

The Influence of Phosphorus Nutrition on the Appearance and Composition of Globoid Crystals in Wheat Aleurone Cells

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ABSTRACT

Cereal Chem. 63(1):14-18

Wheat plants were grown in the greenhouse with contrasting levels of phosphorus (control and low P). Ultrastructure and elemental composition of globoid crystals in aleurone cells were studied in grains at 31 days after pollination (when the low-P grains began to accumulate phytate phosphorus) and at maturity. The structure of aleurone protein bodies was altered considerably by low-P conditions. Transmission electron microscopy showed that protein bodies in aleurone cells of control grains had large, dense globoid crystals. Globoid crystals were difficult to locate in

immature, low-P grains and were small and numerous in mature, low-P grains. Energy-dispersive X-ray analyses of immature and mature grains of control and low-P samples determined that the elemental composition of the globoid crystals was dominated by P, K, and Mg. A significant increase in the K:P ratio was observed in low-P grains. This, together with the small size of the globoid crystals in low-P grains, may have caused the loss of globoids when grains were fixed using traditional aqueous methods.

In whole wheat grain, 38 to 94% of the total phosphorus is bound as phytate (Bassiri and Nahapetian 1977). In grains of wheat, barley, rice, rye, oats, and orchard grass, a large proportion of this phosphorus is found in the aleurone layer, although both aleurone and scutellum are rich in phosphorus (Wada and Maeda 1980). In these tissues, most of the phosphorus is concentrated in electron-dense regions within protein bodies as phytate, the potassium and magnesium salt of myo-inositol hexakisphosphate (Tanaka et al 1974a,b; Lott and Spitzer 1980; Spitzer et al 1981). The composition of the electron-dense globoid crystals appears to be similar in the aleurone and scutellum (Tanaka et al 1976). The starchy endosperm contains no protein bodies with these globoid crystals (Lott and Spitzer 1980). The formation and subsequent breakdown of phytate has been reviewed recently by Loewus and Loewus (1983).

Jennings and Morton (1963) and Williams (1970) reported that phytate forms rapidly as wheat grains reach maximum dry weight. Deposition of phosphorus as phytate at this time may be a mechanism for maintaining the cellular inorganic phosphorus concentration that regulates such biochemical reactions as starch biosynthesis (Michael et al 1980, Jenner 1976).

Nahapetian and Bassiri (1975) found that phytate forms rapidly during the period of leaf chlorophyll destruction. They argued that degradation of chlorophyll would be accompanied by the release of magnesium, which might contribute to additional precipitation of phytate. Grains with a higher total phosphorus concentration have a higher phytate phosphorus concentration (Nahapetian and Bassiri 1976, Lolas et al 1976). However, no reports show the effect of a change in grain phosphorus concentration on the appearance and composition of the globoid crystals.

In this study we examined the effects of changing the grain phosphorus concentration on the ultrastructure and elemental composition of aleurone cells of mature and immature grains. Energy-dispersive X-ray (EDX) analysis was chosen for the study of elemental composition. The advantages of this method of analysis to this study were: the capacity to spot-analyze globoid crystals; the high detection sensitivity of 10^{-17} to 10^{-18} g (Russ 1972); 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 (Lott and Spitzer 1980).

MATERIALS AND METHODS

Growth Conditions and Tissue Sampling

The grains used in this study were from wheat (*Triticum*

aestivum L. cv. Kite) grown in sand culture in the phytotron of the Commonwealth Scientific and Industrial Research Organization (CSIRO) in Canberra, Australia, by Batten (1984). Contrasting grain phosphorus levels were achieved by varying the phosphorus in the Hoagland's nutrient solution applied daily. Control plants were fed a 1 mM P solution, which is the same concentration as that used throughout the phytotron. Low-P plants received a 0.25 mM P solution for 20 days and no additional phosphorus thereafter. Leaves of control plants remained green throughout grain filling, but in low-P plants the retranslocation of nutrients from leaves and glumes led to rapid senescence during grain filling.

Mature, air-dried grains were taken from two replicate plants grown at 15°C during the day (8 hr) and 10°C (15/10, experiment 1) at night. From a second experiment, where plants were grown at 18°C during the day and 13°C at night (18/13), grains were taken from central spikelets of three replicate plants beginning 10 days after pollination (DAP). At harvest the grains were frozen in liquid nitrogen, freeze-dried, weighed, and stored at -25°C.

Whole grains were crushed to a powder in a ball mill. Phosphorus was extracted from 100-mg samples in 5 ml of 0.3M trichloroacetic acid (TCA) with gentle shaking for 45 min. Ten milliliters of distilled water was added, and the 0.1M extract was centrifuged at $3,000 \times g$ for 10 min. A second 0.3M TCA extraction was made over 30 min. After filtering, the combined extracts were placed on a 5×1 cm column of Dowex 1 AG2 (TCA form) resin and eluted with 70 ml of 0.1M TCA to recover inorganic phosphorus and some nonphytate phosphorus. The column was then further eluted with 49 ml of 1M HCl to recover phytate-bound phosphorus (Cosgrove 1980). Phosphorus in 2-ml aliquots of the eluted fractions was mineralized using 200 μ l of 70% HClO₄. The total phosphorus (as orthophosphate) in each fraction was estimated colorimetrically (John 1970).

Electron Microscopic Observation of Aleurone Cells

To ensure that all samples came from the central part of the grain, both the end containing the embryo and the tip end of the grain were removed and discarded. The remaining portions were further dissected so that portions of the grain exterior including the aleurone layer were separated from the bulk of the endosperm. Pieces from individual grains were kept separate to ensure that samples from a number of different grains were studied. Tissue pieces containing aleurone cells from dry grains were fixed for 4 hr in cold 5% glutaraldehyde in 60% EtOH (Lott et al 1984). After fixation, the tissue was gradually dehydrated with absolute ethanol, further dehydrated with propylene oxide, infiltrated with Spurr's epoxy resin, and hardened. Tissue from grains freeze-dried at 31 DAP was placed into 100% propanol and gradually infiltrated with Spurr's resin. This was done because standard aqueous fixation techniques caused the loss of electron-dense particles, especially from low-P tissues.

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Blocks of tissue were sectioned dry with a Reichert OmU2 ultramicrotome. Sections 0.5–1.0 μm thick were picked up from the knife edge with an eyelash and placed onto parlodion-carbon-coated grids moistened with 95% ethanol (Lott et al 1984). Sections were viewed, without post-staining, in a JEOL JEM100S transmission electron microscope operating at 80 kV.

Energy Dispersive X-ray Analysis

Aleurone tissue samples that had been fixed, embedded, and sectioned as described above were studied. A powder preparation procedure, which avoids the use of fixatives, dehydrating agents, and epoxy resin, was also used for both mature dry grains and for 31-DAP, freeze-dried grains. For this procedure, aleurone tissue samples of low-P and control wheat grains were dissected away from the bulk of the endosperm as described previously and then chopped to a powder using a razor blade. Formvar-carbon-coated copper grids were then smeared with the powder, and any unattached particles were shaken loose.

For energy dispersive X-ray (EDX) analysis, grids were placed on carbon holders and inserted into a JEOL JEM-100CX transmission electron microscope operated at 80 kV. In powder samples, round shape and density were used to locate the naturally electron-dense globoid crystals. Elemental composition of globoid crystal regions was determined using a KEVEX 5100 EDX analysis system. A time of 100 sec was used for each spot analysis.

Alternately, powders of low P and control mature wheat grains were examined in a carbon holder in the upper stage of an ISI-DS130 scanning electron microscope operated at 35 kV. EDX analysis of 36 globoid crystals from powders of mature low-P wheat aleurone (18 each from 2 grains) and 36 globoids from mature control wheat aleurone grains (18 each from 2 grains) were obtained with a PGT system IV (Princeton Gamma-Tech, Inc., Princeton, NJ). After 100 sec counting per globoid, background was subtracted using a standard background subtract point file, and integrated counts were calculated for P, K, and Mg ($1.8 \times$ full width at $1/2$ maximum). Statistical significance was determined with a *t* test.

RESULTS

Total Grain Dry Weights and Phytate

The mature control and low-P grain taken from experiment 1 had, respectively, dry weights of 61 and 39 mg/kernel and phytate phosphorus levels of 209 and 26 μg P/kernel. The accumulation of dry weight and phytate phosphorus per kernel for grains taken from experiment 2 are shown in Table I. These data were used to select immature samples which had low levels of phytate. At 31 DAP, low-P grains were just beginning to accumulate phytate and contained only 3 μg phytate P per kernel compared to 63 μg phytate P per control kernel.

Ultrastructure of Aleurone Protein Bodies

Mature grains. In control wheat grains, the protein bodies frequently contained large ($\sim 2.5 \mu\text{m}$ diameter) globoid crystals that occupied a large fraction of the total volume (Fig. 1), although much smaller globoid crystals were also present. In aleurone cells of

low-P wheat grains, the protein bodies tended to have more numerous and smaller ($\sim 0.40 \mu\text{m}$) globoid crystals (Fig. 2). Whereas there was variation in globoid crystal size in the low-P wheat aleurone, larger globoid crystals typical of control samples were not found. Regions lacking globoid crystals were observed in the protein bodies. These were especially noticeable in the low-P samples where the proteinaceous matrix contained numerous small globoid crystals.

Grain 31 DAP. The 31 DAP tissue was not very well preserved, likely resulting from ice crystal formation when whole grains were frozen in liquid N_2 before freeze-drying. It was very difficult to find electron-dense deposits in the low-P samples, and those areas that were found were more diffuse and less dense than in controls.

Elemental Content of Globoid Crystals

Mature grains. EDX analyses of globoid crystals observed in both powder and sectioned preparations from mature grains of both control wheat (Figs. 3 and 4) and low-P wheat (Figs. 5 and 6) revealed the presence of considerable P, K, and Mg. In powder preparations there was a distinct difference in potassium to phosphorus ratios and in magnesium to phosphorus ratios with low-P samples having higher K and higher Mg in relation to P. This can be seen by comparing Figures 5 and 3 and the K:P and Mg:P ratios in Table II. The EDX data for peak-to-background ratios (Table II) also show that low-P growth conditions resulted in a 48% decrease in the P, a 30% decrease in the K, and a slight increase in the Mg peak-to-background ratios. A visual comparison of EDX spectra from powder and sectioned preparations showed that the preparative procedures used to obtain sections of tissue clearly resulted in loss of K from the globoid crystals (compare Figs. 3 and 4, Figs. 5 and 6). Small amounts of sulfur and chlorine were sometimes detected, especially in the powder samples where some tissue adheres to the globoid crystal, and also in cases where the globoid crystals are very small. It thus is probable that most of the S and Cl are located in material adjacent to the globoid crystals. Very small traces of iron, calcium, and zinc were also detected in some globoid crystals from both control and low-P grains. It is possible that the Fe and Zn peaks are artifacts.

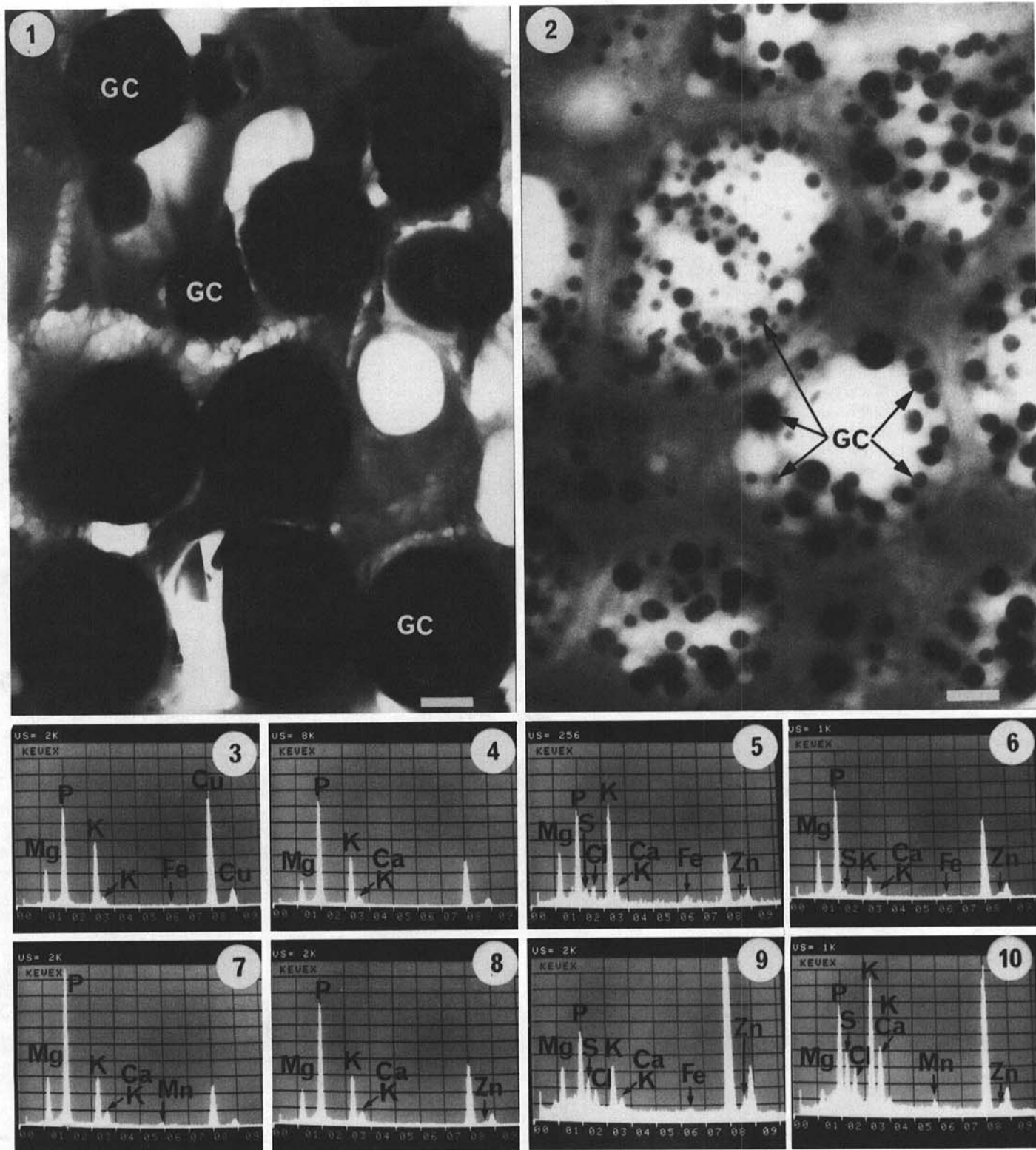
Grain 31 DAP. In both control (Figs. 7 and 8) and low-P samples (Figs. 9 and 10) Mg, P, and K were always present in the dense deposits from both powder and sectioned preparations. Electron-dense regions were much easier to locate in control than in low-P samples. In the low-P samples, the dense areas were rather diffuse and likely contained a good deal of protein or other cellular materials. Perhaps as a result of this, there was usually a considerable S peak and often some Cl in the low-P samples (Figs. 9 and 10). The height of the K peak, relative to P, varied in 31-DAP samples as it did in mature samples. The K peak was never higher than the P peak in control samples, but in eight out of fifteen spectra of low-P grains, K was higher than P. Because the freeze-dried, 31-DAP samples, unlike the mature, air-dried grain samples, could be infiltrated anhydrously, there was not the loss of K found with mature grain samples fixed in 60% alcohol. In both control and low-P samples at 31 DAP, there often was some Ca present, and there was a great deal of variation in the amounts of Fe, Mn, and Zn detected. In some samples all three were present, whereas in

TABLE I
Accumulation of Phytate Phosphorus in Kernels of Wheat Grown at 18°C (day)/15°C (night)

	Days After Pollination													SE ^a	
	10	14	21	26	31	34	37	40	44	47	51	60	67		82
Control															
Kernel dry weight (mg)	6	13	25	34	43	...	52	52	59	63	62	61	70	65	3.6
Phytate P (μg P/kernel)	<1 ^b	2	22	43	63	...	99	118	132	148	150	163	211	222	13
Low P															
Kernel dry weight (mg)	6	11	18	27	32	34	38	40	38	39	44	...	42	...	2.6
Phytate P (μg P/kernel)	<1	<1	<1	<1	<3	7	9	15	19	32	37	...	30	...	4.1

^aSE = Pooled standard error.

^bLess than 1 μg P detected.



Figs. 1 and 2. Transmission electron micrographs of portions of aleurone cells from mature control (1) and low-P (2) wheat grains. Both sections were prepared in parallel and are 0.5–1 μm thick and unstained. Control globoid crystals (GC) are commonly found in the protein bodies and electron density of GC is evident. In low-P grains GC are smaller and more numerous. Bar represents 1 μm . **Figs. 3–10.** Energy dispersive X-ray analysis spectra of portions of globoid crystals. Major elements present, principal emission lines and emission levels in kiloelectronvolts are as follows: calcium K_{α} at 3.69 and K_{β} at 4.01 (10% of K_{α}); chlorine K_{α} at 2.62; iron K_{α} at 6.40; magnesium K_{α} at 1.25; manganese $K_{\alpha,1,2}$ at 5.89; phosphorus $K_{\alpha,1,2}$ at 2.01 and $K_{\alpha,4}$ at 2.03; potassium $K_{\alpha,1,2}$ at 3.31 and K_{β} at 3.59 (10% of $K_{\alpha,1,2}$ peak); sulfur K_{α} at 2.31; zinc K_{α} at 8.63. The copper peaks ($K_{\alpha,1,2}$ at 8.04 and K_{β} at 8.90) are artifacts of copper grid usage. **3,** Globoid crystal region in powder of aleurone from a mature control wheat grain. **4,** Globoid crystal region in a section of aleurone layer from a mature control wheat grain. **5,** Globoid crystal region in a powder of aleurone from a mature, low-P wheat grain. **6,** Globoid crystal region in a section of the aleurone layer from a mature, low-P wheat grain. **7,** Globoid crystal in a powdered tissue preparation from a control wheat grain at 31 days after pollination (DAP). **8,** Globoid crystal in a section of an aleurone layer from a control wheat grain at 31 DAP. **9,** Dense area in a powdered tissue preparation from a low-P wheat grain at 31 DAP. **10,** Dense area in a section of aleurone layer from a low-P wheat grain at 31 DAP.

others there was no detectable Fe, Mn, or Zn. Traces of Fe, Mn, and Zn were more common in the 31-DAP samples than in mature samples.

DISCUSSION

The whole grains in this study contained from less than 3 to 222 μg phytate P per kernel. The amounts in mature control kernels were similar to those reported in greenhouse-grown plants by Williams (1970). The amounts of phytate phosphorus in low-P kernels were similar to those in field-grown wheat samples studied by Jennings and Morton (1963).

The comparison of protein bodies in low-P wheat grains with those from control grains showed structural differences. Control samples had a range of globoid crystal sizes, but large globoids usually occupied most of the diameter of a protein body. Such large globoid crystals were not found in aleurone layer protein bodies in the low-P grains. The low-P grains had numerous small globoid crystals in the proteinaceous matrix of aleurone cell protein bodies. Regions lacking globoid crystals were observed in the protein bodies. These were especially noticeable in the low-P samples where the proteinaceous matrix contained numerous small globoid crystals. It is possible that these regions are the niacin-rich Type II inclusions reported for wheat aleurone protein bodies by Morrison et al (1975) and further characterized by Fulcher et al (1981). The grains examined by Morrison et al (1975) were grown under conditions similar to those used to grow the control plants in our study.

The preparation procedures used here were different from those commonly used for studying plant cell ultrastructure (O'Brien and McCully 1981). Because the purpose of this work was to study the location and elemental composition of the mineral reserves, procedures were used which promoted retention of the globoid crystals. An anhydrous preparation procedure for dry grains would have been ideal, but, as shown by Yatsu (1983), epoxy resins do not easily infiltrate through the cell walls of dry seeds. The procedures used here are thus designed to retain minerals while still allowing some infiltration with epoxy resins. Because globoid crystals tend to shatter and chip out of thin sections, the traditional thin section is not as useful as the thicker sections used here. Dry sectioning also promotes retention of water-soluble materials. For a more complete discussion of retention of soluble phytates in tissue samples prepared for electron microscopy see Lott et al (1984).

EDX analyses showed that in both control and low-P conditions the globoid crystals in aleurone cells were rich in P, K, and Mg. These results are consistent with the globoid crystals being rich in a K and Mg salt of phytic acid. Despite the wide range of phytate phosphorus per grain and the observed structural changes, the mineral composition of the globoid crystals remained quite similar. The main shift noted was a significantly higher K:P ratio in the low-P samples compared to controls, but the Mg:P ratio was also higher. This observation may help to explain the difficulty in retaining globoid crystals in low-P grain samples after conventional aqueous fixation. K and Na phytates are more easily soluble in water than are phytates with higher proportions of divalent and trivalent cations.

The patterns of accumulation of phytate phosphorus were clearly different in control and low-P plants. In control grains phytate P accumulated rapidly beginning 14 DAP. Only in the low-P grains did it accumulate rapidly as the maximum kernel dry weight was reached. All other plant nutrients were supplied daily to both control and low-P plants, so we concluded that the composition of protein body globoid crystals is controlled largely by the amount of phosphorus available to the grain.

This work, together with data presented by Batten (1984), supports the suggestion that phytate phosphorus forms to buffer the concentration of inorganic phosphorus in the grain. The evidence does not support the argument of Nahapetian and Bassiri (1975) that senescence releases magnesium that induces the formation of phytate.

ACKNOWLEDGMENTS

This work formed part of the first author's Ph.D. thesis. A Natural

TABLE II
Energy Dispersive X-ray Analysis Study of Globoid Crystals from Control and Low-Phosphorus Wheat Aleurone^a

Ratio	Control	Low Phosphorus	<i>t</i> Test ^b (<i>P</i> = 0.01)
K:P	0.69	0.92	S
Mg:P	0.22	0.52	S
K peak-to-background	8.34	5.85	S
P peak-to-background	11.50	6.01	S
Mg peak-to-background	2.79	3.17	NS

^a Values given are average ratios of X-ray counts (peak-to-background or element-to-element) based upon analysis of 36 globoid crystals from mature grain produced under each growth condition.

^b S = Significant, NS = not significant.

Science and Engineering Research Council of Canada Travel Grant permitted J. N. A. Lott to visit the CSIRO Division of Plant Industry in Canberra, where most of this study was carried out. We acknowledge the cooperation and helpful comments provided by D. J. Goodchild, Stuart Craig, and I. F. Wardlaw. Technical assistance was provided by Celia Miller, who first devised the dry cutting procedure used here. Plants were grown with help from the staff of the Canberra Phytotron. J. Carson and P. Kerr kindly provided assistance for the scanning electron microscopy studies at McMaster University.

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[Received October 30, 1984. Revision received June 20, 1985. Accepted June 28, 1985.]