

# CHEMICAL CONSTITUTION OF THE PRIMARY CELL WALLS OF AVENA COLEOPTILES<sup>1,2</sup>

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In studies of the structure and development of primary walls of plant cells, little detailed attention has been given to the non-cellulosic components of the wall. Indeed, as Bonner (6, p. 130) has indicated, only rarely have thorough analyses of plant walls of any type been made. For the most part, characterization of wall constituents has depended on solubility properties and staining tests, the specificities of which are open to doubt. With the development of newer techniques such as paper chromatography, means are now available for a much more accurate characterization of components in the walls than has hitherto been possible.

*Avena* coleoptiles are frequently the object of studies relating to primary walls and in view of this, a detailed analysis of their walls has been carried out. As will be seen, the results differ quite strikingly in some respects from those obtained by Thimann and Bonner (20) 25 years ago.

## METHODS AND RESULTS

Seedlings of *Avena sativa* var. Lanark were germinated on damp Kleenex tissue in the dark at about 25° C. Coleoptiles were harvested when from 20 to 35 mm long. At this length, the parenchyma cells are elongating and have only primary walls (18). A small amount of secondary wall is present in the vascular bundles but was not considered separately in the study. Primary leaves were removed from fresh coleoptiles which were placed in water until sufficient material had been accumulated. The bulk of the water was then removed by suction and the wet coleoptiles weighed. Roughly 200 g of this material was ground with water in a Potter-Elvehjem homogenizer. The larger particulate matter (mainly wall fragments) was centrifuged, washed three times with cold distilled water and dried. The supernatant plus washings and the dried residues were fractionated according to the schemes shown in figures 1 and 2 respectively.

The initial treatment of the coleoptiles just described was designed to remove the bulk of the cytoplasm from the wall fragments and is similar to the scheme employed by Thimann and Bonner (20). The subsequent fractionation was essentially that commonly used for isolating carbohydrates from plant material (23). Throughout the fractionation, evaporations were carried out under diminished pressure at 40° C or less. Solid fractions, both precipitates and residues, were dried by the same procedure except where otherwise stated; the solid was washed several times with absolute ethanol, then with ether, and dried at a pressure of 0.03 mm Hg over anhy-

drous calcium chloride for at least 18 hours. Hydrolyses were carried out by heating samples of 10 to 20 mg of the various fractions with 1 ml *N* hydrochloric acid in a sealed tube at 100° C for 8 hours. Chromatograms were run by the descending method (17) using one of the following solvent systems: (A) pyridine : ethyl acetate : water—1 : 2 : 2 (11); (B) ethyl acetate : acetic acid : water—3 : 1 : 3 (11); (C) *n*-butanol : pyridine : water—6 : 4 : 3 (10). Sugars were detected on the chromatograms by the *p*-anisidine hydrochloride spray reagent (10) and were chromatographically identified by running samples of known sugars on the same paper sheet. At least two solvent systems were used to establish this identification. Nitrogen was determined on 15 to 25 mg samples by the micro-Kjeldahl method and protein was taken as 6 times the micro-Kjeldahl value.

The supernatant and washings from the ground coleoptiles were dialyzed in Visking cellophane tubing against fresh distilled water for three 24-hour periods. A precipitate which formed inside the dialysis tube was centrifuged, washed once with water and dried to give Fraction 1 (0.5323 g; N, 9.85 % = 59 % protein). After hydrolysis, chromatography (solvents A and C) revealed the presence of arabinose, xylose, glucose and galactose in the approximate ratios shown in table I.

The supernatant liquor from Fraction 1 was concentrated to 1/20 its volume and ethanol was added until precipitation was complete. The precipitate was centrifuged and dried to give Fraction 2 (0.3166 g; N, 5.26 % = 32 % protein). Hydrolysis and chromatography (solvents A and C) showed the presence of arabinose, xylose, glucose and galactose (table I).

The ethanolic mother liquors from Fraction 2 were evaporated to dryness and the residue was redissolved in ethanol. Addition of ether caused precipitation of Fraction 3 which was washed once with ether and dried (0.2360 g; N, 3.6 % = 22 % protein). Arabinose, xylose and glucose were detected chromatographically (solvents A and C) after hydrolysis. Evaporation of the supernatant liquor from Fraction 3 left no residue indicating that Fractions 1, 2 and 3 together represented all of the non-dialyzable material washed from the coleoptiles by water.

The washed sediment from the original ground coleoptiles was dried in air, ground through a Wiley mill (20-mesh screen), and dried to constant weight at a pressure of 0.03 mm Hg over phosphorus pentoxide. For convenience, this dried product (Fraction 4; 4.8286 g after removal of 0.0255 g for N determination; N, 1.58 % = 9.5 % protein) was regarded as the total primary cell wall material to which Fractions 5 to 9 are referred in terms of percent by dry weight (see discussion and table I).

Fraction 4 was extracted in a Soxhlet apparatus

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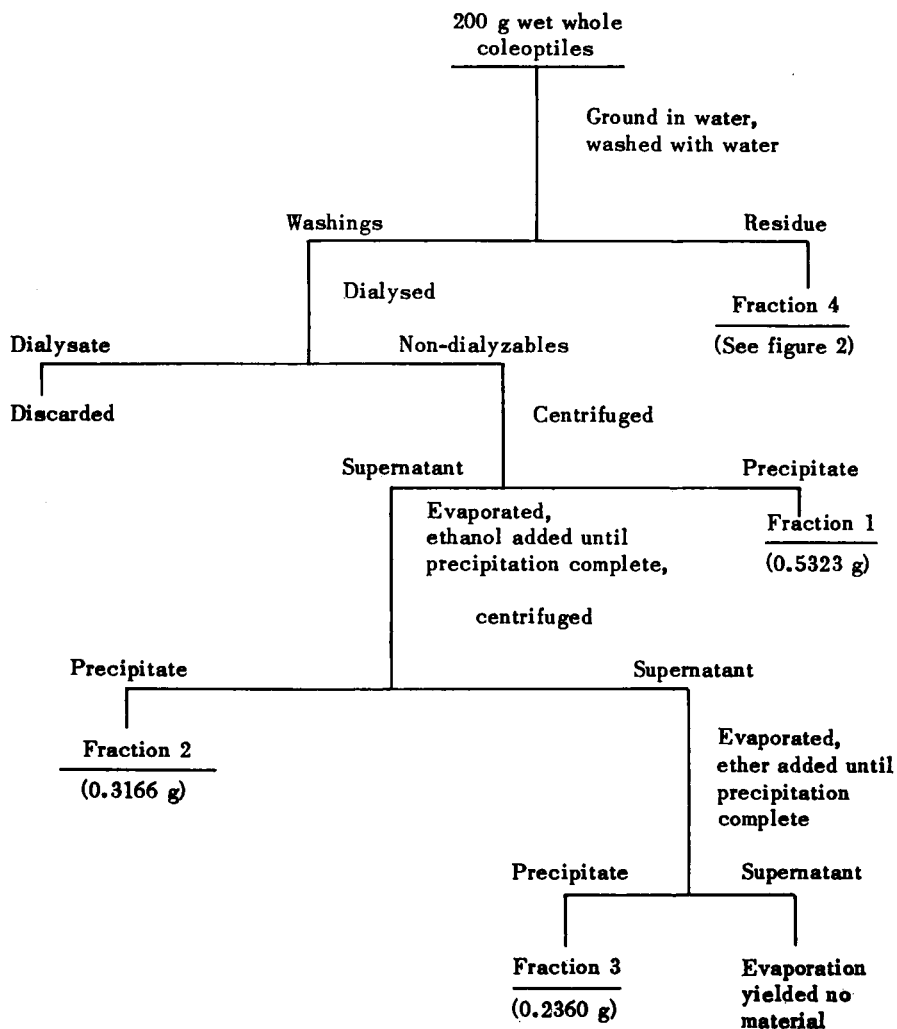


FIG. 1. Separation of polymeric material in the water washings of *Avena* coleoptiles.

with benzene : ethanol—2 : 1. Extractions were repeated until no more material was removed as determined by evaporating each extract to dryness. Four extractions were required, each one lasting 24 hours. The combined extracts were evaporated to give Fraction 5 (0.2008 g; 4.2%). No carbohydrate material could be detected in this fraction by hydrolysis and chromatography. This fraction, which consisted of a crystalline and a waxy component, gave the following X-ray diffraction spacings in Å: 46, 7.8, broad band of scatter from 5.2 to 4.5, 4.25 (strong), 3.85 (strong), 3.05, 2.55 and 2.32.

The residue of Fraction 4 (4.6517 g) remaining after benzene : ethanol extraction was stirred with 70 ml of aqueous ammonium oxalate : oxalic acid (0.25% of each) at 75° C for one hour (5). The mixture was centrifuged and the residue was repeatedly extracted in the same way until no precipitate was obtained when the supernatant liquor was diluted with 8 volumes of ethanol; four extractions were required.

The combined extracts were diluted with two volumes of ethanol and the precipitate which formed was centrifuged and dried to give Fraction 6 (0.0859 g, 1.8%). Hydrolysis of this fraction and chromatographic examination of the hydrolysate (solvents A and B) showed the presence of arabinose, xylose, glucose and a hexuronic acid. Fraction 6 was warmed gently in 15 ml of water and the mixture was centrifuged to remove sub-fraction 6 a. The supernatant liquor was diluted carefully with ethanol until a distinct precipitate formed which was centrifuged and classed as sub-fraction 6 b. When more ethanol was added to the supernatant liquor from sub-fraction 6 b a voluminous, gelatinous precipitate was formed. This precipitate was recovered by centrifuging and dried to give sub-fraction 6 c as a white powder.

Sub-fraction 6 a (0.0100 g, 0.2%) was a dark brown powder, insoluble in water and yielding only traces of xylose, arabinose and glucose on hydrolysis. Sub-fraction 6 b was a white, water-soluble powder

(0.058 g, 1.2 %) having  $[\alpha]_D^{25} = +29^\circ \pm 1^\circ$  (C, 1.5 % in water), which gave a blue coloration with iodine-potassium iodide solution and on hydrolysis yielded mainly glucose with traces of arabinose and xylose as detected chromatographically. Sub-fraction 6 c (0.0165 g, 0.3 %) was a water-soluble powder showing  $[\alpha]_D^{25} = +220^\circ \pm 4^\circ$  (C, 0.4 % in water) and yielding on hydrolysis only a hexuronic acid detected by chromatography in solvents A, B and C. The hexuronic acid was chromatographically identified as galacturonic acid by specific spray reagents and by the

fact that no lactone could be found on the chromatograms (7). Both glucuronic and mannuronic acids readily form lactones which run much faster than the parent acids on paper chromatograms. Galacturonic acid does not form a lactone. Identification of galacturonic acid was confirmed by simultaneous hydrolysis—oxidation (5) of a portion of fraction 6 c (0.010 g) by nitric acid (5 ml, specific gravity 1.15) to yield mucic acid with a melting point of 212 to 213° C (d).

The supernatant liquor from the precipitation of Fraction 6 was concentrated and ethanol was added

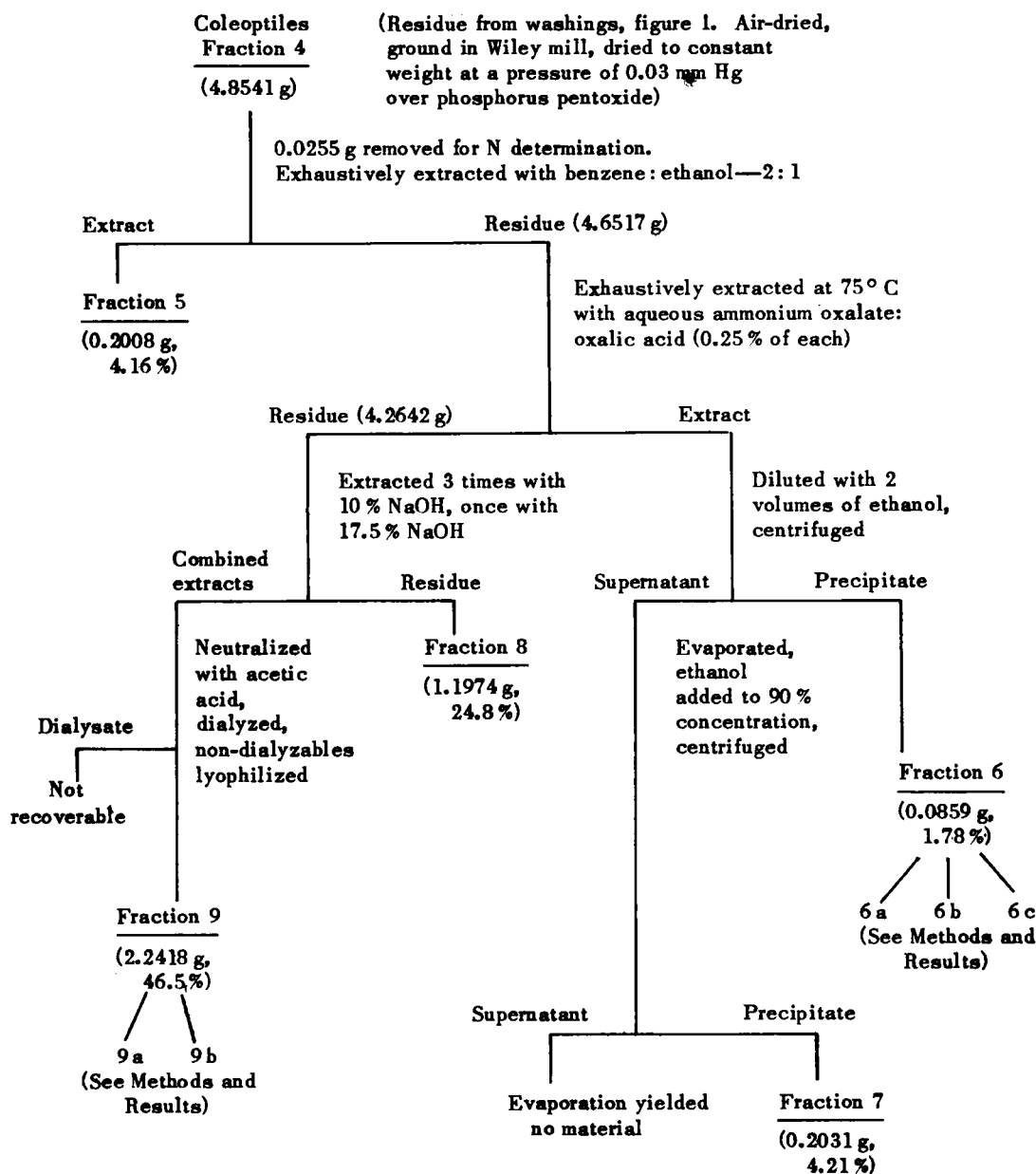


FIG. 2. Fractionation of *Avena* coleoptile walls.

TABLE I  
ANALYSIS OF AVENA COLEOPTILES

FRACTION	CLASS OF MATERIAL	PERCENTAGE OF DRY PRIMARY WALL	MONOSACCHARIDES RELEASED ON HYDROLYSIS				
			GALACTOSE	GLUCOSE	ARABINOSE	XYLOSE	HEXURONIC ACID
1 } 2 } 3 }	Water washings of coleoptiles	...	++	+++	++	++	...
	Protein and carbohydrate	...	++	+++	++	++	...
		...	+	+++	++	++	...
4	Washed sediment from ground coleoptiles (not hydrolyzed)	100	...	...	...	...	...
5	Fats, waxes, pigments	4.2	...	...	...	...	...
6 a	Polysaccharide	0.2	...	+	+	+	...
6 b	Starch-like polysaccharide	1.2	...	+++	+	+	...
6 c	Pectic substances	0.3	...	...	...	...	+++
7	Non-cellulosic polysaccharides <sup>c</sup>	4.2	...	+	+++	+++	...
8	$\alpha$ -cellulose	24.8	...	see text			...
9	Non-cellulosic polysaccharide	46.5	6.7 %	34.3 %	29.1 %	29.8 %	Trace
9 a	Water-soluble	About $\frac{2}{3}$ Fraction 9	8.9 %	27.8 %	31.2 %	32.1 %	...
9 b	Water-insoluble	About $\frac{1}{3}$ Fraction 9	4.5 %	41.0 %	27.0 %	27.6 %	...
	Protein	9.5					
	Total recovery	90.9					

to a concentration of 90%. The precipitate which formed was centrifuged and dried giving Fraction 7 (0.2031 g; 4.2%). Hydrolysis and chromatography (solvents A and C) of this fraction revealed the presence of arabinose, xylose and a trace of glucose. Evaporation of the supernatant liquor from this fraction yielded no further material.

The residue (4.2642 g) from the ammonium oxalate: oxalic acid extraction was shaken for three 18-hour periods with fresh portions of 70 ml of 10% aqueous sodium hydroxide, and for one 18-hour period with 60 ml of 17.5% aqueous sodium hydroxide. The residue was filtered with suction on a sintered glass funnel and washed thoroughly with water. It was then washed successively with 5% aqueous acetic acid, water, 95% ethanol, absolute ethanol, and ether before being dried as Fraction 8 (1.1974 g; 24.8%; N, nil), the  $\alpha$ -cellulose of *Avena coleoptiles*. The  $\alpha$ -cellulose was completely hydrolyzed and the large excess of glucose in the hydrolysate was removed by fermentation with *Saccharomyces cerevisiae*, N.R.C. no. Y7 as described elsewhere (1). Chromatography (solvents A and C) of the glucose-free hydrolysate revealed the presence of galactose, mannose, arabinose, xylose and a trace of rhamnose.

The alkali extracts and washings, up to and including the 95% ethanol washing, were combined and dialysed in Visking cellophane tubing against distilled water for four days, the water being changed twice a day. The non-dialyzable solution was then lyophilized to give Fraction 9 (2.2418 g; 46.5%), the non-cellulosic polysaccharides of the cell wall. This fraction had  $[\alpha]_D^{25} = -9.0^\circ \pm 2^\circ$  (C, 1.06% in N sodium hydroxide) and a number-average degree of polymerization of 16, determined by alkaline hypo-

iodite oxidation (13). On hydrolysis the polysaccharides yielded a mixture of sugars consisting of galactose, 6.7%; glucose, 34.3%; arabinose, 29.1%; and xylose, 29.8%. For this quantitative estimation the sugars were separated as bands on paper chromatograms (solvent A). The relative amounts of the separated sugars were then determined by measuring the area and intensity of their reaction with silver nitrate using a recording densitometer (14).

In a separate experiment the non-dialyzable material (Fraction 9) was separated, immediately after dialysis, into water-soluble and insoluble portions. The soluble portion (Fraction 9 a) represented two thirds by weight of Fraction 9, had  $[\alpha]_D^{25} = -36.0^\circ \pm 2^\circ$  (C, 1.3% in N sodium hydroxide), degree of polymerization 16, and on hydrolysis yielded a mixture of sugars consisting of galactose, 8.9%; glucose, 27.8%; arabinose, 31.2%; and xylose 32.1%. The insoluble portion (Fraction 9 b) constituted one third the weight of Fraction 9, had  $[\alpha]_D^{25} = +50^\circ \pm 2^\circ$  (C, 0.5% in N sodium hydroxide), degree of polymerization 8, and on hydrolysis yielded the following mixture of sugars: galactose, 4.5%; glucose, 41.0%; arabinose, 27.0%; and xylose, 27.6%.

The dialyzate of the alkali extract contained such large amounts (80 to 90 g) of sodium acetate that it was not feasible to recover any carbohydrate material which may have dialyzed.

To check recoveries in the fractionation, the residues from each extraction shown in figure 2 were recovered and dried. In this way it was found that recovery from the benzene: ethanol extraction, i.e., Fraction 5 plus residue, was 100.5%. Recovery from the ammonium oxalate: oxalic acid extraction, Fractions 6 and 7 plus their residue, was 98%. Recovery

in the alkali extraction was 81 % (Fractions 8 plus 9). If it is assumed that the protein (9.5 %) in the original dried wall fragments was lost by hydrolysis and dialysis during the alkali extraction, an assumption that was supported by the lack of nitrogen in Fraction 8, then the overall recovery in the fractionation was 90.9 %.

**COMPLETE HYDROLYSIS OF INTACT AVENA COLEOPTILES:** To check for the presence of any large amounts of galacturonic acid which might have been lost during the detailed analysis just described, a sample (8.0 g) of wet, freshly harvested coleoptiles, equivalent to approximately 0.25 g dry weight, was completely hydrolyzed. The sample was dissolved in 20 ml of 72 % sulphuric acid and the solution was allowed to stand at room temperature for 1.5 hours. Water was then added until the acid concentration was 3 % and this mixture was refluxed for three hours. Acid was removed as barium sulphate and the salts were filtered and washed with hot water. The combined washings and filtrate were concentrated to about 25 ml and were passed over a column of Amberlite IR-120 exchange resin to remove barium. The column eluate was then concentrated and chromatographed on a full size sheet of Whatman no. 1 filter paper (solvent C). After guide strips had been cut and sprayed to locate the separated components, the hexuronic acid band was cut out and eluted with water, the eluate being evaporated to dryness giving a syrup (4.2 mg) having  $[\alpha]_D^{23} = +57^\circ \pm 5^\circ$  (C, 0.42 % in water). This product was refluxed for 8 hours with 8 % methanolic hydrogen chloride (1 ml). The product was recovered by evaporating the solution and was reduced with sodium borohydride to the corresponding hexose glycoside. Sodium was removed from the reduction by Amberlite IR-120 exchange resin and borate was distilled off as its methanol complex. The residue from this reduction was hydrolyzed with *N* hydrochloric acid on a boiling water bath for 6 hours and the hydrolyzate was chromatographed (solvents A and C). Both galactose and glucose were found on the chromatograms in approximately equal proportions.

## DISCUSSION

The main results on which the discussion centres are summarized in table I.

The initial separation of ground coleoptiles into sediment and supernatant, designed to remove protoplasmic material from the walls, was only partially successful. The non-dialyzable carbohydrate material found in Fractions 1 to 3 of the supernatant (about 630 mg) almost certainly represented wall material since its monosaccharide composition closely resembled that of the major constituents of the wall. Non-dialyzable protein was divided roughly equally between sediment and supernatant with about 450 mg in each. On a percentage dry weight basis, the amount associated with the sediment (Fraction 4) was similar to that reported by Thimann and Bonner (20). However, it is uncertain how much of this was contaminating cytoplasm and how much can be regarded as

true wall material. Undoubtedly a large proportion of this protein will be due to dense plasmodesmata which have been found frequently to penetrate even thick epidermal walls (4).

No detailed analysis was made of the fats, waxes and pigments (Fraction 5) extracted from the residue (Fraction 4) with benzene-ethanol, but it is interesting to note that the large X-ray spacing of 46 Å found in this fraction corresponds within experimental error to that of 44 Å detected previously in whole coleoptiles (2). Again, this differs significantly from the value of 60 Å reported earlier by Gunderman et al (9) for waxes extracted from *Avena* coleoptiles.

Removal of materials in Fraction 5 left the remaining tissue open to penetration by hydrophilic solvents. The ammonium oxalate : oxalic acid extraction was designed to remove pectic substances. In recent years it has been shown that ammonium oxalate : oxalic acid solution is a more efficient and selective extracting medium for pectic materials than ammonium oxalate solution alone (5). According to the latest rules of nomenclature "pectic substances" are defined as "those complex, colloidal carbohydrate derivatives which . . . contain a large proportion of anhydrogalacturonic acid units . . . . The carboxyl groups of polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralized by one or more bases" (23, p. 162). When applied to coleoptiles the ammonium oxalate : oxalic acid extraction removed a mixture of polysaccharides which were separated by a sub-fractionation with ethanol-water. By this procedure the pectic material (Fraction 6 c) was separated from a starch-like polysaccharide containing largely glucose, which gave a blue coloration with iodine (Fraction 6 b) and from non-cellulosic polysaccharides (Fractions 6 a and 7) containing arabinose and xylose. The high positive optical rotation of  $+220^\circ$  (cf. 23, p. 185) and the identification of galacturonic acid as the only product of hydrolysis characterized Fraction 6 c as pectic material. The absence of hexuronic acid in hydrolysates of the other fractions, except for a trace in Fraction 9, indicated that the small amount of pectic material in the coleoptiles was removed in this extraction.

The low pectic content of these walls was confirmed by complete hydrolysis of intact coleoptiles, the results of which showed that all hexuronic acids represent only about 1.6 % of the total dry weight of coleoptiles and this is composed of roughly equal quantities of glucuronic and galacturonic acids. This total quantity of hexuronic acids which undoubtedly includes some soluble sugars corresponds sufficiently closely with what was detected in Fractions 6 c and 9 of the detailed analysis to indicate that no large quantity of pectic material (polygalacturonic acid) could have been lost.

By far the largest single class of materials in the coleoptiles were the non-cellulosic polysaccharides, usually termed hemicelluloses, extracted by alkali. These were composed of the monosaccharide units galactose, glucose, arabinose and xylose and the differ-

ences in optical rotation and monosaccharide composition of the subfractions indicated that the material was a mixture of several polysaccharide species.

The alkali extraction and subsequent isolation of  $\alpha$ -cellulose and non-cellulosic polysaccharides was the only step in the analysis where loss of material occurred. Part of this was caused by alkaline hydrolysis of the 9.5% protein in the coleoptiles and its loss as amino acids during dialysis. The remaining loss was probably caused by dialysis of short-chain polysaccharide material having the same composition as the polysaccharide material that was recovered. The non-cellulosic polysaccharides that were recovered (Fraction 9) were of quite short chain length (degree of polymerization, 8 to 16) and it is possible that polysaccharides with a degree of polymerization of less than 8 would pass through the dialysis membrane and be lost. The very large amounts of sodium acetate in this dialyzate precluded any attempts to check this point by recovering the carbohydrate material from the dialyzate. It is also possible that the strong alkali used in the extraction caused degradation of some of the non-cellulosic polysaccharides to fragments sufficiently small to be lost on dialysis (22).

The remainder of the wall consisted of  $\alpha$ -cellulose (Fraction 8) which was present in a concentration (25%) generally lower than that reported for other primary walls (6, p. 134). Traces of monosaccharide residues other than glucose were present in the  $\alpha$ -cellulose. Such residues are commonly found in  $\alpha$ -cellulose and their significance has been discussed previously (1, 3).

The picture this analysis gives of the chemical composition of primary walls of *Avena* coleoptiles is comparatively simple. The walls consist principally of the relatively inert substances, cellulose and non-cellulosic polysaccharides. The latter materials, although they may be numerous and may differ in detailed structure, appear to be composed almost entirely of similar residues.

The most important findings in the study concerned the pectic content, which was considerably lower than that reported in other analyses of primary walls (6, 21). It is probable that the primary walls of different tissues or species vary markedly in pectic content. However, the sole evidence for the presence of pectin has often been the ability of ammonium oxalate to extract material from the wall. The present results show that significant quantities of polysaccharides other than pectin, including starch-like material, can be extracted with this solvent.

More significantly, however, these results show that an appreciable proportion of pectic material is not in fact a prerequisite for rapidly extending walls. Because of its ability to form gels, pectin has usually been vested with the role of supplying flexibility to cell walls. Kerr (12) has described it as the "continuous phase" of the wall, the "discontinuous phase" being provided by the cellulose skeleton. Furthermore, recent results from studies on *Avena* coleoptiles (15, 16, 19) have suggested that the way in which

auxin plasticizes walls to permit increased elongation of cells is by affecting the degree of methyl esterification of the pectic materials in the walls. Other authors (8) have inferred from effects of auxin on the binding of pectin methyltransferase in the wall that in promoting elongation, auxin acts through pectin. However, it is difficult to imagine how the minute pectic content found here could have significant influence on the physical properties of the walls: hexuronic acid residues, either as pectic substances or as components in non-cellulosic polysaccharide chains, could possibly have a disproportionate effect in plasticizing the wall by occupying strategic positions in the wall structure, but the degree of economy and order in the distribution of these residues required to achieve such a system would appear to be so high as to make it improbable.

If the non-cellulosic portion of the primary wall is to be considered an active agent in wall structure, it seems that attention must be turned to the preponderant non-cellulosic polysaccharides. These compounds do not have the obvious capacity for reaction which could be ascribed to the ester groups of pectin. However, the most fruitful approach to a better understanding of the physiological aspects of the structure and metabolism of primary cell walls is probably a more thorough study of these substances.

#### SUMMARY

The primary walls of *Avena* coleoptiles were fractionated chemically and the constituent monosaccharides of the carbohydrate components were identified by paper chromatography and other methods. On a dry weight basis, at least 51% of the walls was a mixture of hemicelluloses containing principally residues of galactose, glucose, arabinose and xylose;  $\alpha$ -cellulose represented 25%, fats, waxes and pigments 4% and protein 9%. The fraction obtained by ammonium oxalate: oxalic acid extraction was composed largely of a starch-like polysaccharide (1.2% of the dry walls); only a small amount of pectic substances was obtained representing 0.3% of the dry walls. The very low pectic content of the walls was confirmed by the finding of less than 1% galacturonic acid on a dry weight basis in a hydrolysate of fresh coleoptiles.

The results suggest that the physical properties of these elongating walls depend more on the preponderant hemicelluloses than on the negligible quantity of pectic substances.

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## EFFECT OF LIGHT ON THE CO<sub>2</sub> ABSORPTION AND EVOLUTION BY KALANCHOË, WHEAT AND PEA LEAVES<sup>1,2</sup>

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A common technique for the determination of the true photosynthetic rate is to measure the apparent CO<sub>2</sub> intake in light and then add to this the amount of CO<sub>2</sub> respired by comparable material in darkness. The validity of this technique is based on the assumption that light does not affect respiration.

Within the last years support of this assumption has come from several experiments. Davis observed that light had no effect on the consumption of oxygen

by a *Chlorella* mutant, which had lost its ability to carry on photosynthesis (3). In a series of experiments with oxygen isotopes, Brown has shown that when leaves of several higher plants or *Chlorella* were illuminated in air enriched with O<sup>18</sup>, their consumption of heavy oxygen was unaffected by light (1, 5). Photoinhibition at higher light intensities was observed, however, for several algae (5). In *Anabaena*, photoinhibition was observed at oxygen concentration below 0.5% in the gas phase, while photostimulation was apparent at higher light intensities (2).

In all these experiments, however, respiration was measured by oxygen consumption and it is desirable to inquire whether the same results are obtained when respiration is measured by CO<sub>2</sub> production.

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