STUDIES ON WHEAT ENDOSPERM

II.* PROPERTIES OF THE WALL COMPONENTS AND STUDIES ON THEIR ORGANIZATION IN THE WALL

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Abstract

Water- and alkali-soluble arabinoxylans from isolated wheat endosperm cell walls were examined by ammonium sulphate fractionation and gel-exclusion chromatography. Both arabinoxylans were polydisperse with similar molecular size ranges, although the alkali-soluble material was slightly less soluble in ammonium sulphate solutions. It was concluded that the differences in size or xylose : arabinose ratios were not of sufficient magnitude to account for the different solubility properties of the two arabinoxylans.

The possibility that the water-insoluble arabinoxylans were bound to one another or to other cell wall components was investigated by chemical and enzymic extraction of water-extracted cell walls. Much of the protein associated with the isolated and water-extracted walls could be dissolved by protein-dispersing agents and the remainder could be removed enzymically without altering the solubility properties of the arabinoxylans. Mild alkaline extraction and neutral hydroxylamine treatment removed the water-insoluble arabinoxylans, suggesting that ester-like linkages are involved in rendering the arabinoxylans insoluble. Esters were shown to be present in water-extracted cell walls. The possible presence of interpolysaccharide ester linkages involving uronic acids or phenolic acids is discussed.

The composition and organization of wheat endosperm cell walls is compared with that of primary plant cell walls.

I. INTRODUCTION

In Part I of this series (Mares and Stone 1973*a*) it was shown using electron microscopy that wheat endosperm walls had a similar ultrastructure to primary plant cell walls, having a microfibrillar phase embedded in an amorphous matrix. Chemical studies showed that isolated walls were largely composed of polysaccharide but that some protein was also present. The predominant polysaccharides in the walls are arabinoxylans, of which one third are soluble in water, the remainder requiring alkaline reagents to bring them into solution.

It has been suggested by Montgomery and Smith (1955) that differences in the degree of branching of the arabinoxylans, as measured by their xylose : arabinose

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ratio, are responsible for the differences in solubility, but this idea is not supported by more recent work of Medcalf *et al.* (1968) or by the results presented in Part I (Mares and Stone 1973*a*).

It has also been proposed that the solubility properties of the pentosan fractions could be related to molecular size differences (Montgomery and Smith 1956), whilst Kulp (1968) has concluded, as a result of studies on the enzymic digestion of pentosan fractions, that the insoluble fraction is closely associated either physically or chemically with cellulose. This paper describes an investigation of the factors responsible for the solubility properties of the arabinoxylans of wheat endosperm cell walls.

The endosperm walls as isolated contain 1·7% (w/w) nitrogen which is present largely in the form of peptides or protein (Mares and Stone 1973*a*), and since the protein components of plant cell walls may play an important role in wall structure (Lamport 1970) their significance in endosperm walls has been investigated by the use of selective solvents and by enzymic digestion.

The occurrence of galactose-rich polysaccharides in the endosperm and their properties are described in Part III (Mares and Stone 1973*b*).

II. Methods

Wheat endosperm cell walls and cell wall fractions were prepared as described by Mares and Stone (1973*a*). Barley endosperm cell walls were prepared by Mr. P. Costello (Costello and Stone 1968) and *Lolium multiflorum* endosperm cell walls were prepared as described by Mares and Stone (1973*c*). Total polysaccharide and monosaccharide analyses, protein estimations, and Bio-Gel P-2 chromatography were performed as described by Mares and Stone (1973*a*).

(*a*) *Sepharose 4B Chromatography*

The amount of polysaccharide to be fractionated was limited to 15 mg or less, since excessively viscous solutions altered the flow rate and the separation characteristics of the Sepharose 4B columns. Samples of arabinoxylans (10–15 mg) were dispersed in 1 ml of 0·3% NaCl containing 0·05% NaN_3 by heating for 10 min in a boiling water-bath. The resulting solution was cooled, allowed to stand at room temperature for 3 hr (Blake and Richards 1971), and, unless otherwise specified, loaded onto a column of Sepharose 4B (49·5 by 3·0 cm) and eluted with NaCl–NaN_3 solution. The column temperature, flow rate, and the height of the eluting solvent head were maintained at constant values throughout. Fractions of approximately 7 ml were collected and assayed for total carbohydrate using the phenol–sulphuric acid method (Dubois *et al.* 1956) and for protein by the method of Lowry *et al.* (1951). The breakthrough and bed volumes of the column were 80 and 265 ml respectively, and were estimated by measuring the elution volumes of *Escherichia coli* cells and inorganic phosphate applied to the column with the sample. The apparent molecular weight of the arabinoxylan fractions was estimated from the molecular weight "selectivity" curves for polysaccharides (Pharmacia Fine Chemicals, Uppsala; Handbook 1969).

(*b*) *Ammonium Sulphate Fractionation*

To enable direct comparison of the $(NH_4)_2SO_4$-insoluble fractions with those isolated by Preece and Hobkirk (1953, 1954) identical conditions of fractionation were employed. Equal portions of the water- and alkali-soluble pentosan (see Fig. 1, Mares and Stone 1973*a*) were dispersed in distilled water. To every 100 ml of pentosan solution 30 g of $(NH_4)_2SO_4$ was added with stirring and the precipitate removed by centrifugation. Subsequent additions of 10-g lots of $(NH_4)_2SO_4$ to the supernatant gave precipitates corresponding to $(NH_4)_2SO_4$ concentrations of 40, 50, 60, and 70 g per 100 ml of original solution. Solutions of the precipitated fractions and the supernatant obtained after bringing the pentosan solution to an $(NH_4)_2SO_4$ concentration of 70 g/100 ml of original solution were dialysed against distilled water at 4°C. Following dialysis, polysaccharides

were precipitated by addition of 4 vol. of ethanol, recovered by centrifugation, and dried by solvent exchange. Samples of each fraction were analysed for total carbohydrate and monosaccharide composition and were chromatographed on Sepharose 4B.

(*c*) *Cell Wall Extraction Procedures*

Samples of water-extracted cell walls (5–10 mg) were extracted by shaking three times with 10 ml of solvent for 16 hr at 27°C. After centrifugation extracts were dialysed extensively against cold distilled water and the protein and total carbohydrate contents measured. The phenol–acetic acid-water (PAW) (1 : 1 : 1 w/v/v) protein solvent of Synge (1957) was buffered to pH 4·5 by the addition of pyridine to prevent loss of acid-labile linkages in polysaccharides and was used under the conditions described above. Before analysis the extracts were dialysed against 40% acetic acid, followed by distilled water. Hydroxylamine hydrochloride solutions were freshly prepared and buffered to pH 5 and 6 with sodium acetate–acetic acid and to pH 7 and 8 with sodium phosphate. Extraction of cell walls with anhydrous ethylene diamine at 37°C for 3 days was performed according to the method of Korn and Northcote (1960).

(*d*) *Phenol–Water Partitioning* (*Westphal and Jann 1965*)

Material to be fractionated was suspended in water at 65–68°C, an equal volume of 90% phenol (preheated to 65–68°C) was added, and the resulting mixture was shaken vigorously and allowed to stand for 10–15 min at 65°C. The mixture was cooled to 10°C in an ice-bath and centrifuged at 1300 *g* for 30 min. The aqueous layer was removed and the procedure repeated following the addition of an equal volume of preheated distilled water to the phenol phase. The combined aqueous phases and the phenol phase were dialysed against 40% acetic acid and then exhaustively against cold distilled water before analysis for protein and carbohydrate.

(*e*) *Amino Acid Analysis*

Analyses were performed as previously described (Mares and Stone 1973*a*). Hydroxyproline, which is not resoved from aspartic acid at the normal pH of 3·28, was separated at pH 3·08 (Mashburn and Hoffman 1970). Hydroxyproline was also estimated by the colorimetric procedure of Leach (1960).

(*f*) *Enzymic Digestion*

Water-extracted endosperm cell walls (5 mg) were incubated with enzyme (substrate to enzyme ratio = 35 : 1 by weight) at 40°C for 48 hr. Proteolytic enzymes were dissolved in sodium phosphate buffers (0·02M, pH 6·8) and carbohydrase preparations were dissolved in sodium acetate buffers (0·02M, pH 5·0). Following incubation, the enzyme–substrate mixtures were centrifuged and the supernatant fractions analysed for total carbohydrate and protein. An aliquot of the supernatant was chromatographed on a column of Bio-Gel P-2 to estimate the size of the hydrolysis products. Samples of the cell wall residues were taken for monosaccharide analysis, for extraction with 6M guanidine hydrochloride and neutral hydroxylamine hydrochloride, and for examination by electron microscopy as described by Mares and Stone (1973*a*).

(*g*) *Estimation of Esters*

The hydroxamic acid test was used in the qualitative and semiquantitative estimation of esters in wall preparations as follows. Cell walls (*c.* 50 mg) were mixed with 2 ml of 0·05M hydroxylamine hydrochloride in 95% ethanol and 0·4 ml of 6M aqueous sodium hydroxide was added. The mixture was heated to boiling, cooled slightly, 4 ml of 1M HCl added, and the mixture filtered. Ester-containing preparations gave a burgundy colour following the addition of 0·1 ml of 5% $FeCl_3$ solution, whereas the control solutions were yellow. Control tests (Vogel 1970) indicated that no interfering substances were present. To obtain a semiquantitative estimate of esters in the wall samples the optical density of the solutions was read at 540 nm and compared with a standard curve prepared using known amounts of ethyl acetate.

(h) Extraction and Examination of Polyphenolic Compounds (Geissman 1970)

Cell wall fractions were extracted with 0·5M KOH at 60°C, under nitrogen, for 90 min. The extract was acidified to pH 3·4 with HCl and extracted with ethyl acetate. The emulsion formed by shaking the extract with ethyl acetate was centrifuged and the upper layer removed, dried over Na_2SO_4, and evaporated to dryness. Since methanol failed to completely dissolve the residue, n-butanol was used as a solvent. The acidified aqueous phase was further extracted with n-butanol, dried, evaporated to dryness, and then dissolved in dimethyl sulphoxide. Both the n-butanol and dimethyl sulphoxide extracts were examined by u.v. irradiation of a spot on filter paper (Hillis 1966), thin-layer chromatography (Geissman 1970), and gas–liquid chromatography of trimethylsilyl derivatives (Pellizarri *et al.* 1969). Total polyphenolic residues were measured by the Folin–Ciocalteau method using the procedure of Lee (personal communication).

III. Results

(a) Comparison of Water- and Alkali-soluble Arabinoxylans

(i) *Sepharose 4B Chromatography*

Elution profiles for arabinoxylans extracted from isolated wheat endosperm cell walls with water and 24% KOH solution are shown in Figure 1. The arabinoxylans are clearly polydisperse with apparent molecular weights in the range 8×10^4–5×10^6 but the distribution of polysaccharide within this range appeared to be similar for each of the arabinoxylan fractions. The elution profile of the water-soluble pentosan

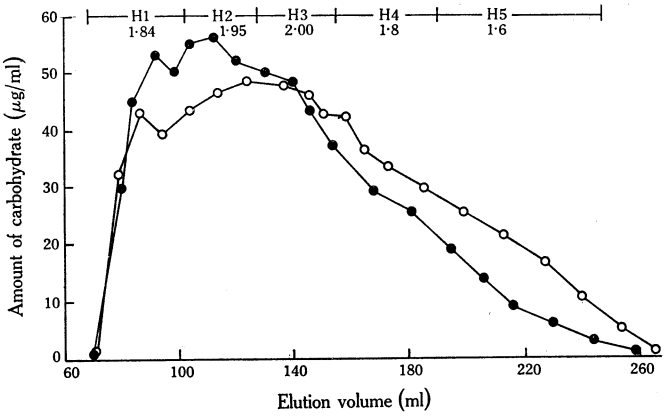


Fig. 1.—Elution profiles of endosperm cell wall arabinoxylans on Sepharose 4B. ● Water-soluble arabinoxylan. ○ Potassium hydroxide-soluble arabinoxylan. Fractions of the water-soluble arabinoxylan were pooled and designated H1, H2, H3, H4, and H5; their xylose : arabinose ratios, calculated from monosaccharide analyses, are given beneath the designations.

was not altered by treatment with 24% KOH containing 0·1% $NaBH_4$ under conditions identical to those used in the extraction of the alkali-soluble pentosan, indicating that this treatment did not cause degradation of the arabinoxylan. The water-soluble pentosan was divided arbitrarily into five fractions as shown in Figure 1 and each was analysed for monosaccharide composition. The xylose : arabinose ratio for each fraction is also shown in Figure 1. The changes observed in this ratio with increasing elution volume were reproducible and similar changes were observed with equivalent fractions obtained by chromatography of the alkali-soluble pentosan.

Molecular aggregation of arabinoxylans may occur during the equilibration period following dispersion by heating (Blake and Richards 1971). The possible aggregation of the water- and KOH-soluble arabinoxylans under such conditions was therefore examined by chromatography of equal portions of the water- and alkali-soluble pentosans which had been equilibrated at room temperature for 5 min and 30 hr following dispersion by heating. For both pentosans the longer equilibration period resulted in a small but reproducible shift of the elution profile to the higher molecular-size region.

(ii) *Ammonium Sulphate Fractionation*

Water- and KOH-extracted arabinoxylans from cell walls were fractionated according to their solubilities in ammonium sulphate. The monosaccharide composition, xylose : arabinose ratio, and proportion of the total polysaccharide represented by each of the fractions are shown in Table 1.

TABLE 1

AMMONIUM SULPHATE FRACTIONATION OF ENDOSPERM CELL WALL ARABINOXYLANS

All samples were digested with salivary α-amylase prior to fractionation. The ammonium sulphate (AS) fractionation procedure is described in Section II(*b*). Monosaccharide analyses were made by gas–liquid chromatography of alditol acetates (Mares and Stone 1973*a*)

Fraction	Percentage of total carbohydrate (by wt.*)	Monosaccharide composition (% by wt.*)				Xyl : Ara ratio
		Ara	Xyl	Gal	Glu	
Water-soluble pentosan						
Unfractionated	—	33	67	—	—	2·03
30% AS-insoluble	14	30·5	69·5	—	—	2·28
40% AS-insoluble	55·4	31	69	—	—	2·22
50% AS-insoluble	23·8	40	60	—	—	1·5
60% AS-insoluble	6	40	60	—	—	1·5
70% AS-insoluble	—	—	—	—	—	—
70% AS-soluble	0·8	39	52	9	—	1·33
Alkali-soluble pentosan						
Unfractionated	—	33·5	66·5	—	—	1·97
30% AS-insoluble	52	32	68	—	—	2·12
40% AS-insoluble	21	33·6	66·4	—	—	1·97
50% AS-insoluble	23·5	40	60	—	—	1·5
60% AS-insoluble	2	—	—	—	—	—
70% AS-insoluble	—	—	—	—	—	—
70% AS-soluble	1·5	—	—	—	—	—

* All weights refer to the dry weights of the fractions.

The general decrease in xylose : arabinose ratio of the wall pentosans with increasing solubility was consistent with the results reported by Preece and Hobkirk (1953). Gel filtration indicated that each fraction was polydisperse with respect to molecular size [Figs. 2(*a*) and 2(*b*)]. The individual profiles showed some differences,

especially among the water-soluble arabinoxylan fractions. The volume of solvent required to elute half the applied polysaccharide was 116, 128, 136, and 138 ml for the 30, 40, 50 and 60% ammonium sulphate-precipitated fractions respectively, indicating that the molecular size distribution shifted towards the low molecular-size region as the solubility increased. On the other hand, values of the same parameter for the ammonium sulphate-precipitated alkali-soluble arabinoxylan were not significantly different and the volumes of solvent required to elute half the applied polysaccharide were all in the range 115–125 ml.

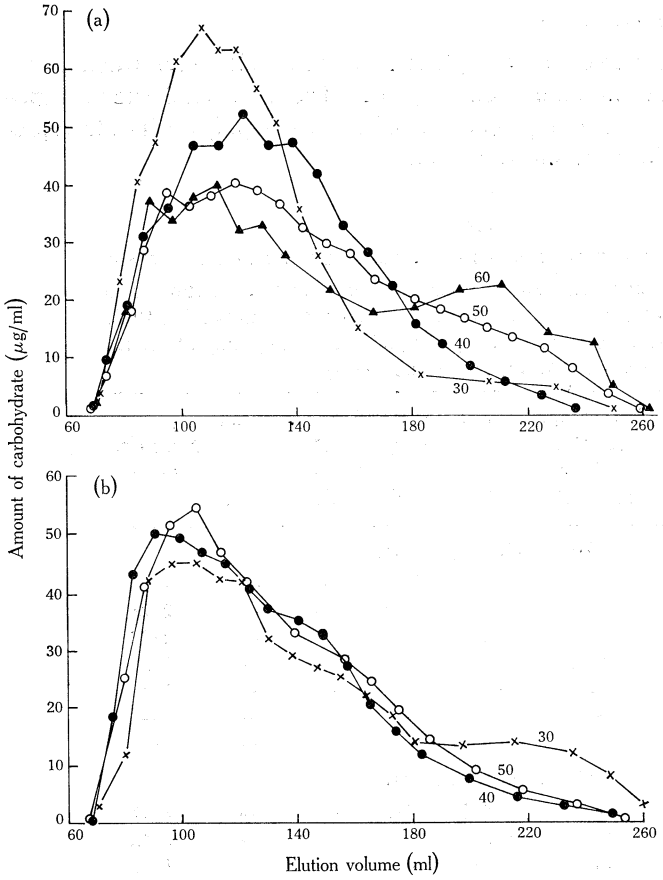


Fig. 2.—Sepharose 4B elution profiles of ammonium sulphate-insoluble fractions of arabinoxylans described in Table 1 and obtained as outlined in Section II(*b*). (*a*) Water-soluble arabinoxylan precipitated by 30% (×), 40% (●), 50% (○), and 60% (▲) ammonium sulphate solution. (*b*) Potassium hydroxide-soluble arabinoxylan precipitated by 30% (×), 40% (●), and 50% (○) ammonium sulphate solution.

(*b*) *Extraction of Cell Walls and Analysis of Extracts*

(i) *Chemical Extraction*

A number of extraction procedures (Table 2) were tested to obtain information on the way in which the protein or peptide found in the cell wall preparations was associated with the polysaccharides of the cell wall.

Phenol, PAW, ethylene carbonate, and sodium chloride solutions were ineffective in solubilizing either protein or polysaccharide. Costello (1968) used PAW extractability as a criterion for the presence of cytoplasmic protein associated with

endosperm cell walls prepared from barley flour. However, the nitrogen content of the isolated wheat endosperm cell walls was 1·7% and of water-extracted walls 2·2% (Mares and Stone 1973*a*) as compared with 0·79% for barley walls and 0·78% for wheat endosperm cell walls which had been prepared by an aqueous method (Costello 1968). This suggests that contaminating cytoplasmic protein may

TABLE 2

EXTRACTION OF PROTEIN AND POLYSACCHARIDE FROM WATER-EXTRACTED ENDOSPERM CELL WALLS

Extraction procedures used are described in Section II(*c*)

Extractant	Percentage of total carbohydrate extracted (by wt.)	Percentage of total protein extracted (by wt.)
Phenol	6	2
Phenol–acetic acid–water (pH 4·5)	5	4
Aqueous ethylene carbonate	3	8
Sodium chloride 1M	3	16
Lithium bromide 8M	7	37
Urea 8M	9	23
Guanidine hydrochloride 6M	11	44
Aqueous calcium thiocyanate 50% w/w	23	43
Ethylenediamine	52	100
Sodium hydroxide 0·2M	79	100
Bicarbonate buffers 0·2M (pH 11)	70	100
(pH 10)	20	65
(pH 9)	8	35
Hydroxylamine hydrochloride 1M (pH 7·2)	69	83

be present despite the failure to extract protein with PAW. The wheat endosperm cell walls were dried with ethanol, methanol, and finally n-pentane following their isolation, and this treatment may have altered the solubility properties of any cytoplasmic protein present. Maes (1962) and Graebner *et al.* (1965) found that there was less extractable nitrogen and protein in wheat flour if water-saturated butanol was used to remove lipid prior to the extraction of salt-soluble proteins. No prior lipid extraction was used in the present experiments.

Protein-disaggregating solvents such as urea, guanidine hydrochloride, lithium bromide, and calcium thiocyanate removed appreciable amounts of protein and smaller amounts of polysaccharide from the walls (Table 2). Since these reagents do not disrupt covalent bonds the results suggest that the protein so extracted, representing 23–44% of the total, may have been adherent or loosely attached protein rather than wall protein. Furthermore, Maddy and Kelly (1971) showed that 6M guanidine hydrochloride does not dissociate protein aggregates formed during some methods of membrane-protein isolation and suggest that its disruptive effects on globular proteins may not necessarily apply to other systems such as membrane proteins.

All of the protein and a large proportion of the wall polysaccharide was removed by extraction with dilute alkali and with bicarbonate buffer at pH 11, but

even under the mild conditions of these extractions, peptide linkages could have been broken and certainly any ester-like linkages and *O*-glycosidic linkages involving sugar residues and the β-hydroxyl group of threonine or serine would have been split (Neuberger and Marshall 1966).

(ii) *Amino Acid Analyses of Protein in Water-extracted Walls and in Extracts from the Walls*

The results of amino acid analysis of the protein associated with water-extracted wheat endosperm cell walls and of the protein obtained by extraction with potassium hydroxide and by the successive extraction of the water-insoluble residue with guanidine hydrochloride, hydroxylamine hydrochloride, and bicarbonate buffer pH 11 are shown in Table 3. The analyses indicate that the fractions are similar to one

TABLE 3

AMINO ACID COMPOSITION (MOLE %) OF THE PROTEIN IN WATER-EXTRACTED ENDOSPERM CELL WALLS AND OF PROTEIN EXTRACTED BY VARIOUS SOLVENTS (SEE TABLE 2)

The amino acids were determined as described in Section II(*e*)

Amino acid	Water-insoluble residue	KOH-soluble fraction		6M Guanidine extract	1M Hydroxylamine extract (pH 7·2)	Bicarbonate buffer extract (pH 11)
		I*	II*			
Lysine	5·0	5·7	7·9	5·7	3·9	7·5
Histidine	3·3	2·6	2·3	2·3	1·6	8·6
Arginine	5·2	5·6	7·6	8·4	2·7	7·0
Hydroxyproline	—	—	—	—	—	—
Aspartic acid	6·8	7·1	6·7	6·9	9·3	7·4
Threonine	4·7	5·1	4·0	5·4	5·8	5·4
Serine	7·2	7·2	5·6	9·7	8·5	8·6
Glutamic acid	18·8	14·3	14·7	17·3	20·6	14·8
Proline	6·6	8·0	5·4	8·7	16·5	4·4
Glycine	11·4	13·1	7·3	12·2	13·5	12·7
Alanine	7·6	8·1	8·0	6·6	4·7	7·1
½Cystine	—	—	—	—	—	—
Valine	9·4	7·1	6·7	4·7	3·8	5·0
Methionine	—	—	—	—	—	—
Isoleucine	2·4	2·5	4·2	2·2	1·8	2·6
Leucine	6·5	6·8	9·8	5·5	4·0	5·2
Tyrosine	2·8	3·4	2·1	2·1	1·5	1·5
Phenylalanine	2·5	3·5	3·9	2·4	2·0	2·3

* I, fraction soluble in 80% ethanol; II, fraction insoluble in 80% ethanol.

another in amino acid composition except for the hydroxylamine extract which was enriched in proline and to a lesser extent in the acidic amino acids. The amino acid composition was also similar to those of water-soluble proteins of wheat flour cited by McMasters *et al.* (1971) and of the protein associated with isolated barley endosperm cell walls (Costello 1968). No cysteine or cystine was detected, although a peak with the elution characteristics of cysteic acid was present in all chromatograms and presumably arose by oxidation of cysteine and cystine (Schram *et al.* 1953).

Although the hydrolyses were performed in a large excess of acid (Dustin *et al.* 1953; Schram *et al.* 1953; Neuberger and Marshall 1966), some destruction of

protein and carbohydrate during hydrolysis was indicated by the brownish colour of the hydrolysates and by the presence of a levulinic acid peak in the amino acid chromatograms. No attempt was made to correct the analyses for the losses of specific amino acids which are known to occur during acid hydrolysis.

(iii) *Hydroxyproline Content*

In view of the suggested importance of hydroxyproline in primary plant cell walls (Lamport 1970) and the finding of hydroxyproline-rich glycoproteins in aqueous extracts of wheat flour (Fincher and Stone 1971), endosperm cell walls were analysed for this imino acid. No hydroxyproline was detected by ion-exchange chromatography of amino acids in acid hydrolysates or by a specific colorimetric test [see Section II(*e*)].

(iv) *Hydroxylamine Hydrochloride and Guanidine Hydrochloride Extraction*

The extent of solubilization of protein and carbohydrate by 1M hydroxylamine hydrochloride at different pH values was measured and compared with the effect of buffers at the same pH but in which the hydroxylamine hydrochloride was replaced by 1M NaCl. The results are shown in Figures 3(*a*) and 3(*b*). The ratio of solubilized protein to solubilized carbohydrate was dependent on pH, the ratio decreasing with decreasing pH in the range 8–5. At pH 5, hydroxylamine hydrochloride extracted considerable quantities of carbohydrate but was barely more efficient than buffer at the same pH as a protein extractant.

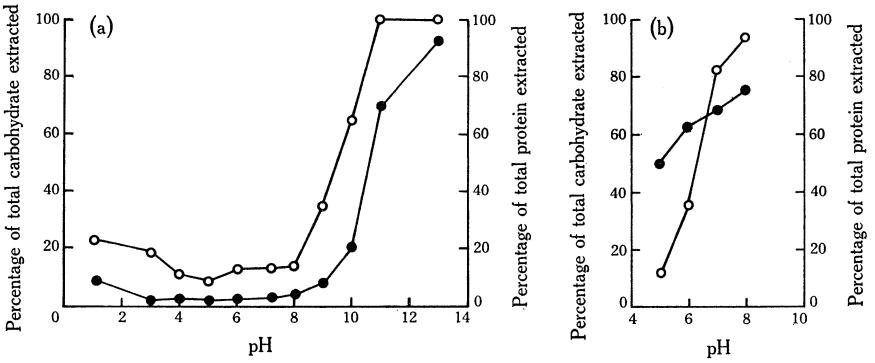


Fig. 3.—Extraction of polysaccharide (●) and protein (○) components from water-extracted endosperm cell walls. (*a*) Extraction with 0·2M sodium acetate–acetic acid (pH 5 and 6) and 0·2M sodium phosphate buffers (pH 7 and 8) containing 1M sodium chloride (3 × 16 hr at room temperature). (*b*) Extraction with 1M hydroxylamine hydrochloride solution buffered to pH 5 and 6 with 0·2M sodium acetate-acetic acid and to pH 7 and 8 with 0·2M sodium phosphate (3 × 16 hr at room temperature).

The monosaccharide composition of the hydroxylamine hydrochloride-extracted polysaccharide (arabinose 34% and xylose 66%) was very close to that of the alkali-soluble arabinoxylan with which it may possibly be identified (see Mares and Stone 1973*a*, Tables 1 and 3). A possible association between polysaccharide and protein in the hydroxylamine hydrochloride extract was investigated by gel filtration chromatography and by phenol–water partitioning. The elution profile from Sepharose

4B is shown in Figure 4 and from these results it can be calculated that at least 70% of the eluted protein was not associated with polysaccharide. Phenol–water partitioning of the extract also separated similar proportions of protein and carbohydrate.

Guanidine hydrochloride extracted 44% of the total protein and 11% of the total carbohydrate. On extraction of the residue with hydroxylamine hydrochloride a further 44% of the total protein and 58% of the total carbohydrate was extracted. The Sepharose 4B profiles of the protein and carbohydrate extracted by these two solvents were qualitatively comparable and similar to the profile of the hydroxylamine hydrochloride extract shown in Figure 4. Since it has been shown that guanidine

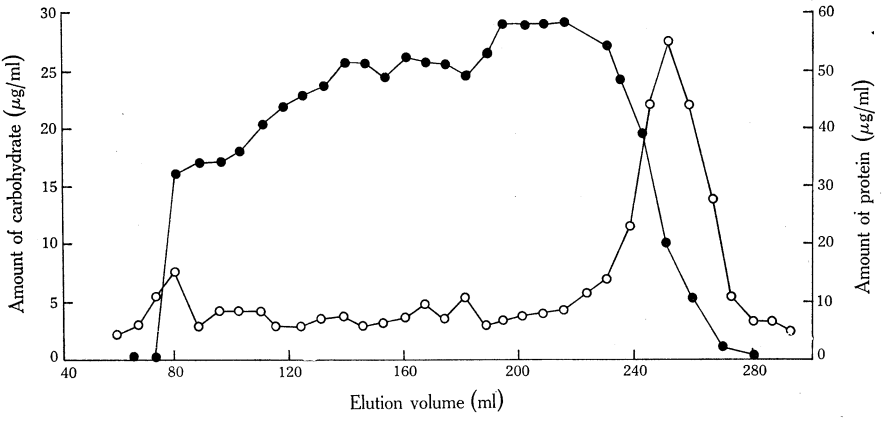


Fig. 4.—Elution profiles from Sepharose 4B for arabinoxylan (●) and protein (○) released from water-extracted endosperm cell walls with 1M hydroxylamine hydrochloride solution at pH 7·2.

hydrochloride does not cleave covalent bonds (Tanford 1968) about half of the protein in the wall preparations is non-covalently bonded, but of the remainder, which is extracted by hydroxylamine hydrochloride, part could be held in the wall by hydroxylamine hydrochloride-sensitive linkages or perhaps is insoluble in guanidine hydrochloride because of its higher proline content. A small part of the total protein appears to be closely associated with the polysaccharide extracted by hydroxylamine hydrochloride and also with the residual wall fraction.

The dramatic effect of pH on the extraction of protein by hydroxylamine hydrochloride remains unexplained. A decrease in the solubility of the protein with decreasing pH, the reaction of amino acid side chains with hydroxylamine hydrochloride, or a combination of these two effects may have been involved. The first possibility is supported by the observation that the protein component of the guanidine hydrochloride extract irreversibly precipitated if the extract was dialysed free of guanidine hydrochloride, concentrated, and stored in the cold.

The finding that neutral hydroxylamine hydrochloride solutions can solubilize a substantial polysaccharide component from water-extracted wheat endosperm walls and that this polysaccharide was quantitatively and qualitatively similar to the fraction solubilized with dilute alkali prompted a comparison of the action of hydroxylamine hydrochloride and 0·2M sodium hydroxide on barley and *Lolium* endosperm cell walls. These wall preparations were first extracted with 8M urea to remove a

β-glucan component (Costello and Stone 1968; Smith and Stone 1973) and then for 30 hr at room temperature with either 0·2M sodium hydroxide containing 0·1% sodium borohydride or 1M hydroxylamine hydrochloride (pH 7·2). The latter solution extracted 43 and 35% and the sodium hydroxide 50 and 41% by weight of the barley and *Lolium* walls respectively. The urea-extracted walls of both barley (Costello and Stone 1968) and *Lolium* endosperm (Smith and Stone 1973) consist of polysaccharides composed of arabinose, xylose, and glucose together with some protein.

(v) *Effect of Protein and Polysaccharide Hydrolases*

The proportions of total wall protein and polysaccharide solubilized by a range of protein and polysaccharide hydrolases and the approximate size of the digestion products are shown in Table 4. The proteolytic enzymes solubilized protein without

TABLE 4

ENZYMIC DEGRADATION OF WATER-EXTRACTED ENDOSPERM CELL WALLS

Walls were digested for 50 hr at 40°C with protein hydrolase preparations dissolved in 0·02M sodium phosphate buffer (pH 6·8) and polysaccharide hydrolase preparations dissolved in 0·02M sodium acetate–acetic acid buffer (pH 5·0)

Enzyme	Percentage of total protein solubilized	Percentage of total carbohydrate solubilized	Bio-Gel P-2 chromatography (exclusion limit 2600)	
			Protein	Carbohydrate
Pronase	99	7	Most retarded	Excluded
Chymotrypsin	99	9	Part excluded, part retarded	Excluded
Trypsin	98	10		Excluded
Cellulosin	56	89		Retarded
Sclerotium rolfsii enzymes	4	59	—	Most excluded
Macerozyme	57	73	Part excluded, part retarded	Most excluded

substantial extraction of wall polysaccharide and Bio-Gel P-2 chromatography of the tryptic and chymotryptic digests indicated that 30–40% of the material released had a molecular weight greater than 2000. Pronase, however, reduced the wall protein to small peptides and amino acids. The polysaccharide hydrolase preparations Cellulosin and Macerozyme solubilized approximately 60% of the wall polysaccharide, most of which was excluded from Bio-Gel P-2 and therefore had an apparent molecular size > 2000. These enzyme mixtures also solubilized somewhat more than half of the protein associated with the wall.

The insoluble residues obtained following digestion with Pronase and with *S. rolfsii* enzymes were extracted successively with guanidine hydrochloride (6M) and neutral hydroxylamine hydrochloride solutions. The former removed some protein from the residue left after digestion with *S. rolfsii* enzymes but had little effect on the polysaccharide. Hydroxylamine hydrochloride removed polysaccharide from the Pronase-insoluble residue and some protein from the *S. rolfsii*-insoluble residue. These results indicated that protein and polysaccharide components of the water-

extracted endosperm cell wall could be released independently of one another without affecting the solubility of the components remaining in the wall structure, and that proteolytic enzymes could be used to prepare cell walls which were substantially free of protein.

Samples of the residues of water-extracted walls following enzymic hydrolysis were examined in the electron microscope with the following results:

1. *S. rolfsii*-insoluble residue. The microfibrillar phase was not affected but the matrix materials appeared to have been removed and the surface of the residue resembled that of cell walls extracted with alkali (Mares and Stone 1973*a*, Figs. 8 and 9).
2. Macerozyme-insoluble residue. The residual material resembled that from the *S. rolfsii* digestion.

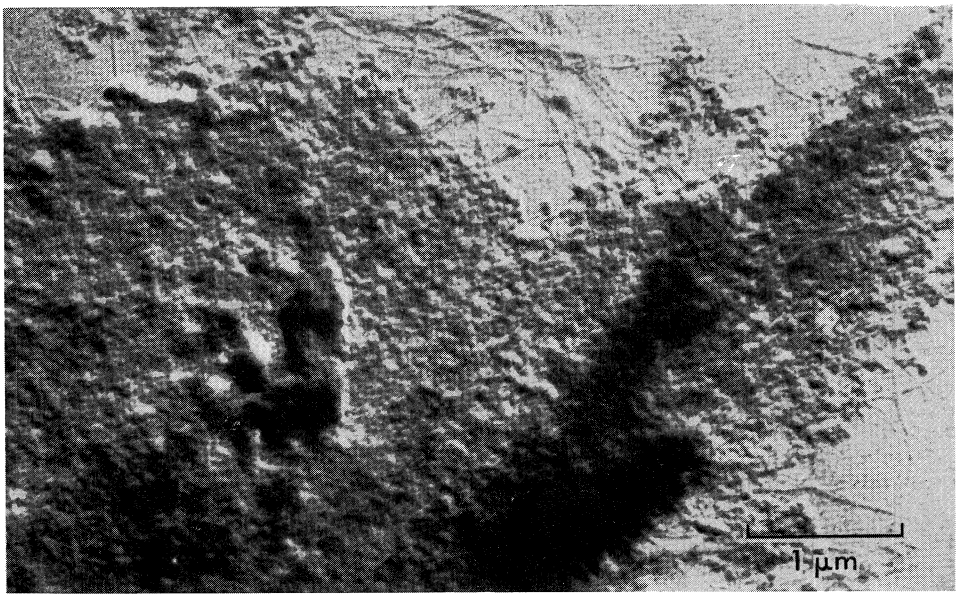


Fig. 5.—Electron microscopy of Cellulosin-digested cell walls. Air-dried samples were shadowed with platinum–palladium as described by Mares and Stone (1973*a*). Only a few microfibrils are present at the edges of a network of globular particles which represents the cell wall residue.

3. Cellulosin-insoluble residue. After 5 hr digestion the microfibrillar phase had been considerably reduced (Fig. 5) and later the microfibrils disappeared altogether and the residue had the appearance of a mat of globular particles. Monosaccharide analysis of the polysaccharides in the residue revealed the presence of glucose and mannose and smaller amounts of xylose and arabinose.
4. Proteolytic enzyme-insoluble residues. Micrographs of these residues resembled those of water-extracted cell walls (Mares and Stone 1973*a*) except for the almost complete absence of particles.

As judged by the extent of chemical conversion and the microscopic appearance of the residue, Cellulosin appeared to be the most effective preparation for degrading isolated wheat endosperm cell walls. However, as shown in Table 4, the solubilized polysaccharides and proteins were not completely converted to monosaccharides and amino acids respectively, even after prolonged incubation.

(*c*) *Ester Content*

Determination of the ester content [Section II(*g*)] of wheat endosperm cells extracted with guanidine hydrochloride and barley and *Lolium* endosperm cell walls which had been extracted with urea gave values of 0·9, 0·7, and 1·6 mg ethyl acetate equivalents per 100 mg of wall sample respectively. Examination of water-extracted wheat endosperm cell walls, before and after digestion with Pronase, indicated that the ester content was not affected by the removal of protein.

(*d*) *Extraction and Estimation of Polyphenols*

(i) *Qualitative Colour Tests*

Ferulic acid, diferulic acid, and cell wall extracts [Section II(*h*)] were examined under u.v. light (Hillis 1966) and after spraying with diazotized benzidine (Randerath 1966). The colours observed are described in Table 5.

TABLE 5

REACTIONS OF EXTRACTS FROM ENDOSPERM CELL WALLS TO ULTRAVIOLET LIGHT AND DIAZOTIZED BENZIDINE SOLUTION

Sample	Colour under u.v. light	Colour with diazotized benzidine
Ferulic acid	Blue-white	Light brown
Diferulic acid	Maroon	Dark brown
Water-insoluble residue of endosperm cell wall		
Ethyl acetate-soluble	Maroon with white border	Yellow-orange
n-Butanol-soluble	Pale yellow	Light brown

(ii) *Total Polyphenolic Residues*

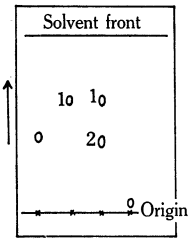
The total polyphenolic residues in the water-soluble fraction of endosperm cell walls, in the combined ethyl acetate and n-butanol extracts of the water-insoluble residue, and in extracts of the non-endospermic cell walls discarded during the isolation of endosperm cell walls were 0·14, 0·58, and 0·66 mg ferulic acid equivalents per 100 mg of sample respectively.

(iii) *Separation of Polyphenolic Residues*

Thin-layer chromatography and gas–liquid chromatography of trimethylsilyl derivatives were used to separate polyphenolic components in extracts of endosperm cell wall fractions. The results are shown in Figures 6 and 7 respectively. The water-

soluble pentosan of wheat endosperm cell walls contained only one component which did not appear to be strongly bound to polysaccharide, since it was released by extracting acidified solutions (pH 3·5) of water-soluble pentosans with ether or ethyl acetate. The water-extracted endosperm cell walls contained at least two components, neither of which was ferulic or diferulic acid. Component 1 appeared to be identical with the compound isolated from the water-soluble fraction whilst component 2 had a longer retention time in gas–liquid chromatography than ferulic acid, indicating that this compound had a higher molecular weight or more polar groups than ferulic acid or both (Pellizzari *et al.* 1969). However, since it was observed that the dried residues of extracted materials were only slightly soluble in methanol and ethyl acetate, the possibility remains that other polyphenolic components, although present, were not extracted under the conditions used. Control experiments in which ferulic and diferulic acids were treated as described above indicated that no significant destruction of these compounds occurred during extraction.

Fig. 6



Component	Colour	R_F
1	yellow	0·59
2	brown	0·38
Ferulic acid	brown	0·41
Diferulic acid	brown	0·04

Fig. 7

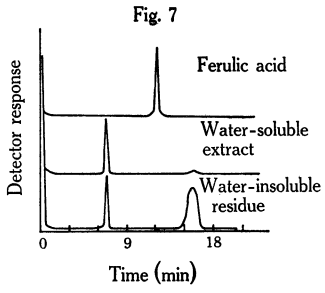


Fig. 6.—Thin-layer chromatography of endosperm cell wall extracts. Extracted compounds were chromatographed on silica gel plates with n-butanol–pyridine–water (14 : 4 : 5 v/v) (Geissman 1970) and developed by spraying with diazotized benzidine (Randerath 1966). The colour with diazotized benzidine and the R_F values of the components are shown in the accompanying table.
Fig. 7.—Gas–liquid chromatography of endosperm cell wall polyphenols and standard compounds. Trimethylsilyl derivatives were separated on a 6-ft column containing Gas Chrom Q coated with 3% OV-1 operated at 180°C.

Although ferulic acid was not found in endosperm cell walls, unidentified polyphenols were detected in extracts of non-endosperm cell walls and may have included ferulic acid which has been reported in aleurone cell walls (Fulcher *et al.* 1971).

IV. Discussion

(*a*) *Arabinoxylan Fractions from Cell Walls*

It is evident from gel-filtration studies that the water- and alkali-soluble arabinoxylans from the cell walls are polydisperse but have similar apparent molecular size distributions ($8 \times 10^4 - 5 \times 10^6$). Since the profiles [Figs. 2(*a*) and 2(*b*)] were asymmetric the polysaccharide fraction eluting at the void volume of the column was presumably outside the fractionation limit of the gel and hence the upper size limit of the arabinoxylan was probably somewhat greater than 5×10^6.

Blake and Richards (1971) have presented evidence that hemicellulose fractions from sugarcane bagasse, guinea grass, and Townsville lucerne undergo molecular aggregation, this property being dependent on salt concentration and on the thermal history of the polysaccharide solution. Similar behaviour was observed in solutions of the arabinoxylans from wheat endosperm cell walls following dispersion by heating, so that standard conditions are essential for the preparation of samples for gel-filtration chromatography or other analyses dependent on physical characteristics.

In ammonium sulphate fractionation of wall arabinoxylans the proportions of the total polysaccharide precipitated at each concentration and the xylose : arabinose ratios in these precipitates were generally consistent with the results obtained by Preece and Hobkirk (1953, 1954). The xylose : arabinose ratio decreased with increasing solubility in ammonium sulphate solution and the alkali-soluble arabinoxylan was slightly less soluble in ammonium sulphate solutions than its water-soluble counterpart. The differences in molecular size and solubility in ammonium sulphate between the water- and alkali-soluble arabinoxylans were not, however, of sufficient magnitude to account for the different conditions required to extract them from the cell wall, suggesting that these differences might be better explained in terms of chemical interactions with other wall components.

(*b*) *Interactions between Polymers in the Cell Wall*

(i) *Proteins*

The amino acid composition of the protein present in the isolated wall is very similar to that of soluble cytoplasmic proteins from wheat endosperm. Under the conditions of cell wall isolation (Mares and Stone 1973*a*) the protein bodies carrying the gluten-type proteins would be physically removed, but some of the cytoplasmic and membrane proteins which are not dispersed in the 70% ethanol suspending medium are likely to remain in the preparation.

The evidence obtained indicates that the protein present in the isolated walls is probably not cross-linked to polysaccharides. Proteolytic enzymes removed nearly all the protein without affecting the solubility of the residual polysaccharides and examination of the cell wall material extracted with guanidine hydrochloride and neutral hydroxylamine hydrochloride solutions showed that the major portion of the protein was not covalently bound to polysaccharide. At least half of the "associated" protein appeared to be adherent protein of cytoplasmic origin which may be identified with the irregularly shaped particles observed in electron micrographs of the surface of cell wall fragments. However, it could not be determined whether the residual protein was evenly distributed through the wall structure or was concentrated in particular areas. Most workers (Lamport 1965, 1969, 1970; Thompson and Preston 1967, 1968; Mitchell and Taylor 1969; Sadava and Chrispeels 1969; Albersheim and Anderson 1971) agree that plant cell walls generally contain protein as a structural component as well as in the form of wall-bound enzymes. The structural protein has been shown to be rich in hydroxyproline and to be glycosidically linked via this residue to arabinose (Lamport 1965, 1969, 1970; Albersheim and Anderson 1971; Heath and Northcote 1971). However, in wheat endosperm cell walls as prepared in these studies, it has not been possible to demonstrate the presence of hydroxyproline or protein–polysaccharide cross-links.

(ii) *Polysaccharides*

The finding that water-insoluble arabinoxylans could be extracted from cell walls with dilute NaOH, HCO_3^- buffers, and neutral hydroxylamine hydrochloride but not with concentrated guanidine hydrochloride or urea solutions was consistent with their being anchored in the wall by alkali-labile ester-like cross-links rather than by simple physical entrapment. Although in solutions with high pH values the polysaccharide framework of the wall could swell and release trapped or hydrogen-bonded components, this is probably not the cause of solubilization by the bicarbonate buffers which were below the pH at which sugar hydroxyl groups become ionized (pH 12–13) (Capon and Overend 1960). Certainly neutral hydroxylamine, which was an effective solubilizing agent, would not be expected to alter the conformation of the wall polysaccharides.

Lamport (1970) has proposed ester bonding between the carboxyl group of a uronic acid and the hydroxyl group of a sugar residue as a possible inter-polysaccharide linkage in cell walls. Such a linkage could occur in endosperm cell walls since a small amount of uronic acid has been detected in the alkali-soluble and -insoluble cell wall fractions (Cole 1967; Mares and Stone 1973*a*).

Geissman (1970) has also proposed that ferulic acid residues esterified to arabinoxylans could cross-link adjacent pentosan chains by dimerization and make them insoluble in water. He gave support to this possibility by demonstrating that diferulic acid was formed when solutions of certain water-soluble pentosans from wheat flour or of a chemically synthesized feruloylguaran were oxidized with hydrogen peroxide in the presence of peroxidase. While ferulic or diferulic acids were not detected in extracts of endosperm cell walls, it is possible that one or other of the presumptive polyphenolic components present in the wall extracts could play a similar role. Due to their low concentration, and the difficulties encountered in extracting these "polyphenolics" from wheat endosperm cell wall fractions, these compounds have not been positively identified.

Whatever the nature of the cross-links between the arabinoxylan chains or between arabinoxylans and other cell wall polymers it is clear that only a few bridges would be necessary to render the large molecules insoluble.

An increase in the amount of water-extractable polysaccharides occurs in barley (Preece and MacKenzie 1952) and possibly other cereals (Pomerantz 1961) during germination, and it has been suggested that these are derived from their water-insoluble counterparts. If ester linkages are, in fact, important in endosperm cell walls, esterases may be involved in the release of soluble polysaccharides during germination. Simple esters have been reported as substrates for esterases in wheat aleurone and germ (Engel 1947), in barley endosperm (Prentice *et al.* 1971), and in malted barley grain (Burger *et al.* 1970), so that enzymes which could account for physical and chemical alterations in cell wall polysaccharides during germination are present, but further examination is necessary to assess their role.

(*c*) *Comparison of Endosperm Cell Wall Composition with that of Primary Plant Cell Walls*

The endosperm cell walls isolated from Insignia wheat flour are composed of cellulose, glucomannan, arabinoxylan, and protein. The protein appears to be

largely of cytoplasmic origin. Neither the protein nor the water-soluble portion of the arabinoxylan appear to be bound to the other cell wall polymers and there is evidence that the water-soluble polysaccharides are located at the surface of the cell wall (Mares and Stone 1973*a*). The water-insoluble part of the arabinoxylan, on the other hand, is held in the cell wall structure by ester-like linkages between adjacent arabinoxylan molecules, between arabinoxylans and other cell wall polysaccharides, or both. Evidence that "ester-like" linkages are present in endosperm walls isolated from barley flour and from cultured *Lolium multiflorum* endosperm suggests that these linkages may be a common feature of gramineous endosperm cell walls.

A comparison of endosperm cell wall composition with that reported for primary plant cell walls (Setterfield and Bayley 1961) emphasizes the simple nature of the endosperm walls, the pectic component being absent and the amount of cellulose markedly reduced. The microfibrils in the endosperm wall are largely composed of cellulose but also contain glucomannan which can only be separated with difficulty. This situation is analogous to that for wood glucomannans which are also reported to be closely associated with the surface of the cellulosic microfibrils (Timell 1965).

The relatively simple composition and organization of wheat endosperm walls may reflect an adaptation to their function in the compact storage tissue of the grain. Structural rigidity is not of prime importance in the mature grain where the cells are completely filled with a mass of protein bodies and starch granules embedded in a proteinaceous matrix. During development, while starch and protein are accumulating, some structural rigidity is required and this could be provided by the microfibrillar framework. At germination the wall provides a barrier to easy movement of the enzymes mobilizing protein and starch reserves in the cells, but the arabinoxylan which forms the bulk of the wall matrix can be readily solubilized and depolymerized to allow access of enzyme molecules. The wall thus appears well adapted for its transient structural role during endosperm development and for ready dissolution during germination.

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