RELATIONSHIP AMONG PHYTIC ACID, PHOSPHORUS, AND ZINC DURING MATURATION OF WHEAT GRAIN

by

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B. S., Kansas State University, 1970

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Agronomy

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1970

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INTRODUCTION

Phosphorus metabolism in seeds of cereal grains is closely related to phytic acid and the enzyme phytase, which releases inorganic phosphate from phytic acid. Phytic acid comprises up to 80% of the total P in mature grain (Asada, Tanaka, and Kasai, 1968). Three physiological roles for phytic acid have been suggested (Williams, 1970). It might serve as a phosphorus store (Hall and Hodges, 1966), an energy store (Biswas and Biswas, 1965; Morton and Raison, 1963), or an activator of dormancy (Sobolev and Rodionova, 1966).

Phytase has been reported to be synthesized during seed germination (Mandal and Biswas, 1970; Sartirana and Bianchetti, 1967; Sobolev, 1962). The enzyme also exhibited characteristics of a nonspecific phosphomonoesterase requiring no metal cofactors (Nagai and Funahashi, 1962).

In an effort to elucidate phytic acid-phytase relationships during maturation, the time of synthesis of phytic acid and phytase in maturing wheat was examined. The relationship of phytase activity and phytic acid synthesis during maturation to P and Zn nutrition was also determined.

LITERATURE REVIEW

Phytin, the mixed Ca and Mg salt of inositol hexaphosphate, is the principal storage form of P in nearly all seed and comprises up to 80% of the total P (Asada, Tanaka, and Kasai, 1969; DeTurk, Holbert and Howk, 1933; Earley and DeTurk, 1944; Koller, et al., 1962). Small amounts have also been found in the vegetative parts of a few species of grass, wheat straw, and the underground portion only of carrots, parsnips, potatoes, and Jerusalem artichokes (Earley and DeTurk, 1944). The transport of P to the grain, and subsequent storage as phytin, appeared to be the primary function of phytin in the plant. Recently, a transphosphorylation between phytic acid and ADP (adenosine diphosphate) through the action of a phosphotransferase enzyme has been suggested (Morton and Raison, 1963). Biswas and Biswas (1965) established the occurrence of an enzyme catalyzing transphosphorylation between GDP (guanosine diphosphate) and phytic acid. These observations suggested the participation of phytin as a phosphagen. Raison and Evans (1968), however, concluded phytic acid lacked a phosphate group of sufficiently high energy to act as a phosphoryl donor after complete hydrolysis by the enzyme phytase. Enthalpy changes during hydrolysis were the basis for their conclusions.

Phytic Acid Assays

Quantitative determination of phytic acid has undergone numerous refinements. Huebner and Stadler (1914) devised one of the first methods which involved the titration of phytic acid in HCl solution with standard $FeCl_3$. Determining the titration end-point was difficult because of the colloidal ferric phytate precipitate formed during titration. Phytic acid

forms insoluble salts with Ba, Cu, and Ca in addition to Fe. Young (1935) used the ferric precipitation method modified so that excess Fe was added and determined colorimetrically as thiocyanate. McCance and Widdowson (1935) modified the ferric phytate precipitation method by directly determining P in the ferric phytate precipitate. Several factors cause inaccurate estimation of phytic acid content by these methods. Reducing substances cause high values by reducing Fe (Samotus and Schwimmer, 1962). Marrese, Duell, and Sprague (1961) found the ferric precipitation method gave values of phytic P over 100% by precipitating lower inositol phosphate esters in addition to inositol hexaphosphate. Endogenous Fe in extracts caused incomplete precipitation of ferric phytate by forming soluble Fe complexes (Anderson, 1963).

In an effort to correct the inaccuracies of the above methods, Smith and Clark (1952) developed an anion exchange chromatographic method for separating the various inositol phosphate esters. That method, preceeded by Ba precipitation to partially purify the extract before chromatography, was employed by Asada et al. (1968).

Phytic Acid Synthesis and Accumulation

Earley and DeTurk (1944) found minute phytin concentrations in the pistillate structure prior to fertilization in corn. Percent phytin P increased from the second to the fifth week after pollination and then synthesis ceased. Synthesis resumed during the seventh week, after which there was no further formation of phytin. Phytin synthesis accompanied cellular structure formation of the ovule.

Phytic acid content increased with maturity of Pinto beans, while total acid-soluble P decreased (Makower, 1969). Nearly half the total acid-soluble seed P was accounted for by phytic acid.

Asada and Kasai (1962) found the maximum inositol level in rice occurred the 14th day after anthesis, but phytic acid P increased up to the 25th day. Inositol was evidently undergoing phosphorylation between the 14th and 25th day after anthesis.

Phosphorylation of inositol to phytic acid has been examined in detail, but the actual mechanism is still in question. Myoinositol kinase, an enzyme catalyzing formation of inositol monophosphate, has been reported (Dietz and Albersheim, 1965; English, Dietz, and Albersheim, 1966). Although the product of the reaction catalyzed by this enzyme could be an intermediate in phytic acid biosynthesis, Asada et al. (1968, 1969) proposed that phosphorylation of inositol occurred through a mechanism other than the usual kinase reaction. Only inositol mono- and hexaphosphates were found in maturing seeds of several plants (Asada et al., 1968; Roberts and Loewus, 1968; Saio, 1964; Sobolev, 1964). Those findings led to the hypothesis that inositol might be phosphorylated directly to the hexaphosphate (Asada and Kasai, 1962). A mechanism by which direct phosphorylation might occur has been proposed (Asada et al., 1968, 1969).

Phytase Enzyme Properties

Following the initial report of phytase in rice by Suzuki et al. in 1907 (Nagai and Funahashi, 1962), numerous investigators have studied phytase in peas (Pierpoint, 1957), lettuce (Mayer, 1958), beans (Gibbins and Norris, 1963; Mandal and Biswas, 1970), peanuts (Davis, 1969), various microorganisms (Theodorou, 1968; Yamada, Minoda, and Yamamoto, 1968), and particularly rice and wheat (Nagai and Funahashi, 1962, 1963; Peers, 1953; Sartirana and Bianchetti, 1967).

Peers (1953) reported for wheat phytase an optimum pH of 5.15, temperature of 55 C, Km of 3×10^{-4} M, and a slight activation by 0.002 M Mg after prolonged dialysis. Nagai and Funahashi (1962) purified phytase enzyme 1500-fold. The purified preparation had broad substrate specificity, which led to classification of phytase as a nonspecific acid phosphomonoesterase. A plant enzyme that had activity toward phytic acid also hydrolyzed the phosphomonoester bonds of phenylphosphate, pyrophosphate, and nucleic acids (Pierpoint, 1957).

The mechanism of dephosphorylation of phytic acid by phytase has been examined by Sobolev (1962). Phytase attacked phytic acid preferentially, but the lower phosphate esters of inositol are eventually hydrolyzed to inositol and inorganic P in the absence of phytic acid (Nagai and Funahashi, 1963). Tomlinson and Ballou (1962) confirmed the stepwise nature of dephosphorylation, determined the order of phosphate ester bond hydrolysis, and proposed structures for the intermediates.

Phytase Synthesis

Eastwood, Tavener, and Laidman (1969) reported increased phytase activity in aleurone tissue during germination apparently resulted from activation of an existing inactive form. Several protein synthesis inhibitors did not affect phytase activation. Peers (1953) reported phytase increased from 3.17 to 20.6 units upon germination of a soft wheat. Several other investigators reported the synthesis of phytase during the germination process (Bianchetti and Sartirana, 1967; Mandal and Biswas, 1970; Sartirana and Bianchetti, 1967; Sobolev, 1962). Phytase activity was not observed in unsoaked bean cotyledons, but occurred after beans were soaked 12 hours (Mandal and Biswas, 1970). Repression of phytase reported by the same investigators

occurred when bean seeds were germinated in the presence of cycloheximide, a protein synthesis inhibitor, and chromomycin A_3, an RNA synthesis inhibitor. Similar results were obtained with other inhibitors and with inorganic P (Bianchetti and Sartirana, 1967). Similar types of enzyme induction during germination are known to occur.

MATERIALS AND METHODS

'Parker' wheat (Triticum aestivum L.) was seeded October 25, 1969, at the rate of 101 kg/ha in 2.5-m x 8.0-m plots of the Kansas State University Agronomy Farm, Manhattan, Kansas. 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.

Sampling Procedure

Two plots were combined for each replicate. Plants from four replicates were sampled weekly for total P and Zn per plant 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 from each replication and dried at 50 C. Grain was separated from the glumes for assays made after the anthesis stage.

Total P and Zn per Plant Assay

Dry weights of the plant samples were obtained after drying to constant weight at 60 C. The dried material was ground through a 20-mesh seive in a Wiley mill with stainless steel parts. After mixing thoroughly, 0.5-g sub-samples were dry-ashed at 200, 400, and 600 C, respectively, for 2 hours at each temperature. Each sample was dissolved in 0.2 N HCl, filtered through Whatman No. 42 paper, and brought to volume in 50-ml flasks. Phosphorus was assayed by the acid-molybdate method of Fiske and Subbarow (1925). Two ml of acid-molybdate (25 g of $Na_2MoO_4 \cdot 2H_2O$ per liter of 2.5 N H_2SO_4) were added to a 1-ml sample aliquot. That was followed 30 minutes later by 2-ml of reducing

solution (10 g of 1-amino-2-naphthol-4-sulfonic acid per liter of 3% $NaHSO_3$). The solutions were brought to volume in 50-ml flasks and, after 30 minutes, absorbance was measured at 660nm using a Beckman DB spectrophotometer.

Zinc concentrations in the diluted filtrates were determined with a Perkin-Elmer 303 atomic absorption spectrophotometer at 214 nm.

Phytic Acid Assay

Phytic acid was extracted and assayed by a modification of the method described by Asada et al. (1968). Five to 8.0 g of grain were ground with mortar and pestle in 20 ml of 0.5 N HCl and shaken in the same solution for 2 hours, decanted, and shaken again in 10 ml additional 0.5 N HCl. The extract was cleared by centrifugation at 6800 x g for 10 minutes. 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 x 8 (Cl^-) resin, 1.2 x 20 cm, which had been washed with 3 N HCl and deionized H_2O. Linear gradient elution was employed, using a mixing chamber containing 1 liter of deionized water and a reservoir containing 1.2 liter 1.5 N HCl. A flow rate of 0.9 ml per minute was maintained. Twenty-ml fractions were collected.

Phytic acid-containing fractions had been identified previously by paper chromatography using Anderson's (1956) method. The method was modified by using n-propanol, NH_4OH, and H_2O (5:4:1) as solvent following neutralization with 4 N NH_4OH. Chromatograms were developed with the Hanes and Isherwood (1949) perchloric acid-molybdate solution.

Phytic acid-containing fractions from each sample were combined and a 50-ml aliquot was removed, evaporated to dryness, and dry-ashed at 300 C for 1.5 hours and at 550 C for 1.5 hours. Inorganic phosphate was determined by a modified Fiske and Subbarow (1925) method. Three ml of 0.2 N HCl, 1 ml of acid-molybdate, 1 ml of reducing solution, and 5 ml H_2O, respectively, were added to the hydrolyzed precipitate. Absorbance was read at 660 nm 20 minutes after developing the color.

Phytase Purification and Activity Assay

The assay for phytase activity was patterned after methods of Nagai and Funahashi (1962) and Peers (1953). Purification procedures were conducted in a crushed ice bath. Both fresh kernels and dried and germinated kernels were homogenized at 16,000 rpm in a Sorvall Omni Mixer in 20 ml of 0.1 M acetate buffer (pH 5.2). The homogenate was centrifuged at 6800 x g for 10 minutes. Thirty g of solid $(NH_4)_2SO_4$ was added per 100 ml of supernatant and the precipitate was allowed to form for 1.5 hours. 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 hours 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 obtained above. After incubation at 40 C for 15 minutes, 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 the incubation period. Inorganic phosphate released was assayed using a modified Fiske and Subbarow

(1925) method. The TCA precipitate was removed by centrifugation and 1 ml of acid-molybdate, 1 ml of reducing solution, and 3 ml of water were added to 5 ml of reaction mixture. Absorbance was read at 660 nm after 20 minutes. Absorbance of the sample blanks was subtracted from the absorbance of the enzyme assays.

RESULTS

The increase in dry weights of wheat grain and vegetation during maturation is shown in Fig. 1. Excluding the fourteenth day following anthesis, dry weight of both grain and vegetation increased each week as maturity approached. Three weeks prior to anthesis mean weight per plant was 0.31 g, reached a peak 4.45 g per plant the fourth week following anthesis, and decreased at maturity. The dry weight increase indicated a fairly uniform rate of growth.

Figures 2 and 3 show the P content per plant in vegetation and the Zn content in both vegetation and grain, respectively. As the plants matured the trend was toward increased P and Zn contents in the plant vegetation until the final week during maturation. Heavy rainfall during the second week after anthesis might account for the decrease in dry weight of vegetation, which caused P and Zn contents to decrease.

A correlation of 0.88 was found between changes in P and Zn contents in the vegetation. Correlations of Zn content of grain with time, as represented by sampling date, was 0.84. All correlations greater than 0.407 are significant at the 5% level.

Phytic acid content and total P content per grain are presented in Fig. 4. Phytic acid synthesis began during the second week and reached a maximum the fourth week after anthesis. Total P per grain did not change as markedly. Most of the P was apparently translocated to the grain immediately after anthesis. The only increase after that was during the second week following anthesis, which appeared to coincide with the time of most rapid phytic acid synthesis. Phosphorus is transported to the grain from vegetation to be

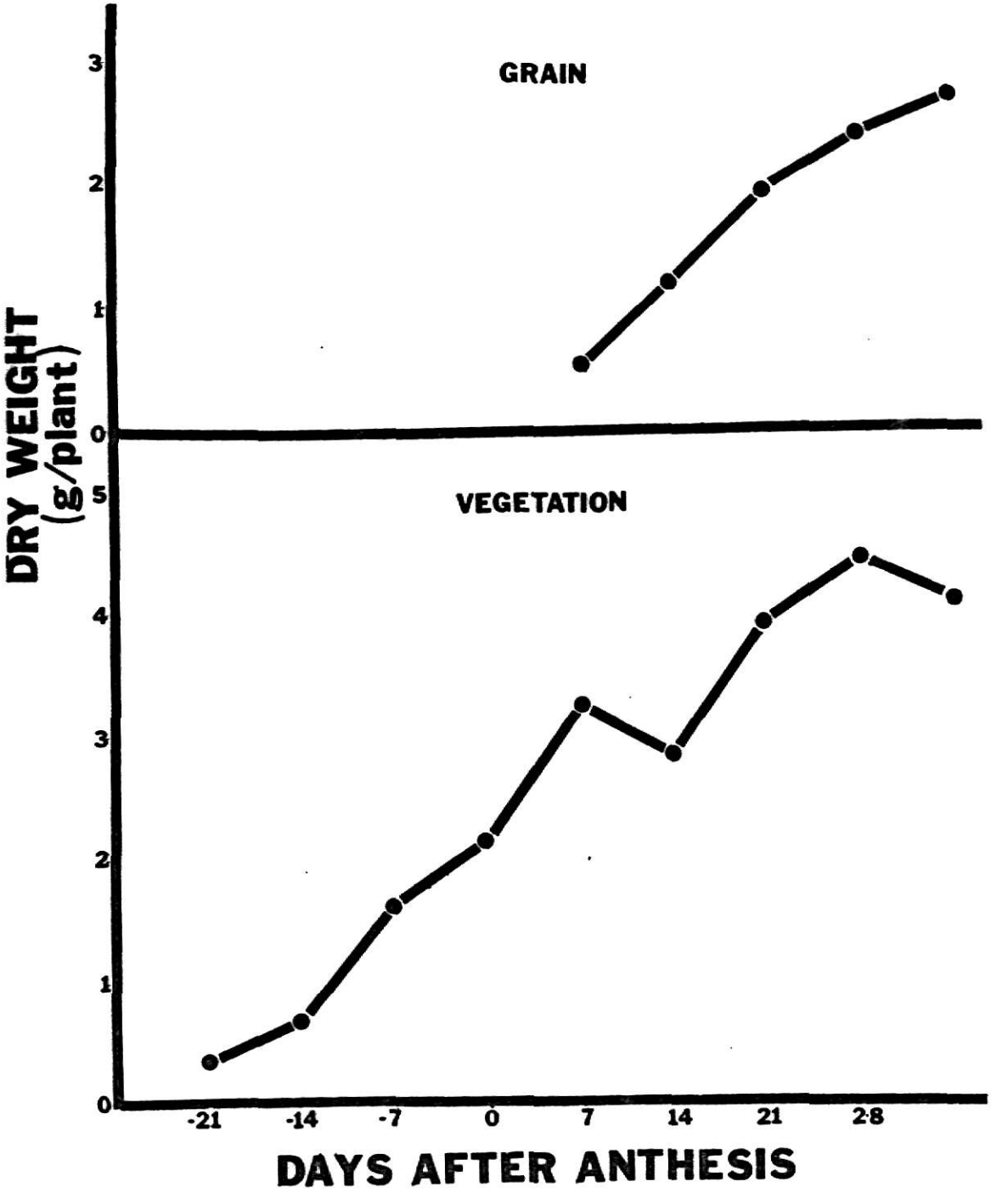

Fig. 1. Changes in dry weight of wheat (Triticum aestivum L. var. 'Parker') grain and vegetation with increasing maturity.

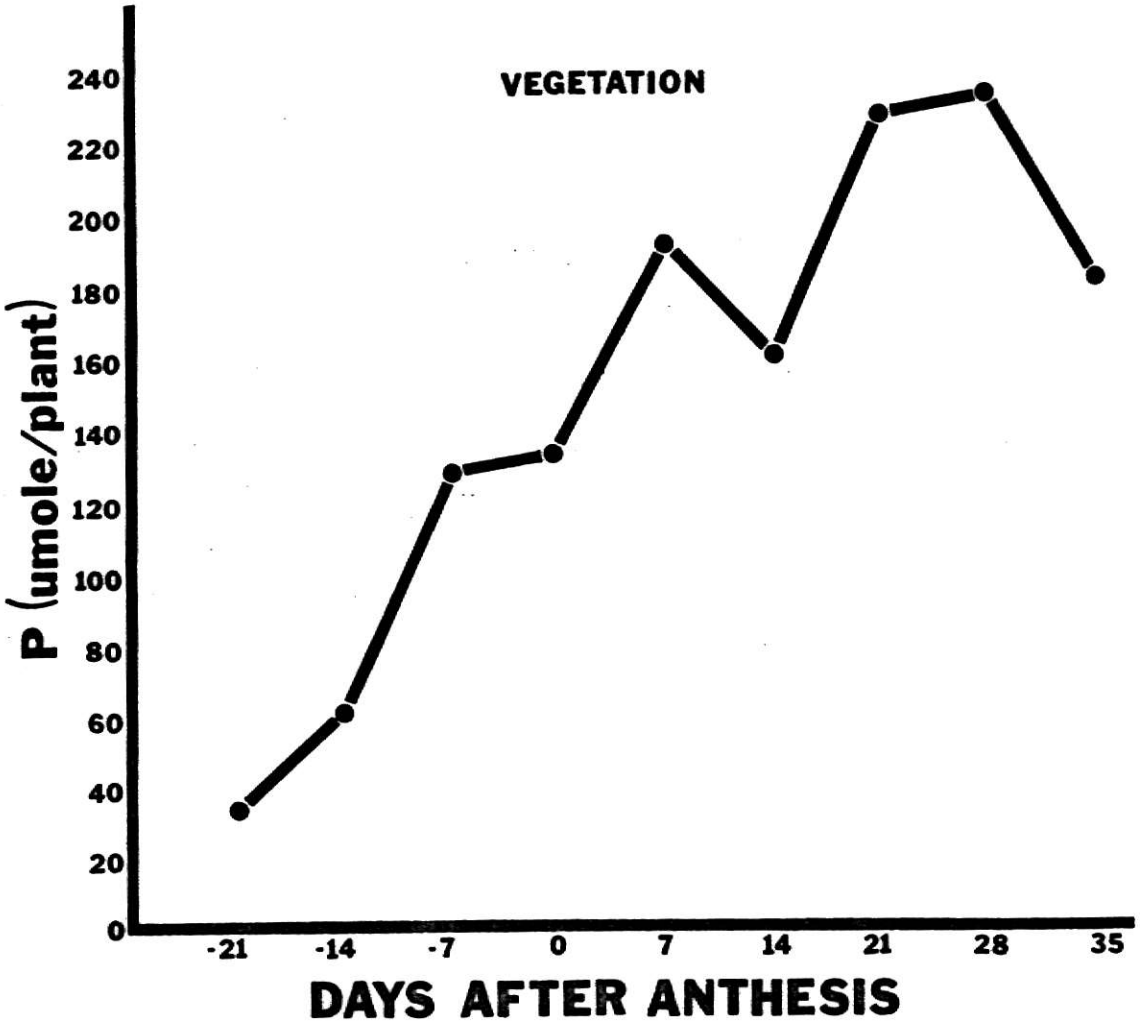

Fig. 2. Changes in P content in wheat (Triticum aestivum L. var. 'Parker') vegetation with increasing maturity.

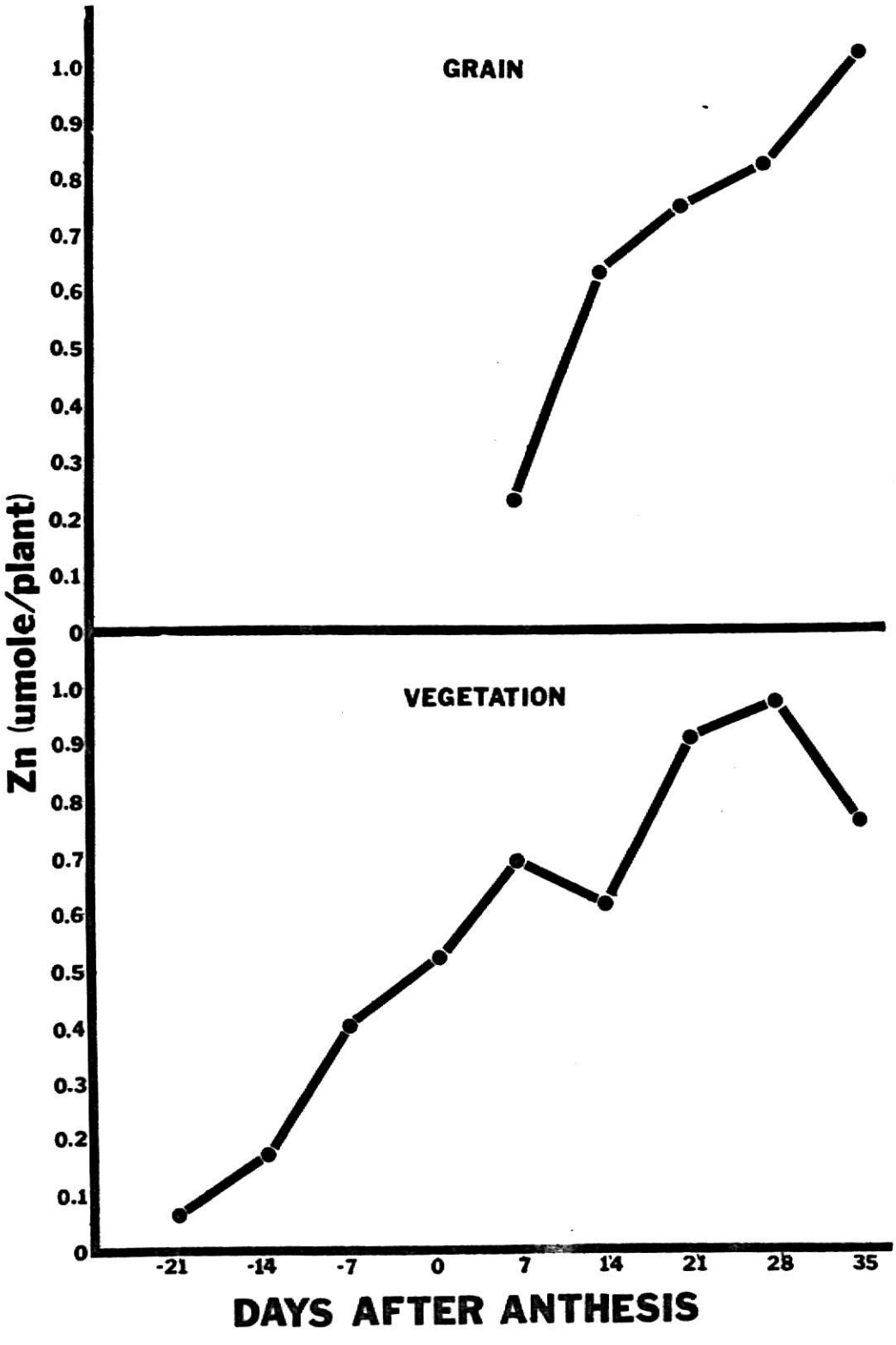

Fig. 3. Changes in Zn content in wheat (Triticum aestivum L. var. 'Parker') grain and vegetation with increasing maturity.

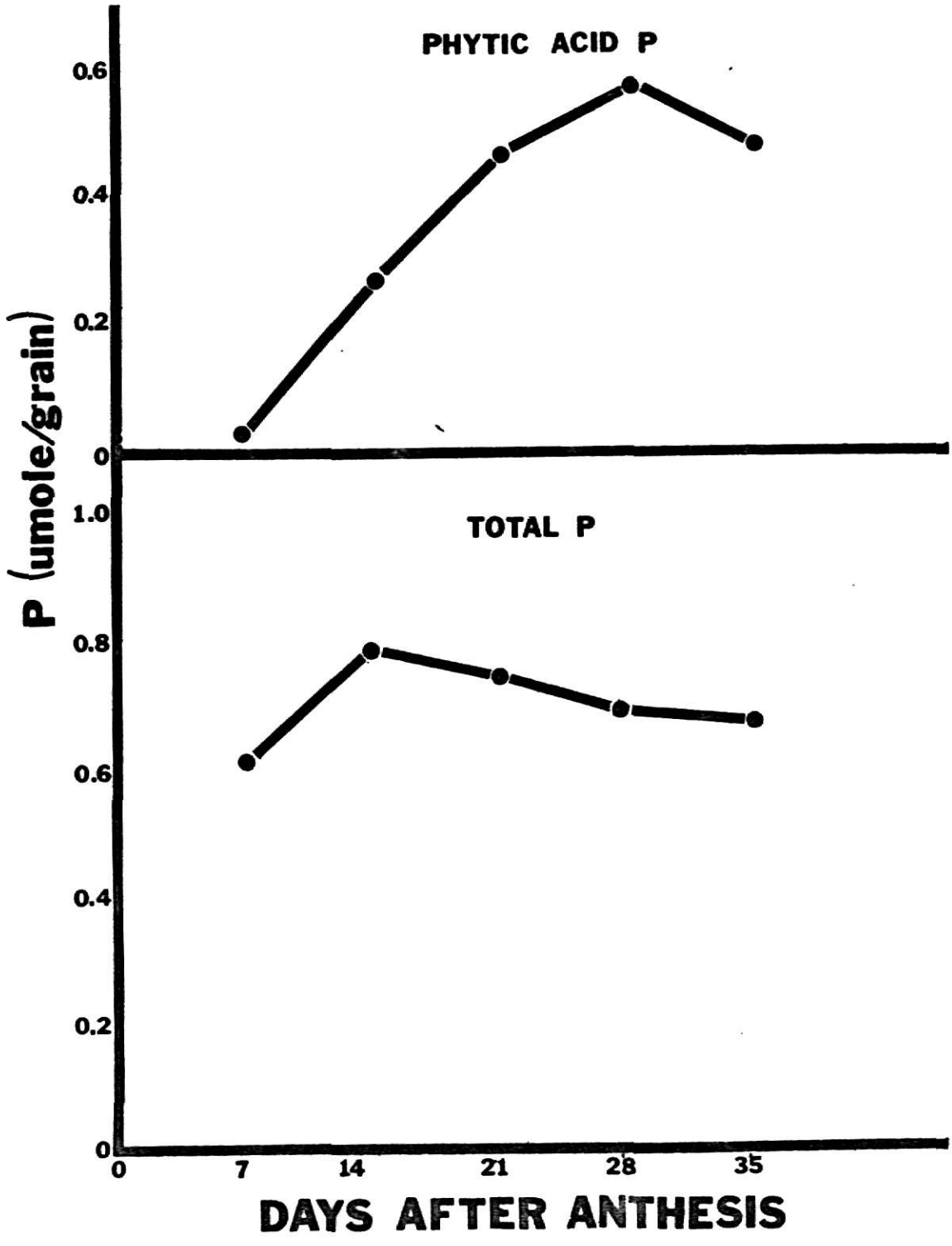

Fig. 4. Changes in phytic acid P and total P content in wheat (Triticum aestivum L. var. 'Parker') grain.

utilized in synthesis of phytic acid (Asada et al., 1969). Phytic acid comprised 8% of the total P at anthesis, 82% 28 days after anthesis, and 72% at maturity. Asada et al. (1969) also reported a decrease in phytic acid content of rice during the latter stage of maturity. Phytic acid content was correlated significantly with sampling date (0.73), total Zn per plant (0.50), and Zn per grain (0.59).

Phytase activity in fresh and germinated grain is shown in Fig. 5. Activity in fresh grain reached a peak the first week after anthesis and remained fairly constant during maturation. Phytase activity in fresh grain was not correlated significantly with any of the other constituents.

Phytase activity in germinated grain increased moderately up to 3 weeks after anthesis. Phytase activity in germinated grain during this period was lower than that in fresh grain, which indicated drying and germinating inactivated the enzyme from fresh grain. From the third week to maturity activity increased markedly faster. Phytase activity in germinated grain was correlated significantly with sampling date (0.80), Zn per grain (0.74), and phytic acid per grain (0.47). The correlation with time as represented by sampling date indicated more phytase was synthesized as the grain matured.

No significant difference was found at the 5% F-test level between phytase activity in fresh and germinated grain when all sampling dates combined were compared. Differences were significant, however, when individual sampling dates were compared. That was because of a significant interaction between sampling date and treatment (fresh and germinated). The 5% LSD between treatments (4.08) showed activity in fresh grain was significantly higher the first week following anthesis, while activity in germinated grain was significantly higher during later stages of maturity.

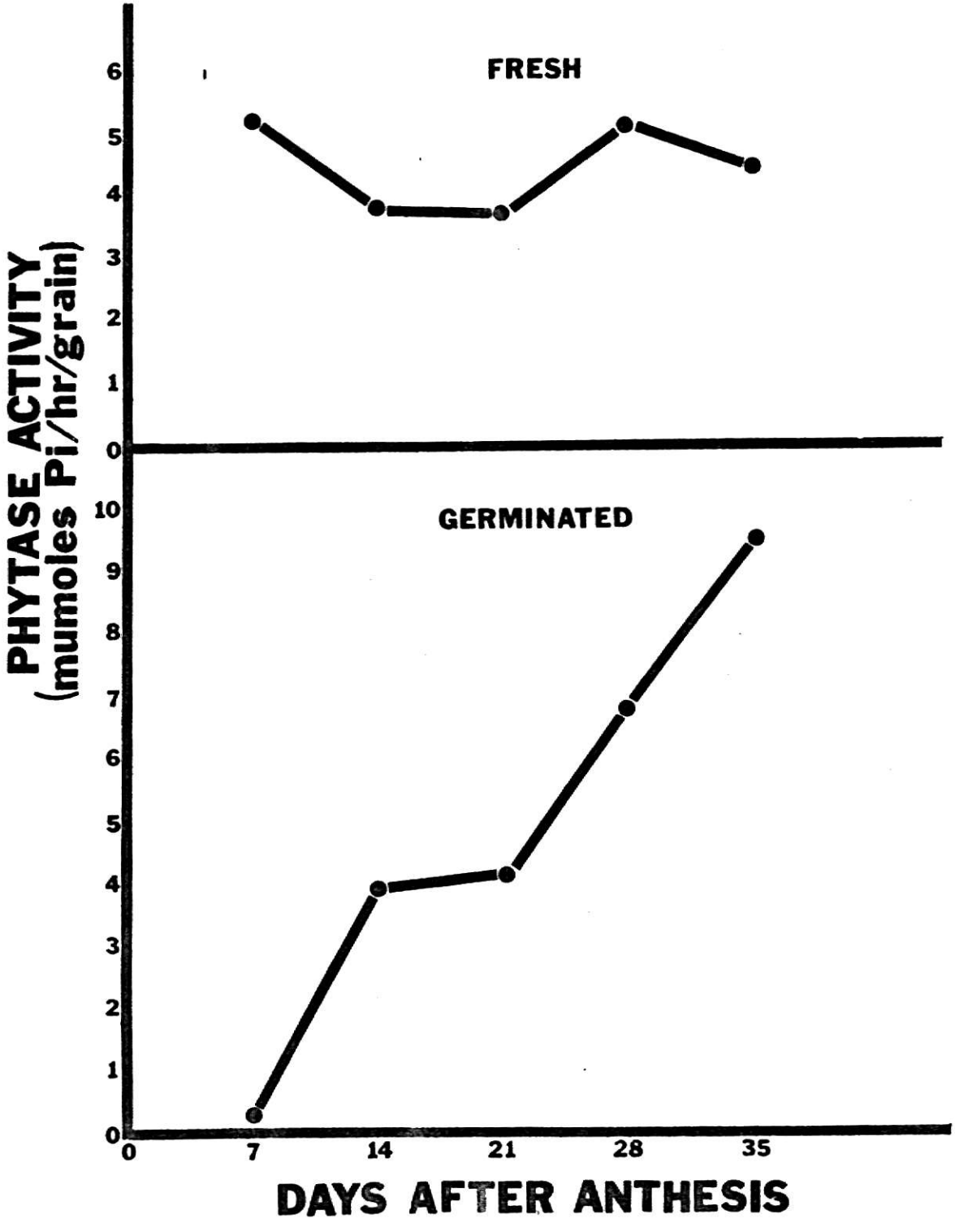

Fig. 5. Changes in phytase enzyme activity in fresh and germinated wheat (Triticum aestivum L. var. 'Parker') grain.

DISCUSSION

The objectives of this study were to determine the time of synthesis of phytic acid and phytase in maturing wheat grain, the relationship of phytase activity with phytic acid synthesis, and the relationship among P, Zn, and phytic acid during maturation. Zinc content was assayed in grain and vegetation to determine if phytic acid complexes Zn in plant P-induced Zn deficiency making it unavailable for metabolism (L. A. Rudgers, 1969. Site and mechanism of phosphorus-zinc interaction in corn (Zea mays L.) seedlings. Ph.D. Thesis. Kansas State University, Manhattan.) Rudgers postulated that with high P levels, plants might synthesize sufficient phytic acid to render Zn deficient. Phytic acid in vegetation was not assayed in this study, and that would be necessary to examine the hypothesis closely. The correlation of 0.59 between phytic acid content and Zn content of grain, while significant at the 5% level, was too low to form any definite conclusions. In addition, wheat has not been found to be susceptible to P-induced Zn deficiency.

Nagai and Funahashi (1962) reported a strong inhibition of *in vitro* phytase enzyme activity by Zn^{++}. If that inhibition occurred *in vivo*, Zn complexed by phytic acid might inhibit enzymatic breakdown of phytic acid and result in more pronounced Zn deficiency.

Phytase activity in germinated grain was positively correlated (0.74) with Zn content of grain. Dialysis during the purification procedure for phytase in this study removed endogenous Zn and prevented inhibition of the enzyme. While the possibility exists that Zn is utilized or required in phytase synthesis, the figures illustrating changes in Zn content in grain and phytase activity show the period of Zn translocation to the grain coincided

with the weekly increases of phytase activity in germinated grain as maturity was approached. This would explain the correlation, although further study might be conducted on the requirement for Zn in phytase synthesis.

The high correlation (0.88) between P and Zn content in wheat vegetation could be attributed to similarity in time of uptake of each element by the plants during maturation. The results shown in Figs. 2 and 3 indicate this, although a proportionally greater P concentration was taken up.

From this study there appeared to be little relationship between phytic acid accumulation and phytase activity during maturation. Saio (1964) and Asada et al. (1968) found only phytic acid and inositol monophosphate were present during phytic acid synthesis in rice. Phytase was reported to hydrolyze the phosphate groups of inositol hexaphosphate one-by-one in a stepwise fashion (Nagai and Funahashi, 1963). A mixture of inositol polyphosphate esters would be found if phytase hydrolyzed phytic acid synthesized previously. The absence of lower esters of inositol hexaphosphate would indicate phytic acid is stable once it is synthesized. Phytic acid formed during maturation is not in a dynamic state where the phytase found in fresh grain releases inorganic P for further metabolism or reutilization in phytic acid synthesis. The correlation between phytase activity in fresh grain and phytic acid was not significant at the 5% level.

A significant correlation (0.47) was found between phytase activity in germinated grain and phytic acid content, but it was very low and probably associated with coincident increases in synthesis with increasing maturity.

Synthesis of phytase enzyme during grain germination has been reported by numerous investigators (Bianchetti and Sartirana, 1967; Mandal and Biswas, 1970; Peers, 1953; Sobolev, 1962). In this study a higher level of activity was found in fresh grain than in grain germinated 48 hours during the early

stages of maturation. Only at physiological maturity was phytase activity in germinated grain significantly higher. Peers (1953) reported a 6.5-fold increase in soft wheat phytase activity for wheat germinated 5 days, although the 5-day germination period would allow greater time for phytase synthesis.

Phytic acid might be hydrolyzed by two enzyme systems, one acting at early stages of maturation in fresh grain, and the other being synthesized and functioning during germination. Phytase in fresh grain was synthesized during the first week after anthesis and remained at a constant level to maturity. Phytase synthesized during germination, however, increased steadily. The germinated assay samples were maintained at 50 C for at least 72 hours to break dormancy of fresh grain. This temperature might have been high enough to inactivate the enzyme that exhibited activity in fresh grain. Peers (1953) found slight thermal inactivation of wheat phytase occurred at 55 C in vitro, while whole grain maintained at 80 C for 10 minutes decreased in phytase activity. This would explain the significantly greater activity found in fresh grain the seventh day following anthesis.

Peers (1953) reported more than half the phytase activity in ungerminated grain was in endosperm and aleurone tissue. No data are available on the location of phytase activity in germinated grain. After phytase in fresh grain was inactivated by heat, phytase could be synthesized in the endosperm during germination. As the grain matured increasingly greater amounts of phytase were synthesized during germination. Without the germination stimulus only a small amount of phytase was synthesized, and remained at a constant level.

Eastwood et al. (1969) postulated an inactive form of phytase in aleurone tissue is activated during germination. From this study it would appear that the phytase activity reported by Eastwood et al. (1969) might

already be present before germination.

Studies conducted by Peers (1953) and Nagai and Funahashi (1962, 1963) with phytase utilized wheat bran and other seed components that had not undergone germination stimulus. From these observations, phytase activity is present, at least in certain components of the grain, before germination.

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. Gary M. Paulsen and Dr. Roscoe Ellis, Jr., co-advisors, for their assistance in planning and conducting the experiments, interpreting the data, and preparing the thesis.

Appreciation is extended to Dr. H. L. Mitchell, member of the advisory committee, and to Dr. R. L. Vanderlip for assistance in conducting the statistical analysis.

Sincere thanks also go to coworkers in this laboratory for their assistance.

A special thanks, also, to my wife, Carolyn, for her understanding and assistance in preparing the manuscript.

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RELATIONSHIP AMONG PHYTIC ACID, PHOSPHORUS, AND ZINC DURING MATURATION OF WHEAT GRAIN

by

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B. S., Kansas State University, 1970

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Agronomy

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1970

ABSTRACT

Changes in phytase activity, phytic acid, P and Zn concentrations in vegetation and grain, and dry weight were analyzed weekly in 'Parker' winter wheat (Triticum aestivum L.) to determine their interrelationships during maturation.

Phosphorus and Zn contents and dry weights of vegetation and grain increased linearly until maturity, when slight decreases occurred. Total P content in grain remained constant during maturation, but inorganic P decreased and phytic acid P content increased. Phytase activity in germinated grain increased with maturity, while activity in fresh grain remained constant. Significant positive correlations were found between P and Zn contents in vegetation and between phytase activity in germinated grain and Zn content of grain. Phytase activity in germinated grain, Zn content of grain, and phytic acid content of grain were correlated with sampling date.

Phytic acid comprised 8% of the total P in grain at anthesis and increased to 82% 28 days after anthesis. There appeared to be little direct relationship between phytic acid accumulation and phytase activity during maturation.

Phytase activity was significantly higher in fresh grain than in germinated grain during early stages after anthesis. During later stages after anthesis germinated grain had significantly higher activity. The results suggested that activity against phytic acid was caused by different enzymes in fresh and germinated grain. Phytase in fresh grain was synthesized during the first week after anthesis and remained at a constant level through maturity. Phytase synthesized during germination appeared to be the typical

phytase enzyme. As the grain matured, increasingly greater amounts of phytase were synthesized.