METABOLISM OF THE PECTIC SUBSTANCES

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Abstract

This thesis examines pectic metabolism by oat coleoptile sections. Pectin accounts for 5 per cent of the dry weight of such sections. Approximately 5 per cent of the pectin is cold water soluble--70 per cent alcohol insoluble. Approximately 50 per cent of the carboxyl groups of this fraction are methyl esterified. Hot water solubilizes from the cell wall a highly esterified pectin fraction which represents 15 per cent of the total. In the remaining hot water insoluble pectin, only 30 per cent of the pectic carboxyl groups are combined as esters.

Pectins, once formed, are metabolized very slowly. There is little or no mixing of the various pectic fractions during a 15-hour period.

The methyl ester group of pectin is supplied by the methyl group of methionine. Oat coleoptile sections, either intact or as homogenates, form S-methylmethionine and methionine sulfoxide from methionine. Both of these compounds are also active as methyl donors for the formation of pectic esters.

Indoleacetic acid accelerates both incorporation of the methyl of methionine into methyl ester moieties and the incorporation of glucose into galacturonic acid residues of water soluble pectins. This increase is a measure of an accelerated rate of pectin synthesis. Indoleacetic acid does not increase the rate of synthesis of the water insoluble pectins.

Evidence is presented which favors the supposition that the methyl ester groups are formed before polymerization of the galacturonic acid residues.

In vitro incorporation of the methyl of methionine into methyl ester groups of pectin has not been achieved. Limited success has been obtained with the in vitro incorporation of glucose into galacturonic acid residues of water soluble pectins.

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INTRODUCTION

General

Perhaps one half of the organic material formed each year upon the surface of the earth consists of the cell walls of plants. Yet less is known of the formation, composition, and metabolism of the plant cell wall than of any other system unique to plants. At present we can account for little more than half of the wall in terms of known constituents. Even for these we know principally the component breakdown products and very little concerning the chemical and physical structure of these same substances as they are arranged in the intact wall. The notable exception is the predominant constituent of plant cell walls--cellulose. The structure of cellulose is known in detail. The early studies on this fibrous polymer indeed form the basis of our modern chemistry of macromolecules.

This thesis concerns a second wall component, the poly-galacturonic acid known as "pectin." Pectin is unique among recognized wall components and among glycosidic polymers in general in that it contains a large number of ionizable groups. Because it contains these reactive sites, pectin has been thought of as a controlling factor in the expansion of matur-ing cell walls. It would seem that a prerequisite for a profitable study of the expansion of plant cell walls would be an increased knowledge of this active constituent.

Characterization of pectin.--In 1917 Saurez (1) recognized that pectin contains an isomer of glucuronic acid. During the same year Ehrlich (2) was able to show that the basic building block of pectin is, indeed, galacturonic acid. Ehrlich stated that pectin is a polygalacturonic acid but he was unable to determine its mode of polymerization. Even before Ehrlich's contribution, Fellenberg (3) found that pectin can be split enzymatically into pectinic acid and methanol. He recognized that the methanol is derived from the methyl ester group of the galacturonic acid residues. Fellenberg went even farther and stated that, in fruits, the pectic substances are the only compounds from which methanol can be derived.

A practically pure (95-99%) source of pectin was soon made available commercially by the California Fruit Growers Exchange (4). Morell, Baur and Link (5), with the aid of this commercial preparation, were the first to show that pectic acid is a linear α-glycosidic polymer. In 1937, Levene and Kreider used periodic acid oxidation of polygalacturonic acid to establish that galacturonic acid residues are ring compounds with either 1,4 or 1,5 glycosidic linkages (6). Confirmation that in pectin, pyranose D-galacturonic acid residues are linked through positions 1 and 4 came shortly thereafter (7,8,9).

The length of the pectin chain seems to depend on the isolation procedure and the method of assay. Morell, Baur, and

Link found the chains to be 8 to 10 residues long (5). Jansen et al. (10) have found by end group analysis that the polymers average 21 units. The osmotic pressure measurements of these investigators suggest a 30 unit chain while diffusion determination indicated a chain of 32 galacturonic acid residues.

There are still questions concerning the composition of pectin. Extracted pectins frequently contain, in addition to galacturonic acid, arabinose, galactose, and sorbose. These sugars are considered by many workers to be integral parts of the pectin molecule (11,12,13). The presence of acetyl groups in pectin has also been reported (14,15). Other workers consider true pectic acid to contain only galacturonic acid and none of the above constituents (16). This thesis will only be concerned with the galacturonic acid residues and their methyl ester groups.

Pectic enzymes.--The early workers in this field recognized the existence of three enzymes capable of degrading pectin (17). Two of these enzymes attack glycosidic bonds of the chain itself while the third hydrolyzes the methyl ester groups. The history of the glycoside splitting enzymes has been confused by non-homogeneous substrates, poor methods of product analysis and failure to separate the enzymes physically. Some workers contend that there is but one polygalacturonase (18, 19,20). Phaff and Demain, for example, found a single poly-

galacturonase in yeast. They report that this enzyme has the ability to attack both the middle and the ends of the chain (21). Other workers, however, claim to have detected two (22,23), three (24,25), and even four (26) different polygalacturonases.

Purr et al. (27) have finally proved the existence of at least two polygalacturonases. The two enzymes, β-polygalacturonase (β-PG) and γ-polygalacturonase (γ-PG), are separated by continual flow electrophoresis. Purr's group has shown that the two enzymes are different from one another in several ways. γ-PG contains no detectable basic amino acids while β-PG contains histidine, lysine, and arginine. β-PG cannot degrade a fully esterified pectin chain but is active on sodium pectate with a pH optimum of 4.1. It is completely inactive at any pH over 7. γ-PG can attack fully esterified pectin and has a pH optimum of 8.3-8.4. γ-PG is also more heat stable than β-PG. Both enzymes quickly reduce the viscosity of a pectin solution. β-PG liberates monogalacturonic acid immediately but γ-PG yields detectable monogalacturonate only after 92 hours of hydrolysis.

The work of Purr et al. correlates well with previous findings. Presumably β-PG is an exopolygalacturonase which degrades pectic acid and γ-PG an endopolygalacturonase which is capable of attacking fully esterified pectins (25,28).

The existence of an enzyme which in the presence of Ca ions converts pectin to a gel was described by Frémy (29) in 1840. Only after Fellenberg (30) recognized the presence of

methyl ester groups in pectin could the chemical action of this enzyme be understood. Although Frémy named the enzyme "pectase," it now seems more suitable to refer to this enzyme, which hydrolyzes the methyl ester groups of pectin, as pectin methylesterase or just pectinesterase (PE) (31).

PE, in most higher plants, is largely adsorbed on the cellular solids. The enzyme is solubilized in 0.25 M salt or buffer at pH 7-8 (31,14). Recognition of this fact greatly enhanced the purification of PE. Citrus, tomato, and fungal PE have been obtained in a highly purified state. Purified citrus PE has been shown to be free of the other pectic enzymes. The pH optimum of higher plant PE is about 7 to 8 while fungal PE has a pH optimum in the region 4.6 to 5.5 (31).

McCready and Seegmiller (19) have found that purified citrus PE readily attacks polygalacturonic acid polymethyl esters with chain lengths of 10 and above. This enzyme however does not hydrolyze the ester groups of the diester of digalacturonic acid, the diester of methyl digalacturonic acid, the triester of methyl trigalacturonic acid or the half ester of digalacturonic acid.

PE is quite specific. It will not hydrolyze the glycol or glycerin esters of pectic acid and attacks the methyl ester of pectin at least 1000 times as fast as it does any of some 50 non-galacturonide esters tested. PE will however hydrolyze the ethyl ester of polygalacturonic acid at a rate of 8 per cent of that observed for the methyl ester (31).

Biogenesis of the galacturonic acid moiety.--It has been clearly shown by Seegmiller and his co-workers (13,32) that in in vivo experiments glucose and galactose are equally good precursors of the galacturonic acid moiety of pectin. This work also provides evidence that the hexoses are incorporated into uronic acid without cleavage of the hexose carbon skeleton.

Leloir's group had previously demonstrated the interconversion of galactose and glucose. In 1948, they discovered the enzyme galactokinase in yeast. This enzyme catalyzes a transphosphorylation between adenosine triphosphate (ATP) and galactose to form galactose-1-phosphate (Gal-1-P) (33). A thermostable factor was found to be necessary for the formation of glucose-1-phosphate (Gl-1-P) from Gal-1-P (34). This cofactor, which is omnipresent in the interconversion of sugars, is uridine diphosphate α-glucose (UDPG) (35,36). It has now been established that mung bean seedlings and a number of other plants contain pyrophosphorylases capable of catalyzing the reversible formation of sugar nucleotides from uridine triphosphate (UTP) and a number of sugar-1-phosphates (S-1-P). According to Neufeld et al., UDPG and UDPGalactose,

$$UTP + S\text{-}1\text{-}P \overset{*}{\rightleftharpoons} PP + UDP^{*}S$$

are formed in this reaction from α-D-Gl-1-P and α-D-Gal-1-P respectively. A bivalent metal ion (Mg^{++}, Mn^{++}, or Co^{++}) is required. The enzyme UDP Glucose pyrophosphorylase has been shown to be distinct from UDP Galactose phosphorylase (38).

Mung bean seedling extracts also contain a galactowaldenase which catalyzes the interconversion of UDPG and UDPGal.

Soon after the finding of UDPG by Caputto et al., Dutton and Storey isolated a rat liver factor necessary for the production of glucuronic acid (39). Characterization of this compound showed it to be uridine diphosphate glucuronic acid (UDPGluA). The enzyme, uridine diphosphoglucose dehydrogenase, which catalyzes the formation of UDPGluA from UDPG is diphosphopyridine nucleotide (DPN) dependent and has been purified by Strominger's group from rat liver, peas and yeast (40,41). Smith et al. have found a similar enzyme in a strain of Pneumococcus (42).

In earlier work Smith and her coworkers (43) were able to isolate UDP Galacturonic acid (UDPGalA) from Pneumococcus. It is now evident that not only Pneumococcus (44) but also mung beans, asparagus, radish, and spinach (45) possess uronic acid epimerase which converts UDP glucuronic acid to UDP galacturonic acid.

Recent evidence obtained by Hassid's group shows that mung bean seedlings contain a pyrophosphorylase which catalyzes the reversible formation of UDP uronic acids from UTP and uronic acid-1-phosphate (46).

$$\text{UDP-GluA} + \text{PP} \rightleftharpoons \text{Glucuronic acid-1-P} + \text{UTP}$$

$$\text{UDP-GalA} + \text{PP} \rightleftharpoons \text{Galacturonic acid-1-P} + \text{UTP}$$

It is not known whether the above reactions are catalyzed by different or by the same enzymes. However, Ginsburg (38) has shown that the plant UDP-uronic acid pyrophosphorylases are distinct from UDP-glucose pyrophosphorylase.

Loewus *et al.* (47) have shown that D-glucuronic acid is incorporated *in vivo* into the D-galacturonic acid moiety of pectin. One may speculate that plants contain a kinase which phosphorylates glucuronic acid and that the above reaction can then convert the glucuronic-1-P to UDPGluA. The uronic acid epimerase could then transform the UDPGluA to UDPGalA.

Evidence for the condensation of UDPGalA to pectin has not been obtained. Analogous reactions, however, have been reported. Cellulose is synthesized by an *Acetobacter xylinum* cell free particulate system from UDPG but not from glucose-1-P nor from free glucose (48). Rat liver (49) and rat muscle (50) enzymes are capable of forming glycogen from UDPG. However, in these systems a primer is needed and the preparation must be free of amylase. The only polysaccharide of higher plants which has been enzymatically synthesized from UDPG is callose, a β-1,3-linked glucan (51). It would seem probable however that pectin is formed by transfer of galacturonic acid residues from UDPGalA to a growing pectic chain. It appears probable too that the UDPGalA, to be active in this reaction, must first be methyl esterified.

Biogenesis of the methyl ester group of pectin.--Sato (52) and Ordin et al. (53) have shown that the methyl group of pectin can arise from that of methionine via transmethylation. In a later paper, Sato et al. (54) showed that the methyl group of methionine is transferred intact, i.e. without loss of hydrogens, to form the methyl ester of pectin. Other precursors of the methyl ester group include formic acid and formaldehyde (55). The carbon of glycine and β-carbon of serine are incorporated to a lesser extent.

Part I of this thesis will describe studies on the pathway of transmethylation from methionine to pectin. Part II will discuss the metabolism of pectin and will show that methylation of galacturonic acid occurs before and not after polymerization.

Influence of indoleacetic acid on pectin metabolism.--This thesis will also bear on the effect of the auxin indoleacetic acid (IAA) on pectin metabolism. It is established that IAA increases rate of cell elongation in the Avena coleoptile by causing the cell walls to become more easily stretched, more plastic. This was first demonstrated by Heyn (56) who showed that coleoptiles previously treated with auxin are more readily stretched by an applied weight than coleoptiles not so treated. Heyn's observation has been confirmed and extended by Tagawa and Bonner (57).

A second type of experiment which also leads to the conclusion that the effect of auxin is upon the properties of the cell

wall is the osmotic analysis of coleoptile growth (58,59). Application of auxin to coleoptile sections decreases wall pressure but does not directly affect osmotic concentration in the tissue. The influence of auxin in decreasing cell wall resistance to expansion may in fact be separated in time from the act of expansion itself (60). In such a two-stage experiment, auxin is allowed to do its work in sections which are prevented from expanding by being immersed in a solution isosmotic with the cell contents. They are then transferred to water under anaerobic conditions, in which auxin is ineffective. Sections pretreated in auxin expand more in the second anaerobic stage in water than sections not pretreated with auxin. The effect of auxin in increasing deformability of the coleoptile under external mechanical load also takes place with sections incubated in isosmotic conditions (61). Thus the influence of auxin on cell wall plasticity takes place whether or not the cell actually takes up water and grows.

Since auxin affects cell wall properties, the next question is how does it do so? The effect requires the participation of metabolism since as indicated above, auxin does not do its work under anaerobic conditions (62) nor in the presence of inhibitors such as HCN. Some aspect of the auxiniferous act takes place in the cytoplasm since plasmolized cells, in which wall and cytoplasm are not in contact do not exhibit wall softening under the influence of auxin (61). Some

controversy exists over whether net synthesis of cell wall components is increased in the presence of auxin. It is clear that in growing seedlings of oat (63) and corn (64) coleoptile, cell wall synthesis normally keeps pace with cell expansion. The same is true of excised oat coleoptile sections growing at 26°C (63), of pea epicotyl sections (65), excised tobacco pith in tissue culture (66), and of potato tuber discs (67,68). All of these tissues require added auxin to grow and in all of them, auxin-induced growth is accompanied by a parallel net increase in cell wall substance. It does not appear however that there is any direct and obligatory coupling between the two processes. Thus net cell wall synthesis of oat coleoptile sections is suppressed at a temperature of 4°C, although auxin-induced growth proceeds at this temperature (63). In wheat roots also, auxin-induced changes in growth rate are not paralleled by changes in rate of cell wall synthesis (69). That auxin does not directly increase net cell wall synthesis is however most clearly evident from experiments in which incorporation of C^{14}-labeled sugars into cell wall materials has been followed over brief (3 hour) time periods (70,71,53,72). In general, such incorporation is unaffected or even slightly decreased by the presence of auxin.

It has been observed that the rate of incorporation of the methyl carbon of methionine into hot water soluble pectin of the cell wall is increased in the presence of auxin (53,72). This effect of auxin is one which appears rapidly, is inhibited

by antiauxins, high auxin concentrations, and ethionine, in all of which respects it is similar to the influence of auxin on cell wall plasticity. This thesis includes a study in quantitative detail of the auxin-induced changes in the pectin of the cell walls of oat coleoptiles. It will be shown that auxin increases the rate of pectin synthesis without changing the degree to which the pectin is esterified.

PART I

Metabolism of Methionine and Pectin Esterification in a Plant Tissue*

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It is known that the methyl group of methionine serves as the source of the methyl ester groups of pectin and protopectin (1, 2). The methionine methyl group also functions in the synthesis of the methoxyl groups of lignin (3) and of the methyl groups of various plant alkaloids (4–6). Although the transfer of methyl from methionine to nitrogen in the synthesis of plant alkaloids thus resembles in principle the methyl transfers that have been studied so extensively in animal tissues by du Vigneaud (7), Borsook and Dubnoff (8), and Vignos and Cantoni (9), transfers of methyl to methoxyl and methyl ester groups by the plant are not yet recognized in animal biochemistry. It is of interest, therefore, to study these types of methyl transfers, since it is possible that elucidation of the mechanism by which they take place may clarify the whole question of the mechanism of methyl transfer. As a first step in the study of these matters, an investigation has been made of the pathways of metabolism of methionine by a plant tissue.

METHODS AND MATERIALS

The experimental tissue consisted of 5 mm. sections cut 3 mm. below the tip of oat seedlings grown as previously described (10). 1 gm. of sections floating on 5 ml. of solution in a Petri dish, containing 25 μg. of indoleacetic acid, 90 μg. of catalase, and the desired quantity of metabolite under study, was used for experiments *in vivo*. After 4 hours of incubation at room temperature in the dark room, the sections were rinsed with water and ground in a Potter-Elvehjem homogenizer. The homogenate was made 70 per cent with ethanol and filtered. The residue, washed with 95 per cent ethanol, was used for the isolation of pectic substances, and the combined filtrate and ethanol wash was used for the isolation of ethanol-soluble metabolites.

For experiments *in vitro*, 1 gm. of sections was homogenized with 2 ml. of 0.1 M Tris[1] buffer at pH 6.95, and the homogenate was then incubated with 90 μg. of catalase and the desired amount of metabolite. After 1 hour at 30° in a constant temperature bath, the homogenate was made 70 per cent with ethanol and filtered, and the filtrate and residue were examined in the same way as in the experiments *in vivo*.

In experiments in which pectin and protopectin were studied, these cell wall constituents were isolated by a modification of a procedure described by Kertesz (11). The residues from either

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[1] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

of the types of experiments described above were distributed in 40 ml. centrifuge tubes so that the contents of each tube represented about 0.5 gm. of the original tissue; 20 ml. of water were added to each tube, and the mixture was extracted in a boiling water bath for 0.5 hour. The mixture was centrifuged, the supernatant fluid was poured off, and the residue was again extracted twice for 10 minutes with 10 ml. of water per tube. The water extracts were combined, concentrated under reduced pressure, and then transferred to a 12 ml. centrifuge tube. 1 mg. of nonradioactive carrier, similar to the radioactive substrate, and water were added to the clear solution to make 1 ml., which was then made 70 per cent with ethanol, and 1 drop (0.02 ml.) of 1 M HCl was added. This mixture was stirred and kept in an ice bath for about 1 hour. A precipitate formed which was centrifuged and washed three times with 95 per cent ethanol. This precipitate is that classically considered to be pectin. The residue after the isolation of pectin was next treated with 0.05 M HCl in order to extract the protopectin fraction which was isolated from the extract in the manner described for pectin. In studies in which the methyl ester groups of pectin and protopectin were hydrolyzed, solutions of the materials were made 6 M with NH_3 and allowed to stand at room temperature for 1 hour.

For experiments with radioactive metabolites, L-methionine-methyl-C^{14} was obtained from the Isotopes Specialties Company, Inc., and L-methionine-S^{35} was purchased from the Abbott Laboratories. Paper chromatography revealed that radioactive methionine always contains a small percentage (about 1.5 per cent) of methionine sulfoxide and a lesser quantity (about 0.1 per cent) of methionine sulfone. Labeled and nonlabeled methionine sulfoxide and methionine sulfone were prepared from methionine according to the procedure of Roper and McIlwain (12). Sulfur- and methyl-labeled methionine methyl sulfonium salts, referred to hereafter as S-methylmethionine or simply as methylmethionine, were synthesized by the method of Floyd and Lavine (13). Catalase was obtained from the Armour Laboratories, Lot R500650-AD4.

The isolated radioactive materials were counted with a micromil window gas flow Geiger-Müller tube (Nuclear-Chicago Corporation). Substances separated by paper chromatography were in some instances counted on the paper with a paper strip counter (Nuclear-Chicago Corporation). The radioactivities were then calculated from the areas under the curves as determined with a polar planimeter (Keuffel & Esser Company, No. 4236M). In all experiments, the amount of labeled substrate used was arranged so as to supply a total of 1×10^6 or 2×10^6 c.p.m.

RESULTS

Oxidation of Methionine—Tissue of oat seedlings possesses the ability both *in vivo* and *in vitro* to convert methionine to methionine sulfoxide and to methionine sulfone. In typical experiments, summarized in Table I, homogenates converted 5.5 to 7.8 per cent of 1.34 μmoles of methionine to the sulfoxide in 1 hour. The methionine sulfoxide was characterized in the following way. Homogenates containing methionine as substrate were incubated for 1 hour and then extracted with 70 per cent ethanol. The extracts were chromatographed in lutidine-water (65:35). The band corresponding to methionine sulfoxide, determined by reference to authentic standards and by the S reagent (nitroprusside in aqueous methanol) of Toennies and Kolb (14), was eluted, and 100 mg. of authentic nonradioactive methionine sulfoxide were added as carrier. This material was crystallized several times, the sulfoxide being dissolved each time in water and precipitated by the addition of absolute ethanol. After two crystallizations, the specific activity of the isolated methionine sulfoxide remained unchanged upon further crystallization. Chromatography and cochromatography of the isolated material with authentic methionine sulfoxide in three solvents (butanol-acetic acid-water, 60:15:25; lutidine-water, 65:35; and ethylacetate-acetic acid-water, 10:5:6) indicated the similarity of the two materials. The methionine sulfone was characterized by cochromatography with an authentic sample of the material.

The conversion of methionine to its sulfoxide by oat tissue *in vitro* may be mediated by H_2O_2, since the reaction is suppressed to some extent by the addition of catalase to the system. The conversion of methionine to its sulfoxide would appear to be irreversible, or essentially so, since, when radioactive methionine sulfoxide labeled either in the methyl carbon or in the sulfur atom was incubated in a homogenate, essentially no radioactive methionine could be detected in the incubation mixture.

In intact oat sections *in vivo*, methionine was also found to be converted to its sulfoxide, the conversion amounting to about 10 per cent of 1.34 μmoles of methionine in 4 hours. Roughly

TABLE I

*Conversion of methionine to its sulfoxide and sulfone and to S-methylmethionine by oat shoots**

Substance	Intact tissue experiments				Homogenate experiments	
	Catalase added		No catalase		Catalase added	No catalase
	External solution	Tissue	External solution	Tissue		
	%	%	%	%	%	%
Methionine	40	13	33	16	92	68
S-Methylmethionine	<0.2	12	0.1	9.1	0.24	2
Sulfoxide	4.4	5.2	5.5	5.4	5.5	7.8
Sulfone	<0.5	1.5	0.2	3.9	1.2	2.6

* For experiments with intact sections, 1 gm. of tissue was incubated for 4 hours at 25° in 5 ml. of solution containing metabolite. For homogenate experiments, 1 gm. of homogenized tissue was incubated in 3 ml. of reaction mixture for 1 hour at 30°.

Data expressed as per cent recovered of the methionine-C^{14} originally supplied (1.34 μmoles, 1×10^6 c.p.m.).

one-half of the sulfoxide is found in the tissue, the remainder being in the external medium. The identity of the methionine sulfoxide in this case was determined by chromatography and cochromatography, with an authentic sample of methionine sulfoxide in the three solvents mentioned above. As noted above and in Table I, methionine sulfoxide appears in the external medium in which oat sections are floated. Addition of catalase to the medium containing the sections decreased the formation of methionine sulfoxide in the external medium, but appears to have had no effect upon oxidation of methionine within the section.

Formation of S-Methylmethionine—A further major product of methionine metabolism in oat tissue is S-methylmethionine. Thus, when methionine-methyl-C^{14} was incubated with a homogenate of oat sections for 1 hour, or when intact sections were incubated with this material for 4 hours, radioactive S-methylmethionine could be isolated from the reaction mixture. This substance was characterized as follows: After the incubation period, the sections were homogenized in absolute ethanol to yield a homogenate 70 per cent in ethanol. This was centrifuged, and the residue was washed twice with 95 per cent ethanol. The combined 70 per cent ethanol extract and the 95 per cent ethanol washings were concentrated *in vacuo* to about 1 ml. and chromatographed in lutidine-water. The band corresponding to S-methylmethionine was eluted and again chromatographed in butanol-acetic acid-water. The band corresponding to S-methylmethionine was again eluted, and 100 mg. of synthetic nonradioactive authentic S-methylmethionine were added as carrier. 1 ml. of a saturated solution of phosphotungstic acid was introduced to precipitate the methylmethionine. The phosphotungstate was then dissolved in 95 per cent acetone in water, and methylmethionine bromide was recovered by the procedure of Floyd and Lavine (13). The bromide was crystallized several times by solution in water and precipitation with ethanol. The specific activity of the product remained constant after the first crystallization. Paper chromatography in three solvents (butanol-acetic acid-water, 60:15:25; lutidine-water, 65:35; and ethylacetate-acetic acid-water, 10:5:6) indicated identity of the radioactivity of the isolated material with authentic S-methylmethionine. In typical experiments, as summarized in Table I, about 9 to 12 per cent of the radioactivity of the original methionine supplied was recovered from the system as S-methylmethionine.

A similar procedure was used for identification of the methylmethionine formed in oat section homogenates incubated with methionine-methyl-C^{14}. With such homogenates, however, the yield of methylmethionine was lower than with intact sections, typically about 0.2 to 2 per cent, the higher yields being obtained in the absence of catalase.

As might be expected, methionine-S^{35} is converted by oat section tissue to S-methylmethionine, both *in vivo* and *in vitro*. In these instances also, the methylmethionine formed was identified by chromatography and cochromatography with authentic material in a variety of solvents.

Methionine Sulfoxide as Methylating Agent—The experiments to be summarized below show that methionine sulfoxide can act as a methyl donor. Thus the methyl group of methionine sulfoxide-methyl-C^{14} can be used by the tissue for the methyl esterification of pectin and protopectin just as has already been shown to be the case for methionine itself (1, 2). L-methionine sulfoxide-methyl-C^{14} was incubated for 4 hours under conditions

TABLE II

*Utilization of methyl group for methyl esterification of pectin and protopectin by intact oat sections**

Substrate	Methionine		Sulfoxide		S-Methylmethionine	
Cell wall fraction	Incorporated	Saponifiable	Incorporated	Saponifiable	Incorporated	Saponifiable
	c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*	*c.p.m.*	*c.p.m.*
Pectin.....	14,600	7,980	3,460	1,290	3,740	2,780
Protopectin...	32,800	6,730	10,100	1,830	3,290	990

* Counts per minute recovered per 10^6 c.p.m. of substrate supplied per gm. of sections.

similar to those of the methionine-methyl-C^{14} experiments. Pectin and protopectin were isolated after incubation, as described above. The distribution of radioactivity in these cell wall fractions in a typical experiment is summarized in Tables II and III. It can be seen that methionine sulfoxide is about one-fourth and one-third as effective as methionine as a methylating agent for pectin and protopectin, respectively. It is possible, however, that only one of the two optical isomers of the synthetic sulfoxide is biologically active. In the case of the next lower homologue of methionine sulfoxide, S-methyl-L-cysteine sulfoxide, only the (+)-isomer is of natural occurrence in the turnip root (15). If methionine sulfoxide behaves in an analogous manner, then the activity of the active isomer would be twice as great as that indicated for the synthetic mixture.

Pectic materials are not known to contain sulfur. Nonetheless, a detectable amount of radioactivity was found to be associated with the pectic fractions isolated from tissue which had been incubated with sulfur-labeled methionine or sulfur-labeled methionine sulfoxide. This activity was much less than that of similar material isolated from tissues incubated with methyl-labeled substrates. The way in which this sulfur is associated with the pectic fractions, or whether it is in fact associated with pectic material or with other substances in the fraction, is not known. The radioactivity is not removed by repeated washing in aqueous alcohol in which methionine sulfoxide is soluble, and no major portion of it is removed by saponification with 6 M ammonia (Table III).

That methionine sulfoxide can act as a methylating agent is evident too from experiments on the formation of S-methylmethionine. These experiments, summarized in Table IV, show that in homogenates the methyl-C^{14} of the sulfoxide is as effective in the formation of S-methylmethionine as is the methyl group of methionine itself. In intact sections, on the other hand, the methyl group of the sulfoxide is only one-tenth as effective as that of methionine in the formation of methylmethionine. The large difference in effectiveness of methionine and its sulfoxide *in vivo* may be due to at least three different factors: (*a*) Methionine-methyl-C^{14} can be methylated by a second similar molecule. Methionine sulfoxide-methyl-C^{14}, on the contrary, must use an unlabeled methionine as its methyl-C^{14} acceptor. (*b*) The endogenous supply of methionine available to accept methyl-C^{14} from the sulfoxide may be limited. (*c*) Only one of the two isomers of L-methionine sulfoxide-methyl-C^{14} may be biologically active.

Unlike the methionine sulfoxide-methyl-C^{14}, methionine sulfoxide-S^{35} does not participate significantly in the formation of S-methylmethionine, being about one-twentieth as effective as the methyl-labeled sulfoxide. The sulfur of methionine-S^{35},

TABLE III

*Association of S^{35} of methionine-S^{35} and methionine sulfoxide-S^{35} with purified pectin and protopectin fractions of oat sections**

Substrate	Methionine		Sulfoxide	
Cell wall fraction	Incorporated	Saponifiable	Incorporated	Saponifiable
	c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*
Pectin	835	226	449	97
Protopectin	2,420	18	1,640	0

* Counts per minute recovered per 10^6 c.p.m. of substrate supplied per gm. of sections.

TABLE IV

*Formation of methylmethionine by oat sections**

Substrate	Methylmethionine formed†	
	Homogenate	*In vivo*
	c.p.m.	*c.p.m.*
Methionine-$C^{14}H_3$	2,400	120,000
Sulfoxide-$C^{14}H_3$	5,000	12,000
Methionine-S^{35}	1,500	
Sulfoxide-S^{35}	260	

* Catalase supplied throughout. Conditions of incubation, as in Table I.

† Counts per minute recovered per 10^6 c.p.m. of substrate supplied per gm. of sections.

TABLE V

*Fate of methylmethionine in oat sections**

Substrate	Material recovered		
	Products	Internal	External
		%	%
Methylmethionine-$C^{14}H_3$	Methionine	0.075	0
	Methylmethionine	40	10
	Sulfoxide	0.17	0.7
	Sulfone	0	0
Methylmethionine-S^{35}	Methionine	23	0
	Methylmethionine	23	0.8
	Sulfoxide	16	0.2
	Sulfone	0	0

* Catalase added throughout. Conditions of incubation, as in Table I. Data expressed as per cent recovered of the methionine-C^{14} or methionine-S^{35} originally supplied (1.34 μmoles, 1×10^6 c.p.m.).

on the other hand, does participate in the formation of S-methylmethionine. These results clearly indicate that methionine sulfoxide, although able to donate its methyl group to a suitable acceptor such as methionine, is unable to act as a methyl acceptor to form methylmethionine.

Methylmethionine is, as might be expected, converted to a variety of related substances by oat sections. Table V summarizes data on this matter. When C^{14}-methyl-labeled methylmethionine is supplied to such tissue *in vivo*, the methyl carbon is recovered principally as unchanged S-methylmethionine, and additional smaller amounts are recovered as methionine and its sulfoxide. The sulfur of S^{35}-labeled methylmethionine is, how-

ever, recovered to a much larger extent as methionine and as the sulfoxide. The total recovery of the methyl group of methyl-labeled S-methylmethionine in the forms itemized in Table V is also less than the recovery of the sulfur of S^{35}-labeled material. Evidently the methyl groups of S-methylmethionine are used in some reaction in which the sulfur does not participate.

Methylmethionine as Methyl Donor—It can be seen from Table II that methylmethionine is only about one-fourth and one-tenth as active as methionine as a methyl donor to pectin and protopectin, respectively. However, in view of the large amount of methylmethionine synthesized within the oat section, the apparent inefficiency of methylmethionine as a methyl donor may be due to large pool size and the presence of only one labeled methyl group per molecule. In addition, although methionine sulfoxide resembles methionine as a methyl donor, transferring its methyl group more effectively to protopectin than to pectin, methylmethionine, on the contrary, yielded its methyl group equally to pectin and to protopectin. That methylmethionine differs from methionine as a methyl donor has also been shown by Stekol (16), who found that methylmethionine is as effective as methionine in the methylation of dimethylethanolamine and guanidinoacetic acid, but that it is less effective in the methylation of homocysteine.

DISCUSSION

The present data concern the interconversion of methionine and related materials in oat shoots and the relative effectiveness of these as methyl donors in the formation of the ester groups of pectic substances. What we really wish to know, however, is the identity of the material which is responsible for this methyl esterification. Methionine itself, although an excellent methyl esterification agent in the present system, is not essential to the process, since methionine sulfoxide is also an effective donor and is transformed to methionine to a negligible extent. Neither methionine sulfoxide nor S-methylmethionine, which is also active, is so effective in the methyl esterification function as methionine itself. The possibility that S-adenosylmethionine may function in the present system as in that of Vignos and Cantoni (9) has been investigated, both by looking for the compound as a native metabolite and by attempting to bring about its enzymatic formation. Neither approach has been successful. It has been impossible either to detect S-adenosylmethionine in oat shoots or to demonstrate its formation *in vivo* or in homogenates. It has, however, been possible to demonstrate the enzymatic formation of S-methylmethionine. In this reaction, which consists of the methylation of methionine, methionine sulfoxide acts highly effectively as the methyl donor, although it is not able to function as the methyl acceptor.

SUMMARY

1. Oat sections, both *in vivo* and *in vitro*, oxidize methionine to its sulfoxide. This reaction is essentially nonreversible.

2. Methionine sulfoxide donates its methyl group to pectin and to protopectin and approaches methionine in effectiveness in this respect.

3. Methionine sulfoxide also yields its methyl group to methionine with the formation of methylmethionine. Methionine sulfoxide cannot, however, serve as a methyl acceptor in the formation of methylmethionine.

4. S-methylmethionine acts as a methyl donor in the formation of pectin and protopectin. It is, however, less effective in this function than either methionine or methionine sulfoxide.

5. Sulfur-labeled methionine and methionine sulfoxide were shown to be incorporated into the pectin and protopectin fractions.

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PART II-A

METABOLISM OF THE PECTIC METHYL ESTER GROUPS IN GROWING CELL WALLS

Introduction

Are the carboxyl groups of pectin esterified before polymerization of the galacturonic acid residues or can the methyl groups be attached and removed after the chain has been formed? Parts II-A and II-B of this thesis will attempt to answer this question. Use will be made of the fact that indoleacetic acid (IAA) increases incorporation of the methyl carbon of methionine into the hot water soluble pectin of the cell wall (53,72).

Part II-A includes, also, a quantitative investigation of the pectic and methyl ester contents of the cell wall as well as a preliminary look at other wall constituents. The growing cell wall consists of polysaccharide and polyuronide components which are in general poorly defined. Earlier determinations of coleoptile cell wall composition by Thimann and Bonner (73), Wirth (64), and Nakamura and Hess (74) agree in suggesting that α-cellulose makes up to 40 per cent of the cell wall dry weight, pectic materials 8 to 12 per cent, and waxes, probably located in the cuticle, 10 to 20 per cent. The balance of the wall material is non-cellulosic polysaccharides. These are however at best rough estimates based as they are solely on solubility properties of the wall materials.

The experiments in Part II-A were carried out in cooperation with Eugene F. Jansen and Rosie Jang, United States Department of Agriculture, Western Regional Research and Development Laboratory.

Materials and Methods

Culture of seedlings.--Oat seeds of the variety Siegeshafer were germinated in vermiculite, moistened with distilled water, and contained in stainless steel trays. The plants were grown at a temperature of 25°C and in low intensity orange light until they had reached an average height of 3 cm (96 hrs). At this stage the seedlings were harvested for use.

Preparation of sections.--Twelve mm sections were cut from each coleoptile, the apical 3 mm tip being discarded. Primary leaves were not removed.

Where sections were to be incubated in auxin solution, they were floated in 0.0025 M potassium maleate buffer, pH 4.8. Indoleacetic acid at a final concentration of 2.8×10^{-5} M was added to the incubation solutions as indicated.

Preparation of cell walls.--Sections were ground in an Omnimixer at 0°C with 4 times their weight of 0.15 N acetate buffer, pH 4.4. Previous work (60) has shown that at this pH no deesterification of pectin by pectinesterase (PE) takes place during homogenization. The homogenate was next centrifuged, the supernatant decanted, and the pellet washed by

resuspension in water at 0°C. The cell fraction was washed in this way 5 times with water, followed by several washes in acetone. The acetone washed cell wall material was finally dried in vacuo and stored for analysis.

The above supernatant and first water wash were combined, heated to 90°C, filtered, and concentrated under reduced pressure. This fraction contains the water soluble pectic material.

Methods of analysis.--Pectic substance was measured by the determination of anhydrouronic acid (AUA) following the pectinase-carbazole method of McCready and McComb (76) and McComb and McCready (77). This method has been calibrated by McCready and McComb (76) with the absolute 12 per cent HCl decarboxylation method.

Methanol was determined by the method of Boos (78). In this determination the solution containing methyl alcohol is distilled, and the distillate collected and diluted to volume. The methanol in the distillate is oxidized to formaldehyde, and the formaldehyde measured colorimetrically with chromotropic acid.

Formaldehyde was determined on the above distillates by applications of the chromotropic acid colorimetric method without prior oxidation.

The Zeisel analyses of total alkyl groups were done by the Clark Modification of the original procedure (79). This method determines all labile simple alkyl groups, including

those attached to oxygen, to nitrogen, and to sulfur.

Radioactivity was in general determined with samples on planchets counted under a micromil window tube in an atmosphere of Q gas. Radioactivity of whole cell walls was however determined by the wet combustion method using the Van Slyke-Folch reagent (80), and counting the CO_2 so formed in the gas phase (81). Aqueous samples were analyzed by a persulfate wet combustion technique (82).

Nomenclature of pectic substances.--Pectic substances occur in plant tissue in three forms which are distinguished on the basis of solubility (83). These are:

1. Pectic substances soluble in cold water and therefore considered not to be associated with cell walls.

2. Pectic substances associated with the cell wall fraction from which they are removed by hot but not by cold water. This is classically known as the pectin fraction.

3. Residual pectic material associated with cell walls but not removed by hot water extraction. This includes the fractions known as protopectin and pectate.

It has been suggested in the past that pectin may differ from protopectin in possessing a shorter chain length (84). It has however been shown by Owens et al. (14) that the hot water insoluble residual cell wall pectic material is completely soluble in hot solutions of sequestering agents. The residual pectic fraction and the soluble pectin fraction may differ from

one another appreciably in chain length but do differ, as will be shown below, in degree of methyl esterification.

Results

Composition of Avena coleoptile tissue and cell walls.-- Whole coleoptiles and the cell walls prepared therefrom can be analyzed for their content of pectic substances (AUA) and the degree to which these are esterified. Typical results are given in Table 1.

The AUA content of the wall fraction is 5.1 per cent. Comparison of this value with that for the whole coleoptile indicates that 91 per cent of the tissue AUA accompanies the cell wall fraction.

The methyl ester content of the cell wall, 0.40 per cent, corresponds to an average degree of esterification of the pectic carboxyl groups of 43 per cent. Only 42 per cent of the tissue methyl ester content is in wall preparations.

The above figures for oat coleoptile cell walls may be compared to those obtained for glycerol-extracted corn coleoptile cell walls. A sample was sent to the author by Dr. A. Kivilaan of Michigan State University. This material was isolated by a non-aqueous technique and quite different therefore from the technique used by the present author. The sample was found to contain 8.3 per cent AUA and 0.93 per cent methanol. In the case of the corn coleoptile pectin, the

Table 1

Composition of Whole Avena Coleoptile Tissue and of Avena Coleoptile Cell Wall

Constituent	Whole Tissue	Cell Wall	Per Cent of Each in Cell Wall
	per cent[a]	per cent[a]	
AUA	0.76	5.1[c]	91
CH_3OH (by alkali)	0.125	0.40[c]	42
CH_3OH (by PE	--	0.37	--
Alkyl groups[b]	--	0.85	--
CH_2O	0.025	0.013	52

[a] On a dry weight basis.

[b] By Zeisel analysis.

[c] Corresponding to a molar ratio of CH_3OH/AUA = 0.43.

average degree of esterification is 63 per cent.

In order to show that the methyl ester content of the cell wall is in fact present as esters of the pectin carboxyl groups, cell wall suspensions were hydrolyzed with pectinesterase (PE). For this purpose 100 mg samples of cell walls were suspended in 10 ml of 0.05 M phosphate buffer at pH 7.8 containing 0.1 PE unit (a dialyzed and lyophilyzed orange PE preparation (85)) and 1 mg of merthiolate (as preservative). After 2 to 3 days of incubation at room temperature, the suspension was filtered, 5 ml of the filtrate distilled, and methanol determined in the distillate. Only 90 per cent as much methanol was obtained by PE hydrolysis as by alkali saponification. This is in keeping with previous results on the extent of hydrolysis of isolated apple and citrus pectins by PE (86).

Cell walls contain a considerable amount of labile alkyl groups other than methyl ester as shown by Zeisel analysis. This method determines any alkyl group on O, N, or S provided only that the alkyl iodide is relatively volatile.

Small but significant amounts of formaldehyde are found in coleoptile tissue. In cell walls the formaldehyde is not present in the free state but is liberated either by acid hydrolysis (1 N HCl for 30 minutes) or by alkaline hydrolysis (1 N NaOH for 30 minutes) followed by acidification prior to distillation. Distillates from unhydrolyzed cell walls

(water suspensions) contain no detectable formaldehyde. Forty-four per cent of the total tissue formaldehyde is found in the cell wall.

Cellulose is known to compose about 25 to 40 per cent of Avena coleoptile walls (73,64,74,87). The AUA makes up a further 5 per cent. About 55 to 70 per cent of the wall is therefore of unknown nature. A preliminary exploration of the remaining cell wall constituents was carried out. A suspension of cell walls (0.75 g dry weight) in 135 ml of 0.2 M pH 5.0 acetate buffer containing 0.15 per cent versene was hydrolyzed with commercial pectinase (Nutritional Biochemicals Corporation) until analysis showed that all of the AUA had passed into solution. The suspension was then centrifuged, the supernatant decanted and the pellet washed by suspension in water and again centrifuged. The supernatant and washings were combined and evaporated under reduced pressure to approximately 10 ml. Basic constituents were removed by passage through a Dowex-50 (H^+) column. The effluent was then passed through a Dowex-1-formate column to remove acidic substances. The effluent contained, as indicated by paper chromatography in butanol-acetic acid-water (60:15:25), ribose, galactose, glucose, xylose, and arabinose, with the latter two sugars predominating. In addition, at least one ultraviolet absorbing and one ultraviolet fluorescing substance were present. The Dowex-1 column was eluted with 1 N formic acid and the eluate evaporated *in vacuo* to dryness.

Chromatography of the residue indicated the presence of galacturonic acid, two ultraviolet absorbing and two ultraviolet fluorescing substances.

It was shown in Part I that the radioactivity of the pectin fractions from Avena sections previously incubated with L-methionine-methyl-C^{14} was seldom more than 50 per cent saponifiable. An effort was made to rid Avena coleoptile walls of the non-saponifiable fraction. Repeated washings with 50:50 ether-ethanol, 1 M NaCl, acidified 6 M urea, and water failed however to eliminate the non-saponifiable activity. A sample of the highly washed wall material was hydrolyzed with commercial pectinase and yielded upon chromatography in n-butanol-acetic acid-water, five ultraviolet absorbing compounds and two ultraviolet fluorescing compounds. Three of the compounds corresponded to adenosine, guanosine, and uridine. These spots were eluted and the ultraviolet spectra (Beckman, Model DK-2) obtained at pH 1, 7, and 11. These were identical with those of authentic samples of the three compounds.

An effort was then made to quantitatively determine the ribonucleotides in the versene extract. Three hundred and sixty grams of Avena shoots were harvested and the walls isolated by the above procedure. The purified walls were then extracted three times with 0.3 per cent versene (pH 6.0), each time for twenty minutes at 100°C. After each extraction the residue was spun down, the supernatant decanted and

filtered through Whatman No. 1 paper. The supernatants were combined and concentrated to 100 ml. The RNA content of the wall extract was estimated by comparison of its optical density at 260 mμ with that of a standard RNA solution. The ribonucleotide content of the wall extract was calculated to be 11 mg, corresponding to 0.15 per cent of the dry weight of Avena walls. Organic phosphorus was then determined by the method of Allen (88). The phosphorus content was 1.01 mg which is equivalent to 0.9 phosphorus atoms for every RNA base. The nitrogen content, as determined by the colorimetric Nessler assay (89), was found to be 13.7 mg. Thus there is only 1 phosphorus atom for every 30 nitrogens. This is eight times more nitrogen than would be contained in the ribonucleotides. Undoubtedly the excess nitrogen is due to the presence of protein in the versene extract (73).

Kivilaan (90) reports finding RNA in glycerol-extracted corn coleoptile walls in amounts similar to those found in Avena walls. It would be interesting to know if this RNA is truly a wall constituent or an artifact of the isolation procedure. These observations, which are by no means complete, do illustrate the complexity of the cell wall.

IAA effect independent of methyl ester content of cell wall pectic substances.--The methyl ester content of Avena coleoptile section pectic material is not detectably influenced by incubation with IAA. This is shown by the following

experiment.

Twenty grams of sections were incubated in one l of 0.0025 M potassium maleate buffer, pH 4.8, at 25° for 18 hours in low intensity orange light. A second 20 g of sections were incubated under similar conditions except that the incubating solution was made 5×10^{-5} M with respect to IAA. The presence of the IAA induced growth of the sections. Dried cell walls were prepared from each group of sections. The analyses (Table 2) for methyl ester, total labile alkyl groups and AUA clearly demonstrate that, under the conditions used, the presence of IAA does not affect methyl ester content or degree of pectic esterification.

The AUA contents of the preparations of the experiment of Table 2 are lower than those reported elsewhere in this paper. This is related to the long period of incubation of the material as excised sections.

In further experiments, the effect of IAA-incubation on the incorporation of the methyl carbon of methionine-$C^{14}H_3$ into cell walls was studied. Sections were floated in 100 ml of buffered solution containing 2 mg catalase and methionine-$C^{14}H_3$. In the first experiment methionine-$C^{14}H_3$ had a specific activity of 0.85 mc/mM. Incubation in the presence or absence of IAA was for 5 hours. The sections were then well washed with water and cell walls prepared from them. The results (Table 3) show that IAA caused a small increase in the total incorporation of radioactive methyl into cell walls.

Table 2

Composition of Cell Walls of Avena Coleoptile Sections after Incubation in the Presence or Absence of IAA[a]

	Control	IAA
AUA	4.2%	4.0%
CH_3OH	0.42%	0.42%
Zeisel	0.91%	0.87%
Molar ratio CH_3OH/AUA	0.55	0.58

[a] Incubation time - 18 hours.

Table 3

Composition of Cell Walls of Sections Incubated with Methionine-$C^{14}H_3$ and in the Presence or Absence of IAA[a]

	Experiment 1[b]		Experiment 2[c]		Experiment 3[c]	
	Control	IAA	Control	IAA	Control	IAA
cpm/mg C	7,900	8,600	27,000	27,200	27,400	33,000
AUA	5.2%	5.1%	5.7%	5.3%	5.1%	5.3%
CH_3OH	0.37%	0.37%	0.43%	0.38%	0.36%	0.36%
Molar ratio CH_3OH/AUA	0.40	0.39	0.41	0.40	0.39	0.37

[a] Incubation time - 5 hours.

[b] 1.3 μc/gm section (0.235 mg methionine possessing 0.85 mc/mM).

[c] 2.7 μc/gm section (0.105 mg methionine possessing 3.9 mc/mM).

However, as in the experiment of Table 2, no net change in methyl ester content resulted from IAA treatment. The molar ratio of methyl ester to pectic substance was also unaffected by the presence of IAA.

Accelerated methyl ester incorporation induced by IAA.-- Although IAA does not cause a measurable change in the ester content of coleoptile pectic substances, it does cause an increased incorporation of methyl into the ester groups of the cell wall. This is shown below by isolation of the methanol obtained by saponification of cell walls of sections incubated in methionine-$C^{14}H_3$ and in the presence or absence of IAA. The dimedone derivative, prepared from formaldehyde formed by oxidation of the methanol was then counted. Cell walls from experiment 2 and 3 were used for the purpose. One hundred mg samples were saponified for 30 minutes with 4 ml of 1 N NaOH after which 4.25 ml of 1 N HCl were added to each (to facilitate distillation) and the methanol distilled. Distillation was continued until one half of the initial volume had been collected. To each distillate was added 1 ml of solution containing 3,950 µgm of reagent grade methanol. The distillates were then oxidized by adding 15 drops of 5 per cent phosphoric acid and 25 drops of 5 per cent potassium permanganate. After 20 minutes the excess permanganate was discharged by the drop-wise addition of 5 per cent sodium thiosulfate, and the pH of the reaction mixture adjusted to

5.3 with 2 N sodium hydroxide. The solutions were then centrifuged. After the supernatants had been decanted the residual manganese dioxide was washed with 3 ml of water and again centrifuged. These supernatants were combined with the previous ones. To each were added 20 ml of 0.38 per cent dimedone solution. The reaction mixtures were allowed to stand one day at room temperature and one day at 4° after which the crystalline dimedone derivative of formaldehyde was filtered off, dried, and dissolved in 3.0 ml of ethanol. Aliquots of the ethanol solutions were dried on planchets and counted. The dimedone derivatives were recrystallized by adding 20 ml of water to the remainder of the ethanol solutions. Experiments 2 and 3 yielded 5 and 8 mg respectively. All samples had melting points of 189-190°. The 5 mg sample was dissolved in exactly 2 ml of ethanol and the 8 mg sample in 5 ml of ethanol. One hundred µl aliquots were dried on planchets for counting and 0.5 ml aliquots used for dry weight determinations. The recrystallization failed to change the specific radioactivities of the dimedone derivatives. The radioactivities of the derivatives are reported in Table 4. It is apparent that the specific activities of the material from the IAA treated sections are greater than those from the control sections. The ratio of the specific activities, IAA/control, were 1.4 and 1.7 in the two experiments. Accordingly, even though IAA does not cause a net change in methyl ester content of the pectic substances, it does cause an increased

Table 4

Specific Radioactivity of Formaldehyde-dimedone Compound Prepared from the Methyl Ester of Cell Walls of Sections Incubated in the Presence or Absence of IAA

Experiment No.		Activity of diluted Dimedone Derivative	Dilution Factor	Activity on Undiluted Basis	Specific Activity[a]	Ratio
		cpm/mg		cpm/mg	μc/mM	IAA/Control
2	Control	6620	10.2	67,700	22	
	IAA	8280	11.4	94,400	31	1.4
3	Control	3615	12.0	43,300	14	
	IAA	5910	12.3	72,700	24	1.7

[a] Calculated on basis of 40 per cent efficiency gas-flow, thin-window Geiger counter.

incorporation of radioactivity into the ester groups. The fact that the dimedone derivatives possessed specific activities approximately one-half per cent of that of the methionine-$C^{14}H_3$ suggests that a fairly direct pathway exists in the transfer of methyl from methionine to the methyl ester of the pectic substances.

The data of Table 4 apply to the cell walls as a whole. The same conclusions as to IAA-induced increase in radioactivity also apply to the hot water soluble (classical pectin) fraction of the wall. It is in this fraction that Ordin, Cleland and Bonner (53,72) have shown an auxin-induced increased rate of incorporation of labeled carbon derived from methionine-$C^{14}H_3$. The results presented below confirm their observations and show that in this fraction, too, net synthesis of methyl ester groups is not detectable.

Hot water extracts of the cell wall preparations from the three experiments (Table 3) were prepared by adding 7 ml of water to 150 mg of cell wall preparation. Each was heated for 1 hour in a boiling-water bath, cooled, centrifuged, and the supernatant (ca. 3 ml) removed. The extractions were repeated for an additional 30 min with another 6 ml of water. The material was again cooled, centrifuged and the supernatant combined (ca. 9 ml). The extracts were made up to 10 ml. Aliquots of these extracts were used for dry weight analyses. The extracted cell wall residues were washed several times with water, once in acetone, and dried <u>in vacuo</u> (to be used in residual

pectin studies).

The hot water soluble portion amounts to ca. 3.5 per cent of the total weight of the cell wall (Table 5). The soluble portion contains, however, 15 per cent of the total cell wall AUA and an even larger fraction, 35 per cent of the total cell wall methyl ester. The methanol/AUA ratio of the hot water soluble material is approximately 0.9. Hot water therefore extracts a highly esterified fraction of the cell wall pectic material. The residual hot water insoluble material, by contrast, possesses a methanol/AUA ratio of 0.3 (Table 6). Although the hot water soluble fraction corresponds to the classical pectin in its high degree of esterification, it does not consist solely of pectin. In fact, as shown in Table 5, only 21 to 27 per cent of the fraction can be accounted for as AUA.

The specific activities of the hot water soluble fractions of the cell walls of the three experiments in which sections had been previously incubated in methionine-$C^{14}H_3$ are given in Table 5. In each case the specific and total activities are greater in the preparation from IAA treated sections than in that from control sections. The ratio of specific activities, IAA/control, was 1.2 for experiment 1 and 1.4 for experiments 2 and 3. The ratios for the hot water soluble pectic material was in general agreement with the ratios of the dimedone derivatives representing the total cell wall methyl ester (Table 4).

Table 5

Composition of Hot-H_2O-soluble Fraction of Cell Walls

Experiment No.	Sample	Per cent of Total Wall Component Solubilized			Molar Ratio	Per cent AUA in Extracted Material[a]	cpm/mg Dry Wt. of Extract	cpm/mgAUA	Ratio of Specific Activities IAA/Control
		Dry Weight	AUA	CH_3OH	CH_3OH/AUA				
1	Control	3.7	14	32	0.9	21	7,900	37,600	
	IAA	3.2	13	30	0.9	21	9,600	45,700	1.2
2	Control	3.7	18	45	1.0	27	20,600	76,300	
	IAA	3.4	16	37	0.9	25	26,200	104,700	1.4
3	Control	3.5	17	35	0.8	25	21,700	86,000	
	IAA	3.3	16	35	0.8	25	31,200	124,000	1.4

[a] On a dry weight basis.

Table 6

Analysis of Hot-water-extracted Residues of Cell Walls of Sections Incubated with and without IAA in Presence of Methionine-$C^{14}H_3$

	Experiment 1		Experiment 2		Experiment 3	
	Control	IAA	Control	IAA	Control	IAA
cpm/mg C	6,200	6,300	23,600	23,900	23,300	25,100
AUA (per cent)	4.0	4.0	4.9	4.3	4.2	4.1
CH_3OH (per cent)	0.24	0.22	0.24	0.22	0.20	0.17
Molar ratio CH_3OH/AUA	0.33	0.30	0.27	0.28	0.26	0.23

Aliquots of the hot water soluble material were saponified and recounted. The method used for saponification consisted of adding 0.5 ml of concentrated ammonium hydroxide to 1 ml of extract. After the solutions had stood at room temperature for 90 minutes, they were heated at 100°C for 60 minutes. Saponification was complete since repeating the above procedure failed to further decrease the specific radioactivity of the residue. The losses of radioactivity on saponification of hot-water extracts of cell walls from control and IAA treated sections were 85 and 89 per cent respectively (experiment 2) and 91 and 94 per cent respectively (experiment 3). The activity of the hot water soluble portion resides therefore principally in saponifiable material, presumably methyl ester. Hot water soluble pectin, similar to total wall pectin, incorporates more radioactivity in the presence of IAA.

Analyses of the cell wall residue after hot water extraction are given in Table 6. The data show in confirmation of the expectation based on analysis of the hot water extracts that approximately 80 per cent of the pectic substance remains in the cell walls after hot water extraction. The degree of esterification of this residual pectic substance is approximately 30 per cent. It is this residual pectic fraction which would classically be known as protopectin. It is apparent that the material possesses a low degree of esterification and differs from soluble pectin in this respect.

Furthermore, 5 hour incubation in IAA does not cause an accelerated incorporation of radioactivity into the methyl ester of the pectic substances remaining in the cell walls after hot water extraction. This was determined on the hot-water extracted cell walls of experiment 3 by saponification in the following manner. To 65 mg of hot-water extracted cell walls from control sections and from those incubated with IAA were added 4 ml of 1 N NaOH. Saponifications were allowed to proceed for one-half hour at 25° after which 4.25 ml of 1 N HCl was added to each and the liberated methanol was removed by distillation. The saponification mixtures were filtered, the residue washed several times with water, dried with acetone and then in a vacuum desiccator. The filtrates and water washings were combined and diluted to 25 ml. The loss of radioactivity on saponification was 42 and 38 per cent respectively for the control and IAA treated hot-water extracted cell walls. The total loss of counts was 232,000 for the former and 204,000 for the latter, corresponding to the ratio, IAA/control, of 0.9. On the assumption that all saponifiable counts are due to ester, a very reasonable assumption as will be shown later, IAA has little or no effect on the methyl ester of the pectic substances of this fraction. It is interesting to note that the alkali treatment solubilized the major portion of the non-saponifiable radioactive material of the cell walls (Table 7).

Table 7

Saponifiable Counts in Hot-Water-Extracted Residues of Cell Walls of Sections With and Without IAA in Presence of Methionine-$C^{14}H_3$ [a]

IAA	Material	cpm/mg C	Total mg C	Total Counts	Per cent Loss on Saponification
-	Hot-water-extracted Residue	23,300	23.4	545,000	
-	Saponification Supernatant [b]	22,000	13.3	293,000	
-	Saponification Residue [c]	1,700	12.2	20,700	42
+	Hot-water-extracted Residue	25,100	21.5	540,000	
+	Saponification Supernatant [b]	31,000	10.0	310,000	
+	Saponification Residue [c]	2,100	12.3	25,700	38

[a] Experiment 3.

[b] Material solubilized by saponification procedure.

[c] Cell wall fraction which was not solubilized by saponification procedure.

IAA effect not due to change in degree of acetylation of cell walls.--The methyl groups of methionine-$C^{14}H_3$ are not transferred to acetyl or similar acyl groups of the cell walls. Further IAA does not cause a net change in the unlabeled acyl group of cell walls. This was demonstrated, using cell walls of experiment 3, in the following manner. To cell wall from control sections (150 mg) and to a like amount of cell walls from sections incubated in IAA were added 25 ml 1 N HCl in glass tubes. The tubes were sealed off and heated in a steam bath for 1 hour after which they were cooled and opened. The residual cell material was removed in each case by filtration and the filtrates were steam distilled to give 50 ml of distillates. Each distillate required 0.17 meq of NaOH for neutralization. Accordingly, IAA does not cause a net change in the acetyl (or similar acyl groups) content of cell walls.

The neutralized steam distillates were evaporated in vacuo to dryness. The residues were dissolved in 5 ml of water and 100 µl aliquots plated for counting. Only 70 cpm were observed from the aliquots of both solutions. This radioactivity was less than 1 per cent of that solubilized by the acid hydrolysis. Therefore little, if any, of the methyl of methionine-$C^{14}H_3$ is incorporated into the acetyl groups of cell walls.

IAA-induced accelerated incorporation of radioactivity into the methyl ester in the cold water soluble, 70 per cent alcohol precipitable fraction of Avena coleoptile sections.-- In the preparations of the cell walls, the tissue was first ground in acetate buffer and the cell wall material then centrifuged off. Some pectic material remains however in the supernatant from the centrifugation. This will be called the cold water soluble pectin, and was prepared as follows: The supernatant was in each case combined with the first water washing of the cell wall material. The water soluble material was then heated to 90° to denature protein, filtered through Whatman No. 1 paper and concentrated *in vacuo* to a volume of 20 ml. Sixty ml of 95 per cent ethanol were then added. After several days at 4°C, the resultant precipitate was centrifuged from the solution, washed with acetone and dried *in vacuo*. From each 30 gm of original sections, 35-40 mg of material were obtained.

The cold water soluble, 70 per cent alcohol insoluble fraction was prepared as described above for all experiments. The dried material was in each case resuspended in 10 ml of water. In the case of experiment 1, 66 per cent of this material proved to be soluble; in the case of experiments 2 and 3, 75 per cent. Analyses of the resultant solutions are given in Table 8, expressed in terms of the dry weight of soluble material. The concentration of pectic substance present was low and the limited amount of material available

Table 8

Characteristics of the Cold-Water-Soluble "Pectic" Substances from Sections Incubated in the Presence of Methionine-$C^{14}H_3$ and in the Presence and Absence of IAA

	Experiment 1		Experiment 2		Experiment 3	
	Control	IAA	Control	IAA	Control	IAA
AUA (per cent	4.3	4.9	3.5	3.2	2.4	3.0
CH_3OH (per cent)	~ 0.5	~ 0.5	—	—	~ 0.2	~ 0.3
Molar Ratio CH_3OH/AUA	~ 0.5	~ 0.5	—	—	~ 0.5	~ 0.5
Specific Activity (cpm/mg AUA)	174,000	217,000	309,000	531,000	333,000	557,000
Ratio of Specific Activities[a] (per mg AUA)		1.2		1.7		1.7
Loss on Saponification (per cent)	66	74	52	67	55	65
Specific Activity of Saponifiable (per mg AUA)	115,000	161,000	161,000	357,000	183,000	362,000
Ratio of Saponifiable Specific Activities[a] (per mg. AUA)		1.4		2.2		2.0
Specific Activity of Saponification Residue (per mg AUA)	59,000	56,000	148,000	174,000	150,000	195,000
Ratio of Specific Activities of Saponification Residues[a] (per mg AUA)		1.0		1.2		1.3

[a] Ratios are IAA/control.

permitted only an approximate determination of methyl ester (CH_3OH) content. However, the specific activities per mg dry weight of soluble material are close to those of the hot water soluble material in spite of the fact that they represent only approximately one-fifth as much pectic substances. Specific activities were calculated on an AUA basis. With the exception of experiment 1, the ratios of specific activities were even higher than the corresponding ones for the hot-water-soluble material (Table 5). IAA treatment apparently causes a greater acceleration in incorporation of radioactive methyl ester into this particular fraction than into the cell walls themselves.

The cold water soluble, alcohol precipitable fractions were next saponified as described above and aliquots of the dried residues recounted. The data (Table 8) indicate that the IAA-induced increase in specific activity of this fraction is almost wholly due to an increase in amount of a saponifiable, volatile material, presumably methyl ester. The residues do contain, however, non-saponifiable activity.

As further evidence that the above saponifiable material was methyl ester, the methanol resulting from saponification was collected by distillation, diluted with reagent grade methanol, oxidized to formaldehyde, the dimedone derivative prepared, and the radioactivity determined. The cold-water soluble "pectin" from experiment 3, both that from the control sections and that from the sections incubated in IAA, was used

for this purpose. The method of preparation of the dimedone derivative was the following. Solutions (3 ml) of each possessing a potential 25 μg of methanol were saponified at 25°C with 2 ml 1 N NaOH. After one-half hour 2.25 ml 1 N HCl were added and the reaction mixtures distilled so as to give 3 ml of distillate. Methanol solution (1 ml containing 3,960 μg of CH_3OH) was added to each and each was oxidized and the dimedone derivative of the formaldehyde prepared as above. Yields of approximately 9 mg of the twice crystallized dimedone derivative were obtained in each case. The second crystallization failed to alter the specific radioactivity of the derivatives. The preparation of the derivatives was repeated using solutions of each containing potentially 38 μg of methanol. The ratio of specific activities, IAA/control, was 2.3 (Table 9). This value is comparable with that obtained for the saponifiable specific activities (Table 8) of the same preparations. The specific activity of that from the control sections was 2 per cent and that from the IAA incubated 4 per cent of the methionine. Thus the methyl group of methionine which is incorporated into the cold water soluble pectic methyl ester appears to be either less diluted or to follow a more direct pathway than the transfer to the ester of the cell walls.

Table 9

Specific Radioactivity of Formaldehyde-dimedone Compound Prepared from the Methyl Ester of Cold-Water-Soluble "Pectic" Substances from Sections Incubated in the Presence of Methionine-$C^{14}H_3$ and in the Presence and Absence of IAA[a]

Preparation	Specific Activity cpm/mg	Dilution Factor	Activity on Undiluted Basis cpm/mg	Specific Activity μc/mM[b]	Ratio IAA/Control
No. 1 Control	1400	160	224,000	74	
IAA	3210	160	513,000	170	2.3
No. 2 Control	2240	105	235,000	78	
IAA	5330	105	533,000	176	2.3

[a] Cold-water-soluble "pectic" substances from Experiment 3 were used.

[b] Calculated on basis of 40 per cent efficiency gas-flow, thin-window Geiger counter.

Discussion

Avena coleoptile cell walls of the kind studied in this work contain on the average 5.1 per cent of pectic material, measured as AUA. That AUA, as determined by the pectinase-carbazole method used, actually is galacturonic rather than some other uronic acid has been shown by paper chromatography on the acidic fraction of the cell wall hydrolysate. The distribution of Avena coleoptile cell wall pectic material between fractions of different solubility properties as well as the degree of methyl esterification of each fraction is summarized in Table 10. Although over 90 per cent of the AUA is associated with cell walls, a small portion is not clearly so but is recovered from the original tissue homogenate as cold water soluble, 70 per cent alcohol insoluble material. It is possible that this material is actually a cell wall constituent, liberated by the homogenization and washing procedure. Alternatively it may be a cell wall precursor as is suggested by the very high specific activity of its methyl ester groups. The hot water soluble pectin which makes up approximately one-seventh of the tissue pectic substance is 90 per cent esterified. The residual hot water insoluble pectin on the contrary, is methyl esterified only to the extent of 30 per cent. This material is apparently held in the wall as the water insoluble salt of inorganic ions such as calcium. That this is so is indicated by the facts (a) that the residual pectin is essen-

Table 10

Distribution of Pectic Substances in Avena Coleoptile Tissue

	Per Cent AUA in Fraction	Per Cent of Total AUA	Per Cent Esterified
Cell walls	5.3	91	40
Hot water-soluble fraction of cell walls	23	14[a]	90
Residue after hot water extraction	4.3	78	31
Cold water soluble, 70 per cent ethanol insoluble	4.0	~ 5	~ 50

[a] This corresponds to 15 per cent of cell wall AUA.

tially completely extracted by hot aqueous solutions of the calcium sequestering agent, ethylenediamine tetraacetic acid, and (b) that the cell walls of Avena coleoptiles prepared as described in this paper contain bound calcium. This calcium which is not readily removed, amounts to ca. 0.03 meq per gm of cell walls and is sufficient to form the calcium salt of one-fifth of the free pectic carboxyl groups present (91).

The data show clearly that when Avena coleoptile section tissue is supplied with IAA, neither the net amount of cell wall pectic substance, its distribution between forms of different solubility, nor its net degree of esterification, is measurably affected. They also show however that the presence of IAA does influence one significant aspect of pectic metabolism, namely, rate of incorporation of pectic methyl ester groups. This rate of incorporation is increased to the greatest extent in the cold-water soluble pectin but is also observed in the hot water soluble pectin. This increase in incorporation of methyl ester into pectin may be either a measure of increased turnover of the methyl ester groups after polymerization of the galacturonic residues or it may be simply a measure of "de novo" synthesis of pectin, too small to be detected by the analytical methods used.

The effect of auxin, IAA, in increasing cell wall plasticity cannot be simply due to increasing the proportion of pectic carboxyl groups which are esterified. This is ruled out by the data of Tables 2 through 7, which show no effect

of auxin treatment on net cell wall methyl ester content.

In the work presented above, it has been clearly shown that IAA does bring about a chemical change in a particular cell wall constituent, namely, increased rate of methyl ester incorporation into pectic material. Whether this is responsible for the growth promoting effects of IAA has not yet been established. Part II-B of this thesis will help to clarify this problem.

Summary

1. Avena coleoptile cell walls have been found to contain, on a dry weight basis, 5.1 per cent of pectic substances as measured by the carbazole method. Methyl ester determinations by alkaline hydrolysis have shown the pectic material to be, on the average, 40 per cent esterified. Methyl ester determination by pectinesterase yields a value approximately 90 per cent of that found by alkaline hydrolysis. The cell walls contain, by Zeisel analysis, a considerable amount of labile alkyl groups other than methyl ester. The nature of these alkyl groups is not known. Hydrolysis of cell walls produced small but significant amounts of formaldehyde. Pectinase hydrolysis of cell walls followed by ion exchange separations and paper chromatography have been used to identify the component sugars and uronic acids of the cell wall. The sugar fraction has been found to contain ribose, galactose, glucose, xylose and arabinose, with the latter two sugars predominating, as well as at least one ultraviolet absorbing and one ultraviolet fluorescing material. The acidic fraction has been found to contain two ultraviolet absorbing and two ultraviolet fluorescing substances in addition to galacturonic acid. Ribonucleotides, equivalent to 0.15 per cent of the cell wall dry weight, was identified in a hot-versene extract of Avena cell walls.

2. Incubation of coleoptile tissue in an auxin, IAA, fails to cause any detectable change in cell wall content of pectic substance, in methyl ester content, or in degree of pectic esterification.

3. Incubation of coleoptile tissue in IAA does induce an accelerated incorporation of methyl into the methyl ester of the pectic substances of cell walls. This is deduced from experiments in which tissue was incubated in methionine-$C^{14}H_3$ and in the presence or absence of IAA, followed by isolation of the pectic methyl ester groups as methanol. The ratio of rate of incorporation amounts to as much as 1.4 both for the total ester of the cell walls as well as for the hot water soluble pectin fraction of the cell wall pectic substances.

4. IAA failed to cause an accelerated incorporation of methyl into the ester groups of the pectic fraction remaining in the cell walls after hot water extraction.

5. Little or no incorporation of the methyl group of methionine occurred in the acetyl (or similar acyl) groups of the cell walls.

6. No net change in the acetyl (or similar acyl) groups resulted from incubation of coleoptile tissue in IAA.

7. Incubation of coleoptile tissue in IAA accelerated incorporation of methyl into the methyl ester groups of the cold water soluble "pectin" fraction to a greater extent than into the pectin of the cell walls. The high specific radio-

activity of the cold water soluble "pectin" fraction suggests a relatively direct pathway of the transfer of the methyl group of methionine to the methyl ester of pectic substances.

PART II-B

METABOLISM OF PECTIC GALACTURONIC ACID RESIDUES IN GROWING CELL WALLS

Introduction

If auxin-induced increase in incorporation of the methyl of methionine into the methyl ester of pectin is a result of an increased turnover of pre-existing methyl ester groups then auxin should have no effect on the incorporation of galacturonic acid precursors into the polygalacturonic acid chain. Conversely, if the effect of auxin is an actual increase in rate of pectin synthesis then auxin should increase incorporation of galacturonic acid precursors into the chain just as it increases rate of methyl incorporation. To test this question use will be made of the fact that glucose is readily incorporated into the galacturonic moiety of pectin (13,32).

It was observed in Part II-A that the specific activity of cold water soluble pectic methyl ester groups is 5 to 6 times greater than that of the corresponding methyl groups in the wall pectins. In this section experiments are described which attack the problem of whether the cold water soluble pectin is in fact a precursor of the wall materials.

Materials and Methods

Culture of seedlings, preparation of sections and of cell walls, and methods of analysis for pectic substance and for radioactivity were similar to those used in Part II-A.

Hydrolysis of pectin.--Samples to be analyzed were suspended in 20 ml of 0.5 per cent versene at pH 6.0. After one hour, 0.5 ml of 1 N NaOH was added. Saponification was allowed to proceed for 0.5 hours after which the pH was adjusted to 5.0 with 2-3 drops of glacial acetic acid. Purified citrus PG was then added and the mixture stirred at a slow speed for three hours (last 0.5 hour with toluene). The reaction mixture was then allowed to stand at room temperature for three days after which the digested cell walls were filtered off.

The author is indebted to Eugene F. Jansen, United States Department of Agriculture, Western Utilization Research and Development Division, for the purified PG used in these experiments.

Isolation of galacturonic acid.--The filtrates of the enzymic hydrolysis were concentrated under reduced pressure (60-70°C) to near dryness. The concentrate was taken up in 4.0 ml of 1 N HCl and heated in a boiling water bath for 15 minutes. Galacturonic acid is not appreciably affected by this treatment. Any glucuronic acid which may be present forms a stable lactone under these conditions (44). The acid

hydrolysate was evaporated to dryness *in vacuo*.

The dried samples were dissolved in water, filtered, passed through a Dowex-50 (hydrogen) column and once again concentrated at 60-70°C. The concentrated sample was adjusted to pH 9.0 with dilute ammonium hydroxide and placed on a Dowex-1 (formate) column. The column was washed with three to four bed volumes of water and eluted with 0.5 *M* formic acid. The eluate was concentrated at 60-70°C and then evaporated to dryness *in vacuo*.

The samples were then subjected to paper chromatography in three successive solvents: isopropanol, pyridine, acetic acid, water (8:8:1:4); ethyl acetate, acetic acid, water (10:5:6); and n-butanol, acetic acid, water (60:15:25). Galacturonic acid was detected by the CD-1 (0.1 *M* 2-amino-biphenyl hydrogen oxalate) solution of Gordon *et al.* (92). Whatman paper No. 1, previously washed with dilute acetic acid, was used for all chromatography. Radiopurity of each chromatogram was determined by use of Nuclear Chicago's Model C-100A Actigraph II as recorded by Texas Instruments' "recti/riter." After chromatography in the first and second solvents, the strip corresponding to galacturonic acid was eluted with water and evaporated to dryness *in vacuo*. Following chromatography in the third solvent, the galacturonic eluate was made to volume for determination of specific activity.

Radioactive compounds.--D-Glucose, uniformly labeled with C^{14} (Isotope Specialties Co., Inc., Burbank, Calif.), was the only radioactive compound used in these experiments. The specific activity of the glucose in experiments 4 and 5 was 1.87 mC/mM while the specific activity of that used in experiment 6 was 3.0 mC/mM.

Results

Effect of IAA on incorporation of D-glucose into pectic galacturonic acid residues.--IAA does cause an increased incorporation of glucose into the galacturonic acid residues of pectin. This is shown below by determination of the radioactivity in galacturonic acid isolated from enzymic hydrolysates of the various pectic fractions of sections previously incubated in glucose (uniformly labeled with C^{14}) and in the presence or absence of IAA.

In experiment 4, fifteen grams of sections were incubated in 75 ml of 2.5×10^{-3} M potassium maleate buffer, pH 4.8. The incubation took place at 25°C for 5 hours in low intensity orange light. The incubation mixture was 2.5×10^{-4} M with respect to glucose-C^{14} and 3×10^{-4} M with respect to L-methionine. The glucose-C^{14} corresponded to 1.3×10^{6} cpm per g fresh weight of sections. A second fifteen grams of sections were incubated under similar conditions except that the incubating solution was made 2.8×10^{-5} M with respect to IAA.

Experiment 5 was similar to experiment 4 except that two 15 g batches of sections were used as control and two for IAA treatment. In experiment 5, the incubation solutions were 1.6×10^{-4} M with respect to glucose-C^{14} (0.9×10^{6} cpm per g fresh weight of sections).

The amounts of purified galacturonic acid isolated (as AUA), as well as the radioactivity of each pectic fraction, are reported in Tables 11 and 12 for experiments 4 and 5 respectively. The residual pectin fraction represents only about one-fourth of the total pectin in the sections used. The data of Tables 11 and 12 show that the specific activity of the cold water soluble pectin as well as that of the hot water soluble pectin are increased in the presence of IAA. IAA has little or no apparent effect on the incorporation of these substances into residual pectin.

The data also show a marked difference between the specific activity of the cold water soluble, 70 per cent alcohol insoluble pectin and that of the other pectic fractions. A similar difference was observed in the C^{14} methyl-labeled methionine experiments of Part II-A. Again it must be asked whether the cold water soluble pectin is a precursor of the wall pectins.

Metabolic turnover of pectin.--The data of Tables 13 and 14 concern an experiment (No. 6) in which sections were incubated for 15 hours in an excess of unlabeled glucose following a 5-hour pretreatment in C^{14} uniformly-labeled glucose. These

Table 11

Specific Activity of Galacturonic Acid Prepared from the Pectic Fractions of Oat Coleoptiles Incubated in Glucose-C^{14} in the Presence or Absence of IAA (Experiment 4)

Pectic Fraction	Treatment	γ AUA Isolated	cpm	cpm/mgAUA	IAA/Cont
Cold Water Soluble, 70% Alcohol Insoluble	Control	25	630	25,200	
	IAA	—	—	—	
Hot Water Soluble	Control	160	636	3,980	1.5
	IAA	124	714	5,760	
Residual	Control	175	530	3,030	1.0
	IAA	190	588	3,090	

Table 12

Specific Activity of Galacturonic Acid Prepared from the Pectic Fractions of Oat Coleoptiles Incubated in Glucose-C^{14} in the Presence or Absence of IAA (Experiment 5)

Pectic Fraction	Treatment	γ AUA Isolated	cpm	cpm/mgAUA	IAA/Cont
Cold Water Soluble, 70% Alcohol Insoluble	Control	100	1,224	12,200	1.7
	IAA	80	1,666	20,800	
Hot Water Soluble	Control	597	1,292	2,160	1.3
	IAA	396	1,050	2,790	
Residual	Control	283	554	1,960	1.2
	IAA	505	1,168	2,310	

Table 13

Specific Activity of Galacturonic Acid Prepared from the Pectic Fractions of Oat Coleoptiles (5-Hour Pretreatment, 1.2×10^{-4} M Glucose-C^{14}, -IAA; 15-Hour Incubation, 0.1 M Nonradioactive Glucose ±IAA)

(Experiment 6)

Pectic Fraction	Treatment	γ AUA Isolated	cpm	cpm/mgAUA
Cold Water Soluble 70% Alcohol Insoluble	Control	103	1,832	17,800
	-IAA	~5	114	~ 22,800
	+IAA	34	722	21,200
Hot Water Soluble	Control	144	572	3,970
	-IAA	1,028	3,250	3,160
	+IAA	1,215	3,482	2,870
Residual	Control	387	1,012	2,620
	-IAA	853	2,856	3,350
	+IAA	366	1,024	2,800

Table 14

Radioactivity per Pectic Fraction in 100 mg of Oat Coleoptile Cell Walls

(5-Hour Pretreatment, 1.2×10^{-4} M Glucose-C^{14}, -IAA;

15-Hour Incubation, 0.1 M Nonradioactive Glucose ±IAA)

(Experiment 6)

Pectic Fraction	Treatment	cpm/mgAUA	mg of AUA in 100 mg Cell Walls	cpm in AUA of 100 mg of Cell Walls
Cold Water Soluble, 70% Alcohol Insoluble	Control	17,800	0.205[a]	3,650
	-IAA	22,800	0.260[a]	5,930
	+IAA	21,200	0.350[a]	7,430
Hot Water Soluble	Control	3,970	0.542	2,170
	-IAA	3,160	0.644	2,040
	+IAA	2,870	0.708	2,030
Residual	Control	2,620	2.69	7,050
	-IAA	3,350	2.86	9,580
	+IAA	2,800	2.87	8,040
Total (above three fractions)	Control	3,740[b]	3.44	12,860
	-IAA	4,670[b]	3.76	17,550
	+IAA	4,450[b]	3.93	17,500

[a] Calculated on the basis of 600 mg of cell walls per 30 g fresh weight of sections.

[b] Weighted average of the above three fractions.

data indicate that there is essentially no turnover of pectin in 15 hours and, that, therefore, the cold water soluble pectin cannot be a precursor of the wall materials.

In this experiment, six 15 g batches of sections were first incubated for 5 hours in 75 ml of solution 1.2×10^{-4} M in glucose (10^6 cpm/g fresh wt of sections), 3×10^{-4} M in L-methionine and 2.5×10^{-3} M in potassium maleate (pH 4.8).

Two 15 g batches were harvested at the end of 5 hours. These sections constituted the control. The remaining 15 g lots were rinsed with distilled water. Two of these batches were then placed in 75 ml of solution 0.1 M with respect to unlabeled D-glucose, 3×10^{-4} M with respect to L-methionine and 2.5×10^{-3} M with respect to potassium maleate (pH 4.8). The remaining two batches were placed in a similar solution except that the incubate was made 2.8×10^{-5} M with respect to IAA.

Table 13 summarizes the specific activities of the various pectic fractions in experiment 6. The "control" lot is that harvested at the end of the 5-hour pretreatment in glucose-C^{14} in the absence of IAA. The "-IAA" treated sections received an additional 15-hour incubation in an excess of non-radioactive glucose, still in the absence of IAA. The "+IAA" treated sections also received the additional 15-hour incubation in non-radioactive glucose, but in the presence of IAA. The data of Table 13 show that during the 15-hour incubation, both in the presence and in the absence of IAA, the

specific activity of the cold water soluble, 70 per cent alcohol insoluble pectin as well as of the residual pectin, tends to increase while the specific activity of the hot water soluble pectin tends to decrease. This finding, which is somewhat in contrast to that of the previous experiment, will be discussed more fully below.

Table 14 includes data on the AUA content and total radioactivity incorporated into the pectic fractions of experiment 6. These data show that during the 15-hour incubation in 0.1 M glucose there is a 9.3 per cent increase in total AUA in the absence of IAA, and a 14.2 per cent increase in AUA in the presence of IAA. The increase in total AUA due to the presence of IAA is found only in the water soluble fractions; IAA has no influence on the synthesis of residual pectin. Thus the IAA-induced increase in specific activity of the water soluble pectins is actually a measure of an increased rate of pectin synthesis.

During the post-incubation in non-radioactive glucose, there is a substantial increase of total radioactivity in the pectic substances. This increase in radioactivity may be attributed to labeled precursors (including glucose-C^{14}) present in the sections at the end of the control period but not yet incorporated into the polygalacturonic acid chains. The hot water soluble pectin is the only fraction which, after 15-hour incubation, does not exhibit a greater total radioactivity than the control. While IAA does not influence this fraction, it has a marked influence on the radioactivity of

the cold water soluble and residual pectins. IAA causes an increased incorporation of labeled precursors into cold water soluble pectin but a decreased incorporation into the residual pectin during the 15-hour incubation in non-radioactive glucose.

Discussion

IAA causes an increase in the incorporation of glucose into the galacturonic acid residues of the water soluble pectins (Tables 11 and 12). This effect is compared, in the data of Table 15, with the effect of IAA on the incorporation of the methyl group of methionine into the methyl ester of pectin. The data for this table have been compiled from the various experiments of Part II. The striking similarity between the effect of IAA on the incorporation of methyl ester and of galacturonic acid suggests the possibility that IAA may enhance the incorporation of both into the pectic moiety, i.e., simultaneously and as a single unit.

That the effect of IAA is not a measure of accelerated turnover of methylgalacturonate residues but is due to an actual increase in pectin synthesis is shown by the data of Table 16. The values in this table represent the changes in AUA content and in AUA radioactivity during a 15-hour period immediately following a 5-hour incubation in glucose-C^{14}. During this period there is a substantial increase both in total AUA and in its total radioactivity. One may conclude

Table 15

A Comparison of the Influence of IAA on the Incorporation of Glucose-C^{14} into the Galacturonic Acid Residue and the Incorporation of the Methyl of Methionine-$C^{14}H_3$ into the Methyl Ester of Pectin

Pectic Fraction	Ratio of IAA Treated to Control Sections	
	Galacturonic Acid	Methyl Ester
Cold Water Soluble, 70% Alcohol Insoluble	1.7	1.9
Hot Water Soluble	1.4	1.4
Residual	1.1	0.9

Table 16

Change in Content and Radioactivity of AUA during 15-Hour Incubation in 0.1 M Non-radioactive Glucose, ± IAA Following, 5-Hour Pretreatment in 1.2×10^{-4} M Glucose-C^{14}, -IAA (Derived from Table 15)

Pectic Fraction	Treatment	mg of AUA[a]	% of Total	cpm in AUA[a]	% of Total
Total (following three fractions)	-IAA	0.32	100	4,690	100
	+IAA	0.49	100	4,640	100
Cold Water Soluble, 70% Alcohol Insoluble	-IAA	0.055	17	2,280	49
	+IAA	0.145	30	3,780	82
Hot Water Soluble	-IAA	0.102	32	-130	-3
	+IAA	0.166	34	-140	-3
Residual	-IAA	0.17	53	2,530	54
	+IAA	0.18	37	990	21

[a] In excess of control in 100 mg of cell walls.

therefore that there is no major turnover of pectin during the experiment. The increase in total AUA is distributed among the three pectic fractions. An accelerated increase in AUA content due to the presence of IAA is to be found only in the water soluble pectic fractions. The synthesis of residual pectin is not enhanced by IAA.

These conclusions are even more apparent in the case of the distribution of the incremental radioactivity after the 15-hour incubation. In the absence of IAA, the incremental radioactivity is divided equally between the cold water soluble pectin (49%) and the residual pectin (54%). In the presence of IAA, the rate of cold water soluble pectin synthesis is increased so that this fraction incorporates four times (82%) as much radioactivity as the residual pectin (21%).

The pectins of the present experiment appear, then, to possess a considerable reserve of pectic precursors which become labeled during the initial 5-hour incubation in glucose-C^{14}. These precursors are made into the various kinds of pectin during the 15-hour incubation in 0.1 M unlabeled glucose. IAA controls the fate of the labeled precursors directing more into cold water soluble pectin, less into residual pectin.

There is an apparent association between the metabolism of the cold water soluble pectin and the residual wall pectin. The hot water soluble pectin, on the other hand, seems to be unrelated to the other pectic fractions. IAA does enhance

the rate of synthesis of hot water soluble pectin. There is, however, no apparent incorporation of radioactive precursors into this fraction after the sections have been removed from an external source of glucose-C^{14}. This may be the result of a "washing out" of existing precursors or of an actual turnover within the fraction itself. The former seems more likely. IAA enhancement of glucose-C^{14} incorporation (1.4) into this fraction is approximately equal to the IAA enhancement of AUA synthesis (1.6). If IAA caused a greater turnover of the pectin one would expect a larger affect on incorporation of glucose-C^{14} than on AUA synthesis.

It is not surprising to find that the various pectic fractions are not equally and intimately influenced in their metabolism. The material used in these experiments contains both the primary leaf and the coleoptile. Perhaps the hot water soluble pectin, which is 90 per cent esterified, is of different origin from the less esterified fractions. The data of Table 17 represent the distribution of dry weight and of total pectic substance between the coleoptile and leaf.

The fact that the synthesis of both the methyl group and the galacturonic acid residue of pectin is enhanced, and to the same degree, by IAA, supports the hypothesis that methylation of uronic acid residues may occur before polymerization. This conclusion is substantiated by a comparison of specific activities. The specific activity of the cold water soluble pectin is much greater than that of the other pectic

Table 17

Distribution of Dry Weight and of Total Pectic Material Between the Coleoptile and the Primary Leaf of the Avena Sections Used in the Present Experiments

	Per cent of Dry Weight	Per cent of Total AUA
Leaves	42.5	36.3
Coleoptiles	57.5	63.7

fractions both with methionine as the labeling substrate and with glucose. In both cases, also, the specific activity of the hot water soluble pectin is slightly higher than that of the residual pectin. Thus, the methyl group and the uronic acid of the pectic fractions behave similarly both with regard to specific activity and with regard to influence by IAA.

The formation of derivatives of UDP-hexoses has been recognized for some time (93,94). It seems possible that the methylation of galacturonic acid may occur while the uronic acid is attached to UDP. According to this hypothesis, polymerization of galacturonic acid to pectin would then be possible only after esterification of the monomer.

The role of IAA in pectic metabolism is truly a regulatory one. IAA controls the balance between two distinctly different forms of pectin--the cold water soluble on the one hand and the hot water insoluble (residual) on the other. Whether an increased proportion of water soluble pectin in the tissue can or does influence cell wall plasticity has not as yet been demonstrated. What has been demonstrated is that very low concentrations of IAA markedly affect the metabolism of specific wall substances.

Summary

1. Incubation of coleoptile tissue in IAA induces an accelerated incorporation of glucose into the galacturonic acid residues of the water soluble pectins.

2. IAA effects no increase in incorporation of glucose into the galacturonic acid residues of the hot water insoluble (residual) pectin.

3. The influence of IAA on the incorporation of glucose into the galacturonic acid residues is compared to the effect of IAA on the incorporation of methyl into the ester moiety of pectin.

4. IAA causes an accelerated synthesis of the water soluble pectins of Avena coleoptile sections during a 15-hour incubation in 0.1 M glucose.

5. There is, under similar conditions, no IAA induced increase in hot water insoluble pectin content.

6. The metabolic associations of the pectic fractions are considered.

7. Evidence is presented to support the hypothesis that the methyl esters of pectin are formed before polymerization of galacturonic acid residues.

8. The regulatory role of indoleacetic acid is affirmed.

PART III

IN VITRO SYNTHESIS OF PECTIN

Introduction

Further elaboration of the mode of synthesis of pectin requires an in vitro, cell free system. Such a system could help, too, in further studies on the mechanism of auxin action. Cleland (61) has attempted the synthesis of pectin methyl ester groups from methionine-$C^{14}H_3$ in Avena homogenates. Since the galacturonic acid residues are apparently esterified before polymerization, Cleland was actually attempting to measure pectin synthesis. The present section, Part III, describes similar experiments. This section also includes the results of an in vitro experiment in which glucose-C^{14} was introduced as the pectic precursor.

Materials and Methods

Culture of seedlings, preparation of sections and of cell walls, and the method of analysis for radioactivity were similar to those used in Part II-A. The procedures for hydrolysis of pectin and isolation of galacturonic acid were similar to those used in Part II-B.

Preparation of homogenates.--Sections were homogenized at 3°C with the aid of a glass mortar and pestle. A minimum amount of distilled water or buffer was used to facilitate homogenization. Cell walls were removed by a two step process. The homogenate was first pressed through orlon fabric and the

resultant filtrate then centrifuged at 500 g for 15 min at 3°C (Servall refrigerated centrifuge). The supernatant was decanted and used as cell wall free homogenate.

Analysis of pectic substance.--The galacturonic acid isolated from the pectin of the glucose-C^{14} experiment was measured in a manner different from the carbazole method used in Part II. Aliquots of both standard and unknown galacturonic acid solutions were placed on Spinco No. 300-028 electrophoretic filter paper strips. The strips were dipped in the CD-1 solution of Gordon et al. (92), air dried for 5 min, and finally heated for 5 min at 90°C. The densities of the resultant purple spots were estimated by use of the Spinco Model R Analytrol. The concentrations of the standard solutions were found to be proportional to the values obtained. It is possible to measure, within 10% accuracy, 1-2 γ of galacturonic acid.

Radioactive compounds.--Methionine methyl-C^{14} with a specific activity of 4.52 mC/mM and D-glucose, uniformly labeled with C^{14}, possessing a specific activity of 3 mC/mM were the radioactive isotopes used in these experiments. Both compounds were obtained from Isotope Specialties Co., Inc., Burbank, California.

Results

In vitro incorporation of the methyl of methionine-$C^{14}H_3$ into the methyl ester of pectin.--The methyl of methionine-$C^{14}H_3$ is rapidly incorporated into the methyl ester of pectin by whole coleoptile sections. Homogenates of such sections have not been induced to carry out this reaction. Liberation from the vacuoles of salts during homogenation as well as the buffer used in the incubation mixture may cause elution of pectinesterase (PE) from the cell wall material (31). This enzyme, once liberated, could hydrolyze the pectic esters formed during incubation. To circumvent this possible trouble the sections were homogenized in distilled water and the cell walls then quickly removed. The reaction mixtures in these experiments contain only the water soluble pectins.

Incubation solutions for "control" homogenates were made 7×10^{-3} M in potassium phosphate buffer (pH 6.5) and 2.4×10^{-4} M in methionine-$C^{14}H_3$ (10^6 cpm per g fresh weight). Other treatments were made 2.8×10^{-5} M with respect to IAA, and/or 0.7×10^{-4} M with respect to adenosine triphosphate (ATP) and 1.4×10^{-4} M with respect to magnesium chloride. Still another treatment contained all of the above and in addition UDP glucuronic acid, 2.8×10^{-4} M. All treatments contained the equivalent of five grams of sections in a total of 15 ml of solution.

The reaction mixtures were placed in 40 ml conical glass

centrifuge tubes and agitated in a Dubnoff Shaker for 2 hours at 30°C in weak orange light. Reaction was stopped by boiling followed by concentration at 60-70°C. The concentrates were made 70 per cent in alcohol and left overnight at -20°C. The 70 per cent alcohol insoluble material was collected by centrifugation and washed once with 80 per cent alcohol at 3°C in an attempt to rid the material of unreacted methionine. There remained in all cases considerable non-saponifiable radioactivity. The data of Table 18 concern the per cent saponification of the activity of the 70 per cent alcohol insoluble material. The proportion of saponifiable radioactivity is not only low but not reproducible.

In vitro incorporation of glucose-C^{14} into galacturonic acid residues of pectin.--Glucose is incorporated by Avena homogenates into galacturonic acid residues of water soluble, 70 per cent alcohol insoluble, pectin, both in the presence and in the absence of the cell walls. The data of Table 19 give the specific activities of the various fractions.

For this experiment, 30 g of sections were homogenized at 3°C in 10^{-2} M potassium phosphate, pH 6.5. The cell walls were removed from one half of the homogenate. Each 15 g equivalent was incubated in 40 ml of a solution 10^{-2} M with respect to the buffer, 3×10^{-4} M with respect to L-methionine, and 7×10^{-4} M with respect to glucose-C^{14} (10^{6} cpm per g fresh weight of sections). The samples were incubated for

Table 18

Per Cent Saponification in Various In Vitro Experiments Using Methionine-$C^{14}H_3$ as the Methyl Donor

Treatment	Expt. 1	Expt. 2	Expt. 3
Boiled	--	0.6	-2.6
Control	2.3	-3.1	--
+IAA	--	1.9	--
+ATP, $MgCl_2$	--	0.0	--
+IAA, ATP, $MgCl_2$	--	-4.5	--
+UDPGluA, IAA, ATP, $MgCl_2$	5.4	--	-3.0

Table 19

Specific Activity of Galacturonic Acid Isolated from the Indicated Pectic Fractions Following a 2 Hour *In Vitro* Incubation in the Presence of Glucose-C^{14}

Pectic Fraction	γ AUA Isolated	cpm	cpm per mg of AUA
Cold Water Soluble (incubated without cell walls)	10	26	2600
Cold Water Soluble (incubated with cell walls)	52	179	3400
Hot Water Soluble	278	24	<100
Residual	166	13	<100

2 hours at 30°C in weak red light while being agitated by a Dubnoff Shaker.

Isolation of cell walls and of galacturonic acid therefrom was by the standard procedure. After chromatography in three solvents the galacturonic acid from the cold water soluble fractions was rechromatographed in the ethylacetate, acetic acid, water solvent to assure purity.

Although the cold water soluble pectins incorporated glucose carbon into their galacturonic acid residues there was essentially no incorporation of glucose into the wall pectins.

Discussion

A successful *in vitro* incorporation of the methyl of methionine into the methyl ester of pectin has not been achieved. The failure to find such a reaction may in part be due to the presence of pectic enzymes in the incubate. Before further such experiments are carried out it would be desirable to study the fate of the water soluble pectins during the incubation period. Do the ester groups remain intact or are they hydrolyzed by PE? Are the polygalacturonic acid chains broken by the action of glycosidases?

The same questions apply to studies of the incorporation of glucose into the galacturonic acid residues. This assay, which is more sensitive than that used for methyl incorporation

indicates that some *in vitro* pectin synthesis does take place.

A much improved system for the *in vitro* synthesis of pectin is necessary for the study of auxin action.

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