STUDIES ON WHEAT ENDOSPERM

I. CHEMICAL COMPOSITION AND ULTRASTRUCTURE OF THE CELL WALLS

By D. J. MARES*† and B. A. STONE*‡

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Abstract

A method is described for the isolation of wheat endosperm cell walls free from non-endospermic cell walls in a 70% ethanol medium which prevents the loss of water-soluble polymeric components. The isolated walls have been fractionated by successive extractions with water, 0·05M EDTA, 4·27M KOH, and 4·38M NaOH containing 0·81M H_3BO_3. The cell walls, as isolated, contain 14–15% protein and 75% polysaccharide, 85% of which is arabinoxylan, whilst the remainder consists of equal amounts of β-glucan and β-glucomannan. Walls prepared from Insignia, Olympic, and Wren wheat flours were very similar, both with respect to the proportions of the polymeric components and to the monosaccharide composition of the walls and wall fractions.

The appearance of endosperm cell walls *in situ* and in wall isolates was examined by light microscopy, scanning and transmission electron microscopy, and a technique is described for the scanning electron microscopy of walls in endosperm sections. Scanning electron micrographs show the apparent moulding of the walls on the cell contents and the different fracture patterns of prismatic and central cells. The cell walls have a microfibrillar skeleton embedded in the amorphous matrix components.

I. INTRODUCTION

The endosperm of wheat has been the subject of numerous studies with respect to the commercially valuable intracellular starch and protein components, but few investigations have been made of the endosperm cell walls. The details of their chemistry and organization is of significance in understanding some of the processes occurring during the germination of the grain when an alteration of the wall accompanies the mobilization of the starch and protein. In milling, and in other manipulations of wheat products such as cooking, the walls are ruptured or disorganized and a knowledge of their composition and construction could foreshadow useful modifications of these processes. The importance of endosperm cell walls in relation to milling quality remains uncertain. Wolf *et al.* (1952) and Larkin *et al.* (1952) examined several Pacific Northwest wheats and were able to show that, whilst quantitative differences in water-insoluble hemicelluloses were not correlated with different milling qualities, the thickness of the endosperm cell walls in the subaleurone regions appeared to be important.

Although much is known of the chemistry and physical properties of the non-starchy polysaccharides of endosperm (see D'Appolonia *et al.* 1971 for a summary), it

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is not certain which of these are cell wall components since in most studies the polymers have been extracted from whole wheat flour. To obtain direct information concerning the cell walls, it is necessary to isolate walls which can be identified as originating from endosperm cells and to separate them from their intracellular contents and non-endosperm cell walls by methods which do not alter their composition or structural integrity.

Wolf *et al.* (1952) described a micromethod for the isolation of wheat endosperm cell walls but this procedure is not applicable to the preparation of quantities suitable for chemical analysis and fractionation. Costello and Stone (1968) developed a method, based on the earlier procedure of Greer *et al.* (1961), for the recovery of large quantities of cell walls from the "tailings" fraction of barley flour from which the starch and protein had been removed by the "batter" process (D'Appolonia *et al.* 1971). This method suffers the disadvantage that water-soluble polymeric components present in the native walls are lost during the isolation, and unsuccessful attempts were made to overcome this by using non-aqueous isolation media such as chloroform–methanol. Subsequently a method was developed using 70% aqueous ethanol as the isolation medium. This paper reports the details of this method, the chemical characteristics of the isolated walls from three varieties of wheat of different milling quality, and describes the ultrastructure of the walls. In later papers further details of the chemistry of the wall components are given (Mares and Stone 1973*a*) and the occurrence of galactose-rich endosperm polysaccharides is discussed (Mares and Stone 1973*b*). The ontogeny of endosperm walls during the early stages of endosperm development has also been studied (Mares and Stone, unpublished data).

II. Materials and Methods

(*a*) *Materials*

Wheat (*Triticum aestivum* L. cv. Insignia, Olympic, and Wren) samples were supplied by Mr. K. Mander, Victorian Department of Agriculture. Sugars used for preparing standards for gas–liquid chromatography (g.l.c.) were recrystallized commercial preparations. D-Glucuronic acid was obtained from Pfanstiehl Laboratories Inc., Waukegan, Illinois. Cellulosin AP was a gift of Ueda Chemical Industry Co., Osaka, Japan. *Sclerotium rolfsii* enzymes were prepared according to the general method of Reese and Mandels (1966) using *Lolium multiflorum* endosperm cell walls as a carbon source. Salivary amylase was prepared from human saliva by the procedure of Bernfeld (1955) up to the acetone precipitation stage. *Aspergillus niger* glucoamylase was prepared according to Fleming and Stone (1965). *Pseudomonas syringae* uronate dehydrogenase was prepared by the method of Bateman *et al.* (1970). Glucose oxidase (Sigma type II) was purchased from Sigma Chemical Co., St. Louis, Mo. Lactoperoxidase was prepared according to Hogg and Jago (1970). Nylon bolting cloth, heavy quality, linear pore size 75 μm, was from Henry Simon, St. Kilda, Vic. Araldite epoxy resin hardener (HY964) and accelerator (DY 064) were obtained from Ciba Co. Pty. Ltd., Lane Cove, N.S.W.

(*b*) *Microscopy*

(i) *Light Microscopy*

Isolated walls and wall fractions were directly examined with phase-contrast optics or were stained with congo red prior to examination.

(ii) *Transmission Electron Microscopy*

Metal-shadowed preparations.—Walls and wall fractions were air-dried onto Formvar-coated copper grids and shadowed with platinum–palladium (30 : 70) at an angle of approximately 30° to the horizontal.

Thin sections.—Walls and wall fractions were fixed for 12 hr in absolute ethanol and embedded in Araldite. Sections were stained with lead citrate or treated with sodium methoxide to remove the Araldite and the residue shadowed with platinum–palladium. Sections were examined in a Hitachi HS-8 electron microscope at 50 kV.

(iii) *Scanning Electron Microscopy*

Median cross-sections of the kernel were cut from dry grains with a razor blade or from grains which had been fixed with 10% acrolein for 2–12 hr on a Reichert freezing microtome. In some instances the grains were pretreated in boiling water for 10 min to gelatinize starch granules in the endosperm. Some sections were treated with salivary amylase for 2 days or with 0·01M NaOH for 1 hr before drying in ethanol and mounting on metal stubs for examination in a JEOLCO JSM U-3 scanning electron microscope at 15–20 kV.

(c) *Analytical Methods*

(i) *Nitrogen*

Nitrogen was determined by the microKjeldahl method by the Australian Microanalytical Service, University of Melbourne.

(ii) *Protein*

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

(iii) *Carbohydrate Analysis*

The method used was an adaptation of the procedure of Feingold *et al.* (1958). A sample of walls or wall fraction (2–3 mg) was weighed into a glass vial, chilled, and 1 ml of HCl, saturated with HCl gas (in a bath of acetone–dry ice), was added. The contents of the vial were immediately frozen and the vial sealed. After incubation at 40°C for 1 hr the vials were refrozen, opened, and the contents neutralized with 2·5M NaOH (bromphenol blue end point). After appropriate dilution the reducing sugars present were estimated by the method of Somogyi (1952) and Nelson (1944) using a 20-min heating period and a xylose standard. Hexoses were quite stable under the hydrolysis conditions, but pentoses, particularly xylose, were subject to losses and corrections were made, although these did not exceed 15%.

(iv) *Hydrolysis and Analysis of Monosaccharides in Hydrolysates*

For samples containing cellulose or glucomannan the saturated HCl hydrolysis procedure was used. Other samples were hydrolysed in 0·5M HNO_3 containing 0·5% (w/v) urea (Jermyn and Isherwood 1956). Standard mixtures of sugars corresponding to the expected wall composition were treated under identical conditions. Following hydrolysis the samples were neutralized with NaOH.

Alditol acetate derivatives of monosaccharides were prepared essentially following the method of Albersheim *et al.* (1967) and analysed by g.l.c. The alditol acetate mixtures were separated by chromatography in a stainless steel column (6 ft by 0·125 in. outer diam., 0·016 in. wall) packed with a mixture of equal parts of 100–200 mesh Gas Chrom Q (Applied Science Laboratories, College Park, Pa.) coated with 3·4% silicone oil (XF 1150) (General Electric) and 2·6% ethylene glycol succinate (Applied Science Laboratories, College Park, Pa.) (Dr. D. G. Widmark, Institute of Analytical Chemistry, University of Stockholm, personal communication) on a F & M series 5750 gas chromatograph equipped with a flame ionization detector. Isothermal analyses were run at 180°C with a nitrogen carrier gas flow rate of 25 ml/min, injection port temperature 280°C, detector temperature 320°C, and at a sensitivity of 10 × 32. A glass insert liner was used at the injection port. The proportion of each individual alditol acetate in the mixtures was determined relative to an internal inositol acetate standard by reference to a calibration curve of peak height alditol acetate/peak height inositol acetate versus amount of monosaccharide present. These curves were constructed by submitting known mixtures of monosaccharides and inositol to the complete procedure for forming derivatives and were linear for each sugar over the range of concentrations used in the analyses.

Precision data for the method was obtained by subjecting a sample containing five monosaccharides together with an inositol internal standard to reduction, acetylation, and assay as described. The sample was injected a total of 20 times. The calculated mean percentage composition of the sample and associated standard deviations of the mean were as follows: L-arabinose, $18{\cdot}42 \pm 0{\cdot}41$; D-xylose, $17{\cdot}55 \pm 0{\cdot}22$; D-mannose, $20{\cdot}67 \pm 0{\cdot}17$; D-galactose, $19{\cdot}91 \pm 0{\cdot}27$; D-glucose, $23{\cdot}60 \pm 0{\cdot}51$.

(v) *Uronic Acids*

Samples for assay were hydrolysed by incubation with a mixture of Cellulosin and *Sclerotium rolfsii* enzymes in a 0·2M acetate buffer (pH 5·0) for 3–4 days at 40°C in the presence of toluene. Aliquots were evaporated to dryness and total uronic acids determined using *Pseudomonas syringae* uronate dehydrogenase which oxidizes both glucuronic and galacturonic acids. The assay system based on the reduction of NAD^+ was essentially that of Kilgore and Starr (1959). D-Galacturonic acid was used as a standard.

(vi) *Starch*

Walls and wall fractions were incubated with salivary amylase at 40°C for 3 days in maleic acid–sodium maleate buffer (10 mM, pH 6·5) containing 10 mM NaCl and 1 mM $CaCl_2$ (Walker and Hope 1963). The incubation mixture was centrifuged in a MSE bench centrifuge at 1000 *g* for 15 min, the supernatant removed, and the pellet washed. The combined supernatant and washings was incubated with *Aspergillus niger* glucoamylase (Fleming and Stone 1965) in acetic acid–sodium acetate buffer (0·2M, pH 4·5) at 40°C until no further reducing sugars were liberated. The glucose released from the starch was estimated by the glucose oxidase procedure of Lloyd and Whelan (1969), using lactoperoxidase instead of horseradish peroxidase.

(vii) *Amino Acid Analysis*

Samples containing 1 mg of protein were weighed into 250-ml glass vials and 200 ml of constant boiling HCl added. After freezing in an acetone–dry ice bath, the tubes were repeatedly evacuated and flushed with nitrogen using a two-way tap. The vials were finally sealed in an atmosphere of nitrogen, thawed, and hydrolysed at 110°C for 24 hr. The contents of each tube were filtered through glass-wool, evaporated to dryness, dissolved in 5 ml of citrate buffer (pH 2·2), passed through a Millipore filter (Millipore Corp., Bedford, Mass.), and analysed on a Beckman–Spinco amino acid analyser.

(viii) *Hexosamines*

Hexosamines in the hydrolysates of walls and wall fractions were detected and estimated in the amino acid chromatograms. Under the elution conditions used, no differentiation between glucosamine and galactosamine could be made.

(ix) *Bio-Gel P-2 Chromatography*

Samples of cell wall extracts for analysis were dissolved in 1 ml of 0·5% sodium azide and loaded onto a 47·5 by 1·5 cm column of Bio-Gel P-2 (200–400 mesh) at room temperature. Each sample was eluted in 0·5% sodium azide and 3–4 ml fractions were collected and assayed for protein and carbohydrate. Bio-Gel P-2 separated peptides and proteins with molecular weights in the range 200–2000.

(d) Preparation of Grain for Wall Isolation

(i) *Pearling*

Prior to milling, wheat grains were pearled in a Satake grain testing mill (Satake Engineering Co. Ltd., Tokyo). Samples of grain (150 g) were pearled for 2 min at 105 r.p.m. using a medium abrasive stone with a resultant loss of 23% of the grain mass. Loose fragments of husk and stalks were removed by hand and the grain cleaned by blasting with dry compressed air.

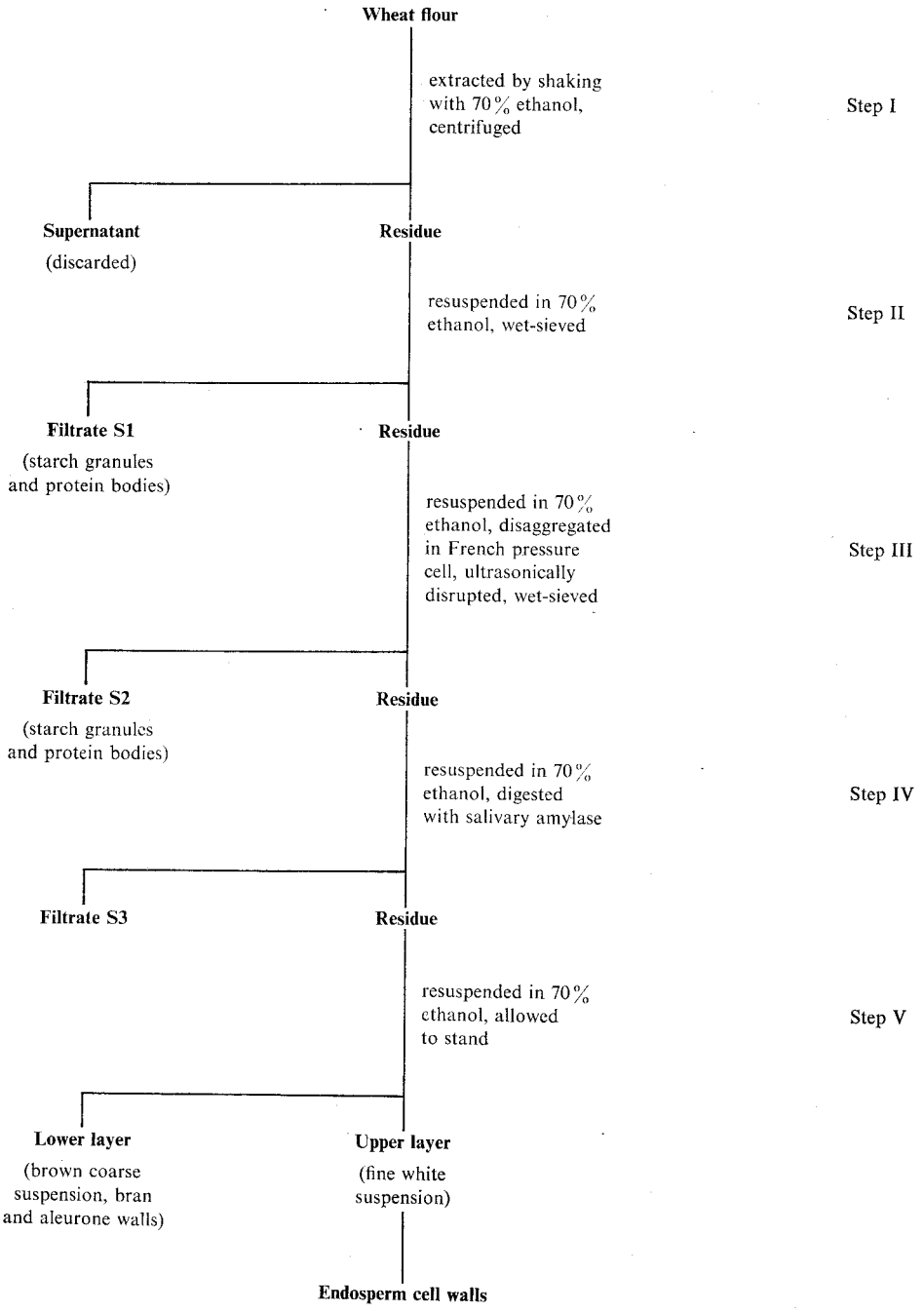


Fig. 1.—Outline of the procedure for the isolation of wheat endosperm cell walls in 70% ethanol.

(ii) *Milling*

Pearled wheat grain was milled in a Buhler Automatic Mill model type MLU-202 (Buhler Brothers, Uzwill, Switzerland) at 100 g/min after adjusting the moisture content to 13·5%. The clearances on the three break rollers were set at 0·5, 0·1, and 0·06 mm and all reduction rollers were set at 0·1 mm. The sieves beneath the reduction rollers were removed so that break flour passed through the first reduction roller only and was then collected. The reduction flour, which accounted for 77·5% of the pearled grain, was used in subsequent isolation steps.

(*e*) *Isolation of Endosperm Cell Walls*

The procedure is summarized in Figure 1 and the details of the individual steps are given below.

Step I.—Reduction flour was extracted with 70% ethanol, with shaking, for 1 hr at 4°C. The solids (starch, protein, and walls) were collected by centrifugation and the clear yellow supernatant containing soluble materials discarded.

Step II.—The residue was resuspended in 70% ethanol, shaken in the cold for 4 hr, and wet-sieved on nylon bolting material (linear pore size 75 μm). Fraction S1 was the material which passed the sieve.

Step III.—The solid material retained on the sieve was resuspended in 70% ethanol, disrupted in a French pressure cell (Aminco Bowman Ltd., Silver Springs, Md., U.S.A.) at 3–5 tons/in.^{2} (0·47–0·78 $\times$ 10^7 Pa) and the cold suspension treated in an MSE 150-W ultrasonic disintegrator (19 mm probe) adjusted to maximum amplitude, for three 5-min periods with cooling between each period, then wet-sieved. Starch granules and aggregates of protein passed through the sieve (fraction S2).

Step IV.—The sieve residue was again resuspended in 70% ethanol and digested with human salivary amylase in 70% ethanol containing 10 mM maleic acid–sodium maleate, 10 mM sodium chloride, and 1 mM calcium chloride (pH 6·5) at 40°C for 1 week. Partially digested starch was further loosened by ultrasonic disruption and removed by wet-sieving. The sieve residue was washed thoroughly with 70% ethanol. Fraction S3 was the material passing the sieve.

Step V.—The sieve residue was resuspended by ultrasonic treatment in 70% ethanol in a 2-litre beaker, allowed to stand for 5 min, and the upper, white suspension removed. This process was repeated several times until the residue from step IV had been divided into a white suspension of endosperm cell walls and a coarse brown fraction which was predominantly non-endosperm cell walls but contained some endosperm cell walls.

Step VI.—The endosperm walls were dried by solvent exchange by washing them successively with absolute ethanol, absolute methanol, and dry n-pentane and stored in a desiccator over silica gel.

(*f*) *Examination of the Purity of the Endosperm Cell Wall Preparation*

Throughout the isolation procedure the light microscope was used to visually check the progress of the purification. Initially sheets of cell walls from a number of adjacent cells were present, but these were largely disrupted in the French pressure cell. Small fragments of endosperm cell walls were found in the sieve filtrates and wall fragments heavily contaminated with starch and protein were observed to sediment with the non-endospermic cell walls.

The homogeneity of the final endosperm cell wall preparation was estimated by a particle count under the light microscope which indicated that less than 4% non-endosperm cell walls were present. Some wall fragments were observed to carry groups of small starch granules which had resisted amylase digestion and loosening by ultrasonic disruption. In general, the cell wall fragments appeared clean and smooth.

(*g*) *Fractionation of Isolated Endosperm Cell Walls*

Endosperm cell walls isolated by the procedure described in Section II(*e*) and Figure 1 were fractionated by chemical extraction according to the procedure outlined in the flow diagram (Fig. 2) which follows established methods for plant cell wall fractionation. Sodium borohydride was employed in all alkaline extraction steps to prevent degradation of polysaccharide chains by the alkaline peeling reaction (Head 1955; Whistler and BeMiller 1958). Potassium hydroxide extracts were neutralized with perchloric acid at 4°C and the resultant precipitate of potassium perchlorate

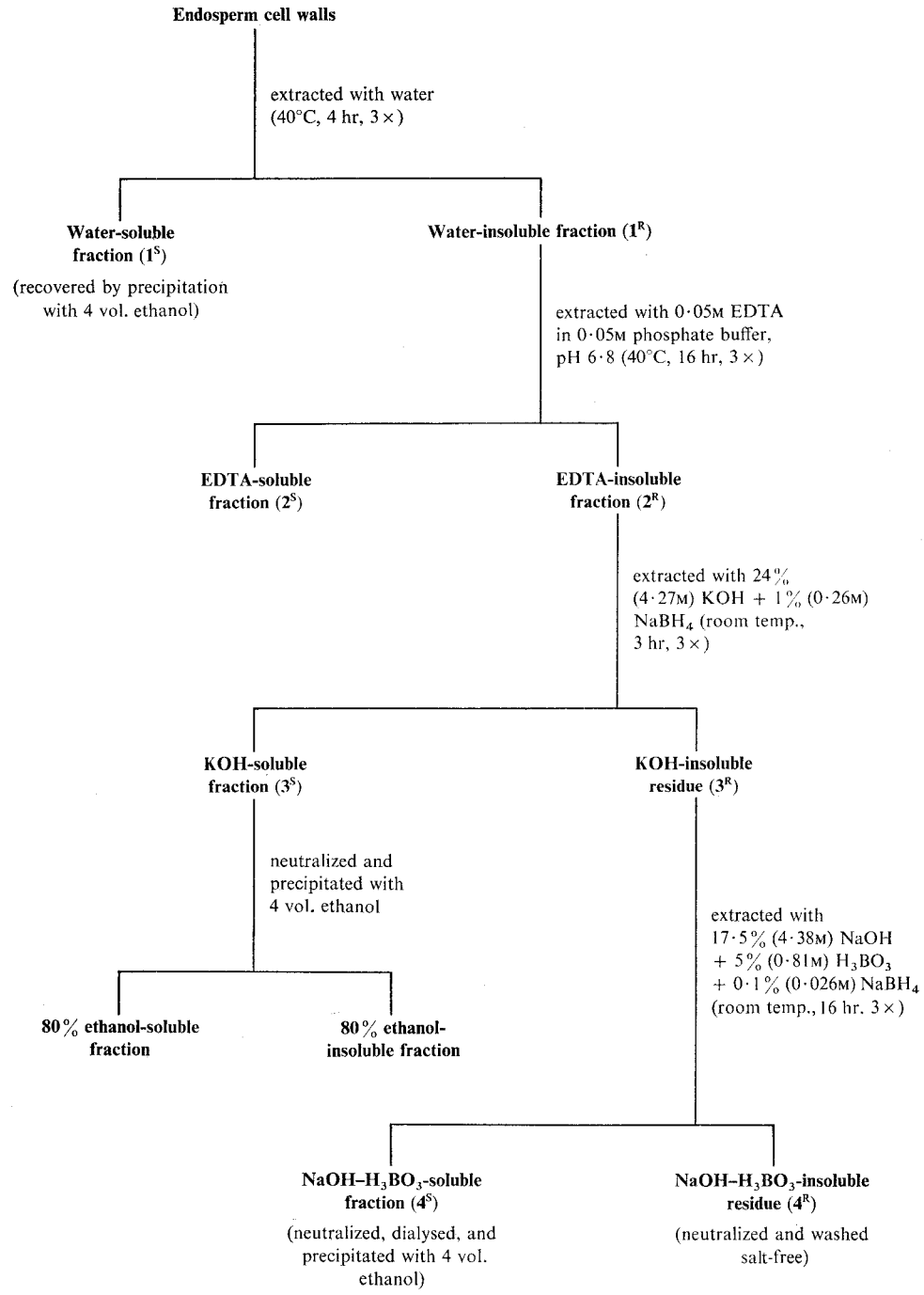


Fig. 2.—Flow diagram showing procedure for the fractionation of isolated wheat endosperm cell walls.

was removed by centrifugation. Extracted polysaccharides were precipitated following exhaustive dialysis against cold distilled water by adjusting to 80% (v/v) with respect to ethanol. Precipitated polysaccharides were washed successively with absolute ethanol, absolute methanol, and dry n-pentane before final drying in a vacuum desiccator over silica gel. Extracted residues were similarly washed and dried.

III. Results

(*a*) *Composition of Insignia Endosperm Cell Wall and Cell Wall Fractions*

Table 1 shows the percentage by weight, the nitrogen content, and the monosaccharide composition of the cell walls and wall fractions from Insignia wheat. Since some residual starch was present in the final preparation, the walls and fractions were treated exhaustively with salivary amylase and the analyses are corrected for starch content.

Table 1

Composition of Insignia endosperm cell wall and cell wall fractions

Fraction	Designation*	Percentage of total wall (by wt.†)	Nitrogen in fraction (% w/w†)	Relative monosaccharide composition (% by wt.†):				
				Ara	Xyl	Man	Gal	Glu
Whole wall			1·72	34	53·5	7	2·5	3
Water-soluble	1^S	23	0·13	34	64·5	1	0·5	—
Water-insoluble	1^R	70	2·2	33	51	9	2	5
EDTA-soluble	2^S	5	4·31	38	62	—	—	—
EDTA-insoluble	2^R	66	2·24	30	49	10	3	8
KOH-soluble	3^S							
80% ethanol-soluble		14·5	5·4					
80% ethanol-insoluble		36	0·35	34·5	63·5	2	—	—
KOH-insoluble	3^R	10	—	11	12	28	2	47
$NaOH–H_3BO_3$-soluble	4^S	3–4	—	11	8	59	2·5	19·5
$NaOH–H_3BO_3$-insoluble	4^R	5–6	—	9·5	7	3·5	1	79

* This column refers to the designation of the fractions obtained as outlined in Figure 2.
† All weights refer to the dry weight of the wall or wall fraction.

The nitrogen analyses for each of the wall fractions (Table 1) show that the major portion of the nitrogen in the wall preparation resisted extraction by water and 0·05M EDTA, but was extracted by 24% KOH and was then soluble in 80% ethanol. This fraction was evaporated to dryness and dissolved in distilled water. A positive Folin phenol reaction and the amino acid analyses indicated that the material was protein or peptide. The elution profile on Bio-Gel P-2 (Fig. 3) showed that this fraction contained low-molecular-weight peptides and little or no higher molecular-weight protein, and further experiments (Mares and Stone 1973*a*) suggested that these may have arisen, in part at least, by alkaline degradation of the protein or peptide

present in the wall preparation. On the basis of amino acid analyses the nitrogen associated with the water-soluble, EDTA-soluble, and KOH-soluble polysaccharides was also mostly protein in origin.

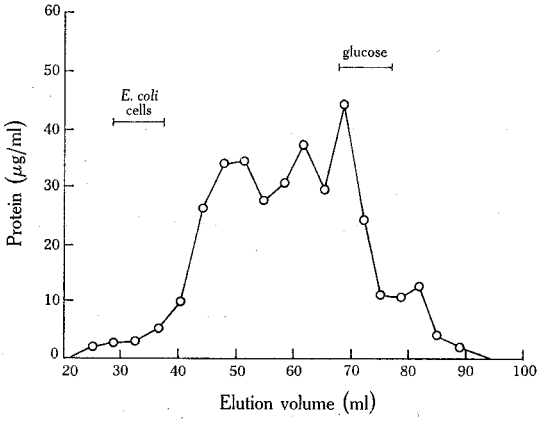


Fig. 3.—Bio-Gel P-2 chromatography of material extracted by 24% KOH and soluble in 80% ethanol. The sample (2–3 mg Folin protein) was applied in 1 ml of water containing sodium azide (0·05%), *E. coli* cells (0·2 mg Folin protein), and glucose (0·2 mg).

Total carbohydrate analyses as described in Section II(*c*)(iii) indicated that approximately 75% by weight of the isolated wall was polysaccharide and that approximately 85% of this polysaccharide was arabinoxylan. Of this arabinoxylan, 36% was soluble in water, 8% was soluble in 0·05M EDTA, and 56% was extracted with KOH solution. The small amounts of arabinose and xylose which remained associated with the KOH-insoluble fraction could be removed by treatment with 0·5M nitric acid at 100°C for 10 min without removing any glucose or mannose. Once extracted from the cell walls, EDTA- and alkali-soluble pentosans could be dissolved in water to give viscous solutions and resembled the water-soluble pentosans with respect to their xylose : arabinose ratio. In general, samples of pentosans required 10 min heating in a boiling water-bath for complete dissolution. The alkali-soluble pentosans sometimes gave cloudy solutions even after heating.

Extraction of the KOH-insoluble residue with $NaOH–H_3BO_3$ solution solubilized a fraction which appeared to be a glucomannan or a galactoglucomannan. This polysaccharide could be precipitated from a neutralized extract by the addition of $Ba(OH)_2$ solution. The glucose to mannose ratio of the extracted polysaccharide was 1 : 3.

Uronic acids have been found associated with the water-soluble polysaccharides, the hemicellulose fraction, and cellulosic polysaccharides from a variety of plant cell walls (Jermyn and Isherwood 1956; Thornber and Northcote 1961*a*, 1961*b*; Ray 1963; Timell 1964, 1965). Analysis of Insignia endosperm cell wall fractions following enzymic digestion with a mixture of Cellulosin and *S. rolfsii* enzymes gave the following results:

Fraction	Uronic acid content (% by wt.)
Water-soluble	0
EDTA-soluble	1·3
KOH-soluble	1·5
KOH-insoluble residue	1·4

The assay method failed to detect uronic acid in whole-wall hydrolysates, and analysis of hydrolysates of native wall containing 0·12 μmoles of added D-galacturonic acid also failed to show the presence of uronic acid. This suggested that the uronate dehydrogenase may have been inhibited by some component of the native wall which had been solubilized during the enzymic hydrolysis. This would be analogous to the suggestion by Albersheim and Anderson (1971) that various plant cell walls contain a protein component which inhibits polygalacturonase.

Although the enzymic assay method failed to estimate uronic acids present in hydrolysates of whole walls, apparently due to the presence of an inhibitor of uronate dehydrogenase, small amounts of uronic acid were found in the EDTA- and KOH-soluble fractions and the KOH-insoluble residue but none was present in the water-soluble polysaccharide. The results obtained should be regarded as minimum values in view of the uncertainty introduced by possible enzyme inhibition; they are, however, consistent with the values obtained by Cole (1967).

Hexosamines were found associated with the KOH-soluble arabinoxylan. Smaller amounts of hexosamines were also found in the water- and EDTA-soluble fractions but none was present in the alkali-insoluble material.

(*b*) *Comparison of the Composition of Insignia, Olympic, and Wren Endosperm Cell Walls and Fractions*

Endosperm cell walls were isolated from flours prepared from Olympic and Wren wheats, using the procedure developed for Insignia wheat flour [Sections II(*d*) and II(*e*); Fig. 1] and fractionated as described in Section II(*g*). Table 2 indicates that there were no significant differences in the monosaccharide composition of the whole cell walls. The percentages of the total wall by weight, represented by each of the fractions, were not significantly different for any of the varieties tested and the monosaccharide compositions were also very similar.

(*c*) *Light Microscopy*

Isolated endosperm cell walls and cell walls which had been extracted with 24% KOH were examined by phase-contrast microscopy. Figure 4 shows fragments of endosperm cell walls free from starch and non-endosperm cell walls which have been isolated from Insignia wheat flour. Successive extraction of the isolated walls with water, 0·05M EDTA, and 24% KOH removed approximately 90% of the wall mass, but, as shown in Figure 5, a recognizable wall structure was still present.

(*d*) *Transmission Electron Microscopy*

Isolated Insignia endosperm cell walls and wall fractions were examined in the transmission electron microscope in thin sections and in surface view. The surface

detail observed in cell wall fractions is shown in Figures 6–9. Initially no microfibrils were visible (Fig. 6) and the surface of the wall fragments had an amorphous texture with occasional depressions and holes where some material had been torn away. Presumably these latter features resulted from the close adpression of the cell walls to the cellular constituents and the subsequent rupture of the wall–matrix association during milling and cell wall preparation.

TABLE 2

COMPARISON OF THE MONOSACCHARIDE COMPOSITION OF OLYMPIC, INSIGNIA, AND WREN WHEAT ENDOSPERM CELL WALLS AND THEIR FRACTIONS

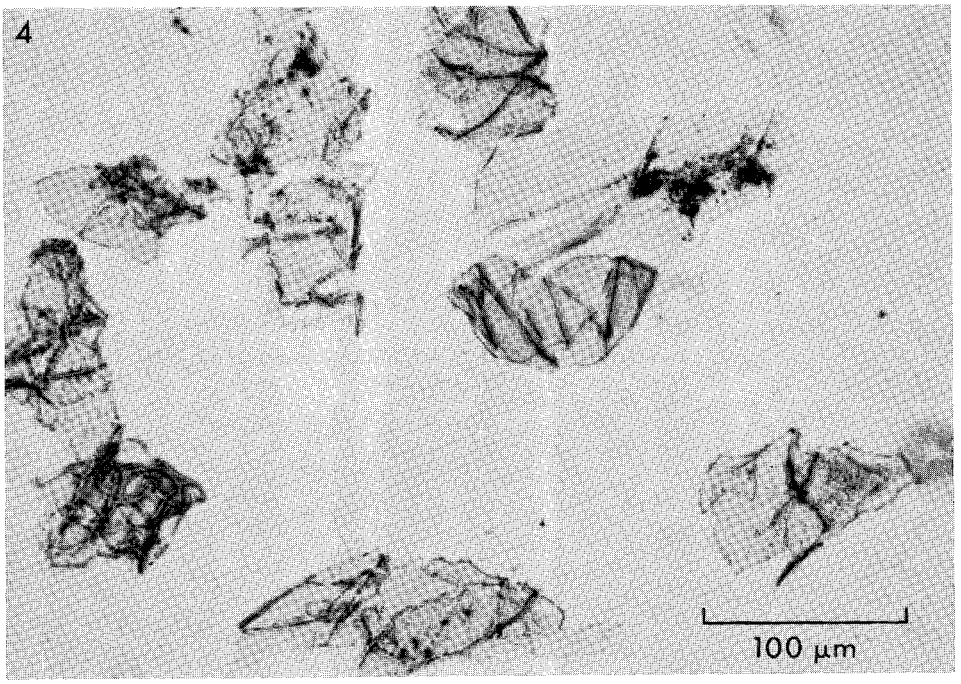
Walls were isolated from Insignia (I), Olympic (O), and Wren (W) wheats and fractionated as described in Sections II(*e*) and II(*g*). Corrections were made for starch content as described in Section II(*c*)(vi)

Fraction	Cultivar	Percentage of total wall (by wt.*)	Relative monosaccharide composition (% by wt.*)				
			Ara	Xyl	Man	Gal	Glu
Whole wall	I		34	53·5	7	2·5	3
	O		35	55	6	—	4
	W		34·5	55	7	—	3·5
Water-soluble	I	23	34	64·5	1	0·5	—
	O	22	33·3	64	2·8	—	—
	W	21·7	34·5	62·8	2·7	—	—
EDTA-soluble	I	4·5	38	62	—	—	—
	O	4·5	34·2	65·7	—	—	—
	W	4·2	37·6	62·4	—	—	—
KOH-soluble							
80% ethanol-soluble	I	14·5					
	O	17					
	W	19·5					
80% ethanol-insoluble	I	36	34·5	63·5	2	—	—
	O	38	34·3	63·8	2	—	—
	W	37	33	63·2	3·8	—	—
$NaOH–H_3BO_3$-soluble	I	4–5	11	8	59	2·5	19·5
	O	4	7·5	6·5	63	—	23
	W	4	3·5	3	68	2	23·5
$NaOH–H_3BO_3$-insoluble	I	5–6	9·5	7	3·5	1	79
	O	4·5	16	14·5	11·5	2	56
	W	5·2	17	15	10·4	2	57·5

* All weights refer to the dry weight of the wall fraction.

As progressively more and more of the wall material was removed (Figs. 7, 8, and 9) the microfibrillar elements became more prominent so that in the alkali-insoluble residues (Figs. 8 and 9) a random orientation of microfibrils was apparent.

Two types of particles were observed on the surface of cell walls. These particles appeared to be irregular in distribution and spatial arrangement and were completely absent from many wall fragments. The first type was large and irregular in shape and was present on both unextracted and water-extracted endosperm walls. These particles were readily removed by proteolytic enzymes (Mares and Stone 1973*a*).



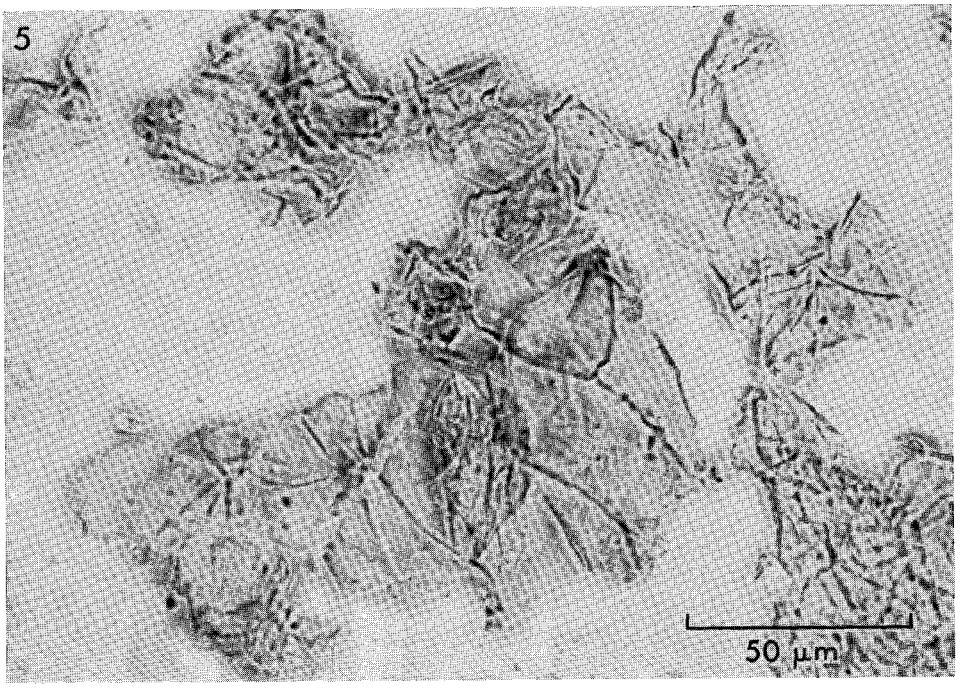
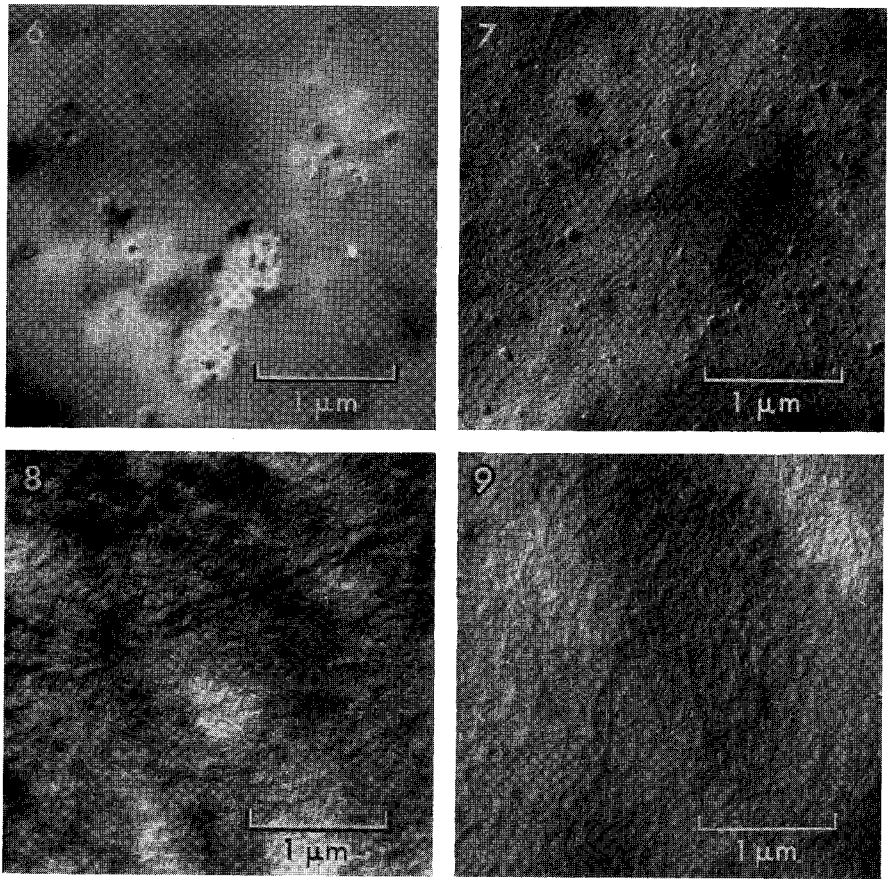


Fig. 4.—Isolated Insignia wheat endosperm cell wall fragments (phase contrast).
Fig. 5.—Cell wall fragments after treatment with 24% KOH (phase contrast).

The second type was smaller, more regular in shape, and was observed on water-extracted walls and to a lesser extent on unextracted walls. They appeared, in general, to be covered by the water-soluble material and there were indications that they were



Figs. 6–9.—Transmission electron micrographs of surfaces of walls and wall fractions which had been air-dried onto copper grids and shadowed with platinum–palladium at an angle of 30°.

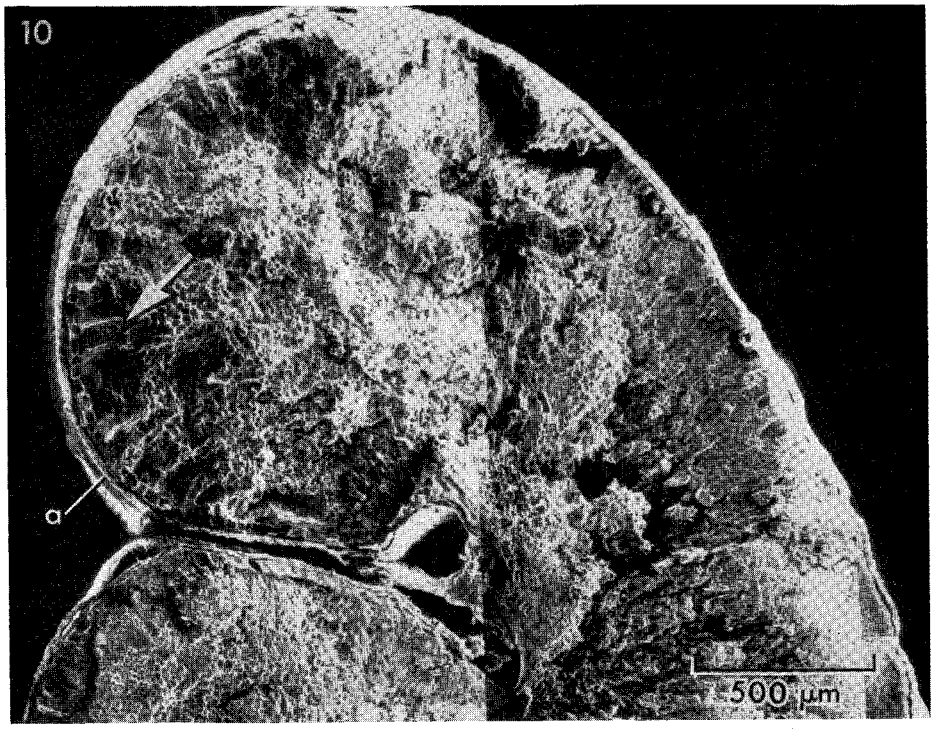
Fig. 6.—Isolated walls. The amorphous texture is interrupted by areas where the surface material has been torn away. Some large, irregular particles are present.

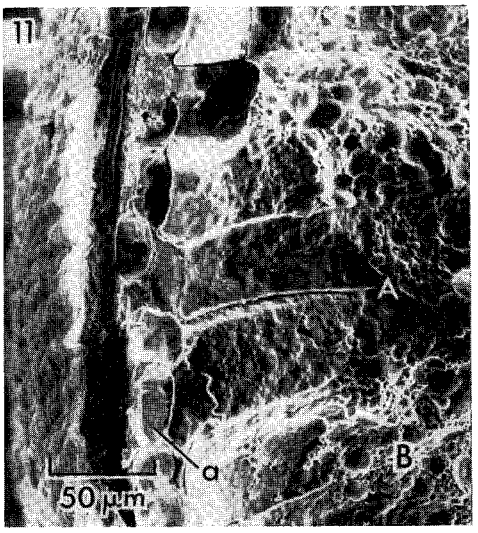
Fig. 7.—Appearance of walls after extraction with water. Microfibrils and small, regular particles are evident.

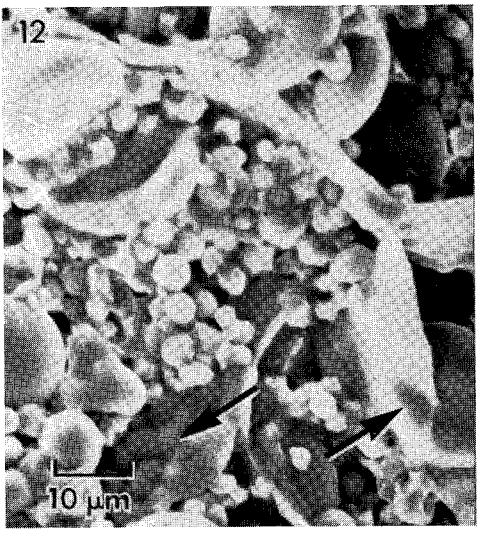
Fig. 8.—Appearance of walls after extraction with KOH.

Fig. 9.—Appearance of walls after extraction with $NaOH–H_3BO_3$. The microfibrils show a random orientation typical of many primary cell walls.

distributed throughout the depth of the wall, since they were sometimes partially obscured by microfibrils. It could not be conclusively demonstrated that these particles were removed by proteolytic enzymes. Both types of particles have also been observed on the surface of isolated *Lolium multiflorum* endosperm cell walls (Mares and Stone 1973*c*).







Figs. 10–12.—Scanning electron micrographs of sections of wheat grain.

Fig. 10.—Median cross-section of dry, mature wheat grain. Subaleurone cells (arrow head) are fractured along the plane of the cell walls while central endosperm cells have fractured through the body of the cell. *a*, Aleurone layer.

Fig. 11.—Subaleurone area from Figure 10. Some cells appear to have fractured along the plane between adjacent cell walls (area *A*), whilst in other cells (area *B*) the contents have been torn from the cell wall leaving small fragments of cellular components adhering to the wall surface.

Fig. 12.—Central endosperm region. This shows the three-dimensional arrangement of cellular components and the moulded appearance of the endosperm cell wall (arrow heads).

Thin sections of endosperm cell walls and wall fractions did not show evidence of distinct wall layers. However, if the Araldite embedding medium was first removed from the sections (Major *et al.* 1961) and the residue shadowed with metal, some structural detail could be distinguished. This was particularly true for alkali-extracted walls where microfibril patterns corresponding to those seen in metal-shadowed surfaces of walls were seen.

(*e*) *Scanning Electron Microscopy*

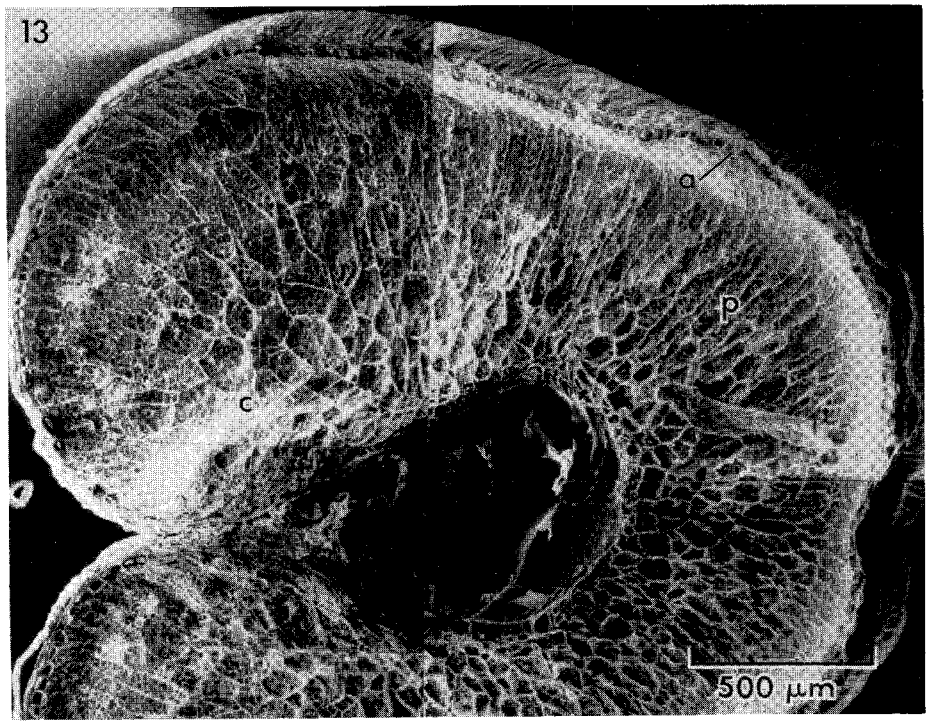
The thin endosperm cell walls were difficult to detect in cross-sections prepared by freehand sectioning of dry grain or freeze-sectioning of acrolein-fixed tissue, since they were generally obscured by masses of starch granules and matrix protein. Walls could be regularly seen only at the periphery of sections of dry grain, where cells tended to fracture along the plane of the cell wall (Fig. 10). This effect was not observed in sections of wet tissue. Surfaces appeared to be fracture faces, since intact starch granules were observed protruding from the cellular matrix and sectioned granules were very rare or completely absent.

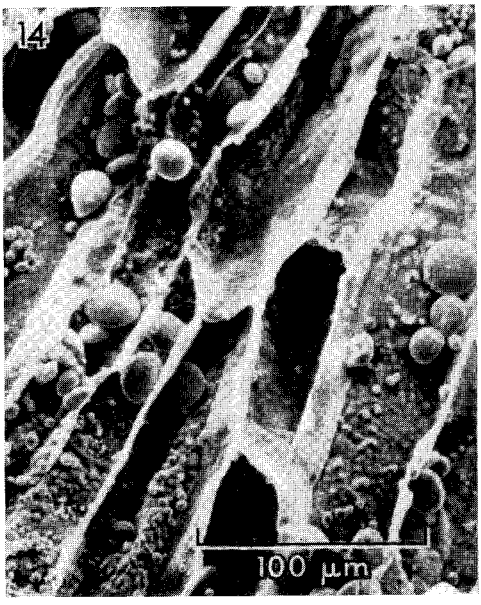
A close examination of subaleurone layers of endosperm (Fig. 11) in sections of dry grain showed that the smooth wall surface was distorted by the cell contents, in particular by the starch granules. In some cases the line of fracture appeared to be between adjacent cell walls whilst in other cases the presence of small particles adhering to the wall surface indicated that the cellular constituents had been torn away from the cell wall. Examination of endosperm cell walls from the central regions of the endosperm (Fig. 12) also suggested that the wall had been moulded around the cell contents, perhaps during the dehydration process of grain maturation.

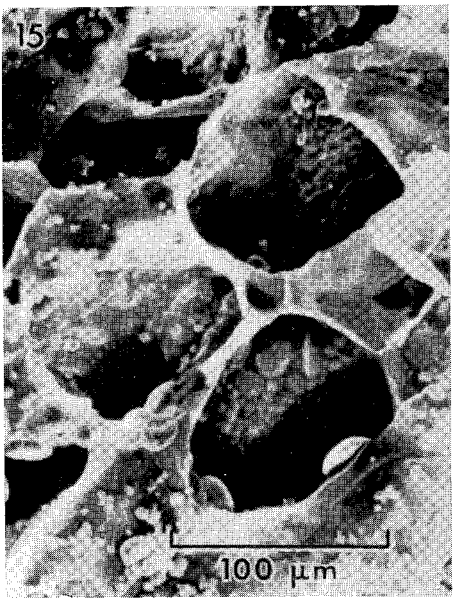
In an attempt to remove the endosperm cell contents and thus expose the cell walls, sections of grain were treated with salivary amylase or 0·01M NaOH or both. Each of these treatments removed the contents from central endosperm cells but not from peripheral or prismatic cells. A more uniform removal of cell contents from sections was obtained with boiling water followed by digestion with salivary amylase, which removed starch from endosperm cells throughout the cross-section, leaving a web of matrix material and exposing the endosperm cell walls (Fig. 13). Cell types corresponding to those observed by Bradbury *et al.* (1956) were seen, the major portion of the endosperm being composed of central endosperm cells. Peripheral and prismatic cells appeared to be absent from the areas adjacent to the modified aleurone of the crease. Indeed the presence of peripheral cells could not be convincingly demonstrated along many parts of the aleurone layer. Figures 14 and 15 emphasize the differences in shape and size between prismatic and central endosperm cells. In Insignia wheat sections central endosperm cells extended around the crease from the central endosperm of one cheek into the other cheek. Bradbury *et al.* (1956) observed a similar pattern in Pawnee wheat, whereas Greer *et al.* (1951) found central endosperm cells only in the cheeks of Manitoba wheat.

IV. Discussion

The isolation procedure adopted has allowed the collection of quantities of endosperm cell walls from mature wheat grains, free from non-endospermic cell walls, and without the loss of water-soluble polymeric components. Preliminary pearling







Figs. 13–15.—Scanning electron micrographs of sections of wheat grain.
Fig. 13.—Median section from a wheat grain which had been heated in boiling water for 10 min. Treatment of the section with salivary amylase removed cell contents and exposed cell walls. Aleurone cells (*a*), prismatic endosperm cells (*p*), and central endosperm cells (*c*) are clearly distinguished.
Fig. 14.—Prismatic endosperm cell profiles.
Fig. 15.—Central endosperm cell profiles.

of the grain removed the germ and much of the outer seed coats and, when combined with a modified milling procedure, allowed the collection of a flour which had a much reduced quantity of non-endospermic components. Nevertheless, intermediate products contained considerable amounts of non-endosperm and aleurone cell walls which could be detected in bulk samples by their brownish colour and could be readily separated from the endosperm walls by differential sedimentation. Efficient separation of endosperm cell walls from other walls was not possible while the walls were contaminated with starch grains. These were difficult to remove by physical means, but as salivary amylase retains activity in 70% ethanol this allowed the removal of a substantial proportion of the adherent starch without the loss of wall components. In the early stages of the isolation long sheets of walls were present but these tended to break up during the isolation procedure and some losses of wall fragments were inevitable. For this reason the procedure was not quantitative nor did it discriminate between walls from the several types of endosperm cells present in the mature endosperm.

The walls themselves are mainly composed of polysaccharides but, as isolated, are associated with some 14–15% protein as estimated by the Folin phenol method. Arabinoxylans accounted for 85% of the polysaccharide and the remaining 15% was made up of approximately equal proportions of β-glucan and β-glucomannan. These results indicate that the arabinoxylans isolated from wheat flour by previous workers (Pence *et al.* 1950; Perlin 1951; Preece and Hobkirk 1953, 1954; Montgomery and Smith 1955*a*, 1955*b*; Kundig *et al.* 1961*a*, 1961*b*; Tracey 1964; Cole 1967; Lin and Pomerantz 1968; Medcalf *et al.* 1968) are in fact largely, if not entirely, derived from endosperm cell walls. It should be emphasized, however, that not all non-starchy polysaccharides found in wheat flour extracts necessarily arise from the cell walls (Mares and Stone 1973*b*).

The presence of alkali-insoluble residues after extraction of wheat flour "tailings" has been reported by Perlin and Suzuki (1965) and these probably correspond, in part, to the alkali-insoluble fraction described here. The glucomannan extracted from the KOH-insoluble residue by $NaOH–H_3BO_3$ corresponds in glucose : mannose ratio to similar polysaccharides associated with cellulose in wood pulp (Nelson 1961; Timell 1964, 1965). The presence of small and variable amounts of arabinose and xylose in the alkali-insoluble fraction probably represents arabinoxylan strongly adsorbed onto the surface of the microfibrils (Bayley and Bishop 1958). It was of interest that only very small amounts of uronic acids were found associated with the endosperm walls and this was distributed between the EDTA-soluble, alkali-soluble, and alkali-insoluble fractions. Clearly, pectic substances are of negligible importance as constituents in endosperm walls and this is further suggested by the absence from the EDTA-soluble fraction of rhamnose and galactose, which are normally part of the pectin complex (Jermyn and Isherwood 1956; Stoddard *et al.* 1967). The uronic acids present may represent single units attached to the arabinoxylan, glucomannan, or glucan polymers (Timell 1964, 1965; Aspinall 1970).

The nitrogen in the isolated wall is mainly accounted for by amino acids in the form of protein or peptide and by small amounts of hexosamines. Alkaline extraction successfully removed the major portion of the nitrogenous components which had not been extracted by water and EDTA solutions. The nature and origin of the protein in the wall preparations is discussed in Part II (Mares and Stone 1973*a*).

No significant differences in monosaccharide composition of the isolated walls of the three wheat varieties was apparent (Table 2). Nor was there a difference in the proportions of the total walls present in each fraction or in the monosaccharide composition of the fractions. These results emphasize the similarities in the chemistry of the cell walls of these wheats of diverse milling quality, and suggest that neither variations in the proportions of the component polysaccharide fractions nor in their composition is responsible for the differences in milling quality. However, the total amount of cell walls was not measured and this parameter, which would be related to wall thickness, could be significant in determining varietal differences in milling quality. The analytical data presented here for the three varieties of the one wheat type support the results obtained by Medcalf *et al.* (1968) from a study of the pentosans of several varieties of hard red spring and durum wheats. These workers showed that water-soluble and water-insoluble pentosans from the same wheat type had a similar degree of branching although the degree of branching was different for pentosans from the two different wheat types investigated.

Endosperm cell walls which had been extracted with water and alkali retained their integrity, and their basic framework consisted of randomly oriented microfibrils typical of many primary cell walls. The apparently closely packed arrangement shown in alkali-insoluble residues may not represent a true picture of the situation in the unextracted walls. The removal of water, which may represent approximately 60% of the native wall (Setterfield and Bayley 1961), and of matrix components presumably results in the collapse of the originally sparse microfibrillar phase. However, the orientation of the microfibrils in the alkali-insoluble residues does seem to be related to the orientation in the unextracted wall (Frey-Wyssling *et al.* 1948; Preston *et al.* 1948; Frey-Wyssling 1959; Roelofsen 1965). The microfibril pattern of wheat endosperm walls closely resembles that of cultured *Lolium multiflorum* endosperm cell walls (Mares and Stone 1973*c*).

The amorphous texture, the indentations in the surface, and the failure to observe microfibrils on the surface of the isolated endosperm cell walls suggested that the water-soluble polysaccharides may be layered onto the surface of the wall rather than distributed through the wall structure. The results obtained with other plant seeds suggest that this may be a common feature of the cell walls in storage tissues. MacLeod *et al.* (1964) were able to show that the endosperm cell wall of *Bromus sterilis* was composed of more than one distinct layer, the outermost of which appeared to be continuous with the matrix around the starch granules. The composition of this layer was not determined, but MacLeod and MacCorquodale (1958) demonstrated that the quantity of water-soluble material was much greater in *Bromus* than in barley or wheat, which had much thinner endosperm cell walls than *Bromus*. Kooiman (1957) has shown that the xyloglucan, or "amyloid", of certain plant seeds occurs as a thickening on the inner surface of the cell wall, adjacent to the cell contents, and apparently acts as a food reserve since it disappears during germination. Other seed polysaccharides, which include mannans, arabinoxylans, galactomannans, and cereal β-glucans, may also occur as cell wall thickenings (Gould *et al.* 1971). These polysaccharides may have a dual function acting as food reserves for germination and also reinforcing the cell wall against crushing or cracking during dehydration and dormancy. However, the extent of their contribution to the energy requirements of germination has not been determined.

The particles observed on the surface of the endosperm cell walls appear to be aggregates of protein. The large, irregular particles may be fragments of cytoplasmic protein or perhaps of membranes, adhering to the surface of the isolated cell walls. The nature of the smaller particles which were not observed in all wall fragments is uncertain.

Following the removal of cell contents from the endosperm cells the variation in cell shape and size, previously reported by Bradbury *et al.* (1956), could be clearly demonstrated in the scanning electron microscope. The endosperm cell walls showed a roughened or crinkled texture and no discontinuities or intercellular connections were observed at the magnifications employed. Scanning microscopy also showed that in the mature grain the endosperm cell wall has the appearance of being moulded over the intracellular contents. This is clearly shown by the impressions left in the thin cell walls by the adpression of large starch granules.

The scanning micrographs also revealed that the fracturing pattern of the endosperm during sectioning with a razor-blade is dependent on the endosperm zone being cut. Cells at the edge of the endosperm in the dry kernel often fractured along the plane of the wall, suggesting that the bonding between adjacent cell walls, and between cell walls and cell contents, was weaker than the bonding between intracellular components. Chemical studies have shown that, whilst all endosperm cells appear to have the same total amount of protein, the central endosperm cells contain much more starch so that the protein concentration is in effect reduced by dilution (Hinton 1959; Evers 1970). These findings would explain the greater resistance of the cell contents of prismatic endosperm cells to removal by amylase and alkali and suggest why, in the central endosperm cells with a higher starch density, the plane of least strength was through the central body of the cells.

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