Relationship among Phytic Acid, Phosphorus, and Zinc during Maturation of Winter Wheat

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Changes in P, Zn, phytic acid, and phytase enzyme in vegetation and grain were observed in winter wheat (*Triticum aestivum* L. var. "Parker") as it matured. Vegetation dry weight and contents of P and Zn increased steadily except for a brief decline after anthesis. Most of the grain P was translocated from the vegetation soon after anthesis and gradually was synthesized into phytic acid. Zinc in the grain increased steadily through maturity. Evidence was obtained for two enzymes or forms of enzymes active against phytic acid in wheat grain. Phytase in fresh grain was constant through maturation; however, phytase in successively dried and germinated grain increased steadily in activity until maturity.

Phytin, the mixed Ca and Mg salt of inositol hexaphosphate (phytic acid), is the principal storage form of P in many seeds and comprises up to 80% of the total P (Asada *et al.*, 1968). Small amounts have been found in aerial parts of several grass species and underground parts of carrots, potatoes, and artichokes (Earley and DeTurk, 1944). Three physiological roles for phytic acid in plants have been suggested (Williams, 1970). It might serve as a P store (Hall and Hodges, 1966), an energy store (Biswas and Biswas, 1965; Morton and Raison, 1963), or as an activator of dormancy (Sobolev and Rodionova, 1966).

Phytase (EC.3.1.3.8), which releases inorganic P from phytic acid, occurs in low amounts in vegetation and in high amounts in germinating seeds (Nagai and Funahashi, 1962; Peers, 1953; Sartirana and Bianchetti, 1967). Increased phytase activity in germinating seeds apparently results from activation of inactive forms (Eastwood *et al.*, 1969). Wheat phytase has an optimum pH of 5.15, optimum temperature of 55°, Km of 3×10^{-4} *M*; it is slightly activated by Mg + 2 (Peers, 1953). Because highly-purified wheat phytase has a broad substrate specificity, it is classified as a nonspecific acid phosphomonoesterase (Nagai and Funahashi, 1962).

Zinc interacts closely with plant inorganic P nutrition. High P levels in growth media induce Zn deficiency by immobilizing Zn or by altering physiological balances between P and Zn (Viets, 1966). The mode of interaction between P and Zn has not been elucidated, although some evidence suggests that it might be mediated through phytic acid. Phytic acid complexes dietary Zn in forms that are insoluble and nonabsorbable by the animal gastrointestinal tract (Oberleas *et al.*, 1966). Phytase enzyme is strongly inhibited by Zn + 2 (Nagai and Funahashi, 1962) and repressed by PO_4^{-3} (Sartirana and Bianchetti, 1967). High amounts of soil P, a proportion of which exists as phytic acid (Caldwell and Black, 1958), induce Zn deficiency in plants (Viets, 1966). We studied changes in phytic acid, phytase, P, and Zn in winter wheat during its growth and maturation to determine relationships among these constituents.

EXPERIMENTAL SECTION

Plant Culture. "Parker" wheat (*Triticum aestivum* L.) was seeded Oct 25, 1969, at the rate of 101 kg/ha in 2.5 m × 8.0 m plots on the Kansas State University Agronomy Farm, Manhattan, Kan. Nitrogen (100 kg/ha) as NH_4NO_3 was top-dressed April 23, 1970. Anthesis began May 26, 1970. Maturity was delayed slightly by cool, rainy weather.

P and Zn Assays. Plants from four replicates were sampled weekly for total P and Zn analysis from May 6, 1970, to maturity. Samples taken before jointing stage contained ten plants; samples taken after jointing stage contained five plants selected at random from each replication. Heads were selected at random weekly from anthesis to maturity and grain was separated from the glumes.

Plant samples were dried to constant weight at 60° and weights were recorded. The dried material was ground through a mill with stainless steel parts to pass through a 20-mesh sieve, and then mixed thoroughly; 0.5-g subsamples were dry-ashed at 200, 400, and 600°, respectively, for 2 hr at each temperature. Each sample was dissolved in 0.2 *N* HCl, filtered through Whatman No. 42 paper, and brought to 50-ml volume. Phosphorus was assayed by the acid-molybdate method (Fiske and Subbarow, 1925) and Zn was determined by atomic absorption spectrophotometry.

Phytic Acid Assay. Phytic acid was extracted and assayed by a modification of the method described by Asada *et al.* (1968, 1969). Five to 8.0 g of plant tissue were ground in 20 ml of 0.5 *N* HCl and shaken in the same solution for 2 hr, decanted, and shaken again in 10 ml additional 0.5 *N* HCl (Anderson, 1963). The extract was cleared by centrifugation at 6800 × *g* for 10 min. Excess $Ba(C_2H_3O_2)$ was added to the extract, the pH was adjusted to 10 with 5 *N* NaOH, and the precipitate was collected by centrifugation. The precipitate was suspended in 5 ml of deionized water. Excess Dowex 50 (H^+) resin was added to dissolve the barium precipitate (barium-precipitable P). The resin was removed by filtration and the filtrate was applied to a column of Dowex 1 × 8 (Cl^-) resin (1.2 × 20 cm) washed previously with 3 *N* HCl and deionized water. Linear gradient elution was employed, using a mixing chamber containing 1 l. of deionized water and a reservoir containing 1.2 l. of 1.5 *N* HCl. A flow rate of 0.9 ml per min was maintained. Twenty-milliliter fractions were collected. Phytic acid-containing fractions were identified by paper chromatography. The method was modified by using 2-propanol, NH_4OH, and H_2O (5:4:1) as solvent following neutralization with 4 *N* NH_4OH. Chromatograms were developed with perchloric acid-molybdate solution (Hanes and Isherwood, 1949). Phytic acid-containing fractions from each sample were combined and a 50-ml aliquot was evaporated to dryness and dry-ashed at 300° for 1.5 hr and at 550° for 1.5 hr. Inorganic phosphate was determined (Fiske and Subbarow, 1925).

Phytase Purification and Activity Assay. The assay for phytase activity was patterned after methods of Nagai and Funahashi (1962) and Peers (1953). The grain samples obtained each date were assayed in two ways: immediately after they were obtained, and after they were dried at 50° for 72 hr and germinated for 48 hr. Fresh tissue or dried and germinated kernels were homogenized in 20 ml of 0.1 *M* acetate buffer (pH 5.2). The homogenate was centrifuged at 6800 × *g* for 10 min. Thirty grams of solid

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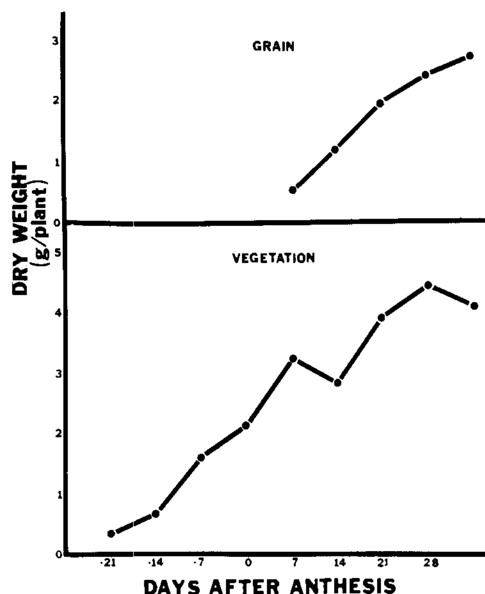

Figure 1. Changes in dry weight of wheat (*Triticum aestivum* L. var. "Parker") grain and vegetation during maturation.

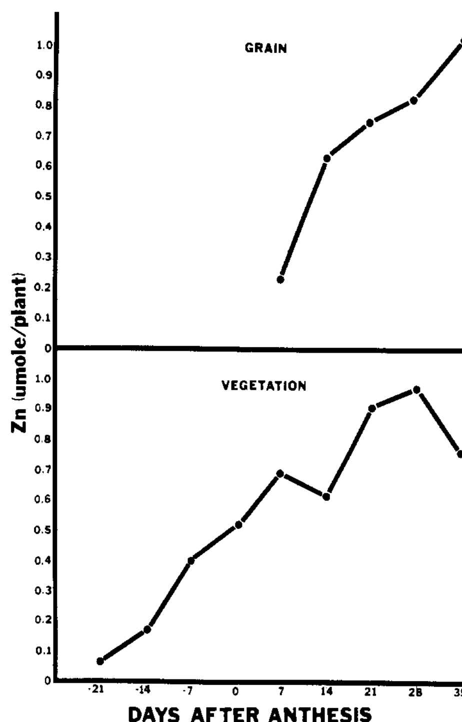

Figure 3. Changes in Zn content in wheat (*Triticum aestivum* L. var. "Parker") grain and vegetation during maturation.

$(NH_4)_2SO_4$ was added per 100 ml of supernatant and the precipitate was allowed to form for 1.5 hr. The precipitate was removed by centrifugation and 65 g of solid $(NH_4)_2SO_4$ per 100 ml of supernatant was added. That precipitate contained the phytase activity. The precipitate was taken up in 20 ml of 0.1 *M* acetate buffer (pH 5.2) and dialyzed 16 hr against 0.05 *M* NaCl and 0.1 *M* acetate buffer (pH 5.2).

Phytase activity was assayed in a reaction medium containing 2 ml of 0.1 *M* acetate buffer (pH 5.2), 1 ml of 5×10^{-3} *M* Na phytate, and 1 ml of enzyme extract. After incubation at 30° for 15 min, the reaction was stopped with 1 ml of 20% TCA (trichloroacetic acid). To determine the endogenous inorganic phosphate present in the enzyme extract, sample blanks were prepared by adding 1 ml of 20% TCA before incubation. Inorganic phosphate released was assayed (Fiske and Subbarow, 1925).

RESULTS

The increase in dry weight of wheat during maturation indicated a uniform rate of growth (Figure 1). A slight decrease in vegetative dry weight during the second week after anthesis coincided with initiation of grain development and with heavy rainfall. Vegetation dry weight decreased slightly again at maturity.

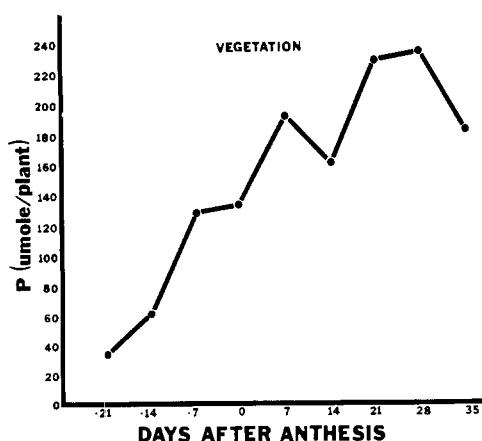

Figure 2. Changes in total (including phytic acid) P content in wheat (*Triticum aestivum* L. var. "Parker") vegetation during maturation.

Phosphorus and Zn contents of the vegetation followed patterns similar to that of the dry weight (Figures 2 and 3). Only slight traces of phytic acid were detected in the vegetation; most of the P occurred in inorganic form or organic forms other than phytic acid. Zinc content of the grain continued to increase through physiological maturity (Figure 3). Changes in P and Zn contents of the vegetation were highly correlated ($r = 0.88$) (Table I). The correlation of Zn content of grain with advance in maturity, as represented by days after anthesis, was 0.84.

Most of the grain P was translocated from the vegetation soon after anthesis (Figure 4). Phytic acid synthesis began during the second week after anthesis and the maximum content occurred the fourth week. Phytic acid comprised 8% of total grain P at anthesis, 82% 28 days after anthesis, and 72% at maturity. The slight increase in phytic acid during the last week was similar to that reported for rice (Asada *et al.*, 1969). Phytic acid content of the grain was correlated significantly with sampling date ($r = 0.73$) and Zn content of grain ($r = 0.59$)(Table I).

Phytase activity was observed in freshly sampled grain (Figure 5). The activity in fresh grain was maximum the first week after anthesis and remained constant during maturation. In contrast, phytase activity in dried and ger-

Table I. Significant (0.05 Level) Correlation Coefficients among Phytic Acid, Phytase Enzyme, and Zn during Maturation of Wheat (*Triticum aestivum* L. var. "Parker")

Comparison	Correlation coefficient
P *vs.* Zn in vegetation	0.88
Zn in grain *vs.* time	0.84
Phytic acid in grain *vs.* time	0.73
Phytic acid in grain *vs.* Zn in grain	0.59
Phytase in germinated grain *vs.* time	0.80
Phytase in germinated grain *vs.* Zn in grain	0.74
Phytase in germinated grain *vs.* phytic acid in grain	0.47

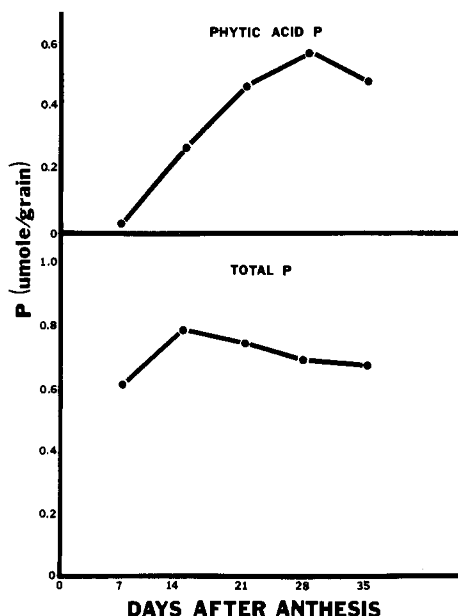

Figure 4. Changes in phytic acid P and total P content per individual wheat (*Triticum aestivum* L. var. "Parker") grain during maturation.

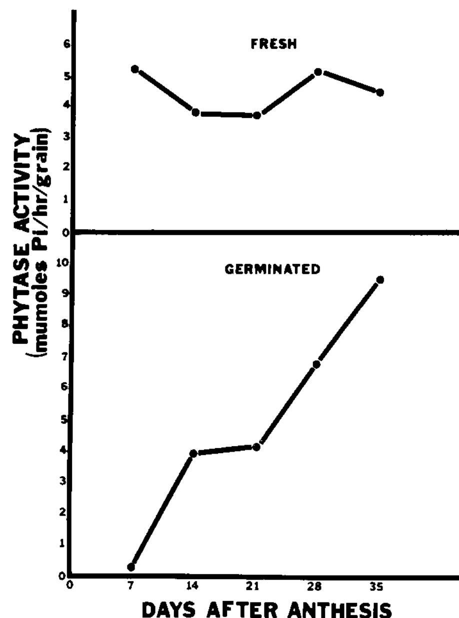

Figure 5. Changes in phytase enzyme activity per individual fresh and germinated wheat (*Triticum aestivum* L. var. "Parker") grain during maturation.

minated grain was low 1 week after anthesis, but increased steadily to a maximum as the grain matured. Phytase activity in fresh grain was not correlated significantly with any other constituents; activity in dried and germinated grain was correlated significantly with days after anthesis (r = 0.80), Zn content of grain (r = 0.74), and phytic acid content of grain (r = 0.47) (Table I). All three constituents increased steadily during maturation to yield the significant associations. Phytase enzyme was not detected in the vegetation.

No significant difference was found at the 5% F-test level between phytase activity in fresh and germinated grain averaged over all sampling dates. Differences between fresh and germinated grain were significant, however, when individual sampling dates were compared. The 5% LSD between treatments (4.08) showed activity was significantly higher in fresh grain than in dried and germinated grain the first week following anthesis; activity was significantly higher in germinated grain than in fresh grain during later stages of maturity.

DISCUSSION

Direct evidence was not found for any functional relationship between P and phytic acid or Zn and phytic acid in wheat vegetation. The level of phytic acid at any time was too low to be a significant reservoir of stored P or to affect Zn availability or solubility. Accumulation patterns of P and Zn in the vegetation, however, were closely associated. That relationship could as well have been coincidental as functional. Phosphorus and phytic acid on the one hand, and Zn and phytic acid on the other, were more closely associated in the grain. High levels of phytic acid in grain apparently explain why grain diets, but not other diets of comparable Zn content, cause animal Zn deficiency symptoms (Oberleas *et al.*, 1966).

Wheat grain apparently contains two enzymes or two forms of one enzyme that exhibit activity against phytic acid. Considerable phytase activity was present in fresh grain obtained soon after anthesis. That activity was lost after the wheat was dried at 50°, which is below the thermal inactivation point of wheat phytase (Peers, 1953), and germinated. At subsequent dates, however, phytase activity in dried and germinated grain equaled or exceeded activity in fresh grain. Most of the phytase activity in ungerminated wheat grain is in aleurone and endosperm tissue (Peers, 1953). It has been speculated that an inactive form of phytase in aleurone tissue is activated during germination (Eastwood *et al.*, 1969). Thus, fresh grain apparently contains an active phytase that is formed early, and an inactive phytase that is formed as the grain matures. These might be two enzymes or isozymes, or they might be active and inactive forms of the same enzyme. *De novo* synthesis of phytase might also cause presence of the enzyme in germinating wheat. Phytase activation in wheat grain aleurone tissue was not affected by several protein synthesis inhibitors (Eastwood *et al.*, 1969), but the enzyme from wheat grain embryo tissue was repressed (Bianchetti and Sartirana, 1967).

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