STUDIES ON THE ALEURONE LAYER

I. CONVENTIONAL AND FLUORESCENCE MICROSCOPY OF THE CELL WALL WITH EMPHASIS ON PHENOL–CARBOHYDRATE COMPLEXES IN WHEAT

By R. G. Fulcher,* T. P. O'Brien,* and J. W. Lee†

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Abstract

In the ungerminated seed, the cell walls of the aleurone layer of wheat (*Triticum aestivum* cv. Heron) are strongly autofluorescent and are stained by aniline blue, resorcinol blue, toluidine blue, and the periodic acid–Schiff's reaction. Thin-layer chromatography of acid or alkaline extracts and microspectrofluorimetry indicate that the fluorescent component of the wall is ferulic acid. During germination, the ferulic acid disappears from the aleurone cell wall in a specific pattern and its loss is accompanied by loss of stainable substance except in the innermost layer of the wall. These facts suggest that the aleurone cell wall contains a ferulic acid–carbohydrate complex which renders it relatively resistant to the action of the hydrolases which digest the endosperm.

I. Introduction

Recently, Taiz and Jones (1970) described the changes which occur in barley aleurone cell walls in the presence of gibberellic acid. There appears to be a localized digestion of carbohydrate components of the wall accompanying the release of β-1,3-glucanase. Their micrographs show that the walls are distinctly two-layered when stained with either the periodic acid–Schiff's (PAS) or the periodic acid–silver methenamine reactions. In addition, Jacobsen and Knox (1971) observed autofluorescence in the cell walls of barley aleurone in the course of fluorescent antibody studies.

We have observed a pattern of polysaccharide staining in wheat aleurone cell walls which is similar to that reported by Taiz and Jones (1970), and observations with the fluorescence microscope indicate the presence of intensely autofluorescent material which corresponds in location to the PAS-positive substances.

It is well known that many phenolic compounds fluoresce intensely in ultraviolet light (see Ibrahim and Towers 1960) and fluorescence microscopy has been used to localize phenolic compounds in plant tissues (e.g. Hepler, Fosket, and Newcomb 1970). Fausch, Kundig, and Neukom (1963) demonstrated the presence of ferulic acid in a glycoprotein from wheat flour and Painter and Neukom (1968) have studied the oxidative gelation of this type of complex.

* Botany Department, Monash University, Clayton, Vic. 3168. (Address for reprints.)

† Wheat Research Unit, CSIRO, North Ryde, N.S.W. 2113.

Our observations of the fluorescence of the aleurone cell wall and its staining reaction with toluidine blue suggested that phenolic compounds might be concentrated in the walls of this tissue. We have attempted to clarify the relationship between the fluorescent material and the polysaccharide component of the wall by conventional and fluorescence microscopy and to identify the nature of the fluorescent material by extractions that liberate phenols and by microspectrofluorimetry.

These studies on dry and germinating seeds indicate that the fluorescence of the aleurone cell wall is due to a ferulic acid–carbohydrate complex which may also be responsible for the resistance of the wall to enzymatic degradation.

II. Materials and Methods

(*a*) *Microscopy*

Seeds of *Triticum aestivum* cv. Heron were surface-sterilized with 0·1% (w/v) silver nitrate solution for 5 min, rinsed in several changes of 0·5% (w/v) NaCl and distilled water, and germinated in the dark on moist filter paper at 23°C. At various intervals after initial soaking, a few seeds were removed from the germination chamber, cut in half transversely, and fixed for 24 hr in a solution of 6% glutaraldehyde in 0·025M sodium phosphate buffer, pH 6·8, at 4°C. For comparison, dry ungerminated seeds were fixed in the same manner without prior sterilization or soaking. The tissue was then dehydrated, embedded in glycol methacrylate (GMA) (see Feder and O'Brien 1968), and sectioned at 1–2 μm with glass knives. Sections were mounted on glass slides and for conventional microscopy were stained by one of the following procedures:

(1) *Toluidine blue* (0·05% in benzoate buffer, pH 4·5)—see Feder and O'Brien (1968);

(2) *Periodic acid–Schiff's reaction*, including aldehyde blockade—see Feder and O'Brien (1968);

(3) *Resorcinol blue* (Eschrich 1964). This stain demonstrates β-1,3-glycans and also reacts with lignified walls. After staining, some sections were treated with dilute HCl at pH 4 for 10 min. β-1,3-glycans remain coloured blue after this treatment while lignified walls stain red (see Eschrich 1964);

(4) *Fluorescence microscopy*. GMA sections were mounted unstained in fluorescence-free immersion oil or in 1–2 drops of 0·05% aniline blue in 0·06M K_2HPO_4 adjusted to pH 9·5 with K_3PO_4 (Eschrich 1964). Sections treated with buffer alone were also examined. Thick sections (*c.* 10–20 μm) of ungerminated seeds were cut by hand with a razor-blade and observed dry on glass slides to check for possible loss of fluorescent material during embedding. A Zeiss fluorescence microscope equipped with glass optics was used with an HBO-200W mercury arc lamp as the source of radiation.

To aid in the interpretation of results obtained with both resorcinol blue and aniline blue, both fresh hand-cut and fixed GMA-embedded sections of cotton (*Gossypium* spp.) stems were stained in a similar manner to determine the colours produced by the callose found in the sieve tubes.

For electron microscopy, aleurone segments from ungerminated seeds were fixed in phosphate-buffered 6% glutaraldehyde for 24 hr at 0°C, post-fixed in 2% OsO_4, dehydrated in alcohol, and embedded in epoxy resin (Spurr 1969). Sections were stained with uranyl acetate and lead citrate and viewed in an Hitachi HU 11E electron microscope.

(*b*) *Microspectrofluorimetry*

Sections of dry ungerminated wheat seeds were cut 10–20 μm thick by hand with a razor-blade and extracted under reflux with boiling 80% (v/v) ethanol for 2 hr to remove simple phenolic glycosides. This treatment did not affect the fluorescence of the aleurone cell wall in any way. Extracted sections and crystals of pure ferulic acid (4-hydroxy-3-methoxycinnamic acid) were

mounted in fluorescence-free immersion oil on glass slides. The material was examined with a Leitz fluorescence microscope equipped with a xenon lamp as light source and recording microspectrofluorimeter accessories (Schoeffel Instrument Corp., Westwood, N.J., U.S.A.). The microscope was equipped with glass optics. The excitation monochromator slit was set at 0·5 mm and the emission monochromator slit at 0·2 mm. Instrument correction factors have been applied to the recorded fluorescence emission spectra.

(c) *Purification and Extraction of Aleurone Layers*

Approximately 0·5 mm of the brush end and all embryonic tissue was removed from a sufficient number of ungerminated grains to yield about 10 g of tissue. The tissue was surface-sterilized and soaked in distilled water for 24 hr at 4°C. After soaking, the endosperm was scraped from the aleurone layer and the region containing the pigment strand (the groove tissues) was removed with a scalpel. This preparation of aleurone layers with pericarp and some residual endosperm was homogenized in distilled water for 10–20 min using a Virtis 23 homogenizer at medium speed. The endosperm and all but the tube cells of the pericarp (see Esau 1965, Fig. 19.2) are removed from the aleurone layer by this process yielding preparations of intact aleurone–tube cell layers and separate sheets of the remaining pericarp tissue. The aleurone–tube cell and pericarp sheets were separated by hand, dehydrated in ethanol, dried under vacuum, and retained for extraction of phenolic components.

Separate 100-mg lots of aleurone and pericarp layers were finely ground in a Wylie mill and extracted with either 0·5N KOH at 60°C for 90 min in a nitrogen atmosphere (Fausch, Kundig, and Neukom 1963) or 2N HCl on a steam bath for 1 hr under reflux (Ibrahim and Towers 1960). Both the HCl and KOH hydrolysates (after acidification to pH 2·0) were extracted with ether for 6 hr. The ether extracts were washed with 5% aqueous $NaHCO_3$ which was re-extracted with ether. The final ether extracts were evaporated to dryness and the residues dissolved in 1 c.c. of methanol. The methanolic solutions of the extracted material and of pure ferulic acid were spotted on glass plates coated with Merck Kiesegel H and developed by ascending chromatography in a mixture of benzene–methanol–acetic acid (90 : 16 : 8 v/v). The chromatograms were air-dried and observed under ultraviolet radiation. The major fluorescent spot in the alkaline aleurone extract and the chromatographed ferulic acid were eluted from the chromatogram with methanol or aqueous 0·001N NaOH and their ultraviolet absorption spectra compared using a Zeiss ultraviolet spectrophotometer.

Samples of aleurone tissue were removed at all stages of the isolation and extraction procedures, dehydrated in ethanol, and embedded in GMA for conventional and fluorescence microscopy. GMA sections of isolated aleurone layers were also extracted with 2N HCl at 100°C for various times.

Approximately 5 mg of ferulic acid crystals were subjected to the alkaline extraction procedure and all subsequent steps. Aqueous solutions of ferulic acid were also tested for PAS positivity by the method of Dahlqvist, Olsson, and Norden (1965).

III. Observations and Results

(a) *Microscopy of the Aleurone Cell Wall in the Ungerminated Seed*

When viewed with the fluorescence microscope, the cell walls of the aleurone layers of ungerminated seeds exhibit intense autofluorescence in both fixed and unfixed sections. It should be noted that the colour and intensity of the autofluorescence vary considerably with the filter combination used, but the combination of exciter filter UG5 and barrier filter 41 produces maximum intensity in the aleurone cell wall and minimum intensity in the endosperm and aleurone cytoplasm (Fig. 1). With this combination, the aleurone cell walls are a bright blue colour. From spectral transmission curves supplied by the manufacturer (Carl Zeiss, W. Germany), it can be determined that maximum fluorescence occurs in the range 410–460 nm under these

conditions. The fluorescence intensity is decreased distinctly if barrier filters are used which transmit only above 460 nm.

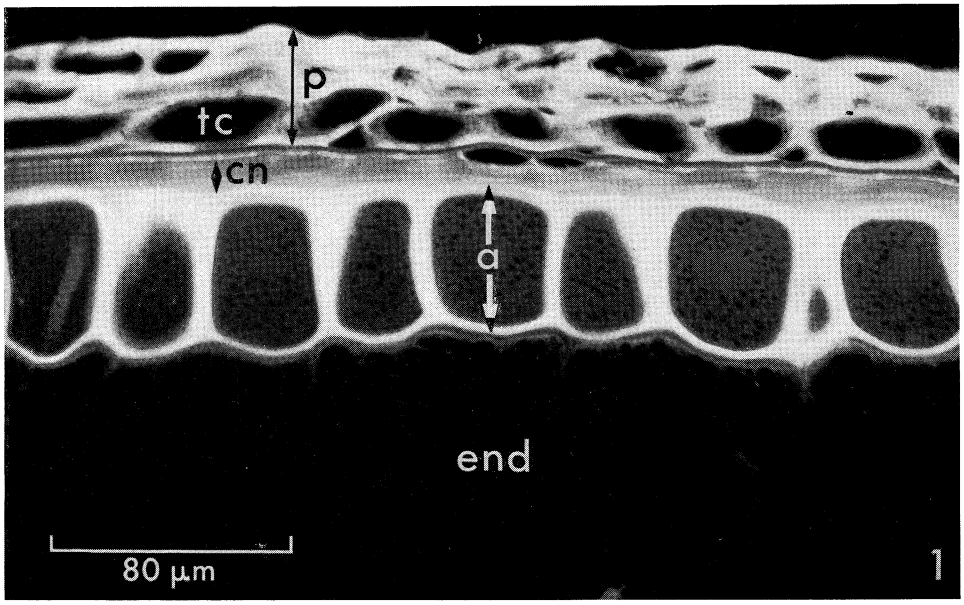
In GMA sections, the crushed nucellar cells which are adjacent to the seed-coat side of the aleurone layer exhibit weak fluorescence of a similar colour. The pericarp cell walls fluoresce strongly but the colour is distinctly different (greenish blue compared to bright blue).

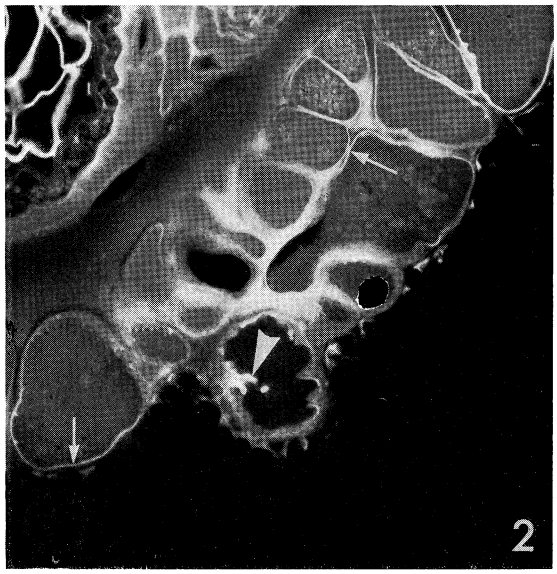
The majority of the aleurone cell walls are uniformly fluorescent throughout their thickness. The fluorescence pattern is somewhat modified in the aleurone cells which occur in the groove of the seed (Fig. 2). The walls in this region often possess projections which penetrate into the cell to varying degrees, and the fluorescent portion of the walls is often much thinner than that of normal aleurone walls. The walls of the scutellar parenchyma and epithelium fluoresce a similar colour quite intensely (Fig. 3).

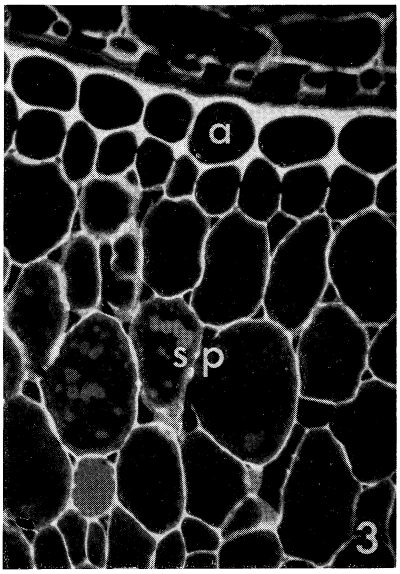
Toluidine blue stains the aleurone wall a light green colour which is generally less intense than that of the nucellar and pericarp walls (Fig. 4). The coloration is uniform through the thickness of the aleurone wall except that there is a much more intense green colour associated with a very thin layer adjacent to the cytoplasm. It is more readily resolved in the isolated and homogenized aleurone layers where the cytoplasm often pulls away from the wall to reveal the thin layer (Fig. 4). PAS-treated sections provide a staining pattern in the aleurone wall which is identical to that of toluidine blue—a uniformly stained wall with an intensely stained inner layer (Fig. 5). Electron microscopy of the wall indicates that there is an electron-dense inner wall layer approximately 150–200 nm thick (Fig. 5, inset).

When stained with resorcinol blue, aleurone cell walls in the ungerminated seed are uniformly stained a light blue colour, with no apparent differentiation of the two-layered wall seen with other stains. After treatment of the sections with dilute HCl at pH 4·0, the previously blue walls become red in colour. It is important to note that the blue colour of the aleurone walls does *not* match the colour of deposits of sieve-tube callose in sections of cotton stem stained in the same way. The latter were stained an intense bright blue colour (presumably the "cobalt blue" described by Eschrich 1964) which did not change to red when treated with dilute HCl at pH 4·0. The xylem vessels and other lignified walls in the cotton stem were stained a colour which closely matched that of the aleurone cell walls. These also became red when differentiated at pH 4·0.

When viewed with the fluorescence microscope utilizing exciter filter BG12 and barrier 53, the aleurone cell wall appeared to stain significantly with aniline blue, producing a bright green fluorescence. With this filter combination, aleurone wall autofluorescence is only faint. This combination also produces the most intense fluorescence (bright yellow) in callose deposits in cotton stem sections stained with aniline blue. The green fluorescence induced by the aniline blue solution in the aleurone wall is indistinguishable from the fluorescence induced in lignified tissues of cotton stem. However, sections mounted in phosphate buffer at pH 9·5 will produce fluorescence in the aleurone cell wall which is identical to that produced by 0·005% aniline blue in phosphate buffer at pH 9·5, the working solution recommended by Eschrich (1964) for fixed tissues.





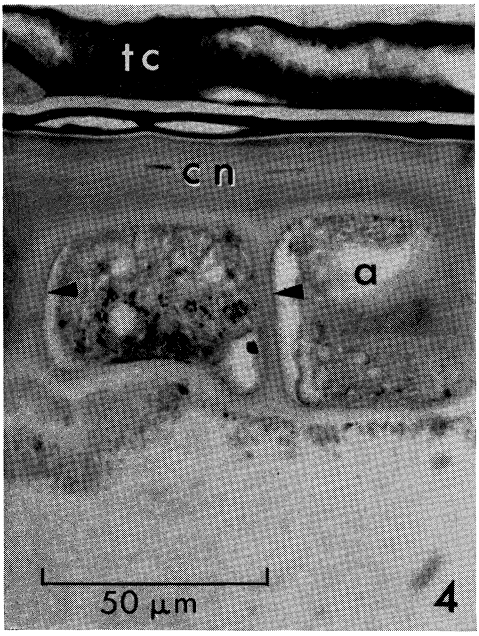


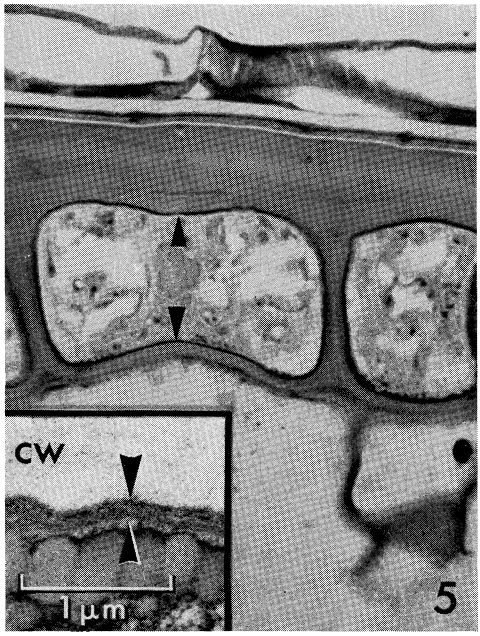
Figures 1–3 are fluorescence micrographs of transverse sections through the outer layers of ungerminated wheat seeds. Scale applies to all three figures.

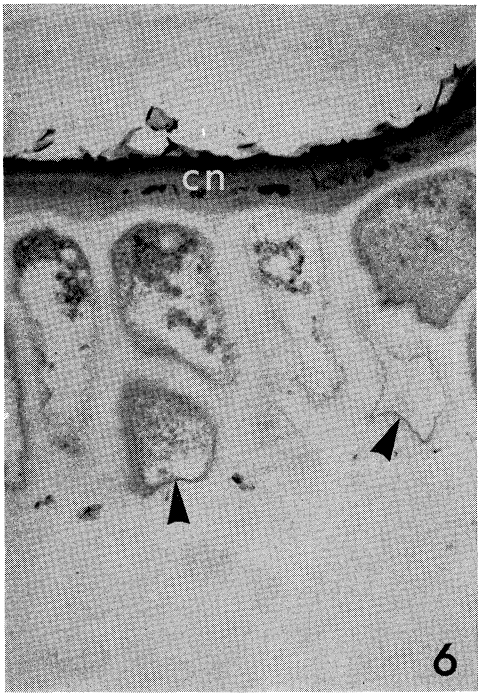
Fig. 1.—A normal aleurone layer (*a*) and associated outer layers. Note intense fluorescence of the aleurone cell walls. *cn*, crushed nucellar cells; *end*, endosperm; *p*, pericarp; *tc*, tube cell.

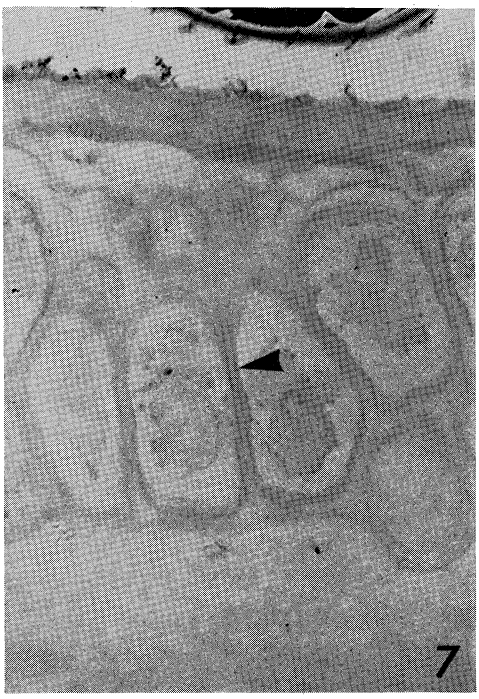
Fig. 2.—The aleurone layer in the groove region of the seed showing thin cell walls (small arrows) with projections (large arrowhead).

Fig. 3.—A portion of the scutellar parenchyma (*sp*) and adjacent aleurone layer (*a*) showing fluorescence in the cell walls of both tissues.









Figures 4–7 are transverse sections of isolated aleurone layers with adhering tube cells. Figure 5 (inset) is an electron micrograph.

Fig. 4.—Section stained with toluidine blue. Note inner wall layer (arrowheads). Abbreviations as in Figure 1.

(*b*) *Extraction and Chromatography*

Thin-layer chromatography of alkaline and acid extracts of purified aleurone and pericarp tissues yielded blue fluorescent spots under ultraviolet radiation (when viewed with the same barrier filter used for fluorescence microscopy) with R_F values shown in the following tabulation (t = trace):

Treatment	R_F
Purified aleurone (KOH extract)	0·48; 0·39t; 0·24t; 0·13t
Purified aleurone (HCl extract)	0·48t; 0·39t
Purified pericarp (KOH extract)	0·48t; 0·39
Purified pericarp (HCl extract)	0·48t; 0·39
Pure ferulic acid (KOH treatment)	0·48 0·24t; 0·13t
Pure ferulic acid	0·48

From the above tabulation it is apparent that the major compound isolated from aleurone tissue by these methods has an R_F value similar to that of pure ferulic acid (R_F 0·48), and at least two of the slower-moving blue fluorescent compounds may be degradation products of ferulic acid formed during alkaline hydrolysis (R_F 0·24 and 0·13). The compound with R_F 0·39, while not identified, appears to be specific to the pericarp tissue since a single layer of pericarp cells adheres to the aleurone layers (Fig. 4).

When the major fluorescent spot from the aleurone alkaline extract (R_F 0·48) was compared spectrophotometrically with the pure ferulic acid eluted from the chromatogram, the ultraviolet absorption spectra were found to possess identical absorption maxima and minima, and produced similar spectral shifts in 0·001N NaOH, as indicated below:

Treatment	Absorption maxima (nm)
Alkaline aleurone extract in methanol (R_F 0·48 spot)	230, 291,* 321
Pure chromatographed ferulic acid in methanol	230, 291,* 321
Alkaline aleurone extract in 0·001N NaOH (R_F 0·48 spot)	245, 305,* 345
Pure chromatographed ferulic acid in 0·001N NaOH	245, 305,* 345

* Shoulder.

When examined with the fluorescence microscope, all of the fluorescence originally present in the aleurone wall is absent from both the alkaline and acid extracted material. This corresponds to a similar loss of stainable material in the aleurone wall. The inner wall layer, however, is still stained to some degree by both toluidine blue (Fig. 6) and the PAS reaction (Fig. 7). The nucellar and pericarp tissues also retain much of the material stained by the PAS reaction and toluidine blue (Figs. 6 and 7) and some of the original fluorescence.

Fig. 5.—Section stained by the PAS reaction. Arrowheads indicate inner wall layer. Magnification as in Figure 4. *Inset*: A portion of a normal aleurone cell wall (*cw*) showing an electron-dense inner layer adjacent to the cytoplasm.

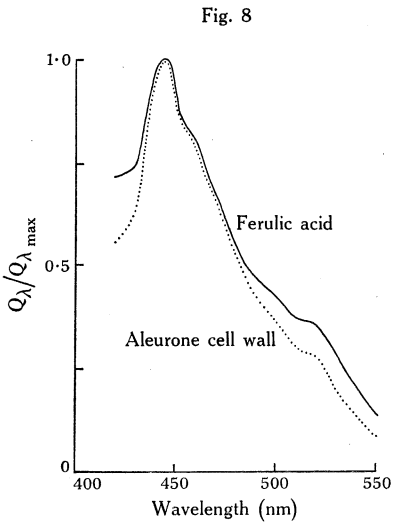
Fig. 6.—Section of alkali-extracted tissue stained with toluidine blue. The inner wall layer (arrowheads) and crushed nucellar cells (*cn*) are stained. Magnification as in Figure 4.

Fig. 7.—As in Figure 6, but stained by the PAS reaction. Magnification as in Figure 4.

When treated with 2N HCl at 100°C, the fluorescence and toluidine blue and PAS stainability of the aleurone cell wall (with the exception of the inner layer) were removed almost completely within 5 min. Pure ferulic acid in aqueous solution failed to show any PAS-positivity.

(c) *Microspectrofluorimetry*

Since quartz optics were not available, aleurone cell walls and ferulic acid crystals could not be excited at wavelengths shorter than approximately 350 nm. The material was therefore scanned at excitation wavelengths of from 350 to 430 nm to determine fluorescence maxima under these conditions. It was found that pure ferulic acid crystals excited at 380 nm produced a fluorescence emission spectrum with a maximum at 445 nm. Examination of a fluorescent portion of the aleurone cell wall under identical conditions produced a fluorescence spectrum comparable to that of ferulic acid crystals (Fig. 8). Pericarp and cytoplasm produced similar fluorescence spectra, but the intensity of the fluorescence at 445 nm in both cases was much less than for the aleurone wall in the same section. Figure 9 shows the relative fluorescence intensities of these three tissue components as determined in the same section.



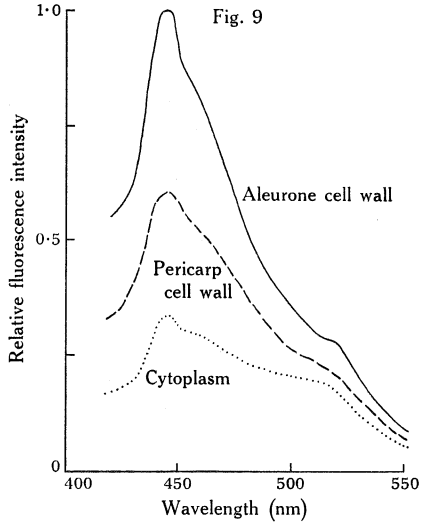


Fig. 8.—Microspectrofluorimetric comparison of $Q_\lambda/Q_{\lambda max}$ for pure ferulic acid crystals and aleurone cell walls. ($Q_\lambda/Q_{\lambda max}$ = ratio of fluorescence intensity at any given wavelength to maximum fluorescence intensity.) Maximum intensity of both materials occurs at 445 nm (excitation wavelength 380 nm).

Fig. 9.—Comparison of fluorescence intensities of the aleurone cell wall, aleurone cytoplasm, and pericarp cell wall in one section (excitation wavelength 380 nm).

(d) *Changes in the Fluorescence of the Aleurone Cell Wall during Germination*

For several hours after the initial soaking, there are no apparent changes in either the pattern of autofluorescence or of the reaction to any of the stains used in this study. The first change is detectable approximately 4 days after germination begins when a significant loss of fluorescent material has occurred in the aleurone cell

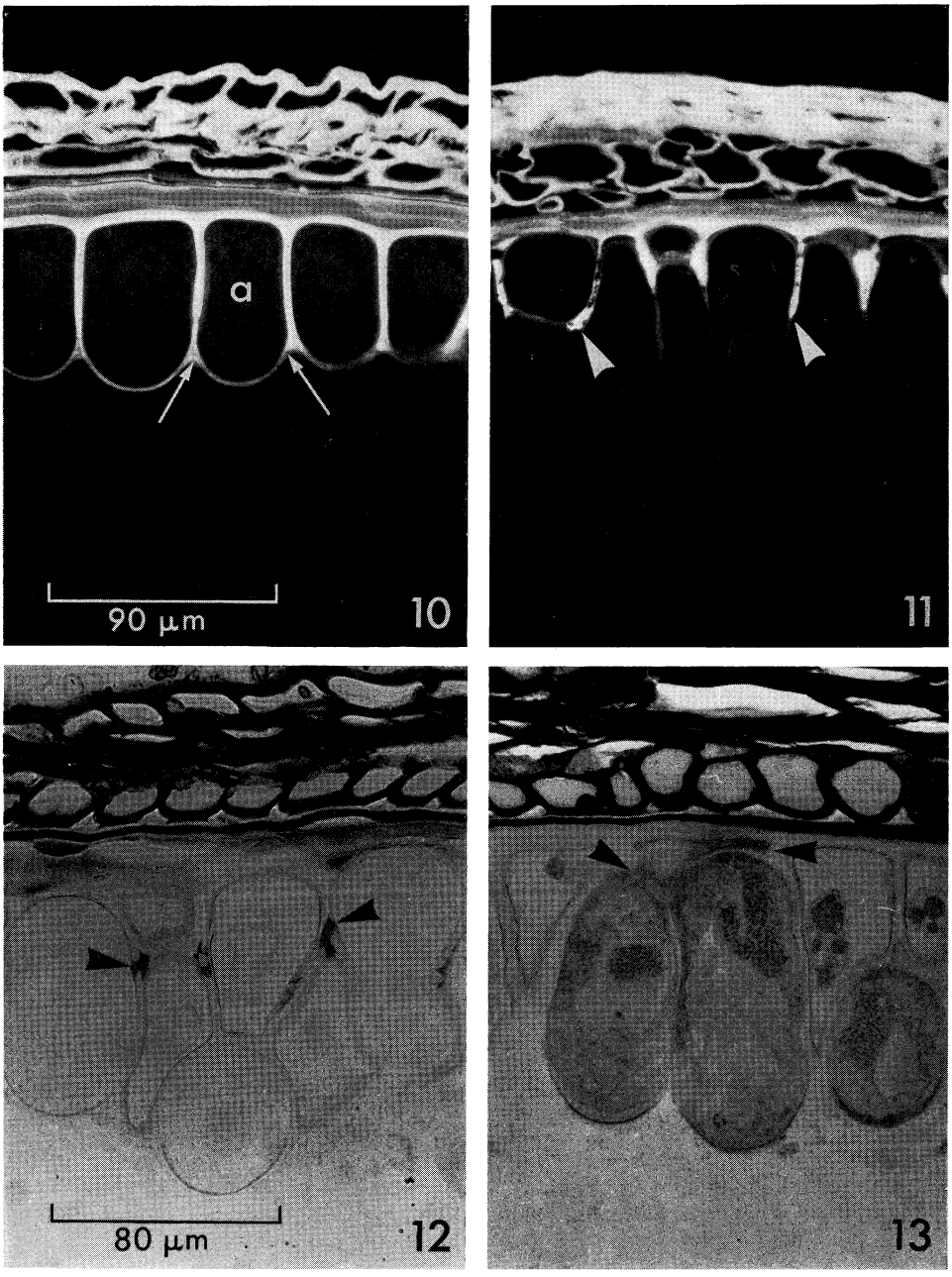


Fig. 10.—Fluorescence micrograph of a transverse section of an aleurone layer (*a*) from seed germinated for 4 days. Fluorescence has decreased in the aleurone cell wall (between arrows) nearest the endosperm.

Fig. 11.—As in Figure 10 but seed germinated for 12 days. Only small patches of fluorescence remain in the cell walls (arrowheads). Magnification as in Figure 10.

Figures 12 (PAS staining) and 13 (toluidine blue staining) are transverse sections of aleurone layers from seeds germinated for 12 days. Arrowheads indicate areas where stainable material has not yet been digested. The inner wall layer is intact in both cases. Scale applies to both figures.

walls on the side of the cell nearest to the endosperm (Fig. 10). When stained by toluidine blue, the PAS reaction, resorcinol blue, and aniline blue the staining pattern again mimics the fluorescence pattern, except that the inner wall layer remains well stained with toluidine blue and PAS even on the endosperm side of the cell (Figs. 11 and 12). It is notable that by this stage of germination, the endosperm has been almost completely digested but the aleurone cell cytoplasm is still intact (Fig. 10).

With continued germination, the fluorescent material in the aleurone walls is gradually digested, from the endosperm side of the cell to the pericarp side, until after several more days of germination, only small regions of fluorescence remain in the walls (Fig. 11). These regions again correspond to the staining patterns of the dyes, and the inner PAS- and toluidine-blue-positive layer is still apparently intact after 12 days of germination (Figs. 12 and 13). Even at this advanced stage of germination, the cell walls of the aleurone layer, while modified as we have indicated, are still relatively intact (Figs. 12 and 13) although the endosperm has been completely digested. The nucellar layer and pericarp layers undergo no detectable changes during this process.

IV. Discussion

At the present time, we may only speculate upon the physiological significance of accumulations of phenolic materials in aleurone cell walls. The occurrence of ferulic acid as a component of wheat flour and other plant tissues is now well established (Ibrahim and Towers 1960; Fausch, Kundig, and Neukom 1963; Levand and Heinicke 1968), but no direct evidence is available which provides for any functional significance. Fausch, Kundig, and Neukom (1963) have suggested that esterification of ferulic acid and other phenolic compounds to cell wall polysaccharides might provide a means of detoxifying these compounds in some plants.

Since the aleurone layer is not digested at the same rate as the endosperm during germination (although it produces some of the hydrolases responsible for mobilizing storage materials), it is not inconceivable that ferulic acid exerts some control over the rate at which the aleurone layer is digested. Certainly the cell walls are very resistant to hydrolysis and it seems that this is due to the phenolic materials embedded in the wall. It would be of interest to determine what enzymes are responsible for removing ferulic acid from the wall.

In addition, the accumulation of quantities of phenolic compounds effectively inhibits invasion by microorganisms. Wheat roots, for example, deposit fluorescent (and probably phenolic) compounds in response to infection by the soil fungus, *Ophiobolus graminis* (Sacc.) (Holland and Fulcher 1971). Turner (1960) has described the occurrence of phenolic glycosides in oat roots which effectively inhibit fungal growth. Preliminary observations in this laboratory indicate that the presence of ferulic acid in culture media at concentrations of 0·5–2·0 g/l significantly inhibits the growth of a variety of fungi isolated from stored wheat seeds. If the two major outer layers of the seed (pericarp and aleurone layer) are different in their phenolic constituents (as these observations suggest), the aleurone cell wall might form an effective secondary barrier to infection by organisms which are capable of penetrating the outer layers.

Levande and Heinicke (1968) have suggested tentatively that phenolic acids such as ferulic acid may play a role in water retention in pineapple stem tissue. If the impregnation of cell wall polysaccharides by phenolic compounds forms a barrier to the passage of water, then the aleurone cell wall might effectively form a barrier to water penetration in addition to that formed by the pericarp and cuticle surrounding the seed.

Resorcinol blue and aniline blue are well-known stains used to demonstrate the presence of β-1,3-linkages in polysaccharides, and are thought to be quite specific, especially for β-1,3-glucans such as callose (Eschrich 1964). Taiz and Jones (1970) have concluded by staining with aniline blue and lacmoid (a stain similar to resorcinol blue), that barley aleurone cell walls contain sufficient β-1,3-linkages to be detectable by these methods. However, our results in wheat cast considerable doubt on their findings. We have shown that the effect of aniline blue solution is due to the high pH of the buffer in which it is dissolved and the blue colour with resorcinol blue turns to red at pH 4·0, whereas sieve-tube callose stained with this dye does not. We conclude that stainable amounts of β-1,3-linkages are not present in the wheat aleurone cell wall, and that the reactions produced by both stains are due solely to the presence of phenolic materials.

As in barley, the cell wall of wheat aleurone is PAS-positive throughout its thickness in the ungerminated seed. This indicates that the wall contains considerable quantities of 1,4-linked polysaccharides, since the specificity of the PAS reaction depends on the presence of free vicinal hydroxyl groups in the sugar polymer (Hotchkiss 1948; Pearse 1968). The PAS reaction should not stain β-1,3-linked polysaccharides since the requisite vicinal hydroxyl groups are not available.

Superficially, it would appear that the inner wall layer is somewhat similar chemically to the remainder of the wall since it stains with toluidine blue and the PAS reaction. However, there is no evidence that its autofluorescence or its aniline blue and resorcinol blue stainability are any more or less marked than the remainder of the wall in ungerminated seeds. Neither acid nor alkaline hydrolysis completely removes the PAS and toluidine blue stainability of the layer which indicates that the layer might be lignified. In addition, the digestive processes which gradually remove the PAS- and toluidine-blue-positive material from the rest of the wall have little or no effect on this layer (Figs. 12 and 13). Since this layer appears to remain intact during germination in both barley (see Taiz and Jones 1970) and wheat, its permeability to hydrolases becomes a matter of considerable interest.

Fausch, Kundig, and Neukom (1963) have demonstrated ferulic acid as a component of a glycoprotein in wheat flour, a component which can only be removed from the complex by hydrolysis. Our results support the presence of a similar complex in aleurone cell walls of which both the polysaccharide (PAS-positive) and phenolic (fluorescent and toluidine-blue-positive) moieties are simultaneously extracted both enzymatically during germination and by acid and alkaline hydrolysis.*

Note added in proof.*—Macko *et al.* (*Science, N.Y.*, **173, 835–6, 1971) have shown recently that methyl ferulate is a potent inhibitor of uredospore germination in stem rust of wheat.

V. Acknowledgments

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