Microchemical Detection of Niacin, Aromatic Amine, and Phytin Reserves in Cereal Bran[1]

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ABSTRACT

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Methods are described for microscopic detection of niacin, aromatic amines (possibly aminophenol), and phytin in mature cereal grains. Niacin and amino compounds are detected by fluorescence microscopy after reacting tissue sections with cyanogen bromide or dimethylaminobenzaldehyde, respectively. Phytin is readily demonstrated by polarizing optics or metachromatic staining with toluidine blue. Phytin crystals are found in the protein bodies (aleurone grains) of both the scutellar parenchyma and the aleurone layer. Niacin and aromatic amines occur only in the protein bodies of the aleurone layer.

Wheat bran is one of the richest natural sources of niacin, yet a large proportion of the vitamin occurs in cereals in a bound form, which renders it nutritionally unavailable (Kodicek 1940). In wheat, rice, and maize, bound niacin is associated with a variety of complex macromolecules. Kodicek and Wilson (1960) suggested that wheat niacin is esterified primarily to a carbohydrate polymer containing substituted cinnamic acids and an aromatic amine. In contrast, Guha and Das (1957) and Das and Guha (1960) isolated niacin-rich substances from both wheat and rice brans that were largely polypeptide and contained no carbohydrate. Christianson et al (1968) then demonstrated an intermediate complex in maize containing protein and carbohydrate, as well as aromatic nitrogen compounds. Mason et al (1973) and Mason and Kodicek (1973a, 1973b) have since found wheat niacin associated with a range of polymers varying in molecular weights and containing differing proportions of carbohydrate, cinnamic acids, protein, and *o*-aminophenol.

The chemical information suggests that the vitamin may be sequestered in different complexes in varying cellular locations in cereal bran or perhaps even in different tissues. In order to clarify the chemical and possible spatial variations associated with niacin residues, we have devised a range of microchemical methods for localizing the vitamin and associated compounds by microscopic and chemical procedures.

MATERIALS AND METHODS

Mature, dry grains of wheat (*Triticum aestivum* L. cv. Heron), barley (*Hordeum vulgare* L. cv. Betzes), oats (*Avena sativa* L. cv. Hinoat), and sorghum (*Sorghum vulgare* Pers.) were processed for microscopic analysis by three methods and analyzed chemically by two methods. (Unless otherwise stated, all solutions are expressed as w/v in distilled water.)

Transmission Electron Microscopy

Small (< 1 mm^3) segments of aleurone and adjacent tissues were removed from dry wheat grains (or wheat grains imbibed 12 hr to facilitate fixative and resin penetration) are fixed for 24–48 hr in 6% glutaraldehyde and 3% acrolein in 0.025*M* potassium phosphate buffer, pH 6.8–7.0, on ice. Tissues were postfixed in osmium tetroxide and prepared for examination as described previously (Fulcher et al 1972a).

Bright-Field Microscopy

Sections (approximately 1 mm thick) were removed from the midregion of unimbibed grains and fixed on ice for a minimum of 48 hr in 6% glutaraldehyde in 0.025*M* potassium phosphate buffer, pH 6.8–7.0. After dehydration, they were embedded in glycol methacrylate (GMA) plastic (Feder and O'Brien 1968), sectioned 1–2 μm thick with glass knives, and affixed to microscope slides. Sections were examined directly using crossed polarizers (to detect anisotropic substances) or after staining by one of two methods.

Toluidine Blue Staining. Sections were stained 1–2 min in 0.05% toluidine blue and rinsed in running water (Feder and O'Brien 1968).

Periodate/Schiff's Staining. This reaction, including aldehyde blockades, was employed as recommended by Feder and O'Brien (1968) for the demonstration of carbohydrates with adjacent hydroxyl groups.

Fluorescence Microscopy

GMA-embedded or hand-cut sections (~ 20 μm thick, cut with a clean razor blade) were examined with a fluorescence microscope (Carl Zeiss Ltd.) equipped with a IIIRS epi-illuminating condenser and 200-W mercury arc illuminator. Two exciter/barrier fluorescence filter combinations with excitation maxima at 470 nm (FC I) and 546 nm (FC II) were used to analyze reactions to the following staining methods.

Niacin Detection. Chromatographic (Kodicek and Reddi 1951, Wang and Kodicek 1943) and spot (Feigl 1975) tests for detection of niacin and related compounds were modified for in situ detection of the vitamin. Sections (on glass slides) were suspended in a sealed staining jar at room temperature over 10–20 ml of freshly prepared cyanogen bromide (CNBr) solution. The solution was prepared on ice by dropwise addition of 10% potassium cyanide to 5–10 ml of saturated bromine water until the solution was decolorized.

CNBr rapidly cleaves the pyridine ring of niacin, and the reaction product, glutaconic aldehyde, can be detected after the addition of aromatic amines to produce polymethine dyes (Feigl 1975). Thus, CNBr-reacted sections were immersed for 1–5 min in a solution of 1) 2% *p*-aminobenzoic acid in 0.5*N* HCl containing 25% v/v ethanol (PABA) or 2) barbituric acid, saturated in distilled water (BA). After staining, the sections were rinsed briefly in ethanol, air-dried, and mounted under cover glasses in fluorescence-free

TABLE I
Microscopic Characteristics of Aleurone Grain Inclusions

Test	Inclusion Type I	Inclusion Type II
TEM[a] staining	Electron transparent	Electron dense
Toluidine blue	Red	Green
PAS[b]	–	+
Polarizing optics	Birefringent	Nonbirefringent
CNBr reactivity	–	+
DAB[c] reactivity	–	+

[a] Transmission electron microscopy.
[b] Periodate/Schiff's staining.
[c] 2,4-dimethylaminobenzaldehyde.

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immersion oil. PABA staining was examined with FC I, BA staining with FC II. Control sections were treated with CNBr alone or with PABA or BA alone.

The reaction occurs over CNBr *vapor* and is dependent in part upon section thickness and temperature. Therefore, as a simple test of reaction progress, a niacin-impregnated filter paper was placed in the reaction vessel with the sections and spotted with a drop of PABA or BA at 5-min intervals. Maximum color development (yellow-orange with PABA, orange-pink with BA) usually occurs within 10–20 min, and the sections are then ready for staining.

Amine Detection. Mason et al (1973) and Mason and Kodicek (1973a, 1973b) employed 2,4-dimethylaminobenzaldehyde (DAB) as a chromatographic spray reagent to detect aminophenol residues in wheat bran hydrolysates. Therefore, tissues were tested microscopically for similar components by adding a few drops of DAB solution (0.5% DAB in ethanol containing 1% v/v concentrated HCl) to sections and evaporating to dryness at 40–50° C.

Alternatively, to ensure that amines are not mobilized during treatment, additional sections were suspended over the DAB

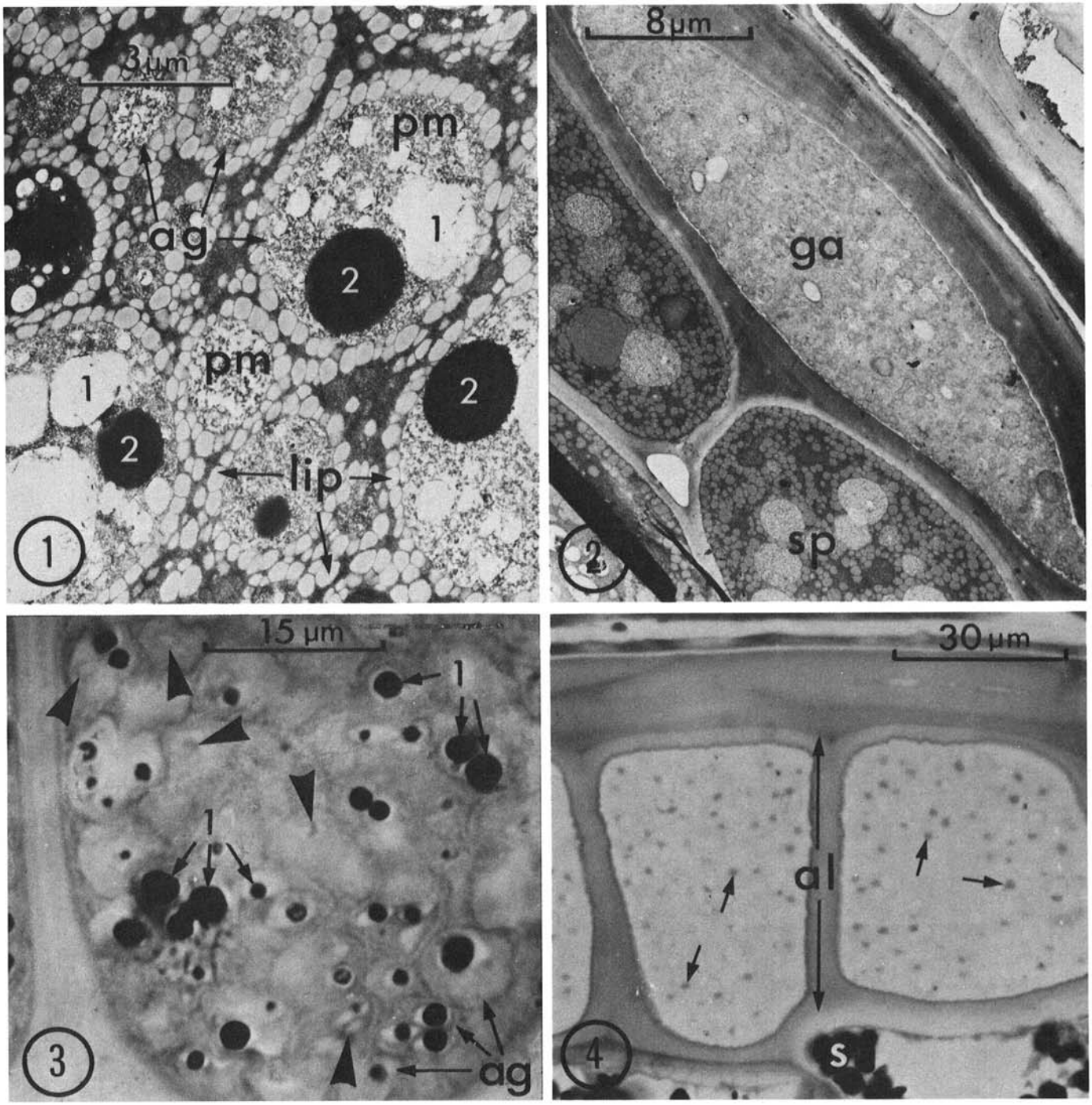

Fig. 1. Electron micrograph of wheat aleurone cell contents in the middorsal region of the grain. Each cell is packed with aleurone grains **(ag)** composed of a protein matrix **(pm)** and including two distinctly different types of inclusions **(1** and **2)**. Each aleurone grain is surrounded by lipid droplets **(lip). Fig. 2.** Electron micrograph of an aleurone cell **(ga)** and adjacent scutellar parenchyma cells **(sp)** taken from the midgerm region of wheat. **Fig. 3.** Light micrograph of wheat aleurone cell embedded in glycol methacrylate after toluidine blue staining, showing two types of inclusions **(small arrows, large arrowheads)** in each aleurone grain **(ag). Fig. 4.** Light micrograph of wheat aleurone cell embedded in glycol methacrylate and stained by periodate/Schiff's reaction to show periodate-sensitive structures **(arrows)** in the aleurone layer **(al)**. Cell walls—and starch **(s)** in the adjacent endosperm—are also stained.

solution at 60–80° C in a closed container for 1–2 hr. Sections were mounted in immersion oil and reaction products examined by fluorescence microscopy using FC I. Control sections were left untreated or treated with acidified ethanol only.

Chemical Assay—Niacin

To confirm that GMA embedding allows retention of significant portions of niacin in tissues, approximately 50 mg of wheat aleurone cell contents prepared by the method of St. Clair (1970) were fixed, dehydrated, and infiltrated with GMA as for normal microscopy. The material was then rehydrated with several changes of water over 4–6 hr, air-dried, and assayed for niacin content. (Assays were conducted by Roche Products Vitamin Laboratories, Sydney, Australia.) An unprocessed sample was also assayed.

Chemical Assay—Amine Extraction

Whole grain samples of wheat, oats, barley, and sorghum were milled to crude bran and flour fractions using the Ottawa Research Station miniature mill. Portions (20 g) of each fraction were suspended in 80% ethanol and extracted under reflux for 3 hr. The

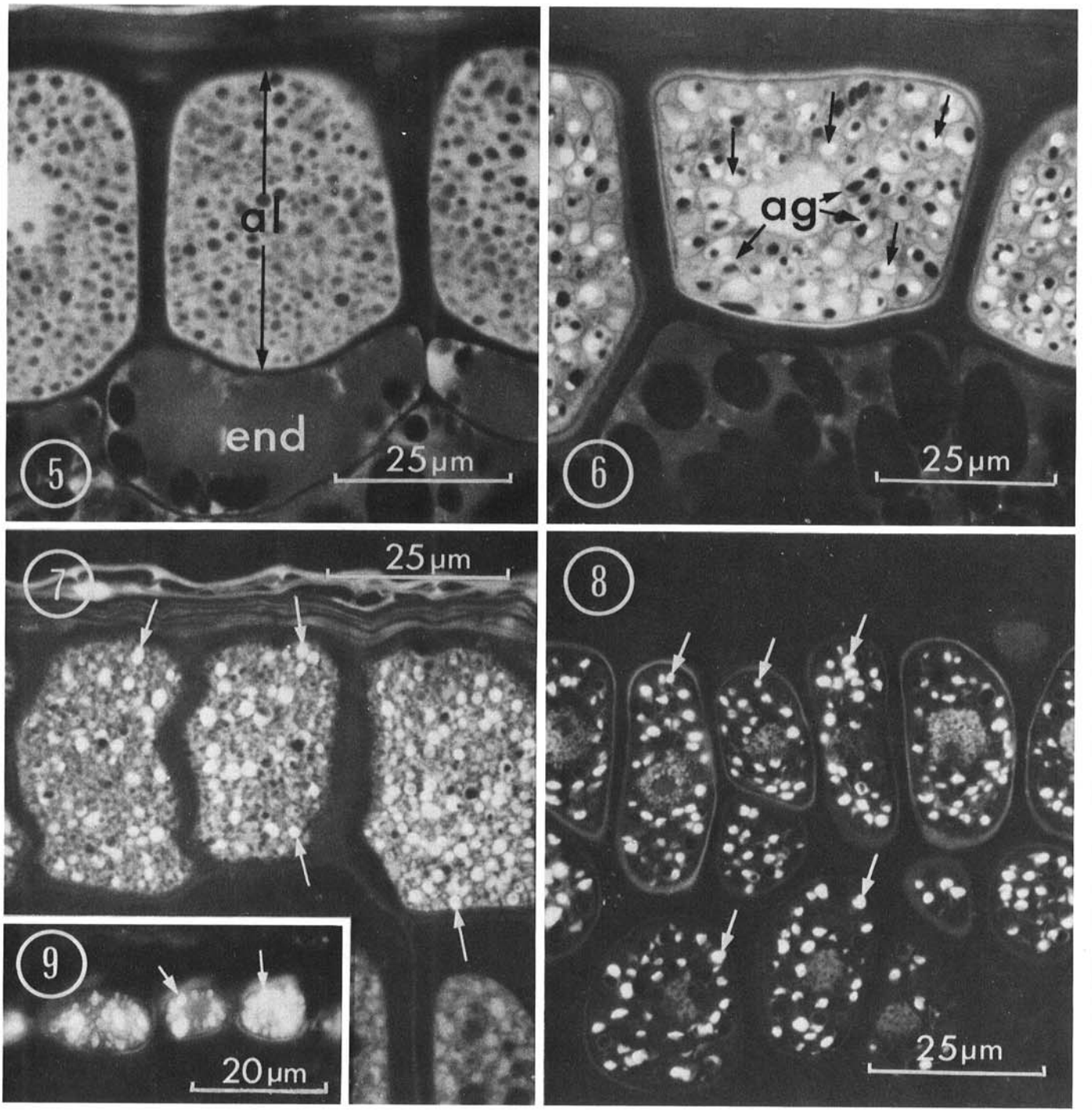

Fig. 5. Fluorescence micrograph of untreated control section of wheat showing autofluorescence. (Tissue embedded in glycol methacrylate, analysis by filter combination I). **Fig. 6.** Fluorescence micrograph of wheat and of oat **(Fig. 7)** sections treated with CNBr/*p*-aminobenzoic acid, showing intensely fluorescent deposits **(arrows)** within the aleurone grains **(ag).** (Tissues embedded in glycol methacrylate, analysis by filter combination I). **Fig. 8.** Fluorescence micrograph of barley material treated with CNBr/barbituric acid, showing intense reaction products **(arrows)** in aleurone cells. (Tissue embedded in glycol methacrylate, analysis by filter combination II). **Fig. 9.** Fluorescence micrograph of sorghum material treated with CNBr/barbituric acid, showing intense reaction products **(arrows)** in aleurone cells. (Material hand-sectioned, analysis by filter combination II.)

extracts were filtered to remove solids and evaporated to dryness in vacuo at 40° C. The residues were then hydrolyzed in boiling 2.5*N* NaOH for 6 hr. The hydrolysates were neutralized, evaporated to dryness, and extracted with 100 ml of ethanol, which was finally reduced to 5 ml for chromatography. Unhydrolyzed residues were also extracted with ethanol and examined chromatographically in comparison with an ethanolic solution of *o*-aminophenol (purified by sublimation). All solutions were spotted on silica gel thin-layer chromatography plates (Eastman Chromagram, or Analtec Inc. Uniplate) and developed in *n*-butanol/acetic acid/water (4:1:1; system I), benzene/methanol/acetic acid (45:8:3; system II), or chloroform/methanol (90:10; system III). Developed chromatographs were sprayed with DAB. Isolated aleurone cell contents were also tested. Chromatographed aminophenol and equivalent R_f regions of developed cereal samples were scraped from unsprayed TLC plates, extracted with methanol, and their ultraviolet absorption spectra obtained.

RESULTS

Microscopy

Transmission electron microscopy (TEM) of wheat aleurone cells shows two distinct types of inclusions in each aleurone grain or protein body (Fig. 1). They are similar structurally to those found in barley by Jacobsen et al (1971). Both inclusions (designated Type I and Type II) are common to all wheat aleurone cells except where the aleurone layer subtends the germ (Fig. 2). The latter cells contain few if any aleurone grains.

Table I summarizes the microscopic characteristics of the Type I and II inclusions. The Type I inclusion is birefringent (anisotropic) using polarizing optics and shows a strong metachromatic (red) color after toluidine blue staining (Fig. 3). It is relatively electron-transparent, presenting a fragmented or fractured appearance by TEM (Fig. 1), and may be removed from tissue sections by extraction with dilute acid (0.1–1.0*N* acetic acid, 5–15 min). One or several Type I inclusions may exist in each aleurone grain (Figs. 1 and 3) and range from 0.5 to 5.0 μm in diameter. Although we have examined only wheat bran by TEM, Type I inclusions can easily be detected in aleurone grains of all four cereals (wheat, oats, barley, and sorghum) using optical methods. The Type I inclusions are clearly the phytin globoids (crystals of calcium and magnesium double salt of *myo*-inositol hexaphosphate) that have been completely defined in barley (Jacobsen et al 1971).

The Type II inclusion (found in wheat, oats, and barley but not in sorghum) is chemically more complex. It is extremely electron dense (Fig. 1); it stains a faint but positive green color with toluidine blue (Fig. 3), which empirically indicates the presence of phenolic compounds (Feder and O'Brien 1968, Fulcher et al 1972a); and it is positively stained by the periodate/Schiff's reaction (Fig. 4), indicating a carbohydrate component with vicinal hydroxyl groups (Feder and O'Brien 1968). The inclusion is not anisotropic, and other tests for protein or lipid have failed to produce a positive reaction in the structure (Fulcher and Wong 1980, Hargin et al 1980).

CNBr reaction of GMA-embedded sections followed by PABA or BA treatment for niacin induces faint yellow or orange reaction products in the Type II inclusions. The coloration is often difficult to detect by conventional microscopy, although it is somewhat more visible in thicker hand-cut sections. However, in comparison with untreated control tissue (Fig. 5), the inclusions are demonstrated dramatically using fluorescence microscopy on even the thinnest GMA sections of wheat (Fig. 6), oats (Fig. 7), and barley (Fig. 8). CNBr alone also induces some yellow-green fluorescence in the inclusions (with FC I), and distinguishing between CNBr-induced fluorescence and the CNBr/PABA-induced fluorescence of Figs. 6 and 7 is occasionally difficult. However, the CNBr/BA method provides bright orange-red fluorescence with FC II (Figs. 8 and 9) for unequivocal demonstration of the complete reaction sequence and has the added advantage of providing greater contrast.

In wheat, oats, and barley, generally only one Type II inclusion, ranging from 1 to 5 μm in diameter, is found in each aleurone grain, and Type I structures are unstained (nonfluorescent). Sorghum, while reacting strongly with the niacin methods, shows no evidence of distinct Type II inclusions. Instead, the fluorescent reaction product appears to be distributed uniformly throughout the protein matrix of the aleurone grains (Fig. 9).

Treatment of hand-cut sections with DAB for amine residues also produces an intensely fluorescent reaction product in wheat (Fig. 10), barley (Fig. 11), and oats (Figs. 12 and 13) when examined with FC I. The deposits are characteristic of Type II inclusions. (Type I structures may be extracted with dilute acid before staining or viewed as separate entities by polarizing optics after staining.) Thin GMA sections show only faint fluorescence after treatment with DAB, indicating partial extraction of the amine substrate during processing or low fluorescence efficiency of the fluorochrome. Solution and vapor phase staining methods yield similar results, indicating that the substrate is not mobilized in sections during solution staining. Sorghum sections do not show significant fluorescence in the aleurone layer after DAB treatment.

The various fluorogenic tests for niacin and *o*-aminophenol were also conducted on filter papers impregnated with the two compounds. Fluorescence colors of the reacted authentic samples were identical to those of tissue deposits in all instances. Furthermore, *o*-aminophenol does not react in the niacin test, nor does niacin in the amine test.

Chemical Assays

Niacin. As shown by microbiological assays, isolated wheat aleurone cell contents contained approximately 1,500 μg/gm of niacin. Equivalent material that had been processed for GMA embedding retained significant concentrations of the vitamin (approximately 650 μg/gm), indicating that GMA sections are quite adequate for high resolution detection of niacin.

Amine Extraction. None of the unhydrolyzed ethanolic flour or bran extracts liberated substances similar in R_f to *o*-aminophenol in any of the three chromatographic solvent systems, although DAB-positive (yellow) material appeared at the origin and, inconsistently, as slower moving spots (R_f 0.65) when solvent system I on Uniplates was used. However, hydrolysis of wheat, oats, and barley bran extracts liberated significant quantities of material that cochromatographed with authentic *o*-aminophenol in all three solvent systems (R_f 0.76 in solvent I, Uniplate; and R_f 0.40 and R_f 0.27 in solvents II and III, respectively, on Chromagram sheets). Color development with DAB was identical to that of *o*-aminophenol, and unsprayed material removed from the chromatograms was also similar in ultraviolet absorption spectra to *o*-aminophenol (λ_{max} 285, 234 in methanol). Similar tests

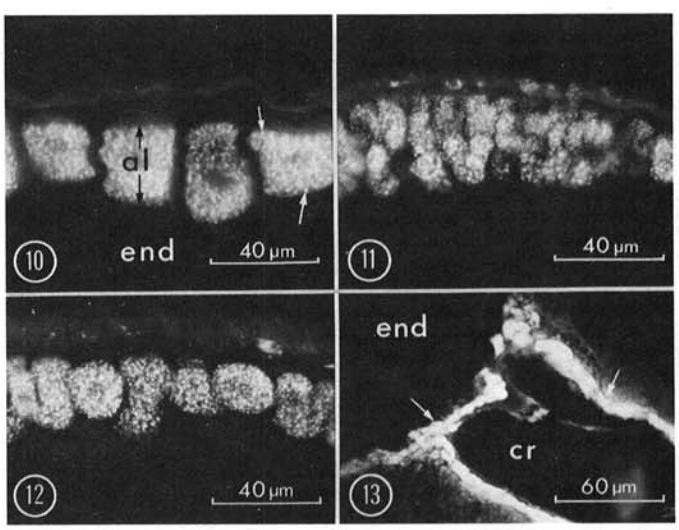

Fig. 10. Fluorescence micrograph of wheat and of barley (**Fig. 11**) and oat (**Fig. 12**) sections treated with 2,4-dimethylaminobenzaldehyde, showing fluorescent deposits (**arrows**) in the aleurone layer of each. No significant fluorescence is visible in the endosperm. (Material hand-sectioned, analysis by filter combination I.)

on sorghum were inconclusive because of large amounts of interfering pigments, and hydrolyzed flours of wheat, oats, and barley liberated only traces of aminophenol, which confirms the theory that the compound is confined primarily to the bran, as indicated by microscopic tests (Figs. 10–13).

DISCUSSION

The Type I inclusion in the aleurone grains is undoubtedly the ubiquitous phytin globoid first described by Pfeffer (1872). It is readily distinguished from the Type II inclusion by toluidine blue staining, polarizing optics, acid solubility, and a range of other assays including tests for calcium (Fulcher 1972) and phosphate content (Jacobsen et al 1971). The Type II inclusion, however, has variously been described as containing flavonoids (Chaze 1933, 1934), protein (Jones 1969), protein crystalloid (Buttrose 1971), and protein-carbohydrate (Jacobsen et al 1971). Otherwise, no suggestion has been made regarding the storage or physiological significance of the structure nor any additional information offered relating to its chemical composition. Our results suggest strongly that the Type II inclusion represents the major site of niacin deposition in wheat, oats, and barley.

The microscopic chemistry of the inclusion is compatible with that of the bound niacin residues described in some detail previously. In addition to niacin, Kodicek and Wilson (1960), Mason et al (1973), and Mason and Kodicek (1973a, 1973b) have all associated proteins, carbohydrates, substituted cinnamic acids, and *o*-aminophenol with the nutritionally unavailable vitamin complexes in wheat bran. Although we have been unable to detect protein residues in the inclusions even with very sensitive protein-specific fluorochromes (such as anilinonaphthalenesulfonic acid; Fulcher and Wong 1980), the remaining components are indicated by CNBr (for niacin), PAS (for carbohydrate), DAB (for aromatic amines) and toluidine blue (for phenolic compounds such as substituted cinnamic acids). Because the inclusion is embedded within a particularly dense protein matrix (and may have some structural continuity with it), protein residues in isolated preparations might be expected. The high concentrations of aromatic amines in the inclusions may in part explain earlier microscopic results showing positive reactions with acid dyes such as naphthol yellow and bromophenol blue (Jacobsen et al 1971). Such dyes have a particular affinity for amino groups.

In our view, the certain identity of *o*-aminophenol as a common bran constituent is not yet established. Although *o*-aminophenol and *o*-aminophenyl glycoside have been found in hydrolysis products of bran constituents (Mason and Kodicek 1973b), to our knowledge neither free *o*-aminophenol nor undegraded *o*-aminophenol complexes have been obtained from any plant tissue. In contrast, a group of similar compounds, the benzoxazinones, are well-known constituents of cereal vegetative tissues (Willard and Penner 1976). These hydroxamic acid derivatives have not yet been identified in cereal grains, but they are capable of hydrolysis to *o*-aminophenol (Hietala and Virtanen 1960). In either case, the DAB-positive residues in the Type II inclusion should be viewed with some interest—*o*-aminophenol possesses a particularly high redox potential and germination-modifying properties, as we have observed, and the benzoxazinones are strong microbial inhibitors (Willard and Penner 1976).

In view of the spectacular concentrations of niacin in cereal bran, the fact that the compound occurs in identifiable deposits in wheat, oats, and barley is not surprising. Sorghum, however, does not exhibit typical Type II inclusions, and the possibility arises that one or more additional patterns of niacin deposition may exist in other cereals and grasses. Indeed, the aleurone layers of some rice varieties apparently are devoid of Type II inclusions (Bechtel and Pomeranz 1977), although at least one sample contains crystalloid inclusions (Bradbury et al 1980) similar to the Type II inclusions of wheat, oats, and barley. More detailed analyses are required to fully define the variety of niacin complexes in cereals.

The identification of niacin in the protein matrix or discrete inclusions *within* aleurone grains raises further questions regarding nutritional characteristics of bran. Although isolated niacin complexes are themselves unavailable nutritionally (Mason and Kodicek 1973a), consideration must also be given to other structural constraints on digestibility. These may be conferred by a relatively tough and impermeable phenolic-enriched aleurone cell wall (Fulcher et al 1972a), a continuous coating of lipid droplets around each aleurone grain (Hargin et al 1980), or embedment in the dense, basic amino acid-enriched protein matrix (Fulcher et al 1972b) characteristic of most aleurone grains. It is no wonder that Girard (1884) found bran to be relatively impervious to digestive activity!

Finally, niacin deposits are found only in the aleurone layer of the four cereals tested, whereas phytin inclusions are common to both the aleurone and scutellar parenchyma. Thus a test for distinguishing aleurone layer and scutellar aleurone grains is available. Niacin deposits in single aleurone grains can be identified easily in flour and other processed material.

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