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X-ray Analysis Studies of Elements Stored in Protein Body Globoid Crystals of *Triticum* Grains[1]

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ABSTRACT

Energy-dispersive x-ray analysis was used to investigate the elemental storage within protein bodies, specifically the globoid crystals, in grains of wheat. Areas of the grain investigated included various parts of the embryo, the aleurone layer plus starchy endosperm near the embryo and the aleurone layer plus starchy endosperm farthest from the embryo. Variations did occur grain-to-grain, cell-to-cell and, in certain regions, intracellularly. No protein bodies with electron-dense globoid crystals were found in the starchy endosperm. Generally globoid crystals contained P, K, and Mg in all areas investigated. Globoid crystals from the aleurone layer farthest from the embryo on occasion contained Ca, whereas aleurone globoid crystals near the embryo sometimes contained Fe. In most of the embryo regions examined, a few globoid crystals contained Ca along with P, K, and Mg. No specific pattern to the Ca distribution could be found. Well-defined elemental distribution occurred with Mn. Manganese was found only in globoid crystals located in the base and midregions of the stele in the radicle. Thus, in wheat there is some specific distribution of minerals dependent upon cell type and/or position in the grain.

Seeds generally store sufficient reserves to allow successful establishment of seedling plants. The reserves of organic compounds, such as lipids, proteins, and carbohydrates, when combined with stores of necessary mineral nutrients, ensure that almost all materials needed during germination and early seedling growth are contained within the seed.

Protein bodies (aleurone grains), which are very important subcellular structures involved in storage in seed tissues, show considerable structural diversity. Depending upon the plant species and the tissue involved, protein bodies may consist of: (*a*) structurally amorphous proteinaceous matrix only, (*b*) proteinaceous matrix plus electron-dense globoid crystals, (*c*) proteinaceous matrix plus globoid crystals plus protein crystalloids, (*d*) proteinaceous matrix plus globoid crystals plus soft globoids plus protein crystalloids, (*e*) proteinaceous matrix plus druse crystals (6, 7, 16, 20, 33).

Although much of the volume of protein bodies is occupied by proteinaceous reserves, the protein bodies are also responsible for storage of most of the seed's supply of minerals. Mineral reserves occur mainly within the electron-dense globoid crystal portion of protein bodies (14, 22, 23, 39, 40). These mineral reserves seem to be mainly in the form of phytin, a cation salt of inositol hexaphosphoric acid (1, 8, 23, 46). Although Mg and K are the most commonly occurring cations found in globoid crystals, a range of other cations, including Ba, Ca, Fe, Mn, and Na, have been demonstrated (5, 9, 17, 19, 22, 37, 47).

Since phytin contains such potentially useful molecules as inositol and a range of minerals used by animals, one might be tempted to think that the presence of phytin in seeds would add to their nutritive value. This does not appear to be the case, however. Since phytin is a chelator, it can bind cations, such as Ca, Mo, Zn, Fe, and Mg, in the digestive tract of animals and lead to mineral deficiencies even though the intake of such minerals is seemingly adequate (see reviews in refs. 3 and 12). Since phytin can form complexes with certain proteins, it is also known to inhibit peptic digestion.

We believe that studies of possible tissue-to-tissue and cell-to-cell variations in mineral storage are important both for an understanding of how cereal grains develop initially and for an understanding of the events occurring during germination and seedling growth. For several reasons, EDX[2] analysis was chosen as the investigative probe for our studies of mineral storage in wheat grains. Advantages of the EDX analysis system for such studies include: the capacity to spot-analyze chosen cell regions; the high detection sensitivity of 10^{-17} to 10^{-18} g (34); simultaneous analysis of all elements of interest including P, K, Mg, Ca, Fe, and Mn; and the capacity to easily detect any unexpected mineral, should it occur.

To date, x-ray analysis studies of seed protein bodies have concentrated upon dicotyledonous plants. The monocotyledonous species, whose seed protein bodies have received at least some study with x-ray analysis, include *Hordeum vulgare* (11), *Triticum aestivum* (47) and *Oryza sativa* (31, 44, 47).

In cereals, the most advanced characterization of the mineral storage system is in rice. These studies from the Research Institute for Food Science at Kyoto University have concentrated upon the aleurone layer and scutellum of rice. Phytin-containing particles have been isolated and chemically characterized (29, 30, 46), the accumulation site of phytic acid has been demonstrated with autoradiography (48), and selected tissues have been studied with microprobe x-ray analysis or EDX analysis (31, 44, 45, 47). In the rice tissues studied, the phytin deposits were mainly Mg and K salts of phytic acid.

A number of studies have demonstrated the presence of phytin in wheat grains (3, 13, 24, 27, 49). Autoradiography has been used to demonstrate that ripening wheat grains accumulate the phytin precursor [^{3}H]myoinositol into protein bodies of the aleurone layer (48). Also, electron microprobe x-ray analysis has been used to

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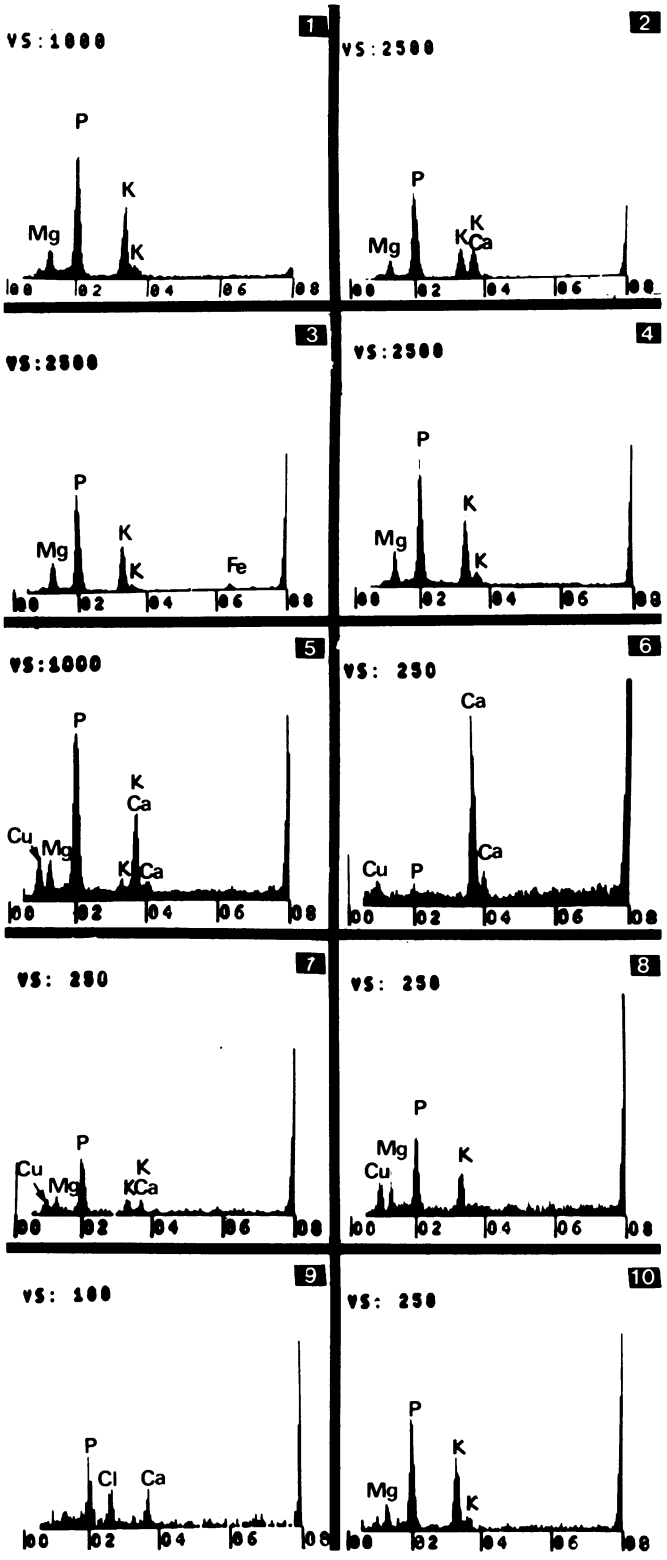
[2] Abbreviation: EDX: energy-dispersive x-ray.



Selected tissues from wheat grains were prepared either as freeze-dried powders (Fig. 16) or were fixed in glutaraldehyde in distilled H_2O, dehydrated, embedded in Spurr's low viscosity resin and sectioned (all but Fig. 16) prior to EDX analysis of globoid crystals. Copper peaks, when present, are an artifact of copper grid usage. Elements present in globoid crystals, energy levels in kev and principal emission lines are as follows: calcium 3.690, $K_{\alpha 1,2}$ and 4.012, K_β (10% of $K_{\alpha 1,2}$); chlorine 2.621, $K_{\alpha 1,2}$; copper 0.930, L_α and 8.040, $K_{\alpha 1,2}$; iron 6.398, $K_{\alpha 1,2}$; magnesium 1.253, K_α; manganese 5.894, $K_{\alpha 1,2}$; and 6.489 $K_{\beta 1}$ (13% of $K_{\alpha 1,2}$); phosphorus 2.013, $K_{\alpha 1,2}$ and 2.028, $K_{\alpha 4}$ (10% of $K_{\alpha 1,2}$ peak) and 2.137 K_β (4% of $K_{\alpha 1,2}$ peak); potassium 3.312, $K_{\alpha 1,2}$ and 3.589, K_β (10% of $K_{\alpha 1,2}$ peak); sulfur 2.307, $K_{\alpha 1,2}$ and 2.322, $K_{\alpha 4}$ (50% of $K_{\alpha 1,2}$). Note that the K_α peak for calcium at 3.690 kev is overlapped by the K_β peak of potassium at 3.589 kev. Since the minor potassium K_β peak is 10% of the major K_α peak for potassium at 3.312 kev, subtraction will reveal the true calcium value. Where Ca is labeled on the spectra its presence was verified by numerical methods involving background subtraction and calculation of integrated peak heights.

FIGS. 1 and 2. EDX analysis spectra of sections of globoid crystals in aleurone layer cells positioned at the end of the grain farthest from the embryo.

FIGS. 3, 4 and 5. EDX analysis spectra of sections of globoid crystals in aleurone cells located near the embryo.

FIG. 6. EDX analysis spectrum of some tiny globoid crystals in a subaleurone cell located at the end of the grain near the embryo.

FIGS. 7, 8, and 9. EDX analysis spectra of sections of globoid crystals in coleoptile cells at a position midway between the base and the tip. Figures 7 and 9 were from the outer epidermal cells, whereas Figure 8 was from a cell three layers of cells in from the outer epidermis.

FIG. 10. EDX analysis spectrum of a globoid crystal from a cell inside a young foliage leaf.

show that P, K, and Mg are concentrated into wheat aleurone layer protein bodies (47).

MATERIALS AND METHODS

Seeds. Seeds of spring wheat (*Triticum aestivum* L. cv. Glenlea) were obtained from Dr. M. Webber, Canada Centre for Inland Waters, Burlington, Ontario.

Tissue Fixation and Embedding. Prior to fixation, the fused pericarp and testa of wheat grains were removed. Four regions then were dissected from each seed as follows: scutellum; embryo axis, including coleoptile and coleorhiza; aleurone plus starchy endosperm from the end of the grain farthest from the peduncle. To avoid any possible extraction of elements due to osmium tetroxide usage (18), the tissue pieces were fixed only in glutaraldehyde according to the procedure of Lott (14). Briefly, the tissue was fixed at 1 C for 1.5 h in 5% (v/v) glutaraldehyde in distilled H_2O (pH 7.1), rinsed in distilled H_2O, dehydrated using an ethanol series, further dehydrated in propylene oxide, infiltrated with Spurr's resin, and polymerized. Selected tissue regions were sectioned in the 150- to 170-nm range and then mounted on carbon-Formvar-coated copper grids.

Freeze-dried Tissue Powders. Freeze-dried tissue powders were used to check for possible extraction of elements from globoid crystals during fixation, dehydration, and embedding. Selected portions of wheat grains were carefully dissected out, then frozen in liquid N_2, fractured while frozen, and low temperature freeze-dried using the procedure of Lott and Vollmer (22). The resulting freeze-dried powders were dusted or pressed onto Formvar and carbon-coated grids. Globoid crystals were located by their electron density.

Energy Dispersive X-ray Analysis. EDX analysis of globoid crystals was conducted with a Philips EM300 transmission electron microscope on which was fitted an EDAX International, Inc. model 606 x-ray spectrometer, a model 707A detector, and an EDIT data improvement system. An accelerating voltage of 80 kv and an analysis time of 60 s were used for all analyses. Background subtraction and calculation of integrated peak height values, which were made according to a procedure described by Lott *et al.* (19), allowed confirmation of the presence or absence of Ca. To reveal if any calcium was present, 10% of the potassium $K_{\alpha 1,2}$ peak value was subtracted from the combined value for the potassium K_β peak and Ca $K_{\alpha 1,2}$ peak.

Three seeds fixed in 5% (v/v) glutaraldehyde, embedded in Spurr's, and sectioned were used as a check of seed-to-seed

variation. In a given seed, at least five globoid crystals were analyzed for each cell type found in the various endosperm and embryo regions studied. The exception was the epiblast where globoid crystals were rare. For the epiblast, only six globoid crystals were located in sections from the three seeds combined. Areas of the seed, such as the radicle, which appeared to show specific elemental composition patterns were EDX-analyzed much more extensively. At least 10 globoid crystals were EDX-analyzed in each of the aleurone, scutellum, and embryo freeze-dried samples taken from a single seed.

RESULTS

SECTIONS OF FIXED AND EMBEDDED TISSUE

Aleurone and Starchy Endosperm. Cells of the aleurone layer contained protein bodies with large globoid crystals. Generally, no globoid crystals were identified in protein bodies of starchy endosperm cells inside the aleurone layer. Endosperm samples analyzed were either from the vicinity of the embryo or from the region farthest from the embryo. Globoid crystals in protein bodies of the aleurone layer cells farthest from the embryo generally contained P, K, and Mg but no Ca (Fig. 1). In one of the three seeds examined, a few globoid crystals containing P, K, Mg, and Ca were found (Fig. 2). No Fe or Mn were found in globoid crystals from aleurone cells farthest away from the embryo. Globoid crystals from aleurone layer cells near the embryo generally contained P, K, Mg, and sometimes Fe, but they lacked Ca (Figs. 3 and 4). Fe content of globoid crystals from aleurone cells near the embryo varied from seed to seed and within one seed. Seed 1 samples contained no Fe in these globoid crystals. Seed 2 samples generally contained some Fe (Fig. 3), and seed 3 samples contained traces of Fe in some and no Fe in others (Fig. 4). In seed 3, a few globoid crystals were found which contained P, Mg, and Ca but little K (Fig. 5). One subaleurone cell was found which had tiny crystals. These small crystals, which were not as electron-dense as normal globoid crystals, were high in Ca (Fig. 6).

Coleoptile. Coleoptile samples were obtained for only two of the three seeds studied. Globoid crystals in coleoptile protein bodies were small in size and, thus, some proteinaceous matrix was present in most analyses. Subepidermal cells of the coleoptile generally had globoid crystals containing P, K, and Mg but no Ca (Fig. 7). Globoid crystals from epidermal cells of seed 1 contained either P, K, Mg, and Ca (Fig. 8) or P, K, and Ca, whereas those of seed 2 contained P and Ca but no K or Mg (Fig. 9). Chlorine values varied from absent to quite distinct (Fig. 9). The smallest globoid crystals had the highest Cl values, which suggests that the Cl was present in the proteinaceous matrix or in the epoxy resin used for embedding. Globoid crystals from both seeds examined lacked Fe, but one subepidermal cell globoid crystal was found that contained some Mn.

Young Foliage Leaves. Protein bodies in the interior cells of young foliage leaves contained small globoid crystals. EDX analysis of these globoid crystals revealed the presence of P, K, and Mg. No Ca was present in the globoid crystals of seeds 1 and 3 (Fig. 10), but traces of Ca were found in some globoid crystals from seed 2. Within one embryo there seemed to be no distinctive differences between the base, center, and tip of a given foliage leaf or between the outermost foliage leaf and the smallest distinct leaf near the shoot apex. No globoid crystals were found in epidermal cells of the specimens examined.

Stem. The stem consisted of the area bounded by the following tissue regions: epiblast, radicle scutellum, coleoptile young foliage leaves, and shoot apex. Globoid crystals throughout the stem were small in size. EDX analysis of globoid crystals from various parts of the stem in all three seeds revealed the presence of P, K, and Mg (Fig. 11). Ca was usually absent. However, one cell was found that contained globoid crystals with P, Mg, and Ca but little K. One globoid crystal containing some Mn was found.

Epiblast. Cells of the epiblast, which is a small structure on the opposite side of the embryo axis from the scutellum attachment point, contained few, if any, globoid crystals. Sections of the epiblast of seed 2 contained no globoid crystals. Those globoid crystals that were present were small in size. Globoid crystals in the epiblast of seed 1 contained P, K, and Mg but no Ca, Fe, or Mn. The one globoid crystal found in the center and base of the epiblast of seed 3 contained P, K, Ca, and a trace of Fe.

Scutellum. Globoid crystals were commonly found in scutellum cells. These globoid crystals ranged in size with those of the epidermal cells being the smallest. In two of the seeds examined, the scutellum globoid crystals commonly contained either P, K, and Mg (Fig. 12) or P, K, Mg, and traces of Ca. The globoid crystals with traces of Ca occurred in less than one-quarter of all globoid crystals analyzed in these two seeds. The globoid crystals of seed 3 contained P, K, Mg, and variable amounts of Ca. A few globoid crystals lacked Ca, but most had at least traces of Ca (Fig. 13). Globoid crystals of epidermal cells contained P, K, Mg, and variable Ca levels (Figs. 14 and 15). About one-third of the epidermal globoid crystals contained no detectable calcium (Fig. 14). No Fe or Mn peaks were found in any areas of the scutellum.

Radicle. In the base and midregions of the radicle, a difference in globoid crystal composition was noted between stele and cortical regions. In the stele regions, globoid crystals usually contained P, K, Mg, and Mn but no Ca (Fig. 17). In the cortical regions, the globoid crystals always contained P, K, and Mg, whereas Ca levels varied (Figs. 18 and 19). In about one-third of the globoid crystals examined, Ca was present. Mn was usually absent. Radicle epidermal cells contained very small globoid crystals that usually contained P, K, and Mg, but no Ca or Mn. The radicle apical meristem was located in only one of the three seeds studied. Globoid crystals in radicle apex cells contained P, K, Mg, and variable Ca levels (Figs. 20 and 21). No pattern of Ca distribution was found in relation to position within the radicle apex. Mn was generally not found in the globoid crystals of the radicle apex. Root-cap cells contained tiny globoid crystals. Analysis of these small globoid crystals, including some proteinaceous matrix, revealed P, S, Cl, Ca, little or no Mg, and no K or Mn (Fig. 22).

Coleorhiza. Definite coleorhiza tissue was located in two seeds. Considerable size variation in globoid crystals occurred, even within one cell. Elemental content appeared to be related to globoid crystal size. Large globoid crystals were composed of P, K, and Mg and lacked Ca (Fig. 23). As globoid crystal size decreased, the K and Mg content decreased and Ca content increased (Fig. 24). Thus, a range of Ca levels occurred even within the globoid crystals of one cell (Figs. 23 and 24). Mn and Fe were not generally present.

FREEZE-DRIED TISSUE POWDERS

Globoid crystals were examined in freeze-dried tissue powders made from the following tissue regions: scutellum; embryo axis, including coleoptile and coleorhiza; and endosperm near the embryo. The endosperm samples were shaved off the edge of the endosperm and, thus, any globoid crystals present were presumably from aleurone cell protein bodies. The major difference between the freeze-dried powders and the fixed tissue related to K levels. In all tissues examined, the K levels were generally higher in freeze-dried powders than in sectioned tissue, which indicates that considerable K was removed during the fixation, dehydration, and embedding process used. The only illustration of this difference presented here is for scutellum tissue (compare Fig. 16 with Figs. 11–13).

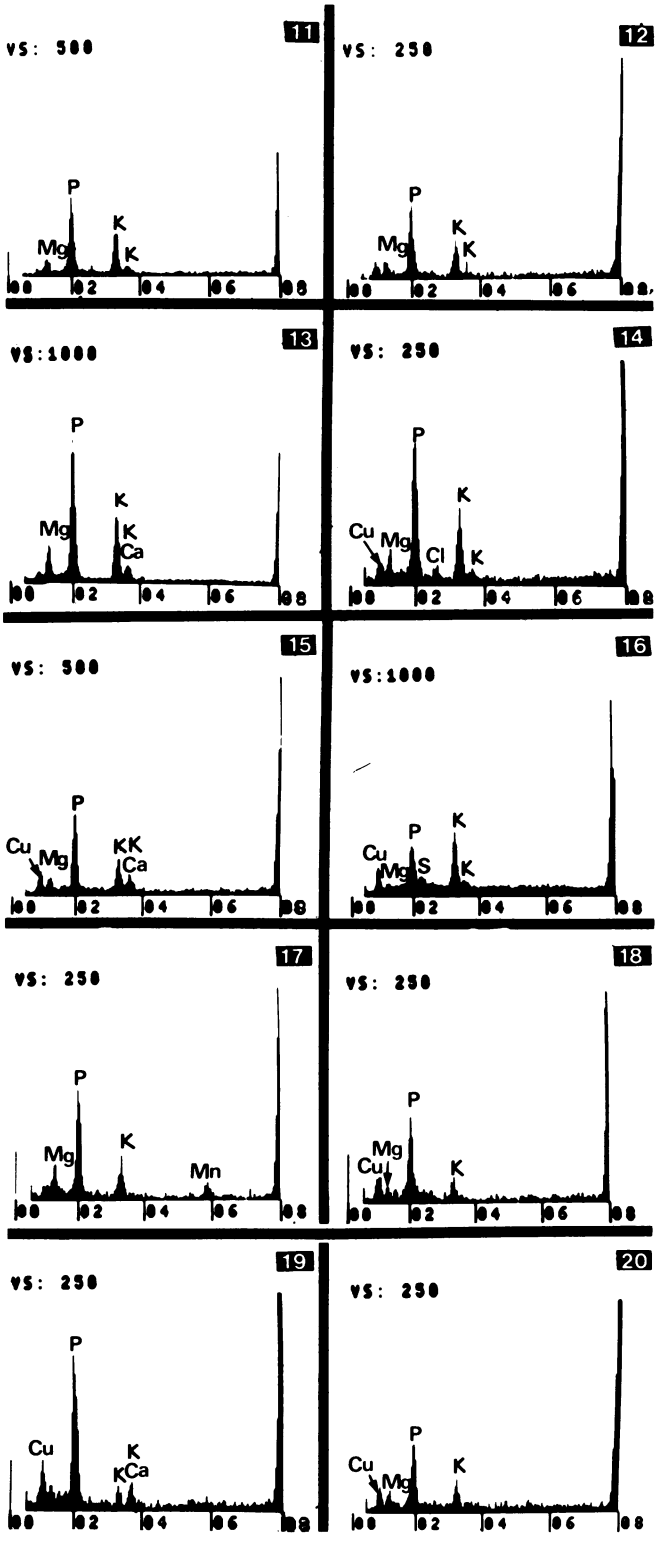
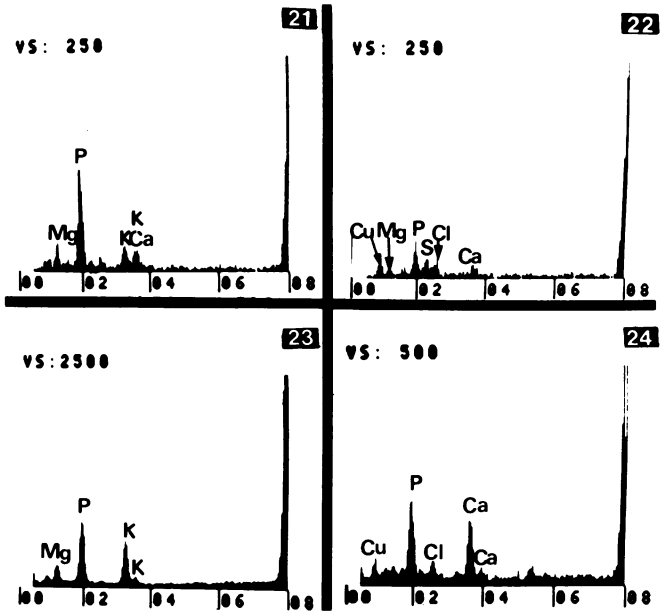


FIG. 11. EDX analysis spectrum from a section of a globoid crystal from the stem region at the first node level.

FIGS. 12 to 16. EDX analysis spectra of globoid crystals from the scutellum. Figures 14 and 15 were from epidermal cells, Figure 12 was in a subepidermal cell on the endosperm facing side of the scutellum, and Figure 13 was from a cell many cells in from the epidermis. Since Figure 16 was found in a freeze-dried powder of scutellum tissue the exact location where it originated cannot be determined.



FIGS. 17 to 22. EDX analysis spectra of sections of globoid crystals from radicles. Figure 17 was from a stele cell, Figures 18 and 19 were from cortical cells, Figures 20 and 21 were from cells in the apical meristem, and Figure 22 was from a root cap cell.

FIGS. 23 and 24. EDX analysis spectra of globoid crystals in coleorhiza tissue. The globoid crystal examined for Figure 23 was a large one, whereas the globoid crystal analyzed for Figure 24 was a small one.

DISCUSSION

Protein bodies have been considered in a number of published ultrastructural studies of wheat grains. Past studies have concentrated upon endosperm, including aleurone (2, 4, 10, 25, 26, 36, 38), and scutellum (28, 32, 41, 42, 43), but studies of other embryo regions have also been made (32). From the studies listed above, it is clear that distinct globoid crystals are commonly found in protein bodies in aleurone cells and embryo cells but are lacking in starchy endosperm cell protein bodies. The results of the current study provide additional evidence for such a globoid crystal distribution pattern. In this study, where identification of globoid crystals in tissue fixed with glutaraldehyde only depends upon the native electron density of the globoid crystals, generally no globoid crystals were found in starchy endosperm cell protein bodies. Our studies, however, do not rule out either the possibility that minute globoid crystals occur in such protein bodies or the existence of minute electron-transparent globoids.

When using fixed and sectioned tissue for EDX analysis, the possibility of extraction or movement of compounds is of great concern. X-ray analysis studies of globoid crystals in dry seeds have distinct advantages over most other biological systems. One major advantage is having a method which permits estimation of any elemental loss due to preparative procedures. Because globoid crystals are electron-dense, they can be identified without the addition of a fixative, such as osmium tetroxide. Through the use of freeze-dried powders, it is possible to obtain data on elemental composition of globoid crystals in an unfixed state. Using such procedures, it has been possible to document major extraction of elements from globoid crystals due to use of OsO_4 (18). Fixative-based loss of a readily soluble K compound from legume seed protein bodies has also been shown (15, 22). Use of freeze-dried powders of portions of wheat grains leads us to believe that the glutaraldehyde fixation, dehydration, and embedding procedure used here does cause some differential extraction of K. Although some K is extracted, considerable K remains. No attempt has been made here to quantify overall losses of elements but, in a quali-

tative study such as this one, it is differential element extraction that is of greatest concern. The tendency of K to be somewhat more easily removed than other elements in globoid crystals has also been reported for *Cucurbita maxima* (19).

When examining EDX analysis spectra to obtain comparison of the amounts of different elements that are present, it is important to realize that the number of x-rays produced, and hence the peak heights, are not the same for equal concentrations of all elements. P values, which give the relative intensity of x-rays from each element at the same concentration, have been prepared from standards (35). The P values of 1.0, 0.94, and 0.93 for Mn, Ca, and K, respectively, indicate that peak heights for these three elements would be similar if these elements were present in equal concentrations. In comparison with Mn, Ca, and K, the peak heights for phosphorus are underestimates (P value of 0.75), and those of Mg are major underestimates (P value of 0.47).

That specific deposition patterns for certain minerals can occur in some species is evident from previous work on *Cucurbita* (19, 21, 22) and tomato seeds (39). The results of this study on wheat now provide evidence of a specific distribution pattern in a monocot. The positioning of Mn deposits in parts of the radicle is very specific indeed.

Even though we have observed specific distributions of certain minerals in certain seeds, it is not clear how such distributions are controlled. Although seed size may be one influence (22), that alone cannot account for the results. For example, why is it that *Cucurbita* species seem to control distribution of Ca, whereas, in tomato seeds and wheat grains, the Ca distributions are seemingly random, but Mn and Fe deposits are specifically located? There is a great need for information on the events that occur during seed formation. Although it is tempting to suggest that the observed distributions are due to differences in time of synthesis, the observations of Ogawa *et al.* (31) on developing rice aleurone indicates that ion accumulation may change with stage of development. In rice aleurone, Mg and P began to concentrate about 12 days after flowering, whereas K concentrations began to occur at the 19th day after flowering.

With regard to the wheat aleurone layer, the findings reported here on cultivar Glenlea are similar to those reported by Tanaka *et al.* (48) from cultivar Shirasagi. In both cultivars, the cations concentrated in protein bodies of the aleurone layer were mainly K and Mg. Some Mn and Fe was concentrated in the aleurone layer of cultivar Shirasagi. We discovered some Fe in aleurone layer globoid crystals, but this seemed to vary from seed to seed.

In the studies reported here, Ca was often not present in wheat grain globoid crystals. Where Ca was located, it was often present in only trace amounts or, when present in greater amounts, was found in only a small proportion of the globoid crystals in a tissue. Based upon our findings, we would predict that a bulk analysis of wheat grains for the cations associated with phytin would show considerable K and Mg with lesser amounts of Ca and a trace of Mn and Fe. Chemical analysis of wheat phytin shows 12.3% Ca, 1.5% Mg, and 0.1% Mn (1). This apparent disparity between Ca and Mg levels could be for several reasons. As pointed out by Ashton and Williams (1), the composition of prepared phytin may be determined to some extent by the conditions under which it is precipitated. It may be that the previous chemical analysis work does not give a true picture of the state of phytin in the grain. Alternatively, it may be that considerable Ca phytate is present in wheat pericarp or in cell areas within embryo and endosperm regions other than in electron-dense globoid crystals. It seems unlikely that major deposits of this type exist within embryo and endosperm regions since major deposits would probably have been observed during our EDX analysis studies or during previous autoradiographic studies of accumulation of [^{3}H]-myoinositol in developing wheat grains (48). Although the pericarp was not investigated in this study, the autoradiographic results presented in Tanaka *et al.* (48) show no accumulation of [^{3}H]-myoinositol in the pericarp of developing rice and wheat grains. The x-ray microprobe studies of Liu and Pomeranz (11) show some K and Ca in barley pericarp samples but no detectable P. This observation indicates that phytin cannot be concentrated in barley pericarp tissues since the P from the hexaphosphate unit would be readily detected. Other possibilities that could contribute to this difference include: that some of the mineral reserves which we locate with EDX analysis are not in the form of phytin, that there is considerable cultivar-to-cultivar differences, or that differences are due to differences in growth conditions. In future studies, such possibilities could be investigated by obtaining quantitative determinations of elemental composition in selected embryo, endosperm, and pericarp regions through use of methods, such as neutron activation analysis.

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