaf intact.

b) Glucose-U-C^{14}.--Uniformly labeled glucose was supplied at a final concentration of 4×10^{-5} M with respect to total glucose. The pH 4.4-

soluble fraction of roots was prepared and the radioactivity contained in the 70% ethanol-insoluble precipitate was determined without further purification.

Methods of analysis.--Protein determinations were conducted according to the standard biuret method (103). Carbazole carbohydrate was measured using a modification of the reaction of carbohydrate with carbazole in the presence of 87% sulfuric acid described in detail as part of Appendix B. Hexose, determined by reaction with anthrone, is reported in terms of galactose equivalent. The procedure followed is that of Scott and Melvin (104). As a general reaction for hexose, the carbazole-water reaction was also employed. The quantitative aspects of this test are discussed in Appendix B. Absorption spectra of the chromogen formed on reaction of the cytoplasmic fraction with carbazole in sulfuric acid were obtained using a Cary recording spectrophotometer balanced against a reagent blank.

Results

Nature of IAA-inhibition of intact roots.--Two interesting features emerge from the time course curves shown in figure 4. The initial effect of high IAA concentration is to reduce the overall growth rate of the tissue segment under observation. However, at the intermediate and lowest concentration of IAA, the decrease in growth rate does not become pronounced until after about two hours. However, the primary response

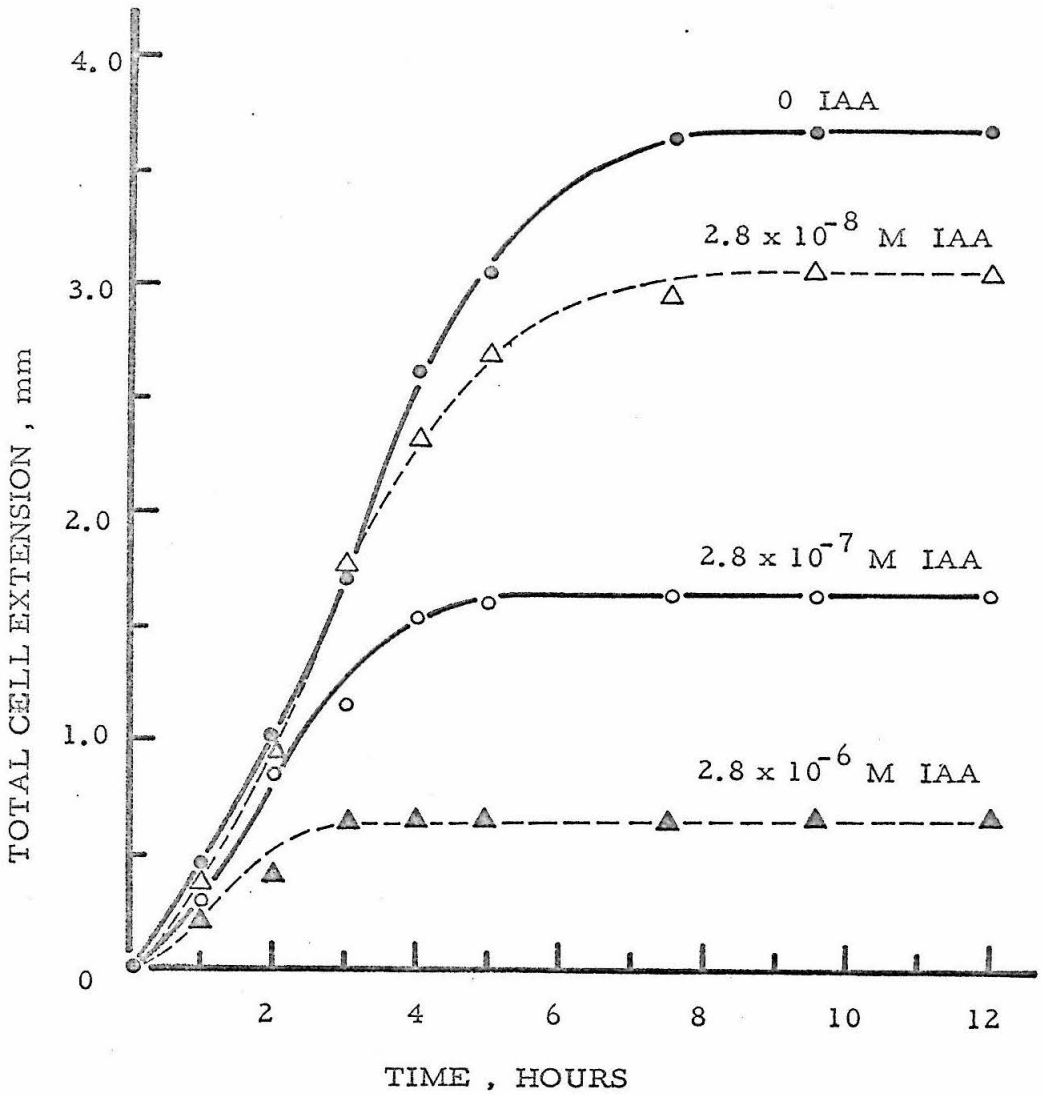


Figure 4. Time course of cell extension of intact maize roots as influenced by IAA concentration. Basal medium of 0.01 M potassium maleate buffer, pH 4.5.

contributing to the overall inhibition of root growth is a concentration-dependent shortening of the period during which the roots elongate.

Although growth rate of the elongating zone of intact roots was not observed to be noticeably increased at any time by IAA over the concentration range tested, increased radial enlargement was consistantly observed in all experiments involving concentrations of IAA greater than 5×10^{-7} M. In some experiments a distinct radial enlargement was observed within two hours after treatment at concentrations between 5×10^{-6} and 10^{-5} M IAA.

Mechanical properties of roots as influenced by indoleacetic acid.--Figure 5 illustrates the time course of the mechanical deformation of roots before and after treatment with IAA. At t = 0, the weight was applied and the root section was found to bend rapidly. This initial deformation was found to be reversible and is referred to as initial elasticity. In the case of untreated roots (figure 5a), the initial deformation is followed by a steady-state deformation which continues for at least 300 seconds or to a total angle of 60°. When the load is removed, the root returns toward its initial position and a portion of the total deformation is recoverable.

The second part of the diagram (figure 5b) presents results obtained from roots treated for one hour with 5×10^{-6} M IAA. The increase in total deformability is due entirely to a very rapid stretching during the first 80 seconds after application of load. Following completion of the rapid deformation, the bending curve assumes the slope of the steady-state curve characteristic of control roots. The transition between the

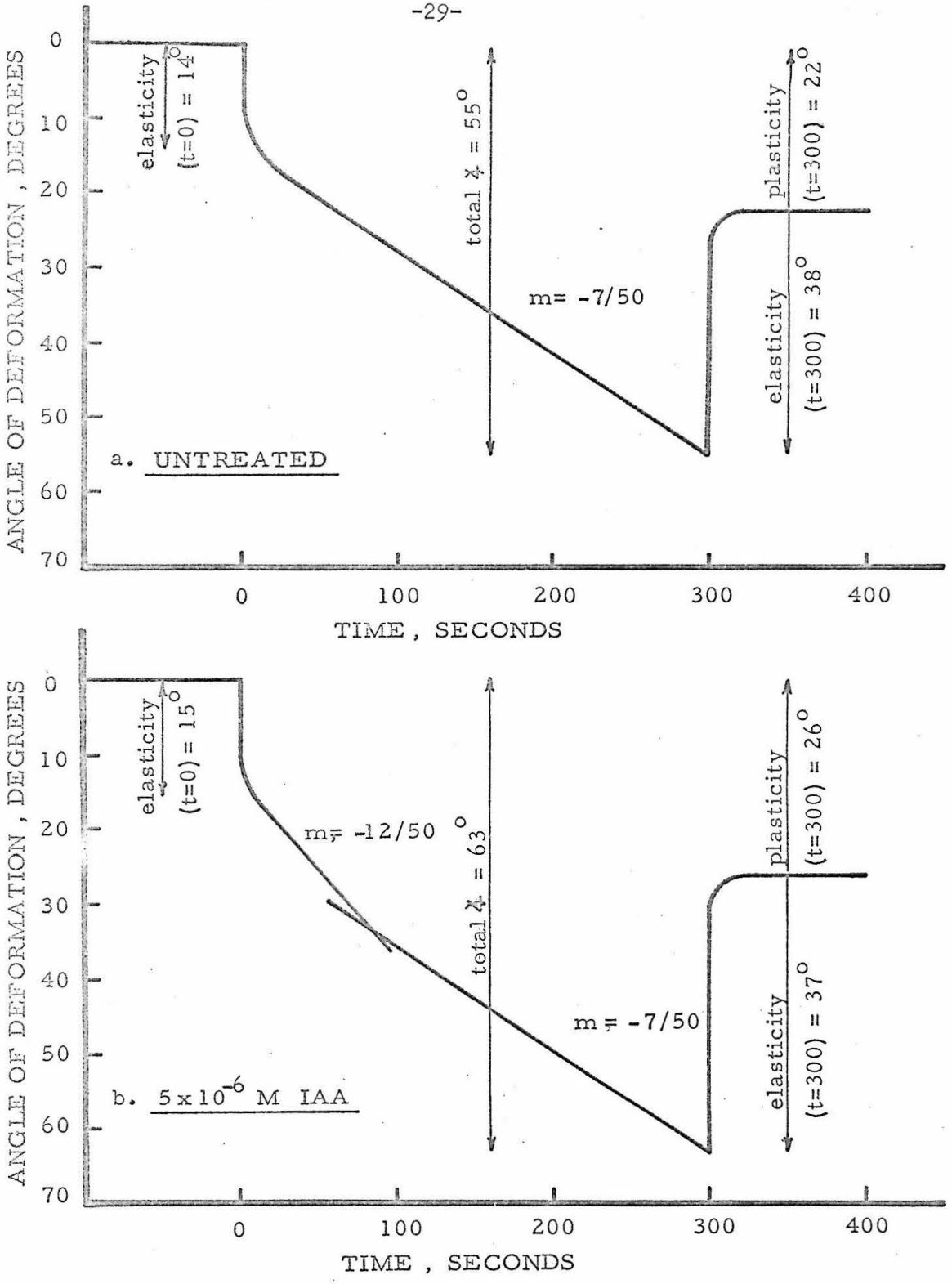


Figure 5. Time course of deformation under load of the elongating zone of maize roots comparing sections cut from IAA-treated and -untreated roots.

two curves is completed well within one of the 10-second time intervals upon which the results are based. The increased initial slope would seem to be specifically an effect of exogenously supplied IAA and is not discernible in the case of untreated roots.

That the IAA-induced deformability is composed of both a plastic and an elastic deformability component is also evidenced by the data of figure 5, with total deformability distributed approximately equally between the two components. This may be a characteristic feature of the deformability of maize root cell walls since the steady-state deformation is also found to consist of both an elastic and a plastic component of nearly equal magnitude. Reversible deformation observed at the end of the experiment is considerably increased as compared to that observed initially. Since recovery of total deformation angle is not complete, the residual angle or plastic deformation remaining after removal of the load increases in a parallel fashion. Since each root must be treated individually in the device used to measure the time course of deformation, the number of roots which could be examined following a short term incubation was severely restricted. The results are based on data from five experiments, each composed of measurements from three roots.

Although the IAA-induced deformation was complete within approximately 80 seconds after application of load, measurements in subsequent experiments were obtained over a period of two minutes as a matter of convenience. The irreversible deformability of sections cut from intact roots previously treated for various times with IAA is shown in figure 6 for two IAA concentrations. Measurements were obtained using the multiple bending apparatus

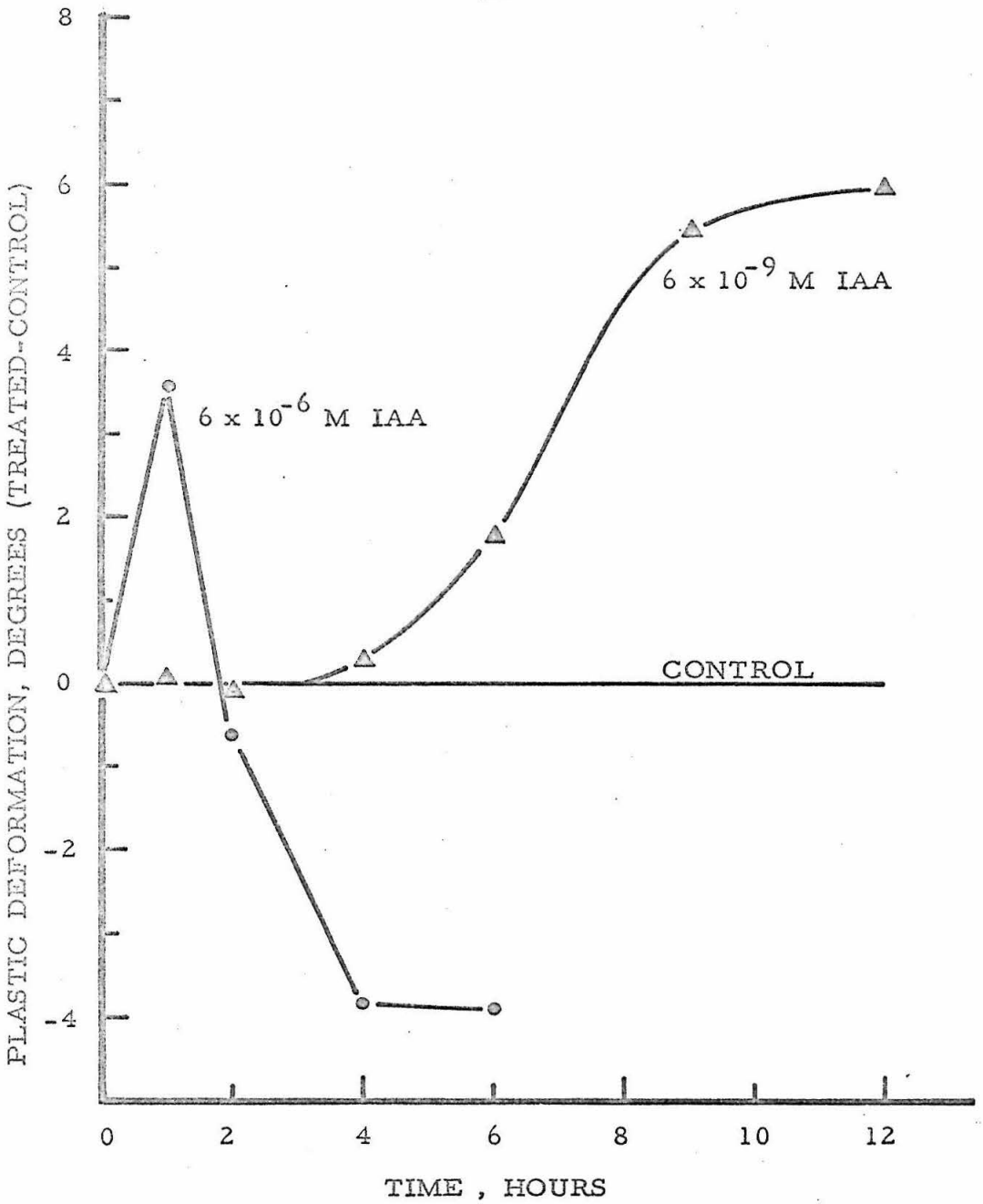


Figure 6. Changes in plastic deformability of the elongation zone of maize roots observed with increasing incubation time in the presence of the IAA concentrations indicated.

shown in figure 1. Root tips were fixed to the support at 15-second intervals and initial curvature was determined. After two minutes, weights were removed and the final curvature was also determined at 15-second intervals. Only the plasticity component (final curvature minus initial curvature) of deformability is reported.

At a concentration of 6×10^{-6} M of IAA, a marked increase in plasticity is observed after treatment for one hour (figure 6). At the end of the second hour of incubation, the plasticity component is no longer observable and with still longer times of treatment, is replaced by a rigidity or negative plasticity with respect to untreated roots. Negative plasticity, therefore, refers to wall stiffening.

By way of contrast, 6×10^{-9} M, a concentration normally considered to be in the range of growth promoting concentrations of IAA for root tissue, resulted in no measurable effect on wall plasticity until after about four hours after the initiation of treatment. Deformability was then observed to increase with time, rising to a plateau between 10 and 12 hours after treatment.

The concentration dependence of the formation of the IAA-induced plasticity component was determined and data for a one-hour pretreatment with varying concentrations of IAA are presented in figure 7. Maximal plasticity after one hour was achieved with IAA concentrations in the range of 2 to 5×10^{-6} M. Concentrations greater than 5×10^{-6} M result in a slightly reduced plasticity, decreasing gradually with increasing concentration. Concentrations greater than 10^{-5} M were not further tested. The concentration necessary to elicit half-maximal

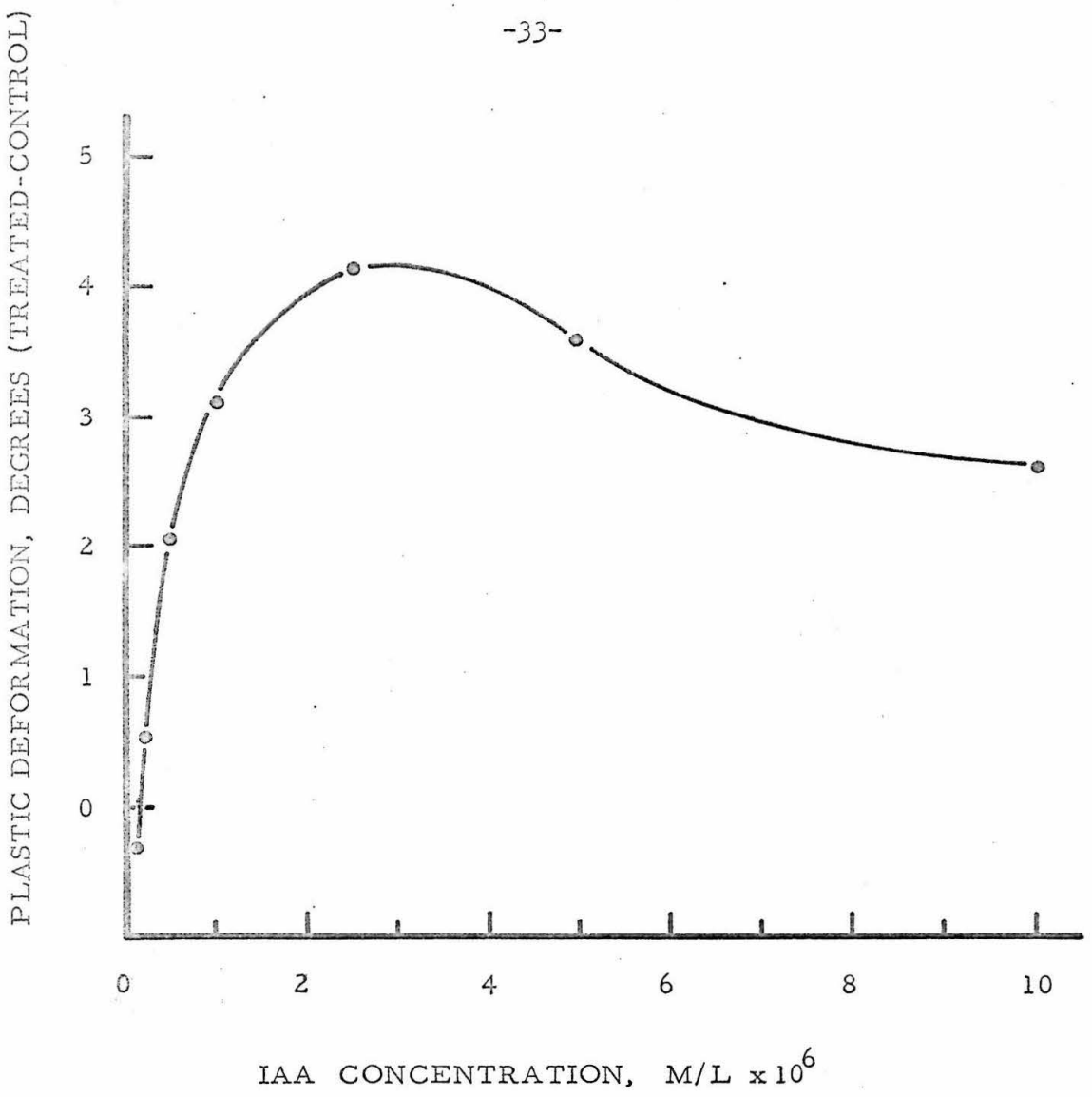


Figure 7. Plastic deformation of the elongating zone of maize roots as a function of IAA concentration. Intact roots were treated with IAA solutions for one hour prior to excision of the zone for deformability measurements. Load applied for two minutes.

plasticity is approximately 5 X 10^{-7} M and the deformabilities resulting from concentrations of IAA of 10^{-8} M or less are not significantly different from the control. Data summarized in figure 7 are based on a total of 1500 individual measurements obtained over a period of several months. The magnitude of the response, although small, was reproducible to within $\pm$ 1° between experiments.

As indicated by the data of figure 6, the plasticity resulting from a one-hour treatment declined rapidly during the second hour and subsequent experiments showed that the decline was observable within 15 minutes after the maximum response at one hour. However, since lower concentrations of IAA became increasingly more effective with longer times of treatment, the overall effect of increasing times of treatment is a shifting of the K_s (concentration for half-maximal response) toward still lower concentrations of indoleacetic acid.

At times of less than one hour, the form of the concentration curve is presumed to be the same, with only the magnitude of the response being reduced. Measurements taken between 30 and 45 minutes after treatment are summarized in table 1. Since the standard deviation based on a large number of seedlings (1500) is of the order of $\pm$ 1°, no significance could be attached to these determinations other than the qualitative fact that a small increase in plasticity was observed.

As discussed initially, the short time response seems to be of greatest significance in terms of a more general mechanism. Therefore, subsequent experiments involving use of IAA were limited to a one-hour pretreat-

TABLE 1.

Plastic deformation of maize roots measured between 30 and 45 minutes after beginning of IAA treatment.

IAA Concentration	Plastic Deformation (Treated - Control)[1]
5×10^{-7} M	1.6 ± 1.3
1×10^{-6} M	1.7 ± 1.2
2.5×10^{-6} M	2.0 ± 1.1
5×10^{-6} M	1.8 ± 1.3
1×10^{-5} M	2.8 ± 0.8

[1] Values are based on 2 experiments composed of 12 to 14 seedlings each. Since treatments were distributed over a period of several hours, a control sample was measured at each concentration tested. Plastic deformations of untreated roots varied between 0 and 2°.

ment with IAA, at the concentration for half-maximal plasticity of 5 X 10^{-7} M.

Effect of ions on the mechanical properties of roots.--Calcium sulfate at a concentration of 10^{-4} M reduces the deformability of roots when supplied during the 12-hour preincubation period (table 2). This effect is partially reversed in the presence of 0.01 M potassium or sodium maleate. When supplied in the absence of calcium, the sodium or potassium buffers do not appreciably influence deformability. Calcium sulfate was effective in reducing deformability over a wide range of concentrations, 10^{-2} M to 10^{-7} M (table 3). Lower concentrations of calcium were not tested.

Evidence for a carbohydrate-containing, cytoplasmic constituent, increased in amount as a result of IAA treatment.--Preliminary experiments on the effects of IAA on the various pectic fractions of roots, revealed the presence of a carbazole-active material soluble in 0.15 M acetate buffer, pH 4.4 and insoluble in 70% ethanol. With excised root sections, this fraction is increased 2- to 3-fold in amount in the presence of 5 X 10^{-7} M IAA (table 4). The response was obtained within the first hour after addition of IAA.

The fraction was found to react with carbazole in a very characteristic manner. The absorption spectra of the carbazole adducts prepared from samples obtained from both IAA treated and untreated tissues are shown in figure 8. The nature of the spectra reveals the presence of secondary hexose maxima and it is not possible to decide from spectral properties alone whether the peak in the vicinity of 535 mμ is hexose or uronic acid

TABLE 2.

Changes in the mechanical properties of maize roots as a result of a 12-hour preincubation in the presence of various salt solutions. Residual angle remaining after a 6 minute deformation.

Salt Solution	Residual Angle, Degrees (Treated - Control)
10^{-4} M $CaSO_4$	-8.4 ± 0.7
10^{-2} M K Maleate	-0.3 ± 1.0
10^{-4} M $CaSO_4$ + 10^{-2} M K Maleate	-1.0 ± 1.5
10^{-2} M Na Maleate	+0.4 ± 0.9
10^{-4} M $CaSO_4$ + 10^{-2} M Na Maleate	-1.5 ± 1.0

TABLE 3.

Influence of a 12-hour preincubation in the presence of varying concentrations of calcium supplied as calcium sulfate on the plastic deformability of the primary root of maize. Residual angle resulting from a 5 minute deformation.

Calcium Sulfate Concentration, M/L	Residual Angle, Degrees (Treated - Control)
10^{-2}	-4.0 ± 1.1
10^{-4}	-4.4 ± 0.2
10^{-5}	-7.1 ± 1.0
10^{-6}	-3.2 ± 0.9
10^{-7}	-3.2 ± 0.1

TABLE 4.

Total 70% ethanol-insoluble carbazole carbohydrate of the pH 4.5-soluble fraction prepared from the elongation zone of maize roots. After incubation for 1 hour in the presence and absence of IAA.

IAA Concentration	Expt.	µg Carbazole Carbohydrate[1]
None	I	50
	II	106
	III	80
	IV	96
	V	88
	Ave	84
5×10^{-7} M	I	270
	II	180
	III	240
	IV	168
	V	176
	Ave	207

[1] Anhydrouronic acid equivalent based on material from three g fresh weight of 5 mm root sections.

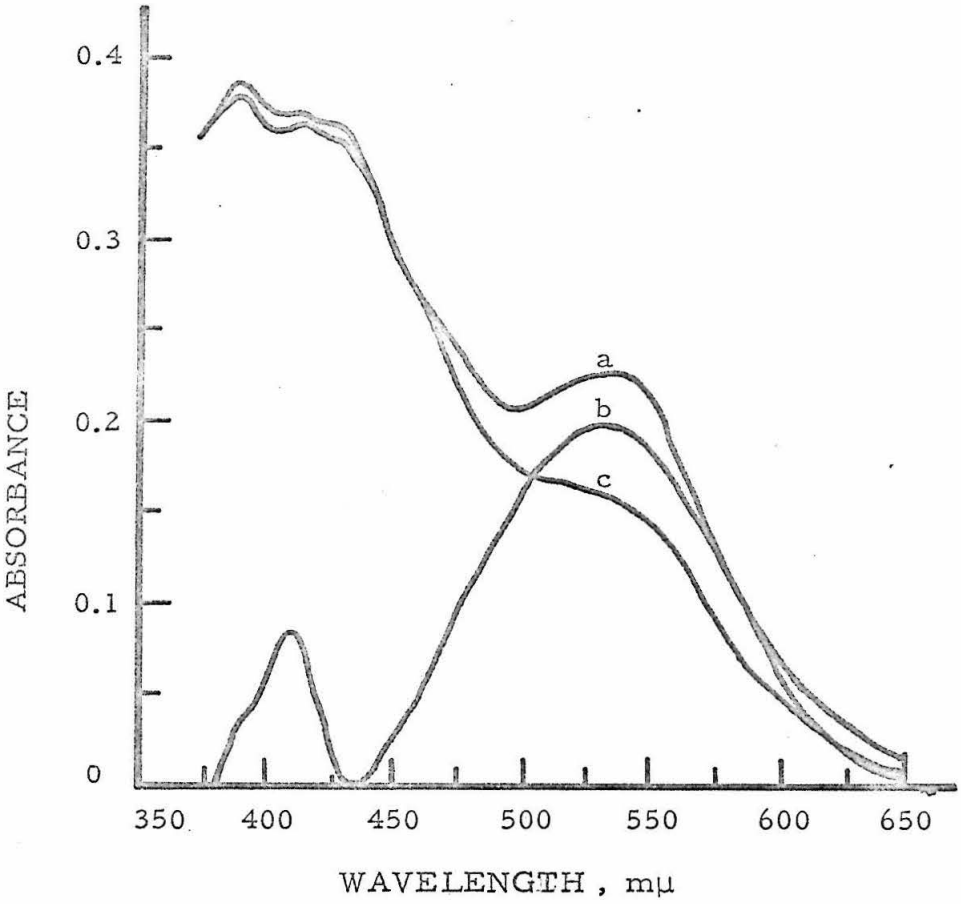


Figure 8. The absorption spectrum of the carbazole adduct prepared from material soluble in 0.15 M acetate, pH 4.4 and insoluble in 70% ethanol of homogenates prepared from 5 mm excised maize root sections. Spectra obtained from IAA-treated (curve a) and -untreated (curve c) preparations and 40 μg per ml of galacturonic acid monohydrate (curve b) are included for comparison. Root sections were IAA-treated for one hour at the concentration of IAA resulting in half-maximal plasticity of 5×10^{-7} M.

derived. The small peak near 395 mμ appears to be characteristic of this type of substance and has not been reproduced by any material thus far subjected to reaction with carbazole in sulfuric acid (Appendix B). Although the IAA-treated samples yield a greater absorbance at the wavelength characteristic of uronic acids (535 mμ), the two curves cross and in the portion of the spectrum attributable to the secondary absorption maxima of hexoses, the extracts from untreated tissue exhibit the strongest absorbance. This fact would suggest a change in composition of the fraction as a result of IAA treatment rather than a net increase in total carbohydrate.

That this may be so is evidenced by the fact that under conditions in which carbazole carbohydrate reflected at least a two-fold increase due to IAA treatment, total hexose as estimated by the carbazole-water reaction was unchanged (table 5). Similarily, anthrone carbohydrate was unchanged by IAA treatment (or slightly decreased in occasional experiments).

A slight turbidity of resuspended 70% ethanol-insoluble precipitates was noted although repeated attempts to sediment the carbazole reacting material by low-speed centrifugation (2,400 rpm, clinical centrifuge) proved unsuccessful. However, since the substance was precipitated in the presence of 7.5% TCA and ammonium sulfate at 50% saturation, the carbohydrate was presumed to be in combination with protein. In addition, a small but reproducable increase in heat-stable protein was consistently associated with the fraction (table 5), raising the possibility of IAA-induced formation of a heat-stable, uronic acid-containing carbohydrate-protein complex.

TABLE 5.

Total carbohydrate and protein content of the cold pH 4.5 buffer-soluble, 70% ethanol-insoluble fraction prepared from maize roots. Sections incubated for 1 hour in the presence and absence of IAA.

	Mg per 3 g Fresh Weight		
IAA Concentration	Anthrone Carbohydrate (as Galactose)	Total Hexose (Carbazole-water Reaction)	Biuret Protein
None	2.0	1.8	1.97
5×10^{-7} M	2.0 (1.95)	1.8	2.30

That some form of growth occurred as a result of IAA treatment is indicated by the increase in fresh weight of the IAA-treated sections during the course of the one-hour incubation in the presence of 5×10^{-7} M IAA (table 6). The increase in fresh weight of 500 5-mm sections (initial weight ca. 3 g) was 163 ± 76 mg, although the precision of the count of sections was probably no better than ± 10 sections or a deviation of ± 60 mg. A parallel increase in weight of dry cell wall was also obtained.

The IAA-induced change in growth rate of root sections.--A uniform lot of roots was carefully chosen and exactly 500 root sections were cut for each time of treatment. Since the time of cutting was a major limitation, duplicate samples were cut with one lot serving as an untreated

TABLE 6.

Increase in total fresh weight of tissue and dry weight of cell wall as a result of treatment for 1 hour with 5×10^{-7} M IAA. Each sample consisted of 500 5-mm root sections weighing ca. 3.0 g.

Treatment	Fresh Weight mg[1]	Weight of Dry Cell Walls, mg[2]
None	3,096	101
5×10^{-7} M IAA	3,259	109
Increase due to IAA	163 ± 76	8 ± 5

[1] An average of six experiments.

[2] An average of four experiments.

control. After 0.5, 1, 2 and 4 hours of incubation in the presence and absence of 5×10^{-7} M IAA, the sections were weighed and the increase in weight due to IAA treatment was determined.

The data shown in figure 9 are expressed in terms of the rate of change of the IAA-induced growth rate in units of mg per hour per 500 root sections where

$$\Delta(\Delta \text{Fr. Wt.}) = \frac{(\text{weight}_{IAA} - \text{weight}_{Cont})t_2 - (\text{weight}_{IAA} - \text{weight}_{Cont})t_1}{(t_2 - t_1)}.$$

The data demonstrate a rapidly increasing IAA-induced increase in fresh weight between 0 and 1 hour after treatment. Between 1 and 2 hours, the difference between the IAA-treated and control already indicates a decreasing IAA-induced growth rate. After 4 hours of incubation, the rate decreased sufficiently so that the difference between IAA-treated and -untreated lots was negative (-40 mg). The final fresh weight weight difference at the end of 12 hours was -173 mg and the overall effect of IAA treatment was, therefore, expressed as a net inhibition of total growth.

Effect of IAA on incorporation of radioactive metabolites.--The incorporation of methionine-$C^{14}H_3$ into the fraction of maize roots soluble in cold acetate buffer, pH 4.4 and insoluble in 70% ethanol was not increased in the presence of 5×10^{-7} M IAA over a period of 1 hour (table 7). This is in contrast to the control experiment conducted with Avena coleoptiles where a small increase in radioactivity was obtained under similar conditions. Incorporation of glucose into this fraction from roots may have been increased slightly by IAA treatment (table 8).

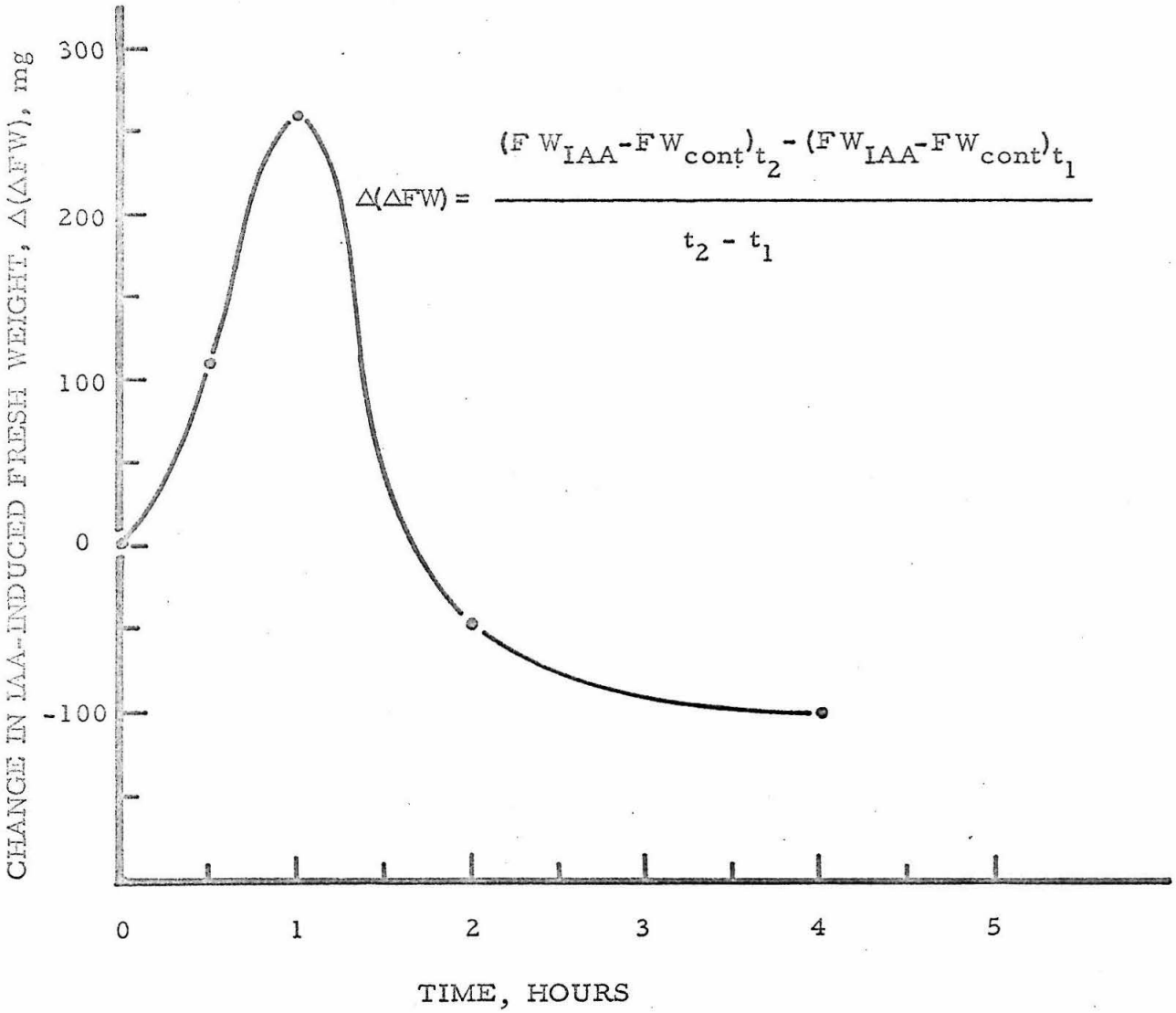


Figure 9. The change in IAA-induced fresh weight of excised maize root sections as a function of incubation time. Basal medium consisted of 0.01 M potassium maleate buffer, pH 4.5. Each determination is based on 500 5 mm sections.

TABLE 7.

Radioactivity of the cold buffer-soluble, 70% ethanol-insoluble fraction prepared from 5 mm subapical sections of maize roots and Avena coleoptiles (3 g fresh weight). Incubated 1 hour with and without IAA in the presence and absence of methionine-$C^{14}H_3$.[1]

Plant Material	IAA Concentration	Expt	Cpm Total	Cpm Lost on Saponification[2]
Maize Roots	None	I	810	100
		II	782	78
	5×10^{-7} M	I	870	73
		II	731	64
Avena Coleoptiles	None	II	1,370	410
	5×10^{-7} M	II	1,600	660

[1] 6×10^5 cpm, 5×10^{-6} M methionine.

[2] 0.5 N NH_3, 24 hours, 24° C.

TABLE 8.

Radioactivity of the pH 4.5 soluble fraction prepared from 5-mm maize root sections incubated with and without IAA for a period of 1 hour in the presence of uniformly labeled glucose C^{-14}.[1]

IAA Concentration	cpm Total	cpm 70% Ethanol-Insoluble
None	20,300	800
5×10^{-7} M	22,260	960

[1] 6×10^{5} total cpm, 4×10^{-5} M with respect to glucose.

Data obtained from incorporation of methionine-$C^{14}H_3$ into the hot water-soluble and hot versene-soluble fractions of the cell wall are summarized in table 9. These data show no effect of IAA on methionine methyl into saponifiable methyl groups under the conditions of the short term incubations. The absence of a fraction from maize roots corresponding to the hot water-soluble pectin fraction of Avena coleoptiles has been verified using both sections cut from the elongating region of roots (table 9) and whole roots. The hot versene-soluble fraction was found to contain a detectable amount of carbazole carbohydrate, which when expressed in terms of anhydrouronic acid (pectin) would account for approximately 0.06% of the dry cell wall. Results of extracting pectic substances from roots using more drastic methods are given as part of Appendix B.

Discussion

According to the scheme proposed for shoots, IAA and other auxins influence cell extension by increasing the plastic properties of the cell wall. Burström (17) has not subscribed entirely to this scheme and, as a result of extensive studies on root elongation, has modified the proposal to include two distinct phases of extension. The first phase consists of a passive stretching of the wall due to decreased wall pressure resulting from increased plasticity. The second phase would involve active formation of new cell wall material. The first phase is characterized by being augmented by auxin and inhibited by coumarin (105). In contrast, the second phase is inhibited by auxin, insensitive to the action of coumarin and promoted by calcium. Similarly, the antiauxin α-(p-chlorophenoxy)iso-butyric acid (PCIB) would also appear to affect the two phases differently,

TABLE 9.

Radioactivity of two cell wall fractions prepared from 100 mg dry cell wall from 5-mm maize root sections. Sections previously incubated with and without IAA for a period of 1 hour in the presence of methionine-$C^{14}H_3$. Fractions correspond to the hot water- and hot versene-soluble, 70% ethanol-insoluble pectin fractions of Avena coleoptiles (2, 46).

HOT WATER-SOLUBLE FRACTION

	Pectin[1]		Methionine-$C^{14}H_3$[2]	
IAA Concentration	µg Total	% of Cell Wall	cpm Total	cpm lost on Saponification[3]
None	10	0.01	20	0
5×10^{-7} M	10	0.01	20	0

HOT VERSENE-SOLUBLE FRACTION

	Pectin[1]		Methionine-$C^{14}H_3$[2]	
IAA Concentration	µg Total	% of Cell Wall	cpm Total	cpm lost on Saponification[3]
None	56	0.06	80	0
5×10^{-7} M	64	0.06	80	0

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1 As anhydrouronic acid determined by reaction with carbazole.

2 6×10^5 total cpm, 5×10^{-6} M methionine.

3 0.5 N NH_3, 24 hours, 24° C.

inhibiting the first phase and stimulating the second (106). Apparently not all antiauxins affect root growth in this manner (94, 107). Hansen (108), for example, has shown that certain auxin analogues which oppose auxin action in shoots result in the same growth responses in roots as obtained with IAA itself, i.e., a growth increase at low concentration and a decrease in growth at high concentration.

The experiments reported in this section have been concerned primarily with the apparent inhibition of the second phase of elongation. Some insight into the nature of this process was initially derived from measurements of the growth rate of IAA-treated and -untreated roots.

The normal time course for cell extension of roots was studied by Brown et. al. (109) but Burström (110) was the first to point out that cell extension does not fit a complete sigmoid curve with the usual initially increasing and subsequently decreasing rate of elongation. Although initially increasing over a period of several hours, growth stops abruptly.

The results of experiments where roots were grown in the presence of growth-inhibitory concentrations of IAA confirm those previously reported by Burström (105), in that the primary effect contributing to an overall inhibition of root growth is a shortening of the period during which the root elongates. Growth rate may also be reduced by IAA if the concentration is sufficiently high. However, at low concentrations, what superficially appears as a reduced rate of elongation is a more rapid termination of cell extension. Experimentation has not progressed sufficiently to provide a basis for the interpretation of these results, although one might intuitively anticipate that the factors which become abruptly limiting

to cell extension during normal root growth, become abruptly limiting sooner following IAA treatment.

That the growth regulating effect of indoleacetic acid is associated with changes in resistance of the cell wall was first suggested by the experiments of Heyn and those of Söding, cf. Heyn (1). The mechanical analysis of this problem has involved bending (18, 20, 111), stretching (31, 111) and plasmolytic methods (cf. 112). In the case of experiments involving bending methods, auxin has been reported to increase plastic extensibility of stems and shoots and to have no effect on elastic extensibility (18, 20). However, the majority of results obtained by plasmolytic methods indicate that elastic extension may also be increased following auxin treatment (112). Preston and Hepton (31) have reached similar conclusions using stretching techniques.

Roots do not grow uniformly throughout their length and, therefore, only the deformability properties of the zone of cell extension were measured. In the case of normally growing roots, this zone was found to bend rapidly after application of load. The initial deformation is reversible and provides a measure of initial elasticity. Initial deformation is then followed by a steady-state deformation which in Avena coleoptiles is primarily irreversible and, by definition, plastic deformation (18). With maize roots, however, this steady-state deformation was shown to be comprised of both an elastic and a plastic component of approximately equal magnitude.

That root cell walls normally contain both a plastic and an elastic component is also implicit in the discussions of Burström (17, 113),

Amlong (114) and more recently that of Odhnoff (115). Epidermal cells of wheat roots, studied by Burström, were found to attain a final length of about 300 μ. Plastic stretching of the cell walls could account for only part of the total extension and appeared only when elongation was reduced to below 200 μ. Burström (17) concluded that plastic stretching can account for extension up to this limit and no more. Although Heyn (1) and Pohl (116) conclude that elastic extensibility of the wall is not a factor in elongation but rather the result of actual elongation, it has been suggested (113, 116, 117) that elastic extension may also be converted into irreversible elongation. The manner in which this might be accomplished is unknown, although insertion of new microfibrils and cross-linking of existing microfibrils could conceivably stabilize the cell wall irreversibly following osmotically-induced extension. Odhnoff considers the values reported for plasticity of bean roots as being uncertain and prefers to represent the sum of elastic and plastic extension as total extensibility. In the present experiments, an attempt was made to separate possible plastic and elastic components of IAA-treated roots using conventional bending techniques.

Short-term incubations of roots in the presence of IAA demonstrate a change in both plastic and elastic deformability within the first hour after treatment. The IAA-induced increase in deformability was found to be a transient response that may involve only a portion of the total cell wall. This is evidenced from the fact that the difference in deformability between IAA-treated and -untreated roots is entirely due to a very rapid stretching which occurs during the first 80 seconds after application of load. Then, within ten seconds, the deformation curve assumes the slope

of the plastic-elastic steady-state curve characteristic of untreated roots.

The meaning of this phenomenon is terms of a physical model cannot be critically evaluated without additional information regarding the specific components affected. However, a change in properties of the cell wall is implicit in these observations. The model which would seem intuitively most correct is the IAA-induced formation of a readily extendible component which is interdispersed in a constant-bending, plastic-elastic matrix. Since the readily extendible component is absent or at least very much reduced in the absence of growth substance, these findings provide additional evidence for cell wall components either specifically synthesized or somehow altered in the presence of IAA. A dissolution of existing cell wall, which would presumably have the same effect by reducing the number of restraining cross linkages between wall components, seems inadequate in view of additional changes which occur during and following the initial surge of growth promotion. A theory of cell wall softening based on dissolution of the cell wall was considered by Matchett and Nance (53) in the case of pea stem sections. The results, however, are inconclusive (118).

Regarding the changes which follow wall plasticization, the situation almost immediately becomes complicated by a cell wall stiffening and termination of cell extension. However, the plasticizing effect is associated with a temporary increase in fresh weight of excised root sections. Plasticity reaches a maximum after about one hour following initiation of IAA-treatment, corresponding to the observed maximum in IAA-induced increase in fresh weight. An increase in length of roots

in the presence of high auxin concentrations has not been demonstrated as a regular and reproducable phenomenon even in the case of intact roots (cf. 95). Therefore, the fresh weight increases may result in part from some form of radial enlargement. An advanced symptom of IAA-inhibition of root growth is the characteristic swellings of the cortical cells in the zone of cell extension. These enlargements are believed to result from isodiametric or even primarily radial enlargement as opposed to the normal polar extension (95).

Although there is no apparent reason why the mechanism of cell extension of roots should differ in any fundamental way from that of shoots, inhibition of growth has been suggested to reflect the manner of organization of new cell wall material deposited after stretching cf. Åberg (95). However, it would seem equally possible that growth inhibition results from the accelerated termination of a series of processes involving perhaps even normal changes within the cell wall. In effect, the root cell might be considered to complete its entire biochemical "life cycle" with sufficient rapidity that the passive processes of cell extention are unable to keep pace. This is another way of saying that roots are more sensitive to IAA than shoots.

That cell wall synthesis may be accelerated during IAA-induced growth promotion of root sections is indicated from the comparison of the quantity of dry cell wall of IAA-treated and -untreated root sections. The increase following a 1-hour treatment with the IAA concentration

necessary to elicit half-maximal plasticity is even somewhat greater than that which would have been predicted on the basis of the increase in fresh weight.

The results, therefore, suggest that IAA-treatment of roots initiates a stretching of the cell wall in much the same manner as in shoots. In fact, the concentration of IAA necessary to evoke a half-maximal plasticity response after one hour, i.e., 5×10^{-7} M corresponds to that reported for steady-state growth of Avena coleoptiles (68) of 3.6×10^{-7} M. The deformability measurements, although indicative of a similarity between IAA-action in both roots and stems, offer no definite evidence that IAA normally limits root cell extension and, in fact, would appear to offer evidence to the contrary.

The ability of roots to respond to very low concentrations of IAA in the form of a delayed increase in cell wall plasticity was also noted. This phenomenon, similar to that reported by Amlong (114), has not been studied in detail but may be related to a continued accumulation of IAA by means of active uptake (119). Larsen (98) has observed that increased growth resulting from treatment with low concentrations of auxin, 10^{-8} to 10^{-13} M, is usually observed only after several hours, normally 17 hours or more. Roots are also apparently able to adapt in some manner to an excess of externally supplied auxin, since the inhibition of root elongation by both IAA (120, 121) and 2,4-D (120) has been shown to decrease with time of treatment.

Roots grown in the presence of a wide concentration of calcium sulfate (10^{-7} to 10^{-2} M) were characterized by decreased deformability. The calcium-induced stiffening was reversed by addition of potassium buffers which do not affect deformability in the absence of added calcium. Similar ionic interactions have been observed in the case of Avena coleoptiles (18, 30). It has been suggested that growth inhibition by divalent ions results from a reduced wall plasticity due to increased ionic binding between pectic carboxyl groups (2, 16, 18, 24, 56).

Ordin et al. (44, 45) supplied methionine-$C^{14}H_3$ to Avena coleoptiles and found that IAA increased the rate of incorporation of C^{14} into methyl esters of the pectic substances soluble in hot water. The nature of this effect was later modified by Jansen et al. (46) to include the possibility of increased turnover rather than net synthesis of methyl ester. Cleland (59) was unable to demonstrate that IAA stimulated incorporation of C^{14} from methyl-labeled methionine into pectic substances of corn mesocotyls and proposed that either IAA-induced methylation of pectic substances is not a general phenomenon or that different methyl donors are used in mesocotyl tissue.

Although providing little support for IAA-induced methylation of pectic substances, experiments with roots have not been conclusive. The classical hot water-soluble pectin fraction appears to be absent (less than 0.01% of the total cell wall) in maize roots and, therefore, an IAA-increased labeling of this fraction in the presence of added methionine-$C^{14}H_3$ would not be expected. Hot versene extraction yielded a small amount of ethanol-insoluble, carbazole-active material which, when converted to anhydrouronic acid, accounts for 0.06% of the cell wall.

The corresponding fraction prepared from Avena coleoptiles yields anhydrouronic acid equivalent to ca. 0.4% of the cell wall (Appendix B).

Similar difficulty in extracting maize root-pectins was reported by Keller and Deuel (122). The usual methods of pectin extraction, i.e., dilute hydrochloric acid, sodium polyphosphate and versene extracted only a small amount of pectin from roots, even after decomposition with dilute sodium hydroxide at room temperature. Extraction with 0.5% ammonium oxalate was effective. As reported in Appendix B, extraction of roots with alkaline versene followed by prolonged digestion with the enzyme pectinase did release quantities of carbazole-active materials comparable to those obtained from other tissues. It is of interest to note that Keller and Deuel (122) also report that graminaceous roots (maize and wheat) were found to contain less pectin and less free carboxyl groups than dicotyledenous roots (bean, tomato and tobacco). The degree of esterification of root pectins was found to be relatively small in all species examined and in tobacco roots, the pectic acid was unesterified.

More recently, Albersheim and Bonner (123) and Jansen et al. (46), using labeled glucose and methyl-labeled methionine, have shown that IAA increased the rate of synthesis of a fraction soluble in cold acetate buffer which was suggested to represent a pectin fraction soluble in cold water. In the case of roots there appeared to be no increased labeling of this fraction when the tissue was supplied with methionine-$C^{14}H_3$. A parallel experiment conducted with Avena coleoptiles did reflect an IAA-induced increase in the portion of the radioactivity lost upon saponification.

Further investigation of the cold-buffer soluble, 70% ethanol-insoluble fraction of maize roots revealed that the portion of this fraction reacting with carbazole in 87% sulfuric acid was increased in amount following treatment with IAA. Treatment for one hour with the concentration of IAA necessary to elicit half-maximal plasticity, i.e., 5×10^{-7} M IAA, resulted in a 2- to 3-fold increase in the carbazole carbohydrate content of this fraction as compared to root sections incubated in buffer for the same period of time. An analysis of the absorption spectrum of the chromogen formed after reaction with carbazole revealed the presence of interfering levels of hexose. However, the hexose content of the fraction appeared to be largely unaffected or perhaps even slightly reduced in amount following IAA treatment. These results could suggest a change in composition of the fraction rather than an overall increase in total carbohydrate. In addition to carbohydrate, presumably containing uronic acid residues, the fraction was also characterized by a small quantity of heat-stable protein also increased in amount following IAA treatment. Since the carbohydrate containing moiety was precipitated in the presence of 7.5% TCA and ammonium sulfate at 50% of saturation, the carbohydrate was presumed to be in combination with protein, raising the possibility of the IAA-induced formation of a heat-stable, uronic acid-containing carbohydrate-protein complex.

Considering a possible relationship between the increased amount of this material and changes in cell wall deformability, subsequent investigations relating to the biochemistry of cell extension have been primarily concerned with isolation of a similar fraction from stems and shoots. These results, as well as further discussion relating to characterization and cellular localization are presented as parts III and IV.

Conclusions

1. The primary response contributing to an overall inhibition of root growth by IAA is a concentration dependent shortening of the period over which the roots elongate.

2. Deformation of root cell walls under constant, externally imposed load is composed of a plastic and an elastic component. These components are separable under the usual conditions of mechanical analysis.

3. Treatment of intact roots for one hour in the presence of concentrations of IAA that normally promote shoot growth and, therefore, inhibit root growth, results in formation of a concentration dependent, IAA-induced plasticity component, not observable in untreated roots. The change in deformability may be indicative of specific wall components either synthesized or altered in the presence of IAA.

4. Half-maximal plasticity is induced by ca. 5×10^{-7} M of IAA, a concentration comparable to that for Avena coleoptiles.

5. Deformability induced by IAA-treatment is transient, reaching a maximum after approximately one hour.

6. That increased deformability may be associated with increased root growth is indicated by measurements of fresh weight changes of excised root sections in the presence and absence of IAA. The IAA-induced increase in fresh weight is also transient with a maximum after approximately one hour. The overall effect of IAA-treatment of root sections is to reduce growth.

7. Ionic regulation of cell wall deformability is found to be similar to that reported for stem tissues. Addition of divalent calcium ions results in cell wall stiffening, an effect which is reversed in the presence of an excess of monovalent sodium or potassium ions.

8. Incorporation of C^{14} from methionine-$C^{14}H_3$ into saponifiable methyl groups of root cell wall fractions soluble in hot water and fractions insoluble in hot water but soluble in hot versene are unaffected by IAA treatment. However, only a small portion of the total pectic substances of the root cell wall are extracted under these conditions.

9. Associated with increased plasticity is an increased amount of a carbohydrate-containing fraction soluble in 0.15 M sodium acetate, pH 4.4 and precipitable in the presence of 70% ethanol, 7.5% TCA and ammonium sulfate at 50% of saturation. The fraction reacts with carbazole in a very characteristic manner. Absorbance measured at wavelengths corresponding to the absorption maximum for uronic acids suggests a 2- to 3-fold increase in amount as a result of IAA-treatment.

10. The pH 4.4-soluble, 70% ethanol-insoluble carbohydrate is associated with a small quantity of heat-stable protein. This protein is also increased in amount as a result of IAA treatment.

11. The possibility of an IAA-induced formation of a heat-stable, uronic acid-containing, carbohydrate-protein complex, therefore, is suggested.

III. A SPECIFIC CYTOPLASMIC FRACTION REFLECTING THE IAA-INDUCED INCREASE IN GROWTH RATE

A. PRELIMINARY ISOLATION AND CHARACTERIZATION

In the preceeding section, evidence was presented for the rapid formation or continued maintenance of a readily solubilized component of the cell associated with the IAA-induced increase in growth rate of maize root sections. This component was presumed to contain carbohydrate as an integral part of the molecule and to be bound to cytoplasmic protein. Since the elongating zone of roots is restricted to a region of only a few millimeters in length, a more abundant source of material was sought before further characterization was attempted.

The general procedure followed was to isolate protein-complexed carbohydrate by a variety of simple preparative techniques and from tissues grown in the presence or absence of IAA. A number of different tissues were examined including Avena coleoptiles, intact maize roots, pea stems, pea embryo axes and mung bean hypocotyls. Of these tissues, Avena coleoptiles were the first to provide additional evidence for the presence of a protein-bound cytoplasmic fraction containing a carbohydrate moiety and with the additional property of being increased in amount by treatment with IAA.

Materials and Methods

Plant material.--Oat seeds of the variety Siegeshafer were soaked in distilled water with continuous aeration for a period of four hours. Germination was continued in vermiculite, moistened with distilled water and contained in stainless steel trays. The seedlings were grown at a temperature of 25° C and in low intensity red light for a period of approximately 96 hours. At this time the coleoptiles had attained an average length of 2.5 to 3.0 cm. Intact coleoptiles were harvested by cutting them just below the coleoptilar node. Fresh weight was determined and any plant material not used immediately was quick frozen and stored in a freezer. Procedures for obtaining other plant materials are given elsewhere.

Experiments involving a comparison of Avena tissue grown in the presence and absence of IAA were conducted using 13 mm sections cut 3 mm from the apex of the coleoptile. Primary leaves were not removed. Sections to be incubated were floated in 50 ml of 0.0025 M potassium maleate buffer, pH 4.5 contained in 20 X 150 mm petri plates. Incubations were for periods of five to eight hours and the indoleacetic acid concentration employed was 5×10^{-6} M. All operations prior to grinding were conducted in low intensity red light. Pea stem sections were incubated in a similar manner.

Preparation of pH 4.4-soluble extracts.--Fresh or frozen coleoptile tissue was ground with a pre-chilled mortar and pestle in the presence of 0.15 M sodium acetate buffer, pH 4.4 in a ration of one gram of tissue to four millimeter of buffer. Cell walls, etc. were removed by filtration through miracloth and the pH 4.4-insoluble cytoplasmic proteins removed by centrifugation for 30 minutes at 13,000 X g. All operations were conducted at 0 to 4° C.

Ammonium sulfate fractionation.--In preliminary isolation procedures, the pH 4.4-soluble fraction remaining after centrifugation was further treated by addition of sufficient solid ammonium sulfate to provide a final concentration of 50% of saturation. Ammonium sulfate additions were based on the tabular data of Green and Hughes (124). The solid ammonium sulfate was added slowly with continuous stirring until solution was complete. The solutions were equilibrated for an additional 20 to 30 minutes at 0° C and the precipitates then collected by a 20-minute centrifugation at 8,000 X g in the cold. The supernatent was discarded and the pellet resuspended in acetate buffer. Any insoluble material was removed by further centrifugation. Further purification was achieved by dialysis against 0.15 M sodium acetate buffer, pH 4.4 or distilled water. During dialysis an insoluble precipitate formed which could be collected by centrifugation at 27,000 X g for 30 minutes. The precipitate began to form during the first few hours but dialysis was normally continued for 12 hours against three changes of medium.

The procedure was later modified to include only the fraction precipitating at ammonium sulfate concentrations between 0 and 30% of saturation. This fraction was found to be largely soluble in chloroform-methanol (2:1). Chloroform-methanol-insoluble material (including protein) was removed by centrifugation or by filtration through fine sintered glass.

Preparation of soluble protein from pea stems and maize roots.--Sections were ground in a pre-chilled mortar and pestle in two volumes of 0.2 M sodium acetate, pH 5.4 in the case of maize roots and in 0.5 M sucrose containing 0.001 M EDTA in the case of pea stems. The extracts

obtained from peas were then centrifuged for one hour at 24,000 X g. Root extracts were filtered through fine sintered glass.

A simple isolation procedure for protein-bound glycolipide from pea stems and maize roots based on alkali insolubility.--Aliquots of the 24,000 X g supernatent from peas were transferred to 18 X 32 mm heavy wall centrifuge tubes and boiled for two minutes over a 100° C water bath to denature protein. The tubes were then cooled for ten minutes in an ice bath during which time a heavy coagulum formed. A readily suspensible pellet was obtained by centrifugation, accelerating to 27,000 X g over a period of 3 to 5 minutes. The denatured protein and associated materials, so obtained, were resuspended in 1 N sodium hydroxide. Addition of a small amount of biuret reagent served to assist in the suspension of protein. Root extracts were treated in a similar fashion.

During suspension the solutions became turbid. As soon as suspension was complete, the insoluble material was collected by centrifugation for 15 minutes using a clinical centrifuge (ca. 2,400 rpm). Absence of turbidity in the supernatent after centrifugation was taken to indicate the quantitative recovery of the alkali-insoluble material. The precipitate was next acidified to pH 2 with hydrochloric acid or to pH 4.4 with a small amount of sodium acetate buffer and the fraction was further purified by precipitation either from 95% ethanol or by addition of several volumes of acetone. After several hours in the cold, the precipitates were collected by centrifugation and subjected to further analysis.

As an alternative procedure, crude extracts were treated by addition of an equal volume of 1 M TCA followed by equilibration at 0° C for two to

three hours. Insoluble materials, including protein, were collected by centrifugation and the alkali-insoluble fraction isolated as described above.

Estimation of protein.--Routine protein determinations were conducted according to the standard biuret method (103). The test was calibrated with bovine serum albumin (Nutritional Biochemicals Corporation), a sample of crystalline ribonuclease (Armour) and purified Avena protein (pH 4.4-insoluble fraction). The values obtained with ribonuclease and Avena protein agreed within 5% and further calibrations were based on the ribonuclease preparation.

Total TCA-insoluble protein was obtained by treatment of the extracts with an equal volume of 1 M aqueous trichloroacetic acid (TCA) in the cold for several hours. The insoluble material was collected by centrifugation and resuspended in water. This procedure was repeated a total of four times. The final resuspended pellet was dialyzed 24 hours at 0° C against three changes of distilled water. After dialysis, the solution was lyophylized and stored in a desiccator over sulfuric acid prior to weighing. A similar procedure was followed in obtaining the Avena protein preparation used in calibration of the biuret reaction.

Determination of carbohydrates.--The general procedures followed are summarized in the article by Dische (125).

a) Total carbazole carbohydrate.--The general procedures and limitations of various modifications of the carbazole reaction are discussed as part of Appendix B. Data is reported in terms of galacturonic equivalent

as determined from parallel determinations using known concentrations of galacturonic acid monohydrate.

b) Reactions for determination of hexose.--Hexose determined by reaction with anthrone is reported in terms of glucose equivalent. The procedure followed is that of Scott and Melvin (104). The extinction coefficient of galactose is about 0.5 of that of glucose. That of mannose is reported to be lower (125), while fructose reacts as glucose. Although glycogen, dextran and cellulose behave like equivalent amounts of glucose, even without prior hydrolysis, anomalous results were occasionally obtained with crude extracts. In the case of side reactions producing yellow and brown colors, the hexose specific contribution to the absorption was determined from the entire absorption spectrum obtained with the Cary recording spectrophotometer.

As a general qualitative test for the presence of interfering levels of hexose, the carbazole-water reaction (126) was used routinely in combination with the reaction of carbohydrate with carbazole in 87% sulfuric acid (127). This reaction was later quantitated and the details of the procedure are included as part of Appendix B.

Estimation of phosphate.--Total phosphate was determined by the method of Allen (128) following oxidation in the presence of perchloric acid and 30% hydrogen peroxide. Inorganic phosphate was determined directly by the procedure of Sumner (129).

Incorporation of glucose-U-C^{14}.--Fifteen grams of 13 mm Avena coleoptile sections were incubated for eight hours in the presence of 50 µc (specific activity of 30 µc per µM)

of glucose-U-C^{14} (Calbiochem) with and without IAA. The sections were incubated in 15 ml of 0.0025 M potassium maleate under low intensity red light. The concentration of IAA used was 7.4×10^{-7} M. Radioactivities were measured with a Packard Tri-Carb liquid scintillation spectrometer system. For this purpose, 0.1 ml aliquots for counting were mixed with 10 ml of a solution containing 3.5 grams of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxazoly)-benzene (Dimethyl POPOP) in 500 ml of toluene made to one liter with absolute ethanol. PPO and dimethyl POPOP were scintillation grade obtained from the Packard Instrument Company, La Grange, Illinois.

Results

Isolation from Avena coleoptiles by ammonium sulfate fractionation.--The ammonium sulfate fractionation pattern of untreated Avena coleoptiles is shown in figure 10. A similar pattern was obtained from five subsequent experiments in which IAA-treated and -untreated tissues were compared. A further addition of ammonium sulfate between 75 and 90% of saturation normally resulted in the precipitation of a fraction high in carbohydrate but of low protein content. Since the bulk of the carbazole-active carbohydrate of Avena coleoptiles is precipitated between 0 and 50% of saturation of ammonium sulfate, this fraction was chosen for further study in preliminary experiments.

The initial choice of ammonium sulfate fractionation as a method for the isolation of the IAA-induced cytoplasmic fraction was based on the observation that the carbazole carbohydrate distribution between various protein fractions is markedly different following IAA treatment of the

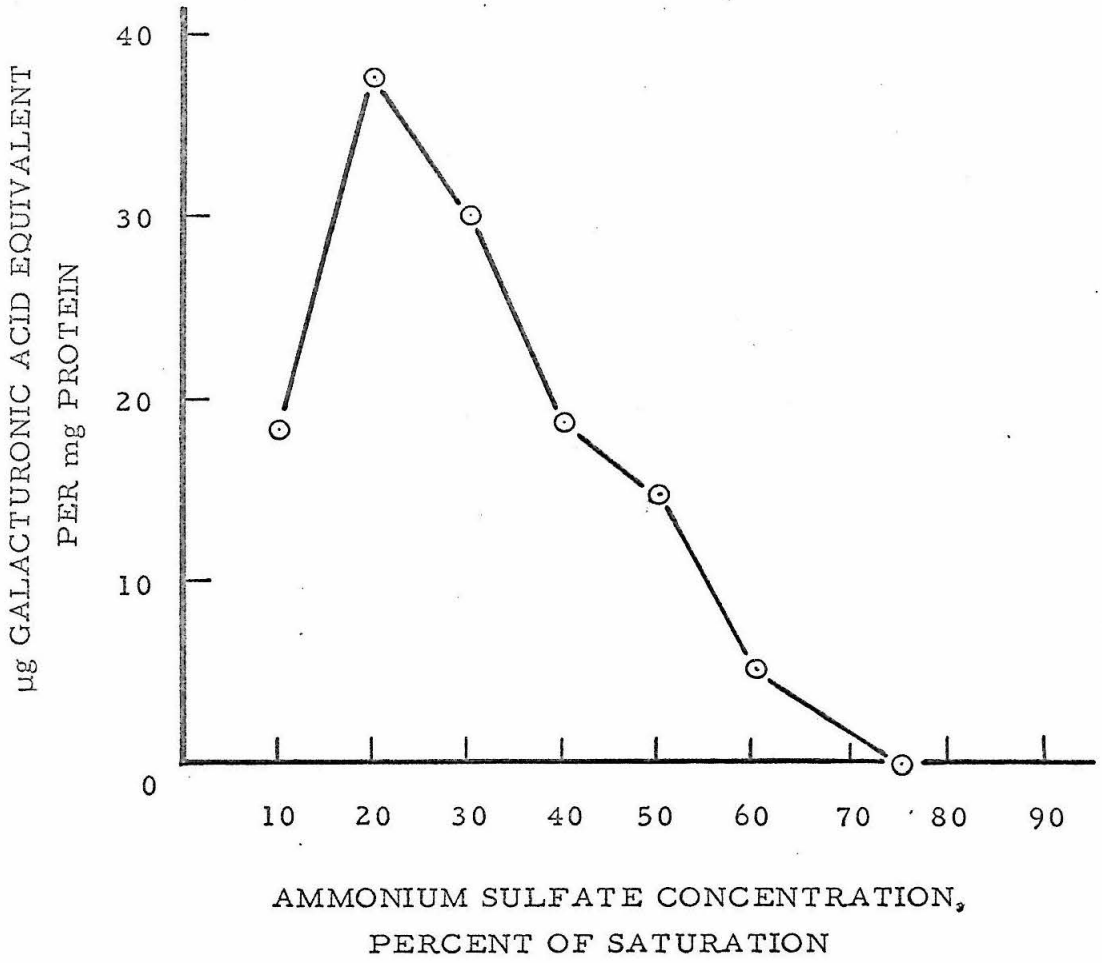


Figure 10. The ammonium sulfate fractionation pattern of carbohydrate associated with protein of the pH 4.4-soluble fraction of Avena coleoptiles. Distribution is expressed in terms of μg galacturonic acid equivalent per mg of biuret protein.

tissue. These differences are obtained under conditions where protein distribution is unchanged (figure 11). An extensive study of fractionation patterns from pea stem sections, mung bean hypocotyl sections and Avena coleoptiles proved these differences reproducable only in a qualitative sense. The presence of substances interfering in the carbazole reaction appeared to contribute to the lack of reproducability and other means of study were sought. The ammonium sulfate fraction pattern of a later experiment is shown in figure 12. These data were obtained with sections previously incubated for 8 hours in the presence of 50 µc of uniformly labeled glucose-U-C^{14}.

In order to establish that the carbohydrate was bound in the form of a relatively stable complex with protein, a fraction was dialyzed against sodium acetate buffer, pH 4.4. The total amounts of both carbazole carbohydrate and biuret-active material were reduced as a result of dialysis. However, the ratios of the two substances remained constant. These data are presented in table 10. Further use of dialysis as a means of purification was temporarily curtailed by the fact that an insoluble precipitate formed during dialysis. Precipitation occurred with equal rapidity with solutions resuspended in buffer and stored at 0° C for the same period of time. Upon exposure to air, the fraction assumed a brick-reddish color. In addition, the properties of the fraction are markedly altered by exposure to extremes in pH which results in the fraction becoming insoluble in all aqueous solvents thus far tested. Turbidity of aqueous suspensions is increased by the addition of alkali. However, after dialysis of crude ammonium sulfate precipitates, resuspension in alkali provided a sufficiently stable mixture to permit removal of uniform samples by pipetting. A

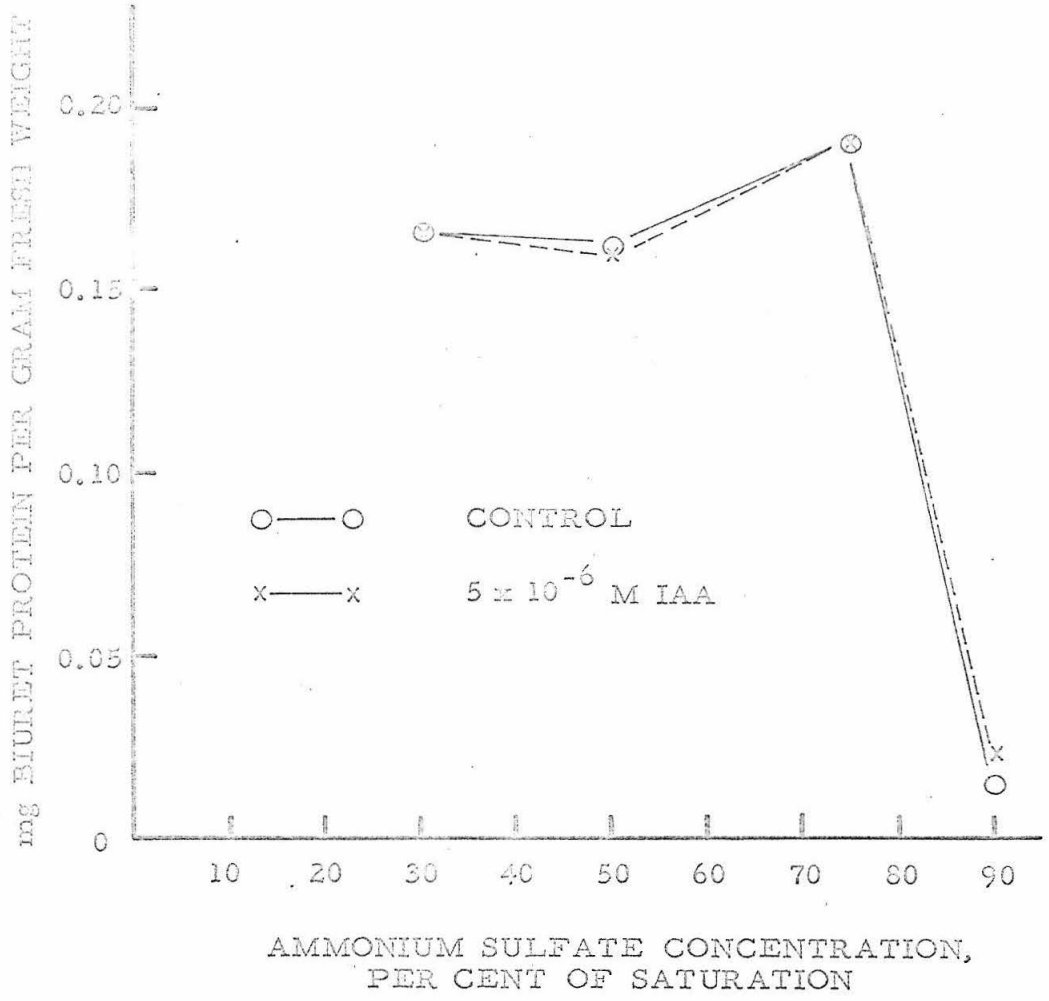


Figure 11. Distribution of protein of the pH 4.4-soluble fraction of Avena coleoptiles as a function of ammonium sulfate concentration comparing preparations obtained from IAA-treated and -untreated 13 mm coleoptile sections.

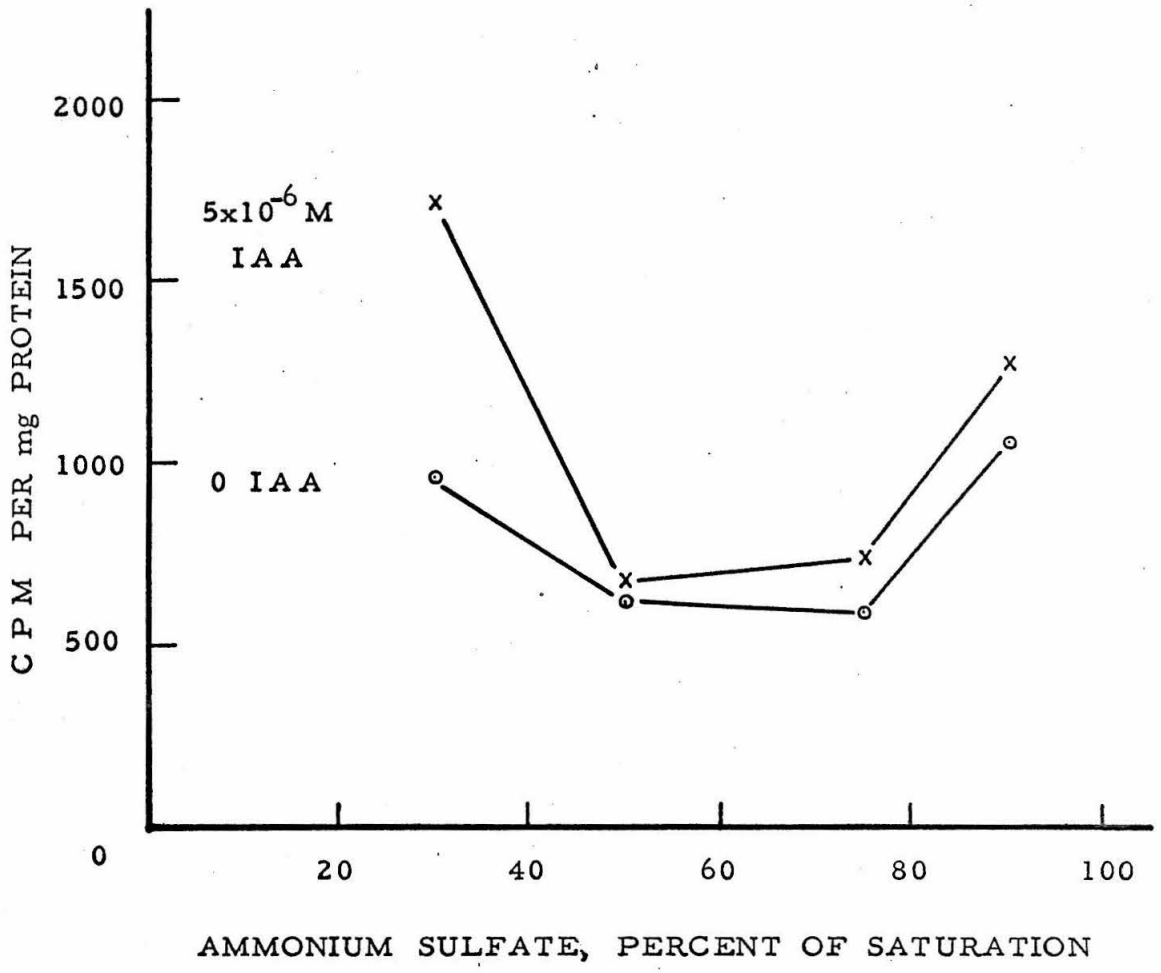


Figure 12. Distribution of radioactivity from glucose-C^{14} among the various pH 4.4-soluble protein fractions of Avena coleoptiles obtained by ammonium sulfate fractionation. A total of 20 g of 13 mm sections were incubated for 8 hours in the presence of 50 µc of uniformly labeled glucose-C^{14} with or without IAA. The fraction precipitated between 0 and 30% of saturation of ammonium sulfate is most influenced by IAA treatment.

TABLE 10.

Total carbazole carbohydrate and biuret protein precipitated between 0 and 50% of saturation of ammonium sulfate from the pH 4.4-soluble fraction from 50 g fresh weight of Avena coleoptiles. Results obtained before and after dialysis for 12 hours against 0.15 M sodium acetate buffer, pH 4.4 are given. Extracts were centrifuged for 30 minutes at 37,000 X g prior to dialysis in order to sediment material not resolubilized following ammonium sulfate precipitation.

Fraction	Mg Biuret Protein	µg Carbazole Carbohydrate as Galacturonic Acid	
		Total	Per mg Protein
Before Dialysis	4.0	336	84
After Dialysis	3.4	288	85
% Recovered	85	86	--

typical absorption spectrum in the ultraviolet region of such an alkaline suspension is shown in figure 13. Other than the turbidity contribution, the spectrum is typically that of protein with a shoulder at 292 mµ, in addition to the maximum at 282 mµ (130). The 292 mµ shoulder is known to be a characteristic of the alkali shift of tyrosine and tryptophan-containing residues (130).

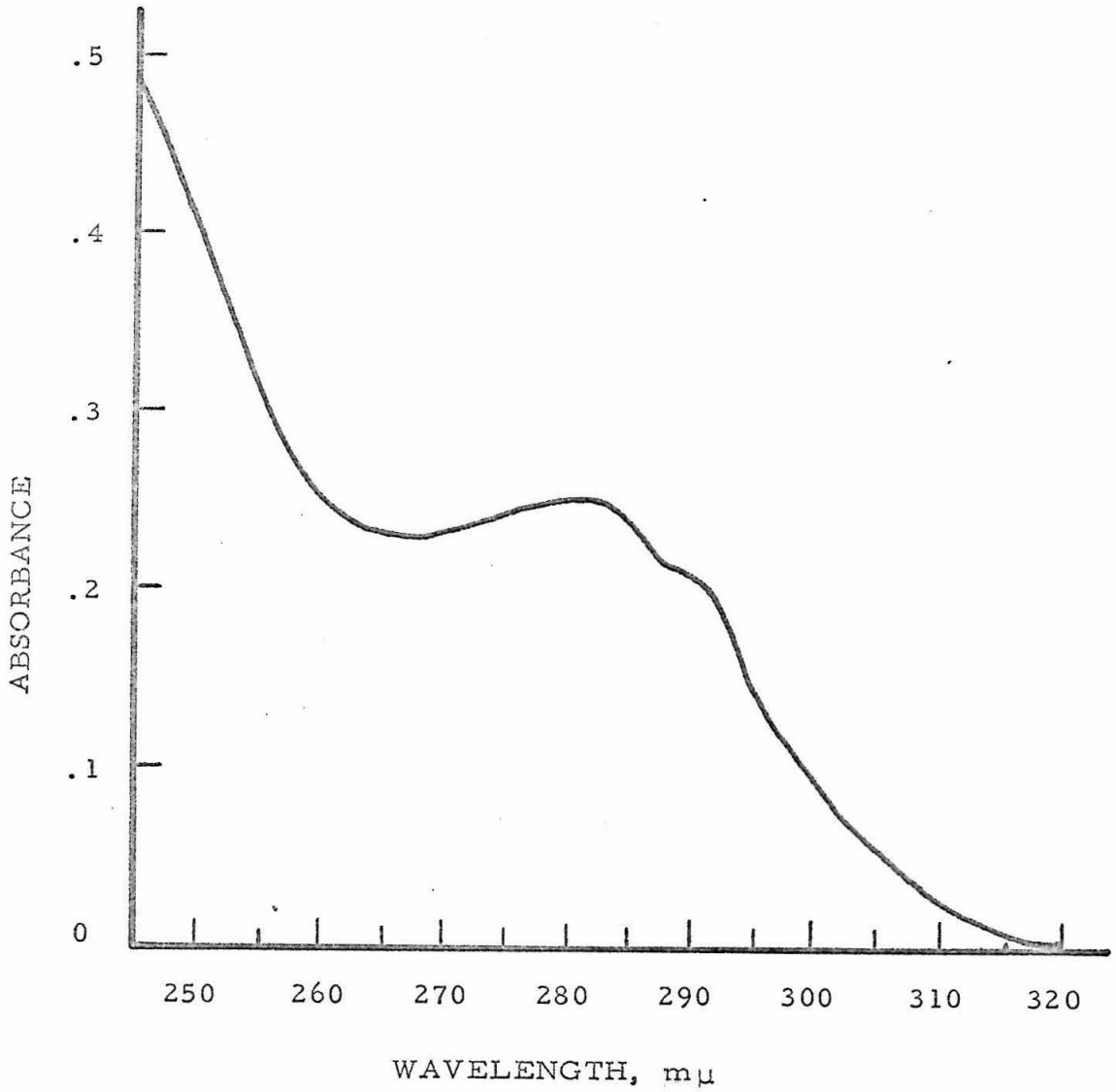
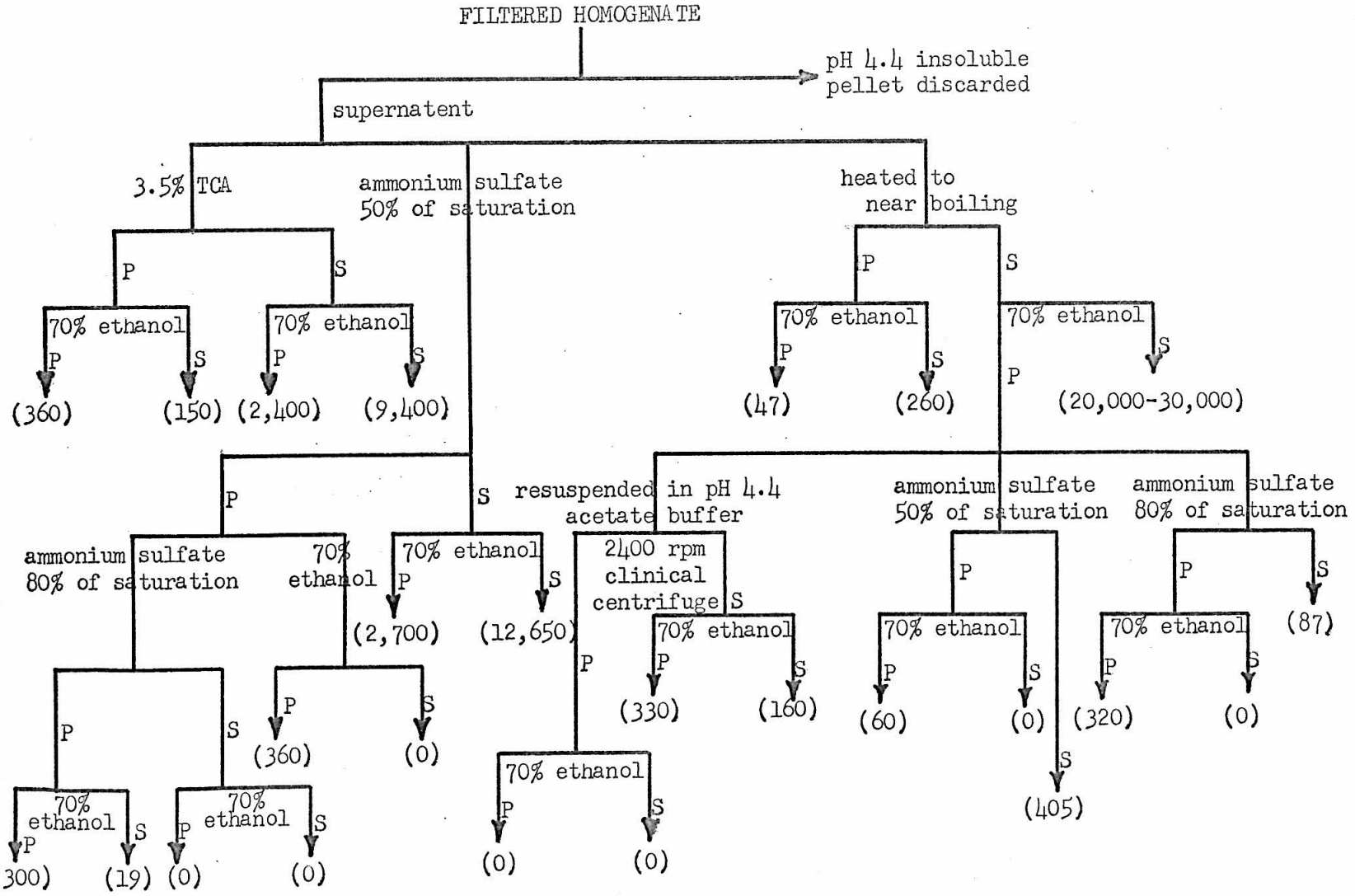


Figure 13. Ultraviolet absorption spectrum of the pH 4.4-soluble, ammonium sulfate-precipitable, carbohydrate-containing fraction of Avena coleoptiles. The insoluble material after dialysis was resuspended in 3 ml of 1 N sodium hydroxide. Total fraction equivalent to approximately 12 g fresh weight of tissue.

The carbazole carbohydrate content of the insoluble material after dialysis normally ranged from 100 to 250 µg of galacturonic acid equivalent per mg of biuret protein, a purification of 4- to 10-fold as compared to the initial ammonium sulfate precipitates. A major portion of this variability is attributable to varying amounts of contaminating protein. Difficulties encountered with the carbazole reaction in the presence of non-linear interfering materials, particularily hexose, are discussed in Appendix B.

A summary of the precipitation reactions conducted with the carbazole carbohydrate of the pH 4.4-soluble fraction of Avena coleoptiles is shown in figure 14. In addition to ammonium sulfate, the material was found to be precipitated by TCA and in the presence of protein or ammonium sulfate, by 70% ethanol at 4° C. The carbazole reacting material was re-soluble in 0.15 M sodium acetate buffer following ammonium sulfate precipitation at 50% of saturation, and the carbohydrate and protein appear to precipitate as a complex in the presence of concentrations of ammonium sulfate as high as 80% of saturation. The situation with regard to ethanol solubility is somewhat more complicated and appears to depend upon a number of factors. After subjection to acid or alkali precipitation, the fraction is found to form a relatively stable suspension in the presence of 70% ethanol at room temperature (not resolvable by low speed centrifugation even after standing at 4° C for several days). The fraction was subsequently shown to be soluble (except for a small amount of residual protein) in chloroform methanol (2:1) and could be precipitated from chloroform methanol by addition of a large excess of acetone.

Figure 14. Distribution of carbazole carbohydrate as a result of various protein precipitation procedures. P = insoluble material (precipitate or pellet); S = soluble or supernatent. Numbers in parenthesis represent µg galacturonic acid equivalent per 50 g fresh weight of Avena coleoptiles.



That the carbazole and biuret-reacting material are not readily dissociable was also verified by dialyzing a preparation for two hours to remove a portion of the salt and then chromatographing the fraction still in suspension on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column with 0.15 N sodium acetate as the eluting agent (131, 132). At least two major components were retained on the column (figure 15). These are accompanied by biuret-reacting material upon elution. The fact that material was retained on Sephadex G-25 would suggest association of carbohydrate with a relatively small protein or polypeptide (131, 132). That this may be so is also indicated from the relatively low number of ninhydrin-active zones observed following paper electrophoresis of tryptic digests. The results presented diagrammatically as figure 16, also reveal the presence of both carbohydrate- and ninhydrin-active material. A portion of the carbohydrate-reacting material was characterized by a mobility of about 0.5 with respect to picric acid and appeared to be accompanied by ninhydrin-active material. Under these conditions, free uronic acids were found to move somewhat faster with a $R_{picrate}$ of 1.0. The remaining carbohydrate-reacting material remained near the point of origin.

That this fraction is increased in amount by treatment of the tissue with IAA is indicated by the data of table 11. The results are based on washed pellets precipitated between 0 and 30% of saturation of ammonium sulfate. The IAA-induced increase appears to be largely restricted to this fraction. Weights were obtained following lyophilization and desiccation under nitrogen

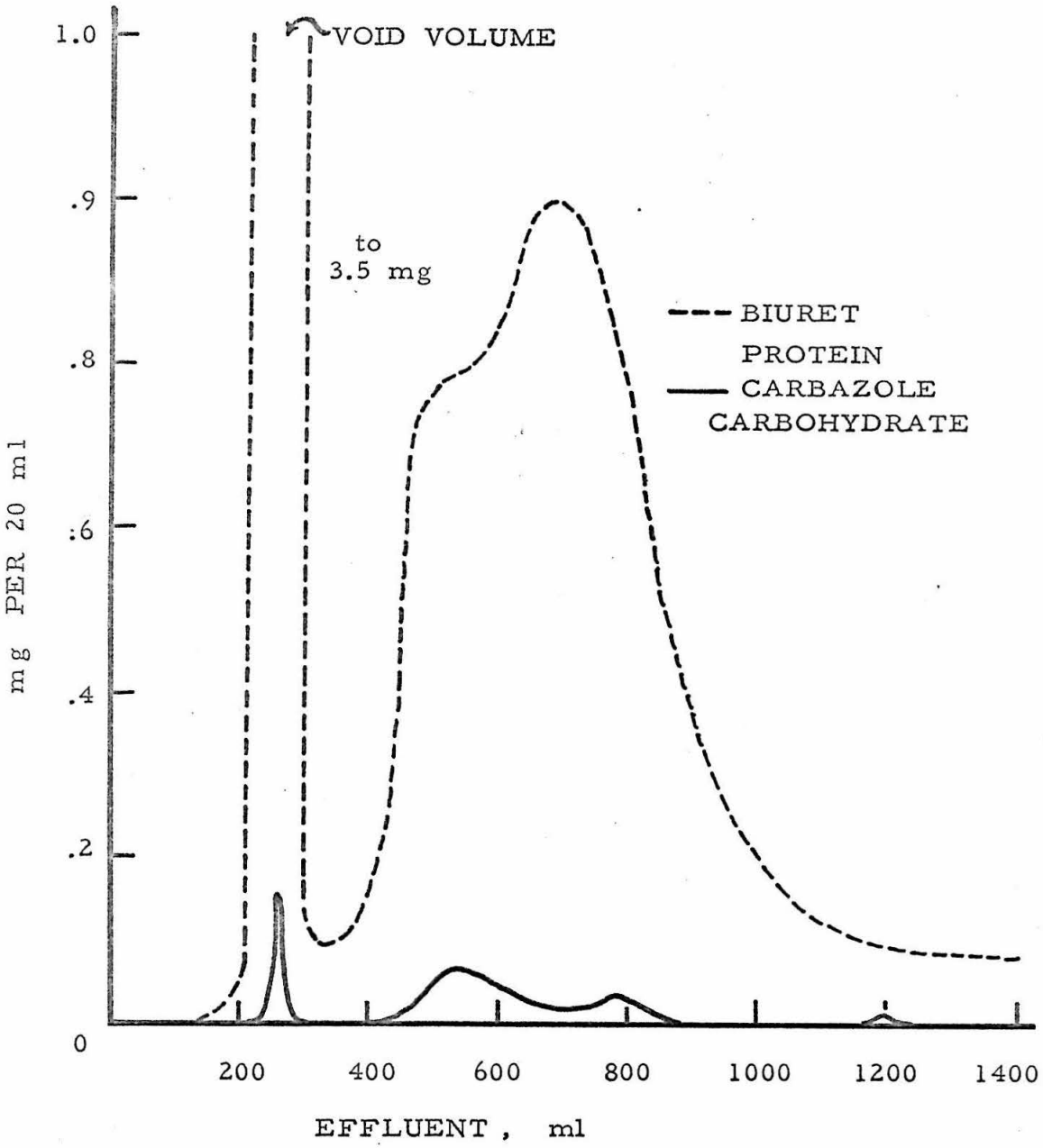


Figure 15. Sephadex G-25 fractionation of protein-complexed carbazole carbohydrate precipitated between 0 and 50% of saturation of ammonium sulfate from the pH 4.4-soluble fraction of Avena coleoptiles. The eluant consisted of 0.15 M acetate, pH 4.4 and the fractionation was conducted at 4° C.

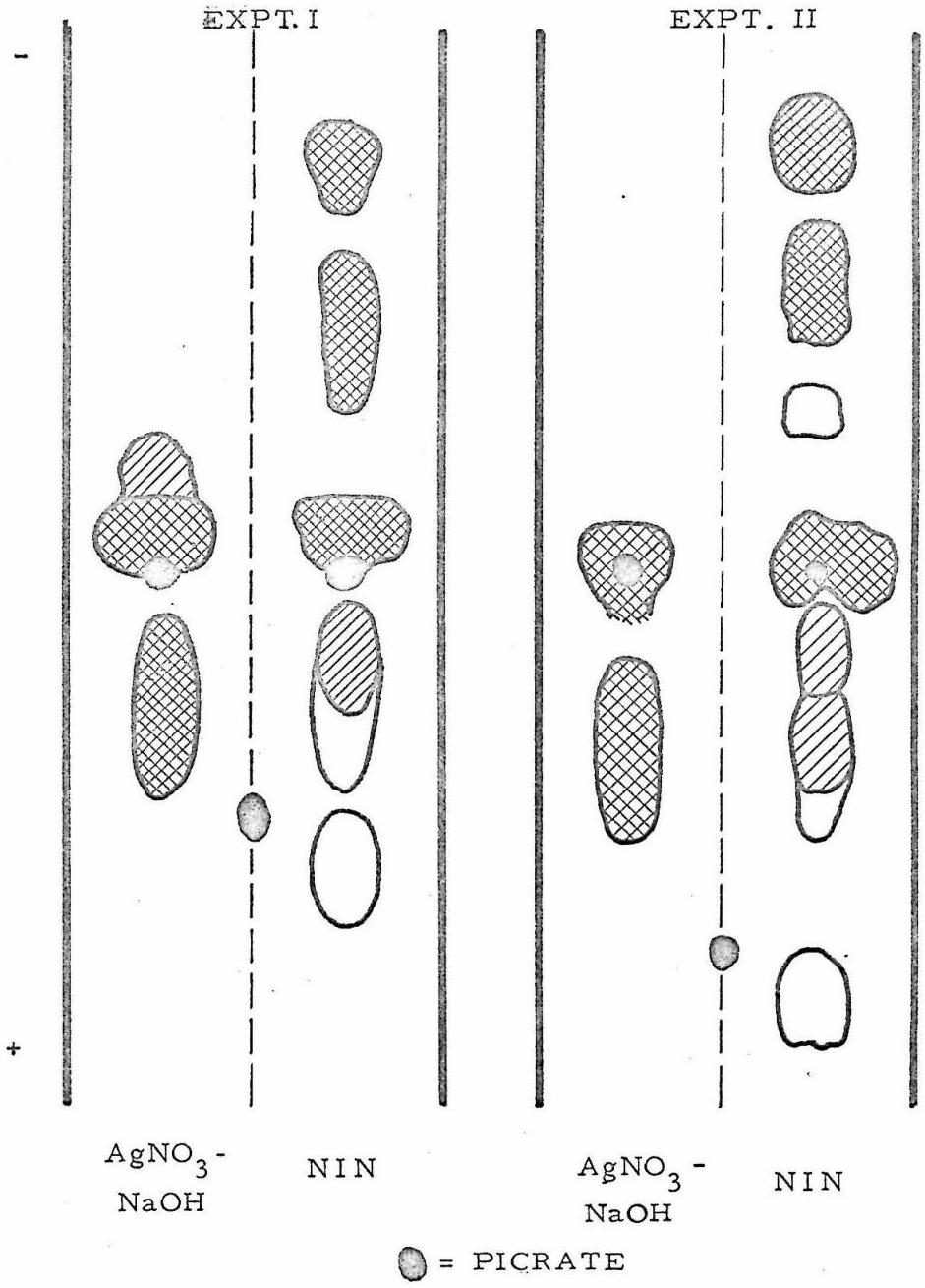


Figure 16. Paper electrophoretic separation of tryptic digests of the fraction from Avena coleoptiles soluble in pH 4.4 acetate and precipitable by ammonium sulfate at 50% of saturation. The fraction was dialyzed 14 hours prior to digestion with trypsin. Electrophoretic system E-I, Appendix E. Samples were applied in duplicate. $AgNO_3$-NaOH = silver nitrate reagent for detection of reducing sugars (199). NIN = ninhydrin. The degree of crosshatching provides an indication of the relative intensity of the spots.

TABLE 11.

Increase by weight as a result of treatment for 8 hours in the presence of 5×10^{-6} M IAA of the pH 4.4-soluble, 0 to 30% ammonium sulfate precipitable, chloroform-methanol- (2:1) soluble fraction of Avena coleoptiles.

	Mg per 50 g Fresh Weight			
IAA Concentration	Expt I	Expt II	Expt III	Ave.
None	18	17	12	16
5×10^{-6} M	27	24	16	22
Ratio (IAA/Cont)	1.5	1.4	1.3	1.4

Expt I = alkali-insoluble

Expt II = chloroform-methanol-soluble

Expt III = chloroform-methanol-soluble, alkali-insoluble

Although preliminary evidence for IAA-induced increases were based on carbazole carbohydrate content and estimates of the relative volume of alkali-insoluble precipitates, anthrone carbohydrate (hexose) and total phosphate may also be increased as a result of IAA treatment. These results are given as table 12.

TABLE 12.

IAA-induced increase in components of the alkali-insoluble fraction of Avena coleoptiles prepared by ammonium sulfate fractionation. Sections incubated for 8 hours in the presence of 5×10^{-6} M IAA. Based on 50 g fresh weight of tissue.

IAA Concentration	µg Anthrone Carbohydrate as Galactose	µM Phosphate	Mg Biuret Protein[1]
None	205	3.5	2.27
5×10^{-6} M	430	5.0	2.67
Ratio (IAA/Cont)	2.1	1.4	1.18

[1] Associated alkali-soluble protein for comparison.

Alkali-insoluble fraction from pea stem protein.--On the premise that the material increased by addition of IAA was protein-bound, the protein of the 24,000 X g supernatent fraction obtained from pea stem sections was isolated by heat or TCA precipitation. The protein was then dissolved in alkali with the addition of biuret reagent to aid in solution of protein and in dissociation of complexed lipide. This procedure was initially applied to similar extracts prepared from Avena coleoptiles and was abandoned due to extreme difficulties in solubilizing the denatured proteins. Results with peas, however, were sufficiently promising to warrant continued investigation. The alkali-insoluble fraction was subsequently found to

have solubility properties similar to that of the previously described fraction from Avena coleoptiles and to contain esterified phosphate (Section C) and a number of ninhydrin-active materials (Section D). Preliminary carbohydrate analyses, comparing IAA-treated and -untreated tissue are given in table 13. The absorption spectrum of the products formed after reaction with carbazole in 87% sulfuric acid are presented in figure 17.

Alkali-insoluble fraction from maize root sections.--The total carbazole carbohydrate of the alkali-insoluble fraction of maize root extracts was also found to be increased by the addition of IAA to the incubation medium (table 14). The tissue was treated for one hour in the presence and absence of 5×10^{-7} M IAA, the concentration shown to result in half-maximal IAA-induced wall deformability. Essentially the same results were obtained both before and after precipitation of the protein by heat. The absorption spectrum of the colored products forming as a result of reaction with carbazole in 87% sulfuric acid are given in figure 18.

Discussion

The existence of a readily soluble fraction of plant tissue which is increased (or maintained) in amount in the presence of physiological concentrations of IAA has been extended to include Avena coleoptile tissue and subapical sections of pea epicotyl tissue. The specific nature of the fraction is unknown but it appears to be complexed with protein. Some form of carbohydrate would appear to form an integral part of the complex as already determined from initial experiments with root sections (Part II). The solubility properties of the fraction suggest a rather

TABLE 13

The effect of IAA on the alkali-soluble protein content of the 24,000 X g supernatent fraction from subapical pea epicotyl sections and the carbohydrate content of the associated alkali-insoluble fraction. Based on 50 g fresh weight of tissue treated for 8 hours in the presence and absence of 5×10^{-6} M IAA.

IAA Concentration	Mg Biuret Protein[1]	µg Galacturonic Acid Equivalent[2]	µg Hexose[3]
None	39	215	900
5×10^{-6} M	41	600	1,575
Ratio (IAA/Cont)	1.0	2.8	1.75

1 Total protein by TCA precipitation.

2 Reaction with carbazole in 87% sulfuric acid.

3 Carbazole-water reaction, as glucose.

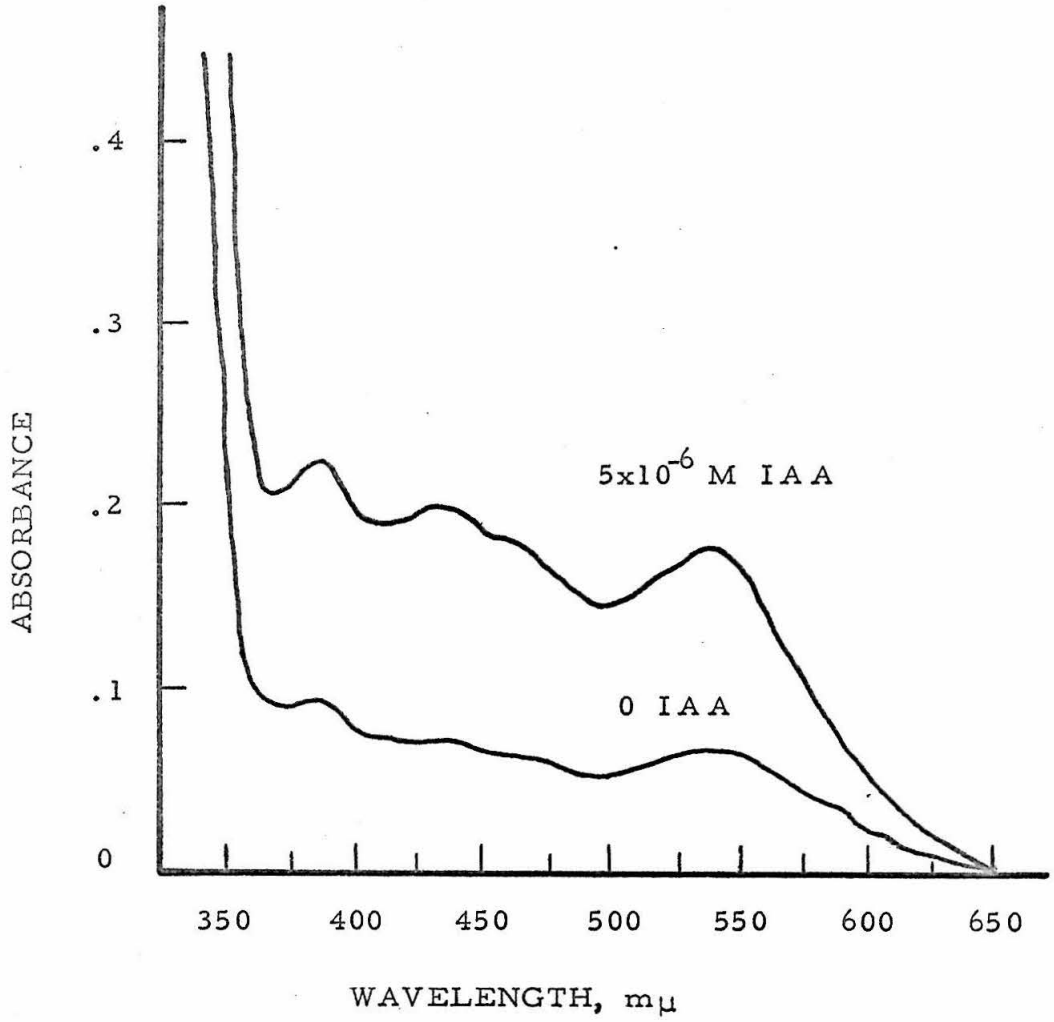


Figure 17. Absorption spectrum of the products formed after reaction of the alkali-insoluble material of the 24,000 X g supernatent fraction of homogenates from subapical pea epicotyl sections with carbazole in 87% sulfuric acid. Sections were incubated for 8 hours in the presence and absence of 5 X 10^{-6} M IAA.

TABLE 14.

The effect of IAA on the alkali-soluble protein content of extracts and the carbohydrate content of the associated alkali-insoluble fraction prepared from 5 mm maize root sections before and after heat precipitation of the proteins. Sections were incubated for 1 hour in the presence and absence of 5×10^{-7} M IAA. Data based on 3 g fresh weight (approximately 500 sections).

Fraction	IAA Concentration	Mg Biuret Protein[1]	µg Galacturonic Acid Equivalent[2]	µg Glucose[3]
Total by Direct Alkali Precipitation	None	12.10	60	318
	5×10^{-7} M	12.15	162	330
	Ratio (IAA/Cont)	1.00	2.70	1.04
Total by Heat Denaturation Followed by Alkali Precipitation	None	12.50	47	328
	5×10^{-7} M	12.25	141	352
	Ratio (IAA/Cont)	0.99	3.00	1.08

1 Total protein by TCA precipitation.

2 Reaction with carbazole in 87% sulfuric acid.

3 Anthrone carbohydrate reaction.

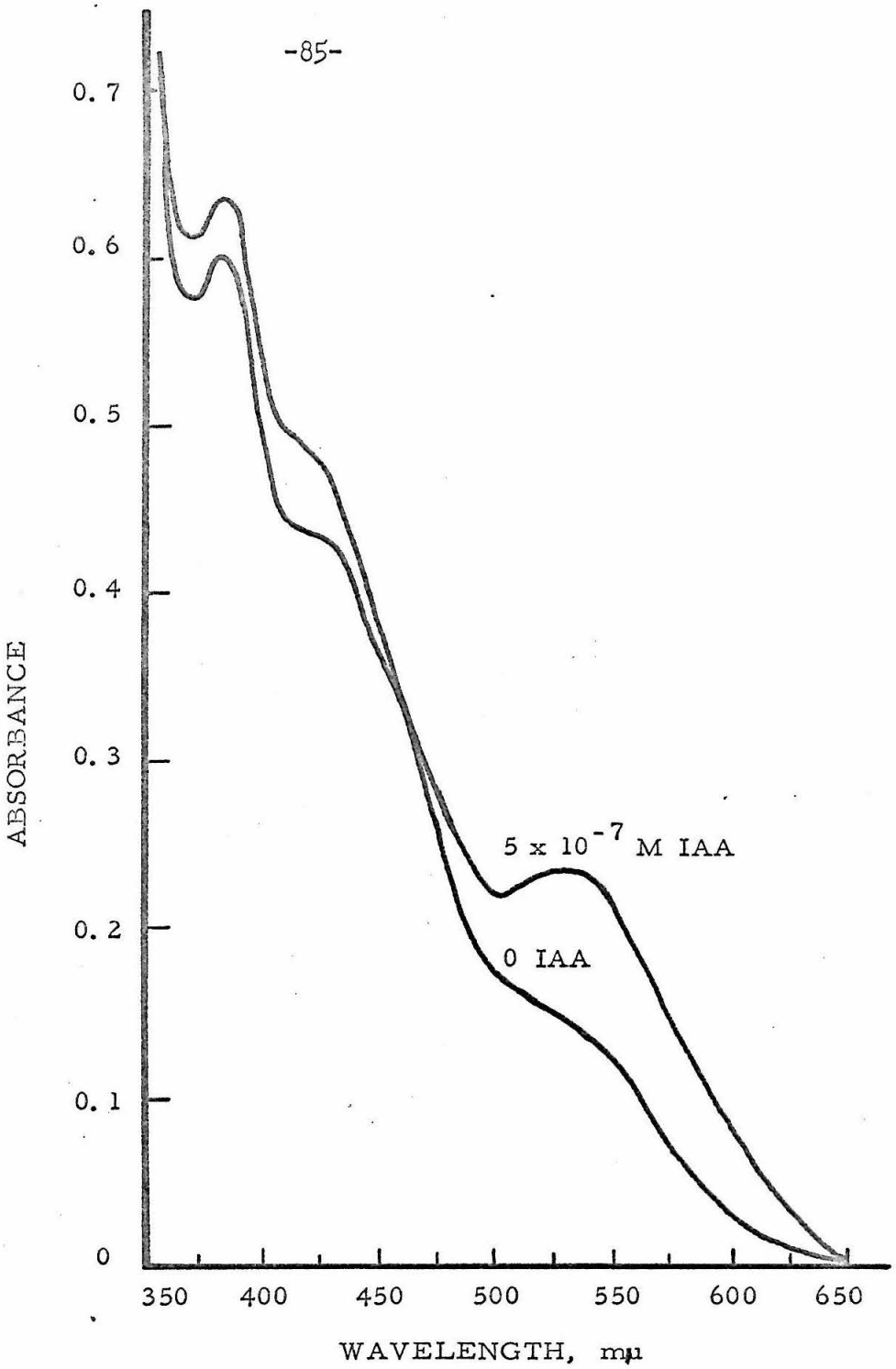


Figure 18. Absorption spectrum of the products formed from the alkali-insoluble material of maize root homogenates after reaction with carbazole in 87% sulfuric acid. Root sections were incubated for 1 hour in the presence and absence of 5 X 10^{-7} M IAA. Compare with figure 8, page 40.

high proportion of lipide, a fact accounting for many of the difficulties observed in preliminary isolation attempts.

There is little evidence that lipides and proteins ordinarily combine through primary covalent linkages (133). The combination seems to be due more to interactions between similar types of functional groups in the two classes of compounds as between non-polar fatty acid residues of lipides and similar residues of certain side groups of proteins. Lipoproteins are known to be sensitive to the usual agents which alter the characteristics of native protein molecules such as heat and extremes of pH. The relatively great solubility in chloroform-methanol and the relative insolubility of the "dissociated" lipide-like material in water, suggests tentative classification of the fraction as a proteoglycolipide, an adaptation of the term proteolipide used by Folch and Lees (134)

The clarity of the initial extracts would also suggest that the lipides are not initially present as large aggregates. The fractions considered in this section, like the lipoproteins of plasma (135), are soluble in aqueous salt solutions and show solubility characteristics of proteins. When the native structure is disrupted, such particles may coalesce and agents which tend to disrupt the lipoprotein complexes, such as heat, extremes of pH, solvents, etc., lead to an aggregation of the lipides into larger particles which increase the turbidity of the solution. (135).

The extent of degradation of the material isolated by alkali precipitation has not been determined nor is the manner of disruption understood. As early as 1934, Bensley and Hoerr (136) reported that

extraction with strong alkali results in essentially complete disruption of cell structure and yields a protein fraction containing fatty substances. More recently Dallam (137) has utilized alkali dispersion as a means of obtaining lipide material from various cell fractions of animal tissues. The alkali-insoluble fraction is reported to contain a glycoprotein (138). Thomas and co-workers (139) have pointed out that the technique of dispersion in alkali is accompanied by some decomposition, although reproducible results were obtained. They found that the cellular lipoprotein is dispersed, but not dissolved, in alkaline solution. The material could be brought out of solution with acid and re-dispersed by dilute alkali.

The initial selection of criteria upon which to base quantitation of the IAA response, as well as further purification of the fraction, is complicated by the fact that lipides occur as complex mixtures, combining not only with protein but with carbohydrates and various other cell metabolites as well. Both phosphate and carbohydrate measurements of the alkali-insoluble fractions were found to reflect the IAA-induced increase in amount and were observed to parallel turbidity estimates. Therefore, these criteria were selected as a basis for further purification and characterization of the fraction.

For purposes of subsequent discussion, both the fractions obtained by ammonium sulfate fractionation and the alkali-insoluble material dissociated from protein will be referred to as proteoglycolipide (PGL). However, specific preparations also will be designated according to the method of preparation or characterization actually used.

Conclusions

The evidence presented for the existence of a readily soluble fraction of plant tissue which is increased or maintained in the presence of physiological concentrations of IAA has been extended to include Avena coleoptiles and subapical sections of pea epicotyls. Preliminary characterization studies suggest the material to be of lipoprotein origin, although the final chloroform-methanol-soluble fractions isolated contain little or no biuret-active materials. The materials isolated also contain carbohydrates (and phosphate) which reflect the IAA-induced increase in amount. For discussion purposes, these fractions have been designated as proteoglycolipides (PGL).

B. CELLULAR DISTRIBUTION

With the recognition of a cellular component which appears to be increased in amount by the addition of IAA, it becomes of interest to determine its intracellular location. The low shear homogenization technique of Rho and Chipchase (140) was utilized in these experiments to distinguish between a cytoplasmic origin of the component and its possible extraction from the cell wall under the influence of cell dispersion.

Materials and Methods

Plant material.--Pea seedlings were grown in the dark in vermiculite for a period of 7 days at 25° C. Subapical sections, 13 mm long, were harvested and treated for eight hours in the dark in the presence or absence of 5×10^{-6} M IAA. The incubation medium consisted of 40 ml of 0.0025 M potassium maleate buffer, pH 4.5, contained in 20 X 150 mm petri plates. After incubation, the sections were washed in cold distilled water and stored at 4° C for approximately 30 minutes prior to homogenization.

Whole tissue fractionation (pea stems).--The cytoplasmic contents were released from the cell by the low shear homogenization procedure of Rho and Chipchase (140). The apparatus is illustrated in figures 19 and 20. The procedure consisted of passing the stem sections between two spring-loaded, counter-rotating rollers. The liberated cell contents were collected in 0.5 M sucrose containing 0.001 M EDTA. The tissue

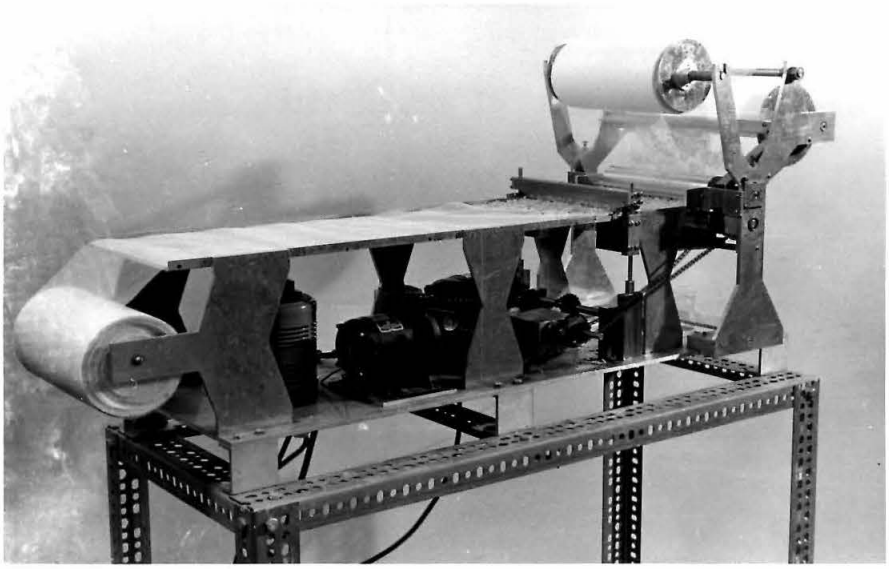


Figure 19. An overall view of the apparatus used in preparation of the low shear homogenates. Photograph courtesy of Drs. J. H. Rho and M. I. Chipchase.

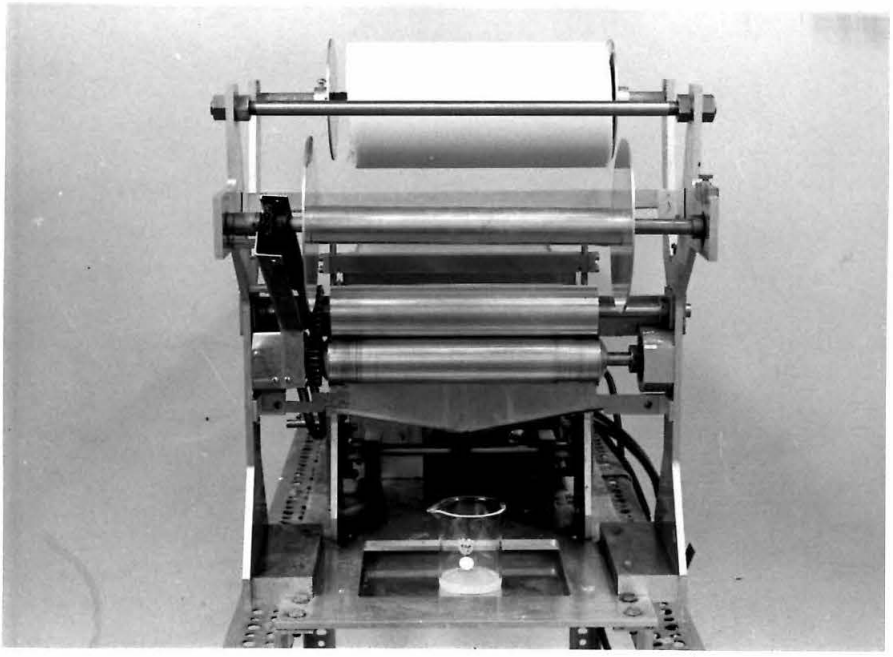


Figure 20. A side view of the apparatus used in preparation of the low shear homogenates illustrating general construction of the tissue squeezing rollers. The homogenate is collected into a beaker from the triangular-shaped stainless steel scraper. Photograph courtesy of Drs. J. H. Rho and M. I. Chipchase.

debris, including cell walls, was retained between the two layers of nylon mesh which pass between the squeezing rollers.

The tissue recovered from the first low shear homogenization was transferred to a pre-chilled mortar and exhaustively ground to further extract non-disrupted tissue and cell walls. Both homogenizations were conducted using an approximate ratio of two ml of grinding medium to one g fresh weight of tissue. Homogenates were centrifuged for one hour at 24,000 X g at 0° C. The pellet from the residual fraction containing cell walls and cell debris was discarded.

Cell fractionation (pea embryo axes).--Cell fractionation was carried out using the low shear homogenates obtained from 200 g of 48-hour old embryonic pea axes supplied by Dr. M. L. Birnstiel (141). The tissue was incubated for one hour in the dark in the presence of 6 μc of P^{32} (specific activity ca. 0.3 μc per μM) and with or without 5×10^{-7} M of IAA. The media were prepared in 0.01 M potassium maleate buffer and incubations were carried out in large Pyrex baking dishes with a ratio of one g of tissue per two ml of incubation medium. At the end of the incubation, the material was collected by filtration through gauze and was washed briefly with water at 0° C. Prior to homogenization, the tissue was diluted with an equal quantity of unlabeled and untreated tissue to serve as carrier. Homogenization was again conducted according to the procedure of Rho and Chipchase (140).

The sample of purified cell nuclei obtained from the 300 X g pellet was supplied for analysis by Dr. Birnstiel. Methods used in the preparation of these nuclei are described elsewhere (142). The 300 X g supernatent

had a final concentration of approximately 0.5 M sucrose-0.002 M calcium chloride and a pH of 6.3. This supernatent was further fractionated into three particulate fractions obtained by centrifugation for one hour at the forces indicated. The various pellets obtained were suspended in buffer and dialyzed for 20 hours against two changes of distilled water. All operations were conducted at temperatures between 0 and 4° C.

The 30,000 X g supernatent remaining after isolation of the particulate fractions was heated to near boiling to precipitate protein and the coagulated material was removed by centrifugation, accelerating to 37,000 X g for three to five minutes. The protein so obtained was resuspended in 1 N sodium hydroxide and the radioactivity of the alkali-insoluble fraction determined as being a measure of the PGL fraction influenced by IAA.

Determination of radioactivity.--Samples for counting were prepared by evaporating an aliquot of the suspended material on a glass planchet. Radioactivity was determined to ± 3% accuracy by means of a Nuclear Chicago D-47 gas flow, proportional counter fitted with a "micromil" window.

Methods of analysis.--Determination of anthrone carbohydrate (104), carbazole carbohydrate (Appendix B) and protein (103) are discussed in the preceeding section. Protein was determined after precipitation from 0.5 M TCA for several hours at 0° C.

Nucleic acid concentration was estimated from absorbance at 260 mμ and 280 mμ taken from the ultraviolet absorption spectrum as obtained with the Cary recording spectrophotometer.

Results

Whole tissue fractionation (pea stems).--The distribution of nucleic acid and protein among the three tissue fractions obtained is given in table 15. Total protein is found to be largely unchanged by IAA action and there appears to be no significant increase in protein in the fraction obtained by low shear homogenization. The latter would have been expected if IAA served to markedly decrease the resistance of the cell to disruption. Nucleic acid, expressed as mg of RNA, was increased slightly in the presence of IAA, due to an apparent doubling of the amount in the 24,000 X g pellet. The nucleic acid content of the remaining fractions was not affected. These quantities of RNA would not react to any significant extent in any of the carbohydrate assays employed.

Table 16 summarizes the results obtained by reacting the alkali-insoluble precipitates with carbazole in 87% sulfuric acid. The total quantities obtained were found to be increased by a factor of 2.5 as a result of IAA treatment. The cell contents, obtained by low shear homogenization, accounted for nearly 50% of the total carbazole carbohydrate isolated and for the greater portion of the IAA-induced increase in amount.

The results obtained by reacting the fractions with anthrone are expressed in terms of glucose standards (table 17). The IAA-induced increases in anthrone carbohydrate were found to be reflected in all three fractions; the largest quantities being derived from the initial low shear homogenates. The anthrone carbohydrate content of the alkali-insoluble fraction from the 5 to 24,000 X g pellet appeared to be most

TABLE 15.

The distribution of nucleic acid and protein among the 3 tissue fractions obtained by a combination of low shear homogenization, centrifugation and further extraction of the residue remaining after low shear homogenization.

		Mg per 50 g Fr. Wt.	
Tissue Fraction	IAA Concentration	Nucleic Acid as RNA	TCA-Insoluble Protein by Biuret
Low Shear Homogenate 24,000 X g Supernatent	None	0.018	22.54
	5×10^{-6} M	0.018	22.92
Low Shear Homogenate 5 to 24,000 X g Pellet	None	0.009	11.70
	5×10^{-6} M	0.018	11.34
Residual Fraction (Cell Wall, Unbroken Cells) 24,000 X g Supernatent	None	0.048	48.66
	5×10^{-6} M	0.048	49.08
Total	None	0.075	60.00
	5×10^{-6} M	0.084	60.78

TABLE 16.

The distribution of total alkali-insoluble carbazole carbohydrate comparing extracts obtained by low shear homogenization and the fraction extracted from the residue remaining after low shear homogenization.

Tissue Fraction	Mg as Galacturonic Acid Equivalent per 50 g Fr. Wt.		
	0 IAA	5×10^{-6} M IAA	Ratio IAA/Cont
Low Shear Homogenate 24,000 X g Supernatent	0.09	0.36	4.0
Low Shear Homogenate 5 to 24,000 X g Pellet	0.03	0.13	4.3
Residual Fraction (Cell Wall, Unbroken Cells) 24,000 X g Supernatent	0.17	0.24	1.4
Total	0.29	0.73	2.5

TABLE 17.

The distribution of anthrone carbohydrate expressed in terms of glucose standards comparing extracts obtained by low shear homogenization and the fraction extracted from the residue remaining after low shear homogenization.

Tissue Fraction	Mg as Glucose per 50 g Fr. Wt. 0 IAA	5×10^{-6} M IAA	Ratio IAA/Cont
Low Shear Homogenate 24,000 X g Supernatent	0.47	1.0	2.1
Low Shear Homogenate 5 to 24,000 X g Pellet	0.06	0.52	8,7
Residual Fraction (Cell Wall, Unbroken Cells) 24,000 X g Supernatent	0.27	0.54	2.0
Total	0.80	2.07	2.6

dramatically altered by the treatment with IAA, however. Since the residual fraction representing material extracted from the cell walls accounted for only about one-third of the total hexose, these data are also considered as being in support of a cytoplasmic origin for the IAA-induced fraction. It is of interest to note that there appears to be no direct correlation between the relative amounts of carbazole carbohydrate and anthrone carbohydrate. Although substances which interfere non-linearily are a potential source of error in experiments of this sort; it is nonetheless, more probable that the data reflect the presence of small quantities of absorbed sucrose which would increase the amount of anthrone positive material in all fractions. The results serve, however, as an indication of the IAA-induced response and of the general distribution of the glycolipide fraction with respect to the tissue fractionation employed. In all cases, the relative amounts of the alkali-insoluble fraction isolated, as based on turbidity estimates, paralleled carbazole carbohydrate content.

Preliminary cell fractionation of P^{32}-labeled pea embryo axes.--
The protein content of the various fractions obtained by centrifugation of the low shear homogenates of embryonic pea axes is given in table 18. Total protein was not measured since the fraction sedimenting between 0 and 300 X g was discarded during preparation of the purified nuclei. There appears to be a slight increase in protein content of the 18,000 to 30,000 X g particulate fraction, however, as well as in that of the 30,000 X g supernatent. An interpretation of these results is possible on the basis of increased stability of the protein associated with PGL as discussed in section C of part III and Appendix C.

TABLE 18.

The distribution of biuret protein among various subcellular fractions isolated. Particulate fractions were dialyzed prior to analysis. Pea embryo axes (200 g) incubated for 1 hour in 0.01 M potassium maleate buffer, pH 4.5, in the presence and absence of IAA.

Fraction	Mg Total Protein		Ratio
	0 IAA	5×10^{-7} M IAA	IAA/Cont
300 to 5,000 X g Particulate	127	134	1.05
5,000 to 18,000 X g Particulate	74	79	1.07
18,000 to 30,000 X g Particulate	15	17	1.13
30,000 X g Supernatent	50	58	1.16
Total Minus 0 to 300 X g Particulate	266	288	1.08

Distribution of radioactivity from P^{32}-labeled orthophosphate among the various subcellular fractions isolated are given in table 19. The particulate fractions were dialyzed prior to determination of the radioactivity reported. Prior to dialysis, no significant difference in radioactivity of these fractions could be detected as a result of IAA treatment nor were large differences apparent in either total extractable radioactivity or among any of the supernatent fractions.

After dialysis, the radioactivity of the purified nuclear fractions (nuclei and 300 to 5,000 X g pellet) was similarily unaffected. However, dialyzed particulate fractions sedimenting between 18,000 and 30,000 X g and, to a lesser extent, the fractions sedimenting between 5,000 and 18,000 X g were found to contain increased P^{32}-label as a result of prior IAA treatment.

The small differences observed between IAA-treated and -untreated tissue in radioactivity of heat denatured protein prepared from the final 30,000 X g supernatents is apparently the result of increased labeling of the alkali-insoluble fraction. If this material does represent a true measure of the IAA-induced PGL fraction of the tissue, it is possible that a two- to three-fold increase in labeling from P^{32} resulted from IAA treatment.

Discussion

The experiments reported in this section support the thesis that the fraction of interest is localized in the cytoplasm and is retained in the 30,000 X g supernatent fraction during centrifugation in 0.5 M sucrose.

TABLE 19.

The distribution of radioactivity from P^{32}-labeled orthophosphate among the subcellular fractions isolated. Particulate fractions were dialyzed prior to determination of radioactivity.

Fraction	Cpm P^{32} 0 IAA	Cpm P^{32} 5×10^{-7} M IAA	Ratio IAA/Cont
Total Extract (Non-dialyzed)	107,800	102,200	0.95
Purified Nuclei	117	116	0.99
300 to 5,000 X g Particulate	1,421	1,345	0.95
5,000 to 18,000 X g Particulate	670	813	1.21
18,000 to 30,000 X g Particulate	117	174	1.49
30,000 X g Supernatent			
Protein by Heat Precipitation	1,574	1,820	1.15
Alkali-Insoluble PGL	236	612	2.59

Preliminary experiments of this nature were considered to be necessary in order to proceed with further isolation and characterization studies. A particulate origin within the cytoplasm has not been ruled out, although the cell nuclei and other particles sedimenting between 0 and 5,000 X g in 0.5 M sucrose would appear unlikely as sites of origin. No net increase in ribosomes was reflected in measurements of nucleic acid content of the alkali-insoluble glycolipide, however, such a fraction has thus far not been purposely isolated and analyzed.

A number of points require further clarification including the nature of the particulate fractions sedimenting between 5 and 30,000 X g which would appear to reflect in some manner the effects of the IAA treatment. Furthermore, it has not been definitely established that the particular criteria upon which the results are based serve as a true measure of the actual fraction increased by IAA. These questions can be answered with assurance only if the nature of the fraction is known with a greater degree of certainty.

Although the cell wall has become firmly fixed as the final locale of auxin action in the phenomena of IAA-induced growth, occasional statements relating the effects of IAA and other plant growth regulators to particulate fractions do appear in the literature (for example, the review by Galston and Purves (3)). Among the effects noted are changes in cytoplasmic properties (viscosity, streaming, vacuolation), oxygen consumption (presumable a reflection of mitochondrial activity), altered permeability patterns and effects on nucleic acid metabolism (often interpreted as a direct effect on the cell nucleus (82).

The evidence relating to the role of auxins in nucleic acid metabolism has been reviewed by Chrispeels and Hanson (82). These effects, by and large, appear to be long term responses and a two- to three-fold increase in nucleic acid content of plant tissue treated with auxin would not be expected during the first hour after application. As noted by Key and Hanson (143), the increase in total RNA due to 2,4-D treatment is more rapid during the second 24-hour period after application.

Conclusions

The results obtained suggest a cytoplasmic origin of the fraction whose formation or maintenance is IAA-dependent. Of the cell particulates, nuclei and other particles sedimenting between 0 and 5,000 X g do not appear to reflect the IAA-induced increase in amount. Soluble preparations from pea epicotyls did not appear to contain sufficient 260 mμ absorbing material to consider nucleic acid as a major source of error. However, on the basis of a number of difficulties encountered in the interpretation of the results, it is concluded that further studies of this sort must await more specific and reliable methods of isolation and assay.

C. INCREASED HEAT STABILITY OF ASSOCIATED PROTEINS

That indoleacetic acid exerts an influence on the physical state of proteins was proposed by Galston and Kaur (80, 81) on the basis of decreased heat coagulability. Their results demonstrated in the case of pea stems that auxins induce a decrease in the heat coagulability of cytoplasmic proteins under conditions in which total protein content is not altered. This effect is not produced when the auxins are added *in vitro*.

Preliminary results already presented suggested that the IAA-influenced glycolipide may be present in extracts as a lipoprotein complex. That the increased heat stability of proteins might also be attributable to an association between cytoplasmic proteins and the glycolipide fraction (PGL), therefore, was investigated.

Materials and Methods

Plant material.--Alaska peas were obtained from two sources, Ferry-Morse Seed Company, Los Angeles, California (Seed Lot I) and Associated Seed Growers, New Haven, Connecticut (Seed Lot II). Seeds were soaked in water overnight (approximately 15 hours) and sown in water-soaked vermiculite contained in stainless steel containers. Seedlings were then grown in darkness for seven days at a temperature of 25° C. Subapical stem sections, 13 mm in length were then excised from the third internode using a double-bladed cutting tool.

Incubation of sections.--Approximately 20 to 25 g of 13 mm subapical pea stem sections were placed in 50 ml of the appropriate growth medium

contained in 20 X 150 mm petri plates. The sections were incubated for eight hours in the presence and absence of 5×10^{-6} M IAA in the dark. This is in contrast to the 18-hour incubation used routinely by Galston and Kaur (80). Total fresh weight of the sections was obtained before and after incubation and the increase in length was estimated from measurements of a random sample of 20 to 40 sections. After harvest the sections were rinsed and frozen until analysis.

Preparation of extracts.--The procedure for isolation of cytoplasmic protein was only slightly modified from that given by Galston and Kaur (80). Frozen sections were homogenized in a pre-chilled mortar and pestle with 35 ml of 0.5 M sucrose containing 0.001 M of ethylenediaminetetraacetic acid (EDTA). The homogenate was filtered through a single layer of miracloth and centrifuged for one hour at 24,000 X g. All operations were conducted at temperatures between 0 and 3° C. The 24,000 X g pellet was discarded and aliquots of the supernatent were removed for analysis.

Preparation of protein fractions.--Heat precipitation was achieved by placing 10 ml aliquots contained in heavy-wall centrifuge tubes in a boiling water bath for periods of 1 to 10 minutes. The tubes were cooled to ice bath temperature and the precipitated protein collected by centrifugation, acceleration to 27,000 X g for 3 to 5 minutes. This fraction is referred to as heat-denatured protein or HDP.

Total protein and heat-stable protein following removal of HDP were obtained by adding to the extracts an equal volume of 1 M TCA. The resulting mixture was equilibrated at 0° C for several hours and the denatured protein was collected by centrifugation (3 to 5 minutes, 27,000 X g).

Isolation of protein-bound glycolipide (PGL).--For estimation of PGL, protein was obtained by exhaustive heat precipition, boiling the extracts for 5 to 15 minutes. The tubes were then cooled and the protein collected by centrifugation.

Release of the glycolipide fraction from protein was achieved in these experiments by relying on the insolubility of PGL under alkaline conditions. Under these conditions, the bulk of the cytoplasmic proteins of pea stem sections are solubilized and readily separable. Suspension of protein was found to be greatly facilitated by addition of a small amount of biuret reagent. Heat-denatured protein equivalent to approximately 10 g fresh weight was suspended in 5 ml of an equal mixture of 1 N sodium hydroxide and standard biuret reagent (103). PGL remains insoluble as a turbid precipitate which can be collected quantitatively by means of a low speed centrifugation (5 minute, 2,400 rpm) within about 10 minutes after resuspension. PGL suspended in alkali has a tendency to float and frequently is not precipitated from dilute solutions. These considerations, as well as the severity of the treatment, greatly curtail the usefulness of alkali insolubility as an isolation procedure. The white amorphous precipitate was further purified by acidification to pH 2 with 0.1 N HCl and re-precipitation from 95% ethanol in the cold.

Results

The data presented in table 20 were obtained in an experiment designed to compare the heat coagulability of proteins of IAA-treated and -untreated pea stem sections. Total protein obtained by TCA precipitation

TABLE 20.

The effect of an 8-hour pretreatment in the presence or absence of 5×10^{-6} M IAA on the quantity of heat-stable and total protein of subapical pea epicotyl sections (Seed Lot I). Results based on 10 g fresh weight of tissue.

	Mg Biuret Protein/10 g Fresh Weight	
IAA Concentration	Heat Precipitable (10 min, 100° C)	TCA Precipitable (0.5 M TCA, 0° C, 3 hrs)
None	11.58	11.70
5×10^{-6} M	8.16	12.18

was found to be unchanged in amount as a result of IAA treatment. The fraction precipitable by boiling for 10 minutes (HDP) is, however, reduced in extracts prepared from tissue treated for eight hours with 5×10^{-6} M IAA. The first indication that the stabilization of protein observed as a result of IAA treatment may be due to the presence of PGL was suggested by the fact that the turbidity of the extracts upon addition of base was often several times greater in the case of IAA-treated tissue than in control tissue. This difference was confirmed by analysis of both the carbohydrate and phosphate content of the fraction isolated.

Carbohydrate determinations on 95% ethanol precipitates of crude PGL prepared from both HDP and TCA-insoluble protein from two experiments are

given in table 21. The protein values represent an amount of starting material equivalent to 5 g fresh weight. Both carbazole carbohydrate and anthrone carbohydrate bound to proteolipide are found to be increased by the IAA treatment. The agreement between the two experiments is not unreasonable considering the inherent variability of the carbohydrate reactions and the uncertainty of the preparative procedure.

Total phosphate content of the crude HDP was determined and the results, based on the protein samples indicated, are given in table 22. That the phosphate present is largely alkali-lable phosphate is suggested by the data of tables 23 and 24.

The two seed lots utilized in these experiments exhibited widely different IAA-induced growth responses. This fact was further reflected not only in their ratios of PGL (Control vs. IAA) but in the heat stability of the accompanying protein as well. Data obtained with sections grown from seed lot II are given in table 25. Although the heat-stable protein levels indicated are increased by a factor of greater than two as a result of IAA treatment, it was necessary to reduce the time of boiling from 10 minutes (table 20) to 1 minute in order to obtain this measure of heat stability. No indication of heat stability could be obtained in these experiments if the time of heating was increased beyond 2 minutes.

The correlation between IAA-induced growth rate and increase in PGL is represented graphically as figure 21. That the compared ratios show a direct correspondence cannot be generalized without further data comparing both time of treatment and different concentrations of indoleacetic acid.

TABLE 21.

The effect of IAA on soluble protein and of subapical pea epicotyl sections (Seed Lot I) and the carbohydrate content of the associated alkali-insoluble glycolipide fraction. Based on 10 g fresh weight of tissue treated for 8 hours in the presence and absence of 5×10^{-6} M IAA.

Expt I

IAA Concentration	Mg Biuret Protein[1]	µg Uronic Acid Equivalent[2]	µg Glucose Equivalent[3]
None	8.12	45	76
5×10^{-6} M	7.80	181	350
Ratio (IAA/Cont)	0.96	4.0	4.6

Expt II

IAA Concentration	Mg Biuret Protein[1]	µg Uronic Acid Equivalent[2]	µg Glucose Equivalent[3]
None	9.60	40	93
5×10^{-6} M	9.60	160	196
Ratio (IAA/Cont)	1.00	4.0	2.1

---

1 Total protein by exhaustive heat precipitation.

2 Reaction with carbazole in 87% sulfuric acid (Appendix B).

3 Reaction with anthrone (104).

TABLE 22.

Total protein-bound phosphate as influenced by treatment of pea epicotyl tissue (Seed Lot II) for 8 hours in the presence or absence of 5×10^{-6} M IAA. Total protein obtained by exhaustive heat denaturation.

IAA Concentration	Mg Total Biuret Protein	µM Total Phosphate[1]
None	10.02	1.0
5×10^{-6} M	10.06	2.1
Ratio (IAA/Cont)	1.0	2.1

[1] Method of Allen (128).

TABLE 23.

Alkali saponifiable phosphate as influenced by treatment of pea epicotyl tissue (Seed Lot II) for 8 hours in the presence or absence of 5×10^{-6} M IAA. Total protein obtained by exhaustive heat denaturation.

IAA Concentration	Mg Total Biuret Protein	µM Saponifiable Phosphate[2]
None	8.0	0.78
5×10^{-6} M	9.6	1.47
Ratio (IAA/Cont)	1.16	1.90

[1] Method of Sumner (129).

[2] 0.5 N ethanolic potassium hydroxide, 2 hours, 100° C.

TABLE 24.

Alkali saponifiable phosphate contained in the alkali-insoluble glycolipide prepared from 10 g fresh weight of untreated subapical pea epicotyl sections (Seed Lot II). Sections were harvested and frozen immediately.

Treatment	µM Inorganic Phosphate[1]
None	0
0.5 N Ethanolic Potassium Hydroxide 2 Hrs, 100° C.	1.51

[1] Method of Sumner (129).

TABLE 25.

The effect of an 8 hour pretreatment in the presence and absence of 5×10^{-6} M IAA on the quantity of heat-stable and total protein of subapical pea epicotyl sections (Lot II). Based on 10 g fresh weight.

Protein Fraction	IAA Concentration	Mg Biuret Protein per 10 g Fr. Wt.	Ratio IAA/Cont
1. Heat Precipitable (1 min 100° C)	None	11.50	0.79
	5×10^{-6} M	9.12	
2. Heat Stable (1 min 100° C by TCA Precipitation)	None	2.56	2.18
	5×10^{-6} M	5.56	
Total (1 + 2)	None	14.06	1.06
	5×10^{-6} M	14.68	
3. Heat Precipitable (10 min 100° C)	None	13.20	0.97
	5×10^{-6} M	12.80	
4. Total (by TCA Precipitation)	None	14.08	1.02
	5×10^{-6} M	14.40	

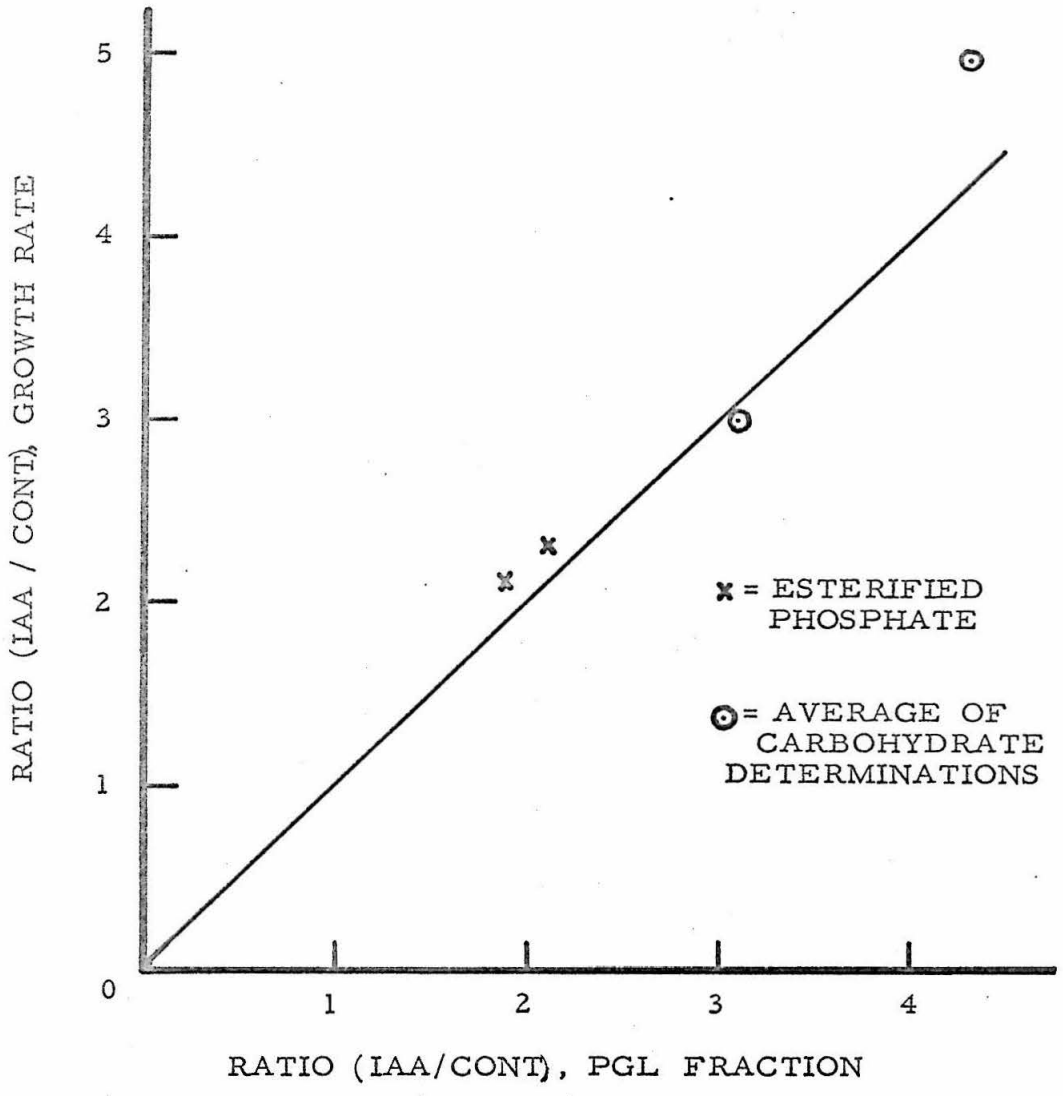


Figure 21. Correlation between IAA-induced growth rate and IAA-induced increase in the protein-bound glycolipide fraction of subapical pea epicotyl sections.

Discussion

The alterations of heat stability of protein as a result of prior IAA treatment of the extracted tissues was found to be less dramatic than the earlier reports (80, 81) would seem to indicate. However, observations of the extracts during the course of heat treatment consistently revealed differences between extracts prepared from IAA-treated and -untreated tissues. Coagulum formed rapidly in extracts from untreated tissue and the protein precipitated in the form of large aggregates. That of the IAA-treated tissue remained turbid longer and eventually formed a finer aggregation. These differences were found to be difficult to quantitate due to the fact that both types of protein coagulum were readily sedimented.

The phenomenon of IAA-induced heat stability of proteins was first attributed to an auxin-induced alteration of the physical state of the cellular proteins (80). That this result may be attributable to the binding of PGL to cytoplasmic proteins either during isolation or intracellularly would seem likely on the basis of present experiments. The presence of protein-bound PGL is indicated from the fact that a material isolated from the heat stabilized protein fraction of peas showed compositional and solubility properties similar to that of PGL obtained from Avena coleoptiles and maize roots. An IAA-induced increase in PGL was observed, as well, being reflected in both total carbohydrate and esterified phosphate, the two parameters measured.

Multiple binding of polyelectrolytes (and other molecules containing one or more charged groups) to proteins is well known (144) and can often

either stabilize or weaken the native configuration. The association of lipides with proteins has been considered from the standpoint of naturally occurring lipoprotein systems (133, 135, 145). The possible contributions of both protein and lipide to structure are discussed. According to Putnam (146), one of the most significant examples of protection against denaturation by heat, urea and guanidine hydrochloride is, in fact, the stabilization offered to serum albumin by long-chain organic anions. Of these, low concentrations of fatty acids anions, anionic detergents or other anions with large non-polar groups are effective in retarding the heat coagulation of bovine serum albumin and preventing the viscosity rise in heated solutions (147, 148). The protective effect is observed on both sides of the isoelectric point and is not given by cations with non-polar groups. Furthermore, the resistance of intact low-density lipoprotein preparations to denaturation by urea has been interpreted by Avigan (149) as a stabilizing effect of extractable lipide. The resistance to urea denaturation was abolished after treatment with ether.

A heat stabilizing effect is also possible with carbohydrates and other polyhydroxy compounds (144). Certain protein-carbohydrate compounds known as mucoproteins generally do not coagulate when heated in aqueous solution. The polypeptide chains are apparently stabilized by carbohydrate against heat denaturation even though the chemical reactions of mucoproteins are predominantly those of the protein (150, 151). In studies of the stabilizing effect of steroid glycosides, however, the carbohydrate portion is not required for combination and the aglucones give comparable effects (152).

On the basis of the above discussion, it would seem reasonable to assume that a phosphate containing glycolipide would possess the potentiality of altering the heat stability of certain cytoplasmic proteins. However, a direct demonstration of the stabilizing properties of PGL has not proceeded beyond preliminary experiments. These results, although not necessarily indicative of a biologically specific interaction between PGL and protein, would appear to substantiate the suggestion that such an interaction could account for the observed effect. A similar interpretation would also appear to account for a portion of an apparent IAA-induced increase in acid phosphatase activity of certain plant tissues (see Appendix C).

The effect of various auxins and auxin analogues on growth and heat coagulability of proteins of etiolated pea epicotyl sections has been reported by Galston and Kaur (80). The most active auxins (IAA and 2,4-D) were found to produce the greatest reduction in heat denaturable protein, whereas p-chloroisobutyric acid, an antiauxin analog of 2,4-D, resulted in no effect on the amount of protein precipitated and was accompanied by only a small change in growth of the sections. Phenylacetic acid and 2,3,5-triiodobenzoic acid, compounds suggested to be only slightly active as plant growth regulators (153), were considerably less effective in envoking the heat stability response. That there is an increasing effect on the proteins with increasing concentration of growth regulator was also established by Galston and Kaur (80) in the case of 2,4-D. It is of some interest to observe that in these experiments, between 10^{-6} and 10^{-5} M 2,4-D, the fresh weight was found to increase from 114% to 130%, whereas the heat-stable protein increased from 13.3 g to 31.7 mg. With a higher

concentration of 2,4-D, i.e., 10^{-4} M, the system apparently became saturated with respect to promotive effects on growth and total growth decreased slightly from 130% to 124%. However, the factors responsible for the 2,4-D induced heat stabilization of protein continued to increase. This same effect was also observed in the case of non-etiolated sections where fresh weight decreased from 93% to 86%.

On the basis of other experiments described in less detail, Galston and Kaur (80) conclude that the auxins are practically inactive when applied *in vitro* and that the effect resides mainly in the proteins of the non-particulate fraction since boiling of the uncentrifuged homogenates yields essentially the same results. Furthermore, with pea roots, these authors report effects on heat stabilization of proteins by 10^{-8} M 2,4-D under conditions where total growth was inhibited.

Data are presently unavailable on similar relationships among different growth regulators in the case of the protein-bound glycolipide. However, the observations that both phenomena may be manifestations of the same general mechanism would suggest the likelihood that similar relationships exist in the case of the PGL. The possibility that PGL concentration may reach a plateau at high auxin concentrations is of considerable interest in relation to the concentration dependence of cell wall deformability. In the case of both Avena coleoptiles (67) and pea stem sections (154), growth rate decreases with increasing concentration of indoleacetic acid above a certain level (supra-optimal or growth-inhibitory concentrations). These results have been interpreted kinetically as a competitive inhibition for auxin receptor sites, i.e., Foster *et al.* (67).

However, IAA-induced cell wall plasticity fails to parallel the growth response over the high concentration range and reaches a plateau or perhaps increases slightly over the growth-inhibitory range in the case of both Avena coleoptile sections (155) and pea epicotyl sections (Unpublished). Thus, the data of Galston and Kaur (80) suggest the possibility that the intracellular PGL concentration and wall plasticity are parallel, IAA-induced responses--proportional to IAA concentration and independent of actual growth.

Whether or not the phenomenon of increased heat stability has physiological meaning cannot be established on the basis of the results thus far obtained. Galston and Kaur (80) have suggested that this phenomenon could account for such hitherto unexplained facets of auxin actions as the decreased viscosity of the cytoplasm described by Northen (76) and the effects of cytoplasmic streaming reported by Sweeney and Thimann (78), as well as those of the cell wall (1) and nucleus (82). However, protein binding of PGL may be both non-specific and reversible. Therefore, that the phenomena is simply the result of a mixing of cell contents as a result of homogenization must be considered, as well as alternative explanations for the above described phenomena.

Conclusions

The report of Galston and Kaur (80) that auxins induce a decrease in the heat coagulability of cytoplasmic proteins has been verified. Associated with the increased heat stability is a 2- to 4-fold increase in the amount of protein-bound PGL. A phosphate-containing glycolipide moiety is

potentially capable of increasing the heat stability of certain cytoplasmic proteins. Therefore, it is suggested that the auxin-induced alteration of the physical state of cellular proteins may be a manifestation of the maintenance of the intracellular levels of PGL.

D. FURTHER CHARACTERIZATION OF THE IAA-INFLUENCED GLYCOLIPIDE FRACTION

Evidence for approximately equimolar amounts of carbohydrate and esterified phosphate in PGL has already been presented. The lipide nature of the PGL fraction has also been deduced from its extreme insolubility in aqueous solvents and its solubility in chloroform-methanol (2:1). In the following section, additional evidence concerning the lipide nature of PGL will be summarized.

Experimental

Isolation of the PGL fraction.--PGL was obtained from Avena coleoptiles, pea stem sections and pea embryo axes. The 48-hour pea embryo axes used consisted predominantly of root tissue and, therefore, were auxin-treated in the manner described for root sections, i.e., 5×10^{-7} M IAA for one hour. Other tissues were untreated.

a) Avena coleoptiles.--Coleoptiles were grown and harvested as outlined in section A. The fraction precipitated between 0 and 30% of saturation of ammonium sulfate of the pH 4.4-soluble supernatent was dialyzed against distilled water (ca. 12 hours) and then lyophylized. The lyophylized material was extracted with chloroform-methanol (2:1)

in a ratio of approximately 2 ml per mg of lyophylized residue. Solvent was removed by evaporation under nitrogen and the preparation was then lyophylized a second time. The residue obtained was dried over sulfuric acid for several days under nitrogen. Following a final re-extraction with chloroform-methanol, the material was recovered by removal of solvent, dried and weighed.

b) Pea epicotyl sections and pea embryo axes.--Extracts were prepared by low shear homogenization (section B) and the alkali-insoluble fraction was obtained from the heat precipited protein as previously described (sections A and B). The alkali treatment is presumed to disrupt the glycolipide-protein complex with the bulk of the protein being alkali-soluble.

Acid hydrolysis and analysis by paper chromatography.--Partially purified alkali-insoluble PGL obtained from approximately 10 mg of heat precipitated protein was acid hydrolyzed in sealed ampules under nitrogen with other conditions as specified. Sulfuric acid was neutralized by addition of barium carbonate and the insoluble barium sulfate was removed by centrifugation. Hydrochloric acid was removed under a stream of nitrogen. Measured aliquots of the concentrated samples were applied to 35 mm strips of Whatman No. 1 filter paper and subjected to analysis by paper chromatography using appropriate solvent systems (described in Appendix E).

a) Ninhydrin positive components.--When alkali-insoluble PGL was hydrolyzed in the presence of 2 N hydrochloric acid for 6 hours at 103° C, paper cnromatography of the neutralized hydrolyzates revealed at

least two ninhydrin zones as being characteristic of all PGL preparations examined (tables 26 to 28). The R_fs in DIPWA corresponded to those of ethanolamine or hexosamine (ca. 0.45) and to those of the leucine, isoleucine, norleucine group (ca. 0.55 to 0.60). Varying amounts of these components were also obtained following hydrolysis in the presence of 0.1 N hydrochloric acid for 75 minutes at 100° C, 88% formic acid for 10 hours at 103° C and 1 N sulfuric acid for 5 hours at 103° C. In addition, a low R_f streak was consistently observed. With longer times of hydrolysis (2 N hydrochloric acid, 12 hours, 100° C; 6 N hydrochloric acid, 12 hours, 103° C), the low R_f streak was replaced by a number of ninhydrin positive materials with R_fs corresponding to those of known amino acids. These materials, therefore, appear to be of polypeptide origin.

The identity of the remaining two ninhydrin positive components has not been established, however, the component corresponding to hexosamine or ethanolamine does not appear to be identical with hexosamine. This is evidenced from the fact that the indole-hydrochloric acid reaction for hexosamine (156) of the R_f 0.45 material purified by paper chromatography is negative (table 29). However, there appears to be material present in all hydrolyzates with an R_f corresponding to ethanolamine using several different solvent systems. The second component (DIPWA R_f of 0.55 to 0.60) is most probably not identical to one of the leucine, isoleucine, norleucine group, as is evidenced by the R_f observed in solvent systems other than DIPWA, i.e., EtAc-Pyr (table 27).

TABLE 26.

Paper chromatographic analysis of ninhydrin positive materials in acid hydrolyzates of an Avena glycolipide fraction.[1] Hydrolysis conducted for 7 hours at 100° C in the presence of 2 N hydrochloric acid.

Materials	R_f DIPWA	Color
Reference Compounds		
Ethanolamine	0.49	violet
Hexosamine	0.48	violet
Norleucine	0.56	violet
Hydrolyzate		
	0.08	red to blue-violet streak
	0.17	only partially resolved
	0.29	
	0.46	blue-violet
	0.62	intense violet

[1] Chloroform-methanol (2:1)-soluble material of the fraction precipitated between 0 and 30% of saturation of ammonium sulfate from the pH 4.4-soluble supernatent prepared from Avena coleoptiles.

TABLE 27.

Paper chromatographic analysis of ninhydrin positive materials contained in acid hydrolyzates of the alkali-insoluble fraction prepared from heat-denatured protein of subapical pea stem sections.

Material	R_f DIPWA	$R_{Norleu.}$ EtAc-Pyr	R_f BAW
Reference Compounds			
Serine	0.23	0.21	0.27
Ethanolamine	0.45	0.55	0.43
Hexosamine	0.42	0.32	0.26
Choline	0.50	0.32	----
Norleucine	0.59	1.00	0.66
Dehydrophytosphingosine[1]	0.90	----	----
Hydrolyzate			
Streak	0.08-0.16	0.0-0.17	0.19-0.42
Violet	0.46	0.54	----
Intense Violet	0.56	0.22	0.64

[1] Gift of Dr. H. E. Carter, University of Illinois, Urbana, Illinois.

TABLE 28.

Paper chromatographic analysis of ninhydrin positive materials contained in acid hydrolyzates of the alkali-insoluble fraction prepared from heat-denatured protein of pea embryo axes.

Materials	R_f DIPWA, After Hydrolysis: 2 N Sulfuric Acid, 6 Hrs.	R_f DIPWA, After Hydrolysis: 2 N Hydrochloric Acid, 12 Hrs.	R_f IAW, After Hydrol.: 2 N Hydrochloric Acid, 12 Hrs.
Reference Compounds			
Serine	0.20	0.22	0.36
Ethanolamine	0.45	0.44	0.56
Hexosamine	0.45	0.45	0.33
Choline	0.47	0.49	0.68
Norleucine	0.58	0.57	0.72
Dehydrophyto-sphingosine[1]	----	0.90	0.92
Hydrolyzate			
Streak	0.15	----[2]	----[3]
Violet	0.32	0.32	
Violet	0.45	0.45	
Intense Violet	0.57	0.60	

[1] Gift of Dr. H. E. Carter, University of Illinois, Urbana, Illinois.

[2] Replaced by 10 recognizable spots corresponding to R_fs of known amino acids.

[3] Approximately 12 ninhydrin positive spots were recognizable.

TABLE 29.

Apparent hexosamine content of extracts before and after paper chromatography. Samples equivalent to 10 g fresh weight of pea embryo axes.

Fraction	IAA Concentration	µM Hexosamine[1]	Ratio (IAA/Cont)
Total After Acid Hydrolysis[2]	None	0.36	2.2
	5×10^{-7} M	0.80	
Total After Acid Hydrolysis[3] and Purification by Paper Chromatography	None	0	---
	5×10^{-7} M	0	

---

1 Indole-Hydrochloric acid reaction (159).

2 2 N sulfuric acid, 6 hours, 100° C.

3 DIPWA, elution 3X with water.

In general, the intensity of the ninhydrin zones of the paper chromatograms paralleled the total amount of alkali-insoluble PGL isolated, being higher from IAA-treated tissues than from -untreated tissues. The relative distribution of ninhydrin intensities estimated from paper chromatograms are given in table 30, in the case of pea embryo axes.

b) Reducing sugars.--A zone chromatographically equivalent to uronic acid has been observed following mild acid hydrolysis (e.g., 0.1 N HCl, four hours, 103° C) of all fractions examined including the PGL fraction prepared by ammonium sulfate fractionation. A zone corresponding to hexose on DIPWA chromatograms has not been resolved further.

c) Glycerol.--A zone corresponding to glycerol has not been observed on chromatograms of acid hydrolyzates. Glycerol would normally be detectable on those chromatograms sprayed with silver nitrate followed by sodium hydroxide (157). Furthermore, glycerol analysis by conversion to acrolein (158) has failed to reveal detectable quantities either before or after acid hydrolysis.

Determination of sialic acid and hexosamine.--Sialic acid content of the various fractions was determined by the direct Erlich reaction as described by Gottschalk (159). The fractions tested were approximately equivalent to material from 10 g fresh weight of tissue and the method was calibrated using known quantities of a sample of N-acetylneuraminic acid (Sigma). Hexosamines were measured by reaction of the deaminated hexosamines with indole and hydrochloric acid (156). Tests for sialic

TABLE 30.

Relative ninhydrin intensities (absorbance at 560 mμ) of the color eluted from a series of 5 DIPWA chromatograms. Ninhydrin positive materials contained in acid hydrolyzates of the alkali-insoluble fraction prepared from heat denatured protein of pea embryo axes.

R_f(DIPWA)	Relative Ninhydrin Intensity
0.15 (streak)	52
0.45	8
0.57	40

acid were negative for both purified and crude preparations. Occasional small positive reactions for amino sugars were observed with crude extracts, however, these differences were lost during purification (table 29).

Salkowski reaction for sterols.--A chloroform solution of lyophylized chloroform-methanol extract was treated with concentrated sulfuric acid as a qualitative test for the presence of sterols (160). If the reaction is positive, the chloroform solution turns red and a green fluorescence appears in the sulfuric acid layer. Chloroform solutions of PGL from both Avena coleoptiles and pea stems yielded a red reaction with sulfuric acid upon exposure to air, however, the color was retained in the sulfuric acid layer and the tests are considered as being negative for sterols.

Qualitative detection of fatty acids.--Preliminary evidence for the presence of fatty acids was obtained by mixing an aliquot of a benzene extract of the material to be tested with a saturated solution of rhodamine B. The mixture was then shaken with a 1% solution of uranyl acetate. Higher carboxylic acids react with uranyl ions to form small quantities of undissociated salts (161). The undissociated salts then react further with the quinone form of the dye to yield benzene-soluble, red, addition compounds which are further characterized by an orange fluorescence in ultraviolet light (161). Following hydrolysis, all fractions yielded a positive reaction for fatty acids. No measurable reaction was observed before hydrolysis.

Methanolysis and analysis of fatty acid esters.--Approximately 12 mg of heat denatured protein from pea embryo axes (cell fractionation study of section B) was resuspended in 5 ml of an equal mixture of 1 N sodium hydroxide and biuret reagent and the alkali-insoluble PGL removed by centrifugation. The precipitate, dried in vacuo, was resuspended in 20 ml of absolute methanol containing 1 ml of concentrated sulfuric acid and hydrolyzed under reflux for six hours. The solution was cooled to room temperature and the fatty acids and esters were removed by shaking with four 10-ml volumes of petroleum ether (B. P. 60-71° C). The petroleum ether was removed in vacuo and the residue re-extracted with chloroform. Vapor phase chromatographic analysis of the concentrated chloroform-soluble extracts were performed by L. A. Rolle of the United States Department of Agriculture, Fruit and Vegetable Chemistry Laboratory, Pasadena, California. The vapor phase chromatographic system was that previously described by Ikeda, et al. (162). The unit was equipped with a 1/4 inch by 10 feet

stainless steel column packed with 60 to 80 mesh firebrick coated with a glycol-adipate polymer (LAC-446).

The chromatographic analysis of the methanolyzates revealed the presence of a number of volatile components tentatively interpreted as being methyl esters of higher fatty acids. These results are presented in figure 22.

A semilogarithmic plot of retention time of the fatty acid methyl ester standards [methyl laurate (C-12), methyl palmitate (C-16) and methyl stearate (C-18)] provided a basis upon which to predict the retention time of fatty acids of greater chain lengths. The volatile components of the hydrolyzates were found to form two homologous series as shown in figure 22. Components a, c, e and g corresponded approximately to the series of saturated fatty acids containing an even number of carbon atoms between C-22 and C-26. It is of interest to note that the second series, components b, d, f and h, would also correspond to the same homologous series if odd numbered fatty acids are included. Such fatty acids are now known to occur naturally in many lipide fractions (163, 164). No distinct fatty acid peaks were observed to coincide with saturated fatty acids having chain lengths of less than 20 carbons.

The area under the peaks corresponding to these volatile components were estimated after correcting for the rather high baseline (origin unknown). These data are given in table 31. The second (b, d, f and h) series appeared to be most influenced as a result of IAA-treatment, although all components show a small increase.

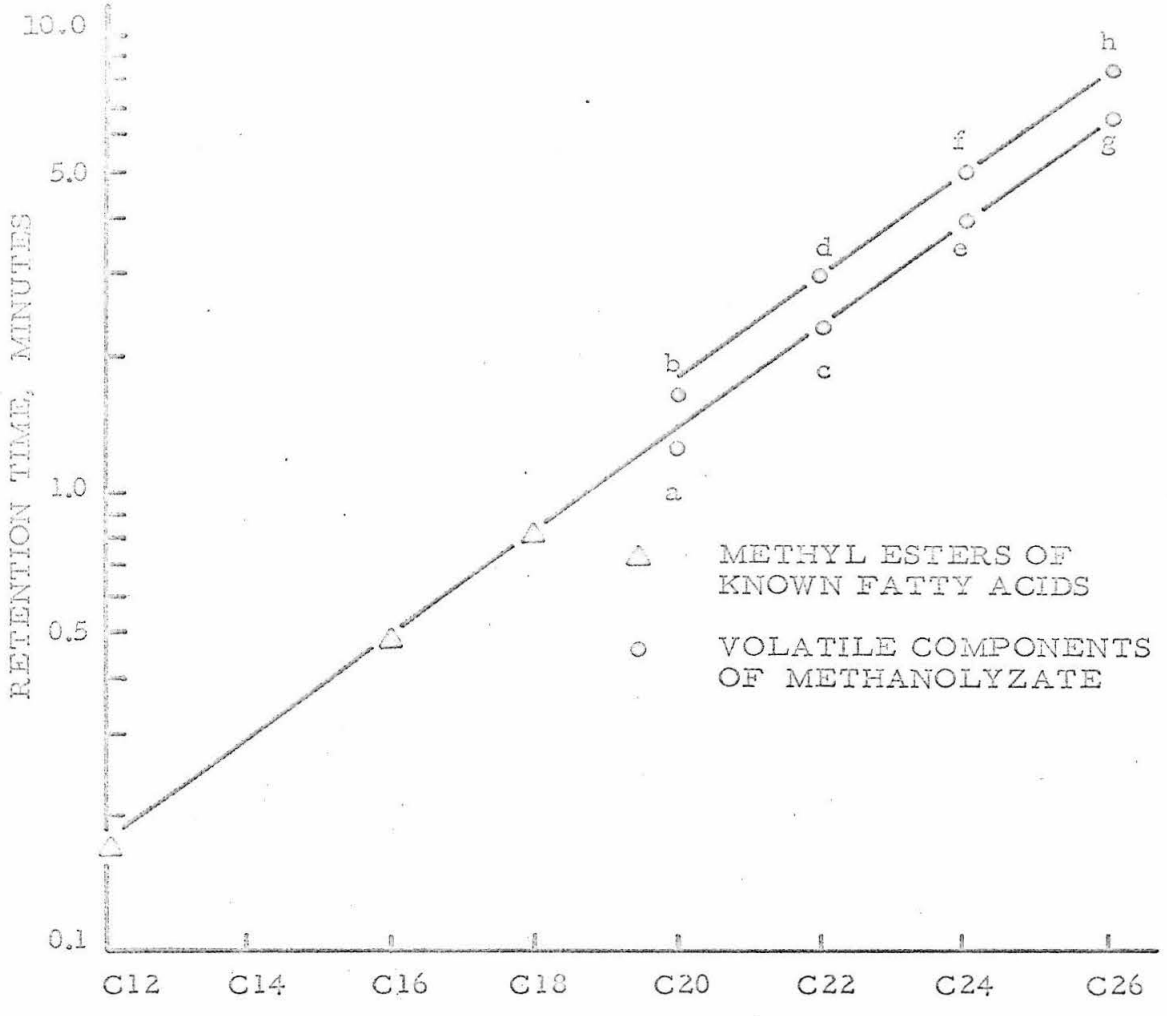


Figure 22. Results of a vapor phase chromatographic analysis of methanolyzates of the alkali-insoluble fraction prepared from heat denatured protein of pea embryo axes. The results show the correspondence between observed retention times of the volatile, petroleum ether-soluble components of the methanolyzates and the predicted retention times of the higher saturated fatty acids. Log plot. Column: 10 ft IAC-446. Column temperature: 210° C. Inlet pressure: 20 psig.

TABLE 31.

Relative amounts of volatile materials corresponding to methyl esters of long chain fatty acids as estimated from the areas under the vapor phase chromatography trace. The designated components refer to figure 22. Tissue was treated for 1 hour in the presence or absence of 5×10^{-7} M IAA.

Designation (in Order of Increasing Retention Time)	Area Percent (Minus Background)		Ratio
	Control	IAA	(IAA/Cont)
a	2.8	3.0	1.07
b	8.8	20.0	2.28
c	12.1	18.0	1.49
d	10.4	24.7	2.38
e	10.0	12.0	1.21
f	5.8	12.5	2.16
g	4.9	6.7	1.37
h	---	3.1	----

Lipide-soluble hydrolysis products.--Extraction of both acid and acidified alkaline hydrolyzates with a number of organic solvents, i.e., ethyl ether, chloroform, petroleum ether, revealed the presence of lipide-soluble hydrolysis products. Upon addition of acetone to concentrates of such extracts, a precipitate of a white waxy material formed (ninhydrin negative, M. P. 50-60° C). The material yellowed rapidly with age. The infra-red spectrum of this fraction in chloroform solution or as a solid film revealed the presence of distinct absorption bands at 3.4, 3.5 and

6.8 μ, with a recognizable band at 5.75 μ and a weak absorption in the region of 2.8 to 3.1 μ. These wavelength positions are consistent with the observed absorption bands of higher fatty acids (165).

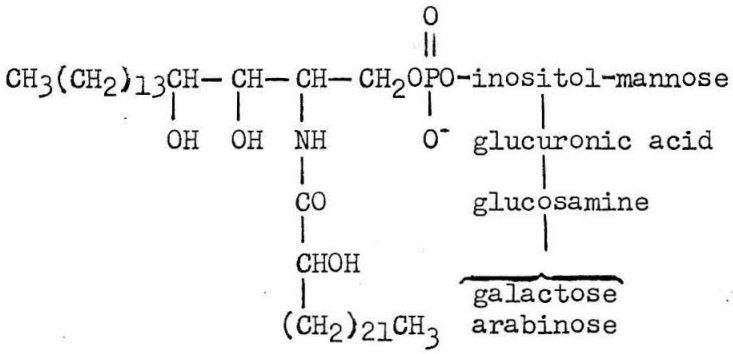
Discussion and Conclusions

Evidence presented is in support of the lipide nature of PGL. In addition to carbohydrate and esterified phosphate, the complex is suggested to contain one or more nitrogenous bases and possibly fatty acids. The appearance of amino acids following extended acid treatment may represent the hydrolysis of small amounts of contaminating protein, although a polypeptide component cannot be excluded. Hexosamine, sialic acid and Salkowski-positive sterols would appear to be absent or present only in trace quantities. However, these results were obtained with milligram-quantities of PGL and must be considered as being of a preliminary nature.

The presence of glycerol in the fraction has not been established, however, a number of complex glycolipides in which glycerol is absent have been reported to occur in plant tissues (166 to 169). Most glycolipides, nevertheless, contain glycerol in addition to phosphate, nitrogenous bases, fatty acids and various sugars. Proteolipides, compounds in which the peptide moiety and lipide are linked covalently, have been isolated from certain microorganisms (166, 170). The occurrence of authentic lipoproteins in plant tissue has not been definitely established (166).

A substance exhibiting solubility properties similar to those of PGL and with certain compositional similarities has been isolated and partially characterized by Carter and coworkers (171, 172). This material, in

which glycerol is replaced by a long chain amino-alcohol, phytosphingosine (173), is obtained by mild saponification of crude plant seed phosphatide mixtures. In addition to phytosphingosine, the sphingolipide contains fatty acid (e.g., cerebronic acid), phosphate, inositol, a hexuronic acid, D-glucosamine, arabinose, galactose and mannose. Degradation gives phytosphingosine, N-cerebronylphytosphingosine, N-cerebronylphytosphingosyl-1-phosphate, inositol phosphate and an oligosaccharide (172). A partial structural representation is given below:



Carter et al. (172) have suggested that this material may be an artifact of isolation and that the native lipides may be even more complex.

Kahn and Colvin (174) have also reported a non-glycerol glycolipide containing glucose, an unknown base, a fatty acid and phosphate. This substance is suggested to function as the precursor of cellulose microfibrils and has been obtained from extracts of Acetobacter xylinum (174, 175). Indirect evidence for the occurrence of similar cellulose precursors in higher plants has been obtained as well (176).

Other metabolic studies of complex non-glycerol lipides have been thus far largely restricted to sphingolipides of animal origin (sphingomyelins, cerebrosides and more complex substances). These materials contain the long chain amino-alcohol, sphingosine. Animal sphingolipides are of particular interest in experimentation concerning brain chemistry, metabolism and function. Sphingolipides are present in particularily high concentration in brain and nerve tissue and may accumulate during certain metabolic disorders (177).

The possibility that PGL may contain long chain fatty acids is of additional significance in view of reports of growth-promoting activity of long chain fatty acids and long chain fatty alcohols as measured with the Avena internode assay (178-180) and of long chain alcohols on maize roots (97). Crosby and Vlitos (178-180) have isolated a biologically-active long chain alcohol and long chain unsaturated fatty acid from tobacco. These authors have suggested that these substances may serve to replace indole auxins as growth regulators. Similar observations have been made with cuticular waxes of sugar cane (181) and Struckmeyer and Roberts (182) have also found a higher alcohol which they believe interacts with auxin. The origin of these higher fatty acids and alcohols has been ascribed to the plant waxes which may be localized within the cytoplasm (183-186), as well as within the cuticle.

In addition to direct growth-promoting effects of higher fatty acids and alcohols, a synergistic growth-promoting effect between IAA or gibberellic acid (or both), sucrose and certain lipides has been described in the case of pea epicotyl tissue (187 to 191). These results have

been summarized by Stowe (190). Fatty acid methyl esters (C-12 to C-20), certain triglycerides and fatty alcohols (C-18 to C-22) were found to be active in concentrations ca. 6 to 30 times greater than that of the added auxin. Free fatty acids were inactive in this system and no effect was produced in the absence of an external supply of IAA. The lipide effect is increased by sucrose and can be replaced by vitamins E and K (190, 191), an effect attributable to the long chain hydrocarbon substituent. Stowe (189, 191) has suggested that the lipides activate the cytochromes of pea tissue, however, evidence in support of this postulate is indirect. Vitamin K has also been reported to enhance IAA-induced root formation (192) but to exert no effect in the absence of an external supply of IAA. Another plant hormone bioassay, that for wound hormones (193), also responds to lipides.

Therefore, it would appear that lipides in small quantities may interact with auxin in promoting growth. However, lipides do not appear to be limiting in all tissues, since cell extension of Avena coleoptile tissue is not enhanced by addition of lipide (187).

E. SUMMARY

1. Evidence is presented for the existence of a readily soluble fraction of plant tissue which is increased or maintained in the presence of physiological concentrations of indoleacetic acid (IAA). The tissues studied most extensively include maize roots, Avena coleoptiles, pea epicotyls and pea embryo axes.

2. The materials isolated contain carbohydrates (uronic acid and hexose) and esterified phosphate. These components are increased 2- to 4-fold in amount as a result of prior IAA treatment.

3. The materials isolated also contain one or more nitrogenous bases and possibly fatty acids which also reflect the IAA-induced increase in amount.

4. The glycolipide fraction is isolated from aqueous solution in association with protein. Following disruption of the complex (and/or denaturation of associated protein), the glycolipide fraction is found to be insoluble in aqueous solvents but readily soluble in chloroform-methanol (2:1). The fraction is suggested to be of lipo-protein origin.

5. The cytoplasmic proteins associated with the glycolipide fraction exhibit increased stability against denaturation by heat. A phosphate containing glycolipide moiety is potentially capable of such stabilization.

6. Results of cell and tissue fractionation studies support the hypothesis that the fraction of interest is localized in the cytoplasm. Of the

cell particulates, nuclei and other particles sedimenting between 0 and 5,000 X g do not appear to reflect the IAA-induced increase in amount.

7. Possible physiological and biochemical significance of these findings is discussed.

IV. AN ANALYSIS OF THE pH 4.4 BUFFER-SOLUBLE, 70% ALCOHOL-INSOLUBLE (CBSEI) PECTIN FRACTION PREPARED FROM AVENA COLEOPTILES

Albersheim and Bonner (123) and Jansen, Jang, Albersheim and Bonner (46) reported that incubation of oat coleoptile tissue with 2.8×10^{-5} M indoleacetic acid (IAA) induces an accelerated incorporation of glucose into galacturonic acid residues of a pectin fraction soluble in cold acetate buffer, pH 4.4 and insoluble in 70% ethanol. The specific activity of galacturonic acid isolated by paper chromatography from this fraction was found to be several times greater than that of other pectic fractions. It was also demonstrated that in short term experiments, the specific activity of methyl groups from methionine incorporated as alkali lable methyl was five to six times greater in the case of the cold water-soluble pectic fraction than that of the corresponding methyl groups of the hot water-soluble and residual pectins (46).

That there does not appear to be a detectable IAA-induced enhancement of the incorporation of methyl groups of methionine into the cold buffer-soluble, 70% ethanol-insoluble fraction of maize roots is shown by the results presented in Part II. However, the fact that the method of preparation of the IAA-increased fraction of maize roots is nearly identical to that utilized in the preparation of the cold water-soluble pectin from Avena coleoptiles, strongly suggests a relationship between the two fractions. In order to test this possibility, experiments were designed

to attempt the isolation and purification of cold water-soluble pectins (CWSP) from the pH 4.4 buffer-soluble, 70% ethanol-insoluble (CBSEI) fraction of Avena coleoptiles.

A. PREPARATION AND PRELIMINARY CHARACTERIZATION OF THE CBSEI-FRACTION OF AVENA COLEOPTILES

The procedures followed in the preparation of the CBSEI-fraction of Avena coleoptiles follow closely those of Jansen et al. (46) and of Albersheim and Bonner (123) with only minor modifications adjusted to fit existing conditions.

Materials and Methods

Plant material.--Oat seeds of the variety Siegeshafer were soaked in distilled water with continuous aeration for a period of four hours. Germination was then continued in vermiculite soaked in distilled water, and contained in stainless steel trays as described in Part III-A. Seedlings were grown in low intensity red light at a temperature of 25° C until the coleoptiles had attained an average length of 2.5 to 3.0 cm.

Oat coleoptiles for preliminary analysis were harvested by cutting just below the coleoptilar node. Lots of approximately 50 g fresh weight each were sealed in polyethylene bags, quick-frozen with dry ice and stored in a deep freeze. As an alternative procedure, coleoptile sections, 13 mm long, were cut 3 mm from the apex with a double-bladed cutting tool. Primary leaves were not removed. All operations prior to homogenization were conducted in low intensity red light.

Preparation of the CBSEI fraction.--The coleoptile tissue was ground at 0° C with a pre-chilled mortar and pestle in the presence of 0.15 M sodium acetate buffer, pH 4.4 in a ratio of 1 g tissue to 4 ml of buffer. These particular conditions were initially chosen (46) since at this pH the de-esterification of pectin by pectin esterase is negligible (194).

Cell walls and the pH 4.4-insoluble cytoplasmic proteins were removed by a 30 minute centrifugation at 13,000 X g using a refrigerated centrifuge. Occasional difficulty was experienced in obtaining complete removal of cell walls and wall fragments by centrifugation. In order to circumvent this difficulty, a step was introduced in the procedure immediately following the initial homogenization in which the bulk of the cell walls and tissue fragments were removed by filtration through a single layer of miracloth.

In preliminary experiments, the cell wall fraction was resuspended in water at 0° C, followed by re-centrifugation. This procedure was repeated a total of five times. The combined supernatents were then heated to near boiling to denature protein which was removed by a low speed centrifugation (usually, 15 minutes at 5,000 X g). In the case of preparations involving 50 g fresh weight or more of Avena coleoptiles, the total volume of extract became excessive and the washing steps were omitted with little loss in total yield of carbazole positive carbohydrate.

The extracts, after removal of heat-denaturable protein, were concentrated approximately 15-fold under reduced pressure, using a Rinco rotary evaporator. As a means of hastening the evaporation of solvent, the extracts were heated during concentration; the temperature not exceeding 60° C.

The syrupy concentrate was then transferred to 15 ml conical centrifuge tubes and the alcohol concentration made to 70% by addition of the necessary volume of absolute ethanol. A final ratio of concentrate from 1 g fresh weight of tissue per ml of 70% ethanol provided reproducable results. Precipitation was conducted at 6° C for a period of 12 to 24 hours and the precipitate was collected by centrifugation (clinical centrifuge, 2,400 rpm, 5 minutes). The fraction was then re-precipitated from 70% ethanol to yield a white flocculant material relatively free of 70% ethanol-soluble carbohydrate. The yield was approximately 1 mg per g fresh weight of Avena coleoptiles.

Methods of analysis.--

a) Cold water-soluble pectin.--In preliminary experiments, total pectin was estimated by the pectinase-carbazole procedure of McComb and McCready (127) and McCready and McComb (195) as described in Appendix B. Materials to be analyzed were de-esterified by treatment of the crude extracts for 30 minutes at room temperature with an equal volume of 1 N sodium hydroxide. The pH was adjusted to between 5 and 5.5 with acetic acid and an equal volume of a pectinase solution (Nutritional Biochemicals Corp.) containing 0.1 mg crude pectinase per ml was then added. After one to three hours at room temperature, aliquots of the hydrolyzed samples were analyzed by reaction with carbazole in 87% sulfuric acid. Results are corrected for interfering colors derived from reaction of the fractions with sulfuric acid alone and for color derived from the pectinase solution. Results are expressed as total carbazole carbohydrate in terms of anhydrogalacturonic acid equivalent.

b) Protein.--Routine protein determinations were conducted according to the standard biuret method calibrated according to the procedure outlined in Part III-A.

c) Nucleic acid.--Total RNA of the pH 4.4-insoluble fraction was determined by the procedure of T'so and Sato (196). Nucleic acid content of the 70% ethanol-insoluble precipitate of the pH 4.4-soluble fraction was estimated from the ratio of the absorbance at 260 mμ and 280 mμ as determined from the complete absorption spectrum in the ultraviolet region.

d) Dry cell wall.--Measurements of total dry cell wall conform to the definition of cell wall as given by Jansen et al. (46). The procedure is described briefly as part of Appendix B.

Deproteinization of the CBSEI fraction.--A solution of chloroform-isoamyl alcohol, 24:1 (v/v) was used to remove heat-stable ethanol-insoluble protein according to the method of Sevag, Lackmann and Smolens (197). The final ethanol-insoluble precipitates were resuspended in 3 ml of 0.15 M sodium acetate buffer, pH 4.4. The resulting suspension was shaken with an equal volume of the chloroform-isoamyl alcohol mixture in a stoppered tube for 30 minutes using a wrist action shaker (Burell) operating at several hundred strokes per minute. The resulting emulsion was separated into three layers by centrifugation for 5 to 15 minutes at 13,000 X g. The upper aqueous phase was carefully removed using a pasteur pipette and was placed under a stream of nitrogen to remove traces of the chloroform-isoamyl alcohol mixture. The chloroform causes surface denaturation of the proteins and by carefully removing the bottom chloroform layer by pipetting,

the protein layer was found to adhere to the side of the tube, thus permitting recovery of approximately 90% of the added chloroform. The protein and chloroform fractions were evaporated to dryness under nitrogen prior to analysis.

Results and Discussion

Total carbazole carbohydrate.--The carbazole reaction when applied to the analysis of the crude CBSEI fraction tended to yield variable results, even when applied to the same sample. The reproducibility of the entire extraction procedure was found to lie in the range of 10 to 15% variation based on analyses representing the average of a minimum of three carbazole determinations per sample (table 32). Each value represents a separate analysis of the CBSEI fraction obtained from a 3 g sample of 13 mm Avena coleoptile sections.

Although the values obtained are consistently lower than those reported by Jansen et al. (46), the absolute amounts are somewhat dependent upon the type of corrections applied, as well as the dilution at which the determinations are carried out.

Carbohydrates of the 70% ethanol supernatent fraction.--Chromatographic analysis (DIPWA, Appendix E) and reaction with carbazole in 87% sulfuric acid still revealed the presence of interfering levels of monosaccharides in the ethanol-soluble phase following a second precipitation from 70% ethanol. No ethanol-soluble uronic acids were detected either before or after treatment with base and pectinase. A third precipitation failed to solubilize sufficient amounts of monosaccharide to warrant its inclusion in the general procedure.

TABLE 32.

Total carbazole carbohydrate content of the CBSEI fraction prepared from Avena coleoptile sections. Expressed in terms of anhydrouronic acid equivalent based on total cell wall.

Avena Preparation	Mg/100 mg Total Cell Wall
I	0.13
II	0.12
III	0.11
IV	0.09
V	0.10
VI	0.12
Ave.	0.11 ± 0.014

Distribution of protein and nucleic acid.--Preliminary experiments revealed that protein was present in all preparations of the CBSEI fraction although in amount it represented only a small fraction of the total soluble protein of the initial pH 4.4 buffer-soluble extract (table 33). The major fraction of the protein solubilized during homogenization and extraction was sedimented during the initial 13,000 X g centrifugation, as well as the bulk of the total cell nucleic acid. The RNA content of the pH 4.4 pellet remaining after removal of the cell wall and debris by filtration was found to account for approximately 50% of the total cell RNA, whereas, the nucleic acid content of the final 70% ethanol-insoluble precipitate was estimated to account for less than 0.1% of the total.

The amount of pH 4.4-soluble protein was found to vary between 16 and 32 mg per 50 g fresh weight, but that portion stable to precipitation by heating was relatively constant in amount.

Distribution of carbohydrate following Sevag deproteinization.--

a) Carbazole carbohydrate.--After removal of protein by Sevag deproteinization, the total amount of carbazole carbohydrate was reduced by a factor of nearly two (table 34), a fact that remains incompletely understood. This finding was initially misleading since the carbohydrate was presumed to have remained complexed with the protein fraction. Subsequent analysis of the protein fractions by both the carbazole procedure and by paper chromatographic methods following acid hydrolysis, indicate that this is not so.

TABLE 33.

Distribution of protein among various fractions obtained during isolation of the CBSEI fraction of Avena coleoptiles. As total TCA-insoluble protein.

Protein Fraction	Mg Protein/50 g Fr. Wt.
pH 4.4 Insoluble	350
pH 4.4 Soluble, Precipitable by Boiling	29
pH 4.4 Soluble, Heat Stable (Biuret)	2

Interference from organic solvents has been eliminated by evaporating the samples to dryness prior to the determination of carbazole carbohydrate. Cysteine, glutathione and other S-H compounds, when present in solution do increase the intensity of the color obtained with uronic acids, as well as with hexoses and pentoses (Appendix B). Such materials derived from protein could account for the loss in carbazole intensity as a result of Sevag treatment, although conclusive proof is lacking.

b) Total hexose.--The distribution of hexose in various fractions obtained after Sevag deproteinization are summarized in table 35. The results were obtained by means of the carbazole-water reaction applied directly to the samples referred to in table 34. Recovery of total hexose was essentially complete with the bulk of the hexose being retained

TABLE 34.

Distribution of total carbazole carbohydrate[1] in various fractions obtained following removal of heat-stable protein by the Sevag procedure.

Deproteinization Treatment	µg Carbazole Carbohydrate as Anhydrogalacturonic Acid Equivalent			
	Expt I	Expt II	Expt III	Ave.
None	432	540	640	537
Sevag				
Aqueous Phase	210	252	384	282
Protein Phase	28	34	53	39
$CHCl_3$ Phase	5	5	5	5
Total	238	286	438	321
% Recovery	55	53	53	57

[1] Reaction with carbazole in 87% sulfuric acid.

TABLE 35.

Distribution of total hexose[1] in various fractions obtained following removal of heat stable protein by the Sevag procedure.

Deproteinization Treatment	µg Total Hexose as Glucose Equivalent			
	Expt I	Expt II	Expt III	Ave.
None	1805	1765	1815	1795
Sevag				
Aqueous Phase	1565	1535	1658	1586
Protein Phase	207	200	205	204
$CHCl_3$ Phase	5	5	5	5
Total	1772	1735	1863	1790
% Recovery	97	98	102	100

[1] Carbazole-water reaction (Appendix B).

in the aqueous phase after Sevag deproteinization. Subsequent experimentation revealed that approximately 80% of the hexose of the aqueous phase was no longer ethanol-insoluble following Sevag treatment. Similar results were obtained by precipitation of the protein in the presence of cold 7.5% TCA. It is of interest in this regard that similar treatments involving chloroform-isoamyl alcohol mixtures are known to disrupt carbohydrate-protein complexes, including supposed mucopolysaccharides (198).

Carbohydrates of the chloroform-extractable fraction.--The total chloroform-extractable material following Sevag deproteinization from 50 g fresh weight of tissue was concentrated under nitrogen to ca. 20 mg of a viscous, yellow-brown liquid. Chromatography in DIPWA revealed the presence of hexose as well as an additional component with an R_f of 0.8 to 0.9 also reacting with silver nitrate-sodium hydroxide spray reagent for reducing sugars (199) but not corresponding to any of the carbohydrate standards tested. This material was found to become highly radioactive following administration of uniformly labeled glucose-C^{14} to the tissue. The zone did not react with ninhydrin or with a molybdate dip test for phosphate (200). Following acid hydrolysis only the presence of hexose was observed.

Conclusions

The procedure as outlined was found to yield preparations of cold buffer-soluble, 70% ethanol-insoluble material corresponding to approximately 1 mg per g fresh weight of tissue. Expressed on this basis, the

average carbazole carbohydrate content as anhydrouronic acid was 29 µg per g fresh weight and protein accounted for an additional 40 to 60 µg. The major fraction of the extracted nucleic acid was sedimented with the pH 4.4-insoluble fraction.

Deproteinization of the CBESI fraction by means of either the Sevag procedure or precipitation in the presence of 7.5% TCA resulted in the formation of an aqueous fraction containing the bulk of the carbohydrate, 80% of which was no longer precipitable by 70% ethanol. Hexose appeared as a major carbohydrate constituent of the CBSEI fraction. These facts, in addition to the presence of an apparent "amplification" factor lost during deproteinization, would seem to invalidate any form of quantitative interpretation of total carbazole carbohydrate in terms of pectin-bound uronic acid.

B. A CHROMATOGRAPHIC ANALYSIS OF THE pH 4.4 BUFFER-SOLUBLE, 70% ALCOHOL-INSOLUBLE PECTIN FRACTION (COLD WATER-SOLUBLE PECTIN)

Due to a number of difficulties encountered in interpretation of the results of carbazole carbohydrate analysis in terms of anhydrouronic acid (pectin), it became necessary to follow further purification of cold water-soluble pectin using assays based on paper chromatographic methods. Discussion will be restricted to the analysis of crude 70% ethanol-insoluble precipitates.

Materials and Methods

Hydrolysis of pectin.--Samples to be analyzed were treated with an equal volume of 2 N ammonium hydroxide for four hours at room temperature in tightly stoppered tubes. Excess ammonia was removed under a stream of nitrogen and the pH adjusted to 5.0 to 5.5 with acetic acid. The samples were then treated for varying times with citrus polygalacturonase in a ratio of 0.1 mg of enzyme per mg of 70% ethanol-insoluble precipitate. The sample of purified citrus polygalacturonase was obtained through the courtesy of E. F. Jansen, Western Regional Research Laboratory, Albany, California.

Paper chromatography.--Enzymatic hydrolyzates were subjected to paper chromatography using a descending solvent system containing isopropanol, pyridine, water and acetic acid; 8:8:4:1 (v/v) (DIPWA) for the isolation and purification of uronic acids. This particular solvent separates galacturonic acid from glucuronic acid as well as from all neutral sugars

tested (Appendix E). Neutral sugars are not resolved sufficiently to permit identification and are characterized by R_f values near 0.5. Therefore, the zone of the paper chromatograms from R_fs of 0.4 to 0.6 is referred to as the neutral sugar zone. The zone from R_fs of 0.18 to 0.28 is designated as the uronic acid zone. All preparative chromatography was conducted using Whatman 3 mm filter paper previously washed for three days with a mixture of ethyl acetate, acetic acid and water (10:5:4, (v/v)). Sugars were detected using the silver nitrate reagent of Trevelyan (199).

When carbohydrates were determined directly from paper chromatograms, the triphenyltetrazolium chloride method was used as discussed in Appendix B. For this purpose, washed Whatman No. 1 filter paper was substituted in the above chromatographic system.

Paper electrophoresis.--Paper electrophoretic separations of both crude and enzymatically hydrolyzed CBSEI fractions were achieved by the electrophoresis systems described in Appendix E.

Results and Discussion

The electrophoretic patterns (System E-I, Appendix E) of the crude preparations of the CBSEI fractions of Avena coleoptiles are shown in figure 23. Treatment with trypsin at pH 8 (201), for a period of eight hours resulted in the liberation of a number of ninhydrin positive spots not present initially. The carbohydrate distribution was of particular interest, since the bulk of the reducing materials remained near the point of origin both before and after treatment with pectinase. The

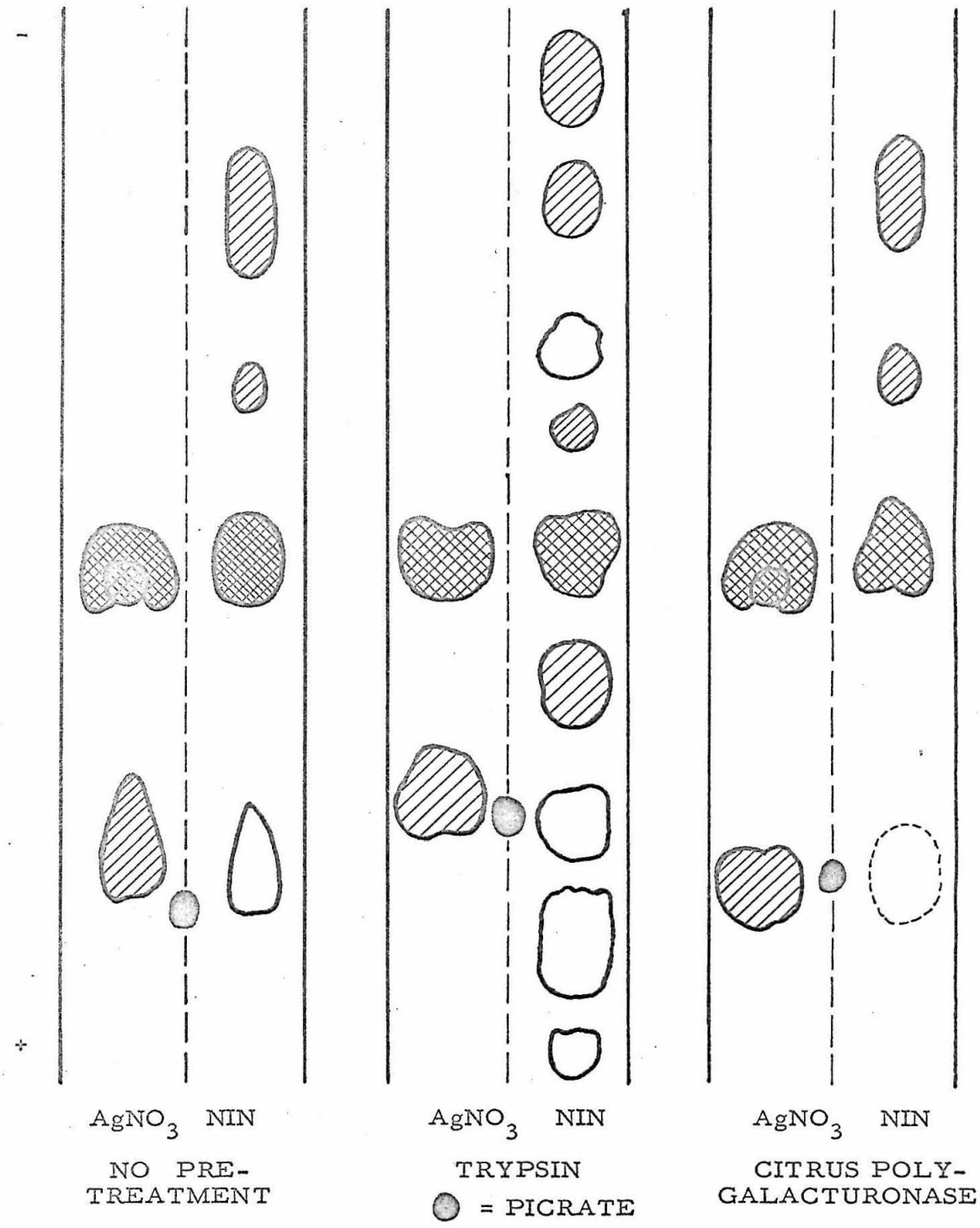


Figure 23. Electrophoretic separation of the components of the CBSEI fraction of Avena coleoptiles before and after enzymatic treatment with purified trypsin and citrus polygalacturonase. System E-I, Appendix E. Samples were applied in duplicate. $AgNO_3$ = silver nitrate reagent for detection of reducing sugars (199). NIN = ninhydrin. The degree of cross-hatching provides an indication of the relative intensity of the spots.

electrophoretic properties of the lighter reducing zone, with a mobility approximately that of picric acid, were little changed by enzymatic treatment.

In order to provide an estimate of the relative electrophoretic distribution of materials after treatment with pectinase, the time of electrophoresis was reduced to one hour and the resulting electrophoretogram was eluted in three sections corresponding to acidic, neutral and basic materials. The results obtained by reaction of the eluates with carbazole in 87% sulfuric acid are given in table 36. Expressed in terms of uronic acid equivalent, acidic materials (to include any uronic acids present) accounted for only 10% of the total carbazole carbohydrate. The calculated amount based on 0.25% of the cell wall as reported by Jansen et al. (46) is included for comparison. Similar results obtained by the tetrazolium assay (electrophoretic separation: System E-II, Appendix E) both before and after deproteinization by the Sevag procedure (197) are given in table 37.

In order to determine whether or not any of the uronic acid zones observed as the result of paper chromatographic and electrophoretic separation were derived from authentic pectin, an experiment was designed to test the effect of treatment with various pectic enzymes on the amount of uronic acid isolated. Polygalacturonase treatment was extended to a period of three days under toluene. The results based on the tetrazolium assay are given as table 38. These data show, certainly, that the amount of tetrazolium positive material contained in the uronic acid zone of the paper chromatograms was increased 2- to 3-fold by treatment with purified citrus polygalacturonase, following a preliminary treatment to insure

TABLE 36.

Electrophoretic distribution of total carbazole carbohydrate of the CBSEI fraction of Avena coleoptiles after treatment with base and pectinase.[1] Electrophoresis conducted for a period of 1 hour (System E-I, Appendix E).

Zone Eluted	Carbazole Carbohydrate as µg Galacturonic Acid Equivalent
Acid Materials	4
Basic Materials	0
Neutral Materials	40

[1] Starting material equivalent to 64 µg anhydrouronic acid based on 0.25% of the cell wall (46).

TABLE 37.

Distribution of total reducing carbohydrate of the CBSEI fraction of Avena coleoptiles electrophoretically equivalent to uronic acids (System E-II, Appendix E) as determined before and after deproteinization of the extracts by the Sevag procedure[1]. Reaction with triphenyltetrazolium chloride.

Deproteinization Treatment	µg Anhydrogalacturonic Acid Equivalent	Relative Tetrazolium Intensity
None	62	100
Sevag		
Aqueous Phase	71	114
Protein Phase	7	12
$CHCl_3$ Phase	7	12
Total	85	137

[1] Starting material equivalent to 660 µg anhydrouronic acid based on 0.25% of the cell wall (46).

TABLE 38.

Effect of various chemical and enzymatic pretreatments on the release of reducing materials from the CBSEI fraction of Avena coleoptiles chromatographically equivalent to uronic acids. Each analysis is derived from material equivalent to 260 µg anhydrouronic acid based on 0.25% of the cell wall (46).

Pretreatment	Enzymatic Hydrolysis	µg Anhydrogalacturonic Acid Equivalent Isolated	
		Expt I	Expt II
None	None	10	10
None	Polygalacturonase	10	10
1 N NH_3	None	37	34
1 N NH_3	Polygalacturonase	36	33
Pectin Esterase, pH 8	None	21	33
Pectin Esterase, pH 8	Polygalacturonase	26	32

de-esterification (ammonium hydroxide or pectin esterase). However, the same effect was obtained by treatment with ammonium hydroxide alone or with pectin esterase for a period of four hours at pH 8. Treatment with polygalacturonase for three days at pH 4.4 was apparently ineffective.

Since the samples were treated for three days under toluene in order to insure complete hydrolysis, the possibility that galacturonic acid was also destroyed was investigated. A known sample of 100 µg of galacturonic acid as the monohydrate was added to an aliquot of the CBSEI fraction resuspended in pH 4.4 acetate buffer. The resulting mixture and a parallel aliquot of the CBSEI fraction containing no added uronic acid were then treated with base followed by purified citrus polygalacturonase for three days in the manner of the previous experiment. Within the experimental error of the tetrazolium assay, complete recovery of the added galacturonic acid was achieved (table 39) under these conditions.

In the case of electrophoretic separation, no pretreatment was necessary in order to obtain maximal tetrazolium intensity at the zone corresponding to uronic acid (table 40). In all experiments, the zone reacting most intensely with tetrazolium corresponded to neutral sugars.

That the material remaining at the origin in these chromatographic analysis does not contain large quantities of unhydrolyzed pectin is indicated by the data of table 41. In these experiments, the total uronic acid after treatment with base and pectinase was determined by two modifications of the carbazole reaction for uronic acids. The first

TABLE 39.

Recovery of uronic acid added to the CBSEI fraction of Avena coleoptiles and treated for 3 days with a preparation of citrus polygalacturonase. Uronic acid estimated from paper chromatograms following reaction with triphenyltetrazolium chloride.

Fraction[1]	µg Carbazole Carbohydrate Isolated as Anhydrogalacturonic Acid Equivalent
CBSEI	46 ± 10
CBSEI + 100 µg Galacturonic Acid	128 ± 30

[1] CBSEI equivalent to 320 µg anhydrogalacturonic acid based on 0.25% of cell wall (46).

reaction conducted at 100° C in 87% sulfuric acid reacts with both hydrolyzed and unhydrolyzed pectic materials to about the same degree (Appendix B). The second reaction, conducted at 60° C with 83% sulfuric acid (also described in Appendix B) is known to react with free uronic acid but not with pectin-bound uronic acid (125, 202). The two determinations were found to provide comparable results.

The chemical identity of the uronic acid-like reducing substance has not been determined although certain evidence would suggest glucuronic

TABLE 40.

Effect of treatment with ammonia and citrus polygalacturonase on the release of reducing sugars from the CBSEI fraction of Avena coleoptiles electrophoretically equivalent to uronic acids (System E-II, Appendix E).[1]

Pretreatment	Enzymatic Hydrolysis	µg Anhydrogalacturonic Acid Equivalent Released
None	None	39
1 N NH_3	None	40
1 N NH_3	Citrus Poly-galacturonase	35

[1] Aliquot analyzed equivalent to 64 µg anhydrouronic acid based on 0.25% of the cell wall (46).

acid as a possibility. The absorption spectrum of the carbazole adduct of the material eluted from paper chromatograms and following electrophoresis qualitatively and quantitatively resembles that of a hexuronic acid. The chromatographic and electrophoretic data based on a large number of experiments are summarized in table 42.

The chromatographic system utilized in these experiments was selected on the basis of the possibility of separating glucuronic acid from galacturonic acid, the common constituent of pectin. Inorganic

TABLE 41.

A comparison of total carbazole carbohydrate of the CBSEI fraction from Avena coleoptiles as determined by 2 modifications of the carbazole-sulfuric acid reaction for uronic acids.

Basis For Estimation	µg Carbazole Carbohydrate as Anhydrogalacturonic Acid Equivalent[1]
Carbazole Reaction-87% H_2SO_4 (Reacts With Both Free and Pectin Bound Uronic Acids)	361
Carbazole Reaction-83% H_2SO_4 (Reacts With Free But Not With Pectin Bound Uronic Acids)	358
As 0.25% of Cell Wall	640

[1] Determined after standard treatment with alkali followed by pectinase.

phosphate, salts of various organic acids and certain amino acids were found to coincide with the uronic acid-like material on paper chromatograms. Of the substances tested in the electrophoretic systems, nucleotide monophosphates and glutamic and aspartic acids were characterized by similar electrophoretic mobilities. In most experiments employing either chromatography or electrophoresis but not both, the uronic acid zone was found to also contain ninhydrin active materials and acid labile

TABLE 42.

Chromatographic and electrophoretic properties of the uronic acid-like substance from the CBSEI fraction of Avena coleoptiles as compared with those of authentic samples of glucuronic acid and galacturonic acid.

Material Analyzed	R_f (DIPWA)	$R_{picrate}$ (E-II)
Galacturonic Acid	0.19-0.21	0.85-0.89
Glucuronic Acid	0.24-0.26	0.96-1.00
Uronic Acid-like Substance From CBSEI Fraction	0.20-0.25	0.94-1.10

phosphate. Both of these properties were lost following a combined separation including both chromatography and electrophoresis, in either order. Essentially no loss of reducing sugar occurred when the elutions were conducted carefully and the eluates concentrated under nitrogen.

Preliminary observations as to the chromatographic distribution of CBSEI carbohydrate after pectic hydrolysis.--A final series of experiments was conducted in which the distribution of tetrazolium positive reducing carbohydrate of the entire DIPWA chromatograms was analyzed. These results are summarized in table 43. The regions of highest intensity were located in a region near the point of application, the uronic acid zone and a zone corresponding to neutral sugars. The zone between R_f 0

TABLE 43.

Distribution of carbohydrate following paper chromatography (DIPWA) of the CBSEI fraction of Avena coleoptiles. Resuspended in 0.5 N ammonia and neutralized to pH 5 with acetic acid prior to chromatography.

Deproteinization Treatment	µg Anhydrogalacturonic Acid Equivalent, R_f		
	0-0.13	0.18-0.28	0.40-0.60
None	152	42	11
Sevag			
Aqueous Phase	121	50	20
Protein Phase	---	15	--
$CHCl_3$ Phase	---	11	--
Total	121	76	20

and 0.13 represented a streak concentrated at the origin which was largely localized in the aqueous phase following Sevag deproteinization. The intensity of the hexose zone was approximately doubled following deproteinization and was subsequently shown to be present in both the chloroform and protein fractions by means of the more sensitive silver nitrate spray reagent (157,.199).

Conclusions[1]

It is concluded that the pectin-bound uronic acid content of the cold buffer-soluble, 70% ethanol-insoluble fraction as prepared from Avena coleoptiles is less than 0.6 µg per g fresh weight, an amount equivalent to less than 0.0025% based on dry cell wall. Therefore, the existence of a cold water-soluble, 70% alcohol-insoluble pectin fraction as defined would seem doubtful. A small amount of an uronic acid-like material, ca. 6 µg per g fresh weight is present, however, presumably in the form of a relatively stable association at pH 4.4. The material was found to be dissociated under relatively mild conditions of electrophoresis at pH 5.4 and at alkaline pH.

[1] The results reported in this section were verified using radioactive tracers and extended as part of a study conducted in cooperation with Dr. A. C. Olsen (49). The incorporation of radioactivity from glucose-U-C^{14} into the CBSEI fraction of Avena coleoptiles in the presence and absence of IAA does reflect labeling changes in both an acidic fraction, as well as neutral carbohydrate. Of the neutral carbohydrates, the labeling of galactose was most influenced by IAA. The acidic fraction was shown to contain various salts of malic acid as a major component which in many solvents are characterized by chromatographic properties similar to those of the uronic acids. Malic acid was identified from the chromatographic properties of the free acid and its salts in a variety of solvents, co chromatography and co-electrophoresis with authentic malic acid and by the preparation of its p-toluidine derivative. The IAA-induced increase in incorporation of label from glucose into this acid varied between experiments but was normally represented by a 1.5- to 2.0-fold increase. The rate of labeling of the uronic acid-like material was largely unaffected by treatment of the tissues with IAA.

C. SUMMARY OF THE INFLUENCE OF INDOLEACETIC ACID (IAA) ON THE RELATIVE DISTRIBUTION OF NON-URONIDE CONSTITUENTS OF THE CBSEI FRACTION OF AVENA COLEOPTILES

During the course of the investigations reported in Part IV, Sections A and B, the CBSEI fraction of Avena coleoptiles was found to contain a variety of non-uronide components, some of which have been partially characterized. In view of the apparent heterogeneity of the fraction, these results will be presented in summary form only.

Materials and Methods

Acid hydrolysis and chromatography of the neutral sugar fraction.-- Unless stated otherwise, the carbohydrate sample to be hydrolyzed was placed in a sealed ampule in the presence of 0.1 N sulfuric acid for periods of time varying between 15 minutes and 6 hours. The hydrolyzed sample was neutralized with barium carbonate and the insoluble barium sulfate removed by centrifugation. The barium sulfate precipitate was then washed with water and the combined supernatent fractions concentrated under nitrogen prior to chromatographic analysis.

Measured aliquots of the concentrated samples were applied to 35 mm wide strips of Whatman No. 1 filter paper and irrigated for 24 hours with an ascending solvent system consisting of ethylacetate-pyridine-water, 12:5:4 (v/v). In order to maintain solvent flow, each strip was fitted with several additional layers of Whatman 3 mm paper to serve as a reservoir for excess solvent reaching the top of the chromatograms.

Methods of detecting the carbohydrate zones on the paper chromatograms included the use of a silver nitrate reagent (157, 199), an aniline-diphenylamine reagent (157, 203) and 0.1% triphenyltetrazolium chloride in alkaline solution (204).

Resolution of carbohydrate and protein by cesium chloride density gradient separation.--A concentrated suspension of the CBSEI fraction prepared from 50 g of Avena coleoptiles and resuspended in 0.15 M sodium acetate buffer, pH 4.4 was added to sufficient cesium chloride to yield a final concentration of 4 M with respect to cesium chloride in a total volume of 12 ml. The mixture was centrifuged in lusteroid tubes in the model L Spinco preparative centrifuge at 30,000 rpm in the SW-39 rotor at 2° C for 44 hours. The tubes were fractionated by securing the tubes firmly in the vertical position and dripping the contents into collection tubes through a small hole bored in the bottom of the tube with a small gauge needle. The collected fractions were analyzed for protein and anthrone carbohydrate content. The concentrations of cesium chloride remaining after dilution of the sample did not interfere appreciably in either assay.

Determination of non-uronide constituents.--The analytical procedures followed are cited in conjunction with the results summarized in table 44.

Results and Discussion

Distribution of non-uronide constituents of the CBSEI fraction.--The data summarized in table 44 represent analyses conducted over a period of several months utilizing the CBSEI fractions from a number of

TABLE 44.

Summary of the non-uronide constituents of the CBSEI fraction of Avena coleoptiles as influenced by treatment of the tissue in the presence and absence of 5 X 10^{-6} M IAA. The mean ratio, based on 5 to 10 preparations is given in parenthesis.

Fraction Analyzed	Quantity in mg Control	IAA	Ratio IAA/Control
Weight of Sections[1]	53,300	62,700	1.2
Protein Precipitable by Boiling[2]	16-32	18-36	1.1
CBSEI Fraction[3]	32-53	43-64	1.1-1.3 (1.2)
7.5% TCA-insoluble, Heat Stable Protein[2]	1.9-2.8	3.4-4.0	1.3-2.0 (1.5)
Nonprotein Nitrogen[4]	0.5	---	---
Anthrone Carbohydrate[5]	1.4-1.6	1.4-1.8	1.0-1.3 (1.1)
Carbazole-water Carbohydrate[6]	1.6-1.8	1.6-2.0	1.0-1.3 (1.1)
Orcinol Carbohydrate[7]	Not Calibrated		1.0
Inorganic Phosphate[8]	7.7-9.7	9.1-12.8	1.1-1.3 (1.3)
Nucleic Acid[9]	.15	.15	---
Total Accounted For	11.0-14.1	13.6-18.6	

---

[1] Fresh weight of 50 g of 13 mm Avena coleoptile sections after treatment for 8 hours in the presence and absence of 5 X 10^{-6} M IAA. [2] Protein determined by means of the standard biuret method (103). [3] Total 70% ethanol precipitate after lyophilization. [4] According to the procedure of Eggman (205). [5] In terms of glucose equivalent. Procedure of Scott and Melvin (104). [6] In terms of glucose equivalent. Procedure outlined in Appendix B. [7] Method of Dische and Schwarz (206). [8] Method of Allen (128). [9] Absorption at 260 mμ.

different Avena preparations. The components analyzed; carbohydrate, protein and inorganic phosphate; account for nearly one-third of the total material present, with inorganic phosphate representing the major component measured. Since none of the components measured, except heat stable proteins, reflected a significant IAA-induced increase in total amount, further investigations as to the nature of the remaining components of the fraction were discontinued. Preliminary results indicated that a variable amount of lipide may be present as well.

Acid hydrolyzates of the neutral sugar fraction were found to contain, in addition to an uronic acid-like material, reducing sugars chromatographically equivalent to glucose, galactose, arabinose and xylose. The presence of mannose has not been excluded, however, due to the coincidence of the R_f of mannose with that of other sugars in the solvent systems utilized. The identity of glucose as a regular constituent of the CBSEI fraction of Avena coleoptiles was confirmed by a secondary cysteine reaction characteristic for glucose (207) and by electrophoresis in 1 N NaOH according to the procedure described by Kowkabany (208). Neither ribose nor sugar alcohols, including glycerol, were detected either before or after acid hydrolysis. Tests for hexosamine and sialic acid were negative.

Varying amounts of fructose occurred in the extracts as evidenced by chromatography in DIPWA. As an aid to the identification of fructose, guide strips were sprayed with a solution of aniline-diphenylamine and heated at 95 to 100° C for two to three minutes. Under these conditions, fructose was found to yield a characteristic brown-colored spot. /Uronic

acids yielded a reddish-colored spot while glucose, galactose, arabinose and mannose reacted to give blue-green spots. Fructose appeared in hydrolyzates with times of hydrolysis as short as 15 minutes in the presence of 0.1 N sulfuric acid at 100° C, indicative of a furanoside linkage (209).

The carbohydrates of the CBSEI fraction of Avena coleoptiles were largely readily hydrolyzed to monosaccharides. Chromatographic estimates of total reducing activity indicated that hydrolysis was relatively complete after four to six hours in the presence of 0.1 N sulfuric acid at 100° C. Hydrolysis for six hours in the presence of 1 N sulfuric acid resulted in the destruction of between 50 and 70% of the total carbohydrate as estimated from measurements of radioactivity of preparations obtained from tissue treated in the presence of glucose-U-C^{14}.

The consistent increase (1.3- to 2.0-fold) in heat stable protein of the CBSEI fraction of Avena coleoptiles as a result of treatment for eight hours in the presence and absence of IAA may be the result of the heat stabilizing effect of the IAA-induced glycolipide fraction (Part III). The possibility that at least a portion of the carbohydrate of the 70% ethanol-insoluble fraction of Avena coleoptiles might occur in a relatively stable association with protein was investigated as a possible explanation to account for the heat stability of the protein associated with the CBSEI fraction. The cesium chloride density gradient technique, introduced by Meselson, Stahl and Vinograd (210), make it possible to separate carbohydrate, protein and protein-complexed carbohydrate under relatively mild conditions. Under the conditions utilized, 4 M cesium chloride, pH 4.4,

carbohydrate would be expected to concentrate at the bottom of the centrifuge tube, whereas, proteins would float as a skin on the top. Any complexed material would have been expected to band at an intermediate density. The results of such an experiment are shown in figure 24. No evidence of a discrete carbohydrate-protein band was obtained since the protein skins were found to be essentially devoid of anthrone carbohydrate and the zones containing carbohydrate were without detectable quantities of protein.

Observation of the presence of the IAA-increased glycolipide fraction as a component of the CBSEI precipitates of Avena coleoptiles.--There are a number of indications that varying quantities of a material with properties similar to that of the IAA-increased glycolipide fraction may be precipitated from 70% ethanol with the CBSEI fraction of Avena coleoptiles. Alkali-insoluble precipitates have been observed with several preparations including the one subjected to cesium chloride density gradient analysis. An IAA-induced increase in ninhydrin materials corresponding chromatographically to those of the IAA-increased glycolipide fraction lead to early speculation concerning the possible presence of a hexosamine containing mucopolysaccharide. These observations proved to be exceptional, however.

Conclusions

The bulk of the CBSEI fraction of Avena coleoptiles appears to consist of a complex mixture of substances which apparently co-precipitate in the form of a relatively stable association in the presence of 0.15

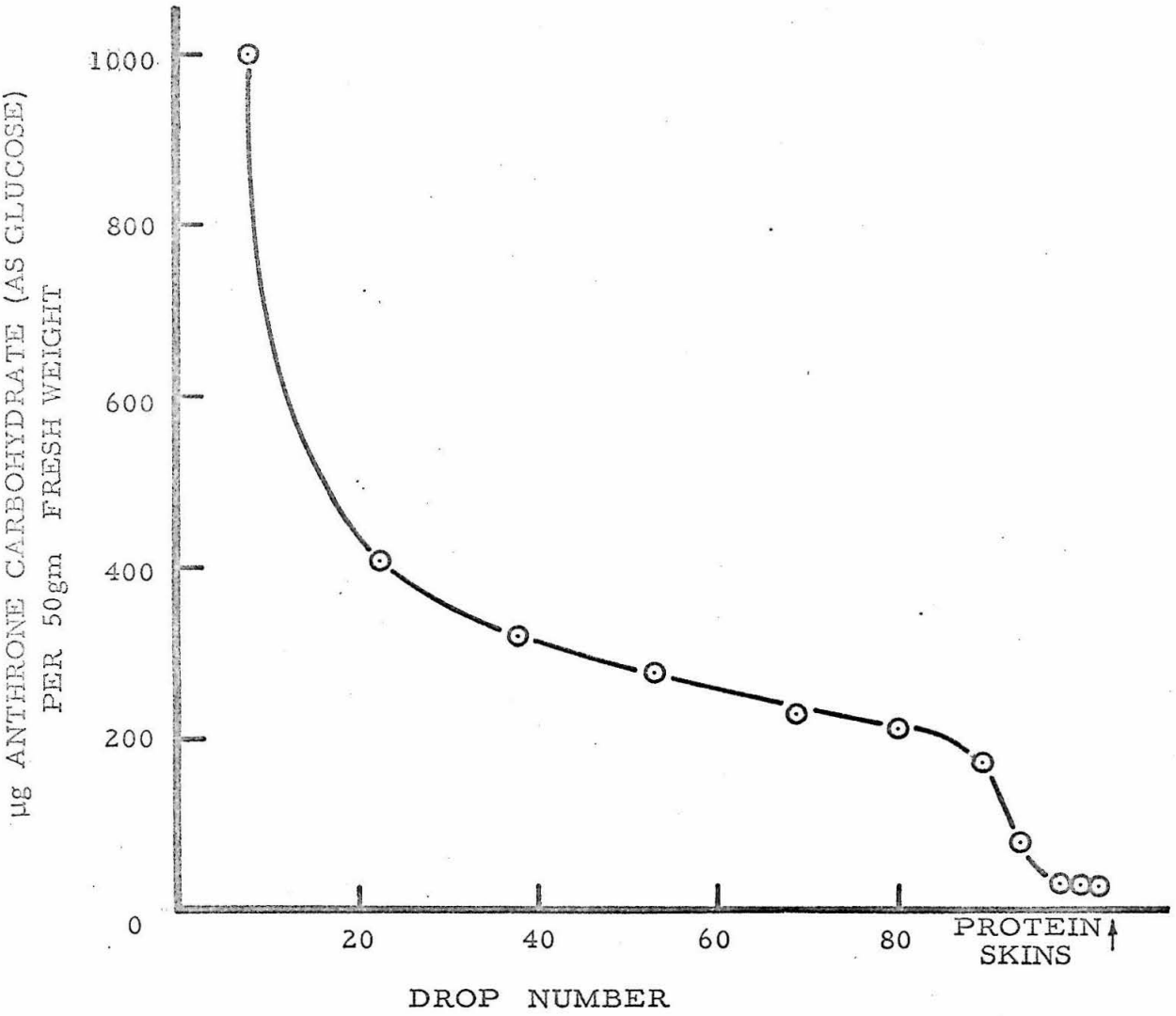


Figure 24. Cesium chloride density gradient separation of anthrone carbohydrate and protein of the CBSEI fraction of Avena coleoptiles. The protein skins were also found to be devoid of measurable carbazole carbohydrate.

M sodium acetate pH 4.4. In addition to the uronic acid-like material, neutral carbohydrates appear to be present giving rise to a variety of monosaccharides upon relatively mild acid hydrolysis.

A comparison of indoleacetic acid-treated and -untreated sections revealed that although all components of the CBSEI fraction examined were increased in amount by the IAA treatment, all but one of these changes can be accounted for on the basis of increased tissue as a result of IAA-induced growth. The single exception is in the case of heat stable protein (30 to 100% increase due to IAA). A cesium chloride density separation provided no evidence for a stable carbohydrate-protein complex. Preliminary efforts to identify the IAA-induced glycolipide with this fraction have also been inconclusive.

V. AN ELECTRON-MICROSCOPIC SURVEY OF IAA-INDUCED CHANGES IN CYTOPLASMIC ORGANELLES[1]

While the main morphological features of the various structural components of the higher plant cell have been described in considerable detail (211), a role for certain cytoplasmic organelles in cell wall deposition has been indicated only recently (212-215). By means of electron microscopy, Porter and Machado (212) have indicated the possible involvement of portions of the endoplasmic reticulum in cell wall formation. This conclusion, however, has been inferred largely from the observed orientation of the endoplasmic reticulum adjacent to expanding walls and from its proximity to the newly forming cell plate during division.

In the case of the Desmid, Micrasterias rotata, Drawert and Mix (213) have suggested a direct participation of the Golgi apparatus (dictyosomes) in the laying down of certain portions of the cell wall; a suggestion also derived from electron microscopic observations. Mollenhauer et al. (214, 215) have reported somewhat similar observations concerning a secretory function of the Golgi apparatus of the outer rootcap cells of maize.

[1] The experiments reported in this section were performed in cooperation with Mr. Jack M. Widholm. The author wishes to express sincere appreciation for permission to reproduce a portion of the results.

The finding that the formation (or maintenance) of a cytoplasmic component is influenced by treatment of the tissue with IAA (Part III), prompted an investigation of cytoplasmic changes observable by electron microscopy. For this purpose, electron micrographs prepared from sections of IAA-treated tissues were compared with those prepared from untreated tissues. Since quantitative changes, rather than qualitative changes, might also be expected as a result of IAA treatment, the appearance or disappearance of vesicular structures was chosen as one criterion for analysis, cf. Cocking (88, 89).

Materials and Methods

Avena coleoptiles were grown as described in Section A of Part III and 5 mm subapical sections were treated for varying times in the presence and absence of 5.7×10^{-6} M IAA. To provide an indication of the appearance of the cell in intact coleoptiles, one lot of sections was fixed immediately after sectioning. The coleoptile sections were fixed for 45 minutes at room temperature using 3% potassium permanganate (216) containing 0.1 M sodium chloride and 0.028 M sodium barbital buffered at pH 7.4. In addition, primary roots of 60-hour old maize seedlings, grown and IAA-treated (5×10^{-7} M IAA, one hour) on filter paper, were prepared for electron microscopy by fixation with a 2% aqueous solution of potassium permanganate (214, 216) for two hours at room temperature.

Results and Discussion

The electron microscope revealed no major structural change in any of the plant organelles studied--nucleus, mitochondria, plastids,

endoplasmic reticulum, Golgi apparatus, vacuole or cell wall--as a result of IAA treatment of the tissue. However, there may be a significant increase in the number of vesicles contained in the cytoplasm and/or associated with the central Golgi structure. The number of these vesicles has been suggested by Whaley et al. (211) to vary with cell activity.

The Golgi apparatus has been observed in all plant tissues thus far examined (211-215) and is in many respects similar in structure to that found in certain types of animal cells (219, 220), cf Whaley et al. (218). The Golgi apparatus is interpreted structurally as a stack of flattened cisternae or disks. During periods of cellular activity, vesicles are observed at the edges of these cisternae and may eventually become free in the cytoplasm (214, 215, 218). The number of these vesicles in the vicinity of the central Golgi structure as observed by electron microscopy has been taken as a measure of Golgi activity. Typical Golgi apparatus of maize roots (figure 25) and of Avena coleoptiles (figure 26), showing the cisternae and associated vesicles, illustrate the ease with which one can distinguish these organelles in plant tissue.

Counts of the number of vesicles per central Golgi structure of Avena coleoptile parenchyma cells are given in figure 27. The number of vesicles observed per Golgi structure of IAA-treated tissue shows an initial rise during the first hour following excision and then is observed to decline to a level more nearly characteristic of intact coleoptiles. In the absence of an external supply of IAA, the number of associated vesicles is found to decrease with time during the first three hours to an average of approximately two per cisternae. Removal of the coleoptile tip by excision is normally considered to result in a

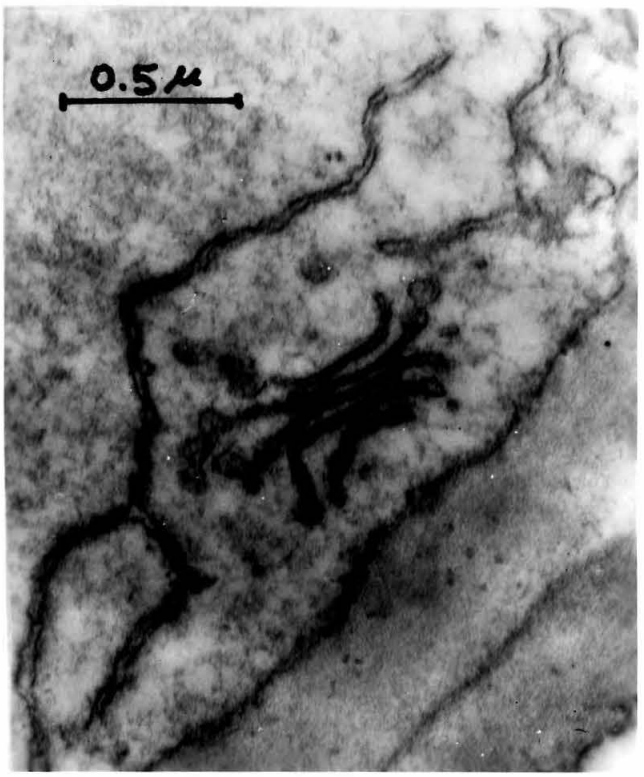
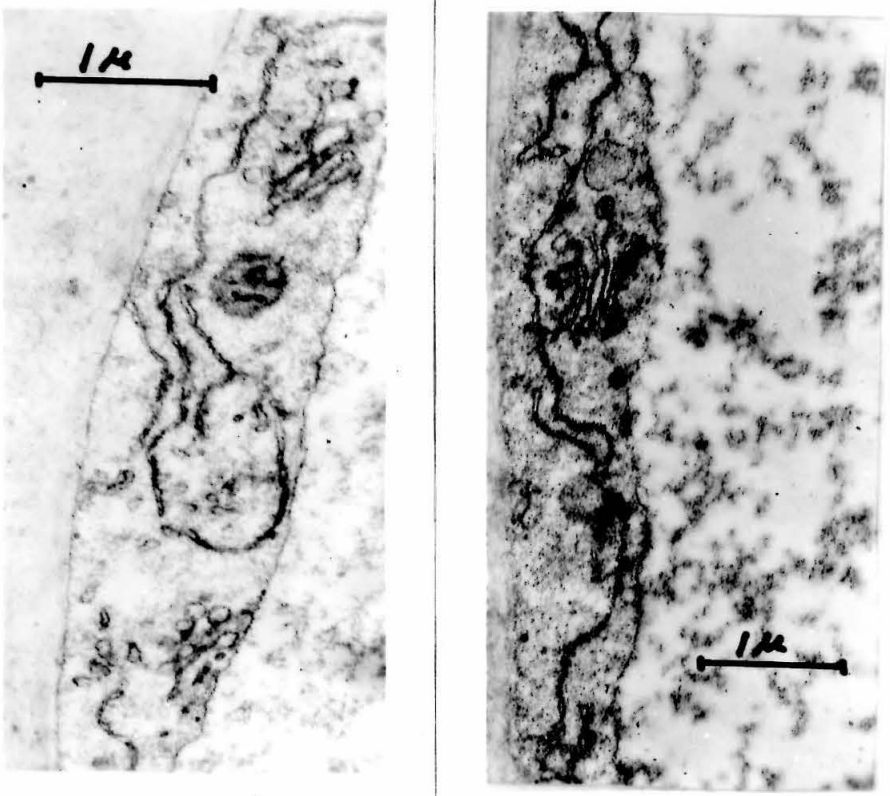


Figure 25. The Golgi apparatus of a cortical cell from the zone of cell extension of maize root tissue. Vesicles are associated with the cisternae of the central Golgi structure. $KMnO_4$ fixed.



a b

Figure 26. Golgi apparatus of vacuolated parenchyma cells of Avena coleoptile tissue. $KMnO_4$ fixed.

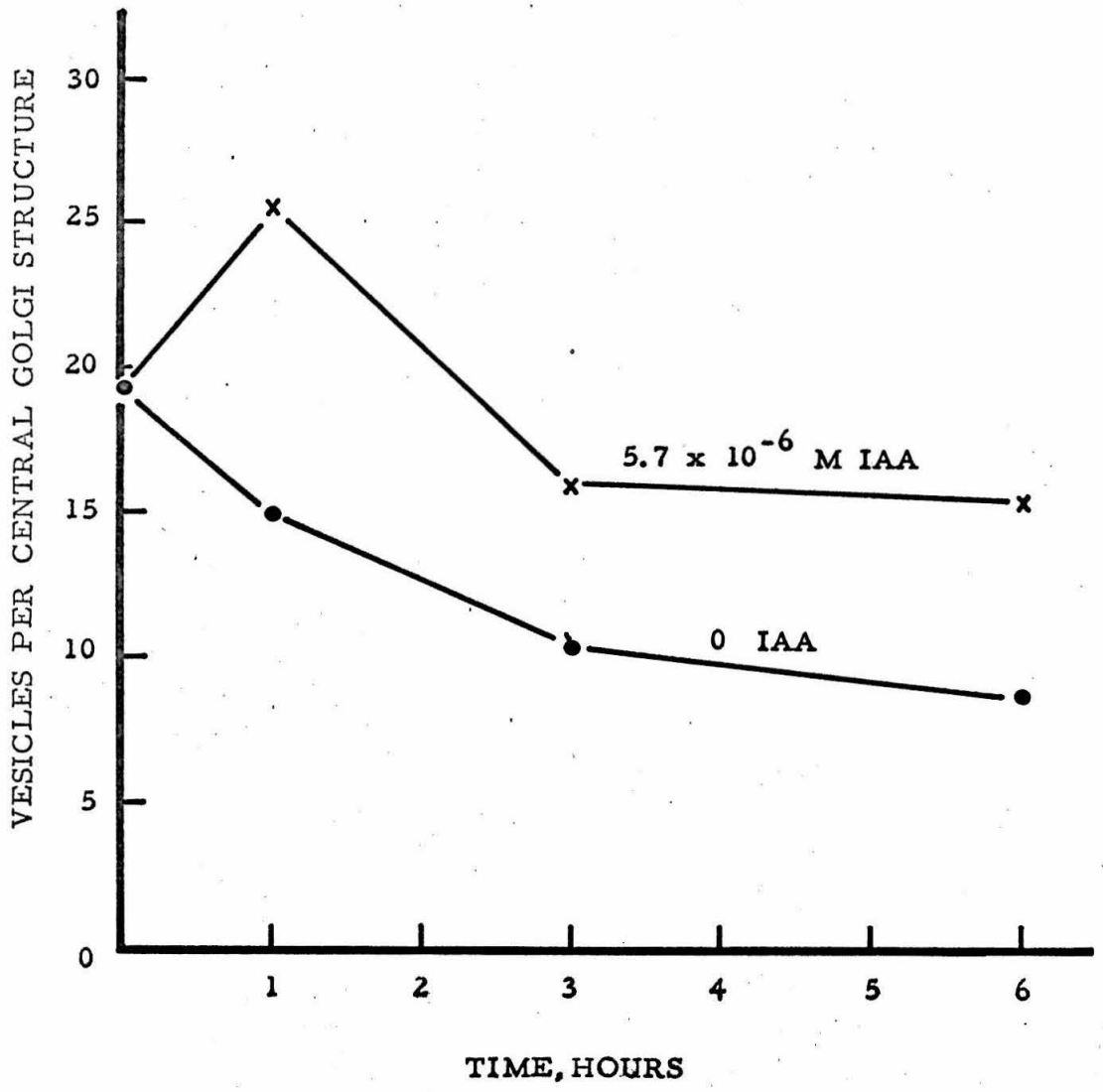


Figure 27. Number of vesicles per central Golgi structure of Avena coleoptile parenchyma cells. Tissue treated for varying times in the presence or absence of 5.7 X 10^{-6} M IAA.

gradual depletion of the endogenous supply of auxin (99). However, since the growth rate of the IAA-treated coleoptile sections was found to be nearly constant over this same time period, Golgi activity does not exactly parallel changes in IAA-induced growth. The number of cisternae remained relatively constant (4 to 6) and appeared to be unchanged as a result of IAA treatment, as did the total number of Golgi structures encountered per thin section.

Counts of the number of vesicles per central Golgi structure from two regions of the primary root of maize seedlings are presented in table 45. These results would indicate that Golgi activity may be as much as doubled in this tissue following a 1-hour treatment with 5×10^{-7} M IAA; a change more nearly equal to the IAA-induced change in growth rate of excised root sections (Part II). In the case of roots, the number of vesicles observed in the vicinity of the Golgi structures was found to be a highly variable quantity, even within a single cell. Therefore, count distributions are presented in figure 28a-d.

Although the evidence for an involvement of the Golgi structure in cell wall formation is somewhat indirect, recent reports by Mollenhauer et al. (214, 215) have provided evidence for the formation of large vesicles by the hypertrophied portion of the Golgi cisternae of outer rootcap cells (figure 29a). These vesicles have been observed to be transported from their apparent site of origin and to pack between the surface of the protoplast and the surface of the cell wall. A similar phenomenon has also been observed to occur in the case of more central rootcap cells in the present study (figure 29b). Ultimately, however, there appears to be a disappearance of these masses, an increase in

TABLE 45.

Influence of a 1 hour incubation in the presence or absence of 5×10^{-7} M IAA on the number of vesicles per central Golgi structure of maize root cells.

Tissue	IAA Concentration	Number of Golgi Structures Examined	Number of Vesicles per Central Golgi Structure	
			Ave	Distribution
Root Tip (Meristematic and Transition Zones)	None	39	10	4 to 17
	5×10^{-7} M	22	19	8 to 35
Root (Zone of Cell Extension)	None	15	16	9 to 27
	5×10^{-7} M	45	27	18 to 61

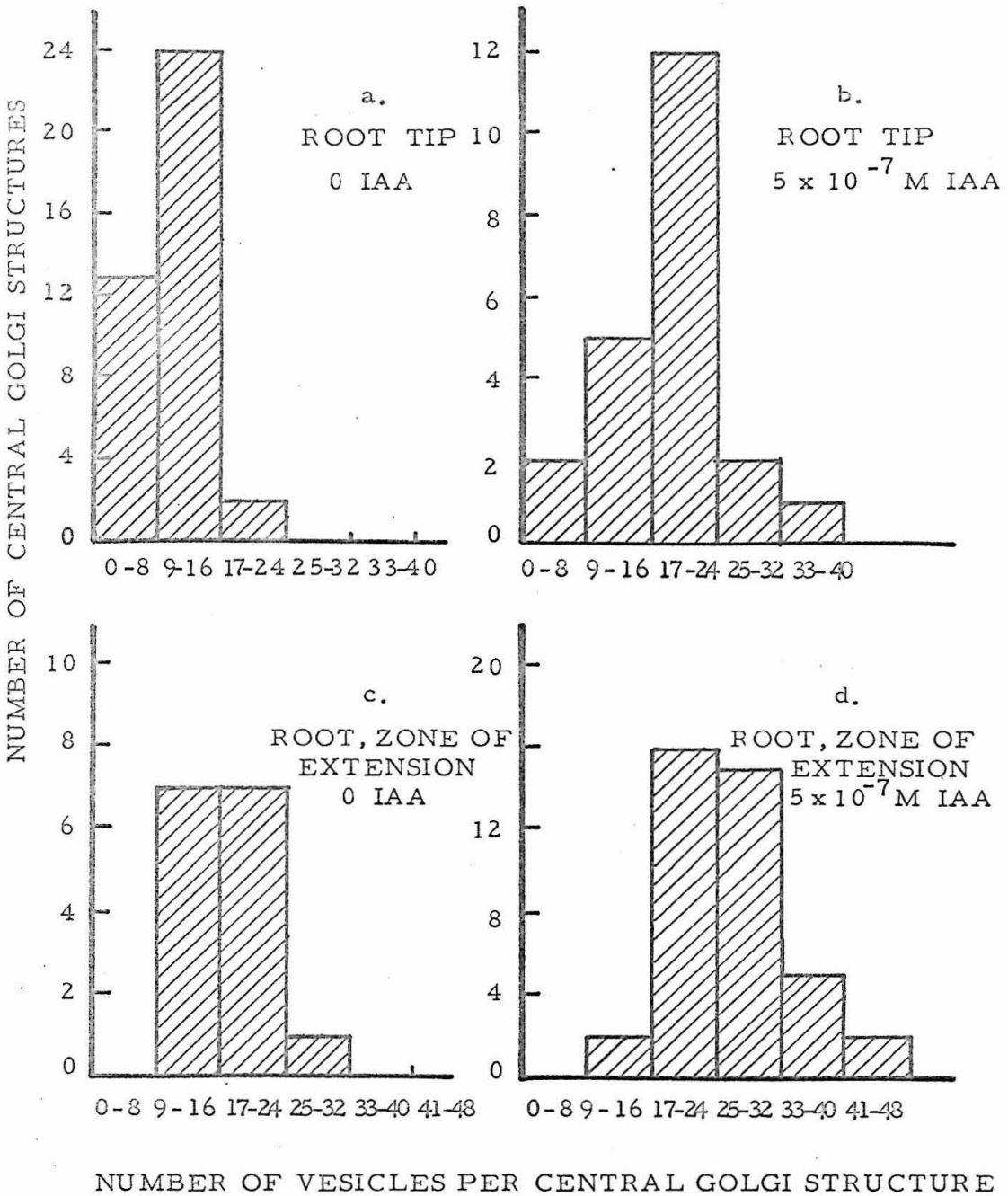


Figure 28. Size class (number of vesicles per central Golgi structure) distribution of the central Golgi structures of two regions of the maize root tip comparing IAA-treated and -untreated tissues.

Figure 29a. The Golgi apparatus of an outer root-cap cell of maize showing the production of large vesicles: $KMnO_4$ fixed.

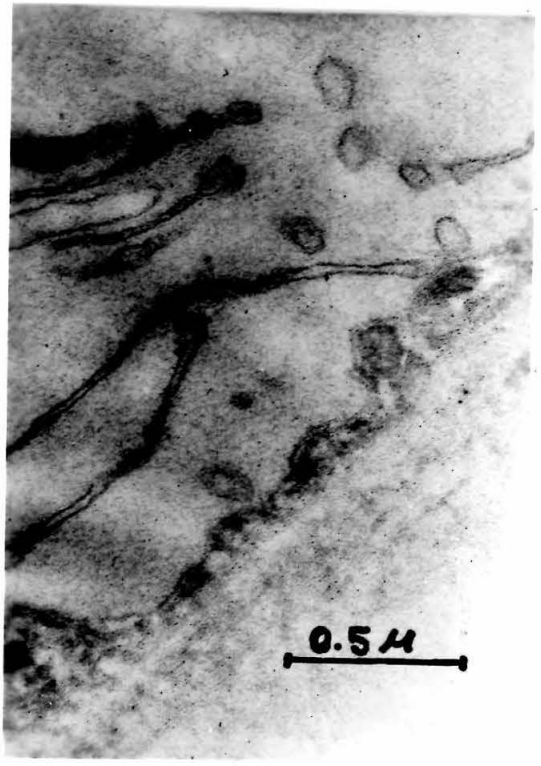


Figure 29b. A portion of a centrally located rootcap cell illustrating extra-protoplastic vesicular material. $KMnO_4$ fixed.

cell volume and a return of the Golgi apparatus to an unhypertrophied state when the secretory function is complete (214, 215). Subsequently, the secreted material would appear to be incorporated into an increased volume of wall but may also contribute to the slime which is normally associated with the outer surface of the rootcap (215).

In the case of cell plate formation, Porter and Machado (212) consider the vesicles to be small reservoirs of products of synthesis which are discharged into the plate region. A similar view has been expressed in the case of the somewhat specialized wall formation observed by Drawert and Mix (213) with Micrasterias rotata.

Although the integrity of the Golgi structure is maintained until final breakdown of the cell (215), the relation between a deposition form of wall synthesis (as suggested for the Golgi structure) and wall formation in the rapidly expanding cell is not clear. Examination of the region between the cell wall and the surface of the protoplast of rapidly expanding cells of both Avena coleoptiles and maize roots has revealed only occasional structures which resemble vesicular material in this extra-protoplastic position. Furthermore, conclusive evidence for fusion of the limiting membrane of the Golgi vesicles with the cell membrane thus far has been obtained only in the case of cells of the rootcap (214, 215).

Also, there is presently no evidence relating the IAA-induced glycolipide fraction (PGL) with the increased Golgi activity. Electron microscopic examination of thin sections prepared from permanganate-fixed Avena PGL obtained by ammonium sulfate fractionation (0 to 30% of

saturation) of the pH 4.4-soluble supernatent fraction, suggest that at least a portion of the crude PGL fraction consists of structures resembling membranes (figure 30a). However, these structures would appear to be more typical of the endoplasmic reticulum as seen in thin section (compare figures 30a and 30b) rather than Golgi derived.

It is still conceivable, however, that membrane-bound structures (the Golgi apparatus as well as portions of the endoplasmic reticulum) may be involved in a general pattern of wall formation along the following lines:

1. A secretion of pre-formed materials which are discharged in the form of membrane-bound vesicles.--These vesicles would then fuse in a manner giving rise to a new cell plate or middle lamella. Such processes have been observed to take place during the course of cell division (212) and in the case of more specialized forms of wall growth of the outer rootcap (215) and of Micrasterias rotata (213).

2. Wall formation by surface growth in which deposition occurs at the cell wall protoplast interface.--This form of synthesis has been advocated by Preston (90, 91, 221) and would be dependent upon contact between the wall and protoplast. A process of this sort might also involve intermediary substances possessing lypophilic properties (e.g., the cellulose precursor described by Kahn and Colvin (174) or perhaps even PGL). In this case membrane-bound vesicles still provide an attractive mechanism of transporting lipide-like materials through the aqueous regions of the cytoplasm (222).

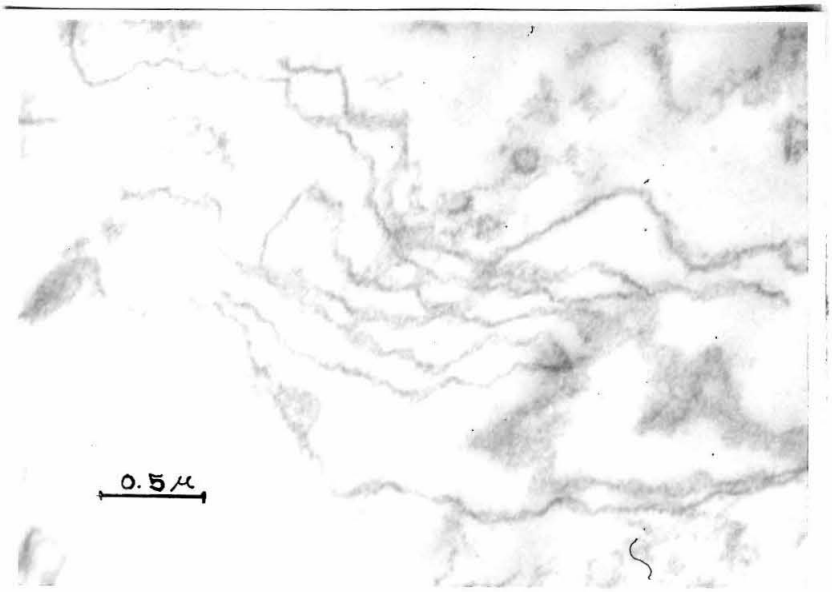


Figure 30a. Avena PGL. $KMnO_4$ fixed.

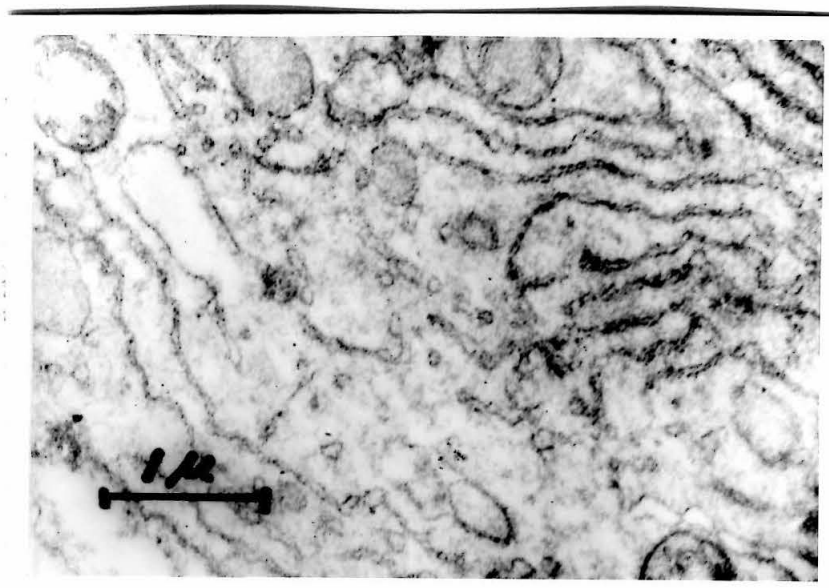


Figure 30b. A portion of a parenchyma cell of Avena coleoptile tissue showing a concentration of endoplasmic reticulum. $KMnO_4$ fixed.

Summary

A comparison of electron micrographs of thin sections prepared from IAA-treated and -untreated tissues (permanganate fixation) revealed no major structural change in any of the cytoplasmic organelles studied. An apparent IAA-induced increase in the number of vesicles associated with the central Golgi structure of maize roots and Avena coleoptiles is reported.

APPENDIX A

STUDIES ON THE GROWTH OF EXCISED ROOT SECTIONS

The plant material was obtained from a single lot (No. 5CC4) of a single-cross maize hybrid (H-23 X H-22) obtained from the Agricultural Alumni Seed Improvement Association, Lafayette, Indiana. This source of plant material was chosen from a number of white maize single-cross hybrid selections tested in preliminary experiments. The growth rate of the intact primary root was found to be steady-state, maintaining a growth rate of slightly more than 1 mm per hour for periods in excess of 72 hours.

Seeds were surface sterilized by means of a dry treatment with approximately 200 mg of a commercial preparation (Orthocide) of 50% Captan (N-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide) per kg of seeds. The treated seeds were then stored in a sealed flask for a minimum period of 24 hours prior to the beginning of germination as part of the sterilization process. Germination was conducted asceptically in enamel instrument pans (20 3/4" X 12 3/4" X 4 1/8"). Seeds were placed germ side down in rows spaced ca. 3 cm apart on a layer of Whatman 3 mm filter paper moistened with sterile deionized water. The trays were then covered with metal foil and placed on a 5° slant with the root axes directed downward. Germination and all incubations were carried out in

the dark in a room maintained at 85 to 90% relative humidity and at a temperature of 25° C. Preparation of plant material and all measurements were performed under weak incandescent light. No marked influence of this light on subsequent growth was noted.

Growth of the intact root.--Preliminary experiments indicated that growth of the primary root of maize was limited to the first 5 mm of the tip. The growth of this region was followed during a 12-hour growth period using four-day old seedlings. In order to facilitate growth measurements of zones within the intact root, India ink marks were placed on the epidermis of each root at 0.8 mm intervals beginning 2.4 mm from the distal end of the root cap, a technique first utilized in the study of root growth by Sachs (223). The device for marking the roots consisted of five nylon threads under tension spaced 0.8 mm apart within a supporting frame. The threads were coated with India ink using a camel's hair brush and each root was stamped individually. The width of the resulting lines was approximately 0.1 mm, and measurements were taken to the center of the mark. Roots were sized according to length of the primary root, randomized and transferred to 20 X 100 mm petri plates for subsequent incubations. The petri plates were fitted with circles of Whatman No. 1 filter paper, which was moistened with 5 ml of 0.01 M potassium maleate buffer, pH 4.5. Displacements were estimated at hourly intervals using a binocular microscope and coordinate paper.

As shown in figure 31a, the region just behind the root cap (2.4 to 3.2 mm from the distal end of the root cap) was characterized by rapidly increasing growth rate followed by a gradually decreasing growth rate. The

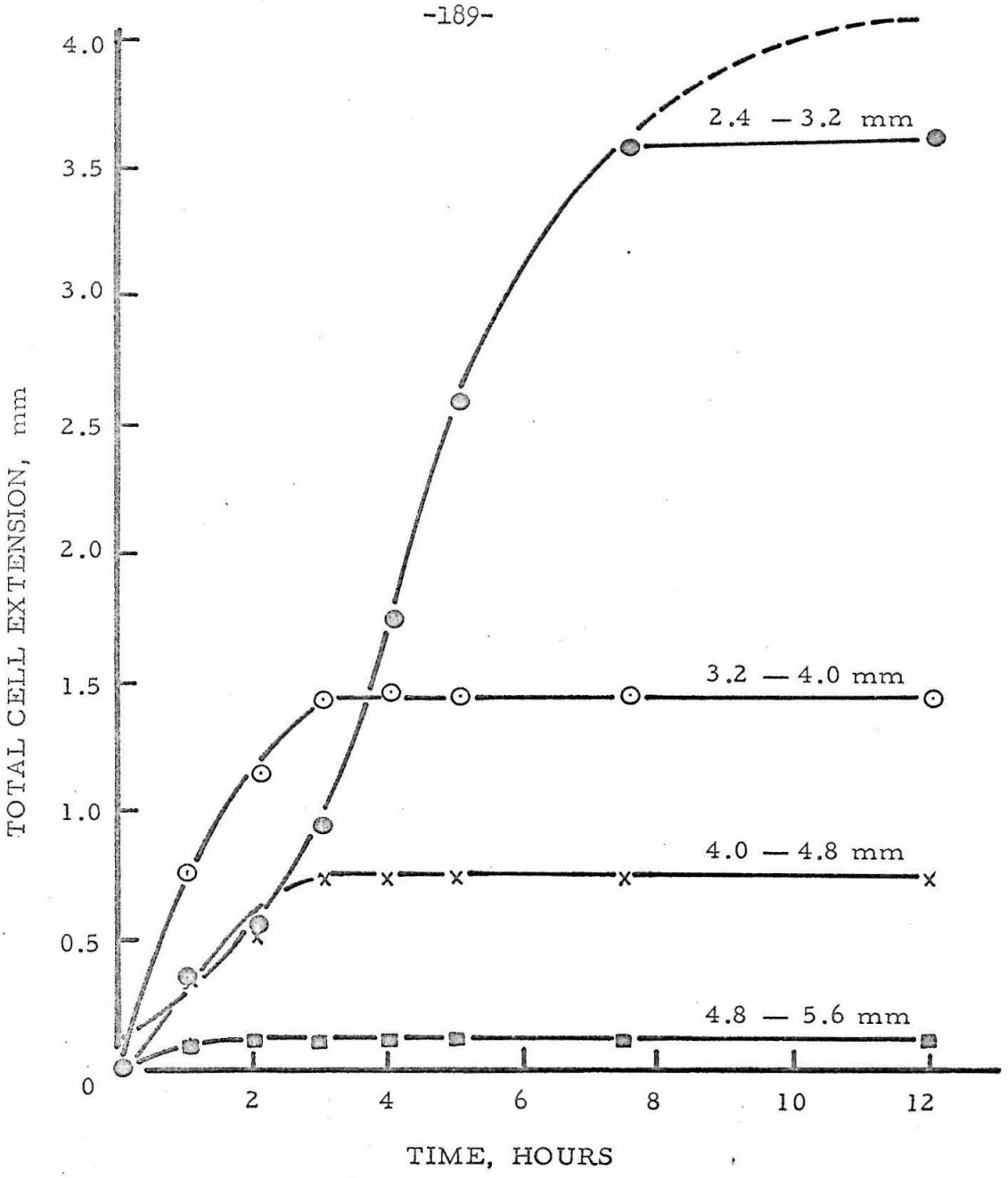


Figure 31a. Growth as a function of time comparing various regions of the zone of cell extension of the intact maize root. The growth curve of the region 2.4-3.2 mm from the tip is extended as a broken curve to indicate that growth does not form an ideal sigmoid curve. Medium consisted of 0.01 M potassium maleate buffer, pH 4.5.

growth between three and six hours would form the central region (grand period) of the normal sigmoid curve although it is evident that the hourly growth rate was continuously changing and could not be characterized as steady-state. This region accounted for over 60% of the total elongation of the marked section and growth was terminated after about eight hours.

The region 3.2 to 4.0 mm from the tip was found to contain cells corresponding to the decreasing growth rate portion of the time curve of the previous increment. Here growth was terminated after about three hours. The 4.0 to 4.8 and 4.8 to 5.6 mm zones accounted for only about 14% of the total extension of the entire region and growth was also terminated during the first three hours of the experiment.

A characteristic feature of the data presented in figure 31a is that the curve representing cell elongation versus time does not form a complete ideal grand period with an initially and subsequently decreasing growth rate. As first pointed out by Burstrom (17), the progress curve of root growth is characterized by an initially rapid exponential increase in rate followed by an abrupt cessation of growth. This fact is also shown by the data of Brown et al. (109) and of Hejnowicz (224).

The earlier work of Baldovinos (225) and of Erickson and co-workers (226) with maize, as well as that of Goodwin et al.(227,228) and of Brumfield (229) with Phleum has established a morphogenetic pattern for growth in the primary root. With the particular strain of maize used by Baldovinos, the so-called apical meristem extended for a distance of 1.25

mm back from the root cap. The cells contained in this zone were of uniform size and at 25° C were dividing at a rate of 0.055 cells per cell per hour, or one complete division about every 18 hours.

The second millimeter of the root was considered as a transition zone in which the first 0.25 mm was yet a part of the apical zone, the next 0.25 mm a region of very rapid cell division and rapidly enlarging cells, and the last half a region restricted to cell elongation. The division rate for the entire section was 0.092 cells per cell per hour at 25° C, or a generation of ca. ten hours.

The third one-millimeter section was completely characterized by elongation without cell division. Growth was complete in the fourth millimeter.

On the basis of these considerations and the fact that growth is always terminated between six and eight hours after marking the zone, it is concluded that the growth of the region beginning 2.4 mm from the distal end of the root cap (corresponding to the end of the transition zone of Baldovinos) is the result of the elongation of pre-existing cells. Elongation of cells formed by subsequent divisions is of a sufficiently infrequent occurrence to be undetectable under the conditions of these experiments. Furthermore, since the overall growth of intact roots of this maize variety is known to be steady-state, this must be the net result of a continuous steady-state production of new cells by division. These cells would then become a part of the zone of elongation with a corresponding increase in length of the intact root. A corollary of this observation is that both

the zone of elongation and the zone of cell division do not change appreciably in length during the course of elongation. On the other hand, prolonged treatment of the root with high concentrations of indole-acetic acid, a treatment that results in a shortening of the time period over which the root can elongate (105), will also result in a proportionate shortening of the region of cell elongation.

Growth of excised roots and root sections.--In the experiments utilizing excised root section, roots were germinated as in the case of experiments with intact roots. Five millimeter sections were cut approximately 2.5 mm in back of the distal end of the root cap using a double bladed cutting tool. The region of active cell division in maize roots is delineated by a readily discernable concentration of yellow pigments. By cutting the sections 0.25 mm distal to the pigmented region, it was possible to remove much of the variability due to differences in length of the root cap.

Sections were incubated asceptically in 25 ml Erhlenmeyer flasks plugged with cotton and containing 5 ml of the solutions to be tested. Unless otherwise stated, the solutions were buffered with 0.01 M potassium maleate buffer, pH 4.5. This particular concentration of buffer was shown to be optimal for the growth of maize roots (97). Solutions were prepared using Pyrex distilled water.

Results with root sections are expressed in terms of a growth index which is defined as the ratio of growth relative to that obtained in

distilled water. Growth in distilled water was in the order of 1 mm per 5 mm root section in all experiments reported. Experiments were conducted with a minimum of five replicate samples of ten sections each.

A comparison of the growth curve for 5 mm root sections grown in buffer alone (figure 31b) with the growth curves for the same region of the intact root (figure 31a) shows that not only does excision reduce the overall growth rate of the section but the time during which the section elongates is also reduced from eight hours to five hours. This phenomenon is further illustrated in figure 32. During a six-hour growth period, the intact root (initially 32 mm long) grew 6.6 mm. When excised at the point of attachment to the endosperm (seed), the total growth of the 32 mm excised roots (tip intact) was reduced to 2.9 mm (56% inhibition). No further reduction of growth was observed until the excised root (tip still intact) was shortened to 8 mm. The 8 mm root apex grew only 1.9 mm (71% inhibition), growth comparable to that of the 5 mm sections prepared from the same lot of roots. A similar observation has been reported by Brown and Wightman (230) who found, in the case of pea roots, that a lag phase preceeding the onset of cell division is longer and the peak rate of division occurs later, the shorter the initial tip excised (beyond a certain critical length).

The data of table 46 further illustrate the growth reduction obtained during excision of maize roots. In these experiments, it was found that the sections with the root tip (meristematic zone and root cap) intact, elongated to the same extent during a six hour growth period as did

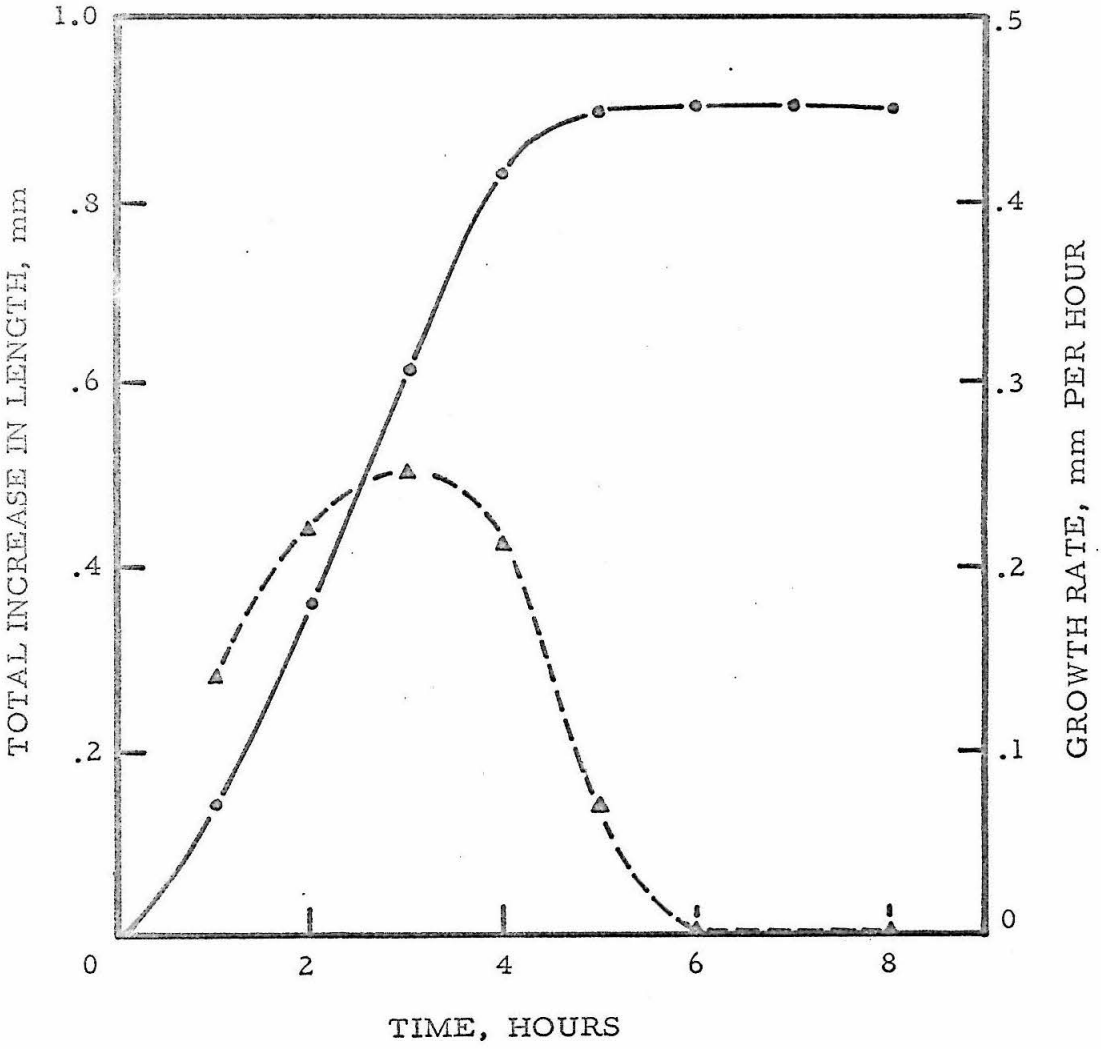


Figure 31b. Growth of a function of time of the excised 5 mm zone of elongation of maize roots. Solid curve = total increase in length. Broken curve = growth rate in mm per hour. Medium consisted of 0.01 M potassium maleate buffer, pH 4.5.

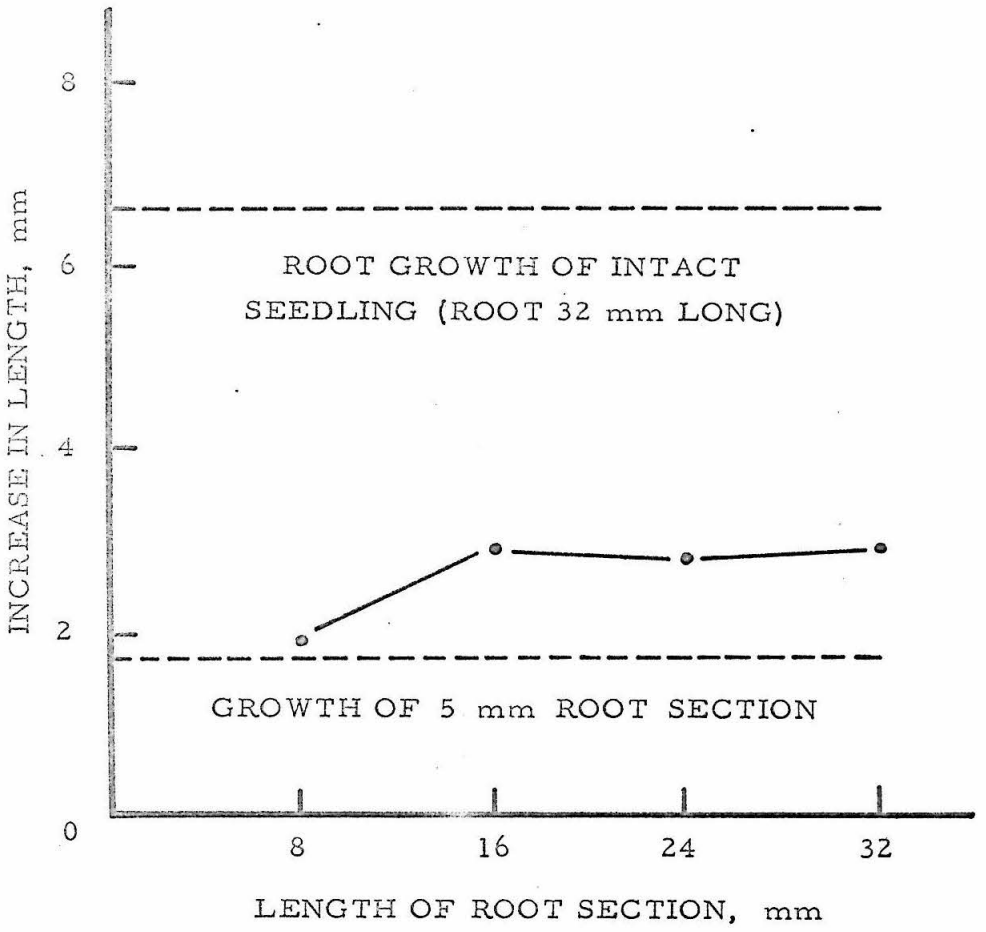


Figure 32. Growth of maize roots as influenced by various degrees of excision of the zone of cell extension. Medium consisted of 0.01 M potassium maleate buffer, pH 4.5.

TABLE 46.

Cell extension of maize roots as influenced by various degrees of excision of the zone of elongation.

Treatment	Relative Growth
Root Intact (Attached to Seed)	1.00
1/2 Vascular Connection from Seed to Elongating Zone Severed	0.88
Root Excised	0.44
2.4 mm Tip Excised (Remainder Intact)	0.40
1/2 Tip Excised (Remainder Intact)	0.23
5 mm Section + 2.4 mm (Tip Intact)	0.25
5 mm Section	0.25

sections with the tip excised. If only one-half of the vascular connections between the root and the endosperm (seed) were severed, the reduction in growth rate was much less severe than if the root were completely severed. This would indicate that at least a portion of the inhibition which results from excising the root is of a nutritional nature. Excised monocotyledenous roots are notoriously difficult to maintain in culture (231) and the experiments of Bonner and co-workers (232) have shown that roots, in general, are dependent upon the shoot for a variety of nutrients and growth substances including the vitamins thiamine, pyridoxine and niacin. According to the classical view of in vivo auxin relations (99), the plant root is growing under supra-optimal endogenous auxin levels supplied by cells located in the root tip. Accordingly, it has been reported that if the root tip is removed, the growth of the elongation zone is increased over that of the control (99, 101). This view has not received universal acceptance (95) and the more recent experiments of Younis (100) have failed to confirm these observations in the case of roots of Vicia faba. In the experiments summarized in table 46, roots with the apical 2.4 mm grew 60% less than comparable control roots over a four hour period. The decapitated roots developed an abvious increase in thickness and a dense coat of root hairs. Experiments in which the exposed surface was covered with lanolin gave identical results, and replacement of the tip was without effect. At least a portion of the growth retardation following removal of the root tip may be the expression of an injury phenomenon, since removal of only one half of the tip served to intensify the growth inhibition. In general, it was found that the injury resulting from the removal

of portions of the growing tip was more nearly proportional to the amount of cut surface than to the total amount of tissue removed.

Therefore, it is concluded that the reduction in growth which occurs as a result of excision of the 5 mm elongating zone of maize roots (as compared to intact roots) is the result of a combination of at least two major disturbances: 1) the generally known fact that roots are dependent upon the shoot for various metabolites and 2) an injury phenomena, occurring perhaps at the cut surface.

Effect of sucrose on the growth of root sections.--The nutritional requirements of excised root sections of maize have been studied previously by Brown and Sutcliffe (233) who report that of the substances normally promoting the growth of excised roots in culture, only sucrose and potassium were of any significance in increasing the growth of excised root sections. In the present experiments unautoclaved sucrose was found to result in a small growth promotion between 0.025 M and 0.1 M during the eight hour incubation period tested. The most striking response to sucrose, however, occurred only when the solutions were autoclaved prior to introduction into the growth media. Initially, a 3% solution of sucrose was prepared in 0.01 M potassium maleate buffer, pH 4.5 and autoclaved for 15 minutes at 15 pounds pressure. The concentration-response curve exhibits a maximum in the region of 0.05 to 0.08 M (figure 33). It was, therefore, concluded that the effect of autoclaving sucrose was the result of a substance or group of substances derived from sucrose during the autoclaving process. That this is so became clear during the course of investigations regarding the nature of the substance formed during autoclaving.

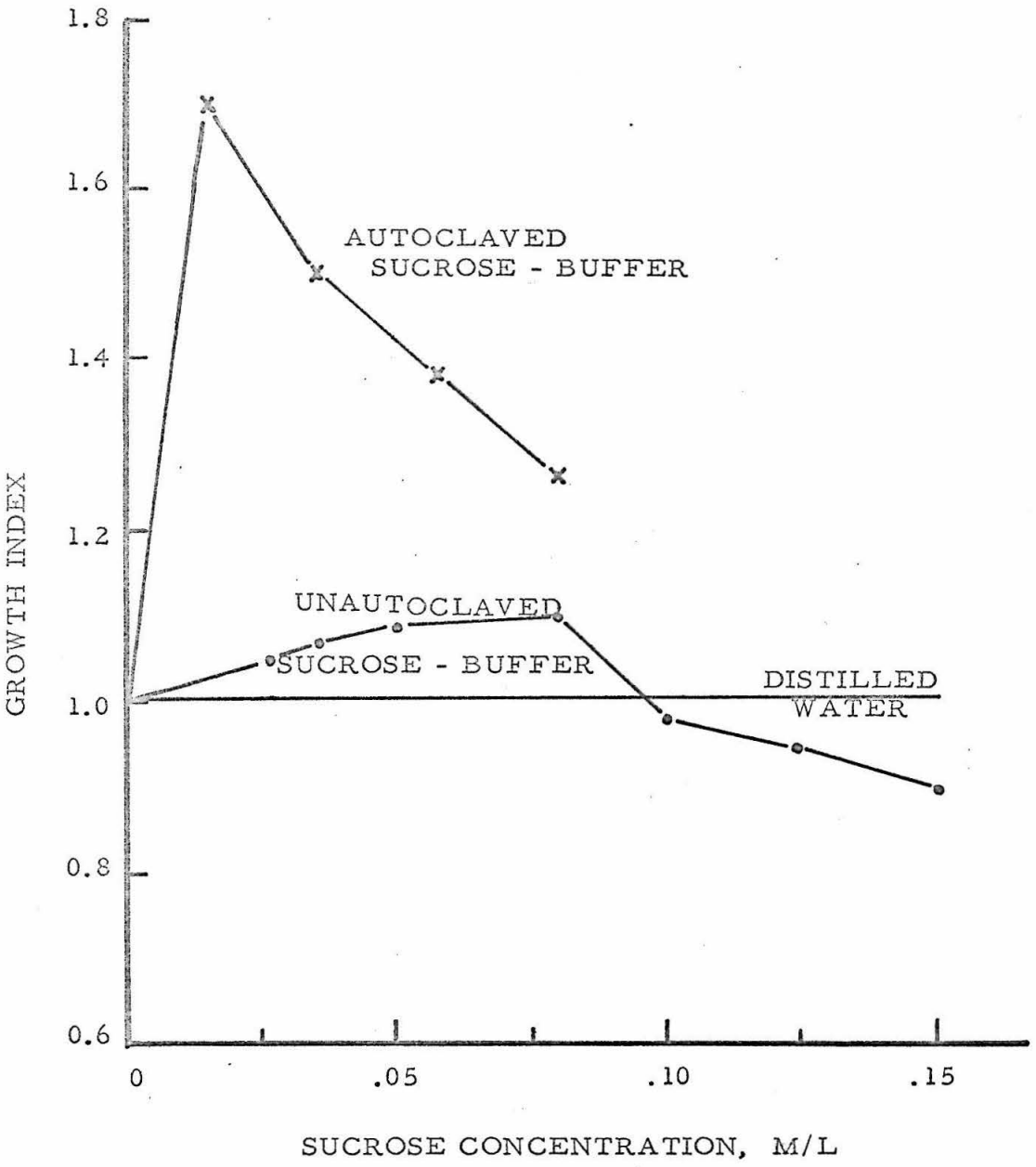


Figure 33. Effect of autoclaving (15 min, 15 lbs pressure) growth media containing sucrose on the subsequent growth of excised maize root sections. Basal medium consisted of 0.01 M potassium maleate buffer, pH 4.5.

Promotion of the growth of excised root sections by 5-hydroxymethyl-2-furaldehyde derived from sucrose solutions as a result of autoclaving at low pH.--Both growth promotions and growth inhibitions have been observed following the sterilization of sucrose solutions by autoclaving (and have been attributed to a number of causes (234,235 , 236), including the general category of formation of toxic substances (234). In the case of excised root sections of maize, the growth promotion observed is comparable to that obtained from other growth promotatory fractions. This fact is illustrated by the growth response of excised 5 mm root sections in the presence of a dilution series of autoclaved sucrose-buffer (figure 33) as compared with a similar dilution series of the neutral fractions prepared from maize roots and maize liquid embryos (figure 34). The acidic and basic fractions from both sources were inhibitory. Preliminary experiments with various types of "complete" growth media indicated that the liquid embryo fraction from maize was much superior basal medium for growth of root sections than various dilutions of White's (237) complete media (which contain glycine; the vitamins: thiamine, niacin and pyridoxine; and sucrose in addition to various mineral salts). Similar results were reported by Brown and Sutcliffe (233) for excised maize root sections in that B-complex vitamins were observed to have no measurable effect on growth. The significant feature of the data of figure 34, is that the growth promotions obtained from autoclaved sucrose-buffer are, in fact, comparable to those obtained from the relatively effective "complete" medium consisting of the neutral fraction of maize liquid embryo.

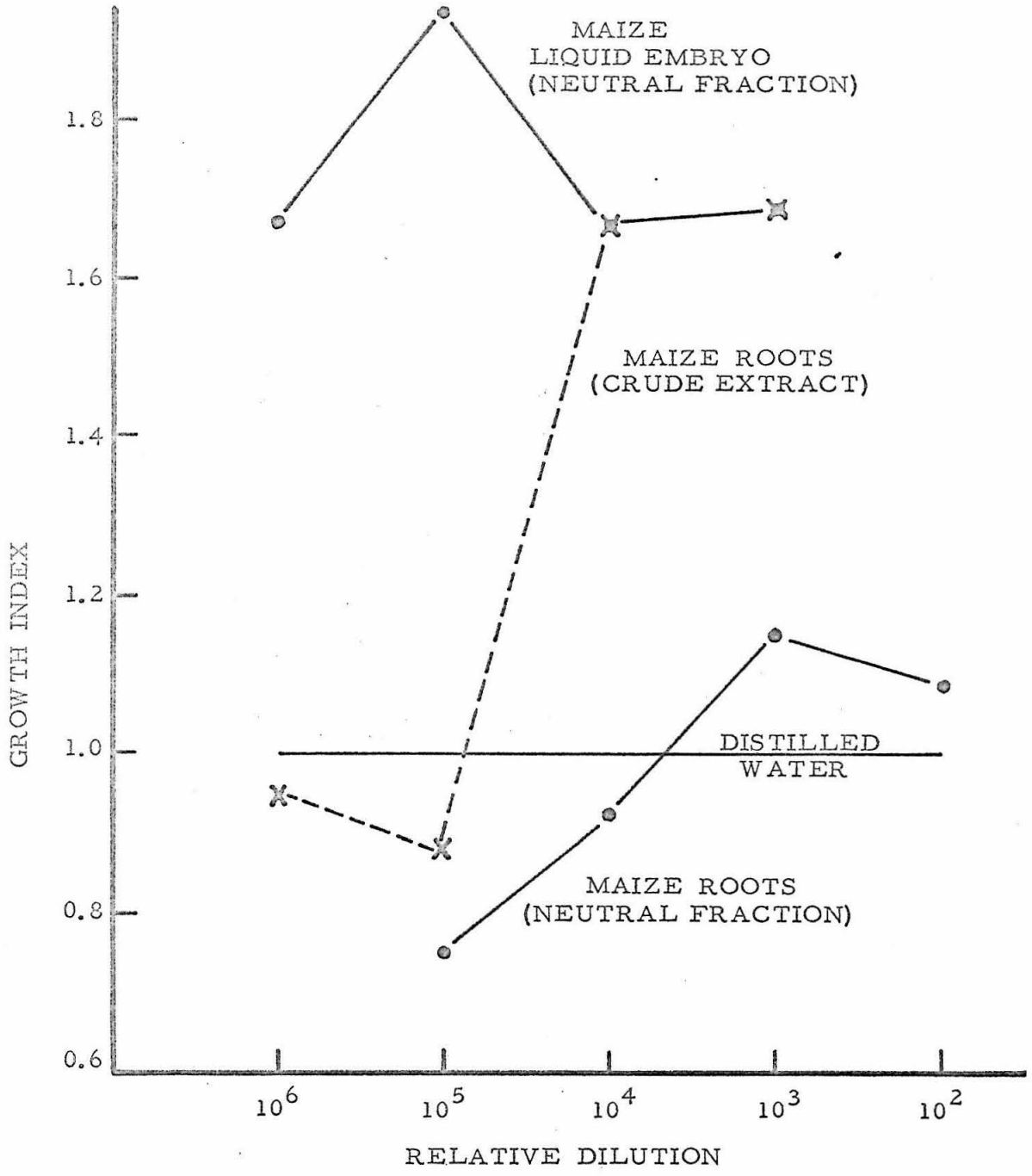


Figure 34. Growth index resulting from various dilutions of extracts which promote the growth of excised root sections. Data are presented for comparison with autoclaved sucrose buffer (figure 33, page 199).

In an effort to establish the chemical identity of the growth factor from autoclaved sucrose-buffer, the well known acid catalyzed breakdown sequence of sucrose (238) was investigated.

Sucrose --→ Glucose + Fructose --→ 5-hydroxymethylfurfural

5-Hydroxymethylfurfural or 5-hydroxymethyl-2-furaldehyde (HMF) is known to be produced under the action of organic acids (e.g., oxalic) on either fructose or glucose at high temperature and under pressure. However, it is apparently more readily obtained from the ketose (239). Montgomery and Wiggins (240) have studied the effect of acids other than oxalic on sucrose degradation and found that not only acids such as maleic, fumaric and phosphoric (which have dissociation constants of the same order as that of oxalic acid) but also hydrochloric acid (in a dilution where the pH of the solution was the same as that of the other acids) produced HMF. Furthermore, when sucrose was heated in water at a temperature of 130 to 170° C, sufficient acidity developed to cause inversion and subsequent degradation to HMF (240). Although HMF would be expected to be the first stable intermediate in the potassium maleate, pH 4.5, catalyzed breakdown of sucrose (other than glucose or fructose), it is also known to be further converted to levulinic acid (238, 239, 241).

In the experiments summarized in table 47, 0.01 M potassium maleate buffer, pH 4.5 was found to increase the growth of excised root sections but to not account for the promotion derived from autoclaved sucrose-buffer. This stimulation is probably derived more from the presence of the maleate

TABLE 47.

Promotion of root section growth by various acid degradation products of sucrose as influenced by autoclaving.

Growth Media	Growth Index	
	Unautoclaved	Autoclaved
Deionized or Deionized Distilled Water	1.00	1.00
0.01 M Potassium Maleate Buffer, pH 4.5	1.28	1.28
3×10^{-2} M Sucrose + Buffer	1.10	1.55
5×10^{-3} M Glucose + Buffer	1.23	1.58
5×10^{-3} M Fructose + Buffer	1.36	1.58
5×10^{-5} M 5-Hydroxymethyl-furfural (HMF)	1.73	1.71
2.5×10^{-4} M Levulinic Acid	1.26	1.18

moiety than from the presence of potassium since both fumarate and succinate were effective, whereas, potassium phosphate at a comparable pH was not. This is contrary to the finding of Brown and Sutcliffe (233) and reminiscent of an earlier report of Thimann and Bonner (242) of growth promoting effects by organic acids in the case of Avena coleoptiles and pea internodes. Autoclaving the buffer was without effect. Growth promotions obtained from unautoclaved sucrose, as well as that of glucose and fructose, the first breakdown products, were comparable to that of buffer. Autoclaving appeared to restore the full growth promoting capacity although only the concentrations shown were tested. Therefore, a commercial sample of HMF (K and K Laboratories, Jamaica, New York) was tested and was found to possess growth promotatory activity equivalent to that of autoclaved sucrose-buffer at a concentration of 5×10^{-5} M (table 47). Further testing revealed that purified levulinic acid (Nutritional Biochemicals Corp.) was completely inactive and that no growth promoting activity could be obtained by autoclaving.

It is suggested, although not conclusively established, that 5-hydroxymethyl-2-furfural (HMF) is the chemical substance responsible for the growth promotatory effects of autoclaved sucrose in the case of 5 mm excised root sections. HMF is ineffective in promoting the growth of Avena coleoptile sections as well as intact roots.

APPENDIX B

DETERMINATION AND EXTRACTION OF PECTIC SUBSTANCES

Pectic substances is a group designation for various complex, colloidal, carbohydrate materials obtained from plants which contain a large proportion of anhydrogalacturonic acid units (243). The galacturonic acid units are normally linked beta 1,4 and the carboxyl groups may be partly esterified with methyl groups or neutralized by a variety of bases. Pectic substances are known to be widely distributed as a component of the cell wall of higher plants (2, 4) and as a constituent of the cementing materials between the cells, particularily in the case of fruit tissues (183, 244, 245). The absolute pectic content of rapidly growing plant tissues, as well as the role of pectic substances in regulating the physical properties of the plant cell wall, continue to remain as basic considerations in current analyses of the growth process (2-9).

Methods used for the determination of pectic substances include precipitation with alcohol, organic solvents and metallic salts (243), titration of carboxyl groups before and after de-esterification (243), reaction with carbazole in sulfuric acid (127, 246) and decarboxylation by heating in mineral acids and measurement of the carbon dioxide evolved (122, 247). Methods relating to the extraction of pectic substances have not been standardized and vary from tissue to tissue. A common

procedure involves the extraction of the tissue in turn with hot water, dilute mineral acid (pH 2) and cold alkali or ammonium oxalate (243, 244). The three fractions isolated each presumably represent a different solubility class. Esterified pectins are water soluble and constitute the classical pectin fraction of the older literature (243). Acid promotes solution of the protopectin and alkali or oxalate dissolves the pectins and pectates of low methyl ester content (195). Overlapping solubilities of the various pectic substances is a major difficulty. For example, sodium pectate is soluble in water, alkali and ammonium oxalate and partly soluble in hot dilute acid (195). In addition, considerable amounts of non-pectic carbohydrate may be extracted as well and quantitative interpretation of the results is often questionable.

As a partial solution to these problems, mild extraction procedures have been introduced which involve the use of a calcium sequestering agent, ethylenediaminetetraacetic acid (Versene or EDTA) and the enzyme pectinase (46, 195). Generally two fractions are obtained. The first, corresponding to the classical pectin fraction, is extracted by hot water. The second fraction, solubilized with hot EDTA, has been suggested to contain the bulk of the protopectin and calcium pectates (2, 46). McCready and McComb have combined pectinase and EDTA extraction (without heating) to yield a single fraction representing total pectin (195). In the present work the extraction procedures of Jansen et al. (46) have served as a basis for continuing investigation. These workers have defined a third pectin fraction, solubilized in cold acetate buffer, pH 4.5, referred to as cold water-soluble pectin.

As a means of estimating the uronic acid content of the isolated pectins, the carbazole reaction remains the method of choice (125, 126, 246). Stark (248) applied Dische's (126) carbazole reaction to the determination of pectic substances in cotton fibers. Since then, the test has received widespread adaptation and a number of modifications have appeared (125, 127, 202, 249-252). The reaction is based on the production of 5-carboxy-2-formylfuran from uronic acids (253) in the presence of sulfuric acid.

Basically, the reaction is carried out by two different procedures (125, 246). In the first, the uronic acid is treated with 87% sulfuric acid at 100° C before the addition of the carbazole reagent. In this procedure, the extinction coefficients of glucuronic acid and galacturonic acid do not differ greatly. The color produced by galacturonides and uronide-containing polysaccharides are nearly equivalent to the color produced by the free acids (see also 125, 126, 127). However, according to McComb and McCready (127), pectic substances containing methyl ester groups give low values. De-esterification before carbazole analysis brings the values into agreement.

In the second type of procedure, the temperature and time of heating are reduced and the reaction is conducted in 83% sulfuric acid. Under these conditions differences between different hexuronic acids are intensified and some polyuronides do not react. Comparisons of extinction coefficients obtained from other uronic acid reactions may provide additional information on uronide components of fractions of unknown composition (252).

Reaction with carbazole in 87% sulfuric acid.--The conditions of the carbazole reaction in 87% sulfuric acid adapted from the procedure of Dische (125) and McComb and McCready (127) are as follows:

1) Three ml of concentrated sulfuric acid (DuPont, reagent grade) are pipetted into 18 X 150 mm test tubes and allowed to equilibrate to ice bath temperature.

2) 0.5 ml of an aqueous solution containing 5 to 50 µg of uronic acid is layered over the sulfuric acid and the tubes swirled to insure complete mixing and returned to the ice bath. In addition to a water blank, standard samples containing 5, 10 and 20 µg of galacturonic acid monohydrate are included in each determination.

3) The tubes containing both the unknown and the control samples are then heated for 15 to 20 minutes utilizing a 100° C water bath.

4) Immediately after heating, the tubes containing the samples are immersed in running tap water for five minutes after which the samples are returned to room temperature.

5) 0.25 ml of an ethanolic solution containing 0.15% of carbazole (Eastman Kodak, reagent grade) is added to the appropriate tubes and the samples are thoroughly mixed.

6) After 25 minutes, the absorbance is determined at 535 mµ.

A plot of absorbance (optical density) versus concentration of uronic acid results in a linear relationship over the range 5 to 50 µg per ml. With the absorbance of the water blank subtracted, the resulting straight line passes through the abscissa at a point corresponding to

between 1 and 2 µg galacturonic acid monohydrate at zero absorbance. In reporting the uronic acid composition of pectic substances, the values are commonly expressed in terms of anhydrouronic acid. To convert galacturonic acid monohydrate to anhydrouronic acid equivalent, the values are reduced by the factor 176/212 = 0.83.

Although daily variations in the order of 20% occur with fractions known to be of pectic origin, these variations probably originate from non-pectic contaminants. In the case of non-uronide carbohydrates, daily variations in excess of 200% are not uncommon. Furthermore, the color intensity resulting from the presence of these substances is not proportional to dilution. Therefore, it is advisable to determine the anhydrouronic acid content of samples of unknown composition as a function of dilution. This is especially true when the absorbance of the sample is only slightly more than doubled by the addition of carbazole.

The specificity of the carbazole reaction.--Although hexoses have been reported to interfere in the carbazole reaction (125, 127, 244, 254), McComb and McCready (127) indicate that a number of substances (including glucose, fructose, arabinose, malic acid and citric acid) do not interfere with the quantitative aspects of the test within the concentration range 50-400 µg per ml. The nature of the hexose interference at higher concentrations is not clear from their report. Dische (125) states that hexoses may produce a brown-red color which can be confused with the color characteristic of hexuronic acids.

A number of substances have been tested in the carbazole reaction as possible sources of interference and the results are summarized in table 48. The substances were tested over the range 10 to 1,000 µg per ml and a molar extinction coefficient calculated from the midpoint of a linear portion of the curve. In the case of non-uronide materials, the values chosen are somewhat arbitrary since absorbance is often non-linear. For convenience, the concentration estimated to result in 50% transmission is also given.

Glucose and fructose produced red-brown colors with spectral properties easily confused with those of galacturonic acid (figure 35). The resulting color is the result of a mixture of a brown color produced with solutions of hexose by acid alone and a red color from the chromogen produced after additions of carbazole. The resulting spectra shows two maxima in the vicinity of 540 and 425 mµ. In the case of fructose the second maximum is shifted to longer wavelengths. Galactose forms predominantly a yellow-brown chromogen. Pentoses normally produce a yellow color with carbazole, xylose being an exception. Xylose reacted at 200 and 400 µg per ml to yield a reddish-orange color. Below 100 µg per ml and above 500 µg per ml of xylose the yellow color was again predominant.

A further consideration in evaluating the interferences resulting from hexoses is the fact that in no case was the relationship between absorbance and concentration either reproducible or linear over a wide range of concentrations. Sample concentration curves are presented in figure 36 a-d. Below 100 µg per ml, the color produced was nearly equal to that resulting from the reaction with acid and could be eliminated by an appropriate blank. Above 500 µg per ml yellow colors predominated

TABLE 48.

Summary of carbohydrates interfering in the reaction of uronic acids with carbazole in 87% sulfuric acid.

Material Tested	Molar Extinction Coefficient, $\epsilon \times 10^{-2}$	µg/ml for 50% Transmittance
Galacturonic Acid	12.0	50
Glucuronic Acid	10.0	42
Glucose	1.2	390
Fructose	1.3	430
Galactose	1.6	340
Glucosamine	0.3	1000
Xylose	1.0	375
Ribose	0.5	1000
Arabinose	0.4	1000
Rhamnose	0.1	1000
Ascorbic Acid	0.1	1000

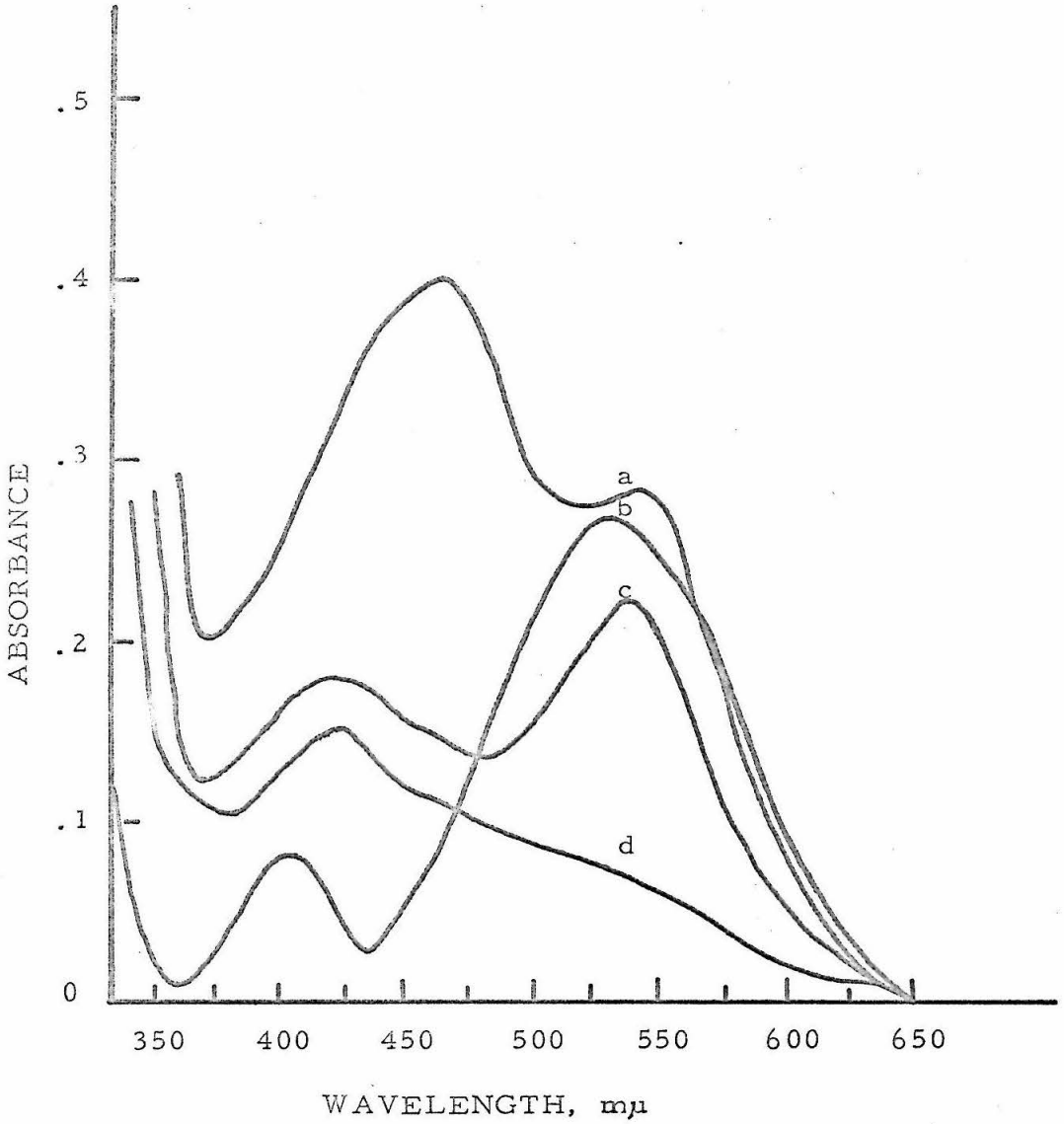


Figure 35. Absorption spectra of the products formed after reaction of several monosaccharides with carbazole in 87% sulfuric acid. a. Fructose, 400 μg per ml. b. Galacturonic acid, 40 μg per ml. c. Glucose 200 μg per ml. d. Galactose, 100 μg per ml.

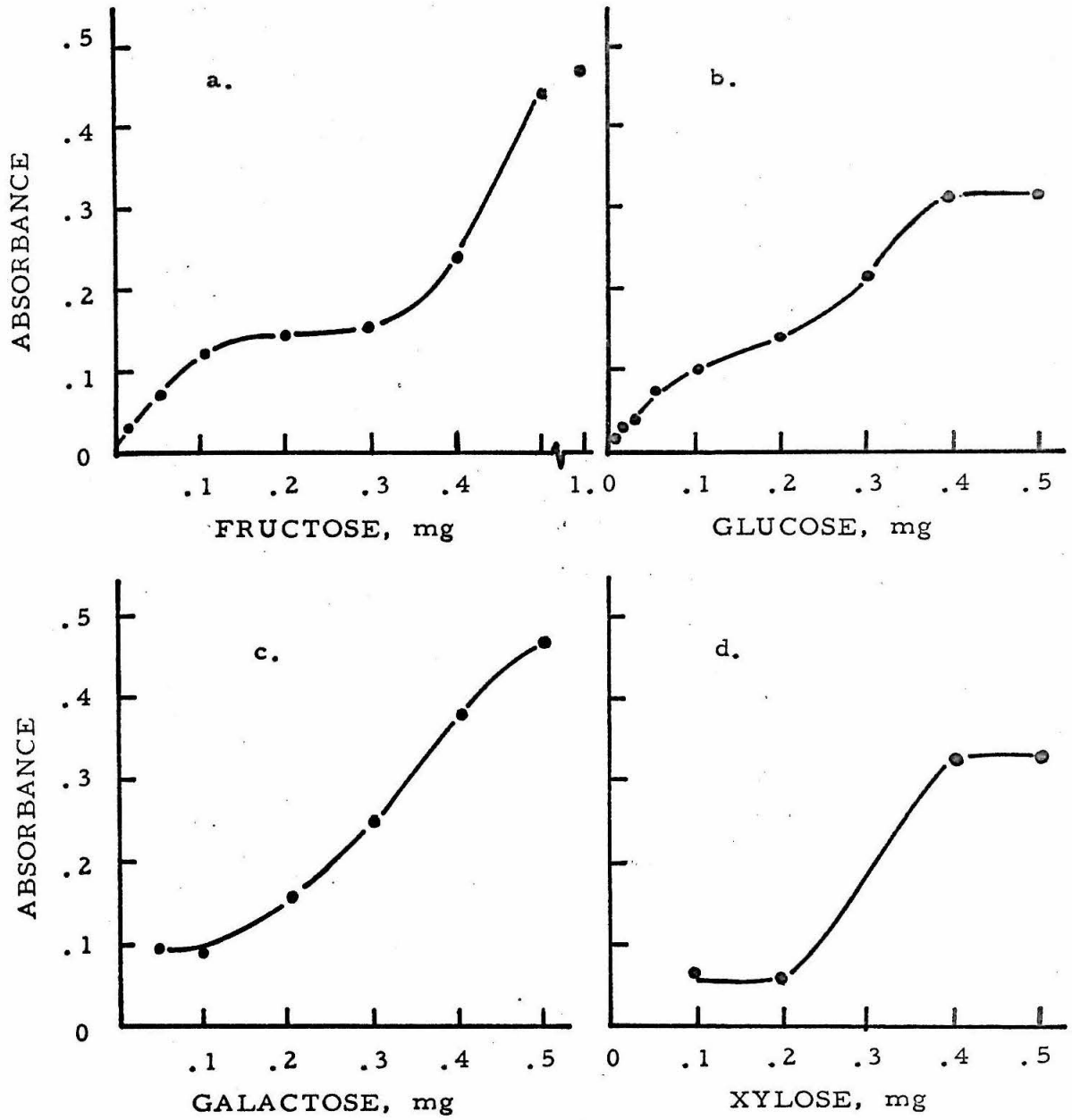


Figure 36. Non-linear concentration dependency of the absorbance of fructose, glucose, galactose and xylose solutions after reaction with carbazole in 87% sulfuric acid. Quantities refer to 1.0 ml of solution employed in the carbazole reaction.

and absorbance was occasionally found to decrease with increasing concentration of sugar. The interfering colors produced over the concentration range 100 µg and 500 µg per ml were found to be impossible to resolve in terms of a valid correction, giving rise to variations of as much as 200% in the results obtained.

Cysteine, glutathione and other sulfhydryl compounds, when present in solution, increase the intensity of the color obtained with uronic acid. According to Dische (125) colors produced from hexoses and pentoses tend to be affected to a greater degree. Effects of small amounts of sulfhydryl can be evaluated in part by establishing a reference curve from known quantities of uronic acid added to the solution in question. Proteins apparently do not represent a serious source of interference (126).

Determination of hexuronic acids in polyuronides by the reaction with carbazole in 87% sulfuric acid.--Although polyuronides react in the carbazole reaction without prior hydrolysis, this fact may be useful only in the case of samples of defined composition. The nature of the glycosidic linkage is known to influence the extinction coefficient to a certain degree (125). Therefore, it is a common practice to treat de-esterified samples of pectic substances with the enzyme pectinase prior to reaction with carbazole (46). The procedure adopted in the present study was to treat the pectin sample with an equal volume of 1 N NaOH for 30 minutes at room temperature, after which the pH of the solution was adjusted to pH 5.0 to 5.5 with acetic acid. The neutralized volume was then diluted with an equal volume of unbuffered pectinase solution containing 0.1 mg per ml of the enzyme preparation. After one hour, the uronic acid

content of the hydrolyzate was determined by the carbazole procedure. The uronic acid content was not increased by further treatment with the enzyme. All commercial pectic enzyme preparations tested were found to contain a carbazole-positive impurity. This contribution to total absorbance may be subtracted by means of an enzyme blank.

Absorption spectra of the carbazole adduct prepared from a hot water-soluble pectic fraction and a versene-soluble pectic fraction are shown in figure 37. The general form of the spectra are comparable to that obtained from authentic galacturonic acid.

Due to difficulties involved in estimating uronic acids in the presence of hexose impurities, a true measure of the uronic acid content of many crude pectic extracts can only be obtained after further purification. Preliminary separation is achieved using a general chromatography solvent (DIPWA, Appendix E). After elution of the zones corresponding to authentic galacturonic acid, the quantities present may be determined directly by the carbazole reaction, or the eluate may be further purified by electrophoresis (System E-II, Appendix E).

Pectic samples purified by paper chromatography were de-esterified using 1 N ammonium hydroxide. The time of hydrolysis was extended to four hours and the samples were contained in tightly stoppered tubes during de-esterification. Prior to addition of pectinase, the ammonia was removed under a stream of nitrogen and the final pH adjusted to 5.0 to 5.5 with acetic acid. The sample of purified citrus polygalacturonase used for chromatographic analyses was obtained through the courtesy of E. F. Jansen, Western Regional Research Laboratory, Albany, California.

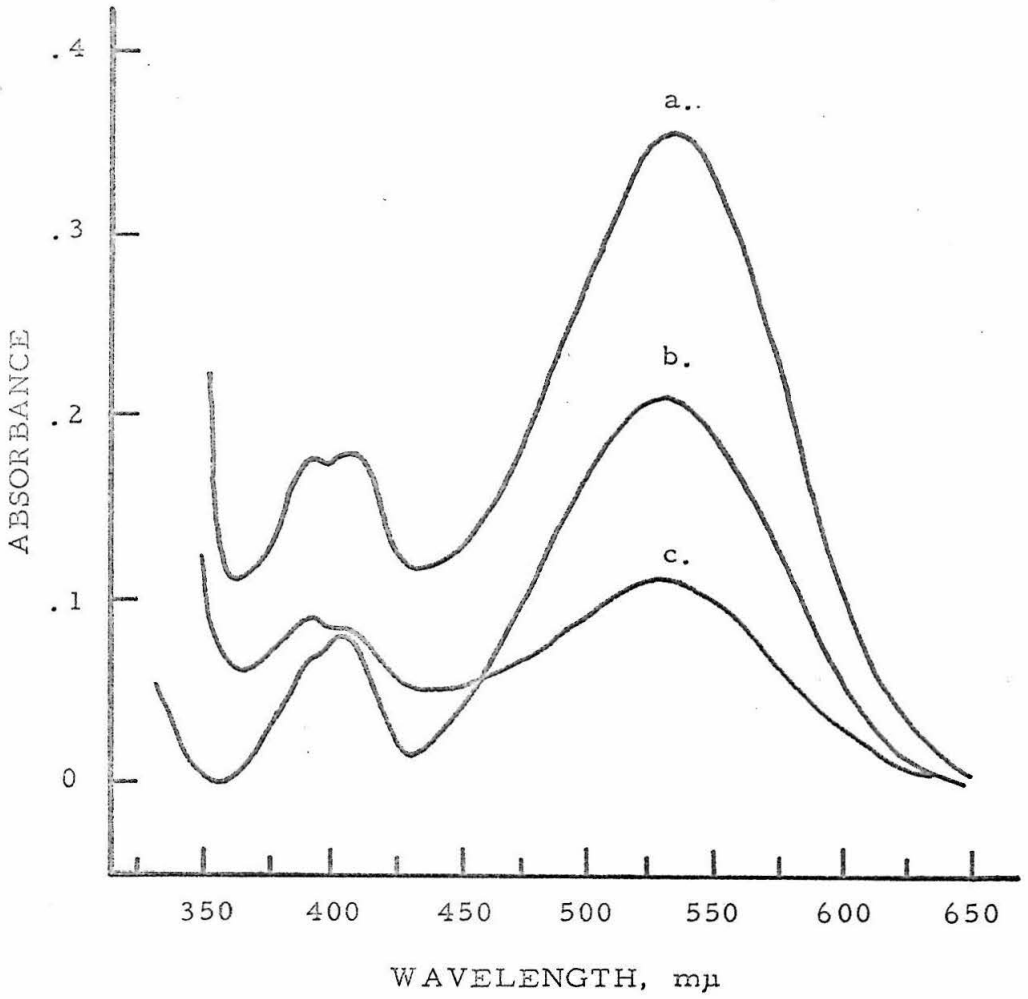


Figure 37. Absorption spectra of products formed after reaction with carbazole in 87% sulfuric acid of polyuronides extracted from Avena coleoptile cell walls. a. Hot water-soluble pectin (55 μg anhydrouronic acid per ml). b. Hot versene-soluble pectin (16 μg anhydrouronic acid per ml). c. Galacturonic acid monohydrate (80 μg per ml).

Determination of hexuronic acids directly from paper chromatograms by reaction with triphenyltetrazolium chloride (tetrazolium assay).--A somewhat simplified procedure was developed for routine estimation of uronic acids directly from paper chromatograms. For this purpose, known quantities of galacturonic acid were applied to one-inch strips of Whatman No. 1 filter paper and equilibrated three to five hours in a saturated atmosphere of the chromatography solvent. The strips were then irrigated 10 hours (DIPWA, Appendix E) and dried in an air stream overnight. The chromatograms were cut into two one-half inch strips and sprayed separately with an alkaline solution of 0.1% triphenyltetrazolium chloride. Color development was achieved by heating the strips for 10 minutes at 135° C. The color so obtained was stable for several days if not exposed to direct sunlight. Each zone corresponding to uronic acid was cut from the strips and fitted between one face of an empty cuvette and the inside face of the cell holder of a Beckman model B spectrophotometer with the center of the spot in the light path of the instrument. The absorbance at 490 mμ was determined with the strip in this position and then redetermined with the strips rotated through 90°, thus providing a total of four readings from the two halves of each chromatogram. A semilogarthmic plot of the average of the four absorbance readings versus total quantity of galacturonic acid was found to be linear in the range of 10 to 100 μg of galacturonic acid applied to the chromatograms (figure 38). The range of variation of the method as determined from a comparison of known quantities of galacturonic acid is of the order of ± 20%. A similar procedure has also been described by Wohnlich (255).

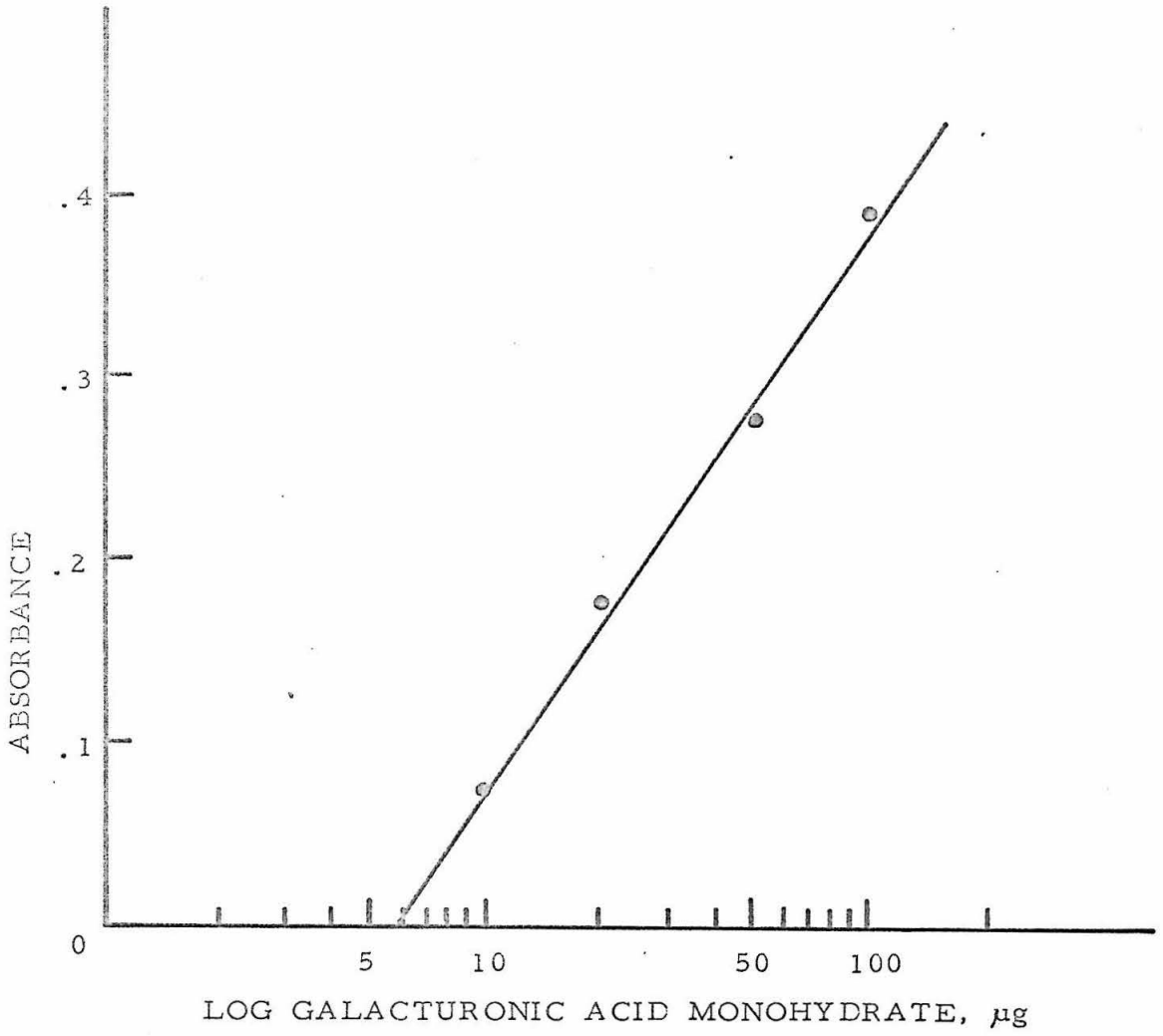


Figure 38. Calibration curve for direct determination of hexuronic acids from paper chromatograms. Following chromatography, the strips were sprayed with an alkaline solution of 1% triphenyltetrazolium chloride and were then heated for 10 minutes at 135° C. Quantities refer to the total amount of material uniformly applied to one inch strips of Whatman No. 1 filter paper. Absorbance was measured at 490 mµ.

As a further verification of the applicability of the procedure, aliquots of a hot water-extractable pectic fraction prepared from Avena coleoptiles were subjected to various hydrolytic treatments followed by chromatographic analysis (table 49). Each aliquot contained a total of 50 µg anhydrouronic acid as determined by reaction with carbazole. De-esterification was accomplished either by a four hour treatment with 1 N ammonium hydroxide at room temperature or treatment with an equal volume of a 0.1 mg per ml solution of tomato pectin esterase (Sigma) for four hours at pH 8. Essentially quantitative recovery of galacturonic acid was obtained when de-esterified pectin was further treated for three days under toluene with the preparation of purified citrus polygalacturonase. When the de-esterification step was omitted, a low R_f zone was observed on the paper chromatograms in addition to a discrete spot corresponding to galacturonic acid. The low R_f zone may represent partially hydrolyzed oligouronides. The aliquot treated with pectin esterase alone appeared to contain sufficient free galacturonic acid to suggest the presence of a polygalacturonase contaminant in the pectin esterase sample utilized.

Carbazole reaction differentiating hexuronic acids from polyuronides.--A modification of the carbazole reaction has been described by Dische (125, 202) which is carried out under relatively mild conditions. Pectic substances and certain other polyuronides do not react. In this procedure 0.4 ml of the unknown is added to 5.4 ml of a mixture of one volume of water and six volumes of 87% sulfuric acid. The mixture is heated for 90 seconds in a 60° C water bath, cooled and 0.2 ml of a 0.1% solution of carbazole is then added. The color is measured after one hour.

TABLE 49.

Effect of various chemical and enzymatic pretreatments on the release of reducing materials chromatographically equivalent to uronic acids from a hot water-soluble pectin fraction prepared from Avena coleoptiles. Each analysis is derived from material equivalent to 50 µg anhydrouronic acid based on the reaction with carbazole in 87% sulfuric acid.

Pretreatment	Enzymatic Hydrolysis	µg Anhydrogalacturonic Acid Isolated	
		Expt I	Expt II
None	None	9	8
None	Polygalacturonase	21[1]	14[1]
1 N NH_3	None	9	8
1 N NH_3	Polygalacturonase	48	--[2]
Pectin Esterase, pH 8	None	22[1]	20[1]
Pectin Esterase, pH 8	Polygalacturonase	55	50

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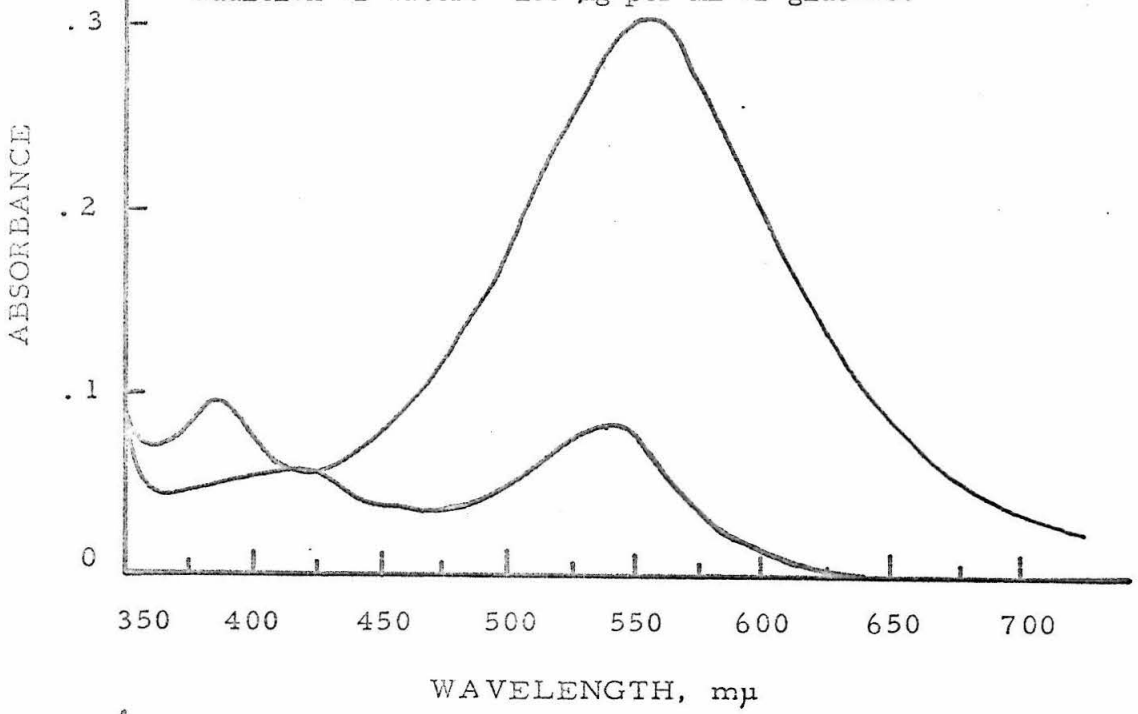
[1] In addition, a low R_f zone was observed corresponding to partially hydrolyzed oligouronides.

[2] Sample lost during analysis.

Free hexose was found to interfere in this modification of the reaction but the colors develop slowly. The test is useful over the range 50 to 500 µg per ml of galacturonic acid and according to Dische (125), galacturonic acid has an extinction coefficient 20 times higher than that of glucuronic acid.

A modified carbazole reaction providing a measure of both uronic acid and hexose content of pectic extracts.--Dische (125) reported that water added to the mixture resulting from the reaction with carbazole in 87% sulfuric acid attacks the colored compound produced by various sugars in different ways. The addition of 1 ml of water after normal carbazole development results in the complete disappearance of the color from hexuronic acids over the concentration range 10 to 40 µg per ml. Solutions containing hexose, however, rapidly develop in intense purple color with an absorption maximum near 550 mµ (figure 39a). The absorbance of the chromogen developed after 10 minutes is proportional to hexose concentration over the range of 20 to 200 µg per ml and slowly increases in intensity for several hours (figure 39b). For convenience in using the Beckman spectrophotometer, all measurements were carried out at 535 mµ, the absorption maximum of the carbazole adduct formed with uronic acids. Although providing a reasonably accurate measure of the hexose content of a sample in the presence of uronic acid, no simple assessment of the hexose contribution to uronic acid absorbance is yet available. The presence of low concentrations of versene in the reaction mixture result in the formation of a blue color similar to but not identical with that produced by hexoses.

Figure 39a. Absorption spectra of the reaction products before (lower curve) and after (upper curve) addition of water. 100 µg per ml of glucose.



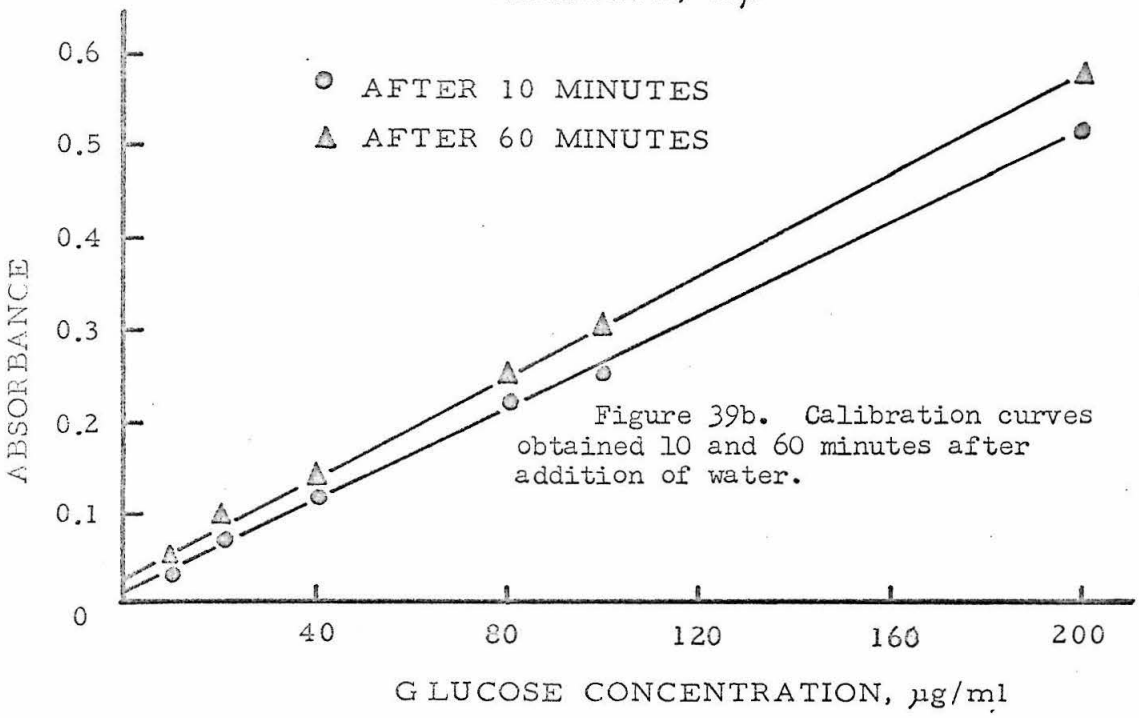


Figure 39b. Calibration curves obtained 10 and 60 minutes after addition of water.

Figure 39. Quantitation of the carbazole-water reaction as a means of determining levels of hexose interference in the reaction with carbazole in 87% sulfuric acid.

The following procedure was finally adopted for routine analysis of pectic extracts of unknown composition. An aliquot of the hydrolyzed material was reacted with carbazole in 87% sulfuric acid and the absorption spectra of the carbazole adduct recorded after 25 minutes of color development. One ml of water was then added to the reaction mixture and after 10 minutes the absorbance of the purple chromogen was determined at 535 mμ to estimate hexose content. With samples in which the hexose content was found to be less than 100 μg, a simple correction based on a no-carbazole blank was sufficient for routine determinations. If the hexose content exceeded 100 μg, the extracts were further purified by paper chromatography and the uronic acid content determined either directly from the chromatograms after treatment with tetrazolium chloride or by means of the carbazole reaction following elution.

Versene-pectinase extraction of cell wall constituents from whole tissue.--Fresh or fresh frozen tissue was ground with a mortar and pestle in the presence of 95% ethanol in a ratio of 1 g of tissue to 25 ml of ethanol according to the procedure of McCready and McComb (195). The cell walls were filtered off and washed twice with 75% ethanol. Aliquots of the combined ethanol filtrates were concentrated and dried to constant weight. The ethanol-washed walls were transferred to a beaker and treated with a 0.5% versene solution at pH 11.5 (8 ml per g fresh weight) for 30 minutes to de-esterify the pectic substances and sequester calcium ions. The pH of the cell wall suspension was then adjusted to pH 5 to 5.5 with acetic acid and treated with pectinase (8 mg per g fresh weight of starting material) with occasional stirring for one hour. The insoluble residue was then filtered off and the total carbohydrate was estimated in terms

of anhydrogalacturonic acid equivalent by means of the carbazole reaction. The residual material was washed with water and acetone, dried in vacuo and weighed. The weight of the material solubilized by the pectinase versene treatment was determined from aliquots of the filtrate and combined washes corrected for added pectinase and versene.

Data obtained using maize roots, maize coleoptiles, mung bean roots and mung bean hypocotyls are presented in table 50. It is readily apparent that a large proportion of the material extracted as a result of the versene-pectinase treatment cannot be accounted for on the basis of pectic anhydrouronic acid.

Versene-pectinase extraction of cell wall constituents of isolated cell wall.--Plant material was grown as described in previous sections. Cell wall fractions were prepared from maize roots, 13 mm maize coleoptile sections with primary leaves removed, maize primary leaves, whole mung bean roots, 13 mm mung bean hypocotyl sections (cut 5 mm from the hook) and 13 mm Avena coleoptile sections with primary leaves intact. The procedure for preparation of cell walls is that of Jansen et al. (46).

The plant material was ground with a mortar and pestle in four volumes by weight of 0.15 M acetate buffer, pH 4.4. The homogenate was centrifuged, the supernatent liquid decanted and the pellet washed by resuspension in water at 0° C followed by centrifugation. This procedure was repeated five times and followed by three washes with acetone. The acetone-washed cell wall material was dried in vacuo and stored for analysis.

TABLE 50.

An analysis of the versene-pectinase method[1] of extraction of pectic substances from fresh tissue.[2]

Plant Material	Material Soluble in 75% Ethanol mg	Material Solubilized by Versene-Pectinase Treatment mg	Total Pectin by Carbazole mg	Residual Cell Wall mg	Total mg	Oven Dry Weight mg	% Recovery
Maize Roots	123	27	1.4	206	356	374	95
Maize Coleoptiles	100	90	4.1	173	363	370	98
Mung Bean Roots	131	51	9.6	112	294	296	99
Mung Bean Hypocotyls	122	60	8.0	36	218	226	96

1 Procedure of McCready and McComb (195).

2 Based on 3 g fresh weight of tissue.

The combined supernatent liquid and subsequent washes were concentrated 25-fold using a Rinco evaporator. This concentrate was used for the preparation of the cold water-soluble 70% alcohol-precipitable fraction (46).

Finely ground dried cell wall was added to 0.5% versene solution, pH 11.5 in a ratio of 100 mg per 20 ml of solution. The solution was neutralized and the extraction continued according to the procedures outlined in the preceeding section. Composition values presented in table 51 are based on parallel measurements of total cell wall and dry weight. The values for total anhydrouronic acid equivalent of Avena coleoptiles agree reasonably well with that reported by Jansen et al. (46). Those obtained for other tissues are in the same order of magnitude (1 to 10%) as currently accepted pectic compositional data (4). The composition of the cold water-soluble, 70% ethanol-precipitable fraction has been discussed (Part IV).

Extraction of pectic substances from cell walls of Avena coleoptiles.-- The general procedures are those followed by Jansen, Jang, Albersheim and Bonner (46). The uronic acid content was determined using the modified pectinase-carbazole method.

Oat seeds of the variety Siegeshafer were germinated in vermiculite contained in stainless steel trays. Coleoptiles were harvested when they had reached an average height of 3 cm (96 hours). Whole coleoptiles were chopped and ground in a mortar and pestle with four times their weight of 0.15 M acetate buffer, pH 4.4. Jansen et al. (194) have shown that at this pH no de-esterification of pectin by pectin esterase takes place

TABLE 51.

Comparison of the carbazole carbohydrate content of the fraction soluble in cold sodium acetate buffer, pH 4.4 and the fraction obtained by versene-pectinase extraction of the isolated cell wall.

	% Carbazole Carbohydrate as Anhydrouronic Acid[1]			
	Cold Water-Soluble, 70% Ethanol Precipitable[2]		Versene-Pectinase Extractable[3]	
Tissue	Dry Weight	Cell Wall	Dry Weight	Cell Wall
Maize Roots	0.12	0.24	0.7	1.2
Maize Coleoptiles	0.10	0.28	0.9	2.6
Maize Primary Leaves	0.06	0.18	0.9	2.6
Mung Bean Roots	0.13	0.36	3.2	7.9
Mung Bean Hypocotyls	0.14	0.57	3.7	9.2
Avena Coleoptiles	----	0.11	---	4.7

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1 Reaction with carbazole in 87% sulfuric acid.

2 Procedure of Jansen *et al.* (46).

3 Procedure of McCready and McComb (195).

during homogenization. The walls were removed by centrifugation, the supernatent liquid decanted and the pellet washed by resuspension in water at 0° C followed by centrifugation. This procedure was repeated five times and followed by three washes with acetone. The acetone-washed cell wall material was dried in vacuo and stored for analysis.

Hot water extraction was achieved by treating 100 mg dry cell wall with 10 ml portions of water at 100° C for 30 minutes. At the end of each 30 minute extraction period, the cell walls were filtered through coarse sintered glass and were recovered for re-extraction. The filtrates were concentrated and the extracted carbohydrates precipitated from 70% ethanol. Hot versene extractions were accomplished in a similar manner using instead of water, 10 ml portions of 0.5% sodium versenate.

Experiment I of table 52, represents four hot water extractions followed by two extractions with hot versene. In the case of experiment II, the walls received a total of six hot water extractions followed by two extractions with hot versene. The additional hot water extraction of experiment II increased the total quantity of hot water-soluble anhydrouronic acid equivalent by nearly 50%, whereas, the total amount remaining in the versene-extractable fractions was essentially unchanged. Although the values corresponding to hot water-soluble pectin resemble closely those reported by Jansen et al. (46), the total quantity extracted accounted for less than 2% of the total cell wall. In an effort to resolve this discrepancy, the efficiency of extraction with time was studied in some detail.

TABLE 52.

Preliminary analysis of the distribution of anhydrouronic acid between two cell wall pectic fractions of Avena coleoptiles. Anhydrouronic acid content determined by reaction with carbazole in 87% sulfuric acid.

	Percent of Cell Wall		
Fraction	Expt I[1]	Expt II[2]	Data of Jansen et al. (46)
Hot Water-Soluble	0.93	1.33	0.8
Hot Versene-Soluble	0.42	0.42	4.5[3]
Total	1.35	1.75	5.3

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[1] Four hot water extractions followed by two extractions with hot versene.

[2] Six hot water extractions followed by two extractions with hot versene.

[3] Residual fraction of Jansen et al. (46).

In the experiments represented by the data of figure 40, hot water extractions were continued until carbazole-positive material could no longer be detected in the extracts. The first five 30-minute extractions were nearly equivalent and the initial rate of increase seems to be related to the relatively slow hydration of the acetone-dried walls. The extraction efficiency as determined from eight 30-minute extraction periods was still nearly 50% of the maximum rate of extraction. A total of 9 1/2 hours of continuous reflux was required to reduce the quantity extracted to below the limits of detection of the carbazole reaction. The dotted portion of the hot water-soluble pectin curve is drawn so that the area conforms to the total quantity of galacturonic acid equivalent extracted. The exact form of the curve in this region is not known.

In two separate experiments (figure 40), versene extractions were initiated after the eighth hot water extraction and after 13 hours of continuous reflux with 260 ml of hot water, respectively. Since the area between the hot water extraction curve (as drawn) and versene curve a corresponds very nearly to the area under versene curve b, it is concluded that the hot versene-soluble uronide components of the cell wall represent a distinct solubility class. Furthermore, the hot versene-soluble fraction does not appear to be solubilized to a significant extent by continued extraction with hot water. All hot water-soluble fractions were found to contain non-polyuronide contaminants by application of the analytical procedures outlined in the preceeding sections.

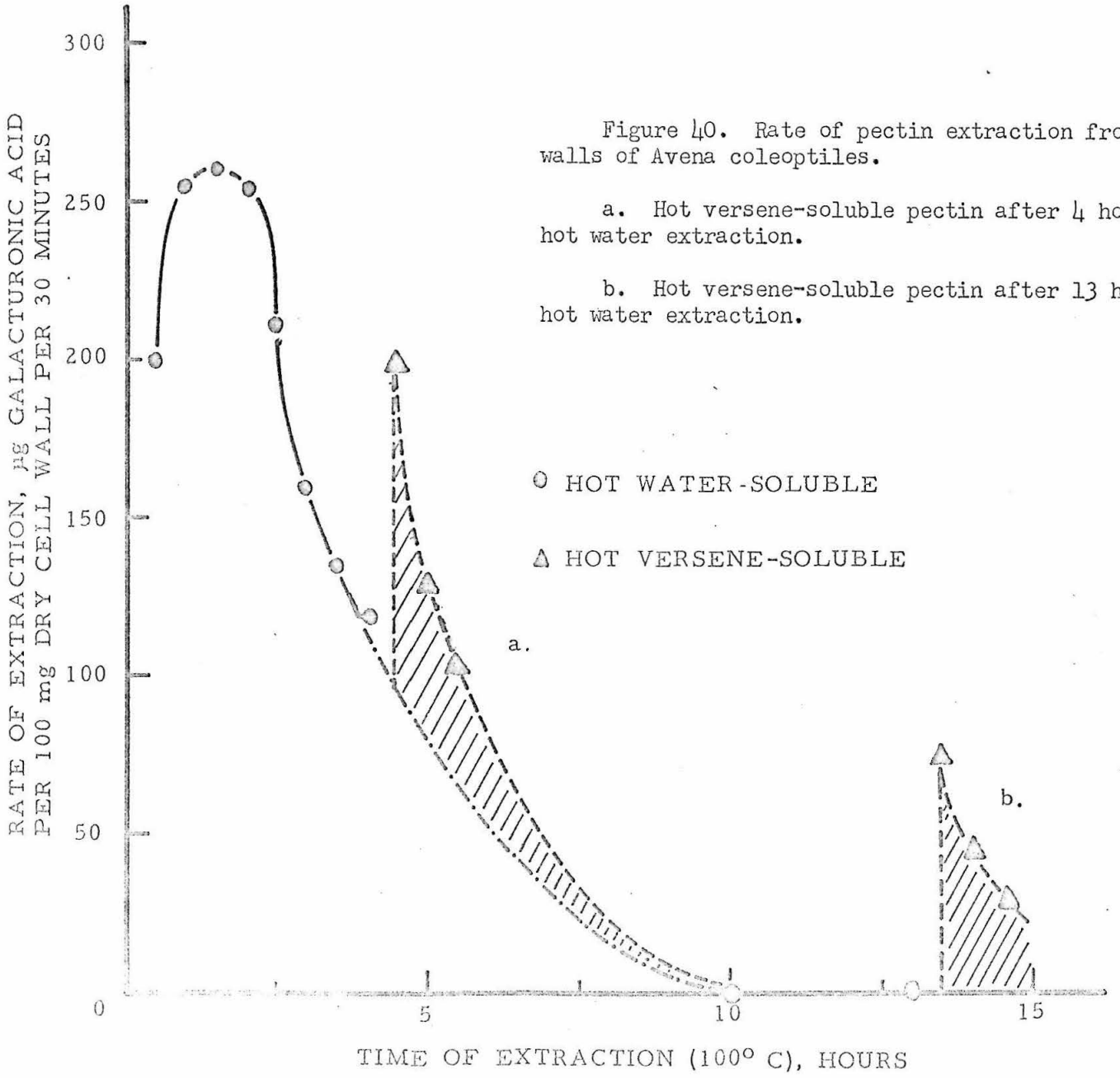

Figure 40. Rate of pectin extraction from cell walls of Avena coleoptiles.

a. Hot versene-soluble pectin after 4 hours of hot water extraction.

b. Hot versene-soluble pectin after 13 hours of hot water extraction.

Summary data based on the above extraction experiments are given in table 53, in terms of mg anhydrouronic acid and compared with the data of Jansen et al. (46). Although the quantity of hot water-extractable materials was increased nearly 2.4-fold through continued extraction to account for 1.9% of the cell wall, the versene-soluble fraction would appear to account for less than 10% of the anhydrouronic acid of the residual material available for further extraction.

Summary.--Several routine procedures for the extraction and determination of plant pectic substances are described and discussed. Hexoses were shown to interfere in the carbazole reaction for uronic acids in a non-linear fashion and a simplified procedure for direct evaluation of hexose interference was developed.

The fact that pectic substances solubilized by EDTA (versene-soluble pectin) constitute a solubility fraction distinct from hot water-soluble pectin has been established. It is suggested, however, that the conditions for extraction of the versene-soluble fractions do not correspond to those of the classical residual pectin fraction.

TABLE 53.

Summary of the distribution of anhydrouronic acid among several cell wall pectic fractions of Avena coleoptiles as determined by reactions with carbazole in 87% sulfuric acid.

	Percent of Cell Wall	
Fraction	Summary of Present Study[1]	Data of Jansen et al. (46)
Hot Water-Soluble	1.9	0.8
Hot Versene-Soluble	0.2	4.5[2]
Total	2.1	5.3
Versene-Pectinase-Extractable	4.7	---

[1] One g fresh weight of tissue was found to yield 27 to 28 mg of dry cell wall.

[2] Residual fraction of Jansen et al. (46).

APPENDIX C

A PRELIMINARY ANALYSIS OF THE IAA-INDUCED INCREASE IN ACID PHOSPHATASE ACTIVITY

In a report by Olsen (256), the phosphatase activity of maize roots was shown to be increased strikingly as a result of the addition of 1.5 ppm of 2,4-D to the culture solution; a concentration of 2,4-D which also results in the inhibition of root growth. In these experiments effects were apparent in as short a time as three hours. An increase in acid phosphatase activity of extracts prepared from IAA-treated Avena coleoptiles has also been observed (155).

These results are of particular interest in view of the cytochemical localization of acid phosphatase activity in certain membrane-bound structures (e.g., Golgi apparatus (219, 257, 258)). The increase in acid phosphatase activity as a result of IAA treatment was, therefore, reinvestigated in the hope that the increase might be localized in a specific subcellular fraction.

Materials and Methods

Method of assay.--The nitrophenyl phosphate method as adapted by Axelrod (259) was used as the primary method of assay. The reaction mixture was modified to include 1.0 ml of 0.2 M sodium acetate buffer, pH 5.4, 1.5 ml of an aqueous solution containing 4 μM p-nitrophenylphosphate

and 0.5 ml of the enzyme solution prepared in 0.2 M acetate buffer, pH 5.4. The final buffer concentration was, therefore, 0.1 M with respect to sodium acetate. The unit of enzyme activity is that defined by Kilsheimer and Axelrod (260), i.e., that quantity of enzyme which will liberate 1.0 µM of p-nitrophenol per ml of reaction mixture per minute. Enzyme preparations were normally diluted to contain between 0.05 and 0.1 units of enzyme.

After temperature equilibration, (24° to 25° C), the enzyme was added and the course of the reaction followed at 0.5 minute intervals for a total time of 10 minutes. The initial rate of reaction was obtained by extrapolation of the linear portion of the curve to zero time. The quantity of liberated p-nitrophenol was determined by measuring the optical density of the reaction mixture at 400 mµ using a Beckman model B spectrophotometer. Corrections for spontaneous hydrolysis of substrate were found to be unnecessary. In experiments involving the comparison of extracts of IAA-treated and -untreated tissues parallel determinations of activity were conducted, in which the course of the reaction in each of the two samples was measured at equally spaced intervals.

The rate of hydrolysis of β-glycerol phosphate was determined by measuring the liberated inorganic phosphate by the method of Sumner (129). Time of hydrolysis and enzyme concentration were adjusted to obtain linear rates. The data obtained were corrected on the basis of both a substrate and enzyme blank, included in each determination.

Enzyme preparation.--Enzyme solutions used were crude extracts prepared from the following tissues: Avena coleoptile sections, Alaska pea subapical stem sections, maize root tips, maize root sections (5 mm zone of elongation) and mung bean hypocotyl sections. Mung beans were pregerminated in running tap water for a period of 48 hours and then transferred to enamel instrument trays lined with moistened filter paper. The trays were covered with foil and transferred to a dark, humid, constant temperature room (25° C) for an additional 72 hours. Subapical sections 13 mm in length were cut from hypocotyls which had attained a length of 2 to 3 cm. Other plant material was grown as described in previous sections.

Enzyme readily extracted from the tissue was obtained by light homogenization using a mortar and pestle followed by decantation of the press juice in the case of Avena coleoptiles and maize roots. Approximately equal volumes of extract were collected from both IAA-treated and parallel control samples. With pea epicotyls and mung bean hypocotyls, initial extracts were obtained by low shear homogenization (described in Part III, Section B). In both situations homogenization was conducted in the presence of 0.2 M sodium acetate buffer, pH 5.4 in the ratio of 1 ml of buffer per g fresh weight of tissue. An aliquot of the crude homogenate was removed for assay and the remainder centrifuged (500 X g) to remove cell debris.

Residual acid phosphatase was obtained by exhaustive grinding (mortar and pestle) of the residue remaining after initial extraction. Extraction was achieved with a volume of buffer equal to that of the first homogenization. The extracts so obtained were then centrifuged to remove cellular debris.

Substrates.--Commercial preparations of p-nitrophenylphosphate and β-glycerolphosphate (Calbiochem) were used. Stock solutions were kept refrigerated and diluted with water prior to use.

Results and Discussion

Avena coleoptile tissue was investigated first and typical results are given in table 54. Since the two substrates were found to yield comparable activity ratios between IAA-treated and -untreated tissues, the nitrophenylphosphate assay was selected in subsequent analysis due to the greater activity and ease of estimation. The acid phosphatase activity obtained as a result of the initial homogenization was found to be 20% greater in the extracts prepared from IAA-treated tissues. Specific activity, units per mg protein, was unchanged by IAA treatment and the difference in total activity was completely removed when the tissue was homogenized a second time. These results are similar to those obtained by Bonner (155) and were first interpreted as being due to differences in ease of grinding between IAA-treated and -untreated tissue. Subsequent experiments revealed increases of only 15%, 10% and 9% under conditions of homogenization similar to those giving rise to the 20% increase of table 54.

Preliminary experiments with other tissues yielded similar variable results with the increases in acid phosphatase activity varying between 0 and 35%. In all cases these differences were restricted to fractions obtained by light homogenization. These data are summarized in table 55 ;

TABLE 54.

The distribution of acid phosphatase activity between two sequential homogenizations of Avena coleoptile sections, comparing IAA treated and untreated tissues.[1]

		Substrate			
		β-glycerolphosphate		p-nitrophenylphosphate	
Fraction	IAA Concentration	Units per g Fr. Wt.	Ratio IAA/Cont	Units per g Fr. Wt.	Ratio IAA/Cont
First Extraction	None	0.010	1.20	0.146	1.20
	5×10^{-6} M	0.012		0.175	
Second Extraction	None	0.013	0.85	0.118	0.85
	5×10^{-6} M	0.011		0.100	

[1] Tissue incubated for 8 hours in the presence of 0.0025 M potassium maleate buffer, pH 4.5.

Unit = that quantity of enzyme which will liberate 1.0 µM of substrate per ml of reaction mixture per minute.

Reaction Mixture = enzyme contained in 0.1 M sodium acetate buffer, pH 5.4 + 4 µM substrate. Total volume of 3 ml.

TABLE 55.

A comparison of the readily solubilized and residual acid phosphatase activities of various tissues treated for 8 hours in the presence and absence of 5×10^{-6} M IAA.

		Readily Soluble Activity			Residual Activity		
Tissue	Treatment	Units per g Fr. Wt.	Ratio IAA/Cont	Units/mg Protein	Units per g Fr. Wt.	Ratio IAA/Cont	Units/mg Protein
Pea Epicotyl Sections	None	0.144	1.26	0.073	0.388	1.07	0.200
	IAA	0.182		0.083	0.415		0.206
Avena Coleoptile Sections	None	0.115	1.22	0.163	0.187	0.91	0.350
	IAA	0.140		0.160	0.171		0.353
Mung Bean Hypocotyl Sections	None	0.103	1.35	0.156	0.228	0.99	0.116
	IAA	0.139		0.167	0.225		0.116
Maize Root Tips	None	0.108	1.27	0.073	0.565	0.99	0.060
	IAA	0.137		0.070	0.560		0.060
Maize Root Sections; 5 mm Elongation Zone	None	0.306	1.21	0.180	0.470	1.00	0.193
	IAA	0.372		0.187	0.471		0.193

Unit = that quantity of enzyme which will liberate 1.0 µM of p-nitrophenol per ml of reaction mixture per minute.

Reaction Mixture = enzyme contained in 0.1 M sodium acetate buffer, pH 5.4 + 4 µM p-nitrophenylphosphate. Total volume of 3 ml.

the experiments showing the largest IAA-induced increase in acid phosphatase are reported.

The data of table 55, considered in total, suggest a general pattern of response. 1) The increase in acid phosphatase activity, when observed, is restricted to the readily soluble fraction obtained either by light or low shear homogenization. 2) The quantity of p-nitrophenol released per mg protein is essentially unchanged as a result of IAA treatment reflecting a net increase in total protein. 3) The residual activity is but little affected as a result of IAA treatment. And 4) the sum of the readily soluble and the residual activity is in general increased by the presence of IAA, unlike the preliminary experiment with Avena coleoptiles.

A partial explanation of the results obtained comes from measurements of the enzyme activity of the readily extracted fraction before and after centrifugation at room temperature. These results are summarized in table 56. Centrifugation was conducted for a period of 10 minutes in a clinical centrifuge (ca. 2,400 rpm). In the cases of pea epicotyl sections and maize root tips, the activity ratios between IAA-treated and control tissues were initially near unity. If the extracts were centrifuged immediately, the ratios were observed to change as a result of a decreased acid phosphatase activity in the extracts prepared from untreated tissue. A similar, although less dramatic, result was also observed with Avena coleoptile extracts.

Data obtained using a protein preparation from mung beans is illustrated graphically in figure 41. The initial acid phosphatase

TABLE 56.

The change in acid phosphatase activity associated with the IAA-induced stabilization of proteins. Tissues treated for 8 hours in the presence and absence of IAA.

		Acid Phosphatase Activity			
		Crude Extract		Centrifuged Extract	
Tissue	IAA Concentration	Units per g Fr. Wt.	Ratio IAA/Cont	Units per g Fr. Wt.	Ratio IAA/Cont
Avena Coleoptile Sections	None	0.093	1.18	0.074	1.32
	5×10^{-6} M	0.110		0.098	
Pea Epicotyl Sections	None	0.173	1.04	0.143	1.21
	5×10^{-6} M	0.180		0.173	
Maize Root Tips	None	0.137	0.97	0.110	1.21
	5×10^{-6} M	0.133		0.133	

Unit = that quantity of enzyme which will liberate 1.0 µM of p-nitrophenol per ml of reaction mixture per minute.

Reaction Mixture = enzyme contained in 0.1 M sodium acetate buffer, pH 5.4 + 4 µM p-nitrophenylphosphate. Total volume of 3 ml.

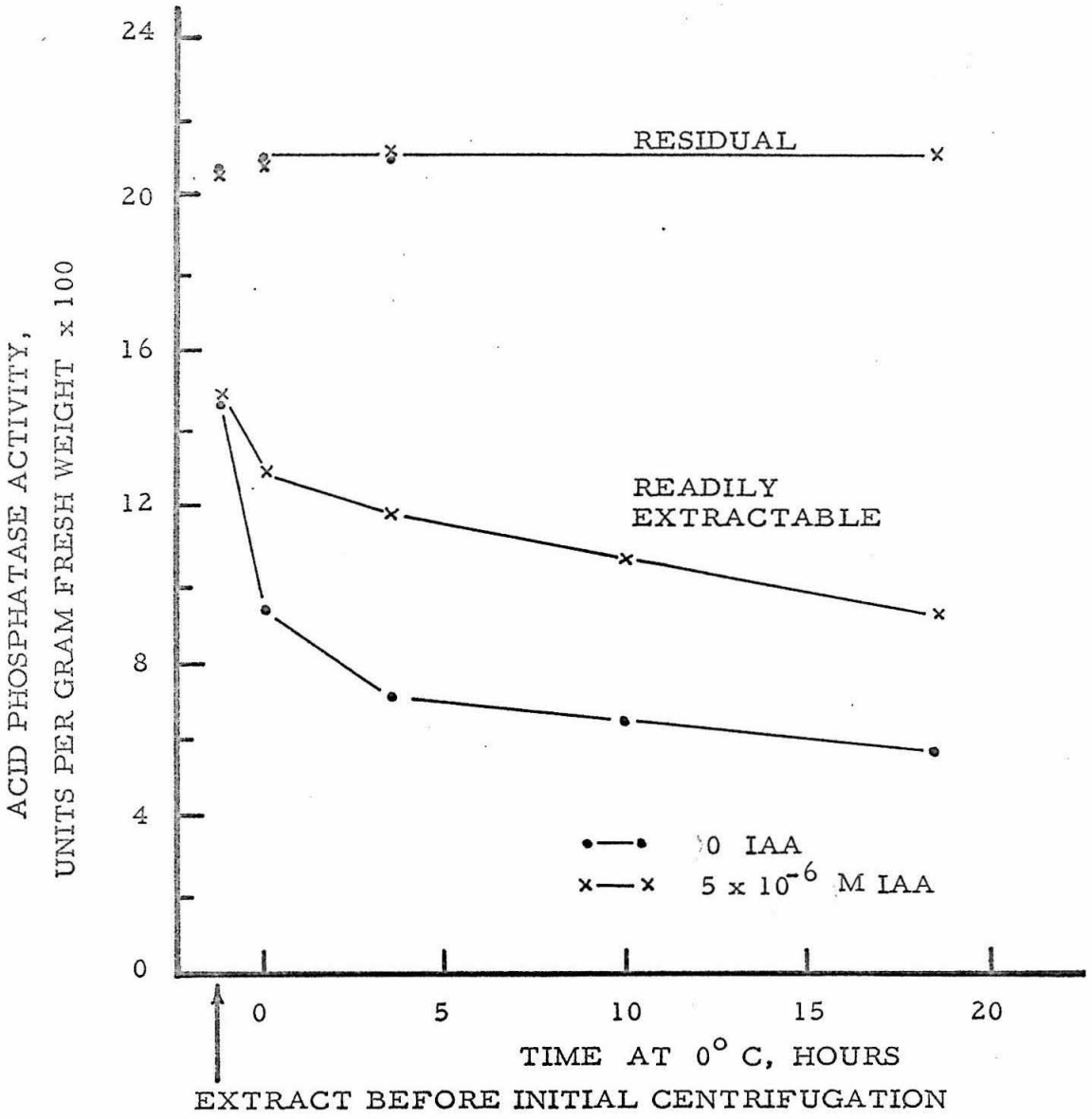

Figure 41. A graphical representation of the IAA-induced stabilization of the readily solubilized acid phosphatase activity of mung bean hypocotyls.

activities of readily extractable fraction of both IAA-treated and control tissues were nearly equal immediately after preparation. An initial centrifugation at room temperature increased the ratio (IAA/control) to 1.35, resulting primarily from a loss of activity in extracts from IAA-untreated tissue. The enzyme preparations were then stored at a temperature of 0° C and sampled over a period of several hours. Between 0 and 3 1/2 hours, the ratio increased still further to 1.60 but thereafter remained constant although the activities of both preparations continued to decrease. The residual activity in the case of this particular preparation of mung beans appeared quite stable and was unaffected by the centrifugation pretreatment. This was the case in general for all residual preparations studied. The ratio was found to remain constant even with extensive heat denaturation.

On the basis of these observations, it would seem reasonable to interpret the IAA-induced increase in acid phosphatase activity as a stabilization phenomenon similar to that discussed in Part III, Section C. The response of acid phosphatase to IAA-induced protein stabilization, however, would appear highly dependent upon external factors. Since the IAA-induced glycolipide (PGL) fraction was also found to be contained largely in the readily extractable fraction, it is possible to account for the increase in acid phosphatase activity on the basis of increased PGL. The apparent lack of response to IAA in certain extracts as well as the parallel stability of extracts after the initial centrifugation suggest the possibility that complex formation occurs during grinding. In either case, some protein not stabilized after grinding would be lost during centrifugation. The fact that the residual acid phosphatase activity shows no differences due to IAA-treatment would provide support

for either explanation. Additional stabilization of the residual fraction could result from the extraction of other stabilizing agents released by the more extensive homogenization.

Conclusions

The increase in acid phosphatase activity associated with indole-acetic acid-induced growth was re-investigated and the response was found to be a characteristic of all plant tissues studied. However, the response is restricted to a readily soluble enzyme fraction with total enzyme activity being generally unchanged. Since the differences obtained are augmented by a centrifugation at room temperature prior to acid phosphatase assay, the results are interpreted in terms of increased stabilization of protein as a result of IAA treatment. The nature of the stabilization cannot be deduced from these experiments but is suggested to arise from complex-formation with a stabilizing agent extracted with the protein.

APPENDIX D

INCORPORATION OF C^{14}-GLUCOSE INTO INSOLUBLE CELL WALL MATERIAL IN SINGLE CELL DISPERSIONS OF HIGHER PLANT TISSUES

Isolated plant cells or plant cell suspensions produced in culture have been used in the study of a variety of physiological and biochemical matters, including cell division (261-267), susceptibility to virus infection (268, 269), cellular differentiation (270, 271), production of cell metabolites (272), food production (273) and cell wall composition (274, 275, 276). Various aspects of the use of suspension cultures of higher plant cells have been recently reviewed by Torrey and Reinert (277).

For the study of the *in vivo* biosynthesis of cell wall constituents it would be highly advantageous to have a system in which added substrate could enter all cells equally rapidly with minimum delay between application and absorption. Such a system would greatly facilitate short term and pulse experiments with radioactive materials. In addition, single cell suspensions could be transferred by pipette and uniform aliquots removed for analysis at closely spaced time intervals in much the same manner as with bacterial cell suspensions. Dispersion cultures would offer additional advantages due to the rapidity and ease of their preparation and the fact that maintenance of stock cultures would not be required

during periods when cells were not routinely used. The technique has been applied successfully to the isolation of single cells of apple fruits (278, 279) and of tobacco leaf cells capable of supporting virus multiplication (269). The procedure used in these studies is based on the enzymatic degradation of the intercellular cementing substances in combination with the chelating agent ethylenediaminetetraacetic acid (EDTA).

Preparation of cell dispersions.--Cucumber fruits were purchased locally, surface sterilized for 30 minutes in a solution of 1% sodium hypochlorite (Chlorox), peeled and the pericarp cut into strips of uniform thickness. The strips were then cut into blocks 1/2 inch X 1/2 inch X 1/4 inch using a commercially available food dicer ("Villa" potato chipper); each block weighing 1.135 g $\pm$ 0.025 g. The blocks were cut into four equal parts with a sharp blade and transferred to the dissociation medium in the proportion of 1 g of tissue to 20 ml of solution. The dispersion medium was buffered at pH 7 with 0.01 M potassium phosphate buffer. Erlenmeyer flasks containing tissue in an appropriate dispersion medium were placed on a laboratory shaker with a reciprocating motion (200-250 excursions per minute) for a period of four hours. All preparative operations were conducted asceptically. Enzyme solutions were filtered through 0.22 u Millipore filters using a pre-sterilized filter and stainless steel pressure filter holder (Millipore XX4004700) to remove contaminating micoorganisms.

Cells were collected from the dispersion medium by filtering through gauze or nylon mesh. Undissociated clumps of cells remained on the filter,

whereas, a large proportion of the dissociated cells passed through. Isolated cells remaining on the filter were washed through with a small amount of buffer. Cells were collected by centrifugation (800 rpm, 5 minutes) in a clinical centrifuge. The cells were resuspended and washed three times with phosphate buffer employing volumes corresponding to the original amount of dispersion medium. The number of cells retaining an intact protoplast and the amount of debris was appraised from the appearance of the cells under the light microscope.

Preparation of cell walls.--Cell walls, relatively free of cytoplasmic contamination, could be prepared by subjecting the isolated cells to additional buffer extractions. Cells were centrifuged at 3,000 X g for 15 minutes and resuspended in 0.01 M phosphate buffer, pH 7.0. Centrifugation and resuspension was continued until the cell wall, as seen under the microscope, was free of visible signs of adhering protoplast. Normally three to five additional centrifugations resulted in a relatively pure cell wall preparation when mature cucumber fruits constituted the starting material. Most of the walls appeared intact but occasional wall fragments and fragments of vascular elements were observed.

Effect of pectinase concentration.--The use of the enzyme pectinase as an aid in the dispersion of plant tissues into single cells has been used successfully in the case of root tips by Chayen (280, 281) and in the case of tobacco leaves by Zaitlin (269). Therefore, buffered tissue blocks were added to varying concentrations of a commercial preparation of pectinase (Nutritional Biochemicals Corp.). The flasks were then placed on the reciprocating shaker. After a period of four hours, the dispersions were filtered and the material which was retained on the

gauze was collected, blotted and weighed. Percent of dispersion derived from the initial and final weights of the blocks is given as a function of pectinase concentration in figure 42. The concentration for half-maximal dispersion was established as 1.25 mg NBC pectinase per ml and maximum dispersion during the four hour incubation period was found to be near 85%.

Crude pectinase preparations are known to contain other enzymes which would be expected to cooperate in cell wall degradation such as hemicellulases, amylases, cellulases and pectin esterases (282). As shown in table 57, the addition of pectin esterase increases the effectiveness of pectinase, presumably by de-esterification in hydrolyzing methlated pectins (283). This effect was most pronounced in the case of a purified pectinase preparation, Sigma pectinase (Sigma Chemical Company, Lot P21B-222). In the latter case the dispersion was increased from 36% to 64% by the addition of 0.1 mg per ml tomato pectin esterase (Sigma Chemical Company) to a solution containing 1.25 mg per ml Sigma pectinase and 0.005 M EDTA. Unfortunately, the additional effectiveness of the pectin esterase was also reflected in an increased tendency toward fragmentation of the cells, the net result being a reduction in the amount of intact cells and/or cell walls isolated (table 57). Therefore, pectin esterase was not used routinely as a component of the dispersion medium.

Effect of pH.--The effect of pH of cell dispersion has been studied by Ginzburg (284, 285) and by Letham (278). The results do not lend themselves to clear interpretation in terms of any single parameter. The effect of pH on cell dispersion of mature cucumber fruits in the presence of 1.25 mg per ml of NBC pectinase is shown in figure 43. The

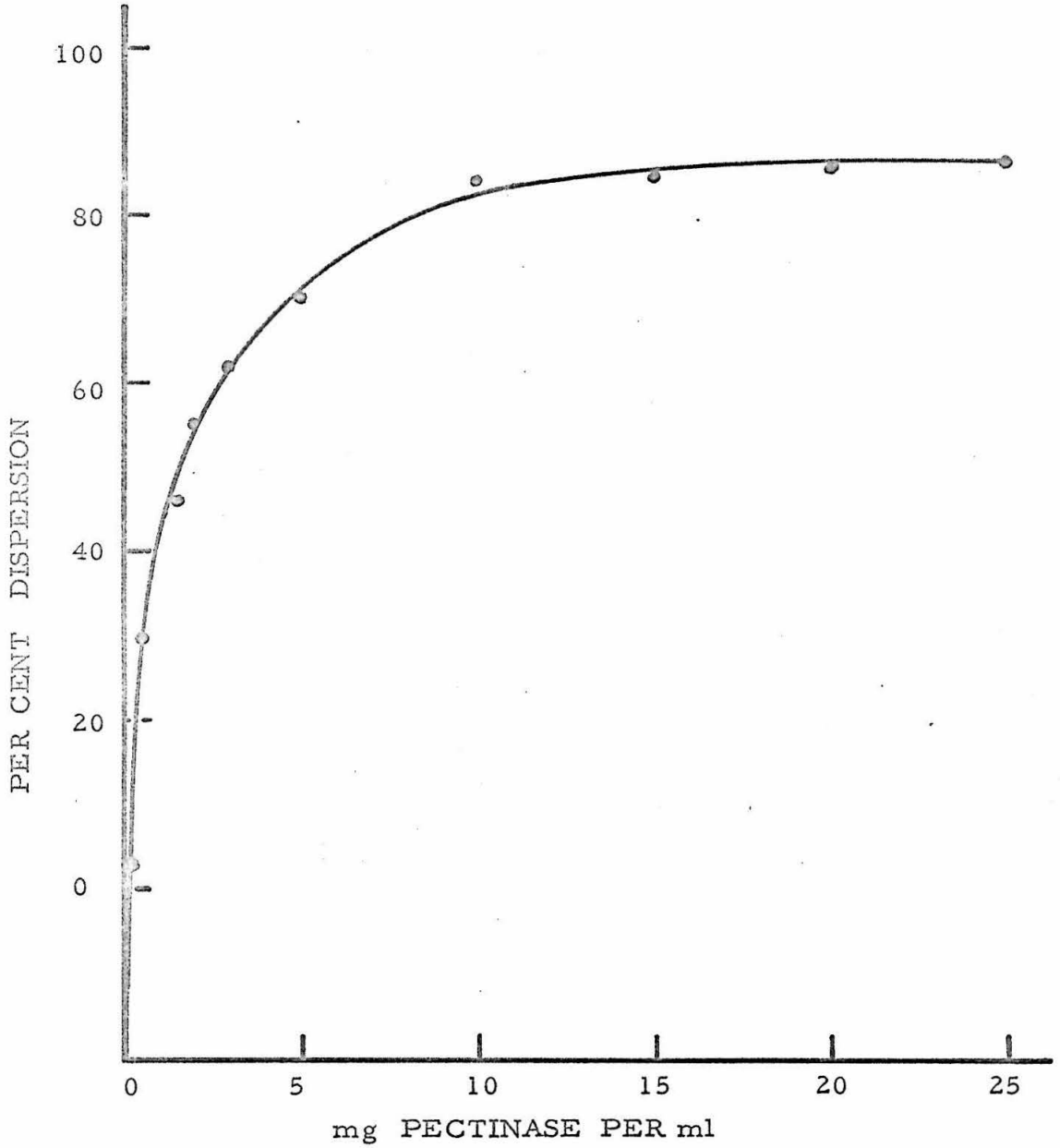

Figure 42. Cell dispersion as a function of pectinase concentration. Tissue blocks shaken for four hours at 24° C, pH 7.

TABLE 57.

Enhancement of cell dispersion by the addition of pectinase and pectin esterase. Basal media contained 0.005 M versene in 0.01 M K phosphate buffer pH 7.0. After shaking for 4 hours.

Enzyme Addition	% Dispersion	Relative Volume of Isolated Cell Walls
None	9	---
1.25 mg/ml NBC Pectinase	43	1.8
1.25 mg/ml Sigma Pectinase	36	1.1
1.25 mg/ml Sigma Pectinase + 0.1 mg/ml Sigma Tomato Pectin Esterase	64	1.0

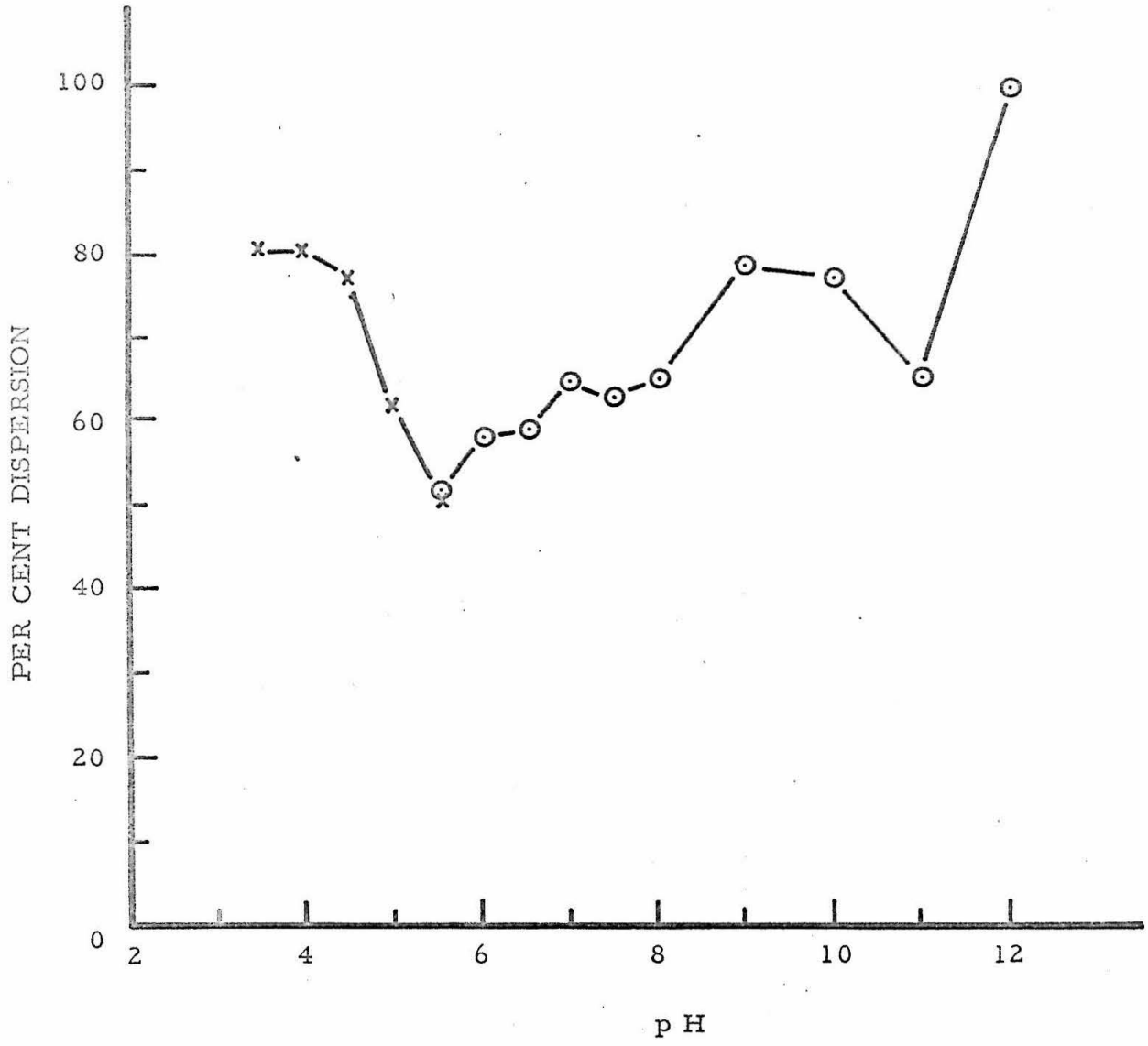

Figure 43. Cell dispersion as a function of pH in the presence of 1.25 mg per ml of pectinase. Tissue blocks shaken for four hours at 24° C.

pH optimum for cell dispersion does not correspond to the pH optimum for pectinase activity but rather appears at both pH extremes. The data more closely resemble those obtained by Ginzburg (285) with pretreatment in buffers of varying pH and subsequent separation of cells in 0.1 M EDTA, pH 8.0. The results of Ginzburg (285), obtained with pea root tips, were interpreted as evidence for an amphoteric component of the intercellular cement, possibly protein. The evidence presented by Ginzburg for protein as an integral component of the intracellular cement has been seriously challenged by Letham (278) since similar results were not obtained in the case of apple fruit tissue.

The increased dispersion observed below pH 4 and particularly that above pH 12 might well be the result of enhanced extraction of hemicelluloses and related materials from the cell wall. At alkaline pH the tissues become very soft and relatively easy to disperse.

Enhanced separation of cells with ethelenediaminetetraacetic acid (EDTA).--Since the cementing substances between individual cells is thought to be composed of insoluble salts (predominantly calcium) of pectic acids (183), a reagent chelating calcium was used to augment dispersion. In these experiments EDTA was combined with pectinase at pH 7. EDTA has been employed as an aid in the maceration of plant cells (278, 279, 284, 285) and has been used extensively in work with animal cells in culture (for example, the reports of Zwilling (286) and Anderson (287)).

At pH 7.0 and in the presence of 1.25 mg per l of pectinase, the maximum effect of EDTA was exerted at the surprisingly low concentration of 0.004 M (figure 44). Effectiveness during the four hour shaking

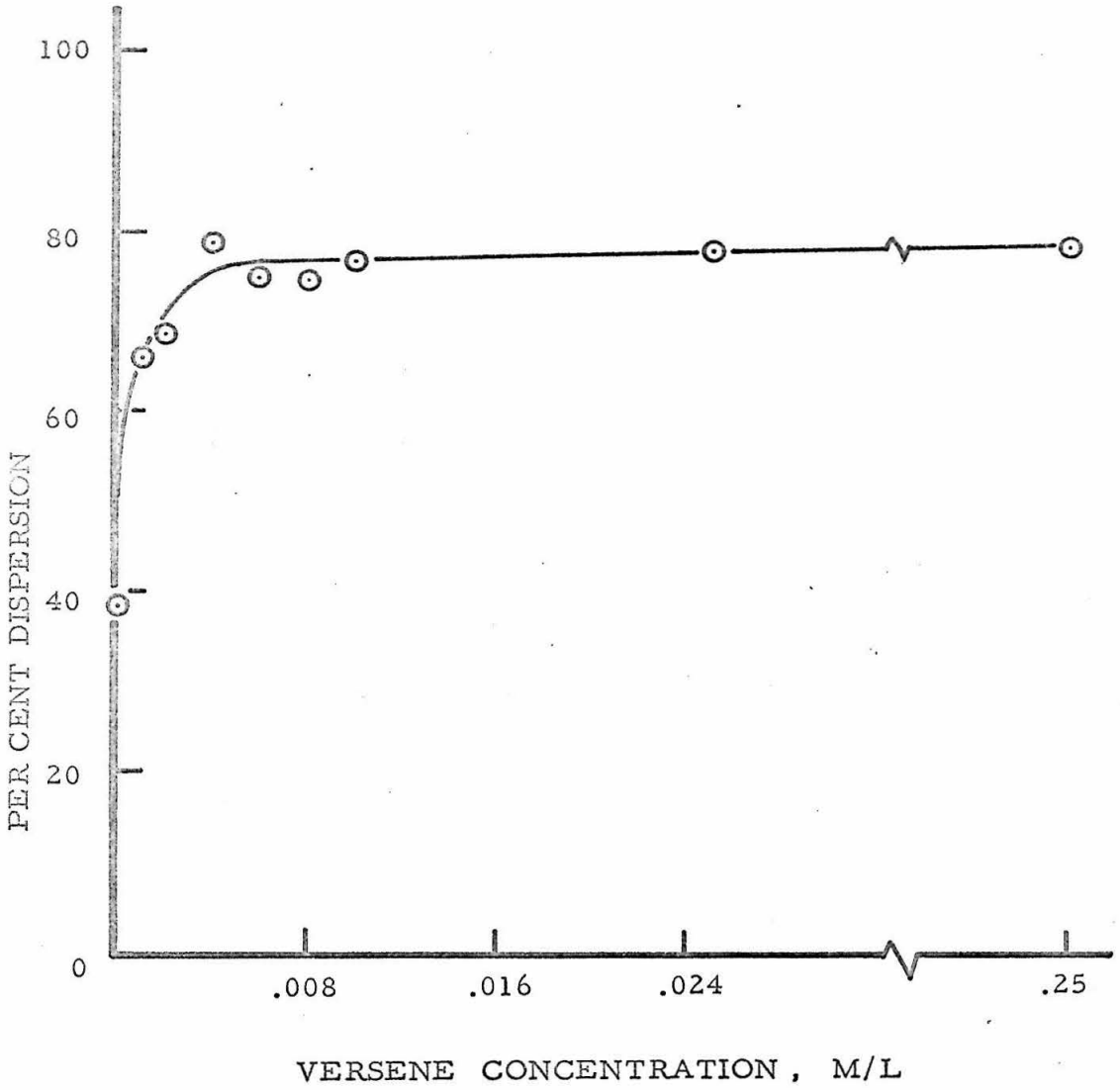

Figure 44. The effect of versene (EDTA) concentration on cell dispersion in the presence of 1.25 mg per ml of pectinase. Tissue blocks shaken for four hours at 24° C, pH 7.

period was increased only slightly by increasing the concentration to as much as 0.25 M. Ginzburg (284) obtained maximum separation of pea root tips at a concentration of 0.1 M EDTA, pH 8.0 and Letham (278), working with apple fruit tissue reported optimum concentrations between 0.03 and 0.07 M at pH 10.6.

In preliminary experiments with Letham's procedure (278), dispersion of cucumber tissue was increased only slightly (less than 10%) between pH 6 and pH 10 using 0.05 M EDTA at 25° C, with optimum dispersion at pH 7. In the absence of EDTA (buffer only) maximal dispersion over the pH range 6 to 10 was observed at pH 10. Dispersions of cucumber tissues under these same conditions of pH and EDTA concentration proceeded very slowly at 6° C and the number of cells produced was negligible even after one month of continuous shaking. A clear temperature dependency of cell separation under the influence of EDTA had been previously established by Letham (278) and Ginzburg (284).

Microscopic survey of tissue dispersion.--In the case of cucumber pericarp; over 80% of the dispersed cells were isolated as individuals, reasonably free of debris. Fragmented vascular elements were found occasionally and groups of two to five cells appeared as well. Microscopic inspection showed that a high proportion of the cells prepared from immature cucumber fruits (10-15 g) retained their protoplast intact (table 58). Cells of mature cucumber fruits (200 g or more), however, contain large vacuoles and the dispersions consisted mainly of cell "ghosts" devoid of visible protoplast or cells with only traces of adhering protoplasm. Intact nuclei could be observed in less than 20% of these cells.

TABLE 58.

A comparison of various tissues as a source of material for single cell dispersion cultures. Basal medium contained 1.25 mg/ml NBC pectinase, 0.005 M versene and 0.01 M potassium phosphate adjusted to pH 7.0. After shaking for 4 hours.

Tissue	% Dispersion	Observations
Mature Cucumber Fruits (Pericarp)	~50	Largely Cell Wall Ghosts
Immature Cucumber Fruits (Pericarp)	~50	40-50% Intact Cells
Avocado Fruit	5	Fragmentation
Carrot Storage Root	45	High Proportion of Intact Cells
Sugar Beet Storage Root (Lignified)	< 1	Fragmentation
Avena Coleoptile Sections	5	Fragmentation
Onion Leaf Scales	16	Cell Wall Ghosts
Mung Bean Hypocotyl Sections	4	Fragmentation

The preparations from mature fruits were, however, ideally suited to the production of protoplast-free cell walls. By conducting the dispersion and subsequent cell isolation in the presence of 0.35 M sucrose, an adaptation of the procedure of Zaitlin (269), the proportion of intact nuclei could occasionally be increased to exceed 25%.

In addition to cucumber fruits, cell dispersions prepared from carrot storage root also were found to contain a significant number of intact cells. Vascular tissue, epidermal tissue and lignified tissues (sugar beet storage root) were resistant to dispersion. In the case of onion leaf scales stripping off the epidermis greatly facilitated dispersion but only cell ghosts were observed. Avocado fruit, Avena coleoptiles and mung bean hypocotyls (Phaseolus aureus) yielded only cell fragments under the conditions tested.

Incorporation of radioactivity from glucose-U-C^{14} into insoluble cell wall of isolated cells and cell walls in the presence and absence of added cofactors.--A dispersion prepared from mature cucumber fruits, consisting predominantly of cell ghosts (ca. 20% of the cells with apparently intact nuclei), was incubated with 3 µc U-C^{14}-glucose (100 µg per µM), in 25 ml of 0.01 M potassium phosphate buffer, pH 7, equally distributed among five 15 ml conical centrifuge tubes. At intervals of 0.5, 1, 2 and 4 hours, the cells were collected from the appropriate tube by centrifugation and resuspended in 5 ml of buffer. The washing procedure was repeated four times. The final volume of packed cell wall was 2.6 ± 0.15 ml per sample. The cells were washed onto circles of filter paper, dried under a heat lamp and the radioactivity was determined to 3%

accuracy using a Nuclear Chicago D-47 gas-flow planchet radiation counter fitted with a "micromil" window. The results derived from this experiment are presented in table 59. Since it is possible that some contamination from microorganisms occurred during addition of the radioactive substrate, the slight increase in radioactivity between two and four hours cannot be considered as indicating a synthetic capacity of the dispersion.

The results of a parallel experiment conducted with immature cucumber fruits are shown in figure 45. The fruits were grown in the Erhart Plant Research Laboratory and supplied through the courtesy of Dr. Ezra Galun. Dispersion was initiated within one hour after harvest. Conditions of the incorporation were identical to that of the previous experiment with the dispersion from mature tissue with the exception that at the end of the incubation, the recovered cells received two additional centrifugation-resuspension cycles in order to obtain protoplast-free walls. Incorporation of C^{14} glucose into buffer-insoluble cell walls was found to be nearly linear for a period of at least four hours and the total radioactivity incorporated at the end of four hours (200 cpm per 10 mg dry cell wall) accounted for 0.07% of the total radioactivity, an amount comparable to incorporation levels obtained with Avena coleoptile sections (49). The dry weight of the material was estimated from the relation that 1 ml of packed cell walls is equal to 7.5 $\pm$ 0.1 mg dry cell, derived from independent determinations.

Preliminary experiments of Albersheim (288) indicated the possibility that a cell free synthesis of certain cell wall components, tentatively identified as pectin, might be possible in the presence of added

TABLE 59.

Incorporation of radioactivity from 3 μc glucose-U-C^{14} into water insoluble cell wall materials of isolated cell walls of mature cucumber fruits.

Incubation Time hrs	cpm per 10 mg Dry Cell Wall
0	46
.5	46
1	47
2	48
4	58

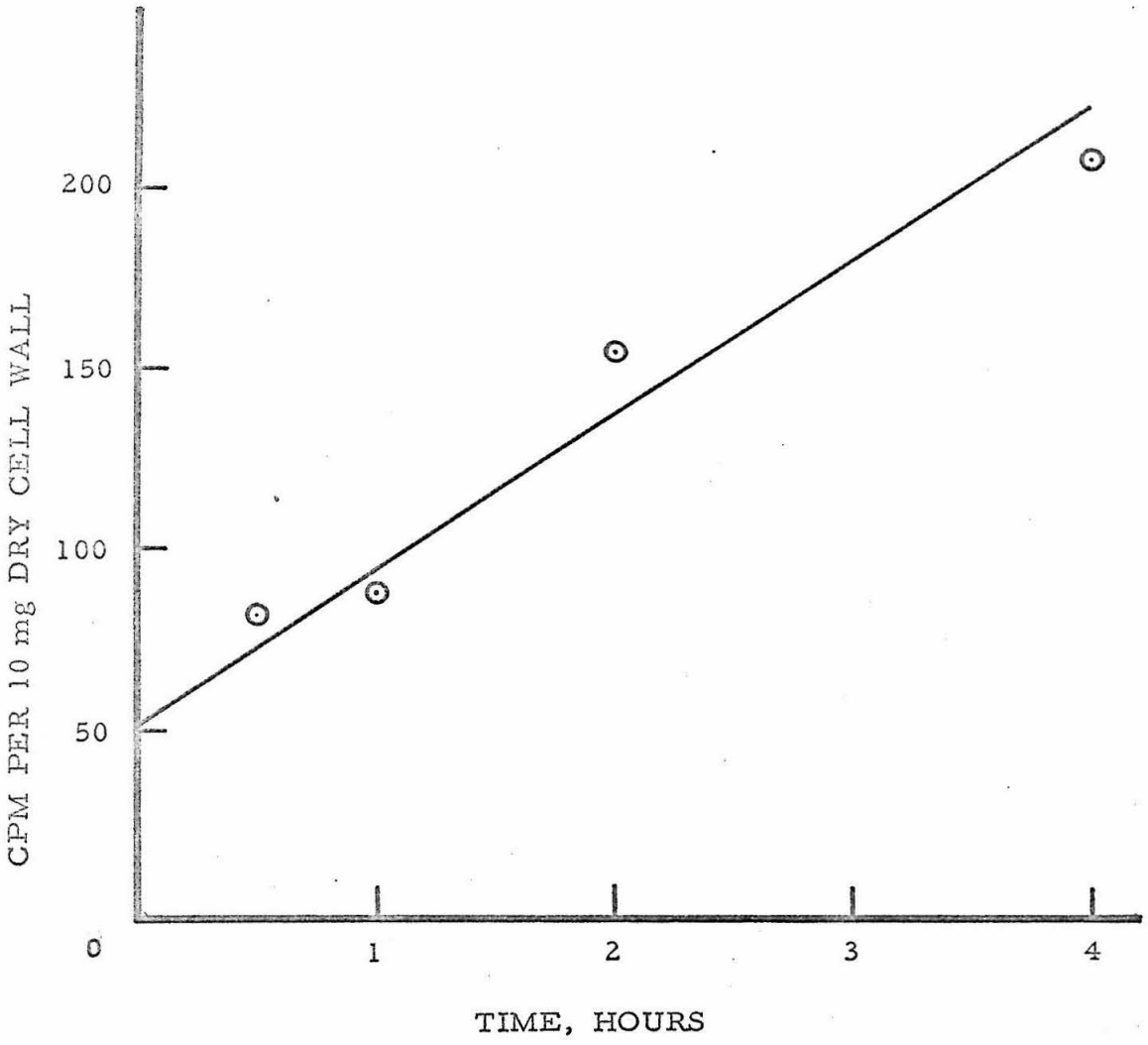

Figure 45. Incorporation of radioactivity from glucose-U-C^{14} into insoluble cell wall of cell dispersions prepared from immature cucumber fruits.

cofactors. The possibility that such incorporation might also be enhanced by the presence of pre-formed cell walls to serve as a primer or template for further synthesis was investigated. Mung beans are known to be an excellent source of many enzymes associated with carbohydrate transformations (289). Therefore, an enzyme preparation was obtained from 100 g of 40 hour old mung bean axes with seed coats removed prior to grinding. The tissue was ground in two 40-ml portions of 0.01 M phosphate buffer, pH 7.3 and centrifuged 10 minutes at 10,000 X g. The lipide layer which formed during centrifugation was removed and the combined supernatents spun for an additional 30 minutes at 12,500 X g. The final supernatent was diluted with buffer to give a ratio of 1 ml enzyme solution per g of fresh tissue. At the end of four hours, the incubation was terminated by the addition of ethanol to give a 70% solution. The 70% ethanol pellet collected by centrifugation was exhaustively extracted with 0.01 M potassium phosphate buffer, pH 7.3 by means of 12 centrifugation-resuspension cycles. The radioactivity measured in the final wash was not significantly above background levels. All radioactivity measurements in this experiment were obtained using a Packard Tri-Carb Scintillation counter. Aliquots of the samples to be counted were mixed with 10 ml of the liquid scintillator mixture described on page 67. The results are summarized in table 60. In the absence of the soluble mung bean enzyme preparation, the radioactivity of the isolated cell walls was comparable to that obtained in the absence of cofactors (experiment summarized in table 59). The addition of the cell wall preparation served to significantly decrease the final radioactivity of insoluble residue in the presence of added cofactor and enzyme. In all

TABLE 60.

Incorporation of radioactivity from glucose-U-C^{14} into 70% ethanol-insoluble materials using a combination of cell walls isolated from mature cucumber fruits and a soluble enzyme preparation from mung beans in the presence and absence of added cofactors.

Reaction Mixture	Cpm Total
Complete[1]	17,633
-Enzyme	411
-Cofactors	3,710
-IAA	20,300
-Walls	38,950

[1]Complete = 10^{-4} M of ATP, UTP, TPN, DPN, Mg and Methionine, 5 X 10^{-7} M indoleacetic acid, 1 ml soluble enzyme solution prepared from mung bean axes, cucumber cell walls equivalent to 7.5 mg dry cell wall and 20 µc glucose-U-C^{14} (100 µc per µM) in 5 ml 0.01 M potassium phosphate buffer pH 7.3. Incubated 4 hours at 25° C.

cases, the incorporation was cofactor dependent, however, elimination of the indoleacetic acid may have resulted in a slight increase in level of incorporation. As a means of further examining the products formed, the washed residue was treated with 1 N sodium hydroxide at room temperature for several hours followed by neutralization with acetic acid to pH 5. The resulting solution was then treated with purified citrus polygalacturonase for three days under toluene according to the procedure of Jansen, Jang, Albersheim and Bonner (46).

Purification of the pectic digests by paper chromatography (DIPWA, Appendix E) and by paper electrophoresis (System E-II, Appendix E) did not reveal any radioactive material characterized by having both the chromatographic R_f and the electrophoretic $R_{picrate}$ of galacturonic acid. The chromatographic and electrophoretic properties of the major radioactive component resembled closely those of a material isolated under similar conditions by Pollard and Olsen (290). The chemical identity of this material has not been completely illucidated but was shown to contain a mixture of sugar phosphates.

The cell walls incubated in the absence of enzyme were extracted separately and two fractions corresponding to hot water-soluble pectin and versene-soluble pectin (46) were obtained. Paper chromatography of these fractions following de-esterification and pectinasing showed no detectable radioactivity corresponding to galacturonic acid; the recoverable radioactivity appeared at an R_f corresponding to hexose. A guide strip from each of the chromatograms was treated with silver nitrate-sodium hydroxide (157, 199). Under these conditions reducing sugars are detectable in very low concentration (1 µg per strip) as dark

brown to yellow spots. No spot corresponding to uronic acid was observed in the hot water-soluble pectic fraction derived from the dispersed cell walls. However, the versene-soluble pectic fraction appeared to contain a substantial quantity of non-radioactive reducing sugar corresponding chromatographically to uronic acid.

Summary.--Cells of single cell dispersions prepared from immature cucumber fruits retained their protoplasts intact and demonstrated a capacity for steady-state incorporation of C^{14} glucose into insoluble cell wall material for a minimum period of four hours after isolation. The cells were obtained by treating blocks of intact tissue with a mixture of buffered EDTA and the enzyme pectinase. In contrast to several tissues studied, the cell walls of cucumber fruit tissue remain intact under these conditions, whereas, the cementing material between the walls is solubilized.

Isolated cell walls did not incorporate significant amounts of radioactivity into insoluble cell wall material either in the presence or absence of added cofactors or in combination with a soluble enzyme preparation obtained from mung beans.

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APPENDIX E

PAPER CHROMATOGRAPHIC AND PAPER ELECTROPHORETIC SYSTEMS

Paper.--Whatman No. 1 and 3 mm papers were used for paper chromatography. The paper was washed for a period of 2 to 3 days with a mixture of ethyl acetate-acetic acid-water (10:5:2; v/v), descending flow, and were dried for a minimum period of 24 hours at 60 to 80° C prior to use.

Apparatus.--Ascending chromatograms were developed in pyrex glass jars (diameter 5 1/2 inches, height 18 inches). The paper strips were attached to a glass-T, the height of which could be adjusted to permit equilibration of strips. Paper sheets were rolled into cylinders and fastened with polyethylene clips. Paper cylinders were then developed without prior equilibration or supported by means of a metal hanger and a magnet during the equilibration period.

Descending chromatograms were irrigated in glass jars (diameter 12 inches, height 24 inches) fitted with standard glass troughs to contain the solvent. Chromatograms were equilibrated with the vapor phase of the solvent prior to addition of the solvent to the troughs.

Solvents.--Solvents are listed alphabetically according to the first letter of the abbreviated designation. All solvents were used as ascending systems with the exception of DIPWA.

1. BAW.--n-Butanol-acetic acid-water (4:1:1.8; v/v) (292). A monophasic solvent useful for many routine separations including carbohydrates and organic acids. Equilibration time: 4 to 5 hours. Development time: 8 to 10 hours.

2. DIPWA.--(Descending) iso-propanol-pyridine-acetic acid-water (8:8:4:1; v/v) (291). A useful solvent for preliminary separations and preparative work. Will separate glucuronic acid and galacturonic acid but hexose mixtures normally are not resolved. Equilibration time: 4 to 5 hours. Development time: 6 to 10 hours, usually 8 hours.

3. EtAc-Pyr.--Ethyl acetate-pyridine-water (12:5:4; v/v). Solvent corresponds to the upper phase of the 2-1-2 two-phase mixture of Jermyn and Isherwood (157, 293). Provides excellent separation of carbohydrate mixtures. No equilibration necessary. Development time: 24 hours.

4. IAW.--iso-Propanol-acetic acid-water (3:1:1; v/v) (171).

Electrophoretic separations.--

1. System E-I.--The apparatus and general procedure are similar to those described by Ingram (201). The electrolyte used consisted of pyridine-acetic acid-water (50:2:450; v/v), pH 6.4 (201).

2. System E-II.--Separations were carried out with a Spinco-Durrum-type apparatus (294) at room temperature using as an electrolyte 0.1 M ammonium acetate, pH 5.8.

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