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The influence of 2,4-dichlorophenoxyacetic acid on the phosphorus metabolism of cranberry bean plants (Phaseolus vulgaris). Plant Physiol. 29: 490–491. 1954.

PECTIC METABOLISM OF GROWING CELL WALLS [1,2]

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INTRODUCTION

It is established that auxin, indoleacetic acid, increases the rate of cell elongation in *Avena coleoptile* by causing the cell walls to become more easily stretched, more plastic. This was first demonstrated by Heyn (16), who showed that coleoptiles treated with auxin were more readily stretched by an applied weight than coleoptiles not so treated. Heyn's observation has been confirmed and extended by Tagawa and Bonner (33).

Osmotic analysis of coleoptile growth (22, 27) also leads to the conclusion that auxin affects cell wall properties. 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 (13). In a 2-stage experi-

ment, auxin was allowed to act upon sections which were prevented from expanding by immersion in a solution isosmotic with the cell contents. The sections were then transferred to water under anaerobic conditions in which auxin is ineffective. Sections pretreated in auxin expanded more in the second anaerobic stage in water than did untreated sections. The effect of auxin in increasing deformability of the coleoptile under external mechanical load also has been demonstrated with sections incubated under isosmotic conditions (12). Thus auxin affects cell wall plasticity 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 participation of metabolism, since, as indicated above, auxin works neither under anaerobic conditions (3) nor in the presence of inhibitors such as HCN. Some aspect of the auxin effect occurs in the cytoplasm, since plasmolyzed cells, in which wall and cytoplasm are not in contact, do not exhibit wall softening under the influence of auxin (12).

It is debatable whether the net synthesis of cell wall components is increased or not in the presence of auxin. It is clear that coleoptile cell wall synthesis normally keeps pace with cell expansion in growing seedlings of oat (4) and corn (38). The same is

true of excised oat coleoptile sections growing at 25° C. (4) ; of pea epicotyl sections (10) ; of excised tobacco pith in tissue culture (37), and of excised potato tuber discs (9, 36). All 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 substances. 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 4° C., although auxin-induced growth proceeds at this temperature (4). In wheat roots, also, auxin-induced changes in growth rate are not paralleled by changes in rate of cell wall synthesis (8). 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 for brief (3 hr) time periods (7, 28, 29, 30). In general, such incorporation is unaffected or even slightly decreased by the presence of auxin.

The growing primary cell wall consists of a mixture of polysaccharide and polyuronide components which are in general poorly defined. Earlier determinations of coleoptile cell wall composition by Thimann and Bonner (34), Wirth (38), and Nakamura and Hess (26) agree in suggesting that a-cellulose makes up 25 to 40 % of the cell wall dry weight, pectic materials 8 to 12 %, and waxes, probably located in the cuticle, 10 to 20 %. The rest of the wall material is non-cellulosic polysaccharides. These are, at best, rough estimates based solely on solubility properties of the wall materials.

Thus far, chemical changes associated with auxin action have been found only in pectic fraction of the oat coleoptile wall. The methyl ester group of pectin is known to be derived, in part at least, from the methyl group of methionine (29, 32). Rate of incorporation of the methyl carbon of methionine into the hot-water-soluble pectin of the cell wall is increased in the presence of auxin (29, 30). This effect of auxin consists of one which appears rapidly and is inhibited by antiauxins, high auxin concentrations, and ethionine. In all of these respects, the response is similar to the influence of auxin on cell wall plasticity.

The purpose of the present paper is to study in quantitative detail the auxin-induced chemical changes in pectic and other components of the cell walls of oat coleoptiles. It will be shown that auxin increases rate of formation of the methyl groups of cell wall pectin without detectably changing the degree to which the pectin is esterified. Auxin treatment of coleoptiles does not, however, appear to bring about major changes in total content of pectic material such as have been reported for tobacco pith (37) and for potato tuber tissue (9). It will also be shown that auxin increases the rate of formation (or turnover) of the methyl ester groups of the pectic material not directly associated with the cell wall to a much greater extent than that of the cell wall.

MATERIALS AND METHODS

CULTURE OF SEEDLINGS: Oat seeds of the variety Siegeshafer were germinated in vermiculite moistened with distilled water, and held 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 AND CELL WALLS THEREFROM: 12 mm sections were cut from each coleoptile, the apical 3 mm tip being discarded. Primary leaves were not removed. Sections were ground in an Omnimixer[4] at 0° C with four times their weight of 0.15 N acetate buffer, pH 4.4. Previous work (18) has shown that at this pH no deesterification of pectin by pectinesterase (PE) takes place during homogenization. The homogenate was next centrifuged, the supernatant liquid decanted, and the pellet washed by resuspension in water at 0° C. The cell wall fraction was washed in this way five times with water, followed by several washes in acetone. Finally, acetone washed cell wall material was dried in vacuo and stored for analysis.

The supernatant liquid in each case was combined with that from the first water washing and used for preparation of the cold-water-soluble, 70 % alcohol-precipitable fraction of *Avena coleoptile* sections (see below).

METHODS OF ANALYSES: Pectic substance was measured by determining the uronic acid content using the pectinase-carbazole method of McCready and McComb (24) and McComb and McCready (25), expressing the pectic substance values as anhydrouronic acid (AUA). This method has been calibrated with the 12 % HCL-decarboxylation method.

For determining methanol (methyl ester) content of cell wall preparations, 100 mg samples were saponified with 4 ml of 1 N NaOH for 30 minutes at room temperature, and then 4.25 ml of 1 N HCl were added. Solutions of pectic substances were similarly saponified. The methanol so formed was determined by the method of Boos (6). In this determination, the solution containing methyl alcohol is distilled, the distillate collected and diluted to volume. The methanol distillate is oxidized to formaldehyde, and the formaldehyde measured colorimetrically with chromotropic acid.

For separate determination of formaldehyde in the distillate, the chromotropic acid colorimetric method was used, but with the oxidation step omitted.

The analyses of total alkyl groups were done by the Clark method (11). This method determines all labile simple alkyl groups such as methyl, ethyl, isopropyl, etc., including those attached to oxygen, nitrogen, or sulfur.

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[4] Mention of specific products does not imply endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

Radioactivity of samples in solution was determined first by drying them on planchets, and then making counts under a micromil window tube in an atmosphere of Q gas. Radioactivity of whole cell walls was determined by the wet combusion method using the Van Slyke-Folch reagent (35), and counting the CO_2 formed in the gas phase (1). Aqueous samples were analyzed by a persulfate wet combustion technique (20).

NOMENCLATURE OF PECTIC SUBSTANCES: Pectic substances occur in plant tissue in three forms which are distinguished on the basis of solubility (5). These are:

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

2) Pectic substances associated with the cell wall 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 that pectin may differ from protopectin in possessing a shorter chain length (15). Owens et al (31) showed, however, that the hot-water-insoluble cell wall pectic material in some plant tissues is completely soluble in hot solutions of sequestering agents. The residual pectic fraction and the soluble pectin fraction may or may not differ in chain length, but as will be shown, they do differ in degree of methyl esterification.

RESULTS

COMPOSITION OF AVENA COLEOPTILE TISSUE AND CELL WALLS: Whole coleoptiles and cell walls prepared therefrom were analyzed for their content of pectic substances (AUA) and the degree to which these are esterified. Typical results are given in table I.

TABLE I

COMPOSITION OF WHOLE AVENA COLEOPTILE TISSUE AND OF AVENA COLEOPTILE CELL WALLS

CON- STITUENT	WHOLE AVENA COLEOPTILES	CELL WALLS	% OF CONSTITUENT OF WHOLE COLEOPTILES RECOVERED IN CELL WALLS
	%[1]	%[1]	
AUA	0.76	5.1[2]	91
CH_3OH (by alkali)	0.125	0.40[2]	42
CH_3OH (by PE)	...	0.37	...
Alkyl groups	...	0.85	...
Formaldehyde	0.025	0.013	50

[1] On a dry weight basis.

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

The AUA content of the wall fraction was 5.1 %. As will be seen below, cell wall preparations from many different lots of coleoptile sections possessed AUA contents of slightly more than 5 %. Comparison of this value with that for the whole coleoptile indicates that 91 % of the tissue AUA accompanies the cell wall fraction.

The methyl ester content of the cell wall, 0.40 %, corresponds to an average degree of esterification of the pectic carboxyl groups of 43 %. Coleoptile tissue contains non-cell-wall methyl ester groups; in fact, only 42 % of the methyl ester content of tissue is localized in the wall.

That the methyl ester of the cell wall is in fact present as pectin is shown by hydrolysis of cell wall suspensions 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 lyophilized orange PE preparation (23)) and 1 mg of merthiolate. After two to three days of incubation at room temperature, the suspension was filtered, 5 ml of the filtrate distilled, and methanol determined in the distillate. Only 90 % 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 (19). In these cases, PE fails to hydrolyze 0.5 % of the methyl ester of the pectins. This corresponds to 90 % hydrolysis of low ester pectins and to 95 % hydrolysis of high ester pectins.

Cell walls contain a considerable amount of labile alkyl groups other than methyl ester, as shown by total alkyl group analysis. This method determines any alkyl group on O, N, or S, provided only that the alkyl iodide is relatively volatile. Nothing is now known in these laboratories concerning the nature of these other alkyl groups.

Small but significant amounts of formaldehyde are found in coleoptile tissue. In cell walls (and presumably in the whole coleoptile), the formaldehyde is not present in the free state but can be liberated either by acid hydrolysis (1 N HCl for 30 minutes) or by alkaline hydrolysis (in NaOH for 30 minutes) followed by acidification prior to distillation. Distillates from unhydrolyzed cell walls (water suspensions) contained no detectable formaldehyde. Approximately 50 % of the total tissue formaldehyde is found in the cell wall.

Since the AUA content of coleoptile cell walls is low (5.1 %), a preliminary exploration was made of the nature of the remaining cell wall constituents. Cellulose is known to constitute about 25 to 40 % of Avena coleoptile walls (2, 26, 34, 38). Therefore, approximately 55 to 70 % of the wall is of unknown nature. 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 % disodium ethylenediamine tetraacetic acid was hydrolyzed with commercial pectinase until analyses showed that all of the AUA had passed into solution. The suspension was centrifuged, the

supernatant liquid decanted, and the pellet washed by suspension in water, and again centrifuged. The supernatant and washings were combined and evaporated in vacuum 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.

As indicated by paper chromatography using butanol-acetic acid-water solvent, the effluent contained galactose, glucose, xylose, arabinose, and possibly ribose. 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. These observations, which are by no means complete, illustrate the complexity of the cell wall.

CATION EXCHANGE CAPACITY: The calcium ion-induced inhibition of coleoptile growth is mediated by exchangeable bound ions (14). The same is true of the calcium-induced stiffening of coleoptiles as measured by resistance of tissue to mechanical deformation (33). It is therefore important to determine what component of the tissue binds ions in such a way as to determine growth rate and mechanical properties.

The cation exchange capacity of the *Avena coleoptile* cell wall preparation was determined as follows. Water suspensions of cell walls were mixed with a series of CaCl₂ solutions so as to give final concentrations between 0 and 10 meq/l. After 1 hour of equilibration, the solution was removed by filtration, the residue washed with cold water to remove any un-

bound material, and the residue then dried. The dried samples were analyzed for calcium content by flame spectrophotometry.

Essentially complete saturation of the exchange capacity was obtained with a Ca^{++} concentration of 10 meq/l. At this concentration the cell wall preparation bound 0.18 meq per g dry weight (table II). That the exchange capacity of coleoptile cell walls can be accounted for by their content of non-esterified pectic carboxyl groups is shown by the second entry in table II. This is calculated from the data of table I, by use of the AUA content, and the fact that the methyl ester/AUA ratio is 0.43. The cation exchange capacity of fresh living coleoptile sections was determined by Cooil and Bonner (14) using Ca^{45}. The cation binding sites of the intact tissue, like those of the cell wall, were essentially saturated at a Ca^{++} concentration of 10 meq/l. The third and fourth items of table II are, respectively, the measured cation exchange capacity of intact coleoptile sections and that which would be expected if all exchange capacity of the living tissue were due to the cell walls. The fifth item is that expected if all exchange capacity were due to the free pectic carboxyl groups of the cell walls. It is clear that the exchange capacity of the cell walls can account quantitatively for the exchange capacity of the living tissue. It is also evident that the exchange capacity of the living tissue can be quantitatively accounted for by the number of free, non-esterified, pectic carboxyl groups which its cell walls contain. This conclusion has also been reached for roots (killed, however) by Keller and Deuel (21).

METHYL ESTER CONTENT OF CELL WALL PECTIC SUBSTANCES AFTER INCUBATION WITH AND WITHOUT INDOLEACETIC ACID (IAA): The effect of IAA incubation on the incorporation of the methyl carbon of methionine-$C^{14}H_3$ into cell walls and the methyl ester content thereof were studied. Sections were floated in 100 ml of buffered solution (0.0025 M potassium maleate at pH 4.8) containing 2 mg catalase and methionine-$C^{14}H_3$ at 25° C. In the case of sections incubated in the presence of IAA, its concentration was 2.8×10^{-5} M. In the first experiment, methionine-$C^{14}H_3$ of activity 0.85 mc/mM was used, and in the second two experiments material of specific activity 3.9 mc/mM. In the presence or absence of IAA, incubation was for 5 hours. Cell wall samples were prepared after sections were washed thoroughly with water.

IAA caused an increase in total incorporation of radioactive carbon into the cell walls (table III) and this increase varied considerably between the three experiments. No detectable net change in methyl ester content resulted, however, from the IAA treatment. The molar ratio of methyl ester to pectic substance also was unaffected by the presence of IAA. Evidently the elongation of sections under the influence of IAA is not associated with any detectable change in ester content of the pectic substance.

TABLE II

CATION EXCHANGE CAPACITY OF COLEOPTILE SECTIONS AND OF COLEOPTILE CELL WALLS

MATERIAL	EXCHANGE CAPACITY
1. Cation exchange capacity of cell walls [1]	0.18 meq/gm dry wt
2. Cation exchange capacity of cell walls calc. on basis of free pectic carboxyl groups	0.165 meq/gm dry wt
3. Cation exchange capacity of fresh sections [2]	3.14×10^{-3} meq/gm fr. wt
4. Cation exchange capacity of fresh sections calc. from item 1 (1 gm sections = 20 mg cell wall)	3.6×10^{-3} meq/gm fr. wt
5. Cation exchange capacity of fresh sections calc. from item 2	3.3×10^{-3} meq/gm fr. wt

[1] Calcium determinations were made by Dr. H. C. Lukens, Western Utilization Research and Development Division, Agricultural Research Service, U.S.D.A., Albany, California.
[2] From Cooil and Bonner (14)

TABLE III

COMPOSITION OF CELL WALLS FROM SECTIONS INCUBATED[1] WITH METHIONINE-$C^{14}H_3$
AND IN PRESENCE OR ABSENCE OF IAA

| | EXPERIMENT 1[2] | | EXPERIMENT 2[3] | | EXPERIMENT 3[3] | |
	CONTROL	IAA	CONTROL	IAA	CONTROL	IAA
Radioactivity, 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

[1] Incubation time, 5 hrs.
[2] 1.3 μc/g section (0.235 mg methionine possessing 0.85 mc/mM).
[3] 2.7 μc/g section (0.105 mg methionine possessing 3.9 mc/mM).

ACCELERATED METHYL ESTER FORMATION INDUCED BY IAA: Although IAA does not cause any analytically detectable net change in the ester content of coleoptile pectic substance, it does cause an increased rate of formation (or turnover) of such ester groups. This conclusion is based upon isolation of the methanol obtained by saponification of cell walls from sections incubated in methionine-$C^{14}H_3$ and in the presence or absence of IAA. The dimedone derivative was prepared from the formaldehyde formed by oxidation of the methanol, and the radioactivity was then counted.

Cell walls from experiments 2 and 3 were used. One hundred mg samples were saponified for 30 minutes at room temperature with 4 ml of 1 N NaOH; then 4.25 ml of 1 N HCl were added to each, so as to minimize foaming, and the methanol distilled. Distillation was continued until half of the initial volume had been collected. To each distillate was added 1 ml of an aqueous solution containing 3,950 μg of reagent grade methanol. The distillates were oxidized by adding 15 drops of 5 % phosphoric acid and 25 drops of 5 % potassium permanganate. After 20 minutes, the excess permanganate was discharged by dropwise addition of 5 % sodium thiosulfate, and the pH of the reaction mixture was adjusted to 5.3 with 2 N NaOH. 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 % dimedone solution. The reaction mixtures were allowed to stand one day at room temperature and one day at 4° C; then the crystalline dimedone derivative of formaldehyde was filtered, dried, and dissolved in 3.0 ml of ethanol. Aliquots of the ethanol solutions were dried on planchets and the radioactivity counted. The dimedone derivatives were recrystallized by adding 20 ml of water to the remainder of the ethanol solutions. Yields of 5 and 8 mg from experiments 2 and 3, respectively, were obtained; all samples had melting points of 189° to 190° C. The 5 mg samples were dissolved in exactly 2 ml of ethanol and the 8 mg samples 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 IV. It is apparent that the specific activities of the material from IAA treated cell walls are greater than those from the controls. The ratio of specific activities, IAA/control, was 1.4 and 1.7 in the two experiments. Accordingly, even though IAA does not cause a detectable net change in methyl ester content of pec-

TABLE IV

SPECIFIC RADIOACTIVITY OF FORMALDEHYDE-DIMEDONE COMPOUND PREPARED FROM
METHYL ESTER OF CELL WALLS FROM SECTIONS INCUBATED IN
PRESENCE OR ABSENCE OF IAA

EXPERIMENT NO.	ACTIVITY OF DILUTED DIMEDONE DERIVATIVE CPM/MG	DILUTION FACTOR	ACTIVITY ON UNDILUTED BASIS CPM/MG	SPECIFIC ACTIVITY[1] μC/MM	RATIO IAA/CONTROL
2 Control	6,620	10.2	67,700	22	
IAA	8,280	11.4	94,400	31	1.4
3 Control	3,615	12.0	43,300	14	
IAA	5,910	12.3	72,700	24	1.7

[1] Calculated on basis of 40 % efficiency gas flow, thin window Geiger counter.

TABLE V

COMPOSITION OF HOT-H₂O-SOLUBLE FRACTION OF CELL WALLS

| EXPERI-MENT NO. | SAMPLE | CELL WALL MATERIAL SOLUBILIZED, % | | | MOLAR RATIO CH₃OH/AUA | AUA IN EXTRACTED MATERIAL %[1] | RADIOACTIVITY OF EXTRACT | | RATIO OF SPECIFIC ACTIVITIES, IAA/CONTROL |
		DRY WEIGHT	AUA	CH₃OH			CPM/MG DRY WT	CPM/MG 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	86,800	
	IAA	3.3	16	35	0.8	25	31,200	124,000	1.4

[1] On a dry weight basis.

tic substances, it does cause an accelerated formation (or turnover) of the ester group. The fact that the dimedone derivatives possessed specific activities of approximately 0.5 % that of the methionine-C¹⁴H₃ supplied suggests that there is a fairly direct pathway for the transfer of methyl from methionine to the methyl ester of the pectic substances.

The data of table IV apply to the cell walls as a whole. Conclusions as to IAA-induced increase in rate of methyl ester formation (or turnover) apply also to the hot-water-soluble (classical pectin) fraction of the wall. It is in this fraction that Ordin, Cleland, and Bonner (29, 30) showed an auxin-induced increased rate of incorporation of labeled carbon derived from methionine-C¹⁴H₃. Data presented below confirm earlier observations and show in addition that either methyl ester formation or turnover is involved.

Hot water extracts of the cell wall preparations from the three experiments (table III) were prepared by adding 7 ml of water to 150 mg of cell wall preparation. Each was heated 1 hour in a boiling water bath, cooled, centrifuged, and the supernatant (ca. 3 ml) removed. The extractions were repeated for an additional 30 minutes with another 6 ml of water. The material was again cooled, centrifuged, and the supernatants 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 and once in acetone, then dried in vacuo.

The hot-water-soluble fraction amounts to ca. 3.5 % of the total weight of the cell wall (table V). The soluble portion contains, however, 15 % of the total cell wall AUA and an even larger fraction, 35 %, 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 VI). 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, only 21 to 27 % of the fraction can be accounted for as AUA (table V), the balance yielding principally xylose and arabinose after hydrolysis (see above).

Specific activities of the hot-water-soluble fractions of the cell walls are given in table V. 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 are in agreement with the ratios for the dimedone derivatives representing the total cell wall methyl ester (table IV).

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

TABLE VI

ANALYSIS OF HOT-WATER-INSOLUBLE RESIDUES OF CELL WALLS FROM SECTIONS INCUBATED WITH AND WITHOUT IAA IN PRESENCE OF METHIONINE-C¹⁴H₃

| | EXPERIMENT 1 | | EXPERIMENT 2 | | EXPERIMENT 3 | |
	CONTROL	IAA	CONTROL	IAA	CONTROL	IAA
Radioactivity, cpm/mg C	6,200	6,300	23,600	23,900	23,300	25,100
AUA, %	4.0	4.0	4.9	4.3	4.2	4.1
CH₃OH, %	0.24	0.22	0.24	0.22	0.20	0.17
Molar ratio, CH₃OH/AUA	0.33	0.30	0.27	0.28	0.26	0.23

solutions had stood at room temperature for 90 minutes, they were heated at 100° C for 60 minutes. Saponification was complete since repetition of the above procedure failed to decrease further the specific radioactivity of the residue. The losses of radioactivity from saponification of hot-water extracts of cell walls were 85 % for the controls and 89 % for IAA-treated sections from experiment 2, and 91 and 94 %, respectively, from experiment 3. The activity of the hot-water-soluble portion resides, therefore, principally in saponifiable material, presumably methyl ester. In the case of the hot-water-soluble pectin, an accelerated incorporation of methionine methyl into methyl ester results from IAA treatment, just as in the case of the cell wall pectic material as a whole.

Analysis of the cell wall residues after hot water extraction shows that approximately 80 % of the pectic substance remains in the cell walls (table VI). This is in good agreement with analysis of the hot water extracts. The degree of esterification of the residual pectic substance is only about 30 %. It is this residual pectic fraction which would be known classically as protopectin. It is apparent, however, that the material possesses a low degree of esterification and differs from soluble pectin in this respect.

Treatment with IAA does not cause any significant acceleration in incorporation of methionine methyl into the ester of pectic substances which remain in the cell walls after hot-water extraction.

This was determined on cell walls of experiment 3 after saponification in the following manner. To 65 mg each of hot-water-extracted cell walls from control and IAA-treated sections were added 4 ml of 1 N NaOH. Saponification was allowed to proceed for one-half hour at 25° C; then 4.25 ml of 1 N HCl were added and the liberated methanol removed by distillation. The saponification mixtures were filtered, the residues washed several times with water, and dried first with acetone and then in a vacuum desiccator. Filtrates were combined with the water washings and diluted to 25 ml.

The loss of radioactivity upon saponification was

42 % and 38 %, respectively, for the control and the 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 a ratio, IAA/control, of 0.9. If all the radioactivity lost upon saponification were due to ester (a very reasonable assumption as will be shown later), then IAA had little or no effect on incorporation of methyl ester in the pectic substances of this fraction. It is interesting to note that the alkali treatment solubilized (but did not make volatile) the major portion of the non-saponifiable radioactive material of the cell walls (table VII).

ABSENCE OF IAA EFFECT ON CELL WALL ACETYLATION: The methyl groups of methionine-$C^{14}H_3$ are not transformed to acetyl or similar acyl groups of the cell walls. Further, IAA does not cause any net change in the unlabeled acyl group. This was demonstrated with cell walls of experiment 3 in the following manner. To 150 mg each of cell wall preparation from control and IAA-treated sections were added 25 ml of 1 N HCl. The materials were sealed in glass tubes and heated in a steam bath for 1 hour. The tubes were cooled and opened. The residual cell material was removed by filtration and the filtrates were steam distilled to yield 50 ml of distillates. Each distillate required 0.17 meq of NaOH for neutralization. Apparently IAA caused no net change in the acetyl (or similar volatile acyl group) content of cell walls, and the effect of IAA in promoting cell elongation is probably not due to any change in degree of acetylation of pectic substances.

The neutralized steam distillates were evaporated in vacuum to dryness. The residues were dissolved in 5 ml of water and 100 μl aliquots were dried and counted. Only 70 cpm were observed on the residues from either solution. The total radioactivities of the distillates were less than 1 % of those solubilized by the respective acid hydrolysis determined in the same manner. Little if any of the methyl of methionine-$C^{14}H_3$ had been incorporated into acetyl groups of cell walls.

TABLE VII

LOSS IN RADIOACTIVITY ON SAPONIFICATION OF HOT-WATER-INSOLUBLE RESIDUES OF CELL WALLS FROM SECTIONS INCUBATED WITH AND WITHOUT IAA IN PRESENCE OF METHIONINE-$C^{14}H_3$[1]

IAA	MATERIAL	RADIOACTIVITY, cpm/mg C	TOTAL C, MG	TOTAL COUNTS	% LOSS ON SAPONIFICATION
—	Hot-water-insoluble residue	23,300	23.4	545,000	
—	Saponification supernatant[2]	22,000	13.3	293,000	
—	Saponification residue[3]	1,700	12.2	20,700	42
+	Hot-water-insoluble residue	25,100	21.5	540,000	
+	Saponification supernatant[2]	31,000	10.0	310,000	
+	Saponification residue[3]	2,100	12.3	25,700	38

[1] Experiment 3.
[2] Material solubilized by saponification procedure.
[3] Cell wall fraction was not solubilized by saponification procedure.

EFFECT OF IAA ON METHYL ESTER OF COLD-WATER-SOLUBLE, 70 % ALCOHOL PRECIPITABLE FRACTION OF AVENA COLEOPTILE SECTIONS: To prepare cell walls, the tissue was first ground in acetate buffer and the cell wall material centrifuged. Some pectic material remains, however, in the supernatant liquid from centrifugation. This is the cold-water-soluble pectin. It was prepared as follows: the supernatant liquid in each case was combined with the first water washing of the cell wall material. This was heated to 90° C to denature the protein, filtered through paper, and concentrated in vacuo to a volume of 20 ml. Sixty ml of 95 % ethanol were then added. After several days at 4° C, the resultant precipitate was centrifuged, washed with acetone, and dried in vacuo. From each 30 g of original plant tissue, 35 to 40 mg of alcohol-precipitable material was obtained.

The cold-water-soluble, 70 % alcohol insoluble fraction was prepared as described above for all experiments. The dried material in each case was resuspended in 10 ml of water. In experiment 1, 66 % of the material proved to be soluble, in experiments 2 and 3, 75 %. Data from the analyses of the resultant solutions are given in table VIII. The concentration of pectic substance present (3.2 to 4.9 %) was

low, and the limited amount of material available 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 about one-fifth as much pectic substance. For comparison, specific activities are calculated on an AUA basis. With the exception of experiment 1, in which methionine of lower activity was used, the ratios of specific activities, IAA/control, are even higher than the corresponding ratios for the hot-water-soluble material (table V). Therefore, IAA treatment apparently causes a greater acceleration in methyl ester incorporation in this particular fraction than in the cell walls themselves.

The cold-water-soluble, alcohol precipitable fractions were next saponified, as described earlier, and aliquots of the dried residues counted. The IAA-induced increase in specific activity of this fraction is, for the most part, due to an increase in amount of a saponifiable, volatile material, presumably methyl ester (table VIII). The residues contain radioactivity which did not become volatile as a result of saponification. In experiments 2 and 3 (where more highly

TABLE VIII

CHARACTERISTICS OF COLD-WATER-SOLUBLE PECTIC SUBSTANCES FROM SECTIONS INCUBATED IN PRESENCE OF METHIONINE-$C^{14}H_3$, WITH AND WITHOUT IAA

	EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3	
	CONTROL	IAA	CONTROL	IAA	CONTROL	IAA
AUA, %[1]	4.3	4.9	3.5	3.2	2.4	3.0
CH_3OH, %[1]	~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[2] (AUA basis)	...	1.2	...	1.7	...	1.7
Loss of radioactivity on saponification, %	66	74	52	67	55	65
Specific activities of volatile material resulting from saponification (AUA basis)	115,000	161,000	161,000	357,000	183,000	362,000
Ratio of specific activities of volatile material resulting from saponification[2] (AUA basis)	...	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[2] (AUA basis)	...	1.0	...	1.2	...	1.3

[1] Percentages are on basis of dry weights of soluble material.
[2] Ratios are IAA/control.

radioactive methionine-$C^{14}H_3$ was employed), IAA induced an increase in this residual radioactive material. The nature of this material, presumably a nonsaponifiable methyl group attached to certain residues, is at present unknown.

To verify that the saponifiable material is methyl ester, the methanol resulting from saponification was distilled, diluted with reagent grade methanol, oxidized to formaldehyde, the dimedone derivative thereof prepared, and the radioactivity determined. The cold-water-soluble pectin fractions from experiment 3, both from the control sections and from sections incubated with IAA, were tested.

The following method of preparation was used. Solutions (3 ml) 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 to give 3 ml of distillate. Methanol solution (1 ml containing 3,960 μg of CH_3OH) was added, the mixture was oxidized, and the dimedone derivative of the formaldehyde prepared. Yields of approximately 9 mg of the twice-crystallized dimedone derivatives were obtained in each case. The second crystallization failed to alter the specific radioactivity of the derivatives. Preparation of the derivatives was repeated with solutions containing potentially 38 μg of methanol.

The ratio of specific activities, IAA/control, was 2.3 (table IX). This value is in good agreement with that obtained for the specific activities (table VIII) of the volatile material resulting from saponification of the same preparations. The specific activity of the dimedone derivative from the control sections was 2 % and that from the IAA-incubated 4 % of that of the methionine supplied. Thus the pathway of transfer of the methyl group from methionine to the cold-water-soluble pectic methyl ester appears to be even more direct than the transfer to the ester of the cell walls.

DISCUSSION

Avena coleoptile cell walls studied in this work contain an average of 5.3 % pectic material, meas-

TABLE X

DISTRIBUTION OF PECTIC SUBSTANCES IN AVENA COLEOPTILE TISSUE

	AUA IN FRACTION %[1]	TOTAL % AUA	DEGREE OF ESTERIFI- CATION %
Cell walls	5.3	91	40
Hot-water-soluble fraction of cell walls	23	14[2]	90
Residue after hot water extraction	4.3	78	31
Cold water soluble, 70 % ethanol insoluble	4.0	~5	~50

[1] On dry weight basis.
[2] This corresponds to 15 % of cell wall AUA.

ured as AUA. That AUA, as determined by the pectinase-carbazole method, actually is galacturonic rather than some other uronic acid has been shown by paper chromatography of the acidic fraction of the cell wall hydrolyzate. The distribution of *Avena coleoptile* cell wall pectic material among fractions of different solubility properties, as well as the degree of methyl esterification of each fraction, is summarized in table X. Although over 90 % of the AUA is associated with the cell walls, a small portion definitely is not, but is recovered from the original tissue homogenate as cold-water-soluble, 70 % alcohol-insoluble material. It is possible that this material is actually a cell wall constituent, liberated by the homogenization and washing procedure. In any case, it may be a precursor of cell wall pectin as is suggested by the 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 almost completely esterified. The residual hot-water-insoluble pectin, on the contrary, is methyl esterified only to the extent of ca. 30 %. The material is, apparently, held in the wall as the water-insoluble salt of inorganic ions such as calcium. This idea is supported by the observations that (a) cell walls of *Avena coleoptiles* contain bound calcium and (b)

TABLE IX

SPECIFIC RADIOACTIVITY OF FORMALDEHYDE-DIMEDONE COMPOUND PREPARED FROM METHYL ESTER OF COLD-WATER-SOLUBLE PECTIC SUBSTANCES FROM SECTIONS INCUBATED IN PRESENCE OF METHIONINE-$C^{14}H_3$ WITH AND WITHOUT IAA[1]

PREPARATION	SPECIFIC ACTIVITY CPM/MG	DILUTION FACTOR	ACTIVITY ON UNDILUTED BASIS CPM/MG	SPECIFIC ACTIVITY μC/MM[2]	RATIO IAA/CONTROL
No 1 control	1,400	160	224,000	74	
IAA	3,210	160	513,000	170	2.3
No 2 control	2,240	105	235,000	78	
IAA	5,330	105	533,000	176	2.3

[1] Cold-water-soluble pectic substances from experiment 3 were used.
[2] Calculated on basis of 40 % efficiency gas flow, thin window Geiger counter.

residual pectin in other plant tissues can be extracted, essentially completely, with hot aqueous solutions of ethylenediamine tetra-acetic acid, a calcium sequestering agent (31). The calcium in *Avena coleoptiles* is not readily removed. It amounts to ca. 0.03 meq per g of cell walls and is sufficient to form the calcium salt of one-fifth of the free pectic carboxyl groups present.

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. However, the presence of IAA does influence one significant aspect of pectic metabolism, namely, rate of formation of pectic methyl ester groups. This rate is increased as much as 70 % for cell wall methyl ester as a whole, as well as for methyl ester of the hot-water-soluble pectin. Rate of methyl ester group formation in the cold-water-soluble pectin is increased by the presence of IAA to an even greater extent, more than two-fold.

Is the increased rate of pectin methyl ester formation directly related to auxin-induced growth? Although this question cannot be answered on the basis of the present work, the answer can be approached first by summarizing the evidence on the role of pectic materials in cell wall plasticity. It has already been shown that the plasticity of *Avena coleoptile* cell walls, as measured either by growth rate or by susceptibility to mechanical deformation, is greatly influenced by inorganic cations. The presence of calcium ions stiffens the wall; the presence of potassium ions softens it. The ions which are effective in this function act as though they are exchangeably bound (14, 33). The present paper demonstrates that the cation binding capacity of living coleoptile tissue is accounted for quantitatively by the free (nonesterified) pectic carboxyl groups of the cell wall. The exchangeably bound ions which influence cell wall mechanical properties are, therefore, ions which are exchangeably bound to cell wall pectic carboxyl groups. Ionic bonds between adjacent pectic chains can, apparently, serve as cross linkage points between them and serve to make the entire cell wall structure more rigid.

The effect of auxin, IAA, in increasing cell wall plasticity cannot be due simply to increasing the proportion of pectic carboxyl groups which are esterified. This is ruled out by the evidence in tables III through VII, in which no effect of auxin treatment on the net cell wall methyl ester content can be detected.

The data show that IAA treatment does not alter the distribution of pectic material, as between forms of different solubility. Hence, there is no reason to suppose that the IAA effect, for example, is one of breaking long chains into short ones. The one clearly demonstrable effect of IAA treatment is the considerable increase in rate of pectin methyl ester group formation in the ester groups of the more soluble pectic materials. IAA, in promoting cell elongation, may have to do with the synthesis of these ester groups (and indeed the galacturonic acid residues themselves) of the soluble pectic material.

SUMMARY

1. Avena coleoptile cell walls contained, on a dry weight basis, 5.1 % of pectic substances as measured by the carbazole method. Methyl ester determination by alkaline hydrolysis showed that, on the average, 40 % of the pectic material was esterified. Methyl ester determination by pectinesterase yielded a value approximately 90 % of that found by alkaline hydrolysis. The cell walls contained a considerable amount of labile alkyl groups other than methyl ester. The nature of these alkyl groups is not now 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, were used to identify the component sugars and uronic acids of the cell wall. The sugar fraction contained ribose, galactose, glucose, xylose, and arabinose, with the last two sugars predominating, as well as at least one ultraviolet absorbing and one ultraviolet fluorescing material. The acidic fraction contained two ultraviolet absorbing and two ultraviolet fluorescing substances in addition to galacturonic acid.

2. The cation exchange capacity of coleoptile cell walls equaled the number of free pectic carboxyl groups. The cation exchange capacity of living *Avena coleoptile* sections, previously determined, is likewise equal to the number of free pectic carboxyl groups which the tissue contains. Accordingly, it is concluded that the cation exchange capacity of coleoptile sections is determined by the deesterified portion of pectic substances present.

3. Incubation of coleoptile tissue with IAA induces an accelerated formation of the methyl ester of the pectic substances of cell walls, although no analytically detectable net change in cell wall content of pectic substance, in methyl ester content, or in degree of pectic esterification was found. This is deduced from experiments in which tissue was incubated for 5 hours 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 specific activity of the pectic methyl ester groups of IAA-treated tissue was as much as 1.7 times that of the non-auxin treated tissue. The IAA-induced accelerated formation of methyl ester occurred exclusively in the hot-water-soluble pectic fraction of the cell walls which represents 15 % of the total pectic substance of the cell walls.

4. Incubation of coleoptile tissue with IAA caused a greater increase in incorporation of methionine methyl into the methyl ester groups of the cold-water soluble, 70 % alcohol-precipitable pectic fraction than into the pectin of the cell walls themselves. The high specific radioactivity of the methyl ester groups of the cold-water-soluble pectin fraction suggests a relatively direct pathway for the transfer of methionine methyl to the methyl ester of pectic substances. These observations also suggest that the cold-water-soluble pectic material may be a precursor of cell wall pectic substances.

5. Little or no incorporation of the methyl group

of methionine into the acetyl (or similar acyl) groups of the cell walls could be found.

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

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